01789 U.S. PTO
111605

PTO/SB/05 (05-05)
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UTILITY PATENT APPLICATION TRANSMITTAL

(ONLY FOR NEW NONPROVISIONAL APPLICATIONS UNDER 37 CFR 1.53(B))

Attorney Docket No.	EGYP 3.0-034
First Inventor	Frédéric Robert
Title	METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND, etc.
Express Mail Label No.	EV313689525US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [X] **Fee Transmittal Form** (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [X] **Applicant claims small entity status.** See 37 CFR 1.27.
3. [X] **Specification** [*Total Pages* 16]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. [X] **Drawing(s)** *(35 U.S.C. 113)* [*Total Sheets* 13]
5. **Oath or Declaration** [*Total Sheets*]
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 18 completed)*
      i. [ ] DELETION OF INVENTOR(S)
      Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. [X] **Application Data Sheet.** See 37 CFR 1.76
7. [ ] **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   [ ] Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. – c. are required)*
   a. Computer Readable Form (CRF)
      i. [ ] Computer Readable Form (CRF)
      ii. [ ] Transfer Request (37 CFR 1.821(e))
   b. Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ADDRESS TO: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. [ ] **Assignment Papers** (cover sheet & document(s))
   Name of Assignee:
10. [ ] **37 CFR 3.73(b) Statement** *(when there is an assignee)* [ ] **Power of Attorney**
11. [ ] **English Translation Document** *(if applicable)*
12. [X] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [X] Copies of foreign patent documents, publications, & other information (13 references, 9 enclosed)
13. [ ] **Preliminary Amendment**
14. [X] **Return Receipt Postcard** (MPEP 503) *(Should be specifically itemized)*
15. [X] **Certified Copy of Priority Document(s) (FR-0412263, 11/18/04)** *(if foreign priority is claimed)*
16. [ ] **Nonpublication Request** under 35 U.S.C.122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. [ ] Other:

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111605

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*
    [ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No.:
    Prior application information: Examiner *Art Unit:*

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 000530 *OR* [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email Address	

Signature		Date	November 16, 2005
Name (Print/Type)	Michael H. Teschner	Registration No. (Attorney/Agent)	32,862

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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

[X] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 930.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Frédéric Robert
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	EGYP 3.0-034

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[X] Deposit Account Deposit Account Number: 12-1095 Deposit Account Name: Lerner, David, Littenberg, Krumholz & Mentlik, LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[X] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17
[X] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	500.00
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
30 - 20 =	10 x	25.00 =	250.00

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
180.00	180.00

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
3 - 3 =	x	=	

3. APPLICATION SIZE FEE

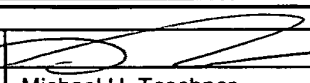
If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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29 - 100 =	/50	(round up to a whole number) x	=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge):	

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	32,862	Telephone	(908) 518-6313
Name (Print/Type)	Michael H. Teschner			Date	November 16, 2005

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    Prior application information: Examiner *Art Unit:*

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First Named Inventor	Frédéric Robert
Examiner Name	Not Yet Assigned
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Provisional	200	100	0	0	0	0	

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Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

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Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
180.00	180.00

Indep. Claims		Extra Claims	Fee ($)		Fee Paid ($)
3	- 3 =		x	=	

3. APPLICATION SIZE FEE

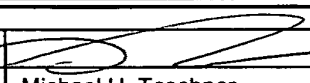
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SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	32,862	Telephone	(908) 518-6313
Name (Print/Type)	Michael H. Teschner			Date	November 16, 2005

METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

The present invention relates to a method for separating haemoglobin by capillary electrophoresis, to buffer compositions for use in said separation, and to kits for analyzing haemoglobin by capillary electrophoresis.

When analyzing haemoglobin A_2 and haemoglobin variants, in particular in biological liquids such as blood, for analytical and in particular for diagnostic purposes, and thereby to separate haemoglobins by electrophoresis, the use of capillary electrophoresis (CE) techniques is known. The term "haemoglobins" means any normal or abnormal haemoglobin and variants of said haemoglobins. One advantage of capillary electrophoresis resides in the fact that only very small quantities of biological liquids to be analyzed are necessary. Further, separation by that technique can be very rapid provided that high voltages can be used without heating the sample too much during separation.

The technique of choice is analysis by capillary isoelectrofocusing (CIEF). This method can produce a high resolution between the various forms of haemoglobin (Hempe) (7). However, it is difficult to automate and, because coated capillaries have to be used to prevent electro-osmotic flow, it is difficult to make it compatible with analyses carried out in series.

A further technique known as "dynamic double coating" may be carried out with commercial kits sold as the "Analis HbA_2-CE kit" or "CEofix HbA_2-CE kit" from Analis. That technique involves initial rinsing of the capillary with a solution containing a polycation at a pH of 4.7 followed by a second rinse with an analysis buffer containing a polyanion, at a pH of 8.7. In that "double coating" method, the quantity of negative charge present on the internal wall of the capillary is higher than on a naked capillary, and so the electro-osmotic flow is even greater. That dynamic double coating method cannot, however, separate HbA_2, HbC and HbE fractions sufficiently well, rendering quantitative analysis of the HbA_2 fraction impossible in the presence of HbC or HbE variants. Further, since HbS and HbD fractions have the same electrophoretic position, complementary analysis in an acidic medium is necessary to determine the type of

variant present in the sample. Finally, the double coating has to be produced again between each sample analysis, rendering the method costly and difficult to employ for mass-produced tests.

Further, free solution haemoglobin separation has been described, but it does not satisfy the expected criteria for accuracy, resolution or rapidity to be able to rationalize EC haemoglobin analyses. Ishoka (1) (1992) described the separation of haemoglobins using a borate buffer (100 mM) at a pH of 9.98 with a migration time of the order of 50 minutes, i.e. incompatible with currently expected times for analyzing haemoglobins. The same type of buffer under similar pH and concentration conditions (Jenkins) (3), (4) and (5) can only produce insufficient resolutions between the HbA, HbF and HbS fractions. Sahin (2) describes more acidic pH conditions, but obtained deeply unimpressive results for resolution between the HbA, HbF, HbS and HbA_2 fractions with lower concentrations (20 mM) of borate or with barbital (50 mM) at a pH of 8.5, and also deeply unimpressive results for resolution between the HbA, HbS and HbA_2 fractions for Tris buffer (1 M) at a pH of 8.0. Further, Tris/arginine combinations (Shihabi) (6) have been used and even though they allowed HbA/HbS separation, HbC/HbE and HbA_2 fractions were not resolved. Finally, United States patents US-A-5 202 006 and US-A-5 439 825, which describe the use of barbital or ethylbarbital, could only produce low resolutions between the principal haemoglobins, namely HbA, HbF, HbS and HbC.

Thus, there exists a need for a method for analyzing haemoglobin and in particular haemoglobin A_2 which allows single step analysis without a double coating, which can be carried out automatically and in series, and which guarantees satisfactory resolution between the HbA_2, HbC, HbD, HbE, HbS, HbF and HbA forms in particular.

The Applicant has now demonstrated that by using a zwitterionic analysis buffer associated with a flow inhibitor, it is possible to obtain greatly improved separation of the fractions mentioned above, in a single step, thus avoiding complementary separations and without a double coating, which simplifies its implementation.

Thus, the present invention concerns separating haemoglobins in biological samples by capillary electrophoresis, in which method the biological sample comprising said haemoglobins is passed through a capillary containing an analysis buffer, comprising at least one step in which the sample is introduced into a capillary tube containing a solution of analysis buffer, and in which the buffer is of the zwitterionic type and is associated with at least one flow inhibitor. Said step is generally followed by separation of haemoglobins, migration and detection of the various variants.

The zwitterionic buffer used in the invention is a zwitterionic buffer buffering between pHs of 8 and 10, comprising at least one amine function and at least one acidic function and at least one hydroxyl function in the position opposite to the acidic function. The term "acidic function" as used here means the carboxylic acid function or the sulphonic acid function. Said zwitterionic buffer may be formed by one or two molecules: in the case in which the amine function is carried by a first molecule without an acidic function, said first molecule is associated with a second molecule carrying an acidic function, in particular a carboxylic or sulphonic acid function or an amino acid. An example of an amino acid which may be cited is glycine.

According to the invention, the flow inhibitors are of the aliphatic or cyclic diamine or polyamine type. They are selected from aliphatic diamines or polyamines and/or cyclic diamines or polyamines, for example. Aliphatic diamines or polyamines are preferred. Examples of aliphatic diamines which may be cited are 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, N,N'-dimethyl-1,6-hexanediamine, N,N,N',N'-tetramethyl-1,4-butanediamine and their acceptable derivatives and salts. Examples of aliphatic polyamines which may be cited are diethylenetriamine, spermine, tetraethylenepentamine and their acceptable derivatives and salts. The flow inhibitors may be used as a mixture.

Acceptable salts which may be cited are the hydrochloride salts or the like. Examples of derivatives which may be cited are derivatives of the above compounds wherein one or more

carbon atoms of the aliphatic chain is/are substituted with one or more alkyl groups and/or one of the hydrogen(s) of the free amines is/are substituted with one or more alkyl groups.

The analysis buffer solution may also comprise other additives, in particular other additives intended to improve the separation of the various haemoglobins.

Further, the invention concerns the use of compounds known for their electrophoretic flow inhibiting activity in free solution EC in association with at least one zwitterionic buffer.

As will become apparent from the following examples, using the associations of the invention can greatly improve the resolution of haemoglobin and haemoglobin variants. It can thus improve the accuracy and precision of qualitative and quantitative analysis of the variants compared with analyses carried out using known methods. It can also allow HbA_2 to be quantified even in the presence of HbC or HbE.

The zwitterionic buffer – flow inhibitor associations of the invention are of particular use in analyzing samples in which normal or variant haemoglobins of the HbA_2, HbC, HbD, HbE, HbS, HbF and/or HbA type are present, to detect and/or quantify them.

Finally, the invention pertains to kits for analyzing haemoglobin A_2 and haemoglobin variants in a biological sample by EC, comprising at least one analysis buffer solution containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor, and an optional pH modifier. Thus, the kits may comprise at least one analysis buffer and a flow inhibitor according to the invention, and one or more solution(s) for rinsing capillaries and/or dilution segments and/or one (or more) diluent(s) for the sample to be analyzed. They may also comprise at least one haemolysing solution. In said kit, the buffer and the inhibitor(s) and diluent(s) or other additives may be stored separately for mixing extemporaneously, or stored as a mixture. This kit also optionally comprises instructions for carrying out the analysis and/or software support information.

Other advantages and characteristics of the invention will become apparent from the following description of examples made with reference to the accompanying drawings.

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0008

Figure 1 shows an electrophoregram of normal human blood (HbA, HbA_2) analyzed by capillary electrophoresis using a buffer solution of the invention;

Figures 2 to 5 each show an electrophoretogram of blood analyzed by capillary electrophoresis using the same buffer solution. The blood respectively comprises the following variants: Fig 2 = HbF and HbS; Fig 3 = HbC; Figure 4 = HbE; Figure 5 = HbS and HbD-Los Angeles;

Figures 6a, b, c, d each show an electrophoretogram of β-thalassemic blood analyzed by capillary electrophoresis using four different buffer solutions based on four different flow inhibitors;

Figures 7a, b, c, d each show an electrophoretogram of blood comprising HbF and HbS analyzed by capillary electrophoresis using four different buffer solutions based on four different flow inhibitors.

The conditions for carrying out capillary electrophoresis are known in the art. They usually include rinsing the capillaries with a rinsing solution, rinsing with the analysis buffer solution, optionally diluting the sample once or more, injection of the sample, migration and detection. Said steps may be carried out using automated machines.

Sample conditions for carrying out capillary electrophoresis are conditions suitable for using the automated Capillarys apparatus (SEBIA).

Examples of zwitterionic buffers for use in the invention which may be cited are "Tris" type buffers having several hydroxyl groups, specific examples of which are the following buffers: Tris (2-amino-2-[hydroxymethyl]-1,3-propanediol), tricine (N-tris[hydroxymethyl]methylglycine), TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulphonic acid), TABS (N-tris[hydroxymethyl]methyl-4-aminobutanesulphonic acid), or AMPD (2-amino-2-methyl-1,3-propanediol) or bis Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane), the last two and Tris possibly being associated with an amino acid.

Other molecules having a smaller number of hydroxyl groups may also be suitable, such as AMPSO (3-[(1,1-dimethyl-2-hydroxymethyl)amino]-2-hydroxy-propanesulphonic acid), bicine (N,N-bis[2-hydroxyethyl]glycine), HEPBS (N-[2-hydroxyethyl]piperazine-N'-[4-butanesulphonic] acid), in particular.

A preferred zwitterionic buffer from those cited above is tricine.

These buffers are known and commercially available. They may also be used as a mixture.

Preferred flow inhibitors from those cited above are 1,4-diaminobutane (DAB), 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine (DETA) and N,N,N',N'-tetramethyl-1,4-butanediamine. The inhibitors may also be used as a mixture.

1,4-diaminobutane hydrochloride is preferably associated with the tricine.

The term "sample of the invention" means the biological sample to be analyzed, i.e. any biological liquid comprising red corpuscles from healthy or sick patients. The human biological liquids may be blood, which may be normal or abnormal, washed, decanted, centrifuged or whole. Further, the blood may be haemolyzed.

In addition to human biological samples, samples of animal origin may also be analyzed. The samples may also be of synthetic origin, and the method of the invention may then be aimed at monitoring production, for example.

The sample may be initially diluted with a suitable diluting solution, a haemolyzing solution or an analysis buffer solution, for example.

The EC methods employing zwitterionic buffer/flow inhibitor associations of the invention are of particular use in analyzing blood and separating haemoglobin and its variants in human samples.

According to the invention, the pH of the analysis buffer solution is in the range 8 to 11, preferably in the range 8 to 10.

The analysis buffer solutions of the invention may also comprise at least one pH modifier. Examples of pH modifiers which may be used are compounds selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri- or tetra-alkyl ammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

According to the invention, the buffers are used in analysis buffer solutions under the usual conditions and concentrations, namely of the order of 20 to 500 mM, preferably 100 to 250 mM.

The flow inhibitors are used in the buffer solutions in concentrations in the range from about 0.01 to 50 mM, preferably in the range about 0.10 to 20 mM.

Further, the buffer solution may comprise one or more additives which may modify the ionic strength.

Examples of such compounds which may be cited are salts such as sulphates, chlorides, for example, and mixtures thereof.

The haemolyzing solution can carry out haemolysis of red corpuscles containing haemoglobin A_2 and haemoglobin variants. It is also used to dilute the sample before EC analysis. Depending on its composition, it may allow complete lysis of red corpuscles using a slight additional mechanical movement (vortex, agitation, ...)

The haemolyzing solution comprises an analysis buffer as mentioned above (zwitterionic buffers), the usual cell lysis additives, such as Triton X100 and/or saponine and other optional additives intended to produce a positive effect on the resolution between certain close-lying haemoglobins. An example which may be cited is Sébia's "Hydragel hémoglobine" haemolysing solution.

The pH of the haemolysing solution is in the range 8 to 11, preferably in the range 8 to 10.

The buffer solutions of the invention are prepared in the usual manner for analysis buffer compositions, namely by adding the constituents in the liquid form or the solid form for dilution, to an acceptable support. The support is usually water, which may be distilled or demineralized.

The materials employed for the capillaries are those normally used in capillary electrophoresis. Fused silica capillaries may be used. Their internal diameter may be from 5 to 2000 μm. Preferably, capillaries with an internal diameter of 200 μm, preferably less than 100 μm, are used. Preferably, capillaries with an untreated internal surface are used. The skilled person will be able to adapt the nature of the capillary and its size to the requirements of the analysis.

The use of such naked capillaries constitutes an advantage of the invention.

The haemoglobins may be analyzed at a wavelength of about 200 nm, on haemolyzed blood obtained from washed, decanted or centrifuged blood. However, to avoid interference with plasma proteins, they are preferably analyzed at a wavelength of about 415 nm, using haemolyzed blood obtained from washed, decanted, centrifuged or whole blood.

EXAMPLES

MATERIALS AND METHODS

A) <u>Capillary electrophoresis</u>

Capillary electrophoresis of clinical samples was carried out on EC apparatus equipped with a fused silica capillary with an internal diameter of 25 microns.

Detection was carried out under optimized conditions, at about 415 nm.

The samples were placed in the Capillarys apparatus (SEBIA) and automatically injected by hydrodynamic injection. The samples were separated in less than 8 minutes by applying an electric field of about 550 V/cm. The capillary was rinsed before each analysis with 0.25 M sodium hydroxide then with a solution of analysis buffer.

Analysis buffers:

The chemical products used were analytical grade product.

- The 200 mM tricine – 15 mM 1,4-diaminobutane (A) buffer was prepared by dissolving 35.84 g of tricine in about 900 ml of demineralized water then by adding 2.32 g of 1,4-diaminobutane hydrochloride (DAB). The pH was adjusted to 9.37 at 22°C with about 38.3 ml of 5M NaOH and the volume of the buffer solution was adjusted to 1 l with demineralized water.
- The 200 mM tricine – 15 mM 1,5-diaminopentane (B) buffer was prepared by dissolving 35.84 g of tricine in about 900 ml of demineralized water then by adding 2.62 g of 1,5-diaminopentane hydrochloride. The pH was adjusted to 9.40 at 22°C with about 38.4 ml of 5M NaOH and the volume of the buffer solution was adjusted to 1 l with demineralized water.
- The 200 mM tricine – 20 mM diethylenetriamine (C) buffer was prepared by dissolving 35.84 g of tricine in about 900 ml of demineralized water then by adding 2.06 g of diethylenetriamine (DETA). The pH was adjusted to 9.40 at 22°C with about 32.4 ml of 5M NaOH and the volume of the buffer solution was adjusted to 1 l with demineralized water.
- The 200 mM tricine – 8 mM N,N,N',N'-tetramethyl-1,4-butanediamine (D) buffer was prepared by dissolving 35.84 g of tricine in about 900 ml of demineralized water then by adding 1.15 g of N,N,N',N'-tetramethyl-1,4-butanediamine. The pH was adjusted to 9.19 at 22°C with about 34.7 ml of 5M NaOH and the volume of the buffer solution was adjusted to 1 l with demineralized water.

B) <u>Clinical samples</u>

For EC, human blood, decanted or centrifuged, was diluted to $1/6^{th}$ in the haemolyzing solution. This was prepared by dissolving 1.00 g of Triton X100, 2.50 g of saponine, 3.63 g of Tris in about 900 ml of demineralized water. The pH was adjusted to 8.70 at 22.0°C and the

volume of the solution was adjusted to 1 l with demineralized water. The chemical products employed were of analytical grade.

Examples 1 to 5

A solution of tricine/DAB.2HCl analysis buffer was prepared as described above.

Electrophoresis was carried out using the above method, on human blood.

Example 1: analysis of normal human blood with HbA as the principal peak and HbA_2 as the smaller fraction (Figure 1).

Example 2: analysis of diseased human blood, which shows a smaller HbA fraction, an augmented HbF fraction, a strong HbS fraction and a normal HbA_2 fraction. Heterozygous HbS was the origin of drepanocytosis (sickle cell anaemia) (Figure 2).

Example 3: analysis of diseased human blood presenting A/C heterozygosity. The HbC fraction leaves just before the HbA_2 fraction from which it is well resolved. This good resolution authorizes quantification of A_2 which means that the diagnosis could be orientated towards a case of β-thalassaemia, i.e. with an augmented HbA_2 fraction (Figure 3).

Example 4: analysis of human blood presenting A/E heterozygosity. The HbE fraction leaves just after the HbA_2 fraction from which it is completely resolved. Thus, there is no problem in quantifying HbA_2 in the presence of HbE (Figure 4).

Example 5: analysis of a mixture of blood presenting A/S heterozygosity and blood presenting A/D Los Angeles heterozygosity. The HbS and HbD fractions are partially resolved, which means that they can be distinguished from each other without the need for complementary analysis.

Example 6: analysis of β-thalassaemic human blood, i.e. having an augmented percentage of HbA_2 function and having a small HbF fraction.

Four analysis buffer solutions A, B, C and D were prepared as above.

Electrophoresis was carried out using the method described above.

Figure 6a) shows the results with buffer A (tricine/DAB.2HCl). Figure 6b shows the results with buffer B (tricine/1,5-diaminopentane.2HCl). Figure 6c shows the results with buffer C (tricine/DETA). Figure 6d shows the results with buffer D (tricine/N,N,N',N'-tetramethyl-1,4-butanediamine).

A comparison of these four figures shows that in all cases, separation of each of the fractions was good and the small HbF and HbA_2 fractions are well focused.

Example 7: analysis of human blood presenting drepanocytary (sickle cell) heterozygosity (HbS/HbA).

Four analysis buffer solutions (A: tricine/DAB.2HCl; B: tricine/1,5-diaminopentane.2HCl; C: tricine/DETA; D: tricine/ N,N,N',N'-tetramethyl-1,4-butanediamine) were prepared as above.

Electrophoresis was carried out using the method described above.

The order of the figures corresponds to the buffers mentioned above. Here again, comparable profiles were observed regardless of the buffer used.

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2. Ahmet Sahin et al, Journal of Chromatography A, 709 (1995) 121-125.
3. Margaret A Jenkins, Michael D Guerin, Journal of Chromatography B, 682 (1996), 23-34.
4. Margaret A Jenkins, Jean Hendy, Ian L Smith, J CAP ELEC. 004: 3 (1997), 137-143.
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CLAIMS

1. A method for separating haemoglobins in biological samples by capillary electrophoresis, in which the sample comprising said haemoglobins is passed through a capillary containing an analysis buffer, comprising at least one step in which the sample is introduced into a capillary tube containing a solution of analysis buffer, characterized in that the buffer is of the zwitterionic type and is associated with at least one flow inhibitor.

2. A method according to claim 1, characterized in that it further comprises separating haemoglobins by migration and detecting said haemoglobins.

3. A method according to claim 1 or claim 2, characterized in that the sample is a sample of blood which may be normal or abnormal, washed, decanted, centrifuged or whole blood.

4. A method according to one of claims 1 to 3, characterized in that the sample is a sample of haemolyzed blood.

5. A method according to one of claims 1 to 4, characterized in that the haemoglobins are haemoglobin A_2 and C, D, E, S, F and/or A haemoglobins.

6. A method according to one of claims 1 to 5, characterized in that the zwitterionic buffer is formed by one or two molecules comprises at least one amine function, at least one acid function and at least one hydroxyl function in the position opposite to the acid function.

7. A method according to one of claims 1 to 6, characterized in that the buffer is selected from tricine, TAPS, TABS, AMPSO, bicine or HEPBS, or an association of Tris, AMPD or bis Tris propane with an amino acid.

8. A method according to one of claims 1 to 7, characterized in that the buffer is tricine.

9. A method according to one of claims 1 to 8, characterized in that the flow inhibitor is of the aliphatic or cyclic diamine or polyamine type.

10. A method according to one of claims 1 to 9, characterized in that said flow inhibitor is selected from aliphatic diamines, aliphatic polyamines and their acceptable derivatives and salts, and mixtures thereof.

11. A method according to one of claims 1 to 10, characterized in that said flow inhibitor is selected from 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine, spermine, N,N'-dimethyl-1,6-hexanediamine, tetraethylenepentamine, N,N,N',N'-tetramethyl-1,4-butanediamine and their acceptable derivatives and salts, and mixtures thereof.

12. A method according to one of claims 1 to 11, characterized in that said flow inhibitor is selected from 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine and N,N,N',N'-tetramethyl-1,4-butanediamine, and their acceptable derivatives and salts, and mixtures thereof.

13. A method according to one of claims 1 to 12, characterized in that the flow inhibitor is 1,4-diaminobutane.

14. A method according to one of claims 1 to 13, characterized in that the flow inhibitor is 1,4-diaminobutane in the hydrochloride form.

15. A method according to one of claims 1 to 14, characterized in that the concentration of buffer in the buffer solution is in the range 20 mM to 500 mM.

16. A method according to one of claims 1 to 15, characterized in that the concentration of buffer in the buffer solution is in the range 100 mM to 250 mM.

17. A method according to one of claims 1 to 16, characterized in that the concentration of flow inhibitor in the buffer solution is in the range 0.01 to 50 mM.

18. A method according to one of claims 1 to 17, characterized in that the concentration of flow inhibitor in the buffer solution is in the range 0.10 mM to 20 mM.

19. A method according to one of claims 1 to 18, characterized in that the buffer solution further comprises a pH modifier.

20. A method according to claim 19, characterized in that the pH modifier is selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, and mono-, di-, tri- or tetra-alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

21. A method according to one of claims 1 to 20, characterized in that the capillary is naked.

22. A method according to one of claims 1 to 21, characterized in that the capillary is formed from fused silica.

23. A method according to one of claims 1 to 22, characterized in that the pH of the analysis buffer solution is in the range 8 to 10.

24. Use of a flow inhibitor selected from aliphatic and cyclic diamines and polyamines, in a method for capillary electrophoresis of haemoglobins, in association with at least one zwitterionic buffer in a buffer solution.

25. Use according to claim 24, in which the flow inhibitor is selected from aliphatic diamines, aliphatic polyamines, their acceptable derivatives and salts, and mixtures thereof.

26. Use according to claim 24 or claim 25, in which the zwitterionic buffer is present in a concentration of about 20 to 500 mM, and the inhibitor is present in a concentration of about 0.01 to 50 mM.

27. A capillary electrophoresis haemoglobin analysis kit comprising at least one analysis buffer containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor, and optionally a pH modifier.

28. An analysis buffer kit according to claim 27, characterized in that it further comprises a haemolyzing solution.

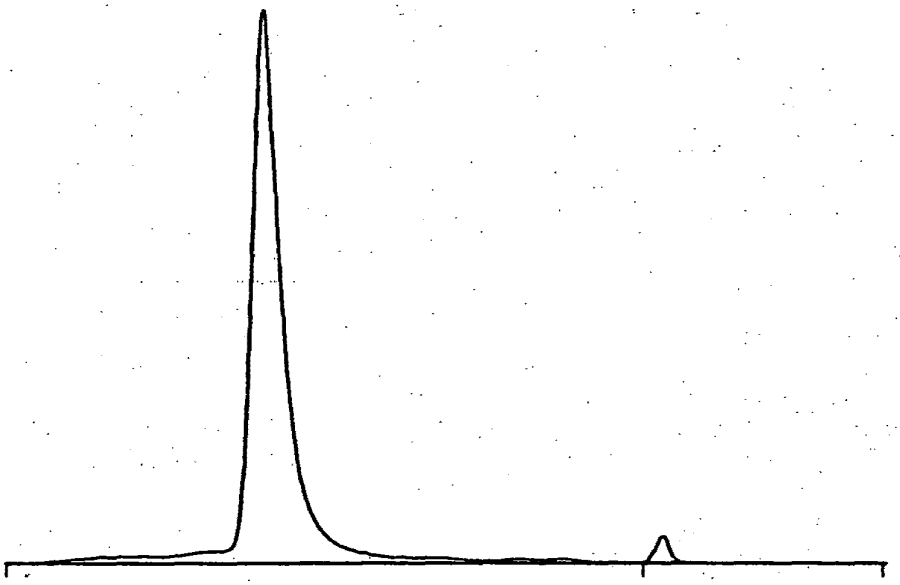
METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

ABSTRACT

The invention concerns a method for free solution capillary electrophoresis at an alkaline pH to analyze samples comprising haemoglobin, in which the sample is passed through a capillary containing an analysis buffer, comprising at least one step in which the sample is introduced into a capillary tube containing a solution of analysis buffer, characterized in that the buffer is of the zwitterionic type and in that it is associated with at least one flow inhibitor.

It also concerns the use of EC flow inhibitors associated with at least one zwitterionic buffer, and a kit for analyzing haemoglobin by capillary electrophoresis.

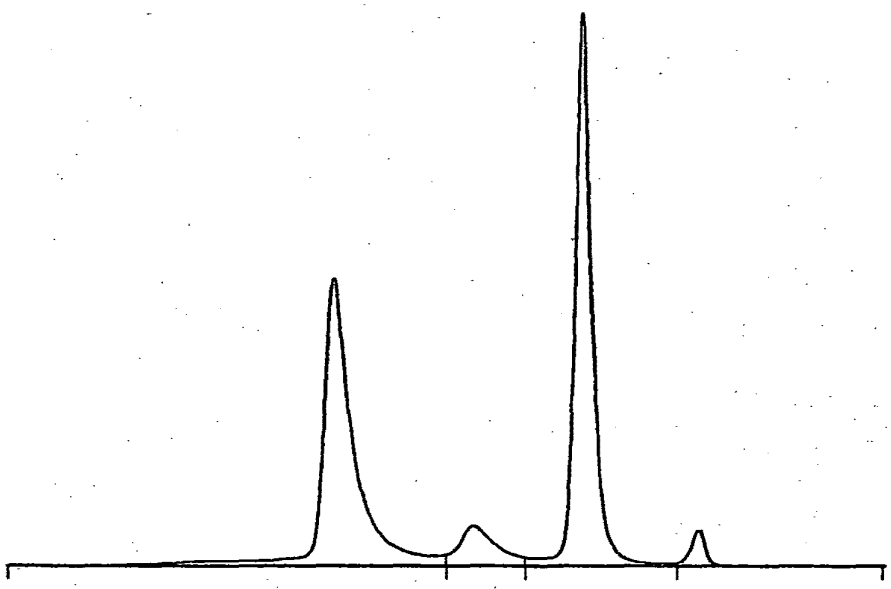
Helena Laboratories Corp.
Exhibit 1003
Page 1003-0020



Nom	%
HbA	98,2
HbA2	1,8

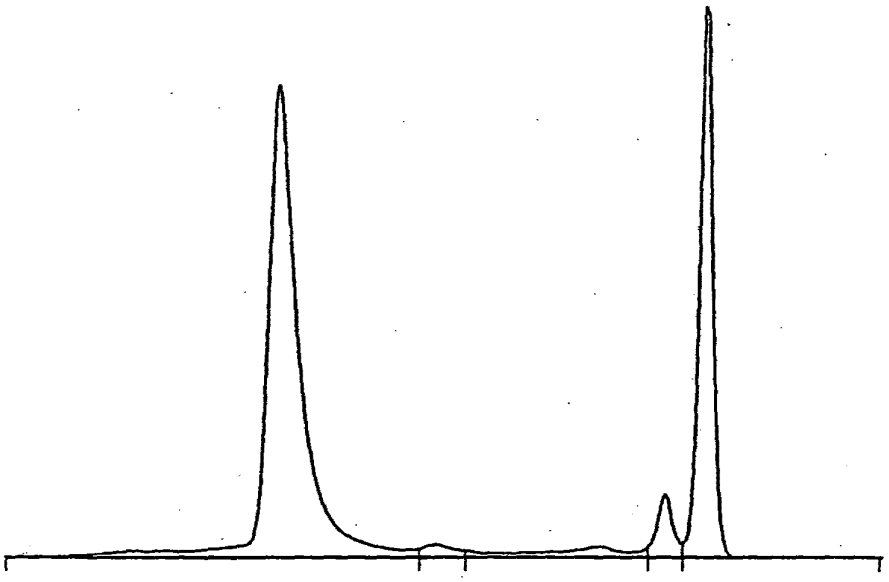
FIG 1

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Exhibit 1003
Page 1003-0021



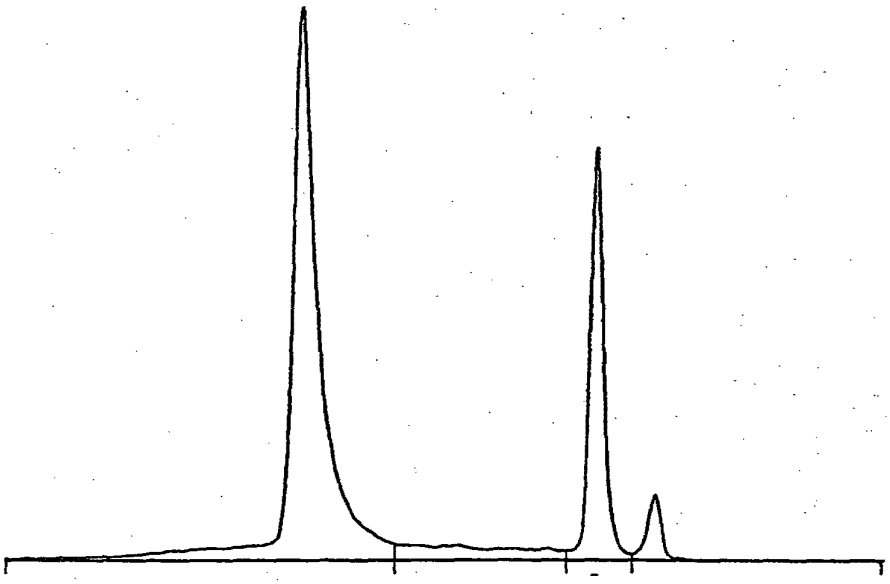
Nom	%
HbA	44,0
HbF	7,1
HbS	46,5
HbA2	2,4

FIG 2



Nom	%
HbA	60,9
HbF	1,6
MetHb	3,9
HbA2	4,0
HbC	29,6

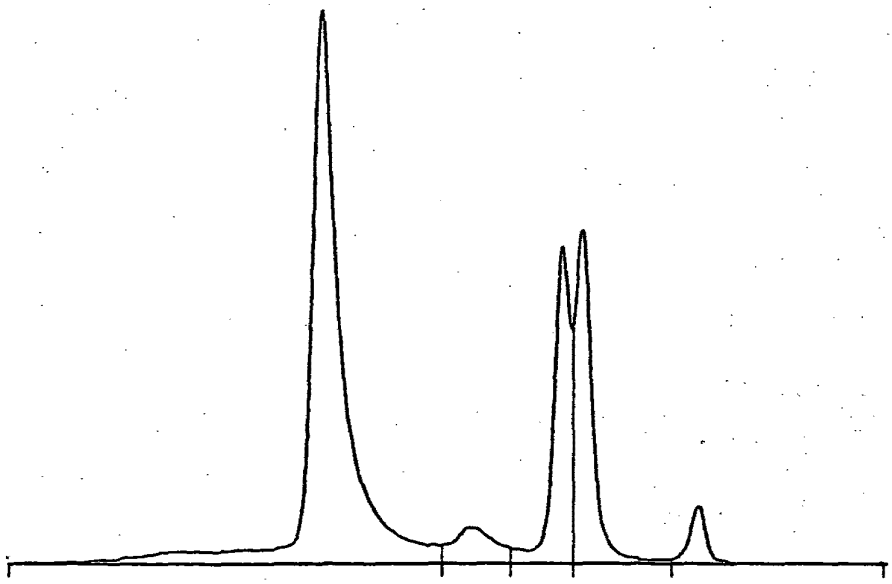
FIG 3



Nom	%
HbA	65,2
MetHb	7,4
HbE	23,5
HbA2	3,9

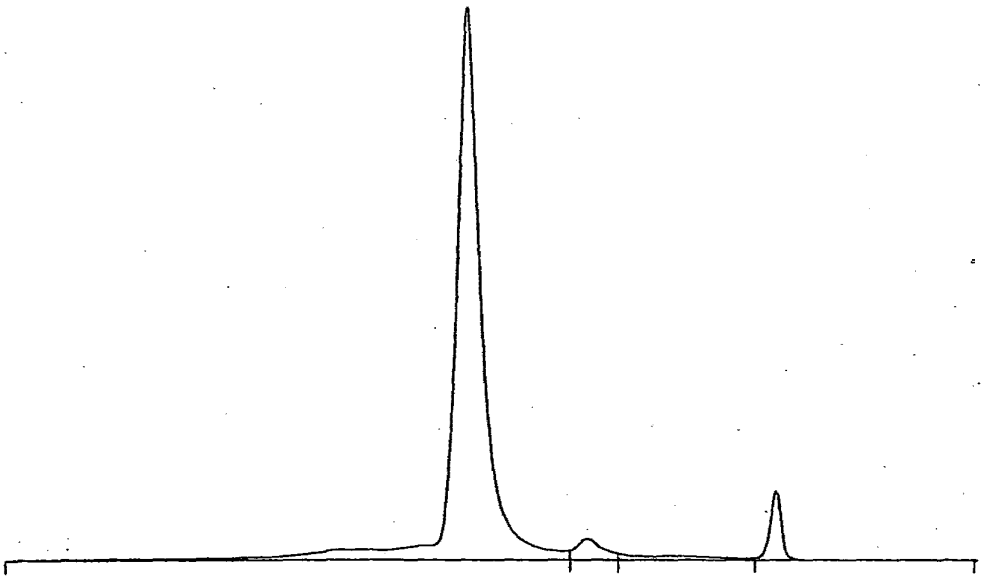
FIG 4

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Exhibit 1003
Page 1003-0024



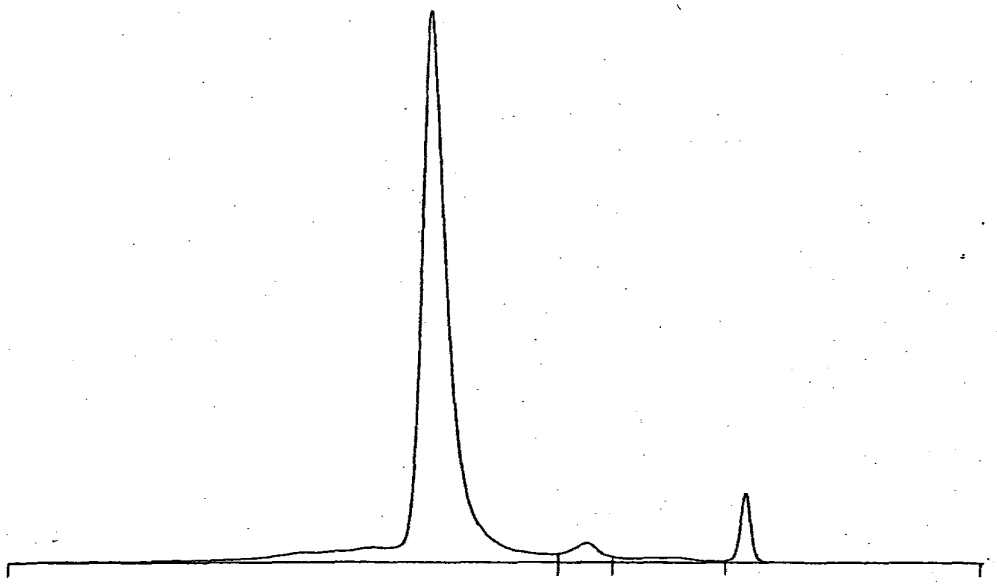
Nom	%
HbA	55,6
HbF	4,8
HbD	18,4
HbS	18,2
HbA2	3,0

FIG 5



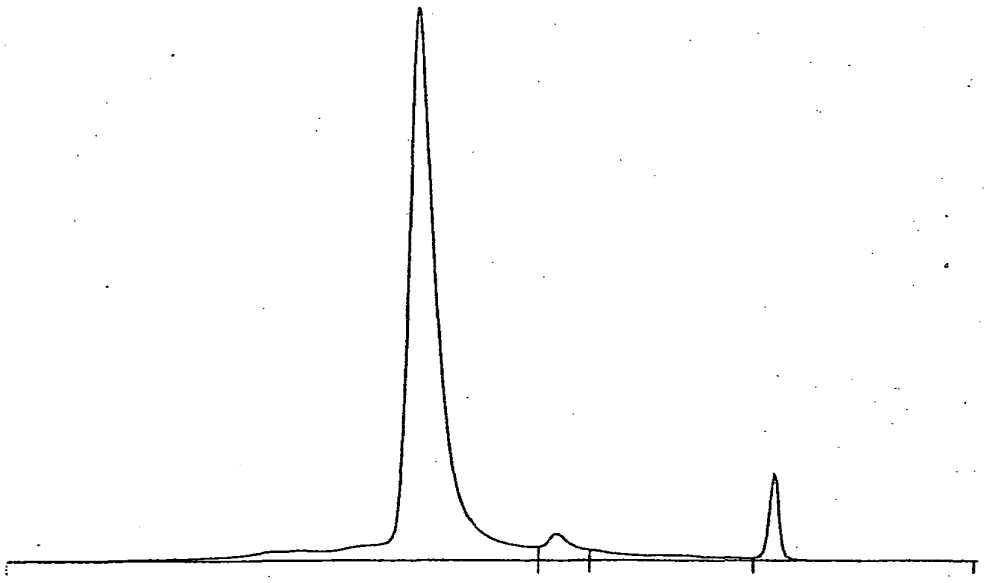
Nom	%
HbA	90,3
HbF	3,2
MetHb	2,2
HbA2	4,3

FIG 6A



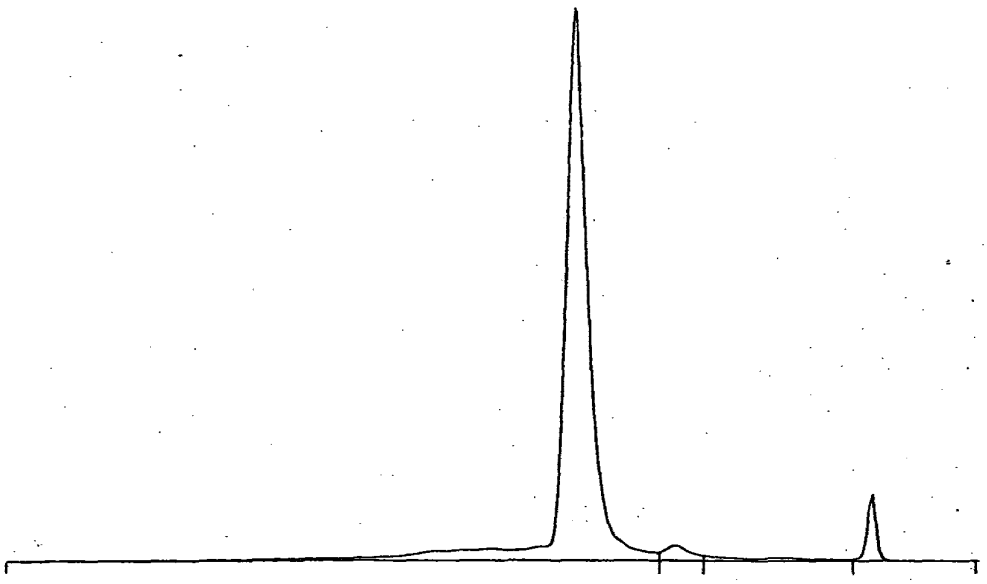
Nom	%
HbA	90,4
HbF	3,3
MetHb	2,0
HbA2	4,3

FIG 6B



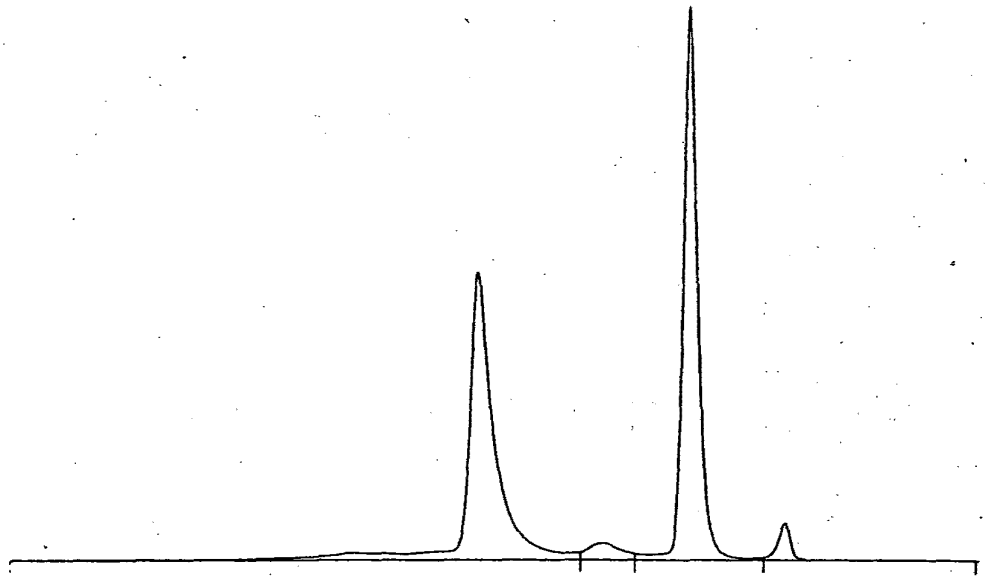
Nom	%
HbA	88,2
HbF	3,9
MetHb	3,4
HbA2	4,5

FIG 6C



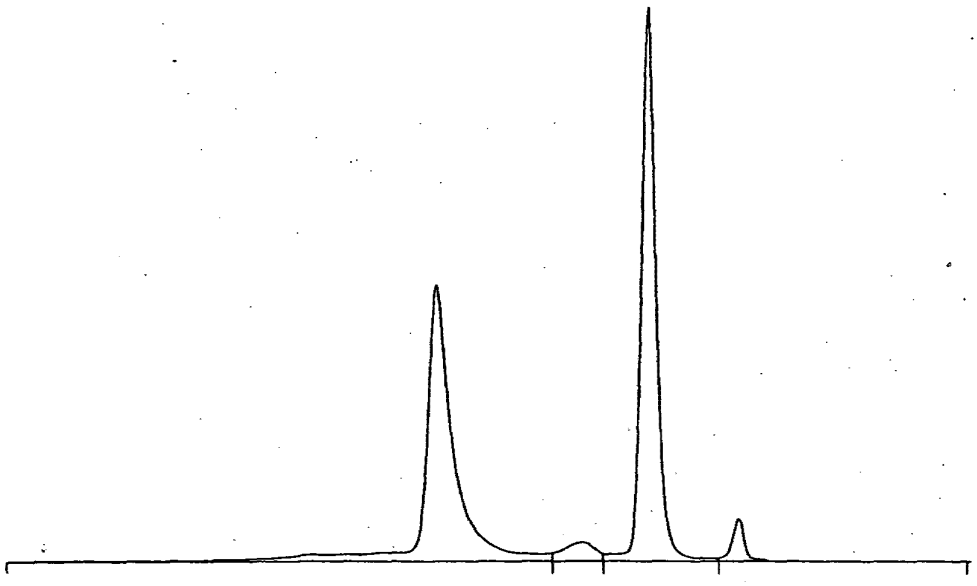
Nom	%
HbA	91,6
HbF	2,6
MetHb	1,9
HbA2	3,9

FIG 6D



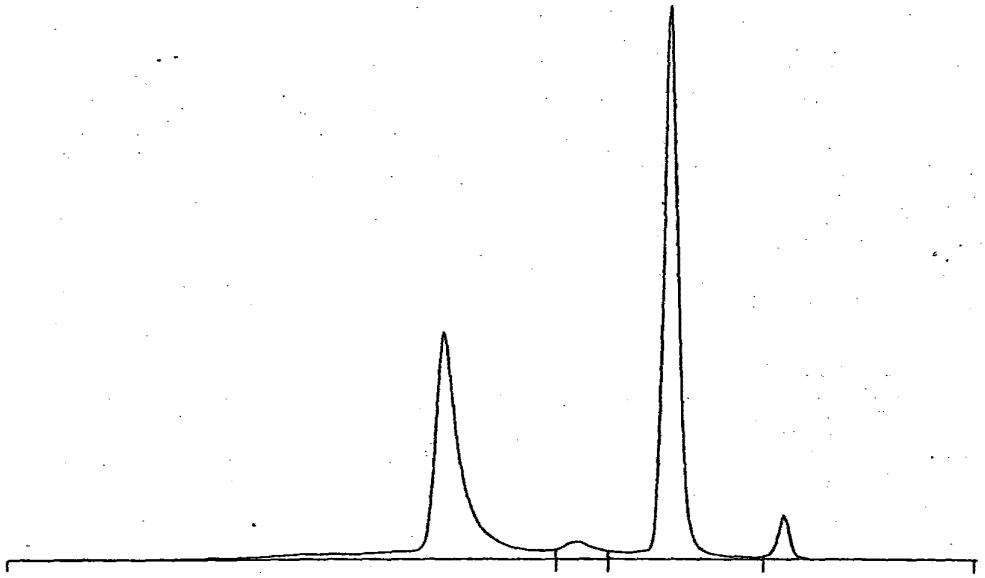
Nom	%
HbA	46,2
HbF	3,3
HbS	47,7
HbA2	2,8

FIG 7A



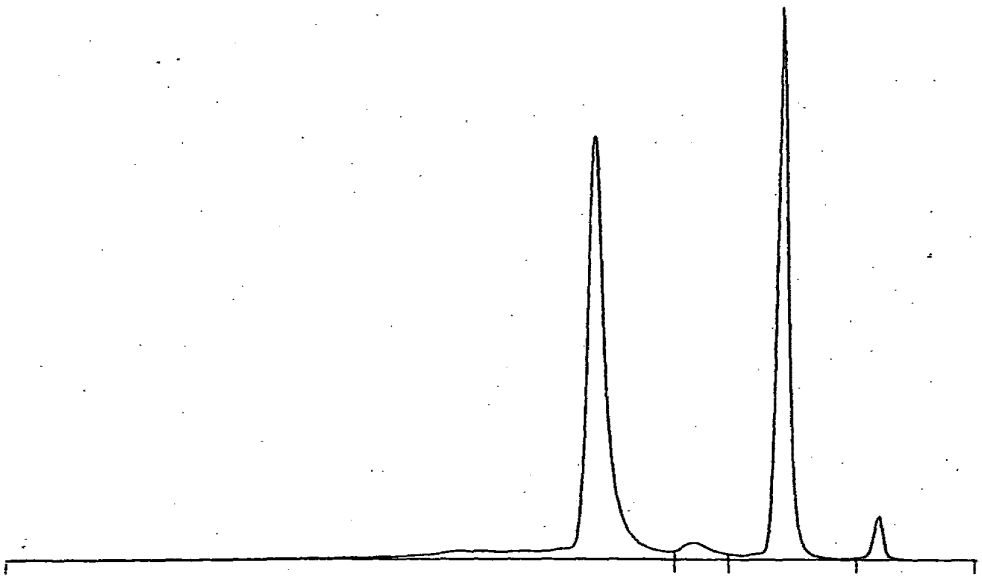
Nom	%
HbA	46,4
HbF	3,5
HbS	47,1
HbA2	3,0

FIG 7B



Nom	%
HbA	42,1
HbF	3,7
HbS	51,3
HbA2	2,9

FIG 7C



Nom	%
HbA	55,5
HbF	3,1
HbS	38,8
HbA2	2,6

FIG 7D

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

SERIAL NO.	FILING DATE
APPLICANT(S)	

CLAIMS

	AS FILED		AFTER 1ST AMENDMENT		AFTER 2ND AMENDMENT	
	IND	DEP	IND	DEP	IND	DEP
1	1					
2						
3		2				
4		0				
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TOTAL IND.	3					
TOTAL DEP.	27					
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TOTAL CLAIMS						

PTO-1360 (Rejection only) (3/03)

PATENT APPLICATION SERIAL NO _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

11/18/2005 FFANAEIA 00000055 121095 11280025

01 FC:2011	150.00	DA
02 FC:2111	250.00	DA
03 FC:2311	100.00	DA
04 FC:2202	250.00	DA
05 FC:2203	180.00	DA

PTO-1556
(5/87)

*U.S Government Printing Office: 2002 — 466-267/60033

PTO/SB/06 (12-04)
Approved for use through 7/31/2006. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875 Effective December 8, 2004

Application or Docket Number: 112 8ee25

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	150.00		N/A	300.00
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	$250		N/A	$500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	$100		N/A	$200
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	10	X$ 25 =		OR	X$50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	0	X100 =	250		X200 =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			+180=	180		+360=	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	930		TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD
Attorney Docket Number::	EGYP 3.0-034
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	13
Small Entity?::	Yes
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	France
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Page 1003-0037

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Representative Information

Representative Customer Number:: 000530

Domestic Priority Information

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::
France	0412263	11/18/04	Yes

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Express Mail Label No. EV313689525US Dated: November 16, 2005

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Robert et al.

Application No.: Not Yet Assigned

Group Art Unit: N/A

Filed: Concurrently Herewith

Examiner: Not Yet Assigned

For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Dear Sir:

It is respectfully requested that the references listed on the enclosed form be made of record and considered with respect to the above-referenced U.S. patent application. A copy of each non-US reference is enclosed. Submission of the present Information Disclosure Statement should not be taken as an admission that the cited references are legally available prior art or that the same are pertinent or material.

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Dated: November 16, 2005

Respectfully submitted,

By_____

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LD-458\

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Sheet 1 of 1

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Frédéric Robert
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	EGYP 3.0-034

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-5,447,612-A	09-05-1995	Bier et al.	
	AB*	US-5,599,433-A	02/1997	KEO ET AL.	
	AC*	US-5,439,825-A	08-08-1995	Chen	
	AD*	US-5,202,006-A	04-13-1993	Chen	

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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	BA	FR-1.554.045-A	01-17-1969	LKB-Produkter AB		√
	BB	WO-97/04308-A1	02-06-1997	Waters Investments Limited		√

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NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	ISHIOKA et al., "Detection of Abnormal Haemoglobin by Capillary Electrophoresis and Structural Identification", Biomedical Chromatography, Vol. 1, pgs. 224-226, 1992	
	CB	SAHIN et al., "Haemoglobin analysis by capillary zone electrophoresis", Journal of Chromatography A, Vol. 709, pgs. 121-125, 1995	
	CC	JENKINS et al., "Capillary electrophoresis as a clinical tool" Journal of Chromatography B, Vol. 682, pgs. 23-34, 1996	
	CD	JENKINS et al., "Evaluation of hemoglobin A_2 quantitation assay and hemoglobin variant screening by capillary electrophoresis", J.Cap.Elec. Vol. 004, No. 3, pgs. 137-143, 1997	
	CE	JENKINS et al., "Capillary isoelectric focusing of haemoglobin variants in the clinical laboratory", Clinica Chimica Acta, Vol. 289, pgs. 121-132, 1999	
	CF	SHIHABI et al., "Hemoglobin A_2 quantification by capillary zone electrophoresis", Electrophoresis 2000, Vol. 21, pgs. 749-752, 1999	
	CG	HEMPE et al., "Separation of hemoglobin variants with similar charge by capillary isoelectric focusing: Value of isoelectric point for identification of common and uncommon hemoglobin variants", Electrophoresis 2000, Vol. 21, pgs. 743-748	

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FR 1 554 045 partial translation

Page 1, left column, last paragraph

This process has been of great importance in fractionating biological substances, particularly inferior peptides and amino acids obtained by hydrolysis of the proteins. However, no particularly selective process was found to date for the resolution of a mixture of ampholytes having isoelectric points rather close to each other. Its importance was therefore limited to the separation of ampholytes of three principal groups: acid ampholytes, neutral ampholytes and basic ampholytes. Tiselius arrived at the same conclusion on the limited use of the process when trying it on a protein mixture (Svensk Kemisk Tidskr. 53, 305, 1941). Two proteins as different as egg albumin (pI=4.6) and hemoglobin (pI=7.0) which are very easy to separate with other processes could not be separated by stationary electrolysis, but only enriched.

Page 4, left column, paragraph 3 – right column, paragraph 5

The present invention concerns also the composition of polyproteic ampholytes which isoelectric points are distributed in the pH range which is narrower than pH 3 – pH 11 as above described. These separations, which generally extend on ranges of 2 to 3 pH-units are very useful for ultrafine preparation of proteins with isoelectric points close to each other, for example antibody systems, hemoglobins and myoglobins. In stationary electrolysis of compositions of ampholytes with isolectric points between pH 6 and pH 8, the obtained anodic pH is of about 6 and the cathodic pH is of about 8 (without secondary effects) and, therefore, the whole volume of the electrolytic cell can be used for separating this narrow pH range.

The system of supporting ampholyte as above described satisfies all the conditions of such a system. It comprises individual ampholytes which isoelectric points are well dispersed in the pH range which cannot be obtained with base-acid systems. The buffering system and the electric conductibility of these ampholytes support at isoelectric stage are satisfactory. They are colorless and do not present a significant absorption of light at 2800 Å. They do not as well affect any of the other methods usually employed to analyze proteins. As described thereafter, the raw material for the production is not expensive.

None of the individual ampholyte according to the invention is available on the market of chemical compounds. The present invention concerns also process for producing these compositions. The process is synthetic but, as the process of partial hydrolysis, it directly gives a complete system of supporting ampholyte. Thus, the process of the invention does not comprise any synthesis of pure chemical product, nor the ultimate mixture of determined quantities of chemical products because such a process would have been too expensive.

The mixture of supporting ampholytes of the present inventions preferably produced by reaction of an organic compound of low molecular weight containing at least two amino and/or imino groups with an acid containing at least a double carbon/carbon bond.

The organic nitrogen base of low molecular weight has (a)normally (sic) a molecular weight of less than 500 and preferably less than 300 it can be substituted on one or more of the nitrogen atoms with group(s) such as methyl, ethyl, ethanolamino or hydroxy. The compound has preferably not more than three carbon atoms between two of the N atoms and preferably only two carbon atoms. Thus, the compound comprises preferably the structure:

$$-NH-R-NH-$$

wherein R represents $-(CH_2)_3-$ and preferably:

$$-CH_2-CH_2-.$$

The amine should furthermore have a pK_s which is not less than 8. The amine is preferably aliphatic and the particularly preferred are ethylenediamine, diethylenetriamine, triethylenetetramine, tetraelylenepentamine and pentaethylenehexamine. The amine can also be alicyclic, heterocyclic or contain aromatic groups. However, in this later case, the mixture of supporting ampholytes presents generally an absorption in the critical zone at 2800 Å. Mixtures of amines can advantageously be used and the mixture can contain one or more monoamines.

The unsaturated acid as used according to the invention contains preferably only one double bond and preferably not more than ten carbon atoms. The double bond should preferably be in an alpha or beta position. The acid is preferably an unsaturated aliphatic acid, an acrylic acid, methacrylic acid or crotonic acid being appropriate. The acid can contain more than one carboxylic group, for example maleic or itaconic acid.

RÉPUBLIQUE FRANÇAISE

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SERVICE
de la PROPRIÉTÉ INDUSTRIELLE

BREVET D'INVENTION

P.V. n° 136.005 — N° 1.554.045

Classification internationale : C 07 c

BIBLIOTHÈQUE DE L'UNIVERSITÉ DE LAVAL

Procédé de préparation d'ampholytes supports et produits obtenus.

Société dite : LKB-PRODUKTER AB résidant en Suède.

Demandé le 15 janvier 1968, à 15h 3m, à Paris.
Délivré par arrêté du 9 décembre 1968.
(*Bulletin officiel de la Propriété industrielle,* n° 3 du 17 janvier 1969.)

La présente invention concerne un procédé de préparation d'ampholytes supports convenant pour l'utilisation dans le fractionnement isoélectrique des ampholytes de poids moléculaire élevé (en particulier les toxines, antitoxines, antigènes bactériens, anticorps, hormones, enzymes) par électrolyse stationnaire (c'est-à-dire par électrolyse effectuée jusqu'à ce que les concentrations des substances dans les différentes parties de la cellule d'électrolyse soient sensiblement constantes) et les ampholytes supports ainsi obtenus.

Le procédé de caractérisation et de fractionnement des ampholytes par électrolyse stationnaire a été découvert par Williams et Waterman (Broc. Soc. Exp. Biol. Med. 27, 56-59, 1929). Leur principe est basé sur un nouveau développement du fait connu que la soude pure et l'acide sulfurique pur peuvent être obtenus par électrolyse du sulfate de sodium si l'on empêche l'alcali formé à la cathode et l'acide formé à l'anode de se recombiner. Ceci s'applique à d'autres acides et bases qui sont résistants à l'oxydation anodique et à la réduction cathodique. Williams et Waterman ont pensé que si l'on effectue de la même manière l'électrolyse sans diffusion d'un mélange d'ampholytes, il s'accumulera à l'équilibre d'autant plus d'ampholytes basiques au voisinage de la cathode qu'il s'accumulera d'ampholytes acides au voisinage de l'anode. Comme chaque ampholyte tend à donner à la solution un pH voisin de son point isoélectrique, le résultat sera que le courant par son action d'électrolyse formera un gradient de pH, le pH augmentant de l'anode vers la cathode. La variation de pH entre les électrodes dépend des quantités et des propriétés électrochimiques des électrolytes dans le système.

Ce procédé a eu beaucoup d'importance dans le fractionnement des substances biologiques, en particulier les peptides inférieurs et les aminoacides obtenus par hydrolyse des protéines. Cependant, on n'a pas encore trouvé le procédé particulièrement sélectif pour la résolution d'un mélange d'ampholytes ayant des points isoélectriques assez voisins. Son importance s'est donc limitée à la séparation des ampholytes en trois groupes principaux : ampholytes acides, ampholytes neutres et ampholytes basiques. Tiselius est arrivé à la même conclusion sur l'utilisation limitée du procédé en l'essayant sur un mélange de protéines (Svensk Kemisk Tidskr. *53*, 305, 1941). Deux protéines aussi différentes que l'albumine de l'œuf (point isoélectrique pI=4,6) et l'hémoglobine (pI=7,0) qui sont très faciles à séparer par d'autres procédés, n'ont pas pu être séparées par électrolyse stationnaire, mais seulement être enrichies.

Une explication de ce phénomène est donnée par Svensson (Acta Chem. Scan. 15, 325, 1961), qui a montré dans une analyse théorique que dans un gradient de pH stationnaire formé par un courant électrique, il ne peut exister nulle part d'ampholytes à l'état pur si l'on part d'un mélange. Chaque ampholyte, même à son maximum de concentration, est contaminé par les ampholytes ayant des points isoélectriques voisins. Il a été en outre montré que la concentration d'un ampholyte dans une zone étroite est d'autant meilleure que la constante de diffusion est plus faible et que la dérivée de la mobilité ionique u par rapport au pH, soit $du/d(\mathrm{pH})$, au point isoélectrique est plus élevée. On sait maintenant que la plupart des amino-acides et des peptides inférieurs ont des constantes de diffusion élevées et que la dérivée $du/d(\mathrm{pH})$ a une faible valeur au point isoélectrique. On peut donc en conclure que, théoriquement, la séparation fine des ampholytes de faible poids moléculaire par électrolyse stationnaire est impossible.

D'autre part, les protéines et autres ampholytes de poids moléculaire élevé sont connus comme ayant de faibles constantes de diffusion et des valeurs élevées de la dérivée $du/d(\mathrm{pH})$ au point isoélectrique. Svensson en a conclu qu'il devait être possible de mettre au point le procédé sous forme d'un procédé très sélectif d'analyse et de fractionnement pour les protéines, pourvu que l'on puisse obtenir un gradient de pH stable couvrant les points isoélectriques du système de protéines. Cette dernière condition peut être obtenue en utilisant cer-

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tains types d'ampholytes de bas poids moléculaire qui doivent être présents pendant l'électrolyse. C'est de là que vient la notion d'ampholytes supports. Un ampholyte support est un ampholyte qui, pendant l'électrolyse, conjointement avec d'autres ampholytes semblables, est capable de provoquer un léger gradient de pH entre les électrodes et également un faible gradient de conductibilité. Cette dernière condition est souhaitable pour que l'intensité du champ ne varie pas trop, l'intensité du champ étant une fonction inverse de la conductibilité. Les ampholytes supports permettent de séparer complètement les protéines l'une de l'autre. Les protéines purifiées sont facilement séparées des ampholytes supports, par dialyse ou tout autre procédé reposant sur des différences de dimensions moléculaires. Dans des cas particuliers, il peut bien sûr y avoir d'autres procédés possibles pour la séparation des ampholytes supports.

Dans un second ouvrage (Acta Chem. Scand. *16*, 456 [1962]), Svensson a encore développé les propriétés d'un ampholyte support utile, au-delà de la condition qu'il doit remplir d'être facilement séparable des autres ampholytes. Cette recherche a abouti à une définition plus facilement compréhensible et plus utile de l'ampholyte support. Conformément à cette définition, un ampholyte de bas poids moléculaire est un bon ampholyte support s'il a au moins deux valeurs de pK_s au voisinage de son propre point isoélectrique (pI). Ainsi, l'histidine qui a un pI de 7,6 et les pK_s les plus voisins à 6,10 et 9,18 est un bon ampholyte support, tandis que la glycine avec un pI de 6,1 et des pK_s de 2,35 et 9,78 est un mauvais ampholyte support (comme tous les autres amino-acides neutres). Un inventaire des ampholytes supports de bas poids moléculaire vendu dans le commerce montre qu'il n'y en a pas qui soient isoélectriques entre pH 4 et pH 7 et qu'il y en a seulement deux qui soient isoélectriques entre pH 7 et pH 8, tandis qu'il y en a seulement quelques-uns qui sont isoélectriques au-dessus de pH 8. Certains des ampholytes supports en vente sont très coûteux, ce qui limite fortement leur utilisation dans ce procédé, mais un inconvénient plus grand est le fait que dans certaines gammes de pH il n'y a pas d'ampholyte support disponible dans le commerce.

Dans un troisième ouvrage (Arch. Biochem. Biophys. Supp. *1*, 132, 1962), Svensson montre que la séparation fine des protéines qui sont isolées dans des régions de pH où l'on peut disposer de bons ampholytes supports, est expérimentalement possible conformément aux prévisions théoriques. On a montré que deux protéines ayant des points isoélectriques différant seulement de 0,1 unité de pH, peuvent être totalement séparées l'une de l'autre, résultat qui a été confirmé et amélioré par la présente invention.

Pour la région de pH comprise entre 0 et 3, on n'a pas besoin d'employer de support car on peut obtenir un gradient de pH utile couvrant cette région par électrolyse d'un système d'acides de forces différentes (des acides forts aux acides très faibles). La même chose s'applique à la région de pH comprise entre 11 et 14 qui peut être dominée en utilisant un système de bases de forces différentes. Cependant, la majorité des protéines sont isoélectriques entre pH 3 et pH 11. Pour pouvoir les séparer de façon satisfaisante, il est nécessaire, conformément aux faits ci-dessus, d'avoir un système d'ampholytes supports dont les points isoélectriques sont distribués uniformément entre pH 3 et pH 11. L'analyse et la séparation effective des protéines par ce procédé dépendent entièrement de la possibilité de disposer d'un tel système d'ampholytes supports.

Jusqu'à présent, on a fait face au manque d'ampholytes supports par deux voies fondamentalement différentes, à savoir la synthèse chimique organique d'ampholytes supports individuels, ou bien l'hydrolyse partielle de protéines effectuée de telle manière que le produit d'hydrolyse consiste essentiellement en peptides inférieurs.

Le premier procédé est fortement gêné par le fait que la plupart des ampholytes organiques facilement disponibles ont des valeurs de pK_s se situant dans des régions de pH assez étroites. Ainsi, presque tous les groupes carboxyliques monovalents se dissocient entre pH 4 et pH 5, presque toutes les amines aliphatiques monovalentes entre pH 9 et pH 10, et la plupart des amines aromatiques à environ pH 5, etc. La production par synthèse d'ampholytes supports individuels « sur mesures » ayant des points isoélectriques aux valeurs désirées est très difficile, même pour un chimiste habile. Les essais de synthèse effectués jusqu'à présent n'ont pas eu beaucoup de succès et on peut dire avec certitude que ces produits synthétiques seraient plutôt coûteux.

Le second procédé a eu davantage de succès car les produits de l'hydrolyse partielle de protéines après dessalage contiennent des quantités considérables d'ampholytes supports intéressants. L'hydrolyse partielle des protéines se distingue du procédé de synthèse expliqué ci-dessus en ce qu'elle fournit directement le système d'ampholytes supports qui est nécessaire pour le fractionnement isoélectrique des protéines, ce qui est un avantage car on économise ainsi la nécessité de recristallisations longues et coûteuses et d'autres procédés de purification pour chaque substance chimique. L'hydrolyse partielle des protéines a eu une grande importance dans le développement ultérieur de l'électrolyse stationnaire et a permis un grand nombre de procédés élégants de séparation de protéines.

L'utilisation de l'hydrolyse partielle des protéines

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pour la production d'ampholytes supports n'est pas satisfaisante pour les raisons suivantes. La fraction des produits d'hydrolyse partielle qui est isoélectrique entre pH 5 et pH 6,5, possède de mauvaises propriétés comme ampholytes supports. Ainsi, pendant l'électrolyse, elle a une très faible conductibilité dans cette gamme de pH, avec surchauffe locale, et l'intensité de champ à l'extérieur de cette gamme de pH est trop faible. Un autre inconvénient de l'utilisation de l'hydrolyse partielle est que les produits sont rarement tout à fait incolores. Donc, les ampholytes supports donnent naissance à des zones colorées, ce qui est un inconvénient dans la séparation isoélectrique des protéines colorées (par exemple : hémoglobines, myoglobines).

Ce dernier fait est seulement un aspect d'une propriété qui devrait être très intéressante dans les ampholytes supports mais qui n'a pas été obtenue par les procédés antérieurs. Dans le fractionnement isoélectrique des protéines, il n'est pas suffisant que l'électrolyse soit complète, c'est-à-dire que l'état stationnaire soit atteint; il doit également être possible de recueillir les protéines après l'électrolyse et de mesurer quantitativement la quantité de protéine qui a été accumulée aux différents points entre les électrodes. Pour cette raison, il est très important que les ampholytes supports n'interfèrent pas avec les procédés utilisés pour l'analyse des protéines. Seuls les procédés de coagulation et les réactions spécifiques des protéines (activité enzymatique, activité antigène, activité toxique, fonction d'anticorps, etc.), peuvent être utilisés en présence d'ampholytes supports consistant en peptides. Pour tous les autres types d'analyses des protéines, les peptides doivent d'abord être séparés. Dans la plupart des cas, ceci peut être effectué par dialyse, procédé qui est simple mais a le défaut d'être long. La méthode générale d'analyse des protéines la plus simple et appropriée consiste à mesurer l'extinction à 2 800 Å. On a donc essayé de produire un système d'ampholytes supports qui soit transparent à la lumière à cette longueur d'onde et aux longueurs d'ondes supérieurs. Le procédé d'hydrolyse partiel ne satisfait aucunement cette condition.

Selon la présente invention, on obtient un système d'ampholytes supports consistant en un mélange d'acides amino-carboxylique polyprotiques, chacun contenant au moins quatre groupes protolytiques, l'un au moins étant un groupe carboxylique et l'un au moins un atome d'azote basique, les composés ne contenant pas des liaisons peptidiques. La composition est caractérisée en ce qu'elle contient un certain nombre de composés, c'est-à-dire au moins trois substances chimiques différentes qui diffèrent l'une de l'autre par le nombre de groupes acides et basiques présents ou bien qui sont des isomères ou des homologues, et d'autre part, lorsque chaque substance chimique contient au moins 4 et de préférence, 4 à 10 groupes protolytiques faibles. Une composition préférée selon l'invention ne contient pas d'ampholytes ayant des noyaux aromatiques, mais seulement des substances de caractère aliphatique ou alicyclique. Un tel produit n'absorbe pas la lumière à 2 800 Å ou au-dessus et convient particulièrement bien pour l'utilisation avec des systèmes de protéines que l'on désire analyser par absorption des rayons ultraviolets.

Comme la composition contient un grand nombre d'ampholytes différents ayant des groupes protolytiques faibles, il présentera un spectre très riche de points isoélectriques différents dans la région de pH comprise entre 3 et 11, propriété nécessaire d'un système d'ampholytes supports. En raison du caractère polyprotique des ampholytes individuels, la plupart d'entre eux auront de bonnes propriétés d'ampholytes supports.

Il est bien connu d'après la chimie-physique organique que deux groupes protolytiques dans la même molécule influencent fortement la dissociation de l'un et de l'autre. Cette influence mutuelle a pour effet que l'intervalle des pK_s dans une molécule biprotique est supérieur à ce que l'on pourrait attendre sans cette interaction. C'est l'une des raisons pour lesquelles les tentatives de synthèse d'ampholytes supports biprotiques n'ont pas conduit au résultat désiré. L'effet de répulsion entre les différentes valeurs de pK_s d'un protolype n'est pas limité aux différents groupes protolytiques qui sont présents dans l'ampholyte biprotique; ceci se produit également à un degré égal ou supérieur entre des groupes protolytiques identiques dans la même molécule. Ainsi, il est en théorie impossible que les pK_s d'un acide bivalent ou d'une base bivalente soient rapprochés de moins de 0,6 unité de pH et en général la différence est beaucoup plus grande. Ceci est évident d'après les exemples ci-dessous. Les valeurs de pK_s sont valables à 25 °C.

	pK_1	pK_2	pK_3	pK_4	pK_5
Éthylènediamine	6,85	9,9			
Diéthylènetriamine	4,43	9,13	9,94		
Triéthylènetétramine	3,32	6,67	9,20	9,92	
Tétraéthylènepentamine	2,65	4,25	7,87	9,08	9,92

Avec les composés organiques ayant des groupes amino et/ou imino, on peut obtenir plusieurs homologues et isomères ayant des valeurs de pK_s différentes en introduisant des substituants sur l'atome d'azote. Par exemple, on peut facilement produire différentes éthanolamines ayant des pK_s différents.

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L'influence du groupe éthanol sur le pK_s pour le groupe amino ressort clairement pour la série homologue suivante :

	pK, à 25 °C
Monoéthanolamine	9,5
Diéthanolamine	9,0
Triéthanolamine	7,9

Selon la présente invention, ce fait physico-chimique peut être utilisé en formant un produit dont les molécules ont au moins 4 et de préférence jusqu'à 10 groupes protolytiques faibles. On peut donc obtenir une compression des différentes étapes de dissociation sur l'échelle de pH, et on trouve dans l'ampholyte un couple de valeurs de pK_s au voisinage du point isoélectrique. Il est presque impossible d'atteindre cette condition pour des ampholytes bi- et triprotiques qui sont isoélectriques entre pH 4 et pH 7.

La présente invention vise également les compositions d'ampholytes polyprotiques dont les points isoélectriques sont distribués dans des régions de pH plus étroites que la gamme de pH 3 à pH 11 décrite ci-dessus. Ces préparations, s'étendant de préférence sur des intervalles de seulement 2 à 3 unités de pH, quelquefois même seulement une unité de pH, sont très utiles pour la préparation ultra-fine des protéines ayant des points isoélectriques très voisins, par exemple des systèmes d'anticorps, d'hémoglobines et de myoglobines. Dans l'électrolyse stationnaire d'une composition d'ampholytes ayant des points isoélectriques compris entre pH 6 et pH 8, on obtient un pH anodique d'environ 6 et un pH cathodique d'environ 8 (sans effets secondaires) et par conséquent, le volume de toute la cellule d'électrolyse peut être utilisé pour la séparation dans cette gamme étroite de pH.

Le système d'ampholytes supports décrit ci-dessus satisfait à toutes les conditions concernant un tel système. Il comprend des ampholytes individuels dont les points isoélectriques sont bien dispersés dans la gamme de pH qui ne peut pas être obtenue avec des systèmes d'acides ou des systèmes de bases. Le pouvoir tamponnant et la conductibilité électrique de ces ampholytes supports à l'état isoélectrique sont satisfaisants. Ils sont incolores et présentent une absorption insignifiante de la lumière à 2 800 Å. De même, ils n'affectent aucune des autres méthodes d'analyse habituellement utilisées pour les protéines. Comme il est décrit ci-après, les matières premières pour la production ne sont pas très coûteuses.

Aucun des ampholytes individuels selon l'invention n'est disponible dans le commerce sur le marché des produits chimiques. La présente invention vise également un procédé de production de ces compositions. Le procédé est synthétique mais, comme le procédé d'hydrolyse partiel, il donne directement un système complet d'ampholytes supports. Donc, le procédé selon l'invention ne comporte pas la synthèse de produits chimiques purs et le mélange ultérieur de quantités données de ces produits chimiques car un tel procédé serait beaucoup trop coûteux.

Le mélange d'ampholytes supports selon l'invention est de préférence produit par réaction d'un composé organique de bas poids moléculaire contenant au moins deux groupes amino et/ou imino avec un acide contenant au moins une double liaison carbone/carbone.

La base organique azotée de bas poids moléculaire a anormalement un poids moléculaire de moins de 500 et de préférence moins de 300. Elle peut être substituée sur un ou plusieurs des atomes d'azote par des groupes tels que méthyle, éthyle, éthanolamino ou hydroxyle. Le composé n'a de préférence pas plus de trois atomes de carbone entre deux des atomes d'azote et de préférence seulement deux atomes de carbone. Ainsi, le composé comporte de préférence la structure :

$$- NH - R - NH -$$

dans laquelle R représente un groupe $-(CH_2)_3-$ ou encore mieux :

$$- CH_2 - CH_2 -.$$

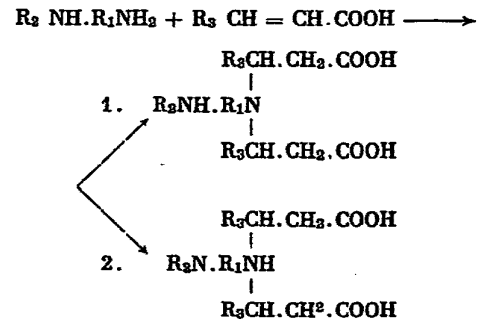
L'amine utilisée doit en outre avoir de préférence un pK_s qui n'est pas inférieur à 8. L'amine est de préférence aliphatique, et les amines particulièrement préférées sont l'éthylènediamine, la diéthylènetriamine, la triéthylènetétramine, la tétraéthylènepentamine et la pentaéthylènehexamine. L'amine utilisée peut également être alicyclique, hétérocyclique, ou contenir des groupes aromatiques. Cependant, dans ce dernier cas, le mélange d'ampholytes supports produit présente souvent une absorption de la lumière dans la zone critique à 2 800 Å. Les amines peuvent avantageusement être utilisées en mélange et dans ce cas le mélange peut contenir également une ou plusieurs monoamines.

L'acide insaturé utilisé dans l'invention contient de préférence seulement une double liaison et de préférence un maximum de dix atomes de carbone. La double liaison doit de préférence être en position alpha, bêta. L'acide est de préférence un acide aliphatique insaturé, un acide acrylique, méthacrylique ou crotonique s'étant révélé des acides appropriés. L'acide peut contenir plus d'un groupe carboxylique. par exemple l'acide maléique et l'acide itaconique.

La réaction entre l'amine et l'acide dans un cas particulier est illustrée par le schéma réactionnel suivant :

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dans lequel R_1 représente un groupe bivalent qui peut contenir un ou plusieurs atomes d'azote, par exemple un groupe alkylène ou $-(CH_2)_n-NH-(CH_2)_n-$, R_2 représente un groupe monovalent tel qu'un atome d'hydrogène ou un groupe alkyle qui peut contenir un ou plusieurs atomes d'azote, par exemple $CH_3-NH-(CH_2)_2-$, et R_3 représente un groupe alkyle ayant de un à dix atomes de carbone. Les réactions 1 et 2 sont lieu simultanément et le nombre de combinaisons possibles augmente avec le nombre d'atomes primaires et/ou secondaires dans l'amine de départ et le nombre de groupes acide carboxylique qui sont ajoutés.

Une base azotée organique peut en outre copuler avec un ou plusieurs acides insaturés, le nombre maximum de molécules d'acide carboxylique par molécule de base azotée correspondant au nombre d'atomes d'hydrogène liés à l'azote dans la base azotée. Par un choix convenable des proportions molaires de la base azotée et de l'acide, on peut obtenir une distribution des produits qui constitue une série homologue avec différents nombres de groupes acide carboxylique et ainsi différentes valeurs de ce pI.

En outre, avec un nombre donné de groupes acides et basiques, il se forme un grand nombre d'isomères, en partie en raison du fait que l'addition peut être faite sur l'un ou l'autre des atomes de carbone de la double liaison dans l'acide insaturé et en partie parce que l'acide peut s'additionner sur les groupes amino et/ ou imino situés dans différentes positions dans la molécule. Ceci est d'une importance essentielle pour que les groupes carboxyliques et amino et/ou imino agissent respectivement les uns sur les autres de telle manière que l'on obtienne des pK_s différents dans les positions différentes. Il est évident que les possibilités d'isomérie augmentent fortement avec le nombre de groupes protolytiques.

La réaction est effectuée dans la plupart des cas avec une quantité d'acide plus faible que celle nécessaire pour saturer tous les atomes d'azote primaires et secondaires de sorte que l'on obtient un mélange convenable de différents acides aminocarboxyliques et de leurs isomères. La réaction entre la polyamine et l'acide carboxylique insaturé s'effectue de préférence à une température élevée et de préférence en présence d'un solvant. La température de réaction est ordinairement comprise entre 30 et 150 °C, de préférence entre 40 et 100 °C. Lorsqu'on utilise des solvants, on effectue souvent la réaction à la température de reflux du solvant. Le solvant peut être l'eau ou un solvant organique, par exemple un alcool ou un hydrocarbure. On peut également utiliser des catalyseurs pendant la réaction, par exemple des bases, des sels d'acides, tels que le phosphate de sodium primaire, le chlorure de zinc et le chlorure d'aluminium. On peut utiliser l'hydroquinone comme inhibiteur de polymérisation de l'acide insaturé.

Les pI des ampholytes formés dans ce procédé se situent généralement entre pH 3 et pH 10. Ceci peut être expliqué partiellement par les différentes positions de ces groupes carboxyliques dans la molécule.

Dans la séparation isoélectrique, on est souvent intéressé à un intervalle particulier de pH selon les points isoélectriques des substances, tels que les protéines, à essayer et à séparer. Le mélange d'ampholytes supports utilisé doit donc couvrir cet intervalle par son spectre de pI et il est donc souvent nécessaire de séparer le mélange d'ampholytes supports produit par le procédé de l'invention suivant les valeurs de pI. Cette séparation peut être effectuée par séparation isoélectrique de préférence dans un dispositif comportant un certain nombre de cellules séparées par un diaphragme évitant la diffusion (par exemple selon Williams et Waterman, référence citée). Le mélange d'ampholytes supports produits selon l'invention et divisé en différents intervalles de pI peut être séché ou cristallisé. Ce procédé de division est également convenable lorsque l'on détermine le rendement en ampholytes supports dans un certain intervalle de valeurs de pI. Lorsqu'on utilise ce procédé, le procédé de la réaction peut être modifié pour obtenir un plus grand partage du rendement total dans une certaine région de pI. Ceci est effectué par exemple en faisant varier les quantités relatives d'amine et d'acide carboxylique la quantité et le type de catalyseur, et le solvant.

Lorsque le mélange d'ampholytes supports produit selon la présente invention a été divisé en intervalles de pI et séché, on peut déterminer son utilité comme mélange comme ampholytes supports en mesurant son pouvoir tampon et sa conductibilité, ces valeurs étant de préférence rapportées à 1 mg de mélange d'ampholytes sec. Les ampholytes produits selon la présente invention sont divisés pendant l'électrolyse de telle manière qu'à l'équilibre, ceux ayant un plus faible pI (qui sont les plus acides) soient situés au voisinage de l'anode et ceux ayant un pI plus élevé s'accumulent au voisinage de la cathode. La distribution à l'équilibre, lorsque chaque ampholyte présente un pic de concentration

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au pI de l'ampholyte, se présente comme des courbes normales de distribution (courbes de Gauss) se recouvrant partiellement l'une l'autre. A l'équilibre, la valeur de pH dans une certaine section du bain d'électrolyse est ainsi à peu près égale au pI des ampholytes qui sont présents dans cette section. Un moyen simple de mesurer le pI de l'ampholyte est donc la mesure du pH.

L'exemple suivant illustre la présente invention sans toutefois en limiter la portée.

Exemple. — Dans un ballon à fond rond muni d'un réfrigérant à reflux, d'un agitateur, d'un thermomètre et d'une ampoule à brome, on charge 100 parties molaires de triéthylènetétramine pure en solution aqueuse à 50 %. On plonge le récipient dans un bain-marie à une température de 50 °C. On ajoute par l'ampoule à brome, par petites portions, une solution aqueuse d'acide acrylique à 50%, purifié au préalable par distillation sous vide et contenant 1 % d'hydroquinone. La quantité totale d'acide acrylique dans les différents essais est de 200, 250, 300 et 350 parties molaires. Les additions sont faites de telle manière que la température dans le ballon ne dépasse pas 65 °C (réaction exothermique). Lorsqu'on a ajouté la totalité de l'acide acrylique, on prélève une fraction de 0,5 ml du mélange réactionnel. On dissout cette fraction dans 10 ml d'eau pour la mesure du pH. Cette solution aqueuse est ensuite acidifiée par l'acide chlorhydrique jusqu'à un pH d'environ 2 et extraite par l'éther. On évapore l'éther et on titre le résidu par une solution de $KMnO_4$ pour donner le nombre de doubles liaisons présentes. Ceci donne une mesure de la quantité d'acide acrylique qui n'a pas réagi. Pour compléter la réaction, on chauffe le ballon en agitant et on le maintient à 80-100 °C pendant 2 à 6 heures jusqu'à ce que le test de l'acide acrylique devienne négatif. Ainsi, on produit un mélange d'ampholytes supports selon la présente invention.

Pour une étude plus détaillée des résultats de la réaction de copulation, on place le mélange d'ampholytes supports dans un appareil d'électrolyse ayant vingt cellules séparées par des diaphragmes. On dissout une quantité du mélange réactionnel correspondant à environ 40 g d'ampholytes supports dans environ 540 ml d'eau distillée et on verse la solution dans l'appareil d'électrolyse. Pour éviter la destruction des ampholytes par oxydation anodique ou réduction cathodique, il est important d'effectuer l'hydrolyse d'une manière telle que les ampholytes soient le moins possible en contact avec l'anode et avec la cathode.

On effectue l'électrolyse sous une tension de 400 à 800 volts correspondant à une intensité moyenne de champ de 20 à 40 volts/cm et on obtient en général l'état d'équilibre en 48 heures, après quoi l'intensité de courant ne change plus si l'on maintient la tension constante.

On vide ensuite simultanément les cellules de l'appareil d'électrolyse dans des récipients individuels. On obtient ainsi un certain nombre de fractions correspondant au nombre de cellules. On mesure le pH des fractions individuelles. Les valeurs de pH correspondent sensiblement au pI des ampholytes. On combine les fractions dont les pH sont dans des intervalles convenables et on évapore à siccité. On dissout ensuite le résidu dans le méthanol ou l'éthanol bouillant. On évapore le solvant et les ampholytes souvent cristallisent.

Si ceci n'a pas lieu, on répète le traitement pour séparer toute l'eau. Les résidus obtenus sont séchés dans un exsiccateur. Le rendement dans les différents intervalles de pH est déterminé par pesée des ampholytes secs. Le pouvoir tamponnant et la conductibilité des ampholytes sont mesurés et rapportés à 1 mg de substance sèche.

On trouve dans les essais que plus de 90 % du rendement total des ampholytes présentent un pI compris entre 3,5 et 10. Les ampholytes obtenus ont ainsi des pI situés dans toute la région intéressante pour le procédé de séparation isoélectrique. Il est également possible de modifier le rendement dans certaines régions de pI.

Le mélange ampholytes supports produit dans cet exemple est pratiquement incolore. Son absorption de la lumière à 2 800-2 850 Å peut être négligée devant l'absorption des protéines.

On peut remplacer la triéthylènetétramine par l'éthylènediamine, la diéthylènetriamine ou la tétraéthylènepentamine. On peut remplacer l'acide acrylique par l'acide méthacrylique, l'acide crotonique, l'acide maléique, l'acide fumarique ou l'acide itaconique.

Selon la présente invention, on obtient un bon rendement d'ampholytes supports intéressant d'une manière simple et peu coûteuse. Les pI de ces ampholytes présentent un spectre qui couvre le domaine utilisé dans le procédé de séparation isoélectrique.

RÉSUMÉ

La présente invention concerne :

I. Un mélange d'ampholytes supports pour l'application dans le fractionnement isoélectrique et l'analyse des ampholytes de poids moléculaire élevé par électrolyse stationnaire, ledit mélange étant caractérisé par les points suivants pris isolément ou en combinaisons diverses :

1° Il comprend un mélange des acides aminocarboxyliques polyprotiques, chaque acide contenant au moins 4 groupes protolytiques dont l'un au moins est un groupe carboxylique et un au moins comporte un atome d'azote basique;

2° Les points isoélectriques des ampholytes se situent dans une gamme étroite de pH;

3° Les ampholytes contiennent des groupes hydroxyles;

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4° Les ampholytes sont des composés non aromatiques.

II. Un procédé pour la préparation d'un mélange d'ampholytes supports tel que spécifié sous I, ledit procédé étant caractérisé par les points suivants considérés isolément ou en combinaisons diverses :

1° On fait réagir un composé organique contenant au moins deux groupes amino ou imino, avec un acide carboxylique contenant au moins une double liaison carbone/carbone;

2° L'acide carboxylique insaturé est un acide mono-carboxylique;

3° L'acide monocarboxylique est l'acide acrylique, l'acide méthacrylique ou l'acide crotonique;

4° L'acide carboxylique insaturé est un acide dicarboxylique;

5° L'acide dicarboxylique est l'acide maléique ou l'acide itaconique :

6° On utilise plus d'un acide carboxylique insaturé;

7° L'acide carboxylique est un acide carboxylique alpha, bêta insaturé;

8° Le composé organique azoté n'a pas plus de trois atomes de carbone entre les atomes d'azote des groupes amino ou imino;

9° Le composé organique azoté est l'éthylènediamine, la diéthylènetriamine, la triéthylènetriamine ou la tétraéthylènepentamine;

10° On fait réagir un mélange contenant plus d'un composé organique azoté avec au moins un acide carboxylique insaturé;

11° Le mélange de composés organiques azotés contient au moins une monoamine primaire ou secondaire;

12° Le composé organique azoté est un composé aliphatique;

13° Le composé organique azoté est un composé alicyclique ou hétérocyclique;

14° On fait réagir le composé organique azoté et l'acide carboxylique insaturé à une température de 30 à 150 °C;

15° On fait réagir le composé organique azoté et l'acide carboxylique insaturé à une température de 40 à 100 °C;

16° La réaction a lieu en présence d'eau ou d'un solvant organique;

17° La réaction a lieu en présence d'un catalyseur;

18° La réaction a lieu en présence d'hydroquinone.

III. L'application d'un mélange d'ampholytes supports préparé par le procédé tel que spécifié sous II, pour le fractionnement isoélectrique et l'analyse des ampholytes de poids moléculaire élevé par électrolyse stationnaire.

IV. A titre de produits industriels nouveaux, les ampholytes de poids moléculaire élevé obtenus par séparation par fractionnement électrique pendant l'électrolyse stationnaire en utilisant un mélange d'ampholytes supports tel que spécifié sous I et III.

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BNSDOCID: <FR 1554045A I >

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 27/447 — A1

(11) International Publication Number: **WO 97/04308**

(43) International Publication Date: 6 February 1997 (06.02.97)

(21) International Application Number: PCT/US96/11973

(22) International Filing Date: 18 July 1996 (18.07.96)

(30) Priority Data:

08/503,462	18 July 1995 (18.07.95)	US
08/585,049	11 January 1996 (11.01.96)	US

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(54) Title: BUFFER/ADDITIVES ELECTROLYTE COMBINATIONS FOR ELECTROKINETIC CHROMATOGRAPHY

(57) Abstract

The invention is an electrolyte comprising a buffer/additive combination for use in electrokinetic chromatography ("EKC"). The EKC buffer/additive combinations of this invention make practical the use of larger diameter capillaries, therefore allowing higher resolution, faster analysis time, and improved detectability compared to buffer/additive combinations currently used in EKC. The invention is a capillary electrophoresis electrolyte for separating analyte components of interest contained in a substantially aqueous sample, comprising a substantially aqueous phase, a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase. The substantially aqueous phase may also include an organic modifier such as methanol or acetonitrile. The protonated additive may be a surfactant, and for chiral separations, a chiral surfactant. The invention may also be used with an achiral surfactant. A preferred embodiment is the chiral surfactant (S)-N-dodecoxycarbonylvaline. The free polyamine refers to an amine molecule having two or more amine moieties, such as a diamine. A preferred embodiment of a diamine is bis-tris propane.

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Buffer/Additives Electrolyte Combinations for Electrokinetic Chromatography

This application is a continuation in part of application Ser. No. 08/503,462, filed July 18, 1995.

1. Field of the Invention

The invention is in the field of electrokinetic chromatography. In particular, the invention relates to improved buffer/additive combinations for electrokinetic chromatography which afford fast, high resolution separations with increased detectability.

2. Description of the Prior Art

Although a relatively new addition to the field of separations techniques, capillary electrophoresis ("CE") is already being used to separate a wide variety of solutes. Separations are performed in thin glass capillaries, as opposed to the 2-dimensional surfaces (such as gels or paper) which have traditionally been used in electrophoresis. CE has the ability to perform fast, high resolution separations in a simple experimental apparatus. Fast, high resolution separations result from the high efficiencies afforded by this technique.

High efficiency is obtained in CE because under ideal conditions, the sole source of band broadening is longitudinal diffusion (*Jorgenson, J.W. and Lukas, K.D.* Analytical Chemistry, 1981, **(53)** 1298-1302). Efficiency in separations systems is commonly expressed as theoretical plates (N). The larger the value of N, the higher the efficiency of the system. In capillary electrophoresis, under conditions where diffusion is the sole source of band broadening, the expression for N was derived by Jorgenson (*Jorgenson, J.W. and Lukas, K.D., supra*):

$$N = \frac{\mu V l}{2DL} \qquad \text{equation 1}$$

where μ is the solute's apparent mobility, V is the applied voltage, l is the length of capillary from injection to detection, D is the solute's diffusion coefficient, and L is the total capillary length.

Equation 1 predicts that by increasing the applied voltage, higher efficiency (and therefore higher resolution) will be obtained. Furthermore, higher voltages cause the solute to move through the capillary at greater velocity, resulting in faster

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separation. Therefore, in theory, increasing the voltage will lead to faster analysis time and higher resolution. However, there is a limit to how high the voltage can be increased before resolution begins to degrade (*Knox, J.H.*, Chromatographia, **26:** 329-333 (1988); *Grushka, E., McCormick, R.M. and Kirkland, J.J.*, Analytical Chemistry **61:** 241-245 (1989) . This limit occurs when the heat which is generated during application of the separation voltage causes thermal gradients in the capillary. These thermal gradients lead to band broadening and loss of resolution. To understand both how thermal gradients are generated and how they generate a mechanism for band broadening and subsequent loss of resolution, we will review the basic theory.

As the voltage increases, the current also increases, as defined by Ohm's Law:

$$V = IR \qquad \text{equation 2}$$

where V is the voltage, I is the current, and R is the electrical resistance of the capillary. Temperature gradients in the capillary are related to the heat generation rate or power density Q (*Nelson, R.J., Paulus, A., Cohen, A.S., Guttman, A. and Karger, B.L.*, Journal of Chromatography, **480**: 111-127 (1989):

$$Q = \frac{VI}{\pi r^2 L} \qquad \text{equation 3}$$

where r is the capillary internal bore radius and L is the total capillary length. Thermal gradients exist when there is a difference in temperature between the buffer in the center of the capillary and the buffer near the wall. Since heat flows from warmer to cooler bodies, the temperature at the capillary wall will be lower because it is in contact with the cooler surrounding environment. Such temperature gradients lead to viscosity gradients within the capillary, with the viscosity being lowest in the center of the tube (where the buffer is warmest). Since the electrophoretic mobility of a solute is inversely proportional to the viscosity of the electrolyte, a solute molecule in the center of the tube will have a higher velocity than a solute molecule near the wall. This non-uniform velocity profile causes band broadening, which is deterimental to overall performance.

For a given electrolyte and capillary length, thermal gradients are proportional to the square of the capillary radius and the square of the applied voltage. To maximize V (and therefore analysis speed and resolution) capillaries with very small internal diameters must be employed (i.e., 1 μm) to increase heat dissipation so that thermal

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gradients are not a substantial factor. However, small diameter capillaries lead to poor detectability when using on-column UV detection. The present state of the art in CE is to use capillaries with internal diameters of 50 or 75 μm to improve detection while keeping the band-broadening effect of the thermal gradients at an acceptable level.

Another way to reduce the deleterious effects of temperature gradients is to use electrolytes with low electrical conductivity (k). The most common electrolytes used in CE are prepared with inorganic salts as the buffering agents, such as sodium phosphate, sodium borate, etc. Because these salts dissociate completely in aqueous solution into highly mobile inorganic ions, the resulting electrolytes have high electrical conductivities. Lower concentrations of the salt can be used, but buffering capacity is reduced. Recently, the use of electrolytes prepared with zwitterionic buffers has gained acceptance in CE (*Weinberger, R.*, "Practical Capillary Electrophoresis", Academic Press, San Diego, CA, Chapter 2, pp. 37-39 (1993)). Such buffers have excellent capacities but low conductivities when used at their pI (where their net charge is zero). Using these buffers, large voltages can be applied for fast separations without the corresponding temperature gradients, band broadening, and subsequent resolution losses afforded by equivalent concentrations of non-zwitterionic buffers.

Electrokinetic chromatography (EKC), believed to be first reported by Terabe, is a subset of capillary *electrophoresis (Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando, T.*, Analytical Chemistry, **56**: 111-113 (1984). In EKC, analytes partition between the bulk aqeous buffer and an additive. EKC electrolytes consist of a buffering agent and an additive which can interact with the solutes. Additives which have been used in EKC include micelles (MEKC or MECC; *Terabe, S. et al., supra*), cyclodextrins (*Terabe, S., Ozaki, H., Otsuka, K. and Ando, T.*, Journal of Chromatography, **332**:211-217 (1985)), polymer ions (*Terabe, S., Isemura, T.*, Analytical Chemistry, **62**: 652-656 (1990)), and proteins (*Yang, J. and Hage, D.S.*, Analytical Chemistry, **66**: 2719-2725 (1994). Resolution of two analytes is achieved in EKC by one or both of the following mechanisms:

1) differences in their mobilities in the bulk aqeous phase (capillary zone electrophoresis), and/or

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2) differences in their partitioning between the bulk aqeous phase and the additive, with the further requirement that the mobility of the analyte-additive complex is different from the mobility of the analyte in the bulk aqeous phase.

The second mechanism requires the existence of a "migration window" in EKC. The migration window is the length of time between the point in time at which a neutral species elutes with no partitioning (t_0), and the elution time of an analyte compound which completely partitions into the additive. For instance, MEKC is usually performed with sodium dodecyl sulfate (SDS) micelles. SDS micelles are anionic and have an electrophoretic mobility towards the anode. Uncoated fused silica capillaries are typically used in MEKC, and a bulk electroosmotic flow toward the cathode is produced at pHs > 2.0. Above pH 6.0, the electroosmotic velocity is usually faster than the electrophoretic velocity of the SDS micelles, causing the micelles to have a net movement toward the cathode. This situation leads to a migration window, which for neutral analytes, is defined by the electroosmotic flow marker (no partitioning) and the micelle marker (complete partitioning). All neutral analytes must migrate between these two boundaries.

The existence of a migration window leads to an additional term in the resolution equation for MEKC compared to the standard resolution equation for chromatography. As developed by Terabe (*Terabe, S., Otsuka, K. and Ando, T.* Analytical Chemistry, 1985, (57) 834-841), the resolution equation for neutral analytes in MEKC is:

$$Rs = \left(\frac{N^{\frac{1}{2}}}{4}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2}{k_2+1}\right)\left(\frac{1-\frac{t_0}{t_{mc}}}{1+\frac{t_0}{t_{mc}}k_1}\right) \qquad \text{equation 4}$$

where N is the theoretical plate count, α is the selectivity term, k_1 and k_2 are the capacity factors for the two analytes, t_o is the electroosmotic flow time, and t_{mc} is the micelle marker time.

The last term in equation 4 is known as the migration window term. Under conditions where the micelle and electroosmotic mobilities are directed toward the same electrode, reduction of the electroosmotic flow will lead to an increase in resolution. Therefore, MEKC electrolytes which lead to low electroosmotic flow are advantageous from a resolution standpoint.

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The majority of MEKC separations have employed sodium dodecyl sulfate as the micelle forming agent. Like other forms of CE, the most common buffering agents are inorganic salts like sodium phosphate and sodium borate. Electrolytes prepared with these buffering agents are desirable from a resolution standpoint, since their high ionic strength reduces electroosmotic flow (Weinberger, R. *supra*). However, the electrolytes have high conductivity due to the high concentrations of mobile inorganic ions. To perform fast, high resolution separations with such electrolytes, capillary internal diameters of 50 and 75 μm are employed. The tradeoff is lower detectability when using on-column UV detection. The use of 100 μm capillaries in MEKC has been shown to lead to detection limits and limits of quantitation ten times lower than with 75 μm capillaries when all other variables (i.e., injection time, voltage, etc.) are kept constant (*Thomas, B.R., Fang, X.G., Chen, X., Tyrrell, R.J. and Ghodbane, S.* Journal of Chromatography, 1994, (657) 383-394). The improvement was attributed to higher injected amount and increased path length with on-column UV detection. However, the improvement in detectability came at the price of some sacrifice in resolution.

MEKC electrolytes using SDS as the surfactant have been prepared with zwitterionic buffering agents such as 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (*Weinberger, R., Sapp, E. and Moring, S.*, Journal of Chromatography, 1990, (516) 271-279). However, there is still a large concentration of sodium present from the SDS. Furthermore, electrolytes prepared with a zwitterionic buffer at a given concentration in MEKC have lower resolution than those prepared with the same concentration of an inorganic salt. The electroosmotic flow is much faster with low ionic strength electrolytes, so the migration window term in the resolution equation is decreased.

The present state of the art of MEKC electrolytes is limited by the tradeoff which exists between good detectability and good separation performance. There is a need for an MEKC electrolyte that would have low conductivity so that large capillary i.d.s could be employed (i.e., 100 μm) for improved detectability and also allow the application of high voltages for fast, high resolution separations. Furthermore, it should give EOF rates similar to those now obtained with high conductivity electrolytes.

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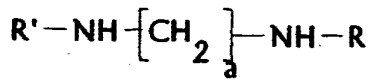
Summary of the Invention

The invention is directed to an electrolyte comprising a buffer/additive combination for use in electrokinetic chromatography ("EKC"). The EKC buffer/additive combinations of this invention make practical the use of larger diameter capillaries, therefor allowing higher resolution, faster analysis time, and improved detectability compared to buffer/additive combinations currently used in EKC.

The invention is also directed to a capillary electrophoresis electrolyte for separating analyte components of interest contained in a substantially aqueous sample, comprising a substantially aqueous phase, a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase. The substantially aqueous phase may also include an organic modifier such as methanol or acetonitrile. The protonated additive may be a surfactant, and for chiral separations, a chiral surfactant. The invention may also be used with an achiral surfactant. A preferred embodiment is the chiral surfactant (S)-N-dodecoxycarbonylvaline. The free polyamine refers to an amine molecule having two or more amine moieties, such as a diamine. A preferred embodiment of a diamine is bis-tris propane.

The invention is also directed to a method for separating analyte components of interest contained in a substantially aqueous sample comprising the step of contacting the sample with an effective amount of a combination of a protonated additive and a free amine, under electrokinetic chromatographic conditions, the combination comprising a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase.

The invention is also directed to a capillary electrophoresis electrolyte for separating analyte components of interest contained in a substantially aqueous sample, comprising a substantially aqueous phase, a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase, the free polyamine having the following structural formula:



wherein

a is from about 1 to 8 methylenes; and

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R and R' are the same or different and may be alkyl, alkenyly or alkynyl substituents, branched or straight chain, substituted or unsubstituted, having from about 1 to 8 carbons, and may have one or more hydrophilic moities such as hydroxy, sulfhydryl, or amine.

Therefore, it is an object of the invention to provide a low-conductivity buffer useful in electrokinetic chromatography.

It is another object of the invention to enhance detectability by making practical the use of larger diameter capillary columns for electrokinetic chromatography.

These and other objects and advantages of the invention will become apparent in the following detailed description of the preferred embodiments, in conjunction with the drawings.

Brief Description of the Drawings

FIG. 1 is the chemical structure of (S)-N-dodecoxycarbonylvaline, the surfactant used for the MEKC separations.

FIG. 2 are chromatograms showing the separation of 1% (-)-ephedrine in the presence of (+)-ephedrine using 25 mM (S)-N-dodecoxycarbonylvaline and 25 mM Na_2HPO_4/25 mM $Na_2B_4O_7$, pH 8.5, in a 100 μm i.d. capillary, with hydrostatic injection times of 2 and 5 seconds.

FIG. 3 are chromatograms showing the separation of 1% (-)-ephedrine in the presence of (+)-ephedrine using 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM bis-tris propane, pH 8.5, in a 100 μm i.d. capillary, with hydrostatic injection times of 2 and 5 seconds.

FIG. 4 is a chromatogram showing the separation of 0.1% (-)-ephedrine in the presence of (+)-ephedrine using 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM bis-tris propane, pH 8.5, in a 100 μm i.d. capillary.

FIG. 5 is the chemical structure of bis-tris propane.

FIG. 6A is a chromatogram showing the separation of norephedrine enantiomers using 25 mM (S)-N-dodecoxycarbonylvaline and 25 mM Na_2HPO_4/25 mM $Na_2B_4O_7$, pH 8.5, in a 50 μm i.d. capillary with 15 kV applied voltage.

FIG. 6B is a chromatogram showing the separation of norephedrine enantiomers using 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM bis-tris propane, pH 8.5, in a 50 μm i.d. capillary with 15 kV applied voltage.

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FIG. 7 is a chromatogram showing the separation of norephedrine enantiomers using 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM bis-tris propane, pH 8.5, in a 50 μm i.d. capillary with 30 kV applied voltage.

FIG. 8 are the chemical structures of polyamines other than bis-tris propane demonstrated to be useful in this invention.

FIG. 9 is a chromatogram showing the baseline separation of metoprolol enantiomers and was obtaiend using an electrolyte containing 25 mM (S)-N-dodecoxycarbonylvaline and 25 mM N,N'-bis(2-aminoethyl)-1,3-propanediamine.

FIG. 10 is a chromatogram showing the baseline separation of benzoin enantiomers and was obtained using an electrolyte containing 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM pentrol.

FIG. 11 is a chromatogram showing the baseline separation of norphenylephrine enantiomers and was obtained using an electrolyte containing 25 mM (S)-N-dodecoxycarbonylvaline and 15 mM pentaethylenehexamine.

FIG 12 is a chromatogram of the baseline separation of metoprolol enantiomers and was obtained using an electrolyte containing 25 mM (S)-N-dodecoxycarbonylvaline and 20 mM 4,7,10-trioxa-1,13-tridecanediamine.

FIGS. 13A-E are the chemical structures of the additives, which include two anionic cyclodextrin derivatives (carboxymethylated-β-cyclodextrin and succinylated-β-cyclodextrin), a chiral crown ether (18-crown-6-tetracarboxylic acid), and two achiral surfactants (lauric acid and dodecyl sulfate).

FIG. 14A is a chromatogram of the separation of the enantiomers of verapamil and was obtained using an electrolyte containing 0.1% (w/v) carboxymethyl-β-cyclodextrin and 25 mM bis-tris propane.

FIG. 14B is a chromatogram of the separation of the enantiomers of verapamil and was obtained using an electrolyte containing 0.1% (w/v) carboxymethyl-β-cyclodextrin and 25 mM sodium tetraborate.

FIG. 15A is a chromatogram of the separation of the enantiomers of verapamil and was obtained using an electrolyte containing 0.1% (w/v) succinylated-β-cyclodextrin and 25 mM bis-tris propane.

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FIG. 15B is a chromatogram of the attempted separation of the enantiomers of verapamil and was obtained using an electrolyte containing 0.1% (w/v) succinylated-β-cyclodextrin and 25 mM sodium tetraborate.

FIG. 16A is a chromatogram of the separation of the enantiomers of methylbenzylamine, resolution of 1.5 was obtained using an electrolyte containing 5 mM 18-crown-6-tetracarboxylic acid and 25 mM bis-tris propane.

FIG. 16B is a chromatogram of the attempted separation of the enantiomers of methylbenzylamine, and was obtained using an electrolyte containing 5 mM 18-crown-6-tetracarboxylic acid and 25 mM sodium tetraborate.

FIG. 17 is a chromatogram showing the baseline separation of the diastereomers of nadolol using an electrolyte containing 25 mM lauric acid and 50 mM bis-tris propane, pH adjusted to 8.5 with phosphoric acid.

FIG. 18 is a chromatogram that shows no separation for the diastereomers of nadolol, and was obtained with an electrolyte containing 25 mM lauric acid, 25 mM disodium phosphate/25 mM sodium tetraborate, pH 8.5 with phosphoric acid.

FIG. 19 is a chromatogram showing the separation of eleven priority pollutant phenols using an electrolyte containing 43 mM protonated dodecylsulfate and 43 mM bis-tris propane, pH 8.6 (unadjusted).

FIG. 20 is a chromatogram showing the separation of the same sample using an electrolyte containing 43 mM sodium dodecylsulfate and 21.5 mM disodium phosphate/21.5 mM sodium tetraborate, pH adjusted to 8.6 with phosphoric acid.

Detailed Description of the Invention

In the present invention, electrolytes are prepared with anionic EKC additives in the protonated form (as opposed to the conjugate base salt form) and free polyamino (as opposed to the conjugate acid salt form) compounds as the buffering agent. These EKC buffer/additive combinations are characterized by low conductivity, since there are no highly mobile inorganic ions present. Furthermore, the electroosmotic flow is low since these free polyamines greatly reduce it. Surprisingly, separations can be performed at higher voltages in larger internal diameter capillary columns than are typically used in EKC. Higher resolution is obtained because of the higher voltages and lower electroosmotic flow, *and* greatly improved detectability is realized because of the capillary column's larger internal diameter.

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Figure 1 is a structural formula depiction of a preferred embodiment of the invention, the anionic chiral surfactant additive class which was used in some of the separations which follow, specifically (S)-N-dodecoxycarbonylyvaline, described further in US Pat. App. Ser. No. 08/124,681, filed Sept. 20, 1993, and PCT/US94/10655, filed Sept. 20, 1994, both of which are incorporated herein by reference. This surfactant contains a carboxylate head group and is fully anionic at pHs above 6.5. The surfactant is used in its protonated form, and is available from Waters Corporation, 34 Maple St., Milford, MA 01757. A short list of other chiral surfactants disclosed in both applications cited immediately above includes, but is not limited to: (S)-2-[(1-oxododecoxy)amino]-3-methyl-1-sulfooxybutane; (R)-N-dodecoxycarbonylvaline; (S)-N-dodecoxycarbonyl-tert-leucine; (S)-N-tetradecoxycarbonylvaline; and (S)-N-dodecoxycarbonylphenylglycine; (S)-N-dodecoxycarbonylserine, (S)-N-dodecoxycarbonylalanine, (S)-N-dodecoxycarbonylleucine, and (S)-N-dodecoxycarbonylproline.

However, the electrolyte combination of protonated additive and buffer is not limited to chiral surfactants as additives, but may also be used in combination with other protonated EKC additives such as carboxylic and sulfonic acid derivatives of: cyclodextrin; macrocyclic antibiotics (e.g. vancomycin); achiral and chiral synthetic surfactants (examples given above); achiral and chiral polymers; and biomolecules (e.g. amino acids, peptides, proteins, oligosaccharides, nucleic acids, oligonucleotides, etc.).

In examples 8-11, polyamines other than bis-tris propane (structures given in Figure 8) were used in combination with (S)-N-dodecoxycarbonylvaline to resolve the enantiomers of several chiral compounds with low EOF and wattage. These examples illustrate that electrolytes with low EOF and wattage can be obtained with (S)-N-dodecoxycarbonylvaline in combination with a variety of polyamines, i.e., the invention is not limited to bis-tris propane. It should be apparent to those skilled in the art that improved detectability would be obtained when using the surfactant with these polyamines vs. inorganic buffers.

Specifically with reference to the additive/polyamine buffer combination of a diamine and a chiral surfactant, their structures are disclosed in Figures 1 and 5. Fig. 1 is the structural formula of the protonated chiral surfactant (S)-N-dodecoxycarbonylvaline. In solution the chiral surfactant will form micelles above its

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critical micellar concentration (cmc), the outer periphery of the micelle presenting the anionic portion of the head group to the aqueous environment. The charged anionic head group of the surfactant will give up its proton to a more basic amine, and so will be present in its charged form. In a preferred embodiment, the polyamine used herein is bis-tris propane (Fig. 5), a diamine buffer that is available as the free amine. The pKas of the two amine moieties of the diamine are 6.8 and 9.0, respectively. It has good buffering capacity over the pH range of 6.3-9.5. When dissolved in water, bis-tris propane causes the pH to increase since it is a base. The additive surfactant, which is in the acid form, donates its proton to the solution, causing the pH of the solution to decrease. It is hypothesized that a complex forms comprising the negatively-charged anionic surfactant and the positively charged polyamine. By choosing the proper concentration ratio of bis-tris propane to (S)-N-dodecoxycarbonylvaline, which is well within the skill of one of ordinary skill in the art, an electrolyte pH which is within the buffering range of bis-tris propane can be achieved. The conductivity of the resulting electrolyte is lower than that afforded by electrolytes prepared with inorganic buffers, because there is no contribution from the inorganic counterions prevalent in salts, such as sodium or chloride. It was surprising to find that the electrolyte used in the chromatograms of Figures 3 and 4, despite the adjustment of pH with phosphoric acid, could be used at high field strength in 100 μm capillaries with minimal joule heat generated.

Another benefit of bis-tris propane when used with protonated additives in EKC is that the electroosmotic flow ("EOF") is reduced, and it has been shown previously that diamino compounds alone reduce the electroosmotic flow in untreated fused silica capillaries (Landers, J.P., Oda, R.P., Madden, B.J. and Spelsberg, T.C. Analytical Biochemistry, 1992, (205) 115-124; Song, L., Ou, Q. and Yu, W. Journal of Chromatography, 1993, (657) 175-183). However, it is surprising that detection limits can be significantly reduced (a full order of magnitude) as a result of both the low conductivity afforded by these buffer/additive combinations, and as well as the increase in resolution due to the EOF reduction. Both factors contribute to the ability to use greater quantities of analyte for improved sensitivity. This simultaneous improvement in 1) resolution and 2) lowered conductivity results in a synergistic improvement in sensitivity, demonstrated herein.

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To demonstrate that the advantages of polyamine buffers in EKC are not limited to (S)-N-dodecoxycarbonylvaline, bis-tris propane was used in combination with several other EKC additives. The resulting electrolytes were compared to ones generated with the same additive in combination with inorganic buffers, and are shown in Examples 12-16. Note that for all additives the electrolyte prepared with bis-tris propane offered superior resolution and substantially lower wattage. The additives include two anionic cyclodextrin derivatives (carboxymethylated-β-cyclodextrin and succinylated-β-cyclodextrin, examples 12-13), a chiral crown ether (18-crown-6-tetracarboxylic acid, example 14), and two achiral surfactants (lauric acid and dodecyl sulfate, examples 15-16). All of these anionic additives, with the exception of dodecyl sulfate, are commercially available in the protonated form.. Chemical structures of the additives are given in Figure 13. At the end of this section is a table summarizing the results for each additive with bis-tris propane and the inorganic buffer.

Other polyamine compounds may also come within the spirit and scope of this invention. For instance, many amines are available commercially that could be used to gain the effect demonstrated herein. Such compounds include, but are not limited to: ethylenediamine; 1,3-diaminopropane; 1,2-diaminopropane; 1,4-diaminobutane; 1,2-diamino-2-methylpropane; (+/-)-1,3-diaminopentane; 1,5-diaminopentane; 2,2-dimethyl-1,3-propanediamine; 1,6 -hexanediamine; 2-methyl-1,5-pentanediamine; 1,7-diaminoheptane; 1,8-diaminoctane; 1,9-diaminononane; 1,10-diaminodecane; 1,12-diaminododecane; n-methylethylenediamine; n-ethylethylenediamine; n-propylethylenediamine; n-isopropylethylenediamine; n,n-dimethylethylenediamine; n,n-diethylethylenediamine; n,n-diisopropylethylene diamine; n,n-dibutylethylenediamine; n,n,n-trimethylethylenediamine; n,n-dimethyl-n'-ethylethylenediamine; n,n-diethyl-n'-methylethylenediamine; n,n,n-triethylethylenediamine; n,n,n,n-tetramethylethylenediamine; n,n,n,n-tetraethylethylenediamine; n-methyl-1,3-propanediamine; n-propyl-1,3-propanediamine; n-isopropyl-1-3-propanediamine; 3-dimethylaminopropylamine; 3-diethylaminopropylamine; 3-(dibutylamino)propylamine; n,n'-dimethyl-1,3-propanediamine; n,n'-diethyl-1,3-propanediamine; n,n'-diisopropyl-1,2-propanediamine; n,n,n'-trimethyl-1,3-propanediamine; n,n,n,'n'-tretramethyl-1,3-propanediamine; n,n,n,'n'-tetraethyl-1,3-propanediamine; n,n,n,'n'-tetramethyl-1,3-butanediamine; n,n, 2,2-tetramethyl-1,3-

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propanediamine; n,n,n,'n'-tetramethyl-1,4-butanediamine; 2-amino-5-diethylaminopentane; n,n'-dimethyl-1-6-hexanediamine; n,n,n,'n'-tetramethyl-1,6-hexanediamine; tris(dimethylamino)methane; diethylenetriamine; n,n,n,'n,'n'-pentamethyldiethylenetriamine; n-(2-aminoethyl)-1,3-propanediamine; 3,3'-diamino-n-methyldipropylamine; 3,3'-iminobispropylamine; 3,3'-iminobis(n,n-dimethylpropylamine); spermidine; bis(hexamethylene)triamine; triethylenetetramine; 1,1,4,7,10,10-hexamethyltriethylenetetramine; n,n'-bis(3-aminopropyl)-ethylenediamine; n,n'-bis(2-aminoethyl)-1,3-propanediamine; n,n'-bis(3-aminopropyl)-1,3-propanediamine; spermine; tris(2-aminoethyl)amine; tetraethylenepentamine; pentaethylenehexamine; 5-amino-2,2,4-trimethyl-1-cyclopentanemethylamine mixture of isomers; 4,4'-methylenebis(cyclohexylamine); 4,4'-methylenebis(2-methylcyclohexylamine); 1,2-diaminocyclohexane; cis-1,2-diaminocyclohexane; (+/-) trans-1-2-diaminocyclohexane; (1S,2S)-(+)1,2-diaminocyclohexane; (1R,2R)-(-)-1,2-diaminocyclohexane; trans-1,4-diaminocyclohexane; 1,3-cyclohexanebis-(methylamine); n-cyclohexyl-1-3-propanediamine; 1,8-diamino-p-menthane; n,n'-diethyl-2-butene-1,4-diamine; n,n,n',n'-tetramethyl-2-butene-1,4-diamine; tetrakis(dimethylamino)-ethylene; 2,2'-oxybis(ethylamine); 4,9-dioxa-1,12-dodecanediamine; 4,7,10-trioxa-1, 13-tridecanediamine; 1,3-diamino-2-hydroxypropane; 2-(2-aminoethylamino)ethanol;1,3-bis(dimethylamino)-2-propanol; n,n'-bis(2-hydroxyethyl)ethylenediamine; n,n,n,'n'-tetrakis(2-hydroxy-propyl)ethylenediamine; pentrol; 2-(aminomethyl)-1-ethylpyrrolidine; 2-(2-aminoethyl)-1-methylpyrrolidine; (S)-(+)-1,(2-pyrrolidinylmethyl)pyrrolidine; dipiperidinomethane; 1,1'-methylenebis(3-methylpiperidine); 4-piperidinopiperidine; 4,4'-ethylenedipiperidine 4.4'-trimethylenedipiperidine; 4,4'-trimethylenebis(1-methylpiperidine); 4,4'-trimethylenebis(1-piperidineethanol); 1-(2-aminoethyl)-piperidine; 1-(3-aminopropyl)-2-pipecoline; 1-methyl-4-(methylamino) piperidine; 4-(aminomethyl) piperidine; 4-amino-2,2,6,6-tetramethylpiperidine; 4-dimethylamino-2-2,6,6-tetramethylpiperidine; 2-methyl-2-imidazoline; 4,4-dimethyl-2-imidazoline; 2-methylthio-2-imidazoline; piperazine; 1-methylpiperazine; 1,4-dimethylpiperazine; 2-methylpiperazine; 2,6-dimethylpiperazine; trans-2,5-dimethylpiperazine; 4-(dimethylamino)-1,2,2,6,6-pentamethylpiperidine; 1-(3-chloropropyl)piperazine; 1-(2-hydroxyethyl)piperazine; 1,4-bis(2-hydroxyethyl)piperazine; 1-(2-(2-

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hydroxyethoxy)ethyl) piperazine; 1-amino-4-methylpiperazine; 1-amino-4-(2-hydroxyethyl)piperazine; 1-(2aminoethyl)piperazine; 1,4-bis(3-aminopropyl)-piperazine hexetidine; mixture of stereoisomers, 1,4,5,6-tetrahydropyrimidine; homopiperazine; 1,3,5-trimethylhexahydro-1,3,5-trizaine; 1,3,5-triethylhexahydro-1,3,5-triazine; 1,4,7-triazacyclononane; 1,4,7-trimethyl-1,4,7-trizacyclononane; 1,5,9-triazacyclododecane; 1,5,9-trimethyl-1,5,9-triazacyclododododecane; cyclen; 1,4,8,11-tetraazacyclotetradecane; 1,4,8,11-tetramethyl-1,4,11-tetraazacyclotetradecane; 1,4,8,12-tetraazacyclopentadecane; hexacyclen trisulfate; 1,4,7,10,13,16-hexamethyl-1,4,7,10,13,16-hexaazacyclooctadecane; 1,4-diazabicyclo(2.2.2)octane; Dabco 33-LV; (-)sparteine sulfate pentahydrate; 1,3,4,6,7,8-hexahydro-2h-pyrimido(1,2-a)pyrimidine; 1,3,4,6,7,8-hexahydro-1-methyl-2H-pyrmidido(1,2-A)-pyrimidine; hexamethylenetetramine; 4-(2-aminoethyl)morpholine; 4-(2-dimethylamino)ethyl)morpholine; 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane; 4,7,13,18-tetraoxa-1,10-diazabicyclo(8,5,5)eicosane; 4,7,13,16,21,22-pentaoxa-1,10-diazabicyclo(8,8,5)-tricosane; and 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo(8,8,8)-hexacosane.

Having now generally described this invention, the same will become better understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified. All US patents and patent applications cited herein are fully incorporated by reference.

Examples

Experimental Apparatus and Conditions for Examples: Capillary electrophoretic separations were performed with a Waters Quanta® 4000E CE unit, Waters Corporation, Milford, MA. Separations were performed in fused silica capillaries, the dimensions specified in the specific examples. Likewise, the magnitude of the voltages are also described in the specific examples. Injection was achieved by raising the inlet end of the capillary immersed in the sample solution to a height of 10 cm above the outlet end for either 2 or 5 seconds. On-column UV detection was performed at 214 nm. Data collection was achieved with Waters Millennium® software v.2.1(Waters Corporation, Milford, MA).

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Example 1. Separation of 99% (+) and 1% (-) Ephedrines in normal buffer, 2-second injection. Figure 2 shows the separation of 1% (-)-ephedrine in the presence of (+)-ephedrine with 25 mM (S)-N-dodecoxycarbonylvaline and common MEKC buffering agents, 25 mM Na_2HPO_4/25 mM $Na_2B_4O_7$. The pH of 25 mM Na_2HPO_4/25 mM $Na_2B_4O_7$ is 9.5 in the absence of the surfactant. However, because the surfactant is in the protonated form, when it is dissolved in the electrolyte, the pH drops to 8.8. The electrolyte pH is then adjusted to 8.5 with 1.4 M phosphoric acid. The separation was performed in a 60 cm x 100 μm i.d. capillary with a voltage of +15,000 volts. The current was 275 μA. The sample was 0.01 mg/mL (-)-ephedrine and 0.99 mg/mL (+)-ephedrine in water.

In the top separation, a hydrostatic injection time of 2 seconds was employed. The resolution was 1.3. The signal for the minor peak was 0.5 mAU, while the noise, measured as peak-to-peak from 9.0 to 9.5 minutes, was 0.25 mAU. Therefore, the S/N was 2, which is generally defined as the detection limit.

Example 2. Separation of 99% (+) and 1% (-) Ephedrines in normal buffer, 5-second injection. To increase detectability, a 5 second hydrostatic injection time was employed. This separation is shown in the bottom of Figure 2. Expectedly, resolution was not achieved at this higher sample load.

The poor resolution and high background noise (0.25 mAU) for both examples 1 and 2 with the surfactant/phosphate/borate combination is due to the fact that the power generated under these experimental conditions was 6.9 W/m. It has been recommended that power levels be kept below 1 W/m to minimize band broadening due to Joule heating (Sepaniak, M.J. and Cole, R.O. Analytical Chemistry, 1987, (59) 472-477). Therefore, a power level of 6.9 W/m would be expected to decrease resolution. Furthermore, with forced air convection and a power level of 6.9 W/m, the temperature inside the capillary has been calculated to be 35°C above the temperature outside the capillary (25°C in this case) (Nelson, R.J. et al. *supra*). At a temperature of 60 °C, the electrolyte may outgas, evaporate, and/or degrade, which would explain the high noise level. Furthermore, reproducibility would suffer due to electrolyte evaporation and degradation.

Example 3: Separation of 99% (+) and 1% (-) Ephedrines with bis-tris propane buffer, 2-second injection. Figure 3 shows the separation of 1% (-)-

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ephedrine in the presence of (+)-ephedrine with 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM bis-tris propane (see Fig. 5), a biological buffer available from Aldrich Chemical Co. Inc., 1001 W. St. Paul Ave., Milwaukee, WI 53233. In the absence of surfactant, the pH of 50 mM bis-tris propane is 10.6. When the electrolyte is made 25 mM in the surfactant, the pH drops to 9.3. The electrolyte pH was then adjusted to 8.5 with 1.4 M phosphoric acid. The separation was performed in a 60 cm x 100 μm i.d. capillary with a voltage of +15,000 volts. The current was 35 μA.

The top separation in Figure 3 employed a 2 second hydrostatic injection. The resolution was 3.5. The signal for the minor peak was 0.5 mAU, while the noise, measured as peak-to-peak from 19.0 to 19.5 minutes, was 0.07 mAU. The S/N was 7.1, 3X the S/N obtained with the phosphate/borate buffer (Figure 2, top separation). The improved S/N is due to the decreased noise with the bis-tris propane buffer (0.07 mAu vs. 0.25 mAU). The power level with the bis-tris propane buffer was 0.9 W/m, below the recommended maximum of 1 W/m. Furthermore, the temperature rise above ambient was calculated to be 4.5 °C at a power level of 0.9 W/m with forced air convection (Nelson, R.J. *supra*). The lower power level affords higher resolution and better detectability.

Example 4. Separation of 99% (+) and 1% (-) Ephedrines in bis-tris propane buffer, 5-second injection. The bottom separation in Figure 3 employed a 5 second hydrostatic injection. Note that sufficient resolution (Rs = 1.4) is maintained while the S/N increases to 18.6. The higher resolution obtained with the surfactant/bis-tris propane combination allows more sample to be injected for better detectability while still maintaining sufficient resolution.

Example 5. Separation of 99.9% (+) and 0.1% (-) Ephedrines in bis-tris propane buffer, 5-second injection. This point is further illustrated in Figure 4, where a 0.1% level of (-)-ephedrine can be detected with a 5 second hydrostatic injection. In this case, the S/N was 2.2. Due to a lower noise level and higher resolution, the surfactant/bis-tris propane combination offers an order of magnitude decrease in detection limit compared to the one buffered with an equivalent concentration of phosphate/borate (0.1% vs. 1.0%).

Examples 6-7. Separation of racemic (+/-) Norephedrines in both buffers. Figures 6A and 6B show the separation of norephedrine enantiomers using 25 mM (S)-

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N-dodecoxycarbonylvaline and 25 mM Na_2HPO_4/25 mM $Na_2B_4O_7$ (Fig. 6A), and the identical separation using 50 mM bis-tris propane buffer (Fig. 6B), both at pH 8.5. The voltage was 15 kV, the capillary 50 μm i.d. x 60 cm, the hydrostatic injection time 2 seconds, and the sample 0.4 mg/mL racemic norephedrine dissolved in electrolyte.

With phosphate/borate (Fig. 6A), the current was 45 μA, resulting in a power level of 1.1 W/m. The resolution was 3.0 and the electroosmotic mobility was 5.7×10^{-4} cm^2/Vs. With bis-tris propane (Fig. 6B), the current was 10.5 μA, resulting in a power level of 0.3 W/m. The resolution was 3.9 and the electroosmotic mobility was 3.6×10^{-4} cm^2/Vs. Higher resolution was obtained with the surfactant/bis-tris propane combination due to the lower electroosmotic flow. Furthermore, because the conductivity of the surfactant/bis-tris propane combination is low, the voltage can be increased to 30 kV with the wattage only increasing to 1.1 W/m (22 μA). Analysis time is decreased from 19.8 minutes to 9.1 minutes, while the resolution actually increases to 4.0 (Figure 7). When the voltage is increased to 30 kV with the surfactant/phosphate/borate combination, the wattage increases to 7.1 W/m (142 μA) and the resolution decreases to 2.0.

Table 1 summarizes the data generated from the above examples.

TABLE 1

Separation of Ephedrine Enantiomers Using Two Surfactant/Buffer Combinations

Buffer Type	Buffer Conc. (mM)	Surfactant Conc. (mM)	Injection Time (Sec)	Ephedrine Ratio (+/-)	Power Level (w/m)	S/N (Minor Peak)	Resolution
Phosphate/ Borate	50	25	2	99/1	6.9	2	1.3
Phosphate/ Borate	50	25	5	99/1	6.9	.....	0
Bis-Tris Propane	50	25	2	99/1	0.9	7.1	3.5
Bis-Tris Propane	50	25	5	99/1	0.9	18.6	1.4
Bis-Tris Propane	50	25	5	99.9 / 0.1	0.9	2.2	1.4

The detection limit is a full order of magnitude lower for the invention (see last row,

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S/N 2.2 for 0.1% ephedrine in bis-tris propane buffer, vs. top row, S/N 2.0 for 1% ephedrine in phosphate/borate buffer) than for the present state-of-the-art.

Example 8: (S)-N-Dodecoxycarbonylvaline with N,N'-bis(2-aminoethyl)-1,3-propanediamine.

The electrolyte contained 25 mM (S)-N-dodecoxycarbonylvaline and 25 mM N,N'-bis(2-aminoethyl)-1,3-propanediamine, pH 9.6 (unadjusted). The capillary was 75 μm i.d. x 35 cm length. With an applied voltage of +20 kV, the current was 27 μA, for a power level of 2 W/m. The electroosmotic flow was 1.9×10^{-4} cm^2/Vs, a three to four-fold reduction in the normal EOF at this pH. These conditions permitted the baseline separation of metoprolol enantiomers in 7 minutes, Figure 9.

Example 9: (S)-N-Dodecoxycarbonylvaline with pentrol.

The electrolyte contained 25 mM (S)-N-dodecoxycarbonylvaline and 50 mM pentrol, pH 8.0 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +30 kV, the current was 25 μA, for a power level of 1.25 W/m. The electroosmotic flow was 2.1×10^{-4} cm^2/Vs, a three to four-fold reduction in the normal EOF at this pH. These conditions permitted the baseline separation of benzoin enantiomers, Figure 10.

Example 10: (S)-N-Dodecoxycarbonylvaline with pentaethylenhexamine.

The electrolyte contained 25 mM (S)-N-dodecoxycarbonylvaline and 15 mM pentaethylenehexamine, pH 9.6 (unadjusted). The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +30 kV, the current was 6 μA, for a power level of 0.3 W/m. The electroosmotic flow was 3.8×10^{-4} cm^2/Vs, a two-fold reduction in the normal EOF at this pH. Note that this reduction was achieved with only 15 mM of this hexaaamine, and that the current generated was extremely low. These conditions permitted the baseline separation of norphenylephrine enantiomers in 6 minutes, Figure 11.

Example 11: (S)-N-Dodecoxycarbonylvaline with 4,7,10-trioxa-1,13-tridecanediamine.

The electrolyte contained 25 mM (S)-N-dodecoxycarbonylvaline and 20 mM 4,7,10-trioxa-1,13-tridecanediamine, pH 9.5 (unadjusted). The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +30 kV, the current was 10 μA, for a power level of 0.5 W/m. The electroosmotic flow was 3.4×10^{-4} cm^2/Vs, a two-fold

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reduction in the normal EOF at this pH. Note that this reduction was achieved with only 20 mM of this diamine, and that the current generated was extremely low. These conditions permitted the baseline separation of metoprolol enantiomers, Figure 12.

Example 12: Carboxymethyl-β-cyclodextrin with bis-tris propane vs. sodium tetraborate.

The top separation (Figure 14A) was obtained using an electrolyte containing 0.1% (w/v) carboxymethyl-β-cyclodextrin and 25 mM bis-tris propane, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 25 μA, for a power level of 1.0 W/m. The electroosmotic flow was 4.4 x 10^{-4} cm^2/Vs. The enantiomers of verapamil were separated with a resolution of 1.6. The bottom separation (Figure 14B) was obtained using an electrolyte containing 0.1% (w/v) carboxymethyl-β-cyclodextrin and 25 mM sodium tetraborate, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 140 μA, for a power level of 5.8 W/m. The electroosmotic flow was 8.2 x 10^{-4} cm^2/Vs. The enantiomers of verapamil were separated with a resolution of only 0.2, and were not fully resolved from the EOF marker. Therefore, the electrolyte containing bis-tris propane offered an increase in resolution by a factor of eight (due to lower EOF and wattage) while decreasing the power level six-fold.

Example 13: Succinylated-β-cyclodextrin with bis-tris propane vs. sodium tetraborate.

The top separation (Figure 15A) was obtained using an electrolyte containing 0.1% (w/v) succinylated-β-cyclodextrin and 25 mM bis-tris propane, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +30 kV, the current was 20 μA, for a power level of 1.0 W/m. The electroosmotic flow was 4.4 x 10^{-4} cm^2/Vs. The enantiomers of verapamil were separated with a resolution of 3.0. The bottom separation (Figure 15B) was obtained using an electrolyte containing 0.1% (w/v) succinylated-β-cyclodextrin and 25 mM sodium tetraborate, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +30 kV, the current was 195 μA, for a power level of 9.8 W/m. The electroosmotic flow was 8.3 x 10^{-4} cm^2/Vs. The enantiomers of verapamil were separated with a resolution of only 0.4. Therefore, the electrolyte

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containing bis-tris propane offered an increase in resolution by a factor of seven (due to lower EOF and wattage) while decreasing the power level ten-fold.

Example 14: 18-Crown-6-tetracarboxylic acid with bis-tris propane vs. sodium tetraborate.

The top separation (Figure 16A) was obtained using an electrolyte containing 5 mM 18-crown-6-tetracarboxylic acid and 25 mM bis-tris propane, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 26 μA, for a power level of 1.1 W/m. The electroosmotic flow was 4.2 x 10^{-4} cm^2/Vs. The enantiomers of methylbenzylamine were separated with a resolution of 1.5. The bottom separation (Figure 16B) was obtained using an electrolyte containing 5 mM 18-crown-6-tetracarboxylic acid and 25 mM sodium tetraborate, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 120 μA, for a power level of 5.0 W/m. The electroosmotic flow was 8.4 x 10^{-4} cm^2/Vs. The enantiomers of methylbenzylamine were not separated at all. Therefore, the electrolyte containing bis-tris propane offered baseline resolution compared to none (due to lower EOF and wattage) while decreasing the power level five-fold.

Example 15: Lauric acid with bis-tris propane vs. sodium phosphate/sodium tetraborate.

Figure 17 shows the baseline separation of the diastereomers of nadolol using an electrolyte containing 25 mM lauric acid and 50 mM bis-tris propane, pH adjusted to 8.5 with phosphoric acid. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 25 μA, for a power level of 1.0 W/m. The electroosmotic mobility was 3.8 x 10^{-4} cm^2/Vs. Figure 18 shows no separation for the diastereomers of nadolol, and was obtained with an electrolyte containing 25 mM lauric acid, 25 mM disodium phosphate/25 mM sodium tetraborate, pH 8.5 with phosphoric acid. With an applied voltage of +25 kV, the current was 270 μA, for a power level of 11 W/m. The electroosmotic mobility was 5.9 x 10^{-4} cm^2/Vs. In this example, bis-tris propane afford baseline resolution vs. zero resolution, and an 11-fold reduction in power level. Furthermore, S/N is improved due to lower noise and increased signal due to sharper peaks.

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Example 16: Protonated Dodecylsulfate with bis-tris propane vs. Sodium Dodecylsulfate with sodium phosphate/sodium tetraborate.

Figure 19 shows the separation of eleven priority pollutant phenols using an electrolyte containing 43 mM protonated dodecylsulfate and 43 mM bis-tris propane, pH 8.6 (unadjusted). Eleven peaks can be distinguished, with all baseline resolved except the pair at 15.5 minutes. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 24 μA, for a power level of 1.0 W/m. The electroosmotic mobility was 4.4 x 10^{-4} cm^2/Vs. Figure 20 shows the separation of the same sample using an electrolyte containing 43 mM sodium dodecylsulfate and 21.5 mM disodium phosphate/21.5 mM sodium tetraborate, pH adjusted to 8.6 with phosphoric acid. Only eight peaks can be distinguished. The capillary was 75 μm i.d. x 60 cm length. With an applied voltage of +25 kV, the current was 200 μA, for a power level of 8.0 W/m. The electroosmotic mobility was 7.3 x 10^{-4} cm^2/Vs. In this example, the protonated version of dodecylsulfate in combination with bis-tris propane offered improved resolution (due to lower EOF and lower wattage) and an eight-fold reduction in the power level.

TABLE 2:

Summary of Different Additives with Bis-Tris Propane vs. Inorganic Buffers

	Bis-Tris Propane			Inorganic Buffer		
Additive	**EOF[1]**	**Rs**	**Power[2]**	**EOF[1]**	**Rs**	**Power[2]**
carboxymethyl-β-CD	4.4	1.6	1.0	8.2	0.2	5.8
succinylated-β-CD	4.4	3.0	1.0	8.3	0.4	9.8
18-crown-6-tetracarboxylic acid	4.2	1.5	1.1	8.4	0	5.0
lauric acid	3.8	1.5	1.0	5.9	0	11
dodecylsulfate	4.4	11 peaks	1.0	7.3	8 peaks	8.0

1- units of 10^{-4} cm^2/Vs
2- units of Watts/m

Although the foregoing invention has been described by way of illustration and

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example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the invention, as limited only by the scope of the appended claims.

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We claim:

1. A capillary electrophoresis electrolyte for separating analyte components of interest contained in a substantially aqueous sample, comprising a substantially aqueous phase, a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase.

2. The electrolyte of claim 1 wherein the substantially aqueous phase includes an organic modifier.

3. The electrolyte of claim 1 wherein the additive comprises a protonated surfactant.

4. The electrolyte of claim 1 wherein the additive comprises a protonated chiral surfactant.

5. The electrolyte of claim 1 wherein the additive comprises a protonated achiral surfactant.

6. The electrolyte of claim 1 wherein the additive is lauric acid.

7. The electrolyte of claim 1 wherein the additive is dodecyl sulfate.

8. The electrolyte of claim 1 wherein the additive is (S)-N-dodecoxycarbonylvaline.

9. The electrolyte of claim 1 wherein the additive is a protonated cyclodextrin acid derivative.

10. The electrolyte of claim 9 wherein the additive is carboxymethyl-β-cyclodextrin.

11. The electrolyte of claim 9 wherein the additive is succinylated-β-cyclodextrin.

12. The electrolyte of claim 1 wherein the additive is a protonated crown ether acid derivative.

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13. The electrolyte of claim 12 wherein the additive is 18-crown-6-tetracarboxylic acid.

14. The electrolyte of claim 1 wherein the free polyamine is a diamine.

15. The electrolyte of claim 1 wherein the diamine is bis-tris propane.

16. The electrolyte of claim 1 wherein the free polyamine is N,N'-bis(2-aminoethyl)-1,3-propanediamine.

17. The electrolyte of claim 1 wherein the free polyamine is pentrol.

18. The electrolyte of claim 1 wherein the free polyamine is pentaethylenhexamine.

19. The electrolyte of claim 1 wherein the free polyamine is 4,7,10-trioxa-1,13-tridecanediamine.

20. A method for separating analyte components of interest under capillary electrophoretic conditions contained in a substantially aqueous sample, comprising the step of contacting said sample with an effective amount of a combination of a protonated additive and a free polyamine dispersed in a substantially aqueous phase.

21. The method of claim 20 wherein the substantially aqueous phase includes an organic modifier.

22. The method of claim 20 wherein the additive is selected from the group consisting of dodecyl sulfate, (S)-N-dodecoxycarbonylvaline, carboxymethyl-β-cyclodextrin, and succinylated-β-cyclodextrin.

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23. The method of claim 20 wherein the free polyamine is selected from the group consisting of bis-tris propane, N,N'-bis(2-aminoethyl)-1,3-propanediamine, pentrol, pentaethylenhexamine, and 4,7,10-trioxa-1,13-tridecanediamine.

24. A kit for separating analyte components of interest contained in a substantially aqueous sample, the kit being compartmentalized to receive in close confinement one or more containers which comprise separately or in combination a protonated additive to be dispersed in the substantially aqueous phase, and a free polyamine to be dispersed in the substantially aqueous phase.

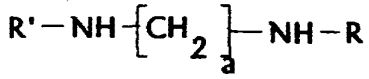
25. The kit of claim 24 wherein the substantially aqueous phase includes an organic modifier.

26. The kit of claim 24 wherein the additive comprises a protonated surfactant.

27. The kit of claim 24 wherein the additive is selected from the group consisting of dodecyl sulfate, (S)-N-dodecoxycarbonylvaline, carboxymethyl-β-cyclodextrin, and succinylated-β-cyclodextrin.

28. The kit of claim 24 wherein the free polyamine is selected from the group consisting of bis-tris propane, N,N'-bis(2-aminoethyl)-1,3-propanediamine, pentrol, pentaethylenhexamine, and 4,7,10-trioxa-1,13-tridecanediamine.

29. A capillary electrophoresis electrolyte for separating analyte components of interest contained in a substantially aqueous sample, comprising a substantially aqueous phase, a protonated additive dispersed in the substantially aqueous phase, and a free polyamine dispersed in the substantially aqueous phase, the free polyamine having the following structural formula:



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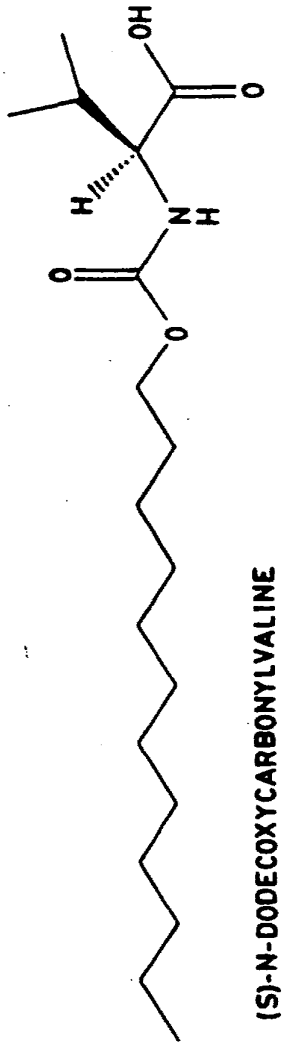
wherein

a is from about 1 to 8 methylenes; and

R and R' are the same or different and may be alkyl, alkenyly or alkynyl substituents, branched or straight chain, substituted or unsubstituted, having from about 1 to 8 carbons, and may have one or more hydrophilic moities such as hydroxy, sulfhydryl, or amine.

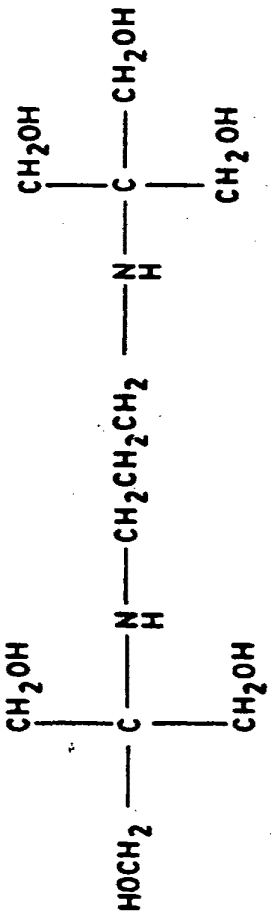
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(S)-N-DODECOXYCARBONYLVALINE

FIG. 1



BIS-TRIS PROPANE

FIG. 5

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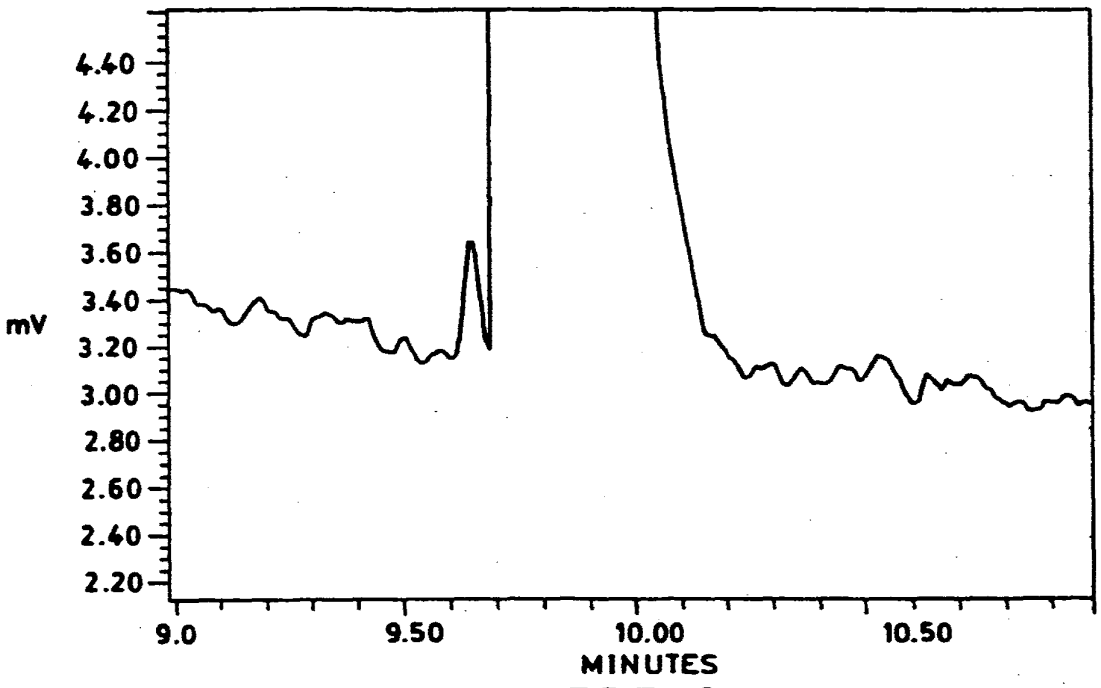


FIG. 2A

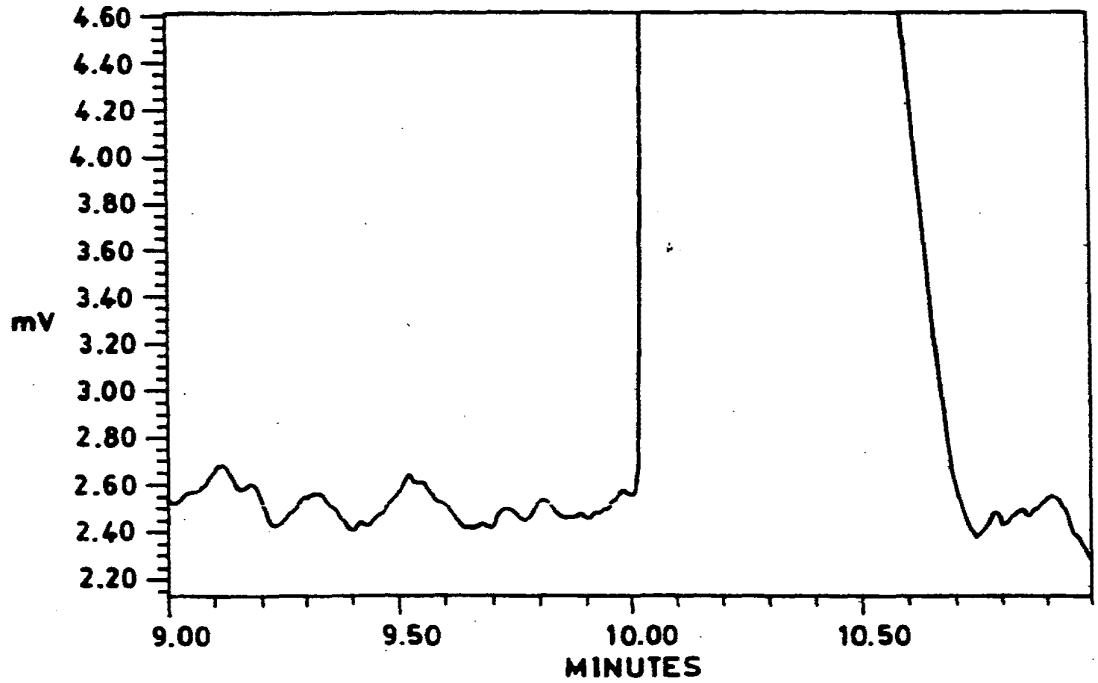


FIG. 2B

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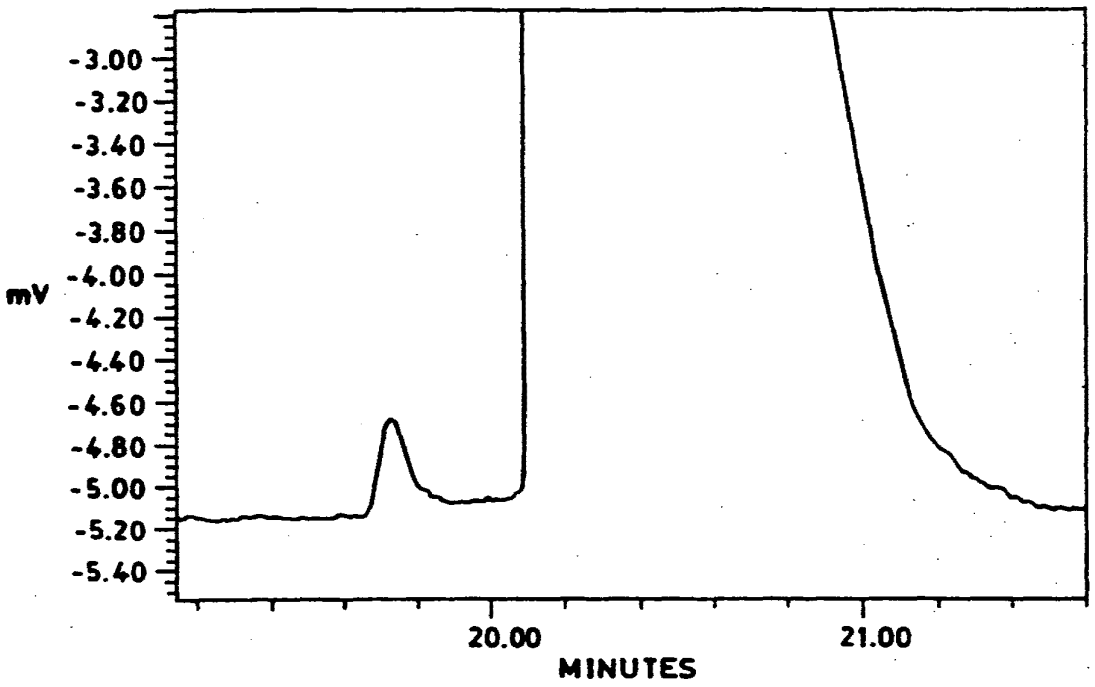


FIG.3A

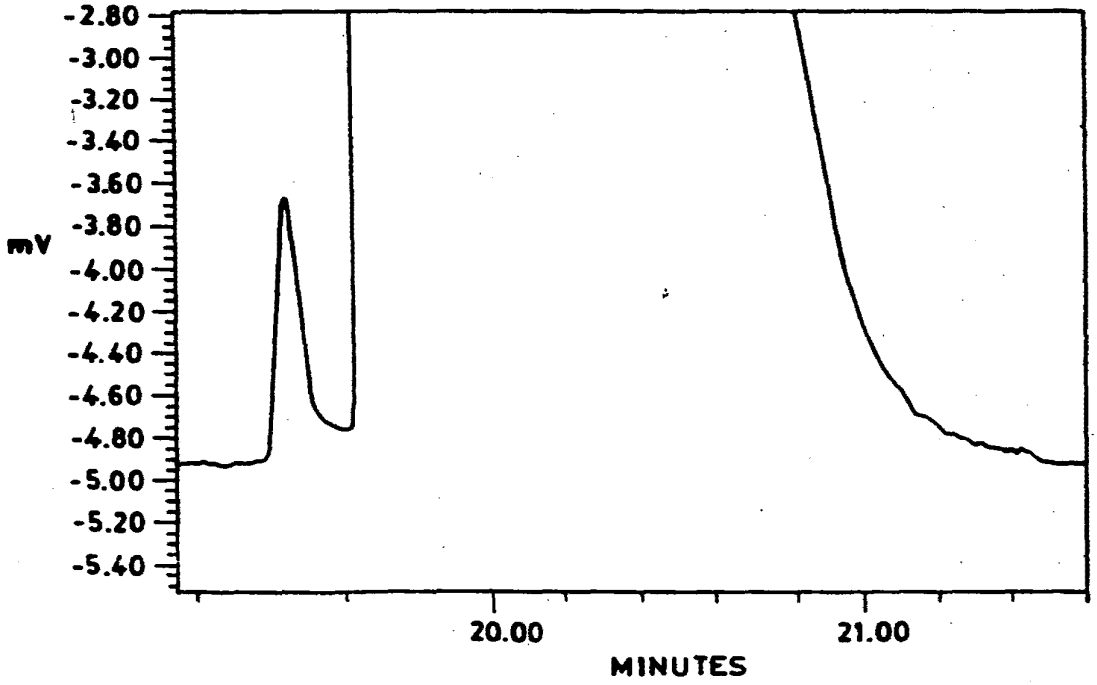


FIG. 3B

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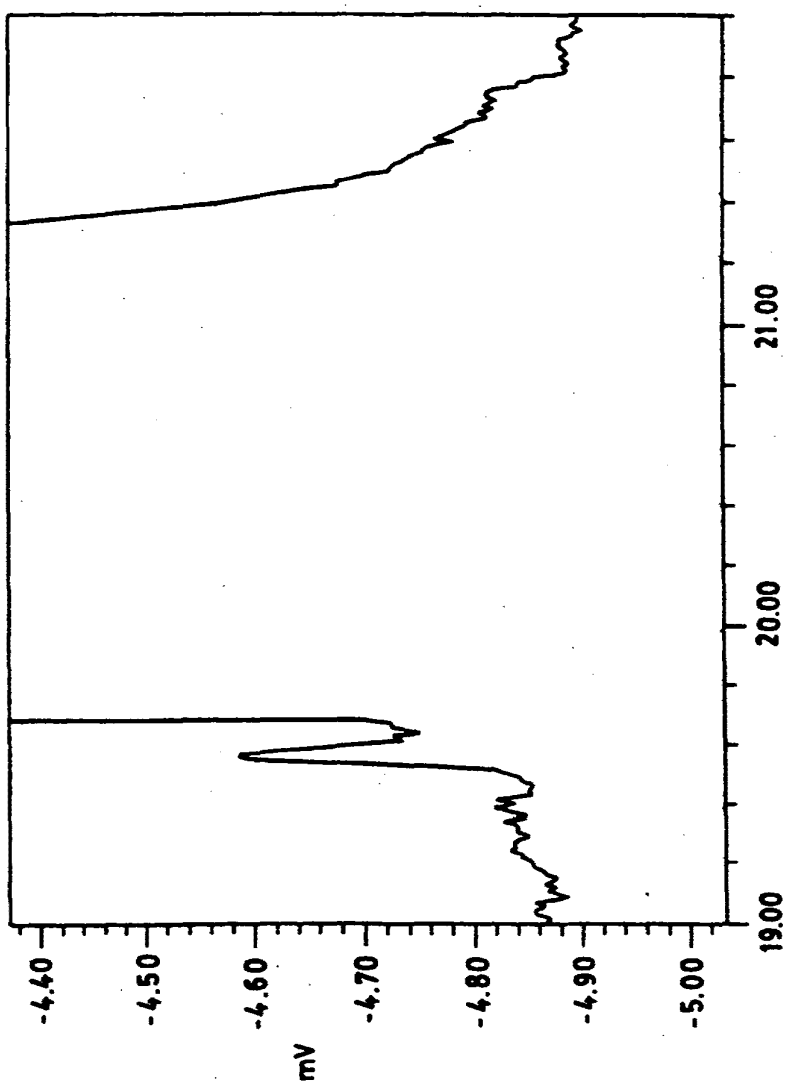


FIG. 4

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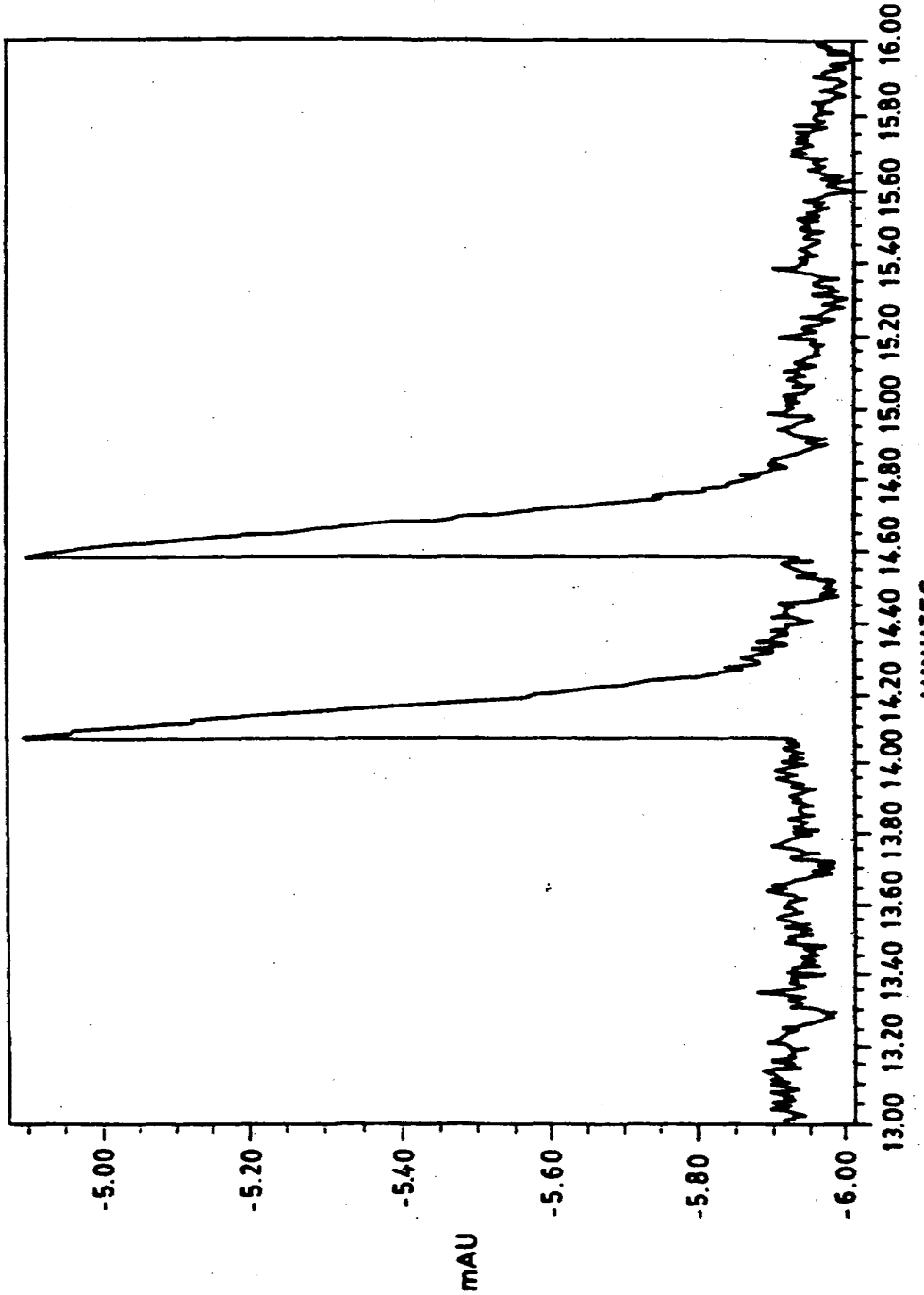


FIG. 6A

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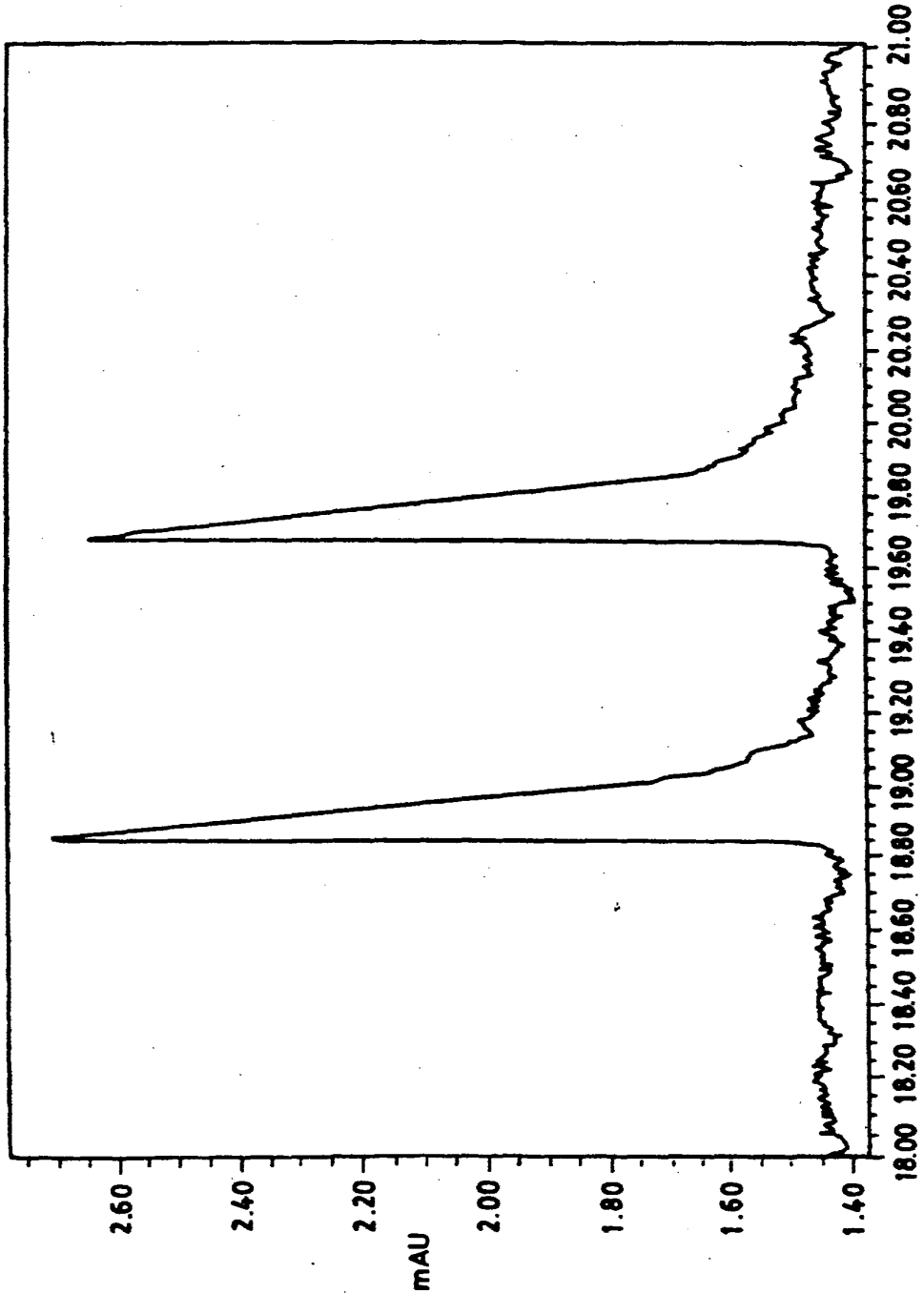


FIG. 6B

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Page 1003-0086

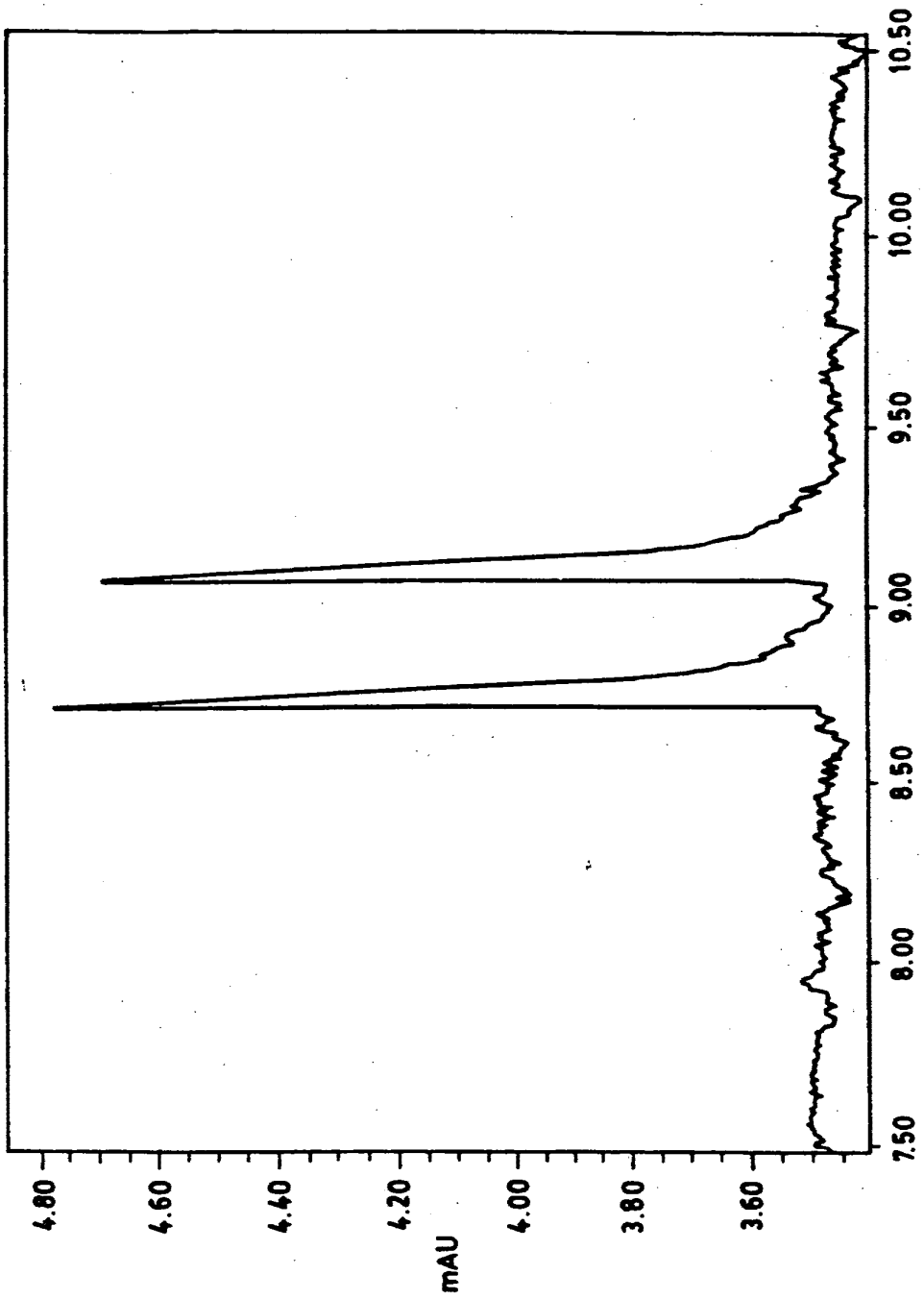


FIG.7

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INSDOCID: <WO_____9704308A1_I_>

$NH_2CH_2CH_2NHCH_2CH_2CH_2NHCH_2CH_2NH_2$

N,N'-bis-(2-aminoethyl)-1,13-propanediamine

$CH_3CH(OH)CH_2N(CH_2CH_2N[CH_2CH(OH)CH_3]_2)_2$

pentrol

$NH_2CH_2CH_2NHCH_2CH_2NHCH_2CH_2NHCH_2CH_2NHCH_2CH_2NH_2$

pentaethylenehexamine

$O[CH_2CH_2O(CH_2)_3NH_2]_2$

4,7,10-trioxa-1,13-tridecanediamine

FIG. 8

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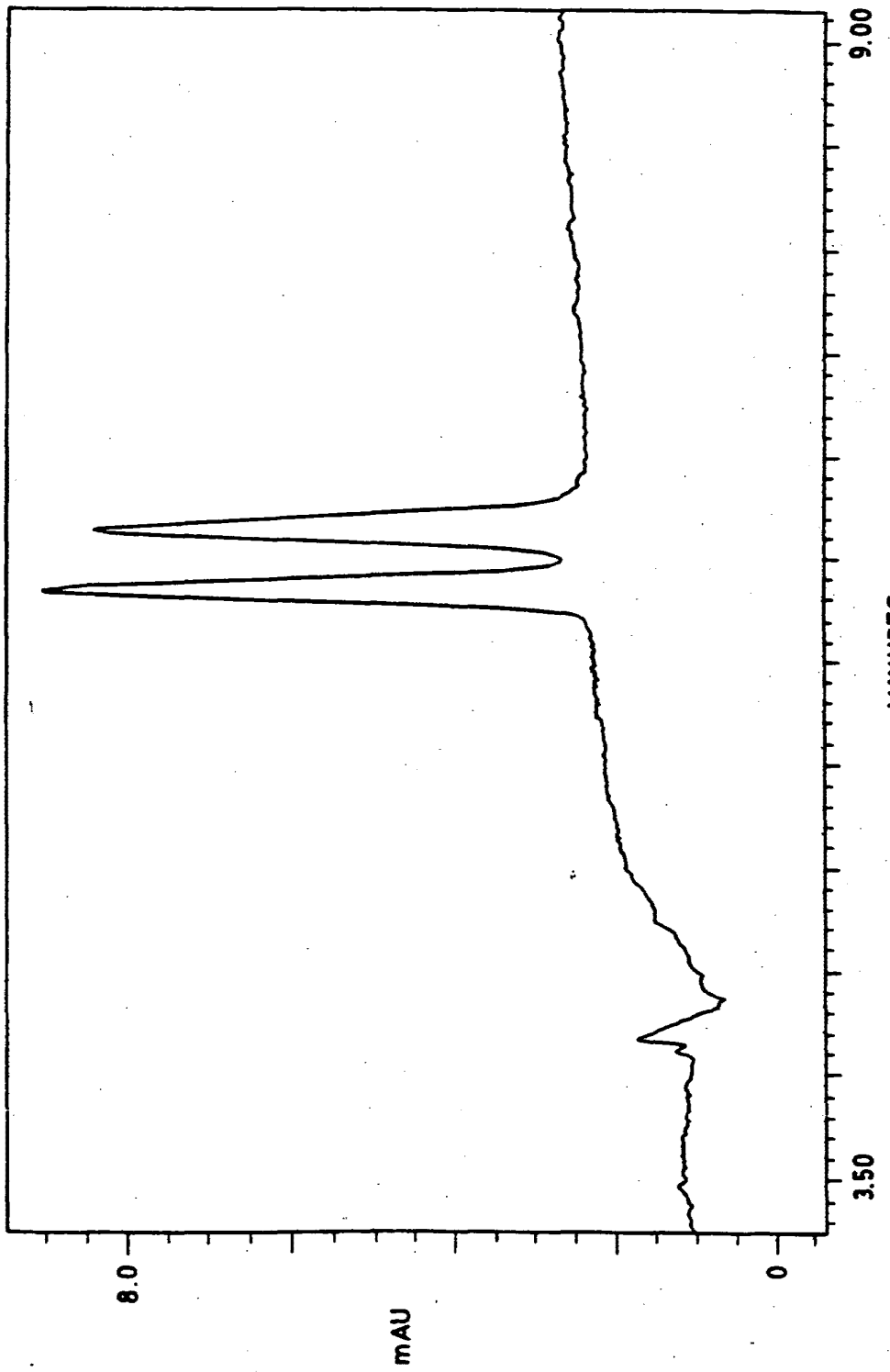


FIG. 9

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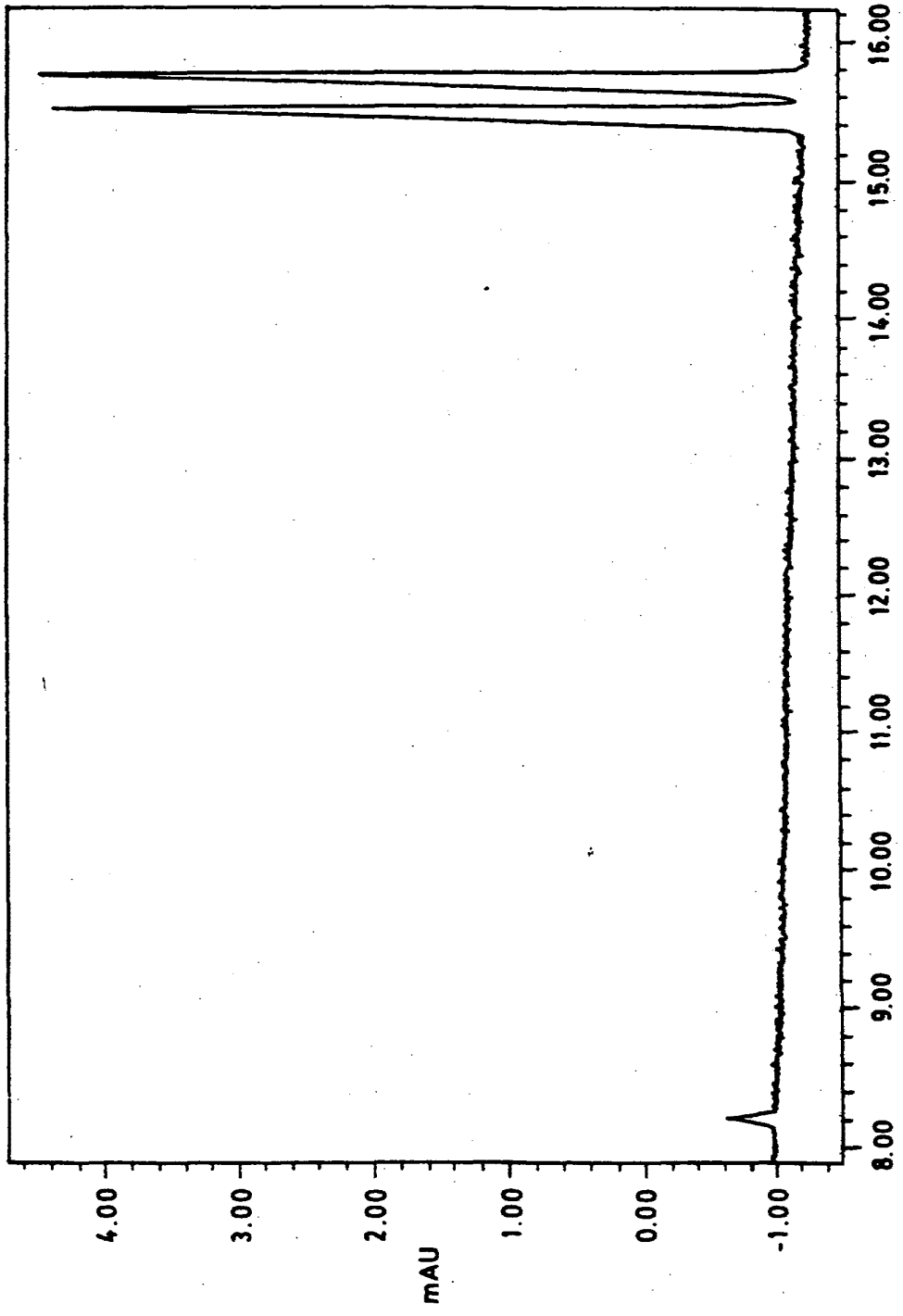


FIG.10

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Page 1003-0090

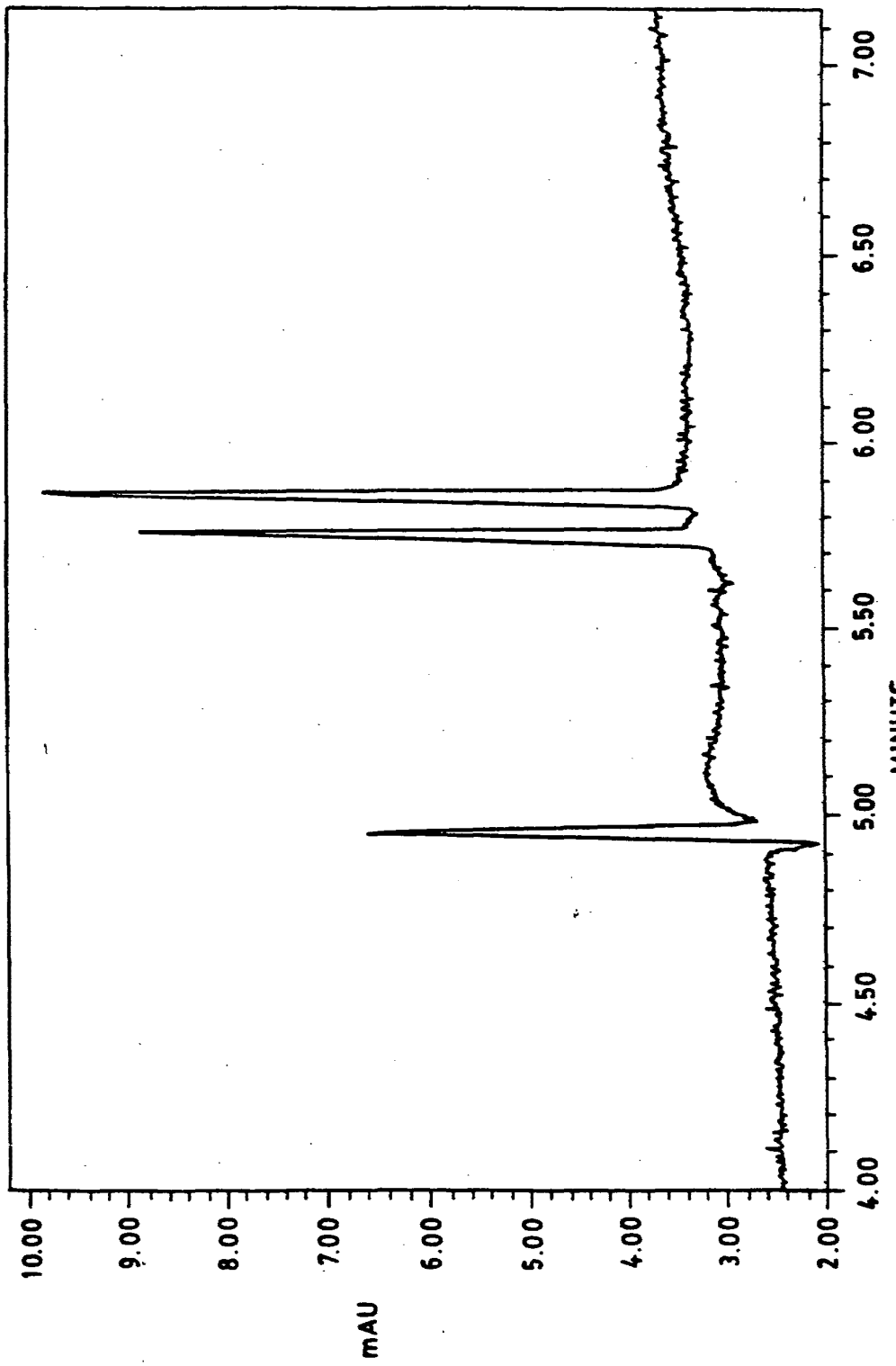


FIG. 11

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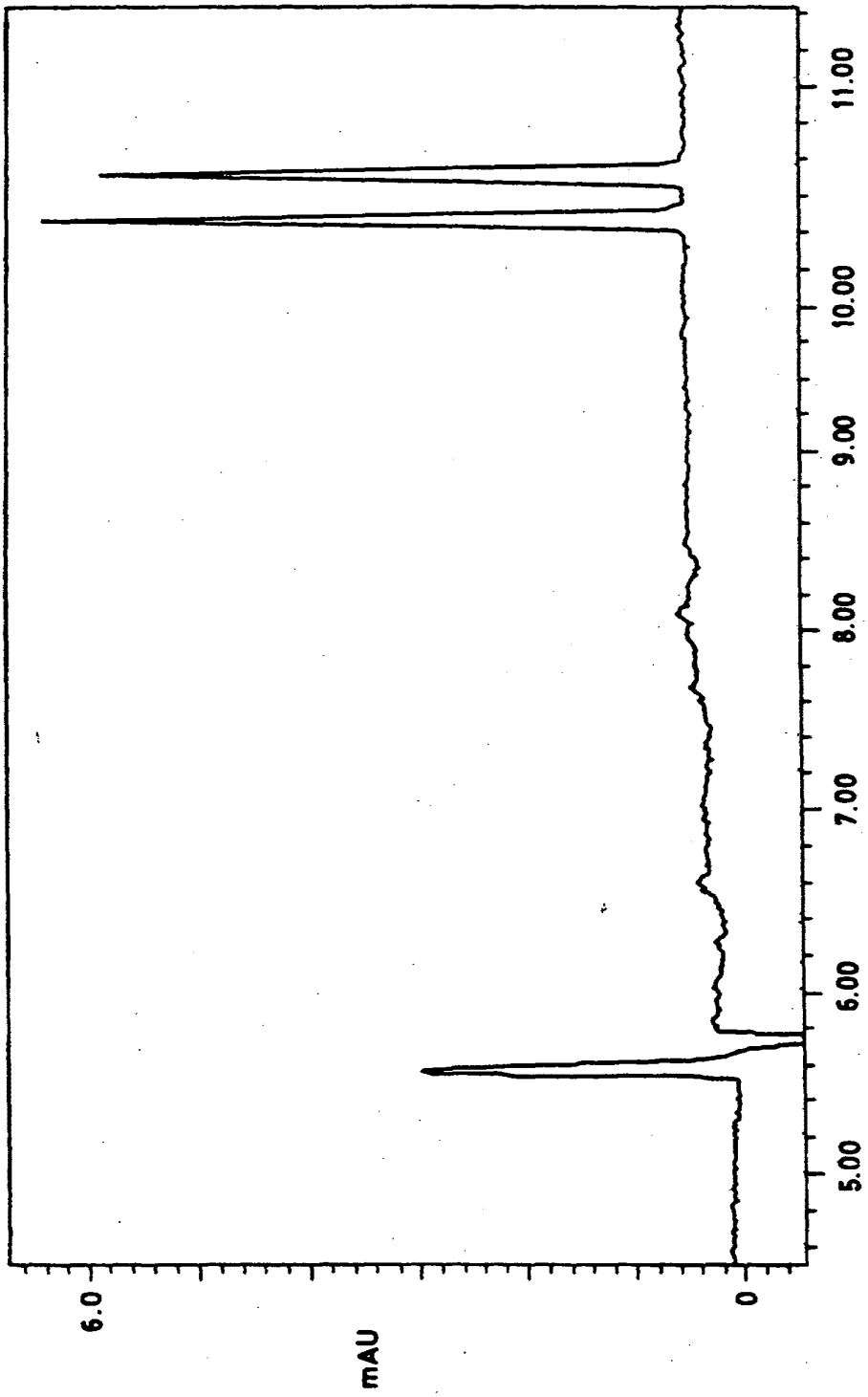


FIG. 12

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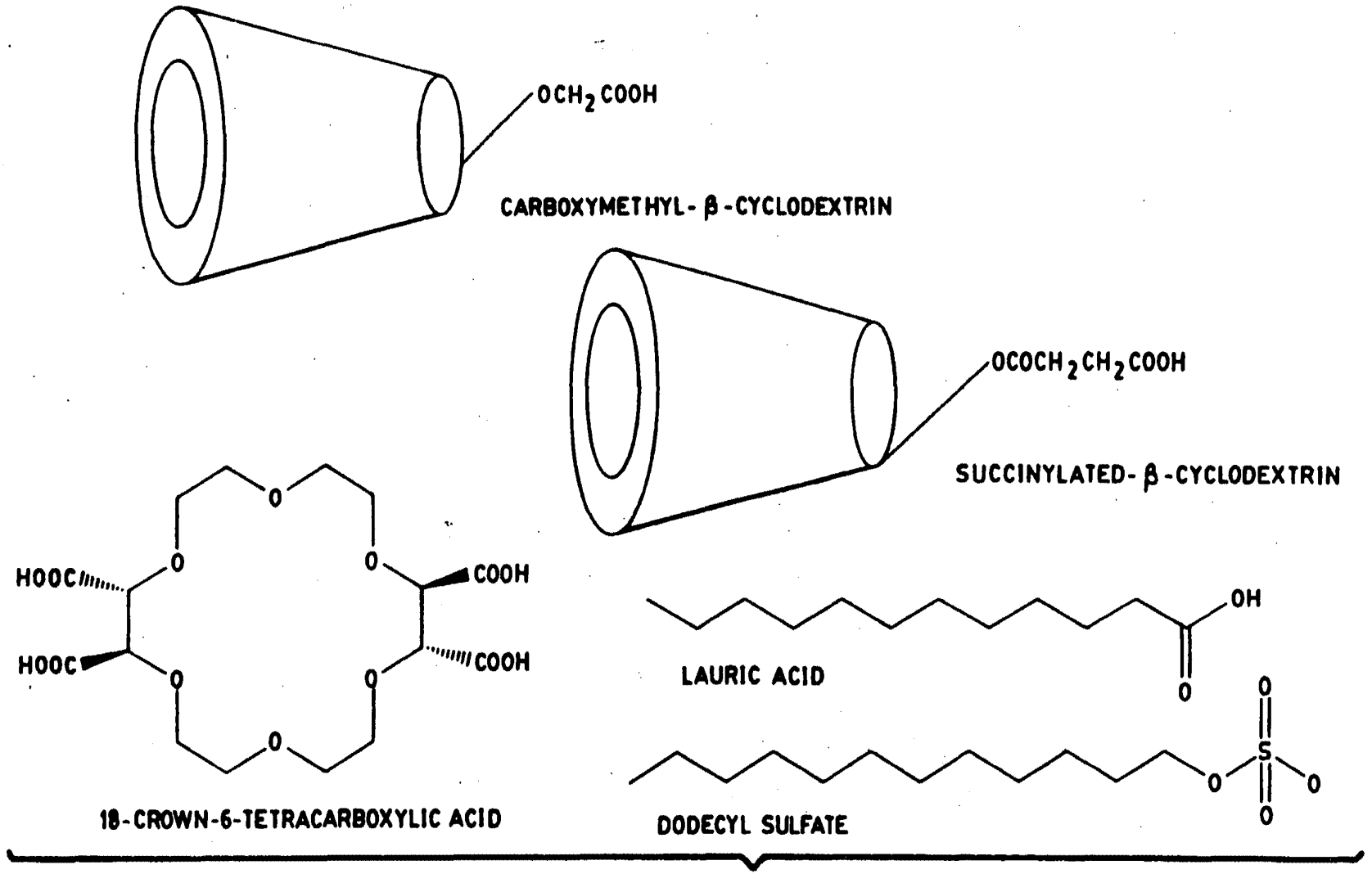


FIG. 13

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FIG.14A

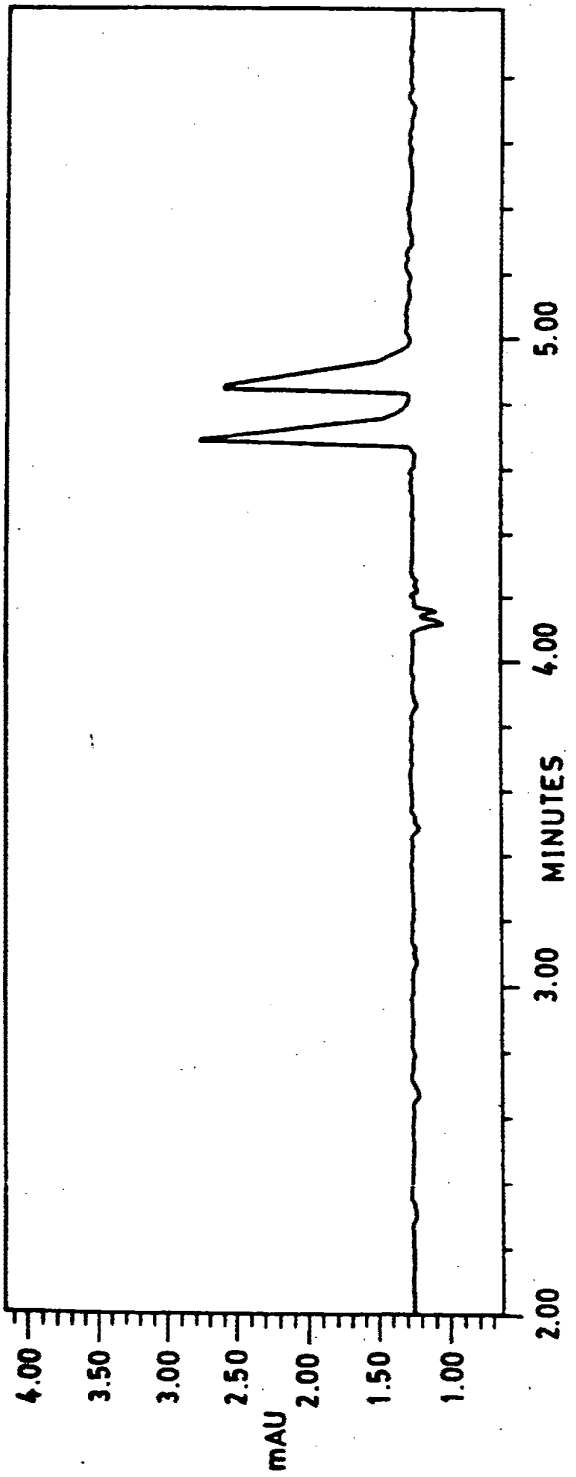
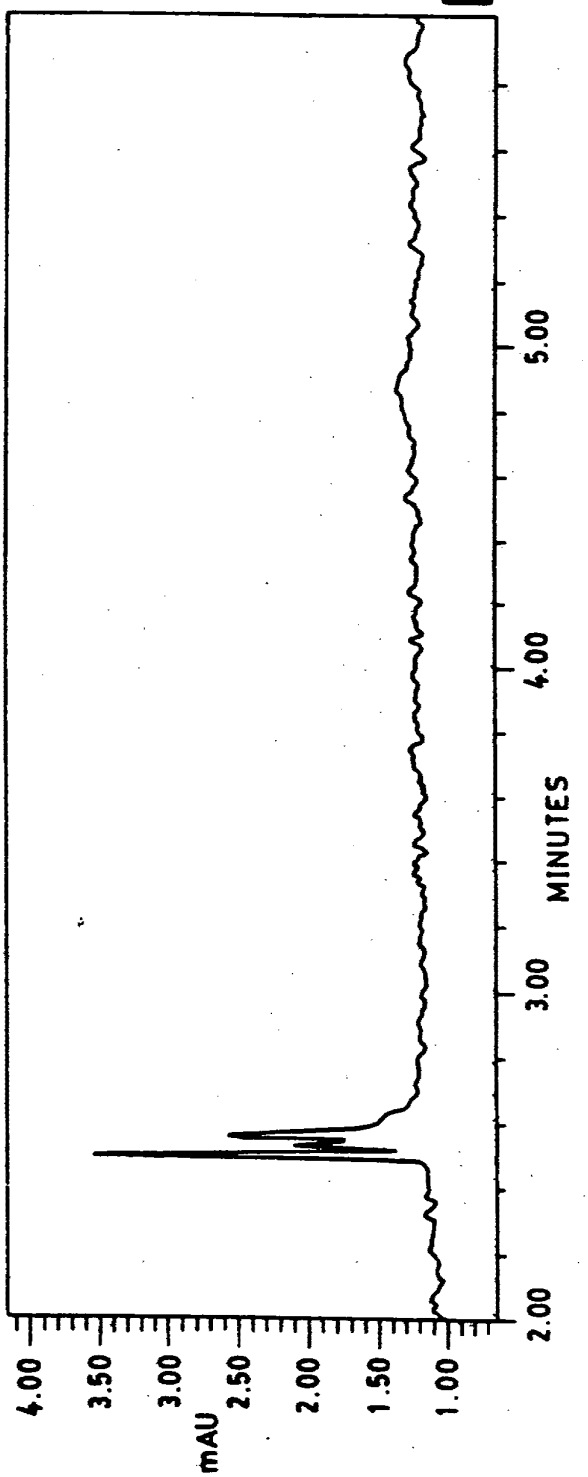


FIG.14B



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FIG.15A

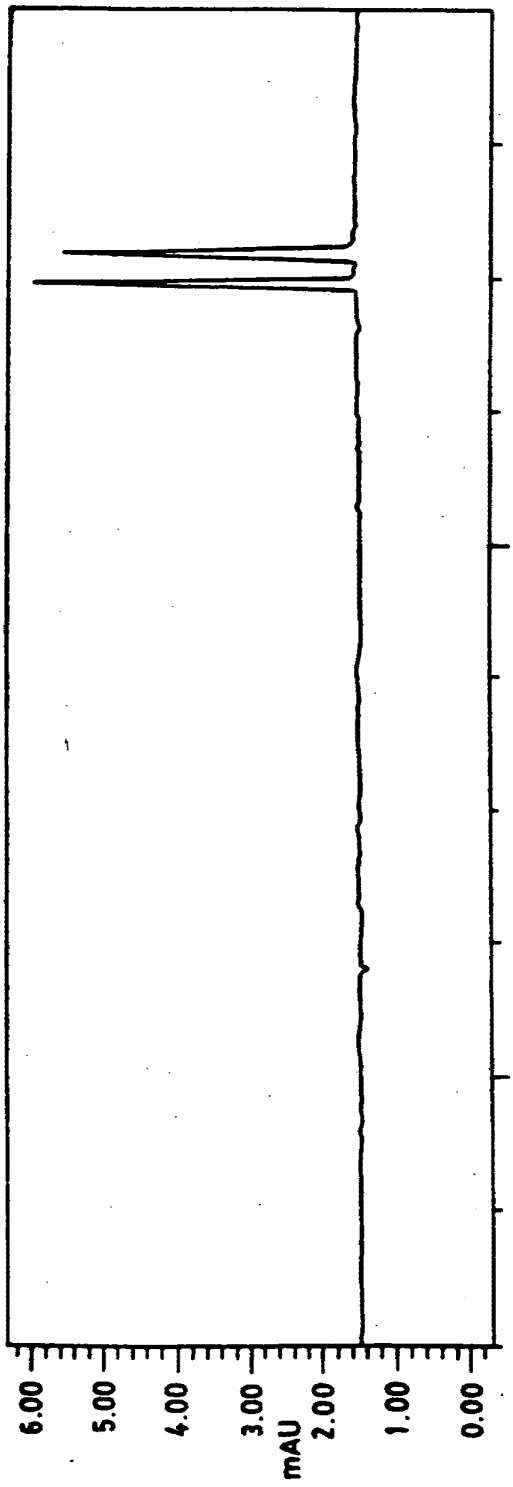
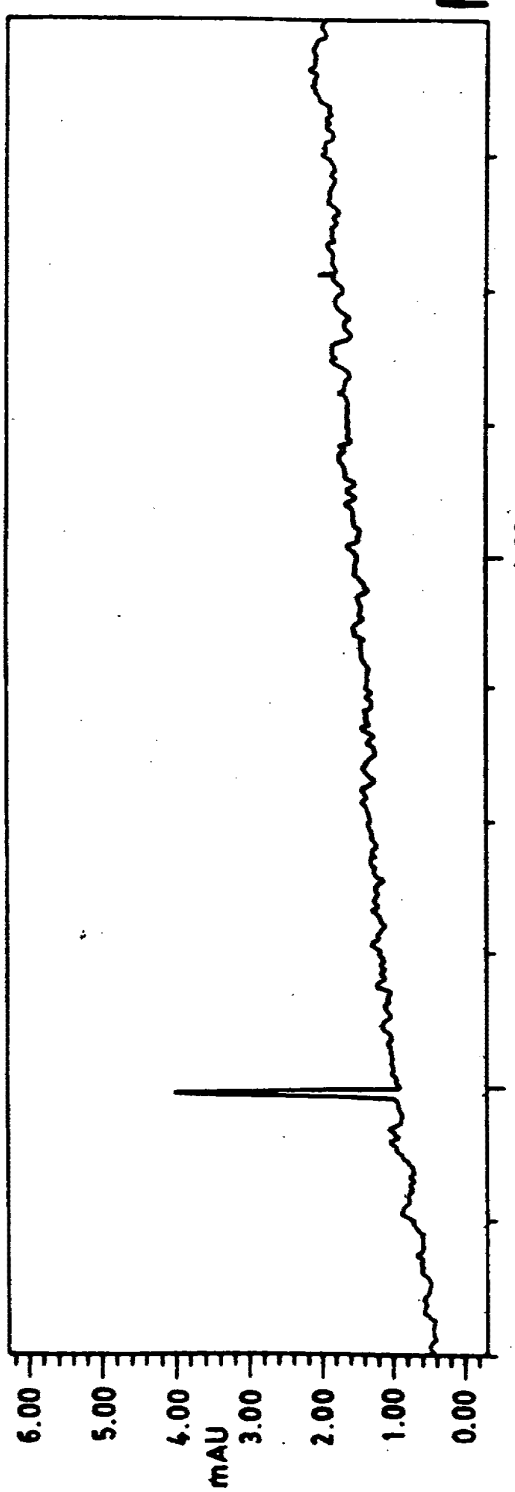


FIG.15B



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FIG.16A

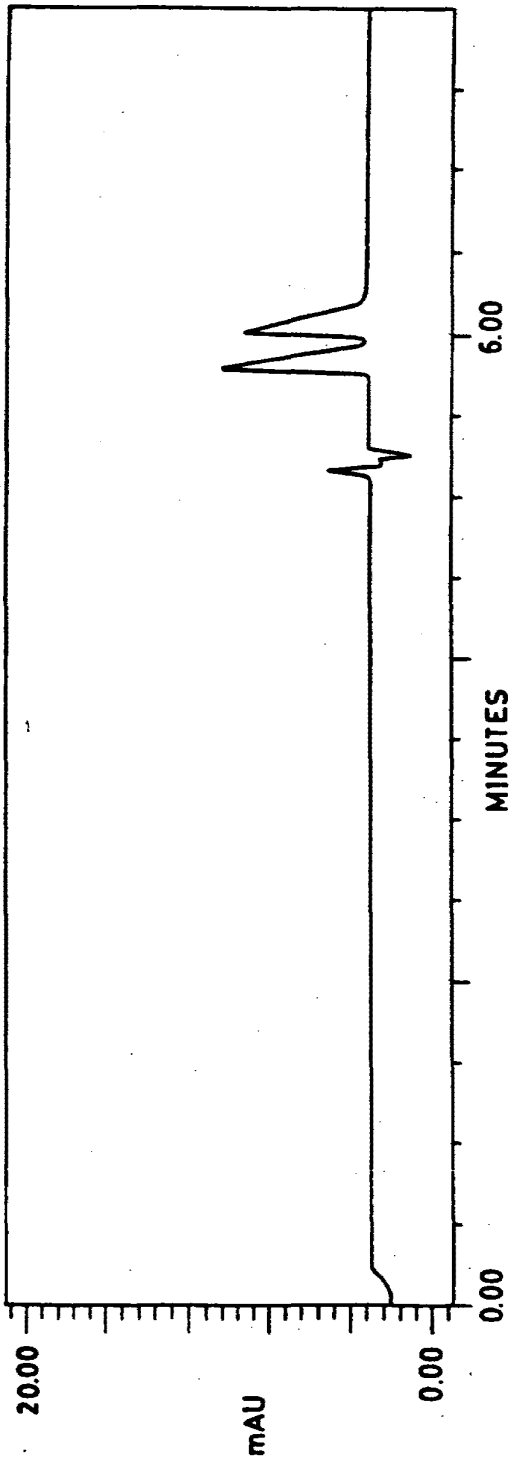
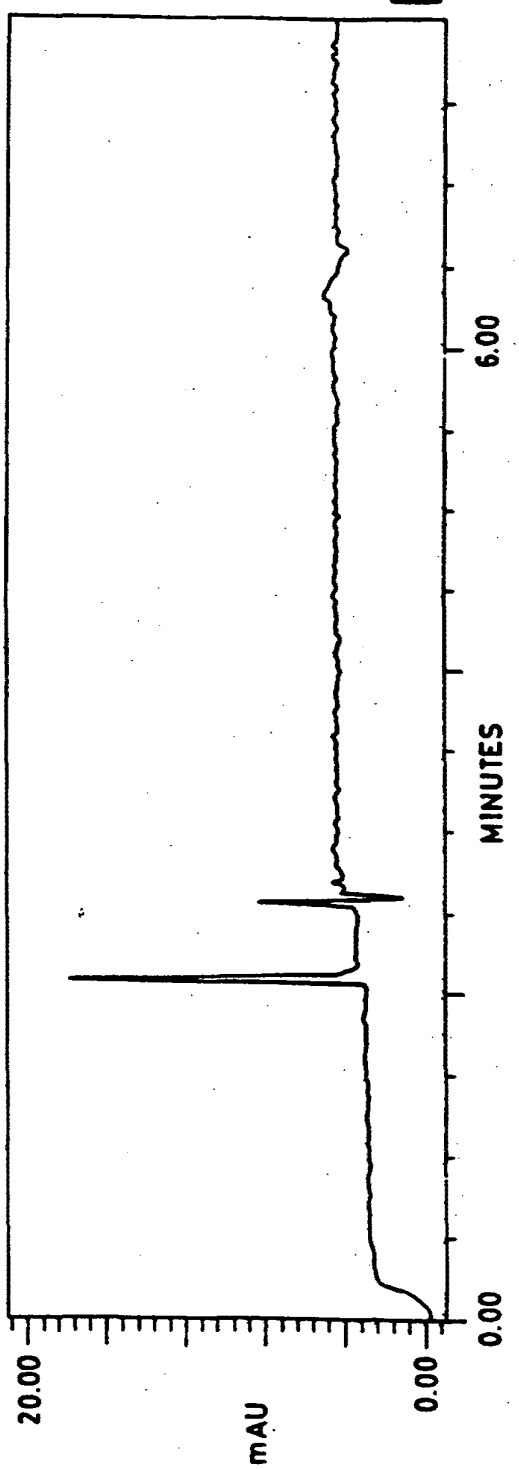


FIG.16B



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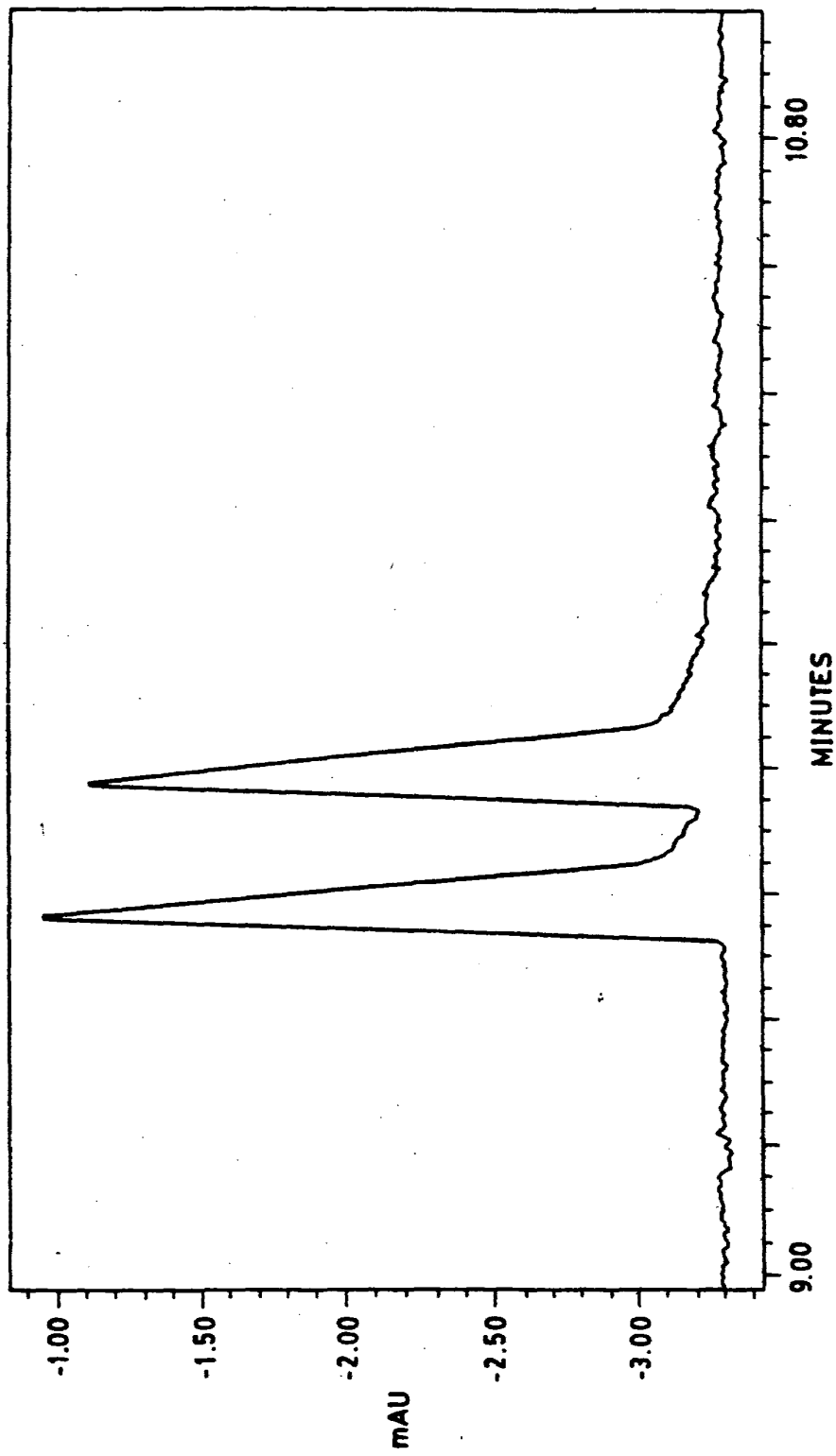


FIG. 17

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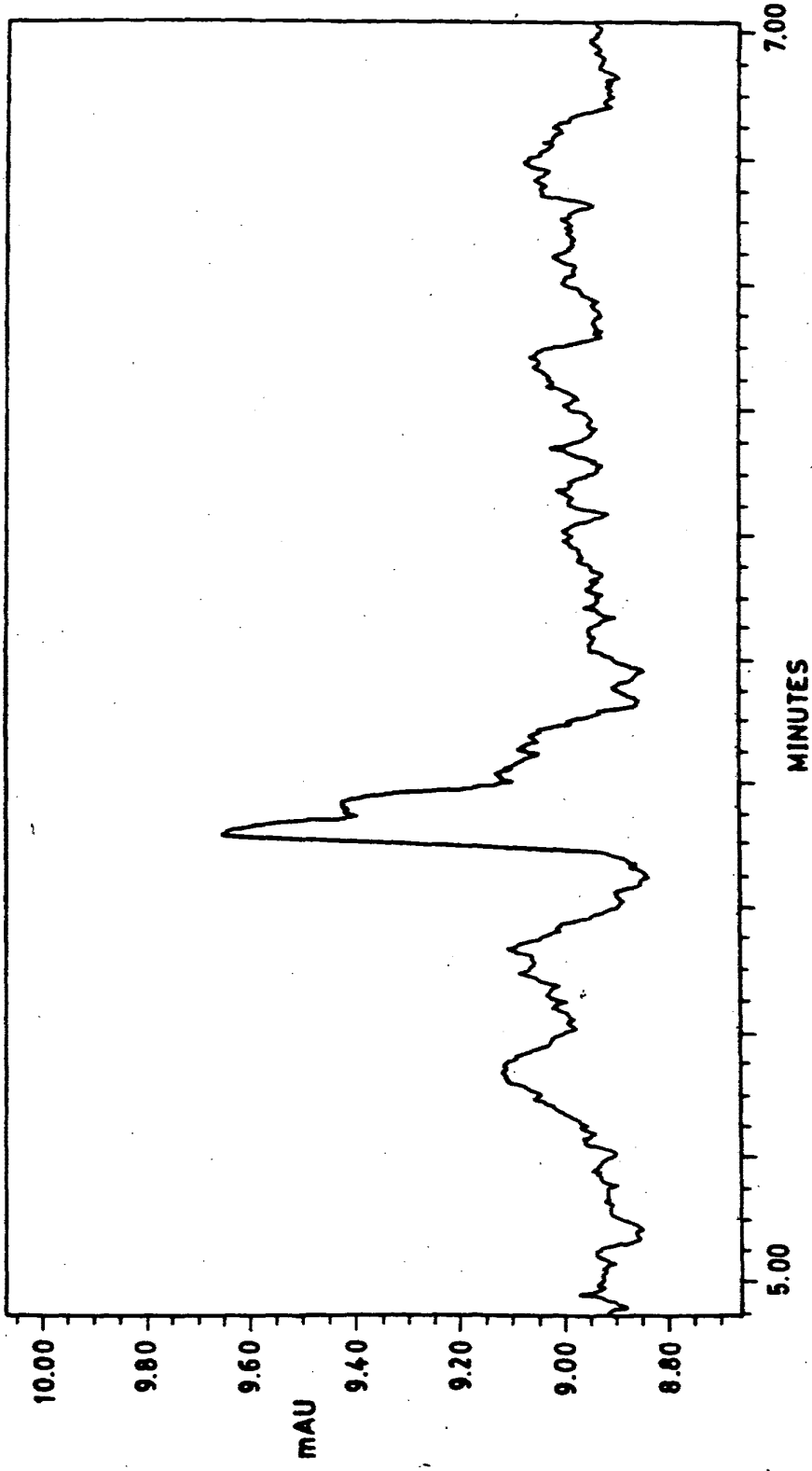


FIG. 18

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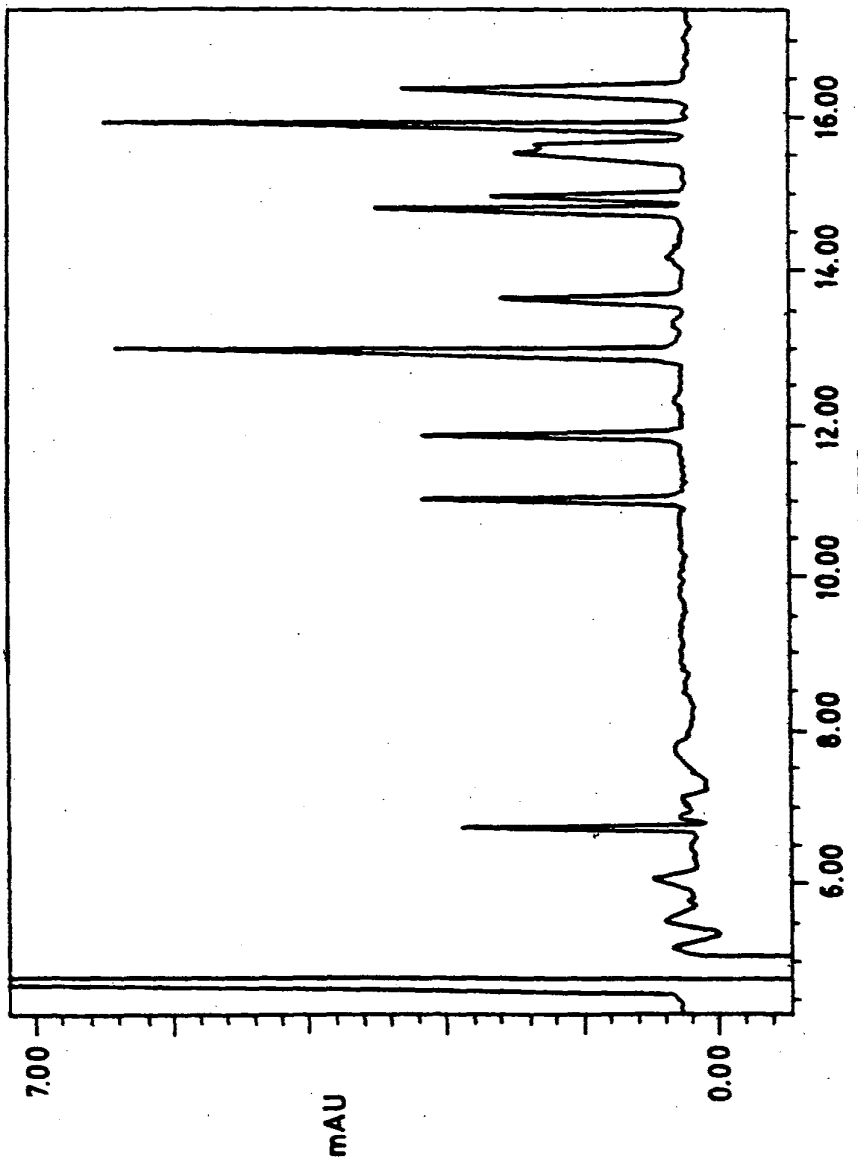


FIG. 19

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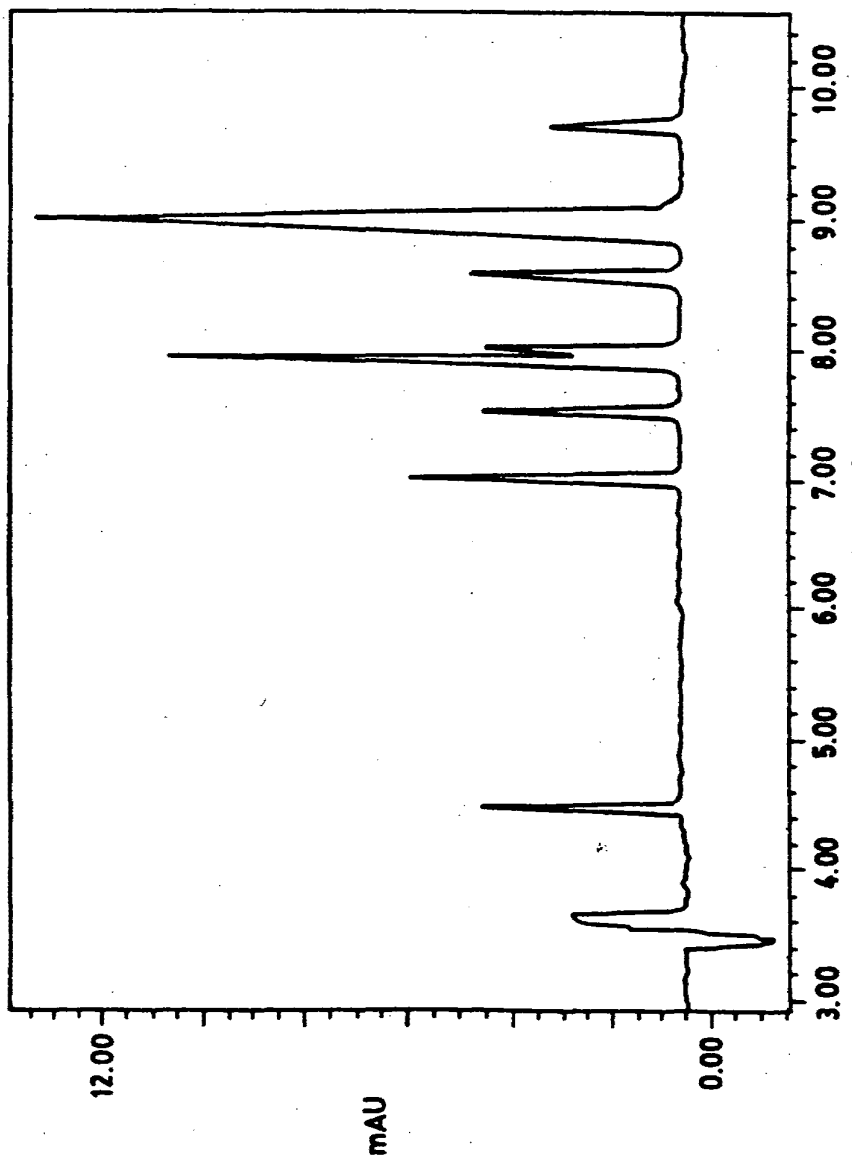


FIG. 20

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/11973

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N27/447

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHROMATOGRAPHIA, vol. 37, no. 9/10, November 1993, pages 475-481, XP000607488 T. SCHMITT: "CHARGED AND UNCHARGED CYCLODEXTRINS AS CHIRAL SELECTORS IN CAPILLARY ELECTROPHORESIS" see abstract ---	1
A	EP,A,0 292 837 (ABBOTT LABORATORIES) 30 November 1988 see claim 9 ---	1
A	WO,A,93 05389 (BECKMAN INSTRUMENTS, INC.) 18 March 1993 see claim 5 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1996	29. 11 96

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CHROMATOGRAPHY, vol. 194, no. 1, 1980, AMSTERDAM, NL, pages 11-19, XP000608555 D. KANIANSKY: "ROLE OF THE CHARGE NUMBER OF THE COUNTER-IONIC CONSTITUENT IN THE SEPARATION OF ANIONS BY ISOTACHOPHORESIS" see abstract ---	1
A	CHROMATOGRAPHIA, vol. 33, no. 1/2, January 1992, pages 32-36, XP000607489 R. KUHN: "CHIRAL SEPARATIONS BY HOST-GUEST COMPLEXATION WITH CYCLODEXTRIN AND CROWN ETHER IN CAPILLARY ZONE ELECTROPHORESIS" see abstract ---	1
A	ANALYTICAL CHEMISTRY, vol. 66, no. 23, 1 December 1994, WASHINGTON, DC, US, pages 4121-4126, XP000485685 D. C. TICKLE: "GLUCOPYRANOSIDE-BASED SURFACTANTS AS PSEUDOSTATIONARY PHASES FOR CHIRAL SEPARATIONS IN CAPILLARY ELECTROPHORESIS" see page 4121 - page 4122 ---	1
A	ANALYTICAL CHEMISTRY, vol. 57, no. 4, April 1985, pages 834-841, XP000608538 S. TERABE: "ELECTROKINETIC CHROMATOGRAPHY WITH MICELLAR SOLUTION AND OPEN-TUBULAR CAPILLARY" cited in the application see the whole document -----	1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte[illegible]nal Application No
PCT/US 96/11973

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-292837	30-11-88	CA-A-	1319007	15-06-93
		DE-A-	3883288	23-09-93
		DE-T-	3883288	09-12-93
		JP-A-	1065444	10-03-89
		US-A-	4936963	26-06-90
WO-A-9305389	18-03-93	US-A-	5259939	09-11-93
		AU-B-	657164	02-03-95
		AU-A-	2424592	05-04-93
		EP-A-	0556352	25-08-93
		JP-T-	6503179	07-04-94

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Electrophoresis 2000, *21*, 743–748

James M. Hempe[1,3]
Randall D. Craver[1,2,3]

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[2]Department of Pathology
Louisiana State University
School of Medicine, New
Orleans, LA, USA
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Orleans, LA, USA

Separation of hemoglobin variants with similar charge by capillary isoelectric focusing: Value of isoelectric point for identification of common and uncommon hemoglobin variants

Clinical assays for the primary evaluation of congenital hemoglobin (Hb) disorders must detect and identify a variety of Hb variants. We analyzed hemolysates containing Hb variants with similar charge to evaluate the diagnostic sensitivity and specificity of automated capillary isoelectric focusing (CIEF). Peak separation was observed for each variant in samples containing Hb S, D, and G. The calculated isoelectric points (p*I*) of these variants were significantly different such that each could be identified in a single run with p*I* as the sole criterion of identification. The p*I* of Hb C was significantly different from that of Hb E, C-Harlem, and O-Arab. Hb E, C-Harlem, and O-Arab had similar p*I* and were not readily differentiated. Hb Koln, M-Saskatoon, Aida, and S/Aida hybrid were readily separated from common Hb variants and detected by CIEF. We conclude that CIEF exhibits both diagnostic sensitivity and specificity, and that p*I* is an objective and specific criterion of Hb variant identification.

Keywords: Hemoglobin variant / Hemoglobinopathy / Congenital hemoglobin disorder / Capillary electrophoresis / Capillary isoelectric focusing / Isoelectric focusing / Clinical diagnosis EL 3820

1 Introduction

Laboratory diagnosis of congenital hemoglobinopathies and thalassemias requires both identification of abnormal hemoglobin (Hb) variants and quantification of major and minor variants over a wide range of concentrations [1–3]. Most clinical laboratories use a progression of analytical methods to evaluate these disorders, beginning with alkaline electrophoresis and followed for more precise identification as needed by acid electrophoresis, gel isoelectric focusing, and/or HPLC. These assays are commonly supplemented by minicolumn ion exchange chromatography and/or alkali denaturation if quantification of the minor Hb variants A_2 and F is needed. Quantification of Hb A_2 and F is often used to help diagnose thalassemia syndromes, or to longitudinally follow changes in Hb F concentration in patients with sickle cell disease that are treated with hydroxyurea.

The combination of alkaline and acid electrophoresis is used in most laboratories as primary and secondary analytical methods for structural Hb variant identification because the assays are well characterized, easy to perform, and relatively inexpensive. Both methods must be used, however, because the separation of Hb variants with similar charge is relatively poor with either method alone. For example, Hb S cannot be differentiated from Hb D or G, and Hb C cannot be differentiated from Hb E, C-Harlem, or O-Arab by alkaline electrophoresis alone [2]. This lack of diagnostic specificity means that laboratories using alkaline electrophroesis as their primary assay must perform a secondary assay, usually by acid electrophoresis, to positively identify Hb S, C, and E, the three most common abnormal Hb variants in the world.

The need for multiple assays increases turn-around-time for results and also adds to test costs for labor, supplies, and administrative management of multiple procedures. Also, because both alkaline and acid electrophoresis are labor-intensive methods, they are not amenable to automation of either sample handling or postanalytical data processing. Automation of clinical assays not only decreases labor costs, but also makes possible the use of computerized diagnostic algorithms for objective interpretation of analytical results. The use of peak elution time as a criterion of Hb variant identification, for example, is one reason for the commercialization and increasing use of HPLC for primary hemoglobinopathy assessment [4–8]. Some HPLC methods, however, require separate programs to identify and quantify both major (*e.g.*, Hb A, S, E, and C) and minor (*e.g.*, Hb A_2 and F) variants. Also, capital equipment and reagent costs are relatively high for HPLC compared to conventional electrophoresis meth-

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E-mail: jhempe@lsumc.edu
Fax: +504-733-4036

Abbreviations: **CAP**, College of American Pathologists; **Hb**, hemoglobin; **RBC**, red blood cells

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ods, although the cost increase is somewhat offset by the lower labor costs afforded by automation.

We have previously reported that computer-assisted capillary isoelectric focusing (CIEF) is an excellent method for the clinical assessment of structural hemoglobinopathies and thalassemias because it is an automated, high resolution, and microanalytical (therefore low use of consumables) assay with good precision for simultaneous quantification of both major and minor Hb variants [9–12]. The experiments described in this report highlight the use of CIEF as a primary analytical method for the specific identification of common and uncommon structural Hb variants. We emphasize the value of isoelectric point (p*I*) as a reproducible, objective, and specific criterion of Hb variant identification for automated post-analytical data processing and results interpretation.

2 Materials and methods

2.1 Samples

Blood was obtained from Children's Hospital Laboratory in accordance with the ethical guidelines of that institution from patients with normal Hb phenotype, sickle cell trait, Hb E trait, Hb O-Arab trait, Hb S trait/Hb Aida trait, Hb S/C-Harlem disease, or Hb S/C disease transfused with normal blood. The sample from the patient with Hb S trait/Hb Aida trait was sent to a commercial reference laboratory (Mayo Medical Laboratories, Rochester, MN) to verify the identify of the Hb variants. Proficiency testing samples, from the College of American Pathologists (CAP; Northfield, IL), containing unusual Hb variants (Hb Koln trait and Hb M-Saskatoon trait) were also analyzed, as were lyophilized commercial controls (Isolab, Akron, OH) containing Hb A and either Hb D-Punjab or Hb G-Philadelphia. Hemolysates were prepared from blood by mixing 10 µL of packed red blood cells (RBC) with 200 µL of hemolyzing reagent (10 mmol/L KCN, 5 mmol/L EDTA) as previously described [13]. Lyophilized controls were reconstituted with hemolyzing reagent. Where necessary, hemolysates were stored at –70°C prior to analysis (storage at –20°C is not suitable). In one study, twenty-four samples were prepared by mixing variable amounts of hemolysate containing Hb S, D, or G ($n = 11$ each). Different mixture ratios were used to produce samples containing two abnormal variants (*i.e.*, SD, SG, or DG) at different concentrations to preclude identification based on proportions or electropherogram similarity. The samples in this study were randomly analyzed over a 5-day period. In a second study, hemolysate from a patient with Hb S/C disease transfused with normal blood (containing Hb A, S, and C) was mixed with hemolysate containing either Hb E, O-Arab, or C-Harlem ($n = 10$ each) to assess the separation of these more positively charged Hb variants by CIEF. All of the samples in this study were randomly assayed in a single analytical run.

2.2 Capillary electrophoresis

CIEF (pH 6–8/3–10, 10:1) was conducted using a P/ACE 2200 capillary electrophoresis system and System Gold software (Beckman Instruments, Fullerton, CA) as described elsewhere [12, 13]. The proportions of different Hb variants present in normal and abnormal controls were compared to expected values to verify assay performance with each analytical run. Quantification was based on the integration of peak area absorbance at 415 nm and expressed as a percent of total Hb.

2.3 Estimation of p*I* with external standards

In each study, p*I* was estimated using a control hemolysate stored at –70°C as an external standard. For each analytical run, the migration times and p*I* of Hb variants present in the control (Hb A_2, S, F, A, and A_{1c} using p*I* of 7.412, 7.210, 7.060, 6.973, and 6.935, respectively) were entered into the System Gold software. Linear regression analysis was used to calculate the p*I* of Hb variants in subsequent unknown samples based on corrected migration times using Hb A as a reference peak as previously described [13]. In each study, differences between estimated p*I* for different Hb variants were determined by one-way ANOVA (SAS Institute, Cary, NC). *Post hoc* comparison of treatment means was by least-squares analysis with significant differences between treatments determined at $p < 0.05$.

3 Results

3.1 Identification of common Hb variants by CIEF

Figure 1 shows Hb variant separation by CIEF in samples containing Hb A and Hb variants S and D, S and G, or D and G. Despite the small differences in net surface charge between Hb S, D, and G, each Hb variant was separated from each of the other abnormal variants. Near-baseline resolution was observed between Hb S and G while Hb D and G were separated only at the peak. Some peak separation was apparent in all three samples analyzed containing Hb D and G, even when the D:G ratio was disproportionate (2:1 or 1:2). Identification of α-variants like Hb G-Philadelphia is facilitated by the presence of the α-variant of Hb A_2 (also called Hb $\alpha A_2'$ or G_2).

Figure 2 shows Hb variant separation by CIEF in samples containing hemolysate from the patient with Hb S/C dis-

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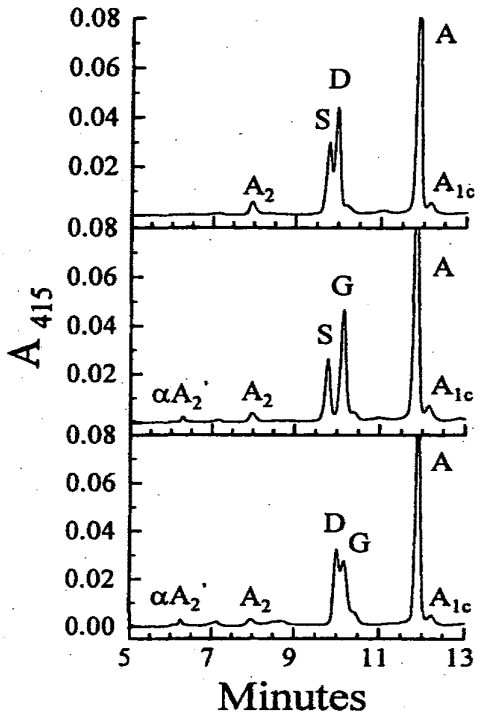


Figure 1. CIEF of Hb S, D, and G. Hemolysates containing Hb A and either Hb S, D, or G were mixed and analyzed. Peaks representing each of these common Hb variants were identifiable regardless of the combination of Hb variants present in the sample.

ease transfused with normal blood (containing Hb variants A, S, and C) mixed with hemolysate containing either Hb E, O-Arab, or C-Harlem. In each case, Hb C was separated from the other abnormal variant in the sample with baseline or near-baseline resolution. The Hb variants E, O-Arab, and C-Harlem, in contrast, all migrated in approximately the same positions. Neither peak separation nor abnormal peak shape was observed in samples containing mixtures of these hemolysates, *i.e.*, containing Hb E and C-Harlem, C-Harlem and O-Arab, or E and O-Arab (data not shown).

3.2 Precision of estimated p*I* for Hb variant identification

Table 1 shows the precision of p*I* estimates using Hb variants in the abnormal control as external standards for each analytical run. The p*I* estimates for Hb S, D, and G were all significantly different from each other and there was no overlap in the observed ranges. The calculated p*I* of Hb S differed from that of the Hb D and G by 0.025 and 0.042 pH units, respectively. The calculated p*I* of Hb D and G differed by 0.017 pH units. In conducting this study with randomized and blinded samples, the medical technologist operating the instrument correctly identified all 33 abnormal Hb variants present in 24 samples based on p*I*.

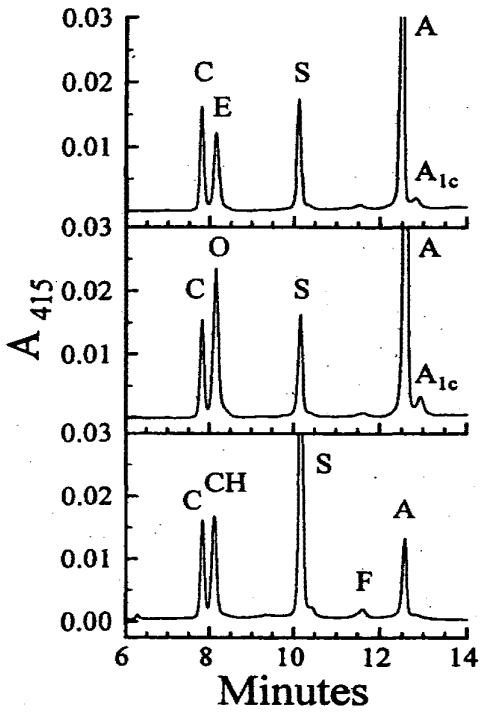


Figure 2. CIEF of Hb C, E, O-Arab, and C-Harlem. Hemolysate prepared from blood collected from a transfused patient with Hb S/C disease (containing Hb A, S, and C) was mixed with hemolysate containing Hb A and either Hb E, O-Arab, or C-Harlem prior to analysis by CIEF. Hb C was readily separated from the other abnormal Hb variants. Peak migration for Hb E, O-Arab, and C-Harlem was similar.

Table 1. Precision of p*I* estimates for common Hb variants with similar charge

Hb variant	p*I* mean (SD)	95% Tolerance interval mean ± 2 SE	Observed range
Experiment 1 (n = 11 each)			
S	7.209 (0.004)[a]	7.206–7.211	7.202–7.215
D	7.183 (0.003)[b]	7.181–7.185	7.178–7.190
G	7.166 (0.004)[c]	7.164–7.169	7.162–7.174
Experiment 2 (n = 10 each)			
C	7.440 (0.003)[a]	7.439–7.442	7.434–7.447
C-Harlem	7.410 (0.002)[b]	7.408–7.411	7.406–7.413
O-Arab	7.408 (0.002)[b c]	7.407–7.410	7.406–7.412
E	7.406 (0.002)[c]	7.405–7.407	7.404–7.410

a) b) c) Values within an experiment having different superscripts are significantly different (p < 0.05).

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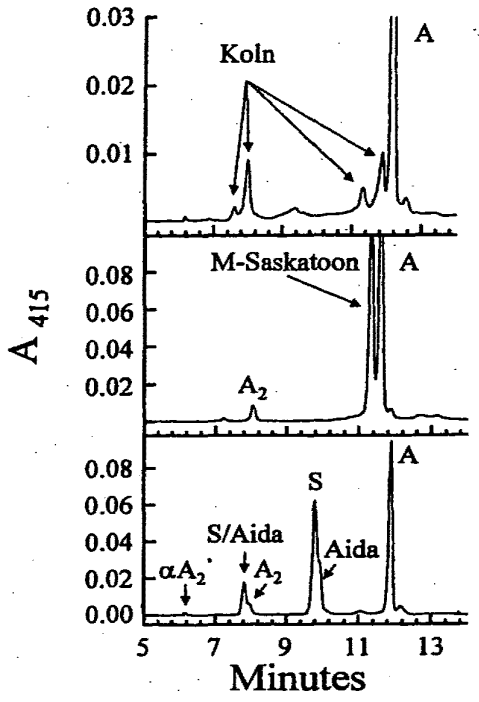


Figure 3. CIEF of Hb Koln, M-Saskatoon, Aida, and S/Aida hybrid. Analysis of hemolysates containing these rare Hb variants demonstrates the excellent separation and diagnostic sensitivity of CIEF for unusual Hb variants with p*I* similar to that of more common Hb variants.

In contrast, the estimated p*I* for Hb C was significantly higher than that of Hb C-Harlem, O-Arab, or E. The estimated p*I*s for Hb C-Harlem and E were also significantly different, but overlap in the observed p*I* ranges indicates lack of specificity for this identification based on p*I* alone. The p*I* for Hb C-Harlem and E were not significantly different from that of Hb O-Arab.

3.3 Identification of uncommon Hb variants by CIEF

Electropherograms from three unusual Hb variants analyzed in our laboratory are shown in Fig. 3 to further demonstrate the value of CIEF for primary Hb variant analysis. Hb Koln is an unstable Hb variant that arises from a mutation in the β-globin gene (98 β-Val→Met) and is typically found in individuals of European ancestry [1–3]. In this example from a patient with Hb Koln trait, Hb A was evident, as were four peaks (p*I* 7.428, 7.407, 6.993, 7.056) attributable to Hb Koln, presumably related to the loss of β globin heme. Hb A_2 was not measurable because of comigration with the p*I* 7.407 Hb Koln peak. Hb M-Saskatoon results from a mutation in the β-globin gene (63 β-His→Tyr) that produces an abnormal variant (p*I* 7.003) with high oxygen affinity and a surface charge between that of Hb F and A. Only 12% of participating laboratories correctly identified Hb M-Saskatoon as an Hb M variant in the CAP proficiency survey from which this sample was obtained (HG-B, 1997). The presence of Hb M-Saskatoon was detected by only 8% of laboratories using either alkaline electrophoresis alone or the combination of alkaline and acid electrophoresis, 21% of those using HPLC, and 55% of laboratories using some form of IEF.

The Hb S trait/Aida trait is a rare double heterozygous condition with a clinical presentation similar to that of Hb S trait. In the case presented here, the affected patient had one normal β-globin allele, one abnormal β-globin allele (Hb S, 6 β-Glu^{-}→Val), three normal α-globin alleles, and one abnormal α-globin allele (Hb Aida, 64 α-Asp^{-}→Asn). This combination of normal and abnormal alleles produced four major αβ Hb variants, including Hb A, S, Aida ($\alpha^{Aida}\beta^{A}$, p*I* 7.188, apparent as a large shoulder on Hb S), and S/Aida hybrid ($\alpha^{Aida}\beta^{S}$, p*I* 7.430, apparent as a small peak cathodic to Hb A_2). Two minor αδ Hb variants are also apparent, including normal Hb A_2 and an α-variant of Hb A_2 (Hb $\alpha A_2'$, $\alpha^{Aida}\gamma$, p*I* 7.617).

4 Discussion

Hb S, E, and C, respectively, are the three most common structural Hb variants in the world and are frequently encountered in most clinical hematology laboratories [1, 2]. Although the gene frequencies for Hb D, G, C-Harlem, and O-Arab are far lower, these variants are nevertheless occasionally encountered. The results show that Hb C, S, D, and G can be confidently identified by CIEF in a single analytical run based on automated postanalytical p*I* calculation. Analysis of several thousand patient samples by CIEF in our laboratory confirms that p*I* is a reproducible, objective, and specific criterion for identification of these four common abnormal Hb variants, and that confirmation by a second assay is unnecessary. In contrast, Hb E, C-Harlem, and O-Arab have similar p*I* and are not readily differentiated by CIEF alone. Further testing may be necessary when an abnormal Hb variant with a p*I* $\cong$ 7.410 is encountered, but the extent and manner of the testing should be decided on a case-by-case basis. For example, Hb C-Harlem can be differentiated from Hb E and O-Arab in the absence of Hb S by a positive sickle solubility test. Patient history may also help logically discriminate between these variants since Hb E is more common in individuals of Asian heritage while Hb C-Harlem is usually encountered in individuals of African heritage. However, conventional acid electrophoresis or another assay may be needed to firmly differentiate between Hb E, C-Harlem, and O-Arab.

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The fundamental role of a primary assay for Hb variant analysis is to rapidly and inexpensively identify individuals with a structural hemoglobinopathy or thalassemia syndrome. The assay must exhibit a high probability of detecting an abnormal condition (diagnostic sensitivity) even if the specific underlying mutation (diagnostic specificity) cannot always be immediately determined [13]. A low-cost primary assay that can rapidly and specifically identify all of the more than 600 known structural Hb variants [1–3] is technically unfeasible at present. Such a highly specific assay is also clinically unnecessary since many abnormal Hb variants are extremely rare and unlikely to be encountered by most laboratories in years of operation except as part of a proficiency testing program. With CIEF, most abnormal Hb variants are readily separated from normal adult Hb A and thus structural hemoglobinopathies are readily detected. The diagnostic sensitivity of CIEF for unusual Hb variants is clearly evidenced by the analyses of Hb Koln trait, Hb M-Saskatoon trait, and the double heterozygous condition Hb S trait/Aida trait. The diagnostic specificity of CIEF for rare Hb variants is presently limited only because many variants have not been analyzed by this method and information about their p*I* is not yet available. The p*I* of 46 Hb variants analyzed in our laboratory were previously reported [12], and the diagnostic specificity of the assay can be expected to improve as information about more unusual Hb variants becomes available.

A major advantage of CIEF over other analytical methods is that it economically combines excellent diagnostic sensitivity with a level of diagnostic specificity suitable for primary assessment of most common congenital Hb disorders in a single automated assay. It is important to note that this includes simultaneous detection of both structural hemoglobinopathies and thalassemia syndromes. The data presented in this report verify the use of p*I* for specific diagnosis of common structural hemoglobinopathies. We have previously demonstrated that CIEF is characterized by precise and simultaneous quantification (CV < 5%) of both major and minor Hb variants [10, 12]. The assay is therefore also useful for the diagnosis of thalassemia syndromes, especially β-thalassemias where elevated amounts of normal minor variants (Hb A_2 and F) are typically diagnostic. Low levels of Hb A_2 are also precisely measured by CIEF, but the use of these data for the detection of mild α-thalassemia syndromes has not been studied. CIEF can, however, identify Hb H and thus detect the more severe forms of α-thalassemia that cause Hb H disease [12, 14].

A number of other investigators have also reported the use of various capillary electrophoresis methods for the analysis of Hb variants [14–21]. In our opinion, however, none of these assays offer the sensitivity, specificity, precision, or throughput of the assay presented here. The CIEF method used in these experiments was optimized to meet the needs of a small to average-size hospital laboratory with a sample throughput of less than 40 samples per day. This is the approximate number of samples that can be analyzed in one day, by one technologist (~ 3 h actual labor time required), using one instrument operating at the rate of three samples per hour with some unattended overnight instrument operation. This throughput routinely permits same day or next day results and is more than sufficient for most laboratories since more rapid reporting of results does not normally influence patient outcome or cost efficiency. Rapid laboratory results are sometimes desirable, however, to minimize the hospital stay of some patients, specifically those with sickle cell disease undergoing multiple transfusions to optimize the ratios of Hb A and S prior to surgical intervention. In such cases, we have often found it advantageous that the assay is simple to set up even for analysis of a single sample. Also, unlike conventional gel electrophoresis or IEF, additional samples can be easily added to a run in progress.

Finally, we would like to note that the experiments described in this report were initiated to address questions posed by the hematology checklist of the CAP Laboratory Accreditation Program. Specifically, question 02:3812 requires that all samples identified to contain Hb S should be "further examined to ascertain whether the 'Hb S' band or peak contains solely Hb S or both Hb S and Hb D or Hb G." Our results show that CIEF can readily detect the simultaneous presence of Hb S and either Hb D or G, and that further examination of a sample showing a single peak identified as Hb S is unnecessary. Furthermore, question 02:3814 asks "are all samples that appear to have Hb S in the primary screening (by whatever method) further examined to confirm the presence of Hb S by solubility testing or other acceptable methods?" Again, because Hb S can be readily differentiated from Hbs D and G by CIEF based on p*I*, misidentification of Hb S is highly unlikely and has not been encountered in over five years of using CIEF in our laboratory. Failure to comply with either of these checklist requirements, however, represents a phase II deficiency that could lead to loss of laboratory accreditation. We therefore routinely perform a sickle solubility each time a patient with Hb S is initially identified in our laboratory. Based on the results presented here, however, we would submit that compliance with this rule is unnecessary and represents an added expense and a competitive disadvantage for laboratories using CIEF or other high resolution separation methods like HPLC [4–6]. We therefore recommend that the CAP guidelines should be amended to provide dispensation from these rules for laboratories using more advanced analytical technologies.

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This research was supported by Children's Hospital of New Orleans.

Received October 15, 1999

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Electrophoresis 2000, *21*, 749–752

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Hemoglobin A_2 quantification by capillary zone electrophoresis

Hemoglobin A_2 (HbA_2) comprises about 2.2% of the total hemoglobin in the erythrocytes. The separation and quantitation of this minor hemoglobin by capillary electrophoresis (CE) using an arginine Tris buffer is described. Some of the variables affecting the accuracy and precision of HbA_2 quantification are investigated. Furthermore, the quantification of this hemoglobin by CE is compared to that of a microcolumn chromatography method. The CE method is better suited than the microcolumn method for measuring HbA_2 in the sickle cell trait.

Keywords: Hemoglobin A_2 / β-thalassemia / Hemoglobin variants EL 3815

1 Introduction

Hemoglobin A (Hb A) is the most common hemoglobin in the normal adult. It is composed of two normal α- and two normal β-chains and it is present in most individuals at about 95–98% of the total hemoglobin. On the other hand, HbA_2 is composed of two α- and two δ-chains. In the normal individual it comprises only about 1.5–3% (median 2.2%) of the total hemoglobin. Because of the lack of β-chain synthesis in β-thalassemia there is an increase in HbA_2 with a decrease in the normal mean corpuscular volume (MCV). A mild hypochromic and microcytic anemia characterizes this clinical condition. An elevated HbA_2 level, in the presence of a normal serum iron level, is used clinically to aid in the diagnosis of β-thalassemia [1]. HbA_2 can also increase slightly in other clinical disorders such as in hyperthyroidism, hemoglobin S (Hb S) trait, and megaloblastic anemia in absence of β-thalassemia. There is also a reciprocal relationship between HbA_2 and Hb F (the fetal hemoglobin). From the analytical aspects, HbA_2 separates well on agarose gel electrophoresis; however, the densitometric scans are not accurate enough to detect the small changes in the quantity of HbA_2 [2, 3]. Microcolumn chromatography [2, 3] and HPLC [4, 5] are also used but they are either complicated or time-consuming.

Previously, HbA_2 as well as the major hemoglobin variants (A, F, S, and C) were separated by CE using a concentrated Tris buffer in a narrow diameter capillary [6]. However, the two major problems in HbA_2 are the accuracy and the reproducibility of the quantification of a very minor peak (~ 2%) in the presence of a very large peak. The precision of the HbA_2 should be < 6% for clinical diagnosis. Jenkins *et al.* [7] found CE to be suitable for Hb A_2 quantification with a CV of 8.4%. Cotton *et al.* [8] used a commercial buffer of unknown composition which coats the capillary with special propriety polyions to achieve good reproducibility. The quantification of a minor small peak in the presence of a large one is problematic in most separation methods and especially CE. In CE, several factors affect the precision and accuracy of such quantification, *e.g.*, sample size, wall interaction, integrator software, and voltage [9]. Here it is demonstrated that the major hemoglobin variants A, F, S, C, and A_2 can be separated well by CE in an arginine-Tris buffer. Some of the variables, which affect the precision and accuracy of hemoglobin A_2 quantitation, are investigated and, furthermore, the result of the CE method is compared to that of a commercial microcolumn method.

2 Materials and methods

2.1 CE Instrument

A 33 cm × 50 μm (ID) capillary was used in a Model 4000 Quanta (Waters Associates, Milford, MA) equipped with a 405 nm filter. The capillary was washed for 1.5 min between samples with 2 M NaOH and for 5 min before the instrument was shut down. The capillary was also rinsed for 1.5 min with the electrophoresis buffer between sample runs. The sample was injected hydrodynamically for 7 s and electrophoresed at 6 kV for 15 min for quantitation (or 9 kV for 10 min for qualitative separation). The data were collected using the software Peak Simple of SRI Instruments (Torrance, CA).

2.2 Electrophoresis buffer and sample preparation

Arginine (2.8 g) and Tris (2.4 g) were dissolved in 100 mL water and the pH was adjusted to 8.5 (if needed). Hemolysis solution (20 mL) was added to the buffer (stored

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Abbreviations: **Hb**, hemoglobin; **MCV**, mean corpuscular volume

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CE and CEC

refrigerated). Hemolysis solution was prepared as follows: Saponin (100 mg) was added to 4.8 g/L Tris buffer and the pH was adjusted to 8.5 with 6 N HCl (stored refrigerated). For sample preparation red blood cells were collected in EDTA tubes (10 µL) and vortex-mixed with 400 µL hemolysis solution. The hemolysates were injected on the capillary. For comparison, some samples were measured with the Beta-Thal HbA_2 Quick Column Kit (Helena Laboratories, Beaumont, TX).

3 Results and discussion

The separation of the major hemoglobin, including A, F, S, and C, is illustrated in Fig. 1. In CE as well as in the majority of the electrophoretic and chromatographic systems, HbA_2 comigrates with Hb C. Few previous studies investigated the separation of the hemoglobin variants by CE (6–8, 10, 11). The Hb F variant, which did not separate well from Hb A at pH 8.5 in the Tris buffer [6], separates better in the arginine-Tris buffer.

For the qualitative analysis of the major variants, a 9 kV voltage for 10 min gave a good separation within a short time. However, for quantification of the HbA_2, the electrophoresis voltage has to be decreased to 6 kV. As a result, of course, the separation time is lengthened to 15 min; this was necessary, however, in order to increase the relative area under the HbA_2 peak for better precision of the integration. Usually, the larger the area under the peak, the better the precision, especially for analytes at low concentration [8]. However, in spite of these conditions, we found the precision of the peak height to be much better than that of the area (Table 1) as has been found previously, mainly because of the integrator software [9]. Increasing the sample size to 20 s to further increase the peak area led to a decrease in precision due to the inadequate capillary wash of the excess adsorbed proteins. However, as far as accuracy is concerned, the peak area is more accurate and more linear than the peak height. Since the peak area was constantly lower than the peak height by an average of 8% (with the software we used) this factor was used to correct the peak height for better accuracy. Cotton *et al.* [8] found a similar CV for HbA_2 using proprietary reagents, while Jenkins *et al.* [7], using a borate buffer, found a CV of 8.4%. To speed up and improve the separation, a narrower capillary, 30 µm × 33 cm, was tried but the precision of the quantification

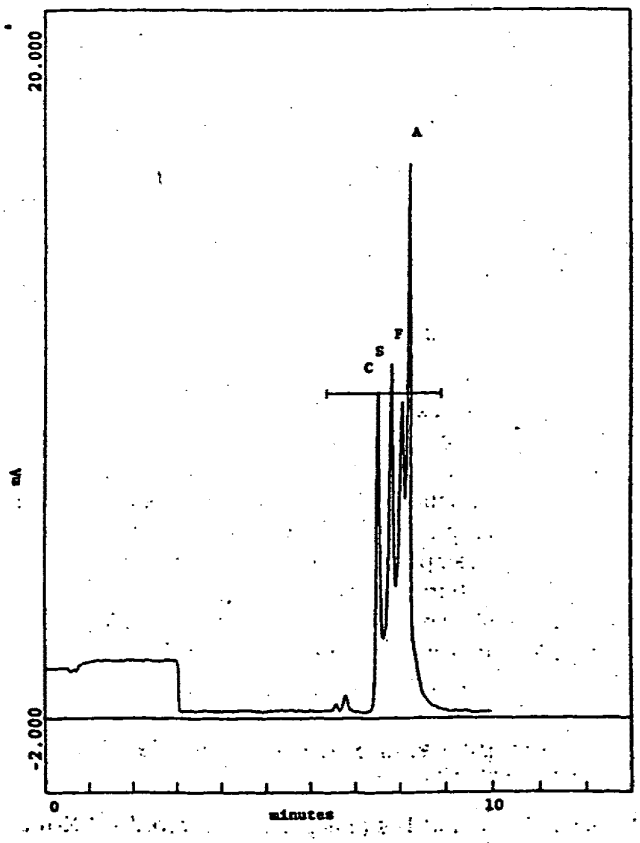


Figure 1. Separation of the major hemoglobin variants by CE at 9 kV.

Table 1. CV for HbA_2 based on area (corrected for the migration) 6tjl;and height at 7 s sample at 6 and 9 kV (n = 10).

	Area (corrected)		Height	
kV	Percentage	CV	Percentage	CV
6	2.5	9.6	2.7	5.4
9	2.6	10.7	2.7	5.7

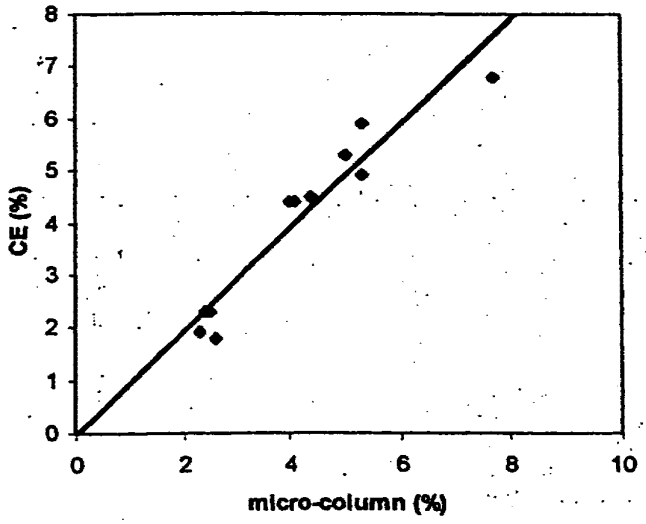


Figure 2. Comparison of HbA_2 by CE and the microcolumn method.

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was not as good as that of the 50 μm because of the decreased signal-to-noise ratio.

Since Hb C behaves similarly to hemoglobin HbA_2 in migration in this method, it can be used for checking the linearity, with some reservations. Note that when HbA_2 is over 10% this high ratio indicates that the hemoglobin is not HbA_2. Hemoglobin E, which is very common in the Far East, comigrated with Hb C and also with HbA_2 in this system, similar to that observed in the agarose electrophoresis. The described CE method was compared to that of the microcolumn (Helena Lab) for patients with normal Hb A. The correlation is relatively good ($r = 0.95$; Fig. 2) similar to the findings of Jenkins *et al.* [7]. HbA_2, which eluted from the microcolumn from a normal individual, gave a corresponding single peak on the CE as expected (Fig. 3A). However, the eluent of the microcolumn from patients who carry the Hb S variant gave two closely migrating peaks, one for HbA_2 and another one for a small amount of the Hb S (Fig. 3).

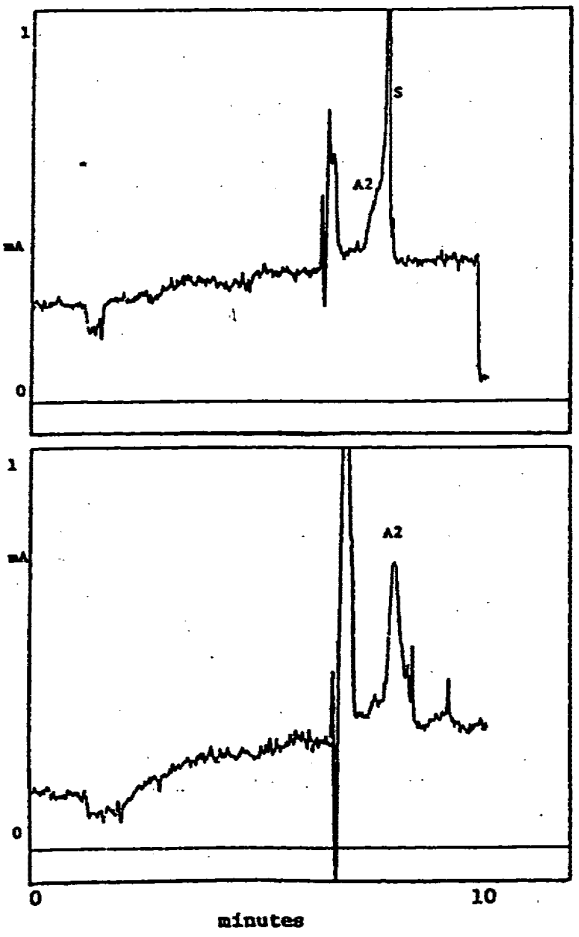


Figure 3. Separation of HbA_2 eluted from the microcolumn by CE from (top) a patient with S trait (Hb S); and (bottom) a normal patient (50 s injection).

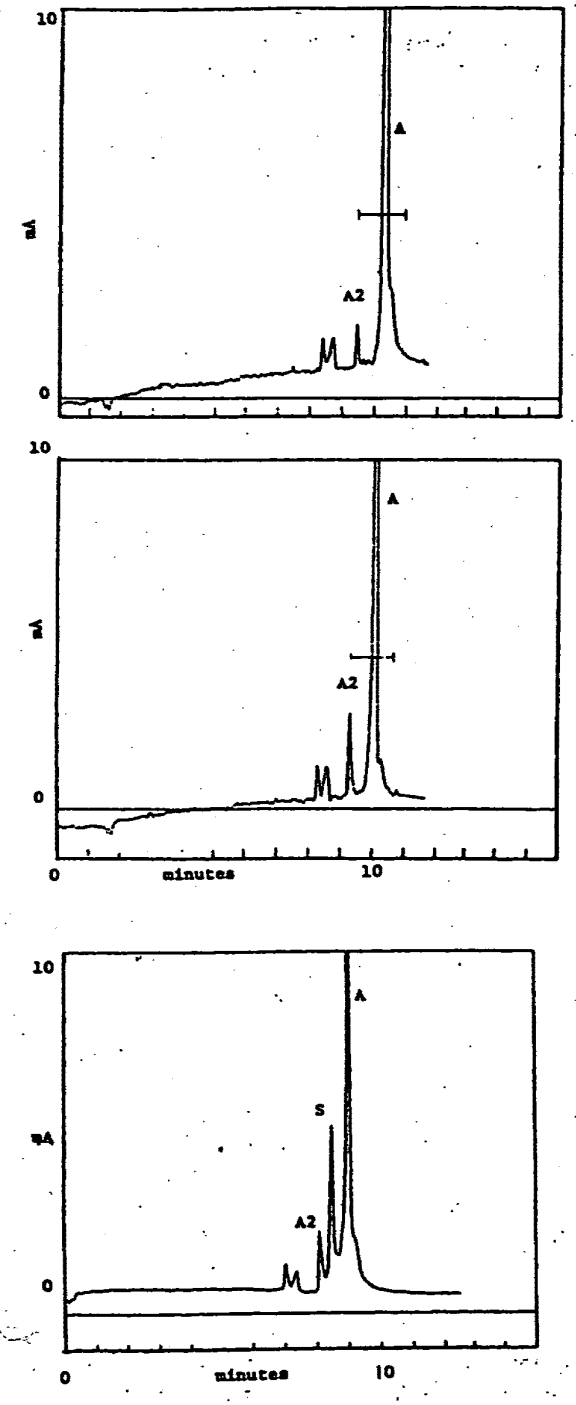


Figure 4. HbA_2 from (top) a normal patient; (middle) a patient with β-thalassemia with a low MCV, and (bottom) a patient with S trait.

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This indicated that small amounts of Hb S coeluted with the HbA_2 on the microcolumn. Peak trailing for HbA_2 has been reported earlier [3]. The coelution in the microcolumn probably depends on several factors, such as temperature, speed, lot number, *etc.* Because of the very low concentration of HbA_2 in the elution, the injected sample size on the CE was increased to 50 s, leading to poor separation between HbA_2 and Hb S in addition to a noisy baseline (Fig. 3). Thus, the CE method is more suited for measuring HbA_2 in the sickle cell trait than the microcolumn method.

The reference interval for 30 individuals with normal MCV is 1.6–3.1, which is close to that reported for this hemoglobin [1]. Figure 4 illustrates HbA_2 from a normal patient, a patient with β-thalassemia (low MCV), and a patient with the S trait. A good separation was obtained in all these cases. Several workers [12–14] have used CE with isoelectric focusing for separation and quantitation of the hemoglobin variants. These methods usually give better resolution than capillary zone electrophoresis but the quantitation and reproducibility, especially of HbA_2, can be more problematic. The advantages of CE over the agarose and the microcolumn are the speed, the low cost of operation, and the full automation. Another advantage of CE is the ability to detect visually any abnormal or possibly interfering peaks, especially Hb S.

Received July, 21, 1999

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Clinica Chimica Acta 289 (1999) 121–132

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Capillary isoelectric focusing of haemoglobin variants in the clinical laboratory

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Received 28 May 1999; received in revised form 23 August 1999; accepted 25 August 1999

Abstract

For capillary isoelectric focusing (CIEF) to be accepted in the clinical laboratory, it must be reproducible and cost effective. The advent of polyAAEE coated capillaries (Bio-Rad Laboratories, Hercules, CA, USA) has provided the means of obtaining over 100 runs per capillary, something which previously had not always been possible with coated capillaries. Using the Clinical Data Management computer program on the BioFocus 2000 Capillary Electrophoresis System (Bio-Rad Laboratories), we have used a one-step salt mobilization to achieve focusing of haemoglobin variants. Washed red cells are diluted, haemolyzed and separated in the capillary at 8 kV using 1.3% Pharmalyte ampholytes (pH 6.6–7.7/pH 6–8 2:1) in 0.40% methylcellulose. The separated haemoglobins were detected by adsorption at 280 nm. Using published values of haemoglobin variants, we investigated the use of pI markers to confirm the pI of haemoglobin variants detected. CIEF, though more expensive than capillary electrophoretic separations of haemoglobin variants, has greater resolution due to the fact that the separation of variants from pI 6.95 to 7.42 occurs over 4 min, whereas the electrophoretic separation is over 60 s. CIEF is quicker than gel IEF, and shows real-time results as the sample is being processed. The potential for CIEF in the clinical laboratory is not limited to haemoglobin variants, and the technique should become increasingly popular in the near future. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Capillary electrophoresis; Clinical laboratory; Capillary isoelectric focusing; Haemoglobin variants

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PII: S0009-8981(99)00167-9

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1. Introduction

Isoelectric focusing (IEF) separates proteins according to their isoelectric points, pI's, by the use of a medium which incorporates ampholytes (amphoteric substances of low molecular weight). On application of a voltage, the ampholytes form a pH gradient and deposit proteins at their pI at which there is no net positive or negative charge on the protein. In gel IEF, the separated proteins are visualised by stains after the ampholytes have been removed by washing.

Capillary isoelectric focusing (CIEF) was first used to separate proteins by Hjerten in 1981 [1]. It has not become popular in the clinical laboratory since then because of problems with several varieties of coated capillaries. However, with the advent of polyAAEE coated capillaries (Bio-Rad Laboratories, Hercules, CA, USA), we decided to investigate the possibility of CIEF for the identification of haemoglobin variants on the BioFocus 2000 Capillary Electrophoresis System (Bio-Rad Laboratories).

Most capillary electrophoresis (CE) instruments (e.g. Applied Biosystems 270A-HT Capillary Electrophoresis System, Perkin Elmer, Foster City, CA, USA) perform CIEF in two steps: focusing of the separated proteins by application of a voltage and then mobilization past the detector using voltage plus pressure. However, the BioFocus 2000 is unable to perform voltage plus pressure at the same time. Hence, we decided to use a one step focusing separation on the BioFocus.

Briefly, the CIEF method for the separation of haemoglobin variants is as follows: ampholytes are incorporated into the buffer and form a pH gradient when a voltage is applied. Washed red cells are diluted, haemolysed and injected onto the column and separated at 8 kV. The separated haemoglobins are detected by absorption at 280 nm. The proteins of lower pI have longer retention times. The peaks are identified by the known pI's of the abnormal haemoglobin originally established by Lehmann on polyacrylamide slabs [2]. Known pI markers can be injected with the sample to aid identification.

2. Materials and methods

2.1. Instrument

For CE, a BioFocus 2000 CE System (Hercules, CA, USA) with 24 cm × 50 μm ID polyAAEE coated capillary was used. IEF was performed using the CDM2.0A software package which had been specifically written by Biorad to allow the BioFocus to be used as an open instrument. The anode solution was 100 mmol/l H_3PO_4 in 0.40% methylcellulose whilst the cathode solution was 20 mmol/l NaOH, 20 mmol/l NaCl in 0.40% methylcellulose. The control or

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sample was injected for 30 psi's, and separated at 8 kV. The separation current was initially 1 μA, but fell to approximately 0.2 μA by the end of each run. Components passing the detector were measured at 280 nm.

2.2. Washing procedure

Between samples the capillary was washed with 10 mmol/l phosphoric acid for 60 s, 1% Brij for 60 s and distilled water for 120 s. Ampholytes 1.3% (Pharmalyte pH 6.6–7.7 and pH 6–8 mixed 2:1) were introduced for 120 s before the sample.

2.3. Patient samples

The patient samples were collected into potassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. One drop of EDTA blood was washed three times in phosphate buffered saline in an automatic cell washer. The blood was further diluted 1:5 in normal saline before 1 μl was added to the haemolyzing/methylcellulose/ampholyte mix used for the control sample.

2.4. Control

Two microlitres of AFSC Control (containing HbA, HbF, HbS and HbC) (Helena Laboratories, Beaumont, Texas) was mixed with 100 μl haemolyzing reagent (10 mmol/l KCN/5 mmol/l EDTA), 100 μl 0.40% Profocus Methylcellulose solution (Applied Biosystems, Foster City, CA 94404), 5 μl Pharmalyte ampholytes (pH 6.7–7.7/pH 6–8 2:1).

2.4.1. Haemoglobin separation by electrophoresis for comparison with the CIEF method

Measurements were carried out on a model 270A-HT CE instrument (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) equipped with a variable wavelength absorbance detector. A 72 cm long (50 cm to detector) fused silica capillary 50 μm i.d. was used. Electrophoresis was performed for 19 min at 10 kV constant voltage with detection at the cathodic end by on column measurement of absorbance at 200 nm.

The capillary was conditioned prior to each separation by sequentially rinsing for 2 min with 100 mmol/l sodium hydroxide, 2 min with distilled water, and 3 min with assay buffer (100 mmol/l boric acid, pH 9.98). The buffer was kept at 4°C and used for 3 weeks.

Patients' specimens were collected into potassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The red cells were washed three times in phosphate buffered saline in an automatic cell washer, and then diluted 1 to 60

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in 10 mmol/l KCN/5 mmol/l EDTA. Aliquots (300 μl) were loaded into individual sample cups in the 50 space autosampler. Each sample was introduced to the capillary by a 1 s 1.27×10^2 mm vacuum injection.

2.5. pI markers

We used an isoelectric focusing calibration kit Pharmacia LKB Biotechnology (Uppsala, Sweden) Code No 17-0471-01 to aid identification of isoelectric points of haemoglobin variants. The lyophilysed material was dissolved in 30 μl of distilled water. This kit was designed for isoelectric point determination of proteins in the range pI 3–10. We substituted ampholytes pH 3–10 for the pH 6.6–7.7/pH 6–8 mix when using these pI markers.

3. Results

Using CIEF we ran the AFSC control and obtained five peaks, the HbA1c separating from the HbA (see Fig. 1). The proportion of HbS and HbF were slightly elevated compared to the figures published by Helena laboratories for their Titan 111-H control. The HbC was acceptable and the HbA (including the

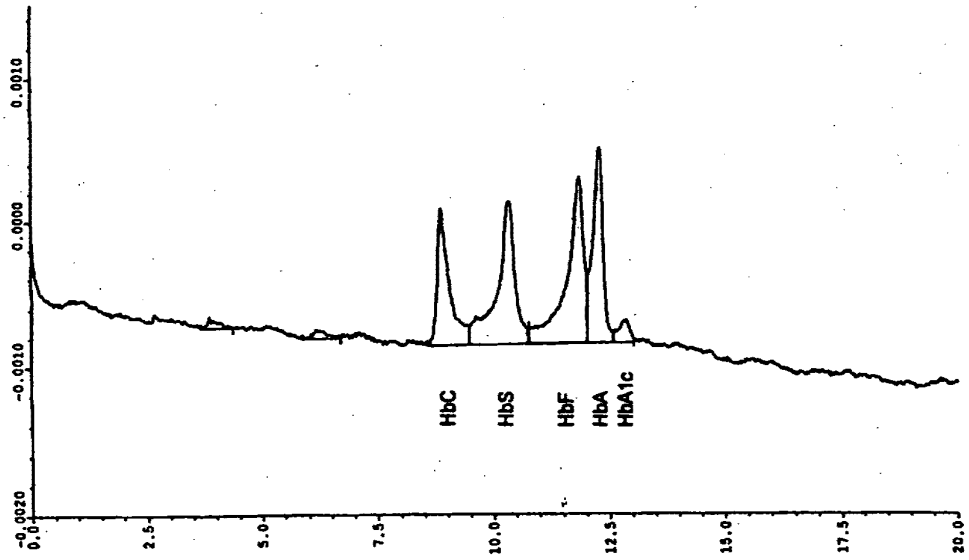


Fig. 1. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control showing from left HbC, HbS, HbF, HbA and HbA1c. Conditions of separation: 1.3% Pharmalytes (pH 6.6–7.7/6–8 2:1) in 0.04% methylcellulose, voltage of separation: 8 kV, separation time 20 min.

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HbA1c) slightly less than expected. However, there were no published figures for the technique of capillary electrophoresis for comparison purposes. To show that the method was isoelectric focusing as distinct from electrophoresis, we ran the AFSC sample by our published haemoglobin A_2 quantitation method (see Fig. 2) [3,4]. The sequence of appearance of bands by CIEF was HbC (pI 7.42), HbS (pI 7.21), HbF (pI 7.06), and HbA (pI 6.98) whereas by CE the bands appeared in the order HbC, HbS, HbA and HbF.

To the AFSC control material we added 1 μl of washed EDTA cells from a known HbS patient. We obtained once again 5 peaks, the proportion of HbA and HbS increasing as expected (see Fig. 3).

With another AFSC sample we added 1 μl of washed cells from a patient with a known HbE (pI 7.41) haemoglobinopathy. This time we observed a new peak between the HbC and HbS peaks as well as a greatly increased HbA (see Fig. 4).

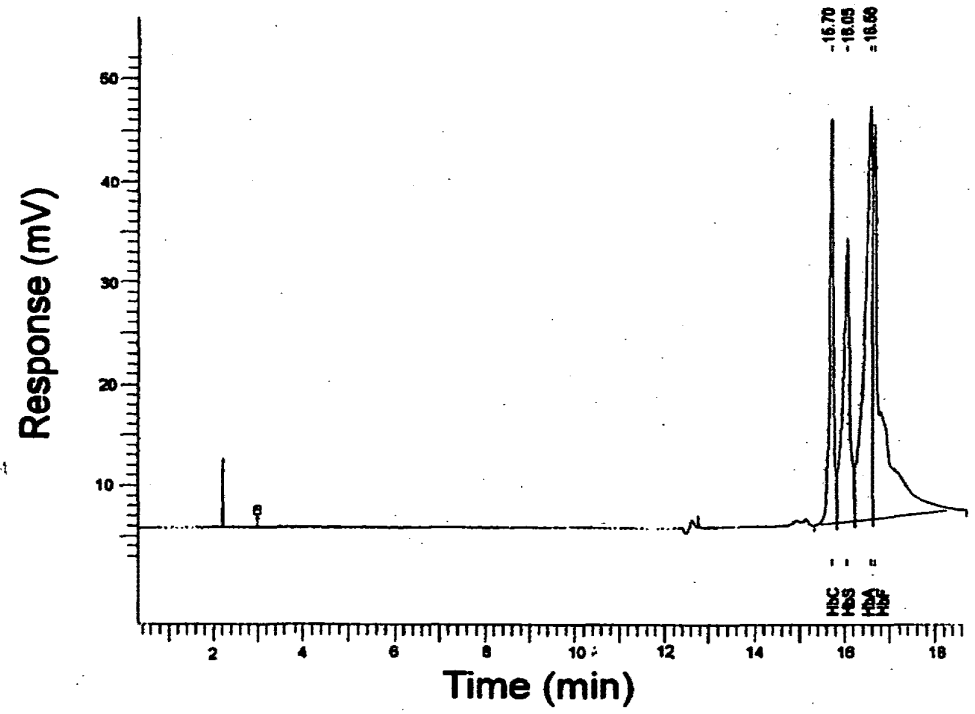


Fig. 2. shows capillary electrophoretic separation of Helena Titan III-H ASFC hemo control. From left HbC, HbS, HbA and HbF. Conditions of separation: Buffer: 100 mM Boric acid pH 9.98, voltage of separation 10 kV, separation time 19 min. Note incomplete separation of HbA and HbF.

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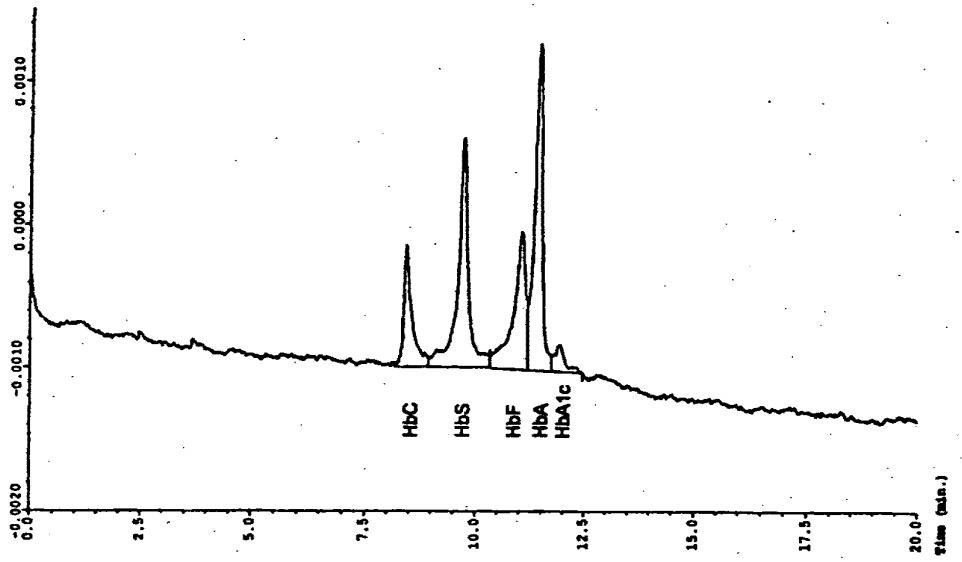


Fig. 3. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control with red cells from known HbS patient added. From left, HbC, HbS, HbF, HbA and HbA1c peaks. Note increased HbS and HbA peaks compared to Fig. 1. Conditions similar to Fig. 1.

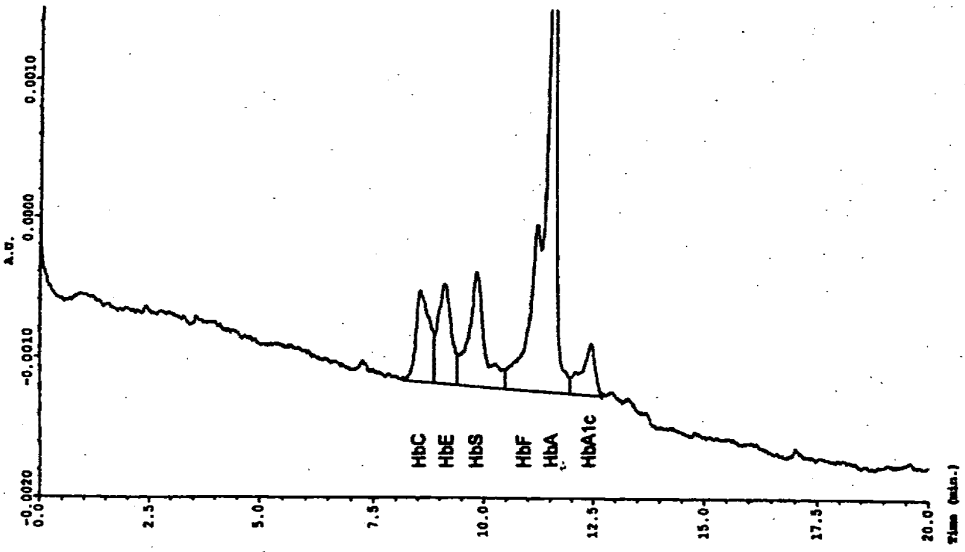


Fig. 4. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control with red cells from known HbE patient added. From left, HbC, HbE, HbS, HbF, HbA and HbA1c peaks. Conditions similar to Fig. 1.

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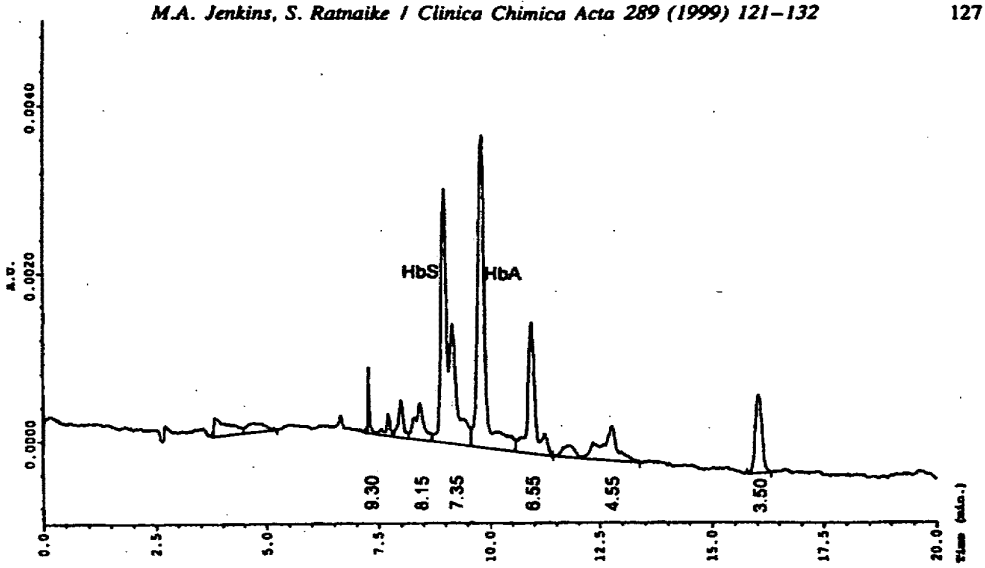


Fig. 5. Electropherogram of Pharmacia isoelectric calibration kit markers pH 3–10 plus sample from known HbS patient. pI of HbS is given as 7.21. From electropherogram, marker 7.35 appears to have slightly lower pI than HbS peak. Ampholytes changed to pH 3–10 for this electropherogram only.

We used 10 μl of Pharmacia isoelectric focusing calibration kit in haemolyzing/methylcellulose/ampholyte mixture. We added 1 μl of a sample from a patient with a HbS variant. The electropherogram obtained is shown in Fig. 5. From this electropherogram the pI 7.35 marker is incorrect with respect to the HbS variant (pI 7.21). These Pharmacia markers were designed for gel pI determination, and do not appear to transfer to CE perfectly.

We have also tried BioMark Synthetic pI markers and have found these markers to be approximately 0.4 pI unit too high (see Fig. 6). Whilst these markers have the advantage of coming in individual vials, the extremely expensive nature of the product plus their variable stability makes them unsuitable for the clinical laboratory.

4. Discussion

CIEF methods have several advantages over gel IEF. They provide automation, an ability to analyze very small amounts of protein, and can also allow quantitation of focused components. Focusing is performed in a coated capillary since effective focusing is prevented by electroosmotic flow which is characteristic of uncoated fused silica capillaries. Focused components are mobilized past the detector while the relationship of the zones is maintained: this is best

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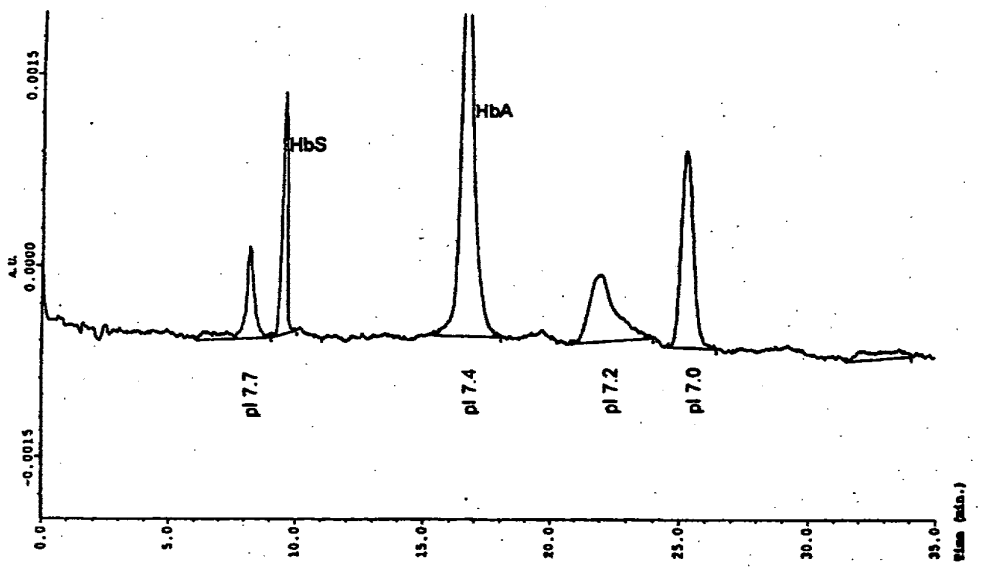


Fig. 6. Electropherogram of Bio-Mark pI markers 7.0, 7.2 and 7.7 with added sample from known HbS patient. HbA and marker pI 7.4 appear to coeluate. pI of HbA has been previously reported as 6.98 (2). Time has been extended to try to separate marker and HbA.

done by the application of voltage plus pressure at the same time. The capillary format makes direct measurement of pH gradient impractical. However, pI markers can be used as indicators of pH gradients achieved [5].

4.1. Resolution

CIEF also has advantages over electrophoretic separations by capillary electrophoresis for analysis of haemoglobin variants. There are about 600 known haemoglobin variants that show abnormal electrophoretic behaviour: over 95% of these have an amino acid substitution involving a change of charge that allows separation from HbA [3]. In a previously published electrophoretic separation for quantitation of HbA_2, the haemoglobin variant peaks have been shown to occur in a window of approximately 60 s [3]. Hence with 600 known haemoglobin variants, it is not possible to assign an unique individual time difference from HbA for each known variant. Also, using electrophoresis, there is a range of up to 6 s for each common variant peak from HbA. Since the finding of a variant may involve clinically significant human haemoglobin variants such as the unstable haemoglobins, haemoglobins with abnormal oxygen affinity, and M haemoglobins (which produce familial cyanosis), it is

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important that a second technique such as CIEF be used to assign a definitive label to a patient's haemoglobin variant peak.

4.2. Quantitation

Quantitative isoelectric focusing (IEF) on gels is not possible due to the residual ampholytes which are often not completely washed out of the gel. Residual ampholytes can be stained by Coomassie BB R-250, and when scanned at 600 nm. will show up as small peaks. Quantitation by CIEF is possible however, as the wavelength of detection is 280 nm, which does not measure residual ampholytes.

4.3. Problems

We have identified two distinct problems with CIEF which have delayed its routine acceptance in the clinical laboratory. In this paper we have discussed how these problems could be overcome.

4.3.1. Instrument

Unfortunately with the BioFocus it is not routinely possible to apply voltage and pressure at the same time. Hence other alternative mobilization techniques have to be utilized. These include salt mobilization [6], employment of a Zwitterion [7], or applying pressure of liquid height differential (referred to as gravity) with an applied voltage [8].

The method of focusing presented here does not have two distinct steps for focusing and mobilization. It is a one-step mobilization of focused components using salt mobilization [6].

4.3.2. Capillaries

Fused silica capillaries are generally not used for CIEF because of the need to eliminate or reduce electroosmotic flow. To reduce electroosmotic flow, coated capillaries (coated according to a modified Hjerten method) [9], or DB-1 capillaries (dimethylpolysiloxane-coated 0.05 · μm coating thickness, J&W Scientific, Folsom, CA), or eCAP Neutral Capillary (Beckman Instruments Inc. Fullerton, CA 92634-3100) have been used for CIEF. In the clinical setting, Hempe and Craver have used DB-1 capillaries to show excellent separations of haemoglobin variants [10]. Several industry-oriented papers have also been published using coated capillaries for IEF [6,11].

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4.4. Cleaning problems

The coating of coated capillaries is easily ruined by any strong alkali used to wash off traces of protein. Also there was a predisposition towards blockages with DB-1 capillaries, often after less than 10 runs. These blockages were often impossible to remove. Coupled with these problems was the fact that coated capillaries were often 10 times as expensive as fused silica capillaries. Hence it has been uneconomic to pursue CIEF. The poly-AAEE capillaries used here have in all cases given at least 100 runs, more often twice this number.

PolyAAEE capillaries used for CIEF cannot be cleaned with strong alkali. However, we found that a standard regime of 10 mM phosphoric acid 1 min, 1% Brij 1 min, distilled water 2 min between samples, from the first sample, substantially increased the life of the polyAAEE capillary. Our original strategy was to clean the polyAAEE capillary with distilled water for 2 min between samples. However, we found that the protein of the diluted/haemolyzed red cells was incompletely removed by the distilled water, and that fewer samples per capillary were obtained.

4.5. pI markers

pI markers in CIEF are used as indicators of the pH gradient as the capillary format makes direct measurement of the pH gradient impractical. Commercial IEF kits such as the ABI or Beckman kit provide 4 markers, Bio-Rad provide a kit (Bio Mark Synthetic pI marker kit) which contains 19 markers. We found the Pharmacia isoelectric calibration kit suitable for running with CIEF for the pH range 3–10. However, it was unsuitable for running with narrow range CIEF separations.

The AFSC control is normally stored at 4°C, and used for traditional electrophoresis techniques for up to 6 months without much deterioration being evident. Using CIEF we noted substantial deterioration of the AFSC control after a period of only 2 months (see Fig. 7). As the freezing of red cells does not produce satisfactory results by either CE or CIEF, perhaps alternative commercial control material should be investigated.

The initial laboratory investigation of thalassemias/haemoglobinopathies has, traditionally, been by electrophoresis on cellulose acetate at alkaline pH, while the use of citrate agar electrophoresis at acid pH permits further separation of several common variants that migrate similarly on cellulose acetate. Anion-exchange cellulose chromatography columns have been routinely used for HbA_2 quantitation. The consumable and labour costs involved in a traditional haemoglobin electrophoresis run of five samples, including HbA_2 quantitation by anion-exchange columns and that for CIEF/CE quantitation of HbA_2 is outlined in Table 1. We note that for a run of five samples, a traditional

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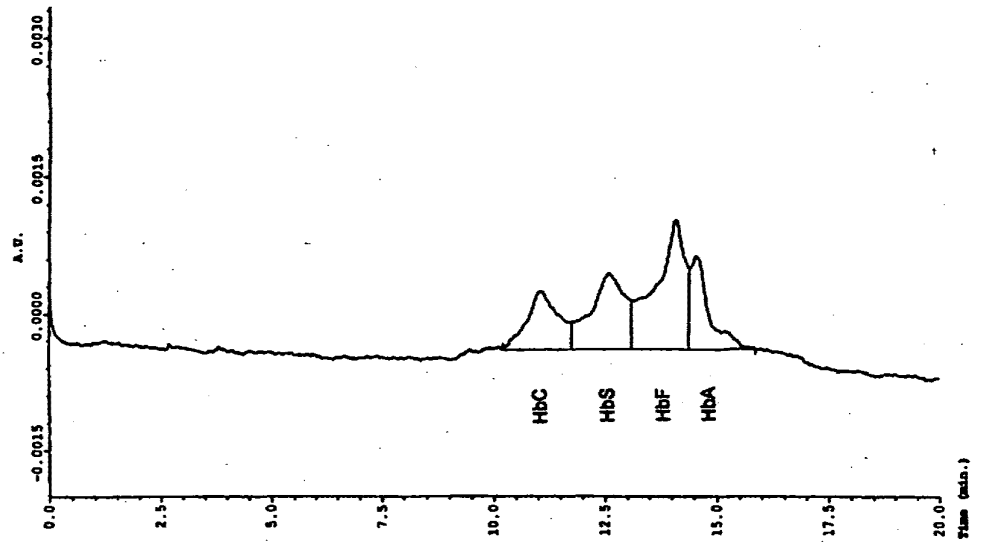


Fig. 7. Electropherogram of Helena AFSC Control stored for 2 months at 4°C. Comparison to Fig. 1 suggests this material might not be suitable for capillary electrophoresis assays after this length of storage.

Table 1
Consumable and labour costs for a run of five samples by traditional gel technology (cellulose acetate, citrate agar and anion exchange columns for quantitation of HbA_2) compared to capillary electrophoretic HbA_2 quantitation and CIEF analysis

	Consumable costs per specimen ($A)	Labour costs ($A) for run of five specimens	Total costs for run of five specimens
Cellulose acetate electrophoresis pH 9.2	$3	$14.40	$29.40
Citrate agar electrophoresis pH 6.0	$6.20	$19.20	$50.20
Anion-exchange columns	$7.24	$9.60	$45.80
CIEF	$3.35	$8.64	$25.39
CE HbA_2 quantitation	$0.15	$5.12	$5.87

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haemoglobin gel electrophoresis run with column quantitation cost $A125.40 versus $A31.26 for the CIEF/CE quantitation of HbA_2.

In conclusion, we have presented a novel approach to isoelectric focussing on the BioFocus, which has not been published previously. In this paper we have discussed how the erstwhile disadvantages of CIEF could be overcome, using the separation of haemoglobin variants as a model of protein separation. CIEF should prove a good, cost effective method in the routine clinical laboratory.

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Evaluation of hemoglobin A_2 quantitation assay and hemoglobin variant screening by capillary electrophoresis

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Abstract

To critically assess the method of capillary electrophoresis, we examined 97 clinical samples submitted for hemoglobin electrophoresis by both conventional methods (electrophoresis on cellulose acetate and citrate agar for detection of abnormal variants, ion-exchange chromatography for quantification of HbA_2, alkali denaturation for quantification of HbF, supravital stains for detection of HbH) and CE. CE was performed using a 72 cm (50 cm to detector) × 50 µm i.d. fused-silica capillary with detection of absorbance at 200 nm. Of the 97 samples examined, 34 contained a hemoglobin variant. Migration time, the difference of the variant peak to that of HbA, was used in an attempt to identify the hemoglobin variant by CE. We found the method to be suitable for the quantification of HbA_2, useful as a screening technique for the presence of hemoglobin variants, but unsuitable for the quantification of HbF.

Normal mammalian hemoglobin consists of two alpha and two nonalpha globin chains, each of which is covalently linked to a central heme group. Adult hemoglobin is >95% HbA, which contains two alpha chains and two beta chains. The minor constituents of normal adult blood consist of HbA_2, which contains two alpha chains and two delta chains, and HbF, which contains two alpha chains and two gamma chains. Disorders of hemoglobin can arise by either the production of an abnormal hemoglobin or by an aberrant production of one of the normal globin chains. The thalassemias are due to abnormal regulation of globin expression, whereas the production of an abnormal hemoglobin is referred to as a hemoglobinopathy.[1]

Clinically significant human hemoglobin variants include the unstable hemoglobins, hemoglobins with abnormal oxygen affinity, M hemoglobins (which produce familial cyanosis), and an inbalance in globin chain production that results in a thalassemic phenotype. These may include the Lepore hemoglobins, as well as chain termination mutants such as Hb Constant Spring. Less than half of the clinically significant variants have an amino acid substitution that alters the overall charge of the protein. Often there are changes in structure, such as elongation of alpha chain subunits in the case of Hb Constant Spring. Alternatively, there may be disruption of secondary structure, as demonstrated in some of the unstable hemoglobins.[2]

There are about 600 known hemoglobin variants that show abnormal electrophoretic behavior; over 95% of these have an amino acid substitution involving a change of charge that allows their separation from HbA.

The initial laboratory investigation of thalassemias/hemoglobinopathies has, traditionally, been by electrophoresis on cellulose acetate at alkaline pH, while the use of citrate agar electrophoresis at acid pH permits further separation of several common variants that migrate similarly on cellulose acetate.

Less frequently, isoelectric focusing on polyacrylamide gels and identification of certain common vari-

Indexing terms

Hemoglobin, HbA_2, HB Variants, thalassemia, hemoglobinopathy, capillary electrophoresis

Abbreviations

PCR, polymerase chain reaction; IEF, isoelectric focusing; cIEF, capillary isoelectric focusing; EDTA, ethylenediaminetetraacetic acid

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ants with monospecific antibodies have been employed.[3] The definitive diagnosis of hemoglobin variants requires either amino acid sequencing to identify the amino acid substitution or, more recently, DNA analysis. Several approaches to DNA analysis are possible, and the method chosen may depend, to some extent, on preliminary data obtained from routine screening procedures. Thus, allele specific oligonucleotide probing or restriction enzyme digestion of polymerase chain reaction (PCR) products would be the method of choice to verify the identity of a common variant.[4] DNA sequencing of the abnormal globin gene, either directly from DNA amplified by PCR, or indirectly from RNA following reverse transcriptase PCR, would be the method of choice for definitive identification of unusual variants.[5,6] These procedures, which are time consuming, labor intensive, and relatively expensive, are available only in specialized laboratories. Furthermore, they are required only for a relatively small number of samples in which an abnormality has been detected, and confirmation and identification are required. For the majority of clinical laboratories performing routine screening for thalassemias/hemoglobinopathies, a rapid, accurate, and cost-effective means of quantitating HbA_2 and HbF and for detecting hemoglobin variants, if present, is required.

In this respect, CE, because of its cost effectiveness, automation, and reproducibility, would appear to offer an effective alternative to traditional methods of screening for thalassemias and hemoglobinopathies.

Previously, both conventional electrophoresis and isoelectric focusing by CE have been used to separate hemoglobin variants. An electrophoretic approach was used by Chen et al.,[7] who chose a fused-silica capillary and a proprietary buffer at pH 8.6. Measurements of HbA, S, and C were made at 415 nm, separation of HbF being obtained with modified (unstated) conditions. The two electropherograms shown illustrate good separation of hemoglobin components. Unfortunately, the lack of detail on the buffer composition makes reproduction of this method difficult.

Ong et al.[8] have also used a fused-silica separation of hemoglobin components in normal individuals and thalassemia patients. Using a high pH phosphate buffer, the analysis time for hemoglobin variants was less than 8 min. Ishioka et al.,[9] using boric acid buffer, demonstrated the use of CE to separate two abnormal variants, HbC and HbS, in 40 min. Measurements were made at 200 nm, which is an unusual wavelength for the measurement of hemoglobin. However, at this wavelength, the peptide bond of the heme proteins was presumably being measured.

Classical gel isoelectric focusing (IEF) is labor intensive and time consuming. However, IEF by commercial CE instruments can be automated by computer-controlled sample injection, on-line detection, and data acquisition. Using coated capillaries for IEF, Hempe and Craver[10] have been able to use CE to separate HbA, A1c, F, D, S, and A_2 in less than 15 min, with a rapid screening method to separate components in 4 min. Electropherograms show excellent separation of abnormal hemoglobin variants with similar pI values. Molteni et al.[11] used a capillary isoelectric focusing (cIEF) approach in fused-silica capillaries, choosing a mixture of ampholytes that had more than twice the concentration used by Hempe and Craver. Published results appear to be of similar quality by the two methods.

Our aim was to evaluate the use of CE for the quantitation of HbA_2 and for the detection and quantification of hemoglobin variants using a prospective comparison of 97 clinical specimens by conventional hemoglobin electrophoretic methods.

Experimental

CE instrumentation

Measurements were carried out on a model 270A-HT CE instrument (Perkin-Elmer/Applied Biosystems, Foster City, CA, U.S.A.) equipped with a variable wavelength absorbance detector. A 72-cm-long (50 cm to the detector cell) fused-silica capillary of 50 µm i.d. (Scientific Glass Engineering, Ringwood, Australia) was used. Electrophoresis was performed for 19 min at 10 kV constant voltage with detection at the cathodic end by on-column measurement of absorbance at 200 nm. Quantification of the various parameters was by calculation of the area under the curve utilizing the Turbochrom IV software package (Perkin-Elmer/Applied Biosystems) supplied with the instrument.

The capillary was conditioned prior to each separation by sequentially rinsing for 2 min with 100 m*M* sodium hydroxide, 2 min with distilled water, and 3 min with assay buffer (100 m*M* boric acid, pH 9.98). The buffer was kept at 4 °C and used for three weeks. The buffer vial was replaced after every six runs to diminish "tailing" after the HbA.

Sample preparation

Whole blood specimens were collected into potassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The red cells were washed three times in normal saline in an automatic cell washer, and then diluted 1 to 60 in 10 m*M* KCN/5 m*M* EDTA. Aliquots (300 µL) were loaded into individual sample cups in the 50-space autosampler. Each sample was introduced to the capillary by a 1-sec $1.27.10^2$ mm vacuum injection.

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Control material

Commercial elevated HbA_2 material (Helena Laboratories, Beaumont, TX, U.S.A., cat. 5333), elevated HbF (Realm Biomedical, Brisbane, Q, Australia, cat. HbF-E), and AFSC control (Helena, cat. 5331) were used for HbA_2, HbF, and electrophoretic techniques, respectively. These control materials were also used for capillary electrophoresis.

Conventional Hb electrophoresis techniques

Cellulose acetate electrophoresis was performed using commercially prepared Super Sepraphore Strips (Gelman Sciences, Ann Arbor, MI, U.S.A., cat. 51040) in Tris-EDTA-glycine buffer (Gelman, cat. 51126) to which 11 g sucrose were added and the pH adjusted to 9.2.

Citrate agar electrophoresis was performed using Titan IV Citrate Agar plates (Helena, cat. 2400) in citrate buffer (Helena, cat. 5121) adjusted to pH 6.0.

HbA_2 quantitation was performed using commercial anion-exchange cellulose chromatography columns, pH 8.3 (Helena, cat. 5334). In this technique, control of the buffer pH causes different hemoglobins to manifest varying net negative charges. These negatively charged hemoglobins are attracted to the positively charged resin and bind accordingly. Following binding, the hemoglobins are removed selectively from the resin by altering the pH or ionic strength of the elution buffer (developer). HbA_2 and HbS are eluted with the A_2 developer and HbS developer, respectively. The HbA_2 fraction is eluted first and compared to the total hemoglobin by determining the absorbance of each using a spectrophotometer and then calculating the percentage of HbA_2. HbS may be quantitated in the same manner by subsequent elution of the column.

HbF was quantitated by a commercial (Sigma Diagnostics, St. Louis, MO, U.S.A., cat. 525-2) manual Betke alkali denaturation method, the HbF being measured at 415 nm.[12] The pH of the prepared Drabkin's reagent was 9.2. The International Committee for Standardization in Haematology has stated that the technique was suitable for HbF values >2% or <40%.[13] However, when HbF levels are lower than 2% or higher than 40%, this test may not give accurate or reproducible results.[14] More accurate values may be achieved by other methods such as chromatography, radioimmunoassay, enzyme-linked immunosorbent assay, or amino acid analysis. At Austin & Repatriation Medical Centre, results greater than 10% for HbF are checked by a 1 to 4 dilution with normal adult lysate.

Results

The comparison of the quantitation of HbA_2 is shown in *Figure 1*. The correlation between the two methods (excluding the HbE specimens) was 0.863. The equation $y = 0.9 x + 0.6$ indicated that the value for A_2 obtained by CE is approximately 10% higher than that obtained by chromatography. Results for HbA_2 obtained by CE for the Australian RCPA Quality Assurance Program correlated well with the commercial anion-exchange cellulose chromatography column results. CE gave baseline separation of the HbA_2 from HbE variants in contrast to HbA_2 anion-exchange columns, cellulose acetate, or citrate agar electrophoresis, none of which will separate HbA_2 from HbE variants.

The electropherograms obtained by CE for heterozygous HbS, HbE, HbD variants, and a sample from a six-week-old child with a greatly increased HbF are shown in *Figure 2*. These variants were identified by CE by the difference in migration time of the variant compared to the migration time for HbA. The variation in migration time differences for each variant are shown in *Table 1*. For homozygous HbE variants, the identity of

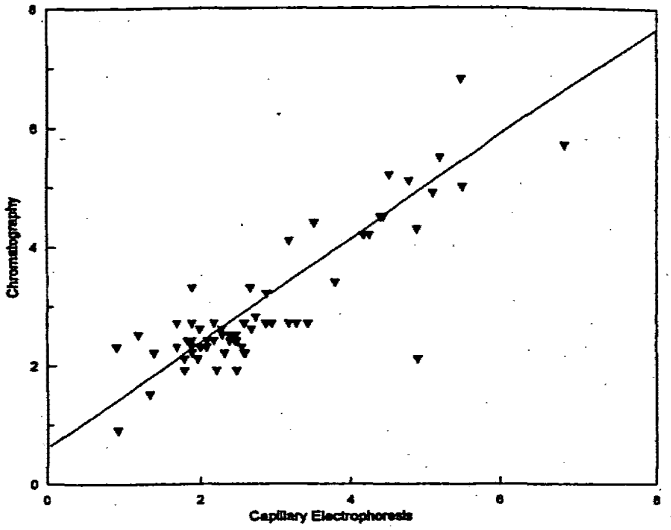


FIGURE 1 *Comparison of quantification of HbA_2 by anion-exchange cellulose chromatography columns compared to CE quantification. The equation was $y = 0.9 x + 0.6$, $p < 0.0005$, $r = 0.863$. HbE samples were excluded from comparison.*

Table 1
TIME DIFFERENCE (IN MIN) IN HEMOGLOBIN VARIANT PEAKS FROM HbA

Variant	Time difference (in min) of peak from HbA
HbC	0.93
HbE	0.66–0.79
HbS	0.55–0.62
Hb Lepore	0.53–0.59
HbD	0.48
Hb Koln	1st peak: 0.76–0.78
	2nd peak: 0.29–0.33
HbF	+0.09

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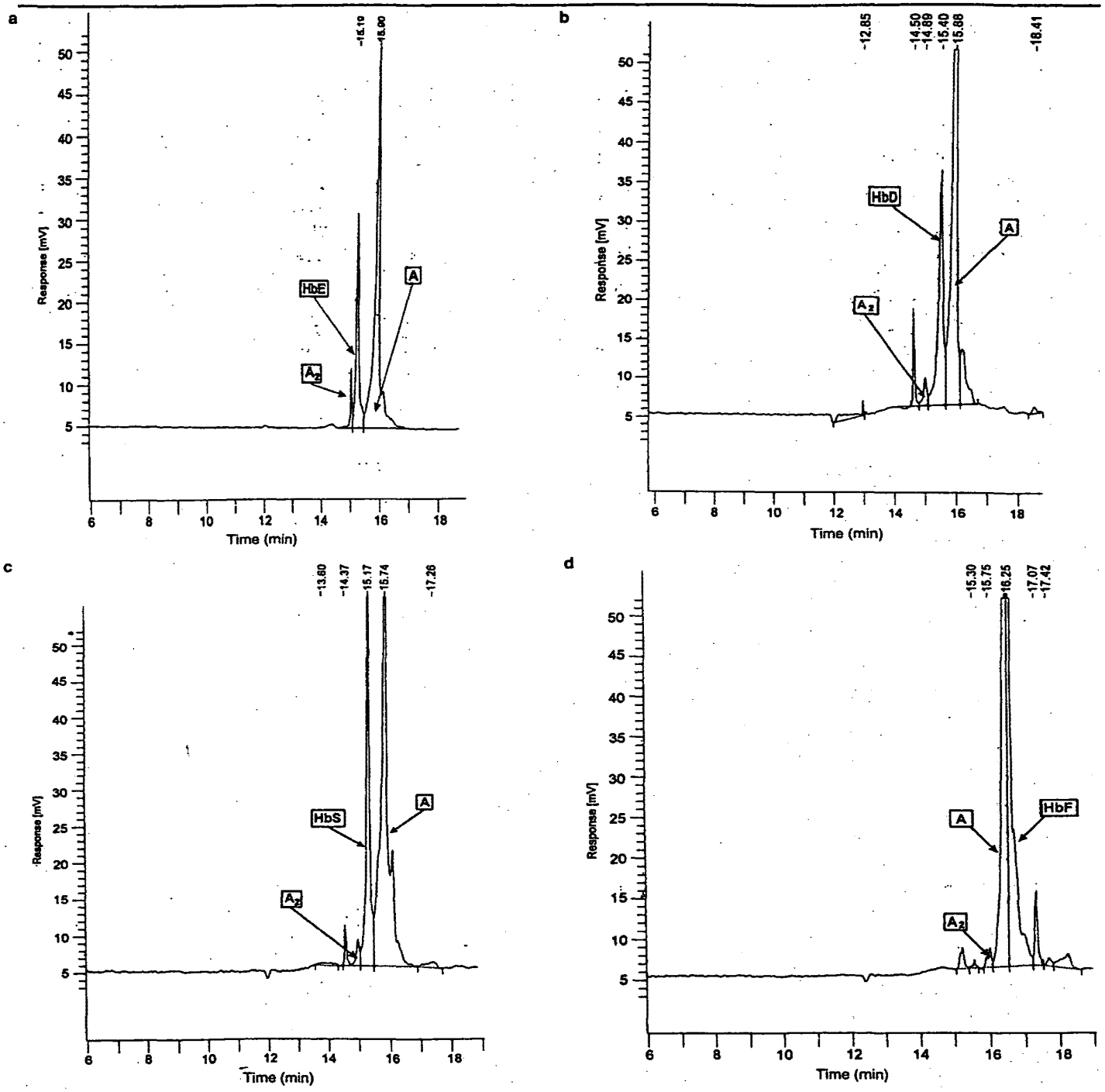


FIGURE 2 *Capillary electropherograms of a) HbE variant, b) HbD variant, c) HbS variant, and d) sample with 37% HbF from a six-week-old child. HbA and HbA_2 components are marked on each tracing. Electrophoretic conditions: 100 mM boric acid buffer, pH 9.98; injection 1 sec, 1.27×10^2 mm vacuum injection; voltage 10 kV; measurement 200 nm.*

the variant was confirmed by rerunning the sample diluted with equal amount of a normal hemoglobin sample. (See *Figure 3.*)

Of the 97 clinical samples studied, 34 contained a hemoglobin variant with a concentration ranging from 9% to 91%. The number of different hemoglobin variants detected by conventional methods is seen in *Table 2.* HbE was the most common variant detected. All of the variant hemoglobins detected by the traditional methods were also detected by CE.

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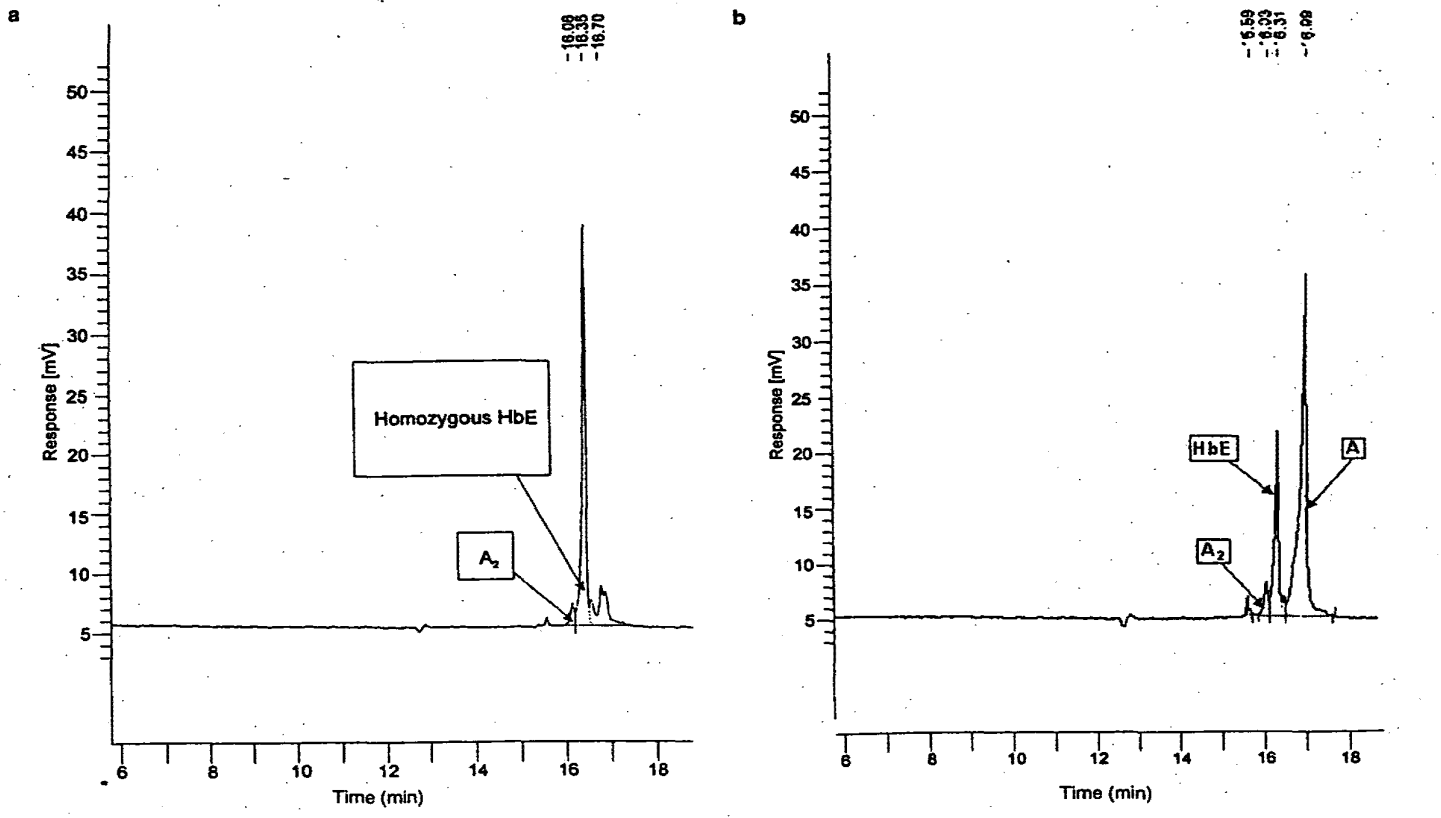


FIGURE 3 *Capillary electropherograms of a) homozygous HbE and b) homozygous HbE blood mixed with an equal quantity of blood from a normal individual. Retention time for peaks is shown at the top of the electropherograms. Electrophoretic conditions as in Figure 2.*

Table 2
DISTRIBUTION OF DIFFERENT TYPES OF HEMOGLOBIN VARIANTS DETECTED, NUMBER OF EACH DETECTED, AND WHETHER HOMOZYGOUS OR HETEROZYGOUS FOR EACH VARIANT

Variant	Number detected	Homozygous/heterozygous
HbC	1	0/1
HbE	13	5/8
HbS	11	1/10
Hb Lepore	4	0/4
HbD	1	0/1
Hb Koln	2	0/2
HbF	1	0/1

Table 3
COEFFICIENTS OF VARIATION OF HbA_2 CONTROL MATERIAL BY ANION-EXCHANGE CELLULOSE CHROMATOGRAPHY COLUMNS AND HbA_2 BY CE (N REFERS TO NUMBER OF RUNS IN WHICH SAMPLE WAS ASSAYED)

HbA_2 control	n	Concentration found	CV (%)	Time difference A–A_2
A_2 columns	16	5.00–6.60	7.74	Not applicable
CE	20	4.69–6.42	8.44	0.91–1.02

CE detected an extra peak in the sample from the six-week-old child, which also contained 37% HbF. This peak was anodal to HbA, and was also found using conventional Hb electrophoresis. The suggestion was that it could be HbH.

Since CE is a new technique for Hb variants, values have not been previously published for control material. Reproducibility studies for both methods for HbA_2 are detailed in *Table 3*. CE quantitation for a control containing the hemoglobin types A, F, S, and C is detailed in *Table 4*. However, gel quantification of the control containing the hemoglobin types A, F, S, and C was not performed.

Discussion

Correct identification of abnormal hemoglobin variants is important both clinically and for genetic counseling. Traditional screening techniques for identi-

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Table 4
COEFFICIENTS OF VARIATION OF AFSC CONTROL MATERIAL BY CE (N REFERS TO NUMBER OF RUNS IN WHICH SAMPLE WAS ASSAYED)

AFSC control	n	Concentration found	CV (%)
C	16	14.53–20.03	7.03
S	16	19.28–23.35	5.34
A	16	28.07–35.24	5.61
F	16	20.62–36.17	13.60

Table 5
ESTIMATED CONSUMABLE COST (IN A$) AND TIME EMPLOYED IN ROUTINE ASSAYS FOR DETECTION OF HEMOGLOBIN VARIANTS

Method	Consumable cost per sample	Time per run
Cellulose acetate electrophoresis (pH 9.2)	$3	35 min
Citrate agar electrophoresis (pH 6.0)	$6.20	120 min
HbA_2 anion-exchange columns	$7.24	30 min (pH 8.3) +60–90 min for HbS spec
HbF alkali denaturation	$2	45 min
Microscopic techniques for HbH	$1	60-min incubation, then 10–20 min per slide

fying hemoglobin variants and quantifying levels are time consuming and can be influenced by the operator. The current methods include ion-exchange chromatography for quantitation of HbA_2 (and separation of HbS), alkali denaturation for quantitation of HbF, supravital stains for detection of HbH, and electrophoresis on cellulose acetate and citrate agar for detection of abnormal variants. The performance of all of these methods is necessary for an adequate screen for either the presence of an abnormal variant or to detect the presence of one of the thalassemias. The times and costs of these tests are shown in *Table 5*. The total run time for CE was 19 min, with 7 min washing between samples.

Methodologies chosen in clinical laboratories must provide rapid, reliable, and cost-effective tests. This is often best achieved by the use of automated methods. While commercial CE provides reliable instrumentation, we have encountered a severe problem with dimethylpolysiloxane-coated capillaries (DB-1). Using capillaries from several different companies, we averaged only one-tenth the number of runs we were able to obtain with fused-silica capillaries. Hence, we chose a fused-silica separation for hemoglobin variants over a coated capillary method. The method described in this paper for detection and quantification of abnormal variants is estimated to have a consumable cost of less than $1.00 per sample.

With this CE method, we are able to use the same capillary with slightly different pH and ionic strength buffer to run serum protein electrophoresis, urine protein electrophoresis, and Hb variant samples. Therefore, we have automated three techniques that had previously been labor-intensive using gel technology. Using the one capillary, it is possible to change from one analysis to another with only a 5-min buffer flush between methods. Thus, in a working day, one can easily run three different types of analyses, depending on specimen demand.

The technical skills required for CE are limited to the correct dilution of the sample and preparation of the buffer. We have overcome any adhesion of proteins to the fused-silica capillary by choosing a buffer at an extreme pH at which adhesion is less likely.[15] Also, by strictly maintaining our washing protocol between runs, we have been able to achieve up to 650 runs per capillary.

The instrument is a well-engineered, automated, fully programmable analyzer, capable of running 10 different methods. The turntable has space for 50 sample vials and 8 buffer vials. It is possible to run wash cycles with each different chemistry method or a washing regime at the end of the run. Dilution of the samples is by manual pipetting. The sample vials are then placed on the turntable of the analyzer. Using a predetermined method for these hemoglobin samples, a sequence is then set in place covering the number of samples to be analyzed, and a washing protocol at the end.

With CE, HbF did not separate well from HbA for normal samples. Where the HbF was elevated, a "kink" was seen to the right of HbA. An elevated HbF of greater than 10% with no apparent hematological disorder may indicate the presence of a gene for hereditary persistence of fetal hemoglobin. It is interesting that Castagnola et al.,[16] in their fused-silica separation of HbA, HbS, and HbC, do not mention separation of HbF. From Table 4 it can be seen that the CV for HbF in the AFSC control material is roughly twice the CV for either HbC, HbS, or HbA. Our conclusion at this time is that this CE method is not suitable for quantitation of HbF.

Conclusion

As with other techniques for the detection of hemoglobinopathies, CE cannot provide definitive identification of hemoglobin variants. This is due to the fact that if the 600 known variants are examined, many variants are likely to have identical migration times. However, from the results obtained, we conclude that CE can provide a reliable and inexpensive method both for HbA_2 quantitation and for detection of the presence of hemoglobin variants in the setting of a routine clinical laboratory.

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Acknowledgment

The authors would like to thank John Styzinski, Catherine Pynta, and Sue Layh (Austin and Repatriation Medical Centre Division of Laboratory Medicine) for their technical assistance.

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Manuscript received August 4, 1997.

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Tp borate pH 10 - J6 CE/RG FR (3)

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Journal of Chromatography B, 682 (1996) 23–34

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Capillary electrophoresis as a clinical tool

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Received 28 June 1995; revised 20 November 1995; accepted 29 November 1995

Abstract

Clinical laboratories must produce accurate results for patients with a minimum turn-around time. Automated commercial capillary electrophoresis instrumentation has been available to the clinical laboratory for the past five years. Our laboratory has utilised capillary electrophoresis (CE) to automate serum protein electrophoresis. We have used the technique of CE to produce clinical results for nearly two years. CE methods are also available for the quantitation of haemoglobin variants, by both isoelectric focusing and free solution techniques. Micellar electrokinetic separations by CE have been developed for some specialised drug assays and for B-group vitamin analysis, while gel-filled capillaries have the capability to separate DNA fragments, such as PCR products. Isoenzyme analysis has shown possibilities by CE, but quantitative results are needed to be clinically useful. Analysis of amino acids for newborn screening programs and as an arterial clotting indicator are being developed. The next five years should see a proliferation of clinical laboratory methods using automated CE.

Keywords: Reviews; Proteins

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SSDI 0378-4347(95)00552-8

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1. Introduction

In the modern medical environment, patient diagnosis and treatment relies heavily on investigation procedures. With the introduction of productivity-based funding of laboratories, they are now required to provide rapid, cost-effective tests. This is often best achieved by the use of automated methods.

Capillary electrophoresis (CE) involves a separation of charged molecules in a buffer-filled capillary by the application of a very high voltage. The outstanding characteristic of CE is the high separation efficiency which can be achieved: 100 000 theoretical plates are able to be attained, at an applied voltage of 30 kV [1]. This allows an analysis time in the order of min [2], although some assays may take over 1 h for all components to be detected [3].

Areas of instrument design, injection methods and detection systems have been previously well described by a number of authors [4–6]. With commercially available CE instrumentation, automation of the assays is now available. Some instruments provide up to 50 position sample carousels, thus allowing sufficient space for a full overnight run.

The reproducibility of CE for serum protein assays in the authors' laboratory were of the order of 2.5% for intra-assay and 5.5% for inter-assay [7]. Other authors have obtained similar or slightly better precision [8].

As CE uses only nanolitres of buffer (often made in-house), the main consumable costs relate to the capillaries and sample cups. The automation of the system allows for considerably lower labour costs compared with other electrophoretic methods, including agarose gels.

The analytical capabilities of CE allow clinical laboratorians to consider automation of tests previously performed only on a manual basis. These include serum and urine protein electrophoresis, haemoglobin electrophoresis and vitamin studies. Assays for several newer drugs by CE could have significant advantages over the sometimes difficult HPLC techniques currently used.

2. Serum protein separations

The primary use of protein analysis by electrophoresis in the clinical laboratory is for the diagnosis of myeloma, a malignant disorder found in greater than 3% of the population, aged over 70 years. Myeloma is usually characterised by the finding of a monoclonal protein in the serum or the presence of monoclonal free light chains in the urine. For over twenty years, the presence of these monoclonal bands in serum and urine have been diagnosed by high resolution agarose gel electrophoresis, a procedure which is technically demanding and costly, due to it being labour intensive. Whilst there are automated instruments dedicated to gel electrophoresis, these are relatively expensive both in capital costs and consumables.

Tiselius [9], in his original "moving boundary" electrophoresis experiments in 1937, separated algae proteins and serum proteins. From his description, three peaks were obtained, one representing albumin and the other two representing globulin peaks. Jorgenson and Lukacs [1], in 1983, separated serum proteins by CE into six peaks, using a surface-modified capillary and UV detection at 230 nm. The comment was made that the classic serum bands, including albumin and globulin were apparent in the electropherogram.

Hjertén [10], in 1990, described a high resolution method for the separation of human serum in a multi-pH buffer system. Five protein peaks were detected at 215 mm using a 0.75 *M* Tris–HCl buffer system, the pH of the leading buffer being 9.7, while that of the stacking buffer was pH 6.8.

Through this early work with proteins, it was recognised that zone broadening of peaks could, in part, be related to adsorption of proteins to the walls of fused silica capillaries due to endosmotic effects related to exposed silanol groups. Two alternative approaches emerged as a possibility for minimising this adsorption: firstly, to use a coated capillary which in effect covered the silanol group and secondly, a possibility was to use fused silica capillaries, but to optimise the conditions so that adsorption is minimised.

The use of "coated" capillaries (where the silanol groups are reacted with various chemical reagents) provided a relatively simple solution to the problem of endosmosis. However, coated capillaries also introduced their own problems: it was difficult to find a compound to coat the capillary which was stable over a wide pH range. Also, reproducibility within batch and between batches of capillaries was

difficult to guarantee. In addition, certain washing procedures, such as the use of sodium or potassium hydroxide, tended to strip parts of the coating off the capillary.

Hjertén and Kubo [11] have described a high stability coating for capillaries which is attached to the silica surface via Si-O-Si-C bonds. This coating permits electrophoresis experiments to be carried out at pH extremes (such as at pH 12 or pH 0.3). The solution used for coating was a bifunctional silane (such as γ-methacryloxypropyltrimethoxysilane) mixed with acetic acid. Subsequently, the free methacryl groups were coupled to molecules containing allyl or acryl groups. Model proteins separated at pH 9.8 in these coated capillaries showed no adsorption in 50 runs performed over sixteen days.

Marked progress over the past four years has been made with serum protein separations in fused silica capillaries. In 1991, Chen et al. [12], in an industry-oriented paper, described serum protein separation into five bands, absorbance measurements being made at 214 nm. Whilst the composition of the proprietary buffer was not stated, the use of pH 10.0 ensured repulsion of the proteins from the silanol wall of the capillary.

At about the same time, Gordon et al. [13] described an approach for the minimisation of wall adsorption of proteins, by adding ethylene glycol to the protein sample which was in boric acid buffer at an acid pH, and using sodium borate decahydrate at pH 9.6 or above as the running buffer. A mixture of five individual proteins with a p*I* range of 4.6 to 6.2, as well as serum proteins from a patient with IgG myeloma, were separated using the system. Ethylene glycol multipeaks were identified either prior to the protein peaks or within the serum protein peaks. The authors felt that the resolving power of capillary zone electrophoresis (CZE) was superior to standard agarose gel electrophoresis, and also concluded that, without ethylene glycol, the electropherograms were not reproducible. Perhaps the stringent capillary washing that the authors followed also impeded the protein adsorbing to the capillaries walls. In addition, the molarity of borate and the higher pH also probably enhanced resolution. However, the electropherograms displayed were not similar to those produced by Chen et al. [12].

Later, Kim et al. [14] using a "home-made" CE apparatus similar to that described by Jorgenson and Lucas [1], assayed 37 samples, comparing the results to agarose gel electrophoresis and SDS–PAGE under non-reducing conditions. The 37 samples included normal samples as well as those from patients with various disease states, such as polyclonal gammopathy, liver cirrhosis, arthritis and chronic renal failure. All exhibited almost identical patterns in both CE and agarose gel electrophoresis. No serum from paraproteinaemic patients was reported. Linearity of response at 200 nm was established, the correlation between CE and agarose gels suggesting that CE was a reliable and reproducible technique for the clinical diagnosis of serum protein abnormalities.

Kim et al. [14] examined linearity plots of relative peak areas *versus* amount of serum proteins, and concluded that albumin and gamma fractions gave reasonable agreement compared to agarose electrophoresis. The comment was also made that the better resolution by CE compared to agarose gel electrophoresis would make it possible for more accurate quantitative analysis of serum proteins.

In another paper, Chen used a higher strength proprietary buffer at pH 10.0 to obtain up to ten peaks or inflections which related to various serum protein components [8]. This was obtained using an applied voltage of 10 kV in less than 4.5 min, absorbance measurements being made at 200 nm.

Our laboratory looked at the aspect of quantitation of serum proteins, particularly in regard to paraproteins, when we commenced investigations of serum proteins by CE [7]. By using a suitable dilution and injection time, we were able to calibrate the fused silica capillary, and quantitate the paraproteins by peak area, thus dispensing with the need for densitometry of high resolution agarose gel electrophoresis. For these measurements, 50 m*M* boric acid buffer, pH 9.7, was used, with 1 m*M* calcium lactate additive improving separation of the beta components into transferrin and C3. Monoclonal IgAs were often easier to identify, as they were separated from transferrin and C3 (see Fig. 1). Overall, the separation obtained resembled that of commercial high resolution agarose gel electrophoresis.

We then embarked on a prospective correlation of 1000 samples from patients, comparing CE and high resolution agarose gel electrophoresis (HRAGE) [15]. These specimens included 362 monoclonal paraproteins with concentrations ranging from 1 to 71 g/l. HRAGE gave slightly higher results for the

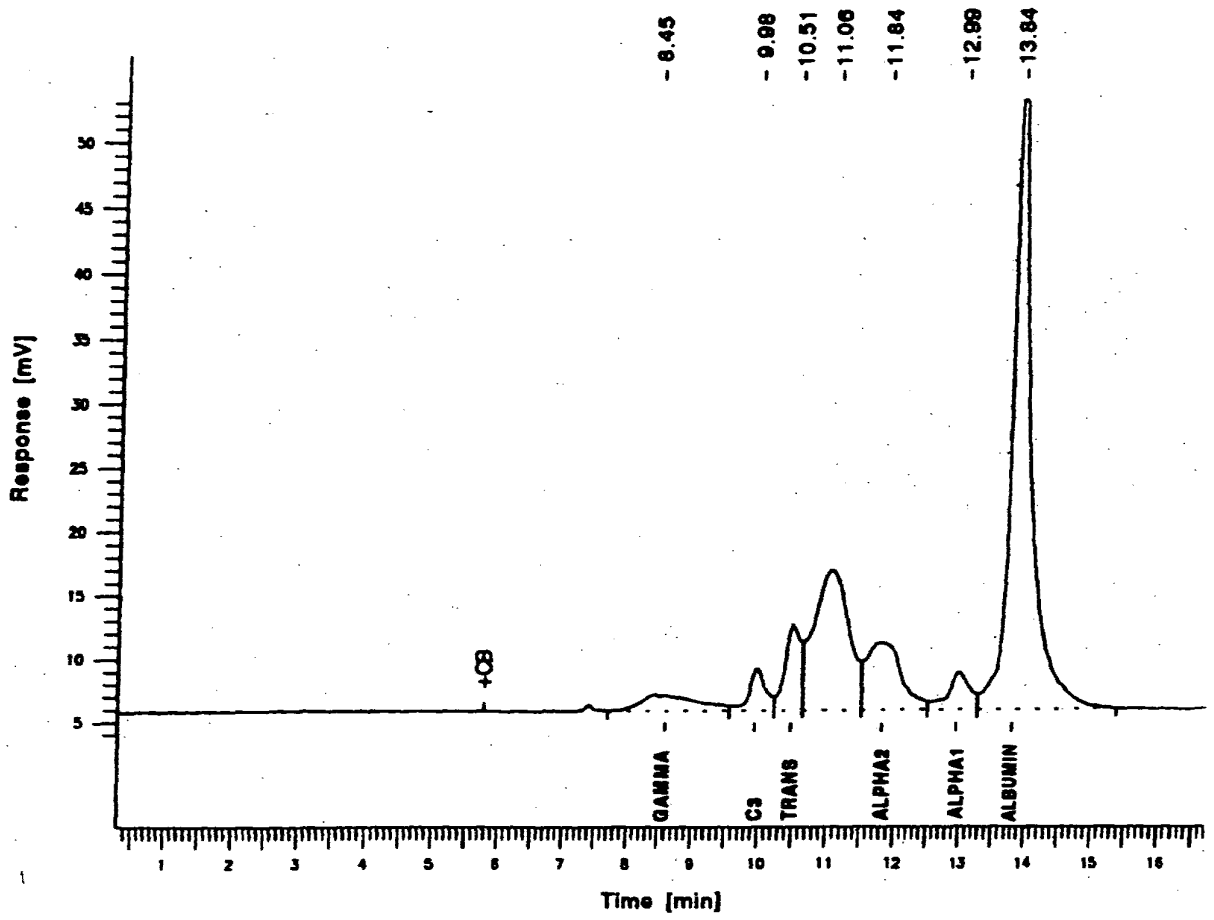


Fig. 1. Electropherogram of a monoclonal IgA detected by capillary electrophoresis showing separation from $\alpha 2$ and transferrin components. Conditions: Fused-silica capillary, 72 cm × 50 μm I.D.; detection, 200 nm; applied voltage, 18 kV; buffer, 50 m*M* boric acid, pH 9.7, containing 1 m*M* calcium lactate.

monoclonal bands, the quantitation of IgG and IgA paraproteins showing a statistically significant difference between the methods. Our conclusions indicated that automation of serum protein electrophoresis by CE was a cost-effective reality, and has been used routinely by our laboratory for nearly two years.

Identification of paraproteins after detection of a monoclonal band on agarose gel has routinely been achieved by immunofixation of either electrophoretic gels or isoelectric focusing [16–18]. Using CE, the identification of paraprotein can be based on immunosubtraction, an original concept proposed by Aguzzi and Poggi [19], in 1977. For CE identification of the paraprotein, the serum is first incubated with immunoglobulin-specific antibodies bound to solid supports, such as beads. When the specific protein is removed from the serum, a change is apparent when the treated serum is rerun [20]. This method is suitable for identification of IgG, IgA, IgM, κ and λ light chains. However, a number of complexities with the methodology lead one to question the cost-effectiveness of the procedure.

Analysis of proteins in human urine was alluded to

by Jorgenson and Lucas [21] when they separated dansyl amino acids in human urine. They were unable to identify the resulting peaks, as was Chen [12] when he dialysed urine through a membrane with a molecular mass cut-off of 14 000 [12]. Guzman et al. [22] demonstrated the co-elution of urea and creatinine. Our laboratory later identified albumin, Bence Jones Protein as well as a number of other proteins and quantitated the albumin and Bence Jones Protein by peak areas [23] (Fig. 2). This led to the correlation of albumin and Bence Jones Proteins in 71 patients by CE and high resolution agarose gels with correlation coefficients of 0.93 and 0.95, respectively. CE is now used routinely for the analysis of human urine proteins on a quantitative basis in our laboratory.

3. Haemoglobin variants

The diagnosis of haemoglobinopathies, by identification of haemoglobin variants, has relied on a variety of techniques. These include electrophoresis on cellulose acetate at alkaline pH and citrate agar at acid pH for common normal and abnormal bands. Ion exchange or affinity chromatography may be

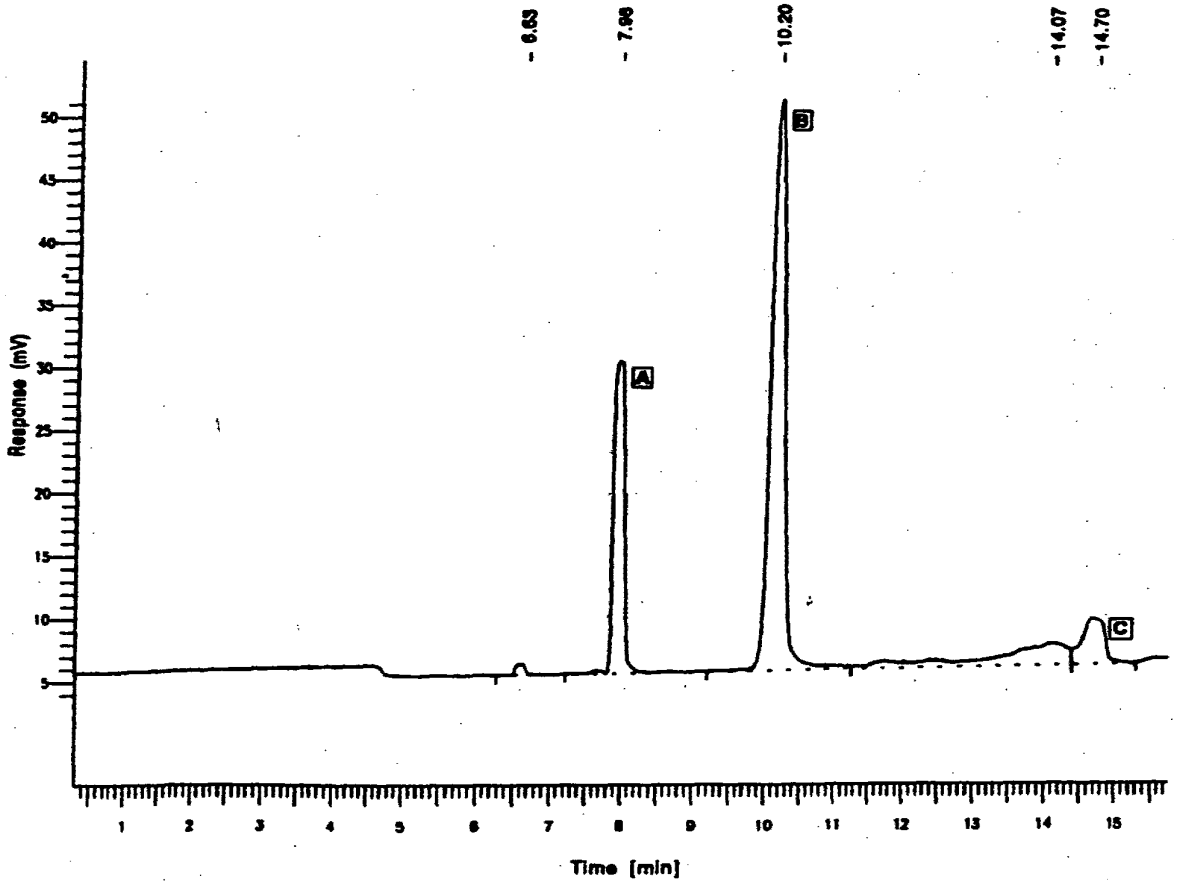


Fig. 2. Electropherogram of human urine proteins showing the components: A = Urea/creatinine; B = Bence Jones protein and C = albumin. Conditions: fused-silica capillary, 72 cm × 50 μm I.D.; detection, 200 nm; applied voltage, 18 kV; running buffer, 150 m*M* boric acid–1 m*M* calcium lactate, pH 9.7.

used for minor constituents such as HbA_2 or $HbA1_c$. HbF is characteristically resistant to alkali denaturation. Microscopic techniques have been employed for the detection of sickle haemoglobins, whilst supra-vital stain, to visualise "golf ball" cells, has been diagnostic for HbH. Isoelectric focusing and HPLC have been used to detect a wide range of variants. Using several techniques is costly due to the labour involved, and may also introduce unnecessary errors.

Two approaches have been made to the separation of haemoglobin variants by CE, these using either conventional electrophoresis or isoelectric focusing. An electrophoretic approach was used by Chen et al. [12], who chose a fused-silica capillary and a proprietary buffer at pH 8.6. Measurement of HbA, S and C was made at 415 nm, separation of HbF being obtained by slightly modified (unstated) conditions. Unfortunately, because of a lack of detail on the buffer composition, reproduction of this method would be difficult.

Ong et al. [24] have also used a fused-silica separation of haemoglobin components in normal individuals and thalassemia patients. Using a high pH phosphate buffer, the analysis time for haemoglobin variants was less than 8 min.

Classical gel IEF is labour-intensive and time-consuming. However, IEF by commercial CE instruments can be automated by computer-controlled sample injection, on-line detection and data acquisition. Using IEF, Hempe and Craver [25] have been able to use CE to separate HbA, $A1_c$, F, D, S and A_2 in less than 15 min, with a rapid screening method to separate components in 4 min. The described separations appeared excellent and should be able to be reproduced on different instrumentation with only minor modification.

One problem we have encountered with coated capillaries is the short lifetime, approximately one tenth the number of runs as compared to fused-silica capillaries. This repeated problem encouraged us to use fused-silica capillaries for the separation of haemoglobin variants. Following the work of Ishioka et al. [26], we have separated commercial haemoglobin variant control material as well as samples from patients, using a 72 cm fused-silica capillary in 12 min (see Fig. 3).

Other recent studies have demonstrated the separation of Hb variants by CE [27]. Molteni et al. [28] used a CIEF approach in fused-silica capillaries, choosing a mixture of ampholytes that had over twice the concentration used by Hempe and Craver [25]. Results published appear to be of similar quality by the two methods.

A micro chromatograph approach to the separation of haemoglobins has been made by Hjertén et al. [29]. These authors used a 0.32 nm continuous bed column prepared from piperazine diacrylamide and methacrylamide, eluting haemoglobins C, S, A and F by use of a NaCl gradient in Tris–HCl buffer. The other novel aspect of this paper is the description of a desalting and concentrating technique for small volume biological samples, using polyacrylamide gels with pores so small that only low-molecular-mass compounds could penetrate. The authors have applied this concentrating technique to a number of biological samples, including human urine, amniotic fluid and cerebrospinal fluid.

A side benefit of the haemoglobin variants work mentioned earlier was that $HbA1_c$ could also be identified. This constituent of haemoglobin reflects long-term control in diabetic patients. There is no reason why the technique for identifying haemoglobin variants could not be used for quantitating $HbA1_c$. In Hempe and Craver's pH 6–8 gradient system [25], separation of HbA and $HbA1_c$ only differ in p*I* by 0.038 pH units and were separated by 0.35 min. Methods for quantitation of $HbA1_c$ at the moment involve ion-exchange chromatography on disposable columns which are expensive, or use of monoclonal antibody haemoglobin $A1_c$ reagent cartridges, which are even more expensive. The third alternative available at present is the use of the Diamat, an instrument dedicated to analysing only $HbA1_c$, the drawback here being the initial cost of the instrument. Recently, however, a further modification has allowed expanded use of the instrumentation for haemoglobin variants.

4. Drug analysis

Routine drug analyses in the clinical laboratory are usually performed by immunoassay methods using four groups of labels, radio-isotopes, enzyme, fluorescent and chemiluminescent. A number of

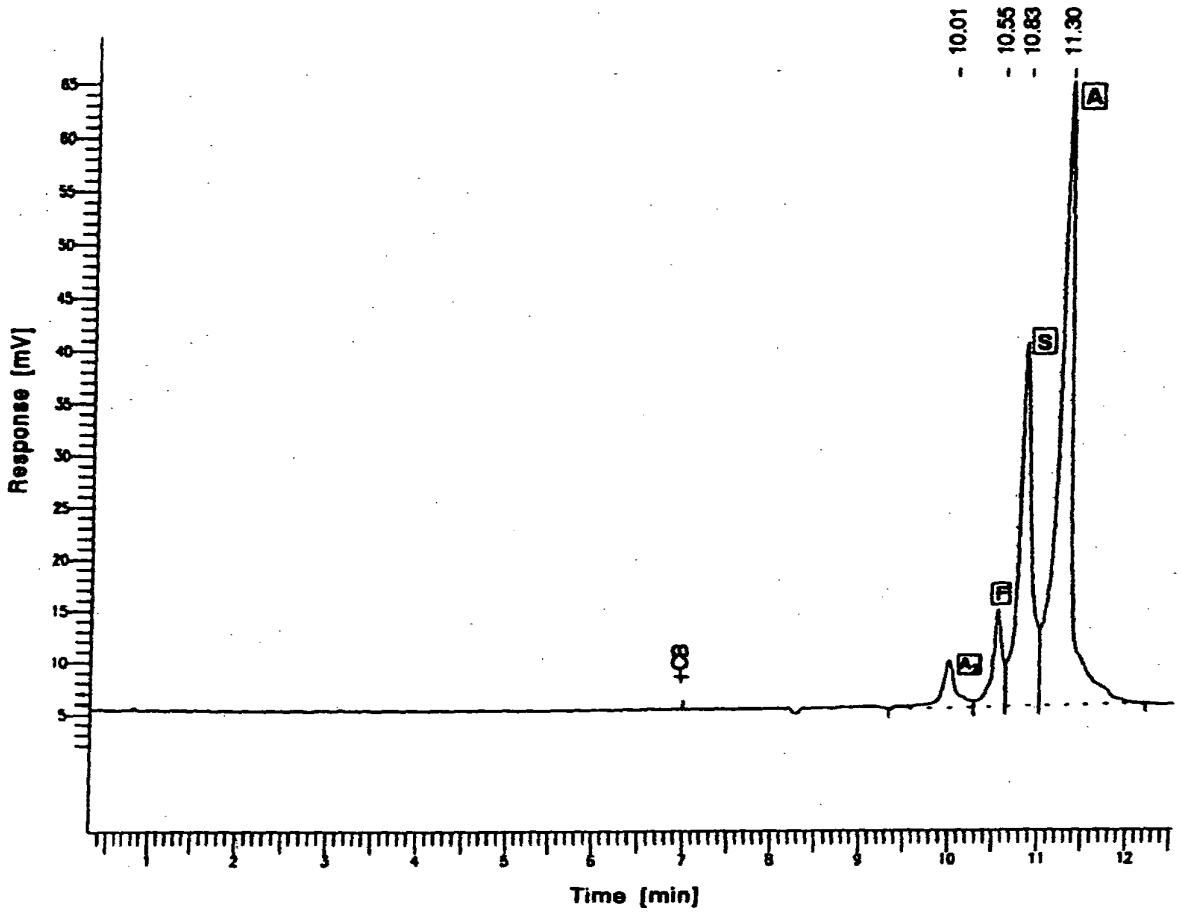


Fig. 3. Electropherogram of a Biorad haemoglobin A_2 control, showing haemoglobin A, S, F and A_2 as marked. Conditions: Applied Bi[illegible]ystem fused-silica capillary, 72 cm × 50 μm I.D.; detection, 200 nm; applied voltage, 15 kV; injection, 1 s; running buffer, 0.1 *M* boric a[illegible] pH 9.98.

these assays are now available on random access instrumentation and data-reduction is performed with automated curve fitting procedures on computers.

Newer drugs, however, are still generally analysed by HPLC. It is in this particular area, where cost is a priority, that CE may play a significant role. Following the original work of Terabe et al. [30], on micellar electrokinetic capillary chromatography (MECC) separations for neutral compounds, a substantial amount of developmental work has already been done for various drug separations by CE.

Evenson and Wiktorowicz [31] described separations of barbiturates, cardio-active drugs, benzodiazepines, anticonvulsants, diuretics and xanthines. Nishi and Terabe [32] described the analysis of penicillin and cephalosporin antibiotics. McLaughlin et al. developed practical guidelines for pharmaceutical drug separation by CE [33].

Other authors have developed methods for the separation of a single drug such as felbamate, a new anticonvulsant drug that was recently approved for the treatment of partial seizures [2], or cefpiramide, a third generation cephalosporin [34]. Shihabi [35] developed a serum pentobarbital analysis on acetonitrile deproteinized serum using CE. 3-Isobutyl-1-methyl xanthine was used as an internal standard, the

separation being monitored at 254 nm. Details of the commercial buffer were not given, which makes reproduction of the work almost impossible. In the same paper, Shihabi also demonstrated serum iohexol analysis by CE. The same internal standard was used as for the pentobarbital separation, the pH of the unstated buffer changing from 8.5, with serum pentobarbital separation, to pH 8.8, for serum iohexol.

One of the major drawbacks in analysing drugs by CE is related to serum protein interference. Nakagawa et al. [34] first reported the determination of antibiotics in human plasma by micellar separations using a direct injection method, similar to micellar HPLC. Protein interference is partly eliminated by MECC separations, as the migration time of plasma proteins is increased with an increase in surfactant concentration. Addition of SDS also eliminates the adsorption of plasma proteins to the capillary wall, thus enabling determination of drugs in plasma by the direct injection method.

An alternative approach for protein elimination of serum samples is by solvent extraction acetonitrile deproteinisation. The use of a fluorimetric detector could be beneficial for direct drug analysis from serum samples.

5. Vitamin analysis

Vitamin analyses by CE originally used MECC separations similar to the SDS preparations which Terabe et al. [30] had used to separate phenol derivatives.

Fujiwara et al. [36] reported separation of B group vitamins and vitamin C by CE, and optimised their SDS–phosphate MECC separation at pH 9.0. Although the absorption maximum of the vitamins varied from 210 nm to 270 nm, they selected 254 nm for measurements and went on to investigate quantification of vitamins using an internal standard in preference to peak-area measurements.

Nishi and Terabe [37] successfully separated eleven water-soluble vitamins in 20 min, the absorbance being measured at 210 nm.

Later, Ong et al. [3], using SDS modified with γ-cyclodextrin, separated five B group vitamins plus vitamins C–H and fat-soluble vitamins A and E. The disappointing aspect of this paper was that the separation took over 70 min per sample.

Jegle separated water-soluble vitamins in phosphate buffer at pH 7.0, using a CZE separation without assistance from any micelles [38]. He went on to analyse an over-the-counter vitamin preparation, comparing the tabled analysis to his reference sample containing eight vitamins.

Koh et al. [39] advocated a fused-silica separation for vitamin C, using 100 m*M* tricine buffer, pH 8.8, with absorbance measurements at 254 nm. A stereoisomer of ascorbic acid, isoascorbic acid, was used as the internal standard. The technique was demonstrated to be suitable for clinical evaluation of human vitamin C status.

6. Isoenzyme analysis

Isoenzymes are multiple forms of an enzyme that all possess the ability to catalyze the enzyme's characteristic reaction, but differ in structure because they are encoded by distinct structural genes. Isoforms are non-genetically determined multiple forms of isoenzymes, which result from post-translational modification of the molecule as a result of changes to glycosylation, sialation, deamidation, etc.

Isoenzyme components of lactic dehydrogenase (LDH) are known to have a pH of between 8.3 and 8.55. Wiktorowicz and Colburn [40] have shown, by CE, increased separation of these isoenzymes of LDH, using the principle of surface charge reversal, which achieves separations of basic proteins by reversing the charge on the capillary surface. Results described in their paper would indicate that this is superior to methods using fused-silica capillaries.

Trace amounts of three LDH isoenzymes have also been separated by Xue and Young [41], using laser-induced fluorescence detection and monitoring the product of the enzyme-catalysed reaction between lactate and NAD.

A third method has been reported by Klein and Jolliff [20]. These authors added substrate for the particular isoenzyme to the running buffer which was used at the pH required for optimal enzyme activity. After application of the electric field for a short time to separate the isoenzymes, the applied voltage was interrupted to allow the resolved isoenzymes to

catalyse the conversion of the substrate in the running buffer. When the electric field was re-established, the isoenzymes formed were quantitated at the detector window, by measuring absorbance at the wavelength optimum for the product.

The ability of CIEF to resolve proteins which may differ by as little as 0.005 of a *pI* unit has been demonstrated. Since isoenzymes are composed of a combination of subunits [liver and heart LDH isoenzymes, and M and B protein subunits for creatine kinase (CK)], perhaps the logical approach for the isoenzymes analysis by CE should be by IEF. As stated previously (Section 3), IEF measurement by commercial CE instruments can be automated by computer-controlled sample injection, on-line detection and data acquisition.

7. DNA separations

The usual mechanism for separation and analysis of DNA fragments has been by submarine or slab gel electrophoresis. The basis of separation is related to the size of the DNA fragments, since the charge density on large individual fragments is similar. Slab gel electrophoresis can be tedious for the application of samples and often non-quantitative and cumbersome in the isolation of individual fragments.

While submarine gels have been routinely used in the past for delineation of PCR products or DNA endonuclease digests, the possibility now exists for using gel-filled capillaries for processing these samples. An example where gel-filled capillaries could be used in the clinical laboratory is in the determination of deletions in cystic fibrosis.

Hjertén [42] introduced gel filled capillaries in 1983. Polyacrylamide gel-filled columns have been used for the separation of oligonucleotides differing by a single base. Cohen et al. [43] have separated restriction fragments using open-tube CE filled with Tris–borate–EDTA–urea–1% SDS buffer. The authors separated nineteen fragments ranging in size from 72 to 23 130 base pairs.

Karger et al. [44], using a similar Tris–borate–EDTA–urea–0.1% SDS buffer, observed that injection of heated samples proved most effective in the high resolution of deoxyoligonucleotides. Another approach for CE separation of restriction fragments is to add a cationic surfactant to the buffer [45]. This is an example of electrokinetic chromatography in which ion pairing and micelles are employed for oligonucleotide separation.

A further possible application of gel-filled capillaries is in automated DNA sequencing. Luckey et al. [46] have developed a CE instrument using a fluorescence detector capable of measuring fluorescence at four wavelengths. Detection sensitivity is the key factor in this paper, the optimum sample size being 0.4 pmol or a femtomole of DNA per band. DNA sequencing is currently a research tool but, with the introduction of molecular biology techniques to the routine clinical laboratory, could, by its nature, see the introduction of CE as well.

8. Metabolic disorders

These disorders are suspected in children with clinical indicators such as failure to thrive, unexplained metabolic acidosis, failure of neurological development or significant family history. Usually, urine or serum samples are analysed by thin-layer chromatography and paper chromatography to determine mucopolysaccharides and carbohydrates. Previously, HPLC or an automated amino acid analyser have been used for quantitative amino acid analysis and to diagnose amino acidopathies.

Amino acid analysis involving separation of phenylalanine and tyrosine in newborn screening programs is another clinical application of CE. Patients undergoing dietary treatment for phenylketonuria (PKU) may be monitored by CE [47]. CE analysis of sulphur-containing amino compounds such as cysteine, homocysteine and glutathione derivatized with monobromobimane, and detected fluorimetrically is a simple alternative to classical amino acid analysis [48]. The association between plasma homocysteine concentrations and extracranial carotid artery stenosis has recently been recognised [49].

Methods have been developed by Araki and Sako [50] for the determination of free and total homocysteine in human plasma by HPLC with fluorescence detection. Stamler and Loscalzo [51] have demonstrated a simple, rapid and reproducible method for separating cysteine and homocysteine by CE, using a phosphate buffer at pH 2.5. With detection at

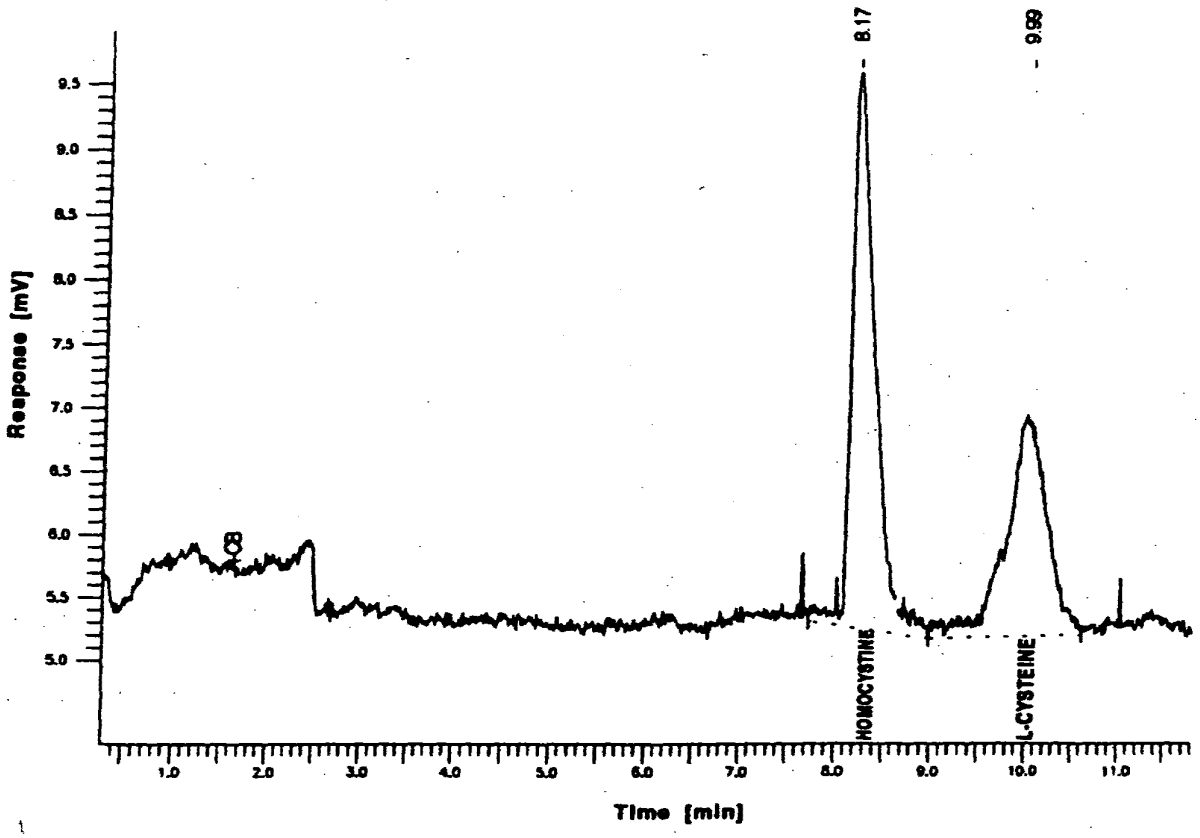


Fig. 4. Electropherogram showing the separation of homocystine (100 μM) and L-cysteine (100 μM). Conditions: fused-silica capillary, 62 cm × 100 μm I.D.; detection, 200 nm; applied voltage, 15 kV; buffer, 10 m*M* phosphate buffer, pH 2.5.

200 nm, the authors were able to show electropherograms of 250 μM homocysteine and 500 μM cysteine. We have been able to reproduce the separation of biological thiols on different equipment with a slight improvement in sensitivity (see Fig. 4). Hence, large-scale testing for plasma homocysteine may become important in the near future. A fast, sensitive and direct method has also been developed for the determination of glutathione peroxidase activity (both selenium and non-selenium dependent) [52].

9. Porphyria

The porphyrias are due to a deficiency, usually inherited, of one of the enzymes of the haem pathway, resulting in impaired haem production.

The symptoms of porphyria correlate well with the biochemical abnormalities; skin lesions varying from mild photosensitivity to severe blistering, whilst neurological disturbances such as peripheral neuritis and abdominal pain may be found. The porphyrins are classified according to whether the main site of porphyrin accumulation is in the liver or in the erythropoietic system. Diagnosis has been made previously by examination of urine, faecal or blood specimens. CE has been used by Weinberger et al. [53] for analysis of urinary porphyrins by MECC using either absorbance at 400 nm or by their natural fluorescence (excitation 400 nm; emission at 550 nm). Fluorescence was found to be about ten fold more sensitive than absorbance for the six test porphyrins studied.

10. Discussion

Automated CE instrumentation will be commonplace in the routine clinical laboratory within three to five years. Depending on the size of the hospital, it may be cost-effective solely for serum and urine protein electrophoresis. For smaller workloads, the initial cost of the instrumentation could probably be justified if haemoglobin variants and/or vitamin assays were run overnight, one or two nights per week.

We have not attempted in this review to assess available instrumentation. Prior to 1989, when a number of the current CE commercial instruments were developed, CE was a research tool, not a routine tool in the clinical laboratory. However, since 1989, clinical applications have been developed for CE. Perhaps it is time for the commercial CE companies to consider the development of a third-generation CE analyser.

Clinical laboratories would welcome any modification of instrumentation that allowed two capillaries to be inserted into the instrument, each with its own detector system. This would allow the throughput to be double. Cost analysis with electrophoretic and manual techniques would then be overwhelmingly favourable to CE.

The other area for CE instrument development must be in the area of modular instruments, particularly in the use of various detectors. If the UV–VIS detector system was able to be removed and a fluorescence detector inserted in its place, the number of analytes considered for CE in a clinical laboratory could be considerably increased.

In conclusion, there is a place for CE in the clinical laboratory. Significantly more developmental work is necessary for a number of analytes.

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Journal of Chromatography A, 709 (1995) 121–125

JOURNAL OF CHROMATOGRAPHY A

Haemoglobin analysis by capillary zone electrophoresis

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Abstract

The analysis of haemoglobin is routine in medical laboratories for the purpose of assessing blood disorders and related pathologies. Haemoglobin is structurally diverse and possesses many variant forms, some disease-producing. With recent improvements in technology, capillary electrophoresis is now being adapted in the clinical laboratory. This paper describes the analysis of haemoglobin variants using uncoated fused-silica capillaries. The effects of using different buffer salts at different concentrations with different buffer pH values to separate haemoglobin variants in these capillaries are described.

1. Introduction

Analysis of haemoglobin (Hb) variants from patients' blood samples is of medical importance and, for this reason, is an essential procedure in the clinical laboratory. Hb is a heterotetramer which is formed from the association of two pairs of identical subunits. The predominant Hb variant in normal adult blood is Hb A1, a heterotetrameric protein with two α-chains and two β-chains. Hb A2 is also a normal variant present in smaller amounts. Hb F is a form composed of two α- and two γ-globin chains and is normally expressed during foetal development; its presence is gradually replaced by Hb A usually 6 months after birth. The Hb S and C variants are isoelectric variants as a result of single amino acid changes in one of the globin chains (β), and such changes induce alterations in the structural–functional relationships of the Hb subunits to one another, such that at certain blood pH and level of blood oxygenation, the Hb tetramer undergoes an irreversible conformational shift which manifests itself as a change in the shape of the red blood cell (the classical sickle shape in sickle-cell anaemia). Hundreds of Hb variants, some producing no symptomatic disease, have been identified by various analytical methods.

The demands for clinical laboratories to provide accurate analyses with timely results makes new methodologies which achieve such goals very welcome. Capillary zone electrophoresis (CZE) is finding many uses in clinical chemistry applications [1–6], and there is the potential to apply CZE in Hb variant analysis also. CZE offers some important advantages over other conventional electrophoretic techniques. Besides requiring very small amounts of sample and electrolyte, its potential for automation makes this technique time and labour effective for clinical laboratories.

Several reports have already appeared in which normal and pathological states were assessed based on Hb variant analysis using capillary electrophoretic methods. Particular methods

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focused on the analysis of intact Hb molecules [2], globin chains [3] or tryptic digests of haemoglobin molecules [4]. Hb variant analysis has also been examined by isoelectric focusing methods using coated [5] and uncoated capillaries [6]. In this study we attempted to establish optimum conditions for Hb variant analysis using CZE with uncoated capillaries.

2. Experimental

The capillary electrophoresis system was a BioFocus 3000 (Bio-Rad, Richmond, CA, USA) equipped with a fused-silica capillary [36 cm (30 cm to the detector) × 50 μm I.D.]. Sample injection was by positive pressure [$34.45 \cdot 10^7$ Pa (5 p.s.i.) for 2 s], with sample loaded at the anode end of the capillary. The applied potential was 10 kV for all analyses. All reagents for preparing electrolytes were of analytical-reagent grade (Merck, Darmstadt, Germany). A capillary wash solution (Bio-Rad) was used to purge the capillaries between runs. Preparation of the capillary for each run involved an automated purging cycle with capillary wash solution, water and running electrolyte in that order, for 1 min each. Detection was at 415 nm using a tungsten lamp source.

Human Hb samples were prepared from whole blood collected in EDTA-containing evacuated tubes. Red blood cells were washed with normal (0.9%) saline four times. An aliquot of a washed cell suspension was mixed with four aliquots of distilled water. Haemolysis was made complete by freezing in a deep freezer for 10 min. Samples were centrifuged at ca. 600 *g* for 10 min. The supernatant was diluted tenfold in running electrolyte, and the sample was made ready by filtering it through 0.45-μm cellulose ester (Syrfil-MF; Costar, Cambridge, MA, USA).

As an external reference for haemoglobin species variants, the Beckman Paragon electrophoresis system and Hb variant standard kit (Beckman Instruments, Brea, CA, USA) was also used to analyse samples.

3. Results and discussion

The basis for the separation of the Hb variants using CZE is the charge differences in the globin polypeptides. The structure of all variants are such that they possess a net negative charge, under the conditions of CZE separation, and thus the inherent electrophoretic mobility of the Hb species will be directed towards the anode. However, the use of uncoated fused silica at pH values in the neutral or alkaline range creates a significant electro-endoosmotic flow (EOF) directed towards the cathode. The magnitude of the EOF is such that the net mobility of all Hb species is towards the cathode. This property means that Hb species having higher isoelectric points (p*I* values) (i.e., species with less net negative charge) should be detectable first inasmuch as the electrostatic repulsive forces in the opposite direction are less than for Hb species having lower p*I* values, and they would show a higher net mobility towards the cathode.

Satisfactory separation of analytes using CZE is highly dependent on a suitable electrophoresis buffer–electrolyte. In addition to ensuring overall stability of the system, the buffer determines the migration behaviour of analytes. Several different buffers have been used for haemoglobin separations in traditional free zone electrophoresis applications. Some methods employ a single weak acid or base to provide the buffering at the running pH; others consist of a mixture of weak acids or bases. As the p*I* values of haemoglobin species variants are between 7.00 and 7.50 (Hb A1, 7.15; Hb A2, 7.40; Hb F, 7.15; Hb S, 7.25), preparation using buffers with the pH adjusted above these values becomes necessary. Tris and borate buffers are commonly selected because of their buffering capacity ranges between pH 8.0 and 9.5. Veronal (barbital) buffers also possess a buffering capacity in this range and are commonly used in electrophoretic systems involving the use of commercially available agarose gels to analyse Hb variants. We tested Tris, borate and veronal buffers at three different concentrations and at different pH values. Analysis with a Tris–HCl electrolyte was carried

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out at 0.5, 1.0 and 1.5 *M*, sodium borate was tested at 20, 30 and 40 m*M* and veronal (sodium barbiturate) at 25, 50 and 75 m*M*. For each of these concentrations for each of the indicated salts, three different pH values were tested, namely pH 8.0, 8.5 and 9.0.

The electropherogram in Fig. 1 illustrates how the molecular charge difference between the Hb S and Hb A1 species affects their separation by CZE. This separation of these variants was also generally observed under a variety of conditions in which all permutations for the three different buffer salts, concentrations and pH were tested. The Hb A2 species is seen to migrate ahead of Hb S in Fig. 1. We found that the degree of separation of Hb S from Hb A1 was not significantly different for any change in the pH (8.0, 8.5, 9.0) of the salts tested, but there were certainly changes in migration times, an increase in pH producing shorter migration times for all species. The inset in Fig. 1 shows a densitometric scan of stained haemoglobin variants observed using the Beckman agarose gel electrophoresis system. Although we did not confirm the identity of Hb variant species during the CZE analysis of our samples using certified standards, the agarose gel analysis operates under identical principles to CZE and the densitometric scan of stained proteins in the gel compares favourably with the absorbance changes during CZE in which detection is specific for the Hb prosthetic group.

The variant Hb F has no significant charge difference from Hb A1 (only 0.05 pH unit separates these variants), and this presents the most difficult challenge to manipulating conditions to separate common variants. Fig. 2 shows the separation of Hb F and Hb A1 from a patient's sample using a 50 m*M* veronal system buffered at pH 8.5. An observable separation of these species was achieved for all pH values tested, but the resolution appeared better at pH 8.0 and 8.5 than at pH 9.0, demonstrating an effect of pH on this separation. Again, the

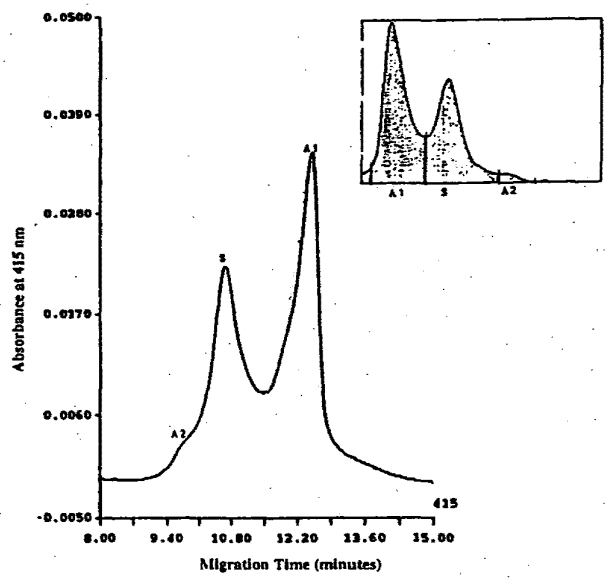


Fig. 1. Electropherogram of a sample from a patient with sickle-cell disease. Electrolyte, 1.0 *M* Tris–HCl (pH 8.0). Other conditions as described under Experimental.

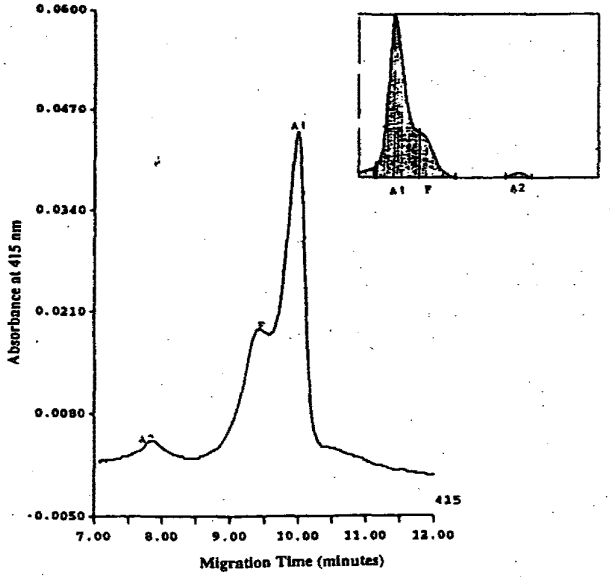


Fig. 2. Electropherogram of a patient's sample with a significant Hb F fraction. Electrolyte, veronal (sodium barbital) (pH 8.5).

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proportions of the amounts of Hb species present in this sample agreed with the agarose gel determination (inset).

Fig. 3 is an electropherogram of another patient's sample in which Hb variants were separated using 20 m*M* sodium borate (pH 8.5). The Hb variants A2, S, F and A1 were all detectable. These particular conditions resolved these four variants the best, although we did observe the separation of these variants using this buffer at other pH values. For selecting particular buffer salts to be used in such CZE separations, we found the best concentration for Tris to be 1.0 *M*, for veronal 50 m*M* and for borate 20 m*M*.

Variations in the migration times of the species for replicate analyses were a significant problem and make routine clinical use difficult, in that species identification depends on consistent migration times (much as component identification in high-performance liquid chromatography depends on consistent retention times). We made attempts to stabilize the EOF, which we believe is the factor responsible for such irreproducibility, but we were unable to obtain more optimum conditions. As the Hb A1 variant is usually a constant in all analyses, its identification can be made initially and then used as a reference for identifying other variants. Fig. 4 is an electropherogram of a normal adult blood sample with Hb A1 present as the major species. There are other cases in which Hb A1 may not be the major species present, as shown in Fig. 5 (identification of Hb species in CZE were made by comparison with separated zones seen in agarose gel electrophoresis, a routine method in our laboratory as well as in many others). Such cases might present some confusion for the analyst. Nevertheless, the experienced analyst would know the relative order of migration of the typical species present in the population, and variations in routine electropherogram patterns can be easily discerned. Alternatively, unknown patients' samples can be run with internal standards (e.g., with commercially available Hb variant standards) to make Hb variant identification unambiguous.

Changes to the conditions of buffering (selection of the ideal salt and therefore of the ideal

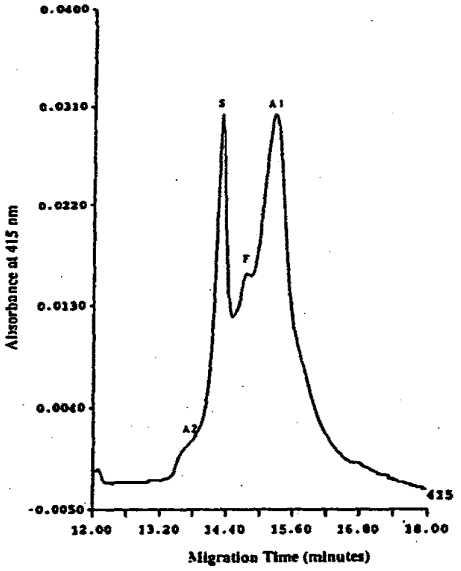


Fig. 3. Electropherogram of a patient's sample containing significant proportions of variants Hb A1, A2, S and F. Buffer, sodium borate (pH 8.5).

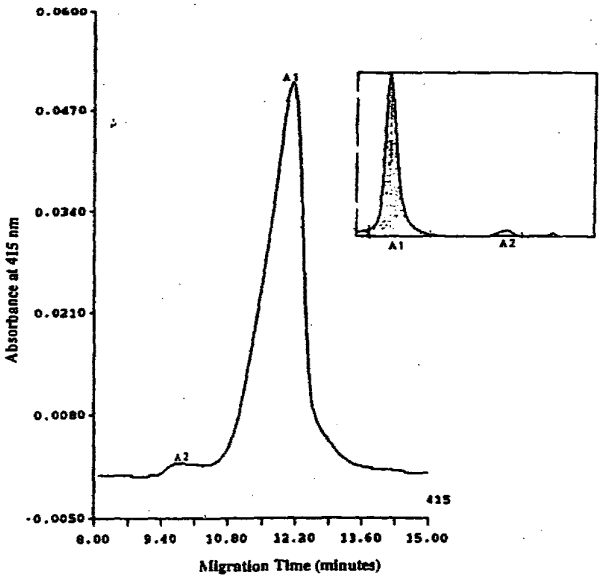


Fig. 4. Electropherogram of a sample obtained from a healthy adult. Buffer, veronal (pH 8.5).

Absorbance at 415 nm

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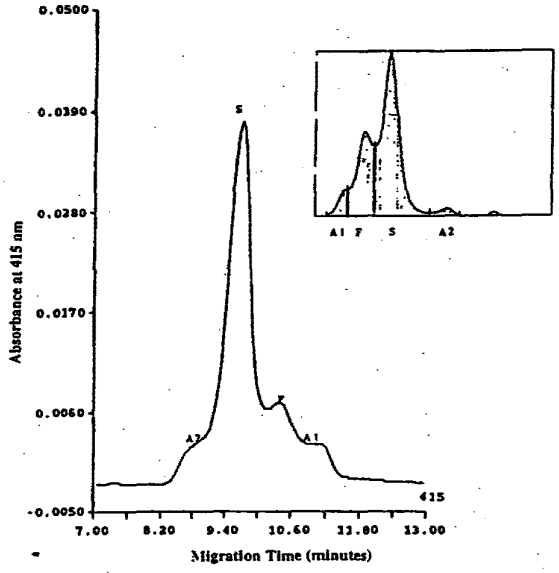


Fig. 5. Electropherogram of a sample from a patient with sickle-cell disease. Buffer, veronal (pH 8.5).

pK_a, electrolyte pH and buffer concentration), in addition to selection of modifying agents which dynamically coat the capillary walls in order to alter protein adsorption and to reduce the EOF to negligible levels, are future considerations for further work to understand and control the considerable variations of migration times observed in replicate analyses.

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BIOMEDICAL CHROMATOGRAPHY, VOL. 6, 224–226 (1992)

Detection of Abnormal Haemoglobin by Capillary Electrophoresis and Structural Identification

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Haemoglobin obtained from a male adult Ghanian with retinopathy, which was probably caused by haemoglobinopathy was analysed by capillary electrophoresis (CE) for clinical diagnosis. Two major peaks, which were in the ratio of nearly one, were detected. The elution times of these peaks (HbXI and HbXII) were faster than that of normal haemoglobin (HbA). The existence of two different abnormal types of haemoglobin was clear in the patient blood. The following sequence analysis revealed that the first peak (HbXI) was HbC and the second (HbXII) was HbS on the electropherogram, and that the patient was a heterozygote of HbS and HbC (HbSC disease). One of the diagnostic processes in a haemoglobin disease was shown by the combined use of CE, HPLC and a protein sequencer.

INTRODUCTION

Capillary electrophoresis (CE) is a powerful and widely utilized technique for analysis of biologically important molecules (Jorgenson 1984; Jorgenson and Lukacs, 1981[a,b], 1983; Tsuda *et al.*, 1983; Gassmann *et al.*, 1985; Lauer and McManigill, 1986[a,b]; Cohen *et al.*, 1987; Gordon *et al.*, 1988; McCormick 1988; Lüdi *et al.*, 1988; Ewing *et al.*, 1989; Kennedy *et al.*, 1989; Grossman *et al.*, 1989; Gross and Yeung, 1990; Zhu *et al.*, 1990; Chen *et al.*, 1991). Furthermore, CE analysis has become feasible for the direct detection of biological microenvironments, specifically at the single cell level (Ewing *et al.*, 1989; Kennedy *et al.*, 1989), and has been used to separate small variations in the primary and/or secondary structure of peptides and proteins (McCormick, 1988; Lüdi *et al.*, 1988).

Since the discovery of haemoglobin S (HbS) abnormal haemoglobin has been studied intensively for medical and genetic purposes. The most popular methods for analysis of abnormal haemoglobin are gel electrophoresis and high performance liquid chromatography (HPLC) on ion exchangers. Electrophoretic analysis has made possible the discovery of numerous haemoglobin variants. The recent developments of the CE procedure offer a new approach for the rapid analysis of a micro amount of abnormal haemoglobin for clinical diagnosis (Chen *et al.*, 1991).

In this study, we employed the CE method prior to the popular and conventional methods to analyse and confirm the existence of haemoglobin variants in the blood of a patient who was a suspected case of haemoglobinopathy because of the presence of leptocytes and characteristic retinopathy.

EXPERIMENTAL

Materials. Haemoglobin solutions were prepared from the blood of both a male adult Ghanian with retinopathy in a suspected case of haemoglobinopathy and a normal adult Japanese. L-(Tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was obtained from Nacalai Tesque (Kyoto, Japan) and trifluoroacetic acid (TFA; sequence grade) was from Wako Pure Chemical Industries (Osaka, Japan). Biofine IEC-CM (7.5 mm i.d. × 75 mm) for a cation exchange column was from Japan Spectroscopic Co. (Hachioji, Japan) and Develosil 300 ODS-7 (4.6 mm i.d. × 250 mm) for a reversed phase column was from Nomura Kagaku (Seto, Japan).

Capillary electrophoresis. CE was performed on the Model 270A capillary electrophoretic system from Applied Biosystems (Foster City, MI, USA). An uncoated fused-silica capillary (50 μm i.d. × 122 cm length) was used. Before electrophoresis, the capillary was washed with 1 N NaOH, rinsed with distilled water, washed again with 1 N HCl, rinsed with distilled water, and equilibrated with 0.1 M sodium borate buffer (pH 9.98). Three nanolitres of haemoglobin solution (3 mg/mL in water) was introduced into the capillary by vacuum injection for 1 s. Electrophoresis ran at 30 KV constant voltage for 50 min at 30°C using 0.1 M sodium borate buffer (pH 9.98), and was monitored simultaneously at 200 nm.

Sequence analysis. The sequence analysis of globin chains was performed by the methods described previously (Ishioka *et al.*, 1986). The purified globin chains from abnormal haemoglobin were reduced and carboxymethylated and then digested with TPCK-trypsin in 0.1 M ammonium bicarbonate solution at 37°C for 24 h. Tryptic peptides were separated by reversed phase HPLC using conditions described in detail previously (Ishioka *et al.*, 1990). Elution for tryptic peptides from α chain was for 40 min with a linear-gradient of 0–42% acetoni-

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0269–3879/92/050224–03 $06.50
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Received 21 January 1992
Accepted 9 March 1992

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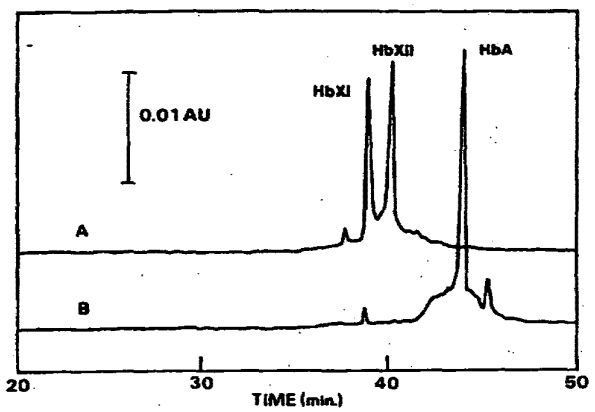


Figure 1. Capillary electropherograms of haemoglobin from a patient with retinopathy (A) and a normal volunteer (B). The electrophoretic run is described in the Experimental Section. HbXI and HbXII in (A) showed two types of abnormal haemoglobin, and HbA in (B) was a normal type of haemoglobin.

trile and for β chain was for 60 min with a linear gradient of 0–25% acetonitrile. The peptides were identified by their amino acid composition and the variant peptide of abnormal haemoglobin was identified by its amino acid sequence. Amino acid sequence determination was performed with an Applied Biosystems Model 471A protein sequencer.

RESULTS

Analysis of the patient's haemoglobin

The capillary electropherogram of the patient's haemoglobin is shown in Fig. 1. Two major peaks were detected under alkaline conditions (HbXI and HbXII in Fig. 1A). On the other hand, HbA was detected as one major peak, and the elution time of HbA was later than those of HbXI and HbXII (HbA in Fig. 1B). The amounts of HbXI and HbXII were almost the same (the ratio of HbXI was 47.4% and that of HbXII was 52.6%). The theoretical plate number was an average of 210,000. This abnormal haemoglobin presumably consists of two different types, and, under alkaline conditions, each net charge of these haemoglobin variants has less negative than the net charge of HbA.

Peptide mapping and sequence analysis of globin chains in abnormal haemoglobin

Two components of abnormal haemoglobin were separated by cation exchange HPLC. As shown in Fig. 2, two major peaks were obtained in the ratio of nearly one, which corresponded to the peaks of the capillary electropherogram except that HbXII was eluted faster than HbXI. The globin chains of HbXI and HbXII were also separated by cation exchange chromatography after being denatured in 8 M urea (not shown), subjected to peptide mapping and to sequence analysis. The HPLC patterns of peptide mapping of the normal β chain are shown in Fig. 3. About 12 major peaks are obtained on each chromatogram, in which all the expected peptides were included. A peptide βT1 disappeared from the chromatogram of βI and βII (βI from HbXI and βII from HbXII in Fig. 3), and one new

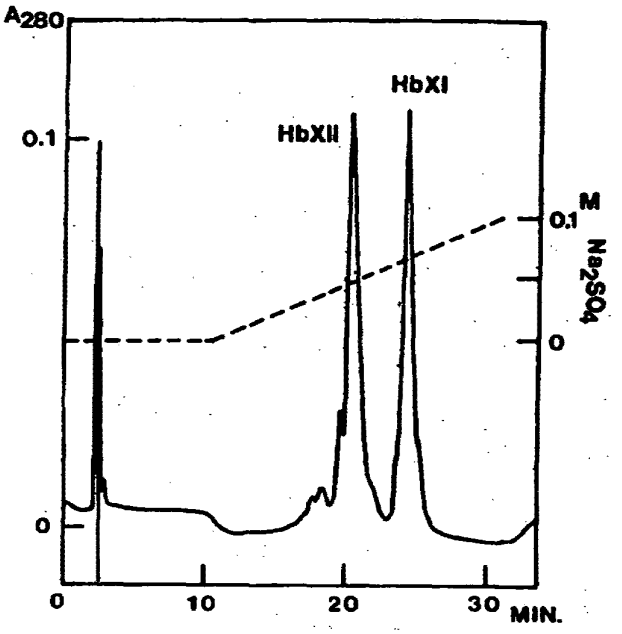


Figure 2. Separation of two components of abnormal haemoglobin on a cation exchange column high performance liquid chromatograph. Two types (HbXI and HbXII) of abnormal haemoglobin were separated at room temperature by a linear gradient of 0.1 M Na_2SO_4 (0–100%) in 0.02 M K_2HPO_4 (pH 6.5) at a flow rate of 1.0 mL/min for 20 min.

peak, βIx and βIIx, appeared instead of βT1, respectively. The βIx and βIIx seem to derive from the *N*-terminal portion, because the βT1 is the *N*-terminal peptide of the β chain. The maps obtained from each α chain also compare closely to one another. However, there were no significant differences on the chromatograms (not shown). It became apparent that the variants existed only in the β chains and not in the α chains. Amino acid composition and sequence analysis verified that Glu-6 was replaced with Lys-6 in the peptide βIx,

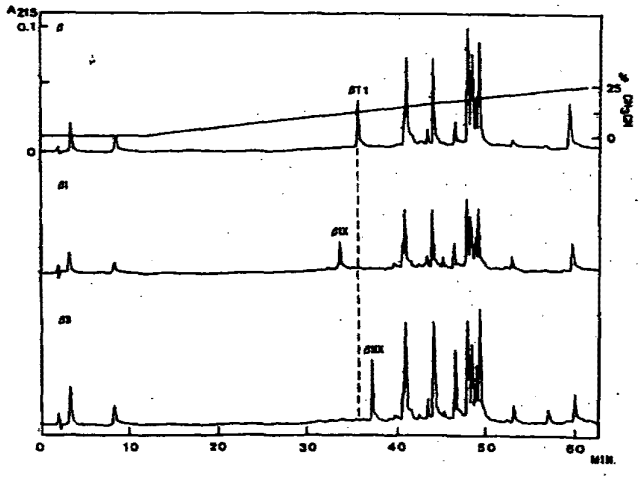


Figure 3. Peptide mappings of β globin chains. The tryptic digests of β globin chains were applied to a reversed phase column and eluted as described in the Experimental Section. β, βI, and βII in the figure showed the maps from HbA, HbXI and HbXII, respectively. A βT1 showed the *N*-terminal peptide of the normal β chain. βIx and βIIx showed new peptide peaks in βI and βII, respectively, instead of βT1.

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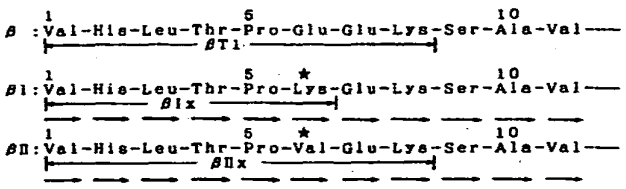


Figure 4. Amino acid sequence analysis of the tryptic peptides with the substitution obtained from abnormal haemoglobin. Amino acid residues identified by automated Edman degradation are indicated by small arrows and (★) shows the substitution sites.

as for HbXI (this [B6(A3)Glu→Lys] substitution type is HbC), and that Glu-6 was also replaced with Val-6 in the peptide βIIx, as for HbXII (this [B6(A3)Glu→Val] is HbS). These results were also supported by direct sequence determination of the *N*-terminal portion of the βI and βII chains (Fig. 4). The above results led to the conclusion that this patient was a heterozygote for haemoglobin S and haemoglobin C (HbSC disease).

DISCUSSION

Separation of proteins by CE in uncoated fused silica capillaries is limited by adsorption and variable rates of electroosmotic flow. Operation at pH values above 8 to minimize these problems is useful (Lauer and McManigill, 1986[a,6]; Lüdi *et al.*, 1988; Zhu *et al.*, 1990; Chen *et al.*, 1991), although some basic proteins can still adsorb, and the resolution may be reduced by inducing a strong electroosmotic flow. In the present study, pH 9.98 was employed and this pH value was above the isoelectric point of haemoglobin. Under these alkaline conditions, an optimal separation of haemoglobin variants was achieved, although it took a relatively long time for analysis. The analysis time might be reduced by using a short capillary, and a good example for separation of haemoglobin variants was presented by Chen *et al.* (1991). The potential, clinical and diagnostic utility of CE has been demonstrated (Jorgenson and Lukacs 1981[b], 1983; Chen *et al.*, 1991) and the effectiveness of analyses for serum proteins and haemoglobin variants was also established by comparison with conventional agarose gel-based electrophoresis (Chen *et al.*, 1991).

In this report, we have demonstrated that CE analysis is useful for clinical diagnosis of haemoglobinopathy of an actual patient with retinopathy, and we have also shown one of the diagnostic processes for the abnormality of unknown clinical samples by the combined use of CE, HPLC and a protein sequencer.

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3 TITRE DE L'INVENTION (200 caractères ou espaces maximum)

PROCEDE D'ANALYSE DE L'HEMOGLOBINE PAR ELECTROPHORESE CAPILLAIRE, TROUSSE POUR ELECTROPHORESE CAPILLAIRE ET UTILISATION DE RALENTISSEUR DE FLUX DANS UN TEL PROCEDE.

4 DÉCLARATION DE PRIORITÉ OU REQUÊTE DU BÉNÉFICE DE LA DATE DE DÉPÔT D'UNE DEMANDE ANTÉRIEURE FRANÇAISE

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7 INVENTEUR (S)		**Les inventeurs sont nécessairement des personnes physiques**
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La loi n°78-17 du 6 janvier 1978 relative à l'informatique, aux fichiers et aux libertés s'applique aux réponses faites à ce formulaire. Elle garantit un droit d'accès et de rectification pour les données vous concernant auprès de l'INPI.

La présente invention concerne un procédé de séparation de l'hémoglobine par électrophorèse capillaire ainsi que des compositions de tampon utiles pour cette séparation, et des trousses d'analyse de l'hémoglobine par électrophorèse capillaire.

Pour analyser l'hémoglobine A_2 et les variants de l'hémoglobine, notamment, dans des liquides biologiques, comme du sang, en ayant des visées analytiques et notamment diagnostiques, et pour donc séparer des hémoglobines par électrophorèse, il est connu d'utiliser des techniques d'électrophorèse capillaire (EC). Par hémoglobines, on entend ici toute hémoglobine normale ou pas, et les variants de ces hémoglobines. L'un des intérêts de l'électrophorèse capillaire réside dans le fait que seules de très petites quantités des liquides biologiques à analyser sont nécessaires. De plus, la séparation par cette technique peut être très rapide, dans la mesure où de forts voltages peuvent être utilisés sans que l'échantillon ne s'échauffe trop lors de la séparation.

Ainsi, la technique de choix est l'analyse par isoélectrofocalisation capillaire (cIEF). Cette méthode permet d'obtenir une résolution élevée entre les différentes formes d'hémoglobine (Hempe) (7). Toutefois son automatisation est difficile et, la nécessité d'utiliser des capillaires revêtus, encore appelés capillaires « coatés » afin de supprimer le flux électro-osmotique, la rend difficilement compatible avec des analyses effectuées en série.

Une autre technique dite technique de « double coating dynamique » peut être mise en œuvre notamment avec les trousses commercialisées sous le nom de « Analis HbA_2-CE kit » ou « CEofix HbA_2-CE kit» d'Analis. Cette technique implique un premier lavage du capillaire par une solution contenant un polycation à un pH de 4,7, suivi d'un second lavage avec un tampon d'analyse contenant un polyanion, à un pH de 8,7.

Dans cette méthode de « double-coating » ou double revêtement, la quantité de charges négatives présentes sur la paroi interne du capillaire est encore plus élevée que sur un capillaire nu, si bien que le flux électro-osmotique est encore plus important. Cette méthode de double-coating dynamique ne permet toutefois pas la séparation suffisante entre les fractions HbA_2, HbC et HbE. Ceci rend l'analyse quantitative de la fraction HbA_2 impossible en présence de fractions HbC ou HbE. De plus, comme les fractions HbS et HbD ont la même position électrophorétique, une analyse complémentaire en milieu acide est nécessaire afin de déterminer le type de variant présent dans l'échantillon. Enfin, le revêtement double doit être refait entre chaque analyse d'échantillon, ce qui rend cette méthode lourde et difficile à employer pour des essais en grande série.

Par ailleurs, des séparations des hémoglobines en solution libre ont été décrites, mais elles ne répondent pas aux critères de précision, de résolution ou de rapidité attendus pour rationaliser les analyses des hémoglobines en EC. Ainsi, Ishoka (1)(1992) décrit la séparation d'hémoglobines en utilisant un tampon borate (100mM), à un pH de 9,98 dans des temps de migration de l'ordre de 50 minutes, c'est-à-dire incompatibles avec la durée des analyses des hémoglobines comme elles sont menées à l'heure actuelle. Le même type de tampon, à des conditions similaires de concentration et de pH (Jenkins)(3), (4) et (5) ne permet que des résolutions insuffisantes entre les fractions HbA, HbF et HbS. Sahin (2) décrit des conditions de pH plus acide, mais des résultats très insuffisants pour la résolution entre les fractions HbA, HbF, HbS et HbA_2 avec des concentrations (20mM), moindres en borate, ou avec du barbital (50mM), à un pH de 8,5, et également très insuffisants pour la résolution entre les fractions HbA, HbS et HbA_2 pour le tampon Tris (1M) à un pH de 8,0. De plus, des associations Tris/Arginine (Shihabi) (6), ont été utilisées et même si elles permettent la séparation HbA/HbS, les fractions HbC/HbE et HbA_2 ne sont pas résolues. Enfin, les brevets US 5 202 006 et US 5 439 825 qui décrivent l'utilisation du barbital ou de l'éthylbarbital ne permettent d'obtenir

que de faibles résolutions entre les principales Hbs que sont les HbA, HbF, HbS et HbC.

Il subsiste donc un besoin pour une méthode d'analyse de l'hémoglobine et notamment de l'hémoglobine A_2 qui permette l'analyse en une unique étape, et sans double revêtement, qui puisse être mise en œuvre automatiquement et en séries, et qui garantisse une résolution satisfaisante entre les formes HbA_2, HbC, HbD, HbE, HbS, HbF et HbA, notamment.

La demanderesse a maintenant mis en évidencé qu'en utilisant en EC en solution libre, un tampon d'analyse zwitterionique associé à un ralentisseur de flux, il est possible d'obtenir une séparation largement améliorée des fractions évoquées ci-dessus, en une étape unique, évitant ainsi les séparations complémentaires, et sans double revêtement, ce qui simplifie la mise en œuvre.

Ainsi, la présente invention concerne la séparation par électrophorèse capillaire des hémoglobines dans des échantillons biologiques, procédé dans lequel on fait passer l'échantillon biologique comportant lesdites hémoglobines dans un capillaire contenant un tampon d'analyse, comportant au moins une étape où l'on introduit l'échantillon dans un tube capillaire contenant une solution de tampon d'analyse, et dans lequel le tampon est du type zwitterionique et est associé à au moins un ralentisseur de flux. Cette étape est en général suivie de la séparation des hémoglobines, par migration et détection des différents variants.

En tant que tampon zwitterionique, on utilise selon l'invention un tampon zwitterionique tamponnant entre les valeurs de pH 8 et 10, et comprenant au moins une fonction amine, et au moins une fonction acide, et au moins une fonction hydroxyle en position opposée à la fonction acide. Par fonction acide, on entend ici la fonction acide carboxylique ou la fonction acide sulfonique, notamment. Ce tampon zwitterionique peut être formé d'une ou de deux molécules : pour le cas où la fonction amine est portée par une première molécule sans fonction acide, cette première

molécule est associée à une seconde molécule portant une fonction acide, notamment une fonction acide carboxylique ou sulfonique ou telle qu'un acide aminé. On peut citer, à titre d'exemple, la glycine comme acide aminé.

Selon l'invention, les ralentisseurs de flux sont du type diamine ou polyamine aliphatique ou cyclique. Ils sont choisis parmi les diamines ou polyamines aliphatiques et/ou les diamines ou polyamines cycliques, par exemple. On préfère les diamines ou polyamines aliphatiques. A titre de diamine aliphatique, on peut citer à titre d'exemple le 1,3-diaminopropane, le 1,4-diaminobutane, le 1,5-diaminopentane, le 1,6-diaminohexane, la N,N'-diméthyl-1,6-hexanediamine, la N,N,N',N'-tétraméthyl-1,4-butanediamine et leurs dérivés et sels acceptables. A titre de polyamines aliphatiques, on peut citer à titre d'exemple la diéthylènetriamine, la spermine, la tétraéthylènepentamine, et leurs dérivés et sels acceptables. Les ralentisseurs de flux peuvent être utilisés en mélange.

Comme sel acceptable, on peut citer les sels de chlorhydrate ou similaires. Comme dérivés, on peut citer par exemple, les dérivés des composés ci-dessus dont un ou des atomes de carbone de la chaîne aliphatique est ou sont substitué(s) par un ou des groupements alkyles et/ou un ou des hydrogène(s) des amines libres est ou sont substitué(s) par un ou des groupements alkyles.

La solution de tampon d'analyse peut en outre comporter d'autres additifs, notamment d'autres additifs visant à améliorer la séparation des différentes hémoglobines.

De plus, l'invention concerne l'utilisation de composés connus pour leur activité de ralentisseur de flux électrophorétique, en EC en solution libre en association avec au moins un tampon zwitterionique.

Comme cela apparaît dans les exemples qui suivent, l'utilisation des associations selon l'invention permet une résolution très améliorée de l'hémoglobine et des variants de l'hémoglobine. Elle permet de ce fait d'améliorer la justesse et la précision de l'analyse qualitative et quantitative

des variants, par rapport aux analyses réalisées avec les procédés connus. Elle permet également de quantifier HbA_2, même en présence de HbC ou HbE.

Les associations tampon zwitterionique-ralentisseur de flux de l'invention sont particulièrement utiles pour l'analyse d'échantillons où les hémoglobines normales ou variants du type HbA_2, HbC, HbD, HbE, HbS, HbF et/ou HbA sont présents, pour les déceler ou les quantifier.

Enfin, l'invention a pour objet des trousses (ou kits) d'analyse par EC de l'hémoglobine A_2 et des variants de l'hémoglobine dans un échantillon biologique, comprenant au moins une solution de tampon d'analyse contenant au moins un tampon d'analyse du type zwitterionique ou un tampon zwitterionique et au moins un ralentisseur de flux, et éventuellement un modificateur de pH. Ainsi, les trousses peuvent comprendre au moins un tampon d'analyse et un ralentisseur de flux selon l'invention, et une ou des solution(s) de lavage des capillaires et/ou des barrettes de dilution et/ou un (ou des) diluant(s) de l'échantillon à analyser. Elles peuvent comporter en outre au moins une solution hémolysante. Dans ce kit, le tampon et le ou les ralentisseurs(s) et diluant(s) ou autres additifs peuvent être stocké(s) séparément pour être mélangés extemporanément, ou stockés en mélange. Ce kit comprend éventuellement, en outre, des indications d'utilisation pour réaliser l'analyse et/ou un support de logiciel d'identification.

D'autres caractéristiques et avantages de l'invention apparaîtront à la lecture de la description détaillée qui va suivre, des exemples et figures annexées.

La figure 1 représente un électrophorégramme d'un sang humain normal (HbA, HbA_2) analysé par électrophorèse capillaire en utilisant une solution de tampon selon l'invention.

Les figures 2 à 5 représentent chacune un électrophorégramme d'un sang analysé par électrophorèse capillaire en utilisant la même

solution de tampon. Le sang comporte respectivement les variants suivants : fig. 2 :HbF et HbS ; fig. 3 : HbC ; fig. 4 : HbE ; fig. 5 : HbS et HbD-Los Angeles.

Les figures 6a, b, c, d représentent chacune un électrophorégramme d'un sang β-thalassémique, analysé par électrophorèse capillaire en utilisant quatre solutions de tampon différentes basées sur quatre ralentisseurs de flux différents.

Les figures 7a, b, c, d représentent chacune un électrophorégramme d'un sang comportant HbF et HbS, analysé par électrophorèse capillaire en utilisant quatre solutions de tampon différentes basées sur quatre ralentisseurs de flux différents.

Les conditions de réalisation d'une électrophorèse capillaire EC sont connues de l'homme de l'art. Elles peuvent comporter usuellement un lavage des capillaires par une solution de lavage, un lavage avec la solution de tampon d'analyse, une ou des dilution(s) éventuelle(s) de l'échantillon, l'injection de l'échantillon, la migration et la détection. Ces étapes peuvent être réalisées par des automates.

Des conditions de réalisation d'une éléctrophorèse capillaire sont par exemple les conditions appropriées pour utiliser l'automate Capillarys (SEBIA).

A titre de tampon zwitterionique utile selon l'invention, on peut citer les tampons de type « Tris » pourvus de plusieurs groupements hydroxyle et notamment à titre d'exemples, les tampons Tris (2-amino-2-[hydroxyméthyl]-1,3-propanediol), Tricine (N-tris[hydroxyméthyl]méthylglycine), TAPS (acide N-tris[hydroxyméthyl]méthyl-3-aminopropanesulfonique), TABS (acide N-tris[hydroxyméthyl]méthyl-4-aminobutanesulfonique), ou encore AMPD (2-amino-2-méthyl-1,3-propanediol) ou Bis Tris Propane (1,3-bis[tris(Hydroxyméthyl)méthylamino]propane), ces deux derniers et le Tris devant être associés à un acide aminé.

D'autres molécules comptant un nombre de groupements hydroxyle moindre conviennent également, telles que l'AMPSO (acide 3-[(1,1-diméthyl-2-hydroxyméthyl)amino]-2-hydroxy-propanesulfonique), la Bicine (N,N-bis[2-hydroxyéthyl]glycine), l'HEPBS (acide N-[2-hydroxyéthyl]pipérazine-N'-[4-butanesulfonique]), notamment.

Parmi les tampons zwitterioniques cités ci-dessus, on préfère la tricine.

Ces tampons sont connus et disponibles dans le commerce. Ils peuvent en outre être utilisés en mélange.

Comme ralentisseur de flux, parmi ceux déjà évoqués, on préfère le 1,4-diaminobutane (DAB), le 1,5-diaminopentane, le 1,6-diaminohexane, la diéthylènetriamine (DETA) et la N,N,N',N'-tétraméthyl-1,4-butanediamine. Les ralentisseurs peuvent également être utilisés en mélange.

En association avec la tricine, on préfère le chlorhydrate de 1,4-diaminobutane.

Par échantillon selon l'invention, on entend l'échantillon biologique à analyser c'est-à-dire tout liquide biologique comportant des globules rouges provenant de patients sains ou malades. Ainsi, les liquides biologiques humains peuvent être du sang, normal ou non, lavé, décanté, centrifugé ou total. De plus, le sang peut être hémolysé.

Outre les échantillons biologiques humains, on peut analyser des échantillons d'origine animale. Les échantillons peuvent aussi être d'origine synthétique, et le procédé de l'invention peut alors avoir des visées de contrôle de production par exemple.

L'échantillon peut être préalablement dilué avec une solution de dilution appropriée, une solution hémolysante ou une solution de tampon d'analyse, par exemple.

Les procédés EC mettant en œuvre les associations tampon zwitterionique/ralentisseur de flux selon l'invention sont particulièrement

utiles pour les analyses de sang et la séparation de l'hémoglobine et de ses variants dans des échantillons humains.

Selon l'invention, le pH de la solution de tampon d'analyse est compris entre 8 et 11, de préférence entre 8 et 10.

Les solutions de tampons d'analyse selon l'invention peuvent en outre comporter au moins un composé modifiant le pH. A titre de modificateur de pH, on peut utiliser un composé choisi parmi l'hydroxyde de lithium, l'hydroxyde de sodium, l'hydroxyde de potassium, l'hydroxyde de rubidium, l'hydroxyde de césium, l'hydroxyde de francium, un hydroxyde de mono-, di-, tri- ou tétra-alkyl ammonium ayant de 1 à 8 atomes de carbone dans la partie alkyle.

Selon l'invention, les tampons sont utilisés dans les solutions de tampon d'analyse dans les conditions et concentrations usuelles, à savoir de l'ordre de 20 à 500 mM, de préférence 100 à 250 mM.

Les ralentisseurs de flux sont utilisés dans les solutions de tampon, à ces concentrations comprises entre environ 0,01 et 50 mM, de préférence environ 0,10 et 20 mM.

De plus, la solution de tampon peut comporter un ou des additifs propre(s) à modifier la force ionique.

A titre de tels composés, on peut citer des sels tels que des sulfates, des chlorures, par exemple, et leurs mélanges.

La solution hémolysante permet de réaliser l'hémolyse des globules rouges contenant l'hémoglobine A_2 et les variants de l'hémoglobine. Elle est également utile à la dilution de l'échantillon avant analyse en EC. Elle peut permettre, selon sa composition, une lyse totale des globules rouges en usant d'un faible mouvement mécanique additionnel (vortex, agitation, ...).

La solution hémolysante comprend un tampon d'analyse tels que ceux déjà évoqués (tampons zwitterioniques), des additifs usuels de lyse cellulaire, comme par exemple le Triton X100 et/ou la saponine et éventuellement d'autres additifs visant à amener un effet positif sur la

résolution entre certaines hémoglobines proches. A titre d'exemple, on peut citer la solution hémolysante « Hydragel hémoglobine » de Sébia.

Le pH de la solution hémolysante est compris entre 8 et 11, de préférence entre 8 et 10.

Les solutions de tampon de l'invention sont préparées de façon usuelle pour des compositions de tampon d'analyse, à savoir par adjonction des constituants sous forme liquide, ou solide à diluer, à un support acceptable. De façon usuelle, le support est de l'eau, distillée ou déminéralisée.

Du point de vue des matériaux utilisés pour les capillaires, ceux-ci sont usuels en électrophorèse capillaire. Ainsi, on peut utiliser des capillaires de silice fondue. Leur diamètre interne peut aller de 5 à 2 000 µm. De façon préférée, on utilise selon l'invention des capillaires de diamètre interne inférieur à 200 µm, de préférence inférieur à 100 µm. On utilise de préférence des capillaires à surface intérieure non traitée. Le spécialiste saura adapter la nature du capillaire et sa taille aux besoins de l'analyse.

L'utilisation de tels capillaires nus constitue un des avantages de l'invention.

Les hémoglobines peuvent être analysées à une longueur d'onde d'environ 200 nm, sur du sang hémolysé obtenu à partir de sang lavé, décanté ou centrifugé. Néanmoins, pour éviter des interférences avec les protéines plasmatiques, elles sont de préférence analysées à une longueur d'onde d'environ 415 nm, sur sang hémolysé obtenu à partir de sang lavé, décanté, centrifugé ou total.

Exemples

MATERIEL ET METHODES

A) Electrophorèse capillaire

L'électrophorèse capillaire d'échantillons cliniques est réalisée sur un appareil d'EC équipé d'un capillaire en silice fondue de diamètre interne 25 microns.

La détection est réalisée dans des conditions optimales autour de 415 nm.

Les échantillons sont placés dans l'appareil Capillarys (SEBIA) et automatiquement injectés par injection hydrodynamique. La séparation des échantillons est réalisée en moins de 8 minutes en appliquant un champ électrique d'environ 550 V/cm . Le capillaire est lavé avant chaque analyse par la soude 0,25 M, puis par la solution de tampon d'analyse.

Tampons d'analyse :

Les produits chimiques utilisés sont de grade analytique.

. Le tampon Tricine 200 mM –1,4-diaminobutane 15 mM (A) est préparé en dissolvant 35,84 g de Tricine dans environ 900 ml d'eau déminéralisée, puis en ajoutant 2,32 g de chlorhydrate de 1,4-diaminobutane (DAB). Le pH est ajusté à 9,37 à 22°C par environ 38,3 ml de NaOH 5M et le volume de la solution de tampon ajusté à 1 l par de l'eau déminéralisée.

. Le tampon Tricine 200 mM –1,5-diaminopentane 15 mM (B) est préparé en dissolvant 35,84 g de Tricine dans environ 900 ml d'eau déminéralisée, puis en ajoutant 2,62 g de chlorhydrate de 1,5-diaminopentane. Le pH est ajusté à 9,40 à 22°C par environ 38,4 ml de NaOH 5M et le volume de la solution de tampon ajusté à 1 l par de l'eau déminéralisée.

. Le tampon Tricine 200 mM –diéthylènetriamine 20 mM (C) est préparé en dissolvant 35,84 g de Tricine dans environ 900 ml d'eau déminéralisée, puis en ajoutant 2,06 g de diéthylènetriamine (DETA). Le pH

est ajusté à 9,40 à 22°C par environ 32,4 ml de NaOH 5M et le volume de la solution de tampon ajusté à 1 l par de l'eau déminéralisée.

. Le tampon Tricine 200 mM – N,N,N',N'-Tétraméthyl-1,4-butanediamine 8 mM (D) est préparé en dissolvant 35,84 g de Tricine dans environ 900 ml d'eau déminéralisée, puis en ajoutant 1,15 g de N,N,N',N'-Tétraméthyl-1,4-butanediamine. Le pH est ajusté à 9,19 à 22°C par environ 34,7 ml de NaOH 5M et le volume de la solution de tampon ajusté à 1 l par de l'eau déminéralisée.

B) Echantillons cliniques :

Pour l'EC, le sang humain, décanté ou centrifugé, est dilué au 1/6ème dans la solution hémolysante. Celle-ci est préparée en dissolvant 1,00 g de Triton X100, 2,50 g de saponine, 3,63 g de Tris dans environ 900 ml d'eau déminéralisée. Le pH est ajusté à 8,70 à 22, 0°C et le volume de la solution ajusté à 1 l par de l'eau déminéralisée. Les produits chimiques utilisés sont de grade analytique.

Exemples 1à 5

Une solution de tampon d'analyse Tricine/DAB.2HCl est préparé comme ci-dessus.

L'électrophorèse a été réalisée selon la méthode ci-dessus sur un sang humain.

Exemple 1 : analyse d'un sang humain normal avec HbA en pic principal et HbA_2, fraction plus petite. (Figure 1)

Exemple 2 : analyse d'un sang humain malade qui montre une fraction HbA plus faible, une fraction HbF augmentée, une fraction HbS forte et une fraction HbA_2 normale. Il s'agit d'une HbS hétérozygote à l'origine de la maladie de drépanocytose. (Figure 2)

Exemple 3 : analyse d'un sang humain de malade présentant une hétérozygotie A/C. La fraction HbC sort juste avant la fraction HbA_2 dont elle est bien résolue. Cette bonne résolution autorise la quantification

de A_2 ce qui permet d'orienter le diagnostic en cas de β-thalassémie, c'est-à-dire de fraction HbA_2 augmentée. (Figure 3)

Exemple 4 : analyse d'un sang humain présentant une hétérozygotie A/E. La fraction HbE sort juste après la fraction HbA_2 dont elle est totalement résolue. Il n'y a donc aucun problème pour quantifier HbA_2 en présence d'HbE. (Figure 4)

Exemple 5 : analyse d'un mélange d'un sang présentant une hétérozygotie A/S et d'un sang présentant une hétérogygotie A/D Los Angeles. Les fractions HbS et HbD sont partiellement résolues ce qui permet de les distinguer l'une de l'autre sans nécessité d'une analyse complémentaire.

Exemple 6 : analyse d'un sang humain β-thalassémique, c'est-à-dire présentant un pourcentage de la fonction HbA_2 augmenté et présentant une petite fraction HbF.

Quatre solutions de tampons d'analyse A, B, C et D sont préparées comme ci-dessus.

L'électrophorèse a été réalisée selon la méthode décrite ci-dessus.

La figure 6a) montre le résultat avec le tampon A (Tricine/DAB.2HCl). La figure 6b) montre le résultat avec le tampon B (Tricine/1,5-diaminopentane.2HCl). La figure 6c) montre le résultat avec le tampon C (Tricine/DETA). La figure 6d) montre le résultat avec le tampon D (Tricine/N,N,N',N'-Tétraméthyl-1,4-butanediamine).

La comparaison de ces quatre figures permet d'observer, dans tous les cas, une bonne séparation de chacune des fractions ainsi qu'une bonne focalisation des petites fractions HbF et HbA_2.

Exemple 7 : analyse d'un sang humain présentant une hétérozygotie drépanocytaire (HbS/HbA).

Quatre solutions de tampons d'analyse (A : Tricine/DAB.2HCl ; B : Tricine/1,5-diaminopentane.2HCl : C : Tricine/DETA ; D :

Tricine/N,N,N',N'-Tétraméthyl-1,4-butanediamine) sont préparées comme ci-dessus.

L'électrophorèse a été réalisée selon la méthode décrite ci-dessus.

L'ordre des figures correspond aux tampons déjà évoqués. Là encore, on peut observer des profils comparables quel que soit le tampon utilisé.

Références

1. Noriaki Ishioka et al., Biomedical Chromatography, vol. 6, 224-226 (1992).
2. Ahmet Sahin et al., Journal of Chromatography A, 709 (1995) 121-125.
3. Margaret A. Jenkins, Michael D. Guerin, Journal of Chromatography B, 682 (1996) 23-34.
4. Margaret A. Jenkins, Jean Hendy, Ian L. Smith, J. CAP. ELEC. 004 : 3 (1997) 137-143.
5. Margaret A. Jenkins, Sujiva Ratnaike, Clinica Chimica Acta 289 (1999) 121-132.
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7. James M. Hempe, Randall D. Craver, Electrophoresis, 21 (2000) 743-748.

REVENDICATIONS

1. Procédé de séparation par électrophorèse capillaire des hémoglobines dans des échantillons biologiques, dans lequel on fait passer l'échantillon comportant lesdites hémoglobines dans un capillaire contenant un tampon d'analyse, comportant au moins une étape où on introduit l'échantillon dans un tube capillaire contenant une solution de tampon d'analyse,
caractérisé en ce que le tampon est du type zwitterionique et est associé à au moins un ralentisseur de flux.

2. Procédé selon la revendication 1, caractérisé en ce qu'il comporte en outre la séparation des hémoglobines par migration et la détection de ces hémoglobines.

3. Procédé selon la revendication 1 ou 2, caractérisé en ce que l'échantillon est un échantillon de sang, normal ou non, lavé, décanté, centrifugé ou total.

4. Procédé selon l'une des revendications 1 à 3, caractérisé en ce que l'échantillon est un échantillon de sang hémolysé.

5. Procédé selon l'une des revendications 1 à 4, caractérisé en ce que les hémoglobines sont l'hémoglobine A_2 et les hémoglobines C, D, E, S, F et/ou A.

6. Procédé selon l'une des revendications 1 à 5, caractérisé en ce que le tampon zwitterionique est formé d'une ou deux molécules, comporte au moins une fonction amine, au moins une fonction acide et au moins une fonction hydroxyle en position opposée à la fonction acide.

7. Procédé selon l'une des revendications 1 à 6, caractérisé en ce que le tampon est choisi parmi Tricine, TAPS, TABS, AMPSO, Bicine ou HEPBS, ou l'association de Tris, AMPD ou Bis Tris Propane avec un acide aminé.

8. Procédé selon l'une des revendications 1 à 7, caractérisé en ce que le tampon est la Tricine.

9. Procédé selon l'une des revendications 1 à 8, caractérisé en ce que le ralentisseur de flux est du type diamine ou polyamine aliphatique ou cyclique.

10. Procédé selon l'une des revendications 1 à 9, caractérisé en ce que ledit ralentisseur de flux est choisi parmi les diamines aliphatiques, les polyamines aliphatiques, et leurs dérivés et sels acceptables, et leurs mélanges.

11. Procédé selon l'une des revendications 1 à 10, caractérisé en ce que ledit ralentisseur de flux est choisi parmi le 1,3-diaminopropane, le 1,4-diaminobutane, le 1,5-diaminopentane, le 1,6-diaminohexane, la diéthylènetriamine, la spermine, la N,N'-diméthyl-1,6-hexanediamine, la tétraéthylènepentamine, la N,N,N',N'-tétraméthyl-1,4-butanediamine et leurs dérivés ou sels acceptables, et leurs mélanges.

12. Procédé selon l'une des revendications 1 à 11, caractérisé en ce que ledit ralentisseur de flux est choisi parmi le 1,4-diaminobutane, le 1,5-diaminopentane, le 1,6-diaminohexane, la diéthylènetriamine, la N,N,N',N'-tétraméthyl-1,4-butanediamine et leurs dérivés ou sels acceptables, et leurs mélanges.

13. Procédé selon l'une des revendications 1 à 12, caractérisé en ce que le ralentisseur de flux est le 1,4-diaminobutane.

14. Procédé selon l'une des revendications 1 à 13, caractérisé en ce que le ralentisseur de flux est le 1,4-diaminobutane sous forme chlorhydrate.

15. Procédé selon l'une des revendications 1 à 14, caractérisé en ce que la concentration en tampon dans la solution de tampon est comprise entre 20 mM et 500 mM.

16. Procédé selon l'une des revendications 1 à 15, caractérisé en ce que la concentration en tampon dans la solution de tampon est comprise entre 100 mM et 250 mM.

17. Procédé selon l'une des revendications 1 à 16, caractérisé en ce que la concentration en ralentisseur de flux dans la solution de tampon est comprise entre 0,01 et 50 mM.

18. Procédé selon l'une des revendications 1 à 17, caractérisé en ce que la concentration en ralentisseur de flux dans la solution de tampon est comprise entre 0,10 mM et 20 mM.

19. Procédé selon l'une des revendication 1 à 18, caractérisé en ce que la solution de tampon comporte en outre un modificateur de pH.

20. Procédé selon la revendication 19, caractérisé en ce que le modificateur de pH est choisi parmi l'hydroxyde de lithium, l'hydroxyde de sodium, l'hydroxyde de potassium, l'hydroxyde de rubidium, l'hydroxyde de césium, l'hydroxyde de francium, un hydroxyde de mono-, di-, tri- ou tétra-alkyl ammonium ayant de 1 à 8 atomes de carbone dans la partie alkyle.

21. Procédé selon l'une des revendications 1 à 20, caractérisé en ce que le capillaire est nu.

22. Procédé selon l'une des revendications 1 à 21, caractérisé en ce que le capillaire est en silice fondue.

23. Procédé selon l'une des revendications 1 à 22, caractérisé en ce que le pH de la solution de tampon d'analyse est compris entre 8 et 10.

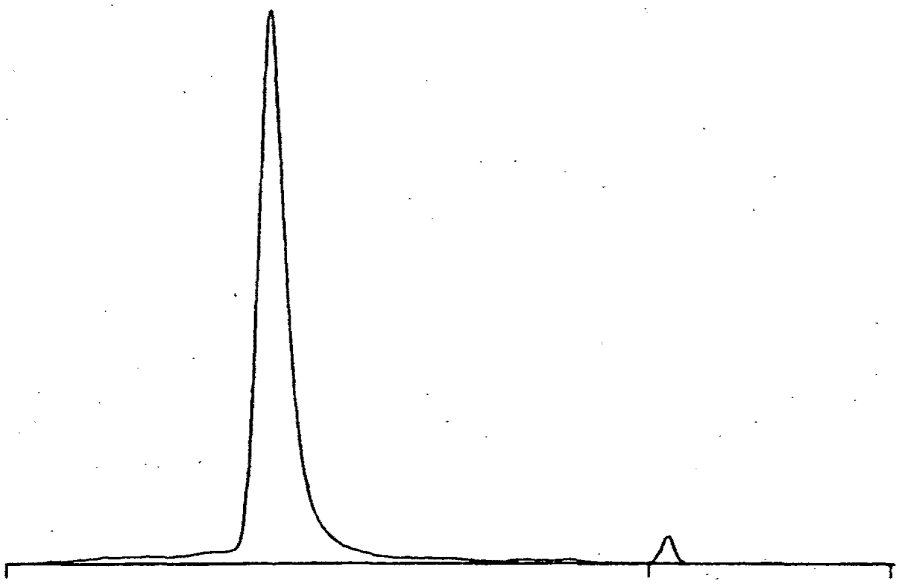
24. Utilisation d'un ralentisseur de flux choisi parmi les diamines et polyamines aliphatiques et cycliques, dans un procédé d'électrophorèse capillaire des hémoglobines, en association avec au moins un tampon zwitterionique dans une solution de tampon.

25. Utilisation selon la revendication 24, dans laquelle le ralentisseur de flux est choisi parmi les diamines aliphatiques, les polyamines aliphatiques, leurs dérivés et sels acceptables, et leurs mélanges.

26. Utilisation selon la revendication 24 ou 25, dans laquelle le tampon zwitterionique et présent à une concentration d'environ 20 à 500 mM, et le ralentisseur est présent à une concentration d'environ 0,01 à 50 mM.

27. Trousse d'analyse de l'hémoglobine par électrophorèse capillaire, comprenant au moins une solution de tampon d'analyse contenant au moins un tampon d'analyse du type zwitterionique ou un tampon zwitterionique et au moins un ralentisseur de flux, et éventuellement un modificateur de pH.

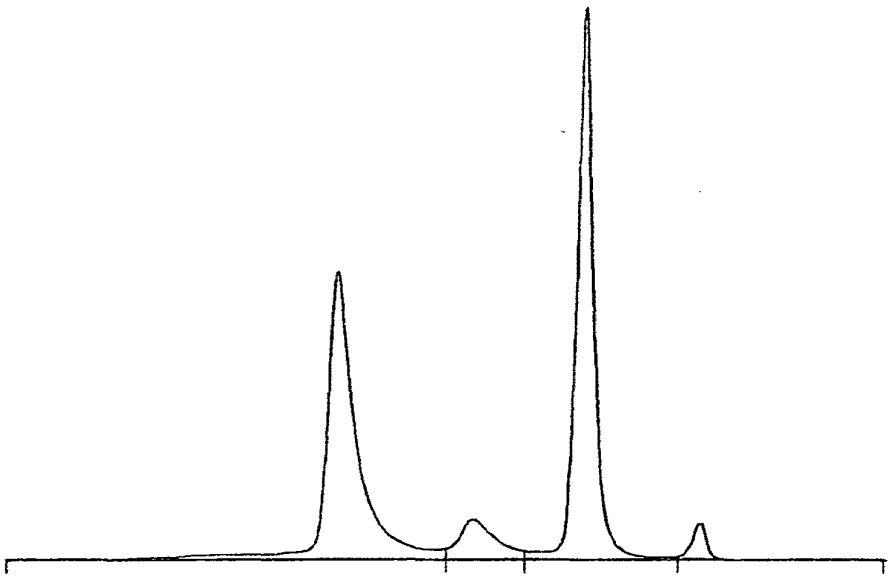
28. Trousse un tampon d'analyse selon la revendication 27, caractérisée en ce qu'elle comporte en outre une solution hémolysante.



Nom	%
HbA	98,2
HbA2	1,8

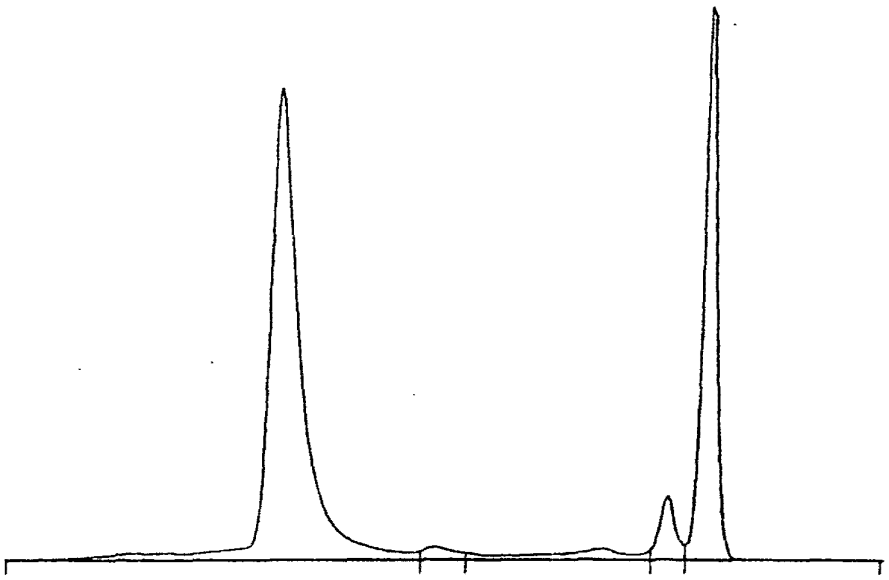
FIG 1

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Exhibit 1003
Page 1003-0177



Nom	%
HbA	44,0
HbF	7,1
HbS	46,5
HbA2	2,4

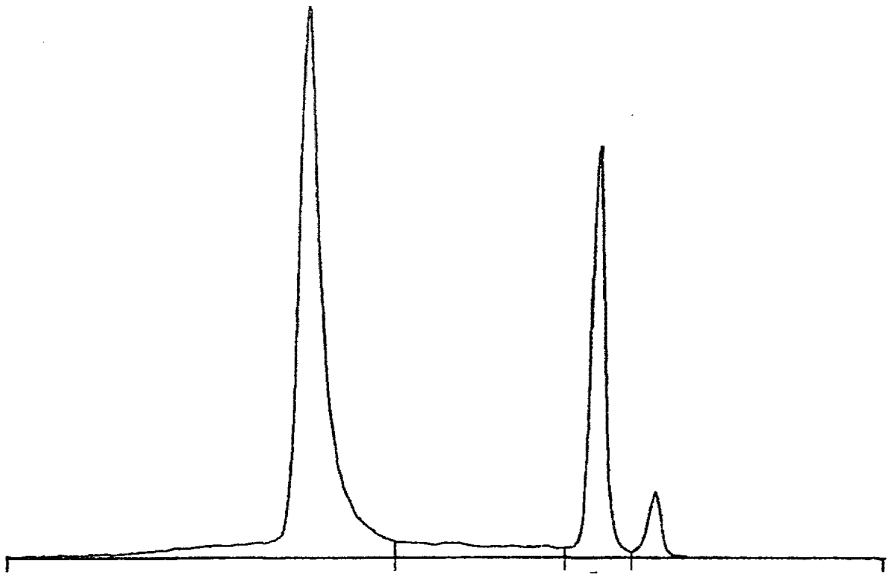
FIG 2



Nom	%
HbA	60,9
HbF	1,6
MetHb	3,9
HbA2	4,0
HbC	29,6

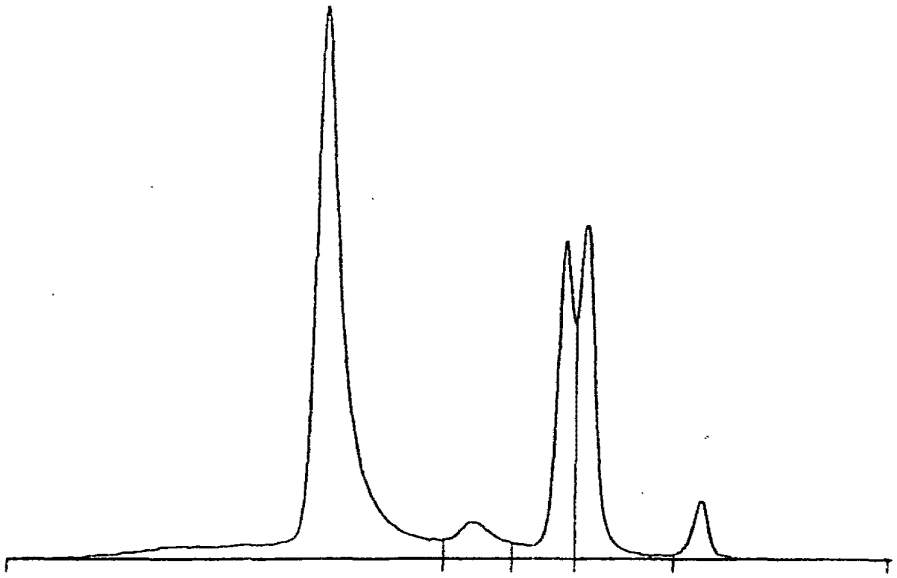
FIG 3

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Exhibit 1003
Page 1003-0179



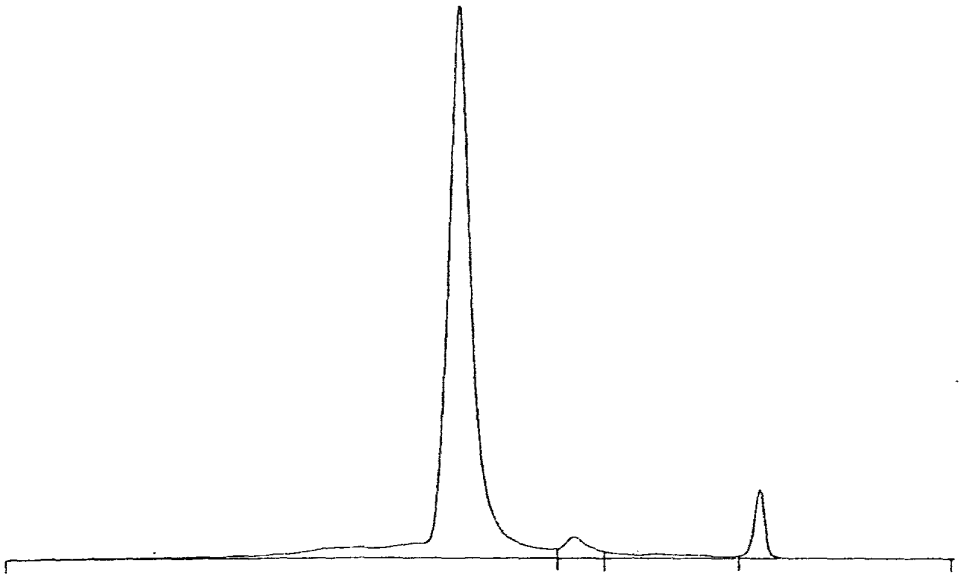
Nom	%
HbA	65,2
MetHb	7,4
HbE	23,5
HbA2	3,9

FIG 4



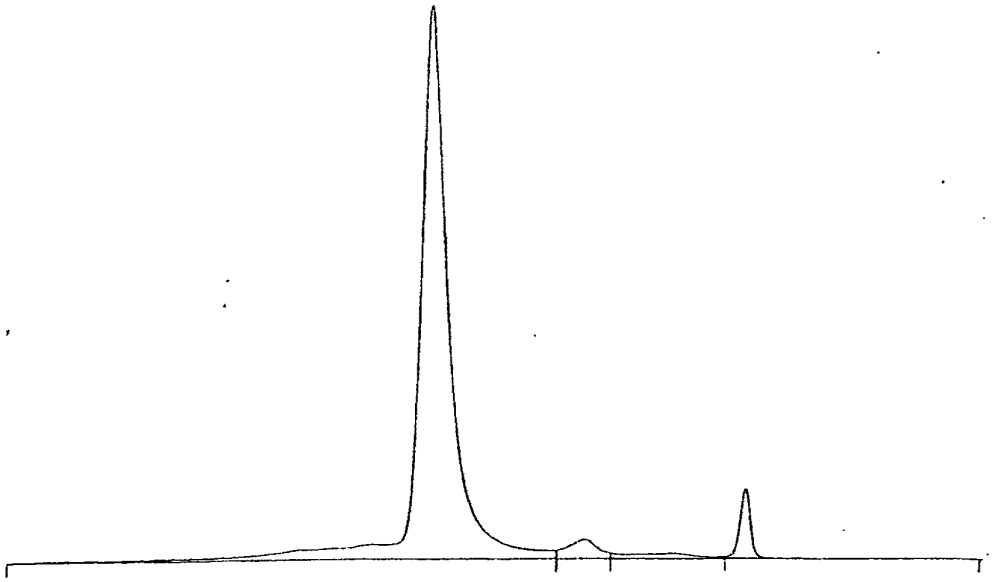
Nom	%
HbA	55,6
HbF	4,8
HbD	18,4
HbS	18,2
HbA2	3,0

FIG 5



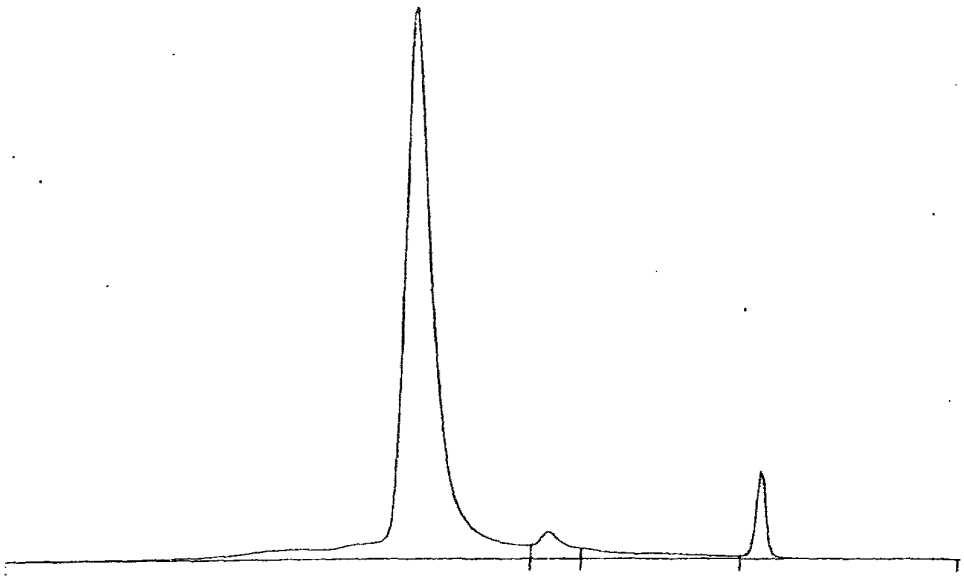
Nom	%
HbA	90,3
HbF	3,2
MetHb	2,2
HbA2	4,3

FIG 6A



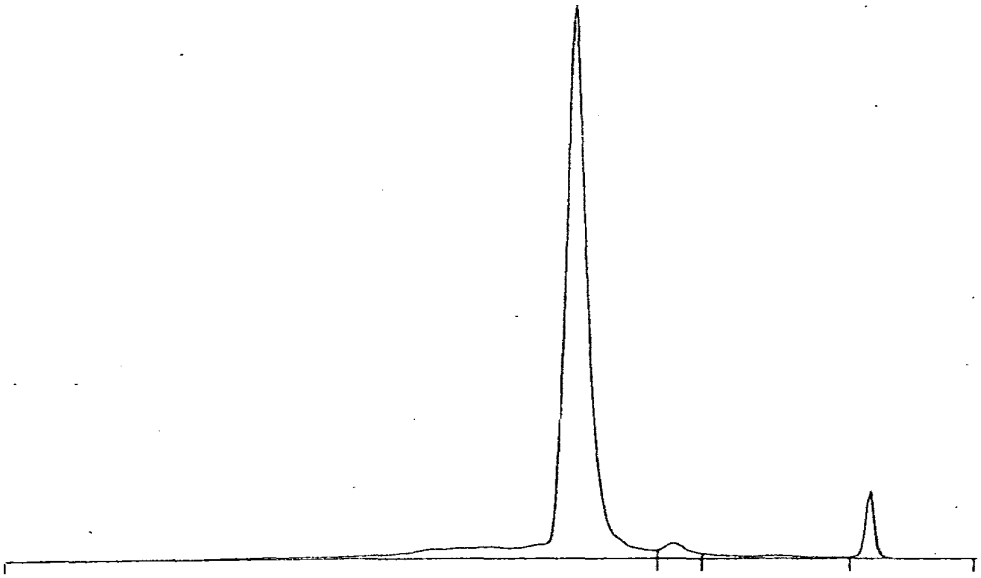
Nom	%
HbA	90,4
HbF	3,3
MetHb	2,0
HbA2	4,3

FIG 6B



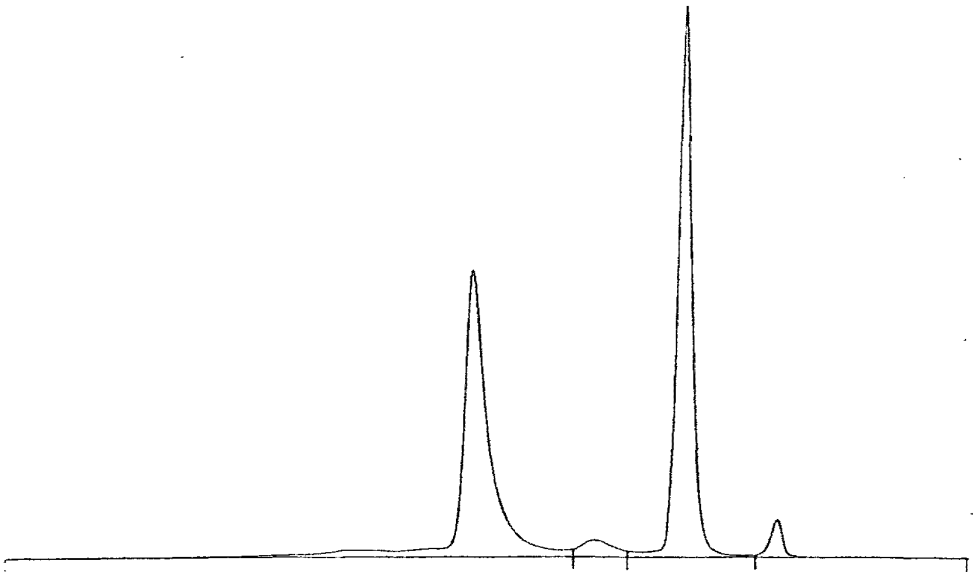
Nom	%
HbA	88,2
HbF	3,9
MetHb	3,4
HbA2	4,5

FIG 6C



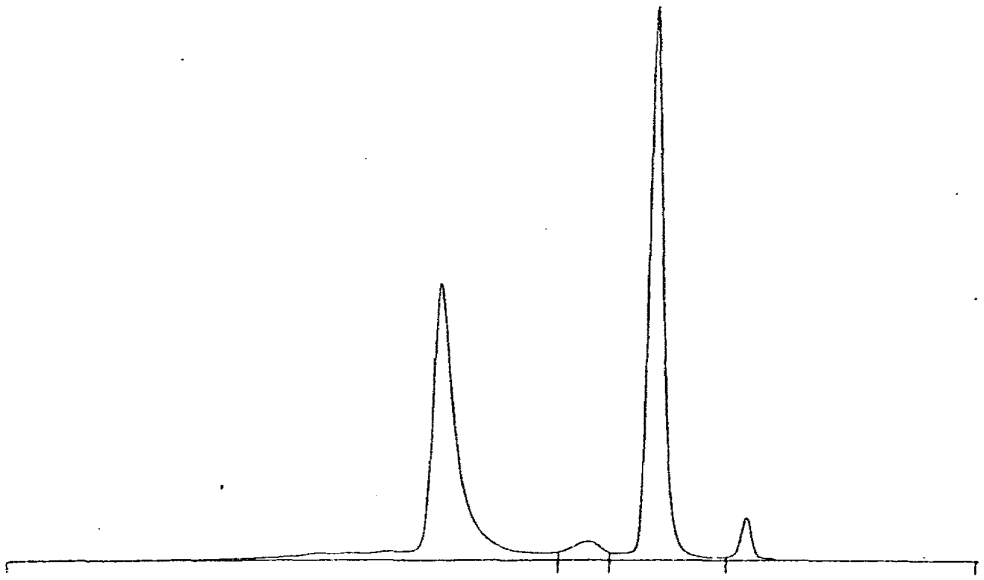
Nom	%
HbA	91,6
HbF	2,6
MetHb	1,9
HbA2	3,9

FIG 6D



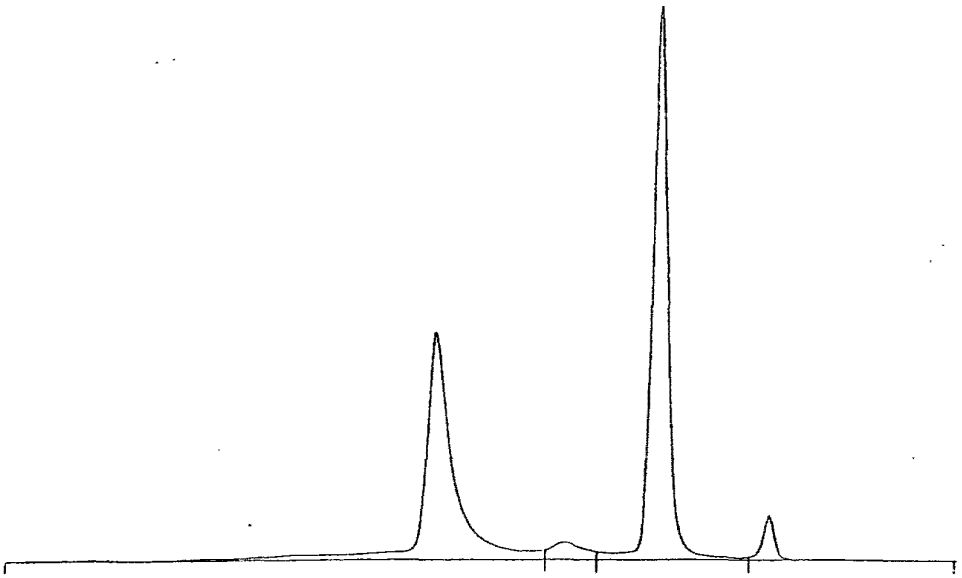
Nom	%
HbA	46,2
HbF	3,3
HbS	47,7
HbA2	2,8

FIG 7A



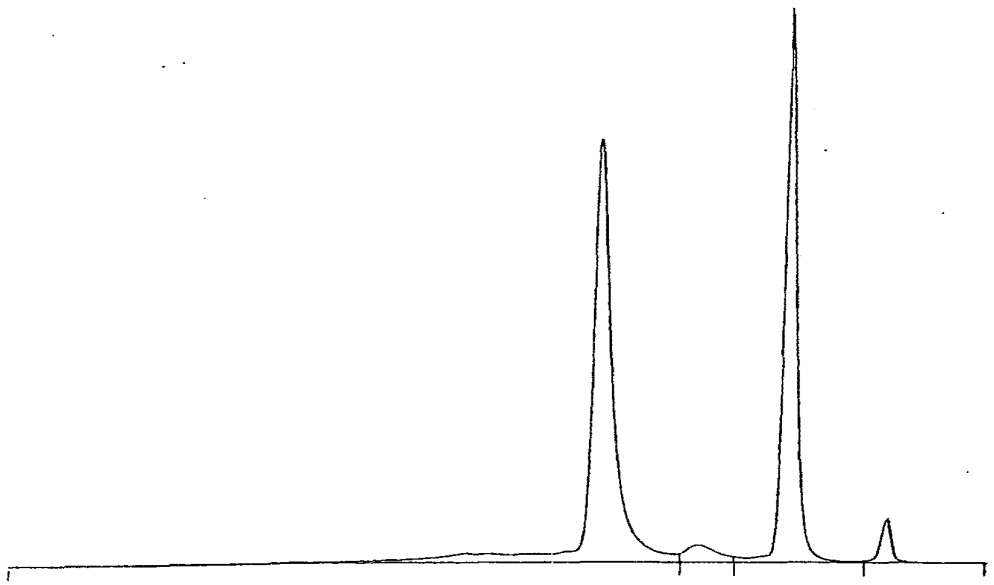
Nom	%
HbA	46,4
HbF	3,5
HbS	47,1
HbA2	3,0

FIG 7B



Nom	%
HbA	42,1
HbF	3,7
HbS	51,3
HbA2	2,9

FIG 7C



Nom	%
HbA	55,5
HbF	3,1
HbS	38,8
HbA2	2,6

FIG 7D

reçue le 28/12/04

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INV

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Vos références pour ce dossier *(facultatif)*	B6086 - JV
N° D'ENREGISTREMENT NATIONAL	0412263

TITRE DE L'INVENTION (200 caractères ou espaces maximum)

PROCEDE D'ANALYSE DE L'HEMOGLOBINE PAR ELECTROPHORESE CAPILLAIRE, TROUSSE POUR ELECTROPHORESE CAPILLAIRE ET UTILISATION DE RALENTISSEUR DE FLUX DANS UN TEL PROCEDE.

LE(S) DEMANDEUR(S) :

SEBIA

Representée par : ERNEST GUTMANN-YVES PLASSERAUD S.A.
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DATE ET SIGNATURE(S)
DU (DES) DEMANDEUR(S)
OU DU MANDATAIRE
(Nom et qualité du signataire)

Paris, le 28 décembre 2004

VAILLANT Jeanne
CPI 97.0801

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APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034

CONFIRMATION NO. 8408

000530
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

FORMALITIES LETTER

Date Mailed: 12/20/2005

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FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The oath or declaration is missing. *A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.*
  Note: If a petition under 37 CFR 1.47 is being filed, an oath or declaration in compliance with 37 CFR 1.63 signed by all available joint inventors, or if no inventor is available by a party with sufficient proprietary interest, is required.

The applicant needs to satisfy supplemental fees problems indicated below.

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- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.16(f) of $65 for a small entity in compliance with 37 CFR 1.27, must be submitted with the missing items identified in this letter.

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APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034

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01 FC:2051 65.00 DA

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Filing Date Granted

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Dated: January 6, 2006 Signature: (Michael H. Teschner)

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Robert et al.

Application No.: 11/280,025 Group Art Unit: 1753

Filed: November 16, 2005 Examiner: Not Yet Assigned

For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

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Dear Sir:

Enclosed for filing please find a Declaration and Power of Attorney in connection with the above-identified application. Please charge our Deposit Account No. 12-1095 in the amount of $65.00 covering the fee set forth in 37 CFR 1.16(e). Also enclosed is a copy of PTO form entitled "NOTICE TO FILE MISSING PARTS OF APPLICATION."

If the Examiner has any questions concerning this application, he or she is requested to call applicant's attorney at (908) 654-5000. If any additional fees are required by the

present Communication, the Examiner is hereby authorized to charge them to our Deposit Account No. 12-1095.

Dated: January 6, 2006

Respectfully submitted,

By _____

Michael H. Teschner
Registration No.: 32,862
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 654-5000
Attorney for Applicant

LD-468\

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0196

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

[ ] Declaration Submitted with Initial Filing OR [x] Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	EGYP 3.0-034
First Named Inventor	Frédéric Robert
COMPLETE IF KNOWN	
Application Number	11/280,025
Filing Date	November 16, 2005
Group Art Unit	N/A
Examiner Name	Not Yet Assigned

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am an original and first inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

(Title of the Invention)

the specification of which

[ ] is attached hereto

OR

[x] was filed on (MM/DD/YYYY) 11/16/2005 as United States Application Number or PCT International Application No. 11/280,025 and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
0412263	FR	11/18/2004	[ ]	[ ]	[x]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (03-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION — Utility or Design Patent Application

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Customer Number 000530

Direct all correspondence to: [X] Customer Number or Bar Code Label	000530	OR [ ] Correspondence address below	
Name			
Address			
City	State	ZIP	
Country	Telephone	Email	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR: [ ] A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Frédéric	Family Name or Surname	Robert
Inventor's Signature		Date	21/12/2005
Residence: City	Mennecy	State	
Country	France	Citizenship	France
Mailing Address:	16 rue des Bergeronnettes		
City	Mennecy	State	
ZIP	91540	Country	France

NAME OF SECOND INVENTOR: [ ] A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Denis	Family Name or Surname	Simonin
Inventor's Signature	Simonin.	Date	21/12/05
Residence: City	Evry	State	
Country	France	Citizenship	France
Mailing Address:	42 rue A. Daudet		
City	Evry	State	
ZIP	91000	Country	France

[X] Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

LD-537\

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0198

OIPE IAP79
JAN 09 2006
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PTO/SB/02A (09-04)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Jean-Baptiste		Clement	
Inventor's Signature			Date 21/12/05
Residence: City Etiolles	State	Country France	Citizenship France
Mailing Address: 7 rue de l'Epiphanie			
City Etiolles	State	Zip 91450	Country France
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Inventor's Signature			Date
Residence: City	State	Country	Citizenship
Mailing Address:			
City	State	Zip	Country
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Inventor's Signature			Date
Residence: City	State	Country	Citizenship
Mailing Address:			
City	State	Zip	Country
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Inventor's Signature			Date
Residence: City	State	Country	Citizenship
Mailing Address:			
City	State	Zip	Country

01789 U.S. PTO
111605

PTO/SB/17 (12-04v2)
Approved for use through 7/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no person are required to respond to a collection of information unless it displays a valid OMB control number.

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

[X] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 930.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Frédéric Robert
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	EGYP 3.0-034

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[X] Deposit Account Deposit Account Number: 12-1095 Deposit Account Name: Lerner, David, Littenberg, Krumholz & Mentlik, LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[X] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, except for the filing fee
[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17
[X] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	500.00
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
30 - 20 =	10	x 25.00	= 250.00

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
180.00	180.00

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
3 - 3 =		x	=

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
29 - 100 =	/50	(round up to a whole number) x	=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge):	

SUBMITTED BY

Signature		Registration No. (Attorney/Agent) 32,862	Telephone (908) 518-6313
Name (Print/Type)	Michael H. Teschner	Date	November 16, 2005

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SEP 19 2006
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DPW

AMENDMENT TRANSMITTAL LETTER

Docket No.
EGYP 3.0-034

Application No.	Filing Date	Examiner	Art Unit
11/280,025-Conf. #8408	November 16, 2005	Not Yet Assigned	1753

Applicant(s): Frédéric Robert, Denis Simonin, and Jean-Baptiste Clement

Invention: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED

	Claims Remaining After Amendment	Highest Number Previously Paid	Number Extra Claims Present	Rate	
Total Claims	27	- 30 =		x	
Independent Claims	2	- 3 =		x	
Multiple Dependent Claims (check if applicable)	☐				
Other fee (please specify):					
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					0.00

☐ Large Entity ☒ Small Entity

☒ No additional fee is required for this amendment.

☐ Please charge Deposit Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 12-1095 as described below. A duplicate copy of this sheet is enclosed.

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

Dated: September 15, 2006

Michael H. Teschner
Attorney/Agent Reg. No.: 32,862

LERNER, DAVID, LITTENBERG, KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 518-6313

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 15, 2006 Signature: _____ (Michael H. Teschner)

693005

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 15, 2006 Signature: _____ (Michael H. Teschner)

Docket No.: EGYP 3.0-034 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Robert et al.	
Application No. 11/280,025	Group Art Unit: 1753
Filed: November 16, 2005	Examiner: Not Yet Assigned
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Dear Sir:

Preliminary to initiation of the prosecution of the above-identified pending U.S. patent application, the following amendments and remarks are respectfully submitted.

683822

IN THE CLAIMS

1-28 (cancelled)

29. (new) A method for separating haemoglobins in a biological sample by capillary electrophoresis comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electophoresing said biological sample in said analysis buffer and said at least one flow inhibitor whereby said haemoglobins are separated by migration.

30. (new) The method according to claim 29, further comprising detecting said haemoglobins.

31. (new) The method according to claim 29, wherein said sample is a sample of blood which may be normal or abnormal, washed, decanted, centrifuged or whole blood.

32. (new) The method according to claim 31, wherein said sample is a sample of haemolyzed blood.

33. (new) The method according to claim 29, wherein the haemoglobins are a haemoglobin A2 and a C, a D, an B, a S, a F and/or an A haemoglobin.

34. (new) The method according to claim 29, wherein the zwitterionic buffer is formed by one or two molecules and comprises at least one amine function, at least one acid function and at least one hydroxyl function in the position opposite to the acid function.

35. (new) The method according to claim 29, wherein said analysis buffer is selected from tricine, TAPS, TABS, AMPSO, bicine or HEPBS, or an association of Tris, AIVIPD or bis Tris propane with an amino acid.

36. (new) The method according to claim 35, wherein said analysis buffer is tricine.

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37. (new) The method according to claim 29, wherein said at least one flow inhibitor is of an aliphatic or a cyclic diamine or a polyamine type.

38. (new) The method according to claim 29, wherein said at least one flow inhibitor is selected from aliphatic diamines, aliphatic polyamines, their acceptable derivatives and salts, and mixtures thereof.

39. (new) The method according to claim 29, wherein said at least one flow inhibitor is selected from 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine, spermine, N,N'-dimethyl-1,6-hexanediamine, tetraethylenepentamine, N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

40. (new) The method according to claim 39, wherein said at least one flow inhibitor is selected from 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine and N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

41. (new) The method according to claim 40, wherein said at least one flow inhibitor is 1,4- diaminobutane.

42. (new) The method according to claim 41, wherein said at least one flow inhibitor is 1,4-diaminobutane in the hydrochloride form.

43. (new) The method according to claim 29, wherein said analysis buffer has a concentration from about 20mM to about 500 mM.

44. (new) The method according to claim 29, wherein said analysis buffer has a concentration from about 100mM to about 250 mM.

45. (new) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.01 to about 50 mM in said analysis buffer.

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46. (new) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.10mM to about 20mM in said analysis buffer.

47. (new) The method according to claim 29, wherein said analysis buffer further comprises a pH modifier.

48. (new) The method according to claim 47, wherein said pH modifier is selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, and mono-, di-, tri- or tetra-alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

49. (new) The method according to claim 29, wherein said capillary tube is naked.

50. (new) The method according to claim 29, wherein said capillary tube is formed from fused silica.

51. (new) The method according to claim 29, wherein said analysis buffer has a pH from about 8 to about 10.

52. (new) The method according to claim 29, wherein said capillary electrophoresis is of the free solution capillary electrophoresis type.

53. (new) A capillary electrophoresis haemoglobin analysis kit comprising at least one analysis buffer containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor.

54. (new) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a pH modifier.

55. (new) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a haemolyzing solution.

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IN THE DRAWINGS

Attachment: Replacement Sheets

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REMARKS

The above-noted cancellation of claims 1-28, and addition of new claims 29-55, as well as the submission of replacement drawings, are respectfully submitted prior to initiation of the prosecution of this application in the U.S. Patent and Trademark Office.

The above-noted new claims are respectfully submitted in order to more clearly and appropriately claim the subject matter which applicant considers to constitute his inventive contribution. No new matter is included in these amendments. In addition, the revisions to the drawings are submitted in order to clarify them, as the originally filed drawings contained the French word "NOM". No new matter is included in these amendments.

In view of the above, it is respectfully requested that these amendments now be entered, and that prosecution on the merits of this application now be initiated. If, however, for any reason the examiner does not believe such action can be taken, it is respectfully requested that he telephone applicant's attorney at (908) 654-5000 in order to overcome any objections which he may have.

If there are any additional charges in connection with this requested amendment, the examiner is authorized to charge applicant's Deposit Account No. 12-1095 therefor.

Dated: September 15, 2006 Respectfully submitted,

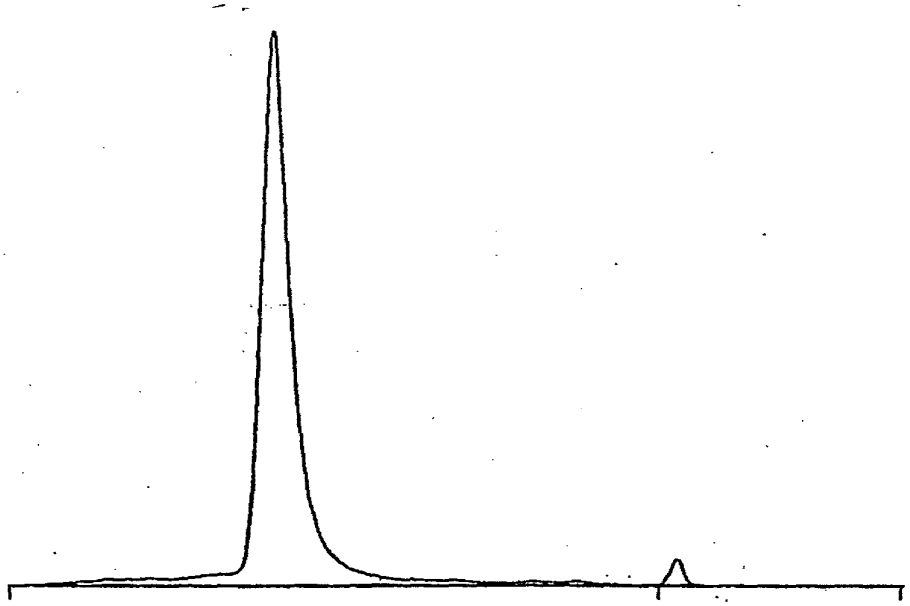
By _____

Michael H. Teschner
Registration No.: 32,862
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 654-5000
Attorney for Applicant

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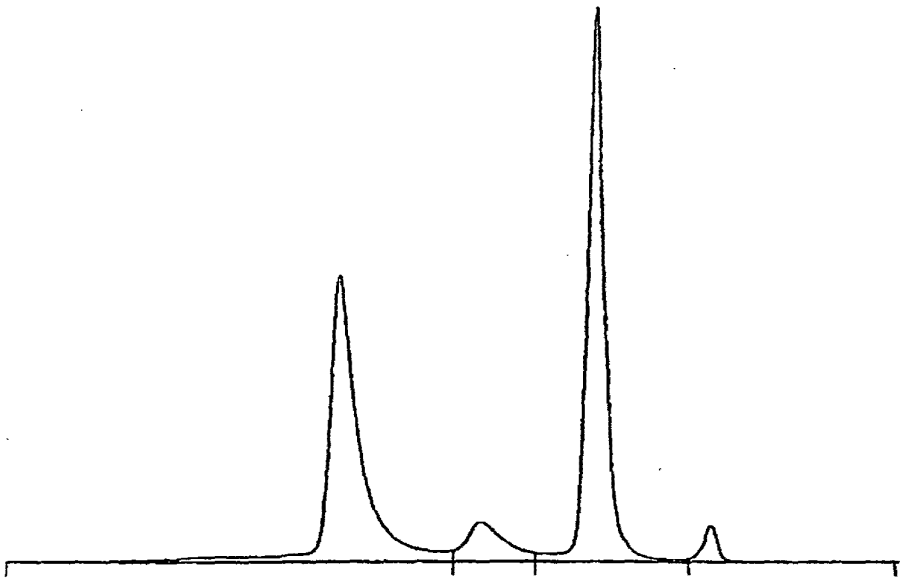
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Page 1003-0207

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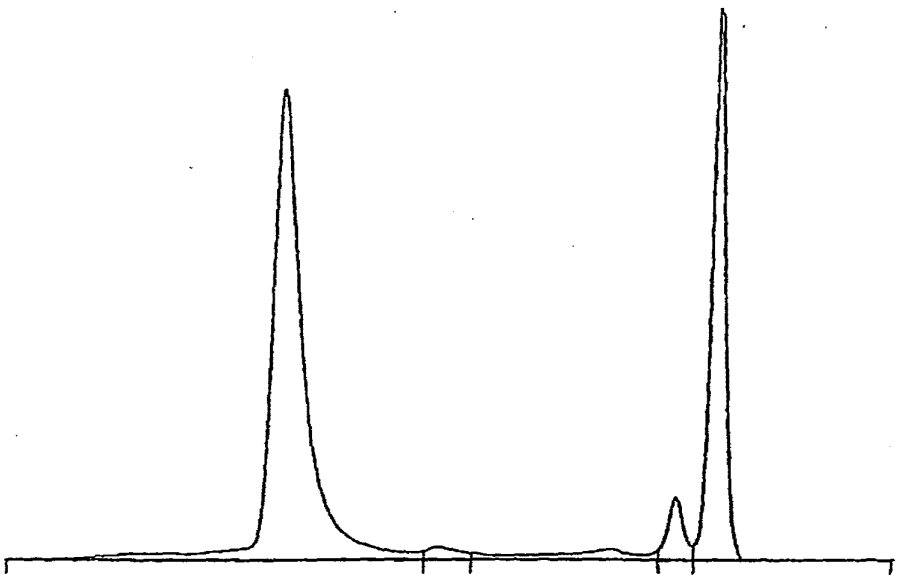
	%
HbA	98,2
HbA2	1,8

FIG 1



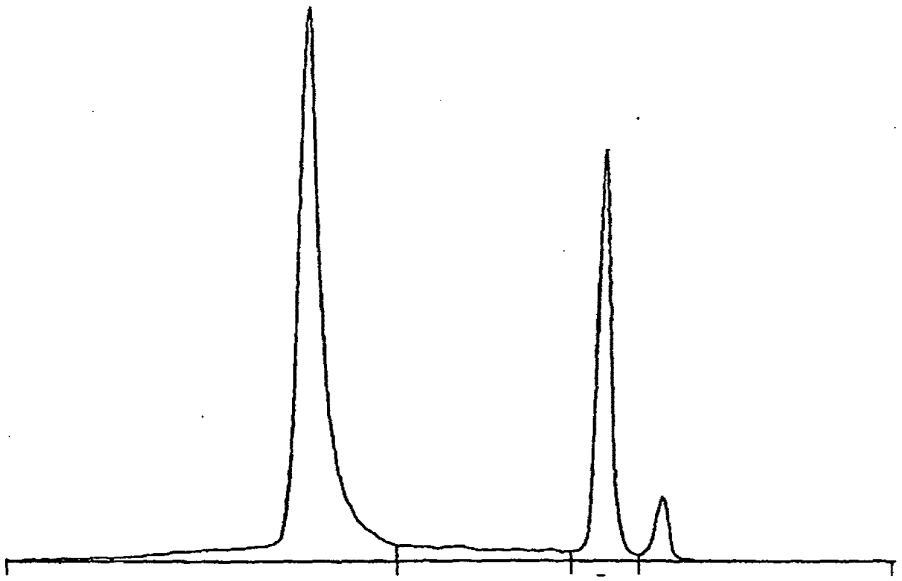
	%
HbA	44,0
HbF	7,1
HbS	46,5
HbA2	2,4

FIG 2



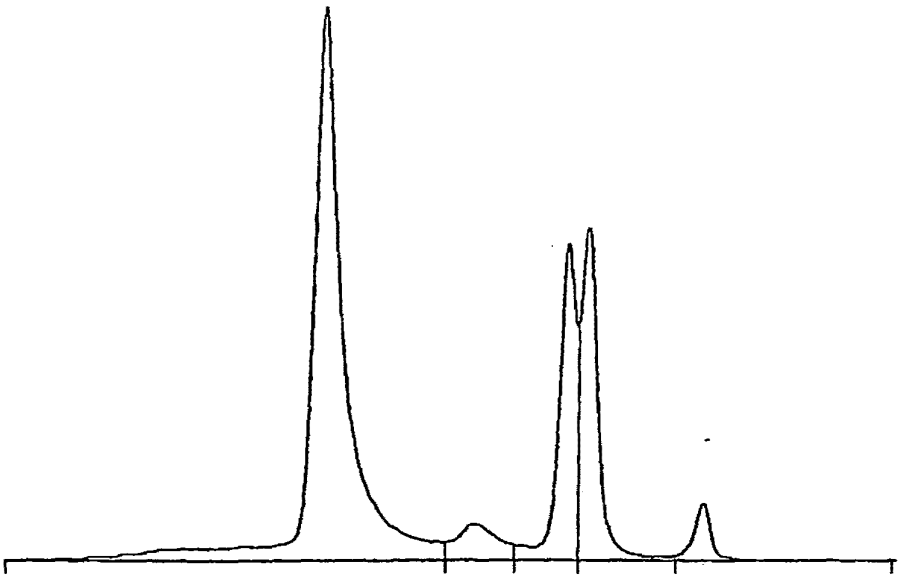
	%
HbA	60,9
HbF	1,6
MetHb	3,9
HbA2	4,0
HbC	29,6

FIG 3



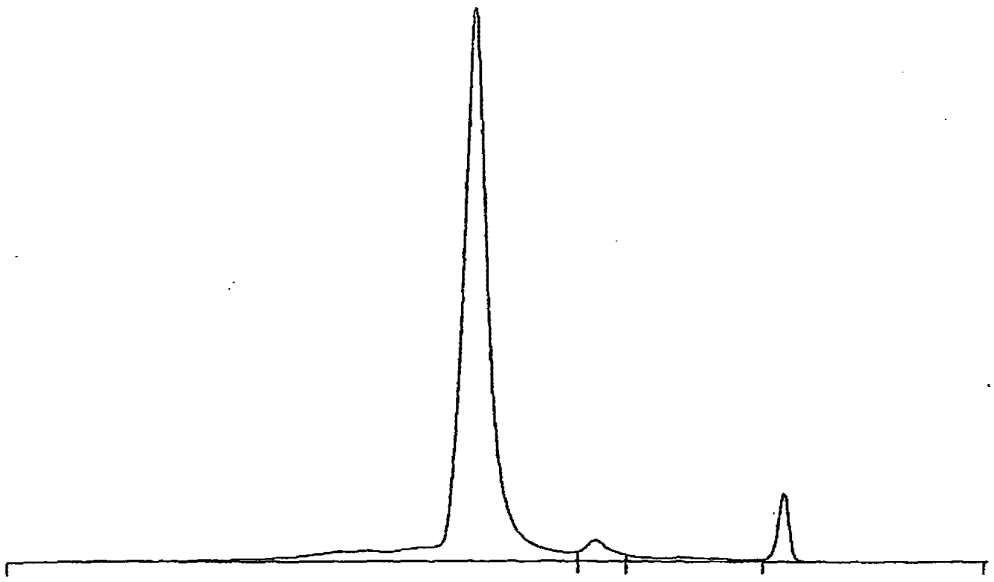
	%
HbA	65,2
MetHb	7,4
HbE	23,5
HbA2	3,9

FIG 4



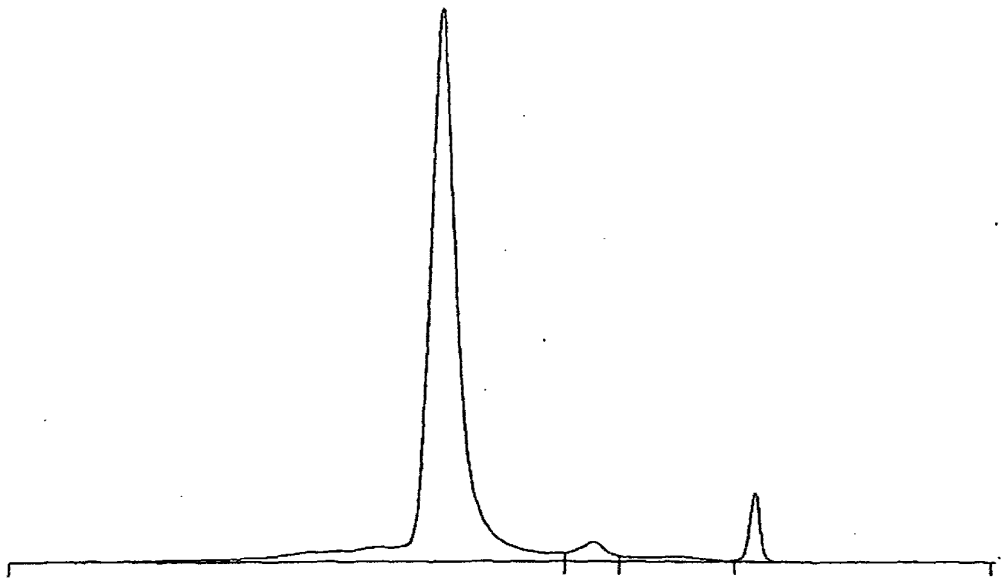
	%
HbA	55,6
HbF	4,8
HbD	18,4
HbS	18,2
HbA2	3,0

FIG 5



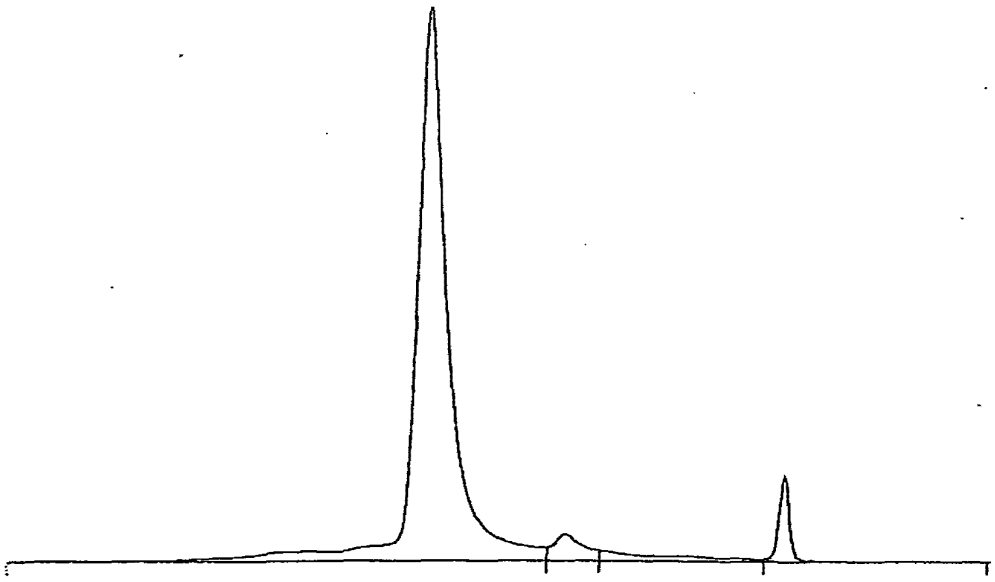
	%
HbA	90,3
HbF	3,2
MetHb	2,2
HbA2	4,3

FIG 6A



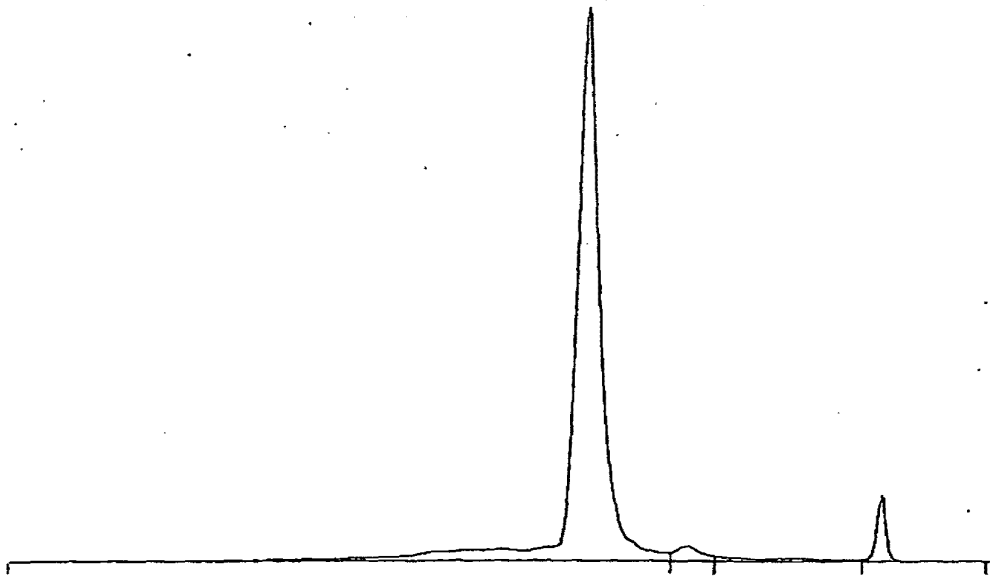
	%
HbA	90,4
HbF	3,3
MetHb	2,0
HbA2	4,3

FIG 6B



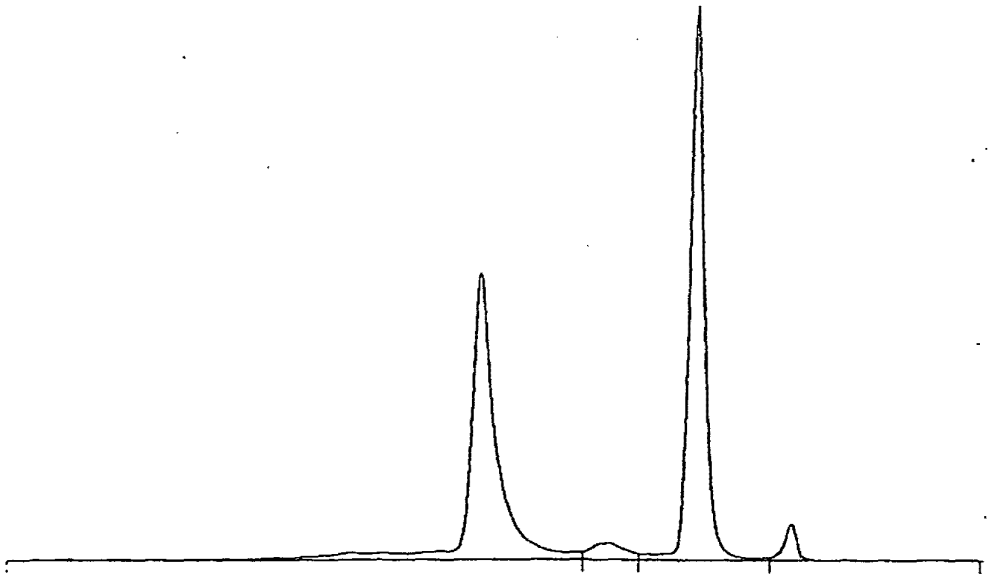
	%
HbA	88,2
HbF	3,9
MetHb	3,4
HbA2	4,5

FIG 6C



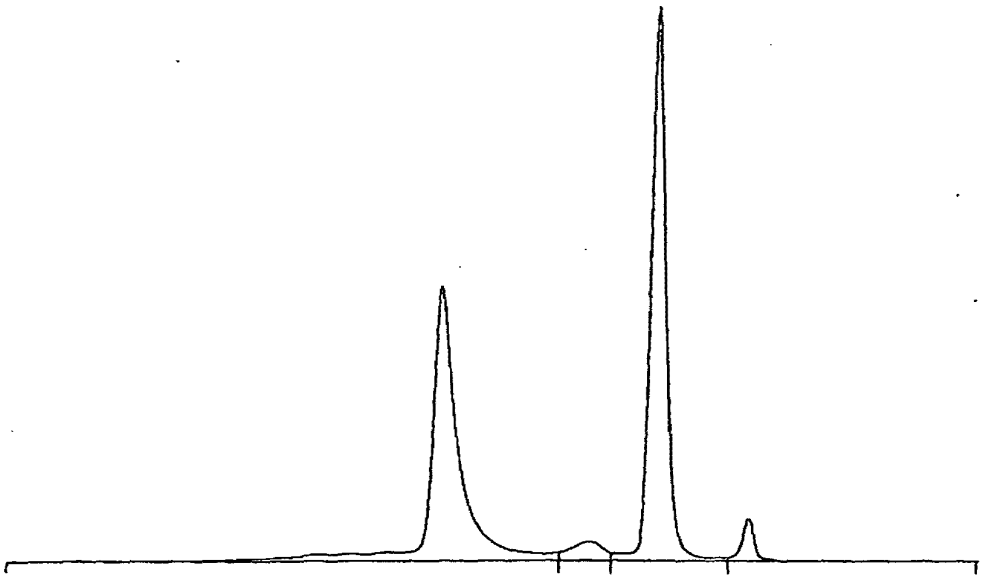
	%
HbA	91,6
HbF	2,6
MetHb	1,9
HbA2	3,9

FIG 6D



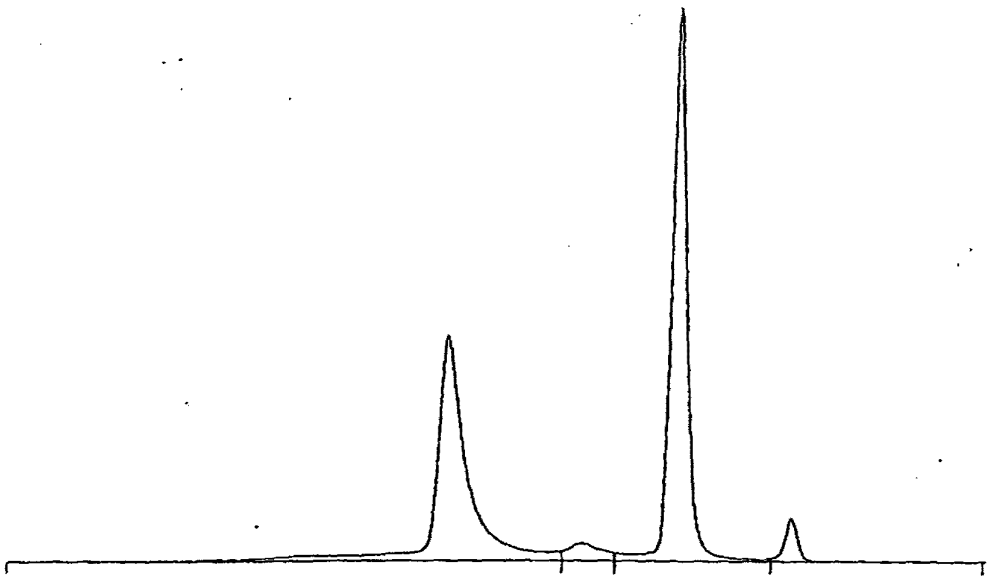
	%
HbA	46,2
HbF	3,3
HbS	47,7
HbA2	2,8

FIG 7A



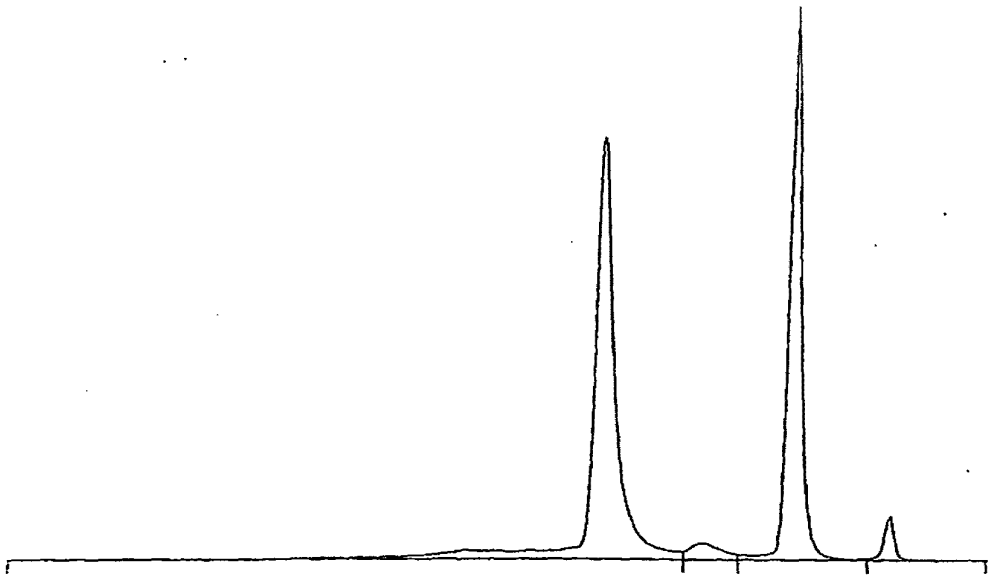
	%
HbA	46,4
HbF	3,5
HbS	47,1
HbA2	3,0

FIG 7B



	%
HbA	42,1
HbF	3,7
HbS	51,3
HbA2	2,9

FIG 7C



	%
HbA	55,5
HbF	3,1
HbS	38,8
HbA2	2,6

FIG 7D

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 15, 2006 Signature: _____ (Michael H. Teschner)

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Robert et al.

Application No.: 11/280,025 Group Art Unit: 1753

Filed: November 16, 2005 Examiner: Not Yet Assigned

For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Dear Sir:

It is respectfully requested that the references listed on the enclosed form be made of record and considered with respect to the above-referenced U.S. patent application. Submission of the present Information Disclosure Statement should not be taken as an admission that the cited references are legally available prior art or that the same are pertinent or material. This reference was identified in an unsolicited e-mail from a third party and is being submitted as the third party thought that it was relevant to the claimed invention.

In the event that any fee is due in connection with the present Information Disclosure Statement, the Commissioner is hereby authorized to charge the same to our Deposit Account No. 12-1095.

Dated: September 15, 2006

Respectfully submitted,

By _____

Michael H. Teschner
Registration No.: 32,862
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP
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Attorney for Applicant

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PTO/SB/08a/b (07-05)
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Substitute for form 1449A/B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	1	of	1

Complete if Known	
Application Number	11/280,025-Conf. #8408
Filing Date	November 16, 2005
First Named Inventor	Frédéric Robert
Art Unit	1753
Examiner Name	Not Yet Assigned
Attorney Docket Number	EGYP 3.0-034

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-6,766,817-A1	07-27-2004	da Silva et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature		Date Considered	

677105

PTO/SB/06 (12-04)
Approved for use through 7/31/2006. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875 Effective December 8, 2004

Application or Docket Number: 11280225

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	150.00		N/A	300.00
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	$250		N/A	$500
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	$100		N/A	$200
TOTAL CLAIMS (37 CFR 1.16(i))	30 minus 20 =	10	X$ 25 =		OR	X$50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	9	X100 =	250		X200 =	
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))			+180=	180		+360=	
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	930		TOTAL	

APPLICATION AS AMENDED – PART II

9-19-06

AMENDMENT A	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 27	Minus	** 30	= 1	X$ 25 =		OR	X$50 =	
Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 1	X100 =		OR	X200 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X$ 25 =		OR	X$50 =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X100 =		OR	X200 =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					+180=		OR	+360=	
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P. . Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PLUS Search Results for S/N 11280025, Searched Fri Oct 24 11:51:47 EDT 2008

The Patent Linguistics Utility System (PLUS) is a USPTO automated search system for U.S. Patents from 1971 to the present PLUS is a query-by-example search system which produces a list of patents that are most closely related linguistically to the application searched. This search was prepared by the staff of the Scientific and Technical Information Center, SIRA.

5748114 79
5812706 79
20010034057 79
20030034356 79
20030056720 79
20040196859 79
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20060102478 77
3620652 61
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

530 7590 11/14/2008

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

EXAMINER	
DIETERLE, JENNIFER M	
ART UNIT	PAPER NUMBER
4111	

MAIL DATE	DELIVERY MODE
11/14/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/280,025	ROBERT ET AL.	
	Examiner	Art Unit	
	Jennifer Dieterle	4111	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *1* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *29-55* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *29-55* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0226

DETAILED ACTION

Election/Restrictions

Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 29 - 52, drawn to the method of separating haemoglobins in a biological sample, classified in class 204, subclass 451.

II. Claims 53 - 55, drawn to a kit containing a zwitterionic type buffer, classified in class 204, subclass 451.

The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case, the product as claimed can be used in a materially different process of using that product. For example, the kit containing a zwitterionic type buffer can be used in a plaque assay (see attached article by Love).

Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

A telephone call was made to Michael H. Teschner on November 6, 2008, to request an oral election to the above restriction requirement, but did not result in an election being made.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder. All claims directed to a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder**. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder. All claims directed to a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process

claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder**. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Dieterle whose telephone number is (571) 270-7872. The examiner can normally be reached on Monday thru Friday, 8am to 5pm (EST).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brian Sines can be reached on (571) 272-1263. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

JMD

/Brian J. Sines/
Supervisory Patent Examiner, Art Unit 4111

Notice of References Cited	Application/Control No. 11/280,025	Applicant(s)/Patent Under Reexamination ROBERT ET AL.	
	Examiner Jennifer Dieterle	Art Unit 4111	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Daria Love, THE UTILIZATION OF ZWITTERIONIC BUFFER SYSTEM IN THE PLAQUE ASSAY OF A FELINE CALICIVIRUS, 1973, AJEBAK 51 (Pt. 2), 263-266.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	4111

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	11/06/2008								
	29	÷								
	30	÷								
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AJEBAK 51 (Pt. 2) 263-266 (1973)

Brief Communication:

THE UTILIZATION OF ZWITTERIONIC BUFFER SYSTEM IN THE PLAQUE ASSAY OF A FELINE CALICIVIRUS

by DARIA N. LOVE

(From the Department of Veterinary Pathology, University of Sydney, Sydney, N.S.W.)

(*Received June 26, 1972. Accepted for publication September 11, 1972.*)

In 1971 feline picornaviruses were placed with vesicular exanthema virus in the genus Calicivirus within the family Picornaviridae (Wildy, 1971). Several publications utilizing the tube culture technique for infectivity titrations of feline caliciviruses have appeared (Burki, 1965; Burki and Pichler, 1971). Kahn and Gillespie (1970) mentioned plaque formation by strains of feline caliciviruses but no plaque assay technique has previously been described. Although Good, Winget, Winter, Connolly, Izawa and Singh (1966) first reported the development and the possible application of certain zwitterionic amino acids as buffering systems for biological research, most virus plaque assay techniques still utilize either closed or open systems with carbon dioxide supplementation. However, Richter (1967) reported the use of a zwitterionic buffer N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES) in an open system which gave results comparable to bicarbonate-carbon dioxide buffering in air. This communication reports on the use of a zwitterionic amino acid buffer system in the development of a plaque technique for feline caliciviruses.

Materials and Methods.

The strain of feline calicivirus used was isolated from a nasal swab from a cheetah which exhibited signs of glossitis and conjunctivitis (Sabine and Hyne, 1970). It was passaged twice in kitten kidney cell culture before plaque purification (Dulbecco and Vogt, 1954). Primary kitten kidney cells were used throughout the study. The cell culture technique of Youngner (1956) was used and cells were established in growth medium (Table 1). Primary kitten kidney cells were seeded at the rate of $2{\cdot}4 \times 10^6$ cells in 8 ml growth medium per 60 mm petri dish. Preliminary experiments showed it was necessary to use this volume and number of cells to produce consistent growth in all dishes. Cells formed confluent monolayers after 5-7 days at 37° in a humidified incubator. Prior to infection, growth medium was removed and the cells were washed twice with 5 ml each of phosphate buffered saline pH 7·4. Triplicate dishes at each dilution (Cooper, 1961) were inoculated with 0·1 ml virus diluted in maintenance medium (Table 1) and adsorbed at 22° or 37° for various times up to 1 h. At the end of the adsorption period excess inocula were removed and 6 ml overlays (Table 1) added. The incorporation of the specific agarose mentioned was shown in preliminary experiments to be superior to either oxoid agarose or oxoid agar, both in gel strength at lower concentrations and plaque detection, without staining or the addition of a second overlay containing neutral red or other stain.

One molar N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES) stock was dissolved in distilled water and sterilized by Millipore filtration and used as described in Table 1, with correction of pH by one molar sodium hydroxide.

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Page 1003-0235

TABLE 1.

Media for primary kitten kidney cell culture.

Growth medium— Constituent	Vol. (ml)
Gibco G-11 medium (5x conc.)	20
Serum (bovine)	10
TES IM	1
NaOH IM	0·8
Antibiotics	100 μm Streptomycin/ml 100 IU Penicillin/ml 1 μg Fungizone/ml
Double distilled water	to 100

Maintenance medium—constituents as above with serum reduced to 2 ml.
Overlay medium—20 ml Behringwerke AG Agarose (2·5% agar) were added to the maintenance medium prior to addition of the distilled water.

Antiserum to the feline calicivirus was prepared in rabbits. Purified virus was emulsified with an equal volume of Freund's complete adjuvant and 1 ml of the mixture was inoculated intramuscularly into two separate sites. This was repeated 1 month later. Sterile serum was then collected after a further 2 weeks and heat inactivated at 56° for 30 min. Plaque neutralisation tests demonstrated titres in rabbit serum of 1:5250 and 1:7630 to a challenge dose of 65 p.f.u./0·1 ml virus.

RESULTS.

Following virus infection of the primary kitten kidney monolayers, plaques first appeared macroscopically at 24 h as distinct opaque foci 0·5 mm diam. when viewed by oblique light against a dark background. Microscopically, these areas appeared as foci of characteristic picornavirus cytopathic effect. The number and sizes of plaques increased until the second day when the size of the plaques averaged 2-3 mm diameter. From day 2 onwards the number of plaques remained constant but the size increased to 5-6 mm by the third day. With continued incubation plaques increased in size until confluent cytopathic effect resulted.

Plaques were easily demonstrable macroscopically at all times without the addition of a second overlay containing vital dyes. On some occasions the accuracy of counting was checked by removal of the overlay and staining of the cell sheet with 0·3% aqueous solution of Giemsa stain. On each occasion no difference in the plaque count resulted. Parallel titration of virus samples in different batches of primary cells gave little difference in virus titre.

A linear relationship between plaque count and virus concentration was established using two-fold virus dilutions of a stock virus preparation of known titre (Fig. 1). Each point is the average of three dishes. The demonstration of a linear relationship between plaque count and virus concentration is necessary to establish the usefulness of a system as a particle counting technique (Cooper, 1961).

Virus was allowed to adsorb to monolayer cultures at 22° or 37° for 10, 20, 30 and 60 min. At the end of each period unadsorbed inocula were withdrawn and overlays added before incubation at 37° for 2 days. Adsorption profiles at both temperatures were similar, but in each of three separate experiments optimum adsorption occurred at 22° after 30 min. Although the adsorption profile was similar in appearance to other published reports, Bachrach, Callis, Hess and Patty (1957) and others have found greater efficiency of adsorption at higher temperatures. At least two factors may have contributed to my results. The small inoculum volume (0·1 ml) allowed cell damage to occur over the adsorption period at 37°.

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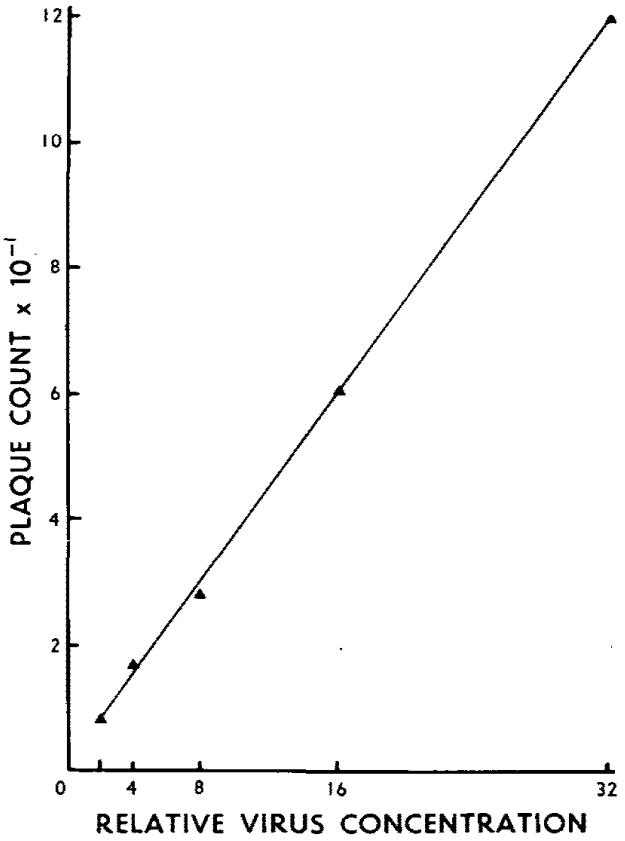


Fig. 1. Relationship between plaque count and concentration of feline calicivirus titrated in primary kitten kidney cell culture monolayers.

Cell damage in the form of cytoplasmic stranding and cell rounding was seen by phase-contrast microscopy of cell layers after 30 min at 37°. Also, this virus has been found to lose 1·2 logs of infectivity after 8 h at 37° (unpublished results).

Plaque formation could be prevented by pretreatment of the virus with virus specific antiserum prepared in rabbits. Pretreatment of virus with normal rabbit or normal cat serum did not prevent plaque formation. Volumes of a standard virus dilution were mixed with equal volumes of serum dilutions and allowed to react at 37° for 2 h before the addition of 0·3 ml of the mixture to each of three monolayer cultures per serum dilution. The results seen in Table 2 are typical of many plaque neutralisation experiments conducted.

TABLE 2.

Suppression of plaque formation by specific antivirus serum.

Type of serum	Serum dilution	Av. number of plaques
Rabbit anticalicivirus serum	undiluted	0
	1 : 1	0
	1 : 2	6·6
	1 : 4	16·0
	1 : 8	27·0
	1 : 16	54·0
Normal rabbit serum	undiluted	64·0
Normal cat serum	undiluted	67·0

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DISCUSSION.

A plaque assay system has been developed for feline caliciviruses. The assay is statistically acceptable by the criteria described by Dulbecco and Vogt (1954) and Cooper (1961). Some technical aspects of the assay were investigated and optimal conditions defined.

It has been shown that a zwitterionic amino acid buffer (TES) successfully supported both growth of primary kitten kidney cell monolayers and plaque production by the virus in an open plaque assay system. Good *et al.* (1966) investigated several zwitterionic buffers which would function most effectively at pK_a values close to the pH of biological systems. These workers found that two buffers, TES and HEPES (N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid) were superior to conventional buffers and were able to maintain optimal pH values in the range of 7·2 to 7·4 in both open and closed cell culture systems. Since then these buffers have been used successfully by Richter (1967), Williamson and Cox (1968) and Shipman (1969).

Although both TES and HEPES buffers were able to maintain pH and allow plaque formation in the present system, it was not possible to obtain consistent monolayer growth using HEPES buffer. Cells tended to clump together and did not migrate rapidly from their point of original attachment. Morris (1971) reported that chick neural retinal cells showed a reduction in cell spreading and increased cell-to-cell binding in HEPES buffer which did not occur when cells were grown in TES buffer.

The method described in this communication has been successfully applied to plaque production with three other strains of feline calicivirus and three feline herpesvirus isolates.

Acknowledgements. I am indebted to Dr. M. McClain for her excellent instruction and original suggestions in this study and to Dr. M. Sabine for discussion throughout this work.

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PATENT & TRADEMARK OFFICE

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 12, 2008 Signature: _____
(Mitchell J. Mehlman)

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Robert et al.	
Application No.: 11/280,025	Group Art Unit: 4111
Filed: November 16, 2005	Examiner: J.M. Dieterle
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	

RESPONSE TO RESTRICTION REQUIREMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

This communication is in response to the Office Action mailed November 14, 2008, setting forth a Restriction Requirement in the above-identified application. In the Office Action, the Examiner required restriction to one of the following inventions under 35 U.S.C. §121:

I. Claims 29-52, drawn to the method of separating haemoglobins in a biological sample, classified in class 204, subclass 451.

II. Claims 53-55, drawn to a kit containing a zwitterionic type buffer, classified in class 204, subclass 451.

The Examiner contends that these inventions are distinct for a number of reasons.

In response, as required, Applicants provisionally elect Invention I, of claims 29-52, method for separating haemoglobins in a biological sample by capillary electrophoresis with traverse.

In particular, while the Examiner has taken the broad position that an invention drawn to a method and a kit are essentially independent and distinct *per se*, Applicants suggest that a review of the specific facts in this case demonstrates that there would not be a serious burden on the Examiner if restriction were not required in this case. Comparing claims 29 and 53, for example, it can be seen that each of these claims require a zwitterionic type analysis buffer and at least one flow inhibitor.

Further, each of the classes and subclasses selected by the Examiner are identical. Each of these classes and subclasses, including indented subclasses, identify processes including capillary electrophoresis processes and products including, kits, electrolytes and buffer compositions used for capillary electrophoresis. For example, compositions used in a kit for capillary electrophoresis and capillary electrophoresis processes would likely be classified in the identified class and subclass. Therefore, a search for one would necessarily be co-extensive with a search for the other. It is thus submitted that a single search will suffice for all pending claims.

Far from being a serious burden on the Examiner, the restriction of this case places a serious burden on the Applicants to separately pursue two sets of claims drawn to different patentable aspects of the same invention.

In any event, in view of the provisional election of claims made hereinabove, it is clear that this application is now in condition for examination on the merits, and such action is therefore respectfully solicited.

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In the event any fee is due in connection with the present response, the Examiner is authorized to charge Deposit Account No. 12-1095 therefor.

Dated: December 12, 2008

Respectfully submitted,

By _____

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

530 7590 01/08/2009

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DIETERLE, JENNIFER M	
ART UNIT	PAPER NUMBER
4111	

MAIL DATE	DELIVERY MODE
01/08/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/280,025	ROBERT ET AL.	
	Examiner	Art Unit	
	Jennifer Dieterle	4111	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *29-55* is/are pending in the application.

4a) Of the above claim(s) *53-55* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *29-52* is/are rejected.

7)☒ Claim(s) *29,33,43-45 and 51* is/are objected to.

8)☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *19 September 2006* is/are: a)☐ accepted or b)☒ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *11/16/2005, 09/19/2006*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

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DETAILED ACTION

Election/Restrictions

Applicant's election with traverse of claims 29-52 in the reply filed on December 28, 2008 is acknowledged. The traversal is on the ground(s) that since the there would not be a burden on the examiner to examine all claims and that the subclasses are identical. This is not found persuasive because the product (a kit containing a zwitterionic type buffer) as claimed can be used in a materially different process than the method of use claimed in the application. For example, the kit containing a zwitterionic type buffer can be used in a plaque assay making the product and process of using that product distinct. While the product and process of use may be classified in the same class/subclass, the product as claimed can be used in a materially different process than that claimed in the application. See MPEP § 806.05(h).

Furthermore, applicant's argument is not found persuasive because the primary classification of subject matter is merely one indication of the burdensome nature of the search requirements and a serious burden on the examiner may also be shown by appropriate explanation of the field of search (see MPEP § 803). As discussed in the restriction requirement, the different inventions incorporate different features that would involve different considerations in determining patentability. The restriction requirement merely refers to the primary classification for the inventions, not the complete scope of the prior art search in additional art class and subclass classifications that would be required in determining patentability. Clearly, different searches and patentability determination issues are involved in the examination of each invention.

The requirement is still deemed proper and is therefore made FINAL.

Claims 53 – 55 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention.

Drawings

The drawings are objected to because all figures lack axis labels. Corrected drawings in compliance with 37 CFR 1.121(d) are required in reply to the Office Action to avoid abandonment of the application. Any amended replacement drawing sheet should include all of the figures appearing on the immediate prior version of the sheet, even if only one figure is being amended. The figure or figure number of an amended drawing should not be labeled as "amended." If a drawing figure is to be canceled, the appropriate figure must be removed from the replacement sheet, and where necessary the remaining figures must be renumbered and appropriate changes made to the brief description of the several views of the drawings for consistency. Additional replacement sheets may be necessary to show the renumbering of the remaining figures. Each drawing sheet submitted after the filing date of an application must be labeled in the top margin as either “Replacement Sheet” or “New Sheet” pursuant to 37 CFR 1.121(d). If the changes are not accepted by the examiner, the applicant will be notified and informed of any required corrective action in the next Office Action. The objection to the drawings will not be held in abeyance.

Specification

Applicant is reminded of the proper language and format for an abstract of the disclosure.

The abstract should be in narrative form and generally limited to a **single paragraph** on a separate sheet within the range of 50 to 150 words. It is important that the abstract not exceed 150 words in length since the space provided for the abstract on the computer tape used by the printer is limited. The form and legal phraseology often used in patent claims, such as "means" and "said," should be avoided. The abstract should describe the disclosure sufficiently to assist readers in deciding whether there is a need for consulting the full patent text for details.

The abstract of the disclosure is objected to because the abstract in the instant Application contains two paragraphs. Correction is required. See MPEP § 608.01(b).

The specification is objected to because of the following informalities: The abbreviation “EC” used throughout, however, the abbreviation is not defined in the specification. Appropriate correction is required.

Claim Objections

The application is objected to because of the following informalities: the heading for the claims section states "IN THE CLAIMS." The correct heading format is "CLAIM or CLAIMS." See MPEP § 608.01(i)-(p). Appropriate correction is required.

Claim 29 is objected to because of the following informalities: a comma is required after the phase “flow inhibitor” in line 9.

In addition, the word “said” (third word in on line 9) should be moved before the phrase “flow inhibitor” in line 9. Appropriate correction is required.

Claim 33 is objected to because of the following informalities: the terms “A2, and a C, a D, an B, a S, a F, and/or an A haemoglobin” are unclear. Refer to the original filed claim 5 for guidance. Appropriate correction is required.

Claims 43, 44, and 51 are objected to because of the following informalities: the term “said analysis buffer” is vague and indefinite. Appropriate correction is required.

Claim 45 is objected to because of the following informalities: the lower range concentration measurement does not contain a unit of measurement. Appropriate correction is required.

Claim 33, is objected to because the term “and/or” is improper Markush language. It is suggested that this is changed to “selected from the group consisting of” or that the “and" is removed. Appropriate correction is required.

Claims 38-40 and 48, are objected to because the term “selected from” is improper Markush language. It is suggested that this is changed to “selected from the group consisting of”. Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 33 and 35 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement.

Claim 33 was added in the preliminary amendments filed 09-19-2006. Claim 33 contains the subject matter concerning haemoglobin B which was not described in the specification or in the originally filed claims in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. On page 3 and 5, lines 21 and 5, respectively, of the specification filed 11-16-2005, haemoglobin E is discussed, however, haemoglobin B is not mentioned.

In addition, Claim 35 was also added in the preliminary amendments filed 09-19-2006. Claim 35 contains the subject matter concerning AIVIPD which was not described in the specification or in the originally filed claims in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 29-31, 34-36, and 43-52 are rejected under 35 U.S.C. 102(b) as being anticipated by Nouadje et al. (U.S. 2002/0162744 A1).

Regarding claims 29-31, Nouadje et al. teaches a method of analyzing biological samples using capillary electrophoresis comprising the following:

- Separating the constituents of blood proteins (page 1, section [0003])(it is well know to those skilled in the art that haemoglobin is an oxygen carrying protein in red blood cells), which can be normal or abnormal (page 2, section [0036]) by capillary electrophoresis in which the sample is introduced into a capillary tube containing a buffer system (page 1, section [0009]) (typically a zwitterionic biological buffer system (page 2, section [0032])).
- further comprising at least one additive that is capable of hydrophobic interaction with one or more protein constituents and capable of providing said protein constituents with one or more negative charges thereby reducing the electrophoretic mobility of said protein (page 1, section [0007]),
- wherein, once the protein constituents are separated, they are detected (page 1, section [0007]).

With respect to claims 34-36, Nouadje et al. teaches an analysis buffer that can be selected from bis-Tris PROPANE with an amino acid, TRICINE, TAPS, TABS, AMPSO, Tris, BICINE, HEPBS (page 2, section [0040]). With respect to a TAPS zwitterionic buffer, this buffer has an acid function on one end and a hydroxyl function in the opposite position to the acid function with an amine in the middle (Roy et al., *Buffer Standards*, page 552, section I).

With respect to claims 43 and 44, Nouadje et al. teaches an analysis buffer that has a concentration between 10 mM to 500 mM (page 3, section [0043]). This range covers the ranges given in the current application's claim of 20 mM to 500 mM.

With respect to claims 45 and 46, Nouadje et al. teaches that the additives concentration be between 0.1 mM to 500 mM (page 3, section [0044]). This range encompasses the current application's claim of 0.1mM to 50mM.

With respect to claims 47 and 48, Nouadje et al. teaches that the analysis buffer may contain a pH modifying compound selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri-, or tetra- alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion (page 3, section [0042]).

With respect to claims 49 and 50, Nouadje et al. teaches a method of capillary electrophoresis using a capillary tube formed from fused silica (page 3, section [0051]). Nouadje et al. also teaches that a person skill in the art will be capable of adapting the nature and size of the capillary to the analytical requirements. These requirements may include utilizing a naked or untreated internal capillary surface (page 3, section [0049]).

With respect to claim 51, Nouadje et al. teaches that the analysis buffer has a pH between 2 and 12 (page 3, section [0041]). This pH range encompasses the current application claimed range of 8 to about 10.

With respect to claim 52, Nouadje et al. teaches the method wherein said capillary electrophoresis is of the free solution capillary electrophoresis type (page 1, section [0007]).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claim 32 is rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. in view of Keo et al. (U.S. Pat. No. 5,599,433).

Regarding claim 32, Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoresis as discussed above. However, Nouadje does not teach the separation of haemolyzed blood.

Keo et al. recognizes the use of haemolyzed red blood cells in capillary electrophoresis.

Therefore, the claimed method involves applying the known technique of capillary electrophoresis to a known product of haemolyzed blood to yield the predictable result of facilitating effective separation and subsequent analysis as shown by the cited prior art. The Supreme Court has decided that applying a known technique to a known product ready for improvement to yield predictable results is likely to be obvious. See *KSR International Co. v. Teleflex Inc.*, 550 U.S. __,__, 82 USPQ2d 1385, 1395 – 97 (2007) (see MPEP § 2143, D.).

Therefore, it would have been obvious to a person of ordinary skill in the art to use haemolyzed blood in capillary electrophoresis.

Claim 33 is rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. in view of Chen (U.S. Pat. No. 5,202,006).

Regarding claim 33, Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoresis as discussed above. However, Nouadje does not teach the specific haemoglobin A2 and a C, a D, an B, a S, a F and/or an A haemoglobin.

Chen recognizes the use of capillary electrophoresis of haemoglobin A, A2, F which comprise normal adult blood (col. 1, lines 26-30), and haemoglobin S (col. 2, lines1-5).

Consequently, a person of ordinary skill in the art would accordingly have recognized the suitability, reasonable expectation for success, and subsequent predictability of utilizing capillary electrophoresis in effectively separating and analyzing the above haemoglobins.

The prior art can be modified or combined to reject claims as *prima facie* obvious as long as there is a reasonable expectation of success. See *In re Merck & Co., Inc.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986) (see MPEP § 2143.02).

Furthermore, the claimed method involves applying the known technique of capillary electrophoresis to a known product comprising haemoglobin to yield the predictable result of facilitating effective separation and subsequent analysis as shown by the cited prior art. The Supreme Court has decided that applying a known technique to a known product ready for improvement to yield predictable results is likely to be obvious. See *KSR International Co. v. Teleflex Inc.*, 550 U.S. __,__, 82 USPQ2d 1385, 1395 – 97 (2007) (see MPEP § 2143, D.).

Therefore, it would have been obvious to a person of ordinary skill in the art to use haemoglobin variants such as A2 and a C, a D, an B, a S, a F and/or an A.

Claims 37-42 are rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. in view of Grover et al. (U.S. Pat. No. 6,083,372).

Regarding claims 37-42, Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoresis as discussed above. However, Nouadje et al. fails to specifically teach the recited flow inhibitor.

Grover et al. teaches the use of flow inhibitors that can encompass polyamine compounds and many amines that are available commercially (col. 11, lines 10-13). Grover et al. teaches that such compounds include, but are not limited to 1,4-diaminobutane (col. 11, line 15). The selection of a known material, which is based upon its suitability for the intended use, is within the ambit of one of ordinary skill in the art. See *In re Leshin*, 125 USPQ 416 (CCPA 1960) (see MPEP § 2144.07). Additionally, the combination of familiar elements is likely to be obvious when it does no more than yield predictable results. Furthermore, the simple substitution of one known element for another is likely to be obvious when predictable results are achieved. See *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. __, __, 82 USPQ2d 1385, 1395 (2007)(see MPEP § 2143 and 2143.02). Therefore, it would have been obvious to one skilled in the art to use various polyamine compounds such as 1,4- diaminobutane as a flow inhibitor for separating haemoglobin by capillary electrophoresis.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Dieterle whose telephone number is (571) 270-7872. The examiner can normally be reached on Monday thru Friday, 8am to 5pm (EST).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brian Sines can be reached on (571) 272-1263. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

JMD

/Brian Sines/
Supervisory Patent Examiner, Art Unit 4111

Notice of References Cited	Application/Control No. 11/280,025	Applicant(s)/Patent Under Reexamination ROBERT ET AL.	
	Examiner Jennifer Dieterle	Art Unit 4111	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2002/0162744	11-2002	Nouadje et al.	204/451
*	B	US-6,083,372	07-2000	Grover et al.	204/451
*	C	US-5,202,006	04-1993	Chen, Fu-Tai A.	204/451
*	D	US-5,599,433	02-1997	Keo et al.	204/451
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	Roy et al., Buffer Standards for the Physiological pH of the Zwitterionic Compound, TAPS, From5 to 55◦C, Journal of Solution Chemistry, Vol. 35, No. 4, April 2006, page 552 (see chemical strucutre)
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	4111

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	12/29/2008	12/29/2008							
	1	-								
	2	-								
	3	-								
	4	-								
	5	-								
	6	-								
	7	-								
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	22	-								
	23	-								
	24	-								
	25	-								
	26	-								
	27	-								
	28	-								
	29	✓								
	30	✓								
	31	✓								
	32	✓								
	33	✓								
	34	✓								
	35	✓								
	36	✓								

Helena Laboratories Corp.
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Page 1003-0258

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	4111

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	12/29/2008	12/29/2008							
	37	✓								
	38	✓								
	39	✓								
	40	✓								
	41	✓								
	42	✓								
	43	✓								
	44	✓								
	45	✓								
	46	✓								
	47	✓								
	48	✓								
	49	✓								
	50	✓								
	51	✓								
	52	✓								
	53	N								
	54	N								
	55	N								

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	4111

SEARCHED			
Class	**Subclass**	**Date**	**Examiner**
204	450, 451, 468, 601	12/29/2008	JMD

SEARCH NOTES		
Search Notes	**Date**	**Examiner**
Inventor Search, Google, NPL	12/29/2008	JMD

INTERFERENCE SEARCH			
Class	**Subclass**	**Date**	**Examiner**

/Jennifer Dieterle/ Examiner.Art Unit 4111	

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	59	204/674.ccls.	US-PGPUB; USPAT	OR	ON	2008/12/29 15:17
L2	1	l1 and zwitterionic	US-PGPUB; USPAT	OR	ON	2008/12/29 15:18
L3	1	11/280025	US-PGPUB; USPAT	OR	ON	2008/12/29 15:21
L4	707	204/451.ccls.	US-PGPUB; USPAT	OR	ON	2008/12/29 16:04
L5	71	204/468.ccls.	US-PGPUB; USPAT	OR	ON	2008/12/29 16:05
L6	860	204/601.ccls.	US-PGPUB; USPAT	OR	ON	2008/12/29 16:05
L7	0	2002/0162744	US-PGPUB; USPAT	OR	ON	2008/12/29 16:21
L8	0	2002/0162744	US-PGPUB; USPAT	OR	ON	2008/12/29 16:21
L9	6	Nouadje.in.	US-PGPUB; USPAT	OR	ON	2008/12/29 16:21
S1	821	204/450.ccls.	US-PGPUB; USPAT	OR	ON	2008/10/27 16:37
S2	606	S1 and (electrophoresis or cze or ce)	US-PGPUB; USPAT	OR	ON	2008/10/27 16:38
S3	41	S2 and zwitterionic	US-PGPUB; USPAT	OR	ON	2008/10/27 16:38
S4	327333	capillary electrophoresis or CE	US-PGPUB; USPAT	OR	ON	2008/10/28 11:46
S5	31	S4 and "zwitterionic buffer".clm.	US-PGPUB; USPAT	OR	ON	2008/10/28 11:46
S6	0	S5 and "flow inhibitor$"".clm"	US-PGPUB; USPAT	OR	ON	2008/10/28 11:47
S7	1	S5 and "flow inhibitor".clm.	US-PGPUB; USPAT	OR	ON	2008/10/28 11:47
S8	327333	S4 and py<"2005"	US-PGPUB; USPAT	OR	ON	2008/10/28 11:50
S9	31	S5 and py<"2005"	US-PGPUB; USPAT	OR	ON	2008/10/28 11:50
S10	0	("2006/0102478").URPN.	USPAT	OR	ON	2008/10/28 11:52
S11	0	("2006/0102478").URPN.	USPAT	OR	ON	2008/10/28 11:52
S12	0	("2006/0102478").URPN.	USPAT	OR	ON	2008/10/28 11:52
S13	0	("2006/0102478").URPN.	USPAT	OR	ON	2008/10/28 11:52

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S16	1	("20060102478").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 13:14
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S18	1	("5447612").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 13:17
S19	1	("5599433").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 13:17
S20	1	("5439825").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 13:18
S21	0	("5439825").URPN.	USPAT	OR	ON	2008/10/28 13:19
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S23	1	("5202006").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 13:21
S24	5	("5202006").URPN.	USPAT	OR	ON	2008/10/28 13:22
S25	147088	zwitterionic buffer.clm.	US-PGPUB; USPAT	OR	ON	2008/10/28 15:54
S26	74645	S25 and flow "inhibitor.clm"	US-PGPUB; USPAT	OR	ON	2008/10/28 15:58
S27	0	S25 and "flow inhibitor.clm"	US-PGPUB; USPAT	OR	ON	2008/10/28 15:58
S28	0	S25 and "hemoglobin.clm"	US-PGPUB; USPAT	OR	ON	2008/10/28 15:59
S29	0	S25 and "haemoglobin.clm"	US-PGPUB; USPAT	OR	ON	2008/10/28 15:59
S30	0	S25 and "haemoglobin.clm."	US-PGPUB; USPAT	OR	ON	2008/10/28 16:00
S31	2	haemoglobin.clm. and zwitterionic.clm.	US-PGPUB; USPAT	OR	ON	2008/10/28 16:00

S32	445	diaminobutane.clm.	US-PGPUB; USPAT; USOCR	OR	ON	2008/10/28 16:07
S33	0	S32 and zitterionic	US-PGPUB; USPAT; USOCR	OR	ON	2008/10/28 16:08
S34	327333	capillary electrophoresis or CE	US-PGPUB; USPAT	OR	ON	2008/10/28 16:17
S35	31	S34 and "zwitterionic buffer".clm.	US-PGPUB; USPAT	OR	ON	2008/10/28 16:17
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S37	237	"flow inhibitor"	US-PGPUB; USPAT	OR	ON	2008/10/28 16:44
S38	1	("6766817").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/10/28 16:54
S39	1483	S25 and "aliphatic diamine" or cyclic adj diamine or polyamine adj type	US-PGPUB; USPAT	OR	ON	2008/10/28 16:57
S40	9	S39 and "204"/$.ccls.	US-PGPUB; USPAT	OR	ON	2008/10/28 16:58
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S42	0	"silva.inv."	US-PGPUB; USPAT	OR	ON	2008/10/29 07:39
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S44	11	S43 and "204"/$.ccls.	US-PGPUB; USPAT	OR	ON	2008/10/29 07:40
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S50	2	("5938930").PN.	US-PGPUB; USPAT; USOCR; DERWENT	OR	OFF	2008/10/30 13:13
S51	2	("5567282").PN.	US-PGPUB; USPAT; USOCR; DERWENT	OR	OFF	2008/10/30 13:50
S52	267127	haemoglobin capillary electrophoresis	US-PGPUB; USPAT	OR	ON	2008/10/30 14:37

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S58	0	"11/280025".ptan.	US-PGPUB; USPAT	OR	ON	2008/10/30 14:39
S59	0	"11/280,025".ptan.	US-PGPUB; USPAT	OR	ON	2008/10/30 14:39
S60	0	"280025".ptan.	US-PGPUB; USPAT	OR	ON	2008/10/30 14:39
S61	0	"11280025".ptan.	US-PGPUB; USPAT	OR	ON	2008/10/30 14:39
S62	225	haemoglobin capillary electrophoresis	US-PGPUB; USPAT	AND	ON	2008/10/30 14:40
S63	0	S62 and zitterionic	US-PGPUB; USPAT	AND	ON	2008/10/30 14:40
S64	4	S62 and tricine	US-PGPUB; USPAT	AND	ON	2008/10/30 14:40
S65	236	haemoglobin capillary electrophoresis	US-PGPUB; USPAT; JPO; DERWENT	AND	ON	2008/10/31 15:04
S66	327842	capillary electrophoresis or CE	US-PGPUB; USPAT	OR	ON	2008/10/31 15:05
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S79	16634	haemoglobin A2.clm.	US-PGPUB; USPAT	OR	ON	2008/11/04 09:53
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S81	89	S79 and "204"/$.ccls.	US-PGPUB; USPAT	OR	ON	2008/11/04 09:54
S82	4	capillary adj electrophoresis adj kit	US-PGPUB; USPAT	OR	ON	2008/11/04 14:08
S83	5	("5792330").URPN.	USPAT	OR	ON	2008/11/04 14:15
S84	224489	haemolobing or heamoglobin near analysis nea kit	US-PGPUB; USPAT	OR	ON	2008/11/04 14:16
S85	0	haemolobing or heamoglobin near analysis near kit	US-PGPUB; USPAT	OR	ON	2008/11/04 14:16
S86	222765	haemolobing or heamoglobin near analysis ajd kit or commercial adj kit	US-PGPUB; USPAT	OR	ON	2008/11/04 14:16
S87	115	S86 and zwitterionic adj buffer	US-PGPUB; USPAT	OR	ON	2008/11/04 14:16
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S91	8	S90 and zwitterionic adj buffer	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/04 14:39
S92	31	"1,4 diaminobutane" and capillary adj electrophoresis	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:18
S93	7112	spermine	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:22
S94	156056	S93 and capillary electrophoresis	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:22
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S96	1014	S95 and py>"2004"	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:22
S97	1013	S95 and (1,4- diaminobutane or "1,4" diamino butane)	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:23
S98	312	S95 and @py>"2004"	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:26
S99	311	S97 and @py>"2004"	US-PGPUB; USPAT; JPO; DERWENT	OR	ON	2008/11/05 13:26
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S101	35	("3909380" | "4680201" | "4865706" | "4865707").PN. OR ("5181999").URPN.	US-PGPUB; USPAT; USOCR	OR	ON	2008/11/05 14:45
S102	5778	diaminobutane	US-PGPUB; USPAT; USOCR	OR	ON	2008/11/05 14:51
S103	42	S102 and "capillary electrophoresis"	US-PGPUB; USPAT; USOCR	OR	ON	2008/11/05 14:51
S104	1119	diamine and "capillary electrophoresis"	US-PGPUB; USPAT; USOCR	OR	ON	2008/11/05 14:56
S105	35580	S103 abd zwitterionic	US-PGPUB; USPAT; USOCR	OR	ON	2008/11/05 14:57
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S111	1	("6268161").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2008/11/06 16:31

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BIB DATA SHEET

CONFIRMATION NO. 8408

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/280,025	11/16/2005	204	4111	EGYP 3.0-034

APPLICANTS
Frederic Robert, Mennecy, FRANCE;
Denis Simonin, Evry, FRANCE;
Jean-Baptiste Clement, Etiolles, FRANCE;

**** CONTINUING DATA **************************

**** FOREIGN APPLICATIONS **************************
FRANCE 0412263 11/18/2004

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED **** ** SMALL ENTITY **
12/19/2005

Foreign Priority claimed ☑ Yes ☐ No 35 USC 119(a-d) conditions met ☑ Yes ☐ No Verified and Acknowledged /JENNIFER M DIETERLE/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		FRANCE	13	28	3

ADDRESS

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090
UNITED STATES

TITLE

Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method

FILING FEE RECEIVED		
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/280,025-Conf. #8408
Filing Date	November 16, 2005
First Named Inventor	Frédéric Robert
Art Unit	1753
Examiner Name	Not Yet Assigned
Attorney Docket Number	EGYP 3.0-034

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-6,766,817-A1	07-27-2004	da Silva et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

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[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature	/Jennifer Dieterle/	Date Considered	12/30/2008

677105

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.D./

PTO/SB/08a/b (07-05)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Frédéric Robert
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	EGYP 3.0-034

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-5,447,612-A	09-05-1995	Bier et al.	
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	BA	FR-1.554.045-A	01-17-1969	LKB-Produkter AB		√
	BB	WO-97/04308-A1	02-06-1997	Waters Investments Limited		√

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NON PATENT LITERATURE DOCUMENTS

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	CA	ISHIOKA et al., "Detection of Abnormal Haemoglobin by Capillary Electrophoresis and Structural Identification", Biomedical Chromatography, Vol. 1, pgs. 224-226, 1992	
	CB	SAHIN et al., "Haemoglobin analysis by capillary zone electrophoresis", Journal of Chromatography A, Vol. 709, pgs. 121-125, 1995	
	CC	JENKINS et al., "Capillary electrophoresis as a clinical tool" Journal of Chromatography B, Vol. 682, pgs. 23-34, 1996	
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Journal of Chromatography A, 709 (1995) 121–125

JOURNAL OF CHROMATOGRAPHY A

Haemoglobin analysis by capillary zone electrophoresis

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Abstract

The analysis of haemoglobin is routine in medical laboratories for the purpose of assessing blood disorders and related pathologies. Haemoglobin is structurally diverse and possesses many variant forms, some disease-producing. With recent improvements in technology, capillary electrophoresis is now being adapted in the clinical laboratory. This paper describes the analysis of haemoglobin variants using uncoated fused-silica capillaries. The effects of using different buffer salts at different concentrations with different buffer pH values to separate haemoglobin variants in these capillaries are described.

1. Introduction

Analysis of haemoglobin (Hb) variants from patients' blood samples is of medical importance and, for this reason, is an essential procedure in the clinical laboratory. Hb is a heterotetramer which is formed from the association of two pairs of identical subunits. The predominant Hb variant in normal adult blood is Hb A1, a heterotetrameric protein with two α-chains and two β-chains. Hb A2 is also a normal variant present in smaller amounts. Hb F is a form composed of two α- and two γ-globin chains and is normally expressed during foetal development; its presence is gradually replaced by Hb A usually 6 months after birth. The Hb S and C variants are isoelectric variants as a result of single amino acid changes in one of the globin chains (β), and such changes induce alterations in the structural–functional relationships of the Hb subunits to one another, such that at certain blood pH and level of blood oxygenation, the Hb tetramer undergoes an irreversible conformational shift which manifests itself as a change in the shape of the red blood cell (the classical sickle shape in sickle-cell anaemia). Hundreds of Hb variants, some producing no symptomatic disease, have been identified by various analytical methods.

The demands for clinical laboratories to provide accurate analyses with timely results makes new methodologies which achieve such goals very welcome. Capillary zone electrophoresis (CZE) is finding many uses in clinical chemistry applications [1–6], and there is the potential to apply CZE in Hb variant analysis also. CZE offers some important advantages over other conventional electrophoretic techniques. Besides requiring very small amounts of sample and electrolyte, its potential for automation makes this technique time and labour effective for clinical laboratories.

Several reports have already appeared in which normal and pathological states were assessed based on Hb variant analysis using capillary electrophoretic methods. Particular methods

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SSDI 0021-9673(95)00229-4

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focused on the analysis of intact Hb molecules [2], globin chains [3] or tryptic digests of haemoglobin molecules [4]. Hb variant analysis has also been examined by isoelectric focusing methods using coated [5] and uncoated capillaries [6]. In this study we attempted to establish optimum conditions for Hb variant analysis using CZE with uncoated capillaries.

2. Experimental

The capillary electrophoresis system was a BioFocus 3000 (Bio-Rad, Richmond, CA, USA) equipped with a fused-silica capillary [36 cm (30 cm to the detector) × 50 μm I.D.]. Sample injection was by positive pressure [$34.45 \cdot 10^7$ Pa (5 p.s.i.) for 2 s], with sample loaded at the anode end of the capillary. The applied potential was 10 kV for all analyses. All reagents for preparing electrolytes were of analytical-reagent grade (Merck, Darmstadt, Germany). A capillary wash solution (Bio-Rad) was used to purge the capillaries between runs. Preparation of the capillary for each run involved an automated purging cycle with capillary wash solution, water and running electrolyte in that order, for 1 min each. Detection was at 415 nm using a tungsten lamp source.

Human Hb samples were prepared from whole blood collected in EDTA-containing evacuated tubes. Red blood cells were washed with normal (0.9%) saline four times. An aliquot of a washed cell suspension was mixed with four aliquots of distilled water. Haemolysis was made complete by freezing in a deep freezer for 10 min. Samples were centrifuged at ca. 600 *g* for 10 min. The supernatant was diluted tenfold in running electrolyte, and the sample was made ready by filtering it through 0.45-μm cellulose ester (Syrfil-MF; Costar, Cambridge, MA, USA).

As an external reference for haemoglobin species variants, the Beckman Paragon electrophoresis system and Hb variant standard kit (Beckman Instruments, Brea, CA, USA) was also used to analyse samples.

3. Results and discussion

The basis for the separation of the Hb variants using CZE is the charge differences in the globin polypeptides. The structure of all variants are such that they possess a net negative charge, under the conditions of CZE separation, and thus the inherent electrophoretic mobility of the Hb species will be directed towards the anode. However, the use of uncoated fused silica at pH values in the neutral or alkaline range creates a significant electro-endoosmotic flow (EOF) directed towards the cathode. The magnitude of the EOF is such that the net mobility of all Hb species is towards the cathode. This property means that Hb species having higher isoelectric points (p*I* values) (i.e., species with less net negative charge) should be detectable first inasmuch as the electrostatic repulsive forces in the opposite direction are less than for Hb species having lower p*I* values, and they would show a higher net mobility towards the cathode.

Satisfactory separation of analytes using CZE is highly dependent on a suitable electrophoresis buffer–electrolyte. In addition to ensuring overall stability of the system, the buffer determines the migration behaviour of analytes. Several different buffers have been used for haemoglobin separations in traditional free zone electrophoresis applications. Some methods employ a single weak acid or base to provide the buffering at the running pH; others consist of a mixture of weak acids or bases. As the p*I* values of haemoglobin species variants are between 7.00 and 7.50 (Hb A1, 7.15; Hb A2, 7.40; Hb F, 7.15; Hb S, 7.25), preparation using buffers with the pH adjusted above these values becomes necessary. Tris and borate buffers are commonly selected because of their buffering capacity ranges between pH 8.0 and 9.5. Veronal (barbital) buffers also possess a buffering capacity in this range and are commonly used in electrophoretic systems involving the use of commercially available agarose gels to analyse Hb variants. We tested Tris, borate and veronal buffers at three different concentrations and at different pH values. Analysis with a Tris–HCl electrolyte was carried

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out at 0.5, 1.0 and 1.5 *M*, sodium borate was tested at 20, 30 and 40 m*M* and veronal (sodium barbiturate) at 25, 50 and 75 m*M*. For each of these concentrations for each of the indicated salts, three different pH values were tested, namely pH 8.0, 8.5 and 9.0.

The electropherogram in Fig. 1 illustrates how the molecular charge difference between the Hb S and Hb A1 species affects their separation by CZE. This separation of these variants was also generally observed under a variety of conditions in which all permutations for the three different buffer salts, concentrations and pH were tested. The Hb A2 species is seen to migrate ahead of Hb S in Fig. 1. We found that the degree of separation of Hb S from Hb A1 was not significantly different for any change in the pH (8.0, 8.5, 9.0) of the salts tested, but there were certainly changes in migration times, an increase in pH producing shorter migration times for all species. The inset in Fig. 1 shows a densitometric scan of stained haemoglobin variants observed using the Beckman agarose gel electrophoresis system. Although we did not confirm the identity of Hb variant species during the CZE analysis of our samples using certified standards, the agarose gel analysis operates under identical principles to CZE and the densitometric scan of stained proteins in the gel compares favourably with the absorbance changes during CZE in which detection is specific for the Hb prosthetic group.

The variant Hb F has no significant charge difference from Hb A1 (only 0.05 pH unit separates these variants), and this presents the most difficult challenge to manipulating conditions to separate common variants. Fig. 2 shows the separation of Hb F and Hb A1 from a patient's sample using a 50 m*M* veronal system buffered at pH 8.5. An observable separation of these species was achieved for all pH values tested, but the resolution appeared better at pH 8.0 and 8.5 than at pH 9.0, demonstrating an effect of pH on this separation. Again, the

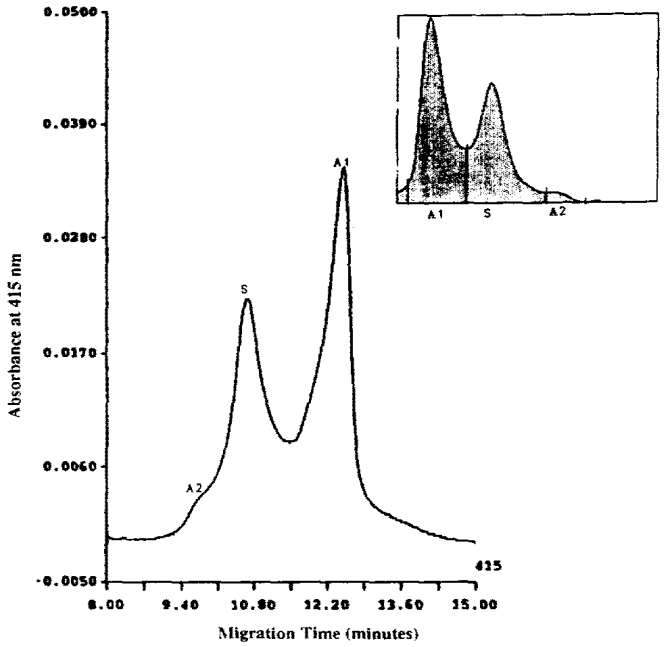


Fig. 1. Electropherogram of a sample from a patient with sickle-cell disease. Electrolyte, 1.0 *M* Tris–HCl (pH 8.0). Other conditions as described under Experimental.

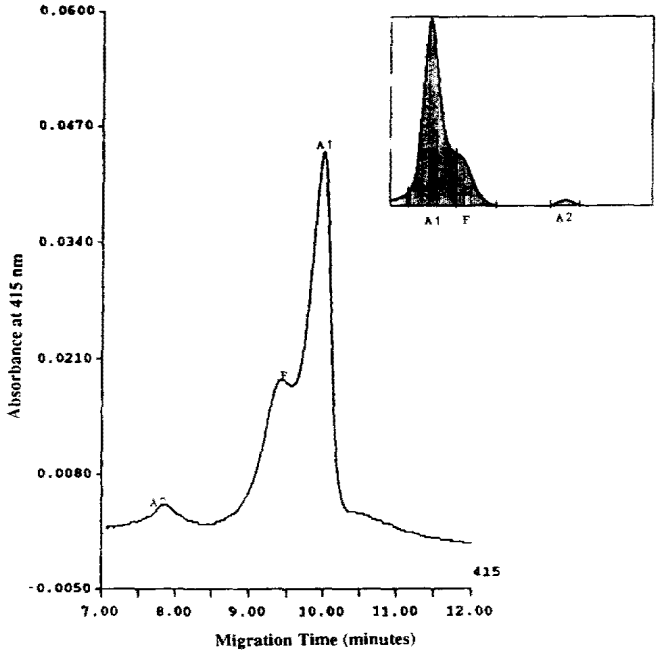


Fig. 2. Electropherogram of a patient's sample with a significant Hb F fraction. Electrolyte, veronal (sodium barbital) (pH 8.5).

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proportions of the amounts of Hb species present in this sample agreed with the agarose gel determination (inset).

Fig. 3 is an electropherogram of another patient's sample in which Hb variants were separated using 20 m*M* sodium borate (pH 8.5). The Hb variants A2, S, F and A1 were all detectable. These particular conditions resolved these four variants the best, although we did observe the separation of these variants using this buffer at other pH values. For selecting particular buffer salts to be used in such CZE separations, we found the best concentration for Tris to be 1.0 *M*, for veronal 50 m*M* and for borate 20 m*M*.

Variations in the migration times of the species for replicate analyses were a significant problem and make routine clinical use difficult, in that species identification depends on consistent migration times (much as component identification in high-performance liquid chromatography depends on consistent retention times). We made attempts to stabilize the EOF, which we believe is the factor responsible for such irreproducibility, but we were unable to obtain more optimum conditions. As the Hb A1 variant is usually a constant in all analyses, its identification can be made initially and then used as a reference for identifying other variants. Fig. 4 is an electropherogram of a normal adult blood sample with Hb A1 present as the major species. There are other cases in which Hb A1 may not be the major species present, as shown in Fig. 5 (identification of Hb species in CZE were made by comparison with separated zones seen in agarose gel electrophoresis, a routine method in our laboratory as well as in many others). Such cases might present some confusion for the analyst. Nevertheless, the experienced analyst would know the relative order of migration of the typical species present in the population, and variations in routine electropherogram patterns can be easily discerned. Alternatively, unknown patients' samples can be run with internal standards (e.g., with commercially available Hb variant standards) to make Hb variant identification unambiguous.

Changes to the conditions of buffering (selection of the ideal salt and therefore of the ideal

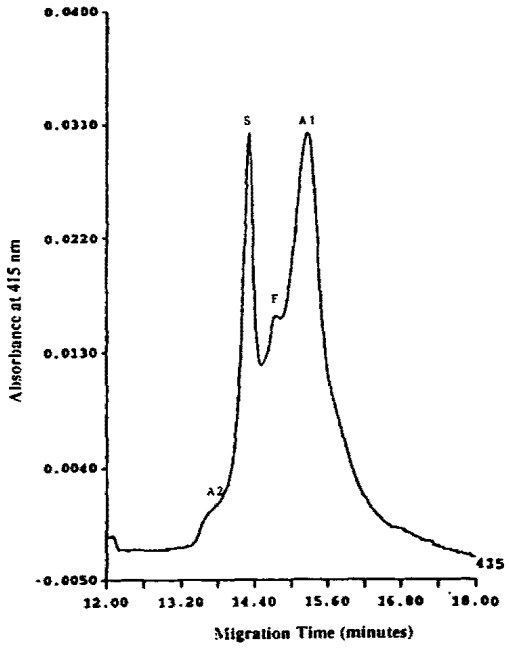


Fig. 3. Electropherogram of a patient's sample containing significant proportions of variants Hb A1, A2, S and F. Buffer, sodium borate (pH 8.5).

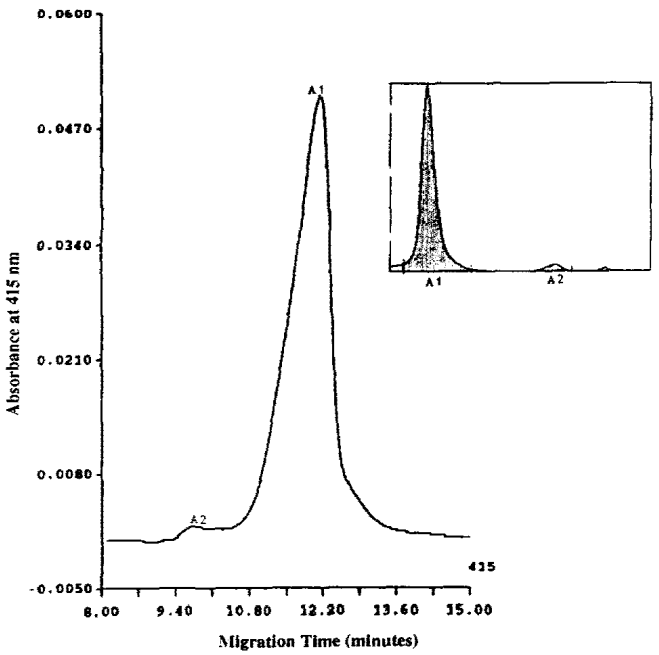


Fig. 4. Electropherogram of a sample obtained from a healthy adult. Buffer, veronal (pH 8.5).

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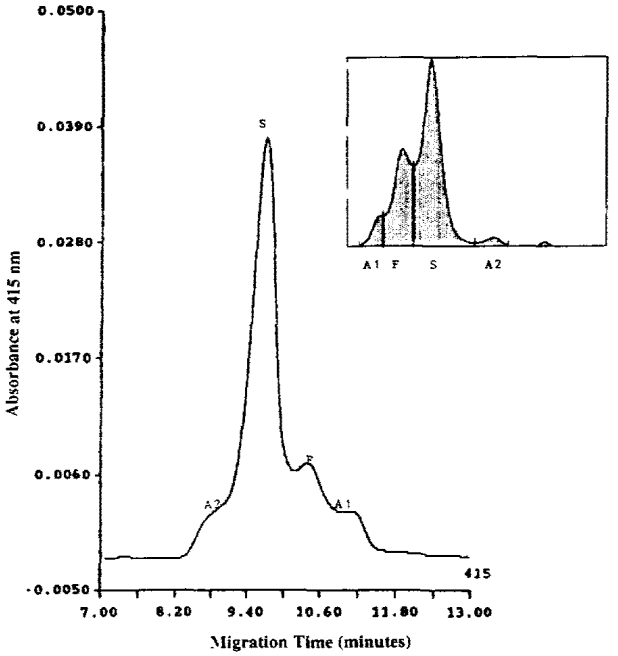


Fig. 5. Electropherogram of a sample from a patient with sickle-cell disease. Buffer, veronal (pH 8.5).

pK_a, electrolyte pH and buffer concentration), in addition to selection of modifying agents which dynamically coat the capillary walls in order to alter protein adsorption and to reduce the EOF to negligible levels, are future considerations for further work to understand and control the considerable variations of migration times observed in replicate analyses.

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Journal of Solution Chemistry, Vol. 35, No. 4, April 2006 (© 2006)
DOI: 10.1007/s10953-005-9010-0

Buffer Standards for the Physiological pH of the Zwitterionic Compound, TAPS, From 5 to 55 °C

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Received June 6, 2005; revised October 19, 2005
Published Online: May 5, 2006

The values of the second dissociation constant, pK_2, and related thermodynamic quantities of *N*-[tris(hydroxymethyl)methyl-3-amino]propanesulfonic acid (TAPS) have already been reported at 12 temperatures over the temperature range 5–55 °C, including 37 °C. This paper reports the results for the pH of five equimolal buffer solutions with compositions: (a) TAPS (0.03 mol·kg^{-1}) + NaTAPS (0.03 mol·kg^{-1}); (b) TAPS (0.04 mol· kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}); (c) TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}); (d) TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}); and (d) TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}). The remaining eight buffer solutions consist of saline media of the ionic strength I = 0.16 mol·kg^{-1}, matching closely to that of the physiological sample. The compositions are: (f) TAPS (0.04 mol-kg^{-1}) + NaTAPS (0.02 mol-kg^{-1}) + NaCl (0.14 mol·kg^{-1}); (g) TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}); (h) TAPS (0.6 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}); (i) TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}); (j) TAPS (0.04 mol· kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}); (k) TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}) + NaCl (0.11 mol·kg^{-1}); (l) TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}); and (m) TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}) + NaCl (0.08 mol·kg^{-1}). These buffers are recommended as a pH standard for clinical measurements in the range of physiological application. Conventional pH values, designated as pH(s), for all 13 buffer solutions from 5 to 55 °C have been calculated. The operational pH values with liquid junction corrections, at 25 and 37 °C for buffer solutions, designated above as (b), (c), (d), (e), (j), (l), and (m); have been determined based on the difference in the values of the liquid junction potentials between the accepted phosphate standard and the buffer solutions under investigation.

KEY WORDS: Buffers; TAPS; liquid junction; ionic strength; emf; zwitterions; pH; acidity function.

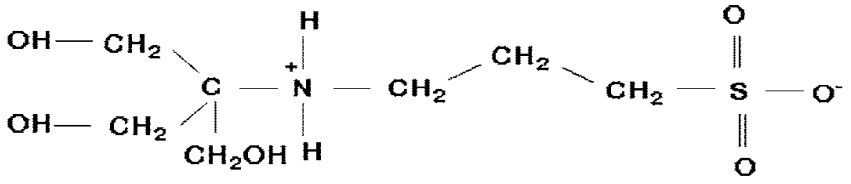
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1. INTRODUCTION

For measurements of the pH of blood and biological specimens, it is necessary to know accurate values of pK_2 of TAPS. Very recently, we have reported the pK_2 values of *N*-[tris(hydroxymethyl)methyl-3-amino]propanesulfonic acid (TAPS)[1] at temperatures from 5 to 55 °C including 37 °C. This zwitterionic buffer system has been recommended by Good and coworkers[2,3] for use as a physiological buffer. The structure is as follows:



TAPS

Standardization of the calibrating electrodes of the pH meter assembly at a point close to the pH of blood (that is, between 7 and 8) can be obtained within the framework of the former National Bureau of Standards (NBS) by using physiological phosphate pH buffer as a primary standard.[4] The pH of this physiological phosphate buffer standard is 7.415 at 25 °C and 7.395 at 37 °C, and has been widely accepted and used as an integral part of clinical diagnoses.

Nevertheless, there are some problems regarding the use of the phosphate buffer as an ideal pH standard of ionic strength, $I = 0.16$ mol· kg^{-1} for biological specimens. The reasons[2,3] are as follows: (i) phosphates interact unfavorably with biological media, (ii) phosphate precipitates with Mg^{2+} and Ca^{2+} present in blood, and (iii) the temperature coefficient of the phosphate buffer is −0.0028 pH unit/°C, which is almost twice that of whole blood (−0.015 pH unit/°C) and plasma (0.01 pH unit/ °C).[5]

Good and his associates[2,3] provided about 25 primarily new hydrogen ion buffers which are mostly compatible with common biological media. They outlined some criteria for the evaluation of these materials. Wu and coworkers[6] have published the values of pK_2 and pH of the zwitterionic buffer, *N*-(2-hydroxyethyl) piperazine-*N*-2-ethanesulfonic acid (HEPES), and a second zwitterionic buffer, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO).[7] Both the HEPES and MOPSO buffers have been certified by the National Institute of Standards and Technology (NIST) as primary reference standards. In this work as well as previously published work, we have provided data for a number of important zwitterionic buffer materials. Just to cite three specific systems, Roy *et al.*[8] reported results for pK_2 and pH for 3-(*N*-morpholino)propanesulfonic acid (MOPS); 4-*N*-(morpholino)butanesulfonic acid (MOBS);[9] and *N*-tris(hydroxymethyl)-4-aminobutanesulfonic acid (TABS).[10] The pH of these solutions closely matches

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that of the common biological media. In 1973, Bates *et al.*[11] suggested the use of tris(hydroxymethyl)methylglycine (Tricine) as a buffer standard for the physiological range of pH 7.2–8.5. The pH of 0.06 mol· kg^{-1} Tricine + 0.02 mol· kg^{-1} sodium Tricinate buffer solution at 37 °C is 7.407, matching exactly the pH of blood. Goldberg *et al.*[12] in their recent review article of the thermodynamic quantities of the biological buffers, indicated that no results of pK_2 and pH are available in the literature for TAPS.

In order to provide very accurate and reproducible pH values for physiological pH standards, we have studied the buffer compound, TAPS, with the following compositions on the scale of molality, m, where m = mol· kg^{-1}:

A. TAPS (0.03 mol·kg^{-1}) + NaTAPS (0.03 mol·kg^{-1}), I = 0.03 mol·kg^{-1}
B. TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}), I = 0.04 mol·kg^{-1}
C. TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}), I = 0.05 mol·kg^{-1}
D. TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}), I = 0.06 mol·kg^{-1}
E. TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}), I = 0.08 mol·kg^{-1}
F. TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.02 mol·kg^{-1}) + NaCl (0.14 mol·kg^{-1}), I = 0.16 mol·kg^{-1} (isotonic saline media).
G. TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
H. TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
I. TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
J. TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
K. TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}) + NaCl (0.11 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
L. TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}), I = 0.16 mol·kg^{-1}
M. TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}) + NaCl (0.08 mol·kg^{-1}), I = 0.16 mol·kg^{-1}

The detailed procedure for the preparation of these buffer solutions of TAPS is described in the experimental section.

2. EXPERIMENTAL

TAPS was obtained from Sigma Chemical Co. (St. Louis, Missouri). The details of the purification by further crystallization as well as the determination of the assay have been reported in an earlier paper.[1] The assay showed that the TAPS was 0.9994 ± 0.0003 pure. All buffer solutions were prepared by weighing the

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buffer substance TAPS, NaCl (ACS reagent grade), a standard solution of NaOH to prepare NaTAPS and finally calculated amounts of CO_2-free doubly distilled water. Air buoyancy corrections were applied for all masses used.

The preparation of the hydrogen electrodes and the silver—silver chloride electrodes of the thermal electrolytic type,[13] the design of the all-glass cells, the purification of the hydrogen gas, and preparation of the solutions have been described previously.[1,8] Details about the control of the temperature (within ± 0.005 K) using a digital platinum-resistance thermometer (Guildline Model 9540), a digital voltmeter (Hewlett-Packard 2000 multimeter), and other experimental procedures, will also be found elsewhere.[1]

A correction for the liquid-junction potential is required if accurate pH values are to be achieved. Thus the cells studied were the following:

$$\text{Pt(s), } H_2\text{(g, 1 atm)|TAPS}(m_1) + \text{NaTAPS}(m_2) + \text{NaCl}(m_3)\text{|AgCl(s), Ag(s)} \quad (1)$$

where m_1, m_2, and m_3 indicate molalities of the respective species, and 1 atm = 101.325 kPa in SI units. The cell (1) is known as the Harned-type cell. The flowing junction cell (2), was used for the evaluation of the liquid junction potential at the contact between the buffer solution and the heavier saturated KCl solution shown with a double vertical line.

$$\text{Pt(s), } H_2\text{(g, 1atm)|TAPS}(m_1) + \text{NaTAPS}(m_2), \text{NaCl}(m_3)\text{||KCl(sat)} \times \text{|}Hg_2Cl_2\text{(s), Hg(l)} \quad (2)$$

where the abbreviations (s), (l), and (g) denote solid, liquid, and gaseous state, respectively. In routine laboratory measurements, the hydrogen electrode is commonly replaced by a glass electrode.

For cell (3), the phosphate salts were NIST standard reference materials with the composition [KH_2PO_4 (0.008695 mol·kg^{-1}) + Na_2HPO_4 (0.03043 mol·kg^{-1})] and its solutions are recommended for pH measurements in physiological solutions.

$$\text{Pt(s); } H_2\text{(g, 1atm)|phosphate buffer||KCl(sat)|}Hg_2Cl_2\text{(s), Hg(l)} \quad (3)$$

The values of the standard electrode potential, E°_{SCE}, of the saturated calomel electrode were taken as: −0.2415 and −0.2335 V at 25 and 37 °C, respectively. The values of the liquid junction potential, E_j, for the physiological phosphate solutions and other buffer solutions of TAPS from cell (2) were obtained[6,8] using the following equation:[8]

$$E_j = E + E^\circ_{SCE} - k\text{pH} \quad (4)$$

where $E^\circ_{SCE} = -0.2415$ V, $k = 0.059156$, and pH is 7.415 (physiological phosphate buffer solution) at 25 °C; $E^\circ_{SCE} = -0.2335$, $k = 0.061538$, and pH is 7.395 at 37 °C. We have attempted to calculate a value of the liquid junction potential for each buffer solution. This may be of interest to some solution chemists, but actually the presumed difference in E_j between the accepted phosphate standard and each

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experimental buffer solution will be a factor when different standards are selected to obtain the values of the operational pH for an unknown medium. This error can be estimated by the operational definition of pH, designated as pH(x), and is:

$$\mathrm{pH(x)} = \mathrm{pH(s)} + \frac{E_\mathrm{x} - E_\mathrm{s} + \delta E_\mathrm{j}}{k} \tag{5}$$

where x refers to the unknown buffer TAPS + NaTAPSate; s is the reference solution (NIST physiological phosphate buffer) of known pH, and $\delta E_j = E_{j(x)} - E_{j(s)}$. If $\delta E_j = 0$, then Eq. (5) takes the form:

$$\mathrm{pH(x)} = \mathrm{pH(s)} + \frac{E_\mathrm{x} - E_\mathrm{s}}{k} \tag{6}$$

3. METHODS AND RESULTS

Accurate values of the second dissociation constants, pK_2, and related thermodynamic quantities have been reported earlier.[1] The emf data for cell (1) containing five equimolal buffer solutions and eight dilute buffer solutions in which NaCl had been added to make $I = 0.16$ mol·kg^{-1}, have been corrected to a hydrogen pressure of 1 atm. The values of the emf at 25 °C are the average of at least two readings (at the beginning and the middle) and sometimes at the end of the temperature sequence. The silver—silver chloride electrodes were used throughout the experiments in order to determine pH values of buffer solutions. Duplicate cells usually gave readings on the average within (0.03 ± 0.01) mV in the temperature range 5–55 °C. All these results are listed in Tables I and II, respectively.

3.1. The pH of TAPS Buffer

Conventional pH values have been evaluated by the method of Bates *et al.*[11,14,15] for 13 standard buffer solutions with the following compositions in units of molality:

(a). TAPS (0.03 mol·kg^{-1}) + NaTAPS (0.03 mol·kg^{-1}), $I = 0.03$ mol·kg^{-1}
(b). TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}), $I = 0.04$ mol·kg^{-1}
(c). TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}), $I = 0.05$ mol·kg^{-1}
(d). TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}), $I = 0.06$ mol·kg^{-1}
(e). TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}), $I = 0.08$ mol·kg^{-1}
(f). TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.02 mol·kg^{-1}) + NaCl (0.14 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1} (isotonic saline media).
(g). TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}
(h). TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}

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Table I. Emf of Cell A: Pt; H_2(g, 1 atm)|TAPS(m_1), NaTAPS(m_2), NaCl(m_3)|AgCl(s), Ag (s)

$m_1 = m_2$[a]	m_3[a]	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	37 °C	40 °C	45 °C	50 °C	55 °C
0.03	0.005	0.86031	0.86106	0.86174	0.86224	0.86252	0.86297	0.86314	0.86322	0.86330	0.86318	0.86313	0.86298
0.03	0.01	0.84370	0.84412	0.84449	0.84468	0.84473	0.84481	0.84475	0.84463	0.84462	0.84438	0.84397	0.84348
0.03	0.015	0.83405	0.73432	0.83448	0.83455	0.83435	0.83432	0.83407	0.83391	0.83370	0.83322	0.83258	0.83195
0.03	0.02	0.82709	0.82726	0.82722	0.82719	0.82688	0.82679	0.82644	0.82626	0.82594	0.82536	0.82461	0.82369
0.04	0.005	0.85988	0.86067	0.86132	0.86183	0.86233	0.86265	0.86287	0.86290	0.86292	0.86295	0.86294	0.86270
0.04	0.01	0.84363	0.84411	0.84448	0.84460	0.84481	0.84490	0.84490	0.84478	0.84464	0.84435	0.84395	0.84334
0.04	0.015	0.83414	0.83451	0.83469	0.83463	0.83471	0.83456	0.83434	0.83417	0.83395	0.83349	0.83229	0.83218
0.04	0.02	0.82756	0.82765	0.82771	0.82752	0.82742	0.82719	0.82693	0.82670	0.82641	0.82578	0.82524	0.82424
0.05	0.005	0.86052	0.86112	0.86173	0.86211	0.86247	0.86272	0.86283	0.86292	0.86298	0.86283	0.86284	0.86276
0.05	0.01	0.84389	0.84430	0.84463	0.84475	0.84484	0.84481	0.84478	0.84473	0.84451	0.84436	0.84370	0.84348
0.05	0.015	0.83442	0.83463	0.83483	0.83481	0.83472	0.83456	0.83412	0.83410	0.83384	0.83340	0.83274	0.83211
0.05	0.02	0.82761	0.82778	0.82788	0.82778	0.82761	0.82734	0.82698	0.82680	0.82647	0.82591	0.82504	0.82448
0.06	0.005	0.86016	0.86089	0.86177	0.86200	0.86263	0.86274	0.86298	0.86310	0.86316	0.86307	0.86293	0.86290
0.06	0.01	0.84384	0.84434	0.84457	0.84482	0.84505	0.84502	0.84495	0.84495	0.84480	0.84447	0.84406	0.84365
0.06	0.015	0.83427	0.83458	0.83476	0.83470	0.83475	0.83464	0.83441	0.83433	0.83411	0.83358	0.83298	0.83247
0.06	0.02	0.82758	0.82785	0.82785	0.82772	0.82753	0.82738	0.82703	0.82690	0.82658	0.82597	0.82526	0.82449
0.08	0.005	0.86111	0.86190	0.86241	0.86288	0.86329	0.86351	0.86369	0.86364	0.86375	0.86367	0.86348	0.86333
0.08	0.01	0.84463	0.84506	0.84531	0.84545	0.84552	0.84546	0.84535	0.84529	0.84511	0.84469	0.84421	0.84369
0.08	0.015	0.83485	0.83514	0.83525	0.83532	0.83533	0.83521	0.83491	0.83469	0.83448	0.83391	0.83328	0.83254
0.08	0.02	0.82797	0.82819	0.82822	0.82815	0.82798	0.82774	0.82734	0.82712	0.82678	0.82612	0.82543	0.82456

[a] Units of m, mol·kg^{-1}.

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Table II. Emf of Cell A: Pt; H_2(g, 1 atm)|TAPS(m_1), NaTAPS(m_2), NaCl(m_3)|AgCl(s), Ag(s) for TAPS Buffer in Isotonic Saline Solution ($I = 0.16$ mol·kg^{-1})

m_1[a]	m_2[a]	m_3[a]	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	37 °C	40 °C	45 °C	50 °C	55 °C
0.04	0.02	0.14	0.76839	0.76735	0.76623	0.76476	0.76324	0.76142	0.75955	0.75883	0.75752	0.75543	0.75308	0.75089
0.05	0.04	0.12	0.78043	0.77978	0.77897	0.77805	0.77697	0.77587	0.77464	0.77410	0.77331	0.77184	0.77037	0.76882
0.06	0.04	0.12	0.77575	0.77496	0.77417	0.77325	0.77202	0.77080	0.76952	0.76899	0.76802	0.76648	0.76501	0.76335
0.08	0.06	0.10	0.75686	0.75580	0.75459	0.75307	0.75148	0.74977	0.74804	0.74720	0.74618	0.74431	0.74238	0.74043
0.04	0.04	0.12	0.78907	0.78831	0.78733	0.78614	0.78484	0.78339	0.78204	0.78137	0.78051	0.77894	0.77739	0.77602
0.05	0.05	0.11	0.78800	0.78747	0.78682	0.78600	0.78508	0.78409	0.78300	0.78250	0.78178	0.78057	0.77915	0.77777
0.06	0.06	0.10	0.78985	0.78942	0.78877	0.78809	0.78713	0.78620	0.78512	0.78472	0.78414	0.78284	0.78162	0.78044
0.08	0.08	0.08	0.79561	0.79521	0.79468	0.79402	0.79319	0.79217	0.79118	0.79082	0.79009	0.78885	0.78768	0.78666

[a]Units of m, mol·kg^{-1}.

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(i). TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}
(j). TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}
(k). TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}) + NaCl (0.11 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}
(l). TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.10 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}
(m). TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}) + NaCl (0.08 mol·kg^{-1}), $I = 0.16$ mol·kg^{-1}

In order to calculate pH(s) values for all 13 buffer solutions, calculations of the values of the acidity function $p(a_H\gamma_{Cl})$ were made in the temperature range 5–55 °C, from the emf (E) listed in Tables I and II, the molality of the chloride ion, and E°, the standard potential of the silver – silver chloride electrode.[1] The commonly used equation[11,13] is given by:

$$p(a_H\gamma_{Cl}) = \frac{E - E^\circ}{k} + \log_{10} m_{Cl} \tag{7}$$

where k is the Nernst slope.

From the plot of $p(a_H\gamma_{Cl})$ for each buffer solution against the molality of the chloride ion employing linear regression analysis, the intercept, $p(a_H\gamma_{Cl})^\circ$, at $m_{Cl} = 0$ was obtained. These values for five equimolal buffers listed above from (a) to (e) are given in Table III. The acidity function, $p(a_H\gamma_{Cl})$ for eight

Table III. Acidity Function $p(a_H\gamma_{Cl})^\circ$ for Equimolal (TAPS + NaTAPS) Buffer Solutions From 5 to 55 °C Computed Using Eqs. (7), (8)[a]

t(°C)	0.03 m TAPS + 0.03 m NaTAPS $I = 0.03$ m	0.04 m TAPS + 0.04 m NaTAPS $I = 0.04$ m	0.05 m TAPS + 0.05 m NaTAPS $I = 0.05$ m	0.06 m TAPS + 0.06 m NaTAPS $I = 0.06$ m	0.08 m TAPS + 0.08 m NaTAPS $I = 0.08$ m
5	9.045	9.031	9.045	9.039	9.060
10	8.905	8.894	8.903	8.899	8.920
15	8.774	8.761	8.768	8.769	8.783
20	8.575	8.633	8.637	8.636	8.653
25	8.520	8.512	8.513	8.518	8.530
30	8.403	8.394	8.393	8.395	8.409
35	8.287	8.279	8.278	8.281	8.294
37	8.243	8.235	8.234	8.238	8.248
40	8.179	8.169	8.168	8.173	8.183
45	8.072	8.064	8.062	8.066	8.076
50	7.971	7.971	7.961	7.963	7.972
55	7.874	7.863	7.863	7.867	7.874

[a]Units of m, mol· kg^{-1}.

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Table IV. $p(a_H\gamma_{Cl})$ of (TAPS + NaTAPS) Buffer Solutions From 5 to 55 °C Computed Using Eq. (7)[a]

t (°C)	0.04 m TAPS + 0.02 m NaTAPS + 0.14 m NaCl $I = 0.16$ m	0.05 m TAPS + 0.04 m NaTAPS + 0.12 m NaCl $I = 0.16$ m	0.06 m TAPS + 0.04 m NaTAPS + 0.12 m NaCl $I = 0.16$ m	0.08 m TAPS + 0.06 m NaTAPS + 0.10 m NaCl $I = 0.16$ m
5	8.826	8.977	8.893	8.471
10	8.685	8.839	8.753	8.333
15	8.549	8.705	8.621	8.199
20	8.415	8.577	8.494	8.068
25	8.288	8.453	8.369	7.943
30	8.162	8.335	8.251	7.822
35	8.041	8.221	8.137	7.706
37	7.995	8.176	8.093	7.660
40	7.924	8.111	8.026	7.595
45	7.812	8.005	7.920	7.490
50	7.701	7.904	7.820	7.388
55	7.597	7.806	7.722	7.290

[a] Units of m, mol·kg^{-1}.

buffers (f)–(m) listed above are entered in Tables IV and V from 5 to 55 °C. The uncertainty (mean deviation) introduced in this type of graphical extrapolation appeared to be slightly greater than 0.001 from the lines drawn. Conventional pH(s) values determined from the emf of cells without liquid junction for the solution without the presence of chloride ion were determined by the equation:

$$\text{pH(s)} = p(a_H\gamma_{Cl})^\circ + \log_{10}\gamma_{Cl}^\circ \tag{8}$$

where the single-ion activity coefficient, γ_{Cl}°, cannot be measured experimentally. The estimation of γ_{Cl}° for the calculation of pH(s) by Eq. (8) has been outlined before.[8] The pH values obtained from the liquid junction cell are indicated by pH, whereas the "conventional" pH calculated from Eq. (8) is designated as pH(s).

The convention is not subject to any proof, but the form (Eq. 6) of a "pH convention"[4] based on an extended Debye–Hückel equation has been widely used for this purpose. In the assignment of pH(s) values for the NIST standard,[6,7,14–16] Eq. (9) has been used. The calculation of $\log_{10}\gamma_{Cl}^\circ$ for all of the buffer-chloride solutions were made by using the following equation:

$$\log_{10}\gamma_{Cl}^\circ = -\frac{A\sqrt{I}}{1 + Ba^\circ\sqrt{I}} + CI \tag{9}$$

where I is the ionic strength of the buffer solution, A and B are the Debye–Hückel constants, hydrolysis of the buffer species is negligible, C is an adjustable

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Table V. $p(a_H\gamma_{Cl})$ of (TAPS + NaTAPS) Buffer Solutions From 5 to 55 °C Computed From Eq. (7)[a]

t(°)	0.04 *m* TAPS + 0.04 *m* NaTAPS + 0.12 *m* NaCl $I = 0.16$ *m*	0.05 *m* TAPS + 0.05 *m* NaTAPS + 0.11 *m* NaCl $I = 0.16$ *m*	0.06 *m* TAPS + 0.06 *m* NaTAPS + 0.10 *m* NaCl $I = 0.16$ *m*	0.08 *m* TAPS + 0.08 *m* NaTAPS + 0.08 *m* NaCl $I = 0.16$ *m*
5	9.134	9.077	9.069	9.076
10	8.991	8.938	8.931	8.937
15	8.851	8.805	8.797	8.804
20	8.716	8.676	8.670	8.675
25	8.586	8.552	8.546	8.551
30	8.460	8.434	8.428	8.430
35	8.342	8.320	8.313	8.315
37	8.294	8.275	8.270	8.272
40	8.227	8.209	8.206	8.205
45	8.117	8.105	8.100	8.098
50	8.013	8.003	8.000	7.998
55	7.916	7.905	7.905	7.904

[a]Units of *m*, mol·kg^{-1}.

parameter, and Ba° was assumed to be 1.38[8] kg$^{1/2}$·mol$^{-1/2}$ for all the experimental temperatures, corresponding to an (ion-size parameter) a° of 4.2 Å.[8] The empirical equation for the calculation of the parameter C[6,8] is given below:

$$C = C_{25} + 6.2 \times 10^{-4}(t - 25) - 8.7 \times 10^{-6}(t - 25)^2 \quad (10)$$

where $C_{25} = 0.032$[6] kg·mol^{-1} at 25 °C and t is the Celsius temperature.

The values of pH(s), listed in Tables VI, VII, and VIII, respectively, for equimolal buffer solutions of TAPS without and with NaCl were computed from Eqs. (7)–(10) and are represented by the following equations:

For TAPS (0.03 mol·kg^{-1}) + NaTAPS (0.03 mol·kg^{-1}):

$$\text{pH(s)} = 8.451 - 2.4387 \times 10^{-2}(t - 25) + 9.04 \times 10^{-5}(t - 25)^2 \quad (11)$$

For TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}):

$$\text{pH(s)} = 8.433 - 2.4270 \times 10^{-2}(t - 25) + 8.56 \times 10^{-5}(t - 25)^2 \quad (12)$$

For TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}):

$$\text{pH(s)} = 8.428 - 2.4654 \times 10^{-2}(t - 25) + 9.62 \times 10^{-5}(t - 25)^2 \quad (13)$$

For TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}):

$$\text{pH(s)} = 8.424 - 2.4403 \times 10^{-2}(t - 25) + 8.92 \times 10^{-5}(t - 25)^2 \quad (14)$$

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Table VI. pH(s) of (TAPS + NaTAPS) Buffer Solutions From 5 to 55 °C Computed Using Eqs. (11)–(15)[a]

t(°)C	0.03 m TAPS + 0.03 m Na TAPS $I = 0.03$ m	0.04 m TAPS + 0.04 m Na TAPS $I = 0.04$ m	0.05 m TAPS + 0.05 m Na TAPS $I = 0.05$ m	0.06 m TAPS + 0.06 m Na TAPS $I = 0.06$ m	0.08 m TAPS + + 0.08 m TAPS $I = 0.08$ m
5	8.976	8.954	8.961	8.949	8.960
10	8.836	8.816	8.818	8.808	8.820
15	8.704	8.683	8.683	8.678	8.683
20	8.575	8.555	8.552	8.546	8.553
25	8.449	8.433	8.427	8.422	8.428
30	8.332	8.314	8.307	8.303	8.307
35	8.216	8.199	8.191	8.188	8.191
37	8.172	8.155	8.147	8.145	8.145
40	8.107	8.088	8.081	8.079	8.080
45	8.000	7.983	7.973	7.972	7.972
50	7.898	7.880	7.872	7.869	7.867
55	7.800	7.780	7.774	7.771	7.768

[a] Units of m, mol·kg^{-1}.

For TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}):

$$\text{pH(s)} = 8.429 - 2.4699 \times 10^{-2}(t - 25) + 9.01 \times 10^{-5}(t - 25)^2 \qquad (15)$$

where $5 \leq t \leq 55$ °C. The standard deviations of regression for the pH(s) of the chloride-free buffer solutions, obtained from the fits with Eqs. (11)–(15), are 0.0010, 0.0011, 0.0010, 0.0014, and 0.0012, respectively.

For eight buffer solutions containing NaCl in an isotonic saline medium of the ionic strength $I = 0.16$ mol·kg^{-1}, the values of pH(s) listed in Tables VII and VIII are expressed by the equations:

For TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.02 mol·kg^{-1}) + NaCl (0.14 mol·kg^{-1}):

$$\text{pH(s)} = 8.161 - 2.5465 \times 10^{-2}(t - 25) + 7.65 \times 10^{-5}(t - 25)^2 \qquad (16)$$

For TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}):

$$\text{pH(s)} = 8.454 - 2.4294 \times 10^{-2}(t - 25) + 9.09 \times 10^{-5}(t - 25)^2 \qquad (17)$$

For TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}):

$$\text{pH(s)} = 8.244 - 2.4369 \times 10^{-2}(t - 25) + 8.73 \times 10^{-5}(t - 25)^2 \qquad (18)$$

For TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.1 mol·kg^{-1}):

$$\text{pH(s)} = 7.816 - 2.4675 \times 10^{-2}(t - 25) + 9.24 \times 10^{-5}(t - 25)^2 \qquad (19)$$

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Table VII. pH(s) of (TAPS + NaTAPS) Buffer Solutions from 5 to 55 °C Computed Using Eqs. (16)–(19)[a]

t (°C)	0.04 m TAPS + 0.02 m NaTAPS + 0.14 m NaCl $I = 0.16$ m	0.05 m TAPS + 0.04 m NaTAPS + 0.12 m NaCl $I = 0.16$ m	0.06 m TAPS + 0.04 m NaTAPS + 0.12 m NaCl $I = 0.16$ m	0.08 m TAPS + 0.06 m NaTAPS + 0.10 m NaCl $I = 0.16$ m
5	8.701	8.852	8.767	8.346
10	8.559	8.713	8.628	8.207
15	8.423	8.579	8.495	8.074
20	8.289	8.451	8.368	7.942
25	8.161	8.327	8.243	7.816
30	8.035	8.208	8.124	7.695
35	7.913	8.093	8.009	7.579
37	7.867	8.048	7.965	7.532
40	7.795	7.982	7.897	7.467
45	7.683	7.876	7.791	7.360
50	7.571	7.774	7.690	7.258
55	7.466	7.674	7.590	7.159

[a] Units of m, mol·kg^{-1}.

For TAPS (0.04 mol·kg^{-1}) + NaTAPS (0.04 mol·kg^{-1}) + NaCl (0.12 mol·kg^{-1}):

$$\text{pH(s)} = 8.459 - 2.5531 \times 10^{-2}(t - 25) + 10.1 \times 10^{-5}(t - 25)^2 \quad (20)$$

For TAPS (0.05 mol·kg^{-1}) + NaTAPS (0.05 mol·kg^{-1}) + NaCl (0.11 mol·kg^{-1}):

$$\text{pH(s)} = 8.426 - 2.4395 \times 10^{-2}(t - 25) + 8.96 \times 10^{-5}(t - 25)^2 \quad (21)$$

For TAPS (0.06 mol·kg^{-1}) + NaTAPS (0.06 mol·kg^{-1}) + NaCl (0.1 mol·kg^{-1}):

$$\text{pH(s)} = 8.420 - 2.4315 \times 10^{-2}(t - 25) + 9.28 \times 10^{-5}(t - 25)^2 \quad (22)$$

For TAPS (0.08 mol·kg^{-1}) + NaTAPS (0.08 mol·kg^{-1}) + NaCl (0.08 mol·kg^{-1}):

$$\text{pH(s)} = 8.424 - 2.4519 \times 10^{-2}(t - 25) + 9.21 \times 10^{-5}(t - 25)^2 \quad (23)$$

where t is the temperature in °C. The standard deviations for regression of the "observed" results from Eqs. (16) to (23) are 0.0007, 0.0009, 0.001, 0.0009, 0.001, 0.0009, 0.0007, and 0.0008, respectively.

The pH values at 25 and 37 °C were evaluated from cells with liquid junctions (cells (2) and (3)) by means of the flowing junction cell. The emf values of the cells (2) and (3) at 25 and 37 °C are given in Table IX. Also, the values of E_j from Table X were obtained by using Eqs. (4) and (5). As evident from the data of Table XI, there is a wide variation in pH (as high as ±0.03 pH units). There is no known method for accurately determining the single-ion activity coefficient, $\log_{10} \gamma^{\circ}_{Cl}$, which is the major source of error. Another cause of uncertainty is in

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Table VIII. pH(s) of (TAPS + NaTAPS) Buffer Solutions From 5 to 55 °C Computed Using Eqs. (20)–(23)[a]

t (°C)	0.04 m TAPS + 0.04 m NaTAPS + 0.12 m NaCl $I = 0.16$ m	0.05 m TAPS + 0.05 m NaTAPS + 0.11 m NaCl $I = 0.16$ m	0.06 m TAPS + 0.06 m NaTAPS + 0.10 m NaCl $I = 0.16$ m	0.08 m TAPS + 0.08 m NaTAPS + 0.08 m NaCl $I = 0.16$ m
5	9.008	8.951	8.943	8.951
10	8.865	8.812	8.806	8.812
15	8.725	8.679	8.671	8.678
20	8.590	8.550	8.544	8.549
25	8.460	8.426	8.419	8.425
30	8.333	8.307	8.301	8.303
35	8.214	8.192	8.185	8.187
37	8.166	8.147	8.141	8.144
40	8.098	8.081	8.077	8.076
45	7.988	7.976	7.971	7.969
50	7.883	7.873	7.870	7.867
55	7.784	7.774	7.773	7.772

[a] Units of m, mol·kg^{-1}.

the liquid junction potential measurement. However, the agreement is excellent (within ±0.001) between the calculated pH values and the values obtained from the E_j corrections. The total uncertainty for the pH values was evaluated by combining the various uncertainties due to the: (i) assumption for the calculation

Table IX. Emf of Cell B for TAPS Buffer

			E, V	
$m1$	$m2$	$m3$	25 °C	37 °C
0.04	0.04	0.00	0.74231	0.73775
0.06	0.06	0.00	0.74186	0.73709
0.08	0.08	0.00	0.74212	0.73693
0.04	0.04	0.12	0.74231	0.73636
0.06	0.06	0.10	0.73998	0.73499
0.08	0.08	0.08	0.74034	0.73517

Emf of Cell C[a]

Cell C	E, V	
0.008695 mol·kg^{-1} KH_2PO_4 + 0.03043 mol·kg^{-1} Na_2HPO_4	25 °C	37 °C
	0.68275	0.69147

[a] Corrected to a hydrogen pressure of 1 atm, for physiological phosphate buffer solutions (primary reference standard buffer) at 25 and 37 °C.

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Table X. Values of the Liquid Junction Potentials for TAPS Buffer at 25 and 37 °C, From Eq. (3)

System	E_j^a,mV	
	25 °C	37 °C
Physiological phosphate (0.008695 mol·kg^{-1} KH_2PO_4 + 0.03043 mol·kg^{-1} NaCl)	2.6	2.9
0.04 mol·kg^{-1} TAPS + 0.04 mol·kg^{-1} NaTAPS + 0.0 mol·kg^{-1} NaCl	2.3	2.5
0.06 mol·kg^{-1} TAPS + 0.06 mol·kg^{-1} NaTAPS + 0.0 mol·kg^{-1} NaCl	2.2	2.4
0.08 mol·kg^{-1} TAPS + 0.08 mol·kg^{-1} NaTAPS + 0.0 mol·kg^{-1} NaCl	2.1	2.3
0.04 mol·kg^{-1} TAPS + 0.04 mol·kg^{-1} NaTAPS + 0.12 mol·kg^{-1} NaCl	0.4	0.5
0.06 mol·kg^{-1} TAPS + 0.06 mol·kg^{-1} NaTAPS + 0.10 mol·kg^{-1} NaCl	0.5	0.6
0.08 mol·kg^{-1} TAPS + 0.08 mol·kg^{-1} NaTAPS + 0.08 mol·kg^{-1} NaCl	0.5	0.6

[a] $E_j = E + E^\circ_{SCE} - k$pH from Eq. (4), E is the emf from Table IX, k = Nernst slope with values 0.059156 at 25 °C and 0.061538 at 37 °C; the pH of the primary reference standard phosphate buffer is 7.415 and 7.395 at 25 and 37 °C; E°_{SCE} = electrode potential of the saturated calomel electrode = −0.2415 and −0.2335 V at 25 and 37 °C, respectively.

of the $\log_{10} \gamma^\circ_{Cl}$ (±0.004 pH unit), (ii) extrapolation to $p(a_H \gamma_{Cl})^\circ$ at $m_{Cl} = 0$ (less than ±0.002 pH unit), (iii) liquid junction potential measurement using the flow junction cell, and (iv) error in the experimental emf measurement (±0.03 mV). Thus, the overall estimated uncertainty is ±0.009 pH unit for buffers without the presence of NaCl, and ±0.014 pH unit for buffer solutions with the ionic strength $I = 0.16$ mol·kg^{-1}. The operational pH values at 25 and 37 °C (Table XI) for buffer solutions having the compositions (with and without the presence of NaCl) are

Table XI. Values of pH at 25 and 37 °C for TAPS Buffer Solutions Using Data for E_j Corrections From Table X

Cell B			25 °C			37 °C		
$m_1 = m_2$	m_3	Ionic strength, I	without[a] E_j corr	with[b] E_j corr	Calc[c]	without[a] E_j corr	with[b] E_j corr	Calc[c]
0.04	0.00	0.04	8.427	8.432	8.433	8.148	8.154	8.155
0.06	0.00	0.06	8.414	8.421	8.422	8.137	8.145	8.145
0.08	0.00	0.08	8.419	8.427	8.428	8.134	8.144	8.145
0.04	0.12	0.16	8.422	8.459	8.460	8.125	8.164	8.166
0.06	0.10	0.16	8.382	8.418	8.419	8.103	8.140	8.141
0.08	0.08	0.16	8.389	8.424	8.425	8.106	8.143	8.144

[a] pH = 7.415 + [(E−0.68275)/0.059156 at 25 °C, and pH = 7.395 + [(E−0.69144)/0.061538 at 37 °C; the emf values (Table IX) are 0.68275 and 0.69144 V at 25 and 37 °C for the physiological phosphate buffer standard solution.

[b] Values obtained from Eq. (5) and E_j data of Table X.

[c] Obtained from Tables VI and VII using Eqs. (12), (14), (15), (20), and (22), (23).

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recommended as primary standards for calibrating electrodes for pH measuring systems in physiological fluids.

ACKNOWLEDGMENTS

The authors are grateful to Dr. R. G. Bates for useful discussions and important suggestions incorporated in this revised manuscript; and the grant fund from the National Institutes of Health (AREA), under grant R15 GM 066866-01.

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EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	52	capillary adj electrophoresis same (haemoglobin or hemoglobin)	US-PGPUB; USPAT	OR	ON	2009/01/05 10:54
L2	17	L1 and @py<"2002"	US-PGPUB; USPAT	OR	ON	2009/01/05 10:54
L3	1	("6766817").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2009/01/05 11:03
L4	1	("5447612").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2009/01/05 11:05
L5	1	("5599433").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2009/01/05 11:06
L6	0	capillary adj electrophoresis same (haemolyzed adj blood or hemolyzed adj blood)	US-PGPUB; USPAT	OR	ON	2009/01/05 11:41
L7	1	capillary adj electrophoresis same (haemolyzed or hemolyzed)	US-PGPUB; USPAT	OR	ON	2009/01/05 11:42

1/5/2009 12:12:51 PM

OIPE IAP40 APR 13 2009 PATENT & TRADEMARK OFFICE

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: April 8, 2009 Signature: Diane P. Tso (Diane P. Tso)

Docket No.: EGYP 3.0-034 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Robert et al.	
Application No.: 11/280,025	Group Art Unit: 4111
Filed: November 16, 2005	Examiner: J.M. Dieterle
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	

AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

In response to the Office Action mailed January 8, 2009 in the above-identified application, Applicants submit the following amendments and remarks.

04/14/2009 MBELETE1 00000013 121095 11280025
01 FC:2201 110.00 DA

IN THE SPECIFICATION

On Page 2, please replace the first full paragraph with the following amended paragraph:

Further, free solution haemoglobin separation has been described, but it does not satisfy the expected criteria for accuracy, resolution or rapidity to be able to rationalize ~~EC~~ CE haemoglobin analyses. Ishoka (1) (1992) described the separation of haemoglobins using a borate buffer (100 mM) at a pH of 9.98 with a migration time of the order of 50 minutes, i.e. incompatible with currently expected times for analyzing haemoglobins. The same type of buffer under similar pH and concentration conditions (Jenkins) (3), (4) and (5) can only produce insufficient resolutions between the HbA, HbF and HbS fractions. Sahin (2) describes more acidic pH conditions, but obtained deeply unimpressive results for resolution between the HbA, HbF, HbS and HbA_2 fractions with lower concentrations (20 mM) of borate or with barbital (50 mM) at a pH of 8.5, and also deeply unimpressive results for resolution between the HbA, HbS and HbA_2 fractions for Tris buffer (1 M) at a pH of 8.0. Further, Tris/arginine combinations (Shihabi) (6) have been used and even though they allowed HbA/HbS separation, HbC/HbE and HbA_2 fractions were not resolved. Finally, United States patents US-A-5 202 006 and US-A-5 439 825, which describe the use of barbital or ethylbarbital, could only produce low resolutions between the principal haemoglobins, namely HbA, HbF, HbS and HbC.

On Page 4, please replace the second full paragraph with the following amended paragraph:

Further, the invention concerns the use of compounds known for their electrophoretic flow inhibiting activity in

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free solution ~~EC—~~ CE in association with at least one zwitterionic buffer.

On Page 4, please replace the fifth full paragraph with the following amended paragraph:

Finally, the invention pertains to kits for analyzing haemoglobin A_2 and haemoglobin variants in a biological sample by ~~EC~~ CE, comprising at least one analysis buffer solution containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor, and an optional pH modifier. Thus, the kits may comprise at least one analysis buffer and a flow inhibitor according to the invention, and one or more solution(s) for rinsing capillaries and/or dilution segments and/or one (or more) diluent(s) for the sample to be analyzed. They may also comprise at least one haemolysing solution. In said kit, the buffer and the inhibitor(s) and diluent(s) or other additives may be stored separately for mixing extemporaneously, or stored as a mixture. This kit also optionally comprises instructions for carrying out the analysis and/or software support information.

On Page 6, please replace the ninth full paragraph with the following amended paragraph:

The ~~EC—~~ CE methods employing zwitterionic buffer/flow inhibitor associations of the invention are of particular use in analyzing blood and separating haemoglobin and its variants in human samples.

On Page 7, please replace the sixth full paragraph with the following amended paragraph:

The haemolyzing solution can carry out haemolysis of red corpuscles containing haemoglobin A_2 and haemoglobin variants. It is also used to dilute the sample before ~~EC—~~ CE analysis.

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Depending on its composition, it may allow complete lysis of red corpuscles using a slight additional mechanical movement (vortex, agitation, ...)

On Page 8, please replace the fifth full paragraph with the following amended paragraph:

Capillary electrophoresis of clinical samples was carried out on ~~EC~~ CE apparatus equipped with a fused silica capillary with an internal diameter of 25 microns.

On Page 9, please replace the first full paragraph, bridging top of Page 10 with the following amended paragraph:

For ~~EC~~ CE, human blood, decanted or centrifuged, was diluted to $1/6^{th}$ in the haemolyzing solution. This was prepared by dissolving 1.00 g of Triton X100, 2.50 g of saponine, 3.63 g of Tris in about 900 ml of demineralized water. The pH was adjusted to 8.70 at 22.0°C and the volume of the solution was adjusted to 1 l with demineralized water. The chemical products employed were of analytical grade.

On Page 10, please replace the seventh full paragraph with the following amended paragraph:

Example 5: analysis of a mixture of blood presenting A/S heterozygosity and blood presenting A/D Los Angeles heterozygosity. The HbS and HbD fractions are partially resolved, which means that they can be distinguished from each other without the need for complementary analysis (Figure 5).

On Page 11, please replace the fourth full paragraph with the following amended paragraph:

Four analysis buffer solutions (A: tricine/DAB.2HCl; B: tricine/1,5-diaminopentane.2HCl; C: tricine/DETA; D: tricine/ N,N,N',N'-tetramethyl-1,4-butanediamine) were prepared as above (Figure 7).

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IN THE CLAIMS

1. - 28. (cancelled)

29. (currently amended) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electophoresing said biological sample in said analysis buffer and ~~said~~ at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

30. (previously presented) The method according to claim 29, further comprising detecting said haemoglobins.

31. (currently amended) The method according to claim 29, wherein said blood sample is selected from the group consisting of ~~a sample of blood which may be~~ normal blood, ~~or~~ abnormal blood, washed blood, decanted blood, centrifuged blood ~~or~~and whole blood.

32. (currently amended) The method according to claim 31, wherein said blood sample is a sample of haemolyzed blood.

33. (currently amended) The method according to claim 29, wherein the haemoglobins are a haemoglobin HbA2 and an HbC, an HbD, an ~~B~~HbE, an HbS, an HbF ~~and/~~or an HbA haemoglobin.

34. (previously presented) The method according to claim 29, wherein the zwitterionic buffer is formed by one or two molecules and comprises at least one amine function, at

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least one acid function and at least one hydroxyl function in the position opposite to the acid function.

35. (currently amended) The method according to claim 29, wherein said analysis buffer is selected from tricine, TAPS, TABS, AMPSO, bicine or HEPBS, or an association of Tris, ~~AIVIPD~~ AMPD or bis Tris propane with an amino acid.

36. (previously presented) The method according to claim 35, wherein said analysis buffer is tricine.

37. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is of an aliphatic or a cyclic diamine or a polyamine type.

38. (currently amended) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of aliphatic diamines, aliphatic polyamines, their acceptable derivatives and salts, and mixtures thereof.

39. (currently amended) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine, spermine, N,N'-dimethyl-1,6-hexanediamine, tetraethylenepentamine, N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

40. (currently amended) The method according to claim 39, wherein said at least one flow inhibitor is selected from the group consisting of 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine and N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

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41. (previously presented) The method according to claim 40, wherein said at least one flow inhibitor is 1,4-diaminobutane.

42. (previously presented) The method according to claim 41, wherein said at least one flow inhibitor is 1,4-diaminobutane in the hydrochloride form.

43. (currently amended) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 20mM to about 500 mM.

44. (currently amended) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 100mM to about 250mM.

45. (currently amended) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.01 mM to about 50 mM in said analysis buffer solution.

46. (currently amended) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.10mM to about 20mM in said analysis buffer solution.

47. (previously presented) The method according to claim 29, wherein said analysis buffer solution further comprises a pH modifier.

48. (currently amended) The method according to claim 47, wherein said pH modifier is selected from the group consisting of lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, ~~and~~ mono-alkylammonium hydroxide, di-alkylammonium hydroxide, tri-alkylammonium hydroxide ~~or~~and tetra-alkylammonium hydroxide said alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

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49. (previously presented) The method according to claim 29, wherein said capillary tube is naked.

50. (previously presented) The method according to claim 29, wherein said capillary tube is formed from fused silica.

51. (currently amended) The method according to claim 29, wherein said analysis buffer solution has a pH from about 8 to about 10.

52. (cancelled)

53. (withdrawn) A capillary electrophoresis haemoglobin analysis kit comprising at least one analysis buffer containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor.

54. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a pH modifier.

55. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a haemolyzing solution.

56. (new) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said sample being selected from the group consisting of normal blood, abnormal blood, washed blood, decanted blood, centrifuged blood, whole blood and haemolyzed blood, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

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electophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

57. (new) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, said flow inhibitor being an aliphatic or a cyclic diamine or a polyamine type, and

electophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

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IN THE ABSTRACT

Please replace the Abstract with the following amended Abstract:

The invention concerns a method for free solution capillary electrophoresis at an alkaline pH to analyze samples comprising haemoglobin, in which the sample is passed through a capillary containing an analysis buffer, comprising at least one step in which the sample is introduced into a capillary tube containing a solution of analysis buffer, characterized in that the buffer is of the zwitterionic type and in that it is associated with at least one flow inhibitor. It also concerns the use of CE flow inhibitors associated with at least one zwitterionic buffer, and a kit for analyzing haemoglobin by capillary electrophoresis.

~~It also concerns the use of EC flow inhibitors associated with at least one zwitterionic buffer, and a kit for analyzing haemoglobin by capillary electrophoresis.~~

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IN THE DRAWINGS

Please replace Figures 1-7D with the replacement sheets provided herein.

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REMARKS

In response to the Examiner's objection to the Specification, Applicants submit herein a replacement Abstract which has been amended to contain only one paragraph.

Furthermore, in response to the Examiner's objection, Applicants have amended the specification to replace EC (Electrophorèse Capillaire) with CE (Capillary Electrophoresis) where necessary throughout the application. Support for this amendment may be found in the specification on page 1, line 6. No new matter has been added.

In response to the Examiner's objection, Applicants submit herewith new drawings as replacement sheets in compliance with 37 CFR 1.121(d) which have been amended to contain x and y axes.

The Examiner has objected to the heading for the claims section, alleging that the heading states, "IN THE CLAIMS". Applicants cannot find this heading in the application as filed. The Preliminary Amendment did contain the heading "IN THE CLAIMS" at the beginning of the amended claim set, however, and possibly the Examiner may have mistaken this section heading for the heading format of the claims in the application. Clarification of this outstanding objection is respectfully requested.

In response to the objections of Claim 29, Applicants have included a comma and have amended this claim to place "said" before "flow inhibitor" as requested.

In response to the objections regarding Claim 33, Applicants have amended this claim to provide appropriate

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clarification and to correct a typographical error, replacing "B" with "E", support for which may be found, for example, at Page 2, lines 18-21 and in claim 5 as originally filed. Applicants have also amended Claim 33 to include proper Markush language.

In response to the Examiner's objections, Claims 38-40 and 48 have also been amended to include proper Markush language.

Claim 35 has been amended herein to replace "AIVIPD" with "AMPD". Support for this amendment may be found at Page 5, line 24 in the application as filed as well as in Claim 7 of the published application, US 2006/0102478.

Claims 43, 44 and 51 have been objected to because the term "said analysis buffer" has been alleged to be vague and indefinite. In response, Applicants respectfully submit that antecedent basis for this term can be found in claim 29. In addition, Applicants have now amended these claims, as well as claims 45-47, to refer to "in said analysis buffer solution" to more precisely define the concentration and to render this objection moot. Support for these amendments may be found, for example, at Page 6, line 24 bridging Page 7, lines 1-8, of the application as filed.

In response to the objection to Claim 45, Applicants have amended this claim to contain the unit of measurement "mM" after "0.01".

Claim 48 has been amended herein to include the full chemical name of the alkylammonium hydroxides as indicated, for the sake of clarity.

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Claims 29, 31 and 32 have been amended herein to clarify the subject matter of the present invention.

Finally, claim 52 has been cancelled and new claims 56 and 57 have been added. Support for these new claims may be found throughout the specification.

No new matter has been added.

Rejections under 35 U.S.C. § 112

Claims 33 and 35 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Applicants respectfully submit that the above amendments to Claims 33 and 35 render the rejections moot. Specifically, Applicants have amended these claims to remove typographical errors such that "E" has been changed into "B" and "AIVIPD" into "AMPD".

Rejections under 35 U.S.C. § 102

Claims 29-31, 34-36, and 43-52 are rejected under 35 U.S.C. § 102(b) as being anticipated by Nouadje et al. (US 2002/0162744 A1).

In response, Applicants respectfully submit that the aim of the methods of the instant invention is to analyze haemoglobin variants in biological liquid such as blood using free solution capillary electrophoresis. Applicants have amended the claims of the instant invention, specifically claims 29, 31 and 32, to clarify this fact. No new matter is introduced by these amendments; support may be found, for example, at Page 1, lines 4-11 ([0002] of the published application), Page 4, lines 5-6 ([0013]), Page 6, lines 13-15 (0030]) as well as in claims 31, 32 and 52.

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The emphasis of Applicants' invention concerns the analysis of haemoglobins in blood samples. In contrast, Nouadje et al. essentially discloses the analysis of serum proteins in serum samples. More precisely, Nouadje et al. discloses the analysis of albumin and globulins, which are serum proteins, in biological liquids such as serum or plasma.

According to the Examiner, Nouadje et al. teach "separating the constituents of blood proteins" … "which can be normal or abnormal". The Examiner refers to [0003] and [0036] of US 2002/0162744. It should be noted, however, that in [0036] "normal or abnormal" is followed by "serum".

Applicants respectfully submit that the Examiner could be misled in his reading of Nouadje et al. Specifically, Applicants note that Nouadje et al. was corrected during its prosecution (USSN 10/052,931) such that the word "blood" was replaced by the word "serum" or "seric" as appropriate in both specification and claims. The reason for this correction is that the French word "proteines sériques" which means "serum proteins" had been erroneously translated into "blood proteins" at some places. Applicants submit herein for the Examiner's convenience relevant sections of "Remarks" and "Amendments" as filed during the prosecution of USSN 10/052,931 (published as US 2002/0162744; granted as US 7,297,244). See also, particularly [0037] and [0038] of US 2002/0162744 compared with lines 60 to 65 of US 7,297,244, which correctly mention "serum".

In contrast to the Examiner's allegation, Applicants respectfully submit that Nouadje et al. specifically discloses the analysis of seric protein, and fails to teach the methods of the instant invention, e.g., wherein a flown inhibitor will

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separate the various haemoglobin variants in blood samples. Indeed, it appears that Nouadje et al. discloses the separation of serum proteins, particularly that the fractions to be separated correspond to albumin, α_1, α_2, β and γ-globulin in serum samples. The Examiner's attention is respectfully drawn to [0003], lines 3-6; [0006], lines 2-3; [0008] and [0036] of US 2002/0162744 as well as the examples disclosed therein, which are all carried out with serum.

Applicants respectfully submit that Nouadje et al. does not disclose the separation of haemoglobin variants. Applicants respectfully submit that, as acknowledged by the Examiner, haemoglobins are in blood. Nouadje et al. does not mention haemoglobins but exclusively albumin and globulin fractions. To this end, Applicants enclose herein a copy of several pages from "Fundamentals of Clinical Chemistry" and in particular, respectfully direct the Examiner's attention to Table 7-6 which lists serum proteins. Applicants note that while albumin and globulins appear to be present in serum, the list does not mention haemoglobin.

In light of the above arguments, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 29-31, 34-36, and 43-52 under 35 U.S.C. § 102(b).

Rejections Under 35 U.S.C. § 103

Claim 32 is rejected as being obvious over Nouadje et al. in view of Keo et al., US Patent 5,599,433. According to the Examiner, Nouadje et al. teaches a method for separating haemoglobins, but does not teach the separation of haemolysed blood. The Examiner alleges that Keo et al. recognizes the use of haemolysed red blood cells in capillary electrophoresis, thus Applicants claimed invention is obvious.

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In response, Applicants respectfully submit that the Examiner's assertion that Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoreses is not correct. As discussed above, Nouadje et al. does not disclose nor teach the separation of haemoglobins. According to [0003] of US 2002/0162744, "the fraction to be separated corresponds to albumin, α_1, α_2, β and γ-globulin" and a fair reading of [0008] of Nouadje et al. confirms that Nouadje et al. concerns a method for separating albumins and said fraction α_1, α_2, β and γ-globulins. Applicants respectfully submit that there is nothing in the teachings of Nouadje et al. concerning the separation of albumin/globulin fraction which teaches the separation of haemoglobin variants as disclosed in the instant application.

Furthermore, Keo et al. concerns the analysis of normal haemoglobin including glycated or glycosylated haemoglobins (or glyco-haemoglobins). In contrast, the haemoglobins to be separated according to the present invention are haemoglobin variants, for example HbA_2, HbC, HbD, HbB, HbE, HbF or HbA, irrespective of their glycosylation. According to Keo et al., for specifically determining the amount of glycosylated protein, a buffer comprises a sugar complexing compound, a zwitteronic compound and base leading to a pH of 9-12.

When viewed together, Applicants respectfully submit that there is nothing in the teachings of Nouadje et al. in combination with the teachings of Keo et al. such that one of skill in the art would consider Applicants' use of a flow inhibitor to improve the separation of the haemoglobin variants in a blood sample as claimed in the instant invention predictable, let alone obvious under 35 USC 103(a).

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Claim 33 is rejected as being unpatentable over Nouadje et al. in view of Chen, US Patent 5,202,006.

In response, as discussed above, Applicants respectfully submit that the Examiner's assertion that Nouadje et al. teach a method for separating hemoglobins is not correct. Indeed, the Examiner acknowledges that the specific haemoglobins and their separation is not taught by Nouadje et al. Indeed, Applicants submit that Nouadje et al. fails to teach the separation of any haemoglobin variant in blood samples, let alone that a flow inhibitor may be used for this purpose.

The teaching of Chen adds nothing to Nouadje et al. such that one of ordinary skill in the art would arrive at the claimed invention. Chen merely defines some haemoglobin variants and their separation by CE in the presence of barbituric acids. There is no mention of the analysis buffer of the invention comprising a zwitterionic buffer and a flow inhibitor.

Taken together, Applicants respectfully submit that one of skill in the art would not be motivated to combine the techniques disclosed in Nouadje et al. (albumin/globulin separation without flow inhibitor) together with Chen (haemoglobin variants) to arrive at the claimed invention, nor do these references, either alone or in combination, provide the reasonable expectation of success and subsequent predictability necessary to render the claimed subject matter *prima facie* obvious.

Claims 37-42 are rejected as unpatentable over Nouadje et al. in view of Grover et al., US Patent 6,083,372.

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As discussed above, Applicants respectfully submit that Nouadje et al. fails to teach the separation of any haemoglobin variant in blood samples, let alone that a flow inhibitor may be used for this purpose.

While the Examiner alleges that Grover et al. teaches the use of polyamine compounds as a flow inhibitor, Applicants respectfully submit that Grover et al. describes the use of these compounds in an analysis method which is completely different from free solution capillary electrophoresis (FSCE). For example, Grover's capillary is filled with a stationary phase and an electrolyte. Also, the electrokinetic chromatography (EKC) technique disclosed in the cited reference is carried out in a micellar phase with a surfactant in the buffer. These techniques are completely different from FSCE.

Furthermore, Grover et al. does not teach the separation of haemoglobin variants using the disclosed polyamine compounds. Indeed, Grover et al. discloses the separation of phenols, verapamil and metoprolol which are completely different from haemoglobins and thus cannot fairly be compared with haemoglobins. As such, as discussed above, the methods disclosed by Grover et al. of separating these molecules in additive/buffer combination is different from the buffer system of the present invention.

Taken together, Applicants respectfully submit that claims 37-42 are not obvious given Nouadje et al. in view of Grover et al. Simply put, one of skill in the art could not fairly find Applicants' claimed invention, including the use of various polyamine compounds as flow inhibitors suitable for use in the claimed methods, predictable or obvious given the limited disclosures of the two cited references.

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In view of the arguments presented above, Applicants respectfully request reconsideration and withdrawal of all outstanding claim rejections under 35 U.S.C. § 103.

As it is believed that all of the rejections set forth in the Official Action have been fully met, favorable reconsideration and allowance are earnestly solicited.

If, however, for any reason the Examiner does not believe that such action can be taken at this time, it is respectfully requested that she telephone Applicants' attorney at (908) 654-5000 in order to overcome any additional objections which she might have.

In the event any fee is due in connection with the present response, the Examiner is authorized to charge Deposit Account No. 12-1095 therefor.

Dated: April 8, 2009

Respectfully submitted,

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REMARKS

Entry of the foregoing and reexamination and reconsideration of the above-identified application, as amended hereby, pursuant to and consistent with 37 C.F.R. § 1.112, and in light of the remarks that follow, are respectfully requested.

Applicants acknowledge the withdrawal of the prior objections to the specification and to claims 1-12, based on informalities. Applicants also acknowledge that the rejection of claims 9, 15 and 26, pursuant to 35 U.S.C. § 112, second paragraph, has been overcome and that the prior rejections pursuant to 35 U.S.C. § 102(b) and § 103(a) have been withdrawn.

The specification is now objected to because of the presence of "en" on page 6, line 9. This has been corrected. Applicants also wish to draw the examiner's attention to changes to replace the word "blood" with "seric" and "serum" as appropriate in both the specification and claims. The French version of the priority application as filed recites "serum" and this was erroneously translated into "blood" and "blood proteins."

Claims 1, 8, 9, 19 and 24 are currently objected to because of minor typographical informalities. These have all been corrected. Claim 3 was objected to as being in improper dependent form for failing to further limit the claim from which it depended. While applicants respectfully disagree, the claim has been canceled hereby and thus the objection is moot.

Claims 25, 29, 30 and 33 are now rejected pursuant to 35 U.S.C. § 112, second paragraph as allegedly being indefinite. Applicants have amended claim 25 as the examiner suggested and thus the rejection should be moot.

Before discussing the individual references, applicants respectfully point out that nothing in the references applied teaches or suggests the methods claimed as none of the references teaches or suggests the idea of associating a particular species in a buffering solution with a particular

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IN THE SPECIFICATION

Page 6, second paragraph:

The above di- and tri-carboxylates, di- and tri-sulphonates and carboxysulphonates are thus combinations of one or more carboxylate or sulphonate functions on C_6 to C_{22} alkyl chains. Non limitative examples thereof are the 1,2,3-nonadecanetricarboxylic acid (three carboxylate functions and a ~~en~~ C_{19} alkyl chain), the 2-methyl-2-sulfooctadecanoic acid (one carboxylate function and one sulfonate function and a C_{18} alkyl chain) and the 1,12-dodecanedicarboxylic acid (two carboxylate functions and a C_{12} alkyl chain).

Page 7, fifth paragraph:

The additives of the invention are of particular application for analysing serum, and for separating ~~blood~~ seric proteins in samples from humans.

Page 7, sixth paragraph:

In ~~blood~~ serum samples, the ~~blood~~ seric proteins to be separated are primarily albumin and the α_1; α_2; β (or β_1 and β_2);and γ globulin fractions.

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fundamentals of Clinical Chemistry

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Fundamentals of Clinical Chemistry ISBN 0-7216-8866-7

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Library of Congress catalog card number 73-91279.

Last digit is the print number: 9 8 7 6 5 4 3 2

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SECTION THREE

THE PROTEINS OF BODY FLUIDS

by Gregor H. Grant, M.A., B.M., F.R.C. Path.,
and John F. Kachmar, Ph.D.

PLASMA AND SERUM PROTEINS

Blood is made up of particulate cell forms suspended in a fluid medium called *plasma*, a very complicated mixture of inorganic and simple and complex organic materials dissolved in water. If the blood is collected without anticoagulant, in vessels with siliconized or non-polar surfaces, the fluid separating is referred to as *native plasma*. This approximates very closely the plasma actually present in circulating blood. It is the practice, however, to use such anticoagulants as oxalate, citrate, EDTA, and heparin to prepare specimens of plasma for study or analysis. This differs from native plasma because it is modified by the presence of the added chemicals and by the partial loss of calcium bound by oxalate, if this is used. If blood is permitted to clot, the fluid separating is referred to as *serum*. It lacks the protein fibrinogen present in plasma, the fibrinogen having been transformed into insoluble fibrin in the clotting process. The fibrinogen constitutes only some 3 to 6 per cent of the total plasma proteins. It is satisfactory and much more convenient to use serum rather than plasma in most clinical chemical studies.

About 93 per cent of plasma or serum is solvent water. The remaining 7 per cent, the solutes, consists almost entirely of proteins, but because of their large molecular masses their molecular concentration is only about 1 mmol/l, compared with a mass concentration of 70 g/l. In clinical work the concentration of proteins is given in grams per 100 ml or per liter of serum volume. It would be more meaningful to express it in terms of serum water, a value about 1.07 times greater.

FUNCTIONS AND ORIGINS OF PLASMA PROTEINS[17]

As described in this and later chapters, many individual plasma proteins have specific functions, for example as enzymes, as antibodies, as factors in blood coagulation, or as transport proteins: many vital metabolites, metal ions, hormones, and lipids are transported around the body bound to carrier proteins.

Plasma proteins collectively also serve a number of different functions. They play a nutritive role inasmuch as they constitute a portion of the amino acid pool of the body; thus, the proteins are a form of storage amino acids. If needed, these proteins can be broken down in the liver to produce amino acids for use in building other proteins. Alternatively, they can be deaminated to give keto acids which can be mobilized to provide caloric energy, or they can be transformed into carbohydrates and lipids.

Another important function of proteins is a physicochemical one. The plasma proteins, being large, colloidal molecules, are nondiffusible; i.e., they cannot move through the thin capillary wall membranes as can most other plasma solutes. They are thus entrapped in the vascular system and exert a *colloidal osmotic pressure*, which serves to maintain a normal blood volume, and a normal water content in the interstitial fluid and the tissues. The albumin fraction is most important in maintaining this normal colloidal osmotic or *oncotic*

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TABLE 7-6 ELECTROPHORETIC FRACTIONS

Electrophoretic Bands	*Normal Range (depth of staining of electrophoretic fractions), g/100 ml*	*Main Constituents and Their Molecular Masses in Daltons*	
Albumin	3.5–5.0	*Albumin* (Alb)	65,000
α_1	0.17–0.33	α_1-*anti-trypsin* (α_1ATr) Cause of band on protein staining.	45,000
		α_1-*lipoprotein* (α_1Lp) (apoprotein A) Cause of band on fat staining.	200,000
		Orosomucoid (α_1AGp) (α_1-acid-glycoprotein, seromucoid) Stains only faintly with protein stain.	44,000
α_2	0.42–0.90	α_2-*macroglobulin* (α_2M)	820,000
		Haptoglobin (Hpt)	From 85,000 up to more than 1,000,000 in higher polymers.
		Pre-β-lipoprotein (VLDL) (apoprotein A, B, and C) Seen only on fat staining.	Very large
β_1	0.52–1.05	*Transferrin* (Tr) (siderophilin)	80,000–90,000
		Hemopexin (Hpx) Stains poorly.	80,000
		β-lipoprotein (βLp) (apoprotein B) Requires fat stain.	About 3,000,000
β_2	0.08–0.14 (only present in fresh serum)	β_{1C}	
γ	0.71–1.65	Immunoglobulin G (IgG, γG)	150,000
		Immunoglobulin A (IgA, γA)	180,000 (higher when polymerized)
		Immunoglobulin M (IgM, γM)	900,000 (higher when polymerized)

* Some levels vary with season, age, or sex.[51] Values may also differ in accordance with the method employed (e.g., electrophoresis vs. immunochemical techniques).

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...IONS ... AND THEIR MAIN CONSTITUENTS[47,77]

Average Values[a] and Approximate Normal Ranges of Individual Proteins g/100 ml	Biological Function	Main Pathological Abnormalities
4.4 (3.5–5.0)	Transport of fatty acids, ect. Regulation of plasma volume by colloid osmotic pressure. Provides main protein reserve.	Reduced in cirrhosis and nephrotic syndrome, malnutrition, protein losing enteropathy and hemodilution.
0.25 (0.2–0.4)	Proteinase inhibitor.	Increased in acute phase reaction. Its congenital deficiency leads to emphysema and neonatal liver necrosis.
0.36 (0.29–0.77)	Transport of lipids, fat-soluble vitamins, and hormones.	Reduced in liver disease.
0.09 (0.05–0.14)	Not established.	Increased in acute phase reaction, chronic inflammation, rheumatoid arthritis and malignancy.
Males: 0.24 (0.15–0.35) Females: 0.29 (0.17–0.42)	Plasmin inhibitor.	Increased in nephrosis, liver disease, and diabetes.
0.1–0.3 (depending on type)	Binds hemoglobin, preventing loss of iron.	Reduced in hemolysis and liver failure. Increased in acute phase reaction.
	Transports lipids, especially triglycerides.	Increased in primary and secondary hyperlipidemias Fredrickson Type IV.
0.29 (0.2–0.4)	Iron transport.	Increased in iron deficiency. Reduced in nephrotic syndrome.
0.10 (0.07–0.13)	Heme binding.	Reduced in hemolytic anemia.
0.53 (0.29–0.95) (depending on age and sex)	Lipid transport, especially cholesterol, fat-soluble vitamins, and hormones.	Increased in primary and secondary hyperlipoproteinemia Fredrickson Type II.
0.11 (0.08–0.14)	Complement Factor C3. On standing converted to β_{1A}.	Reduced in autoimmune diseases, e.g., glomerulonephritis, and SLE.
1.16 (125 IU/ml) (0.8–1.4)	Antibodies.	Polyclonal increase in liver disease and chronic infections. Monoclonal increase in myeloma. Reduced in immune paresis.
0.21 (116 IU/ml) (0.09–0.45)	Antibodies, especially in secretions.	Increased in portal cirrhosis. Otherwise similar to IgG.
0.1 (140 IU/ml) (0.06–0.25)	Antibodies formed first; confined to blood.	Monoclonal increase in Waldenström's macroglobulinemia. Otherwise similar to IgG.

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SEPARATION OF PROTEINS BY ELECTROPHORESIS[77,78]

A mixture of protein molecules of differing net charges can be separated into fractions by an electric field. Two categories of supporting media are used. The first group includes paper (Durrum, 1950), agar gel (Gordon, 1949), and cellulose acetate (Kohn, 1957); these all have pores wide enough for the protein molecules to move freely. Methods employing such supports give four to seven fractions similar to those of the original moving boundary method (Tiselus, 1937). The second group of support media includes starch gel (Smithies, 1955) and acrylamide (Ornstein and Davis, 1964); they have pore diameters so small that they act as molecular sieves. Techniques employing these materials fractionate proteins according to their molecular volumes as well as their charges and are able to separate serum proteins into some 20 or more fractions. Their resolving power has proved of great value in the study of genetic protein variants and some isoenzymes, as these cannot be distinguished immunochemically. In routine work, however, the many bands tend to cause confusion rather than provide useful additional information.

In the clinical laboratory, paper has now been superseded by cellulose acetate[44] or agar gel,[95] which have a more uniform structure and provide faster and sharper fractionation. These media can be made transparent, permitting better scanning. Agar gel[47] is probably best for lipoprotein electrophoresis, but for routine investigation of serum proteins, cellulose acetate is simpler and more popular. An example of its use is described in detail below.

Barbital (veronal) buffer at pH 8.6 is the standard buffer for routine serum protein electrophoresis. If calcium lactate is added, a total of seven bands (prealbumin, albumin, α_1, α_2, β_1, β_2, and γ) is obtained with fresh serum. Other buffers are only used for special work, such as the separation of glycoproteins or of hemoglobin variants.

There are many alternative dyes available for staining the protein bands, such as Amido Black 10B, Ponceau S, Nigrosin, or Lissamine green. Glycoproteins can be detected by staining a duplicate strip for carbohydrate by the periodic acid Schiff's reagent method (p. 334), but this is seldom required in routine work. Lipoproteins can be demonstrated with fat stains: four bands are seen which do not correspond exactly to the protein-stained bands (see below and Chap. 10).

Only 15 proteins in normal serum are present in sufficient concentration to influence the electrophoretic patterns seen after staining for protein or for fat.[47] With the exception of prealbumin, they are listed in Table 7–6 in order of mobility, with molecular masses, serum concentrations, functions (if known), and principal alterations in disease.

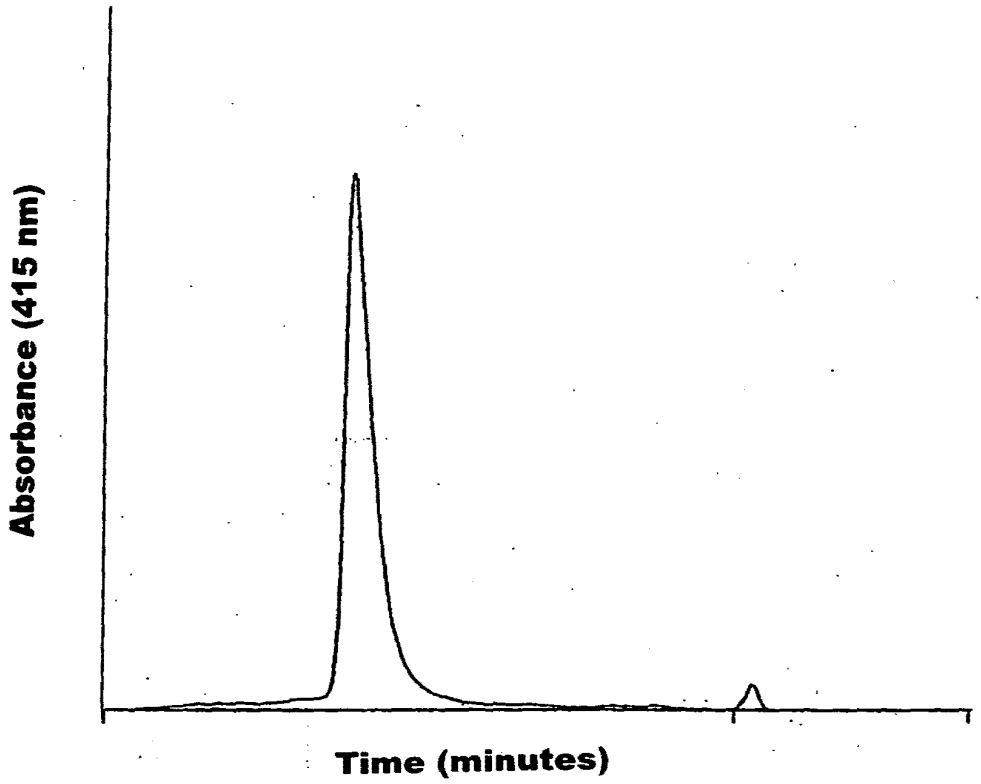
Because several of them have similar mobilities, they give rise to only six clear bands when stained for protein. These bands are listed in the table; one of them (the γ band) is broad and ill-defined because of the heterogeneity of the immunoglobulins. The β_2 band is peculiar in that it is clearly separated from the β_1 band only when the buffer contains Ca

---

Figure 7–10 A set of cellulose acetate electrophoretic patterns obtained with the Beckman microzone system. A, albumin; B, α_1-globulin; C, α_2-globulin; D, β-globulin; and E, γ-globulin region.

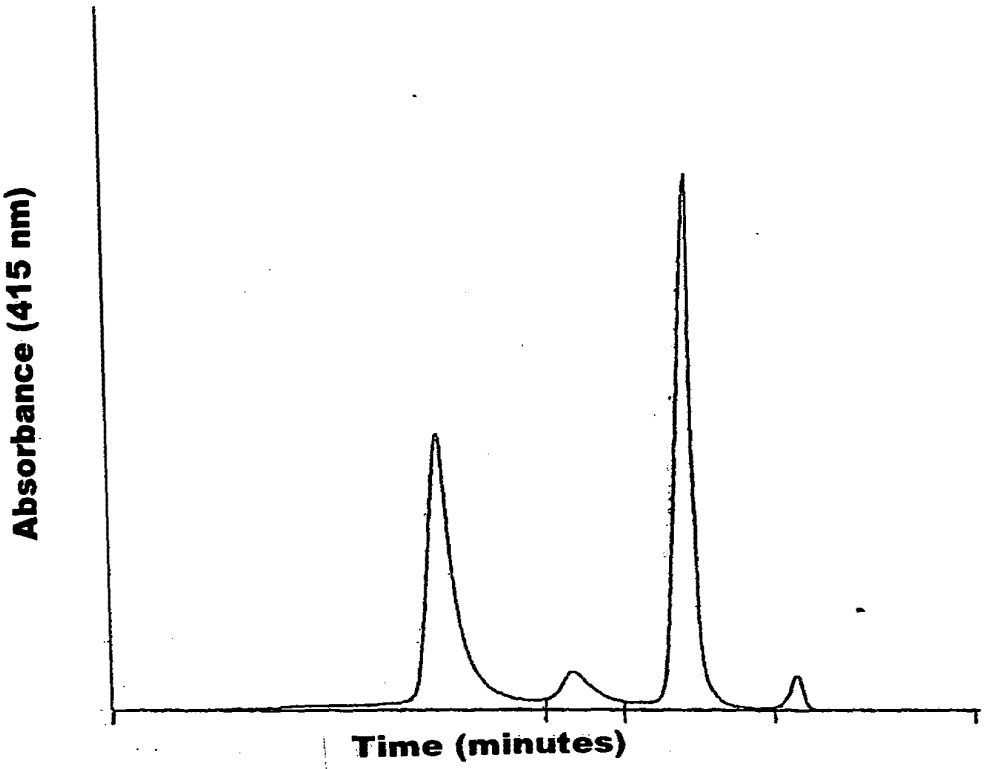
Pattern 1, *Normal* serum (Reconstituted Metrix Control Serum). Pattern 2, *Chronic infection* (hepatitis): a relative decrease in albumin with a notable elevation in γ globulin. Pattern 3, *Destructive lesion* (acute reaction): pattern associated with a myocardial infarction with relative increased levels of α_2 and α_1-globulins. Pattern 4, *Hypogammaglobulinemia*: the considerable drop in γ-globulin is readily observed. A slight increase in α_2-globulin is also seen on this pattern, but is not a common finding in hypogammaglobulinemia. Pattern 5, *Nephrotic syndrome*: the very low level of albumin is striking, as is the extremely elevated peak for α_2-globulin, the moderate rise in β-globulin and the fall in γ-globulin. Pattern 6, *Cirrhosis*: the elevation in both γ- and β-globulin, with a partial fusion of these bands, is present, along with a decrease in albumin. Pattern 7, *Multiple myeloma*: the clone of myeloma proteins is associated entirely with the β-globulins. Pattern 8, *Multiple myeloma*: the myeloma protein is entirely in the γ-globulin area. The fall in albumin is only apparent. It is interesting to compare this γ-globulin region with those in patterns 2, 4, 6, and 7. Note the homogeneous peak in patterns 7 and 8 and the heterogenous peak in pattern 2.

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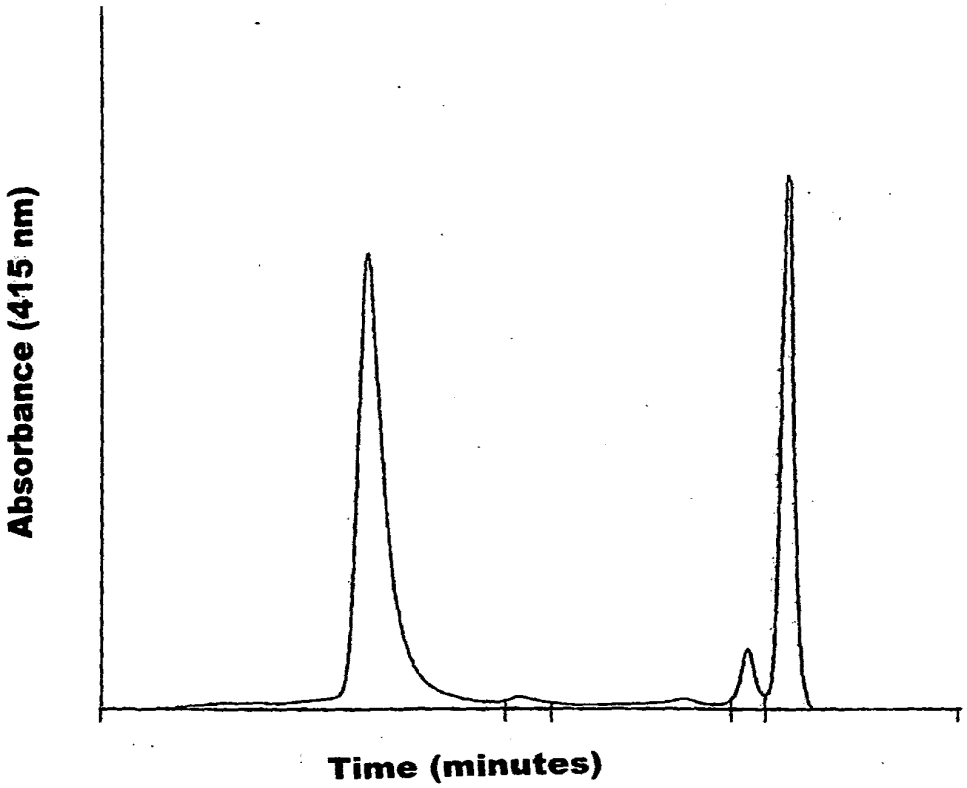
	%
HbA	98,2
HbA2	1,8

FIG 1



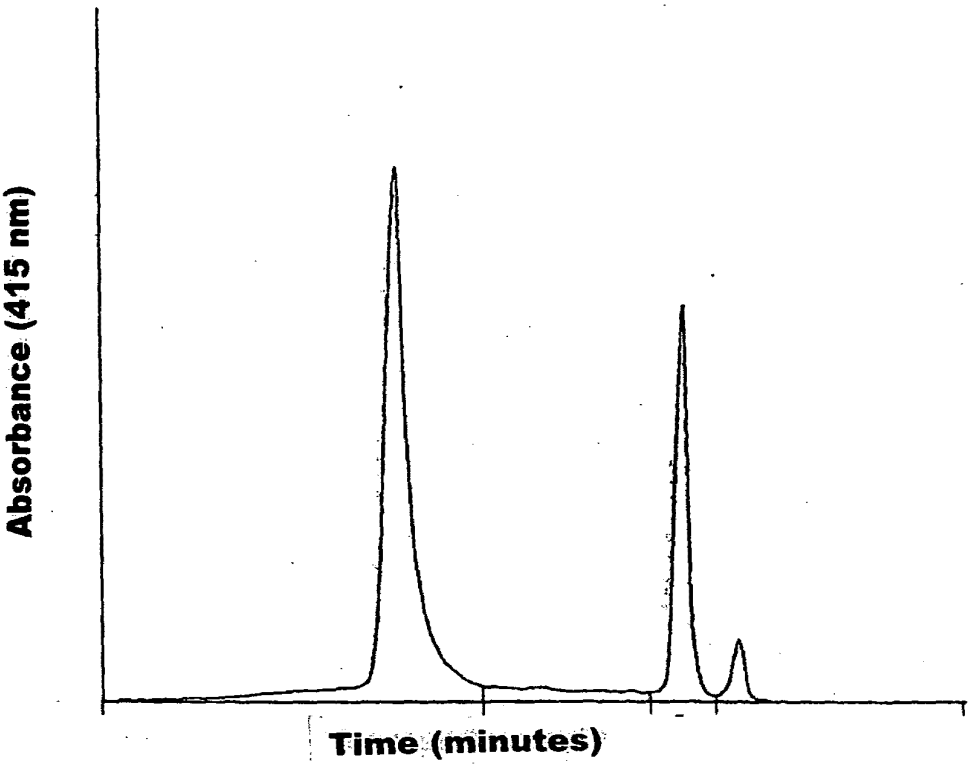
	%
HbA	44,0
HbF	7,1
HbS	46,5
HbA2	2,4

FIG 2



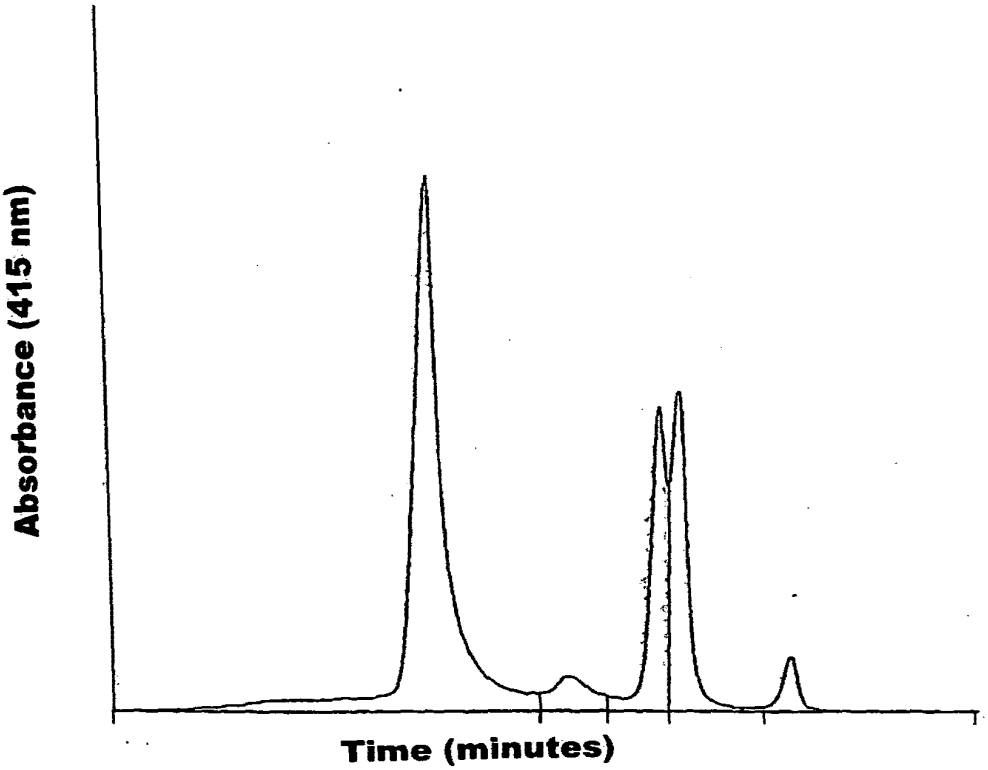
	%
HbA	60,9
HbF	1,6
MetHb	3,9
HbA2	4,0
HbC	29,6

FIG 3



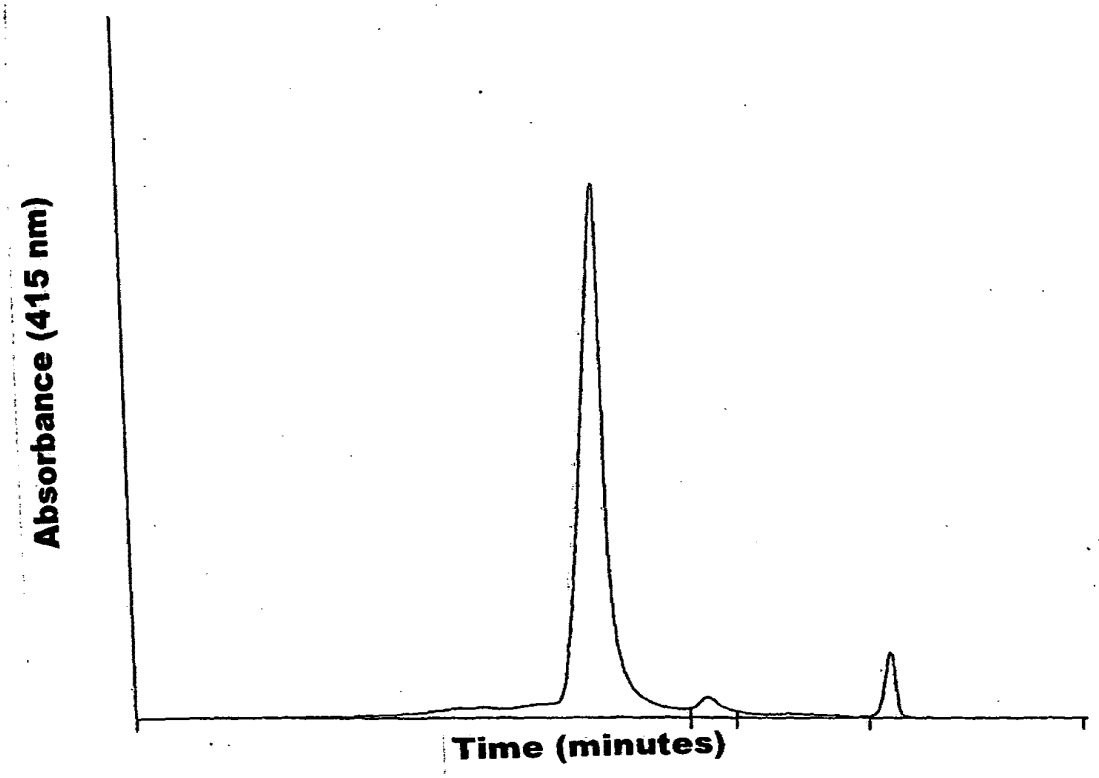
	%
HbA	65,2
MetHb	7,4
HbE	23,5
HbA2	3,9

FIG 4



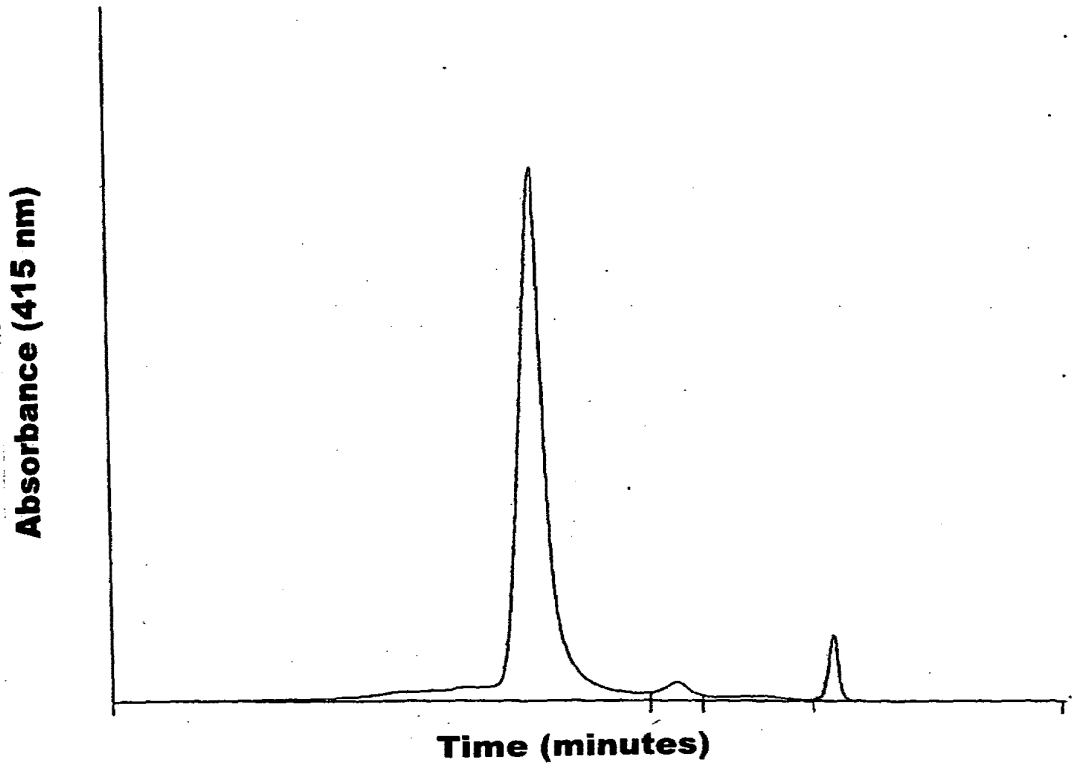
	%
HbA	55,6
HbF	4,8
HbD	18,4
HbS	18,2
HbA2	3,0

FIG 5



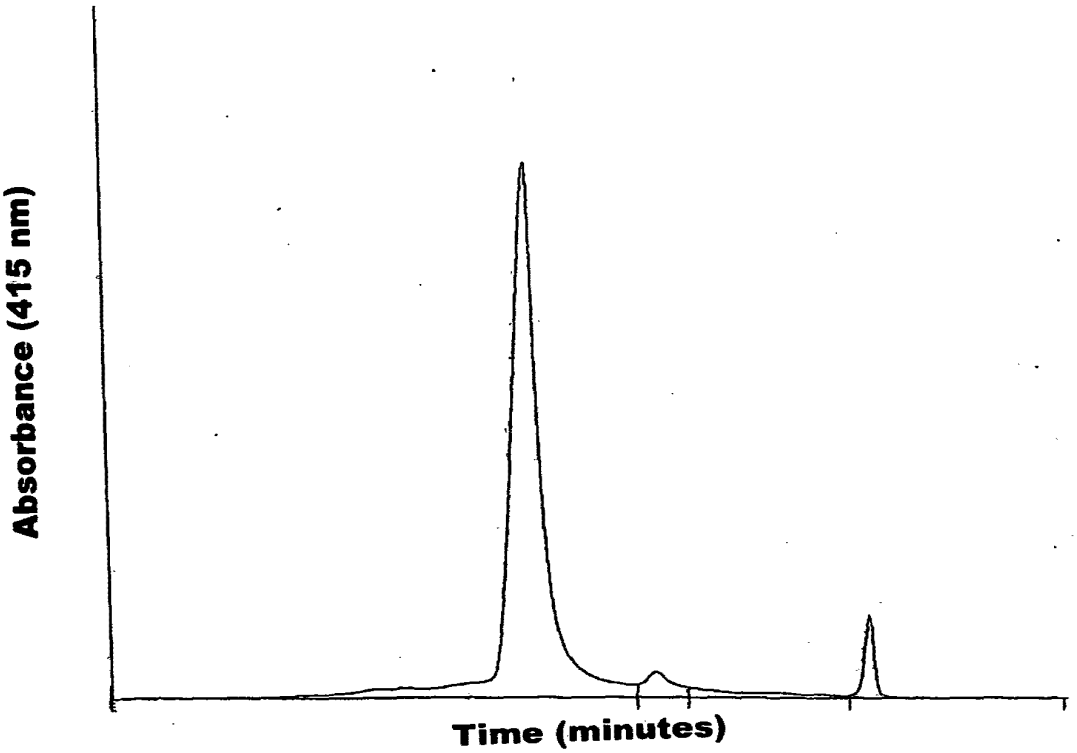
	%
HbA	90,3
HbF	3,2
MetHb	2,2
HbA2	4,3

FIG 6A



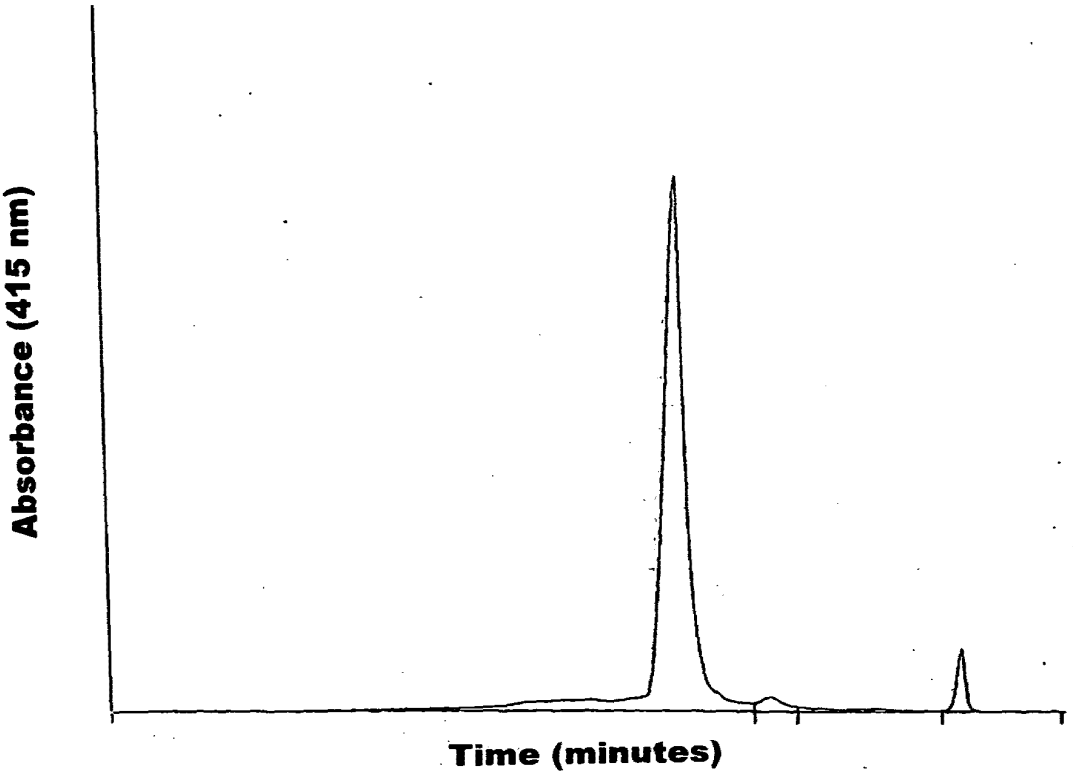
	%
HbA	90,4
HbF	3,3
MetHb	2,0
HbA2	4,3

FIG 6B



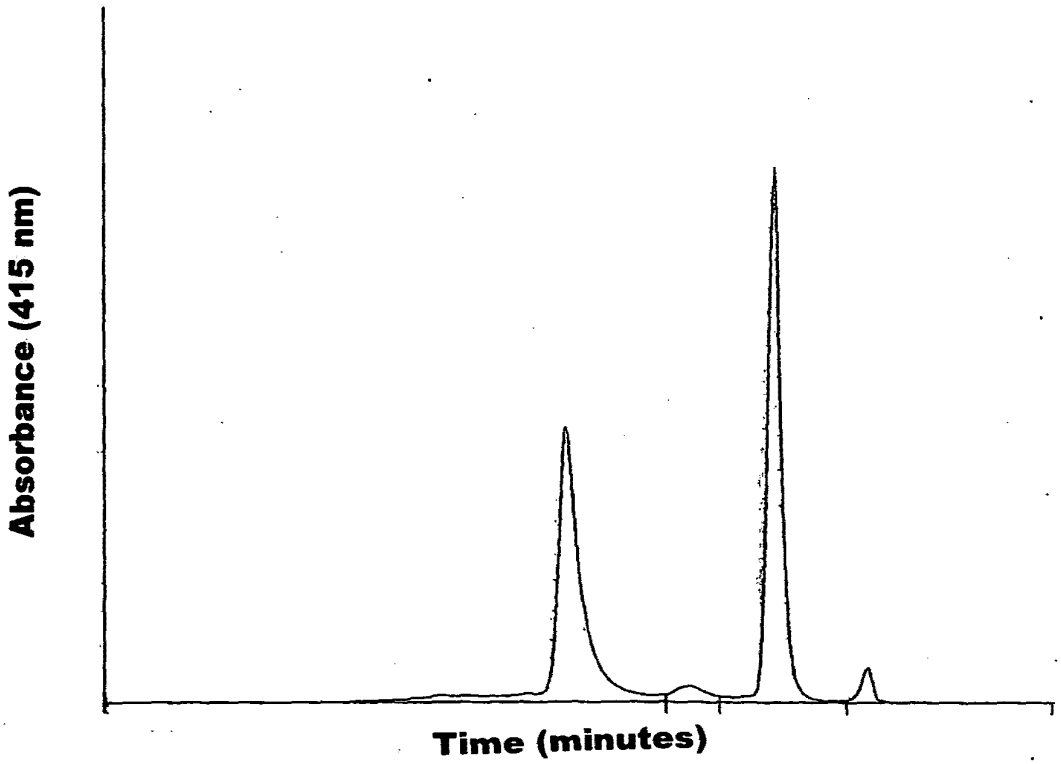
	%
HbA	88,2
HbF	3,9
MetHb	3,4
HbA2	4,5

FIG 6C



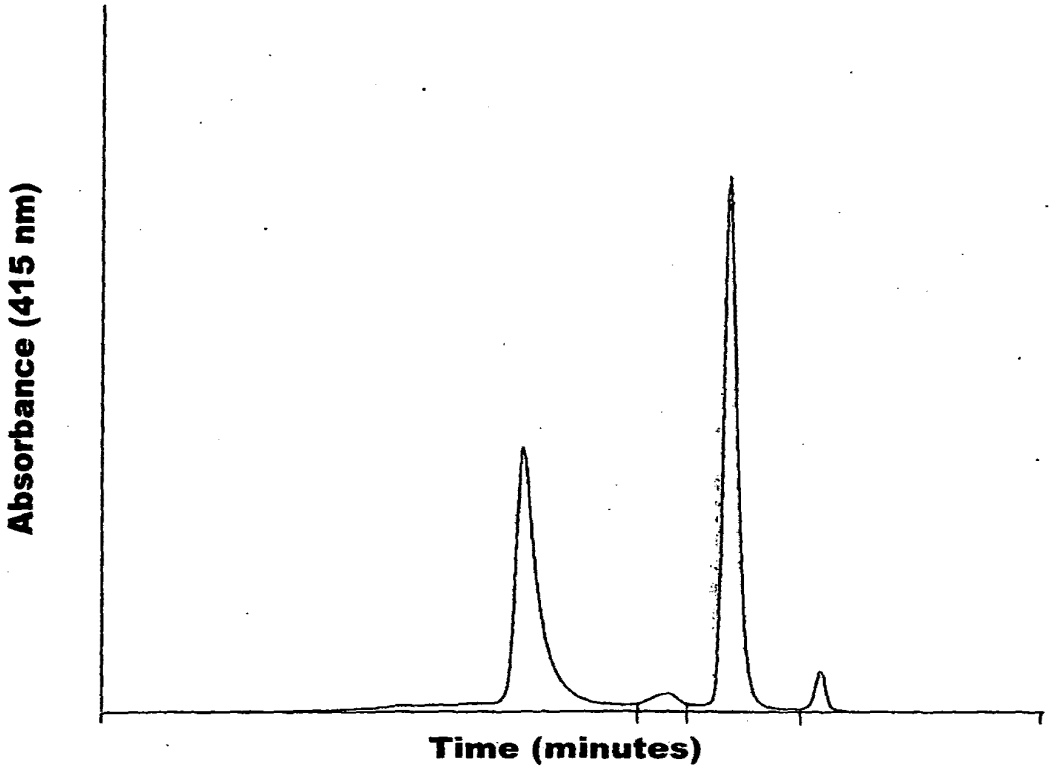
	%
HbA	91,6
HbF	2,6
MetHb	1,9
HbA2	3,9

FIG 6D



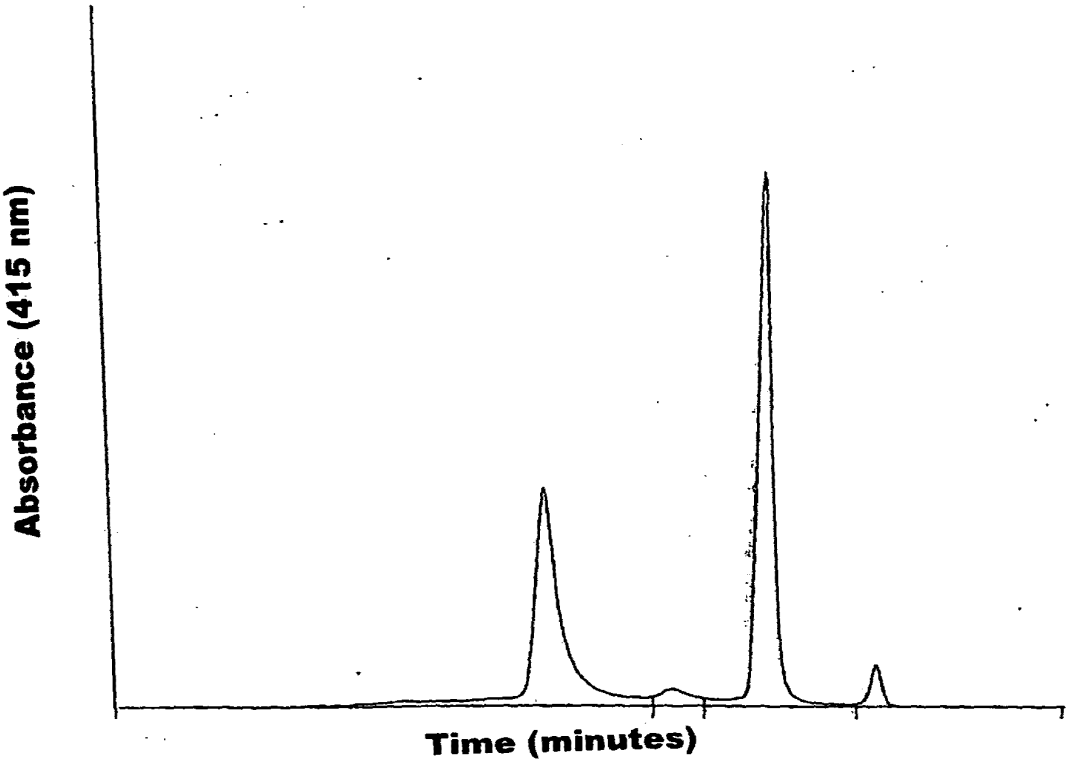
	%
HbA	46,2
HbF	3,3
HbS	47,7
HbA2	2,8

FIG 7A



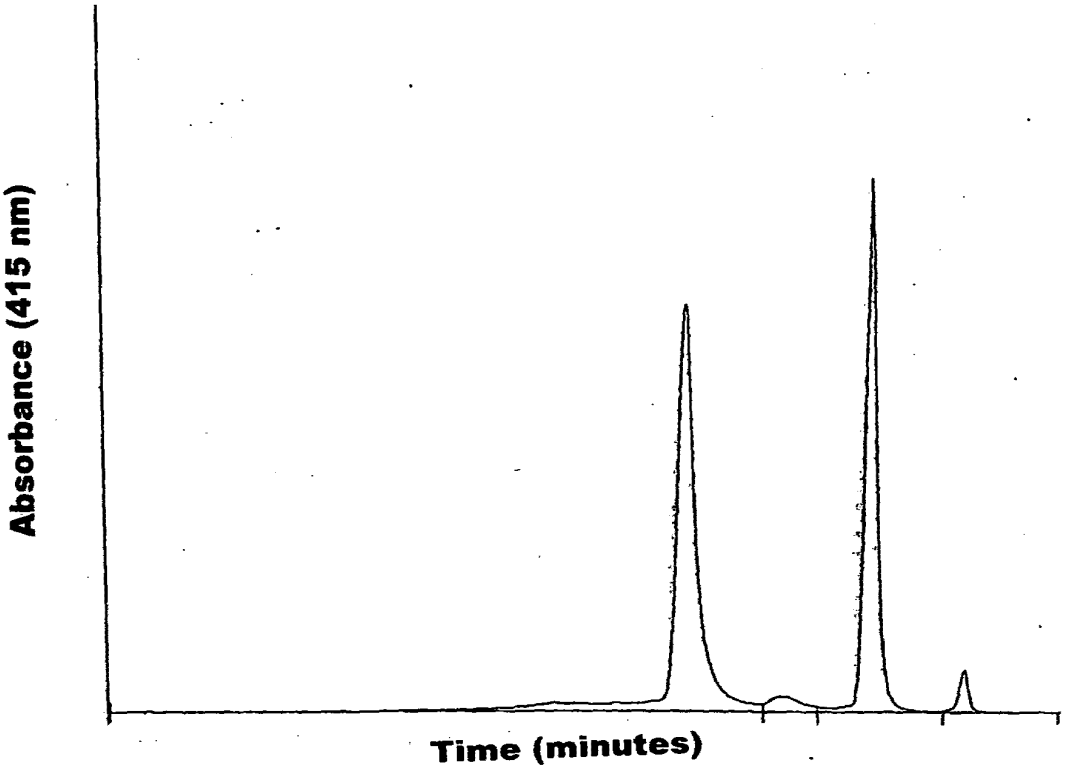
	%
HbA	46,4
HbF	3,5
HbS	47,1
HbA2	3,0

FIG 7B



	%
HbA	42,1
HbF	3,7
HbS	51,3
HbA2	2,9

FIG 7C



	%
HbA	55,5
HbF	3,1
HbS	38,8
HbA2	2,6

FIG 7D

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IFW

AMENDMENT TRANSMITTAL LETTER

Docket No. EGYP 3.0-034			
Application No. 11/280,025-Conf. #8408	Filing Date November 16, 2005	Examiner J. M. Dieterle	Art Unit 4111

Applicant(s): Frédéric Robert et al.

Invention: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED

	Claims Remaining After Amendment	Highest Number Previously Paid	Number Extra Claims Present	Rate	
Total Claims	28	- 30 =	0	x 26.00	0.00
Independent Claims	4	- 3 =	1	x 110.00	110.00
Multiple Dependent Claims (check if applicable)	☐				
Other fee (please specify):					
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					110.00

☐ Large Entity ☒ Small Entity

☐ No additional fee is required for this amendment.

☒ Please charge Deposit Account No. 12-1095 in the amount of $ 110.00.

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 12-1095 as described below. A duplicate copy of this sheet is enclosed.

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

Diane P. Tso

Dated: April 8, 2009

Diane P. Tso
Attorney/Agent Reg. No.: 46,012

LERNER, DAVID, LITTENBERG, KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 654-5000

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: April 8, 2009 Signature: Diane P. Tso (Diane P. Tso)

PTO/SB/08a (01-09)
Approved for use through 02/28/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11280025
Filing Date	2005-11-16
First Named Inventor	Frédéric Robert
Art Unit	4111
Examiner Name	J. M. Dieterle
Attorney Docket Number	EGYP 3.0-034

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5536382		1996-07-16	Sunzeri	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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	1							☐

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	11280025
	Filing Date	2005-11-16
	First Named Inventor	Frédéric Robert
	Art Unit	4111
	Examiner Name	J. M. Dieterle
	Attorney Docket Number	EGYP 3.0-034

	1		☐

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	11280025
Filing Date	2005-11-16
First Named Inventor	Frédéric Robert
Art Unit	4111
Examiner Name	J. M. Dieterle
Attorney Docket Number	EGYP 3.0-034

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☒ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☐ None

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Diane P. Tso/	Date (YYYY-MM-DD)	2009-04-14
Name/Print	Diane P. Tso	Registration Number	46012

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	11280025
Filing Date:	16-Nov-2005
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Filer:	Scott Evan Charney/Brian McManus
Attorney Docket Number:	EGYP 3.0-034

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	5290669
Application Number:	11280025
International Application Number:	
Confirmation Number:	8408
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Customer Number:	00530
Filer:	Scott Evan Charney/Brian McManus
Filer Authorized By:	Scott Evan Charney
Attorney Docket Number:	EGYP 3.0-034
Receipt Date:	06-MAY-2009
Filing Date:	16-NOV-2005
Time Stamp:	22:27:47
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$180
RAM confirmation Number	6950
Deposit Account	121095
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Information Disclosure Statement (IDS) Filed (SB/08)	EGYP46_-_Information_Disclosure_Statement_Fillable_PDF.PDF	601297 1c34f52703c4f65728f9cdb9538c24c5f3737f09	no	4
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	30458 28997caeb3656f2d84dbd8536aa1d1f7b3af28c2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	631755		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/280,025	11/16/2005	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 04/13/2009	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 28	Minus	** 30	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/FELICIA FARMER/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

530 7590 06/22/2009

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

EXAMINER	
DIETERLE, JENNIFER M	

ART UNIT	PAPER NUMBER
1795	

MAIL DATE	DELIVERY MODE
06/22/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/280,025	ROBERT ET AL.	
	Examiner	Art Unit	
	Jennifer Dieterle	4111	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *4/13/09*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *29-51 and 53-57* is/are pending in the application.

4a) Of the above claim(s) *52, 53-55* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *29-51, 56-57* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☒ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *5/6/09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0344

DETAILED ACTION

Response to Amendment

Applicant's amendment of 4/13/2009 does not render the application allowable.

Comments

1. The objection to the drawings has been overcome by Applicant's amendment drawings.

2. The objection to the specification has been overcome by Applicant's amendment thereof.

3. The objections to claims 29, 33, 38-40, 43, 44, 45, 48 and 51 have been overcome by Applicant's amendments.

4. The rejection of claims 33 and 35 under 35 USC 112, second paragraph, has been overcome by Applicant's amendment of these claims.

Claim Status

Claims 29-51 and 53-57 are pending.

Claims 29, 31-33, 35, 38-40, 43-46, 48 and 51 were amended.

Claims 56 and 57 were added in the amendment.

Claims 53-55 are withdrawn.

Claims 1-28 and 52 are canceled.

Claims 29-51 and 56-57 are being examined.

Status of the Rejections

All rejections from the previous office action are withdrawn in view of Applicant's amendment. New grounds of rejections under 35 U.S.C. 102(b) are necessitated by amendments. Alter et al. (US 5,753,094) is being cited and relied on for the first time in this office action. Its use was necessitated by the amendment to the claims.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation

under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

5. Claims 29-31, 34-36, 43-52 and 56 are rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. (US 2002/0162744 A1) in view of Alter et al (US 5,753,094).

Regarding claims 29-31 and 56, Nouadje et al. teaches a method of analyzing biological samples using free solution capillary electrophoresis (section 0007) comprising the following:

- Separating the constituents of protein serums (section 0003)(it is well know to those skilled in the art that haemoglobin is an oxygen carrying protein in red blood cells), which can be normal or abnormal (section 0036) by free solution capillary electrophoresis (section 0007) in which the sample is introduced into a capillary tube containing a buffer system (section 0009) (typically a zwitterionic biological buffer system (section 0032), that separates by migration (section 0007).
- further comprising at least one additive that is capable of hydrophobic interaction with one or more protein constituents and capable of providing said protein constituents with one or more negative charges thereby reducing the electrophoretic mobility of said protein (section 0007), the inhibitor can be a cyclic amine (section 0025).

- wherein, once the protein constituents are separated, they are detected (section 0007).

Nouadje et al. does not teach the use of blood, but does teach the use of protein serum which is a component of blood.

Alter et al. teach that whole blood, serum, plasma, cerebrospinal fluid and urine can be separated and detected using electrophoresis (col. 3, lines 51-53).

Therefore, it would have been obvious to one skilled in the art to modify the method of Nouadje et al. to utilize blood as the biological sample of interest as taught by Alter et al. because blood is a larger sample base then its component serum. It is known serum is a component of blood. It is also known that using a larger sample base, such as blood which contains the two major protein groups: albumin and globulin, will provide a broader range of data then just separating a component of blood such as serum.

Regarding claims 34-36, Nouadje et al. teaches an analysis buffer that can be selected from bis-Tris PROPANE with an amino acid, TRICINE, TAPS, TABS, AMPSO, Tris, BICINE, HEPBS (section 0040). With respect to a TAPS zwitterionic buffer, this buffer has an acid function on one end and a hydroxyl function in the opposite position to the acid function with an amine in the middle (Roy et al., *Buffer Standards*, page 552, section I).

Regarding claims 43 and 44, Nouadje et al. teaches an analysis buffer that has a concentration between 10 mM to 500 mM (section 0043). This range covers the ranges given in the current application's claim of 20 mM to 500 mM.

Regarding claims 45 and 46, Nouadje et al. teaches that the additives concentration be between 0.1 mM to 500 mM (section 0044). This range encompasses the current application's claim of 0.1mM to 50mM.

Regarding claims 47 and 48, Nouadje et al. teaches that the analysis buffer may contain a pH modifying compound selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri-, or tetra- alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion (section 0042).

Regarding claims 49 and 50, Nouadje et al. teaches a method of capillary electrophoresis using a capillary tube formed from fused silica (section 0051). Nouadje et al. also teaches that a person skill in the art will be capable of adapting the nature and size of the capillary to the analytical requirements. These requirements may include utilizing a naked or untreated internal capillary surface (section 0049).

Regarding claim 51, Nouadje et al. teaches that the analysis buffer has a pH between 2 and 12 (section 0041). This pH range encompasses the current application claimed range of 8 to about 10.

Regarding claim 52, Nouadje et al. teaches the method wherein said capillary electrophoresis is of the free solution capillary electrophoresis type (section 0007).

6. Claim 32 is rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. and Alter et al. in view of Mohammad et al. (Clinical Application of Capillary Isoelectric Focusing on Fused Silica Capillary for Determination of Hemoglobin Variants, Clinical Chemistry 1997, 43: 1798-1799).

Regarding claim 32, Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoresis as discussed above. However, Nouadje does not teach the separation of haemolyzed blood or that the hemoglobin HbA2 and an HbC, an HbD, HbE, an HbS, an HbF or an HbA hemoglobin.

Mohammad et al. teach the use of hemolyzed blood in capillary electrophoresis for identification of hemoglobin variants such as: C, S, F, and A.

Therefore, it would have been obvious to one skilled in the art to modify the method of Nouadje et al. to use hemolyzed blood cells wherein the hemoglobin is C, S, F, and A as taught by Mohammad et al. because it is known that hemolyzed blood is found within the red blood cells and disrupting the plasma membranes of the red blood cells would lead to faster and more accurate measurements.

7. Claim 33 is rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. and Alter et al. in view of Chen (U.S. Pat. No. 5,202,006).

Regarding claim 33, Nouadje et al. teaches a method for separating hemoglobin utilizing capillary electrophoresis as discussed above. However, Nouadje does not teach that the hemoglobin HbA2 and an HbC, an HbD, HbE, an HbS, an HbF or an HbA hemoglobin.

Chen recognizes the use of capillary electrophoresis of hemoglobin A, A2, F which comprise normal adult blood (col. 1, lines 26-30), and hemoglobin S (col. 2, lines1-5).

Therefore, it would have been obvious to a person of ordinary skill in the art to modify the method of Nouadje et al. by using hemoglobin HbA2 and variants such as

C, a D, an B, a S, a F and/or an A as taught by Chen because it is well known that hemoglobin HbA2, A and F comprise the majority of hemoglobin contained in normal adult blood and it is known that in order to account for abnormalities in any scientific test, one must have a standard base (i.e. normal hemoglobin) in order to detect the abnormalities.

8. Claims 37-42 and 57 are rejected under 35 U.S.C. 103(a) as being unpatentable over Nouadje et al. and Alter et al., as applied to claim 29 above, in view of Grover et al. (U.S. Pat. No. 6,083,372).

Regarding claims 37-42 and 57, Nouadje et al. teaches a method for separating haemoglobins utilizing capillary electrophoresis as discussed above. However, Nouadje et al. fails to specifically teach the recited flow inhibitor.

Grover et al. teaches the use of flow inhibitors that can encompass polyamine compounds and many amines that are available commercially (col. 11, lines 10-13). Grover et al. teaches that such compounds include, but are not limited to 1,4-diaminobutane (col. 11, line 15).

The selection of a known material, which is based upon its suitability for the intended use, is within the ambit of one of ordinary skill in the art. See *In re Leshin*, 125 USPQ 416 (CCPA 1960) (see MPEP § 2144.07). Additionally, the combination of familiar elements is likely to be obvious when it does no more than yield predictable results. Furthermore, the simple substitution of one known element for another is likely to be obvious when predictable results are achieved. See *KSR Int'l Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1395 (2007)(see MPEP § 2143 and 2143.02). Therefore, it would have been obvious to one skilled in the art to use various polyamine compounds such as 1,4- diaminobutane as a flow inhibitor for separating haemoglobin by capillary electrophoresis.

Response to Arguments

Applicant's arguments with respect to claims 29-51 and 53-57 have been considered but are moot in view of the new ground(s) of rejection.

9. Applicant's arguments filed 4/13/2009 have been fully considered but they are not persuasive. Applicant's response that Nouadje et al fails to teach the biological sample being a blood sample is acknowledged. In light of Applicant's amendments to the claims, new references, such as Alter et al., were introduced and teach the use of "blood" in "free solution" capillary electrophoresis as shown in the amended claims. Applicant's response is no longer persuasive in light of the new art.

10. Applicants' argue that Chen et al. adds nothing to Nouadje et al. such that one of ordinary skill in the art would arrive at the claimed invention; however, the Court's have

held the combination of familiar elements is likely to be obvious when it does no more than yield predictable results. Furthermore, the combination of a known technique to a product is likely to be obvious when predictable results are achieved. See *KSR Int'l Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1395 (2007)(see MPEP § 2143 D). Therefore, the motivation provided by the examiner is that applicant used a known technique with a known product.

11. Applicants' argue that Grover adds nothing to Nouadje et al. such that one of ordinary skill in the art would arrive at the claimed invention however, the Court's have held the combination of familiar elements is likely to be obvious when it does no more than yield predictable results. Furthermore, the combination of a known technique to a product is likely to be obvious when predictable results are achieved. See *KSR Int'l Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1395 (2007)(see MPEP § 2143 D). Grover et al. specifically teach that electrokinetic chromatography is a subset of capillary electrophoresis and the motivation provided by the examiner is that applicant used a known technique with a known product.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Dieterle whose telephone number is (571) 270-7872. The examiner can normally be reached on Monday thru Friday, 8am to 5pm (EST).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Brian Sines can be reached on (571) 272-1263. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

JMD
6/10/09

/Brian J. Sines/
Supervisory Patent Examiner, Art Unit 1795

Notice of References Cited	Application/Control No. 11/280,025	Applicant(s)/Patent Under Reexamination ROBERT ET AL.	
	Examiner Jennifer Dieterle	Art Unit 4111	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,753,094	05-1998	Alter et al.	204/451
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Mohammad et al. (Clinical Application of Capillary Isoelectric Focusing on Fused Silica Capillary for Determination of Hemoglobin Variants, Clinical Chemistry 1997, 43: 1798-1799).
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	3	"11280025"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:26
L2	3	"20020162744"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:27
L3	14	flow adj inhibitor and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:36
L4	11	"10052931"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:44
L5	7321	electrophoretic near3 mobility and (cyclic or aliphatic or polyamine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:45
L6	7262	electrophoretic adj mobility and (cyclic or aliphatic or polyamine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:45
L7	1068	(electrophoretic adj mobility) and (cyclic or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
L8	688	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
L9	0	(electrophoretic adj mobility) near5 (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
L10	0	(electrophoretic adj mobility) with (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
L11	0	(electrophoretic adj mobility) near (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46

L12	688	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
L13	0	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis) and bloood	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:47
L14	12	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis) and zwitterion	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:47
S1	173	faradaic adj reaction	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:41
S2	102	S1 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:41
S3	4639	reaction near4 electrode near4 surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:57
S4	3228	reaction near3 electrode near3 surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:57
S5	471	reaction near electrode near surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58
S6	43	S5 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58
S7	28	S6 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58
S8	4	S7 and ferrocyanide	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:44
S9	14	S2 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59
S10	3	S9 and ferrocya$	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59
S11	3	S9 and ferrocya$4	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59

S12	12	glucose and mediator and enzyme and faradaic adj reaction and shuttle	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 17:28
S13	2	("5922183").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/07 17:38
S14	733	ferricyanide near5 mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:26
S15	0	ferricyanide and ("PF. sub.6" near5 mediator)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S16	0	ferricyanide and ("Os (MeBpy.sub.2.(Im)sub.2" near5 mediator)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S17	0	ferricyanide and "Os (MeBpy.sub.2.(Im)sub.2"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S18	0	ferricyanide and "Os (MeBpy.sub.2.(Im)sub.2."	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S19	0	ferricyanide and "Os (MeBpy.sub.2(Im)sub.2."	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S20	0	ferricyanide and "Os (MeBpy.sub.2(Im)sub.2"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S21	0	ferricyanide and "Os (MeBpy)"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S22	1837	monodentate adj ligand	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S23	1497	S22 and Os	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S24	38701	S22 and Os or osmium	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S25	1558	S22 and (Os or osmium)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S26	198	S25 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S27	2	S26 and (ferri or fefficyanide)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:29

S28	7	S26 and (ferri or ferricyanide)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:29
S29	2	"20050258035"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:33
S30	4752	glucose and (ferri$7 and ferro$7) and (os or osmium)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:59
S31	2665	S30 and ((polyethylene adj glycol or polyethyleneglycol) or erythiritol)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 14:00
S32	50	S31 and (GOX or GOD)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 14:00
S33	2	("5,682,884").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/08 14:19
S34	101	(os or osmium) near4 redox near2 mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S35	80	S34 and ferr$8	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S36	75	S35 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S37	35	S36 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S38	11	dual adj mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:07
S39	104	two adj mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15
S40	58	S39 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15
S41	39	S40 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15
S42	2	("6826966").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/08 17:25

S43	3	"11280025"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/09 18:51
S44	3	"20020162744"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/09 19:02
S45	2	("5753094").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 10:12
S46	0	hawmoglobin	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:48
S47	1	haemoglobin and free adj solution adj capillar$3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S48	6280	haemoglobin andcapillar$3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S49	110	haemoglobin and capillar $3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S50	24	S49 and @py<"2004"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:50
S51	2	haemolyzed adj blood and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 13:43
S52	2	("4764279").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 13:49
S53	2	("5,599,433").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 13:53
S54	17	hemolyzed adj blood and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 14:21

6/10/2009 5:41:02 PM
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Clinical Chemistry



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Clinical Chemistry 43: 1798-1799, 1997;

(*Clinical Chemistry.* 1997;43:1798-1799.)

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Oak Ridge Poster Sessions

Clinical Application of Capillary Isoelectric Focusing on Fused Silica Capillary for Determination of Hemoglobin Variants

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Hemoglobin is composed of two α and two ß polypeptide chains. Any modifications in the amino acid sequence, which may either be congenital or acquired, affects the oxygen-carrying ability of hemoglobin, resulting in a series of hemoglobinopathies. To date, >600 structurally different human hemoglobins are known (1)(2). Identification of abnormal hemoglobin is very important in the differential diagnosis of hemoglobinopathies. Currently, most clinical laboratories have a battery of hemoglobin assays that includes gel electrophoresis, ion-exchange or affinity chromatography, and isoelectric focusing (1)(3). Of all the available methods, computer-operated cation-exchange HPLC is the most widely used for identifying and quantifying major and minor hemoglobins (4)(5)(6)(7). The main disadvantage of a HPLC system is expensive instrumentation and the high cost of columns and reagents. Although an excellent resolution is achieved with gel isoelectric focusing (8), it is labor intensive, time consuming, and not readily amenable for routine screening.

The use of capillary electrophoresis (CE) for identification of hemoglobin variants has been reported by several authors (9)(10)(11)(12). In 1994, Hempe and Craver demonstrated the applicability of CE for quantification and identification of hemoglobin variants in clinical samples (13). Capillary isoelectric focusing (cIEF) was performed on a dimethylpolysiloxane (DMS)-coated fused silica capillary having an i.d. of 50 μm. The method is rapid, requires low sample volume, and gives excellent resolution for all major and most of the minor hemoglobin variants. The main

drawback of this method is the use of DMS-coated capillary. In our laboratory we found that coated capillaries are not very stable and show considerable lot-to-lot variation, thereby resulting in irreproducible migration times. cIEF can also be done on a fused silica capillary provided electroosmotic flow (EOF) is eliminated or reduced. This can be achieved by addition of hydrophilic polymers such as methyl cellulose (MC) (11). MC (2 g/L) reduces EOF significantly but not to the extent seen in a neutral DMS-coated capillary, thus resulting in poor precision for migration times.

We have overcome this problem of irreproducible migration time on a fused silica capillary by introducing two external pI markers purchased from Bio-Rad bracketing the pI gradient 6.6 and 7.7 formed by the ampholine (pI 6.6–7.7). The cIEF is performed on a 57 cm x 50 μm (i.d.) fused silica capillary. The electrophoresis is performed on a PrinCE system manufactured by Helena Labs., and data evaluation was done on Ceaser software. NaOH (20 mmol/L) and H_3PO_4 (100 mmol/L) constituted catholyte and anolyte solutions, respectively. The run buffer consisted of 50 mL/L ampholine prepared in 3 g/L MC solution. Sample preparation involved dilution of 50 μL of citrated whole blood to 1 mL with hemolyzing reagent (10 mmol EDTA and 5 mmol KCN). Two-hundred microliters of hemolyzed blood is further diluted to 400 μL with 2 g/L MC containing 30 mL/L ampholine and 2 μL of individual pI markers (1 g/L). Before focusing, the capillary is flushed with run buffer for 1 min at 200 kPa (2000 mbar) pressure. A sample plug is injected for 0.4 min at 20 kPa (200 mbar) pressure, followed by injection of run buffer at 20 kPa (200 mbar) pressure for 0.4 min. Focusing is carried out at 30 kV for 5 min and separated hemoglobin variants are mobilized past the detector window by applying low pressure [8 kPa (80 mbar)] under an applied voltage of 30 kV.

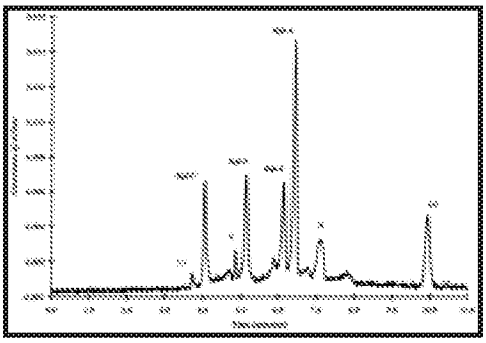
Figure 1 shows the separation of the four most common hemoglobin variants (C, S, F, and A). The pI marker 7.7, being most alkaline, migrates first and the pI marker 6.6 is seen last. These two markers define the beginning and end of the pH gradient. We overcame the problem of irreproducible migration times on fused silica capillaries by relating the migration times of the individual hemoglobins to these two markers. This is done by defining a new term called migration index, which is the ratio of the distance of a hemoglobin variant from two markers and is calculated as shown in Eq. 1:

$$\text{Migration index} = t_{hgb} - t_{7.7}/t_{6.6} - t_{hgb} \quad (1)$$

where t_{hgb}, $t_{7.7}$, and $t_{6.6}$ are the migration times of the hemoglobin variant and pI markers 7.7 and 6.6, respectively. The migration index is a dimensionless quantity and is constant for a given set of conditions. This is readily seen from the CVs shown in Table 1. Furthermore, the migration index calculated for hemoglobins C, S, F, and A in a commercial control were used to create a calibration plot. The regression equation derived from this curve is then used to calculate the pI of the hemoglobin present in a patient's sample. Table 1 shows a comparison between the calculated and the literature pI for hemoglobins C, S, F, and A. By using this equation we are able to identify these four most commonly seen hemoglobins unambiguously in 100 patient samples previously identified by classical gel isoelectrophoresis. Because of the existence of a considerable EOF, we are unable to consistently resolve hemoglobin C from A_2 and E.

Even with this shortcoming, the method is robust, fast, and can be used as a rapid screen for detecting abnormal hemoglobins in clinical samples. It makes use of easily available fused silica capillary, and the concept of using migration index instead of migration times for positive identification makes this method readily adaptable to any CE system available in the market.

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Figure 1. cIEF separation of hemoglobins C, S, F, and A.

X denotes degraded hemoglobin.

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Table 1. Comparison between migration time, migration index, and pI.

Footnotes

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First Named Inventor	Frédéric Robert
Art Unit	4111
Examiner Name	J. M. Dieterle
Attorney Docket Number	EGYP 3.0-034

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Application Number	11280025	Filing Date	2005-11-16	Docket Number (if applicable)	EGYP 3.0-034	Art Unit	1795
First Named Inventor	Frédéric Robert			Examiner Name	J. M. Dieterle		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
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☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

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Ann Biol Clin 2006 ; 64 (4) : 367-80

Proposition de commentaires interprétatifs prêts à l'emploi pour l'électrophorèse des protéines sériques

A proposal of ready-made interpretative comments applicable to serum protein electrophoresis

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J.-P. Couaillac[3]
C. Gibaud[4]
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Article reçu le 2 janvier 2006, accepté le 14 mars 2006

Résumé. L'analyse des protéines sériques par électrophorèse est une analyse utile dans de nombreuses situations pathologiques pour orienter un diagnostic, préciser la gravité d'une maladie ou suivre l'efficacité d'une thérapeutique. L'obligation légale est faite au biologiste d'accompagner chaque résultat d'un commentaire. Le but est de favoriser l'interprétation du résultat et d'en assurer la pleine exploitation par le clinicien, voire de satisfaire les patients qui souhaitent bénéficier d'une double information : du prescripteur et du biologiste. Afin d'aider les biologistes à répondre à ces objectifs, notre groupe de biologistes, sous l'égide du Collège national de biochimie des hôpitaux (CNBH) a établi, une liste de commentaires prêts à l'emploi que nous proposons comme outil facilitant la validation des électrophorèses des protéines sériques.

Mots clés : électrophorèse, protéine, sérique, Capillarys®, commentaire

Abstract. Zone electrophoresis for separation and quantification of serum proteins is useful in numerous pathological situations to make clinical diagnostics, to follow the evolution of a disease or to evaluate the efficiency of a treatment. The biologist must apply professional recommendations according to the official nomenclature of biological analysis. He must attach a comment to each result in order to help the physician to perform the best interpretation of the results. To answer those needs, a work of standardization of these comments has been realized by a group of biologists. They are members of the national college of biologists (CNBH). All the commentaries are assembled in a thesaurus which could be a base of ready-made comments, favouring the interpretation of serum protein electrophoresis results.

***Key words:** serum, protein, electrophoresis, Capillarys®, comment*

Le principe de l'électrophorèse des protéines est connu depuis les premiers travaux de Tiselius dans les années 1930. Il met en jeu le déplacement des protéines ionisées lorsqu'elles sont soumises à un champ électrique dans des conditions définies de force ionique, de pH, de durée et d'intensité du courant électrique appliqué. L'électrophorèse des protéines a été adaptée pour la biologie clinique dans les années 1940. Cette méthode s'est progressivement perfectionnée, miniaturisée et standardisée pour atteindre sa maturité vers la fin des années 1990. Depuis une dizaine d'années, en 1994 est apparue une nouvelle méthode : l'électrophorèse capillaire développée par la société Beckman Coulter sous la dénomination Paragon CZE 2000®. Ce principe a été aussi développé plus récemment par la société Sebia qui commercialise depuis 2001 un nouvel appareillage le Capillarys®. Ce matériel semble

Tirés à part : A. Szymanowicz

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bien répondre à l'attente des biologistes et gagne régulièrement du terrain sur l'électrophorèse traditionnelle sur gel. Dans des conditions techniques automatisées bien maîtrisées [1], les résultats des analyses par électrophorèse sont devenus reproductibles et précis. Cela est bien confirmé par les résultats des campagnes de contrôle de qualité externes régionaux et nationaux, même si certains sérums de contrôle révèlent des pics artéfactuels par électrophorèse capillaire. Ces anomalies ne sont actuellement pas encore complètement expliquées. L'électrophorèse capillaire, comme l'électrophorèse sur gel d'agarose, permet la séparation de 6 fractions de protéines. L'ordre de migration est inversé par rapport à l'électrophorèse sur gel car le déplacement des protéines utilise en fait le courant d'électroendosmose [1]. L'ordre traditionnel a cependant été conservé par le fournisseur afin de ne pas perturber les habitudes des utilisateurs biologistes et médecins. L'image du profil obtenu est présenté sur la *figure 1*. Cet ordre des fractions est celui le plus largement utilisé pour la représentation des profils d'électrophorèse des protéines du sérum.

L'électrophorèse des protéines sériques bénéficie d'indications cliniques consensuelles et d'autres plus subjectives dépendantes des écoles de formation des médecins, ce qui implique la nécessité de pouvoir disposer de recommandations de bonne pratique de prescription et d'interprétation de cet examen.

L'indication principale et incontestable de l'électrophorèse est le dépistage, à faible coût, d'une immunoglobulinopathie. La recommandation de pratiquer cet examen dans ce cadre figure d'ailleurs dans nombre de guides de bonne pratique [2-4]. Lorsque le diagnostic de malignité est posé et le traitement mis en œuvre, le suivi de l'efficacité thérapeutique est également assuré par l'électrophorèse le plus souvent. Cette dernière recommandation [2] est d'ailleurs en passe d'être officiellement confirmée par la *National academy of clinical biochemistry* [5]. De même, le suivi de l'évolution des gammapathies de signification indéterminée (*monoclonal gammopathy of undetermined significance* (MGUS) ou gammapathies quiescentes idiopathiques) est habituellement effectué par l'électrophorèse semestrielle puis annuelle en situation stable du pic monoclonal, certains cliniciens y ajoutant éventuellement un myélogramme lors de la découverte initiale de l'anomalie.

L'électrophorèse donne, d'autre part, un reflet panoramique de l'ensemble des protéines sériques et peut à ce titre dépister, notamment un syndrome inflammatoire (zones alpha-1 et alpha-2), une carence martiale (zone bêta), une hémolyse intra-vasculaire (zone alpha-2), une cirrhose (zone bêta-gamma), une maladie infectieuse, auto-immune ou de système (zone alpha-1, alpha-2, bêta et surtout gamma), un déficit congénital ou acquis des immunoglobulines ou d'autres protéines (albumine, alpha-1 antitrypsine et gammaglobulines notamment) ou d'évaluer le retentissement d'une pathologie connue, voire d'en assurer le suivi : atteinte hépatique (dont une cirrhose ou une hépatite), atteinte rénale (dont un syndrome néphrotique), digestives, etc. [6, 7].

Les résultats de cet examen doivent légalement être accompagnés d'un commentaire qui favorise la bonne interprétation de l'analyse et assure sa pleine exploitation par le clinicien, tout en satisfaisant l'information des patients [8-12]. Il est toutefois démontré que les commentaires interprétatifs ne répondent pas toujours très bien à l'attente des cliniciens ou des patients, ce qui soulève d'ailleurs des débats dans les pays où la biologie est exercée par des professionnels moins qualifiés qu'en France [12, 13]. Dans ce contexte nous avons élaboré, au sein d'un groupe de travail du CNBH, un catalogue de propositions de commentaires les plus précis et didactiques possibles. Ces commentaires ont été validés par un biologiste, spécialisé dans le domaine de l'électrophorèse et des protéines sériques et n'appartenant pas au CNBH.

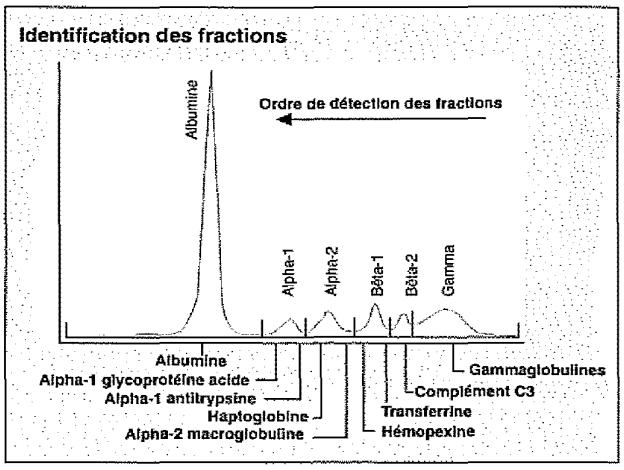


Figure 1. Profil d'électrophorèse des protéines sériques obtenu par Capillarys.

Matériels et méthodes

Dans une première étape, nous avons établi la liste des commentaires habituellement utilisés dans leur pratique professionnelle par les membres du groupe de travail. Le coordonnateur en a assuré une mise en forme commune correspondant à la version I, adressée ensuite aux membres du groupe. Les modifications proposées par ces derniers ont été réintégrées par le coordonnateur dans une version II et renvoyées pour validation. La version II a

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ensuite été soumise pour validation à un biologiste référent pour notre groupe : le docteur Didier Le Carrer (laboratoire de biochimie spécialisée, 9 quai Moncousu, B.P. 1005, 44093 Nantes Cedex 1) qui a proposé des améliorations. Celles-ci ont été prises en compte par le coordonnateur aboutissant à la version III qui a été finalement diffusée auprès des membres du groupe. Ce thésaurus enrichi a été soumis à l'épreuve de l'utilisation pratique pendant 4 années [14] et réactualisé en juin 2005 par le coordonnateur dans une version IV. Celle-ci a été revalidée une dernière fois par l'ensemble du groupe. Tout au long de ce processus d'élaboration, la revue régulière de la littérature et l'utilisation des textes ainsi proposés ont permis de conforter par certains éléments de preuves la grande majorité des commentaires. C'est cette version IV qui est présentée dans l'article. L'utilisation de ces textes prêts à l'emploi peut s'appuyer très utilement sur le paramétrage du système informatique du laboratoire (SIL) selon les axes décrits dans la partie résultats et dans les *tableaux 1 à 5*. Dans cette perspective, ces textes sont saisis dans le dictionnaire des textes codés du SIL. Ils peuvent alors être sélectionnés selon les besoins par le biologiste, au cours de l'étape de validation des électrophorèses. Cette sélection est très aisée grâce au principe de la boite de dialogue, disponible aujourd'hui dans tous les SIL. Le cas échéant, le biologiste conserve toute son initiative pour compléter les commentaires par des textes libres si aucun des textes prêts à l'emploi ne convient.

La génération de la majorité des commentaires prêts à l'emploi proposés doit s'appuyer sur des renseignements cliniques et nécessite donc un dialogue clinico-biologique efficace. Celui-ci permet éventuellement de mieux adapter les textes aux pratiques locales et aussi en fonction de la méthode d'électrophorèse retenue. Les particularités migratoires de certaines fractions ou protéines, propres à chaque système d'électrophorèse, doivent être connues de chaque utilisateur. Nous ne pouvons dans cet article en faire une revue générale. Toutefois il nous semble utile de préciser que le tampon de migration « 6 protéines » utilisé par le système Capillarys®, entraîne la migration des bêta-lipoprotéines et des chylomicrons avec l'albumine. Cette option, retenue par Sebia, est celle qui minimise le plus les interférences des lipides avec l'intégration des fractions protéiques dont notamment les alpha-1. De même, il nous paraît utile de rappeler que sur gel d'agarose, les bêta-lipoprotéines migrent en position bêta-1 prenant une forme particulière, dite « en moustache ».

Le préalable à la bonne réalisation de l'électrophorèse ainsi qu'à la qualité de son interprétation est de travailler sur un échantillon de sang veineux, recueilli sur tube sec impérativement et sur un patient à jeun. L'analyse n'ayant aucun caractère d'urgence doit cependant être pratiquée dans la journée chaque fois que possible. Cela évite ainsi la dégradation plus ou moins importante des protéines les plus fragiles (fractions du complément et lipoprotéines). Le sérum, après coagulation d'une heure, est obtenu par centrifugation de 15 minutes à 3 000 rpm. L'aspect du sérum doit être limpide (absence de chylomicrons), exempt d'hémolyse et de fibrine [15]. Dans l'article nous mentionnerons les raisons principales qui doivent inciter le biologiste à faire respecter impérativement ces bonnes conditions préanalytiques.

Résultats

Qualité de l'échantillon

La présence d'une hémolyse franche, d'un aspect lactescent ou d'une formation renouvelée de fibrine sur un sérum doit conduire le biologiste à refuser l'échantillon impropre pour la réalisation d'une électrophorèse fiable.

Toute autre anomalie visuelle moins flagrante du sérum, doit être signalée par un commentaire accompagnant le résultat si toutefois l'analyse doit être réalisée, pour des motifs cliniques ou organisationnels validés par le biologiste (patient sous héparine ou très difficile à prélever ou ayant déjà quitté le service). Des pics artéfactuels provoqués par la présence de produits de contraste iodés sont mis en évidence par le système Capillarys et ont été identifiés grâce aux travaux de Didier Le Carrer lors de la période de validation de la méthode Capillarys® [1]. Le Visipaque®, l'Omnipaque®, le Xénétix® induisent un pic vers la fin des alpha-2. L'Héxabrix® provoque un double pic dans cette même zone. En bêta-1 l'Ioméron® et en bêta-2 l'Optiject® provoquent également un pic artéfactuel. L'association pipéracilline tarzobactam peut induire un pic en bêta et le sulfaméthoxazole peut induire un pic précédant celui de l'albumine. La raison de ces interférences est due au fait que ces molécules absorbent à la longueur d'onde de 200 nm choisie pour la détection des protéines. L'injection de gammaglobulines sous forme de Tégéline® ou de substitut de plasma à base de gélatine fluide modifiée telle la Gélofusine® augmente artificiellement la zone des gammaglobulines [16]. Cette incidence était déjà connue avec l'électrophorèse traditionnelle sur gel, utilisant la révélation par les colorants. La connaissance de ces artefacts est importante. Ainsi, pour éviter toute interprétation erronée des résultats, le biologiste devra demander des renseignements cliniques, des renseignements concernant les traitements et examens subis par le patient dans les jours précédents. Les textes prêts à l'emploi concernant la qualité de l'échantillon et le signalement d'artefacts sont présentés dans le *tableau 1*.

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Fraction albumine

L'albumine est la seule fraction de composition homogène séparée par l'électrophorèse sur gel ou sur acétate de cellulose. Ce dogme n'est plus vrai dans le cas de la technique Capillarys® car dans les conditions de migration du tampon « 6 protéines », les bêta-lipoprotéines accompagnent l'albumine. Malgré cela, la concentration de l'albumine obtenue par l'intégration, doit être normalement très bien corrélée avec son dosage spécifique par technique immunologique. L'inexactitude ne doit pas dépasser 6 % [1]. L'albumine est aussi, en général, assez bien corrélée avec le dosage des protéines totales mais des discordances peuvent exister dans des situations pathologiques : infections sévères, myélome multiple, maladie de Waldenström, syndrome néphrotique, états de dénutrition sévère. Toute anomalie significative doit être mentionnée par un commentaire accompagnant le résultat de l'électrophorèse. D'autre part, la présence rare d'une bisalbuminémie doit être mentionnée. Ces dédoublements du pic sont rencontrés dans les très rares mutations héréditaires qui sont sans expression pathologique connue à ce jour. Signalons que la deuxième fraction d'albumine d'une bisalbumine peut être de migration plus rapide ou plus lente que l'albumine du sujet témoin normal. La vitesse de migration de l'albumine mutée est dépendante de l'origine ethnique du patient. Par contre, les bisalbuminémies acquises transitoires sont plus fréquentes et en majorité elles sont induites soit par la fixation de bêtalactamines soit par la lyse partielle par les protéases pancréatiques dans les pancréatites chroniques associées à une fistulisation d'un faux kyste du pancréas. L'exceptionnelle absence d'albumine [6] doit aussi être signalée par un commentaire, d'autant qu'elle se manifeste par des œdèmes et une augmentation compensatrice de toutes les globulines.

Tableau 1. Textes prêts à l'emploi concernant l'aspect visuel du sérum, l'aspect général du profil et la présence d'artefacts.

N°	Texte	Argument déclenchant
1	Profil qualitatif et quantitatif de l'électrophorèse sans anomalie notable	Taux et concentration des fractions normaux, absence de bande étroite
2	Sérum très hémolysé, l'électrophorèse ne peut être réalisée sur un tel échantillon. Renvoyer un échantillon pour contrôle si nécessaire	Sérum rouge
3	Sérum légèrement hémolysé, résultats sous réserve. Renvoyer un échantillon pour contrôle si nécessaire	Migration de HbHp en alpha-2 et de l'hémoglobine en bêta-1 Sérum rosé
4	Sérum lactescent, l'électrophorèse ne peut être réalisée sur cet échantillon. Renvoyer un échantillon après normalisation des triglycérides si nécessaire	Sérum lactescent ou très lactescent
5	Hypoprotéinémie globale par fuite urinaire, compatible avec le contexte clinique	Protéines < 55 g/L Albumine < 30 g/L Protéinurie > 5 g/L
6	Hypoprotéinémie globale par dénutrition, compatible avec le contexte clinique	Protéines < 55 g/L Albumine < 30 g/L Protéinurie < 1 g/L
7	Hypoprotéinémie globale par fuite digestive, compatible avec le contexte clinique	Protéines < 55 g/L
8	Hypoprotéinémie globale par insuffisance hépatique, compatible avec le contexte clinique	Protéines < 55 g/L
9	Hypoprotéinémie globale par dilution compatible avec le contexte clinique	Protéines < 55 g/L Hématocrite < 0,35
10	Pic probable de fibrinogène au niveau des gammaglobulines. Préciser le traitement anticoagulant en cours actuellement. Redemander si nécessaire l'analyse, à distance d'un traitement par l'héparine	Bande étroite à marge un peu floue en gamma CRP normale
11	Pic très probable de CRP au niveau des gammaglobulines, confirmé par le taux élevé de CRP associé à un syndrome inflammatoire, non attribuable à une bande mince d'immunoglobuline monoclonale	Bande très fine en gamma, CRP > 300 mg/L si hyper-gamma CRP > 200 mg/L si gamma normale CRP > 100 mg/L si hypo-gamma
12	Sérum de couleur brune signant la présence probable de methémalbumine, témoin d'une hémolyse intravasculaire récente ou d'un déficit d'élimination de la bilirubine ou des deux à la fois ou d'interférence médicamenteuse. SVP, veuillez nous transmettre les renseignements cliniques afin que nous les enregistrions dans le dossier biologique	Sérum de couleur brune Vérifier le contexte avec le prescripteur
13	Sérum opalescent, résultats sous réserve. À vérifier si nécessaire dans quelques jours lorsque la lipémie sera normalisée	Sérum opalescent
14	Bien que l'électrophorèse ne montre aucune anomalie qualitative ni quantitative des gammaglobulines, l'immunotypage sera réalisé conformément à la confirmation de la prescription et compte tenu du contexte clinique	Taux des fractions normal, absence de bande mince Seuil de détection d'une bande mince > 0,2 g/L

HbHP : complexe hémoglobine-haptoglobine.

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Tableau 2. Textes prêts à l'emploi concernant les fractions des alpha-globulines.

N°	Texte	Argument déclenchant (g/L)
1	Profil compatible avec un syndrome inflammatoire modéré	Alpha-1 > 4 g/L < 6 g/L et/ou alpha-2 > 9 g/L < 12 g/L
2	Profil compatible avec un syndrome inflammatoire modéré et diminution de l'albumine	Alpha-1 > 4 g/L < 6 g/L et/ou alpha-2 > 9 g/L < 12 g/L Albumine < 35 g/L > 30 g/L
3	Profil compatible avec un syndrome inflammatoire modéré et diminution importante de l'albumine	Alpha-1 > 4 g/L < 6 g/L et/ou alpha-2 > 9 g/L < 12 g/L Albumine < 30 g/L
4	Profil compatible avec un syndrome inflammatoire important	Alpha-1 > 6 g/L et/ou alpha-2 > 12 g/L
5	Profil compatible avec un syndrome inflammatoire important et diminution de l'albumine	Alpha-1 > 6 g/L et/ou alpha-2 > 12 g/L Albumine < 35 g/L > 30 g/L
6	Profil compatible avec un syndrome inflammatoire important et diminution importante de l'albumine	Alpha-1 > 6 g/L et/ou alpha-2 > 12 g/L Albumine < 30 g/L
7	Profil électrophorétique compatible avec un syndrome inflammatoire accompagné d'une réaction immunitaire	Alpha-1 > 6 g/L et/ou alpha-2 > 9 g/L et gamma > 15 g/L
8	Diminution importante de la zone des alpha-1-globulines, compatible avec un déficit en alpha-1-antitrypsine. Le dosage et le phénotype Pi ont été rajoutés compte tenu du contexte clinique	Alpha-1 < 1,5 g/L

Les hypoalbuminémies sont la conséquence d'une diminution de synthèse dans l'insuffisance hépatocellulaire, la malnutrition et les états inflammatoires.

Elles peuvent aussi résulter de fuites urinaires dans le syndrome néphrotique en particulier, digestives dans les gastroentéropathies exsudatives ou cutanées dans le cas des brûlures étendues. Elles résultent parfois de l'hypercatabolisme dans les endocrinopathies acquises telles que la thyréotoxicose et le syndrome de Cushing ou dans les syndromes tumoraux dans lesquels une inflammation peut aussi être présente. Le profil correspondant à ces deux syndromes concomitants est parmi les plus fréquemment rencontrés en pratique. Cela justifie de ce fait, la proposition des textes (mixtes) prêts à l'emploi correspondants que nous avons regroupés dans le *tableau 2.*

L'hyperalbuminémie ne peut se rencontrer que dans le cadre d'une hémoconcentration ou suite à une perfusion d'albumine. Il s'agit donc toujours d'une fausse hyperalbuminémie sans conséquence pathologique. Signalons que l'excellente corrélation entre dosage immunologique et quantification électrophorétique est mise en défaut lorsque la proportion albumine/globulines est très diminuée (immunoglobuline monoclonale > 20 g/L ou syndrome néphrotique avec albumine < 20 g/L) car dans ce cas le dosage des protéines totales par le biuret surévalue la concentration des protéines de l'échantillon. Cette surestimation est induite par la plus grande réactivité des globulines vis-à-vis du biuret comparée à la réactivité de l'albumine [1]. Ce déséquilibre albumine/globulines entraîne une moins bonne corrélation entre les résultats des deux techniques mentionnées d'autant que ce phénomène est amplifié par la migration des bêta-lipoprotéines au même niveau sur Capillarys®. De par leur masse moléculaire importante, ces bêta-lipoprotéines sont augmentées dans le syndrome néphrotique et vont donc accentuer la discordance. Alors, des différences pour l'albumine de - 10 % par la technique immunochimique peuvent être constatées. Lorsque les discordances des résultats entre l'électrophorèse et le dosage immunologique de l'albumine pour les taux inférieurs à 15 g/L dépassent 10 %, il convient de vérifier la linéarité du dosage immunologique. En effet, la linéarité du dosage immunologique peut être prise en défaut pour ces taux très bas, dont l'exactitude est rarement explorée en pratique courante.

Les textes prêts à l'emploi relatifs aux commentaires sur la fraction albumine sont présentés dans le *tableau 3.*

La fraction alpha-1 globulines

C'est une fraction hétérogène qui contient principalement, l'alpha-1 antitrypsine et l'alpha-1 glycoprotéine acide (orosomucoïde). Cette fraction est augmentée dans les syndromes inflammatoires associés généralement à l'augmentation des alpha-2 globulines. Plus exceptionnellement cette fraction sera diminuée lors des états de dénutrition sévères ou dans l'insuffisance hépatocellulaire. Plus exceptionnellement encore, sa diminution sera le témoin d'un déficit génétique homozygote en alpha-1 antitrypsine ou plus modéré chez les sujets hétérozygotes. Ce déficit génétique est parfois associé à une atteinte hépatique chez l'enfant et pulmonaire chez l'adulte. Notons que dans le cas des techniques d'électrophorèse capillaire, cette fraction est à un taux relativement plus important car le signal de détection par spectrophotométrie est plus juste que l'intégration après coloration. En effet, celle-ci sous-estime habituellement cette fraction riche en acide sialique [1] qui possède moins d'affinité pour les colorants que les

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Tableau 3. Textes prêts à l'emploi concernant l'albumine.

N°	Textes	Argument déclenchant
1	Hypoalbuminémie modérée	Albumine < 35 g/L > 30 g/L
2	Hypoalbuminémie importante	Albumine < 30 g/L
3	Profil en faveur d'un syndrome néphrotique compatible avec le contexte clinique. À confirmer par le dosage de la protéinurie des 24 heures	Protéines < 60 g/L Albumine < 30 g/L Alpha-2 > 11 g/L
4	Augmentation de l'albumine, hémoconcentration très probable, compatible avec le contexte clinique	Albumine > 50 g/L
5	Bisalbuminémie secondaire à un traitement par des bêtalactamines (pénicillines ou céphalosporines). Compatible avec les renseignements cliniques. Analyse à refaire si nécessaire après arrêt du traitement	Forte dose de pénicillines ou céphalosporines
6	Bisalbuminémie secondaire à la protéolyse par les enzymes pancréatiques libérées en excès, compatible avec le contexte clinique	Pancréatite chronique compliquée d'un faux kyste fistulisé du pancréas
7	Bisalbuminémie congénitale probable compatible avec les renseignements cliniques	Pic dédoublé d'albumine vers les alpha-1. Absence de causes secondaires
8	Absence d'albumine : analbuminémie congénitale avec augmentation de toute les fractions globuliniques	Absence du pic d'albumine Augmentation de toutes les autres fractions globulines

autres glycoprotéines. L'aspect de dédoublement des alpha-1 peut être consécutif à un variant hétérozygote de l'alpha-1 antitrypsine [17].

La fraction alpha-2 globulines

Elle correspond principalement à l'alpha-2 macroglobuline (A2M) et à l'haptoglobine (Hp). C'est dans cette même zone que va migrer le complexe que forme l'hémoglobine (Hb) avec l'haptoglobine (HbHp) dans le cas de l'hémolyse *in vitro*. Le pic des alpha-2 peut alors être déformé par un épaulement plus ou moins important correspondant à ce complexe moléculaire stœchiométrique stable développant une activité peroxydasique. Celle-ci a été notamment exploitée pour l'étude des différents types génétiques d'haptoglobine [18] et pour la mise au point des premiers dosages automatisés de l'haptoglobine. Notons qu'en cas d'hémolyse pathologique *in vivo* le complexe HbHp qui se forme irréversiblement est rapidement capté et détruit par le foie avec récupération du fer. Cette élimination très rapide du complexe HbHp (demi-vie de 20 minutes) entraîne une diminution importante de la fraction alpha-2 en cas d'hémolyse intravasculaire et une diminution plus modérée dans les hémolyses extravasculaires. Dans ces circonstances pathologiques l'Hp joue son rôle protecteur vis-à-vis de l'effet toxique rénal de l'Hb [19]. Le dosage de l'Hp effondré est par ailleurs un excellent indicateur dans le cas d'une hémolyse intravasculaire même modérée, il doit cependant être corrélé avec la concentration de l'alpha-1 glycoprotéine acide afin de permettre une interprétation fiable d'un processus d'hémolyse chronique associant un syndrome inflammatoire.

Cette zone peut parfois être le siège de la migration de chaînes légères monoclonales et très exceptionnellement celui des chaînes lourdes alpha révélateur d'une maladie des chaînes lourdes alpha. Il convient aussi de signaler que le pic des alpha-2 peut être nettement dédoublé dans le cas d'un patient dont l'haptoglobine (Hp) est de phénotype Hp 1-1 et plus discrètement s'il s'agit d'un patient de phénotype Hp1-2 [19]. Ce dédoublement est moins net dans le cas des électrophorèses sur agarose que pour celles réalisées par les techniques capillaires. Dans l'insuffisance hépatocellulaire et dans la dénutrition, les alpha-2 sont diminuées. Dans le syndrome néphrotique le profil devient caractéristique par l'augmentation relative très marquée de la fraction alpha-2 correspondant alors à la macromolécule A2M qui ne s'échappe pas par le rein devenu perméable à la plupart des protéines de masse moléculaire plus faible. Notons que sur le gel d'agarose la fraction bêta sera aussi augmentée par l'augmentation des bêta-lipoprotéines dans ce cas, selon le même mécanisme car il s'agit aussi de macroprotéines ne franchissant pas la barrière glomérulaire même partiellement lésée.

Dans le syndrome inflammatoire, la fraction alpha-2 est augmentée en parallèle généralement à la fraction alpha-1 mais lui est toujours supérieure. L'examen attentif de cette zone est donc important pour une interprétation correcte du profil.

Les commentaires applicables aux fractions alpha sont rassemblés dans le *tableau 2*.

La fraction bêta-1 globulines

Cette fraction contient principalement la transferrine, l'hémopexine et les bêta-lipoprotéines dans le cas des gels d'agarose mais principalement la transferrine et l'hémopexine sur Capillarys®. Les diminutions de cette fraction sont dues à l'insuffisance hépatocellulaire, la surcharge martiale, la dénutrition ou les fuites protéiques d'origine

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digestive ou rénale ou à des transfusions répétées entraînant une diminution importante de la transferrine. L'augmentation est corrélée avec l'importance de la carence martiale qui entraîne une hypertransferrinémie adaptative. La transferrine peut aussi voir sa synthèse augmentée lors d'un traitement œstroprogestatif, mais dans de moindres proportions que lors d'une carence martiale, surtout quand on se situe au stade de l'anémie ferriprive. C'est dans cette zone aussi que migre l'hémoglobine libérée par hémolyse le plus souvent artéfactuelle *in vitro*. Sa présence se manifeste sous forme d'un double pic en bêta-1 corroboré par l'aspect franchement hémolysé du sérum, la limite de détection visuelle se situe vers 0,2 à 0,3 g/L d'hémoglobine libre. Entre les zones alpha-2 et bêta-1 globulines vont migrer un certain nombre de produits de contraste utilisés en imagerie, absorbant à 200 nm et qui ne perturbent pas le profil électrophorétique sur agarose coloré par l'amidoschwarz ou le rouge ponceau.

La fraction bêta-2 globulines

Elle renferme les fractions du complément C3 et C4 et les IgA largement prépondérantes. Elle est augmentée modérément dans les hypercomplémentémies d'origine inflammatoire ou secondaire à une obstruction biliaire intra- ou extrahépatique. Une diminution sera associée à une hypocomplémentémie (sérum vieilli, consommation du complément, présence d'un C3Nef – anticorps anti C3 – ou rare déficit en C3). Signalons aussi la possibilité de déformation de cette zone par la présence d'une immunoglobuline monoclonale IgA le plus fréquemment mais qui pourra aussi être d'un autre isotype. Toute fraction bêta-2 supérieure à la fraction bêta-1 devra être interprétée avec prudence pour ne pas méconnaître une gammapathie monoclonale de migration bêta, IgG ou IgM, voire IgA, chaînes légères libres monoclonales ou encore plus rarement IgD ou IgE. La fusion de la zone bêta-2 avec les gamma sera aussi à signaler car elle traduit l'augmentation de synthèse des IgA polyclonales, consécutive à un état de cirrhose éthylique le plus souvent. Cette fusion des zones bêta et gamma donne un aspect traditionnellement qualifié de bloc bêta-gamma. Un aspect tout à fait semblable peut, plus rarement être dû à la synthèse accrue d'une sous-classe d'IgG, les IgA étant normales dans ce cas. Dans la zone de début des gamma peut migrer le fibrinogène, formant un épaulement après le pic des bêta-2 lorsque la coagulation *in vitro* n'a pas été possible ou pas complète pour différentes raisons : patient sous héparine, patient de dialyse, prélèvement à partir d'un cathéter *in situ*, échantillon prélevé sur un tube avec anticoagulant et transvasé dans un tube sec, temps de coagulation insuffisant avant la centrifugation, etc. Notons que l'artefact produit par la fibrine ne peut se confondre avec celui produit par un taux élevé de CRP. Dans le cas de la CRP, la bande ou le pic sont faibles et extrêmement étroits. Une concentration supérieure à 200 mg/L peut aussi simuler une bande mince migrant dans la zone de début des gammaglobulines [20]. Cet aspect est bien visible, notamment en cas d'hypogammaglobulinémie car dans ce cas ce seuil de détection de la CRP est de 100 mg/L environ. Cet artefact est notamment lié à l'amélioration des capacités séparatives des méthodes au cours de ces 10 dernières années [20].

Bien évidemment un nouvel échantillon sur tube sec doit être demandé pour s'assurer de l'artefact provoqué par la fibrine tandis que le dosage de la CRP permettra de conforter cette dernière hypothèse. Avec la même logique, lorsque ces deux hypothèses ne sont pas validées par les tests précédents, l'identification immunologique du pic est déclenchée après concertation avec le clinicien.

Les commentaires concernant les fractions bêta sont présentés dans le *tableau 4*.

La fraction gammaglobulines

Elle correspond aux immunoglobulines (Ig) prépondérantes IgG, IgA et IgM et celles de très faibles concentrations de classe IgD et IgE. Ces dernières Ig sont habituellement non visibles sur le profil à l'exception des très rares myélomes de ces 2 isotypes. L'observation de cette zone est très importante et toute anomalie significative devra être clairement signalée au clinicien ou faire l'objet d'un complément d'examen rajouté à l'initiative du biologiste, comme le préconise le texte de la nomenclature. Il nous paraît préférable de faire ce rajout après concertation avec le prescripteur afin de ne pas prendre le risque d'effectuer des examens complémentaires inutilement coûteux pour la collectivité. Les résultats de ces compléments d'examens peuvent ne pas influencer la décision médicale ou être déjà connus car effectués dans un autre laboratoire, voire être contraires à l'éthique médicale. En effet, il ne faut pas prendre le risque qu'un patient puisse être informé directement d'un diagnostic par la simple lecture d'un document écrit en provenance du laboratoire sans que le médecin en charge du patient n'en ait été lui même informé. C'est cette concertation qui permet aussi d'établir la liste des examens complémentaires à réaliser : calcémie (si pas déjà effectuée), identification immunologique et dosage des immunoglobulines IgG, IgA, IgM (afin de quantifier l'éventuelle répression de synthèse des Ig non monoclonales) [2]. Dans un second temps, l'électrophorèse avec l'identification immunologique urinaire et le dosage des chaînes légères libres dans le sérum [21, 22], peuvent être utiles notamment en cas de myélome à chaînes légères. Ces examens ont pour intérêt de mettre respectivement en évidence une éventuelle protéinurie dite de Bence-Jones, toxique pour le rein, ou un excès de chaînes légères libres dans le sérum avec déséquilibre du rapport kappa/lambda.

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Tableau 4. Textes codés concernant les fractions bêtaglobulines.

N°	Texte	Argument déclenchant
1	Les bêta-2 globulines sont supérieures aux bêta-1 avec diminution des gammaglobulines. Cet aspect est compatible avec la présence d'une bande mince monoclonale de migration bêta. L'identification immunologique et le bilan complémentaire*** ont été rajoutés en accord avec le prescripteur	Gamma < 5 g/L Bêta-2 > bêta-1 Âge > 45 ans
2	Bloc bêta gamma débutant	Comblement partiel β-γ
3	Bloc bêta gamma important	Bêta + gamma > 20 g/L < 30 g/L
4	Bloc bêta gamma avec augmentation polyclonale importante des immunoglobulines	Bêta + gamma > 30 g/L
5	Augmentation des bêta-1 globulines (transferrine) compatible avec une sidéropénie ou une imprégnation œstogénique. À compléter éventuellement par le bilan de carence martiale, en fonction des données cliniques, si cette sidéropénie n'est pas déjà connue	Femme Hb < 12 g/dL TCMH < 27 pg
6	Augmentation des bêta-1 globulines (transferrine) compatible avec une sidéropénie et confirmée par la diminution du taux d'hémoglobine, associée à une microcytose. Le bilan de carence martiale a été rajouté en fonction des données cliniques	Homme Hb < 12 g/dL TCMH < 27 pg
7	Aspect dissymétrique des bêta-2 globulines avec diminution des gammaglobulines, compatible avec la présence d'une bande mince monoclonale de migration bêta. L'identification immunologique et le bilan complémentaire*** ont été rajoutés en fonction des données cliniques	Gamma < 5 g/L Bêta-2 > 8 g/L Âge > 45 ans
8	Augmentation modérée des bêta-1 globulines	Bêta-1 > 6 g/L < 8 g/L Absence d'anémie
9	Augmentation modérée des bêta-2 globulines	Bêta-2 > 4 g/L-< 8 g/L
10	Diminution importante des bêta-2 globulines consécutive à l'activation de la voie alterne et ou classique du complément, soit une dénutrition grave, soit une insuffisance hépatocellulaire sévère. Veuillez nous faire parvenir les renseignements cliniques afin que nous les enregistrions dans le dossier biologique	Bêta-2 < 2 g/L
11	Augmentation importante des bêta-2 globulines, syndrome inflammatoire important et pérennisé, évolution à surveiller. Veuillez nous faire parvenir les renseignements cliniques afin que nous les enregistrions dans le dossier biologique	Bêta-2 > 8 g/L
12	Augmentation importante des bêta-2 globulines, compatible avec une cholestase biliaire, évolution à surveiller. Veuillez nous faire parvenir les renseignements cliniques afin que nous les enregistrions dans le dossier biologique	Bêta-2 > 8 g/L

*** Ce bilan complémentaire, décidé par accord entre le biologiste et le prescripteur peut comprendre les analyses précisées dans le texte.

Le dosage de la bêta-2 microglobuline et de la CRP peuvent éventuellement également être rajoutés. L'avantage de ces dosages réside essentiellement dans le suivi de l'évolution de la maladie ou de l'efficacité thérapeutique si un traitement doit être instauré. La bêta-2 microglobuline devra naturellement être interprétée en fonction de la créatinine car ces deux marqueurs sont augmentés dans l'insuffisance rénale. Quant au myélogramme et/ou la biopsie ostéomédullaire, ils sont effectués en fonction, des critères indispensables au diagnostic de la pathologie suspectée.

Tout au long du présent article, nous utilisons volontairement le terme « d'identification immunologique » plutôt qu'immuno-électrophorèse ou immuno-fixation ou immuno-soustraction car, conformément aux recommandations d'un groupe de travail de la Société française de biologie clinique (SFBC) [2], nous laissons à chaque biologiste le soin de choisir la technique la mieux adaptée à sa pratique. Signalons que la technique d'immunosélection est indispensable à mettre en œuvre pour l'identification d'une maladie des chaînes lourdes et que seul un très petit nombre de laboratoires spécialisés sont en mesure de la réaliser.

Les commentaires pour cette fraction gamma utilisent des termes qui sont d'usages parfois ambigus. Cela nous a conduit à en proposer une définition présentée dans le *tableau 5*.

Les hypogammaglobulinémies

Elles peuvent être physiologiques chez le nourrisson. En effet le taux de gammaglobulines dépend de l'âge et les valeurs de l'adulte ne seront atteintes que vers l'adolescence. Elles peuvent révéler des déficits immunitaires primitifs de l'enfant et de l'adulte ou être secondaires aux traitements : corticoïdes, immunosuppresseurs, chimio- et radiothérapies. Mais elles sont aussi révélatrices de certaines pathologies comme le myélome à chaînes légères dont la preuve sera apportée par la caractérisation des chaînes légères libres monoclonales dans les urines ou de manière plus sensible récemment, par le dosage des chaînes légères libres dans le sérum et le rapport kappa/lambda [22]. L'hypogammaglobulinémie permet aussi de définir une situation relativement fréquente correspondant au déficit immunitaire commum variable (DICV) dont l'étiologie doit être systématiquement recherchée.

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Les hypergammaglobulinémies

Elles sont le plus souvent polyclonales accompagnant les pathologies hépatiques, infectieuses, parasitaires ou auto-immunes. Elles peuvent parfois présenter un aspect monoclonal qui est associé aux immunoglobulinopathies malignes telles que le myélome multiple (maladie de Kahler) ou la maladie de Waldenström, l'amylose AL (A pour amylose, L pour *light chain*) ou une hémopathie lymphoïde B. Selon le taux de gammaglobulines, le seuil de détection d'une IgG monoclonale peut varier de 0,2 à 0,75 g/L [23]. Certaines Ig monoclonales peuvent migrer, par électrophorèse capillaire, en dehors de la fenêtre de captation, heureusement très rarement, et ne seront détectées qu'en gel d'agarose [24]. Dans le cadre des immunoglobulinopathies malignes l'électrophorèse est aussi d'un grand intérêt pour la surveillance de l'évolution de la maladie et de l'efficacité thérapeutique. En effet l'intégration du pic étroit permet une quantification plus fiable et plus exacte de l'immunoglobuline monoclonale que le dosage immunologique [2], notamment pour l'isotype IgM et à un moindre degré pour l'IgA et l'IgG. Cet avis, validé par la NACB [5], est unanimement partagé par notre groupe de travail. Certaines immunoglobulinopathies de malignités suspectes mais non confirmées sont étiquetées myélomes indolents. Elles nécessiteront une surveillance rapprochée tous les 2 à 4 mois, en vue de la mise en route du traitement dès que les signes de malignité apparaissent. Certaines peuvent aussi être dites d'accompagnement dans les pathologies systémiques auto-immunes, les hépatopathies chroniques, les infections chroniques et les déficits immunitaires. Plus rarement, l'hypergammaglobulinémie monoclonale peut être associée à une leucémie lymphoïde chronique, un lymphome ou à un cancer épithélial. D'autre part, il est fréquent de découvrir des MGUS chez le sujet âgé notamment à partir de 75 ans. Ces gammapathies de faible taux le plus souvent, ont la capacité de se transformer en gammapathie maligne. Il y a un consensus pour en suivre l'évolution à intervalle régulier de 4 à 6 mois [25].

Les gammaglobulines peuvent présenter des aspects très polymorphes plus ou moins hétérogènes ou plus ou moins oligoclonaux ou pauciclonaux [26] en relation avec de nombreuses pathologies, notamment les syndromes lymphoprolifératifs, les cancers, les maladies auto-immunes, l'amylose, les hépatites virales B et C, les infections à VIH, les infections à virus Epstein-Barr [27]. Les profils oligoclonaux sont fréquents chez les patients infectés par le VIH.

La recherche des profils oligoclonaux peut être aussi utile chez les patients greffés et traités par immunosuppresseurs. Dans ce contexte, la survenue d'un syndrome lymphoprolifératif peut être précocement dépistée, en particulier par l'émergence d'une Ig monoclonale nettement prépondérante dans un contexte de profil oligoclonal post-greffe. Ce constat permet au clinicien de diminuer le traitement immunosuppresseur et d'instaurer en complément un traitement antiviral. Cet ajustement du traitement va permettre de résorber le syndrome lymphoprolifératif qui est réversible dans ce cas particulier pris à un stade précoce [28]. La survenue d'un syndrome lymphoprolifératif peut être décelée précocement par la détection et la surveillance régulière de l'évolution d'un aspect oligoclonal des gammaglobulines [29].

Tableau 5. Définitions des termes concernant l'aspect de la zone des gammaglobulines.

Polyclonales : séparation symétrique et homogène des gammaglobulines comparable à une courbe de Gauss.

Monoclonale : pic étroit correspondant à la synthèse d'un idiotype d'immunoglobuline par un clone de plasmocytes et un seul.

Biclonales : 2 pics étroits correspondant à la synthèse de 2 idiotypes d'immunoglobulines par 2 clones de plasmocytes.

Pluriclonales : plus de 2 pic étroits correspondant à la synthèse de plusieurs idiotypes d'immunoglobulines ou formes moléculaires d'immunoglobulines à différents degrés de polymérisation (ce terme est très peu utilisé dans la littérature et la pratique et n'est pas utilisé dans cet article).

Oligoclonales : plus de 2 pics étroits en général de faible amplitude correspondant à la synthèses de quelques idiotypes d'immunoglobulines par une sélection d'un petit nombre de plasmocytes.

Restriction d'hétérogénéité : la répartition gaussienne des gammaglobulines n'est plus respectée avec perte de symétrie du profil des gamma sans bande étroite. La courbe d'intégration montre plusieurs points d'inflexion. Ce terme est moins souvent utilisé bien que le mieux approprié pour signaler la diminution de la diversité de synthèse des immunoglobulines traduisant une induction de synthèse plus ou moins sélective (par exemple de certaines sous-classes d'IgG).

Homogènes : la répartition gaussienne des gammaglobulines reflète la grande diversité des immunoglobulines qui se répartissent sur une zone relativement large et par conséquent la grande variété des principales classes IgG, IgA et IgM. Ce terme est parfois employé à contre sens pour signaler l'aspect normal des gammaglobulines.

Hétérogènes : la répartition gaussienne des gammaglobulines n'est plus respectée avec plusieurs courbes qui se superposent correspondant à la synthèses de plusieurs familles d'immunoglobulines entraînant une ondulation de la courbe des gammaglobulines. Ce terme est souvent improprement utilisé dans la pratique, c'est pourquoi nous lui avons préféré le terme de restriction d'hétérogénéité tel que recommandé par l'un des relecteurs.

Bloc bêta gamma : fusion des fractions bêta et gamma par remplissage de la vallée entre les bêta et les gammaglobulines généralement par des IgA produites par synthèse hépatique accélérée. Il peut aussi exister des blocs bêta gamma dus à une augmentation polyclonale des sous-classes d'IgG migrant à ce niveau avec une synthèse d'IgA normale dans ce cas). Ce terme encore largement utilisé n'est pas scientifiquement recommandé.

Bande étroite : migration sur une zone réduite des immunoglobulines témoin de l'origine monoclonale de leur synthèse (1 seul isotype de chaîne lourde et légère). Selon les écoles médicales on lui préfère parfois le terme de bande mince. Sur le profil d'enregistrement de l'électrophorèse cette anomalie donne un pic qualifié plus précisément de pic étroit dans le cas de l'électrophorèse capillaire.

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Les textes prêts à l'emploi, associés à la fraction gamma sont présentés sur les *tableaux 6 à 8* en fonction respectivement de l'aspect quantitatif ou qualitatif et de l'évolution de la zone de migration relative aux gammaglobulines.

Discussion

L'électrophorèse des protéines sériques est un examen très ancien dont la pertinence nous semble pouvoir être améliorée. Certains biologistes jugeant en effet cette analyse peu fiable du point de vue quantitatif, ne lui affectent qu'un rôle d'étape de dépistage des gammapathies monoclonales [2, 30, 31]. Cette attitude a été notamment diffusée par les équipes, qui en France, ont développé le profil protéique [32, 33]. Certains biologistes ont réussi cependant à fédérer les deux approches [34] qui peuvent parfaitement être réalisées successivement en fonction des besoins cliniques. D'autres biologistes considèrent que cette analyse manque de spécificité et ne présente donc qu'un faible intérêt pour une interprétation semi-quantitative commentée.

Depuis les années 2000, les techniques sur gel se sont bien perfectionnées et le développement récent des techniques

Tableau 6. Textes prêts à l'emploi concernant la fraction des gammaglobulines pour l'aspect quantitatif des gammaglobulines.

N°	Texte	Argument déclenchant
1	Discrète diminution des gammaglobulines. Évolution à surveiller en fonction du contexte clinique	Gamma < 8 g/L > 5 g/L Âge > 45 ans
2	Augmentation polyclonale modérée des gammaglobulines	Gamma > 15 g/L < 20 g/L
3	Augmentation polyclonale importante des gammaglobulines	Gamma > 20 g/L
4	Très importante diminution des gammaglobulines, l'identification immunologique sérique, le dosage des chaînes légères libres kappa et lambda et l'électrophorèse des protéines urinaires ont été rajoutés en accord avec le prescripteur	Gamma < 5 g/L Âge > 45 ans Hb < 12 g/dL
5	Vallée prononcée entre les fractions bêta et gamma, en faveur d'un déficit en IgA, le dosage spécifique des IgA a été rajouté en cohérence avec le contexte clinique	IgA < 0,5 g/L

Tableau 7. Textes prêts à l'emploi concernant la fraction des gammaglobulines pour l'aspect qualitatif des gammaglobulines.

N°	Texte	Argument déclenchant
1	Profil électrophorétique quantitatif et qualitatif normal : absence de pathologie clonale visible	Gamma > 8 g/L < 15 g/L Courbe de Gauss homogène
2	Aspect de restriction d'hétérogénéité des gammaglobulines. Évolution à surveiller en fonction du contexte clinique	Plusieurs zones plus ou moins visibles ou dissymétrie de la courbe des gamma Gamma > 8 g/L < 15 g/L
3	Aspect de restriction d'hétérogénéité des gammaglobulines qui sont diminuées, évolution à surveiller. Éventuellement, en fonction de la clinique, demander les analyses complémentaires*** en vue du bilan d'immunoglobulinopathie	Gamma <5 g/L Âge > 45 ans
4	Augmentation des gammaglobulines avec aspect oligoclonal à intégrer dans le contexte clinique	Gamma >15 g/L < 20 g/L Plusieurs bandes étroites
6	Profil oligoclonal des immunoglobulines sans augmentation de leur taux. Évolution à surveiller en fonction du contexte clinique	Plusieurs bandes étroites sur un fond d'Ig polyclonales conservées > 11% < 18 %
7	Profil oligoclonal des immunoglobulines sans augmentation de leur concentration. Évolution à surveiller en fonction du contexte clinique	Plusieurs bandes étroites sur un fond d'Ig polyclonales conservées > 8 g/L < 15 g/L
8	Aspect oligoclonal très marqué des gammaglobulines. L' identification immunologique et le bilan complémentaire*** ont été rajoutés en accord avec le prescripteur	Plusieurs bandes minces nettes en gamma sur un fond d'Ig polyclonales diminuées
9	Présence d'une bande étroite d'aspect monoclonal migrant dans la zone gamma. Identification immunologique et bilan complémentaire*** rajoutés en accord avec le prescripteur	Pic étroit suspect inhabituel
10	Profil anormal de l'électrophorèse. Identification immunologique et bilan complémentaire*** rajoutés en accord avec le prescripteur	Hypogamma importante < 5 g/L Bêta ou alpha dissymétriques

*** Ce bilan complémentaire, décidé par accord entre le biologiste et le prescripteur peut comprendre les analyses précisées dans le texte.

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Tableau 8. Textes prêts à l'emploi concernant la fraction des gammaglobulines pour l'évolution des gammaglobulines par comparaison de 2 électrophorèses consécutives d'intervalle rapproché (1 mois en général ou moins en fonction du traitement) dans les pathologies malignes ou espacées (de 4 à 6 mois et plus) dans le cadre d'une surveillance en absence de signe de malignité.

N°	Texte	Argument déclenchant
1	Importante diminution des gammaglobulines, compatible avec l'efficacité du traitement	Diminution de 25 % du taux en 15 jours
2	Importante diminution des gammaglobulines, compatible avec l'efficacité du traitement par plasmaphérèse	Diminution de 25 % du taux en 2 jours
3	Disparition complète du pic monoclonal sur l'électrophorèse, compatible avec l'efficacité de la chimiothérapie	Absence de pic étroit
4	Disparition complète du pic monoclonal sur l'électrophorèse, compatible avec l'efficacité du traitement par greffe de moelle osseuse	Absence de pic étroit
5	Diminution du pic monoclonal depuis l'électrophorèse précédente du jj/mm/aa d'environ XX %	Calcul en % d'évolution du taux du pic
6	Stabilité du pic monoclonal depuis l'électrophorèse précédente du jj/mm/aaaa	Évolution < 10 %
7	Augmentation du pic monoclonal depuis l'électrophorèse précédente du jj/mm/aa d'environ XX %	Calcul en % d'évolution du taux du pic
8	Pic monoclonal déjà identifié. L'identification immunologique ne sera pas refaite *a priori*. Demander le duplicata du résultat de l'identification s'il n'est pas accessible dans le dossier médical du patient	Identification immunologique déjà réalisée profil non modifié
9	Pic monoclonal déjà identifié dans un autre laboratoire. L'identification immunologique ne sera pas refaite *a priori*. Faites nous parvenir un duplicata du résultat en vue du suivi ultérieur de votre patient	Identification immunologique déjà réalisée profil non modifié

d'électrophorèse capillaire a permis de démontrer l'excellente corrélation entre les dosages immunologiques de certaines protéines et les fractions séparées par l'électrophorèse [1, 23]. Ces résultats sont notamment bien vérifiés pour l'albumine et les immunoglobulines polyclonales. Des corrélations satisfaisantes avaient déjà été constatées avec l'électrophorèse sur gel d'agarose et aussi plus anciennement, sur acétate de cellulose. Aujourd'hui l'électrophorèse des protéines sériques doit retrouver sa place parmi les examens utiles, notamment en médecine interne, en cancérologie et en pédiatrie. Cette analyse peut fournir, avec une excellente fiabilité, un nombre important d'informations au clinicien pour un coût raisonnable (B60 soit 16,20 €). Ce faible coût explique-t-il que certains biologiste ne prennent pas toujours le temps de commenter cet examen. Pourtant, afin d'optimiser le rendement de cette analyse, le biologiste doit apporter toute son expertise et accompagner les résultats quantitatifs exprimés en g/L et en %, d'un commentaire le plus explicite possible. Ce commentaire doit être une aide pour le clinicien et pour le patient et non pas source de questionnements inutiles, voire néfastes. Nous pensons, pour notre part, que c'est plutôt la difficulté de produire rapidement un commentaire pertinent qui est la cause d'interprétation non systématique des électrophorèses. C'est précisément pour répondre à cette problématique que nous proposons ce catalogue de textes prêts à l'emploi.

Les *tableaux 9 et 10* permettent de définir les termes quantitatifs attribuables en fonction des deux techniques les plus fréquemment utilisées. Ils doivent être adaptés pour les valeurs de référence de chaque laboratoire.

Bien évidemment, en l'absence de renseignements, le biologiste ne peut pas se passer du dialogue avec le clinicien pour commenter les anomalies majeures qu'il constate sur le profil d'électrophorèse, voire compléter le bilan par d'autres analyses. Pour notre part, nous pensons que le biologiste doit toujours passer par ce contact car la réalisation d'examens complémentaires à sa seule initiative peut se révéler tout à fait inutile et donc coûteuse. D'autres auteurs [35-37] ont suggéré qu'une telle pratique, d'ajout direct d'examens par le biologiste, peut être utile aux patients. Il s'agit, dans ces études, de professionnels exerçant en Grande Bretagne, où les biologistes sont nettement moins nombreux qu'en France et sont presque tous des médecins. D'autre part, l'addition d'analyses complémentaires concernait des pathologies beaucoup plus légères, liées à des carences (anémie ferriprive ou carences vitaminiques) et donc de diagnostic plus simple et aussi plus facilement curables.

Il nous semble que la réalisation par exemple de l'identification immunologique d'une bande étroite doit être faite après concertation avec le prescripteur car le patient peut être déjà connu dans une autre structure de soins, ou bien

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Tableau 9. Seuils de définition des termes quantitatifs pour les résultats sur « Capillarys protéine 6 » pour un taux de protéines sériques de référence de 70 g/L.

Fractions (g/L)	Diminution importante	Diminution	Valeurs de référence	Augmentation	Augmentation importante
Albumine	< 30	30-35	39-46,3	> 50	
Alpha-1	< 1,5	1,5-2	2,1-3,4	4-6	> 6
Alpha-2	< 3	3-4,9	5,0-8,3	9-12	> 12
Bêta-1	< 2	2-3,2	3,3-5,0	6-8	> 8
Bêta-2	< 1,5	1,5-2,1	2,2-4,5	5-8	> 8
Gamma	< 5	5-7,7	7,8-13,2	15-20	> 20

Tableau 10. Seuils de définition des termes quantitatifs pour les résultats sur agarose, valeurs standardisées pour un taux de protéines de 70 g/L.

Fractions (g/L)	Diminution importante	Diminution	Valeurs de référence	Augmentation	Augmentation importante
Albumine	< 30	30-35	38-46	> 50	
Alpha-1	< 0,5	0,5-0,8	0,8-2,3	3-5	> 5
Alpha-2	< 3	3-5	5,8-11	12-15	> 15
Bêta-1	< 3	3-4,5	4,6-8,1	9-12	> 12
Bêta-2	< 1,0	1,0-1,7	1,8-5,0	5-8	> 8
Gamma	< 3	3-4,9	5,0-14	15-20	> 20

le contexte clinique ne va pas modifier la décision médicale par la connaissance des résultats. D'autre part, dans le cas d'une immunoglobulinopathie monoclonale déjà connue ailleurs, la question posée en fait au laboratoire est le suivi de la concentration de l'Ig monoclonale. Celle-ci doit être appréciée par intégration du pic, délimité toujours dans des conditions comparables. De même, dans le cas d'une surveillance d'une MGUS, l'électrophorèse sera le meilleur moyen de suivre l'évolution au fil des années. Une variation est considérée comme significative d'un risque de transformation maligne lorsqu'elle dépasse 25 % sur deux examens consécutifs répétés à trois ou quatre mois d'intervalle [25]. Des travaux récents démontrent l'intérêt d'utiliser aussi le dosage des chaînes légères libres kappa et lambda et du rapport K/L pour la recherche d'une probable immunoglobulinopathie [38], notamment dans les myélomes peu sécrétants, les myélomes à chaînes légères et l'amylose AL [22]. Dans le cas des myélomes à chaînes légères, le dosage des chaînes libres sériques peut avantageusement remplacer l'électrophorèse urinaire avec identification immunologique [22].

Certains commentaires que nous proposons peuvent être générés automatiquement par un SIL à partir de règles d'expertises à établir par le biologiste qui le souhaite. Pour notre part, nous pensons qu'il revient à chaque professionnel de choisir les commentaires qui peuvent être présents dans le dictionnaire des textes codés de son SIL et donc être disponible pour une saisie simplifiée par le biologiste au moment de la validation. Afin de rendre le travail de sélection plus simple, nous proposons que les commentaires soient classés en autant d'axes que de tableaux présentés dans le présent article. Cela permet de décrire l'analyse commentaire comme un groupement contenant chacun des sept items que nous avons retenus. L'initiative appartient entièrement au biologiste, de choisir selon les circonstances le texte prêt à l'emploi ou de créer un texte libre. Cela lui permettra de faire le commentaire le mieux adapté à une situation rare, non prévue dans cet article. En effet, le travail présenté ici ne prétend pas avoir envisagé toutes les circonstances cliniques possibles eu égard à la diversité des pathologies connues ou à découvrir.

Notre but est avant tout de proposer des textes prêts à l'emploi, pertinents et applicables dans la grande majorité des cas rencontrés dans la pratique professionnelle du plus grand nombre de nos confrères.

Conclusion

L'électrophorèse est un examen biologique riche d'enseignements lorsqu'il est prescrit à bon escient et accompagné par les commentaires de biologistes bien formés [13]. Cette interprétation du biologiste est primordiale car son expérience vis-à-vis de cette analyse le place dans une situation privilégiée. En effet, il est amené dans sa pratique professionnelle à interpréter en moyenne 15 à 20 fois plus d'électrophorèses qu'un clinicien. Le but des commentaires est de valoriser le résultat de l'électrophorèse et

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d'en exploiter de manière optimale toutes les informations qualitatives et quantitatives. Cela permet en outre, d'être en conformité avec la NABM (Nomenclature des actes de biologie médicale), le GBEA (Guide de bonne exécution des analyses de biologie médicales) ainsi qu'avec les recommandations de la HAS (Haute autorité de santé) dans le cadre de l'accréditation des établissements de santé. Selon nous, le biologiste sera d'autant plus efficace qu'il utilisera un thésaurus de commentaires prêts à l'emploi et didactiques bien compréhensibles par les cliniciens. Cependant, le biologiste ne doit pas méconnaître les limites de sensibilité et de spécificité de cet examen rappelées dans cet article. Bien évidemment, ces commentaires sont modulables en fonction aussi de la méthode d'électrophorèse mise en œuvre. Il appartient ensuite au clinicien de décider, à partir de ces informations, de la suite à donner à la démarche diagnostique ou de prendre la décision thérapeutique adéquate, en accord avec le patient.

Nous espérons que notre thésaurus sera utilisé, sous cette forme ou une autre, par les biologistes et retiendra également l'intérêt des cliniciens et des patients. Afin de répondre à l'état de l'art, il devra régulièrement être réactualisé en fonction de l'évolution des connaissances scientifiques, techniques et médicales. La première version en a été présentée en 2001 [39]. Sa réactualisation paraissait indispensable à la suite du développement récent important de l'électrophorèse capillaire. En effet, le passage d'une technique en gel d'agarose vers une technique capillaire n'est pas une simple commutation. Par conséquent, les textes prêts à l'emploi présentés ne sont applicables qu'avec les principales nuances détaillées dans cet article. Nous espérons que la qualité et la quantité des commentaires sur les résultats d'électrophorèse va augmenter suite à la publication du présent article ce qui légitimera la démarche entreprise par ce groupe de travail.

Remerciements. Nous remercions la société Sebia pour son assistance scientifique et la mise à notre disposition de l'importante documentation portant sur l'électrophorèse capillaire. Nous remercions aussi le docteur Didier Le Carrer pour ses avis expérimentés et la communication de ses nombreux résultats personnels. Nos remerciements vont aussi à Renaud Laurain pour la traduction anglaise du résumé. Nous remercions profondément les relecteurs de cet article dont les très nombreuses remarques ont démontré l'intérêt et la difficulté ce travail et en ont permis une amélioration importante. Nous remercions notamment le docteur Joseph Watine (hôpital de Rodez) pour l'excellence de ses conseils et les nombreuses références internationales qu'il nous a communiquées pour argumenter et consolider la démarche proposée par notre groupe de travail du CNBH.

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) EGYP 3.0-034
Application Number 11/280,025-Conf. #8408	Filed November 16, 2005
For METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	
Art Unit 1795	Examiner J. M. Dieterle

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
X	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 555.00
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 12-1095 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ attorney or agent of record. Registration Number 46,012

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Diane P. Tso/	December 22, 2009
Signature	Date
Diane P. Tso	(908) 654-5000
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

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Dated: December 22, 2009 Electronic Signature for Diane P. Tso: /Diane P. Tso/

Electronic Patent Application Fee Transmittal

Application Number:	11280025
Filing Date:	16-Nov-2005
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Filer:	Arnold H. Krumholz/Aisha Irvis
Attorney Docket Number:	EGYP 3.0-034

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	2253	1	555	555

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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	2801	1	405	405
		Total in USD ($)		**960**

Electronic Acknowledgement Receipt

EFS ID:	6689576
Application Number:	11280025
International Application Number:	
Confirmation Number:	8408
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Customer Number:	00530
Filer:	Arnold H. Krumholz/Aisha Irvis
Filer Authorized By:	Arnold H. Krumholz
Attorney Docket Number:	EGYP 3.0-034
Receipt Date:	22-DEC-2009
Filing Date:	16-NOV-2005
Time Stamp:	14:58:24
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$960
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Arguments/Remarks Made in an Amendment	Amendment__122209.PDF	70607 a52c2e39f25eeb0f53d57d13dcdc869dfa1b777d	no	15
Warnings:					
Information:					
2	Miscellaneous Incoming Letter	Amendment_Transmittal_122209.PDF	33295 22d1af525d5f133a2b6f16b730d0ecddb74bf1b7	no	1
Warnings:					
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3	Request for Continued Examination (RCE)	EGYP46_-_Request_for_Continued_Examination_Fillable_PDF.PDF	697486 ebfce8e8f09e4198649c3812114d738e5b3ae8e7	no	3
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	Szymanowicz_et_al_Ann_Biol_Clin_2006_644_367-80.PDF	921235 d14ed03708dfea895766a3c62bc2e8fd93401a25	no	14
Warnings:					
Information:					
5	Extension of Time	Three_Month_Extension_Petition_122209.PDF	30546 7ef0ccf9cb242d67542fd9b4f3c29ea15df43902	no	1
Warnings:					
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6	Fee Worksheet (PTO-875)	fee-info.pdf	32323 6cd82057bcc65c726349f66ea723ff7acffb7b02	no	2
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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Dated: December 22, 2009
Electronic Signature for Diane P. Tso: /Diane P. Tso/

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	
Robert et al.	Group Art Unit: 1795
Application No.: 11/280,025	Examiner: J. M. Dieterle
Filed: November 16, 2005	
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	

REQUEST AND AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action dated June 22, 2009, finally rejecting claims 29-51 and 56-57, Applicants have filed concurrently herewith a Request for Continued Examination and submit the following amendments and remarks.

IN THE CLAIMS

1. - 28. cancelled.

29. (currently amended) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electrophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration, and wherein said haemoglobins are a haemoglobin HbA2 and an HbC, an HbD, an HbE, an HbS, an HbF or an HbA haemoglobin.

30. (previously presented) The method according to claim 29, further comprising detecting said haemoglobins.

31. (previously presented) The method according to claim 29, wherein said blood sample is selected from the group consisting of normal blood, abnormal blood, washed blood, decanted blood, centrifuged blood and whole blood.

32. (previously presented) The method according to claim 31, wherein said blood sample is a sample of haemolyzed blood.

33. cancelled.

34. (previously presented) The method according to claim 29, wherein the zwitterionic buffer is formed by one or two molecules and comprises at least one amine function, at least one acid function and at least one hydroxyl function in the position opposite to the acid function.

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35. (previously presented) The method according to claim 29, wherein said analysis buffer is selected from tricine, TAPS, TABS, AMPSO, bicine or HEPBS, or an association of Tris, AMPD or bis Tris propane with an amino acid.

36. (previously presented) The method according to claim 35, wherein said analysis buffer is tricine.

37. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is of an aliphatic or a cyclic diamine or a polyamine type.

38. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of aliphatic diamines, aliphatic polyamines, their acceptable derivatives and salts, and mixtures thereof.

39. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine, spermine, N,N'-dimethyl-1,6-hexanediamine, tetraethylenepentamine, N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

40. (previously presented) The method according to claim 39, wherein said at least one flow inhibitor is selected from the group consisting of 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine and N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

41. (previously presented) The method according to claim 40, wherein said at least one flow inhibitor is 1,4-diaminobutane.

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42. (previously presented) The method according to claim 41, wherein said at least one flow inhibitor is 1,4-diaminobutane in the hydrochloride form.

43. (previously presented) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 20mM to about 500 mM.

44. (previously presented) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 100mM to about 250mM.

45. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.01 mM to about 50 mM in said analysis buffer solution.

46. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.10mM to about 20mM in said analysis buffer solution.

47. (previously presented) The method according to claim 29, wherein said analysis buffer solution further comprises a pH modifier.

48. (previously presented) The method according to claim 47, wherein said pH modifier is selected from the group consisting of lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, mono-alkylammonium hydroxide, di-alkylammonium hydroxide, tri-alkylammonium hydroxide and tetra-alkylammonium hydroxide said alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

49. (previously presented) The method according to claim 29, wherein said capillary tube is naked.

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50. (previously presented) The method according to claim 29, wherein said capillary tube is formed from fused silica.

51. (previously presented) The method according to claim 29, wherein said analysis buffer solution has a pH from about 8 to about 10.

52. cancelled.

53. (withdrawn) A capillary electrophoresis haemoglobin analysis kit comprising at least one analysis buffer containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor.

54. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a pH modifier.

55. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a haemolyzing solution.

56. (previously presented) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said sample being selected from the group consisting of normal blood, abnormal blood, washed blood, decanted blood, centrifuged blood, whole blood and haemolyzed blood, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

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57. (previously presented) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, said flow inhibitor being an aliphatic or a cyclic diamine or a polyamine type, and

electophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

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REMARKS

Claims 29-51 and 56-57 currently stand rejected.

In a sincere effort to expedite prosecution of this patent application toward allowance, Applicants have cancelled claim 33 and have incorporated the language of this claim into claim 29 herein to specify the haemoglobin variants separated according to the method of the present invention. No new matter has been added. Thus, entry of these claim amendments is respectfully requested.

Applicants note that under "Status of the Rejections" on Page 3 of the final Office Action the Examiner indicates that "new grounds of rejection under 35 U.S.C. §102(b) are necessitated by [Applicants'] amendments", however, the only rejections that follow in the Action are made in view of 35 U.S.C. §103(a). As Applicants have addressed each obviousness rejection presented by the Examiner in detail below, Applicants believe that the instant submission is fully responsive to all outstanding rejections. Applicants respectfully request clarification as to any specific rejection of the pending claims that the Examiner may have intended to make under 35 USC §102(b).

REJECTIONS UNDER 35 U.S.C. §103

Rejection of Claims 29-31, 34-36, 43-52 and 56:

Claims 29-31, 34-36, 43-52 and 56 are rejected under 35 U.S.C. §103(a) as being unpatentable over Nouadje *et al.* (US 2002/0162744 A1, hereafter "Nouadje") in view of Alter *et al.* (US 5,753,094, hereafter "Alter"). In response, Applicants respectfully submit the following arguments.

Electrophoresis is a method for separating various protein fractions in a liquid. As far as seric proteins are concerned,

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five zones (six regions when β_1-globulins and β_2-globulins are considered separately) are well known from the usual agarose technique and are still considered in the electrophoretic profiles as usually obtained by capillary electrophoresis (CE). The seric protein profile usually identifies albumin and various globulins, and no haemoglobin variants. (*See, e.g.*, Figure 1, page 368 of Szymanowicz *et al.*, Ann Biol Clin 2006: 64 (4): 367-80; copy attached herein).

Applicants respectfully submit that a person skilled in the art is aware of the fact that seric proteins have physical and chemical properties different from those of the haemoglobins. The respective natures of seric proteins and haemoglobins have an impact on the principle of their separation. For example:

(1) A seric protein differs from the other seric proteins by their entire structure. A seric protein can be shifted and therefore better separated from other seric proteins by using specific additives which interact with specific seric proteins. Any additive changing the ratio charge/weight can be used. Amongst these additives, due to the specific hydrophobicity of a seric protein, additive having a hydrophobic portion can be used (*see* Nouadje).

The analysis of seric proteins is commonly conducted with serum or serum like biological liquid.

(2) Haemoglobin (Hb) is not known as a "seric protein". As already argued, "haemoglobin" is a large group of blood proteins classified as various variants. The variants differ from each other in specific regions of the molecule. Haemoglobins variants are difficult to distinguish from each other because they can vary by only one amino acid, the remaining part of the molecule being exactly the same as in the other variants. The variants are distinguished from each other by their isoelectric point.

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When an additive is added to a blood sample for improving the separation of Hb variants, the variants remain in the same order when separated. In other words, the transformation of the profile is homothetic. To the contrary, the seric protein peaks in an electrophoregram are shifted under the influence of the additives having an influence on the ratio charge/weight, for example additives intended to interact with the hydrophobic portion of the seric protein. Thus, the separation principle is:

- Hydrophobic interaction in the case of seric proteins: the shift of one specific peak corresponding to a specific globulin is the consequence of the use of the additive having a hydrophobic portion.

- Flow inhibition in the case of haemoglobin variants: the resolution is improved as a consequence of the use of a flow inhibitor, which acts on all the variants.

With regard to the specific rejection of claims 29-31, 34-36, 43-52 and 56, it is acknowledged that Nouadje discloses the separation of seric proteins by CE with an additive which reduces the mobility of said proteins (one or more constituent which mobility is reduced compared to the other constituents).

The principle of the separation disclosed by Nouadje is to interact with specific seric proteins, however, not to reduce the flow in order to resolve variants.

The hydrophobic portion of said additives that are capable of interaction with the hydrophobic part of albumin (hereinafter hydrophobic-active additive), can comprise amine functions, which are known not to essentially modify the hydrophobic nature of said portion (*see* Nouadje at [0025]: "…*this hydrophobic portion can comprise residues or functions that do not*

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essentially modify its hydrophobic nature, such as one or more hydroxyl or amine functions, for example.").

Hydroxyl is -OH, amine function is $-NH_2$. The Examiner purports that [0025] refers to cyclic amines. Applicants respectfully submit, however, that [0025] does not refer to cyclic amines, but only refers to the potential presence of $-NH_2$ on the hydrophobic portion of the additive.

Taken together, Applicants respectfully submit herein that Nouadje does not teach the separation of blood samples, but of seric proteins. In addition, Nouadje fails to teach that the resolution of haemoglobin variants can be improved. Furthermore, According to Nouadje, the only additives to be included in the separation buffer are hydrophobic-active additives, pH modifiers (*see* Nouadje at [0042]), and sodium sulphate (*see* Nouadje at [0047]).

With regard to Alter, Applicants submit that Alter discloses a storage buffer or diluent, beside the running buffer (borate). The medicinal sample to be stored or diluted can be blood, however, typically, serum protein electrophoresis or immunosubtraction analysis is conducted after the storage or dilution. No Hb variant resolution is suggested nor carried out according to Alter.

Applicants respectfully submit that Alter fails to teach that a better resolution of haemoglobin variants can be obtained by using any additive or any flow inhibitor.

In addition, a person skilled in the art would understand that in order to analyse Hb, the diluant should contain a surfactant for the lysis of the blood cells or alternatively, have a very low conductivity. If no lysis of blood occurs, no Hb is in solution and as a consequence, the analysis of Hb

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variants is not possible. Applicants respectfully submit that Alter discloses no surfactant. Also, Alter discloses that the diluant conductivity should be the same as the conductivity of the running buffer, and should be high. In addition, the electrophoresis running buffer has a high conductivity (*see* Alter at column 6, lines 26-29). As a consequence, Applicants submit that Alter cannot be considered as adapted for the resolution of Hb variants.

Thus, in contrast to the Examiner's opinion, Applicants respectfully submit that it would not have been obvious to one skilled in the art to modify the method of Nouadje to utilize blood as the biological sample of interest in view of the teachings of Alter and predictably arrive at Applicants' method of Hb variant resolution as claimed in the instant invention. Thus, Applicants respectfully request that the rejection of claims 29-31, 34-36, 43-52 and 56 be withdrawn.

Rejection of Claim 32:

Claim 32 is currently rejected under 35 USC 103(a) as being unpatentable over Nouadje and Alter in view of Mohammad *et al*. (Clinical Application of Capillary Isoelectric Focusing on Fused Silica Capillary for Determination of Hemoglobin Variants, Clinical Chemistry 1997, 43: 1798-1799).

In response, Applicants submit that, for the reasons presented in detail above, Nouadje in view of Alter does not render obvious the invention of the claims from which claim 32 depends, and nothing in Mohammad *et al*. corrects these deficiencies.

Specifically, Applicants respectfully submit that Mohammad relates to the separation of some Hb variants. According to Mohammad, the run buffer consists of ampholine in a methyl

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cellulose solution. The hemolyzed blood is diluted in a solution of methyl cellulose containing ampholine as well as pI markers. The run buffer of Mohammad is therefore different from the buffer according to the present invention.

In addition, Mohammad states that they were "unable to consistently resolve haemoglobin C from A_2 and E" and this is an acknowledged shortcoming of Mohammad's method (*see* Mohammad, at last line of penultimate paragraph, first line of last paragraph). In contrast, the methods of the present invention permit analysis of Hb, and in particular HbA_2. Indeed, Applicants' specification clearly states that the methods of the present invention "can also allow HbA_2 to be quantified even in the presence of HbC or HbE" (published application at [0014]) and this is clearly supported by Applicants' data (*see, e.g.*, Figure 3 which depicts that HbA_2 can be efficiently separated from HbC).

Rejection of Claim 33:

Claim 33 is rejected under 35 USC 103(a) as being unpatentable over Nouadje and Alter in view of Chen (US Patent No. 5,202,006).

As amended herein, claim 33 has been cancelled, and the limitations from this claim have been incorporated into claim 29.

Applicants submit that, for the reasons presented in detail above, Nouadje in view of Alter does not render obvious the invention of claim 29 as amended herein, and nothing in Chen corrects these deficiencies. Specifically, Chen teaches that only poor resolution (separation) of the principal haemoglobin variants have been achieved in the past by prior art methods, and discloses a separation method comprising the use of a buffer

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comprising barbituric acid (*see e.g.,* Chen at column 8, lines 58-68). The haemoglobin variant buffers of Chen, however, do not teach or suggest the zwitteronic buffer disclosed in Applicants' invention.

Applicants respectfully submit that the combination of the elements disclosed in Nouadje, Alter and Chen do not predictably yield the methods of the instant invention, and respectfully request reconsideration and withdrawal of any rejection of the claims of the instant invention based on any combination of these cited references.

Rejection of Claims 37-42 and 57:

Claims 37-42 and 57 are currently rejected under 35 USC §103(a) as being unpatentable over Nouadje and Alter in view of Grover *et al*. (US Patent No. 6,083,372). Specifically, the Examiner alleges that Grover teaches the use of 1,4-diaminobutane as a flow inhibitor in capillary electrophoresis.

In response, Applicants submit that, for the reasons presented in detail above, Nouadje in view of Alter does not render obvious the invention of the claims from which claims 37-42 depend, and nothing in Grover *et al*. corrects these deficiencies. Similarly, for all the reasons presented hereinabove, Applicants also submit that the combination of Nouadje in view of Alter does not render obvious the invention of claim 57, and as nothing in Grover *et al*. corrects these deficiencies, the rejection of this claim should likewise be withdrawn.

Grover relates to electrokinetic chromatography (EKC), and discloses the separation of compounds (phenols, verapamil, metoprolol) which cannot fairly be compared to Hb variants. The

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technical field of Hb variants separation is a specific technical field. In addition, even if Grover generally indicates in the description of the prior art that EKC is a subset of capillary electrophoresis, the focus of the teachings of Grover are directed to EKC. Taken together, there is in Grover no motivation to separate Hb variants, let alone by capillary electrophoresis. Thus, Applicants submit that, in contrast to the Examiner's opinion, it would not have been obvious to one skilled in the art to use 1,4-diaminobutane as a flow inhibitor for separating haemoglobin by capillary electrophoresis as claimed in the instant invention.

Thus, Applicants respectfully submit that the combination of the elements disclosed in Nouadje, Alter and Grover do not predictably yield the methods of the instant invention, and earnestly solicit reconsideration and withdrawal of the rejection of claims 37-42 and 57.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejections of the claims and to pass this application to issue. If, however, for any reason the Examiner does not believe that such action can be taken at this time, it is respectfully requested that she telephone Applicants' attorney at (908) 654-5000 in order to overcome any additional objections which she might have.

Submitted herewith is a Petition for a Three Month Extension of Time, and the Examiner is authorized to charge Deposit Account No. 12-1095 accordingly. If there are any additional charges in connection with this Request and Amendment, the Examiner is authorized to charge Deposit Account No. 12-1095 therefor.

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Dated: December 22, 2009

Respectfully submitted,

Electronic signature: /Diane P. Tso/

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Attorney for Applicants

1086764_1.DOC

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AMENDMENT TRANSMITTAL LETTER

Docket No. EGYP 3.0-034			
Application No. 11/280,025-Conf. #8408	Filing Date November 16, 2005	Examiner J. M. Dieterle	Art Unit 1795

Applicant(s): Frédéric Robert, Denis Simonin, and Jean-Baptiste Clement

Invention: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED

	Claims Remaining After Amendment	Highest Number Previously Paid	Number Extra Claims Present	Rate	
Total Claims	27	- 30 =	0	x 26.00	0.00
Independent Claims	4	- 4 =	0	x 110.00	0.00
Multiple Dependent Claims (check if applicable)	☐				
Other fee (please specify):					
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					0.00

☐ Large Entity ☒ Small Entity

☒ No additional fee is required for this amendment.

☐ Please charge Deposit Account No. _____ in the amount of $ _____ .

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 12-1095 as described below. A duplicate copy of this sheet is enclosed.

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

/Diane P. Tso/ Dated: December 22, 2009

Diane P. Tso
Attorney/Agent Reg. No.: 46,012

LERNER, DAVID, LITTENBERG, KRUMHOLZ & MENTLIK, LLP
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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: December 22, 2009 Electronic Signature for Diane P. Tso: /Diane P. Tso/

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/280,025	11/16/2005	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **04/13/2009**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 28	Minus	** 30	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 4	Minus	***3	= 1	X $110 =	110	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	110	OR	TOTAL ADD'L FEE	

AMENDMENT **12/22/2009**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 27	Minus	** 30	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 4	Minus	*** 4	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/CAROL BARNES/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

530 7590 01/29/2010

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EXAMINER	
DIETERLE, JENNIFER M	

ART UNIT	PAPER NUMBER
1795	

MAIL DATE	DELIVERY MODE
01/29/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/280,025	ROBERT ET AL.	
	Examiner	Art Unit	
	Jennifer Dieterle	1795	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *22 December 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *29-32, 34-51 and 53-57* is/are pending in the application.

4a) Of the above claim(s) *53-55* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *29-32, 34-51, 56 and 57* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

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DETAILED ACTION

Claim Status

Claims 53-55 are withdrawn.

Claims 1-28, 33 and 52 are canceled.

Claims 29-32, 34-51, 56 and 57 are being examined.

Continued Examination Under 37 CFR 1.114

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 12/22/09 has been entered.

Status of the Rejections

2. All rejections from the previous office action are withdrawn. Sunzeri (US 5,536,382 is being cited for the first time.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

3. Claims 29-32, 34-36, 43, 49, 50 and 56 are rejected under 35 U.S.C. 102(b) as being anticipated by Sunzeri (US 5,536,382) with evidence from Camilleri, Capillary Electrophoresis, 1997.

Regarding claims 29-32, 34-36 and 56, Sunzeri teaches a method utilizing capillary electrophoresis for separating and detecting (claim 30) hemoglobin in a lysed (i.e. haemolyzed) blood sample (claims 31 and 32) utilizing a plain capillary and a 50mM tricine buffer (col. 11, lines 25-50, claims 34-36).

Sunzeri teaches that the silica walls, which carry negatively charged silane groups, give a net negative charge to the inner surface and the action of positive counterions will cause the bulk flow of liquid known as electroosmotic flow because the charge shielding produces a cylindrical "shell" of positively charged ions in the electrophoresis medium near the surface wall of the capillaries (col. 1, lines 35-50).

Sunzeri teaches that a quaternary amine (i.e. CTAB) can be used for electroosmotic flow reversal (col. 8, lines 13-15, i.e. giving “flow inhibitor” its broadest reasonable interpretation)(see Camilleri, Capillary Electrophoresis, which evidences that CTAB can be added to a buffer for capillary electrophoresis in order to reverse the charge on the silica capillary surface, thus creating a positively charged surface, see page 371).

Regarding claim 43, Sunzeri teaches utilizing a 50mM tricine buffer (col. 11, lines 25-50, claims 34-36).

Regarding claims 49 and 50, Sunzeri teaches the use of an uncoated fused silica capillary (col. 7, lines 30-35).

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was

not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

4. Claims 37-42, 45, 46 and 57 are rejected under 35 U.S.C. 103(a) as being obvious over Sunzeri (US 5,536,382) in view of Camilleri (Capillary Electrophoresis, 1997, pages 370-371) with evidence from Hempe et al. (*Capillary isoelectric focusing of hemoglobin variants in the pediatric clinical laboratory,* Electrophoresis 1997, 18, 1785-1795).

Regarding claims 37-42, 45, 46 and 57, Sunzeri teaches the use of capillary electrophoresis for the separation and detection of hemoglobin utilizing a zwitterionic buffer and additional additives such as a quaternary amine (i.e. CTAB) that can be used for electroosmotic flow reversal (col. 8, lines 13-15, i.e. giving “flow inhibitor” its broadest reasonable interpretation).

Sunzeri teaches the separation of hemoglobin HA1c. As evidenced by Hempe et al., hemoglobin HA1c has a pI point of 6.935 (page 1794). Given that hemoglobin HA1c has a pI point of 6.935 and Sunzeri uses a zwitterionic buffer with a pH of 7.2, the hemoglobin species will contain a net negative charge and given that fused silica tubes carry negatively charged silane groups, the charge shielding produces a cylindrical "shell" of positively charged ions in the electrophoresis medium near the surface wall (page 3, lines 1-5).

Sunzeri does not specifically teach the use of a flow inhibitor of an aliphatic or a cyclic diamine or a polyamine type (i.e. 1,4 diaminobutane).

Camilleri teaches that capillary electrophoresis can be performed without chemically modifying the capillary using a zwitterionic buffer and additives such as 1,4 diaminobutane, with concentrations of up to 60mM, which will suppress capillary surface interactions (page 370) because the amine will compete with the negatively charged protein for absorption sites on the capillary wall (page 370)(i.e. a hemoglobin species that contains a net negative charge will have the same capillary surface interaction as a negatively charged protein).

Therefore, it would have been obvious to one skilled in the art to modify the buffer of Sunzeri to contain the flow inhibitor 1,4 diaminobutane as taught by Camilleri because the flow inhibitor will function to suppress capillary surface interactions of the negatively charged hemoglobin by competing for absorption sites on the capillary wall thus modifying the charge on the silica capillary surface creating a positively charged surface which will reverse or inhibit electroosmotic flow.

5. Claims 44 and 51 are rejected under 35 U.S.C. 103(a) as being obvious over Sunzeri (US 5,536,382).

Regarding claim 44, Sunzeri teaches the use of 50mM tricine (col. 11, lines 25-50).

In the case where the claimed ranges "overlap or lie inside ranges disclosed by the prior art" a prima facie case of obviousness exists. See *In re Wertheim*, 541 F.2d

257, 191 USPQ 90 (CCPA 1976); *In re Woodruff*, 919 F.2d 1575, 16 USPQ2d 1934 (Fed. Cir. 1990); MPEP 2144.05 I. Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical. "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." See *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955); MPEP 2144.05 II A. Therefore, applicants' note that while tricine concentrations of 100mM to about 250mM are an ideal range, concentration in the range of 20mM to about 500mM will provide effective separation thus indicating that concentration is not critical.

Regarding claim 51, Sunzeri teaches that the buffer has a concentration of about 7.2 (col. 11, lines 40-45).

Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical. "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." See *In re Aller*, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955); MPEP 2144.05 II A. Therefore, since applicants'' claim states "about" 8, a pH of 7.2 would fall generally within the claimed range.

6. Claims 47 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Sunzeri in view of Nouadje et al. (US 2002/0162744 A1).

Regarding claims 47 and 48, Sunzeri teaches that a variety of additives can be added to a buffer in order to achieve the desired ionic strengths of buffers.

Nouadje et al. teaches that the analysis buffer may contain a pH modifying compound selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri-, or tetra- alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion (section 0042).

Therefore, it would have been obvious to one skilled in the art to modify the buffer of Sunzeri to comprise a pH modifier as taught by Nouadje et al. because achieving the optimal isoelectric point of hemoglobin in a buffer solution will affect its charge and its interaction with the capillary surface depending on the separation desired.

Response to Arguments

Applicant's arguments with respect to claims 29-32, 34-51, 56 and 57 have been considered but are moot in view of the new ground(s) of rejection.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Dieterle whose telephone number is (571) 270-7872. The examiner can normally be reached on Monday thru Friday, 8am to 5pm (EST).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Nam Nguyen can be reached on (571) 272-1342. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Nam X Nguyen/
Supervisory Patent Examiner, Art Unit 1753

JMD
1/21/10

Notice of References Cited	Application/Control No. 11/280,025	Applicant(s)/Patent Under Reexamination ROBERT ET AL.	
	Examiner Jennifer Dieterle	Art Unit 1795	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,536,382	07-1996	Sunzeri, Franklin J.	204/451
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Camilleri (Capillary Electrophoresis, 1997, pages 370-371)
	V	Hempe et al. (Capillary isoelectric focusing of hernoglobin variants in the pediatric clinical laboratory, Elecrrophoresis 1997, 18, 1785-1795).
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	1795

SEARCHED

Class	Subclass	Date	Examiner
204	450, 451, 468, 601	12/29/2008	JMD

SEARCH NOTES

Search Notes	Date	Examiner
Inventor Search, Google, NPL	12/29/2008	JMD
EAST, Google	6/10/09	JMD
Consulted with Alex Noguerola, EAST, Google	1/5/10	JMD

INTERFERENCE SEARCH

Class	Subclass	Date	Examiner

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Capillary isoelectric focusing of hemoglobin variants in the pediatric clinical laboratory

We have used capillary isoelectric focusing (cIEF) to diagnose and monitor hemoglobinopathies in children for over three years. This report describes a major revision of our original method (*Clin. Chem.* 1994, *40*, 2288–2295) that improves the analysis of hemoglobin (Hb) variants by cIEF, especially capillary performance and quantitation precision for minor variants. The revised method uses mixed ampholytes (2% pH 6–8:3–10; 10:1), lower viscosity methylcellulose (0.375%) solution, between-sample capillary conditioning with methanol, and hemolysates prepared from red blood cells (RBC) instead of whole blood. Collectively, these changes prolonged capillary life by minimizing capillary exposure to NaOH, standardized the sample matrix, and improved the precision of peak autointegration. The between-run quantitation imprecision (% relative standard deviation) of the revised method was 0.1–3.5% for all diagnostically important major and minor Hb variants present at normal or abnormal levels. The results show the use of the revised method for (i) post-translationally modified Hb present at low concentrations in normal blood, (ii) Hb oxidation products produced by improper sample storage, (iii) differential diagnosis of S/β^+ thalassemia, G-Philadelphia trait, S/C-Harlem disease, and Hb H disease, (iv) sensitive detection of minor variants like Hb A_2' as indicators of an α globin mutation, and (v) neonatal screening using dried blood collected on filter paper. The results show that high-efficiency separation and precise quantitation of Hb variants over a wide range of concentrations makes cIEF a comprehensive assay that can be used without adjunct analyses for the automated primary evaluation of hemoglobinopathies and thalassemias.

1 Introduction

Development of new clinical laboratory methods and devices is prompted by the need for optimal patient care within the cost confinements of the prevailing health care system. In this cost-conscious era of managed care, capillary electrophoresis (CE) is an attractive technological advancement for the clinical laboratory because it can be used for automated, sensitive, and highly discriminatory clinical diagnostics at low cost [1–3]. Perhaps nowhere is the clinical application of CE more practical or the value more straightforward than in the use of capillary isoelectric focusing (cIEF) for the analysis of hemoglobin (Hb) variants [4–9]. The identity and proportion of different Hb variants in human blood is diagnostic of many congenital hemoglobinopathies and thalassemias [10, 11]. For example, the presence of Hb S is used to diagnose sickle cell disease or trait. The proportion of Hb A_2, a minor variant present at low concentrations, is widely used in the diagnosis of thalassemia syndromes, especially those caused by mutations in the β globin gene. Thus even clinical laboratories providing only basic hemoglobinopathy evaluation must discriminate between common and unusual Hb variants and also precisely quantify both major and minor variants that are present at widely different concentrations. Conventional clinical laboratories require four tests to meet these basic needs, usually including alkaline and acid electrophoresis for analysis of major Hb variants, minicolumn chromatography for Hb A_2 quantitation, and alkali denaturation for measuring low levels of Hb F. We have previously shown that cIEF alone can largely replace these four assays because quantitation is precise over the wide range of Hb concentrations needed to quantify Hbs A, F, and A_2, and because high-efficiency separation of structural Hb variants allows positive identification of Hbs S and C without a subsequent confirmatory test [4, 5].

We have now used cIEF in a pediatric clinical laboratory for over three years with excellent diagnostic success. In our original method [4], narrow-range carrier ampholytes (pH 6–8) were used to separate Hb variants in 27 cm DB-1 capillaries (20 cm from the inlet to the detector). Under these conditions, however, a backflush of cathode solution containing NaOH was needed to make Hb A_2 focus anodal to the detector window prior to mobilization. The capillary was also rinsed between samples with 100 mM NaOH to remove adsorbing sample material, but the efficacy of this rinse had not been determined and was not apparent. Both the backflush and the rinse repeatedly exposed the internal coating of the capillary to potentially damaging NaOH and could explain the sometimes erratic performance of the assay and relatively frequent and unexplained capillary failure.

As a concequence, a primary goal of the cIEF method revision described in this report was to limit capillary exposure to NaOH with the expectation of improved capillary life and performance. A second goal was to improve quantitation precision for minor Hb variants; between-run imprecision using our original method was

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Nonstandard abbreviations: **AMC,** carrier ampholyte-methylcellulose; **cIEF,** capillary isoelectric focusing; **Hb,** hemoglobin; **MC,** methylcellulose; **RBC,** red blood cells

Keywords: Hemoglobin / Capillary electrophoresis / Capillary isoelectric focusing / Analytical clinical chemistry / Pediatrics

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9.6% (relative standard deviation, %RSD) for normal blood containing 2.0% Hb A_2 [4]. Through continued use of the assay it became clear that automation of post-analytical data processing had a profound effect on quantitation precision because auto-integration precision was adversely affected by erratic capillary performance and by differences in sample matrix. Method revision, therefore, focused on controlling variables like peak size and shape that markedly affect auto-integration of peak area using preset integration parameters. Because the results of our original method correlated well with conventional clinical methods [4], a further objective throughout these experiments was to obtain quantitative accuracy similar to that of the original method.

2 Materials and methods

2.1 Samples

Whole blood was collected in EDTA from both normal subjects and patients with hematological disorders, and obtained from Children's Hospital Laboratory in accordance with the approved ethical standards of that institution. Whole blood was stable for at least 1 week for Hb variant analysis by cIEF when stored at 4°C. In these experiments, hemolysates were prepared by mixing either 5, 10, or 15 μL of whole blood or 10 μL of packed red blood cells (RBC, 5 min at 1500 × g) with 200 μL of hemolyzing reagent: EDTA, 5 mmol/L; KCN (Aldrich, Milwaukee, WI), 10 mmol/L. RBC hemolysates were prepared by directly pipetting RBC through the plasma layer of centrifuged whole blood and thoroughly rinsing the inside of the pipette tip in the hemolyzing reagent. Some samples containing unusual variants were obtained from the hemoglobinopathy proficiency testing program of the College of American Pathologists (CAP, Northfield, IL). Samples of neonatal blood dried on filter paper were obtained from the newborn screening quality assurance program of the Centers for Disease Control and Prevention (US Department of Health and Human Services, Atlanta, GA). Hemolysates were prepared from dried blood samples by placing two filter spots (1/8 in diameter) into 200 μL of hemolyzing reagent and mixing after 15 min. Using cIEF, lyophilized commercial controls were observed to contain oxidation products that affect quantitation results. Consequently normal and abnormal controls were prepared from fresh RBC and stored as hemolysates in KCN/EDTA at −70°C until use (hemolysates were stable at least 2 years under these conditions; storage of hemolysates at −20°C is not acceptable). Abnormal controls were made by blending hemolysates prepared using blood from two subjects, one with sickle cell disease and elevated Hb F and one with β thalassemia and elevated Hb A_2. Hb C was not included in the abnormal control because it migrates near Hb A_2 and interferes with Hb A_2 quantitation by cIEF.

2.2 Reagents

Unless otherwise noted, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Methylcellulose (MC) solution was prepared by a revision of our previous procedure that appears to improve solubility and results in better batch-to-batch viscosity reproducibility. First, 250 mL of deinonized, distilled water in a glass bottle was heated to 60–70°C in a microwave oven (~90 s). A suspension was prepared by adding 0.9375 g (0.375% w/v) of MC (1500 cp at 20 g/L) to the water and mixing at room temperature (~25°C) on an electromagnetic stirrer. After 5 min, the suspension was placed in ice water with continuous stirring until the MC entered solution and the mixture reached 5–10°C (~30 min). The MC solution was removed from the ice water, stirred until it again reached room temperature, and was then vacuum filtered (0.45 μm pore size filter; Gelman, Ann Arbor, MI). Anode solution was 100 mmol/L H_3PO_4 in 0.375% MC solution. Cathode solution was 20 mmol/L NaOH in deionized, distilled water. Anode and cathode solutions were prepared in 100 mL batches from 5 N H_3PO_4 and 1 N NaOH stock solutions in water, respectively. A stock ampholyte mixture of pH 6–8 and 3–10 carrier ampholytes (10:1 v/v) was prepared by mixing 1 mL of pH 6–8 carrier ampholyte (Pharmalyte) with 0.1 mL of pH 3–10 carrier ampholytes. Carrier ampholyte-MC solution (AMC) was prepared by mixing 2% v/v of the stock carrier ampholyte mixture in MC solution. The anode solution, MC solution, and all ampholyte solutions were stored at 4°C and were used directly from storage without first equilibrating to room temperature. All other reagent solutions were stored at room temperature. All solutions were observed to be stable at least 6 months under these conditions.

2.3 Capillary conditioning

All analyses used 50 μm (ID) × 27 cm total length (20 cm to the detector, total volume of ~530 nL) dimethylpolysiloxane-coated capillaries (DB-1, 0.05 or 0.2 μm coating thickness; J.&W. Scientific, Folsom, CA). Detector windows were prepared on capillaries removed from bulk purchase lots by heating the external coating after first manually filling the capillary with water. New capillaries were rinsed at high pressure (138 kPa) with methanol, H_2O, and AMC for 10 min each. The same rinse sequence was maintained for daily pre-run conditioning using 5 min rinses, and for between-sample conditioning using 1.0, 0.5, and 1.0 min, respectively. After each run, the capillary was rinsed with H_2O for 5 min and stored on the instrument at 20°C with both ends of the capillary in H_2O.

2.4 cIEF analysis

cIEF was conducted with a P/ACE 2200 capillary electrophoresis system equipped with ultraviolet (UV) detector and System Gold software (Beckman Instruments, Fullerton, CA). The instrument was operated with anode and cathode at the inlet and outlet reservoirs, respectively, and with capillary cooling at 20°C. Prior to each assay, the capillary was rinsed at high pressure with AMC solution for 1 min. Hemolysate was then introduced into the capillary by low-pressure injection (3.5 kPa) for 10–30 s (< 50 nL of sample), focused for 5 min at 30 kV (~ 1.5 μA), then mobilized past the detector by simultaneous voltage and low pressure for

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10 min. Between assays, the capillary was rinsed at high pressure with methanol and H_2O as described above. Total assay time including rinses was 18 min.

2.5 Variant identification

Abnormal and normal controls were analyzed at the beginning of each run of samples to assess assay performance. We have consistently observed that between-run migration time variability is greater than within-run variability. Thus the migration times of Hbs A_2, S, F, A, and A_{1c} in the abnormal control were entered into the peak identification and p*I* calculation tables for each run to obtain more precise daily estimates of p*I* in unknown samples. Linear regression was used to calculate p*I* based on the observed migration times and using p*I* of 7.412, 7.210, 7.060, 6.972, and 6.935 for Hbs A_2, S, F, A, and A_{1c}, respectively. We have observed that using Hb A as a reference peak to calculate corrected migration time improves the precision of p*I* estimates. Variants can be identified based on p*I* with high predictive probability if the calculated p*I* equals the expected value ± 0.01 pH units. More precise determination of the p*I* of an unknown variant was achieved by simultaneous cIEF analysis of a mixed hemolysate containing both the abnormal control and the sample containing the unknown. In this configuration, the variants in the abnormal control serve as internal standards and the actual migration times of the known variants can be used to more precisely calculate the p*I* of the unknown peak. Known variants can be excluded from the differential diagnosis of a patient sample if they are resolved from the unknown peak when the patient sample is analyzed simultaneously with a commercial control containing the known variant. Although capillary rinses began 15 min after sample injection, detector output was collected until 15.5 min to permit the detection but not quantitation of major Hb variants with p*I* lower than that of Hb A_{1c}. Evidence of an unusual spike in the capillary rinse would prompt a re-analysis of the unknown sample using a 20 min mobilization step.

2.6 Hb variant quantitation

Quantitation was based on peak detection and integration of absorbance at 415 nm, with values expressed as percent of total Hb (peak area for individual Hb variants divided by total peak area). Peak threshold was preset at 0.004 for the entire analysis. Peak width was set at 0.2 from 0 min until after the migration time of Hb A_2, changed to 0.8 surrounding the migration time of Hb S (to include partially-resolved glycosylated Hb S in the calculation of total Hb S), and reset to 0.25 prior to the migration time of Hb F until the end of the analysis. These integration parameters allowed the detection of all diagnostically important major and minor Hb variants while ignoring minor constituents that are not important for the diagnosis of hemoglobinopathies. The times at which the integration parameters took effect were changed when necessary, based on the migration times of Hb variants in the abnormal control analyzed with each run.

3 Results

3.1 Revised cIEF method

Extensive revision of our original cIEF method included the use of a mixture of narrow- and wide-range carrier ampholytes (2% pH 6–8:3–10; 10:1), 0.375% rather than 0.4% MC solution, removal of MC from the cathode solution, between sample capillary conditioning with methanol, and the use of RBC instead of whole blood to prepare hemolysates. Collectively, these changes eliminated the need for a cathode backflush and limited exposure of the capillary to potentially damaging NaOH. Using mixed ampholytes with 0.4% MC solution excessively narrowed the width of the focusing zone (monitored as Δt_m, the difference in the migration times of Hbs A_2 and A_{1c}) and decreased peak resolution. Lower viscosity 0.375% MC solution restored the size and shape of Hb peaks to that observed with the narrow-range ampholytes in 0.4% MC solution. We have experienced markedly fewer capillary failures since adopting these changes. In addition to improving capillary performance, the revised method also gave results similar to that of the original method. The electropherogram depicted in Fig. 1 shows that despite the many method changes, peak size and shape, the migration times of common variants, and Δt_m in an abnormal control sample were all similar to those previously described [4]. Replicate analysis of 20 abnormal controls each by the original and revised methods showed no differences in the results when compared by paired *t*-test ($P > 0.05$ for all variants).

3.2 Quantitation precision

Although the original and revised methods gave similar quantitative results, the precision of the revised method was markedly better than that reported with the original cIEF procedure. Between-run imprecision measured as %RSD (Table 1) was determined using fifty replicate analyses of the normal and abnormal controls performed

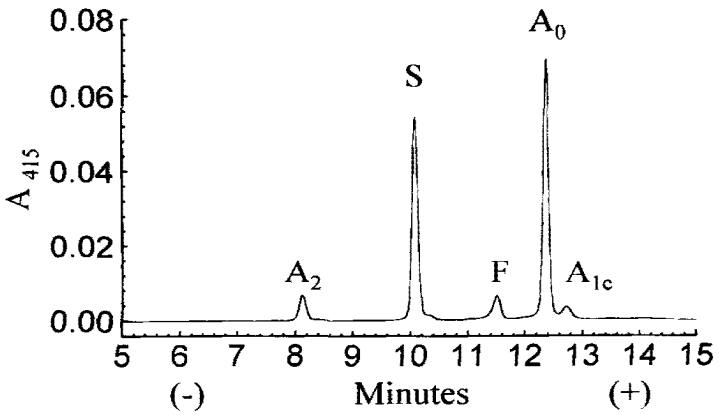


Figure 1. Separation of common Hb variants using the revised cIEF protocol. The sample was an abnormal control formulated to resemble blood from a patient with sickle cell trait, but also with Hb A_2 in the range normally observed in patients with β thalassemia. The control was prepared as an RBC hemolysate and contained elevated amounts of Hb A_2, S, and F and low amounts of Hb A and A_{1c}. This sample was stored at −70°C for over 1 year prior to analysis.

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Table 1. Between-run cIEF precision using abnormal and normal controls[a)]

	Hemoglobin variant					
Quantitation (% of total Hb)	A_2	S	F	A_0	A_{1c}	A_{tot}[b)]
Abnormal control	5.20 (2.3)	40.02 (1.1)	5.85 (2.2)	45.28 (0.8)	3.65 (4.3)	48.93 (0.8)
Normal control	2.74 (3.5)	–	–	89.72 (0.4)	7.54 (4.7)	97.26 (0.1)
Migration time (min)	A_2	S	F	A_0	A_{1c}	Δt_m[c)]
Abnormal control	8.06 (2.4)	10.01 (2.8)	11.42 (2.9)	12.30 (3.0)	12.63 (3.1)	4.56 (4.6)
Normal control	8.10 (2.5)	–	–	12.38 (3.1)	12.73 (3.1)	4.63 (4.7)

a) Values are means (%RSD) determined using control hemolysates stored at −70°C. Each value is based on 50 different analyses performed on 50 different days over a 5-month period using three different DB-1 capillaries and two different lots of methylcellulose solution
b) Total Hb A (Hb A_0 + A_{1c})
c) Indicator of the width of the focusing zone measured as the difference in the migration times of Hb A_2 and A_{1c}

Table 2. Effect of sample matrix on cIEF precision[a)]

		RBC		WB, 5 µL		WB, 10 µL		WB, 15 µL		Wb, 5–15 µL[b)]	
		Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Migration time (t_m)	A_2	7.73	0.63	7.64	0.62	7.71	0.73	7.78	0.96	7.71	1.04
	S	9.60	0.74	9.45	0.68	9.55	0.84	9.64	1.15	9.55	1.21
	F	11.00	0.86	10.73	0.80	10.90	0.93	11.03	1.25	10.89	1.51
	A	11.90	0.83	11.66	0.78	11.80	0.96	11.92	1.27	11.79	1.36
	A_{1c}	12.21	0.88	11.81	0.76	12.09	0.99	12.23	1.29	12.04	1.78
	Δt_m[c)]	4.48	1.36	4.17	1.06	4.38	1.47	4.46	1.89	4.34	3.20
Peak area	A_2	0.316	11.8	0.175	9.0	0.344	7.6	0.515	9.9	0.345	42.0
	S	3.302	12.0	1.757	6.2	3.406	4.6	8.031	6.6	3.398	40.4
	F	0.543	13.2	0.224	15.2	0.459	7.0	0.707	6.6	0.463	43.9
	A	6.792	11.0	3.796	6.4	7.130	4.4	10.265	6.1	7.063	38.4
	A_{1c}	0.538	13.2	0.186	11.3	0.470	7.3	0.768	8.0	0.475	51.4
% of total Hb	A_2	2.75	2.2	2.85	5.8	2.91	3.7	2.97	4.0	2.91	4.8
	S	28.72	1.0	28.63	0.8	28.84	0.7	29.10	0.8	28.86	1.0
	F	4.72	5.8	3.64	11.0	3.89	4.3	4.09	1.6	3.9	8.0
	A	59.13	0.7	61.85	0.8	60.38	0.4	59.40	0.5	60.5	1.8
	A_{1c}	4.68	4.6	3.03	6.1	3.98	3.4	4.44	2.5	3.8	16.0

a) RBC or whole blood (WB, 5, 10, or 15 µL) was added to 200 µL of hemolyzing reagent and analyzed by cIEF. Samples were pressure-injected for 10 (RBC) or 20 (all WB) seconds. Four replicate samples were prepared for each treatment and each replicate was analyzed 3 times for a total of 12 results per treatment
b) Results from samples containing 5, 10, or 15 µL of whole blood were combined (n=36) to show the adverse effect of variable sample volume on precision
c) Indicator of the width of the focusing zone measured as the difference in the migration times of Hb A_2 and A_{1c}

over a five-month period using multiple capillaries and solution lots. The results show that imprecision for quantitation of Hb A_2 using the revised method was between 2 and 3 times lower than that previously reported (6.2% and 9.6% in abnormal and normal controls, respectively). Quantitation precision for the more abundant major variants (Hb A and S, present at concentrations 25–50 times greater than Hb A_2) was similar to that previously observed (1.3% and 1.2% for Hbs A and S, respectively, in the abnormal control). Migration time imprecision for all variants was $\leq$ 3.1%, but the results indicate that migration time was more variable for later-migrating Hbs, *i.e.*, those with lower p*I*, like Hb A_{1c}. Why some variants have more variable migration time is unclear, but could be related to the variable effect of pH on electroendosmotic flow over the pH gradient inside the capillary.

Using RBC instead of a variable amount (5–15 µL) of whole blood to prepare hemolysates also markedly improved cIEF precision (Table 2). Estimates of imprecision (%RSD) for migration times, peak areas, and proportions (%, variant peak area/total peak area) of all variants were lower for the RBC hemolysates. Most importantly, the data show that assay imprecision for Hb A_2 determination was much lower when RBC (2.2%) rather than 5–15 µL of whole blood (4.8%) were used to prepare hemolysates. In clinical application of our original method we could not use a fixed blood:hemolyzing reagent ratio because of the dilution effect of the variable Hb concentrations in blood from anemic and normal patients on peak autointegration efficiency. Instead, we used whole blood in variable blood:hemolyzing reagent ratios to prepare hemolysates with approximately similar Hb concentrations. These results, however, show that sample matrix markedly affected quantitation precision and thus that the true improvement in precision using the revised method with RBC was actually more pronounced than indicated in Table 1 where between-run imprecision was based on replicate analysis of identical hemolysates.

3.3 HB constituents in normal blood

Hb structural variants normally present in blood include Hbs A_2, F, and A, each containing two α globin chains, and two δ, γ, or β globin chains, respectively. However, a wide variety of post-translational modifications of Hb A can be observed in normal blood using sensitive, high-resolution assays like HPLC [12] or cIEF. Figure 2 is an

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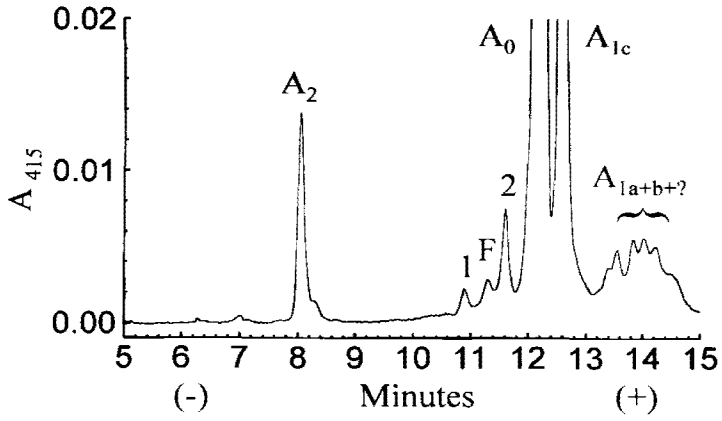


Figure 2. Minor Hb variants in normal blood. Hemolysate prepared from a sample of normal blood was overinjected (30 s) to emphasize the presence of minor constituents. Small peaks with p*I* of 7.12 and 7.04 (labeled 1 and 2 on either side of Hb F, p*I* 7.06) were consistently observed in normal blood and could be mistaken for or included with Hb F by other analytical methods. The poorly resolved group of peaks migrating anodal to Hb A_{1c} consists primarily of post-translationally modified products of Hb A_0, including minor glycosylated Hb A_{1a} and A_{1b}, aging band, and acetylated Hb A. Minor peaks other than Hb A_2, A_2', A_{1c}, F, and F_{ac} are not integrated or typically useful in quantitative assessment for the diagnosis of hemoglobinopathies.

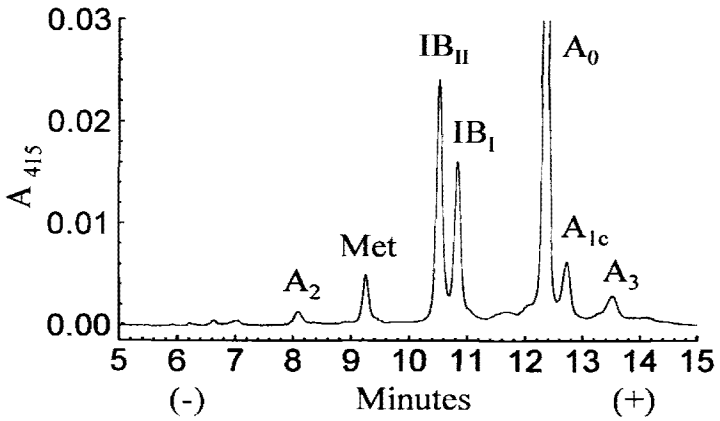


Figure 3. Hemoglobin oxidation products in normal blood. Hemolysates prepared in H_2O from normal RBC were incubated at 37°C for 24 h, then stored at −20°C for one week prior to analysis. The normal Fe oxidation state of Hb A_0 is $\alpha_2^{2+}\beta_2^{2+}$. Large amounts of fully oxidized methemoglobin (Met, $\alpha_2^{3+}\beta_2^{3+}$) and partially oxidized intermediates (IB_I, $\alpha_2^{2+}\beta_2^{3+}$; IB_{II}, $\alpha_2^{3+}\beta_2^{2+}$; the designation IB is derived from the term intermediate band from conventional gel IEF literature) were observed under these conditions. Hb A_3 or "aging band" is a glutathione adduct of Hb A_0 that is typically associated with poor storage conditions. Oxidation products can interfere with peak integration and have different extinction coefficients than Hb in the reduced state. Oxidation products of minor variants like Hb A_2 or A_{1c} also occur but in such low concentrations that they appear only as minor baseline perturbations that are not readily integrated. The presence of oxidation products should be considered grounds for rejection of clinical samples where quantitation is important.

electropherogram of normal blood that emphasizes the presence of ultraminor constituents by sample overload and by narrowing the *y*-axis of the graph. The identities of Hbs A_2, F, A_0 and A_{1c} (a glycosylated form of Hb A) were based on p*I* determination. The other peaks presumably are among the reported complexes of Hb A with a variety of carbohydrates and other compounds that have been closely examined using HPLC [12]. For example, although the exact identities of the poorly resolved minor peaks eluting after Hb A_{1c} are uncertain, the group is presumed to include Hbs A_{1a} and A_{1b} based on the reported focusing patterns of glycosylated Hbs using classical gel IEF [13]. Acetylated Hb A and "aging band" also focus in this pH range [14], but carbamylated Hb A and acetylated Hb F (Hb F_{ac}) partially comigrate with Hb A_{1c}. The identities of the peaks labeled 1 and 2 are unknown, but peak 2 is undoubtedly another glycosylated form of Hb A because the proportion of this minor constituent increased with Hb A_{1c} in diabetic patients. Under normal sample loading and auto-integration conditions, these ultraminor peaks are omitted from the quantitative analysis used to diagnose congenital hemoglobin disorders.

Degradation products were also observed in normal blood, especially in samples subjected to improper storage conditions. For example, the "aging band" typically associated with poor sample storage is a glutathione adduct of Hb A_0 [14] and identified in Fig. 3 as Hb A_3, a designation often seen in the classical gel IEF literature [15–17]. Three of the more stable methemoglobins were also identified by cIEF (Fig. 3). Methemoglobins are formed by the full (Met) or partial (IB_I and IB_{II}) oxidation of the four heme Fe^{++} atoms in Hb A_0 [15, 18, 19]. Methemoglobin formation can be inhibited and/or reversed by including KCN in the hemolyzing reagent. The presence of oxidation products in a sample analyzed by cIEF should be considered evidence of excessive sample degradation and grounds for sample rejection, especially if quantitation of Hb A_2 is important for the diagnosis.

3.4 Differential diagnosis of hemoglobin disorders using cIEF

Unlike conventional clinical assays, cIEF alone provides sufficient data for a comprehensive primary assessment of the more commonly encountered genetic mutations that cause hemoglobinopathies or thalassemias. This is because cIEF quantitation precision is excellent over the wide range necessary to assess both Hbs A and A_2, and because cIEF gives high-efficiency separation of structural Hb variants that is superior to that obtained with conventional electrophoresis. Figure 4 highlights why simultaneous identification and quantitation of both major and minor Hb variants is diagnostically important and a major advantage of cIEF. Hb S/β^+ thalassemia is a double heterozygous condition where one of the two β globin genes produces β^S globin and thus Hb S (hemoglobinopathy mutation), while the other gene produces normal β^A globin and thus Hb A, but at a lower rate (thalassemia mutation) than the wild-type allele. High resolution and identification of the abnormal Hb S structural variant is important for the diagnosis of the hemoglobinopathy mutation, whereas quantitation (Hb S > Hb A, Hb $A_2 > 4\%$) is important for the differential diagnosis of the β^+ thalassemia mutation. Conventional clinical analysis would require at least three separate assays to make the same interpretation.

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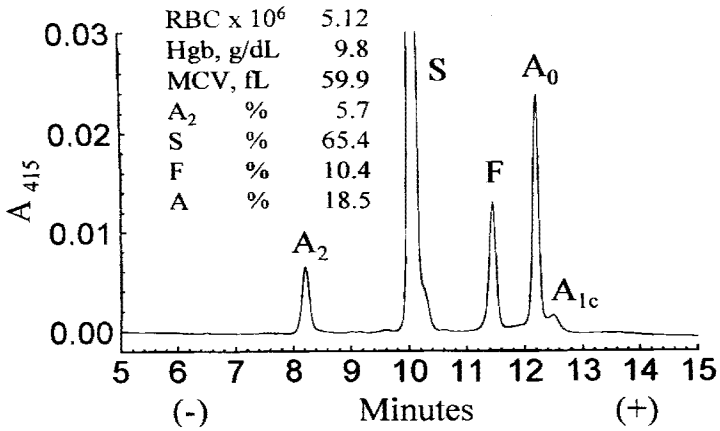


Figure 4. Hb S/β⁺ thalassemia. Both high-efficiency separation for identification of Hb variants and quantitation of major and minor variants are needed for accurate interpretation of the underlying double heterozygous hemoglobinopathy and thalassemia. RBC is the red blood cell count, Hgb is the hemoglobin concentration, and MCV is the mean corpuscular volume obtained by complete blood count (CBC). In this case, elevated Hb A_2 helps differentiate the β⁺ thalassemia syndrome from other potential causes (*e.g.* iron deficiency) of elevated RBC (erythrocytosis), low Hgb (anemia), and low MCV (microcytosis) observed in this patient.

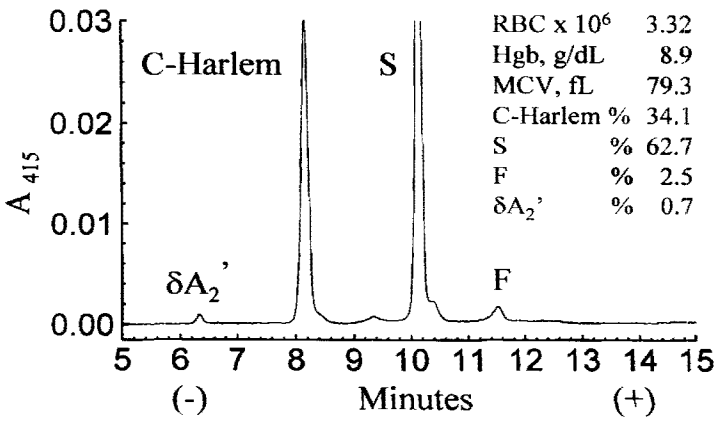


Figure 6. Hemoglobin S/C-Harlem disease. Hb C-Harlem, E, and O-Arab are β globin variants that focus in the same location and cannot be differentiated by cIEF. All three, however, are easily separated from and not confused with Hb C. Migration of these major variants near Hb A_2 interferes with Hb A_2 quantitation for thalassemia evaluation. Also present in this sample is Hb $\delta A_2'$, a δ variant of Hb A_2 that is readily detected by cIEF. RBC is the red blood cell count, Hgb is the hemoglobin concentration, and MCV is the mean corpuscular volume obtained by complete blood count (CBC).

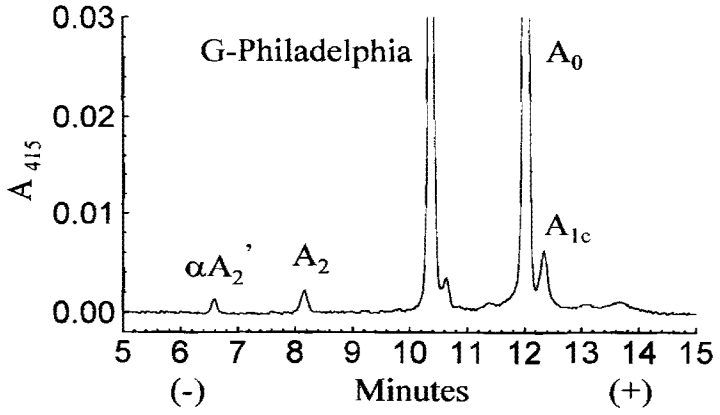


Figure 5. HB G-Philadelphia trait. Hb G-Philadelphia migrates in the vicinity of Hb S but is readily differentiated without further analysis based on calculated p*I* (7.168). The high sensitivity of cIEF also shows the presence of Hb $\alpha A_2'$, an α globin variant of Hb A_2 that helps confirm the presence of an α globin gene mutation.

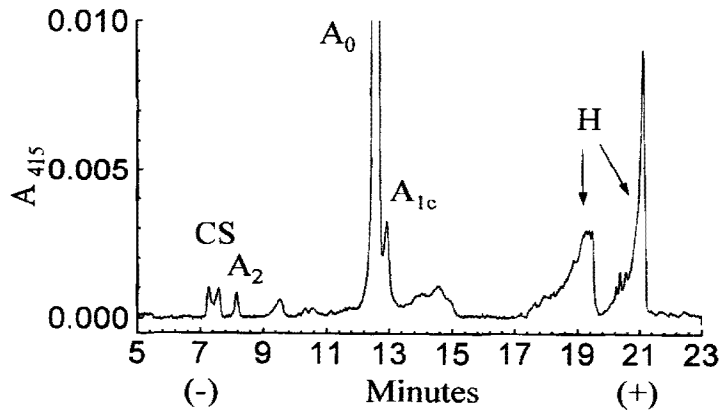


Figure 7. Hemoglobin H disease with Hb Constant Spring. Extending the pressure mobilization step to 20 min permits the identification of Hb variants with lower p*I* like Hb H. The shape of the Hb H peaks shown in the electropherogram is characteristic of that typically observed for these variants on cIEF. The sample also contained Hb Constant Spring and minor oxidation products due to long sample storage prior to analysis.

Another diagnostic advantage of cIEF is the easy identification of minor structural variants of Hb A_2 that help determine the chromosomal location of some hemoglobinopathy mutations. Although most structural hemoglobinopathies arise from mutations in the β globin gene on chromosome 11, there are also a few relatively common mutations in the α globin genes located on chromosome 16 [10, 11]. With cIEF, identification of the major α variant in Fig. 5 as Hb G-Philadelphia can be made with high probability based simply on the calculated p*I* (7.168). The presence of a minor α variant of Hb A_2 ($\alpha A_2'$), however, is further evidence of an α globin mutation and thus increases the confidence of the interpretation. Unlike conventional electrophoresis methods, cIEF is sufficiently sensitive to identify minor variants like Hb $\alpha A_2'$ (~1% of total Hb) with high proficiency. Care must be taken, however, not to confuse Hb $\alpha A_2'$ with Hb $\delta A_2'$, another minor variant that arises from mutations in the δ rather than α globin gene (Fig. 6). Mutations of δ globin are relatively common, occurring in ~2% of persons of African heritage [11].

Figure 6 also highlights other diagnostic benefits as well as limitations of Hb variant analysis by cIEF. Hbs C-Harlem, E, and O-Arab are β globin mutations with similar p*I* that cannot be separated or positively identified by either cIEF or alkaline electrophoresis without confirmation by acid electrophoresis. Hb C, the second most common structural Hb variant after Hb S, is also not separated from these three variants by alkaline electrophoresis, but can be positively identified by cIEF based on calculated p*I* without a subsequent confirma-

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tory test [4]. In the homozygous state or when heterozygous with Hb A, Hb C-Harlem can be differentiated from Hbs E and O-Arab by a positive sickle solubility test. As is the case with the example shown in Fig. 6, however, the sickle solubility test cannot be used to identify Hb C-Harlem in the presence of another sickling Hb like Hb S. The figure also shows that, as with conventional methods, Hbs C-Harlem, E, and O-Arab interfere with quantitation of Hb A_2. Hb C partially comigrates with Hb A_2 and thus also interferes with Hb A_2 quantitation by cIEF [4] as it does with conventional methods for measuring Hb A_2.

Hemoglobin H (Fig. 7) is an unstable tetramer formed by the assembly of four β globin subunits in the absence of sufficient α globin in patients with severe α thalassemia [11]. Hb Bart's (not shown) is the neonatal γ globin equivalent of Hb H and is thus also important for the diagnosis of α thalassemia. Both are typically seen as smears on conventional gel electrophoresis, and appear as similar characteristically uneven peaks with protracted leading edges and sharp trailing edges on cIEF [9]. As shown in the figure, Hb H is sometimes evident as two peaks, perhaps indicating differential tetramer stability in aging samples. In our limited experience using cIEF to analyze these variants, Hbs H and Bart's were indistinguishable and estimates of p*I* were variable. The sample shown in Fig. 7 was shown by other methods to also contain Hb Constant Spring, a minor extended Hb that focuses cathodic to Hb A_2. In this electropherogram two incompletely resolved peaks are present in this region, one of which is presumed to be Hb Constant Spring. The other may be an oxidation product of either Hb A or H since small amounts of other methemoglobins due to long storage of the refrigerated whole blood are also apparent.

Hb variants with low p*I* like Hb H present a diagnostic dilemma for the routine use of cIEF. As shown in Fig. 7, the mobilization step of the revised cIEF assay can be extended to 20 min to permit identification of diagnostic Hb variants with low p*I*. Using the longer mobilization step for all routine samples, however, would add a prohibitive 10 min to each assay. We therefore adopted a compromise strategy that used the more cost-effective and shorter mobilization step, but only detected low p*I* Hb variants if they were present at higher concentrations. This was accomplished by continuing to collect detector output after beginning the between-sample capillary rinse with methanol; under these conditions Hbs N-Baltimore or H present at > 25% of total Hb were readily apparent on the electropherogram as unusual spikes. Note, however, that this strategy proved ineffective for Hb H present at low concentrations (< 5%) because the variant was not detected during rapid transit through the detector.

3.5 Neonatal screening

Common Hb variants were readily identified by cIEF in blood samples dried on filter paper for neonatal hemoglobinopathy screening (Fig. 8). Compared to fresh blood, baseline variation was extensive in hemolysates prepared from filter paper (probably due to extensive

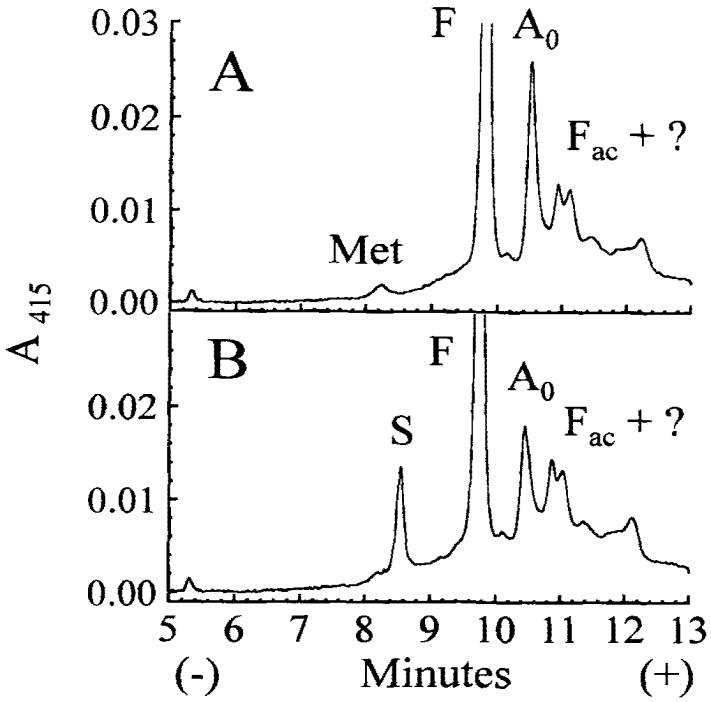


Figure 8. Neonatal screening by cIEF. Hemolysates prepared from whole blood dried on filter paper for a neonatal screening proficiency testing program were analyzed by cIEF. Hbs F, A, and S were readily identified in samples from a normal infant (A) or one with sickle cell trait (B) despite extensive baseline variability that is probably attributable to oxidation of samples prepared and stored in this manner.

sample degradation) but did not affect the identification of Hbs S or A even at the low levels present in neonates (~5–15%). Automated p*I* calculation facilitated the identification of uncommon Hb variants, but, more importantly, also helped discriminate between Hb S and methemoglobin that may be present at > 10% in severely degraded samples. These results show that cIEF can be used for neonatal hemoglobinopathy screening but will require method or instrumental advances (*e.g.*, development of a multi-channel cIEF analyzer) to cost-effectively increase sample throughput to accommodate the large number of neonatal samples processed by mass-screening programs.

3.6 Assay cost

The following parameters were used to calculate direct laboratory costs for quantitation of Hb variants by cIEF using the methods described above. Labor: salary, $35 000/year; fringe benefits, 24%; h/year, 2080; min/day for assay setup and instrument maintenance, 45; min/sample for data entry and sample preparation, 5. Instrument: purchase price, $50 000 (approximate list price of a current model CE instrument) amortized over 5 years, maintenance contract, $3000/year. Consumables: actual costs were used for reagents, nitrogen, and capillaries (with a conservative estimate of 250 analyses/capillary). Results are based on analyzing ten patient samples with two controls and 5% repeats three times a week. Under these conditions, the cost of the assay is estimated to be $12.05 with 30% of the cost attributable to labor, 1% to materials, and 69% to instrument purchase

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and maintenance. Because instrument cost is fixed and a high proportion of the assay cost, greater sample throughput would markedly decrease assay cost/sample. Potential throughput using the revised method in an 8 h day is approximately 20 samples, but considerably more if overnight analyses are performed unattended.

4 Discussion

These results further demonstrate the utility of Hb variant analysis by cIEF for the primary assessment of hemoglobinopathies and thalassemias in the clinical laboratory. High efficiency separation and precise quantitation over a wide range of hemoglobin concentrations make cIEF a comprehensive assay that can replace multiple conventional assays. The revised method described in this report decreases erratic capillary performance and capillary failure. The method also markedly improves quantitation precision for minor variants like Hb A_2, primarily by optimizing assay conditions to maximize signal-to-noise ratios while also promoting uniform peak size and shape between samples for more precise peak auto-integration.

4.1 Hb variant quantitation precision

Precise quantitation is most critical for minor Hb variants because the proportion of Hb A_2, along with evidence of anemia, microcytosis, and erythrocytosis, is widely used to help diagnose thalassemia syndromes. Because cIEF is highly sensitive, however, information is also available about other minor variants that are not relevant to the diagnosis of congenital hemoglobin disorders. As was shown in Fig. 2, even normal blood contains minor constituents that are not detected or reported by conventional methods. We have observed that using integration parameters that include these non-diagnostic minor peaks in the determination of total peak area did not improve quantitation precision. Rather, the excessive information simply increased the amount of post-analytical data reduction required to present the data in a format recognizable by the clinician. All experiments to optimize quantitation precision, therefore, used peak detection/integration parameters (see Section 2.6) that included only diagnostically important major and minor Hb variants in the estimate of total peak area.

Automation of post-analytical data processing is important for clinical diagnostics because it reduces labor costs and avoids potential operator bias or error. It is important to note, however, that automation of peak detection and integration also imposes limits on cIEF quantitation precision. This is because auto-integration depends on preset peak width and threshold parameters that are used in software algorithms to measure peak areas. Optimal inegration parameters differ for peaks of different sizes and shapes, and thus must be predetermined for different regions of the electropherogram depending on the size and shape of the typical Hb variant of interest that focuses in that region. Unfortunately, significant sample-to-sample variation in peak size or shape from that of the template peak markedly affects the accuracy and precision of peak area auto-integration. This is especially true for smaller peaks because a low signal-to-noise ratio (*i.e.* low detector response relative to baseline variation) makes it difficult for the algorithm to reproducibly detect the leading and trailing edges of a peak. Consequently, the precision of peak auto-integration and therefore Hb variant quantitation by cIEF can be improved primarily by optimizing assay variables that (i) maximize the signal-to-noise ratio of minor variants, or (ii) promote relatively uniform peak size and shape between samples.

One way to increase the signal-to-noise ratio for Hb A_2 would be to increase the amount of sample injected into the capillary. Sample loading is limited, however, by the high relative concentration of Hb A and the limited linear range of the UV detector. Hb A affects the amount of sample and Hb A_2 that can be loaded because clinical quantitation of Hb variants is expressed in proportions determined by dividing the calculated area of each individual variant peak by the total area of all Hb peaks. Underestimation of Hb A peak area when the peak height exceeds the limit of detector linearity also results in underestimation of total peak area and thus overestimation of the percentage of Hb A_2. We evaluated this effect using well-characterized controls and observed that the proportion of Hb A_2 was indeed erroneously elevated when Hb A peak height exceeded the detector limit (0.2 AU) of the Beckman P/ACE. Normal blood contains approximately 98% Hb A and 2% Hb A_2. Thus when the height of the Hb A peak approaches 0.2 AU, the height of the Hb A_2 peak is only about 0.004 AU. This approximately represents the optimal Hb A_2 signal without sample overload, and typically resulted in an acceptable signal to noise ratio of about 50 using normal blood.

Because of the limiting effect of Hb A on sample loading, the only way to optimize the Hb A_2 signal-to-noise ratio was to maximize the size of the Hb A peak without exceeding the detector limit. At the same time, however, it was also important to maintain a relatively uniform peak size and shape between samples to further optimize auto-integration precision. Consistent sample loading could not be achieved using whole blood to prepare hemolysates because of the variable Hb concentration of blood from normal and anemic subjects, and because of sample matrix effects on auto-integration when variable ratios of blood hemolyzing reagent were used. These problems were minimized in the revised method, however, by using RBC to prepare sample hemolysates with consistent Hb concentrations and more similar sample composition. The extra time required to centrifuge the samples to obtain the RBC was minimal and well worth the added labor cost for the improved precision.

4.2 Capillary performance

It should be emphasized that cIEF quantitation precision was also markedly affected by capillary performance. Long-term correlation of Hb A_2 quantitation with likely indicators of assay performance using well-characterized controls eventually showed that deviations from the expected mean Hb A_2 level were almost always asso-

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ciated with deviations in Δt_m. Furthermore, Hb A_2 was underestimated compared to the established mean when Δt_m was wider than usually observed, and overestimated when Δt_m was more narrow. The apparent inverse relationship between the proportion of Hb A_2 and Δt_m was attributable to the effects of variable peak width on the auto-integration of minor peaks. We observed, for example, that as Δt_m increased, peak width also increased because each protein eluted over a longer period. Under these conditions, however, preset integration parameters optimized for a narrower template peak tended to underestimate peak area. Through experience it became increasingly clear that Δt_m was an excellent indicator of assay performance because it was also an excellent indicator of capillary performance in cIEF.

Using Δt_m to monitor performance, we found that remarkable changes in Δt_m and the estimated proportion of Hb A_2 were almost always attributable to deteriorating capillary performance and were usually restored by replacing the capillary. Instrument problems were relatively uncommon and usually due to low coolant level or pump malfunction, leading to poor control of capillary temperature and fluid viscosity. Reagents prepared and stored as described above were never identified as a source of Δt_m variability. We considered that variable Δt_m might be a characteristic peculiar to the DB-1 capillary, but pilot studies to evaluate other commercially available capillaries revealed similar Δt_m variability. We chose therefore, to optimize capillary conditioning for reproducible Δt_m using the much less expensive DB-1 capillary.

It seemed likely that variable Δt_m and deteriorating capillary performance using our original method were attributable to capillary exposure to potentially damaging NaOH and/or inappropriate capillary conditioning. Adding wider-range ampholytes to the pH 6–8 carrier ampholytes pushed the focus point of Hb A_2 further from the detector window and eliminated the need for a backflush of cathode solution. Using methanol instead of NaOH for between-sample capillary conditioning also reduced capillary exposure to high pH conditions. Unfortunately, it was not practical or cost-effective to comprehensively evaluate the effects of either NaOH or methanol on capillary performance. However, our records clearly indicated fewer capillary failures and more consistent Δt_m after instituting these changes. Note that although the capillary conditioning used in the revised method inproves capillary performance, variation in Δt_m between new capillaries or for the same capillary on different days was reduced but not eliminated. Also, mild migration time drift within a run of samples was still observed, and resulted in slightly increased Δt_m during the run that, for unclear reasons, returned to normal by the next scheduled run.

4.3 Clinical application of cIEF

Routine application of cIEF for the analysis of Hb variants in our pediatric clinical laboratory included the analysis of a normal and abnormal control to assess analytical run suitability. Hb variant quantitation and Δt_m for the controls were used as criteria of acceptable performance if within predetermined ranges (means ± 2 SD). Each sample report and electropherogram were also subjected to performance checks based on (i) acceptable Δt_m, (ii) all peak heights < 0.2 AU, (iii) typical integration of minor and major peaks, and (iv) the absence of unusual spikes in the methanol rinse. The rare presence of a spike possibly representing a low p*I* variant in the methanol rinse was addressed by reanalyzing the sample using a longer (20 min) mobilization step. Note, however, that anomalous spikes in the capillary rinse were not uncommon and longer analyses for all samples were not cost-effective. Prior notification that a sample was suspected to contain Hb H or Bart's based on clinical indications would save time and improve the probability of an accurate diagnosis of these rare conditions. The presence of oxidation peaks was infrequent, but, when encountered, was cause for sample rejection.

Identification of a cIEF peak as a common variant, including Hb A_2', C, A_2, S, F, A, A_{1c}, or F_{ac}, was routinely accepted without further evaluation if the observed quantity was within the expected range for that variant and if the p*I* was within ± 0.01 pH units of the expected value (Table 3). To comply with regulatory guidelines, a sickle solubility test was performed to verify the presence of Hb S in all new sickle cell disease or trait patient samples, but in our experience has been completely redundant and unnecessary. To make the data resemble that reported by conventional methods, the proportions of Hb A and A_{1c} were combined to compute total Hb A in the final report. Similarly, the amounts of Hb F and F_{ac} (usually present at ~10% of Hb F) were also combined in the absence of Hb A. When Hb A was present, Hb F_{ac} partially comigrated with Hb A_{1c} and could not be measured separately. Under these circumstances, Hb F_{ac} was included in the estimate of total Hb A because the amount of Hb F_{ac} was not important for an accurate interpretation of the results. As with conventional methods, Hb A_2 was not resolved from Hb C and was included with the estimate of Hb C where applicable.

The presence of an uncommon variant in a sample was indicated by the presence of a major or minor cIEF peak with a p*I* different from that of the common variants listed above. The need for a confirmatory analysis to positively identify the variant depended on which Hb variant was encountered. For example, the calculated p*I* of an unusual peak was first compared to those previously determined by cIEF in our laboratory using either verified controls, CAP proficiency samples, or patient samples with results confirmed by a reference laboratory. Our current p*I* library (Table 3) contains 21 Hb variants and six post-translational products of Hb A or F. We have had sufficient experience with Hb D-Punjab and G-Philadelphia to indentify these variants in an unknown sample based on p*I* without a confirmatory analysis. We have also encountered Hb C-Harlem, E, and O-Arab enough to report a probable identification based on the presence of a major variant with a p*I* of 7.40 ± 0.01. In these cases, a positive sickling test in the absence of Hb S confirms the presence of Hb C-Harlem. Tentative identification of an uncommon Hb variant not included in the p*I* library can sometimes be made by

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Table 3. Library of Hb variants analyzed by cIEF[a)]

Variant	pI	%[b)]	Comment
$\delta A_2'$	7.624	Minor	δ variant present in ~2% of persons of African descent
$\alpha A_2'$ (G-Philadelphia)	7.597	Minor	p*I* of α variants of Hb A_2 will vary
Constant Spring	7.487	Minor	Second peak with p*I* 7.459 observed in only sample analyzed that also contained Hb H
C	7.445	Major	Interferes with Hb A_2 quantitation, resolved from Hbs C-Harlem, E, and O-Arab
A_2	7.412	Minor	Increases in most β thalassemias
E	7.405	Major	Interferes with Hb A_2 quantitation, cannot be differentiated from Hb O-Arab
O-Arab	7.405	Major	Interferes with Hb A_2 quantitation, cannot be differentiated from Hb E
C-Harlem	7.401	Major	Interferes with Hb A_2 quantitation, differentiated from Hbs E and O-Arab by positive sickling test if Hb S not present
Methemoglobin	7.295	nd	Fully oxidized hemoglobin suppressed by KCN in hemolyzing reagent
Zurich	7.236	Major	
S	7.210	Major	Most common structural Hb variant
D-Punjab	7.182	Major	Peak resolved from Hb S in mixed sample
G-Philadelphia	7.168	Major	Peak resolved from Hb S in mixed sample, Hb $\alpha A_2'$ also present
Lepore	7.165	Major	Unusually broad peak
IB_{II}	7.162	nd	Methemoglobin intermediate suppressed by KCN in hemolyzing reagent
P-Galveston	7.137	Major	
IB_I	7.131	nd	Methemoglobin intermediate suppressed by KCN in hemolyzing reagent
F	7.060	Variable	Normally not detected unless > 0.5%, high in neonates and hereditary persistence of fetal Hb
Koln (3)	6.996	Major/ Minor	Multiple smaller peaks (p*I* 7.428, 7.410), interferes with Hb A_2 quantitation
A (A_0)	6.972	Major	Normal adult Hb
A_{1c}	6.935	Minor	Labile A_{1c} sometimes evident as shoulder with lower p*I*
F_{ac}	6.935	Minor	Present at ~10% of Hb F level, interferes with Hb A_{1c} if Hb A is present
A_3	6.854	nd	Aging band due to poor sample storage
Malmo	6.844	Major	High oxygen affinity
I	6.614	Major	Peak partially resolved from Hb N-Baltimore in a mixed sample
N-Baltimore	6.544	Major	
H	6.058	Variable	Present as asymmetric peak or peaks with variable p*I*

a) The p*I* of Hb variants other than Hb A_2, S, F, A, and A_{1c} were determined where possible by simultaneous analysis of a sample containing the variant listed with an abnormal control as described in the text. Samples were verified commercial controls, CAP proficiency samples, or patient samples with results verified by a commercial reference laboratory

b) Designation: minor, < 10%; major, ususally > 25%; variable, 0–100%; nd, not detected under normal conditions

comparing relative focusing positions on cIEF to published maps of Hb variants analyzed by conventional IEF [15–17] or alkaline electrophoresis [10].

Clearly, an exact and more comprehensive p*I* library is both practical and highly desirable. Comparable estimates of p*I* for uncommon variants can be obtained from any laboratory by simultaneous analysis of a control containing Hb A_2, S, F, A, and A_{1c} as internal standards (using the same p*I* listed in this report), and a sample containing a variant with undetermined p*I*. With a programmable CE, mixing the two samples at the time of injection can be automated, and the observed migration times of the known variants can be used to exactly determine the p*I* of the uncommon variant. Unlike migration or elution times, p*I* determined in this way is a measurable parameter of variant identification that is relatively universal between laboratories and instruments. This potential for automation and demystification of Hb variant identification, especially at the primary analytical level, is a strong argument for widespread clinical application of cIEF.

The quality of Hb variant analysis by cIEF should continue to improve with continued method development. Creation of an extensive p*I* library, and perhaps the use of external p*I* markers, would facilitate identification of Hb variants based on p*I*. Better capillary performance may follow the development of new capillaries with more stable coatings, but more likely will result from better capillary conditioning protocols that further minimize Δt_m variability. Faster assays may be possible if capillary temperature, solution viscosity, and/or ampholyte range and concentration are optimized. Advances in instrument technology can also be expected to improve cIEF analysis of Hb variants, especially through better control of hydrodynamic mobilization, improved detection with extended linear range and lower noise, and software modifications specific for clinical application. Rather than wait for the development of a dedicated commercial analyzer for cIEF analysis of Hb variants, however, we suggest that hospital laboratories with good special chemistry sections may wish to adopt the revised method described in this report as a comprehensive method for the primary assessment of hemoglobinopathies and thalassemias.

Received April 8, 1997

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EAST Search History

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S7	21	cermet with (pd or palladium and pt or platinum and alumina) with (sinter$5 or vitrif $5 or calcif$5)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:25
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S13	100	S12 and (pd or palladium)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:36
S14	90	S12 and ((pd or palladium) and (alumina or zirconia or yttria or lanthana))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:37
S15	11	S12 and ((pd or palladium) with (lead or via or pad or resistor or plane))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:38
S16	1848	((pd or palladium) with (thick near5 film))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:51
S17	2	((pd or palladium) with (thick near5 film) with (sinter or calcify or vitrify))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/13 16:52
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S20	1	"12035206"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 10:25
S21	0	hemaglobin with (zwitterionic or amine or hydroxyl or tricine or taps or tabs or hepbs or bicine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 10:30
S22	284	hemoglobin with (zwitterionic or amine or hydroxyl or tricine or taps or tabs or hepbs or bicine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 10:30

S23	19	hemoglobin with (zwitterionic or tricine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:02
S24	6	hemoglobin with (zwitterionic or tricine) and electrophores$5	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:12
S25	780	(sickle near3 cell near3 anemia) with (test$5 or detect$5 or sensor)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:21
S26	779	(sickle near cell near anemia) with (test$5 or detect$5 or sensor)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:21
S27	852	(sickle near cell near anemia) with ((apparatus or device or sensor) near$5 (test $5 or detect$5))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:22
S28	477	S27 and electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:22
S29	117	S27 and (capillary near5 electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:22
S30	3	S29 and (tricine or zwitterionic)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 11:22
S31	379	(serum near protein) with hemoglobin	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:04
S32	304	(serum adj protein) with hemoglobin	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:04
S33	1	((serum adj protein) with hemoglobin) and electrphoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:04
S34	75	((serum adj protein) with hemoglobin) and capillary	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:09
S35	233	hematocrit and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:22
S36	0	hematocrit with (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 12:22
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S41	2	"20060234379"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 14:10
S42	15462	microfluidic and (gate or channel or branch)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 14:12
S43	269	microfluidic and (gate or channel or branch) and (particle adj sort $5)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 14:13
S44	6	microfluidic and (gate or channel or branch) and (particle adj sort $5) and (field adj cage)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/19 14:20

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S8	1	(control near3 (circuit or unit)) near5 (disconnect$5 or disengag$5) near5 electrolyte	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 12:52
S9	1	(control near3 (circuit or unit)) near5 (disconnect$5 or disengag$5) near5 (electrolyte or "gas sensing")	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 12:52
S10	1	("gas sensor") near5 (disconnect$5 or disengag $5) near5 (electrolyte or "gas sensing")	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 12:53
S11	0	("gas sensor") near5 (disconnect$5 or disengag $5) near5 (electrolyte or "gas sensing") near5 (control or unit)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 12:53

S12	13	(disconnect$5 or disengag $5) near5 (electrolyte or "gas sensing") near5 (control or unit)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 12:54
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S20	2	(hemoglobin or heamoglobin) with "flow inhibitor"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:08
S21	2	(hemoglobin or heamoglobin) with ("flow inhibitor" or cyclic adj amine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:08
S22	3	"11280025"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:08
S23	344	(hemoglobin or heamoglobin) with ("flow inhibitor" or 1,4-diaminobutane or 1,5-diaminopentane or1,6-diaminohexane or diethylenetriamine or N,N,N',N'-tetramethyl-1,4-butanediamine or 1,4-diaminobutane hydrochloride or tricine or amine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:10

S24	5	S23 and "capillary electrophoresis"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:10
S25	172	(hemoglobin or heamoglobin) with ("flow inhibitor" or 1,4-diaminobutane or 1,5-diaminopentane or1,6-diaminohexane or diethylenetriamine or N,N,N',N'-tetramethyl-1,4-butanediamine or 1,4-diaminobutane hydrochloride or tricine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:11
S26	168	(hemoglobin or heamoglobin) with ("flow inhibitor" or 1,4-diaminobutane or 1,5-diaminopentane or1,6-diaminohexane or diethylenetriamine or N,N,N',N'-tetramethyl-1,4-butanediamine or 1,4-diaminobutane hydrochloride)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:11
S27	37	S26 and electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:11
S28	10	(capillary near2 electrophoresis) with ("flow inhibitor" or 1,4-diaminobutane or 1,5-diaminopentane or1,6-diaminohexane or diethylenetriamine or N,N,N',N'-tetramethyl-1,4-butanediamine or 1,4-diaminobutane hydrochloride)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:15
S29	2	(hemoglobin or heamoglobin) with (zwitterionic or tricine) with "capillary electrophoresis"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:30
S30	2	(hemoglobin or heamoglobin) with (zwitterionic or tricine) with electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/01/07 14:30
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Library of Congress Cataloging-in-Publication Data

Capillary Electrophoresis / edited by Patrick Camilleri. — 2nd. ed.
p. cm. — (New directions in organic and biological chemistry)
Includes bibliographical references and index.
ISBN 0-8493-9127-X (alk. paper)
1. Capillary electrophoresis. I. Camilleri, Patrick. II. Series.
[DNLM: 1. Hepatitis B virus. QW 710 G289h]
QP519.9.C36C373 1997
543'.0871—dc21

97-22491
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International Standard Book Number 0-8493-9127-X
Library of Congress Card Number 97-22491
Printed in the United States of America 2 3 4 5 6 7 8 9 0
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Methylcellulose	Hjerten	*J. Chromatogr.* 347, 191 (1985)
Polyacrylamide (through Si-O-Si-C bonds)	Hjerten	*J. Chromatogr.* 347, 191 (1985)
Polyacrylamide (through Si-C bonds)	Novotny	*Anal. Chem.* 62, 2478 (1990)
3-glycidoxypropyltrimethoxysilane	Jorgenson	*Science* 222, 266 (1983)
Epoxy-diol, maltose	Poppe	*J. Chromatogr.* 480, 339 (1989)
Polyethyleneglycol	Poppe	*J. Chromatogr.* 471, 429 (1988)
Polyvinylpyrrolidone	McCormick	*Anal. Chem.* 60, 2322 (1988)
Aryl-pentafluoro(aminopropyltrimethoxy)silane	Swerdberg	*Anal. Biochem.* 185, 51 (1990)
α-lactalbumin	Swerdberg	*J. High Res. Chromatogr.* 14, 65 (1991)
Polyether	El Rassi	*J. Chromatogr.* 559, 367 (1991)
Hydrophilic, C1, C8, C18	Dougherty	*Supelco Reporter*, Vol. X, No. 3 (1991)
Ion exchangers, polyacrylamide	Engelhardt	*J. Microcol. Sep.* 3, 491 (1991)
Neutral, hydrophilic	Karger	*J. Chromatogr.* 652, 149 (1993)
Hydrophilic & hydrophilic polymers	Lee	*Anal. Chem.* 65, 2747 (1993)

Hence, the separation of protein species varying only in subtle structural differences would be difficult, if not impossible, to achieve.

If the optimum for the separation is found at around neutral pH conditions, strategies other than the extreme pH approach should be considered. One of these is to coat the capillary surface either dynamically (using the rinse capability of the CE instrument) or covalently (by using chemical derivatization procedures prior to inserting the capillary in the instrument). The goal of these procedures is to minimize, eliminate, or reverse the surface charge on the capillary wall.

Covalent coating is generally performed by attaching hydrophilic polymers to the silanol groups on the surface. Robust and reproducible covalent coatings are often not easily made in the laboratory. However, nowadays several companies offer such coated capillaries.[5] They are typically developed for specific applications and are usable in a given pH range. Table 8.1 lists a selection of some of the coatings which have appeared in the CE literature.[6]

Instead of chemically modifying the capillary surface to avoid adsorption of the proteins, one can perform a "dynamic" coating by adding various substances to the electrophoresis buffer while using the rinse cycles of the CE instrument to equilibrate the columns. One simple way of doing this is to increase the electrophoresis buffer concentration or to add various salts to the buffer.[6,17] As the salt and protein compete for adsorption sites on the capillary wall, the higher the salt-to-protein ratio, the higher the probability of salt adsorption. A drawback of this approach is that the higher the salt concentration, the higher the conductivity of the buffer, and therefore the higher the power induction (at a constant applied voltage) and temperature increase in the capillary tube. This is especially pronounced in CE instruments lacking the ability to adequately remove Joule heating, i.e., with simple configurations based on ambient air-cooling of the capillaries.

The power induction can be greatly reduced by using zwitterionic salts such as tricine, CAPS, CHES, and betaine instead of ionic salts such as phosphate and borate.[18] The zwitterions yield very low conductivity, and therefore they may be used at rather high concentrations (up to more than 1 *M*, depending on their solubility) without causing high power inductions. As buffer components, zwitterions are frequently used in FSCE.

Cationic amines such as 1,4-diaminobutane (DAB) and 1,5-diaminopentane (DAP) have also been used to suppress protein-capillary surface interactions at concentrations up to 60 m*M*.[6] As mentioned above for the "high salt" approach, the idea is that the amine competes with the protein for adsorption sites on the capillary wall. The larger the protein, the higher the concentration of cationic amine must be in order to obtain the desired effect.

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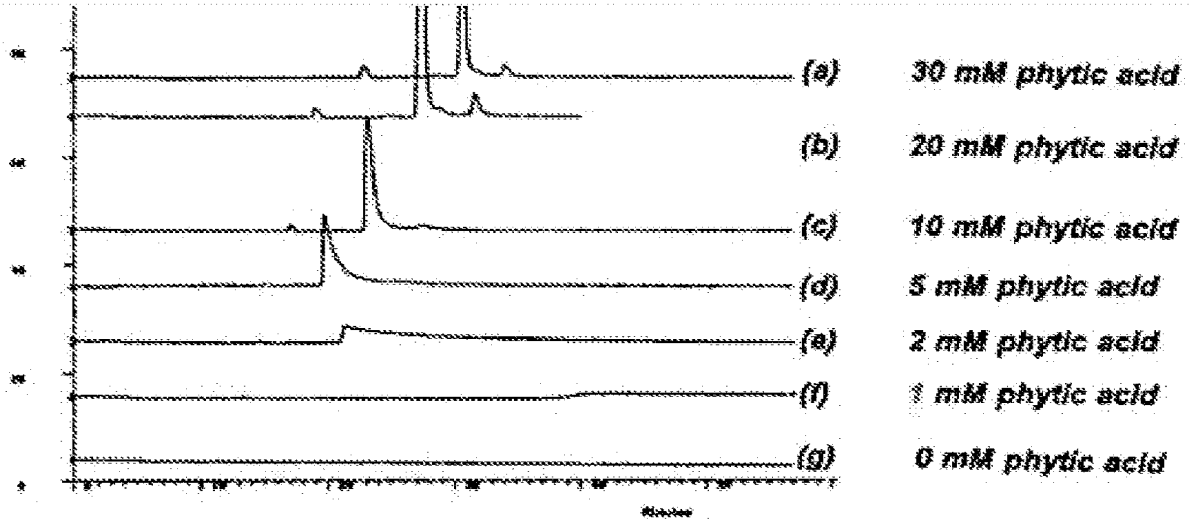


Figure 8.4 FSCE analysis of aprotinin by the use of phytic acid as ion-pairing reagent. Analysis was performed in 25 m*M* phosphate buffer at pH 7.0 with increasing amounts of phytic acid (0, 1, 2, 5, 10, 20, 30 m*M*, from bottom to top) added to the buffer. (From Okafo, G. N., et al., *Electrophoresis*, 16, 1917, 1995. With permission.)

Various cellulose derivatives (e.g., methylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose — generally added at concentrations below 1%), polyethylene oxides, and ethyleneglycol (10 to 30%) decrease the zeta potential (i.e., a measure of the electric potential at the shear plane set up in the surface region of the electrical double layer) on the surface of the capillary wall. These additives also increase the viscosity of the buffer, thereby decreasing the tendency for protein adsorption.

Protein adsorption may also be reduced by addition of cationic surfactants such as cetyltrimethylammonium bromide (CTAB) to the buffer. Increasing the concentration first reduces, then eliminates, and finally reverses the charge on the capillary surface, thus creating a positively charged surface (the latter condition also causes a reversal of the EOF).

Finally, one can perform ion-pairing CE in order to suppress or eliminate protein adsorption. The anionic ion-pairing reagent, which is added to the buffer, reacts with cationic sites on the protein molecule, thus making it electrophoretically net negatively charged. An example of that approach is shown in Figure 8.4,[38] using phytic acid as the ion-pairing reagent. This molecule contains six phosphate groups with pK_a values in the range of 1.9 to 9.5, which allows one to manipulate its charge over a wide pH range. Aprotinin is a highly basic protein (pI ~ 10.5, MW 6511 Daltons, 58 amino acid residues). Initial FSCE analysis of aprotinin in a 25 m*M* phosphate buffer at pH 7.0 resulted in the protein being totally adsorbed onto the capillary surface. When phytic acid was added to the buffer, aprotinin eluted as a distinct peak — first severely tailed, but with increasing phytic acid concentrations the aprotinin peak became more symmetrical, even separating several aprotinin variants.

Ion pairing with phytic acid can also be used to optimize separation of peptides and predict their basic nature.[39] This is illustrated by the following example: Aprotinin was unfolded, reduced, pyridylethylated, and finally digested with endoproteinase Lys-c. In this way the expected five peptide fragments were formed: fragment 1, RNNFK (pI ~ 11.4); fragment 2, SAEDCMRTCGGA (pI ~ 4.4); fragment 3, AGLCQTVYGGCRAK (pI ~ 9.2); fragment 4, ARIIRYFYNAK (pI ~ 11.3); fragment 5, RPDFCLEPPYTGPCK (pI ~ 5.9). The digest was subjected to CE analysis in a 100 m*M* sodium phosphate buffer (pH 7.0) with varying concentrations of the dodecasodium salt of phytic acid. The mobilities of fragments 1, 4, and 3 (all with basic pI values) decreased with increasing concentration of phytic acid,

AMENDMENT TRANSMITTAL LETTER

Docket No. EGYP 3.0-034			
Application No. 11/280,025-Conf. #8408	Filing Date November 16, 2005	Examiner J. M. Dieterle	Art Unit 1795

Applicant(s): Frédéric Robert, Denis Simonin, and Jean-Baptiste Clement

Invention: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED

	Claims Remaining After Amendment	Highest Number Previously Paid	Number Extra Claims Present	Rate	
Total Claims	27	- 30 =	0	x 26.00	0.00
Independent Claims	4	- 4 =	0	x 110.00	0.00
Multiple Dependent Claims (check if applicable)	☐				
Other fee (please specify):					
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					0.00

☐ Large Entity ☒ Small Entity

☒ No additional fee is required for this amendment.

☐ Please charge Deposit Account No. _____ in the amount of $ _____ .

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 12-1095 as described below. A duplicate copy of this sheet is enclosed.

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

/Diane P. Tso/ Dated: July 21, 2010

Diane P. Tso
Attorney/Agent Reg. No.: 46,012

LERNER, DAVID, LITTENBERG, KRUMHOLZ & MENTLIK, LLP
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(908) 654-5000

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Dated: July 21, 2010 Electronic Signature for Diane P. Tso: /Diane P. Tso/

PTO/SB/22 (07-09)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) EGYP 3.0-034
Application Number 11/280,025-Conf. #8408	Filed November 16, 2005
For METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	
Art Unit 1795	Examiner J. M. Dieterle

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
X	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 555.00
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 12-1095 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ attorney or agent of record. Registration Number 46,012

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Diane P. Tso/	July 21, 2010
Signature	Date
Diane P. Tso	(908) 654-5000
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☐ Total of 1 forms are submitted.

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Dated: July 21, 2010 Electronic Signature for Diane P. Tso: /Diane P. Tso/

Electronic Patent Application Fee Transmittal

Application Number:	11280025
Filing Date:	16-Nov-2005
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Filer:	Robert B. Cohen/Sharon Mcgee
Attorney Docket Number:	EGYP 3.0-034

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	2253	1	555	555

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Page 1003-0448

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**555**

Electronic Acknowledgement Receipt

EFS ID:	8059171
Application Number:	11280025
International Application Number:	
Confirmation Number:	8408
Title of Invention:	Method for analyzing haemoglobin by capillary electrophoresis, a kit for capillary electrophoresis, and use of a flow inhibitor in said method
First Named Inventor/Applicant Name:	Frederic Robert
Customer Number:	00530
Filer:	Robert B. Cohen/Sharon Mcgee
Filer Authorized By:	Robert B. Cohen
Attorney Docket Number:	EGYP 3.0-034
Receipt Date:	21-JUL-2010
Filing Date:	16-NOV-2005
Time Stamp:	12:59:36
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$555
RAM confirmation Number	8252
Deposit Account	121095
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Arguments/Remarks Made in an Amendment	Amendment_72110.pdf	64311 323898cc598c861928656658d640f7fcfad764a9	no	14
Warnings:					
Information:					
2	Miscellaneous Incoming Letter	Amendment_Transmittal_72110.pdf	33287 c60d089c31faf18112fc9a255041bdddbc5eafaa	no	1
Warnings:					
Information:					
3	Extension of Time	Three_Month_Extension_Petition_72110.pdf	30540 07bba79c6b8797eda203af66999b3f9089aefca2	no	1
Warnings:					
Information:					
4	Fee Worksheet (PTO-875)	fee-info.pdf	30340 7a8452c7c56af425e1efbadac2cfd4245dfbf3f6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	158478		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: July 21, 2010
Electronic Signature for Diane P. Tso: /Diane P. Tso/

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	
Robert et al.	Confirmation No. 8408
Application No.: 11/280,025	Group Art Unit: 1795
Filed: November 16, 2005	Examiner: J. M. Dieterle
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT

Dear Sir:

In response to the Official Action mailed January 29, 2010, Applicants submit the following amendments and remarks.

IN THE CLAIMS

1. - 28. cancelled.

29. (previously presented) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electrophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration, and wherein said haemoglobins are a haemoglobin HbA2 and an HbC, an HbD, an HbE, an HbS, an HbF or an HbA haemoglobin.

30. (previously presented) The method according to claim 29, further comprising detecting said haemoglobins.

31. (previously presented) The method according to claim 29, wherein said blood sample is selected from the group consisting of normal blood, abnormal blood, washed blood, decanted blood, centrifuged blood and whole blood.

32. (previously presented) The method according to claim 31, wherein said blood sample is a sample of haemolyzed blood.

33. cancelled.

34. (previously presented) The method according to claim 29, wherein the zwitterionic buffer is formed by one or two molecules and comprises at least one amine function, at least one acid function and at least one hydroxyl function in the position opposite to the acid function.

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35. (previously presented) The method according to claim 29, wherein said analysis buffer is selected from tricine, TAPS, TABS, AMPSO, bicine or HEPBS, or an association of Tris, AMPD or bis Tris propane with an amino acid.

36. (previously presented) The method according to claim 35, wherein said analysis buffer is tricine.

37. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is of an aliphatic or a cyclic diamine or a polyamine type.

38. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of aliphatic diamines, aliphatic polyamines, their acceptable derivatives and salts, and mixtures thereof.

39. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor is selected from the group consisting of 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine, spermine, N,N'-dimethyl-1,6-hexanediamine, tetraethylenepentamine, N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

40. (previously presented) The method according to claim 39, wherein said at least one flow inhibitor is selected from the group consisting of 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, diethylenetriamine and N,N,N',N'-tetramethyl-1,4-butanediamine, their acceptable derivatives and salts, and mixtures thereof.

41. (previously presented) The method according to claim 40, wherein said at least one flow inhibitor is 1,4-diaminobutane.

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42. (previously presented) The method according to claim 41, wherein said at least one flow inhibitor is 1,4-diaminobutane in the hydrochloride form.

43. (previously presented) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 20mM to about 500 mM.

44. (previously presented) The method according to claim 29, wherein said analysis buffer has a concentration in said analysis buffer solution from about 100mM to about 250mM.

45. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.01 mM to about 50 mM in said analysis buffer solution.

46. (previously presented) The method according to claim 29, wherein said at least one flow inhibitor has a concentration from about 0.10mM to about 20mM in said analysis buffer solution.

47. (previously presented) The method according to claim 29, wherein said analysis buffer solution further comprises a pH modifier.

48. (previously presented) The method according to claim 47, wherein said pH modifier is selected from the group consisting of lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, mono-alkylammonium hydroxide, di-alkylammonium hydroxide, tri-alkylammonium hydroxide and tetra-alkylammonium hydroxide said alkylammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

49. (previously presented) The method according to claim 29, wherein said capillary tube is naked.

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50. (previously presented) The method according to claim 29, wherein said capillary tube is formed from fused silica.

51. (previously presented) The method according to claim 29, wherein said analysis buffer solution has a pH from about 8 to about 10.

52. cancelled.

53. (withdrawn) A capillary electrophoresis haemoglobin analysis kit comprising at least one analysis buffer containing at least one zwitterionic type analysis buffer or a zwitterionic buffer and at least one flow inhibitor.

54. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a pH modifier.

55. (withdrawn) The capillary electrophoresis haemoglobin analysis kit according to claim 53, further comprising a haemolyzing solution.

56. (currently amended) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said sample being selected from the group consisting of normal blood, abnormal blood, washed blood, decanted blood, centrifuged blood, whole blood and haemolyzed blood, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, and

electrophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

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57. (currently amended) A free solution capillary electrophoresis method for separating haemoglobins in a biological sample by capillary electrophoresis, said biological sample being a blood sample, said method comprising:

introducing said biological sample into a capillary tube comprising a solution of an analysis buffer, wherein said analysis buffer is of the zwitterionic type and at least one flow inhibitor, said flow inhibitor being an aliphatic or a cyclic diamine or a polyamine type, and

electrophoresing said biological sample in said analysis buffer and at least one said flow inhibitor, whereby said haemoglobins are separated by migration.

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REMARKS

Applicants acknowledge with appreciation the Examiner's withdrawal of all rejections from the previous Office Action.

Claims 29-32, 34-51, 56 and 57 currently stand rejected.

Claims 56 and 57 are currently amended herein to correct typographical errors. No new matter has been added. Entry of these claim amendments is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 102(b)

Claims 29-32, 34-36, 43, 49, 50 and 56 are rejected under 35 U.S.C. § 102(b) as being anticipated by Sunzeri (US 5,536,382, hereafter "Sunzeri") with evidence from Camilleri, Capillary Electrophoresis, 1997 (hereafter "Camilleri"). Specifically, with regard to the rejection of claims 29-32, 34-36 and 56, the Examiner has cited *Sunzeri* at col. 11, lines 25-50 as allegedly teaching a method utilizing capillary electrophoresis for separating and detecting hemoglobin in a lysed (*i.e.*, hemolysed) blood sample utilizing a plain capillary and a 50mM tricine buffer. *Sunzeri* is also cited for teaching use of 50mM tricine buffer (claim 43) and use of an uncoated fused silica capillary (claims 49, 50). These rejections are traversed for the reasons stated below.

Applicants respectfully submit that *Sunzeri* teaches a method for the detection of an analyte of interest in a biological sample by mixing the sample with a labeled reagent ("binding pair member") which specifically binds the analyte, and then employing capillary electrophoresis to resolve the bound from unbound reagent. The sample constituents are then quantitated by measuring directly or indirectly the amount of bound reagent. *Sunzeri* at col. 2, lines 24-29. *See also Sunzeri*, col. 2, lines 39-40 which teaches that "[c]apillary electrophoresis is . . . used to resolve bound, *e.g.* reagent

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displaced from its normal migration position, from unbound reagent, and the constituents detected." As disclosed in *Sunzeri*, the analyte may be any analyte having a binding site for a specific binding pair member, but preferably binding pairs of labeled antibodies as detector molecules and antigen complexes are used. *Sunzeri* at col. 2, lines 64-66. Quantitation of the analyte (antigen) will depend on the ability to resolve uncomplexed antibody from antibody-antigen complex. *Sunzeri* at col. 3, lines 7-9.

The text in *Sunzeri* at col. 11, lines 25-50 cited by the Examiner describes the detection of glycosylated hemoglobin (hemoglobin A1c) in human blood. Specifically, *Sunzeri* teaches that in a first step, a blood sample is centrifuged to separate the red cells, which are then lysed, and diluted. A labeled antibody mouse anti-human hemoglobin A1c, which is specific for hemoglobin A1c, is added to the cell lysate, and then incubated overnight. (Applicants note that as taught at col. 6, lines 34-36, the incubation period should be sufficient to allow the complementary binding pair members to form complexes.) Incubated samples are resolved by capillary electrophoresis and distinct fluorescent peaks corresponding to antibody and antibody-antigen complexes are detected.

Applicants respectfully submit that *Sunzeri* does not anticipate the claims of the present invention. *Sunzeri* clearly teaches the detection of a single specific analyte using a labeled reagent which specifically binds the analyte of interest. The particular example referred to by the Examiner describes the detection of a single hemoglobin variant. In contrast, the claims of the present invention are directed to separating multiple hemoglobin variants in a biological sample using capillary electrophoresis. Applicants respectfully submit that *Sunzeri* clearly does not teach such separation method. Indeed, the only separation taught in *Sunzeri* is the resolution

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of bound from unbound reagent; thus, in the cited example, *Sunzeri* only teaches the separation of mouse anti-human hemoglobin A1c monoclonal antibody-hemoglobin A1c complexes from unbound antibody.

Moreover, Applicants respectfully point out that *Sunzeri's* method only permits the analysis of a single constituent in a biological fluid per electrophoretic run; the detection and separation of more than one analyte-reagent complex is not taught. In contrast, Applicants' invention successfully achieves the separation of multiple hemoglobin variants in a single electrophoretic run, *i.e.*, hemoglobins HbA2, HbC, HbD, HbE, HbS, HbF and HbA. This separation of multiple hemoglobin variants is clearly depicted in Figures 1-7 of the instant application. *See, e.g.*, Applicants published application Pub. No. US 2006/0102478 A1.

Applicants respectfully submit that one of skill in the art would easily recognize the significant differences which distinguish the teachings of Sunzeri from Applicants' invention. First, *Sunzeri's* multi-step method requires that "the reaction mix of sample, detector and optionally competitor will be incubated for a period of time sufficient to allow the complementary binding pair members to form complexes." *Sunzeri* at col. 6, lines 34-37. In contrast, Applicants' methods may be carried out in a single step; notably, the present invention does not require performing an incubation step during which the hemoglobin variants are bound to a labelled reagent (*e.g.*, antibody). Rather, the methods of the present invention can be carried out directly on the biological sample to be analyzed, *e.g.*, simply using washed, decanted, centrifuged or whole blood samples. *See, e.g.*, Applicants published application Pub. No. US 2006/0102478 A1 at [0030], [0046] and [0056].

Furthermore, Applicants note that according to *Sunzeri*, the labelled bound reagent is separated from labeled unbound reagent

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because the bound reagent is shifted or displaced from its normal migration position (*i.e.*, preferential electrophoretic mobility). *See Sunzeri*, col. 2, lines 39-40. In contrast, the methods of the instant invention comprise the use of an analysis buffer of the zwitterionic type which comprises at least one flow inhibitor. The flow inhibitor is not specific for a particular analyte or a complex to be separated, but interacts with the capillary, thus *reducing* the electroosmotic flow (EOF) and displacing each compound present in the capillary in an homothetic manner, *i.e.*, the variants remain in the same order when separated.

Applicants take this opportunity to emphasize that the present invention comprises the use of an analysis buffer of the zwitterionic type which comprises at least one flow inhibitor which is used to *decrease* the charge density on the capillary surface, and thus *reduce* the EOF. In support of the rejection of the claims under § 102 (b), however, the Examiner alleges that *Sunzeri* discusses the concept of EOF and teaches that the quaternary amine, CTAB, can be used for *EOF reversal*. The Examiner relies on *Camilleri* for evidence that CTAB can be added to capillary electrophoresis buffer in order to *reverse* the charge on the silica capillary surface, thus creating a positively charged surface. Based on these teachings, and by "giving 'flow inhibitor' its broadest reasonable interpretation", the Examiner apparently concludes that because CTAB can be used for EOF reversal, *Sunzeri* and *Camilleri* teach the use of CTAB as a *flow inhibitor in capillary electrophoresis*. *See* Office Action at page 3. Applicants respectfully disagree with the Examiner's conclusion for the reasons stated below.

Applicants submit that *reversal* of EOF by CTAB as disclosed in the prior art is not the same as *reduction* of EOF, and thus the art does not in fact teach or even suggest the use of CTAB

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as a flow inhibitor such as described in the methods of the present invention. Specifically, *Camilleri* teaches that one can reduce the protein adsorption on a capillary surface by increasing the concentration of CTAB in the buffer used during capillary electrophoresis. The addition of CTAB causes a reversal of charge on the capillary surface, thus creating a *positively* charged surface, which causes a reversal of the EOF. As disclosed by Applicants, however, the flow inhibitor is used to *decrease* the charge density, and thus *reduce* the EOF - *not to reverse EOF*. Indeed, Applicants submit that hemoglobin variants have isoelectric points between about 6 and 7.7; thus at alkaline pH, hemoglobins are *negatively charged, as is the capillary surface*. If the capillary surface is positively charged, which is the case when reversal is caused, no separation would occur because of the interaction between the positively charged capillary surface and the negatively charged proteins.

In view of the above, Applicants respectfully submit that *Sunzeri* alone, or in combination with *Camilleri*, does not anticipate the methods of the present invention. Thus, reconsideration and withdrawal of the rejection of claims 29-32, 34-36, 43, 49, 50 and 56 under 35 U.S.C. § 102(b) is earnestly solicited.

REJECTIONS UNDER 35 U.S.C § 103

Rejection of Claims 37-42, 45, 46 and 57:

Claims 37-42, 45, 46 and 57 are rejected under 35 U.S.C. § 103(a) as being obvious over *Sunzeri* in view of *Camilleri* with evidence from Hempe et al. (*Capillary isoelectric focusing of hemoglobin variants in the pediatric clinical laboratory*, Electrophoresis, 1997, 18, 1785-1795, hereafter "Hempe").

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In response, Applicants respectfully submit the following arguments in traverse.

Applicants submit that, for the reasons presented in detail above, the teachings of *Sunzeri* in view of *Camilleri* do not teach the separation of hemoglobin variants as claimed by Applicants in the instant invention. Specifically, *Sunzeri* fails to teach the actual detection and separation of hemoglobin A1c, but instead only discloses the resolution by capillary electrophoresis of hemoglobin A1c-hemoglobin A1c monoclonal antibody complexes from unbound hemoglobin A1c monoclonal antibody. Significantly, *Sunzeri* also fails to disclose the actual claimed hemoglobin variants, nor does it teach or even hint at the possibility that one could separate these or any other hemoglobin variants by free solution capillary electrophoresis. Indeed, since the methods taught by *Sunzeri* only permit the analysis of a single analyte at a time, the separation of multiple proteins is not even possible, nor is it even remotely contemplated, in this reference.

Applicants further submit that nothing in *Hempe* corrects these deficiencies. Indeed, the Examiner only cites *Hempe* for teaching that hemoglobin HA1c has a pI point of 6.935. Applicants take this opportunity to point out that, in fact, *Hempe* concerns capillary isoelectric focusing (cIEF), and the gist of the principles and methods disclosed therein for the separation of hemoglobin are very different from Applicants' free solution capillary electrophoresis (FSCE) method.[1]

[1] In cIEF, coated capillaries have to be used to prevent EOF. According to *Hempe*, separation is carried out in the presence of methylcellulose (used as a coating) and ampholytes. NaOH is used as a cathode solution. As disclosed, a two step separation process is carried out. In a first step, the hemoglobin variants are immobilized due to the presence of ampholytes; in a further step, the content of the capillary is translated and analysis is carried out as the content of the capillary is displaced out of the capillary.

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Thus, taken together, Applicants believe that the teachings of *Sunzeri* in view of *Camilleri* and with evidence from *Hempe* do not predictably lead one of skill in the art to the methods of the instant invention, and respectfully request reconsideration and withdrawal of the rejection of claims 37-42, 45, 46 and 57 of the instant invention based on any combination of these cited references.

Rejection of Claims 44 and 51:

Claims 44 and 51 are rejected under 35 U.S.C. § 103(a) as being obvious over *Sunzeri*. Specifically, *Sunzeri* is cited for teaching the use of 50mM tricine (claim 44) and also that the buffer has a "concentration" (pH) of about 7.2 (claim 51). In response, Applicants submit that, as discussed in detail above, *Sunzeri* does not render obvious any of the claimed methods of the instant invention, including the method of claim 29 from which claims 44 and 51 depend. As such, reconsideration and withdrawal of these rejections are earnestly solicited.

Rejection of Claims 47 and 48:

Claims 47 and 48 are rejected under 35 U.S.C. § 103(a) as being obvious over *Sunzeri* in view of Nouadje et al., US 2002/0162744 A1 (hereafter "Nouadje").

In response, Applicants submit that, for the reasons presented in detail above, *Sunzeri* does not render obvious the methods of the present invention. Moreover, nothing in *Nouadje* corrects these deficiencies.

Specifically, Applicants submit that *Nouadje* relates to the separation of seric proteins by capillary electrophoresis with an additive which reduces the mobility of said proteins (one or more constituent which mobility is reduced compared to the other constituents). The principle of the separation disclosed by *Nouadje* is to interact with specific seric proteins, however, not to reduce the flow in order to resolve variants. Applicants have previously distinguished the teachings of *Nouadje* from the

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instant invention in detail, and respectfully direct the Examiner's attention to Applicants' Request and Amendment filed December 22, 2009.

In this case, Applicants respectfully submit that, in contrast to the Examiner's allegation, the disclosure of *Sunzeri* in combination with the disclosure of pH modifiers such as provided in *Nouadje* at [0042] is insufficient to predictably yield the methods of the claimed invention, and respectfully request reconsideration and withdrawal of the instant rejection of claims 47 and 48.

As it is believed that all of the rejections set forth in the Official Action have been fully met, favorable reconsideration and allowance are earnestly solicited.

If, however, for any reason the Examiner does not believe that such action can be taken at this time, it is respectfully requested that she telephone Applicants' attorney at (908) 654-5000 in order to overcome any additional objections which she might have.

Submitted herewith is a Petition for a Three Month Extension of Time, and the Examiner is authorized to charge Deposit Account No. 12-1095 accordingly. If there are any additional charges in connection with this requested amendment, the Examiner is authorized to charge Deposit Account No. 12-1095 therefore.

Dated: July 21, 2010

Respectfully submitted,
Electronic signature: /Diane P. Tso/
Diane P. Tso
Registration No.: 46,012
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 654-5000
Attorney for Applicants

1148875_1.doc

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Page 1003-0465

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/280,025	11/16/2005	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	07/21/2010	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 27	Minus	** 30	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 4	Minus	***4	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/NICOLLE L. SCRIVNER/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

530 7590 10/12/2010

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

EXAMINER	
DIETERLE, JENNIFER M	
ART UNIT	PAPER NUMBER
1795	

DATE MAILED: 10/12/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

TITLE OF INVENTION: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	01/12/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0467

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

530 7590 10/12/2010

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

TITLE OF INVENTION: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	01/12/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
DIETERLE, JENNIFER M	1795	204-451000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively, 1 _____

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____ 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frederic Robert	EGYP 3.0-034	8408

530 7590 10/12/2010

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

EXAMINER	
DIETERLE, JENNIFER M	
ART UNIT	PAPER NUMBER
1795	

DATE MAILED: 10/12/2010

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)

(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 466 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 466 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

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Page 1003-0469

Notice of Allowability	Application No.	Applicant(s)	
	11/280,025	ROBERT ET AL.	
	Examiner	Art Unit	
	Jennifer Dieterle	1795	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *7/21/10*.

2. ☒ The allowed claim(s) is/are *29-32, 34-51, 56 and 57*.

3. ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☒ All b) ☐ Some* c) ☐ None of the:
      1. ☒ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

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Page 1003-0470

DETAILED ACTION

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Diane Tso on September 22, 2010.

The claims have been amended as follows:

Claims 53-55 have been canceled.

Claim 29, line 2, after "separating" inserted --- and resolving ---

Claim 31, line 4, after "centrifuged blood," inserted --- haemolyzed blood, ---

Claim 56, line 2, after "separating" inserted --- and resolving ---

Claim 57, line 2, after "separating" inserted --- and resolving ---

Allowable Subject Matter

Claims 29-32, 34-51, 56 and 57 are allowed.

The following is an examiner's statement of reasons for allowance:

The instant application is allowable over other art because the instant application teaches a free solution capillary electrophoresis method for separating and *resolving* haemoglobins in a biological sample. The method comprises electrophoresing utilizing an analysis buffer that is a zwitterionic type and at least one flow inhibitor.

The closest prior art of record, Sunzeri (US 5,536,382) teaches a method of capillary electrophoresis for separating and detecting heamoglobin in a lysed blood sample utilizing a tris tricine buffer. Sunzeri mixes the sample with a labeling reagent and detects the labeling reagent's displacement from its normal migration position from unbound reagent. Sunzeri teaches the detection of a single specific analyte, not multiple haemoglobins. In Sunzeri, only the resolution of bound from unbound reagent is taught. However, the present invention not only separates haemoglobins, it also provides for the resolving of the different haemoglobin components such as HbA2, HbC, HbD, etc.

Additionally, Nouadje et al. (US 2002/0162744) was cited, however, while Nouadje et al. teach a method of electrophoresis for separating blood components which includes haemoglobin, Nouadje et al. does not specifically teach the resolution of the different haemoglobin constituents. Rather Nouadje et al. only teach the detection

of haemoglobin in the sample, it does not resolve the different haemoglobin constituents.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jennifer Dieterle whose telephone number is (571) 270-7872. The examiner can normally be reached on Monday thru Friday, 8am to 5pm (EST).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Alexa Neckel can be reached on (571) 272-1446. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

JMD
9/22/10

/Alexa D. Neckel/

Supervisory Patent Examiner, Art Unit 1795

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
11280025	ROBERT ET AL.
Examiner	**Art Unit**
Jennifer Dieterle	1795

ORIGINAL		INTERNATIONAL CLASSIFICATION	
CLASS	SUBCLASS	CLAIMED	NON-CLAIMED
204	451	G 0 1 N 27 / 447 (2006.0)	

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
204	468	601			

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		17		33	10	49								
	2		18	3	34	11	50								
	3		19	15	35	12	51								
	4		20	16	36		52								
	5		21	4	37		53								
	6		22	5	38		54								
	7		23	19	39		55								
	8		24	20	40	23	56								
	9		25	21	41	24	57								
	10		26	22	42										
	11		27	6	43										
	12		28	7	44										
	13	1	29	8	45										
	14	2	30	9	46										
	15	13	31	17	47										
	16	14	32	18	48										

/Jennifer Dieterle/ Examiner.Art Unit 1795	9/22/10	**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	24	
/ALEXA D NECKEL/ Supervisory Patent Examiner.Art Unit 1795	09/29/2010	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	2

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11280025	ROBERT ET AL.
	Examiner	**Art Unit**
	Jennifer Dieterle	1795

SEARCHED			
Class	**Subclass**	**Date**	**Examiner**
204	450, 451, 468, 601	12/29/2008	JMD
204	600-616,400,403.01-403.15,450-468	9/22/10	JMD

SEARCH NOTES		
Search Notes	**Date**	**Examiner**
Inventor Search, Google, NPL	12/29/2008	JMD
EAST, Google	6/10/09	JMD
Consulted with Alex Noguerola, EAST, Google	1/5/10	JMD
Google, EAST, Inventor	9/22/10	JMD

INTERFERENCE SEARCH			
Class	**Subclass**	**Date**	**Examiner**
204	600-616,400,403.01-403.15,450-468	9/22/10	JMD
	Interference text search of claim language	9/22/10	JMD

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	8749	204/600-616,400,403.01-403.15,450-468.ccls.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 13:48
L3	152	(frederic with robert).in. or (denis with simonin).in. or (jean-baptiste with clement).in.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 13:48
L4	23510	"2" and (capillary with electrophore$5)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 13:49
L5	10	l3 and (capillary with electrophore$5)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 13:49
L6	1	l3 and (haemoglobin or hemaglobin)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 13:49
L8	46	4 and ((hemoglobin or haemoglobin) with capillar $3 with electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 14:05
L9	18	1 and ((hemoglobin or haemoglobin) with capillar $3 with electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2010/09/22 14:06
S1	173	faradaic adj reaction	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:41
S2	102	S1 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:41
S3	4639	reaction near4 electrode near4 surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:57
S4	3228	reaction near3 electrode near3 surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:57
S5	471	reaction near electrode near surface	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58
S6	43	S5 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58
S7	28	S6 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 15:58

S8	4	S7 and ferrocyanide	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:44
S9	14	S2 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59
S10	3	S9 and ferrocya$	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59
S11	3	S9 and ferrocya$4	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 16:59
S12	12	glucose and mediator and enzyme and faradaic adj reaction and shuttle	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/07 17:28
S13	2	("5922183").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/07 17:38
S14	733	ferricyanide near5 mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:26
S15	0	ferricyanide and ("PF. sub.6" near5 mediator)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S16	0	ferricyanide and ("Os (MeBpy.sub.2.(Im)sub.2" near5 mediator)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S17	0	ferricyanide and "Os (MeBpy.sub.2.(Im)sub.2"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S18	0	ferricyanide and "Os (MeBpy.sub.2.(Im)sub.2."	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S19	0	ferricyanide and "Os (MeBpy.sub.2(Im)sub.2."	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S20	0	ferricyanide and "Os (MeBpy.sub.2(Im)sub.2"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S21	0	ferricyanide and "Os (MeBpy)"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:27
S22	1837	monodentate adj ligand	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S23	1497	S22 and Os	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28

S24	38701	S22 and Os or osmium	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S25	1558	S22 and (Os or osmium)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S26	198	S25 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:28
S27	2	S26 and (ferri or fefficyanide)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:29
S28	7	S26 and (ferri or ferricyanide)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:29
S29	2	"20050258035"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:33
S30	4752	glucose and (ferri$7 and ferro$7) and (os or osmium)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 13:59
S31	2665	S30 and ((polyethylene adj glycol or polyethyleneglycol) or erythiritol)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 14:00
S32	50	S31 and (GOX or GOD)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 14:00
S33	2	("5,682,884").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/08 14:19
S34	101	(os or osmium) near4 redox near2 mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S35	80	S34 and ferr$8	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S36	75	S35 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S37	35	S36 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:00
S38	11	dual adj mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:07
S39	104	two adj mediator	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15

S40	58	S39 and glucose	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15
S41	39	S40 and @py<"2006"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/08 17:15
S42	2	("6826966").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/08 17:25
S43	3	"11280025"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/09 18:51
S44	3	"20020162744"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/09 19:02
S45	2	("5753094").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 10:12
S46	0	hawmoglobin	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:48
S47	1	haemoglobin and free adj solution adj capillar$3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S48	6280	haemoglobin andcapillar$3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S49	110	haemoglobin and capillar $3 adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:49
S50	24	S49 and @py<"2004"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 12:50
S51	2	haemolyzed adj blood and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 13:43
S52	2	("4764279").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 13:49
S53	2	("5,599,433").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 13:53
S54	17	hemolyzed adj blood and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 14:21

S55	3	"11280025"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:26
S56	3	"20020162744"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:27
S57	14	flow adj inhibitor and capillary adj electrophoresis	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:36
S58	11	"10052931"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:44
S59	7321	electrophoretic near3 mobility and (cyclic or aliphatic or polyamine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:45
S60	7262	electrophoretic adj mobility and (cyclic or aliphatic or polyamine)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:45
S61	1068	(electrophoretic adj mobility) and (cyclic or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
S62	688	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
S63	0	(electrophoretic adj mobility) near5 (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
S64	0	(electrophoretic adj mobility) with (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
S65	0	(electrophoretic adj mobility) near (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46
S66	688	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:46

S67	0	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis) and bloood	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:47
S68	12	(electrophoretic adj mobility) and (cyclic adj diamine or aliphatic or polyamine) and (capillary adj electrophoresis) and zwitterion	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	ON	2009/06/10 16:47
S69	2	("5447612").PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2009/06/10 17:55

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L2	4992	204/600-616,400,403.01-403.15,450-468.ccls.	USPAT; UPAD	OR	ON	2010/09/22 13:48
L7	4	((hemoglobin or haemoglobin) with capillar $3 with electrophoresis).clm.	USPAT; UPAD	OR	ON	2010/09/22 13:53
L10	6	2 and ((hemoglobin or haemoglobin) with capillar $3 with electrophoresis)	USPAT; UPAD	OR	ON	2010/09/22 14:19
L11	4	(free with solution with capillary with electrophoresis).clm.	USPAT; UPAD	OR	ON	2010/09/22 14:20

9/22/2010 2:21:16 PM
C:\Documents and Settings\JDIETERLE\My Documents\EAST\Workspaces\11280025.wsp

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: January 3, 2011
Electronic Signature for Diane P. Tso: /Diane P. Tso/

Docket No.: EGYP 3.0-034
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Robert et al.	Allowed: October 12, 2010
Application No.: 11/280,025	Confirmation No.: 8408
Filed: November 16, 2005	Art Unit: 1795
For: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD	Examiner: J. M. Dieterle

"COMMENTS ON STATEMENT OF REASONS FOR ALLOWANCE"

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Included in the Notice of Allowance which recently issued in the above-identified patent application is an "Examiner's Statement of Reasons for Allowance". In this Statement, the Examiner makes the following observation:

> ...Nouadje et al. (US 2002/0162744) was cited, however, while Nouadje et al. teach a method of electrophoresis for separating blood components which includes haemoglobin, Nouadje et al. does not specifically teach the resolution of the different haemoglobin constituents. Rather Nouadje et al. only teach the detection of haemoglobin in the sample; it does not resolve the different hemoglobin constituents.

Under 37 CFR § 1.104(e), Applicants are permitted to submit "Comments on Statement of Reasons for Allowance" along with the payment of the issue fee, and thus respectfully submit the

following comments in view of the Examiner's characterization of Nouadje *et al.*

Applicants respectfully submit that the Examiner's observation of Nouadje *et al.* is inconsistent with what Applicants have argued during prosecution, *e.g.*, that Nouadje *et al.* teaches the detection and resolution of seric proteins in a sample and it does not resolve different haemoglobin constituents. While blood may contain haemoglobins, Nouadje *et al.* does not mention these proteins, but instead mentions albumin and globulin fractions. Indeed, haemoglobin is not known as a seric protein; a seric protein profile usually identifies albumin and various globulins, but no haemoglobin variants.

Applicants note that Nouadje *et al.* mentions "separating blood proteins" (at [0037]) and "In blood samples, the blood proteins to be separated…" (at [0038]). The phrase "blood protein" was an erroneous translation of "proteins sériques" (seric proteins). This error was dealt with during prosecution and the phrase replaced with "seric proteins" before grant of Nouadje *et al.* as U.S. Patent No. 7,297,244. The granted patent correctly mentions "separating seric proteins" at col. 3, line 61 (which corresponds to [0037] in the published application); similarly, "In serum samples, the seric proteins to be separated…" is found at col. 3, line 63 of the granted patent (which corresponds to [0038] in the published application). Applicants also note that claim 4 of U.S. Patent No. 7,297,244 recites "serum proteins" compared to claim 5 of the published patent application which recites "blood protein".

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0484

Relevant pages of Nouadje *et al*. and corresponding U.S. Patent No. 7,297,244 are enclosed herewith.

Dated: January 3, 2011

Respectfully submitted,
Electronic signature: /Diane P. Tso/
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Helena Laboratories Corp.
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Page 1003-0485

US 20020162744A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2002/0162744 A1

Nouadje et al. (43) Pub. Date: Nov. 7, 2002

(54) CAPILLARY ELECTROPHORESIS SYSTEMS AND ADDITIVES

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(21) Appl. No.: **10/052,931**

(22) Filed: **Jan. 18, 2002**

(30) **Foreign Application Priority Data**

Jan. 19, 2001 (FR) 0100762

Publication Classification

(51) **Int. Cl.**[7] **G01N 27/26**; G01N 27/447

(52) **U.S. Cl.** **204/451**

(57) **ABSTRACT**

Capillary electrophoresis systems, reagents, and processes for analyzing a sample which includes at least one protein constituent. The processes involve introducing the sample into a capillary tube containing a buffer system, wherein said buffer system includes at least one additive having an hydrophobic interaction with said at least one protein constituent and providing said at least one protein constituent with at least one negative charge thereby modifying the electrophoretic mobility

[0023] **FIG. 10** shows an electropherogram of serum from a patient presenting with an acute inflammatory syndrome, obtained by agarose gel electophoresis.

[0024] **FIG. 11** shows the mobility of the α_1 globulin fraction and that of the albumin fraction for various lengths of the carbon chain of an allylsulphonate added to a borate buffer.

[0025] Additives for the buffer in accordance with the invention that are capable of interacting with the hydrophobic portion of albumin that can be cited are compounds comprising an anionic pole with a pH of more than 9 and a hydrophobic portion. The hydrophobic portion can be composed of at least one alkyl chain, which may or may not be branched, containing 4 to 22 carbon atoms, namely 4 to 20 carbon atoms, and/or at least a combination of 1 to 10 cyclic groups, which groups may be aromatic or non aromatic. Preferably, combinations of 1 to 4 cyclic groups are used. As will be readily understood by the skilled person, this hydrophobic portion can comprise residues or functions that do not essentially modify its hydrophobic nature, such as one or more hydroxyl or amine functions, for example.

[0026] The anionic pole can be constituted by one or more of the chemical groups or functions from the following list: sulphonates, carboxylates, sulphates, phosphates, carbonates.

[0027] The following can in particular be cited: cholates, C_6 to C_{22} alkyl-mono-, di- or tri- sulphonates, tetradecenesulphonate, naphthalenesulphonates, C_6 to C_{22} alkylmono-, di- or trioxylates, C_6 to C_{22} alciboxysulphonates, naphthalenecarboxylates, C_4 to C_{14} alkylsulphates, C_4 to C_{14} alkylcarbonates, benzenesulphonates and benzenecarboxylates.

[0028] The above di- and triarboxylates, di- and tri-sulphonates and carboxysulphonates are thus combinations of one or more carboxylate or sulphonate functions on C_6 to C_{22} alkyl chains. Non limitative examples thereof are the 1,2,3-nonadecanetricarboxylic acid (three carboxylate functions and a en C_{19} alkyl chain), the 2-methyl-2-sulfooctadecanoic acid (one carboxylate function and one sulfonate function and a C_{18} alkyl chain) and the 1,12-dodecanedicarboxylic acid (two carboxylate functions and a C_{12} allyl chain).

[0029] C_4 to C_{10} alkylsulphonates are more specifically cited amongst the C_6 to C_{22} alkyl-mono-, di- or tri- sulphonates and the C_4 to C_{10} alkylcarboxylates amongst the C_6 to C_{22} alkyl-mono-, di- or tri- carboxylates,

[0030] Within the above denominations, the alkyl radicals are preferably linear.

[0031] Biological buffers can be used as additives according to the invention. Particularly zwitterionic buffer of the Good type can be cited, as CAPS (3-cyclohexylamino-1-propanesulphonic acid) and CHES (2-(N-cyclohexylamino-)ethanesulphonic acid).

[0032] Other zwitterionic biological buffer can be used within the context of the invention. The amino acid buffers are however not intended as a buffer or additive according to the present invention.

[0033] Preferred additives from those cited above are C_6 to C_{10} alkylsulphonates, and of the C_6 to C_{10} alkylsulphonates, octanesulphonate is preferred.

[0034] These compounds are known per se and are commercially available. They can be in the acid or salt form.

[0035] The term "sample in accordance with the invention" means the biological sample to be analysed, diluted with a suitable diluting solution or buffer system, for example, or pure.

[0036] The sample for analysis can be any biological liquid from healthy humans or human patients. The human biological liquids can be normal or abnormal serum, and also haernolysed serum, plasma, urine, or cerebro-spinal fluid. In addition to human biological samples, it is possible to analyse samples of animal origin. The samples can also be synthetic proteins, and the method of the invention is then intended for production control, for example.

[0037] The additives of the invention are of particular application for analysing serum, and for separating blood proteins in samples from humans.

[0038] In blood samples, the blood proteins to be separated are primarily albumin and the α_1; α_2; β (or β_1 and β_2); and γ globulin fractions.

[0039] The buffer system can be any known buffer system adapted to the desired separation, for use in electrophoresis in general and capillary electrophoresis in particular. Examples that can be cited are borate, phosphate and carbonate buffers, buffers based on amino acids and buffers known as biological buffers.

[0040] Examples of biological buffers that can be cited are those known as bis-TRIS (2-bis[2-hydroxyethyl]amino-2-hydroxymethyl-1,3-propanediol), ADA (N-[2-acetamido]-2-iminodiacetic acid), ACES (2-[2-acetamino[-2-aminoethanesulphonic acid), PIPES (1,4-piperazinediethanesulphonic acid), MOPSO (3-[N-morpholino]-2-hydroxypropanesulphonic acid), bis-TRIS PROPANE (1,3-bis[tris(hydroxymethyl)methylamino-propane]), BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid), MOPS (3-[N-motpholino]propancsulphonic acid), TES (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulphonic acid), HEPES (N-[2-hydroxyethyl]piperazine-N'-(2-ethanesulphonic)acid), DIPSO (3-N,N-bis[2-hydroxyethyl]amino-2-hydroxypropanesulphonic acid), MOBS (4-N-morpholinobutanesulphouic acid), TAPSO (3[N-tris-hydroxymethylmethylamino]-2-hydroxypropanesulphonic acid), TRIS (2-amino-2-[hydroxymethyl]-1,3-propanediol), HEPPSO (N-[2-hydroxyethyl]piperazine-N'-[2-hydroxypropanesulphonic]acid), POPSO (piperazie-N,N'-bis[2-hydroxypropanesulphonic]acid), TEA (triethanolamine), EPPS (N-[2-hydroxyethyl]-piperazine-N'-[3-propanesulphonic]acid), TRICINE (N-tris[hydroxymethyl]methylglycine), GLY-GLY (diglycine), BICINE (N,N-bis[2-hydroxyethyl]glycine), HEPBS (N-[2-hydroxyethyl]piperazine-N'-[4-butanesulphonic]acid), TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulphonic acid), AMPD (2-amino-2-methyl-1,3-propanediol), TABS (N-tris[hydroxymethyl]methyl-4-aminobutanesulphonic acid), AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid), CHES (2-(N-cyclohexylamino)ethanesulphonic acid), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid), AMP (2-amino-2-methyl-1-propanol), CAPS (3-cyclohexylamino-1-propanesulphonic acid) and CABS (4-[cyclohexylamino]-1-butanesulphonic acid), preferably AMPD, TABS, AMPSO, CRES, CAPSO, AMP, CAPS or CABS.

[0067] The electrophoresis was carried out as described in Example 1.

[0068] As can be seen in **FIG. 3**, the electropherogram obtained exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin and albumin fractions respectively.

Example 4

[0069] Octanesulphonate in a concentration of 2.5 mM was added to the buffer system of Example 4.

[0070] The electrophoresis was carried out as described in Example 1.

[0071] As can be seen in **FIG. 4**, the electropherogram obtained exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin and albumin fractions respectively. Separation between the two fractions, α_1 globulin and albumin, is substantially improved, and the return to the base line is improved.

Example 5 (Comparative)

[0072] The electropherogram of **FIG. 5** was obtained by analysing the same serum as in the preceding examples using method B above. Comparison with the result obtained in Examples 2 and 4 shows that these implementations can produce a resolution that is substantially comparable to the resolution obtained with an agarose gel.

Example 6 (Comparative)

[0073] A 150 mM glycine buffer system was prepared.

[0074] Serum with high α_1 and α_2 globulin contents was analysed.

[0075] The electrophoresis was carried out as described in Example 1.

[0076] As can be seen in **FIG. 6**, the electropherogram obtained exhibits five successive peaks, attributed to γ, β, α_2, α_1 globulin and albumin fractions respectively.

Example 7

[0077] Octanesulphonate in a concentration of 2.5 mM was added to the buffer system of Example 7.

[0078] The electrophoresis was carried out as described in Example 6.

[0079] As can be seen in **FIG. 7**, the electropherogram obtained exhibits five successive peaks, attributed to γ, β, α_2, α_1 globulin and albumin fractions respectively. Comparison with the result of Example 6 shows that the separation between the two fractions, α_1 globulin and album is substantially improved, and the return to the base line is improved.

Example 8 (Comparative)

[0080] The procedure of Example 6 was followed, the buffer being a 150 mM borate buffer.

[0081] The electrophoresis was carried out as described in Example 6.

[0082] As can be seen in **FIG. 8**, the electropherogram obtained exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin ad albumin fractions respectively.

Example 9

[0083] Octanesulphonate in a concentration of 2.5 mM was added to the buffer system of Example 8.

[0084] The electrophoresis was carried out as described in Example 6.

[0085] As can be seen in **FIG. 9**, the electropherogram obtained under the same conditions exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin and albumin fractions respectively. Separation between the two fractions, α_1 globulin and albumin, is substantially improved, and the return to the base line is improved. The α_1 fraction can be seen to be composed of two peaks, one of which corresponds to the orosomucoid and, in the absence of octanesulphonate, being merged with the albumin peak.

Example 10 (Comparative)

[0086] The electropherogram of **FIG. 10** was obtained by analysing the same serum as in Examples 6 to 9 using method B above. Comparison with the result obtained in Examples 7 and 9 showed that these implementations can produce a resolution that is substantially comparable to the resolution obtained with an agarose gel.

Example 11

[0087] The comparative mobility (mn^{-1}) of α_1 globulin and albumin were measured in a borate buffer (150 mM) with a pH of 10, using method A above. Alkylsulphonates with an increasing alkyl chain length (n) (n represents 4, 6, 8 and 10 respectively for C_4, C_6, C_8 and C_{10}, and n=0 corresponds to a buffer with no alkylsulphonate) were added to the borate buffer in a concentration of 2.5 mM. The mobilities of the alpha-1 fractions and the albu fractions were calculated and are shown on the graph of **FIG. 11**. A substantial drop in the mobility of the albumin (■) compared with that of the alpha-1 fraction (♦) can be seen beyond a C6 chain.

1. An alkaline pH, free solution capillary electrophoresis process for analyzing a sample comprising at least one protein constituent, said method comprising: introducing the sample into a capillary tube containing a buffer system, wherein said buffer system comprises at least one additive having an hydrophobic interaction with said at least one protein constituent and providing said at least one protein constituent with at least one negative charge thereby modifying the electrophoretic mobility.

2. The method of claim 1, which further comprises separating said at least one protein constituent by migrating and detecting said at least one protein constituent.

3. The method of claim 1, wherein the sample is a biological sample.

4. The method of claim 1, wherein the sample is blood, hemolyzed blood, plasma, urine or cerebrospinal fluid.

5. The method of claim 1, wherein said at least one protein constituent is blood protein.

6. The method of claim 1, wherein said at least one protein constituent is selected from albumin, α_1-globulin, α_2-globulin, β-globulin, β_1-globulin, β_2-globulin and γ-globulin.

7. The method of claim 1, wherein said at least one additive comprises an anionic pole with a pH of more than 9 and a hydrophobic portion.

US007297244B2

(12) United States Patent
Nouadje et al.

(10) Patent No.: US 7,297,244 B2
(45) Date of Patent: Nov. 20, 2007

(54) **CAPILLARY ELECTROPHORESIS SYSTEMS AND ADDITIVES**

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(73) Assignee: **Sebia** (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 162 days.

(21) Appl. No.: **10/052,931**

(22) Filed: **Jan. 18, 2002**

(65) **Prior Publication Data**

US 2002/0162744 A1 Nov. 7, 2002

(30) **Foreign Application Priority Data**

Jan. 19, 2001 (FR) 01 00762

(51) **Int. Cl.**
G01N 27/447 (2006.01)

(52) **U.S. Cl.** **204/451**; 204/468

(58) **Field of Classification Search** 204/451, 204/468, 601; 435/7.1; 436/517; 252/62.2
See application file for complete search history.

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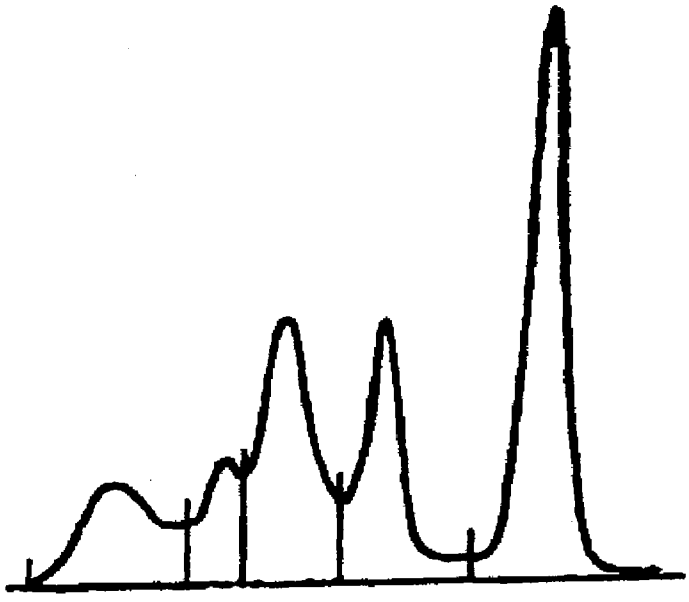
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(57) **ABSTRACT**

Capillary electrophoresis systems, reagents, and processes for analyzing a sample which includes at least one protein constituent are disclosed. The processes involve introducing the sample into a capillary tube containing a buffer system, wherein said buffer system includes at least one additive having a hydrophobic interaction with said at least one protein constituent and providing said at least one protein constituent with at least one negative charge thereby modifying the electrophoretic mobility.

24 Claims, 3 Drawing Sheets



Helena Laboratories Corp.
Exhibit 1003
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least one alkyl chain, which may or may not be branched, containing 4 to 22 carbon atoms, namely 4 to 20 carbon atoms, and/or at least a combination of 1 to 10 cyclic groups, which groups may be aromatic or non aromatic. Preferably, combinations of 1 to 4 cyclic groups are used. As will be readily understood by the skilled person, this hydrophobic portion can comprise residues or functions that do not essentially modify its hydrophobic nature, such as one or more hydroxyl or amine functions, for example.

The anionic pole can be constituted by one or more of the chemical groups or functions from the following list: sulphonates, carboxylates, sulphates, phosphates, carbonates.

The following can in particular be cited: cholates, C_6 to C_{22} alkyl-mono-, di- or tri- sulphonates, tetradecenesulphonate, naphthalenesulphonates, C_6 to C_{22} alkylmono-, di- or trioxylates, C_6 to C_{22} alciboxysulphonates, naphthalenecarboxylates, C_4 to C_{14} alkylsulphates, C_4 to C_{14} alkylcarbonates, benzenesulphonates and benzenecarboxylates. The above di- and tri-carboxylates, di- and tri-sulphonates and carboxysulphonates are thus combinations of one or more carboxylate or sulphonate functions on C_6 to C_{22} alkyl chains. Non limitative examples thereof are the 1,2,3-nonadecanetricarboxylic acid (three carboxylate functions and a C_{19} alkyl chain), the 2-methyl-2-sulfooctadecanoic acid (one carboxylate function and one sulfonate function and a C_{18} alkyl chain) and the 1,12-dodecanedicarboxylic acid (two carboxylate functions and a C_{12} alkyl chain).

C_4 to C_{10} alkylsulphonates are more specifically cited amongst the C_6 to C_{22} alkyl-mono-, di- or tri- sulphonates and the C_4 to C_{10} alkylcarboxylates amongst the C_6 to C_{22} alkyl-mono-, di- or tri- carboxylates,

Within the above denominations, the alkyl radicals are preferably linear.

Biological buffers can be used as additives according to the invention. Particularly zwitterionic buffer of the Good type can be cited, as CAPS (3-cyclohexylamino-1-propanesulphonic acid) and CHES (2-(N-cyclohexylamino)ethanesulphonic acid).

Other zwitterionic biological buffer can be used within the context of the invention. The amino acid buffers are however not intended as a buffer or additive according to the present invention.

Preferred additives from those cited above are C_6 to C_{10} alkylsulphonates, and of the C_6 to C_{10} alkylsulphonates, octanesulphonate is preferred.

These compounds are known per se and are commercially available. They can be in the acid or salt form.

The term "sample in accordance with the invention" means the biological sample to be analysed, diluted with a suitable diluting solution or buffer system, for example, or pure.

The sample for analysis can be any biological liquid from healthy humans or human patients. The human biological liquids can be normal or abnormal serum, and also haemolysed serum, plasma, urine, or cerebro-spinal fluid. In addition to human biological samples, it is possible to analyse samples of animal origin. The samples can also be synthetic proteins, and the method of the invention is then intended for production control, for example.

The additives of the invention are of particular application for analysing serum, and for separating seric proteins in samples from humans.

In serum samples, the seric proteins to be separated are primarily albumin and the α_1; α_2; β (or β_1 and β_2); and γ globulin fractions.

The buffer system can be any known buffer system adapted to the desired separation, for use in electrophoresis in general and capillary electrophoresis in particular. Examples that can be cited are borate, phosphate and carbonate buffers, buffers based on amino acids and buffers known as biological buffers.

Examples of biological buffers that can be cited are those known as bis-TRIS (2-bis[2-hydroxyethyl]amino-2-hydroxymethyl-1,3-propanediol), ADA (N-[2-acetamido]-2-iminodiacetic acid), ACES (2-[2-acetamino[-2-aminoethanesulphonic acid), PIPES (1,4-piperazinediethanesulphonic acid), MOPSO (3-[N-morpholino]-2-hydroxypropanesulphonic acid), bis-TRIS PROPANE (1,3-bis[tris(hydroxymethyl)methylaminopropane]), BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid), MOPS (3-[N-motpholino]propanesulphonic acid), TES (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulphonic acid), HEPES (N-[2-hydroxyethyl] piperazine-N'-(2-ethanesulphonic)acid), DIPSO (3-N,N-bis [2-hydroxyethyl]amino-2-hydroxypropanesulphonic acid), MOBS (4-N-morpholinobutanesulphouic acid), TAPSO (3[N-tris-hydroxymethyl-methylamino]-2-hydroxypropanesulphonic acid), TRIS (2-amino-2-[hydroxymethyl]-1,3-propanediol), HEPPSO (N-[2-hydroxyethyl]piperazine-N'-[2-hydroxypropanesulphonic]acid), POPSO (piperazie-N, N'-bis[2-hydroxypropanesulphonic]acid), TEA (triethanolamine), EPPS (N-[2-hydroxyethyl]-piperazine-N'-[3-propanesulphonic]acid), TRICINE (N-tris[hydroxymethyl]methylglycine), GLY-GLY (diglycine), BICINE (N,N-bis[2-hydroxyethyl]glycine), HEPBS (N-[2-hydroxyethyl] piperazine-N'-[4-butanesulphonic]acid), TAPS (N-tris [hydroxymethyl]methyl-3-aminopropanesulphonic acid), AMPD (2-amino-2-methyl-1,3-propanediol), TABS (N-tris [hydroxymethyl]methyl-4-aminobutanesulphonic acid), AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid), CHES (2-(N-cyclohexylamino)ethanesulphonic acid), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid), AMP (2-amino-2-methyl-1-propanol), CAPS (3-cyclohexylamino-1-propanesulphonic acid), and CABS (4-[cyclohexylamino]-1-butanesulphonic acid), preferably AMPD, TABS, AMPSO, CRES, CAPSO, AMP, CAPS or CABS.

The pH of the biological liquid in the buffer system, including the additive, can be between 2 and 12 However, for alkaline pH capillary electrophoresis, the pH is in the range 8 to 12, preferably in the range 9 to 11, more particularly preferably at about 10.

The buffer systems of the invention can also comprise at least one pH-modifying compound. The pH-modifying compound can be a compound selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri- or tetra-alkyl ammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

In accordance with the invention, the biological buffers are used under the usual conditions, at concentrations in the buffer system of the order of 10 to 500 mM, preferably 20 to 200 mM.

The additives of the invention are used in concentrations of 0.1 mM to 500 mM without, however, exceeding their critical micellar concentration in the buffer system.

This value of the critical micellar concentration is applicable to additives that are surfactants.

When octanesulphonate is used, its concentration in the buffer is of the order of 1 to 5 mM, preferably 1 to 5 mM; preferably, the concentration is about 2.5 mM.

In the methods of the invention, the buffer system can also comprise sodium sulphate.

The electrophoresis was carried out as described in Example 6.

As can be seen in FIG. 7, the electropherogram obtained exhibits five successive peaks, attributed to γ, β, α_2, α_1 globulin and albumin fractions respectively. Comparison with the result of Example 6 shows that the separation between the two fractions, α_1 globulin and album is substantially improved, and the return to the base line is improved.

Example 8 (Comparative)

The procedure of Example 6 was followed, the buffer being a 150 mM borate buffer.

The electrophoresis was carried out as described in Example 6.

As can be seen in FIG. **8**, the electropherogram obtained exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin ad albumin fractions respectively.

Example 9

Octanesulphonate in a concentration of 2.5 mM was added to the buffer system of Example 8.

The electrophoresis was carried out as described in Example 6.

As can be seen in FIG. **9**, the electropherogram obtained under the same conditions exhibits six successive peaks, attributed to γ, β_2, β_1, α_2, α_1 globulin and albumin fractions respectively. Separation between the two fractions, α_1 globulin and albumin, is substantially improved, and the return to the base line is improved. The α_1 fraction can be seen to be composed of two peaks, one of which corresponds to the orosomucoid and, in the absence of octanesulphonate, being merged with the albumin peak.

Example 10 (Comparative)

The electropherogram of FIG. **10** was obtained by analysing the same serum as in Examples 6 to 9 using method B above. Comparison with the result obtained in Examples 7 and 9 showed that these implementations can produce a resolution that is substantially comparable to the resolution obtained with an agarose gel.

Example 11

The comparative mobility (mn^{-1}) of α_1 globulin and albumin were measured in a borate buffer (150 mM) with a pH of 10, using method A above. Alkylsulphonates with an increasing alkyl chain length (n) (n represents 4, 6, 8 and 10 respectively for C_4, C_6, C_8 and C_{10}, and n=0 corresponds to a buffer with no alkylsulphonate) were added to the borate buffer in a concentration of 2.5 mM. The mobilities of the alpha-1 fractions and the albu fractions were calculated and are shown on the graph of FIG. **11**. A substantial drop in the mobility of the albumin (■) compared with that of the alpha-1 fraction (♦) can be seen beyond a C6 chain.

The invention claimed is:

1. An alkaline pH, free solution capillary electrophoresis process for analyzing a human biological sample comprising serum protein constituents including albumin and at least one other constituent selected from α_1-globulin, α_2-globulin, β-globulin, β_1-globulin, β_2-globulin and γ-globulin, said method comprising: introducing the human biological sample into a capillary tube containing a buffer system, wherein said buffer system comprises a buffer and at least one additive having a hydrophobic interaction with said albumin constituent and providing said albumin constituent with at least one negative charge thereby reducing the electrophoretic mobility of said albumin.

2. The method of claim **1**, which further comprises separating said protein constituents by migrating and detecting said constituents.

3. The method of claim **1**, wherein the sample is serum, hemolyzed blood, plasma, urine or cerebrospinal fluid.

4. The method of claim **1**, wherein said constituents are serum proteins.

5. The method of claim **1**, wherein said at least one additive comprises an anionic pole with a pH of more than 9 and a hydrophobic portion.

6. The method of claim **1**, wherein said additive comprises a hydrophobic portion composed of at least one linear or non linear alkyl chain containing 4 to 22 carbon atoms, and/or at least a combination of 1 to 10 aromatic or non-aromatic cycles, and an anionic pole constituted by one or more groups selected from sulphonates, carboxylates, sulphates, phosphates and carbonates.

7. The method of claim **1**, wherein said additive is selected from cholates, C_6 to C_{22} alkyl-mono-, di- or tri-sulphonates, tetradecenesulphonate, naphthalenesulphonates, C_6 to C_{22} alkymono-, di- or tri-carboxylates, C_6 to C_{22} alkylcarboxysulphonates, naphthalenecarboxylates, C_4 to C_{14} alkylsulphates, C_4 to C_{14} alkylcarbonates, benzenesulphonates and benzenecarboxylates.

8. The method of claim **1**, wherein said additive is a C_6 to C_{10} alkylsulphonate.

9. The method of claim **1**, wherein said additive is octanesulphonate.

10. The method of claim **1**, wherein said additive has a concentration in said buffer system in the range of 0.1 mM to 500 mM.

11. The method of claim **10**, wherein said additive in said buffer system does not exceed the critical micellar concentration of said additive in said buffer.

12. The method of claim **1**, wherein said additive has a concentration in the range of 1 mM to 4 mM in said buffer system.

13. The method of claim 1, wherein said additive has a concentration of about 2.5 mM in the buffer system.

14. The method of claim **1**, wherein said buffer system has a pH in the range of 9 to 11.

15. The method of claim **1**, wherein the capillary tube is fused silica.

16. The method of claim **1**, wherein said buffer system further comprises at least one pH-modifying agent.

17. The method of claim **16**, wherein the pH-modifying agent is selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, cesium hydroxide, francium hydroxide, or a mono-, di-, tri- or tetra-alkyl ammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

18. The method according to claim **1**, wherein said additive is a zwitterionic biological buffer.

19. The method of claim **1**, wherein said additive is a linear C_6-C_{10}-alkylsulphonate.

20. The method of claim **1**, wherein said additive is n-octylsulphonate.

21. A method for separating protein constituents in a human biological sample comprising albumin and at least one serum protein selected from α_1-globulin, α_2-globulin, β-globulin, β_1-globulin, β_2-globulin and γ-globulin, said method comprising passing said serum protein constituents into a capillary containing a buffer system comprising at

Electronic Acknowledgement Receipt

EFS ID:	9149166
Application Number:	11280025
International Application Number:	
Confirmation Number:	8408
Title of Invention:	METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD
First Named Inventor/Applicant Name:	Frederic Robert
Customer Number:	00530
Filer:	Arnold H. Krumholz/Aisha Irvis
Filer Authorized By:	Arnold H. Krumholz
Attorney Docket Number:	EGYP 3.0-034
Receipt Date:	03-JAN-2011
Filing Date:	16-NOV-2005
Time Stamp:	12:14:36
Application Type:	Utility under 35 USC 111(a)

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1	Miscellaneous Incoming Letter	Comments_on_Statement_of_Reasons_for_Allowance_1311.pdf	39583 3f30b96464231c24ee79fdcc025a46630fab58c2	no	3

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2	Miscellaneous Incoming Letter	US_20020162744.pdf	552496 e19065ebe521e589f5894868b77e4af43f80b773	no	3
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3	Miscellaneous Incoming Letter	US_7297244.pdf	665904 9f1cbdab8b9bdf5731a79fadcf77cfded21be991	no	3
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	11/16/2005	Frédéric Robert	EGYP 3.0-034	8408

TITLE OF INVENTION: METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
Non-Provisional	yes	$755.00	$300.00	$1,055.00	01/12/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
J. M. Dieterle	1795	204-451000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- [ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- [X] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Lerner, David, Littenberg, Krumholz & Mentlik, LLP
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: SEBIA

(B) RESIDENCE: (CITY and STATE OR COUNTRY): France

Please check the appropriate assignee category or categories (will not be printed on the patent) : [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. The following fee(s) are enclosed:
- [X] Issue Fee
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4b. Payment of Fee(s):
- [ ] A check in the amount of the fee(s) is enclosed.
- [ ] Payment by credit card. Form PTO-2038 is attached.
- [X] The Director is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number 12-1095

5. **Change in Entity Status** (from status indicated above)
- [ ] a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
- [ ] b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

The Director of the USPTO is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.
NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Diane P. Tso/ Date January 3, 2011

Typed or printed name Diane P. Tso Registration No. 46,012

Helena Laboratories Corp.
Exhibit 1003
Page 1003-0494

Application No. (if known): 11/280,025 Attorney Docket No.: EGYP 3.0-034

Certificate of Electronic Filing Under 37 CFR 1.8

I hereby certify that this correspondence is being transmitted via the Office electronic filing system in accordance with 37 CFR 1.6(a)(4):

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on January 3, 2011
Date

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Signature

Diane P. Tso
Typed or printed name of person signing Certificate

46,012
Registration Number, if applicable

(908) 654-5000
Telephone Number

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Issue Fee Transmittal (1 page)
Charge $1,055.00 to deposit account 12-1095

Electronic Patent Application Fee Transmittal

Application Number:	11280025
Filing Date:	16-Nov-2005
Title of Invention:	METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD
First Named Inventor/Applicant Name:	Frederic Robert
Filer:	April M. Capati/Arleen Taylor
Attorney Docket Number:	EGYP 3.0-034

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Publ. Fee- early, voluntary, or normal	1504	1	300	300
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	2501	1	755	755

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1055**

Electronic Acknowledgement Receipt

EFS ID:	9149484
Application Number:	11280025
International Application Number:	
Confirmation Number:	8408
Title of Invention:	METHOD FOR ANALYZING HAEMOGLOBIN BY CAPILLARY ELECTROPHORESIS, A KIT FOR CAPILLARY ELECTROPHORESIS, AND USE OF A FLOW INHIBITOR IN SAID METHOD
First Named Inventor/Applicant Name:	Frederic Robert
Customer Number:	00530
Filer:	April M. Capati/Arleen Taylor
Filer Authorized By:	April M. Capati
Attorney Docket Number:	EGYP 3.0-034
Receipt Date:	03-JAN-2011
Filing Date:	16-NOV-2005
Time Stamp:	12:44:30
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1055
RAM confirmation Number	9639
Deposit Account	121095
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Change of Address	EGYP46_-_Fee_Address_Indication_Form_PTO_SB-47.pdf	25999 c0f0f7008469fd0d7dba4e07420a4cc5f91d8d22	no	1
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	EGYP46_-_Fee_Transmittal_--_Part_B_PTOL-85.pdf	55629 2222338b8355c8e76684496d13a26cf68de17902	no	2
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	31997 d3e155eadda647cb21f22929ab00e227c5cebd99	no	2
Warnings:					
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		Total Files Size (in bytes):	113625		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/47 (03-09)
Approved for use through 03/31/2012. OMB 0651-0016
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"FEE ADDRESS" INDICATION FORM

Address to:
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Fax to:
571-273-6500

- OR -

INSTRUCTIONS: The issue fee must have been paid for application(s) listed on this form. In addition, only an address represented by a Customer Number can be established as the fee address for maintenance fee purposes (hereafter, fee address). A fee address should be established when correspondence related to maintenance fees should be mailed to a different address than the correspondence address for the application. **When to check the first box below:** If you have a Customer Number to represent the fee address. **When to check the second box below:** If you have no Customer Number representing the desired fee address, in which case a completed Request for Customer Number (PTO/SB/125) must be attached to this form. For more information on Customer Numbers, see the Manual of Patent Examining Procedure (MPEP) § 403.

For the following listed application(s), please recognize as the "Fee Address" under the provisions of 37 CFR 1.363 the address associated with:

[X] Customer Number: 000033248

OR

[ ] The attached Request for Customer Number (PTO/SB/125) form.

PATENT NUMBER (if known)	APPLICATION NUMBER
	11/280,025

Completed by (check one):

[ ] Applicant/Inventor — /Diane P. Tso/ (Signature)

[X] Attorney or Agent of record — 46,012 (Reg. No.) — Diane P. Tso (Typed or printed name)

[ ] Assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96) — (908) 654-5000 (Requester's telephone number)

[ ] Assignee recorded at Reel _____ Frame _____ — January 3, 2011 (Date)

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

[ ] ***Total of** 1 **forms are submitted.**

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/280,025	02/15/2011	7887686	EGYP 3.0-034	8408

530 7590 01/26/2011

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK
600 SOUTH AVENUE WEST
WESTFIELD, NJ 07090

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 866 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Frederic Robert, Mennecy, FRANCE;
Denis Simonin, Evry, FRANCE;
Jean-Baptiste Clement, Etiolles, FRANCE;