



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

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

sample was injected for 30 psi's, and separated at 8 kV. The separation current was initially 1 μ A, but fell to approximately 0.2 μ A 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 μ l 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 μ l haemolyzing reagent (10 mmol/l KCN/5 mmol/l EDTA), 100 μ l 0.40% Profocus Methylcellulose solution (Applied Biosystems, Foster City, CA 94404), 5 μ l 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 μ m 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

in 10 mmol/l KCN/5 mmol/l EDTA. Aliquots (300 μ l) 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 lyophilised material was dissolved in 30 μ l 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

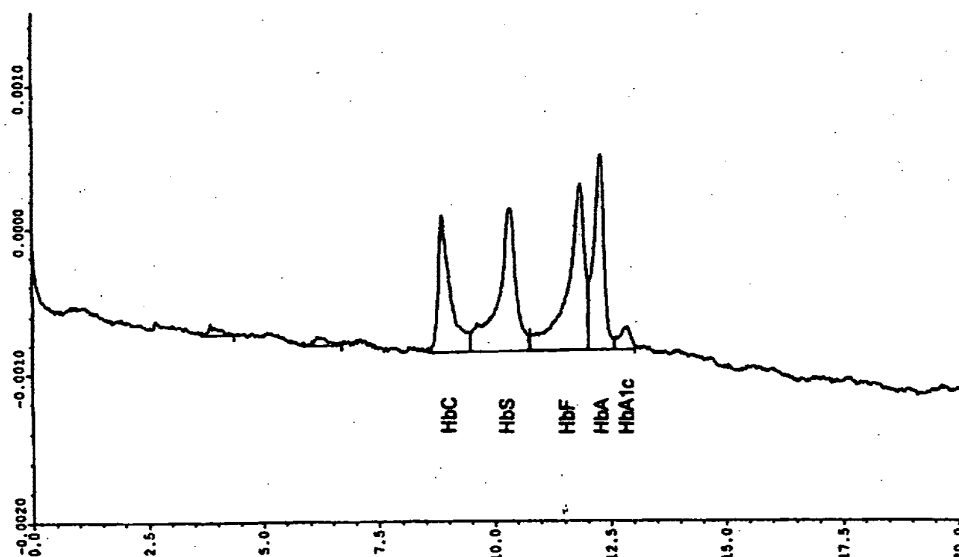


Fig. 1. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control showing from left HbC, HbS, HbF, HbA and HbA1c. Conditions of separation: 1.3% Pharmalytes (pH 6.6–7.7/6–8 2:1) in 0.04% methylcellulose, voltage of separation: 8 kV, separation time 20 min.

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 A₂ 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 μ l 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 μ l 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).

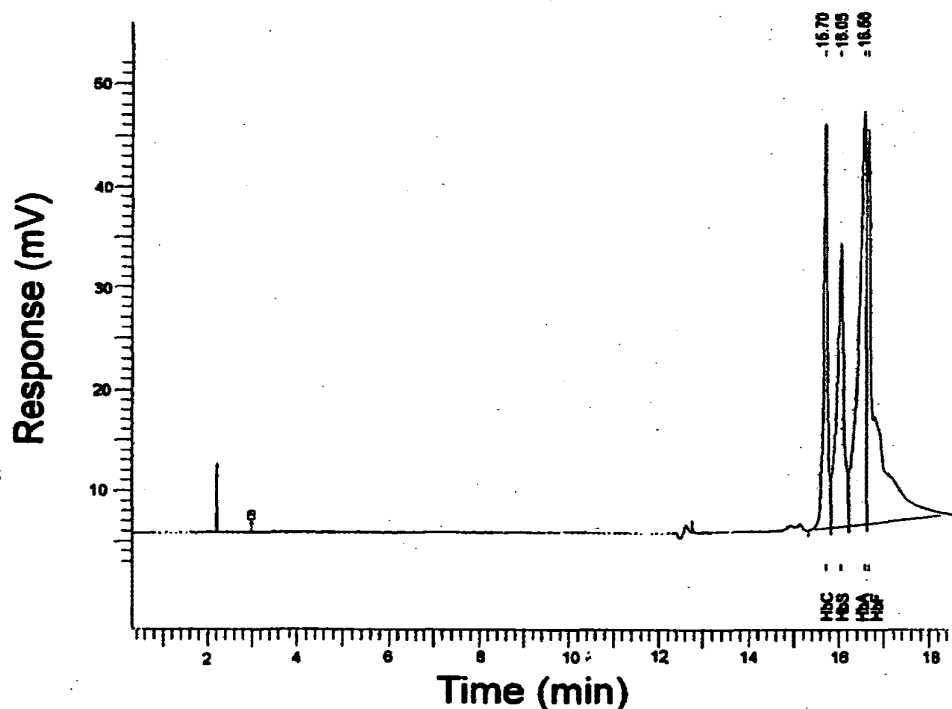


Fig. 2. shows capillary electrophoretic separation of Helena Titan III-H ASFC hemo control. From left HbC, HbS, HbA and HbF. Conditions of separation: Buffer: 100 mM Boric acid pH 9.98, voltage of separation 10 kV, separation time 19 min. Note incomplete separation of HbA and HbF.

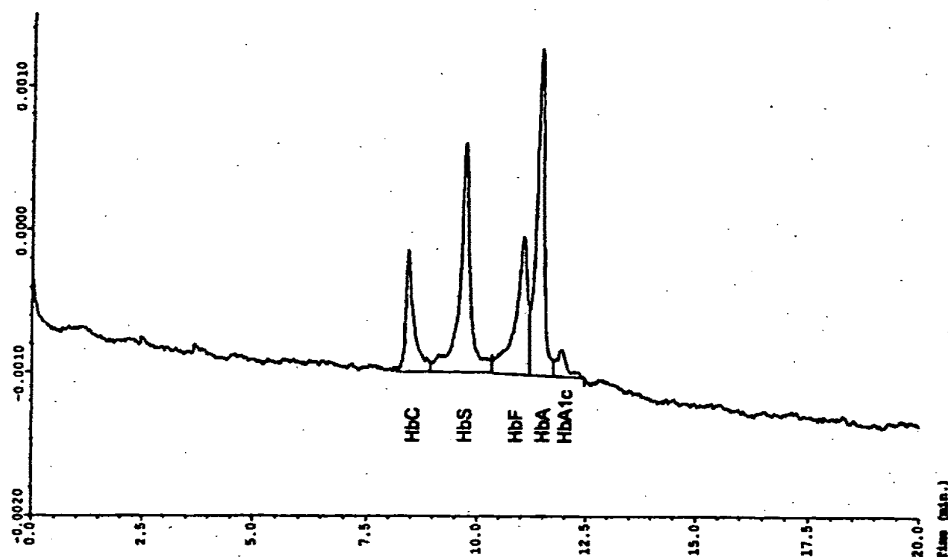


Fig. 3. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control with red cells from known HbS patient added. From left, HbC, HbS, HbF, HbA and HbA1c peaks. Note increased HbS and HbA peaks compared to Fig. 1. Conditions similar to Fig. 1.

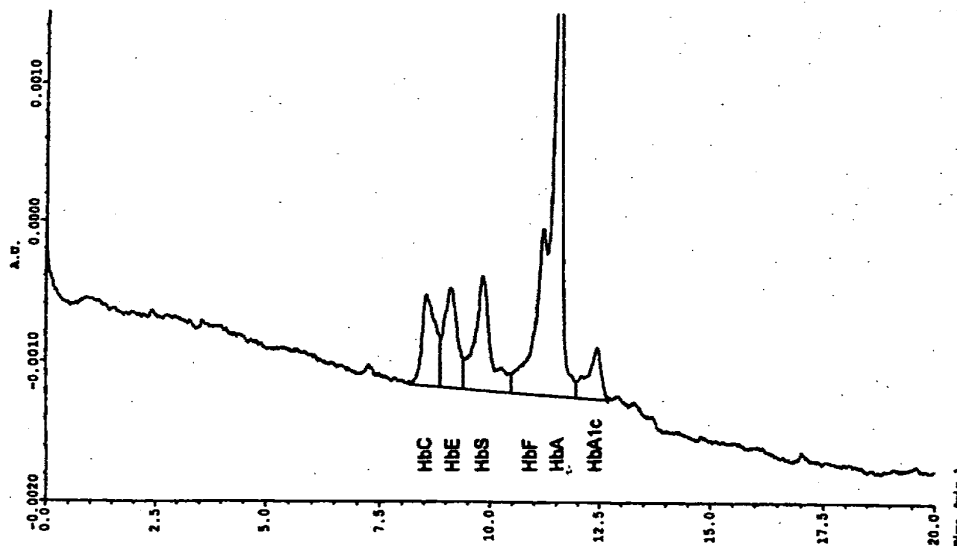


Fig. 4. Electropherogram of capillary isoelectric focusing of Helena Titan III-H ASFC Hemo Control with red cells from known HbE patient added. From left, HbC, HbE, HbS, HbF, HbA and HbA1c peaks. Conditions similar to Fig. 1.

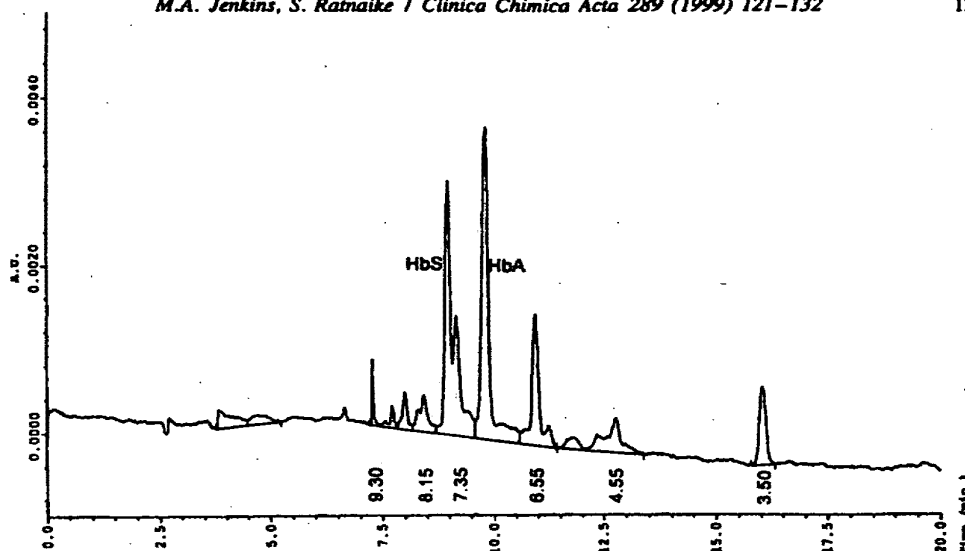


Fig. 5. Electropherogram of Pharmacia isoelectric calibration kit markers pH 3–10 plus sample from known HbS patient. pI of HbS is given as 7.21. From electropherogram, marker 7.35 appears to have slightly lower pI than HbS peak. Ampholytes changed to pH 3–10 for this electropherogram only.

We used 10 μ l of Pharmacia isoelectric focusing calibration kit in haemolyzing/methylcellulose/ampholyte mixture. We added 1 μ l 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

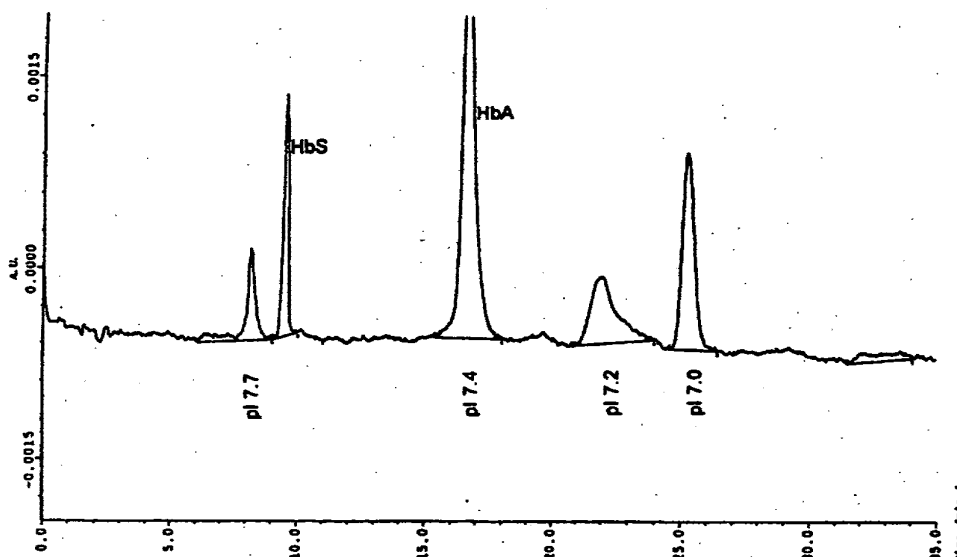


Fig. 6. Electropherogram of Bio-Mark pI markers 7.0, 7.2 and 7.7 with added sample from known HbS patient. HbA and marker pI 7.4 appear to coelute. pI of HbA has been previously reported as 6.98 (2). Time has been extended to try to separate marker and HbA.

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 HbA₂, 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

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].

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 HbA₂ quantitation. The consumable and labour costs involved in a traditional haemoglobin electrophoresis run of five samples, including HbA₂ quantitation by anion-exchange columns and that for CIEF/CE quantitation of HbA₂ is outlined in Table 1. We note that for a run of five samples, a traditional

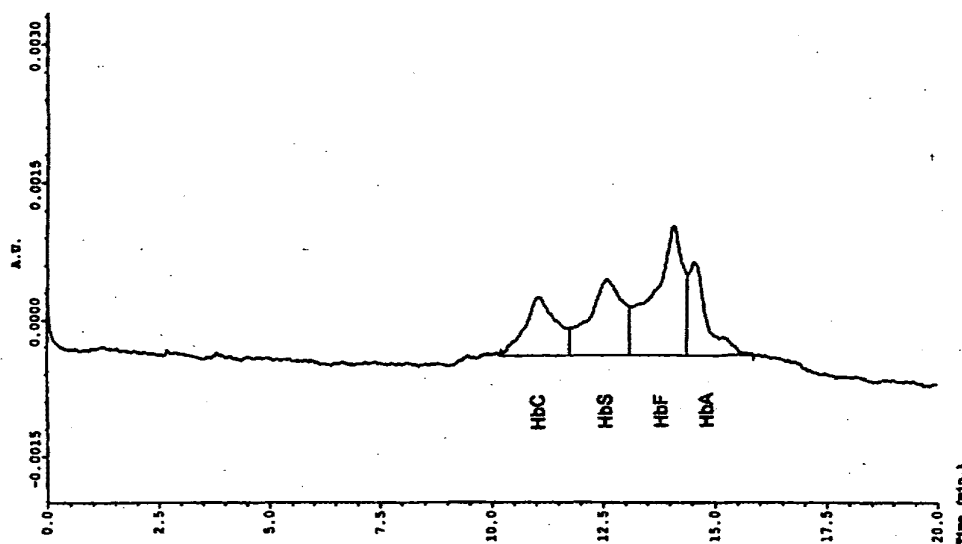


Fig. 7. Electropherogram of Helena AFSC Control stored for 2 months at 4°C. Comparison to Fig. 1 suggests this material might not be suitable for capillary electrophoresis assays after this length of storage.

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 HbA₂) compared to capillary electrophoretic HbA₂ 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

haemoglobin gel electrophoresis run with column quantitation cost \$A125.40 versus \$A31.26 for the CIEF/CE quantitation of HbA₂.

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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