

Evaluation of hemoglobin A₂ 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 HbA₂, 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 HbA₂, useful as a screening technique for the presence of hemoglobin variants, but unsuitable for the quantification of HbF.

Normal mammalian hemoglobin consists of two α -pha and two nonalpha globin chains, each of which is covalently linked to a central heme group. Adult hemoglobin is >95% HbA, which contains two α -pha chains and two beta chains. The minor constituents of normal adult blood consist of HbA₂, which contains two α -pha chains and two delta chains, and HbF, which contains two α -pha 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 imbalance 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 α -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, HbA₂, HB Variants, thalassemia, hemoglobinopathy, capillary electrophoresis

Abbreviations

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

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 HbA₂ 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 commer-

cial CE instruments can be automated by computer-controlled sample injection, on-line detection, and data acquisition. Using coated capillaries for IEF, Hempe and Craver¹⁰ have been able to use CE to separate HbA, A1c, F, D, S, and A₂ 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 HbA₂ 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 mM sodium hydroxide, 2 min with distilled water, and 3 min with assay buffer (100 mM 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 mM KCN/5 mM 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 1.27.10² mm vacuum injection.

Control material

Commercial elevated HbA₂ 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 HbA₂, 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.

HbA₂ 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). HbA₂ and HbS are eluted with the A₂ developer and HbS developer, respectively. The HbA₂ 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 HbA₂. 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 HbA₂ is shown in Figure 1. The correlation between the two

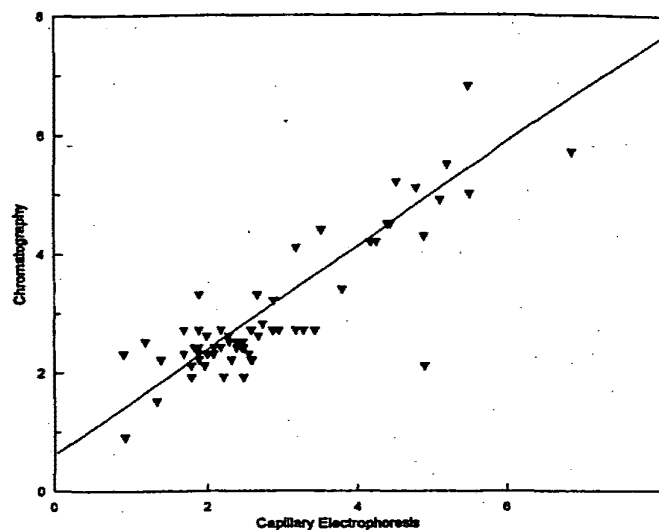


FIGURE 1 Comparison of quantification of HbA₂ by anion-exchange cellulose chromatography columns compared to CE quantification. The equation was $y = 0.9x + 0.6$, $p < 0.0005$, $r = 0.863$. HbE samples were excluded from comparison.

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 Köln	1st peak: 0.76-0.78
	2nd peak: 0.29-0.33
HbF	+0.09

methods (excluding the HbE specimens) was 0.863. The equation $y = 0.9x + 0.6$ indicated that the value for A₂ obtained by CE is approximately 10% higher than that obtained by chromatography. Results for HbA₂ 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 HbA₂ from HbE variants in contrast to HbA₂ anion-exchange columns, cellulose acetate, or citrate agar electrophoresis, none of which will separate HbA₂ 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

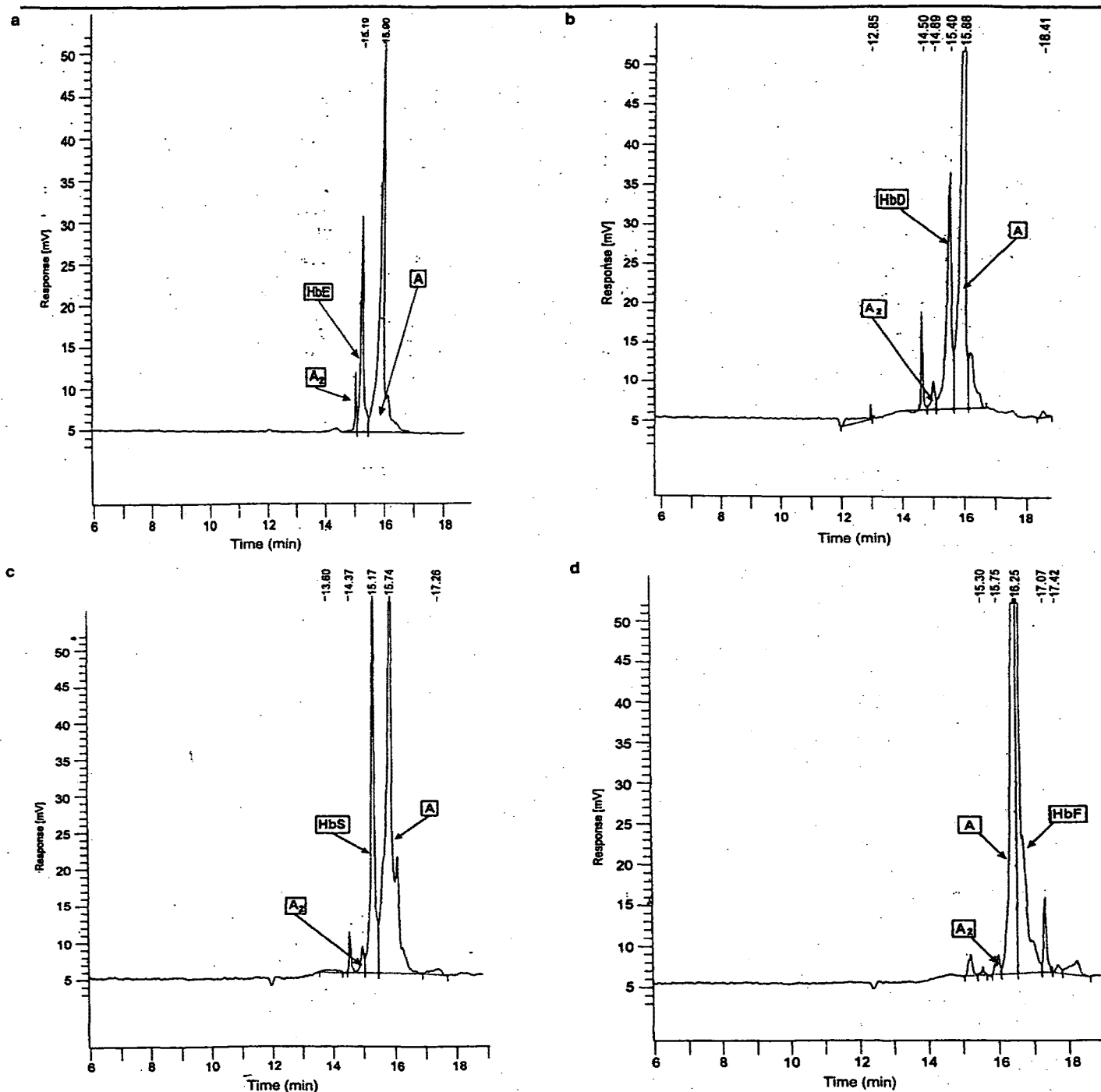


FIGURE 2 Capillary electropherograms of a) HbE variant, b) HbD variant, c) HbS variant, and d) sample with 37% HbF from a six-week-old child. HbA and HbA₂ components are marked on each tracing. Electrophoretic conditions: 100 mM boric acid buffer, pH 9.98; injection 1 sec, 1.27×10^2 mm vacuum injection; 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.

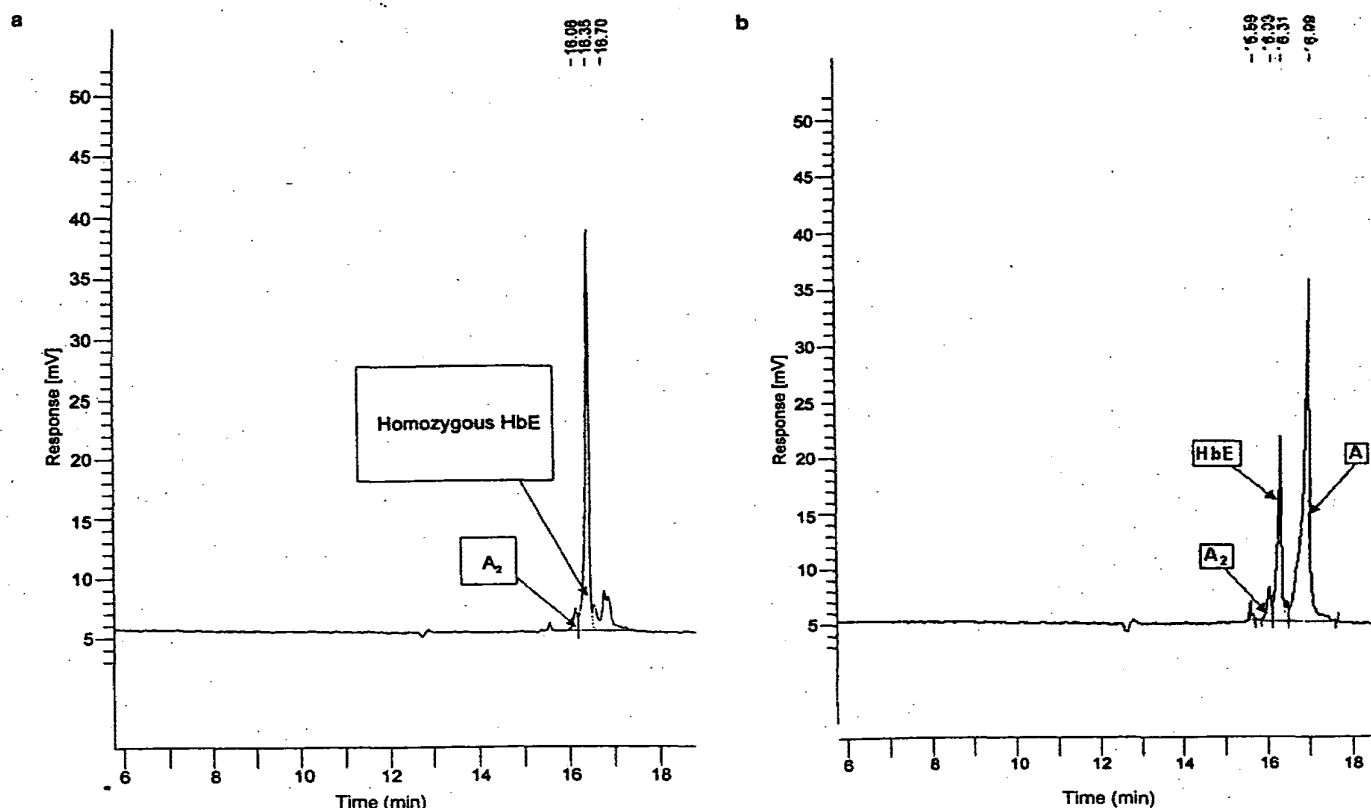


FIGURE 3 Capillary electropherograms of a) homozygous HbE and b) homozygous HbE blood mixed with an equal quantity of blood from a normal individual. Retention time for peaks is shown at the top of the electropherograms. Electrophoretic conditions as in Figure 2.

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 Köln	2	0/2
HbF	1	0/1

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 ma-

Table 3

COEFFICIENTS OF VARIATION OF HbA₂ CONTROL MATERIAL BY ANION-EXCHANGE CELLULOSE CHROMATOGRAPHY COLUMNS AND HbA₂ BY CE (N REFERS TO NUMBER OF RUNS IN WHICH SAMPLE WAS ASSAYED)

HbA ₂	n	Concentration found	CV (%)	Time difference A-A ₂
control ^a				
A ₂ columns	16	5.00-6.60	7.74	Not applicable
CE	20	4.69-6.42	8.44	0.91-1.02

terial. Reproducibility studies for both methods for HbA₂ 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-

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 HbA₂ (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 HbA₂ quantitation and for detection of the presence of hemoglobin variants in the setting of a routine clinical laboratory.

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