Journal of Chromatography A, 652 (1993) 119–129
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 066

Capillary electrophoresis of abnormal hemoglobins associated with α-thalassemias

Mingde Zhu, Tim Wehr,* Viktorya Levi, Roberto Rodriguez, Kathleen Shiffer and Zhu A. Cao

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547 (USA)

ABSTRACT

Capillary electrophoresis was evaluated for separation of hemoglobin species associated with α-thalassemias, and for identification of hemoglobin variants commonly found in the same human populations. Separation of hemoglobins was achieved using capillary isoelectric focusing with chemical mobilization; visible-wavelength absorbance detection was used to identify hemoglobins against a background of nonheme-containing proteins. This technique could easily differentiate hemoglobins Bart's and H (associated with α-thalassemias) from hemoglobin variants. Analysis of globin chains derived from intact hemoglobins was performed by free zone capillary electrophoresis under denaturing conditions. This technique was useful for distinguishing Hb Bart's and Hb H, and for confirming the identity of hemoglobin variants.

INTRODUCTION

Thalassemias are blood disorders associated with reduced production of the globin chains of hemoglobin. In α-thalassemias, reduction of α-globin production most commonly arises from deletion of one or more of the four α-globin genes, resulting in anemias of varying degrees of severity from asymptomatic to lethal [1,2]. As a consequence of reduced α-globin chain production, α-thalassemias are characterized by appearance of abnormal hemoglobin species in varying amounts depending upon the nature and severity of the genetic defect. In the most severe form of α-thalassemia, hemoglobin Bart's *hydrops fetalis*, there is no α-globin chain production and the fetus dies before or soon after birth. Hemoglobin Bart's is characterized by the appearance of a tetramer composed of four chains of γ-hemoglobin. Hemoglobin H disease, usually manifested by milder forms of anemia, is characterized by the presence of Hb H, a tetramer composed of four β-globin molecules.

Particular hemoglobin disorders are known to occur at higher frequencies in certain geographical areas and within certain ethnic groups. Thalassemias are prevalent in Southeast Asia, and α-thalassemia gene frequencies approach 40% in northern Thailand and Laos [2]. A number of hemoglobin variants are also common in Southeast Asian populations, and these may give rise to anemias which mimic the symptoms of thalassemias or may be coinherited with α-globin gene deletions and potentiate their effects [2]. Hemoglobin E, a variant found in high frequency in the region joining Thailand, Laos and Cambodia, results from a replacement of glutamic acid by lysine in position 26 of the β-globin gene. The hemoglobin E mutation results in reduced β-globin production and a β-thalassemia phenotype. The hemoglobin Constant Spring variant (Hb CS) arises from a mutation in the α-globin chain termination codon which results in a 24–26 amino acid addition to the α-globin polypeptide. The Hb CS globin mRNA is unstable, and the resulting reduction in α-globin production leads to an α-thalassemia condition. Coinheritance of hemoglobin E or

* Corresponding author.

0021-9673/93/$06.00

Constant Spring mutations with α-globin chain deletions results in complex phenotypes with varying levels of Hb Bart's and H, Hb CS and Hb E.

In a previous report [3] we described the separation of normal hemoglobins and hemoglobin variants using capillary isoelectric focusing (cIEF), and separation of globin chains by free zone capillary electrophoresis under denaturing conditions. In this report we have extended the study using cIEF to characterize the hemoglobin species associated with α-thalassemias and hemoglobin variants common in Southeast Asian populations; globin chain analysis was also performed using free zone capillary electrophoresis. In addition, hemoglobins and globin chains from a patient carrying the G Philadelphia and C variants were analyzed; these mutations (an asparagine-to-lysine replacement in position 48 of the α-globin chain, and a glutamic acid-to-lysine replacement in position 6 of the β-globin chain, respectively) are common among some populations in the USA.

EXPERIMENTAL

Materials

Hemoglobin AF electrophoresis control was obtained from Isolab (Akron, OH, USA). Hemoglobin AFSE reference standard, Bio-Lyte pH 3–10 ampholytes, and AG 501-X8 resin were obtained from Bio-Rad Labs. (Hercules, CA, USA). Reduced Triton X-100 was obtained from Aldrich (Milwaukee, WI, USA). Patient samples of hemoglobin E were generously provided by Childrens Hospital, Oakland, CA, USA. Patient samples of hemoglobin Bart's, hemoglobin H, hemoglobin G Philadelphia/C, and hemoglobin Constant Spring were generously provided by Kaiser Permanente Hospital, Oakland, CA, USA.

Gel isoelectric focusing of hemoglobins

Whole blood samples (50 μl) were lysed by 1:10 dilution with deionized water. Hemoglobin content was measured by conversion of cyanomethemoglobin using Drabkin's reagent [4] and spectrophotometric determination at 540 nm. Samples were diluted to 17 mg/ml hemoglobin, and 170 μg hemoglobin were applied to each lane of the gel. Isoelectric focusing was carried out using Pharmacia Ampholine PAG plate isoelectric focusing gels, pH 5.5–8.5 (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). The anolyte solution was 0.5 *M* acetic acid + 0.1% KCN and the catholyte solution was 0.5 *M* ethanolamine + 0.1% KCN. Following isoelectric focusing, heme protein-containing bands were selectively stained with *o*-dianisidine in the presence of hydrogen peroxide [1]. In this procedure, *o*-dianisidine is oxidized by the heme portion of the hemoglobin molecule, producing an insoluble brown precipitate which is proportional to the amount of hemoglobin present.

Preparation of hemoglobin samples for capillary electrophoresis

Whole blood samples (50 μl) containing hemoglobin Bart's, hemoglobin E, hemoglobin G Philadelphia C, or hemoglobin Constant Spring were added to 250 μl isotonic saline (0.9% sodium chloride) and centrifuged at 2000 *g* for 10 min. After removal of the supernatant, erythrocytes were lysed by the addition of 1 ml of deionized water. Whole blood samples (50 μl) containing hemoglobin H were lysed directly by addition of 1 ml deionized water. Hemoglobin content was measured by conversion to cyanomethemoglobin using Drabkin's reagent [4] and spectrophotometric determination at 540 nm. All samples were diluted with deionized water to a final hemoglobin concentration of 2.4 mg/ml.

Preparation of globin chains

One volume of hemoglobin sample was mixed with 20–40 volumes of acidic acetone [2% concentrated hydrochloric acid (36%) in acetone] and stirred briefly. The mixture was held at 4°C for 30 min, then centrifuged for 2 min in a microcentrifuge. After drawing off the supernatant, the precipitate was washed twice with acetone and dissolved in 10 m*M* sodium phosphate buffer (pH 2.5) + 7 *M* urea + 0.1% reduced Triton X-100. Prior to use, the buffer + urea solution was stirred with AG-501-X8 resin to remove urea impurities.

Capillary electrophoresis

All separations were performed with the BioFocus 3000 automated capillary electrophoresis system (Bio-Rad Labs.). All capillaries used in this study were coated internally with a covalently attached hydrophilic linear polymer [5]. Capillaries were enclosed in a cartridge format and thermostated at 20°C by liquid cooling. The distance from the monitor point to the capillary outlet was 4.6 cm.

Isoelectric focusing of hemoglobins was carried out using 17 cm × 25 μm I.D. coated capillaries. Capillaries were purged with water and 10 m*M* phosphoric acid between separations. Hemoglobin samples were mixed with pH 3–10 ampholytes to a final ampholyte concentration of 2% and total hemoglobin concentration of 240–480 μg/ml. The sample + ampholyte mixtures were pressure-injected into the capillary at 100 p.s.i. (689 476 Pa) for 60 s. Focusing was carried out at 10 kV constant voltage for 5 min using 40 m*M* sodium hydroxide as catholyte and 20 m*M* phosphoric acid as anolyte. Cathodic mobilization was performed by replacing the catholyte was a proprietary zwitterionic solution (Bio-Rad Labs.). Mobilization voltage was 10 kV. Detection was at 415 nm. Free zone electrophoresis of globin chains was carried out using 36 cm × 50 μm I.D. coated capillaries. The electrophoresis buffer was 100 m*M* sodium phosphate (pH 2.5) + 7 *M* urea + 1% reduced Triton X-100. Samples were pressure-injected for 5 p.s.i. · s and separated at 8 kV constant voltage. Detection was at 210 nm.

RESULTS AND DISCUSSION

Capillary isoelectric focusing of hemoglobins

cIEF provides extremely high resolution of proteins based on small differences in their isoelectric points. This is due to the use of high field strengths (typically 400–600 V/cm) combined with good heat dissipation using narrow-bore (25 μm I.D.) capillaries with liquid cooling. Once focused, protein zones may be mobilized past the detector monitor point by changing the composition of the anolyte or catholyte solutions [6]. In this investigation, proteins were mobilized towards the cathode by exchanging the 40 m*M* NaOH for a zwitterion solution; in this process proteins across the full pH range from 4 to 10 are efficiently mobilized [7], and sharp zones are maintained by application of high voltage during the mobilization process. The use of coated capillaries eliminated electroosmosis which would otherwise disrupt the focusing process. The resolving power of cIEF is seen in the separation of a hemoglobin reference standard containing hemoglobins A, F, S and E (Fig. 1). These species have isoelectric points of 7.10, 7.15, 7.25 and 7.42, respectively. In this and other cIEF separations of hemoglobins, detection was performed at 415 nm, which allowed high-sensitivity detection of hemoglobins against a background of non-heme-containing proteins (Fig. 2).

CE of hemoglobin Bart's

The cIEF profile of a sample from a patient with hemoglobin Bart's disease revealed a major peak for normal adult hemoglobin A_0 and an elevated level of fetal hemoglobin F (Fig. 3). In addition, there were a series of minor peaks migrating late in the separation (18.5–19.5 min), corresponding to heme-containing proteins with isoelectric points much lower than normal hemoglobins and known hemoglobin variants. These were observed in all Hb Bart's samples separated by cIEF, and are thought to include the γ-chain tetramer characteristic of this α-

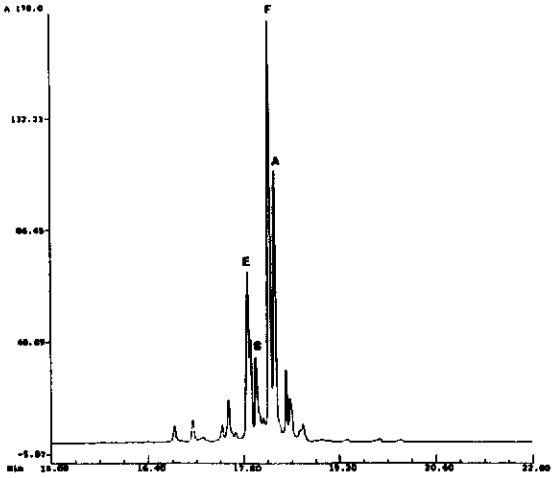

Fig. 1. Capillary isoelectric focusing of hemoglobin A, F, S and E reference standard.

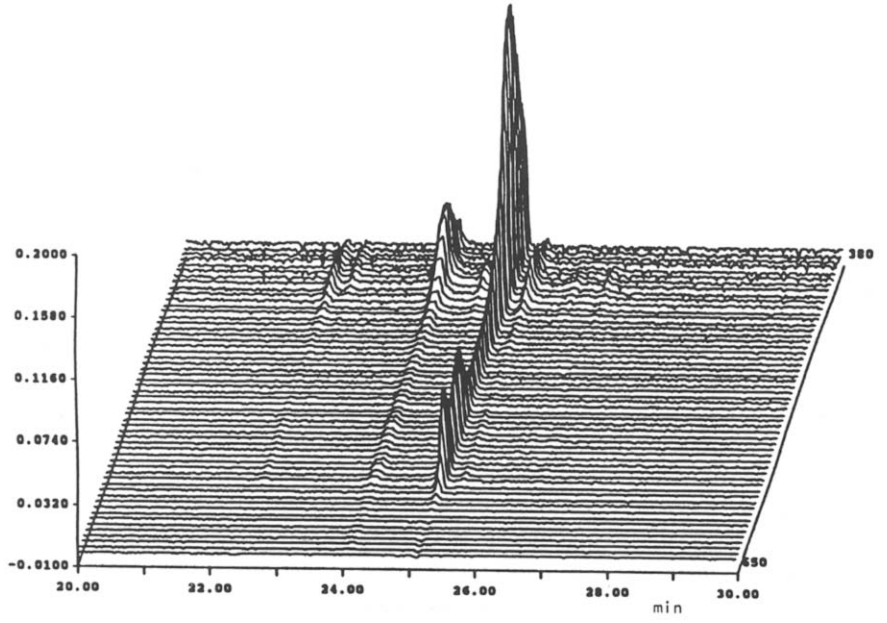

Fig. 2. Capillary isoelectric focusing of hemoglobin A using scanning detection in the 380–650 nm spectral region. The major peak migrating at 25 min is hemoglobin A_0 and the minor peak migrating at 24 min is hemoglobin A_2. The hemoglobin A_0 spectrum exhibits a maximum at 415 nm. *x*-Axis, time (min); *y*-axis, absorbance; *z*-axis, wavelength (nm).

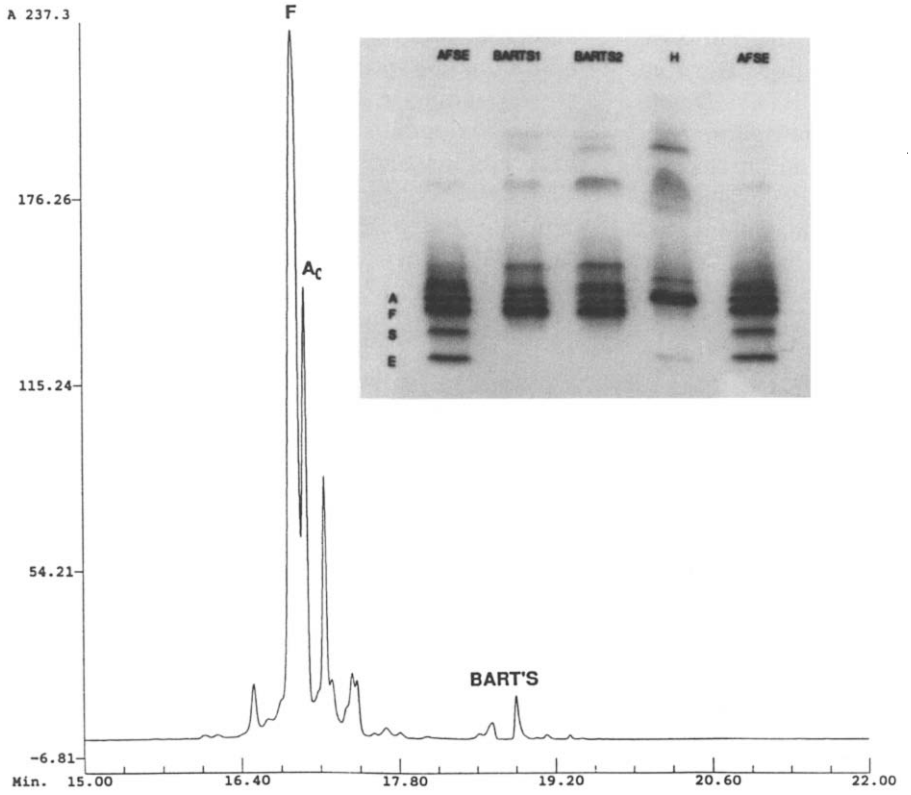

Fig. 3. Capillary isoelectric focusing of hemoglobins from a patient with hemoglobin Bart's disease. Inset: isoelectric focusing gel of hemoglobin samples stained with *o*-dianisidine (1st and 5th lanes: hemoglobin AFSE reference standard; 2nd and 3rd lanes: hemoglobin Bart's samples; 4th lane: hemoglobin H). The sample separated by cIEF is the one shown in lane 3 of the IEF gel.

thalassemia. The series of peaks migrating after Hb A_0 (17–18 min) have not been identified, but probably represent glycated forms of Hb A_0, acetylated Hb F, and degradation products.

Comparison of the cIEF profile with the band pattern on a conventional isoelectric focusing gel (Fig. 3 inset, 2nd and 3rd lanes) revealed similar patterns. The gel showed major bands for Hb A_0 and F, a series of minor bands focusing above the Hb A_0 band, and an Hb Bart's band focusing in the acidic portion of the gel, corresponding to an approximate pI of 6. A comparison of the two Hb Bart's samples indicated a higher level of Hb Bart's in the 3rd lane, which was in agreement with cIEF peak area percent values obtained for the two samples (data not shown).

The globin chain content of the Hb Bart's sample was analyzed by free zone capillary electrophoresis under denaturing conditions. This separation was carried out in the presence of 7 *M* urea with detection at 210 nm; at this wavelength the globin chains could be detected with satisfactory sensitivity and minimal interference from the urea in the electrophoresis buffer (Fig. 4). The electropherogram showed the presence of the normal α-, β- and γ-globin chains but in altered ratios (Fig. 5), with γ-globin elevated relative to β-globin.

CE of hemoglobin H

The cIEF profile of a sample from a patient with hemoglobin H disease exhibited a major peak for normal adult hemoglobins A_0 and A_2 and a small amount of Hb F (Fig. 6). In addition, a late-migrating doublet peak was observed which was assumed to represent the β-chain tetramer associated with Hb H disease. The isoelectric focusing gel pattern of this sample (Fig. 6 inset, 4th lane) showed the same major band for Hb A_0 and a band focusing in the acidic part of the gel. There were two differences observed between the IEF pattern and the band pattern of the conventional IEF gel. First, the gel exhibited a diffuse band focusing in the same position as Hb Bart's, which may represent degradation products. This material was either not detected in cIEF, or co-migrated with Hb H. Second, an Hb A_2 band was not observed in the

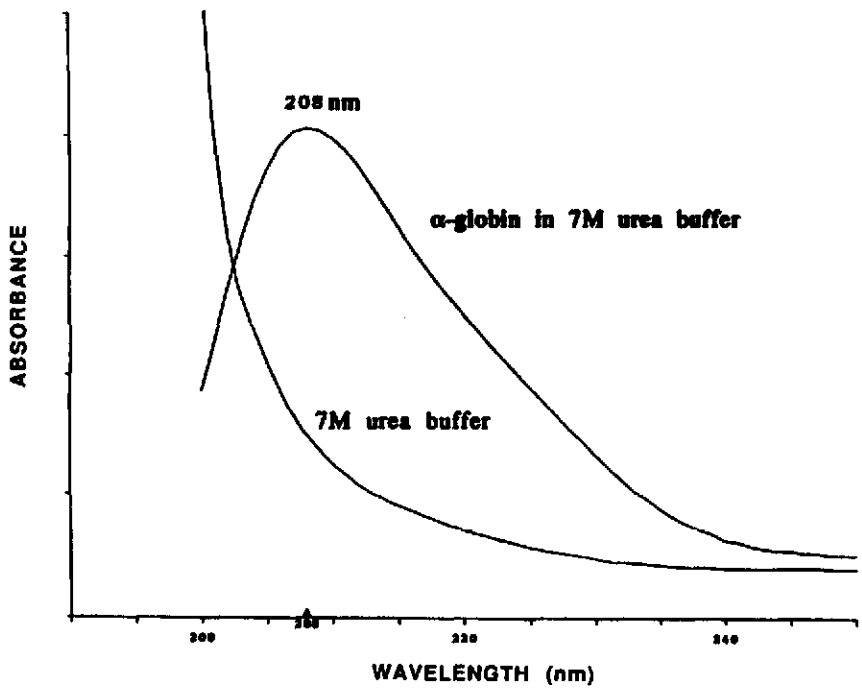

Fig. 4. UV spectra obtained during free zone capillary electrophoresis with scanning detection. (Lower trace) background absorbance of electrophoresis buffer (100 m*M* sodium phosphate, pH 2.5, +0.1% reduced Triton X-100 + 7 *M* urea). (Upper trace) spectrum of α-globin in electrophoresis buffer.

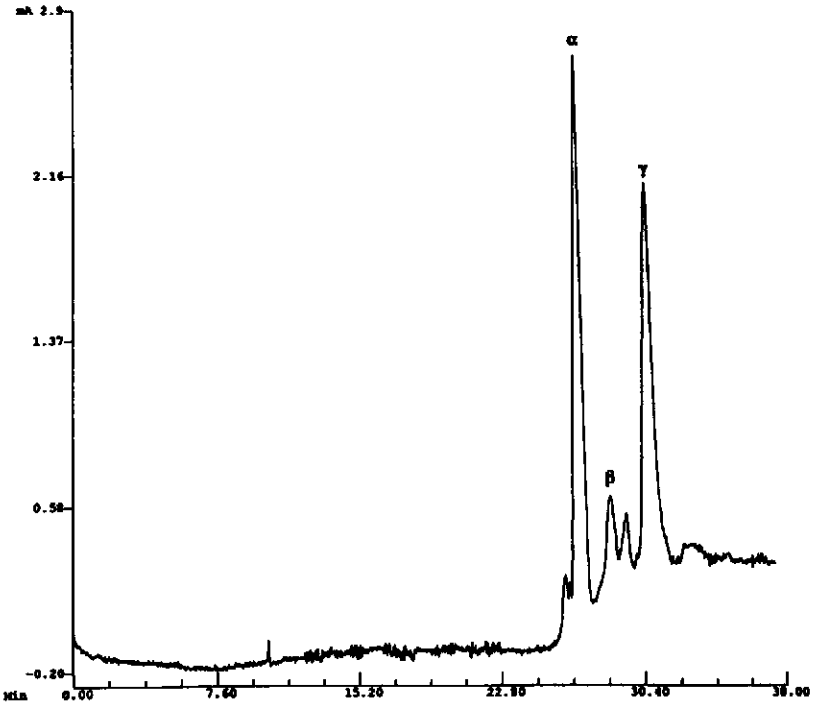

Fig. 5. Free zone capillary electrophoresis separation of globin chains from the hemoglobin Bart's sample shown in Fig. 3.

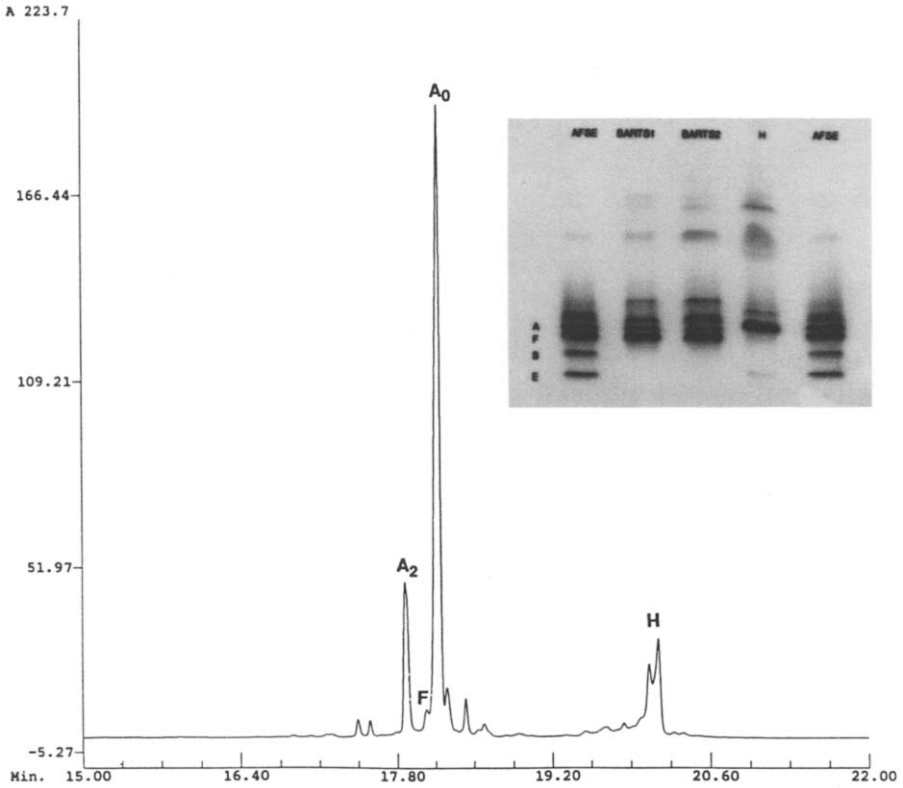

Fig. 6. Capillary isoelectric focusing of hemoglobins from a patient with hemoglobin H disease. Inset: isoelectric focusing gel of hemoglobin samples stained with *o*-dianisidine (1st and 5th lanes: hemoglobin AFSE reference standard; 4th lane: hemoglobin H sample; 2nd and 3rd lane: hemoglobins Bart's).

gel pattern, while a distinct peak migrating in the position of Hb A_2 was observed in the cIEF electropherogram. We have no explanation for this difference. Globin chain analysis of the Hb H sample (in contrast to the results obtained with Hb Bart's) showed major peaks for α- and β-globin, with only a minor amount of γ-globin (Fig. 7).

CE of hemoglobin E

The cIEF profile for a sample obtained from a patient homozygous for the Hb E mutation exhibited a single major peak for hemoglobin E and two minor peaks (Fig. 8). Since both β-globin genes carry the Hb E mutation, only β-globin polypeptides with the glutamic acid-to-lysine replacement at position 26 are synthesized, and no Hb A_0 should be formed. The two minor peaks probably represent glycated Hb E and fetal Hb F. The identity of the second minor peak was confirmed in another homozygous Hb E sample by capillary isoelectric focusing of the sample following addition of an Hb AF electrophoresis control (Fig. 9).

The gel IEF profile (Fig. 8, inset) exhibited a band pattern similar to the cIEF peak profile, *e.g.* a single major band for Hb E and indistinct minor bands. Free zone CE globin chain analysis of the Hb E sample exhibited only a single major peak (Fig. 10); we believe this to represent comigration of the mutated β-globin chains with α-globins due to increased β-globin mobility imparted by the substitution of the basic lysine residue for glutamic acid.

CE of G Philadelphia C

This patient carried two hemoglobin mutations, Hb G Philadelphia (asparagine to lysine replacement at position 68 in the α-globin gene) and Hb C (glutamic to lysine replacement at position 6 in the β-globin gene). Therefore at least four hemoglobin species would be expected: $\alpha\beta$, $\alpha_G\beta$, $\alpha\beta_C$ and $\alpha_G\beta_C$. The cIEF profile showed four major peaks (Fig. 11) which agreed with the gel IEF band pattern observed for the same sample (Fig. 11 inset). Globin chain analysis also displayed the expected profile of four peaks (Fig. 12). Since reference standards are not readily available, the peak assignments for

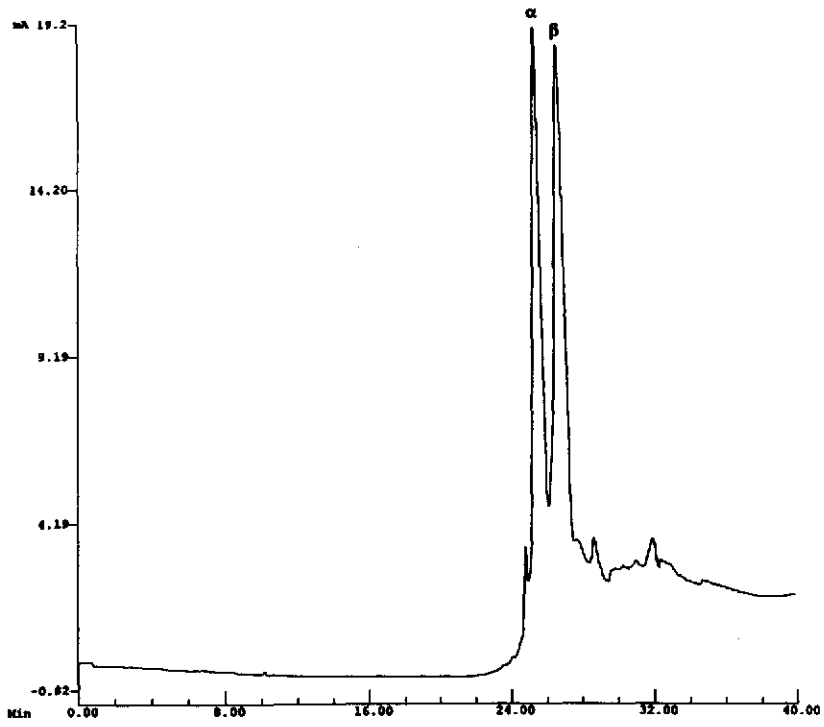

Fig. 7. Free zone capillary electrophoresis separation of globin chains from the hemoglobin H sample shown in Fig. 6.

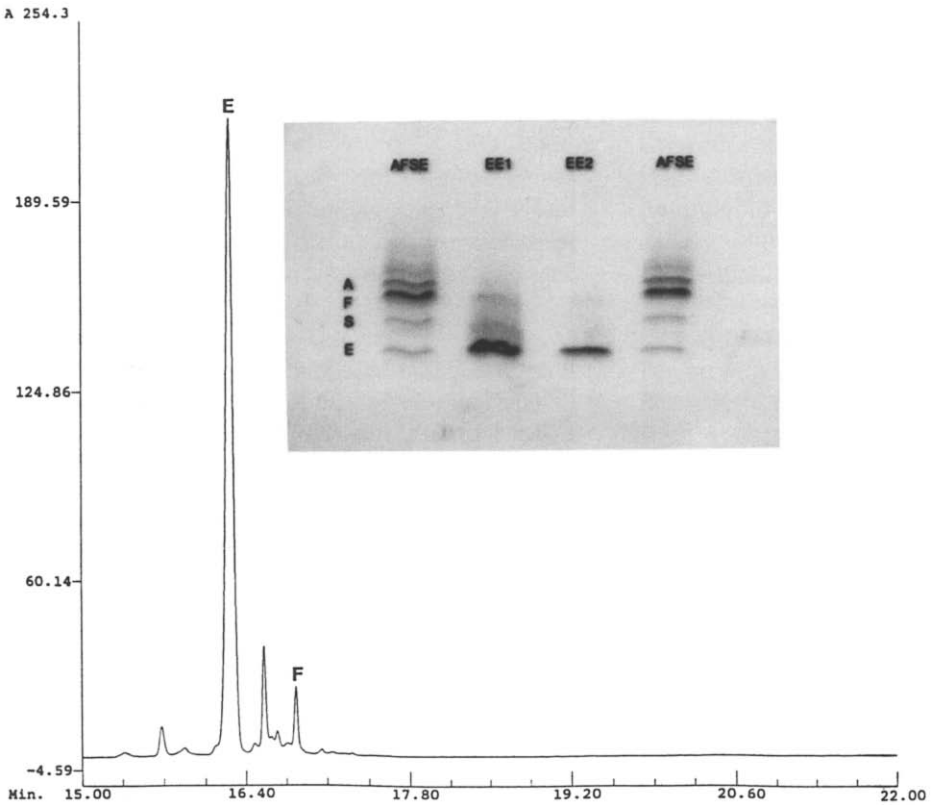

Fig. 8. Capillary isoelectric focusing of hemoglobins from a patient homozygous for the hemoglobin E mutation. Inset: isoelectric focusing gel of hemoglobin samples stained with *o*-dianisidine (1st and 4th lanes: hemoglobin AFSE reference standard; 2nd and 3rd lanes: hemoglobin E samples). The sample separated by cIEF is the one shown in the 2nd lane of the IEF gel.

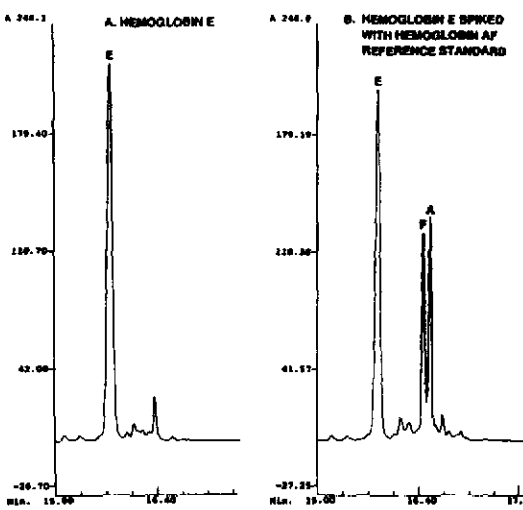

Fig. 9. Capillary isoelectric focusing of the Hb E sample shown in Fig. 8 after addition of a hemoglobin AF electrophoresis control.

β_C and α_G in Fig. 12 are tentative and based on assumptions drawn from the amino acid substitutions.

cIEF of hemoglobin Constant Spring

In conventional isoelectric focusing gels, hemoglobin Constant Spring focuses towards the very basic end of the pH gradient with an apparent pI of about 7.47 [8]. We have obtained preliminary cIEF results with a sample containing about 1% Hb Constant Spring which indicated a minor component migrating in the basic region of the electropherogram (Fig. 13). However, because of the low concentration of this species and its instability, confirmation of this component will require additional work with samples containing larger concentrations of Hb Constant Spring.

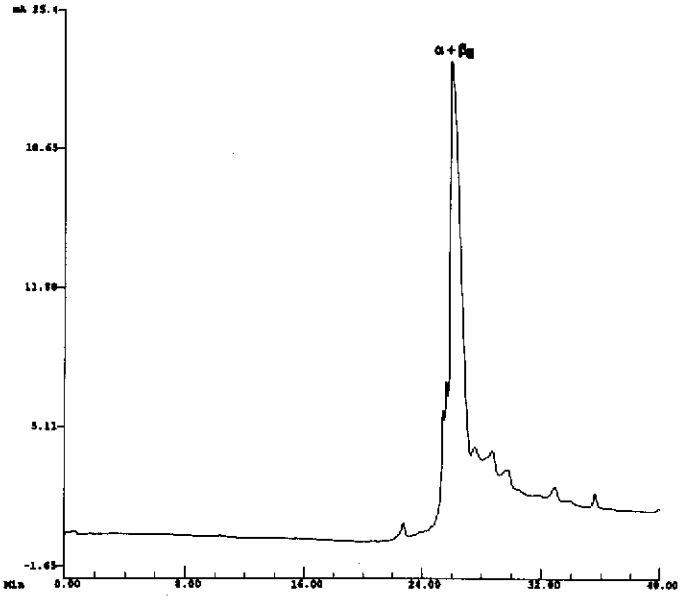

Fig. 10. Free zone capillary electrophoresis separation of globin chains from the hemoglobin E sample shown in Fig. 8.

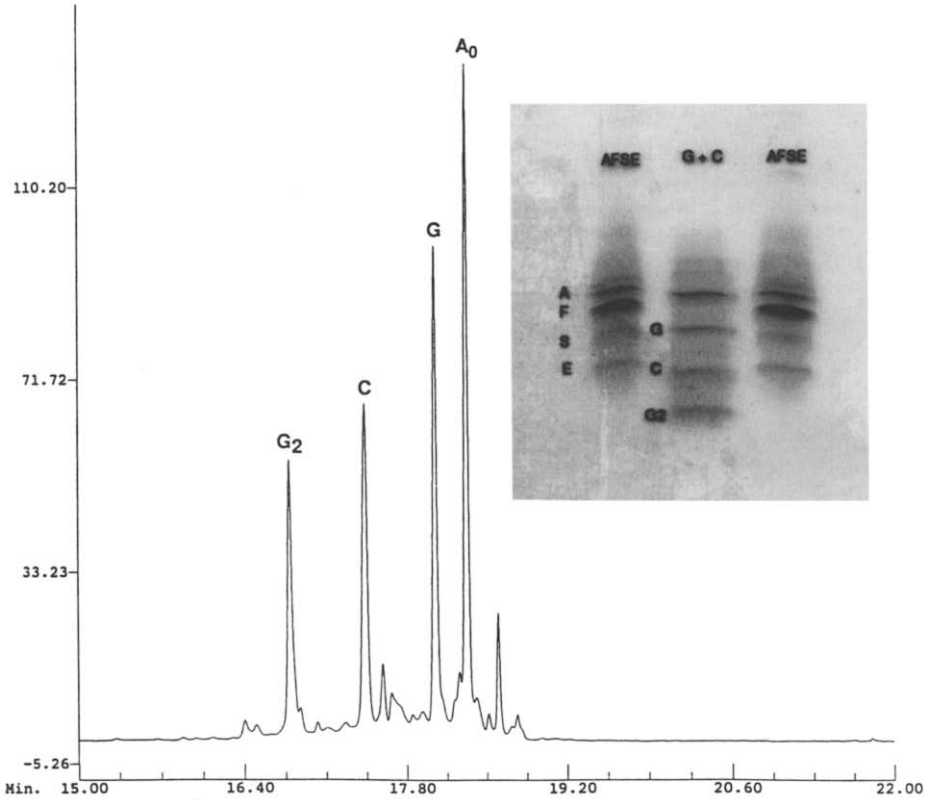

Fig. 11. Capillary isoelectric focusing of hemoglobins from a patient carrying the Hb G Philadelphia and Hb C mutations. Inset: isoelectric focusing gel of hemoglobin samples stained with *o*-dianisidine (1st and 3rd lanes: hemoglobin AFSE reference standard; 2nd lane: hemoglobin G Philadelphia C sample).

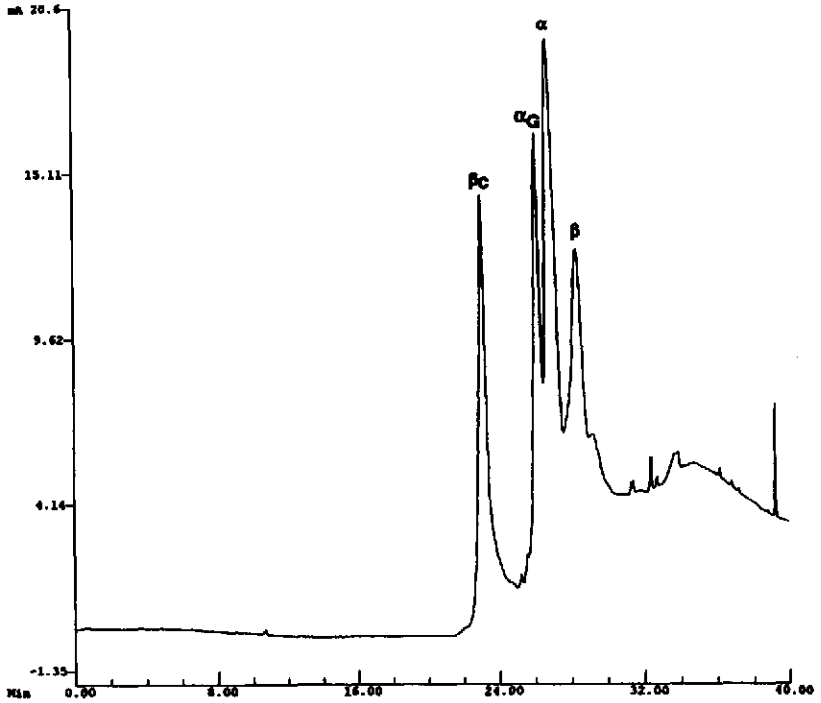

Fig. 12. Free zone capillary electrophoresis separation of globin chains from the Hb G Philadelphia C sample shown in Fig. 11.

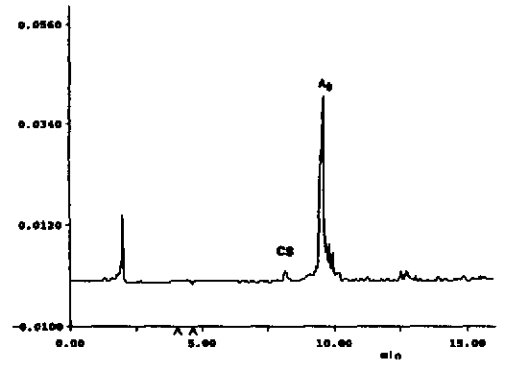

Fig. 13. Capillary isoelectric focusing of hemoglobins from a patient carrying the Hb Constant Spring mutation. *x*-Axis, time (min); *y*-axis, absorbance.

CONCLUSIONS

cIEF provides rapid, high-resolution separation of hemoglobin species, and specific detection of heme proteins in the visible region is highly sensitive and free of interferences. Hemoglobins Bart's and H, characteristic of α-thalassemia conditions common in Southeast Asia, can be easily distinguished by late-migrating components in the acidic portion of the pH gradient. Normal hemoglobins and known hemoglobin variants do not migrate in this region in cIEF. Hemoglobin H and hemoglobin Bart's samples were differentiated from each other by the distribution of globin chains analyzed by free zone electrophoresis under denaturing conditions. Hb Bart's samples exhibited major peaks for α- and γ-globin, while predominantly α- and β-globin chains were observed in Hb H samples.

The common hemoglobin variants could be distinguished from α-thalassemia samples (and from each other) by the presence of early-migrating species in the cIEF profile. Hemoglobin variants E, S, C, G Philadelphia, and Constant Spring all have isoelectric points higher than normal hemoglobins A_0 and F. Our results indicate that hemoglobins E, S, C, and G. Philadelphia are resolved from Hb A_0 and F. In addition, hemoglobins E, C, G Philadelphia can be distinguished by altered mobility of the mutant

globin chains using free zone capillary electrophoresis under denaturing conditions. This confirmatory analysis could be particular useful for identification of Hb E, which co-focuses with normal hemoglobin A_2 in conventional gel IEF.

REFERENCES

1 V.F. Fairbanks (Editor), *Hemoglobinopathies and Thalassemias, Laboratory Methods and Case Studies*, Brian C. Dekker, New York, 1980.
2 S. Fucharoen and P. Winichagoon, *Southeast Asean J. Trop. Med. Public Health*, 23 (1992) 647.
3 M. Zhu, R. Rodriguez, T. Wehr and C. Siebert, *J. Chromatogr.*, 608 (1992) 225.
4 E.J. van Kampen and W.G. Zijlstra, *Adv. Clin. Chem.*, 8 (1965) 141.
5 S. Hjertén, *J. Chromatogr.*, 247 (1985) 191.
6 S. Hjertén, J.-L. Liao and K. Yao, *J. Chromatogr.*, 387 (1987) 127.
7 M. Zhu, R. Rodriguez and T. Wehr, *J. Chromatogr.*, 559 (1991) 479.
8 P. Basset, F. Braconnier and J. Rosa, *J. Chromatogr.*, 227 (1982) 267.