CHROM. 24 337

Capillary electrophoresis of hemoglobins and globin chains

Mingde Zhu, Roberto Rodriguez, Tim Wehr and Chris Siebert

Analytical Systems Division, Life Science Group, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547 (USA)

ABSTRACT

Capillary isoelectric focusing (cIEF) and free zone capillary electrophoresis were evaluated for separation of native hemoglobins and globin chains. High-resolution separations of adult human hemoglobin A, fetal human hemoglobin F, and hemoglobin variants S and C were obtained using cIEF with cathodic mobilization. Absorbance detection in the UV and visible regions were compared, and on-line fast UV or visible-wavelength scanning detection was used to obtain spectral information on separated components. Globin chain analysis was performed on the same hemoglobin species by free zone capillary electrophoresis following precipitation of the protein with acidic acetone. Free zone separations were carried out at low pH in the presence of 7 M urea.

INTRODUCTION

Analysis of the hemoglobin composition of human blood is of major clinical interest because of the number of disorders associated with abnormal blood hemoglobin content [1,2]. These diseases are grouped into anemias arising from the presence of deleterious genes coding for defective sequence variants of hemoglobins, and thalassemias characterized by abnormal levels of globin chains. Because of the prevalence of genetically-derived anemias in many populations, routine clinical screening for defective hemoglobins has been implemented in many areas of the world. For example, the state of California in the USA has mandated screening of all newborns for the presence of defective hemoglobins in blood samples collected at birth. A second major interest in clinical hemoglobin analysis is the determination of glycosylated hemoglobins as a means of monitoring long-term blood glucose levels in diabetic patients.

A variety of analytical methods have been employed for hemoglobin determination including immunoassay, gel electrophoresis, and gel isoelectric focusing. More recently, high-performance cation-exchange chromatography has been applied to routine screening of blood hemoglobins [3]. This method has the advantages of rapid analysis times, automated processing of large numbers of samples, and quantitative analysis. High-sensitivity detection is possible by the use of on-line absorbance detection of native hemoglobins at visible wavelengths where non-heme proteins do not interfere.

We are interested in the use of capillary electrophoresis (CE) as an alternative technique for hemoglobins analysis. CE shares with high-performance liquid chromatography (HPLC) the advantages of automation and on-line detection, and offers separation modes which would be complementary to chromatography. In addition, CE is truly a microscale analytical method, consuming minute amounts of sample per analysis compared to HPLC. We have evaluated capillary isoelectric focusing as a method for separation of intact hemoglobins, and investigated the use of UV and visible scanning detection to identify and differentiate hemoglobin species. In addition, we have used free zone electrophoresis under denaturing conditions

Correspondence to: Dr. T. Wehr, Analytical Systems Division, Life Science Group, Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA.

to separate the globin chains derived from hemoglobins.

EXPERIMENTAL

Materials

Hemoglobins A, F, S and C were obtained from Isolab (Akron, OH, USA). Bio-Lyte pH 3–10 ampholytes and AG 501-X8 resin were obtained from Bio-Rad Labs. (Richmond, CA, USA). Triton X-100 reduced was obtained from Aldrich (Milwaukee, WI, USA). Reference standards of hemoglobin A, hemoglobins A + S, hemoglobins A + F, and hemoglobins A + C were the generous gift of Dr. Ken Dobra of the Bio-Rad Diagnostic Group.

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 mM sodium phosphate buffer (pH 3.2) + 7 M urea + 0.1% reduced Triton X-100. Prior to use, the buffer and 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, Richmond, CA, USA). All capillaries used in this study were coated internally with a covalently-attached hydrophilic linear polymer [4]. 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 4.5 cm.

Isoelectric focusing of hemoglobins was carried out using 17 cm × 25 μm I.D. coated capillaries. Capillaries were purged with water and 10 mM 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 about 1 mg/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 7 kV constant voltage

for 5 min using 40 mM sodium hydroxide as catholyte and 20 mM phosphoric acid as anolyte. Cathodic mobilization was performed by replacing the catholyte with a proprietary zwitterionic solution (Bio-Rad Labs.). Mobilization voltage was 8 kV. Single wavelength mode detection was at 280 nm; in scanning mode, spectra were acquired at 5-nm intervals.

Free zone electrophoresis of globin chains was carried out using 35 cm × 25 μm I.D. coated capillaries. The electrophoresis buffer was 100 mM sodium phosphate (pH 3.2) + 7 M urea + 1% reduced Triton X-100. Samples were loaded electrophoretically at 8 kV for 8 s and separated at 8 kV constant voltage. Detection was at 210 nm .

RESULTS AND DISCUSSION

Human hemoglobin is a tetramer consisting of two α-globin and two β-, δ- or γ-globin chains, to each of which is bound a heme group. The β chain contains 141 amino acid residues while the other chains contain 146 residues. Normal adult human blood contains hemoglobin A ($\alpha_2\beta_2$) and hemoglobin A_2 ($\alpha_2\delta_2$). Fetal blood contains hemoglobin F ($\alpha_2\gamma_2$) as the predominant species, and during the sixth months following birth hemoglobin F is replaced by hemoglobin A. Several hundred genetic variants of the hemoglobin molecule are known, many of which are associated with blood disorders. Since there are only two copies of the β-globin gene in the human genome, most of the known hemoglobinopathies are associated with mutations in the β-globin molecule. For example, sickle cell disease arises from a glutamic acid to valine transition at position 6 in the β-globin gene. In the homozygous state, this results in the production of the mutant hemoglobin S ($\alpha_2\beta_{S_2}$) which causes a characteristic sickling morphology of erythrocytes. A glutamic acid to lysine transition at the β^6 position results in production of hemoglobin C. Although benign in the heterozygous state, the β_C allele in the homozygous state or in the $\beta_C\beta_S$ heterozygote causes moderate to severe sickling.

Capillary isoelectric focusing (cIEF) of hemoglobin variants

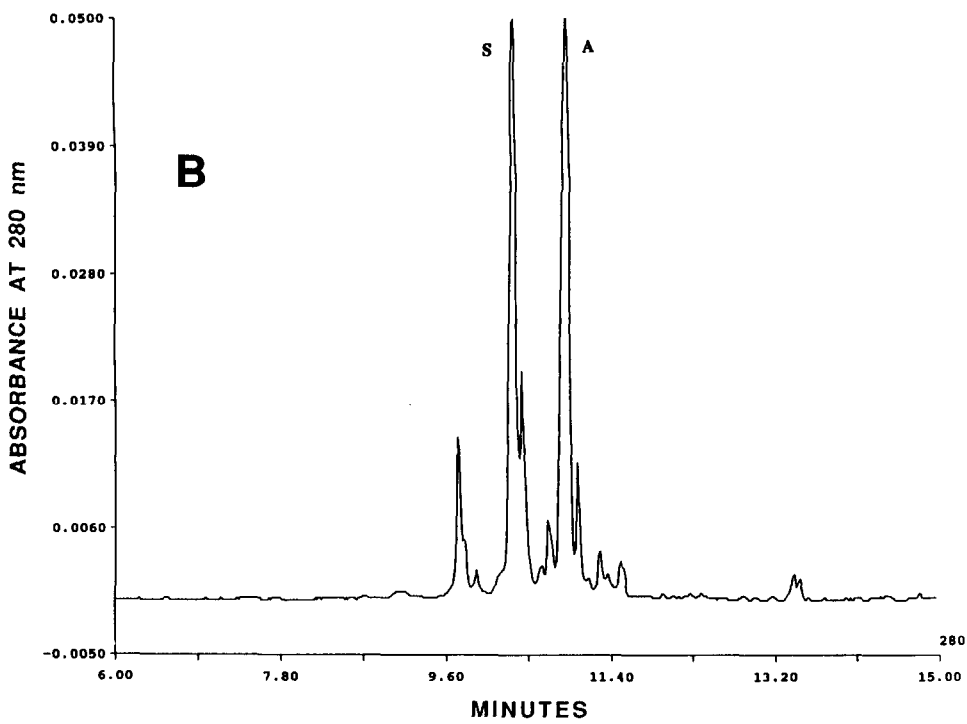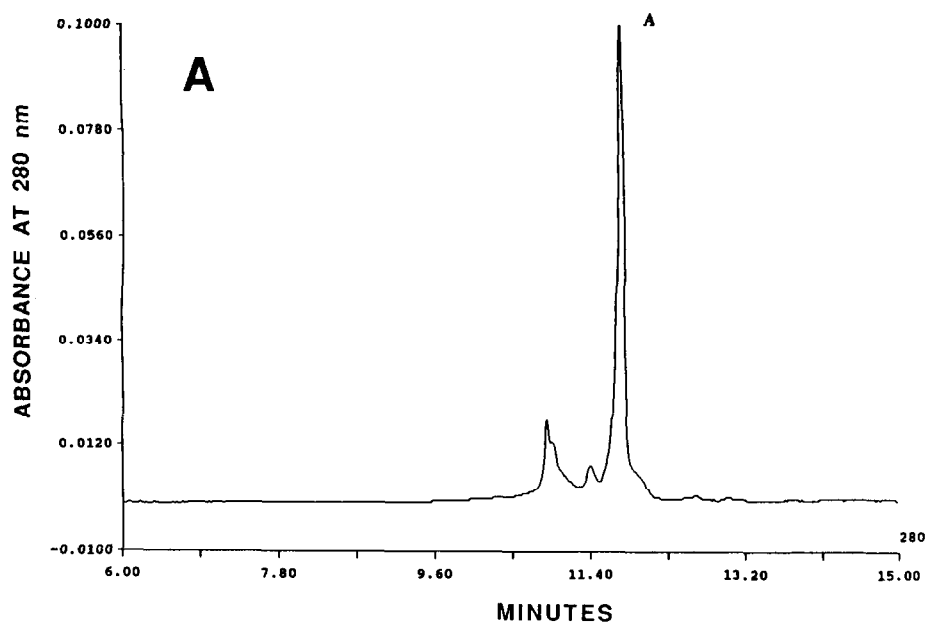Hemoglobin variants are excellent candidates for analysis by cIEF. This technique can resolve pro-
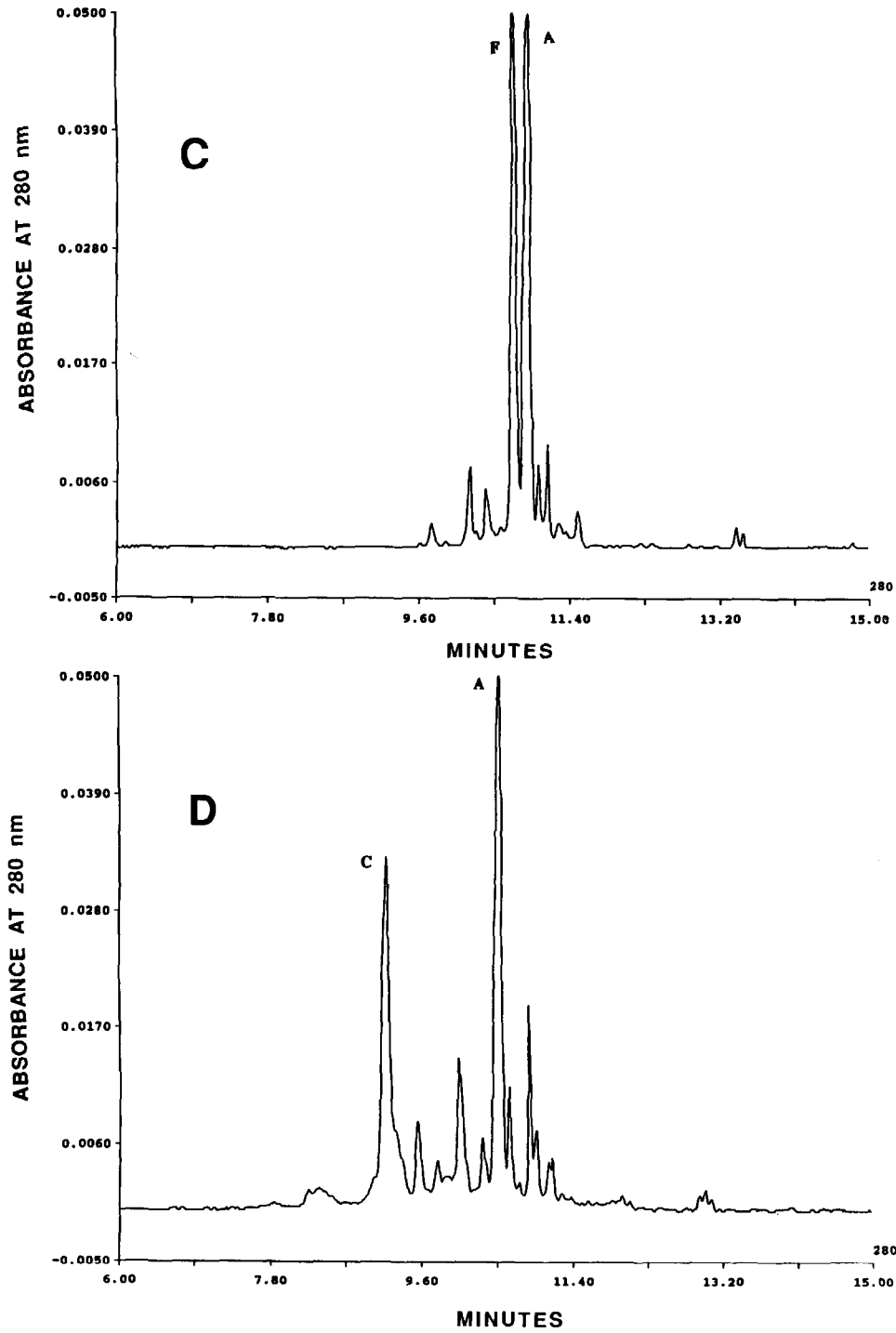
Fig. 1.

Fig. 1. Capillary isoelectric focusing of (A) hemoglobin A, (B) hemoglobins A and S, (C) hemoglobins A and F and (D) hemoglobins A and C.
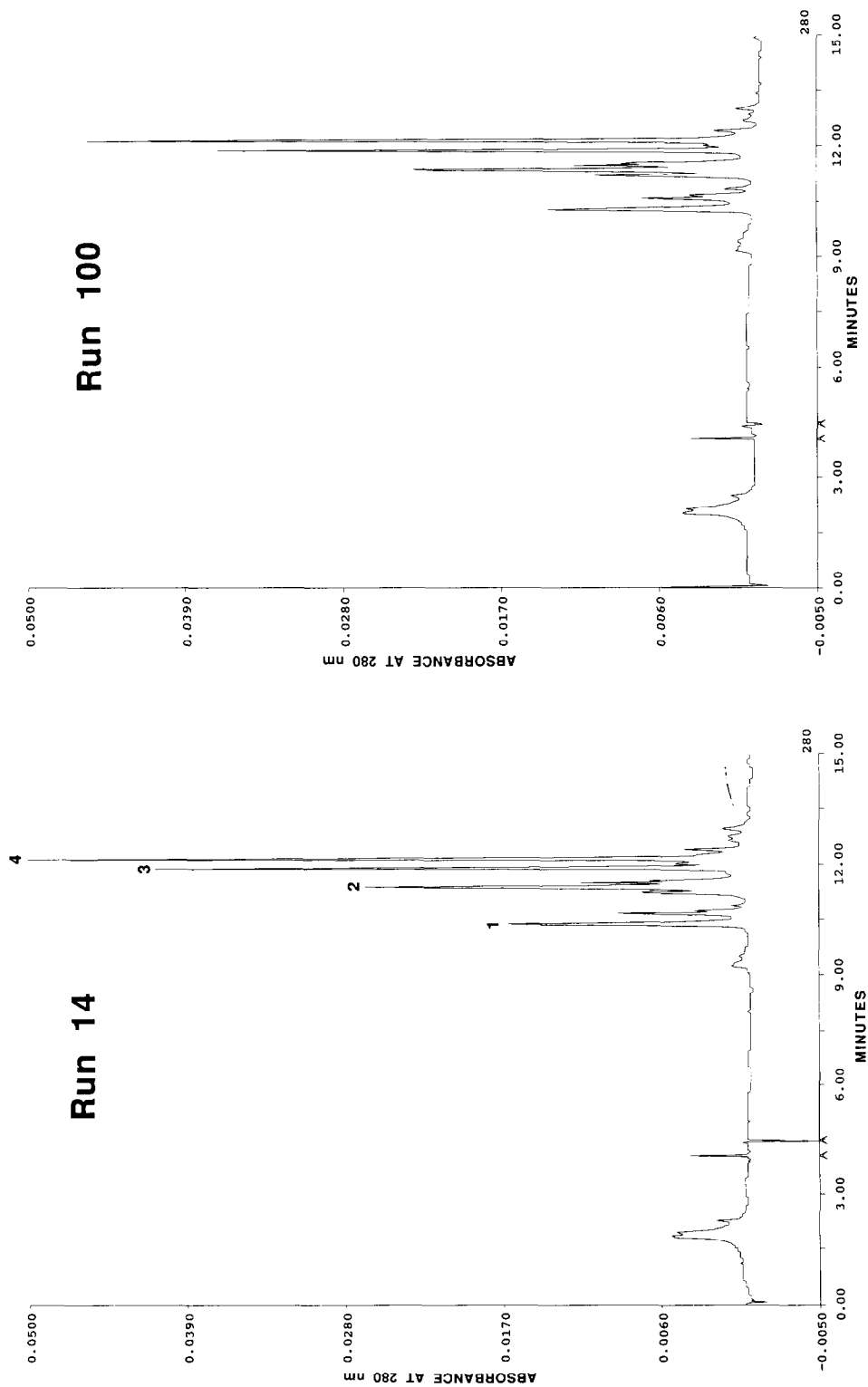
Fig. 2. Repetitive analysis of hemoglobin reference standard using a coated capillary. Peak 1 = hemoglobin C; peak 2 = hemoglobin S; peak 3 = hemoglobin F; peak 4 = hemoglobin A.
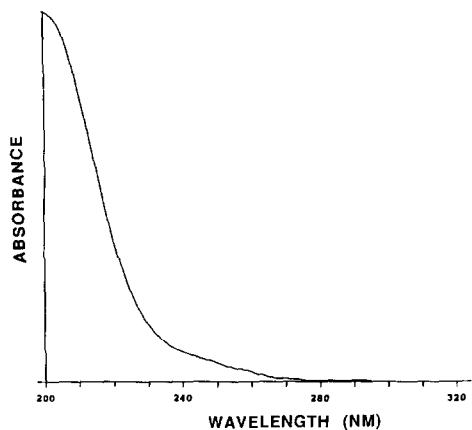
Fig. 3. Ultraviolet spectrum of Bio-Lyte pH 3–10 ampholytes.

teins differing in isoelectric points by as little as 0.02 pH units [5], and the amino acid substitutions in the major hemoglobin variants cause p*I* shifts of this magnitude or greater. The hemoglobin molecule is highly water soluble, and occurs within the erythrocyte at concentrations in excess of 300 mg/ml.

Therefore it should be much less susceptible than other proteins to precipitation when focused into sharp zones during the cIEF process.

Human fetal hemoglobin F has an isoelectric point of 7.15, and the p*I* values of the hemoglobin variants S and C are 7.25 and 7.50. These three species are easily resolved from normal human hemoglobin A (p*I* 7.10) by cIEF using a wide-range ampholyte blend (Fig. 1). In this separation, cathodic mobilization moves focused proteins past the monitor point in order of decreasing isoelectric point. The high resolution observed in this technique is due to the use of high field strengths and the absence of electroendosmosis. High field strengths (> 400 V/cm), providing strong focusing forces, are possible because the modest current levels (0.2–5 μA) during the separation minimize the amount of Joule heat. The excellent heat dissipation qualities of the small-bore 25 μm I.D. capillaries used in these separations further reduces any thermal zone distortion. Coating the capillaries with a hydrophilic polymer eliminates electroosmotic flow, which would otherwise disrupt the focusing process and
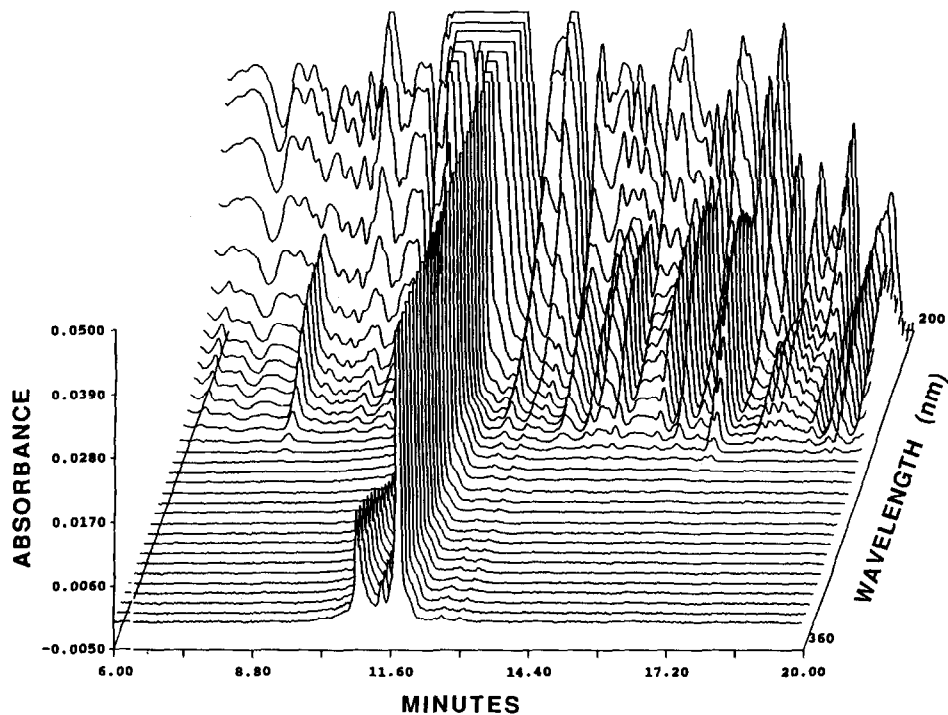

Fig. 4. Capillary isoelectric focusing separation of hemoglobin A using scanning detection in the 200–360 nm UV region. Peaks below 280 nm in the mobilization electropherogram are due to background absorbance of the ampholytes.
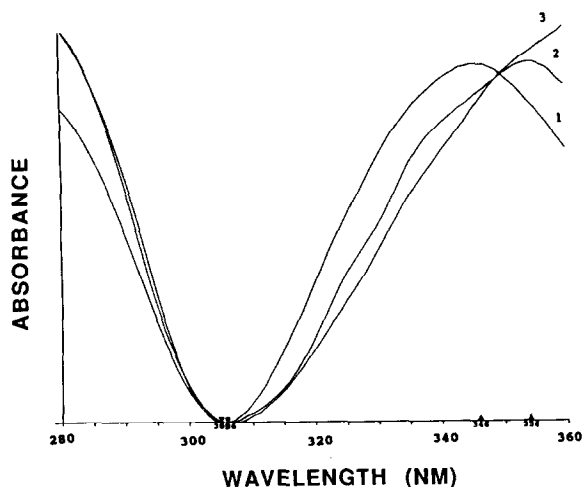
Fig. 5. UV spectra of hemoglobin A (spectrum 1) and two minor components (spectra 2,3) acquired during the cIEF separation shown in Fig. 1A. Spectrum 3 is taken from the fastest migrating minor peak, spectrum 2 is taken from the slower minor peak.

sweep proteins from the capillary before focusing was achieved. Separations are reproducible, and over one hundred repetitive runs have been achieved with no significant change in the mobilization profile (Fig. 2). Attempts to obtain good separation of native hemoglobins using free zone capillary electrophoresis were not as successful as cIEF.

The use of a high-speed scanning detector enables additional information to be obtained during the separation. The ampholytes themselves absorb in the ultraviolet region below 280 nm (Fig. 3), and when scanning detection in the 200–360 nm wavelength range is used during cIEF, a series of extra peaks is observed in the low UV region due to ampholyte absorption (Fig. 4). This pattern is reproducible and characteristic of the ampholyte blend. These peaks may be used as internal standards, and, if correlated with pI values by using external protein standards, could be used to estimate isoelectric
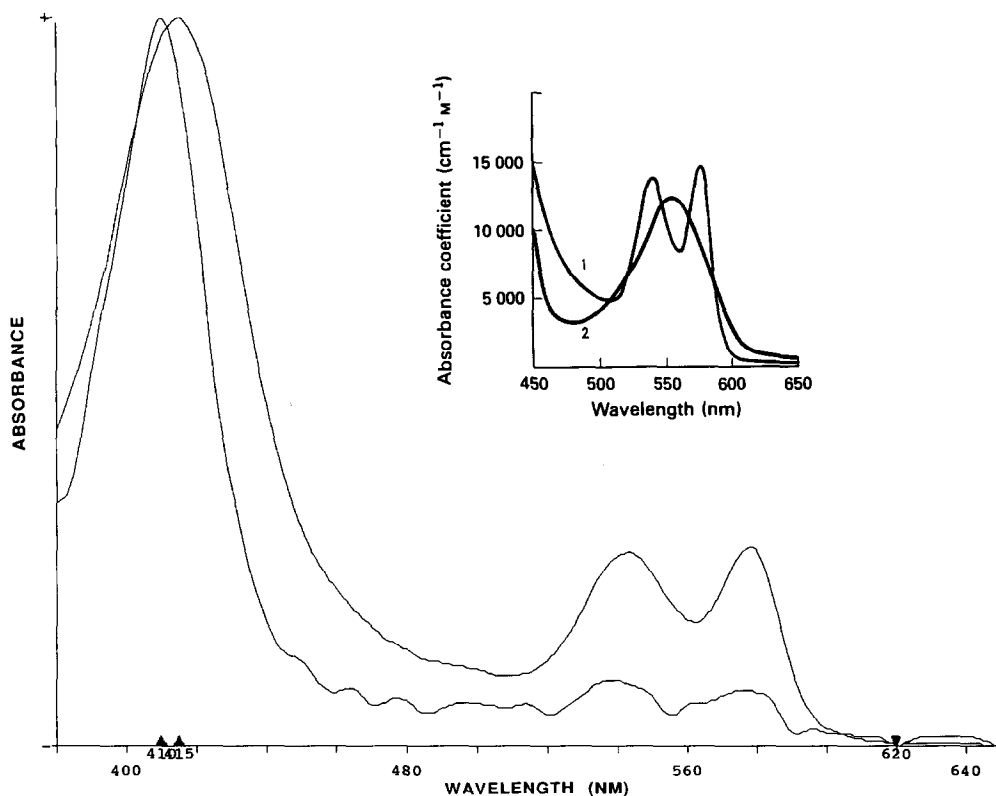

Fig. 6. Visible spectra of hemoglobin A (major peak in Fig. 1A) and minor component (first peak in Fig. 1A). Inset shows spectra of oxyhemoglobin (1) and deoxyhemoglobin (2) for reference.

points of separated proteins. Using scanning detection in the UV region above 280 nm, spectral data can be used to distinguish different components. For example, the spectra of hemoglobin A and two minor peaks exhibit different absorbance maxima

in the 300–360 nm region, suggesting structural differences for these species (Fig. 5).

The strong absorbance of the heme group in the visible region enables high-sensitivity detection of hemoglobins; the absorbance of hemoglobin A at
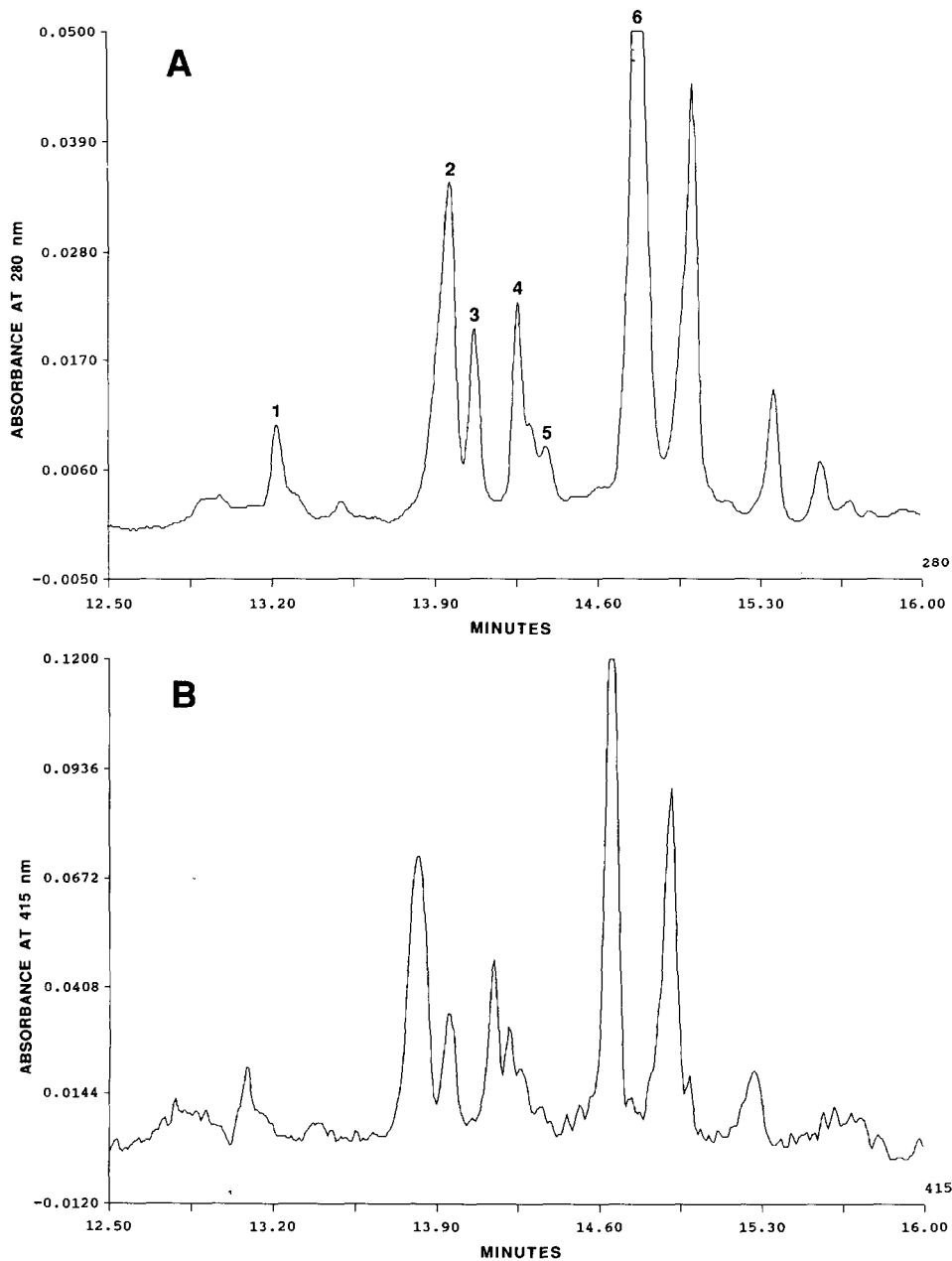

Fig. 7.

415 nm is 2.7-fold greater than at 280 nm. Moreover, there is no interference from non-heme proteins at this wavelength. The shape of the hemoglobin visible spectrum depends upon the oxidation state of the heme-bound iron and the presence of bound oxygen, and this information can be extracted from spectra acquired during the separation. For example, the spectrum of oxyhemoglobin exhibits two maxima at 542 and 578 nm, while the deoxyhemoglobin spectrum shows a single maximum at 550
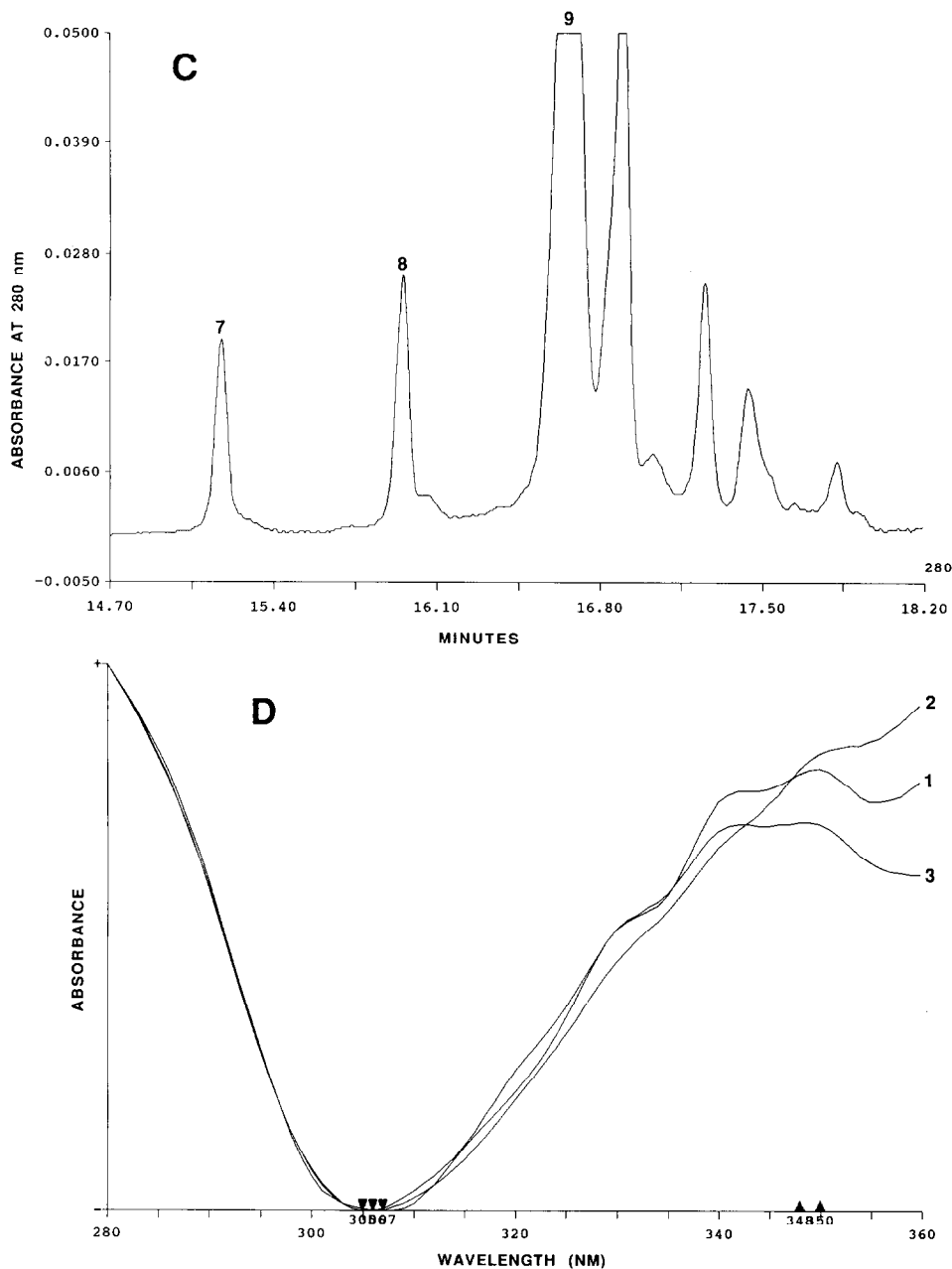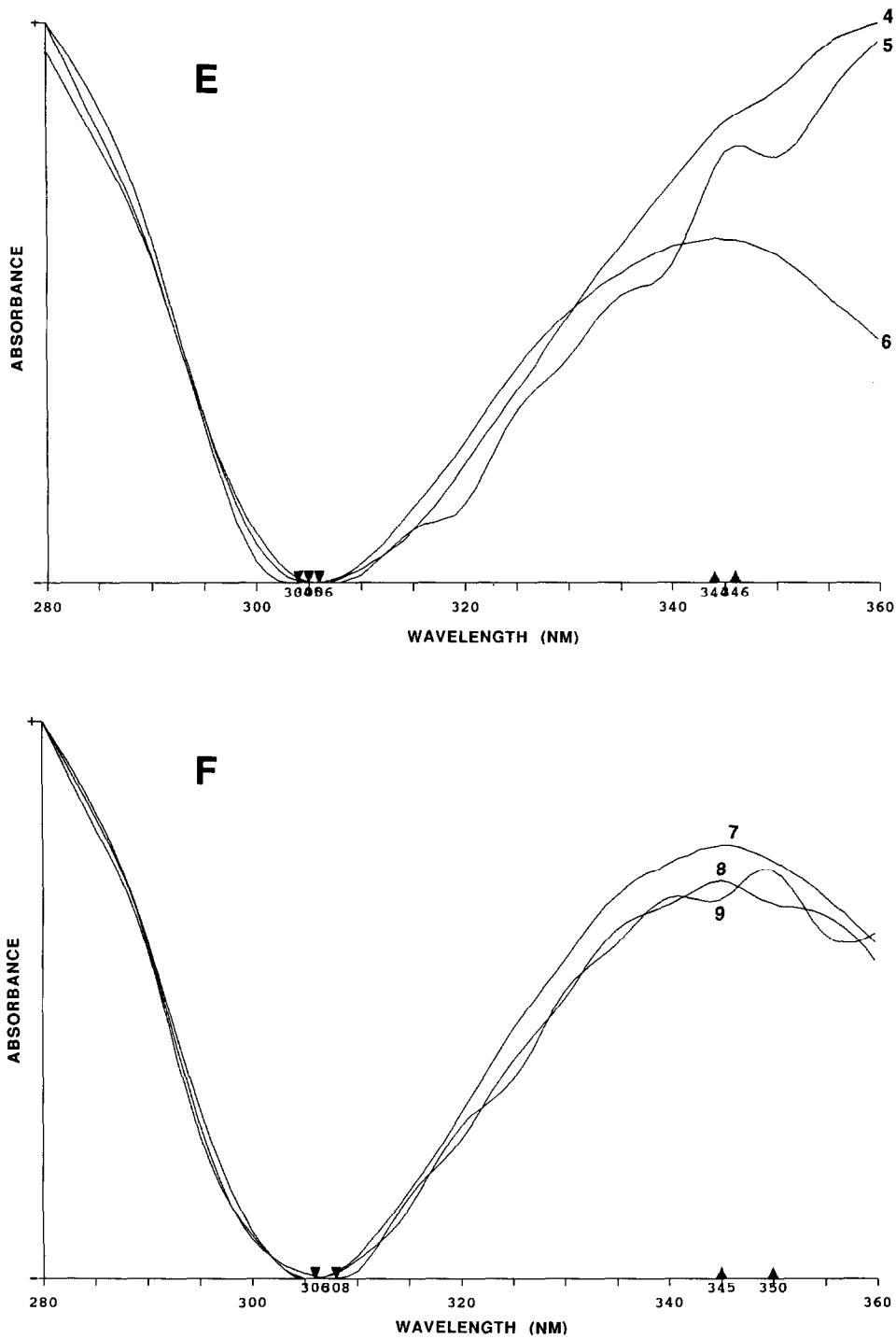

Fig. 7.

Fig. 7. Capillary isoelectric focusing of hemoglobin reference standard (containing hemoglobins C, S, F and A) using (A) detection at 280 nm, (B) detection at 415 nm, (C) detection at 280 nm following treatment of sample with 0.1% KCN. (D–F) UV spectra of peaks 1–9.

nm. Inspection of the visible spectra in Fig. 6 indicates that both the major peak and the minor peak in Fig. 1A contain primarily oxyhemoglobin.

Separation of a commercial reference standard containing hemoglobins A, F, S and C shows several minor components using detection at 280 nm (peak 1, 3 and 5 in Fig. 7A). The UV spectra of the major and minor peaks (Fig. 7D and 7E) exhibit significant differences, suggesting that they are structurally different. The electropherogram obtained with detection at 415 nm (Fig. 7B) shows the same pattern of peaks as Fig. 7A, indicating that the minor peaks are also heme-containing proteins. It is well known that hemoglobin can exist in a variety of complexed states, for example oxyhemoglobin, deoxyhemoglobin, carboxyhemoglobin, etc. Cyanide ion binds tightly to the hemoglobin molecule, and treatment with potassium cyanide converts all species to cyanohemoglobin. In the electro-

pherogram obtained after treatment of the sample with KCN (Fig. 7C) most of the minor peaks have disappeared, and the UV spectra of the separated components are similar (Fig. 7F). These results suggest that several of the minor components are different complexed states of the four major hemoglobins.

Free zone capillary electrophoresis of globin chains

Globin chains prepared by treatment of hemoglobins with acidic acetone could be resolved by free zone capillary electrophoresis under denaturing conditions (Fig. 8). Generally UV adsorption of proteins is greater at 200 nm than at longer wavelengths. However, the presence of urea in the electrophoresis buffer at high concentration introduces a high background absorbance below 210 nm. Therefore 210 nm was the best wavelength for detection of globin chains (Fig. 9). These separations
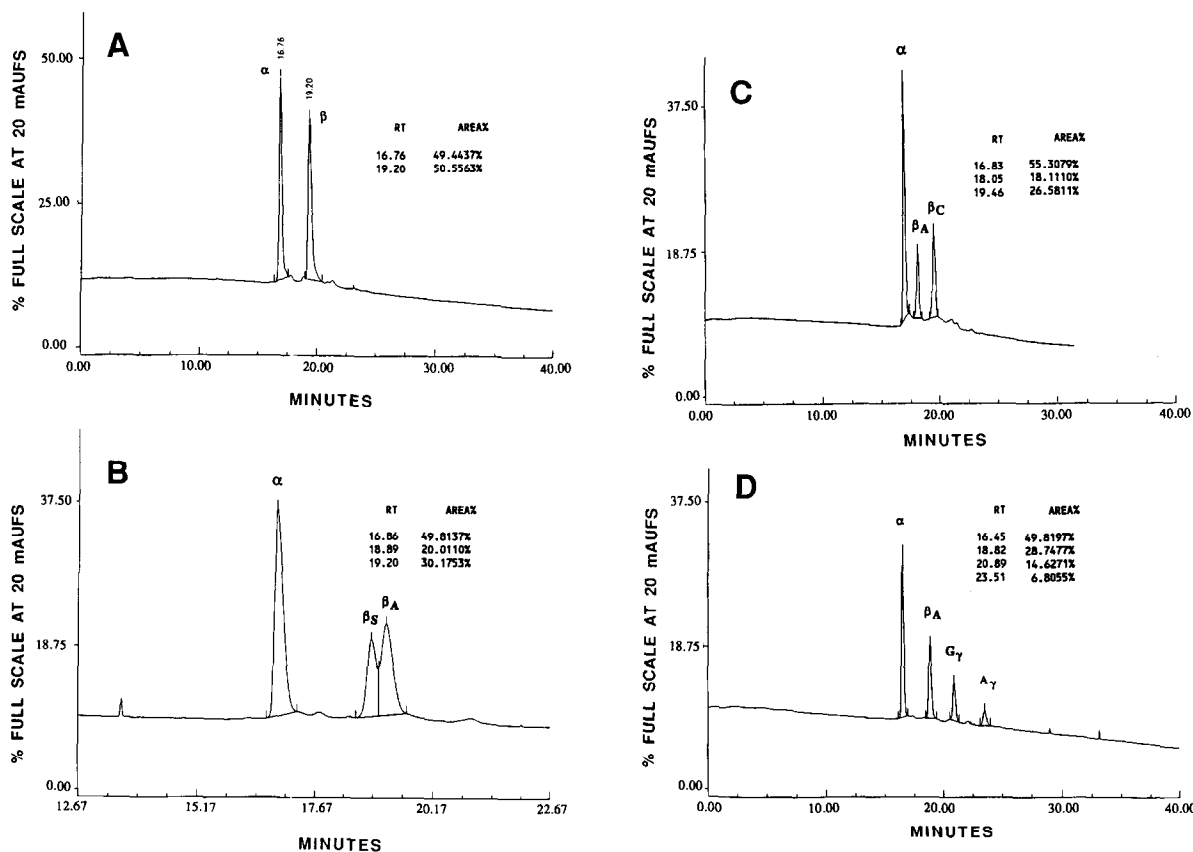

Fig. 8. Free zone electrophoresis of globin chains derived from (A) hemoglobin A, (B) hemoglobins A and S, (C) hemoglobins A and C, and (D) hemoglobins A and F. RT = Retention time in min.
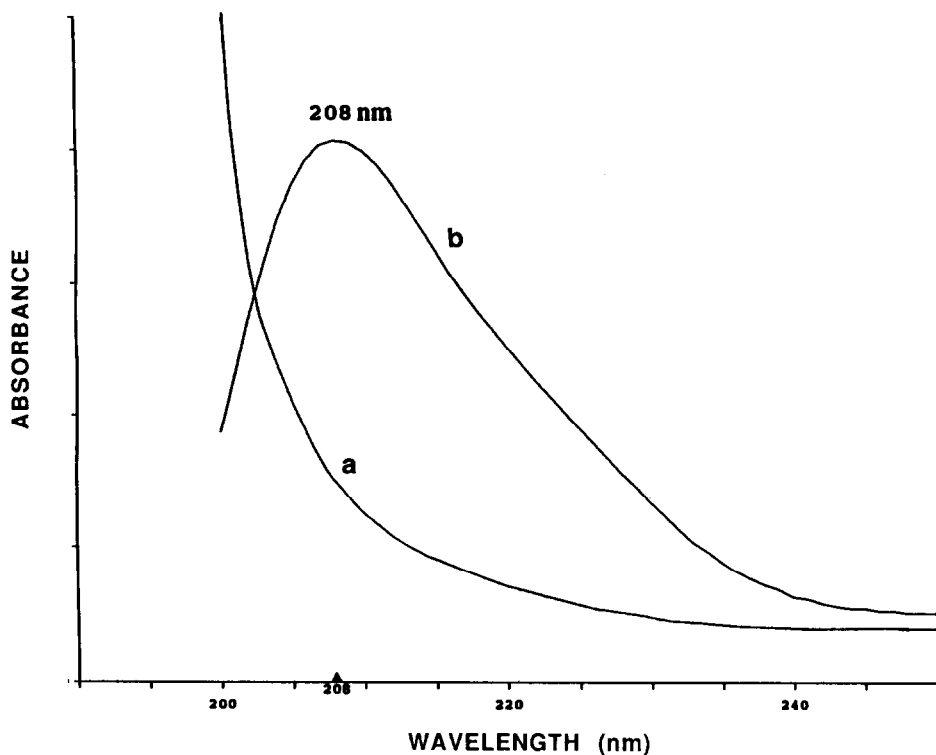
Fig. 9. UV spectra of (a) free zone electrophoresis buffer (100 mM sodium phosphate, pH 3.2, + 0.1% reduced Triton X-100 + 7 M urea), and (b) α-globin in electrophoresis buffer.

were performed at pH 3.2 using positive (inlet) to negative (detector) polarity. Even though the buffer pH is well below the pK of the side-chain carboxylic acid group of glutamic acid, the substitution of valine for glutamic acid in hemoglobin S changes the mass-to-charge ratio of the β_S globin chain relative to β_A sufficiently to permit partial resolution of these globin chains (Fig. 8B). Replacement of glutamic acid by lysine in hemoglobin C results in sufficient gain in charge to allow baseline resolution of β_C from β_A (Fig. 8C).

Electrophoresis of globin chains derived from adult hemoglobin A and fetal hemoglobin F resolves the $^G\gamma$ and $^A\gamma$ globins from β_A and from each other (Fig. 8D). These two molecules differ by the occurence of glycine and alanine in position 136 of the γ chain, respectively. A comparision of the areas of these two peaks indicates the two chains are present in a ratio of 68% $^G\gamma$:32% $^A\gamma$, which is close to the 75:25 ratio typical of fetal blood [1].

CONCLUSIONS

Due to the high solubility of hemoglobins, excellent results can be obtained in the separation of hemoglobin variants using cIEF. The technique provides high resolution, reproducible separation patterns, and analysis times of less than 20 min. Absorbance detection in the visible region is highly sensitive and free of interferences from non-heme proteins. High-speed scanning detection in either the visible or UV region enables acquisition of additional information on separated hemoglobins. Following denaturation of the hemoglobin tetramer into monomers with 7 M urea, free globin chains can be analyzed by capillary zone electrophoresis.

While the methods described in this study may not be rapid enough to compete with cation-exchange HPLC for high-throughput screening for hemoglobin disorders, capillary electrophoresis offers several advantages as a secondary or confirma-

tory technique. These include high-resolution separations with selectivities complimentary to HPLC, automation and quantitative analysis.

ACKNOWLEDGEMENTS

REFERENCES

1 H. F. Bunn and B. G. Forget, *Hemoglobin: Molecular, Genetic and Clinical Aspects*, W. B. Saunders, Philadelphia, PA, 1986.
2 V. F. Fairbanks (Editor), *Hemoglobinophathies and Thalassemias, Laboratory Methods and Case Studies*, Brian C. Dekker, New York, 1980.
3 S. J. Loomis, M. Go, L. Kupeli, D. J. Bartling, and S. R. Binder, *Am. Clin. Lab.*, October (1990).
4 S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
5 M. Zhu, R. Rodriguez and T. Wehr, *J. Chromatogr.*, 559 (1991) 479.