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Separation of human globin chains by micellar electrokinetic capillary chromatography

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Abstract

A new separation method of human globin chains by micellar electrokinetic capillary chromatography (MECC) is described. In this method, a 25 m*M* phosphate buffer (pH 2.5) containing 7 *M* urea and 1% (w/v) reduced Triton X-100 buffer system was used. All experiments were performed in a 47 cm×50 μm I.D. uncoated fused-silica capillary. The separation voltage was set at 19 kV. Normal globin chains derived from normal adults and newborns, α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ globin chains as well as common variant globin chains were successfully separated within 20 min. High reproducible migration times of globin chains (CVs of intra- and inter-assay were less than 1% and 2% respectively), and quantification of $^{G}\gamma$ and $^{A}\gamma$ chains (CVs for intra- and inter-assay were less than 5% and 10%, respectively) were obtained. This new MECC method provides primary information on structural modification of globin chains. It can be an important diagnostic tool in clinical laboratory practice in the field of hemoglobinopathies.

Keywords: Hemoglobin; Globin chains

1. Introduction

Congenital defects of hemoglobin (Hb) synthesis include the production of abnormal globin chains and unbalanced synthesis of normal globin chains. The prognosis and the treatment of these diseases require a precise diagnosis related to an exact identification and an accurate quantification of the concerned globins. Separation of globin chains by gel electrophoresis or high-performance liquid chromatography (HPLC) have their respective advantages and disadvantages [1–3]. In the polyacrylamide gel electrophoresis method, several samples can be analyzed on one gel. It shows clearly the migration positions of normal and variant bands. However, this method is labour intensive and time consuming. Meanwhile, quantification of $^{G}\gamma$ and $^{A}\gamma$ is usually difficult since the concentrations of both chains are low in adults. Recent developments of HPLC methods have enabled the separation of variant and normal globin chains, such as separation of $^{A}\gamma^{I}$ and $^{A}{}_{G}{}^{T}$ chains and integration of these minor fractions. In protein analysis, capillary electrophoresis (CE) is an emerging methodology, which is becoming increasingly popular, owing to its clinical sensitivity, rapidity and efficacy. In the particular field of globins, free solution capillary electrophoresis (FSCE) has been successfully used for separation of normal or abnormal globins [4–8].

FSCE cannot separate uncharged species. However, neutral and charged species can be separated by

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micellar electrokinetic capillary chromatography (MECC). In MECC, separation is based on hydrophobic and electrostatic interactions of proteins with surfactant micelles present in the buffer medium [9]. This method is therefore very useful for the separation of normal or abnormal globin chains which have a very similar structure and which cannot be well separated by FSCE.

2. Experimental

2.1. Chemicals

Reduced Triton® X-100 was purchased from Sigma (St. Louis, MO, USA), Ultrodex® and ampholines (pH 6–8) from Pharmacia Biotech (Uppsala, Sweden), and other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Micellar electrokinetic capillary chromatography

The analyses were carried out with a Beckman P/ACE 5500 capillary electrophoresis system equipped with a diode array detector (Beckman, Fullerton, CA, USA). Peaks detected at 214 nm were integrated with the P/ACE station software. The fused-silica capillary (47 cm×50 μm, length=40 cm), was enclosed in a cartridge format and maintained at 25°C by liquid cooling. The running buffer was a 25 m*M* phosphate buffer (pH 2.5) containing 7 *M* urea and 1% (w/v) reduced Triton X-100. The buffers were prepared as follows: 100 m*M* sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) was mixed with 100 m*M* phosphoric acid (H_3PO_4) to reach pH 2.5. Twenty-five millilitres of this phosphate buffer were mixed with 1 g of reduced Triton X-100 and 42.042 g urea. After dissolving completely, the volume was adjusted to 100 ml. A similar procedure was used to prepare sample buffer which was 5 m*M* phosphate buffer containing 7 *M* urea and 0.5% reduced Triton X-100. Prior to use, the capillary was conditioned by high pressure rinse (138 kPa) with 1 *M* HCl, followed with deionised water, for 10 min each. Before each separation, the capillary was filled with running buffer. The sample was injected into the capillary with low pressure (3.4 kPa) for 10 s. The separation voltage was set at 19 kV (≈400 V/cm). The common current level was 11.5 μA. After each run, the capillary was rinsed with 1 *M* HCl for 5 min then with deionised water for 5 min.

2.3. Sample preparation

Blood samples (anti-coagulated with ethylenediaminetetraacetic acid, EDTA) were obtained from patients who entered our laboratory for hemoglobin analysis. All blood samples were subjected to Hb A_2 and Hb F analyses by an ion-exchange HPLC method (BioRad Labs, Hercules, CA, USA), and screened for common Hb variants (Hb C, E, O, S, D, J-Baltimore, G-Philadelphia and O-Indonesia) using standard techniques i.e. isoelectric focusing (IEF) (Isolab, Akron, OH, USA), citrate agar gel electrophoresis at pH 6.5 and cellulose acetate electrophoresis at pH 8.6.

Adult Hb (Hb A, $\alpha_2\beta_2$), fetal Hb (Hb F, $\alpha_2^G\gamma_2$ and $\alpha_2^A\gamma_2$) and Hb A_2 ($\alpha_2\delta_2$) were purified by preparative IEF [10]. $^A\gamma^I$ and $^A\gamma^T$ chains were separated by HPLC [11]. Heme-free globin chains were prepared by the acetone–acid method described by Clegg et al. [12]. Red blood cells were washed with NaCl (0.9%) three times and lysed in the same volume of deionised water. Red cell membranes were eliminated by mixing with chloroform and centrifuged for 5 min at 19 000 *g*. The clear hemolysate (supernatant) was used for preparation of globin chains. One volume of hemolysate containing 2 g/l Hb was mixed with 20 volumes of cold acid acetone (−20°C) and globin chains were precipitated by centrifugation at 3000 *g* for 5 min. The supernatant was discarded. The precipitated globin chains were then washed for several times till the pellet was white. After drying under a nitrogen flow, the globins were weighed and reconstituted in the sample buffer to reach a final protein concentration of 1 or 2 g/l.

2.4. Reproducibility

To evaluate the reproducibility of the migration time of normal globin chains and quantification of $^G\gamma$ and $^A\gamma$ globin chains, three samples were select-

ed. They were obtained from patients with various hemoglobinopathies, thus containing different levels of Hb F and Hb A_2 (Sample A: Hb F=5.6% and Hb A_2=2.2%; sample B: Hb F= 2.7% and Hb A_2= 6.4%; and sample C: Hb F= 13.9% and Hb A_2= 3.6%). These samples were chosen to get different Hb F levels and to study their influence on the measurement of the $^{G}\gamma/^{A}\gamma$ ratio. Intra-assay and inter-assay coefficients of variation (CVs) were performed using sample A or samples A, B and C, respectively. The levels of $^{G}\gamma$ and $^{A}\gamma$ chains are expressed in percentage of total γ chain, $^{G}\gamma+^{A}\gamma$.

3. Results

3.1. Separation of normal globin chains

All normal adult and fetal globins were clearly separated in 20 min (Fig. 1). The elution sequence was as follows: α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ globin chain peaks. The method did not allow the separation of $^{A}\gamma^{I}$ and $^{A}\gamma^{T}$ (data not shown).

To confirm the elution sequence of α, β, $^{G}\gamma$ and $^{A}\gamma$ globin chain, MECC of samples containing purified Hb A and Hb F was performed (Fig. 2A). The elution position of the δ chain was confirmed by performing either MECC of purified Hb A_2 or samples containing various levels of Hb A_2 (high, usual and low) (Fig. 2B).

Table 1 summarises the intra-assay and inter-assay CVs of migration times of α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ chains. CVs were always less than 1% and 2%, respectively. The reproducibility of the quantification of $^{G}\gamma$ and $^{A}\gamma$ chains is shown in Table 2. The intra-assay CVs were 2.2% and 4.1%, respectively. The inter-assay CVs ranged from 4% at high Hb F levels through 14.8% at levels of Hb F lower than 4%.

3.2. Separation of common variant globin chains

In order to identify the peaks of variant globin chains, we compared the elution profiles of the variants with normal globin chains. The extra peaks were identified as the globin chain variants. Fig. 3 shows the separation of five common β chain variants β^{E}, β^{C}, $\beta^{O\text{-Arab}}$, β^{S} and $\beta^{J\text{-Baltimore}}$. β^{C} chain is fully separated from β^{A} chain and migrates faster

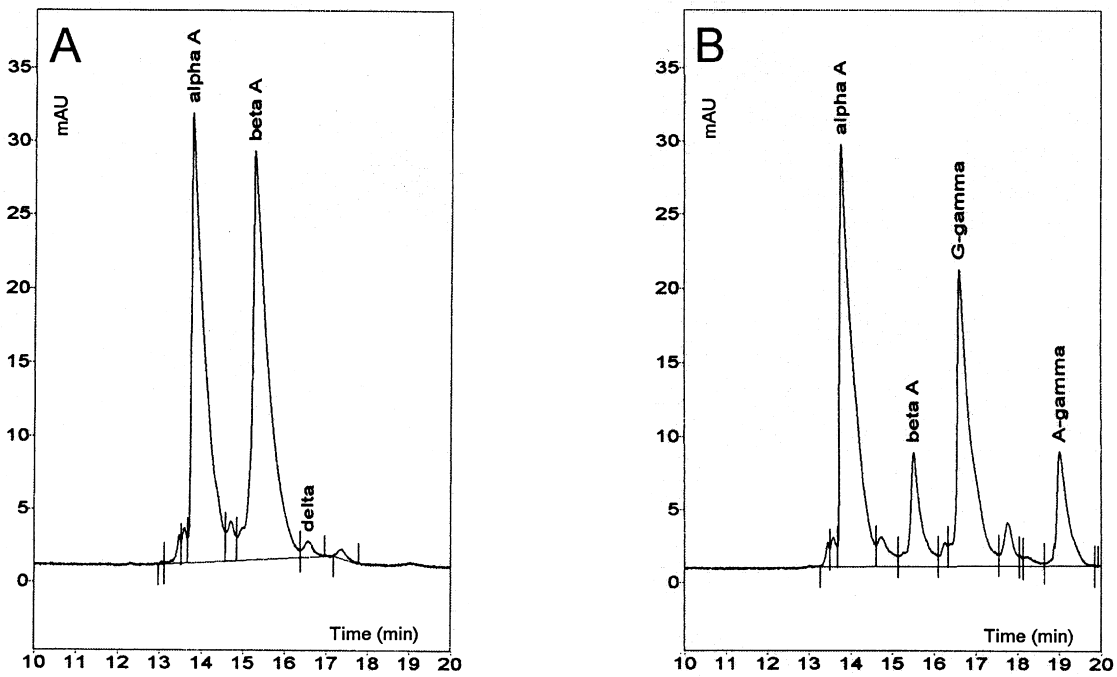

Fig. 1. Separation of normal globin chains from: (A) Normal adult,(B) normal newborn. Separation conditions are described in Section 2.

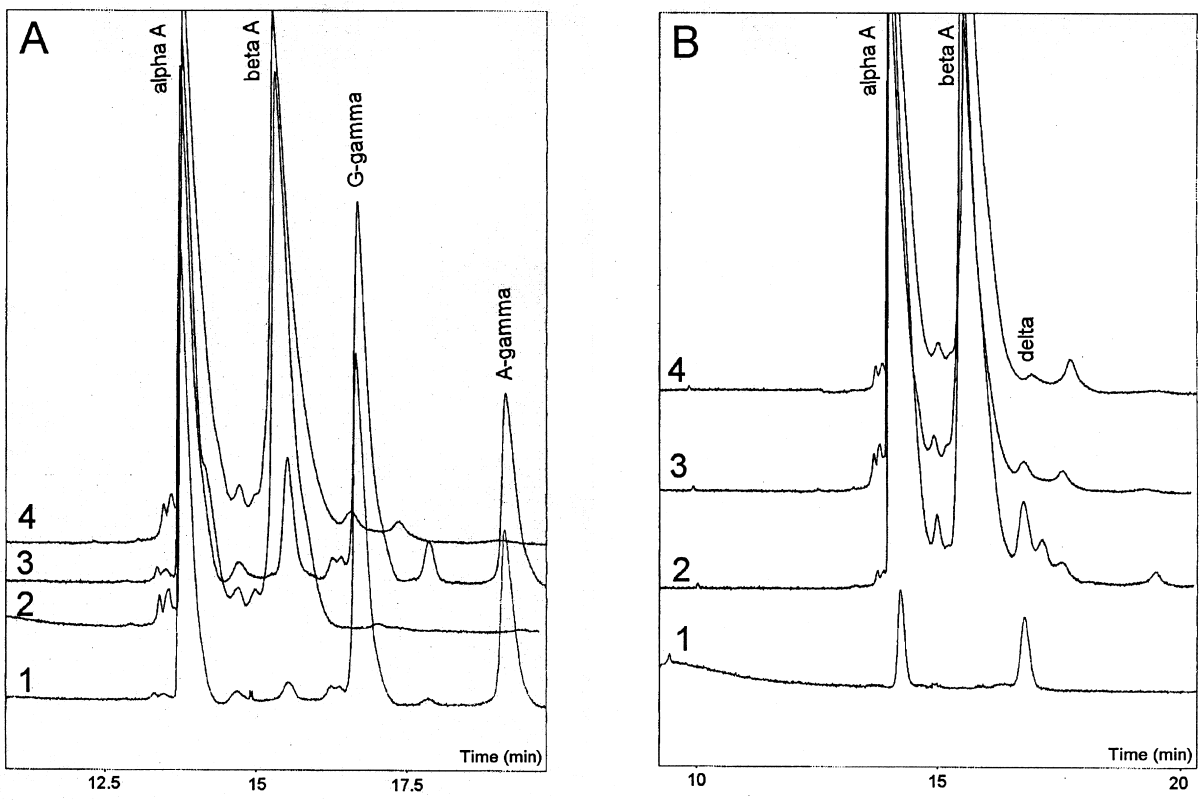

Fig. 2. Identification of α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ chains. (A) Globin chains derived from (1) purified Hb F; (2) purified Hb A; (3) normal newborn; and (4) normal adult. (B) Globin chains from (1) Hb A_2; (2) high; (3) normal; and from (4) low Hb A_2 (Hb A_2=1.3, 3.1 and 6.4% respectively);. Separation conditions are described in Section 2.

than $\beta^{O\text{-}Arab}$ chain. The β^{E} chain is only partially separated from the α chain (see also Fig. 5). Fig. 3D shows the migration, just before the β^{A} chain, of the β^{S} chain. The $\beta^{J\text{-}Baltimore}$ chain migrates after the β^{A} chain (Fig. 3E).

Fig. 4 shows the separation of two α chain

Table 1
Reproducibility of migration time of globin chain

		Migration time of globin chains (min)				
		α chain	β chain	δ chain	$^{G}\gamma$ chain	$^{A}\gamma$ chain
Intra-assay						
(Sample A)[a]	Mean	13.9	15.5	16.8	17.3	19.6
(n=9)	CV %	0.4	0.4	0.5	0.7	0.5
Inter-assay						
(Sample A, B and C)[a]	Mean	13.8	15.4	16.7	17.1	19.4
(*n*=15)	CV %	1.2	1.2	1.1	1.2	1.3

[a] Sample A: Hb F=5.6%, Hb A_2=2.2%; Sample B: Hb F=2.75, Hb A_2=6.4%; Sample C: Hb F=13.9%, Hb A_2=3.65.

Table 2
Reproducibility of quantification of γ chains

		$^{G}\gamma/(^{G}\gamma+^{A}\gamma)\times100$	$^{A}\gamma/(^{G}\gamma+^{A}\gamma)\times100$
Intra-assay ($n=9$)	Mean	65.1	34.9
	CV %	2.2	4.1
Inter-assay			
Sample A[a] ($n=5$)	Mean	63.3	36.7
	CV %	4.4	7.6
Sample B ($n=5$)	Mean	63.2	36.8
	CV %	8.6	14.8
Sample C ($n=3$)	Mean	43.7	56.3
	CV %	7.8	6.1

[a] Sample A: HbF=5.6%; Sample B: HbF=2.7%; Sample C: HbF=13.9%.

mutants, $\alpha^{\text{O-Indonesia}}$ and $\alpha^{\text{G-Philadelphia}}$. The $\alpha^{\text{G-Philadelphia}}$ chain migrates faster than the normal α chain, but slower than the $\alpha^{\text{O-Indonesia}}$ chain. The elution profile of normal and variant globin chains are summarised in Fig. 5.

4. Discussion

The CE separation technique is rapid, very small amounts of sample and reagents are required, and compared to HPLC, the capillary and reagents are inexpensive. Even so, clinical application of CE for globin chain analysis is still not used as widely as reversed-phase HPLC in particular for quantification of $^{G}\gamma$ and $^{A}\gamma$ chains [2,3]. We have described here a new, inexpensive and rapid MECC technique to separate normal and abnormal globin chains. Compared with a 50-min typical separation time by reversed-phase HPLC or two days by polyacrylamide gel electrophoresis, this technique can compete with them since it allows within 20 min the identification of α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ chains as well as variant globin chains. It is worthwhile mentioning that even the present method is not as fast as some FSCE methods [4,5], but it is the only CE method available for separation of some globin chains, esp. $^{G}\gamma$ and $^{A}\gamma$ chains, which cannot be separated by FSCE methods.

Most of the techniques developed to separate globin chains use coated capillaries [5–8]. Indeed, using a bared silica capillary, the main problem is protein adhesion onto the capillary wall. Nevertheless, the coating may also affect the separation as proven by the different migration order of globin chains obtained in different studies. Moreover, all these techniques were unable to separate $^{G}\gamma$, $^{A}\gamma$ and/or δ chains. One author [8] suggests that on theoretical grounds, globin chains in the presence of detergents would resolve on the basis of their different hydrophobicity. This kind of detergent-modified CE procedure of separation was described by Zhu et al. [6] who have used urea and reduced Triton X-100 with phosphate buffer to separate different globin chains. The same authors [7] modified the pH of the phosphate buffer and the capillary but the separation of $^{G}\gamma$ and $^{A}\gamma$ chains was not achieved, even some common globin variants were not separated, for example the β^{E} globin chain.

In one study, separation of globin chains was achieved using an uncoated capillary and a strong alkaline buffer (pH 11.8) [4]. This method induces a strong and constant electroosmotic flow (EOF) and achieves a separation of α, β, and γ chains in less than 10 min. When distinct peaks of α, β, and γ chains were resolved, separation of δ or $^{G}\gamma$ and $^{A}\gamma$ chains were not obtained. No information was given concerning the resolution of variant globin chains.

We have developed a new capillary technique for the separation of globin chains based on the differences in hydrophobic and electrostatic interactions of proteins with micelles. We use a inexpensive uncoated capillary and strongly acidic buffer (pH 2.5) to solve the problem of protein adhesion on the capillary wall. Urea at high concentration ($>4M$) was used as denaturing agent to prevent globin chain hybridisation. Terabe et al. [13] found that in SDS–

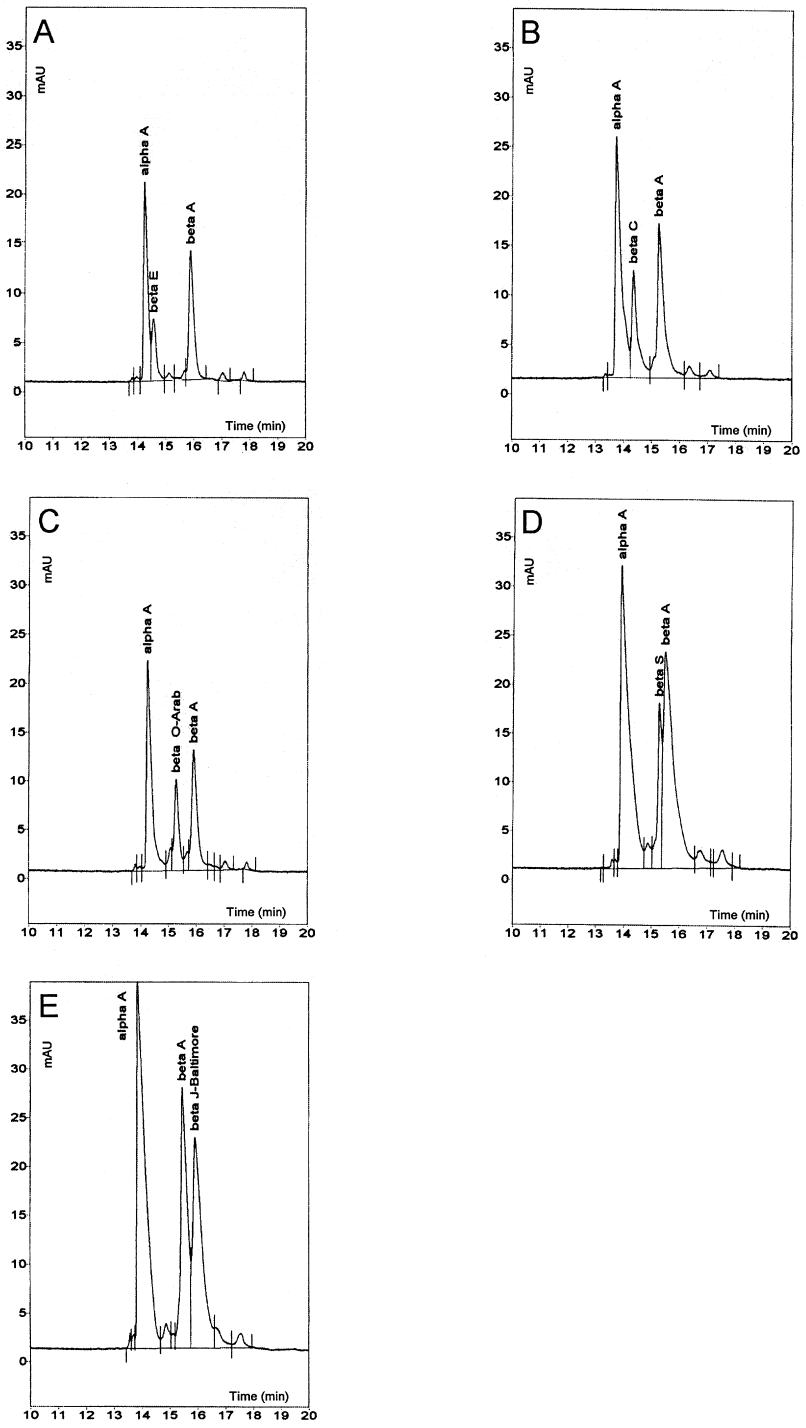

Fig. 3. Separation of five β chain variants: (A) β^{E}, (B) β^{C}, (C) $\beta^{O\text{-}Arab}$, (D) β^{S}, and (E) $\beta^{J\text{-}Baltimore}$. Separation conditions are described in Section 2.

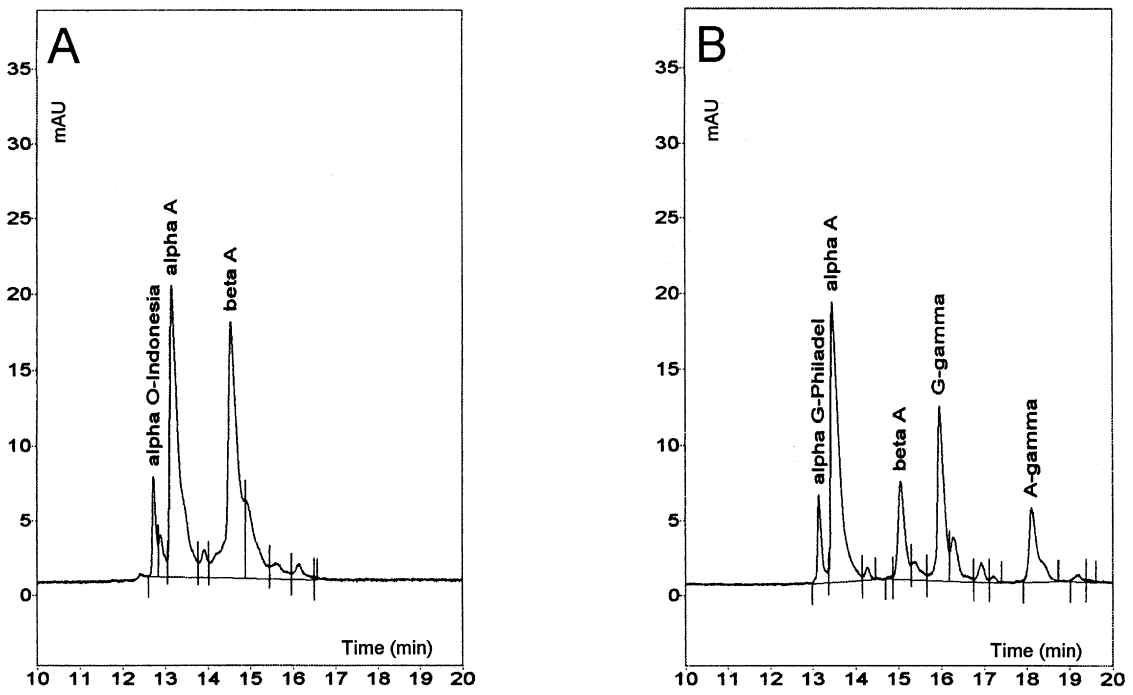

Fig. 4. Separation of two α variant globin chains; (A) $\alpha^{\text{O-Indonesia}}$ and (B) $\alpha^{\text{G-Philadelphia}}$. Separation conditions are described in Section 2.

MECC, the capability factor decreases logarithmically with increasing urea concentration. In addition, the elution time window was extended and resolution was enhanced by the addition of urea. The neutral surfactant added in the buffers, reduced Triton® X-100, does not cause denaturation of the proteins but it can split the gamma-chain into two types, G-gamma and A-gamma chains. The detergent mi-

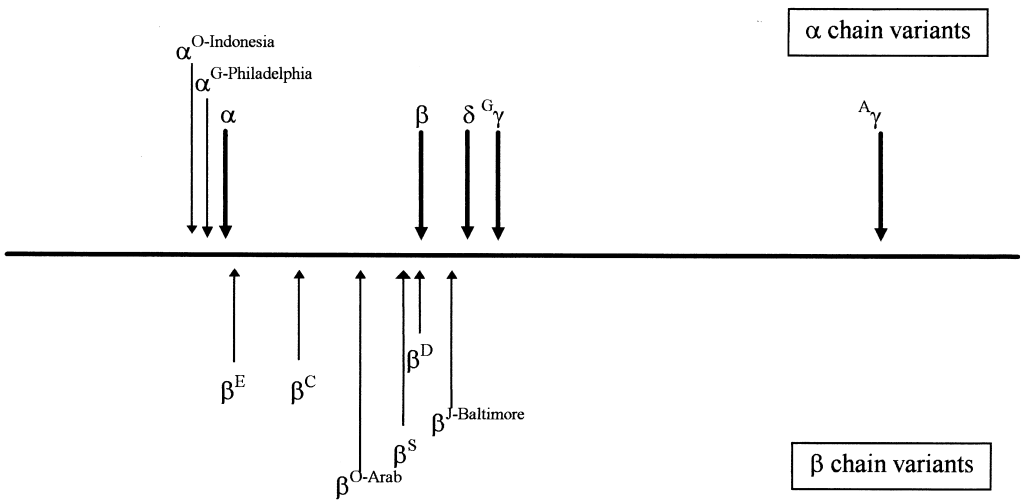

Fig. 5. Illustration of the relative migration times of normal and variant globin chains.

celles bind preferentially to the hydrophobic stretch of nine amino acids (No. 133–141) in $^{A}\gamma$ chain[14]. A balance of association and disassociation of proteins and detergent in the buffer during electrophoresis may thus exist. During electrophoresis, globin chains will inevitably interact with detergent micelles and be separated by their hydrophobic characteristics. We were thus able to separate all the normal globin chains in the migration order of α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ chains as well as variant globin chains which have the same charge but different hydrophobicity compared to normal globin chains.

With MECC, the γ chain splits into two fractions: the $^{G}\gamma$ and the $^{A}\gamma$ chains, which can be quantified. Nevertheless, quantification of minor components like $^{G}\gamma$ and $^{A}\gamma$ chains in normal adults with fused-silica capillary is usually difficult. Protein fouling onto the inner surface of the capillary causes significant decrease in reproducibility of quantification of the two types of γ chains. Higher sample concentration and on-line stacking can be helpful in solving this problem. A sufficient rinse with HCl and deionised water after each analysis ensures full reconditioning of the capillary and improves the reproducibility of the $^{G}\gamma$ and $^{A}\gamma$ peaks. The δ chain migrates closely to the $^{G}\gamma$ chain, but is well separated from it. The separation of these different peaks permits a diagnosis of some inherited diseases of fetal globins.

This MECC method is also powerful for the separation of variant globin chains. In the IEF method, Hb E, Hb C and Hb O-Arab are focused close to Hb A_2 and it is difficult to identify them even coupling IEF with other standard methods for Hbs separation. With MECC, it is possible to identify variant globin chains, like β^{E}, β^{C} and $\beta^{O\text{-}Arab}$ by their peak positions.

5. Conclusion

We have presented a new capillary technique for the separation of globin chains based on the differences of hydrophobic and electrostatic interactions of proteins with micelles, namely, MECC. By this method, normal globin chains, α, β, δ, $^{G}\gamma$ and $^{A}\gamma$ chains, and a number of variant globin chains, were successfully separated. This method also provided highly reproducible results for migration time of globin chains as well as quantification of $^{G}\gamma$ and $^{A}\gamma$ chains. In addition, the MECC method can provide some primary information on structural modifications of globin chains which are important for identification or for further structural studies of the protein concerned. It is reasonable to conclude that this new method is suitable for the diagnosis of fetal hemoglobin inherited diseases or other hemoglobinopathies in clinical laboratories.

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