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Short Communication

Separation of globins using free zone capillary electrophoresis

Choon-Nam Ong*, Lian Soon Liau and Her Yam Ong

Department of Community Medicine, National University of Singapore, National University Hospital, Kent Ridge, Singapore 0511 (Singapore)

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ABSTRACT

This paper describes the use of high-performance capillary electrophoresis for the separation of globin chains. Adult and newborn haemolysates from normal individuals and children suspected of having thalassaemia were analysed using free zone electrophoresis. Separation of globins was accomplished using a 25 m*M* phosphate buffer at pH 11.8. Distinct peaks of α-, β- and γ-chains were resolved within 8 min. The coefficient of variation for within-day and between-day runs was 4.1% and 5.7%, respectively. This method is simple and rapid, and it can be used to screen for thalassaemia and for the clinical study of various haemoglobinopathies.

INTRODUCTION

Electrophoresis using cellulose acetate at alkaline pH and citrate agar at acidic pH has been used in most haematology laboratories for the diagnosis of thalassaemia. The accuracy of this procedure is low, and confirmation is usually required. This is usually achieved by using low-pressure column chromatography or macro-column chromatography, using a weak cation-exchange column. This procedure takes 2–3 days for a complete separation of haemoglobin variants [1]. Isoelectric focusing (IEF) has also been used for the identification of haemoglobin variants. However, haemoglobin is not easily quantitated by this technique, which is not commonly performed in a routine haematology laboratory. Further structural characterization is based upon reversed-phase high-performance liquid chromatography (RP-HPLC) for separation of a tryptic digest of the globin chains, followed by the characterization of the peptides. This technique has been confined primarily to research laboratories. Nevertheless, the recent development of the immobilized pH gradient (IPG) technique combined with isoelectric focusing (IPG–IEF) may be an alternative method of screening for thalassaemias [2].

Since detection of haematological abnormalities requires a fast and sensitive technique that is capable of separating the different globin variants, simple methods are needed for diagnosis and genetic counselling.

High-performance capillary electrophoresis (HPCE) is an emerging methodology which has high sensitivity and selectivity [3]. This technique combines the advantages of HPLC and electrophoresis, such as detection and quantification,

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with a superior separating power. This paper reports the separation of globin chains by using a free zone electrophoretic (FZE) technique on an uncoated fused-silica capillary. The use of this technique reduces the separation time significantly. In the present study this technique was used for screening thalassaemia carriers and a group of newborns.

EXPERIMENTAL

Reagents and chemicals

Disodium phosphate and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA) and acetone and diethyl ether were obtained from E. Merck (Darmstadt, Germany).

All reagents used for electrophoresis were filtered through a 0.45-μm filter before use.

Instrumentation

The apparatus consisted of a Beckman PACE 2000 system with autoinjection function and a 42-cm open-tube uncoated fused-silica capillary. Detection and quantitation were carried out with a UV detector at 214 nm. Integration was performed by a Beckman System Gold chromatography data system version 6.0 for peak area measurement.

Sample preparation

Blood collected in EDTA or heparin was first centrifuged at 2500 *g* to remove the plasma. It was then washed three times with an equal volume of saline. The washed red cells were then lysed in two volumes of deionized water and vortexed. Globin was prepared according to method used by Clegg *et al.* [4] with slight modifications. The hamolysate was added drop by drop into cold acidified acetone at -20°C (one volume of haemolysate was added to twenty volumes of the acidified acetone). During addition of the haemolysate, the tube was shaken vigorously. The white flocculent precipitate formed was separated by centrifugation at 2500 *g* for 3–4 min. It was then washed three times with 20°C acetone (ten volumes of acetone to one volume of haemolysate used). The precipitate was then purified with 2–3 ml of diethyl ether. The mixture was then dried with a clean stream of air to isolate the globin and stored at -10°C.

Prepared globin was weighed and dissolved in deionized water to a concentration of less than 10 mg/ml. It was further diluted with running buffer (30 μl of the globin solution was added to 200 μl of running buffer) and used for analysis.

Prior to use, the HPCE capillary column (42 cm $\times$ 75 μm I.D.) was primed overnight with 25 m*M* sodium phosphate buffer at pH 11.80. The column was rinsed with the same buffer for 2 min before each run. Samples were injected into the capillary under pressure. Each injection was carried out for 3 s. The components of the protein were separated at 40 μA, at a voltage limit of 20 kV, and the temperature was set at 22°C. In order to ensure optimal performance of the column, the capillary was rinsed with 0.1 *M* sodium hydroxide for 1.2 min and then with deionized water for 1.5 min. At the end of each run, the capillary was rinsed again with the phosphate buffer for 1.5 min.

RESULTS AND DISCUSSION

Chain separation

Globin polypeptides are known to be difficult to separate by FZE as proteins tend to stick to the capillary wall, leading to incorrect migration time and zone broadening. However, progress has been made in recent years, with the development of coated capillaries making protein analysis more feasible. Theoretically, by using coated capillaries, homologous proteins such as globins can easily be resolved by preventing the proteins from adhering to the capillary wall. Unfortunately, the system used in the present investigation did not have coated capillaries. Nevertheless, through manipulation of buffer composition, pH and current, it was possible to resolve the three rather similar polypeptides with very subtle differences in charge densities.

Optimal conditions

After preliminary experiments, 40 μA was selected for the analysis, as separation carried out

at this current provided best resolution of the three globin chains.

It has been reported that the separation of proteins under alkaline conditions causes complications such as an increased electroendosmosis rate, which affects the resolution and reproducibility, solute adsorption, which causes poor peak shape, reduced response or no elution [5]. In order to overcome this problem the capillary was rinsed with sodium hydroxide to wash away any protein sticking to the wall of the capillary after each run.

Furthermore, the injection time was kept at 3 s to prevent overloading which might result in band broadening. Low sample sizes have also been found helpful in improving the column efficiency [6,7].

As shown in Figs. 1–3, the peak with the highest mobility, corresponding to the more cationic

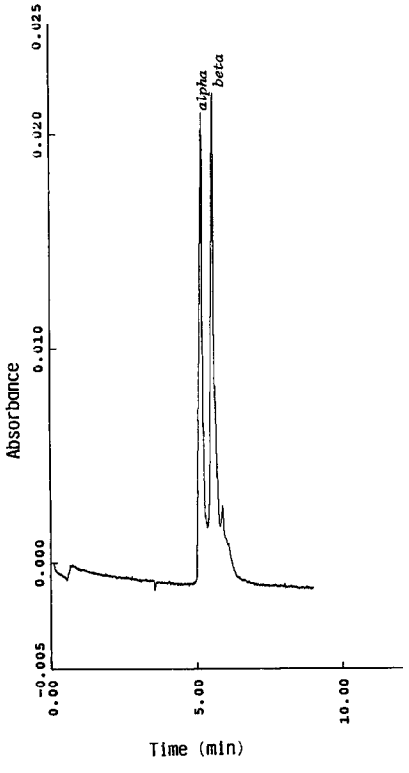

Fig. 1. Electropherogram of globins from a normal subject. Electrophoretic solution: 25 m*M* sodium phosphate buffer at pH 11.8 using an uncoated capillary column of 42 cm × 75 μm I.D. Voltage limit: 20 kV at 40 μA.

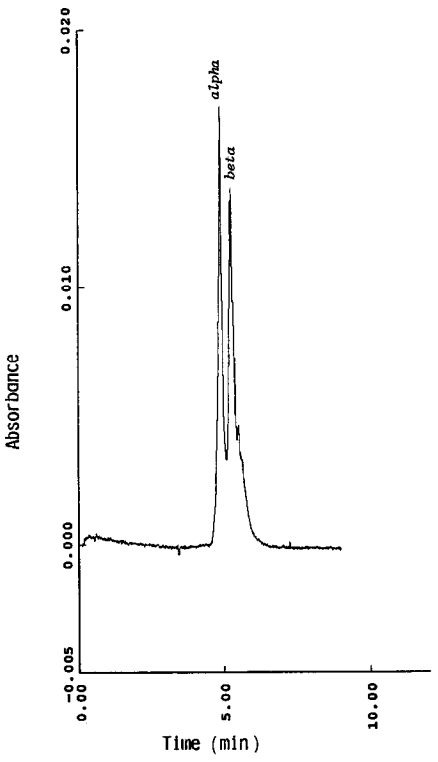

Fig. 2. Electropherogram of globins from a carrier of the β-thalassaemia trait. Electrophoretic conditions same as for Fig. 1.

α-chain, was detected at 5.2 min, while the slower one corresponded to the β-chain at 5.6 min. The main peak found in cord blood sample was the least cationic γ-chain, which was detected at 6.5 min. The total time required for the separation under the above conditions was less than 8 min, a relatively short time when compared with the electrophoretic techniques, which often require more than 10 h.

Migration in capillary electrophoresis is governed primarily by solute charges, so manipulation of the electrophoresis buffer is the most direct means of optimizing separation [7]. Grossman *et al.* [8] found that pH is also an important parameter affecting electroendosmosis and protein–silica interaction. For peptide analysis, it has been reported that under low-pH conditions osmotic flow and electrostatic interactions with proteinaceous solutes are reduced [5]. However, it is also well known that many proteins aggregate and precipitate under acidic conditions.

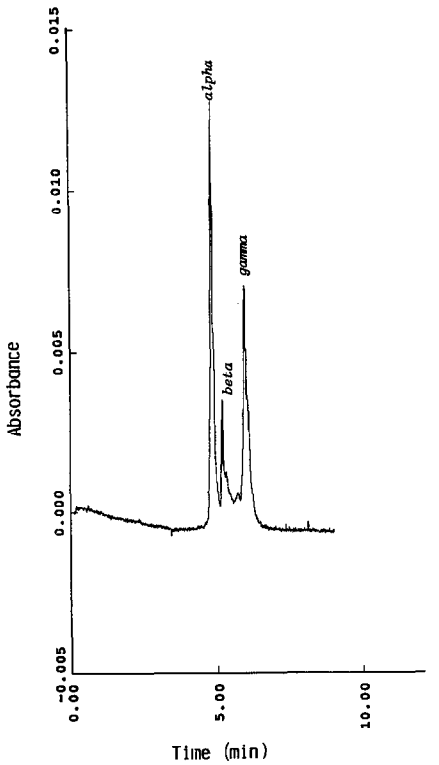

Fig. 3. Electropherogram of foetal haemoglobin.

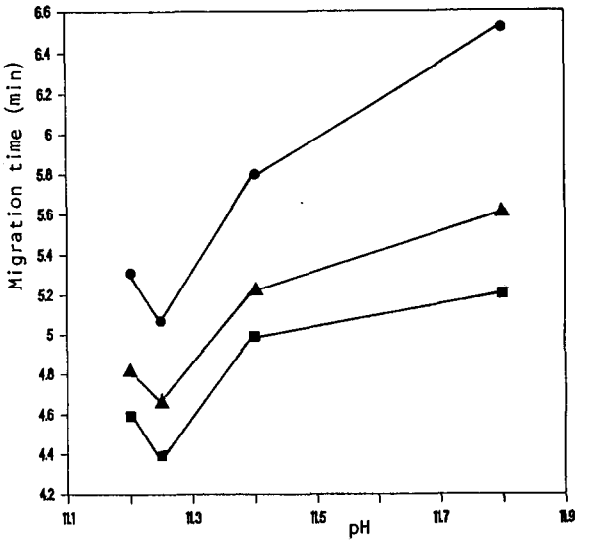

Fig. 4. Plot of migration time *versus* pH. All these experiments were conducted at a voltage of 20 kV across an uncoated 42 cm × 75 μm I.D. capillary column. Globins: ■ = α-; ▲ = β-; ● = γ-chain.

There may be also insufficient difference in their mass-to-charge ratios to achieve good separation. McCormick [9] has recently shown that subunits of haemoglobin could be associated in the column at pH 2.1, leading to multiple peaks for a single pure species.

At high pH values, it is believed that the peptide and silica surfaces exhibit high negative charge density, and adsorption through electrostatic interaction should be reduced. Furthermore, as shown in the present investigation, by using a sodium hydroxide solution to purge the capillary between runs globin contamination in the capillary can be minimized.

Fig. 4 shows the migration time for the three globin chains at different pH values. From the results, it can be seen that the migration time for each globin increased with increasing pH from 11.25. This observation was consistent with previous investigations of protein [10]. High pH caused the capillary walls and proteins to be negatively charged, thus reducing the adsorption of the proteins on to the capillary wall. It was also noted that the migration order for all the globins remained unchanged throughout the range of values examined. This observation suggested that there were no major changes in the extent of charges for all three polypeptides at all the pH values investigated. The more ionic α-chain migrated faster than the least cationic γ-chain.

Reproducibility

The decision to work at a fixed current resulted in much better reproducibility than when running at a fixed voltage. In the latter case, it was noted that the pattern was reproducible but retention times varied from run to run. In addition, it was noted that the baseline was less stable. This resulted in difficulties in the rapid identification of peaks.

When working at constant current, sequential injections of the same globin samples of adult haemoglobin gave almost identical retention times and area percentage of the two globin chains, α and β. The coefficient of variation for within-day run and between-day runs (based on the ratio β/α) was 4.1% and 5.7%, respectively.

Capillary column life

The capillary used in the present investigation was uncoated, and no additives were used to enhance the resolution or separation. Although the lifetime of the column used under the above conditions was not estimated, no significant deterioration of performance was noted over 4 weeks of continuous electrophoresis and separation of over 100 samples.

Clinical screening

Blood samples from 41 healthy children and 25 children suspected for having β-thalassaemia (one of the most common genetic diseases in Singapore) were analysed by the present method. The average β/α area ratio of healthy subjects was 1.07 $\pm$ 0.07 (range 0.97–1.21). The mean ratio for β-thalassaemia carriers was 0.88 $\pm$ 0.11 (range 0.62–0.99) and none of the subjects had a β/α ratio greater than 1. The globin sample prepared was kept at -10°C and was reported to be stable for months without any loss of its physiochemical properties [11].

Although HPCE is a relatively new technique in the field of analytical separation, it has been shown to be extremely useful for the separation of biomolecules (for review see refs. 3, 9 and 12–15). The average sample preparation time using the above-described method was about 95 min for 20 samples. The analysis time was 14.2 min per injection using an autosampler. About 25 samples could be analysed within a day, and only 100 ml of the running buffer were required.

CONCLUSION

The results here clearly show that the separation of the globin chains can be successfully achieved using capillary electrophoresis. Free zone electrophoresis using un uncoated capillary is a fast and sensitive method for the detection of similar charge globin variants. The speed and the simplicity of the method should make it an attractive alternative to the conventional time-consuming procedures.

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