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Haemoglobin analysis by capillary zone electrophoresis

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Abstract

The analysis of haemoglobin is routine in medical laboratories for the purpose of assessing blood disorders and related pathologies. Haemoglobin is structurally diverse and possesses many variant forms, some disease-producing. With recent improvements in technology, capillary electrophoresis is now being adapted in the clinical laboratory. This paper describes the analysis of haemoglobin variants using uncoated fused-silica capillaries. The effects of using different buffer salts at different concentrations with different buffer pH values to separate haemoglobin variants in these capillaries are described.

1. Introduction

Analysis of haemoglobin (Hb) variants from patients' blood samples is of medical importance and, for this reason, is an essential procedure in the clinical laboratory. Hb is a heterotetramer which is formed from the association of two pairs of identical subunits. The predominant Hb variant in normal adult blood is Hb A1, a heterotetrameric protein with two α-chains and two β-chains. Hb A2 is also a normal variant present in smaller amounts. Hb F is a form composed of two α- and two γ-globin chains and is normally expressed during foetal development; its presence is gradually replaced by Hb A usually 6 months after birth. The Hb S and C variants are isoelectric variants as a result of single amino acid changes in one of the globin chains (β), and such changes induce alterations in the structural–functional relationships of the Hb subunits to one another, such that at certain blood pH and level of blood oxygenation, the Hb tetramer undergoes an irreversible conformational shift which manifests itself as a change in the shape of the red blood cell (the classical sickle shape in sickle-cell anaemia). Hundreds of Hb variants, some producing no symptomatic disease, have been identified by various analytical methods.

The demands for clinical laboratories to provide accurate analyses with timely results makes new methodologies which achieve such goals very welcome. Capillary zone electrophoresis (CZE) is finding many uses in clinical chemistry applications [1–6], and there is the potential to apply CZE in Hb variant analysis also. CZE offers some important advantages over other conventional electrophoretic techniques. Besides requiring very small amounts of sample and electrolyte, its potential for automation makes this technique time and labour effective for clinical laboratories.

Several reports have already appeared in which normal and pathological states were assessed based on Hb variant analysis using capillary electrophoretic methods. Particular methods

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focused on the analysis of intact Hb molecules [2], globin chains [3] or tryptic digests of haemoglobin molecules [4]. Hb variant analysis has also been examined by isoelectric focusing methods using coated [5] and uncoated capillaries [6]. In this study we attempted to establish optimum conditions for Hb variant analysis using CZE with uncoated capillaries.

2. Experimental

The capillary electrophoresis system was a BioFocus 3000 (Bio-Rad, Richmond, CA, USA) equipped with a fused-silica capillary [36 cm (30 cm to the detector) × 50 μm I.D.]. Sample injection was by positive pressure [$34.45 \cdot 10^7$ Pa (5 p.s.i.) for 2 s], with sample loaded at the anode end of the capillary. The applied potential was 10 kV for all analyses. All reagents for preparing electrolytes were of analytical-reagent grade (Merck, Darmstadt, Germany). A capillary wash solution (Bio-Rad) was used to purge the capillaries between runs. Preparation of the capillary for each run involved an automated purging cycle with capillary wash solution, water and running electrolyte in that order, for 1 min each. Detection was at 415 nm using a tungsten lamp source.

Human Hb samples were prepared from whole blood collected in EDTA-containing evacuated tubes. Red blood cells were washed with normal (0.9%) saline four times. An aliquot of a washed cell suspension was mixed with four aliquots of distilled water. Haemolysis was made complete by freezing in a deep freezer for 10 min. Samples were centrifuged at ca. 600 *g* for 10 min. The supernatant was diluted tenfold in running electrolyte, and the sample was made ready by filtering it through 0.45-μm cellulose ester (Syrfil-MF; Costar, Cambridge, MA, USA).

As an external reference for haemoglobin species variants, the Beckman Paragon electrophoresis system and Hb variant standard kit (Beckman Instruments, Brea, CA, USA) was also used to analyse samples.

3. Results and discussion

The basis for the separation of the Hb variants using CZE is the charge differences in the globin polypeptides. The structure of all variants are such that they possess a net negative charge, under the conditions of CZE separation, and thus the inherent electrophoretic mobility of the Hb species will be directed towards the anode. However, the use of uncoated fused silica at pH values in the neutral or alkaline range creates a significant electro-endoosmotic flow (EOF) directed towards the cathode. The magnitude of the EOF is such that the net mobility of all Hb species is towards the cathode. This property means that Hb species having higher isoelectric points (p*I* values) (i.e., species with less net negative charge) should be detectable first inasmuch as the electrostatic repulsive forces in the opposite direction are less than for Hb species having lower p*I* values, and they would show a higher net mobility towards the cathode.

Satisfactory separation of analytes using CZE is highly dependent on a suitable electrophoresis buffer–electrolyte. In addition to ensuring overall stability of the system, the buffer determines the migration behaviour of analytes. Several different buffers have been used for haemoglobin separations in traditional free zone electrophoresis applications. Some methods employ a single weak acid or base to provide the buffering at the running pH; others consist of a mixture of weak acids or bases. As the p*I* values of haemoglobin species variants are between 7.00 and 7.50 (Hb A1, 7.15; Hb A2, 7.40; Hb F, 7.15; Hb S, 7.25), preparation using buffers with the pH adjusted above these values becomes necessary. Tris and borate buffers are commonly selected because of their buffering capacity ranges between pH 8.0 and 9.5. Veronal (barbital) buffers also possess a buffering capacity in this range and are commonly used in electrophoretic systems involving the use of commercially available agarose gels to analyse Hb variants. We tested Tris, borate and veronal buffers at three different concentrations and at different pH values. Analysis with a Tris–HCl electrolyte was carried

out at 0.5, 1.0 and 1.5 *M*, sodium borate was tested at 20, 30 and 40 m*M* and veronal (sodium barbiturate) at 25, 50 and 75 m*M*. For each of these concentrations for each of the indicated salts, three different pH values were tested, namely pH 8.0, 8.5 and 9.0.

The electropherogram in Fig. 1 illustrates how the molecular charge difference between the Hb S and Hb A1 species affects their separation by CZE. This separation of these variants was also generally observed under a variety of conditions in which all permutations for the three different buffer salts, concentrations and pH were tested. The Hb A2 species is seen to migrate ahead of Hb S in Fig. 1. We found that the degree of separation of Hb S from Hb A1 was not significantly different for any change in the pH (8.0, 8.5, 9.0) of the salts tested, but there were certainly changes in migration times, an increase in pH producing shorter migration times for all species. The inset in Fig. 1 shows a densitometric scan of stained haemoglobin variants observed using the Beckman agarose gel electrophoresis system. Although we did not confirm the identity of Hb variant species during the CZE analysis of our samples using certified standards, the agarose gel analysis operates under identical principles to CZE and the densitometric scan of stained proteins in the gel compares favourably with the absorbance changes during CZE in which detection is specific for the Hb prosthetic group.

The variant Hb F has no significant charge difference from Hb A1 (only 0.05 pH unit separates these variants), and this presents the most difficult challenge to manipulating conditions to separate common variants. Fig. 2 shows the separation of Hb F and Hb A1 from a patient's sample using a 50 m*M* veronal system buffered at pH 8.5. An observable separation of these species was achieved for all pH values tested, but the resolution appeared better at pH 8.0 and 8.5 than at pH 9.0, demonstrating an effect of pH on this separation. Again, the

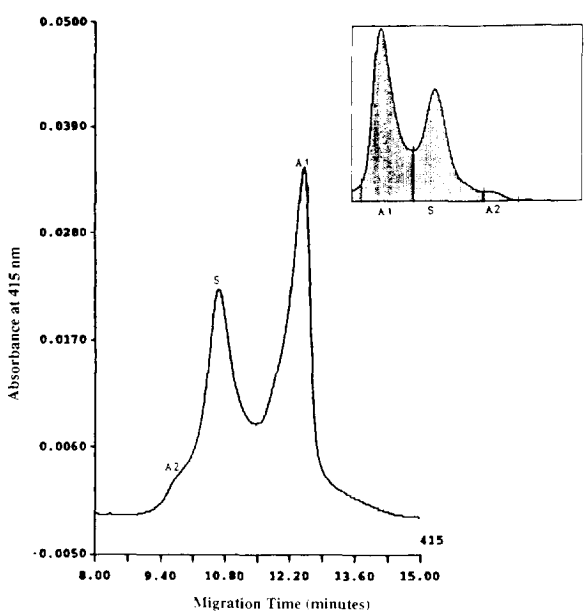

Fig. 1. Electropherogram of a sample from a patient with sickle-cell disease. Electrolyte, 1.0 *M* Tris–HCl (pH 8.0). Other conditions as described under Experimental.

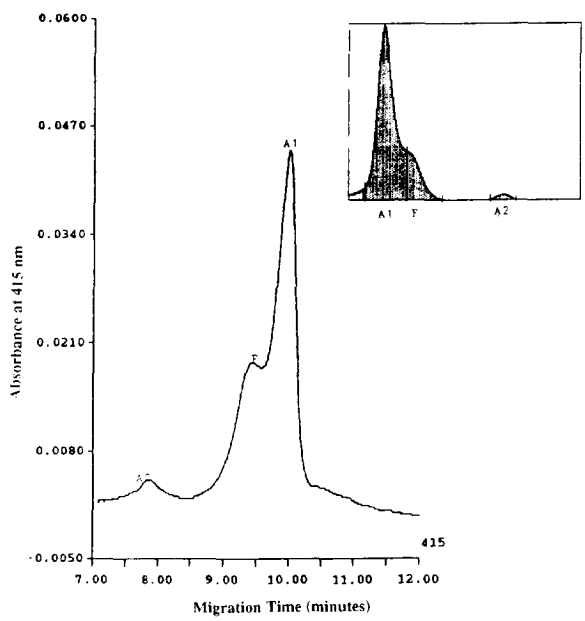

Fig. 2. Electropherogram of a patient's sample with a significant Hb F fraction. Electrolyte, veronal (sodium barbital) (pH 8.5).

proportions of the amounts of Hb species present in this sample agreed with the agarose gel determination (inset).

Fig. 3 is an electropherogram of another patient's sample in which Hb variants were separated using 20 m*M* sodium borate (pH 8.5). The Hb variants A2, S, F and A1 were all detectable. These particular conditions resolved these four variants the best, although we did observe the separation of these variants using this buffer at other pH values. For selecting particular buffer salts to be used in such CZE separations, we found the best concentration for Tris to be 1.0 *M*, for veronal 50 m*M* and for borate 20 m*M*.

Variations in the migration times of the species for replicate analyses were a significant problem and make routine clinical use difficult, in that species identification depends on consistent migration times (much as component identification in high-performance liquid chromatography depends on consistent retention times). We made attempts to stabilize the EOF, which we believe is the factor responsible for such irreproducibility, but we were unable to obtain more optimum conditions. As the Hb A1 variant is usually a constant in all analyses, its identification can be made initially and then used as a reference for identifying other variants. Fig. 4 is an electropherogram of a normal adult blood sample with Hb A1 present as the major species. There are other cases in which Hb A1 may not be the major species present, as shown in Fig. 5 (identification of Hb species in CZE were made by comparison with separated zones seen in agarose gel electrophoresis, a routine method in our laboratory as well as in many others). Such cases might present some confusion for the analyst. Nevertheless, the experienced analyst would know the relative order of migration of the typical species present in the population, and variations in routine electropherogram patterns can be easily discerned. Alternatively, unknown patients' samples can be run with internal standards (e.g., with commercially available Hb variant standards) to make Hb variant identification unambiguous.

Changes to the conditions of buffering (selection of the ideal salt and therefore of the ideal

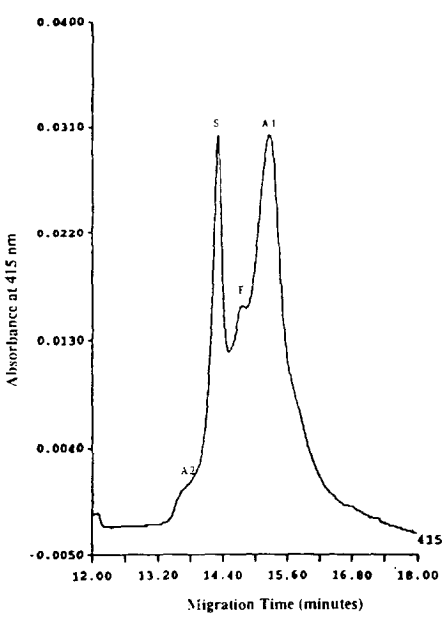

Fig. 3. Electropherogram of a patient's sample containing significant proportions of variants Hb A1, A2, S and F. Buffer, sodium borate (pH 8.5).

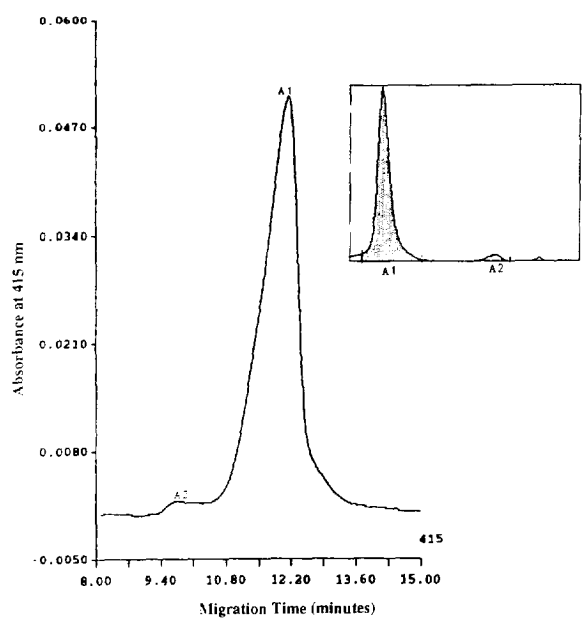

Fig. 4. Electropherogram of a sample obtained from a healthy adult. Buffer, veronal (pH 8.5).

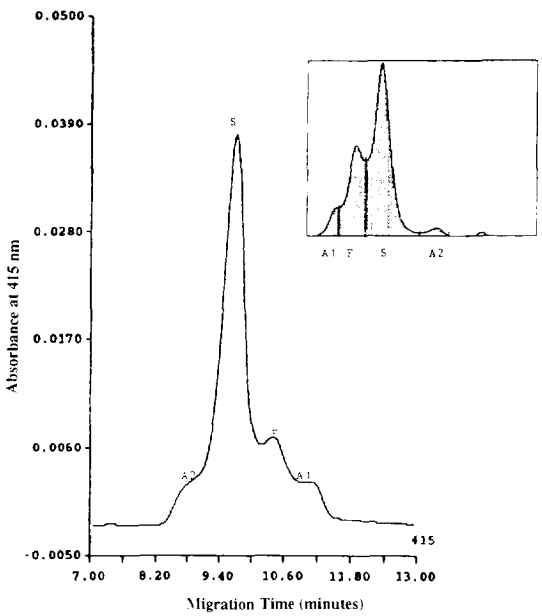

Fig. 5. Electropherogram of a sample from a patient with sickle-cell disease. Buffer, veronal (pH 8.5).

pK_a, electrolyte pH and buffer concentration), in addition to selection of modifying agents which dynamically coat the capillary walls in order to alter protein adsorption and to reduce the EOF to negligible levels, are future considerations for further work to understand and control the considerable variations of migration times observed in replicate analyses.

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