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Note

Carrier-ampholyte displacement chromatography (chromatofocusing) on ion-exchange papers

Application to the separation of haemoglobin variants

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Ion-exchange chromatography is the most frequent method used to perform separations of proteins. Proteins fixed on a chromatographic support are eluted either by changing the pH and/or ionic strength of eluent, or by using an elution reagent able to displace proteins selectively from the support. The use of carrier ampholytes in displacement chromatography on an ion-exchange column for liquid chromatography has been described [1—6]. This paper describes carrier ampholytes as developers in ion-exchange paper chromatography applied to the study of human haemoglobins. The behaviour of some human haemoglobins in the presence of carrier ampholytes in an ion-exchange column for liquid chromatography has been previously described [7].

EXPERIMENTAL

All haemoglobin samples were prepared from red cell lysates as described

elsewhere [7]. However, pure foetal haemoglobin components F_0 and F_1, used for controls, were obtained by preparative isoelectric focusing of cord blood hemolysates [8]. Haemoglobin concentrations were adjusted to 4% (w/v) with distilled water.

The chromatographic support was an anion-exchange paper with diethylaminoethyl (DEAE) groups (Whatman DE-81). Paper strips were cut to the specific size of 200 mm × 45 mm. The equilibration buffer was tris(hydroxymethyl)aminomethane (Tris), 0.05 *M* HCl (pH 8.9), KCN 0.01%. Aqueous solutions of carrier ampholytes used as developers were prepared from commercial Ampholines^R (LKB, Bromma, Sweden) or Pharmalytes^R (Pharmacia, Uppsala, Sweden); the pH values of the solutions were not adjusted.

Chromatography was performed as follows. Haemoglobin samples were diluted down to 1/50 with the equilibration buffer; 2 μl of each diluted sample were deposited on the chromatographic paper in saturating conditions [9] at point 0, 20 mm from the lower edge of the paper. Ascending development was made at 4°C for 12—14 h in a chromatographic chamber in saturating conditions.

RESULTS AND DISCUSSION

Aqueous solutions of Ampholine in the pH range 6—8 (1.2%, w/v) and Pharmalyte in the pH range 6.5—9 (3%, w/v) have been compared as developers. A typical separation obtained between some haemoglobin variants with the above Ampholine solution is shown in Fig. 1. The corresponding R_F values are given in Table I.

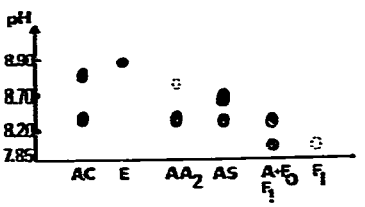

Fig. 1. Paper chromatogram of some haemoglobin variants. Solvent: Ampholine pH range 6—8 solution (1.2%, w/v). Equilibration buffer: 0.05 *M* Tris · HCl (pH 8.9), 0.01% KCN. Spots with broken line: haemoglobin variants. R_F values increase in the order: HbF_1, HbA (HbF_0), HbS, HbA_2, HbC, HbE.

TABLE I

R_F VALUES OBTAINED WITH AMPHOLINE SOLUTIONS IN THE pH RANGE 6—8

Ampholine concentration (pH 6—8)	HbA	HbA_2	HbF_0	HbS	HbC
1.2% (w/v)	0.13	0.24	0.13	0.19	0.26
4.0% (w/v)	0.45	0.60	0.42	0.54	0.61

No separation was obtained with the Pharmalyte solution tried; i.e. the pH varying from 8.16 (point 0: initial application) to 8.90 (solvent from at 60 mm). However, a clear-cut separation of haemoglobins, similar to that obtained with Ampholine in the pH range 6—8, was obtained with another Pharmalyte solution: 6% (w/v), pH range 6.5—9. The pH values detected varied from 8.13 to 9.00 for a maximal migration distance of 180 mm.

The influence of the pH range of the carrier ampholytes was studied with the haemoglobin samples previously shown in Fig. 1. Optimal conditions of separation were obtained with Ampholine of pH range 6—8. The effect of the concentration of the carrier ampholytes on R_F values was also determined, and the results are shown in Table I.

As an additional assay, a pre-run was done before the application of the haemoglobin samples and the pH determined on the chromatographic support. But there was no definite improvement in the resulting power of resolution, although the solutions for pre-run and elution were the same.

The influence of the position of the sample application on the chromatographic paper was tested using a mixture of HbC and HbS. With an Ampholine solution of pH range 6—8 (1.2%, w/v) the separation between the haemoglobins was clear-cut at the initial point 0 (pH determined was 7.50—7.60), but the separation was partial when application was made at 30 mm (pH 8.30—8.40), and no separation occurred with initial applications at 60 mm, 90 mm, 120 mm, where the respective pH values measured were 8.60, 8.80—8.90 and 8.90—9.00.

In ampholyte-displacement chromatography (ADC) proteins fixed on the chromatographic support are selectively displaced by carrier ampholytes. The interactions between carrier ampholytes and ion exchanger induce the formation of an "internal" pH gradient and a displacement of counter-ions (i.e. chloride ions) by ampholytes. The migration order obtained by carrier ampholytes on ion-exchange paper is identical to that obtained for the same haemoglobins on an ion-exchange column with either DEAE-cellulose or DEAE-Sephadex supports and an "external" pH gradient maker [10].

A clear-cut separation between HbA (or HbF_0) and HbF_1 is shown in Fig. 1, but no separation is obtained between HbA and HbF_0 when ion-exchange papers are equilibrated with Tris · HCl, KCN (pH 8.9) buffer and the Ampholine solution of pH range 6—8 (1.2%, w/v) is the developer. The equilibration of ion-exchange paper with 0.2 *M* glycine, 0.01% KCN buffer [11] increases the power of resolution of Ampholine 6—8 solvent between HbA and HbF_0.

Isoelectric points, p*I*, of the two components of foetal haemoglobin differ by 0.3 pH units. The p*I* of HbA and HbF_0 differ by 0.2 pH units [12, 13]. An approach to the influence of p*I* on the migration order of haemoglobins in paper ADC may be obtained by introducing relative mobilities, R_M, of the haemoglobins. A linear relationship exists between R_M and p*I* for two different Ampholine pH range 6—8 concentrations: 1.2% (w/v) and 4% (w/v) (Fig. 2). The p*I* values of haemoglobin variants are reported elsewhere [12, 13].

Carrier ampholytes as developers in ion-exchange paper chromatography strongly diminish tailings [14]. The carrier ampholyte solutions are usable for several runs. The ADC method avoids an exposure of proteins to large pH

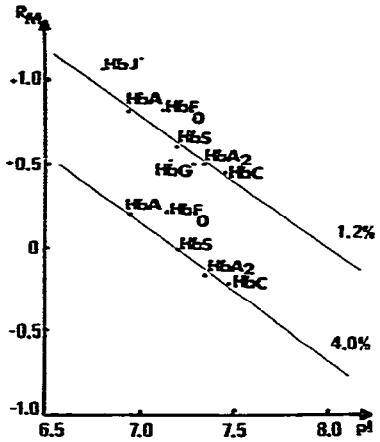

Fig. 2. Relative mobilities, R_M, as related to isoelectric points, pI, of some haemoglobin variants, for two different concentrations of Ampholine solutions, 1.2% (w/v) and 4% (w/v), in the same pH range 6—8. Equilibration buffer: 0.05 M Tris · HCl (pH 8.9), 0.01% KCN. HbJ* = haemoglobin variant with HbJ Oxford mobility in isolelectric focusing. HbG° = haemoglobin G Philadelphia.

variations during the chromatographic process and, as compared with conventional chromatographic techniques, the effects of temperature changes are also reduced.

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