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Note

Chromatofocusing of human hemoglobins

NICHOLAS M. ALEXANDER* and WILLIAM E. NEELEY

Division of Clinical Pathology, Department of Pathology, University of California, San Diego, CA 92103 (U.S.A.)

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Chromatofocusing is a relatively new technique in which a pH gradient is produced on an ion exchanger by utilizing the buffering action of the charged groups on the ion exchanger [1, 2]. This article describes separation of the commonly occurring human hemoglobins A_2, E, C, S, A_0, F_0, and F_1 by this chromatographic technique.

EXPERIMENTAL

Reagents

Polybuffer 96, polybuffer exchanger PBE 94, and columns were obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). All other chemicals were commercial analytical grade products.

Hemoglobin preparations

Human red blood cells were collected from whole blood in EDTA anticoagulant by centrifugation and washed three times with 5 volumes of 0.15 *M* sodium chloride. Cord blood cells were the source for fetal hemoglobin. The washed red cells were lysed with 2 volumes of deionized water.

Hemoglobin electrophoresis

Hemoglobin electrophoresis was performed on cellulose acetate at pH 8.4 and on citrate agar at pH 6.2 with an apparatus from Helena Laboratories (Beaumont, TX, U.S.A.) [3].

Chromatofocusing

Chromatofocusing was performed with 0.9 cm diameter columns containing

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21—54 cm of anion-exchanger gel PBE 94. The eluates were continuously monitored (percent transmission) at 540 nm with a Model 100-10 spectrophotometer and flow-through cuvette (Hitachi, Tokyo, Japan) that was interfaced with a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.).

The anion exchanger was equilibrated with starting buffer, 0.025 *M* imidazole · HCl, pH 7.4 or pH 8.0, by passing several column bed volumes of buffer through the column. The eluting ampholyte polybuffer, PBE 96, was prepared by diluting the stock with 10 volumes of deionized water, adjusting the pH to 6.0 with 1 *M* hydrochloric acid, and adding water to a final dilution of 1:13. All buffer solutions were degassed under vacuum (water aspirator) immediately prior to use.

A mixture of hemoglobin variants was prepared by combining erythrocyte lysates that contained hemoglobins E, C, A_2, S, A_0, F_0, and F_1. One hundred microliters of this mixture containing 4—5 g/dl hemoglobin were applied to the column. Elution with polybuffer 96 was performed by gravity flow at a rate of 14—24 ml/h. Electrophoretic analysis of the fractions was performed after concentrating them in an A25 Amicon macrosolute concentrator (Amicon, Lexington, MA, U.S.A.).

RESULTS

With a 54 × 0.9 cm column excellent separations of hemoglobins E plus A_2, S, A_0, F_0, F_1 were achieved within 18 h, but hemoglobins A_2 and E failed to separate (Fig. 1). Hemoglobin C was not available for this separation. The hemoglobin type in each peak was confirmed by electrophoresis, although neither chromatofocusing nor electrophoresis resolved hemoglobins E and A_2. Hemoglobins F_0 and F_1 were further identified by their resistance to denaturation in alkali [5, 6].

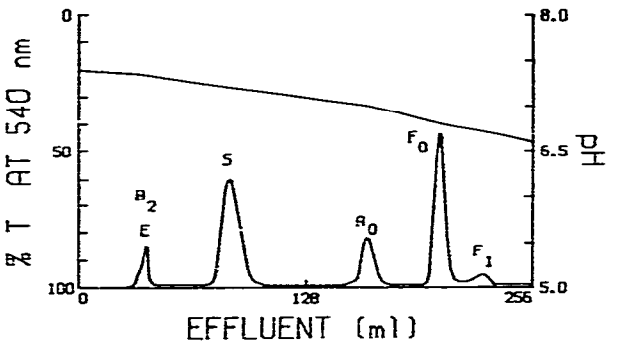

Fig. 1. Chromatofocusing of human hemoglobin variants A_2, E, S, A_0, F_0, and F_1 on a long column. A mixture of oxyhemoglobins (A_2, E, S, A_0, F_0, F_1) containing a total concentration of 4.7 mg hemoglobin was chromatographed on a PBE gel column, 54 × 0.9 cm, starting at pH 7.4, 0.025 *M* imidazole · HCl and eluting by gravity with pH 6.0 polybuffer 96—HCl at a flow-rate of 14.3 ml/h.

A similar pattern (Fig. 2) with high resolution can be achieved in about 6 h using a shorter column (26 × 0.9 cm). Hemoglobins S, A_0, F_0 and F_1 were clearly resolved from each other and from a heterogeneous peak that consisted

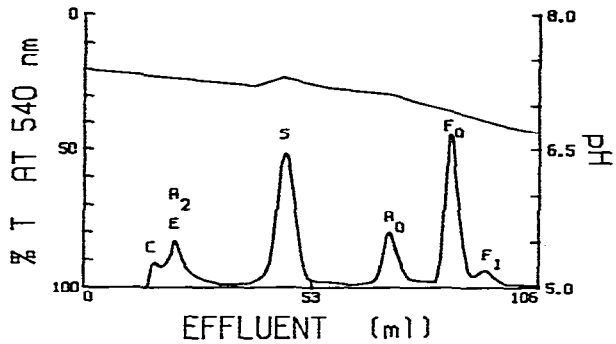

Fig. 2. Chromatofocussing of human hemaglobin variants C, A_2, E, S, A_0, F_0 and F_1 on a short column (pH 7.4 to 6.0). Conditions were the same as in Fig. 1 except that the column dimensions were 26 × 0.9 cm with a flow-rate of 17.5 ml/h and hemoglobin C was added to the mixture.

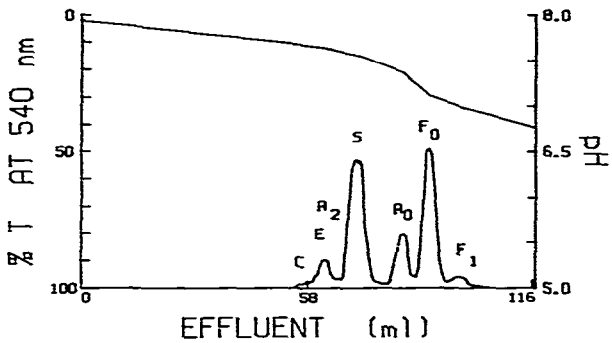

Fig. 3. Chromatofocusing of human hemoglobin variants C, A_2, E, S, A_0, F_0, and F_1 on a short column (pH 8.1 to 6.0). Conditions were the same as in Fig. 1 except that the column dimensions were 21 × 0.9 cm with a flow-rate of 23.6 ml/h and the starting buffer was at pH 8.1.

of hemoglobins A_2, E and C, although C migrated slightly faster than A_2 and E.

When the elution was performed with pH 8.1 rather than pH 7.5 polybuffer, resolution of the hemoglobin variants was less satisfactory and elution of the peaks occurred later (Fig. 3).

A comparison of the isoelectric points of the hemoglobin variants and the pH at which they chromatofocused showed that the hemeproteins emerged at a pH close to their p*I*, and in the order of their isolelectric points, except that hemoglobin F_0 emerged after A_0 even though F_0 has a more basic p*I* (Table I). Moreover, we observed that the pH at which the hemoglobins focused varied with the pH of the starting buffer. When the starting pH was at 8.0 the more basic hemoglobins A_2, C, E, S and A_0 eluted slightly above their p*I* values, while F_0 and F_1 eluted near their p*I* values, whereas with a starting buffer at pH 7.4, hemoglobins A_2, E, C, S, F_0 and F_1 eluted near their p*I* values, while A_0 emerged from the column at a pH above its p*I*.

Attempts to separate hemoglobins C, A_2 and E by starting at pH 7.8 and eluting with either pH 7.2 or pH 6.8 failed to resolve these hemeprotein variants.

TABLE I

ISOELECTRIC POINTS OF HEMOGLOBIN VARIANTS COMPARED WITH ELUTION pH IN CHROMATOFOCUSING

Hemoglobin species	p*I**	Elution pH***	
		Gradient pH 7.4 to 6.0	Gradient pH 8.1 to 6.0
C	7.40	7.36	7.63
E	7.40	7.34	7.63
A_2	7.40	7.34	7.63
S	7.25	7.26	7.55
A_0	6.95	7.06	7.36
F_0	7.15	6.86	7.11
F_1	6.90	6.74	6.98

*Obtained by isoelectric focusing in polyacrylamide gel [4] except for hemoglobin E which was estimated from our data.
**From Figs. 1—3.

DISCUSSION

Protein purification by chromatofocusing is dependent on eluting a given protein at, or near, its p*I* [1, 2], and thus, the choice of a narrow pH interval from 7.4 to 6.0 was sufficient to separate most of the commonly occurring human hemoglobins as sharp peaks with 0.05 pH units at half width. These hemeproteins emerged from an anion-exchange column in order of their isoelectric points, with the exception of fetal hemoglobin, which behaved as if it has a more acidic p*I*, a phenomenon that was also observed with pH-gradient chromatography on DEAE-Sephadex [7].

By using a pH interval of 8.1 to 6.0 for chromatofocusing, the hemoglobins emerged from the column at pH values somewhat higher than their p*I* (except for hemoglobin F_0). This was probably due to some separation by ampholyte displacement because the hemeproteins were eluted after 4 to 8 column bed volumes of eluents had passed through the column (Fig. 3) when ambient buffer concentration increased and ampholyte displacement was more pronounced [8].

Others have utilized ampholyte displacement chromatography to separate human hemoglobin variants [9]. Mixtures of A_2 and A_0, A_2, S and A_0, and C, S, and F were resolved but no experiments with a complete mixture of all the variants were reported. Although ampholyte displacement chromatography is an effective procedure for separating some hemoglobin variants, its major limitation is the high cost of the carrier amphoteric buffers which are used in many-fold greater concentrations than in chromatofocusing [8, 9].

The method described in this report is especially useful for preparative isolation of hemoglobin species. But its application as a routine analytical procedure would not appear to be as convenient as gel electrophoresis which is faster and less expensive than chromatofocusing.

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