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# SEPARATION OF HAEMOGLOBINS USING A MONODISPERSE CATION EXCHANGER

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## SUMMARY

In order to separate haemoglobin variants in one step, a chromatographic method using Mono S (Pharmacia) monodisperse cation exchanger was developed The separation of haemoglobins (A, S, E, D, C and F) on a short analytical column (50 × 5 mm I.D.) was accomplished in 30 min, including the regeneration, using linear buffer gradient elution (lithium chloride in 0.01 *M* sodium malonate, 0 to 100%). For haemoglobin A, two peaks, probably corresponding to different molecular forms, were found. This systematic double peak affected the recognition and the measurement of haemoglobins. By filling the test-tube with carbon monoxide before injecting the haemolysate, we obtained reproducible results with regard to both retention times and peak shape, and also great stability.

The long-term stability and the high speed of the separation seem to be adequate for automation, making the method suitable for routine clinical laboratory use.

## INTRODUCTION

Most abnormal haemoglobins were discovered by electrophoresis. The most widely used technique for separating haemoglobins S, F, C, $A_1$ and $A_2$ is cellulose acetate electrophoresis at alkaline pH[1]. If an S band is present, a solubility test or sickling test must be performed. In order to provide the ready separation of haemoglobins that migrate together on cellulose acetate, citrate agar electrophoresis at pH 6.0–6.5[2] is essential (separation of S from D and G, and C from E and O)[3,4]. However, none of these methods will give satisfactory results when the percentage of abnormal haemoglobin is low.

The introduction of ion-exchange chromatography, isoelectric focusing and chromatofocusing have allowed considerable refinement in isolating abnormal haemoglobins[5,6]. In this paper, we describe a chromatographic method for the separation of haemoglobin variants in one step, using Mono S monodisperse cation exchanger (Pharmacia)[7].

## EXPERIMENTAL

### *Sample preparation*

Blood samples were drawn from normal subjects and patients with haemoglobinopathy into heparinized collection tubes. Immediately after collection, specimens were stored at 4°C until a haemolysate was prepared.

The samples (1 ml) were centrifuged (1320 *g*, 5 min) and all of the supernatant fluid was aspirated and discarded, leaving packed erythrocytes. The cells were incubated at 37°C for 5 h with 5 ml of isotonic saline. Such incubation of samples eliminates the labile fractions. After incubation, the samples were centrifuged and the saline was aspirated. Samples were haemolysed in 3 ml of distilled, deionized water and glycolipids were then extracted into 0.5 ml of carbon tetrachloride by vigorous shaking. To separate the organic phase, cell ghosts and haemolysate, the mixture was centrifuged (1320 *g*, 30 min). the supernatant was collected in a rubber-stoppered tube and the air inside was replaced by carbon monoxide. The haemolysate was then diluted 1:5 with a buffer (malonate, 0.01 *M*, pH 5.7).

In order to study the separation of haemoglobin variants, a mixture of their haemolysates was prepared. The quantitation of haemoglobin variants was carried out by electrophoresis (Helena Laboratories). The composition of the mixture was HbF 2.5%, $HbA_1C$ 4%, $HbA_1$ 28%, HbD 14.5%, HbE 15%, HbS 24%, HbC 12%.

### *Chromatographic system*

The chromatographic system [Pharmacia Fast Protein Liquid Chromatography (FPLC) system] consisted of two P 500 pumps, an LCC 500 gradient programmer, an MV7 injector valve and a UV-1 detector fitted with an HR-10 flow cell.

The haemolysates were injected into a monobead column (Mono S HR 5/5) by means of an injection valve equipped with a 50-$\mu$l sample loop. The eluent was as follows: buffer A, 0.01 *M* malonate–NaOH, pH 5.7; buffer B, 0.01 *M* malonate–NaOH, pH 5.7 + 0.3 *M* LiCl. Detection was effected at 405 nm, 0.5 a.u.f.s. The sample was 50 $\mu$l of haemolysate.

An appropriate gradient was chosen for each separation: for haemoglobins $A_1$, S, C, D, E, F and H, a linear buffer gradient (0–100% B), time gradient 50 min, flow-rate 1 ml/min, maximum pressure 4.5 MPa; for haemoglobin $A_1C$, time gradient 25 min (to obtain a better separation of HbF), flow-rate 2 ml/min.

### *Evaluation procedure*

Preparations of blood specimens drawn from patients with haemoglobinopathy were subjected to electrophoresis on cellulose acetate (Helena Laboratories) to detect the abnormal haemoglobin. The haemoglobin electrophoresis controls were provided by Gelman Sciences. The common adult HbA, HbS, HbC, HbD and HbE were identified and separated from haemoglobin F. Each characterized haemoglobin variant was analysed with the Pharmacia FPLC system. The relative migration of each haemoglobin variant was determined.

The concentration of haemoglobin F was also determined for comparison by radial immunodiffusion assay (RID, HbF–Quiplate, Helena Laboratories).

## RESULTS AND DISCUSSION

### *Influence of carbon monoxide*

Initial experiments with the FPLC system yielded chromatograms that showed two peaks of haemoglobin A (Fig. 1). This systematic double peak affected the recognition and subsequent measurement of haemoglobins. To resolve this problem, we compared the chromatograms obtained with the untreated haemolysate and the CO-saturated haemolysate. By filling the test-tube with carbon monoxide before injecting the haemolysate, we obtained reproducible results with regard to both retention times and peak shape, and also greater stability (Fig. 2).

### *Influence of the separation time*

Mono S, a strong cation exchanger, enables useful separations of different haemoglobins to be carried out within 20 min (Fig. 3). However, a series of experiments were conducted in which the gradient times were varied. The resulting chromatograms show that the resolution actually improved with an increased gradient time (50 min). Fig. 4 shows a better separation of haemoglobins D and E.

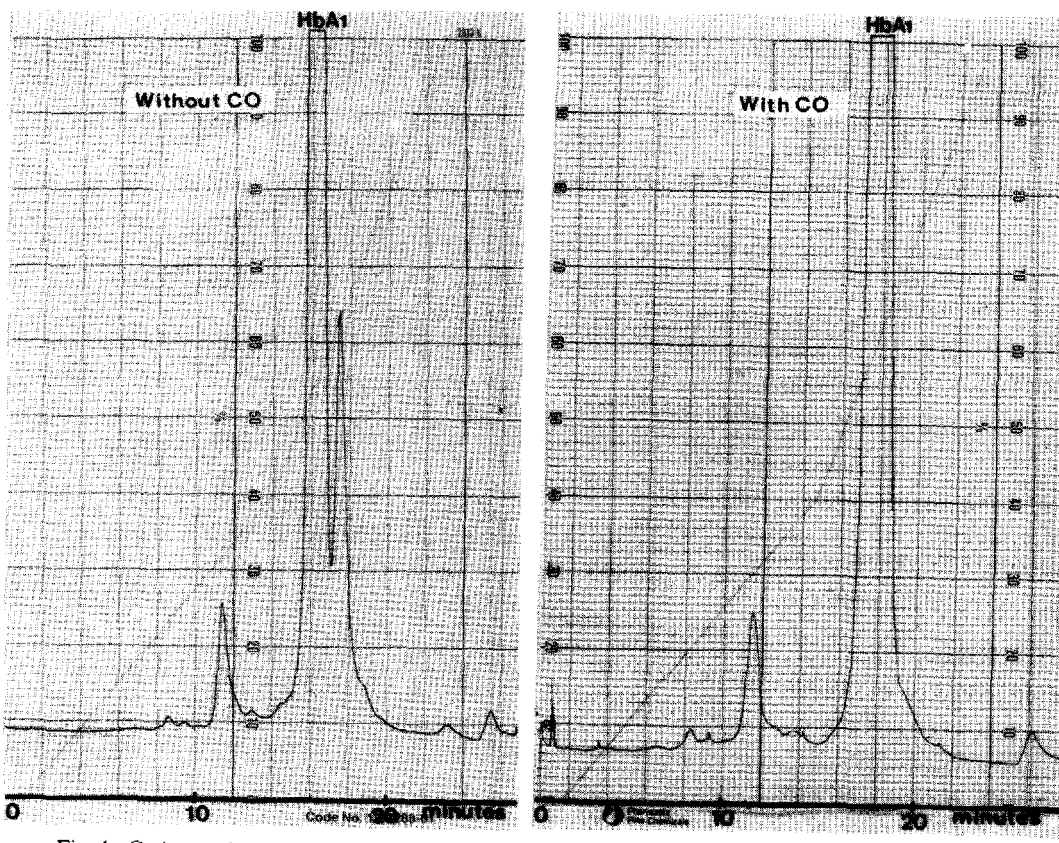


Fig. 1. Cation-exchange chromatography of untreated haemolysate.

Fig. 2. Cation-exchange chromatography of CO-saturated haemolysate.

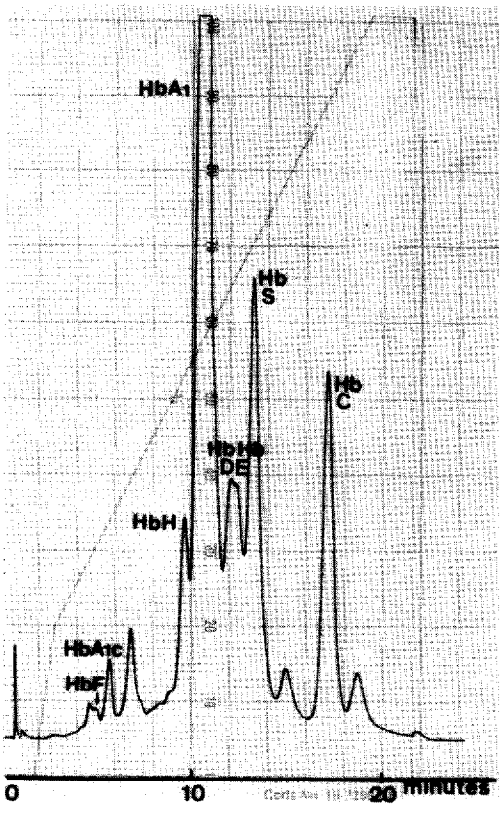


Fig. 3. Separation of haemoglobin variants with a gradient time of 20 min. **Sample: CO-saturated haemolysate.**

The quality of the separation that can be achieved by this method is very good. Normal haemoglobin and the main haemoglobin variants are clearly resolved, especially F, which is clearly separated from A and S. The retention volume for each haemoglobin variant is HbF = 3.50 ml, $HbA_1C$ = 4 ml, $HbA_1$ = 20.40 ml, HbD = 22.00 ml, HbE = 23.50 ml, HbS = 26.50 ml and HbC = 34.00 ml. Results for HbF obtained by FPLC were comparable to those obtained by radial immunodiffusion assay.

These results indicate that a monobead column provides distinct advantages over the use of acetate cellulose and a valuable base matrix for screening purposes. Although the basic chemistry involved in FPLC separations is analogous to that in conventional ion-exchange techniques, there is a considerable reduction in the separation times.

## CONCLUSION

Our study offers a practical approach in obtaining rapid and high-quality separations of HbA, HbF, HbS, HbE, HbD and HbC and relatively simple and sensitive evaluations of $HbA_1C$.

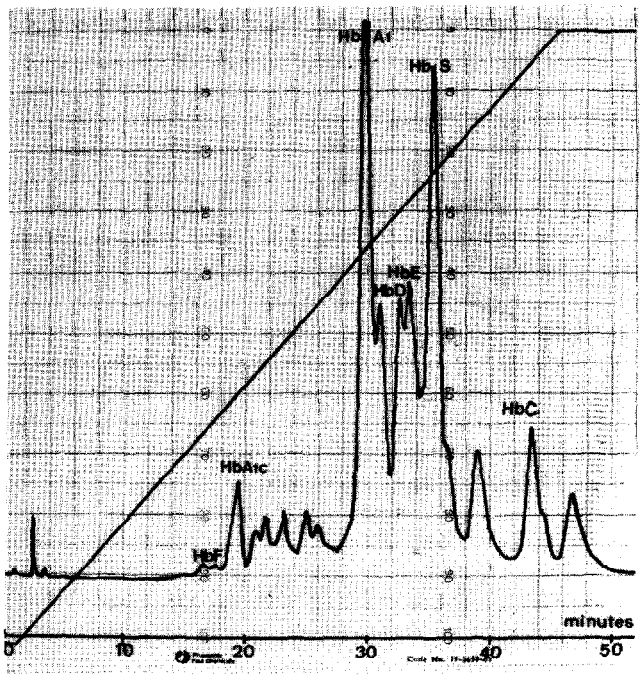


Fig. 4. Separation of haemoglobin variants with a gradient time of 50 min. Sample: CO-saturated haemolysate.

The long-term stability and the high speed of the separation seem to make it adequate for automation and thus suitable for clinical laboratory use.

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