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SEPARATION OF HEMOGLOBIN VARIANTS BY ION-EXCHANGE CHROMATOGRAPHY ON MONOBEAD RESINS

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SUMMARY

Our studies on the separation of hemoglobin variants using Monobead* ion exchangers show that these ion exchangers will resolve variants that heretofore could not be resolved. Procedures with these ion exchangers, therefore, extend our ability to detect hemoglobinopathies. Since it is also important to study the physicochemical properties of variants, their isolation is of critical importance to the researcher. The methods described in this report enable variants to be isolated in quantitative amounts so that such studies can be performed. Most isolations can be accomplished within 1 h, under mild conditions with aqueous buffers. Elution gradients can be changed conveniently to optimize resolution of most variants. These methods should serve as a valuable adjunct in studying the structure–function relationship of hemoglobin and other proteins.

INTRODUCTION

Because of their clinical importance, a large number of methods have been developed for detecting hemoglobin variants. These methods, which include electrophoretic procedures[1], isoelectric focusing (IEF)[2], ion-exchange chromatography[3], and high-performance liquid chromatography (HPLC)[4,5], have allowed investigators to make presumptive identifications and to quantitate variants. However, to do structural and functional studies, it is often necessary to isolate the variants. The method of choice for isolating variants is generally ion-exchange column chromatography[3], a procedure that employs mild conditions with aqueous buffers. Unfortunately, separating hemoglobins on a preparative scale using this procedure can take several days and can result in hemoglobin degradation and formation of excessive amounts of methemoglobin. In addition, variants that are clearly resolved by electrophoretic procedures may not necessarily be completely resolved by ion-exchange chromatography. Therefore, it would be advantageous to have a procedure that not only permits rapid resolution of hemoglobin variants for presumptive diagnosis, but, more

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importantly, provides adequate quantities for structural and functional analyses. In this report we present data on a new series of Monobead (Pharmacia) ion exchangers that we feel meet the above criteria.

EXPERIMENTAL

The structure of the hemoglobin variants used in this study were determined in our laboratory. Washed cells were lysed by adding an equal volume of distilled water and a quarter volume of carbon tetrachloride; cellular debris was removed by centrifugation. The hemolysates were stored either at 4°C in the presence of potassium cyanide and carbon monoxide or in liquid nitrogen as oxyhemoglobin. All samples were desalted on a G25M disposable Sephadex column (Pharmacia PD-10) and syringe filtered through a Gelman ACRO LC3A filter (0.45-μm pore size) prior to chromatography at room temperature (22°C).

The equipment used in this study was a Pharmacia fast protein liquid chromatography (FPLC) system composed of two P-500 pumps, a LCC-500 controller, a UV-1 monitor, and a MV-7 or V-7 valve. The columns used were Mono Q HR 5/5 (5 × 0.5 cm I.D.) and 16/10 (10 × 1.6 cm I.D.) (anion exchanger) and Mono S HR 5/5 (5 × 0.5 cm I.D.) (cation exchanger). The clean-up procedures followed the manufacturer's instructions: 1–4 ml 50–75% acetic acid for the Mono Q column, and 1–5 ml 0.5% sodium dodecyl sulfate (SDS), followed by 80–100% methanol to remove the SDS from the Mono S column.

For separations on the Mono Q column, buffer A was 50 m*M* tris–HCl, pH 8.5, and buffer B was 50 m*M* Tris–HCl, pH 6.5. Both buffers contained 0.01% potassium cyanide. The buffers were filtered through a membrane filter with 0.45-μm pore size and then sonicated for approximately 5 min. The first gradient which was 0 to 40% B in 20 min, 40 to 75% B in 20 min, and 100% B for 5 min, was used primarily to separate variants with electrophoretic mobilities cathodic to Hb A (slow variants). The second gradient which was 0 to 60% B in 15 min, 60 to 100% B in 15 min, and 100% B for 5 min, was used to separate variants with electrophoretic mobilities anodic to Hb A (fast variants). The flow-rate was 1 ml/min, and the eluent was monitored at 280 nm. The sensitivity can be further increased by monitoring in the Soret band.

For separations on the Mono S column, buffer A was 10 m*M* sodium phosphate, pH 6.5, and buffer B was 10 m*M* sodium phosphate, pH 8.5. The buffers were filtered and sonicated as above. The gradient used was 0% B for 1 min, 30 to 70% B in 34 min, and 100% B for 5 min. The flow-rate was also 1 ml/min, and monitoring was at 280 nm.

After each experiment the columns were flushed with buffer B for 5 min and then equilibrated for 5 min with buffer A.

RESULTS

Fig. 1 shows that the Mono Q resin can be used to screen cord blood samples. The bottom trace depicts a sample of Hb S trait, the middle trace a sample of Hb C trait, and the top trace a sample of Hb Bart's. All variants are clearly resolved. These variants are difficult to detect in cord blood on cellulose acetate at pH 8.5, because

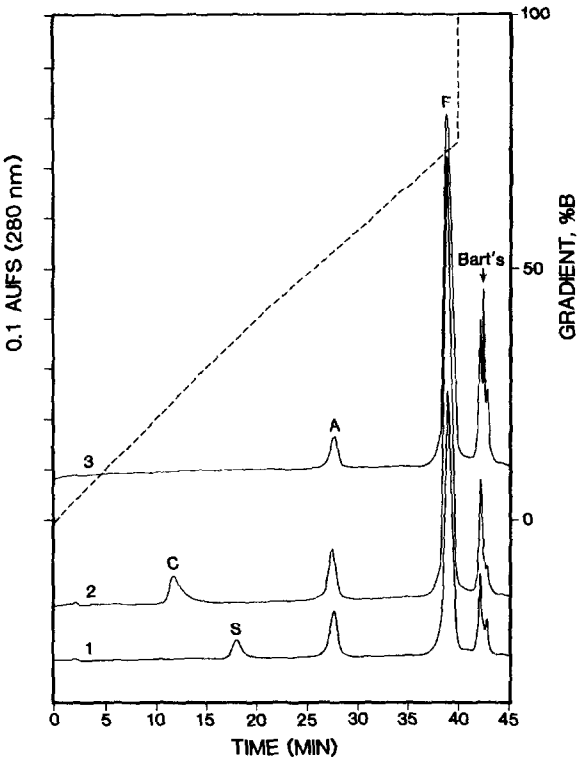

Fig. 1. Resolution of cord bloods on the Mono Q column. Experimental details are in the text. The first gradient was used. Bottom trace, Hb S trait; middle trace, Hb C traits; top trace, Hb Bart's. The three chromatograms are superposed. The two small peaks in the region of Hb Bart's were not identified but could represent the acetylated forms of Hb F.

of the heavy sample load that must be applied to the membrane. Under these conditions Hb C can be detected, but Hb S may be obscured by the heavy band of Hb F that migrates close to the Hb S position. Another problem encountered in this method is the distinction between homozygous Hb S, Hb S trait, and S/β^+-thalassemia, because acetylated Hb F and Hb A have the same electrophoretic mobility. In the chromatographic method described, Hb F and its acetylated derivatives have retention times greater than that of Hb A, so that these conditions can be clearly distinguished.

Alternatively, Hbs S and C can be resolved on citrate agar at pH 6.2, and Hb Bart's can be detected by electrophoresis at pH 7. The one-step chromatographic method is more convenient for a reference laboratory where limited numbers of samples are processed. Electrophoresis on both cellulose acetate and citrate agar and IEF, which will resolve these variants are more suitable for multiple screening. Other HPLC methods can also be used to screen cord blood samples[4,5].

Apart from the importance of screening cord blood samples to determine the at-risk infants in a newborn population, it is also important to determine the physicochemical properties of variants. Although many methods have been developed for detecting and resolving variant hemoglobins, ion-exchange column chromatography

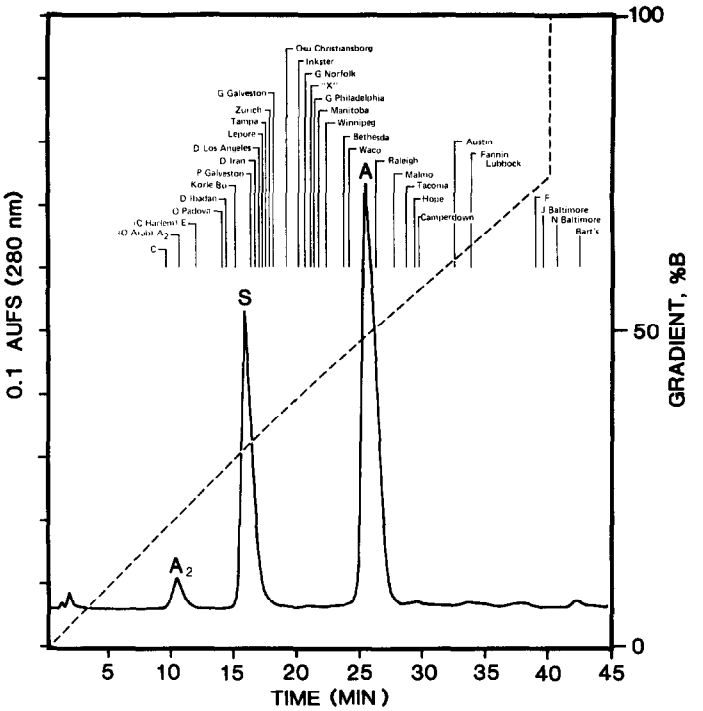

Fig. 2. Resolution of variant hemoglobins on the Mono Q column. Experimental details are in the text. The first gradient was used.

has been used almost exclusively to isolate variants in amounts large enough to be used in other studies[6,7]. The main disadvantage of these procedures is that separations generally extend over several days, during which hemoglobin degradation can take place and high concentrations of methemoglobin can be generated. To overcome this problem, several investigators have used HPLC in an attempt to improve resolution and reduce chromatography time[4,5].

Chromatography of hemoglobins using the FPLC system with Monobead columns indicates that this method is effective for separating variant hemoglobins and can be used for the presumptive diagnosis of hemoglobinopathies. In addition, this procedure is suitable for generating quantitative amounts of variants that can be used for structural and functional studies. Fig. 2 shows the separation on the Mono Q column (anion exchanger) of several variants that migrate cathodic to Hb A at pH 8.5. The non-α chain variants (Hbs Lepore, Osu Christiansborg, D Los Angeles, G Galveston, and P Galveston) and the α-chain variants (Hbs G Philadelphia, G Norfolk, and Winnipeg) can be resolved from each other as well as from Hb S. These variants cannot be distinguished from Hb S solely by electrophoresis on cellulose acetate. Likewise, Hbs C and E, C Harlem, and O Padova can be resolved from Hb A_2, but not Hb O Arab which has the same retention time as Hb A_2. The figure shows other variants that can be resolved; noteworthy among these is Hb Bethesda, which cannot be resolved from Hb A except on citrate agar, and Hb Malmo, which differs from Hb A only in the slight anodic mobility of the variant chain at pH 6.2.

Fig. 3 shows the results that were obtained for variants that migrate anodically to Hb A at pH 8.5. Hemoglobins New York, Hope, and Camden migrate very close to Hb A on cellulose acetate, and frequently Hb Hope cannot be resolved from Hb A. The same is also true for Hbs Austin and Waco. The presence of these variants is often suggested by the broad Hb A band that is observed. These pairs of variants are completely resolved on the Mono Q column. Hemoglobins Fannin-Lubbock and K Woolwich have approximately the same retention times but can be resolved on cellulose acetate. Hemoglobin J Baltimore is clearly resolved from Hb N Baltimore and from other "J-like" variants, which in turn can be distinguished from each other.

IEF has been suggested as a single-step method of screening for hemoglobin variants[2]. A comparison of the results described in this report with those obtained by IEF on variants common to both studies shows that both procedures are similar in resolving the above variants from Hb S, except for Hbs G Galveston and G Norfolk. However, IEF cannot differentiate among Hbs Lepore, P Galveston, G Philadelphia, and Winnipeg, nor between G Galveston and G Norfolk, nor between Mobile and Korle Bu. The results for variants in the Hb A_2 position are the same

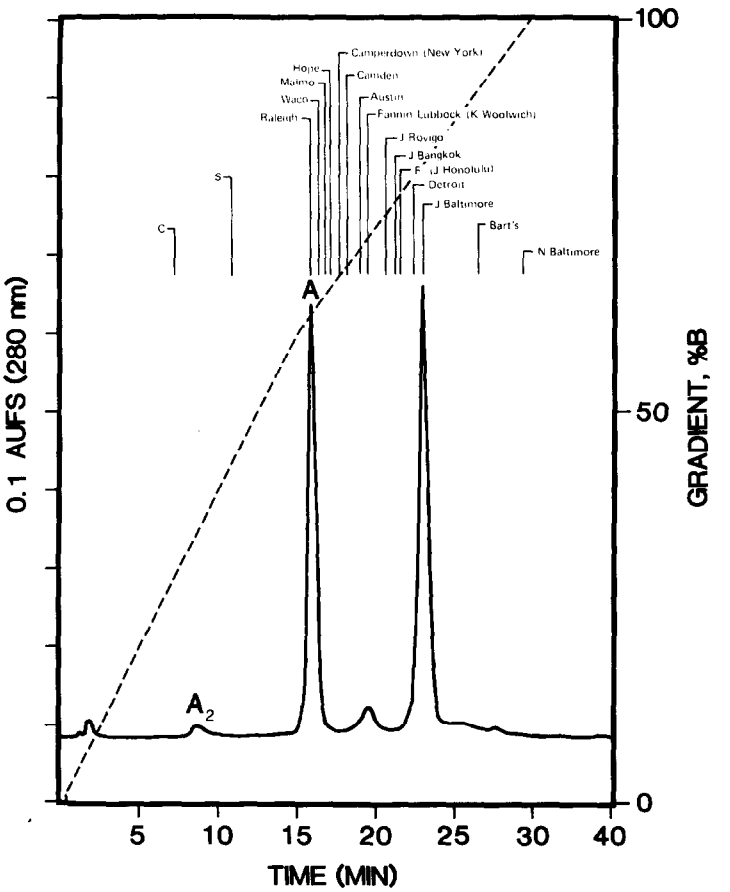

Fig. 3. Resolution of hemoglobin variants on the Mono Q column. Experimental details are in the text. The second gradient was used.

for both methods, except that IEF can resolve Hb O Arab from Hb A_2. Although we cannot resolve Hb O Arab from Hb A_2, we can resolve Hbs E and C Harlem, variants that co-isoelectric focus with Hb O Arab.

Fig. 4 shows the resolution of variants on the Mono S column (cation exchanger). Since most variants will resolve on the Mono Q column, the Mono S column was used only in instances in which there were incomplete separations. Hemoglobins Raleigh, Bethesda, and Malmo, which migrate like Hb A on cellulose acetate, all separated. Although Hbs Waco (β40 Lys-Arg) and Austin (β40 Lys-Ser) can be resolved on the Mono Q column, the Mono S column will only resolve Hb Waco.

These results demonstrate that the columns will resolve some variants with neutral substitutions (Hb Waco and Hb "X", α138 Ser-Pro, unpublished data), a property that is invaluable for characterizing variants. Other neutral variants like Hb Cheverly (β45 Phe-Ser) are slightly resolved on Mono S whereas Hb Brockton (β138 Ala-Pro) is not, on either exchanger. These and other studies demonstrate that resolution of variants is dependent not only on net charge, but also on the location of the substitution and its effects on the 3-dimensional structure of the molecule.

These columns may also be used to detect and prepare hybrid mixtures. In individuals with Hb ASG Philadelphia and Hb Richmond, the mixed hybrides are clearly resolved. In addition, since Hb A_2 is well resolved on the Mono Q column, this method should provide an accurate means of quantitating this hemoglobin.

Samples as large as 10 mg hemoglobin have been loaded onto an HR 5/5 Mono

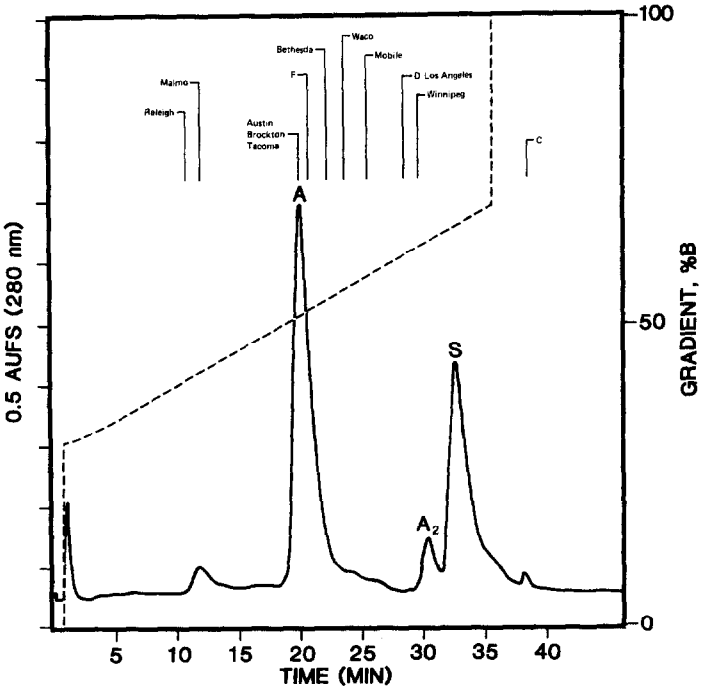

Fig. 4. Resolution of hemoglobin variants on the Mono S column. The experimental details are in the text.

Q column (1 ml) without any substantial loss in resolution. The retention time is altered slightly as the load level is progressively increased. For variants that are well separated and for which conditions of separation have been optimized much higher loads can be applied without affecting resolution. In other studies, samples as large as 50 mg have been applied, though the columns are rated for a maximum of approximately 25 mg or 5 mg per single peak[8]. Preparative columns (HR 16/10) are also available with load capacities of 200–500 mg per column.

The recovery of hemoglobin from the Mono Q resin was 90%, compared with recoveries of 60% from DEAE-Sephadex-A50 and 70% from DEAE-Sephacel. We did not observe any differences in the resolution of samples stored with potassium cyanide and carbon monoxide at 4°C compared to those stored in liquid nitrogen.

DISCUSSION

Chromatography on Monobead resins offers an easy and reliable means for diagnosing hemoglobinopathies and complements both the cellulose acetate and IEF methods. The uniform particle size of the resin generates very low back pressures, and the strong exchanger groups ensure superior resolving power compared with weaker exchangers such as DEAE-Sephadex or DEAE-Sephacel. These features, coupled with favorable load capacities, contribute to the advantages of this method. Most variants can be resolved discretely in a relatively short time (usually within 1 h) and in quantitative amounts under mild conditions. This is an important consideration, particularly for variants that have electrophoretic mobilities between Hbs A, F, and S and in instances in which the variant chain cannot be completely resolved from the normal chain by the Clegg procedure[9]. Invariably, attempts to separate these variants on anionic exchangers such as DEAE-Sephadex or DEAE-Sephacel result in some contamination with HbA. With the far superior resolving power of the Monobead resins, complete resolution of variants (even some with neutral substitutions) can be achieved, so that structural analysis is facilitated and functional properties can be accurately determined on isolated samples chromatographed within an hour rather than over a period of several days. Rapid chromatography also ensures that recoveries are consistently higher than in conventional chromatographic procedures. From a practical standpoint, isolation and functional studies can be carried out on the same day, thus eliminating or minimizing possible artifacts that may be generated over a longer time. On DEAE-Sephadex or DEAE-Sephacel, some hemoglobin degradation takes place, and the concentration of methemoglobin can increase over the several days of chromatography.

We have found it convenient to determine chromatographic conditions for a particular separation using the 1 ml analytical column. However, for preparing larger quantities, the procedure can be scaled up with a preparative column without any loss of resolution. With an automated gradient generating system, experimental conditions can easily be altered to maximize most separations.

An added feature of this method, which is also applicable to similar column chromatographic procedures, is the ability to quantitate hemoglobins by integration of peak areas. The relative mobilities of hemoglobins can also be determined according to the method of Schneider and Barwick[10] by direct measurement of relative distances between peaks on the chromatograms. The direct measurement of relative

peak distances on the chromatogram, rather than from a densitometric scan of a cellulose acetate strip, ensures that distances can be estimated with greater precision, particularly for bands that migrate close together and/or are not sharply defined. In addition, Hb A_2 is present in all adult samples, and its peak position is clearly defined. Thus it serves as an internal marker for calculating the ratio that is normally derived from a separate external measurement of an Hb C standard. However, because this method will resolve Hbs A_2 and C, samples with Hb C require an external measurement of Hb A_2.

The clear disadvantage of this system is the initial cost. The columns are expensive, but since they are stable ion exchangers they should last a long time with proper care and handling. Laboratories with HPLC systems can implement this method by placing the columns in-line. At a flow-rate of 1 ml/min the HR 5/5 column generates approximately 150 p.s.i., so that pumps and accessories rated at lower pressures can be used. Another disadvantage is that, unlike cellulose acetate electrophoresis and IEF, multiple analyses cannot be done simultaneously, although the system can be automated. On the other hand, with this method quantitative amounts of hemoglobin variants can be prepared conveniently under optimal physiologic conditions, a feature that should be advantageous to many researchers.

The development of new methods has enhanced our ability to detect hemoglobinopathies and to resolve different proteins, but it is still necessary to do several tests to make a reasonable presumptive diagnosis of many hemoglobinopathies. Other factors, such as ethnic origin, clinical data, and the availability of hemoglobin standards are also important in differentiating variants, particularly in those instances where differences in retention times between variants are small. α-Chain variants can readily be distinguished from β-chain variants by the presence of the variant Hb A_2. Where ambiguities exist, structural studies will have to be performed. We feel that this chromatographic procedure will be an additional means for evaluating variants and should prove invaluable to investigators interested in studying the structure–function relationship of hemoglobin and other proteins.

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