Journal of Chromatography, 266 (1983) 197–205
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 044

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN HEMOGLOBINS ON A NEW CATION EXCHANGER

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SUMMARY

We have investigated the use of a high-performance liquid chromatographic (HPLC) column packed with a unique weak cation exchanger prepared by coating silica with poly(aspartic acid) for hemoglobin analysis. The complete separation of hemoglobin Bart's F, A_0, A_2, S, C, D, E, G, SG, Winnipeg and Sealy was achieved by gradient elution within 30 min. The high resolution made it possible to distinguish hemoglobin variants such as Bart's, AC, AD, AE, AG, AS, ASG, CC, SC, SS, Winnipeg, Sealy and β-chain variants with thalassemia such as S/β^+, S/β^0 and S(C)-β^+ thalassemia. Comparison of DEAE-cellulose column chromatography and our HPLC method for the quantitation of hemoglobin A_2 yielded a good correlation. Hemoglobins A_2, C and E are completely resolved on PolyCAT A columns in contrast to both cellulose acetate electrophoresis and DEAE-cellulose column chromatography. The high resolution of the system and the accuracy of the method combined with complete automation make this procedure useful for diagnosis of hemoglobin disorders in both a research and clinical laboratory environment.

INTRODUCTION

Various analytical schemes including electrophoretic and chromatographic methods have been developed for human hemoglobin analysis[1]. The most widely utilized method for hemoglobin analysis is cellulose acetate electrophoresis at alkaline pH. It is rapid, reproducible and capable of separating common hemoglobin variants, such as S, F, A_0 and A_2, but hemoglobins A_2, C, O and E are unresolved, as are hemoglobins S, D and G. Consequently, citrate agar electrophoresis is needed for the identification of the aforementioned hemoglobins. Nevertheless, electrophoretic methods will not separate hemoglobins E and O or D and G[2]. During the past 20 years the use of ion-exchange chromatography has become increasingly important for the separation of hemoglobin variants. Unfortunately, these procedures are laborious and require days in some cases to achieve suitable separations. As a conse-

0021-9673/83/$03.00

quence, these methods are not applicable to the routine clinical laboratory. Recently, high-performance liquid chromatography (HPLC) has been applied to the analysis of hemloglobin variants α and β subunits and tryptic digests of hemoglobin chains[3]. HPLC of human hemoglobin molecules has been carried out successfully on an anion exchanger prepared by crosslinking of low-molecular-weight poly(ethylenimine)[3–5]. The resolution of this anion-exchange HPLC method is sufficient to diagnose hemoglobin disorders such as AS, AC, SS, CC, SC and S(C)-β^+ thalassemia but lacks the resolving capacity to classify other hemoglobin disorders[6]. Recently, Alpert[7] has synthesized a weak cation exchanger by coating silica with poly(aspartic acid). We report the use of this column packing material for hemoglobin analysis. Complete separation of hemoglobins Bart's, F, A_0, A_2, S, C, D, E, G, SG and other α variants such as Winnipeg and Sealy can readily be achieved by gradient elution from this column within 30 min. The column packing material was stable for at least 300 analyses without serious deterioration of resolution or efficiency. The high resolution achievable with this system permits accurate quantitative analysis of the hemoglobin fractions. This new packing material makes HPLC of hemoglobins a practical and useful tool for efficient and accurate diagnosis of common hemoglobin disorders in both the routine clinical and research laboratories.

EXPERIMENTAL

Materials

2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (Bis-Tris) and potassium cyanide were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acrodisc filter units were obtained from Gelman (Ann Arbor, MI, U.S.A.). AFSC hemoglobin control containing hemoglobins A, F, S, C and A_2 was from Helena Laboratories (Beaumont, TX, U.S.A.). Various abnormal hemoglobin specimens were the generous gifts of Dr. Winston F. Moo-Penn, Host Factors Division, Center for Infectious Diseases, Center for Disease Control (Atlanta, GA, U.S.A.) and Dr. Titus H. J. Huisman, Sickle Cell Center, Medical College of Georgia (Augusta, GA, U.S.A.).

Apparatus

The chromatographic apparatus consisted of a Varian Model 5060 ternary gradient liquid chromatograph interfaced with a Vista 401 Data System (Varian Instruments, Palo Alto, CA, U.S.A), a Waters Intelligent Sample Processor (WISP 710B; Waters Assoc., Milford, MA, U.S.A.) for sample injections and a Waters Model 440 UV detector. A 20 × 0.46 cm column packed with 5-μm microparticulate poly(aspartic acid)–silica (PolyCAT A) was obtained from Custom LC (Houston, TX, U.S.A.). The column effluent was monitored at 436 nm, and the peak areas were used for the quantitation of individual hemoglobin peaks.

Procedures

Hemolysates were prepared by mixing the packed red cells from a given patient with two volumes of doubly-distilled water for 30 sec. After lysis, 60 μl of hemolysate were added to 1 ml of mobile phase A, the mixture was filtered through a 0.45-μm Gelman Acrodisc filter, and 20 μl of the filtrate were applied to the column. The chromatographic separation of hemoglobins was achieved by gradient elution.

Gradient programs

The gradient was made up of mobile phase A, containing 40 m*M* Bis-Tris and 4 m*M* KCN (pH 6.5) and mobile phase B, containing 40 m*M* Bis-Tris, 4 m*M* KCN and 0.2 *M* NaCl (pH 6.8). Using a flow-rate of 1 ml/min, the column was rinsed with mobile phase B for at least 10 min and then equilibrated with mobile phase containing 22% B. Elution of hemoglobins was accomplished by increasing the percent of B to 56% and 100% at 16 and 22 min, respectively and then decreasing to 22% at 24 min. Before application of the next sample, the column was equilibrated with 22% mobile phase B for at least 6 min.

Quantitation of hemoglobin A_2

DEAE-cellulose columns for hemoglobin A_2 (Hb A_2) quantitation were purchased from Isolab (Akron, OH, U.S.A.). The method utilized was based on the analytical procedure of Abraham *et al.*[8], as modified by Huisman *et al.*[9].

RESULTS AND DISCUSSION

Chromatographic system

Separation of human hemoglobins on CM-cellulose or CM-Sephadex with either a salt or pH gradient has been thoroughly described by Schroeder and coworkers[10,11]. Because the differences in the p*I* values of the hemoglobin variants are so small, the chromatography requires prolonged, shallow gradients to achieve adequate resolution. Under these conditions, significant bandbroadening occurs, re-

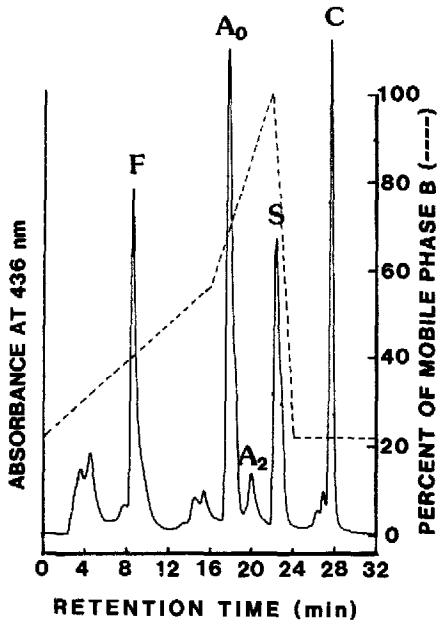

Fig. 1. Cation-exchange chromatography of an AFSC hemo control containing hemoglobin A_0, A_2, F, S and C. Solid line is the monitoring of absorbance at 436 nm; dotted line is the elution program in terms of percent of mobile phase B.

sulting in the loss of resolution of minor peaks. Superior resolution is possible when a small-volume, combined pH and salt gradient system is applied to the PolyCAT A column, because this column has a significantly greater number of theoretical plates per meter. Fig. 1 illustrates the elution profile of a commercial AFSC hemoglobin control. The resolution of major and minor peaks of multiple variants, within the same column run, provides an indication of the superiority of this chromatographic system for hemoglobin analysis and its potential for protein studies.

Applications

One of the significant advantages of HPLC analysis of hemoglobins over traditional electrophoretic methods is a significant improvement in sensitivity as well as resolution. This is clearly illustrated in Figs. 2 and 3 which show the chromatographic patterns of hemoglobin samples from normal children of various ages and children with sickle cell trait and one infant with hemoglobin Bart's. Fig. 3 and Panel 1 in Fig. 2 represent the relative hemoglobin concentrations of A_0, F, Bart's, A_2 and S present in cord and blood samples from infants. By standard electrophoretic techniques, the presence of A_0, A_2 and S would not have been detectable. As such, this chromatographic system has a unique potential to provide accurate screening of high risk newborn populations.

Clear distinctions among hemoglobins E, D and SD were readily achieved by this chromatographic system as shown in Fig. 4. In routine practice, both cellulose acetate and citrate agar electrophoretic methods are necessary to differentiate hemo-

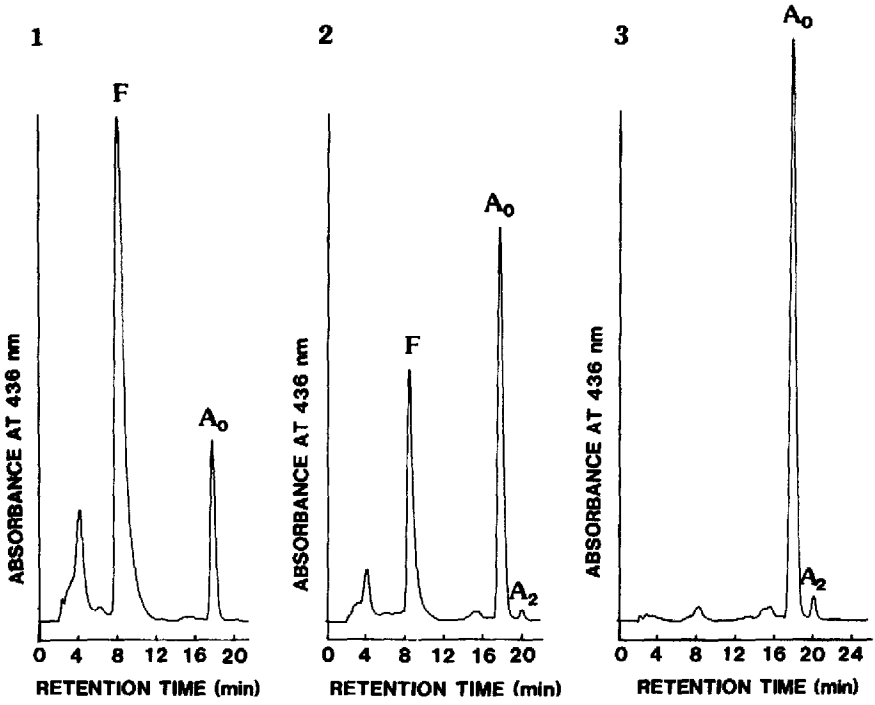

Fig. 2. Chromatographic patterns of normal hemoglobins from cord blood (1), a one-month-old child (2) and an adult (3).

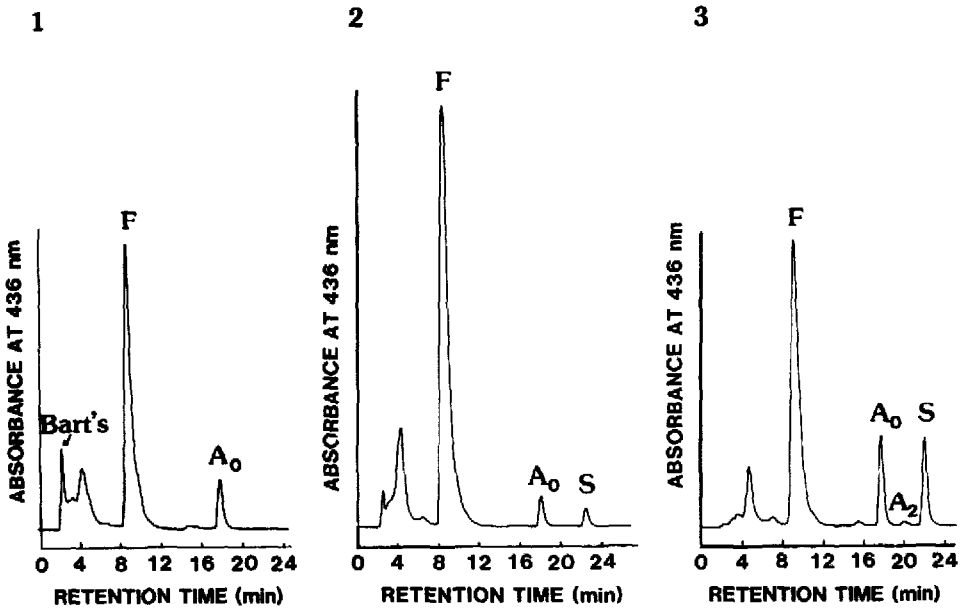

Fig. 3. The chromatographic patterns of hemoglobins from a cord blood sample with hemoglobin Bart's (1), a newborn with sickle cell trait (2) and a one-month-old child with sickle cell trait (3).

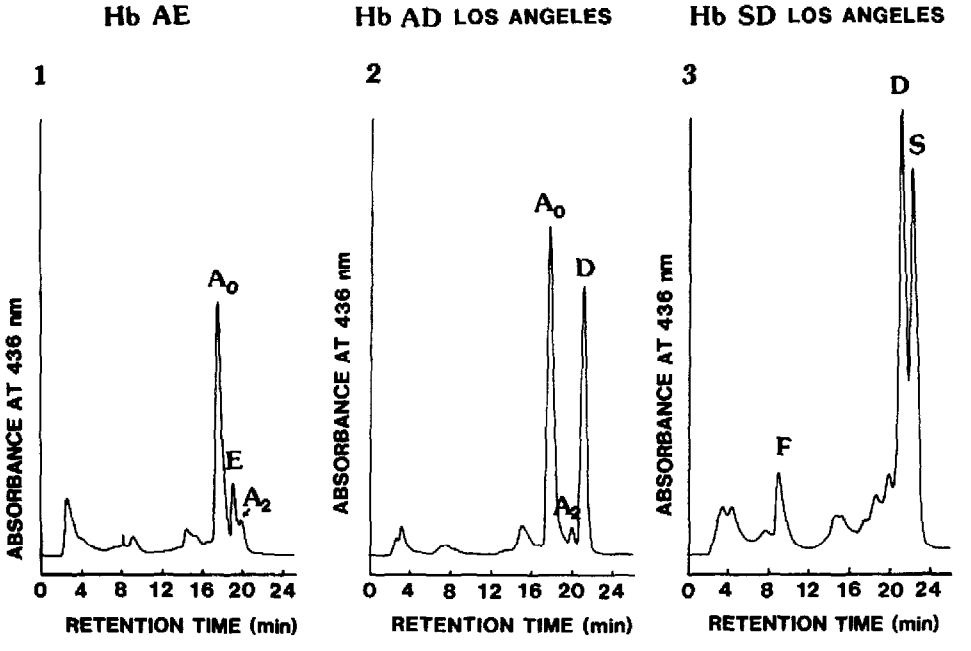

Fig. 4. Hemoglobin profile of patients with hemoglobin E trait (Hb AE) (1), hemoglobin D Los Angeles (Hb AD Los Angeles) (2) and sickle cell hemoglobin D disease (Hb SD Los Angeles) (3).

globins S, D and E[12,13]. For the purpose of quantitation of hemoglobin E, A_2, D and S, a more shallow gradient would be desirable.

Because quantitative measurement of hemoglobin A_2 is an essential adjunct to the diagnosis of β-thalassemia, the accuracy of the chromatographic system was compared with that of DEAE-cellulose chromatography, which is considered to be the reference method for Hb A_2 quantitation. Comparison of the two chromatographic systems in the analysis of samples containing hemoglobins A_0, A_2 and F showed a good correlation. Linear regression analysis of the data yielded $y = 1.04x - 0.29$, $n = 39$, $r = 0.964$ and $Sy \cdot x = 0.27$ where x = DEAE-cellulose chromatography and y = HPLC. However, when hemoglobin S or C was present, inaccurate results were obtained with commercially prepared DEAE-cellulose columns due to partial or complete contamination of Hb A_2 by these variants. Even with hemoglobin S or C present, the PolyCAT A column provided complete resolution of A_2 and accurate quantitation.

An unknown minor component (labeled as ?) was observed, having the same mobility as hemoglobin S in conjunction with hemoglobin C (Fig. 5). This minor component was also observed by Schroeder *et al.*[11] and was designated as Cx hemoglobin when CM-cellulose column chromatography was used. This minor component is probably glycosylated hemoglobin C[14].

The resolving power of the chromatographic system is demonstrated by elution

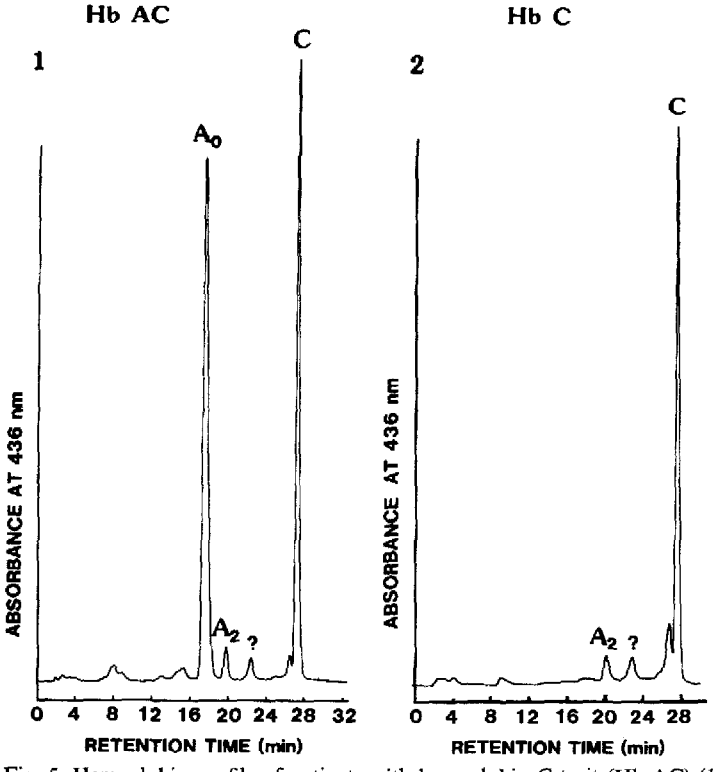

Fig. 5. Hemoglobin profile of patients with hemoglobin C trait (Hb AC) (1) and hemoglobin C disease (Hb C) (2). The label ? presumably is Cx as described by Schroeder *et al.*[11].

profiles of hemoglobin AG Philadelphia and hemoglobin ASG Philadelphia as shown in Fig. 6. Since hemoglobin G Philadelphia is an α chain variant, hemoglobin S a β chain variant and Hb A_2 a δ-chain variant, up to eight chain combinations are possible. Seven different hemoglobins including A_0, A_2, G, S, SG hybrid, a shoulder peak of A_0 and a small peak between A_0 and A_2 are resolved by the chromatographic system. A possible chain combination with $\alpha_2{}^{68\ Lys}\delta_2$ is presumably present but obscured by its being eluted with hemoglobin S. The $\alpha_2{}^{68\ Lys}\delta_2$ form is seen in the chromatographic pattern for Hb AG (Fig. 6, Panel 1). A major peak eluted in 14 min (Fig. 6, panels 1 and 2) may be a naturally occurring minor hemoglobin or a decomposed product of hemoglobin G in patients with hemoglobin AG or ASG Philadelphia. All the α-chain hemoglobin variants examined (Fig. 7), including hemoglobin Winnipeg, Sealy and AG Philadelphia had an $\alpha_2^{variant}\ \delta_2$ minor component (labeled as ?) which was eluted after the primary peaks of the α-chain hemoglobin variant analogous to the situation with A_0 followed by A_2. This observation provides a unique and useful means for distinguishing α-chain hemoglobin variants from β-chain hemoglobin variants.

The storage of hemoglobin hemolysate may have a slight effect on the chro-

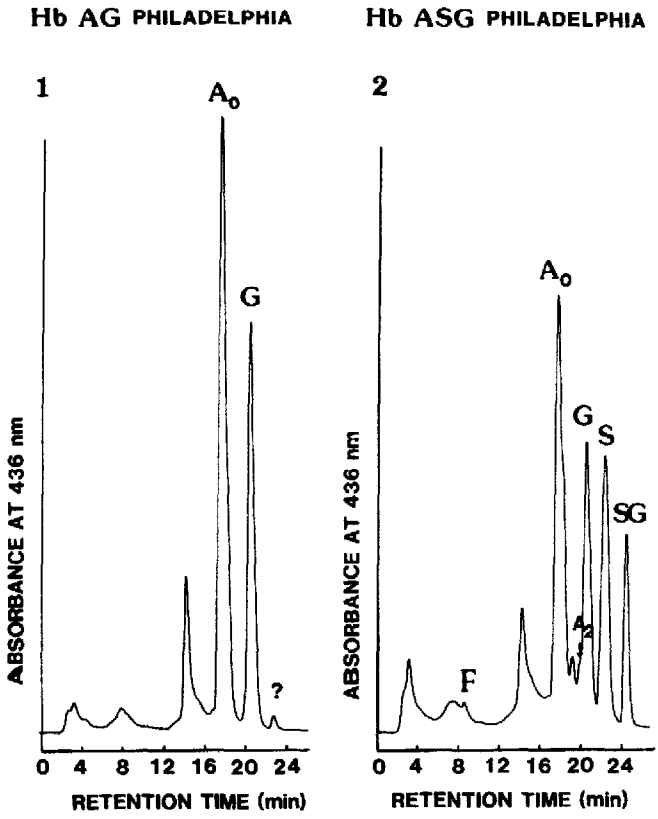

Fig. 6. The chromatographic separation of hemoglobins from patients with hemoglobin G Philadelphia (Hb AG Philadelphia) (1) and sickle cell-hemoglobin G Philadelphia disease (Hb ASG Philadelphia) (2). The peak labeled ? is presumably an $\alpha_2{}^{68\ Lys}\ \delta_2$ hemoglobin variant.

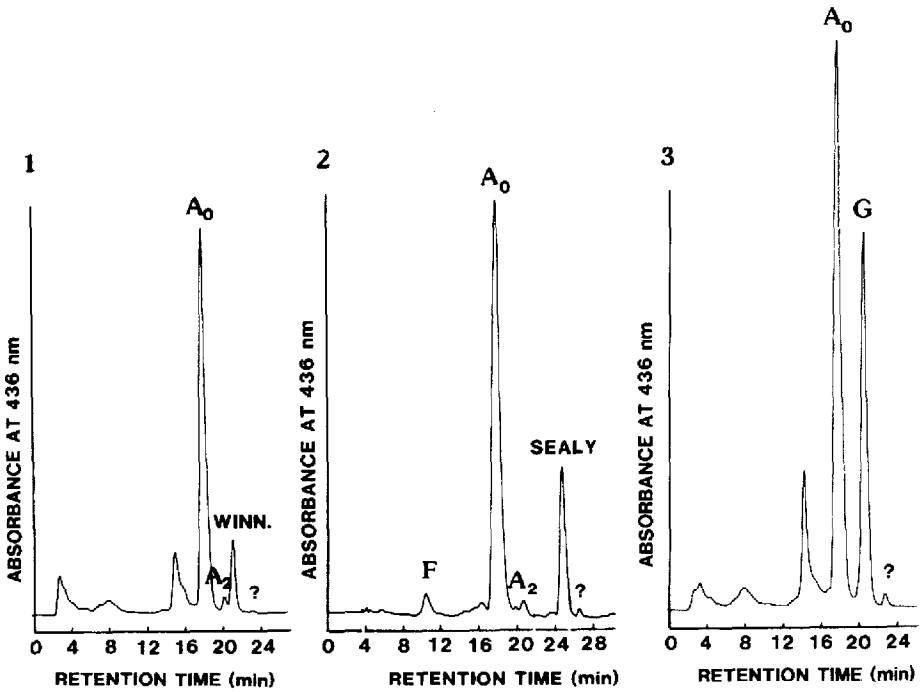

Fig. 7. The chromatographic separation of α-chain hemoglobin variants from patients with hemoglobin Winnipeg (1), Sealy (2) and G Philadelphia (3).

matographic pattern. As shown in Fig. 2, panels 1 and 2, Figs. 1, 3, 4 and 6 and Fig. 7, panels 1 and 3, the aging of the hemolysate causes an increase of the concentration of minor peaks in the region around 3–4 min of elution time. When a freshly prepared hemolysate is used, a very small quantity of these minor components is observed (Fig. 2, panel 3; Fig. 5; and Fig. 7, panel 2). Nevertheless, the aging of the hemolysate does not affect the chromatographic resolution at all.

In summary the chromatographic condition developed gave excellent resolution, allowing the separation of all of the frequently encountered normal and hemoglobin variants including Bart's, F, A_0, A_2, S, C, D, E, G, Winnipeg and Sealy. This made it possible to identify hemoglobin variants such as Bart's, AC, AD, AE, AG, AS, ASG, CC, SC, SS, Winnipeg, Sealy and β-chain variants with thalassemia such as S/β^+, S/β^0 and S(C)-β^+ thalassemia. The superior sensitivity of the system also makes it an excellent method for the screening of high risk newborn populations for hemoglobinopathies. Further modification of the chromatographic condition should increase its resolving power and make the study of other hemoglobin variants possible. Because this system requires a minimum of technician's time and is fully automated, its adoption by reference laboratories and even some clinical laboratories is feasible. We believe this chromatographic system is the first of its kind and heralds a new era in protein separation for clinical applications.

ACKNOWLEDGEMENTS

We thank Dr. Winston F. Moo-Penn and Mr. Danny L. Jue from Centers for Disease Control, Atlanta, GA, and Dr. Titus H. J. Huisman from Medical College of Georgia, Augusta, GA, for their generous gifts of various hemoglobin variants. We also thank Dr. Jerome S. Wilkenfeld from Spring Branch Memorial Hospital, Houston, TX, for the generous donation of hemoglobin Sealy. Excellent technical assistance from Ms. Marty Cooner, Becky Grove, Carol Harker and Dee Ann Walker is greatly appreciated.

This work is supported by a research grant from the Moran Foundation, Department of Pathology, Baylor College of Medicine, Houston, TX, U.S.A.

REFERENCES

1 P. Basset, F. Braconnier and J. Rosa, *J. Chromatogr.*, 227 (1982) 267–304.
2 R. M. Schmidt and E. F. Brosious, *Basic Laboratory Methods of Hemoglobinopathy Detection,* HEW Publ. No. (CDC) 77-8266, U.S. Dept. Health, Education, and Welfare, Center for Disease Control, Atlanta, 6th ed., 1976.
3 S. M. Hanash and G. J. Brewer (Editors), *Progress in Clinical and Biological Research*, Vol. 60, Alan R. Liss, Inc., New York, 1981.
4 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375–392.
5 S. M. Hanash and D. N. Shapiro, *Hemoglobin*, 5 (1982) 165–175.
6 M. B. Gardiner, J. Carver, B. Abraham, J. B. Wilson and T. H. J. Huisman, *Hemoglobin*, 6 (1982) 1–13.
7 A. J. Alpert, *J. Chromatogr.*, 266 (1983) 21.
8 E. C. Abraham, A. Reese, M. Stallings and T. H. J. Huisman, *Hemoglobin*, 1 (1976–1977) 27–44.
9 T. H. J. Huisman, W. A. Schroeder, A. N. Brodie, S. M. Mayson and J. Jakway, *J. Lab. Clin. Med.*, 86 (1975) 700–702.
10 W. A. Schroeder and T. H. J. Huisman (Editors), *The Chromatography of Hemoglobins*, Marcel Dekker, New York, 1980.
11 W. A. Schroeder, L. A. Pace and T. H. J. Huisman, *J. Chromatogr.*, 118 (1976) 295–302.
12 R. Brier, T. Golias and J. G. Batsakis, *Amer. J. Clin. Pathol.*, 44 (1965) 695.
13 P. F. Milner and H. Gorden, *Amer. J. Clin. Pathol.*, 64 (1975) 58.
14 R. T. Jones, personal communication, 1983.