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REVIEW

SEPARATION OF HEMOGLOBINS AND HEMOGLOBIN CHAINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

Human hemoglobin (Hb) abnormalities have been studied for many years, particularly since 1949 when the Hb S was discovered in sickle cell anemia. Since then the number of Hb variants has increased to 485 (September, 1986) while numerous forms of thalassemia and related conditions have been evaluated. The

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structural analysis of these proteins, the clinical evaluation of the hemoglobinopathies, and direct DNA analyses have provided details that are invaluable for a better understanding of the basis of various hematological abnormalities.

Normal adult Hb (Hb A) is composed of two α and two β chains ($\alpha_2\beta_2$). The primary structures of these two types of chains were determined in the early 1960s. A second adult Hb (Hb A_2) consists of two α and two δ chains ($\alpha_2\delta_2$); the primary structure of the δ chain differs from that of the β chain at 10 positions. The human fetus and newborn produce mainly fetal Hb (Hb F) which is composed of two α and two γ chains ($\alpha_2\gamma_2$); the β and γ chains differ at 39 positions. Additional chains have been observed in the early human embryo; the primary structures of the embryonic ζ chain and ϵ chain have been established. Newborn babies have two types of γ chain; the $^{G}\gamma$ chain has a glycyl and the $^{A}\gamma$ chain an alanyl residue at position 136. These two chains are the products of duplicated γ globin genes. Thus, seven different types of Hb chains exist in the human, namely ζ, α, ϵ, $^{G}\gamma$, $^{A}\gamma$, δ, and β chains.

The first abnormal Hb (Hb S), discovered in 1949, was characterized in 1959 as having a Glu→Val substitution at position 6 of the β chain. Most of the hundreds of variants, discovered during the past 30–40 years, are the result of single base substitutions in the DNA of either the $^{G}\gamma$, $^{A}\gamma$, δ, β, or α globin genes, but other variants with hybrid chains, extended chains, chains with deletions, etc., have also been discovered. Several extensive reviews have appeared during recent years; the reader is referred to the most recent book by H.F. Bunn and B.G. Forget, entitled "Hemoglobin: Molecular, Genetic and Clinical Aspects" [1] for detailed information. Some of the abnormal Hbs are discussed later in the text, and are listed in Table II. Fig. 29 attempts to illustrate the three-dimensional structures of the α and β chains, while some variants and their specific amino acid substitutions are also listed in this figure.

Numerous procedures have been used to identify and/or characterize the normal and abnormal Hbs. Most common are electrophoretic methods, isoelectric focusing (IEF) techniques, and macro-chromatographic procedures. These various methods have been described in detail in manuals such as "The Hemoglobinopathies Techniques of Identification" (by T.H.J. Huisman and J.H.P. Jonxis) [2] and "The Hemoglobinopathies" (edited by T.H.J. Huisman) [3].

During the past several years high-performance liquid chromatography (HPLC) has influenced all aspects of analytical chemistry and it is therefore not surprising that different types of HPLC have also been applied to the study of the hemoglobinopathies. This review will discuss two major applications that have most affected the analyses of hemoglobinopathies in patients with different abnormalities. The methodologies listed are primarily the ones in use in the laboratories of the author. Some of these procedures have been reviewed before, while modifications and/or alternative approaches have also been presented [4–14]. Other applications of HPLC methodology were directed towards the detection and quantitation of minor Hb components; these methods will not be reviewed here and the reader is invited to consult the listed publications [15–21].

2. GENERAL INFORMATION

2.1. Instrumentation

Our general equipment consists of a Perkin-Elmer Series 3 microprocessor HPLC unit, a Rheodyne sample injector, a Perkin-Elmer LC-55B UV–VIS digital detector, a Perkin-Elmer Model 23 dual-pen recorder, a Hewlett-Packard Model 3390A recording integrator, and an LKB Model 2070 ultra fraction collector. The Perkin-Elmer series 3 and LC-55B units have recently been replaced by more advanced equipment such as the Series 4 or Series 400 LC solvent delivery system, and Model LC-85 or LC-95 spectrophotometric detector (all from Perkin-Elmer). Comparable equipment can be obtained from various other companies. Equipment useful for mass-testing programs are also available.

The following HPLC columns have been used.

(a) SynChropak CM 300 (SynChrom, Linden, IN, U.S.A. Cat. No. CCM 103-25), a 250 mm×4.1 mm I.D. cation-exchange column and guard column filled with SynChropak CSC (Cat. No. C00) packing material. SynChropak CM 300 is a weak cation-exchange HPLC support; it is a 6.5-μm macroporous spherical silica with a bonded polyamide coating derivatized with carboxymethyl groups. The average pore size is 300 Å. The 1–5 cm guard column is filled with non-porous macroparticulate beads with the same coating. Guard columns can be purchased prepacked from various HPLC vendors, but it may be advisable to purchase empty guard columns which can readily be dry-packed with the appropriate material. The guard column life is greatly affected by the condition of the samples being analyzed; clean samples will require less frequent changes.

(b) Other cation-exchange HPLC columns, which possibly require slight gradient changes, are: (1) WP-CBX Baker-Bond wide-pore column, (J.T. Baker, Phillipsburg, NJ, U.S.A.); (2) CX-300 Aquapore wide-pore weak cation exchanger (Brownlee Labs., Santa Clara, CA, U.S.A.); (3) TSK-CM-2-SW IEX 530CM ion-exchange column (Toya Soda, Tokyo, Japan).

(c) SynChropak AX 300 (Cat. No. Ca 103-10), a 100 mm × 4.1 mm column. This weak anion exchanger is a 6.5-μm macroporous spherical silica with a bonded polymeric layer of amines. The SynChropak AX 300 is compatible with aqueous solvents in the pH range 2–8, as well as with many organic solvents. The system requires a guard column filled with SynChropak ASC (Cat. No. A00) packing material and a SynChrosorb AX A-300 packed preguard column, which is inserted before the injector for solvent conditioning and prolongs the life-time of analytical columns operating in the pH range above pH 8.0.

(d) Other anion-exchange HPLC columns, which may work equally well as the SynChropak AX 300 column are: (1) BIO-SIL TSK-IEX-540 DEAE, weak anion exchanger (Cat. No. 125-0501); 300 mm×4 mm (Bio-Rad Labs., Richmond, CA, U.S.A.); (2) PEI-WAX Baker-Bond wide-pore columns (J.T. Baker); (3) AX-300 Aquapore (Brownlee Labs.); (4) Vydac 10 ANEX PP 301 TP 4.6 column (Separation Group, Hesperia, CA, U.S.A.).

2.2. Reagents

Any HPLC-grade chemical provided by a reputable supplier is satisfactory. In our laboratories the following reagents are used: acetic acid (No. 9195), sodium acetate (No. 3466), sodium chloride (No. 3624), potassium cyanide (No. 3080), all from J.T. Baker; Bis-Tris (No. B9754), from Sigma (St. Louis, MO, U.S.A.); Tris [tris(hydroxymethyl)aminomethane] or Tham (No. 395), from Fisher Scientific (Pittsburgh, PA, U.S.A.); and deionized water with a megohm resistance of > 15.

2.3. Blood samples

These are collected with EDTA as an anticoagulant. Red cell lysates are prepared by hemolysis of thrice washed cells (with 0.9 g/dl sodium chloride), using an equal volume of distilled water and a half volume of carbon tetrachloride. Red cell lysates are used as fresh as possible or stored at a low temperature, preferably in liquid nitrogen at $-70\,^{\circ}C$. Cord blood samples can be collected on filter paper, air-dried, and shipped to the laboratory at room temperature up to seven days after collection.

3. SEPARATION OF HEMOGLOBINS BY ANION-EXCHANGE HPLC

The anion exchanger SynChropak AX 300 was introduced by Gooding et al. [15] and Hanash and co-workers [4,13] for the separation of normal and variant

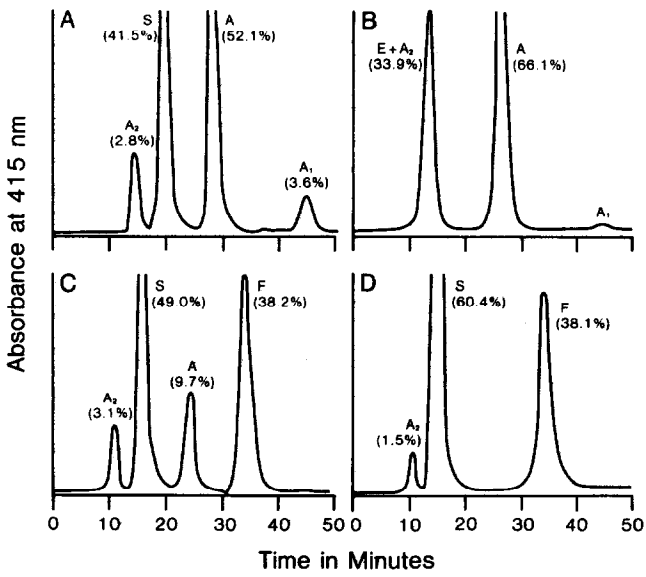

Fig. 1. Separation of Hb types present in adult red cell lysates by anion-exchange HPLC using SynChropak AX 300. (A) Hb S heterozygote; (B) Hb E heterozygote; (C) Hb S-β^+-thalassemia with a large quantity of Hb F; (D) SS patient, aged eight months. All chromatograms were developed with a 0–0.05 *M* sodium acetate gradient (from ref. 7).

Hb types. We modified their method [5,10] and used the following procedure: 80–300 μg of Hb, contained in 8–30 μl of red cell lysate, is injected and the chromatogram is developed at room temperature with developer A (0.015 *M* Tris, 0.0015 *M* potassium cyanide, 0.05 *M* sodium acetate, pH 8.6) and developer B (0.015 *M* Tris, 0.0015 *M* potassium cyanide, pH 8.6 adjusted with 25% acetic acid). A gradient is applied which gradually increases the sodium acetate to allow the sequential elution of the Hb components; the steepness of the gradient (i.e. the percentage developer A added to the elution solvent per min) depends on the Hb components to be separated. The flow-rate is usually kept at 1.0 ml/min and the wavelength is 415 nm. The developers are filtered and sonicated before being used. An optimal separation of Hb components in this system requires a careful monitoring of the column pressure; a significant increase in the column pressure will result in an incomplete elution and in broadening of the peaks.

Fig. 1 illustrates the separation of different Hb types including those in red cell lysates of a Hb S heterozygote (Fig. 1A), of a Hb E heterozygote (Fig. 1B), of a patient with Hb S-β^+-thalassemia (Fig. 1C), and of an eight-month-old SS (sickle cell anemia) patient with a high level of Hb F (Fig. 1D). Separation of the Hbs A and F is important; however, the elution times of Hb F and the minor Hb A_1 components are about the same, which prevents the quantitation of Hb F in adult red cell lysates with a low Hb F percentage. The Hbs A_2 and E also cannot be separated.

Identification and quantitation of adult Hb abnormalities in cord blood samples are possible, particularly for the β chain variants Hb S and Hb C (Fig. 2). Hb D-Los Angeles and Hb S, however, can only be incompletely separated by this procedure although a slower gradient will improve the separation of these variants. The method is particularly useful for the quantitation of Hb Kenya which is composed of α chains combined with $^A\gamma\beta$ hybrid chains (Fig. 3).

Other types of weak anion-exchange HPLC columns may provide improved resolutions. Examples are the BIO-SIL TSK-540-DEAE column and the Baker-Bond PEI-WAX column; a chromatogram developed with these columns is best obtained using a slow gradient between the 0.015 *M* Tris–potassium cyanide, pH 8.6 developer and an 0.015 *M* Tris–potassium cyanide, 0.3 *M* sodium chloride, pH

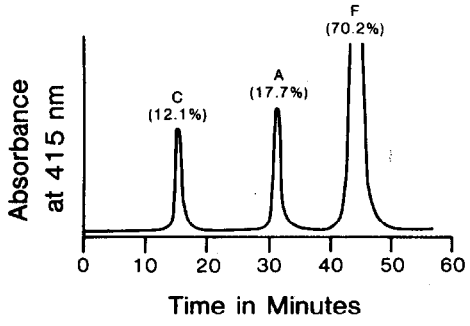

Fig. 2. Separation of Hb types present in a cord blood red cell lysate of a baby with an Hb C heterozygosity using a column of SynChropak AX 300. The chromatogram was developed with a gradient between 0 and 0.01 *M* sodium acetate.

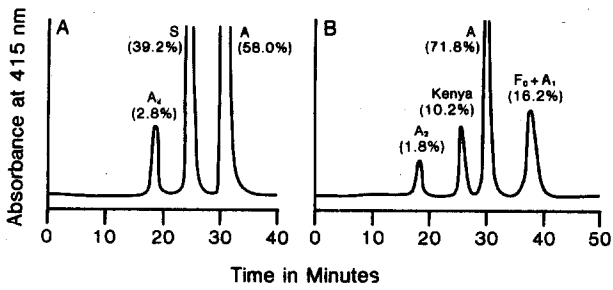

Fig. 3. Separation of a few Hb variants by HPLC on a BIO-SIL TSK column using a 0.03 *M* Tris–potassium cyanide buffer system and a 0 to 0.3 *M* sodium chloride gradient. (A) Hb S heterozygote; (B) a heterozygote for Hb Kenya, a hybrid Hb containing α and $^{A}\gamma\beta$ chains (from ref. 7).

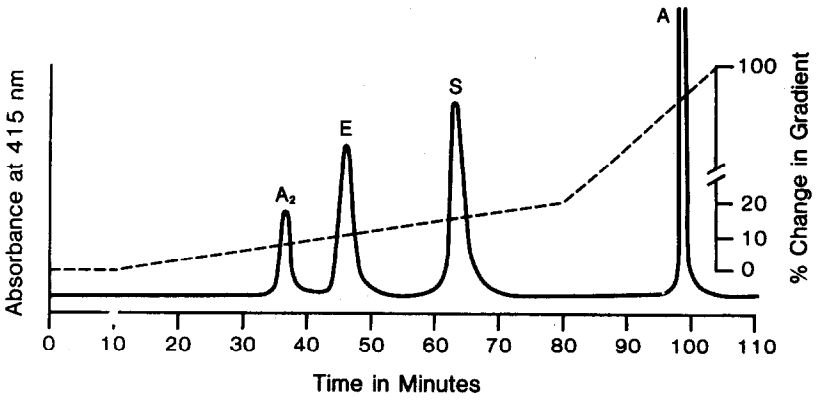

Fig. 4. Separation of the Hbs A_2, E, S, and A in an artificial mixture. A slowly developing gradient between 0 and 0.3 *M* sodium chloride was applied to a Baker-Bond PEI-WAX column (from ref. 7).

8.6 developer (percentage change per min: 0.75). When this gradient is further reduced (percentage change per min: 0.15), it even becomes possible to separate the Hbs A_2 and E (Fig. 4).

4. SEPARATION OF HEMOGLOBINS BY CATION-EXCHANGE HPLC

Separation of the normal Hbs and their numerous variants by cation-exchange HPLC has become most popular during the past several years. In our laboratories we successfully use (modifications of) the following procedures (see also refs. 7, 21 and 22). About 200–400 μg of Hb, dissolved in 20–40μl, is chromatographed on a 250 mm$\times$4.1 mm I.D. SynChropak CM 300 column using a sodium acetate gradient (0–0.15 *M*) in 0.03 *M* Bis-Tris–potassium cyanide (0.0015 *M*), pH 6.40 developers. A freshly prepared red cell lysate or an eluent of a spot of whole dried blood (on SS No. 903 filter paper) can be used. One punch-out of this paper (D=4–5 mm) is soaked in developer B (0.2 ml of 0.03 *M* Bis-Tris–potassium cyanide, pH 6.40) for 30 min, whereafter 10–20 μl of the eluent is applied to the column. The eluent is centrifuged in a simple table-top centrifuge to remove solid particles. The column can be attached to any suitable HPLC equipment. The

slope of the gradient is determined by the types of Hb component to be analyzed. A small integrator is used for the calculation of the relative quantities of the various proteins.

Fig. 5 illustrates separations that can be obtained. Fig. 5A depicts the separation of the normally occurring minor Hb A_1 and Hb A components; the blood sample was collected from a severely affected, uncontrolled, diabetic patient and contained large quantities of Hb A_{1c} (=glycosylated Hb A; normal value 4–7%) and Hb A_{1d} (normally not observed or present in small quantities; this fraction is Hb A with glucose attached to more than one amino acid residue, including some of the α chain). Hb A_2 elutes after Hb A_0 and is completely separated from this major Hb, thus allowing its quantitation. As shown in Fig. 5B, Hb E does not separate from Hb A_2, while the minor Hbs elute in front of the major Hb E component behind two Hb F zones, i.e. the major Hb F_0 and its acetylated derivative Hb F_1. Fig. 5C and D concerns samples from patients with a homozygous Hb S or Hb C condition. The elution characteristics of these β chain variants readily allow the quantitation of Hb F ($=F_0+F_1$) and Hb A_2 in one chromatographic

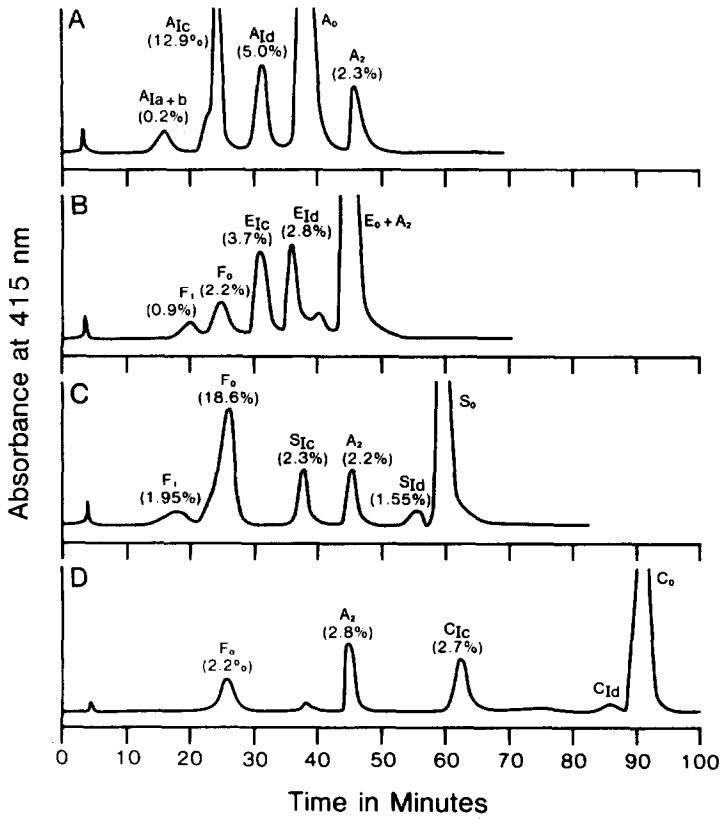

Fig. 5. Separation of Hb components in red cell lysates of (A) a normal person with uncontrolled diabetes, (B) a Hb E homozygote, (C) a Hb S homozygote, and (D) a Hb C homozygote. The separation of the minor Hbs is greatly improved by the use of a slower gradient. Column: SynChropak CM 300 (from ref. 7).

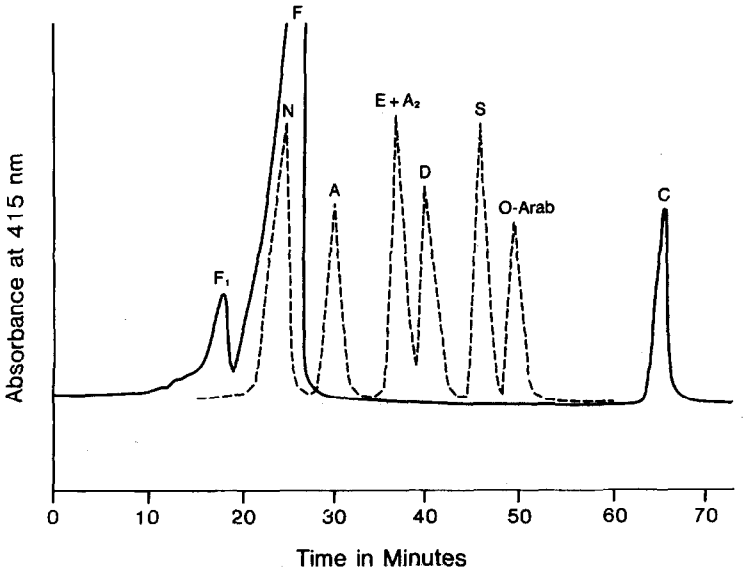

Fig. 6. Positions of different adult Hb types in chromatograms of red cell lysates from newborn babies. Column: SynChropak CM 300 (from ref. 7).

experiment. Fig. 6 illustrates the positions of various β chain abnormal Hbs in this type of chromatography. It is interesting to note that variants with identical substitutions (Glu→Lys) but in different positions of the β chain (C: β6; E:β26; O-Arab: β121) elute at completely different elution times, indicating that the chromatographic properties of these variants are greatly influenced by the location of this Glu→Lys substitution in the β chain.

Variations in the methodology have been introduced and concern mainly the use of shorter columns and of columns of different types and different manufacturers. Shorter columns certainly have advantages, particularly in a laboratory where many samples have to be analyzed. A disadvantage is some loss of resolution. Fig. 7 demonstrates three chromatograms: one is from a subject with Hb H disease (i.e. a deficiency in α chain production because of the loss of three α globin genes: $-\alpha/--$), a second is from a subject with the electrophoretically fast-moving Hb Pontoise, and the third is from an Hb E homozygote. Although separations appear adequate (Hb H elutes within 2 min, well separated from Hb A and its minor derivatives; Hb Pontoise separates from Hb A; Hb E elutes at its expected position together with Hb A_2), the decrease in resolution makes the quantitation of Hb H less definite, disallows the separation of the minor Hb A_1 and the minor Hb Pontoise derivatives, and makes it impossible to quantitate Hb F in the Hb E homozygote. The decrease in column size does not affect the quantitation of Hb F and Hb A_2 in blood samples from patients with sickle cell anemia or sickle cell β-thalassemia; the chromatograms depicted in Fig. 8 show an adequate separation of all Hb fractions. This is also the case for diseases involving the Hb C variant (Fig. 9). Thus, the short 30–50 min program allows adequate differentiation between conditions such as SS, S-β^+-thalassemia (with Hb A eluting at 14–18 min), S-β°-thalassemia, SC, CC, and C-β°- or β^+-thalassemia.

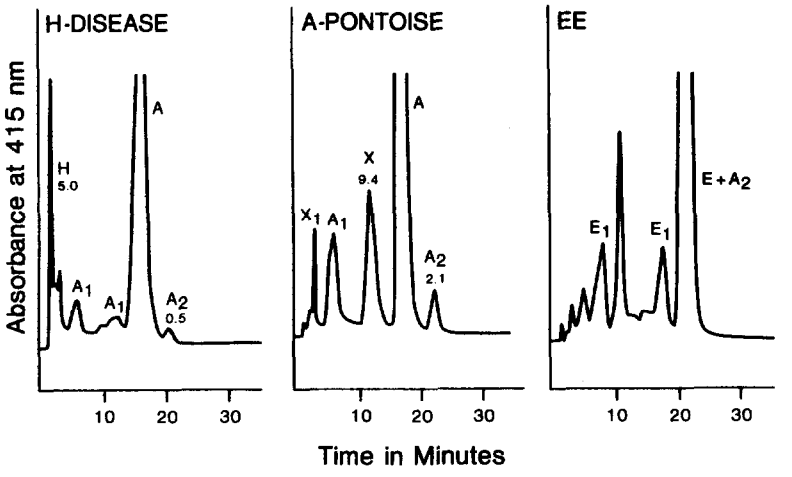

Fig. 7. Separation of Hb types using a shorter (100 mm×4.6 mm) SynChropak CM 300 column and a faster gradient. Left: a patient with Hb H disease; Middle: a person with Hb Pontoise or $\alpha_2$63(E12)Ala→Aspβ_2; right: a Hb E homozygote.

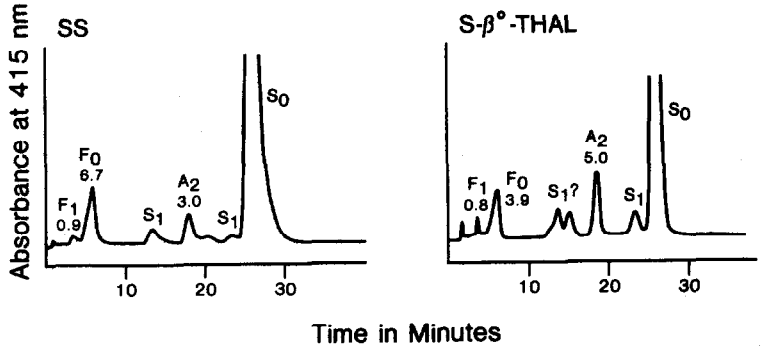

Fig. 8. Separation of Hb types using the 100 mm×4.6 mm SynChropak CM 300 column. This faster procedure adequately separates Hb A, Hb A_2, and Hb S, and allows the differentiation between the two conditions listed.

This procedure is ideally suited for the quantitation of Hb A in blood of SS patients who are undergoing blood transfusion therapy.

An abnormality in the α chain affects all types of Hb. When the Hb G-Philadelphia substitution is present [α68(E17)Asn→Lys], variants of Hb A ($\alpha_2{}^G\beta_2$), of Hb A_2 ($\alpha_2{}^G\delta_2$), of Hb F ($\alpha_2{}^G\gamma_2$), and of the minor Hbs, for instance, $\alpha_2{}^G\beta_2$ (A_{1c}), will be present which will greatly complicate the separation. Moreover, when a β chain variant is also present, additional variants can be expected, such as $\alpha_2{}^G\beta_2{}^X$. Fig. 10 depicts the Hb fractions obtained from red cell lysates of one person with a Hb S trait as well as a Hb G-Philadelphia heterozygosity ($ASAG_\alpha$) and of a second person with a similar combination but with Hb C ($ACAG_\alpha$). The chromatograms show (i) a complete separation of the major Hb G and Hb S components; (ii) quantitation of Hb A_2 and Hb G_2 ($=\alpha_2{}^G\delta_2$); and (iii) the elution of the hybrid Hbs ($SG=\alpha_2{}^G\beta_2{}^S$ and $CG=\alpha_2{}^G\beta_2{}^C$) at elution times of 70 and

100 min, respectively. Minor Hbs do not resolve well from Hb A and Hb A_2. The resolution of the various Hbs becomes less definitive when a shorter column and program are used, although separation of the major Hbs A_0 and G_0, and of the minor Hbs A_2 and G_2 is still possible (Fig. 11).

The original cation-exchange HPLC methodology was most useful for the separation of abnormal Hbs in blood from newborn babies. Fig. 12 provides numerous examples. The major advantage of this method is the fast elution of Hb F (F_0+F_1) well separated from Hb A and the most common β chain variants. Their

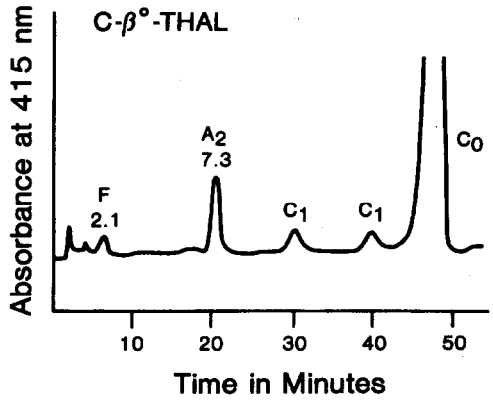

Fig. 9. Separation of the Hb components in a red cell lysate of an adult with Hb C-β°-thalassemia using a 100 mm×4.6 mm SynChropak CM 300 column. Complete separation of Hb F, Hb A_2, the minor Hb C_1 components, and Hb C makes it possible to diagnose the condition as a Hb C-β°-thalassemia.

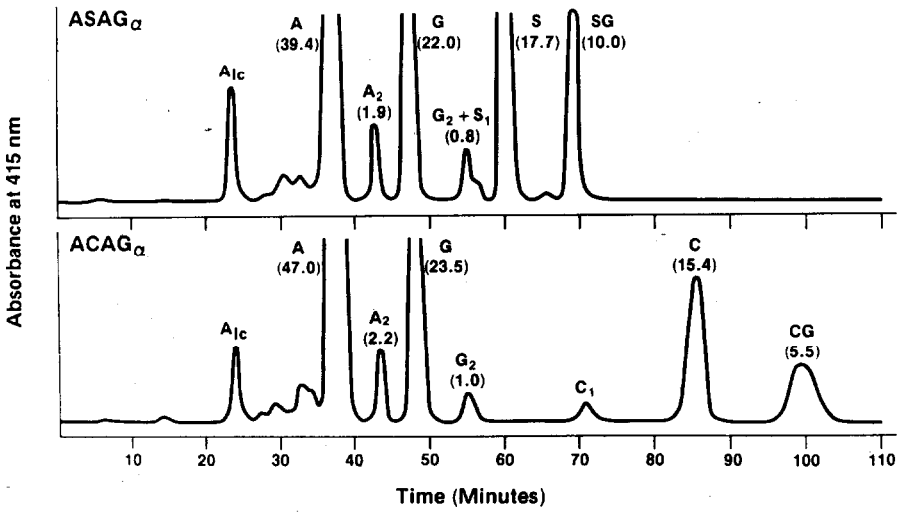

Fig. 10. Separation of major and minor Hb components in red cell lysates of two patients with a Hb G-Philadelphia heterozygosity and either Hb S ($ASAG_\alpha$) or Hb C ($ACAG_\alpha$). The numbers between parentheses are percentages. Minor Hbs are not named unless specific for the condition. Column: SynChropak CM 300 (250 mm×4.6 mm).

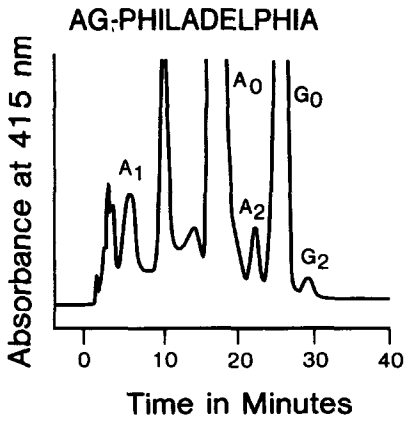

Fig. 11. Separation of Hb components in a red cell lysate of a Hb G-Philadelphia heterozygote. Column: SynChropak CM 300 (100 mm×4.6 mm). The separation should be compared with that given in Fig. 10.

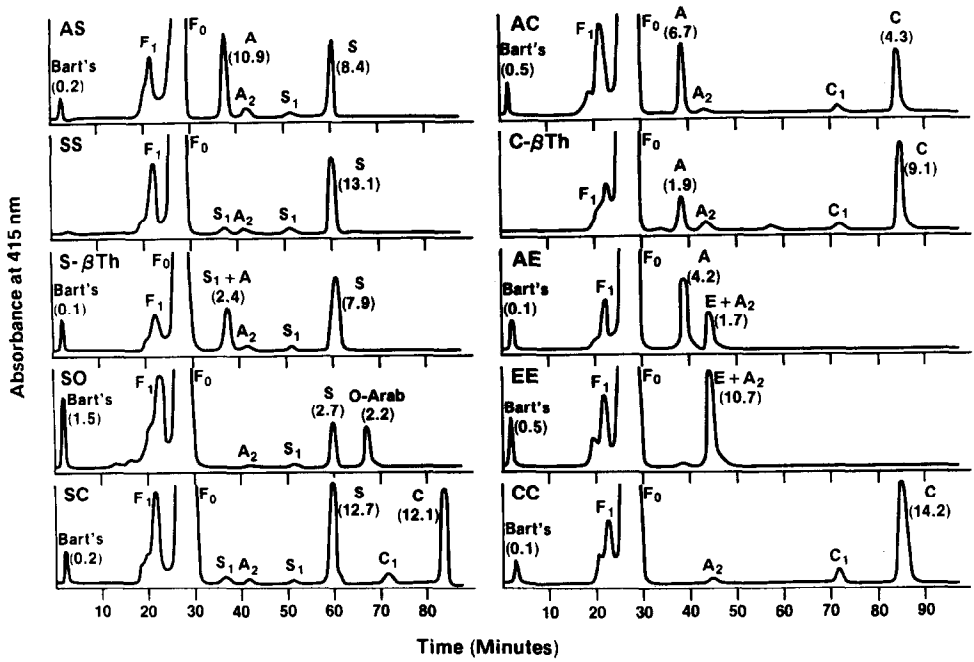

Fig. 12. Separation of Hb F and adult Hbs in lysates of red cells from cord blood of ten babies with different Hb abnormalities. The numbers between parentheses are percentages. Minor Hbs are not named unless specific for the condition. Column: SynChropak CM 300 (250 mm×4.6 mm) (from ref. 21).

elution is preceded by that of Hb Bart's (or γ_4); the presence of a notable quantity of this unusual variant is indicative for the presence of an α chain deficiency or α-thalassemia. A review of the various chromatograms, and particularly of the positions of the different abnormal β chain Hbs, show that a differential diagnosis between conditions such as AS, SS, and S-β^+-thalassemia; SS, S-O-Arab,

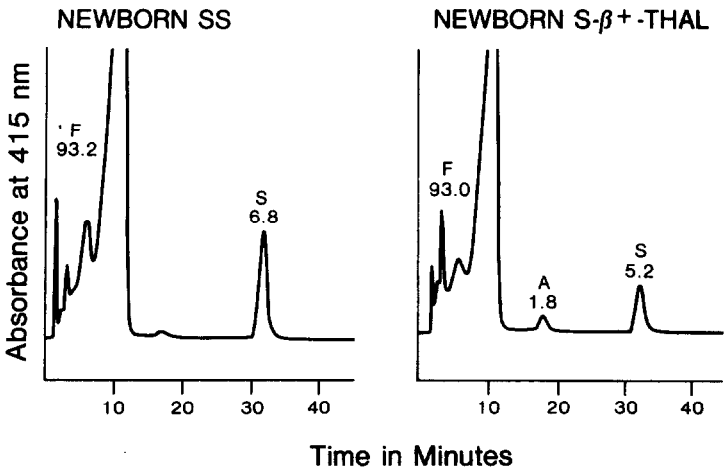

Fig. 13. Separation of Hb F and adult Hbs in lysates of red cells from cord blood samples using a 100 mm×4.6 mm SynChropak CM 300 column. A differential diagnosis of SS and S-β^+-thalassemia can readily be made.

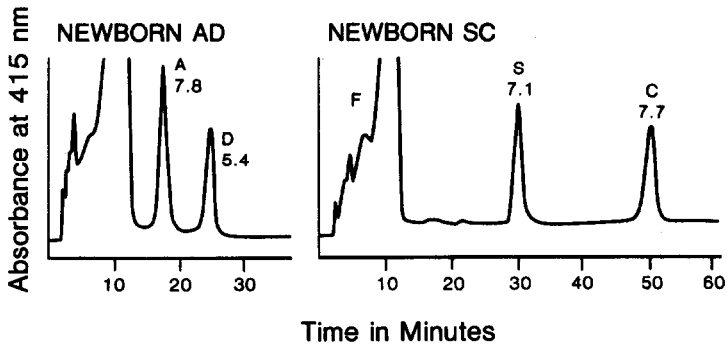

Fig. 14. Separation of Hb F and adult Hbs in red cell lysates from newborn babies with a Hb D-Los Angeles [$\alpha_2\beta_2$121(GH4)Glu→Gln] heterozygosity (left) and with SC disease (right). Column: 100 mm×4.6 mm SynChropak CM 300.

and SC; and AC and C-β^+-thalassemia; AE, AC, EE, and CC can readily be made. It is worth noting that Hb A_2 is present in all chromatograms (except in those with Hb E which has the same mobility); minor adult Hbs are also observed, but their presence does not seriously interfere with reaching a diagnosis.

Shortening of the column and the program does not significantly interfere with the separation of the β chain variants in cord blood red cell lysates. Figs. 13 and 14 illustrate results obtained with a 100 mm×4.6 mm column. The fetal Hb components elute within 10–12 min and the different elution times of Hb A (18 min), Hb D (25 min), Hb S (30–32 min), and Hb C (50 min) adequately allow characterization and quantitation of slowly moving β chain variants. A differential diagnosis of SS or S-β^+-thalassemia can also be made (Fig. 13). Most recently, this method was further simplified by using a 30 mm×4.6 mm column (Brownlee-3CM cartridge; Aquapore CX-300, X03-GU, Brownlee Labs.) and a linear

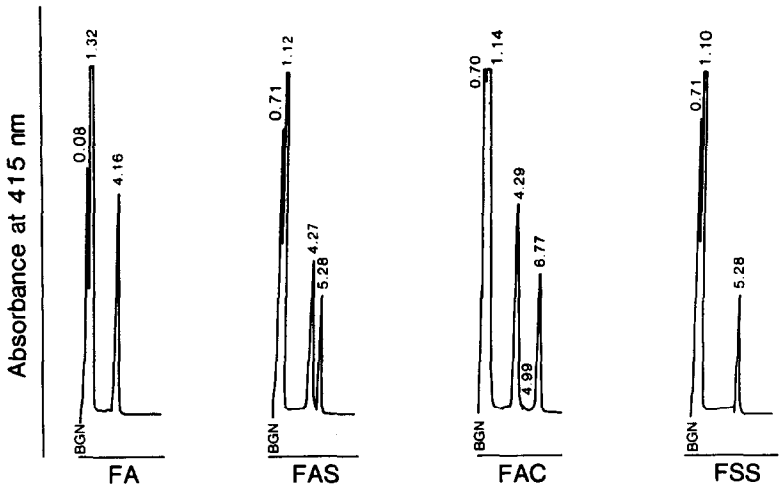

Fig. 15. Elution patterns and times of Hb components in cord blood hemolysates obtained from a normal baby (Hb AA) and from babies with Hbs AS, AC, and SS. A program of less than 10 min was applied to a 30 mm×4.6 mm Brownlee cartridge type of column (from ref. 23).

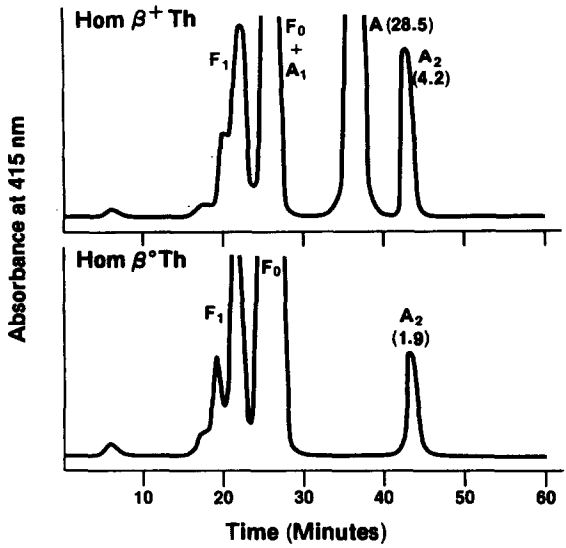

Fig. 16. Separation of major and minor Hb components in red cell lysates of one patient with homozygous β^+-thalassemia and a second with homozygous β°-thalassemia. The numbers between parentheses are percentages. Column: SynChropak 250 mm×4.6 mm (from ref. 21).

but faster gradient, which is applied for only 5 min (for details, see ref. 23). The chromatogram is completed in less than 10 min with an adequate resolution of the Hbs A, S, and C (Fig. 15). This new approach is relatively cheap (the column price is below US$ 100 and it can be used for several hundred analyses), is reproducible, can readily be automated, and thus be used for mass testing programs. Its obvious limitation is its decreased resolution and a differential diagnosis of

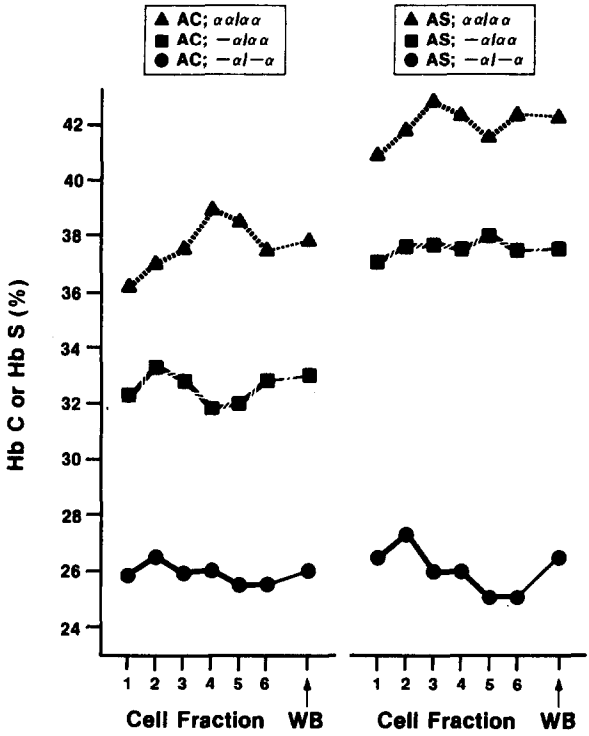

Fig. 17. Levels of Hb C and Hb S in lysates of adult red cells isolated by a Dextran density gradient (WB = whole blood). Determinations were made on a 250 mm × 4.6 mm SynChropak CM 300 column. For details see text (from ref. 22).

conditions such as SC and S-O-Arab, AS and DS, and others, will require additional experiments.

The excellent separation of Hb F and Hb A makes cation-exchange HPLC the method of choice to analyze and quantitate Hbs F, A, and A_2 in patients with homozygous β°- or β^+- or $(\delta\beta)^\circ$-thalassemia. Fig. 16 gives two examples. It is unfortunate that Hb F cannot be separated from the minor Hb A_1 (mainly A_{1c}) which complicates matters considerably, notably when the level of Hb F is below 10%.

Quantitation of β chain variants in red cell lysates from adult subjects with an α-thalassemia (i.e. α-thalassemia-2 heterozygosity or $-\alpha/\alpha\alpha$, or homozygosity or $-\alpha/-\alpha$) is often useful because these percentages are decreased when an α chain deficiency is present. This is particularly the case for Hb S and Hb C because their respective β chains (β^S and β^C) do not combine with α chains as easily as do the β^A chains of Hb A [24–26]. Thus, the quantity of Hb S or Hb C in a (freshly prepared) red cell lysate of heterozygotes is indicative for the presence of such deficiencies. Fig. 17 illustrates some data for Hb C and Hb S heterozygotes. These results also show that the quantitative differences do exist in all cell fractions, irrespective of age. The slight differences between the Hb S and Hb C heterozygotes remain unexplained.

5. SEPARATION OF HEMOGLOBIN CHAINS

Although different techniques have been developed [6,27–32], the one introduced by Shelton et al. [33] has proven to be most useful. For analytical purposes, a 250 mm×4.0 mm large-pore Vydac C_4 column is used which can be attached to any type of HPLC equipment. The two developers are mixtures of acetonitrile–water–trifluoroacetic acid (TFA). Developer A is acetonitrile–water (60:40) with TFA added to a final concentration of 0.1%. Developer B is comparable except that the mixture is composed of acetonitrile–water (20:80) (with 0.1% TFA). For most HPLC equipment it is possible to regulate the flow of the two developers so that appropriate mixtures can be made. We equilibrate the column for 10 min with 50% developer A (+ 50% developer B) and apply 10–200 μl hemolysate containing approximately 60–120 μg of Hb (equipment is available to automate the application of multiple samples). The first step in the development of the chromatogram is through the application of a gradient of developer A (50→60%) with a corresponding decrease in developer B (50→40%) for a period of 80 min. A shallower gradient (60→62% developer A) is applied for an additional 20 min, which is important for the optimal separation of the different γ chains. The column is finally purged with 99% developer A for 5 min, and next reequilibrated for 10 min with 50% developer A. The flow-rate is kept at 1 ml/min; the effluent is monitored at 220 nm, and calculation of the relative concentrations is made with a simple computer–integrator. Slight variations in the gradient system are often introduced to improve or facilitate the elution of certain chains. The same column can be used for several hundred analyses; its life-span is greatly increased by the use of ultra-clean reagents and red cell lysates and by occasionally turning the column upside-down to remove most of the trash that has accumulated on its top surface.

5.1. Adult blood samples

Red cell lysates are prepared in the usual manner and some 60–120 μg (15–20 μl) are applied to the column. Because of the acidity of the developing system (pH 2.0–2.2) the heme is removed from the globin and the proteins are dissociated in their constituent α and β, γ or δ chains. Fig. 18 illustrates two chromatograms; the one on the left is from a normal adult and the one on the right from a person with a homozygosity for Hb E [or $\alpha_2\beta_2$26(B8)Glu→Lys]. Several protein zones can be detected: in the AA chromatogram, heme is eluted first (usually in less than 10 min), followed by the β chain and its pre-β chain derivative, the δ chain, a non-Hb fraction (NHP), the α chain, and the γ chains. Quantitation of Hb A_2 (as δ chain) is possible by this procedure and its accuracy is only slightly less than that seen for micro-column chromatographic methods [8,34]. The γ chains are usually not observed as their concentrations are too low to allow detection. However, small but noticeable peaks are seen in this AA sample; the first corresponds to $^{A}\gamma^{T}$ ($=\gamma$75Thr, 136Ala) and the second to $^{G}\gamma$ ($=\gamma$75Ile, 136Gly). The elution patterns for the Hb E homozygote are comparable, although a slight modification in the gradient resulted in a faster elution of the δ, NHP, and α zones. Most interesting is the fast elution of the β^{E} chain, well separated from

the δ chain, which allows the quantitation of Hb A_2 (as δ) in persons with this Hb abnormality. Data from analyses of several Hb E homozygotes and Hb E-β°-thalassemic individuals have been reported [8]; Hb A_2 values in EE subjects varied between 4.4 and 6.1%, while those in E-β°-thalassemia subjects were higher at 7.2–10%.

Several β chain variants with different types of substitutions can be identified by this procedure. Fig. 19 illustrates some chromatograms; the β chain of Hb City of Hope [β69(E13)Gly→Ser] elutes at about the same position as the β chain of Hb S [β6(A3)Glu→Val], and quite different from the position of the β chain of Hb C [β6(A3)Glu→Lys]. Abnormalities in the α chain can also often be discovered with this procedure (see also ref. 8). Fig. 20 illustrates the elution of two variant α chains, namely those of Hb Chicago or $\alpha_2$136(H19)Leu→Metβ_2 and of Hb G-Georgia or $\alpha_2$95(G2)Leu→Proβ_2. The large differences in elution time for these two variant α chains clearly illustrate the effect by the specific amino acid residue that is introduced.

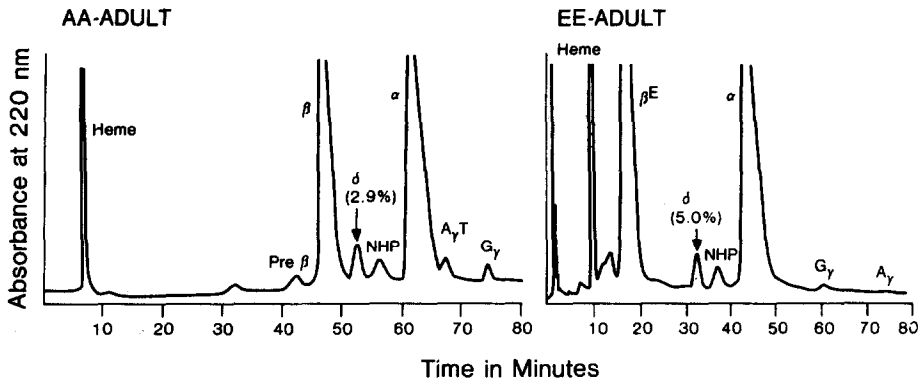

Fig. 18. Separation of globin chains by reversed-phase HPLC using a 250 mm×4.0 mm large-pore Vydac C_4 column. Left: adult red cell lysate; Right: red cell lysate of a Hb E homozygote.

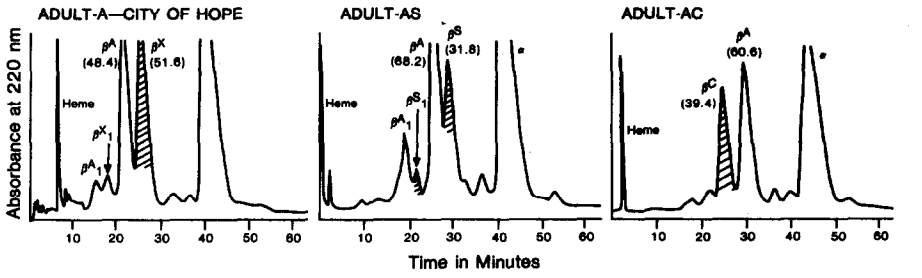

Fig. 19. Separation of abnormal globin chains by Vydac C_4 reversed-phase HPLC. Left: hemolysate from a person with a heterozygosity for Hb City of Hope [$\alpha_2\beta_2$69(E13)Gly→Ser]; middle: same of a Hb S heterozygote; right: same of a Hb C heterozygote.

The reversed-phase HPLC procedure [33] has been used extensively in the study of the γ chain heterogeneity. Nearly twenty years ago, Schroeder et al. [35] observed the existence of two types of γ chain which are the products of dupli-

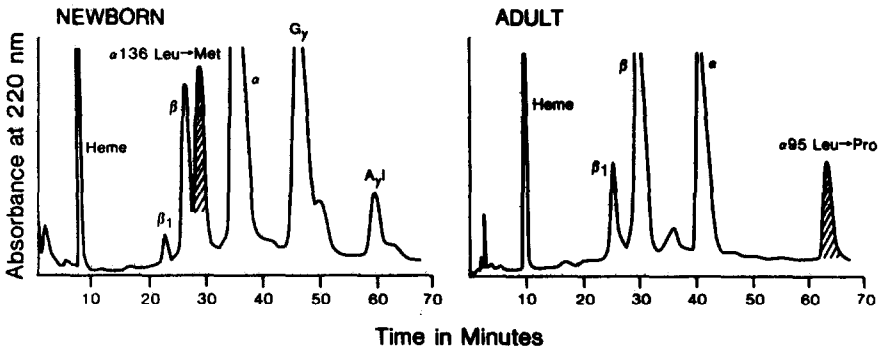

Fig. 20. Separation of abnormal globin chains by Vydac C_4 reversed-phase HPLC. Left: red cell lysate from a newborn with Hb Chicago or $\alpha_2 136(H19)Leu \rightarrow Met\beta_2$; right: red cell lysate from an adult with Hb G-Georgia or $\alpha_2 95(G2)Leu \rightarrow Pro\beta_2$.

cated γ globin genes. The $^{G}\gamma$ chain has a glycine residue at position $\gamma 136$ and the $^{A}\gamma$ chain has an alanine at that position. Later studies [36–39] identified a common $^{A}\gamma$ variant, the $^{A}\gamma^{T}$ chain, with an Ile→Thr substitution at position $\gamma 75$. Analyses of Hb F from adults often require isolation of this protein, as its concentration in the adult red cell lysates is too low for an accurate evaluation. Such isolation is usually done by a macro-chromatographic method, such as DEAE-cellulose chromatography [40], which is simple and relatively inexpensive. The isolated Hb F, which is contaminated with adult Hb(s), can readily be analyzed by the reversed-phase HPLC method [33].

Figs. 21–24 illustrate the separation of the γ chains in various blood samples. Fig. 21 shows the results for three adult patients with a homozygosity for a $(\delta\beta)^{\circ}$- or (β°)-thalassemia; these subjects are unable to produce normal β chains, and α and γ chains are the important chains found in the chromatograms. Red cell lysates can be analyzed without further purification, provided the patient did not receive a blood transfusion. The data for the three untransfused patients, shown in Fig. 21, are of interest because of the different ratios between the $^{G}\gamma$ and $^{A}\gamma$ chains. For instance, the subject with the $(\delta\beta)^{\circ}$-thalassemia homozygosity had 100% Hb F with a $^{G}\gamma$-to-$^{A}\gamma$ ratio of about 4:6. This ratio is much higher in the two β°-thalassemia homozygotes; one patient produced nearly all $^{G}\gamma$ chains. Such data are important in the evaluation of the genetic condition causing the thalassemic abnormality; for instance, the $(\delta\beta)^{\circ}$-thalassemia was caused by a deletion which kept both γ globin genes functioning, while one of the β°-thalassemia patients was homozygous for a β°-thalassemia being located on a chromosome with four γ globin genes of the $-^{G}\gamma-^{G}\gamma-^{G}\gamma-^{A}\gamma-$ type, explaining the high $^{G}\gamma$ chain production (third chromatogram).

Fig. 22 illustrates $^{G}\gamma$ and $^{A}\gamma$ data for three patients with sickle cell anemia. In each case, Hb F was isolated by DEAE-cellulose chromatography, which explains the small β^{S} peaks observed in the three chromatograms. Of interest are the differences in $^{G}\gamma$-to-$^{A}\gamma$ ratios, such as 3:7, 1:1, and 7:3. These differences are due to structural changes in the DNA of regions 5′ to the $^{G}\gamma$ globin gene which influences the rate of synthesis of the $^{G}\gamma$ chains (for review, see ref. 41).

Most adult red cell lysates contain less than 1% Hb F which greatly complicates the determination of the relative quantities of the different types of γ chain. Hb F isolation can be attempted by DEAE-cellulose preparative chromatography [40]; however, this isolation is successful in only one third of all cases. The two

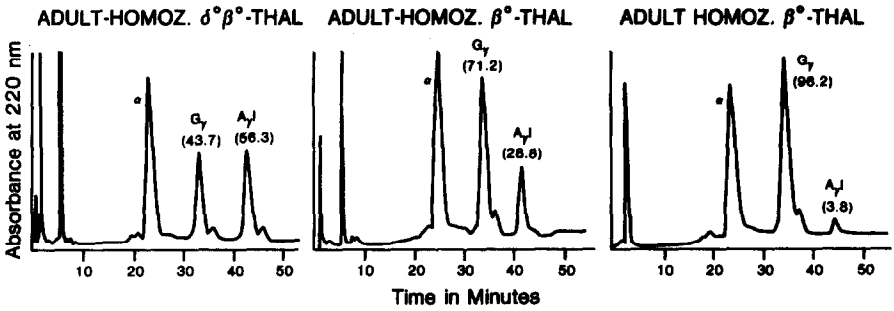

Fig. 21. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Red cell lysates from three adults with different types of homozygous $(\delta\beta)^\circ$- or β°-thalassemia were analyzed. See text for details.

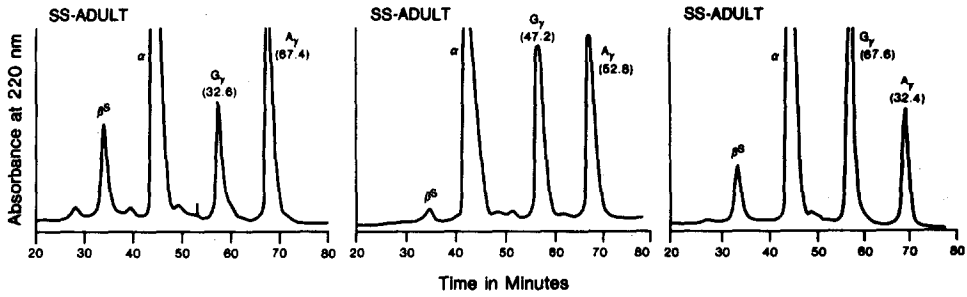

Fig. 22. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Hb F samples isolated from red cell lysates of three adult SS patients were analyzed. See text for additional information.

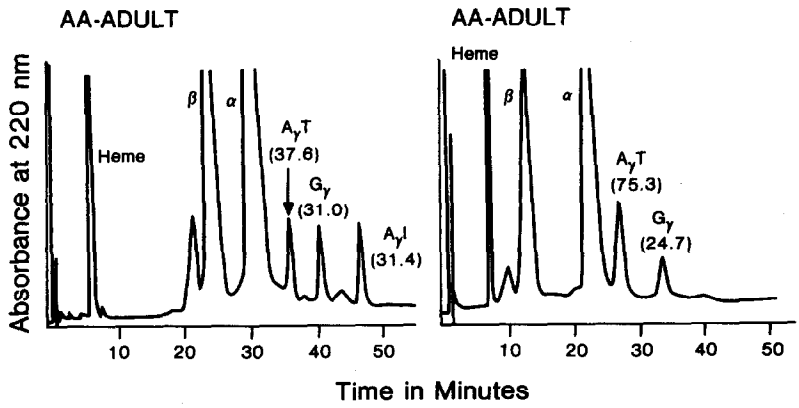

Fig. 23. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Hb F was isolated from red cell lysates of two normal adults by macro-chromatography and these impure Hb F fractions were analyzed. See text for additional information.

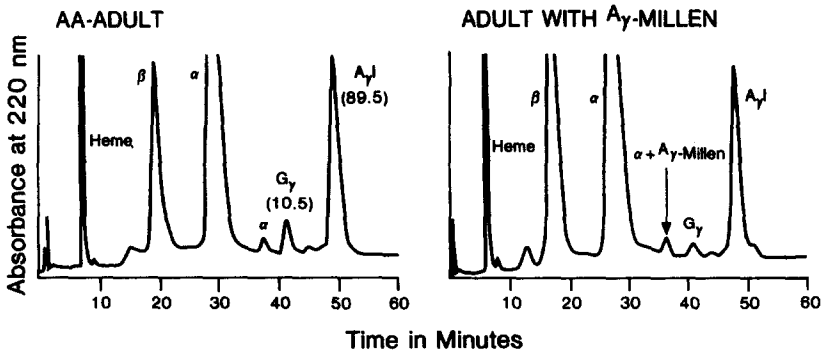

Fig. 24. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Hb F was isolated by DEAE-cellulose chromatography from red cell lysates of a normal adult (left) and of a person with an elevated Hb F level (right) who, moreover, produced the variant Hb F-Millen or $\alpha_2{}^{A}\gamma_2$141(H19)Leu→Met. See text for further details.

chromatograms of Fig. 23 illustrate the γ chain composition of the Hb F of two normal adults; the subject shown on the left is an $^{A}\gamma^{T}$ heterozygote with the expected low $^{G}\gamma$ value of some 30%, while the subject shown on the right is an $^{A}\gamma^{T}$ homozygote. The level of $^{G}\gamma$ chain in the Hb F of normal adults seems to vary considerably and is to a large extent associated with specific chromosomal characteristics, known as haplotypes [42]. A recent study by Hattori et al. [43] offered evidence that at least four different haplotypes can be found in adults with low levels of Hb F but with varying $^{G}\gamma$ percentages. One such an analysis is depicted in the left chromatogram of Fig. 24. This normal person with an extremely low $^{G}\gamma$ level of only 10% had two chromosomes with identical haplotypes (for additional discussion, see ref. 43).

The condition of the subject with the $^{A}\gamma$-Millen abnormality (Fig. 24) is quite different. This person was found to have an elevated level of Hb F (10–15%) with γ chains mainly of the $^{A}\gamma$ type. However, a small (5–10%) zone was observed in front of the normal $^{G}\gamma$ chain which could be identified as an altered $^{A}\gamma^{I}$ chain but with a Leu→Met substitution at γ141. Thus, the C_4 chain HPLC is an excellent procedure to detect variant γ chains, even when present in low quantities.

5.2. Newborn blood samples

The C_4 chain HPLC procedure is indeed most suitable for the characterization of the γ chain composition of the Hb F of newborn babies. As the Hb F level is usually in excess of 70%, analyses can be made on red cell lysates without further preparations and some 5–15 μl containing 60–120 μg Hb are applied. Normal chromatograms are depicted in Fig. 25. Each γ chain zone is usually followed by a much smaller zone containing the same γ chain as in the preceding major zone [44]. The $^{G}\gamma$-to-$^{A}\gamma$ ratio in normal babies averages at 7:3 and is not different in babies with an $^{A}\gamma^{T}$ heterozygosity or homozygosity (Fig. 25). Abnormalities in the γ globin gene arrangement have been observed, such as γ globin gene triplications ($\gamma\gamma\gamma$) and quadruplications ($\gamma\gamma\gamma\gamma$), as well as a γ globin gene deletion (γ) or γ-thalassemia [45–48]. Quantitation of the $^{G}\gamma$-to-$^{A}\gamma$ ratio (or $^{G}\gamma$-to-$^{A}\gamma^{I}$-

to-$^{A}\gamma^{T}$ ratio) will often be helpful in deciding the types of γ globin(s) present in these anomalies. Fig. 26 illustrates three chromatograms of the Hbs from infants with different conditions, as determined by globin gene mapping analysis. Newborn No. IV was heterozygous for the $^{A}\gamma^{T}$ chain with the mutated $^{A}\gamma$ globin gene being located on the normal chromosome ($-^{G}\gamma-^{A}\gamma^{T}-$), while the second chromosome carried a γ globin gene triplication of the type $-^{G}\gamma-^{G}\gamma-^{A}\gamma-$. The relative quantities of the different γ chains were quite different: the high level of 78% $^{G}\gamma$ chain is the result of the presence of three $^{G}\gamma$ genes, while the difference in the levels of the $^{A}\gamma^{T}$ chain and the $^{A}\gamma^{I}$ chain (17.5 versus 9.5%) is significant to the extent that it indicates a decreased expression of the $^{A}\gamma^{I}$ gene on the chromosome with the triplicated γ globin gene arrangement. This suggestion is supported by data obtained for newborn No. V who is homozygous for the $-^{G}\gamma-^{G}\gamma-^{A}\gamma-$ triplication; less than 10% of the γ chain was of the $^{A}\gamma$ type. Newborn No. VI had a γ globin gene quadruplication on one chromosome ($-^{G}\gamma-^{G}\gamma-^{G}\gamma-^{A}\gamma-$) while one $^{G}\gamma$ gene and one $^{A}\gamma$ gene was present on the normal chromosome. This baby with six γ globin genes ($-^{G}\gamma-^{G}\gamma-^{G}\gamma-^{A}\gamma-/-^{G}\gamma-^{A}\gamma-$) had an Hb F with only some 15% $^{A}\gamma$ chains, again suggesting only a minor contribution by the $-^{A}\gamma-$ globin gene of the γ gene quadruplication.

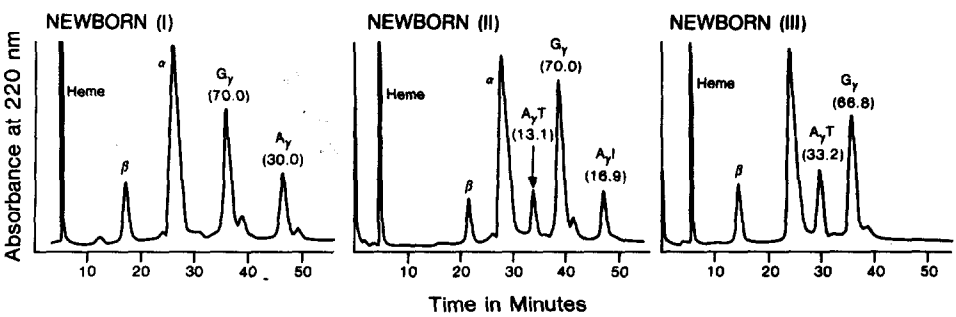

Fig. 25. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Red cell lysates from three babies with or without the $^{A}\gamma^{T}$ variant were analyzed. Newborn I: normal; newborn II: an $^{A}\gamma^{T}$ heterozygote, newborn III: an $^{A}\gamma^{T}$ homozygote.

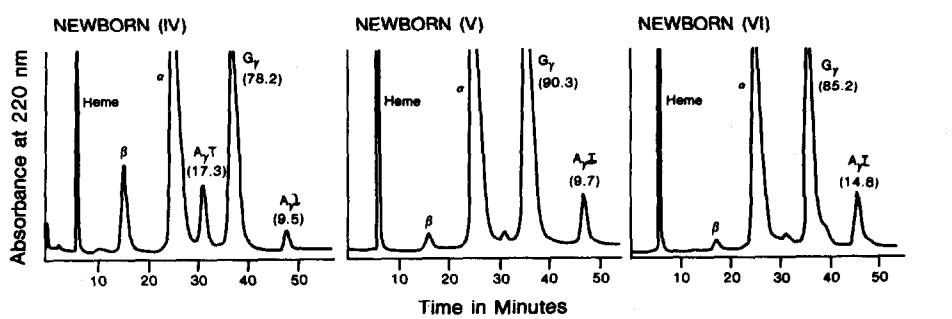

Fig. 26. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Red cell lysates from three babies were analyzed. Newborn IV: an $^{A}\gamma^{T}$ heterozygote with a γ globin gene triplication in trans; newborn V: a homozygote for a γ globin gene triplication; newborn VI: a baby heterozygous for a γ globin gene quadruplication.

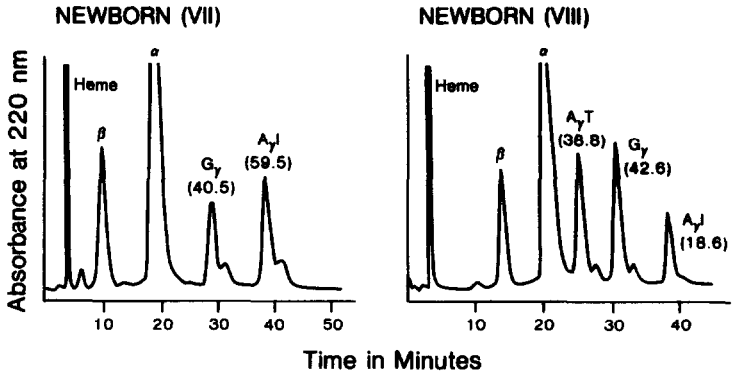

Fig. 27. Separation of globin chains by Vydac C_4 reversed-phase HPLC. Red cell lysates from two babies were analyzed. Newborn VII: a baby with a γ-thalassemia heterozygosity; newborn VIII: same, except that the hybrid gene carried the $^{A}\gamma^{T}$ mutation.

Fig. 27 presents chromatograms for two babies with a γ globin gene deletion or a γ-thalassemia heterozygosity. The one γ globin gene present on the abnormal chromosome regulates the synthesis of the $-^{A}\gamma-$ chain, which explains the low $^{G}\gamma$ level in baby No. VII with the $-^{G}\gamma-^{A}\gamma-/-^{A}\gamma^{*}-$arrangement. Baby No. VIII had a similar condition except that the γ globin gene on the deleted chromosome was responsible for the synthesis of the variant $^{A}\gamma^{T}$ chain. Thus, baby No. VIII with the $-^{G}\gamma-^{A}\gamma^{I}-/-^{A}\gamma^{T*}-$ globin gene arrangement had a Hb F with a $^{G}\gamma$-to-$^{A}\gamma$ ratio of 42.6:18.6% or 70:30, as found in a normal newborn (the synthesis of the two normal γ chains is directed by γ genes located on the normal chromosome). The high percentage of the $^{A}\gamma^{T}$ chain of almost 39% is similar to that of the $^{G}\gamma$ chain, which suggests that the hybrid γ gene on the chromosome with the γ globin gene deletion promotes the synthesis of $^{A}\gamma^{T}$ chain at a high level.

5.3. Preparative reversed-phase HPLC for chain separation

When structural analyses are required it is often useful to isolate the individual chains by a preparative procedure. A considerably larger column is used, and a BIO-SIL TSK-ODS column of 30 cm×2.15 cm is most suitable, as this column will accept a load of at least 20 mg Hb. The chromatogram is developed with two solutions: developer A consists of acetonitrile–water (52:48) (with 0.1% TFA, final concentration) and developer B consists of acetonitrile–water (35:65) (with 0.1% TFA, final concentration). The gradient applied at a flow-rate of 6 ml/min is from 50 to 74% developer A (with a corresponding decrease in developer B from 50 to 26%) in a period of 150 min. This is followed by a second gradient of 75 to 85% developer A in an additional 80 min. The column is purged for 30 min with 99% developer A and equilibrated for 30 min with 50% developer A. The wavelength used to detect the protein is 220 nm.

This procedure has been used successfully for the isolation of variant globin chains, such as the β chain of Hb St. Louis or $\alpha_2\beta_2$26(B8)Leu→Gln [49,50], Hb $^{A}\gamma$-Millen (see above), and others. Fig. 28 illustrates the successful isolation of a $^{G}\gamma$ variant, both by a C_4 analytical system (top chromatogram) as well as by

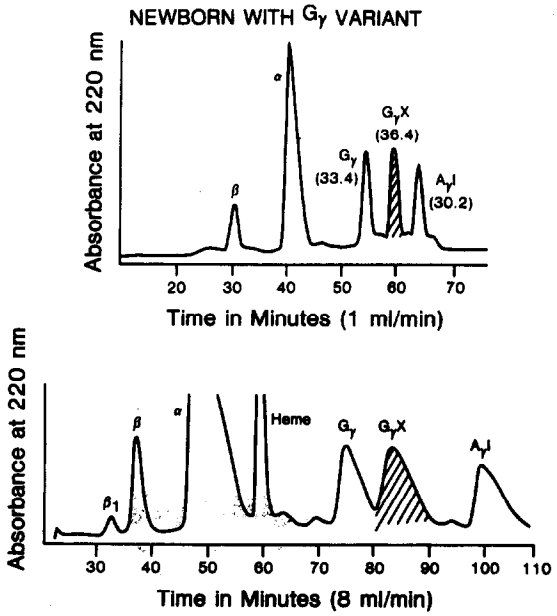

Fig. 28. Separation of α, β, and γ chains by preparative reversed-phase HPLC. Column: BIO-SIL TSK-ODS-120T, 300 mm×21.5 mm. Insert: same but on an analytical scale using the Vydac large-pore C_4 column. The numbers listed above the three chains are percentages (from ref. 51).

preparative C_{18} HPLC, using the above mentioned BIO-SIL TSK-ODS column. The chain of this newly discovered abnormal fetal Hb was identified as a $^{G}\gamma$ chain variant with a Lys→Asn substitution at position 65 (details about these analyses are given in ref. 51). Elution of the $^{G}\gamma$ chain between the normal $^{G}\gamma$ and $^{A}\gamma$ chains greatly facilitated its isolation in pure form. Because of limited amount of blood available, only four chromatograms could be developed; of the 60 mg Hb applied, some 3 mg of the $^{G}\gamma^{X}$ chain were isolated in pure form; this, however, was sufficient for the characterization of the amino acid substitution. The use of volatile developers greatly facilitates the recovery of the desired protein (usually by lyophilization).

6. GENERAL COMMENTS AND CONCLUSIONS

Separation and quantitation of many Hb variants is best achieved on one of the many cation-exchange type of HPLC columns, and Figs. 5–17 provide multiple examples. The data show that the general rule of the higher the positive charge, the longer the elution time, is also applicable in this type of chromatography. However, this is not the only factor. Fig. 29 (which is from ref. 9) illustrates the locations and types of substitution in six different α chain and ten different β chain variants; the figure also lists the relative elution times (RETs) (relative to Hb A). Please note that variants with the same type of substitution (Glu→Lys in Hbs C, E, O-Arab) or with comparable substitutions (Glu→Val; Asp→Asn; Asn→Lys; His→Arg) often exhibit different RET values. These and other data suggest that, as a rule, Hb variants resulting from substitutions occur-

ring within a helical structure (the helices B, D, E, and G of the β chain; the E and G helices of the α chain) are eluting much faster than those due to substitutions in a prehelical region or in an interhelical segment (Hb Richmond with an Asn→Lys substitution at the amino terminus of the G helix of the β chain is a notable exception, probably because the β102 Asn→Lys substitution interferes with an important α_1–β_2 contact). Most illustrative are the high RET values for Hb S (β6 Glu→Val), Hb C (β6 Glu→Lys), Hb Dunn (α6 Asp→Asn), and Hb C-Harlem (the β6 Glu→Val substitution in this variant is responsible for a high RET value, while the second substitution at β73 Asp→Asn, which occurs in the E helix, results in only a modest additional increase in RET value; see also Hb Korle Bu). Thus, the availability of the extra positive charge(s) on each of the two α or β chains to participate in the chromatographic separation process plays an important role. Moreover, the possibility of the formation of contacts, as for instance salt bonds which eliminate the extra positive charges, cannot be excluded. Indeed, it may explain the differences in RET values of Hbs Montgomery and Russ. These two variants have a substitution in the region between the C and E

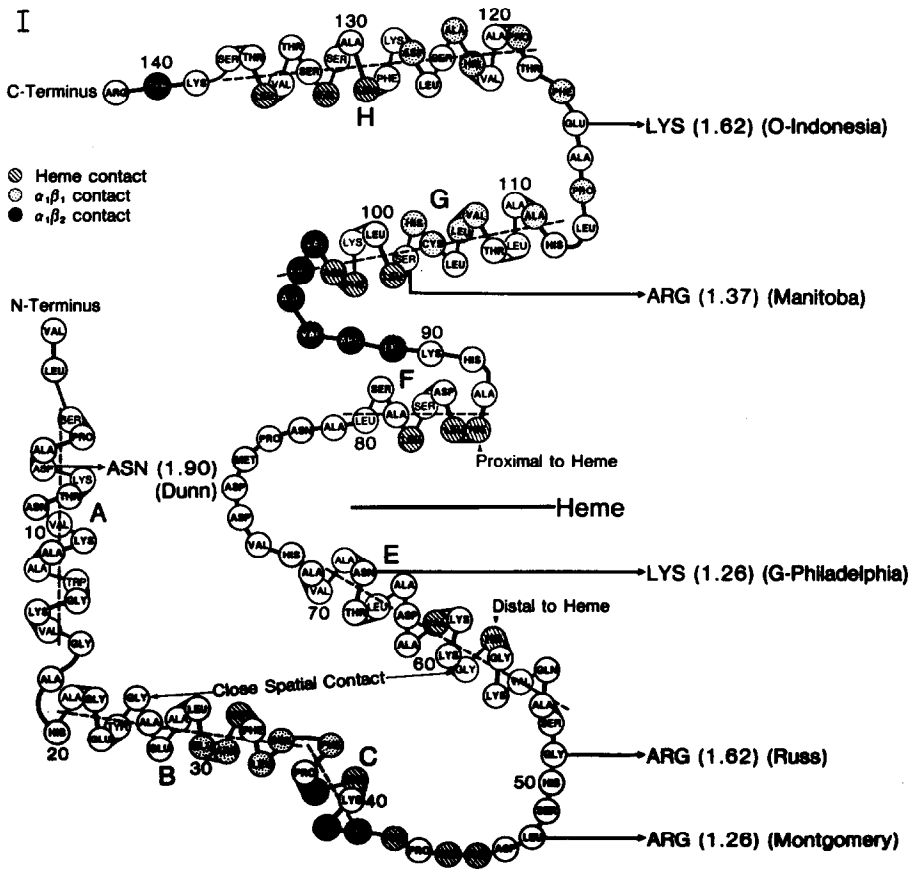

Fig. 29.

(Continued on p. 300)

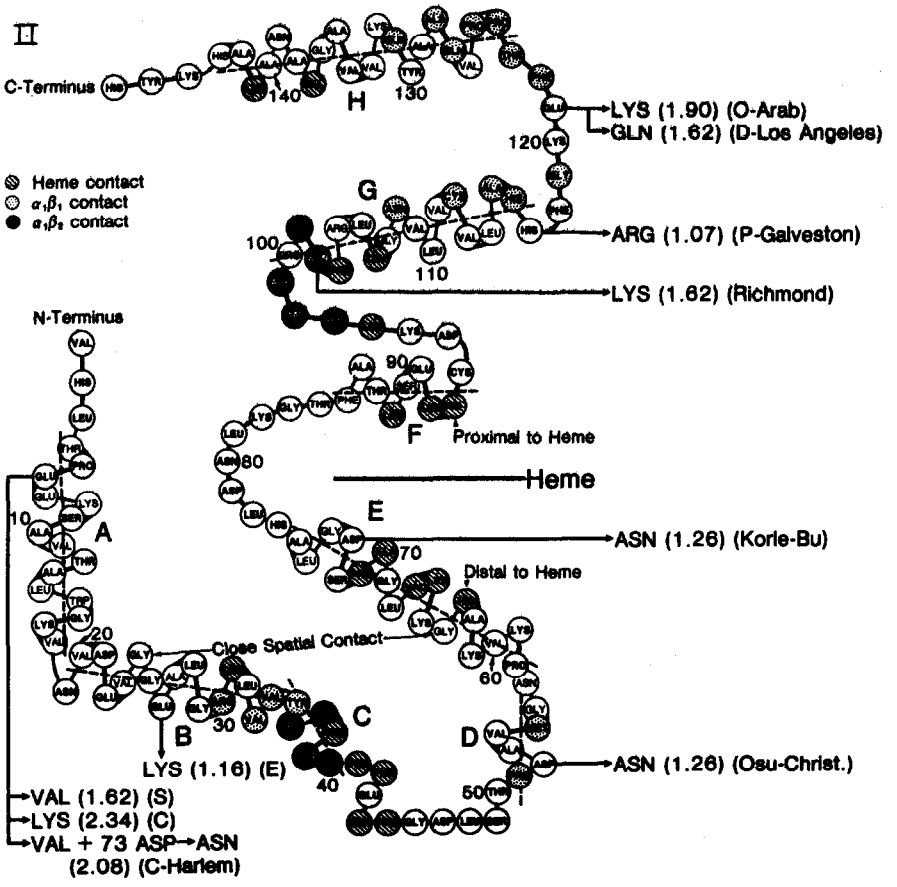

Fig. 29. Positions and types of substitutions of six α chain variants (I) and of ten β chain variants (II). The numbers between parentheses refer to the elution times relative to that of Hb A. Details are given in ref. 9.

helices of the α chain, but the RET value of the first variant with an α48 Leu→Arg substitution is a low 1.26, while that of Hb Russ with an α51 Gly→Arg substitution is the expected 1.62. The differences in RET values are reproducible for native Hb types and are of considerable importance for the chromatographic identification of certain Hb abnormalities.

The practical value of cation-exchange HPLC cannot be overestimated. The classical types of cation-exchange and anion-exchange chromatography have been completely replaced by this procedure in many laboratories. Data obtained with large (250 mm×4.6 mm) and smaller (100 mm×4.6 mm) columns are nearly comparable. It appears likely that a further simplification of this method will soon be adopted by clinical chemistry or clinical hematology laboratories. Moreover, its recent modification, which makes use of the simple cartridge column, is presently being tested in different institutions where large-scale cord blood screening programs are conducted.

TABLE 1

RANKING OF AMINO ACID RESIDUES BASED ON POLARITIES, SIZES, AND pK VALUES AT pH VALUES BELOW 3

Courtesy of Dr. T.A. Stoming (Augusta, GA, U.S.A.); modified after Meek [52]. These data indicate that substitutions such as a leucine residue by arginine will greatly decrease the retention time of the polypeptide chain. An Asn→Asp substitution will have (almost) no effect, while an His→Tyr replacement will increase the retention time of the variant polypeptide chain.

Amino acid	Abbreviation	Amino acid	Abbrevation
1 Tryptophan	Trp	11 Glycine	Gly
2 Phenylalanine	Phe	12 Aspartic acid	Asp
3 Isoleucine	Ile	13 Glutamic acid	Glu
4 Leucine	Leu	14 Asparagine	Asn
5 Tyrosine	Tyr	15 Cystine	Cys
6 Proline	Pro	16 Glutamine	Gln
7 Methionine	Met	17 Serine	Ser
8 Valine	Val	18 Histidine	His
9 Threonine	Thr	19 Lysine	Lys
10 Alanine	Ala	20 Arginine	Arg

The separation of globin chains of the normal and the numerous abnormal Hbs by reversed-phase HPLC at low pH has been useful for their detection, identification, and quantitation. The scaled-up procedure has been used successfully for the isolation of such quantities of chain that structural analyses can be made. Separation under the conditions described is probably based on the differences in polarities and pK_a values between the amino acid residues that are deleted from and introduced into the polypeptide chain. Table 1 ranks the twenty amino acids in a sequence of increasing polarity at a pH below 3; thus, substitution of a residue such as leucine by an arginine residue as in Hb Borås or $\alpha_2\beta_2$88(F4) Leu→Arg will greatly decrease the RET of the abnormal polypeptide chain. Substitutions such as Asp→Asn (as in Hb G-Copenhagen or $\alpha_2\beta_2$47(CD6) Asp→Asn) will not affect the RET, while a His→Tyr substitution (as in Hb M-Hyde Park or $\alpha_2\beta_2$92(F8) His→Tyr) will greatly extend the elution of the variant chain.

Table 2 lists the many α, β, and γ variants that have been studied in the author's laboratory; the table includes RET values and the changes predicted from the ranking given in Table 1. An excellent correlation between predicted and observed changes can be noted although there are some exceptions. When evaluating the data for variants with a Glu→Lys substitution (three β variants, one α variant, two $^{G}\gamma$, and one $^{A}\gamma$ variants) it becomes evident that the location of the substitution within the polypeptide chain can have a profound effect on the RET value. Moreover, substitutions involving a glycine residue such as the Glu→Gly substitution in the β-G-San Jose chain (7 Glu→Gly), the Gly→Asp substitutions in β-J-Baltimore (16 Gly→Asp) and α-J-Oxford (15 Gly→Asp), and particularly the Gly→Ser substitution in β-City of Hope (69 Gly→Ser), results in higher than expected RET values when the glycine is replaced and in lower than expected RET values when the glycine is introduced. Nevertheless, the list of amino acids,

TABLE 2

RELATIVE ELUTION TIMES (RET) FOR DIFFERENT ABNORMAL GLOBIN CHAINS IN REVERSED-PHASE HPLC

RET is the elution time of the variant relative to that of the normal α^A, β^A, $^G\gamma$, or $^A\gamma$ chain. Information and references concerning the different Hb variants can be found in publications provided by the International Hemoglobin Information Center in Augusta, GA, and in ref. 53.

Hb variant	Substitution	RET	ΔX^*	Hb variant	Substitution	RET	ΔX^*
α Chain variants				*β Chain variants*			
Chicago	136 Leu→Met	0.83	−	St. Louis	28 Leu→Gln	0.55	− − −
Q-Thailand	74 Asp→His	0.88	− −	Borås	88 Leu→Arg	0.65	− − −
G-Philadelphia	68 Asn→Lys	0.95	− −	E	26 Glu→Lys	0.68	− −
O-Padova	30 Glu→Lys	0.95	− −	O-Arab	121 Glu→Lys	0.68	− −
Q-Iran	75 Asp→His	0.96	− −	C	6 Glu→Lys	0.90	− −
Hasharon	47 Asp→His	0.97	− −	D-Iran	22 Glu→Gln	0.91	−
Daneshgah-Tehran	72 His→Arg	1.00	−	D-Los Angeles	121 Glu→Gln	0.92	−
J-Pontoise	63 Ala→Asp	1.00	−	G-San Jose	7 Glu→Gly	0.94	+
I-Philadelphia	16 Lys→Glu	1.08	+ +	J- (not named)	86 Ala→Asp	1.00	−
J-Sardegna	50 His→Asp	1.09	+ +	Osu-Christiansborg	52 Asp→Asn	1.00	−
Le Lamentin	20 His→Gln	1.14	+	G-Copenhagen	47 Asp→Asn	1.00	−
J-Oxford	15 Gly→Asp	1.27	−	Koln	98 Val→Met	1.00	0
G-Georgia	95 Pro→Leu	1.53	+	J-Baltimore	16 Gly→Asp	1.05	−
				Hamilton	11 Val→Ile	1.07	+ +
$^G\gamma$ Chain variants				S	6 Glu→Val	1.08	+ +
F-Kingston	55 Met→Arg	0.74	− − −	City of Hope	69 Gly→Ser	1.15	− −
F-La Grange	101 Glu→Lys	0.84	− −	Complutense	127 Gln→Glu	1.15	+
F-Oakland	26 Glu→Lys	0.94	− −	J-Amiens	17 Lys→Asn	1.16	+ +
F-Malta-I	117 His→Arg	0.98	−	J-Antakya	65 Lys→Met	1.28	+ + +
F-Port Royal	125 Glu→Ala	1.00	+	Beograd	121 Glu→Val	1.32	+ +
F-Auckland	7 Asp→Asn	1.00	−	N-Baltimore	95 Lys→Glu	1.35	+ +
F-Clarke	65 Lys→Asn	1.08	+ +	M-Hyde Park	92 His→Tyr	1.69	+ + +
$^A\gamma$ Chain variants							
F-Cobb	37 Trp→Gly	0.73	− − −				
F-Hull	121 Glu→Lys	0.80	− −				
F-Dickinson	97 His→Arg	1.00	−				
F-Beech Island	53 Ala→Asp	1.00	−				

*ΔX=predicted change; + + +, + +, +, 0, −, − −, and − − − indicate changes in RET as predicted from the ranking of the amino acid residues given in Table 1. + + + =greatest increase in RET; − − − =greatest decrease.

as ranked in Table 1, might serve as a helpful guide in a search for structural variations in abnormal globin chains which readily separate from their normal counterparts. The usefulness of this method to detect variant chains with neutral substitutions has been emphasized before [8], and quite a few Hb variants with a decreased stability or increased oxygen affinity have been identified after isolation of the chain by reversed-phase HPLC. Such variants had substitutions like Leu→Gln (β-Saint Louis), Leu→Met (α-Chicago), His→Gln (α-Le Lemantin); however, β chains of important unstable variants like Hb Köln (β98 Val→Met) cannot be separated from the normal β^A chain because of the near equal polarities of the substituted and introduced amino acid residues.

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8. SUMMARY

This paper evaluates various high-performance liquid chromatographic (HPLC) procedures useful for the detection and evaluation of numerous hemoglobin abnormalities in adults and in newborn infants. Two major approaches are discussed. The first concerns the analyses of hemoglobins in red cell lysates by anion- and cation-exchange chromatography; this type of HPLC has great potential in a routine laboratory, as quantitative data for numerous abnormalities can readily be obtained. The second concerns the separation of globin chains by reversed-phase HPLC that is most useful for the identification of hemoglobinopathies in adults, newborns, and fetuses.

REFERENCES

1 H.F. Bunn and B.G. Forget, Hemoglobin: Molecular, Genetic and Clinical Aspects, Saunders, Philadelphia, PA, 1986.
2 T.H.J. Huisman and J.H.P. Jonxis, The Hemoglobinopathies Techniques of Identification, Marcel Dekker, New York, 1977.
3 T.H.J. Huisman (Editor), The Hemoglobinopathies, Churchill Livingstone, Edinburgh, 1986.
4 S.M. Hanash, M. Kavadella, A. Amanullah, K. Scheller and K. Burnell, in S.M. Hanash and G.J. Brewer (Editors), Advances in Hemoglobin Analysis, Alan R. Liss, New York, 1981, p. 53.
5 T.H.J. Huisman, M.B. Gardiner and J.B. Wilson, in S.M. Hanash and G.J. Brewer (Editors), Advances in Hemoglobin Analysis, Alan R. Liss, New York, 1981, p. 69.
6 T.H.J. Huisman, B. Webber, K. Okonjo, A.L. Reese and J.B. Wilson, in S.M. Hanash and G.J. Brewer (Editors), Advances in Hemoglobin Analysis, Alan R. Liss, New York, 1981, p. 23.
7 J.B. Wilson and T.H.J. Huisman, in T.H.J. Huisman (Editor), The Hemoglobinopathies, Churchill Livingstone, Edinburgh, 1986, p. 46.
8 F. Kutlar, A. Kutlar and T.H.J. Huisman, J. Chromatogr., 357 (1986) 147.
9 T.H.J. Huisman and J.B. Wilson, in H. Peeters (Editor), Protides of the Biological Fluids, Pergamon Press, Brussels, 1984, p. 1029.
10 T.H.J. Huisman and J.B. Wilson, in M. Aksoy and T.H.J. Huisman (Editors), Proceedings of the North-Cyprus Symposium on Abnormal Hemoglobin and Thalassemia, Scientific and Technical Research Council of Turkey, Ankara, 1984, p. 22.
11 C.-N. Ou, G.J. Buffone, G.L. Reimer and A.J. Alpert, J. Chromatogr., 266 (1983) 197.
12 B.B. Roberts, R.A. Wessels, C.-N. Ou and G.J. Buffone, Am. J. Clin. Pathol., 84 (1985) 671.
13 S.M. Hanash and D.N. Shapiro, Hemoglobin, 5 (1981) 165.
14 A. Amanullah, S. Hanash, K. Bunnell, J. Strahler, D.L. Rucknagel and S. Ferruci, Anal. Biochem., 123 (1982) 402.
15 K.M. Gooding, K.C. Lu and F.E. Regnier, J. Chromatogr., 164 (1979) 506.
16 E.C. Toren, Jr., D.N. Vacik and P.B. Mockridge, J. Chromatogr., 266 (1983) 207.
17 R.M. David and Z.K. Shihabi, J. Liq. Chromatogr., 7 (1984) 2875.
18 E.C. Abraham, A. Abraham and M. Stallings, J. Lab. Clin. Med., 104 (1984) 1027

19 U.-H. Stenman, K. Pesonen, K. Ylinen, M.-L. Huhtala and K. Teramo, J. Chromatogr., 197 (1984) 327.
20 M.B. Gardiner, J. Carver, B.L. Abraham, J.B. Wilson and T.H.J. Huisman, Hemoglobin, 6 (1982) 1.
21 J.B. Wilson, M.E. Headlee and T.H.J. Huisman, J. Lab. Clin. Med., 102 (1983) 174.
22 A. Kutlar, F. Kutlar, J.B. Wilson, M.G. Headlee and T.H.J. Huisman, Am. J. Hematol., 17 (1984) 39.
23 J.B. Wilson, R.N. Wrightstone and T.H.J. Huisman, J. Lab. Clin. Med., 108 (1986) 138.
24 E.C. Abraham and T.H.J. Huisman, Hemoglobin, 1 (1977) 861.
25 A. Felice, E.C. Abraham, A. Miller, N. Cope, M. Gravely and T.H.J. Huisman, in G.J. Brewer (Editor), The Red Cell, Alan R. Liss, New York, 1978, p. 131.
26 A.E. Felice, B. Webber, A. Miller, S.M. Mayson, H.F. Harris, J.B. Henson, M.E. Gravely and T.H.J. Huisman, Am. J. Hematol., 6 (1979) 91.
27 J.B. Shelton, J.R. Shelton and W.A. Schroeder, Hemoglobin, 3 (1979) 353.
28 L.F. Congote, H.P.J. Bennett and S. Solomon, Biochem. Biophys. Res. Commun., 89 (1979) 851.
29 T.H.J. Huisman and J.B. Wilson, Am. J. Hematol., 9 (1980) 225.
30 L.F. Congote, in S.M. Hanash and G.J. Brewer (Editors), Advances in Hemoglobin Analysis, Alan R. Liss, New York, 1981, p. 39.
31 K. Shimizu, J.B. Wilson and T.H.J. Huisman, Hemoglobin, 4 (1980) 487.
32 J.B. Shelton, J.R. Shelton, W.A. Schroeder and J. DeSimone, Hemoglobin, 6 (1982) 451.
33 J.B. Shelton, J.R. Shelton, and W.A. Schroeder, J. Liq. Chromatogr., 7 (1984) 1969.
34 W.A. Schroeder, J.B. Shelton, J.R. Shelton and V. Huynh, Hemoglobin, 10 (1986) 253.
35 W.A. Schroeder, T.H.J. Huisman, J.R. Shelton, J.B. Shelton, E.F. Kleihauer, A.M. Dozy and B. Robberson, Proc. Natl. Acad. Sci. U.S.A., 60 (1968) 537.
36 G. Ricco, U. Mazza, R.M. Turi, P.G. Pich, C. Camaschella, G. Saglio and L.F. Bernini, Hum. Genet., 32 (1976) 305.
37 T.H.J. Huisman, W.A. Schroeder , A. Reese, J.B. Wilson, H. Lam, J.R. Shelton, J.B. Shelton and S. Baker, Pediatr. Res., 11 (1977) 1102.
38 W.A. Schroeder, T.H.J. Huisman, G.D. Efremov, J.R. Shelton, J.B. Shelton, R. Phillips, A. Reese, M. Gravely, J.M. Harrison and H. Lam, J. Clin. Invest., 63 (1979) 268.
39 T.H.J. Huisman, in A. Cao, U. Carcassi and P.T. Rowley (Editors), Thalassemia: Recent Advances in Detection and Treatment, Birth Defects, Vol. 18, Alan R. Liss, New York, 1982, p. 83.
40 W.A. Schroeder and T.H.J. Huisman, The Chromatography of Hemoglobin, Marcel Dekker, New York, 1980.
41 J.G. Gilman, T. Harano, T. Nakatsuji, I. Bakioglu, A.L. Reese, M.B. Gardiner and T.H.J. Huisman, Ann. N.Y. Acad. Sci., 445 (1985) 235.
42 S.H. Orkin, H.H. Kazazian, Jr., S.E. Antonarakis, S.C. Goff, C.D. Boehm, J.P. Sexton, P.G. Waber and P.J.V. Giardina, Nature, 296 (1982) 627.
43 Y. Hattori, F. Kutlar, C.J. Mosley, S.M. Mayson and T.H.J. Huisman, Hemoglobin, 10 (1986) 185.
44 W.A. Schroeder, J.B. Shelton, V. Huynh and J.R. Shelton, Hemoglobin, 10 (1986) 239.
45 P.K. Sukumaran, T. Nakatsuji, M.B. Gardiner, A.L. Reese, J.G. Gilman and T.H.J. Huisman, Nucleic Acids Res., 11 (1983) 4635.
46 T. Harano, T.H.J. Huisman, Y.T. Zeng and S.Z. Huang, Am. J. Hematol., 19 (1985) 349.
47 K. Harano, T. Harano, F. Kutlar and T.H.J. Huisman, FEBS Lett., 190 (1985) 45.
48 K.G. Yang, J.Z. Liu, F. Kutlar, A. Kutlar, C. Altay, A. Gurgey and T.H.J. Huisman, Blood, 68 (1986) 1394.
49 M. Cohen-Solal, M. Seligmann, J. Thillet and J. Rosa, FEBS Lett., 33 (1973) 37.
50 B.F. Wiedermann, K. Indrak, J.B. Wilson, B.B. Webber, K.G. Yang, F. Kutlar, A. Kutlar and T.H.J. Huisman, Hemoglobin, in press.
51 A. Kutlar, F. Kutlar, J.B. Wilson, B.B. Webber, J.M. Gonzales Redondo and T.H.J. Huisman, Hemoglobin, in press.
52 J.L. Meek, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 1632.
53 International Hemoglobin Information Center Variant List, Hemoglobin, 10 (1986) 261.