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SEPARATION OF NORMAL AND ABNORMAL HEMOGLOBIN CHAINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

F. KUTLAR, A. KUTLAR and T. H. J. HUISMAN*
Comprehensive Sickle Cell Center, Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912 (U.S.A.)
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SUMMARY

A review is presented of the elution patterns on reversed-phase columns of the normal and abnormal globin chains of different hemoglobin types, including 16 β-chain variants, 7 α-chain variants, 9 γ-chain variants, and 4 variants with fusion or hybrid chains. Separations appear to be based primarily on differences in hydrophobicity. The method is ideally suited for the detection of abnormal globin chains, their quantitation and their isolation. Semi-quantitative data based on the calculation of the δ/non-α ratios allow the detection of β-thalassemic conditions in situations where the quantitation of hemoglobin A_2 by other procedures is impossible or complicated.

INTRODUCTION

Recently Shelton *et al.*[1] described a new reversed-phase high-performance liquid chromatography (HPLC) procedure for the separation and elution of globin chains of hemoglobins (Hb) present in adult and newborn red cell lysates. The method which uses the Vydac C_4 wide-pore column and acetonitrile–water-trifluoroacetic acid developers, allows completion of the chromatogram in 60–80 min. During the past two years we have used this method in our studies of abnormal globin chains, and are summarizing our experiences in this communication.

MATERIALS AND METHODS

Blood samples

These were collected in vacutainers with EDTA as anticoagulant. Many samples were received from different investigators requesting the identification of the abnormal Hb that was present. Such studies followed procedures routinely in use in our laboratories[2,3], and the results of the structural analyses will not be presented in detail here.

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Procedures

Lysates were prepared by mixing packed red cells, washed three times with 0.9 g/dl sodium chloride solution, with an equal volume of distilled water and 0.4 volume of carbon tetrachloride. Membranes and other debris were removed by centrifugation. The clear Hb solution was stored at 4°C for not longer than three weeks, or in liquid nitrogen for an indefinite time. Separation of the globin chains followed the procedure described by Shelton *et al.*[1]. HbA_2 quantitation by microcolumn chromatography and by cation-exchange HPLC has been described before[4,5].

RESULTS AND DISCUSSION

Red cell lysates from adults and newborn babies with 36 different Hb variants have been studied. The abnormal globin chains, either α-, β-, γ- or hybrid chains, from several variants occupied specific positions in the chromatogram; presentation of the data will take the form of a review of elution patterns in a few composite drawings.

The normal adult

This chromatogram is shown in Fig. 1. The elution pattern, which is highly reproducible, shows the appearance of the heme group in about 10 min, followed in 26–28 min by a pre-β^A peak (presumably a β-globin-glutathion complex[6]), in 31–35 min by the β-chain of HbA, in 36–38 min by the δ chain of the HbA_2, and in 43–48 min by the α-chain. The small peaks between the δ and α globin chains are non-Hb proteins[6], while the two normal γ-chains (not observed in a normal adult red cell lysate) are eluted at 55–57 min ($^G\gamma$) and at 65–66 min ($^A\gamma$) (see also Fig. 4). The relative quantity of each peak can readily be calculated from the data obtained with a simple integrator attached to the HPLC equipment. Comparison of the areas for the α globin chain and the pre-β + β + δ globin chains provides a ratio between the α- and the non-α-chains which can be expected to approach 1; the average data for 10 different lysates was 0.96 ± 0.03. Slight variations in the composition of the developers will not alter the general elution pattern, but only the elution times.

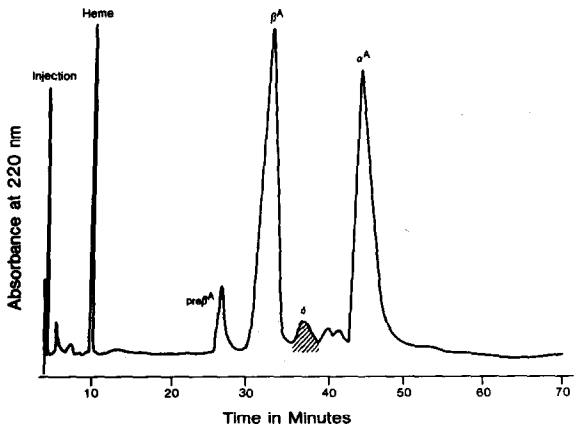

Fig. 1. Separation of the globin chains in a normal adult red cell lysate by reversed-phase HPLC.

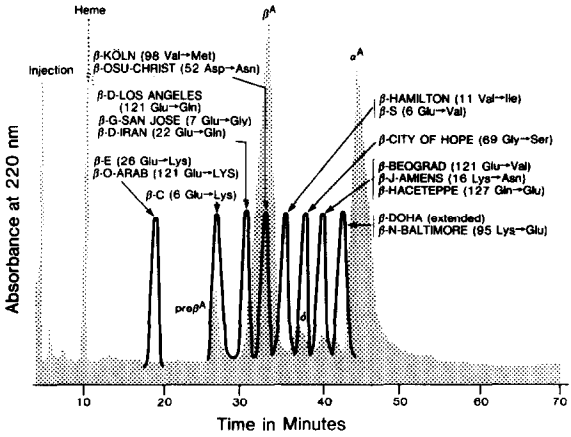

Fig. 2. The positions of abnormal β-globin chains of 16 different Hb variants in the reversed-phase HPLC chromatogram.

β-Chain variants

Sixteen different variants were analyzed (details about the variants can be obtained from the International Hemoglobin Information Center, Medical College of Georgia, Augusta, GA, U.S.A.; see also ref. 7). Of these, nine were electrophoretically slow-moving variants due to an Asp → Asn, a Glu → Gly, a Glu → Gln, a Glu → Val, or a Glu → Lys substitution. Six of the abnormal β-globin chains eluted in front of the normal β-chain (Fig. 2). The position of the substitution in the chain apparently can have an effect on the elution pattern as is evident from the differences in elution times of the β-E- and β-O-Arab-chains *versus* the β-C-chain (the three chains have a Glu → Lys substitution). The β-globin chain of Hb Osu-Christiansborg (β52 Asp → Asn) did not separate from the normal β-A-chain, while the two globin chains with a Glu → Val substitution eluted after the normal β-A-chain; the β-S-globin chain (6 Glu → Val) eluted between the β^A and δ-chains and the Hb Beograd chain (121 Glu → Val) was found between the δ- and α-chains. The increase in hydrophobicity due to the introduction of an extra valine residue apparently affects the elution pattern to a major extent. This is also evident from the data obtained for two of the Hbs with neutral substitutions; the β-Hamilton globin chain (11 Val → Ile) eluted just behind the β-A-chain while that of Hb City of Hope (69 Gly → Ser) occupied the position of the δ-chain. The β-chains of the two "fast-moving" variants J-Amiens (β16 Lys → Asn) and Hacetteppe (β127 Gln → Glu) occupied a position in the chromatograms, which was, remarkably enough, the same as that of the β-Beograd chain (β121 Glu → Val). The positions of the β-globin chains of Hb N-Baltimore (β95 Lys → Glu) and of Hb Doha (an extended β-chain with an acetyl-Met-Glu-His sequence[8]) were as expected.

The level of HbA_2

The complete separation of the β- and δ-globin chains allowed quantitation of HbA_2 in red cell lysates through a calculation of the percentages of the δ-chains in the total non-α-chains (*i.e.*, pre-β + β^A + β^X + δ). This was particularly of interest for samples containing Hb variants such as HbC, HbE, and HbO-Arab, which are

TABLE I

THE QUANTITIES OF HbA_2 IN SOME NORMAL AND ABNORMAL CONDITIONS DETERMINED BY VYDAC C_4 REVERSED-PHASE HPLC

N.D. = Not determined.

Condition	*n*	$\frac{100 \cdot \delta}{(\delta + \beta^A + \beta^X)}$	*HbA_2*	
			Microcolumn chromatography (ref. 4)	*Cation-exchange HPLC (ref. 5)*
Normal	10	3.8 ± 0.3 (3.2–4.3)	2.6 ± 0.5 (1.9–3.6)	N.D.
A-β-Thal	2	4.8–5.6	4.2–4.5	N.D.
AE	5	4.05 ± 0.9 (2.4–5.0)	N.D.	N.D.
EE	12	5.3 ± 0.45 (4.4–6.1)	N.D.	N.D.
E-β^0-Thal	4	8.6 ± 1.0 (7.2–10.0)	N.D.	N.D.
CC	11	3.0 ± 0.45 (1.6–4.6)	N.D.	3.7 ± 0.75 (2.1–4.6)

difficult to separate from HbA_2 by electrophoresis, isoelectrofocusing, or by some types of ion-exchange chromatography. The data are listed in Table I; whenever possible, the levels were compared with those obtained with other procedures. In general, the HbA_2 values were significantly higher than those by microcolumn chromatography perhaps because of the relatively high background in the small δ-globin chain peak (see Fig. 1). However, the increase in HbA_2 level, known to occur in patients with β-thalassemia, was readily observed. HbA_2 levels were elevated in patients with homozygous HbE disease (average values 5.3% as compared to 3.8% for normal controls) but were significantly less than those seen for patients with HbE-β^0-thalassemia (average 8.6%).

The hybrid Hb chains

Four such globin chains were studied, namely the two $\delta\beta$-chains of Hb Lepore-Boston-Washington (crossover between positions 87 and 116) and of Hb Lepore-Baltimore (crossover between positions 50 and 86), the $\beta\delta$-chains of Hb P-Nilotic (crossover between residues 22 and 50), and the $\gamma\beta$-hybrid chain of Hb Kenya (crossover between residues 81 and 86) (for references see ref. 7). The positions of the chains in the chromatogram, shown in Fig. 3, were about as expected. The position of the $\beta\delta$-hybrid chain of Hb P-Nilotic suggests that the carboxy-terminal segment of the δ-chain with its Thr → Ser, Ala → Ser, Thr → Gln, His → Arg, His → Asn, Pro → Gln, and Val → Met substitutions at positions 50, 86, 87, 116, 117, 124, and 126 ($\beta \rightarrow \delta$) is responsible for the increased hydrophobicity of the δ chain while the Ser → Thr, Thr → Asn, and particularly the Glu → Ala replacements at positions 9, 12, and 22 ($\beta \rightarrow \delta$) result in a faster elution of the $\delta\beta$-hybrid chains of Hb Lepore.

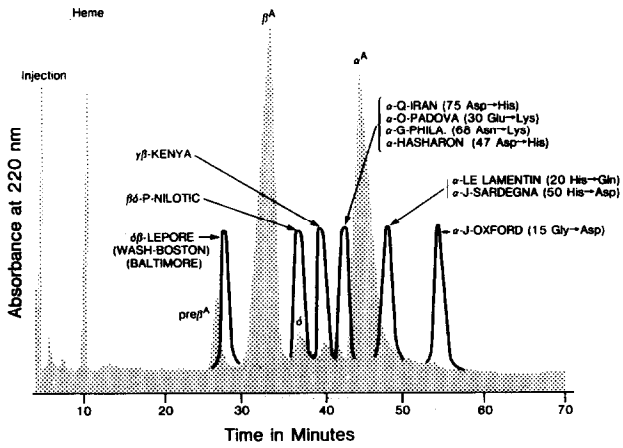

Fig. 3. The positions of four hybrid globin chains ($\delta\beta$, $\beta\delta$, $\gamma\beta$) and seven abnormal α-globin chains in the reversed-phase HPLC chromatogram.

α-Chain variants

Of the seven abnormal hemoglobins analyzed, four were slow-moving variants with Asp → His, Asn → Lys, or Glu → Lys substitutions. The α-globin chains of these four eluted ahead of the normal α chain (Fig. 3). The α^X-chains of the three fast-moving variants eluted later than the normal α-A-chain; substitution of a glycine residue in the α-J-Oxford chain by the much larger aspartic acid residue greatly increased the elution time of this variant chain.

γ-Chain variants

Two different series of variants have been discovered; in one, substitutions occur in the $^G\gamma$-chain with glycine at position γ-136 while in the other these are present in the $^A\gamma$-chain with alanine at position γ-136. The $^A\gamma$- and $^G\gamma$-chains are the products

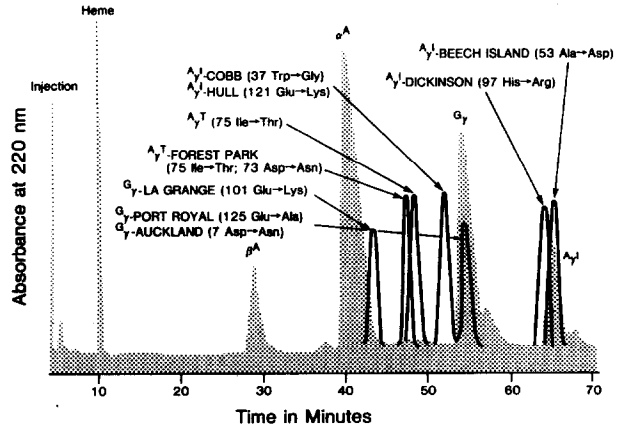

Fig. 4. The positions of the γ-globin chains of several $^A\gamma$ and $^G\gamma$ variants in the reversed-phase HPLC chromatogram.

of two non-allelic genes and are present in the blood of all normal newborn babies (for a review see ref. 9). The $^{G}\gamma$- and $^{A}\gamma$-globin chains are readily separated from each other; the replacement of the glycine residue by the alanine residue apparently increased the hydrophobicity of the $^{A}\gamma$-chain considerably, resulting in an increased elution time (Fig. 4). A most common variant of the $^{A}\gamma$-chain is the $^{A}\gamma^{T}$ in which the isoleucine residue at $^{A}\gamma$ 75 is replaced by a threonine residue. This substitution decreases the hydrophobicity of the $^{A}\gamma$-globin chain considerably. This and other $^{A}\gamma$-chain variants are listed in Fig. 4. Substitutions such as Ala → Asp (F-Beech Island), His → Arg (F-Dickinson), and Asp → Asn (the $^{A}\gamma^{T}$ chain of F-Forest Park) did not change the chromatographic mobility of the γ-globin chain to any major extent, while the Trp → Gly (F-Cobb) and Glu → Lys (F-Hull) substitutions had the expected effect. Similar conclusions can be drawn from the mobilities of the three $^{G}\gamma$-chain variants; the Glu → Lys substitution in the γ-chain of Hb F-LaGrange resulted in a much faster elution while the Glu → Ala and Asp → Asn substitutions in the γ-chains of F-Port Royal and F-Auckland did not effect mobilities of the altered γ-globin chains.

CONCLUSIONS

The reversed-phase HPLC method, developed by Shelton *et al.*[1], has many applications in hemoglobin research. It allows the separation of numerous abnormal α-, β- and γ-globin chains, even those with neutral substitutions, thus allowing their initial detection. The separation of closely related proteins appears to be primarily based upon differences in hydrophobicity; the replacement, for instance, of a glycine residue by serine (β-City of Hope), by aspartic acid (α-J-Oxford), or by alanine (the $^{A}\gamma$ *versus* the $^{G}\gamma$-chain) greatly increases the elution times of the abnormal Hb chains. The position of the substitution in the globin chain also plays a role as is evident from the different elution times of the β-chains of HbE (26 Glu → Lys) and HbC (6 Glu → Lys), and of HbS (6 Glu → Val) and Hb Beograd (121 Glu → Val). Quantitative data obtained with this method have been widely used to evaluate the percentages of the $^{G}\gamma$- and $^{A}\gamma$-chains in numerous cord blood samples (refs. 10 and 11 and references quoted) while the (semi) quantitative data presented in Table I are useful to establish the possible presence of a thalassemia condition. Lastly, the method readily allows the isolation of an aberrant globin chain for structural analyses; it often is necessary to run several chromatograms to obtain sufficient material.

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