Separation of Adult Chains of Abnormal Haemoglobin: Identification by Reversed-Phase High-Performance Liquid Chromatography

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Rare abnormal haemoglobin (Hb) often poses a problem in the diagnosis of relative electrophoresis mobility. The rare neutral Hbs interact with the Hb S and thus can increase the severity of sickle cell anaemia. In the present study, we investigated the use of reversed-phase high-performance liquid chromatography (RP-HPLC) as a method for the identification of neutral or unstable Hb, and compared it with the well-known techniques urea-triton electrophoresis (UT) and isoelectrofocusing

(IEF). The results of our experiments showed that Bucuresti Hb was separated without ambiguity by RP-HPLC. To date, no other electrophoresis techniques have been able to isolate this Hb. We also examined the Hb Nouakchott, which was the most hydrophobic Hb. Moreover, the adult and foetal chains of globins (especially the A γ^t and A γ^l chains) were quantified on the same chromatogram. J. Clin. Lab. Anal. 18:65–69, 2004.

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INTRODUCTION

Many investigators have used electrophoresis techniques to separate globin chains. Moreover, reversed-phase high-performance liquid chromatography (RP-HPLC) has been used to separate several types of chains. These methods of analysis appear to be useful for the diagnosis of sickle cell anaemia (1–3), and have been extended to the separation of globin chains in adults (4). Some profiles of expression appear to have shapes that are clinically better tolerated than others (5,6). Several methods had been proved to be perfectly congruent for the separation of chains of globins. In the present study, we used Leonie et al.'s (7) method as modified by Groff et al. (8). This method has a good power of resolution to analyse rare abnormal haemoglobins (Hbs), since no species had been separated by the use of conventional electrophoresis methods.

The aim of this study was to evaluate RP-HPLC in comparison with isoelectrofocusing (IEF) and urea triton (UT) X-100 electrophoresis for the identification

of rare, abnormal variants that are difficult to separate, and the quantification of neutral or unstable Hbs.

MATERIALS AND METHODS

A collection of 100 mutant Hbs conserved in the Laboratoire des Hemoglobines, Hôpital Henri Mondor, were either characterised in a precise way or identified in a presumptive way by electrophoresis, as described by Schneider and Barwick (9). These methods were chosen for the analysis of globin chains. In our study, a Lichrospher 100 RP8 column (Merck, Darmstadt, Germany) grafted in C8 of spherical particles of 5 μ M,

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100 Å of porousness, was set up at the beginning of the column. The column thermostat was stabilised at 45°C. The techniques used for structural characterisation were as previously detailed by Wajcman et al. (10). We used the RP-HPLC technique described by Yapo et al. (11) to analyse the abnormal chains.

First, 5 µL of red blood cell sediment was haemolysed in 1 mL of distilled water. After centrifugation to 1,200 rpm for 15 min, 20 µL of dithiotrietol (dt) at 1 mg/mL were added to 100 µL of haemolysate. The dt protected the thiol groups, and 25 µL and 50 µL of haemolysate were employed for injection to the column. Solvent A (acetonitrile-methanol-NaCl [0.143 M adjusted to pH 2.7 by HCl 1N] 24V/38V/36V) and solvent B (acetonitrile-methanol-NaCl [0.143 M adjusted to pH 2.7 by HCl 1N] 55V/6V/39V) were employed for the elution.

A linear gradient from 10% to 70% of solvent B was used for 90 min at 0.7 mL/min of flow. The detection was performed at a 280-nm wavelength, since no important variation of the baseline was observed.

Chemicals

Acetonitrile and methanol were purchased from Hoechst AG (Darmstadt, Germany). The dt was purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals were reagent grade.

Sample Analysis

The sample analysis was made comparatively with a standard solution of β and α globin chains.

The arbitrary mobilities (on a linear part of the gradient) of the β^A and α^A chains were 10 and 20, respectively. For α and β , the chain mobility was calculated with regard to the β^A chain as follows:

$$N = \frac{\text{distance from } \beta^A \text{ to X of globin chain mutated}}{\text{distance from the } \beta^A \text{ chain to } \alpha^A \text{ chain} \times 10} + 10$$

If X was eluted before the β^A chain, X mobility was <10. However, if X was eluted after the β^A chain, its mobility was >10.

We analysed the values using GraphPad software (GraphPad Software Inc., San Diego, CA). All values in the text and illustrations are presented as means. We evaluated significant differences ($P < 0.05$) between the groups by Student's *t*-test.

RESULTS

Figure 1 illustrates the RP-HPLC chromatogram of the β chain of Hb Porto Alegre (β^9 Ser $\rightarrow$ Cys) in the presence and absence of dt. The addition of dt in our haemolysate provided a good separation of the Hb Porto Alegre. This product protected the thiol group of globins by preventing them from oxidizing. Hb Porto

Fig. 1. RP-HPLC chromatographic profile of the Hb Porto Alegre: analysis of the haemolysate containing Hb Porto Alegre in the (a) presence and (b) absence of dt.

Alegre was eluted with the dtt in a conventional way just after the β^A chain. The migration depended on the hydrophobicity of the cys in the experimental condition defined previously by Yapo et al. (11). In comparison with dtt-treated chains, the elution of the Hb Porto Alegre was not only delayed, but was also carried out after the α^A chain in the absence of dtt. The experiments revealed the importance of dtt in our haemolysate for masking potential, unknown globin variants in the system.

The relative mobilities of common abnormal Hb were obtained by three methods (UT, IEF, and RP-HPLC) and compared (Table 1).

The three methods were also employed for the identification of frequent pathologic Hb. The comparison of values of the relative mobilities of rare neutral β mutants, which are difficult to separate with conventional electrophoresis systems (e.g., UT and IEF), and those obtained by RP-HPLC are shown in Table 2.

All of the Hbs had the same migration value in UT electrophoresis (values = 20). Hb Bucuresti was not observed with the IEF and UT techniques, but it was clearly detected by the use of RP-HPLC on a Lichrospher column (Merck, Darmstadt, Germany (Fig. 2).

Similar results were observed in the rare α mutant group (Table 3). Hb Fontainebleau, Hb Lisbon, Hb Bois-Guillaume, and Hb Aztec were not distinguishable in UT electrophoresis (values = 10). Furthermore, Hb Fontainebleau had the same mobility as Hb Lisbon in IEF (values = 18–19.5). All variants that were undetected by the UT or IEF techniques were both identified and clearly separated by RP-HPLC. The latter technique, which used a Lichrospher column, separated the abnormal Hb with a good power of resolution (Tables 2 and 3).

The latter observations required a precise identification by a complementary structural study. RP-HPLC and electrophoresis analysis of globins used to detect them and to distinguish one from another. Furthermore, this method was used to identify foetal Hb in the same chromatogram.

DISCUSSION

In general, under physiological conditions, proteins have a preferential configuration. Unfortunately, no

Table 1. Electrophoresis parameters of common abnormal Hb observed UT, IEF, and RP-HPLC

Places	Mutation	Name	RP-HPLC	IEF	UT
6	Glu → Lys	C	7.1	−16	16.3
6	Glu → Val	S	11.4	−8.5	19.4
26	Glu → Lys	E	3.5	−15.1	11.9
121	Glu → Gln	D Punjab	8.6	−7.6	19
121	Glu → Lys	O Arab	3.7	−15.1	17

Table 2. Electrophoresis parameters of rare neutral β mutants difficult for separation in UT and IEF*

Places	Mutation	Name	RP-HPLC	IEF	UT
28	Leu → Pro	Genova	6.5	−0.1	20
42	Phe → Leu	Bucuresti	12.8	—	—
67	Val → Ala	Sydney	7.7	−0.5	20
76	Ala → Pro	Calais	5.7	1.9	20
86	Ser → Asn	Créteil	8.6	1.6	20
89	Ser → Thr	Villaverde	11.5	0.01	20
103	Phe → Ile	St Nazaire	13.1	0.1	20
124	Pro → Gln	Ty Gard	17.4	1.2	20
124	Pro → Ser	Tunis	13.4	0.01	20
126	Val → Ala	Beiruth	4.9	0.4	20
128	Ala → Pro	Mt St Aignan	4.3	1.3	20
134	Val → Ala	Yaoundé	11.7	0.0	20

*Values are given for the relative mobilities of globins for β chains.

Fig. 2. RP-HPLC chromatographic profile of Hb Beiruth and Hb Bucuresti: comparative study of both Hbs.

Table 3. Electrophoresis parameters of rare neutral α mutants difficult for separation in UT, IEF*

Places	Mutation	Name	RP-HPLC	IEF	UT
21	Ala → Pro	Fontainebleau	18	1.2	10
23	Glu → Asp	Lisbon	19.5	1.2	10
65	Ala → Val	Bois-Guillaume	20.3	−0.7	10
76	Met → Thr	Aztec	16.3	−0.7	10
114	Pro → Leu	Nouakchott	57.9	−3.3	4.5

*Values are given for the relative mobilities of globins for β chains.

physiological conditions must often be used during protein isolation. Thus, the selected protein may be denatured and require refolding to regain the native structure (12). We recommend the use of dtt because it provides good resolution for the isolation of Hb Porto Alegre and Hb S ($\beta 9$ Glu $\rightarrow$ Val).

The use of RP-HPLC, the most frequent abnormal Hb with mutations on codon 6 on β chain like Hb S (Glu $\rightarrow$ Val) Hb C (Glu $\rightarrow$ Lys) or on codon 21 on β chain like Hb D Punjab (Glu $\rightarrow$ Gln), Hb O arab (Glu $\rightarrow$ Lys), didn't superpose among themselves or to other variants of globins. Three variants (Hb C, Hb O, and Hb E) have a [Glu $\rightarrow$ Lys] substitution and were observed at different elution times. The comparison of the techniques showed that RP-HPLC performs better than UT or IEF, as previously reported (1,3,4,13), and that it offers good separation and quantification of normal and abnormal Hbs.

Tables 2 and 3 show the results from the study of relative mobility by RP-HPLC for the rare variants that

are difficult to separate by UT and IEF. Our study provides additional information for the diagnosis of rare Hbs and the quantification of unstable Hbs. Some of the rare neutral mutants are present in African populations and interact with major sickle cell anaemia (5). These interactions (such as with Hb S Antilles [6 Glu $\rightarrow$ Val, 23 Val $\rightarrow$ Ile]) may increase the severity of sickle cell anaemia. Other rare neutral mutants, such as Hb C Harlem (6 Glu $\rightarrow$ Val, 73 Asp $\rightarrow$ Asn), can lessen the severity of sickle cell anaemia. RP-HPLC was able to separate without ambiguity foetal chains ($A\gamma^1 A\gamma^1$, $G\gamma$) that were imperceptible by UT electrophoresis. RP-HPLC was also useful for quantifying the three most common types of human Hb F chains in a single analysis. The heterogeneity of Hb F with sickle cell anaemia was described in a previous study (2). We were also able to separate the Hb Nouakchott ($\alpha 114$ Pro $\rightarrow$ Leu) (Fig. 3) by the same method. This was the most hydrophobic Hb, as previously demonstrated by Wacjman et al. (15).

Fig. 3. RP-HPLC chromatographic profile of Hb Nouakchott.

Recently, we proposed the use of RP perfusion chromatography for chain analysis as a complementary tool for the characterization of Hb neutral variants. This method offers the same sensitivity as polyacrylamide gel IEF. Moreover, RP-HPLC can identify abnormal adult Hb in a blood sample obtained in a neonatal screening program. Electrophoresis parameters of rare neutral α mutants (Hb Fontainebleau, Hb Lisbon, Hb Bois-Guillaume, and Hb Aztec) were difficult to separate with other techniques (UT and IEF; Table 3).

The use of RP-HPLC, under well-controlled experimental conditions, should answer many questions. This technique is not only a useful tool for the characterization of Hb variants, it is also more effective than other methods (e.g., electrophoresis) for the quantification of HbA2 through α chain measurements in patients carrying HbE.

Another advantage of this method is that it provides good separation of the Bucuresti Hb (β 42 Phe $\rightarrow$ Leu) (Fig. 2), which has not been achieved heretofore. In a previous study, Von Planta et al. (16) reported that only half of the clinically significant unstable Hbs could be detected by IEF. It is important to detect this mutation because the Phe residue at 42 position on chain appears to be highly conserved through species, suggesting that it has an important role in Hb function. Phe acts as a pacer in the heme pocket, and supports the heme group in an inclined position corresponding to the oxy structure. Furthermore, close-fitting contacts of this Phe-residue prevent water invasion into the heme pocket, resulting in a drop out of the heme group. Thus, the stability and oxygen-binding functions of the Hb are altered, depending on the size and hydrophobicity of the substituted amino acid (16).

The present study showed the advantages of RP-HPLC analysis, including improved separation of Hb, which is difficult to achieve with electrophoretic systems such as UT and IEF. This method also allows the dosage of neutral mutants and unstable Hbs.

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