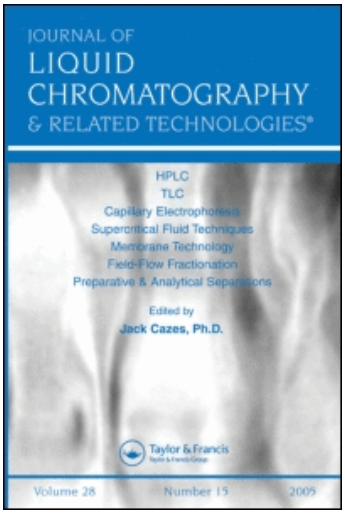

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HYDROPHOBIC INTERACTION CHROMATOGRAPHY IN THE DETECTION OF HEMOGLOBINOPATHIES

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ABSTRACT

We describe a rapid method of high-performance liquid chromatography that utilizes the principle of hydrophobic interaction to separate the mutant globin ß chains found in certain hemoglobinopathies. We used this method to analyse blood globin chains in patients with all the known diagnostized hemoglobinopathies found in Finland, that is $Hb_{Helsinki}$, $Hb_{Linköping}$, Hb_{Vaasa}, $Hb_{Hijiyama}$ and $Hb_{Syracuse}$. As reference we also analyzed normal adult and fetal blood hemoglobins. Our method allowed good separation of the mutant ß globin chains from intact chains in the heterozygous patients as well as from α globin chain and fetal γ globin chains. We used two different systems of liquid chromatographic columns for the analyses, each

column giving a slightly different elution selectivity and complementing the other. The analyses we describe are rapid and automated and allow hemoglobinopathies to be diagnosed precisely and, owing to the different principles of separation, complement other methods in common use.

INTRODUCTION

Hemoglobinopathies are a group of hematological disorders in which the presence of structurally abnormal hemoglobins (Hb) may cause alterations in red blood cell (RBC) function and morphology, accompanied by definite clinical manifestations. The molecular basis for hemoglobinopathies can mostly be explained by point mutations and more rarely by deletions, inversions or other disorders in gene coding some of the globin chains resulting in a changed amino acid sequence of the mature globin protein (1). The abnormal primary structure may change the tertiary structure of hemoglobin, thus altering physical properties such as stability, oxidation, oxygen affinity or solubility.

Methods for diagnosing of hemoglobinopathies involve analysis of the oxygen dissociation curve or electrophoresis on cellulose acetate or on agarose, or isoelectric focusation (2-6). Electrophoresis and isoelectric focusation are useful techniques for screening the electrophoretically changed hemoglobins. Some of the known hemoglobinopathies such as $Hb_{Linköping}$, however, are electrophoretically silent (4). For certainty, diagnosis of hemoglobinopathies generally requires a combination of methods.

Recently, high-performance liquid chromatography (HPLC) has become a valuable tool in protein and peptide chemistry, allowing for rapid and sensitive separation and isolation of various biomolecules (7). HPLC with ion-exchange chromatography (based on a principle utilizing weak cation exchangers) has been used to analyze the variants of hemoglobin (8-10). Separations of a number of both the α and ß chain variants of hemoglobin have been

demonstrated using these chromatography supports by gradient elution within 30 to 60 minutes (8-10).Reversed phase-technique (RP-HPLC) on a C18 column has been used to separate various globin chains (11,12,13), but there have been difficulties resolving the various chains and the chromatographic runs have been rather lengthy. The applicability of HPLC in detecting mutant Hb chains in hemoglobinopathies has been reported by Leone et al. (14) who described a RP-HPLC method involving C8 phase for separation of the normal Hb chains and some mutant chains, and Jeppson et al. (4), who used a new C1/C8 phase to separate intact ß chain from mutant chain in $Hb_{Linköping}$.

The aim of this study was to analyze globin chains in the red blood cells of patients with all the known Finnish hemoglobinopathies, using HPLC and based on hydrophobic interaction chromatography. To obtain diagnostic chromatographic profiles for the mutant globins, we used to different HPLC columns and two HPLC systems.

MATERIALS AND METHODS

Materials

HPLC grade acetonitrile and methanol as well as the other chemicals used, were supplied by E. Merck AG (Darmstadt, F.R.G). Water was purified by Millipore^{R} Q-system.

METHOD 1

Sample preparation

Peripheral blood samples were taken from normal adults and infants and from patients who are heterozygous for $Hb_{Helsinki}$,

Hb_{Vaasa}, $Hb_{Linköping}$, $Hb_{Syracuse}$, and $Hb_{Hijiyama}$. Heparinized or EDTA blood samples were centrifuged (10 min, 3000 g) and the cells were washed three times with 0.9 % NaCl (3 x blood cell volume). Cells were lysed with Millipore water (3 x blood cell volume), agitated with a horizontal shaker for 30 min and kept in chilled water for 30 min. Hemolyzate was centrifuged (30 min, 4°C, 4000 g) and the Hb concentration of supernatant was adjusted to about 10 g/l with Millipore water. Samples were used immediately for analyses.

Chromatography

For chromatographic separation, a Perkin-Elmer Series 4 liquid chromatograph (Norwalk, CT, USA) fitted with a P-E ISS-100 autoinjector, a P-E LC-95 variable UV-detector, and LCI-100 integrator were used. Blood lysates (5 µl) were injected in onto a single C_8 7 µm reversed-phase column (MN Nucleosil 7 C8, 250 x 4 mm, without end capping, Düren, FRG).

The best separation of globin chains was achieved with a gradient of 0.155 M sodium chloride, pH 2.7 - methanol - acetonitrile - 0.077 M sodium chloride, pH 2.7 (1:33:26:41 at the beginning and 10:23:42:25 at the end of the run). The temperature of the column was adjusted to 48°C and the flow rate was 1.0 ml/min. The eluted globin peaks were detected at 215 min.

METHOD 2

Sample preparation

Heparinized blood (2.0 ml) was centrifugated (10 min, 3000 g), and the cells were washed four times with 4.0 ml of 0.9 % NaCl. To induce hemolysis, 3.0 ml of water and 1.25 ml of carbon tetrachloride were added. After centrifugation (30 min, 3500 g), the supernatant solution was stored at -70°C. Before assay, the

hemolysate was diluted 1:40 with the elution solvent A; and after 30 min, when the heme was dissociated and the chains were disaggregated, 20 µl of diluted hemolysate was injected into the column.

Chromatography

For chromatographic separations, a Varian 5000 Liquid Chromatograph with variable wavelength UV-100 detector (Palo Alto, CA, USA) was used. Globin chains were separated by RP-HPLC on a Pharmacia (Uppsala, Sweden) HR 5/10 proRPC-column (C_1/C_8, particle size 5 µm). A modification of a previously described elution system was used (4). Samples were eluted with a 30 min linear gradient from 0 to 80 % solvent B at a flow rate of 0.4 ml/min. Solvent A was 39 % acetonitrile-0.3% trifluoroacetic acid, and solvent B was 50 % acetonitrile. Globin chains were detected at 280 nm.

RESULTS

Normal hemoglobin chains

The methods described above were applied to study Hb chains from normal adults, newborn babies and from persons with different hemoglobinopathies (Table I).

The major hemoglobins of a normal adult are HbA ($\alpha_2\beta_2$, more than 90 %), HbA_2 ($\alpha_2\delta_2$, less than 2 %) and HbF ($\alpha_2\gamma_2$, less than 2 %). Newborn babies normally have HbF as the major form of hemoglobin. It rapidly falls to normal adult level within the first year of life.

Figure 1. shows the chromatograms of the Hb chains of a normal adult HbA homozygote person with the major α and ß chains.

Table I. Hemoglobinopathies found in Finland and the respective ß chain amino acid mutations.

Hb variant	Amino Acid mutation	Reference
$Hb_{Helsinki}$	ß 82 Lys → Met	2
$Hb_{Hijiyama}$	ß 120 Lys → Glu	5
$Hb_{Linköping}$	ß 36 Pro → Thr	4
$Hb_{Syracuse}$	ß 143 His → Pro	6
Hb_{Vaasa}	ß 39 Gln → Glu	3

The chains are well separated from each other, and from the G_γ and A_γ chains of a newborn baby. Retention times of the various chains are presented in Table II. Free heme eluted at about 7.2 min and 12.0 min on the Pharmacia and Nucleosil columns respectively. The between-day coefficients of variation (CV) of the absolute retention times were less than 3.7 % for the various globin chains.

Hb chains in hemoglobinopathies

All the hemoglobinopathies found so far in the Finnish population are mutations of the globin ß chain (2-6), and all the patients with these mutations are heterozygous. That is, about half of their globin ß chains are normal (Table I). Figure 2 presents chromatograms of the globin chains in various hemoglobinopathies using the Nucleosil C8-column. The retention times for the various chains are shown in Table II. This system gave good separation and resolution for all the chains, with the exceptions of Hb_{Vaasa} and $Hb_{Hijiyama}$, in which the mutant ß chain coeluted with the α chain. The presence of an abnormal Hb was, however, evident because of an asymmetric α peak together with a

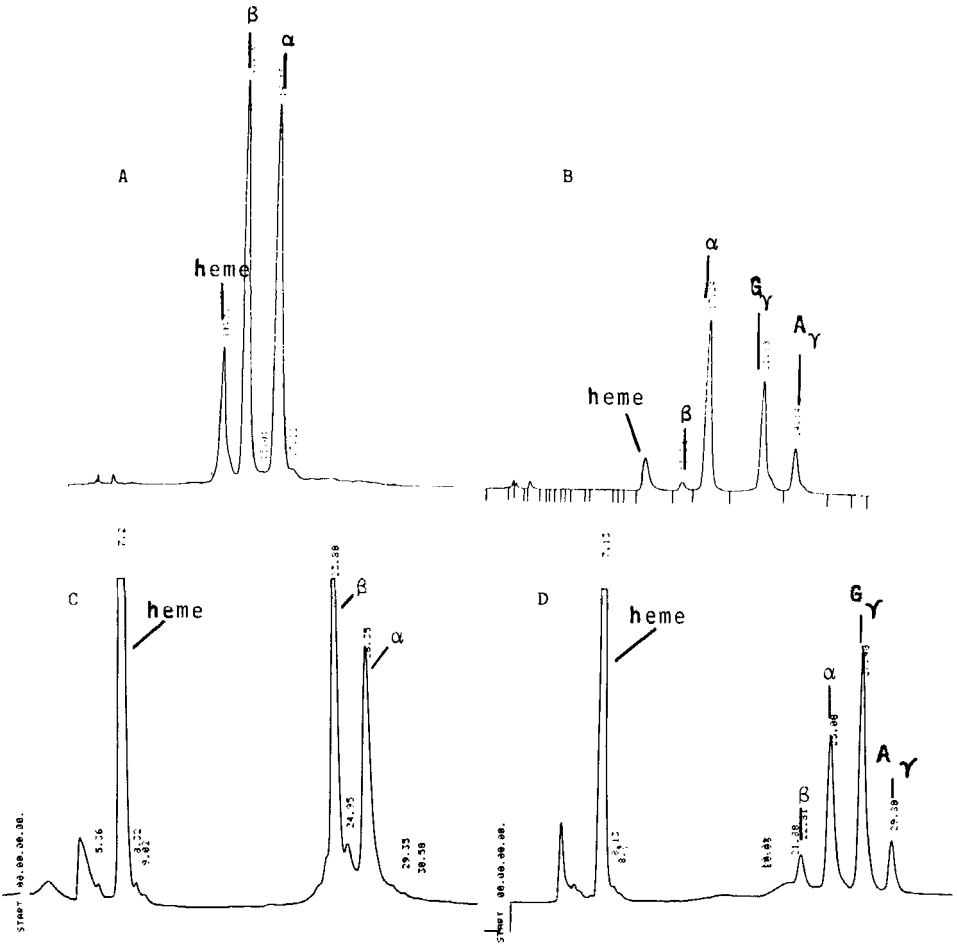

Fig. 1. Separation of different globin chains in red cell lysates by hydrophobic interaction chromatography. Chromatograms A and C represent elution of Hb chains from a normal adult HbA homozygote, and B and D Hb chains from a new-born baby. The columns used were Nucleosil C8 in A and B, and Pharmacia C1/C8 in C and D (for details see text).

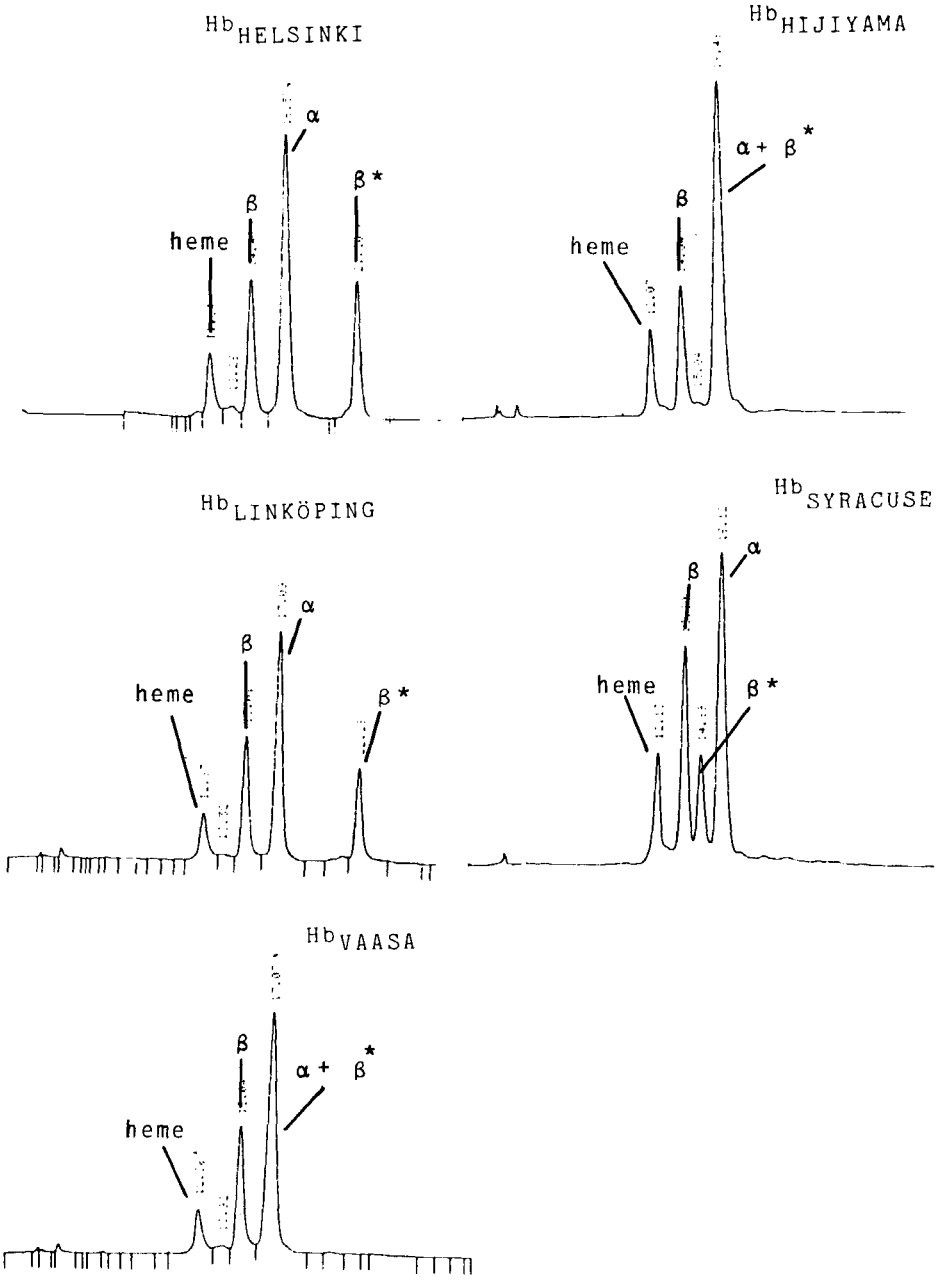

Fig. 2. Separation of different globin chains in red cell lysates of patients with different hemoglobinopathies, as indicated in the figure. The Nucleosil C8 column was used.

Table II. Retention times (t_R) of the various globin chains and relative retention times (r_R) of the globin chains normalized to α-chain in the HPLC systems used.

Hb variant	chain	Nucleosil C8		Pharmacia C1/C8	
		t_R	r_R	t_R	r_R
HbA	α	17.04	1.000	26.35	1.000
	ß	14.96	0.878	23.88	0.906
HbF	α	17.24	1.000	25.34	1.000
	G_γ	21.60	1.253	27.87	1.100
	A_γ	24.14	1.400	30.07	1.187
$Hb_{Helsinki}$	α	16.57	1.000	25.87	1.000
	ß	14.43	0.870	23.53	0.909
	ß*	21.20	1.279	28.16	1.088
$Hb_{Hijiyama}$	α	16.48	1.000	26.03	1.000
	ß	14.04	0.852	23.83	0.915
	ß*	16.48	1.000	25.20	0.968
$Hb_{Linköping}$	α	17.09	1.000	26.37	1.000
	ß	15.01	0.878	24.14	0.915
	ß*	22.28	1.304	29.57	1.121
$Hb_{Syracuse}$	α	16.12	1.000	26.03	1.000
	ß	13.80	0.856	23.73	0.912
	B*	14.89	0.924	23.73	0.912
Hb_{Vaasa}	α	17.07	1.000	26.29	1.000
	ß	15.04	0.881	23.99	0.913
	ß*	17.07	1.000	24.72	0.940

α, ß and $^{G}\gamma$ and $^{A}\gamma$ denote for the normal globin chains and ß* for the mutant ß chain.

reduced ß peak. Hb_{Vaasa} and $Hb_{Hijiyama}$ were separated better in the Pharmacia column system.

In Figure 3 are chromatograms obtained using the Pharmacia C1/C8 column system for the aforementioned patients, and retention times for the various chains are shown in Table II. This system separated all the chains, the only exception being $Hb_{Syracuse}$, in which the mutant ß chain coeluted with the intact ß chain.

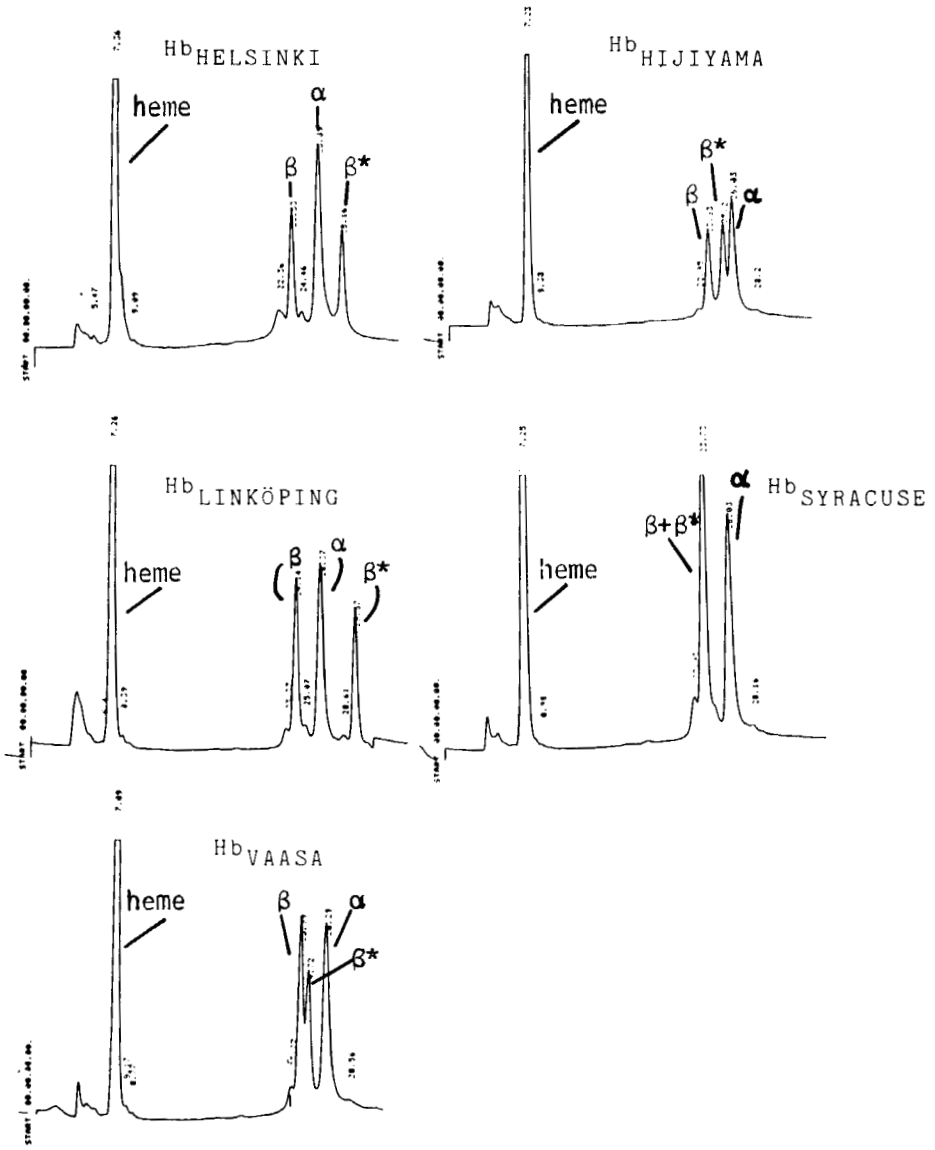

Fig. 3. Separation of different globin chains in red cell lysates of patients with different hemoglobinopathies, as indicated in the figure. The Pharmacia C1/C8 column was used.

The Pharmacia C1/C8 column system clearly gave different resolution for the various mutant chains compared to the Nucleosil C8-column. The Nucleosil column had longer relative retention times for the mutant ß globins when normalized to the α globin (Table II). Both chromatographic systems were needed for complete separation of the various Hb ß chains.

DISCUSSION

Hydrophobic interaction chromatography refers to the interaction between the hydrophobic domains of the solute protein and the bonded phase that, in this case, was either C8 in the Nucleosil column or C1/C8 in the Pharmacia column. In addition, hydrophilic interactions can be expected between the polar aminoacids of the globin chains and the free silanol groups since at least the Nucleosil column packing has no end-capping. Therefore, the C8 phase contains free unsubstituted silanol groups. The retention mechanism of the globin chains is thus not purely due to hydrophobic interaction (14). Compared to the Pharmacia column, the Nucleosil column clearly had longer relative retention times for the mutant globin ß chains.

The retention times of our chromatographic system involving the Nucleosil column are about half of those described by Leone et al. (14) and about one-third of those described by Jeppson et al. (4). We used an elution rate of 1 ml/min compared to the 0.8 ml/min (14) or 0.2 ml/min (4) used in these previous studies. The resolution and elution order of the various globin chains are essentially similar to the earlier results (4,14).

The hemoglobinopathies studied here involve mostly hydrophobic substitutions, as illustrated in Table I. This was also comfirmed in our chromatographic systems by increased retention times for the mutant chains. The only exception was

that the Pharmacia column failed to separate the mutant and normal ß chains in $Hb_{Syracuse}$. Both chromatographic systems were thus needed to completely separate the various Hb ß chains.

In conclusion, hydrophobic interaction chromatography allows for rapid, specific and sensitive separation of various normal and mutant globin chains and is suitable for routine use in diagnosing hemoglobinopathies. Because of the different separation principle, the method complements well electrophoretic techniques and other chromatographic methods used in the detection of hemoglobinopathies.

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