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Sensitive detection of human globin chains by microbore high-performance liquid chromatography and its forensic application

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Abstract

This paper describes a simple and sensitive high-performance liquid chromatographic (HPLC) method for the detection of human globin chains in blood and bloodstains. The method involves direct injection of the filtered samples of dilute hemolysates or bloodstain extracts onto a microbore C₄ reversed-phase column (2.1 mm I.D.) with UV detection at 220 nm. Microbore HPLC offers a significant improvement in sensitivity with little loss of the resolution of globin chains and only small variations in the determination of γ chain composition. The detection limit of hemoglobin (Hb) was 0.1 μg , which is equivalent to about 1 nl of fresh whole blood. Umbilical cord blood could be differentiated from adult blood in stains that were up to twenty weeks old, by the presence of γ globin chains. The present method will be useful for detection of abnormal Hbs and for the determination of γ chain composition in clinical laboratories, as well as in the practice of forensic science for the analysis of minute amounts of blood and bloodstains.

Keywords: Hemoglobin; Globin chains

1. Introduction

High-performance liquid chromatography (HPLC) has become a generally applied tool for the separation, purification and quantification of proteins from biological materials. Relatively large volumes of a diluted protein solution can be separated and concentrated with gradient elution on ion-exchange or reversed-phase columns. For analytical applications the sensitivity of detection can be increased by using lower flow-rates, but in order to maintain the separation efficiency the gradient time has to be increased concomitantly, which results in much

longer analysis times [1]. Increasing attention is focused on the use of microbore HPLC for a wide variety of applications in analytical chemistry [2–4] and biochemistry [1,5–7], due to several inherent advantages that it has over conventional HPLC, such as smaller sample requirements, reduced mobile-phase consumption and lower costs [7].

It is well known that most vertebrate hemoglobins (Hbs) are tetramers consisting of two α and two non- α chains, each containing the heme group. In humans, adult hemoglobin (Hb A), fetal hemoglobin (Hb F) and Hb A₂ are characterized by non- α chains, i.e., β , γ and δ chains, respectively. In clinical laboratories, electrophoresis, isoelectric focusing and HPLC have been employed for Hb

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separations [8–12] and are useful for the screening and diagnosis of genetic diseases including diabetes mellitus. Reversed-phase HPLC [11,12] can separate normal, abnormal and three types of γ chains by direct injection of hemolysate samples onto the column.

In cases of infanticide, criminal abortion and concealed delivery, identification of fetal and/or neonatal bloodstains is of great importance. The usefulness of reversed-phase HPLC for this purpose has already been described [13,14]. However, in forensic science, very small amounts of blood and/or bloodstains can be submitted for examination and procedures should be rapid, simple, reliable and sensitive. In this study, we used a microbore system for the separation of human globin chains and to determine performance characteristics such as resolution, detection limit, effects of injection volume and detector flow-cell volume and reproducibility of retention times, which were compared with those obtained by a conventional HPLC system. The γ chain composition in umbilical cord hemolysates was determined with both systems. A forensic application of the present method is also described.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (TFA, amino acid sequencing-grade) and acetonitrile (HPLC-grade) were obtained from Wako Pure Chemicals (Osaka, Japan). Water for HPLC was treated with a Milli-Q Labo purification system (Millipore, Bedford, MA, USA). Hb S was from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

The liquid chromatograph consisted of two LC-9A pumps, an SCL-6A system controller, an SIL-6A auto-injector equipped with a 1-ml injection loop and a CTO-6A column oven (Shimadzu, Kyoto, Japan). The column eluent was passed through an SPD-6A UV absorbance detector (Shimadzu) equipped with a conventional 8- μ l flow-cell with a 10-mm optical path length and it was monitored at 220 nm. A 0.6- μ l microflow-cell with a 3-mm path length was also used in experiments to determine the resolution

and sensitivity using a 2.1 mm I.D. column. The HPLC system was attached to a C-R4A integrator (Shimadzu), programmed to compute peak areas and retention times.

Either a microbore (2.1 mm I.D.) or a conventional bore (4.6 mm I.D.) SynChropak RP-4 column (250 mm length, 6.5 μ m particle size, 300 Å pore; SynChrom, Lafayette, IN, USA), protected by a 2- μ m precolumn filter (Millipore), was used for this study. Neither precolumns nor guard columns were used. A static solvent mixing chamber with a volume of 500 μ l was employed for the 2.1 mm I.D. column. When the 4.6 mm I.D. column was used, a static mixing chamber with a volume of 2 ml was added.

The outlet of the mixing chamber was connected to the auto-injector by a stainless-steel tube of 0.3 mm I.D. A PEEK tube (polyetherether ketone; Upchurch Scientific, Oak Harbor, WA, USA) of 0.25 mm I.D. was employed to connect the injector to the column and the column to the detector.

2.3. Chromatographic conditions

Chromatographic conditions for a 4.6 mm I.D. column were as described previously [13]. Solvent A was 0.1% TFA in 20% acetonitrile and solvent B was 0.1% TFA in 60% acetonitrile. The gradient was from 44 to 50% B in 30 min, from 50 to 52% B in 20 min and from 52 to 56% B in 20 min. The solvent system used for a microbore column in the early stage of this study was the same as that used for a conventional column and the gradient was from 43 to 53% B in 70 min. In the latter half of the study, different TFA concentrations were used for both A and B solvents (A=0.107%; B=0.094%). The flow-rate for the 2.1 mm I.D. column was 0.2 ml/min to obtain the same mobile-phase linear velocity as that of the 4.6 mm I.D. column (1.0 ml/min). All solvents were degassed with helium prior to use. The column temperature was set at 35°C in both systems.

2.4. Blood and bloodstain samples

Venous blood samples from eight human adults and umbilical cord blood samples from eight neonates were collected in EDTA. They had no detectable Hb abnormalities. Washed cells were hemolyzed with water and toluene, and cellular debris was removed by centrifugation. The Hb concentrations of

hemolysates were determined by a methemoglobin-cyanide method with modified Van Kampen-Zijlstra's reagent [15]. Hb A, Hb F and Hb A₂ were isolated by DEAE-cellulose (DE-52, Whatman, Kent, UK) chromatography, as described by Abraham et al. [16]. Hb S was used without further purification.

Bloodstain samples were prepared by dropping whole blood on filter paper (Toyo Roshi, No. 1, Tokyo, Japan) which was then dried and stored at room temperature. A piece of bloodstain (equivalent to about 5 μ l of fresh blood) was cut into small fragments and immersed in 250 μ l of distilled water. The extract was centrifuged at 11 750 g for 20 min.

2.5. Determination of fetal hemoglobin

In cord samples, the ratios of Hb F to total Hb were determined by measuring relative peak areas of non- α globin chains (β and γ) on the chromatogram. The δ chain was omitted from the calculation because it was too small to detect as a definite peak.

3. Results and discussion

3.1. Separation of globin chains

Fig. 1 illustrates typical chromatograms of diluted umbilical cord hemolysates, obtained with each of the two columns of different I.D. The use of the equivalent flow-rates through each column and a minor modification of the gradient profile resulted in separations of very similar quality with the two columns with different I.D.s. The sensitivity of detection of the 2.1 mm I.D. column was about five times greater than that of the 4.6 mm I.D. column.

The Hb F level and the globin chain composition on each of the columns are compared in Table 1. There seems to be a slight increase in the Hb F levels and in the G γ ratios obtained from the 2.1 mm I.D. column compared with those from the 4.6 mm I.D. column. Ratios between non- α and α chains were also very close to unity, as expected. Similar results were obtained from the other four samples.

3.2. TFA concentration in the mobile phase

The slope of the baseline increased with the increase in detector sensitivity because of acetonitrile

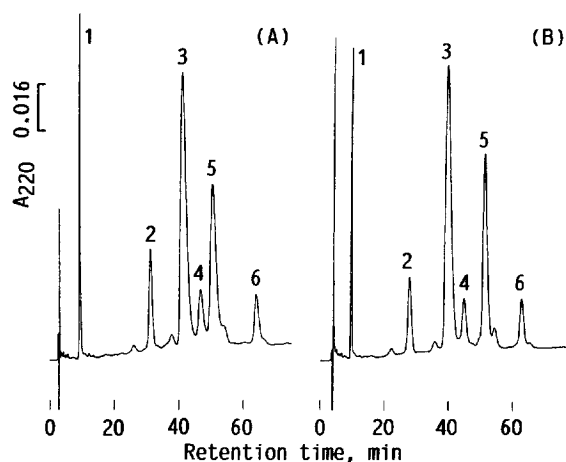


Fig. 1. Separation of globin chains in a cord hemolysate. Peaks: 1=heme; 2= β^A ; 3= α ; 4= $A\gamma^T$; 5= $G\gamma$; 6= $A\gamma^I$. Chromatographic conditions: solvent A, 0.1% TFA in 20% acetonitrile; solvent B, 0.1% TFA in 60% acetonitrile; column temperature, 35°C. (A) Column, 250 \times 4.6 mm I.D.; linear gradient from 44–50% B in 30 min, from 50–52% B in 20 min and from 52–56% B in 20 min at a flow-rate of 1.0 ml/min; a 20- μ l volume of 2 mg/ml Hb was applied to the column. (B) Column, 250 \times 2.1 mm I.D.; linear gradient from 43–53% B in 70 min at a flow-rate of 0.2 ml/min; a 20- μ l volume of 0.4 mg/ml Hb was applied to the column.

gradient elution at 220 nm. When the detector sensitivity was set at 0.01 absorbance units full scale (AUFS), a gradual increase of the baseline during gradient elution occurred rising to about 30% AUFS.

Table 1

Comparison of the levels of Hb F and the γ globin chains in cord hemolysates for columns of different diameters.

Sample number	Hb F (%)	γ -Chain composition (%)			Non- α / α
		$A\gamma^T$	$G\gamma$	$A\gamma^I$	
<i>4.6 mm I.D. column</i>					
1	81.7	—	68.2	31.8	0.99
2	90.3	12.2	69.9	17.9	0.93
3	78.7	—	52.3	47.7	1.00
4	80.6	12.9	68.4	18.6	0.98
<i>2.1 mm I.D. column</i>					
1	84.3	—	70.2	29.8	1.02
2	91.3	10.8	73.3	15.9	1.01
3	81.6	—	54.1	45.9	1.05
4	82.1	12.7	70.0	17.3	1.06

Samples 1 and 3 are from $A\gamma^I$ homozygotes and samples 2 and 4 are from $A\gamma^T/A\gamma^I$ heterozygotes.

Values represent the mean of three determinations on different days.

Chromatographic conditions are as in Fig. 1.

An attempt was made to improve the TFA concentration in the mobile phase to compensate for the increase in baseline absorbance.

At first, the effect of changes in TFA concentration in the mobile phase on Hb separation was evaluated. As shown in Fig. 2, TFA concentration had little influence on the retention times of heme over the range investigated (0.085–0.115%). In contrast, there was an increase in the retention times of globin chains with increasing TFA concentration at a rate of about 5 min per 0.01% TFA. When the solvent A was 0.107% TFA in 20% acetonitrile and B was 0.094% TFA in 60% acetonitrile, the baseline became almost flat, even at 0.01 AUFS. TFA was added as a 0.4% aqueous solution to stabilize the TFA concentrations in the mobile phases. Globin chain separation in normal cord hemolysate and in an artificial mixture of Hb A, Hb F, Hb A₂ and Hb S is shown in Fig. 3A and Fig. 3B, respectively. Separation of the globin chains was satisfactory, although the resolution between α and $\Lambda\gamma^T$ chains decreased slightly. Using this solvent system with a gradient from 43 to 53% solvent B, the TFA concentration in the mobile phase changed from 0.10122 to 0.09995% during gradient elution. In this study, no attempt was made to separate the globin chains in the microbore column using a triphasic linear gradient, as used for the conventional bore column. A detailed investigation of the gradient profile might improve the separation between the α and $\Lambda\gamma^T$ peaks. Hereafter,

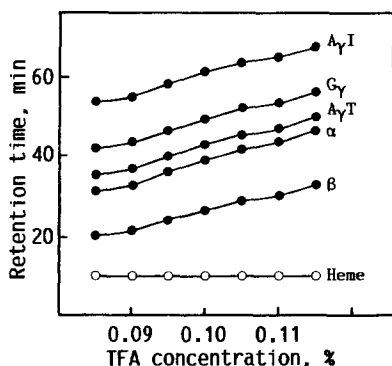


Fig. 2. Influence of TFA concentration on retention times of heme and globin chains. Chromatographic conditions: solvent A, 0.085–0.115% TFA in 20% acetonitrile; solvent B, 0.085–0.115% TFA in 60% acetonitrile; column, 250×2.1 mm I.D. Other conditions are as in Fig. 1.

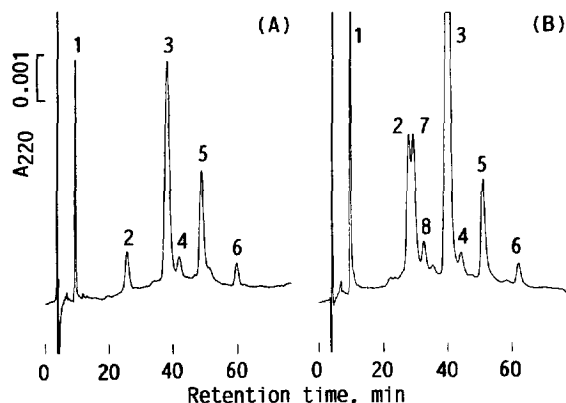


Fig. 3. Separation of globin chains (A) in a cord hemolysate and (B) in an artificial mixture of Hb A, Hb F, Hb A₂ and Hb S. Peaks: 1=heme; 2= β^A ; 3= α ; 4= $\Lambda\gamma^T$; 5= $G\gamma$; 6= $\Lambda\gamma^I$; 7= β^S ; 8= δ . Chromatographic conditions: column, 250×2.1 mm I.D.; solvent A, 0.107% TFA in 20% acetonitrile; solvent B, 0.094% TFA in 60% acetonitrile; linear gradient from 43–53% B in 70 min; flow-rate, 0.2 ml/min; column temperature, 35°C. Sample volume: (A) 10 μ l of 40 μ g/ml Hb; (B) 10 μ l of 0.1 mg/ml Hb.

the improved solvent system (A=0.107% TFA in 20% acetonitrile; B=0.094% TFA in 60% acetonitrile) was used for the analyses that were carried out using the 2.1 mm I.D. column.

3.3. Hb F level and globin chain composition at high detector sensitivity

Table 2 shows the Hb F level and the globin chain composition using the 2.1 mm I.D. column at high detector sensitivity. The values were very similar to those in Table 1, with the exception of the ratios between non- α and α chains, which were

Table 2
233Hb F level and globin chain composition in cord hemolysates analyzed on a 2.1 mm I.D. column at high detector sensitivity (0.01 AUFS)

Sample number	Hb F (%)	γ chain composition (%)			Non- α/α
		$\Lambda\gamma^T$	$G\gamma$	$\Lambda\gamma^I$	
1	84.4	—	69.5	30.5	0.83
2	90.0	10.1	74.1	15.9	0.85
3	80.5	—	51.8	48.2	0.84
4	81.9	10.0	74.0	16.0	0.83

Values represent the mean of three determinations on different days. Chromatographic conditions are as in Fig. 3A.

significantly lower than might be expected. A satisfactory explanation has not yet been found, but this may be the result of slight differences in separation of the minor components, mainly derived from the post-translational modifications of Hb A and Hb F [12,17,18]. The detection limit for Hb was 0.1 µg, based on a signal-to-noise ratio of twenty (as a heme peak).

3.4. Sample concentration and injection volume

Table 3 shows the effect of injection volume on peak width for globin chains. It is apparent that over a range of injection volumes, no peak broadening occurs. It seems reasonable to assume that heme and globin chains are adsorbed and concentrated at the top of the column by non-eluting solvent at injection, then eluted and separated by the increased acetonitrile concentration in the mobile phase [6].

3.5. Detector flow-cell volume

The area response obtained with the 0.6-µl flow-cell decreased to about one-third of that obtained using the 8-µl cell, while there was no difference between the peak widths obtained (Table 4). It is generally accepted that the use of microflow-cells provides reduced band broadening. With photometric detectors, sub-microliter flow-cell volumes are obtained by reducing the optical path length, which

Table 3
Effect of injection volume on peak width for globin chains on a 2.1 mm I.D. column

Hb concentration (µg/ml)	Injection volume (µl)	Peak width (µl)				
		β	α	^A γ ^T	^G γ	^A γ ^I
200	2	447	580	560	500	513
80	5	450	573	550	493	493
20	20	463	580	590	480	480
8	50	410	570	583	477	507
4	100	467	580	580	493	500
50	2	500	583	550	450	490
50	5	477	573	580	490	500
50	20	473	563	537	480	507
50	50	460	580	527	483	493
50	100	440	613	493	493	477

Values represent the mean of three determinations on different days. Chromatographic conditions as in Fig. 3A.

Table 4
Comparison of different detector flow-cell volumes on peak width and area response

Globin chain	Peak width (µl)		Area response	
	Flow-cell volume (µl)		Flow-cell volume (µl)	
	8	0.6	8	0.6
β	428	426	14898	5198
α	554	538	96200	32720
^A γ ^T	532	520	6209	2001
^G γ	480	478	50819	16473
^A γ ^I	494	498	10593	3684

Values represent the mean of five determinations. Chromatographic conditions are as in Fig. 3. A 20-µl volume of 50 µg/ml Hb was applied to the 2.1 mm I.D. column.

limits absorbance, i.e., the concentration sensitivity of such systems [6,19]. In this study, it seemed that the flow-cell volume had no effect on peak width due to relatively large peak volumes of globin chains, although the concentration sensitivity was reduced in proportion to the optical path length. It is also noted that the 0.6-µl flow-cell had an appreciably higher noise level than the 8-µl cell.

3.6. Reproducibility of retention times

Table 5 summarizes the results of a comparison of the reproducibility of retention times of heme and globin chains obtained with the microbore system and those with the conventional system. Good reproducibility was obtained with the microbore column as well as with the conventional column.

Table 5
Retention time precision in Hb analysis

Column I.D. (mm)	Coefficient of variation (%)					
	Heme	β	α	^A γ ^T	^G γ	^A γ ^I
<i>Within-day assay (n=8)</i>						
4.6	0.3	0.4	0.4	0.4 ^a	0.3	0.3
2.1	0.2	1.3	1.0	0.7 ^a	0.9	0.8
<i>Between-day assay (n=3)</i>						
4.6	0.4	1.2	0.8	1.0	1.1	0.8
2.1	0.3	1.6	0.9	1.2	0.9	0.8

Chromatographic conditions for the 4.6 mm I.D. column are as in Fig. 1 and those for the 2.1 mm I.D. are as in Fig. 3A. Data from the between-day assay were determined within two weeks.

^a n=4.

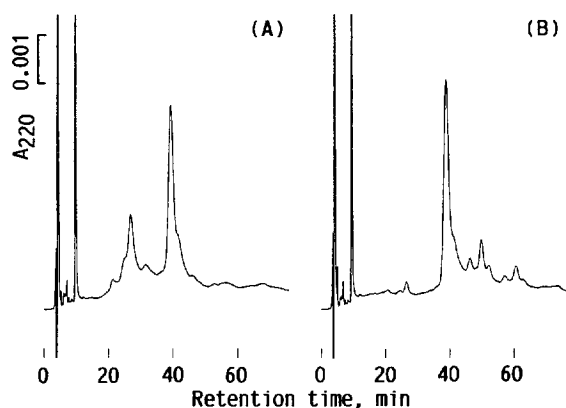


Fig. 4. Chromatograms from extracts of (A) adult and (B) cord bloodstains that were 20 weeks old. Chromatographic conditions are as in Fig. 3. A 20- μ l volume of 100-fold-diluted stain extracts (diluted with distilled water) was applied onto the column.

3.7. Forensic application

Fig. 4 shows chromatograms from extracts of bloodstains from human adult and umbilical cord that had been stored for twenty weeks at room temperature. No clear peaks from plasma components were observed on the chromatograms. With older stains, extra peaks appeared before and after the native globin chains and were often only partially separated from the native major ones, as shown previously [13,14]. However, the chromatographic profiles of cord bloodstains could easily be distinguished from those of adults in stains that were up to twenty weeks old. Although further investigations were not performed on older stains, it could be applicable to bloodstains up to at least 32 weeks old, as far as can be estimated from the previous study [14].

Microbore HPLC on the 2.1 mm I.D. column using the modified solvent system offered a significant improvement in sensitivity with little loss of the resolution of globin chains and only small variations in the determination of γ -chain composition. The significance of determining Hb F levels for forensic application has already been discussed [13]. The minimum detectable quantity of Hb is 0.1 μ g, which is equivalent to about 1 nl of fresh whole blood. The present method is more sensitive than previous

methods that used antisera to human Hb A and Hb F, such as thin-layer immunoassay [20] and the microtiter latex agglutination test [21], where detection limits are 10 nl of blood and 6–60 μ g of dried blood, respectively. In view of previous reports [12,22], microbore HPLC could also detect abnormal Hbs, though only Hb S was tested as an example of an abnormal Hb. The present method will be useful for the detection of abnormal Hbs and for the determination of γ -chain composition in clinical laboratories and for their determination from minute blood samples and bloodstains for the practice of forensic science.

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