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Identification of fetal hemoglobin in blood stains by high performance liquid chromatography

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Summary. A new method for the identification of fetal hemoglobin (Hb F) in blood stains by reverse-phase high performance liquid chromatography is described. Differentiation between fetal and adult blood stains is based on the existence of γ-chain peaks which are characteristic of Hb F. Very few γ chains appeared on chromatograms of all the adult blood stains examined. The level of Hb F could be determined by measuring the total of chromatogram γ-globin chain areas, and expressing it as a percentage of total Hb. Levels in six cord blood stains on filter paper ranged from 81.1% to 91.3% and remained constant for at least 12 weeks. This method is of great value for its simplicity, sensitivity and speed, and most importantly for its reliability in the field of forensic medicine.

Key words: Fetal hemoglobin, identification in blood stains – Blood stains, identification of fetal hemoglobin

Zusammenfassung. Es wurde eine neue Methode zur Identifizierung von fetalem Hämoglobin (Hb F) in Blutspuren mit der umgekehrten Phase-Hochleistungsflüssigkeitschromatographie durchgeführt. Die Unterscheidung zwischen fetalen Blutspuren und denen von Erwachsenen erfolgte aufgrund der Existenz der Spitzen der γ-Ketten von Hb F. In den Chromatogrammen der untersuchten Blutspuren von Erwachsenen traten fast keine γ-Ketten auf. Diese Methode ermöglichte ferner die Bestimmung des Hb F-Wertes mit dem Flächenverhältnis der Globin-Ketten auf den Chromatogrammen. Hb F-Werte der sechs Fälle von Nabelschnurblutspuren auf dem Filterpapier lagen zwischen 81.1% und 91.3%. Die vorliegende Untersuchung zeigt, daß sich der Hb F-Wert für mindestens 12 Wochen nicht ändert. In der Praxis der Rechtsmedizin ist diese Methode im Hinblick auf die Einfachheit, hohe Empfindlichkeit, Schnelligkeit und Zuverlässigkeit sehr wertvoll.

Schlüsselwörter: Fetales Hämoglobin, in Blutspuren – Blutspuren, Nachweis von fetalem Hämoglobin

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Introduction

In cases of infanticide, criminal abortion, and concealed delivery, identification of fetal and/or neonatal blood stains is of great importance. Since fetal hemoglobin (Hb F) is the major component of total hemoglobin (Hb) in fetal and/or neonatal blood, numerous methods have been developed for the detection of Hb F in blood stains. Making use of the various physicochemical characteristics of this protein, these methods include UV absorption [1], thin-layer immunoassay using anti Hb F serum [2], pyrolysis-gas-liquid chromatography [3], and electrophoresis on cellulose acetate [4, 5]. It is well known, however, that normal adult blood still contains a certain percentage of Hb F, which is elevated in some diseases, such as anemias, thalassemias, hereditary persistence of Hb F (HPFH), and various hereditary or acquired hemoglobinopathies [6–10]. Because the methods mentioned above only measure Hb F qualitatively or semiquantitatively, discrimination may be ambiguous or unreliable. Elevations of Hb F levels are generally low (below 30%), although large increases are seen in patients with β-thalassemia major, homozygotes for African Negro type of HPFH and certain leukemias. These conditions, however, are very rare [6, 9, 10]. Based on the above considerations, it was necessary to develop a reliable method for measuring Hb F levels in blood stains to distinguish more precisely between fetal and adult blood.

Hb consists of four polypeptide chains attached to the heme group, two of which are α chains. Hb A, Hb F, and Hb A_2 are characterized by β, γ and δ chains, respectively. In clinical laboratories high performance liquid chromatography (HPLC) has been employed for Hb separations. This is useful for the diagnosis and screening of genetic diseases and diabetes [11–14]. Some methods [12, 13] make use of a reverse-phase column, which is suitable for the analysis of normal and abnormal globin chains in hemolysates. We describe a method for identifying and quantifying Hb F in fetal and/or neonatal blood stains by reverse-phase HPLC using a large pore C_4 column.

Materials and methods

Preparation of hemoglobin solution and blood stains

Ten venous blood samples from adults and six umbilical cord blood samples from newborns were collected in EDTA or heparin. Erythrocytes were washed 3 times with ten volumes of physiologic saline. The cells were then lysed with 1.5 volumes of distilled water and well shaken with a half volume of toluene. Cellular debris was removed by centrifugation at 3000 rpm for 20 min. An aliquot of the clear supernatant was diluted to a Hb concentration of about 3 mg/ml.

Blood stain samples were made by dropping whole blood on filter paper (Toyoroshi, No. 1, Tokyo, Japan). Additionally, five of the adult and three of the cord blood samples were also used to stain cotton gauze. The samples were then dried under atmospheric conditions and stored at room temperature.

Extraction of hemoglobin from blood stains

A piece of blood stain (about 5 × 5 mm in area on filter paper, or about 5 × 10 mm on cotton gauze) was immersed in 250 μl of distilled water for 2 h at 37°C. The extract was then diluted, if necessary, with distilled water to a suitable Hb concentration (1–3 mg/ml).

High performance liquid chromatography

The method used is a modification of the procedure described by Shelton et al. [13] and the details are as follows. Equipment used in this study was an LKB Ultrochrom GTi system consisting of a Model 2150-020 HPLC pump, a Model 2152-010 LC controller, a Model 2040-203 gradient mixing valve, a Model 2154-002 sample injector, and a Model 2151-001 variable wavelength monitor. The column used was a SynChropak RP-4 (250 × 4.6 mm I.D., 300 Å pore, SynChrom, Inc., Indiana). Neither pre-columns nor guard columns were used. Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA, amino acid sequencing grade) were obtained from Wako Pure Chemical Industries, Ltd. Water was purified with a Milli-Q Labo (Millipore Corporation). Solvent A was an 80:20 mixture of 0.1% aqueous TFA and 0.1% TFA in acetonitrile and solvent B consisted of these solutions in a 40:60 ratio. Solvents were degassed with helium prior to use. The gradient was 44%–50% B in 30 min, 50%–52% B in 20 min, 52%–56% B in 20 min. The solvents were maintained at this concentration for 5 min and then returned to starting conditions in 2 min. Re-equilibration was carried out for 20 min. The flow rate was 1.0 ml/min, and the column effluent was monitored at 220 nm. All separations were carried out at ambient temperature. The HPLC system was attached to a Shimadzu C-R6A integrator, programmed to compute peak areas and retention times. The percentage of Hb F was determined by planimetry, i.e., $\text{Hb F}(\%) = ({}^{G}\gamma + {}^{A}\gamma^{I}) \times 100/(\beta + {}^{G}\gamma + {}^{A}\gamma^{I})$.

Samples applied to the column varied in volume from 10 to 100 µl, depending on the Hb concentration. The column was stored in 100% acetonitrile without TFA.

Results

Figure 1 shows chromatograms of hemolysates from adult and cord blood. The major peaks contributed by α, β and two γ chains were well separated from each

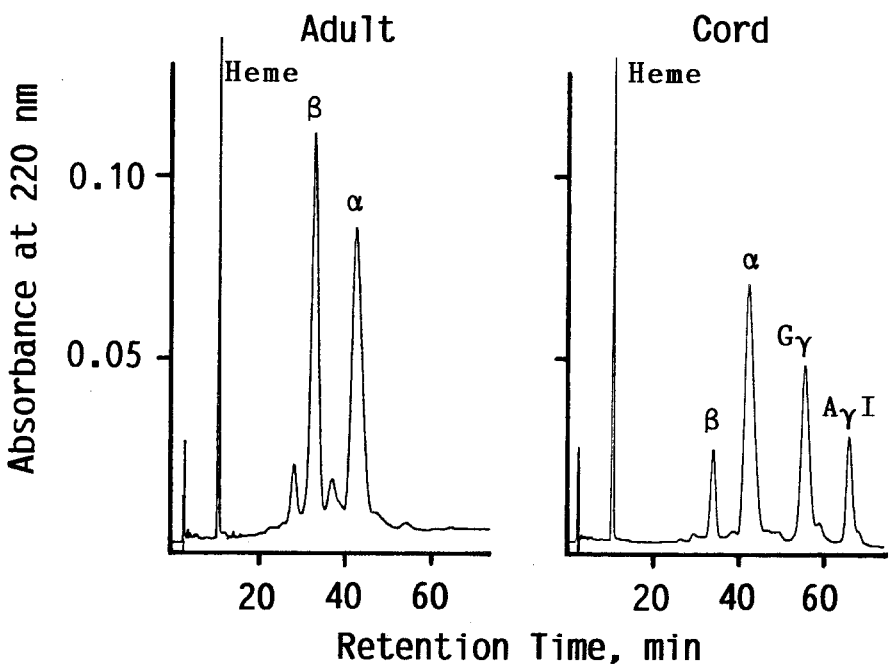

Fig. 1. Separation of globin chains in adult and cord hemolysates by reverse-phase HPLC. Twenty microliters of approximately 3 mg/ml hemoglobin was applied to the column

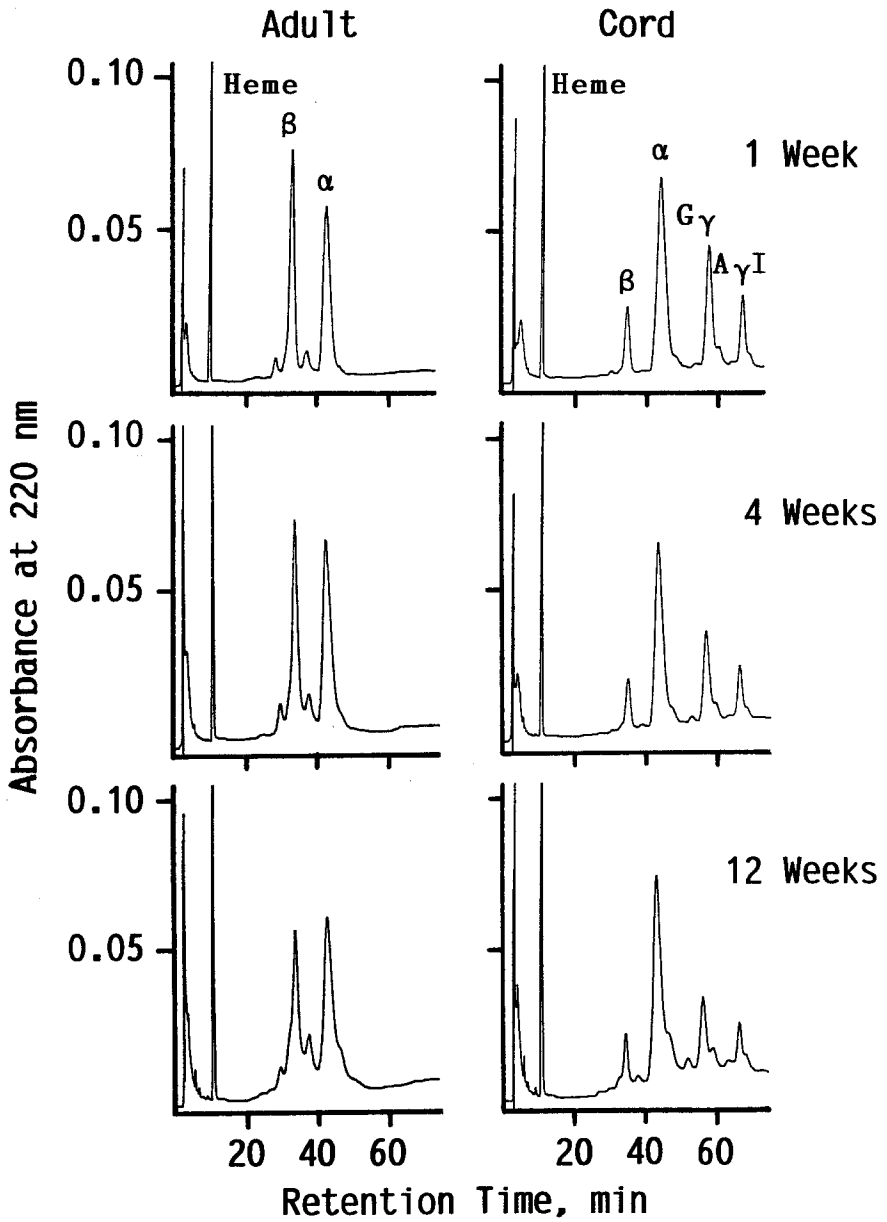

Fig. 2. Separation of globin chains in extracts of adult and cord blood stains aged for varying periods. The *top* of each heme peak on the chromatograms is deleted

other. Some minor peaks also appeared before and after the major ones and one or more of these eluted together with the δ chain in a region between the β and α chains. The δ chain was not detected as a definite peak on the chromatograms. The level in the cord hemolysate was 83.5%. On the chromatograms obtained with adult blood stains, traces of γ chains were detected but these were impossible to quantitate.

Table 1. Levels of Hb F in hemolysates from cord blood and in extracts of blood stains on filter paper aged from 1 to 12 weeks. Values of Hb F are expressed as percentages of total Hb

Sample no.	Hemolysate	Weeks after staining				
		1	2	4	8	12
1	83.5	81.1	80.7	82.3	80.3	77.6
2	85.4	82.8	82.2	83.4	78.7	78.3
3	88.7	85.2	85.1	86.1	79.6	85.4
4	85.7	82.6	82.0	82.2	83.5	80.7
5	90.3	91.3	91.0	89.2	90.3	91.2
6	89.9	88.0	85.2	88.1	89.2	91.0
Mean	87.3	85.2	84.4	85.2	83.6	84.0
SD	2.8	3.9	3.7	3.0	5.0	6.1

Table 2. Changes with time in Hb F levels from cord blood stains on cotton gauze. Hb F values are expressed as percentages of total Hb

Sample no.	Weeks after staining				
	1	2	4	8	12
1	82.4	80.4	81.5	78.9	80.6
2	83.2	82.5	83.2	78.9	78.3
3	86.2	86.4	87.3	84.5	86.0
Mean	83.9	83.1	84.0	80.8	81.6
SD	2.0	3.0	3.0	3.2	4.0

Figure 2 shows chromatograms of extracts from adult and cord blood stains aged for various periods. In both adult and cord samples, there was little difference in chromatograms of hemolysates and those of extracts of blood stains aged 1 week. It appears that the tailing observed preceding heme is a mixture of various plasma components which are either not absorbed or only weakly absorbed to the column. Deformations in chromatographic patterns obtained with older samples were observed. This suggests that the globin chains in blood stains gradually degrade. We had no problem, however, to clearly distinguish between adult and cord blood stains.

Table 1 shows percentages of Hb F to total Hb of six cord hemolysates and the time-dependent changes in Hb F levels of blood stain extracts on filter paper. The levels in six cord hemolysates ranged from 83.5% to 90.3% (87.3% ± 2.8%; mean ± SD). We also measured the levels in hemolysates of seven cord blood and seven peripheral blood samples of neonates less than 1 week old. The levels in the former ranged from 77.1% to 89.5% (83.3% ± 4.3%) and those in the latter, from 76.8% to 89.4% (83.8% ± 5.0%). Each blood stain sample showed little change in Hb F levels up to 12 weeks.

Table 2 shows changes with time in the Hb F levels of three blood stain samples prepared on cotton gauze. No differences were observed between the filter paper and the cotton gauze.

Discussion

A number of methods have been described for the identification of fetal and/or neonatal blood stains by employing Hb F as a specific marker [1–5]. However, these methods only measure Hb F qualitatively or semi-quantitatively. It has been reported that the Hb F level in the blood of newborns is 75% to 90%, whereas it is less than 2% in adults [6]. The Hb F level is elevated in adult patients suffering from certain diseases, and the elevations are generally below 30%. Even in such cases, an adult blood stain could be mistaken for one of fetal origin by qualitative analysis. Although elevated levels of more than 75% have been reported in some diseases, such as β-thalassemia major, homozygotes for African Negro type of HPFH, and a certain leukemia, these cases are quite rare. Therefore, a quantitative determination of Hb F affords a more reliable means for distinguishing between blood stains of adult and fetal origin. Recently, Katsumata et al. [15] attempted to determine the relative amount of Hb F in blood stains using an alkali-denaturation method. Although no special reagents and instruments are necessary for the method, the levels decrease rapidly with the age of the blood stains as compared to those of the original blood tested. It is consequently unreliable to use the alkali-denaturation method to examine older blood stains. The present method is superior to others described previously for quantification of Hb F in blood stains and our results demonstrate its reliability. Blood stains up to 12 weeks old have been studied, and changes in Hb F levels with older blood stains are now under investigation.

In medico-legal practice the age or the amount of blood stain initially present is occasionally uncertain [16]. Also, the amount of Hb extractable gradually decreases with time. Therefore, even though Hb is extracted from the same quantity of stain, using the same procedure, the extract concentration may vary. In the present study, it must be emphasized that the Hb F level was not affected by age, the initial amount of blood stain, nor the extent of extraction because the calculation is based on the ratio of the areas of β and γ chains in the extract chromatogram. Blood stains were prepared on cotton gauze as well as filter paper, and similar results were obtained with both kinds of materials.

We took 2 h to extract sufficient Hb from the stains. However, for relatively fresh stains, the required quantity of Hb for HPLC analysis was extracted in 20 min. Therefore, this procedure requires less than 2 h to perform for blood stains less than 1 month old.

Human adult hemolysate contains minor Hbs, namely Hb A_{Ia1}, A_{Ia2}, A_{Ib}, and A_{Ic} [17]. Similarly, minor Hbs, designated F_{Ia1}, F_{Ia2}, F_{Ib}, and F_{Ic}, have been separated from hemolysates of newborn children and from an adult homozygous for HPFH [18]. Almost all of these minor Hbs are glycosylated or acetylated derivatives of Hb A and Hb F [17–19]. On our chromatograms, some minor peaks also appeared before and after the major ones produced by α, β,

and γ chains. These were probably derivatives of the major chains [13] and were excluded from the calculation. According to Fadel et al. [19], the amount of minor Hb F compared to total Hb F and that of minor Hb A to total Hb A in normal cord blood were about 17% and 12%, respectively. The levels of Hb F calculated in this study may be slightly lower, but this is not believed to impair the identification of fetal blood stains.

It has been shown that the γ chain of Hb F possesses either an alanine ($^{A}\gamma$) or a glycine ($^{G}\gamma$) residue at position 136 [20]. In addition, a new type of Hb F called Hb F Sardinia, in which isoleucine ($^{A}\gamma^{I}$) in position 75 of the $^{A}\gamma$ chain is replaced by a threonine ($^{A}\gamma^{T}$) residue, was found in patients with β-thalassemia [21], and was also present in normal newborns and premature infants [22]. One of the cord samples used for staining (shown as no. 4 in Table 1) had the $^{A}\gamma^{T}$ chain, which eluted between the α and $^{G}\gamma$ chains. When the cord blood stain was kept for more than 2 weeks, an extra peak appeared with a similar retention time as that of the $^{A}\gamma^{T}$ chain and occasionally overlapped with it. Because this peak was absent on any chromatogram from adult blood stains, it is thought to be a denaturation product of either the $^{G}\gamma$ or $^{A}\gamma^{I}$ chain. In the case of the blood stain possessing the $^{A}\gamma^{T}$ chain, its value was included in the calculation of the Hb F level in the hemolysate and blood stain less than 2 weeks old, but was omitted for the blood stain 2 weeks old or more. However, the Hb F levels of sample no. 4 were not influenced by omitting calculation of the $^{A}\gamma^{T}$ peak area from samples up to 12 weeks.

As stated earlier, the HPLC method presented here is quick, simple, and sensitive. Moreover, it is of particular importance with regard to the field of forensic medicine as it provides an extremely reliable means of identifying fetal and/or neonatal blood stains.

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