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Identification of fetal hemoglobin and simultaneous estimation of bloodstain age by high-performance liquid chromatography

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Summary. A method using reverse-phase high-performance liquid chromatography (HPLC) for the identification of fetal hemoglobin (Hb F) and the simultaneous estimation of bloodstain age is described. Umbilical cord and neonatal bloodstains can be differentiated from adult stains by the presence of γ-globin chains which are characteristic of Hb F. With this method, cord and neonatal blood could be distinguished from adult blood in stains up to 32 weeks old. The age of the stain was estimated from the ratio of the peak area of the α-globin chain to that of heme on the same chromatogram. The ratio decreased gradually with an increase in the age of the stain up to 20 weeks old. Studies performed at each time period revealed no significant difference in the ratios of cord and neonatal bloodstains or in the ratios of cord and adult stains. The regression equation calculated from the ratios (y) and the ages of stains in weeks (x) expressed logarithmically is $y = 2.5758 - 0.2497 \ln(x)$ and the coefficient of correlation is -0.7491 ($n = 252$, $P < 0.001$). The present method, having the advantages of simplicity, speed and sensitivity, should be of great value to forensic science.

Key words: Bloodstain age – Fetal hemoglobin – HPLC – Bloodstain identification

Zusammenfassung. Beschrieben wird eine „reverse-phase" – HPLC-Methode für die Identifizierung von fetalem Hämoglobin (Hb F) und zur gleichzeitigen Bestimmung des Blutspuren-Alters. Blutspuren von Blut aus Nabelvenen und von Neugeborenen können von Blutspuren erwachsener Personen durch die Anwesenheit der γ-Globin-Ketten differenziert werden, welche für Hb F charakteristisch sind. Mit Hilfe dieser Methode gelang die Differenzierung zwischen bis zu 32 Wochen alten Blutspuren aus Nabelschnüren und Neugeborenen einerseits und von Erwachsenen andererseits. Das Alter der Spur wurde bestimmt aus dem Verhältnis der Peak-Fläche, der α-Globin-Kette zu jener des Häm auf demselben Chromatogramm. Das Verhältnis dieser beiden Flächen nahm mit zunehmendem Alter der Blutspur graduell ab, – dies bis zu einem Spurenalter von 20 Wochen. Studien, welche zu jenem Zeitpunkt durchgeführt wurden, deckten keine signifikanten Unterschiede in diesen Peak-Verhältnissen von Nabelvenen- und Neugeborenen-Blutspuren und auch nicht in den Verhältnissen von Nabelvenen und Erwachsenen-Spuren auf. Die Regressions-Gleichung, welche von den Verhältnissen (y) und dem Alter der Spuren in Wochen (x) errechnet und logarithmisch ausgedrückt wird, lautet: $y = 2.5758 - 0.2497 \ln(x)$ und der Korrelations-Koeffizient beträgt -0.7491 ($n = 252$, $P < 0.001$). Die vorliegende Methode, welche einfach, schnell und empfindlich ist, sollte für die forensischen Wissenschaften von großem Interesse sein.

Schlüsselwörter: Blutspurenalter – Fetales Hämoglobin – HPLC – Blutspurenidentifizierung

Introduction

Estimation of bloodstain age is of great importance in forensic science. Over the years, various methods have been developed, such as chloride reaction [1], solubility test [2], spectrophotometry with several kinds of extracts [2–5], surface absorption spectrophotometry [6, 7], immunoelectrophoresis [8], and methods utilizing changes in the ratio of the albumin level to that of hemoglobin (Hb) [9] or changes in enzyme activities [10, 11]. In recent years, a new electron spin resonance technique has been proposed by Miki et al. [12]. Identification of fetal and/or neonatal bloodstains is also important, especially in cases of infanticide, criminal abortion or concealed delivery. We have previously described a method for the identification of fetal hemoglobin (Hb F) in bloodstains by high-performance liquid chromatography (HPLC) and discussed its usefulness [13]. In the present paper, we describe a one-step HPLC method which can simultaneously identify Hb F in bloodstains and be used to estimate bloodstain age.

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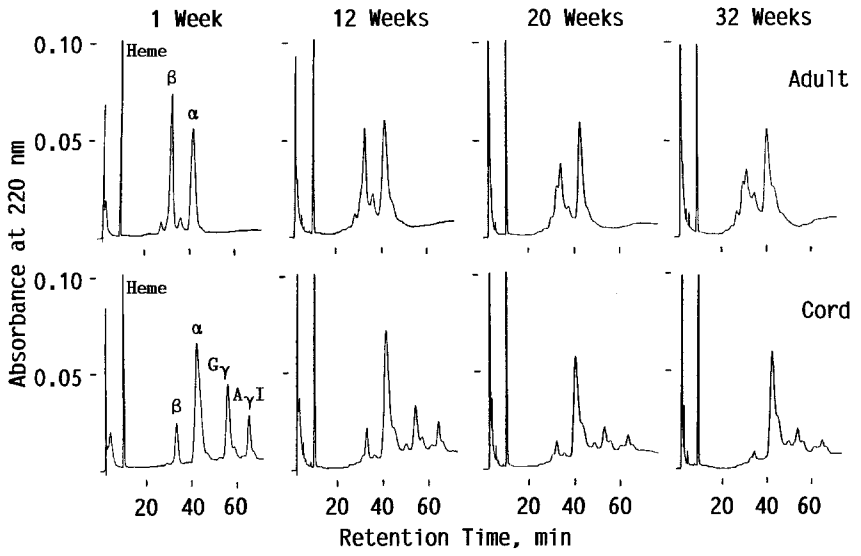

Fig. 1. Separation of globin chains in extracts of adult and cord bloodstains at various times using reverse-phase HPLC

Materials and methods

Sample preparation. Six umbilical cord blood samples from newborns, 25 peripheral blood samples from infants various numbers of weeks age (including 5 neonates) and 5 venous blood samples from adults were collected in EDTA or heparin. Hemolysates were prepared by lysing the washed erythrocytes with distilled water and toluene and cellular debris was removed by centrifugation. An aliquot of each hemolysate was diluted to a Hb concentration of about 3 mg/ml. Bloodstains were prepared on filter paper (Toyo Roshi, No. 1, Tokyo, Japan) and kept at room temperature. Hb was extracted from bloodstains with distilled water at various time intervals, after which the extract was diluted if necessary with distilled water and filtered (Ultrafree C3-GV, 0.22 μm, Millipore Corp., Bedford, MA).

High-performance liquid chromatography. An LKB Ultrochrom GTi system was used with a 300 Å pore SynChropak RP-4 column (250 × 4.6 mm I.D., SynChrom, Lafayette, Ind.). Neither precolumns nor guard columns were used. Solvent A was an 80:20 mixture of 0.1% aqueous trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile, and solvent B consisted of these solutions in a 40:60 ratio, as described by Shelton et al. [14]. Solvents were degassed with helium prior to use. The gradient ranged 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. After each gradient, the solvents were maintained at final percentages of solvent B for 5 min and then returned to starting conditions in 2 min. Re-equilibration was carried out for at least 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 ratio of Hb F to total Hb was determined by measuring relative peak areas of non-α-globin chains on the chromatograms. Other technical details have been described elsewhere [13].

Statistical analysis. Statistical analysis was performed by multiple comparison and the regression line was calculated using the least-square method.

Results

Figure 1 illustrates typical chromatograms from extracts of adult and umbilical cord bloodstains after varying periods of aging. Only small differences between the chromatographic profiles of hemolysates and those of stain extracts aged 1 week could be observed for either adult or cord samples, except for the tailing preceding heme on the chromatograms of the extracts. An increase in the relative peak area of β-chains and decreases in those of γ-chains were observed relative to the age of the blood donor, especially in the case of infants. With older bloodstains, extra peaks appeared before and after the native globin chains and were often only partially separated from the native major chains. However, the chromatographic profiles of cord and neonatal bloodstains could easily be distinguished from those of adults in stains up to 32 weeks old.

Table 1 shows levels of Hb F in hemolysates from cord and neonatal samples and in the bloodstain extracts. Samples numbered 1–6 were from cord blood and those numbered 7–11, from peripheral blood of neonates less than 3 weeks old. Data from cord samples up to 12 weeks old have been reported in a previous paper [13]. The

Table 1. Levels of Hb F in hemolysates and extracts of bloodstains from umbilical cord (samples 1–6) and neonatal (samples 7–11) samples. Values of Hb F are expressed as percentages of total Hb

Sample no.	Hemolysate	Weeks of storage						
		1	2	4	8	12	16	20
1	83.5	81.1	80.7	82.3	80.3	77.6	82.1	77.7
2	85.4	82.8	82.2	83.4	78.7	78.3	82.5	71.9
3	88.7	85.2	85.1	86.1	79.6	85.4	82.3	82.8
4	85.7	82.6	82.0	82.2	83.5	80.7	81.5	82.7
5	90.3	91.3	91.0	89.2	90.3	91.2	87.8	91.9
6	89.9	88.0	85.2	88.1	89.2	91.0	90.0	90.0
7	88.4	84.5	84.0	86.6	81.1	77.2	82.2	81.5
8	84.6	73.5	75.4	74.0	74.4	76.0	71.8	69.0
9	88.0	86.1	84.8	86.3	83.9	79.5	81.1	86.4
10	82.2	78.8	78.4	75.6	76.9	75.7	68.0	69.7
11	88.5	86.1	89.0	87.0	87.9	87.4	86.7	79.9

Table 2. Changes in Hb F levels of bloodstains from infants aged 6–18 weeks over time. Values of Hb F are expressed as a percentage of total Hb

Sample no.	Weeks after birth	Hemolysate	Weeks of storage						
			1	2	4	8	12	16	20
12	6	74.9	67.2	67.4	67.1	51.7	43.5	53.9	56.9
13	6	74.5	68.8	72.6	72.1	69.4	67.7	52.4	55.5
14	7	59.9	48.7	53.0	47.6	38.8	34.5	28.9	27.5
15	9	57.7	47.5	45.2	42.4	44.2	29.2	29.0	28.8
16	8	52.9	44.2	44.9	40.5	27.6	29.7	17.3	30.7
17	12	37.9	25.2	27.0	21.0	17.0	11.5	19.5	15.2
18	9	35.5	23.5	22.2	21.8	21.1	14.4	3.9	N.D.
19	11	32.9	28.4	24.9	25.1	21.8	16.0	7.6	12.8
20	18	21.9	16.5	13.9	12.1	8.0	8.1	7.5	N.D.
21	17	18.9	13.8	12.7	12.0	7.8	5.1	4.7	1.7

N.D.; not determined

Table 3. Changes in ratios of the α-chain area to the heme area over time. Values represent the mean ± SD of the ratios

Group	*n*	Weeks of storage						
		1	2	4	8	12	16	20
Cord	6	2.61 ± 0.12	2.56 ± 0.21	2.43 ± 0.19	2.09 ± 0.06	1.94 ± 0.08	1.78 ± 0.14	1.64 ± 0.09
Neonate	5	2.57 ± 0.14	2.50 ± 0.06	2.29 ± 0.09	2.07 ± 0.21	1.93 ± 0.16	1.85 ± 0.14	1.85 ± 0.39
Infant	20	2.48 ± 0.15	2.30 ± 0.12	2.19 ± 0.19	1.96 ± 0.22	1.90 ± 0.25	1.91 ± 0.24	1.86 ± 0.39
Adult	5	2.65 ± 0.04	2.62 ± 0.03	2.53 ± 0.07	2.42 ± 0.07	2.03 ± 0.12	1.96 ± 0.16	1.75 ± 0.09

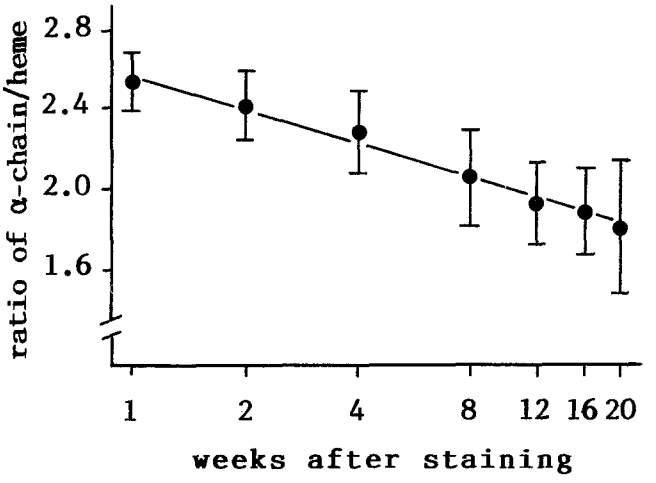

Fig. 2. Relationship between stain age and the ratio of α-chain area to heme area. Weeks after staining are plotted on a logarithmic scale. Each *point* represents the mean ± SD of 36 values (6 cord, 5 neonate, 20 infant and 5 adult samples)

chromatographic profiles illustrate that globin chain denaturation proceeds gradually in bloodstains but little change is noted in Hb F levels for up to 20 weeks.

Table 2 shows levels of Hb F in hemolysates from 10 infants ranging in age from 6 to 18 weeks and time-dependent changes in the levels of the bloodstain extracts. In contrast to results from cord and neonatal samples, Hb F levels decreased gradually with time. The Hb F levels of hemolysates from the other 10 infants examined in this study, whose ages were 19–47 weeks, ranged from 7.8% to 0.7%. Using 4-week-old stains, no Hb F was detected in 4 of the 10 samples; with 20-week-old stains, no Hb F was observed in any of the samples. Adult Hb F levels could not be determined from the chromatograms because of the low levels of γ-chains.

Table 3 shows changes over time in the ratios of the α-chain area to the heme area on the chromatograms. They decreased gradually over time. No significant difference in the ratios of cord and neonatal groups or between cord and adult groups was observed in the time periods examined. At 2 and 4 weeks, the ratios from the infant group were statistically lower than those from the cord and adult groups ($P < 0.05$). At 8 weeks, ratios from the infant group were lower than those from the adult group ($P < 0.001$). At 12 weeks or older, there was no significant difference in this ratio between any of the 4 groups.

Figure 2 illustrates the relationship between stain age and the ratio of α-chain area to heme area. The total number of samples examined was 252. The ratios show good linearity when time is plotted on a natural logarithmic scale. The regression equation calculated from the ratios (y) and the stain age in weeks (x) expressed logarithmically is $y = 2.5758 - 0.2497 \ln(x)$, and the coefficient of correlation is -0.7491 ($P < 0.001$).

Discussion

Bloodstains are an important and useful material in the field of forensic science. Stains discovered at the scene of an accident or crime provide the basis for a wide range of investigative studies. The first and most important

step is to determine whether a stain is actually of blood origin. The second step is to determine the species. Then it is often classified with regard to sex, age, fetal blood, menstrual blood, various blood groups, etc. as summarized by Lee [15] and Oepen [16]. In some cases, stains submitted for examination may be very small and procedures require speed, simplicity, reliability and sensitivity. Many forensic scientists have therefore attempted to develop methods for the examination of bloodstains with these considerations in mind.

In this study, we developed a one-step HPLC method for the identification of Hb F and the simultaneous estimation of bloodstain age. Our method can distinguish cord and neonatal bloodstains from those of adults for up to 32 weeks by comparing their characteristic profiles. Hb F levels in cord and neonatal bloodstains remained constant for up to 20 weeks, while levels in infants above 6 weeks of age decreased gradually with time. With these older infants, it is thought that peak areas of the native globin chains on the chromatograms gradually lose precision. This is probably due to extra peaks and an unsteady baseline, both of which appeared as time elapsed. The extra peaks, which eluted in the proximity of the native globin chains, are thought to be denaturation products of these chains and often separated incompletely.

The chromatograms were also used to reveal bloodstain age by measuring the ratios of the α-chain area to the heme area. This ratio is theoretically unaffected by levels of Hb F or whether the sample was of adult or cord origin, because 2 of the 4 subunits constituting normal Hb are always α-globin chains. In the present study, no significant difference in this ratio was found between cord and neonatal bloodstains for the time periods examined, and none between cord and adult stains. However, the present data also show that the ratios obtained with infant bloodstains differed from those of cord and adult stains at certain periods. This may also result from the extra peaks and unsteady baselines, as mentioned above. These extra peaks and distortions of the chromatographic profiles have also been observed in animal samples [17]. The ratios of the α-chain area to the heme area on the chromatograms from animal bloodstains might also decrease in the same manner. Technical improvements to ensure clearer separation of the native globin chains from the denatured ones are necessary for a more precise estimate of the age of a stain and to determine the level of Hb F in infant bloodstains.

In the present study, bloodstains were made from blood samples collected in EDTA or heparin. According to Schroeder and Huisman [18], the type of anticoagulant is unimportant for chromatography, for qualitative and quantitative separation of normal and abnormal Hb, and for microchromatographic methods. It is therefore normally sufficient to prepare the samples from blood diluted with several volumes of water. It was equally conceivable that anticoagulant had no effect on the determination of Hb F or changes over time in the chromatographic profiles.

It is generally known that the antigenic reaction of Hb extracted from bloodstains with antiserum specific for the protein decreases with the lapse of time. The present study shows that deformation of β-chains progresses faster than that of α-chains, as determined by chromatographic profiles. Structural changes of native globin chains in bloodstains over time are under investigation.

There have been numerous reports on abnormal Hb [19]. Most abnormal Hb is a result of point mutations in globin chains. For example, sickle cell Hb (Hb S) and Hb C differ from the normal adult Hb (Hb A) by a single substitution in the respective β-chain at the 6th residue from the N terminus, although the α-chains are quite normal. On the basis of results from Shelton et al. [14] and Kutlar et al. [20], it is assumed that our HPLC procedure can separate normal α-chains from abnormal β-chains, such as β^S, β^C, β^E, $\beta^{O\text{-}Arab}$, etc. Therefore, the present method for estimation of bloodstain age should be suitable for β-chain variants.

Many investigators have observed that their methods for determination of bloodstain age are affected by various environmental conditions during storage, such as temperature, humidity, exposure to sunlight and substrate [3, 5, 7, 10–12]. Most groups observed changes in Hb using spectrophotometry or electron spin resonance and utilized these changes as an index for estimating the age of a bloodstain. Likewise, we determined the level of native α-chains and correlated it with the heme level. This ratio would also be influenced to some extent by these environmental conditions. This influence must be studied to ensure more exact estimation of bloodstain age.

Although further investigations are necessary for perfecting this technique for practical use, the HPLC method, with its advantages of simplicity, speed and sensitivity, should prove to be of great value to forensic science.

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