CHROMBIO 4537

QUANTITATION OF HEMOGLOBINS BART'S, H, PORTLAND-I, PORTLAND-II AND CONSTANT SPRING BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

FERDANE KUTLAR, LI HAO GU, HUAIYU HU and TITUS H.J. HUISMAN*

Department of Cell and Molecular Biology, Laboratory of Protein Chemistry, Medical College of Georgia, Augusta, GA 30912-2100 (U.S.A.)

SUMMARY

We introduce a new high-performance liquid chromatographic procedure that uses a specific anion-exchange column for the separation of hemoglobin (Hb) Bart's (γ_4), Hb H (β_4), Hb Portland-I ($\zeta_2\gamma_2$), Hb Portland-II ($\zeta_2\beta_2$), and the abnormal Hb Constant Spring (α_2 extended β_2) in cord blood and adult red cell lysates. The method provides quantitative data for Hb Bart's in cord blood that correlate well with the α-globin gene status of the babies and can be used for an initial identification of α-thalassemic conditions. Quantitation of Hb Bart's from cord blood samples that are collected on filter paper and submitted as dried blood spots is unreliable. The separation of Hb H and Hb Bart's allows an evaluation of the synthesis of these two hemoglobin components in patients with Hb H disease.

INTRODUCTION

Several unusual hemoglobins (Hbs) can be observed in blood samples from newborn and/or adult patients with (severe) α-thalassemia (α-thal). These variants are Hb Bart's or γ_4 [1], Hb H or β_4 [2], the embryonic Hb Portland-I or $\zeta_2\gamma_2$ [3], Hb Portland-II or $\zeta_2\beta_2$ [4], Hb Portland-III or $\zeta_2\delta_2$ [4], and Hb Constant Spring (Hb CS or $\alpha_2^{CS}\beta_2$) [5], a variant which is characterized by an α-chain that is 31 residues longer than the normal α-chain. These Hb types can be detected by (a combination of) electrophoretic and/or chromatographic procedures. Here we describe a rather simple and reproducible high-performance liquid chromatographic (HPLC) procedure that allows the rapid isolation and quantitation of these variants in less than 50 min. The method makes use of a weak anion exchanger, Tris buffer–KCN mobile phases, and an NaCl gradient for elution. The procedure appears to be most useful for the quantitation of Hb Bart's and the

embryonic Hbs in cord blood samples, and of Hb H, Hb Bart's, and Hb CS in samples from adults with certain forms of α-thal.

EXPERIMENTAL

Blood samples

Cord blood samples from Black newborns were collected in vacutainers with EDTA as anticoagulant. A similar blood sample was collected from an East-Asian stillborn with hydrops fetalis; this material was shipped in wet ice from Honolulu, HI, U.S.A. to Augusta, GA, U.S.A. and was received within 48 h. Samples from subjects with Hb H disease were from adult patients living in Macedonia, Yugoslavia and in Turkey; these samples were shipped in wet ice, airmail special delivery. Samples from members of a family with Hb CS were shipped in a similar manner from Nanning, P.R. China. Blood samples were stored at $4\,^\circ$C, while red cell lysates were kept at $-70\,^\circ$C. Informed consent was obtained.

Hb analyses

All red cell lysates were analyzed by isoelectric focusing (IEF) [6], by cation-exchange HPLC [7], and many by reversed-phase HPLC (RP-HPLC) to evaluate the composition of the globin chains [8–10]. Fetal Hb was quantitated by alkali denaturation [11], and Hb A_2 by cation-exchange HPLC [7] and by microcolumn chromatography [12].

Separation of Hb Bart's (γ_4), Hb H (β_4), Hb Portland-I $(\zeta_2\gamma_2)$, Hb Portland-II $(\zeta_2\beta_2)$, and Hb CS $(\alpha_2$ extended $\beta_2)$ by anion-exchange HPLC

The method used a 250 mm $\times$ 4.6 mm I.D. BIO-SIL-TSK-DEAE-SW column (125 Å; 5 μm) obtained from Bio-Rad (Richmond, CA, U.S.A.). Mobile phase A was 0.03 M Tris buffer, 0.15 M NaCl, and 100 mg KCN per 1000 ml (pH 8.3). Mobile phase B was 0.03 M Tris buffer and 100 mg KCN per 1000 ml (pH 8.3). Each chromatogram required 50 min to complete at a flow-rate of 0.7 ml/min; the program involved the following gradients: T_1 (17 min) 40% A→45% A (60% B→55% B); T_2 (15 min) 45% A→95% A (55% B→5% B); purge (8 min) with 99% A (1% B); equilibration (10 min) with 40% A (60% B). A slower gradient was applied for the separation of Hb CS from Hb A_2 and Hb A; this was achieved by extending the time during which certain gradients were developed. For instance, Hb CS can readily be separated from Hbs A and A_2 by applying a gradient of 15% mobile phase A (85% B) to 70% mobile phase A (30% B) over a period of 45 min at a flow-rate of 0.7 ml/min. Equilibration of the column is done for 10 min with a mixture of 15% mobile phase A and 85% mobile phase B.

DNA analyses

The number of α-globin genes was determined by gene mapping using methodology described before [13,14]; the same procedures were followed in identifying the large deletions on both chromosomes which resulted in hydrops fetalis.

RESULTS

Identification of Hb Bart's and the Portland Hbs by anion-exchange HPLC

The initial detection of Hb Bart's was by IEF which allowed the selection of cord blood samples to be used in this study. Fig. 1 (top) provides a chromatogram of the Hb from baby No. 3091 who was found to have a "large quantity" of Hb Bart's by IEF and 1% ζ-chain by RP-HPLC. Hb Bart's was eluted after the Hbs A, F_0, F_1, and X (to be identified below) and was present for 6%. The component was nearly pure when studied by RP-HPLC (Fig. 1, bottom); some 95% was $^G\gamma$- and $^A\gamma$-chains, while only a small amount of α-chain and minute quantities of β- and ζ-chains were present. The method gives quite reproducible data (not shown), and removal of plasma proteins, although desirable, is not required (Fig. 2).

The quantity of Hb Bart's in these samples is dependent upon the degree of α-chain deficiency (α-thal) which can be evaluated by gene mapping. Baby No.
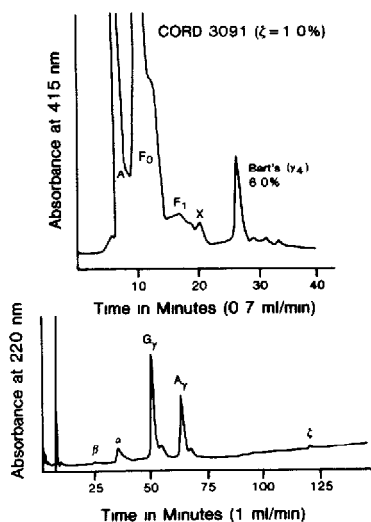

Fig. 1. Top: separation of Hbs from a cord blood sample of baby No. 3091 with $-\alpha/-\alpha$ by anion-exchange HPLC; X = $\zeta_2\gamma_2$. Bottom: chain composition analysis by RP-HPLC of the isolated Hb Bart's component.
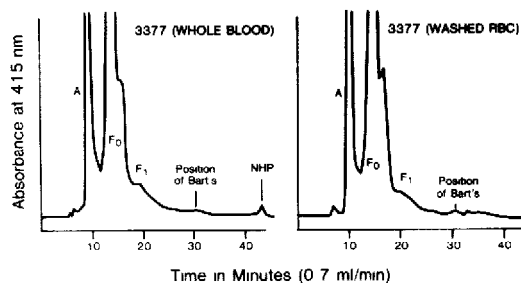

Fig 2 Separation of Hbs and non-heme proteins (NHP) from a cord blood sample of baby No. 3377 with $\alpha\alpha/\alpha\alpha$ by anion-exchange HPLC. RBC = red blood cells.

3091 (Fig. 1) with 6% Hb Bart's was found to have an α-thal-2 homozygosity [$-\alpha(-3.7)/-\alpha(-3.7)$]. Baby No. 3419 with 2.8% Hb Bart's had the same α-thal-2 homozygosity, while baby No. 3270 with 0.7% Hb Bart's had a heterozygosity for this deficiency [$-\alpha(-3.7)/\alpha\alpha$] and baby No. 3268 (with nearly no Hb Bart's) was normal ($\alpha\alpha/\alpha\alpha$; Fig. 3).

The method has been compared with a simple microchromatographic procedure that uses small CM-cellulose (CMC) columns and mobile phases of 0.03 M Bis–Tris, 0.01% KCN, and HCl (pH 6.2) with 0.001 M NaCl added for the elution of Hb Bart's and 0.2 M NaCl added for the elution of the remaining Hb [15]. This method is most useful for distinguishing lower quantities of Hb Bart's (as in the $-\alpha/\alpha\alpha$ deficiency) from larger quantities (as in the $-\alpha/-\alpha$ deficiency) but has the disadvantage that non-Hb proteins which absorb slightly at 415 nm are calculated as Hb Bart's, thus erroneously increasing the "baseline Hb Bart's level". Fig. 4 provides the data for more than 70 cord blood samples (Black babies only) obtained by the micro-CMC procedure and by the new anion-exchange HPLC technique; DNA data are available for 37 newborns (16 had $\alpha\alpha/\alpha\alpha$; 5
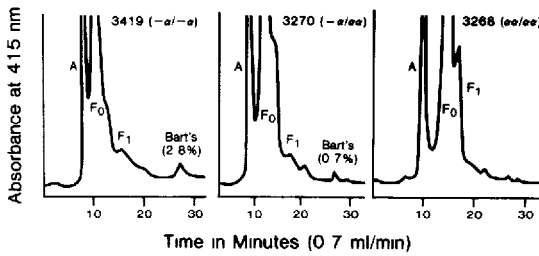

Fig. 3. Separation of Hbs from three cord blood samples by anion-exchange HPLC. Baby No. 3419 had an α-thal-2 homozygosity ($-\alpha/-\alpha$) with a low level of Hb Bart's; baby No. 3270 had an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$), and baby No. 3268 was normal ($\alpha\alpha/\alpha\alpha$).
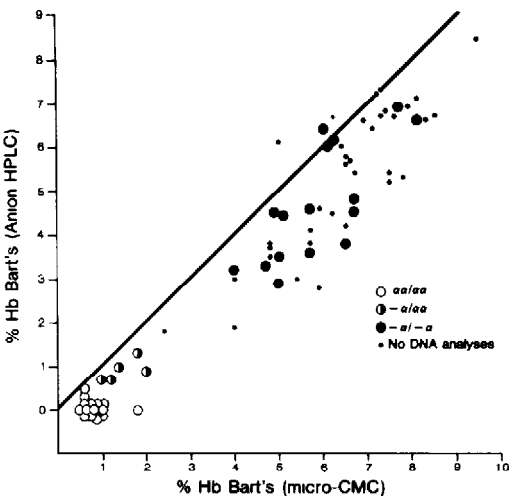

Fig. 4. Correlation of the quantities of Hb Bart's determined by anion-exchange HPLC and by micro-CMC chromatography. Total number of babies. 71.

had $-\alpha/\alpha\alpha$; 16 had $-\alpha/-\alpha$). As expected, the values by CMC are always higher ($\sim 1\%$ "Hb Bart's") than those by the HPLC procedures. The Hb Bart's level in normal babies ($\alpha\alpha/\alpha\alpha$), determined by the new anion-exchange HPLC method, is mostly zero (none detectable in 14 of 16 babies; 2 babies had 0.2 and 0.5%, respectively), that in babies with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$) varied between 0.7 and perhaps 2%, while that in babies with the α-thal-2 homozygosity ($-\alpha/-\alpha$) varied from 2.8 to 8.4%.

Cord blood samples with notable quantities of Hb Bart's usually contain between 0.5 and 3% of the embryonic ζ-chain (as percent of total $\alpha + \zeta$; see ref. 10); the level of ζ-chain apparently increases with a decrease in the number of α-globin genes. Fig. 5 compares similar data for more than 60 newborn babies; all babies without Hb Bart's had an average value for ζ of 0.02%, while higher ζ-chain levels are apparent for babies with higher levels of Hb Bart's (i.e. with an α-thal-2 homozygosity or $-\alpha/-\alpha$). This ζ-chain is present as Hb Portland-I or $\zeta_2\gamma_2$ [3] which can be observed in many chromatograms although this Hb type does not separate well from the modified Hb F_1 components. Fig. 6 provides three examples; component X was identified as $\zeta_2\gamma_2$ through chain analysis of isolated Hb by RP-HPLC (data not shown).

The mobilities of two embryonic Hbs, Hb Portland-I or $\zeta_2\gamma_2$ and Hb Portland-II or $\zeta_2\beta_2$, in this chromatographic system are amply demonstrated in Fig. 7. The chromatogram on the left concerned the Hb of a red cell lysate from a stillborn fetus with homozygous α-thal-1 ($--/--$), a condition in which no α-chains
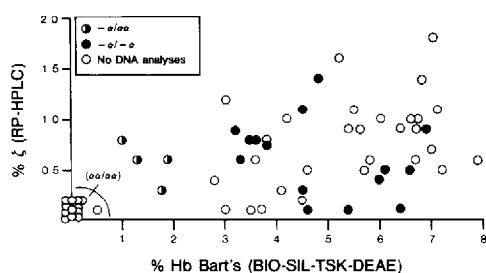

Fig. 5. Relationship between the levels of Hb Bart's (by anion-exchange HPLC) and those of the ζ-chain (by RP-HPLC) for 63 cord blood samples.
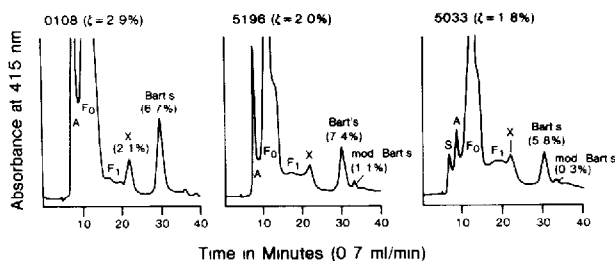

Fig. 6. Separation of Hbs by anion-exchange HPLC. The three babies had an α-thal-2 homozygosity ($-\alpha/-\alpha$) with high levels of ζ-chain and Hb Bart's. All three samples contained component X that was (partially) separated from Hb F_1; separation can be improved by using a slower gradient. Component X in the sample from baby No. 0108 was identified as $\zeta_2\gamma_2$ by RP-HPLC.
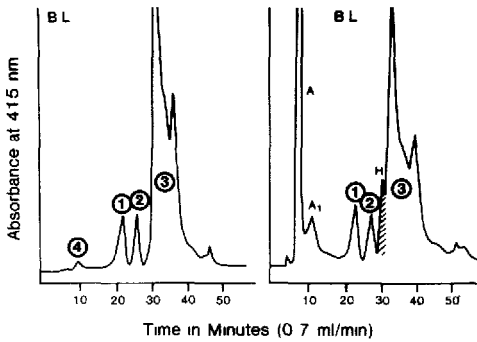
Fig. 7 Left: separation of the Hb components present in a blood sample from fetus B.L. with hydrops fetalis ($- - / - -$) by anion-exchange HPLC. Component 1 = Hb Portland-I ($\zeta_2\gamma_2$); component 2 = Hb Portland-II ($\zeta_2\beta_2$); component 3 = Hb Bart's or γ_4; component 4 = Hb Portland-III or $\zeta_2\delta_2$. Right: the same but for a mixture of the sample with a sample from an adult patient with Hb H disease.
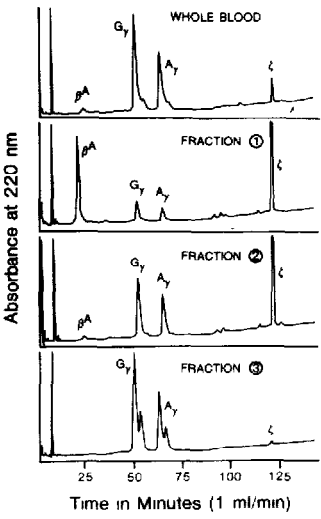

Fig. 8. Identification of isolated Hb components by RP-HPLC. The fractions refer to the Hb zones of Fig. 7.

are synthesized. Three Hb components were identified (labeled 1, 2, and 3) which were further analyzed by RP-HPLC (Fig. 8). Component 1 consisted of ζ- and β-chains (with some $^G\gamma$- and $^A\gamma$-chains as impurities) and can therefore be identified as Hb Portland-II. Component 2, consisting primarily of ζ- and $^G\gamma$- and $^A\gamma$-chains, was identified as Hb Portland-I (a mixture of $\zeta_2{}^G\gamma_2$ and $\zeta_2{}^A\gamma_2$). Fraction 3 contained $^G\gamma$- and $^A\gamma$-chains only (Hb Bart's). When a small quantity of an adult lysate containing Hb H was added to baby B.L.'s hemolysate and the mixture was chromatographed, it was possible to compare the mobilities of these embryonic Hbs with that of Hb H (Fig. 7, right) indicating distinct differences between the chromatographic properties of these Hb components. The small zone, labeled 4 in the chromatogram of Fig. 7 (left), was found to be impure Hb Portland-III ($\zeta_2\delta_2$) [4].

Separation of Hb H and Hb Bart's in adult red cell lysates

The isolation and quantitation of Hb H (or β_4), which is found in blood from subjects with a severe form of α-thal $(-\alpha/--)$, was readily possible by the anion-exchange HPLC procedure; this Hb eluted at about 25 min, well separated from the adult Hb components (Fig. 9, left). When the same sample was mixed with the cord blood red cell lysate from baby No. 3091 which contained 6% Hb Bart's (Fig. 1), a nearly complete separation of the two components was noted (Fig, 9, right). Attempts to obtain similar separations on various other weak anion-exchange HPLC columns were not successful. The successful separation of Hb H and Hb Bart's made it possible to evaluate the presence of these two Hb types in adult subjects with Hb H disease. Samples from 20 subjects were available. Red cell lysates had been stored at $-70\,^\circ$C for various periods of time; under these conditions the Hb Bart's level is not affected, while that of Hb H gradually decreases due to the considerable instability of this β_4 tetramer. Fig. 10 provides two chromatograms; the one on the left is from a sample with Hb H only and that on the right is from a sample of a patient who had both Hb H and Hb Bart's. Of the twenty cases studied, six had Hb H (1.1–9.3%) without Hb Bart's, two had no Hb H and Hb Bart's only (1.4 and 8.5%), and twelve had Hb H (1.1–13.4%) and Hb Bart's (0.3–6.1%). Variations like these are due to technical difficulties in obtaining and shipping the blood samples (reducing the level of Hb H) as well as to the existence of various genotypes resulting in different phenotypes (to be discussed elsewhere).
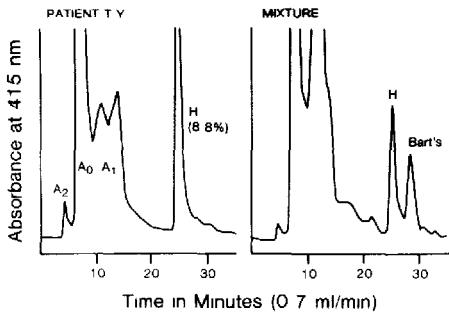

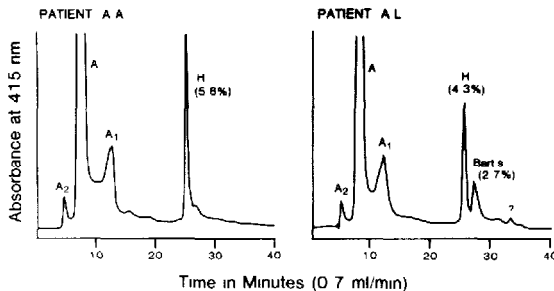Fig 9. Separation of Hb H and Hb Bart's by anion-exchange HPLC.

Fig. 10. Quantitation of Hb H and Hb Bart's by anion-exchange HPLC. The two patients suffer from Hb H disease $(--/-\alpha)$.

Quantitation of Hb CS

This Hb abnormality is characterized by the presence of an extended α-chain (with 172 residues instead of the normally occurring 141 residues) due to a mutation within the terminating codon [5]. In the heterozygote, Hb CS is present in quantities of less than 0.5%. Because of its decreased synthesis and increased instability, a carrier of Hb CS has α-thalassemic features, which is particularly evident in the homozygote ($\alpha\alpha^{CS}/\alpha\alpha^{CS}$). Hb CS readily separated from Hb A$_2$ and Hb A by anion-exchange HPLC provided an extended gradient was used (see methodology). Fig. 11, left and top, illustrates a chromatogram for a Chinese patient with an Hb CS homozygosity. The Hb CS level was as high as 3%, while the level in the heterozygous parents was 0.2–0.3% (Fig. 11). The method is rather sensitive and allows the detection of a small quantity of Hb Bart's in the homozygotous patient. Chain analysis of the isolated peak confirmed its identity; the possible presence of Hb H (β_4) in the blood sample of this patient cannot be excluded as this Hb type might have precipitated within the cells during the rather long journey between Nanning, P.R. China and Augusta, GA, U.S.A.

The "slow-moving" Hb components which include Hb A$_2$ and Hb CS could also be isolated from the red cell lysate of the propositus using macro-DEAE-
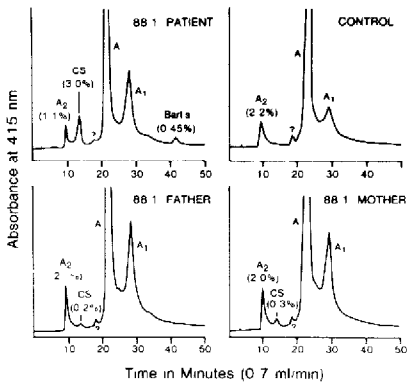

Fig. 11. Separation and quantitation of Hb CS by anion-exchange HPLC. Family 88 is from Nanning, P.R. China. The separation of Hb A$_2$ and Hb CS was possible when a slower gradient was used (see text).
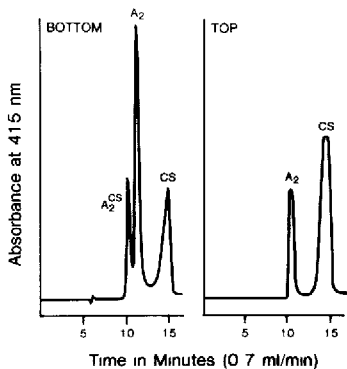

Fig. 12. Separation of the Hbs A$_2$, CS, and A$_2^{CS}$ (or $\alpha_2^{CS}\delta_2$) by anion-exchange HPLC. The "top" and "bottom" fractions were isolated by preparative DEAE-cellulose chromatography

cellulose chromatography [16]. The resulting Hb zone was collected as two fractions which were analyzed by the anion-exchange HPLC procedure (Fig. 12). The bottom zone contained mainly Hb A_2 and Hb CS, while the elution of Hb A_2 was preceded by that of an Hb that is believed to be composed of α^{CS}- and δ-chains (A_2^{CS} or $\alpha_2^{CS}\delta_2$). The top fraction consisted primarily of Hb CS or $\alpha_2^{CS}\beta_2$ and some Hb A_2.

DISCUSSION

Quantitation of Hb Bart's is often used to evaluate the α-globin gene status of a newborn, because the level of this tetramer of the γ-chain is directly proportional to the number of active α-globin genes. Methods used are scanning after electrophoretic separation, elution from the cellulose acetate strip after electrophoretic separation, and micro-CMC chromatography. The method described here is another simple procedure that can also be used for this purpose. It has the added advantage that the presence of the embryonic Hb Portland-I ($\zeta_2\gamma_2$) is readily indicated. Material to be used for this purpose should be red cell lysates from freshly collected blood samples or lysates or red cell suspensions that have been stored at $-70\,^\circ$C for an indefinite time.

There are numerous hemoglobinopathy testing programs in the U.S.A. and elsewhere that use blood samples collected on filter paper. This approach greatly facilitates the collection procedure, particularly when it is combined with testing programs for other inborn errors. Numerous investigators have objected to this methodology because of a decreased accuracy in detecting (and identifying) common Hb variants (Consensus Meeting for Testing for Hemoglobinopathies, Washington, DC, 1987). Similar problems have been encountered when a few cord blood samples were stored under various conditions; as indicated by the data listed in Table I, Hb Bart's cannot be accurately quantitated by the new HPLC procedure when the blood is collected on filter paper and red cell lysates are prepared by elution of the dried blood spots.

Identification of the Portland Hbs in cord blood samples of certain newborns is greatly facilitated by the anion-exchange HPLC procedure (Fig. 7) and the ability to detect Hb Portland-I ($\zeta_2\gamma_2$) in some cord bloods from babies with an α-thal-2 homozygosity was a pleasant surprise (Fig. 6). The levels of ζ-chain, however, varied greatly (Fig. 5) and it appears doubtful that quantitation of this ζ-chain allows definitive differentiation between babies with two ($-\alpha/-\alpha$) or three ($-\alpha/\alpha\alpha$) active α-globin genes.

The anion-exchange HPLC method can be most useful for the differentiation of the "fast-moving" Hbs in blood from patients with Hb H disease ($-\alpha/--$) because it allows the separation of Hb H (β_4) and Hb Bart's (γ_4) (Figs. 9 and 10). However, it is mandatory to apply this technique immediately after the collection of the sample to avoid any decrease in the level of Hb H. Finally, with a modified gradient that decreases the elution rates of the Hb types it is possible to quantitate Hb CS, an Hb variant which is responsible for certain types of α-thal. This Hb type can even be quantitated in blood samples from simple heterozygotes (Fig. 11).

TABLE I

QUANTITIES OF Hb BART'S IN CORD BLOOD SAMPLES, COLLECTED ON FILTER PAPER AND STORED AT DIFFERENT CONDITIONS

Case No.	Hb Bart's quantity (%)			
	Freshly collected[a]	On Whatman filter paper[b]		
		At room temperature (day 6)[c]	At 4°C (day 6)	At −70°C (day 8)
05935	7.3	1.3	2.1	3.0
05938	6.0	1.4	4.9	2.7
05939	4.7	0.7	?[d]	3.0
05940	6.2	3.1	3.4	4.6
05942	4.4	1.4	3.2	2.4

[a]In vacutainers with EDTA as anticoagulant.
[b]Major complications were irregular baseline which interfered with calculation; occurrence of extraneous zones.
[c]Sample was kept between 20 and 35°C as it was mailed in the summer using regular postal services.
[d]Calculation was not possible.

ACKNOWLEDGEMENTS

REFERENCES

1   J.A. Hunt and H. Lehmann, Nature (London), 184 (1959) 872.
2   R.T. Jones, W.A. Schroeder, J.E. Balog and J.R. Vinograd, J. Am. Chem. Soc., 81 (1959) 3161.
3   G.L. Capp, D.A. Rigas and R.T. Jones, Science, 157 (1967) 65.
4   Z.I. Randhawa, R.T. Jones and L.E. Lie-Injo, J. Biol. Chem., 259 (1984) 7325.
5   J.B. Clegg, D.J. Weatherall and P.F. Milner, Nature (London), 234 (1971) 337.
6   P.G. Righetti, in T.H.J. Huisman (Editor), The Hemoglobinopathies, Churchill Livingstone, Edinburgh, 1986, pp. 47–70.
7   J.B. Wilson, M.E. Headlee and T.H.J. Huisman, J. Lab Clin. Med., 102 (1983) 174.
8   J.B. Shelton, J.R. Shelton and W.A. Schroeder, J. Liq. Chromatogr., 7 (1984) 1969.
9   F. Kutlar A. Kutlar and T.H.J. Huisman, J. Chromatogr., 357 (1986) 147.
10 F. Kutlar, Y.J. Fei, J.B. Wilson, A. Kutlar and T.H.J. Huisman, J. Chromatogr., 394 (1987) 333.
11 K. Betke, H.R. Marti and I. Schlicht, Nature (London), 184 (1959) 1877.
12 C.T H Schleider, S.M. Mayson and T.H.J. Huisman, Hemoglobin, 1 (1977) 503.
13 Y.J. Fei, S. Fujita and T.H.J. Huisman, Br. J. Haematol., 68 (1988) 249.
14 Y.C. Gu, H. Landman and T.H.J. Huisman, Br. J. Haematol., 66 (1987) 245.
15 J.B. Henson, J R. Carver, J.B Wilson and T.H.J. Huisman, J. Chromatogr., 198 (1980) 443.
16 T.H.J. Huisman and J.H.P. Jonxis, The Hemoglobinopathies Techniques of Identification, Marcel Dekker, New York, 1977.