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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF HUMAN HAEMOGLOBINS

SIMULTANEOUS QUANTITATION OF FOETAL AND GLYCATED HAEMOGLOBINS

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SUMMARY

A high-performance liquid chromatographic system, which uses a weak cation exchanger (PolyCATA) together with Bis-Tris buffer (pH 6.47–7.0) and sodium acetate gradients, is described. Samples from adults and newborns were analysed and a clean separation of many minor and major normal and abnormal haemoglobin (Hb) variants was greatly improved. The method allows the separation of minor foetal haemoglobin (HbF) variants and the simultaneous quantitation of HbF and glycated HbA. HbF values correlated well with those obtained by the alkali denaturation method ($r=0.997$). The glycated haemoglobin (HbA_{Ic}) levels measured in patients with high HbF concentrations correlated with the total glycated haemoglobin determined by bioaffinity chromatography ($r=0.973$). The procedure is useful for diagnostic applications and affords an effective and sensitive way of examining blood samples for haemoglobin abnormalities.

INTRODUCTION

Foetal haemoglobin (HbF) is the predominant form of haemoglobin (Hb) in the foetal red blood cells; at birth, the blood of a normal newborn contains up to 80% HbF. After birth the γ-chain synthesis decreases concomitantly with the predominant synthesis of the β-chain, and HbF usually drops to the normal adult level of less than 1% by seven months to two years.

Several neoplastic and normoplastic conditions are associated with the elevated levels of HbF [1,2]. Higher concentrations of HbF are observed in many acquired or congenital anaemias and in leukaemias. Most haemoglobinopathies are accompanied by increased production of HbF. Therefore, an accurate measurement of HbF in the presence of other Hb variants is an essential step in the

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diagnosis and understanding of various haemoglobinopathies. This also holds true for other diseases associated with high or low HbF values.

In the past decade, many procedures have been developed that can be used to identify and quantify normal and abnormal Hbs. Other than the classical celllulose acetate electrophoresis technique, methods reported have involved isoelectric focusing and column chromatography using cation-exchange resins or anion-exchange material [3].

More recently, high-performance liquid chromatographic (HPLC) procedures using ion-exchange hard resin have been devised for the separation, quantitation and characterization of Hb analogues [4–7]. With few exceptions [8], these procedures are not reliable for the quantitation of HbF in samples containing minor adult HbA_I variants. In most cases, HbF co-elutes with minor HbA_I variants, which prevents accurate quantitation. Schroeder et al. [9] developed a method that relies on a dual chromatographic isolation of HbF and amino acid analysis. This procedure may be the most accurate assay for quantifying HbF, but it has the disadvantages of being laborious and not applicable to routine clinical diagnoses. Shukla and Headings [10] developed a radial immunodiffusion technique to quantify HbF using a specific antibody. This method is more sensitive than most, but is more difficult than chromatography.

This report describes an HPLC procedure that yields an excellent separation and quantitation of several normal and abnormal Hb variants of red cell haemolysates from newborns and adults. Minor adult Hb components are clearly separated from each other and from both HbF_I and HbF_0. Many other minor Hb variants not separated by previous chromatographic techniques are also analysed in various red cell haemolysates.

EXPERIMENTAL

Patients

Normal newborns, normal adults, diabetics and patients with a high HbF level, associated with various diseases and haematological disorders, were investigated in this study.

Blood samples

Venous blood was collected in EDTA tubes. Cord blood samples were also collected in EDTA tubes without contamination by maternal blood. The cytochemical staining of HbF (Boehringer Mannheim Kit, F.R.G.) was used to check for the absence of maternal cells. Red cell haemolysates were prepared by haemolysis of red cells washed three times with saline [3].

Electrophoresis of haemoglobins

Red cell lysates were subjected to cellulose acetate electrophoresis at pH 9, as described in ref. 3.

Determination of haemoglobin F by the alkali denaturation method

The procedure used was similar to that described by Pembrey et al. [11].

Acetylation of haemoglobin A

The acetylation of HbA was performed as previously described by Nathan et al. [12]. One volume of washed red cells was incubated at 37°C with four volumes of phosphate-buffered saline (PBS) (pH 6.2) containing 10 mmol/l acetylsalicylic acid. After 6 h, the erythrocytes were washed twice in isotonic saline and the haemolysate was prepared as described above.

HPLC separation of haemoglobin analogues

The equipment consisted of a System L 5000 controller with one pump, equipped with three solvent-delivery systems, a 665A-40 autosampler, a Model L-4200 UV–VIS detector and a D-2000 chromato-integrator, all manufactured by Merck/Hitachi (E. Merck, Darmstadt, F.R.G.). The analytical column was made of stainless-steel tubing (200 mm×4.6 mm I.D.) (Bischoff Analysentechnik und Geräte, Leonberg, F.R.G.). The column was slurry-packed in propan-2-ol with a 5-μm PolyCATA cation exchanger (PolyLC, Columbia, MD, U.S.A.) in our laboratory using a slurry-packer (Shandon Products, U.K.). The chromatographic column was preceded by a guard column filled with the same material. The 20 mm×4.6 mm I.D. guard cartridge and holder were obtained from PolyLC.

Developers and gradient program

Mobile phase A was prepared from 35 m*M* Bis-Tris with 1.53 m*M* (0.1 g/l) potassium cyanide and 3.0 m*M* ammonium acetate. The final pH was adjusted to 6.47 with 20% acetic acid. Mobile phase B contained 35 m*M* Bis-Tris, 1.53 m*M* potassium cyanide, 0.15 *M* sodium acetate and 16.86 m*M* ammonium acetate. The final pH was adjusted to 7.0 with 20% acetic acid.

The column was conditioned with 70% mobile phase A plus 30% mobile phase B for 15 min before and after every analysis. The flow-rate was maintained at 1.0 ml/min, and the pressure of the column was 113 bar. The effluent was monitored at 415 nm, 0.05 a.u.f.s. and 0.25 cm/min (chart-speed). At time 0 (A/B, 70:30) samples (10 μl) containing 100 μg of Hb were applied to the column and Hbs were eluted by increasing the proportion of mobile phase B to 70 and 100% at 68 and 80 min, respectively. At 85 min, the proportion of B was decreased to 30%.

Peak identification

Samples were screened for the presence of common Hbs, such as HbF, HbA, HbS, and HbA_2, by cellulose acetate electrophoresis at pH 9. The retention time of the Hb components separated by HPLC were compared with those of the reference Hb variants isolated by DEAE-cellulose or Bio-Rex 70 chromatography. Further to characterize certain Hb components (HbF, HbA_{Ic}, HbA_{Id3}), the fractions of interest were collected, pooled, concentrated and used for both electrophoresis analysis and separation of the globin chains.

Chain separation

The globin chains present in isolated fractions (HbF, HbA_{Ic} and HbA_{Id3}) were separated by reversed-phase HPLC on a Vydac large-pore C_4 column (25 cm×0.4 cm I.D.). The column was obtained from Macherey-Nagel (Düren, F.R.G.). Two

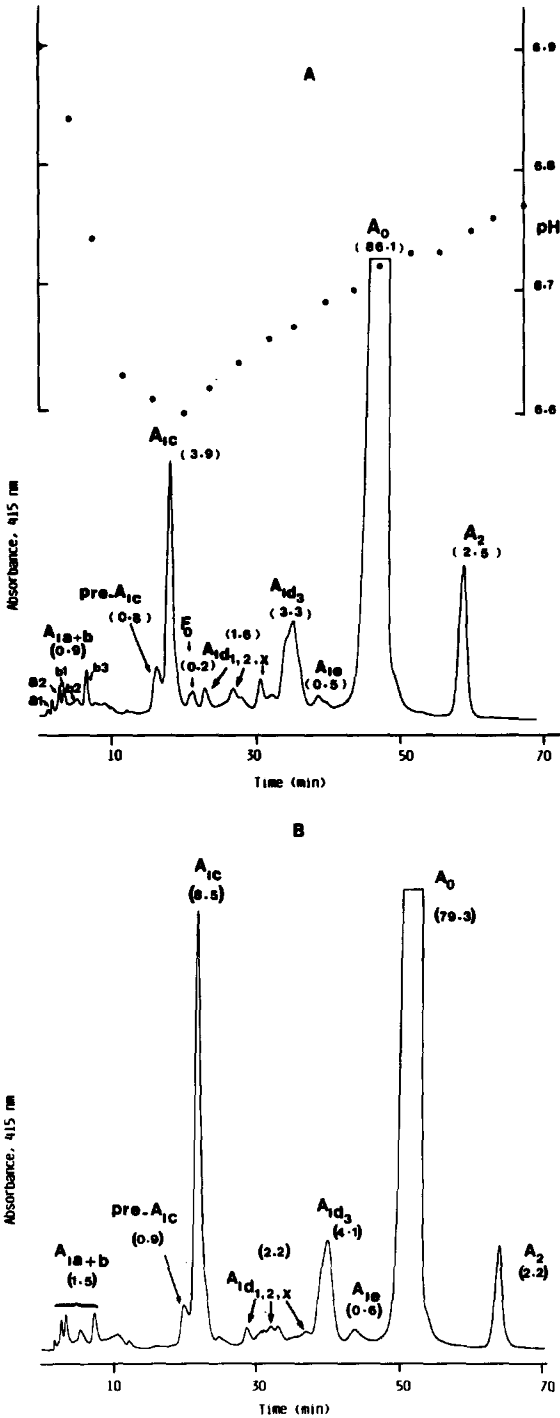
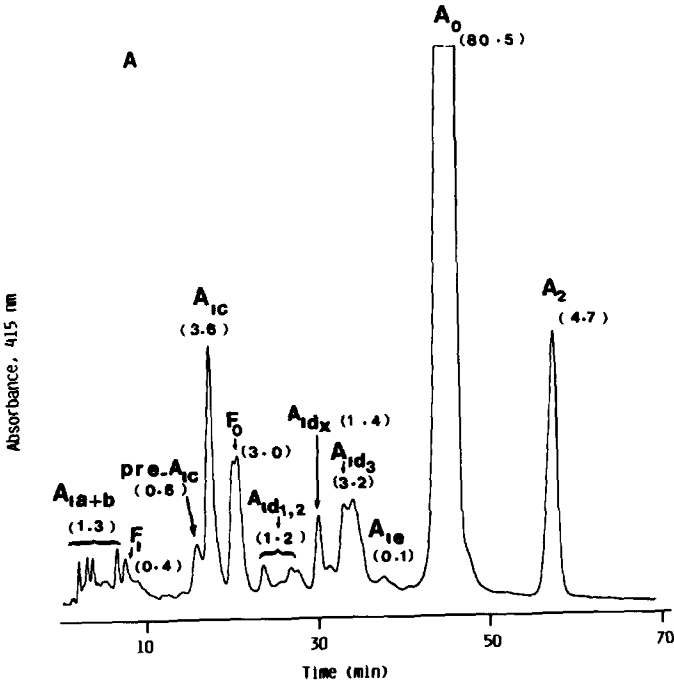


Fig. 1. Separation of Hb variants by ion-exchange chromatography using a combination of pH and ionic gradients. (A) Haemolysate from normal adult; the broken line represents the variation of pH in the effluent. (B) Red cell haemolysate from a diabetic. The numbers in parentheses are percentages.



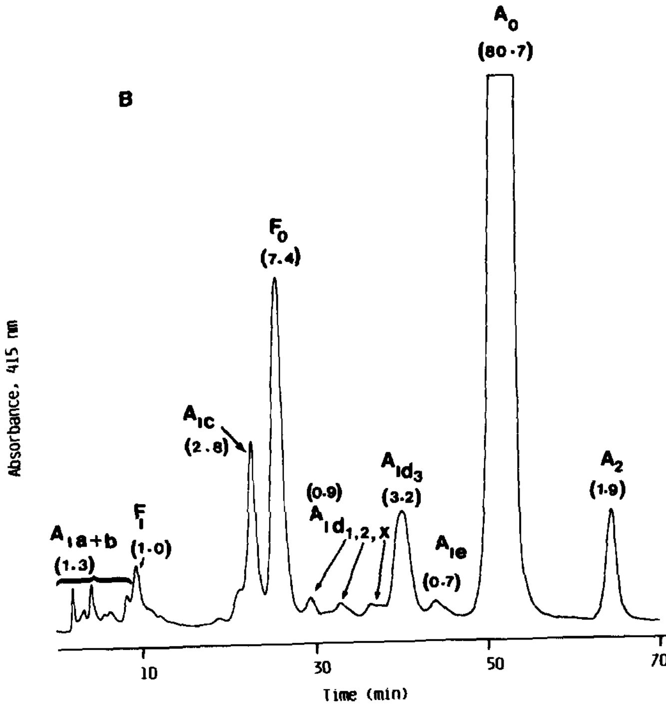


Fig. 2. Separation of Hb components present in freshly prepared haemolysates of (A) a patient with Hb-β-thalassemia and (B) a subject with chronic myeloid leukemia. The numbers in parentheses are percentages.

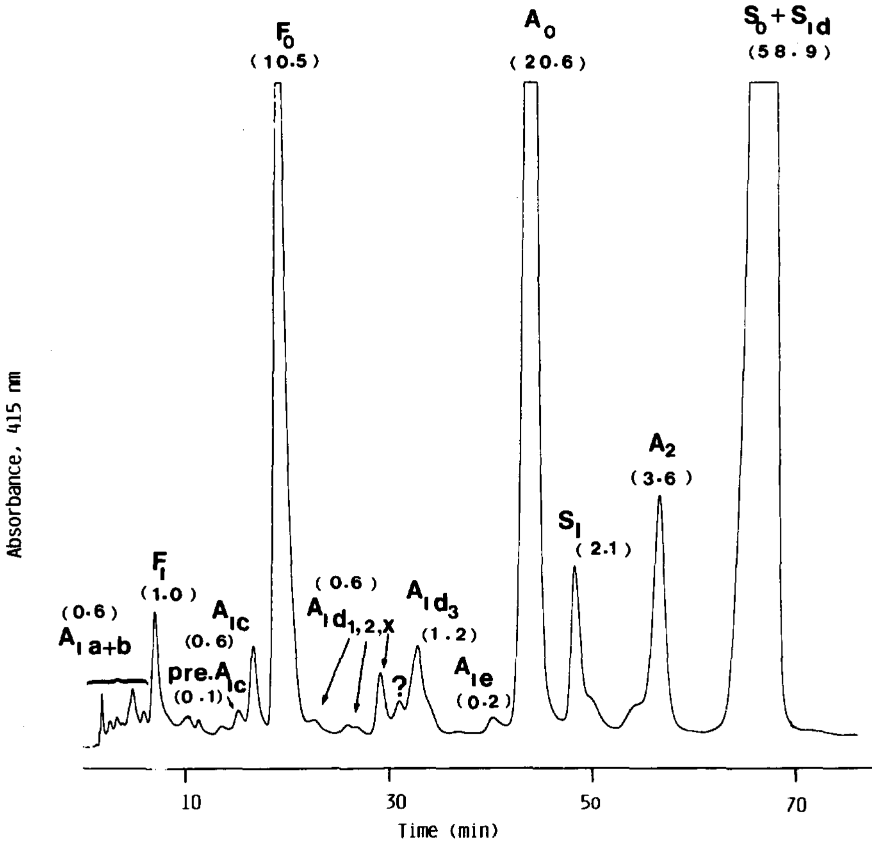


Fig. 3. Separation of minor and major Hb variants present in red cell lysate of a patient with HbS-β^+-thalassemia. The numbers in parentheses are percentages.

eluents were used: eluent A contained 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile (40:60); eluent B contained 0.1% TFA in water and 0.1% TFA in acetonitrile (80:20). The slopes of the gradients were as described before [5,7].

RESULTS

Fig. 1 shows the elution profiles of minor Hbs present in red cell lysate of a normal adult (A) and a diabetic patient (B). Fig. 2 depicts the separation of the Hbs from a subject with β-thalassemia (A) and that of the Hbs of a patient with chronic myeloid leukemia (CML) (B). HbF_I and HbF_o were separated from each other and from the minor HbA_I in both β-thalassemic and CML samples. HbA_{Ic} and HbF_o were also readily separated. The peak close to HbA_{Ic} was designated pre-A_{Ic} and the mean ± S.D. was 0.27 ± 0.34 and 0.62 ± 0.22% in 23 normals and 31 diabetics, respectively. Pre-A_{Ic} vanished partially or completely following treatment at pH 5 [13]. This component increased to twice its initial value after

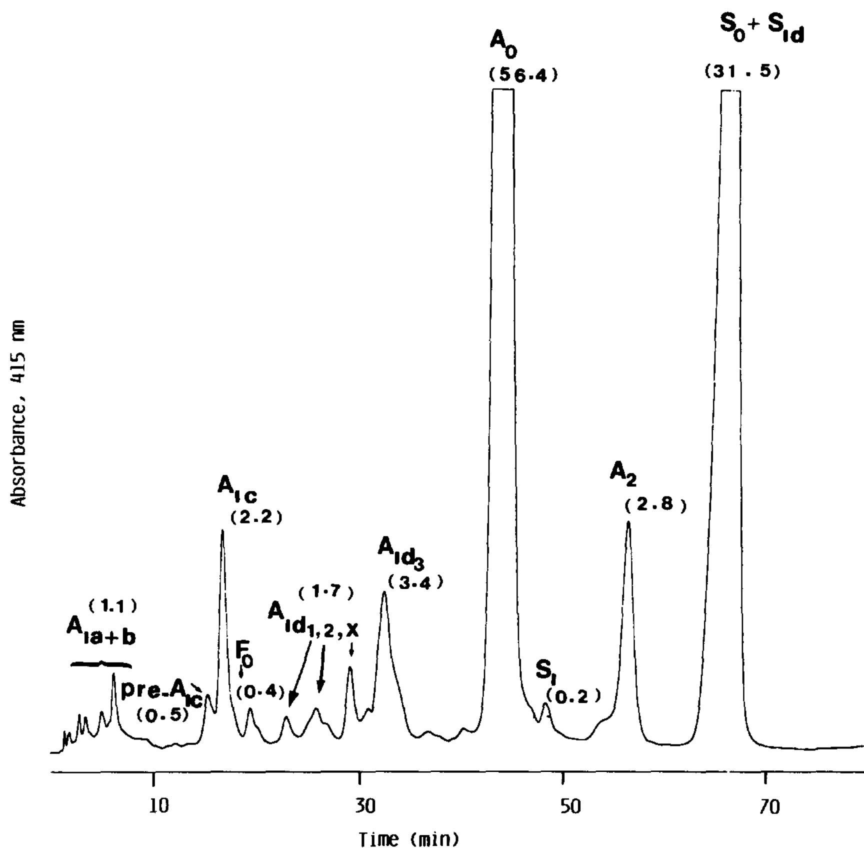


Fig. 4. Separation of minor and major Hb components in freshly prepared red cell lysate of a sickle cell trait (HbAS) subject. The numbers in parentheses are percentages.

incubation of packed erythrocytes, either with 10 m*M* acetylsalicylic acid or with 50 m*M* glucose.

In contrast to the aldimine adduct formed by interaction with glucose, the acetylated HbA increment was eliminated neither by the acidic treatment nor by the incubation of erythrocytes in isotonic saline.

More than three minor Hb components were eluted after HbA_{Ic} or HbF_o. These Hb variants were designated HbA_{Id1}, HbA_{Id2}, HbA_{Idx}, HbA_{Id3} and HbA_{Ie}, based on the order of their elution. HbA_{Id3} was the second largest minor HbA peak after HbA_{Ic}, and accounted for (mean ± S.D.) 3.39 ± 0.22 and 4.5 ± 0.91% in normals and diabetics, respectively.

The presence of α- and β-chains in isolated HbA_{Ic} and HbA_{Id3} was confirmed by reversed-phase HPLC. As expected, the separation of α-, G_γ- and A_γ-chains was achieved in isolated HbF, which was not contaminated with detectable adult Hbs.

As illustrated in Figs. 3 and 4, the procedure exhibits selectivity in the separation of Hb analogues of samples containing HbA, HbF and HbS. The separation of minor HbA_I, HbF_I and HbF_o was possible in both HbAS and HbS-β^+-

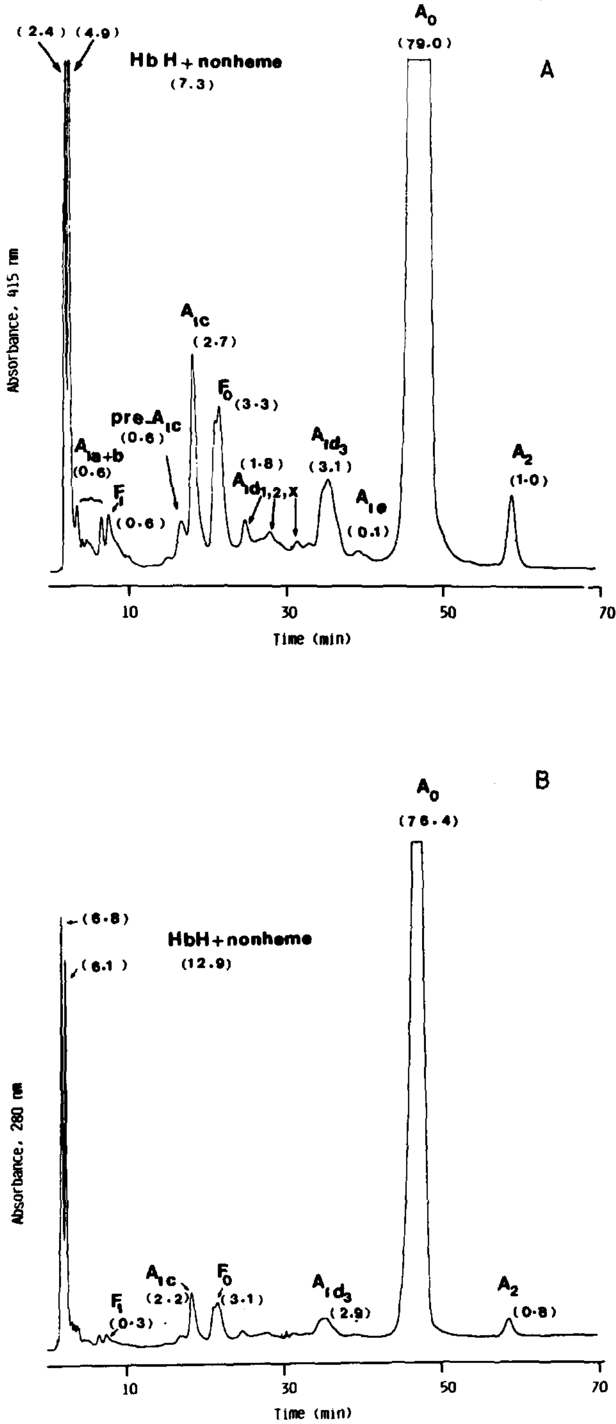


Fig. 5. Cation-exchange chromatograms of haemolysates from a patient with HbH (β_4) disease: de tection at (A) 415 nm and (B) 280 nm. The numbers in parentheses are percentages.

TABLE I

PERCENTAGES OF MINOR AND MAJOR HAEMOGLOBIN COMPONENTS IN RED CELL HAEMOLYSATES OF NORMAL ADULTS, DIABETICS AND PATIENTS WITH HAEMOLYTIC ANAEMIA

Haemoglobin type	Level (mean ± S.D.) (%)		
	Normals (n=23)	Diabetics (n=31)	Haemolytic anaemia (n=35)
A_{Ia1}	0.03 ± 0.02	0.03 ± 0.02	0.06 ± 0.04
A_{Ia2}	0.18 ± 0.06	0.24 ± 0.06	0.12 ± 0.07
A_{Ib1}	0.25 ± 0.14	0.39 ± 0.14	0.16 ± 0.10
A_{Ib2}	0.21 ± 0.10	0.34 ± 0.11	0.16 ± 0.11
A_{Ib3}	0.44 ± 0.11	0.34 ± 0.11	0.25 ± 0.12
pre-A_{Ic}	0.27 ± 0.34	0.62 ± 0.22	0.10 ± 0.19
A_{Ic}	4.14 ± 0.68	8.37 ± 1.68	2.38 ± 0.79
A_{Id1}	0.39 ± 0.13	0.44 ± 0.17	0.28 ± 0.21
A_{Id2}	0.93 ± 0.43	1.11 ± 0.38	0.43 ± 0.37
A_{Idx}	0.56 ± 0.54	0.72 ± 0.42	0.70 ± 0.78
A_{Id3}	3.39 ± 0.27	4.54 ± 0.91	2.61 ± 0.69
A_{Ie}	0.69 ± 0.20	0.70 ± 0.35	0.29 ± 0.27
A_o	85.94 ± 1.66	79.37 ± 2.89	83.63 ± 2.39
A_2	2.29 ± 0.23	2.03 ± 0.25	2.35 ± 0.94
F_I	–	–	0.12 ± 0.23
F_o	0.01 ± 0.04	0.09 ± 0.30	1.13 ± 1.75

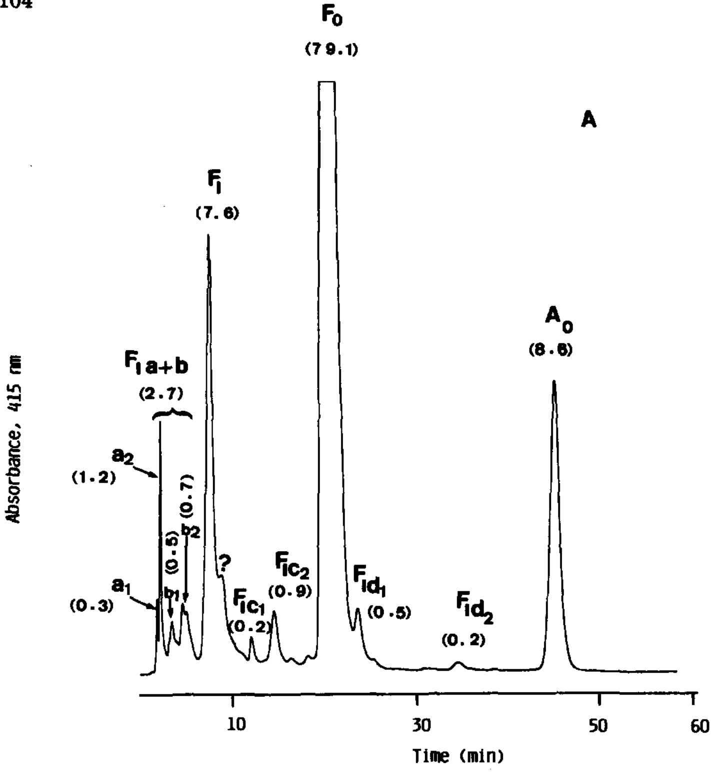
thalassemia. In contrast to previous reports [7], HbA_o and HbS_I were clearly separated from each other. However, HbS_{Id} was eluted together with HbS_o.

Fig. 5 illustrates the separation of Hb variants from a patient with HbH ($\beta 4$) disease. HbH appeared first, within 3 min, and co-eluted with HbA_{Ia1}. Furthermore, a large absorbance at 280 nm indicated that HbH co-eluted with non-haem-carrying proteins, which were slightly different in their chromatographic mobility as monitored at 280 nm (Fig. 5B).

Table I summarizes the percentages of different minor and major Hbs determined in various blood samples. The procedure results in excellent separation of minor and major Hb analogues present in lysates of red cell from cord blood of normal babies. As shown in Fig. 6A, several minor HbFs, designated F_{Ia1}, F_{Ia2}, F_{Ib1}, F_{Ib2}, F_I, F_{Ic1}, F_{Ic2}, F_{Id1} and F_{Id2} eluting in that order, were detected in cord blood samples. Most of these minor HbFs could not be separated by previous chromatographic procedures [3,14].

HbF_I accounts for the major portion of minor HbF, and its concentration ranged from 6.8 to 10.50% in fourteen normal babies. HbF_{Ib2} and HbF_{Ic2}, the next largest component, represented (mean ± S.D.) 1.72 ± 0.71 and 1.46 ± 0.36% of the total HbF, respectively.

The level of HbF_o was (mean ± S.D.) 68.69 ± 5.92%. The percentages of the minor and major hemoglobins in cord blood are listed in Table II.



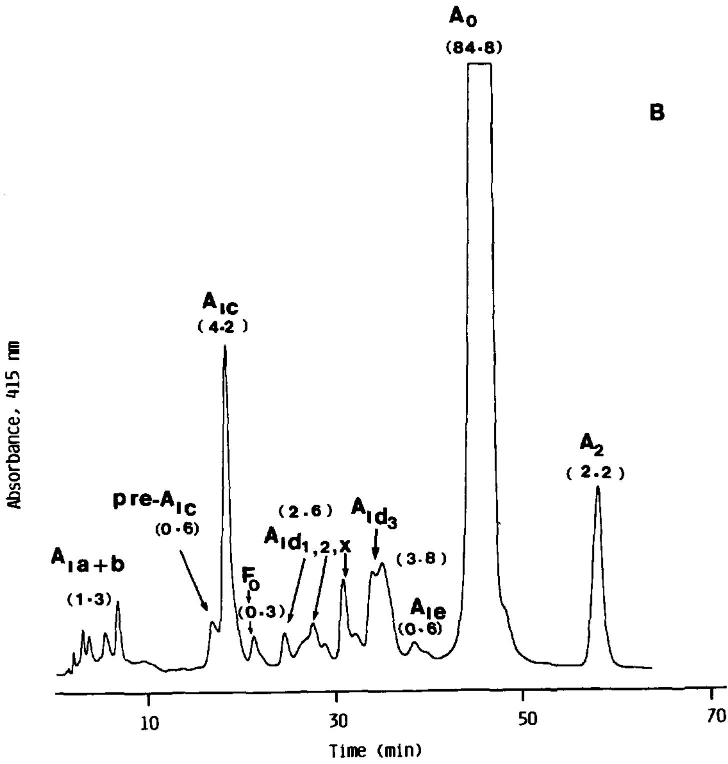


Fig. 6.

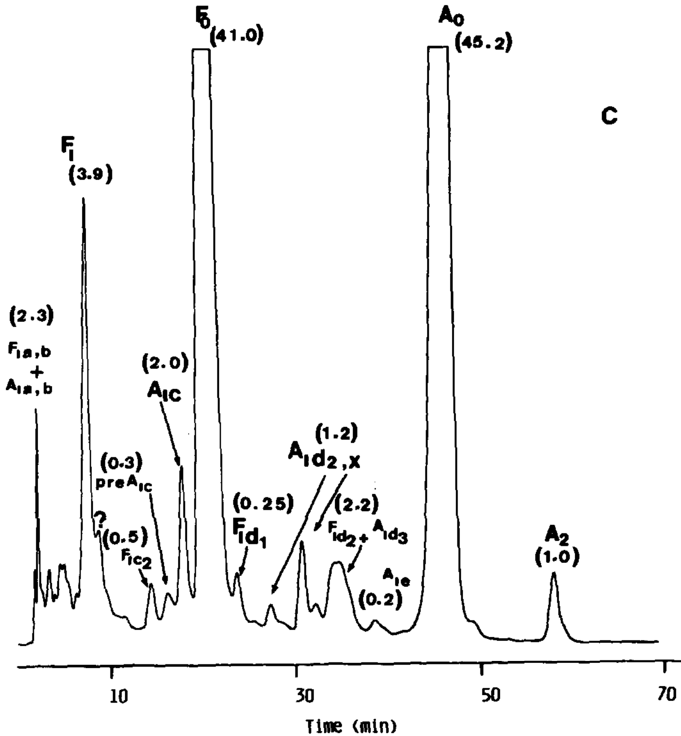


Fig. 6. Separation of minor and major HbF components from each other and from adult Hb variants. (A) Separation of Hb components in haemolysates of red cells from cord blood of a normal newborn. (B) Red cell haemolysate from a normal adult. (C) Chromatogram of a mixture (1:1) of haemolysate from cord blood with adult blood (B) of equal Hb concentration. The numbers in parentheses are percentages.

TABLE II

LEVELS OF HAEMOGLOBIN TYPES IN CORD BLOOD RED CELL LYSATES OF FOURTEEN BABIES

Haemoglobin type	Level (mean ± S.D.) (%)
F_{Ia1}	0.20 ± 0.06
F_{Ia2}	0.42 ± 0.25
F_{Ib1}	0.59 ± 0.14
F_{Ib2}	1.72 ± 0.71
F_I	8.87 ± 1.17
F_{Ic1}	0.13 ± 0.11
F_{Ic2}	1.46 ± 0.36
F_o	68.69 ± 5.92
F_{Id1}	0.23 ± 0.18
F_{Id2}	0.20 ± 0.14
A_o	16.69 ± 5.74
$F_I + F_o$	77.56 ± 5.73

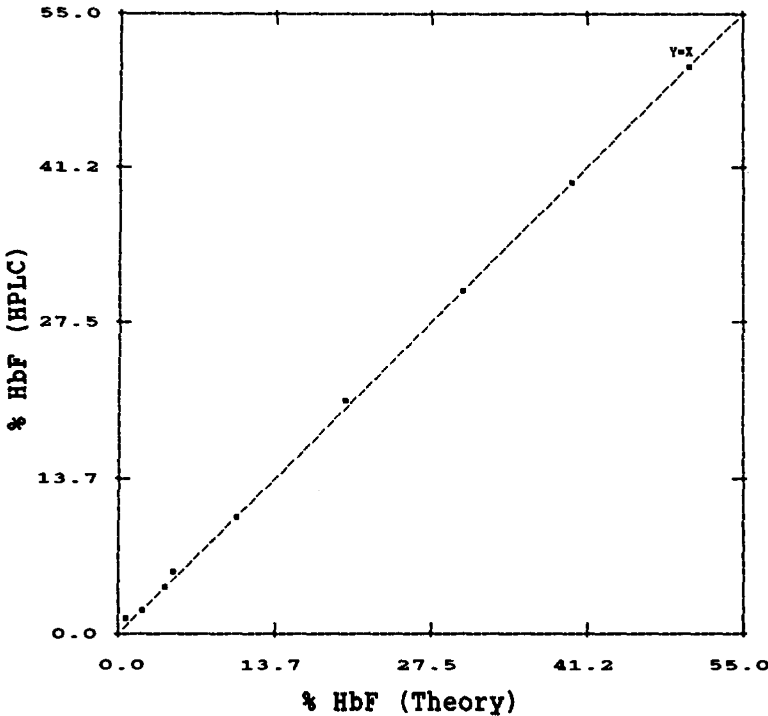


Fig. 7. Linear relation between the theoretical HbF values and those determined by HPLC: $y = 0.998x + 0.25$; $sy \cdot x = 0.14$; $n = 9$.

Application

The procedure was applied to the simultaneous quantitation of HbF and glycated Hb in an artificial mixture and in samples from patients with elevated HbF. The selectivity of the method was tested by mixing, in different proportions, the haemolysate from cord blood with that of a normal adult. Hb from a mixture containing 50% of cord blood and 50% of adult blood yields the elution profile of Fig. 6C. In addition to HbF_I and HbF_o, a number of other minor HbF components separated from the minor HbA_I. However, there was an overlap of minor HbF variants and minor HbA components in the pre-F_I zone.

Fig. 7 shows a good recovery of HbF in artificial mixtures over the whole range considered (0–60%). The recovery was 99.5–105.1% for both HbA_{Ic} and HbF ($HbF_I + HbF_o$).

HbF and HbA_{Ic} values obtained by the present procedure were compared with those obtained by the classical alkali denaturation and bioaffinity chromatography (total glycated Hb), respectively. A comparison of HPLC with alkali denaturation yields a good relationship with a high correlation coefficient ($r = 0.997$). The regression line ($y = 1.08x + 0.02$; $n = 30$) was very close to the line of identity (Fig. 8). Fig. 9 shows that HPLC and bioaffinity chromatography give comparable results, even when samples contain a large amount of HbF and HbA_I. The Pearson correlation coefficient (r) was 0.973 ($y = 0.69x - 0.03$; $n = 25$) with a residual variance ($sy \cdot x$) of 0.54. The mean ± S.D. of glycated Hb (HbA_{Ic}) in 32

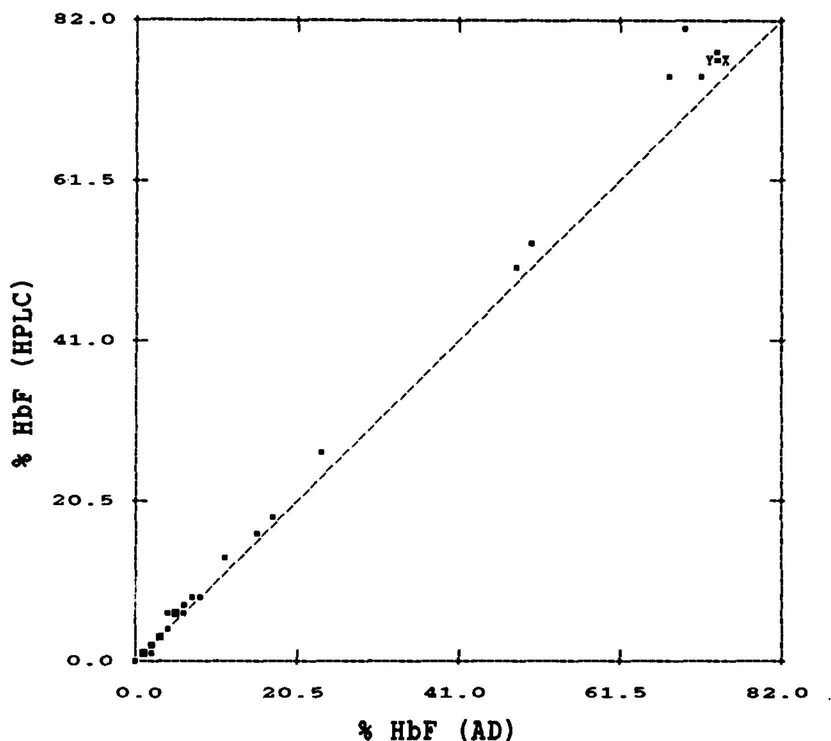


Fig. 8. Comparative determination of HbF by HPLC and by the alkali denaturation (AD) method: $y=1.08x+0.02$; $sy{\cdot}x=1.33$; $n=30$; r (Pearson) $=0.997$.

patients with haemolytic anaemia was 2.38±0.79%, with a range from 0.28 to 3.72%.

DISCUSSION

HPLC applications directed towards the separation and quantitation of various Hb variants have been reviewed recently [5]. Huisman [15] reported an HPLC method that made use of a weak cation-exchange resin (Synchropak CM-300). This procedure uses a variable sodium acetate gradient at constant pH (6.4) and constant Bis-Tris concentration. Unfortunately, the elution times of HbF and the minor HbA_I components were the same. More recently, Brenna et al. [8] described a procedure for the quantitation of glycated minor HbA_I and HbF, using the Diamat™ instrument (Bio-Rad). The Diamat instrument does not include a direct determination of minor HbF_I and, therefore, a value of total HbF (HbF_o and HbF_I) is not estimated. This method also lacks the resolving capacity of quantifying various minor and major Hb variants, such as HbF_I, HbA_2, HbS_I, HbH and HbC.

The present HPLC procedure is a modification of the methods described earlier [7,15]. We have optimized the experimental conditions using a combination of pH and ionic gradients. During the elution a two-sloped pH gradient was formed,

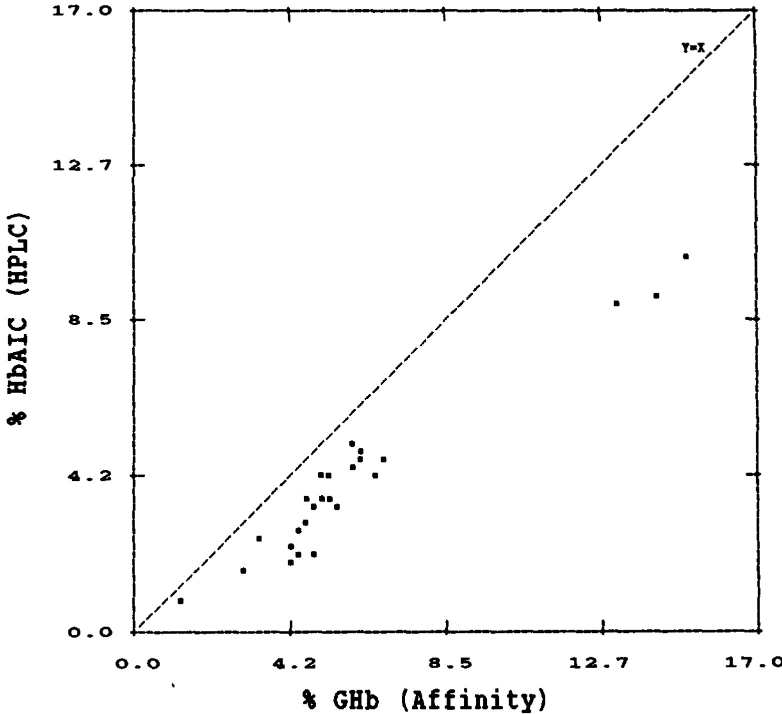


Fig. 9. Correlation of the levels of glycated haemoglobin (HbA_{Ic}) determined by HPLC with the levels of total glycated haemoglobin (GHb) determined by bioaffinity chromatography in patients with high HbF value: $y=0.69x-0.03$; $sy{\cdot}x=0.54$; $n=25$; r (Pearson) $=0.973$.

which has a minimum at pH 6.60 and maximum at 6.9. The addition of ammonium ions to both developers leads to the reproducibility of the point of emergence of Hb variants. These experimental conditions yield an excellent separation of many Hb components and lead to their simultaneous quantitation.

Falsely high values for HbA_{Ic} were reported in patients on high doses of aspirin [12]. Another common problem is the fluctuations in the HbA_{Ic} level owing to the aldimine form or pre-HbA_{Ic} [13]. The present HPLC procedure was unaffected by these interactions.

We have shown that the adduct formed by in vitro acetylation with acetylsalicylic acid (aspirin) co-elutes with the labile aldimine immediately before HbA_{Ic}. Such heterogeneity of pre-A_{Ic} has not been reported before. Acetylated HbA has proved to be resistant to acidic treatment (pH 5). Therefore, it should be pointed out that this Hb variant can be quantitated by HPLC after elimination of labile glycated Hb by either acidic treatment or isotonic saline incubation. Further studies in patients with chronic aspirin ingestion are necessary to elucidate the utility of acetylated HbA.

The lower glycated Hb level in haemolytic anaemias has been reported previously [16] and was confirmed in this study. The values for glycated Hb (HbA_{Ic}) obtained by HPLC in the absence or presence of HbF correlated well with total glycated Hb levels determined by bioaffinity chromatography. The HPLC results

correlated well with that obtained by the classical alkali denaturation. However, the regression line was obviously different from the line of identity at higher HbF concentrations. This is probably due to the poor sensitivity and the lack of reproducibility of the alkali denaturation method. Nearly 100% analytical recovery was observed with artificial Hb mixtures, and the method was linear up to 60% HbF. The present HPLC procedure provides both selectivity and sensitivity and allows the detection of as little as 0.05% HbF in 10 μl of haemolysate containing 100 μg of Hb. Since the method exhibits high resolution the heterogeneous nature of Hb components in adults and newborns becomes more evident. In fact, HbA_{Id} minor adult variant splits into four distinct peaks (d1, d2, dx, d3), which indicate extensive heterogeneity of this Hb component. In most common chromatographic systems minor HbA_{Id} components are not separated from each other or from HbA_o. This is probably due to the small difference in their p*I* values. The separation of these minor Hb variants on a Bio-Rex 70 column [17] needs prolonged gradients (four days) at low temperature (4°C). We have induced slight changes in pH and ionic strength during the elution, and thus increased the interaction between the fixed charge of the resin and the Hb variants. The key parameter of the method is therefore the induction of a two-sloped pH gradient in a very narrow pH interval (6.6–6.9).

Thus, under the stated conditions, the system is able to distinguish slight changes in p*I* values of Hb components during ion-exchange interactions. The excellent separation of HbA_{Id3} from HbA_o is in agreement with the difference in their net charge, as demonstrated by Abraham et al. [17].

HbA_{Id3} seems to be an adduct of HbA formed by interaction with glucose of more than one amino acid, including some of the α-chain [17]. The level of HbA_{Id3} was consistently increased in diabetics (3.39 ± 0.22 versus 4.5 ± 0.91%). Similar increases of this minor HbA_I were reported earlier [7]. It appears probable that HbA_{Id3} could serve as a marker of glycaemic control. This possibility is presently being investigated, and we shall report on these results after more data are obtained. HbA_{Idx}, not observed before, is probably formed by disulphide interchange with oxidized glutathione [17]. The ageing of the haemolysate produces an increase (up to 10%) of HbA_{Idx}, while HbA_{Id3} remains stable. HbA_{Ie} was not or less observed by previous methods [18] and has not been identified yet. We found an appreciable amount of this component in normal adults (0.63 ± 0.20%) and diabetics (0.70 ± 0.35%).

We have further demonstrated the heterogeneity of minor HbFs and their chromatographic similarity to minor adult Hbs (Fig. 6). The most significant minor HbF was HbF_I, which represented 8.87 ± 1.17% of total Hb in cord blood. Chromatographically separable minor HbF variants eluting in the pre-HbF_I zone accounted for ca. 3%, and were approximately three times as high as for minor HbA_{Ia+b} (1.1%) in normal adults. The higher level of minor HbF compared with minor HbA_I may be the result of the simultaneous glycation and acetylation of HbF. Similar observations were made by Abraham et al. [14]. The pre-HbF_I value (4.1%) determined by these authors using Bio-Rex chromatography was slightly higher than that obtained by HPLC (3.0%). This is probably related to the non-haem proteins co-eluting with the fast-moving Hbs on Bio-Rex 70.

Although no detailed study has been made of minor HbF variants, the presented HPLC procedure could be an important tool in studying the switching of Hb synthesis from HbF to HbA. An important part of this study was the separation of minor HbA_I components from each other and from minor and major HbF variants, which leads to an accurate and simultaneous quantitation of glycated HbA_I and HbF. It is of great interest to note that the determination of glycated Hbs by this HPLC ion-exchange system is not thwarted by the presence of common abnormal Hb variants.

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