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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN HAEMOGLOBIN CHAINS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the separation of human haemoglobin chains has been devised. Using a LiChrospher 100 CH-8/2 column and a ternary eluent (acetonitrile–methanol–0.155 *M* NaCl, pH 2.7) improved resolution was achieved between ($\delta\beta$) Lepore, βA, βS, α, Gγ and Aγ chains within a 60-min linear gradient. The AγT chain can also be separated by increasing the gradient time and decreasing the flow-rate. Silanophilic interactions play an important role in the retention mechanism, and NaCl addition was necessary in order to suppress adsorption on free silanols. Increasing the methanol concentration to 10% caused a slight increase in chain retention, probably owing to solvation of the stationary phase.

The recovery was 82% and the reproducibility of retention times was as good as ± 1.5%. Quantitation of chains is likely to be possible by peak area measurement. Owing to its sensitivity, the proposed method may be useful in the diagnosis of haemoglobinopathies and in the study of haemoglobin variants.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become a useful analytical tool in peptide and protein chemistry, as its resolution, sensitivity and rapidity compare favourably with those of the more traditional chromatographic methods (for reviews, see refs. 1–3). Most RP-HPLC studies have dealt with low-molecular-weight polypeptides (mol.wt. up to 20,000), either native[4–6] or generated by cleavage of larger proteins[7], but there are also examples of separations of complex mixtures of proteins up to 200,000 (ref. 8).

A useful application of RP-HPLC is the separation of globin chains (mol.wt. 15,000), which is necessary for the characterization of haemoglobin (Hb) variants and for monitoring *in vitro* haemoglobin synthesis (for a review of haemoglobins and chains structures and of the diagnostic utility of chain separation, see ref. 9).

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Globin chain separation is generally performed by carboxymethylcellulose (CMC) chromatography[10] or, less frequently, by isoelectric focusing (IEF)[11] or electrophoresis on polyacrylamide gels[12]. As far as RP-HPLC methods are concerned, they can be divided into two groups: those derived from the method of Shelton *et al.*[13], subsequently modified by the same group[14,15] and also by other workers[16–20], and those derived from Congote and co-workers[21–24]. Petrides *et al.*[25] proposed a different method that has never subsequently been used in other studies, at least to our knowledge.

All of the methods mentioned above involve the use of similar octadecylsilyl columns but differ in the eluent composition (see Discussion for details). Their resolution is often incomplete because of peak broadening: also the reproducibility, when tested[22], was poor.

Therefore, led by our interest in the evaluation of the synthetic ratio between β and γ chains, for the antenatal diagnosis of thalassaemia, we devised an RP-HPLC method that gives an improved resolution in the β region and a reasonable separation of the principal γ chains.

We describe in this paper the results obtained with foetal, newborn and adult blood samples, heterozygous or homozygous for different haemoglobinopathies, and an evaluation of the recovery, reproducibility and effects of changes in experimental conditions, such as ionic strength, organic solvent composition and temperature.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and methanol (LiChrosolv grade) were supplied by Merck (Darmstadt, G.F.R.). Water was doubly glass-distilled and deionized. Tritiated leucine (L-[4,5-H^3]Leu, 145 Ci/mmole) and scintillation solution (Pico-Fluor 30) were obtained from Amersham International (Amersham, U.K.) and Packard International (Zurich, Switzerland), respectively. Carboxymethylcellulose (CM-52) was purchased from Whatman (Maidstone, U.K.). Ultrafiltration was performed by using an Amicon Centrifree System (Amicon, Denver, CO, U.S.A.). All other chemicals (reagent grade) were supplied by Carlo Erba (Milan, Italy).

Mobile phases were prepared by measuring each solvent volume separately and the mixtures were sonicated briefly (3 min) before use. Eluent A was acetonitrile–methanol–0.155 *M* sodium chloride (acidified to pH 2.7 with hydrochloric acid) (68:4:28) and eluent B was acetonitrile–methanol–0.077 *M* sodium chloride (pH 2.7) (26:33:41). Further details of the eluent composition are discussed below.

Sample preparation

Peripheral blood samples were obtained from normal subjects and from heterozygotes for HbS, Hb Lepore, and from a case of homozygous $\delta\beta$-thalassaemia. Cord blood samples from normal newborns were collected at delivery. Foetal blood samples were obtained at 18–20 weeks gestational age by foetoscopy in pregnancies at risk of β-thalassaemia. When maternal blood contamination was present the samples were purified by selective lysis of adult blood cells[26]. Blood cells were washed in saline and lysed with a double volume of distilled water, freezing and thawing.

The Hb concentration in the haemolysate was adjusted to 1 g/dl with distilled water.

New synthesized labelled haemoglobin was obtained by incubation of fresh blood cells with tritiated leucine (0.5–1 μCi/μl of packed red cells) in a leucine-free amino acid-rich mixture. Globin chains were separated by CMC chromatography after acid-acetone precipitation[10]. Different haemoglobins (HbA, HbA2, HbS, HbF and HbLepore) were obtained by electropohoretic separation on cellulose acetate; single bands from several runs were eluted with saline and concentrated, if necessary, by ultrafiltration.

Samples were stored at −30°C if not measured immediately. Storage for up to 2 months did not affect the results. Some samples were repeatedly measured during five days and stored at 4°C after each measurement; no differences such as the appearance of new peaks or increases in band broadening were noted between runs.

Apparatus

Separations were carried out on a Perkin-Elmer Series 3B liquid chromatograph fitted with a Rheodyne 0932 sample injector, a Perkin-Elmer LC 75 variable-wavelength UV detector, an LCI 100 digital integrator and a Beckman Omniscribe recorder. In a preliminary part of this study we also used a Beckman Model 342 liquid chromatograph, equipped with a Model 160 fixed-wavelength detector operating at 214 nm. Columns were Merck LiChrospher 100 CH-8/2 (5 μm, code 50329, 250 × 4 mm I.D., with pore diameter of about 100 Å and a C_8 bonded phase, without end-capping.

Temperature control was achieved by using a water-jacket and a Lauda Model K2RD thermostat.

Counting was performed in a Packard Tri-Carb 460 scintillation counter.

Chromatographic procedure

Blood lysates, or the ultrafiltrate obtained after electrophoretic separation, were centrifuged at 4400 *g* and then directly injected in variable volumes (1–10 μl) corresponding to about 10–100 μg of total haemoglobin. In order to compensate for the dilution effect, larger volumes (up to 50 μl) were injected when using fractions from CMC chromatography.

The detector was set at 215 nm and the temperature of the column was 48°C. The recorder was operated at a chart speed of 0.25–0.5 cm/min (2.5 cm/min when band widths were measured).

Retention times and peak areas were measured by digital integration, with correction for the baseline drift; minor unresolved peaks were integrated as they emerged from the tail of the dominant peak.

A linear gradient from 20 to 60% solvent A in 60 min was applied, at a flow-rate of 0.8 ml/min. Other conditions will be specified later. At the end of the gradient, the column was washed for 10 min with solvent A and equilibrated for 20 min with the initial solvent composition. No "ghost" peaks were observed in several blank gradient runs. The apparatus and the column were washed overnight with distilled water (30 min, flow-rate 0.8 ml/min) and then methanol (0.2 ml/min).

Peaks were identified by comparing their retention times with those of peaks obtained from known haemoglobin solutions, or from Hb chains collected after CMC chromatography.

RESULTS

Chain separation

Chain separation in normal cord blood and in a foetal blood sample from a β0-thalassaemia homozygous is shown in Fig. 1a and b, respectively. The latter reveals a complete absence of beta chains, as was expected. The good resolution achieved in the beta region is illustrated in Fig. 2a, which shows the separation of an artificial mixture of Lepore, A, S and A2 haemoglobins in approximately equimolar proportions. It was also possible to quantitate the βS chain in a βAβS heterozygous (Fig. 2b), and to identify the ($\delta\beta$) Lepore chain, as a shoulder before the dominant beta peak in an Hb Lepore heterozygous (Fig. 2c).

Other minor peaks were present, but not in all samples. One of these occasionally preceded the beta chain (Figs. 1a and 2b and c) while the others were located immediately after the alpha, Gγ and Aγ chains (Fig. 1).

An additional peak, present in some foetal blood sample (peak X, Fig. 1b), was located before the Gγ chain, and was always associated with an elevated Gγ/Aγ ratio (calculated by peak area measurement), being particularly high when Aγ chain was nearly absent. This suggested that this peak could reflect the AγT chain, a hypothesis which was confirmed by comparison with the elution pattern of a sample from a patient with $\delta\beta$-thalassaemia, homozygous for AγT[27]. The separation of the AγT chain from the Gγ chain can be improved considerably by increasing the gradient time and reducing the flow-rate (Fig. 3).

The recovery was measured by injecting labelled blood samples and collecting 0.2-ml aliquots (Fig. 4). The collection was also continued during the washing time. Neither radioactivity nor increases in absorbance were observed after the elution of

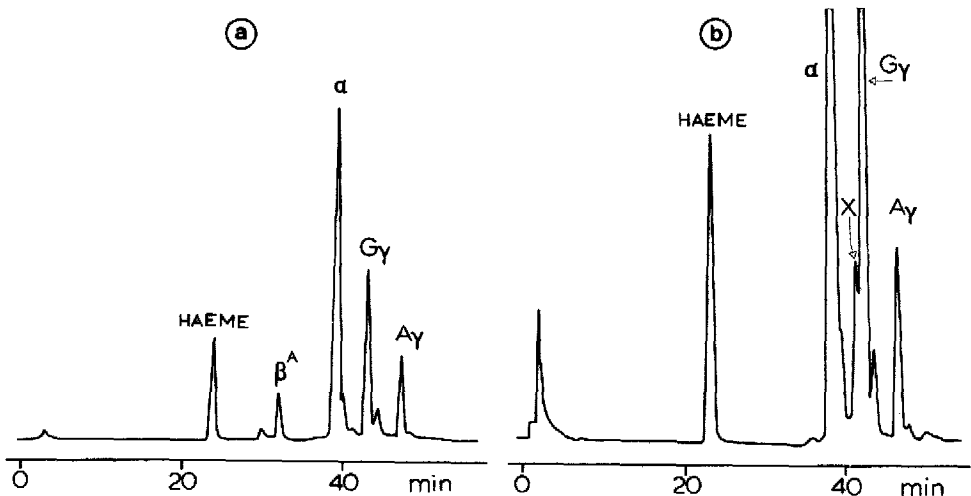

Fig. 1. Chromatograms from (a) normal cord blood, 1 μl of haemolysate, 0.64 a.u.f.s.; (b) foetal blood of a β0-thalassaemia homozygous, 10 μl of haemolysate, 0.16 a.u.f.s. Peak X was identified as AγT (see text). Conditions: eluent A = acetonitrile–methanol–0.155 M sodium chloride (pH 2.7) hydrochloric acid (68:4:28); eluent B = acetonitrile–methanol–0.077 M sodium chloride (pH 2.7) hydrochloric acid (26:33:41). Linear gradient from 20 to 60% A in 60 min; flow-rate, 0.8 ml/min; 215 nm; column temperature, 48°C.

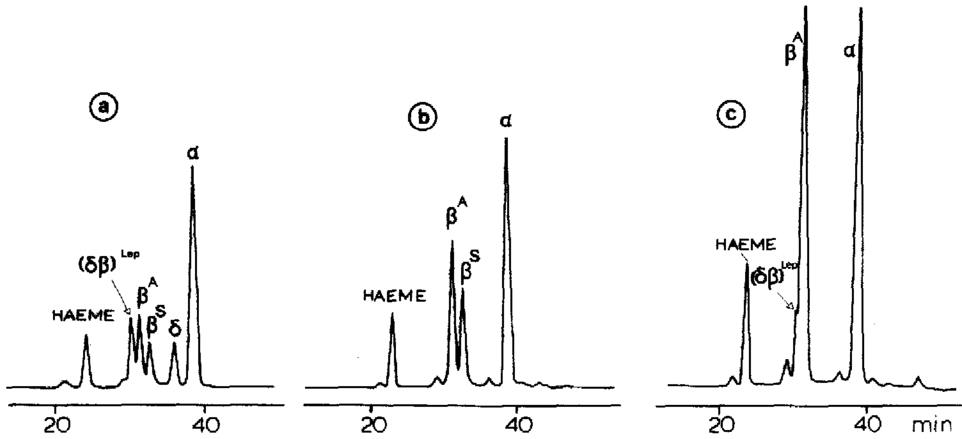

Fig. 2. Chromatograms from (a) an artificial mixture of HbLepore, HbA, HbS and HbA2 (10 μl of haemolysate); (b) peripheral blood from an adult HbS heterozygous (1 μl of haemolysate); (c) peripheral blood from an adult Hb Lepore heterozygous (1 μl of haemolysate). Conditions as in Fig. 1.

the Aγ chain. The overall recovery, including non-retained minor peaks, averaged 82 $\pm$ 7% (n = 10, range 74–95%).

Reproducibility

The mean retention times of the haeme and the main chains are shown in Table I. The reproducibility was assessed from ten separations carried out on the same column on different days and with different cord blood samples. A correct and reproducible eluent preparation is critical if reproducible retention times are to be obtained. As many as 200 separations have been performed to date on each column

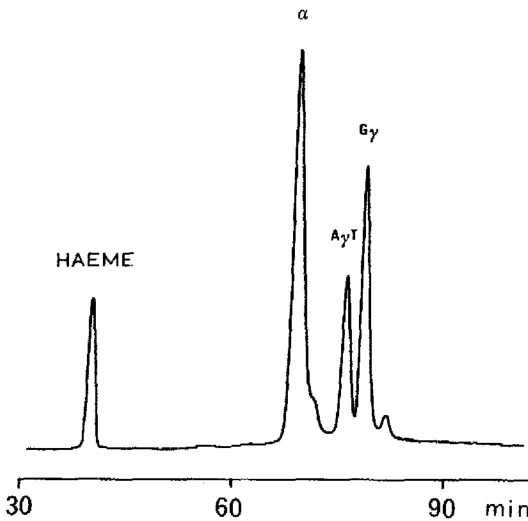

Fig. 3. Chromatogram of peripheral blood from a patient with $\delta\beta$-thalassaemia, homozygous for AγT (1 μl of haemolysate). Mobile phase as in Fig. 1. Gradient time, 150 min; flow-rate, 0.4 ml/min.

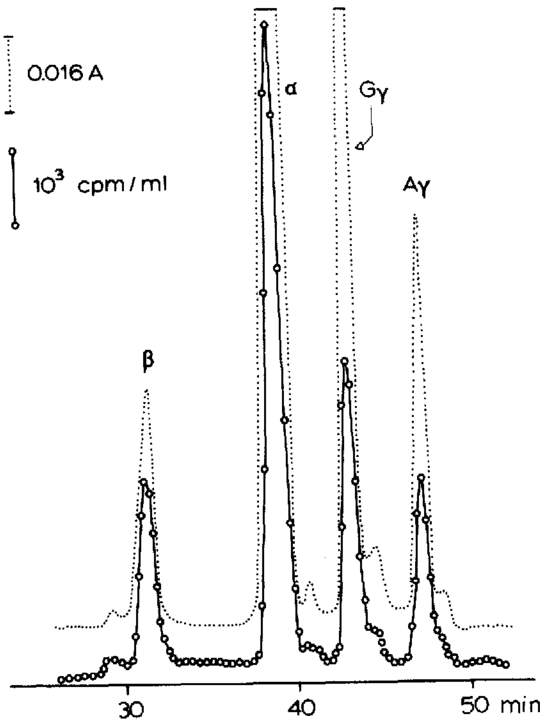

Fig. 4. Radioactivity of fractions (0.2 ml) collected after injection of labelled normal cord blood (2 μl of haemolysate). Condition as in Fig. 1.

without significant loss of reproducibility. However, some differences in selectivity were noted between columns under the same operating and ageing conditions.

Mobile phase composition

In preliminary experiments we used a slight gradient of acetonitrile in water, both containing 0.1% of trifluoroacetic acid, according to Congote *et al.*[21]. Such an eluent has the important advantage, with respect to our and other[13–21] mobile phases

TABLE I

REPRODUCIBILITY OF HAEME AND GLOBIN CHAIN SEPARATIONS

Values are the means ± standard deviations of ten determinations on different days, from different cord blood samples. Experimental conditions as in Fig. 1.

Haeme or chain	*Retention time (min)*	*Coefficient of variation (%)*
Haeme	23.35 ± 0.35	1.51
β chain	31.14 ± 0.49	1.56
α chain	37.97 ± 0.57	1.51
Gγ chain	42.16 ± 0.38	0.90
Aγ chain	46.67 ± 0.42	0.90

containing inorganic ions, of being volatile and hence easily removed from fractions collected during HPLC separation. However, under these conditions separation between α and Gγ chains was inadequate, mainly because of pronounced asymmetry of the α peak. This was attributed to an interaction between solute molecules and free silanol groups of the packing material. Therefore, a strong electrolyte (sodium chloride) and/or a second organic modifier (methanol) were added, which could deactivate the silanol groups via ionic suppression or hydrogen bonding[28].

The mobile phase composition reported under Experimental was derived from these preliminary experiments.

In order to evaluate the effect of changes in mobile phase composition, we operated under a linear gradient of acetonitrile and changed the methanol or sodium chloride concentration between runs, while keeping all other conditions constant (for details, see the legends to Figs. 5 and 6). The samples injected were from the same cord blood pool. Reported values of retention times represent the means of three chromatographic runs.

Methanol (Fig. 5) affected mainly the elution of haeme. The globin chains were eluted more rapidly in the absence of methanol and exhibited a slight increase in their retention times at a methanol concentration of 10%. The resolution was slightly improved, at least up to 15% methanol.

In contrast, the effect of NaCl was more pronounced on the globin chains than on haeme (Fig. 6). Without sodium chloride (*i.e.*, by using only hydrochloric acid, pH 2.7) the globin chains were completely retained and only the haeme was eluted during the gradient time. An increase in sodium chloride concentration up to $3.1 \cdot 10^{-2}$ M shortened the retention times of the globin chains almost exponentially,

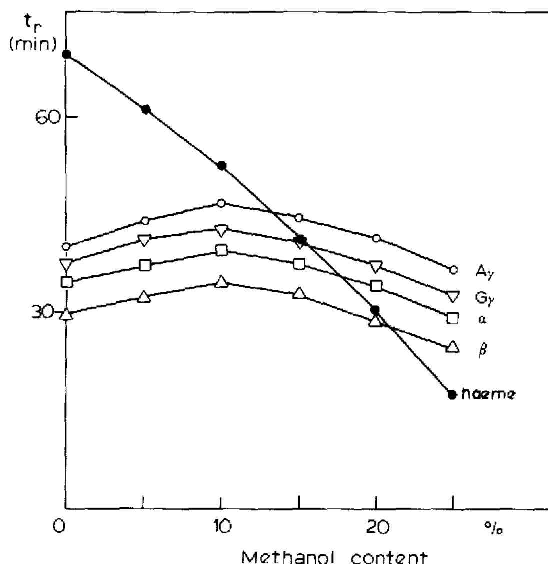

Fig. 5. Relationship between retention times (t_r) of haeme and globin chains and methanol concentration. Conditions: eluent A = 60% acetonitrile–15% 0.155 M sodium chloride (pH 2.7) hydrochloric acid–0–25% methanol; eluent B as eluent A, with water (pH 2.7) instead of acetonitrile. Gradient: 60–100% A in 80 min. Flow-rate, 0.8 ml/min; column temperature, 48°C.

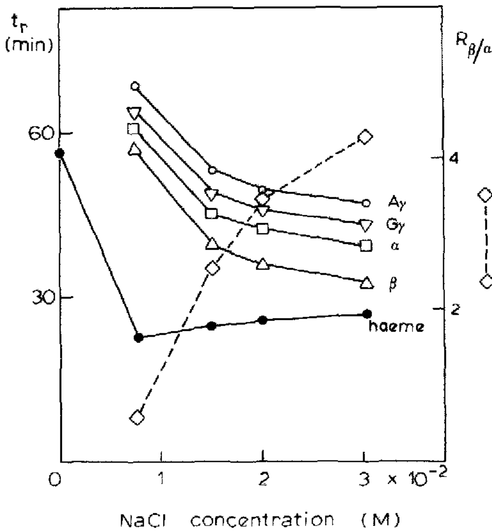

Fig. 6. Influence of sodium chloride concentration on resolution between beta and alpha chains ($R\beta/\alpha$) and on retention times (t_r) of haeme and globin chains. Conditions: eluent A = 55% acetonitrile–20% 0–0.155 *M* sodium chloride–25% methanol; eluent B as eluent A, with water (pH 2.7) instead of acetonitrile. Other conditions as in Fig. 5.

while the retention time of haeme, after an initial decline, remained constant or exhibited a slight increase. The broadening of peaks was greatly reduced, with a consequent improvement in resolution, as shown in Fig. 6 (for clarity only the β/α resolution is reported). At a sodium chloride concentration of $0.77 \cdot 10^{-2}$ *M* the globin

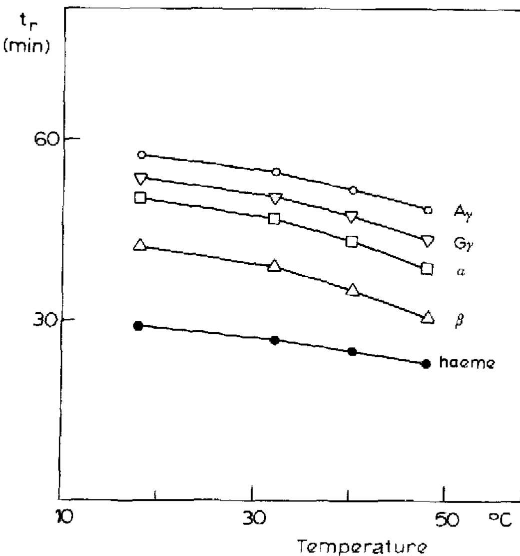

Fig. 7. Relationship between column temperature and retention times (t_r) of haeme and globin chains. Conditions as in Fig. 1.

chains were incompletely separated, whereas adequate resolution was achieved at sodium chloride concentrations between $2 \cdot 10^{-2}$ and $3 \cdot 10^{-2}$ *M*.

Temperature

Temperature had only a slight influence on the separation, at least over the range investigated (18–48°C) (Fig. 7). As expected, there was a decrease in retention times when the temperature was increased, with a slightly better separation between alpha and gamma chains. Neither changes in elution profile nor the appearance of secondary peaks were noted in any of the samples separated at different temperatures. A temperature of 48°C was chosen in order to reduce both the back-pressure and analysis time.

DISCUSSION

It is generally accepted[29–31] that protein separations at pH below 3 on reversed-phase supports are dictated mainly by interactions between the hydrophobic domains of the solute and the bonded phase. This holds true, from a statistical point of view, when comparing peptides[7,32] or proteins[29,33] with a wide range of molecular weights. However, the band broadening observed with peptides containing basic amino acids[34], and some failure to correlate retention times with calculated hydropohobicities[4,31], indicate that the retention mechanisms are likely to be more complex and also to involve hydrophilic interactions between free silanol groups on the silica surface and polar or charged groups on the polypeptide chain, as pointed out by several workers[28,35,36].

The nature of the supports (length of the carbon bonded chain, pore diameter, pre-treatment of the silica surface) can determine which one of the two mechanisms is predominant. Moreover, the same support can behave in different ways, depending on the mobile phase composition, as demonstrated by Hearn and Greco[36].

The stationary phase we used should have a high content of free silanols, allowing for its low carbon content (about 4 μmol C/m^2) and the absence of any form of end-capping performed by the manufacturer. Other physico-chemical characteristics are similar to those exhibited by LiChrosorb C_8 (manufacturer's information). Therefore, it is likely that with such a packing silanophilic interactions will play an important role in retention mechanisms, and indeed the dependence of the retention times of globin chains on the ionic strength (Fig. 6) supports this hypothesis. A similar effect of salt addition on the retention of aromatic amines was noted by Papp and Vigh[37], who postulated a mechanism of ion exchange of the protonated amines with the free silanols of the RP-18 packing they used.

The effect of the methanol is less clear. Hancock and Sparrow[28] noted that pre-washing with methanol improved the elution profile of peptides separated on a column with a relatively light loading of a C_{18} bonded phase. They suggested that high selectivity is achieved when the silica surface contains a high proportion of accessible silanol groups, and that methanol conditioning is useful in overcoming the unwanted effects of mixed-mode mechanism, such as peak broadening or irreversible adsorption.

Probably the water content of the stationary phase is critical in polypeptide separations[28]. Methanol, which is more reactive than acetonitrile in forming hydro-

gen bonds[38], can solvate the bonded phase and also the silanol groups, as demonstrated by Yonker *et al.*[39] in a study on LiChrosorb C_8 support. As a consequence, a more regular conformation of the bonded carbon chain and a decrease in the water solvation of the stationary phase should occur[39], so determining a slight decrease in its polarity. These considerations agree with the effects of methanol addition that we have already observed (Fig. 5). In fact, the globin chains were eluted at a higher acetonitrile concentration when the methanol concentration was increased to 10%, whereas the haeme group, which is smaller and probably less affected by silanol interactions, behaved as expected in reversed-phase separations.

Finally, changes in polypeptide conformation should be taken into account. The elution order of globin chains does not correlate with their hydrophobicities, calculated from sequences[40,41], by the methods of Meek and Rossetti[32] and Sasagawa *et al.*[33]. This is not surprising in view of the fact that the primary structures of the chains are very similar (for example, there is only one amino acid difference between βA and βS, or between Gγ and Aγ chains). Even the presence of amino acids with different basicities in the N-terminal end of the chains cannot by itself explain the observed elution order. It is likely that conformational changes, as determined by solvent composition and type of stationary phase, have a dominant effect in determining the nature and the extent of the interactions involved. It is known[42] that polyelectrolytes contract with an increase in ionic strength. If this also holds true for polypeptides, the effect of sodium chloride can also be explained by a different and possibly reduced exposure of the hydrophobic domains of the chains to the bonded phase.

As far as the separation itself is concerned, a comment is required on the main differences between our and other published methods. As already noted, all previous workers[13–25] used octadecylsilyl columns (mainly Waters μBondapak), with developers containing phosphate buffer, methanol and acetonitrile (PMA developer)[13,16–19], perchlorate, phosphate, methanol and acetonitrile (PPMA developer)[14,20] or acetonitrile–0.1% trifluoroacetic acid[21–24]. Petrides *et al.*[25] used as the eluent 1 *M* pyridine formate in 26–28% *n*-propanol while Shelton *et al.*[15] improved the separation of haeme from globin chains by adding nonylamine to their PPMA developer.

There are differences between methods in the order of elution of the chains, with no obvious relationship with the eluent composition and column type: in some reports[13,16–19,25] the β chains elute after the α chains, whereas in ours and others[14,21–24] the order is the opposite. In one instance[14], different columns gave a reversed order of elution with the same eluent.

The resolution differs widely. Shelton *et al.*[14] succeeded in separating βA, βC, βS, α, Gγ and Aγ chains, but the resolution was incomplete because of broadening and asymmetry of the peaks. Moreover, δ and βS chains eluted together and the AγT were only seen as a shoulder before the Gγ peak. Other workers[16–18] focused their attention mainly on separating the γ chains and their method allowed the quantitation of AγT chains; however, it is time consuming (160–200 min) and exhibits poor resolution between β and other chains. Congote[22] separated βA, α, Gγ and Aγ chains in 30 min but could not achieve resolution between βA, βS and δ chains or between AγT and other γ chains. The reproducibility was poor.

A further comment is required on the presence of minor, unidentified peaks.

This is a common feature with all other published methods: Petrides *et al.*[25] noted a peak after (not before) the β chain peak, but in their method the elution order is opposite to ours. Congote[22] and Shelton *et al.*[14] also reported the presence of minor peaks in the β and γ regions. The identity of these secondary peaks is still unclear. They all reflect labelled material, as demonstrated by injecting labelled samples (Fig. 4); moreover, they are also present in freshly collected samples, ruling out the possibility of denaturation phenomena due to storage. Also, in-column decomposition can be excluded, as single haemoglobin solutions (HbA, HbF), obtained after electropohoretic separation, did not exhibit secondary peaks. Some preliminary experiments suggest that the peaks eluting closely after the γ chains could represent their acetylated forms, but more data are necessary to confirm this. In any event, the presence of these secondary peaks affects the determination of the area of the principal peaks to only a minor extent.

The methods described in this paper should allow quantitation of the relative amount of each chain, simply by peak area measurement. This quantitation procedure has been used already to determine the ratio between the γ chains[17,18,43,44] but it could also be applied to all chains, provided that the relative absorbance coefficients are the same or at least can be calculated, as proposed by Shelton *et al.*[14]. Indeed, it is likely that at the low wavelength we used (215 nm) there is little or no difference between the absorbance coefficients of the different chains, as the contribution of the few aromatic amino acids is less relevant with respect to the many absorbing peptide bonds[45]. Indeed, in a preliminary study on blood samples from normal adults and foetuses, in whom the alpha and non-alpha chains are present in molar equivalents, we found a ratio between alpha and non-alpha areas very close to unity (Table II).

TABLE II

RATIO BETWEEN α AND NON-α CHAIN AREAS

See text for experimental conditions.

Case	*α/non-α area ratio*
Normal adults	
S.D.	1.016
V.G.	1.001
V.D.	0.987
Normal foetal blood (20 weeks)	
1	0.885
2	1.004
3	0.984
4	1.049
5	1.042
6	1.030
7	0.912
8	1.198
Mean ± standard deviation (all samples)	1.01 ± 0.08

CONCLUSION

The proposed method allows the separation of globin chains in less than 1 h, with satisfactory reproducibility, recovery and sensitivity. Hydrophilic interactions contribute to a great extent to the overall retention mechanism, and this could probably explain the high selectivity obtained when matching a stationary phase having a high content of free silanols with a mobile phase containing methanol and a strong electrolyte.

Resolution is adequate in the β region and can be further improved in the γ region by increasing the gradient time. Quantitation appears to be possible simply by peak area measurement, but further research is needed to confirm this. The method can be applied in the study of physiological and clinical problems on haemoglobins and could be usefully employed in prenatal diagnosis of haemoglobinopathies, owing to its sensitivity. Studies on this topic are currently in progress.

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