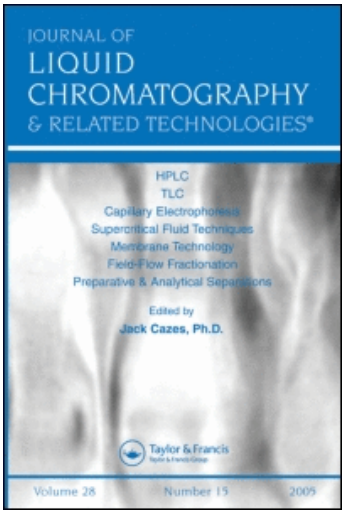

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A Comparative Study of the Separation of the Tryptic Peptides of the β-Chain of Normal and Abnormal Hemoglobins by Reversed Phase High Performance Liquid Chromatography

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A COMPARATIVE STUDY OF THE SEPARATION OF THE TRYPTIC PEPTIDES OF THE β-CHAIN OF NORMAL AND ABNORMAL HEMOGLOBINS BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of the tryptic peptides of the human hemoglobin A β-chain by reversed phase high performance liquid chromatography under different elution conditions on several microparticulate alkylsilica supports is described. Similar methods have been used to separate the tryptic peptides of β-chain hemoglobin variants including HbC, HbE, and Hb(Kempsey). Selectivity differences which can be achieved under the different chromatographic conditions have been exploited to permit the assignment of all the anticipated peptide fragments derived from the tryptic digestion of these β-chain Hb-variants.

INTRODUCTION

A large variety of procedures is now available for the structural characterisation of human hemoglobin (Hb) variants. In many cases, the identification has been based on the analysis of tryptic peptides of the isolated Hb polypeptide chains. Until

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recently, the usual methods of analysis of these tryptic fragments included combinations of cation- and anion- exchange chromatography or alternatively combinations of two dimensional chromatography and electrophoresis on inert support matrices. With the advent of reversed-phase high performance liquid chromatography (RP-HPLC), procedures have now become available for the rapid, high resolution separation of complex peptide mixtures, including those generated by enzymatic digestion of protein samples (1). Several of these procedures have already attracted attention for the peptide mapping of hemoglobin variants. For example, RP-HPLC techniques have been used, in conjunction with amino acid analysis and/or sequence analysis, to distinguish single amino acid replacement differences between hemoglobin variants using acetonitrile-water-orthophosphoric acid elution systems (2-4). Alternative RP-HPLC micro- and semi-preparative methods for the separation of hemoglobin fragments have also been described based on the use of non-volatile as well as completely volatile eluents, including various hydro-organic solvent combinations containing such additives as trifluoroacetic acid, ammonium acetate, triethylammonium acetate or trifluoroacetate, sodium perchlorate-orthophosphoric acid, ammonium bicarbonate or pyridine - acetic acid buffers (1, 5-12).

Most RP-HPLC studies on homology mapping of hemoglobins have largely been devoted to the separation of the tryptic peptides of either intact hemoglobins or the isolated α-, β-, γ- and δ-chains under a single set of chromatographic conditions. However, with complex peptide mixtures, a single elution protocol is rarely sufficient to allow complete resolution, i.e., with $R_s = 1$ of all the components. As a consequence of renewed interest in resolution optimisation for peptides separated on alkylsilicas, attention has recently been directed to examination of the chromatographic selectivity differences known (5, 13, 14) to be induced by different organic solvents as well as the concentration and composition of the buffer components, the pH and associated mobile phase conditions. A knowledge of these effects is clearly needed, if the full potential of RP-HPLC is to be exploited as an analytical tool in the detection of different hemoglobinopathies. The present study was addressed to this issue using as model systems the tryptic peptides of the isolated β-chains of normal and several hemoglobin variants in conjunction with low and neutral pH elution systems separated on different microparticulate alkylsilicas.

EXPERIMENTAL

High performance liquid chromatography was performed on μBondapak - alkylphenyl or -C18 columns (Waters Assoc., Milford, Mass.) and on LiChrosorb RP-8 (E. Merck, Darmstadt, G.F.R.) columns. The chromatographs used were a Waters model 202/401, equipped with a M440 gradient module and a M450 variable wavelength UV detector, and microprocessor-controlled

Spectra-Physics SP 8000B (Spectra-Physics, Santa Clara, Calif.) equipped with a SP 840 variable wavelength UV detector. Eluents and solutions of peptides were filtered using 0.45um type HA membranes from Millipore Corp. (Bedford, Mass.).

The normal hemoglobin A as well as the variants were obtained from blood samples collected in the usual fashion with EDTA as an anticoagulant. The erythrocytes were washed with 0.8% saline and hemolysed at ca. 0 under hypotonic conditions. The cell debris was removed by centrifugation at 2000xg and the supernatent dialyzed at 4 against 3 changes of 50mM Tris-HCl, pH 8.6, buffer (500ml) overnight. The hemoglobins were isolated by anion-exchange chromatography on a DEAE-Sephadex A50 column (64.5 x 1.5 cm) equilibrated with 50mM Tris-HCl, pH 8.6 at a flow rate of 50ml/h. Elution of the non-bound components was achieved with 150ml of the equilibration buffer. The bound hemoglobin components were eluted with a two stage gradient, commencing with a linear gradient of 50mM Tris-HCl (pH 8.5, 150ml) to 50mM Tris-HCl (pH 7.5, 150ml) up to an elution volume of 200ml, followed by a further linear gradient from 50mM Tris-HCl (pH 7.5, 150ml) to 50mM Tris-HCl (pH 6.5, 150ml). Elution of the hemoglobins was monitored at 415nm. The polypeptide chains of the normal and variant hemoglobins were separated by the procedure of Clegg et al. (15).

The isolated β-chains were aminoethylated and digested with trypsin (DPCC-treated) according to established methods (7, 15, 16). The samples were freeze-dried and stored at -20° until required. Individual tryptic peptides of the β-chain of HbA were resolved, as well as variant peptides identified, using conventional two dimensional high voltage electrophoretic-chromatographic procedures (17) for use as comparative solutes in the RP-HPLC experiments. Distilled water was further purified by reverse-osmosis. Acetonitrile was obtained from Burdick and Jackson (Muskegon, Mich.) or Waters Assoc. Orthophosphoric acid was from May and Baker (Dagenham, U.K.), ammonium acetate and ammonia were both Analar grade from B.D.H. (Poole, U.K.) and acetic acid was from Merck.

The reversed-phase columns were conditioned to all new elution conditions for ca. 30 min. and subjected to at least two blank gradient elutions prior to use. In the present study, the following elution protocols were employed: (A), a 60 min. linear gradient from aqueous 15mM orthophosphoric acid to 50% acetonitrile - 50% water - 15mM orthophosphoric acid (16, 19), and (B) a 80 min. linear gradient from 10mM ammonium acetate, pH 6.0, to 40% acetonitrile - 60% water - 10mM ammonium acetate (6). A flow rate of 1.0 or 2.0 ml/min. was used as indicated in the text. Peptide samples were dissolved in the initial eluent of the gradient system immediately prior to use and centrifuged at 5,000xg for 2 min. in a Microfuge. Sample injections were made using Hamilton (Reno, Nev.) model 1010W syringes and ranged from

45-850µg. The bulk solvents and various mobile phases were prepared and degassed as described previously (4).

Recovered peptide fractions from the reversed-phase separations using the (A)-elution system were immediately adjusted to pH 7.0 with 15mM NaOH. These fractions, as well as appropriate fractions obtained with the (B)-elution system, were dried under nitrogen and hydrolysed in vacuo in 6M HCl containing 0.1% phenol at 110° for 24 hr. The hydrolysates were analysed on a Joel amino acid analyser or a Beckman 121MB analyser.

RESULTS AND DISCUSSION

Fig. 1a shows the chromatogram of the tryptic peptides of the aminoethylated normal HbA β-chain eluted under the pH 2.1

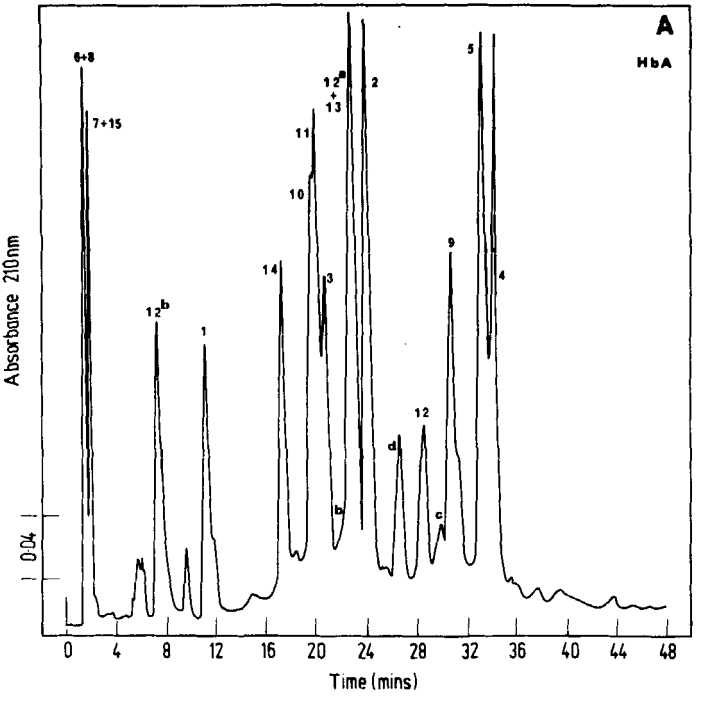

Figure 1(a). Separation of the peptides from the trypsin digestion of the aminoethylated normal human Hb β-chain by RP-HPLC. Chromatographic conditions: column, µBondapak - alkylphenyl; flow rate, 2 ml/min.; linear 60 min. gradient from aqueous 15mM orthophosphoric acid to 50% acetonitrile in aqueous 15mM orthophosphoric acid; sample loading, 810µg injected in a volume of 100µl.

phosphate mediated elution conditions from a μBondapak alkylphenyl column. As was noted in our earlier studies (1, 13, 18, 19) on the separation of tryptic and thermolysin peptides of a variety of globular proteins with aquo-acetonitrile gradients containing phosphate buffers at various molarities and pHs, these conditions permit excellent separation of complex peptide mixtures with little loss of resolution for sample loading up to ca. 500 nmoles on standard analytical (30 x 0.4 cm) reversed-phase columns. Following recovery of all the eluted peaks shown in Fig. 1a individual tryptic peptides were readily identified from their amino acid compositions. It was evident from these compositional data that all the expected major tryptic peptides had been recovered, and that most of the eluted peak zones contained only single peptide fragments. Peptides T-6 (Val^{60}Lys) and T-8 (Lys^{66}) coelute under these conditions as do T-7 (Ala^{62}His Gly Lys) and T-15 (Tyr^{145}His). The two peptides (T-12A and T-12B) corresponding to cleavage at the aminoethylated Cys^{112} were well resolved and a minor peak containing the intact peptide T-12 recovered. In

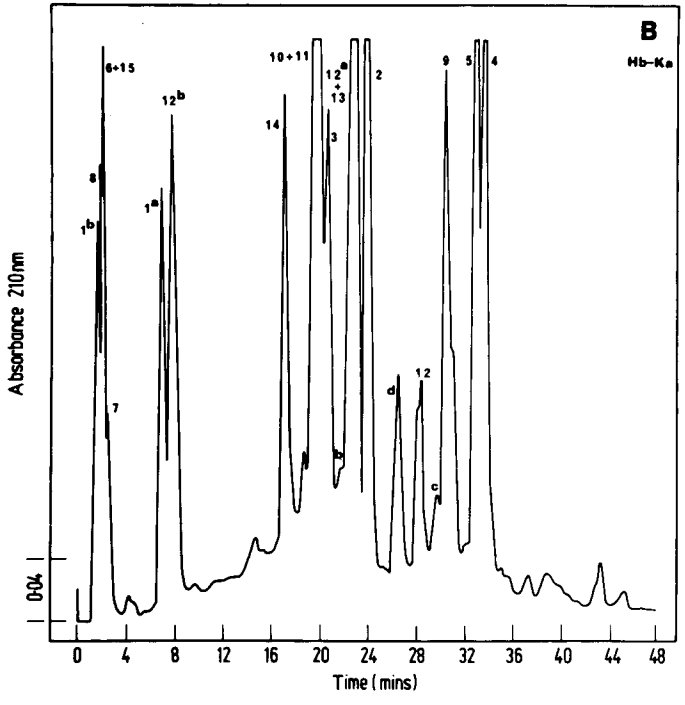

Figure 1(b). Separation of the peptides from the trypsin digestion of the aminoethylated abnormal human Hb(Ka) β-chain by RP-HPLC. Chromatographic conditions as in Fig. 1a; sample loading; 870μg injected in a volume of 100μl.

TABLE

Comparative elution orders for the tryptic peptides of normal Hb β-chain separated under different RP-HPLC conditions

Tryptic Peptide*	Residue †	Sequence**	Condition 1 Elution Order+	Condition 2 Elution Order§	Condition 3 Elution Order∫	Calculated Relative Hydrophobicity $\Sigma f_n\emptyset$	Calculated Relative Hydrophobicity $\Sigma X_n\emptyset\emptyset$
T-1	1-8	VHLTPEEK	6	5	5	4.35	2.21
T-2	9-17	SAVTALWGK	13	13	11	6.52	3.79
T-3	18-30	VNVDEVGGEALGR	10	7	6	4.59	4.25
T-4	31-40	LLVVYPWTQR	15	16	16	9.47	7.32
T-5	41-59	FFESFGDLSTPDAVMGNPK	16	15	14	11.78	16.85
T-6	60-61	VK	1	1	1	1.98	2.09
T-7	62-65	AHGK	3	3	3	0.82	-2.60
T-8	66	K	1	2	2	0.52	1.01
T-9	67-82	VLGAFSDGLAHLDNLK	14	14	14	11.35	15.45
T-10	83-95	GTFATLSELHCDK	8	11	10	7.40	6.71
T-11	96-104	LHVDPENFR	9	8	7	4.23	4.47
T-12A	105-112	LLGNVLVC	11	12	13	9.47	9.64
T-12B	113-120	VLAHHFGK	5	6	9	6.28	-1.04
T-13	121-132	EFTPPVQAAYQK	12	9	8	6.49	2.60
T-14	133-144	VVAGVANALAHK	7	10	12	4.81	1.68
T-15	145-146	YH	3	3	4	1.47	-0.28

* Peptides are numbered in order of their final position in the sequence.

† Amino acid sequence numbers.

** The one letter code for the amino acid as given by M.O. Dayhoff in Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Silver Spring, M.D., U.S.A.

\+ Relative order of elution from a μBondapak-alkylphenyl column with elution system (A).

§ Relative order of elution from a μBondapak C_{18} column with elution system (B).

ʃ Relative order of elution from a LiChrosorb RP-8 column with elution system (B).

Ø Calculated using the Σf_n (amino acid) values from ref. 31 with f_n (Glycine) = 0 and f_n (arginine) =-1.10 respectively. ØØ Calculated using the ΣX_n (amino acid) values from ref. 30

addition, the partial cleavage tryptic peptides corresponding to Lys^{66} Val ... Lys^{82} (T-8 + T-9) and Leu^{105} ... Lys^{132} (T-11 + T-12) were also present. Two peaks, both with amino acid compositions characteristic of peptide T-5 were obtained; the minor peak presumably corresponding to the oxidised Met^{55}-nonadecapeptide. Amino acid compositions of the remaining minor peaks indicated they contained peptides arising from residual chymotryptic cleavage, e.g., β-chain (131-132) or residual α-chain contamination, e.g., α(62-76) and α(77-90) of peptide α-T-9. The identification of even these minor peaks which occur regularly, but are not necessarily predictable in a sample emphasise again the excellent resolution which can be obtained with the current RP-HPLC methods compared to conventional procedures for Hb peptide mapping.

The RP-HPLC profile for the HbA- β-chain tryptic peptides eluted with a water-acetonitrile (0-40%) - 10mM ammonium acetate, pH 6.0, gradient on a LiChrosorb RP-8 column is shown in Fig. 2a. The peak assignments and relative elution order for the individual tryptic peptides recovered from this, and the corresponding μBondapak C18column separation are given in the Table. In both cases, amino acid compositional analyses revealed that the majority of recovered peak zones again contained only single tryptic fragments. Peptides T-6 (Val^{60} Lys), T-7 (Ala^{62} His Gly Lys) and T-8 (Lys^{66}) were only partially resolved on the LiChrosorb RP-8 support and coeluted with the μBondapak C18column. Superficially, the retention behaviour of the other tryptic peptides on the LiChrosorb RP-8 (5μm, spherical particle, ca. 14% carbon loading w/w, with nominal pore size of 6nm and surface area ca. 250 m^2/g) and the μBondapak C18 (10um, irregular particle, ca. 10% carbon loading w/w, with a nominal pore size of 8nm and surface area ca. 350 m^2/g) were similar under these pH 6.0 mobile phase conditions with many of the peptides following the same elution order. However, changes in relative selectivity, that is $\Delta\alpha/\alpha$, were evident for some peptides with the most noticeable elution order differences associated with the characteristic peptide cluster T-10, T-12, T-2 and T-14 which eluted over the intermediate concentration range of the organic solvent modifier (20-30% acetonitrile). Within this volume fraction range of the organic solvent modifier, relative selectivities of polypeptides on alkylsilicas are known to be responsive to stationary phase surface effects other than those due to the w/w percentage of the carbon loading, for example the presence of a more open siloxane-silanol network in the parent silica matrix which gives rise to different solvent extraction isotherms. Such subtle differences in stationary phase characteristics can be revealed from comparative plots of the logarithmic capacity factors for peptides/polypeptides versus the volume fraction of the organic solvent modifier (20).

Although the peptide mixture produced by the tryptic cleavage of the HbA β-chain contains peptides encompassing a wide range of

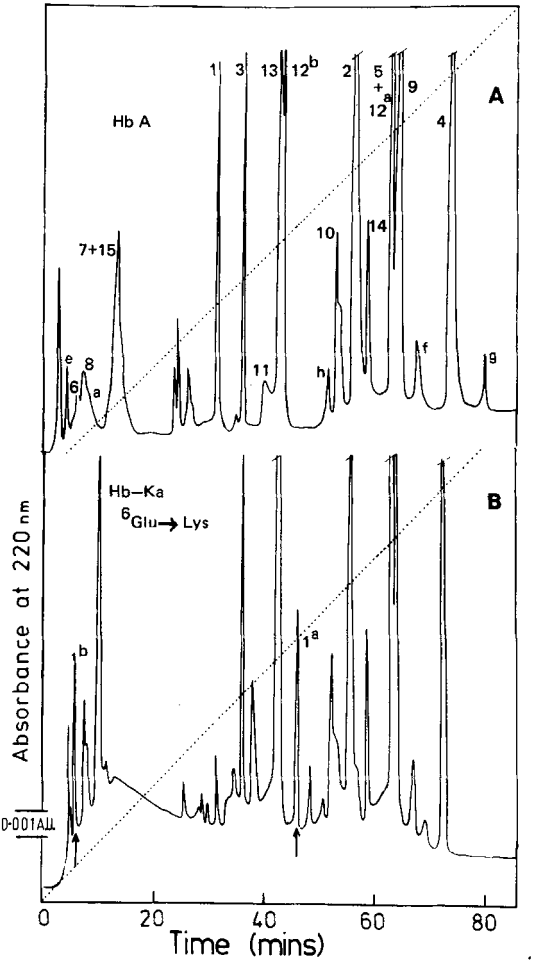

Figure 2. Separation of the peptides from the trypsin digestion of the aminoethylated normal and abnormal (Ka) human Hb β-chain by RP-HPLC. Chromatographic conditions: column, LiChrosorb RP-8; flow rate, 1ml/min.; linear 80 min. gradient from aqueous 10mM ammonium acetate, pH 6.0, to 40% acetonitrile in 10mM ammonium acetate, pH 6.0; sample loading, 100μg in a volume of 10μl.

hydrophobicities, on all three alkylsilica phases examined many of these peptides individually eluted within a narrow and characteristic ranges of organic modifier percentages irrespective whether the 10mM ammonium acetate, pH 6.0, or the 15mM orthophosphoric acid, pH 2.1, primary mobile phase conditions were employed, i.e., the retention of these peptides to a large extent was independent of secondary solution equilibria mediated by difference in the pH or buffer composition. Thus peptide T-2 (SAVTALWGK) eluted near to 25% acetonitrile under the two buffer and pH conditions irrespective of which stationary phase was selected. This type of retention behaviour has numerous precedents with peptides separated on alkylsilicas. The dominant role which the organic solvent exerts on the distribution equilibria for peptides separated under regular reversed-phase conditions (5, 20-23) is well recognised. Many peptides exhibit pronounced dependencies of their logarithmic capacity factors on the volume fraction of the organic solvent in the eluent. Where very steep dependencies exist, the retention behaviour of such peptides may become relatively unresponsive to secondary solution equilibrium effects due to changes in ionisation, solvation or buffer-ion complexation.

As expected, the smaller, more polar peptides were not strongly retained on the various reversed phases under the pH2.1 or the pH6.0 gradient elution conditions. With several Hb tryptic peptides, significant selectivity differences were noted presumably mediated by the different pH and/or buffer ion conditions. For example, peptides T-10 and T-14 elute much rapidly, and peptides T-3 and T-13 are retained longer, with the low pH, phosphate-based eluent when compared to the ammonium acetate conditions. Several studies (5, 24-26) have shown that relatively non-polar peptides (as is the case with T-10 and T-14) have augmented retentions on capped alkylsilica stationary phases with mobile phases near pH 7.0 but that the retention of these peptides is significantly decreased at lower pHs, e.g., pH 2.0-4.0 in the presence of phosphate, perchlorate or acetate ions. This situation is in contrast to peptides containing acidic amino acid residues at the C-terminus or endo-positions in the sequence (as is the case with T-3 and T-5) where retention times are generally smaller at high pHs, but progressively increase as the pH is lowered. It is unlikely that the changes seen in the retention behaviour for the above group of the Hb β-chain peptides under the two elution conditions have their origin solely due to differences in solute ionisation, but more likely reflect composite effects which give rise to variations in the effective molecular hydrophobic contact area of the peptide at the stationary phase interface (5). The observed differences nevertheless highlight the important role which secondary solution equilibria can play in modulation peptide selectivity on alkylsilicas. With tryptic fragmentation of proteins, the commonest occurrence of such pH-dependent selectivity changes on alkylsilicas can be anticipated with peptides containing several internal Asp and/or Glu residues

where potentially the side chain ionisation provides additional control for the optimisation of resolution of a complex peptide mixture.

In Fig. 1b, 2b and 3a are shown representative elution profiles for the tryptic peptides of the β-chains of the two variants Hb(Ka) and Hb(Ha) separated under the different chromatographic conditions. Comparison of the elution profiles for the tryptic peptides of the Hb(Ka) and normal β-chains indicated the presence of two new peaks, with the peak corresponding to peptide T-1 of the normal β-chain absent. Amino acid compositions of the appropriate fractions confirmed the substitution β^{6}- (Glu → Lys), thus identifying this variant as HbC. The peptide T-1A (Val^{1} ... Lys) of this HbC β-chain variant was well resolved from the other tryptic fragments under all the elution conditions examined. The dipeptide T-1B (Glu^{7} -Lys) resulting from the substitution, however, coeluted with peptide T-8 under the low pH phosphate conditions. The variant Hb(Ha) with an amino acid substitution in peptide T-3 was readily identified from the amino acid composition of the two peptides T-3A (Val^{18} ... Lys^{26}) and T-3B (Ala^{27} ... Arg^{30}) indicative of the substitution β^{26}(Glu → Lys) which is characteristic of the HbE variant. Using similar experimental methods, the variant Hb (Kempsey) was found to have a variant peptide T-11 which showed enhanced chromatographic retention. Subsequent analysis confirmed the β-chain amino acid substitution Asp^{99}→ Asn.

Because of the potential diversity in peptide structure, complete resolution of all the tryptic peptides generated by enzymatic cleavage of different hemoglobin variants (and, in general, of other proteins) is unlikely under a single RP-HPLC gradient elution condition. It should, however, be possible to achieve this goal by exploiting the selectivity differences which arise when two (or more) different elution conditions are employed sequentially. Such approaches have been applied to multidimensional separations of peptides using hydrophobic pairing ion systems (5, 28). The separation of partially resolved peptides can be achieved in several ways on reversed-phases. The simplest in terms of equipment requirements involves discrete rechromatography of the recovered zone using alternative elution systems, i.e., as exemplified by the characterisation of the HbC and HbE variants where peptides partially resolved by the phosphate based eluent could be separated by rechromatography using the ammonium acetate eluent under gradient (cf Fig. 1b, 2b) or isocratic conditions. Alternatively, on-line switching valves can be employed to redirect the appropriate portions of the column effluent directly onto a second coupled column pre-equilibrated to a second set of mobile phase conditions. Practical consideration for the use of coupled column strategies for the RP-HPLC separation of polar solutes, including peptides, have been discussed elsewhere (27, 28). Although the latter approach permits shorter overall analysis times for selected components, it

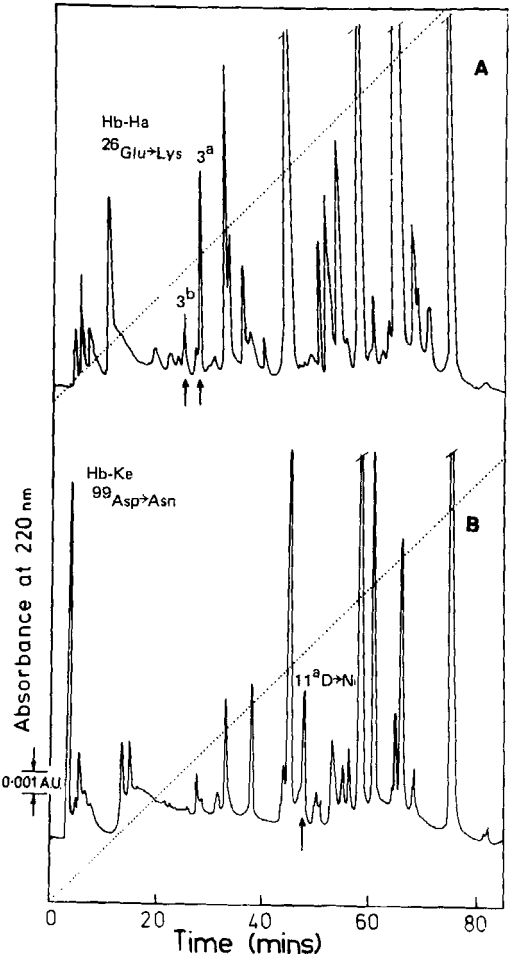

Figure 3. Comparison of the RP-HPLC profiles for the peptides from the tryptic digests of the aminoethylated β-chains of the Hb (Ha) and Hb (Kempsey) variants. Chromatographic conditions as in Fig. 2.

can suffer from reduced detection sensivity and elution behaviour perturbation for small polar peptides due to the effect of larger sample loading volumes.

In conclusion, this publication has further demonstrated that RP-HPLC peptide mapping of the tryptic digests of isolated aminoethylated Hb β-chain variants is a rapid, and extremely versatile technique. Single amino acid substitutions can be observed in the elution profile and readily confirmed by amino acid and/or sequence analysis. The combination of several gradient elution systems used with discrete or coupled column strategies should generally permit unequivocal resolution and assignment to be made for a specific abnormal peptide. Furthermore, Hb variants which do not involve changes in overall charge may now be systematically sought using these rapid RP-HPLC procedures which are becoming of increasing importance for the routine screening of hemoglobinopathies. One benefit of using elution systems of different pH and composition in combination is that advantage can be taken of pH-dependent selectivity changes exhibited peptides on alkylsilica supports. Clearly other combinations of ionic modifiers (e.g., TFA, ammonium bicarbonate) and pH conditions could be used in a similar manner to those employed in the present study to manipulate the resolution of peptide fragments generated in the characterisation of protein variants. The combined use of such alternative systems will be described elsewhere (29).

ACKNOWLEDGEMENTS

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