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Analytical peptide mapping by ion-exchange high-performance liquid chromatography: application to haemoglobin variants

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High-performance liquid chromatography (HPLC) has become increasingly important in the study of amino acid sequences in proteins [1, 2]. A number of advantages of HPLC include convenience and efficiency, high sensitivity, and ready application to both analytical and preparative purposes. Although peptide separations have been commonly made by HPLC with a reversed-phase column, the use of an ion-exchange column provides a new dimension. This report describes our experiences with separation of tryptic peptides of haemoglobins (Hb) by the use of an ion-exchange column and phosphate—acetonitrile gradient elution. The results indicate that this procedure is a useful technique for routine peptide mapping.

MATERIALS AND METHODS

Human haemoglobin variants investigated were those detected in this laboratory [3]. The methods for purification of haemoglobins, preparation of globins, separation and aminoethylation of the α- and β-globin chains were described previously [4, 5]. Tryptic peptides were made from aminoethylated globins in ammonium bicarbonate buffer at pH 8.0 and 37°C for 4 h. A JASCO (Japan Spectroscopic Co., Tokyo, Japan) Model TWINCLE high-pressure liquid chromatograph equipped with a Model GP-30 gradient elution system, a VL-611 variable-loop sample injector, and a UVIDEC 100-III variable-wavelength detector was employed. All separations were performed either on a TSK

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(Toyo Soda Manufacturing, Tokyo, Japan) IEX-510 SP SIL cation-exchange column (5 μm particle size, 0.4 × 30 cm), or on a JASCO FINEPAK SIL C_{18} reversed-phase column (10 μm particle size, 0.46 × 25 cm). Elution of peptides was achieved by the use of a linear gradient of phosphate buffers and acetonitrile. Two solvents were used for ion-exchange HPLC: developer 1 was 49 m*M* potassium dihydrogen phosphate and 5.4 m*M* orthophosphoric acid, pH 2.86; developer 2 was 40% acetonitrile in 50 m*M* potassium dihydrogen phosphate and 50 m*M* disodium hydrogen phosphate, pH 6.8, at a flow-rate of 0.8 ml/min. Developer 1 at pH 2.86 was used to elute the peptides from the reversed-phase column, with a gradient of acetonitrile at a flow-rate of 1.0 ml/min [2]. The elution was monitored at 220 nm. Most of our experiments were with 0.5—1 mg of the lyophilized samples on either of the two columns. No more than 0.1 mg was necessary for a pilot run. All column fractions from ion-exchange HPLC were lyophilized, redissolved in 100—150 μl of developer 1, and rechromatographed on the reversed-phase column. A 0.1% solution of trifluoroacetic acid was also used instead of phosphate buffer [6] when desalting of the final peptide was desired. The presence of phosphate salts in the sample interfered neither with analyses of the amino acid compositions, nor with rechromatographing the peptides by reversed-phase HPLC.

RESULTS

Fig. 1 (A and B) illustrates the separations of tryptic peptides from Hb A by ion-exchange HPLC. The developer sequence was initially a 12-ml isocratic elution with developer 1, and a 102-ml linear gradient from developer 1 to 2, the final level of which was continued isocratically. The peaks are very sharp and symmetrical for the most part in the overloaded chromatograms. Thus, each peptide emerged in a maximum volume of 0.5—1 ml, except for the αT6 peptide, which was eluted in a volume of 2—4 ml because of the substantial trailing pattern. The broader peak was also characteristic of the altered αT6 peptide from Hb Kokura in which a glycine residue is substituted for an aspartic acid residue at position 47. Peptides αT12-13, αT12, and αT13 did not derive from the soluble portion of the α-chain. Fig. 1 shows that these core peptides could have been isolated by ion-exchange HPLC. This knowledge was used to devise methods that may hopefully lead to the isolation of the core peptides. Indeed, these materials from the insoluble portion of the α-chain are barely soluble in the solvents at acidic pH, the majority of which precipitates and is then separated either by centrifugation or by filtration. The difficulty in dissolving these peptides upon application may be overcome by the use of 0.05% nonylamine—33% β-mercaptoethanol as solubilizer in a 0.1% phosphoric acid solution containing 5% methanol. The materials, once dissolved, did not form precipitates during chromatographic runs under the conditions used. Other attempts to isolate the core in pure form by reversed-phase HPLC were unsuccessful. Since problems were encountered with the solubility of the core, special emphasis has not been placed on the determination of the yield of core peptides. In all instances, nearly quantitative elution may be anticipated from chromatography on the cation-exchange column used for the initial study.

Fig. 2 provides a comparison of the positions of tryptic peptides from Hb A

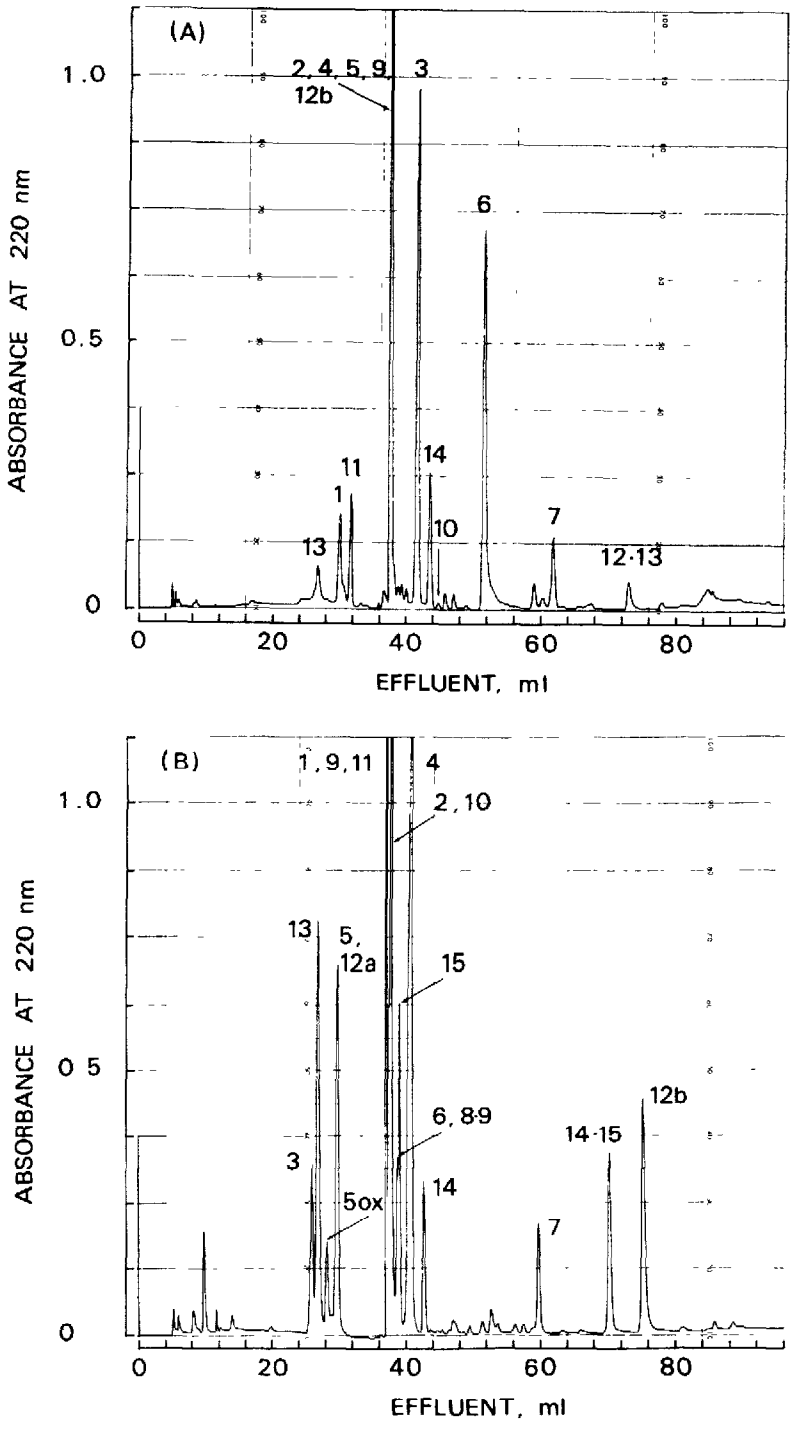

Fig 1. Separation of tryptic peptides from (A) α-globin, and (B) β-globin chains of Hb A on a 0.4 × 30 cm TSK IEX-510 SP SIL cation-exchange column. See text for developer sequence.

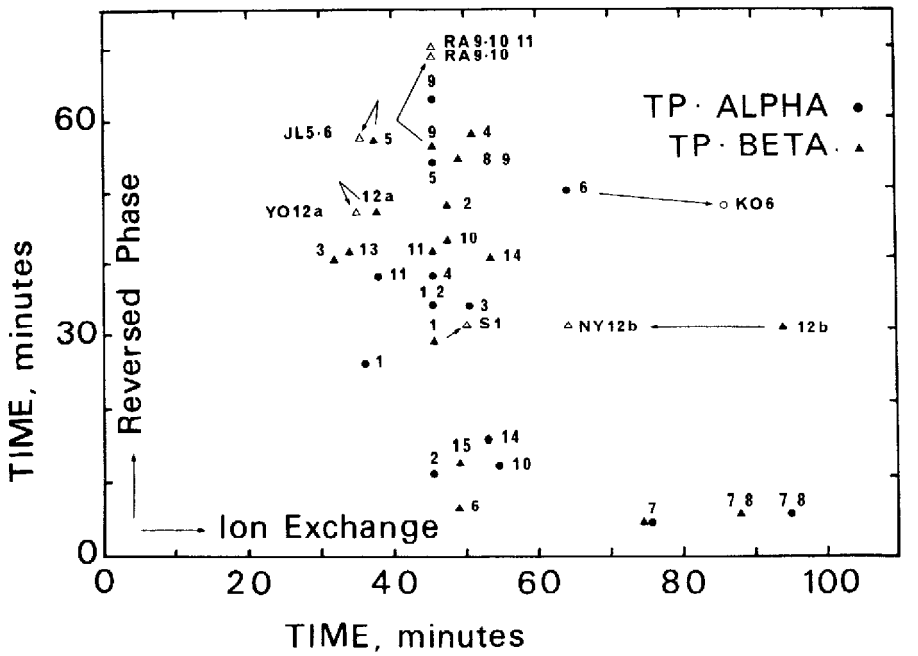

Fig. 2. Comparison of elution volumes of tryptic peptides of Hb A and the variants in two HPLC systems. The compositions of solvents are given in the text. Tryptic peptides (TP) are numbered in the sequence in which they occur in the polypeptide chains. Open symbols denote the positions of altered peptides from haemoglobin variants: RA = Hb Rahere (β82 lysine → threonine); JL = Hb J Lome (β59 lysine → asparagine); YO = Hb Yoshizuka (β108 asparagine → aspartic acid); KO = Hb Kokura (α47 aspartic acid → glycine); S = Hb S (β6 glutamic acid → valine); and NY = Hb New York (β113 valine → glutamic acid). Structural identifications are cited in refs. 3 and 7.

in the two HPLC systems. The open symbols and arrows which connect the positions of an altered peptide from haemoglobin variant with the normal counterpart show how different the movement can be. It is apparent that some electrophoretic variants could be identified more precisely on these two-dimensional maps than on the one-dimensional chromatograms.

DISCUSSION

In applying ion-exchange chromatography to the study of haemoglobin variants, we have tried to extend the unique possibilities of peptide separation by HPLC as much as does the successive use of electrophoresis and chromatography for the peptide mapping on paper support media. As shown, ion-exchange HPLC could identify peptides without much change in the molecular weight, but with alteration in charge. Although the hydrophilic matrices covered with hydroxyl groups have some properties of molecular sieving, the separation of peptides appears to be mainly due to the effect of net charge of the peptide. This was apparent when most of the neutral peptides, including some with +1 net charge, emerged in a single fraction. When this system produces a mixture, rechromatographing by reversed-phase HPLC usually effects the needed separation, since these two systems have entirely

different elution patterns. The substitution of an aspartic acid for an asparagine residue, or of glutamic acid for a glutamine residue, may be identified on ion-exchange HPLC. These substitutions may not cause much change in behaviour on reversed-phase HPLC [2]. Therefore, it is advantageous for the study of electrophoretic variants to use ion-exchange HPLC in the first dimension. If this system failed to show any abnormality, rechromatographing some of the fractions by reversed-phase HPLC could usually detect an alteration in the peptide compositions.

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