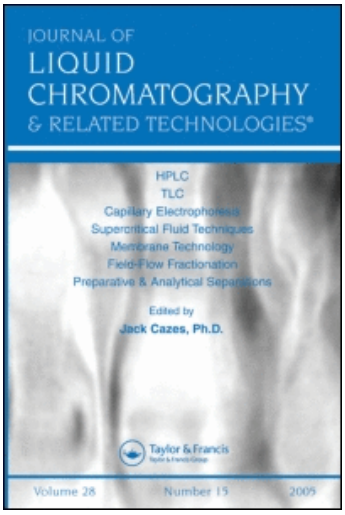

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High Performance Chromatography of Amino Acids, Peptides and Proteins XXIII · Peptide Mapping by Hydrophilic Ion-paired, Reversed-Phase High Performance Liquid Chromatography for the Characterisation of the Tryptic Digest of Haemoglobin Variants

C. A. Bishop[a]; W. S. Hancock[a]; S. O. Brennan[b]; R. W. Carrell[b]; M. T. W. Hearn[c]
[a] Dept. of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand [b] Clinical Biochemistry Dept. Christchurch Hospital, Christchurch, New Zealand [c] Immunopathology Research Unit Medical Research Council of New Zealand Medical School, Dunedin, New Zealand

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HIGH PERFORMANCE CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS XXIII·PEPTIDE MAPPING BY HYDROPHILIC ION-PAIRED, REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE CHARACTERISATION OF THE TRYPTIC DIGEST OF HAEMOGLOBIN VARIANTS[1]

C.A. Bishop and W.S. Hancock

Dept. of Chemistry, Biochemistry and Biophysics
Massey University
Palmerston North
New Zealand

S.O. Brennan and R.W. Carrell

Clinical Biochemistry Dept.
Christchurch Hospital
Christchurch
New Zealand

M.T.W. Hearn

Immunopathology Research Unit
Medical Research Council of New Zealand
Medical School
P.O. Box 913
Dunedin
New Zealand

ABSTRACT

The application of high performance liquid chromatography on reversed-phase columns for the tryptic mapping of haemoglobin variants is reported. The effect of flow rate and gradient shape on resolution and column efficiencies has been examined using acetonitrile-water-orthophosphoric acid elution systems. With these conditions it is possible to recognise sequence changes for variant haemoglobins including HbC, HbS, HbE, HbJ Cambridge and HbD Punjab.

INTRODUCTION

Rapid, sensitive, reproducible peptide mapping is now possible by means of high performance liquid chromatography (HPLC). A number of papers have demonstrated the use of ion-pair, reversed-phase HPLC to separate complex peptide mixtures generated by tryptic digestion of protein samples (1-6).

In order to fully assess the potential of HPLC for peptide mapping a well characterised protein was required with both sequence and structure/function data available. Perhaps the most widely investigated protein to date is haemoglobin, the structural characterisation of which has revealed a large number of functional and non-functional variants (7-10).

A number of well chraracterised haemoglobin variants in which the precise amino acid substitutions had been identified by classical paper fingerprinting and amino acid analysis techniques, were available for study by HPLC. In preliminary studies with the tryptic digests of a variety of haemoglobin variants, we demonstrated (11, 12) that HPLC techniques could be used to distinguish obvious differences between the tryptic peptide maps of HbA and the variants examined. The profiles could be obtained using microgram quantities of the digest, within 30 minutes of the initial injection and with excellent reproducibility.

A number of publications have subsequently confirmed the potential of similar techniques for screening and identifying haemoglobin variants (12-16).

In this paper we present further improvements in this HPLC approach utilising reversed-phase separations based on the hydrophilic ion pairing reagent, phosphoric acid. This chromatographic system was found to be capable of resolving variants which differ from HbA by a single amino acid substitution in one of the chains. Identification of the separated

mutant peptides was readily made by amino acid analysis of the recovered peaks. Excellent reproducibility and resolution have been obtained using this approach.

In addition, the flexibility of the chromatographic system allows easy and rapid manipulation of the mobile phase conditions to maximise resolution in areas of interest in the profile.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector coupled to two M450 variable wavelength UV spectrophotometers (Waters Assoc.) in series and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, Texas, U.S.A.). μBondapak C_{18} columns (10μm, 30 cm x 4 mm I.D.) purchased from Waters Assoc. were used for all analyses. Sample injections were made using a Gastight 1010 W syringe (Hamilton).

Solvents were filtered using a Pyrex filter holder (Millipore, Bedford, Mass., U.S.A.), while peptide samples were filtered using a Swinney Filter (Millipore). Millipore HA grade, 0.45μm filters were used for aqueous solvent and sample preparation.

Chemicals

Water was glass-distilled. Acetonitrile, supplied by Fisher Scientific, was further purified by the method of Walter and Ramaley (17). Orthophosphoric acid was from May and Baker (Dagenham, Great Britain).

Sample Preparation

All the tryptic digest samples used in the analyses were prepared by standard methods (9, 10), freeze dried and stored in

a freezer until required. Samples were made up in 0.1% phosphoric acid prior to use, at concentrations of 10mg/ml.

Method

The flow rate used was 1.5, 1.7 or 3.0 ml/min. All chromatography was carried out at room temperature (ca. 22°C). Sample size varied from 50 - 250 µl. Detection was at 230nm or 254nm. The aqueous component of the solvent was degassed by vacuum aspiration for at least 30 minutes, while the acetonitrile-water mix was degassed for 0.5 minutes prior to use. Orthophosphoric acid was added to the mobile phase at a concentration of 0.1%.

RESULTS AND DISCUSSION

Figure 1 shows three chromatographic profiles developed under different separation conditions of flow rate and column length. Each chromatogram is of a tryptic digest of HbA (see Methods section). A 30 minute linear gradient of 0.1% phosphoric acid to acetonitrile - 0.1% phosphoric acid (1:1) eluted all the peptides present in the mixture within 30 minutes. It is obvious that using two columns in series has markedly improved the resolution of the constituent peptides (compare Figure 1A with 1C). Comparing Figures 1B and 1C shows that resolution is in general improved at lower flow rates, although a decrease in flow rate may also require a corresponding increase in the length of the gradient so that the same amount of mobile phase is used to develop a critical separation, i.e., the rate of change of the organic solvent modifier (%/min/cm^{-3}) is held effectively constant.

Once an area of difference has been observed between the normal and mutant haemoglobin peptide map it is then necessary to maximise resolution in this area in order to facilitate the analysis and the subsequent identification of the variant pep-

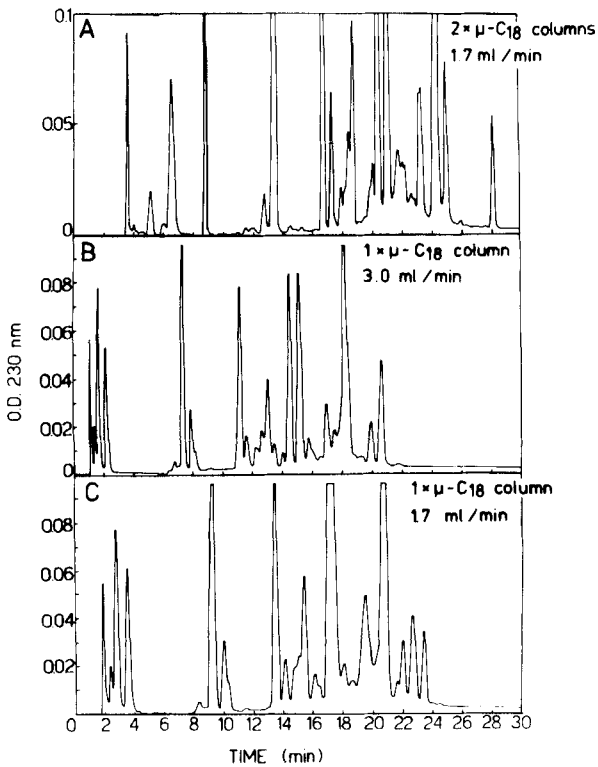

FIGURE 1

The HPLC elution profiles of a tryptic digest of HbA. Chromatographic conditions: in each case a 30 minute linear gradient was used with solvent A, 0.1% phosphoric acid, pH 2.2, and solvent B acetonitrile - 0.1% phosphoric acid, pH 2.2. (A) two μBondapak C_{18} columns connected in series with a flow rate of 1.7ml/min, (B) one μBondapak C_{18} column with a flow rate of 3.0ml/min and (C) one μBondapak C_{18} column with a flow rate of 1.7ml/min.

tide(s). One simple method for doing this, without altering the mobile phase conditions per se, is to manipulate the gradient shape. Figure 2 shows three markedly different elution profiles obtained for the tryptic digest of HbA using the same

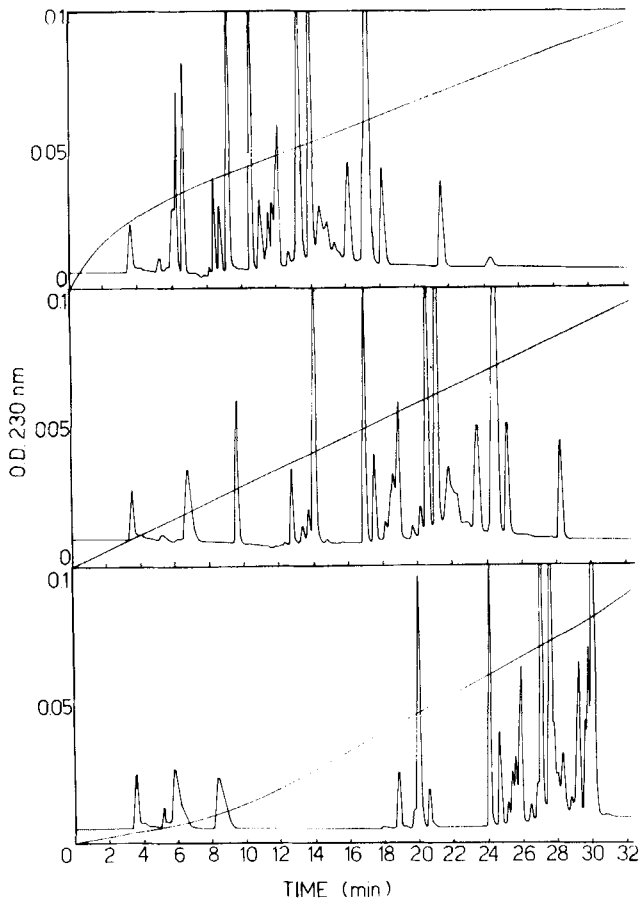

FIGURE 2

The elution profiles obtained for the tryptic digest peptides of HbA developed using three distinct gradient shapes, numbers 3, 6, and 8 on the M660 solvent programmer. In each case two μBondapak C_{18} columns were connected in series, a flow rate of 1.7ml/min was used, with the mobile phase compositions as in Figure 1.

conditions as in Figure 1A but altering the shape of the gradient. In this example the linear gradient, shown in the middle elution profile, gives optimal separation. Similar conclusions regarding the optimal gradient for acetonitrile - water systems have also been reached by Schoenmakers et al (18).

For unambiguous identification of peaks obtained within an HPLC peptide map the peaks must be collected and subjected to amino acid analysis and, if possible, other chemical procedures such as mass spectrometry. However preliminary rapid identifications can be made by simultaneous detection of the eluted peptides at two or more wavelengths, using variable wavelength UV spectrophotometry. Figure 3 shows the detection of HbA tryptic peptides at 230nm and 254nm. Only peptides containing amino acids with aromatic side chains will be observed at the higher wavelength. In addition UV detection at 210nm can be used to gain a significant increase in sensitivity (1,2,11,12). The use of low wavelength detection, however, requires the use of highly purified aqueous and organic solvents in the mobile phase. To guard against the presence of artifactual solvent peaks in the peptide map, blank gradients were always run following any alteration in chromatographic conditions.

Once suitable chromatographic conditions were established, the tryptic digests of a series of mutant haemoglobins were analysed and compared to normal adult HbA. Figure 4 shows one such comparison, between HbA and HbS. The clear difference between the chromatograms is shown by the arrows in Figure 4. A peak with a retention time (R_t) of 13.6 minutes in the Haemoglobin A profile is absent in the Haemoglobin S profile and is replaced by a peak with R_t 14.6 minutes. In order to establish that the different peak did in fact correspond to the known structure of the variant peptide, the peak was collected, freeze-dried, subjected to acid hydrolysis (6M HCl, 24 hours) and amino acid analysis. HbS has a valine residue substituted

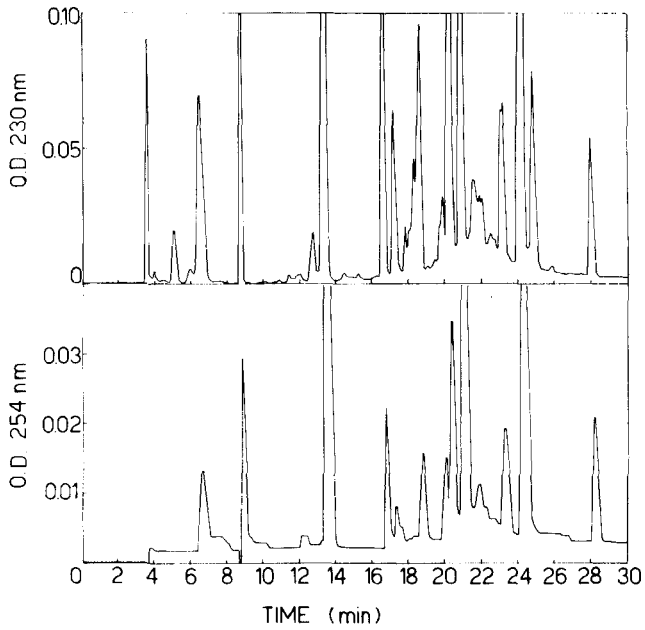

FIGURE 3

The elution profile of the HbA tryptic digest peptides using chromatographic conditions as in Figure 1A with UV detection at 230 nm and 254 nm. The chromatograms were recorded simultaneously using two variable wavelength spectrophotometers connected in series by minimum dead-volume connecting tubing.

for a glutamic acid at position 6 on the β chain of HbA. Such a substitution would make the octapeptide Val-His-Leu-Thr-Pro-Glu-Glu-Lys, (β^A chain, 1-8), in which it occurs more non-polar. This would increase the interaction between the peptide and the hydrophobic stationary phase, hence increasing the observed retention time. Amino acid analyses of the two peaks in question confirmed that the composition of the R_t 14.6 peak was in agreement with the known mutant octapeptide of haemoglobin S. The analysis results obtained were as follows. For the octa-

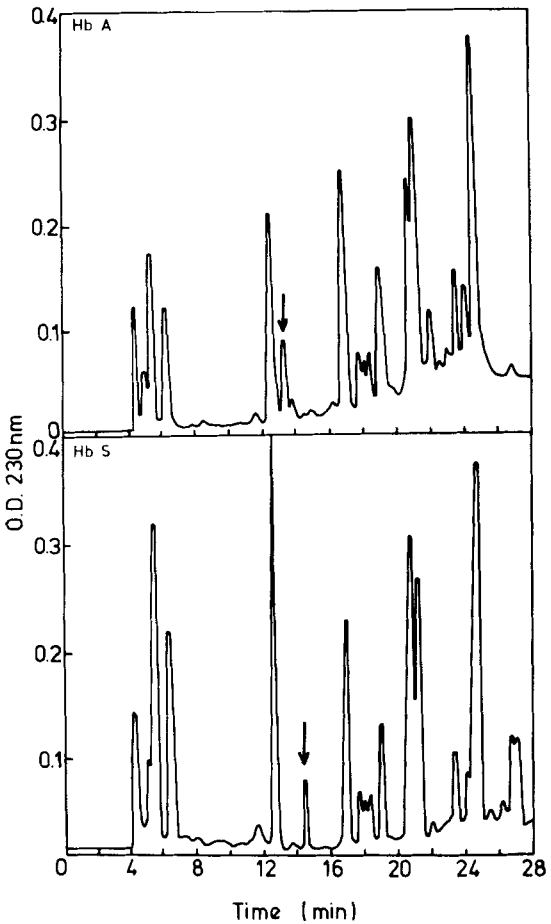

FIGURE 4

The comparative elution profiles of the tryptic digests of HbA and HbS, run using the same chromatographic conditions as in Figure 1A with the exception of the flow rate, which in both cases was 1.5ml/min. The arrow refers to the replacement (β6 Glu→Val) octapeptides.

peptide from haemoglobin A: $Thr_{0.98(1)}Glu_{2.01(2)}Pro_{1.0(1)}Val_{1.1(1.0)}Leu_{1.1(1)}His_{1.0(1)}Lys_{1.0(1)}$. For the octapeptide collected from the haemoglobin S sample: $Thr_{1.02(1)}Glu_{1.0(1.0)}Pro_{1.05(1)}Val_{2.03(2.0)}Leu_{1.15(1.0)}His_{1.08(1.0)}Lys_{1.0(1.0)}$. In general, it has been our experience that the determination of the amino acid composition of peptides, recovered from reversed-phase HPLC under the above elution conditions, is straightforward. Alternatively, the fractionated peptides can be subjected to sequence analysis by standard techniques.

This example represents a relatively major change in the polarity of the variant peptide. However, the identification of more subtle changes in peptide structure are possible by similar means, particularly when the potential flexibility of the chromatographic conditions is taken into account. It is now possible, when investigating a selected area of difference within a chromatogram, to manipulate the elution conditions such as gradient shape, gradient time, pH, type of ion pairing reagent used or temperature, and thus greatly improve resolution of overlapping peaks in that area. In order to demonstrate that these procedures were generally applicable, a range of variant haemoglobins were analysed and compared to HbA. Figure 5 shows a selection of these comparisons. The arrow on each profile indicates the position of a peptide which does not occur in the HbA analysis. Circled letters indicate peaks missing in corresponding chromatograms. The amino acid substitutions occurring in each variant peptide are also indicated.

With the conditions used in this study, excellent reproducibility was observed between analyses of the same tryptic digestion sample run at times differing by up to 6 months. As a cautionary point, however, care must be exercised in accurately reproducing these chromatographic conditions between runs since a change in any of the conditions e.g. pH, can markedly alter a profile. In general, these precautions present

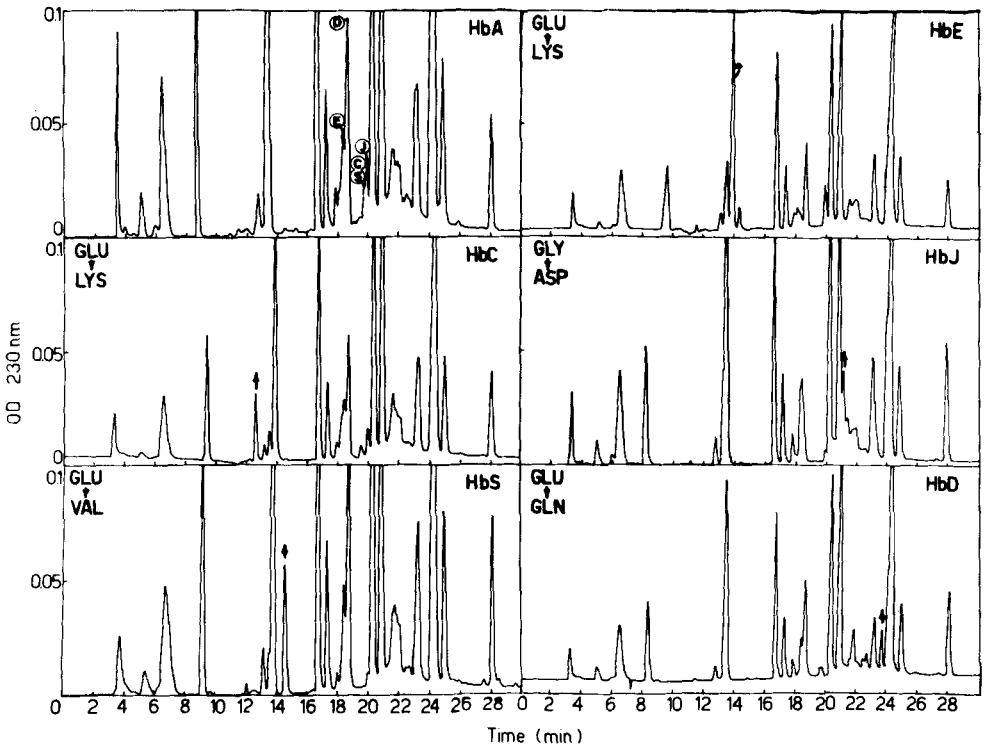

FIGURE 5

The comparative elution profiles of the tryptic digest profiles of HbA and a range of well characterised variants: HbC (β6 Glu→Lys), HbS (β6 Glu→Val), HbE (β26, Glu→Lys), HbJ Cambridge (β69 Gly→Asp), HbD Punjab (β121 Glu→Gln). Chromatographic conditions were in each case identical to those in Figure 1A. Two variants (HbC and HbE) would be expected to give an additional tryptic fragment due to the presence of an additional lysine residue. In both cases, the region which occurs at 13 to 15 minutes in the gradient analysis shows an additional peak, although the major difference in each chromatogram is indicated by an arrow.

no experimental difficulty. We have, however, noted some changes occurring in the chromatograms of haemoglobin digests obtained when different tryptic digestion conditions are used. As it is often difficult to exactly reproduce an enzymatic hydrolysis of a protein, slight variations in the extent of digestion can result in altered relative peak heights and positions. If the tryptic digestion and subsequent chromatographic separation are carried out using carefully standardised procedures, any variation should be limited to small changes in peak heights and not alter the observed order or number of peptides.

CONCLUSIONS

This publication has shown that peptide mapping of tryptic digests of haemoglobin variants is a rapid, sensitive, reproducible and extremely flexible technique.

This conclusion combined with other recent publications (12-16) suggests that reversed-phase HPLC will become an important procedure in the screening for haemoglobin variants. The technique should be particularly useful for examining variants exhibiting minor sequence differences, e.g. Gly→Ala, which may be amenable to separation by reversed-phase HPLC, but not as readily by other current chromatographic procedures. In addition the use of the hydrophilic ion pairing reagent, phosphoric acid combines excellent peak shape with low wavelength UV detection. This should be particularly useful for the analysis of haemoglobin variants which are only available in microgram amounts.

Similar peptide mapping techniques also have many potential applications for the rapid screening of homology similarities of variant proteins, a number of which are presently under investigation.

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FOOTNOTES

1. Part XXII : ref 19.
2. To whom correspondance should be addressed.