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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

II. THE USE OF PHOSPHORIC ACID IN THE ANALYSIS OF UNDERIVATISED PEPTIDES BY REVERSED-PHASE HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The chromatographic properties of a range of peptides varying in size from di- to decapeptide have been investigated by reversed-phase high-pressure liquid chromatography. A new set of conditions, namely, the addition of phosphoric acid to the mobile phase, has been found to have very real advantages in the analysis of underivatised peptides. These conditions allowed marked alterations in retention times, improvement in reproducibility and excellent resolution of peptides differing by as little as a single amino acid. A major advantage of phosphoric acid is that it can be used successfully in the range 195–220 nm which makes it compatible with the use of variable wavelength UV monitors as sensitive detectors in high-pressure liquid chromatography. In addition, the use of phosphoric acid permits the significant lowering of concentrations of organic solvents in the mobile phase, thus reducing the possibility of denaturation or precipitation.

INTRODUCTION

In the past, reversed-phase liquid–liquid partition chromatography has found limited application, but recent developments in the preparation of chemically bonded stationary phases have shown that highly efficient columns can be prepared for a variety of applications which include the analysis of pharmaceuticals[1], metabolites[2], pesticides[3], derivatised amino acids[4–7] and derivatised peptides[8]. Recently the use of

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ammonium acetate[9] and other buffers[10] has been reported for the analysis of peptides by reversed-phase high-pressure liquid chromatography (HPLC).

We recently reported a reversed-phase method for the analysis and resolution of a series of underivatised peptides on 37–50 μm pellicular packings[11]. Improved retention times with consequently improved peak separations should result by using reversed-phase packings of less than 10 μm particle diameter. These fully-porous silica based packings are noted for their significantly higher plate counts. It was found, however, that retention times of known peptides were extremely long and reproducibility was difficult to obtain with these micro-reversed-phase packings. In order to overcome these problems a series of ion-pairing and ionic suppression reagents were examined as additives to the mobile phase. As a result of these studies we found that several reagents, and in particular phosphoric acid, when added to the eluant, gave a marked improvement in reproducibility and retention times of peptides on commonly used reversed-phase systems. In addition, the use of phosphoric acid allows detection of eluted peptides at wavelengths in the range of 200–220 nm.

It is the purpose of this paper to describe the effectiveness of phosphoric acid in the analysis of a variety of peptides.

MATERIALS AND METHODS

High-pressure liquid chromatography

A Waters high-pressure liquid chromatography system was used which included two M-6000 solvent delivery units, an M-660 solvent programmer and a U6K universal liquid chromatograph injector, coupled either to a Cecil 212 variable wavelength UV monitor with an 8 μl flow-through cell and a Linear Instruments Corp. double channel chart recorder, or to a Series 440 Waters UV detector and Rikadenki double channel chart recorder.

The Bondapak C_{18}-Corasil and Bondapak Phenyl-Corasil (37–50 μm) were purchased prepacked in stainless steel columns (61 cm × 2 mm I.D.) from Waters Assoc. (Milford, Mass., U.S.A.). The μBondapak-Fatty acid analysis and μBondapak-C_{18} columns (10 μm, 30 cm × 4 mm I.D.) were also from Waters Assoc. Sample injections were made with a Pressure-Lok liquid syringe, Series B-110 from Precision Sampling (Baton Rouge, La., U.S.A.). Filtration of solvents was carried out using a pyrex filter holder (Millipore, Bedford, Mass., U.S.A.) while peptide samples were filtered using a Swinney Filter (Millipore).

Reagents

All solvents were Analar grade. The methanol was used as supplied by Mallinckrodt (St. Louis, Mo., U.S.A.). The acetonitrile, supplied by Fisher Scientific (Pittsburgh, Pa., U.S.A.) was further purified by the method of Walter and Ramaley[12]. Orthophosphoric acid was from May & Baker (Dagenham, Great Britain), potassium dihydrogen phosphate and benzoic acid from BDH (Poole, Great Britain). Water was glass-distilled and de-ionised. Samples in Fig. 3 (A, B, D) and the protected hexapeptide (Fig. 2) were produced in this laboratory[13] by the solid phase method using standard procedure[14]. The peptides described in Table I were purchased from Research Specialities (Richmond, Calif., U.S.A.). All amino acids were of the L-configuration.

Methods

A flow-rate of 1.5 ml/min was used for each column which was maintained by a pressure of 1100 p.s.i. for the C_{18}-Corasil column, 2000 p.s.i. for the phenyl-Corasil column, 2100 p.s.i. for the μBondapak-C_{18} column and 2300 p.s.i. for the μBondapak-Fatty acid analysis column. All tests were at room temperature (*ca.* 22°). Sample sizes varied between 0.1 and 10 ng of peptide material injected in volumes 1–100 μl. Detection was in the range 205–225 nm, 254 nm or 280 nm depending on the nature of the sample and the mobile phase. All peptides were taken up in de-ionised, distilled water and, prior to injection, made up in the eluting solvent. All bulk solvents were degassed separately for the following times; water for at least 30 min, organic solvents for 1.5 min. The solvents were mixed in the required volumes, degassed for 1.0 min and then equilibrated to room temperature. This preciseness was necessary for reproducibility. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents for at least 30 min. The mobile phase solutions were routinely filtered using 0.5 μm Millipore filters as were all peptide samples.

RESULTS

In Fig. 1 the marked decrease in retention time of a tetrapeptide (Leu-Trp-Met-Arg) on C_{18}-Corasil after the addition of 0.1% phosphoric acid to the mobile phase is shown. The pH of the acetonitrile–water mobile phase decreased from 6.5 to 2.2. These are apparent pH values obtained using a glass electrode. Thus, the addition of phosphoric acid will have a dual effect of a change in pH and of the equilibria of the ionisable groups in the peptide. The very basic guanidino side chain of the arginine residue (pK_a 12.48) will remain protonated over the pH ranges used in reversed-phase chromatography. The α-amino group and C-terminal carboxyl group will, however, both be affected by this change in pH.

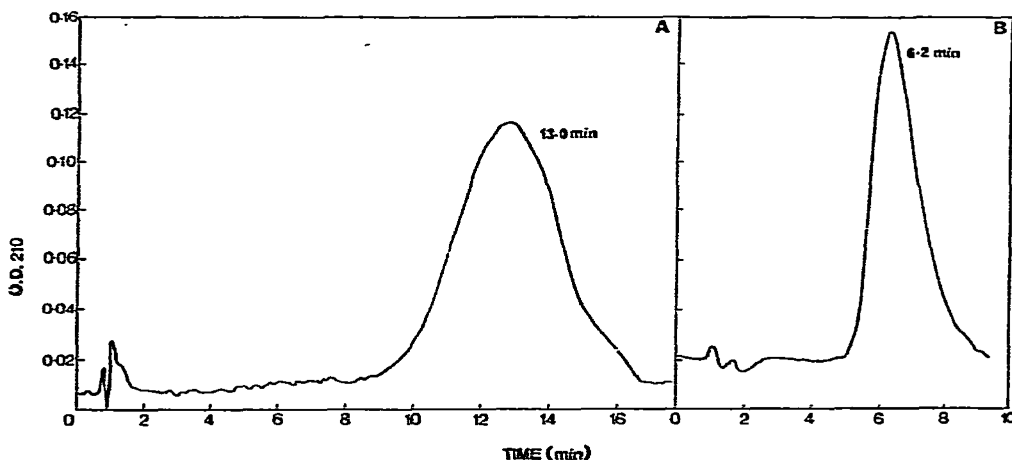

Fig. 1. Elution profile of the tetrapeptide Leu-Trp-Met-Arg on a C_{18}-Corasil column. (A) with acetonitrile–water (1:3) as mobile phase (B) with acetonitrile–water (2.5:97.5) and 0.1% phosphoric acid as mobile phase.

In an attempt to distinguish between these effects a model peptide (a derivative of the amino terminal hexapeptide fragment of Acyl Carrier Protein) Ac-Ser-Thr-Ile-Glu(OBzl pNO$_2$)-Asp(OBzl pNO$_2$)-Arg(NO$_2$)OH, was examined under the same conditions. Since the amino terminus has been blocked by acetylation and all side chain functional groups are protected, the only ionisable group is the C-terminal carboxyl group. In this case decreasing the pH of the mobile phase increases the retention time of the peptide (Fig. 2). Apparently, suppression of the ionisation of the carboxyl group has reduced the polarity of the peptide. A variety of concentrations of added phosphoric acid were tested, and 0.1% (v/v) was found to be optimal and was used in all subsequent studies. A similar result was observed for acetic acid in that at the same pH of the mobile phase, both acetic and phosphoric acid resulted in increased retention times for the peptide. Similar results were obtained in the analysis of the amphoteric molecule, p-aminobenzoic acid[15].

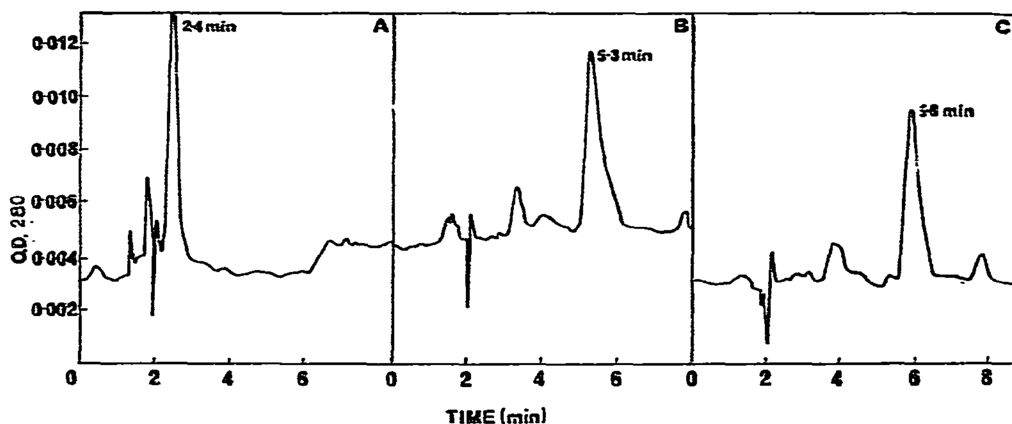

Fig. 2. Elution profile of the protected hexapeptide Ac-Ser-Thr-Ile-Glu (OBzl pNO$_2$)-Asp(OBzl pNO$_2$)-Arg(NO$_2$)OH on a μBondapak-Fatty acid analysis column. In each case the mobile phase was methanol–water (48:52) with the following additions: (B) 0.1% acetic acid; (C) 0.1% phosphoric acid.

The dramatic reduction in retention time for peptides which contain free amino groups was confirmed with a variety of other examples (Column 1, Table I). The retention time of the pentapeptide Leu-Trp-Met-Arg-Phe with a mobile phase consisting of 40% methanol–water decreased from 104 to 12.4 min on the addition of phosphoric acid. Many peptides which contain aromatic amino acids are strongly retained on reversed-phase columns. Such an example is shown in Table II with the peptide linear antamanid where the use of phosphoric acid greatly facilitates the chromatography of such materials. Fig. 3 shows the elution profiles of four peptides on a μBondapak-Fatty acid analysis column. Again in each case the addition of phosphoric acid allowed rapid analysis of the peptide by reversed-phase HPLC.

It is of interest to note that phosphate buffers also mimic the effect of phosphoric acid on retention times. As is shown in Table II with linear antamanid as an example, the effect of the $H_2PO_4^-/HPO_4^{2-}$ system on retention time is greatest at the lower pH values. However, 0.1 M potassium dihydrogen–monohydrogen phosphate at pH 7 still causes a significant decrease in retention time for this peptide from 100 to 4.7 min.

TABLE I

THE EFFECT OF PHOSPHORIC AND ACETIC ACID ON THE RETENTION TIME OF VARIOUS PEPTIDES

The analysis was carried out on a μBondapak alkyl-phenyl column with 50% methanol as the eluent.

*Peptide**	*Retention time*	
	*0.1% H_3PO_4, pH 2.5***	*0.1% CH_3COOH, pH 4**,****
L-W-M-R	2.3	4.1
L-G-M-R-F	5.1	8.3
G-F	2.4	3.5
G-G-Y	1.9	3.0
M-R-F	2.5	3.8
F-S-K-L-G-D-G	2.4	3.8
G-L-Y	2.4	3.6
R-F-A	2.05	2.2

* The code for amino acids is as used by Dayhoff[23]. A = alanine, D = aspartic acid, F = phenylalanine, G = glycine, K = lysine, L = leucine, M = methionine, R = arginine, S = serine, W = tryptophan, Y = tyrosine.

** Apparent pH values measured with a glass electrode.

*** Broad peaks unsuitable for analytical separations were observed with this reagent.

TABLE II

THE RETENTION TIMES OF THE DECAPEPTIDE LINEAR ANTAMANID[1] ON A μBONDAPAK-C_{18} COLUMN

Amino acid sequence Val-Pro-Pro-Ala-Phe-Phe-Pro-Pro-Phe-Phe.

Mobile phase Methanol–water	*Retention time (min)*
50:50	∞
55:45	∞
60:40	100
Methanol–water + 0.1% phosphoric acid	
50:50	6.01
55:45	2.74
60:40	2.37
Methanol–water (55:45) + 0.1% phosphoric acid + 0.1 M potassium dihydrogen dipotassium hydrogen phosphate	
pH 2.5	2.45
pH 3.0	2.70
pH 4.0	3.30
pH 5.1	4.95
pH 7.0	4.07

DISCUSSION

In a recent publication[11] the separation of underivatised peptides on reversed-phase C_{18}- or alkyl-phenyl columns (37–50 μm) was described. Although useful results could be obtained with this system, it was noted that peptides characteristically give broad peak shapes indicating a lower theoretical plate count, N, than those observed

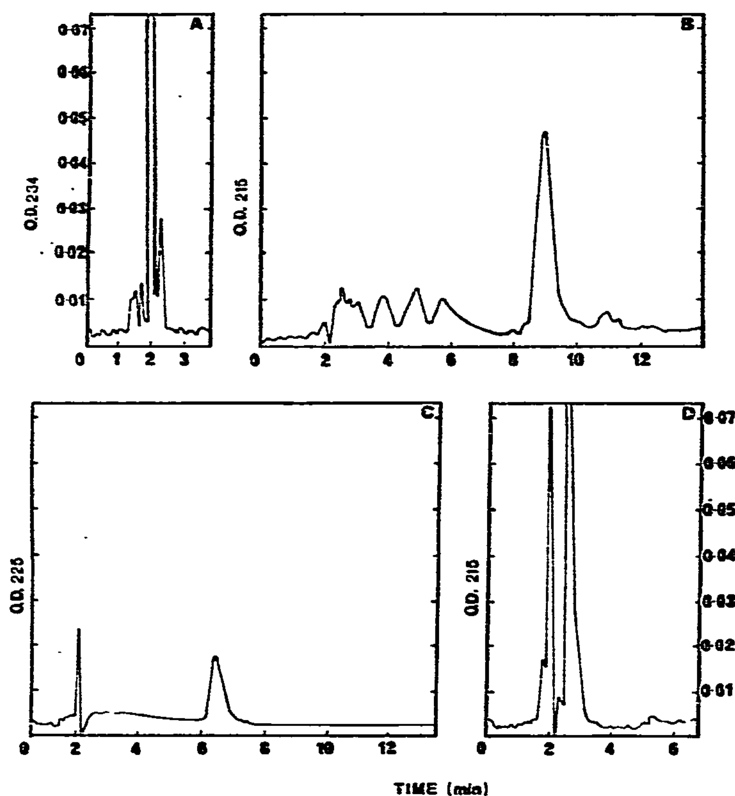

Fig. 3. Elution profiles of four different peptides on a μBondapak-Fatty acid analysis column. (A) The synthetic peptide linear antamanid with a mobile phase consisting of methanol–water (4:1) and 0.1% phosphoric acid. (B) The synthetic peptide Val-Ile-His-Pro-Phe, an angiotensin fragment, with a mobile phase consisting of methanol–water (1:1) and 0.1% phosphoric acid. (C) The tetrapeptide Met-Arg-Phe-Ala with mobile phase acetonitrile–water (1:3) and 0.1% phosphoric acid. (D) The synthetic peptide Glu(OBzl)-Glu(OBzl)-Glu(OBzl)-Glu(OBzl) with mobile phase methanol–water (3:7) and 0.1% phosphoric acid.

for most other organic molecules. For example, benzoic acid has a value of N 2.5 times that of the tetrapeptide Leu-Trp-Met-Arg when compared on the same chromatogram. This broader peak shape can possibly be attributed to the complex contribution of ionic groups to the polarity of the peptide. This phenomenon has been recently observed with other highly polar molecules[16,17].

The use of the micro columns, μBondapak-Fatty acid analysis and μBondapak-C_{18}, has now been investigated in an attempt to solve this problem. These packings have the stationary phase chemically bonded to silica particles of 10 μm. Unfortunately, in preliminary experiments with these micro columns, peptides often gave irreproducible elution times when chromatographed under standard conditions with methanol–water mobile phases. In some cases, very long retention times were obtained, as was the case with linear antamanid which was eluted at 100 min even with a high concentration of an organic solvent in the mobile phase.

The addition of phosphoric acid to the eluant resulted in dramatic decreases

in the retention time of a range of peptides of widely different chemical structures (Figs. 1(b) and 3; Tables I and II) as well as giving good reproducibility. This general phenomenon of decrease in retention time is, presumably, due to an increase in polarity of peptides when analysed by reversed-phase chromatography in the presence of phosphoric acid.

A possible explanation for the effect of phosphoric acid on the polarity of a peptide can be obtained by a consideration of the ionic equilibria involved. In the case of the unblocked peptide Leu-Trp-Met-Arg, addition of acid will not only have the effect of protonating the carboxyl group, but also will simultaneously affect other equilibria, such as:

$$R\text{-}NH_2 \rightleftharpoons R\text{-}NH_3^+ \quad (1)$$

It appears that protonation of the amino group, by driving the equilibrium (1) to the right, overwhelms the contribution of the neutral carboxyl group, the formation of which should make the peptide less polar.

The effect of phosphoric acid cannot, however, be solely explained on the basis of a simple acid effect. As can be seen for the case of linear antamanid the addition of $H_2PO_4^-$ at constant pH causes a large decrease in the retention time (Table II). With methanol–water (60:40) and 0.1% phosphoric acid as the eluant this decapeptide gave a retention time of 2.37 min on the μBondapak-C_{18} column. The addition of 0.1 *M* potassium dihydrogen phosphate to this eluant decreased this retention time further to 1.9 min. Other polar anions such as perchlorates[18,21], picrates[22], and methyl sulphonates[20] cause a similar decrease in retention time of peptides[21] and polar pharmaceuticals[22], which was attributed to ion-pairing between the polar anion and the solute molecules.

By analogy it is possible that the formation of an ion-pair between the peptide $R\text{-}NH_3^+$ and the hydrophilic anion $H_2PO_4^-$ is responsible for the large increase in polarity observed for solute molecules in the present study. Other possible explanations seem less likely, particularly as less polar acids such as acetic acid (column 2, Table I) do not show this dramatic effect.

CONCLUSION

The purpose of this paper has been to report the advantages of phosphoric acid in the analysis of underivatised peptides by reversed-phase HPLC: namely, marked alterations in retention times, improvement in reproducibility and excellent resolution of peptides differing by as little as a single amino acid (Fig. 3 B, C).

Since phosphoric acid can be used successfully in the range 195–220 nm, it is compatible with the use of variable wavelength UV monitors as sensitive detectors in HPLC[11]. In addition phosphoric acid is readily available, inexpensive, and allows the use of significantly lower concentrations of organic solvents in the mobile phase (with less chance of precipitation or denaturation of peptides). The development of a system for the routine analysis of underivatised peptides and amino acids using such reagents is being vigorously pursued.

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REFERENCES

1 F. Bailey, *J. Chromatogr.*, 122 (1976) 73.
2 S. Roggia, G. Grossoni, G. Pelizza, B. Ratti and G. G. Gallo, *J. Chromatogr.*, 124 (1976) 169.
3 J. N. Snieber, *J. Chromatogr.*, 94 (1974) 151.
4 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, *Anal. Chem.*, 48 (1976) 1106.
5 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 75 (1976) 77.
6 M. R. Downing and K. G. Mann, *Anal. Biochem.*, 74 (1976) 298.
7 J. X. de Vries, R. Frank and C. Birr, *FEBS Letters*, 55 (1975) 65.
8 Waters Associates Bulletin, *Proteins: Edman Degradation and Liquid Chromatography.*
9 R. Burgus and J. Rivier, *Peptides 1976, Proceedings of the Fourteenth European Peptide Symposium* (Belgium, 1976), 85.
10 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
11 W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *FEBS Letters*, 72 (1976) 139.
12 M. Walter and L. Ramaley, *Anal. Chem.*, 45 (1973) 165.
13 W. S. Hancock, D. R. K. Harding, J. E. Battersby and C. A. Bishop, unpublished results.
14 R. B. Merrifield, *J. Amer. Chem. Soc.*, 85 (1963) 2149.
15 Waters Associates Bulletin (1976) D61.
16 K. G. Wahlund, *J. Chromatogr.*, 115 (1975) 411.
17 J. H. Knox and G. R. Laird, *J. Chromatogr.*, 122 (1974) 17.
18 B.-A. Persson and B. L. Karger, *J. Chromatogr. Sci.*, 12 (1974) 521.
19 B. L. Karger, S. C. Su, S. Marchese and B. A. Persson, *J. Chromatogr. Sci.*, 12 (1974) 678.
20 B.-A. Persson and P.-O. Lagerstrom, *J. Chromatogr.*, 122 (1976) 305.
21 M. T. W. Hearn, unpublished results.
22 W. Santi, J. M. Huen and R. W. Frei, *J. Chromatogr.*, 115 (1975) 423.
23 M. O. Dayhoff, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Maryland, U.S.A., 1973.