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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

VI. RAPID ANALYSIS OF PEPTIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH HYDROPHOBIC ION-PAIRING OF AMINO GROUPS

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SUMMARY

This report describes the use of hydrophobic ion-pairing reagents in the rapid analysis of peptides by reversed-phase high-pressure liquid chromatography. It was found that combination of a hydrophobic anion such as hexanesulphonate with the cationic groups ($R\overset{+}{N}H_3$) of a peptide resulted in a decreased polarity of the sample. This change in polarity resulted in an increased retention time on a μBondapak-alkylphenyl column. In addition, the use of different ion-pairing reagents allowed dramatic changes in the selectivity of the reversed-phase system. This is demonstrated with peptides which range from tri- to heptapeptides using the following ion-pairing reagents: phosphoric acid, sodium hexanesulphonate and sodium dodecyl sulphate.

INTRODUCTION

We have recently reported[1] that paired-ion or ion-pair chromatography can be applied to the analysis of peptides and proteins by reversed-phase high-pressure liquid chromatography (HPLC). It was found that combination of a small polar anion such as $H_2PO_4^-$ with the cationic groups ($R\overset{+}{N}H_3$) of a peptide or protein allowed the rapid and reproducible analysis of a variety of samples[2,3]. The hydrophilic ion-pair effected an increase in polarity of the sample with a consequent decrease in retention on a reversed-phase column. While this system was particularly useful for smaller hydrophobic peptides, it was found that many protein samples such as bovine insulin, acyl-carrier protein, thermolysin and trypsin were not adequately retained on a C_{18}

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(or an alkylphenyl) column in the presence of phosphoric acid. It was for this reason that the chromatographic properties of a number of peptides were examined on a μBondapak-alkylphenyl column in the presence of different hydrophobic ion-pairing reagents. Previous studies[4,5] have shown that ion-pairing with hydrophobic anions such as hexanesulphonate lead to increased retention times for dye-stuffs, pharmaceuticals and simple amphoteric molecules.

It is the purpose of this report to show that hydrophobic ion-pairing does in fact give an increased retetention time for a variety of peptides. In addition, the use of different ion-pairing anions and different eluent compositions allowed dramatic shifts in the elution order of the series of peptides examined. It is proposed that this selectivity will prove to be invaluable in the analysis of peptides and proteins by reversed-phase HPLC.

EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which included two M6000 solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to a Series 440 UV monitor (Waters Assoc.) and a two-channel chart recorder (Linear Instruments, Costa Mesa, Calif., U.S.A.). The μBondapak-alkylphenyl column (10 μm, 30 cm × 4 mm), purchased from Waters Assoc., has an alkylphenyl stationary phase covalently attached to a fully-porous silica-based packing. 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).

Chemicals

All solvents were Analar grade. The methanol was used as supplied by Mallinckrodt (St. Louis, Mo., U.S.A.). Orthophosphoric acid was from May & Baker (Dagenham, Great Britain); sodium dodecyl sulphate (SDS) (specially pure grade) from BDH (Poole, Great Britain) and sodium hexanesulphonate was prepared from *n*-hexylbromide[6]. The acid forms of the two reagents were prepared by passage of an aqueous solution of the sodium salts down a Dowex 50W-X2 ion-exchange column.

Water was glass-distilled and deionised. The peptides described in Tables I and II were purchased from Research Plus Labs. (Denville, N. J., U.S.A.). All amino acids except glycine were of the L-configuration.

Methods

A flow-rate of 1.5 ml/min was used which was maintained by a pressure of 2000 p.s.i. All tests were at room temperature (*ca.* 22°). Sample sizes varied between 0.1 and 10 μg of peptide material injected in volumes 1–50 μl. All peptides were taken up in deionised, distilled water and, prior to injection, made up in the eluting solvent. Water was degassed for at least 30 min, the solvents were mixed in the required volumes, filtered through a 0.45-μm Millipore filter and then equilibrated at room temperature. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents for at least 30 min.

TABLE I

THE EFFECT OF HYDROPHOBIC ION-PAIRING ON THE RETENTION TIME OF PEPTIDES ON A μBONDAPAK-ALKYLPHENYL COLUMN

Eluent: methanol–water (1:1). The values given are the retention times in min.

*Peptide**	*Ion-pairing reagent*			
	None	*H_3PO_4, pH 2.5, 5 mM*	*Hexanesulphonate sodium salt, pH 6.5, 5 mM*	*SDS, pH 7.15, 5 mM*
L–W–M–R	112	2.3	4.0	16.2
L–W–M–R–F	>120	5.1	10.2	40.5
G–F	2.3	2.3	2.3	2.5
G–G–Y	64.5	1.9	2.4	6.2
M–R–F	32.5	2.4	3.6	>58
G–L–Y	2.5	2.4	2.4	2.7
R–F–A	> 48	2.05	3.0	33.2

* The code for amino acids is used by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Md., U.S.A. A = alanine, D = aspartic acid, F = phenylalanine, G = glycine, K = lysine, L = leucine, M = methionine, R = arginine, S = serine, W = tryptophan, Y = tyrosine.

TABLE II

THE EFFECT OF pH ON THE RETENTION TIME OF PEPTIDES ON A μBONDAPAK-ALKYLPHENYL COLUMN IN THE PRESENCE OF AN ION-PAIRING REAGENT

Eluent: methanol–water (1:1). The values given are the retention times in min.

Peptide	*Ion-pairing reagent*			
	$CH_3(CH_2)_5SO_3Na$		*$CH_3(CH_2)_{11}SO_4Na$*	
	pH 2.1	*pH 6.5**	*pH 2.9*	*7.15**
L–W–M–R	3.2	3 (−)	>25	16.2(−)
L–W–M–R–F	5.6	10.2(+)	—	40.5
G–F	2.5	2.3(−)	8.9	2.5(−)
G–G–Y	2.1	2.4(+)	5.5	6.2(+)
M–R–F	3.1	3.6(+)	—	>58
G–L–Y	2.5	2.3(−)	9.3	2.7(−)
R–F–A	2.5	3 (+)	—	33.2

* The + or − sign indicates whether an increased or decreased retention time is caused by an increase in pH.

RESULTS AND DISCUSSION

The results of the analysis of seven different peptides on a μBondapak-alkylphenyl column are shown in Table I. In these studies hydrophilic (H_3PO_4), moderately hydrophobic [$CH_3(CH_2)_5SO_3Na$] and extremely hydrophobic [$CH_3(CH_2)_{11}SO_4Na$] ion-pairing reagents were used. In this study an eluent with a high concentration of methanol was chosen so as to compare a wide range of ion-pairing reagents. If one wished to analyse the purity of these peptides an eluent with a lower methanol composition could be used in many cases to obtain adequate separation from contaminating peptides.

It can be seen from these results in columns 2–4 (Table I) that hydrophobic ion-pairing does indeed give a longer retention time for these peptides than does a more polar anion such as dihydrogen phosphate. It is likely, therefore, that both sodium hexanesulphonate and SDS will prove to be useful in the analysis of polar peptides and proteins by reversed-phase HPLC. Indeed preliminary experiments in our laboratories have shown that bovine insulin, trypsin and acyl-carrier protein are much more strongly retained on reversed-phase columns in the presence of sodium hexanesulphonate. Such a reagent presumably operates by modifying the polarity of the peptide or protein by the formation of hydrophobic ion-pairs with the $R\overset{+}{N}H_3$ groups of the sample. Other effects such as modification of the stationary phase by the reagent probably also make a contribution to the observed retention time of a peptide. However, we have found the concept of ion-pairing useful, because it has allowed the prediction of many of the observed changes in retention time.

It is not clear why, in most cases, hydrophobic ion-pairing reagents cause a decrease in retention time of peptide samples when compared with the retention time obtained in the same mobile phase without an ion-pairing reagent (*cf.* columns 1 and 3 of Table I). It is possible that the ion-pairing reagent disrupts ionic intra- and intermolecular interactions between the peptide molecules. This explanation would be consistent with the observation that ion-pairing often causes a dramatic improvement in peak shapes and reproducibility obtained for peptide samples (Fig. 1).

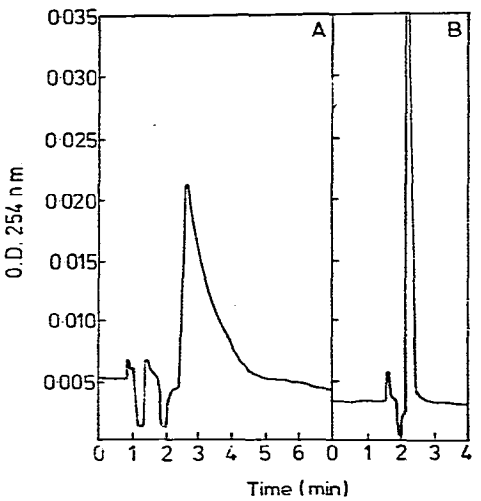

Fig. 1. The elution profile of Gly–Leu–Tyr on a μBondapak-alkylphenyl column in the absence (A) and presence (B) of 5 m*M* sodium hexanesulphonate, pH 7.1. The mobile phase was methanol–water (1:1).

The pH of the eluent can have a dramatic effect on both the retention time and peak shape of a sample, as is shown in Fig. 2. The effect of pH can be related to changes in the state of ionisation of the sample and/or ion-pairing reagent.

The use of SDS in reversed-phase HPLC should prove to be useful in the analysis of hydrophobic peptides which tend to aggregate in the absence of lipid *e.g.*, membrane proteins, apolipoproteins. We have examined the effect of much lower

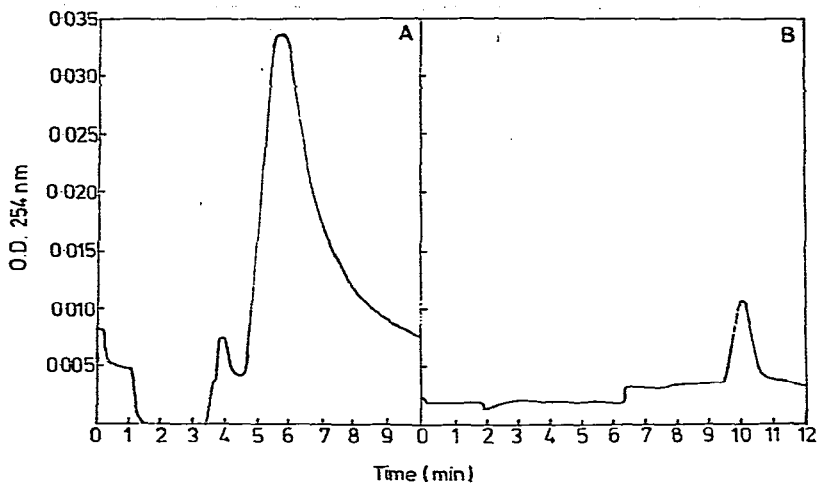

Fig. 2. The elution profile of Leu–Trp–Met–Arg–Phe on a μBondapak-alkylphenyl column. The eluent was methanol–water (1:1) with 5 m*M* sodium hexanesulphonate. The elution profile A was obtained at pH 2.1 and B at pH 7.1.

concentrations of SDS in the mobile phase in an effort to minimize the problems of removal of SDS from the sample after purification. A wide range of peptides have been chromatographed with results similar to those described in Table I with as little as 0.05 m*M* SDS in the mobile phase. Such an observation, however, cannot be

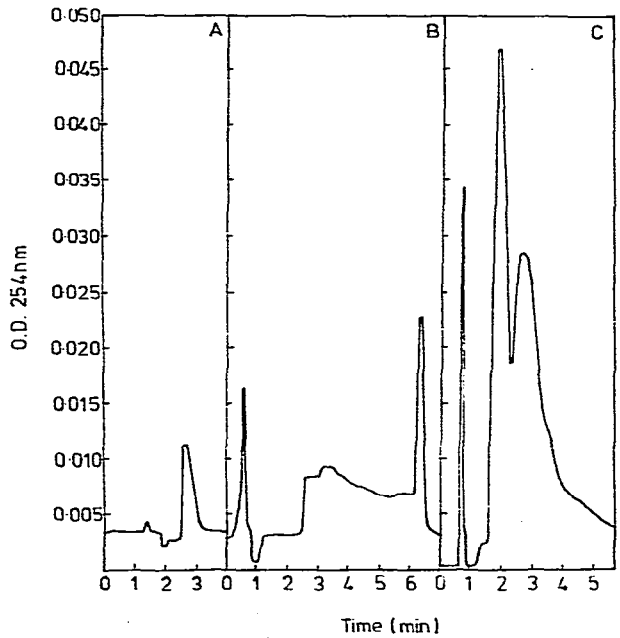

Fig. 3. The elution profile of Gly–Leu–Tyr on a μBondapak-alkylphenyl column with methanol–water (1:1) as eluent. The following concentrations of SDS were used: A, 1 m*M*; B, 0.1 m*M*; C, 0.02 m*M*.

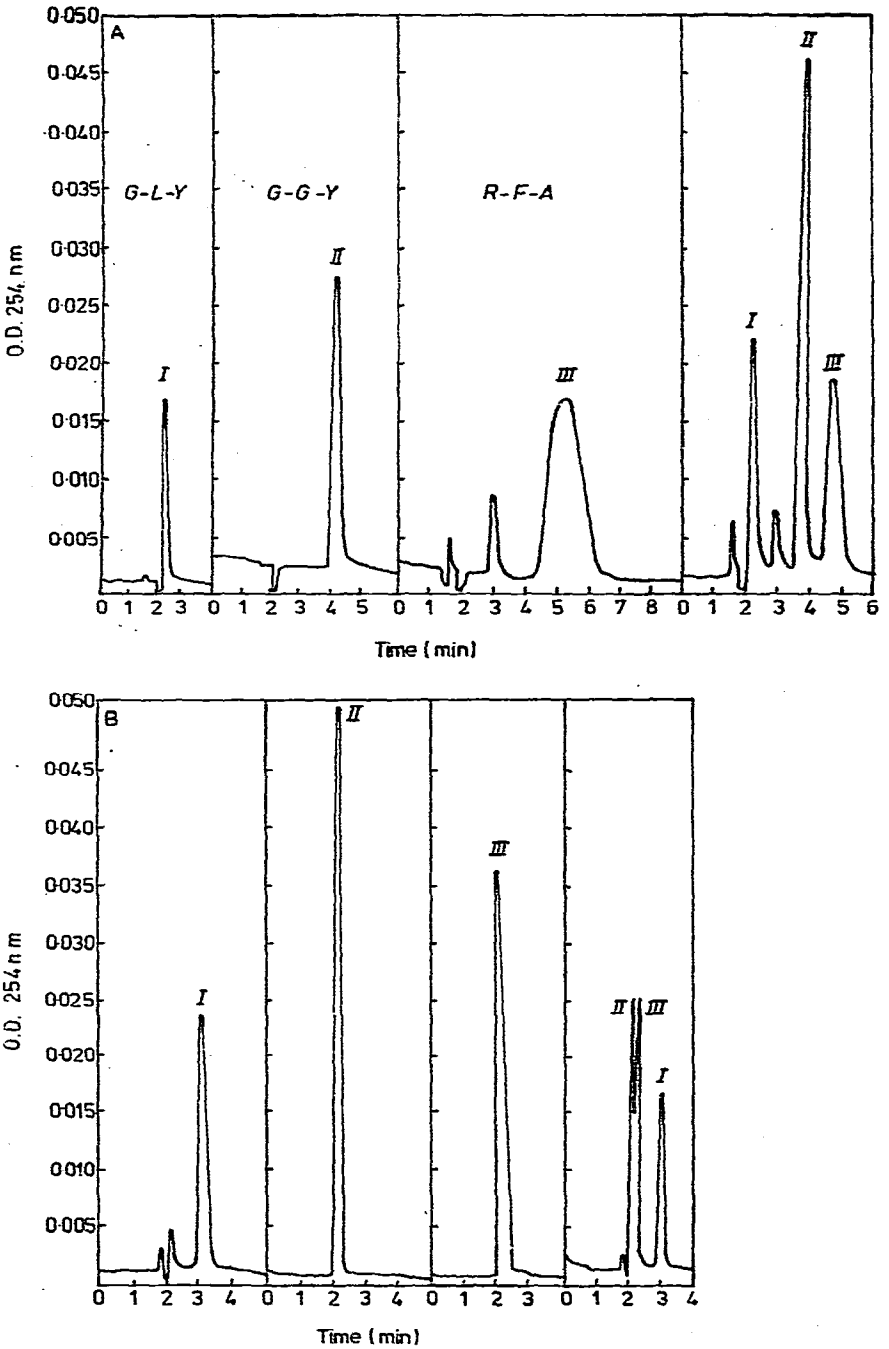

Fig. 4. The elution profiles of three peptides, Gly–Leu–Tyr (I), Gly–Gly–Tyr (II), and Arg–Phe–Ala (III) on a μBondapak-alkylphenyl column with methanol–water (1:1) as eluent. A, 1 m*M* SDS was added to the eluent; B, 0.1% H_3PO_4.

extended to all peptides, as is shown in Fig. 3. The tripeptide Gly–Leu–Tyr exhibits a complex elution profile at concentrations of SDS less than 1 m*M* in the eluent.

Perhaps the most striking result from these studies is the dramatic changes in the selectivity of the chromatographic system one can achieve by the use of different ion-pairing reagents (Fig. 4) or pH (Table II).

It can be seen that the elution order of three peptides, Gly–Gly–Tyr, Gly–Leu–Tyr and Arg–Phe–Ala can be reversed by the substitution of phosphoric acid with SDS in the mobile phase. The examination of the elution profile of a peptide in the presence of several different ion-pairing reagents should provide excellent evidence of purity, just as thin-layer chromatography in different solvent systems is presently used as a criterion of purity.

The potential of ion-pairing in reversed-phase HPLC of peptides is illustrated in Fig. 5. The addition of such reagents allows dramatic improvements in peak shape and elution time as well as the manipulation of retention times depending on the nature of the peptide and ion-pairing reagent.

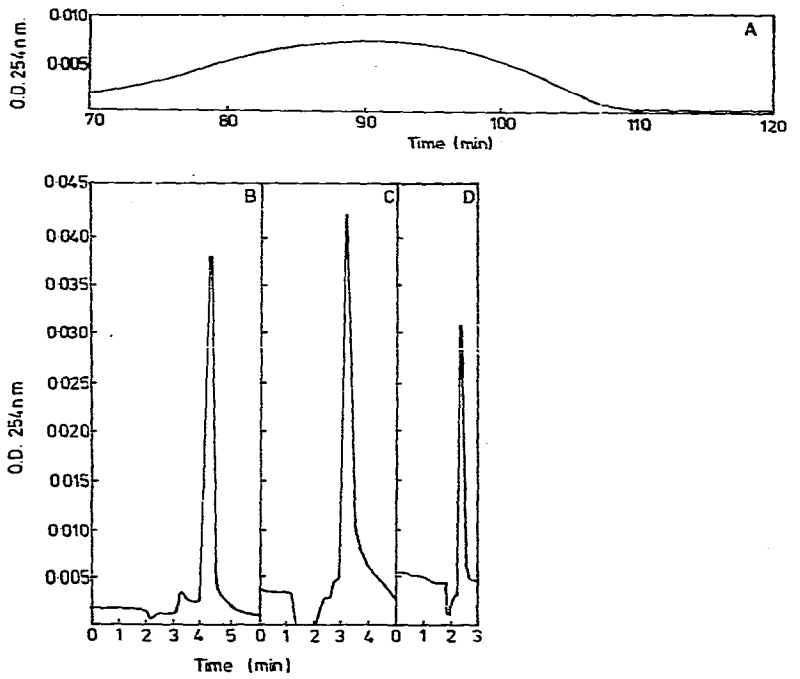

Fig. 5. The elution profile of Leu–Trp–Met–Arg on a μBondapak-alkylphenyl column with methanol–water (1:1) as eluent. A, no ion-pairing reagent was added; B, sodium hexanesulphonate, pH 7.1; C, hexanesulphonic acid, pH 2.1; D, phosphoric acid, pH 2.5.

In a subsequent paper the use of a different form of ion-pairing is described which involves the association of anionic reagents, such as tetra-alkylammonium ions, with the carboxylate anions of proteins.

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