Journal of Chromatography, 168 (1979) 377–384

CHROM. 11,402

HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

XI. THE USE OF CATIONIC REAGENTS FOR THE ANALYSIS OF PEPTIDES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

W. S. HANCOCK*, C. A. BISHOP, J. E. BATTERSBY and D. R. K. HARDING

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North (New Zealand)

and

M. T. W. HEARN*

Immunopathology Group, Medical Research Council of New Zealand, Medical School, P.O. Box 913, Dunedin (New Zealand)

(Received August 18th, 1978)

SUMMARY

This report describes the effect of different cationic reagents (tetraalkylammonium, alkylammonium and inorganic salts) on the retention times of di- to pentapeptides chromatographed on a reversed-phase support (*i.e.* a μBondapak-alkylphenyl column). Several trends are apparent with these reagents which can be explained on the basis of either ion-pairing or ion-exchange interaction of the reagent with the peptide sample. Reagents which generate in solution small highly solvated cations, *e.g.* Li, Na or Mg salts, give retention times similar to those obtained for ammonium salts. Tetraethylammonium salts give a modest increase in retention times relative to ammonium salts. By contrast, hydrophobic cations with long or bulky carbon chains, *e.g.* tetrabutylammonium or dodecylammonium ions, cause substantial decreases in retention times, resulting in very rapid elution of all peptides examined from the reversed-phase column. These observations are consistent with the composite interplay of ion-pair partition and dynamic ion-exchange effects for the cationic reagents. The use of a mixture of dodecylammonium acetate and sodium dodecylsulphate for the analysis of peptides and proteins is described. It is anticipated that such a chromatographic system will be useful for the analysis of proteins which readily aggregate.

INTRODUCTION

In earlier reports[1,2] we have demonstrated that hydrophobic ion-pairing of unprotected amino groups is a useful technique for the analysis and isolation of

* To whom correspondence should be addressed.

peptides by high-pressure liquid chromatography (HPLC). The association of a peptide sample with a hydrophobic anion in the eluent leads to a less polar complex which has an increased retention time on a reversed-phase column. By contrast, the use of a hydrophilic anion such as dihydrogen phosphate results in a decreased retention time for the peptide on the same column[3,4]. Thus, depending on the amino acid composition and sequence of the peptide, selectivity can be achieved by a suitable choice of negatively charged counter ions.

In a similar manner it was expected that ion-pairing of a cationic reagent with the carboxyl groups of a peptide sample would lead to useful modifications of its chromatographic behaviour. Previous studies have used tetraalkylammonium ions in the analysis of polar pharmaceuticals[5,6] and organic carboxylates[7]. In this report the effect of a series of tetraalkylammonium, alkylammonium and inorganic reagents on a variety of peptides is described. The results indicate that, depending on the cationic species involved, the retention time of the peptide can either be significantly increased (*e.g.* tetraethylammonium ion) or decreased (*e.g.* dodecylammonium ion) on a μBondapak-alkylphenyl reversed-phase column.

MATERIALS

High-pressure liquid chromatography

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 Cecil 212 (Cecil, Cambridge, Great Britain) variable wavelength UV monitor with an 8-μl flow-through cell and a Linear Instruments (Costa Mesa, Calif., U.S.A.) double-channel chart recorder.

The μBondapak-alkylphenyl column (10 μm, 30 cm × 4 mm I.D.) was obtained 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 as described previously[3]. Orthophosphoric acid was from May & Baker (Dagenham, Great Britain). Water was glass-distilled and deionised. The peptides were purchased from Research Specialties (Richmond, Calif., U.S.A.). All amino acids were of the L-configuration. The tetraalkylammonium ions were obtained from the following suppliers: tetramethyl (BDH, Poole, Great Britain), tetraethyl (Merck, Munich, G.F.R.), tetrapropyl (Pfaltz Bauer, Flushing, N.Y., U.S.A.) and tetrabutyl (BDH). All other cationic reagents were obtained from BDH and were of reagent grade.

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 1 and 50 μg of peptide material injected in volumes of 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 to room temperature. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents for at least 30 min.

Unless otherwise indicated, the same mobile phase conditions were used, *i.e.* methanol–water (1:1), a 2 m*M* solution of the reagent, acetate as the anion and adjusted to pH 4. A high concentration of methanol was chosen so that a wide range of samples and solute concentrations could be examined with the same chromatographic system. All retention times were reproducible and represent at least two determinations.

RESULTS

The results from the analysis of seven different peptides on a μBondapak-alkylphenyl column with a series of tetraalkylammonium reagents are shown in Table I. The acetate salts were chosen for this study because we had previously shown[3] that the presence of acetate anions caused minimal shifts in the retention of peptides and proteins on reversed-phase columns. As can be seen from Table I there is a small but progressive increase in retention time as the alkyl chain length of the reagent increases. A similar trend has previously been observed[2] with anionic ion-pairing reagents. The tetrabutylammonium reagent, on the other hand, caused decreased retention in each case. The addition of tetrapropylammonium ions to the mobile phase gave rise to double peaks for several of the peptidic samples. It is unlikely that the emergence of a second peak is due to contaminants since these

TABLE I

COMPARISON OF THE EFFECT OF TETRAALKYLAMMONIUM SALTS ON THE RETENTION TIME OF PEPTIDES

The other eluent parameters are described under Methods.

*Peptide**	*Retention time (min)*				
	Ammonium salt ($R_4\overset{+}{N}\overset{-}{O}Ac$)				
	$R = H$	$R = CH_3$	$R = CH_2CH_3$	$R = (CH_2)_2CH_3$	$R = (CH_2)_3CH_3$
L–W–M–R	2.8	3.0	3.05	3.5	2.7
L–W–M–R–F	5.7	6.8	6.5	8.7	4.0
G–F	2.4	2.4	2.6	2.7	2.05
G–G–Y	2.0	2.0	2.2	1.8**, 2.05	1.7
M–R–F	2.6	2.7	2.8	3.0	1.7
G–L–Y	2.4	2.5	2.8	2.7	2.1
R–F–A	2.3	2.3	2.6	2.1**, 2.7	1.8

* The code for amino acids of M. O. Dayhoff[14] is used. A = alanine, D = aspartic acid, F = phenylalanine, G = glycine, K = lysine, L = leucine, M = methionine, R = arginine, W = tryptophan, Y = tyrosine.

** Two peaks were observed.

samples had previously been shown to be homogeneous by several chromatographic procedures and by compositional data. Rather, the presence of two chromatographic species suggests the involvement of two discrete mechanisms operating via different partition effects. It is possible that a transition from ion-pair formation to a dynamic ion-exchange mode occurs with the twelve-carbon tetrapropyl group. Certainly, the shorter retentions observed with the tetrabutylammonium cation are consistent with a different separation mode operating with this reagent compared to the lower homologues.

The alkylammonium salts also show a similar trend with the lower homologues again causing increased retention (Table II). In general, the observed retention follows the order $NH_4^+ < \overset{+}{N}H_3CH_3 \approx \overset{+}{N}H_3CH_2CH_2OH > \overset{+}{N}H_3(CH_2)_3CH_3 > \overset{+}{N}H_3(CH_2)_5CH_3 \gg \overset{+}{N}H_3(CH_2)_{11}CH_3$ (common anion acetate). With the elution system containing dodecylammonium acetate the peaks obtained with the various peptides had very high plate efficiencies even allowing for the short retention times observed.

TABLE II

COMPARISON OF THE EFFECT OF DIFFERENT ALKYLAMINE SALTS ON THE RETENTIOI TIME OF PEPTIDES

The other eluent parameters are described under Methods.

Peptide	*Retention time of amine salt* ($R\overset{+}{N}H_3$)					
	$R = H$	$R = CH_3$	$R = CH_2CH_2OH$	$R = (CH_2)_3CH_3$	$R = (CH_2)_5CH_3$	$R = (CH_2)_{11}CH$
L–W–M–R	2.7	3.05	3.1	2.9	2.4	1.6
L–W–M–R–F	5.7	7.0	6.1	6.8	4.4	2.1
G–F	2.4	2.5	2.4	2.3	2.25	2.0
G–G–Y	2.0	2.2	2.3	2.0	1.8	1.4
M–R–F	2.6	2.75	2.7	2.5	2.2	1.45
G–L–Y	2.4	2.6	2.6	2.45	2.25	2.0
R–F–A	2.3	2.3	2.45	2.1	2.0	1.3

These results contrast with the small effects noted with alkaline metal salts on the retention times of the peptides. In this case (Table III) the observed retention times follow the apparent order Li ≈ Na < K < Cs and with the divalent cations, intermediate between Li and Cs, Mg < Ca (again common anion acetate).

In view of the significant decrease in the retention of these peptides with dodecylammonium ion, reflecting an apparent increase in polarity of the peptidic molecules, it was attractive to use this reagent in conjunction with the anionic detergents which are known[2,8] to involve dynamic ion exchange. Table IV shows the comparative effects of dodecylammonium acetate, sodium dodecylsulphate and an equimolar mixture of these two reagents on the retention times observed for the series of peptides. As we have noted[2] before the eluent containing sodium dodecylsulphate resulted in increased retention times irrespective of whether the reagent was complexing with the amino terminus or side chain cationic group. Eluents containing dodecylammonium acetate, in contrast, showed the opposite effect with invariant increases in apparent polarity of the peptidic samples. Significantly, equimolar mixtures of the anionic and cationic reagents resulted in the rapid elution of peptide (Table IV and Fig. 1) and protein (Fig. 2) samples.

TABLE III

COMPARISON OF THE EFFECT OF METAL IONS ON THE RETENTION TIME OF A SERIES OF PEPTIDES

The other eluent parameters are described under Methods.

Peptide	*Cationic reagent*					
	Li^+	Na^+	K^+	Cs^+	Mg^{2+}	Ca^{2+}
L–W–M–R	2.9	2.9	3.0	3.4	2.8	3.2
L–W–M–R–F	4.7	4.7	4.8	4.8	4.5	4.9
G–F	2.2	2.2	2.35	2.5	2.2	2.5
G–G–Y	2.1	2.1	2.3	2.4	2.2	2.3
M–R–F	2.6	2.6	2.7	2.8	2.6	3.0
G–L–Y	2.3	2.3	2.5	2.7	2.3	2.5
R–F–A	2.3	2.2	2.45	2.8	2.3	2.6

TABLE IV

COMPARISON OF THE EFFECT OF DODECYLAMINE ACETATE AND SODIUM DODECYLSULPHATE ON THE RETENTION TIME OF PEPTIDES

Peptide	*Retention time (min)*		
	$CH_3(CH_2)_{11}SO_3^-Na^+$ (A)*	$CH_3(CH_2)_{11}NH_3^+{}^-OAc$ (B)**	(A) + (B)**
L–W–M–R	16.2	1.6	2.3
L–W–M–R–F	40.5	2.1	4.45
G–F	2.5	2.0	2.2
G–G–Y	6.2	1.4	1.6
M–R–F	>58	1.45	2.1
G–L–Y	2.7	2.0	2.2
R–F–A	33.2	1.3	1.9

* A 2 m*M* solution of this reagent was used in methanol–water (1:1) (pH 6.5).

** The eluent parameters are described under Methods.

DISCUSSION

Peptides and proteins show a wide range of polarities and it would be surprising to find a single ion-pairing reagent suitable for the analysis of such an extensive diversity. However, selectivity can be achieved by taking advantage of free amino or carboxyl groups in peptides or proteins. We recently demonstrated[1–4] that hydrophilic, hydrophobic ion-pairing and dynamic ion exchange of peptides and proteins via amino-group interactions is a valuable aid in the analysis and isolation of these materials. It is the purpose of this study to examine the potential of cationic reagents for ion-pairing of carboxyl groups of peptides and proteins. Such reagents have already been applied to a variety of separations, *e.g.* tetrabutylammonium ions have been used in the analysis of polar pharmaceuticals[5,6], vitamins[7,8] and recently nucleotides[9].

In Tables I, II and III, the effect of a series of tetraalkylammonium, alkylammonium and inorganic salts on the retention time for a number of peptides is described. It should be noted that these changes in retention time were quite repro-

ducible. At lower concentrations of the organic components in the mobile phase larger differences could be observed between the various cationic reagents. A relatively high methanol concentration was used in these studies so that a wide range of eluant conditions and sample polarities could be examined with the same mobile phase. From these results it can be seen that the retention time of peptides can be significantly modified by the addition of various cationic reagents to the mobile phase. For example, the addition of small polar cations, *e.g.* Li^+, Na^+, Mg^{2+}, caused a decrease in retention time while less polar cations, *e.g.* Cs^+, Ca^{2+}, $\overset{+}{N}(CH_2CH_3)_4$ caused an increase in retention time relative to $\overset{+}{N}H_4$. It is likely, therefore, that these cationic reagents will provide a useful addition to the conditions available for the analysis of complex mixtures of peptides and proteins. The results obtained, however, were less dramatic than those obtained for ion-pairing of the amino groups of peptides and proteins and, presumably, reflects the dominant effect of the alkylammonium ions on the polarity of these molecules[3].

The changes in retention time observed for tetramethyl- and tetra-ethylamine, ethylamine, ethanolamine and inorganic salts can be explained on the basis of ion-pairing between carboxyl groups of the peptide sample and the cation added to the mobile phase. For example, if a more polar cation forms an ion pair with the peptide, the resulting complex would be expected to show decreased retention on a non-polar reversed-phase column. This suggestion is supported by a recent observation[10] that the use of trimethylammonium phosphate in the eluent gives excellent resolution and recoveries of peptide and protein mixtures on reversed-phase HPLC. It is possible that both the polar trimethylammonium and phosphate ions associate with the carboxylate and amino functional groups of peptides and proteins respectively and thus allow their facile chromatography.

Several authors have demonstrated, however, that ion-pair reagents can also partion between the stationary and mobile phase[8,9,11–13]. Under such conditions, the ion-pair reagent can be retained and form a dynamic surface coating on the stationary phase. These solvent-generated stationary-phase modifications can permit ionic interactions between the peptide and the stationary phase. This effect appears to be the dominant mechanism with anionic detergents like sodium dodecylsulphate and probably also for cationic detergents like dodecylammonium acetate. At pH 4 a peptide would be predominantly protonated and thus show minimal interaction with a cationic stationary phase. This will be reflected in short retention times as can be seen, for example with dodecylammonium acetates in Tables II and IV and the decrease in retention time observed for alkylamines of increasing C-chain length (see Table II).

The increase and then decrease in retention time for the tetraalkylammonium ion series (Table I) could be explained by considering the possible balance between the ion-pairing and ion-exchange effects of the reagents added to the mobile phase. It is possible that the tetramethyl- and tetraethylammonium ions act predominantly by ion-pairing effects while the tetrabutylammonium ion undergoes little ion-pairing with the solute due to the steric bulk of the cation but acts by forming a surface coating on the stationary phase. Several of the peptides showed two peaks with the intermediate case of the tetrapropylammonium ion (see Table I), an observation which could be explained on the basis of these competing effects. The data in Tables I and II also suggest that an alkyl chain of a significant number of C-atoms and surface bulk is required for an effective coating of the stationary phase.

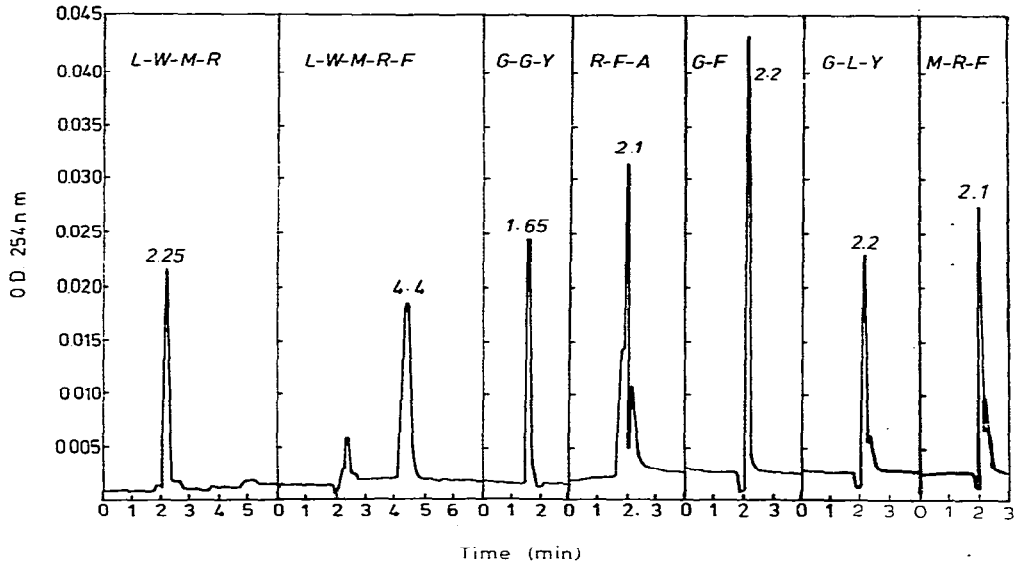

Fig. 1. The analysis of a series of peptides on a μBondapak-alkylphenyl column with an eluent which consisted of 2 mM sodium dodecylsulphate and 2 mM dodecylammonium acetate dissolved in methanol–water (1:1) at pH 4.

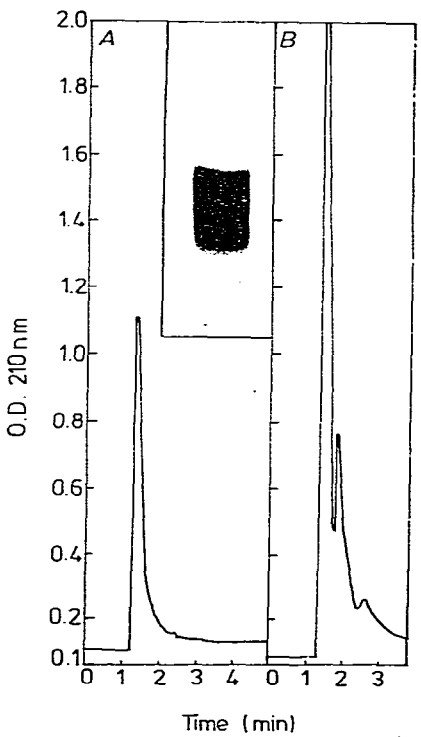

Fig. 2. The elution profile of a sample of sheep liver aldehyde dehydrogenase which was chromatographed on a μBondapak-C_{18} column with an eluent which consisted of isopropanol–water (1:4) 2 mM dodecylammonium phosphate, pH 2.6. (A) 2 μl of a dialysed sample of enzyme; (B) 10 μl of a dialysed sample of enzyme. The inset shows the electrophoretic pattern obtained for the analysis of 100 μg of the protein on a 7% polyacrylamide gel at pH 8.9 in the presence of 0.1% sodium dodecylsulphate.

In Table IV the opposing effects of dodecylamine acetate and sodium dodecylsulphate is shown. Together the two reagents offer a powerful method of increasing or decreasing the retention of peptides on reversed-phase systems. As is shown in Fig. 1 a mixture of the two reagents leads to a useful analytical system in which peptides can be rapidly analysed under dissociating conditions. The use of this system for the analysis of proteins which are sensitive to aggregation, such as membraneous proteins and lipoproteins, is currently under investigation. Fig. 2 shows the analysis of the enzyme sheep liver aldehyde dehydrogenase with an eluent which contained dodecylammonium acetate, pH 4. A minor impurity observed by polyacrylamide gel electrophoresis was also observed in the elution profile shown in Fig. 2 and presumably is caused by a small amount of a contaminating protein. In the absence of dodecylamine the protein sample was indefinitely retained on a reversed-phase column even in the presence of 0.1% H_3PO_4 and high concentrations of organic solvents.

ACKNOWLEDGEMENTS

We wish to thank Mr. L. Meyer for expert technical assistance and Mrs. J. Trow for preparation of the figures. This investigation was supported in part by University Research Committee (New Zealand) Grants No. 72/214, 73/94, Medical Research Council (New Zealand) Grant No. 74/126, National Heart Foundation of New Zealand Award No. 102 and Lottery Distribution Committee Grant No. 20/12508.

REFERENCES

1 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *Science*, 200 (1978) 1168.
2 W. S. Hancock, C. A. Bishop, L. J. Meyer, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
3 W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
4 W. S. Hancock, C. A. Bishop, R. L. Prestidge and M. T. W. Hearn, *Anal. Biochem.*, 89 (1978) 203.
5 S. C. Su, A. V. Hartkopf and B. L. Karger, *J. Chromatogr.*, 119 (1976) 523.
6 B.-A. Persson and P.-O. Lagerstrom, *J. Chromatogr.*, 122 (1976) 305.
7 K. G. Wahlund, *J. Chromatogr.*, 115 (1975) 411.
8 J. C. Kraak, K. M. Jonker and J. F. K. Huber, *J. Chromatogr.*, 142 (1977) 671.
9 N. E. Hoffman and J. C. Liao, *Anal. Chem.*, 49 (1977) 2231.
10 J. Rivier, *J. Liquid Chromatogr.*, 1 (1978) 347.
11 J. H. Knox, in P. F. Dixon, C. H. Gray, C. K. Lim and M. S. Stoll (Editors), *High Pressure Liquid Chromatography in Clinical Chemistry*, Academic Press, New York, 1976, p. 6.
12 P. T. Kissinger, *Anal. Chem.*, 49 (1977) 883.
13 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 142 (1977) 213.
14 M. O. Dayhoff (Editor), *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, Md., 1972, p. D-2.