Journal of Chromatography, 473 (1989) 63–69
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 469

LIQUID CHROMATOGRAPHIC SEPARATION OF ACIDIC PHOSPHOSERINE PEPTIDES ON MACROPOROUS COPOLY(STYRENE–DIVINYLBENZENE) USING AMINES TO REGULATE RETENTION

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(Received January 6th, 1989)

SUMMARY

Using phosphoserine peptides as model substances, we have studied the separation of acidic peptides as substituted ammonium ion-pairs on a macroporous copoly(styrene–divinylbenzene) (PLRP-S) chromatographic column. Various hydrophobic amines were examined, of which long-chain N-methyl- and N,N-dimethyl-*n*-alkylamines proved to be the best with respect to selectivity and peak symmetry. Excellent separations were obtained in ammonium bicarbonate buffer (pH 7.5) with ethanol as organic modifier under isocratic conditions using the corresponding heptyl- to nonylamines.

INTRODUCTION

In recent years, reversed-phase high-performance liquid chromatography (HPLC), owing to its inherent sensitivity, resolution, speed, and general convenience, has become an invaluable tool in the analysis of natural as well as synthetic peptides[1–3]. Among refinements of this technique the application of various ionic or ionizable additives as counter-ions should be emphasized in this context. Additives to have been applied include carboxylates, perfluorinated carboxylates, sulphonates, sulphates and picrate, with a vast number of organic cationic analytes, including drugs and peptides, whereas mainly quaternary ammonium ions have been used for anionic substances[4–6].

Earlier it was shown that for the analysis of basic hydrophilic peptides, such as the arginine-containing serine peptides, prepared as substrates for cAMP-dependent protein kinase[7], hexanesulphonate could be applied as the counter-ion[8]. The peptides were separated on a C_{18} column in phosphate buffer (pH 3–4.5) with ethanol as modifier. Later it was demonstrated that the corresponding phosphoserine peptides could be resolved under the same conditions[9].

In a parallel fashion, attempts were made to resolve mixtures of acidic phosphopeptides on C_{18} microsilica columns. The idea was to apply amines of varying hydrophobicity to regulate the retention but, even under essentially neutral conditions (pH below 7.5), the columns had limited stability and lost their resolving capacity.

0021-9673/89/$03.50

With the advent of new stationary phases such as copoly(styrene–divinylbenzene), which are stable at a higher pH, new attempts were undertaken in this direction.

EXPERIMENTAL

Materials and solvents

To regulate the retention, amines and a quaternary ammonium salt were used. Hexylamine, octylamine, N-ethyldiisopropylamine, N,N-dimethylcyclohexylamine and tributylamine were obtained from Fluka (Buchs, Switzerland), N-methyloctylamine (MOA) and trimethylnonylammonium bromide from Eastman Kodak (Rochester, NY, U.S.A.), N,N-dimethylheptylamine, N,N-dimethylnonylamine and N-methylnonylamine from Pfaltz & Bauer (Waterbury, CT, U.S.A.) and N,N-dimethyloctylamine (DMOA) from ICN Biomedicals (Plainview, NY, U.S.A.). The quaternary ammonium bromide was converted into the corresponding hydroxide by means of an anion exchanger before use.

Isocratic mobile phases were used, consisting of binary aqueous solvents with ethanol or acetonitrile as organic modifier. The acetonitrile used was LiChrosolv from Merck-Schuchardt (Darmstadt, F.R.G.). The ethanol was of spectroscopic quality. The buffers were prepared from ammonium hydrogencarbonate, generally to an ionic strength of 0.1 *M*, and neutralized by carbon dioxide to the required pH. The mobile phases also contained the mentioned amines to serve as counter-ions.

Synthesis of phosphoserine peptides

The phosphopeptide Arg–Ala–Ser(P)–Val–Ala (**1**) was prepared and purified as already described[10]. Employing the earlier established experimental procedures[10], the tetrapeptide Ala–Ser(P)–Val–Ala (**2**) and the tripeptide Ser(P)–Val–Ala (**3**) were obtained in similar yields, as outlined previously[11]. The protected peptide intermediates were thoroughly characterized by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy and amino acid analyses, and their high purity was confirmed with thin-layer chromatography (TLC). The final free phosphopeptides were examined with TLC, HPLC, and amino acid analyses, and exhibited satisfactory purity.

Instrumentation

Our liquid chromatographic system consisted of a Model 6000 A solvent-delivery device and a Model 450 variable-wavelength detector (Waters Assoc., Milford, MA, U.S.A.). The detector head was carefully isolated in order to reduce any influence on the baseline stability at high sensitivity due to variations in the ambient temperature. The flow-cell had a volume of 8 μl. The detection wavelength was 210 nm. A laboratory-made block-heater was used and thermostatted at 30.0 $\pm$ 0.1°C. A Model 7125 sample-loading injector was obtained from Rheodyne (Cotati, CA, U.S.A.).

The separation column (150 $\times$ 4.6 mm I.D.) was obtained from Polymer Labs. (Shropshire, U.K.). It contained a polymeric material, macroporous copoly(styrene–divinylbenzene), PLRP-S, 100 Å, 5 μm.

Procedure

The column was carefully conditioned by pumping the mobile phase through the

system at a flow-rate of 1 ml/min. for 6 h, when the retention times had generally stabilized. In the studies on the effect of the amine concentration, the experiments were conducted in the order from lower to higher concentrations, because otherwise the results would be poorly reproducible. Before changing from one amine to another, the column was carefully washed with 0.1 *M* phosphate buffer (pH 3.0) and distilled water.

Standard chromatographic conditions

Support, PLRP-S, 5 μm; column, 150 × 4.6 mm I.D.; mobile phase, 0.1 *M* ammonium hydrogencarbonate (pH 7.50)–modifier (93:7); flow-rate, 1.0 ml/min; amine, as indicated (otherwise 0.010 *M*); temperature, 30.0 ± 0.1°C; detection wavelength, 210 nm. The three phosphopeptides are indicated in the figures as follows: (■) Arg–Ala–Ser(P)–Val–Ala (**1**); (●) Ala–Ser(P)–Val–Ala (**2**); and (×) Ser(P)–Val–Ala (**3**).

RESULTS AND DISCUSSION

Although various chromatographic systems containing trifluoroacetic acid (TFA) have been successfully used for the separation of many peptides on reversed-phase C_{18} microsilica columns[12], preliminary experiments with peptides **2** and **3** indicated that even in the absence of an organic modifier, poor retention was obtained (capacity factor, $k' < 2$). This led us to consider the possibilities for chromatographing our peptides as anions with cationic counter-ions (amines and quaternary ammonium salts), but these experiments were hampered by the poor stability of this column material. Under the conditions used, at pH 7.0 the resolving power of our columns already deteriorated at an unacceptable rate. In this work we therefore exclusively used copoly(styrene–divinylbenzene) as the support, because it is stable over a much wider pH range and various improved types of this material have been used successfully in recent years for the separation of peptides[13,14]. In the presence of 0.1% TFA, peptides 1–3 passed through the column without any retardation, even in the absence of an organic modifier.

With this new type of column, a number of hydrophobic (C_6–C_{12}) amines were screened for their usefulness in the separation of acidic hydrophilic peptides, using three phosphopeptides as model substances. A preliminary study of several different types of amine was made at this stage (Table I), but this list is by no means complete. Nevertheless, as a result of this screening, two apparently good candidates for further study appeared. With the majority of the amines tested, however, neither satisfactory selectivity nor the required peak symmetry could be obtained. This was the case with the two primary amines studied, hexylamine and octylamine. On the other hand, with N-methyloctylamine the results were dramatically improved, and N,N-dimethyloctylamine proved equally useful. Three other tertiary amines tested in this context did not seem promising. An attempt has been made to summarize the findings in qualitative terms in Table I.

At this stage it was decided to study the influence of N,N-dimethyloctylamine and N-methyloctylamine on the separation of the three model peptides mentioned above in more detail. In this context the effect of pH as well as of ionic strength on k' also had to be investigated, and this was done with the former of these two amines. These results are presented in Fig. 1. Considering the pK_a values of the various groups

TABLE I

SCREENING OF AMINES FOR THEIR USEFULNESS IN THE SEPARATION OF THREE ACIDIC HYDROPHILIC PHOSPHOSERINE PEPTIDES

The code used to grade retardation, selectivity, and peak symmetry: − − (poor); − (less satisfactory); + (satisfactory); + + (good to excellent). For further details, see Standard chromatographic conditions, at the end of the Experimental section.

Amine	*Retardation*	*Selectivity*	*Symmetry*
Hexylamine	+	− −	−
Octylamine	+	− −	+
N-Methyloctylamine	+ +	+ +	+ +
N-Ethyldiisopropylamine	−	− −	− −
N,N-Dimethylcyclohexylamine	−	− −	− −
Tributylamine	−	+	− −
N,N-Dimethyloctylamine	+ +	+ +	+ +
Trimethylnonylammonium (hydroxide)	+ +	− −	+ +

involved[15], the pH 7–8 range was studied. As can be seen from Fig. 1a, k' has a maximum at *ca.* pH 7.5 for the model peptides, but appears to be quite high throughout the pH interval. (It should be noted that this applies to the resolution as well.) On the basis of this result, we chose pH 7.5 as a standard value in subsequent experiments. Correspondingly, in these experiments the ionic strength was kept at 0.1 *M*. As can be seen from Fig. 1b, k' is only modestly influenced by a change above this value, whereas the retention is satisfactory and the resolution quite high.

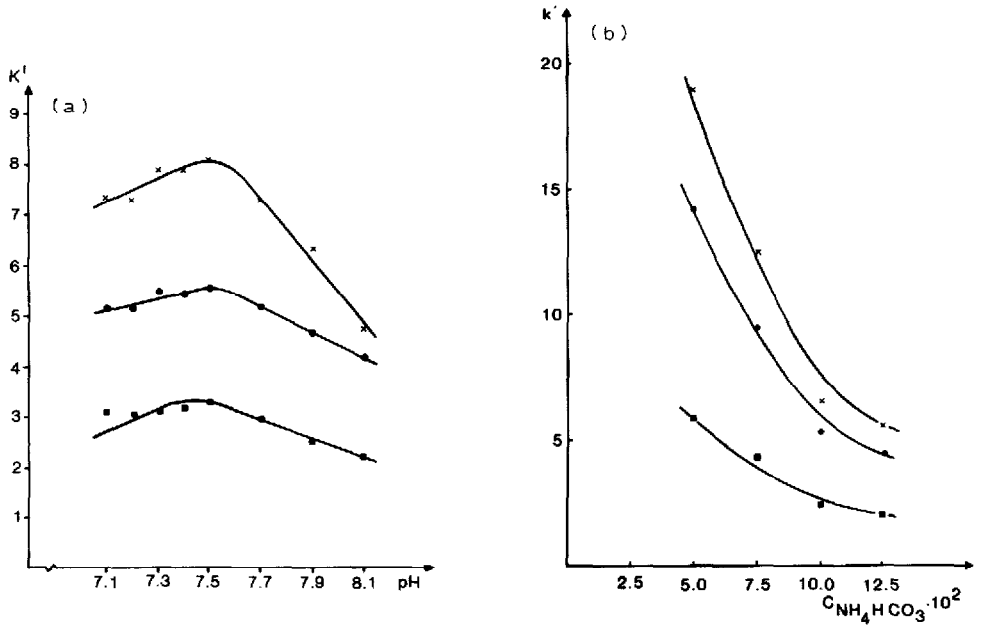

Fig. 1. The influence of (a) pH and (b) ionic strength on k' for peptides 1–3 in the presence of N,N-dimethyloctylamine and NH_4HCO_3 (in the latter, the pH used was 7.60). For further details, see Standard chromatographic conditions, at the end of the Experimental section.

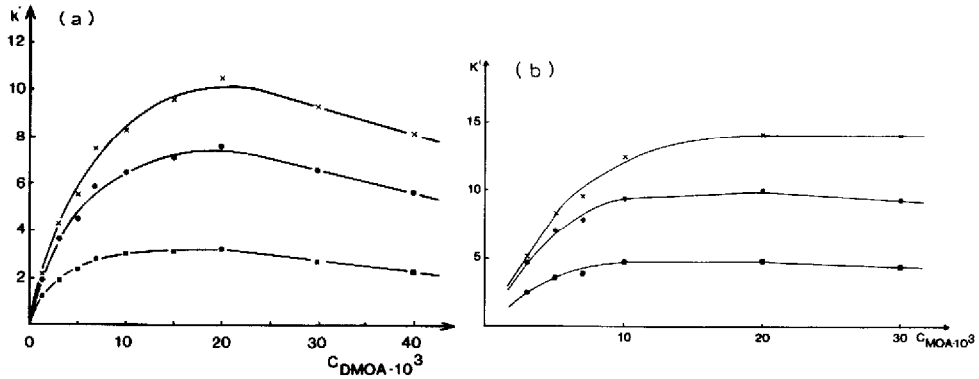

Fig. 2. Effect of the concentrations of (a) N,N-dimethyloctylamine and (b) N-methyloctylamine on k' for peptides 1–3. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

The effect of the concentration of N,N-dimethyloctylamine and N-methyloctylamine on k' is presented in Fig. 2. For both amines, k' for all the model peptides first increases sharply on the addition of amine, reaching its half-maximum value at *ca.* 4 m*M*, to exhibit more or less pronounced maxima at *ca.* 20 m*M*. The resolving capacity is satisfactory (for α values at $C_{amine} = 0.010$ *M*, see Table II), whereas the column efficiency is more modest (Table II) as in all other cases in which this support was used. It should be noted in this context that N-methyloctylamine consistently gives rise to higher k' values than does N,N-dimethyloctylamine (Table II). A typical chromatogram of the three model peptides using N,N-dimethyloctylamine is shown in Fig. 3. The first peak in the chromatogram refers to the pentapeptide, which has one negative charge less than the tetra- and tripeptides, and therefore quite likely binds one fewer molecule of hydrophobic amine. The tripeptide is obviously the most hydrophobic of the three phosphopeptides studied.

In Table II, the results of similar experiments with three additional secondary and tertiary heptyl- and nonylamines are presented, together with data on the two octylamines discussed above. As expected, within each group k' increases with the chain length of the amine. As previously shown for the two octylamines, N-methyl-

TABLE II

EFFECT OF THE AMINES ON THE RETENTION OF THREE ACIDIC HYDROPHILIC PHOSPHOSERINE PEPTIDES

For further details, see Standard chromatographic conditions, at the end of the Experimental section.

Amine	k'_1	H_1	k'_2	α_2	H_2	k'_3	α_3	H_3
N,N-Dimethylheptyl	–	–	1.91	–	0.15	2.21	–	0.14
N,N-Dimethyloctyl	3.27	0.15	5.59	1.71	0.14	8.09	2.47	0.11
N,N-Dimethylnonyl	3.44	0.17	7.67	2.23	0.13	11.21	3.26	0.10
N-Methyloctyl	4.74	0.15	9.37	1.98	0.13	12.53	2.64	0.10
N-Methylnonyl	5.82	0.15	12.40	2.13	0.13	19.15	3.29	0.13

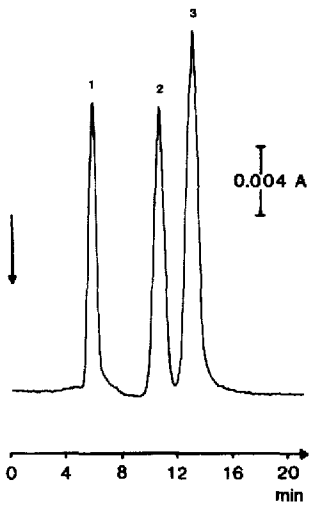

Fig. 3. Separation of three model peptides on a PLRP-S column using N,N-dimethyloctylamine. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

nonylamine gives rise to higher k' values than does N,N-dimethylnonylamine. With respect to the selectivity, the two nonylamines mentioned rank highest, but the octylamines are nearly as good. The column efficiency has approximately the same value for all amines studied, and is rather low in comparison with that of a microsilica column. For any given amine it improves with the k' value.

Table III contains some additional data on N,N-dimethylnonylamine, obtained using two different organic modifiers, acetonitrile and ethanol. As can be seen, the retention order of the three phosphopeptides is identical with both modifiers. Contrary to our previous experience of chromatography of peptides, these phosphopeptides

TABLE III

SUMMARY OF DATA ON THE SEPARATION OF THREE PHOSPHOSERINE PEPTIDES USING N,N-DIMETHYLNONYLAMINE WITH ACETONITRILE AND ETHANOL AS ORGANIC MODIFIERS

Peptide	*Acetonitrile*			*Ethanol*		
	k'	α	H	k'	α	H
Arg–Ala–Ser(P)–Val–Ala[a]	3.43	1	0.15	2.88	1	0.16
Ala–Ser(P)–Val–Ala[a]	7.30	2.13	0.12	6.00	2.08	0.13
Ser(P)–Val–Ala[a]	9.98	2.91	0.09	8.08	2.81	0.10
Arg–Ala–Ser(P)–Val–Ala[b]	3.92	1	0.16	3.44	1	0.17
Ala–Ser(P)–Val–Ala[b]	9.08	2.31	0.12	7.67	2.23	0.13
Ser(P)–Val–Ala[b]	12.80	3.27	0.08	11.21	3.26	0.10

[a] Concentration of N,N-dimethylnonylamine 0.005 *M*.

[b] Concentration of N,N-dimethylnonylamine 0.010 *M*. For further details, see Standard chromatographic conditions, at the end of the Experimental section.

gave higher k' values with acetonitrile than with ethanol. The selectivity appears to be approximately the same for both, whereas the column efficiency is marginally higher for acetonitrile. The amount of modifier was the same, 7%, as in all previous experiments. Because of the high k' values, no further experiments were conducted with a higher concentration of the amine.

Amines have previously been applied as modifiers only in conjunction with microsilica columns[16]. Even after careful capping of their silanol groups, the residual ones can have unfavourable effects on the symmetry of the peaks. The peak symmetry could, however, be improved by minute amounts of a hydrophobic amine or quaternary ammonium salt. Compounds of the latter type were recently used in one case for the separation of amino acids[17]. Up to now, however, amines have not been exploited for the regulation of the retention of anionic peptides, and their widely variable selectivities in this context have also gone unnoticed.

The column has been in regular use for nearly a year without any loss of resolution. It made possible the convenient separation of peptides, otherwise not easily separated, by application of protonated lipophilic amines as counter-ions at a pH not compatible with standard microsilica columns.

ACKNOWLEDGEMENTS

This project was made possible by a research grant from the Swedish Natural Science Research Council.

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