Journal of Chromatography, 364 (1986) 209–214
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 824

EFFECT OF ORGANIC SOLVENT MODIFIER AND ION-PAIR REAGENT (TETRABUTYLAMMONIUM SALT) ON THE CHROMATOGRAPHIC BEHAVIOUR OF PROTECTED PEPTIDES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

E. V. TITOVA*, M. A. CHLENOV* and L. I. KUDRJASHOV
N. D. Zelinsky Institute of Organic Chemistry, U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.)

SUMMARY

The effect of organic solvent modifiers (methanol and acetonitrile) on the retention of synthetic peptides with protective groups in high-performance liquid chromatography (HPLC) was examined. In the HPLC of totally protected peptides, an increase in the organic solvent content in the eluent up to 90% did not result in abnormal changes to the retention mechanism, which did occur in the separation of unprotected peptides. The chromatographic behaviour of partially protected peptides with free amino groups varied on addition of methanol or acetonitrile to the eluent; a parabolic dependence of log k' on acetonitrile concentration with a minimum at *ca.* 80% acetonitrile was obtained, whereas an increase in the methanol concentration in the eluent up to 90% led to a gradual reduction in the k' values of the peptides studied.

The effect of counter ions (Br^- and HSO_4^-) of tetrabutylammonium salts as mobile phase components on the retention of protected peptides is discussed.

INTRODUCTION

Reversed-phase (RP) and ion-pair (IP) high-performance liquid chromatography (HPLC) have been successfully applied to peptides separations. The possibility of varying the eluent composition over a wide range (both the type and concentration of organic solvents and/or ion-pair reagents in the mobile phase) allows the analysis of peptides that are markedly different in structure and size, both natural and synthetic[1,2]. However, the eluent composition is mainly chosen empirically, on the basis of the hydrophobicity of the peptides and the effects of the eluent components and different additives on the retention of the peptides. Some papers have dealt with certain regularities of the RP and IP HPLC behaviour of peptides. For example, the dependence of peptide retention on the percentage of organic modifier in the eluent[3–5], the amino acid composition governing the hydrophobicity of peptides[1,3,6,7],

* Present address: Branch of Research Institute for Biological Tests of Chemical Compounds, Moscow, U.S.S.R.

0021-9673/86/$03.50

TABLE I

PEPTIDES STUDIED

No.	*Peptide*
I	BocValLys(*Z*)ValTyrProOBzl
II	BocValTyrProOBzl
III	HValLys(*Z*)ValTyrProOBzl
IV	HValTyrProOBzl
V	BocArg(NO_2)Arg(NO_2)ProOH
VI	BocArg(NO_2)Arg(NO_2)ProOCH_3
VII	BocValGlyLys(*Z*)Lys(Z)OCH_3
VIII	BocValGlyLys(*Z*)Lys(Z)N_2H_3
IX	BocValGlyLys(*Z*)Lys(Z)Arg(NO_2)Arg(NO_2)ProOH
X	TrtGlu(OBu^t)His(Trt)PheArgTrpGlyLys(Boc)ProOH
XI	HGlu(OBu^t)His(Trt)PheArgTrpGlyLys(Boc)ProValGlyLys(*Z*)Lys(*Z*)Arg(NO_2)-Arg(NO_2)ProValLys(*Z*)ValTyrProOBzl
XII	HValGlyLys(*Z*)Lys(*Z*)Arg(NO_2)Arg(NO_2)ProValLys(*Z*)ValTyrProOBzl
XIII	HValTyrProOBu^t

and the pH, type and concentration of reagents in mobile phase[2,8–10] have been studied. On the basis of the results obtained, possible mechanism of interactions in the chromatographic systems have been suggested. It was demonstrated[4,5] that these mechanisms are often ambiguous or variable and the predominance of a particular mechanism may be controlled by the eluent composition. It should be stressed that these investigations have mainly dealt with unprotected peptides. The applications of HPLC to the analysis of protected synthetic peptides is rare[11–14], and their chromatographic properties have been insufficiently studied.

This work continues previous investigations[15,16] on the application of RP and IP HPLC to the separation of synthetic peptides with protective groups. It considers the effect of organic modifiers on the retention of peptides and the contribution of counter ions of tetrabutylammonium (TBA) salts to the various interactions in the system.

EXPERIMENTAL

Chemicals and reagents

The peptides used (Table I) were obtained from the All-Union Research Institute for Technology of Blood Substitutes and Hormone Preparations. Acetonitrile and methanol (HPLC grade) were purchased from Merck. Doubly distilled water and TBA bromide (pure) were used. TBA hydrogen sulphate (analytical-reagent grade) was purchased from Serva.

Instrumentation and procedure

The chromatographic separation of peptides was carried out on a Zorbax ODS (DuPont) column (250 × 4.6. mm I.D.). The mobile phase was delivered by a Gilson Model 302 pump. A Model BT 3030 variable-wavelength UV spectrophotometer (Biotronik) was used as a detector (λ = 220 nm). The samples were introduced with a Rheodyne Model 7125 injector, loop volume 20 μl.

Chromatographic separations were performed at room temperature. Mixtures of methanol or acetonitrile with water or aqueous TBA salt were used as the mobile phase.

RESULTS AND DISCUSSION

The chromatographic separation of hydrophobic peptides (protected peptides in particular) in the reversed-phase mode requires a high percentage of organic modifier in mobile phase (more than 50–60%). According to some workers[4,5], however, a significant increase in organic solvent concentration often results in "reversion" of the main (reversed-phase) mechanism of interactions in the chromatographic system, which is expressed by a parabolic dependence of log k' on the concentration of organic modifier with a minimum in the region of about 50–60%. This phenomenon was observed with large hydrophobic unprotected peptides. In the present author's opinion, when the concentration of organic modifier in the mobile phase exceeds a critical value, the molecules of this modifier form a solvate shell on the surface of the packing, which hampers the hydrophobic interactions of the solute molecules with the support. Thus the normal-phase mechanism based on the interaction of free amino groups of peptides with the silica surface (silanophilic effect) becomes dominant and the retention of peptides starts to increase with increasing concentration of organic modifier (acetonitrile). Some published results[5] demonstrated that a similar parabolic dependence of log k' on C_{org} occurs with methanol as the organic modifier, but its minimum was at higher concentrations.

In this work we have investigated the effects of the type and percentage of organic modifier on the retention of partially and fully protected synthetic peptides. It would be natural to suggest that the chromatographic behaviour of peptides with free amino groups would be similar to that of unprotected peptides. For fully protected peptides, the possible formation of a solvate shell around a bonded ODS stationary phase should have less effect on their retention (owing to the absence of free amino groups, a higher hydrophobicity and thus successful "competition" of these peptides for hydrophobic binding with bonded-phase groups).

We have investigated the effects of the concentrations of two popular RP-HPLC organic modifiers, methanol and acetonitrile, on the retentions of fully protected peptides (I and II) and peptides with a free N-terminus (III and IV). Fig. 1 demonstrates the dependences of log k' of these peptides on the concentration of organic modifier (C_{org}). With acetonitrile (Fig. 1A) the curves have the expected shape, *i.e.*, an increase in acetonitrile concentration from 50 to 90% results in a reduction in the retention of fully protected peptides, inversion of the mechanism of interaction is not observed and the behaviour of peptides with a free N-terminus is similar to that of unprotected peptides (as reported elsewhere[5]), with a parabolic dependence of log k' on concentration. However, the critical concentration of acetonitrile with partially protected peptides (about 80%) is markedly higher than that for unprotected peptides (50–60%). This seems to be connected with the relatively lower content of free amino groups (per molecule) in partially protected synthetic peptides in comparison with large unprotected polypeptides, and with the presence of hydrophobic protective groups, which strengthen the hydrophobic interaction of peptides with the stationary phase.

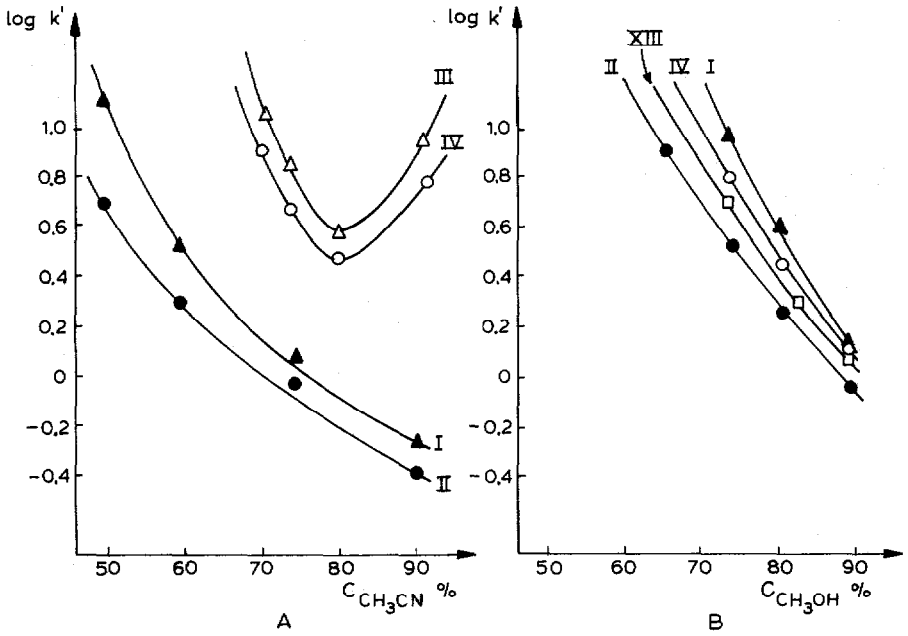

Fig. 1. Retention of synthetic protected peptides *versus* concentration of organic solvent in the eluent: (A) acetonitrile; (B) methanol.

The chromatographic behaviour of both fully and partially protected peptides using methanol as organic modifier is similar over the whole concentration range and agrees with a reversed-phase mechanism (Fig. 1B). The lack of a minimum on the curve appears to be due to the higher polarity of this solvent in comparison with acetonitrile and hence a lower affinity for the ODS support, which correlates with results presented elsewhere[5]. The weak binding of methanol with the bonded ODS phase results, on the one hand, in a higher retention of peptides with free amino groups due to binding with residual silanol groups of the packing (thus peptide III fails to be eluted from the column over the whole range of methanol concentrations) and, on the other, in a lack of "reversion" of the interaction mechanism because of the low probability of solvate shell formation. The first conclusion may be considered as a disadvantage of this eluent, but it can be reduced (*e.g.*, by suppression of the interaction of peptides with silica silanol groups or by reduction of hydrophobic interactions of peptides with the stationary phase). The second conclusion is an advantage, in our opinion, as the problem of the prediction of the chromatographic behaviour of synthetic peptides on variation of the proportions of the components of the mobile phase becomes simpler. Thus, one can suggest that both partially and fully protected peptides will be eluted according to the principles of reversed-phase chromatography over the whole range of concentrations of methanol in the mobile phase.

We conclude that the chromatographic behaviour of protected synthetic peptides in RP-HPLC is different from that of unprotected peptides and the dependence of the retention of protected peptides on the percentage of organic modifier in the

mobile phase is controlled by the presence of free amino groups and the type of organic solvent used.

The effect of ion-pair reagents on the chromatographic properties of protected peptides was also of interest. It was shown earlier[15,16] that the TBA^+ ion, after addition to the mobile phase, modified mainly the surface of the packing, binding to the alkyl groups and thus decreasing its hydrophobicity. Deviations from a reversed-phase mechanism were not observed on analysis of protected peptides with an aqueous methanolic eluent in the presence of TBABr, but the peptides were retained more weakly. An attempt was made to compare the effects of Br^- and HSO_4^- counter ions of TBA salts on the retention of peptides in RP-HPLC.

For this purpose the chromatographic separation of various peptides using eluents of the following composition was carried out (the choice of the separation conditions was discussed in previous papers[15,16]): methanol–0.005 *M* TBABr (75:25); methanol–0.005 *M* $TBAHSO_4$ (75:25); methanol–0.01 *M* TBABr (94:6); methanol–0.01 *M* $TBAHSO_4$ (94:6). The k' values of the peptides are presented in Table II. It can be seen that the retention of fully protected peptides is almost independent of the type of counter ion (except for peptide I). The peptides with free amino groups (III, IV, XI and XII) are eluted faster with the HSO_4^- ion, whereas the peptides with an unprotected C-terminus (V, IX and X) emerge later. It is possible that the pH of the eluents has some effect on the chromatographic behaviour of peptides. Owing to dissociation of the HSO_4^- ion ($HSO_4^- \rightleftharpoons H^+ + SO_4^{2-}$) the acidity of the medium is increased (in comparison with an eluent containing TBABr) and the free functional groups of the peptides are protonated, resulting in an increase in the retention of peptides having free carboxyl groups and a decrease in the retention of peptides with

TABLE II

DEPENDENCE OF THE RETENTION OF PEPTIDES ON THE TYPE OF COUNTER ION OF TBA SALT

Conditions: (1) column, Zorbax ODS; eluent, methanol–0.005 *M* TBABr (75:25); (2) column, Zorbax ODS; eluent, methanol–0.005 *M* $TBAHSO_4$ (75:25); (3) column, Zorbax C_8; eluent, methanol–0.01 *M* TBABr (94:6); (4) column, Zorbax C_8; eluent, methanol–0.01 *M* $TBAHSO_4$ (94:6).

Peptide	k_1'*	k_2'	k_3'	k_4'	$\Delta = k'_{1(3)} - k'_{2(4)}$
I	4.0	4.7	–	–	−0.7
II	1.5	1.6	–	–	−0.1
III	3.0	2.2	–	–	+0.8
IV	1.3	0.9	–	–	+0.4
V	0.0	0.1	–	–	−0.1
VI	0.2	0.2	–	–	0
VII	3.4	3.2	–	–	+0.2
VIII	2.5	2.4	–	–	+0.1
IX	0.8	1.5	–	–	−0.7
X	–	–	1.6	4.6	−3.0
XI	–	–	8.1	2.8	+5.3
XII	–	–	2.0	1.6	+0.4

* All k' values are less than those reported in 1982[15], owing to changes in the packing properties after column operation during the intervening years.

free amino groups. Hence the application of a certain TBA^+ salt may lead to a required selectivity of separation.

We conclude that the chromatographic behaviour of fully protected peptides in RP-HPLC corresponds to basic principles of reversed-phase chromatography and it is less dependent on the eluent composition than that of partially protected peptides. The presence of free functional groups in partially protected peptides leads to additional effects on variation of the eluent composition similar to those observed with unprotected peptides but to a lesser extent. The effects found have resulted in a better understanding of the chromatographic properties of synthetic peptides in RP-HPLC and allow the prediction of these properties on changing the proportions of the components of the eluent.

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