Journal of Chromatography, 353 (1986) 399–408
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 738

LIQUID CHROMATOGRAPHY WITH CROWN ETHER-CONTAINING MOBILE PHASES

VI. MOLECULAR RECOGNITION OF AMINO ACIDS AND PEPTIDES*

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SUMMARY

Host–guest interaction between crown ethers and amino compounds has been incorporated into reversed-phase high performance liquid chromatography, where crown ethers are involved as components of the mobile phase. The capacity factors for amino acids and peptides bearing primary amino group(s) increased significantly in the presence of crown ethers (18-crown-6) in the mobile phase. The extent of the increase, k'/k'_0 (k'_0 is the capacity factor in the absence of 18-crown-6), varied with the configuration around the amino group; α-amino acids showed a lesser increase than corresponding amines, indicating the hindrance caused by the α-carboxyl group in the association of the α-amino group with 18-crown-6, whereas glycine, β-alanine and lysine, having an amino group free from the carboxyl group, showed a marked increase. Imidazolyl, guanidinyl and carbamoyl groups in the lateral chains of histidine, arginine and glutamine, respectively, gave rise to much smaller increases. Proline, which has only a secondary amino group, showed a decreased k' value, possibly because of competition with 18-crown-6 in binding to the hydrophobic stationary phase. The capacity factors of peptides were also affected by addition of 18-crown-6 to the mobile phase. The change in k' values varied, depending on the amino acid residue at the N-terminal, while the elution sequence of dipeptides with a common residue at the N-terminal agreed with the order of hydrophobicity of the amino acid residue at the C-terminal. However, the lysyl residue provided a significant increase in k' value, regardless of its location in the peptide chain. The elution profiles for some amino acids and biologically important polypeptides (bradykinin and lysylbradykinin) were shown in comparison and in combination with those obtained by conventional ion-pair chromatography.

INTRODUCTION

Because of high efficiency and facility, reversed-phase high-performance liquid

* For Part V, see ref. 9.

0021-9673/86/$03.50

TABLE I

HPLC CONDITIONS

Column temperature: 40°C; detection: UV at 200 or 210 nm.

Experiment	*Stationary phase*	*Mobile phase*	*Flow-rate*	*Data given in*
k' of amino acids and dipeptides	Chemcosorb* 5 ODSH (15 cm × 4.6 mm I.D.)	Water (pH 2.5 with hydrochloric acid)	0.7 ml/min	Figs. 1, 2, 4 and 8
k' of aromatic amino acids and amines	Chemcosorb 5 ODSH (15 cm × 4.6 mm I.D.)	Water methanol (80:20) (pH 2.5 with hydrochloric acid)	1.0 ml/min	Fig. 3 and Table II
Separation of basic amino acids	Nucleosil** 5 C_{18} (15 cm × 4.6 mm I.D.)	Water (pH 2.5 with hydrochloric acid)	0.7 ml/min	Fig. 5
k' of dipeptides	Chemcosorb 5 ODSH (15 cm × 4.6 mm I.D.)	Water–methanol (80:20) (pH 2.5 with hydrochloric acid)	1.0 ml/min	Figs. 6 and 7
Retention behaviour of bradykinin and lysylbradykinin	Chemcosorb 300-7 C_{18} (25 cm × 4.6 mm I.D.)	Water–acetonitrile (80:20) (pH 2.9 with phosphoric acid)	1.0 ml/min	Table III

* Manufacturer: Chemco Scientific, Osaka, Japan.
** Manufacturer: Macherey Nagel, Düren, F.R.G.

chromatography (HPLC) has proved to be of wide applicability to the separation of not only hydrophobic but also hydrophilic substances. Recent developments, including microbore columns and the gradient elution technique, seem to surpass conventional ion-exchange chromatography in separating free and derivatized amino acids, polypeptides and proteins[1–3]. Retention characteristics in this type of chromatography indicate, in accordance with solvophobic theory, that the increase in capacity factors could be ascribed to the increase in hydrophobic surface areas of both solute and stationary ligand, and also to the decrease in the polarity of the mobile phase[4]. Although it is quite natural that ion-pair chromatography has become widely used for the separation of ionic (or ionizable) compounds, it affords low specificity in the separation of ionic compounds, since electrostatic interaction occurs less discriminately.

In our previous papers, we investigated the part played by host–guest interactions between crown ethers and amino compounds in reversed-phase HPLC, where a crown ether was involved as a component of the mobile phase[5–9]. Significant changes in the capacity factors of amino compounds resulted from the addition of a crown ether, depending on the nature and concentration of the crown ether, and on protons and added ions in the mobile phase. The degree of change was also specific for the class, number and location of amino groups, as well as for the chemical structure around the amino groups in the guest molecule.

The present paper describes the structure–retention relationships of amino acids and peptides in this host–guest interaction chromatography.

EXPERIMENTAL

Reagents and materials

Amino acids and peptides in the L-form were obtained from commercial sources, and were used as supplied. 18-Crown-6 was a product of Merck (Darmstadt, F.R.G.). Sodium heptanesulphonate, used as an ion-pairing reagent, was purchased from Nakarai Chemicals (Kyoto, Japan). Glass-distilled water and methanol were used to prepare the mobile phases after degassing. Hydrochloric acid of analytical-reagent grade was used to adjust the pH of the mobile phases.

Measurement of capacity factors

A liquid chromatograph (LC-3A, Shimadzu, Kyoto, Japan), equipped with a UV detector (SPD-2A, Shimadzu, Japan), was used for the measurement of capacity factors. The samples were dissolved in a small portion of the mobile phase, and the minimal amount required for detection was injected. The capacity factors were calculated from $(t_R - t_0)/t_0$, where t_R and t_0 are the average retention times of a sample and a non-adsorbed substance (sodium nitrate), respectively, measured repeatedly at the peak of the elution curve.

The operating conditions are given in Table I.

RESULTS AND DISCUSSION

Amino acids

The capacity factors (k') of various amino acids are shown in Fig. 1 as a

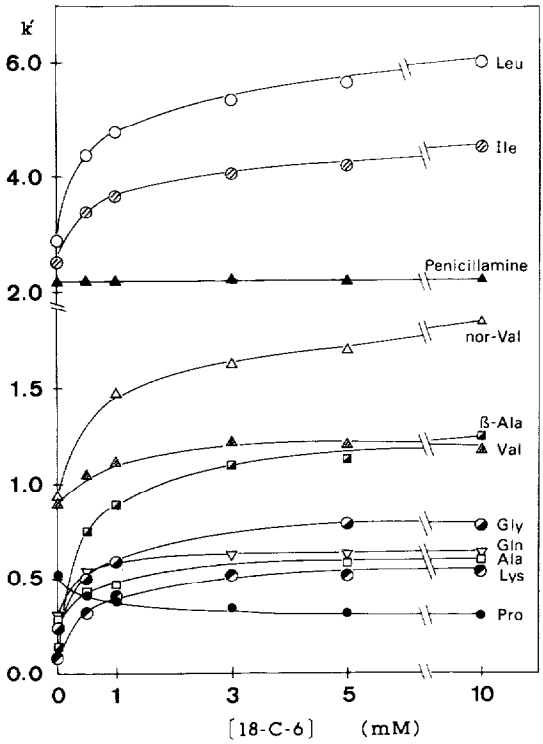


Fig. 1. Effect of 18-crown-6 concentration on the capacity factor (k') of amino acids. HPLC conditions, see Table I. Abbreviations: Leu = leucine, Ile = isoleucine, nor-Val = norvaline, Val = valine, β-Ala = β-alanine, Gly = glycine, Ala = alanine, Pro = proline, Lys = lysine, Gln = glutamine.

function of 18-crown-6 concentration in the mobile phase. It was found that the k' values of all amino acids, except for penicillamine and proline, increased with increasing concentration of 18-crown-6, approaching individually different plateau levels, and that the elution sequence was reversed, in some cases, by addition of low concentrations (< 1 mM) of 18-crown-6. The shape of the k' *vs.* 18-crown-6 concentration curve is well predicted by the capacity factor equation, which was derived from equilibria involved in this particular chromatographic system[5]. The gradual increase in k' values, approaching the plateau level, means that significant interaction takes place between the amino groups of the guest compounds and the relatively hydrophilic crown ether (18-crown-6)[9]. On the contrary, the curve for proline shows a slight decrease in k' values as the 18-crown-6 concentration increases, because 18-crown-6 associates so weakly with proline, which has only a secondary amino group, that it competes with proline in binding to the hydrophobic stationary ligand. The k' *vs.* crown ether concentration profile is generally considered to depend on the two opposing effects mentioned above. When these effects are balanced, the k' value becomes apparently independent of crown ether concentration, as in the case of penicillamine in Fig. 1. The variety of individual profiles seen in Fig. 1 suggests that the specificity due to molecular recognition can be manifested by taking into account the k'/k'_0 value, which is the ratio of a capacity factor to that obtained by

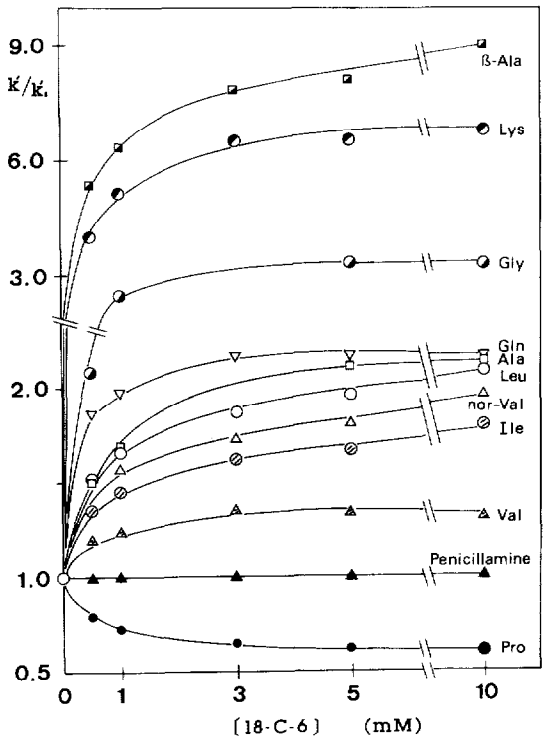
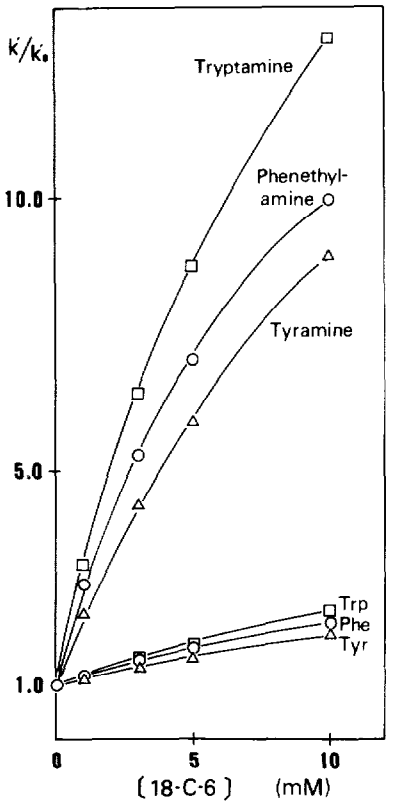


Fig. 2. Effect of 18-crown-6 concentration on the retention enhancement of amino acids. $k'_0 = k'$ without 18-crown-6. For abbreviations, see Fig. 1.

using the mobile phase without crown ether (*i.e.* in the usual reversed-phase mode). Fig. 2 shows the k'/k'_0 values *vs.* concentration of 18-crown-6 curves for the same amino acids as given in Fig. 1. As expected from the molecular structures, the k'/k'_0 values for β-alanine, lysine and glycine are much larger than others. This is obviously because these amino acids have an amino group free from the steric hindrance caused by the carboxyl group; the ω-amino group of lysine and amino group of β-alanine are located apart from the α-carboxyl group so that interference by the α-carboxyl group is avoided. The methylene group of glycine, lacking a bulky side-chain, keeps the amino group free from the hindrance caused by the carboxyl group. The hindrance caused by an α-carboxyl group is clearly shown in Fig. 3, where k'/k'_0 *vs.* 18-crown-6 concentration profiles for tryptophane, phenylalanine and tyrosine are compared with those for the corresponding amines, tryptamine, phenethylamine and tyramine. The k'/k'_0 values for these amines are always larger than those for amino acids in a whole range of 18-crown-6 concentrations, and the order of k'/k'_0 for amino acids (Trp > Phe > Tyr) is the same as that for amines. The actual elution sequence and degree of retention enhancement are shown in Table II, where k'_{10} is the capacity factor in the presence of 10 mM 18-crown-6 in the mobile phase. It is readily found that although amino acids have larger k'_0 values than the corresponding amines, the addition of 18-crown-6 gives rise to far more increase in k' values of amines than in those of amino acids. As a result, amino acids and the



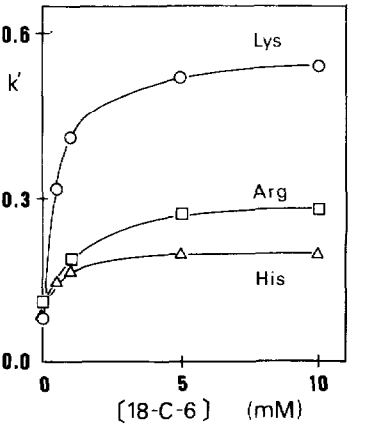


Fig. 3. Effect of 18-crown-6 concentration on the retention enhancement of amino acids and their corresponding amines. Abbreviations: Trp = tryptophan, Phe = phenylalanine, Tyr = tyrosine. HPLC conditions, see Table I.

Fig. 4. Effect of 18-crown-6 concentration on the capacity factor of basic amino acids. Abbreviations: Lys = lysine, Arg = arginine, His = histidine. HPLC conditions, see Table I.

corresponding amines are separated satisfactorily in the reversed elution sequence.

Besides the effect caused by the α-carboxyl group, the side-chain structure of α-amino acid is also reflected in the degree of retention enhancement. The k'/k'_0 values changed, as found in Fig. 2, depending on the class of carbon atom at the β-position,

TABLE II

RETENTION ENHANCEMENT EFFECT OF 18-CROWN-6 ON AROMATIC AMINO ACIDS AND THEIR CORRESPONDING AMINES

k'_0 and k'_{10} are the capacity factors in the absence and in the presence of 10 m*M* 18-crown-6, respectively.

Compound	k'_0	k'_{10}	k'_{10}/k'_0
Trp	2.68	6.28	2.34
Tryptamine	2.12	27.3	12.9
Phe	1.50	3.19	2.13
Phenethylamine	1.40	13.9	9.93
Tyr	0.62	1.19	1.92
Tyramine	0.42	3.73	8.88

i.e. the k'/k'_0 values for amino acids bearing a primary or secondary carbon atom (Ala, Gln, Leu, nor-Val), tertiary carbon atom (Ile, Val) and quaternary carbon atom (penicillamine) became smaller in this order.

Fig. 4 shows a comparison of k' values among basic amino acids. The elution sequence of these amino acids in the absence of 18-crown-6 is Lys $\approx$ His $<$ Arg, although these are scarcely retained on the ODS stationary phase because of their strong charge. However, the addition of 18-crown-6 gives rise to a larger increase in the k' value of lysine than in those of arginine and histidine, causing the elution sequence to be reversed, *i.e.* Lys $>$ Arg $>$ His. This suggests that, unlike the aminobutyl group in the side-chain of lysine, the imidazolyl and guanidinyl groups do not participate in the interaction with 18-crown-6.

Fig. 5 shows such effects on the separation of histidine, arginine and lysine, where the elution profiles are also demonstrated in comparison and in combination with conventional reversed-phase ion-pair chromatography, with heptanesulphonate as a counter-ion. In the ion-pair mode (Fig. 5a), the ion-pair effects on these basic amino acids were of virtually the same order of magnitude, so that the elution sequence (His $\approx$ Lys $<$ Arg) was unchanged, and the peaks of histidine and lysine were not resolved, although their retention times were prolonged. Addition of 3 m*M* 18-crown-6 instead of heptanesulphonate (Fig. 5b) revealed the difference in specificity between them; the peaks were resolved with a selective increase in the capacity factor of lysine, resulting in the elution sequence His $<$ Arg $<$ Lys. The elution time was shortened to about half that in ion-pair chromatography (Fig. 5a), because the crown ether-associated ammonium ion underwent an ion-exclusion effect. When the mobile phase contained both sulphonate and crown ether (Fig. 5c) at the same concentration as in Fig. 5a and b, respectively, a synergistic effect resulted in their complete separation, keeping the elution sequence the same as in Fig. 5b.

Peptides

As a preliminary experiment to the study of the crown ether effects on polypeptides, the retention behaviour of some dipeptides was investigated. The k' values

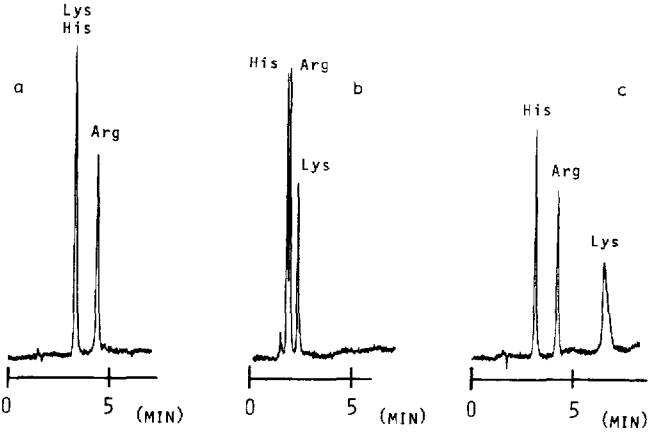
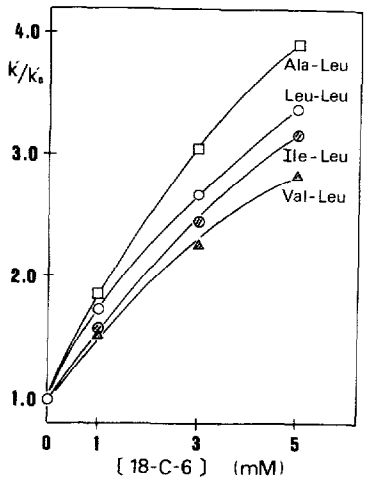


Fig. 5. Separation profiles of basic amino acids. Mobile phase containing (a) 0.1 m*M* sodium heptanesulphonate, (b) 3 m*M* 18-crown-6, (c) 0.1 m*M* sodium heptanesulphonate and 3 m*M* 18-crown-6.

of dipeptides were also affected by the addition of 18-crown-6 in the mobile phase and their k'/k'_0 values were also characteristic of the composition of the amino acid residues in the peptides. Fig. 6 shows the k'/k'_0 *vs.* 18-crown-6 concentration curves for four dipeptides with leucine as a common residue at the C-terminal. The effect of retention enhancement, expressed by k'/k'_0 values, is in the order of the N-terminal residues, Ala- > Leu- > Ile- > Val-, which agreed with that for corresponding amino acids given in Fig. 2. This suggests that the amino acid residue at the N-terminal could solely be involved in association with 18-crown-6. However, the elution sequence depends on the hydrophobicities of these residues at the N-terminal. In this connection, it is interesting to investigate the effect of 18-crown-6 on the capacity factors of dipeptides having a common residue at the N-terminal. As found in Fig. 7, the k' values for leucyl dipeptides increased with the concentration of 18-crown-6, while the elution sequence remained unchanged. This indicates that, since the retention enhancements are of almost the same magnitude among these peptides (this resembles the situation in ion-pair chromatography), the elution sequence (-Leu > -Val > -Ala) is determined by hydrophobicity of the C-terminal amino acid residue. Therefore, the sequence agrees with that found in Fig. 1.

The aforementioned unique properties of lysine and proline are also reflected in the retention behaviour of dipeptides containing these residues. The k' *vs.* 18-crown-6 concentration curves in Fig. 8 show that glycyl-proline and prolyl-glycine react quite contrary to the addition of 18-crown-6. This confirms that the amino acid residue at the N-terminal contributes to the molecular recognition by 18-crown-6. Comparison between glycyl-lysine and glycyl-glycine, which have small and similar k'_0 values, leads to the conclusion that the ω-amino group of the lysine residue at the C-terminal can be another binding site for 18-crown-6, besides the amino group of the glycyl residue at the N-terminal. A similar conclusion may also be derived from



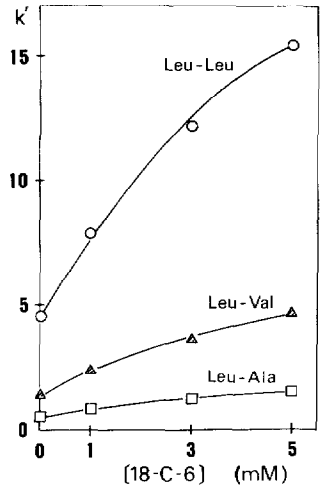


Fig. 6. Effect of 18-crown-6 on the retention enhancement of dipeptides with a leucyl residue at the C-terminal. HPLC conditions, see Table I.

Fig. 7. Effect of 18-crown-6 on the capacity factors of dipeptides with a leucyl residue at the N-terminal. HPLC conditions, see Table I.

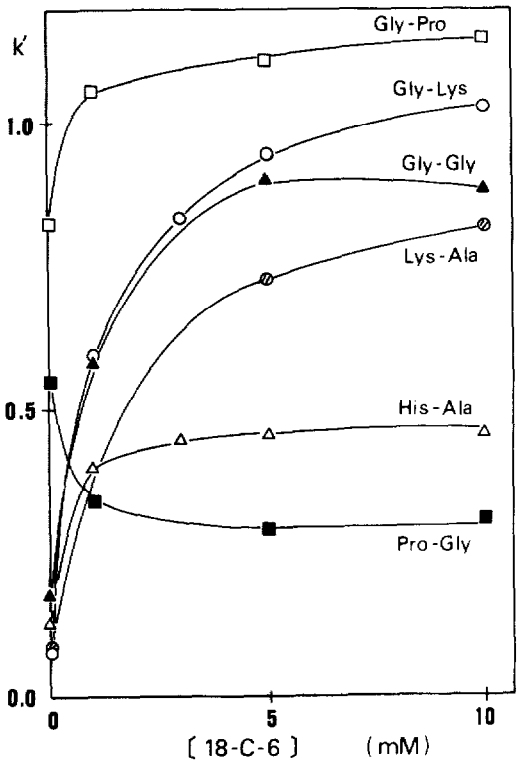


Fig. 8. Effect of 18-crown-6 concentration on the capacity factors of several dipeptides. HPLC conditions, see Table I.

a comparison between lysyl-alanine and histidyl-alanine, where the lysyl residue is located at the N-terminal; the ω-amino group of the lysyl residue seems to be responsible for the larger increase in k' value of lysyl-alanine than of histidyl-alanine. These results suggest that the lysyl residue may provide a significant increase in the capacity factor regardless of its location in a polypeptide chain, and the extent of the increase may reflect the higher structure.

As an example of polypeptides, bradykinin and lysylbradykinin, both hypotensive oligopeptides containing nine and ten amino acid residues, respectively (for structures, see Table III), were investigated with respect to the effects of 18-crown-6 and heptanesulphonate on their retention behaviour. In order to estimate these effects adequately, the mobile-phase conditions given in the legend of Table III were selected. The results in Table III show that when the mobile phase contained neither 18-crown-6 nor heptanesulphonate, the capacity factor of bradykinin was a little larger than that of lysylbradykinin. Addition of 1 m*M* heptanesulphonate caused the capacity factors of both peptides to be increased to almost the same extent (2.4 times), so that their elution order and capacity factor ratio (bradykinin/lysylbradykinin = 1.2) were not changed, and therefore, their separation was not improved. On the contrary, when 20 m*M* 18-crown-6 was added to the mobile phase, the capacity factor of lysylbradykinin was increased by 1.4 times more than that of bradykinin. As a result, these peptides were resolved and their elution order was reversed. When

TABLE III

CAPACITY FACTORS OF BRADYKININ AND LYSYLBRADYKININ

Bradykinin = Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. Lysylbradykinin = Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. HPLC conditions: Chemcosorb 300-7 C_{18} (25 cm × 4.6 mm I.D.). Mobile phases: I = water–acetonitrile (80:20) (pH adjusted to 2.9 with phosphoric acid) containing 10 m*M* lithium sulphate; II = mobile phase I containing 20 m*M* 18-crown-6; III = mobile phase I containing 1 m*M* sodium heptanesulphonate; IV = mobile phase I containing 20 m*M* 18-crown-6 and 1 m*M* sodium heptanesulphonate.

Compound	*Mobile phase*	k'	k'/k'_0
Bradykinin	I	1.23	1
	II	1.36	1.11
	III	2.95	2.40
	IV	3.38	2.75
Lysylbradykinin	I	0.99	1
	II	1.45	1.46
	III	2.40	2.42
	IV	4.01	4.05

the mobile phase contained both 18-crown-6 and heptanesulphonate, the capacity factors of both peptides were increased more than expected from their multiplied effects, resulting in complete separation in the same elution sequence as that obtained by using 18-crown-6 alone. This indicates that the ion-pair between heptanesulphonate and guest ammonium formed with 18-crown-6 is more hydrophobic and structure-recognizable than that formed without 18-crown-6. We expect the present method to be applied to the structure-recognizing separation of amino compounds in various samples by a simple modification of the mobile phase in reversed-phase chromatography.

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