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Manipulation of ion-pairing reagents for reversed-phase high-performance liquid chromatographic separation of phosphorylated opioid peptides from their non-phosphorylated analogues

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Abstract

The use of reversed-phase high-performance liquid chromatography for the separation of a mixture of 14 phosphorylated and non-phosphorylated enkephalins is described. The influence of two homologous series of hydrophobic ion-pairing reagents, consisting of perfluorinated carboxylic (trifluoroacetic, pentafluoropropionic and hexafluorobutyric) acids and sodium salts of sulfonic (butane-, hexane- and heptane-) acids, on the retention of enkephalin peptides was investigated. The incorporation of the phosphate group reduces retention time in proportion with the resulting change in hydrophobicity of the peptide. All peptides exhibit increase in retention time with increase in the counter ion hydrophobicity. The increase is proportional to the number of positively charged groups present in a peptide. Phosphopeptides show small increases in retention times than their corresponding non-phospho derivatives. The near-neighbor effect of the Tyr–O–phosphate group is responsible for suppression of the ion-pairing interaction of the mobile phase counter ions with the positively charged terminal amino group of enkephalins.

1. Introduction

During their transport in the cell body, proteins undergo several covalent modifications, of which phosphorylation is the most important and ubiquitous. It serves as a mechanism to regulate the activity of many proteins, hormones and enzymes. Methionine enkephalin (MetEnk; YGGFM) and leucine enkephalin (LeuEnk; YGGFL) are neuropeptides that play a role in several biological processes, including nociception. Biochemically, they are synthesized in the cell body from a large precursor protein preproenkephalin A (ProEnk A), which also produces several C-terminally extended MetEnk and LeuEnk opioid peptides, including MetEnk–Lys (MetEnk-K), LeuEnk–Lys (LeuEnk-K), MetEnk–Arg (MetEnk-R), MetEnk–ArgPhe (MetEnk-RF) and MetEnk–ArgGlyLeu (Met-

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Enk-RGL). It is important to know whether enkephalin peptides and their precursor molecules undergo phosphorylation at the tyrosine residue. However, before that question can be answered it is essential that an analytical method for their purification and separation is known.

Reversed-phase (RP) high-performance liquid chromatography (HPLC) has proven to be the most versatile method for rapid and effective separation of peptides and proteins and can be credited as a major contributing factor to advances made in several areas of peptide research, including synthesis and biomedical sciences. Selectivity in RP-HPLC analysis of peptides and proteins is achieved by exploiting the presence of free amino and carboxylic acid groups in those solutes. Previous researchers have shown that by taking advantage of the interaction of anionic and cationic counter ions present in the mobile phase with the amino and carboxylic groups of peptides, respectively, retention of peptides can be significantly altered. Typical additives used are perfluorinated carboxylic acids [1–4], alkylsulfonates [5–7], amine salts [8], tetraalkylammonium salts [4,7,9] perchloric acid [10] and phosphoric acid [5,11]. The incorporation of counter ions in the mobile phase has been a successful approach in isolation of several endogenous peptides from body tissues [4,12,13], in peptide mapping [14] and in preparative HPLC [3]; phosphorylated and non-phosphorylated forms of adrenocorticotropin (ACTH) [15] and corticotropic-like intermediate lobe peptide (CLIP) [16] have been resolved; and empirical sets of retention coefficients for amino acid residues have been developed [17–21], from which retention of peptides can be predicted [22].

As a first step in our quest for detection of the phosphorylation state of ProEnk A peptides, we report here the use of RP-HPLC for the analysis of Tyr–O–phosphate esters of the above listed enkephalins. The use of several perfluorinated carboxylic acids (trifluoroacetic, pentafluoropropionic and hexafluorobutyric) and sodium salts of sulfonic (butane-, hexane- and heptane-) acids as anionic ion-pairing reagents was exploited to effectively separate phosphorylated enkephalins from their non-phospho derivatives.

2. Experimental

2.1. Materials

All of the non-phosphorylated peptides were purchased from Sigma (St. Louis, MO, USA) except for MetEnk-R, which was obtained by trypsin digestion of MetEnk-RGL. The phosphorylated peptides were synthesized in our laboratory by solid-phase peptide synthesis protocol using 9-fluorenylmethoxycarbonyl (FMOC) chemistry, the details of which will be published elsewhere. They were purified by semi-preparative RP-HPLC. A further purification involved analytical RP-HPLC column. The purity of those synthetic peptides was checked by liquid secondary ionization mass spectrometry (LSI-MS).

Triethylamine was purchased from Pierce (Rockford, IL, USA), formic acid from Mallinckrodt (Paris, KY, USA), trifluoroacetic acid and heptafluorobutyric acid from Sigma, pentafluoropropionic acid and sodium salts of 1-butane-, 1-hexane- and 1-heptanesulfonic acids from Aldrich (Milwaukee, WI, USA) and acetic acid and acetonitrile from J.T. Baker (Phillipsburg, NJ, USA). All chemicals and solvents were of HPLC grade.

2.2. High-performance liquid chromatography

RP-HPLC was performed on an HP 1050 (Hewlett-Packard, CA, USA) instrument, consisting of a quaternary pumping system, a solvent degassing system, a Rheodyne 7125 manual injection valve, a photodiode-array based multiple-wavelength detector and an LC ChemStation data system. Samples were injected via a 20-μl sample loop onto a Vydac C_{18} analytical column (250 × 4.6 mm I.D. with 5 μm particle size and 300 Å pore size, purchased from Sigma fitted with PLRP-S guard column, containing poly(styrene–divinylbenzene) support (5 × 3 mm

I.D.; Polymer Labs., Amherst, MA, USA). Thus, two different column supports were used in tandem. The solvents were filtered through a 0.45-μm nylon-66 filter membrane (Pierce) and degassed by sparging with helium. The solvent reservoirs were maintained under helium atmosphere throughout the experiment. Chromatography was carried out at ambient temperatures with the mobile phase consisting of water–acetonitrile containing appropriate buffer and ion-pairing reagent flowing at 1.5 ml/min. The UV–visible multiple-wavelength detector was set at 200, 248 and 288 nm to monitor the absorbance due to the peptide bond, Tyr–O–phosphate and tyrosine residue, respectively. Water used was deionized in the laboratory, and was further purified by distillation using the Corning AG 11 system.

2.3. Ion-pairing reagents

The following seven ion-pairing reagent systems were studied: 0.1% trifluoroacetic acid (TFA), 0.1% pentafluoropropionic acid (PFPA), 0.1% heptafluorobutyric acid (HFBA), 0.1% sodium salts of butanesulfonic acid (BSA), hexanesulfonic acid (HxSA) and heptanesulfonic acid (HpSA) and triethylamine formate (TEAF; 0.04 *M* formic acid titrated with triethylamine to pH 3.12). The alkylsulfonates were dissolved in 0.02 *M* acetic acid to give a pH of 3.0.

3. Results

To understand the effect of ion-pairing reagents upon retention time in RP-HPLC, we studied a set of seven phosphorylated and the corresponding seven non-phosphorylated enkephalins. Those peptides, along with their sequence, the number of positive charges and the hydrophobicity/hydrophilicity indices, are listed in Table 1. A simple approach that uses the Bull and Breese (B and B) indices [23] for each individual amino acid is used to calculate the hydrophobicity/hydrophilicity index of a peptide. A more negative value of the B and B index reflects increased hydrophobicity. A num-

Table 1
Peptides studied

Peptide	Sequence of the peptide	Number of positively charged residues	Hydrophobicity/ hydrophilicity index (B and B index, cal/mol)
1	YGGFM	1	−398
1P	Y(P)GGFM	1	−268
2	YGGFL	1	−596
2P	Y(P)GGFL	1	−466
3	YGGFMK	2	−255
3P	Y(P)GGFMK	2	−147
4	YGGFLK	2	−420
4P	Y(P)GGFLK	2	−312
5	YGGFMR	2	−217
5P	Y(P)GGFMR	2	−109
6	YGGFMRF	2	−403
6P	Y(P)GGFMRF	2	−310
7	YGGFMRGL	2	−268
7P	Y(P)GGFMRGL	2	−187

Sequence is given in the single letter code: Y = tyrosine; G = glycine; F = phenylalanine; M = methionine; L = leucine; K = lysine; R = arginine; P in parentheses represents the phosphate group; the B and B index is calculated by adding the ΔF values (taken from ref. [23]) of individual amino acids and dividing the sum by the number of amino acids in the peptide; the contribution of the phosphate group ($\Delta F = +650$ cal/mol) is from ref. [31].

ber of other approaches have also been described in the literature to determine the overall hydrophobicity/hydrophilicity of a peptide [24–26]. The two homologous series of ion-pairing reagents (TFA, PFPA and HFBA and sodium salts of BSA, HxSA and HpSA) being used here are surface-active reagents and offer a range of gradually increasing hydrophobicity in a particular series.

The water–acetonitrile containing TFA as the ion-pairing reagent is the most commonly used RP-HPLC separation system for peptides and proteins because TFA offers the advantages of volatility, low UV transparency and excellent resolving power. Acetonitrile has been shown to be an excellent organic modifier for the analysis of peptides because it provides better resolution and selectivity than other common solvents such as 2-propanol and methanol [21]. The separation of a mixture of enkephalin peptides was first attempted using this system. A linear gradient, where acetonitrile content increased from 12% (at 0 min) at the rate of 0.5%/min, was found to give optimum separation of the complex mixture of 14 phosphorylated and non-phosphorylated enkephalin peptides; only phospho-MetEnk and phospho-LeuEnk-K coeluted under these conditions. The mobile phase contained 0.1% TFA to give pH 2.0. At this low pH, all carboxylic acids in a peptide are protonated and the positively charged amino groups are masked by the trifluoro group, increasing retention of the peptide via increased interaction with the hydrophobic stationary phase of the column. The effect of various other ion-pairing reagents mentioned above on the retention of those peptides was evaluated under the same chromatographic conditions except that the required amounts of those ion-pairing reagents were added to the mobile phase. As a typical example, the chromatogram obtained using the TFA ion-pairing system is shown in Fig. 1.

An aliquot (5 μl) of a mixture containing all of the 14 peptides (8–12 nmol/μl) was injected onto the RP-HPLC column. Fig. 2 is a pictorial depiction of relative retention times obtained using perfluorinated carboxylic acids (upper panel) and sodium alkylsulfonates (lower panel) as ion-pairing reagents. Each peak in the chromatogram was identified by analyzing separately mixtures of non-phosphorylated and phosphorylated enkephalin peptides. Sometimes to resolve ambiguity, individual peptides were injected. Recording of chromatogram at multiwavelengths also helped in identification of peaks. All non-phosphorylated peptides were easily distinguished in the mixture by monitoring the absorption at 288 nm, which is characteristic of tyrosine residue. Although no wavelength was found specific to the Tyr–O–phosphate group, that signal was recorded at 248 nm.

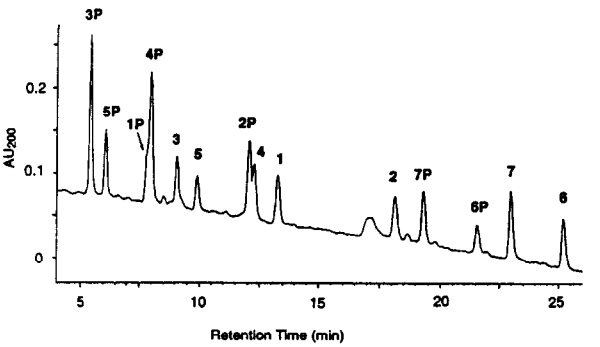

Fig. 1. A typical chromatogram of a mixture of 14 phosphorylated and non-phosphorylated enkephalin peptides. Mobile phase, water–acetonitrile containing 0.1% TFA; gradient, linear from 12% acetonitrile at 0 min to 32% at 40 min. The injection volume was 5 μl and contained 8–12 nmol/μl of each peptide. See Table 1 for peak identification.

With the above linear gradient, a clear separation of some of the peptides was not achieved in the TFA and HFBA systems, whereas the PFPA system completely resolved all of the 14 peptides under the same elution conditions. To obtain a baseline separation in the TFA and HFBA systems, a non-linear gradient was developed and the following gradient was found to provide optimum separations in both the systems: 12–20% acetonitrile, 0–18 min; 20–35% acetonitrile, 18–30 min; 35% acetonitrile, 30–40 min. Similarly, the above linear gradient was adequate to provide separation of all of the 14 peptides in the BSA and HpSA systems, whereas in the HxSA system all but LeuEnk and phospho-MetEnk-RGL were well resolved. In this system, the following non-linear gradient provided a clean separation of all of the peptides studied here: 12–18% acetonitrile, 0–12 min;

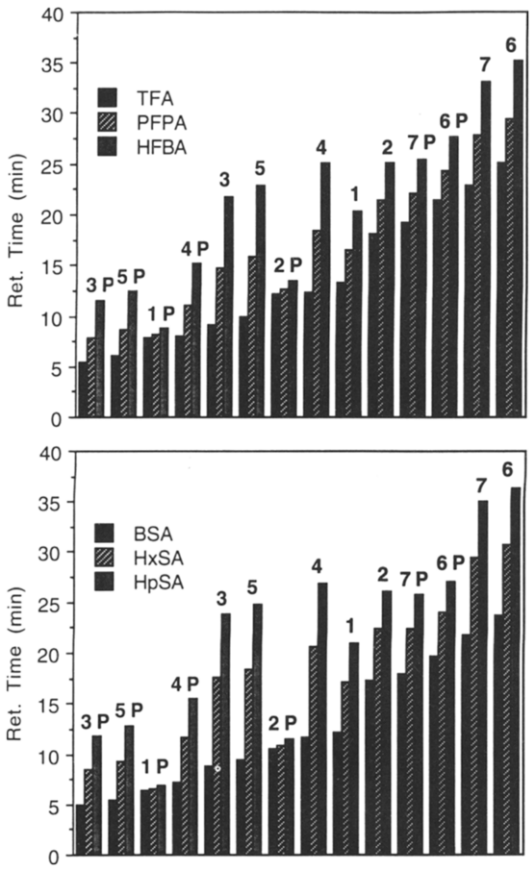

Fig. 2. Graphical representation of relative retention times of 14 phosphorylated and non-phosphorylated enkephalin peptides obtained using perfluorinated carboxylic acids (upper panel) and sodium salts of sulfonic acids (lower panel) as ion-pairing reagents under linear gradient conditions.

18–22% acetonitrile, 12–30 min; 22–35% acetonitrile, 30–35 min; 35% acetonitrile, 35–40 min.

The water–acetonitrile buffered by TEAF (pH 3.1) is frequently used in our laboratory for separation of neuropeptides from tissue extracts [27]. Similar to perfluorinated carboxylic acids, TEAF is also volatile. TEA is a weak hydrophobic ion-pairing reagent. This system was also tested for separation of phospho and non-phospho enkephalins. The following gradient of acetonitrile was found to resolve all of the 14 components of the mixture: 0–10 min, 10%; 10–20 min, 15%; 20–30 min, 15–30%; 30–40 min, 30%. The presence of a peptide in a biological extract is confirmed only when the amino acid sequence of that peptide is demonstrated. Therefore, our research uses MS as a highly specific post-column detector for analysis of peptides because it provides a precise knowledge of the molecular mass and amino acid sequence of the peptide [12,27]. However, it was found that the TEAF system may not be appropriate for MS analysis of phosphopeptides because the phosphate group is known to form adducts with TEA [28]. LSI-MS mass spectra of the lyophilized fractions of phosphopeptides also confirmed that, in addition to the usual $(M+H)^+$ ions of phosphopeptides, monoadducts with TEA are also formed.

4. Discussion

Peptides are polar molecules and contain both hydrophobic (leucine, methionine, phenylalanine, tyrosine, etc.) and hydrophilic (aspartic, glutamic, lysine, arginine, etc.) amino acids. The predominant factor involved in separation of peptides by RP-HPLC is the hydrophobic interaction between the non-polar hydrocarbonaceous matrix of the column material and the hydrophobic groups of the peptide. At any given pH, the RP-HPLC behavior of a peptide is determined by the relative contribution of hydrophobic and hydrophilic amino acids [17–22]. In the case of phosphorylated peptides, the polar phosphate group also contributes to hydrophilicity. Peptides with hydrophobic side chains are preferentially retained on the column material. Therefore, relatively hydrophilic peptides elute earlier with aqueous mobile phase, whereas those strongly retained hydrophobic peptides require increasing concentrations of an organic modifier in the mobile phase.

This initial behavior, however, can be altered by the addition of a suitable ion-pairing reagent [1–11]. Because peptides are charged molecules they form complexes with the auxiliary ions added to the solvent system; the anionic counter ions bind to the positively charged amino groups and the cationic ions bind to the negatively charged acidic groups. The ion-pair complex of a

peptide with the hydrophobic counter ions results in increased affinity of the peptide with the column, whereas the complex with the hydrophilic counter ions reduces that interaction [29]. In addition to the surface adsorption, the dynamic ion exchange can also play a role in determining retention of a solute on the RP-HPLC column [30]. Recently, Patthy [30] has shown that separation of small peptides, amines and acidic compounds in the TFA system is solely governed by surface adsorption, whereas in the HFBA system dynamic ion exchange is an important factor.

4.1. Perfluorinated carboxylic acids

The effect of hydrophobicity of amino acids on retention of peptides

From the data in Fig. 2, it is obvious that the hydrophobicity of peptides has a profound influence on their retention time in perfluorinated carboxylic acid systems. For example, amongst the pentapeptides, LeuEnk (B and B = −596 cal/mol; 1 cal = 4.184 J) elutes later than MetEnk (B and B = −398 cal/mol). All hexapeptides contain an additional hydrophilic basic amino group, and thus elute earlier than MetEnk and LeuEnk. MetEnk-RF and MetEnk-RGL also contain a basic amino acid but are retained more than the hexapeptides mainly due to the presence of additional hydrophobic amino acids (Phe and Leu, respectively). As expected, all phospho derivatives elute earlier than their non-phospho counterparts; the polar phosphate group contributes +650 cal/mol towards the hydrophobicity/hydrophilicity index of a peptide [31].

These observations are consistent with the findings of Guo and co-workers [21,22], who pointed out that RP-HPLC retention of peptides can be correlated with the summated relative retention coefficients of each amino acid residues. Under those experimental conditions, retention time of a peptide can be predicted from its amino acid composition. On the basis of the relative coefficients of each amino acids determined by them [21], MetEnK should elute earlier than LeuEnk, Metenk-K earlier than MetEnk-R and MetEnk-RGL earlier than MetEnk-RF; a similar retention behavior is noted in the present study.

The effect of hydrophobicity of ion-pairing reagents on retention of peptides

From Fig. 2, it is also obvious that retention of peptides of interest here can be significantly modified by the addition of various anionic reagents to the mobile phase. Fig. 3 is a plot of retention time versus length of the alkyl chain in the ion-pairing reagents; the latter is a direct measure of the hydrophobicity of the anionic counter ion. To avoid cluttering, the data for non-phosphorylated (Fig. 3A) and phosphorylated peptides (Fig. 3B) are plotted separately. Those plots illustrate a linear increase in retention time with increasing length of the alkyl

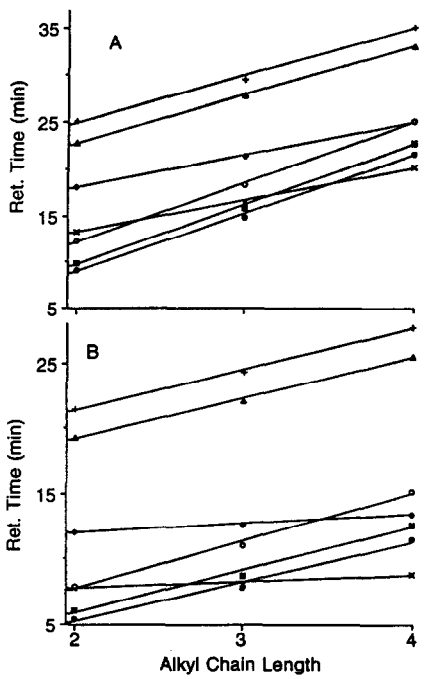

Fig. 3. Effect of counter ion hydrophobicity on the retention of enkephalin peptides, when TFA, PFPA and HFBA are used as the mobile phase additives. (A) Non-phospho enkephalins: × = MetEnk; ◆ = LeuEnk; ● = MetEnk-K; ○ = LeuEnk-K; ■ = MetEnk-R; + = MetEnk-RF; ▲ = MetEnk-RGL; (B) corresponding phospho derivatives.

chain, demonstrating clearly the effect of the hydrophobicity of the counter ions on retention of peptides. In view of the findings of Patthy [30], it is likely that dynamic ion exchange plays an increasing role in determining the retention of peptides when longer-chain perfluorinated carboxylic acid solvent systems are used.

As observed by Guo et al. [29], the magnitude of increase in retention time also depends upon the number of positively charged residues in peptides. For example, non-phosphopeptides that contain basic amino acid residues Arg or Lys show almost twice the increase in retention compared to MetEnk and LeuEnk; the slopes of the plots for peptides MetEnk-K, LeuEnk-K, and MetEnk-R vary from 6.34 to 6.51 and for peptides MetEnk-RF and MetEnk-RGL from 5.04 to 5.14, whereas the corresponding slope for pentapeptides is ca. 3.50.

In contrast, phospho-MetEnk and -LeuEnk show only a marginal change in retention time in moving from TFA to HFBA system; the slopes of their retention time versus counter ion hydrophobicity plots are 0.49 and 0.68, respectively. Similarly, the increase in retention time for other phosphopeptides is not as significant as with their non-phospho analogues. As a result, the elution order of peptides changes in progressing from TFA to HFBA. For example, phospho-MetEnk-K and -MetEnk-R both elute earlier than phospho-MetEnk in the TFA system, whereas in the HFBA system they elute after phospho-MetEnk.

The probable reason for the intriguing behavior for phosphopeptides is that the phosphate group is located in close proximity to the terminal amino group and as a result offers steric hindrance for the ion-pairing interaction of the counter ions with that amino group. Phospho-MetEnk and -LeuEnk behave as if no basic group is present. Likewise, the phosphorylated hexapeptides behave as if they contain only one basic amino acid. Fig. 4 demonstrates that the magnitude of the slope of the plot of retention time versus the counter ion hydrophobicity for these three phosphopeptides is nearly identical to that obtained for MetEnk and LeuEnk, in which the only positively charged site is the N-terminal amino group.

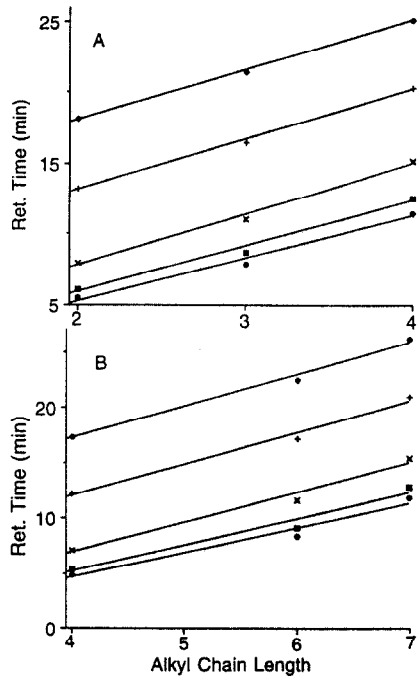

Fig. 4. Graphical representation of the near-neighbor effect on ion-pair interaction. The counter ion hydrophobicity vs. retention time curves for (●) phospho-MetEnk-K, (■) phospho-MetEnk-R and (×) phospho-LeuEnk-K are nearly parallel with those of (+) MetEnk and (◆) LeuEnk, indicating that phosphorylation of the N-terminal tyrosine suppresses the ion-pair interaction with the terminal amino group. (A) Perfluorinated carboxylic acids, (B) sodium salts of sulfonic acids.

The effect of phosphate group on the retention of peptides

The polar phosphate group also influences retention of enkephalin peptides. With the introduction of the phosphate group, the hydrophobic character of a peptide is reduced. Consequently, the interaction between a peptide and the hydrophobic sites on the HPLC column is diminished, resulting in early elution of the phosphopeptides. Introduction of a phosphate group produces a change in the hydrophobicity index of −130.0, −108.3, −92.9 and −81.2 cal/mol for penta-, hexa-, hepta- and octapeptides, respectively. The change in retention time upon phosphorylation is also roughly proportional to the resulting change in hydrophobicity. This aspect is demonstrated in

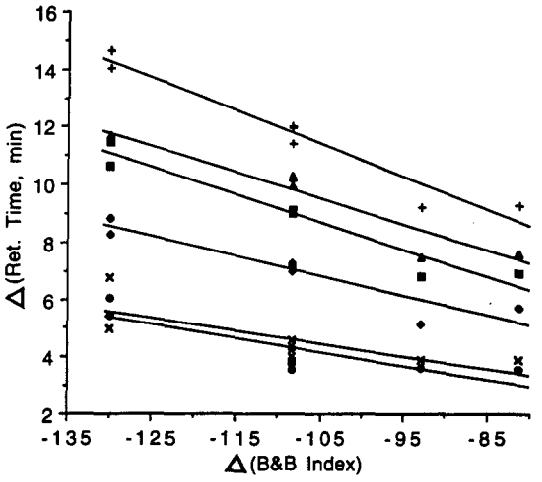

Fig. 5. Effect of phosphorylation on the retention of enkephalin peptides; the decrease in retention time is approximately linear with decrease in hydrophobicity of the peptide. ● = TFA; ◆ = PFPA; ▲ = HFBA; × = BSA; ■ = HxSA; + = HpSA.

Fig. 5. An approximately linear relation is observed between change in retention time and change in hydrophobicity for the ion-pairing systems used here.

4.2. Alkylsulfonates

The RP-HPLC elution profile of phospho and non-phospho enkephalin peptides in the alkylsulfonate solvent system is also parallel to the perfluorinated carboxylic acids solvent system. The hydrophilic peptides elute earlier than peptides with the hydrophobic character (Fig. 2), the increase in retention in progressing from butanesulfonate to heptanesulfonate system is proportional to the hydrophobicity of the counter ion (Fig. 6), and decrease in retention upon phosphorylation is commensurate with the decrease in the hydrophobicity (Fig. 5).

Thus, this study has demonstrated that RP-HPLC can be effectively used to separate phosphorylated and non-phosphorylated enkephalin peptides by either manipulation of the gradient or by changing the ion-pairing system. Although parallel results were obtained with the perfluorinated carboxylic acids and alkylsulfonates, the former system offers advantages because of

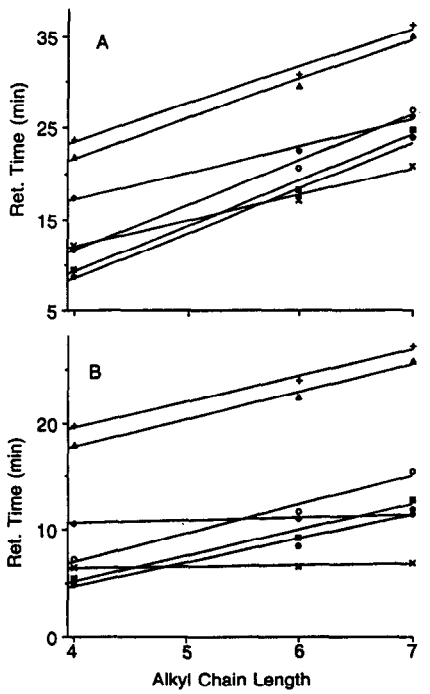

Fig. 6. Effect of counter ion hydrophobicity on retention time of enkephalin peptides, when BSA, HxSA and HpSA are used as the mobile phase additives. (A) Non-phospho enkephalins: × = MetEnk; ◆ = LeuEnk; ● = MetEnk-K; ○ = LeuEnk-K; ■ = MetEnk-R; + = MetEnk-RF; ▲ = MetEnk-RGL; (B) corresponding phospho derivatives.

volatility of the ion-pairing reagents. A volatile reagent does not interfere in the post-column detection of peptides by radioimmunoassay and MS, the two most commonly used detectors by us for analysis of peptides.

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References

[1] W.M.M. Schaaper, D. Voskamp and C. Olieman, *J. Chromatogr.*, 195 (1980) 181.
[2] H.P.J. Bennett, C.A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353.

[3] H.P.J. Bennett, *J. Chromatogr.*, 266 (1983) 501.
[4] D.R.K. Harding, C.A. Bishop, M.F. Tarttellin and W.S. Hancock, *Int. J. Peptide Protein Res.*, 18 (1981) 214.
[5] W.S. Hancock, C.A. Bishop, L.J. Meyer, D.R.K. Harding and M.T.W. Hearn, *J. Chromatogr.*, 161 (1978) 291.
[6] E. Spindel, D. Pettibone, L. Fisher, J. Fernstrom and R. Wurtman, *J. Chromatogr.*, 222 (1981) 381.
[7] Z. Iskandarini, R.L. Smith and D.J. Pietrzyk, *J. Liq. Chromatogr.*, 7 (1984) 111.
[8] J.E. Rivier, *J. Liq. Chromatogr.*, 1 (1978) 342.
[9] W.S. Hancock, C.A. Bishop, J.E. Battersby, D.R.K. Harding and M.T.W. Hearn, *J. Chromatogr.*, 168 (1979) 377.
[10] M. Rubenstein, *Anal. Biochem.*, 98 (1979) 1.
[11] W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, *Science (Washington, D.C.)*, 200 (1089) 1168.
[12] D.M. Desiderio, J.J. Kusmierz, X. Zhu, C. Dass, D. Hilton, J.T. Robinson and H.S. Sacks, *Biol. Mass Spectrom.*, 22 (1993) 89.
[13] A.W. Burgess, J. Knesel, L.G. Sparrow, N.A. Nicola and E.C. Nice, *Proc. Natl. Acad. Sci. U.S.A.*, 79 (1982) 5753.
[14] J.D. Hockenhull-Johnson, M.S. Stern, P. Martin, C. Dass, D.M. Desiderio, J.B. Wittenberg, S.N. Vinogradov and D.A. Walz, *J. Protein Chem.*, 10 (1991) 609.
[15] H.P.J. Bennett, C.A. Browne and S. Solomon, *Biochemistry*, 20 (1981) 4530.
[16] C.A. Browne, H.P.J. Bennett and S. Solomon, *Biochemistry*, 20 (1981) 4538.
[17] J.L. Meek and Z.L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
[18] S.J. Su, B. Grego, B. Niven and M.T.W. Hearn, *J. Liq. Chromatogr.*, 4 (1981) 1745.
[19] C.A. Browne, H.P.J. Bennett and S. Solomon, *Anal. Biochem.*, 124 (1982) 201.
[20] T. Sasagawa, T. Okuyama and D.C. Teller, *J. Chromatogr.*, 240 (1982) 329.
[21] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker and R.S. Hodges, *J. Chromatogr.*, 359 (1986) 499.
[22] D. Guo, C.T. Mant, A.K. Taneja and R.S. Hodges, *J. Chromatogr.*, 359 (1986) 519.
[23] H.B. Bull and K. Breese, *Arch. Biochem. Biophys.*, 161 (1974) 665.
[24] J. Kyte and R.F. Doolittle, *J. Mol. Biol.*, 157 (1982) 105.
[25] D. Eisenberg and A.D. McLachlan, *Nature*, 319 (1986) 199.
[26] J.L. Cornette, K.B. Cease, H. Margalit, J.L. Spouge, J.A. Berzofsky and C. DeLisi, *J. Mol. Biol.*, 195 (1987) 659.
[27] C. Dass, G.H. Fridland, P.W. Tinsley, J.T. Killmar and D.M. Desiderio, *Int. J. Peptide Protein Res.*, 34 (1989) 81.
[28] G. Zardeneta, D. Chen., S.T. Weintraub and R.J. Klebe, *Anal. Biochem.*, 190 (1990) 340.
[29] D. Guo, C.T. Mant and R.S. Hodges, *J. Chromatogr.*, 386 (1987) 205.
[30] M. Patthy, *J. Chromatogr.*, 660 (1994) 17.
[31] L. Poulter, S.G. Ang, D.H. Williams and P. Cohen, *Biochim. Biophys. Acta*, 929 (1987) 296.