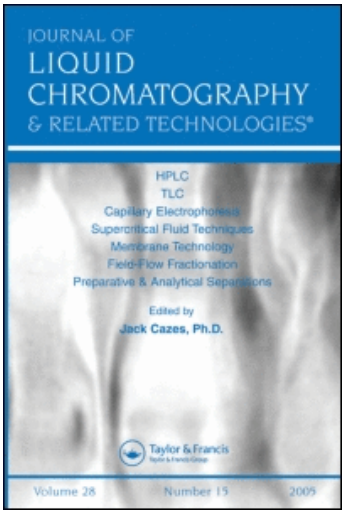

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A Comparative Study on the Effect of Hydrochloric, Phosphoric, and Trifluoroacetic Acid in the Reversed Phase Chromatography of Angiotensins and Related Peptides

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A COMPARATIVE STUDY ON THE EFFECT OF HYDROCHLORIC, PHOSPHORIC, AND TRIFLUOROACETIC ACID IN THE REVERSED PHASE CHROMATOGRAPHY OF ANGIOTENSINS AND RELATED PEPTIDES

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ABSTRACT

This paper examines the effect of hydrochloric acid as an alternative mobile phase additive in reversed phase chromatography of closely related peptides, such as the analogs of human angiotensin I and III, in comparison to the retention behavior and selectivity obtained with the two most popular additives TFA and phosphoric acid. The effect of the concentration of the additive and of the apparent pH of the mobile phase was investigated by isocratic elution of the selected peptides on a totally porous butyl-silica column with water-acetonitrile eluents containing either additive at various concentrations, corresponding to different values of the apparent pH of the mobile phase within the range 1.23 to 2.28. Differences in retention behavior and selectivity observed with the three mobile phase additives were discussed in terms of differences in the ion-pair associations between the ionized peptides and the counterions in the mobile phase.

INTRODUCTION

Reversed-phase chromatography (RPC) is the predominant mode of high-performance liquid chromatography (HPLC) for both analytical and preparative scale separations of peptides (1). Its excellent resolving power and flexibility is mainly due to the wide possibility of manipulation of mobile phase composition, which include the type and the concentration of organic modifier, pH, choice of buffering agent, and use of hydrophilic or hydrophobic ion-pairing reagents (2).

Peptides are charged molecules at most pH values and the presence of different counterions in the mobile phase influences their charge and hydrophobicity to different extent, depending on the nature of both the counterion and peptide. Mobile phases in the range of pH 2-3 are usually selected in order to ensure protonation of hydrophilic acidic groups of peptides and to suppress the ionization of the surface silanols of the silica-based sorbents which are the dominant reversed-phase columns used in peptide RPC. Ionization of peptide carboxyl groups increases the analyte polarity and hence reduce its retention, whereas silanols dissociation is believed to be the main source of the dual functionalities (polar and hydrophobic) exhibited by silica-based reversed-phase sorbents (3-4).

The most commonly used additive for acidic mobile phases employed in RPC of peptides is trifluoroacetic acid (TFA), due to its anionic ion-pairing properties, excellent solubilization characteristics for most peptides, low UV transparency, capability to minimize the ionic effects of free silanols of the sorbent, and volatility, which renders this additive particularly suitable for preparative scale separations (5-6). Acetate, formate and dihydrogen phosphate are also popular alternative choices (2, 7-8). Furthermore, a variety of alkylsulfonates and alkyl sulfates offer a range of anionic ion-pairing reagents with varying degree of hydrophobicity. Cationic ion-pairing additives have also been employed including tertiary alkyl amines (e.g. triethylamine) and quaternary ammonium salts (e.g., tetrabutylammonium phosphate) (9-12). However, using these hydrophobic ion-pairing reagents as mobile phase additives induces an increase in peptide retention time thus requiring the use of higher organic modifier concentrations.

The purpose of this study is to examine the effect of hydrochloric acid in reversed phase chromatography of small peptides, in comparison to the retention behavior and selectivity obtained with the two most popular additives TFA and phosphoric acid. While TFA and to a lesser extent phosphoric and hydrochloric acid were exploited in HPLC of peptides (7-8, 13-14), a systematic study involving the use and examination of the effect of hydrochloric acid in peptide RPC is sorely needed .

This report summarizes the results obtained by comparing the chromatographic behavior of a set of angiotensin analogs, selected as typical models of closely related peptides, on a totally porous 5-μm butyl-silica column which were eluted under isocratic conditions with water-acetonitrile mobile phases containing either TFA or hydrochloric or phosphoric acid at concentration corresponding to various values of the apparent pH of the hydro-organic eluents, ranging from 1.23 to 2.28.

EXPERIMENTAL

Instrument and column

The experiments were carried out with an HPLC unit assembled from two Model 114 solvent delivery pumps, a Model 420 system controller, both from Beckman (Fullerton, CA, USA), a Varian (Walnut Creek, CA, USA) Model Star 9050 variable wavelength UV-vis detector, and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 5-μl sample loop. Chromatograms were obtained with a Spectra-Physics (San Jose, CA, USA) Model SP 4400 integrator. HPLC was performed on a Vydac (The Separations Group, Hesperia, CA, USA) Protein C-4 column (150 x 4.6 mm, I.D.) containing 5-μm butyl-silica.

Materials

The following synthetic bioactive peptides were obtained from Sigma (MIlan, Italy): Angiotensin I from human, bullfrog, and salmon, human angiotensin III, human angiotensin III analogs and des-**Arg**^{1}-bradykinin. The sequence of these peptides are in Table 1. Reagent-grade phosphoric acid and TFA were purchased from Fluka (Milan, Italy).

TABLE 1

Sequence of Peptides Used in This Study.

Peptides	Amino-acid residues	Sequence
Human Angiotensin I	10	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Bullfrog Angiotensin I	10	Asp-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu
Salmon Angiotensin I	10	Asn-Arg-Val-Tyr-Val-His-Pro-Phe-Asn-Leu
Human Angiotensin III	7	Arg-Val-Tyr-Ile-His-Pro-Phe
[Val^4]-Angiotensin III	7	Arg-Val-Tyr-Val-His-Pro-Phe
[Val^4-Ile^7]-Angiotensin III	7	Arg-Val-Tyr-Val-His-Pro-Ile
des-Arg^1-Bradykinin	8	Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Reagent-grade hydrochloric acid as well as HPLC-grade water and acetonitrile (ACN) were obtained from Carlo Erba (Milan, Italy).

Preparation of eluents

The eluents were prepared by mixing in a volumetric flask the required volumes of acetonitrile and additive for the concentration stated. The apparent pH was measured with a glass electrode model HI 1131 and Model HI 9017 Microprocessor pH-Meter, both from Hanna Instruments (Woonsocket, RI, USA), and was taken as the pH of the solution. All solutions were filtered through a Millipore (Milan, Italy) type FH 0.5-μm membrane filter and degassed by sparging with helium before use.

Chromatographic measurements

Acetonitrile-water mixtures having ACN concentration of 19.95% (v/v) and containing either TFA or phosphoric acid or hydrochloric acid at a given concentration were used for isocratic elution at room temperature. The column was allowed to equilibrate for a period of 1-h after each change

of mobile phase composition. The peak obtained by injecting pure acetonitrile was used to evaluate the hold-up time at each mobile phase composition. All measurements of retention data were made in triplicate.

RESULTS AND DISCUSSION

Angiotensin I from three different biological sources (human, salmon, bullfrog), human angiotensin III and two analogs of this peptide, [Val^4]-angiotensin III and [Val^4-Ile^7]-angiotensin III, were selected as typical models of closely related peptides. The dependence of the retention factors, k', of these bioactive peptides on the concentration of TFA in the mobile phase containing acetonitrile as the organic modifier was examined on a Vydac Protein C-4 column and the results are shown in Fig. 1. The peptides were eluted at room temperature under isocratic conditions at a flow rate of 1.0 ml/min with hydro-organic mobile phases containing the same percentage of acetonitrile, 19.95 % (v/v), and TFA at concentration ranging from 5 to 40 mM. The concentration of acetonitrile in the mobile phase was selected so as to obtain accurately measurable retention factors and peptide separation.

As seen in Fig. 1, all of the peptides exhibited increasing retention factors with increasing the TFA concentration in the eluent. The measurement of the apparent pH of the hydro-organic mobile phases revealed that as the concentration of TFA increased from 5 to 40 mM the apparent pH of the hydro-organic eluent decreased from 2.28 to 1.41. Since the pH of the mobile phase can affect the net charge of the peptide, resulting in a variation of the analyte polarity, we decided to examine the effect of the apparent pH of the mobile phase on the retention behavior of the various peptides under investigation. This study was performed by eluting the selected angiotensins with mobile phases containing acetonitrile at the same concentration as in the previous experiments and phosphoric acid at concentrations needed to bring the apparent pH of the hydro-organic mobile phases to the values of 2.28, 2.00, 1.66, and 1.41, which were the values of the apparent pH measured with TFA at concentrations of 5, 13.5, 25, and 40 mM, respectively. Further experiments were carried out using

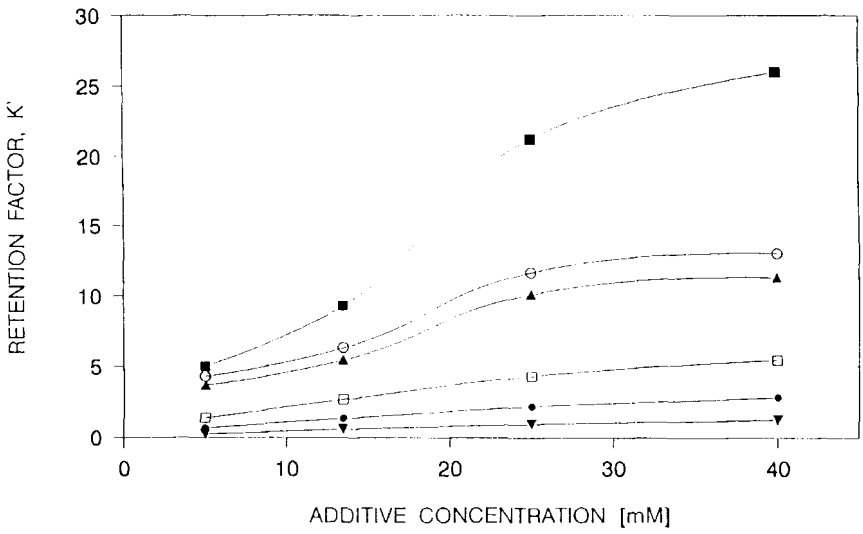

FIGURE 1. Dependence of retention factor on the concentration of TFA in the hydro-organic mobile phase containing 19.95% (v/v) acetonitrile. Column, Vydac Protein C-4 (150 x 4.6 mm I.D.); flow rate, 1.0 ml/min; detection, 214 nm, 0.05 a.u.f.s.; peptides: [Val^4-Ile^7]-angiotensin III (wedge), [Val^4]-angiotensin III (dot), human angiotensin III (square), angiotensin I from human (filled square), bullfrog (circle) and salmon (triangle).

hydro-organic mobile phases with the same content of acetonitrile as above and hydrochloric acid as the acidic additive at concentration ranging from 4.4 to 36.5 mM, the apparent pH of which ranged from 1.23 to 2.0.

The retention factors obtained with the mobile phases containing the three acids are plotted in Fig. 2 against the value of the apparent pH measured by the pH-meter with the glass electrode. It is shown that with either hydrochloric or phosphoric acid the retention factors are only slightly affected by the variations in the apparent pH of the mobile phase. It should be noted that the concentration of the acid in the mobile phase corresponding to a given value of the apparent pH varied with the strength of the acid. For example, the concentration of TFA, hydrochloric, and phosphoric acid corresponding to the apparent pH 2.0 was 13.5, 4.5, and 31 mM, respectively. Consequently, plots of Figure 2 express also the

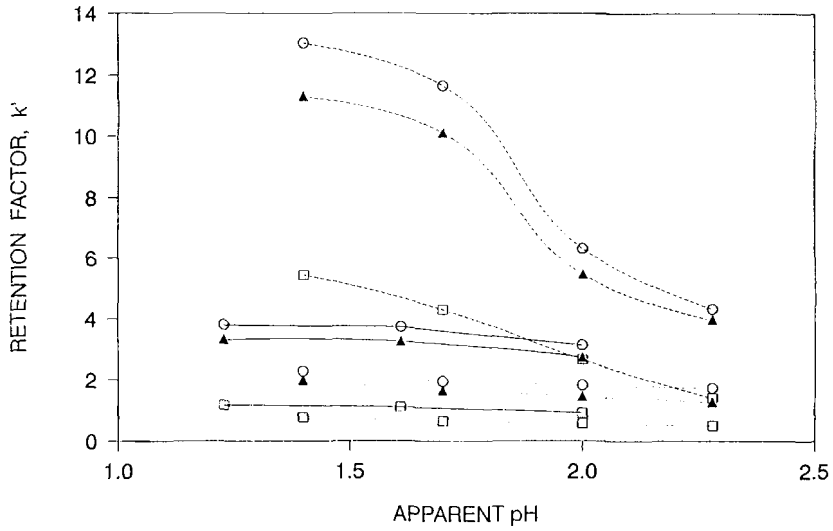

FIGURE 2. Dependence of retention factor on the value of the apparent pH of the mobile phase containing 19.95% (v/v) acetonitrile and either TFA (dashed lines) or phosphoric acid (dotted lines) or hydrochloric acid (solid lines). Symbols and experimental conditions as in Fig. 1.

variations of the retention factors within the different concentration ranges of the three acids in the mobile phase, which varied from 5 to 40, 4.4 to 36.5, and 9.4 to 203 mM for TFA, hydrochloric and phosphoric acid, respectively. Plots of Fig. 2 indicate that for the peptides investigated in this study the apparent pH of the mobile phase, at least within the range from 1.23 to 2.28, may not be a critical parameter in the case of the two inorganic acids (i.e. phosphoric and hydrochloric acid). On the other hand, the increasing retention of peptides observed with TFA is related to the effect of increasing the concentration of this organic acid to lowering the value of the apparent pH of the mobile phase and reflects the more hydrophobic character of TFA than that of phosphoric and hydrochloric acid.

Besides lowering the pH and thus suppressing the ionization of the residual silanol groups at the chromatographic surface, acidic additives are

believed to interact with the amino functions of the peptides via ion-pair formation (2). Furthermore, TFA and phosphoric acid posses high hydrogen-bonding capability (15) which is believed to affect both surface and solute polarity (16). TFA is a proton acceptor via its fluoro atoms and the carboxyl group and is less polar then phosphoric and hydrochloric acid. Extensive hydrogen-bonding of the trifluoroacetate ion to the silica surface and the peptide molecules led to enhancing the hydrophobicity of both the sorbent and the peptide, so that the retention of peptides is greater with TFA then with hydrochloric or phosphoric acid as the additive under otherwise identical conditions.

The peak capacity measured in the presence of the different mobile phase additives was used to compare their effect on the separation of a peptide test mixture, which included des-Arg^1-bradykinin and angiotensin I from three different biological sources: human, salmon and bullfrog. The peak capacity is calculated by dividing the net retention time of the last peak in the chromatogram by the average peak width. It expresses the maximum number of peaks that can theoretically be resolved in the chromatogram (17).

In order to facilitate the comparison, peak capacities were measured with the same mobile phases used to study the effect of the apparent pH on the retention behavior of peptides and the results are reported in Table 2. It is observed that peak capacity increases with the concentration of the additive up to a limiting value which is greater with mobile phases containing TFA. The increased value of peak capacity with increasing the additive concentration is related to both the improved peak efficiency, due to a better masking effect of the free silanol groups on the surface of the chromatographic sorbent and the greater separation distance obtained with increasing the additive concentration, which was larger with TFA due to the longer retention times obtained with this mobile phases additive.

Figure 3 and 4 report the chromatograms of the above peptide test mixture obtained with hydrochloric and phosphoric acid as the additives of the hydro-organic mobile phases. With mobile phases containing hydrochloric acid (see Fig. 3), angiotensin I from salmon elutes after des-Arg1-bradikinin, which elutes first, followed by the peptide from bullfrog and then that from human, which elutes last. It is observed that the chromatographic resolution of the three angiotensins improves as the

TABLE 2

Peak Capacity of the Vydac Protein C-4 Column as Determined by Isocratic Elution of a Peptide Mixture with Hydro-organic Mobile Phases Containing 19.95% (v/v) Acetonitrile and Either Hydrochloric or Phosphoric or Trifluoroacetic Acid at Different Concentrations.

HCl		H_3PO_4		TFA	
Concentration [mM]	Peak Capacity	Concentration [mM]	Peak Capacity	Concentration [mM]	Peak Capacity
4.4	10	9.4	6	5.0	14
8.3	14	31.0	7	13.5	21
19.4	14	90.0	8	25.0	27
36.5	15	203.0	8	40.0	32

concentration of the additive increases. Similar results (not shown) were also obtained with TFA. However, with this additive the retention times were noticeably longer than those obtained with either hydrochloric or phosphoric acid (see Fig. 2). In contrast, dramatic selectivity changes were obtained with the use of phosphoric acid as the mobile phase additive. This is illustrated in Fig. 4 which displays the chromatograms obtained with the hydro-organic mobile phases containing phosphoric acid at concentration ranging from 9.4 to 203 mM. It is of interest to note the reversal of elution order for angiotensin I from bullfrog and from human and the co-elution of this angiotensin with the peptide from salmon observed with the lower concentration of phosphoric acid in the eluent. Furthermore, the chromatographic resolution of these peptides decreases as the concentration of phosphoric acid increases.

The differences in selectivity and resolution observed with hydrochloric and phosphoric acid as the additives can be attributed to differences in the ion-pair associations between the ionized peptides and the counterions in the mobile phase. We have already mentioned that

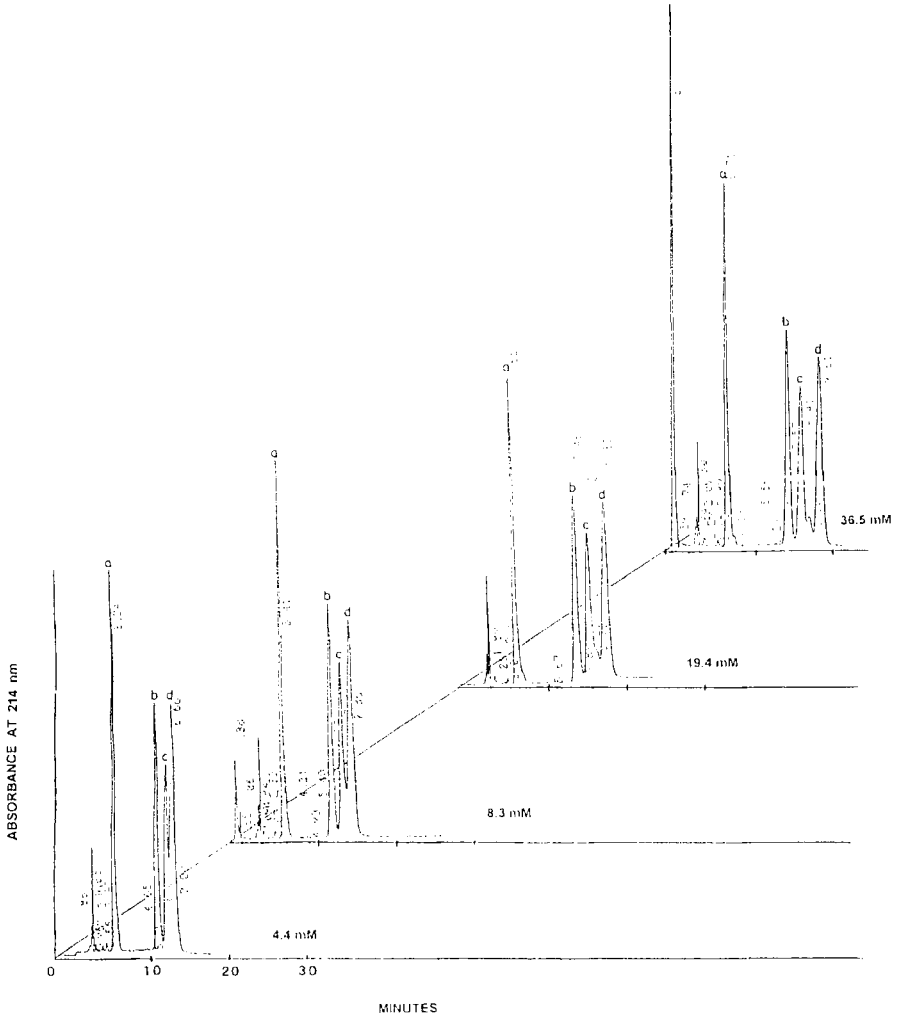

FIGURE 3. Chromatographic separation of des-Arg^1-bradykinin (a) and angiotensin I from salmon (b), bullfrog (c) and human (d) with mobile phases containing 19.95% (v/v) acetonitrile and hydrochloric acid at various concentrations as indicated on the chromatogram. Other conditions as in Fig. 1.

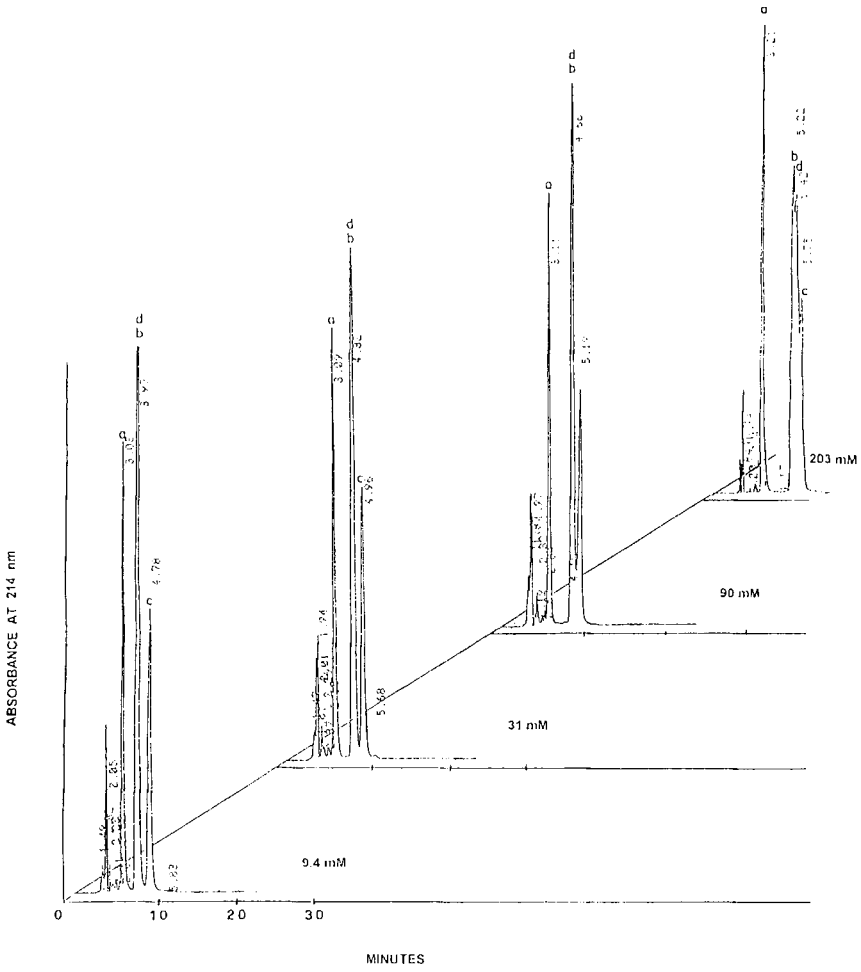

FIGURE 4. Chromatographic separation of des-Arg^1-bradykinin (a) and angiotensin I from salmon (b), bullfrog (c) and human (d) with mobile phases containing 19.95% (v/v) acetonitrile and phosphoric acid at various concentrations as indicated on the chromatogram. Other conditions as in Fig. 1.

phosphoric acid exhibit strong hydrogen-bonding properties, acting both as the donor and the acceptor. In most cases, each oxygen of phosphate ions can enter into more than one hydrogen bond owing to its tetrahedral symmetry (18). Extensive hydrogen-bonding of the phosphate ions to the molecules of the three angiotensins may enhance the virtual polarity of the ion-pair complexes of these closely related peptides to an extent that may level off the selectivity of the hydrophobic stationary phase towards these molecules. In contrast, hydrochloric acid is expected to suppress the ionization of the peptide carboxyl groups without the ion-pairing effect seen with phosphoric acid. Therefore, it can be inferred that the variations of the chromatographic behavior of the selected peptides observed with increasing the concentration of hydrochloric acid is mainly due to the effect of lowering the value of the apparent pH of the hydro-organic mobile phase.

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REFERENCES

1. C.T. Mant, R.S. Hodges, "HPLC of Peptides" in HPLC of Biological Macromolecules, Methods and Applications, K.M. Gooding, F.E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

2. M.T.W. Hearn, "High Performance Liquid Chromatography of Peptides" in High Performance Liquid Chromatography - Advances and Perspectives, Cs. Horváth, ed., Academic Press, New York, 1983, pp. 87-155.

3. A. Nahum, Cs. Horváth, J. Chromatogr., 203: 53-63 (1981)

4. K. E. Bij, Cs. Horváth, W.R. Melander, A. Nahum, J. Chromatogr., 203: 65-84 (1981)

5. H. Hagestam Freiser, K.M. Gooding, BioChromatogr., 2: 186-189 (1987)

6. D. Guo, C.T. Mant, R.S. Hodges, J. Chromatogr., 386: 205-222 (1987)

7. W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, M.T.W. Hearn, Science, 200: 1168-1170 (1978)

8. W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, M.T.W. Hearn, .J. Chromatogr. 153: 391-398 (1978)

9. W.S. Hancock, C.A. Bishop, L.J. Meyer, D.R.K. Harding, M.T.W. Hearn, J. Chromatogr., 161: 291-298 (1978)

10. W.S. Hancock, C.A. Bishop, J.E. Battersby, D.R.K. Harding, M.T.W. Hearn, J. Chromatogr. 168: 377-384 (1979)

11. W.M.M. Schaaper, D. Voskamp, C. Olieman, J. Chromatogr., 195: 181-186 (1980)

12. C.T. Mant, R.S. Hodges, "The Effect of Anionic Reagents on Peptide Retention in Reversed Phase Chromatography", in HPLC of Peptides and Proteins: Separation, Analysis and Conformation, C.T. Mant, R.S. Hodges, eds., CRC Press, Boca Raton, 1990, pp. 327-341.

13. M. J. O'Hare, E. Nice, J. Chromatogr. 171: 209-226 (1979)

14. P.M. Young, T.E. Wheat, J. Chromatogr. 512: 273-281 (1990)

15. G. Zundel, "Easily Polarizable Hydrogen Bonds", in The Hydrogen Bond, P. Schuster, G. Zundel, C. Sandorfy, eds., North-Holland Publishing Company, Amsterdam, 1976, pp. 750-753

16. B. Grego, F. Lambrou, M.T.W. Hearn, J. Chromatogr. 266: 89-103 (1983)

17. A.J. Banes, G. W. Link, L.R. Snyder, J. Chromatogr. 326: 419-431 (1985)

18. D.E.C. Corbridge "Phosphorous: An Outline of its Chemistry, Biochemistry and Technology", Elsevier, Amsterdam, 1990, pp. 959-971

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