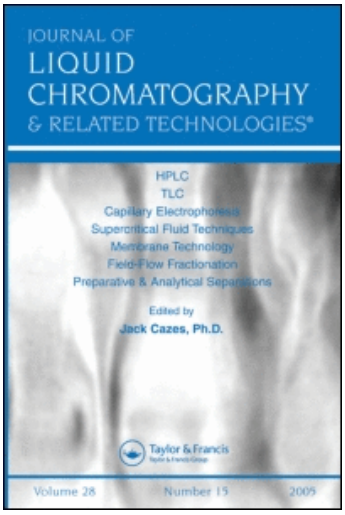

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Micellar HPLC: Investigation of the Retention of Positively Charged Peptides Using Cationic Micellar Mobile Phases

Thomas A. Walker[a]
[a] Hoechst Marion Roussel, Inc., Kansas City, MO

To cite this Article Walker, Thomas A.(1996) 'Micellar HPLC: Investigation of the Retention of Positively Charged Peptides Using Cationic Micellar Mobile Phases', Journal of Liquid Chromatography & Related Technologies, 19: 11, 1715 — 1727
To link to this Article: DOI: 10.1080/10826079608013999
URL: http://dx.doi.org/10.1080/10826079608013999

J. LIQ. CHROM. & REL. TECHNOL., 19(11), 1715-1727 (1996)

MICELLAR HPLC: INVESTIGATION OF THE RETENTION OF POSITIVELY CHARGED PEPTIDES USING CATIONIC MICELLAR MOBILE PHASES

Thomas A. Walker

Hoechst Marion Roussel, Inc.
P. O. Box 9627
Kansas City, MO 64134

ABSTRACT

Many new and complex molecules are being investigated as potential drug candidates. Conventional analytical methodologies may not be suitable for determining the purity, identity, and degradation of these complex molecules. Therefore, new analytical techniques must be developed that address and overcome these problems. Micellar liquid chromatography is such a technique.

Micellar liquid chromatography (MLC) is a technique where a micellar agent is added to a mobile phase that contains a buffer and a small amount of organic modifier. Several advantages are apparent with MLC when compared to reversed-phase liquid chromatography. MLC uses a much lower amount of organic modifier and is therefore less toxic, MLC does not denature peptides and proteins as does RPLC, and gradient MLC is done without the need for long column re-equilibration. In this study, various mobile phase variables were studied to determine the effect that each had on peptide retention.

The variables studied include: the concentration of micellar agent, mobile phase ionic strength, concentration of buffer, concentration of organic modifier and mobile phase pH. The results that were obtained are discussed.

INTRODUCTION

The separation and quantitation of complex molecules has become a very important part of analytical chemistry. Many of these complex molecules are being synthesized as potential new drug candidates. Several different research techniques are being used to identify and develop new drug candidates, including: biotechnology, molecular modeling, and natural product discovery. In many of these cases, the drug candidates are large, complex molecules that are difficult to assay for purity using conventional analytical methodologies. Therefore, new analytical strategies and techniques are required to determine the purity of the drug substance, to identify any impurities or degradation products, and to fully characterize the new chemical entity.

Several separation schemes have been shown to be useful for separating complex molecules and include: high performance liquid chromatography (HPLC), and capillary zone electrophoresis (CE) and, to a much lesser extent, gas chromatography, and supercritical fluid chromatography. Problems have been associated with these separation techniques and, although each holds promise, none have been found to be acceptable for the routine analysis for all types of complex molecules.

Capillary zone electrophoresis (CE) has shown great promise for the separation of various types and sizes of molecules in the biological sciences.[1] In the area of peptide and protein separations, CE can provide analytical chemistry with some very interesting and potentially outstanding separations. These types of analytes can be separated based on charge differences at a given pH.

A drawback to this strategy is that proteins may not be stable in low pH buffers. Another problem is that increased temperatures may result from Joule heating which can denature proteins and give inaccurate purity information.[2] A reliable, consistent injection system is also required before CE will become as routinely used as HPLC. This has been a major stumbling block for the use of CE in QA/QC labs, as has the cost of the instrumentation. Most QA/QC labs already have HPLC instrumentation in place and the addition of a CE unit may be cost prohibitive.

Ion exchange chromatography has been successfully employed for the separation of peptides.[3,4] Ion exchange columns, however, are much less efficient than reversed-phase columns and may not provide the kind of resolution that would be required to separate similar peptides.

An alternative to these analytical techniques would be micellar HPLC (MLC). The advantages of using MLC in place of reversed-phase or ion exchange chromatography are extensive when protein separations are being investigated. Reversed-phase HPLC (RPLC) requires the use of high organic modifier concentrations. This may denature the peptides and prevent complete resolution of the analyte peak of interest and possible impurities. Silica based columns are typically used for these separation and are limited to a pH range of 2.0-7.0. In some cases, a higher pH would provide a better separation for the analyte of interest. A polymeric-based column may be used to overcome the pH limitation of the silica-based columns; however, the efficiency of polymer columns is significantly less than that of silica-based columns.

Several interesting separations have been accomplished using MLC. Cline Love and co-workers[5-7] reported the direct injection of serum and urine into a reversed-phase column with no protein precipitation or pressure build-up problems. This method was used for therapeutic drug monitoring without the requirement of sample cleanup prior to injection. MLC has been shown to be useful for the separation of amino acids and peptides,[8] and proteins.[9] One study found that small changes in the concentration of surfactant produced tremendous changes in the retention of different proteins.[9] Other protein separations that are being pursued include one in which the biological activity of the protein is maintained.[10,11]

The purpose of this research was to determine the effects that each mobile phase variable had on peptide retention and resolution using micellar liquid chromatography. The mobile phase variables that were studied include: the concentration of micellar agent, mobile phase ionic strength, concentration of buffer, concentration of organic modifier and mobile phase pH. The results from these studies are discussed.

EXPERIMENTAL

Reagents and Instrumentation

All of the peptides used in this study were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Phosphoric acid, sodium monobasic

phosphate, and sodium hydroxide were obtained from Mallinckrodt (Paris, KY, USA). Hexadecyltrimethylammonium bromide was purchased from J. T. Baker (Phillipsburg, NJ, USA). HPLC grade water was obtained by passing de-ionized water through a Nanopure II water purification system (Barnstead, Dubuque, IA, USA).

The instrumentation consisted of a Spectra Physics P4000 Quaternary Pump, Spectra Physics AS3000 Autosampler, Spectra Physics Spectra UV1000 detector, and Spectra Physics ISM100 (Fremont, CA, U.S.A.). The Zorbax Rx C-18 column (4.6 x 250 mm, 5 μm) was purchased from Mac-Mod Analytical (Chadds Ford, PA, USA).

Procedures

The peptide samples were prepared at a concentration of 1 mg/g of HPLC-grade water. A flowrate of 1.0 mL/min. was used for all separations along with UV detection at 220 nm, an injection volume of 20 μL and a column temperature of 30 ^{o}C.

RESULTS AND DISCUSSION

Micellar liquid chromatography (MLC) provides a very convenient and reproducible route for separating peptides without the problems associated with CE, RPLC and ion exchange chromatography. MLC mobile phases consist of a surfactant, a buffer, and a low concentration of organic modifier. High column efficiencies are achieved since silica-based reversed-phase columns are used. A major advantage to MLC is that the mobile phases contain a much lower concentration of organic modifier than a reversed phase system and are therefore less toxic.

The surfactants used in MLC consist of two portions that contain distinctly different properties: a polar head group and a hydrocarbon tail. These properties allow the surfactant to adsorb at interfaces (stationary phase) where both the hydrophobic and hydrophilic character can be satisfied. The formation of micelles is the result of opposing forces– hydrophilic and hydrophobic. When the critical micelle concentration is achieved, the surfactant molecules arrange in such a way that the hydrophobic tails are oriented toward the center of the aggregate and the polar heads point outward.[12] The repulsion between the polar head groups is the controlling force that determines the size and shape of the micelles.

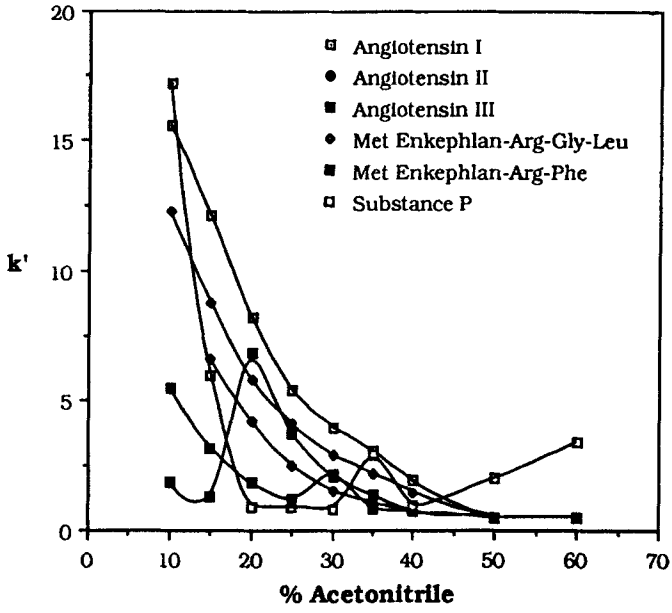

Figure 1. Effect of Organic Modifier Concentration on Peptide Retention. Mobile phase: 5 mM $HDTMA^+Br^-$, 15 mM H_3PO_4, pH 7.0, CH_3CN.

The separation mechanism in MLC is similar to RPLC in that the primary equilibrium of the analyte is between the mobile phase and the stationary phase. In MLC a secondary equilibrium is also involved in the separation. This equilibrium is the partitioning of the analyte between the mobile phase and the micelles.[12,13]

Various mobile phase parameters will have an effect on the retention and separation of organic analytes such as peptides. The parameters that were studied include: concentration of surfactant, buffer concentration, mobile phase ionic strength, concentration of organic modifier and mobile phase pH.

Effect of Organic Modifier Concentration

The amount of organic modifier present in the mobile phase will have an effect on analyte retention. Khaledi and co-workers[14] have shown that elution strength increased with an increase in the organic solvent concentration. A corresponding enhancement in the separation selectivity was also observed. The selectivity enhancement was found to occur systematically and was observed for

a large number of ionic and nonionic compounds with different functional groups, and also for two different surfactants, one anionic and one cationic. The selectivity enhancement was credited to competing partitioning equilibria in micellar HPLC systems and/or to the characteristics of micelles to compartmentalize solutes and organic solvents.[14]

Some concern has been expressed that micellar mobile phases would act like a hydro-organic system at higher concentrations of organic modifier. This, however, was shown not to be the case. It has been demonstrated that a micellar eluent that contains up to 20% isopropanol does not change to a hydro-organic system.[8] The addition of an organic modifier actually enhances the solvent strength and selectivity for some ionic and nonionic analytes. Retention characteristics for a solvent-water-micellar system were also found to be similar to a purely aqueous micellar eluent.[15,16] It was concluded, from these studies, that the micelle influences the role of an organic modifier in the mobile phase.

Figure 1 shows the effect on peptide retention when the amount of acetonitrile added to the mobile phase was changed. When the concentration of acetonitrile was less than 10%, retention of the peptides was extremely high. It was found that the retention of the peptides generally decreased with increasing concentrations of acetonitrile. One peptide, substance P, was found to increase in retention. This increase in retention is most likely attributable to changes in interactions between the micelles and the solvent.[8] The organic modifier concentration must be chosen such that the peptides are resolved yet retention is not excessive.

Effect of Micellar Concentration

When the concentration of a micellar agent was increased in the mobile phase, a corresponding decrease in analyte retention was usually observed.[17] The rate at which the retention of the analyte changes varies with the charge and hydrophobicity of solutes as well as the length of the alkyl chain, charge and concentration of the micelles.[18] A study done by Bailey and Cassidy[19] showed that the efficiency of the micellar system improved for hydrophobic analytes but not for polar analytes as the micellar concentration was increased.

Figure 2 shows how the concentration of hexadecyltrimethylammonium bromide (HDTMABr) influenced peptide retention. As the mobile phase concentration of HDTMABr was increased, peptide retention was found to decrease. This would be expected since at low concentrations of micellar agent, the chromatographic system resembles conventional reversed-phase LC. As the concentration of micellar agent is increased, the number of micelles in the system increases and binding between the analyte and the micelle increases.[20]

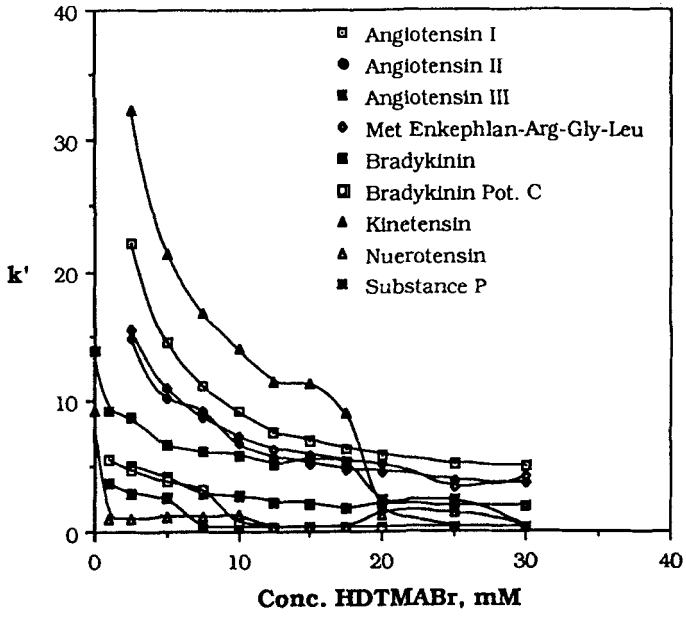

Figure 2. Effect of HDTMABr Concentration on Peptide Retention. Mobile phase: $HDTMA^{+}Br^{-}$, 15 mM H_3PO_4, pH 7.0, 20% CH_3CN.

Changes in elution order were observed and are due to differences in the binding constants of the micelle and the analyte. Selectivity between analytes may change due to the contribution of electrostatic and hydrophobic interactions, which is dependent on the structure of the compound. Selectivity changes have also been observed for diverse pairs of zwitterionic amino acids and peptides with changing micellar concentrations.[21]

The solvent strength of the mobile phase increased at higher concentrations of micellar agent. However, this increase has a negative effect on the efficiency of the chromatographic system.[20] Therefore, care must be taken when choosing an appropriate amount of micellar agent for a desired separation.

In the case of the peptides in this study, a concentration of 5.0 mM HDTMABr appears to provide the best compromise between retention, selectivity, and efficiency.

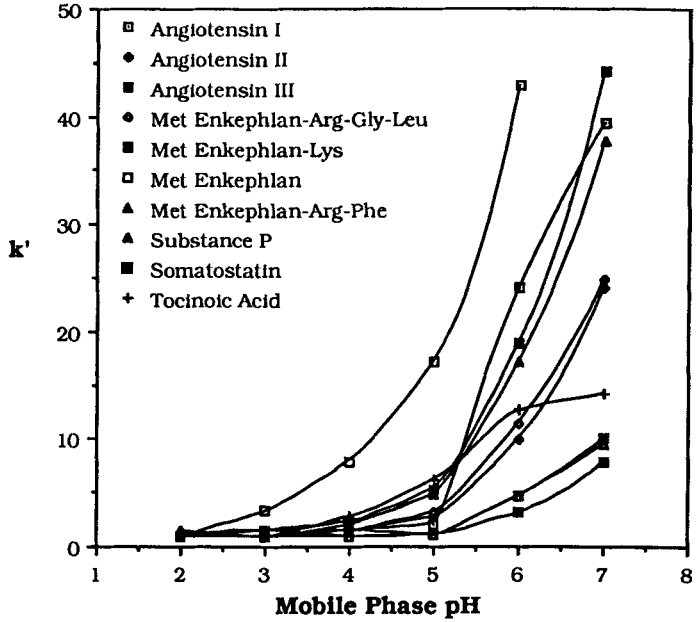

Figure 3. Effect of Mobile Phase pH on Peptide Retention. Mobile phase: 5 mM $HDTMA^{+}Br^{-}$, 15 mM H_3PO_4, 20% CH_3CN.

Effect of Mobile Phase pH

The micellar mobile phase pH will have a dramatic effect on the retention of weak organic acids and bases. Partition coefficients for the micelle-analyte interactions are different for the associated and unassociated forms.

Several studies have shown that small changes in the mobile phase pH will have an effect on retention especially when the mobile phase pH is close to the analyte's pK_a value.[22-24]

Adsorption of anionic surfactant monomers on the surface of a C_{18} stationary phase cause protonated organic bases to be retained for a longer period of time than the neutral free-base form due to electrostatic attraction.[20] Research has also shown that the dependence of k' on pH at a constant concentration of micellar agent is sigmoidal if there is no electrostatic repulsion between any of the acid-base forms and surfactant molecules.[25]

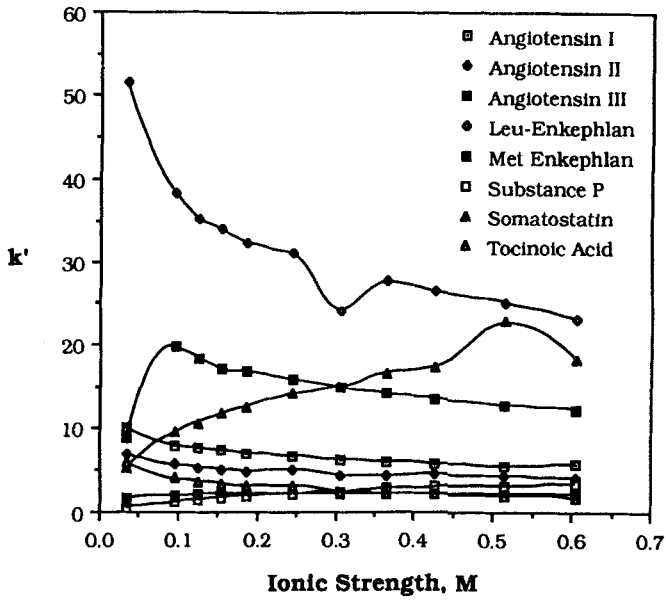

Figure 4. Effect of Mobile Phase Ionic Strength on Peptide Retention. Mobile phase: 5 mM HDTMA $^{+}Br^{-}$, H_3PO_4, pH 7.0, 20% CH_3CN.

The retention of the peptides in this study were found to decrease when the mobile phase pH was lowered (Figure 3). It is interesting to note that this is the opposite of what would be expected if the micellar agent was anionic (sodium dodecyl sulfate) rather than the cationic HDTMABr. Rodgers and Khaledi[26] showed that amino acids increased in retention as the mobile phase pH was lowered from 5.5 to 2.5 when an anionic surfactant, sodium dodecyl sulfate (SDS), was used. This was attributable to electrostatic repulsion between the solute and surfactant at zwitterionic conditions. It was also shown that the retention of amino acids, using nonionic micelles, pass through a retention minima at zwitterionic pH conditions. In both cases, analyte retention decreased with increasing anionic micelle concentration.

The cationic HDTMABr surfactant used in this study repels the positively charged peptides which leads to lower retention times. When the peptides are zwitterionic, interactions between the peptide and the cationic HDTMABr may take place thus leading to higher retentions. Therefore, for the peptides to be retained using a cationic surfactant, a higher mobile phase pH is required.

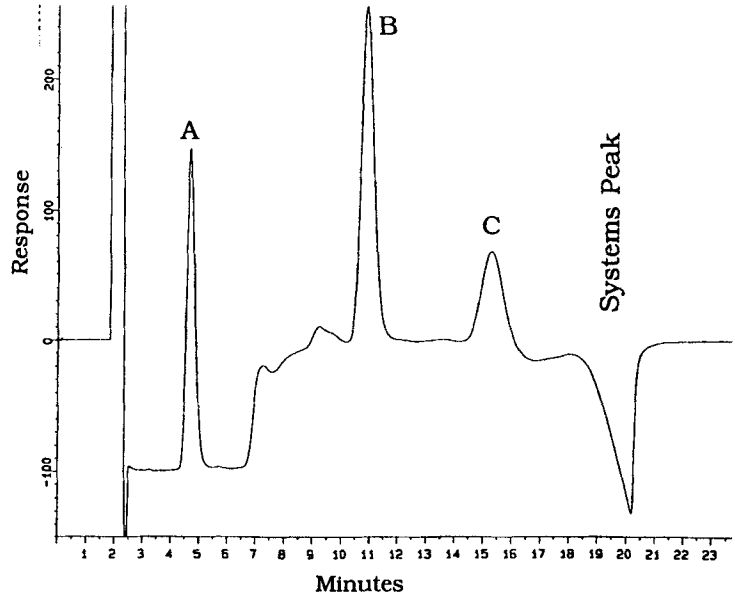

Figure 5. The separation of Angiotensin I, II and III by micellar liquid chromatography. Mobile phase: 5 mM HDTMA $^{+}Br^{-}$, 15 mM H_3PO_4, pH 7.0, 20% CH_3CN. A) Angiotensin III, B) Angiotensin II, C) Angiotensin I.

Effect of Mobile Phase Ionic Strength

In micellar liquid chromatography, electrostatic interactions are involved between a charged analyte and the micelle in the diffuse secondary layer while hydrophobic interactions take place in the hydrophobic inner portion of the micelle. Armstrong and Stine[27] have shown that the thickness of the double layer decreases with increasing ionic strength, which allows hydrophobic interactions to take place between the analyte and the micelle.

Anti-binding analytes (compounds that are strongly excluded or repelled from a micelle) have been found to have increased retention with higher ionic strength mobile phases.[20] For the transition from anti-binding to non-binding to binding to occur, the analyte ion must have enough hydrophobic character to associate with the non-polar portion of the micelle, overcoming electrostatic repulsion. Bromophenol blue has been shown to change from an anti-binding to a binding analyte with a corresponding increase in retention using an SDS mobile phase with 0.02 M NaCl added.[27]

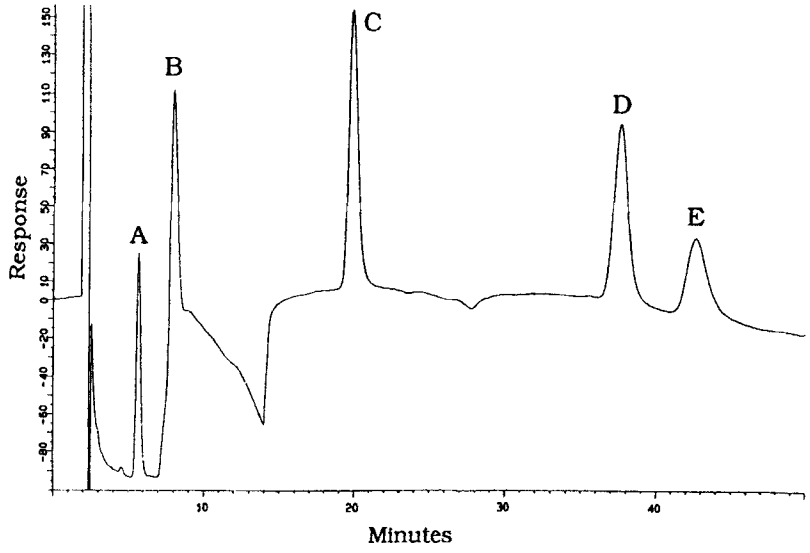

Figure 6. The separation of Enkephalins by micellar liquid chromatography. Mobile phase: 5 mM HDTMA$^+$Br$^-$, 15 mM H_3PO_4, pH 6.0, 20% CH_3CN. A) Met-Enkephalin-Arg-Gly-Leu, B) Met-Enkephalin-Arg-Phe, C) Derm-Enkephalin, D) Leu-Enkephalin, E) Met-Enkephalin.

Several changes were observed for the retention of the peptides when the mobile phase ionic strength was increased (Figure 4). Some of the peptides showed a reduction in retention, several did not show much change in retention and some showed a large increase in retention. The peptides that showed an increase in retention with increasing ionic strength (e.g., somatostatin) are changing from an anti-binding to a binding character, whereas the peptides that are decreasing in retention may be considered to have binding characteristics (e.g., Leu-enkephalin). The peptides that do not change in retention may be considered to have non-binding character. Interesting changes in selectivity and elution order can take place with different ionic strength mobile phases. However, for the separation of the different peptides to take place, lower ionic strength mobile phases are desirable.

Separations

The separation of a mixture of different peptides was accomplished. Figure 5 shows the separation of Angiotensin I, II and III while Figure 6 shows the separation for several Enkephalins. The Angiotensin samples were better

resolved at a mobile phase pH of 7.0 where the peptides were more highly retained, whereas the Enkephalins had a better separation at pH 6.0. At pH 7.0, some of the Enkephalins had extremely long retention times and were not observed after 90 minutes. Therefore, the pH of the mobile phase was lowered so that the Enkephalins would elute in a reasonable amount of time. Overall, the peptides were well resolved and the separations for the complex mixtures were acceptable.

CONCLUSIONS

The use of micellar mobile phases for the separation of peptides was studied. The retention of the different peptides were found to be affected by different mobile phase parameters. The various parameters were identified and studied. It was found that the use of a micellar mobile phase for the separation of short to medium chain peptides is possible. Further studies are ongoing to determine how other types of micellar agents will affect the retention and separation of peptides.

REFERENCES

1. B. L. Karger, A. S. Cohen, A. Guttman, J. Chromatogr., **492**, 585 (1989).

2. R. M. McCormick, Anal. Chem., **60**, 2322 (1988).

3. P. Mychack, J. R. Benson, LC-GC, **4**, 462 (1986).

4. R. A. Bradshaw, O. J. Bates, J. R. Benson, J. Chromatogr., **187**, 27 (1980).

5. F. J. DeLuccia, M. Arunyanart, L. J. Cline Love, Anal. Chem., **57**, 1564 (1985).

6. M. Arunyanart, L. J. Cline Love, J. Chromatogr., **342**, 293 (1985).

7. F. J. DeLuccia, M. Arunyanart, P. Yarmchuk, R. Weinberger, L. J. Cline Love, LC Magazine, **3**, 794 (1985).

8. A. S. Kord, M. G. Khaledi, J. Chromatogr., **631**, 125 (1993).

9. R. A. Barford, B. J. Sliwinski, Anal. Chem., **56**, 1554 (1984).

10. K. Ohbu, N. Jona, N. Miyajima, N. Mizushima, I. Kashiwa, Yakagaku, **29**, 866 (1980).

11. T. Asakura, K. Adachi, E. Schwartz, J. Biol. Chem., **253**, 6423 (1978).

12. D. W. Armstrong, Separation and Purification Methods, **14(2)**, 213 (1985).

13. J. G. Dorsey, M. G. Khaledi, J. S. Landy and J. L. Lin, J. Chromatogr., **316**, 183 (1984).

14. M. G. Khaledi, J. K. Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

15. M. G. Khaledi, E. Peuler, J. Ngeh-Ngwainbi, Anal. Chem., **59**, 2738 (1987).

16. M. G. Khaledi, Anal. Chem., **60**, 876 (1988).

17. D. W. Armstrong, G. Y. Stine, Anal. Chem., **55**, 2317 (1983).

18. A. S. Kord, M. G. Khaledi, Anal. Chem., **64**, 1894 (1992).

19. R. Bailey, R. M. Cassidy, Anal. Chem., **64**, 2277 (1992).

20. M. J. M. Hernandez, M. C. G. Alvarez-Coque, Analyst, **117**, 831 (1992).

21. M. G. Khaledi, J. K Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

22. F. Palmisano, A. Guerrieri, P. G. Zambonin, T. R. I. Cataldi, Anal. Chem., **61**, 946 (1989).

23. L. J. Cline Love, J. Fett, J. Pharm. Biomed. Anal., **9**, 323 (1991).

24. J. Haginaka, J. Wakai, H. Yasuda, J. Chromatogr., **488**, 341 (1989).

25. M. Arunyanart, L. J. Cline Love, Anal. Chem., **57**, 2837 (1985).

26. A. H. Rodgers, M. G. Khaledi, Anal. Chem., **66**, 327 (1994).

27. D. W. Armstrong, G. Y. Stine, J. Am. Chem. Soc., **105**, 6220 (1983).

Received December 20, 1995
Accepted January 10, 1996
Manuscript 4056