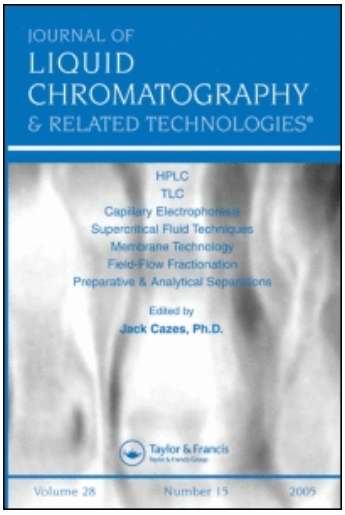

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LC-EC of Endorphins

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LC-EC OF ENDORPHINS

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ABSTRACT

Reversed phase liquid chromatography with electrochemical detection (LC-EC) was used to separate a series of endorphin standards. Chromatographic conditions were manipulated so that methionine- and leucine-enkephalin were clearly resolved from other endorphins of similar hydrophobicity using an isocratic mobile phase. The most significant factors affecting endorphin retention were the concentration and type of organic modifier in the isocratic mobile phase. Hydrodynamic voltammograms were performed for methionine- and leucine-enkephalin to assess their electroactivity. Both enkephalins were oxidized with a glassy carbon electrode only at high potentials (> +.90V vs Ag/AgCl). The effect of these high potentials on the sensitivity of electrochemical detection of endorphins was evaluated.

INTRODUCTION

The discovery of neuropeptides with opiate-like activity focused considerable interest on the isolation, characterization, and localization of these molecules. This research effort has attempted to elucidate the roles of endorphins in normal and disease states. Physiologic studies have implicated endogenous opiates in stress (1) and pain (2), memory and reward behavior (3), psychosis (1) and petit mal seizures (4). At the molecular level, the endorphins may act as neuromodulators (5,6) or in some cases, as neurotransmitters (5-7).

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To evaluate endorphin activities in physiological fluids and tissues, highly specific and sensitive techniques are required. Radioimmunoassay (RIA) has been used to quantify endorphins such as methionine-(ME) and leucine-(LE) enkephalin (8,9) but has been limited for other endorphins by the lack of highly specific antibodies (10,11). The cross-reactivity of the monoclonal antibody to β-endorphin (BE) that was prepared by Herz et al (12) demonstrates the difficulty in achieving highly specific antibodies for RIA of closely-related endorphins. In an effort to improve the specificity of endorphin assay, high performance liquid chromatography (LC) has been used to separate and identify endorphins (10,11,13-15). Most LC techniques use reversed phase chromatography and detect endorphin standards by far UV absorbance (14, 16). However, quantification of endorphins in brain samples is usually performed by collecting chromatographic fractions which are then analyzed by RIA for each endorphin of interest (10,11,17). Although these procedures are time-consuming, expensive and introduce additional experimental variables, they are necessary because the concentration of endorphins in most physiologic samples is below the limits of detection by far UV spectroscopy.

An electrochemical detector (EC), which can measure compounds on the basis of their electroactivity, has been used with LC for the quantification of catechol- and indol-amines in the 10^{-11} gram range from small biological samples (18,19). The electroactivity of ME and LE was originally demonstrated by Meek et al (20) in their study of enkephalin catabolism. Although they showed that ME and LE could be detected electrochemically in 10^{-8} gram quantities, they did not develop LC-EC as an assay technique for endorphins. Using differential pulse voltammetry, Bennett et al (21) reported the electroactivity of other neuropeptides, such as vasopressin and somatostatin, and some amino acids (tyrosine, tryptophan and cysteine). In this report, we have developed conditions for the separation and detection of a series of endorphins standards using LC-EC. A preliminary report of this work has been presented (22).

MATERIALS AND METHODS

ME, LE, [D-Ala^2]-leucine enkephalinamide (LEA), human β-endorphin and glycylglycine were purchased from Sigma Chemical Company, St. Louis, Mo. Dynorphin 1-6 and 1-17, pro-enkephalin, β-neo-endorphin, α-neo-endorphin 1-8 and 1-10, and α-endorphin were obtained from Bachem, Torrance, CA. Reagents included HPLC grade potassium dihydrogen phosphate, ammonium acetate, phosphoric acid (85%), methanol, tetrahydrofuran (THF) and acetonitrile (Fisher Scientific, Pittsburgh, PA). Ultrex acetic acid was from J. T. Baker Co., Phillipsburg, N.J. Water for LC mobile phases was prepared by adding activated charcoal (Sigma) to fresh glass distilled water. After standing overnight, the water was filtered through a 0.2μm or 0.45μm Nylon-66 filter (Rainin Instrument Co., Inc; Woburn, MA) and degassed.

The chromatographic system consisted of Model 6000A pumps (Waters Associates, Milford, MA) and a Model U6K sample injector (Waters Associates). The reversed phase columns included a 5μm Biophase ODS (250 x 4.6mm, Bioanalytical Systems, West Lafayette, IN) protected by a C_{18}/Corasil guard column (Waters), a 5μm Ultrasphere-Octyl (150 x 4.6mm, Altex Scientific, Inc., Berkeley, CA) and a 10μm μBondapak (300 x 3.9mm, Waters). A 10μm Aquapore RP-300 column (250 x 4.6mm, Brownlee Labs, Santa Clara, CA) and a 5μm Sepralyte Octyl column (250 x 4.6mm, Analytichem International, Harbor City, CA) were protected by a Soft Seal guard column (Applied Science Laboratories, Inc., State College, PA) packed with a 10μm Ultrapack-Octyl (Altex). An LC-4 or LC-4B amperometric detector with a TL-5 glassy carbon electrode (Bioanalytical Systems) was set at a potential of +1.05V referenced to a Ag/AgCl electrode unless indicated otherwise. A Topaz Line 2 power conditioner (Topaz Electronics Div., San Diego, CA) was used with the LC-4 and LC-4B detector for all experiments. Only isocratic mobile phases were used and their compositions are described in the figure legends. All mobile phases were filtered through a 0.2μm or 0.45μm Nylon-66 filter and degassed before use. Steel solvent reservoir filters were omitted from mobile phase reservoirs. Where indicated,

the mobile phase reservoir was suspended in a circulating water bath at 27°C. A flow rate of 1.0 ml/min was used for all separations.

Endorphin standards were dissolved in a 30mM KH_2PO_4-27.5μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid). Aliquots were lyophilized and stored dessicated at -20°C. Prior to chromatography, the endorphin standards were dissolved in an appropriate volume of 25mM KH_2PO_4-27.5μM glycylglycine, pH 2.3 and stored for approximately one month at -20°C.

RESULTS

Various chromatographic parameters were manipulated in an effort to optimize ME and LE resolution. These parameters included various analytical reversed phase columns and mobile phase components. Factors which affected the performance and sensitivity of the electrochemical detector were also examined.

Chromatographic Conditions

1. Columns

The reversed phase columns described above were used to separate ME and LE from other endorphin standards. A representative chromatogram is shown in Figure 1. The longest endorphin retention times were obtained with the 5μm Ultrasphere-Octyl and the 5μm Biophase ODS columns. Intermediate retention times were obtained with the 5μm Sepralyte Octyl. The least retention of the endorphins was found with the 10μm μBondapak and the 10μm Aquapore RP-300; there was no significant difference in the chromatographic behavior of the endorphins on either column.

2. Mobile Phase

The effect of changes in the mobile phase composition on the chromatographic behavior of endorphins was investigated. Components of the mobile phase that were examined included buffers, pH, organic modifiers and ion-pairing reagents.

A. Buffer. Several buffers were used for the chromatography of ME, LEA and LE including ammonium acetate, potassium phosphate, sodium phosphate and triethylamine phosphate. Each of these buffers could be used for endorphin chromatography with appropriate adjust-

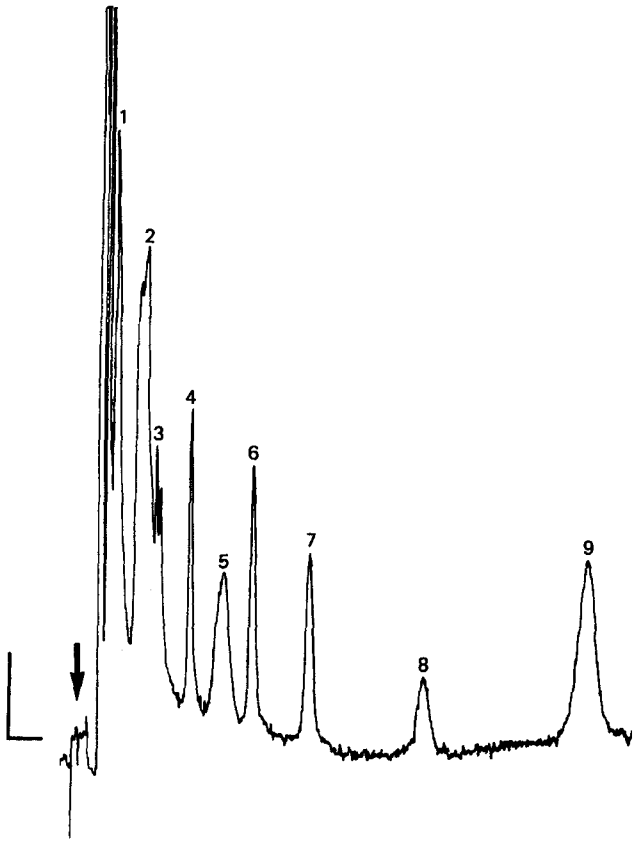

FIGURE 1

Isocratic elution of endorphin standards. Column = Aquapore RP-300; Mobile phase = 128 ml [50mM KH_2PO_4 -55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) containing 29% acetonitrile] diluted to 250 ml with water; Mobile phase temperature = 27°C; Flow Rate = 1.0ml/min.; Applied Potential = +1.05V vs Ag/AgCl; Reference Bars: Ordinate = 0.2nA and Abscissa = 15 min. Peak 1 = 10ng α-neo-endorphin 1-8, 2 = 10ng α-neo-endorphin 1-10, 3 = 10ng dynorphin 1-6, 4 = 6.6ng ME, 5 = 10ng β-neo-endorphin, 6 = 7.34ng LEA, 7 = 6.6ng LE, 8 = 25ng α-endorphin, 9 = 20ng pro-enkephalin.

ments in concentration. The use of a formic acid - pyridine buffer in the mobile phase was unacceptable because it caused a substantial decrease in the sensitivity of the working electrode.

The effect of other salts on endorphin retention was examined by adding potassium chloride or potassium perchlorate to the phosphate buffer-acetonitrile mobile phase. In general, increases in the potassium chloride concentration (0.25mM to 2.5mM, final concentrations) decreased the retention times of ME and LE. Increases in potassium perchlorate concentrations (0.25mM to 2.5mM, final concentrations) had little effect on endorphin retention times.

B. pH. Mobile phases ranging in pH from 2.0 to 7.5 were used to chromatograph ME and LE. Although a decrease in pH caused an increase in ME and LE retention, small changes in pH did not substantially alter the chromatographic behavior of ME or LE. A pH of 2.3 was chosen for the phosphate buffer-acetonitrile mobile phase because retention of the early eluting endorphins (α-neo-endorphin 1-8 and 1-10) was improved at low pH.

C. Organic Modifier. Methanol, acetonitrile, tetrahydrofuran and propanol were used as organic modifiers in the isocratic mobile phase. For each organic modifier, the endorphins could be chromatographed only within a limited concentration range. Organic modifier concentrations above or below this range caused neither retention nor elution of the endorphins. (i.e. using the Aquapore 300 column, the K' of β-neo-endorphin when using 10% acetonitrile in the mobile phase was > 20 but with 14.8% CH_3CN, the K' was reduced to 6.4). This effect was independent of the type of column or buffer used for endorphin chromatography.

It was not possible to substitute one organic modifier for another solely by maintaining an equivalent polarity of the aqueous-organic mobile phase. Table 1 shows that the accuracy of K' predictions for ME and LE from the calculated polarity of the mobile phase was dependent on the type of organic modifier. A comparison of calculated K' ratios vs K' ratios obtained experimentally with aqueous-acetonitrile, aqueous-methanol and aqueous-acetonitrile-tetrahydrofuran mobile phases indicates that ME and LE are not eluted

TABLE 1

Comparison of Experimentally Determined (Exp.) K' Ratios to Calculated (Calc) K' Ratios

Solvent Polarities P_2' ; P_1' (Organic Modifier)	Calc. K' Ratios*	ME Exp. K' Ratios	ME % Calc./Exp.	LE Exp. K' Ratios	LE % Calc./Exp.
9.408 : 9.32 (CH_3CN only)	1.11	1.78	62	1.62	68.5
9.32 ; 9.14 (CH_3CN ; CH_3CN-THF)	1.23	1.17	105	1.04	118
9.14 ; 9.052 (CH_3CN-THF only)	1.11	1.19	93	1.20	92.5
9.052 ; 8.67 (CH_3CN-THF ; MeOH)	1.55	0.32	484	0.26	596
9.408 ; 8.67 (CH_3CN ; MeOH)	2.34	0.86	272	0.58	403

Column = 5µm Biophase ODS; Mobile phase = 10mM ammonium acetate, pH 4.25 (adjusted with glacial acetic acid) + varying amounts of organic modifiers to obtain the P' values indicated above. CH_3CN = acetonitrile; THF = tetrahydrofuran; MeOH = methanol.

K' ratios* calculated by the formula $\frac{K_2'}{K_1'} = 10^{(P_2' - P_1')\div 2}$ (23)

only on the basis of polarity considerations. Aqueous-acetonitrile mobile phases were more efficient in decreasing the K' of ME and LE than other aqueous-organic mobile phases of equivalent polarities.

Another significant factor for the resolution of ME and LE from other endorphins was the total salt concentration in the mobile phase versus the concentration of the organic modifier. Small changes in salt concentration below an optimal level caused a change in peak shape even if the concentration of organic modifier was sufficient to maintain an equivalent K'. The two endorphins most affected were β-neo-endorphin and α-neo-endorphin 1-10.

In Figure 1, the α-neo-endorphin 1-10 peak is split. A sharp single peak was obtained by increasing the final KH_2PO_4 concentration to 30mM in the mobile phase. Figure 2 shows the effect of small changes in the KH_2PO_4 and acetonitrile concentrations on the elution of ME and β-neo-endorphin. The peak shape of ME was essentially unchanged while the peak shape of β-neo-endorphin broadened.

D. Ion-pairing Reagents. Data obtained with trifluoroacetic acid and nonylamine indicated that the retention times of both ME and LE were similarly decreased with increasing ion-pairing reagent concentrations in the mobile phase. Increases in tetrabutylammonium hydrogen sulfate concentration caused small increases in the retention of ME and LE.

After examining the effect of various mobile phase components on endorphin chromatography, the composition of the isocratic mobile phase was adjusted so that the ME and LE peaks were clearly resolved from the other endorphin standards using the Aquapore RP-300 column. As shown in Figure 1, a mobile phase consisting of 128ml [50mM KH_2PO_4-55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) + 29% CH_3CN] diluted to 250ml with water, was warmed to 27°C in a circulating water bath and used for endorphin chromatography. This buffer was suitable as a mobile phase for endorphin chromatography using each of the reversed phase columns described above by making small adjustments in the phosphate or acetonitrile concentrations. Therefore, this mobile phase was used for most of these studies.

Glycylglycine was added to the mobile phase to prevent absorption of the endorphins to residual silanols on the column packing material (10). There was no change in the shape of the endorphin peaks with an increase in glycylglycine concentration in the mobile phase. The order of endorphin elution was α-neo-endorphin 1-8 followed by α-neo-endorphin 1-10, dynorphin 1-6, ME, β-neo-endorphin, LEA, LE, α-endorphin, and pro-enkephalin (Table 2). BE and dynorphin 1-17 was not eluted from the column with this mobile phase. The order of endorphin elution was different from that predicted by the summation of the retention coefficients of component amino acids as determined by Wilson et al (15) and Meek and Rossetti (25) (Table 2).

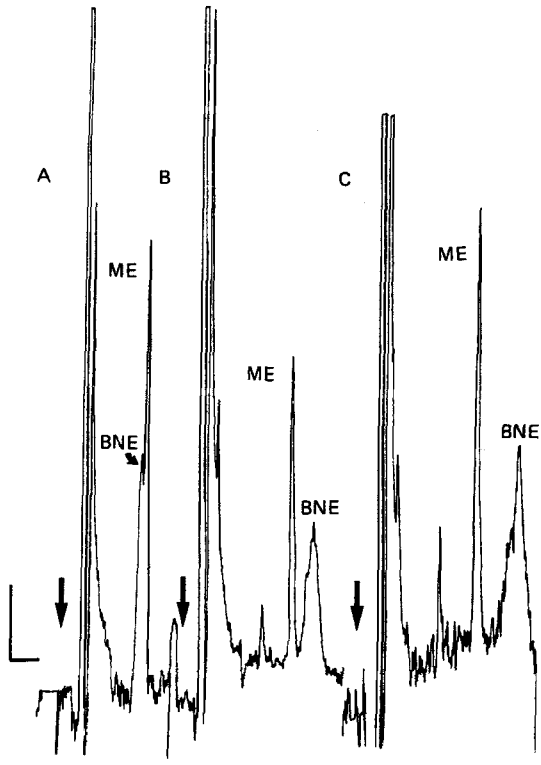

FIGURE 2

Effect of changes in mobile phase on ME and β-neo-endorphin (BNE) elution. Column = Aquapore RP-300; Oxidation Potential = +1.05V vs Ag/AgCl; Flow Rate = 1.0ml/min; Reference Bars: Ordinate = 0.1nA; Abscissa = 4 min.; arrow denotes injection.
A = 128 ml of 52mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 32% CH_3CN diluted to 250 ml with H_2O.
B = 128 ml of 50mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 29% CH_3CN diluted to 250 ml with H_2O.
C = 128 ml of 47mM KH_2PO_4-55μM glycylglycine, pH 2.3 containing 31% CH_3CN diluted to 250 ml with H_2O.

Electrochemistry

1. Hydrodynamic Voltammograms

Hydrodynamic voltammograms for ME, LE and LEA were performed with the mobile phase described in Figure 1. The curves in Figure 3 indicate that ME, LE and LEA are oxidized by the glassy carbon working electrode at potentials above +.90V referenced to Ag/AgCl. The

TABLE 2

Order of Endorphin Elution

Endorphin	This study	Meek & Rossetti (25)	Wilson et al (15)
α-neo-endorphin 1-8	1	4	6
α-neo-endorphin 1-10	2	5	4
dynorphin 1-6	3	2	2
ME	4	1	1
β-neo-endorphin	5	9	7
LEA	6	8	-
LE	7	3	3
α-endorphin	8	7	5
pro-enkephalin	9	6	8

The order of endorphin elution observed in this study was compared with the order calculated by the summation of the retention coefficients (25,15) of the amino acids contained in each endorphin. In this study, endorphins were chromatographed with either the μBondapak or Aquapore RP-300 reversed phase columns at a flow rate of 1.0 ml/min with an isocratic mobile phase consisting of 128 ml of [50mM KH_2PO_4 -55μM glycylglycine, pH 2.3 (adjusted with 85% phosphoric acid) containing 29% acetonitrile] diluted to 250 ml with water. The mobile phase reservoir was suspended in a circulating water bath at 27°C.

plateau region of the curve occurs at approximately +1.09V for LE and LEA. For ME, however, the peak height is still increasing with increasing oxidation potentials. Other endorphins, α-neo-endorphin 1-8 and 1-10, dynorphin 1-6, β-neo-endorphin, α-endorphin and pro-enkephalin were also electroactive at applied potentials above +.90V. A potential of +1.05V was chosen for these studies as a compromise between increasing peak height and increasing noise. (A chelator such as ethylenediamine-tetraacetic acid was not used in the mobile phase due to its oxidation at these potentials).

2. Standard Curves

The detector response was linear from 1 ng to 20 ng of ME, LE or LEA (the concentration range used for these studies).

3. Sensitivity

The most significant factor affecting the sensitivity of electrochemical detection of endorphins was the age of the working electrode.

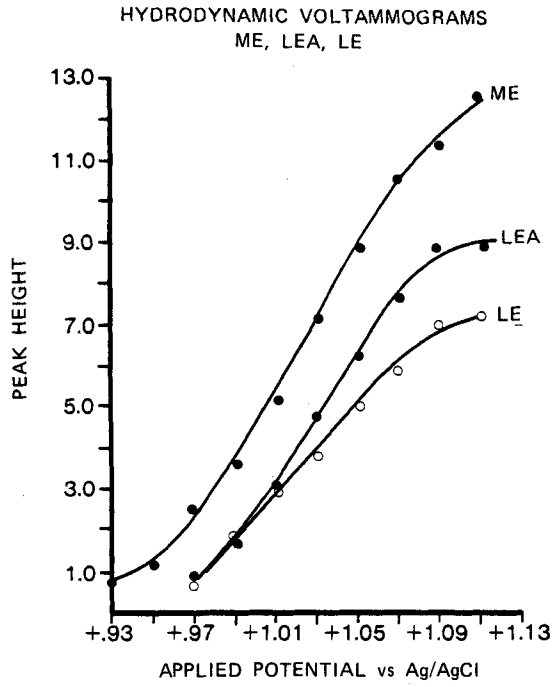

FIGURE 3

Chromatographic conditions as in Figure 1. (Peak height in centimeters; Applied potential in volts; Sensitivity = 2nA/V).

With a new TL-5 glassy carbon electrode (Bioanalytical Systems) approximately 300 picograms of ME (signal to noise = 3; 1.7nA/ng) were detected. The detection limit for LE using the same electrode was approximately 600 picograms. The sensitivity of the electrode gradually decreased with continued use at high potentials (>+1.0V). Repolishing the electrode restored some of the sensitivity but not to its original level. Another factor influencing the sensitivity of EC detection was the composition and pH of the mobile phase. Both ME and LE oxidized more readily in a mobile phase consisting of 10mM ammonium acetate (adjusted to pH 4.25 with glacial acetic acid) containing methanol with an optimal potential of +.99V referenced to Ag/AgCl. The use of a lower potential decreased the background current and baseline noise causing a small increase in sensitivity.

The chromatographic resolution of the endorphins, however, was improved when the phosphate-acetonitrile mobile phase was used.

Other factors aiding EC sensitivity included several minor modifications to the LC system. The mobile phase reservoir was suspended in a circulating water bath at a constant temperature (27°C) to minimize baseline shift. Stainless steel solvent reservoir filters were omitted from all mobile phase reservoirs to eliminate baseline noise due to oxidation of metallic contaminants (personal communication, Dr. Michael Joseph, MRC Clinical Research Centre, U.K.) A Topaz power conditioner was used to protect the electrochemical detector from aberrant power line fluctuations.

DISCUSSION

Several previous studies of endorphin chromatography have used gradient elution to separate and investigate the behavior of a series of endorphin standards on reversed phase columns (10, 14, 15, 24, 25). This study investigated the use of an isocratic mobile phase for the elution of endorphins. Using LC-EC, various chromatographic conditions were manipulated to separate ME and LE from other endorphins of similar hydrophobicity. The chromatographic behavior of the endorphins in response to certain changes in the composition of the isocratic mobile phase was different from the behavior that has been reported for gradient elutions (15, 25, 26). Alterations in the organic modifier and total salt concentration affected endorphin resolution dramatically, either by a change in K' or by a change in peak shape. The role of the total salt concentration in the mobile phase observed in this study for the isocratic resolution of endorphins, however, is in agreement with the results obtained with gradient elution (26). The most important factors for endorphin retention were the concentration and type of organic modifier in the mobile phase. As reported for ACTH-related peptides (26), the peptides used in this study were efficiently chromatographed only within a limited concentration range for each organic modifier. In addition, endorphin retention could not be predicted solely on the basis of polarity considerations when substituting different organic modifiers in the mobile phase (Table 1). Like somatostatin (27), selectivity was increased when acetonitrile was used as the organic

modifier instead of methanol. This is in contrast to what has been observed using gradient elution (26). Reversals in retention order were reported with the use of some organic modifiers but, for the most part, the retention times of the polypeptides were shifted according to the polarity of the solvent (26).

Recently, the elution characteristics of an extensive series of peptides have been investigated in an effort to correlate peptide retention with amino acid composition (15, 25). In both reports, large numbers of peptides (n=95-100) were used to determine the hydrophobicity or retention coefficients for each amino acid. An examination of predicted and actual retention times of peptides using gradient elution, showed a high degree of correlation (Meek and Rossetti (25), correlation coefficient = 0.98; Wilson et al (15) correlation coefficient = 0.83). However, this study demonstrates that these retention coefficients can not be used to predict the order of endorphin elution when using isocratic mobile phases (Table 2). The observation of differences in the chromatographic behavior of endorphins when comparing gradient elution and isocratic elution was not unexpected since some of the endorphins are particularly affected by changes in the mobile phase composition.

The specific aim of this study was to use LC-EC to separate and detect a series of endorphin standards. Therefore, various chromatographic parameters were investigated to develop conditions for the isocratic elution of endorphins. A simple phosphate buffer - acetonitrile isocratic mobile phase was used to separate endorphins on C_8 or C_{18} reversed phase columns. The oxidation characteristics of both ME and LE were examined by hydrodynamic voltammetry and the electroactivity of several other endorphins (α-neo-endorphin 1-8 and 1-10, dynorphin 1-6, β-neo-endorphin, α-endorphin and pro-enkephalin) was demonstrated. These data indicate that the specificity and and sensitivity of LC-EC may provide an efficient and inexpensive alternative to RIA for the assay of endorphins. Application of this technique to the study of brain samples is currently in progress.

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REFERENCES

1. Beddell, C. R., Lowe, L. A., and Wilkinson, S., Endogenous opioid peptides - The enkephalins and endorphins, Prog. Med. Chem., 17, 2, 1980.

2. Panerai, A. E., Martini, A., DeRosa A., Salerno, F., Di Giulio, A. M., and Mantegazza, P., Regulatory Peptides: From Molecular Biology to Function, Costa E. and Trabucchi, M., eds., Raven Press, New York, 1982, p. 139.

3. DeWied, D. and Jolles, J., Neuropeptides derived from pro-opiocortin; Behavioral, physiological, and neurochemical effects, Physiol. Reviews, 62, 976, 1982.

4. Dingledine, R., Possible mechanisms of enkephalin action on hippocampal CA1 pyramidal neurons, J. Neurosci., 1, 1022, 1981.

5. Miller, R. J., Peptides as neurotransmitters: Focus on the enkephalins and endorphins, Pharmac. Ther., 12, 73, 1981.

6. Nathanson, J. A., Neurosecretion and Brain Peptides, Martin, J. B., Reichlin, S., Bick, K. L., eds., Raven Press, New York, 1981, p. 599.

7. Sheehy, M., Schacter, M., Marsden, C. D., Parkes, J. D., Research Progress in Parkinson's Disease, Rose, F. C. and Capildeo, R., eds., Pilman Medical, Kent, 1981, p. 165.

8. Akil, H., Watson, S. J., Berger, P. A., Barchas, J. D., The Endorphins, (Adv. Biochem. Psychopharm., vol. 18), Costa, E., and Trabucchi, M., eds., Raven Press, New York, 1978, p. 125.

9. Przewlocki, R., Höllt, V., Kleber, G., Gramsch, C. H., Haarmann, I., and Herz, A., Long-term morphine treatment decreases endorphin levels in rat brain and pituitary, Br. Res., 174, 357, 1979.

10. Loeber, J. G., Verhoef, J., High-pressure liquid chromatography and radioimmunoassay for the specific and quantitative determination of endorphins and related peptides, Methods Enzymol., 73, 261, 1981.

11. Morris, H. R., Etienne, A. T., Dell, A., and Albuquerque, R., A rapid and specific method for the high resolution, purification and characterization of neuropeptides, J. Neurochem., 34, 574, 1980.

12. Herz, A., Gramsch, C., Höllt, V., Meo, T., Riethmuller, G., Characterization of a monoclonal β-endorphin antibody recognizing the N-terminus of opioid peptides, Life Sci., 31, 1721, 1982.

13. Currie, B. L., Chang, J.-K., Cooley, R., High performance liquid chromatography of enkephalin and endorphin peptide analogs, J. Liq. Chromatogr., 3, 513, 1980.

14. Davis, T. P., Schoemaker, H., Chen, A., Yamamura, H., High performance liquid chromatography of pharmacologically active amines and peptides in biological materials, Life Sci., 30, 971, 1982.

15. Wilson, K. J., Honegger, A., Stotzel, R. P., Hughes G. J., The behavior of peptides on reverse-phase supports during high-pressure liquid chromatography, Biochem. J., 199, 31, 1981.

16. Mousa, S., Mullet, D., Couri, D., Sensitive and specific high performance liquid chromatographic method for methionine and leucine enkephalins, Life Sci., 29, 61, 1981.

17. McDermott, J. R., Smith, A. I., Biggins, J. A., Chyad Al-Noaemi, M. and Edwardson, J. A., Characterization and determination of neuropeptides by high-performance liquid chromatography and radioimmunoassay, J. Chromatogr., 222, 371, 1981.

18. Kissinger, P. T., Bruntlett, C. S., and Shoup, R. E., Neurochemical applications of liquid chromatography with electrochemical detection, Life Sci., 28, 455, 1981.

19. Warsh, J. J., Chiu, A., Godse, D. D., Simultaneous determination of norepinephrine, dopamine and serotonin in rat brain regions by ion-pair liquid chromatography on octyl silane columns and amperometric detection, J. Chromatogr., 228, 131, 1982.

20. Meek, J. L., Yang, H. Y. T., Costa E., Enkephalin catabolism in vitro and in vivo, Neuropharmac., 16, 151, 1977.

21. Bennett, G. W., Brazell, M. P., Marsden, C. A., Electrochemistry of neuropeptides: A possible method for assay and in vivo detection, Life Sci., 29, 1001, 1981.

22. Fleming, L. H., Reynolds, N. C., and Stein, E. A., LCEC of opiate peptides: Separation and quantification from rat brain, Third Int. Symposium on LCEC and Voltammetry, 52, 1982.

23. Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatrophy, Second edition, Wiley, New York, 1979.

24. Meek, J. L., Prediction of peptide retention times in high pressure liquid chromatography on the basis of amino acid composition, Proc. Natl. Acad. Sci. USA., 77, 1632, 1980.

25. Meek, J. L., Rosetti, Z. L., Factors affecting retention and resolution of peptides in high-performance liquid chromatography, J. Chromatogr., 211, 15, 1981.

26. O'Hare, M. J. and Nice, E. C., Hydrophobic high-performance liquid chromatography of hormonal polypeptides and proteins on alkyl-silane-bonded silica, J. Chromatogr., 171, 209, 1979.

27. Abrahamsson, M., and Grőningsson, K., High-performance liquid chromatography of the tetradecapeptide somatostatin, J. Liq. Chromatogr., 3, 495, 1980.