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SEPARATION AND DETECTION OF CLOSELY RELATED ENDORPHINS BY LIQUID CHROMATOGRAPHY—ELECTROCHEMISTRY*

LYNDA H FLEMING* and NORMAN C REYNOLDS, Jr

Department of Neurology, University of Wisconsin Medical School, Milwaukee Clinical Campus, Mount Sinai Medical Center, P O Box 342, Milwaukee, WI 53201 (U S A)

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SUMMARY

The specific aim of this study was to investigate the selectivity of liquid chromatography—electrochemistry for minor variations in the structure of small endorphins Using isocratic mobile phases, chromatographic conditions were established for the separation of a series of closely related endorphins Hydrodynamic voltammetry showed that each peptide exhibited a characteristic oxidative behavior that was also reflected in peak current ratios Changes in a small moiety altered both the chromatographic behavior and electroactivity of these neuropeptides

INTRODUCTION

The discovery of increasing numbers of closely related neuropeptides has made the need for highly selective assay techniques more apparent Currently, the most selective and sensitive technique has used some form of liquid chromatographic (LC) fractionation step followed by radioimmunoassay (RIA) quantification [1—6]. This necessarily restricts the choices of solutions used for the chromatographic portion of the technique to those solutions which are volatile or compatible with the RIA analysis [3] The lack of commercially available antibodies to some of the neuropeptides has caused investigators to prepare and characterize their own antibodies. Differences in antibody specificity make it difficult to compare data between laboratories. The development of a technique in which a series of neuropeptides could be identified and

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quantified from a single sample, would eliminate the present need for multi-step time-consuming separate determinations

Some research efforts to develop a new assay technique for endorphins and other neuropeptides have focused on LC with electrochemical detection (ED) [7—11] Electrochemistry provides additional selectivity to the assay since only those compounds which are oxidizable or reducible at the applied potential will be detected Other advantages of using electrochemistry are its modest cost, both of instrumentation and reagents, and its versatility An LC—ED assay for oxytocin solutions was developed by White [8] Mousa and Couri [9] studied the metabolism of synthetic methionine-enkephalin (ME) by serum and brain aminopeptidases using LC—ED A column-switching LC—ED procedure was used to determine cholecystokinin tetrapeptide and octapeptide sulfate levels from rat brain [10]. This procedure was subsequently modified and used to assay ME and leucine-enkephalin (LE) from rat brain by LC—ED [11]

In a previous paper [12], we investigated conditions for the isocratic elution of a series of endorphins Factors which affected the performance of the electrochemical detector were also examined. The specific aim of this study is to investigate the selectivity of LC—ED for minor variations in endorphin structure

EXPERIMENTAL

The endorphins used in this study are listed in Table I with their abbreviations, amino acid compositions and suppliers Reagents included HPLC-grade potassium dihydrogen phosphate, phosphoric acid (85%), methanol and acetonitrile (Fisher Scientific, Pittsburgh, PA, U S A.). All water used 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 0.2-μm Nylon-66 filter (Rainin, Woburn, MA, U.S.A.) and degassed

The chromatographic system consisted of Waters Assoc (Milford, MA, U S A.) Model 6000A pumps, a Model U6K sample injector and a Bioanalytical Systems (West Lafayette, IN, U S A) LC-4, LC-4B or LC-4B/17 amperometric detector with a TL-5A or TL-5A dual glassy carbon working electrode A

TABLE I

ENDORPHIN STRUCTURE

Endorphin	Abbreviation	Amino acid composition	Supplier*
α Neo endorphin 1 8	αNE 1 8	Tyr Gly-Gly Phe Leu Arg-Lys Tyr	1
α Neo-endorphin 1 10	αNE 1 10	Tyr-Gly-Gly-Phe Leu Arg Lys Tyr Pro-Lys	1
Dynorphin 1 6	Dyn 1 6	Tyr Gly Gly Phe Leu Arg	1
β Neo-endorphin	βNE	Tyr Gly Gly-Phe Leu Arg Lys Tyr Pro	1
[D Ala^2]-leucine-enkephalinamide	LEA	Tyr D Ala-Gly Phe Leu NH_2	3
Leucine-enkephalin	LE	Tyr Gly Gly Phe Leu	3
Methionine enkephalin	ME	Tyr Gly Gly Phe Met	3
Methionine-enkephalinamide	ME NH_2	Tyr Gly-Gly Phe Met NH_2	3
[L Ala^2]-methionine-enkephalin	L Ala^2 ME	Tyr-Ala Gly-Phe-Met	3
[D Ala^2] methionine enkephalin	D-Ala^2-ME	Tyr D Ala Gly Phe-Met	3
[D Ala^2]-methionine-enkephalinamide	D-Ala^2-ME-NH_2	Tyr D-Ala-Gly-Phe Met NH_2	3
Methionine-enkephalin lysine	ME-Lys	Tyr-Gly Gly Phe Met Lys	2
Methionine enkephalin arginine	ME Arg	Tyr Gly-Gly-Phe Met Arg	2

*1 = Bachem (Torrance, CA, U S A), 2 = Peninsula Labs (Belmont, CA, U S A), 3 = Sigma (St Louis, MO, U S A)

Topaz Lme 2 power conditioner (Topaz Electronics, San Diego, CA, U S A) was used with the amperometric detectors for all experiments A Soft Sealguard column (Applied Science Labs , State College, PA, U S A) packed with 10-μm Ultrapack-Octyl (Altex, Berkeley, CA, U S A.) was used with a 10-μm Aquapore RP-300 column (250 mm × 4 6 mm) (Brownlee Labs , Santa Clara, CA, U S A) A Phantom microbore C_8 guard column (C -M Labs , Nutley, NJ, U S.A) and an in-line filter were used with a Zorbax® Golden Series C_8 column (8 cm × 6 2 mm) (DuPont de Nemours, Wilmington, DE, U.S A.). Only isocratic mobile phases were used and their compositions are described in the figure legends All mobile phases were filtered through a 0.2-μm Nylon-66 filter and degassed before use To prevent the formation of microbubbles, the mobile phase was sparged with helium gas (99 995% purity) 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 While establishing chromatographic conditions, an applied potential of +1 05 V vs Ag/AgCl was used for the detection of the endorphins [12]

Endorphin standards were dissolved in 30 m*M* KH_2PO_4--27 5 μM glycylglycine, pH 2 3 (adjusted with 85% phosphoric acid) Aliquots were lyophilized and stored desiccated at −20°C Prior to chromatography, the endorphin standards were dissolved in an appropriate volume of 25 m*M* KH_2PO_4– 27 5 μM glycylglycine, pH 2 3, and stored for approximately one month at −20°C

The TL-5A dual glassy carbon working electrode was cleaned by the alumina-polishing procedure recommended by the manufacturer. To generate hydrodynamic voltammograms with the TL-5A dual glassy carbon working electrode, the two working electrodes were placed in parallel configuration and set at the same potential The mean response for each potential was plotted The TL-5A glassy carbon working electrode was cleaned by the chromic acid procedure of Anton [13] The chromatographic conditions used to generate the hydrodynamic voltammograms are described in the figure legends

Peak current ratios were calculated by placing the TL-5A dual working electrodes in the parallel configuration. Working electrode A was always set at the smaller applied potential and B at the larger applied potential The Aquapore RP-300 column and a mobile phase of 20 8 m*M* KH_2PO_4- 20 μM glycylglycine, pH 2 3 (with H_3PO_4)- acetonitrile (85.2 14 8) at 27°C was used to separate endorphins for generation of peak current ratios

RESULTS

Chromatography

Fig 1 shows the separation of αNE 1-8, αNE 1-10, Dyn 1-6, ME, βNE, LEA and LE on the Aquapore RP-300 column The isocratic mobile phase that gave the best separation of these endorphins was 20 8 m*M* KH_2PO_4- 20 μM glycylglycine, pH 2 3 (with 85% H_3PO_4)- acetonitrile (85 2 14 8) warmed to 27°C in a circulating water bath A characteristic broadened peak shape was obtained for βNE with this mobile phase in agreement with a previous report [12].

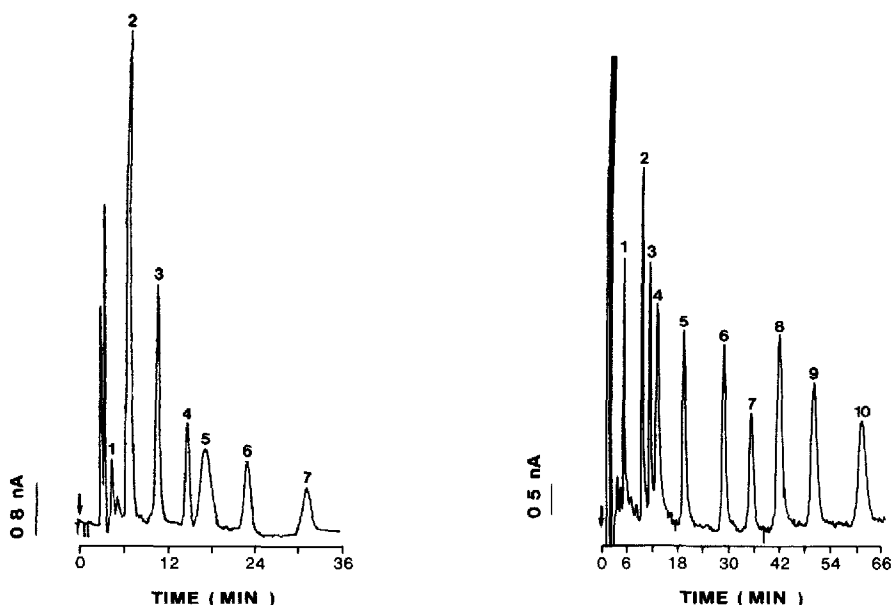


Fig 1 Isocratic elution of endorphins Chromatographic conditions column, Aquapore RP-300, mobile phase, 20 8 m*M* KH_2PO_4—20 μ*M* glycylglycine, pH 2 3 (with H_3PO_4)—acetonitrile (85 2 14 8) at 27°C, flow-rate, 1 0 ml/min, electrode, TL-5A with a glassy carbon auxiliary electrode, applied potential, +1 05 V vs Ag/AgCl Peaks 1 = αNE 1-8, 2 = αNE 1-10; 3 = Dyn 1-6, 4 = ME, 5 = βNE, 6 = LEA, 7 = LE

Fig 2 Isocratic elution of endorphins and ME analogues Chromatographic conditions column, Zorbax Golden Series C_8, mobile phase, 27 8 m*M* KH_2PO_4– 78 μ*M* glycylglycine, pH 2 3 (with H_3PO_4)—acetonitrile (85 4 14 6), flow-rate, 1 0 ml/min, electrode, TL-5A with a stainless-steel auxiliary electrode, applied potential, +1 05 V vs Ag/AgCl Peaks 1 = αNE 1-8, 2 = ME-Lys, 3 = ME-Arg, 4 = αNE 1-10, 5 = ME-NH_2, 6 = ME, 7 = L-Ala^2-ME, 8 = D-Ala^2-ME, 9 = LEA, 10 = LE

Attempts to manipulate the isocratic mobile phase concentration to obtain a longer retention of αNE 1-8 were not successful because other peptides co-eluted or were excessively retained Decreasing the concentration of salt and organic modifier in the mobile phase caused a deterioration in the shape of the later eluting peaks (data not shown)

To examine the selectivity of the chromatographic system for changes in endorphin composition, ME and ME analogues with small moiety differences were separated on the Zorbax® Golden Series C_8 column (Fig 2) The hexapeptides in peaks 2 and 3 differ only in the C-terminal amino acid which is basic in both peptides (lysine and arginine, respectively). Substituting an amide group for the carboxyl terminal group of ME (peaks 5 and 6) decreased the retention time by 6 min Although the pentapeptides in peaks 7 and 8 differ by a single methyl group (alanine is substituted for glycine in position 2 of the ME amino acid sequence), they are well separated in this chromatographic system. Even the most closely-related peptides in this series which differ by stereoisomerism of a single amino acid, L-Ala^2-ME and D-Ala^2-ME are separated by approximately 3 min

Electrochemistry

Hydrodynamic voltammograms. To determine oxidative characteristics of a

series of endorphins, hydrodynamic voltammetry was performed The hydrodynamic voltammograms in Figs 3–5 show that each peptide exhibits characteristic oxidative behavior. In Fig 3, the slope of the curves for αNE 1-10, Dyn 1-6 and βNE are relatively flat, showing only a small change in nA/ng over the range of applied potentials These curves begin to plateau at approximately +1.0 V After an initial steep increase in the nA/ng ratio for αNE 1-8, the rate of change becomes smaller with increasing applied potential without reaching a plateau The slope of the voltammogram for βNE, however, increases sharply between applied potentials +1 15 V and +1 18 V The voltammogram for LE does not plateau but continues to slowly increase the nA/ng ratio with increasing potential In contrast, the oxidative behavior of ME is different from that of the other endorphins shown in Fig 3 There is an approximately linear increase in the nA/ng ratio with increasing potentials. ME also exhibits the greatest electroactivity at higher applied potentials

The hydrodynamic voltammograms for ME and the hexapeptides ME-Arg and ME-Lys are shown in Fig 4 Although arginine and lysine are basic amino acids, they affect the electroactivity of ME differently The electroactivity of ME is enhanced with the addition of a lysyl residue and is slightly decreased by the addition of an arginyl residue A comparison of the hydrodynamic voltammograms for ME in Fig. 3 and 4 shows similar slopes but differences in the oxidative response of ME at higher potentials This difference illustrates the

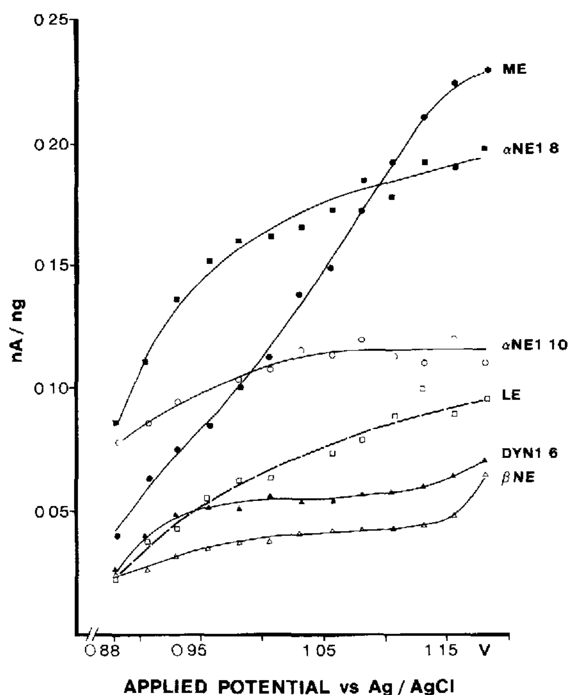
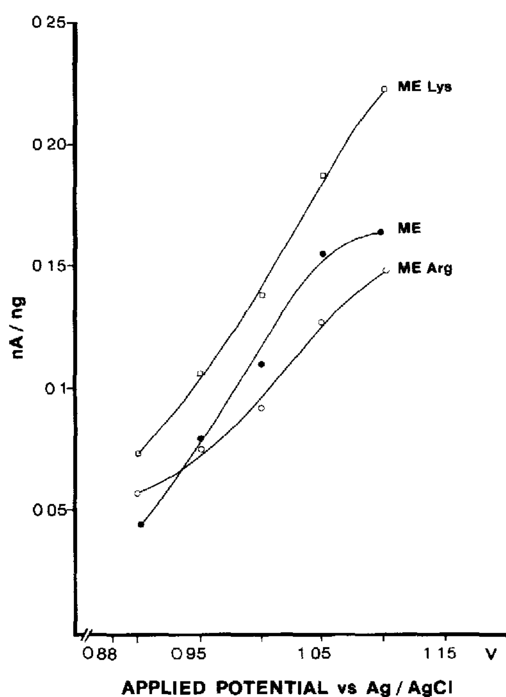


Fig 3 Hydrodynamic voltammograms of endorphins Chromatographic conditions column, Aquapore RP-300, mobile phase, 20 8 mM KH_2PO_4–20 μM glycylglycine, pH 2 3 (with H_3PO_4)–acetonitrile (85 2 14 8) at 27°C, flow-rate, 1 0 ml/min, electrode, TL-5A dual with an additional connecting wire from the auxiliary electrode pin to the stainless-steel outlet tube of the reference electrode compartment



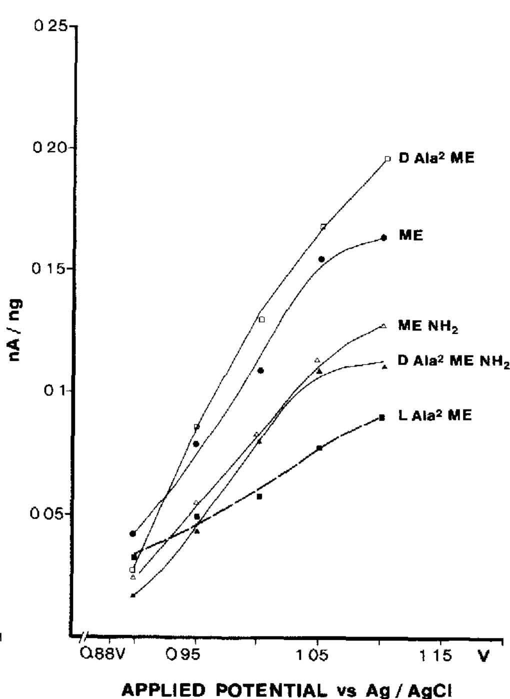


Fig 4 Hydrodynamic voltammograms of ME-related peptides Chromatographic conditions column, Zorbax Golden Series C_8, mobile phase, 25 7 m*M* KH_2PO_4, pH 2 3 (with H_3PO_4)–acetonitrile (86 7 13 3) at room temperature, flow-rate, 1 0 ml/min, electrode, TL-5A with a stainless-steel auxiliary electrode

Fig 5 Hydrodynamic voltammograms of ME analogues Chromatographic conditions column, Zorbax Golden Series C_8, mobile phase, 25 7 m*M* KH_2PO_4, pH 2 3 (with H_3PO_4)–acetonitrile (86 7 13 3) at room temperature, flow-rate, 1 0 ml/min, electrode, TL-5A with a stainless-steel auxiliary electrode

importance of changes in electrode configuration and mobile phase composition in the oxidative behavior of ME The hydrodynamic voltammetry must be re-evaluated for each new working electrode and set of mobile phase conditions

Fig 5 shows the hydrodynamic voltammograms for ME and analogues with small moiety changes Although the substitution of an alanyl residue for a glycyl residue in position 2 of the amino acid sequence of ME adds only a methyl group to the pentapeptide, the electroactivity of L-Ala²-ME is decreased by approximately 50% at the higher potentials Substitution of the D-form for the L-form of alanine, however, increases the electroactivity of the peptide (D-Ala²-ME vs L-Ala²-ME) approximately 100% at higher potentials An amide group substitution at the C-terminal position of ME (ME-NH_2 vs ME) decreases the electroactivity of ME by shifting the hydrodynamic voltammogram to the right

Peak current ratios As an additional approach to evaluating selectivity, peak current ratios were calculated for the endorphins and are listed in Table II The potentials used to generate the peak current ratios were selected by examining the hydrodynamic voltammograms for the greatest differences in

TABLE II

PEAK CURRENT RATIOS

Chromatographic conditions column, 10 μm Aquapore RP-300, mobile phase, 20 8 m*M* KH_2PO_4—20 μ*M* glycylglycine, pH 2 3 (with H_3PO_4)—acetonitrile (85 2 14 8) warmed to 27°C Amperometric detector was a Model LC-4B/17 with TL-5A dual glassy carbon working electrode with an additional connecting wire from the auxiliary electrode pin to the stainless-steel outlet tube of the reference electrode compartment The data are expressed as the mean ± S D

Endorphin	+0 99 V/+1 05 V	+1 030 V/+1 085 V	+1 050 V/+1 085 V
αNE 1-8	—	—	0 78 ± 0 04
αNE 1-10	0 81 ± 0 11	1 21	1 11 ± 0 06
Dyn 1-6	0 48 ± 0 04	0 31 ± 0 01	0 28
ME	0 55 ± 0 03	0 42 ± 0 05	0 68 ± 0 04
βNE	0 64 ± 0 04	1 08 ± 0 02	1 05 ± 0 01
LEA	0 44 ± 0 01	0 21 ± 0 02	0 46 ± 0 01
LE	0 47	0 27 ± 0 04	0 55 ± 0 06

endorphin electroactivity Numerical values from the hydrodynamic voltammograms in Fig. 3 cannot be used to generate the peak-current ratios because these curves represent mean responses of both working electrodes at each applied potential. Working electrode B always gave a larger nA/ng response at a given potential than electrode A for any of the endorphins.

The +0 99 V/+1 05 V peak-current ratios are similar for several of the endorphins, Dyn 1-6, ME, LEA and LE Differences in peak current ratios were the greatest using applied potentials of +1 05 V/+1 085 V with standard deviations averaging approximately 5% No attempts were made to use applied potentials greater than +1.085 V because of increasing baseline noise

DISCUSSION

The potential use of LC-ED for the measurement of endorphins was suggested by the observation of the electroactivity of ME and LE [7]. Subsequently, a voltammetric study [14] showed that several proteins were oxidized by a graphite electrode at a neutral pH at potentials of 0 7—0.8 V vs. SCE To determine which groups in the proteins were oxidized, linear sweep voltammetry and differential pulse voltammetry of free amino acids and model peptides were performed Histidine, methionine, cystine and cysteine showed some electroactivity but tyrosine and tryptophan were the amino acids primarily responsible for the electroactivity of the proteins. The electroactivity of several neuropeptides was shown by Bennett et al [15] using differential pulse voltammetry More recently, several groups [8- 12] have used electrochemistry as the basis for detection of neuropeptides after separation by LC It was our aim to determine the potential selectivity of electrochemistry for detection of closely related small endorphins separated by LC

Changes in small moieties altered both the chromatographic behavior and electroactivity of the endorphin The changes in chromatographic behavior were reflected by shifts in retention times indicating that altering a small functional group in the endorphin alters its hydrophobicity Substitution of an

alanyl residue for a glycyl residue in position 2 in the ME amino acid sequence increased the hydrophobicity of the peptide Stereoisomerism of the alanyl residue (L-Ala^{2}-ME vs. D-Ala^{2}-ME) further increased the hydrophobicity, so that even such closely related pentapeptides were clearly resolved The alterations in the electroactivity of the peptide caused by changes in a small moiety were reflected in the hydrodynamic voltammograms of ME and ME analogues The substitution of an alanyl residue for the glycyl residue in position 2 of the ME amino acid sequence (L-Ala^{2}-ME vs. ME) which adds only a methyl group to the pentapeptide, substantially decreases the electroactivity. The electroactivity of the peptide is dramatically increased by stereoisomerism of that methyl group (D-Ala^{2}-ME vs. L-Ala^{2}-ME) The differences in the electroactivity of LE and Dyn 1-6, ME and ME-Arg suggest an electron-withdrawing effect of arginyl residues. The decrease in electroactivity of αNE 1-10 and βNE by comparison to αNE 1-8, may be an effect of the prolyl residue next to the tyrosyl residue in position 8 of the amino acid sequence The greater electroactivity of ME by comparison to LE suggests that the oxidation of the C-terminal methionine contributes to the electroactivity of the peptide Even the most closely related endorphins exhibited characteristic oxidative behavior which suggests that hydrodynamic voltammetry could be used to determine peak purity in an LC—ED assay of endorphins This approach has been used to verify the identity of the norepinephrine peak in chromatograms of clinical samples [16]

The selectivity that can be obtained with ED has led to the development of multiple-electrode detectors [17—25] Blank [17] published one of the first reports of dual-electrode detection with an electrochemical identification of chromatographically overlapping compounds Subsequently, dual amperometric [18, 20, 21, 23, 25], dual coulometric [24, 25], and dual coulometric—amperometric detectors have been developed with several working electrode configurations [19, 22, 24, 25] These detectors have been used in assays to remove contaminants and to increase sensitivity by improving the signal-to-noise ratio [17—25]. Dual working electrodes can be used to obtain peak current ratios which are analogous to the absorbance ratios obtained with dual wavelength absorbance detectors Peak current ratios, like absorbance ratios, are characteristic for each compound [24] In this study, the parallel adjacent position of dual amperometric electrodes was used to generate peak current ratios for several endorphins Using peak current ratios in an LC—ED assay of endorphins would provide an additional assurance of peak purity

In this study, we have examined the selectivity of LC—ED for minor variations in endorphin structure Using isocratic mobile phases, we have shown that endorphins differing by small moieties such as stereoisomers of a single amino acid residue can be adequately resolved by LC These differences are also reflected in changes in the electroactivity of the peptides determined by hydrodynamic voltammetry and peak current ratios. These data suggest that hydrodynamic voltammetry and peak-current ratios could be used in an LC—ED assay of endorphins to determine peak purity. Application of this technique to biologically relevant samples is currently in progress

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