Journal of Chromatography, 431 (1988) 65–76
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4274

SIMULTANEOUS ANALYSIS OF METHIONINE- AND LEUCINE-ENKEPHALIN FROM RAT BRAIN: QUANTIFICATION BY LIQUID CHROMATOGRAPHY–ELECTROCHEMISTRY*

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(First received January 26th, 1988; revised manuscript received April 18th, 1988)

SUMMARY

This study focuses on the application of liquid chromatography with electrochemical detection (LC-ED) for the analysis of methionine-enkephalin (ME) and leucine-enkephalin (LE) extracted from rat brain regions. The high applied potentials necessary for enkephalin detection required the development of an efficient sample processing protocol. Brain extracts were processed using chromatographic mode sequencing (CMS). The decrease in electroactive interfering substances by CMS improved the chromatographic resolution of ME and LE and the electrode performance. Other qualitative and analytical methods were used to evaluate the enkephalin data obtained by LC-ED for rat brain regions. This study demonstrates that LC-ED provides both the sensitivity and specificity necessary for the analysis of enkephalins from rat brain regions.

INTRODUCTION

The endogenous opioid peptides are derived through proteolytic processing of one of three precursors [1–3] to form a chemically complex system. For a given neuron, differences in precursor processing and post-translational modifications generate distinct sets of closely related peptides with widely differing opioid activities [1]. This chemical complexity, coupled with low physiologic concentrations, makes the analysis of opioid peptides difficult. Although several different assays are available [bioassay, radioreceptor assay, radioimmunoassay (RIA), liquid chromatography (LC)–RIA and LC–mass spectrometry], problems of sensitivity, selectivity or technical complexity limit their utility for investigating

*A preliminary report of this work was presented at the 17th Annual Meeting, Society for Neuroscience, New Orleans, LA, Nov. 16–21, 1987.

0378-4347/88/$03.50

research questions, such as opioid peptide processing and metabolism, which require multi-component analyses.

The potential use of LC with electrochemical detection (ED) for the measurement of opioid peptides was suggested by the observation of the electroactivity of the opioid pentapeptides, methionine-enkephalin (ME) and leucine-enkephalin (LE) [4]. Other voltammetric studies showed that histidinyl, methioninyl, cystinyl and cysteinyl residues had some electroactivity but tyrosyl and tryptophanyl residues were primarily responsible for the electroactivity of peptides [5]. Subsequently, several groups have used electrochemistry as the basis of detection of neuropeptides in brain extracts separated by LC. These neuropeptides include ME, LE [6–10], cholecystokinin tetrapeptide and octapeptide sulfate [9,11], vasopressin [10,12] and oxytocin [10,12,13].

In this study, we have focused on an LC–ED method of analysis for enkephalins extracted from rat brain regions. The high applied potentials ($> +0.90$ V) required for detection of neuropeptides complicates the assay protocol. At these high potentials, other electroactive substances from the brain matrix or contaminants from chromatographic equipment and supplies can also be detected. To minimize coeluting interfering substances, an efficient sample processing protocol was developed. Most of the difficulties in developing this protocol were in three areas: (1) reducing early and late eluting electroactive contaminants from brain samples, (2) discovering the sources and eliminating other contaminating substances and interfering phenomena, and (3) maintaining an adequate recovery of enkephalins to allow detection with the present ED technology.

EXPERIMENTAL

Reagents and chemicals

ME and LE were obtained from Peninsula Labs. (Belmont, CA, U.S.A.) and stored as previously described [14]. Both [tyrosyl-3,5-^{3}H]ME (30 Ci/mmol) and LE (38 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.). All reagents were HPLC grade or the highest purity available, including potassium dihydrogenphosphate, phosphoric acid (85%), methanol and acetonitrile from Fisher Scientific (Pittsburgh, PA, U.S.A.). Trichloroacetic acid (TCA) was obtained from GFS Chemicals (Columbus, OH, U.S.A.) and Ultrex glacial acetic acid was from J.T. Baker (Phillipsburg, NJ, U.S.A.). All water used for chemical solutions and LC mobile phases was prepared by adding activated charcoal (Sigma, St. Louis, MO, U.S.A.) 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. All glassware was washed with Nochromix from Godax Labs. (New York, NY, U.S.A.), an inorganic, non-metallic oxidant which was dissolved in concentrated sulfuric acid.

All LC equipment was powered by an electrical line conditioned by a Powermark frequency converter (Topaz, San Diego, CA, U.S.A.). The LC system included Model 6000A pumps, a Model U6K sample injector (Waters Assoc., Milford, MA, U.S.A.), a Model LP-21 pulse damper (Scientific Systems, State College, PA, U.S.A.) or an MF 4000 pulse damper (Bioanalytical Systems, West

Lafayette, IN, U.S.A.) and an EG & G Princeton Applied Research Model 400 electrochemical detector. A thin-layer single or dual glassy carbon working electrode was used with a stainless-steel auxiliary electrode directly opposed. A 2 cm × 2 mm I.D. guard column packed with 10-μm Ultrapack-Octyl (Altex, Berkeley, CA, U.S.A.) and an in-line filter was used with a Zorbax Golden Series C_8 column (8 cm × 6.2 mm I.D.) (DuPont de Nemours, Wilmington, DE, U.S.A.). The composition of the isocratic mobile phase is described in the figure legends. A flow-rate of 1.0 ml/min was used for all separations.

Glassy carbon working electrodes were cleaned by the chromic acid procedure of Anton [15]. To generate peak current ratios, the dual working electrodes were placed in the parallel adjacent configuration. An applied potential of +1.050 V vs. Ag/AgCl was always used for electrode 1 and an applied potential of +1.085 V vs Ag/AgCl was always used for electrode 2. Peak-current ratios were calculated using peak heights.

Tissue preparation

Male Sprague–Dawley rats were lightly anesthetized with diethyl ether, decapitated, and the brains were dissected by the procedure of Heffner et al. [16]. Frontal cortex and remaining cortex were pooled into one sample, which is referred to as "cortex". The extrapyramidal system consisted of pooled substantia nigra, caudate-putamen and globus pallidus. In experiments using "whole brain", the cerebellum was removed.

To prevent the loss of enkephalins by absorption onto glass, all glassware was siliconized with Sigmacote (Sigma) or PTFE labware was used. This precaution was especially important in any evaporation step where enkephalin loss on untreated glass could be as high as 30%. Enkephalin recovery from Sigmacote-treated glassware and PTFE labware was essentially 100%. Each piece of labware was tested with blank solutions and solutions containing ME and LE standards, then an aliquot was injected onto the LC system. This control was necessary to determine if the use of either PTFE labware or siliconized glassware would interfere with the analysis of ME or LE.

Enkephalin extraction IA

Brain regions were placed in 1.0 ml ice-cold 1.0*M* acetic acid and weighed. They were transferred to centrifuge tubes containing 2.5 ml of boiling 1.0 *M* acetic acid for 10 min. After chilling, each brain region was homogenized and centrifuged at 32 566 *g* for 10 min. The supernatants were saved and the pellets were washed with 1.0 ml of 1.0 *M* acetic acid, then centrifuged at 32 566 *g* for 10 min. The supernantants were combined, lyophilized and frozen at −70 °C.

Enkephalin extraction IB

The lyophilized brain extract was suspended in 2 ml of 10% TCA containing 0.1% sodium metabisulfite and centrifuged at 14 474 *g* for 10 min. The supernatant was then processed in the protocol described below.

Enkephalin extraction II

The smaller brain regions were placed into 2.0 ml ice-cold 10% TCA containing 0.1% sodium metabisulfite and minced. For the larger brain regions, cortex and whole brain, 4.0 ml of 10% TCA containing 0.1% sodium metabisulfite were used. After homogenization, all brain extracts were centrifuged at 14 474 *g* for 10 min. The supernatants were collected and the pellets were washed and centrifuged at 14 474 *g* for 10 min. The supernatants were combined and frozen at −70°C.

Sample processing protocol

To decrease the concentration of TCA and to extract hydrophobic compounds, two volumes of diethyl ether were added to each brain extract and vortexed. The ether layer was discarded and a stream of nitrogen gas was blown over the aqueous layer to remove any remaining diethyl ether. The pH of the aqueous layer was adjusted to approximately 2 with 1.0 *M* sodium hydroxide and potassium phosphate buffer, pH 2.3, was added to obtain a final phosphate concentration of 40 m*M*. The brain extract was added to a 400-mg activated Bond Elut C_8 column with stainless-steel frits (Analytichem, Harbor City, CA, U.S.A.). Large brain samples were split into aliquots so that no more than 300 mg tissue (original wet mass) was added per column. The column was washed with 3.0 ml of 50 m*M* potassium dihydrogenphosphate, pH 2.3 (adjusted with phosphoric acid), 2.0 ml of water–methanol (83:17, v/v), 2.0 ml of water–acetonitrile (92:8, v/v) and 2.0 ml of water–acetic acid (85:15, v/v). A 3.0-ml water wash step was used between each organic wash step. The enkephalin fraction was eluted with 1.0 ml triethylamine phosphate (TEAP)–acetonitrile (50:50, v/v) and dried under a stream of nitrogen. (TEAP = 140 μl of 85% phosphoric acid + triethylamine to pH 3.0 and diluted to a final volume of 25 ml with water.) The enkephalin residue was dissolved in water and added to a 180-mg activated Type W column (resin was obtained from DuPont and packed into an empty column using stainless-steel frits from Analytichem). The column was washed with 3.0 ml of water, 2.0 ml of water–methanol (83:17, v/v), 2.0 ml of water–acetonitrile (93:7, v/v) and 2.0 ml of water–acetic acid (88:12, v/v) with a 3.0-ml water wash step between each organic wash. The enkephalin fraction was eluted with 1.0 ml acetonitrile–water (56:44, v/v) and the eluate was dried under nitrogen. The residue was dissolved in an appropriate volume of 50 m*M* potassium dihydrogenphosphate, pH 2.3 (with phosphoric acid) and an aliquot was injected into the LC system. This sample processing protocol can be automated using a multi-solvent sample processor such as the DuPont Prep II.

All tissue samples were extensively processed as described above and only these "purified" extracts were injected onto the LC system. Since there were no ghost peaks or changes in the retention times of ME and LE standards after the injection of tissue extracts, it was necessary to wash the analytical column only once a week with 100% methanol.

Interferences

ED at high applied potentials imposes restrictions on the labware and chromatographic supplies that can be used for sample processing. Electroactive sub-

stances can leach from filters, precolumns, O-rings, frits, and polyethylene or polypropylene tubes, all of which are commonly used for chromatographic protocols. Some of these substances are leached into aqueous solutions as well as into aqueous–organic solutions. In addition to contributing to the size of the void volume, some of these contaminants can coelute with the enkephalins. Other sources of interferences were procedurally unrelated and environmental in origin. Both electromagnetic fields (EMF) and radiofrequency interference (RFI) caused problems in the quantification of enkephalin peaks. Equipment that contains motors is the most likely source of EMF and was located at a distance from the electrochemical detector.

In our laboratory environment, the Bioanalytical Systems LC-4 and LC-4B electrochemical detectors were susceptible to RFI from citizen band radios (in taxicabs, delivery trucks) and walkie talkies, which produced spikes on the chromatograms. The EG & G Princeton Applied Research Model 400 electrochemical detector was not susceptible to RFI, therefore, it was used for these studies of brain extracts.

RESULTS

Development of sample processing protocol

Initial efforts to develop a rat brain processing protocol for enkephalins focused on the boiling acetic acid extraction method, commonly used for RIA, which is

Fig. 1. Chromatogram of rat brain extracted with boiling acetic acid and processed through a metal-ligand affinity column and C_8 reversed-phase column. This type of chromatogram is representative of the results obtained for brain extracts processed without the use of CMS. The arrows above the peaks denote the positions of ME and LE. RFI is indicated by asterisks. Mobile phase, 128 ml of 50 m*M* potassium dihydrogenphosphate (adjusted to pH 2.3 with phosphoric acid)–29% acetonitrile, diluted to a final volume of 250 ml with LC water. Electrochemical detector, BAS LC-4B.

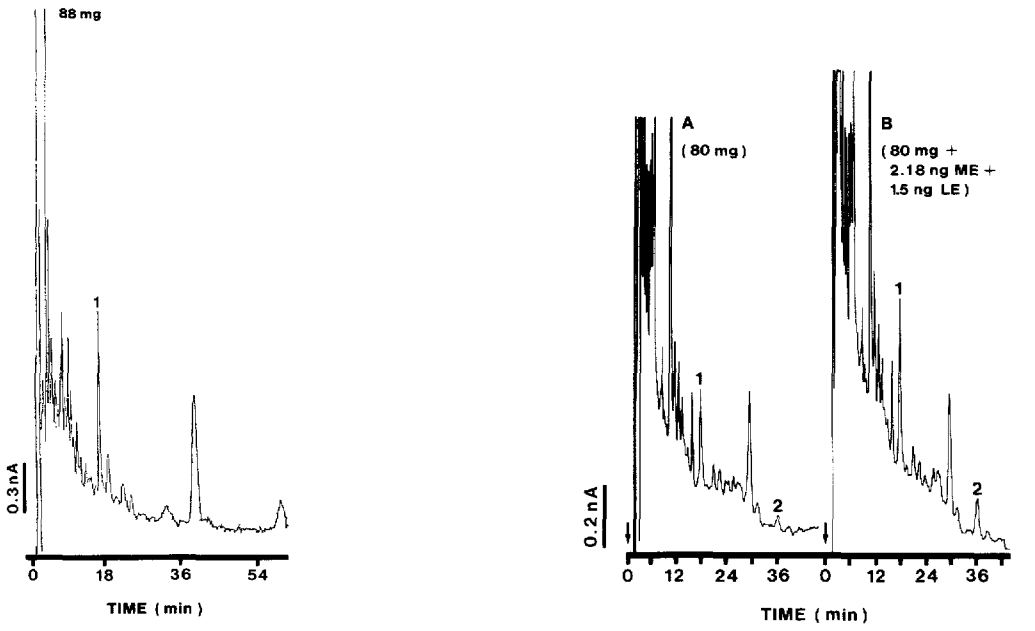

Fig. 2. Chromatogram of 88 mg (wet mass) extracted rat whole brain (minus cerebellum); peak 1 = ME. Mobile phase, 128 ml of 50 m*M* potassium dihydrogenphosphate (adjusted to pH 2.3 with phosphoric acid)–29% acetonitrile, diluted to a final volume of 250 ml with LC water. Mobile phase temperature, ambient.

Fig. 3. (A) Chromatogram of 80 mg extracted rat cortex. (B) Chromatogram of 80 mg (wet mass) extracted rat cortex with 2.18 ng ME and 1.5 ng LE standard added. Peaks: 1 = ME; 2 = LE. Mobile phase, 250 ml of 50 m*M* potassium dihydrogenphosphate (adjusted to pH 2.3 with phosphoric acid)–29% acetonitrile plus 4.4 ml of 0.01 *M* glycyl-glycine diluted to a final volume of 440 ml with LC water. Mobile phase temperature, 29 °C.

described in *Enkephalin extraction IA*. Several extract processing procedures were used in an attempt to develop a technique with minimal sample manipulation, including liquid–liquid extractions, solid-phase extractions, ultrafiltration and metal-ligand affinity chromatography [17]. Chromatograms of brain extracts processed with these techniques were characterized by a large interfering void volume of coextracting and/or contaminating electroactive compounds (Fig. 1). This large void volume made the identification and quantification of ME in brain extracts difficult.

The chromatograms in Figs. 2–4 were obtained with a sample processing protocol designed to eliminate substances with hydrophobicities significantly different from ME and LE while maintaining a high enkephalin recovery. This sample processing protocol was based on chromatographic mode sequencing (CMS*). Two disposable reversed-phase columns were used: a silica-based C_8 column and a resin-based column. With each of these columns, the solvent system was changed in a serial manner using step gradients. The concentration of the elution buffer

*The term CMS is taken from Analytichem International product literature. CMS refers to the manipulation of various mobile and stationary phases to obtain several different chemical interactions to maximize selectivity in sample processing.

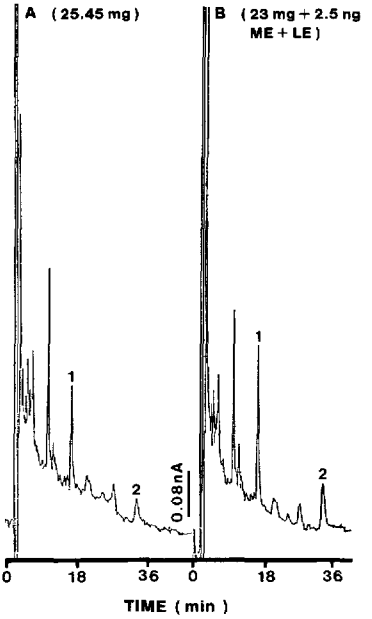

Fig. 4. (A) Chromatogram of 25.45 mg (wet mass) extracted hypothalamus. Peaks: 1 = ME, 2 = LE. Mobile phase, 142 ml of 50 m*M* potassium dihydrogenphosphate (adjusted to pH 2.3 with phosphoric acid)–29% acetonitrile, diluted to a total volume of 250 ml. Mobile phase temperature, 29°C. (B) Chromatogram of 23 mg (wet mass) extracted hypothalamus + 2.5 ng ME standard + 2.5 ng LE standard.

TABLE I

PEAK-CURRENT RATIOS: DIFFERENCE FROM STANDARD RATIO

Peak-current ratios were calculated by taking the ratios of the peak heights as expressed in nA at applied potentials of +1.05 V and +1.085 V. The ratios obtained for ME and LE in tissue extracts are compared to ratios obtained for ME and LE standards. The data are the percentage difference between those ratios.

Tissue	ME		LE	
	Mean	S.D.	Mean	S.D.
Hypothalamus	3.6	0.6	5.6	1.3
Cortex	1.2	1.0	21.1	3.6
Hippocampus	2.2	0.6	9.3	0
Extrapyramidal system (caudate-putamen, substantia nigra and globus pallidus)	1.7	1.0	5.7	2.0
Whole brain (minus cerebellum)	2.0	0.2	–	–
Average	2.1	0.9	10.4	1.7

TABLE II

RIA VERSUS LC-ED

All data expressed as ng/g wet mass. Where necessary, data were converted from pmol/mg protein to ng/g wet mass (10% wet mass of brain = protein; from Scientific Tables, K. Diem and C. Lentner (Editors), Ciba-Geigy, Basle, 1970, p. 576).

Tissue	Compound	Reference*									This paper
		1	2	3	4	5	6	7	8	9	
Cortex	ME	–	95.0	35.5	–	140.0	101.0	–	207.0	48.3	41.3
	LE	–	–	24.4	–	15.0	11.0	–	–	11.2**	12.6
Hypothalamus	ME	–	–	310.0	212.0	450.0	471.0	–	728.0	274.2	235.8
	LE	–	–	22.0	50.0	80.0	134.0	–	–	69.9**	131.5
Hippocampus	ME	–	64.0	52.2	–	200.0	63.0	–	137.0	–	27.7
	LE	–	–	3.3	–	20.0	11.0	10.7	–	–	23.7
Whole brain	ME	62.0	–	50.5	–	–	–	–	–	–	76.1
	LE	17.0	–	11.6	–	–	–	–	–	–	–

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** R.M. Quock, personal communication.

or solvent was carefully adjusted to desorb ME and LE while leaving the more hydrophobic components of the matrix on the column. The use of a single reversed-phase precolumn and serial step gradients did not sufficiently decrease the concentration of early eluting matrix constituents to allow the resolution of ME from the void volume.

The efficiency of the CMS approach to sample processing can be seen by comparing Fig. 1 with Figs. 2–4. The interfering void volume has been significantly reduced and the ME peak is clearly resolved in contrast to Fig. 1. The most difficult sample to process is whole brain because the enkephalin concentration is low by comparison to the other constituents of the matrix. In chromatograms of whole brain (Fig. 2), the ME peak is well separated from void volume components. The most complex chromatograms were obtained from cortex which contained the greatest number of electroactive compounds. However, there was good resolution of both ME and LE. A chromatogram of extracted hypothalamus is shown in Fig. 4. By comparison to whole brain and cortex, hypothalamus is enriched in ME and LE. Data from other laboratories have shown that the hypothalamus is also enriched in other neuropeptides [1] which could potentially interfere with enkephalin analysis. The small number of peaks in this chromatogram (Fig. 4) and the resolution of ME and LE from interfering matrix con-

stituents demonstrates the effectiveness of CMS for the processing of brain regions containing a variety of neuropeptides.

The low concentration of enkephalins in rat brain makes sample loss an area of concern with any multi-step protocol. Therefore, to minimize enkephalin loss, only PTFE or siliconized labware was used (see Experimental). Enkephalin recovery through the extraction and sample processing protocol, calculated by the addition of tritiated ME and LE to brain samples, averaged 78%. There was no difference in enkephalin recovery using extraction procedure I or II. For most of these studies, procedure II was used because less sample manipulation was needed.

After nitrogen evaporation steps, the extracts were dissolved in 50 m*M* potassium dihydrogenphosphate at pH 2.3. Antioxidants were not used at this step because they are electroactive at high applied potentials.

The electrode response did not change throughout the day's analysis as determined by a comparison of peak heights of standards chromatographed at the beginning and end of the day. To maintain maximum sensitivity, the electrode was cleaned at the end of the day using the chromic acid procedure [15] and reequilibrated overnight using low flow-rates.

Peak identity

Enkephalin peak purity was evaluated by other analytical methods. In addition to coelution with endorphin standards, we have used peak-current ratios and RIA data to confirm peak purity. Using dual-electrode detection in the parallel adjacent position and two applied potentials, the peak-current ratios obtained for ME and LE from brain extracts are compared with those obtained for standards. In Table I, the values for brain extracts are expressed as the percentage difference from the ratios obtained for standards. The average difference for the peak-current ratios for the ME peaks from brain regions was approximately 2%. These data indicate that there is no interference with the quantification of ME due to the presence of coeluting electroactive substances. The peak-current ratios for LE from brain regions are more variable and may be due to the low peak area, which would make the ratio more susceptible to random error variance. The percentage difference for LE from cortex is relatively high, which may indicate coelution of a contaminating substance.

In Table II, published data obtained from the quantification of ME and LE from rat brain regions by RIA are compared with the data we obtained by LC–ED. The LC–ED data are within the range of values reported for RIA quantification of equivalent brain regions.

DISCUSSION

Although LC–ED has been used to separate and detect neuropeptides [4–13] including enkephalins, it has not become a technique commonly used by analytical laboratories. Until recently, analytical LC of peptides and proteins was in a developmental phase. The rapid advances in LC technology (column packing materials, etc.) and in understanding of the chromatographic behavior of peptides and proteins has made LC of these compounds a more widely used tech-

nique. The other factor is a concern about the sensitivity and specificity of electrochemistry at the high applied potentials necessary for neuropeptide detection. Background current and baseline noise are significantly greater and selectivity is decreased because more compounds are oxidizable at these high potentials. This decrease in ED selectivity is reflected in the chromatograms of neuropeptides from biologic matrices [8–11]. These chromatograms are characterized by a large interfering void volume caused by the coextraction of contaminating electroactive compounds [8–11]. Attempts to decrease the size of this void volume by column switching [9] or sample processing techniques [10] has met with limited success.

To remove interfering matrix constituents, we developed a brain extract processing protocol based on CMS. Two types of disposable reversed-phase columns were used with a solvent system consisting of a series of step gradients. The specific aim of CMS was to maximize intermolecular interactions between the solvent system and interfering matrix constituents without eluting the enkephalins. Because the chemical nature and quantity of the interfering substances were unknown, we used the solvent triangle of Snyder [18,19] to choose solvents with different selectivities for the step gradients. Methanol was chosen from solvent group II, a proton acceptor group. Acetic acid from group IV is intermediate in selectivity but on the proton donor–proton acceptor side of the triangle. Acetonitrile was chosen from group VI and has the largest dipole moment of the three solvents. Group V and VIII solvents were not used because of solubility limitations in aqueous solutions.

In addition to a series of solvents, two reversed-phase sorbents were used, a C_8 bonded silica and a poly(styrene-divinylbenzene) copolymer resin. A single reversed-phase precolumn or two silica-based reversed-phase precolumns in series did not sufficiently reduce electroactive contaminants to resolve ME from the void volume. The reversed-phase resin column was chosen because of its ability to form π–π interactions [20] causing the adsorption of the enkephalins because of their aromatic group content. This interaction would allow a greater percentage of organic solvent in the step gradients to remove contaminants without eluting the enkephalins. The end result of CMS and subsequent isocratic LC–ED is that oxidizable compounds only within a narrow range of hydrophobicities are seen on the chromatogram. Given the chemical complexity of the opioid peptide system and the large numbers of interfering substances in the brain, this approach provides high resolution for enkephalins.

The decrease in electroactive contaminants also improved the performance of the glassy carbon electrode. In our early studies of brain extracts with large interfering void volumes, the sensitivity of detection decreased as the number of injections increased. This phenomenon was also reported by Dawson et al. [10]. Electrode passivation was not a problem with brain extracts processed through the CMS protocol. These data suggest that a primary factor in electrode passivation is the adsorption of compounds from the matrix, which are eliminated or significantly decreased by CMS.

To ensure peak purity, evaluation of LC–ED by other qualitative and analytical techniques is necessary. In this study, we have confirmed the presence of specific enkephalins in peaks from brain extracts using coelution with ME and LE stan-

dards, peak-current ratios and a comparison of LC–ED and RIA data. Peak-current ratios, analogous to the absorbance ratios obtained with dual-wavelength absorbance detectors, are characteristic for each compound [21]. These ratios are used to provide qualitative information of peak purity [21]. At present, there is no established method for expressing the relationship between peak-current ratios obtained for biologic samples and those ratios obtained for standards. We have chosen to express this relationship as a percentage difference. The close agreement for the ratios obtained for the ME peak from brain extracts versus ME standards indicates that the ME peak is not contaminated by the other electroactive substances. In our previous data, the peak-current ratios for endorphin standards had standard deviations that averaged 5% [14]. Therefore, the percentage differences between LE standard and sample ratios in this study are within an acceptable range of variability with the exception of the data obtained for cortex. The ratio obtained for the LE peak from cortex indicates that there may be a coeluting contaminating substance. This peak would be a good choice for further analysis by fast atom bombardment (FAB)–tandem mass spectroscopy, which would provide unambiguous structural proof of peak identity [22]. For LE, the detection limits of FAB–tandem mass spectroscopy are 20–40 ng, which would require pooling chromatographic fractions from several rat cortices [22].

The data obtained by LC–ED for ME and LE concentrations in rat brain regions were compared to published data obtained by RIA for equivalent regions. All of the LC–ED values are within the range of enkephalin concentrations that have been obtained by RIA. The wide variability in the reported enkephalin concentrations as determined by RIA are reflections of laboratory to laboratory differences in the technical aspects of RIA assay such as antibody specificity [23]. The inconsistency in RIA values is not confined to the assay of enkephalins but is a problem for the quantification of other neuropeptides, as well. To improve assay specificity, LC has been combined with RIA quantification of chromatographic fractions for each neuropeptide [24–27]. The combination of LC and FAB–tandem mass spectrometry provides molecular specificity but is limited in sensitivity for many physiologic samples [22]. The high cost of the equipment and the expertise needed for this technology make its use impractical for the average analytical laboratory. A technique that could incorporate specificity with on-line detection would have many practical advantages. This study demonstrates that LC–ED, evaluated by other qualitative and analytical techniques, can provide both the sensitivity and specificity necessary for the study of enkephalins from brain regions.

ACKNOWLEDGEMENTS

The authors would like to thank Diane M. Pergande for her technical assistance and Sharon A. Neuman for preparing the manuscript. We would also like to acknowledge Dr. Donald B. Nuzzio of EG & G Princeton Applied Research for his cooperation in the loan of the Model 400 electrochemical detector. We also thank the family of David I.M. Fine, M.D. for their contribution in support of this research.

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