Journal of Chromatography, 494 (1989) 67–76
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4876

MEASUREMENT OF METHIONINE ENKEPHALIN AND LEUCINE ENKEPHALIN IN RAT BRAIN REGIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC ELECTROCHEMICAL DETECTION

C KIM* and R CHENG

Addiction Research Foundation, 33 Russell Street, Toronto, Ontario M5S 2S1 (Canada)

and

S R GEORGE

Departments of Medicine and Pharmacology, University of Toronto, Toronto M5S 1A8 (Canada)

(First received February 9th, 1989, revised manuscript received May 18th, 1989)

SUMMARY

A method is described for the determination of two pentapeptides, methionine enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) (ME) and leucine enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) (LE) in discrete rat brain regions Separation and quantitation were performed by reversed-phase high-performance liquid chromatography with coulometric detection Perchloric acid extracts of the tissue after enzyme inactivation by heat treatment were passed through a normal-phase solid-phase extraction diol (COHCOH) column, and endogenous ME and LE were subsequently eluted with methanol The mobile phase was 1-propanol–phosphate buffer (pH 5 5) (9 91) Eluted samples were detected electrochemically using dual coulometric electrodes operated in screen mode Each of these enkephalins gave a linear response over the range 40–160 ng/ml cerebellar homogenate (0 8–3 2 ng absolute amount on column) Analytical recoveries of synthetic ME and LE, added to the homogenates, were 70 ± 3 and 70 ± 10%, respectively, when compared with enkephalins dissolved in water The mean between-assay coefficients of variation for synthetic ME and LE were lower than 10 7 and 7 4%, respectively, over the concentration range studied The within-assay coefficients of variation for synthetic ME and LE were 11 4 and 9 5%, respectively, at the lowest concentration The present method has been applied to a study determining the levels of endogenous ME and LE in discrete rat brain regions

0378-4347/89/$03 50

INTRODUCTION

Two pentapeptides, methionine enkephalin (ME) and leucine enkephalin (LE), with morphine-like biological properties, have been isolated from whole-brain extracts and characterized chemically [1,2]. When the specific sequences, precursor peptides and genes of the enkephalins and other opioid peptides were established, it became clear that ME originated from the processing of proenkephalin A [3] and LE could be derived either from proenkephalin A or prodynorphin (proenkephalin B) processing [4,5] Determination of the precise role of enkephalins in neurobiological processes, however, will undoubtedly rely on specific and sensitive methods for their quantitation in biological materials.

The classical method for the determination of neuropeptides is radioimmunoassay (RIA) [6–10] However, appropriate antibodies have first to be raised for each peptide, with assay procedures usually requiring long incubation times. One serious drawback of currently used RIA techniques is often the lack of specificity for a given opioid peptide; antibodies suitable for RIA are raised with specificity towards a certain sequence in the molecule of interest and may therefore crossreact significantly with other peptides or precursor forms containing the same sequence. By using a combination of high-performance liquid chromatography (HPLC) to effect physicochemical separation and RIA [11–16] or receptor binding assay [17], it is often possible to achieve a specificity and sensitivity unsurpassed by any other analytical technique, except mass spectrometry [18] Usually, a gradient HPLC system is used and the neuropeptide is collected for subsequent RIA.

HPLC in combination with fluorescence and ultraviolet detection to separate and quantitate neuropeptides has been demonstrated. However, conventional HPLC detectors are generally not sensitive enough to quantify the low levels of neuropeptides in biological materials. Electrochemical detection (ED) [19–23] has recently evolved as a versatile, sensitive detection method in HPLC, because many neuropeptides contain oxidizable amino acids. Nabeshima et al [20] showed that the minimum detectable amount for synthetic ME was 2 ng with a recovery of 46% Similarly, Mousa and Couri [21] used HPLC–ED for the determination of several neuropeptides However, they applied an extremely high electrochemical oxidation potential of 1.25 V and therefore precautions had to be taken, such as establishing a calibration graph under each set of experimental conditions and, worst of all, polishing the glassy carbon electrode at least once a day. A two-column switching technique [22] is an alternative to existing methods: on the first column the neuropeptides are separated from large, interfering void peaks whereas the second column separates the neuropeptides from other compounds that have the same or a similar retention time on the first column.

This paper describes a sensitive HPLC–ED method for the determination

of endogenous ME and LE in several regions of rat brain. Perchloric acid extracts of the tissue were passed through a solid-phase extraction (SPE) diol column and the peptides were subsequently eluted with methanol The eluent was dried, reconstituted and injected into the HPLC system

EXPERIMENTAL

Materials

Materials in their purest commercially available forms were obtained from the following sources: ME acetate salt (M-6638), LE formate salt (L-6509) and leucine enkephalinamide acetate salt (LEA, E-3756) were from Sigma (St Louis, MO, U S A), potassium bicarbonate (P-184), anhydrous potassium carbonate (P-208), sodium dihydrogenphosphate (S-369-1) and 70% perchloric acid (PCA, A-229) were from Fisher (Fairlawn, NJ, U S A), methanol (HPLC grade) and 1-propanol (distilled in glass, 8501-2) were from Caledon Labs (Georgetown, Canada) Water was deionized and glass-distilled.

For extraction of ME and LE, the Baker-10 SPE diol (COHCOH) disposable column (Cat. No. 7094) and the Baker-10 SPE system were purchased from Baker (Phillipsburg, NJ, US.A).

HPLC apparatus

Isocratic HPLC determinations were carried out with a Beckman Model 330 liquid chromatograph (Irvine, CA, U S A), a Beckman Model 110A pump, a Model 5100A coulochem detector (ESA, Bedford, MA, U.S.A) with a Model 5020 guard cell containing a single porous graphite working electrode and a Model 5010 analytical cell containing dual coulometric working electrodes made from graphite, and a Hewlett-Packard 3390A recording integrator (Palo Alto, CA, U S A) The guard cell was operated at an oxidation potential of 0 9 V The first and second coulometric detectors were set at 0 45 and 0 8 V, respectively. The gain control used for signal amplification resulting in peak height and area changes was maintained at 50.

Separation by isocratic elution was carried out on a 150×4 6 mm I.D. Nova Pak (C_{18}, particle size 5 μm; Waters Assoc , Milford, MA, U.S.A.) preceded by a guard column (C-135 B precolumn; Upchurch Scientific, Oak Harbor, WA, U S A)

Mobile phase

The mobile phase was 9% (v/v) 1-propanol in a solution (pH 5 5) of 25 m*M* sodium dihydrogenphosphate The mobile phase was filtered through a 0 45-μm filter (Millipore, Bedford, MA, U S A) and then degassed under vacuum before use A flow-rate of 0.9 ml/min at ambient temperature was employed

Preparation of standard solutions

Standard solutions of synthetic ME, LE and LEA (100 ng/ml of the base) were prepared in water, which was previously filtered and degassed These solutions were stored at −70 °C and freshly prepared every 6–8 weeks.

Animal study

Adult male Sprague–Dawley rats (Charles River, Montreal, Canada), weighing 260–285 g, were housed individually in an environmentally controlled room at 21–23 °C and 40% relative humidity, with lighting on from 7:00 to 19.00 h, for one week before use They received food and water ad libitum. The rats were sacrificed by decapitation and the brains were removed rapidly Each brain was dissected on an ice-chilled glass plate, and the striatum, hypothalamus, hippocampus, frontal cortex, anterior lobe of pituitary and neurointermediate lobe of pituitary were separated, as described by Glowinski and Iversen [23]. The tissue was frozen on dry ice, and stored at −70 °C until assayed. The cerebellum was saved to make a pooled homogenate for use in the preparation of the standard curve, as described below.

Preparation of tissue samples

The sample weights (mean ± S D., $n=7$ or 8) were 69 ± 9, 58 ± 13, 106 ± 11, 80 ± 21, 7.2 ± 1.0 and 2 2 ± 0.5 mg for striatum, hypothalamus, hippocampus, frontal cortex, anterior lobe of pituitary and neurointermediate lobe of pituitary, respectively. Individual frozen brain regions were transferred to polypropylene tubes containing 1.0 ml of boiling 0 17 *M* PCA; the tubes were immersed in a boiling water-bath for an additional 7 min The samples were cooled to room temperature, and then homogenized for two 25-s periods with the use of a Polytron at a setting of 7 (Brinkman Instruments, Westbury, NY, U S A.) A sonicator was used for homogenization of the pituitary lobes. To each homogenate, 150 ng of LEA were added as an internal standard and were subsequently centrifuged at 4 °C for 20 min at 35 550 *g*. The supernatants were separated, and the pH of the solutions was adjusted to 8.0 by the addition of 50 mg of potassium bicarbonate and 50 μl of 1 *M* potassium carbonate (pH 10) The solutions were centrifuged for 10 min at 1200 *g* and the clear supernatants were collected into polypropylene tubes for rapid extraction of endogenous ME and LE with a Baker-10 SPE diol column

Column conditioning was as follows: wash with (1) two column volumes of 100% methanol, (2) two column volumes of water and (3) one column volume of 0.2 *M* potassium bicarbonate (pH 8.1–8.5). The neuropeptides of interest were eluted with 100% methanol using a Baker-10 SPE system. The eluent was evaporated under a stream of dry nitrogen. To the dry residues were added 1.0 ml of water, and 20 μl of the resulting aqueous solution was injected into the HPLC system with a 50-μl Hamilton syringe

Standard curve

Known amounts of synthetic ME and LE (40–160 ng) in 1.0-ml aliquots of pooled cerebellar homogenates (70 mg tissue per ml of 0.17 *M* PCA) were taken through the entire procedure. LEA (150 ng) was added to each of these samples as an internal standard A corresponding series of standard samples was also made in 1 0 ml of 0 17 *M* PCA. To construct the standard curve, the ME/LEA and LE/LEA response ratios were plotted against the amounts of each of these enkephalins in ng/ml Actual amounts of ME and LE injected into the HPLC system were in the range 0 8–3 2 ng, whereas the amount of LEA was 3 ng

RESULTS AND DISCUSSION

Representative chromatograms are shown for synthetic ME, LE, and LEA standards in water (Fig 1a), for brain cerebellum homogenate containing an internal standard LEA (Fig 1b) and for ME and LE occurring endogenously in rat striatum (Fig. 1c). No interfering endogenous compounds are observed.

The linearity of the concentration response relationship for synthetic ME and LE was established over the range 40–160 ng in 1.0 ml of cerebellar homogenate (0.8–3.2 ng absolute amount on column). Linear regression analysis from standard curves in water, PCA and cerebellar homogenates indicated that the correlation coefficients for ME and LE were higher than 0 9969 (Table I) The average between-assay coefficients of variation (C V) for ME and LE were 10 7 and 7 4%, respectively, over the concentration range studied (Table II) The average within-assay C.V. for ME and LE were 11 4 and 9 5%, respectively, when measured at 40 ng/ml of homogenate (Table II) Recoveries of synthetic ME and LE (40–160 ng) added to the homogenates were calculated by comparison of peak areas with results obtained with the corresponding samples in water, as well as with that of internal standard LEA. The recoveries for ME and LE were both 70% (Table II) The recovery for the internal standard LEA was found to be 78%. No significant differences in the extraction rates were found between the values of synthetic ME and LE measured in PCA and homogenate.

The isocratic mobile phase that gave the best separation of ME and LE was 25 m*M* sodium dihydrogenphosphate (pH 5.5)–1-propanol (91 9). Higher concentrations of ionic strength (50–100 m*M*) resulted in baseline drift and also gave broader peaks We found improved resolution in the pH range 5.5–6 0 At pH 4 5, the retention times for both ME and LE and the internal standard LEA were lengthened and also caused peak tailing Other organic modifiers, such as methanol and acetonitrile, were tested. The elution of ME and LE with retention times similar to those obtained with 1-propanol required 30% methanol or acetonitrile with high pressure

The hydrodynamic voltammograms for ME and LE indicated that the op-

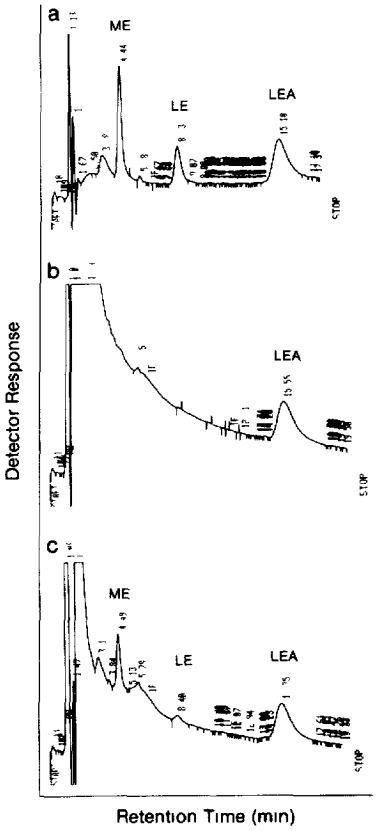

Fig 1 Representative chromatograms of ME, LE and the internal standard LEA (a) Chromatogram of synthetic ME and LE (each 0 8 ng), and LEA (3 ng) in water (b) Chromatogram of extract from cerebellum containing an internal standard LEA (c) Chromatogram of extract from rat striatum, showing peaks of endogenous ME and endogenous LE, and of LEA used as an internal standard

timal oxidation potential for both was 0.8 V However, the electroactivity of ME was higher than that of LE, i.e the detector response of ME is higher than that of LE for the identical mobile phase. It has been suggested that the greater electroactivity of ME compared with LE is due to the fact that the oxidation of the C-terminal methionine contributes to the electroactivity in addition to the electroactivity of tyrosine present in both ME and LE [24]. The behaviour of pentapeptides and hexapeptides in electrochemistry has been described in detail [24].

The present ESA ED system comprises a coulometric guard cell to preoxidize mobile phase contaminants, and dual coulometric working electrodes made from porous graphite The first detector was set at 0.45 V to oxidize undesirable endogenous electroactive compounds. The second detector was set at 0.8 V, the

TABLE I

LINEAR REGRESSION ANALYSIS OF SLOPES AND INTERCEPTS DERIVED FROM STANDARD CURVES

	Slope	y-Intercept	r^2
ME preparation			
Water[a]	0 0166	−0 0495	0 9999
PCA[b]	0 0153	−0 0240	0 9988
Cerebellar homogenate[b]	0 0142	−0 0460	0 9969
LE preparation			
Water[a]	0 0087	−0 0410	0 9999
PCA[b]	0 0094	−0 0210	0 9999
Cerebellar homogenate[b]	0 0081	−0 0265	0 9986

[a]Absolute
[b]After extraction with SPE diol column

TABLE II

RECOVERY AND PRECISION OF THE HPLC DETERMINATIONS OF SYNTHETIC ME AND LE IN CEREBELLAR HOMOGENATES

Compound	Recovery[a,b] (%)	Coefficient of variation (%)	
		Between-assay[c]	Within-assay[d]
ME	70± 3	10 7	11 4
LE	70±10	7 4	9 5

[a]Relative to the value of water
[b]Over the range 40–160 ng/ml of homogenate ($n=5$)
[c]Over the range 40–160 ng/ml of homogenate (at each concentration, $n=5$)
[d]At 40 ng/ml of homogenate ($n=5$)

oxidation potential required for ME and LE, as described above The installation of a guard cell before the injector and dual-electrode system improved the sensitivity The use of a single electrode with an applied oxidation potential of higher than 1.0 V was associated with increased levels of detector noise Other disadvantages in using a high oxidation potential are well illustrated elsewhere [21,24]

The main problem encountered in neuropeptide analysis from biological material by HPLC–ED arises from the coextraction of electrochemically active compounds and blockage of the electrode surface by non-electrochemically active compounds Several sample clean-up procedures have been described, such as organic extraction and application of Porapak-Q, Sep-Pak C_{18} or P-D column (Bio-Beads) In general, these techniques eliminate many endogenous compounds or ions that react with the electrochemical detector, and are reputed to prolong the life of column We found that the use of a normal-phase

TABLE III

LEVELS OF ME AND LE IN RAT STRIATUM AND HYPOTHALAMUS

The PCA extracts equivalent to 1 4 and 1 2 mg for striatum and hypothalamus, respectively, containing 3 ng of LEA as an internal standard, were injected into the HPLC system Results are mean ± S D of the determinations The numbers of rats are cited in parentheses

Brain region	Concentration (ng/g wet tissue)	
	ME	LE
Striatum	467 ± 90 (8)	54 ± 22 (8)
Hypothalamus	291 ± 60 (7)	70 ± 15 (7)

TABLE IV

REGIONAL ME AND LE LEVELS REPORTED IN THE LITERATURE

Compound	Concentration (ng/g wet tissue)		Method	Ref
	Striatum	Hypothalamus		
ME	270	148	RIA	13
	2100	1245	RIA	15
	1300	–	RBA[a]	17
	1217	50	HPLC	20
	701	–	RIA	29
	–	386	RIA	30
LE	289	122	RIA	15

[a]Receptor binding assay

SPE diol column, which is similar to silica gel, rapidly and efficiently cleaned the sample because the void volume is significantly small, with only 30% loss for both ME and LE

Meek and co-workers [17,25] have reported that post-mortem degradation of ME and LE by proteolytic enzymes, such as aminopeptidase, in brain is very active in metabolizing enkephalins Other workers have reported rapid destruction of enkephalins by cleavage of peptide bonds by enkephalinase A, enkephalinase B and angiotensin-converting enzyme [26,27] More than 95% of synthetic ME was decomposed within less than 1 min, as was found in a study of enkephalin catabolism in vivo [17] To eliminate the enzymic activity, tissue supernatants obtained after homogenization were heated to 70–100 °C for 10 min [12,19] In the present study, the weighed frozen samples were transferred to polypropylene tubes, which contained boiling PCA, and boiling was continued by immersing the tubes in a water-bath at 100 °C for an additional 7 min Treatment of samples only by immersion in a boiling water-bath resulted in at least 50% loss of endogenous ME and LE concentrations compared with samples taken through the entire procedure Under the present

experimental conditions, no destruction of synthetic ME and LE was found by heat treatment

Table III presents the levels of ME and LE in brain regions of striatum and hypothalamus. These values are in good agreement with what we have previously documented for ME by RIA in these regions [29,30]. The wide range of levels reported in the literature (Table IV) may indicate methodological problems, such as endogenous interference or lack of sensitivity, difficulties associated with the isolation and detection schemes, and variability in tissue sampling techniques. Other brain regions in the present study included hippocampus, frontal cortex, anterior pituitary and neurointermediate lobe of pituitary. However, only trace amounts of either both or one of the peptides were found in unpooled regions from single animals.

Thus, in summary, we have described the measurement of endogenous ME and LE in certain rat brain regions by HPLC with coulometric ED. The method is straightforward and reproducible, and should enhance the study of enkephalin peptides and their function.

ACKNOWLEDGEMENTS

We thank M Kertesz for help with the animal studies and J Shepperd for typing the manuscript. S.R G. is a Career Scientist of the Ontario Ministry of Health

REFERENCES

1 J Hughes, Brain Res , 88 (1975) 295-308
2 J Hughes, T W Smith, H W Kosterlitz, L A Fother, B A Morgan and H R Morris, Nature (London), 258 (1975) 577-579
3 U Gubler, P Seeburg, B J Hoffman, L P Gage and S Udenfried, Nature (London), 295 (1982) 206-208
4 H Kakidani, Y Furutani, H Takahashi, M Noda, Y Morimoto, T Hirose, M Asai, S Inayama, S Nakanishi and S Numa, Nature (London), 298 (1982) 245-249
5 H Imura, Y Kato, Y Nakai, I Nakao, H Tanaka, H Jingami, T Koh, T Yoshimasa, T Tsukada, M Suda, M Sakamoto, N Mori, H Takahashi, H Tojo and A Sugawara, J Endocrinol , 107 (1985) 147-157
6 J Rossier, A Bayon, T M Vargo, N Ling, R Guillemin and F Bloom, Life Sci , 21 (1977) 847-852
7 J S Hong, H -Y T Yang, W Fratta and E Costa, Brain Res , 134 (1977) 383-386
8 H -Y Yang, J S Hong and E Costa, Neuropharmacology, 16 (1977) 303-307
9 J S Hong, H -Y T Yang, W Fratta and E Costa, J Pharmacol Exp Ther , 205 (1978) 141-147
10 I Lindberg and J L Dahl, J Neurochem , 36 (1981) 506-512
11 J G Loeber, J Verhoef, J P H Burbach and A Witter, Biochem Biophys Res Commun , 86 (1979) 1288-1295
12 P C Emson, A Arregui, V Clement-Jones, B E B Sandberg and M Rossor, Brain Res , 199 (1980) 147-160

13 J Verhoef, V M Wiegant and D De Wied, Brain Res , 231 (1982) 454-460
14 J S Hong, K Yoshikawa and R W Hendren, Methods Enzymol , 103 (1983) 547-564
15 P Giraud, E Castanas, G Patey, C Oliver and J Rossier, J Neurochem , 41 (1983) 154-160
16 M C Chappell, K B Brosnihan, W R Welches and C M Ferrario, Peptides, 8 (1987) 939-942
17 J L Meek and T P Bohan, Adv Biochem Psychopharmacol , 18 (1978) 141-147
18 D M Desiderio, S Yamada, F S Tanzer, J Horton and J Trimble, J Chromatogr , 217 (1981) 437-452
19 S Mousa, D Mullet and D Couri, Life Sci , 29 (1981) 61-68
20 T Nabeshima, M Hiramatsu, S Noma, M Ukai, M Amano and T Kameyama, Res Commun Chem Pathol Pharmacol , 35 (1982) 421-442
21 S Mousa and D Couri, J Chromatogr , 267 (1983) 191-198
22 A Sauter and W Frick, J Chromatogr , 297 (1984) 215-223
23 J Glowinski and L L Iversen, J Neurochem , 13 (1966) 665-669
24 L H Fleming and N C Reynolds, J Chromatogr , 375 (1986) 65-73
25 J L Meek, H -Y T Yang and E Costa, Neuropharmacology, 16 (1977) 151-154
26 J C Schwartz, Trends Neurosci , 8 (1983) 143-151
27 Z Vogel and M Altstein, FEBS Lett , 80 (1977) 332-336
28 J C Gillin, J S Hong, H -Y T Yang and E Costa, Proc Natl Acad Sci U S A , 75 (1978) 2991-2993
29 S R George and M Kertesz, Peptides, 8 (1987) 487-492
30 S R George and M Kertesz, Peptides, 7 (1986) 277-281