*Journal of Chromatography*, 267 (1983) 191–198
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,940

# ANALYSIS OF ENKEPHALINS, β-ENDORPHINS AND SMALL PEPTIDES IN THEIR SEQUENCES BY HIGHLY SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION: IMPLICATIONS IN OPIOID PEPTIDE METABOLISM

SHAKER MOUSA* and DANIEL COURI
*College of Medicine, Department of Pharmacology, Ohio State University, Columbus, OH 43210 (U.S.A.)*
(Received April 21st, 1983)

## SUMMARY

Sensitivity in the 10–100 pg range for enkephalins, β-endorphin, tyrosine (T), tyrosylglycine (T-G) and tyrosylglycylglycine (T-G-G) was attained by using a high-performance liquid chromatographic (HPLC) method with electrochemical detection which is at least 100 times more sensitive than HPLC with UV detection. The chromatographic conditions on a reversed-phase $C_{18}$ silica column were 50 m*M* sodium phosphate buffer (pH 2.1) (A) in acetonitrile–methanol (1:1) (B), isocratic mixture, flow-rate 0.6–1 ml/min, UV detection at 205 nm, electrochemical oxidation potential +1.25 V. The separation of T, T-G and T-G-G was obtained by using 10% B while the separation of the pentapeptide, enkephalins required 40% B. Separation of enkephalins from β-endorphin was attained at a shorter retention time using 60% acetonitrile. Under all of these different conditions the retention times did not exceed 15 min. This method can be used to determine tissue levels and pharmacodynamics of enkephalins and β-endorphin. A highly specific measurement of the different enzymes involved in the metabolism of enkephalin has been achieved.

## INTRODUCTION

Analysis of the total opiate activity was initially performed without any specificity by a bioassay and radioreceptor binding assay[1,2]. The recent development of radioimmunoassay (RIA) methods offered sufficient sensitivity for determining normal biological tissue levels[3,4]. However, despite the sensitivity of RIA, cross-reactivity remains a significant problem unless RIA is coupled with high-performance liquid chromatography (HPLC) for separation purposes[5]. Although the separation of the specific peptides prior to RIA would improve specificity, it requires extra time, expense and possible sample loss.

Earlier we reported a sensitive and specific HPLC method coupled with UV detection (HPLC–UV) for methionine (ME) and leucine enkephalins (LE)[6]. We now report a method with improved sensitivity for enkephalins and β-endorphin, and also the measurement of tyrosylglycylglycine (T-G-G), tyrosylglycine (T-G) and tyrosine (T) using HPLC with electrochemical detection (HPLC–ED).

0021-9673/83/$03.00 

The separation and detection of T, T-G and T-G-G is of great importance in studying the pharmacodynamics of enkephalins and β-endorphin with reference to the specific enzymatic activities involved in their degradation[7].

## EXPERIMENTAL

### *Chemicals*

HPLC-grade acetonitrile, methanol and sodium dihydrogen phosphate were obtained from Fisher Scientific. Phosphoric acid (85% electronic grade) was obtained from the Leheigh Valley Chemical Co. A 50 m*M* sodium dihydrogen phosphate solution was prepared in water purified by deionization, adjusted to pH 2.1 with 85% phosphoric acid and then the phosphate buffer solution was filtered through a Millipore 0.22 μm filter (Type G.S.) and degassed with sonication. Methionine, leucine enkephalins, T, T-G and T-G-G were obtained from Sigma. β-Endorphin was obtained from New England Nuclear.

### *Chromatographic conditions*

Chromatography was accomplished using a Beckman 332 gradient liquid chromatograph system equipped with a Model 420 programmer and a Model 155-00 variable-wavelength detector set at 205 nm. The Model 155-00 spectrophotometer was equipped with a standard analytical flow cell (path length 1 cm and cell volume 20 μl). After the UV cell an amperometric detector with a glassy carbon electrode (Model LC-4, Bioanalytical System) was set at an oxidation potential of +1.25 V referenced to a silver–silver chloride electrode. Samples were injected on to a 5-μm Ultrasphere ODS column (250 × 4.6 mm I.D.) obtained from Beckman using a Model 210 injector with a 20-μl loop. The response was recorded on a Kipp and Zonen dual-pen BD 41 two-channel recorder.

An isocratic mobile phase consisting of 50 m*M* sodium phosphate buffer (pH 2.1) (A) plus acetonitrile–methanol (1:1) (B) mixed in different ratios with a flow-rate of 0.6–1 ml/min was used. The column was conditioned prior to use by passing the mobile phase through the column until the baseline stabilized. Analysis was performed with the column at room temperature (22–23°C). The column was washed after every analysis with water–methanol as described previously[6].

### *Enzymatic determinations*

*Serum aminopeptidase preparations.* Male Sprague–Dawley rats (150–200 g) were killed by decapitation, blood was collected in ice-cold tubes (all the following steps at 0–5°C) and centrifuged at 2400 *g* for 30 min and aliquots of serum were kept frozen until assayed for peptidase activity. The assay procedure for the serum aminopeptidase activity was performed using ME or β-endorphin as substrate; the product, tyrosine, was determined by both electrochemical and UV detection. The enzyme assay consisted of 20 μl (100 ng/μl substrate) + 20 μl of serum (enzyme source) incubated for different times at 37°C; the reaction was terminated by adding 20 μl of 1 *M* hydrochloric acid. Tubes containing the reaction mixture were centrifuged at 2000 *g* for 15 min, the supernatant was carefully decanted and a 5-μl aliquot was removed and analyzed by HPLC. The tyrosine released was measured by HPLC–ED and HPLC–UV and expressed in terms of μmoles of tyrosine formed/mg protein.

Protein in the serum was measured according to the Lowry method[8].

*Brain aminopeptidase preparations.* Rat brain was weighed and homogenized using a cold glass, PTFE pestle homogenizer with 10 m*M* cold sucrose containing 10 m*M* Tris–HCl (pH 7.4) for each gram of brain. The homogenate was centrifuged at 100 *g* for 15 min and the supernatant was then recentrifuged at 17,300 *g* for 30 min. The crude mitochondrial pellet was hypotonically lysed by homogenizing in 5 m*M* Tris–HCl (pH 7.4) and centrifuged at 43,500 *g* for 30 min. The supernatant was used as a source of aminopeptidases[9].

*Synaptosomal membrane enzyme preparations (endopeptidases).* The pellet of aminopeptidase preparation containing the crude synaptosomal membranes was washed with 5 m*M* Tris–HCl (pH 7.4) and centrifuged at 56,000 *g* for 30 min. This washing and centrifugation step was repeated three times. The pellet from the last centrifugation step was taken up in 5 m*M* Tris–HCl (pH 7.4), frozen in aliquots and used as the stock endopeptidase preparation. This preparation retained only residual aminopeptidase activity, which was overcome by adding an aminopeptidase inhibitor, 0.1 m*M* *p*-hydroxymercuribenzoate (PHMB), prior to endopeptidase determinations.

The *in vitro* effect of corticosteroid on aminopeptidase and endopeptidase activity was examined.

*Enkephalin metabolism by tissue peptidases*

*Assay procedure.* The reaction mixture consisted of 20 μl of substrate, ME (100 ng/μl), 20 μl of 50 m*M* Tris–HCl (pH 7.4) or the drug to be tested and 20 μl of serum peptidases. The incubation mixture was incubated for different intervals of time at

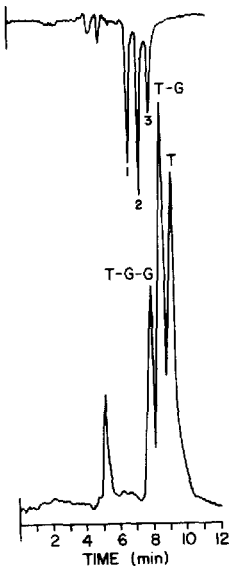


Fig. 1. Representative chromatogram of T, T-G and T-G-G standard. The upper chromatogram (HPLC–UV) and the lower chromatogram (HPLC–ED) show the separation of 55 ng of T-G-G (1), 70 ng of T-G (2) and 35 ng of T (3). HPLC conditions as described in the text; analytes were eluted using 10% B at 0.6 ml/min and detected at 205 nm (0.1 A) and +1.25 V oxidation potential (100 nA).

37°C and the reactions were terminated by adding 20 μl of 1 *M* hydrochloric acid. The rate of ME disappearance and the rate of metabolite formation were quantified by HPLC–ED and HPLC–UV.

## RESULTS

Separation and quantitation of T, T-G and T-G-G were attained by using 10% B at a flow-rate of 0.6 ml/min (Fig. 1). Detection was accomplished by using UV and ED. Calibration graphs were obtained using chemically defined reference compounds chromatographed under the conditions discussed before (Fig. 2). For the separation of either T or T-G or T-G-G from ME and LE, 40% B was used to shorten the ME and LE retention times. A calibration graph for ME was constructed using different concentrations of ME (Fig. 2).

Several precautions have to be taken: (a) establish a calibration graph under

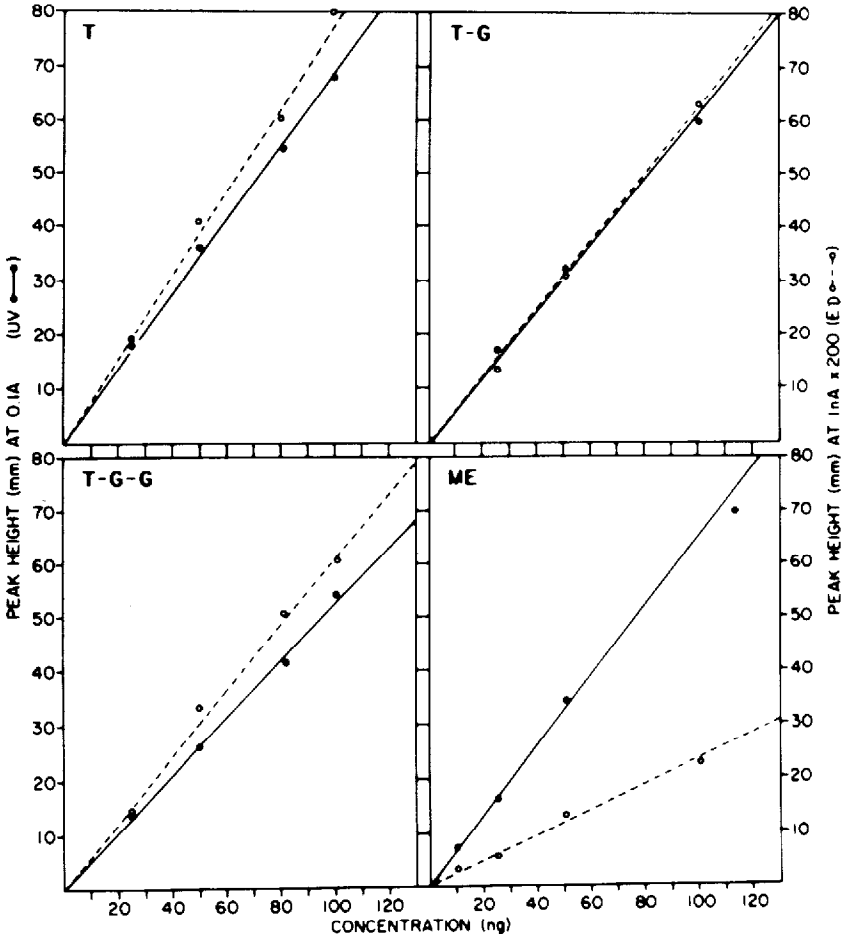


Fig. 2. HPLC–ED and HPLC–UV calibration graphs for T, T-G, T-G-G and ME. HPLC conditions as described in the text, using 10% B for T, T-G and T-G-G and 40% B for ME.

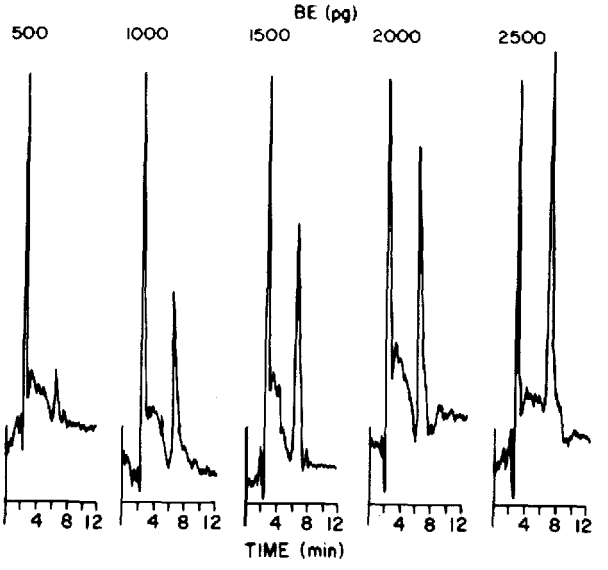


Fig. 3. Representative chromatogram of β-endorphin (BE) standard. HPLC conditions as described in the text; β-endorphin was eluted at a shorter retention time using 60% acetonitrile and detected using ED at +1.25 V oxidation potential.

each set of experimental conditions; (b) polish the glassy carbon electrode with the polishing kit at least once a day; (c) when using a relatively new $C_{18}$ column, the conditions for separation will be as described, but when using an old $C_{18}$ column, the percentage B might have to be reduced to even 5% to separate T, T-G and T-G-G; (d) the pH should be kept acidic, the optimal pH for ED sensitivity being around 2–3.

The elution of β-endorphin with a relatively short retention time required the use of 60% acetonitrile (Fig. 3). A calibration graph was constructed for picogram levels of β-endorphin using ED (Fig. 4). These levels of β-endorphin were not detectable by UV detection.

*Implications in enkephalin and β-endorphin metabolism*

*In vitro effects of corticosteroids on serum aminopeptidase activity.* The effects of

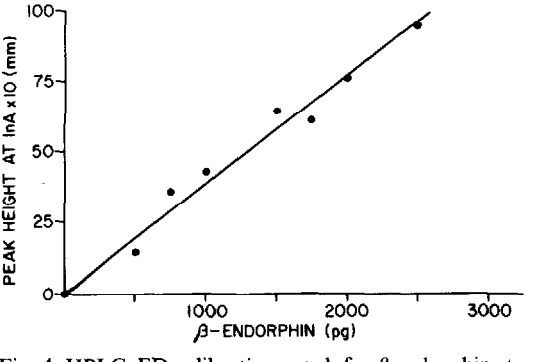


Fig. 4. HPLC–ED calibration graph for β-endorphin standard.

TABLE I

*IN VITRO* EFFECTS OF CORTICOSTEROIDS ON SERUM AMINOPEPTIDASE ACTIVITY

Values given are the means and ranges of two separate determinations.

| *Treatment** control serum | *μmol tyrosine formed/mg protein/h* | |
|---|---|---|
| | *Methionine enkephalin substrate* | *β-Endorphin substrate* |
| Buffer | 1.50 ± 0.11 | 0.28 ± 0.01 |
| Cortisone | 1.37 ± 0.04 | 0.27 ± 0.01 |
| Hydrocortisone | 1.40 ± 0.05 | 0.28 ± 0.02 |
| Dexamethasone | 1.38 ± 0.03 | 0.28 ± 0.01 |

* Corticosteroids were each at $10^{-4}$ *M*.

corticosteroids on serum aminopeptidase activity using enkephalin or β-endorphin as substrate was determined by measuring the tyrosine formed and expressing the data as μmoles of tyrosine formed/mg protein/h. The results showed no significant effect of $10^{-4}$ *M* corticosteroids on aminopeptidase activity (Table I).

*In vitro effects of corticosteroids on brain aminopeptidase activity.* The effect of corticosteroids in comparison with the effect of the classical peptidase inhibitors, bacitracin or PHMB, on brain aminopeptidase activity was examined. The different corticosteroids at a concentration of $10^{-4}$ *M* did not to have any significant effect on the brain aminopeptidase activity using either T-G-G or ME as substrate (Table II).

*In vitro effects of corticosteroids on brain endopeptidases activity.* The effect of corticosteroids on the synaptosomal membrane endopeptidase enzyme was examined by monitoring the T-G-G formed using ME as substrate. The data showed no significant effect of corticosteroids on brain endopeptidase activity (Table III).

*In vitro effects of corticosteroid on enkephalin metabolism.* The effect of corticosteroid on degradation of ME was examined, the disappearance of the substrate (ME) being monitored at different intervals of incubation. Neither corticosteroids nor

TABLE II

*IN VITRO* EFFECTS OF CORTICOSTEROIDS ON BRAIN AMINOPEPTIDASE ACTIVITY

Values given are means and ranges of two separate determinations.

| *Treatment** | *μmol tyrosine formed/mg protein/h* | |
|---|---|---|
| | *Tyrosylglycylglycine substrate* | *Methionine enkephalin substrate* |
| Control: brain soluble enzyme + buffer | 1.13 ± 0.07 | 1.48 ± 0.03 |
| Bacitracin | | 0.72 ± 0.05 |
| PHMB | | 0.54 ± 0.03 |
| Cortisone | 1.01 ± 0.19 | 1.38 ± 0.05 |
| Hydrocortisone | 1.21 ± 0.04 | 1.32 ± 0.05 |
| Dexamethasone | 1.11 ± 0.08 | 1.40 ± 0.06 |

* All drug additions were at $10^{-4}$ *M*.

TABLE III

*IN VITRO* EFFECTS OF CORTICOSTEROIDS ON BRAIN ENDOPEPTIDASES ACTIVITY

Values given are means and ranges of two determinations.

| *Treatment** | *nmol T-G-G formed/mg protein/h: methionine enkephalin substrate* |
|---|---|
| Control: brain synaptosomal membrane enzyme + buffer | 201 ± 8 |
| Cortisone | 197 ± 9 |
| Hydrocortisone | 183 ± 8 |
| Dexamethasone | 187 ± 10 |

* All drug additions were at $10^{-4}$ *M* and included PHMB in each assay.

morphine affected the rate of ME degradation relative to the standard peptidase inhibitor, bacitracin (Fig. 5).

## DISCUSSION

The separation of small and large peptides has been markedly improved by the introduction of reversed-phase HPLC[10–12]. This technique depends on the hydrophobic interactions between a hydrocarbonaceous column and the peptide to be separated; the more lipophilic the compounds, the stronger is its retention by the column. Strongly retained compounds are eluted with higher concentrations of or-

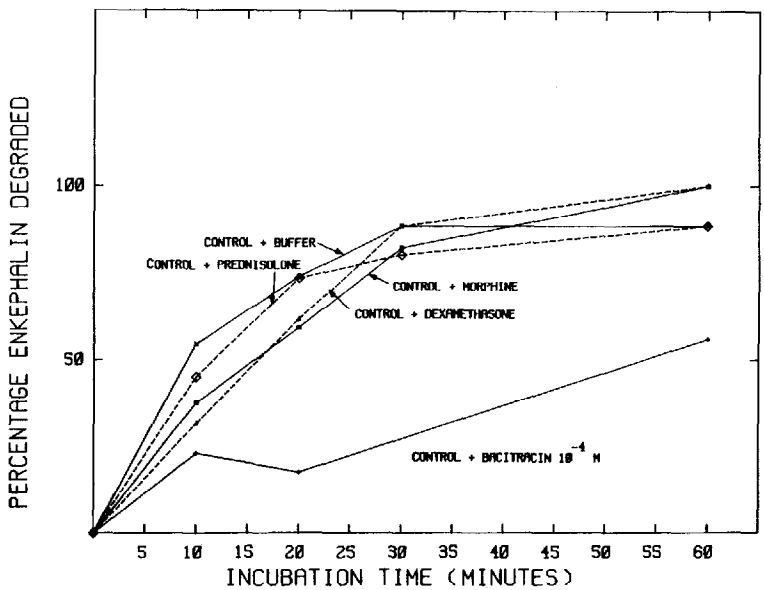


Fig. 5. Percentage of enkephalin degraded as a function of incubation time. The effects of $10^{-4}$ *M* of different corticosteroids, bacitracin and morphine on the rate of enkephalin metabolism are shown.

ganic solvent, *e.g.*, β-endorphin is eluted at 60% acetonitrile, ME or LE at 40% B and T, T-G or T-G-G at 10% B.

The separation of peptides of various size can be achieved in a single run by using a gradient of acetonitrile–methanol and 50 m*M* sodium dihydrogen phosphate (pH 2.1) when using UV detection but not with ED. When using ED, an isocratic mixture of organic and aqueous phases must be used and separation can be accomplished only in a separate run, as mentioned above.

The use of ED improved the sensitivity over UV detection and approached that of RIA.

The chromatographic conditions selected allowed for complete separation of T, T-G, T-G-G, ME and β-endorphin in less than 15 min.

HPLC–ED and HPLC–UV allowed the quantitation of amounts of opioid peptides in biological tissue ranges[6] without the need for derivatization or extensive preparative procedure or the use of RIA.

The technique described provides a means of studying specific enzyme activities and enkephalin/endorphin metabolism.

## REFERENCES

1 J. Hughes, *Brain Res.*, 88 (1975) 295.
2 H. Osburne, V. Halt and A. Herz, *Life Sci.*, 22 (1978) 611.
3 H. Y. T. Yang, J. S. Hong and E. Costa, *Neuropharmacology*, 16 (1977) 303.
4 S. R. Childers, R. Simantov and S. H. Snyder, *Eur. J. Pharmacol.*, 46 (1977) 289.
5 T. Duka, V. Hölt, R. Przewlocki and D. Wesche, *Biochem. Biophys. Res. Commun.*, 85 (1978) 1119.
6 S. Mousa, D. Mullet and D. Couri, *Life Sci.*, 29 (1981) 61.
7 J. L. Meek, H. Y. T. Yang and E. Costa, *Neuropharmacology*, 16 (1977) 151.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 M. Altstein, S. Mittman and Z. Vogel, *Life Sci.*, 28 (1981) 185.
10 K. Krumen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
11 C. A. Bishop, D. R. K. Harding, L. J. Meyer, W. S. Hancock and M. T. W. Hearn, *J. Chromatogr.*, 192 (1980) 222.
12 M. Rubinstein, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4969.