ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
19 (1999) 855–864

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Analysis of leucine enkephalin by high-performance liquid chromatography using enzymatic derivatization by tyrosinase and electrochemical or fluorescence detection

Véronique Larsimont [1], Marja Bogaards, Pascalle Mulleneers, Günther Hochhaus *

Department of Pharmaceutics, University of Florida, Box 100 494 JHMHC, Gainesville, FL 32610, USA

Received 14 January 1998; received in revised form 29 May 1998; accepted 29 May 1998

Abstract

Two tyrosine specific, HPLC methods with either electrochemical (HPLC-ED) or fluorescence (HPLC-FL) detection are described for leucine-enkephalin (LE) in cerebrospinal fluid (CSF). Both approaches involve the hydroxylation of the Tyr[1]-moiety of LE by mushroom tyrosinase. Production of a catechol permitted the selective clean-up of the analyte using boronate gels and produced species which were more readily oxidizable than the LE. The controlled oxidation of the catechol to corresponding chinones enabled the reaction with 1,2-diamino-1,2-diphenylethane (DPE) and subsequent quantification by HPLC-FL. The HPLC-ED and HPLC-FL yielded limits of detection for LE in CSF of 360 and 500 fmol per injection, respectively. Inter-day variability of calibration curve samples was lower than 20% for the HPLC-ED with slightly higher variability for the HPLC-FL assay.

Keywords: HPLC; Fluorescence detection; Electrochemical detection; Tyrosinase

1. Introduction

A considerable body of research has been focused on the development of opioid peptides as therapeutic agents, particularly in the area of analgesia and in the treatment of opiate addiction [1]. The low physiological concentrations of endogenous opioid peptides and those to be expected for therapeutically administered derivatives necessitate the development of specific and ultrasensitive analytical methods to support clinical studies.

Currently, for the most part, opioid peptides are analyzed by radio-or enzyme-immunoassays

* Corresponding author. Tel.: +1 352 8462727; fax: +1 352 3924447; e-mail: hochhaus@cop.ufl.edu

[1] Present address: Zentrallaboratorium Deutscher Apotheker, Ginnheimer Strasse 20, D-65760 Eschborn, Germany.

0731-7085/99/$ - see front matter
PII S0731-7085(98)00141-1

(RIA or ELISA). Although immunoassays can be highly sensitive and reproducible, they are hampered by several disadvantages, including the dependence on antiserum, and low selectivity due to cross-reactivity of the antiserum to structurally similar compounds. HPLC/RIA procedures, although more specific [2] have the disadvantage of being extremely complex and time-consuming.

Instrumental methods which have been used for the analysis of opioid peptides include high-performance liquid chromatography with electrochemical detection (HPLC-ED), fluorescence detection (HPLC-FL) or mass spectrometry (HPLC-MS). The current HPLC-ED methods for opioid peptides [3,4] make use of high oxidation potentials ($+0.9$–1.25 V), thus compromising sensitivity and selectivity as background current and baseline noise are increased significantly.

The majority of HPLC-FL methods in existence for opioid peptides derivatize the N-terminal amino group of the peptide and as a consequence, are not specific for a particular peptide [5,6]. Tyrosine-specific HPLC-FL methods do exist for the determination of opioid peptides [7–10]. These more specific methods have yielded limits of detection for opioid peptides in the 100–500 fmol per injection range.

In the present study, two HPLC assays for opioid peptides are described using leucine enkephalin (LE, Tyr-Gly-Gly-Phe-Leu) as a model peptide. Both assays make use of tyrosine-specific pre-column derivatization by the enzyme tyrosinase. In the HPLC-ED assay, specific hydroxylation of the tryosine group in the 1 position of LE which is preserved in all opioid peptides, presents two analytical advantages. Firstly, the derivatization results in the formation of a catechol which is amenable to specific clean-up using boronate gels, and secondly, this catechol is more easily oxidizable than the parent peptide, thus facilitating electrochemical detection (Figs. 1 and 2A). In the HPLC-FL assay, previously applied to a model dipeptide [11], enzymatic derivatization by tyrosinase renders the peptide amenable to fluorogenic derivatization using 1,2-diamino-1,2-diphenylethane (DPE, Figs. 1 and 2B).

2. Materials and methods

2.1. Materials

Leucine enkephalin and mushroom tyrosinase were obtained from Sigma (St. Louis, MO). Tetrabutylammonium was procured from the Eastman Kodak (Rochester, NY). 1,2-Diamino-1,2-diphenylethane (DPE) was obtained as previously described [11]. ^{3}H-Leu-enkephaline (tyrosyl-3,5-^{3}H(N)-leucine-enkephaline), specific activity: 38.9 Ci $mmol^{-1}$, was purchased from NEN (Wilmington, DE). All other chemicals were of either reagent or HPLC grade. CSF was collected by lumbar puncture from patients undergoing spinal anesthesia. Approval for collection of human CSF was attained by the University of Florida Health Science Center Institutional Review Board and the Subcommittee on Human Studies, Department of Veterans Affairs, VAMC, Gainesville.

2.2. Purification of mushroom tyrosinase

Mushroom tyrosinase was purified prior to use by ultra-filtration using Centricon membrane filters (molecular weight cut off 30000; Amicon, Danvers, MA) as described earlier [11]. The final concentrate was reconstituted in phosphate buffer to give a concentration of 1 mg ml^{-1} of mushroom tyrosinase corresponding to 3870 units of activity per ml of solution. Aliquots were stored at -20°C and defrosted immediately prior to use.

2.3. Characterization of tyrosinase reaction products

For the general characterization of the enzymatic derivatization procedure (identification of reaction products, isolation of main product for electrochemical characterization and optimization of boronate purification), the following incubation mixture was set up in a microcentrifuge tube: 1 mM LE, 135 U ml^{-1} of purified mushroom tyrosinase and 50 mM ascorbic acid in 0.5 M phosphate buffer pH 7.4 (100 μl total volume). The reaction was allowed to proceed at room

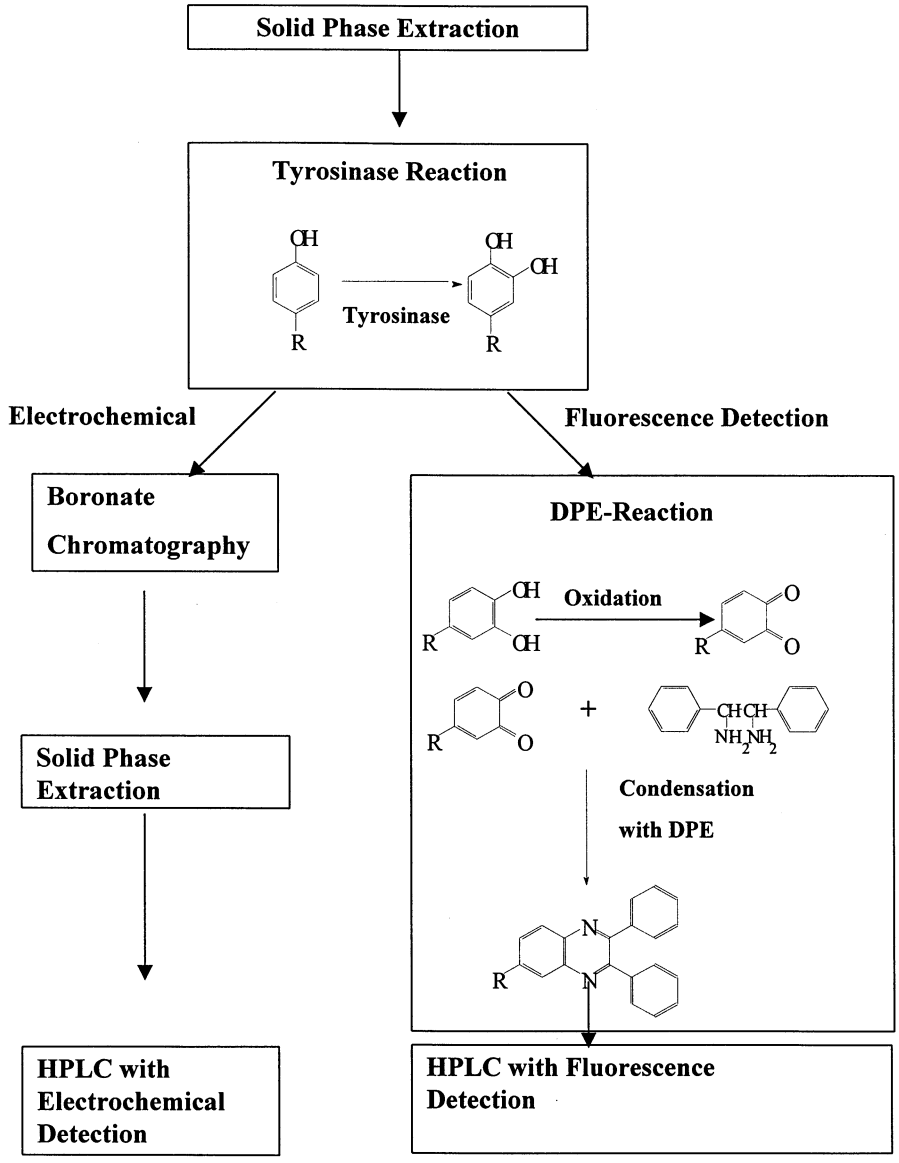

Fig. 1. Scheme of the proposed assays.

temperature with constant shaking for 60 min. Aliquots of this incubation mixture were then applied to an HPLC system with UV-detection at 254 nm using a Partisil 5 ODS-3 column (Whatman Labsales, Hillsboro, OR, 125 × 4.6 mm) and a mobile phase of 12.5% acetonitrile (v/v) in citrate/dipotassium phosphate buffer (pH 5) at a flow rate of 1 ml min^{-1}.

For identification of reaction products, peaks were collected from the HPLC eluent, organic solvent was removed by evaporating under a stream of nitrogen and the collected peaks were concentrated using a preconditioned Sep-Pak C_{18} preparative column (Waters Associates, Milford, MA). Material was used to identify the products by electrospray ionization mass spectrometry us-

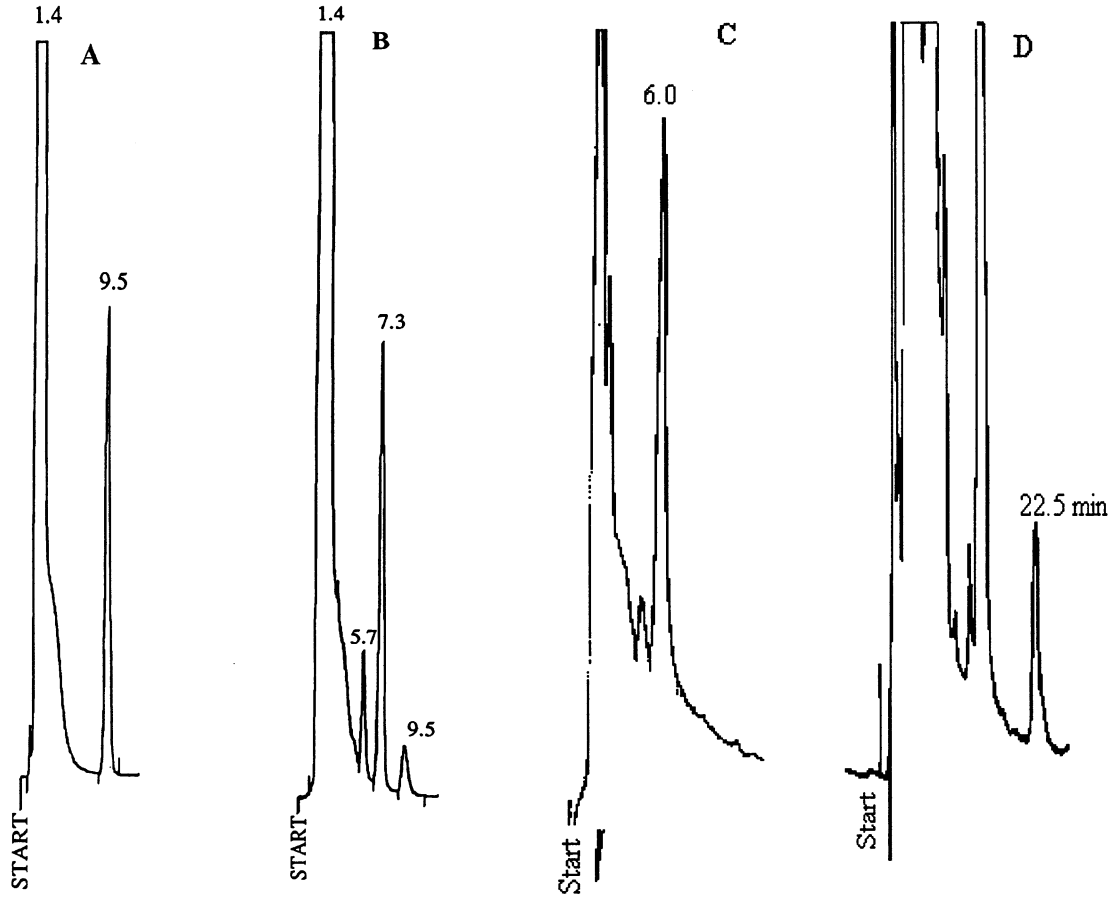

Fig. 2. (A) Chromatograph with UV detection of a control run (no tyrosinase) for the tyrosine reaction showing LE eluting after 9.5 min, distinct from ascorbic acid eluting in the solvent front after 1.4 min, and (B) chromatograph with UV detection of the tyrosine reaction products showing the emergence of two new peaks with retention times of 5.7 and 7.3 min. (C) Sample chromatograph of LE in CSF using the HPLC-ED assay showing the peak of interest eluting after 6 min. (D) Sample chromatograph of LE in CSF using the HPLC-FL assay procedure showing peak of interest eluting after 22.5 min.

ing a Vestec 200 ES instrument (Vestec, Houston, TX) under conditions which allowed collision-induced dissociation. In addition, material corresponding to the peak eluting at 7.3 min in Fig. 2B was collected and used to assess oxidation potentials of the derivatives. This material was also used also in establishing the boronate chromatography (see below).

2.4. Electrochemical assay

In the fully developed HPLC-ED method, calibration curves were constructed for LE in both buffer (170–2500 pmol inj^{-1} in buffer) and CSF (8.8–240 pmol ml^{-1}; equivalent to 360–9600 fmol inj^{-1}). For comparison, CSF spiked with LE in a concentration range of 9–86 pmol inj^{-1}, equivalent to 440–43400 pmol ml^{-1} was assayed after solid phase extraction in a direct assay without derivatization.

Leucine enkephalin was extracted from human cerebrospinal fluid (CSF, 100 μl) through the use of Supelclean LC 18 solid phase extraction columns (Supelco, Bellefonte, PA). The columns were activated with 3 ml each of water and methanol and loaded with 100 μl of spiked CSF

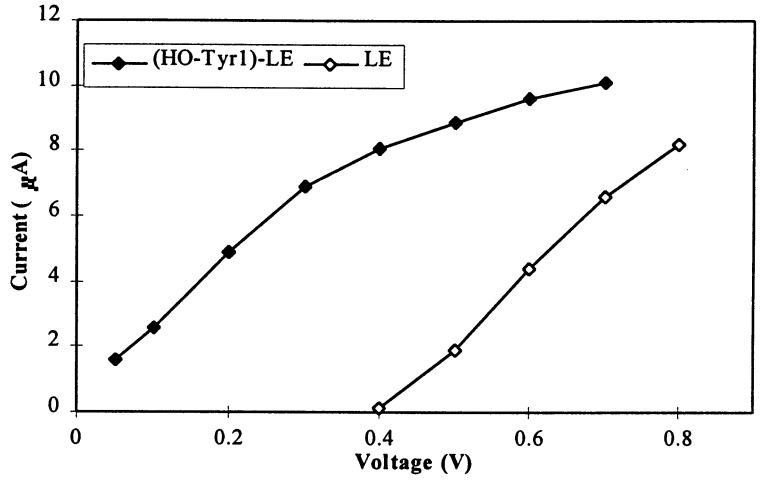

Fig. 3. Current–voltage curves for [HO-Tyr^1]-LE and LE.

or buffer. Subsequently, the columns were washed with 1 ml of water, 3 ml of 0.1 N HCl, 1 ml of water, 3 ml of 0.1 M borate buffer (pH 8.5) and 1 ml of water. The LE rich fraction was then eluted in 2 ml of methanol and evaporated to dryness under a stream of nitrogen. Recovery during solid phase extractions was assessed with CSF sample spiked with 17000 dpm of ^{3}H-LE; and radioactivity retained and eluted under the above assay conditions was determined by liquid scintillation counting using a Beckman LS 5000 TD scintillation counter (Fullerton, CA).

LE (270 μl) in phosphate buffer (without prior extraction) or extracts of spiked CSF reconstituted in 270 μl phosphate buffer (pH. 7.4, 90 μl) were then incubated with tyrosinase in a total volume of 310 μl, (135 U ml^{-1} of purified mushroom tyrosinase, 50 mM ascorbic acid in 0.5 M phosphate buffer pH 7.4). The reaction was allowed to proceed at room temperature with constant shaking for 5 min and the reaction was stopped with 20 μl of 1 N HCl. The reaction products were then purified using hydrated boronate columns.

A hydrated boronate column packed with a 3-ml bed volume of Affigel 601 (BioRad Laboratories, Melville, NY) was used for the clean-up of the enzymatically derived species prior to application to the HPLC-ED system. The column was pre-rinsed with 0.2 M phosphate buffer (pH 8.5) and the incubation mixture (270 μl of the total 330 μl of incubation mixture) was applied. The column was washed with 20 ml of phosphate buffer (pH 8.5) and the analyte was eluted from the boronate column onto a pre-conditioned Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) with 20 ml of aqueous 0.01% (v/v) trifluoroacetic acid (TFA). Here, the Sep-Pak C_{18} cartridge served to concentrate the sample as the analyte was eluted from the boronate column in a relatively large volume of aqueous 0.01% (v/v) TFA. The Sep-Pak C_{18} column was then washed with 10 ml of aqueous 0.01% (v/v) TFA and the analyte was eluted in 2 ml of 0.01% TFA in acetonitrile, evaporated to dryness under a stream of nitrogen and reconstituted in 200 μl mobile phase prior to application to the HPLC-ED system.

Electrochemical detection was effected using a Model 5100A Coulochem multi-electrode electrochemical detector (ESA, Bedford, MA) fitted with a Model 5020 guard cell and a Model 5011 analytical cell. The solvent delivery system was a Milton Roy constaMetric III metering pump (Riviera Beach, FL), with a Rheodyne Model 7125 injector (Cotati, CA) fitted with a 100-μl loop. A Spherisorb ODS2 5 μm 150 × 4.6 mm analytical column (Keystone Scientific, Bellefonte,

PA) was used with a mobile phase of 20% acetonitrile (v/v) in monosodium phosphate buffer (100 mM, pH 5) containing 200 mg l^{-1} of sodium dodecyl sulfate. Mobile phase flow rate was set at 1 ml min^{-1}. The electrochemical detector was used with the following potentials: guard cell 0.4 V, analytical cell, first electrode: −0.1 V; analytical cell, second electrode: 0.3 V. For the experiments with un-derivatized leucine-enkephaline, the following potentials were used: guard cell 0.75 V, analytical cell, first electrode: 0.4 V; analytical cell, second electrode: 0.7 V.

In early experiments, LE and dihydroxyphenyl-LE, the major product of the enzymatic reaction were collected by HPLC and then characterized electrochemically using the HPLC system with electrochemical detection (HPLC-ED) described above. Five nanomoles of analyte per injection were applied to the system and the responses (in μA) obtained at various potentials (+0.05 to +0.80 V) at the analytical cell were recorded to allow the construction of current–voltage curves.

2.5. Fluorogenic assay

Calibration curves for LE in both buffer and CSF (0.5–7.0 pmol inj^{-1}, corresponding to 12–168 pmol ml^{-1}) were constructed within the HPLC-FL assay.

For the tyrosinase reaction, various concentrations of LE (86.8 μl in 0.5 M sodium phosphate buffer pH 7.4) or spiked CSF extracts reconstituted in 0.5 M sodium phosphate buffer pH 7.4 (86.8 μl) were reacted with mushroom tyrosinase (135 U ml^{-1}) in the presence of ascorbic acid (50 mM). The total volume of this incubation mixture was 100 μl. After a 60-min incubation at room temperature with constant shaking, 449 μl of an oxidizing solution containing 9.45 mg ml^{-1} potassium ferricyanide, 17.85 mg ml^{-1} potassium chloride and 55% acetonitrile (v/v) was added followed by 45.5 μl of a solution of DPE containing 20 mg ml^{-1} in 0.1 N HCl. The fluorogenic reaction was allowed to proceed in the dark for 60 min at room temperature with constant shaking. A 250-μl aliquot of this reaction mixture, representing 42% of the total final reaction mixture, was then injected into the above HPLC system using a Nucleosil C_{18} column (5 μm, 150 × 4.6 mm; Keystone Scientific, Bellefonte, PA) and a Perkin Elmer 650S spectrofluorodetector (Norwalk, CT) set at λ_{ex} 345 nm and λ_{em} 480 nm with slit widths of 12.5 nm. The mobile phase contained 32% acetonitrile (v/v) and 67 mM TBA in citrate/dipotassium phosphate buffer (pH 5). The system was operated at a mobile phase flow rate of 1 ml min^{-1}.

3. Results and discussion

Schemes of the developed assays are shown in Fig. 1. In the final assay, leucine-enkephaline (Tyr-Gly-Gly-Phe-Leu) was extracted by solid phase extraction, and hydroxylated via tyrosinase in the presence of ascorbic acid. In the electrochemical assay, the reaction mixture was cleaned using a boronate column, concentrated by solid phase extraction and injected into the HPLC. For the fluorescence assay, the generated catechol was converted into the corresponding chinone, reacted with DPE to yield a highly fluorescence species, followed by HPLC with fluorescence detection.

3.1. Solid-phase extraction

Recovery during solid phase extraction was determined in one experiment to be 91.3%. No further optimization of above procedure was necessary.

3.2. Hydroxylation reaction

Incubation of tyrosinase with LE in the presence of ascorbic acid resulted in two peaks with retention times of 5.7 and 7.3 min, respectively, as compared to the retention time of 9.5 min for leucine-enkephaline (Fig. 2A and B). These two product peaks were identified by electrospray ionization mass spectroscopy as the diphydroxyphenyl- and trihydroxyphenyl derivatives of LE ([$(HO)_2$-Tyr^1]-LE and [HO-Tyr^1]-LE). The relative intensity of the peaks obtained from mass spectrometric analysis showed that [$(HO)_2$-Tyr^1]-LE is a minor product (data not shown).

Table 1
Limits of quantification (LOQ), and correlation coefficients (r^2) for LE in buffer or CSF using either the complete HPLC-ED method or HPLC-ED without derivatization and boronate clean-up

Method	Sample	N	LOQ (fmol inj^{-1})	Slope (peak area pmol^{-1})	r^2
Electrochemical					
Complete HPLC-ED	Buffer	3	170	$28\,071 \pm 2381$	0.9925 ± 0.005
Complete HPLC-ED	CSF	3	360	$22\,057 \pm 1064$	0.9944 ± 0.004
HPLC-ED (no derivatization)	CSF	3	8800	$10\,217 \pm 1163$	0.9690 ± 0.024,
Fluorescence					
Complete HPLC-FL	Buffer	3	280–560	$3.04 \pm 1.7 \times 10^{12}$	0.992 ± 0.008
Complete HPLC-FL	CSF	4	500	$2.54 \pm 0.95 \times 10^{12}$	0.993 ± 0.01

N, number of independent experiments.

A 50-mM concentration of ascorbic acid was found to be adequate to prevent or reverse the tyrosinase-induced formation of *o*-quinones in the enzymatic reaction mixture, thereby inhibiting the subsequent polymerization of the reaction products. The time course of the enzymatic derivatization using a 1-mM concentration of LE showed that the concentration of mushroom tyrosinase (135 U ml^{-1}) used for the enzymatic derivatization of LE allowed the efficient conversion of LE to its dihydroxyphenyl-derivative within 60 min (data not shown). Studies at lower concentrations showed that a reaction time of 5 min is sufficient to reach maximum concentrations of hydroxylated species. This incubation time was used during subsequent HPLC-ED experiments.

3.3. Electrochemical assay

Current–voltage curves for LE and [HO-Tyr^1]-LE (isolated via HPLC) are shown in Fig. 3. When these curves were compared with LE (Fig. 3), [HO-Tyr^1]-LE was seen to be oxidized at considerably lower potentials than LE. As a result of this experiment, the potentials listed in the method section were selected.

By applying 35 pmol of purified dihydroxyphenyl-LE to the boronate clean-up procedure, an average recovery of analyte of 68.7% was achieved when comparing the resulting peak heights with those obtained after direct re-injection of the dihydroxyphenyl-LE species (S.D. = 6.1%, $n = 3$).

A sample chromatograph of dihydroxyphenyl-LE in CSF employing the entire procedure shows the analyte eluting after 6 min (Fig. 2C). Table 1 shows the limits of detection (LOD, defined as twice the baseline noise), slope factors and correlation coefficients (r^2) obtained for the calibration curves constructed for LE in buffer and CSF using the HPLC-ED assay. Table 1 also shows data for calibration curves for LE in CSF using HPLC-ED without enzymatic derivatization and boronate clean-up. The resulting slopes of the calibration curves for buffer and CSF were similar. The limits of detection for LE in CSF correspond to 8.8 pmol ml^{-1} of CSF for the complete HPLC-ED method and 176 pmol ml^{-1} of CSF for analysis by HPLC-ED without derivatization or boronate clean-up. To give an indication of the inter-day variability associated with the complete HPLC-ED method, calibration curves were re-evaluated. The found concentrations were determined from the calibration curve after regression analysis was repeated while omitting the data point under investigation. The relative standard deviation of the found concentrations was found to be smaller than 20% ($n = 3$) and the accuracy was found to be within 6% of the nominal concentration (Table 2).

The evaluated HPLC-ED method for the analysis of LE in CSF compares favorably to existing HPLC methods for opioid peptides with on-line detection [5,12,13] and represents an improvement with respect to limit of detection and practicability when compared to current HPLC-ED methods

Table 2
Nominal concentrations, found concentrations, relative standard deviation (S.D.) and percentage accuracy calculated from three calibration curves for LE in CSF using complete HPLC-ED method

Nominal concentration (pmol 100 μl^{-1})	Found (pmol 100 μl^{-1})	Relative S.D. (%)	% Accuracy
0.88	0.92	20	104
1.73	1.67	12	97
3.33	3.20	12	96
5.24	5.56	1	106
8.57	8.82	9	103

for enkephalins [3,4,14]. The increased practicability of the analytical approach described here is characterized by the minimal precautions required in the preparation of the mobile phase (filtering and degassing under negative pressure with sonication) and is due to the fact that the enzymatic derivatization employed allowed the use of lower applied potentials for electrochemical detection ($+0.3$ V compared to $+0.85$–1.25 V for existing HPLC-ED methods). The low applied potentials allowed the electrochemical detector to be operated at the maximum gain setting since background current and baseline noise were low. These factors, in addition to the observed minimal baseline drift, increased the ease of handling of this analytical approach since minimal precautions could be taken in the preparation of mobile phase and samples. This was demonstrated when a comparison was made between the analysis of LE in CSF using the complete HPLC-ED method incorporating enzymatic derivatization and boronate clean up and the HPLC-ED analysis of LE in CSF without derivatization or boronate clean-up. When an attempt was made to analyze LE in CSF without derivatization or boronate clean-up, considerably higher applied potentials had to be used ($+0.7$ V compared to $+0.3$ V) and as a consequence, background current, baseline noise and baseline drift were greatly increased. Therefore, the maximum gain setting of the instrument could not be used, sensitivity was compromised by a factor of 25 and the linearity of the calibration curves was reduced.

The boronate clean-up procedure used in the HPLC-ED assay described here proved to be an effective and efficient clean-up method for LE in CSF resulting in a clean chromatograph for the analyte (Fig. 2C). To avoid interfering peaks, existing HPLC-ED methods for enkephalins involve complex and relatively non-selective sample clean up procedures (e.g. multiple precipitation, centrifugation and adsorption steps) prior to application of the sample to the HPLC system. By contrast, here the use of a boronate clean-up procedure, which had been previously established for use with catecholamines [15,16], increased the selectivity of this assay as only derivatized peptide incorporating the dihydroxyphenyl group introduced by enzymatic derivatization was retained on the boronate gel column. The limits of detection achieved for LE using this analytical approach (170 fmol inj^{-1} in buffer and 360 fmol inj^{-1} in CSF) also compared favorably to assays for catecholamines using a boronate clean-up method and electrochemical detection (≈ 200 fmol inj^{-1}) although the recovery of dihydroxyphenyl-LE from the boronate gel (69%) was considerably lower than the recovery of catecholamines from the same matrix (80–100%) [15,16].

3.4. Fluorogenic assay

For the fluorogenic reaction, the concentration of reagents used were the same as those used previously for fluorogenic derivatization of tyrosine-containing peptides following enzymatic hydroxylation by mushroom tyrosinase [11]. Here, as before, the concentrations of DPE and KCl used were taken directly from corresponding catechol assays [17,18] but the concentration of potassium ferricyanide was increased to ensure

the formation of the quinone from the dihydroxy-phenyl-LE derivative despite the presence of high concentrations of ascorbic acid. Acetonitrile was included in the reaction mixture to facilitate the condensation reaction by reducing the thermodynamic activity of water.

Fig. 2D shows a typical chromatogram obtained under these conditions. Table 1 shows a summary of the results obtained in the HPLC-FL assay. Limits of detection for LE of at least 500 fmol per injection could be obtained in both buffer samples and spiked CSF samples. This limit of detection corresponds to 12 pmol of LE per ml of CSF. The assay was in general more difficult to perform with a higher number of outliers observed during the assay development when compared to the HPLC-ED assay. Accuracy for read-back calculations of the calibration curves in CSF was between 82 and 118% with relative standard deviations averaging 25%. This indicated that the HPLC-FL is more variable than the HPLC-ED assay. In addition, slope factors obtained for CSF samples were smaller than those obtained in buffer, indicating some interference of the fluorescence species with biological material. Although a lower limit of detection in buffer samples (277 fmol inj^{-1}) was obtained when a 5-min reaction time instead of a 60-min reaction time was used for the tyrosinase reaction, when this modification of the method was attempted in spiked CSF samples, the linearity of the calibration curve could not be preserved.

The HPLC-FL analytical approach for LE described here yielded detection limits of 500 fmol per injection in spiked CSF samples, corresponding to 12 pmol of LE per ml of CSF. This HPLC-FL assay shows similar sensitivity or represents an improvement in the limit of detection by one order of magnitude when compared to existing tyrosine-specific HPLC-FL methods with pre-column fluorescence derivatization [7–10]. The improvement in limit of detection seen with the approach described here may be attributed in part to the fact that in some of these existing tyrosine-specific HPLC-FL methods [7,8], fluorogenic derivatization necessitates the use of harsh reaction conditions which may result in peptide instability and consequently reduced recovery of intact fluorescence-labeled analyte. Although more selective, the HPLC-FL approach described here does not achieve limits of detection for LE as low as those obtained by investigators using methods involving post-column fluorescence derivatization of the N-terminal primary amino group of opioid peptides (36–100 fmol inj^{-1}) [5,19,20]. However, due to lack of selectivity, these methods often involve the use of extensive sample clean-up procedures and complex multi-dimensional chromatographic systems (column switching) to minimize the occurrence of interfering peaks [5]. The higher sensitivity obtained using these methods can also be attributed to the use of laser-induced fluorescence detection [19,20] and a microbore chromatographic system [19]. Incorporating a microbore chromatographic system and laser-induced fluorescence into the analytical approach described here may be expected to reduce considerably the limits of detection obtainable by this HPLC-FL assay.

4. Conclusion

Both the HPLC-ED and HPLC-FL methods for LE described here should be applicable to the entire range of opioid peptides since the N-terminal tyrosine group which is initially derivatized is preserved throughout the entire family of opioid peptides. This approach should also be applicable to the analysis of other tyrosine-containing proteins and peptides as it has been shown previously that the amino acid adjacent to the tyrosine group does not dramatically influence the tyrosinase reaction [11]. Endogenous levels of opioid peptides in human CSF, as determined by radioimmunoassay lie in the fmol ml^{-1} range. Therefore, although in their present form, these methods are inadequate for the determination of endogenous levels of opioid peptides in human CSF, given sufficient sample (>1 ml) they may be adequate for the analysis of the elevated physiological concentrations of opioid peptides to be expected in clinical studies.

Acknowledgements

We thank Dr. Betty L. Grundy (VAMC, Gainesville FL) for collecting the cerebrospinal fluid and Dr. R. Prankerd, Department of Pharmacy, University of Queensland, Australia for providing DPE. This work was supported by a PDA Foundation and Schering-Plough Corporation Foundation Grant in Biotechnology.

References

[1] R.S. Rapaka, F. Porreca, Pharm. Res. 8 (1991) 1–8.
[2] S. Mueller, B. Ho, P.L. Gambus, W. Millard, G. Hochhaus, J. Pharm. Biomed. Anal. 16 (1997) 101–109.
[3] L.H. Fleming, N.J. Reynolds, J. Chromatogr. 431 (1988) 65–76.
[4] C. Kim, R. Cheng, S.R. George, J. Chromatogr. 494 (1989) 67–76.
[5] M. Mifune, D.K. Krehbiel, J.F. Stobaugh, C.M. Riley, J. Chromatogr. 496 (1989) 55–70.
[6] S. Lunte, O. Wong, Liq. Chromatogr. Gas Chromatogr. 7 (1989) 908–916.
[7] J. Ishida, M. Kai, Y. Ohkura, J. Chromatogr. 356 (1986) 171–177.
[8] M. Kai, J. Ishida, Y. Ohkura, J. Chromatogr. 430 (1988) 271–278.
[9] M. Nakano, M. Ohno, M. Kai, Y. Ohkura, J. Chromatogr. 411 (1987) 305–311.
[10] G. Zhang, M. Kai, M. Nakano, Y. Ohkura, Chem. Pharm. Bull. 39 (1991) 126–129.
[11] M. Tellier, R.J. Prankerd, G. Hochhaus, J. Pharm. Biomed. Anal. 9 (1991) 557–563.
[12] P. de Montigny, C.M. Riley, L.A. Sternson, J.F. Stobaugh, J. Pharm. Biomed. Anal. 8 (1990) 419–429.
[13] W.M. Muck, J.D. Henion, J. Chromatogr. 495 (1989) 41–59.
[14] S. Shibanoki, S.B. Weinberger, K. Ishikawa, J. Martinez, J. Chromatogr. 532 (1990) 249–259.
[15] S. Higa, T. Suzuki, A. Hayashi, I. Tsuge, Y. Yamamura, Anal. Biochem. 77 (1977) 18–24.
[16] K. Koike, T. Aono, F. Chatani, T. Takemura, K. Kurachi, Life Sci. 30 (1982) 2221–2228.
[17] A. Mitsui, H. Nohta, Y. Ohkura, J. Chromatogr. 344 (1985) 61–70.
[18] H. Nohta, A. Mitsui, Y. Ohkura, Anal. Chim. Acta 165 (1984) 171–176.
[19] K.J. Dave, J.F. Stobaugh, T.M. Rossi, C.M. Riley, J. Pharm. Biomed. Anal. 10 (1992) 965–977.
[20] C.M.B. van den Beld, U.R.B. Tjaden, N.J. Reinhoud, D.S. Stegehuis, J. van der Greef, J. Control. Release 13 (1990) 129–139.