Journal of Chromatography, 430 (1988) 271–278
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4268

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LEUCINE-ENKEPHALIN-LIKE PEPTIDE IN RAT BRAIN BY PRE-COLUMN FLUORESCENCE DERIVATIZATION INVOLVING FORMYLATION FOLLOWED BY REACTION WITH 1,2-DIAMINO-4,5-DIMETHOXYBENZENE

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(First received January 13th, 1988; revised manuscript received April 14th, 1988)

SUMMARY

Fluorimetrically reactive leucine-enkephalin, one of the opioid peptides in rat brain tissues such as the striatum, cortex and hypothalamus, was assayed by reversed-phase high-performance liquid chromatography with fluorescence detection based on pre-column derivatization of the tyrosyl residue. The tyrosine-containing peptides extracted from the tissue were first formylated with chloroform in an alkaline medium, and the resulting aldehydes were then converted into highly fluorescent derivatives by reaction with 1,2-diamino-4,5-dimethoxybenzene. The derivative of leucine-enkephalin-like peptide in tissue was separated from variuos other fluorescent compounds on a reversed-phase column (TSK gel ODS-120T) by isocratic elution and detected by fluorimetry. The concentrations of the leucine-enkephalin-like peptide in the tissues were 20–245 pmol/g. The method is sensitive enough to permit the quantitative determination of the endogenous peptide at concentrations as low as 5.6 pmol/g in brain tissues.

INTRODUCTION

Leucine-enkephalin (LE; Tyr–Gly–Gly–Phe–Leu) is an opioid peptide that occurs in mammalian brain tissues [1]. Opioid peptides play a role in the control of pain sensation [1]. Most of the opioid peptides are known to have a tyrosyl residue at the N-terminus of their amino acid sequence. The sequencing analysis of enkephalin precursors indicates the existence of various peptides retaining the sequence of either LE or methionine-enkephalin (ME; Tyr–Gly–Gly–Phe–Met) [2].

Many methods, such as radioimmunoassay (RIA), radioreceptor assay, bioassay and mass spectrometry, either alone or in combination with high-perform-

0378-4347/88/$03.50

ance liquid chromatography (HPLC), have been used for the quantitative determination of the opioid peptides in biological samples. RIA methods [3,4] generally offer a high sensitivity for the peptides, but there is still difficulty in the preparation of specific antibodies especially for the oligopeptides [5]. The methods of radioreceptor assay [6] and bioassay [7] are both less selective for the individual opioid peptides. The mass spectrometric method [8] coupled with off-line HPLC can determine the endogenous LE and ME in brain tissues, because the method of detection has a satisfactory sensitivity and structural specificity for the peptides. However, the mass spectrometric method is technically difficult for the simultaneous determination of several opioid peptides.

HPLC can simultaneously separate many synthetic and biological peptides [9,10]. However, the quantification of the biogenic peptides by HPLC is difficult especially for biological samples, because there are more peptides present in a biological extract and biological interferences may occur. Simple detection methods in HPLC are generally based on ultraviolet (UV) absorption at a wavelength between 200 and 230 nm [10] or the electrochemical response [11]. UV detection in HPLC is less selective for peptides. Therefore, several peptides and some other biogenic substances in complex samples often interfere with the detection of target peptides [12]. Although electrochemical detection (ED) is more selective than UV detection, oxidizing and/or reducing substances in biological samples may disturb the sensitive determination of the peptides. On the other hand, fluorimetry with HPLC permits more sensitive detection of biological peptides [13], because therer are fewer fluorescent substances in biological fluids, and also two wavelengths (excitation and emission) are used for measuring the fluorescence intensity.

We previously reported a reversed-phase HPLC method with pre-column fluorescence derivatization for the selective and sensitive determination of the synthetic tyrosine-containing peptides involving LE [14]. This derivatization is based on two reaction steps in which the tyrosyl residue of the peptide is formylated selectively at the *ortho* position of its phenolic moiety by reaction with chloroform in potassium hydroxide solution, and the resulting peptide is then derivatized to a fluorophore by reaction with 1,2-diamino-4,5-dimethoxybenzene (DDB) [15], a fluorogenic reagent for aromatic aldehydes in weakly acidic solution (Fig. 1).

The aim of this study was to develop an HPLC method with fluorescence detection utilizing the above derivatization for the quantification of LE in complex biological samples such as rat brain.

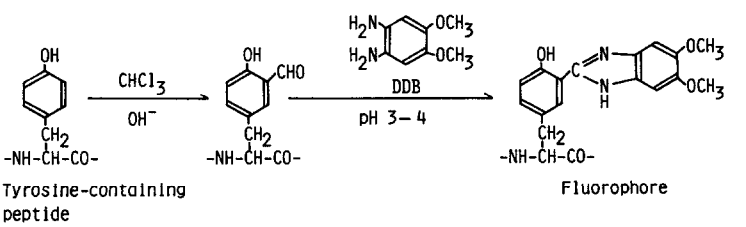

Fig. 1. Fluorescence derivatization of tyrosine-containing peptides with DDB.

EXPERIMENTAL

Chemicals and solutions

The following peptides were obtained from the Protein Research Foundation (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.): LE, ME, Tyr–Ala–Ala–Phe–Met ([Ala^2,Ala^3]ME), Tyr–Gly–Gly–Phe, Tyr–Gly–Gly, Tyr–Gly, Gly–Tyr, Arg–Val–Tyr–Ile–His–Pro–Phe and Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu. DDB monohydrochloride was purchased from Dojindo Lab. (Kumamoto, Japan) and carboxypeptidase A treated with diisopropyl fluorophosphate from Funakoshi (Tokyo, Japan). Water was deionized and distilled before use. The synthetic peptides were dissolved in water and stored at −80 °C. The solutions were usable for at least three weeks. The other chemicals were of analytical-reagent grade.

Peptide extraction

Male Sprague–Dawley rats (220–280 g) were anaesthetized with diethyl ether and killed by dehaematization. Each region of the striatum, cortex and hypothalamus in the brain was quickly removed and stored at −80 °C until used.

A portion (ca. 0.2 g) of the tissue was homogenized at 0–4 °C with 3 ml of 0.1 *M* hydrochloric acid, then a 50-μl portion of 1.0 nmol/ml [Ala^2,Ala^3]ME as internal standard was added to the homogenate. The homogenate was transferred into a centrifuge tube, rinsing with 1 ml of 0.1 *M* hydrochloric acid. The homogenate was deproteinized with 0.5 ml of 2 *M* perchloric acid. After centrifugation at 800 *g* for 10 min, the precipitate was suspended with 2 ml of 0.2 *M* perchloric acid and centrifuged again. The combined supernatant was then neutralized at pH 7–8 with ca. 2 ml of 1 *M* sodium hydrogencarbonate solution. The deproteinized solution was applied to a cartridge (Bond Elut C_{18}; Analytichem International, Harbor City, CA, U.S.A.). In advance, the cartridge was washed with 3 ml each of water and methanol. After loading the sample solution, 1 ml of water, 2 ml of dichloromethane to remove strongly hydrophobic substances, 1 ml of water, 3 ml of 0.1 *M* hydrochloric acid, 1 ml of water, 3 ml of 0.1 *M* borate buffer (pH 8.5) and 1 ml of water were successively passed through the cartridge. Finally, the LE-rich fraction was obtained by the elution with 1 ml of aqueous 90% methanol. After evaporation in vacuo at ca. 30 °C, the residue was dissolved in 200 μl of water. The solution obtained was used for fluorescence derivatization.

Fluorescence derivatization

To a 100-μl portion of the sample solution were added 50 μl of chloroform and 25 μl of 3.0 *M* potassium hydroxide solution. The mixture was warmed at 60 °C for 10 min, then cooled in ice–water for ca. 1 min. To the mixture, 25 μl of 14 *M* acetic acid and 25 μl of 4.6 m*M* DDB were added. The mixture was warmed at 60 °C for 18 min to derivatize the formylated residue, then cooled. A 100-μl portion of the final reaction mixture was analysed by HPLC.

Enzymatic degradation

For the identification of the peptide peaks, a portion (100 μl) of the sample eluted from the cartridge was mixed with 30 μl of 50 m*M* phosphate buffer (pH

7.5) containing $5 \cdot 10^{-4}$ U (0.25 μg) of carboxypeptidase A. The mixture was incubated at 37°C for 30 min. The final mixture (100 μl) was then used for the above fluorescence derivatization.

Apparatus and HPLC conditions

The HPLC system consisted of a Toyo Soda 803 D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Shimadzu RF 530 fluorescence spectrophotometer fitted with a 12-μl flow cell operating at an emission maximum wavelength of 425 nm and an excitation maximum wavelength of 350 nm for the derivatized peptides. A reversed-phase column (250 mm×4.6 mm I.D.) packed with TSK gel ODS-120T (particle size 5 μm) (Tosoh, Tokyo, Japan) was used. The column temperature was ambient (24±4°C). For the separation of the fluorescent derivatives of peptides on the column, mobile phase composed of acetonitrile–50 m*M* phosphate buffer (pH 2.2)–50 m*M* sodium 1-hexanesulphonate (26:64:10, v/v/v) was pumped at a flow-rate of 1.0 ml/min. When the tissue sample was analysed, the column was washed in order to elute more hydrophobic substances retained on the column for ca. 20 min with aqueous 50% acetonitrile after the LE peak had eluted.

Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path length 10×10 mm); spectral band widths of 5 nm were employed for both the excitation and emission monochromators.

RESULTS AND DISCUSSION

Separation

The fluorescent derivatives of the synthetic peptides of LE, ME and [Ala^2,Ala^3]ME were separated within 27 min on the reversed-phase column (TSK gel ODS-120T) by isocratic elution with acetonitrile–50 m*M* phosphate buffer (pH 2.2)–50 m*M* sodium 1-hexanesulphonate (Fig. 2A). In the chromatogram (Fig. 2B), however, many fluorescent peaks other than those of the peptides were observed when the detection was carried out in a range of high sensitivity permitting the detection of picomole amounts. These peaks were formed from the DDB reagent during derivatization.

Sodium 1-hexanesulphonate in the mobile phase was required as an ion-pair reagent for the complete separation of the peptide peaks from the artifacts of the reagent blank as shown in Fig. 2. In its absence, the fluorescent peaks of the synthetic peptides of LE, ME and [Ala^2,Ala^3]ME were eluted earlier, and then the peaks of ME and [Ala^2,Ala^3]ME overlapped with some peaks of the reagent blank.

The acidic buffer in the mobile phase was necessary because the derivatized peptides fluoresced most intensely at pH 1.3–3.5 at 425 nm for maximum emission with irradiation at 350 nm for maximum excitation [14].

The proposed derivatization method gives fluorescent products not only for tyrosine-containing peptides [14] but also for α-keto acids [16] and sialic acids

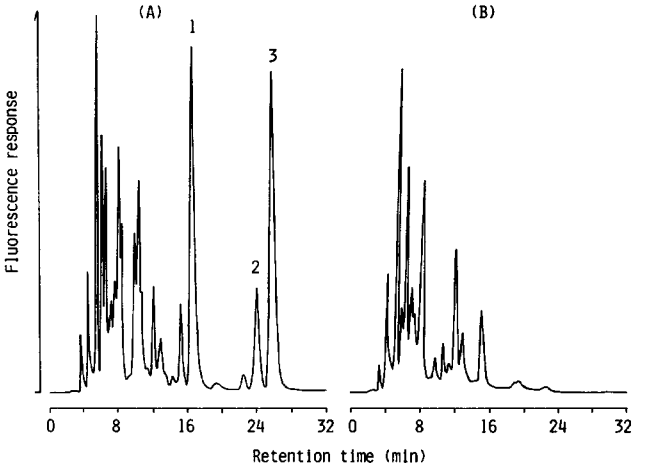

Fig. 2. Chromatograms of (A) a standard mixture of LE, ME and [Ala^2,Ala^3]ME (140 pmol each per 100-μl injection volume) and (B) water for the reagent blank. A 100-μl portion of the solution of the synthetic peptides or water for the blank was treated according to the derivatization procedure and the final reaction mixture (100 μl) was subjected to HPLC. Peaks: 1 = ME; 2 = [Ala^2,Ala^3]ME; 3 = LE.

TABLE I

RETENTION TIMES OF THE DDB FLUORESCENT DERIVATIVES OF TYROSINE-CONTAINING PEPTIDES, TYROSINE, α-KETO ACIDS AND SIALIC ACID

Compound	Retention time (min)	Compound	Retention time (min)
LEK	25.8	Gly-Tyr	6.0
[Ala^2,Ala^3]ME	24.0	Tyr	5.6
ME	16.8	α-Ketoglutaric acid	6.8
Tyr-Gly-Gly-Phe	11.2	Pyruvic acid	6.8
Tyr-Gly-Gly	5.6	Phenylpyruvic acid	14.8
Tyr-Gly	5.6	*p*-Hydroxyphenylpyruvic acid	11.2
		N-Acetylneuraminic acid	8.0

[17], as the DDB reagent reacts directly with these acids, although the optimum reaction conditions of the acids are different from those of the formylated peptides. However, biological substances (e.g., α-ketoglutaric acid, pyruvic acid and N-acetylneuraminic acid) and enkephalin-related peptides could be separated from the peptides of LE, ME and [Ala^2,Ala^3]ME (Table I). In addition, thyrosine-containing peptides such as Arg-Val-Tyr-Ile-His-Pro-Phe and Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu, with larger molecules than LE, were eluted later than LE [14]. The optimum reaction conditions, permitting the production of a single fluorescent derivative for each peptide, were the same as those previously reported [14].

Determination of LE in brain tissues

The extraction of the peptide from rat brain was accomplished by the procedures of homogenization and deproteination as described previously [3,4,6].

The extacts were passed through an ODS mini-cartridge (Bond Elut C_{18}) for clean-up because many biological substances in the sample interfered with the derivatization and the subsequent HPLC separation. This technique has often been used for the partial purification of bioactive peptides in biological samples [18]. The use of the cartridge facilitated not only the removal of large amounts of some interfering substances but also the concentration of the peptides in a small sample size with a high recovery. The recoveries of 50 pmol each of LE and [Ala^2,Ala^3]ME internal standard added to the homogenate were both approximately 80% ($n=5$).

Fig. 3A shows a typical chromatogram of striatum tissue of rat brains obtained by the recommended procedure. Fluorescent peaks at the same retention times as those of synthetic LE and the internal standard were apparently detected. There was no peak corresponding to the internal standard in the chromatogram when the peptide was not added to the homogenate.

The LE-like peptide peak showed the same fluorescence excitation and emission spectra as those of the synthetic LE. Additionally, the peak was not formed when the same derivatization was carried out but the formylation procedure was omitted, as shown in Fig. 3B. To provide further evidence for the identification of the peptide, the same tissue sample was treated with carboxypeptidase before the fluorescence derivatization. The shaded peaks in the chromatogram in Fig. 3A disappeared after the enzymatic degradation. Therefore, these peaks correspond to the digested peptides.

The observation of the endogenous LE-like peptide in other tissue regions such as the cortex and hypothalamus of the rat brains is shown in Fig. 4. However, the detection of the endogenous ME-like peptide was impossible because some other

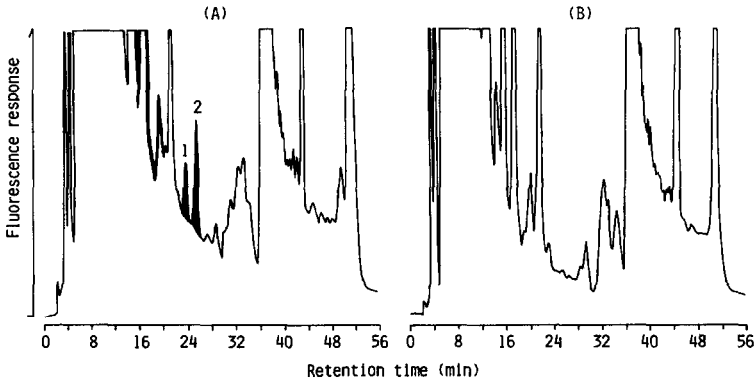

Fig. 3. Chromatograms of striatum tissue of rat brains obtained by (A) the recommended procedure and (B) the same procedure but omitting the formylation. In the fluorescence derivatization procedure for chromatogram B, chloroform was not added to the reaction mixture. Peaks: 1 = [Ala^2,Ala^3]ME; 2 = LE. Shaded areas were not observed when the same sample was treated with carboxypeptidase A as described under Experimental.

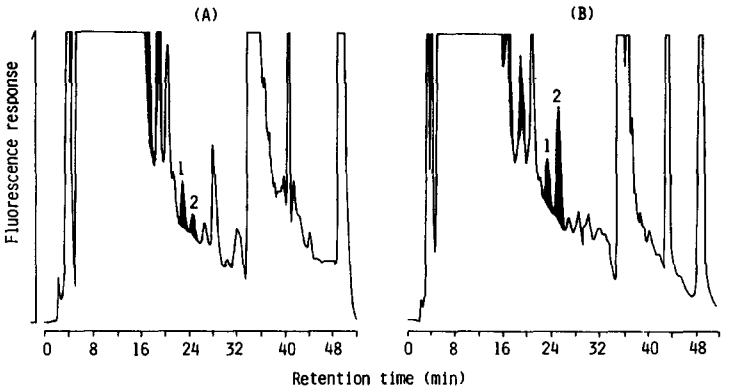

Fig. 4. Chromatograms of (A) cortex tissue and (B) hypothalamus tissue of rat brains. The samples were treated in the same way as in Fig. 3A. Peaks: 1 = [Ala^2,Ala^3]ME; 2 = LE. Shaded areas were not observed after the enzymatic degradation with carboxypeptidase A.

large peak(s), which were not degraded by the enzyme reaction with carboxypeptidase, eluted at the retention time (16.8 min) of the synthetic ME peak. This problem would be better resolved by utilizing HPLC techniques such as gradient elution and/or column switching.

The calibration graph of LE for the striatum tissue, which was constructed by plotting the ratio of the peak height of the spiked LE against the peak height of the internal standard, was linear in the concentration range corresponding to 0–200 pmol of LE added to the tissue homogenate. The equation was $y = 0.011x - 0.179$ and the correlation coefficient (r) was 0.9988. No significant change in the slope of the graph was observed with different tissues. The above results demonstrate that the present internal standard method permits the quantification of endogenous LE.

The concentration of LE in rat brains ($n=5$) was 211 pmol/g in striatum, 20 pmol/g in cortex and 245 pmol/g in hypothalamus. These values are very close to the results obtained by RIA [3].

The sensitivity of this HPLC method is sufficient for the quantitative determination of endogenous LE at the picomole level in brain tissue. The limit of detection at a signal-to-noise ratio of 2 for the peptide in tissues is 5.6 pmol/g, which corresponds to an amount of ca. 500 fmol injected.

This study has provided the first practical HPLC method with fluorescence detection for the quantification of LE-like peptide in such complex biological samples.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. We are also grateful to Miss U. Taguchi for skilful assistance.

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