Pre-column Fluorescence Derivatization High-Performance Liquid Chromatography of Opioid Peptides in Rat Brain and Its Use for Enzymatic Peptide Characterization

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A high-performance liquid chromatographic method involving fluorescence derivatization followed by separation on a reversed-phase polymer (octadecylated polyvinylalcohol copolymer gel) column is described for the determination of opioid peptides in rat brain tissues. The peptides extracted from brain tissues were converted into fluorescent derivatives by reaction with hydroxylamine, cobalt(II) ion and borate. The derivatives were separated on an Asahipak ODP-50 column by gradient elution of acetonitrile in the mobile phase containing borate buffer (pH 9.5). The detection limits ($S/N=3$) for the peptides were 0.33—1.21 pmol per 100 μ l injected. The method actually permit the determination of leucine enkephalin, methionine enkephalin, methionine enkephalin-Arg-Phe and methionine enkephalin-Arg-Gly-Leu in the tissues. The method is also applied to the characterization of the peptides in the tissues by means of enzymatic degradations with carboxypeptidase A and trypsin.

Keywords opioid peptide; brain tissue; pre-column fluorescence derivatization; high-performance liquid chromatography; octadecylated polyvinylalcohol copolymer gel column

Opioid peptides involving leucine enkephalin, (LE, Tyr-Gly-Gly-Phe-Leu) and methionine enkephalin (ME, Tyr-Gly-Gly-Phe-Met)¹⁾ have been found to act *in vivo* as neurotransmitters *via* interaction with opiate receptors.²⁾ These peptides retain the sequence of either LE or ME,³⁻⁵⁾ which have a tyrosyl residue at the N-terminus in their molecules.

Many methods, such as bioassay,⁶⁾ radioimmunoassay (RIA),⁷⁻⁹⁾ enzyme-immunoassay,¹⁰⁾ radioreceptor-assay¹¹⁾ and mass spectrometry,¹²⁾ either alone or in combination with high-performance liquid chromatography (HPLC), have been developed for the determination of the opioid peptides in biological samples. The bioassay, immunoassay and receptor-assay are not very selective.¹³⁾ The mass spectrometric method does not permit the simultaneous determination of opioid peptides, though the structural specificity of each peptide is sufficient.

Intact opioid peptides can be separated simultaneously by HPLC. However, spectrophotometric¹⁴⁾ and electrochemical¹⁵⁾ detections in HPLC are less selective for the opioid peptides. We previously developed a fluorescence derivatization reaction specific for N-terminal Tyr-containing peptides,¹⁶⁾ which uses hydroxylamine, cobalt(II) ion and borate reagents. This reaction provides a single fluorescent product for each of N-terminal Tyr-containing peptides. This reaction was utilized for a post-column fluorescence derivatization HPLC for the determination of several opioid peptides in rat brain.¹⁷⁾ The reaction was also applied to a pre-column fluorescence derivatization method of HPLC for N-terminal Tyr-containing peptides, where the fluorescent derivatives of the peptides were separated on an octadecylated silica gel column.¹⁸⁾ However, the method only allowed the assay of LE and ME in rat brain,¹⁹⁾ presumably due to an increased adsorption of the derivatives at the polar matrix in the support material of the column.

We have recently found that an octadecylated polyvinylalcohol copolymer gel column shows less adsorption of the fluorescent derivatives of the opioid peptides than the silica type of column and can afford an improved separation pattern. Thus, the column was subjected to the use in a pre-column fluorescence derivatization method for HPLC in the hope that endogenous opioid peptides other

than LE and ME in rat brain tissues could be quantified. [D-Ala²]LE-NH₂ was used as an internal standard for the quantification. The method was also utilized for the characterization of the peptides in the tissues by means of enzymatic degradations with carboxypeptidase A and trypsin.

Experimental

Chemicals and Solutions The following synthetic peptides were purchased from Sigma (St. Louis, MO, U.S.A.). The sequences and the abbreviations of named opioid peptides are shown in the parentheses: Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, LE, LE-NH₂, [D-Ala²]LE-NH₂, LE-Arg, ME, ME-Arg, ME-Arg-Phe, ME-Arg-Gly-Leu, α -endorphin (α -EDP, ME-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr), γ -EDP (α -EDP-Leu), β -neoendorphin (β -NED, LE-Arg-Lys-Tyr-Pro), α -NED (β -NED-Lys), dynorphin 1-10 (DNP 1-10, LE-Arg-Arg-Ile-Arg-Pro), DNP 1-9, DNP 1-8, DNP 1-7 and DNP-B (LE-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr). These peptides were dissolved in water. The solutions were stored at -80°C and consumed within 3 weeks. Carboxypeptidase A (EC 3.4.17.1) and trypsin (EC 3.4.21.4) from bovine pancreas were purchased from Sigma. Water was deionized and then distilled before use. Other chemicals were of reagent grade.

Rat Brain Tissues Male Sprague-Dawley rats (220—280 g, 7 weeks) were anesthetized with diethyl ether and killed by dehematization. The tissues of striatum, hypothalamus, pituitary and cortex were obtained in the same manner as described previously.¹⁹⁾ The tissues were stored at -80°C .

Peptide Extraction This was performed according to the previously reported solid-phase extraction method¹⁷⁾ with modifications. Each tissue (0.1—0.2 g) was homogenized at $0-4^{\circ}\text{C}$ with 3 ml of 0.1 M HCl. To the homogenate, 20 μ l of 5.0 μM [D-Ala²]LE-NH₂ as an internal standard and 20 μ l of water (or synthetic peptide solution for calibration graphs) were added. The homogenate was deproteinized with 0.5 ml of 2 M HClO₄ and centrifuged at 2450g for 10 min. The supernatant was neutralized to pH 7—8 with 1 M NaHCO₃ (approximately 2 ml) and mixed with 2 ml of 50 mM ethylenediamine tetraacetic acid (EDTA)·2Na. The mixture was applied to a mini-cartridge packed with 0.2 g of Asahipreggel T C18 (particle size, 9 μm ; Asahi Chemical Ltd., Kawasaki, Japan). The cartridge was preconditioned with 2 ml of methanol, of 0.1 M borate buffer (pH 8.5) and of water. After loading the supernatant, the cartridge was washed by consecutive portions of 1 ml of water, 3 ml of 0.1 M HCl, 3 ml of water, 3 ml of 0.1 M borate buffer (pH 8.5) and 1 ml of water. A fraction containing the peptides was eluted with 1.5 ml of a mixture of methanol and 0.2 M borate buffer (pH 8.5) (4:1, v/v). After evaporation *in vacuo* at 30°C , the residue was diluted with water to 300 μ l and used for the subsequent fluorescence derivatization or enzymatic degradation reaction.

Fluorescence Derivatization This was performed as described previously with minor modifications.¹⁷⁾ To 60 μ l of the sample solution were added 25 μ l of a mixture of 10 mM NH₂OH·HCl and 0.5 mM Co(II) (OAc)₂, and

15 μ l of water. The mixture was heated at 100 °C for 3 min and then added with 25 μ l of 20 mM 2-mercaptoethanol (stabilizer of the derivatives). The reaction mixture was used for HPLC.

Enzymatic Degradation The sample solution (60 μ l) was mixed with 150 mU of carboxypeptidase A or 1100 mU of trypsin in 50 mM phosphate buffer (pH 7.5). The mixture was incubated at 37 °C for 30 min and then subjected to the fluorescence derivatization.

Chromatograph and Its Operation Conditions The HPLC system consisted of Hitachi 655 chromatograph, a Rheodyne 7125 syringe-loading sample injector (100 μ l loop), a Hitachi F-1100 fluorescence spectrophotometer (12 μ l flow-cell) operating at 440 nm (emission wavelength) and 330 nm (excitation wavelength) and an Asahipak ODP-50 column (octadecylated polyvinylalcohol copolymer gel column; 150 $\times$ 6 mm i.d.; particle size, 5 μ m, Asahi Chemical Ltd.). The column temperature was ambient (24 $\pm$ 4 °C). A gradient elution was performed by using two eluents that consisted of acetonitrile, 0.1 M borate buffer (pH 9.5) and water (1:10:39 and 5:2:3, v/v). The variation of acetonitrile concentration in the mobile phase is indicated in Fig. 1. The flow-rate of the mobile phase was 0.8 ml/min up to 25 min, thereafter 1.0 ml/min up to 55 min and again 0.8 ml/min.

Results and Discussion

Figure 1 shows a chromatogram obtained with a standard mixture of seventeen N-terminal Tyr-containing synthetic peptides including the opioid peptides. The derivatives of the peptides except for those of DNP 1-8 and β -NED (peaks coincided) were clearly separated on the reversed-phase polymer column. In addition, the synthetic peptides of Tyr-Gly, Tyr-Gly-Gly and ME-Arg were also derived. Their retention times were 3.2, 3.2 and 16.7 min, respectively, under the same HPLC conditions as for Fig. 1, though these peaks are not shown in Fig. 1. The detection limits ($S/N=3$) for the peptides were in the range 0.33–1.21 pmol on the column.

Figure 2 shows a chromatogram obtained with striatum tissue (approximately 40 mg) of rat brain according to the recommended procedure. The N-terminal Tyr-containing peptides were represented by shaded peaks. Other fluorescent peaks were also observed even if the derivation was omitted. Therefore, these peaks were caused by some fluorescent compounds present in the tissue. Similar chro-

matograms to Fig. 2 were obtained with other tissues of rat brain such as hypothalamus, pituitary and cortex.

The peptide derivatives fluoresce only in a borate buffer at pH 8–10^{16,18}; the recommended mobile phase contains 0.1 M borate buffer of pH 9.5. The pH of the borate buffer in the mobile phase affected the retention times of the peaks. At pH 8.5, the synthetic peptide peaks were eluted slightly earlier than at pH 9.5 and are clearly separated. However, the peptide peaks of [D-Ala²]LE-NH₂ and ME-Arg-Gly-Leu overlapped with some peaks of fluorescent substances in the biological sample eluted at retention times between 22 and 26 min in the chromatogram of Fig. 2.

The opioid peptide peaks in Fig. 2 were identified by chromatographic techniques based on retention time and co-chromatography with their synthetic peptides. For further characterization of the peptides, the enzymatic degradations utilizing carboxypeptidase A and trypsin were carried out for the tissue samples before the fluorescence derivatization. Carboxypeptidase A releases amino acid from the C-terminal end of peptides which do not contain Arg, Lys, Pro, Gly and amino acid amide residues. Trypsin

Fig. 2. Chromatogram of Striatum Tissue of Rat Brain

Shaded peaks were produced only by the fluorescence derivation. Peaks: 1, ME; 2, LE; 3, [D-Ala²]LE-NH₂ (internal standard); 4, ME-Arg-Gly-Leu; 5, LE-Arg-Phe.

Fig. 3. Chromatogram of the Same Sample as for Fig. 2 after the Enzymatic Degradation with Carboxypeptidase A

Arrows 1–5 indicate the retention times of ME, LE, [D-Ala²]LE-NH₂, ME-Arg-Gly-Leu and ME-Arg-Phe, respectively.

Fig. 1. Chromatogram of a Standard Mixture of Synthetic Seventeen N-Terminal Tyr-Containing Peptides

Peaks (amount injected): 1, Tyr-Gly-Gly-Phe (5 pmol); 2, ME (5 pmol); 3, LE (5 pmol); 4, LE-Arg (5 pmol); 5, α -EDP (10 pmol); 6, LE-NH₂ (10 pmol); 7, DNP 1-7 (10 pmol); 8, [D-Ala²]LE-NH₂ (10 pmol); 9, ME-Arg-Gly-Leu (10 pmol); 10, γ -EDP (10 pmol); 11, ME-Arg-Phe (5 pmol); 12, DNP 1-8 (10 pmol); 13, β -NED (50 pmol); 14, α -NED (50 pmol); 15, DNP 1-9 (10 pmol); 16, DNP 1-10 (10 pmol); 17, DNP-B (50 pmol).

Fig. 4. Chromatogram of the Same Sample as for Fig. 2 after the Enzymatic Degradation with Trypsin

Arrows 1—5 indicate the retention times of ME, LE, [D-Ala²]LE-NH₂, ME-Arg-Gly-Leu and ME-Arg-Phe, respectively.

mediates the hydrolysis of peptide bonds at the carboxyl sides of Arg and Lys.

As expected, the peaks corresponding to ME, LE, ME-Arg-Gly-Leu and ME-Arg-Phe disappeared, when the same sample of Fig. 2 was digested with carboxypeptidase A (Fig. 3). On the other hand, some increasing peaks were also observed. The peak at 3.2 min contains probably the peak due to Tyr-Gly-Gly, and the peak at 11.2 min was Tyr-Gly-Gly-Phe, because both peptide fragments were produced from ME and LE by the enzyme digestion. A small peak due to ME-Arg was also observed, because the fragment of ME-Arg was formed from ME-Arg-Phe.

After the degradation of the same sample with trypsin (Fig. 4), the ME-Arg-Gly-Leu and ME-Arg-Phe peaks disappeared, while the peaks due to ME, LE and [D-Ala²]LE-NH₂ (internal standard) remained. Instead, the peak due to the fragment of ME-Arg produced from ME-Arg-Gly-Leu and ME-Arg-Phe was observed.

From these results, the shaded peaks (peaks 1, 2, 4 and 5 in Fig. 2) represent the endogenous peptides corresponding to ME, LE, ME-Arg-Gly-Leu and ME-Arg-Phe, respectively. The same results were obtained from the hypothalamus, pituitary and cortex samples. The other shaded peaks in Fig. 2 could not be characterized successfully.

The peptide extract from brain tissue was passed through a reversed phase mini-cartridge packed with AsahipregelT C18, which consists of the same material as used in the Asahipak ODP-50 column, otherwise marked interferences with biological substances were observed. Additionally the target peptides were concentrated in small volumes.

The recoveries of 100 pmol each of synthetic LE, ME, [D-Ala²]LE-NH₂, γ -EDP, ME-Arg-Phe, ME-Arg-Gly-Leu, DNP 1-8 and DNP-B added to the homogenate of striatum, hypothalamus, pituitary or cortex were 41–45%, ($n=3$). The low recoveries for these peptides were mainly caused by the interference of other sample constituents with the derivatization reaction. Nevertheless, the constant ratio of the recovery of each peptide to that of the internal standard, [D-Ala²]LE-NH₂ was obtained with each treatment.

TABLE I. Regional Concentrations of Opioid Peptides in Rat Brains

Tissue	Concentration ^{a)} (pmol per g of tissue)			
	ME	LE	ME-Arg-Gly-Leu	ME-Arg-Phe
Striatum	541	161	118	128
Hypothalamus	350	100	85	86
Pituitary	185	114	ND ^{b)}	159
Cortex	63	22	34	33

a) The average value of triplicate determinations. b) Could not be determined successfully, because of an interfering peak.

The ratio of the peak height of each of ME, LE, ME-Arg-Phe and ME-Arg-Gly-Leu to that of the internal standard added to the homogenate of striatum, hypothalamus, pituitary or cortex was linear in the range 10–800 pmol per g of the tissue. The correlation coefficients (r) of these calibration graphs were greater than 0.9964 ($n=3$ each plot).

The concentrations values of ME, LE, ME-Arg-Gly-Leu and ME-Arg-Phe in striatum, hypothalamus, pituitary and cortex of rat brain measured by the present method are shown in Table I. The values were calculated by a standard addition method for correcting the loss of recoveries of the detected peptides. The values are almost identical with those measured by the post-column method,¹⁷⁾ but those for ME and LE are slightly lower than those measured by the RIA methods.^{7–9)} The precision of the present method was established by repeated determination ($n=7$) of the same sample of striatum. The relative standard deviations were 3.3, 3.1, 4.1 and 3.7% for ME, LE, ME-Arg-Phe and ME-Arg-Gly-Leu (541, 161, 118 and 128 pmol per g of the tissue), respectively.

The sensitivity of this HPLC method is comparable with the post-column method¹⁷⁾ and other HPLC methods combined with mass spectrometric detection¹²⁾ and electrochemical detection,¹⁵⁾ though it is nearly one order of magnitude lower than the RIA method.^{7–9)} However, the sensitivity and reproducibility of this method are sufficient for the simultaneous quantification of the four peptides in rat brain tissue.

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