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High-performance liquid chromatographic separation of kyotorphin, a basic Tyr–Arg dipeptide, in rat brain tissue and quantification using fluorimetric detection

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Abstract

Two HPLC procedures based on sample derivatization at the N-terminal Tyr moiety with agents yielding fluorescent derivatives were applied to the selective and sensitive detection as well as quantification of the basic kyotorphin, Tyr–Arg dipeptide, in rat brain tissue. The first one is a post-column fluorescence derivatization method, whereby the peptides extracted from the brain tissue are separated on an octadecylsilyl-silica gel column, followed by on-line fluorescence derivatization for detection. The other one is a pre-column derivatization method, where the extracted peptides are first reacted with fluorogenic agents at the N-terminal Tyr moiety to their corresponding fluorescent derivatives, subsequently separated on an octadecyl-poly(vinyl alcohol) copolymer gel column, and signal responses are measured fluorimetrically. Both methods permitted the quantification of the synthetic kyotorphin added to the rat brain tissues. The concentration range of kyotorphin-like biogenic peptide was 60–100 pmol/g in the cortex, striatum and hypothalamus tissues.

Keywords: Kyotorphin; Tyr–Arg dipeptide; Neuropeptides

1. Introduction

Kyotorphin (Tyr–Arg) was originally isolated from bovine brain [1,2], and is considered a member of the neuropeptide family [1–7], because it reveals opiate-like activity via stimulation of the release of methionine enkephalin in the central nervous system [8]. In order to measure its low concentration, a facile and reliable method for the quantitative determination of the peptide is required to study further its physiological actions as well as its biosynthesis.

Peptides and proteins strongly absorb at 210 nm, i.e., the optimum wavelength of the peptide bond and, furthermore, exhibit weak fluorescent responses attributable to the weak native fluorescence of aromatic amino acids such as Tyr, Phe and Trp. However, UV absorption and native-fluorescence measurements in HPLC are susceptible to severe interferences by the complex matrix. Hitherto, a method based on HPLC with electrochemical detection has been developed for the quantitative analysis of kyotorphin [9,10]. The detection system was operated at high potential (+1.0 V vs. Ag/AgCl), and permitted detection of synthetic kyotor-

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phin at picomole level. However, detection at high voltages was only achievable with difficulty due to interferences from the matrix. Furthermore, the baseline noise strongly depends on the composition of the mobile phase used for chromatography.

In this paper, two HPLC methods with fluorescence detection are studied for the quantification of the biogenic kyotorphin in rat brain tissues. In both methods, a unique chemical fluorescence reaction was applied to the selective and sensitive detection of the peptide. This fluorogenic reaction using three reagents, i.e., hydroxylamine, cobalt(II) and borate salts specifically converts only N-terminal Tyr-containing peptides under mild reaction conditions [11,12] into their corresponding fluorescent derivatives and is independent of the chemical structure of the residual peptide chain.

Therefore, we thought that the HPLC method in combination with the fluorescence derivatization reaction would be suitable for the quantification of the target peptide, kyotorphin in biological matrices. In this work, the fluorescence reaction procedure selective for the N-terminal Tyr-containing peptides was applied to both post- and pre-column derivatization methods in HPLC analysis and signal responses measured fluorimetrically (Fig. 1). This study provides the first HPLC method with fluorimetric detection for the quantification of kyotorphin in biological samples.

2. Experimental

2.1. Chemicals and solutions

Synthetic peptides were purchased from Sigma (St. Louis, MO, USA). The peptides (1.0 μmol/ml each) were dissolved in 2-methoxyethanol, and stored at −80°C to avoid their hydrolysis. The solutions were diluted with water at an appropriate concentration before use. Deionized water was purified with a Milli-QII device (Japan Millipore, Tokyo, Japan). Other chemicals were of the highest purity available.

2.2. Peptide extraction and clean-up

Male Sprague–Dawley rats (200–260 g, 7 weeks old) were anesthetized with diethyl ether and killed by bleeding. The striatum, hypothalamus and cortex regions were removed from the brain. The tissues were then washed quickly with cold saline and weighed after removing the saline with filter-paper. They were stored at −80°C and analyzed within a week.

A portion (0.8 g) of the tissue was homogenized with 14 ml of 0.1 *M* HCl; then a 400-μl portion of 1.0 nmol/ml Tyr–Phe as internal standard was mixed with the homogenate. The homogenate was quickly divided into four equal volumes. Synthetic

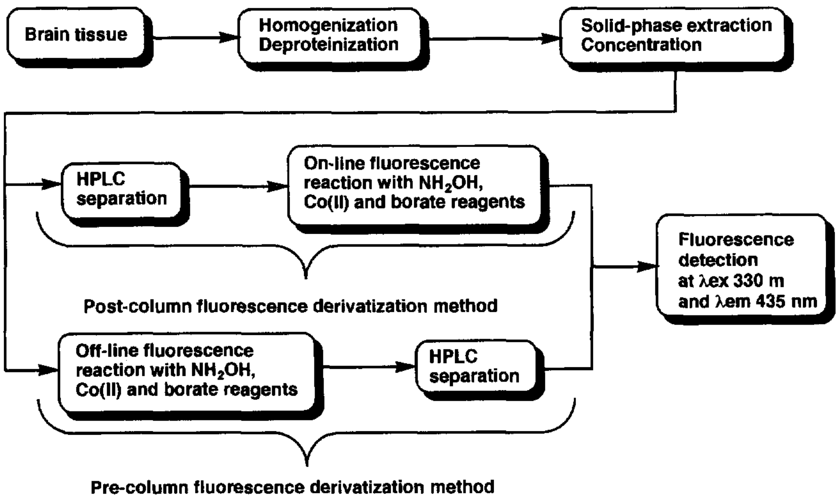

Fig. 1. Scheme of sample preparation and chromatography of kyotorphin (Tyr–Arg).

kyotorphin (100 μl) at the concentrations of 0 (blank), 10, 20 and 40 pmol/tube corresponding to the concentrations of 0, 50, 100 and 200 pmol per g of wet weight of the tissue, and 2.0 *M* $HClO_4$ (0.5 ml) were added to the four volumes.

After centrifugation at 1350 *g* for 5 min, the deproteinized supernatant was mixed with 1.0 ml of 0.2 *M* sodium *n*-hexanesulfonate, and immediately applied to a disposable octadecylsilyl-cartridge (Bon-dElut C_{18}, Analytical International, Harbor City, CA, USA). The cartridge was preconditioned with 2.0 ml of each methanol and water before used. After loading the tissue sample, the cartridge was washed twice with 2.0 ml of water, and then kyotorphin-rich fraction eluted with 2.0 ml of methanol–water (3:1, v/v). After solvent evaporation at reduced pressure the residue was reconstituted in 0.5 ml of water and a 100-μl sample was used for either post- or pre-column derivatization HPLC.

2.3. Post-column derivatization HPLC

As shown in Fig. 1, the native sample was first separated by a gradient elution with acetonitrile as organic modifier on a TSK gel 80TM C_{18}-silica gel column (150×4 mm I.D., 5 μm particle size) obtained from Tosoh (Tokyo, Japan). The percentage of organic modifier is depicted in Fig. 2. Eluents A and B used for the gradient elution were mixtures of acetonitrile, 0.1 *M* boric acid, 5.0 m*M* sodium *n*-hexanesulfonate and water (eluent A: 0:1:1:8; eluent B: 5:1:1:3). The mobile phase was pumped at a flow-rate of 1.0 ml/min. The column temperature was ambient (24±4°C).

The column effluent was introduced into a post-column reactor. In the system, 0.3 *M* borate buffer (pH 9.0) and a mixture of 16 m*M* hydroxylamine oxalate and 0.25 m*M* cobalt (II) acetate (1:1) were added to the eluate stream with two reagent-delivery pumps at a flow-rate of 0.4 ml/min each. The mixture was then passed through a reaction coil (10 m×0.5 mm I.D.; stainless-steel tube) in an electronic heater at 105°C. The fluorescence in the final eluate was monitored at λ_{em} 435 nm (emission maximum) and at λ_{ex} 330 nm (excitation maximum) with a F1000-type fluorescence spectrophotometer from Hitachi (Tokyo, Japan).

2.4. Pre-column derivatization HPLC

A portion (100 μl) of the sample solution was placed in a test-tube (50×5 mm I.D.), to which were

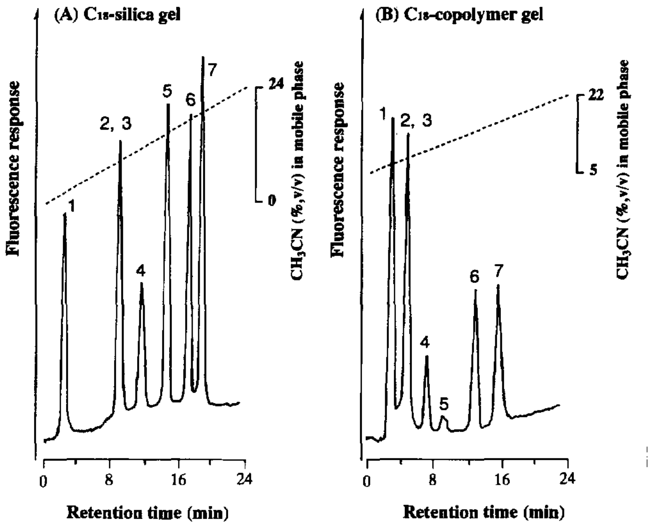

Fig. 2. Chromatograms of a standard mixture of kyotorphin and small N-terminal Tyr-containing peptides by post-column fluorescence derivatization HPLC using (A) C_{18}-silica gel and (B) C_{18}-copolymer gel columns. A mixture (100 μl) of the synthetic peptides (200 pmol/ml each) was injected into the chromatograph. Peaks: 1=Tyr–Glu; 2=Tyr–Gly; 3=Tyr–Gly–Gly; 4=Tyr–Tyr; 5=kyotorphin; 6=Tyr–Phe; 7=Tyr–Gly–Gly–Phe.

added 50 μl each of a mixture of 6.0 m*M* hydroxylamine oxalate and 0.24 m*M* cobalt (II) acetate (1:1) and 0.4 *M* sodium borate buffer (pH 9.0). The mixture was heated in a bath of boiling water for 4 min, and then a 25-μl portion of 20 m*M* 2-mercaptoethanol was added to stabilize the produced fluorescent derivatives. A portion (100 μl) of the mixture was used for HPLC.

The HPLC system was the same as the one used for post-column derivatization HPLC. The fluorescent derivatives were separated on an Asahipak ODP-50 C_{18}-poly(vinyl alcohol) copolymer gel column (150×6 mm I.D., 5 μm particle size) obtained from Asahi Chemical (Tokyo, Japan) by gradient elution with acetonitrile as organic modifier containing 15% (v/v) of 0.1 *M* sodium borate buffer (pH 8.5) and 10% (v/v) of 10 m*M* tetra-*n*-butylammonium chloride. The acetonitrile gradient is indicated in Fig. 3. The mobile phase was pumped at a flow-rate of 1.0 ml/min. The column temperature was ambient (24±4°C). The fluorescence in the eluate was monitored at the same emission and excitation wavelengths as those for the post-column derivatization HPLC.

3. Results and discussion

3.1. Fluorescence derivatization

For fluorescence detection, kyotorphin was derived to a fluorescent compound by reaction with hydroxylamine, Co(II) and borate salts. The conditions of pH, reaction time and temperature for the post- and pre-column derivatization methods for kyotorphin were almost the same as those reported previously for the HPLC determination of leucine- and methionine-enkephalins [13] and their related large opioid peptides [14,15]. However, the optimum concentrations of hydroxylamine (16 and 6 m*M* for the present post- and pre-column reactions, respectively) and Co(II) salt (0.25 and 0.24 m*M* for the post- and pre-column reactions, respectively) were different from those (hydroxylamine: 16 m*M* for post-column reaction and 20 m*M* for per-column reaction; Co (II) salt: 0.4 m*M* for post-column reaction and 1.0 m*M* for pre-column reaction) used for the previous methods [13–15]. This derivatization method was selective for N-terminal Tyr-containing peptides, and provided a single fluorescent

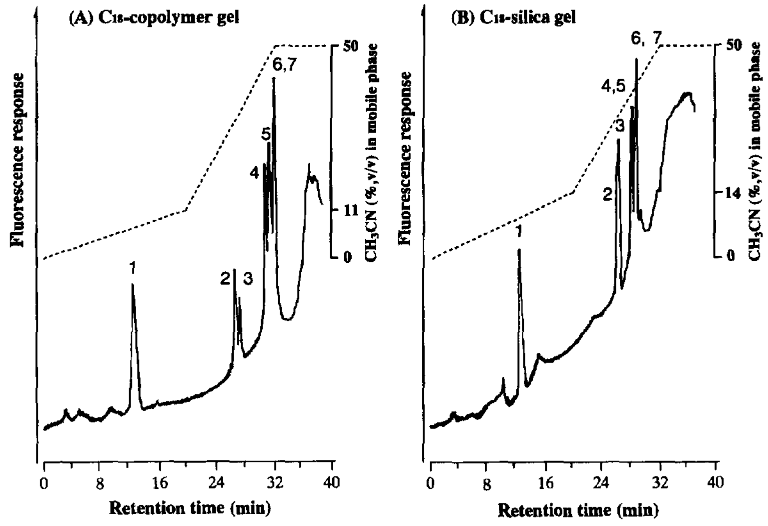

Fig. 3. Chromatograms of a standard mixture of kyotorphin and small N-terminal Tyr-containing peptides by pre-column fluorescence derivatization HPLC using (A) C_{18}-copolymer gel and (B) C_{18}-silica gel columns. A mixture (100 μl) of the synthetic peptides (200 pmol/ml each) was reacted with the fluorogenic reagents, and the reaction mixture (100 μl) was injected into the chromatograph. Peaks: 1=kyotorphin; 2=Tyr–Gly; 3=Tyr–Gly–Gly; 4=Tyr–Tyr; 5=Tyr–Glu; 6=Tyr–Phe; 7=Tyr–Gly–Gly–Phe.

product for each of the peptides. Other peptides without Tyr at the N-terminus (i.e., Gly–Tyr, Phe–Arg–Gly and Arg–Val–Tyr–Ile–His–Pro–Phe), amino acids other than Tyr, sugars, purine and pyrimidine bases, and steroids did not produce the fluorescent derivatives [11,12].

The fluorogenic reaction seems to include the primary amino and hydroxyl functions as well as the peptide bond of Tyr, although the chemical structure of the fluorescent product remains unknown. Thus, most of endogenous substances might not be detected by the proposed HPLC methods. A great advantage of this reaction is that the reagents used for derivatization are all non-fluorescent and that the reaction proceeds rapidly with heating in a weakly alkaline solution at pH 8–9. Therefore, these reagents were usable for both the pre- and post-column derivatization HPLC methods.

3.2. Separation of kyotorphin

In the post-column derivatization system, the dipeptide kyotorphin should be separated from other N-terminal Tyr-containing small oligopeptides (e.g., Tyr–Glu, Tyr–Gly, Tyr–Gly–Gly, Tyr–Tyr, Tyr–Phe, Tyr–Gly–Gly–Phe), since these peptides are present in brain tissues. Two columns were examined for the separation; they are both the reversed-phase columns packed with C_{18}-silica gel or C_{18}-poly(vinyl alcohol) copolymer gel. The synthetic oligopeptides are generally separated on several reversed-phase columns by elution with aqueous acetonitrile containing a weakly acidic buffer. However, the acidic conditions were not acceptable for the separation of kyotorphin and the related small peptides, because they eluted early, near the void volume under similar gradient conditions to those depicted in Fig. 2, when the mobile phase did not contain sodium *n*-hexanesulfonate. Thus, ion-pair chromatography was applied to the separation, because kyotorphin is present as its protonated species in an acidic mobile phase showing only low retention. Therefore, *n*-hexanesulfonate was used as an ion-pairing agent in order to increase the hydrophobicity of the native dipeptide.

Fig. 2 shows the ion-pair chromatograms of kyotorphin and the N-terminal Tyr-containing small peptides, which were obtained by the post-column derivatization HPLC. The synthetic kyotorphin peptide was eluted at 14.5 min and separated from the other oligopeptides on a C_{18}-silica gel column (Fig. 2A). However, kyotorphin was significantly adsorbed on the C_{18}-poly(vinyl alcohol) copolymer column, as revealed by its low signal response as well as peak distortion (Fig. 2B). The data suggested that the pairing of the basic Arg residue of kyotorphin with *n*-hexanesulfonate was responsible for the penetration of this dipeptide into the copolymer matrix. Consequently, we selected the C_{18}-silica gel column for the peptide separation in the post-column derivatization HPLC system.

In the pre-column derivatization HPLC, the separation of the peptides derivatized with hydroxylamine, Co(II) and borate salts was also examined on the two columns. In this method, the aqueous mobile phase should contain a weakly alkaline borate buffer (pH 8–10), because the fluorescent product of kyotorphin did not exhibit any fluorescence in the acidic mobile phase (pH 2–5), even in the presence of the borate salt.

The fluorescent derivatives of kyotorphin and of the tested small peptides were retained and separated on both columns by gradient elution of acetonitrile containing borate buffer (pH 8.5) and tetra-*n*-butylammonium ions as the ion-pair for the carboxyl group of the peptides, in order to increase hydrophobicity and thus retention (Fig. 3). Although the kyotorphin derivative was obviously not adsorbed on these column supports, the baseline profile of the chromatogram obtained with the C_{18}-copolymer column was preferable to that with the C_{18}-silica gel column, probably because the excess of matrix components of the former column is more stable in the alkaline eluent; the copolymer-type column was thus employed in the pre-column derivatization HPLC.

3.3. Clean-up for brain samples

The aim of the clean-up procedures for rat brain tissue samples is to concentrate the target peptide into a small sample volume, and also to remove some biogenic substances interfering with the fluorescence derivatization of kyotorphin, especially in the pre-column derivatization method. However, derivatization failed when kyotorphin added to a brain tissue sample was not treated by solid-phase extraction after acid deproteinization and neutralization with potassium carbonate.

In the recommended procedure for clean-up, the deproteinized sample of brain tissue was quickly passed through an ODS-disposable minicartridge for the clean-up. This treatment additionally suppressed acidic hydrolysis of the dipeptide. In order to retain the peptide on the cartridge support, *n*-hexanesulfonate at higher concentration (0.2 *M*) was required and thus, was added to the extracted sample before applying to the cartridge. In this case, the recovery of the dipeptide spiked to the tissue sample was 92±5% for kyotorphin and 96±4% for Tyr–Phe used as internal standard in the eluted fraction, in contrast to the recovery of 18±6% for kyotorphin and 95±4% for Tyr–Phe without the ion-pair reagent ($n=4$ each).

3.4. Determination of kyotorphin-like biogenic peptide

A satisfactory separation of kyotorphin spiked to the tissue homogenate from other synthetic N-terminal Tyr-containing peptides was obtained by both the post- and pre-column derivatization HPLC methods. Under the applied chromatographic conditions, elution of the larger members of the enkephalin and endorphin family, i.e., enkephalins (pentapeptides), methionine enkephalin–Arg, α- (a hexadecapeptide) and γ- (a heptadecapeptide) endorphins, and dynorphins 1–9, 1–10, 1–13 and B (a tridecapeptide) did not take place within the time window of 25 min chosen for kyotorphin and related peptides in both the post- and pre-column derivatization HPLC methods. After the separation of each tissue sample, the columns were washed with a high concentration (70%, v/v) of the organic modifier for approximately 15 min in order to remove strongly retained biogenic substances, and then reconstituted with the initial mobile phase for at least 20 min.

Fig. 4 shows the chromatograms obtained from rat brain tissues (cortex, hypothalamus and striatum), by use of the post-column derivatization HPLC method. The fluorescent signal attributable to kyotorphin-like biogenic N-terminal Tyr-containing peptide was observed in each of the chromatograms at a retention time of 14.5 min. The signals obtained from derivatization of either a biological sample alone or a biological sample spiked with the synthetic kyotorphin coincided. Therefore, the peak signals are probably due to the endogenous kyotorphin present in each tissue sample. However, these peaks may contain not only endogenous kyotorphin but also other biogenic small N-terminal Tyr-containing peptides, since the present HPLC method has no validation for the purity of an endogenous peptide.

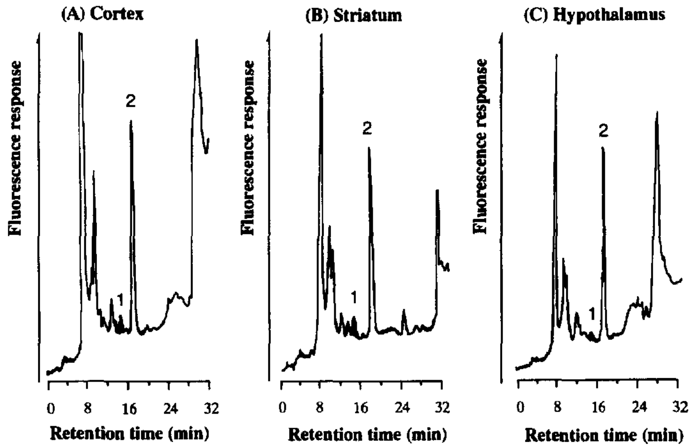

Fig. 4. Chromatograms of (A) cortex, (B) striatum and (C) hypothalamus tissues of rat brain, obtained by post-column fluorescence derivatization HPLC using the C_{18}-silica gel column. Peaks: 1=kyotorphin-like endogenous peptide; 2=Tyr–Phe (I.S.).

On the assumption that the kyotorphin-like peak obtained by the derivatization of the tissue sample alone is completely attributable to endogenous kyotorphin, the concentration of the kyotorphin-like endogenous peptide was determined by the standard addition method using the internal standard (I.S.) of Tyr–Phe in order to compensate for differences in either individual recoveries or sample volumes. The endogenous concentration of Tyr–Phe was below the detection limit. The concentration values of the kyotorphin-like endogenous peptide in the cortex, striatum and hypothalamus tissues were 80, 100 and 60 pmol per g of wet weight of the tissue, respectively. In this experiment, the brain homogenate containing the I.S. was divided into four parts, and the synthetic kyotorphin peptide of 0, 50, 100 or 200 pmol per g of tissue was added to each part for the calibration. A linear relationship was obtained between the peak-height ratio of calibration samples and I.S. For example, the regression equation for the curve of the cortex sample was $y=8.9 \cdot 10^{-4}x+0.0872$, in which y and x represent the peak-height ratio and the spiked kyotorphin concentration, respectively. No significant change in the slope of the graph was observed with different tissues. The correlation coefficients (r) of the curves for each tissue sample were 0.996–0.999, when the two HPLC measurements of each tissue sample were made in duplicate. The lower limit of detection of kyotorphin at a signal-to-noise ratio of 3 was approximately 18 pmol per g of the tissue, which corresponded to the absolute amount of 0.7 pmol per HPLC injection volume.

Additionally, a pre-column derivatization HPLC method was carried out for determination of the endogenous kyotorphin in brain tissues. The purpose of this experiment was to confirm the concentration level of the kyotorphin-like biogenic peptide determined by the post-column derivatization HPLC method.

Fig. 5 shows the chromatograms obtained from brain tissue samples by pre-column derivatization and subsequent HPLC. The fluorescent signal of the derivatized kyotorphin-like peptide observed at a retention time of 12.5 min that was identical to that of the synthetic kyotorphin added to the same tissue samples. In this case, however, the peak due to Tyr–Phe used as I.S. coeluted with the Tyr–Gly–Gly–Phe tetrapeptide as shown in Fig. 3A. The quantification was performed by the standard addition method without the I.S. Nevertheless, the peak height due to kyotorphin showed a linear dependence on the amount of the synthetic kyotorphin added to the tissue homogenate in the range of 0–200 pmol per g of the tissue. The regression equation for the curve of the cortex sample was $y=0.0154x+1.3$, in which y and x represent the peak height (cm) and the spiked peptide concentration, respectively. The slope (0.0121–0.0154) for each calibration curve was slightly changed depending on the tissue species,

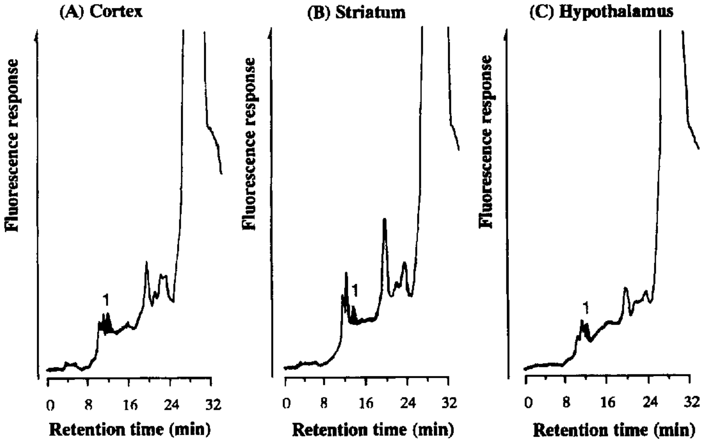

Fig. 5. Chromatograms of (A) cortex, (B) striatum and (C) hypothalamus tissues of rat brain, obtained by pre-column fluorescence derivatization HPLC using the C_{18}-copolymer gel column. Peak: 1 = kyotorphin-like endogenous peptide.

probably because the yield of the fluorescent derivative obtained by the pre-column derivatization reaction was affected by each sample constituent. The correlation coefficients (*r*) of the calibration curves for each tissue sample were 0.990–0.987, when the two HPLC measurements for each tissue sample were carried out by pre-column derivatization reactions in duplicate. The lower limit of detection of kyotorphin at a signal-to-noise ratio of 3 was approximately 26 pmol per g of the tissue. The concentration values of the kyotorphin-like biogenic peptide in cortex, striatum and hypothalamus tissues were 80, 80 and 65 pmol per g of wet weight of the tissue, respectively. They were not significantly different from those determined by the post-column derivatization HPLC method.

4. Conclusions

The HPLC quantification of biogenic peptides in biological samples is generally difficult, due to the presence of similar peptides as well as unknown peptides. Recently, endogenous opioid peptides such as methionine enkephalin have been quantified with molecular specificity by collision-induced dissociation tandem mass spectrometry after HPLC separation [16].

However, the proposed HPLC methods utilizing the selective fluorescence derivatization reaction of N-terminal Tyr-containing peptides have proven useful for the sensitive quantification of the kyotorphin-like endogenous peptide in rat brain. Due to the good sensitivity of detection, the number of rats used for brain tissue generation could be reduced to only three to five. Furthermore, no additional expensive reagents and instrumentation were required. Finally, the post-column derivatization HPLC method proved to be more suitable with respect to either efficiency of separation or sensitivity as well as specificity of detection.

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