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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SUBSTANCE P-LIKE ARGININE-CONTAINING PEPTIDE IN RAT BRAIN BY ON-LINE POST-COLUMN FLUORESCENCE DERIVATIZATION WITH BENZOIN

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SUMMARY

A high-performance liquid chromatographic method with fluorescence detection is described for the determination of substance P, one of the neuropeptides, in the hypothalamus tissue of rat brain. The detection is based on on-line post-column fluorescence derivatization selective for arginine-containing peptides. The endogenous substance P-like arginine-containing peptide extracted from the tissue and [D-Phe^{11}]-neurotensin as an internal standard were separated from various interfering substances on a reversed-phase column (TSKgel ODS-120T) by gradient elution with acetonitrile–phosphate buffer (pH 2.3). The peptides in the eluate were then automatically converted into fluorescent derivatives for detection by reaction with benzoin. Arginine-containing fragments produced by the enzyme reaction of substance P in the chromatographic fraction with trypsin were also detected, for the identification of the endogenous substance P-like arginine-containing peptide. The method was sensitive enough to permit the quantitative determination of the peptide at a concentration as low as 580 fmol/mg of protein in the brain homogenate. The concentration values of the substance P-like arginine-containing peptide in the tissue were 9.45 ± 1.50 pmol/mg of protein (six determinations).

INTRODUCTION

In 1970 substance P (SP; Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was first purified from the hypothalamus tissue of bovine brain [1], and shortly thereafter its amino acid sequence was determined [2]. This undecapeptide appears to act in vivo as a neurotransmitter or neuromodulator in the nervous system [3,4].

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For identifying the detailed physiological function of SP, the determination of the SP-like peptide in various brain tissues has usually been carried out by radioimmunoassay (RIA). The RIA methods [5–7] offer sufficient sensitivity for the peptide, but there is still difficulty in the preparation of specific antibody that discriminates between SP and related peptides, such as the peptide fragments and precursors. Therefore, the current methods have employed a fractionation step by high-performance liquid chromatography (HPLC) followed by RIA [8,9], for the quantitative assay of the SP-like peptide immunoactivity in biological samples. The main problems of the RIA methods combined with HPLC are the lengthy analysis and the use of limited antibodies for SP-related peptides.

HPLC using reversed-phase or ion-exchange columns can simultaneously and rapidly separate the synthetic SP, its fragments and related peptides [8–10]. We previously developed an on-line post-column fluorescence derivatization method using benzoin, a fluorogenic reagent for the sensitive HPLC determination of various Arg-containing peptides [11]. Benzoin reacts selectively with the guanidino moiety of Arg or Arg-containing peptides to give highly fluorescent derivatives, i.e. 2-substituted amino-4,5-diphenylimidazole compounds [12,13]. Therefore, SP and its Arg-containing fragments may be separated and determined by HPLC with post-column fluorimetric detection.

This paper aims to establish a simple method for the determination of the SP-like Arg-containing peptide in rat brain by reversed-phase HPLC with on-line post-column fluorescence derivatization. Enzymic digestion of the endogenous SP-like Arg-containing peptide with trypsin was also investigated for the identification. Synthetic [D-Phe^{11}]-neurotensin ([D-Phe^{11}]-NT) was used as the internal standard for the quantitative determination of the SP-like Arg-containing peptide in the tissue.

EXPERIMENTAL

Chemicals and solutions

The following synthetic peptides were purchased from the Peptide Institute (Osaka, Japan) or Sigma (St. Louis, MO, U.S.A.): SP, neurotensin (NT; pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), [D-Phe^{11}]-NT, vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2), bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), kallidin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), luteinizing-hormone releasing-hormone (LH-RH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), leucine enkephalin-Arg (LE-Arg; Tyr-Gly-Gly-Phe-Leu-Arg), methionine enkephalin-Arg-Phe (ME-Arg-Phe; Tyr-Gly-Gly-Phe-Met-Arg-Phe), ME-Arg-Gly-Leu, α-neoendorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys), β-neoendorphin (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro), dynorphin 1–8 (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile). Benzoin (Wako, Osaka, Japan) was recrystallized from methanol.

Tris(hydroxymethyl)aminomethane (Tris; Wako) was recrystallized from 60% aqueous methanol to remove fluorescent impurities. Water was deionized and then distilled before use. Other chemicals were of the highest purity available. The synthetic peptides were used as received and dissolved in water. The solutions were usable for at least three weeks when stored at −80 °C. The reagent solutions used for the post-column derivatization were prepared as described previously [11].

Peptide extraction

Male Sprague–Dawley rats (220–289 g, seven weeks) were anaesthetized with diethyl ether and killed by dehaematization. The hypothalamus region of brain tissues was quickly removed and stored at −80 °C until use.

The tissue (ca. 0.4 g) was homogenized with 2 ml of 0.1 *M* hydrochloric acid. Then a 20-μl portion of 10 nmol/ml [D-Phe^{11}]-NT was added to the homogenate. The homogenate was deproteinized by mixing with 2 ml of acetone followed by centrifugation at 2450 *g* for 15 min. The supernatant was mixed with 0.22 ml of 1 *M* sodium hydrogencarbonate and 0.5 ml of 0.1 *M* disodium EDTA. The precipitate in the mixture was removed by centrifugation at 2450 *g* for 15 min. After evaporation of acetone in the supernatant, the resulting aqueous solution was diluted with 2 ml of water. The deproteinized extract was applied to a mini-cartridge (Bond Elut C_{18}; Analytichem International, Harbor City, CA, U.S.A.), previously washed with 3 ml each of water and acetonitrile. After loading the sample, 1 ml of water, 3 ml of 0.1 *M* hydrochloric acid, 3 ml of dichloromethane (twice, to remove strongly hydrophobic substances), 1 ml of water, 2 ml of 0.1 *M* phosphate buffer (pH 8.0) and 2 ml of water were successively passed through the cartridge. Finally, the SP-rich fraction was obtained by elution with 2 ml of acetonitrile–0.1 *M* phosphate buffer (pH 2.3) (7:3, v/v). After evaporation in vacuo, the volume of the residue was adjusted with water to 400 μl. A 100-μl portion of the solution was used for HPLC.

The protein in the homogenate that was diluted ten times with water was measured by the method of Lowry et al. [14].

Chromatography and apparatus

Fig. 1 shows a schematic diagram of the HPLC system for the determination of SP. PTFE tubing (0.33 mm I.D.) was used to connect the column outlet to the detection system. SP and [D-Phe^{11}]-NT in a 100-μl portion of the sample solution were separated by gradient elution of acetonitrile in the mobile phase on a reversed-phase column (200 mm × 4 mm I.D.) packed with TSKgel ODS-120T (particle size, 5 μm; Tosoh, Tokyo, Japan). The acetonitrile gradient is indicated in Fig. 2. Eluents A and B used for the gradient elution were mixtures of acetonitrile, 0.3 *M* sodium phosphate buffer (pH 2.3) and water (1:20:79 and 3:1:1, v/v, respectively).

The mobile phase was pumped at a flow-rate of 1.0 ml/min by a Hitachi 638-

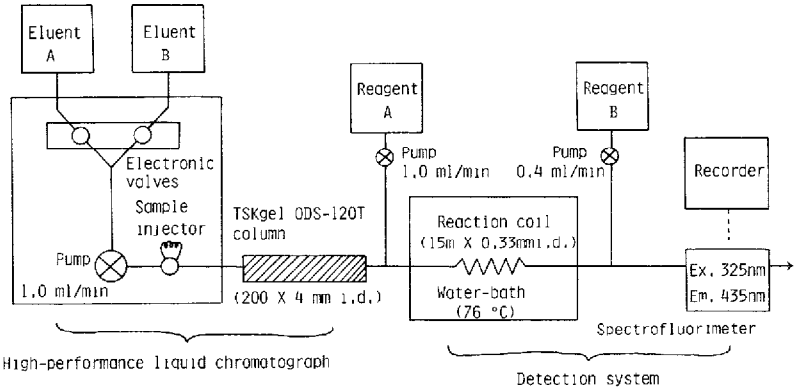

Fig. 1. Schematic diagram of on-line post-column fluorescence derivatization HPLC for the determination of SP.

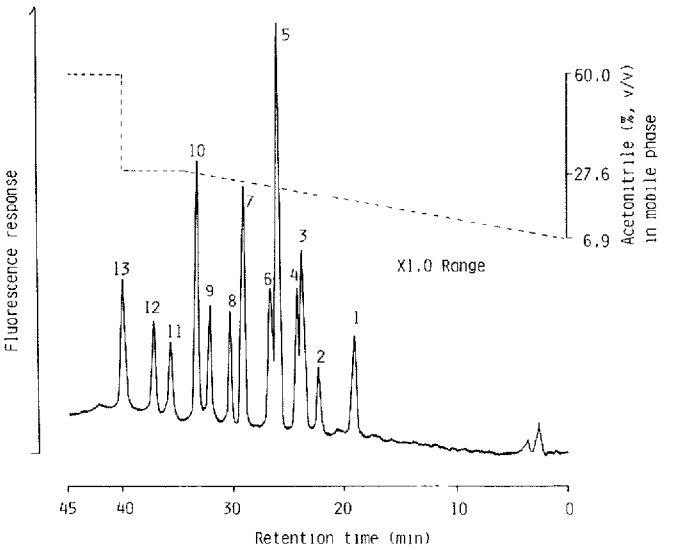

Fig. 2. Chromatogram of a standard mixture of synthetic Arg-containing peptides. Peaks (pmol injected): 1 = vasopressin (100); 2 = α-neoendorphin (200); 3 = kallidin (100); 4 = LE-Arg (100); 5 = bradykinin (100); 6 = β-neoendorphin (200); 7 = LH-RH (25); 8 = dynorphin 1-8 (200); 9 = NT (50); 10 = ME-Arg-Gly-Leu (200); 11 = ME-Arg-Phe (100); 12 = SP (50); 13 = [D-Phe[11]]-NT (50). For HPLC conditions, see Experimental.

30 high-pressure pump fitted with programmed electronic valves for various gradient elutions. The column temperature was ambient (24 ± 4 °C).

The column eluate was led into the detection system. In the system, reagent A (a mixture of 2 m*M* benzoin, 1.6 *M* potassium hydroxide and 0.7 *M* 2-mercaptoethanol as a stabilizer for benzoin) was added to the eluate stream with a Hitachi 633 reagent-delivery pump at a flow-rate of 1.0 ml/min. The mixture

was passed through a PTFE reaction coil (15 m×0.33 mm I.D.) immersed in a water-bath at 76±1°C. After heating, reagent B (a mixture of 0.5 *M* Tris and 2.1 *M* hydrochloric acid) was added to the reaction mixture at a flow-rate of 0.4 ml/min with the same type of reagent-delivery pump as for reagent A.

The fluorescence in the final eluate was monitored at 435 nm (emission maximum) and 325 nm (excitation maximum) with a Hitachi 650-10LC spectrofluorometer equipped with an 18-μl flow-cell.

Fractionation and enzyme reaction

The eluate from the column was fractionated before being led into the HPLC detection system. Acetonitrile in fraction of the SP peak was removed in vacuo at 26°C for ca. 2 h. The residual solution (ca. 200 μl) was neutralized with ca. 15 μl of 0.5 *M* sodium hydroxide (pH 7–8). The mixture was incubated with 20 μl of 40 U/ml trypsin at 37°C for 3 h. The enzyme reaction was terminated by adding 30 μl of 1 *M* hydrochloric acid. The final reaction mixture was analysed by HPLC.

RESULTS AND DISCUSSION

Separation and detection

Fig. 2 shows a chromatogram obtained with a standard mixture of thirteen synthetic Arg-containing peptides on a reversed-phase column (TSKgel ODS-120T) by gradient elution of acetonitrile in the mobile phase containing phosphate buffer (pH 2.3) under the conditions recommended for the determination of the SP-like Arg-containing peptide in rat brain. The synthetic SP and [D-Phe^{11}]-NT were separated from the other tested peptides within 40 min. The separation conditions were similar to those in conventional HPLC [9,10]. The Arg-containing peptides in the column effluent were automatically converted into fluorescent derivatives with benzoin, for the fluorimetric detection. Table I shows the detection limits and the retention times of the Arg-containing peptides. The lower limits of detection at a signal-to-noise ratio of 3 for the peptides were in the range 0.5–13.5 pmol per 100 μl injected.

When the initial concentration of acetonitrile in the mobile phase was increased by ca. 10%, as shown in Fig. 3A, the synthetic SP and the internal standard were also separated from the other tested peptides on the same column within 24 min, though the separation of the thirteen peptides was less satisfactory. The retention times of the synthetic SP and [D-Phe^{11}]-NT were 18.4 and 22.5 min, respectively. These conditions for Fig. 3A were used for the separation and fluorimetric detection of the SP fragments produced by the enzymic digestion with trypsin (see Fig. 5).

The peak of the synthetic SP in the chromatogram of Fig. 2 was fractionated before the on-line fluorescence derivatization with benzoin. Fig. 3B shows the chromatogram of this SP fraction obtained under the same HPLC conditions

TABLE I

RETENTION TIMES AND LOWER LIMITS OF DETECTION OF Arg-CONTAINING PEPTIDES

Peptide	Retention time (min)	Lower limit of detection (pmol injected)
Vasopressin	19.1	4.4
α-Neoendorphin	22.0	13.5
Kallidin	23.6	3.5
LE-Arg	24.0	4.4
Bradykinin	26.2	1.4
β-Neoendorphin	26.8	8.4
LH-RH	29 2	0.5
Dynorphin 1-8	30.5	7 8
NT	32.0	2.0
ME-Arg-Gly-Leu	33.2	3 6
ME-Arg-Phe	35.8	5.6
SP	37.1	2 4
[D-Phe^{11}]-NT	40.0	1.7

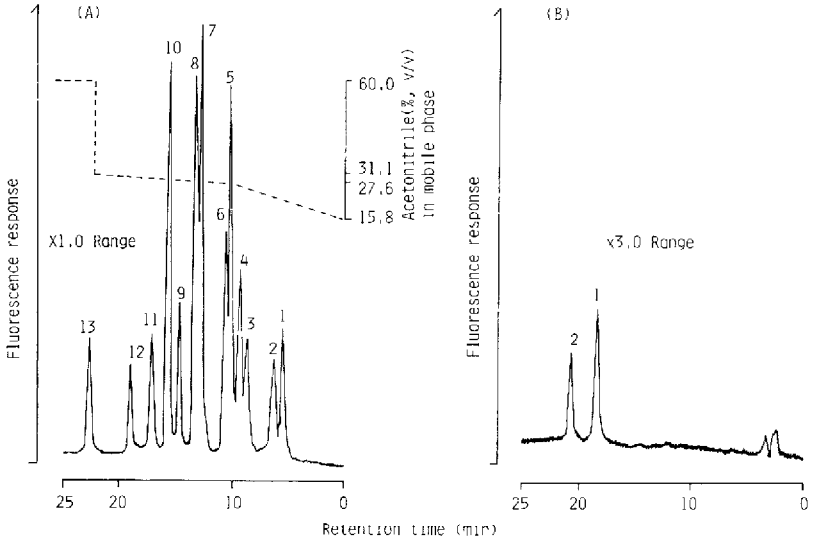

Fig. 3. Chromatograms of (A) a standard mixture of the synthetic Arg-containing peptides and (B) the SP fraction obtained by the chromatography of Fig. 2. The concentration of acetonitrile in the mobile phase was increased as indicated with dotted lines and the other HPLC conditions were the same as for Fig. 2. Peptides (1-13) and their amounts in (A) were the same as in Fig. 2. Peaks in (B) 1 = SP, 2 = probably modified SP.

as Fig. 3A. Two peaks, 1 and 2, were observed at the retention times of 18.4 and 20.6 min, respectively. Peak 1 corresponded to the intact SP. Peak 2 was unknown, though it may have been an SP sulphoxide produced by auto-oxidation of methionyl residue in the molecule or an SP fragment produced by hydrolysis at the C-terminal amide bond of SP, when the SP fraction (pH 2.3) was evaporated for 2 h to remove acetonitrile in the fractionated solution.

Determination of endogenous SP-like Arg-containing peptide

The common extraction of the peptides from brain tissue was carried out by the procedures of homogenization with 0.1 *M* hydrochloric acid and deproteinization with acetone. The extracts were passed through an ODS mini-cartridge (Bond Elut C_{18}) for clean-up, according to the procedure reported previously for enkephalin determination [15]. The use of the cartridge facilitated not only the removal of large amounts of interfering biogenic substances but also the concentration of the peptide in a small sample size.

The recoveries of 200 pmol each of the synthetic SP and [D-Phe^{11}]-NT added to the homogenate were 93 ± 12 and $81 \pm 10\%$, respectively ($n=5$ in each instance). The variation of the recoveries was mainly caused by the treatment with the mini-cartridges. However, a constant ratio of the recovery of SP to that of the internal standard was obtained on each treatment. Thus, the internal standard was used for a precise and facile quantification of SP-like Arg-containing peptides in the homogenate.

When perchloric acid (0.2 *M* in the homogenate) was used for the deproteinization instead of acetone, the recovery of the added SP was low, ca. 38% ($n=2$). This suggests that the acid caused the hydrolysis of SP during the deproteinization.

Fig. 4 shows a typical chromatogram of hypothalamus tissue of rat brain obtained by the recommended procedure. A fluorescent peak at the same retention time as that of the synthetic SP was detected. There was no peak at the retention time (40.0 min) of the internal standard, [D-Phe^{11}]-NT, as this peptide was not added to the homogenate. The peaks of the SP-like Arg-containing peptide and the internal standard were not observed when the fluorogenic reagent, benzoin, was not added to the reactor system.

This reactor system utilizing the benzoin reaction cannot detect various biological substances that have no guanidino moiety in the molecule, as reported previously [11]. In this HPLC method, therefore, separation of the peptides with no arginyl residue was unnecessary. Detailed conditions for the fluorimetric detection were described in the previous report [11].

The SP-like Arg-containing peptide separated by HPLC under the conditions of Fig. 4 was also identified by enzymic fragmentation using trypsin. The other fluorescent peaks in the chromatogram of Fig. 4 were not investigated. Trypsin (EC 3.4.21.4) mediates the hydrolysis of peptide bonds at the carboxyl sites of arginyl and lysyl residues [16]. Therefore, Arg should be produced from

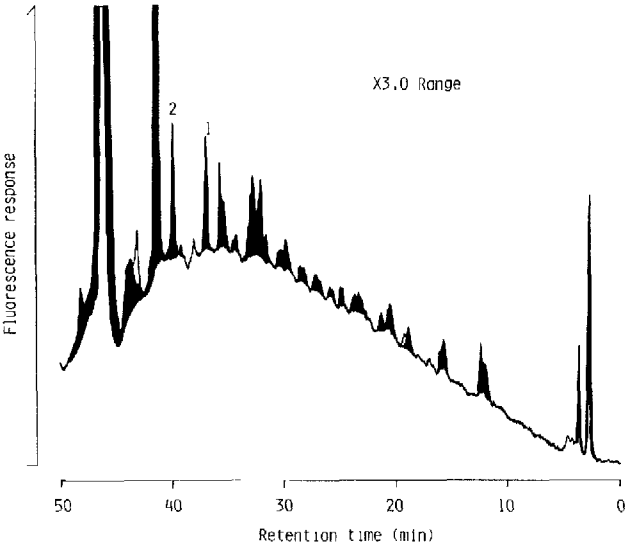

Fig. 4. Chromatogram of hypothalamus tissue of rat brain. HPLC conditions as in Fig. 2. Peaks: 1 = SP; 2 = [D-Phe^{11}]-NT, internal standard. Shaded areas were not observed when the benzoin reagent was not added to the column effluent.

SP by tryptic digestion and Arg-Pro-Lys may be produced when this hydrolysis is incomplete.

Fig. 5 shows chromatograms obtained with the tryptic digests of the HPLC fractions corresponding to the synthetic SP and SP-like Arg-containing peptide in the tissue. Two peaks, 1 and 3, were newly produced by the enzyme reaction with the SP fraction. These two peaks were not observed when the same enzyme reaction was carried out in the absence of the SP fraction. Peak 1 was eluted at the same retention time as that of Arg. Peak 3 is probably the peptide fragment of Arg-Pro-Lys from the points of the retention time and also the time course for the enzymic degradation, because the peak decreased with a prolonged incubation time. Compared with the chromatogram of the synthetic SP fraction, that of the SP fragments obtained with the tissue sample was identical with those of the synthetic SP. Therefore, the present HPLC method could detect the endogenous SP-like Arg-containing peptide in the tissue.

A linear relationship was observed between the ratio of the peak height of the SP-like Arg-containing peptide to that of the internal standard and the amount (0–320 pmol) of the synthetic SP added to the homogenate. The correlation coefficient (r) of the calibration graph was 0.999 ($n=3$ each plot). The lower limit (signal-to-noise ratio of 3) of detection for the SP-like Arg-containing peptide in the tissue was ca. 580 fmol/mg of protein. The precision of the method was established by repeated determinations ($n=6$) of the same

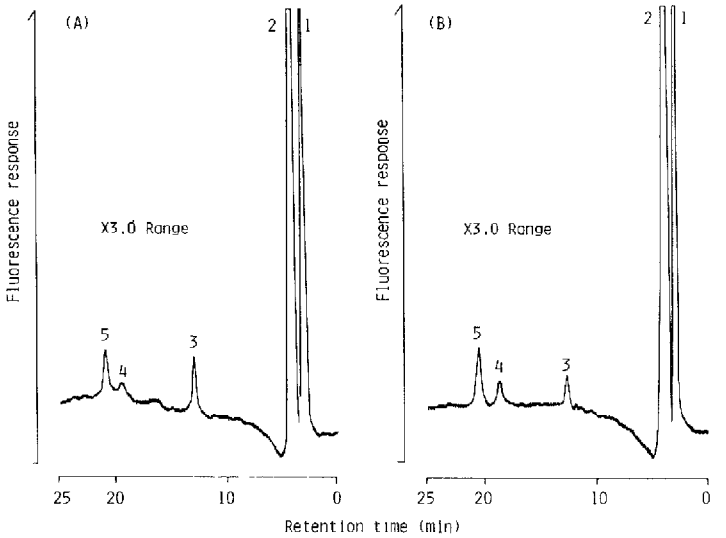

Fig. 5. Chromatograms of the SP fragments after tryptic digestion of the SP fractions obtained by the separation of (A) the synthetic SP and (B) hypothalamus tissue. A 100-μl portion of 250 pmol/ml synthetic SP or the hypothalamus sample was injected. After separation under the same conditions as for Fig. 2, each SP fraction without the fluorescence derivatization was treated with trypsin as the recommended procedure, and then the fragments produced were separated and fluorimetrically detected by the same HPLC method as in Fig. 3. Peaks: 1 = Arg as SP fragment and artifact from trypsin; 2 = artifact from trypsin; 3 = SP fragment (probably Arg-Pro-Lys); 4 = SP; 5 = probably modified SP.

sample. The relative standard deviation was 3.2% for 8.12 pmol/mg of protein in the tissue.

The concentration of the endogenous SP-like Arg-containing peptides in the hypothalamus tissue of rat brain was determined by the present HPLC method. The concentrations (six determinations) were 9.45 ± 1.50 pmol/mg of protein (mean $\pm$ S.D.) which corresponded to 523 ± 82.8 pmol/g of wet tissue. The results are in good agreement with the data obtained by the RIA method coupled with HPLC [9].

The detection system in the present HPLC method showed the necessary selectivity and sensitivity for the determination of the SP-like Arg-containing peptide in brain tissue. This study also provides the first practical HPLC method with fluorescence detection for quantification of the endogenous SP-like Arg-containing peptide.

REFERENCES

1 M.M. Chang and S.E. Leeman, J. Biol. Chem., 245 (1970) 4784.
2 M.M. Chang, S.E. Leeman and H.D. Niall, Nature New Biol., 232 (1971) 86.
3 C.J. Helke, T.L. O'Donohue and P.M. Jacobowitz, Peptides, 1 (1980) 1.

4 B. Pernow, Pharmacol. Rev., 35 (1983) 85.
5 I. Kanazawa and T. Jessell, Brain Res., 117 (1976) 362.
6 L.L. Iversen, T. Jessell, and I. Kanazawa, Nature (London), 264 (1976) 81.
7 M. Otsuka and S. Konishi, Nature (London), 264 (1976) 83.
8 T. Sakakura, P.L. Greves, J. Stewart and T. Terenius, J. Neurochem., 44 (1985) 718.
9 Y. Takano, A. Nagashima, H. Masui, K. Kuromizu and H. Komiya, Brain Res., 369 (1986) 400.
10 J.M. Conlon and B. Göke, J. Chromatogr., 296 (1984) 241.
11 M. Ohno, M. Kai and Y. Ohkura, J. Chromatogr., 392 (1987) 309.
12 M. Kai, M. Yamaguchi and Y. Ohkura, Anal. Chim. Acta, 120 (1980) 411.
13 M. Kai, T. Miura, K. Kohashi and Y. Ohkura, Chem. Pharm. Bull., 29 (1981) 1115.
14 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
15 M. Kai, J. Ishida and Y. Ohkura, J. Chromatogr., 430 (1988) 271.
16 C.-Y. Yang, H.J. Pownall and A.M. Gotto, Anal. Biochem., 145 (1985) 67.