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RESEARCH ARTICLE

Simultaneous Determination of Substance P and CGRP in Rat Brainstem Tissue Using LC-ESI-MS/MS Method

Neva Alasağ[1*], Erol Şener[2] and Dilek Doğrukol-Ak[2]

[1]*Department of Analytical Chemistry, Graduate School of Health Sciences, Anadolu University, Eskişehir, Turkey;* [2]*Pharmacy Faculty, Department of Analytical Chemistry, Anadolu University, Eskişehir, Turkey*

Abstract: ***Background:*** Substance P and CGRP are neuropeptides that belong to the thachykinin and calcitonin family, respectively. They play an important role in neuropathic pain development and regulation.

Methods: In this study, simultaneous analysis of Substance P and CGRP in rat brainstem tissue was conducted by using LC-ESI-MS/MS method. A gradient mobile phase was used with a Zorbax C8 150x2.1 mm, a particle size of 3.5 µm narrow-bore column. Mobile phases consist of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (v/v) (B). Firstly, mass spectrometry parameters were optimized. After achieving chromatographic separation, the method has been validated and applied to rat brainstem samples.

Results: SP and CGRP were eluted at 8.7 min and 10.1 min, respectively. Mass spectrum of neuropeptides was obtained in positive polarity and ion transitions of Substance P and CGRP were monitored at m/z 674.4→[600+254.4] and 952.2→1214.200, respectively. Linearity range for quantification was 5.85-438.52 ng/mL for Substance P and 0.21-155.28 ng/mL for CGRP. The limit of quantification was 5.85 ng/mL for Substance P and 0.21 ng/mL for CGRP. According to tissue samples analysis (n=6), basal endogen concentrations of Substance P and CGRP in brainstem tissue were calculated as 260.57±11.24 ng/g and 22.53±0.75 ng/g respectively.

Conclusion: A novel LC-ESI-MS/MS method was successfully developed and validated according to the bioanalytical validation guideline published by the EMA including precision, accuracy, specificity and linearity. Acceptable results were achieved. Basal endogen concentrations of Substance P and CGRP in brainstem tissue were determined.

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1. INTRODUCTION

Neuropeptides are one of the largest class of signaling molecules in nervous systems to regulate a wide range of physiological processes such as feeding, behavior, anxiety, pain sensing and reproduction [1-4]. Although neuropeptides were initially discovered in the nervous system, every tissue or cell utilizes them to communicate with its environment. They affect neural and non-neural tissues. Also, they can have autocrine, paracrine and hormonal effects. In the nervous system, they act as neurotransmitters or neuromodulators [3].

Substance P (SP) is an 11 aminoacid consisting of short-chain polypeptide belonging to the tachykinin family. Its closed chemical formula is $C_{63}H_{98}N_{18}O_{13}S$ with a molecular weight of 1347.63 g/mole. It was first described by Von Euler and Gaddum in 1931. Once synthesized into a large protein, it is enzymatically converted into active undecapeptide structure (Arg-Pro-Lys-Pro-Gln-Gln-Phe- Phe-Gly-Leu-Met-NH_2) [5, 6].

SP is widely distributed in the central, peripheral nervous systems. It also has been found in the cardiovascular system. It is connected with many of the physiological processes. Additionally, it plays an important role in cell repair, bone marrow fibrous degeneration and wound healing, angiogenesis, pain transmission and inflammation. SP was qualitatively detected in the skin, cerebrospinal fluid, blood plasma, gingiva, sweat glands, saliva, digestive tract texture, mucus and recently in tears. SP has been found to play a key role in the normal secretion of tears, but it has not been determined how it occurs. Because of the difficulty in analyzing, the biological function of this peptide, it is difficult to understand [7].

Alpha-calcitonin gene-related peptide (α-CGRP) is a 37 aminoacid belonging to the calcitonin family of neuropep-

*Address correspondence to this author at the Department of Analytical Chemistry, Graduate School of Health Sciences, Anadolu University, Eskişehir, Turkey; Tel: +90 541 7814707; E-mails: nalasag@anadolu.edu.tr; neva76@gmail.com

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tides. Its closed chemical formula is $C_{163}H_{267}N_{51}O_{49}S_2$ with a molecular weight of 3789.36 g/mole. The amino acid sequence of the rat α-CGRP is shaped like Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH_2 [6]. It is known as the most potent endogenous vasodilatory peptide which is 100-1000 times more potent than substance P or acetylcholine. It also performs a significant function in cardiovascular regulation. Various studies have demonstrated its important role in migraine pathophysiology. In migraine attacks, high CGRP concentrations were observed both in plasma from jugular vein and saliva. It was also reported that people with migraine headaches have increased headache when injected with α-CGRP, intravenously [8-13].

In the central nervous system, CGRP coexists with SP and is co-released from primary sensory neurons, that are involved in blood flow, pain and inflammation [14-16]. It has been reported that there was not change in SP levels but CGRP concentration increased during migraine attacks [17]. Several studies indicated that brain-skin connections and SP and CGRP could be important as the key of stress mediators. Stress increased the number of both SP and CGRP positive sensory nerve fibers in mice model [18].

Since peptides were few in biological samples, the separation and quantification of them were the most challenging issue in peptidomics [19]. For separation, commonly used methods are sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Size-Exclusion Chromatography (SEC), Radioimmunoassay (RIA), Capillary Electrophoresis (CE) and High Performance Liquid Chromatography (HPLC) [5]. Effective peptide quantification from biological samples requires highly selective, sensitive, robust and accurate Liquid Chromatography–Mass Spectrometric (LC–MS) methods. Recently technological developed methods such as quantitative matrix-assisted laser desorption/ionization-mass spectrometric (MALDI-MS) approaches or the coupling of electrospray ionization with high performance liquid chromatography led to the development of new LC-ESI-MS/MS techniques for peptide analysis [20-25]. Neuropeptidomic studies based on mass spectrometry include the identification of endogenous neuropeptides in the nervous system in biological samples and have an increasing prevalence in neuroscience research. New peptides can be discovered using this method, and when applied to disease models and important roles in pathophysiological mechanisms can be identified and used as drug targets or biomarkers [26-28]. Techniques based on Enzyme Linked Immunosorbent Assay (ELISA) methods have been used predominantly for quantification of SP, CGRP in the literature [29-36].

It could be reach just one study on simultaneous determination of SP and CGRP. Such as a study on determination of SP and CGRP from spinal cord of rat with LC-IonTrap/MS. The linearity, precision and accuracy parameters have been shown to be suitable for validation guide. In the rat spinal cord analysis, 110.7 pmol/g for SP and 2541 pmol/g for CGRP were found. It was reported that the developed method was useful for determining neuropeptide regulation in animal models of neuropathic and chronic pain [37].

A new, specific and validated method for the quantification of SP, CGRP in rat brainstem tissues were reported using triple quadrupole tandem mass spectrometer in this study. Separation parameters were optimized successfully and validated according to the bioanalytical validation guideline published by the EMA. Acceptable validation results were achieved. Basal endogen concentrations of Substance P and CGRP in rat brainstem tissue were determined.

2. MATERIALS AND METHOD

2.1. Chemicals and Reagents

SP and CGRP were purchased from Bachem (Torrance, CA, USA). Acetonitrile and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and LiChrosolv® water for chromatography were purchased from Merck (Darmstadt, Germany). All solvents were of analytical grade.

2.2. Solutions

Stock solution of SP and CGRP were prepared as 1 mg/mL in 5% acetic acid and stored at −20°C. Stock solution of SP and CGRP was further diluted in 0.5% acetic acid in acetonitril and used as a standard working solution and stored at 4°C. The calibration solutions were prepared in 3 sets consisting of 5 different concentrations (5.85 ng/mL, 58.5 ng/mL, 146.18 ng/mL, 292.350 ng/mL, 438.52 ng/mL) for SP and 3 sets of 6 different concentrations (0.21 ng/mL, 2.1 ng/mL, 21 ng/mL, 51.760 ng/mL, 103.521 ng/mL, 155.28 ng/mL) for CGRP. Each set was run 3 times.

2.3. Apparatus and Conditions

The Agilent 1290 Series LC system consists of an autosampler, a binary pump and a column oven. A gradient mobile phase was used with a Zorbax C8 150x2.1 mm with a particle size of 3.5 µm narrow-bore column. Binary gradient mobile phase components consist of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (v/v) (B). Flow rate was fixed at 0.2 mL/min. The LC separation was performed by using gradient elution program including initial (0 min) 10% B, 15 min 45% B, 20 min 60% B, 20.01 min 10% B. SP and CGRP eluted at 8.7 min, 10.1 min, respectively.

The LC system was coupled to 6460 triple-quadrupole mass spectrometer equipped with a jet stream electrospray ionization source. Analysis of data were evaluated by Agilent MassHunter B.06.0. Sheath gas flow was 11 L/min and sheath gas temperature was 300°C. Nebulizer at 45 psi and capillary voltage at 3500 V were applied. Mass spectrum was obtained in positive polarity for Substance P and CGRP, and ion transitions were used at m/z 674.4→[600+254.4] and 952.2→1214.200, respectively.

2.4. Preparation of Brainstem Tissue Samples

Wistar rats (n=6) were used in experiments. They were housed in a standard facility with a 12h light/dark cycle under constant temperature and humidity. Rats were fed with pellet feed and they were given free access to food and water. All animal experiments were performed in accordance with the principles of animal use and care approved by the

ethical committee of the Medical Faculty of Abant İzzet Baysal University (Approval File No. 2010/21). Brainstem tissue of rats was rapidly dissected after deep anesthesia with ketamine/xylazine. Following the dissection, tissues were weighted and homogenized by 2M acetic acid. Then, samples were heated at 90°C for 10 min in a thermostatic water bath and then centrifuged by vivacon®500 microcon filter at 7659xg for 30 min. Filtrated supernatant was transferred into an injection vial for analysis.

2.5. Quantification of SP and CGRP in Brainstem Tissue

In order to quantitate SP and CGRP in rat brainstem tissues, calibration solutions were prepared for the construction of external standard curves as shown in Table **2**. LC-MS/MS analysis was performed in the optimum analytical and instrumental conditions and the peak areas against the concentrations were analyzed. Standard addition method was used in order to evaluate matrix effect and recovery values. It was performed by spiking standard SP and CGRP solutions at different concentrations (5.85 ng/mL - 438.52 ng/mL for SP and 2.1 ng/mL - 155.28 ng/mL for CGRP) to supernatant of brainstem tissue samples and another sample without standard addition was prepared by adding 0.5% acetic acid in acetonitrile instead of standard solutions used for dilution into the rat brainstem tissue samples.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS Method Optimization

In order to optimize LC parameters, some different gradient elutions were tried. According to elution times of compounds and clean-up of the column between runs, the gradient elution was performed in the study.

LC-MS/MS analysis parameters such as precursor ion, fragmentor voltage, fragment ion, collision energy and accelerator voltage were investigated to optimize the analysis of neuropeptides. For the determination of the precursor ions of SP and CGRP, a mass scan of *m/z* 100-1400 was carried out. MS scan spectrum was obtained in positive ion mode for neuropeptides. Mass spectrum of SP and CGRP showed the formation of multiple charged ions ($[M+nH]^{n+}$) at *m/z* 674.4 (+2) and *m/z* 952.20 (+4) consistent with the molecular weight of SP and CGRP, respectively. For CGRP, high signal intensity *m/z* 761.90 (+5) was obtained as a precursor ion peak, but MRM signal intensity of *m/z* 761.90 was low (not suitable) to monitor this neuropeptide in the calibration studies and tissue analysis. Therefore, the *m/z* 952.20 has been used as a precursor ion of CGRP in the analysis and *m/z* value of the precursor ion peaks was found to be the same as reported in literature [38, 40, 41]. Representative MS^1 and MS^2 mass spectra of SP and CGRP are shown in Fig. (**1**).

The mass transitions for SP and CGRP quantitation in MRM mode were at *m/z* 674.4→[600.0+254.4] and *m/z* 952.2→1214.20, respectively. Fragmentor voltage determination for SP and CGRP was performed in single ion monitoring (SIM) mode. Since the highest signal intensities were obtained at 143 V and 160 V for SIM spectra of SP and CGRP, these values were assumed for the fragmentor voltage to be used during the experimental study. The collision energy of the LC-MS/MS instrument could be used in the

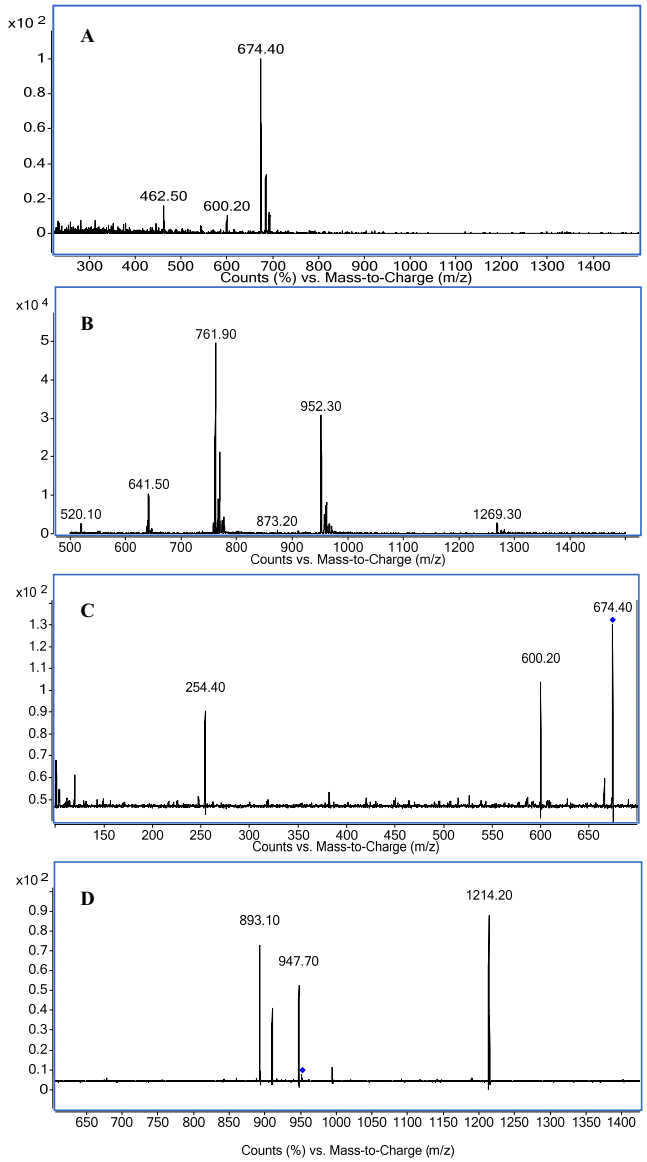


Fig. (1). Mass spectra of the compounds, MS^1 of SP (**A**), MS^1 of CGRP (**B**) MS^2 of SP (**C**), MS^2 of CGRP (**D**).

range of 0-250 V. SP and CGRP could be fragmented at low collision energy. By scanning the values of the collision energy between 20 V - 30 V,suitable values were determined as 24 V and 25 V for the neuropeptides. Optimization parameters are presented in Table **1**.

After optimization of separation parameters, standard SP and CGRP solutions and brainstem tissue samples were analyzed using LC-MS/MS. The retention times for SP and CGRP in brainstem tissues were observed to be the same as 8.7 min. and 10.1 min. for standard solution of SP and CGRP, respectively. MRM chromatograms of SP and CGRP in standard solutions and in brainstem tissue samples can be seen in Fig. (**2**).

3.2. LC-MS/MS Method Validation

After method optimization, quality control samples were prepared for the method validation. Validation parameters

Table 1. MS/MS Optimization Parameters of SP and CGRP.

Optimization Parameter	SP	CGRP
Precursor ion (*m/z*)	674.4	952.20
Product ion (*m/z*)	600.0, 254.4	1214.20
Fragmentor voltage (V)	143	160
Collision energy (V)	24	25
Cell accelerator voltage (V)	3	5

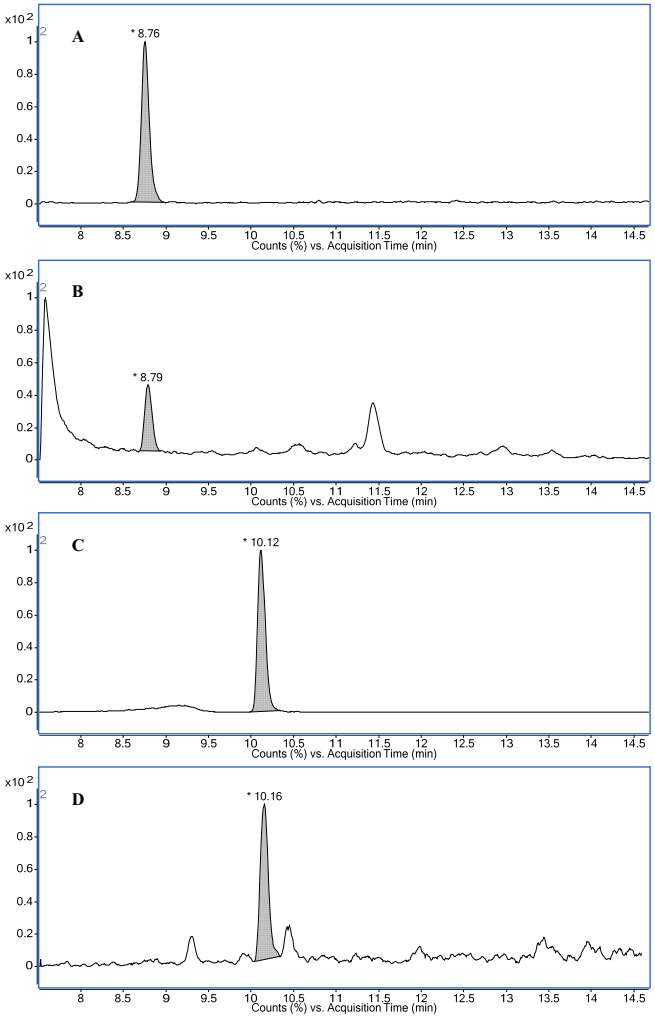


Fig. (2). MRM chromatograms of SP in standard solution (556 ng/mL) (**A**), SP in rat brainstem sample (**B**), CGRP in standard solution (200 ng/mL) (**C**), CGRP in rat brainstem sample (**D**).

including linearity, accuracy and precision, limit of quantification, selectivity, matrix effect and stability were investigated according to bioanalytical validation guideline European Medicines Agency (EMA) [37].

3.2.1. Linearity

Linearity of the developed method for neuropeptides was determined. MRM signals including fragments of *m/z* 600, *m/z* 254.4 for SP and *m/z* 1214.20 for CGRP were used. The linearity range was determined to be 5.85-438.52 ng/mL, 0.21-155.28 ng/mL after plotting the weighted linear regression (1/x) with correlation coefficient (r) ranging between 0.9927-0.9980 and 0.9903-0.9940 for SP and CGRP, respectively. The GraphPad Prism Version 6.0 program was used to evaluate the results.

The inter-day and intra-day linearity were statistically evaluated including slope, intercept, coefficient of correlation (r) and 95% confidence limit of slope and intercept values after weighted linear regression (1/x). The results of statistical analysis are demonstrated in Table **2**. Inter-day calibration curves were used for quantification of the neuropeptides.

3.2.2. Accuracy, Precision and Lower Limit of Quantification

Precision and accuracy were determined by measuring the concentration of SP and CGRP in three replicates of quality control standards at three different concentrations (5.85, 146.18 and 438.52 ng/mL for SP and 0.21, 21 and 155.28 ng/mL for CGRP) for three separate batches. Intra-day precisions for SP and CGRP were lower than 8%, 6%, and calculated recovery was higher than 99% and 97% for SP and CGRP, respectively. For the inter-day studies precisions were about 15% and 11%, and calculated recovery was higher than 98% and 87% for SP and CGRP, respectively. The lower limit of quantification (LLOQ) was 5.85 ng/mL and 0.21 ng/mL for SP and CGRP according to 20% coefficient of variance. Method accuracy and precision are represented in Table **3**.

The method's accuracy and precision in brainstem tissue were determined by spiking three different concentrations of standard solution (58.5, 146.16 and 438.52 ng/mL for SP and 21.0, 51.76, 155.28 ng/mL for CGRP) into brainstem tissue samples. Accuracy in brainstem tissue was shown with recoveries higher than 97%, 95% and precision was shown to be lower than 7% RSD, 13% RSD for SP and CGRP. According to tissue samples analysis (n=6), SP and CGRP basal endogen concentrations in brainstem tissue were calculated as 260.57±11.24 ng/g and 22.53±0.75 ng/g respectively. Accuracy and precision values are shown in Table **4**.

3.2.3. Selectivity and Matrix Effect

0.5% acetic acid solution prepared in acetonitrile used to dilute the peptide solution and 2 M acetic acid solution used for the homogenization of the tissue samples were analyzed

Table 2. Statistical analysis of method linearity.

	SP				CGRP			
Linearity Parametres	**Day1 (n=5)**	**Day2 (n=5)**	**Day 3 (n=5)**	**Inter-day (n=15)**	**Day1 (n=5)**	**Day2 (n=5)**	**Day 3 (n=5)**	**Inter-day (n=15)**
Slope	2.089	2.271	2.289	2.219	23.25	24.07	23.56	23.50
Intercept	-5.360	1.650	-3.595	2.445	2.595	11.75	19.67	13.30
r	0.9966	0.9927	0.9980	0.9967	0.9922	0.9903	0.9923	0.9940
CL of Slope*	±2.016	±2.154	±2.224	±2.143	±22.16	±22.76	±22.42	±22.54
CL of Intercept*	±11.09	±9.518	±8.940	±9.513	±30.18	±35.07	±13.63	±15.69

*CL: confidence limits at 95% probability level.

Table 3. Method accuracy and precision of SP and CGRP.

	Inter-day				Intra-day		
	Added (ng/mL)	**Found (ng/mL)**	**Recovery%**	***RSD %**	**Found (ng/mL)**	**Recovery%**	**RSD %**
SP	5.85	6.32	113.60	7.31	5.83	99.77	14.91
	146.18	145.50	99.54	3.59	143.73	98.32	8.96
	438.52	444.20	101.30	4.84	446.81	101.09	6.09
CGRP	0.21	0.25	119.53	5.74	0.18	87.60	10.30
	21.00	24.65	119.07	3.43	24.75	119.56	3.95
	155.28	150.79	97.11	5.35	151.55	97.60	5.89

*RSD%: Relative Standard Deviation.

Table 4. Accuracy and precision in brainstem tissue.

	Added (ng/mL)	**Found (ng/mL)**	**Recovery %**	***RSD %**
SP	58.50	69.91	119.55	6.05
	146.16	145.53	100.57	5.69
	438.52	325.38	97.43	2.22
CGRP	21	23.25	112.09	10.96
	51.76	55.18	106.54	4.95
	155.28	148.99	95.93	12.36

*RSD%: Relative Standard Deviation.

to determine if there was an interference at the retention time of SP and CGRP from reagent and homogenization process. Interference originated from blank solutions was not found at the retention times of SP and CGRP. In addition to the results of blank samples, recovery results from spiked tissues given in Table **4** indicate that the method is selective for SP and CGRP analysis.

The matrix factor and related RSD% values were calculated by proportioning the peak areas of the calibration curve of tissue samples belonging to each concentration to the peak areas of the calibration curve of the standard SP and CGRP solutions. The RSD% values of all concentrations were observed to be consistent with the values reported by the validation guide. Matrix factor and related RSD% values are shown in Table **5**.

3.2.4. Stability

Stability experiments in standard SP and CGRP solution and brainstem tissue were performed to demonstrate whether SP and CGRP were stable under analysis conditions such as short-term stability (room temperature, 12 hours), freeze-thawing stability (3 freeze-thaw at -20°C) and long-term stability (one month at -20°C). The peak areas of the samples at the beginning and the end of each stability test obtained

Table 5. Matrix factor and RSD% for SP and CGRP (n=6).

	Concentration (ng/mL)	*MF	RSD%
SP	5.85	7.103	14.70
	58.50	3.463	7.61
	146.18	2.821	6.79
	438.50	2.474	3.64
CGRP	2.10	3.831	14.57
	21.10	2.408	5.31
	51.76	2.233	8.23
	155.28	1.791	4.17

*MF: Matrix factor: Peak area in the presence of matrix/Peak area in absence of matrix.

Table 6. Stability tests of SP and CGRP.

Sample	Room Temperature Recovery%	Freeze-thaw Recovery%	Long Term Recovery%
SP in rat brainstem tissue	83.7	79.5	18.1
CGRP in rat brainstem tissue	77.53	67.75	8.6
SP standard solution (58.5 ng/mL)	93.28	87.5	-
CGRP standard solution (2.1 ng/mL)	63.38	-	-

were compared. Instability of the peptides could be originated from modifications such as oxidation, reduction, hydrolysis, and deamination of amino acid residues in peptides and proteins; therefore, they are chemically instable [6]. In order to minimize the stability problem in our study, stock solutions were stored at -20 °C in small portions and freshly prepared each time. After dissection, the tissue was immediately homogenized and analyzed. Table **6** shows the brainstem tissue sample and standard solution of SP and CGRP recovery values for the stability tests.

Compared with the existing literature, the following main advantages can be considered about the developed LC-MS/MS method in this study:

- It was the first full validated study on simultaneous determination of SP and CGRP in brainstem tissue. The validation parameters were investigated according to the guidelines of EMA bioanalytical validation including the matrix effect [37].
- Various sample preparation techniques have been tried to achieve the best signal and signal intensity. A simple and one-step tissue procedure consisting of acetic acid homogenization was used in the LC-MS/MS method developed in this study. The applicability of the two-step acetic acid procedures recommended in the literature is facilitated [26, 33, 38-41].
- For the preparation of standard solutions for the LC-MS/MS analysis of neuropeptides, it was recommended to prepare it in 0.25% TFA-water solution [38-41]. However, standard peptide solution prepared in 0.5% (v/v) acetic acid in acetonitrile in this study contributed to the observation of fragment ions finally obtained.
- Shorter retention time than the literature using a C8 narrowbore column was obtained for the analysis of neuropeptides [25].
- Unlike mass analyzers used in other LC-MS methods such as MALDI-MS, iontrap-MS ect., a triple quadrupole mass analyzer was used in the determination and quantification of SP and CGRP.
- Isotopes of the components targeted for analysis as Internal Standard (IS) in LC-MS/MS methods are usually used. This, because of neuropeptides isotopes, could not be used due to the difficulties of providing these isotope components.
- The quantitative determination of neuropeptides by the LC-MS/MS method was also carried out on the tissue sample being studied [21-23, 28, 29].

CONCLUSION

In this study aimed to perform SP and CGRP in brainstem tissues using LC-MS/MS, because of the presence of neuropeptides in biological samples at very low concentrations. A new fully validated LC-MS/MS method was developed to identify and quantify SP and CGRP in rat brainstem tissue. Generally, RIA and ELISA methods are widely used for quantification of neuropeptides. Although these methods are sensitive, most of the antiserum are not unique to a single peptide and can cross-react with other peptides. In addition to these methods, only a certain number of neuropeptides can

be analyzed at the same time. It limits the recognition of peptides. Using LC-MS/MS, it is possible to simultaneously identify and quantify multiple neuropeptides in a biological sample [26].

A highly sensitive, reliable, selective, rapid LC-MS/MS method has been developed and validated for determination of SP and CGRP in brainstem tissues. It will contribute to diagnostic studies of diseases in the fields of medicine and physiology researchers working in the field of neuroscience. It could be used in illuminating various hypotheses which would be based on measurements in biological tissues.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were performed in accordance with the principles of animal use and care approved by the ethical committee of the Medical Faculty of Abant İzzet Baysal University (Approval File No. 2010/21).

HUMAN AND ANIMAL RIGHTS

No human were used in this research. The reported experiments are in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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