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Direct detection of tetrahydrobiopterin (BH4) and dopamine in rat brain using liquid chromatography coupled electrospray tandem mass spectrometry

Hak Rim Kim, Tae-Hyun Kim, Sung-Hyun Hong, Hyung-Gun Kim *

Department of Pharmacology, College of Medicine, Dankook University, San 29, Anseo-dong, Dongnam-gu, Cheonan, Chungnam 330-714, Republic of Korea

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ABSTRACT

A simple and rapid liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed for the quantification of tetrahydrobiopterin (BH4) and dopamine in rat brain using epsilon-acetamidocaproic acid (AACA) as an internal standard. Proteins in the samples were precipitated with acetonitrile and then the supernatants were separated by a Sepax Polar-Imidazole (2.1 × 100 mm, i.d., 3 μm) column using a mixture of 10 mM ammonium formate in acetonitrile/water (75:25, v/v) as the mobile phase at a flow rate of 300 μl/min. Quantification was performed on a triple quadrupole mass spectrometer employing electrospray ionization with the operating conditions as multiple reaction monitoring (MRM) and positive ion mode from m/z 242.1 → 166.0 for BH4, m/z 154.1 → 90.0 for dopamine and m/z 174.1 → 114.0 for AACA (IS). The total chromatographic run time was for 5.5 min. The method was validated for the analysis of samples: the limit of detection was 10 ng/g. The calibration curve was linear between 10–2000 ng/g for BH4 (r^2 = 0.995) and 10–5000 ng/g for dopamine (r^2 = 0.997) in the rat brain. Thus, good correlated LC–ESI/MS/MS results were obtained and found to be a powerful tool for the quantitative analysis of BH4 and dopamine in the rat brain.

1. Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor for hydroxylation reactions of cyclic amino acids and is synthesized via the de novo pathway in a serial reaction from guanosine triphosphate (GTP) [1,2]. As a cofactor of phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase (TH) and tryptophan-5-hydroxylase (TPH), BH4 is involved in the biosynthesis of tyrosine from phenylalanine, of serotonin from tryptophan, of other critical neurotransmitters such as dopamine, and norepinephrine from tyrosine [2–5]. Also, nitric oxide synthase (NOS), a critical enzyme for normal vascular and cardiac function, requires BH4 as an essential cofactor for the synthesis of the reactive free radical nitric oxide [2,6,7]. The pathogenesis of various cardiovascular diseases like, hypertension, atherosclerosis, diabetes, cardiac hypertrophy, and heart failure had been reported to be associated with the concentration of BH4 in the cardiovascular system [2,7–9]. Moreover, alteration of BH4 metabolism has been reported to be closely related with different diseases including phenylketonuria, renal failure, and Parkinson's disease [4,10–12].

Therefore, it is obviously necessary to determine the levels of bioavailable BH4 with a precise method that can be applied for diagnostic evaluation of related various diseases or for screening of potential therapeutic products to modulate BH4 levels [13,14]. As the importance of measurement of BH4 levels in cells had been recognized, several groups developed HPLC methods to determine the cellular levels BH4, BH2, and other biopterins [15–17].

However, its direct determination has been considered as impossible because of the low sensitivity of previous methods. BH4 coexists with the oxidized BH2 in plasma and urine, but both are sparingly fluorescent species. Therefore, both are indirectly determined by generation of the strongly fluorescent biopterin and pterin, by using an iodine oxidation method [16,17].

Although, an indirect method to determine BH4 levels in plasma has been effectively employed, a more precise and robust method is required for the direct measurement without any oxidation reactions. In the present study, we developed a novel method for the direct measurement of biologically active BH4 by LC–MS/MS in rat brain sections. In addition, we could measure the concentration of dopamine in the samples of rat brain sections. This new method significantly improved the characteristics of the assay performance and will be well-suited for numerous studies for the direct determination of the concentrations of BH4 and dopamine in biological samples.

2. Materials and methods

2.1. Materials

The (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (BH4 2HCl), 7,8-dihydro-L-biopterin (BH2), 6-biopterin and dopamine

* Corresponding author. Fax: +82 41 551 3866.
E-mail address: hgkimm@dankook.ac.kr (H.-G. Kim).

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hydrochloride (dopamine HCl) were purchased from Sigma–Aldrich (St. Louis, MO, USA). epsilon-acetamidocaproic acid (AACA) was donated by Kuhnil pharmaceuticals (Seoul, Korea). Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and used without further purification.

2.2. Preparation of stock solution, calibration standards and quality control samples

The solution of BH4 or dopamine was prepared as a stock (1 mg/mL each) with acetonitrile (50%) and then diluted with pure acetonitrile for each experiment. Standard solutions of BH4 or dopamine for calibration curves were prepared by spiking at prepared blank solution to following amounts but the adding volumes were less than 10% of total volume. The giving final concentrations for standard curve were of 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/g for BH4. In parallel, the final concentrations for standard curve were of 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 ng/g for dopamine. The limit of reliable detection was 10 ng/g for each. The calibration curve was linear between 10–2000 ng/g for BH4 (r^2 = 0.995) and 10–5000 ng/g for dopamine (r^2 = 0.997). All solutions were freshly made for each experiment.

To clarify the internal calibration of our new method, we have spiked BH4 and dopamine into brain homogenate in same amounts that we used in above standard curves. The detected levels were increased by the amounts that we added above the endogenous levels in both BH4 and dopamine (Figs. S1, S2 on electronic supplementary material). Due to the matrix effects, we found that the lower limits of quantification (LLOQ) were 20 ng/g for BH4 and 50 ng/g for dopamine.

2.3. Sample preparation

Brain tissue (50 mg) was homogenized in acetonitrile (500 μL) with 5 μL of IS (250 ng/mL). After homogenizing thoroughly, the BH4 and dopamine from brain tissue were further extracted by sonication for 60 s. The homogenates of brain tissue were centrifuged at 12,000 rpm for 10 min at 4 °C. Supernatants were carefully transferred to 96-well plates and then injected onto the LC–MS/MS system by auto-sampler for subsequent analysis. To make blank matrix, we homogenize the whole brain tissues and take the supernatant after centrifugation. The fresh supernatant has BH4 and dopamine as endogenous levels (data not shown). To take an advantage of unstability of BH4 at room temperature, we kept the supernatant at room temperature more than 24 h and found almost all BH4 oxidated either BH2 or biopterins. It is d-water for dopamine to use as blank matrix.

2.4. Apparatus and chromatographic conditions

The liquid chromatographic system was an Accela system (Thermo Fisher Scientific Inc., Waltham, MA, USA), equipped with a nanospace SI-2 3133 solvent delivery module as an auto-sampler (Shiseido Inc., Japan), and connected to a Discovery Max (Thermo Fisher Scientific Inc.) quadrupole tandem mass spectrometer coupled with electrospray ionization (ESI-MS/MS). System control and data processing were performed using the Xcalibur software (Thermo Fisher Scientific Inc.). The chromatographic separation was achieved by using a hydrophilic interaction chromatography (HILIC) Sepax Polar-Imidazole (2.1 × 100 mm, i.d., 3 μm particle size) HPLC column (Sepax Technologies, Delaware, USA) with a Phenomenex C_{18} guard column (4 × 2 mm, Phenomenex). A nanospace SI-2 3004 column oven (Shiseido, Japan) was used online. The mobile phase consisted of 10 mM ammonium formate (pH 3) in acetonitrile/water (75:25, v/v) mixture. The flow rate was 300 μl/min and the injection volume was 10 μl. The electrospray ionization (ESI) mass spectrometer was operated in the positive ion mode. Multiple reaction monitoring (MRM) of the precursor-product ion transitions was m/z 242.1 → 166.0 for BH4, m/z 154.1 → 90.0 for dopamine, and m/z 174.1 → 114.0 for AACA. Collision energy was 20.0, 24.0, and 14.0 V for BH4, dopamine, and AACA, respectively. The optimized conditions were: ESI needle spray voltage (4000 V), sheath gas pressure (35 unit), auxiliary gas pressure (20 unit), capillary temperature (206 °C), collision gas (Ar) pressure (1.5 mTorr), skimmer offset (5 V), and chrome filter peak width (10 s). The scan was performed in profile mode with SIM width of 0.700 FWHM, scan time of 0.200 s, and scan width of 0.5 Da.

2.5. Method validation

For the determination of linear range, eight non-zero calibration samples were employed. Linear regression of the ratio of peak area

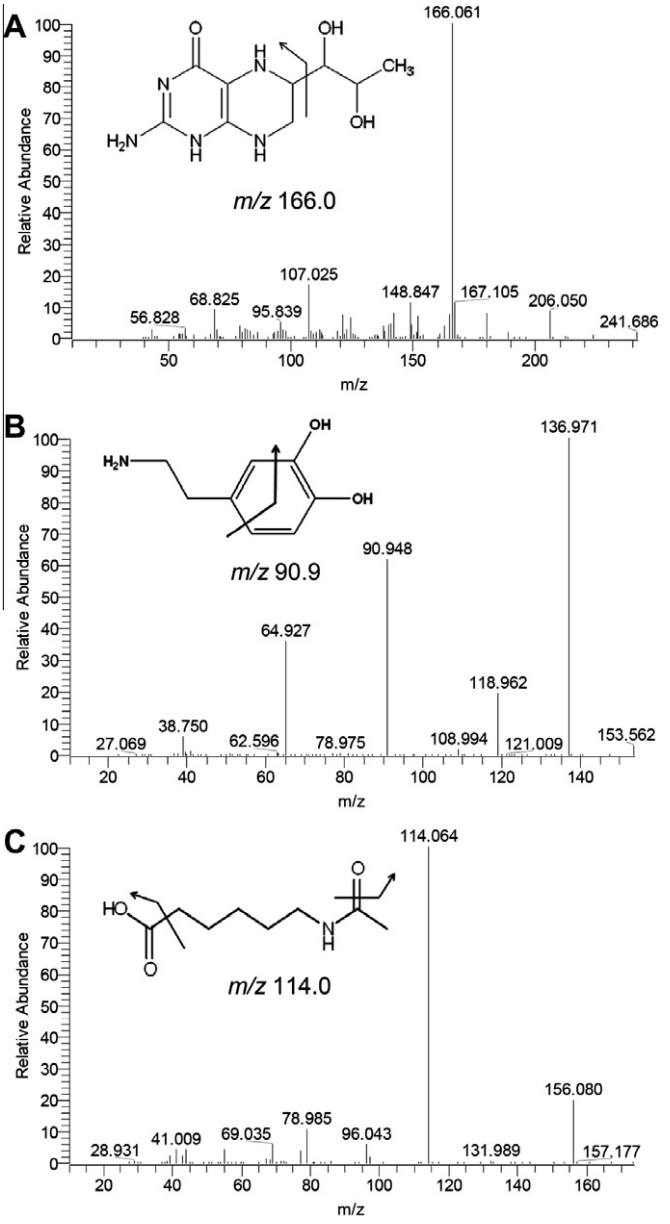

Fig. 1. Product ion mass spectra used in multiple reactions monitoring for (A) BH4 (precursor ion m/z 242.1), (B) Dopamine (precursor ion m/z 154.1), and (C) AACA (precursor ion m/z 174.1).

of BH4 or dopamine were obtained for peak areas of BH4 or dopamine vs peak areas of IS with a weighting of $1/X^2$ (least-squares linear regression analysis, where X is the concentration of the analyte).

2.6. Statistical analysis

All the values given in the text and table are expressed as mean ± SE. Statistical differences between means were evaluated with a two-tailed Student's *t*-test. *P* values of less than 0.05 were taken to be statistically significant.

3. Results

3.1. Sample preparation and liquid chromatography

For simple sample preparation, protein precipitation was attempted using acetonitrile. To prevent sample degradation and oxidation, HCl (0.1 mM) and dithioerythritol (0.01%, w/v) were also added. The peak shapes of BH4, dopamine, and IS were the best, when acetonitrile was used for protein precipitation and as an organic solvent for the mobile phase.

3.2. Mass spectrometry

Our optimized electrospray ionization conditions could sensitively detect BH4, dopamine and IS with positive ion detection mode. The most abundant protonated ion peaks in the Q1 mass spectra of BH4, dopamine and IS were at *m/z* 242.1, *m/z* 154.1 and *m/z* 174.0, respectively, and there was no evidence of fragmentation and adduct formation. The product ions ($[M + H]^+$) in the Q3 mass spectra and proposed fragmentation patterns were BH4, at *m/z* 166.0 (2-amino-7,8-dihydropteridin-4(1H)-one) by loss of propane-1,2-diol (Fig. 1A), dopamine at *m/z* 90.9 (butane-1,2-diol) by loss of (E)-3-methylpent-3-en-1-amine (Fig. 1B), and IS at *m/z* 114.0 ((E)-*N*-ethylidenepentan-1-amine) by loss of both carboxyl and hydroxyl groups (Fig. 1C). Also to confirm the separation between BH4 and other biopterins in biological sample, we did experiments to quantify BH4, BH2, and biopterin in a mixed sample (Fig. 2).

3.3. Sensitivity and specificity

As shown in Fig. 3A, there are no peaks for dopamine or BH4 in the representative blank chromatograms (no exogenous spike of BH4 and dopamine). We used homogenate extract from rat whole brain as the matrix for BH4 and distilled water for dopamine. The validation of samples at the LLOQ (lower limit of quantification) was 10 ng/g for BH4, dopamine, and IS, respectively. No significant interference from endogenous substances was observed in the rat brain from five different individual sources preparation. There was a blank peak at retention time of BH4 (0.4 min), but it was separated from the target peak (1.4 min). There was a blank peak at retention time of dopamine (1.1 min), but this was separated from the target peak (0.9 min). Retention time of the IS was 1.5 min. The peak signal-to noise ratios were 25 for BH4 (10 ng/g), and 20 for dopamine (10 ng/mL), respectively. The area of LC–MS/MS chromatogram at the LLOQ (10 ng/g) was at least five times higher than that of the blank (Fig. 3A and B). We further tested our newly developed method by using corpus striatum from rat, and subsequently confirmed that the chromatogram peaks of both BH4 and dopamine had the same peak shapes and retention times with that of the standard (Fig. 3D).

3.4. Linearity

Eight different concentrations from 10 to 2000 ng/g of BH4 or from 10 to 5000 ng/g of dopamine vs AACA were used for the standard curves. We found that the data from eight points were linear and the correlation coefficient (r^2) of the standard curves were

Biopterins MS/MS Break Down

	Precusor Ion $[M+H]^+$ (*m/z*)	Product Ion $[M+H]^+$ (*m/z*)	CE (V)
Biopterin (B)	238.00	193.86	18
Dihydrobiopterin (BH2)	240.00	196.00	14
Tetrahydrobiopterin (BH4)	242.10	166.00	20

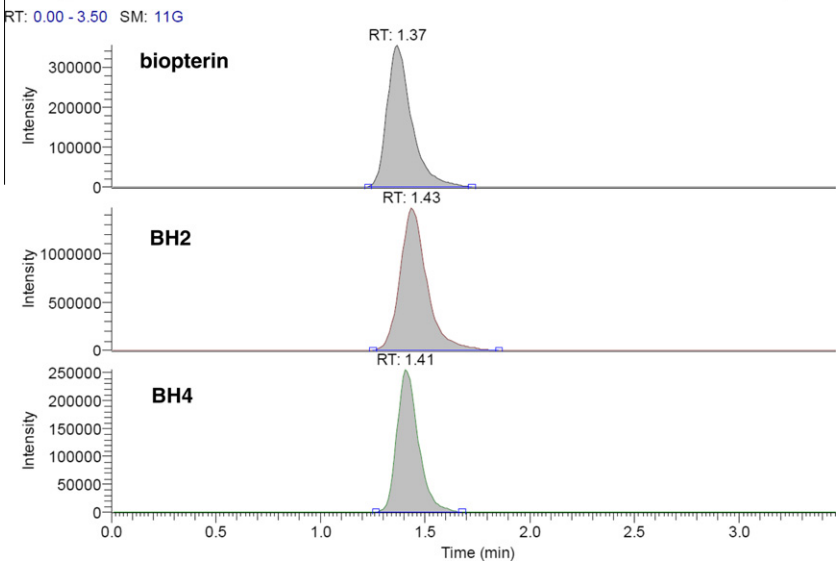

Fig. 2. Biopterin MS/MS break down and representative chromatograms from mixture of biopterin, BH2 and BH4.

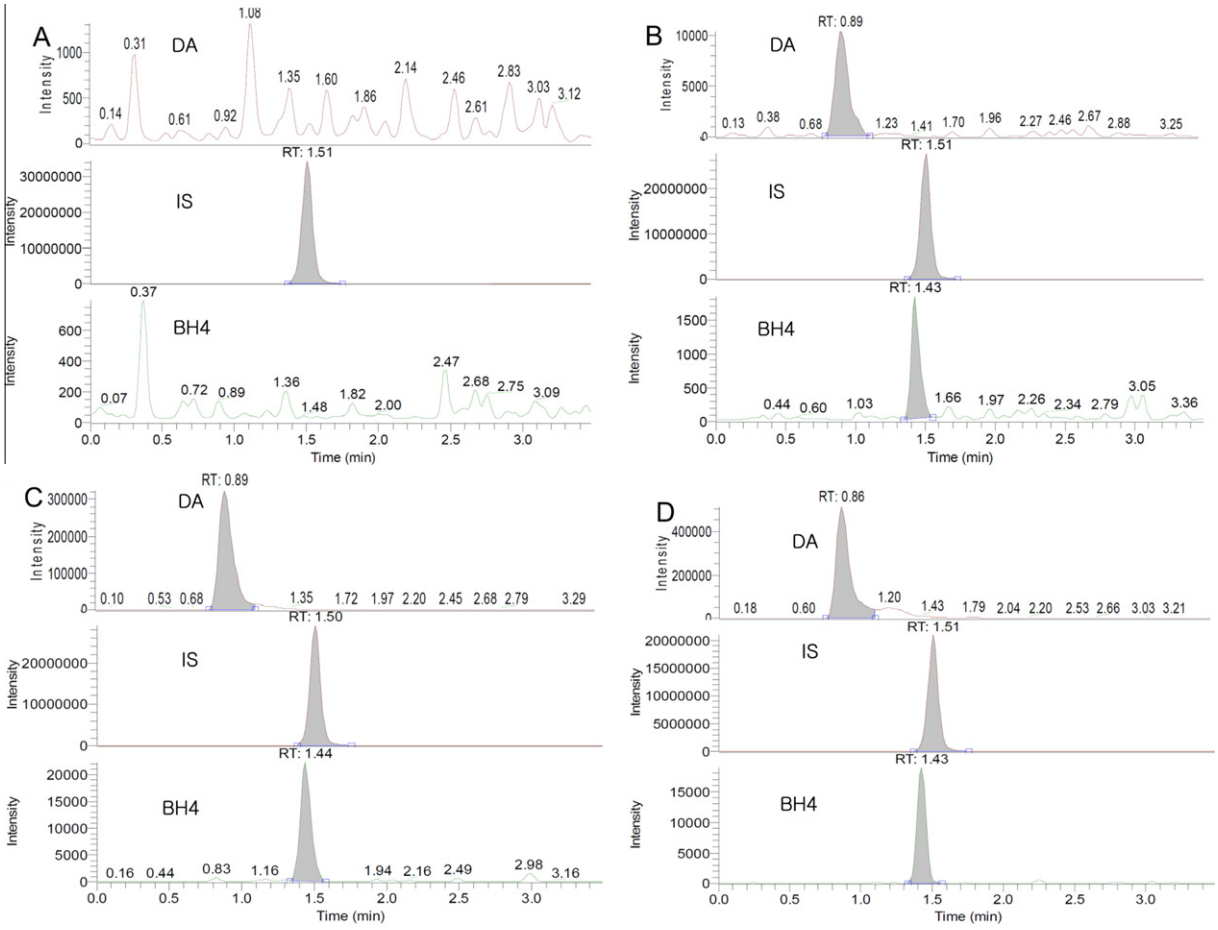

Fig. 3. Representative chromatograms of rat brain homogenant (A: blank), chromatograms of rat brain spiked with BH4 and IS (B: 10 ng/g, C: 200 ng/g), and brain tissue (D: corpus striatum).

Table 1
Concentration of BH4 & dopamine in various brain tissues ($n = 10$).

Brain tissues	BH4 (ng/g)		Dopamine (ng/g)	
	Mean ± SE	BQL* (n)	Mean ± SE	BQL* (n)
Corpus striatum	215 ± 38.2	0	5461 ± 797.7	0
Substantia nigra	157 ± 34.2	0	3504 ± 961.7	0
Mid brain	116 ± 44.7	0	144 ± 54.8	1
Cerebellum	43.8 ± 5.40	0	14.0 ± 5.01	7
Hippocampus	42.0 ± 18.24	4	119 ± 74.2	3
Frontal cortex	19.5 ± 4.02	2	27.8 ± 6.87	3
Occipital lobe	14.2 ± 2.65	3	19.6 ± 4.60	1

*BQL : below the quantification limit.

0.9953 ($y = -0.00003809 + 0.00002105x$) for BH4, and 0.9975 ($y = -0.0001912 + 0.00006037x$) for dopamine.

3.5. BH4 Stability and the measurement of BH4 and dopamine in brain tissue

The concentration of BH4 in the brain tissue treated with only acetonitrile dramatically decreased to 42% of its initial concentration in the brain tissue, when the samples were kept at room temperature for 4 h. However, in the case of acetonitrile treated samples kept at 4 °C, 85% of the initial concentration of BH4 was conserved in the brain tissue. These data suggest that the temperature is one of the critical factors for sample preparation to prevent the degradation of BH4 in brain tissue.

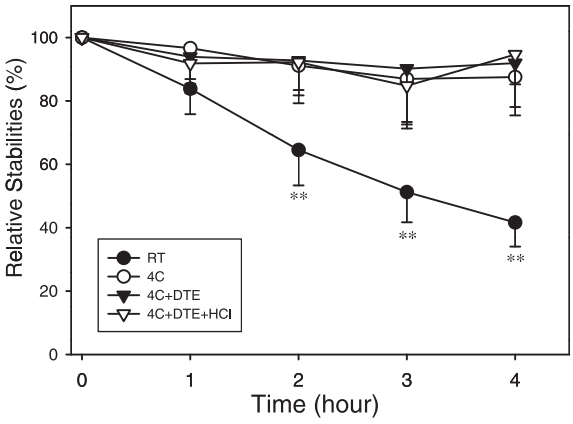

Fig. 4. Comparative relative stability of BH4 with time ($n = 4$). Graph showing the relative stability of BH4 in different conditions (temperature, DTE, DTE + HCl) with time. Data were normalized by the initial concentration of BH4 (time 0). RT, room temperature; 4C, 4 Celsius; HCl, 0.1 mM; DTE, dithioerythritol (0.01%, w/v). $^{**}p < 0.01$ vs 4C.

In this study, we measured the concentration of BH4 and dopamine in several brain sections by using newly developed experimental method (Table 1). The results showed that BH4 concentration was highest at corpus striatum, followed by, in decreasing order: substantia nigra, mid brain, cerebellum, hippocampus, frontal cortex, and occipital lobe. In parallel, dopamine concentration was also highest in corpus striatum, followed by

substantia nigra, mid brain, hippocampus, frontal cortex, occipital lobe, and cerebellum. These data revealed that the concentrations of dopamine were clearly correlated with the concentration of BH4 in brain tissue.

4. Discussion

Tetrahydrobiopterin (BH4), is an essential cofactor for the functions of phenylalanine-4-hydroxylase (PAH), tyrosine-3-hydroxylase (TH), and tryptophan-5-hydroxylase (TPH), and is necessary for the biosynthesis of tyrosine from phenylalanine, of serotonin from tryptophan, and of other critical neurotransmitters such as dopamine and norepinephrine from tyrosine [2–4]. Also, another critical enzyme for normal vascular and cardiac function, is nitric oxide synthase (NOS) which needs BH4 as a cofactor for synthesis of nitric oxide [2,6,7]. Therefore, BH4 has been suggested to play an important role in many diseases and considered as a critical factor in the occurrence of numerous diseases such as Parkinson's disease, depression, phenylketonuria, hypertension, atherosclerosis, diabetes, cardiac hypertrophy, and heart failure [2,4,7,8,10,11].

Therefore, it is necessary to determine the biological levels of BH4 with a precise method for evaluation of various diseases and to screen for potential therapeutic candidates [14]. As the importance of measurement of BH4 levels in cells has been recognized previously, several groups therefore developed HPLC methods to determine the levels of BH4, BH2 and other biopterins with indirect methods [16,17]. Nevertheless, direct determination of BH4 in biological sample was difficult due to low sensitivity and lack of a fluorescent species. Moreover, BH4 is readily oxidized and degraded to biopterin or pterin [18].

In the present study, we developed a novel method for the direct measurement of biologically active BH4 and dopamine in the rat brain tissues by using LC–MS/MS. This new method is able to analyze BH4 and dopamine accurately and quickly in brain tissues. Previously, BH4 concentrations have been indirectly calculated by measuring the levels of biopterin in biological samples [18]. However, this indirect method has the problem of not measuring exact BH4 concentrations due to loss of conversion between biopterin and BH4. Because of oxidation and degradation of BH4, it is difficult to measure BH4 directly. To avoid these difficulties, we found that low temperature is a critical factor to prevent degradation of BH4 in brain tissues (Fig. 4), and the addition of antioxidant (DTE) and/or acid (HCl) to the sample does not affect the stability of BH4 (data not shown). Maintaining the sample at 4 °C is sufficient in keeping BH4 stable for 4 h, which is enough time to complete the entire analysis.

By using HPLC, BH4 and dopamine were separated into single peaks. In previous studies, reverse-phase columns were used to separate BH4 and dopamine, but such type of column had the limitation of separating many hydrophilic materials. Moreover, many unknown materials in the matrix interfered with the analysis of BH4 and dopamine in the biological samples [15]. To avoid those problems, we used a HILIC column in this experiment. The HILIC column was able to separate BH4 and dopamine from interfering matrices with an appropriate retention time (Fig. 3). In addition, using MS/MS could increase the selectivity and sensitivity of BH4 and dopamine in brain samples (Fig. 1).

In this communication, we also present data on the concentration of BH4 and dopamine of seven brain regions, including corpus striatum, substantia nigra, mid brain, cerebellum, hippocampus, frontal cortex, and occipital lobe. The BH4 concentrations have been measured previously by Milstien and Kaufman [19]. The data of BH4 presented here in most cases generally appears to be a good agreement between the present results and those previously reported by analysis of BH4 by HPLC with coulometric detection. The dopamine concentrations also have been measured previously [20–22]. Especially, Versteeg et al. [21] had studied intensively the dopamine concentrations of various regions at rat brain by paper chromatography with spectrometry. The dopamine concentration of substantia nigra from our study has a good agreement with that of Versteeg et al. [21]. However, that of frontal cortex in our study is far less than that of previous study [21]. Although differences of tool for analysis or errors in calibration may account for this large difference, differences in sampling region are also a likely factor.

This new method significantly improved the characteristics of assay performance for BH4 and dopamine and should be well-suited for many biological and clinical studies for the direct determination of the concentration of BH4 and dopamine in biological samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.02.064.

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