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The establishment of a sensitive method in determining different neurotransmitters simultaneously in rat brains by using liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

An effective way to determine the amount of different neurotransmitters is vital to the study of brain function. Here, a highly sensitive HPLC–MS/MS method was developed to simultaneously measure γ-aminobutyric acid, dopamine, epinephrine, norepinepherine, glutamate and serotonin in one sample. The quantification of the neurotransmitters was achieved by a tandem mass spectrometer using the selected reaction monitoring scan mode. The method validation included selectivity, linearity, accuracy, precision, stability, recovery and matrix effect. For the six neurotransmitters, the linear regression analysis was calibrated by deuterated internal standards with a R^2 of over 0.991, and the limit of detection (LOD) and the limit of quantification (LOQ) were from 2.5 to 500 pg/mg and 7.5 to 1000 pg/mg, respectively. This method was employed here to reveal different types and amounts of neurotransmitters simultaneously in adult and embryonic rat brains. Here, the change of dopamine concentration in embryonic and adult brain was from 0.071 to 0.760 ng/mg of brain tissue, GABA was from 207.643 to 445.148 ng/mg, glutamate was from 679.535 to 1408.920 ng/mg, serotonin was from 0.058 to 0.485 ng/mg and norepinepherine was from 0.054 to 0.290 ng/mg. For epinephrine, it was only detected in embryonic stage but not in adult, with the concentration at 0.241 ng/mg.

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1. Introduction

Neurotransmitters are signaling molecules released by the presynaptic nerve cell and transmit the signal to the post-synaptic cell, which plays a vital role in neuron communication of our nervous system [1]. Abnormalities in the function of neurotransmitter systems could lead to a wide range of neurological and psychiatric disorders. Indeed, numerous therapies are established based on those drugs affecting the release and removal of neurotransmitter from synaptic cleft in the brain [2]. Usually, the neurotransmitters could be classified into two categories based on their chemical structures: (i) the small molecules, e.g. dopamine, epinephrine, glutamate, γ-aminobutyric acid (GABA), serotonin, norepinephrine, histamine and endocannabinoids; and (ii) the neuropeptides, e.g. enkephalin, endorphin and substance P [3]. The levels of small molecule neurotransmitters in brains, especially the aromatic monoamines, could be determined by using high-pressure liquid chromatography (HPLC) separation coupled with amperometric electrochemical detection (ECD): this method has been employed for over thirty years, and now which is the routine method of neurotransmitter analysis [4–7]. However, it is still rather difficult to determine different types of neurotransmitters simultaneously in one sample due to the limited ability to accommodate changes in the mobile phase composition. Another difficulty of using this method is that the analytes can only be identified by stable retention time matching.

In contrast to HPLC–ECD, tandem mass spectrometry (MS/MS) can provide high specificity due to additional structure information and high sensitivity. Therefore, it has been commonly used for the quantification of neurotransmitters in the brain by coupling with both gas chromatography (GC) and/or liquid chromatography (LC). For example, serotonin, 5-hydroxyindole-3-acetic acid and other tryptophan metabolites could be quantified by using GC–MS after derivatization [8]. Owing the varied efficiency and time consumption of derivatization, a simplified sample preparation using liquid chromatography coupled with electrospray tandem mass spectrometry (ESI-MS/MS) was widely employed to quantify the neurotransmitters and its metabolites in the brains without derivatization [9–12]. However, such detection is restricted to 1–2 types of neurotransmitters. The simultaneous quantification of multi-

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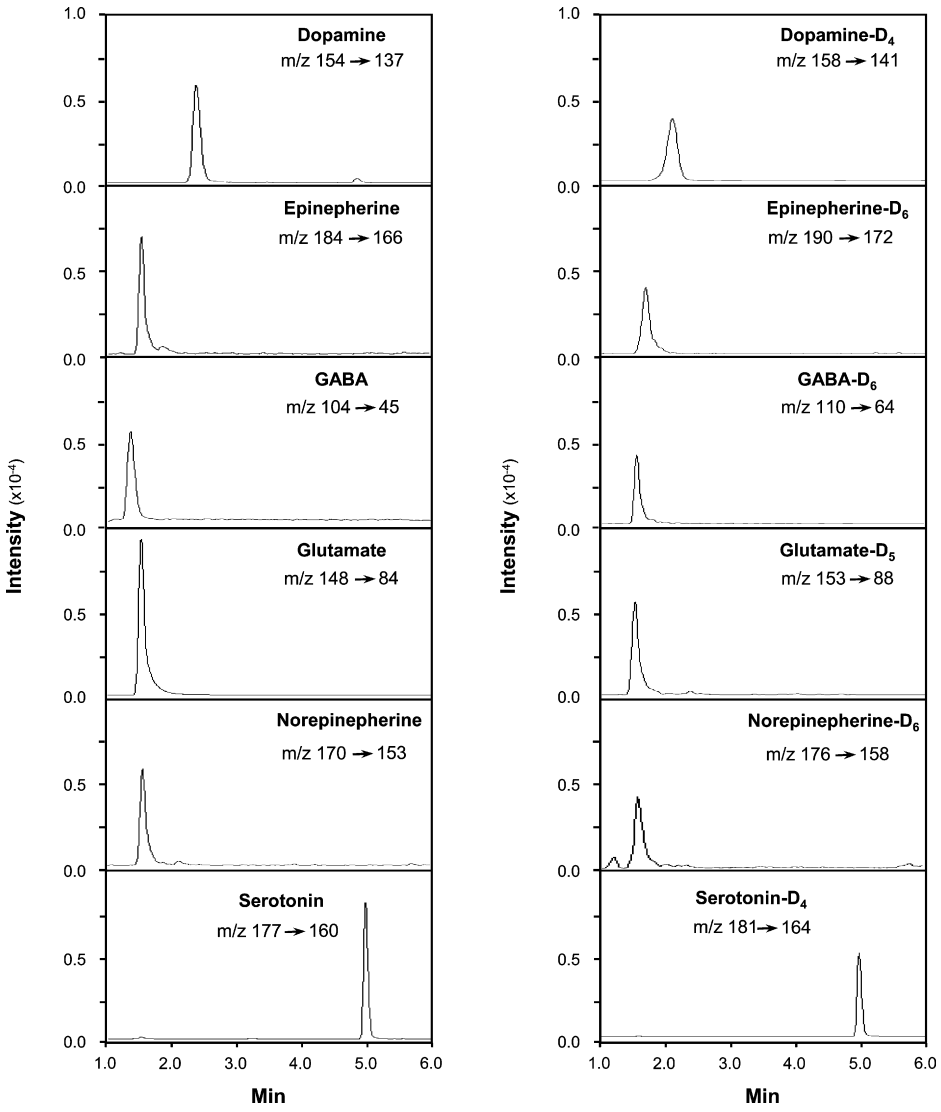

Fig. 1. Chromatograms of neurotransmitter standards. Different neurotransmitters standards and the corresponding isotope labeled internal standards were spiked in the brain tissues (matrix). The selected reaction monitoring scan mode (SRM) was use to detect the compounds in different ion channels. Representative chromatograms are shown, $n = 3$.

ple classes of neurotransmitters has not been fully optimized yet.

Here, we developed a sensitive, simple and simultaneous method to measure the amount of 6 neurotransmitters in the embryonic and adult brains. The present study was performed by a high efficiency reversed-phase HPLC separation and an ESI-MS/MS: this could minimize the sample interferences. At the same time, the selected reactions monitoring (SRM) scan mode was sensitive enough to identify and quantify the neurotransmitters. Moreover, the method validation was carried out to guarantee the precision and accuracy of the analysis.

2. Experimental

2.1. Reagents

Dopamine hydrochloride, epinepherine, γ-aminobutyric acid, glutamate, serotonin hydrochloride and norepinepherine were purchased from Sigma Chemical Co. (St. Louis, MO). Internal standard (IS) with isotope labeling were: 2-(3,4-dihydroxyphenyl)ethyl-1,1,2,2-d_4-amine HCl (dopamine-D_4, 98 at.% D); epinephrine-2,5,6,α,β,β-d_6 (epinephrine-D_6, 98 at.% D); D,L-2-aminobutyric-d_6 acid (γ-aminobutyric acid-D_6, 98 at.% D); L-glutamic-2,3,3,4,4-d_5 acid (glutamate-D_5, 98 at.% D); serotonin-α,α,β,β-d_4 creatinine sulfate complex (serotonin-D_4, 98 at.% D); norepinepherine-2,5,6,α,β,β-d_6 HCl (norepinepherine-D_6, 98 at.% D). All the IS were purchased from CDN Isotopes (Quebec, Canada). LC–MS grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ). Formic acid was purchased from Riedel-de Haën International (Hannover, Germany).

2.2. Preparation of brain extracts

Whole brain derived from adult male Sprague–Dawley (SD) rats (180–220 g) and embryonic day 18 rats were obtained from Animal Care Facility of the University. The handling of animal and the experimental procedures were approved by the ethical committee of the University that was in line with international guidelines of animal care and welfare. The rats were sacrificed by decapitation. Brain tissues were rapidly frozen in liquid nitrogen and kept in −80 °C for storage. For lysate preparation, brain tissues were homogenized in ice-cold 0.5 M formic acid with the concentration of 5 mL/g tissue, in the presence of the IS at 500 ng/g tissue except the 25 μg/g for glutamate and γ-aminobutyric acid.

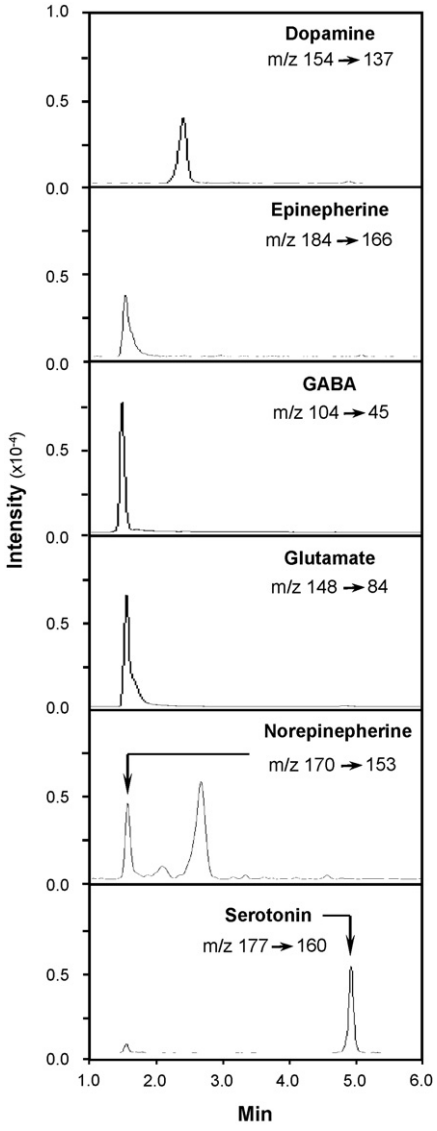

Fig. 2. Chromatograms of detected neurotransmitters in brains. Six neurotransmitters were detected in the brain tissues. Representative chromatograms are shown, $n = 3$.

Lysates were centrifuged 15,000 × *g* for 30 min at 4 °C. The supernatant was separated and stored at −20 °C until the LC–MS analysis for dopamine, epinephrine, norepinephrine and serotonin as the working mixture. For γ-aminobutyric acid and glutamate, the sample was diluted 50-fold with 0.5 M formic acid before the analysis.

2.3. Preparation of standard solutions, calibration curves and QC samples

Individual stock solution of each neurotransmitter and isotope-labeled standard were prepared by accurate weighing from each compound (1 mg/mL in methanol as the stock solution). The standards were spiked in the brain homogenates to prepare the calibrating solutions of dopamine, epinephrine, norepinepherine and serotonin (25, 50, 100, 250, 500, 1000, 2500 pg/mg), and glutamate and γ-aminobutyric acid (1250, 2500, 5000, 12,500, 25,000, 50,000 pg/mg). The solution having known spiked amount of neurotransmitters was defined as the quality control (QC) solution, which was set at three levels of concentration and prepared independently from the calibrators. The analyses of dopamine, epinephrine, norepinepherine and serotonin included 40 pg/mg for low QC solution; 500 pg/mg for medium QC solution and 2000 pg/mg for high QC solution. For the determination of glutamate and γ-aminobutyric acid, the QC solution was 2000 pg/mg, 12,500 pg/mg, 40,000 pg/mg. The QC solutions were stored at −20 °C for 5 days and then analyzed with the calibrators and samples during the method validation. The isotope-labeled neurotransmitter was used as the IS in the calibrators and QC samples, and the concentration was kept consistent at 500 pg/mg except 25,000 pg/mg for glutamate and γ-aminobutyric acid.

2.4. Liquid chromatography

The chromatographic separation was performed on an Agilent UHPLC 1290 series system (Agilent, Waldbronn, Germany), which was equipped with a degasser, a binary pump, an auto-sampler and a thermo-stated column compartment. The brain sample was separated on an ACE C18 column (3.0 μm i.d., 100 mm × 2.1 mm). The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using the following gradient program: 0–2 min, isocratic gradient 1.0% (B); 2–6 min, linear gradient 1.0–90.0% (B); 6–10 min, isocratic gradient 90.0% (B). A pre-equilibration period of 4 min was used between each run. The flow rate was 0.2 mL/min; the column temperature was 25 °C; and the injection volume was 5 μL.

2.5. Mass spectrometry

An Agilent QQQ-MS/MS (6410B) equipped with an ESI ion source was operated in positive ion mode. The following conditions were optimized: drying gas, nitrogen (10 L/min, 325 °C); capillary voltage, 1950 V; scan mode, SRM. The detected ion pairs, the acquired fragmentor and the collision energy were tuned with the aids of Agilent optimization software (B02.01). The mass spectrometry calibration was performed with the auto-feature of Agilent Mass Hunter Chemstation software (version B01.03) using the ESI-L low concentration tuning mix supplied with the apparatus. Agilent Mass Hunter workstation software version B.01.00 was used for data acquisition and processing.

2.6. Method validation

Selectivity, linearity, accuracy, precision, stability, sensitivity, recovery and matrix effect were determined to evaluate the integrity of the developed method. For selectivity, all IS solutions were analyzed by full scan mode of mass spectrometry to determine whether there was any detectable contamination by unlabeled substances. The linearity of analytic method was performed as the follows. For dopamine, epinephrine, norepinepherine and serotonin, the 7 calibrators: 25, 50, 100, 250, 500, 1000, 2500 pg/mg were evaluated, while the concentration of 1250, 2500, 5000, 12,500, 25,000, 50,000 pg/mg were included for glutamate and γ-aminobutyric acid. The deuterated standard was included in all analyses, and the concentration was 500 pg/mg, except 25,000 pg/mg for glutamate and γ-aminobutyric acid. The peak area ratios of the target ions to those of corresponding deuterated IS were calculated. The linearity was evaluated by the method of least squares, and the $1/x$ weighted factor was applied.

Precision and accuracy were evaluated by three different concentrations of QC solutions: Inter-day precision was evaluated for 6 replicates per single concentration on 4 days, equally weighted over 4 batches. The value of accuracy was expressed as the per-

Table 1
The analytic parameters of neurotransmitters in rat brain by HPLC–MS/MS.

Chemical	Precursor ion (*m/z*)[a]	Fragmentor energy[b]	Collison energy[c]	Product ion[d]	Retention time (min)[e]
Dopamine	154	72	5	137	2.34
			25	91	
Dopamine-D_4	158	92	9	141	2.32
			25	95	
Epinepherine	184	72	5	166	1.72
			21	107	
Epinepherine-D_6	190	92	9	166	1.73
			45	107	
GABA	104	72	5	87	1.36
			17	45	
GABA-D_6	110	40	9	64	1.37
			29	46	
Glutamate	148	72	5	130	1.52
			13	84	
Glutamate-D_5	153	92	9	107	1.53
			13	88	
Serotonin	177	72	5	160	4.92
			33	115	
Serotonin-D_4	181	92	9	164	4.93
			33	118	
Norepinepherine	170	40	5	153	1.47
			17	107	
Norepinepherine-D_6	176	92	9	158	1.46
			21	111	

GABA, γ-aminobutyric acid.
[a] The detected chemicals had the greatest responses under the positive mode: the $[M+H]^+$ was used as the precursor ion.
[b] The fragmentor energy was optimized to have the greatest ionize efficiency.
[c] The collision energy was optimized to have the greatest product ion intensity.
[d] Two product ions were used for the MRM analysis. The upper one was used for quantitative analysis, and the lower one was for qualitative analysis. The employment of 2 individual ions was to guarantee the identification of the compounds.
[e] The retention time was a mean of 3 different individual analyses ($n = 3$).

cent of determined concentration from 6 individual tests to the QC concentration.

Stability investigation was carried out in two parts. The stability of 6 individual samples of each low, medium and high QC solutions were analyzed in 24 h after the extraction, which were stored in auto-sampler vials of the LC–MS at room temperature. For the evaluation of freeze–thaw stability, QC solutions (low, medium and high) were analyzed before and after three freeze–thaw cycles. For each freeze–thaw cycle, the samples were frozen at −20 °C for 21 h, thawed, and kept at ambient temperature for 3 h. The concentrations of QC solution were calculated based on the daily calibration curves.

The percentage of drug recovery for each analyte was determined at low, medium and high levels. The recovery was expressed as the mean area of analyte with QC solution added before the sample preparation divided by the mean area of the same QC solution added after the sample preparation.

The sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ) via the two transitions of each compound. The LOD was calculated by using the weaker transition, while the stronger transition was for the LOQ determination. A series of decreasing concentrations of QC solution was analyzed to determine the LOD and LOQ. LOD was determined as the concentration of compound with a signal-to-noise ratio of at least 3, while LOQ was the concentration with a signal-to-noise ratio of at least 10. The acceptable precision of LOQ was 20% and the accuracy was 80–120%, all compared to the correspondent nominal concentration.

The matrix effect was investigated by comparing the peak areas of standard spiked in brain extract versus the peak areas of that prepared in the initial mobile phase at equivalent concentrations. It was determined at the low, medium and high concentrations.

3. Results and discussion

3.1. Calibration and validation

Standards for calibration were prepared in rat brain extract (matrix) spiked with the standards (Fig. 1). The peak areas of the spiked standard were constructed by subtracting the corresponding areas derived from the matrix. Meanwhile, the calibration by using internal standardization with deuterated analogues (i.e. IS

Table 2
The calibration of neurotransmitters in rat brain by HPLC–MS/MS.

Chemical	Internal standard	Equation[a]	Linear range (pg/mg)	Correlation coefficient (R^2)	LOD[b] (pg/mg)	LOQ[c] (pg/mg)
Dopamine	Dopamine-D_4	$y = 1.3728x - 0.0481$	25–2500	0.9940	2.5	7.5
Epinepherine	Epinepherine-D_6	$y = 1.9684x - 0.0571$	25–2500	0.9943	2.5	7.5
GABA	GABA-D_6	$y = 1.0759x + 0.0630$	1250–50,000	0.9909	500	1000
Glutamate	Glutamate-D_5	$y = 2.1771x - 0.0344$	1250–50,000	0.9976	125	500
Serotonin	Serotonin-D_4	$y = 1.9781x - 0.0562$	25–2500	0.9937	2.5	12.5
Norepinepherine	Norepinepherine-D_6	$y = 6.3254x - 0.0639$	25–2500	0.9941	2.5	12.5

GABA, γ-aminobutyric acid.
[a] The calibration curves were constructed by plotting the peak area versus the concentration of each analyte. Each calibration curve was derived from seven data points ($n = 7$).
[b] LOD referred to the limits of detection in pg/mg of brain tissue.
[c] LOQ referred to the limits of quantification in pg/mg of brain tissue.

Table 3
Determination of neurotransmitters by HPLC–MS/MS: validation results on precision, accuracy and recovery.

Chemical[a]	Intra-day precision[b]			Inter-day precision			Accuracy (%)			Recovery (%)[c]		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Dopamine	3.39	3.83	8.32	9.39	2.38	3.36	115.15	90.35	105.79	81.32	100.30	96.35
Epinepherine	5.97	3.06	2.38	11.77	1.53	4.48	109.31	86.60	106.84	90.24	104.36	93.54
GABA	6.75	8.19	1.50	5.34	2.19	3.63	115.67	97.64	105.08	85.32	92.45	91.43
Glutamate	12.41	7.69	3.78	10.50	9.79	5.86	113.29	88.66	108.96	83.14	103.49	96.12
Serotonin	13.83	11.07	12.72	10.15	4.23	1.87	110.39	87.30	105.11	98.10	93.67	106.34
Norepinepherine	9.02	4.07	4.49	6.27	7.44	9.23	101.34	94.79	112.31	91.48	102.73	99.68

In most cases, the values in percentage of control with a mean of 6 individual experiments ($n = 6$). The SD values were less than 5% of the mean, not shown for clarity. GABA, γ-aminobutyric acid.

[a] For dopamine, epinephrine, norepinepherine, serotonin, the low, medium and high control solution were 40 pg/mg, 500 pg/mg, 2000 pg/mg of brain tissue, respectively. For glutamate and γ-aminobutyric acid, those were 2000 pg/mg, 12,500 pg/mg, 40,000 pg/mg of brain tissue.

[b] For both precision tests, the values were in percentage of RSD.

[c] Recovery was expressed as the mean analyte area of samples having the control solution added before extraction divided by the mean analyte area of samples with control solution added after extraction.

solution) was performed here. In biological specimen analysis, the isotope-labeled analogues (the IS) of the targeted analyte is often recommended. Due to their similar physicochemical properties, the variability during sample preparation and ionization efficiency in the transfer of analytes (both native and isotope-labeled) from liquid to gas could be compensated for each other, and they could be differentiated ideally by their distinct mass-to-charge (m/z) ratio (Table 1) [13]. All analytes were subjected to HPLC–MS/MS analysis, and their distinct mass-to-charge (m/z) ratios were determined. The analytic parameters were listed as in Table 1. Two product ions were produced here for the identification of a compound and the sensitivity exploration (Table 1 and Supplymentary Figure). Besides, the employment of two ions was to ensure the precision of analysis. For the selectivity, there were no deuterated IS found to have interference with the analytes. In the linearity analysis, several concentrations of calibrators (QC solutions) were included in the establishment of calibration curve. The linearity was achieved with a squared correlation coefficient (R^2, $1/x$ weighting factor) (Table 2).

The LOD and LOQ values were listed in Table 2, and the LOQ values fulfilled the precision and accuracy acceptance. For dopamine, epinephrine, norepinepherine and serotonin, the LOD and LOQ values were 2.5 pg/mg and 7.5 pg/mg of brain tissue, respectively: the same values of LOD and LOQ were achieved for these neurotransmitters because of their chemical nature, i.e. the catecholamine based. The LOD and LOQ values of acetylcholine were at least 200 folds higher than that of catecholamine, which were 50 pg/mg and 200 pg/mg of brain tissue, respectively. For glutamate and γ-aminobutyric acid, the LOD and LOQ values were higher than that of the others due to the sample dilution (50 folds). Overall speaking, the sensitivity of analytes could be varied greatly according to their chemical nature.

Precision and accuracy were evaluated at low, medium and high concentrations, as described in Section 2. Table 3 shows the results for accuracy and precision testing. The RSD of intra-day precision was less than 15% at low, medium and high concentrations for all analytes. Similarly, the RSD of inter-day precision was less than 12% for all analytes at low, medium and high QC concentrations. Accuracy was calculated as the percent of estimated concentration to that of each analyte at low, medium and high QC concentrations, which ranged from 86.60% to 115.67% of the correspondent concentration.

Stability at room temperature on an auto-sampler for 24 h was also investigated, which ranged from 80.76% to 118.68% of the correspondent concentration. The freeze–thaw stability analysis was carried out according to the design of Section 2, and the effect of freeze–thaw on the calibration was in a range from 87.84% to 115.86% of the correspondent concentration. Usually, the stability was considered to be acceptable at a range of 80–120% of the control sample. Here, the analytes at different QC concentrations fulfilled this criterion (Table 4).

The neurotransmitter recovery during the extraction process was ranged from 81.32% to 106.34% (Table 4), and the acceptable criterion for the biological sample recovery was at least higher than 50%. Matrix suppression was happened in this analysis. Nevertheless, an adequate quantitative assay was achieved: because the deuterated analogues were used for calibration.

After system validation, this method was accepted for simultaneous analysis of 6 neurotransmitters. As compared to the method of neurotransmitters analysis using ECD-HPLC [7], the current method could avoid the usage of saline, which provides better maintenance for the equipment. In addition, the analytic time was shortened to only 10 min, which included the equilibrium time.

Table 4
Determination of neurotransmitters by HPLC–MS/MS: validation results on stability and matrix effect.

Chemical[a]	Stability (%)[b]			Freeze–thaw stability (%)[c]			Matrix effect[d]		
	Low	Mid	High	Low	Mid	High	Low	Mid	High
Dopamine	89.14	98.49	105.04	104.32	107.53	98.16	71.87	55.35	67.15
Epinepherine	87.84	92.97	103.97	96.34	99.20	98.92	62.19	78.34	70.19
GABA	90.84	107.83	107.13	87.84	103.50	103.97	68.74	69.42	73.01
Glutamate	118.68	84.44	98.92	115.86	99.09	97.47	50.14	70.13	68.42
Serotonin	105.11	80.76	89.96	89.14	98.45	105.04	62.69	58.32	71.32
Norepinepherine	100.99	92.19	101.87	96.54	92.19	95.59	72.49	56.96	65.87

In most cases, the values are in percentage of control with a mean of 6 individual experiments ($n = 6$). The SD values were less than 5% of the mean, not shown for clarity. GABA, γ-aminobutyric acid.

[a] For dopamine, epinephrine, norepinepherine and serotonin, the low, medium and high control solution were 40 pg/mg, 500 pg/mg, 2000 pg/mg of brain tissue, respectively. For glutamate and γ-aminobutyric acid, those were 2000 pg/mg, 12,500 pg/mg, 40,000 pg/mg of brain tissue.

[b] Stability was evaluated by exploring the concentration changes of the control samples that were placed in auto-sampler vials for 24 h.

[c] Freeze–thaw stability was evaluated by exploring the concentration changes of the control samples that went through the freeze–thaw cycle.

[d] Matrix effect was calculated by dividing the analyte areas of blank samples spiked with drug after extraction by areas of neat samples in initial mobile phase.

Table 5
The contents of neurotransmitters in rat brain revealed by HPLC–MS/MS.

Chemical	Adult brain (ng/mg)[a]	Embryonic brain (ng/mg)
Dopamine	0.760 ± 0.023	0.071 ± 0.009
Epinephrine	ND[b]	0.241 ± 0.024
GABA	445.148 ± 110.767	207.643 ± 23.745
Glutamate	1408.920 ± 141.825	679.535 ± 75.480
Serotonin	0.485 ± 0.094	0.058 ± 0.002
Norepinepherine	0.290 ± 0.034	0.054 ± 0.024

[a] Values are expressed in ng/mg of brain tissue, mean ± SD, $n = 5$.
[b] ND, not determined. As a control, the amount of epinephrine in adrenal gland, taken from the same rat, was 17.5 ± 0.5 ng/mg of tissue. GABA, γ-aminobutyric acid.

Most importantly, the method sensitivity could be enhanced at least 5–20 folds for different neurotransmitters. Meanwhile, the detection ability of the current method could be enhanced by ~2 folds, as compared to the recently reported analysis of neurotransmitters in brains using similar approach by LC–MS/MS [11].

3.2. Analysis of neurotransmitter in the brain

The developed LC–MS method was used to analyze the levels of neurotransmitters in adult and embryonic rat brains (Table 5). Neurotransmitters including γ-aminobutyric acid, dopamine, epinephrine, norepinepherine, glutamate and serotonin were found both in adult and embryonic rat brain tissues, while epinephrine was only found in embryonic rat brain (Fig. 2). From the quantitative results, the concentrations of 6 neurotransmitters in adult rat brain were significantly higher than that in embryonic rat brain, except for epinephrine. Our current report of the neurotransmitter contents in rat brains was in line to previous studies [11,14].

Neurotransmitters are stored in synaptic vesicles clustered beneath the pre-synaptic membrane of synapses, and which are released into the synaptic cleft for binding to post-synaptic membrane receptors in triggering an action potential [15]. Due to the brain development, the amount of synapses in adult brain is far more than that of embryonic brain. This increase of synaptic development could account for the higher amount of neurotransmitters being found in adult brain. In terms of the amount of neurotransmitter, glutamate was ranked first and followed with γ-aminobutyric acid. In mammalian brain, glutamate is by far the most prevalent neurotransmitter, which is excitatory working on over 90% of the synapses in the brain. The next most prevalent is γ-aminobutyric acid, which is serving as inhibitory role at more than 90% of the inhibitory synapses. This phenomenon is similar to that in human brain. Though the amount of dopamine, epinephrine, norepinepherine and serotonin was lower compared to the glutamate and γ-aminobutyric acid, they were confirmed vital for the pathogenesis of mental diseases and neurodegenerative diseases, such as depression disorder, Parkinson disease and schizophrenia [16]. For epinepherine, it was not detected in adult brain by our method. To test the integrity of the method, the adrenal gland was analyzed for epinephrine, which could be found definitely (Table 4). Thus, the low level of epinephrine in the brain was verified.

4. Conclusion

A simple and convenient method using the HPLC–MS/MS technology for the simultaneous detection of different neurotransmitters was established and validated here. The principal advantages of using LC/MS/MS method included a simple purification procedure and a simple chromatographic condition by using the SRM scan mode. Having the co-eluting isotopically labeled internal standards, the quantitative and confirmatory assurance could be guaranteed. Therefore, the current developed method could be very useful for the brain research in relating to the alternation of the levels of neurotransmitters.

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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.jchromb.2011.02.011.

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