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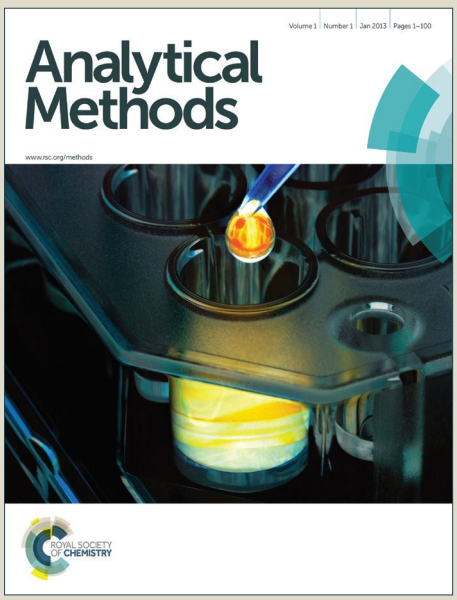

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ARTICLE TYPE

Simultaneous determination of multiple neurotransmitters and their metabolites in rat brain homogenates and microdialysates by LC-MS/MS

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Since neurotransmitters (NTs) and their metabolites are involved in some major neurological disorders, there is a demand of suitable analytical techniques allowing highly sensitive determination of NTs in biological samples. We developed and validated a derivatization method for simultaneous determination of NTs and their metabolites in rat brain homogenates and microdialysates of the prefrontal cortex and hippocampus using high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The NTs and their metabolites were derivatized with dansyl chloride under optimal conditions and separated by an Ultimate XB-C8 column. The method was used for quantification of monoamine NTs like dopamine (DA), norepinephrine (NE) and serotonin (5-HT), and their metabolites including 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), vanilmandelic acid (VMA), 4-hydroxy-3-methoxyphenylglycol (MHPG), and 5-hydroxyindole-3-acetic acid (5-HIAA). Amino acid neurotransmitters like tryptophan (TRP), kynurenine (KYN), kynurenic acid (KA) and glutamate (GLU), glutamine (GLN) and γ-aminobutyric acid (GABA) were also taken into account. This method exhibited good linearity for all of the analytes with regression coefficients higher than 0.99. The intra-day and inter-day precision were no greater than 16%. The lower limit of quantification (LLOQ) values for NTs ranged from 0.4 to 9.7 pmol/mL. The mean recovery ranged from 90.3% to 115.0%. The described LC–MS/MS method was successfully applied for quantification of the NTs and their metabolites in the brain homogenates and microdialysates of rats with good precision and accuracy.

Introduction

Neurotransmitters (NTs) and their metabolites play a direct role in maintaining various brain physiological functions including the control of behaviour, mood and cognition. The metabolic pathways of dopamine, serotonin and kynurenine are closely related to some major neurological disorders including depression, anxiety, schizophrenia and Parkinson' s disease[1-5].

Microdialysis is a reliable sampling technique that can be used to continuously monitor the concentrations of NTs and their metabolites in the extracellular fluid[6]. In addition, microdialysis is a minimally invasive technique allowing experiments to be performed in animals while conscious and freely moving, so that the fluctuation of NTs can be determined more accurately with mirodialysis. Furthermore, the characteristics of measurement and sample preparation of microdialysis provide relatively clean dialysate that is ready for analysis. Sample contamination and dilution can be avoided when further treatments and extraction are

performed. However, the main challenges in monitoring NTs in rodent brain microdialysates are the low basal level, low sample volumes and high inorganic salt concentration[7]. Due to this reason, when analysing microdialysis samples, there is a high potential for matrix effects which could compromise a reliable quantification of the analyte[6, 7].

For the determination of NTs, high performance liquid chromatography (HPLC) or capillary electrophoresis (CE)[8] coupled with various detection methods are widely used, such as ultraviolet (UV) detection[9], fluorescence detection (FD)[10,11], electrochemical detection (ECD)[12-14], and mass spectrometry (MS)[6,15,16]. However, most of these methods have some limitations. For instance, UV detection is not sensitive to monoamines; FD has poor selectivity and ECD tends to lack reproducibility mainly because of the electrode fouling. To overcome these problems, mass spectrometric approaches have been developed. Mass spectrometry offers advantages in analytical selectivity because detection is based on molecular mass and relatively specific ionization and fragmentation behaviors of a certain analyte. Recently, gas chromatography/mass spectrometry (GC–MS)[17], capillary electrophoresis/mass spectrometry(CE–MS)[18], liquid chromatography/mass spectrometry(LC–MS)[6,15,16,19-21] and liquid chromatography/tandem mass spectrometry (LC–MS/MS) techniques[7,22-24,26] have become valuable tools for NTs analyses. Furthermore, LC–MS/MS method combined with other sample preparation techniques could be highly sensitive to trace level quantification of NTs in biological samples. Generally speaking, a multitude of interfering peaks could occur in the low-m/z region when biological samples are analyzed and cause poor sensitivity and specificity. It is crucial to derivatize NTs and their metabolites for the goal of improving sensitivity and specificity by increasing mass. Thus, a derivatization step that modifies a functional group in the molecule by introducing a charged or proton acceptor moiety was developed in this study[16,22,25].

The purpose of the present study was to establish a fast and reliable analytical method for the simultaneous determination of all 14 NTs and their acidic metabolites in rat tissue homogenates and microdialysates samples from the regions of prefrontal cortex and hippocampus by precolumn dansyl chloride derivatization coupled with LC–MS/MS. These techniques facilitate better understanding of complex neurobiology in the central nervous system disorders and have extended uses in drug discovery and clinical investigation of psychiatric and neurological diseases.

Experimental

Animals

Male Sparague-Dawley (SD) rats weighing 250–300 g were used for all experiments (Slac Laboratory Co, Ltd, Shanghai, China). The rats were maintained at free access to food and water, on a 12 h light-dark cycle (light at 7:00 AM), room temperature 22 ± 2℃ and humidity 50–55%. All animal experiments were performed in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee of Central South University. All efforts were made to minimize animal's suffering and the number of animals used for the study.

Chemical and reagents

Dopamine (DA), homovanillic acid (HVA), norepinephrine (NE), vanilmandelic acid (VMA), 5-hydroxtryptamine (serotonin,5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), tryptophan (TRP), kynurenine (KYN), kynurenic acid (KA) and γ-aminobutyric acid (GABA) were purchased from Sigma (St. Louis, MO, USA). Dihydroxyphenylacetic acid (DOPAC), glutamate(GLU), glutamine(GLN) and the internal standards (ISs) 3,4-dihydroxybenzylamine (DHBA) and 5-hydroxyindole-2-carboxylic acid (5-HICA) were purchased from Aldrich (Steinheim, Germany). The IS L-aspartic acid-$^{13}C_4$,^{15}N (L-Asp-$^{13}C_4$,^{15}N) was purchased from Isotec (Miamisburg, OH, USA). 4-hydroxy-3-methoxyphenylglycol (MHPG) was obtained from Fluka (Buchs, Switzerland). Dansyl chloride was obtained from Alfa Aesar (Karlsruhe, Germany). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Formic acid (HPLC grade) was purchased from Tedia (Fairfield, OH, USA). Sodium carbonate and sodium bicarbonate (analytical reagent [AR] grade) were purchased from Shanghai Experiment Reagent (Shanghai, China). Water used in the experiment was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

In vitro rat brain homogenates experiments

Sample preparation

SD rats' prefrontal cortex and hippocampus were obtained on ice immediately after rats being sacrificed in the animal laboratory,

Table 1 MRM Transition parameters of neurotransmitters and internal standards

Compound	MRM Transition (m/z)	Dwell(s)	Cone Voltage(V)	Collision energy(eV)
GLN	380.0>170.0	0.1	22	25
GLU	381.0>170.0	0.1	20	20
GABA	337.0>170.0	0.1	35	42
5-HT	644.0>170.0	0.1	27	50
DA	854.0>170.0	0.1	27	50
NE	870.0>170.0	0.1	23	50
5-HIAA	425.0>171.0	0.1	22	29
DOPAC	635.0>170.0	0.1	26	25
HVA	416.0>171.0	0.1	25	25
MHPG	418.0>171.0	0.1	22	27
VMA	432.0>171.0	0.1	22	27
TRP	439.0>170.0	0.1	37	30
KYN	442.0>170.0	0.1	35	32
KA	424.0>170.0	0.1	25	40
ISs				
L-Asp-$^{13}C_4$,^{15}N	373.0>170.0	0.1	20	20
5-HICA	411.0>171.0	0.1	26	25
DHBA	839.0>170.0	0.1	27	50

and transported in liquid nitrogen to our laboratory and kept at -80℃until biochemical analysis.

1 mL of 85% acetonitrile in water and 10 μL of mixed ISs solution (containing DHBA 0.12 μg/mL, 5-HICA 0.19 μg/mL, L-Asp-$^{13}C_4$,^{15}N 1.41 μg/mL) were added to 0.1 g of rat brain tissue, and the mixtures were homogenized by tissue homogenizer. After vortex mixing for 5 min, the mixture was centrifuged at 4 ℃ for 5 min at 25050 g. The supernatant (500 μL) was then transferred into another Eppendorf tube and subsequently evaporated to dryness under vacuum. For derivatization, 150 μL of dansyl chloride solution(4 mg/mL in acetonitrile) and 50 μL of 0.1 M Na_2CO_3-$NaHCO_3$ buffer (pH 11.0) were added to the residue. Then the mixture was vortexed for 30 s and further reacted at 35℃ for 30 min, avoiding light throughout. After the reaction, the pH of the mixture was adjusted to approximately 7.0 by adding 5 μL of 15% formic acid in water solution. After centrifugation at 25050 g for 5 min, the supernatant was transferred to the vial and 5μL was injected for analysis.

LC-MS/MS Analysis

LC-MS/MS analyses were carried out on a Waters Acquity ultra-performance liquid chromatography system (Waters, Milford, MA,USA) with a Micromass Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with ESI source. The analytes were separated on an Ultimate XB-C8 column, 2.1 mm × 50 mm, 3.0 μm particle size (Welch, Shanghai, China) with the column temperature setting at 40℃. The mobile phase consisted of aqueous phase (A: 20 nM ammonium acetate and 0.1% formic acid in water) and organic phase (B: acetonitrile). To achieve satisfactory separation of analytes, a gradient at a flow rate of 0.25 mL/min was used, starting at 20% B with a linear increase of B until it reached 90% B at 21 min. The initial conditions were restored between 21.1 and 23.0 min and retained 3.0 min for equilibration.

Table 2 Equations of the calibration curves with correlation coefficients (r^2) of rat brain homogenates

Analyte	Regression equation	r^2	Linear range (pmol/mL)	LLOQ (pmol/mL)
GLN	y =0.0031x-0.4522	0.9994	6839.9-683994.5	6.8
GLU	y =0.0020x-6.0047	0.9942	6798.1-679809.7	6.9
GABA	y =0.0091x+5.6659	0.9933	9699.3-969932.1	9.7
5-HT	y =0.0035x-0.0226	0.9995	5.7-4542.9	0.6
DA	y =0.2110x-0.0470	0.9967	6.5-5225.3	0.7
NE	y =0.0079x-0.0138	0.9989	5.9-4728.1	0.6
5-HIAA	y =0.0003x+0.0063	0.9981	5.2-4184.1	0.5
DOPAC	y =0.0057x+0.0077	0.9991	5.9-4759.1	0.6
HVA	y =0.0015x+0.0087	0.9990	5.5-4390.8	0.6
MHPG	y =0.0014x+0.0073	0.9998	3.8-3038.4	0.4
VMA	y =0.0005x+0.0071	0.9992	5.0-4036.3	0.5
TRP	y =0.0046x-2.1675	0.9960	3264.8-39177.3	3.3

KYN	y =0.0022x+0.0081	0.9986	4.8-3842.5	0.5

Samples vials were maintained in the autosampler at 4 ℃ throughout. The source was operated in positive ion mode, and its main working parameters were set as follows: capillary voltage, 3.00 kV; extractor voltage, 3.00 V; source temperature, 120 ℃; desolvation temperature, 450 ℃; desolvation gas flow, 750 L/h (N_2, 99.9% purity); cone gas flow, 50 L/h (N_2, 99.9% purity). Argon (99.9999% purity) used as the collision gas was introduced into the collision cell at a flow rate of 0.16 mL/min. Multiple reaction monitoring (MRM) measurements of the dansylated analytes were performed using individually optimized cone voltage and collision energy (see Table1). The dwell time established for each transition was 0.1 s, and the interscan delay was set at 5 ms. Data acquisition was carried out by Mass Lynx 4.1 software.

In vivo microdialysis experiments and sample collection

Surgical techniques

SD rats weighing 250–300 g were used for all experiments. The rats were anesthetized by an intraperitoneal injection of chloral hydrate solution (400 mg/kg). Heads of the rats were shaved before placing them in a stereotaxic apparatus in a flat skull position with the incisor bar set to −3.2 mm. The body temperature of the rat was controlled by microwaveable heating pad and maintained at +37◦C using a CMA/150 temperature controller (CMA/Microdialysis, Stockholm, Sweden). After exposing the skull, the bregma was located and used as the reference point for positioning the microdialysis probe. A hole for a probe cannula and two holes for the fixing screws were drilled using a fine skull drill. An intracerebral guide cannula was stereotaxically inserted through cranial burr hole, using the following coordinates: hippocampus: −4.3 mm anterior, −4.6 mm lateral and −5.8 mm ventral; prefrontal cortex: 3.2 mm anterior, −0.5 mm lateral and −1.4 mm ventral. All coordinates were relative to bregma and the dura surface, according to the stereotaxic atlas of Paxinos and Watson[27]. The guide cannulae were fixed to the skull using two anchor screws and dental cement. When experimenting, the guide cannula was replaced by the brain microdialysis probes (CMA/12) with 4 mm membranes which were purchased from CMA/Microdialysis AB Inc.(CMA, Stockholm, Sweden). After experiment, the animals were sacrificed by an overdose of isoflurane and dislocation of the neck. The brains were removed, frozen on dry ice and stored at −20◦C for histological examination of the probe position.

Sample collection and preparation

Microdialysis experiments were performed in the home cage of the animal. Rats were given at least 24 h to recover from surgery before starting microdialysis experiments. The probes were perfused with Ringer's solution at a flow rate of 2.0 μL/min using a CMA 102 perfusion pump (CMA, Solna, Sweden) and allowed to equilibrate for 1 h. The composition of the Ringer's solution was 154 mM sodium chloride, 1.6 mM potassium chloride and 2.2 mM calcium chloride. The samples were collected for 8 h in a 30 min interval using an automatic fraction collector (CMA/142; CMA, Solna, Sweden). Samples were kept at −80 ◦C until analysis.

10 μL of mixed ISs (containing DHBA 0.12 μg/mL, 5-HICA 0.19 μg/mL, L-Asp-$^{13}C_4$,^{15}N 1.41 μg/mL) were added to 50 μL microdialysates sample. For derivatization, 50 μL of dansyl chloride solution (4 mg/mL in acetonitrile) and 50 μL of 0.5 M Na2CO3 – NaHCO3 buffer (pH 11.0) were added to microdialysates sample. Then the mixture was vortexed for 30 s and further reacted at 60 ℃ for 15 min, avoiding light throughout. After the reaction, the pH of the mixture was adjusted to approximately 7.0 by adding 5 μL of 15% formic acid-water solution. After centrifugation at 25050 g for 5 min, the supernatant was transferred to the vial and 10 μL was injected for analysis.

Liquid chromatographic and mass spectrometric conditions of microdialysis experiments

Using a column switching valve, before analytical separation and during the first 2 minutes of analytical run the valve is diverted to waste in order to preserve the EPI interface from contamination or clogging from salts. At the same time, another pre-mixed mobile phase consisting of 27% acetonitrile and aqueous phase (20 mM ammonium acetate and 0.1% formic acid) at a flow rate of 0.25 mL/min was eluted from a single pump to facilitate stabilizing the baseline and washing the EPI source. Liquid chromatographic and mass spectrometric conditions were set as described above. The detector was operated in the positive ion mode with ESI using multiple-reaction monitoring (MRM) to monitor the mass transitions. Measurements of the dansylated analytes were performed using individually optimized cone voltage and collision energy (see Table1) with the main working parameters setting as described above.

Method validation

Preparation of Standard Curves and Quality Control(QC) Samples

A mixed standard stock solution of DA, NE, and 5-HT was prepared in acetonitrile-water (3:2) at 1 mg/mL, respectively. A mixed standard stock solution of monoamine metabolites (DOPAC, HVA, VMA, MHPG, and 5-HIAA) was prepared in acetonitrile-water (3:2) at 1 mg/mL for each. A mixed standard stock solution of GLU and GABA was prepared in water at 1mg/mL, respectively. Standard stock solution of GLN was

Table 3 Equations of the calibration curves with correlation coefficients(r^2) of rat brain microdialysates

Analyte	Regression equation	r^2	Linear range (pmol/mL)	LLOQ (pmol/mL)
GLN	y =0.000100x+0.0088	0.9918	68.4-13679.9	6.8
GLU	y =0.000028x-0.0013	0.9970	67.9-1019.7	6.8
GABA	y =0.000800x-0.0228	0.9972	96.9-193986.4	9.7

5-HT	y =0.000075x+0.0009	0.9976	56.8-11357.2	5.7
DA	y =0.000800x-0.0187	0.9996	65.3-13063.4	6.5
NE	y =0.000100x-0.0073	0.9904	59.1-11820.3	5.9
5-HIAA	y =0.000100x+0.0002	0.9979	52.3-10460.3	5.2
DOPAC	y =0.000600x-0.0035	0.9944	59.5-11897.7	6.0
HVA	y =0.000100x+0.0016	0.9976	54.9-10976.9	5.5
MHPG	y =0.000800x-0.0382	0.9940	37.9-7595.9	3.8
VMA	y =0.000300x+0.0010	0.9967	50.5-10090.8	5.1
TRP	y =0.000300x+0.0018	0.9978	49.0- 9794.3	4.9
KYN	y =0.000065x-0.0012	0.9974	48.0-9606.1	4.8
KA	y =0.000022x-0.0043	0.9956	11.8-1712.5	1.2

prepared in water at 1 mg/mL. Standard stock solution of TRP and KYN were dissolved in acetonitrile-water (3:2) at 1 mg/mL. A standard stock solution of KA was prepared in water-methanol (1:1) at 1 mg/mL. A mixed standard stock solution of ISs (DHBA, 5-HICA, and L-Asp-$^{13}C^{4}$,^{15}N was prepared in acetonitrile-water solution (1:1) at 0.12 mg/mL, 0.19 mg/mL, and 1.41 mg/mL, respectively. The analyte stock solutions were then serially diluted with acetonitrile to provide working standard solutions of the desired concentrations.

To prepare the standard curve, 500 µL of blank brain homogenates or 50 µL Ringer's solution were spiked with 10 µL of the internal standard solution (containing DHBA 0.12 µg/mL, 5-HICA 0.19 µg/mL, L-Asp-$^{13}C_4$,^{15}N 1.41 µg /mL) and 10 µL of specific working solutions to generate calibration levels covering a range of analytes (table 2 and 3), respectively. QC samples were prepared following the same procedure giving low, medium, and high analyte concentrations.

Lower Limit of Quantification

The lower limit of quantification (LLOQ) was defined as the concentration level with the signal-to-noise ratio > 10.

Linearity Range, Stability, Matrix effect evaluation, Statistics

To determine the dynamic range of the method, eight batches(n=3, at each concentration level) of calibration samples along with five blank samples were prepared and analyzed following the method

mentioned above. The linearity range was investigated both in

brain homogenates and microdialysates sample. As the inherent existence of the endogenous substances in the matrix of brain homogenates, blank values of them should be subtracted from each calibration point. Calibration curves were constructed by plotting the margin of the analyte/IS peak area ratio (peak area of DA, DOPAC, HVA, NE,VMA, and MHPG/peak area of DHBA; peak area of 5-HT and 5-HIAA/peak area of 5-HICA; peak area of TRP, KYN, KA ,GLU, GLN and GABA/peak area of L-Asp-$^{13}C_4$,^{15}N) between the brain homogenates that spiked standard solutions and the blank brain homogenates with ISs as a function

Table 4 Intra-day and inter-day precision and recovery of rat brain homogenates

Analyte	Added (pmol/mL)	Recovery %	intra -day precision (CV%)	inter-day precision (CV%)
GLN	13679.8	97.3	5.1	3.1
	136798.9	101.4	4.0	1.4
	547195.6	96.9	6.6	2.2
GLU	13596.1	115.0	11.2	9.5
	135961.9	112.1	6.3	2.1
	543847.7	104.5	4.1	1.8
GABA	19398.6	105.8	7.1	1.6
	193986.4	102.3	5.4	2.5
	969932.1	114.5	1.1	1.4
5-HT	11.4	95.2	5.8	5.6
	567.8	93.6	13.8	5.4
	3407.1	100.3	1.8	2.1
DA	13.1	93.4	14.5	5.3
	653.1	106.7	1.9	3.8
	3919.0	98.6	2.5	1.5
NE	11.8	101.5	3.0	5.6
	591.0	106.2	1.6	3.7
	3546.1	102.0	2.3	1.5
5-HIAA	10.4	104.8	1.5	4.3
	523.0	101.1	13.6	4.0
	3138.0	99.6	1.5	9.7
DOPAC	11.9	96.1	1.8	3.8
	594.9	92.5	1.8	3.0
	3569.3	91.8	2.4	1.7

HVA	10.9	106.1	1.6	13.6
	548.8	103.2	6.1	3.2
	3293.1	109.1	1.7	3.9
MHPG	7.6	90.3	1.8	6.4
	379.8	95.2	15.8	2.5
	2278.7	111.9	1.9	1.6
VMA	10.1	95.3	6.1	6.6
	504.5	94.1	5.1	3.5
	3027.2	97.6	2.0	1.8
TRP	4897.2	100.8	8.5	2.7
	14691.5	110.6	6.6	2.5
	29382.9	103.5	9.4	9.0
KYN	9.6	92.8	7.0	10.2
	480.3	103.9	1.1	3.9
	2881.8	99.8	1.2	2.3

of the concentration calibrator. Analyte of blank brain homogenates /IS peak area ratio was set as R_0 , and analyte of spiked brain homogenates /IS peak area ratio was set as R_n (n = 1, 2, ... , 8). Calibration curves were then fitted to the equation y = bx + a by weighted linear regression ($1/x^2$), where y corresponds to the peak area ratio difference ($y_n = R_n - R_0$, n = 1, 2, ... , 8) and x refers to the concentration of analytes added to the brain homogenates.

In this study, the stability of NTs in Ringer's solution and brain homogenate was evaluated under different storage condition. The stability of QC samples was tested after being stored at 4°C for 0.5 h, 1 h, 1.5 h, 3 h, 4 h, 8 h, respectively. QC samples

Table 5 Intra-day and inter-day precision and recovery of rat brain microdialysates

Analyte	Added (pmol/mL)	Recovery %	intra -day precision (CV%)	inter-day precision (CV%)
GLN	273.6	97.6	7.1	13.2
	2051.9	102.4	8.0	11.0
	6839.9	115.0	6.9	8.2
GLU	1019.7	106.3	1.5	7.5
	4078.8	112.1	9.3	12.3
	13596.2	104.5	7.1	3.8
GABA	9699.3	102.8	10.1	1.9
	29097.9	103.3	15.43	12.5
	193986.4	114.9	4.1	6.4
5-HT	227.1	91.8	6.8	4.7
	1703.6	97.6	8.9	3.1
	5678.6	97.9	7.0	12.1
DA	261.3	93.4	4.9	5.6
	1959.5	98.4	11.9	5.9
	6531.7	106.7	6.5	11.5
NE	236.4	102.5	13.0	15.4
	1773.1	96.2	11.6	13.2
	5910.2	98.4.	12.3	6.5
5-HIAA	209.2	99.6	1.6	7.3
	1569.0	111.9	3.8	5.6
	5230.1	108.5	11.2	8.8
DOPAC	237.9	92.1	3.6	7.8
	1784.6	104.5	7.8	5.0
	5948.8	102.8	9.4	1.7
HVA	219.5	103.1	5.6	10.6
	1646.5	106.2	6.7	12.2
	5488.4	99.1	5.7	1.9
MHPG	151.9	100.3	6.8	7.3
	1139.3	97.2	5.7	3.7
	3797.9	101.9	8.6	4.9
VMA	201.8	102.3	7.3	8.5
	1513.6	98.1	6.2	2.7
	5045.4	97.7	3.7	6.3
TRP	4897.2	95.8	9.5	5.1
	14691.5	102.6	5.4	12.3

	29382.9	107.5	4.2	10.0
KYN	192.1	102.8	6.0	12.1
	1440.9	93.9	11.1	4.7
	4803.1	99.8	5.2	5.6
KA	1585.6	98.4	7.3	7.1
	10570.8	96.7	4.5	5.2
	21141.7	109.8	6.1	14.9

were subjected to three freeze-thaw cycles and all stability studies were conducted at three concentrations of NTs .

The matrix effect was evaluated by measuring the peak area ratio of a certain analyte to the corresponding IS in the spiked matrix samples at the same three concentration levels of QC samples (n = 5), The increases in the peak area ratios of the compounds were compared with the respective area ratio measured in each aqueous calibrator to which the same concentrations of analytes had been added.

Table 6 The levels of the targeted neurotransmitters in both brain homogenates and microdialysates hippocampus.

	brain homogenates(ng/g)		microdialysates(pmol/mL)	
Compound	prefrontal cortex	hippocampus	prefrontal cortex	hippocampus
GLN	49836.1 ± 3651.0	38135.7 ± 2245.2	6941.9 ± 1404.6	1243.4 ± 540.8
GLU	90480.9 ± 6496.0	80092.0 ± 3512.6	5082.4 ± 1986.6	2085.9 ± 836.9
GABA	28192.2 ± 2418.0	20407.8 ± 2788.1	261.9 ± 80.6	147.1 ± 16.4
5-HT	976.8 ± 109.0	747.2 ± 32.6	39.3 ± 7	18.3 ± 9.7
DA	551.5 ± 104.1	335.1 ± 26.6	80.3 ± 8.5	84.7 ± 4.0
NE	609.2 ± 65.3	546.6 ± 113.3	242.6 ± 14.4	239.6 ± 11.1
5-HIAA	232.7 ± 31.6	343.1 ± 54.4	339.4 ± 78.4	187.1 ± 27.1
DOPAC	144.4 ± 44.1	30.5 ± 5.0	79.6 ± 11.3	47.0 ± 3.7
HVA	164.9 ± 29.7	28.9 ± 6.8	955.2 ± 366.7	348.6 ± 175.5
MHPG	46.9 ± 7.7	45.8 ± 8.1	174.5 ± 2.9	178.3 ± 9.7
VMA	84.4 ± 10.1	48.7 ± 6	219.8 ± 17.9	73.9 ± 45.4
TRP	5565.5 ± 99.2	5459.6 ± 74.1	469.2 ± 12.9	387.5±9.3
KYN	337.3 ± 23.9	394.4 ± 107.4	107.8 ± 8.1	90.6 ± 8
KA	———	———	8.4 ±0. 3	7.0 ± 0.2

Data are expressed as mean ± SEM (n=8).

Precision and Recovery

The intra-day precision were calculated by analyzing QC samples at three concentrations (n = 5, at each concentration level) on the same day. The inter-day precision were determined by analyzing the three concentrations on five replicates on three separate days. The precision was calculated as the coefficient of variance (CV) of the intraday and interday analytical results. The recovery rate of each analyte was assessed by comparing the difference between QC and blank samples with the added standard.

Results and discussion

Linearity, LLOQ, precision, recovery, stability, and matrix effects

All standard curves showed good linearity both in brain homogenates and microdialysates. The equations of the standard curves, corresponding linear regression coefficients and each calibration ranges are illustrated in Table 2 and 3. Owing to the fact that the targeted analytes herein are endogenous metabolites, the LLOQs were determined by using standard mixtures. The results of LLOQs for each analyte are illustrated in Table 2 and 3.

The data of intra-day precision, inter-day precision and recovery assays are summarized in Table 4 and 5 for all the analytes in both brian homogenates and microdialysates. The expression of results by CVs ranging from 1.1% to 15.8% for intra-day precision and from 1.4% to 15.4% for inter-day precision. The values of the standard addition recoveries and CVs in the quality control samples are also showed in Table 4 and 5.

The stability test of the derivatization products in brain homogenates and microdialysates showed that the derivatives of NE and 5-HT were not stable and must be measured within 8 h after derivatization at 4 ℃ in the dark. The rest of compounds were stable for at least 1 week at 20 ℃. The CVs of QC samples ranged from 3.2% to 17.9% in the dark. The values of matrix effect ranged from 7.4% to 13.9%, which indicates no significant matrix effects existed.

Analyses of NTs in both brain homogenates and microdialysates

The LC-MS/MS method was used for simultaneous determination of neurotransmitters and their metabolites in rats brain homogenates and microdialysates in the prefrontal cortex and hippocampus. The data summarized in Table 6 shows the levels of the analytes ranged from 28.9 ± 6.8 to 90480.9 ± 6496.0 ng/g in brain tissues (n=8), 7.0 ± 0.2 to 6941.9 ± 1404.6 pmol/mL in brain microdialysates (n=8).

Chromatography and mass spectra conditions`

According to previous reports, each of the dansylated compounds investigated here produced a much stronger signal in positive mode using the electrospray ion source[16,22,25]. Thus, all of the analyses were detected under MRM mode. MS optimization was performed in positive ionization mode in order to select the precursor ions that could provide higher sensitivity. Fig. 1 shows parents ion spectra of DA, NE, 5-HT, DHBA, L-Asp-

$^{13}C_4$,^{15}N, 5-HICA, GLN, GABA, GLU, TRP, KYN, KA, DOPAC, HVA, 5-HIAA, MHPG and VMA, respectively. Fig.2 shows daughter ion spectra of all analytes. As compared with the previous studies [22,26], we had simultaneous analyzed more compounds and their metabolites by LC-MS/MS. Moreover, since we dealt with microdialysis samples with high salt level this time, the liquid chromatographic conditions settings were specifically modified. By upgrading these conditions, this method becomes more universal and should be of interest to the researchers who are coping with different types of biological sample. The representative MRM chromatograms for 5-HIAA, 5-HICA , HVA, DOPAC, 5-HT, NE, DHBA, DA, GLN, L-Asp-$^{13}C_4$,^{15}N, GLU, GABA, KYN, VMA, TRP, MHPG are shown in Fig. 3. Fig. 4 shows the MRM ion chromatogram for KA. The collision energy and the cone voltage were optimized for each mass transition (Table.1).

Main challenges in monitoring NTs in rodent brain microdialysates are the low basal level, low sample volumes and high inorganic salt concentration. We used a column switching valve before analytical separation in order to preserve the mass spectrometer from contamination or clogging from salts. A pre-column was also installed to extend its lifetime. Optimization of column switching valve time conditions focused on maximizing the signal intensity of the earlier eluted compounds. For this reason, different switching time points were tested and the switching valve was set to waste before 2.0 min. The chromatograms showed good reproducibility, symmetric peak shape and high peak intensity (Fig. 3).

Optimization of the derivatization conditions

The main factors affecting the derivatization reaction were investigated and optimized, including the pH of the buffer, concentration of the derivitization reagent, temperature and duration of water bath. The optimal conditions for all NTs except KA in brain homogenates were as follows: pH 10.5, 0.1 M of buffer concentration, derivatization temperature 35℃, and 35 min of water bath. However, the derivatization of KA needs more vigorous condition. The optimal conditions for all NTs in microdialysates sample were modified as: pH 10.5, 0.5 M of buffer concentration, derivatization temperature 60℃, and 15 min of water bath. These conditions were used for further experiments. The solution was added with 15% formic acid to stop the reaction and to avoid degradation of the derivatives. The method of derivatization showed good reproducibility.

Conclusions

The currently developed LC-MS/MS method is reliable and accurate for the determination of the NTs and their metabolites spanning dopamine, serotonin, and kynurenine metabolic pathways in the brain. Since NTs and their metabolites play a key role in various diseases, the method will be expected to find its broad applications in both the preclinical and clinical contexts.

Fig1. Parents ion spectra of NTs and internal standards

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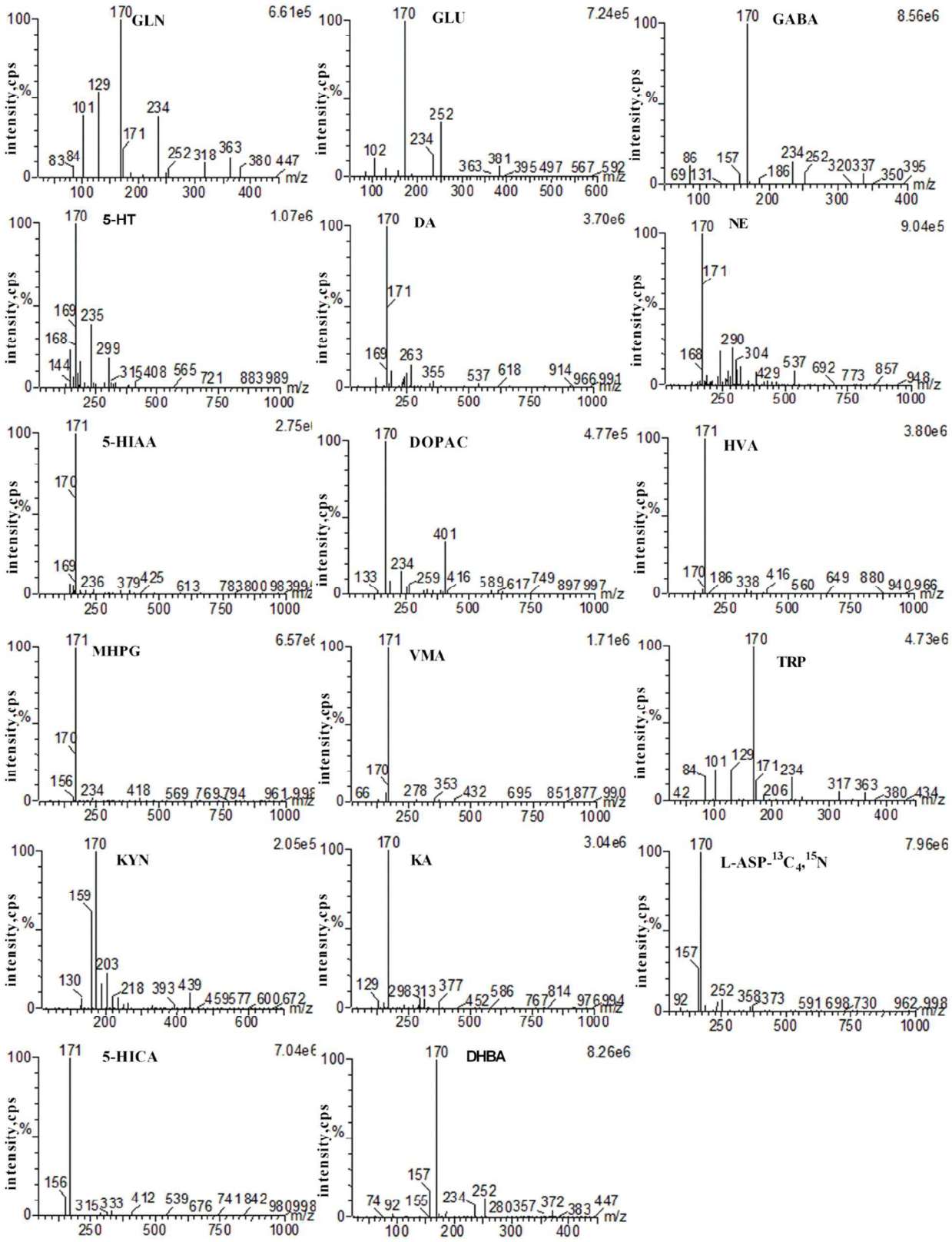

Fig2. Daughter ion spectra of NTs and internal standards

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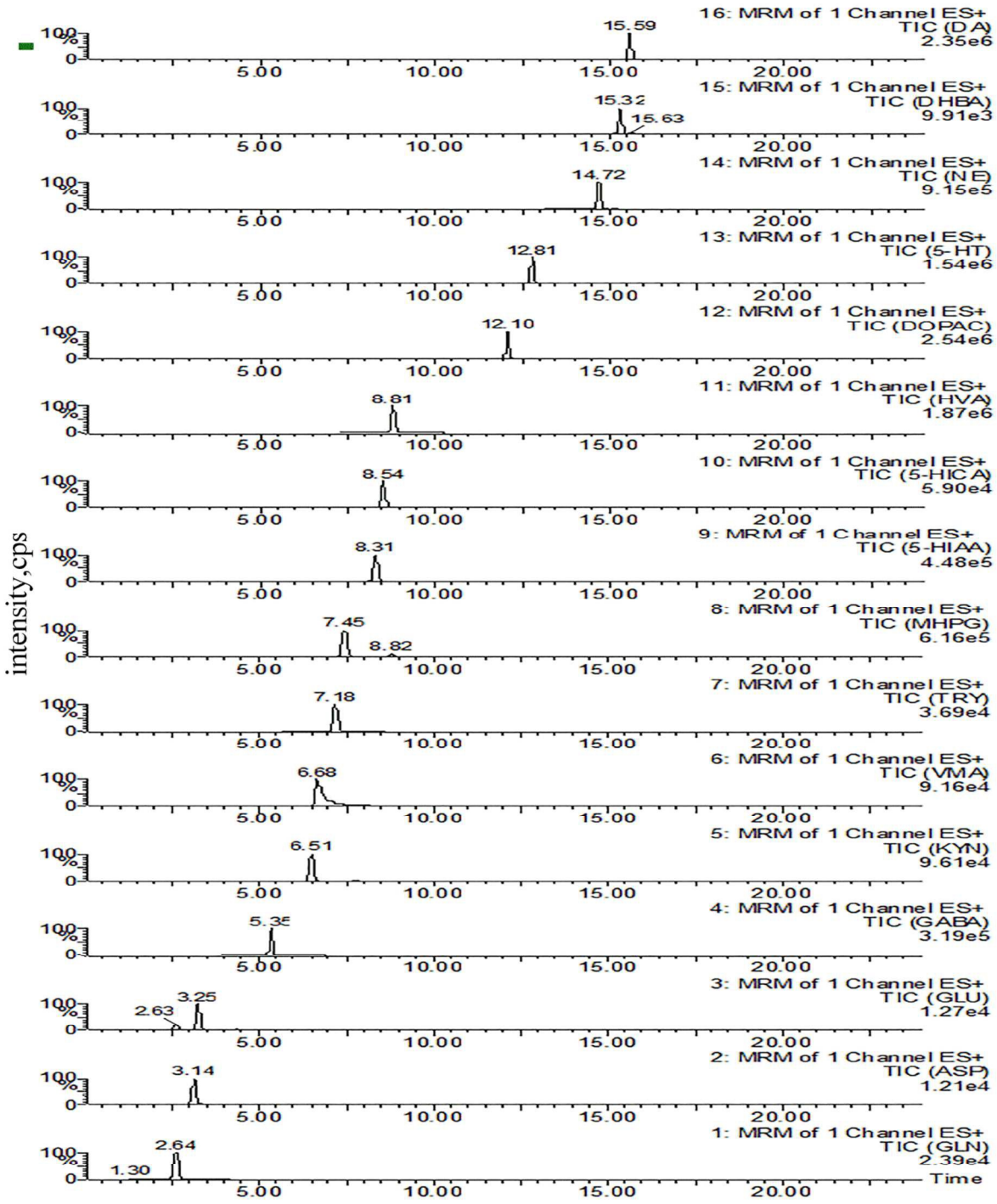

Fig3.MRM ion chromatograms for NTs and internal standards in rat brain homogenates, 500 µL of brain homogenates were spiked with 10 µL of the internal standard solutions (containing DHBA 0.12 µg/mL, 5-HICA 0.19 µg/mL, L-Asp-$^{13}C_4$,^{15}N 1.41 µg/mL)

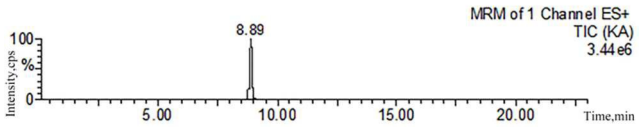

Fig. 4 MRM ion chromatograms for Kynurenic acid in brain microdialysates (50 µL microdialysates sample was derivatized and 10 µL was injected for analysis)

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1 M. A. Kurian, P. Gissen, M. Smith, S. Heales Jr and P. T. Clayton, *Lancet Neurol*, 2011, **10**, 721-733.

2. N. Muller, A. M. Myint and M. J. Schwarz, *Curr Pharm Des*, 2011, **17**, 130-136.

3. D. J. Nutt, *J Clin Psychiatry*, 2008, **69 Suppl E1**, 4-7.

4. J. Ng, S. J. Heales and M. A. Kurian, *Paediatr Drugs*, 2014, **16**, 275-291.

5. P. L. Pearl, P. K. Capp, E. J. Novotny and K. M. Gibson, *Clin Biochem*, 2005, **38**,1051-1008.

6. B. Yu, M. Ruan, X. B. Cui, J. M. Guo, L. Xu and X. P. Dong, *J Pharm Biomed Anal*, 2013, **77**, 128-132.

7. S. Greco, W. Danysz, A. Zivkovic, R. Gross and H. Stark, *Anal Chim Acta*, 2013, **71**, 65-72.

8. Z. D. Sandlin, M. Shou, J. G. Shackman and R. T. Kennedy,

Anal Chem, 2005, **77**, 7702-7708.

9. G. Liu, J. Chen and Y. Ma, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2004, **805**, 281-288.

10. X. E Zhao, Y. R Suo, *Talanta*, 2008, **76**, 690-697.

11. T. Yoshitake, K. Fujino, J. Kehr, J. Ishida, H. Nohta and M. Yamaguchi, *Anal. Biochem*, 2003, **312**, 125-133.

12. B. Ferry, E. P. Gifu, I. Sandu, L. Denoroy and S. Parrot, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2014, **951-952**, 52-57.

13. S. Cheemalapati, S. Palanisamy, V. Mani and S. M. Chen, *Talanta*, 2013, **117**, 297-304.

14. J. Lu, S. Zhang, A. Wang, W. Zhang and L. Jin, *Talanta*, 2000, **52**, 807-815.

15. P. Song, O. S. Mabrouk, N. D. Hershey and R.T. Kennedy,

Anal Chem, 2012, **84**, 412-419.

16. R. Nirogi, P. Komarneni and V. Kandikere, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2013, **913-914**, 41-47.

17. M. Sano, V. Ferchaud-Roucher and C. Nael, *J Mass Spectrom*, 2014, **49**, 128-135.

18. M. Moini, C. L. Schultz and H. Mahmood, *Anal Chem*, 2003, **75**, 6282-6287.

19. K. Rangiah and D. Palakodeti, *Rapid Commun Mass Spectrom*,

2013, **27**, 2439-2452.

20. X. Zhang, A. Rauch, H. Lee, H. Xiao, G. Rainer and N. K. Logothetis, *Rapid Commun Mass Spectrom*, 2007, **21**, 3621-3628.

21. K. Qu, L. Zhao and X. Luo, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2014, **965**, 206-215.

22. H. L. Cai, R. H. Zhu and H. D. Li, *Anal Biochem*, 2010, **396**, 103-111.

23. J. A. Eckstein, G. M. Ammerman, J. M. Reveles and B. L. Ackermann, *J Neurosci Methods*, 2008, **171**, 190-196.

24. X. Zheng, A. Kang and C. Dai, *Anal Chem*, 2012, **84**, 10044-10051.

25. K. Guo and L Li, Anal. Chem. 2009, **81**, 3919–3932.

26. H. L. Cai, R. H. Zhu, H. D. Li, J and Zhang, L. F. Li, *J Chromatogr B Analyt Technol Biomed Life Sci*, 2011, **879**, 1993-1993.

27. G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, 1998.