Short communication

MultiSimplex optimization of chromatographic separation and dansyl derivatization conditions in the ultra performance liquid chromatography–tandem mass spectrometry analysis of risperidone, 9-hydroxyrisperidone, monoamine and amino acid neurotransmitters in human urine

Hua-Lin Cai [a,b,1], Rong-Hua Zhu [a,1], Huan-De Li [a,*], Jun Zhang [a,b], Lan-Fang Li [a,b]

[a] *Clinical Pharmacy & Pharmacology Research Institute, The Second Xiangya Hospital of Central South University, 139# Middle Renmin Road, Changsha 410011, Hunan, PR China*
[b] *School of Pharmaceutical Sciences, Central South University, Changsha 410013, PR China*

ABSTRACT

A pre-column dansylated ultra-performance liquid chromatography–electrospray ionization tandem mass spectrometry (UPLC–MS/MS) method for simultaneous determination of risperidone (RIP), 9-hydroxyrisperidone (9-OH-RIP), monoamine and amino acid neurotransmitters in human urine was developed with the aim of providing data on how neurotransmitters may influence each other or change simultaneously in response to risperidone treatment. MultiSimplex based on the simplex algorithm and the fuzzy set theory was applied to the optimization of chromatographic separation and dansyl derivatization conditions during method development. This method exhibited excellent linearity for all the analytes with regression coefficients higher than 0.997. The lower limit of quantification (LLOQ) values for 9-OH-RIP and RIP were 0.11 and 0.06 ng/ml, respectively, and for neurotrasmitters ranged from 0.31 to 12.8 nM. The mean accuracy ranged from 94.7% to 108.5%. The mean recovery varied between 81.6% and 97.5%. All the RSD of precision and stability were below 9.7%. Finally, the optimized method was applied to analyze the first morning urine samples of schizophrenic patients treated with risperidone and healthy volunteers.

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1. Introduction

The major neurotransmitters (NTs) widely distributed in the central nervous system (CNS) and peripheral body fluids are monoamine and amino acid neurotransmitters. Monoamine neurotransmitters (MANTs) consist of dopamine (DA), norepinephrine (NE), 5-hydroxytryptamine (5-HT) and their respective acidic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), vanilmandelic acid (VMA), 3-methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxytindole-3-acetic acid (5-HIAA). Amino acid neurotransmitters (AANTs) include the most prevalent excitatory neurotransmitter glutamate (Glu), its metabolite the most important inhibitory neurotransmitter γ-aminobutyric acid (GABA), and their major precursor glutamine (Gln). Ever since the discoveries of the NTs and related metabolites, there are accumulating evidences that changes in these compounds within peripheral body fluids including cerebrospinal fluid (CSF), plasma and urine have some sort of indirect associations with certain neuropsychiatric disorders occurred in the brain [1,2]. Given the merits of stability, sensitivity and non-invasiveness in urinary NT testing, it may be the desirable method to analyze nervous system function among all the biological fluids that can be utilized [3]. Besides, psychiatrists have also kept a keen interest in exploring the relationship between the effects of antipsychotics and neurochemical changes, both centrally and peripherally. Based on the above, we attempted to undertake an investigation of the effects of risperidone (RIP) on peripheral NTs metabolism in schizophrenics to enhance our understanding of the biological mechanisms of this atypical antipsychotic drug action and the pathophysiology of schizophrenia. Due to the relatively small sample size we investigated and the pharmacokinetics variation in this heterogeneous population, the data from humoral concentration of RIP and its active metabolite 9-hydroxyrisperidone (9-OH-RIP) may provide a better correlation between parameters instead of the given dose. Consequently, an analytical method for simultaneous determination of the drugs and NTs in urine is urgently needed to facilitate the research.

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* Corresponding author. Tel.: +86 731 85292121; fax: +86 731 82258487.
  E-mail address: lihuande1953@126.com (H.-D. Li).
[1] These authors contributed equally to this work.

Our group has recently developed a sensitive and selective LC–MS/MS method for NT testing in human plasma, employing dansyl derivatization to significantly enhance both ionization efficiency in the electrospray ionization (ESI) source and collision-activated dissociation in the collision cell giving rise to lower detection limits [4]. However, the concentrations of the drug and its active moiety affecting neurotransmissions were not considered, and the separation and dansylation conditions were not thoroughly optimized in the method. Therefore, we here try to upgrade it partly based on our previous work.

MultiSimplex is designed as a true multivariate nonlinear optimization tool that combines the modified simplex method with the fuzzy set theory by means of the membership functions or the point response measure called the aggregated value of membership, providing an efficient and flexible tool for handling different optimization criteria (i.e., maximization, minimization, and target value) [5]. It is easy to follow and has been used in several analytical applications [6,7]. The aim of this work was to further optimize the factors affecting chromatographic separation and dansyl derivatization by MultiSimplex, and to apply the method for simultaneous measurement of RIP, 9-OH-RIP and the NTs in human urine samples.

2. Experimental

2.1. Materials and reagents

2.1.1. Sample collection

The study was approved by the Ethical Committee of the Second Xiangya Hospital of Central South University, and all the subjects signed informed consent before participating. The participants were instructed to collect their first morning midstream urine specimens after overnight fasting. With no added preservative, the collected samples were immediately frozen at $-80\,°C$ until testing was performed.

2.1.2. Chemicals and reagents

RIP and midazolam were purchased from the National Institutes for Food and Drug Control of China. 9-OH-RIP was obtained from Toronto Research Chemical Inc (Toronto, Canada). DA, HVA, NE, VMA, 5-HT, 5-HIAA, Gln and GABA were purchased from Sigma (St. Louis, MO, USA). L-Aspartic acid (L-Asp), DOPAC, Glu, 3,4-dihydroxybenzylamine (DHBA) and 5-hydroxyindole-2-carboxylic acid (5-HICA) were purchased from Aldrich (Steinheim, Germany). MHPG was obtained from Fluka (Buchs, Switzerland). L-Aspartic acid-$^{13}C_4$,^{15}N (L-Asp-$^{13}C_4$,^{15}N) was purchased from Isotec (Miamisburg, OH, USA). Dansyl chloride (Dns-Cl) was obtained from Alfa Aesar (Karlsruhe, Germany). HPLC grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). HPLC grade ammonium acetate (NH$_4$Ac) and formic acid (FA) were purchased from Tedia (Fair Field, USA).

2.1.3. Preparation of standard solutions, calibration curves and quality control samples

Separate solutions containing 0.232 mg/ml of RIP, 0.05 mg/ml of 9-OH-RIP and 1.264 mg/ml of internal standard (IS) midazolam were prepared using acetonitrile, respectively. A mixed stock solution containing 130.6 μM of DA, 118.2 μM of NE and 113.5 μM of 5-HT was prepared in acetonitrile. A mixed stock solution of monoamine metabolites (DOPAC, HVA, VMA, MHPG and 5-HIAA) was prepared in acetonitrile at 118.9 μM, 109.8 μM, 100.9 μM, 108.6 μM and 104.6 μM, respectively. A mixed stock solution of Gln, Glu and GABA was prepared in water at 54.7 mM, 6.39 mM and 0.970 mM, respectively. A mixed stock solution of ISs (DHBA, 5-HICA) was prepared in acetonitrile at 921.9 μM and

Table 1
The preparation of calibration curve, regression equation, correlation coefficient, LOD and LLOQ for each analyte.

Analyte	Point on the calibration curve									Calibration equation	Correlation coefficient	LOD	LLOQ
	1	2	3	4	5	6	7	8	9				
Drugs													
9-OH-RIP (ng/ml)	5.00	10.00	15.00	50.0	100.0	150.0	500	1000	1500	$Y = -1.5105e^{-4} + 1.7955e^{-4}X$	0.9998	0.03	0.11
RIP (ng/ml)	0.58	1.16	1.74	5.80	11.6	17.4	58.0	116	174	$Y = 8.3070e^{-6} + 2.113e^{-4}X$	0.9999	0.02	0.06
NTs													
Gln (μM)	5.47	10.95	16.42	54.7	109.5	164.2	547	1095	1642	$Y = 1.9976e^{-5} + 2.2410e^{-3}X$	0.9973	$3.85e^{-3}$	$12.8e^{-3}$
Glu (μM)	0.64	1.28	1.92	6.4	12.8	19.2	64	128	192	$Y = 1.5421e^{-3} + 2.915e^{-3}X$	0.9995	$1.86e^{-3}$	$6.21e^{-3}$
GABA (nM)	96.97	193.95	290.92	969.7	1939.5	2909.2	9697	19395	29092	$Y = 7.9723e^{-4} + 1.0062e^{-5}X$	0.9985	0.68	2.28
DA (nM)	6.53	13.06	19.59	65.3	130.6	195.9	653	1306	1959	$Y = 1.1821e^{-5} + 1.1487e^{-5}X$	0.9999	0.40	1.33
DOPAC (nM)	5.95	11.89	17.84	59.5	118.9	178.4	595	1189	1784	$Y = 1.5653e^{-4} + 9.3015e^{-5}X$	0.9995	0.13	0.45
HVA (nM)	5.49	10.99	16.47	54.9	109.9	164.7	549	1099	1647	$Y = 1.1236e^{-4} + 4.9746e^{-4}X$	0.9997	0.09	0.31
NE (nM)	5.91	11.82	17.73	59.1	118.2	177.3	591	1182	1773	$Y = 1.2458e^{-6} + 7.1133e^{-6}X$	0.9999	0.36	1.20
VMA (nM)	5.05	10.09	15.14	50.5	100.9	151.4	505	1009	1514	$Y = 1.8134e^{-4} + 9.7998e^{-5}X$	0.9989	0.24	0.80
MHPG (nM)	5.43	10.86	16.29	54.3	108.6	162.9	543	1086	1629	$Y = 1.4449e^{-6} + 5.2255e^{-6}X$	0.9999	0.31	1.02
5-HT (nM)	5.67	11.35	17.02	56.7	113.5	170.2	567	1135	1702	$Y = 3.4828e^{-5} + 2.0792e^{-5}X$	0.9994	0.25	0.83
5-HIAA (nM)	5.23	10.46	15.69	52.3	104.6	156.9	523	1046	1569	$Y = 2.4707e^{-4} + 5.7813e^{-5}X$	0.9994	0.21	0.70

Table 2
Precision, accuracy, recovery and stability in spiked human urine (n=5).

Analyte	Added concentration	Precision R.S.D (%, $n = 5$)		Accuracy (%)	Recovery (%)	Stability R.S.D (%, $n = 5$)		
		Intra-day	Inter-day	Mean ± S.D.	Mean ± S.D.	Processed sample at 4 °C (12 h)	Long-term storage at −80 °C (30 d)	Three freeze–thaw cycles (each at −80 °C for 24 h)
9-OH-RIP (ng/ml)	1.50	2.7	3.1	94.7 ± 2.6	88.2 ± 2.7	3.8	4.3	2.6
	15.0	5.6	3.7	101.6 ± 5.7	81.6 ± 5.4	3.7	6.6	5.2
	120.0	3.3	3.4	105.3 ± 3.4	90.1 ± 2.9	2.6	1.7	1.4
RIP (ng/ml)	1.74	7.4	7.7	97.5 ± 7.2	88.8 ± 6.1	2.0	1.5	2.7
	17.4	3.4	2.4	107.6 ± 3.7	81.9 ± 2.3	2.6	3.5	1.4
	139.2	3.3	3.0	97.6 ± 3.2	84.4 ± 4.0	6.9	4.1	5.4
Gln (μM)	16.42	6.1	7.2	96.7 ± 5.9	88.0 ± 7.8	3.2	8.0	8.1
	164.2	5.5	5.8	101.6 ± 5.6	84.2 ± 4.5	2.7	1.3	5.5
	1313.6	2.7	3.0	99.8 ± 2.6	87.3 ± 1.6	3.4	3.4	1.7
Glu (μM)	1.92	7.3	5.7	98.7 ± 7.2	94.5 ± 9.4	7.6	5.1	6.0
	19.2	2.4	4.6	100.7 ± 2.5	95.9 ± 4.9	1.5	2.1	1.9
	153.6	3.7	3.0	96.2 ± 3.5	87.9 ± 0.7	2.3	1.0	5.4
GABA (nM)	290.92	2.9	5.1	99.2 ± 2.9	95.2 ± 4.1	6.7	3.3	7.2
	2909.2	4.1	5.5	102.9 ± 4.2	92.0 ± 7.2	2.4	4.0	3.0
	23273.6	5.1	3.6	105.7 ± 5.4	89.0 ± 2.0	2.0	4.7	5.4
DA (nM)	19.59	7.2	5.7	96.0 ± 6.9	87.6 ± 5.3	4.6	5.6	6.5
	195.9	3.4	5.1	106.4 ± 3.6	91.9 ± 3.7	2.9	2.9	7.1
	1567.2	4.3	2.5	96.3 ± 4.2	92.6 ± 4.7	1.5	1.9	2.3
DOPAC (nM)	17.84	7.4	7.6	98.6 ± 7.3	88.3 ± 8.4	6.2	4.3	4.2
	178.4	6.3	5.1	102.4 ± 6.4	91.7 ± 5.5	5.8	7.9	6.3
	1427.2	2.0	3.9	99.9 ± 2.0	92.8 ± 2.1	1.2	1.8	2.8
HVA (nM)	16.47	7.1	6.4	103.2 ± 7.3	95.4 ± 8.4	2.5	7.6	2.7
	164.7	5.2	6.6	99.4 ± 5.2	94.2 ± 1.3	7.1	6.0	7.7
	1317.6	2.3	2.8	100.6 ± 2.3	90.3 ± 1.2	3.5	3.5	2.0
NE (nM)	17.73	7.2	6.4	108.5 ± 7.8	96.5 ± 6.5	9.5	5.7	7.1
	177.3	3.0	6.6	96.7 ± 2.9	91.0 ± 2.3	2.5	6.0	3.7
	1418.4	3.6	2.8	97.6 ± 3.5	92.3 ± 3.7	1.4	1.6	0.9
VMA (nM)	15.14	9.1	8.4	100.1 ± 9.1	91.7 ± 8.9	8.2	8.2	6.5
	151.4	7.1	6.4	97.8 ± 6.9	94.9 ± 4.6	3.4	4.4	8.7
	1211.2	4.1	3.5	96.8 ± 4.0	87.9 ± 7.8	1.2	2.2	2.0
MHPG (nM)	16.29	6.3	5.7	100.8 ± 6.3	85.0 ± 5.9	6.9	7.3	2.5
	162.9	2.8	4.5	101.3 ± 2.9	87.4 ± 2.7	3.3	2.2	2.7
	1303.2	8.9	5.1	100.6 ± 9.0	90.0 ± 7.9	3.7	2.6	3.3
5-HT (nM)	17.02	3.7	4.9	99.8 ± 3.7	84.1 ± 9.1	7.3	7.4	6.3
	170.2	5.7	4.4	100.3 ± 5.7	83.4 ± 2.5	6.1	4.1	3.3
	1361.6	4.2	3.9	101.7 ± 4.3	86.0 ± 3.3	2.1	2.5	5.1
5-HIAA (nM)	15.69	8.7	7.3	104.5 ± 9.1	90.2 ± 8.1	2.0	6.5	9.7
	156.9	6.7	7.7	107.0 ± 7.2	97.5 ± 7.7	6.6	4.1	6.5
	1255.2	4.0	2.7	97.1 ± 3.8	95.3 ± 2.8	4.1	1.8	4.6

677.4 μM, respectively. A stock solution of IS L-Asp-^{13}C$_4$,^{15}N at 2.61 mM was prepared in water. A solution of Dns-Cl was prepared in acetonitrile at a concentration of 3.5 mg/ml. Working solutions were obtained by sequentially diluting the stock solutions with acetonitrile or water. All the standard solutions were maintained at −80 °C. Appropriate amount of working solutions including analytes and ISs (30 μl for each of the stock solutions of midazolam, DHBA, 5-HICA and L-Asp-^{13}C$_4$,^{15}N) were spiked into Eppendorf tubes and then evaporated to dryness by vacuum freeze-drying to yield desired concentration points of the calibration curves as shown in Table 1. Quality control samples were prepared at three concentration levels that are summarized in Table 2.

2.2. Derivatization with Dns-Cl

Sample preparation consisted of the addition of 100 μl urine, 100 μl Dns-Cl solution (3.5 mg/ml) and 100 μl NaHCO$_3$–Na$_2$CO$_3$ buffer (0.1 M, pH = 11.0) to the Eppendorf tube containing the residue of ISs. After capping the tube and brief vortex-mixing for 30 s, the mixture was reacted in the dark at 35 °C in a water bath for 30 min. Then, 5 μl of 15% formic acid–water solution was added into the tube to stop the reaction. The mixture was centrifuged at 13,000 × g for 5 min at 4 °C. 5 μl of the supernatant of the reaction mixture was injected into the UPLC system. The sample vials should stay in autosampler at 4 °C throughout the process of analyzing.

Table 3
MRM acquisition settings of the analytes and internal standards.

Compound name	Molecular mass of precursor (Da)	Number of add-on dansyl groups	MRM transition (m/z)	Dwell (s)	Cone voltage (V)	Collision energy (eV)
Drugs						
9-OH-RIP	426	–	427.1 > 207.1	0.1	40	30
RIP	410	–	411.1 > 191.1	0.1	38	28
NTs						
Gln	146	1	379.9 > 169.9	0.1	22	25
Glu	147	1	381.0 > 169.9	0.1	20	20
GABA	103	1	337.0 > 169.8	0.1	20	20
VMA	198	1	432.0 > 171.0	0.1	22	27
MHPG	184	1	418.2 > 170.5	0.1	20	23
5-HIAA	191	1	425.0 > 170.4	0.1	22	29
HVA	182	1	416.1 > 171.0	0.1	25	25
DOPAC	168	2	635.0 > 170.4	0.1	26	35
5-HT	176	2	643.0 > 169.9	0.1	35	42
NE	169	3	869.0 > 170.5	0.1	23	50
DA	153	3	853.0 > 170.5	0.1	27	50
ISs						
Midazolam	326	–	326.1 > 291.3	0.1	40	25
L-Asp-$^{13}C_4$,^{15}N	138	1	372.0 > 169.9	0.1	20	20
5-HICA	177	1	411.4 > 170.9	0.1	26	25
DHBA	139	3	839.0 > 170.4	0.1	27	50

2.3. Apparatus

A Waters Quattro Premier^{TM} XE triple–quadrupole tandem mass spectrometer fitted with an UPLC/Acquity^{TM} system with cooling autosampler and column oven was used for the analyses. Chromatographic separation of the analytes was conducted on an ACQUITY UPLC HSS T3 column (2.1 mm × 50 mm, 1.8 μm) using a binary solvent system to achieve gradient elution. To avoid thermal degradation of the derivatives, separation was operated at room temperature (25 ± 2 °C). During the optimization, the elution conditions varied with each experiment but the nature of the mobile phase remained constant and was constituted with A: water (ammonium acetate and formic acid) and B: acetonitrile. The tandem mass spectrometer was set to collect data in multiple reaction monitoring (MRM) mode applying ESI source operated in positive ion mode during the run. The ionization source parameters were: capillary voltage 3.0 kV; source temperature 120 °C; desolvation gas temperature 450 °C at a flow rate of 750 L/h (N_2) and cone gas at a flow rate of 50 L/h (N_2). Argon was used as collision gas and flow rate was set at 0.16 ml/min. The MRM transitions, cone voltages and collision energies individually optimized for quantification are summarized in Table 3. Data acquisition and processing were performed using MassLynx 4.1 software. The MultiSimplex program was downloaded from www.multiSimplex.com site.

Table 4
Uncorrected and creatinine-corrected concentrations of the drugs and NTs in urine of schizophrenic patients and paired controls.

| Analyte	Schizophrenics (n = 7)			Controls (n = 7)
	Week 0	Week 3	Week 6	
Uncorrected				
9-OH-RIP (ng/ml)	–	401.0 ± 334.0	232.3 ± 161.4	–
RIP (ng/ml)	–	23.1 ± 25.0	14.8 ± 16.1	–
Gln (μM)	321.6 ± 352.2	421.8 ± 310.5	561.6 ± 410.4	1097.5 ± 903.9
Glu (μM)	8.4 ± 13.6	8.9 ± 9.9	11.6 ± 10.1	18.5 ± 22.7
GABA (nM)	1812.3 ± 2228.5	2076.1 ± 2160.1	1614.5 ± 1369.4	4818.9 ± 4207.2
DA (nM)	902.1 ± 765.0	444.2 ± 379.4	129.2 ± 93.5	160.6 ± 126.5
DOPAC (nM)	975.6 ± 1429.6	1016.2 ± 1114.3	716.5 ± 698.6	2215.9 ± 1879.5
HVA (nM)	1132.1 ± 1612.5	1309.6 ± 1430.3	1500.6 ± 1322.5	1445.3 ± 1649.1
NE (nM)	15.8 ± 18.6	23.0 ± 28.7	16.6 ± 12.5	10.5 ± 6.6
VMA (nM)	1841.0 ± 2184.9	1937.1 ± 2238.2	1705.1 ± 1490.8	983.3 ± 885.3
MHPG (nM)	2367.3 ± 5407.5	686.1 ± 1520.2	211.0 ± 434.2	312.6 ± 352.3
5-HT (nM)	99.3 ± 53.5	114.2 ± 61.8	129.3 ± 92.4	143.8 ± 116.5
5-HIAA (nM)	409.0 ± 287.8	696.8 ± 582.3	638.3 ± 353.5	1459.1 ± 1935.4
Creatinine-corrected				
9-OH-RIP (mg/g CRE[a])	–	0.47 ± 0.11	0.51 ± 0.11	–
RIP (mg/g CRE)	–	0.029 ± 0.019	0.030 ± 0.018	–
Gln (μmol/mmol CRE)	35.5 ± 17.3	67.0 ± 29.7	146.8 ± 60.6	89.6 ± 43.3
Glu (μmol/mmol CRE)	0.84 ± 0.71	1.39 ± 0.84	3.93 ± 2.64	1.28 ± 0.89
GABA (nmol/mmol CRE)	227.9 ± 161.6	320.6 ± 163.3	662.5 ± 578.7	487.7 ± 368.0
DA (nmol/mmol CRE)	105.5 ± 73.4	61.3 ± 43.6	44.2 ± 34.5	17.9 ± 15.0
DOPAC (nmol/mmol CRE)	93.6 ± 80.2	128.7 ± 80.1	203.0 ± 136.2	132.3 ± 79.9
HVA (nmol/mmol CRE)	138.4 ± 111.4	227.4 ± 175.5	481.3 ± 302.3	116.2 ± 115.9
NE (nmol/mmol CRE)	1.67 ± 1.04	3.01 ± 1.50	5.29 ± 2.79	1.31 ± 1.16
VMA (nmol/mmol CRE)	202.5 ± 155.9	286.9 ± 167.3	567.7 ± 344.9	131.0 ± 126.0
MHPG (nmol/mmol CRE)	127.0 ± 162.9	59.9 ± 72.7	40.8 ± 58.0	22.6 ± 16.4
5-HT (nmol/mmol CRE)	20.2 ± 18.6	27.2 ± 24.4	59.0 ± 55.5	16.0 ± 11.8
5-HIAA (nmol/mmol CRE)	69.3 ± 55.1	133.3 ± 101.0	266.4 ± 207.3	124.7 ± 97.9

[a] CRE: creatinine.
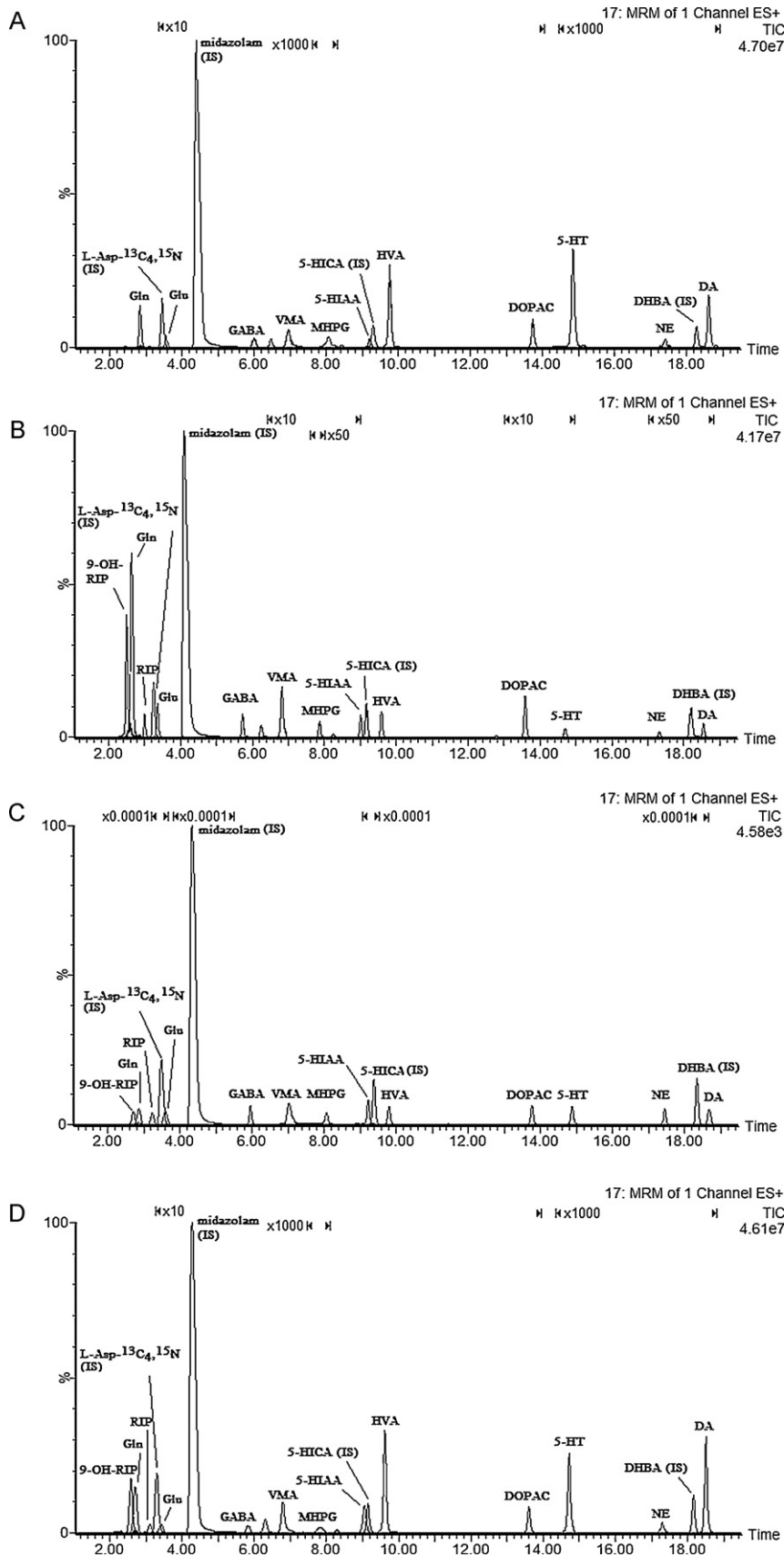
Fig. 1. Overlaid chromatograms of: (A) blank urine sample (Note that the signals of Glu, GABA, VMA, 5-HIAA, HVA and DOPAC have been magnified by a factor of 10, whereas the factors are set at 1000 for MHPG, 5-HT, NE and DA.); (B) blank urine spiked with the analytes at high concentration of QC sample (Note that the signals of VMA, 5-HIAA, DOPAC and 5-HT have been magnified by a factor of 10, whereas those of MHPG, NE and DA have been magnified by a factor of 50.); (C) standards at LLOQ for the analytes (Note that the signals of the ISs have been magnified by a factor of 0.0001.); (D) a typical urine sample of schizophrenic patient at week 3 (Note that the magnification factor settings were the same as the chromatogram of blank urine sample.).

2.4. Validation study

Weighted ($1/X^2$) nine-point calibration curves were constructed by the standard addition method using the blank urine with ISs as a function of the concentration calibrator. Because the pre-existed concentrations of the targeted NTs in blank urine were not authentically known, the limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated at signal-to-noise ratios of 3 and 10, respectively, using aqueous standard solutions derivatized following the same procedure above. Moreover, since the levels of analytes in the tested urine were very much high (Table 4), we had to construct the calibrations with their starting concentrations much higher than the LLOQs in order to cover the expected concentration ranges. Five replicated samples of QCs were analyzed to obtain precision, accuracy and recovery data. Because the matrices contained the targeted analytes (NTs), the recovery and accuracy were therefore calculated upon the subtraction of the averaged levels of each analyte in 'blank' urine. The accuracy was obtained by comparing the difference of the concentration between the analyte found in the spiked urine sample and in the blank urine sample to the concentration theoretically spiked. The recovery was calculated as follows: the peak area difference of the analyte between the spiked sample and the non-added control sample was divided by the peak area of it in the aqueous calibrator. Intra- and inter-day precisions were assessed by performing five duplicate analyses of each level on the same day and over three different days, respectively. The stabilities of the analytes in human urine under various storage conditions shown in Table 2 were also evaluated. In addition, the matrix effect from different sources of urine was investigated with post-column infusion of the derivatized standard mixture, coupled with injection of the derivatized blank urine sample.

2.5. Application of the method

We used the optimized method to determine the urinary concentrations of RIP, 9-OH-RIP, MANTs and AANTs for 7 schizophrenic inpatients from the Second Xiangya Hospital and 7 age- and sex-paired healthy volunteers. The seven patients were prescribed risperidone at a target dose of 3–4 mg/d. In case some of the analytes' concentrations exceeded their calibration ranges, the sample was diluted proportionally with water and re-analyzed. All the concentration data were then corrected by urine creatinine (CRE) that was analyzed by Hitachi 7170A Auto Biochemical Analysis Instrument in clinical laboratory of the Second Xiangya Hospital. Two-tailed Pearson's correlation analyses were applied to examine the relationships between the adjusted concentrations of the drugs and NTs. Data were provided as means ± standard deviations. The significant difference level was $p < 0.05$.

3. Results and discussion

3.1. Optimizations of chromatographic separation and dansylation condition

The optimizations of the chromatographic separation method and dansylation procedure were made on spiked urine samples of high concentration level in order to assure that the differences observed were due to changes in the experimental conditions and not to the uncertainty of the measurements at low concentrations. As urine samples needed a dansylation procedure before introducing them onto UPLC system, a basic dansyl derivatization condition was used as the same (Dns-Cl: 4 mg/ml; temperature: 60 °C; time: 15 min; buffer pH: 11.0) for treating plasma [4].

3.1.1. Selection of column and internal standard

The ACQUITY UPLC HSS T3 columns utilizes a trifunctional C_{18} alkyl phase bonded at a ligand density that promotes polar compound retention and separation via reversed-phase UPLC, while also affords balanced retention for broad analyte mixtures, therefore was used for the present analyses. Cross-talk occurs when ions from one scan event are still present in the collision cell when the second MRM transition is taking place, which could lead to signal artifacts in the next transition's chromatogram [8]. Stable multi-isotope-labeled internal standard L-Asp-$^{13}C_4$,^{15}N was chosen to avoid the cross-talk effect of endogenous Asp [9]. The parent ion of its dansyl derivative containing the labeled portion of the molecule differs from that of Asp by 5 Da, hence use of the specific MRM transition of 372.0–169.9 for the analysis would be expected to minimize cross-talk from endogenous Asp into the L-Asp-$^{13}C_4$,^{15}N channel. The cross-talk from Asp into the L-Asp-$^{13}C_4$,^{15}N channel was evaluated by the analysis of derivatized standard solution containing L-Asp at the concentrations of 2.90 μM, 29.0 μM, 289.7 μM and 2897 μM, respectively. Only very slight responses were observed into the L-Asp-$^{13}C_4$,^{15}N channel at concentrations of 289.7 μM and 2897 μM, which was respectively 0.006% and 0.02% of the area of the L-Asp-$^{13}C_4$,^{15}N internal standard at the concentration used for the assay (782.2 μM). The human urinary reference concentration of Asp reported in literature was 1–25 μM [10], therefore there would be no interference of endogenous Asp with L-Asp-$^{13}C_4$,^{15}N internal standard.

3.1.2. MultiSimplex optimization of chromatographic separation

MultiSimplex software was used to optimize the following parameters: the percentage of FA in the aqueous phase, the concentration of ammonium acetate in the aqueous phase, the initial composition of the mobile phase, the steepness of acetonitrile and the flow rate during the linear gradient elution mode. The summed signal-to-noise ratio, the total resolution and the elution time of the final analyte (tridansyl-DA) were defined as response variables. If the resolution value[2] between two peaks was superior to 1.5 that complete base-line separation was achieved, no matter how high the value was, then the value 1.5 was attributed to total resolution when inferior they kept the calculated value. Besides, the gradient was stopped when the percentage of acetonitrile reached 90% which permitted to have sufficient eluent strength for tri-dansyl derivatives and to decrease the time of equilibration before each following injection. In view of the experiments proposed by the MultiSimplex program and the membership value of each of them (Supplemental Table 1), we decided to stop the optimization after the 14th run. All the defined control and response variables in the optimization of chromatographic separation and the optimum conditions obtained upon the basis of experiment 11 are summarized in Supplemental Table 2. In that way, satisfactory chromatograms (Fig. 1A–D) were obtained when the gradient elution started at an initial percentage of 80% aqueous phase containing 0.15% formic acid and 20 mM ammonium acetate, and reached 10% in 18.7 min at a flow rate of 0.275 ml/min. Then, the mobile phase was turned back to the original state and kept constant for 2.3 min before next run.

3.1.3. Optimization of dansyl derivatization by MultiSimplex

Previously reported conditions for dansyl derivatization differ from group to group [11–13], but have one thing in common: they are optimized by time- and labor-consuming univariate method. However, the factors affecting derivatization are usually involved in dynamic interactions and thus the results obtained from uni-

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[2] This parameter was defined as Rs (resolution) = 2 × (distance of the two peak positions)/(sum of the band widths of the two peaks).

variate method may not be reliable. Therefore, a MultiSimplex optimization of the dansylation procedure was carried out by investigating the influences of Dns-Cl concentration, derivatization temperature, buffer pH and derivatization time, on the output of the dansyl derivatization. Notably, although the drugs would not react with Dns-Cl, the derivatization conditions could impact on their peak intensities, which should also be taken into account. To avoid working with an excess of response variables, the peak areas of the compounds were summated and categorized into three independent response variables: MANT derivatization output, AANT derivatization output and drug peak intensity. The suggested experiments and the evolution of membership value are shown in Supplemental Table 3. The defined parameters of the variables in the dansylation optimization and the optimum results acquired are indicated in Supplemental Table 4. According to our observation, rate of dansyl derivatization increased rapidly with the rising temperature thus shorten the time taken for the reaction to get to equilibrium. In fact, Dns-Cl will inevitably react with the water in mixture, a part of the Dns-Cl is therefore lost by hydrolysis instead of going into reaction with the NTs. Then, the effect of a temperature increase on the dansylation is rather complex since it increases both the rate of the reaction and hydrolysis, and reduces the signals of drugs especially 9-OH-RIP. Related to the above, a relatively low temperature seems to afford a better reaction condition. As reported, the rate of hydrolysis of Dns-Cl is constant and low up to pH 9.5 and above this pH it increased rapidly [14]. However, our results indicate that MANTs containing phenolic groups will react much more slowly at pH 9.5 than it would do above this pH and that maximum reactivity would ideally need a pH of 11.0 or more. Finally, a Dns-Cl concentration of 3.5 mg/ml, a derivatization temperature of 35 °C, a derivatization time of 30 min and a buffer pH of 11.0 were suggested as the compromised condition.

3.2. Basic analytical method validation

3.2.1. Linearity, LOD, LLOQ, precision, accuracy, recovery and stability

The linearity was studied by examining the standard addition calibrations of the analytes. The obtained equations have correlation coefficients (r) higher than 0.997 in all cases using midazolam (for RIP and 9-OH-RIP), L-Asp-$^{13}C_4$,^{15}N (for the AANTs), 5-HICA (for 5-HT and 5-HIAA) and DHBA (for the rest of the MANTs) as the internal standards (Table 1). The LODs and LLOQs are also listed in Table 1. The data of precision, accuracy, recovery and stability are collected in Table 2. The mean accuracy ranged from 94.7% to 108.5%. The mean recovery varied between 81.6% and 97.5%. All the RSD were below 9.7%.

3.2.2. Matrix effect

A derivatized standard solution of the mixed analytes was constantly infused into the eluent from the column via post-column tee connection using a syringe pump at a rate of 10 μl/min. Continuous infusion of the derivatized standards (the same as the aqueous calibrator at medium concentration level) generated a baseline in each MRM channel and derivatized extracts of 0.1 ml of the pooled urine samples obtained from five independent sources were then injected. Three typical chromatograms on the amount of suppression or/and enhancement of the ionization as a result of eluting substances are shown in the upper trace in each panel (Supplemental Fig. 1A–C)). The traces are superimposed on the

MRM signal resulting from analysis of an aqueous calibrator at high concentration level. The majority of the channels presented with ion suppression, lasting until 1 min. However, the channels of 9-OH-RIP, RIP and midazolam were differentiated from those of Gln, L-Asp-$^{13}C_4$,^{15}N, Glu, GABA, VMA, MHPG, 5-HIAA, 5-HICA, HVA, DOPAC and 5-HT by the trend of baseline resulting from gradient elution (Supplemental Fig. 1A–B). For the remainder of the channels (NE, DHBA and DA) no significant ion suppression or enhancement was observed (Supplemental Fig. 1C). The results indicated no significant interference at the retention times of the analytes and ISs.

3.3. Application to urine samples of schizophrenics and healthy volunteers

The values of the analytes corrected for creatinine in the tested urine samples are summarized in Table 4. No urinary levels of NTs at weeks 3 and 6 were related to the dose of risperidone. However, levels of NE at weeks 3 and 6 were correlated with urinary RIP and the sum of RIP and 9-OH-RIP. Moreover, the sum of RIP and 9-OH-RIP was also associated with the urinary HVA at weeks 3 and 6.

4. Conclusions

MultiSimplex program allowed us to reduce the number of experiments needed for optimization as well as the attainment of true optimum sets of conditions. In this work, the separation and dansylation conditions were optimized to ensure fast, selective and sensitive simultaneous determination of RIP, 9-OH-RIP, MANTs and AANTs in human urine by UPLC–MS/MS. Moreover, the method was applied to analyze first morning urine samples of schizophrenics and healthy volunteers, and turned out to be a good screening method for monitoring the NTs and their metabolites. In this sense, the results obtained with this optimized method could be used to envisage the study of observing the changes in NT metabolism profile associated with risperidone treatment and different stages of schizophrenia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.05.006.

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