Neurotransmitters in the Striatum Tissues from Animal Brain: UPLC-MS/MS Method Validation and Application to Animal Models of Parkinson's Disease

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Novel Aspect

Validated UPLC-MS/MS methods for determining dopamine, serotonin, and their metabolites in the striatum tissues from animal models of Parkinson's disease.

Abstract

Simultaneously determining dopamine, serotonin and their metabolites in animal striatum tissues has been a challenge as their concentrations are low, sample size is limited, and many polar endogenous compounds may interfere with their analyses. Because of its unique features of sensitivity, selectivity, and speed (3S), ultra performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS) is increasingly used for the quantitation of biological compounds including small polar neurotransmitters. In this presentation, we describe UPLC-MS/MS method validation and application to the analysis of dopamine, serotonin and their metabolites in the striatum tissues from animal models of Parkinson's disease. The striatum tissue samples were surgically removed from euthanized animals and immediately flash frozen with liquid nitrogen and stored frozen at approximately –70°C until processing. The tissue samples were homogenized in 0.1% formic acid aqueous solution in a ratio of 1:9 [weight (mg) : mL]. In the positive ion multiple reaction monitoring (MRM) mode for norepinephrine (NE), dopamine (DA), serotonin (5-HT), and 5-hydroxyindole-3-acetic acid (5-HIAA), the sample homogenates were filtered through a Supelco HybrideSPE plate and separated on a Restek Ultra II PFP Propyl column (1.9 mm, 50 x 2.1 mm) using mobile phases of 0.1% formic acid in water and in acetonitrile with a 4-minute gradient elution. In the negative ion MRM mode for 3,4-dihydoxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the homogenates were filtered through a Whatman filter plate and separated on a Waters HSS T3 column (1.7 mm 50 x 2.1 mm) using mobile phases of 0.1% acetic acid in water and in acetonitrile in a 4-minute run time. Stable isotope labeled internal standards were used for the 6 endogenous analytes to minimize the matrix effects on their quantitation. The calibration standards were prepared in 0.1% formic acid aqueous solution with ranges from 6.00 to 1200 ng/mL for DA and 5-HT, and from 12.0 to 2400 ng/mL for NE, 5-HIAA, DOPAC, and HVA. The method has been validated using quality control samples prepared in a surrogate matrix, charcoal treated (2X) animal brain homogenates. Finally the validated method has been routinely used to analyze study samples from various animal models.

Introduction

Research in neuroscience and neurodegenerative diseases has been translational in nature, i.e., laboratory animals have been used for investigating biological processes, studying the causes of diseases, and testing new therapeutic means of treating and preventing them. The capability of producing transgenic, knockout, mutation cell lines of animal species has given researches many models linked to human diseases. As small-molecule biomarkers are commonly monitored in neurochemistry studies, reliable bioanalytical methods with high sample throughput are needed for the quantitation of the endogenous biomarkers in biological matrices. We describe our continuing efforts of developing and validating a UPLC-MS/MS method for determining dopamine, serotonin, and their metabolites in striatum tissues from animal (i.e., rat and mouse) models of Parkinson's disease[1].

Neurotransmitters have been analyzed by using liquid chromatography coupled with electrochemical detection (LC-ECD)[2,3], fluorescence detection (LC-FL)[4] or lately electrospray tandem ionization mass spectrometric (ESI-MS/MS) detection[5-6]. The lack of method specificity and sensitivity of LC-ED and FC-FL methods resulted in differences in concentrations of various neurotransmitters reported in literature. The analysis time per sample injection is typically long (20–30 minutes) using the nonspecific LC-ED and LC-FL methods. We describe here a simple, specific, and sensitive UPLC-MS/MS method to quantify dopamine, serotonin and their acidic and basic metabolites in small striatum tissues.

Experimental Section

Calibration standards and quality control samples: Stock solutions of neurotransmitters in Figure 2 were prepared in ice-cold 0.1% formic acid at a concentration of 1.00 mg/mL, and the stocks were subaliquoted into tubes (0.50 mL each) and stored at -80°C. Calibration standards were prepared from the stock solutions with ice-cold 0.1% formic acid at various concentrations on the day of the analysis. Quality control samples were prepared in charcoal treated (2X) rat and mouse brain homogenates spiked with neurotransmitters at four concentration levels (LLQC, LQC, MQC, HQC). Quality control samples were subaliquoted into tubes and stored at -80°C.

Preparation of study samples: Striatum tissues were surgically removed from euthanized animals. After weighing, tissue samples were immediately flash frozen with liquid nitrogen and stored frozen at approximately -80°C until sample processing. During analysis, tissue samples were homogenized on wet ice with ice-cold 0.1% formic acid using a Kontes microgrinder at a ratio of 1:9 [mg (weight): mL]. For the analysis of rat striatum samples, aliquots of 50.0 mL of homogenates were added to a 96-well sample plate (2-mL well size), followed by 50 mL of 0.1% formic acid solution containing internal standards. The samples were further diluted with 350 mL of ice cold 0.1% formic acid (total 450 mL). Aliquots (200 mL) of the diluted samples were transferred and filtered through a Supelco HybridSPE® plate for the positive ion analysis (NE, DA, 5-HT, and 5-HIAA). Second portions (200 mL) of the diluted samples were filtered through a Whatman filter plate for the negative ion analysis (DOPAC and HVA). Because of limited sample size for mouse striatum tissues (6–28 mg), Whatman filter plates were used to prepare the diluted homogenates for both positive ion and negative ion modes of analysis.

Dopamine (a catecholamine), serotonin and their metabolites are known to be unstable in the presence of light, oxygen, metal ions, and low or high pH, as well as sensitive to temperature. Samples should be processed on ice quickly in 0.1% formic acid. Proper collection, processing, and storage of tissue samples are important for generating accurate results.

Triple-stage quadrupole mass spectrometer (API-4000) coupled with Waters Acquity UPLC® systems were used for UPLC-MS/MS analysis.

UPLC conditions (the positive ion mode):

LC System:	Waters Acquity UPLC® System
Column:	Restek Ultra™ PFP-Propyl 50 x 2.1 mm, 1.9 µm
Column Temperature:	60°C
Autosampler Temperature:	4°C
Mobile Phase A:	0.1% Formic acid in water
Mobile Phase B:	0.1% Formic acid on acetonitrile
Strong Wash:	0.1% Formic acid in acetonitrile
Weak Wash:	0.1% Formic acid in water
Seal Wash:	10% Acetonitrile

UPLC gradient program

Time (min)	A%	B %	Flow rate (mL/min)
Initial	100	0	0.40
0.50	100	0	0.40
2.60	70	30	0.40
2.70	10	90	1.00
3.30	10	90	1.00
3.31	100	0	1.00
4.00	100	0	1.00

Mass spectrometric detection parameters

Compound Name	Precursor Ion m/z	Product Ion m/z	Ionization Mode
NE	152	107	Positive
DA	154	91	Positive
5-HT	177	115	Positive
5-HIAA	192	146	Positive
NE-d6	158	111	Positive
DA-d4	158	95	Positive
5-HT-d4	181	118	Positive
5-HIAA-d5	197	150	Positive

UPLC conditions (the negative ion mode):

LC System:	Waters Acquity UPLC® System
Column:	Waters HSS T3, 50 x 2.1 mm, 1.7 µm
Column Temperature:	60°C
Autosampler Temperature:	4°C
Mobile Phase A:	0.1% Acetic acid in water
Mobile Phase B:	0.1% Acetic acid on acetonitrile
Strong Wash:	0.1% Acetic acid in acetonitrile
Weak Wash:	0.1% Acetic acid in water
Seal Wash:	10% Acetonitrile

UPLC gradient program

Time (min)	A%	B %	Flow rate (mL/min)
Initial	100	0	0.40
2.50	75	25	0.40
2.60	5	95	1.00
3.20	5	95	1.00
3.21	5	95	1.00
3.30	100	0	1.00
4.00	100	0	1.00

Mass spectrometric detection parameters

Compound Name	Precursor Ion m/z	Product Ion m/z	Ionization Mode
DOPAC	167	123	Negative
HVA	181	122	Negative
DOPAC-d5	172	128	Negative
HVA-d5	186	127	Negative

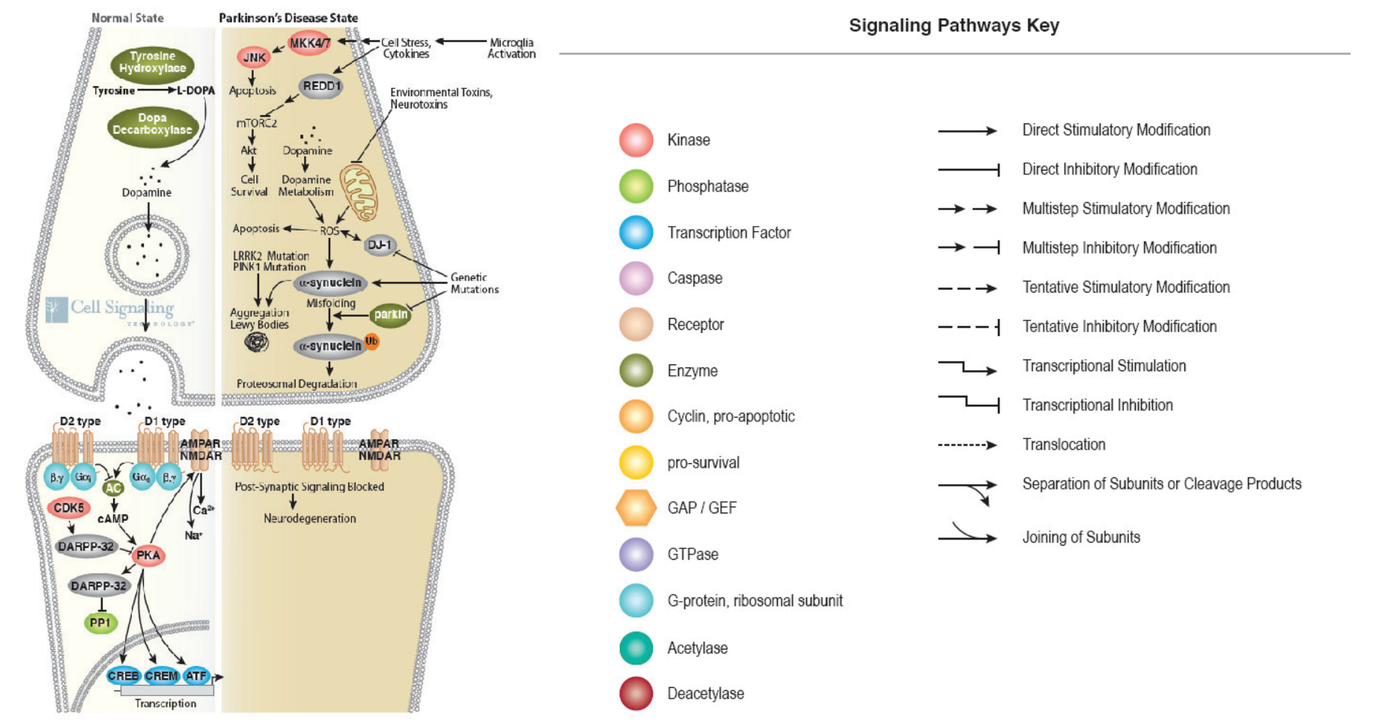

Figure 1. Dopamine signaling in Parkinson's disease (Source: Cell Signaling Technology, www.cellsignal.com).

Table 1. Animal Models of Parkinson's Disease Evaluated at Three Ages (4, 8, 12 months).

Group Number	MJFF-Funded Animal Models Transgenic=Tg; Knochout=KO; Wildtype=WT; Knockin=KI	Species	Vendor	Numer of Animals*
1	C57BL/6J Control	Mouse	Jackson laboratory	15
2	FVB Control	Mouse	Jackson laboratory	15
3	WT LRRK2 on C57BL/6J backgroud	Mouse	Jackson laboratory	15
4	WT LRRK2 on FVB backgroud	Mouse	Jackson laboratory	15
5	G2019S LRRK2 on C57BL/6J background	Mouse	Jackson laboratory	15
6	G2019S LRRK2 on FVB background	Mouse	Jackson laboratory	15
7	R1441G LRRK2 on C57BL/6J backgound	Mouse	Jackson laboratory	15
8	R1441G LRRK2 on FVB backgound	Mouse	Jackson laboratory	15
1	Long Evans WT	Rat	SAGE Labs	15
2	Parkin KO	Rat	SAGE Labs	15
3	Pink1 KO	Rat	SAGE Labs	15
4	LRRK2 KO	Rat	SAGE Labs	15
5	DJ-1 KO	Rat	SAGE Labs	15
6	a-SCNA KO	Rat	SAGE Labs	15

*A subset of six snimal of these models were undergo neurochemistry characterization: Striatal concentations of dopamine, DOPAC, HVA, 5-HIAA, serotonin, and norepinephrine

Table 1. Animal models of Parkinson's disease Evaluated at three ages (4, 8, and 12 months).

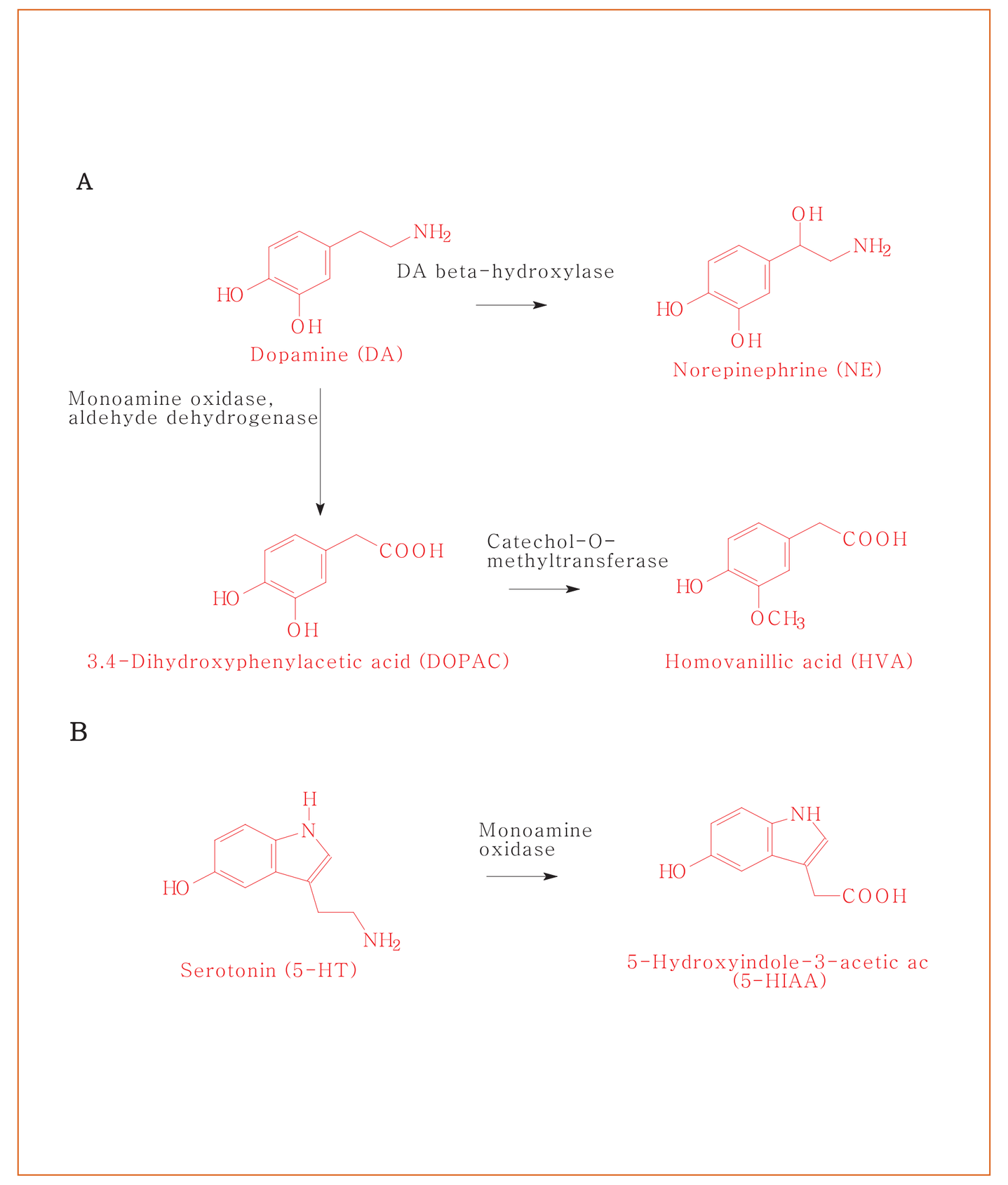

Figure 2. Chemical structures and metabolic pathways of (A) dopamine, (B) serotonin, and their acidic and basic metabolites.

Table 2. Calibration Standard Statistics (Mouse Striatum Assay)

DA (ng/mL)	6.00	12.0	60.0	120	300	600	1080	1200
Mean	6.02	11.9	59.8	119	305	604	1060	1210
%RSD	1.00	4.20	4.00	4.70	2.70	2.40	2.60	2.90
%RE	0.33	-0.83	-0.33	-0.83	1.67	0.67	-1.85	0.83
n	10	10	10	10	10	10	10	10
DOPAC (mg/mL)	12.0	24.0	120	240	600	1200	2160	2400
Mean	12.0	24.0	119	237	611	1210	2150	2400
%RSD	1.8	5.3	4.0	4.7	3.8	3.9	2.5	2.4
%RE	0.00	0.00	-0.83	-1.25	1.83	0.83	-0.46	0.00
n	9	9	9	9	9	9	9	9
HVA (ng/mL)	12.00	24.00	120.00	240.00	600.00	1200.00	2160.00	2400.00
Mean	12.0	24.1	120	239	603	1210	2120	2440
%RSD	2.0	6.0	1.9	4.2	3.4	3.0	2.5	3.0
%RE	0.00	0.42	0.00	-0.42	0.50	0.83	-1.85	1.67
n	9	9	9	9	9	9	9	9
5-HT (ng/mL)	6.00	12.00	60.00	120.00	300.00	600.00	1080.00	1200.00
Mean	5.99	12.2	58.7	121	304	603	1070	1200
%RSD	2.2	4.9	2.7	3.3	3.8	1.9	4.2	3.8
%RE	-0.17	1.67	-2.17	0.83	1.33	0.50	-0.93	0.00
n	11	11	11	10	11	11	11	11
5-HIAA (ng/mL)	12.00	24.00	120.00	240.00	600.00	1200.00	2160.00	2400.00
Mean	12.1	23.8	119	238	610	1210	2150	2400
%RSD	1.8	4.0	3.2	5.8	3.6	3.4	2.6	3.2
%RE	0.83	-0.83	-0.83	-0.83	1.67	0.83	-0.46	0.00
n	9	9	9	9	9	9	9	9
NE (ng/mL)	12.0	24.0	120	240	600	1200	2160	2400
Mean	12.1	23.9	121	236	605	1210	2160	2400
%RSD	1.4	3.7	3.4	3.8	1.8	2.1	2.8	3.0
%RE	0.83	-0.42	0.83	-1.67	0.83	0.83	0.00	0.00
n	9	9	9	9	9	9	9	9

Table 3. Precision and Accuracy of Quality Controle Samples (Mouse Striatum Assay)

	DA (ng/mL)				5-HT (ng/mL)			
	6.00	18.0	600	960	6.00	18.0	600	960
Mean	5.59	18.1	574	911	6.27	19.2	572	907
%RSD	7.5	11.2	6.6	6.5	8.5	10.1	6.1	6.2
%RE	-6.8	0.6	-4.3	-5.1	4.5	6.7	-4.7	-5.5
n	6	10	10	10	6	11	11	11
	5-HIAA (ng/mL)				NE (ng/mL)			
	12.0	36.0	1200	1920	12.0	36.0	1200	1920
Mean	13.3	37.9	1190	1830	10.7	33.8	1170	1880
%RSD	6.4	8.5	4.5	5.4	9.4	24.7	7.3	6.1
%RE	10.8	5.3	-0.8	-4.7	-10.8	-6.1	-2.5	-2.1
n	4	9	9	9	4	9	9	9
	DOPAC (ng/mL)				HVA (ng/mL)			
	12.0	36.0	1200	1920	12.0	36.0	1200	1920
Mean	12.6	38.3	1250	1960	11	36.2	1200	1880
%RSE	7.8	11	5.2	5.3	12.9	8.5	6.5	6.8
%RE	5.0	6.4	4.2	2.1	-8.3	0.6	0.0	-2.1
n	5	9	9	9	5	9	9	9

Tables 2–3. Validation results of mouse homogenate assay.

Table 4. Calibration Standard Statistics (Rat Striatum Assay)

DA (ng/mL)	6.00	12.0	60.0	120	300	600	1080	1200
Mean	5.90	12.5	58.7	120	301	598	1090	1190
%RSD	1.60	3.30	2.50	2.30	1.70	3.40	1.20	1.40
%RE	-1.67	4.17	-2.17	0.00	0.33	-0.33	0.93	-0.83
n	10	10	10	10	10	10	10	10
DOPAC (mg/mL)	12.0	24.0	120	240	600	1200	2160	2400
Mean	11.8	24.9	117	239	599	1220	2130	2420
%RSD	3.1	5.7	5.8	4.2	2.9	5.2	2.8	2.4
%RE	-1.67	3.75	-2.50	-0.42	-0.17	1.67	-1.39	0.83
n	9	9	9	9	9	9	9	9
HVA (ng/mL)	12.00	24.00	120.00	240.00	600.00	1200.00	2160.00	2400.00
Mean	11.9	24.7	118	238	602	1220	2150	2400
%RSD	1.9	4.4	5.1	3.9	6.6	2.0	2.5	2.9
%RE	-0.83	2.92	-1.67	-0.83	0.33	1.67	-0.46	0.00
n	11	11	11	11	11	11	11	11
5-HT (ng/mL)	6.00	12.00	60.00	120.00	300.00	600.00	1080.00	1200.00
Mean	6.03	12.0	59.0	120	303	608	1090	1190
%RSD	2.8	6.1	3.5	2.8	3.6	2.2	1.5	1.5
%RE	0.50	0.00	-1.67	0.00	1.00	1.33	0.93	-0.83
n	11	11	11	10	11	11	11	11
5-HIAA (ng/mL)	12.00	24.00	120.00	240.00	600.00	1200.00	2160.00	2400.00
Mean	12.0	24.3	118	241	601	1210	2140	2410
%RSD	1.5	3.4	2.6	2.4	2.4	3.3	2.9	2.7
%RE	0.00	1.25	-1.67	0.42	0.17	0.83	-0.93	0.42
n	11	11	11	11	11	11	11	11
NE (ng/mL)	12.0	24.0	120	240	600	1200	2160	2400
Mean	11.9	24.7	119	238	602	1210	2170	2380
%RSD	1.9	5.1	4.0	3.9	3.2	3.4	1.7	1.9
%RE	-0.83	2.92	-0.83	-0.83	0.33	0.83	0.46	-0.83
n	10	10	10	10	10	10	10	10

Table 5. Precision and Accuracy of Quality Controle Samples (Rat Striatum Assay)

	DA (ng/mL)				5-HT (ng/mL)			
	6.00	18.0	600	960	6.00	18.0	600	960
Mean	5.67	17.4	583	937	6.33	17.6	588	942
%RSD	3.4	5.0	3.3	3.1	5.5	4.9	4.4	4.8
%RE	-5.5	-3.3	-2.8	-2.4	5.5	-2.2	-2.0	-1.9
n	4	10	10	10	5	11	11	11
	5-HIAA (ng/mL)				NE (ng/mL)			
	12.0	36.0	1200	1920	12.0	36.0	1200	1920
Mean	12.9	36.3	1180	1870	11.7	35.4	1200	1920
%RSD	9.0	4.5	4.2	3.5	4.5	4.8	3.1	4.2
%RE	7.5	0.8	-1.7	-2.6	-2.5	-1.7	0.0	0.0
n	5	11	11	11	4	10	10	10
	DOPAC (ng/mL)				HVA (ng/mL)			
	12.0	36.0	1200	1920	12.0	36.0	1200	1920
Mean	12.3	38.9	1240	2000	11.6	36.1	1170	1860
%RSE	13.6	11	5.3	4.1	10.9	6.5	5.4	5.2
%RE	2.5	8.1	3.3	4.2	-3.3	0.3	-2.5	-3.1
n	5	9	9	9	6	11	11	11

Tables 4–5. Validation results of rat homogenate assay

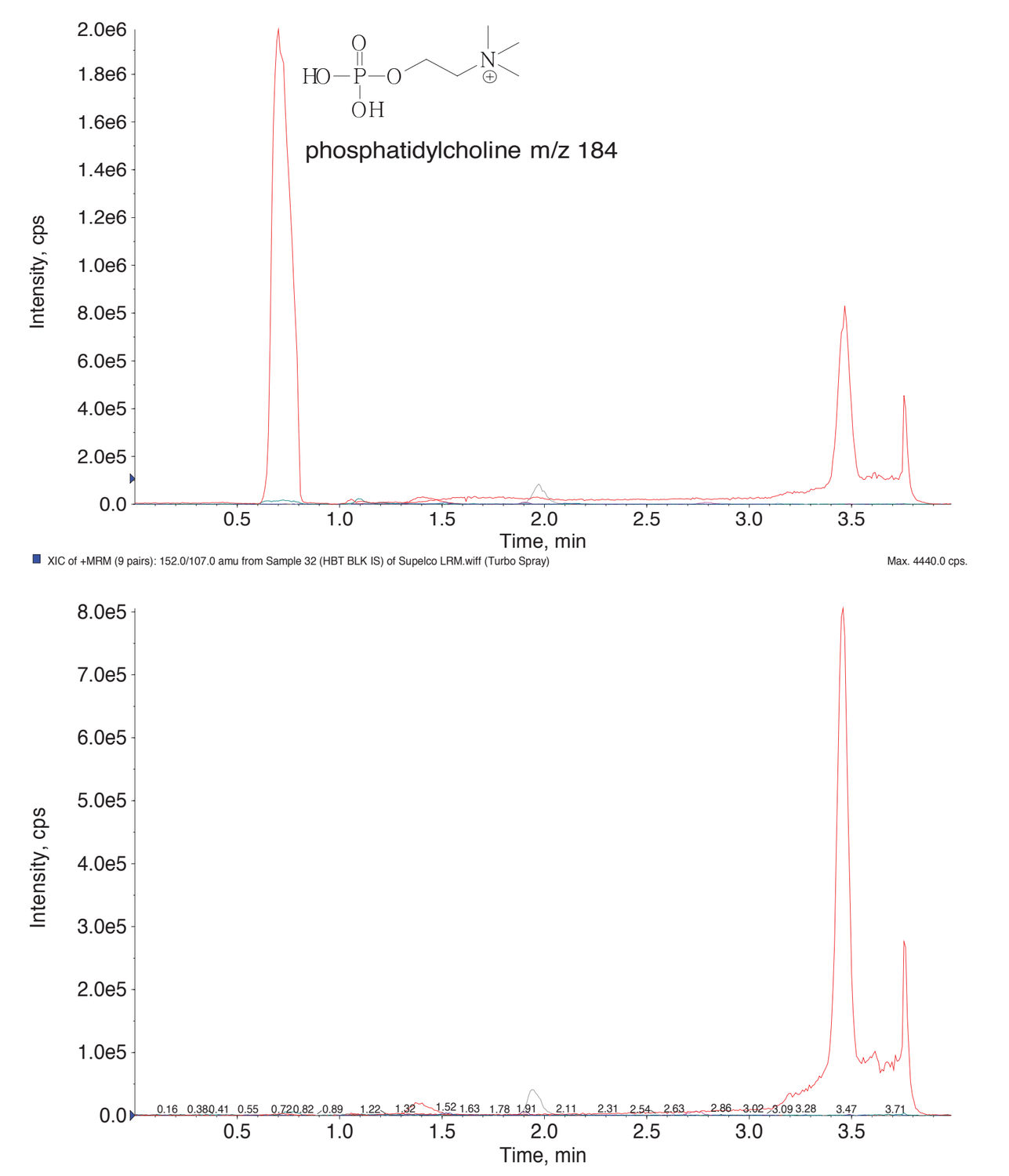

Figure 3. Representative UPLC-MS/MS chromatograms of mouse striatum homogenates. (Top) the homogenate sample without phospholipids removal, (Bottom) the sample extract filtered with a HybridSPE phospholipids plate.

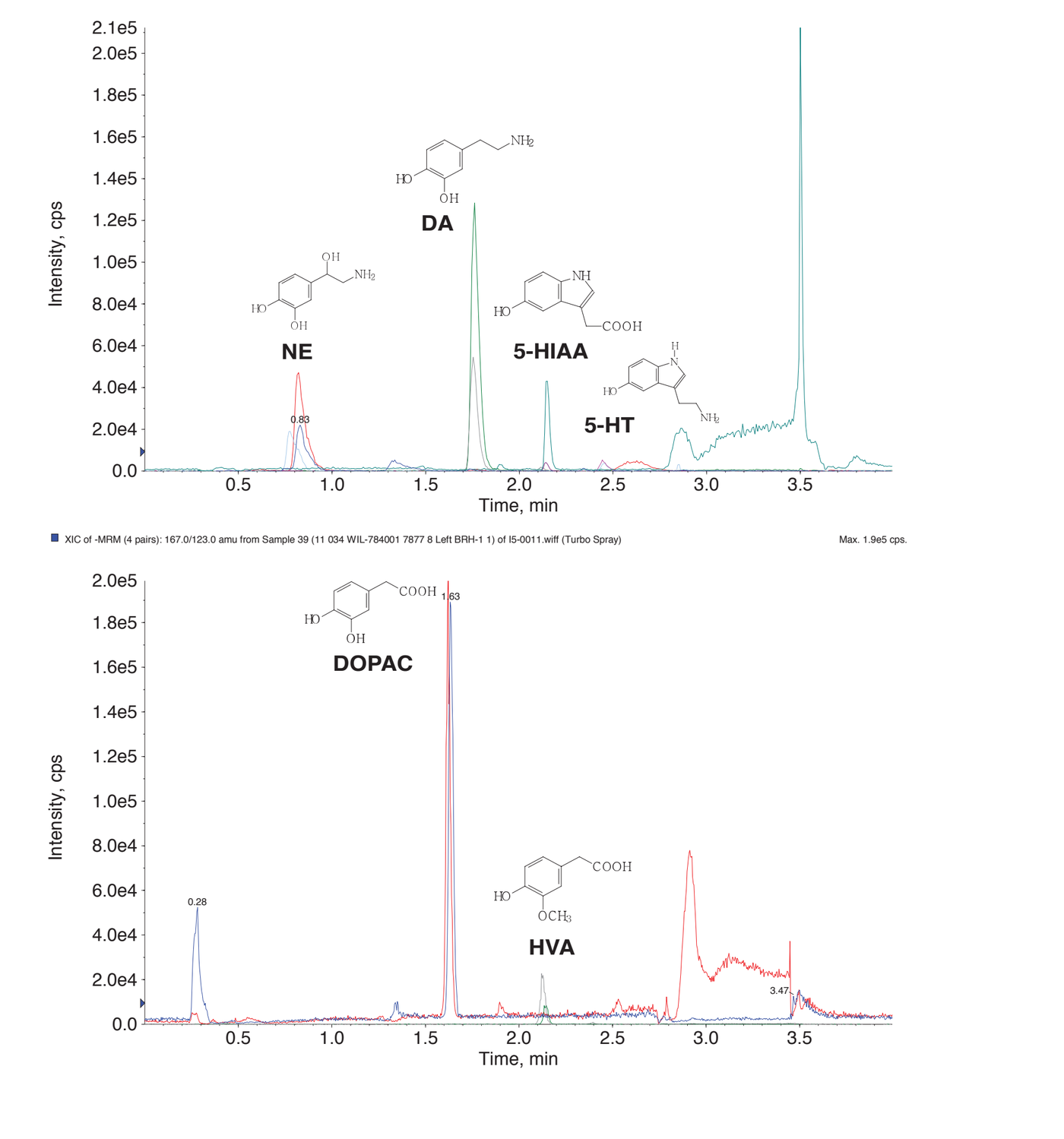

Figure 4. UPLC-MS/MS chromatograms of a mouse striatum homogenate (Top) in positive ion MRM mode and (Bottom) in negative ion MRM mode. Stable isotope-labeled internal standards were added to the sample.

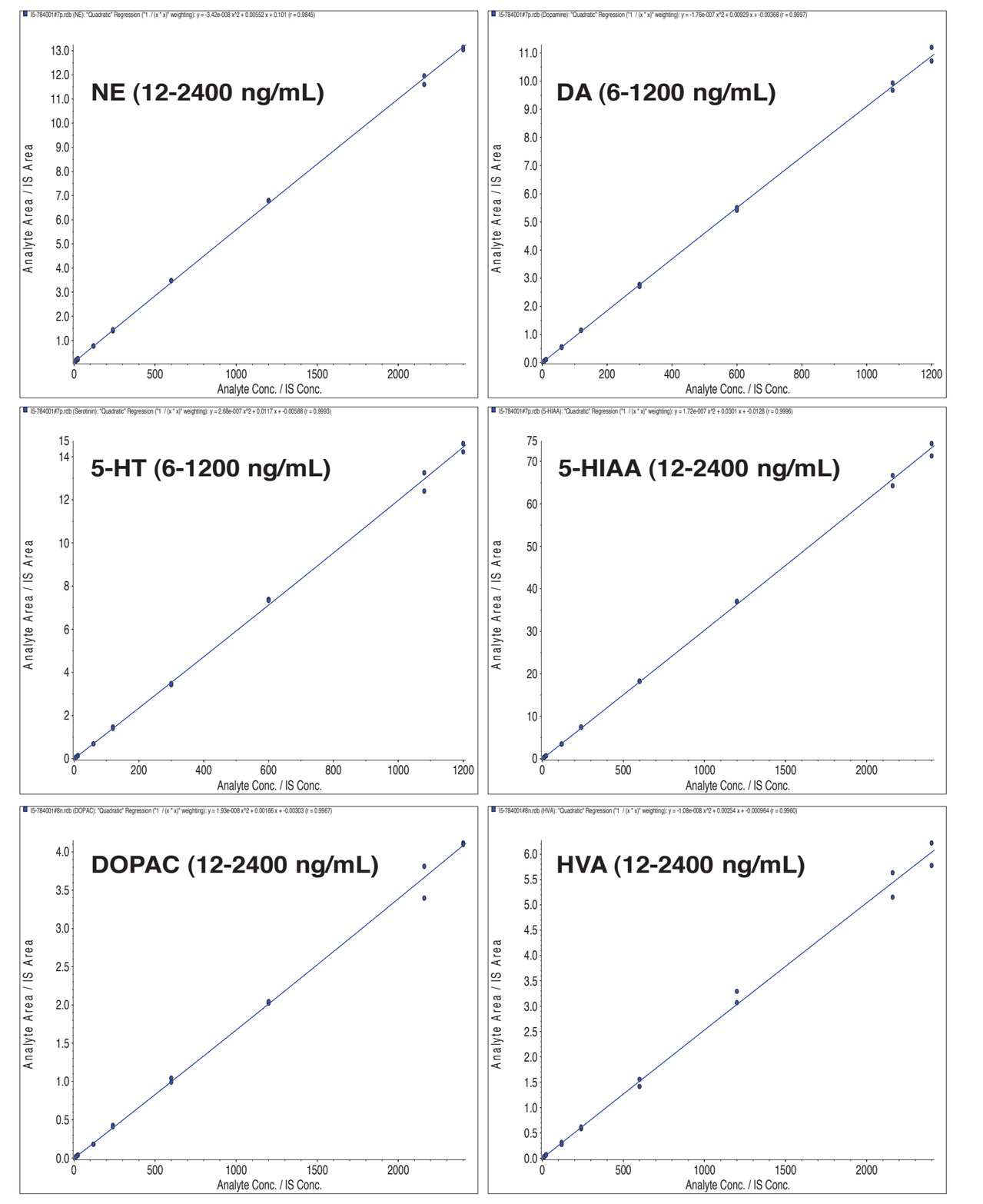

Figure 5. Calibration standard curves of NE (12-2400 ng/mL), DA (5-1200 ng/mL), 5-HIAA (12-2400 ng/mL), 5-HT (6-1200 ng/mL), DOPAC (12-2400 ng/mL), and HVA (24-2400 ng/mL). The curves were prepared in aqueous 0.1% formic acid solution.

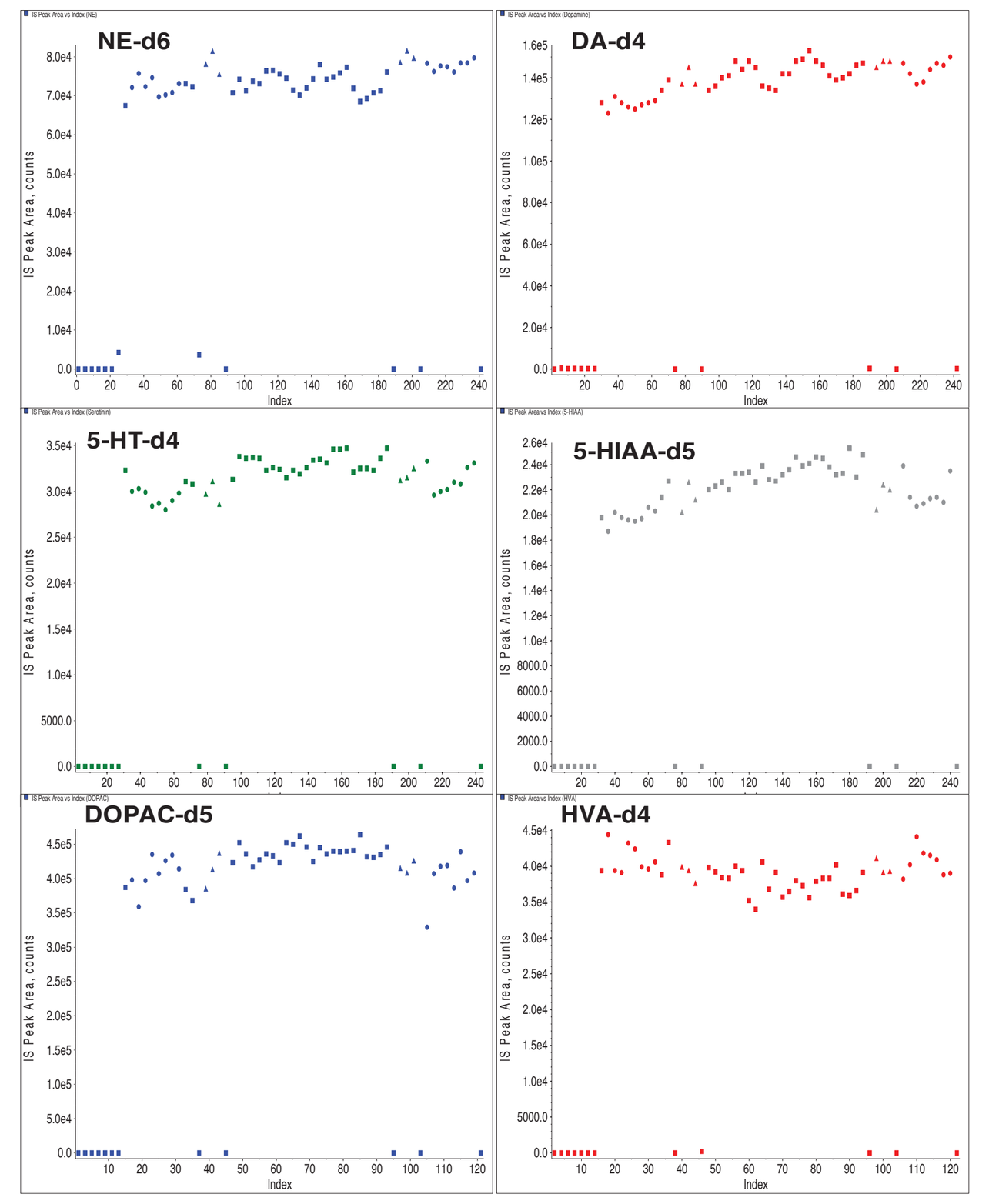

Figure 6. Batch internal standard responses of stable isotope labeled compounds: NE-d6, DA-d4, 5-HT-d4, 5-HIAA-d5, DOPAC-d5, and HVA-d4. (●) calibrators, (▲) quality control samples, and (■) study samples.

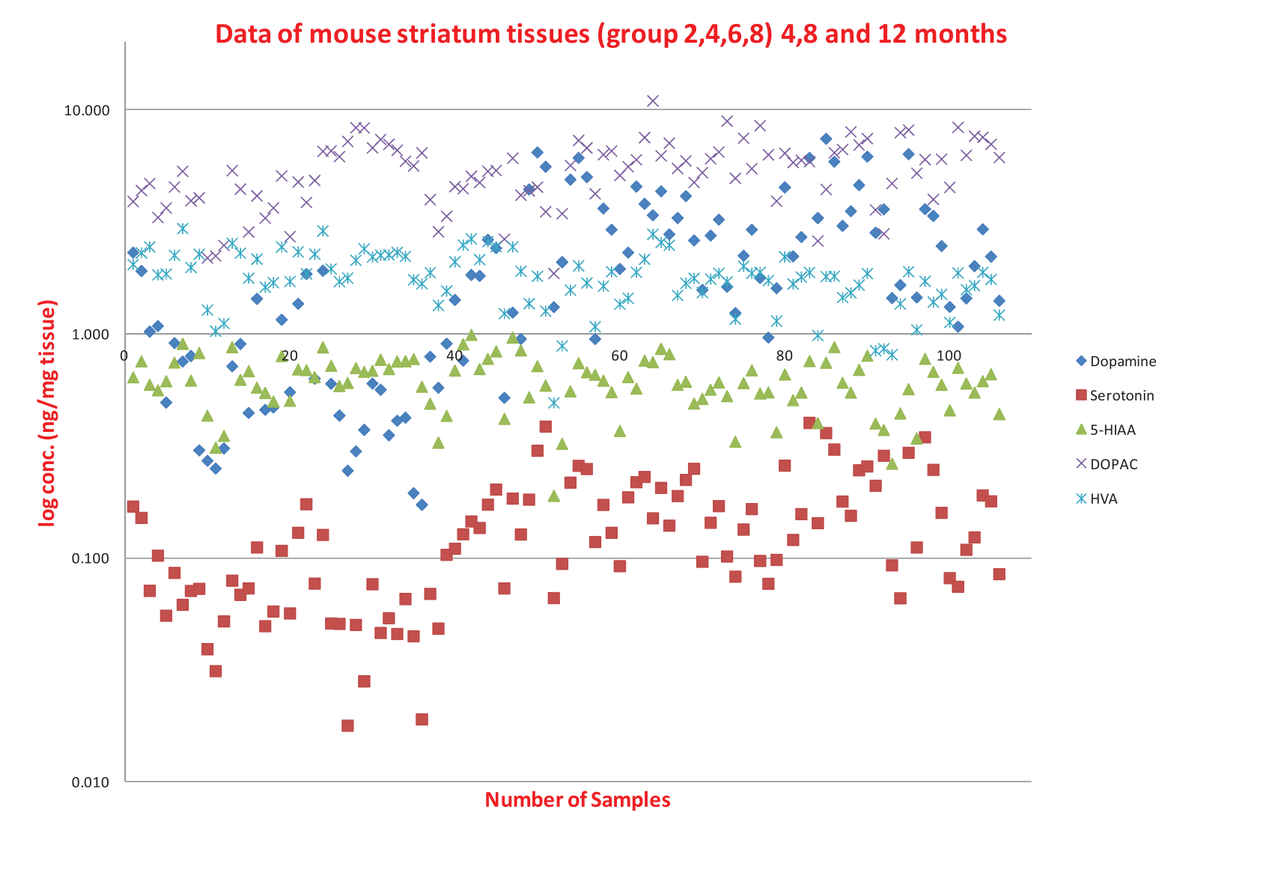

Figure 7. Concentrations (ng/mg tissue) of neurotransmitters in mouse striatum tissues collected from animals of Parkinson's disease models at various ages.

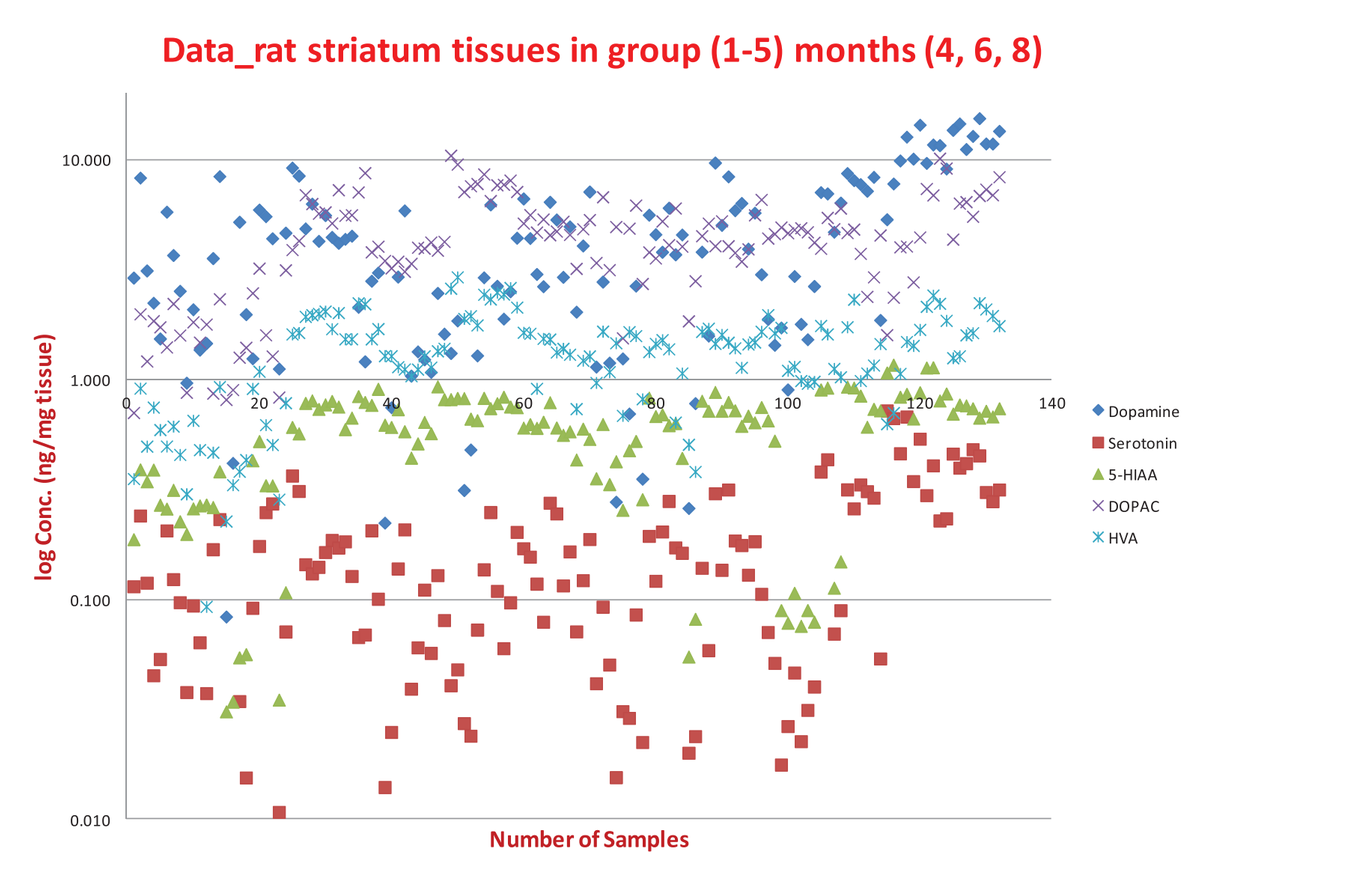

Figure 8. Concentrations (ng/mg tissue) of neurotransmitters in mouse brain tissues collected from study animals.

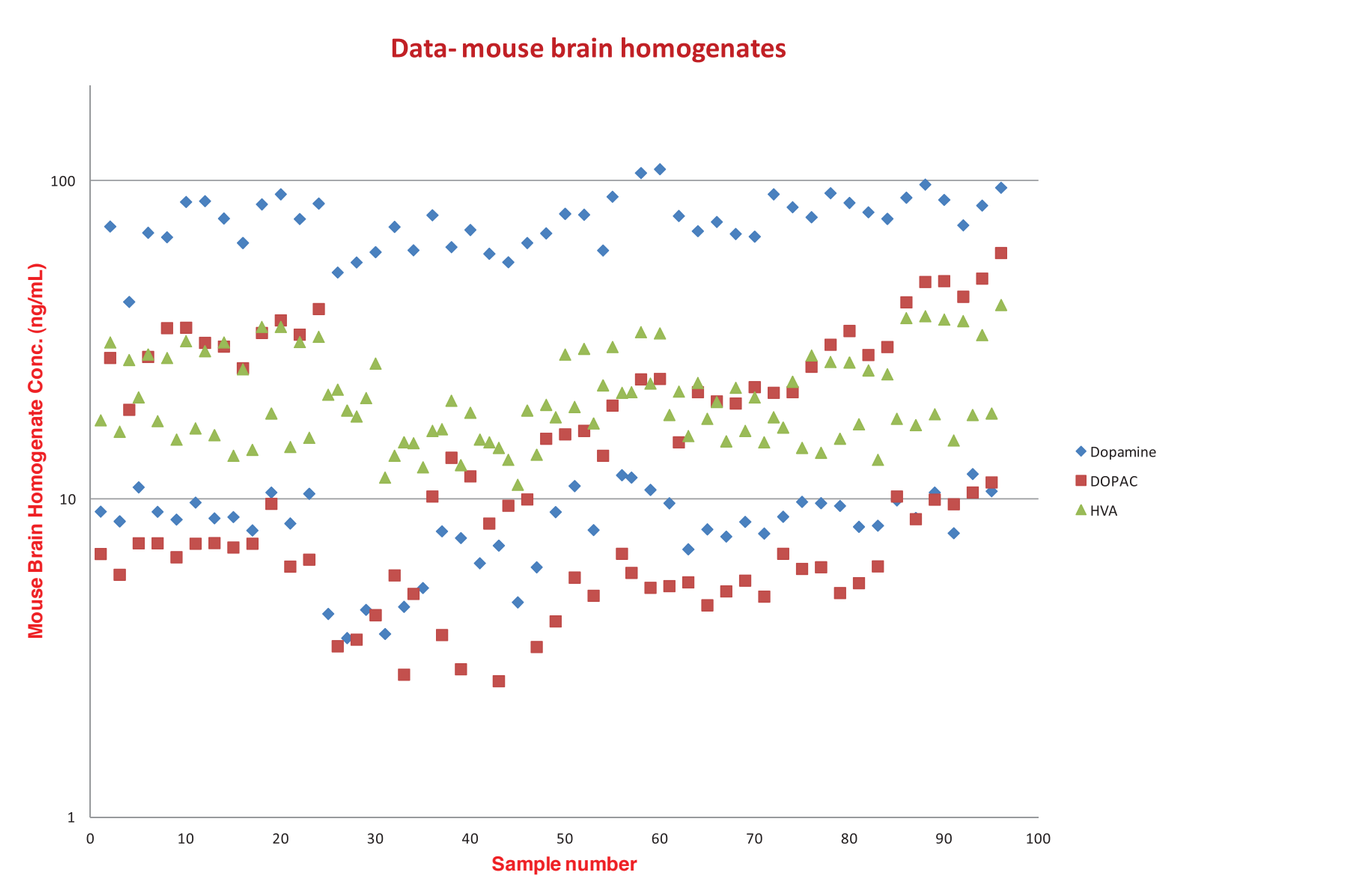

Figure 9. Concentrations (ng/mg tissue) of neurotransmitters in mouse brain tissues collected from study animals.

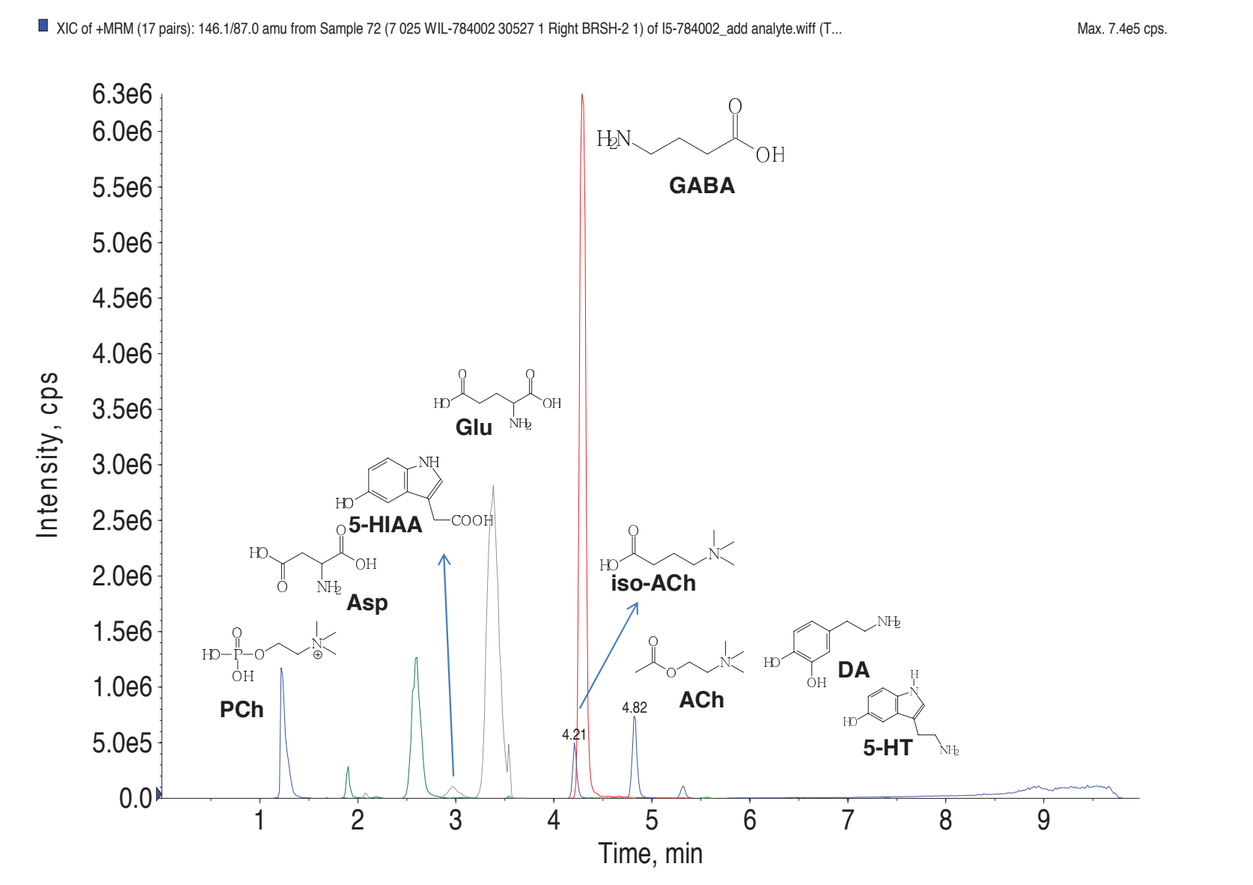

Figure 10. UPLC-MS/MS chromatogram of mouse striatum homogenate. Chromatographic column: Scherzo SS-C18 (150x3 mm, 3µm). Mobile phases: A: 0.5% formic acid, B: 0.5% formic acid and 50 mM ammonium formate in acetonitrile/water (50/50, v/v).

Results and Discussion

Bioanalytical method validation

High performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS) has an extensive history as a quantitative tool for small molecules in biological matrices. The quantitative determination of endogenous (i.e., naturally occurring) compounds in biological samples of limited size (rat or mouse striatum tissues) is more complicated, both analytically and from method validation point of view. We have previously shown that UPLC-MS/MS provides sensitive and selective measurements of dopamine, serotonin and metabolites in the animal striatum tissues homogenates[1]. To demonstrate the validity of a bioanalytical method, we have shown that charcoal treated (2X) animal (rat and mouse) brain homogenates were suitable control matrices for the study samples (striatum tissue homogentates)[1]. Dopamine, serotonin and their metabolites are small polar molecules and the use of more polar pentaflurophenylpropyl (PFP) stationary phase provided adequate retentions without using ion-pairing reagents. While monitoring phospholipids using an MRM transition m/z 184/184, we observed a phosphatidylcholine peak at an extremely high abundance in tissue homogenates samples, but not in calibration standards. Figure 3 shows that phosphatidylcholine derivatives can be completely eliminated using a Supelco HybrideSPE filter plate, which improves the PFP column performance and method robustness significantly. Additionally, Whatman filter plates were used for processing samples for UPLC-MS/MS analysis of DOPAC and HVA in negative ion MRM mode because of low recoveries and instability of DOPAC when using Supelco lipids plates. Meanwhile, for the analysis of acidic DOPAC and HVA, the best UPLC-MS/MS conditions were obtained on a Waters HSS T3 column (2.1x50 mm) using 0.1 % acetic acid in water and acetonitrile mobile phases. Note that Whatman filter plates which partially remove phosphatidylcholine derivatives were used for processing all mouse tissue homogenates due to limited sample volume.

With the suitable control matrices, the above UPLC-MS/MS method for endogenous neurotransmitters was validated following FDA Guidance on Bioanalytical Method Validation. Validation results are summarized in Table 2 through Table 5 for the 6 neurotransmitters in mouse and rat brain homogenates, respectively. Method precision and accuracy were acceptable based on the WIL Research SOP established criteria. Other validation parameters including matrix effects, recovery, and matrix and stock stabilities were also assessed during our method validation efforts.

Method application to study sample analysis

The LC-MS/MS method described above was used for the quantitative analysis of 6 neurotransmitters in animal striatum tissue samples collected from a large number of animal models of Parkinson's disease at various ages. Figure 4 shows representative MRM chromatograms of mouse striatum homogenate with endogenous levels of neurotransmitters. Figure 5 presents typical calibration standard curves in duplicates of the six neurotransmitters prepared in 0.1% formic acid. Figure 6 shows the internal standard (IS) responses of stable isotope labeled neurotransmitters. The IS responses were relatively consistent throughout analysis batch runs for calibrators (in 0.1% formic acid), QCs (in surrogate matrix), and study samples, indicating that matrix effects were insignificant and that reliable concentrations could be obtained with the method.

UPLC-MS/MS analysis results for all study samples collected so far in the on-going studies are shown in Figure 7 (mouse striatum tissues) and Figure 8 (rat striatum tissues). Overall, the endogenous concentration levels in study samples are within the calibration ranges of the assay for 5 neurotransmitters (DA, 5-HT, 5-HIAA, DOPAC, and HVA). The concentrations of NE were detectable, but below the lower limit of quantitation (LLOQ) in most of the study samples. As dopamine metabolism is studied in a separate mouse model study, Figure 9 shows the analysis data of mouse brain homogenates from a mouse model study. In this separate study, the homogenates of mouse whole brain tissues were analyzed for DA and its metabolites (DOPAC and HVA). Unfortunately, large variations of neurotransmitter concentrations were observed among study samples even though the described assay demonstrated good precision and accuracy. The temporal and heterogenic nature of neurotransmitters in the animal brain locations and their chemical instability may play significant roles in assay variations. Therefore, it is very important to improve the precision and accuracy both in sample collection and sample processing steps when dealing with a large number of animal study samples.

Conclusions and future work

A simple, sensitive, specific, and robust UPLC-MS/MS method was validated with good linearity, precision and accuracy for the determination of endogenous dopamine, serotonin and their acidic and basic metabolites in animal striatum tissues. The method has been used successfully to assay samples from animal models of Parkinson's disease and from a separate animal model study. Recently, we have extended the work to include other classes of neurotransmitters such as amino acids, acetylcholine, g-aminobutyric acid (GABA), and monoamines (Figure 10) in animal brain tissues and in microdialysis fluids using UPLC-MS/MS with new generations of mixed-mode chromatographic stationary phases.

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