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Application News

No. SSK_004

LCMS-8050

Automated Derivatization of Neurotransmitters in Plasma Extracts for Liquid Chromatograph Tandem Mass Spectrometry

❑ Introduction

Neurotransmitters are endogenous chemicals that transmit a message from a nerve cell to a target cell. In vivo neurochemical monitoring is important in neuroscience because it allows correlation of neurotransmission with behavior, disease state and drug concentrations in the intact brain. Analytical results of neurotransmitters and their metabolites in a sample such as blood, urine, and cerebrospinal fluid (CSF) can provide information of index compounds related to diseases and biochemical information for diagnosis [1]. However, the high polarity of neurotransmitter compounds often makes chromatographic separations difficult.

In this Application News, auto-precolumn derivatization by benzoyl chloride was employed to overcome difficulties of chromatographic separation given by high polarity of neurotransmitters. In this pretreatment process, neurotransmitter compounds were converted into hydrophobic forms to be suitable for reverse-phase LC-MS/MS.

Figure 1. Shimadzu LCMS-8050

❑ Experimental

A. Analytical system and conditions

Table 1 and 2 show the analytical conditions for the determination of neurotransmitters in plasma extracts using Shimadzu LCMS-8050 shown in Figure 1.

Table 1. Analytical Conditions for the Determination of Neurotransmitters in Plasma Extracts.

LC system	Shimadzu Nexera X2 UHPLC
Flow	0.3 mL/min
Mobile phase A	5 mM ammonium formate and 0.1 % formic acid in water
Mobile phase B	Acetonitrile
Gradient	20 % B (0.5 min) – 70 % B (6.5 – 7.5 min) - 20 % B (7.6 – 10.0 min)
Column	Shim-pack GIST C18 (50 x 2.1 mm i.d., 2 µm)
Column oven	30 ℃
Injection volume	5 µL
MS system	Shimadzu LCMS-8050
Ionization method	ESI, positive
Nebulizing gas flow	3 L/min
Heating gas flow	10 L/min
Desolvation line temp.	250 ℃
Interface temp.	300 ℃
Heat block temp.	400 ℃
Drying gas flow	10 L/min

Table 2. MRM Parameters for Neurotransmitters and Isotope Labelled Internal Standards

Compound	Class	Target ion, *m/z*	Reference ion, *m/z*
3-HK	Target	433.1 > 105.2	433.1 > 162.2
3-MT	Target	376.1 > 105.2	376.1 > 77.1
5-HIAA	Target	313.1 > 105.2	313.1 > 77.2
5-HT	Target	385.0 > 105.2	385.0 > 264.2
DA	Target	466.1 > 105.1	466.1 > 77.2
DOPAC	Target	394.1 > 105.1	394.1 > 77.2
DOPAL	Target	378.1 > 105.2	378.1 > 77.2
E	Target	496.1 > 105.2	496.1 > 478.2
HVA	Target	304.1 > 105.2	304.1 > 137.2
KYN	Target	417.1 > 146.2	417.1 > 122.2
KYNA	Target	294.1 > 105.2	-
L-DOPA	Target	510.1 > 105.1	510.1 > 360.3
NE	Target	482.1 > 105.1	482.1 > 77.2
Salsolinol	Target	388.1 > 105.2	388.1 > 77.2
Trp	Target	309.1 > 105.2	309.1 > 263.2
Tyr	Target	390.1 > 105.1	390.1 > 77.2
GABA	Target	208.0 > 105.0	208.0 > 77.0
Glutamate	Target	252.2 > 105.1	252.2 > 76.9
Glycine	Target	180.2 > 105.0	180.2 > 76.9
3-MT-d4	IS	380.0 > 105.2	380.0 > 77.2
5-HIAA-d5	IS	318.0 > 105.2	318.0 > 77.2
5-HT-d4	IS	389.1 > 105.2	389.1 > 268.3
DA-d4	IS	469.9 > 105.1	-
DOPAC-d5	IS	399.1 > 105.2	399.1 > 77.2
E-d6	IS	502.0 > 105.2	502.0 > 484.2
HVA-d5	IS	309.0 > 105.2	309.0 > 292.3
L-DOPA-d3	IS	513.1 > 105.2	513.1 > 363.2
NE-d6	IS	488.2 > 105.2	488.2 > 470.2
Trp-d8	IS	317.0 > 105.2	317.0 > 269.2
Tyr-d7	IS	397.0 > 105.1	397.0 > 247.2

B. Reaction scheme of benzoylation

Benzoylation of primary or secondary amines or phenols under the alkaline condition using benzoyl chloride is a very attractive and handy known as Schotten-Baumann reaction [2]. Reaction scheme of benzoylation is shown in Figure 2.

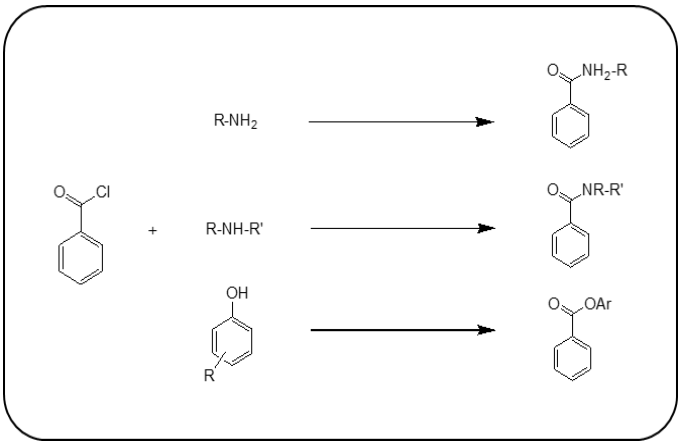


Figure 2. Reaction scheme of benzoylation

C. Preparation of Samples

To remove protein, cold acetonitrile was added to rat plasma samples and centrifuged for 10 minutes at 12,000 *g*. The supernatant was then transferred and reacted under basic condition with benzoyl chloride. The process of benzoylation was carried out by autosampler. Workflow for sample pretreatment is shown in Figure 3, and the detail procedure of preparation in the autosampler is shown in Figure 4.

Protein Precipitation

- Mix 20 µL of rat plasma with 80 µL of cold acetonitrile
- Vortex
- Centrifuge at 12,000 *g* for 10 min

[Automation using SIL-30AC autosampler]

Derivatization

- Transfer 15 µL of sample to a new vial
- Add 10 µL of 100 mM Na_2CO_3
- Add 10 µL of 2% benzoyl chloride

Internal standard

- Add 5 µL of Internal standard in 1% formic acid

Injection to LC-MS/MS

Figure 3. Workflow for sample pretreatment

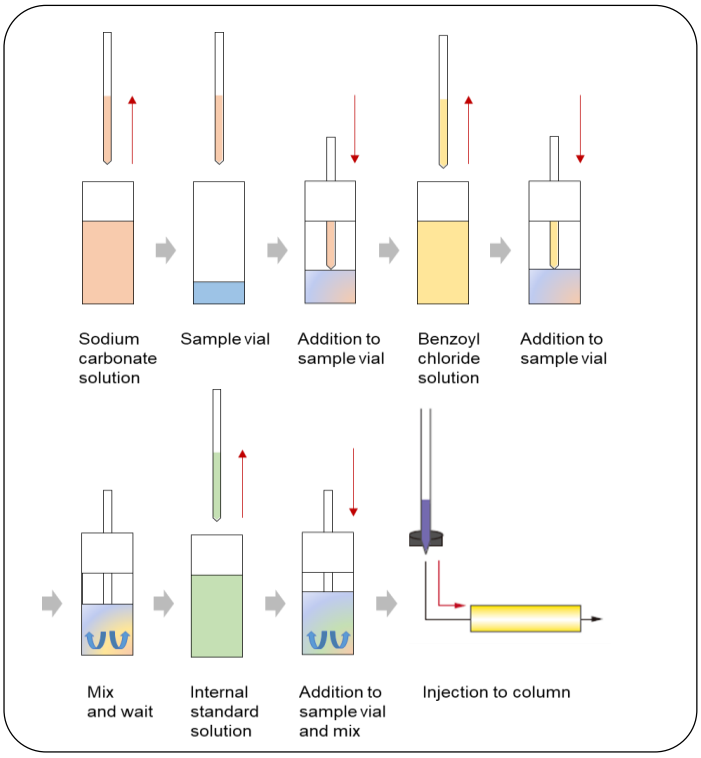
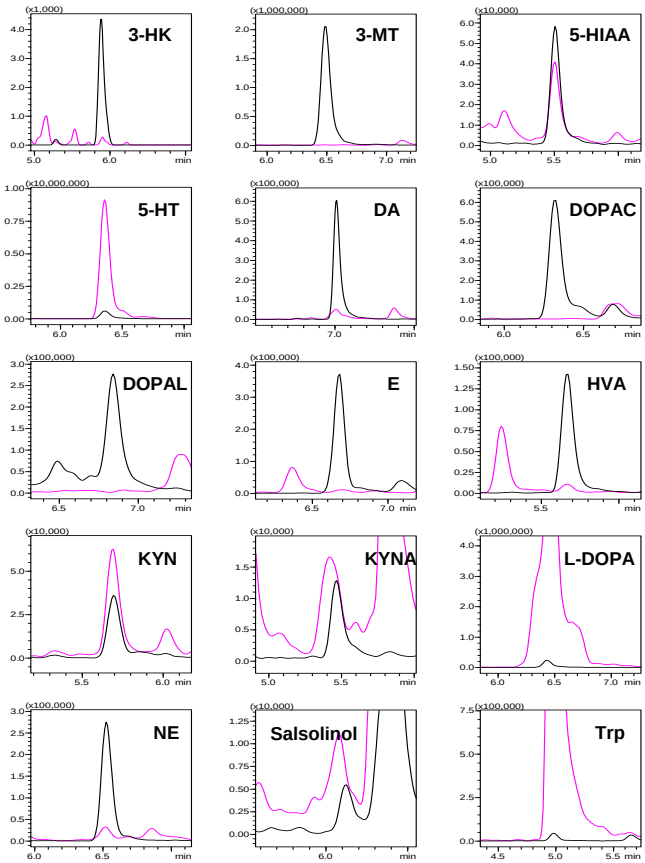


Figure 4. Detail preparation procedure in the autosampler

❑ Results

A. Chromatograms

The overlaid MRM chromatograms of standard solution and plasma sample are shown in Figure 5.



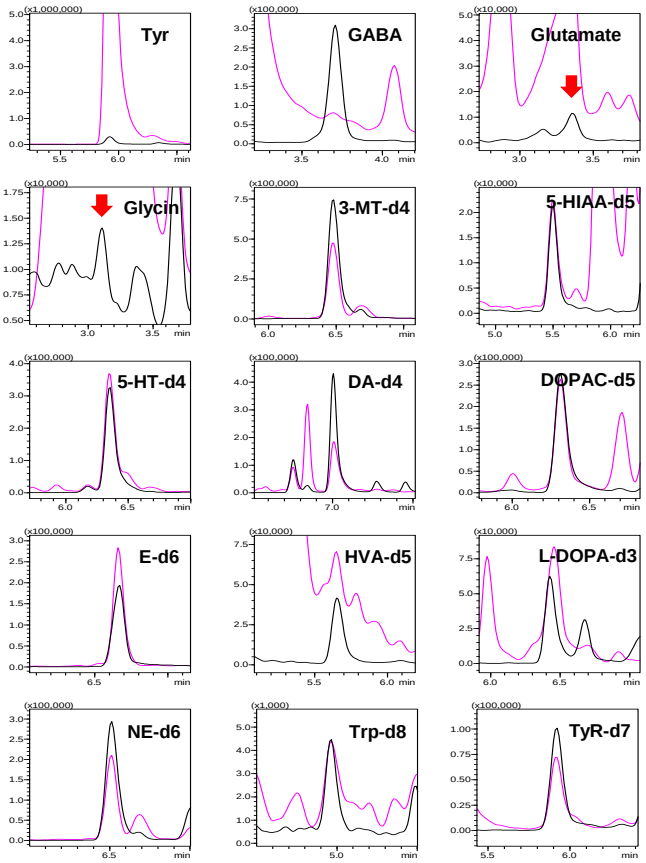


Figure 5. Overlaid MRM chromatograms (Black: STD 10 ng/mL, Pink: Plasma sample)

B. Validation

The calibration curves were generated by internal standard (IS) method for plasma samples covering the range of 1 to 100 ng/mL. The correlation coefficient (r^2) was better than 0.99 in every case.

The repeatability and accuracy (n = 8) of target compounds were evaluated at concentration level of 10 ng/mL. The results of the linearity, accuracy, repeatability and limit of quantitation (LOQ) are shown in Table 3. And the repeatability (n = 4) of internal standards is as shown in Table 4.

Table 3. Evaluation of Method Performance of IS Method

Compound	R^2	Accuracy (%) (n = 8)	% RSD (n = 8)	LOQ (ng/mL)
3-HK	1.000	104	3.7	0.30
3-MT	0.996	106	2.1	0.03
5-HIAA	0.999	118	2.8	1.48
5-HT	0.996	108	3.7	0.08
DA	0.991	115	2.5	0.07
DOPAC	0.998	117	2.4	0.07
DOPAL	0.997	138	4.6	0.22
E	0.992	121	3.5	0.08
HVA	0.993	106	2.8	0.18
KYN	0.999	96	5.6	0.33
KYNA	1.000	102	5.8	5.16
L-DOPA	0.996	129	5.2	0.06
NE	0.991	116	1.7	3.16
Salsolinol	0.992	126	3.5	5.33
Trp	0.995	95	8.0	1.15
Tyr	0.998	95	3.2	0.14
GABA	1.000	110	1.9	0.25
Glutamate	0.994	137	13.7	4.90
Glycine	0.993	138	14.3	5.18

Table 4. Repeatability of Internal Standards

Compound	% RSD (n = 4)
3MT-d4	5.3
5-HIAA-d5	10.4
5-HT-d4	12.7
DA-d4	14.1
DOPAC-d5	6.0
E-d6	12.9
HVA-d5	19.2
L-DOPA-d3	8.5
NE-d6	11.4
Trp-d8	3.8
Tyr-d7	4.7

❑ Conclusions

An automated precolumn derivatization method for determination of neurotransmitters in plasma is established using Shimadzu LCMS-8050 with a heated ESI. A convenient pretreatment process of plasma sample is possible using Shimadzu Autosampler SIL-30AC and high sensitivity and reliable results are obtained as well.

❑ Reference

[1] Lance H. Rodan, K. Michael Gibson, Phillip L. Pearl. Clinical Use of CSF Neurotransmitters. *Pediatric Neurology*, 53, 2015, 277-286.
[2] Somnath Ghosh, Jhantu Das. Benzoylation of Amines sans Alkali: A Green Protocol in Neat Phase. *Organic Chemistry International*, volume 2010, Article ID 743186.

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First Edition: Sep. 2020