A Novel, Sensitive, High-throughput Assay To Quantify Simultaneously Three Neurotransmitter Biomarkers in Human Plasma by Benzoyl Chloride Derivatization Coupled With UPLC-MS/MS

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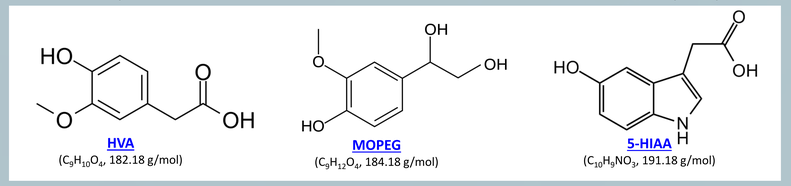
16th WRIB — Where Regulators & Industry Convene

Introduction

- Neurotransmitters are signaling molecules released by a neuron to affect other cells across a synapse. The major types of neurotransmitters include acetylcholine, biogenic amines, and amino acids. Homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MOPEG), and 5-hydroxyindoleacetic acid (5-HIAA) are three major monoamine metabolites (**Figure 1**) and important biomarkers associated with neurological disorders such as schizophrenia and depression.
- Due to the low molecular weight and high hydrophilicity of HVA, MOPEG, and 5-HIAA (**Figure 1**), developing a reliable quantitative assay for these monoamine biomarkers in a biological matrix is very challenging.
- The capability to quantify levels of HVA, MOPEG, and 5-HIAA accurately in human plasma is important in clinical studies. To that end, we developed and validated a novel, robust, sensitive, high-throughput ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method to simultaneously determine these three monoamine neurotransmitters in human sodium heparin plasma.



HVA ($C_9H_{10}O_4$, 182.18 g/mol) **MOPEG** ($C_9H_{12}O_4$, 184.18 g/mol) **5-HIAA** ($C_{10}H_9NO_3$, 191.18 g/mol)

Figure 1: Chemical structures, molecular formulas and molecular weights of HVA, MOPEG, and 5-HIAA.

Method Challenges

HVA, MOPEG, and 5-HIAA have low molecular weights at 182.18, 184.18, and 191.18 g/mol, respectively, and are very hydrophilic due to the multiple hydroxyl groups. Due to the low molecular weight and high hydrophilicity, developing a reliable quantitative assay for these neurotransmitter metabolites in a biological matrix is challenging.

- **Derivatization to improve sensitivity:** The required sensitivity was not originally achieved due to insufficient ionization on MS, which was most likely caused by their low mass and high hydrophilicity. Thus, different derivatization reagents including benzoyl chloride and Dansyl chloride were compared, and the results indicated that benzoyl chloride derivatization (**Figure 2**) significantly improved the sensitivity to achieve the required lower limit of quantification (LLOQ) of 2.00, 1.00, and 2.00 nM for HVA, MOPEG, and 5-HIAA, respectively.
- **Improvement of internal standard (IS):** During method development, HVA-$^{13}C_6{}^{18}O$ and 5-HIAA-$^{13}C_6$ were added at the beginning of the sample extraction and used as the ISs for HVA and 5-HIAA, respectively, while MOPEG-BC-D_5 was synthesized by MOPEG and benzoyl-D_5 chloride was added after the derivatization step during sample extraction (**Figure 3**). With this approach, the big variation of the extraction was observed for MOPEG. In order to resolve this, the IS of MOPEG-D_3 (**Figure 3**) that was added at the beginning of the sample extraction was used.
- **Chromatographic optimization to achieve high-throughput assay:** Challenges were encountered to obtain adequate chromatographic resolution and efficient run time. Different columns and mobile phases were evaluated, and the ACQUITY 130Å UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm, Waters Corporation, Milford, MA) with optimized mobile phases (mobile phase A: 10mM ammonium formate and 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile/methanol 1:1) was selected (**Figure 4**). The run time was shortened from 15 to 6 minutes per sample for the three analytes, and the total elapsed time to process one full 96-well plate was ~8 hours.
- **Overcoming short processed sample stability:** A large amount of salt was used during the derivatization step and then dissolved in the final reconstitution solvent. A precipitate formed in the extracted samples after a short time period (<1 day) under 2–8°C due to the high content of salts, leading to difficulty in establishing processed sample stability. The reconstitution solvent of methanol/dimethyl sulfoxide/water (MeOH/DMSO/H_2O, 2:2:6, *v/v/v*) was identified to resolve this problem, and 8 days of processed sample stability was established.
- **Suitable surrogate matrix:** Human plasma has endogenous levels of HVA, MOPEG, HIAA. Different types of potential surrogate matrices, including phosphate-buffered saline (PBS) buffer, bovine serum albumin, Triton X-100 buffer, and CHAPS, were compared by evaluating dilution linearity (**Table 1**) and recovery (**Table 2**). Non-specific binding (NSB) was also evaluated (**Table 3**) with these potential surrogate matrices, and the results revealed that pure water had a severe NSB issue. Therefore, 5% BSA was selected as the suitable surrogate matrix with a good recovery and parallelism in this assay.

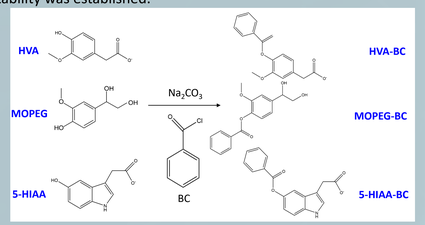
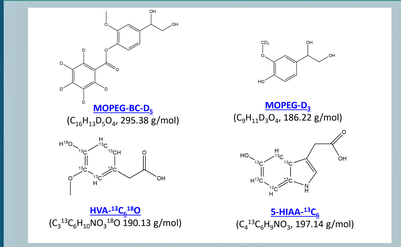


Figure 2: Derivatization of HVA, MOPEG, and HIAA with benzoyl chloride (BC)

Method Challenges (*Cont.*)



MOPEG-BC-D_5 ($C_{16}H_{13}D_5O_4$, 295.38 g/mol) **MOPEG-D_3** ($C_9H_{11}D_3O_4$, 186.22 g/mol)
HVA-$^{13}C_6{}^{18}O$ ($C_3{}^{13}C_6H_{10}NO_3{}^{18}O$ 190.13 g/mol) **5-HIAA-$^{13}C_6$** ($C_4{}^{13}C_6H_9NO_3$, 197.14 g/mol)

Figure 3: Internal standards

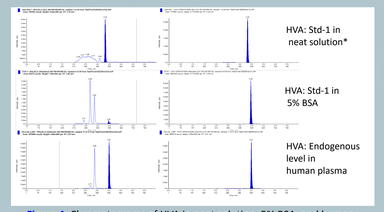


Figure 4: Chromatograms of HVA in neat solution, 5% BSA, and human plasma (*Interference was observed in neat solution).

Table 1: Dilution linearity of HVA, MOPEG, and 5-HIAA in different surrogate matrices

HVA								
Surrogate Matrix Type	Accuracy (%)							
	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8
Water	106.5	90.9	90.2	102.5	99.9	104.9	101.6	101.6
0.1% Triton X-100 in water	101.7	95.4	94.36	98.3	100	91.2	96.1	95.2
5% BSA in water	99.5	100.3	100.9	101.3	99.8	99.7	100	98.5
MOPEG								
Surrogate Matrix Type	Accuracy (%)							
	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8
Water	100.9	102.4	94.5	91.4	101.3	103.9	104.8	99.5
0.1% Triton X-100 in water	69.4	91.0	94.4	101.1	103.6	95.6	100.4	101.8
5% BSA in water	103	99	101.3	101.3	101.6	99.4	99.1	98.2
5-HIAA								
Surrogate Matrix Type	Accuracy (%)							
	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8
Water	103.5	90.6	107.8	88	110.7	98.7	105.6	100.2
0.1% Triton X-100 in water	54.5	83.5	79.04	104.7	85.7	83.5	96.5	98.2
5% BSA in water	100	100.8	101.8	100	100.8	101	99.4	97.5

Table 2: Recoveries of HVA, MOPEG, and 5-HIAA in different surrogate matrices

Surrogate Matrix Type	Recovery of Each Compound		
	HVA	MOPEG	5-HIAA
Water	85%	90%	78%
5% BSA in Water	87%	99%	81%
0.1% Triton X-100 in water	83%	134%	53%
0.1% CHAPS in water	74%	152%	32%

Table 3: Non-specific binding of HVA, MOPEG, and 5-HIAA in different surrogate matrices

Surrogate Matrix Type	Response Difference Between Control vs Five-Time Transfer		
	HVA	MOPEG	5-HIAA
Water	-4.2%	-3.0%	-9.3%
0.1% Triton X-100 in water	4.4%	-3.6%	-5.1%
0.5% Triton X-100 in water	-1.6%	-2.3%	-5.5%
0.1% CHAPS in water	-0.2%	-4.5%	-2.7%
0.5% CHAPS in water	-6.6%	-5.4%	-4.9%
5% BSA in water	2.3%	3.5%	4.8%

Finalized Method

Aliquots (50.0 µL) of human sodium heparin plasma were spiked with a mixture of stable-labeled ISs. The samples were extracted by protein precipitation with acetonitrile, and the resulting supernatant was alkalified by sodium carbonate and then derivatized with benzoyl chloride. After derivatization, the reaction was quenched with 10 µL of 10% formic acid in acetonitrile. The sample extracts were injected onto an ACQUITY BEH C18 column at a column temperature of 40°C. The mobile phase consisted of a solvent mixture of formic acid, ammonium formate, water, and acetonitrile using a shallow gradient at 0.5 mL/min for 6 min. A SCIEX API 5500 UPLC-MS/MS system was used under electrospray ionization mode to monitor HVA, MOPEG, and 5-HIAA at ion transitions of 304.1→137.0, 306.1→167.0 and 313. 1→ 250.1, respectively.

Results

The UPLC-MS/MS assay was successfully developed and fully validated in human plasma within a quantification range of 2.00 to 200 nM for HVA, 1.00 to 100 nM for MOPEG, and 2.00 to 200 nM for 5-HIAA (**Figure 5**). The validation experiments included intra- and inter-day precision and accuracy, sensitivity, selectivity/specificity, matrix effect, various stability tests, recovery, and dilution linearity. Typical chromatograms of the LLOQ quality control (LLOQ-QC) and control-zero (Control-0) are shown in **Figure 6**. The intra-day accuracy (% bias) results for three runs of LLOQ and other QCs ranged from 84.7% to 111.2% for all analytes; the intra-day precision (%CV) results for three runs of LLOQ and other QCs were within 13.5% for all analytes (**Table 5**). Selectivity was successfully determined without observable interference using six different lots of matrix. A total of 21 hours of benchtop stability, four cycles of freeze-thaw stability, ~one month of matrix stability at −20°C, and 88 days of matrix stability at −70°C were successfully established. The extracted sample stability was established for 8 days (**Table 4**). The validated method was successfully applied to a clinical pharmacokinetic study.

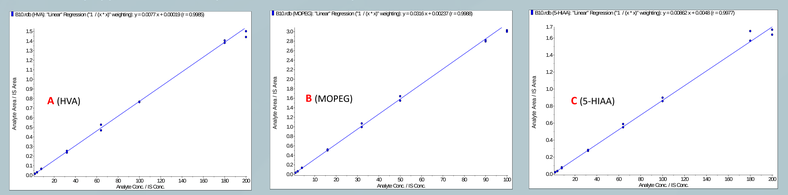


Figure 5: Typical standard calibration curves of HVA (A), MOPEG (B), and 5-HIAA(C), (regression type: linear; weighting factor: $1/x^2$)

Results (*Cont.*)

Table 4: Established stability of HVA, MOPEG, and 5-HIAA

Parameter	Analyte		
	HVA	MOPEG	5-HIAA
Benchtop room temperature (RT) storage stability	21 hours	21 hours	21 hours
Processed sample stability at 6°C	192 hours	192 hours	192 hours
Freeze/thaw stability (– 70°C/wet ice)	4 cycles	4 cycles	4 cycles
Stock solution stability at – 20°C	57 days	57 days	57 days
Working solution stability at– 20°C	22 days	22 days	22 days
Long-term stability at – 20°C	34 days	32 days	34 days
Long-term stability at – 70°C	88 days	88 days	88 days

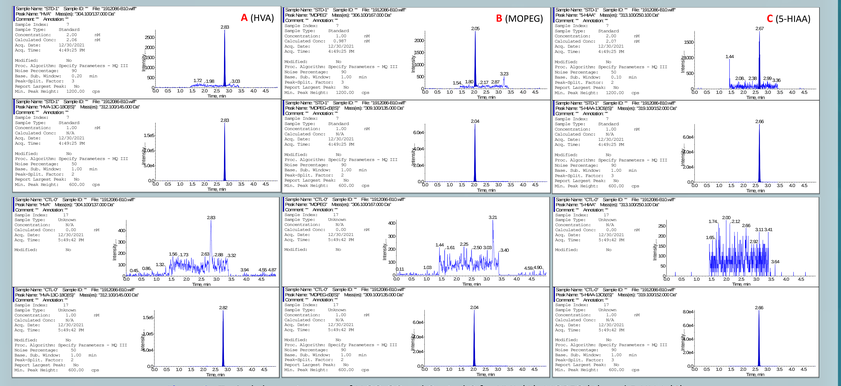


Figure 6: Typical chromatograms of LLOQ-QC and Control-0 for HVA (A), MOPEG (B), and 5-HIAA (C)

Table 5: Inter- and intra-accuracy and precision for HVA (A), MOPEG (B), and 5-HIAA (C)

A (HVA)	Concentration of HVA (nM)								
	LLOQ-QC 2.00	DEV (%)	LQC 6.00	DEV (%)	MQC [a] 44.4	DEV (%)	HQC [b] 144	DEV (%)
10	2.01	0.5	6.67	11.2	41.1	-7.4	128	-11.1
	2.08	4.0	5.68	-5.3	44.2	-0.5	132	-8.3
	1.86	-7.0	5.28	-12.0	40.8	-8.1	136	-5.6
	1.84	-8.0	5.73	-4.5	42.7	-3.8	140	-2.8
	2.13	6.5	5.47	-8.8	44.2	-0.5	144	0.0
	2.25	12.5	5.28	-12.0	44.2	-0.5	139	-3.5
n	6		6		6		6	
Mean	2.03		5.69		42.7		135	
SD	0.159		0.519		1.48		4.64	
CV (%)	7.8		9.1		3.5		3.4	
Accuracy (%)	101.5		94.8		96.2		93.8	
11	2.02	1.0	5.62	-6.3	42.8	-3.6	140	-2.8
	2.14	7.0	5.78	-3.7	43.2	-2.7	139	-3.5
	1.99	-0.5	5.19	-13.5	42.2	-5.0	136	-5.6
	2.04	2.0	5.39	-10.2	43.6	-1.8	139	-3.5
	1.97	-1.5	6.29	4.8	43.8	-1.4	142	-1.4
	1.94	-3.0	6.25	4.2	43.1	-2.9	154	6.9
n	6		6		6		6	
Mean	2.02		5.75		43.1		142	
SD	0.0700		0.448		0.574		6.35	
CV (%)	3.5		7.8		1.3		4.5	
Accuracy (%)	101.0		95.8		97.1		98.6	
13	2.22	11.0	5.68	-5.3	42.1	-5.2	141	-2.1
	2.35	17.5	6.31	5.2	42.6	-4.1	145	0.7
	1.92	-4.0	5.80	-3.3	40.7	-8.3	159	10.4
	2.03	1.5	6.01	0.2	41.6	-6.3	132	-8.3
	1.82	-9.0	5.91	-1.5	41.4	-6.8	131	-9.0
	2.08	4.0	5.84	-2.7	43.4	-2.3	133	-7.6
	N/A		6.19	3.2	45.5	2.5	135	-6.3
	N/A		5.61	-6.5	48.0	8.1	146	1.4
	N/A		5.88	-2.0	44.7	0.7	150	4.2
n	6		9		9		9	
Mean	2.07		5.91		43.3		141	
SD	0.138		0.226		2.35		9.53	
CV (%)	6.7		3.8		5.4		6.8	
Accuracy (%)	103.5		98.5		97.5		97.9	
Inter-assay:								
n	18		21		21		21	
Overall Mean	2.04		5.80		43.1		139	
SD	0.143		0.385		1.70		7.88	
CV (%)	7.0		6.6		3.9		5.7	
Accuracy (%)	102.0		96.7		97.1		96.5	

B (MOPEG)	Concentration of MOPEG (nM)								
	LLOQ-QC 1.00	DEV (%)	LQC 3.00	DEV (%)	MQC [a] 22.7	DEV (%)	HQC [b] 72.7	DEV (%)
10	0.768	-23.2	2.96	-1.3	20.7	-8.8	64.9	-10.7
	0.901	-9.9	2.68	-10.7	21.5	-5.3	65.9	-9.4
	0.888	-11.2	2.71	-9.7	21.1	-7.0	68.1	-6.3
	0.893	-10.7	2.81	-6.3	20.4	-10.1	70.4	-3.2
	1.09	9.0	2.81	-6.3	20.9	-7.9	69.8	-4.0
	1.10	10.0	2.78	-7.3	22.0	-3.1	63.7	-12.4
n	6		6		6		6	
Mean	0.940		2.79		21.1		66.7	
SD	0.130		0.0983		0.576		2.41	
CV (%)	13.8		3.5		2.7		3.6	
Accuracy (%)	94.0		93.0		93.0		91.7	
11	1.03	3.0	2.56	-14.7	23.6	4.0	66.8	-8.1
	0.979	-2.1	2.61	-13.0	22.9	0.9	67.7	-6.9
	0.918	-8.2	2.60	-13.3	21.4	-5.7	67.1	-7.4
	0.883	-11.7	2.69	-10.3	23.7	4.4	68.1	-6.3
	0.955	-4.5	2.89	-3.7	21.8	-4.0	69.8	-4.0
	1.04	4.0	2.85	-5.0	22.8	0.4	72.2	-0.7
n	6		6		6		6	
Mean	0.968		2.70		22.7		68.7	
SD	0.0617		0.139		0.934		2.02	
CV (%)	6.4		5.1		4.1		2.9	
Accuracy (%)	96.8		90.0		100.0		94.5	
13	0.857	-14.3	2.51	-16.3	23.4	3.1	73.5	-1.7
	0.857	-14.3	2.69	-10.3	22.9	0.9	72.1	-0.8
	0.688	-31.2	2.67	-11.0	22.9	0.9	80.9	11.3
	0.767	-23.3	2.86	-4.7	28.6	26.0	65.8	-9.5
	0.984	-1.6	2.88	-4.0	24.6	8.4	66.0	-9.2
	0.926	-7.4	2.66	-11.3	26.2	15.4	66.5	-8.5
	N/A		3.88	29.3	23.0	1.3	69.9	-3.9
	N/A		2.96	-1.3	24.0	5.7	72.3	-0.6
	N/A		4.03	34.3	23.3	2.6	74.2	2.1
n	6		9		9		9	
Mean	0.847		3.02		24.3		71.0	
SD	0.112		0.551		1.93		4.81	
CV (%)	13.2		18.2		7.9		6.8	
Accuracy (%)	84.7		100.7		107.0		97.7	
Inter-assay:								
n	18		21		21		21	
Overall Mean	0.918		2.86		22.9		69.1	
SD	0.111		0.386		1.92		3.89	
CV (%)	12.1		13.5		8.4		5.6	
Accuracy (%)	91.8		95.3		100.9		95.0	

C (5-HIAA)	Concentration of 5-HIAA (nM)								
	LLOQ-QC 2.00	DEV (%)	LQC 6.00	DEV (%)	MQC [a] 36.1	DEV (%)	HQC [b] 136	DEV (%)
10	1.83	-8.5	5.70	-5.0	35.6	-1.4	130	-4.4
	1.76	-12.0	5.70	-5.0	37.1	2.8	133	-2.2
	1.91	-4.5	5.61	-6.5	36.7	1.7	136	0.0
	1.68	-16.0	5.06	-15.7	37.1	2.8	136	0.0
	1.91	-4.5	5.78	-3.7	35.4	-1.9	132	-2.9
	1.63	-18.5	5.46	-9.0	36.0	-0.3	132	-2.9
n	6		6		6		6	
Mean	1.79		5.55		36.3		133	
SD	0.117		0.264		0.752		2.34	
CV (%)	6.5		4.8		2.1		1.8	
Accuracy (%)	89.5		92.5		100.6		97.8	
11	2.27	13.5	6.15	2.5	35.3	-2.2	135	-0.7
	1.94	-3.0	6.42	7.0	37.4	3.6	135	-0.7
	2.04	2.0	6.42	7.0	36.4	0.3	139	2.2
	2.25	12.5	6.50	8.3	36.7	1.7	141	3.7
	2.02	1.0	6.39	6.5	37.9	5.0	139	2.2
	2.02	1.0	6.39	6.5	37.0	2.5	141	3.7
n	6		6		6		6	
Mean	2.09		6.26		36.8		138	
SD	0.136		0.290		0.918		2.73	
CV (%)	6.5		4.6		2.5		2.0	
Accuracy (%)	104.5		104.3		101.9		101.5	
13	1.58	-21.0	6.12	2.0	34.8	-0.8	132	0.0
	1.93	-3.5	6.68	11.3	36.7	1.7	139	2.2
	1.83	-8.5	6.43	7.2	36.3	0.6	150	10.3
	1.72	-14.0	6.40	6.7	37.8	4.7	140	2.9
	1.65	-17.5	6.83	13.8	37.6	4.2	144	5.9
	2.18	9.0	6.70	11.7	45.8	26.9	148	8.8
	N/A		8.00	33.3	36.3	0.6	143	5.1
	N/A		6.61	10.2	40.6	12.5	141	3.7
	N/A		6.22	3.7	38.4	6.4	152	11.8
n	6		9		9		9	
Mean	1.82		6.67		38.4		144	
SD	0.288		0.552		3.14		5.36	
CV (%)	15.8		8.3		8.2		3.7	
Accuracy (%)	91.0		111.2		106.4		105.9	
Inter-assay:								
n	18		21		21		21	
Overall Mean	1.90		6.23		37.3		139	
SD	0.208		0.620		2.28		5.83	
CV (%)	10.9		10.0		6.1		4.2	
Accuracy (%)	95.0		103.8		103.3		102.2	

Conclusions

A rapid, sensitive, high-throughput assay was successfully developed to quantify three monoamine neurotransmitter metabolite biomarkers in human plasma using derivatization coupled with UPLC-MS/MS. The assay has the following advantages:

- **High Sensitivity:** HVA, MOPEG, and 5-HIAA are difficult to ionize using LC-MS due to their low molecular weight and high hydrophilicity. This was solved by adopting he benzoyl chloride derivatization after protein precipitation.
- **Suitable Surrogate Matrix**: Different surrogate matrices were compared by evaluating NSB, recovery, and linearity. Based on results, 5% BSA was selected as a suitable surrogate matrix.
- **High Throughput:** The total chromatography run time was 6 minutes per sample, and the total elapsed time to analyze one full 96-well plate on the instrument was ~8 hours for HVA, MOPEG, and 5-HIAA .
- **Precision and Accuracy:** This assay was fully validated in accordance with U.S. Food and Drug Administration *Bioanalytical Method Guidance for Industry* (2018) and showed good precision and accuracy.