

Simultaneous separation and quantitation of five Biogenic amines in urine by liquid chromatography–ionspray tandem mass spectrometry with multiple-reaction

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Abstract—A reliable, selective and sensitive liquid chromatography tandem mass spectrometry method was developed and validated for the quantification of Biogenic amines in urine. Chromatographic separation was performed on a Shim pack GIST C18, 4.6*75mm, 3µm using lc gradient methanol: 0.1% formic acid solution as the mobile phase at a flow rate of 0.300 mL/min. We describe sample processing and analysis methods for simultaneous quantification of multiple biogenic amines, in urine using LC–MS/MS. A simple extraction procedure technique compatible with each functional group for measuring VMA, HIAA, HVA, Hydroxyproline and 3-Methoxytyramine, with high sensitivity and specificity is also described. The positive and negative ionization mode with multiple reaction monitoring was used for detection. The sensitivity was good with no carry-over detected and the lower limit of quantification range. The method has been successfully verified using authentic case samples that had previously been quantified using different methods. The assay is suitable for clinical utilization and management of patients on these medications.

Index Terms—Biogenic amines; Therapeutic drug monitoring; UPLC-MS/MS; human urine

I. INTRODUCTION

Biogenic amines (Bas) are a specific class of neurotransmitters that are found in a variety of foods, such as fish, meat, cheese, and wines, are made through human biosynthetic pathways, and also made by the gut microbiome. BAs are characterized by vasoactive and psychoactive properties that make them able to interact with human metabolism. The most known intoxications are due to histamine, which causes the “scorn-broid fish poisoning”, and tyramine which is the principal cause of

the “cheese reaction” (Fabio et al.,2012). In addition to the measurement of parental biogenic amines, the measurement of their phase I metabolites provides complementary information regarding changes in biogenic amine metabolites. Stable metabolites of biogenic amines, such as VMA and HVA, for example are used as the biomarkers for the detection of neuroblastoma and similar tumors (M. Tuchman et al., 1985). Similarly, 5-HIAA is used as a biochemical marker of several neuroendocrine tumors (Corcuff et al., 2017). A large fraction of biogenic monoamines and metanephrines further undergoes phase II metabolism to yield respective sulfated and glucuronated forms, which are excreted in urine (Bicker et al., 2013).

Mass spectrometry instruments over the last decade have led to increased utilization of high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as a means of providing assays with increased specificity and sensitivity, with the aim of improving the quality of patient care (Sallustio BC et al., 2010). The use of LC coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use (Silva et al., 2006). UPLC has been evaluated as a faster and more efficient analytical tool compared to current HPLC (Villiers et al., 2006).

Several analytical methods to measure free biogenic monoamines in tissues as well as biological matrices such as serum, plasma, urine, and saliva (Grouzmann et al., 2013, Fang et al., 2012, Alberts et al., 1992, Goldstein et al., 2003, Siegert et al., 2013, Su et al., 2009), high-performance liquid chromatography with UV (Siren et al., 1999), ultra-high performance liquid chromatography (UPLC) with tandem mass spectrometry (MS/MS) (Chan et al., 2000, Jong et al., 1999, Zhengzhi et al., 2018, Magera et al., 2018, Lionetto et al., 2008, Fang et al., 2012)

The focus of this review is the analysis of biogenic amines in biological fluids. We report a quick, accurate and reliable liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantitative determination of Vanillymandelic Acid (VMA), Homovanillic Acid (HVA), Hydroxyindole Acetic Acid (HIAA), Hydroxyproline and 3-Methoxytyramine in human urine samples. The simple extraction procedure and run time is only 8.0 min. Furthermore, all analytes are directly measurable without the need for any additional derivatization step, positive and negative ion modes were used to achieve the best sensitivity and specificity.

II. EXPERIMENTAL CHEMICALS AND REAGENTS.

The Calibrator & Controls standard set lyophilised of biogenic amines (Figure 1) Vanillymandelic Acid (VMA), Homovanillic Acid (HVA), Hydroxyindole Acetic Acid (HIAA), Hydroxyproline and 3-Methoxytyramine were procured from Recipe, Munich, Germany. High purity water used for the LC-MS/MS was prepared from Purelab Flex 3 purification system procured from Synergy medical (Mumbai, India). HPLC methanol and acetonitrile were purchased from Honeywell. All other reagents and solvents were obtained from general commercial suppliers.

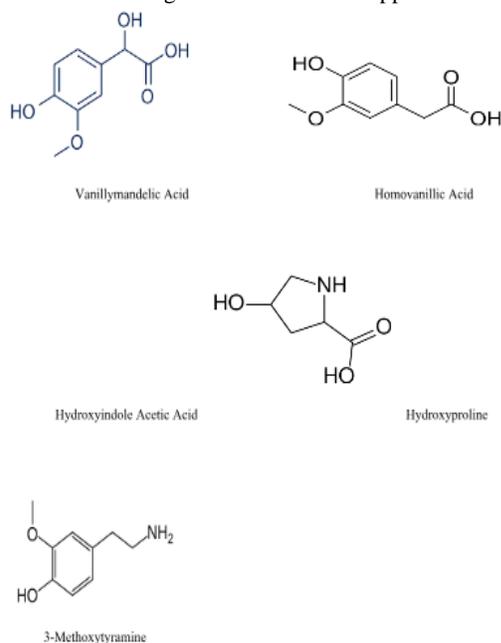


Figure 1. Structure of 05 Biogenic amines drugs.

III. CALIBRATION CURVES

The Calibration curve was established using the recipe clinical calibrator set lyophilised for Vanillymandelic acid, Homovanillic Acid, Hydroxyindole Acetic Acid,

Hydroxyproline and 3-Methoxytyramine (Recipe, Munich, Germany). Only one levels of calibration curve (CC) and two levels of quality controls (QC) were reconstituted as per company recommendation.

IV. SAMPLE PREPARATION

Sample preparation was achieved by simple extraction. Fifty microliters of human urine was mixed with 0.15 mL of 0.1% formic acid. To this 0.15 mL of methanol was added after vortex mixing for 60 s, Centrifuge the sample for 5 minutes at 10000 RPM at $5 \pm 5^\circ\text{C}$. Sample was transferred to an auto sampler vial for LCMS/MS system.

V. CHROMATOGRAPHIC CONDITIONS

Chromatography separation was performed on Nexera UPLC system (Shimadzu, Japan) with cooling auto-sampler and column oven enabling temperature control of the analytical column. The chromatography separations were performed on a Shim pack GIST C18, 4.6*75mm, 3 μm and temperature maintained at 40 $^\circ\text{C}$. Samples were separated under gradient conditions with a mobile phase composed of buffer methanol/0.1% formic acid, a pump operating at a flow rate of 0.300mL/min and the autosampler temperature maintained at 10 $^\circ\text{C}$ and the injection volume was 10 μL . The total running time was 8.0 min for each injection. Mobile phase was used as weak wash and strong wash solvent to avoid any carry over from previous injection.

Time	Flow	Conc. A	Conc. B
0.00	0.30	10.0	90.0
0.20	0.30	10.0	90.0
0.50	0.30	50.0	50.0
3.00	0.30	50.0	50.0
3.10	0.30	90.0	10.0
4.00	0.30	90.0	10.0
4.10	0.30	10.0	90.0
8.00	0.30	10.0	90.0

VI. RESULT AND DISCUSSION

Chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as short run

time. Formic acid buffer in the mobile phase improved the detection of the analytes. It was found that mixture of methanol-0.1% formic acid could achieve this purpose and was finally adopted as the mobile phase. The use of small particles of stationary phase allowed UPLC to push the limits of peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities).

Five channels were used for recording with a retention time of 8.0min, and this met the requirement for a high sample throughput.

VII. MASS SPECTROMETRY

The mass spectrometer was tuned in both positive and negative ionization modes to check for optimum response of biogenic amines. The biogenic amines molecule gave fragment ions at low and high collision energy (CE). The setting of the MS method for the detection of biogenic amines in MRM mode was done with the aim of achieving the best specificity with respect to any other available matrix ion.

The first quadrupole (Q1) of the MS system was given a filter for scanning only this ion with unit resolution and keeping the third quadrupole (Q3) filter to scan the product molecular ion with low resolution. The tandem mass spectrometer was operated in electro spray with multiple reactions monitoring acquisition parameters shown in Table 1. The Desolvation and Heat Block Temperature were set at 200°C and 350°C respectively. Nitrogen was used as nebulizing and drying gas; flow was set at 3.0 and 15.0 L/min respectively.

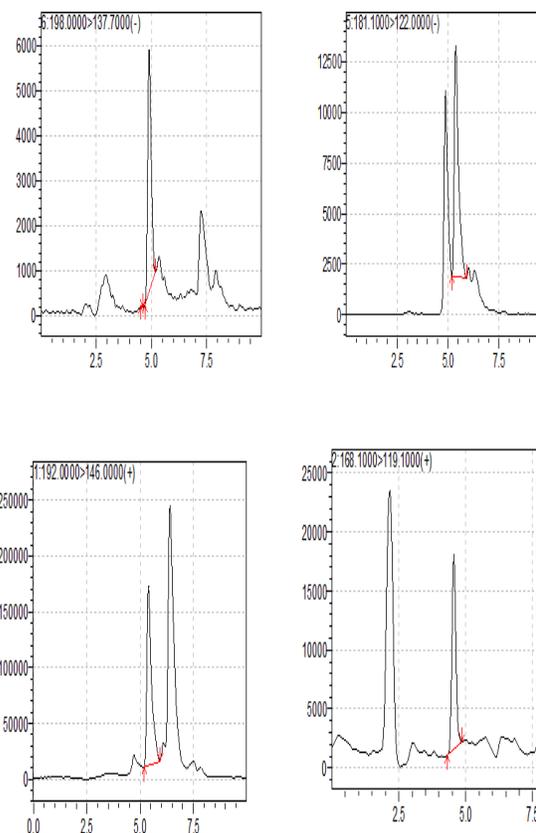
Table 1. Optimization of MRM parameters, Ionization mode Q1 prebias, Collision energy, Q3 pre bias and retention time (RT) of Biogenic amines.

Drugs	VMA	HVA	HIAA	3 Methoxytyramine	Hydroxyproline
Precurso r ion (m/z)	198.00	181.00	192.00	168.10	132.01
Product ion (m/z)	137.70	122.00	146.00	119.10	68.00
Ionizatio n mode	-	-	+	+	+
Q1 pre bias (v)	10.0	21.0	-11.0	-13.0	-14.0

Collision energy (v)	22.0	18.0	-20.0	-10.0	-13.0
Q3 pre bias (v)	12.0	11.0	-15.0	-13.0	-18.0
Dwell time(msec)	50	50	50	50	50
RT (minutes)	4.90	5.38	5.39	4.66	2.68

VIII. SPECIFICITY, SELECTIVITY AND CARRY OVER

Ten blank urine samples from different sources were analyzed for peaks interfering with the detection of the analyte. As shown in Figure 2 no significant direct interference in the blank serum traces were observed from endogenous substances in drug-free human urine at the retention time of the analytes respectively. The carry-over effect was assessed for each ion by injecting blank serum immediately after the highest calibration standard.



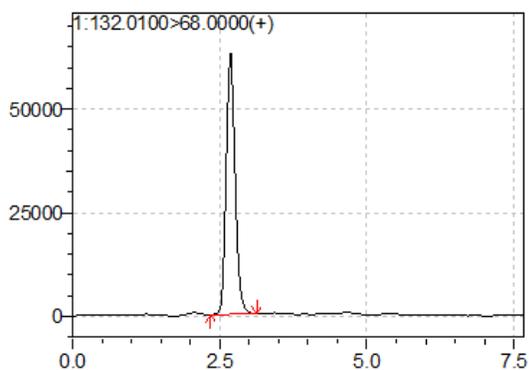


Figure 2: Representative chromatograms of extracted lower limit of quantification urine sample.

IX. LINEARITY AND LOWER LIMIT OF DETECTION (LOD)

The linearity of the target compound peak area versus the calculated concentration was verified in human urine using a 1/x2 weighted linear regression and the linearity was conducted by external calibration, for it is a better way using the specific standard biogenic amines to quantitative themselves. LOD was determined by repeated analyses of spiked samples at decreasing concentrations. Five different sources of matrix samples were spiked at decreasing concentrations and were processed and analyzed by proposed extraction procedure. Table 2 summarizes the calibration range, lower limit of quantification and concentration of LOD.

Table2. Calibration range, LLOQ and LOD for Biogenic amines drugs in human urine.

Drugs	Calibration range	Quality Control		LLOQ	LOD
		Level-1	Level-2		
VMA	11.1 µg/ml	6.32 µg/ml	16.4 µg/ml	6.32 µg/ml	3.16 µg/ml
HVA	11.0 µg/ml	6.09 µg/ml	17.0 µg/ml	6.09 µg/ml	3.05 µg/ml
HIAA	15.9 µg/ml	5.44 µg/ml	31.2 µg/ml	5.44 µg/ml	2.72 µg/ml
3 Methoxytyramin	214.0 ng/ml	156.0 ng/ml	330.0 ng/ml	156.0 ng/ml	78.0 ng/ml
Hydroxyproline	50.5 µg/ml	20.1 µg/ml	97.1 µg/ml	20.1 µg/ml	10.05 µg/ml

X. PRECISION AND ACCURACY

Intra-day precision and accuracy results were calculate using two different batches analyzed on a single day, whereas inter-day results were calculated using five different batches analyzed on a three successive day. The acceptable intra-day and inter day precision and accuracy results of biogenic amines are presented in Table 3.

Table 3. Precision an accuracy of the method for determining Biogenic amines drugs concentration in human urine samples.

Analytes & Unit	Concentration added		Intra-day precision(n=5)		Inter-day precision (n=5)	
			Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
VMA & µg/ml	Level-1	6.32	2.9	100.2	3.9	100.5
	Level-2	16.40	3.7	101.3	4.2	102.1
HVA & µg/ml	Level-1	6.09	4.8	103.7	5.0	104.1
	Level-2	17.0	3.6	102.4	3.9	103.2
HIAA & µg/ml	Level-1	5.44	5.2	98.6	4.8	97.9
	Level-2	31.2	3.7	99.2	4.2	98.6
3 Methoxytyramin & ng/ml	Level-1	156.0	2.0	102.6	3.1	101.8
	Level-2	330.0	2.8	100.4	3.4	101.2
Hydroxyproline & µg/ml	Level-1	20.1	1.9	101.6	2.4	102.5
	Level-2	97.1	3.2	102.7	4.0	103.7

XI. STABILITY

All the stability tests for biogenic amines were studied at two concentration levels. Stability experiments were performed exhaustively to evaluate the stability of biogenic amines in urine samples under different conditions, simulating the same conditions which occurred during study sample analysis: auto sampler stability and bench top stability for human urine. The stability results summarized that biogenic amines drugs spiked into urine were stable for at least 6 h in bench top, for at least 40 h in the mobile phase at 10°C under autosampler storage condition,

XII. CONCLUSION

This LC-MS/MS method was fully validated in urine and allowed as part of a multi-analyte approach, an accurate and precise quantification of five biogenic amines in urine. This method has significant advantages in terms of clean and reproducible simple extraction procedure and a short chromatographic run time is only 8.0 min. The extraction method gave consistent and reproducible recoveries for analytes from urine, with minimum interference and ion suppression. We provide a platform that can be easily adapted to a number of clinical settings, particularly those which have complex and challenging patient populations with relatively low specimen numbers. Practically, this would facilitate economical, single day turnaround time for biogenic amines requiring maintenance of a single assay. This all provides better and faster patient care at lower costs.

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