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Original Article

HPLC analysis of biomarkers of Toluene and Xylene in human urine samples

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ABSTRACT

Introduction: Urinary Hippuric acid (HA) and Methylhippuric acid (MHA) are considered reliable biomarkers for monitoring exposure to toluene and xylenes. A simple and robust HPLC method for simultaneous determination of HA and MHA in urine samples was developed and validated as per Internation Conference on Harmonization (ICH) Q2 guidelines.

Methods: Mobile phase consisting of two solvents A & B, solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. Separation was performed in gradient elution mode and the time program was as follows - Time (min)/%B: 0/20, 4/20, 11/80, 11.1/90, 16/90, 16.1/20, 25/20. The detection wavelength was 205 nm.

Results: The limits of detection (LOD) and quantification (LOQ) of HA in urine were 0.3 and 1.0 mg/L, and for MHA were 0.25 mg/L and 1.0 mg/L respectively and the recovery for HA and MHA were 95% and 98.9% respectively.

Conclusion: The developed method was successfully applied for the analysis of urine samples.

Keywords: Biomarker, Hippuric acid, HPLC, Methylhippuric acid, Method validation

Introduction

Smoking and combustion of fossil fuels are well-established sources of volatile organic compounds (VOCs) containing benzene and substituted benzenes such as toluene and xylenes (have three isomers (m-/p-/o-xylenes). Agency for Toxic Substances and Disease Registry (ATSDR) of the U.S. Department of Health and Human Services published toxicological profiles of toluene and xylenes. VOC exposure is associated with toxic effects on human health and may lead to various diseases but respiratory problems in the case of xylenes and central nervous system (CNS) problems in the case of toluene are most prevalent.[1-4]

Xylenes exposure may happen due to skin permeation as well as inhalation, whereas toluene exposure happens mainly by inhalation. Post-exposure xylene and toluene are absorbed into the blood. In humans major metabolic pathway of toluene proceeds primarily by oxidation to benzyl alcohol catalyzed by Cytochrome P450 enzymes followed by benzoic acid which further converts to hippuric acid (HA) whereas, in the case of xylenes oxidation to methyl benzoic acids followed by methyl hippuric acids (MHA) in liver. HA and MHA are considered primary exposure

biomarkers of toluene and xylenes which are excreted from the body in urine.[3-5] According to the American Conference of Governmental Industrial Hygienists (ACGIH), the permissible exposure limit in workers after 1-day exposure is 1.6 w/w creatinine for urinary HA and 1.5 w/w creatinine for urinary MHA, respectively.[6]

Many chromatographic methods are reported for the determination of HA and MHA in urine samples such as gas chromatography (GC),[7] capillary zone electrophoresis (CZE),[8] ion chromatography (IC),[9] liquid chromatography and each has its own merits and demerits.

There are various efforts to develop novel methods for HA and MHA. Recently, Takeuchi et. al. developed Direct methyl esterification with 2,2-dimethoxy propane for the simultaneous determination of urinary metabolites of toluene, xylene, styrene, and ethylbenzene by gas chromatography-mass spectrometry.[7] Chiu et. al. reported the use of urinary hippuric acid and methyl hippuric acid to evaluate surgical smoke exposure in operating room healthcare personnel.[10] Here we tried to develop and validate a simple high-performance liquid chromatography (HPLC) for analysis of HA and MHA as per ICH Q2 R(1) guidelines in human urine samples because HPLC methods are convenient as it is cost-effective and derivatization is not required.[11]

Methods

Standards of urinary metabolites, HA and MHA were procured from Sigma Aldrich USA. Trifluoroacetic acid (TFA), HPLC grade acetonitrile was purchased from Merck. Ultrapure water obtained by Merck Synergy water purification system.

The HPLC system consists of two LC-10AT vp pumps, a diode array detector (SPD-M10A vp), a column oven (CTO-10AS vp) and a system controller (SLC-10A vp) (all from Shimadzu, Kyoto, Japan), was used. Reversed-phase Thermo ODS-2 Hypersil, 250 x 4.6 mm, particle size: 5 µm column was used for analysis. The chromatographic and the integrated data were recorded using the LG computer system using LC-Solution data acquiring software (Shimadzu, Kyoto, Japan). The mobile phase consisted of two solvents A & B, solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. Separation was performed in gradient elution mode and the time program was as follows –Time (min)/%B: 0/20, 4/20, 11/80, 11.1/90, 16/90, 16.1/20, 25/20. The detection wavelength was 205 nm.

Water and methanol were mixed (1:1) to form a diluent solution. solution: 10.0 mg of HA and MHA standard were taken separately into a 100.0 mL volumetric flask and dissolved in methanol up to mark to prepare 100ppm Stock HA and MHA individually. Working standard solutions of HA and MHA of concentration 1, 2, 3, 5, and 10 mg/L were prepared respectively by diluting 100 µL, 200 µL, 300 µL, 500 µL and 1000 µL of the 100 mg/L stock solution of HA and MHA to 10.0 mL with diluent. The reference solution of HA and MHA was prepared by diluting 500 µL of the 100 mg/L stock solution of HA and MHA to 10.0 mL with diluent. 2.5 g of disodium hydrogen orthophosphate, 2.5 g of sodium dihydrogen orthophosphate and 8.2 g of sodium chloride was dissolved in 950 mL of water. pH of the solution was adjusted to 6.4 with 1 M sodium hydroxide or 1 M hydrochloride acid, if necessary and diluted to 1000.0 mL to prepare phosphate buffer at pH 6.4.

This study was part of our previous "Consortium on Vulnerability to Externalizing Disorders and Addictions (cVEDA)" study and the HPLC method was developed for the analysis of volatile organic compounds (VOCs) in urine samples, is incorporated in cVEDA as a measure of exposure to environmental neurotoxins.[12] Details of sample size and study recruitment centers can be found in this reference However, only three samples were selected randomly and used for method development purposes. Spot urine samples were collected from all subjects after washing their hands to avoid any contamination and collect the mid-stream urine sample in polythene bottles and samples were brought to the laboratory and kept under refrigerated conditions and preserved in a deep fridge at -20°C. All urine samples were obtained after approval from the Human Ethics Committee of the Institute. Before analysis, urine

samples were cleaned up by solid phase extraction (SPE) for removing matrix interferences. Strong anion exchange (SAX) SPE cartridges from Whatman (500 mg per 3 mL cartridge) were used for this purpose., A vac-Elute vacuum elution system was used for SPE. SAX cartridges are a quaternary ammonium bonded phase of the silica matrix, positively charged in the full pH range, and have a strong anion exchange capacity. SAX cartridges are mainly used for extracting weakly acidic compounds such as carboxylic acids. Matrix interferences with strong negative charge bind strongly on SAX cartridges and thus help in eliminating matrix interferences. First, for conditioning, the SAX cartridge was washed with methanol (3 times), followed by washing with pH 6.4 phosphate buffer solution (elution rate was adjusted to between 2 to 3 mL. 5 mL urine sample and 5 mL of phosphate buffer solution was mixed thoroughly and passed through SAX column. The SAX column was washed by 1 mL water, followed by 1 mL phosphate buffer and 1 mL of 1.0% aqueous acetic acid. The column was dried in air and analytes were eluted with 5 mL of 10% aqueous acetic acid.[2]

Results

During HPLC Method Validation following parameters were evaluated:

Selectivity, Calibration Curve and Linearity, Accuracy and Precision, Limit of Detection (LOD) and Quantitation (LOQ), Robustness, and Application of the method to real samples.

During Selectivity evaluation, no endogenous components extracted from blank urine were eluted at the retention time of the peak of HA and MHA (**Fig.1 and 2**). The developed method was found to be selective for the determination of HA and MHA without interference from the endogenous constituents of urine.

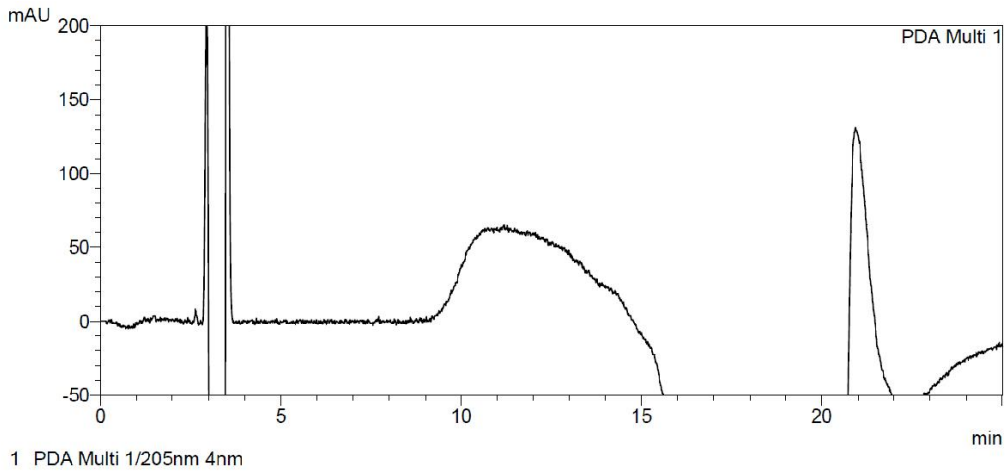

Figure 1: Chromatogram for Blank (Diluent)

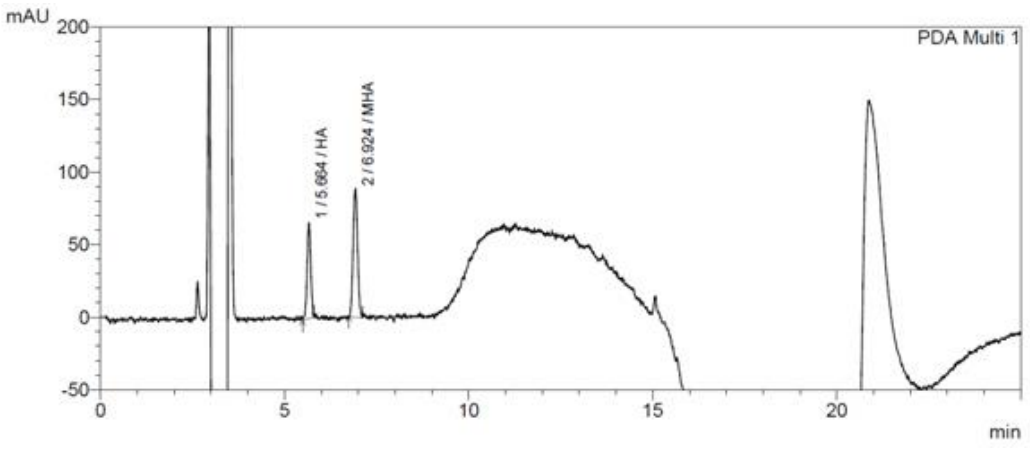

Figure 2: Chromatogram for HA and MHA under optimized conditions.

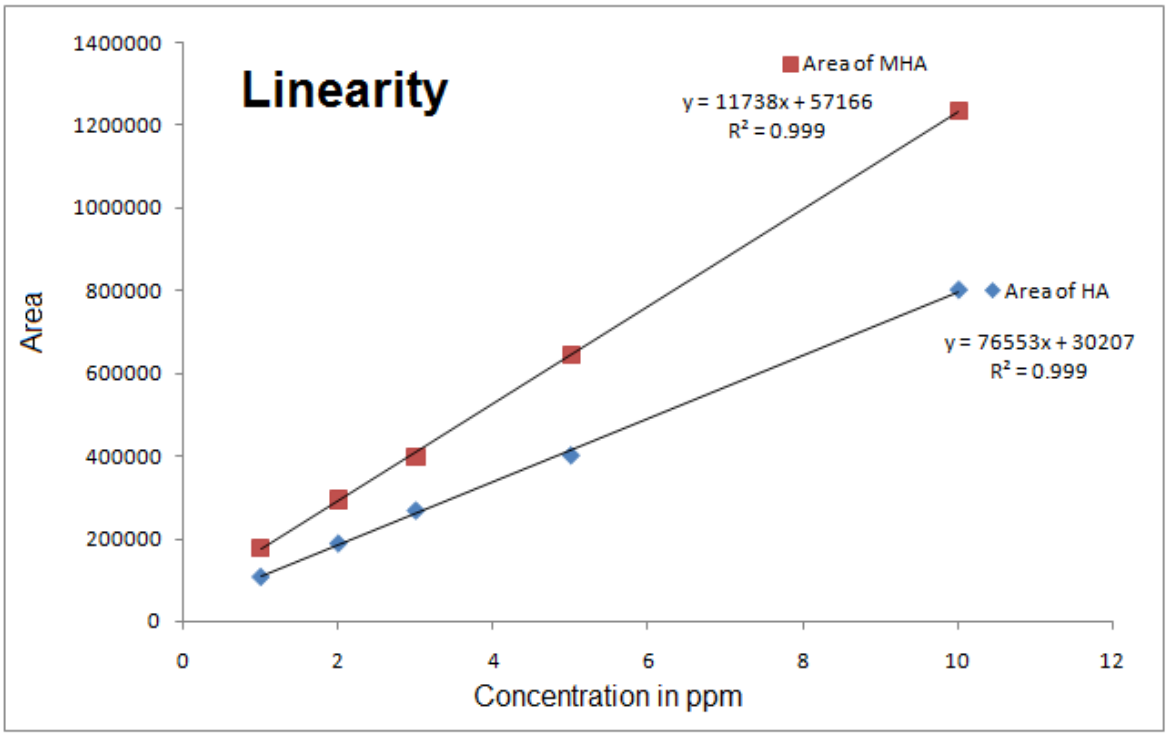

Figure 3: Linearity chart for HA and MHA

Analysis was based on the external standard method. Calibration curve and linearity studies were performed with calibration standards fresh on the day of analysis by diluting the appropriate working solutions with diluent. The standard calibration curve was constructed using blank urine samples spiked with HA and MHA at five different concentrations from 1, 2, 3, 5 and 10 mg/L. The data were subjected to statistical analysis using a linear regression model. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using Microsoft Excel 2007 software. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards. The developed method was linear over the tested concentrations with correlation coefficient $r^2 = 0.999$ (for HA) and $r^2 = 0.999$ (for MHA), the calibration curve (**Fig. 3**) was described by equations y = 76553x + 30207 (for HA) and y = 11738x + 57166 (for MHA).

Accuracy and precision studies were performed with, 5 mg/L concentration of HA and MHA, in three replicates were used to validate the accuracy and precision of the developed method. The results showed that the intra- and inter-day accuracy (% bias) for the method ranged between −14.8% and 1.7%, respectively for HA (**Table 1**). The % CV of intra- and inter-day precision was <8.85% for HA. The results showed that the intra- and inter-day accuracy (% bias) for the method ranged between −16.7% and 14.2%, respectively for MHA (**Table 2**). The % CV of intra- and inter-day precision was <9.23% for MHA. The developed method was thus found to meet generally accepted requirements of accuracy and precision over the studied concentration ranges

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Table 1: Accuracy, Precision and Recovery for HA

			Mean	Std. Deviation	%RSD	
Standard Area (Day-1)			503103	23611.28	4.7	
476445		Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
521385		118852	589554	470702	4.7	93.6
511478			600789	481937	4.8	95.8
			586578	467726	4.6	93.0
	Mean				4.7	94.1
	Std. Deviation				0.07	1.49
	% Relative Std. Deviation				1.58	1.58
Standard Area (Day-2)			481664	6728.629	1.4	
480863		Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
488757		131479	592500	461021	4.8	95.7
475371			604001	472522	4.9	98.1
			621307	489828	5.1	101.7
	Mean				4.9	98.5
	Std. Deviation				0.15	3.01
	% Relative Std. Deviation				3.06	3.06
Standard Area (Day-3)			537854	56275.77	10.5	
540587		Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
480262		139432	683837	544405	5.1	101.2
592714			597473	458041	4.3	85.2
			626738	487306	4.5	90.6
	Mean				4.6	92.3
	Std. Deviation				0.41	8.17
	% Relative Std. Deviation				8.85	8.85
	Overall Recovery					95%

Limit of Detection (LOD) and Quantitation (LOQ) were performed after sample clean-up, and the extracts from spiked urine were injected into the chromatographic systems. The analysis was carried out at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of 3:1. The LOD and LOQ for HA was 0.3 mg/L and 1.0 mg/L respectively and for MHA were 0.25 mg/L and 1.0 mg/L respectively.

According to the ICH guideline Q2A, "robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and indicates its reliability during normal usage".[11] Flow rate and temperature of the optimized method was changed deliberately and no drastic deviation was found by these alterations and the developed method was considered robust.

The developed method was used for three batches of samples that were analyzed after sample preparation and the result has been tabulated below (**Table 3**)

$$Assay = \frac{Area\ of\ analyte\ in\ sample}{Mean\ area\ of\ standard} \times Conc.\ of Standard$$

Table 2: Accuracy, Precision and Recovery for MHA

		Mean	Std. Deviation	%RSD	
Standard Area (Day-1)		768895	36203.59	4.7	
727214	Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
792523	65648	855513	789865	5.1	102.7
786946		863773	798125	5.2	103.8
		796417	730769	4.8	95.0
			Mean	5.0	100.5
			Std. Deviation	0.24	4.78
			% Relative Std. Deviation	4.75	4.75
Standard Area (Day-2)		739690	19424.47	2.6	
724339	Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
761528	51623	838496	786873	5.3	106.4
733204		753579	701956	4.7	94.9
		896272	844649	5.7	114.2
			Mean	5.3	105.2
			Std. Deviation	0.49	9.70
			% Relative Std. Deviation	9.23	9.23
Standard Area (Day-3)		824454	64365.06	7.8	
811230	Sample 3-8	Area of spike sample 3-8	Area spike sample 3-8 corrected	Conc. (mg/L)	Recovery %
767729	76950	851699	774749	4.7	94.0
894405		763767	686817	4.2	83.3
		868881	791931	4.8	96.1
			Mean	4.6	91.1
			Std. Deviation	0.34	6.84
			% Relative Std. Deviation	7.51	7.51
				Overall Recovery	98.9%

Table 3: Assay for HA and MHA in three batches of real urine samples.

Standard	Run 1	Run 2	Run 3	Mean	STDEV	%RSD
HA (5mg/L)	476445	521385	511478	503103	23611.28	4.7
MHA (5mg/L)	727214	792523	786946	768895	36203.59	4.7
HA		Sample 1	Sample 2		Sample 3	
		826443	562865		118852	
Conc. (mg/L)		8.2	5.6		1.2	
MHA		Sample 1	Sample 2		Sample 3	
		267520	90923		65648	
Conc. (mg/L)		1.7	0.6		0.4	

Conclusion

A simple, rapid, and robust HPLC method for the determination of HA and MHA was developed. The experimental results concerning linearity, accuracy, precision, specificity and sensitivity demonstrate the reliability of the procedure for its intended application. The method was successfully applied to test real urine samples for the determination of HA and MHA.

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