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Simultaneous Determination of Urinary Trans, Trans-muconic Acid (t,t-MA) and S-phenyl-mercapturic acid (SPMA) by Liquid Chromatography Tandem Mass Spectrometry

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Abstract: Benzene is an important industrial solvent and a ubiquitous environmental pollutant. Urinary S-phenyl mercapturic acid (SPMA) and trans, trans - muconic acid (t,t-MA) are specific and sensitive biomarkers for the determination of low levels of benzene exposure. This paper describes a specific and sensitive LC-MS/MS method for simultaneous determination of t,t-MA and SPMA from human urine samples using dual columns of two different internal diameters to reduce the matrix effect on ionization. This method was applied for the measurements of t,t-MA and SPMA from the random urine samples of exposed subjects of the footwear industry and comparable control subjects. The recoveries (Mean ± SD) of spiked standards of t,t-MA and SPMA in urine were 95.4 ± 12.3% (n = 6) ranging from 79.4 - 114% and 69.7 ± 9.5% (n=6) ranging from 60.1 - 89% respectively. The Limit of Detection (LOD) was found to be 1 ng/ml and 0.03 ng/ml and the Limit of Quantitation (LOQ) was found to be 5 ng/ml and 0.1 ng/ml for t,t-MA and SPMA at the S/N ratio of 3 and 10 respectively. The observed values of t, t-MA and SPMA in the exposed subjects of footwear industry were below the values of biological exposure indices (BEI) as described by ACGIH for benzene exposure.

Key words: Benzene; t,t-MA; SPMA; LC-MS/MS.

Introduction

Human exposure to benzene is a global health problem. Benzene is an important industrial solvent and a natural component of petroleum and gasoline. It is classified as a group I carcinogen by IARC, known to cause bone marrow damage and cancer after long term exposure to high concentration [1,2]. It is also a ubiquitous environmental pollutant due to its formation in many combustion processes. The industrial emission and traffic exhaust originating from the burning of fossil fuels as well as personal smoking habits or exposure to environmental tobacco smoke (ETS) contribute to the general population and some categories of workers [3,4,5]. The major effect of benzene leads to decrease in red blood cells, resulting in aplastic anemia and is associated with other blood disorders. It also causes leukemia and pre-cancers of the blood [6,7]. Occupational exposure to benzene in developing countries can be one or two magnitude higher than that in the USA [8, 9,10].

In many countries, proposals were made and adopted from the late 1980s, to lower the occupational exposure limit values for benzene from 10 to 1 ppm 8 h. Time weighted averages (TWA)]. In some countries, even lower values have been adopted, e.g. 0.5 ppm in Sweden and 0.3 ppm in USA [11,12]. It has become difficult to assess worker exposure to benzene as the exposure limits have been reduced to such low levels. Therefore, studies of biological monitoring of benzene must include identifying biomarkers

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that are sensitive and reliable at low-level exposure [13,14,15].

Benzene causes toxic effects via metabolism. It is metabolized by cytochrome P450 (CYP) enzymes to benzene oxide, which is the source of all other metabolites. S-phenyl mercapturic acid (SPMA) and trans, trans-muconic acid (t,t-MA) are both minor urinary metabolites of benzene exposure and are recommended for biological monitoring of benzene in the working environment by the DFG [16] and BEI- Committee [17].

Among these two minor metabolites of benzene, SPMA is considered to be a more specific biomarker for low level exposure to benzene because excretion of t,t-MA might be influenced by the uptake of sorbic acid (a widely used food preservative) [5,18,19]. SPMA is formed after conjugation of the highly reactive benzene oxide with glutathione. No dietary or other sources that would lead to the formation of urinary SPMA have been reported so far. Now a days, t,t-MA is utilized as a biomarker only at exposure level above 1 ppm (3.2 mg/m^3) [5]. Comparative investigation has shown that SPMA is suitable for benzene exposures down to 0.3 ppm (0.96 mg/m^3) [18] or 0.1 ppm (0.32 mg/m^3) [19]. Several HPLC and GC-MS methods have been published for the determination of urinary mercapturic acids of benzene. However, most of these methods determine only one metabolite [18,20-26]. Over the past decades, the LC-MS/MS gained widespread use for quantitation of drugs and their metabolites in biological matrices [5,27-34]. Several methods with isotopic dilutions have been developed on LC-MS/MS for the determination of t,t-MA and SPMA in urine samples of workers exposed to low levels of benzene [28,35].

Several studies have indicated that benzene is used as a component of the solvent, glues and paints used in the manufacture of shoes in the shoes making industry [36]. Employees are at risk of high exposure to solvents and glue containing high levels of benzene due to the enclosed working environment. Aplastic anemia, leukemia and other health problems were reported among Chinese footwear workers due to high levels of exposure to benzene, toluene and other toxic solvents contained in the adhesive used in the shoe making process [37].

The footwear industry is a significant segment of the leather industry in India and ranks second among footwear producing countries next to China. According to estimates, as many as 25000 children between the age of 10 and 15 yrs are mainly employed in shoe making both for the domestic and international markets. 80% of the children work for home contracts. Children work on soling (fixing upper portion of shoe to leather or rubber soles) with glue. The children working are exposed to benzene that is used as a solvent in glues. Most children suffer from respiratory problems, lung diseases and skin infections through constant exposure to glue and fumes.

In the present study, a LC-MS/MS method was standardized for simultaneous determination of two urinary metabolites of benzene namely t,t-MA and SPMA. The method was employed for the determination of these two biomarkers of benzene exposure in the urine samples of footwear industry workers mainly child workers.

Experimental

Chemicals & Materials

t,t-MA and SPMA reference standards were procured from Sigma-Aldrich; Methanol (MeOH), Acetonitrile (ACN) and HPLC water of LC grade were obtained from JT Baker Ltd, USA; Isopropanol, di-sodium hydrogen phosphate and mono-sodium dihydrogen phosphate were purchased from Merck (I) Ltd; Ethyl acetate, Creatinine and Formic acid were obtained from Fluka; Acetic acid and Hydrochloric acid were obtained from Across Organic, USA. Durapore Membrane filter from Milipore Ltd. were used for filtering the mobile phase. Quaternary strong anion exchange (QSAX), 100 mg/3cc cartridge for sample clean up and extraction of analytes was procured from Analchem Ltd, India.

Blank Urine

Blank urine sample for method validation was obtained from healthy, non-smoker individual and clean up as described earlier by Doctor et al [38].

Sample Collection

Spot urine samples from randomly selected

exposed child laborers working in the different processes of footwear manufacturing at Agra and matched control groups from school children studying in government school located in the neighboring area were collected in polyethylene container in cool condition and stored at -80°C. Frozen samples were transported in dry ice to the laboratory and preserved at -20°C until analysis.

Sample Clean up and Extraction of Analytes from Urine Samples

One ml urine sample of each subject was passed through a QSAX cartridge (100mg/3cc) that was preconditioned with 1 ml methanol (MeOH) and 1 ml 2 mM phosphate buffer (pH 6.8). Urine sample was applied at the slow flow rate without drying the cartridge. The cartridge was rinsed with 1 ml HPLC water, followed by 1ml 2mM phosphate buffer (pH 6.8) and 1 ml 0.1% aqueous acetic acid under full vacuum for 1 min. The analytes were eluted with 2 x 0.5 ml of 10 % aqueous acetic acid. Final eluant was transferred to 10 ml centrifuge tube. One ml ethyl acetate was added to the eluant and vortex for 2-3 minutes. Upper organic layer (0.5 ml) was separated into a vac-elute tube and evaporated it to dryness under nitrogen stream at room temperature. Dry residues were reconstituted in 0.5 ml of the mobile phase and 5.0 µl was injected for LC-MS/MS analysis.

Liquid Chromatography

Perkin Elmer series 200 system equipped with quaternary pump, auto sampler and Peltier column oven was used for the separation of analytes. HPLC separation was performed using dual columns connected in series to minimize the matrix suppression on ionization. Altima C18 - 50 x 2.1 mm id, 3 µm particle size (Altech) and C18 - 50 x 4.6 mm id, 5 µm particle size (Analchem ltd.) columns were used at the flow rate of 400 µl/min. A phenomenex universal guard column 4.0 x 3.0 mm id was used to protect the columns. Five micro liters of extracts were injected into the HPLC using a mobile phase consisting of HPLC water with 0.1% formic acid (Solvent A) and methanol with 0.1% formic acid (Solvent B). Gradient profile of the mobile phase is given in Table 1.

Mass Spectrometry

An API 3000 triple quadrupole mass spectrometer from Applied Biosystem was used for MS/MS analysis. The mass spectrometer was equipped with an electro spray ionization (ESI) interface. Mass and product ion spectra were acquired in negative ionization mode. MRM transitions were monitored for quantitative analysis of t,t-MA and SPMA. Mass to charged ion ratio (m/z) of 141.1 and 238.3 were selected as precursor ions (Q1), while m/z 96.9 and 109.1 were selected as productions (Q3), for t,t-MA and SPMA, respectively. For each analyte, the compound dependent parameters like declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and collision exited potential (CXP) were optimized by continuous infusion mode and the source dependent parameters like nebulising gas

Table 1. Mobile phase gradient for liquid chromatography

Time (min)	Flow (µl/min)	Solvent A (%) HPLC water with 0.1% Formic acid	Solvent B (%) MeOH with 0.1% Formic acid
0.0	400	40	60
3.0	400	40	60
7.0	400	80	20
9.0	400	80	20
11.0	400	40	60
14.0	400	40	60

(NEB), auxiliary gas (heater gas), and curtain gas (CUR). Ion spray voltage, and source temperature were optimized by flow injection analysis (FIA) mode. The MS parameters are given in Table 2.

Calibration Curve

Calibration curves were prepared by processing spiked blank urine sample of a healthy, non-smoker individual. Calibration standards were prepared by spiking blank urine to final concentrations of 5.0, 10.0, 20.0, 25.0, 40.0, 50.0 and 100.0 ng/ml of t,t-MA and SPMA mix prepared from reference stock standards. Calibration curves were prepared by plotting peak areas versus concentrations of the standards.

Recovery, Precision and Accuracy

The recovery of t,t-MA and SPMA was calculated by comparing the area response of extracted and non-extracted standard solutions containing t,t-MA and SPMA six times. The interday and intraday accuracy and precision were determined from the analysis on three independent QC samples tested on the same day and the five different days of the study. Precision is expressed as the relative standard deviation (RSD %) of the values found over the mean for each concentration.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were defined as three and ten times, respectively the standard deviation of the LC/MS/MS peak areas detected at the retention times of the analytes of interest in the blank urine samples.

Matrix Effect on Ionization

The matrix effects in LC-MS/MS analysis have been reported previously [39]. The matrix effects can decrease the response of analyte, thus affecting the sensitivity or precision of the analytical method. It is important in the analysis of biological samples to reduce the risk of interferences from the matrix itself.

Post column infusion experiments were performed to assess ion suppression. By using an infusion pump, a continuous post column infusion of the standard solution of analytes was introduced into the analytical LC system through a T-connector, during injection of an extract of urine. Ion suppression of the signal of the analytes by the urinary matrix was examined as negative chromatographic peak from the elevated baseline.

Creatinine Measurement

24 hours creatinine clearance is a measure of kidney function. Urinary creatinine provides a good adjustment for the variations in concentration of spot urine samples due to differences in fluid intake among workers. Creatinine of spot or random urine sample is a measure of concentration or dilution of the urine. Therefore, creatinine of all the spot urine samples was measured by the Alkaline Picrate Method [40]. All the results were expressed as t,t-MA and SPMA µg/g creatinine.

Results and Discussion

Total run time of analysis was 14 minutes. t,t-MA was eluted at 3.1 min. and SPMA was eluted at 5.3 min. The extracted ion chromatogram of t,t-MA and SPMA is show in Fig. 1. The calibration curves were linear at concentrations of 10, 20, 30, 40, 50 and 100 ng/ml for t,t-MA (r^2 = >0.99) and 5,10,15,20,40 and 50 ng/ml for SPMA (r^2 = >0.99) (Fig. 2 & 3). The LOD for t,t-MA standard was 1 ng/ml and that for SPMA was 0.03 ng/ml while LOQ for tt-MA was 5 ng/ml and for SPMA was 0.1 ng/ml at the S/N ratio of 3 and 10 respectively. The recovery (Mean ± SD) of spiked standard of t,t-MA (40 ng/ml) in urine was 95.4 ± 12.3 % (n=6), ranging from 79.4 -114 % and for

Table 2. Instrument parameters for mass spectrometry

Analyte	Precursor	Product	DP	FP	CE	CXP
t, t-MA	141.1	96.9	-40	-60	-12	-5.5
SPMA	238.3	109.1	-18	-50	-15	-8

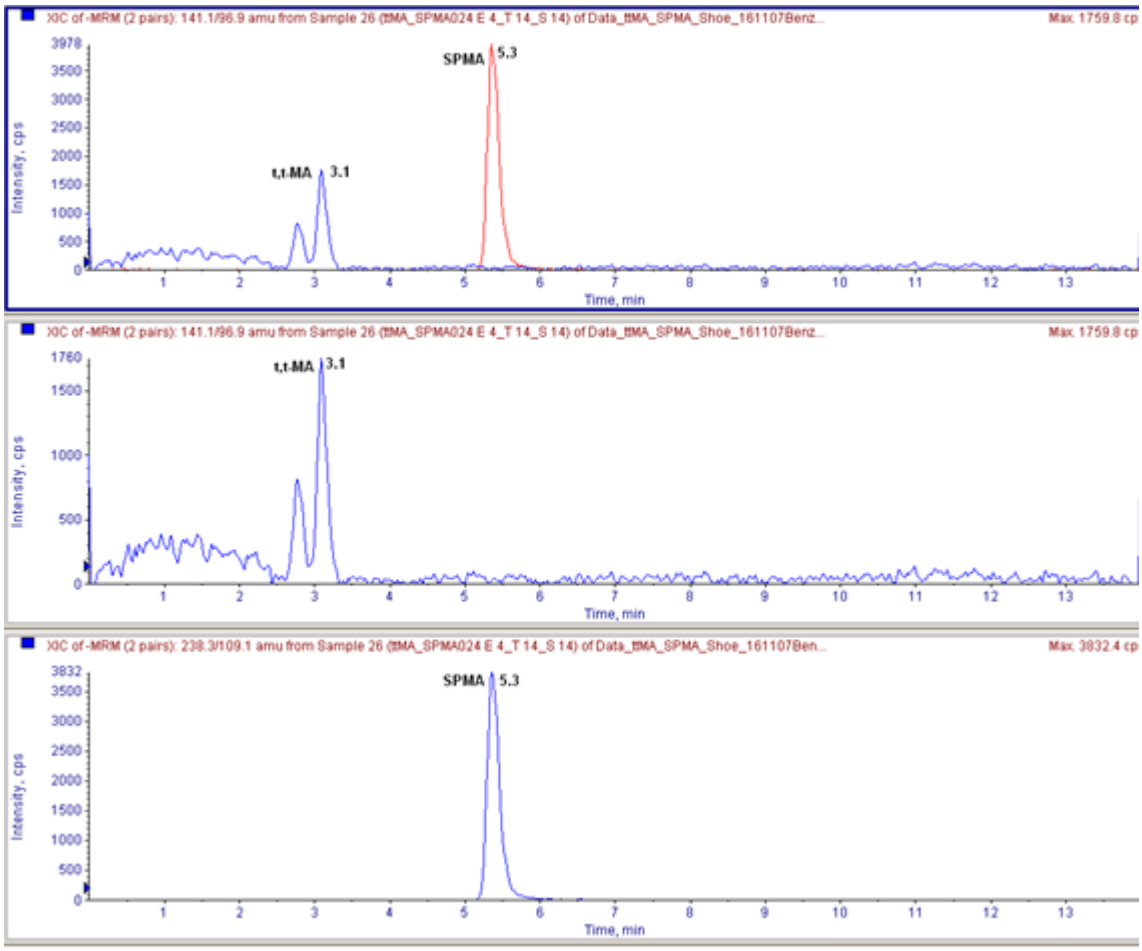

Fig. 1. Extracted ion chromatogram of t,t-MA & SPMA

SPMA (20 ng/ml) it was 69.7 ± 9.5 % (n=6), ranging from 60.1 - 89 %. The intraday and interday coefficient variation (% CV) were below 20 % for both t,t-MA and SPMA, which met the FDA acceptance criteria.

During the investigation of ion suppression effect of urine, the negative peaks revealed that the matrix effect was not the same for all retention time [41]. Therefore, analyte's retention in column was increased by using dual column of different internal diameters, which allows the reduction in the matrix effect on ionization of analyte [39]. Choi et al [42] also observed during their experiment that co-elution of matrix components and analyte from the LC column may be most strongly attributed to column overloading rather than similar analyte and matrix retention behavior. They used two dimensional (LC-LC) separation approaches to address signal suppression effects for the quantitative LC-MS/MS analysis of complex mixture samples.

Quantitation of urinary t,t-MA and SPMA in foot wear industry

Urine samples in which the creatinine concentration was either below 0.3 or above 3.0 g/L were excluded from the data analysis because highly diluted or highly concentrated urine samples are generally not suitable for analysis [43]. Total 50 urine samples, i.e. 30 of exposed child laborers of footwear industry and 20 controls were selected randomly from the collected urine samples for the quantitation of urinary t,t-MA and SPMA. Tables 3, 3-a and 3-b shows the Mean ± SE values of urinary concentration of tt, MA and SPMA from exposed and control subjects in all workers, only male workers and only female workers of footwear industry respectively. Statistical analysis

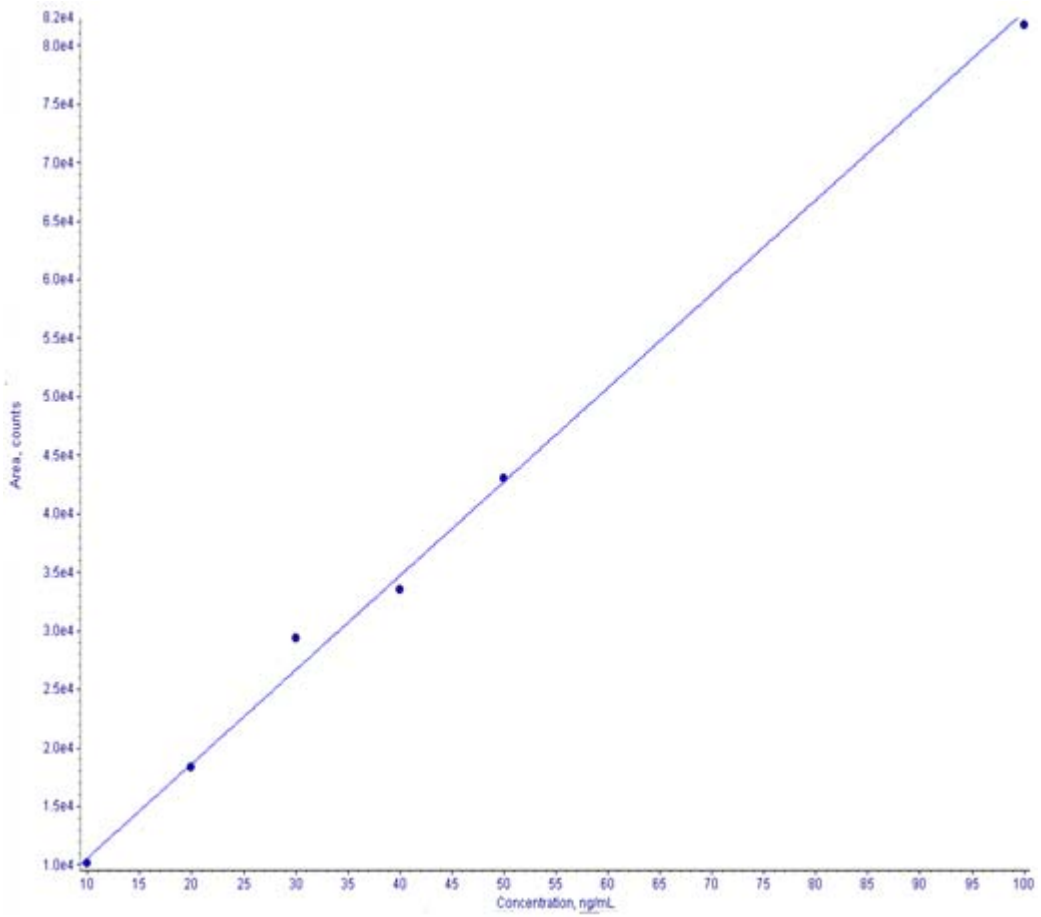

Fig. 2. Calibration curve of t,t-MA.

Table 3. Urinary concentrations of t, t-MA and SPMA in footwear industry worker

Metabolites	Concentration of t,t MA & SPMA (µg/g creatinine)			
	Exposed (30)		Controls (20)	
	Mean ± SE	Range	Mean ± SE	Range
t,t-MA	48.87 ± 7.58	1.51 - 174.52	27.02 ± 4.72	2.09 - 71.2
F value	3.87			
P value	0.0032 (**)			
SPMA	1.53 ± 0.70	0.07 - 21.3	0.96 ± 0.25	0.04 - 4.08
F value	11.5			
P value	< 0.0001 (***)			

of the data was carried out with Graph pad prism 5 software using unpaired‘t’ test and ‘F’ test.

As shown in the Table 3, the mean concentration of urinary t,t-MA in the exposed and control samples was 48.87 ± 7.58 and 27.02 ± 4.72 µg/g creatinine respectively. While the mean concentration of urinary SPMA in the exposed and control samples was 1.53 ± 0.70 and 0.96 ± 0.25 µg/g creatinine respectively. Results show that the concentrations of t,t-MA ($F=3.87$, $p=0.0032$) and SPMA ($F=11.5$, $p < 0.0001$) were significantly higher with respect to control. But in comparison to t,t-MA, the concentration of SPMA was found to be highly significant.

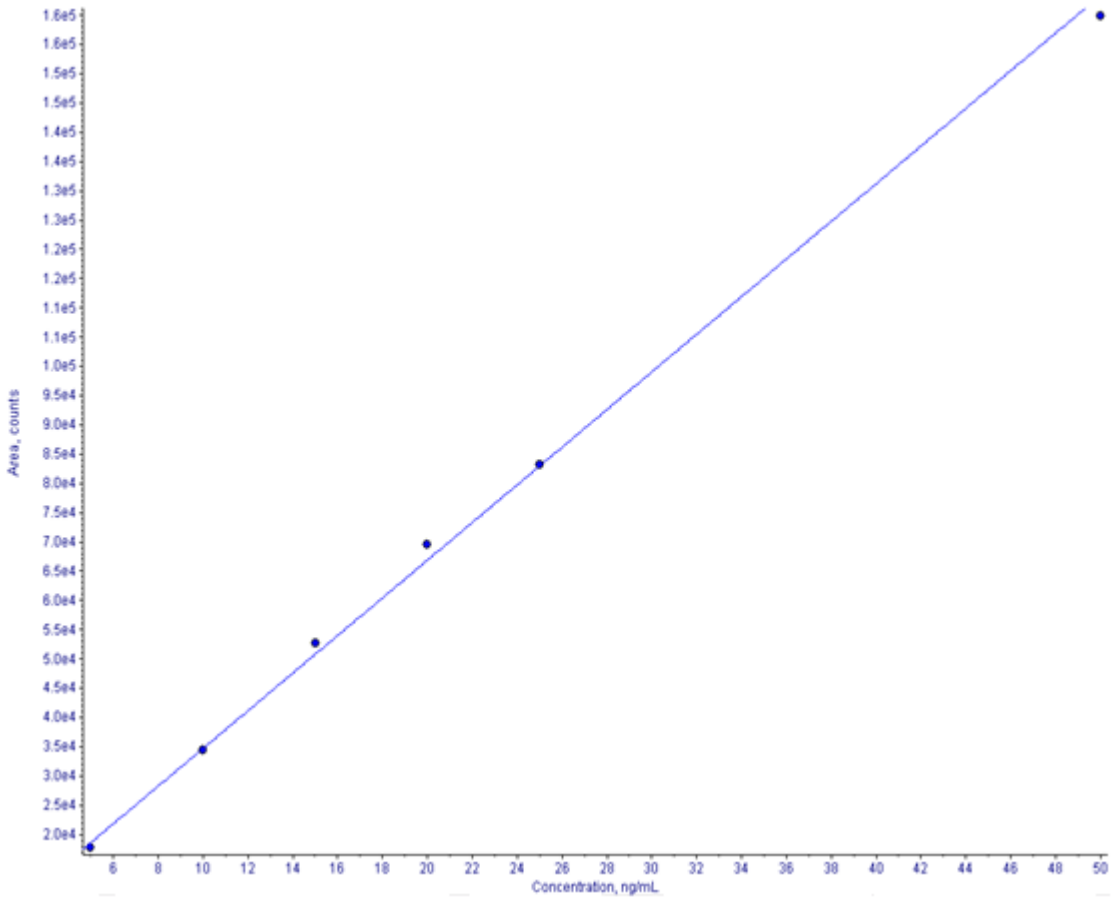

Fig. 3. Calibration curve of SPMA

Table 3a. Urinary concentrations of t, t-MA and SPMA in male workers of footwear industry

Metabolites	Concentration of t,t MA & SPMA (µg/g creatinine)			
	Exposed Male (19)		Control Males (14)	
	Mean ± SE	Range	Mean ± SE	Range
t,t-MA	51.70 ± 10.48	1.51 - 174.52	31.06 ± 6.05	2.35 - 71.2
F value	4.075			
P value	0.0133(*)			
SPMA	1.79 ± 1.10	0.07 - 21.3	0.91 ± 0.27	0.04 - 3.67
F value	23.31			
P value	< 0.0001(***)			

Based on the sex-wise classification of the footwear industry workers, it was found that the concentrations of t,t-MA ($F=4.075$, $p=0.0133$) and SPMA ($F=23.31$, $p < 0.0001$) were significantly higher in the exposed male workers as shown in the Table 3-a. While a non-significant increase in the concentration of t,t-MA was found in case of the female workers and the concentration of SPMA was more or less same with respect to control as shown in the Table 3-b.

In the present study all the subjects were in the age group of 10-15 years and all were non-smokers. In the present, study the urinary concentrations of SPMA in the exposed subjects

Table 3b. Urinary concentrations of t, t-MA and SPMA in female workers of footwear industry

Metabolites	Concentration of t,t MA & SPMA (µg/g creatinine)			
	Exposed Female (11)		Control Female (6)	
	Mean ± SE	Range	Mean ± SE	Range
t,t-MA	44.00 ± 10.45	6.13 - 111.92	17.61 ± 5.91	2.09 - 38.41
F value	5.73			
P value	0.0677 (NS)			
SPMA	1.07 ± 0.27	0.21 - 2.72	1.09 ± 0.61	0.17 - 4.08
F value	2.785			
P value	0.158 (NS)			

NS - Non-significant

were found to be higher which was at the levels of heavy smokers found in a study carried out by Melikan *et al* [5]. The concentrations of urinary SPMA analyzed by GC-MS and HPLC methods, reported in the literature were 3.61 and 9.4 µg/g creatinine in smokers and 1.99 and 1.5 µg/g creatinine in nonsmokers [18,44] and so far t,t-MA was reported in the range of 0.14 to 0.61 mg/g creatinine and 0.05 to 0.21 mg/g creatinine in smokers and nonsmokers respectively [18,20,25,26].

Thus, current values of our study are in agreement with the literature data and it indicates the exposure of benzene among footwear industry workers.

Epidemiological reports from Italy, Turkey and Great Britain have shown an association between employment in shoe and boot manufacturing industry and an increased risk of leukemia mortality among workers [45-48]. The workers were found to be at risk where exposure to solvents and glues containing high levels of benzene was common.

Previous studies have indicated that the exposure level could influence the ratio of benzene metabolites. At low levels of benzene exposure, excretion of t,t-MA can be relatively high even when SPMA excretion is very low [49,50]. Urinary SPMA in workers exposed to 0.5 ppm benzene has been reported in the range from 7.2 to 25 µg/g creatinine while the predicted excretion of t,t-MA ranged from 0.39 to 1.1 mg/g creatinine for 0.5 ppm benzene exposure [5]. As t,t-MA is a metabolite of sorbic acid [27] the values of t,t-MA in urine are strongly influenced by the consumption of food or drinks containing sorbic acid which is used as a preservative, while SPMA is a specific biomarker of benzene exposure at low level. SPMA is formed after conjugation of the highly reactive benzene oxide with glutathione. So far no dietary or other sources that would lead to the formation of urinary SPMA have been reported. Nowadays, t,t-MA is utilized as a biomarker only at exposure level above 1 ppm benzene (3.2 mg/m^3) [5]. Comparative investigation has shown that SPMA is a suitable biomarker for benzene exposures down to 0.3 ppm (0.96 mg/m^3) [18] or 0.1 ppm (0.32 mg/m^3) [19]. It was observed by Wiesel et al [51] that at relatively low benzene exposure levels, the complexity of the urine matrix suppressed the ionization. The suppression of monitored analytes by co-eluting matrix compound was also observed in our method. Therefore, dual columns of different dimensions were used to reduce the matrix suppression on analytes.

Conclusion

Previously, several methods have been described for the determination of t,t-MA and SPMA. But most of these methods determined only one metabolite. In this study, a sensitive and specific LC-MS/MS method has been standardized; matrix suppression on ionization has been minimized by using dual columns of different internal diameters. This method has been used successfully for the simultaneous measurements of two urinary metabolites, t,t-MA and SPMA in

the urine samples of shoe making industry. In the present study, both t,t-MA and SPMA concentrations were found significantly higher with respect to controls but the concentration of SPMA was found to be highly significant. The observed values of t,t-MA and SPMA in this study were found below the values of Biological Exposure Indices (BEI) i.e. 500 µg/g creatinine for t,t-MA and 25 µg/g creatinine for SPMA, described by ACGIH [52] of exposure to benzene. Thus, this study suggests that the workers in shoe manufacturing units in India are exposed to low levels of benzene.

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