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Research Article

DETERMINATION OF HIPPURIC ACID AND METHYL HIPPURIC ACID IN URINE AS INDICES OF TOLUENE AND XYLENE EXPOSURE BY HPLC MS/MS SPECTROMETRY

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ABSTRACT

This main objective of the present investigation was to detect and quantify the concentration levels of hippuric acid and methyl hippuric acid as indices of exposure to toluene and xylenes air pollutants in the urine of subjects exposed to Benzene Toluene Ethyle benzene Xylene (BTEX) -air pollutants. Fifty seven urine samples were collected from individuals working in nine different locations in Khartoum city including six Gasoline stations; two industrial areas and one control site were examined using HPLC MS/MS analysis. Average concentration levels of hippuric acid (HA) and methyl hippuric (MHA) acid 776.0612 ppm, 468.824 ppm were detected, respectively in the urine samples collected from subjects working in gasoline stations and concentration levels of HA and MHA of 60.5531ppm, 36.1945 ppm, respectively were detected for samples collected from subjects working in industrial areas. On the other hand, the urine samples collected in the control site showed an average of 27.6275 ppm, 16.297 ppm for HA and MHA, respectively. Statistical analysis revealed that all of these results were significantly different ($P< 0.05$). More importantly, the results not only revealed the degree of exposure to toluene and xylenes to which these workers were subjected to, but also revealed the levels of these pollutants in the surrounding environment.

Keywords: Hippuric acid, Methyl hippuric acid, HPLC MS/MS, Urine.

INTRODUCTION

There are many definitions of pollution as a serious phenomenon in human surroundings. Generally speaking, pollution is the contamination of the environment incurred by the human activity or natural disasters.There are different kinds of pollution, including air, water, soil, food, and radioactive pollution (Baird, 1998). Toluene and o-, m- and p-xylene have been widely used, separately and as mixtures, as organic solvents, ingredients of thinners, and in the synthesis of otherchemicals. A mixture of the three isomeric xylenes is commonly called 'xylol'. *Invivo,* toluene is oxidized and then conjugated with glycine and excreted in urine as hippuric acid (HA).Toluene is readily absorbed from the gastrointestinal tract after ingestion, and is distributed preferentially in adipose tissue, then the kidneys, liver and brain. The main effect of toluene is on the brain and nervous system, with fatigue and drowsiness being the most obvious symptoms (ATSDR, 2010).*In vivo,*the xylene isomers are oxidized to o-,m-and p-toluic acid, then conjugated with glycine and excreted in urine as o-, m- and p-methylhippuric acid (MHA)(Arthur and Curnock, 1982).Xylenes are readily absorbed after inhalation. Both short- and long-term exposure to high concentrations of xylene can also cause a number of effects on the nervous system, such as headaches, lack of muscle coordination, dizziness, confusion, and changes in one's sense of balance as well as irritate the eyes and respiratory tract (ATSDR 2010, IPCS 1993).

HA and MHA are used as biological markers in studies of occupational exposure and are used to monitor these industrial solvents because they show a good correlation with the level of exposurein workers exposed to organic solvents (Paola Maniniet *et al*. 2004; Kingsbury and Swanson, 1921).

Several methods, including high-performance liquid chromatography (HPLC) with detection by mass spectrometry (MS) combined with mass spectrometric (HPLC MS/MS) detection have been used for the analysis of HA and MHA in urine(Pleasance *et al*. 1992). It is a practical, sensitive and specific laboratory assay, and enables detection of the levels of exposure to aromatic solvents in factory workers and in the general population (Linscheid and Westmoreland, 1994). The applications of HPLC MS/MS includedetection ofenvironmental (Pesticides, Polycyclic Aromatic Hydrocarbons, Organometallic compounds and Technical Products,biomedical (Amino Acids, Nucleosides and Nucleotides, Saccharides, Peptides and Proteins, Bile Acids) and pharmaceuticals (Drug Metabolites, Natural products components) (Perkins and Tomer, 1992).

Within the last two decades diseases (cancer, asthma etc) that are directly related to air pollution are increasing to an alarming rate. Cancer and asthma cases were observed among patients irrespective of the gender or ages. Several conferences and seminars local or international were organized to study this epidemic problem. Presumably, it either be attributed to the feed we eat, the water we drink or the air we breathe.Accordingly, this research work aimed at and meant to address the problems caused by air pollution in Khartoum State, Sudan.

Generally, this study was conducted to run out a short-term pilot industrial hygiene survey to determine, qualitatively & quantitatively, the limits of industrial pollution by toluene and xylene and to estimate the environmental toluene and xylene through the determination of their urinary metabolites in urine samples collected from individuals working in petroleum stations and industrial areas, in addition to students from University of African International University, Sudan. Moreover, the study aimed to raise the awareness of environmentalists, the concerned Government Quarters and the public of the health hazards of these pollutants. The specific aims of the current study are to conduct trials of investigating the other possible metabolites such as isomeric Cresols besides measurements of urinary hippuric acid and methyl hippuric acid as indices of toluene and xylene occupational exposure.

MATERIALS AND METHODS

Samples collection

Fifty seven urine samples were collected from individuals working in nine different locations in Khartoum State that included six petrol stations, two locations in the industrial area and one location in African International University as control. Age, occupational duration, health state and smoking were taken into consideration for each individual. The collected urine samples were kept in -20º C until analysis.

Ethical clearance

Written informed consents signed by participants were obtainedbefore collection of samples. Ethical approval was obtained from Ministry of health, Khartoum, Sudan.

Solid phase extraction (SPE)

SPE is a useful sample preparation technique than liquid / liquid extraction technique. This was clearly documented in the current study in which SPE technique was performed. The instrument had been calibrated via injecting, increasing and varying concentrations of hippuric acid and methyl hippuric acid in separate runs. Linear curves were obtained for both standards which are shown previously (Fig. 1, 2).

Extraction procedure

100µL of each urine sample were diluted 100-fold with distilled water and adjusted to pH ~ 2 with hydrochloric acid. Urine samples were passed through Oasis HLB extraction cartridge with 500mg packing material, from Waters (Milan, Italy), that was preconditioned with 6 ml of MeOH and 6 ml of $H2O$. The cartridge was then eluted with two 5ml aliquots of acetone. The eluate was evaporated to dryness at about 30C under a stream of nitrogen for approximately 15 min, and the dried extract was re-dissolved in 500 µL of water / methanol solution (70:30, v/v) and transferred to an injection vial (Marcheseet *et al*., 2004).

The processed urine samples were then subjected to the general extraction protocol based on 30 mg sorbent mass according to SPE reference manual and user's guide, Phenomenex. The protocol was performed in 5 steps as follows: conditioning, equilibrating, loading, washing and eluting(Zwir-Ference and Biziuk, 2006).

RESULTS AND DISCUSSION

Average concentration levels of hippuric acid (HA) and methyl hippuric (MHA) acid 776.0612 ppm, 468.824 ppm were detected, respectively in the urine samples collected from subjects working in gasoline stations and concentration levels of HA and MHA of 60.5531ppm, 36.1945 ppm, respectively were detected for samples collected from subjects working in industrial areas. On the other hand, the urine samples collected in the control site showed an average of 27.6275 ppm, 16.297 ppm for HA and MHA, respectively.

In the present study statistically significant difference ($P<0.05$) was detected in HA concentration between petrol station workers, industrial area workers and control groups (Table1). The results showed that a significant difference ($P<0.05$) was observed in MHA concentration between three study groups (Table 2).

The prevalence of HA and MHA in the different study groups in Khartoum State, Sudan was showed in (Table 3).

Significant difference ($P<0.05$) was detected in HA and MHA concentrations in urine samples collected from gasoline stations regard to occupational duration and health state as a risk factor (Table 4).

The current study is one part of a wider research project which involves the identification and determination of volatile organic compound (VOCs) and polyaromatic hydrocarbons (PAHs) pollutants in the ambient air in Khartoum city. The former study, which was aimed to the determination of the concentration levels of BTEX pollutants is directly associated with the present investigation in the context that the BTEX-mixture of air pollutants is composed of benzene, ethyl benzene and xylenes (m and p-xylene). Previous research studies have shown that toluene and xylenes are the precursors of the urine metabolites hippuric acid (HA) and methyl hippuric acid (MHA). Accordingly, the main objective of the present study is of two folds. In one hand, the identification and determination of the concentration levels of HA and MHA in the urine of subjects exposed to BTEX-pollutants would reveal the degree of exposure of these subjects to an air which is predominantly polluted with both toluene and xylenes i.e. the mixture of BTEX-pollutant. On the other hand, the present study would also reveal the resulting health hazards these subjects are exposed to. In order to fulfill these two objectives an efficient method of clean up was used, which is the solid phase extraction (SPE) method and an accurate, sensitive and high precision analytical method was chosen and adopted which is HPLC/ MS/MS.

In the current study the mean concentration (ppm) of HA was 776.0612 ± 65.54727, 60.5531 ± 10.63083 and 27.6275 ± 3.73042 in gasoline station workers, industrial area workers and nearby residential area respectively and the mean concentration (ppm) of MHA was 468.824 ± 39.632, 36.1945 ± 6.4259 and 16.297 ± 2.25553 in gasoline station workers, industrial area workers and nearby residential area respectively.

Statistically significant difference ($P< 0.05$) was detected in HA concentration among the three different groups with the highest concentration in gasoline station workers group (776.0612 ± 65.54727). This may be attributed to the fact that gasoline station workers in particular are usually extensively exposed to the toluene more than the other studied groups. Significant difference ($P< 0.05$) was also detected in MHA concentration among studied groups with high concentration in gasoline station workers (468.824 ± 39.632). These results also indicated high levels of these pollutants in the surrounding ambient air.

The current study have also revealed that the percentage prevalence of HA in gasoline stations workers, industrial area workers and nearby residential area was 82.8%, 42.9% and 42.9% respectively whereas, the percentage prevalence of MHA in gasoline stations workers, industrial area workers and nearby residential area was 48.3%, 42.9% and 35.7% respectively.

Risk factor analysis of HA and MHA concentrations in urine samples collected from gasoline stations workers suggested that there was a significant relationship ($P< 0.05$) regard to occupational duration and health state (Table 4).

The extremely high level of concentration of HA and MHA in the urine samples of the gasoline station workers is attributed to the high levels of toluene and xylenes in the ambient air of this site, compared to their lower levels in industrial and residential sites. The latter two sites are far away from the BTEX-source.

CONCLUSIONS

It could be concluded that high level of HA and MHA were detected in examined urine samples as indices of toluene and xylene pollution in petroleum stations and industrial areas workers in Khartoum State. Therefore, authorities and environmental specialists should set regulatory laws that ensure preservation of a healthy atmosphere for the protection of these workers and inhabitants of nearby residential areas.

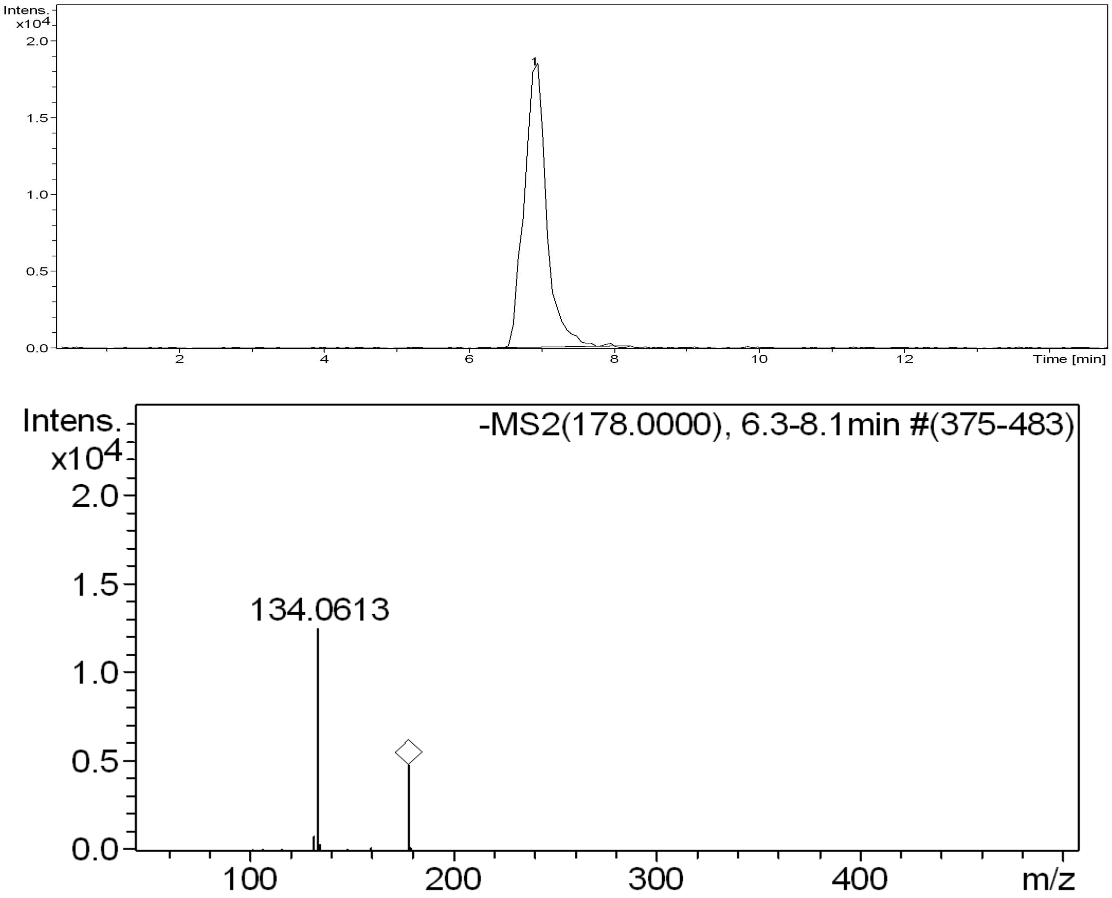
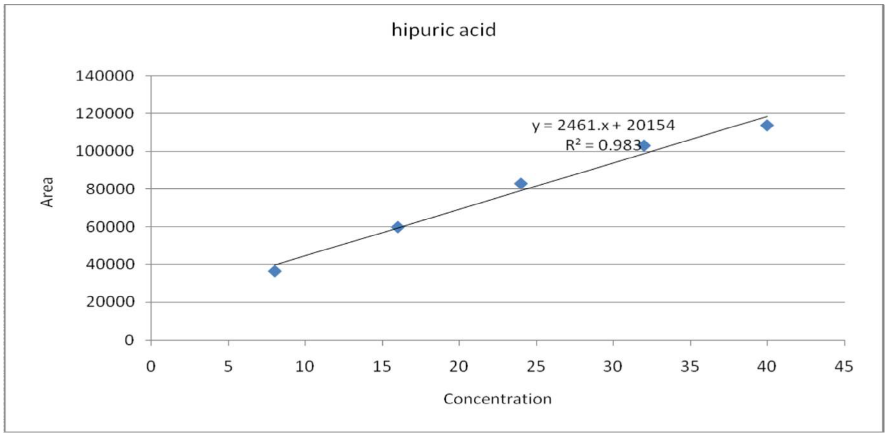


Fig. 1: Standard of hippuric acid



Standard curve of hippuric acid

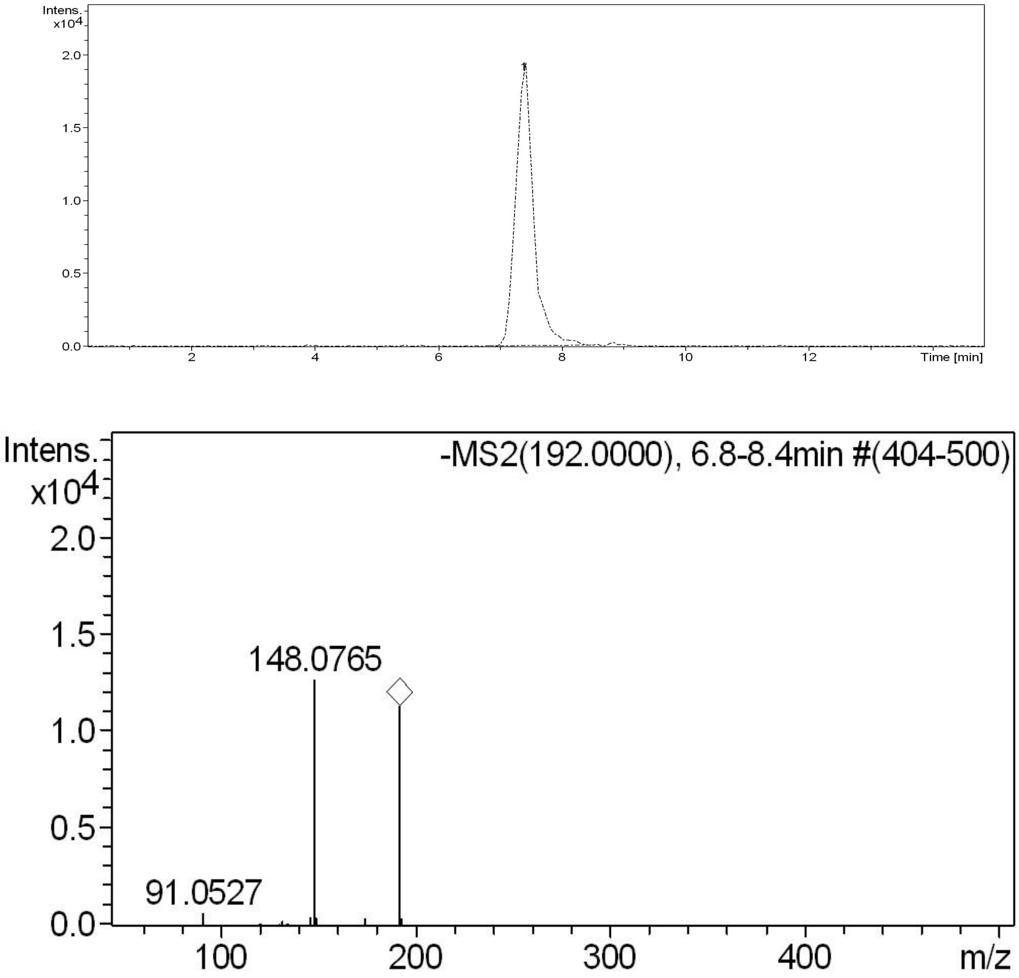
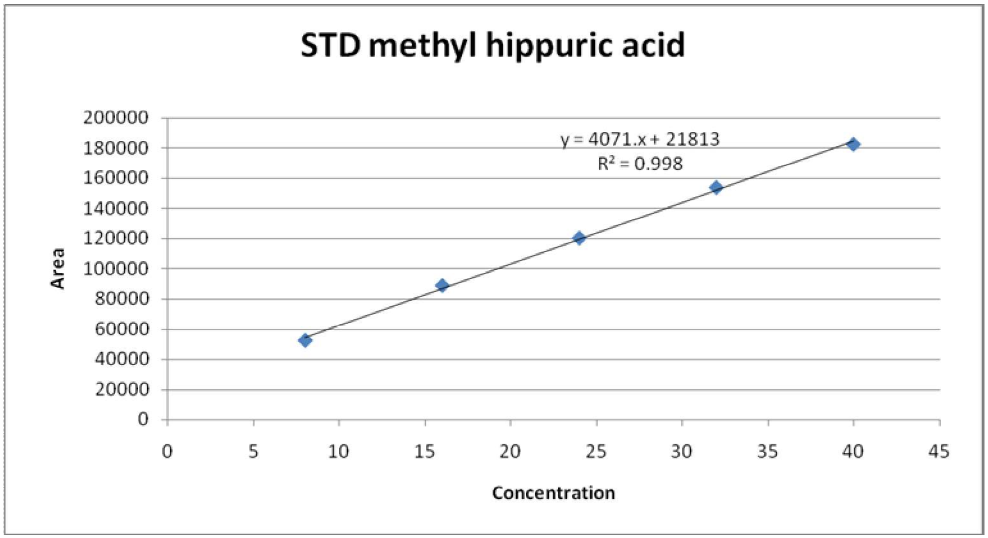


Fig. 2: Standard of methylhippuric acid



Standard curve of methyl hippuric acid

Table 1: Mean concentration (µg/ml) ± SE of Hippuric acid for the three groups in Khartoum State

Group	Number	Mean ± SE	*P* value
Petrol	29	776.0612 ± 65.54727	*P*<0.05*
Industrial	14	60.5531 ± 10.63083	
Control	14	27.6275 ± 3.73042	

* The mean difference between three groups is significant

Table 2: Mean concentration (µg/ml) ± SE of methylhippuric acid for studied three groups in Khartoum State

Group	Number	Mean ± SE	*P* value
Petrol	29	468.824 ± 39.632	*P*<0.05*
Industrial	14	36.1945 ± 6.4259	
Control	14	16.297 ± 2.25553	

* The mean difference between three groups is significant

Table 3: The prevalence of hippuric and methyl hippuric acid in the three study groups in Khartoum State, Sudan

Group	Hippuric acid (%)	Methyl hippuric acid (%)
Petrol	24/29 (82.8%)	14/29 (48.3%)
Industrial	6/14 (42.9%)	6/14 (42.9%)
Control	6/14 (42.9%)	5/14 (35.7%)

Table 4: Influence of selected risk factors on HA and MHA concentrations in urine samples collected from gasoline stations in Khartoum State, Sudan

Variable	Group	HA		MHA		P value
		Mean	SD	Mean	SD	
Age	< 35 yrs	712.88	284.72	430.63	172.15	*P> 0.05*
	> 35 yrs	853.82	421.34	515.84	254.75	
Occupational duration	< 7 yrs	674.01	327.83	407.12	198.22	*P< 0.05*
	> 7 yrs	901.66	354.13	544.77	214.12	
Healthy state	healthy	734.79	329.40	443.87	199.17	*P< 0.05*
	sick	1333.25	25.82	805.72	15.61	
Smoking	Yes	802.86	349.90	485.03	211.57	*P> 0.05*
	No	759.68	363.94	458.92	220.05	

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