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High-performance Liquid Chromatographic Determination of Urinary *Trans*, *Trans*-Muconic Acid Excreted by Workers Occupationally Exposed to Benzene

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Objective To investigate the relationship between t*rans*, *trans*-muconic acid (*tt*MA) as benzene metabolite of occupational workers and benzene concentration in air. **Methods** A rapid and sensitive high-performance liquid chromatography was developed to determine the level of urinary *tt*MA. *tt*MA was extrated from urinary samples in liquid-liquid phase a ODS (2) (5u) column (Φ4.6 mm×150 mm) and detected at wavelength 264 nm in a UV detector using vanillic acid as an internal standard. The mobile phase was acetaticacid/tetrahydrofuran/methanol/water (v/v, 1:2:10:87). The method was validated with 56 urine samples collected from occupationally benzene-exposed individuals. **Results** A correlation coefficient (r = 0.9963) was found for *tt*MA ranging 0.10-10.00 μg/mL. The limit of detection was 0.10 μg/mL. The recovery and reproducibility were generally over 90%. There was a positive correlation between *tt*MA and benzene level in air. The equation was Y=0.859＋0.108C (before work, r=0.6200) or Y=1.980＋0.179C (after work, r=0.7930). **Conclusion** This method can be used to determine and control the level of urinary *tt*MA in those who are occupationally exposed to benzene.

Key words: Benzene; *Trans*; *Trans*-muconic acid; High-performance liquid chromatography

INTRODUCTION

Benzene exists ubiquitously as an environmental contaminant to which the general population are exposed, due to its occurrence in mineral oil and formation in many combustion processes[1]. It is mutagenic and carcinogenic in animal models and interrelates to bone marrow toxicity and leukemia[2-4]. The risk of developing leukaemia has been estimated in approximately six cases per million people who experience lifelong exposure to benzene concentrations of 1 μg/m^3 in air [5]. So monitoring and control of benzene exposure are of great importance[6-8].

The main route for the uptake of benzene at workplace and from environment is *via* inhalation. Benzene is primarily metabolized in the liver to a series of ring-hydroxylated and conjugated metabolites as well as ring-opened products, which are excreted in urine[9]. Benzene is metabolized *in vivo* by transforming to phenol, hydroquinone, and catechol[10-11]. These metabolites are mainly excreted in urine after conjugation with glucuronide and sulphate or undergo further metabolism to 1,4-benzoquinone and 1,2,4-trihydroxybenzene. Benzene is also transformed to *trans, trans*-muconic acid (1, 3-butadiene-1, 4-dicarboxylic acid, *tt*MA) after the ring-opened oxidation process. Among these benzene metabolic products, *tt*MA, a short-chain dicarboxy acid as a main metabolite of benzene ring cleavage, has been used as a potential biomarker for benzene exposure.

*tt*MA as a marker is also determined by gas chromatography-mass spectrometry (GC-MS)[12] and liquid chromatography-mass spectrometry (LC-MS)[13]. Although these methods are highly sensitive, they are not suitable for the routine analysis in industry. The aim of this study was to develop a simple, rapid, sensitive HPLC method for evaluating the level of urinary *tt*MA excreted by workers occupationally exposed to benzene.

MATERIALS AND METHODS

Drugs and Reagents

*tt*MA and vanillic acid (internal standard, IS)

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were purchased from Sigma (St. Louis. Mo. USA). Methanol, acetic acid and tetrahydrofuran were of HPLC grade.

Ten mg of *tt*MA were dissolved in 8 mL of methanol and then deionzed water was added to 10 mL of *tt*MA solution (1 mg/mL). One mL of *tt*MA solution (1 mg/mL) was poured in a volumetric flask, and then dilute hydrochloric acid 1/10(v/v) was added to make 10 mL *tt*MA solution (100 μg/mL). The stock solution was obtained by diluting 5 times the above solution with deionzed water. Vanillic acid stock solution (IS solution, 100 μg/mL) was prepared by the same method. These solutions were stored at 4℃.

Apparatus and Conditions

A Hitachi SPD 6A HPLC system consisting of L-6000 pumps, Nanjing Qianpu system controller, SPD6A ultraviolet spectrophotometer detector, ODS(2)(5u) column (Φ4.6mm × 150mm) (Dalian Yilite) was used in this study. The data were analyzed by Sigma Plot 2000 Microsoft Excel98 software (USA).

The mobile phase components were acetate-acid/ tetrahydrofuran/methanol/water (v/v, 1/2/10/87). Ultraviolet wavelength was 264 nm. The flow-rate was 0.9 mL/min.

Study Design

Workers in iron and steel factory in China occupationally exposed to benzene and other aromatic solvents were enrolled in this study. Controls were selected from those not exposed to benzene and matched by age and sex. The amount of benzene inhaled by the workers for a full work shift was collected with passive dosimeters (TMP 1500) and determined by gas chromatography (Asilent G1540A). The urinary samples were obtained from each volunteer twice a day, at 8:00 AM (second urination, work shift beginning) and at 16:00 PM. Twenty to 50 mL of urine were collected and 3 mL samples were stored at -18℃ until analysis. The level of urine creatinine was determined by the WS/T 97-1996 method.

Extraction Procedures

In brief, 0.5 mL of urine sample, 50 μL of IS (vanillic acid solution, 100 μg/mL) and 100 μL of 2 mmol/L HCl were mixed in a 10 mL tube for 10 s. Then 4 mL of ethyl acetate was added and the tube was vortex-mixed for 20 min followed by centrifugation at 2500 rpm for 15 min. Five mL of organic layer were transferred to another tube and evaporated to dryness below 40℃ under a gentle stream of nitrogen. The residue was dissolved in 500 μL of mobile phase and mixed for 30 s, 10 μL of the above solution were injected into the HPLC system for analysis.

Preparation of Calibration Curve

The working solutions at concentrations of 0.10, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/mL were obtained by further diluting the working stock solution with blank urine, and then 100 μL of 0.05 mol/L HCl were added to the above solutions. The solution of vanillic acid at the concentration of 100 μg/mL was prepared as internal standard. These solutions were used to prepare the calibration curves and for quality control. Determination was carried out based on internal standardization. The calibration curves were drawn by plotting peak area ratio (Y) of the analyte to the internal standard against the concentration (C) of the analyte.

Sample Analyses

*tt*MA was extracted from urinary samples collected from 56 workers exposed to benzene and 24 volunteers not exposed to benzene and was determined according to the above method.

RESULTS

Chromatographic Separation

The chromatogram of determined *tt*MA is shown in Fig. 1. *tt*MA and the internal standard were completely with the rention time of 5.5 min and 13.5 min. There was no interference of impurities. All results showed that the method was specific for determination of *tt*MA in urine.

Calibration, Recovery, and Reproducibility

The calibration curve of *tt*MA was in good linearity over the concentration range of 0.10-10.00 μg/mL, and the coefficient of correlation was 0.9963. The calibration equation was Y=0.014＋0.271C (Y, peak areas of samples -peak areas of blank urine/peak areas of IS; C, concentrations of *tt*MA). The limit of detection was 0.10 μg/mL, and the recovery and reproducibility were generally over 90%. The intra-day and inter-day coefficients of variation were below 10% (Table 1).

Stability of ttMA in Urine

*tt*MA was stable in urine for at least 4 months when stored at -18 ℃ without any chemical preservative, while the same samples stored at room temperature were stable for a week.

TABLE 1

Recovery and Precision Studies of Urine Samples for *tt*MA in Urine

Concentration	Precision		Recovery ($\bar{x} \pm s$, %)	
(μg/mL)	Intra-day	Inter-day	Extraction	Relative
0.5	8.3	9.0	86.8±5.1	104.6±0.1
2.5	4.9	6.4	87.7±6.6	95.1±4.0
5	6.0	6.7	86.1±4.6	96.5±4.5

Urinary Concentrations of ttMA

On the basis of the developed method, the urinary excretion of *tt*MA in 56 workers occupationally exposed to benzene was determined. The values of *tt*MA /creatinine in urine before or after work and the correlation of *tt*MA /creatinine-benzene level exposed in air are shown in Table 2 and Fig. 2. In the present study, the 56 workers occupationally exposed to benzene were divided into three groups according to their benene-exposure levels: <20 mg/m^3 (*n*=21), >20-60 mg/m^3 (*n*=18), and >60 mg/m^3 (*n*=17). The urinary *tt*MA /creatinine under different levels of benzene-exposure are shown in Table 2 and Fig. 2. In the mean time, the results illustrated that there was a positive correlation between *tt*MA and benzene exposure level in air, and the equations were Y=0.859 + 0.108C (before work, r=0.6200) and Y=1.980+0.179C (after work, r=0.7930) (Fig. 2).

TABLE 2

Urinary *tt*MA Levels in Volunteers Under Different Benzene Levels

Level of Benzene (mg/m^3)	Workers (*n*)	*tt*MA Level (mg/g Creatinine) ($\bar{x} \pm s$)	
		Before Work	After Work
<20	21	1.23±1.17	2.78±3.13
20-60	18	4.85±3.40	10.03±4.59
>60	17	11.21±7.02	17.28±6.74

Note. Level of benzene: the amount of benzene from the environment *via* inhalation. The urinary samples were collected from each volunteer twice a day, at 8:00 AM (second urination, work shift beginning) and 8 h later. The levels of urinary *tt*MA were determined by the above HPLC method.

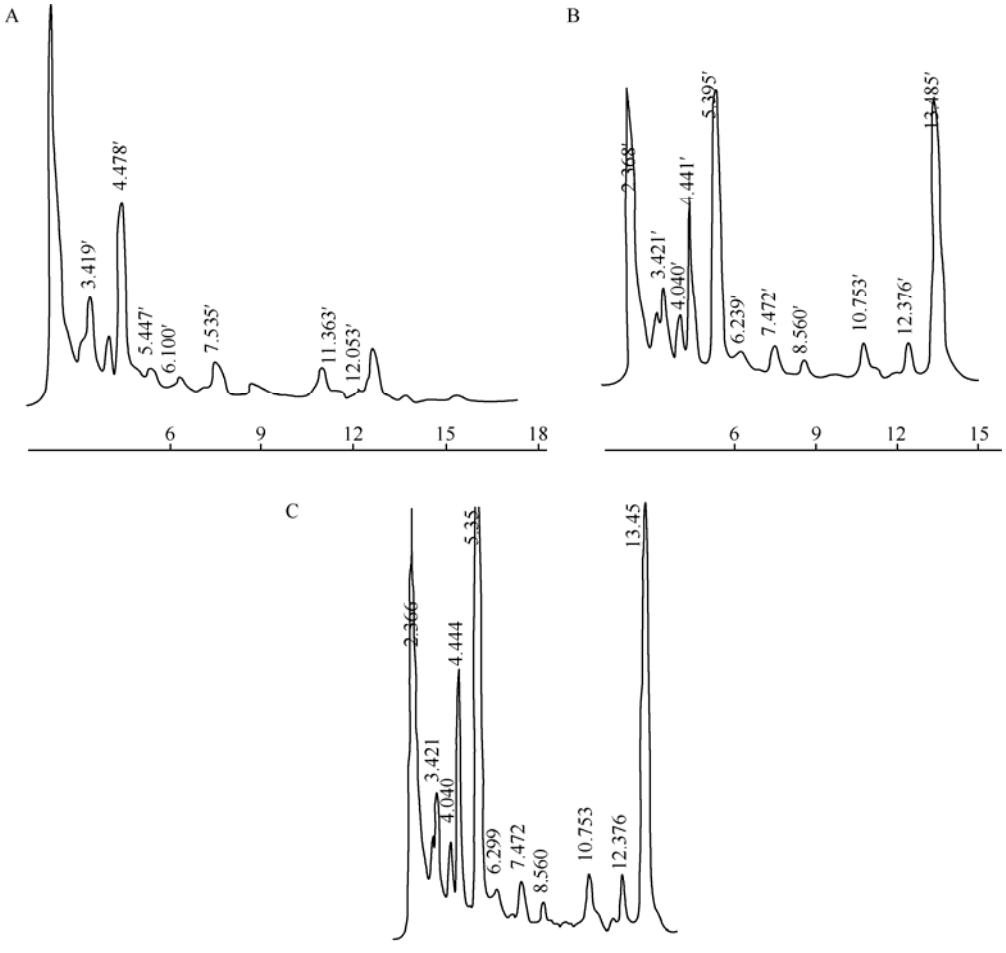

FIG. 1. HPLC of determined *tt*MA (*tt*MA:5.4 min; I.S:13.5 min). A, blank urine; B, standard sample of *tt*MA in blank urine; C, urinary sample of volunteer.

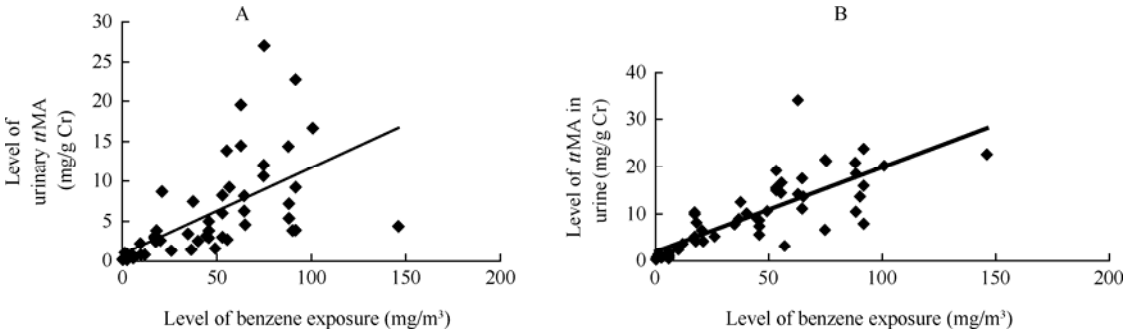

FIG. 2. Correlation between *tt*MA and benzene level in air before (A) or after work (B) in 56 volunteers occupationally exposed to benzene.

DISCUSSION

Occupational exposure to benzene has been causally associated with the increasing risk of aplastic anaemia, myelodysplastic syndrome, and acute non-lymphocytic leukaemia. The method to estimate the biological limit for exposure to occupational or environmental benzene is important for many countries, especially for the developing countries. Excretion of urinary *tt*MA has been widely recommended as a biomarker for estimation of occupational or non-occupational exposure to benzene.

Although the analytical method of LC/MS or GC/MS can provide a better detection mode with high sensitivity, the expensive equipment and complicated techniques limit its application in routine analysis. In the present study, a rapid, sensitive and accurate method was developed, which can be used to monitor the biological limit value for occupational exposure to benzene. Fifty-six volunteers exposed to benzene and other aromatic solvents were included in this study. The results showed that *tt*MA in urine was closely correlated with benzene in air (Y=0.859＋0.108C, before work, r=0.6200) and (Y=1.980＋0.179C, after work, r=0.7930). On the basis of above results, we draw the conclusion that in these workers the median excretion of *tt*MA is 3.05 mg/g creatinine after work and 1.50 mg/g creatinine before work. These experiments provide a linear extrapolation between excretion of *tt*MA and benzene level, which can determine the excretion of *tt*MA according to the inhaled benzene concentration.

In summary, our method can be applied to the determination of *tt*MA in urine in workers occupationally exposed to benzene.

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