

URINE TRANS, TRANS-MUCONIC ACID DETERMINATION FOR MONITORING OF BENZENE EXPOSURE IN MECHANICS

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Abstract. Benzene is an important carcinogenic substance used in many industrial processes. Inhalation of this substance can cause both acute and chronic toxicity. In this study, monitoring of benzene exposure by high-performance liquid chromatography for urine trans,trans-muconic acid (ttMA) determination in 94 subjects, (49 control subjects and 45 mechanics) was performed. The mean urine ttMA level in the control group was 0.116 ± 0.027 mg/g creatinine. The mean urine ttMA level in the mechanics group was 0.280 ± 0.131 mg/g creatinine. There was a significant difference between the groups ($p < 0.05$). Based on this study, we recommend the use of urine ttMA determination for monitoring of benzene exposure in high risk workers.

INTRODUCTION

Benzene is an important chemical agent used in many industrial processes at present. It plays an important role in the pathogenesis of both acute and chronic toxicity. To determine benzene exposure, many techniques have been developed and used. Determination of urine phenol, excreted metabolite of benzene, is a widely used method. But this technique provides rather high false positive rate due to the fact that ingestion of some foods and metabolism of some aromatic amino acids can produce urine phenol.

Therefore, the determination of urine trans, trans-muconic acid has been introduced for detection of benzene exposure. Trans,trans-muconic acid (ttMA) is a non-phenolic metabolite of benzene excreted into the urine (Bechtold *et al*, 1991; Inoue *et al*, 1989; Lee *et al*, 1993). It can be detected by high-performance liquid chromatography (HPLC). This method is considered easy to perform and convenient as to the preparation of standard reagents.

Due to the continuous industrialization in Thailand, many occupations seem to be at high risk for benzene exposure. Surprisingly, according to the review literature, there has been no report

about using urine ttMA determination to monitor the risk of workers.

In Thailand, the occupation of mechanic implies a high risk of benzene exposure. Many processes involved in fixing a machine, these workers have direct contact with the petroleum product, therefore, exposure to benzene cannot be avoided. This study has been set as a pilot study to determine the difference of urine ttMA between non-exposed subjects and mechanics.

MATERIAL AND METHOD

Subjects

A total of 94 subjects was included in this study. The first group, 49 subjects, was the control group with low risk for benzene exposure. All were villagers from rural area without any nearby factories in Chanthaburi and Pathum Thani Provinces, Thailand. All were interviewed for possible exposure to benzene and none report any.

The second group, comprising the study group 45 subjects, was a group of mechanics working in garages in Ladprao and Pathum Wan districts, Bangkok, Thailand. These workers had to work in the garages for 8 hours daily.

All subjects were asked for informed consent. Then 10 ml of random voided urine sample was collected for laboratory analysis from each subject at the time between 14.00-19.00 hr, about 4-8 hours after work.

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Laboratory analysis

High-performance liquid chromatography: The HPLC system used was the SHIMADZU communication bus module and set as outlined below.

Condition of HPLC:

1. Flow rate equal to 1.2 ml/minutes.
2. Mobile phase consists of glacial acetic acid: ethanol: 0.5 mmol/l sodium acetate in a ratio of 10:100:890, pH = 3.2.
3. Stationary phase (column) is a Lichrocart C18 cartridge [120 x 4 mm (id); Merck].
4. Detector is a UV/visible spectrophotometer at a wavelength of 265 nanometers.
5. Injected volume equal to 5 μ l.

Injection and result reading: Injection was performed at an injection volume of 10 μ l, at a flow rate of 1.2 ml/minutes. Reading the results from HPLC was performed by interpretation of retention time (RT) of each substance and calculation of the quantity using the peak area ratio of ttMA and vanillic acid (internal standard).

Calculation of the ttMA quantity: To calculate the concentration of ttMA, the peak areas of both ttMA and vanillic acid (internal standard) were used. At first the concentration of ttMA was calculated in mg/l unit and compared to the creatinine concentration in the same sample to provide the final result in mg/g creatinine.

Reagents: Vanillic acid, Tris base, Tris HCl, Dowex I chloride form and trans,trans-muconic acid were obtained from SIGMA, Thailand. Hydrochloric acid and glacial acetic acid were obtained from JY Baker, Thailand. Methanol, sodium chloride and sodium acetate were obtained from BDH, Thailand, and phosphoric acid was obtained from MERCK, Thailand.

Sample collection: Voided urine samples were collected. Ten ml were added to 100 μ l 6 mol/l hydrochloric acid as a preservative and frozen at -20°C. Ten mm were added to 1 drop of toluene to be used for creatinine determination.

Sample extraction: As an internal standard, 2.0 ml vanillic acid were added to 500 μ l sample, slowly mixed on a vortex and processed by ion-exchange column to exclude interference. The column used is 1 mm in diameter and filled with Dowex I (chloride form, 100-200 mesh) at a 100

mm height.

Two ml of purified water were passed through the column two times. The urine mixed with the vanillic acid standard, 1 ml 1 mol/l sodium acetate buffer (pH=7), and 1 ml purified water were passed through the column.

This was followed by adding altogether 1 ml sodium chloride and methanol at a ratio of 1:1. The resulting product was separated by centrifugation at 1,000 rpm for 1 minute. The supernatant was injected into the HPLC machine.

Standard curve and quantification: Analysis of ttMA in urine was performed based on the internal standard. The internal standard, vanillic acid, was used to correct sample recovery. Both standard and sample were prepared by adding a constant amount of ttMA before extraction. Standard curves were prepared by adding increase amounts of ttMA standard solution. The mixture was extracted step by step as the procedure described above. These solutions were injected into the HPLC system and the peak areas were recorded. The result was calculated based on the ratio of the compound's peak area to that of the internal standard.

Identification and separation by the test: The calibration curve proved linear with a correlation coefficient of 0.99. The detection limit of ttMA, estimated from the lowest concentration at which baseline activity could be discriminated on chromatograms was 0.05 mg/l. Precision analysis yielded a variation coefficient of 13.6%.

Statistical analysis

Mean and standard deviations of urine ttMA levels in both groups were calculated. The average urine ttMA levels of each group were compared using the unpaired Student's *t*-test with level ≤ 0.05 considered statistically significant.

RESULTS

Ninety-four healthy volunteers were included in this study. The mean urine ttMA level in the control group was 0.116 ± 0.027 mg/g creatinine. The mean urine ttMA level in the mechanics group was 0.280 ± 0.131 mg/g creatinine. There was a significant difference between both groups ($p < 0.05$) (Table 1).

Table 1
Urine ttMA levels in control and mechanics groups.

Group	No.	Urine ttMA level (mg/g creatinine)	
		Range	Average
Control	49	0-0.40	0.116±0.027
Mechanics	45	0-2.35	0.280±0.131

DISCUSSION

Benzene is an important carcinogenic substance. Its toxicity is caused by inhalation of the benzene vapors. Therefore, monitoring of its toxicity is very important. Although there is a report (Muttamara *et al*, 1999) on the benzene levels in the air in Bangkok it does not reflect the *in vivo* process.

Determination of benzene metabolite as biomarkers has continuously been developed. Urine ttMA determination has been accepted as the most reliable method at present (Boogaard and van Sittert, 1995; Ong *et al*, 1996). The determination of urine ttMA by HPLC is the easiest method and can be used as a monitoring system for benzene exposure in the workers.

In this study, the feasibility of using HPLC to determine urine ttMA as a marker for benzene exposure was investigated. Comparing the average urine ttMA levels of the mechanics to those of the control group, a significantly higher level was detected in the mechanics group. This result agrees with the study of Lee *et al* (1993), indicating the higher urine ttMA levels among high risk workers in petroleum purification plants.

Mechanics can be classified as an occupation at high risk of benzene exposure. They are constantly in contact with the petroleum product during the machine fixing process. Monitoring of benzene exposure in these workers is still important. Protective equipment for these workers such as gloves and masks are necessary and should be provided.

In Thailand, health education of benzene toxicity to high risk workers is necessary. Annual check up for urine ttMA as a marker for benzene exposure in these workers is recommended and there should be a specific law on this subject.

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