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## Determination of trans, trans-muconic acid in Children Living in Adelaide Based on HPLC Developed Method

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**Abstract:** The objectives of this study were to develop a method for analysis of trans, trans-muconic acid (tt-MA) of urine in group of children living in Adelaide and compare to those residing in a rural zone in the South Australia. tt-MA was extracted from urine by ethyl acetate and analyzed with High Performance Liquid Chromatography (HPLC). The mean recovery of tt-MA from urine for spiked sample was 94 to 99%. There was a significant difference between tt-MA level for children living in Adelaide compare to children living in a rural at South Australia ( $p < 0.05$ ). Also a significant difference was found between tt-MA level for children living less than 100 m from road compare those living more than 100 m ( $p < 0.05$ ). The sensitive and specific HPLC analysis has been developed for the simultaneous measurement of tt-MA and tt-MA can be utilized as a biomarker for exposure to benzene in children.

**Key words:** tt-MA, benzene, children

### INTRODUCTION

Benzene toxicity involves bone marrow depression and leukemogenesis caused by damage to multiple classes of hematopoietic cells and various hematopoietic cell functions<sup>[1]</sup>.

The environmental concentrations of benzene have increased significantly in the last years due to increased road traffic. The major sources of benzene in ambient air of urban area are car exhaust and evaporation loss during handling, distribution and storage of petrol<sup>[2,3]</sup>.

The major metabolite of benzene is urinary phenol, until recently, phenol and other hydroxylated benzene metabolites were the major biomarkers studied in occupational exposure, the primary limitation of these indicators is the lack of specificity for benzene exposure at level near 5 ppm<sup>[4]</sup>. The measurement trans, trans-muconic acid [(tt-MA), 2-4-hexadienedioic acid] and S-phenylmercapturic acid (PMA) were proposed as a sensitive bioindicator of low level benzene<sup>[4-6]</sup>. tt-MA is produced by biotransformation of trans, trans-muconaldehyde which is considered as one of the benzene metabolite responsible for its toxicity<sup>[7]</sup>. Several investigators have reported tt-MA urinary concentrations in subjects who were exposed occupationally to benzene,<sup>[4,8,9]</sup> nevertheless, few data exist for children<sup>[10,11]</sup>.

It is important for researchers to establish the actual levels of benzene exposure in urban area for children. Children are typically exposed to airborne benzene, passive cigarette smoking and spoiled food. They usually do not smoke and they are not exposed occupationally.

The analysis of tt-MA from urine is based on solid phase extraction using of SAX tube and it is expensive<sup>[12-16]</sup>. The method described here is proposed for use in routine analysis by any laboratory equipped with HPLC-UV. The extraction of tt-MA from urine was done by ethyl acetate that is easier than solid phase extraction.

The objective of this study was to develop a method for analysis of tt-MA of urine in group of children living in Adelaide, compare to those residing in a rural zone in the South Australia.

### MATERIALS AND METHODS

**Collection of children urine samples:** Eighty-two urine samples were selected with simple random sampling from a 1300 preschool children in four areas of Metropolitan Adelaide.

All children subjects were preschools, aged 4-5 years. There was a questionnaire, about the possible risk factors for benzene exposure. This questionnaire was completed by children parents. Sterile urine containers together with

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an information sheet and consent forms were distributed to parents via the kindergartens and childcare centers in each of the selected area. All children had no specific exposure to benzene other than that present in the general environment. The analysis of urine samples in this study was performed in January and February 2003.

Urinary samples were acidified as soon as possible after collection and stored at  $-20^{\circ}\text{C}$  until dispatch for analysis.

Stock solution was prepared with dissolve 100 mg tt-MA in 1 L water/acetic acid (90-10)<sup>[12]</sup>.

**Sample preparation:** One milliliter of the urine sample was taken in 12 mL glass-stopper glass test tube and mixed with 0.2 mL of HCl (4 mole). The mixture was extracted with 4 mL ethyl acetate by 30 min vigorous mechanical shaking. The organic phase (ethyl acetate) was transferred to another test tube and dried under a nitrogen stream with warming  $60^{\circ}\text{C}$ . The remaining compounds in test tube extracted with 1 mL mobile phase and injected to HPLC.

**Chromatographic conditions:** The Chromatographic conditions of tt-MA was carried out according to the method of Maestri *et al.*<sup>[14]</sup> and Wiwanitkit *et al.*<sup>[16]</sup>. Analysis was carried out by HPLC (GBC Company) The high performance liquid chromatography column was an APEX ODS II 3  $\mu\text{m}$  (25 cm $\times$ 4.6 mm) analytical column. Chromatography was isocratic in a mobile phase consisting of water-methanol-acetic acid (89:10:1) as mobile phase. The flow rate was set at 1 mL  $\text{min}^{-1}$ . All chemicals and water used were HPLC grade. The UV-Vis detector (GBC, LC1205) was set at 270 nm. The retention time of tt-MA was around 5.2 to 5.4 min.

The urinary creatinine was measured by Jaffe kinetic method with out deproteinization on a Boehringer Mannheim Hitachi 917 automatic analyzer.

Data analysis was performed with SPSS statistical software for windows. Comparison between the tt-MA mean values (creatinine adjusted) was carried out with Mann-Whitney test and for benzene concentration was obtained by the student's t-test.

## RESULTS

For recovery and reproducibility studies, pooled urine samples were spiked with various concentrations. The mean recovery of data was 94 to 99%. Day to day variation of calibration was less than 10% over 5 times. The detection limit of the method was 0.02 mg  $\text{L}^{-1}$ .

There was a significant difference between tt-MA levels for children living in Adelaide compare to children living in a rural at South Australia ( $p < 0.05$ ) (Table 1).

Table 1: Urinary tt-MA results determined in children living in Australia and a ruler in South Australia

Parameter	n	tt-MA level in urine	
		$\mu\text{g L}^{-1}$	$\mu\text{g g}^{-1} \text{ cr}$
Total tt-MA in Adelaide	82	144.81 $\pm$ 110.86*	167.45 $\pm$ 138.38*
Total tt-MA in ruler	26	83.22 $\pm$ 70.38*	101.10 $\pm$ 85.12*

significant difference ( $p < 0.05$ ), with t-student

Table 2: Urinary tt-MA results determined in children living in South Australia

Parameter	n	tt-MA level in urine	
		$\mu\text{g L}^{-1}$	$\mu\text{g g}^{-1} \text{ cr}$
<b>House locality</b>			
More than 100 m from road	52	137.35 $\pm$ 103.77*	166.20 $\pm$ 132.96*
Less than 100 m from road	30	181.80 $\pm$ 129.72*	217.05 $\pm$ 164.16*
<b>Season</b>			
Summer	37	156.18 $\pm$ 100.18**	186.28 $\pm$ 120.67**
Winter	45	143.13 $\pm$ 126.35**	177.37 $\pm$ 144.92**
<b>Passive smoke</b>			
No	62	128.13 $\pm$ 114.17*	151.20 $\pm$ 135*
Yes	20	181.76 $\pm$ 85.61*	261.19 $\pm$ 159.11*

\*significant difference ( $p < 0.05$ ), with t-student, \*\* not- significant difference

Table 3: Levels of tt-MA in different area of Adelaide

Area	n	tt-MA level in urine	
		$\mu\text{g L}^{-1}$	$\mu\text{g g}^{-1} \text{ cr}$
Inter South	19	133.45 $\pm$ 127.29	161.92 $\pm$ 128.84
South suburb	22	142.23 $\pm$ 126.56	176.19 $\pm$ 155.53
West suburb	19	136.84 $\pm$ 106.35	182.73 $\pm$ 169.05
North suburb	22	164.48 $\pm$ 93.41	164.99 $\pm$ 97.65

A significant difference was seen between tt-MA level for children living less than 100 m from road with those living more than 100 m ( $p < 0.05$ ). Correlation coefficient for distance from road and urinary tt-MA was 0.34.

A significant difference was seen for urinary tt-MA in children their parents smoker with non-smoker parents ( $p < 0.05$ ) but no significant difference for urinary tt-MA in children at summer and winter (Table 2).

There was not any significant difference among urinary tt-MA in four-difference area of Adelaide (Table 3).

## DISCUSSION

The sensitive and specific HPLC analysis has been developed for the simultaneous measurement of tt-MA and a biomarker for low benzene exposures in humans. The feasibility and efficiency of this assay have been examined in a pilot study for children living in Adelaide with low benzene exposure and children living in a rural as the control group.

The other method for preparation of tt-MA for analysis was based on solid extraction<sup>[13-19]</sup> the current method described here is based on liquid extraction that is easier, faster and due to less economy is better than solid extraction.

The significant difference between tt-MA in children that living in Adelaide and a ruler in south Australia concern to pollution in Adelaide emitted by vehicles.

The concentration of benzene in recent research was  $1.66 \pm 1.80$  ppb<sup>[20]</sup>. Motor vehicles are considered to main sources of air pollution in the Adelaide metropolitan as the number of 653 motor vehicle per 1000 person<sup>[20]</sup>.

Weaver *et al.*<sup>[21]</sup> measured tt-MA in urban children, the mean tt-MA was  $144 \pm 296.1$   $\mu\text{g L}^{-1}$ . These levels are approximately same in the present study. Ammonia-Cocchieri *et al.*<sup>[22]</sup> measured tt-MA in children living in Campania (Italy) the mean of tt-MA was 98.7 and in ruler study was  $48.4 \pm 71$   $\mu\text{g L}^{-1}$ <sup>[22]</sup>. These tt-MA level was less than data in this study. The difference may concern to use of some foods that have sorbic acid. Weaver *et al.*<sup>[23]</sup> found large increase in tt-MA concentration of volunteers that used sorbic acid-preserved foods. They also found that refrigerated flavored drinks and sweet snack foods resulted in the excretion of large amounts of main adults and children. At low level benzene exposure the use of tt-MA as a benzene biomarkers is complicated by the fact it is not completely specific because tt-MA is also a metabolite of sorbic acid<sup>[24]</sup>. These studies indicate that sorbic acid-preserved foods have the potential to cause substantial interference with tt-MA as a biomarkers for both occupational and environmental exposure in populations, such as in South Australia were consumption of preserved food in common<sup>[23-25]</sup>.

There was not any significant difference between measurement of urinary tt-MA in winter and summer and also among four areas in Adelaide that may concern to benzene concentration in ambient air. In recent research there was not any significant difference among benzene concentration in four geographical situation and seasons<sup>[20]</sup>.

There was a significant difference between tt-MA in urine of children near to road with other children and also significant difference between children their smokers parents with non smokers, these results may concern to benzene emitted from cigarette.

Excretion of tt-MA can also affected by simultaneous exposure to toluene and benzene.

The other important factors that effect to tt-MA concentration in children are basic difference in rate of benzene metabolism among children and difference in renal function<sup>[26]</sup>.

The use of ethyl acetate for extraction of tt-MA was developed in this method. Data shows, that tt-MA can be utilized as a biomarker for exposure to benzene in children. Exposure to benzene is more for children that living near to main road compare to children far from road.

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