High-Performance Liquid Chromatography Method for Urinary trans,trans-Muconic Acid. Application to Environmental Exposure to Benzene

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Abstract

This report describes a specific and precise high-performance liquid chromatography (HPLC) method for the quantification of trans,trans-muconic acid in human urine. The procedure involved a highly efficient Bond-Elut SAX extraction with 20% acetic acid elution. The HPLC analysis used a sodium acetate/methanol mobile phase with a C18 reverse phase column and UV detection at 265 nm. The recovery, precision, linearity, and limits of detection and quantification of the method were determined. Mean absolute recoveries were between 97% and 115%. The calibration curve showed a correlation coefficient of 0.9955 and the limit of detection was determined to be 10.8 pg/L. The method is suitable for evaluation of occupational and environmental benzene exposure in humans. The study of urinary trans,trans-muconic acid of two populations of children to evaluate environmental benzene exposure is presented.

Introduction

Trans,trans-muconic acid (t,t-MA) has been widely used as biomarker of benzene exposure because of its sensitivity and specificity (1–3). Benzene is a common industrial chemical, and it is released into the environment from both natural and anthropogenic sources, the latter accounting for the major part of the emissions. The presence of benzene in gasoline (petrol) and as a widely used industrial solvent can result in significant and widespread emissions to the environment (4). Thus, its use is under severe restrictions.

In humans, the primary route of exposure is the respiratory route (4–6). Dermal and oral exposures are of minimal importance in terms of total daily intake of the general population (4). Benzene metabolism occurs mainly in the liver through the cytochrome P450 II E1 enzyme system and, to a lesser extent, in such target tissues as the bone marrow (4,7). This metabolism involves the formation of a series of unstable reactive metabolites (4), which seemed to be responsible for benzene toxicity. Among these metabolites is the trans,trans-muconaldehyde, a precursor of t,t-MA (5). It is a minor metabolite of benzene (5), the metabolic conversion of benzene to t,t-MA ranged from 2% to 25% and is dependent on the benzene exposure level. The only other known precursor of t,t-MA is sorbic acid, a food additive commonly used as a preservative in juices, soft drinks, and some foodstuffs (8).

Benzene is a well-established human carcinogen. It is known that benzene produces a number of adverse health effects, the most frequently reported health effect is bone marrow depression leading to aplastic anemia (4).

Urinary t,t-MA levels are significantly higher in exposed workers at the end of a work shift than those measured in unexposed subjects (9). Also, t,t-MA has been proposed as a biomarker for environmental benzene exposure (5).

The aim of the present work was to develop and validate a method to extract and quantify urinary t,t-MA. The biological monitoring of benzene exposure (occupational and environmental) seems to be of great importance for the prevention of toxic and carcinogenic effects on humans.

Experimental

Chemicals

t,t-MA was purchased from Sigma (St Louis, MO). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade and purchased from Merck (Merck KGaA, Darmstadt, Germany). Sodium acetate trihydrate was pro analysi (p.a.) and purchased from Merck (Merck KGaA). Acetic acid glacial and hydrochloric acid were p.a. and purchased from Merck Quimica Argentina (Buenos Aires, Argentina). Sodium hydroxide was p.a. and purchased from Mallinckrodt (Mallinckrodt Chemical Works). Double distilled water was obtained with a Figmay™ distillatory system (Córdoba, Argentina).
Bond Elut-SAX cartridges (500 mg of sorbent mass, 3 mL of column reservoir volume) were supplied by Varian (Harbor City, CA).

**Chromatography**

The HPLC system (JASCO Corporation, Tokyo, Japan) consisted of an intelligent HPLC pump (PU-980) with a ternary gradient unit (LG-980-02), an automatic sampler injector (AS-950), a UV/vis detector (UV-975), and an online degassing system (Alltech, Deerfield, IL). Data and chromatograms were collected and analyzed using Borwin Chromatography Software (UMBS Developments, France). The C18 reversed-phase column was a Prevail (Alltech). The length of the separation column was 250 mm × 4.6 mm with a 5-µm particle size.

The mobile phase consisted of methanol and 5 mM sodium acetate buffer (pH 3.5) (15:85, v/v). The injection volume was 15 µL, and the flow rate was 1.5 mL/min. The detection wavelength was 265 nm. The assay was performed at room temperature.

**Standard solutions and urine samples**

Stock solution for t,t-MA was prepared in methanol to a final concentration of 1 mg/mL. Two working standard solutions of 5 and 10 µg/mL in methanol were prepared from the t,t-MA stock solution.

A pool of urine samples was collected from young, healthy, non-smoker adults. The urine samples were spiked with t,t-MA to reach final concentrations of 10, 50, 100, 150, 500, 1000, and 2000 µg/L, and they were stored at pH 2.

**Environmentally exposed population**

Urine samples (25 mL) were obtained from children from two urban-industrial areas and were stored at pH 2. The intake of soft drinks and juices was avoided 72 h prior to urine collection. All solutions and urine samples were stored at 4°C.

**Sample preparation and extraction procedures**

Aliquots (1 mL) of spiked urine samples were raised to pH 7 with 10% sodium hydroxide, and they were loaded onto a Bond Elut-SAX cartridge, previously conditioned with 3 mL of acetonitrile and 3 mL of water. The columns were washed four times with 3 mL of a 1% acetic acid solution and dried. The elution was performed with 3 mL of a 20% acetic acid solution. The eluate was filtrated through a 0.45-µm pore diameter filter (Cameo 13N, MSI, Westboro, MA) prior to injection.

Blank unspiked urine and children urine samples were prepared using the same extraction procedure. Creatinine analysis was performed according to the method described by Jaffé.

**Method validation**

Recovery, precision, and linearity. The intraday (within-run) precision and recovery were determined by the analysis (triplicate determinations) of two sets (150 and 2000 µg/L) of spiked urine samples within the same day. The interday (between-run) precision and recovery were determined by the analysis of the same two sets (150 and 2000 µg/L) of spiked samples in three consecutive days. One blank urine sample was processed simultaneously to subtract pre-existing t,t-MA.

Seven 1-mL urine samples spiked to reach the final concentrations of 10, 50, 100, 150, 500, 1000, and 2000 µg/L were analyzed by duplicated determinations. Linearity was established in these conditions.

Limits of detection (LOD) and quantification (LOQ). Both the LOD (i.e., the smallest concentration that can be reliably detected) and the LOQ (i.e., the smallest concentration that can be quantified with precision and accuracy) can be estimated from the calibration curve, if low concentrations of the analyte were considered, by the extrapolation to zero concentration (10). Briefly, a second calibration curve, from low concentrations of t,t-MA (10, 50, 100, and 150 µg/L, triplicate determinations) was obtained. From the extrapolation to zero concentration, a blank response ($Y_b$) was estimated. Standard deviation (SD) for each point of the curve described previously was determined. A plot of SD versus concentration was obtained, and an SD for zero concentration ($S_b$) was extrapolated.

LOD and LOQ were calculated according to:

$$LOD = (Y_b + 3S_b)/b$$

$$LOQ = (Y_b + 10S_b)/b$$

where $b$ is the slope of the calibration curve.

**Results and Discussion**

An HPLC–UV method for the quantification of t,t-MA was developed. The urinary t,t-MA was quantified by reversed-phase HPLC with an isocratic system. Under these conditions, the mean retention time of t,t-MA was 9.77 min (SD = 0.05) (Figure 1), and the total run time was 13 min. UV spectrum of t,t-MA was obtained for peak identification purpose (Figure 2).

**Method validation**

The anionic exchange solid-phase extraction resulted in clean sample extracts and an excellent efficiency. Mean absolute recoveries assessed at two concentration levels (150 and 2000 µg/L) were between 97.4% and 115.4% (Table I). Intra- and interday coefficients of variation (CV%) are shown in Table I.

The calibration curve was constructed by least squares linear regression analysis and was linear over the concentration range of 50–2000 µg/L. The linear correlation coefficient ($R^2$) was 0.9955 (Figure 3). The LOD for t,t-MA in urine was 10.8 µg/L, and the LOQ was 65.9 µg/L.

The system presented here has been found to provide a sensitive, quantitative, and simple method for the quantification of urinary t,t-MA.

The strong-anionic exchange solid-phase extraction procedure resulted in excellent efficiency, and the eluates showed chromatograms clean enough to avoid the second extraction step (C18 column) proposed by Maestri et al. (11). t,t-MA recovery was also comparable with the one obtained by Maestri et al. (11) for their extraction system.

This method used an isocratic system with one HPLC separation column instead of a system with a "spike" of acetonitrile,
column switching, or the combination of two mobile phases described by other authors (11-13) because no delayed peaks (5,11,12) were observed in the chromatograms for at least 20 min of run length (data not shown). Mobile phase and flow rate conditions were optimized to improve the resolution between \( t,t\)-MA and interfering bands from urine components. The best resolution was achieved with 15% of methanol and 85% of 5 mM sodium acetate buffer and a flow rate of 1.5 mL/min. These conditions also resulted in an optimal run length of 13 min, which proved to be shorter than in other reports (25 to 60 min) (11-13).

The urine standard curve was found to produce excellent linearity over the concentration range of 50 to 2000 \( \mu \)g/L. The LOD was of 10.8 \( \mu \)g/L for an injection volume of 15 \( \mu \)L, and it was comparable with the results obtained by Maestri et al. (11,12) for an injection volume of 50 or 200 \( \mu \)L, but with the advantage that injecting smaller sample amounts into the HPLC column will contribute to longer column lifetime.

Analysis of urine samples from environmentally exposed populations

The developed HPLC assay was successfully applied to urinary \( t,t\)-MA quantification in two populations of children (between 7 and 11 years old) of similar social and economic conditions to evaluate environmental benzene exposure in two urban-industrial areas (Villa I and Villa II) in Buenos Aires, Argentina (14).

The analysis of urinary \( t,t\)-MA of the two groups revealed that 83.9% (99 of 118) of the children from Villa I and 69.7% (99 of 142) of the children of Villa II had \( t,t\)-MA values below the LOQ of 65.9 \( \mu \)g/L. There were no peaks at the same retention time that could interfere with the \( t,t\)-MA peak.

The quantifiable \( t,t\)-MA values were between 66.6 to 1020.5 \( \mu \)g/g creatinine in Villa I and between 48.6 to 1367.8 \( \mu \)g/g creatinine in Villa II. The arithmetic mean of Villa I group was 55.2 \( \mu \)g/g creatinine (SD 203.4, \( n = 118 \)) and of Villa II was 85.6 \( \mu \)g/g creatinine (SD 203.4, \( n = 142 \)). The mean \( t,t\)-MA values for both groups (Villas I and II) were lower than those described by Barbieri et al. (15) and Weaver et al. (16) [55.2 and 85.6 \( \mu \)g/g creatinine versus 147 \( \mu \)g/g creatinine (15) and 177 \( \mu \)g/g creatinine (16)]. The mean benzene air concentrations in Villa I prior to sample collection were between 17 and 155 ppb (0.05 and 0.49 mg/m\(^3\)) and in Villa II were between 38 and 49 ppb (0.12 and 0.16 mg/m\(^3\)) (14). Benzene air concentration levels were higher than those described in bibliography for urban areas (4,15,17).

Requiring a diet free of soft drinks and juices 72 h before

| Table I. Intra- and Interday Precision Assessed at Two Concentration Levels of \( t,t\)-MA (150 and 2000 \( \mu \)g/L) |
|--------------------------------------|--------|--------|
|                                       | 150 \( \mu \)g/L | 2000 \( \mu \)g/L |
| Intraday mean recovery (%)             | 115.4  | 106.6  |
| Intraday precision (%CV)              | 2.06   | 0.72   |
| Interday mean recovery (%)            | 103.6  | 97.4   |
| Interday precision (%CV)              | 11.78  | 7.68   |

\[ y = 52.673x - 430.15 \]

\[ R^2 = 0.9955 \]

Figure 3. Plot of \( t,t\)-MA peak areas versus \( t,t\)-MA concentrations.
urine collection was recommended to minimize the contribution of preservative sorbic acid on urinary \( t,t\)-MA levels. Although animal studies showed that less than 0.5% of the dietary sorbic acid is excreted as \( t,t\)-MA (8).

The urinary \( t,t\)-MA is one of the benzene adopted biological exposure determinants (18). This method provided a simple, fast, and reliable urinary \( t,t\)-MA quantification for biological monitoring of benzene exposed populations and, in consequence, the prevention of toxic and carcinogenic effects of benzene on humans.

References

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