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Determination of urinary *trans,trans*-muconic acid by gas chromatography–mass spectrometry

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Abstract

A sensitive and specific method for the determination of *trans,trans*-muconic acid (t,t-MA) in urine is described. After clean-up on an anion-exchange cartridge, t,t-MA was derivatized with BF_3–methanol to the dimethyl ester and analyzed by gas chromatography–mass spectrometry (GC–MS), with 2-bromohexanoic acid as an internal standard. The limit of detection was 0.01 mg/l, the coefficient of variation for duplicate analysis in a series of urine samples ($n = 50$) was 2.6% and the recovery rate ranged from 93.3 to 106.3%. The between-day and within-day precision for the analysis were 7.4 and 14.6%, respectively. The method was applied to the determination of t,t-MA in urine samples from smokers and non-smokers. The mean concentration of t,t-MA in urine of 10 smokers was 0.09 ± 0.04 mg/g creatinine and was significantly ($p = 0.012$) higher than that found in urine of 10 non-smokers (0.05 ± 0.02 mg/g creatinine). In contrast to the results obtained with the commonly used high-performance liquid chromatographic ultraviolet detection (HPLC-UV) methods, no interference between t,t-MA and other urinary compounds was found. This GC–MS method is both specific and sensitive for biomonitoring of low environmental benzene exposure.

1. Introduction

Compared to phenol and other hydroxylated metabolites of benzene, *trans,trans*-muconic acid (t,t-MA) is only a minor metabolite formed by ring opening to *trans,trans*-mucondialdehyde, a putative genotoxic intermediate [1–4]. About 2% of the total benzene uptake in man is excreted as t,t-MA in urine [5], the half-life of which is reported to be less than 6 h [3]. However, for two reasons t,t-MA appears to be more suitable than the phenolic metabolites as a biomarker for low-level benzene exposure: Firstly, urinary background concentrations are much lower and secondly, there is evidence for the preferential formation of t,t-MA at low-level benzene exposure [3]. However, the specificity of t,t-MA as a marker for benzene exposure may be limited since it is also reported to be a metabolite of sorbic acid [6,7], a widely used food preservative.

Urinary t,t-MA has mainly been determined by HPLC-UV using external calibration [1,5,8–12]. Lee et al. [13] have reported the use of

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vanillic acid as an internal standard. Detection limits between 0.05 and 0.1 mg/l have been reported. A lower detection limit of 0.01 mg/l has been reported using GC–MS after derivatization of t,t-MA to a trimethylsilyl diester [14,15].

In the present study, the use of a GC–MS method for the determination of urinary t,t-MA after ion-exchange extraction and derivatization of t,t-MA to a dimethyl ester is described using 2-bromohexanoic acid as an internal standard.

The method has been applied to the analysis of urine samples from smokers and non-smokers without occupational exposure to benzene.

2. Experimental

2.1. Chemicals

Analytical-reagent grade chemicals were used and ultrapure water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). *trans,trans*-2,4-Hexadienoic acid (t,t-MA) and boron trifluoride–methanol (BF_3–MeOH) were purchased from Sigma (Deisenhofen, Germany). (±)-2-Bromohexanoic acid (2-BHA) was purchased from Aldrich (Steinheim, Germany). Acetic acid was supplied by Fluka (Neu-Ulm, Germany), methanol and heptane from Fisons (Loughborough, UK).

2.2. Gas chromatography–mass spectrometry

GC was performed on a Hewlett-Packard gas chromatograph (Model 5890 II plus) equipped with an automated liquid sampler (Model 7673B), a mass-selective detector (MSD) (Model 5971 A) and a split/splitless injector (Hewlett-Packard, Waldbronn, Germany). Data acquisition and processing were carried out using Hewlett-Packard ChemStation software (1994 release). Chromatographic separation was accomplished on an Ultra-5 30 m × 0.25 mm I.D. fused-silica capillary column with a film thickness of 0.25 μm (Hewlett-Packard). GC conditions were as follows: the temperature of the split/splitless injector was 260°C with an initial helium head pressure of 6.5 p.s.i. (45 kPa) for one min, followed by a 1 p.s.i./min (6.9 kPa/min) pressure increase to 16.5 p.s.i. (114 kPa) which was maintained for 5 min. Helium was used as the carrier gas and was purified by passing through a molecular sieve and copper catalyst traps to remove carbon dioxide, water and oxygen. The carrier gas flow-rate was 0.8 ml/min (35.5 cm/s linear velocity) and the split flow 15 ml/min (split ratio 1:18.7). Sample injections (1 μl) were splitless with the split value on at 0.5 min. The initial column temperature was 80°C, maintained for 1 min and then programmed at 20°C/min to 280°C, and maintained at 280°C for 5 min.

The MSD was automatically tuned prior to sample analysis with perfluorotributylamine (PFTBA) as a standard. For analysis, the MSD was tuned to *m/z* 129, 152 and 154 for 2-bromohexanoic acid methyl ester (internal standard; retention time 4.64 min) grouped from 3.5 to 5.4 min and tuned to *m/z* 111, 139 and 170 for *trans,trans*-muconic acid dimethyl ester (retention time 5.81 min) grouped from 5.4 to 6.7 min. The dwell time for each group was 50 ms.

The areas of the *m/z* 139 and 170 ions were used for quantitation of t,t-MA dimethyl ester and the *m/z* 154 ion for the internal standard. An unspecific response was occasionally obtained in some urine samples for the t,t-MA dimethyl ester *m/z* 111 ion.

2.3. Extraction procedure

A Baker SPE-12 G vacuum manifold (J.T. Baker, Groß-Gerau, Germany) equipped with 3-ml Bakerbond SPE quaternary amine anion-exchange cartridges (J.T. Baker) was used for sample extraction. The cartridges were preconditioned by washing with 4 ml of methanol followed by 3 ml of distilled water (vacuum 100–150 mbar below ambient pressure). An aliquot of the urine sample (2 ml) was loaded onto the conditioned column, which was then washed with 3 ml of 1% acetic acid. t,t-MA was eluted from the column with 4 ml of 10% acetic acid at a pressure difference not exceeding 50 mbar. A portion of the eluent (2 ml) was concentrated by centrifugation under vacuum to

dryness. The internal standard (1 μg of 2-BHA in 20 μl of methanol) was added and the residue reconstituted in 300 μl of BF_3–methanol. Methylation was performed at 56°C for 10 min, after which the sample was extracted three times with 1 ml of heptane. The heptane extracts were combined and reduced to 50–100 μl under a nitrogen stream.

2.4. Calibration procedure

A stock solution of t,t-MA was prepared in distilled water. Internal standard (2-BHA) solution was prepared in methanol (50 mg/l). Calibration standard solutions were prepared by dilution of the t,t-MA stock solution with distilled water to obtain final concentrations of 0.01, 0.05, 0.10, 0.25, 0.50 and 1.00 mg/l. The calibration solutions were analyzed using the internal standard according to the same procedure as described for urine samples. Calibration curves were constructed by plotting the peak-area ratios of t,t-MA to the internal standard against the concentrations of t,t-MA added.

2.5. Extraction efficiency

Recovery was determined with spiked samples, prepared by adding t,t-MA to pooled urine samples with high and low creatinine concentrations (1.69 and 0.96 g/l, respectively), giving concentrations of 0.05, 0.25 and 1 mg t,t-MA/l.

2.6. Urine samples

Urine samples from 10 male smokers and 10 male non-smokers (age: 22–42 yr) were collected overnight for 12 h (8 pm–8 am). The samples were stored at −25°C prior to investigation. Creatinine was determined according to the method of Jaffé using a test kit from Merck (Darmstadt, Germany).

3. Results and discussion

The structures of t,t-MA, 2-BHA and the reaction products after derivatization by methylation are shown in Fig. 1. Mass spectra of 2-bromohexanoic acid methyl ester and *trans, trans*-muconic acid dimethyl ester are shown in Fig. 2. A total-ion chromatogram (TIC) as well as the mass traces of the ions *m/z* 139 and *m/z* 170 obtained from derivatized t,t-MA in a smoker urine sample are shown in Fig. 3.

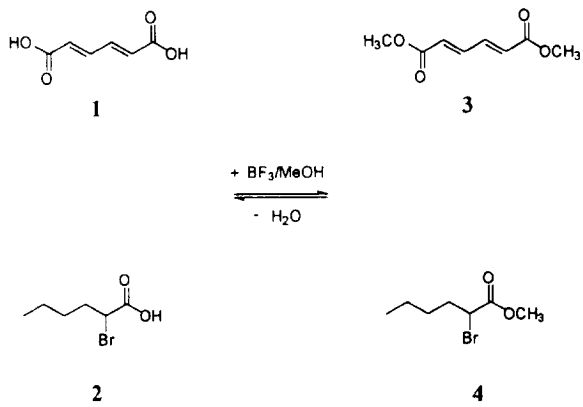

Fig. 1. Derivatization of t,t-MA (1) and 2-bromohexanoic acid (2) to t,t-MA dimethyl ester (3) and 2-bromohexanoic methylester (4).

Inter- and intra-assay precision for the determination of 0.06 mg t,t-MA/l in a pooled urine sample were found to be 14.6 and 7.4%, respectively ($n = 17$). The recovery of 0.1 mg t,t-MA/l urine from the anion-exchange cartridges was 96.9 ± 3.6% ($n = 3$). For the complete extraction procedure the recovery of t,t-MA from spiked urine samples ranged from 93.3 to 106.3% and was independent of the t,t-MA and creatinine concentration (Table 1). For calibration, the coefficient of linear regression was 0.996. The limit of detection, calculated on the basis of 24 measurements was found to be 0.01 mg/l.

Traditionally HPLC-UV methods have been used for determining t,t-MA in urine [1,5,10,11,13]. These methods have a limit of detection of 0.05–0.1 mg t,t-MA/l urine. Chromatograms of urinary extracts contain several compounds that elute in the vicinity of t,t-MA so that false positives are a potential analytical problem. Also, the use of an internal standard is hampered due to the presence of these mainly unidentified compounds. To avoid these prob-

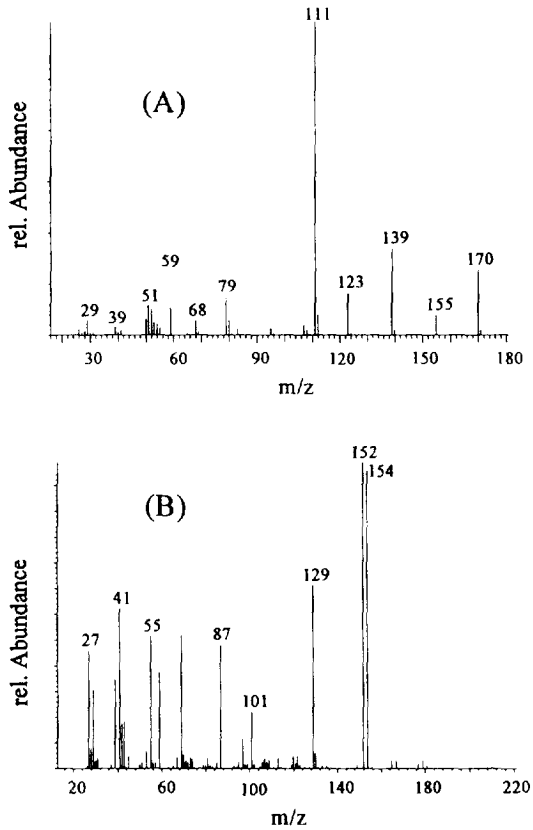

Fig. 2. Mass spectrum of *trans,trans*-muconic acid dimethyl ester (A) and 2-bromohexanoic acid methyl ester (B).

lems, hippuric acid has been used as an endogenous retention reference [16]. Alternatively, [^{14}C]*trans,trans*-muconic acid [17] has been used as an internal standard with liquid scintillation counting to determine the analyte recovery. [^{13}C]*trans,trans*-Muconic acid would be the ideal internal standard for GC–MS determination of t,t-MA and has been used by Bartczak et al. [17]. However, since [^{13}C]*trans,trans*-muconic acid is not commercially available, other aromatic and aliphatic acids were investigated for their suitability as internal standards. No compound fulfilled all criteria for use in the whole clean-up and extraction procedure. Since the recovery of t,t-MA in the clean-up step on an anion-exchange cartridge was highly reproducible (95%–100%), 2-BHA was used as the internal standard to control the derivatization by

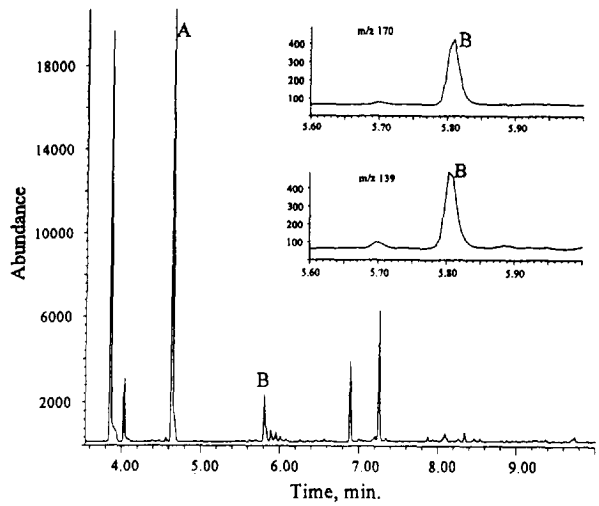

Fig. 3. Total-ion chromatogram of a methylated urine sample from a smoker containing 0.08 mg/l t,t-MA. Peaks: A = internal standard (2-bromohexanoic acid); B = t,t-MA. Insert: Selected-ion monitoring of t,t-MA ions *m/z* 139 and 170. For conditions, see text.

BF_3–methanol and the subsequent extraction and concentration steps.

The method described was used to determine t,t-MA in 20 urine samples from subjects who were not occupationally exposed to benzene. The results are summarized in Table 2. Smokers showed significantly higher urinary t,t-MA concentrations than non-smokers. Previously published studies report urinary t,t-MA concentrations of 0.05–0.21 mg/g creatinine for non-smokers and 0.19–0.61 mg/g creatinine for smokers, with smokers/non-smokers ratios of 1.4–4.8 [12,13,18]. All these studies have used HPLC-UV methods. According to our experience, interference with other urinary compounds occurs, which could produce false positive results at very low t,t-MA concentrations in urine. Although HPLC methods for the determination of t,t-MA in urine are suitable for biomonitoring of occupational benzene exposures, their sensitivity and selectivity for determining environmental exposure are limited. Since the reported method is not subject to interferences caused by other urinary compounds at t,t-MA concentrations as low as 0.01 mg/l, we believe that this GC–MS method is more suitable for biomonitoring en-

Table 1
Recovery rates of two urine samples with low (U1: 0.96 g/l) and high (U2: 1.69 g/l) creatinine concentrations, spiked with *trans,trans*-muonic acid

	t,t-MA (mean ± S.D., $n = 4$) (mg/l)	Expected t,t-MA concentration (mg/l)	Recovery (%)
U1	0.095 ± 0.017		
U1 + 0.05 mg/l t,t-MA	0.153 ± 0.018	0.145	105.5
U1 + 0.25 mg/l t,t-MA	0.375 ± 0.011	0.345	108.7
U1 + 1.0 mg/l t,t-MA	1.063 ± 0.023	1.095	94.6
U2	1.295 ± 0.027		
U2 + 0.05 mg/l t,t-MA	1.255 ± 0.017	1.345	93.3
U2 + 0.25 mg/l t,t-MA	1.558 ± 0.008	1.545	100.8
U2 + 1.0 mg/l t,t-MA	2.440 ± 0.019	2.295	106.3

vironmental benzene exposure than currently reported HPLC methods.

Regardless of the analytical method used to determine t,t-MA as a biomarker of low environmental exposure to benzene, two problems occur which need to be taken into consideration. (1) The conversion rate of benzene to t,t-MA is dependent on the level of benzene exposure with higher conversion rates at lower exposure levels [19]. Although this is an advantage when biomonitoring exposure to low benzene concentrations, it almost certainly increases the inter-individual variation in urinary t,t-MA excretion, thus diminishing the value of this biomarker. (2) Sorbic acid, a widely used food preservative, is also a precursor of t,t-MA [6,7]. The daily dietary intake of sorbic acid is 6–24 mg/day [20]. Preliminary results in our laboratory show that this intake would lead to an excretion of 0.01–0.04 mg/l t,t-MA and could thus interfere with t,t-MA levels predicted to occur after low environmental benzene exposure. Further studies are in progress in our laboratory to investigate this problem in subjects with low benzene exposure.

Table 2
Urinary concentrations of *trans,trans*-muconic acid in 10 non-smokers and 10 smokers

	t,t-MA (mg/l)[a]	t,t-MA (mg/g creatinine)[b]
Non-smokers		
Range	0.023–0.124	0.032–0.081
Mean	0.073	0.054
S.D.	0.027	0.017
Smokers		
Range	0.034–0.313	0.041–0.144
Mean	0.139	0.090
S.D.	0.085	0.035

Smokers vs. non-smokers: $p = 0.040$[a] and $p = 0.012$[b] (Mann–Whitney rank sum test).

4. Conclusions

Capillary GC–MS is a selective and sensitive method for the determination of t,t-MA in urine after anion-exchange extraction and derivatization to *trans,trans*-muconic acid dimethyl ester. This method can be used to determine t,t-MA at concentrations as low as 0.01 mg/l. A disadvantage of this method is the time-consuming sample preparation. However, the absence of interference caused by other compounds in urine during very low-level determination is a major advantage of this method.

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