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Short communication

Simultaneous detection of hippuric acid and methylhippuric acid in urine by empore™ disk and gas chromatography–mass spectrometry

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Abstract

A method is described for the determination of hippuric acid (HA) and *o*-, *m*-, and *p*-methylhippuric acids (*o*-, *m*-, *p*-MHAs) in urine using solid-phase extraction and gas chromatography–mass spectrometry (GC–MS). The extraction procedure uses an Empore™ disk, derivatized into the respective trimethyl silyl derivatives. All metabolites including the internal standard (I.S.) were clearly able to be analyzed by the DB-17 column. The calibration curves for the four acids show linearity in the range of 5–70 μg/ml. The detection limit of each acid was 1.0–2.5 μg/ml.
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1. Introduction

Toluene and *o*-, *m*-, and *p*-xylenes are widely used as organic solvents, a main ingredient of thinner. Although the inhalation of thinner vapor occasionally causes industrial accidents, thinner abuse by the young generation is a social problem in Japan. Toluene and three isomers of xylenes are respectively metabolized to hippuric acid (HA) and *o*-, *m*-, and *p*-methylhippuric acid (*o*-, *m*-, *p*-MHAs) in vivo, and they are excreted in urine. These urinary metabolites are measured as an index of the exposure of toluene and xylenes.

A number of methods for simultaneous identification and quantification of HA and *o*-, *m*-, *p*-MHAs in human urine have been described; these include high performance liquid chromatography (HPLC) [1–6] and gas chromatography (GC) [7–11], all of which utilize liquid–liquid extraction prior to analysis. However, one method using solid phase extraction [9] for the analyses of HA and *o*-, *m*-, *p*-MHAs in urine by GC has been reported.

In a comparison of extraction by conventional solid phase extraction [9] and that by Empore™ disk, the latter allows for extraction using a

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smaller amount of organic solvent in a shorter time. Although the extraction can be carried out by Empore™ disk rapidly and in a small amount of organic solvent, the extraction method of urinary HA and *o*-, *m*-, and *p*-MHAs has not yet been reported. Herein, we report the results of quantitative analysis by gas chromatography–mass spectrometry (GC–MS) after urinary HA and *o*-, *m*-, and *p*-MHAs were extracted using the Empore™ disk.

2. Experimental

2.1. Reagents and materials

N,*O*-bis(trimethylsilyl)acetamide (BSA) and trimethyl chlorosilane (TMCS) were obtained from GL Sciences (Tokyo, Japan). Hippuric acid and *o*-, *m*-, and *p*-MHA were purchased from Wako Pure Chemical, Osaka. *p*-Hydroxybenzoic acid *n*-butyl ester was purchased from Kanto Chemical, Tokyo. All solvents used were of HPLC grade, and all chemicals were of analytical grade. Empore™ disk cartridges C18 (7 mm/3 ml) were purchased from 3 M Filtration Products, MN, USA.

2.2. Extraction procedure

Solid phase extraction of HA and *o*-, *m*-, and *p*-MHAs was performed with an Empore™ disk. The disk was activated by 100 μl of methanol followed by 100 μl of 10% acetic acid. A total of 100 μl of urine was added to 100 μl of 10% acetic acid and 3 μl of *p*-hydroxybenzoic acid *n*-butyl ester (100 μg/ml) as an internal standard (I.S.). After the above sample had been passed through, 100 μl of 5% acetic acid was added to the disk, and the solution was centrifuged for 5 min at 2500 rpm. Then 100 μl of 5% acetic acid/ethanol (80:20) was added to the disk, and the solution was centrifuged for 5 min at 2500 rpm. The analytes were eluted with 100 μl of ammonia solution/methanol (80:20). The extracts were evaporated to dryness under stream nitrogen. The residue was derivatized with 50 μl of BSA with 10% TMCS at 80 °C for 20 min.

2.3. Gas chromatography procedure

A Hewlett-Packard Model 5890 series II gas chromatograph (Palo Alto, CA) fitted with a model 5971 mass spectrometer and a DB-17 capillary column (30 m $\times$ 0.25 mm; 0.25 μm film of (50%-phenyl)-methylpolysiloxane as stationary; J&W Scientific, Folson, CA) was used. The derivatized sample (1 μl) was injected into the DB-17 column. The oven temperature was programmed as follows: initial temperature 150 °C; initial hold 3 min; ramp 10 °C/min to 250 °C, which temperature was held for 3.0 min. The injector temperature and detector temperature were both maintained at 280 °C. The instrument was autotuned daily with perfluotributylamine. The detector was used in electronic impact mode at 70 eV.

3. Results and discussion

Liquid chromatography–mass spectrometry (LC–MS) seems to be more suitable than GC–MS in the analysis HA and *o*-, *m*-, and *p*-MHAs. However, we examined by GC–MS, because the LC–MS sufficiently does not spread in comparison with GC–MS. Some derivatization methods for analyzing HA and *o*-, *m*-, and *p*-MHAs using GC have been reported. Kataoka et al. reported on the alkylation method that used benzene to extract urinary HA and *o*-, *m*-, and *p*-MHAs [11]. Subsequently, Kongtip et al. [7] and de Carvalho et al. [8] reported the derivatization of HA and *o*-, *m*-, and *p*-MHAs using methanol in acid medium (HCl) as a less toxic reagent. However, their methods involved extraction and a long derivatization time. Therefore, for purposes of shortening the derivatization time, we chose trimethylsilyl as the derivatization reagent.

Although HA was derivatized by *N*,*O*-bis(trimethylsilyl)trifluroacetamide (BSTFA) + 1% TMCS, *o*-, *m*-, and *p*-MHAs were not derivatized. However, HA and *o*-, *m*-, and *p*-MHAs were derivatized by adding TMCS to BSA. Fig. 1 shows the result of silylation reaction of these compounds, which was conducted at different TMCS concentrations (2, 4, 10, 20%) in order to

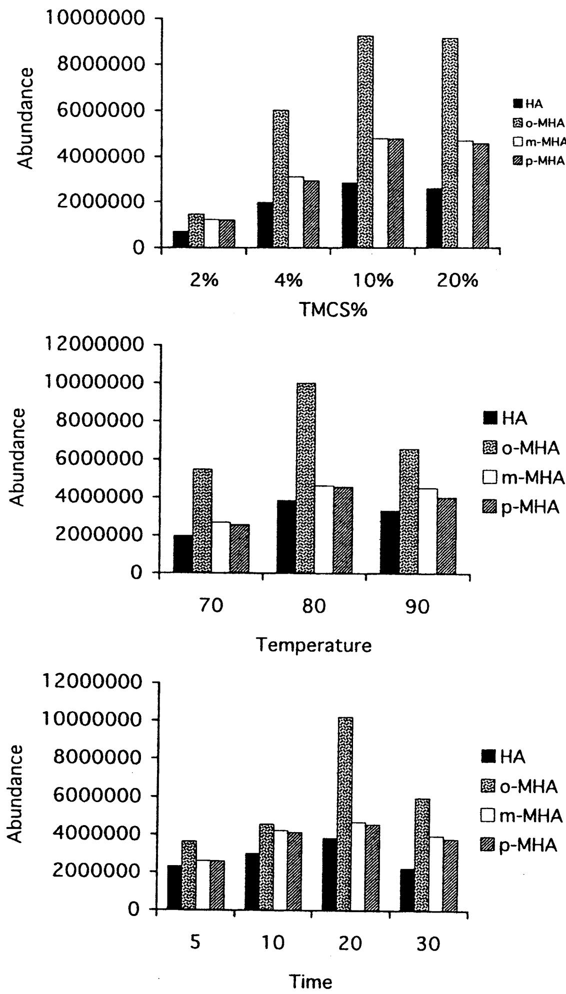

Fig. 1. The examination of TMS derivatization condition for four acids (each 1 μg). (A) The effect of the TMCS proportion for BSA (at 80 °C for 15 min); (B) the effect of the derivatization temperature (BSA + 10% TMCS); (C) the effect of the derivatization time (BSA + 10% TMCS).

efficiently derivatize HA, and *o*-, *m*-, and *p*-MHAs. These compounds were analyzed by GC–MS. The optimal abundance was obtained at BSA + 10% TMCS, under the conditions of 80 °C for 15 min. When the heating temperature of BSA + 10% TMCS was examined at three dif-

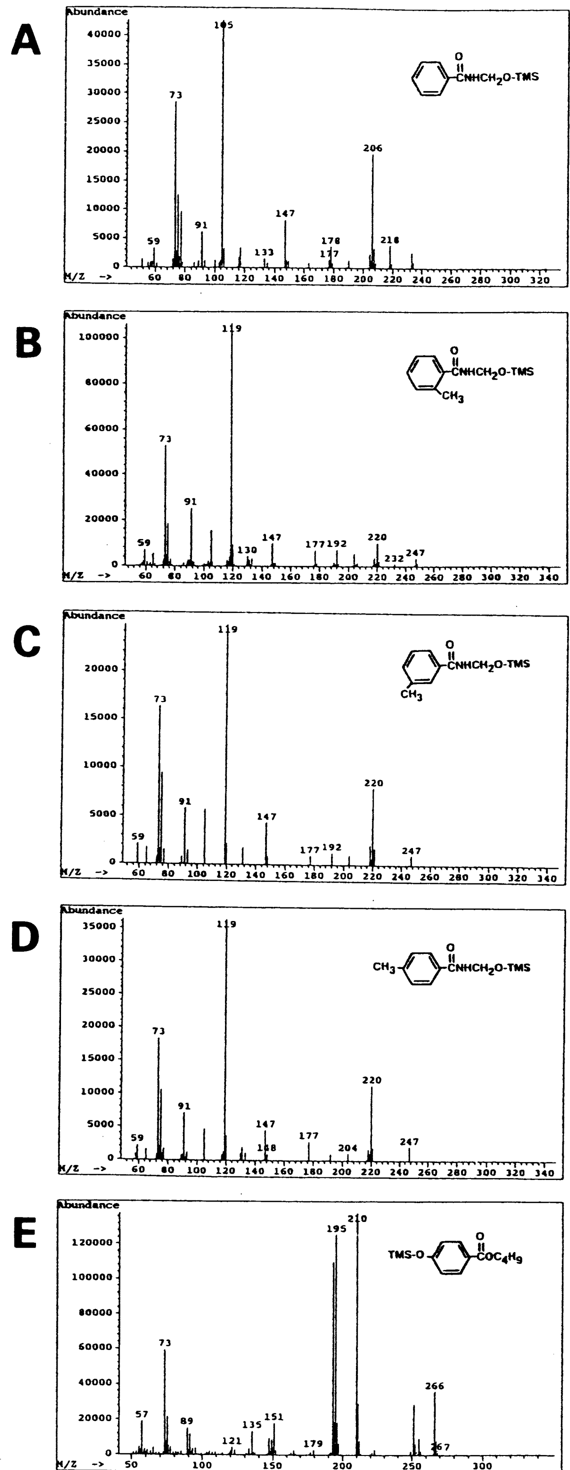

Fig. 2.

Fig. 2. EI mass spectra of each acid and I.S. derivative (TMS = trimethylsilyl). (A) HA; (B) *o*-MHA; (C) *m*-MHA; (D) *p*-MHA; (E) *p*-hydroxybenzoic acid *n*-butyl ester (I.S.).

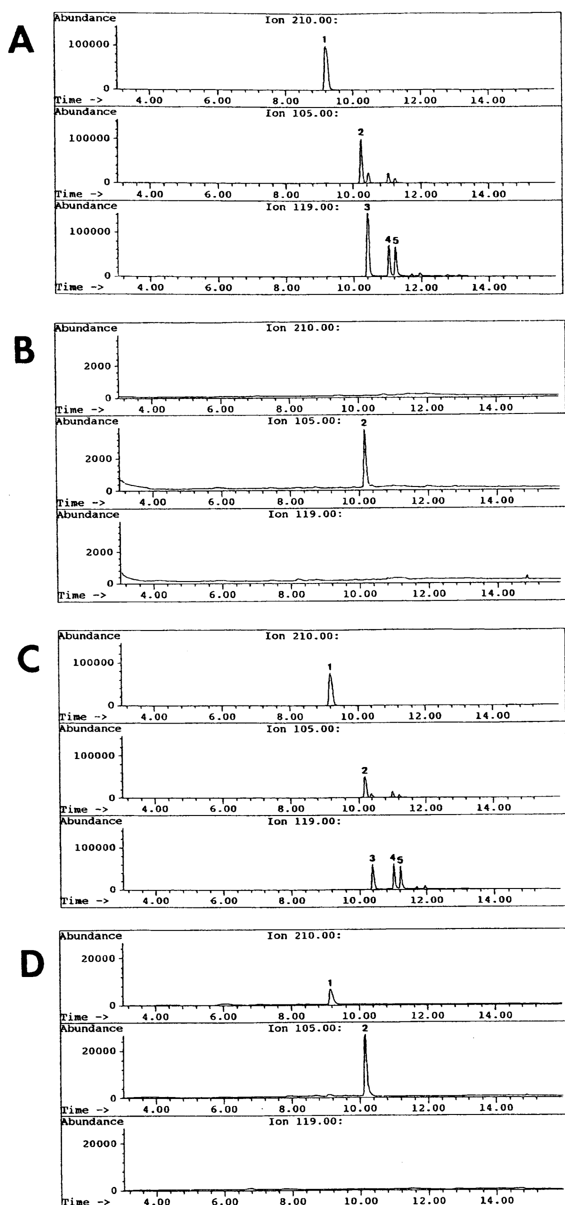

Fig. 3. Gas chromatograms of standard and urine extracts spiked or not spiked with four acids and I.S. (A) Standard solution containing 3 μg of each of four acids (HA and *o*-, *m*-, and *p*-MHAs) and I.S.; (B) extract from unexposed human urine; (C) extract from 100 μl of urine spiked with 3.0 μg of each of four acids and I.S.; (D) the decedent's urine sample. urine sample was diluted 20-fold with water. Peaks: 1 = I.S. (3 μg/ml); 2 = HA; 3 = *o*-MHA; 4 = *m*-MHA; 5 = *p*-MHA.

ferent heating temperatures (70, 80, 90 °C) for 15 min, the optimal abundance was obtained at 80 °C. Finally, at 80 °C, the derivatization time that gave optimal abundance was 20 min. The best silylation condition was obtained in BSA + 10% TMCS at 80 °C for 20 min.

The electron impact mass spectrum of trimethylsilyl derivatized HA, *o*-, *m*-, and *p*-MHAs, and I.S. are shown in Fig. 2. The spectrum of trimethylsilyl derivatized HA was characterized by a [C_6H_5-CO] ion at *m*/*z* 105 and a M^+-77 [C_6H_5] ion at *m*/*z* 147 (Fig. 2A). The spectrum of trimethylsilyl derivatized *o*-, *m*-, and *p*-MHAs were characterized by a [CH_3-C_6H_5-CO] ion at *m*/*z* 119 and a M^+-91 [CH_3-C_6H_5] ion at *m*/*z* 147 (Fig. 2B–D). The spectrum of trimethylsilyl derivatized I.S. was characterized by a M^+ ion at *m*/*z* 267 and a M^+-57 [C_4H_9] ion at *m*/*z* 210 (Fig. 2E). The ions chosen for monitoring were the base peaks at *m*/*z* 105 (HA), 119 (MHAs), and 210 (I.S.). To be considered positive for HA and *o*-, *m*-, and *p*-MHAs, the selected ion monitoring analysis must show coincident peaks in the *m*/*z* 105, 206, and 147 for HA, and in the *m*/*z* 119, 220, and 147 for *o*-, *m*-, and *p*-MHAs, respectively. However, a molecular ion peak (M^+) was not observed for HA and *o*-, *m*-, and *p*-MHAs derivatives. The sensitivity seems to be high, because the fragment-ion of *o*-MHA is lower than other compounds (Fig. 2). The TMS derivatives were stable for at least few days if the containers were kept air-tight.

Typical GC–MS chromatograms for the separation of the HA and *o*-, *m*-, and *p*-MHA derivatives under these experimental conditions are given in Fig. 3A. *p*-Hydroxybenzoic acid *n*-butyl ester, chosen as an I.S., was clearly separated from HA and *o*-, *m*-, and *p*-MHAs.

The relative extraction efficiency from urinary HA and *o*-, *m*-, and *p*-MHAs using this procedure were presented in Table 2. The efficiency of this method for extracting HA and *o*-MHA was lower than that for extracting *m*-, and *p*-MHAs. The difference in extraction efficiency among isomers of MHA cannot be explained. Calibration curves were rectilinear over the ranges of 5–70 μg/ml for HA and *o*-, *m*-, and *p*-MHAs. All had correlation coefficients higher than 0.99, as shown in Table 1. The detection limits of this method, for a single-to-noise ratio of 3:1, were 2.5 μg/ml for HA, 1.0 μg/ml

Table 1
Detection limit and linearity ($n = 5$)

Metabolites	Limit of detection (μg/ml)	Range of linearity (μg/ml)	Linear regression	Correlation coefficient
Hippuric acid	2.5	5.0–70.0	$y = 0.0943x + 0.1722$	0.9931
o-methylhippuric acid	1.0	5.0–70.0	$y = 0.2299x - 0.8155$	0.9948
m-methylhippuric acid	2.5	5.0–70.0	$y = 0.1554x - 0.2438$	0.9934
p-methylhippuric acid	2.5	5.0–70.0	$y = 0.13x - 0.0551$	0.9977

Table 2
Interday precision and recovery in urine ($n = 5$)

Compound	Concentration (μg/ml)	Interday precision (%)	Recovery (%)	Error* (%)
Hippuric acid	10	3.8	42.3 ± 1.7	+4.0
	60	5.6	43.6 ± 1.5	+6.5
o-methylhippuric acid	10	4.4	40.7 ± 1.7	+5.4
	60	5.2	42.5 ± 1.6	+7.3
m-methylhippuric acid	10	3.7	96.3 ± 1.2	+3.6
	60	5.6	96.6 ± 1.6	−3.8
p-methylhippuric acid	10	2.3	97.5 ± 1.5	+4.9
	60	2.9	98.2 ± 1.5	−3.6

* Expressed as the least squares equations.

Table 3
Intraday precision in urine ($n = 5$)

Compound	Concentration (μg/ml)	Intraday precision (%)	Error* (%)
Hippuric acid	10	3.9	+4.2
	60	4.6	−3.4
o-methylhippuric acid	10	3.6	+3.8
	60	5.4	+6.1
m-methylhippuric acid	10	2.2	−4.8
	60	3.1	+3.3
p-methylhippuric acid	10	1.9	+3.1
	60	2.3	−4.8

* Expressed as the least squares equations.

Table 4
Toluene, phenobarbital and hippuric acid concentrations in postmortem samples

Sample	Toluene (μg/ml)	Phenobarbital (μg/ml)	Hippuric acid (μg/ml)
Blood	85.4	4.2	–
Urine	7.2	1.7	458

for *o*-MHA, 2.5 μg/ml for *m*-MHA, and 2.5 μg/ml for *p*-MHA. The interday and intraday coefficients of variation for two different concentrations (10 and 60 μg/ml) in urine were 1.9–5.4 and 2.3–5.6%, respectively (Tables 2 and 3), showing that the proposed method was repeatable and the results were reproducible.

Fig. 3B was prepared from the urine of a non-exposed subject, and no peaks without HA were observed that would interfere with the measurement of *o*-, *m*-, and *p*-MHAs with an I.S. Human urinary HA concentration is largely affected by a meal, even if the subject has not been exposed to toluene. The urinary excretion of HA rises in response to intake of cranberries, plums, and prunes [12–14], and decreases in response to smoking and drinking [15,16]. We examined urinary HA concentration in a non-exposed healthy subject ($n = 6$), and values were within a range of 5.5–15.8 μg/ml. The urinary HA and MHAs concentrations of creatinine in the biological exposure indices of Japan are 2.5 and 1.5 g/g, respectively. A chromatogram from a urine extract (100 μl) containing HA and *o*-, *m*-, and *p*-MHAs with an I.S. is shown in Fig. 3C.

A 28-year-old man, known to be toluene abuser, was found lying dead in his home. At the autopsy, no particular morphological changes were noted except for visceral congestion. Toxicological analyses were performed on blood and urine samples. Toluene and phenobarbital were detected in blood samples and concentrations are presented in Table 4. Generally, the blood concentration is associated with blood toluene concentrations from 10 to 20 μg/ml [17]. In our case, the blood toluene concentration exceeded by more than 4 times the fatal levels. However, the blood phenobarbital concentration was within the range of therapeutic concentrations (16–48 μg/ml) previously reported [18]. Hippuric acid was detected in urine sample and chromatogram is shown in Fig. 3D.

In conclusion, the method for simply and rapid analyzing urinary HA and *o*-, *m*-, *p*-MHAs using Empore™ disk and GC–MS was presented. This method is useful not only for analysis of thinner abuse but also analysis of public health hazards.

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