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Determination of hippuric acid and *o*-, *m*- and *p*-methylhippuric acids in urine by capillary gas chromatography

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Abstract: A capillary gas chromatographic (GC) method for the simultaneous determination of urinary hippuric acid (HA) and *o*-, *m*- and *p*-methylhippuric acids (MHAs), metabolites of toluene and *o*-, *m*- and *p*-xylenes, respectively, is described. These metabolites are converted into their isopropyl derivatives by extractive alkylation with tetrahexylammonium ion as extracting agent and isopropyl bromide as alkylating reagent in benzene. The derivatives are analysed using a chromatograph equipped with hydrogen flame ionization detector, split injection system and DB-17 capillary column. Benzoylleucine is used as an internal standard. The derivatives are well separated within 5 min and no interfering peaks are observed. The calibration curves of HA and MHAs in the range 1–50 μg are linear and sufficiently reproducible for quantitative analysis. Urine can be analysed accurately and precisely by this method without prior clean-up of the sample.

Keywords: *Capillary GC; hippuric acid;* o-, m- *and* p-*methylhippuric acids; extractive isopropylation; metabolites of toluene and* o-, m- *and* p-*xylenes; urinary excretion of hippuric acid and methylhippuric acids.*

Introduction

Toluene and xylene are organic solvents used in industry as a replacement for the carcinogen benzene, but these solvents also exhibit considerable toxicity [1]. Estimation of the exposure levels or inhaled amounts of these solvents is important in monitoring the health of workers exposed to such solvents. However it is difficult to measure the atmospheric concentrations of these solvents in the workplace. The major metabolites of toluene and xylene are hippuric acid (HA) and methylhippuric acid (MHA), respectively [2]. Concentrations of these metabolites in urine of workers quantitatively reflect the solvent vapour concentrations in their workplace, and have been regarded as indices of exposure to these solvents [3–9]. There are many factories where both toluene and xylene are used in a mixture or simultaneously, and xylene often occurs as a contaminant of the toluene used in industry [10]. Xylene used in industry consists of a mixture of *o*-, *m*- and *p*-isomers, and these isomers are metabolized to the corresponding isomers of MHA, respectively. If there are differences in the toxicity of toluene and isomers of xylene, the separate determination of HA and MHAs is necessary. Therefore, the simultaneous determination of urinary HA and MHAs concentrations is useful for occupational health control.

Many methods for the determination of these metabolites in urine have been described, such as thin-layer chromatography [11], gas chromatography (GC) [12–16], isotachophoresis [17, 18] and HPLC [18–26]. However, there is no report describing complete separation of these metabolites, except some HPLC methods [23–26]. These HPLC methods can be performed without pretreatment of sample, however the contamination of columns with other urinary components causes deterioration of column performance.

In the present work, a simple and reliable method for the simultaneous determination of HA and *o*-, *m*- and *p*-MHAs by capillary GC after extractive alkylation is described.

Experimental

Reagents

HA and benzoylleucine as the internal standard (i.s.) were purchased from Nakarai

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Chemicals (Kyoto, Japan). MHA isomers (*o*-, *m*- and *p*-MHAs) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). A standard solution of HA and MHAs was prepared so as to contain 0.1 mg ml^{-1} of each compound in distilled water. Isopropyl bromide was obtained from Nakarai Chemicals. Tetrahexylammonium hydroxide (THA-OH) was prepared as follows: 5 g of tetrahexylammonium iodide (Nakarai Chemicals) were dissolved in 30 ml of 80% methanol and to this solution was added 8 g of silver oxide (Nakarai Chemicals). The mixture was shaken for 1 h at room temperature. After centrifugation, the supernatant was evaporated at 50°C, and the residue reconstituted in methanol to give a 10% (w/v) solution. All other chemicals were of analytical grade.

Urine samples

Normal human urine samples (24 h) were obtained from laboratory personnel. Male Wistar rats weighing 350–450 g were used in experiments. The rats were treated with solvent mixture containing 0.05 ml ml^{-1} each of toluene and *o*-, *m*- and *p*-xylenes in mineral oil (0.5 ml kg^{-1}, b.w., p.o.). Control rats received the same dose of mineral oil without solvent. Each rat was placed in a metabolic cage which allowed separate collection of urine and faeces. Urine samples (24 h) were collected under toluene and kept frozen if not analysed immediately.

Analytical derivatization procedure

Urine (50 μl) was pipetted into a 10 ml pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.1 ml of 0.1 mg ml^{-1} i.s. solution and 0.2 ml of 10% THA-OH, the total volume was made up to 1 ml with distilled water. Immediately after addition of 0.2 ml of isopropyl bromide and 2 ml of benzene, the mixture was shaken with a shaker set at 300 rpm (up and down) for 2 min at room temperature. After centrifugation at 3000 rpm for 1 min, the organic layer was transferred to another tube and the aqueous layer was re-extracted with 1 ml of benzene by shaking for 30 s. The combined organic layers were then heated at 90°C for 10 min and washed with 2 ml of a saturated silver sulphate by shaking for 30 s. After centrifugation, the organic layer was transferred to another tube and evaporated to dryness at 90°C under a stream of dry air. The residue was dissolved in 0.1 ml of ethyl acetate, and a 1 μl of this solution was injected into the GC.

Gas chromatography

GC analyses were carried out by means of a Hewlett–Packard 5890A gas chromatograph equipped with a hydrogen flame ionization detector and a split injection system. The output of the FID was recorded on a Hewlett–Packard 3392A recording integrator. A fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with cross-linked DB-17 (equivalent to OV-17) (J & W, Folson, CA, USA) was used. The helium carrier gas and nitrogen make-up gas flow-rates were 1.0 and 40 ml min^{-1}, respectively. Split ratio was 20:1. The temperatures of injector, detector and column were 250, 280 and 230°C, respectively. The peak heights of HA, MHAs and the i.s. were measured and the peak height ratios against the i.s. calculated for the construction of the calibration curves.

Gas chromatography–mass spectrometry

The Hewlett–Packard 5890A gas chromatograph was operated in connection with HP 5970B mass detector under the following conditions: GC column, identical to that used for GC analysis; ion-source temperature, 250°C; ionization voltage, 70 eV; helium flow rate, 1.0 ml min^{-1}.

Preparation of reference compound

A reference sample of isopropyl hippurate was prepared as follows: 100 mg of hippuric acid was dissolved in 2 ml of 15% BF_3-isopropanol (Tokyo Kasei Kogyo) and the mixture was heated for 5 min in a boiling water bath. After cooling, the reaction mixture was transferred into a separatory funnel with 15 ml of distilled water and this solution was extracted with 20 ml of chloroform. After washing with 20 ml of water, the organic layer was transferred to a round-bottomed flask and the solvent evaporated. The crystalline residue was recrystallized from *n*-hexane to yield long colourless needles; m.p. 84–85°C. The data for elemental analysis were as follows. Calc. for $C_{12}H_{15}O_3N$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.83; H, 6.79; N, 6.22.

Results and Discussion

The extractive alkylation technique [27] introduced by Ehrsson is simple and selective

for the derivatization of acidic compounds prior to GC analysis, and it has been applied to analysis of a great number of organic acids [28, 29]. The extractive alkylation process can be described as a two step reaction, in which acid compound is first extracted as an ion-pair with alkylammonium cation into organic layer, and next alkylated with a nucleophilic reagent such as alkyl halide in the organic layer. In the present work, benzene was selected as the most suitable solvent for ion-pair extraction. Of several alkylammonium ions tested, THA-OH proved to be the most satisfactory counter-ion for the rapid and quantitative extraction of HA and MHAs. As shown in Fig. 1, alkylation of these compounds with isopropyl bromide was accomplished within 10 min at 90°C. THA bromide formed as a by-product in the reaction and its degradation products formed in the injector caused significant peak distortion. However the problem was solved by washing with aqueous silver sulphate solution [30]. The mean derivatization yield of HA throughout the procedure established above was determined to be 96.9% (n = 3) by comparison with the synthetic reference derivative. The derivative preparation took within 30 min, and several samples could be treated simultaneously.

The structures of the derivatives of HA and MHAs were confirmed by gas chromatography–mass spectrometry (GC–MS) and by elemental analysis. As shown in Fig. 2, a molecular ion peak (M^+) was observed for each of the derivatives. Other ion peaks useful for structure elucidation were as follows: $M^+ -59$ [$OCH(CH_3)_2$], $M^+ -87$ [$COOCH(CH_3)_2$] and m/e 77 (C_6H_5), 91 ($CH_3—C_6H_5$), 105 ($C_6H_5—CO$) and 119 ($CH_3—C_6H_5—CO$). The elemental analysis data for HA derivative agreed with the theoretical values calculated for the expected structure. The derivatives were found to be very stable under normal laboratory conditions.

Typical chromatogram of HA and MHAs was shown in Fig. 3(A). Each compound gave a single and symmetrical peak on GC with a DB-17 capillary column. Each peak represents *ca* 0.25 μg. A chromatographic run was completed within 5 min. In order to test the linearity of the calibration curve, various amounts of HA and MHAs ranging from 1 to 50 μg were derivatized in a mixture, and aliquots representing 0.01–0.5 μg of each compound were injected. In each case, a linear relationship was obtained, and the regression lines of HA and *o*, *m*- and *p*-MHAs were $y = 0.0417x - 0.0043$ ($r = 0.9995$, $n = 18$), $y = 0.0535x + 0.0013$ ($r = 0.9994$, $n = 18$), $y = 0.0639x + 0.0177$ ($r = 0.9997$, $n = 18$) and $y = 0.0503x + 0.0017$ ($r = 0.9999$, $n = 18$), respectively, where y is the peak height ratio and x is the amount (μg) of each compound. The minimum detectable amounts of HA and MHAs to give a signal three times that of the average noise level were found to be about 1 ng.

In order to demonstrate the applicability of the method to urinary samples, the contents of HA and MHAs in urines of human and rat treated with toluene and xylene isomers were analysed. As shown in Fig. 3(B–F) HA and

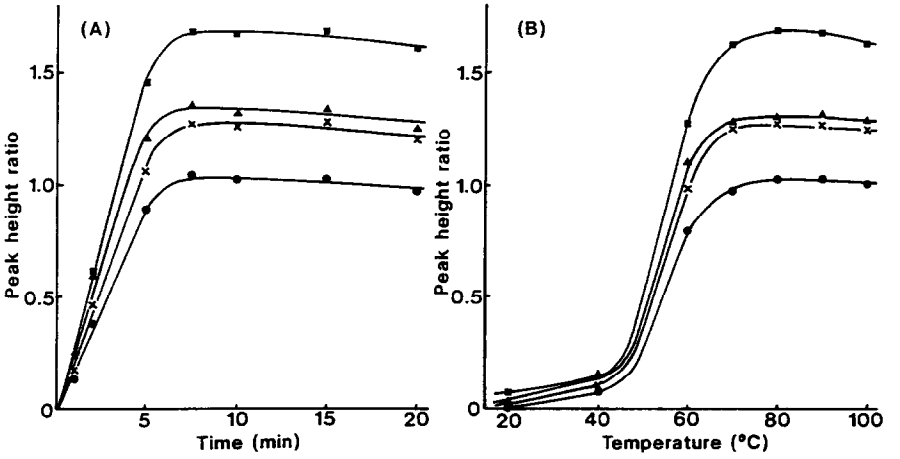

Figure 1
Effect of time (A) and temperature (B) on the isopropylation of hippuric acid and methylhippuric acids. Key: ● = hippuric acid; ▲ = *o*-methylhippuric acid; ■ = *m*-methylhippuric acid; × = *p*-methylhippuric acid.

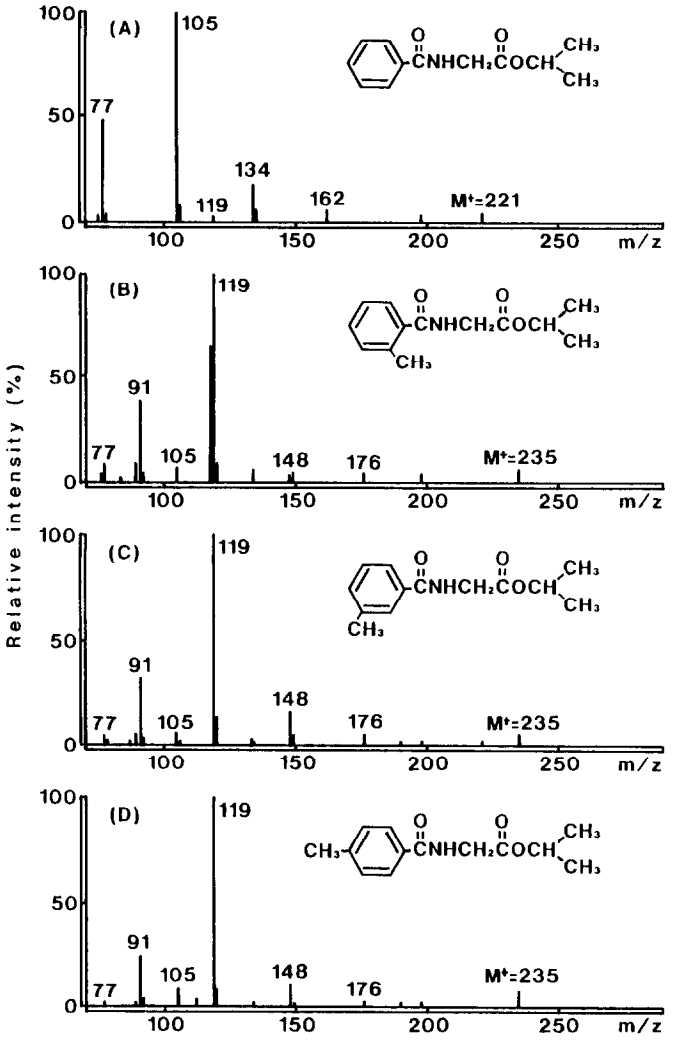

Figure 2
GC–MS spectra obtained for the isopropyl derivatives of (A) hippuric acid, (B) *o*-methylhuppuric acid, (C) *m*-methylhippuric acid and (D) *p*-methylhippuric acid.

Table 1
Recoveries of hippuric acid and *o*-, *m*- and *p*-methylhippuric acids added to human and rat urines

Compound added (0.5 mg ml^{-1})	Amount found (mg ml^{-1})*		Recovery (%)
	Non-addition	Addition	
Human urine			
HA	0.317 ± 0.010	0.801 ± 0.004	96.8
o-MHA	ND†	0.471 ± 0.010	94.2
m-MHA	ND	0.480 ± 0.012	96.0
p-MHA	ND	0.488 ± 0.008	97.6
Rat urine			
HA	ND	0.486 ± 0.018	97.2
o-MHA	ND	0.479 ± 0.018	95.8
m-MHA	ND	0.483 ± 0.015	96.6
p-MHA	ND	0.485 ± 0.020	97.0

*Mean ± SD (n = 3).
†Not detectable.

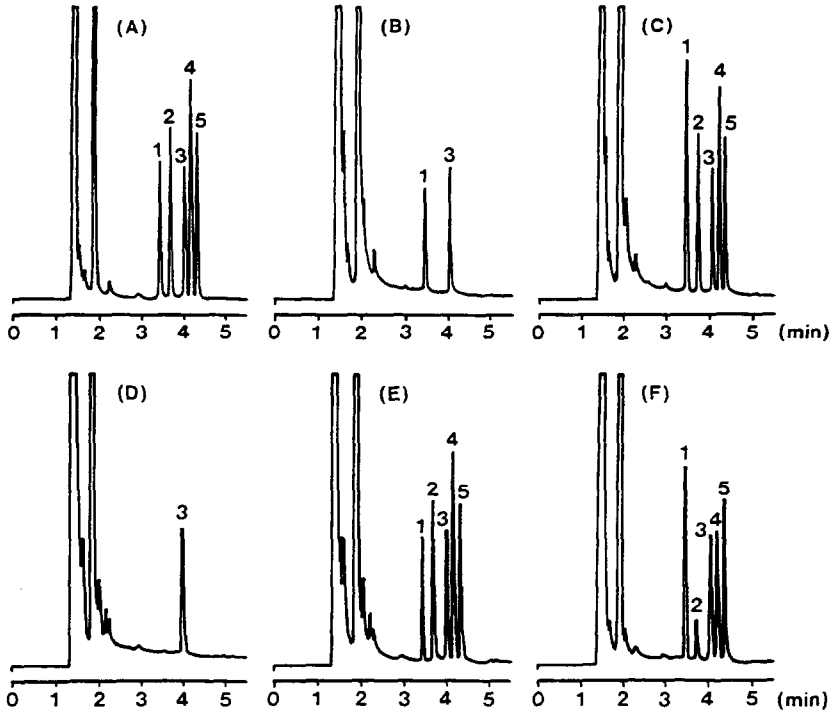

Figure 3
Capillary gas chromatograms of hippuric acid and methylhippuric acids obtained from standard solution and urine samples. (A) Standard solution containing 25 μg each of four acids (hippuric acid and *o*, *m*- and *p*-methylhippuric acids); (B) normal human urine; (C) sample B plus for acid standards; (D) control rat urine; (E) sample D plus four acid standards; (F) solvent-treated rat urine (the rat was treated with solvent mixture containing toluene and *o*-, *m*- and *p*-xylenes in mineral oil, 0.5 ml kg^{-1}, b.w., p.o.). GC conditions: see Experimental. Peaks: 1 = hippuric acid, 2 = *o*-methylhippuric acid, 3 = benzoylleucine (i.s.), 4 = *m*-methylhippuric acid, 5 = *p*-methylhippuric acid.

MHAs were completely separated from urine constituents. GC–MS analysis of the peaks of HA, MHAs and i.s. from urine samples confirmed that each peak was almost uniform. MHAs were not detected in the normal human urine [Fig. 3(B)], and urinary HA excretions were 323–759 mg 24 h^{-1} (n = 5). On the other hand, the control rat excreted neither HA nor MHAs [Fig. 3(D)], but the solvent-treated rats excreted the corresponding metabolites of each solvent [Fig. 3(F)]. As shown in Table 1, the recovery rates of HA and MHAs added to urine samples were 94.2–97.6%, and their relative standard deviations were 0.5–4.1% (n = 3), indicating that this method is accurate and precise.

In conclusion, extractive alkylation and subsequent capillary GC analysis is a simple and reliable method for the simultaneous determination of HA and MHAs in urine. The present study indicates that this method becomes a useful tool for occupational health control, not only in cases of exposure to one solvent but also in cases of exposure to a mixture of solvents.

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