Measurement by gas chromatography of urinary hippuric acid and methylhippuric acid as indices of toluene and xylene exposure

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ABSTRACT A gas chromatographic method was applied to the determination of the urinary glycine conjugates, hippuric, o-, m- and p-methylhippuric acids. These were extracted with ethyl acetate from urine after acidification with hydrochloric acid. The internal standard solution (heptadecanoic acid methanol solution) was added before extraction and a diazomethane-ether-ethanol solution was subsequently added to the dried extracts. The methylated residues were dissolved in methanol and injected into a gas chromatograph as described by Buchet and Lauwerys (1973). By the combined use of gas chromatography and mass spectrometry the methyl esters of hippuric acid and m-methylhippuric acid were identified in the urine of a volunteer who had been exposed to toluene and m-xylene vapours. When the urine specimen contained salicyluric acid (a urinary metabolite of salicylic acid) two sharp peaks were observed. The faster peak coincided with m- or p-methylhippuric acid. The upper limit of urinary hippuric acid concentration in healthy subjects with no occupational exposure was calculated by this method to be 1.026 $\mu\text{g/ml}$ (fiducial limit 5%) after correction to 1.024 for variation in urinary density.

Toluene and xylene are used as industrial solvents. Inhaled toluene and m- or p-xylene are excreted as hippuric and m- or p-methylhippuric acids in the urine. The estimation of these acids in urine is therefore a valuable index of toluene or xylene exposure (Ogata *et al.*, 1970). To estimate exposure by quantitative determination of urinary hippuric and m-methylhippuric acids, several investigators have used methods based on colour development after paper chromatography (Ogata *et al.*, 1969) and gas chromatography (Buchet and Lauwerys, 1973). The gas chromatographic method is very useful, but Buchet and Lauwerys did not examine interference by other urinary metabolites nor did they show the normal range of urinary hippuric acid concentration by their method.

The present paper describes gas chromatographic identification and determination of urinary hippuric acid and methylhippuric acid isomers. The normal range of urinary hippuric acid concentration was calculated by this method, and interference by other urinary glycine conjugates was assessed.

Materials and methods

REAGENTS

Hippuric acid, methanol, ethanol and HCl were obtained from the Wako Pure Chemical Co. Ltd., Japan; ethyl acetate and anhydrous Na_2SO_4 were obtained from Nakarai Chemicals Ltd., Japan; heptadecanoic acid and m-methylhippuric acid were obtained from Tokyo Kasei Industries Ltd., Japan, and salicyluric acid, o- and p-methylhippuric acids were obtained from the Nippon Kanko Shikiso Kenkyusho Co. Ltd., Japan.

URINE SAMPLES

For the identification of urinary hippuric and m-methylhippuric acids, a sample was obtained from a healthy volunteer who had inhaled the vapours of toluene (67 ppm) and m-xylene (83 ppm) for three hours.

In order to determine the normal range of urinary hippuric acid concentration, urine samples were obtained from 48 healthy volunteers (27 men and 21 women, aged from 21 to 50 years) who had not been exposed to toluene vapour. The urine samples were obtained in an early summer (May) afternoon.

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URINE TREATMENT

Urine (0.5 ml), internal standard (0.2 ml of heptadecanoic acid methanol solution in a suitable concentration) and a few drops of HCl (6 mol/l) were shaken with 4 ml of ethyl acetate in a mechanical shaker for about a minute. The organic phase was transferred to another test tube and approximately 0.5 g of anhydrous Na₂SO₄ was added to remove the remaining water. The supernatant was transferred to a third test tube and dried in an air stream from a pump, while the tube was kept at 70°C in a water bath. Diazomethane-ether-ethanol solution was added to the residue until the yellow colour persisted at room temperature. The solvents were then evaporated and the methylesterified residues were dissolved in 0.1-0.2 ml methanol.

GAS CHROMATOGRAPHY

The instrument used was a Hitachi 063 type, FID., with a 1 m × 3 mm internal diameter stainless steel column packed with silicon SE 30 3% or 10% on chromosorb WAW. The oven temperature was 190°C or 200°C, and the injection port and detector temperature was 230°C. The sample size was 1-2 µl, and the carrier gas was N₂ at a flow rate of 40 ml/min.

GAS CHROMATOGRAPH MASS SPECTROMETRY

The instrument was a Shimadzu LKB 9000 with a 1 m × 3 mm internal diameter glass column packed with silicone SE 30 10%. The carrier gas was He at a flow rate of 20 ml/min. The oven temperature was kept constant at 220°C or programmed at 200°C-250°C.

Results

The relative retention times of the glycine conjugates hippuric, o-, m- and p-methylhippuric acids, compared to that of the internal standard (heptadecanoic acid) by this method are shown in the Table. The order of elution was hippuric acid first, followed by o-methylhippuric and m- or p-methylhippuric acids. An example of the gas chromatograph of these glycine conjugates and salicylic acid after their

addition to urine is shown in Fig. 1. This shows that m- and p-methylhippuric acids eluted together as a single peak.

This method was used to analyse a urine sample from a man exposed to toluene (67 ppm) and m-xylene (83 ppm) vapour and the molecular weights of apparent components were measured by gas chromatograph-mass spectrometry. These chromatograph and mass spectra patterns are shown in Figs. 2a and b. The molecular weight and the patterns correspond to the methyl esters of hippuric and methylhippuric acid respectively.

Interference by other metabolites or glycine conjugates was assessed. The gas chromatogram of the urine sample from a healthy man six hours after he had swallowed 1 g of acetylsalicylic acid (aspirin) is shown in Fig. 3a. Two large peaks appeared and the relative retention times of these peaks were 0.39 (SA 1) and 0.57 (SA 2) as shown in the Table. The faster peak had almost the same retention time as methyl-esterified m-methylhippuric acid; the mass spectra patterns of these peaks are shown in Fig. 3b. The molecular weights of these peaks were 209 and 223, and these molecular weights correspond to the mono- and di-methyl esters of salicylic acid respectively.

Urinary hippuric acid concentrations after correction to 1.024 for variation in urinary density in

Fig. 1 Gas chromatographic elution pattern of methylesterified glycine conjugates. (1) = hippuric acid; (2) = o-methylhippuric acid; (3) = m-methylhippuric acid, p-methylhippuric acid or the faster peak of salicylic acid; (4) = the slower peak of salicylic acid; (5) = internal standard (heptadecanoic acid).

Table Relative retention times of methylesterified glycine conjugates

	HA	o-MHA	m-MHA	p-MHA	HD	SA 1	SA 2
1*	0.29	0.33	0.40	0.41	1.00	0.39	0.57
2*	0.27	0.33	0.40	0.40	1.00	0.40	0.58

HA = hippuric acid; MHA = methylhippuric acid; HD = heptadecanoic acid; SA 1 = faster peak of salicylic acid; SA 2 = slower peak of salicylic acid.

1*: values were obtained singly.

2*: values were obtained from injection together (mixture).

healthy subjects showed a logarithmic distribution (Fig. 4). The mean geometric value and the upper 5% fiducial limit of the hippuric acid concentration were 123 $\mu\text{g/ml}$ and 1026 $\mu\text{g/ml}$ respectively.

Discussion

Buchet and Lauwerys (1973) demonstrated gas chromatographic determination of urinary hippuric and m-methylhippuric acids using silicone SE 30 3% as the analytical column. However, no clear separation of hippuric and o-methylhippuric acids (as a minor metabolite of o-xylene) was observed when this method was applied to the determination of urinary

glycine conjugates. We found that the concentration of the liquid phase, SE 30, had to be increased from 3% to 10% to obtain satisfactory separation of these two glycine conjugates. On the other hand, no separation of m- and p-methylhippuric acids was obtained by any concentration of SE 30 from 3% to 10%. The peak height of each methylhippuric acid isomer increased linearly with concentration. Measurement of the combined peak height could therefore be used to estimate the total or additional amount of inhaled m- and p-xylenes. There was no marked difference in the amounts of methylesterified p- or m-methylhippuric acid detected by the use of mass spectra, as shown in Fig. 2b (3).

Fig. 2 Gas chromatographic elution pattern (A) and mass spectra patterns (B) of methylesterified urine extract from a volunteer exposed to toluene and m-xylene vapours. (1) in A and B = hippuric acid; (2) in A and B = m-methylhippuric acid; (3) = internal standard (heptadecanoic acid in A; p-methylhippuric acid in B); M = molecular weight.

Fig. 3 Gas chromatographic elution pattern (A) and mass spectra patterns (B) of methylesterified urine extract from a male volunteer 6 h after ingestion of acetylsalicylic acid. (1) in A and B = salicyluric acid (mono-methylesterified); (2) in A and B = salicyluric acid (di-methylesterified); (3) in A = internal standard (heptadecanoic acid); M = molecular weight.

Fig. 4 Distribution of the levels of urinary hippuric acid in healthy subjects. Values were calculated after correction to 1.024 for variation of urinary density.

The urine sample, collected six hours after oral administration of acetylsalicylic acid, contained a component that had the same retention time as m- or p-methylhippuric acid after methylesterification. Analysis by gas chromatograph mass spectrometry suggested that this component was mono-methylesterified salicyluric acid. When a peak such as this one, with a relative retention time of about 0.57 is detected, the subject should be asked whether he has in fact taken a drug which would be excreted as salicyluric acid.

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References

- Buchet, J. P., and Lauwerys, R. R. (1973). Measurement of urinary hippuric and m-methylhippuric acids by gas chromatography. *British Journal of Industrial Medicine*, **30**, 125-128.
- Ogata, M., Tomokuni, K., and Takatsuka, Y. (1969). Quantitative determination in urine of hippuric acid and m- or p-methylhippuric acid, metabolites of toluene and m- or p-xylene. *British Journal of Industrial Medicine*, **26**, 330-334.
- Ogata, M., Tomokuni, K., and Takatsuka, Y. (1970). Urinary excretion of hippuric acid and m- or p-methylhippuric acid in persons exposed to vapours of toluene and m- or p-xylene as a test of exposure. *British Journal of Industrial Medicine*, **27**, 43-50.