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Note

High-performance liquid chromatographic separation of urinary hippuric and *o*-, *m*- and *p*-methylhippuric acids with a β-cyclodextrin-bonded column

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Toluene and xylene have many industrial applications as solvents. Most of the absorbed toluene and *o*-, *m*- and *p*-xylene is excreted in the urine as hippuric acid (HA) and *o*-, *m*- and *p*-methylhippuric acids (MHA). The determination of these acids is therefore a valuable index of exposure to toluene and/or xylene [1]

We have described a high-performance liquid chromatographic (HPLC) method for the quantitative determination of HA and *m*-MHA in urine [2]. However, this method had little selectivity towards *m*- and *p*-MHA, which have similar dissociation constants. Although separation of *m*- and *p*-MHA has been achieved with HPLC [3] and gas chromatography [4, 5] after derivatization, these methods are time-consuming and elaborate.

An HPLC separation of *o*-, *m*- and *p*-MHA isomers using cyclodextrins as an additive to an aqueous mobile phase has been published recently by Sakai et al. [6] However, Fujimura et al [7] pointed out problems often encountered with this method, such as contamination of solutes in the effluent with cyclodextrins, the loss of cyclodextrins, which are expensive, or partial plugging of the mobile phase delivery line.

In this paper, we report an HPLC method for the simultaneous determination of HA and all three isomers of MHA in urine using a β-cyclodextrin-bonded column.

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EXPERIMENTAL

Materials

HA, *o*-, *m*- and *p*-MHA, mandelic acid and phenylglyoxylic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan) HPLC-grade methanol was obtained from Wako (Osaka, Japan). The other chemicals and solvents used were of analytical-reagent grade.

Apparatus and chromatographic conditions

A Waters Model ALC/GPC 609 liquid chromatograph, consisting of a Model U6K injector, a Model M600 multi-solvent delivery system and a Model 490 programmable multi-wavelength (190–600 nm) detector was used. The analyses were performed on a 250 mm × 4.6 mm I D., 5 μm particle size, β-cyclodextrin-bonded column (Cyclobond I, Advanced Separation Technologies, Whippany, NJ, U.S.A.). The detection wavelength was set at 272 nm with a sensitivity setting of 0 01–0.03 a u.f.s. The chromatograms were recorded on a strip chart recorder (Nihon Denshi Kagaku, Tokyo, Japan) at a full scale of 10 mV and a chart speed of 0.5 cm/min. The mobile phase used was methanol–5 m*M* potassium phosphate–acetic acid (50:50:0 25, v/v) at a flow-rate of 1.0 ml/min at ambient temperature.

Extraction procedure

To 1.0 ml of urine in a glass-stoppered tube were added 0.04 ml of concentrated hydrochloric acid and 0.3 g of sodium chloride This mixture was extracted with 4 0 ml of ethyl acetate by mechanical shaking for 5 min. After centrifugation at 1000 *g* for 5 min, a 0.2-ml aliquot of the supernatant organic phase was transferred to a test-tube. The ethyl acetate was evaporated to dryness in a water-bath at 70 °C. The residue was dissolved in 0.2 ml of the mobile phase, and a 2-μl sample was injected into the chromatograph.

Calibration curves

Standard solutions containing HA and *o*-, *m*- and *p*-MHA were made up in water and in urine to give a range of concentrations of 0.2–1.0 mg/ml.

RESULTS

Separation of HA and o-, m- and p-MHA

The chromatograms obtained for sample extracts are shown in Fig 1, and HA and *o*-, *m*- and *p*-MHA were separated in the same assay. Only HA was present in the urine of a person who had not been exposed to solvent. These acids were also well separated from other normal components in urine. In the urine from a technologist in a histology laboratory exposed to low levels of

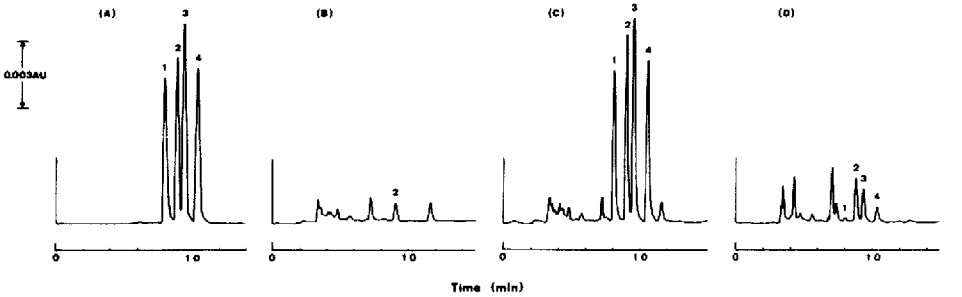

Fig 1 Chromatograms of extracts from (A) standard aqueous solution (1 mg/ml of each acid), (B) control urine, (C) urine supplemented with standard acids (1 mg/ml of each acid) and (D) urine from a technologist in a histology laboratory exposed to xylene Peaks 1 = *o*-methylhippuric acid, 2 = hippuric acid, 3 = *m*-methylhippuric acid, 4 = *p*-methylhippuric acid

xylene (4–50 ppm) for 55 min, the concentrations found were 0.04 mg/ml for *o*-MHA, 0.21 mg/ml for *m*-MHA and 0.11 mg/ml for *p*-MHA (Fig. 1D).

Calibration

As the peaks of the acids were well resolved and symmetrical, peak heights were used to measure the concentrations. Peak heights showed a linear relationship with the HA or MHA concentrations. Urinary solutions were not different from the aqueous solutions, except for HA. As urine already contains some HA, the calibration curve is separated from the water calibration curve, resulting in two parallel lines.

Recovery

Analytical recoveries were determined by comparing the peak heights of each compound extracted from spiked urine with those obtained by direct injection of the compounds. Analytical recoveries for HA and MHA over the concentration range 0.2–1 0 mg/ml are shown in Table I The analytical recoveries were within $100 \pm 5\%$ for the twenty samples

Precision

The precision was evaluated by assaying ten aliquots of control urine samples spiked with 0.5 mg/ml of each compound. The coefficients of variation were less than 2.4% for all substances (range 1.5–2.4%)

Detection limits

The limits of detection, which were determined on the basis of a signal-to-noise ratio of 2, were between 0.01 and 0.02 mg/ml for the four acids in aqueous standards. The detection limits of the four metabolites in urine ranged from 0.03 to 0.04 mg/ml.

TABLE I

ANALYTICAL RECOVERY OF HA AND MHA ADDED TO URINE

Concentration added (mg/ml)	Recovery (%)			
	HA	*o*-MHA	*m*-MHA	*p*-MHA
0 2	105	101	98	99
0 4	97	97	98	99
0 6	96	95	95	98
0 8	102	101	103	103
1 0	99	100	100	100

Interfering substances

Possible interference was studied by chromatographing aqueous solutions of the metabolites of ethylbenzene and styrene (mandelic acid and phenylglyoxylic acid) which were also separated bHqthismethod. They did not interfere with the determination of HA and MHA. The retention times of mandelic acid and phenylglyoxylic acid were ca. 11 and 41 min, respectively.

DISCUSSION

HPLC using a β-cyclodextrin-bonded column allows the determination of HA and *o*-, *m*- and *p*-MHA in urine This method does not necessitate derivatization of the acids, as do gas chromatographic methods [4, 5] and the previously reported HPLC method [3], and requires few and inexpensive solvents and materials. Ethylbenzene is often present in industrially used xylene [1]. Xylene and styrene are sometimes used simultaneously in workplaces. The method also separates HA and MHA from mandelic acid and phenylglyoxylic acid, the metabolites of ethylbenzene and styrene. This procedure can therefore be used for the estimation of exposure to toluene and xylene in cases of workers exposed to a mixture of solvents.

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