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Note

Simultaneous determination of hippuric acid and *o*-, *m*- and *p*-methylhippuric acids in urine by high-performance liquid chromatography

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Toluene and xylene are widely used as organic solvents in industry. The solvents inhaled by workers are mainly eliminated unchanged via the lungs or excreted as metabolites in urine. Major metabolites of toluene and xylene are hippuric acid (HA) and methylhippuric acid (MHA), respectively [1]. Recent study showed that *o*-MHA as well as *m*- and *p*-MHA are major metabolites of the corresponding xylene isomers in humans [2]. Concentrations of the metabolites in urine of workers quantitatively reflect the solvent vapour concentrations in their work-place, and have been regarded as indices of exposure to these solvents [3—8].

In 1977, high-performance liquid chromatography (HPLC) was introduced for the quantitative determination of HA and *m*- or *p*-MHA in urine [9, 10]. The methods, however, can not separately determine each component in a mixture of *m*- and *p*-MHA. Although efforts for separation of *m*- and *p*-MHA were made with HPLC [11] as well as with gas chromatography [12, 13], those methods require complicated pretreatment of urine to form the derivative of each metabolite. A simple method for the separation of xylene metabolites has not been reported yet. We previously reported the determination of total *m*- plus *p*-MHA in urine by HPLC without any pretreatment [14].

The work published so far indicates that the chromatographic application of inclusion compounds such as cyclodextrins allows the solution of specific analytical problems [15]. Here we describe that the use of cyclodextrin as a component of the mobile phase in reversed-phase HPLC leads to effective separation of isomers of MHA, and that simultaneous determination of HA and *o*-, *m*- and *p*-MHA in urine serves as a useful index of solvent exposure.

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EXPERIMENTAL

Chemicals

MHA isomers (*o*-, *m*- and *p*-MHA) and β-cyclodextrin (CD) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). HA, acetonitrile, and acetic acid were purchased from Wako (Osaka, Japan). Acetonitrile was of HPLC grade and other chemicals were of analytical grade.

Automated HPLC

A Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) consisting of a pump (LC-3A), an automatic sample injector (SIL-2AS), a column oven (CTO-2AS), a variable-wavelength spectrophotometer (SPD-1), and an integrator (C-R1A) was used. The column used was Zorbax C8 (250 × 4.6 mm, particle size 5 μm, Dupont Instruments, Wilmington, DE, U.S.A.). A guard column (50 × 4.6 mm) was packed with Zorbax ODS (particle size 7—8 μm, Dupont). The mobile phase was the mixture of 200 ml of acetonitrile, 800 ml of distilled water, 15 ml of acetic acid, and 20 g of CD. The mixture without CD was also used for the separations. The flow-rate and column temperature were set at 1.2 ml/min and 40°C, respectively. Detector wavelength was set at 272.4 nm where *m*- and *p*-MHA showed identical molar absorption [14]. Although *m*- and *p*-MHA were not separated when CD was not added to the mobile phase, *m*- and *p*-MHA standards and their mixture gave the same peak area which was independent of the ratio of the isomers in the mixture. For calculations of urinary HA and MHA concentrations, we used a mixture of HA and *o*-, *m*- and *p*-MHA in equal amount as standards (Fig. 1A).

Urine samples

Samples used here were those obtained from solvent-workers at their work-place and from non-exposed control subjects. Urine (10 μl) was directly injected into the HPLC system without any pretreatment.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic separation of HA, and *o*-, *m*- and *p*-MHA. In the present HPLC system, four metabolites of solvents inhaled are completely separated from urine constituents in less than 10 min. Standard curves were linear in the wide range of concentration of each acid (0—2000 mg/l for MHA and 0—6000 mg/l for HA). The results in Table I show the recoveries of the four acid standards added to six urine samples. Recoveries are close to 100%. Within-run coefficients of variation (C.V.) are listed in Table II. The data indicate good precision of the method. Detection limits for HA and each MHA were found to be 50 mg/l urine and 10 mg/l urine, respectively. In the range of low concentrations the signal-to-noise ratio was more than 3, although the precision was decreased to some extent (C.V. = 17.6%). Since an atmospheric concentration of toluene corresponding to a urinary HA concentration of 8100 mg/l, and that of xylene corresponding to a urinary MHA (*m*- plus *p*-isomer) concentration of 700 mg/l are estimated to be about

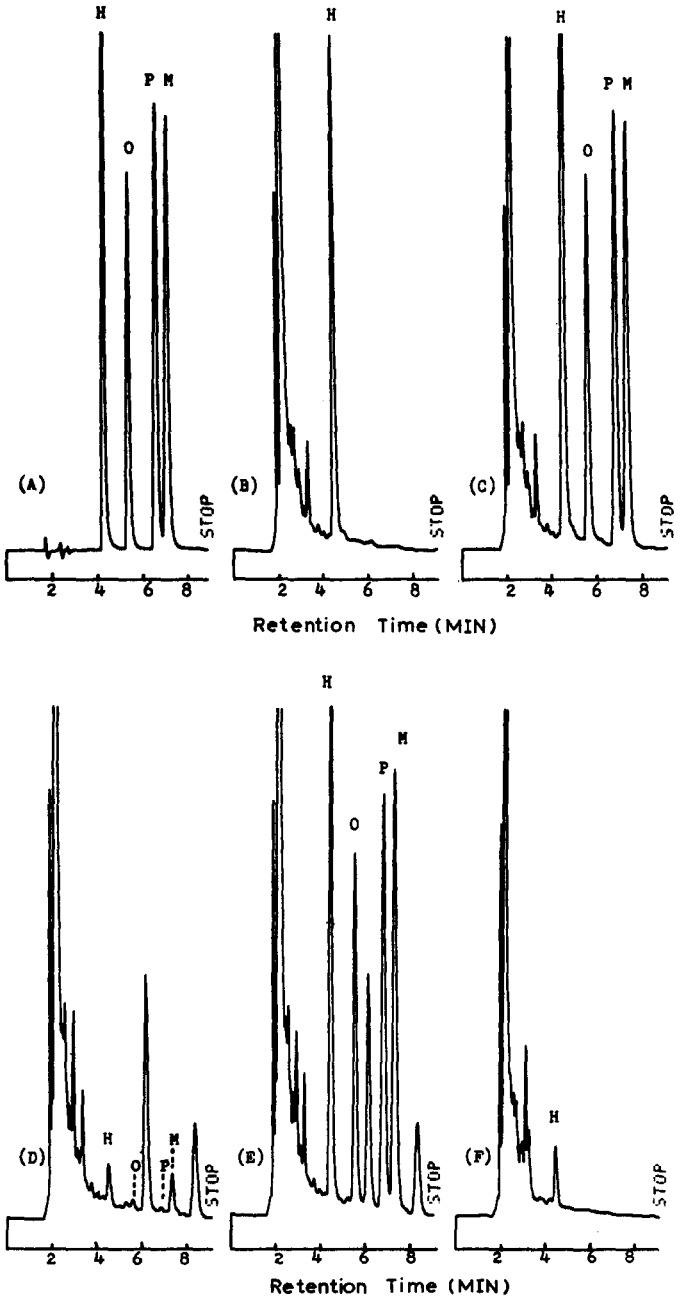

Fig. 1. Chromatographic separations of four metabolites from urine constituents. (A) Four acid standards (H = HA, O = *o*-MHA, P = *p*-MHA, and M = *m*-MHA), 1 g/l of each. (B) Urine from a person exposed to toluene. (C) Sample B plus four acid standards. (D) Urine from a person exposed to xylene. (E) Sample D plus four acid standards. (F) Urine from a control subject not exposed to the solvents.

TABLE I

RECOVERY OF FOUR ACID STANDARDS ADDED TO URINE FROM WORKERS (A—F) EXPOSED TO TOLUENE AND/OR XYLENE

Samples	HA			*o*-MHA			*p*-MHA			*m*-MHA		
	Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)		Original (mg/l)	Added (mg/l)	
		1000	2500		250	1000		250	1000		250	1000
		Recovery (%)			Recovery (%)			Recovery (%)			Recovery (%)	
A	186	95.5	100.6	62	95.5	97.0	20	99.6	97.6	210	98.5	98.6
B	1822	96.0	101.8	0	103.0	99.0	15	103.0	99.4	32	101.9	100.5
C	3005	104.1	102.8	22	99.9	99.6	21	99.3	99.5	151	100.2	97.6
D	3162	99.2	99.2	27	95.0	98.8	24	100.5	99.8	23	104.0	100.2
E	885	100.9	102.5	0	98.2	99.8	0	97.1	99.6	0	95.1	100.4
F	2025	97.2	101.9	0	97.5	98.9	0	100.5	100.0	0	100.0	100.3
Mean		98.8	101.5		98.2	98.9		100.0	99.3		100.0	99.6

TABLE II

PRECISION OF THE METHOD

$n = 10$.

	Mean ± S.D. (mg/l)	C.V. (%)
HA	57 ± 0.86	1.5
	478 ± 3.5	0.7
	3366 ± 23.7	0.7
o-MHA	11.1 ± 1.63	14.7
	92 ± 1.4	1.5
	364 ± 4.0	1.1
p-MHA	9.8 ± 1.72	17.6
	152 ± 1.8	1.3
	361 ± 2.2	0.6
m-MHA	9.5 ± 1.08	11.4
	258 ± 6.3	2.3
	600 ± 7.2	1.2

225 ppm and 15 ppm, respectively [14], the method is sufficiently accurate to detect solvent exposure.

Some samples from control and exposed subjects were analyzed by the present method (with CD in the mobile phase) and by the previously reported method (without CD) [14] (Fig. 2). HA and MHA concentrations determined by the present method agree well with those obtained by the previously reported method. The data indicate that the four acids can be determined simultaneously by the addition of CD to the mobile phase with little change in results. Using the present method we determined urinary HA concentrations in 49 healthy male subjects not exposed to the solvent. Fig. 3 shows the frequency distribution of HA concentrations in non-exposed subjects. Arithmetic and geometric means of urinary HA concentrations are 344.2 and 216.8 mg/l, respectively. The percentile (95th) is 1135 mg/l in non-exposed subjects. MHAs were not detected in the non-exposed subjects.

For the evaluation of occupational exposure to xylene, we have determined the sum of *m*- and *p*-MHA in urine because the mixture of *m*- and *p*-xylene is actually used in industry and the rate of metabolism of *m*-xylene is reported to be similar to that of *p*-xylene [7, 11]. However, the separate determination of *m*- and *p*-MHA is necessary if there is a difference in the toxicity of the isomers [1, 11].

In the method previously reported [14], we did not determine *o*-MHA concentrations because *o*-MHA had been known as a minor metabolite of *o*-xylene [1]. Recent study showed that *o*-MHA was a major metabolite of *o*-xylene in humans [2]. Xylene used in industry is composed of 15–20% *o*-xylene besides *m*- and *p*-isomers. So the determination of *o*-MHA is also necessary for the evaluation of xylene exposure. There are many factories where both xylene

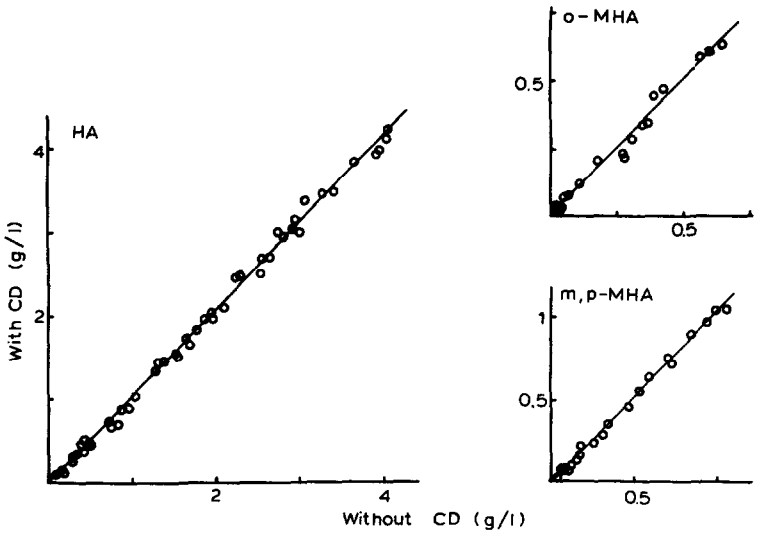

Fig. 2. Comparison of the present method (with CD) with the previously reported one (without CD). HA: $Y = 1.030X - 6.242$ ($n = 49$, $r = 0.999$). *o*-MHA: $Y = 0.996X + 4.442$ ($n = 20$, $r = 0.993$). *m,p*-MHA (*m*- plus *p*-MHA): $Y = 1.049X - 27.94$ ($n = 24$, $r = 0.998$).

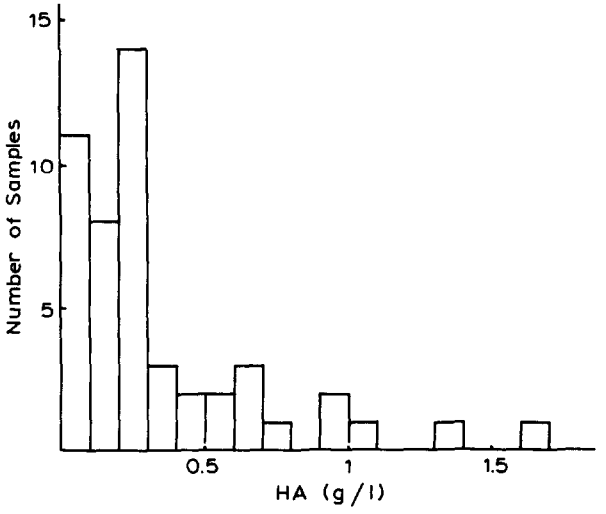

Fig. 3. Frequency distribution of HA concentrations in normal male subjects ($n = 49$).

and toluene are used in a mixture or simultaneously, and xylene often occurs as a contaminant of toluene in industry [16]. Hence the method of simultaneous determination of urinary HA and MHAs would give indicative data from which occupational exposure to either toluene or xylene or both can be monitored.

CONCLUSIONS

A simple method is described for the simultaneous determination of urinary HA, and *o*-, *m*- and *p*-MHAs concentrations by HPLC. The four metabolites were separated from urine components in less than 10 min without any

pretreatment of samples. The detection limits were found to be 50 mg/l and 10 mg/l for HA and MHAs, respectively. The method is sufficiently accurate to detect abnormal exposure to either toluene or xylene, or both.

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