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Simultaneous Determination of Hippuric Acid, *o*-, *m*-, and *p*-Methylhippuric Acid, Phenylglyoxylic Acid, and Mandelic Acid by HPLC

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Xylene is the most widely used solvent only second to toluene (Kumai et al., 1983; Inoue et al., 1983). In practical application, 3 xylene isomers are present together with toluene and ethylbenzene especially when technical grade xylenes are in use. Because toluene and 3 isomers of xylenes are known to be metabolized primarily to hippuric acid (HA) and corresponding isomers of methylhippuric acids (MHA), respectively (Williams, 1959; Lauwerys, 1984) whereas ethylbenzene is biotransformed to 2 metabolites of phenylglyoxylic (PhGA) and mandelic acids (MA), simultaneous determination of the 6 metabolites is requested when comprehensive biological monitoring of exposure to the solvent mixture is attempted by means of urinalysis (Hasegawa et al., 1983; Inoue et al., 1986).

In the present study, a simple high-performance liquid chromatographic (HPLC) procedure is described as a method for simultaneous determination of the 6 metabolites in urine; urine analysis was selected in the present study because of its non-invasive nature in sampling with minimal burden on exposed workers.

MATERIALS AND METHODS

A HPLC system [a product of Japan Spectroscopic (JASCO), Tokyo] was employed. The system consisted of a system controller (801-SC), a degasser (880-50), a gradient unit (880-02), a HPLC pump (880-PU), a liquid autosampler (850-AS), a column oven (860-CO), an UV/VIS detector (870-UV), and a graphic integrator (805-GI). Two columns [4.6 mm in diameter and 250 mm in length, packed with Inertsil ODS-2 (5 um; Gasukuro Kogyo, Tokyo, Japan)] were connected in series so that a total column length of 500 mm was achieved. Under standard conditions, the columns were heated at 42° C, and a

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mobile phase of methanol: acetic acid: water (MtOH:AA:H_2O) =200:8:792 (by volume) was allowed to flow at a rate of 0.85 ml/min. The effluent was monitored at a wavelength of 257 nm, with attenuation set at 64 mV F.S. and with a chart speed of 1 mm/min.

To establish reference values for the 6 urinary metabolite levels, urine samples were collected from 148 non-exposed subjects [101 men aged at 20.1 ± 0.4 years (arithmetic mean ± arithmetic standard deviation) and 47 women at 20.0 ± 0.2 years]. A urine sample from a male worker exposed to industrial xylenes at 41 ppm (11 ppm o-xylene, 21 ppm m-xylene and 9 ppm p-xylene) together with ethylbenzene at 13 ppm) was also employed. Each urine sample was mixed with an equal volume of methanol, and the mixture was spun at 1600 x g for 10 min to eliminate salts. The supernate, 10 ul/injection, was introduced to the HPLC system for analysis. For statistical evaluation, the results were expressed in terms of geometric mean and geometric standard deviation with an assumption of log-normal distribution (Heath, 1967).

RESULTS AND DISCUSSION

Experiences showed that the addition of ß-cyclodextrin in the mobile phase as recommended by Sakai et al. (1989) tended to cause early deterioration of separation capacity of the HPLC column after 100 or more analyses. Accordingly, efforts were made to establish HPLC conditions fit for separation in absence of this additive. It was also found that tailing of peaks was evident when acetic acid in the mobile phase is reduced, i.e., to less than 5 ml/L mobile phase. A titration study with various portion of methanol with a fixed amount (8 ml/L) of acetic acid showed that the separation of p-MHA from its m-isomer is subject to variation as a function of methanol portion. For example, the use of a mobile phase with an increased methanol portion of MtOH:AA:H_2O = 300:8:692 failed to achieve clear separation between m- and p-MHA (Fig. 1A). The experiments with MtOH in a range of 100 to 300 mL/1000 mL mobile phase showed that separation of m- and p-MHAs became less clear when MtOH amount was less or more than 200 mL, and it was indicated that the best separation could be achieved when a MtOH:AA:H_2O ratio of 200;8;792 is employed. Another point of study was column temperature. The increase of column temperature from 40° C to 60° C gradually made the separation of HA from MA and p-MHA from m-MHA rather difficult, although the over-all time necessary for one analysis was shortened.; a case of analysis at a column temperature of 55° C is shown in Fig. 1B.

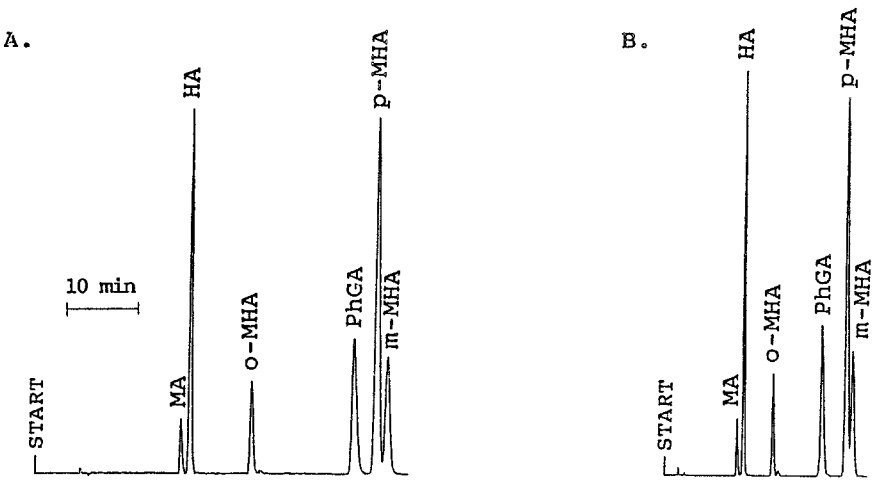

Figure 1. Chromatograms to show difficulties in separation: HA, hippuric acid (600 mg/L); o-, m- and p-MHA, o-, m- and p-methylhippuric acid (550, 300 and 300 mg/L, respectively); PhGA, phenylglyoxylic acid (50 mg/L); MA, mandelic acid (670 mg/L).

A. With a mobile phase of MtOH:AA:H_2O = 300:8:692 (by volume).

B. At a column temperature of 55° C.

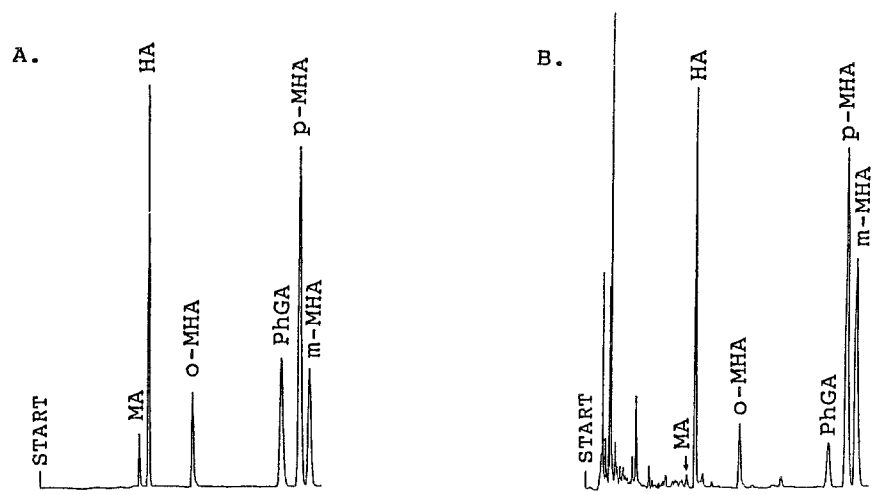

Figure 2. Chromatograms under standard conditions: HA, hippuric acid; o-, m- and p-MHA, o-, m- and p-methylhippuric acid; PhGA, phenylglyoxylic acid; MA, mandelic acid.

A. With the mixture used in Fig. 1A.

B. With a urine sample from a worker exposed to xylenes at 40 ppm and ethylbenzene at 13 ppm.

Table 1. Reproducibility of analysis

Metabolite	Sample 1		Sample 2	
	Conc.[a]	CV[b]	Conc.	CV
HA	300	5.3%	600	1.7%
o-MHA	250	3.3%	500	2.6%
m-MHA	300	2.9%	600	1.8%
p-MHA	150	1.3%	300	1.6%
PhGA	25	2.6%	50	1.7%
MA	300	3.2%	600	1.9%

HA, hippuric acid; o-, m- and p-MHA, o-, m- and p-methylhippuric acid; PhGA, phenylglyoxylic acid; MA, mandelic acid.
a/ Concentration in mg/L.
b/ Coefficient of variation (10 determinations each).

Table 2. Recovery test

Metabolite	Concentration[a]			Recovery
	A	B	C	
HA	0	285	570	96% (4.3%)
o-MHA	0	280	560	101% (2.4%)
m-MHA	0	150	300	103% (1.6%)
p-MHA	0	145	290	105% (1.1%)
PhGA	0	25.5	51	100% (2.8%)
MA	0	335	670	98% (3.3%)

Ten urine samples each were spiked with authentic chemicals at the concentrations described. A regression was calculated from each set of samples at the 3 concentrations. Recovery is calculated as the rate (in %) of the slopes obtained with spiked urine samples over the slope with spiked water samples. The mean rate with the 10 urine samples is shown together with the coefficient of variation in parenthesis.
HA, hippuric acid; o-, m- and p-MHA, o-, m- and p-methylhippuric acid; PhGA, phenylglyoxylic acid; MA, mandelic acid.
a/ Concentration in mg/L.

Accordingly, a standard HPLC condition was established as described in MATERIALS AND METHODS. Typical chromatograms at a column temperature of 42° C are depicted in Fig. 2 [one (Fig. 2A) with a mixture of the 6 authentic compounds, and the other (Fig. 2B) with a urine sample from a worker exposed to 41 ppm industrial xylene mixture and 13 ppm ethylbenzene] to show a clear

Table 3. HA, o-, m- and p-MHA, PhGA and MA levels in the urine of non-exposed Japanese subjects

Metabolite	Sex[a]	Observed[b]	Corrected for Sp. gr.[c]	Corrected for Creatinine[d]
HA	M	192 (3.0) 3936	127 (2.8) 1908	99 (2.8) 1043
	W	228 (3.1) 2901	181 (2.7) 1719	202 (2.6) 1971
o-MHA	M	23.9(3.0) 402	15.7(2.8) 230	12.2(2.7) 179
	W	23.2(2.8) 236	18.4(2.7) 171	20.6(2.6) 149
m-MHA	M	0.16(3.7) 14.4	0.11(3.7) 11.0	0.08(3.8) 12.7
	W	0.23(5.2) 16.6	0.18(5.4) 17.7	0.21(5.9) 19.0
p-MHA	M	0.05(6.1) 24.2	0.04(6.2) 13.8	0.03(6.3) 8.5
	W	0.05(5.5) 3.5	0.04(5.9) 4.7	0.05(6.2) 3.5
PhGA	M	0.05(4.4) 2.1	0.03(4.5) 1.8	0.03(4.6) 1.7
	W	0.06(4.8) 1.4	0.05(4.6) 0.9	0.06(4.8) 2.1
MA	M	115 (3.7) 1937	76 (3.5) 1192	59 (3.5) 911
	W	96 (3.4) 1200	76 (3.3) 711	85 (3.7) 862

The values in the table are geometric mean (geometric standard deviation) and the maximum value.
HA, hippuric acid; o-, m- and p-MHA, o-, m- and p-methylhippuric acid; PhGA, phenylglyoxylic acid; MA, mandelic acid.
a/ Non-exposed subjects, 148 in total [101 men (M) and 47 women (W)] were examined.
b/ Uncorrected values (Unit: mg/L).
c/ Corrected for a specific gravity of 1.016 (Unit: mg/L).
d/ Observed values divided by creatinine concentration (Unit: mg/g creatinine).

separation of HA from MA and m-MHA from p-isomer. When a peak/noise ratio of 2 was employed, the detection limit under the conditions studied was 0.5 mg/L for HA, 2.0, 0.8 and 0.2 mg/L for o-, m- and p-MHA, respectively, 0.1 mg/L for PhGA, and 3 mg/L for MA.

Both peak height and peak space were proportional to the added amounts of each of the 6 compunds in water up to the maximum amount studied (i.e., 2500 mg/L). When mixture preparations of the 6 compounds at 2 different concentrations were analyzed 10 times each (Table 1), the coefficient of variation for the 6 compunds were all 5% or less (Table 1). The recovery was also between 96 and 105% with small coefficients of variation of <5%, when the authentic compounds were added at 3 different concentrations (including zero) to 10 urine samples and slopes were compared with that after addition to water in the place of urine samples (Table 2).

The method under the standard conditions was applied to examine the normal levels of the 6 metabolites in the urine of the non-exposed, as an example to show the applicability of the method for urinalysis. The results are summarized in Table 3, in which a value of 1/10 the detection limit was assumed when the observed peak corresponded less than the detection limit. The values thus observed are essentially the same with the values previously reported for Japanese (Ohtsuji and Ikeda, 1970; Hasegawa et al., 1983; Inoue et al., 1986).

The two major advantages of the proposed method are a long life of the column to permit more than 1000 analyses (in contrast to less than 100 analyses in the presence of ß-cyclodextrin), and the absence of tailing of peaks in the chromatogram (Fig. 2A and B). The relatively long time necessary for one analysis (i.e., some 80 min; Fig. 2B) may be disadvantageous when analyses of multiple samples in a short time is requested, as often encountered in occupational health practice. This difficulty could be dissolved by the elevation of column temperature up to 55° C, because the quantification of both HA and m-MHA will not be disturbed significantly by co-present MA or p-MHA in practice. It is known through the analysis of commercial xylenes (Lauwerys, 1984) that m-xylene (a precursor of m-MHA) is a major component and both p-xylene (p-MHA precursor) and ethylbenzene (a MA precursor) are minor components of xylene preparations used in industries. At the wavelength employed under the present analytical conditions, p-MHA would give a larger peak than m-MHA when compared on an eqimolar basis. Thus, the peaks for p-MHA and MA in a chromatogram would be rather comparable to or smaller than those for m-MHA and probably HA after occupational exposure.

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