Urinary t,t-muconic acid as an indicator of exposure to benzene

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ABSTRACT A method for rapidly determining t,t-muconic acid (MA) by high performance liquid chromatography was developed and successfully applied to urine samples from 152 workers exposed to benzene (64 men, 88 women) and 213 non-exposed controls (113 men, 100 women). The MA concentrations in urine correlated linearly with time weighted average benzene concentrations in the breath zone air of workers. A cross sectional balance study showed that about 2% of benzene inhaled is excreted into the urine as MA. The MA concentrations in the urine of the non-exposed was below the detection limit (< 0.1 mg/l) in most cases, and the 95% lower confidence limit of MA for those exposed to benzene at 5 ppm (5.0 mg/l as a non-corrected value) was higher than the 97.5%-tile values for the non-exposed (1.4 mg/l). In practice, it was possible to separate those exposed to 6-7 ppm benzene from the non-exposed by means of urine analysis for MA. The urinary MA concentration was suppressed by coexposure to toluene.

Benzene, an established human leukaemogen,¹ is an important material in the chemical industry, and it is also known to be present in petrol² and in petroleum distillate solvents of low boiling point.³ Accordingly, efforts have been made to identify exposure markers, preferably in urine, which are sensitive enough to detect benzene exposure at or below 10 ppm.^{4,5}

The present report is an extension of three previous studies on the biological monitoring of exposure to benzene by means of urine analysis for metabolites such as phenol,⁵ catechol, and quinol,⁶ and on the effects of combined exposure to toluene.⁷ A time and work saving method of urine analysis for t,t-muconic acid (MA) has been developed and successfully used for the biological monitoring of workers exposed to 6-7 ppm benzene.

Materials and methods

WORKERS STUDIED AND URINE COLLECTION

Those who participated in this study were 152 workers exposed to benzene (64 men exposed up to 92 ppm, 88 women up to 210 ppm), 55 male workers exposed to a mixture of benzene (up to 116 ppm) and toluene (up to 114 ppm), and 213 non-exposed workers (113 men, 100 women) in factories in China. The jobs of the

exposed workers have been described previously⁸; the benzene workers were mostly shoemakers whereas the workers with mixed exposure were predominantly painters. The urine samples were collected at 1500 in the second half of a working week, when the concentrations of benzene metabolites in urine are expected to reach a maximum.⁹

URINE ANALYSIS FOR t,t-MA

The high performance liquid chromatographic (HPLC) method of Karim *et al*¹⁰ for t,t-MA was simplified for urine analysis as follows: an aliquot of urine sample, kept frozen and thawed immediately before analysis, was well mixed with an equal volume of methanol. The mixture was spun at 3000 rpm for 10 minutes to remove precipitates as in urinary hippuric acid determination.¹¹ The supernatant layer (5 to 10 μ l per injection) was introduced into a HPLC (Hitachi Model 635) equipped with a Spherisorb ODS 5 μ m column (4 mm in inner diameter and 250 mm in length). The mobile phase (one volume of methanol mixed with nine volumes of 1% acetic acid) was allowed to flow at a rate of 1.0 ml/min, and the eluates were monitored at a wavelength of 265 nm.¹⁰ The MA peak appeared at 10 to 11 minutes after the injection with no interference by other urine components (fig 1). The results were presented as observed (in terms of mg/l), or corrected for creatinine concentration (mg/g

Fig 1 HPLC determination of *t,t*-MA in urine. HPLC chromatogram of urine from a benzene worker and of authentic *t,t*-MA dissolved in water.

creatinine)¹² or for specific gravity of urine of 1.016 (mg/l).¹³ The specific gravity was measured refractometrically and the calculation for correction was made according to Rainsford and Lloyd Davies.¹³

As no extraction with solvent was made during the pretreatment of the urine sample, possible interference with conjugated phenols in the determination was considered. The retention time of MA differed from that of phenyl sulphate or phenyl glucuronide (prepared after Dodgson and Spencer¹⁴ and Spencer

and Williams,¹⁵ respectively), and the pretreatment of the urine with Glusulase (Du Pont Pharmaceuticals, Wilmington, Delaware, USA) did not produce any change in the peak, suggesting that there is no interference with conjugated metabolites. The lowest detection limit was 0.1 mg/l. When authentic MA was dissolved in 10 MA-free urine samples at concentrations of 100 and 200 mg/l and the slopes of the calculation lines obtained were compared with that of water, the former (CV = 2.8%) was identical with the latter. When the urine samples with 1.3, 77.6, and 160.5 mg endogenous MA/l were analysed 10 times each, the CV was 5.0, 1.1, and 1.7% respectively, suggesting high reproducibility of the results.

SOLVENT CONCENTRATIONS IN THE BREATHING ZONE

The time weighted average (TWA) concentration of benzene or benzene and toluene in the breathing zone during a workday shift was measured by means of a diffusive sampling method.^{16,17} The values are cited from a previous report.⁸

STATISTICAL ANALYSIS

Regression analysis was conducted using a program supplied by Nippon Electric Co (Tokyo, Japan) for the ACOS 1000 in the Computer Center, Tohoku University.

Fig 2 Distribution histograms of *t,t*-MA concentration in urine of non-exposed subjects. Observed values in 113 men (top) and in 100 women (bottom) are shown. Concentrations in urine of those exposed to benzene at 3–7 ppm are spotted to show that urinary concentrations after exposure at 6 ppm and above are higher than non-exposed concentrations.

Table 1 Concentration of *t,t*-MA in the urine of workers exposed to benzene at various workshops

Workshop	No of workers	Benzene in breath zone air (ppm)	Observed value (mg/l)	Value corrected for	
				Creatinine (mg/g)	Spec gravity (mg/l)
Men:					
A	3	1.0 (1.00)	1.84 (1.880)	2.07 (1.944)	2.32 (2.010)
B	24	11.1 (2.40)	8.77 (2.219)	11.37 (2.344)	9.30 (2.592)
C	2*	7, 76	6.8, 17.4	6.8, 32.8	6.2, 27.8
D	20	32.6 (1.42)	34.45 (1.766)	47.50 (1.463)	38.62 (1.506)
E	15	60.2 (1.28)	79.86 (1.572)	90.10 (1.276)	71.26 (1.251)
Women:					
A	1*	1	0.7	1.4	1.0
B	19	18.1 (2.79)	19.41 (2.963)	26.85 (2.361)	20.11 (2.149)
C	2*	5, 37	0.5, 18.3	0.7, 30.3	0.5, 20.9
D	37	42.4 (2.49)	36.63 (2.227)	45.78 (2.053)	34.68 (2.059)
E	29	76.4 (1.58)	70.91 (1.934)	94.94 (1.674)	71.04 (1.556)

All results are the geometric mean (geometric standard deviation).

*Individual values are shown.

Results

t,t-MA CONCENTRATION IN URINE OF NON-EXPOSED SUBJECTS

The concentration of *t,t*-MA in the urine of 213 non-

exposed subjects (113 men, 100 women) was below the lowest detection limit (< 0.1 mg/l) in most cases (63 of 113 men (56%), 74 of 100 women, or 137 of 213 subjects (64%) when men and women were combined), indicating skewed distributions (fig 2) with

Fig 3 Relation between benzene in breath zone air and *t,t*-MA in urine. Points indicate individual values. Lines and curves are calculated regression line (line in centre), 95% confidence ranges of sample means (curves close to regression line), and 95% confidence ranges of individual samples (outmost lines).

Table 2 Correlation between benzene in breath zone and *t,t*-MA in urine

Measurement group	No of subjects*	A†	B†	r‡
Observed value (mg/l):				
Men + women	365	0.829	3.247	0.816
Men§	177	1.133	0.796	0.831
(Men)	(166)	(0.273)	(0.817)	(0.684)
Women¶	188	0.769	3.873	0.820
Women**	172	1.024	0.842	0.818
Value corrected for creatinine (mg/g):				
Men + women	365	0.989	4.429	0.827
Men§	177	1.223	2.123	0.860
(Men)	(166)	(0.278)	(0.868)	(0.739)
Women¶	188	0.939	5.396	0.814
Women**	172	1.302	0.577	0.858
Value corrected for specific gravity (mg/l):				
Men + women	365	0.699	4.064	0.842
Men§	177	0.987	1.940	0.855
(Men)	(166)	(0.226)	(0.774)	(0.724)
Women¶	188	0.644	4.432	0.854
Women**	172	0.943	0.811	0.901

*Including non-exposed subjects.

†Slope (A) and the intercept on the Y axis (B) in the equation as $Y = AX + B$, where Y is the urinary concentration of catechol or quinol (unit; as described in the table) and X is the breath zone concentration of benzene (ppm).

‡p for correlation coefficient (r) is <0.01 for all groups.

§Exposed up to 92 ppm.

||Exposed also to toluene (up to 114 ppm) in addition to benzene.

¶Exposed up to 210 ppm.

**Women exposed to less than 100 ppm.

both medians and modes at 0 mg/l in the two sexes. The largest value observed was 2.0 mg/l in men and 1.6 mg/l in women.

INCREASE IN *t,t*-MA CONCENTRATION AS A FUNCTION OF INTENSITY OF EXPOSURE TO BENZENE

MA concentrations in urine samples collected from male and female workers exposed to benzene at various concentrations in five workshops were compared with benzene concentration in the breathing zone. Table 1 shows that MA concentration increased with exposure to benzene. Individual MA and benzene values subjected to regression analyses disclosed a linear correlation (fig 3) with correlation coefficients

Table 3 Comparison of the 95% lower confidence limit of *t,t*-MA concentration in the urine of workers exposed to 5 ppm benzene with the 97.5%-tile value of *t,t*-MA concentration in the urine of non-exposed subjects. (Numbers in parentheses)

Sex	Observed value (mg/l)	Value corrected for	
		Creatinine (mg/g)	Spec gravity (mg/l)
Men + women	5.00 (1.61)	6.67 (1.96)	5.75 (1.56)
Men	3.91 (1.96)	5.83 (2.09)	4.83 (1.73)
Women	3.89 (1.24)	5.33 (1.23)	4.78 (0.89)

larger than 0.80 ($p < 0.01$) (table 2). The correction of the metabolite level for either creatinine concentration or specific gravity of urine did not increase the correlation coefficients (table 2). The slope for men appeared to be steeper than for women regardless of correction or non-correction for urine density (table 2). This difference, however, disappeared when women exposed to more than 100 ppm benzene were excluded from the comparison (table 2).

SEPARATION OF THE BENZENE EXPOSED FROM THE NON-EXPOSED BY URINE ANALYSIS FOR *t,t*-MA

As the 95% confidence range for group means was narrow in all cases studied (fig 3), we investigated what would be the lowest benzene concentration at which the MA in the urine of the exposed would be higher than that of the non-exposed. Comparison was made (table 3) between the 97.5%-tile values of MA in the urine of the non-exposed and the 95% lower confidence range of MA in the urine of those exposed to benzene at 5 ppm, half the level of the currently recommended occupational exposure limit in Japan.¹⁸ It was evident that the former values were all smaller (36% at maximum) than the latter in both sexes and with or without correction for urine density. When observed MA values for those exposed to 3–7 ppm benzene (13 men, 6 women) were superimposed in the frequency histograms for the non-exposed, the MA concentration in one woman with 5 ppm exposure (out of six with 3–7 ppm exposure) fell in the non-exposed range (bottom in fig 2), whereas no such case was observed in men (top in fig 2). The findings, one from statistics and the other from observation, suggest that MA urine analysis is useful at a group level to identify those exposed to over 6–7 ppm benzene (as a TWA benzene concentration over a day shift). No difficulty is expected for group monitoring at 10 ppm level. As the lower 95% confidence limit of individual values passed the horizontal axis at 25–50 ppm in all cases examined (fig 3), however, MA analysis is unsuitable for monitoring individual exposure.

DECREASED URINARY *t,t*-MA CONCENTRATION DUE TO COMBINED EXPOSURE TO TOLUENE

The relation between the intensity of exposure to benzene and the urinary MA concentration was examined in male workers exposed to a mixture of benzene and toluene and the results were compared with the findings in the male workers exposed to benzene only. Combined exposure data for women were too few to allow statistical analysis. The comparison showed that the urinary MA concentration was much less in the workers with combined exposure than in the workers exposed to benzene only. The slope of the regression line for the mixture group (0.2–0.3 mg/l

Fig 4 Suppressed urinary excretion of *t,t*-MA after coexposure to toluene. Lines and curves are calculated regression line (line in centre), 95% confidence ranges of sample means (curves close to regression line), and 95% confidence ranges of the individual samples (outmost lines). 95% Confidence ranges of sample means are shown by shades.

urine or g creatinine/ppm; table 2) was about a quarter that of the benzene group (1.0–1.2). The slopes of the regression lines also differed (fig 4) and the correlation coefficients for the mixture group were somewhat smaller than for the benzene group.

Multiple regression analysis was conducted taking MA concentration as a dependent variable and the exposure concentrations of benzene and toluene as independent variables. The multiple correlation coefficients (MCC) and the partial correlation coefficients (PCC) of benzene and toluene thus obtained are summarised in table 4. The fact that the MCC were statistically significant in all three cases (either with or without correction for the density of urine) indicates that the analysis was valid. In confirmation of the results from simple regression analysis described above, the urinary MA concentrations were significantly ($p < 0.01$) and positively (with a

Table 4 Correlation coefficients obtained in regression analyses between *t,t*-MA in urine as a dependent variable and benzene and toluene as two independent variables

<i>t,t</i> -Muconic acid	MCC	PCC	
		Benzene	Toluene
As observed	0.730**	0.726**	-0.219**
As corrected for:			
Creatinine concentration	0.753**	0.748**	-0.258**
Specific gravity (1.016)	0.749**	0.744**	-0.259**

** $p < 0.01$.

MCC: multiple correlation coefficient.

PCC: partial correlation coefficient.

coefficient larger than 0) related to the intensity of the exposure to benzene. Of particular interest is the observation that the MA concentrations relate negatively (with a coefficient smaller than 0) to coexposure to toluene; the correlations were significant ($p < 0.01$) but the coefficients were between -0.2 and -0.3. Thus it is plausible to conclude that the urinary MA concentration is reduced by coexposure to toluene.

Discussion

With assumptions that the absorption rate of benzene through the lungs at the steady state is about 50% and that the respiration rate is 15 l/min, then the amount of benzene absorbed by a worker exposed to benzene at 100 ppm will be 2392.5 $\mu\text{g}/\text{min}$.⁵⁶ When the rate of excretion of urine is assumed to be 1 ml/min it is possible to show from the present findings (table 2) that the amount of benzene excreted into urine as MA may be calculated as

$0.829 \times 100 \text{ (mg/l)} \times 1 \times 10^{-3} \text{ (l/min)} = 82.9 \text{ } \mu\text{g}/\text{min}$ or 45.6 $\mu\text{g}/\text{min}$ as benzene ($= 82.9 \text{ } \mu\text{g}/\text{min} \times 78.11/142.11$) where 78.11 and 142.11 are the molecular weight of benzene and MA, respectively. Based on the cross sectional calculation, therefore, it is possible to estimate that about 1.9% of benzene absorbed will be excreted into the urine as MA. A similar calculation for phenol, catechol, and quinol shows that the amount of absorbed benzene excreted in the form of the three phenolic metabolites is 13.2%, 1.6%, and 10.1%, respectively.⁵⁶ MA is a minor metabolite of benzene in man as it is in rabbits.¹⁹ Nevertheless, MA is the only exposure indicator with which those exposed to 6–7 ppm benzene can be separated on a group basis from those not exposed (fig 3; table 3); this cannot be achieved with the other three metabolites so far studied.⁵⁶ This high sensitivity is primarily due to very low urinary background levels of MA among the non-exposed, which was not the case with phenol,⁵ catechol, or quinol.⁶

While the non-invasive nature of urine analysis for

MA is an apparent advantage over blood analysis for benzene, one major drawback is that the urinary MA concentration is lowered by coexposure to toluene. As toluene is a widely used solvent^{20,21} and benzene in solvent preparations is often combined with that of toluene,^{2,3} this possibility should be taken into account when exposure to benzene is biologically monitored by means of urine analysis for MA.

Regarding the metabolism of benzene, the observation of particular interest is that the coexposure to toluene results in extensive reduction of urinary excretion of MA (table 3) but only a small reduction in catechol.⁶ It is generally believed that benzene is oxidized in vivo to benzene epoxide and then benzene dihydrodiol, and that these two intermediates are common for the formation of both catechol and MA.²² Karim *et al*¹⁰ confirmed that catechol is not a precursor of urinary MA in mice.¹⁰ If this is true toluene coadministered should not interfere with the oxidative formation of benzene epoxide but selectively suppress further oxidation to MA. This hypothesis implies that the oxidation to open the ring of benzene dihydrodiol would be suppressed by coexisting toluene. The possibility is yet to be proved.

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References

- 1 International Agency for Research on Cancer. *Monographs on the evaluation of carcinogenic risk of chemicals to humans*. Vol 29. *Benzene*. Lyon: IARC, 1982:99–148.
- 2 Ikeda M, Kumai M, Watanabe T, Fujita H. Aromatic and other contents in automobile gasoline in Japan. *Ind Health* 1984;22:235–41.
- 3 Kasahara M, Suzuki H, Takeuchi Y, Hara I, Ikeda M. n-Hexane, benzene and other aromatic components in petroleum distillate solvents in Japan. *Ind Health* 1987;25:205–14.
- 4 Lauwerys R. *Industrial biological monitoring of industrial chemicals*. I. *Benzene*. Luxembourg: Health and Safety Directorate, Commission of European Communities, 1979.
- 5 Inoue O, Seiji K, Kasahara M, *et al*. Quantitative relation of urinary phenol levels to breath zone benzene concentrations: a factory survey. *Br J Ind Med* 1986;43:692–7.
- 6 Inoue O, Seiji K, Kasahara M, *et al*. Determination of catechol and quinol in the urine of workers exposed to benzene. *Br J Ind Med* 1988;45:487–92.
- 7 Inoue O, Seiji K, Watanabe K, *et al*. Mutual metabolic suppression between benzene and toluene in man. *Int Arch Occup Environ Health* 1988;60:15–20.
- 8 Yin S-N, Li G-L, Hu Y-T, *et al*. Symptoms and signs of workers exposed to benzene, toluene or the combination. *Ind Health* 1987;25:113–30.
- 9 Ikeda M, Hara I. Evaluation of the exposure to organic solvents by means of urinalyses for metabolites. *Jpn J Ind Health* 1980;22:3–17. (In Japanese with an English abstract.)
- 10 Karim MMG-E, Ramanujam VMS, Legator MS. *trans,trans*-Muconic acid, an open-chain urinary metabolite of benzene in mice. Quantification by high-pressure liquid chromatography. *Xenobiotica* 1985;15:211–20.
- 11 Hasegawa K, Shiojima S, Koizumi A, Ikeda M. Hippuric acid and o-cresol in the urine of workers exposed to toluene. *Int Arch Occup Environ Health* 1983;52:197–208.
- 12 Jackson S. Creatinine in urine as an index of urinary excretion rate. *Health Phys* 1966;12:843–50.
- 13 Rainsford SG, Lloyd Davies TA. Urinary excretion of phenol by men exposed to vapour of benzene: a screening test. *Br J Ind Med* 1965;22:21–6.
- 14 Dodgson KS, Spencer B. Studies on sulphatases. 1. The choice of substrate for the assay of rat-liver arylsulphatase. *Biochem J* 1953;53:444–51.
- 15 Spencer B, Williams RT. Studies in detoxication. 35. Spectrophotometric determination of *beta*-glucuronidase using *p*-chlorophenylglucuronide as substrate. *Biochem J* 1951;48:537–46.
- 16 Hirayama T, Ikeda M. Applicability of activated carbon felt in the dosimetry of solvent vapor mixture. *Am Ind Hyg Assoc J* 1979;40:1091–6.
- 17 Ikeda M, Kumai M, Aksoy M. Application of carbon felt dosimetry in field studies distant from analytical laboratory. *Ind Health* 1984;22:53–8.
- 18 Japan Association of Industrial Health. Recommended occupational exposure limits for 1987. *Jpn J Ind Health* 1987;29:397–415.
- 19 Williams RT. *Detoxication mechanisms*. London: Chapman and Hall, 1959:188–94.
- 20 Inoue T, Takeuchi Y, Hisanaga N, *et al*. A nationwide survey on organic solvent components in various solvent products. Part 1. Homogeneous products such as thinners, degreasers and reagents. *Ind Health* 1983;21:175–83.
- 21 Kumai M, Koizumi A, Saito K, *et al*. A nationwide survey on organic solvent components in various solvent products. Part 2. Heterogeneous products such as paints, inks and adhesives. *Ind Health* 1983;21:185–97.
- 22 Snyder CA. Benzene. Hydrocarbons. In: Snyder R, ed. *Ethyl bromine's toxicity and metabolism of industrial solvents*. Vol 1. 2nd ed. Amsterdam: Elsevier, 1987:3–37.