A rapid colorimetric method for the determination of phenylglyoxylic and mandelic acids

Its application to the urinalysis of workers exposed to styrene vapour

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Ohtsuji, H., and Ikeda, M. (1970). *Brit. J. industr. Med.*, 27, 150-154. A rapid colorimetric method for the determination of phenylglyoxylic and mandelic acids. Its application to the urinalysis of workers exposed to styrene vapour. A rapid colorimetric method has been developed for the determination of the styrene metabolites, phenylglyoxylic and mandelic acids, in urine. Ether extracts of acidified urine containing the two acids were evaporated to dryness in a test tube, and a mixture of sulphuric acid and formalin (100:1, v/v) was added for colour development. Factors necessary to convert optical extinction to gravimetric units were determined. When urine samples from workers exposed to up to 30 ppm of styrene were analysed for phenylglyoxylic and mandelic acids together with hippuric acid, it was found that the phenylglyoxylic acid level provided the most sensitive index of styrene exposure and that the optical extinction at 350 nm was practically proportional to phenylglyoxylic acid concentration. No significant increase in hippuric acid levels was observed.

Recent progress in the synthetic resin industry has resulted in a marked increase in the production and use of styrene monomer (vinylbenzene) (Browning, 1965). El Masri, Smith, and Williams (1958) found hippuric acid, styrene glycol and mandelic acid as urinary metabolites of styrene in rabbits. Bardoděj and Bardodějová (1966) measured polarographically both phenylglyoxylic acid and mandelic acid in the urine of volunteers exposed to styrene. Mandelic acid was also found in the urine of workers exposed to styrene (Bardoděj and Bardodějová, 1966; Hůzl, Sýkora, Mainerová, Janková, Šrutek, Junger, and Lahn, 1967). A colorimetric method convenient for the urinalysis of factory workers was therefore investigated. When a sulphuric acid-formalin mixture (El Masri *et al.*, 1958) was added to phenylglyoxylic acid and mandelic acid in the absence of water, a

colour was produced which was suitable for their quantitative determination.

Materials and methods

Apparatus, reagents and materials

A Shimadzu multipurpose recording spectrophotometer (MRS-50L) and a Shimadzu spectrophotometer (QR-50) with 10-mm cuvettes were used.

Phenylglyoxylic acid was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A. Silica gel GF 254 was used for thin-layer chromatography (Stahl; E. Merck A.G., Darmstadt, West Germany). The sulphuric acid-formalin reagent was a mixture of concentrated sulphuric acid and 40% (w/v) formalin (100:1, v/v) (El Masri *et al.*, 1958). Peroxide-free ether was obtained by distillation in the presence of ferrous sulphate.

Urine samples were collected at about 3 pm from

factory workers exposed to styrene at the concentrations shown in Table 2.

Determination of phenylglyoxylic acid and mandelic acid
A urine sample, 0.5 ml in a 25-ml glass-stoppered bottle, was acidified with 0.05 ml of 1N HCl and shaken vigorously with 5.0 ml of ether for 10 minutes. Some of the ether (1 to 4 ml) in a test tube was evaporated to dryness in a water bath at 70°C. To the tube was added 4 ml of the sulphuric acid-formalin reagent and after 15-60 minutes the extinctions at 350 nm and 450 nm, E 350 and E 450, were measured. For the calculation of the amounts of phenylglyoxylic acid and mandelic acid from E 350 and E 450 see the 'Results' section.

Determination of hippuric acid

This was determined by the method of Ikeda and Ohtsuji (1969).

Determination of styrene

Atmospheric styrene concentrations were determined with Kitagawa detection tubes (Kitagawa, 1960).

Results

Identification of phenylglyoxylic acid and mandelic acid

The ether extracts of the urine of styrene-exposed workers were chromatographed alongside authentic phenylglyoxylic acid on a thin-layer plate, which was developed with *n*-propanol: 28% aq. NH₃ (7/3, v/v). Another thin-layer plate, spotted with the ether extract from the urine and authentic mandelic acid, was developed with the lower layer of the system of *n*-butanol: acetic acid: water in the ratio 4:1:5 by volume. After the plates had been dried, the authentic phenylglyoxylic acid and mandelic acid markers were located as a yellow spot and a dark-brown spot respectively when the plates were treated with the sulphuric acid-formalin reagent. The parts of the plates containing phenylglyoxylic acid or mandelic acid in urine were not treated with

formalin reagent but were scraped into separate bottles, and the acids were eluted with 10% aqueous Na₂CO₃.10H₂O (w/vol). The silica gel was removed by centrifugation, the sodium carbonate solution was acidified with HCl and the organic acid was extracted into ether. The ether layer was concentrated by evaporation and re-chromatographed for identification as shown in Table 1. A portion of each of the two organic acids was treated as described in 'Materials and methods' for spectrophotometry. The absorption spectrum of each of the phenylglyoxylic acid and the mandelic acid isolated from the urine was identical with that of corresponding authentic acid (Fig. 1).

Calculation of acid concentrations from E 350 and E 450
The extinction from phenylglyoxylic acid was greatest at 350 nm and from mandelic acid at 450 nm, and the extinctions were proportional to the amounts of each acid (Figs 1 and 2) and were additive when the mixtures of two acids were investigated (Fig. 2). For mixtures E 350 increased linearly with the phenylglyoxylic acid present. The slopes of the regression lines were constant and independent of the amounts of mandelic acid; and the intercepts at the vertical axis were proportional to the amounts of mandelic acid. The same was true when values of E 450 were plotted against the amounts of mandelic acid, except that the intercepts at the vertical axis due to phenylglyoxylic acid were very small (Fig. 2). The results imply that:

$$E\ 350 = a_1 \times (\mu\text{g phenylglyoxylic acid}) + a_2 \times (\mu\text{g mandelic acid});$$

$$E\ 450 = a_3 \times (\mu\text{g phenylglyoxylic acid}) + a_4 \times (\mu\text{g mandelic acid}),$$

where *a*s are the appropriate constants, which can be calculated from Figure 2. Rearranging gave the

TABLE 1

THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION OF PHENYLGLYOXYLIC ACID AND MANDELIC ACID

Solvent systems	10 × R _f values			
	Phenylglyoxylic acid		Mandelic acid	
	From urine	Authentic	From urine	Authentic
Benzene : acetic acid : H ₂ O ¹ = 1 : 1 : 2	8.0	7.8	9.1	9.0
<i>n</i> -propanol : 28% ammonia water = 7 : 3	6.4	6.3	6.7	6.9
Benzene : tetrahydrofuran : acetic acid = 57 : 35 : 8	7.2	7.0	6.5	6.4
<i>n</i> -butanol : acetic acid : H ₂ O ¹ = 4 : 1 : 5			7.3	7.4

¹Lower layer was used for the development.

In some instances the yellow colour of the phenylglyoxylic acid spot was intensified by exposing the plates to ammonia vapour prior to the treatment with sulphuric acid-formalin reagent.

FIG. 1. Spectra of phenylglyoxylic acid and mandelic acid. A, authentic phenylglyoxylic acid; B, authentic mandelic acid; C, authentic phenylglyoxylic acid plus authentic mandelic acid; D, phenylglyoxylic acid isolated from the urine of a worker exposed to styrene; E, mandelic acid isolated from the urine of a worker exposed to styrene.

following relationships between quantities of the acids and the extinction coefficients:

$$\mu\text{g phenylglyoxylic acid} = 37.5 \times E_{350} - 22.1 \times E_{450} \dots (1)$$

$$\mu\text{g mandelic acid} = 422 \times E_{450} - 16.6 \times E_{350} \dots (2)$$

Efficiency of solvent extraction From acidified aqueous solution into 10 volumes of ether were extracted $95.2 \pm 5.5\%$ (mean $\pm$ SD, $n = 5$) of phenylglyoxylic acid and $97.8 \pm 6.2\%$ ($n = 6$) of mandelic acid. Ordinary constituents of urine did not modify the colour development significantly.

Fading of colour The maximum absorption by mandelic acid at 450 nm shifted to shorter wavelength with time, as shown in Fig. 3, but E 450 did not alter significantly during the period of 15 minutes to 1 hour after the addition of the sulphuric acid-formalin reagent. Two hours after the addition the extinction had decreased 1.6%, and by 4.5% after 3 hours.

FIG. 2. Extinctions from phenylglyoxylic acid and mandelic acid mixtures at 350 nm (A) and 450 nm (B). Lines in the figure are calculated regression lines (least mean square fits).

(A) Amounts of mandelic acid were as follows: A, none; B, 0.09 mg/tube; C, 0.18 mg/tube; D, 0.27 mg/tube; E, 0.36 mg/tube.

(B) Amounts of phenylglyoxylic acid were as follows: A, none; B, 0.04 mg/tube. Lines for 0.01, 0.02 and 0.03 mg/tube of phenylglyoxylic acid were between the two lines shown.

FIG. 3. Variation with time of the spectrum of the colour from mandelic acid.

No such shift in the spectrum was observed in the case of phenylglyoxylic acid. Decrease in E_{350} was not significant at 1 hour, was 3% at 2 hours and 8% at 3 hours after the addition of the reagent.

Application of the method to urine samples from factory workers

The method was applied to urine samples of factory workers exposed to styrene vapour at various concentrations. The results together with hippuric acid

determinations are summarized in Table 2. It is apparent that phenylglyoxylic acid and mandelic acid levels increased with the styrene concentrations in the atmosphere, while hippuric acid levels were essentially independent. In workshop A, where the styrene concentration was highest, the increase from the normal range (*i.e.*, the ratio exposed:normal) was greatest for phenylglyoxylic acid. In agreement with the observation by Bardoděj and Bardodějová (1966), the ratio mandelic acid:phenylglyoxylic acid appeared to vary with the environmental styrene concentration, being greater at higher styrene concentrations.

Discussion

The results in Table 2 indicate that mandelic acid or, better, phenylglyoxylic acid levels in urine are a sensitive index of exposure to styrene vapour. It may be that mandelic acid in urine does not provide a reliable index of absorption when there is simultaneous skin and lung exposure (Dutkiewicz and Tyras, 1967, 1968). At styrene levels of 1 to 20 ppm (*i.e.*, 0.01 to 0.2 of the recommended maximal allowable concentration (MAC) of 100 ppm set by the Japanese Association of Industrial Health) urinary phenylglyoxylic acid levels were significantly increased above normal.

Statistical analyses were conducted to see if it was possible to simplify equations (1) and (2) in the Results section so as to simplify calculation of urinary levels of the two acids from the extinctions

TABLE 2
METABOLITE LEVELS IN URINE OF WORKERS EXPOSED TO STYRENE

Workshops	No. of workers	Styrene concentrations (ppm)	Metabolites in urine (mg/litre)			Ratio mandelic acid Ph-glyoxylic acid
			Hippuric acid	Phenylglyoxylic acid	Mandelic acid	
A	6	10-30	438 (326-588)	381 (298-487)	875 (505-1515)	2.3
B	4	7-20	556 (263-1175)	287 (183-449)	473 (319-702)	1.7
C	4	1-20	494 (238-1022)	201 (153-263)	310 (189-518)	1.5
D	7	< 1	309 (198-482)	98 (46-213)	137 (49-385)	1.4
E	9	0	350 (199-616) ¹	19 (11-34)	92 (47-178)	

¹From Ikeda and Ohtsuiji (1969)

The factory surveyed was making large plastic containers. The mixture of glass-wool and liquid styrene monomer was sprayed over moulds in exhaust chambers at workshops A, B and C, and then heated in ovens for solidification by polymerization. The layer of styrol resin containing glass-wool was then removed from the moulds at workshop D. Those at workshop E were office workers. Styrene in air was determined by Kitagawa detection tubes. Urinary hippuric acid was determined as previously described (Ikeda and Ohtsuiji, 1969a). For statistical analysis data were assumed to be log-normally distributed (Heath, 1967), *i.e.*, geometric means, together with SD ranges in parentheses are given.

FIG. 4. Simplified estimation of phenylglyoxylic acid and mandelic acid. Urine samples from workers exposed to styrene vapour were treated as described in Materials and methods. Urinary levels of phenylglyoxylic acid and mandelic acid, calculated precisely by equations (1) and (2), are plotted against extinction at 350 nm and 450 nm respectively. Each solid circle represents a urine sample. Lines are calculated regression lines (least mean square fits).

at 350 and 450 nm, i.e., from E 350 and E 450. Re-plotting the data summarized in Table 2 showed that E 350 alone could be used as a measure of phenylglyoxylic acid, the two being related by the relationship:

$$\mu\text{g of phenylglyoxylic acid} = 33.0 \times E\ 350 \dots (3)$$

Even though the ratio of phenylglyoxylic acid to mandelic acid varies as a function of environmental styrene concentration, the coefficient of correlation was 0.995 (Fig. 4A).

Similarly, mandelic acid levels could be measured by E 450 (Fig. 4B).

The equation is:

$$\mu\text{g of mandelic acid} = 344 \times E\ 450 - 1.5 \dots (4)$$

The coefficient of correlation was 0.958. The last term is very small, so, mandelic acid levels are given well enough by:

$$\mu\text{g of mandelic acid} = 344 \times E\ 450 \dots (5)$$

Contrary to statements by others (American Industrial Hygiene Association, 1968) and observation in animals such as rabbits (El Masri *et al.*, 1958) and rats (Ikeda and Ohtsuji, unpublished data), no significant increase was observed in hippuric acid levels in the urine from human subjects exposed to styrene. The discrepancies may be accounted for by different intensities of exposure to styrene. It may

also be suggested that man has relatively poor ability to convert mandelic acid (or phenylglyoxylic acid) to benzyl alcohol, the possible precursor of hippuric acid.

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