TLC separation of hippuric, mandelic, and phenylglyoxylic acids from urine after mixed exposure to toluene and styrene

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ABSTRACT A method using thin-layer chromatography is described to determine the concentration of hippuric acid, mandelic acid, and phenylglyoxylic acid present in the urine after occupational mixed exposure to toluene and styrene. These substances are known metabolites of toluene and styrene, and therefore the evaluation to mixed exposure to toluene and styrene may be carried out separating these metabolites beforehand. Procedures are proposed to separate the metabolites as follows: (1) separation of hippuric acid from mandelic acid, (2) separation of mandelic acid from phenylglyoxylic acid, and (3) separation of hippuric acid and mandelic acid from phenylglyoxylic acid. The developing reagent p-dimethylaminobenzaldehyde in acetic acid anhydride was used after separation on Kieselgel and Silicagel. The sensitivity of the method was 6 μg of hippuric acid, 10 μg of mandelic acid, and 7 μg of phenylglyoxylic acid with an average recovery of 94%.

The evaluation of professional exposure to styrene is based on the determination of mandelic acid¹⁻³ or mandelic and phenylglyoxylic acids⁴⁻⁵ in the urine. Ikeda *et al*⁶ and Horigushi *et al*⁷ described the usefulness of hippuric, phenylglyoxylic, and mandelic acid determination in the urine after exposure to styrene. Hippuric acid is the main metabolite of styrene in animals³ and the main metabolite after exposure to toluene⁸⁻⁹; hippuric acid is also found in the urine after exposure to benzylamines.¹⁰ Some separation procedures of mandelic and phenylglyoxylic acids have been described using gas chromatography¹¹⁻¹³; a colorimetric method is described for determining these metabolites using TLC techniques,⁴ but at the same time hippuric acid was analysed separately, according to the method of Ikeda and Ohtsuji.¹⁴

The aim of the present work was to elaborate a simple method for the simultaneous quantitative determination of hippuric, mandelic, and phenylglyoxylic acids by using thin-layer chromatography. There are large discrepancies among various authors concerning the evaluation of metabolites in the urine after professional exposure to styrene, and therefore the present method enables the direct

use in following variations: two from the three analysed compounds or all three compounds.

Material and methods

The general separation procedure was carried out in aqueous solutions of the metabolite mixtures and proved in urine samples, to which given amounts of the analysed compounds were administered. The final results were checked in urine samples collected from individuals exposed to styrene.

For mandelic and phenylglyoxylic acid determination, urine samples were extracted by using ethyl ether. Because of rather low solubility of hippuric acid in ethyl ether, when all three compounds were analysed, chloroform was used for the extraction or urine samples were directly administered to the TLC plates. Such urine samples were evaporated under vacuum (10:1 (v:v)) before use.

ADSORBENTS USED

Chromatographic plates covered with Silicagel LS 5/40 μ , Kieselgel H, Kieselgel HF₂₅₄, Kieselgel H type 60, MN Kieselgel G, and MN Kieselgel H were used. The layer thickness of the sorbent on plates dried at room temperature for 10-15 min was 0.25 mm. The plates were activated for one hour, dried again at 105°C, and stored in a desiccator over CaCl₂.

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Among various developing systems and developing reagents used the following ones are proposed as being in good agreement with quantitative control experiments.

DEVELOPING SYSTEMS

Metabolite mixtures

- I Hippuric acid (HA)-mandelic acid (MA).
- II Mandelic acid (MA)-phenylglyoxylic acid (PGA).
- III Hippuric acid (HA)-mandelic acid (MA)-phenylglyoxylic acid (PGA).

DEVELOPING SYSTEMS IN V/V

- (A) Light petroleum-n-butyl alcohol-chloroform-acetic acid (3:3:4:2).
- (B) Light petroleum-chloroform-acetic acid (3:4:2).
- (C) n-butyl alcohol-formic acid-water (3:2:1).
- (D) Light petroleum-benzene-chloroform-acetic acid (4:1:3:2).
- (E) n-butyl alcohol-formic acid-water (4:1:1).
- (F) Chloroform-acetic acid-water (4:1:1) chloroform layer used after having solvent mixture shaken and separated.
- (G) n-butyl alcohol-chloroform-acetic acid-water (5:2:1:4).
- (H) Light petroleum-benzene-chloroform-acetic acid-water (3:3:4:2:2).

DEVELOPING REAGENTS

The following developing systems were used given that all are able to form coloured compounds with aromatic acids:

- (a) p-dimethylaminobenzaldehyde (4 g was dissolved in 100 ml of acetic acid anhydride together with 1 g of sodium acetate),
- (b) methyl red reagent, ethanol solution 0.1%,
- (c) o-bromophenol red ethanol solution (0.04%), and
- (d) bromophenol blue ethanol solution 0.04%, pH = 6.7.

Spectrophotometric absorption measurements were carried out on the substances taken from plates after reaction with p-dimethylaminobenzaldehyde in

acetic anhydride using a recording Carl Zeiss UV-Vis spectrophotometer.

Procedure—One ml of urine acidified with two drops of concentrated H_2SO_4 was twice extracted with 5 ml of ethyl ether and strongly shaken. The extract was concentrated to a volume of 1 ml. When the person is also exposed to toluene it is suggested that chloroform be used for urine extraction; alternatively, the urine may be concentrated under low pressure to small volumes. Aliquots of 100 μ l were used for each analysis, and the chromatographic plates were developed in one of the solutions described above, depending on the aim of the procedure. The solvent was allowed to evaporate, and the plates were sprayed with p-dimethylaminobenzaldehyde reagent and kept at room temperature for several hours to develop the colour. The obtained azlactones were extracted twice with 1 ml of ethanol, and the absorbance was determined spectrophotometrically against ethanol. Absorption measurements of the orange spots of hippuric acid were carried out at 465 nm and the greenish-yellow spots of mandelic acid and blue-yellow spots of phenylglyoxylic acid at 220 and 228 nm respectively.

Results

As shown in table 1, the R_f coefficients for hippuric and mandelic acids on plates covered with Silicagel LS 5/40 μ , Kieselgel HF₂₅₄, MN Kieselgel G, and MN Kieselgel H in three development systems (B, D, F) are quite different, making the separation procedure simple and giving good reproducibility.

The R_f coefficients for mandelic and phenylglyoxylic acids on several silicagels are presented in table 1. Relatively good separation was obtained when the analysed compounds were separated on MN Kieselgel H and Silicagel LS 5/40 μ in all four developing systems (A, C, G, H). The best separation was obtained when butanol-chloroform-acetic acid and water (G 5:2:1:4) was used. The solvents presented in table 1 are useful when the urinary metabolites of styrene are analysed in the absence of

Table 1 R_f values of hippuric acid (HA), mandelic acid (MA), and phenylglyoxylic acid (PGA) in various solvent systems. (For solvent systems A-H, see text)

No mixtures	Metabolites mixtures	Adsorbent Silicagel LS 5/40 μ								Kieselgel HF ₂₅₄		
		A	B	C	D	E	F	G	H	B	D	E
I	HA		0.13		0.27		0.19			0.26	0.40	
	MA		0.27		0.49		0.32			0.41	0.69	
II	MA	0.89		0.91				0.95	0.20			
	PGA	0.12		0.44				0.13	0.53			
III	HA			0.93		0.78			0.07			0.64
	MA			0.81		0.66			0.16			0.60
	PGA			0.55		0.37			0.46			0.20

Table 2 Colour reactions of hippuric acid (HA), mandelic acid (MA), and phenylglyoxylic acid (PGA) with various developing reagents. (For reagent a-d and solvent system H, see text)

Metabolite	Silicagel LS 5/40 μ				Kieselgel HF 254			
	Reagent				Reagent			
	a	b	c	d	a	b	c	d
HA	Orange spot on light-greenish bgd	Red spot on rose bgd	Light yellow spot on dark-yellow bgd	Yellow spot on greenish bgd	Orange spot	Red spot on rose bgd	Yellow spot on light-brown bgd	Yellow spot on blue bgd
MA	Greenish-yellow spot on light-greenish bgd	Dark red spot on rose bgd	Orange-yellow spot on dark-yellow bgd	As above	Greenish-yellow spot	Dark red spot on rose bgd	As above	As above
PGA	Blue-yellow spot on light-greenish bgd	Violet spot on rose bgd	As above	As above	Light greenish or bluish spot	Violet spot on rose bgd	As above	As above

bgd = Background.

toluene exposure.

The separation of all three compounds produces the greatest difficulties. In table 1 the R_f values for hippuric acid, mandelic acid, and phenylglyoxylic acid on various silicagels and kieselgels are presented when three developing solvents (C, E, H) are used. The best results were obtained when the solvent system H (light petroleum-benzene-chloroform-acetic acid-water (3:3:4:2:2)) was used. The optimisation of colour production when spots of hippuric acid, mandelic acid, and phenylglyoxylic acid are present on the plates are shown in table 2. The best results were obtained when p-dimethylaminobenzaldehyde in acetic acid anhydride was used. Hippuric acid under the described conditions forms an orange-coloured spot, which, after elution with ethanol, gives an absorption maximum at 465 nm when measured versus ethanol. Mandelic acid forms a greenish-yellow spot that after elution with ethanol, shows two maxima at 220 nm and at about 345 nm.

Phenylglyoxylic acid gives a light yellow-blue spot showing absorption in the range between 250 and 350 nm with a maximum of 228 nm. The intensity of the coloured substance produced by mandelic and phenylglyoxylic acids increases, reaching a constant value after a few hours.

As shown in table 2, the analysed metabolites are

detectable as coloured spots on the chromatographic plates after appropriate development. The separations were carried out on Silicagel LS 5/40 μ and Kieselgel HF₂₅₄ in the developing system—(light petroleum-benzene-chloroform-acetic acid-water (3:3:4:2:2)). The best of the developing reagents was p-dimethylaminobenzaldehyde, which produces coloured compounds with both mandelic and phenylglyoxylic acids after a few hours. Bromophenol-blue which gives yellow spots on a blue or green background is also useful because of the almost immediate colour production.

The absorption maxima of the coloured compounds was, for mandelic acid, 218 nm, for hippuric acid, 227 nm, and for phenylglyoxylic acid, 234 nm.

Discussion

We have shown that, after mixed exposure to styrene and toluene, the urinary metabolites of these substances may be separated and quantitatively determined. Satisfactory results were obtained when the analysed metabolites were developed in light petroleum-benzene-chloroform-acetic acid-water (3:3:4:2:2) on Kieselgel HF₂₅₄, MN Kieselgel H, and Silicagel 5/40 μ .

When workers were exposed only to styrene, both

			MN Kieselgel H								MN Kieselgel G			
F	G	H	A	B	C	D	F	G	H		B	D	F	G
0.31				0.38		0.50	0.37				0.45	0.50	0.25	
0.43				0.54		0.68	0.50				0.65	0.77	0.37	
	0.94	0.29	0.78		0.66			0.87	0.24					0.84
	0.07	0.55	0.49		0.52			0.12	0.78					0.09
		0.11			0.72				0.07					
		0.23			0.63				0.23					
		0.85			0.31				0.80					

metabolites (mandelic acid and phenylglyoxylic acid) were satisfactorily separated in n-butyl alcohol-chloroform-acetic acid-water (5:2:1:4) on Kieselgel HF₂₅₄, MN Kieselgel G, MN Kieselgel H, and Silicagel LS 5/40 μ . The developing reagent proposed by Ogata *et al*¹⁵ for hippuric acid determination based on p-dimethylaminobenzaldehyde also enables mandelic acid and phenylglyoxylic acid to develop. The coloured substances produced when mandelic acid and phenylglyoxylic acid were present in the analysed urine sample are probably produced during longer direct contact of these substances with the developing reagent because, as mentioned above, the intensity of the colour increases after prolonged time of development. The present method gives quite good separation of the analysed compounds as well as relatively good and reproducible quantitative results.

This procedure may be used when exposure to both toluene and styrene, or styrene alone, has to be evaluated. The simplified procedure involving TLC techniques may be used in some factory laboratories. The lowest quantities of the analysed substances were 6 μ g for hippuric acid, 10 μ g for mandelic acid, and 7 μ g for phenylglyoxylic acid. When the concentration of these substances in urine is lower than the above mentioned values, the urine samples have to be concentrated by evaporation under low pressure. The exactness of determination was ± 4.7 , ± 9.4 , and $\pm 8.4\%$ for hippuric, mandelic, and phenylglyoxylic acids respectively. The presented accuracy is good enough to evaluate occupational exposure to toluene and styrene.

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