Thin-layer chromatography of hippuric and m-methylhippuric acid in urine after mixed exposure to toluene and xylene

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ABSTRACT The separation of hippuric and m-methylhippuric acid as toluene and m-xylene metabolites present in urine of people exposed simultaneously to toluene and xylene is described. Chloroform was used for hippuric and m-methylhippuric acid extraction. Satisfactory separation of these metabolites was obtained on TLC plates covered with silica gels and developed in chloroform acetic acid-water (4:1:1); p-dimethylaminobenzaldehyde in acetic acid anhydride was applied to develop the colour. The sensitivity of the method was 6 µg hippuric acid per 1 ml urine and recovery was 100% (± 1).

Evaluation of mixed exposure to toluene and xylene may be carried out by measuring the rate of excretion of hippuric acid¹ and m-methylhippuric acid² in the urine, separating the compounds beforehand. Numerous reported methods³⁻⁵ of hippuric acid determination afford combined determination of toluene and xylene metabolites. Among methods for determination and separation of the above metabolites, gas chromatography,⁶⁻⁸ paper chromatography,^{9,10} high-speed liquid chromatography,¹¹ and thin-layer chromatography¹⁰ have been described. The method presented in this paper is simple and highly reproducible. Since the reproducibility of results obtained by the method of Ogata *et al.*¹⁰ was unsatisfactory, the determination of m-methylhippuric acid has been modified by Orłowski⁹ by using paper chromatography where optimal conditions of analysis were described. The accuracy of Orłowski's⁹ method of hippuric and m-hippuric acids estimation is ± 3%, yet the development of the chromatogram is time-consuming. To overcome these difficulties, we have attempted to develop a method of hippuric and m-methylhippuric acids determination in urine by the use of thin-layer chromatography. The problem of determining toluene and xylene metabolites in urine has been detailed by Ogata *et al.*¹⁰ Orłowski,⁹ and Burkiewicz and Zielińska.³

Material and methods

The method of determining hippuric and m-methyl-

hippuric acid concentrations was worked out for standard water solutions within the range of the concentration 0.3-0.4 mg/ml and later checked on urine samples. Toluene and xylene metabolites were extracted according to the methods of Burkiewicz and Zielińska.³ All colour-developing reactions for making identification and quantitative determinations were similar to that described by Ogata *et al.*¹⁰ This system has the highest reproducibility among many others for the separation and identification of hippuric and m- or p-methylhippuric acid. Separation was carried out with the use of toluene-acetic acid-water (100:50:2.5) on silica gel G. In this study, double chloroform extraction of these compounds from urine has been carried out, but the extraction of hippuric and m-methylhippuric acids may be carried out also according to the methods of Ogata *et al.*¹⁰ and Orłowski.⁹

Adsorbents used—Plates with adsorbents already in situ, silufol 254 UV, silufol 254 + 266 UV (made in Czechoslovakia), as well as chromatographic plates covered with kieselgel G (according to Stahl), silicagel GF 254 (Macherey-Nagel), and silicagel LS 5/40 µ were used. The adsorbents on the plates dried at room temperature for 10-15 min were in layers of 0.25 mm. The plates were activated for one hour, dried again at 105°C, and stored in a desiccator over CaCl₂.

Three developing systems and four developing reagents were examined to reach the highest reproducibility and to improve the exactness of determination.

Developing systems—I-chloroform-acetic acid-

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water (4:1:1) chloroform layer used after having solvent mixture shaken and separated. II-toluene-methanol-acetic acid (43:9:5). III-light petroleum-benzene-chloroform-methanol-acetic acid (14:2:2:1:1).

Developing reagents—A *p*-Dimethylaminobenzaldehyde (4 g) was dissolved in 100 ml of acetic acid anhydride with 1 g of sodium acetate.¹⁰ B Pyridine-acetic acid anhydride (5:2 v:v).³ C Methyl red in acetic acid anhydride—1 % solution. D Bromocresol green—1 % solution.

Procedure

One millilitre of urine was acidified with two drops of concentrated H₂SO₄ and twice extracted with 5 ml chloroform. The extract was concentrated to the volume of 1 ml under reduced pressure. Aliquots of 50 μ l were spotted on the activated chromatographic plate-silufol 254 UV and developed with chloroform-acetic acid-water (4:1:1). The degree of separation of hippuric and m-methylhippuric acids obtained by using the above described adsorbents is presented in table 1. The solvent was allowed to evaporate, and the plates were sprayed with *p*-dimethylaminobenz-

aldehyde reagent (A), and heated at 135°C for three minutes to develop the colour. The azlactones were extracted twice with 2 ml and 1 ml of ethanol, and the absorbance was determined spectrophotometrically at 465 nm against ethanol as the blank by the use of the low-range Cary 118 C spectrophotometer.

Results

Table 1 presents the values of R_f coefficients for hippuric and m-methylhippuric acid separated on commercial silufol 254 UV, silufol 254 + 366 UV plates, as well as on home-made plates covered with silica gel type LS 5/40 μ and silicagel type GF 254 and kieselgel G.

Since *p*-dimethylaminobenzaldehyde in acetic acid anhydride irritates the mucose membrane and conjunctivae and therefore is noxious to persons in direct contact with this reagent, attempts were undertaken to find other reagents that would give coloured reactions with intensive and stable colouring. The results of the experiments are presented in table 2.

Stable colouring of the separated compounds was obtained from the application of *p*-dimethylamino-

Table 1 *R_f coefficients of hippuric acid (HA) and m-methylhippuric acid (MHA) developed under described conditions*

Absorbent	Silufol 254 UV		Silufol 254 + 366 UV		Silicagel LS 5/40 μ		Silicagel GF 254		Kieselgel G after Stahl	
	Values									
Developing system	MHA	HA	MHA	HA	MHA	HA	MHA	HA	MHA	HA
Chloroform-acetic acid-water (4:1:1)	0.89	0.64	0.49	0.41	0.45	0.37	0.37	0.27	0.45	0.33
Toluene-methanol-acetic acid (43:9:5)	0.61	0.57	0.46	0.40	NO	NO	NO	NO	0.45	0.32
Light petroleum-benzene-chloroform-methanol-acetic acid (14:2:2:1:1)	NO	NO	0.36	0.30	0.26	0.20	NO	NO	NO	NO

NO = Separation of hippuric and m-methylhippuric acid was not obtained.

Table 2 *Colour reactions of hippuric and m-methylhippuric acids with the use of various developing systems*

Developing reagent	A <i>p</i> -Dimethylaminobenzaldehyde reagent	B Pyridine reagent	C Methyl red reagent	D Bromocresol green solution
Chloroform-acetic acid-water (4:1:1)	Orange against light background stable	Yellow against light background unstable	White against red background stable	Yellow against green background unstable
Toluene-methanol-acetic acid (43:9:5)	As above	No reaction	As above	Whole plate covered with yellow colour
Light petroleum-benzene-chloroform-methanol-acetic acid (14:2:2:1:1)	As above	No reaction	As above	No reaction
Maximum absorbance of the colour compound nm	465	363	Far UV	420

benzaldehyde as well as methyl red in acetic acid anhydride. Coloured compounds were obtained on chromatograms developed with these reagents in all three developing systems as presented in table 1. The absorption maximum of the compound formed during the reaction of hippuric and m-methylhippuric acid with methyl red in the presence of acetic acid anhydride was in the far UV region. It was shown that p-dimethylaminobenzaldehyde, proposed by Ogata *et al.*,¹⁰ is still the best developing solution among those tested, forming stable compounds with intensive orange colouring. The above described method of determination was compared with the colorimetric method of hippuric acid determination presented by Burkiewicz and Zielińska,³ where the hippuric acid concentration was determined in the urine of people exposed to toluene.

One has to take into consideration that the method described by Burkiewicz and Zielińska³ is proposed for hippuric acid determination only. The results are shown in the figure. A high correlation coefficient

Comparison of hippuric acid concentrations in urine analysed by thin-layer chromatography v colorimetric method.

($r = 0.988$ $n = 26$) was obtained when hippuric acid was analysed in urine samples by the use of both independent methods, the colorimetric one and the one proposed in this paper; the correlation was statistically significant ($0.01 > \alpha > 0.001$).

Discussion

The best separation of hippuric and m-methylhippuric acids was achieved using chloroform-acetic acid-water (4:1:1) because of the highest differences

of the R_f values. This system can be applied for all adsorbents listed in table 1. The separation of hippuric and m-methylhippuric acids was most effective on silufol plates type silufol 254 + 366 UV. Similar results were achieved when both acids were separated on silicagel G (after Stahl) and when the developing system of chloroform-acetic acid-water (4:1:1) or toluene-methanolacetic acid (43:9:5) was used. It was shown that the best developing reagent among those tested is still that proposed by Ogata *et al.*¹⁰; p-dimethylaminobenzoic acid gives an intense colour reaction and is stable for several days.

The described separation procedure of hippuric and m-methylhippuric acids enables the selective determination of metabolites derived from toluene and m-xylene to be made when workers are professionally exposed to both compounds simultaneously. The exactness of hippuric acid and m-methylhippuric acid determination is $99\% \pm 3.4\%$ by $6.0 \mu\text{g/ml}$. This procedure may be applied therefore for low quantities of analysed metabolites.

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