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Short communication

Modified method for determination of hippuric acid and methylhippuric acid in urine by gas chromatography

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Abstract

A modified method for the simultaneous determination of hippuric acid (HA) and *o*-, *m*- and *p*-methylhippuric acids (*o*-, *m*- and *p*-MHAs) in urine is described. These metabolites were extracted, derivatized into their methyl ester derivatives and analyzed using a gas chromatograph equipped with flame ionization detector and a DB-1 capillary column. The derivatives of HA, *o*-, *m*- and *p*-MHAs were well separated within 11 min. The accuracy and precision in the present method were sufficient for quantitative analysis, and the results obtained by the GC method were highly correlated with those by the HPLC method (NIOSH 8301).

Keywords: Hippuric acid; Methylhippuric acid

1. Introduction

Toluene and xylenes are widely used as organic solvents in industry. The best estimation for assessment of mixed exposure to toluene and xylenes is the quantitative determination of their metabolites, hippuric acid (HA) and *o*-, *m*- and *p*-methylhippuric acids (*o*-, *m*- and *p*-MHAs), excreted in urine, which show good correlation between the level of exposure and amount of metabolites excreted [1,2].

There are several methods used for analyzing urinary HA and MHA isomers, such as spectrophotometry [3], gas chromatography (GC) [4–7] and high-performance liquid chromatography (HPLC) [8–10]. The HPLC method is considered by the National Institute for Occupational Safety and Health (NIOSH) [11] to be the reference method (NIOSH 8301). This method, however, cannot separately quantitate each isomer of *m*- and *p*-MHAs.

The GC method was considered to be a specific method for determination of HA and MHA [4,5,8]. This method requires a derivatization procedure with some chemicals; for example, several investigators have used diazomethane, an explosive, carcinogenic and toxic reagent [4,5]. Others have used trimethylsilyl derivatives [6]. In 1991, de Carvalho et al. [7] described the derivatization of HA and MHA using methanol in acid medium (HCl), a low cost and less toxic reagent, and analysis by GC equipped with 5% SE-30 on Chromosorb W and a flame ionization

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detector. However, the GC methods have not yet produced a separation of *m*- and *p*-MHAs.

The purpose of this study is to modify the method of de Carvalho et al. [7] for simultaneous determination of HA, *o*-, *m*- and *p*-MHAs by GC. This study can separate the methyl esters of HA, *o*-, *m*- and *p*-MHAs using a gas chromatograph equipped with a DB-1 capillary column and a flame ionization detector. The modified GC method will be compared with the HPLC method (NIOSH 8301).

2. Experimental

2.1. Chemicals and reagents

HA, MHA isomers (*o*-, *m*- and *p*-MHAs), picric acid and heptadecanoic acid were obtained from Sigma (St. Louis, MO, USA). Methanol (max. 0.005% water) was purchased from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade and other chemicals were of analytical grade. 2.0 mg/ml heptadecanoic acid (internal standard) and derivatizing reagent were prepared according to the method described by de Carvalho et al. [7].

2.2. Instrumentation

The Shimadzu GC-14B gas chromatograph with a DB-1 capillary column (30 m×0.53 mm I.D.), equipped with a flame ionization detector and an integrator (Shimadzu C-R7A, Shimadzu, Kyoto, Japan) was used. The carrier gas was helium at a flow-rate of 10 ml/min. The GC condition was isothermal: column, 200°C; injector, 250°C; detector, 250°C.

The HPLC system consisted of a Waters 600S controller, a Waters 626 pump and a Waters 717 plus automatic sampler injector (Waters, Milford, MA, USA). The column was a μBondapak C_{18} steel column (0.39 m×300 mm I.D.; Waters) packed with 10 μm reversed-phase packing and a UV detector set at 254 nm. The mobile phase was distilled water–acetonitrile–glacial acetic acid (900:100:0.2, v/v/v) at a flow-rate of 2.5 ml/min.

2.3. Preparation of standard solutions

Stock standard solutions of HA (1.0 mg/ml) and MHA isomers (0.5 mg/ml) were prepared by dissolving 100 mg of HA and 50 mg of each isomer of MHA (*o*-, *m*- and *p*-MHAs) in 100 ml distilled water. All standard solutions were kept at 4°C.

2.4. Sample preparation

A 1-ml volume of urine sample and 1 ml distilled water were pipetted into a 15-ml screw cap tube, 0.2 ml internal standard solution (heptadecanoic acid) was added. The mixture was acidified with 0.2 ml of 0.5 *M* HCl and extracted with 3 ml ethyl acetate. The organic phase was evaporated to dryness under a compressed air flow at room temperature. The residues were reconstituted with 1 ml derivatizing reagent and left in an oven at 60°C for 45 min. The mixture was allowed to cool to room temperature and extracted with 1 ml chloroform after addition of 2 ml distilled water. A 1-μl volume of chloroform solution was injected into the GC system.

2.5. Matrix calibration

The calibration curves for HA, *o*-, *m*- and *p*-MHAs were obtained from 1-ml urine sample from persons not occupationally exposed to solvents; 1 ml of each of HA and MHA isomers standard solution was added. It was extracted and derivatized according to the method described above. Relative peak area ratios of HA, *o*-, *m*- and *p*-MHAs to heptadecanoic acid (internal standard) was used for the plotting of matrix calibration curve in the range of 0.2–1.0 mg/ml (HA) and 0.1–0.5 mg/ml (*o*-, *m*- and *p*-MHAs).

2.6. Method validation

A 1-ml volume of known HA and MHA isomers standard solution was added to 1 ml pooled urine; then analyzed as described above together with quality control samples prepared by adding known concentrations of HA, *o*-, *m*- and *p*-MHAs in urine. The inter-day precision and the accuracy were calculated as the relative standard deviations (RSDs)

and the percent recovery of concentration found, respectively.

2.7. Comparison of urinary HA and MHA isomers by GC and HPLC methods

Forty urine samples were collected from workers who exposed ($n=30$) and non-exposed ($n=10$) to toluene and xylenes. The urine samples were treated simultaneously and analyzed in duplicate for the GC and HPLC methods.

3. Results and discussion

Fig. 1 shows a typical chromatogram of urine sample of a worker exposed to toluene and xylene in the paint industry. The five acid methyl esters were separated completely and eluted within 11 min. The order of elution was methyl esters of HA, *o*-, *m*- and *p*-MHA and heptadecanoic acid (internal standard), respectively.

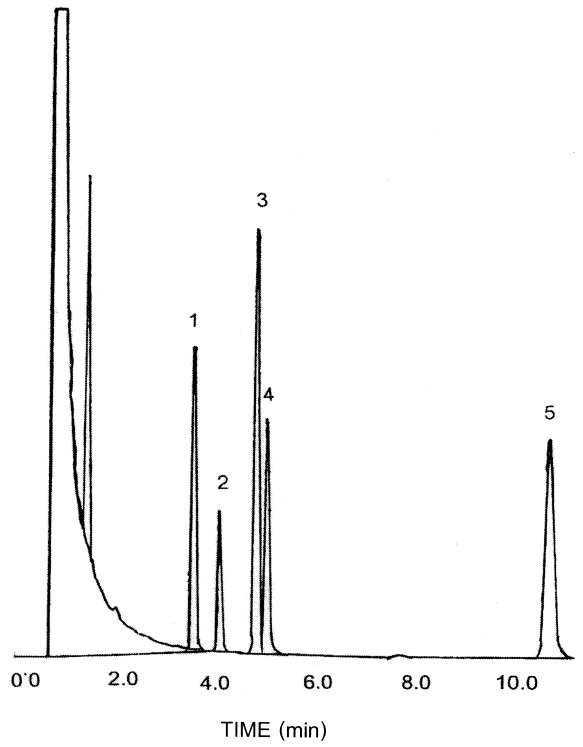

Fig. 1. Chromatogram of methyl esters obtained by analyzing urine sample from an exposed worker; 1=HA, 2=*o*-MHA, 3=*m*-MHA, 4=*p*-MHA and 5=heptadecanoic acid (internal standard).

3.1. Matrix calibration

The matrix calibration curves of HA, *o*-, *m*- and *p*-MHAs were studied in the range of 0.2–1.0 mg/ml for HA and 0.1–0.5 mg/ml for MHA isomers due to the expected range found in the urine samples. Linear correlations were found between the acid concentrations and the relative peak area ratios. The parameters of the linear regression for HA, *o*-, *m*- and *p*-MHAs are given in Table 1.

3.2. Method validation

The RSDs (100×SD/mean) were calculated from 10 days for inter-day precision (Table 2). The calculated precisions were all within 8% RSD and the accuracy of all acids were very high ranging from 93.0 to 104.3%, indicating the reliability of the GC method for determination of HA, *o*-, *m*- and *p*-MHAs. The detection limits of urinary HA could be detected at a concentration of 0.05 mg/ml, and *o*-, *m*- and *p*-MHAs could be detected individually at the same concentration of 0.015 mg/ml.

3.3. Comparison of urinary HA, o-, m- and p-MHAs by the GC and HPLC methods

Forty urine samples obtained from non-exposed workers ($n=10$) and workers exposed ($n=30$) to organic solvents in the paint industry were determined by GC and HPLC methods. The GC method showed that methyl esters of HA, *o*-, *m*- and *p*-MHAs were separated completely, whereas the HPLC method did not resolve *m*- and *p*-MHAs. The

Table 1
Typical calibration parameters of HA, *o*-, *m*- and *p*-MHAs in urine (relative peak area ratios vs. mg/ml)

Compound	Slope	Intercept	Correlation coefficient (r)
HA	0.8995	0.0064	0.9995
o-MHA	1.1114	−0.0017	0.9999
m-MHA	1.1446	−0.0016	0.9998
p-MHA	1.1434	0.0020	0.9998

Table 2
Inter-day precision and accuracy for the determination of HA, *o*-, *m*- and *p*-MHAs in urine

	Concentration added (mg/ml)	Concentration found (mg/ml)	Recovery (%)	RSD (%)
HA	0.3	0.30±0.02	99.3	6.5
	0.5	0.47±0.02	93.4	5.1
	0.7	0.68±0.03	96.9	4.3
o-MHA	0.15	0.15±0.01	102.7	7.0
	0.25	0.23±0.02	93.8	7.8
	0.35	0.33±0.02	95.6	5.0
m-MHA	0.15	0.16±0.01	104.3	6.7
	0.25	0.26±0.02	102.2	7.4
	0.35	0.34±0.02	93.1	5.6
p-MHA	0.15	0.14±0.01	93.0	6.6
	0.25	0.25±0.01	99.0	5.7
	0.35	0.34±0.02	97.3	6.0

results showed that the GC method highly correlated with the HPLC method. The correlation coefficient of HA, *o*-, *m*- and/or *p*-MHAs were 0.992, 0.975 and 0.992, respectively. The equations of HA, *o*-, *m*- and/or *p*-MHAs were $y=1.041x+0.0063$, $y=0.9386x+0.0015$ and $y=0.9278x-0.0056$, respectively; where y was the concentration of HA, *o*- or *m*- and/or *p*-MHAs in mg/ml obtained by the HPLC method and x was that obtained by the GC method. The relationship of analytical results from the two methods is presented for HA in Fig. 2. Considering the mean of HA, *o*-, *m*- and/or *p*-MHAs, a paired *t*-test on the mean of HA showed a significant difference between the two methods at a confidence limit of 95%. Most of HA levels analyzed by the GC method were slightly lower than those by the HPLC method. The mean of urinary *o*-MHA was not statistically different, but the mean of urinary *m*- and/or *p*-MHAs was significantly different at 95%. Most of *m*- and/or *p*-MHA concentrations from the GC method were slightly greater than those from the HPLC method. It can be seen that the line did not pass through origin (Fig. 2). This suggested that other constituents in urine might interfere with determination of HA, *o*-, *m*- and/or *p*-MHAs by the HPLC method, this interference did not appear in the GC method, probably due to the double extraction and derivatization procedure.

4. Conclusion

The present study described a modified GC method for simultaneous determination of HA, *o*-, *m*- and *p*-MHAs in urine, sufficiently accurate and precise to detect the exposure to either toluene or xylenes or both. Moreover, the modified GC method has the advantage of being able to separate *m*- and *p*-MHAs,

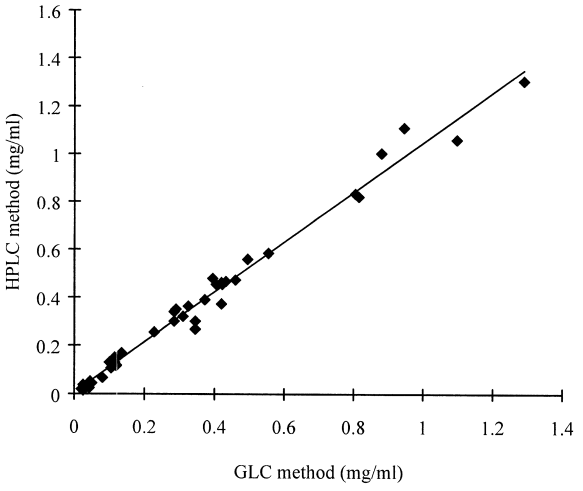

Fig. 2. Comparison between HA analyzed by the GC and HPLC methods. Regression equation: $y=1.041x+0.0063$; $r=0.992$ ($P<0.05$).

whereas the HPLC method does not resolve the *m*- and *p*-isomers of MHA [11].

References

[1] M. Ogata, K. Tomokuni, Y. Takatsuka, Br. J. Ind. Med. 20 (1969) 330.
[2] S.R. Lauwery, J.P. Buchet, IARC Sci. Publ. 85 (1988) 205.
[3] L.D. Pagnotto, L.M. Lieberman, Am. Ind. Hyg. Assoc. J. 2 (1967) 129.
[4] S. Kira, Br. J. Ind. Med. 34 (1977) 305.
[5] J.P. Buchet, R.R. Lauwerys, Br. J. Ind. Med. 30 (1973) 125.
[6] P.B. Van Poosmalen, I. Drummond, Br. J. Ind. Med. 35 (1978) 56.
[7] D. de Carvalho, V.L. Lauchote, P.S. Bonato, C.R.H. Queiroz, A.C. Santos, S.A. Dreossi, Int. Arch. Occup. Environ. Health 63 (1991) 33.
[8] J. Sollenberg, A. Baldesten, J. Chromatogr. 132 (1977) 469.
[9] H. Matsui, M. Kasoa, S. Imamura, J. Chromatogr. 45 (1978) 231.
[10] T. Sakai, Y. Niinuma, S. Yanagihara, K. Ushio, J. Chromatogr. 276 (1983) 182.
[11] C.P. Frederick, NIOSH Manual of Analytical Methods (NMNM), 4th ed., National Institute for Occupational Safety and Health, Cincinnati, OH, 1994, NIOSH 8301.