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Short Communication

High-performance liquid chromatographic determination of 3,5-dimethylhippuric acid in the occupational exposure to trimethylbenzenes

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ABSTRACT

A high-performance liquid chromatographic method for the measurement of urinary 3,5-dimethylhippuric acid (3,5-DMHA) in the human biological monitoring of the occupational exposure to trimethylbenzenes has been developed. 3,5-DMHA was extracted from urine with ethyl acetate. The organic phase was dried under vacuum and the resultant product, dissolved in the mobile phase, was analysed by an isocratic system and a programmable photodiode-array detector adjusted to 205 nm. The mobile phase was water–acetonitrile (80:20, v/v) containing 0.1% acetic acid. 3,5-DMHA was chromatographed on a reversed-phase Supelco C_{18} column (3 μm; 15 cm × 0.46 cm I.D.), and identified by its retention time and ultraviolet spectrum. Quantitation was performed by peak area. The detection limit of the method is 30 ng/ml and the recovery and the accuracy are 96%.

INTRODUCTION

The trimethylbenzenes are aromatic monocyclic hydrocarbons, colourless and flammable liquids, existing in three isomeric forms: 1,3,5-trimethylbenzene (mesitylene), 1,2,4-trimethylbenzene (pseudocumene) and 1,2,3-trimethylbenzene (hemimellitene). They are used in industry as solvents for paints, lacquers, gums and grease, and are considered to be very good substitutes for benzene, especially for their low volatility. This characteristic has caused a certain increase in their use, thereby making necessary an adequate biological control of professional exposure; acute and chronic cases of poisoning after professional exposure have been described [1–3]. Also their toxicity has been demonstrated in laboratory animals [4–7].

Trimethylbenzene metabolism has been studied in rats and rabbits [8–12]. In the organism the trimethylbenzenes can be oxidized to form dimethylbenzoic acids, which in turn can be conjugated with glycine, glucuronic acid and sulphuric acid. The main elimination route is conjugation with glycine, which brings about the formation of the respective dimethylhippuric acids. This ac-

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counts for *ca.* 60% of the total dose of mesitylene absorbed, and conjugation with glucuronic acid and sulphuric acid accounts for 5 and 10%, respectively.

The values for pseudocumene and hemimellitene are 25 and 10% conjugated with glycine, 4 and 8% with glucuronic acid and 9 and 15% with sulphuric acid [11].

A recent study performed on humans demonstrated a correlation between individual exposure to trimethylbenzenes and the urinary elimination of the corresponding metabolites, confirming the results already found in laboratory animals [13].

The commercial products commonly used in factories have variable percentages of the isomeric trimethylbenzenes: *e.g.* in Solvesso 100 [4], mesitylene is present in a smaller proportion than pseudocumene. In any case, relying on the highest percentage of the transformation to dimethylhippuric acid of the mesitylene in respect to pseudocumene, it is still possible to trace this metabolite in considerable amounts in the urine of subjects exposed to trimethylbenzenes.

The analytical methods reported for the determination of the metabolites of trimethylbenzenes use the complicated procedures of thin-layer chromatography [14] and high-performance liquid chromatography [13]. The aim of this study was to develop a sensitive, precise, accurate and simple analytical method for the determination of 3,5-dimethylhippuric acid (3,5-DMHA) in urine.

EXPERIMENTAL

Reagents and chemicals

Standard 3,5-DMHA was synthesized at the Department of Organic Chemistry of the University of Florence, according to Laham *et al.* [15]. All other reagents were form Merck (Darmstadt, Germany): 37% HCl (art. 317), NaCl (art. 6404) and ethyl acetate (art. 9623).

Chromatographic conditions

Chromatographic separation and peak detection of 3,5-DMHA were carried out on a reversed-phase C_{18} column (3 μm, 15 cm × 0.46 cm I.D.) (Supelco, Bellefonte, PA, USA) by a Waters (Millipore, Milford, MA, USA) HPLC system with a 715 Ultra Wisp sample processor, a Model 510 pump, and a 994 programmable photodiode-array detector. Injection volume, 10 μl; mobile phase, water–acetonitrile (80:20, v/v) containing 0.1% acetic acid; flow-rate, 1 ml/min; temperature, 37°C; detector wavelength, 205 nm. 3-Hydroxybenzoic acid was used as internal standard added to obtain a final concentration of 100 mg/l for each sample or standard before the extraction.

Analytical procedure

The urine specimens were obtained from healthy, unexposed men and from workers exposed to trimethylbenzenes in the workshop. The collection was executed at random for unexposed men, and at the beginning and end of a workshift for workers. The urine specimens were stored at 4°C for one day or at −20°C for eight months.

To 1 ml of urine, containing 0.1 ml of internal standard (0.1% solution), were added 0.1 ml of 37% HCl and NaCl to saturation. 3,5-DMHA was extracted with 4 ml of ethyl acetate on a rotatory agitator for 15 min. After centrifugation at 3000 *g* for 15 min, 1.0 ml of the extract was dried

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF 3,5-DMHA IN THE URINE OF SUBJECTS EXPOSED TO TRIMETHYLBENZENES

Sample	Concentration found (mean ± S.D., n = 10) (mg/l)	C.V. (%)
Intra-assay		
1	11.9 ± 1.3	10.8
2	16.0 ± 1.3	8.3
3	54.4 ± 5.1	9.4
4	105.2 ± 4.6	4.3
5	143.6 ± 7.2	5.0
Inter-assay		
1	9.2 ± 0.18	1.9
2	13.9 ± 0.22	1.6
3	45.2 ± 1.03	2.3
4	105.0 ± 1.82	1.7
5	138.7 ± 1.33	1.2

under vacuum or in a stream of nitrogen, and taken up in 0.1 ml of mobile phase. Then 10 μl of this solution were injected into the HPLC system, and peaks were detected at 205 nm.

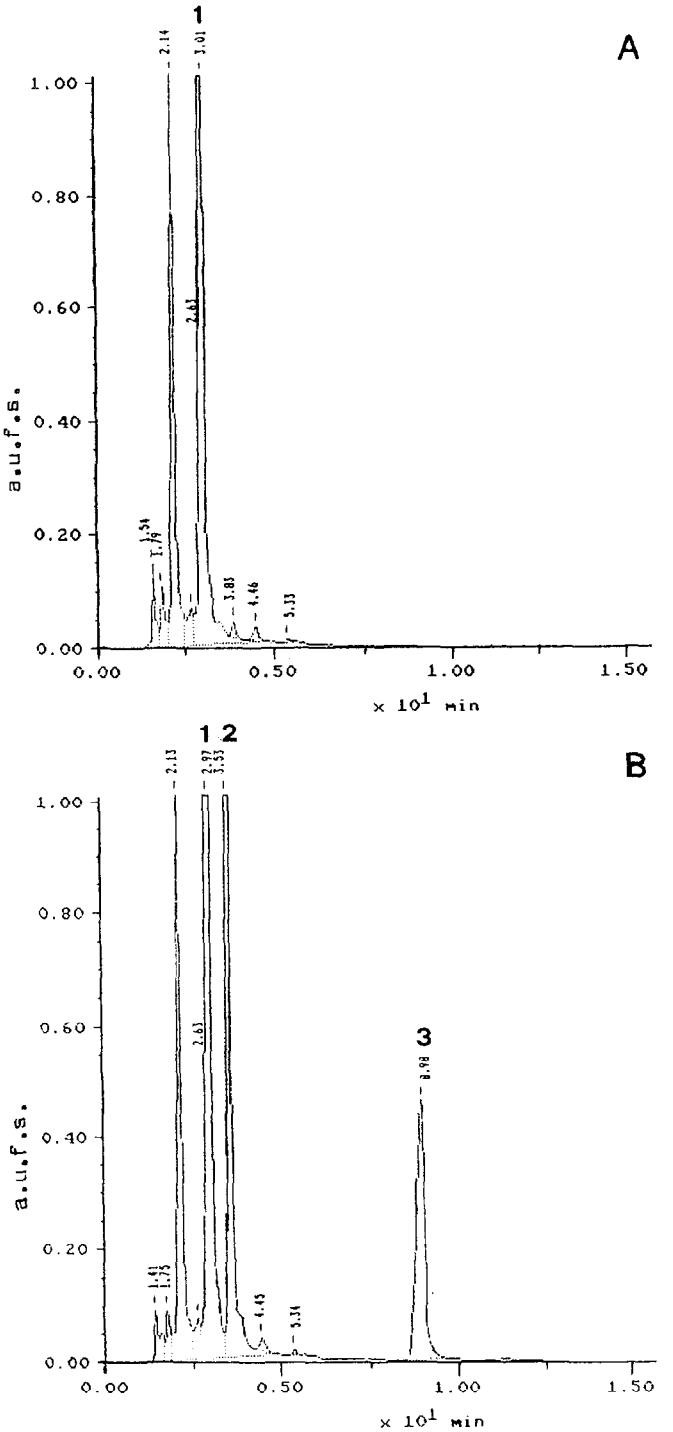

Fig. 1. Chromatograms of the urine of subjects unexposed (A) and exposed (B) to trimethylbenzenes. Peaks: 1 = hippuric acid; 2 = internal standard (100 mg/l); 3 = 3,5-dimethylhippuric acid.

The calibration curve was prepared by adding known amounts of 3,5-DMHA to urine specimens of healthy, unexposed men: the amounts added were 1.0, 5.0, 10, 25, 50, 100 and 200 mg/l, obtained from an aqueous concentrated solution (0.5 g/l) of 3,5-DMHA, stable at 4°C for six months.

RESULTS AND DISCUSSION

The standard curve, obtained using the same procedure as for the other samples, was linear up to at least 200 mg/l 3,5-DMHA, described by the equation $y = 18.1x + 2.05$ (y = concentration of 3,5-DMHA; x = peak area) with $r = 0.999$ and $n = 7$.

The detection limit, defined as the minimum concentration of 3,5-DMHA capable of giving a signal at least double the mean noise oscillation, was 30 ng/l.

Under the experimental conditions described, the recovery from urine was 96% for both the 3,5-DMHA and the internal standard. The results of this study, with a concentration of 100 mg/l internal standard and 20, 50 and 125 mg/l 3,5-DMHA, confirm the validity of the extraction procedure.

The accuracy, tested by examining normal urinary samples with 3,5-DMHA added at concentrations of 10, 50 and 100 mg/l, was 96%.

The reproducibility was tested by examining five urinary samples of subjects exposed to trimethylbenzenes, with different concentrations of 3,5-DMHA, for ten times within-day and for ten days in succession. Table I shows the coefficients of variation obtained: the low values show that the analytical procedure is reproducible, precise and reliable.

Fig. 1 shows a chromatogram obtained from a sample extract: 3,5-DMHA was identified by its retention time, and the quantification was performed by automatic peak-area measurement. Thanks to the photodiode-array detector it was possible to perform a spectrum comparison (FIT[a]) to verify the purity of the 3,5-DMHA peak: the values of the FIT, relative to chromatograms of specimens under examination, confirm the validity of analytical method.

With the proposed method we have analysed the urine of 36 subjects not exposed to trimethylbenzenes: the average of values obtained is 0.42 mg/l, with a range from 0.0 to 2.8 mg/l. The monitoring of occupational exposure to trimethylbenzenes has taken place in five factories for a total of fifty specimens: the values obtained are distributed in a range from 0.0 to 159 mg/l, with higher values in the urine specimens collected at the end of workshift.

CONCLUSION

The HPLC determination proposed is precise, sensitive, accurate and simple to perform. Also it is quick, taking less than 1 h to pretreat up twenty samples and *ca.* 10 min to obtain individual chromatographic results. The determination of 3,5-DMHA is a quantitative evaluation of occupational exposure only to mesitylene, although it can offer good information about the total exposure to the other isomers, since those are normally present with mesitylene in the commercial products.

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[a] FIT is an algorithm of the Waters software that gives a numerical value indicating the degree of match between spectra comparison: $\text{FIT} = (2 - \text{Res}(\sum |S_i - R_i|)/2) \times 1000$, where S_i is the response in the sample spectrum at wavelength i, R_i is the response in the reference spectrum at wavelength i and Res is the spectral resolution of the spectrum.

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