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Short communication

Gas chromatographic method for the determination of toluidines in spiked urine samples

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Abstract

A capillary gas-chromatographic method was developed for the analysis of a mixture of toluidines in urine. The method is based on the extraction of toluidines with toluene and derivatisation with heptafluorobutyric anhydride to form a product for electron capture detection. The procedure gave a linear response at concentrations of 0.02–0.20 μg/ml with sufficient reproducibility. The method is simple, requires little sample pretreatment and is being considered for biomonitoring workers exposed to toluidines.

Keywords: Toluidines; Heptafluorobutyric anhydride

1. Introduction

Toluidines are important industrial chemicals. They are used in chemical and pharmaceutical manufacturing and in herbicide production. *o*-Toluidine is used especially in dye production and as an antioxidant in the rubber industry [1]. Toluidines have been detected in surface water samples as well as in various vegetables [2]. *o*-Toluidine has attracted great interest since it has been recognized by the IARC as an animal carcinogen [3]. Recent evidence points increasingly towards the human carcinogenicity of this compound [1].

Since a number of workers are involved in processes which employ toluidines, a simple and sensitive method is necessary to monitor their exposure to these compounds.

The majority of previously available methods concern the determination of toluidines in media other than biological material, such as in waste-water [4,5], air [6], food additives [7] and in synthesis control [8,9].

Recently, several methods have been proposed for the analysis of toluidines in biological material. The method described by El-Bayoumy et al. [10] enabled the quantification of *o*-toluidine at the parts-per-billion level in urine, but the sample work-up was too time and labour consuming for routine use. Another gas-chromatographic assay was established by Cheever et al. [11] for biotransformation studies on three toluidines. The method was rather semiquantitative, no recovery and precision data were specified. HPLC methods developed by Klein et al. [12] and Pah Phah Rop et al. [13] were used for determination of some local anesthetics like lidocaine and prilocaine and the metabolite of the latter, *o*-toluidine, in urine and plasma.

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Stettler et al. [14] described the method recommended for the biological monitoring of workers simultaneously exposed to *o*-toluidine and aniline. Their method quantified not only unchanged *o*-toluidine in urine but also acetylated metabolites that were converted by hydrolysis back to the parent amine. The compound was isolated by a two-step extraction and subjected to ion-interaction reversed-phase HPLC.

Since this is a single method proposed for biological monitoring and the only one of the cited methods concerns the measurement of the three toluidines together [10], we attempted to develop an alternative, a simple, time and cost effective procedure for the assay of all three toluidines in human urine.

The most commonly prepared derivatives of primary amines are formed by acylation with fluorinated acid anhydrides. One of the most popular perfluoroacyl groups is heptafluorobutyryl which enables the formation of derivatives for electron capture detection (ECD) [15]. Our method employed derivatisation with heptafluorobutyric anhydride (HFBA) followed by capillary gas chromatography using an electron capture (EC) detector. We adopted the derivatisation procedure described by Rosenberg [16] for amines formed from isocyanates in the workplace atmosphere.

2. Experimental

2.1. Instrumentation and chromatographic conditions

A Hewlett-Packard 5890 series II gas chromatograph equipped with a ^{63}Ni EC detector and HP 3396 series II integrator (Hewlett-Packard, Waldbronn, Germany) were employed. The detector was operated at 300°C with nitrogen (40 ml/min) as the make up gas. The injector temperature was 160°C and it was operated in the splitless mode. Chrompack CP-Sil 8CB fused-silica capillary column (30 m× 0.32 mm, film thickness 0.5 μm) was employed. Hydrogen was used as a carrier gas (2.7 ml/min). Temperature programme was as follows: 2 min at 100°C, heating to 150°C at 20°C/min, then heating to 230°C at 10°C/min.

2.2. Reagents and solutions

Ortho- and *para*-toluidine were purchased from E. Merck (Darmstadt, Germany), *m*-toluidine from Sigma (St. Louis MO, USA). *Ortho*- and *meta*-toluidine were distilled before use. HFBA and *p*-chloroaniline (internal standard) were from Sigma–Aldrich (Steinheim, Germany). All other chemicals were obtained from commercial sources and were of reagent-grade. Stock solutions of toluidines mixture (10 mg/ml) were prepared in ethanol and diluted to yield the appropriate concentrations: 0.10 and 0.01 mg/ml. The concentration of ethanolic solution of *p*-chloroaniline was 0.05 mg/ml.

2.3. Effect of alkalinisation on extraction recovery

To samples of physiological urine, standard solution of toluidines (final concentration: 0.02 μg/ml), 4 ml aliquots of NaOH solution at concentrations: 0.1, 1.0, 5.0 M saturated and 1 *M* phosphate buffer pH 10 were added. The analytical procedure was performed as described in Section 2.4. It was found that the recovery was the same for all the NaOH concentrations and the phosphate buffer. The latter was chosen for further assay since the same solution was recommended for removing excess of HFBA [16].

2.4. Assay procedure

To 2 ml of urine, 20 μl of *p*-chloroaniline solution, 4 ml of 1 *M* phosphate buffer, pH 10 and 2 ml of toluene were added. The mixture was shaken vigorously for 2 min. To the separated organic layer, 40 μl of HFBA was added, shaken for 1 min and left for 5 min. To remove the excess of HFBA, 4 ml of phosphate buffer, pH 10, were added and shaken for 2 min. A 0.2 μl aliquot of the toluene layer was injected onto a GC column.

2.5. Extraction recovery

Physiological human urine was spiked with the three toluidines to get the final concentration of 0.1 μg/ml and was carried through the procedure in Section 2.4. For a 100% recovery 20 μl of the

Table 1
Precision, linearity and recovery of toluidines determination in urine

Tested compound	Precision (CV%, $n=6$)			Linearity (Cr) (0.02–0.20 μg/ml)	Recovery (%) mean±SD ($n=6$)
	Concentration (μg/ml)	Within-day	Day-to-day		
o-Toluidine	0.02	11.3	10.9	0.98	101±2
	0.10	4.8	11.2		
	0.20	1.4	7.9		
m-Toluidine	0.02	8.5	9.2	0.99	105±4
	0.10	3.2	9.7		
	0.20	1.8	1.2		
p-Toluidine	0.02	8.7	11.5	0.9	102±2
	0.10	2.9	5.3		
	0.20	1.6	1.4		

toluidines mixture solution (0.01 mg/ml) was added directly to 2 ml toluene and derivatized with HFBA.

2.6. Precision

The standard solution of the toluidines was added to physiological urine to get the following concentrations: 0.02, 0.10 and 0.20 μg/ml. The internal standard concentration was 0.5 μg/ml. The precision was tested by replicate analysis ($n=6$) of the urine pool. Day-to-day and within-day coefficients of variations were calculated (Table 1).

2.7. Calibration curves

The physiological human urine was spiked with increasing concentrations of the three toluidines (0.02, 0.05, 0.10, 0.15 and 0.20 μg/ml) and a fixed concentration of *p*-chloroaniline (0.5 μg/ml). The work-up procedure was performed. For each concentration three determinations were made with a duplicate injection into the gas chromatograph. The ratio of the peak areas of toluidines to that of the internal standard was calculated and plotted against the concentration of the compounds tested.

3. Results and discussion

A typical chromatogram of urine spiked with the three toluidines is shown in Fig. 1. The retention times of the compounds tested were as follows: *o*-toluidine=4.99 min, *m*-toluidine=5.30 min, *p*-toluidine=5.43 min and *p*-chloroaniline=6.23 min. The percentage recovery for all toluidines reached 100% (Table 1). The response was linear in the

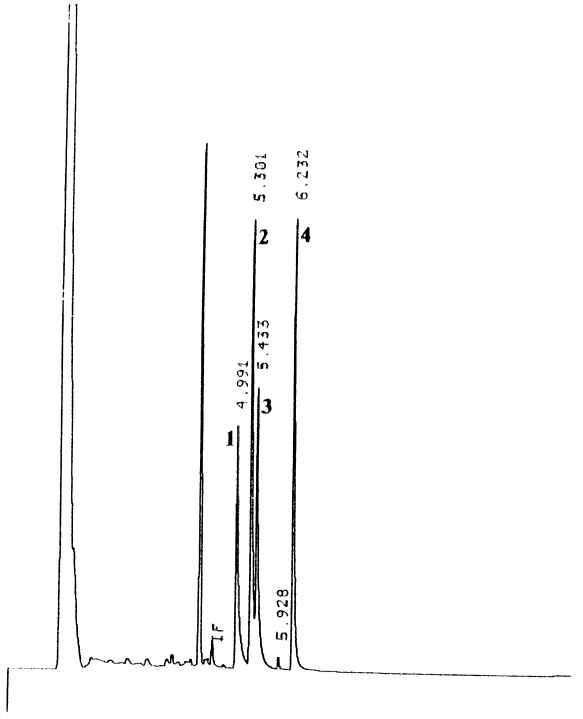

Fig. 1. Gas chromatogram of derivatized toluidines added to urine at the concentration of 0.05 μg/ml. (1) *o*-Toluidine, (2) *m*-toluidine, (3) *p*-toluidine, (4) *p*-chloroaniline (internal standard).

range studied (0.02–0.20 μg/ml) for all compounds. Linear regression equations were as follows: *o*-toluidine $y = 3.49x - 0.044$, *m*-toluidine $y = 4.36x - 0.043$, *p*-toluidine $y = 3.65x + 0.011$. The coefficients of correlation are given in Table 1. The assay was reproducible with a low day-to-day and within-day coefficients of variation (Table 1). Under the described conditions the detection limit was 0.005 μg/ml. It was the concentration of the standard giving a signal-to-noise ratio greater than two.

As mentioned above we adopted some elements of the derivatisation procedure with HFBA described by Rosenberg [16]. Our modifications relied on: (1) changing the volume of HFBA to 40 μl instead of 20 μl; (2) the application of the same phosphate buffer, pH 10 for the derivatisation procedure and for removing excess of HFBA; (3) a different GC capillary column; (4) the introduction of the internal standard, *p*-chloroaniline. The volume of HFBA used in the original method did not seem to be sufficient for our purposes, since the reproducibility was not satisfactory (CV>12%) with such a volume. Scarping et al. [17] recommended even two portions each of 100 μl of HFBA for diisocyanate determination but we found that 40 μl of HBFA as used for piperazine determination [18] was sufficient for our assay.

The general pattern of the method described can be modified to meet particular requirements. The limit of quantitation may be lowered by enrichment of the sample as described by Scarping et al. [17] and Rosenberg and Savolainen [19]. In their procedure the solvent containing acylated amines was evaporated to dryness and the residue was dissolved in a small volume of toluene. Moreover, the hydrolysis procedure can be performed prior to extraction as reported by Stettler et al. [14]. The base hydrolysis enables a quantification of not only the unmetabolised toluidines as in our method but also the *N*-acetyltoluidines that are present in urine of persons exposed to toluidines.

The main advantage of our method is that it is simple and straightforward, requiring little sample pretreatment. After verifying the method in field studies, its use for the routine biomonitoring of occupationally exposed persons could be considered. The range of concentrations in our assay seems to be sufficient for this purpose since post-shift urine levels of *o*-toluidine, reported by Ward et al. [20], were 80 or 104 μg/l as shown by Stettler et al. [14]. We did not find any data about the levels of *meta*- and *para*-isomers in the urine of occupationally exposed persons.

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