

Sensitive gas chromatographic method for determination of mercapturic acids in human urine

T. Einig, L. Dunemann, W. Dehnen*

Medizinisches Institut für Umwelthygiene, Abteilung Biochemie, Auf'm Hennekamp 50, D-40225 Düsseldorf, Germany

Received 5 December 1995; revised 3 June 1996; accepted 5 June 1996

Abstract

A method was developed for sensitive determination of the specific benzene metabolite S-phenylmercapturic acid and the corresponding toluene metabolite S-benzylmercapturic acid in human urine for non-occupational and occupational exposure. The sample preparation procedure consists of liquid extraction of urine samples followed by precolumn derivatization and a clean-up by normal-phase HPLC. Determination of analytes occurs by gas chromatography with electron-capture detection. With this highly sensitive method (detection limits 60 and 65 ng/l, respectively) urinary S-phenylmercapturic and S-benzylmercapturic acid concentrations for non-occupationally exposed persons (e.g. non-smokers) can be measured precisely in one chromatographic run. Validation of the method occurred by comparison with a HPLC method we have published recently.

Keywords: Mercapturic acids; S-Phenylmercapturic acid; S-Benzylmercapturic acid

1. Introduction

Despite the low acute toxicity [1], monitoring of benzene exposure is of great interest in occupational and environmental medicine because it causes cancer to humans [2]. As petrol contains up to 5% benzene, traffic is the main source for benzene in the environment. It is also an important product for the chemical industry [3]. Another main source of non-occupational benzene exposure is cigarette smoke [4]. Sources of toluene are petrol and the widespread use as solvent. There is no evidence yet for carcinogenicity of toluene, but exposure induces changes in the central nervous system and neurotoxic effects [5,6] like several organic solvents. For this reason,

biomonitoring is of interest to study health effects induced by environmental exposure.

Biomonitoring of exposure to volatile organic compounds (VOCs) by determination of these components in blood is well established [7,8]. Some disadvantages of this direct determination are due to short half lives of VOCs in blood and volatility of the compounds, causing possible contamination of the sample from ambient air or loss of analytes during storage. Determination of metabolites of VOCs excreted with urine avoids these disadvantages.

The important metabolic pathways of benzene and toluene are well known [9–11]. Several methods for determination of benzene metabolites as biomarkers for benzene exposure have been published [12–24]. Most of them are dealing with occupational benzene exposure. As phenol is also formed by metabolism of

*Corresponding author.

other xenobiotics and by catabolism of proteins, phenol in urine [12–14] is not a specific biomarker for benzene. Several authors described methods for determination of *trans,trans*-2,4-hexadienedioic acid (*trans,trans*-muconic acid) in urine [14–17], S-phenylcysteine bound to albumin [18] or haemoglobin [19], or S-phenylmercapturic acid in urine [20–24]. Unlike *trans,trans*-2,4-hexadienedioic acid where interference occurs on a low level by metabolism of the food preservative sorbic acid [15,25], the formation of S-phenylmercapturic acid seems to be specific for benzene. Additionally, the conversion rate of benzene to *trans,trans*-2,4-hexadienedioic acid is dependent on the level of benzene exposure [26]. Studies in occupational and environmental medicine published recently show a higher correlation between benzene exposure and S-phenylmercapturic acid excretion compared to *trans,trans*-2,4-hexadienedioic acid [27–29]. Biomonitoring of toluene exposure, via determination of S-benzylmercapturic acid, has been published recently for glue sniffers and exposed workers [30].

The determination of S-phenylmercapturic acid and S-benzylmercapturic acid in urine samples from environmentally exposed persons in one chromatographic run has not been described yet. The published methods are not sensitive enough (detection limit is 1 µg/l [27]) to precisely determine these biomarkers in urine of non-occupationally exposed persons (non-smokers). In this paper, we will describe a method to determine both S-phenylmercapturic acid and S-benzylmercapturic acid, in one chromatographic run. The determination of the toluene metabolite S-benzylmercapturic acid was not possible with the HPLC-method we have published before [23]. The detection limit of our method is sufficiently low (60 and 65 ng/l, respectively) to determine reproducibly the S-phenylmercapturic acid concentrations of 1.0–2.5 µg/l in the urine of environmentally exposed non-smokers.

2. Experimental

2.1. Chemicals

N-Acetyl-S-phenylcysteine (S-phenylmercapturic acid) and N-acetyl-S-benzylcysteine (S-benzylmer-

capturic acid) were supplied by Janssen Chimica (Neuss, Germany). 2-Acetamidoacrylic acid, N-acetylcysteine, α-bromo-4-methyltoluene, diisopropylamine, N,N-dimethylaminopyridine, 4-fluorothiophenol, 4-methylthiophenol and pentafluorobenzyl bromide were purchased from Fluka (Neu-Ulm, Germany); all other chemicals were from Merck (Darmstadt, Germany). All chemicals were of the highest purity available. Creatinine determination was carried out according to the Jaffé method with a test-kit (Boehringer, Mannheim, Germany).

2.2. Chromatographic equipment

Two 114M pumps with an AI 406 interface connected to a personal computer with System Gold Software (Version 7.00) (all Beckman Instruments, Munich, Germany), a 717 autosampler (Waters, Eschborn, Germany), a Latek HMV-P electric switching valve (purchased by Millipore, Eschborn, Germany) and a 168 diode-array detector (Beckman Instruments) were used. HPLC columns were purchased from Hewlett-Packard (Waldbronn, Germany).

Gas chromatography was carried out with a GC 8000/Mega II, equipped with programmed temperature vaporizer (PTV) controlled by a MFA 815, ECD 800 electron-capture detector with EL 980 controlling unit and a CTC A200SE autosampler (all purchased from Sato Electronics, Mönchengladbach, Germany). The system was controlled by a personal computer with Chromcard software (Version 17.0).

2.3. Synthesis of the internal standards

4-Fluorophenylmercapturic acid and 4-methylphenylmercapturic acid were synthesised according to the method published by Behringer and Fackler [31].

4-Methylbenzylmercapturic acid was prepared by the following procedure: a 500-mg quantity of N-acetylcysteine was dissolved in 25 ml acetonitrile. A 50-µl volume of α-bromo-4-methyltoluene and 300 µl of diisopropylamine were added. The reaction was catalysed by 10 mg N,N-dimethylaminopyridine in 1 ml acetonitrile. The mixture was refluxed for 60 min, followed by evaporation of the solvent. The yellowish residue was dispersed in 20 ml water and

acidified with 1 ml concentrated hydrochloric acid. The reaction product was extracted with dichloromethane. The crude product was recrystallized from 1 ml methanol.

Purity of the different reaction products was confirmed by reversed-phase HPLC. Identification was carried out by GC–MS, after esterification with pentafluorobenzyl bromide.

2.4. Sample preparation

4-Fluorophenylmercapturic acid (2 nmol, 512 ng), 4-methylphenylmercapturic acid (2 nmol, 504 ng) and 4-fluorobenzylmercapturic acid (2 nmol, 540 ng) dissolved in 50 μ l acetonitrile were added to 5 ml of urine as internal standards. The mixture was acidified with 0.2 ml concentrated hydrochloric acid and extracted twice using 5 ml ethyl acetate. The solvent was evaporated carefully under a stream of nitrogen.

The dry residue was dissolved in 500 μ l acetonitrile, followed by addition of 100 μ l diisopropylamine and 100 μ l pentafluorobenzyl bromide. Derivatization occurs by heating at 40°C for 35 min, the reaction was stopped by adding 500 μ l water. The derivatized analytes were extracted twice with 500 μ l hexane.

Gas chromatographic separation of these samples shows several peak interferences. Therefore, further purification of the samples was necessary. Purification of the residue is performed by the following clean-up with normal-phase HPLC: a 100 \times 2.1 mm I.D. HPLC column and a 20 \times 2.1 mm I.D. precolumn with Hypersil (particle size 5 μ m) were used; a 100- μ l volume of the extract was injected and purified by a *n*-hexane–2-propanol gradient from 100% *n*-hexane to 5% 2-propanol in 10 min. After 15 min the column was reequilibrated for 5 min. Flow-rate was 1.0 ml/min. A fraction ranging from 6.9 to 8.6 min was cut by an electrically driven 6-way valve and collected in a GC vial.

2.5. Gas chromatographic conditions

GC separation was carried out on a Rtx-50 column (30 m, 0.25 mm I.D., film thickness 0.10 μ m; Restek, Bad Soden, Germany). A 10- μ l volume of the sample was injected and the solvent was evaporated in the PTV injector for 15 s at 55°C. After

closure of the purge valve the injector was heated ballistically to 340°C.

Oven temperature was 70°C at injection. After 3 min, it was raised to 240°C at 20°C/min followed by 35 min isothermal separation at 240°C. At the end of this program, the column was cleaned at 300°C for 10 min. Helium was used as carrier gas at 50 kPa. The ECD was set at 370°C with a pulse height of 50 V and pulse duration of 0.1 ms. Nitrogen was used as make-up gas at 150 kPa.

3. Results and discussion

We established a GC method for determination of the benzene metabolite *S*-phenylmercapturic acid and the corresponding toluene metabolite *S*-benzylmercapturic acid in urine of non-occupationally exposed persons (smokers and non-smokers). *S*-Phenylmercapturic acid and *S*-benzylmercapturic acid were extracted from urine by liquid extraction. After pre-column derivatization with pentafluorobenzyl bromide and a clean-up procedure, the samples were determined with GC–ECD. Fig. 1 describes the complete analytical procedure.

The solvent extraction with ethyl acetate was validated by reversed-phase HPLC. Recovery of the extraction procedure determined for *S*-phenylmercapturic acid was 98.7% with a R.S.D. (relative standard deviation) of 1.2% within-day and 1.5% between-day (concentration was 2.5 mmol/l, experi-

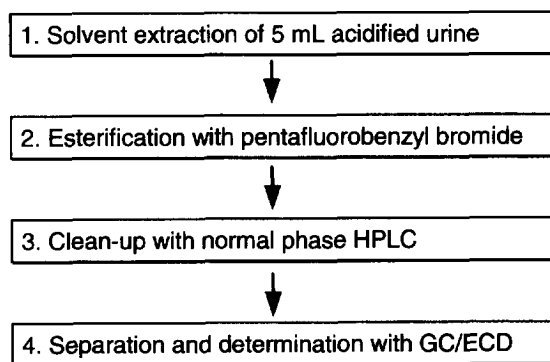


Fig. 1. Setup for sample preparation and determination of *S*-phenylmercapturic acid and *S*-benzylmercapturic acid with GC–ECD.

ments were carried out in five-fold on five different days).

Several experimental conditions were published for esterification with pentafluorobenzyl bromide [32–35]. Experimental design was used to adjust the derivatization procedure for the present problem. The reaction is shown in Fig. 2. The derivatization procedure was optimized by using a Taguchi experimental design for parameter estimation [36]. As a result, diisopropylamine was used for neutralization of the released hydrobromic acid. Further optimization of temperature and time was carried out by using a central composite design (evaluation of the reaction surface by adapting a second order function to the measuring points). As pentafluorobenzyl bromide reacts not only with carboxylic acids but also with phenols, amines, thiols etc., the described clean-up procedure was necessary.

The temperature program for gas chromatographic separation was optimized according to the linear elution strength theory (LES-theory). Calculations were carried out with a spreadsheet program using the described algorithm [37]. The temperature program provides optimum separation of the samples.

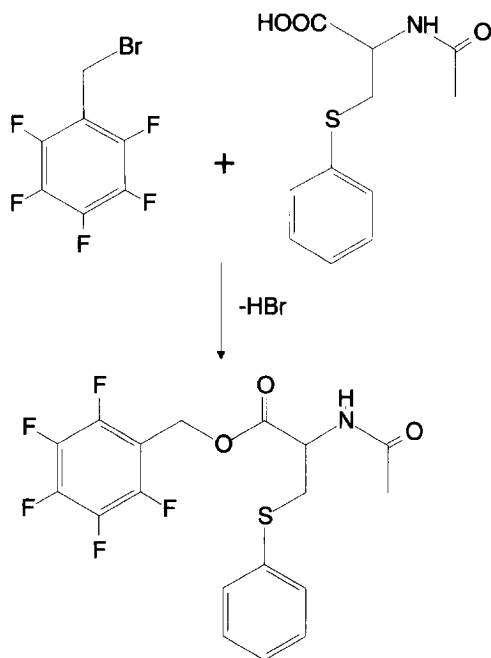


Fig. 2. Esterification of S-phenylmercapturic acid with pentafluorobenzyl bromide.

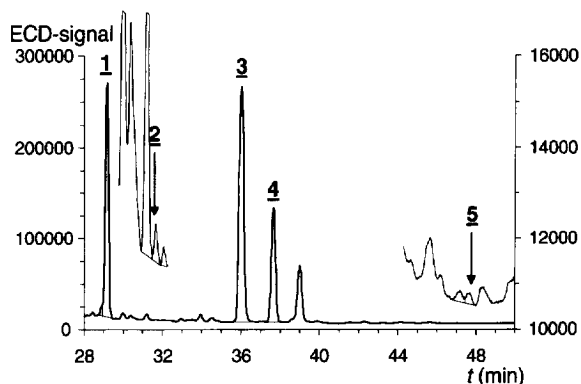


Fig. 3. Chromatogram of a sample from an environmentally exposed person (non-smoker). Peaks: 1, 3 and 4 (left axis) are signals from the internal standards 4-fluorophenylmercapturic acid, 4-methylphenylmercapturic acid and 4-fluorobenzylmercapturic acid. The amplification shows peaks 2 and 5 (right axis) according to S-phenylmercapturic acid and S-benzylmercapturic acid, respectively.

Fig. 3 shows a chromatogram of a urine sample from an environmentally exposed person.

The detection limit of the method [based on a signal-to-noise (S/N) ratio of 3, $n=5$] is 60 ng/l for S-phenylmercapturic acid and 65 ng/l for S-benzylmercapturic acid, which is sufficiently low to analyze urine samples of non-occupationally exposed persons (Fig. 3). It was confirmed that no interferences by coelution with the internal standard occur under the described conditions. Evaluation of the

Table 1

Data for cross-validation of the presented method with a HPLC-method we published elsewhere [23]

Sample	Concentration ($\mu\text{g/l}$)	
	HPLC method	GC method
1	2.4	2.7
2	3.5	3.0
3	7.7	7.2
4	2.1	1.7
5	2.6	2.5
6	13.9	13.5
DL	0.4	0.06
R.S.D.	5/11	3/5

Intercept: -0.12 ; slope: 0.97 ; r : 0.998 .

The concentrations of S-phenylmercapturic acid determined with both methods show a very good correlation. Limit of detection (DL) and relative standard deviation (R.S.D.) of both methods are compared in line 7 and 8.

Table 2
Data from twenty environmentally exposed persons: twelve non-smokers, eight smokers

Parameter	Concentration ($\mu\text{g/l}$)	
	S-Phenylmercapturic acid	S-Benzylmercapturic acid
<i>Non-smokers</i>		
Mean	1.7	1.1
Range	0.9–2.4	0.6–1.5
S.D.	0.8	0.4
<i>n</i>	12	12
<i>Smokers</i>		
Mean	6.1	3.4
Range	2.2–11.3	1.7–6.2
S.D.	5.4	2.4
<i>n</i>	8	8

analyte concentrations with the different internal standards leads to consistent results. For further improvement, each of these standards can be selected as internal standard. Cross-validation with a HPLC method (see below), peak shape and band-width of the peaks indicate that no coelution of the analytes with unknown compounds occurs.

The precision of our method was confirmed by repeated analysis of a urine sample. The reproducibility of the method was 3% R.S.D. within-day and 5% R.S.D. between-day for both analytes. The values were measured with a sample containing 7.1 $\mu\text{g/l}$ S-phenylmercapturic acid and 4.8 $\mu\text{g/l}$ S-benzylmercapturic acid, experiments were carried

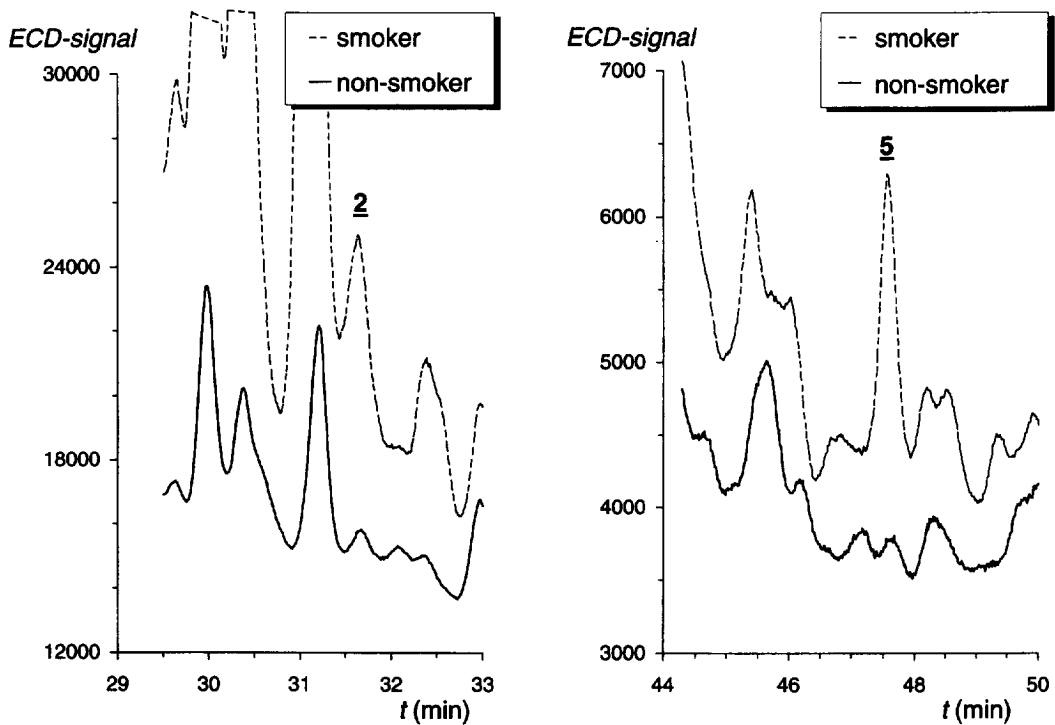


Fig. 4. Comparison of samples from one environmentally exposed person (thick line) and one smoker (dashed line). Exposure levels of non-smokers are significantly lower than those from smokers.

out in five-fold on four different days. Cross-validation with a previously published HPLC method [23], shows a very good correlation between the results for determination of S-phenylmercapturic acid (Table 1).

A number of urine samples from smokers and non-smokers were analysed confirming the values obtained previously (Table 2). Fig. 4 compares chromatograms of samples from an environmentally exposed person with one from a smoker. It can be seen clearly that the exposure level of the smoker is higher. Means of S-phenylmercapturic acid concentrations were 1.7 and 6.1 $\mu\text{g/l}$ (2.2 and 7.7 $\mu\text{g/g}$ creatinine) for non-smokers and smokers, respectively. For S-benzylmercapturic acid, the mean concentrations were 1.1 and 3.4 $\mu\text{g/l}$ (1.4 and 4.3 $\mu\text{g/g}$ creatinine), respectively.

The method presented here combines a lower limit of detection with a higher reproducibility for determination of S-phenylmercapturic acid compared to other methods [20–24]. The possibility to determine S-benzylmercapturic acid also is a further advantage. S-benzylmercapturic acid has not been determined in non-occupational samples before, but was investigated as a biomarker for toluene exposure for glue sniffing and occupational exposure [30]. As metabolism of xylenes and chlorinated alkenes such as tri- and tetrachloroethylene leads also to the formation of mercapturic acids [38–40], further investigations will involve these metabolites because of the high interest of environmental medicine in these parameters.

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