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Sensitive determination of the benzene metabolite S-phenylmercapturic acid in urine by high-performance liquid chromatography with fluorescence detection

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Abstract

A method was developed for the determination of the specific benzene metabolite S-phenylmercapturic acid in urine. The analyte is determined by HPLC with fluorescence detection after solid-phase extraction of urine with C₁₈ material and hydrolysis followed by precolumn derivatization. The samples are separated by a column-switching method with a dual column system. As the method is highly sensitive (detection limit ca. 1 µg/l), urinary S-phenylmercapturic acid concentrations for non-exposed persons (e.g., non-smokers) can also be measured precisely.

1. Introduction

Benzene is a natural occurring compound of relatively low acute toxicity [1]. As petrol contains up to 5% of benzene, traffic is the main source of benzene in the environment. It is also an important product in the chemical industry. It has been shown that the main source of non-occupational benzene exposure is cigarette smoke [2]. Despite the low acute toxicity, monitoring of benzene is of great interest in occupational and environmental medicine because it causes cancer in humans [3]. The important pathways of benzene metabolism are well known (Fig. 1) [4].

Several methods for the determination of

benzene metabolites as biomarkers for benzene exposure have been published [5–16]. As phenol is also formed by the metabolism of other xenobiotics and by catabolism of proteins, the determination of phenol in urine [5–7] is not a specific biomarker for benzene. Several workers have described methods for the determination of *trans,trans*-2,4-hexadienedioic acid in urine [7–10], S-phenylcysteine bound to albumin [11] or haemoglobin [12] and S-phenylmercapturic acid (S-PMA) in urine [13–16] as biomarkers for benzene exposure. As most methods are not sensitive enough to determine these biomarkers in urine of low-exposed persons (non-smokers), we describe here an improved method to determine S-PMA by HPLC with fluorescence detection with a sufficiently low detection limit. The method is based on a procedure published earlier [13].

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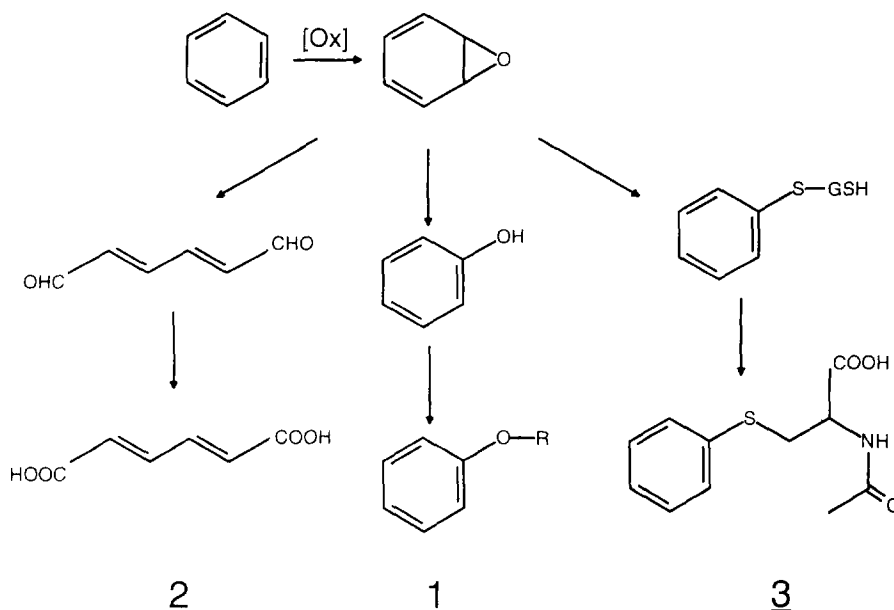


Fig. 1. Important pathways of benzene metabolism (for details see [2]). Most of the benzene uptake is metabolized to phenol (centre), which is excreted as conjugate **1** (R = sulfate or glucuronic acid). Formation of the specific metabolites *trans,trans*-2,4-hexadienedioic acid (**2**) (left) and S-phenylmercapturic acid (**3**) (right) totals only ca. 2%.

2. Experimental

2.1. Chemicals

N-Acetyl-S-phenylcysteine (S-PMA) was supplied by Janssen Chimica (Neuss, Germany), methanol for HPLC by Baker (Bad Soden/Ts., Germany) and monobromobimane by Calbiochem (Gross Gerau, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) and were 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 Model 114M pumps with an AI 406 interface connected to a personal computer with System Gold Software (version 7.00) (all from Beckman Instruments, Munich, Germany), an M6000 pump (Millipore, Eschborn, Germany), a Model 717 autosampler (Millipore, Eschborn, Germany), Latek HMV-P electric switching

valve (purchased from Millipore) and an HP 1046A fluorescence detector (Hewlett-Packard, Waldbronn, Germany) were used. Columns were purchased from Macherey–Nagel (Düren, Germany). The following solid-phase extraction (SPE) cartridges were used: BondElut C₁₈ (Analytichem International; obtained from ICT, Frankfurt, Germany), Bakerbond C₁₈ (Baker) and Sep-Pak C₁₈ (Millipore, Eschborn, Germany).

2.3. Synthesis of the internal standard

S-Acetyl-4-methylthiophenol was synthesized as the internal standard by reaction of 0.5 g of 4-methylthiophenol in 20 ml of acetonitrile with 1 ml of acetic anhydride; 1 ml of N,N-dimethyl-4-aminopyridine in acetonitrile (0.5 mol/l) was added as catalyst. The mixture was refluxed for 60 min and poured in 500 ml of ice–water after cooling to room temperature. The reaction product was extracted three times with 100 ml of dichloromethane. The solvent was evaporated to

dryness and the purity of the product was confirmed by HPLC.

2.4. Solid-phase extraction

For extraction of S-PMA from urine, C_{18} cartridges with 500 mg of sorbent were used. The cartridges were washed with 5 ml of methanol and conditioned with 10 ml of 1% acetic acid. S-PMA was extracted from 2 ml of urine adjusted to pH 1.0 with 25% hydrochloric acid after addition of 50 μ l of internal standard in methanol ($15 \cdot 10^{-6}$ mol/l). The cartridges were then washed with 2 ml of 1% acetic acid. S-PMA was eluted with 2 ml of methanol–ammonium acetate buffer (0.1 mol/l, pH 7.0) (80:20).

2.5. Derivatization

A 100- μ l volume of NaOH (10 mol/l) was added to the extract. In a 5-ml flask with an 8-cm long narrow neck (Dünges flask [17]), the solution was concentrated to ca. 100 μ l at 78°C under nitrogen and hydrolysed for 30 min at 95°C. A 2-ml volume of glycine–NaOH buffer (0.4 mol/l, pH 9.0) was added followed by 350 μ l of phosphoric acid [diluted 1:1 (v/v) with water]. The thiophenol (TP) released from S-PMA by the hydrolysis was derivatized by adding 500 μ l of monobromobimane (MB) [18] ($1 \cdot 10^{-3}$ mol/l) in acetonitrile–water (1:9, v/v). The reaction is shown in Fig. 2.

2.6. Chromatographic conditions

A 50- μ l volume of the above solution was separated under the following conditions. A dual column system was used (see Fig. 3): pre-separation of the sample was carried out on a 50 mm \times 2 mm I.D. column with an 11 mm \times 2 mm I.D. built-in precolumn filled with Nucleosil 120 C_{18} (5 μ m) (column 1). Two minutes before elution of the analyte, the eluent from column 1 was directed to a 125 mm \times 2 mm I.D. column filled with Nucleosil 120 C_{18} (3 μ m) for further separation (column 2). Separation was carried out by gradient elution: column 1 was equilibrated with 10% methanol in 0.1% acetic acid.

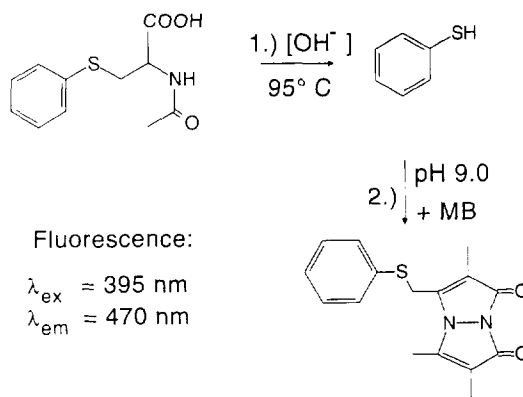


Fig. 2. Precolumn derivatization occurs in two steps: (1) the extract from the Sep-Pak C_{18} cartridge is concentrated, followed by alkaline hydrolysis; (2) the pH is adjusted to 9.0 and the thiophenol released is derivatized with monobromobimane.

After 2 min, the modifier concentration was increased to 50% in 3 min; the flow-rate was 0.2 ml/min. Thiophenol–MB was eluted from column 1 after 24 min under these conditions, and therefore column switching was applied after 22 min. At this time the methanol concentration was increased to 60% and the flow-rate was decreased to 0.1 ml/min to avoid excessively high pressure (both in 1 min). The retention times were 48 and 65 min for thiophenol–MB

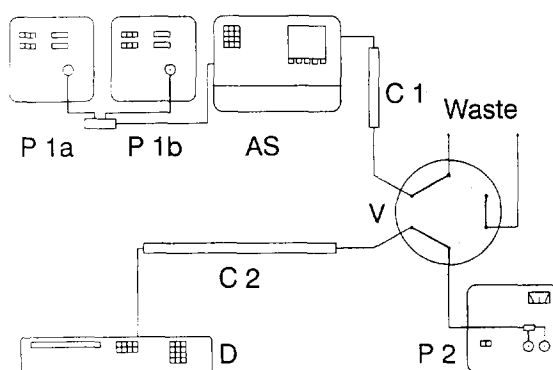


Fig. 3. Diagram for the column-switching procedure. P 1a, P 1b and P 2 = pumps; AS = autosampler; C 1 and C 2 = columns; V = switching valve; D = fluorescence detector. On-column focusing and pre-separation are carried out on column 1 with the valve in position 1 (thick lines). Final separation on column 2 occurs after switching to position 2 (dotted lines).

and 4-methylthiophenol–MB, respectively. After elution of the internal standard, the valve was turned back and column 1 was re-equilibrated for 10 min at a flow-rate of 0.2 ml/min, and meanwhile column 2 was flushed with the M6000 pump isocratically with 50% methanol in 0.1% acetic acid (flow-rate 0.1 ml/min). The excitation and emission wavelengths of the detector were set at 395 and 470 nm, respectively.

3. Results and discussion

An HPLC method was established for the determination of the benzene metabolite S-PMA in urine. The formation of S-PMA seems to be specific for benzene, unlike *trans,trans*-2,4-hexadienedioic acid, where interference may occur from the preservative sorbic acid in samples from “non-exposed” persons, as the metabolism of sorbic acid also leads to low levels of *trans,trans*-2,4-hexadienedioic acid [8,19].

For SPE, three different C_{18} materials were tested. Recoveries of the extraction from urine samples spiked with 250 $\mu\text{g/l}$ of S-PMA using the different C_{18} SPE cartridges were 45% (BondElut), 82% (Bakerbond) and 103% (Sep-Pak). As Sep-Pak C_{18} cartridges showed the highest recovery (103%), they were subsequently used for extraction of the urine samples. The internal standard can also be extracted by the procedure described above. The detection limit of the method (signal-to-noise ratio = 3) is below 1 $\mu\text{g/l}$, which is sufficiently low for the analysis of samples from “non-exposed” persons (for chromatograms, see Fig. 4). The linearity of the calibration graphs indicates a quantifiable range between 1 and 200 $\mu\text{g/l}$. It was confirmed that no interferences with the internal standard occur on co-elution under the described conditions. The peak shape and bandwidth of the TP–MB peak indicate that no co-elution with the analyte occurs.

The precision of the method was confirmed by repeated analyses of a urine sample with internal standard and standard addition. The reproducibility (R.S.D.) of the method was <5% within-day and <10% day-to-day (measured with a

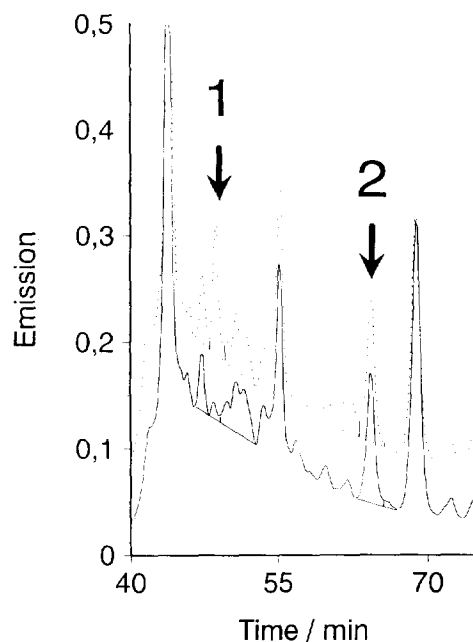


Fig. 4. Chromatogram of a urine sample from a non-exposed person. Peaks: 1 = S-PMA; 2 = internal standard. Straight line = chromatogram of urine without addition of S-PMA; dotted line = chromatogram of the same sample after addition of 40 ng of S-PMA to 2 ml of sample.

sample containing ca. 15 $\mu\text{g/l}$). S-PMA in urine samples from non-smokers was determined less accurately (within day reproducibility 20% for a sample containing 2.5 $\mu\text{g/l}$). A number of urine samples from smokers and non-smokers were analysed, confirming the values obtained previously [13]. The mean results were 2.0 and 7.6 $\mu\text{g/g}$ creatinine for samples from non-smokers and smokers, respectively.

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