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Short Communication

Determination of phenylmercapturic acid in urine of benzene-exposed BDF-1 mice

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ABSTRACT

This paper describes a procedure for the identification of phenylmercapturic acid in urine of benzene-exposed mice. Collected urine of benzene exposed mice was adjusted to pH 7 and applied to an anion exchanger. After extraction with diethyl ether and evaporation to dryness, the sample was dissolved in aqueous phosphoric acid and injected into the HPLC. HPLC conditions included an ODS column and an eluent consisting of tetrabutylammoniumhydrogensulfate–methanol (75:25, v/v), the absorbance wavelength was 255 nm. The detection limit of phenylmercapturic acid was 3 mg/l in mouse urine.

INTRODUCTION

Benzene, an ubiquitous substance, is produced by natural processes and used in huge amounts in industry. Intensive industrial use of benzene as an excellent solvent started late in the 19th century. High benzene concentrations in the workshops soon produced the first adverse effects in humans, hematological disorder and leukemia. Benzene intake also results from smoking of tobacco (20–90 μg benzene/cigarette) [1].

This prompted many studies on the effects of benzene but until now the mechanisms of its toxicity are not completely understood. It is generally agreed that benzene has to be metabolized to an active metabolite to exert its toxic effects.

Various analytical techniques have been reported for the separation, quantification and identification of benzene metabolites. But in most publications only a single metabolite was detected [2], or radiolabelled benzene was used [3], or the time needed for analysis was nearly 2 h [4].

In refs. [5,6] we reported a simple method for determination of the benzene metabolites catechol (C), hydroquinone (H), *trans,trans*-muconic acid (Ma) and phenol (P) in urine of mice using HPLC. In the present paper we describe an analytical HPLC procedure without derivatization to determine phenylmercapturic acid (PhA), an other important benzene metabolite in urine of benzene exposed mice.

EXPERIMENTAL

Chemicals

Methanol was supplied by LAB-Scan (Biotronik, Maintal, Germany); *trans,trans*-muconic

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acid by Fluka (Neu-Ulm, Germany); phenylmercpaturic acid was synthesized according to Behringer and Fackler [7]. All other chemicals were from Merck (Darmstadt, Germany) and had the highest purity available.

Phosphate solution (pH 7) was prepared from an aqueous 5 m*M* K_3PO_4 solution adjusted with concentrated phosphoric acid to pH 7.

Tetrabutylammoniumhydrogensulfate solution (TBAHS, analytical grade, Serva, Heidelberg, Germany) was prepared from an aqueous 5 m*M* K_3PO_4 solution (puriss. Merck) and 1.7 g/l TBAHS (5 m*M*/l), adjusted to pH 4.1 with concentrated phosphoric acid.

Solid-phase extraction columns

Bond Elut extraction cartridges with NH_2 anion exchanger (500 mg) from Varian (Harbor City, CA, USA) were used.

Urine source

Groups of 16 female BDF-1 mice, weighing 20–25 g, 3–5 month of age, were housed in metabolism cages designed for urine collection. Animal rooms were maintained at 24°C, with a relative humidity of 50%, and a 12-h light-dark cycle. Water was provided *ad libitum*. During the inhalation period of 6 h no food was present in the cages. The mice were exposed to 858 μl/l (v/v) benzene in air for 6 h a day, 5 days a week. Urine of all mice was collected over a 24-h period in polyethylene vials containing 30 mg of ascorbic acid to avoid oxidation of urine. Urine was stored at −20°C until analysis.

Chromatographic conditions

Analysis was carried out by HPLC (CM 4000, LDC Analytical, Gelnhausen, Germany) with a column (40 mm × 4.6 mm I.D., 3 μm particle size) (Grom, Herrenberg, Germany) filled with

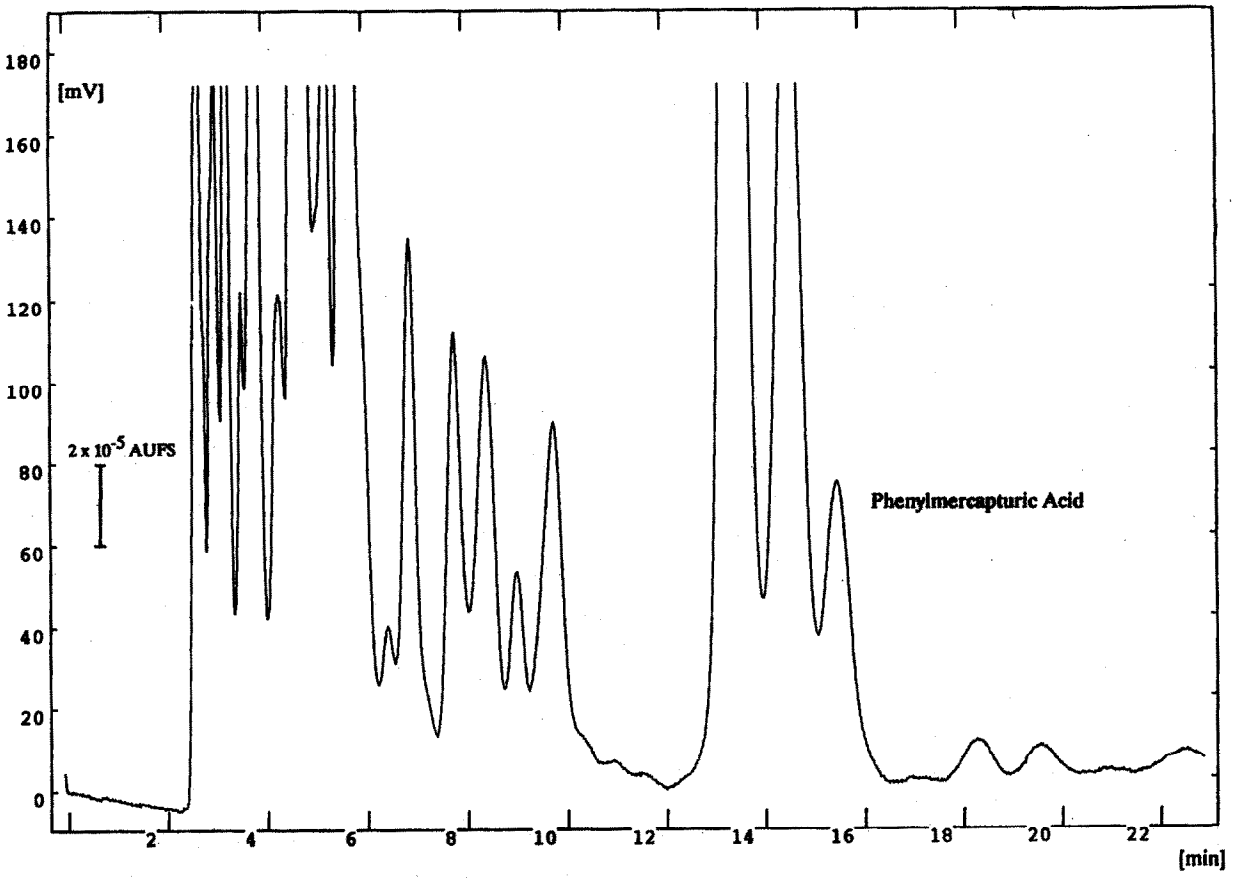

Fig. 1. Chromatogram of mouse urine (858 μl/l, v/v benzene exposure); after clean-up with NH_2 anion exchanger; absorption: 255 nm.

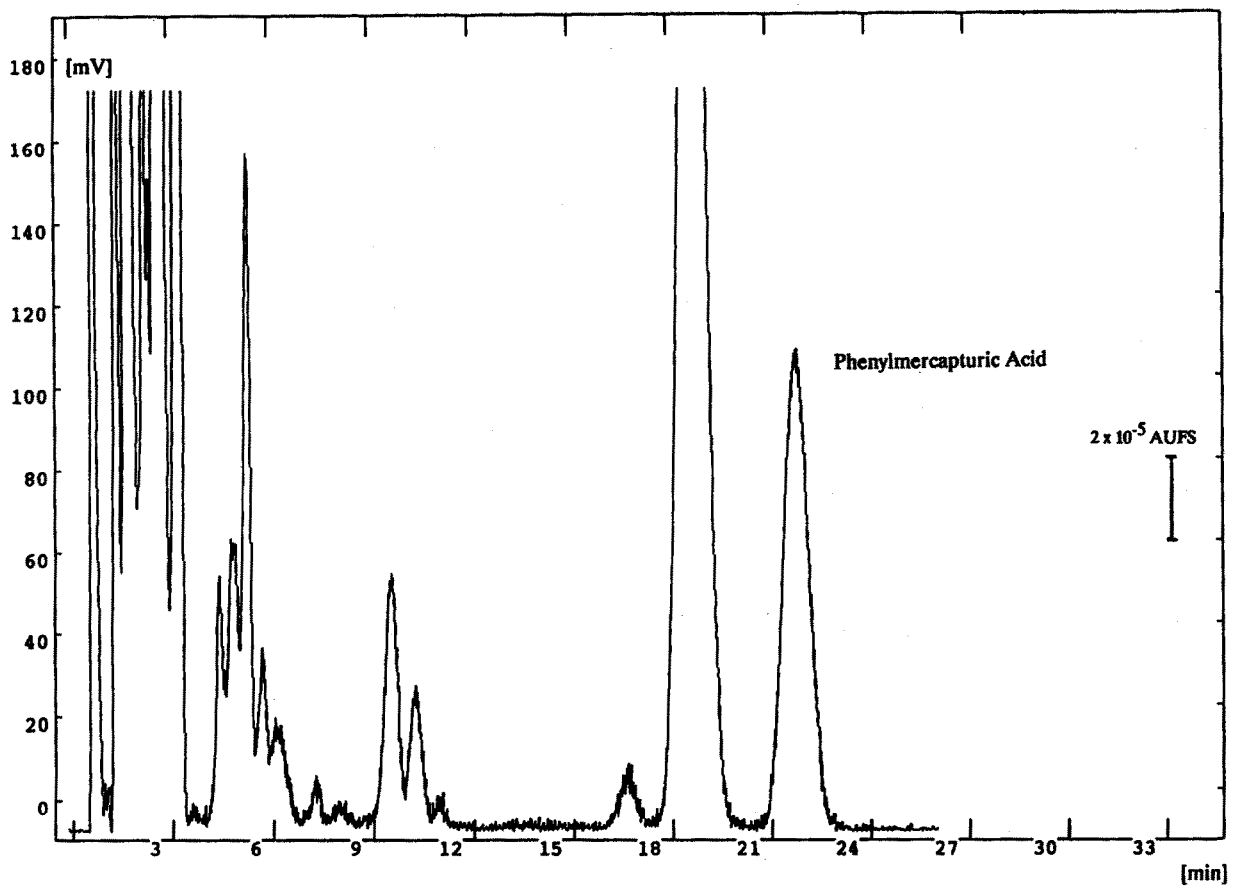

Fig. 2. Chromatogram of mouse urine (858 μl/l, v/v benzene exposure); after clean-up with TBAHS ion pair reagent; absorption: 255 nm.

Nucleosil ODS (Machery and Nagel, Düren, Germany) at a constant temperature of 30°C. The detector (SM 4000, LDC Analytical) was set at 255 nm and 0.001 AUFS. The eluent was TBAHS (pH 4.1)–methanol (75:25, v/v). With a flow-rate of 0.7 ml/min the retention time of phenylmercapturic acid was 22 min. The duration of an analytical cycle was 55 min.

Determination of S-phenylmercapturic acid (PMA)

A 125-μl aliquot of mouse urine was diluted with water to 4 ml and adjusted to pH 7 with concentrated H_3PO_4. The diluted urine was applied to an NH_2 anion exchanger. The anion exchanger was a Bond Elute extraction cartridge filled with 500 mg NH_2 (aminopropyl) sorbent previously conditioned with 3 ml of methanol and 3 ml of phosphate solution pH 7.

After application of the urinary sample the cartridge was eluted with 3 ml of phosphate solution pH 7. This fraction was acidified with concentrated hydrochloric acid to a pH less than 3 and vortex-mixed 3 times with 5 ml diethyl ether. The ether layers were removed, combined and evaporated to dryness at 30°C and $4 \cdot 10^4$ Pa. The residue was dissolved in 1 ml of 1% aqueous phosphoric acid and a volume of 20 μl was injected onto the HPLC. The absorption wavelength was set at 255 nm.

PMA peak purity was tested with spiking and spectral analysis (FastScan-detector, Sykam, Gilching, Germany).

RESULTS AND DISCUSSION

A method was established to determine phenylmercapturic acid, one of the specific metabo-

lites of benzene. Degradation of benzene takes place by three different metabolic pathways. The first and major pathway ends with phenolic compounds, which are mainly conjugated as sulfates or glucuronides in urine. With special hydrolic enzymes in urine the conjugates can be broken up [4]. The unconjugated metabolites can be separated from urinary compounds by a SAX anion exchanger followed by extraction with diethyl ether. After these procedures the benzene metabolites can be detected with HPLC [6].

The main metabolite of the second pathway of benzene is muconic acid. Muconic acid can be separated and detected in the same way as the phenolic compounds [6].

PMA, the metabolite of the third, glutathione pathway, can not be analyzed by this method, because PMA is eluted in two fractions after application onto the SAX anion exchanger. With an aminopropyl (NH_2) anion exchanger the whole amount of PMA is eluted in the first fraction. Urine of benzene exposed mice gives an unknown peak in the HPLC chromatogram which has a similar retention time on an ODS column (250 mm × 4.6 mm, 5 μm) as PMA. This leads to a double peak as shown in Fig. 1. Using a short chromatographic column (40 mm × 4.6 mm, 3 μm), TBAHS as an ion-pair reagent, and with a higher pH of the HPLC solution, it is possible to separate both peaks (Fig. 2). With this method the detection limit of PMA in mouse urine was 3 mg/l. PMA measurement had a maximum standard deviation of 7% and a day-to-day reproducibility of 10%. The range of detection of PMA was up to 2000 times the PMA detection limit and recovery of the clean-up procedure was over 85%.

Since many studies are concerned with benzene, its metabolites, its toxicity and the possibility to interfere with its metabolism, a simple method for the quantitative determination of several benzene metabolites, including PMA, in urine was needed.

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