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Determination of benzene metabolites in urine of mice by solid-phase extraction and high-performance liquid chromatography

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ABSTRACT

A method was developed for quantitative measurement of *trans,trans*-muconic acid, catechol, hydroquinone and phenol in urine. Hydrolysis of esterified and glucuronized phenolic compounds was effected by specific enzymes. The hydrolysed mixture was purified and separated by solid-phase extraction with an anion exchanger, followed by extraction with diethyl ether. By using a clean-up procedure the natural background from mouse urine could be reduced, so that the detection limit of the metabolites was in the range 3–60 mg/l. Optimization of the chromatographic conditions resulted in a short high-performance liquid chromatography analysis time. Phenol had the longest retention time of about 10 min. The clean-up procedure could also be used for phenylmercapturic acid, an additional benzene metabolite, but for sensitive high-performance liquid chromatographic detection of phenylmercapturic acid other conditions are necessary.

INTRODUCTION

Benzene is an important organic chemical, widely used in industry. Chronic exposure to benzene can cause leukaemia in humans, and it is a carcinogen at many organs in animals [1,2]. In the body, benzene is metabolized by the microsomal cytochrome P-450 monooxygenase system into benzene epoxide. This benzene epoxide is metabolized in three different pathways which end in excretion of *trans, trans*-muconic acid, phenylmercapturic acid and different phenols. Various techniques have been reported for the separation, quantitation and identification of benzene metabolites to elucidate the chemical pathogenesis of benzene toxicity. Most of the described procedures could detect only single metabolites. Gad El-Karim *et al.* [3] found a high-performance liquid chromatography (HPLC) method able to analyse the four benzene metabolites in the urine of mice, but the retention times were quite long. Sabourin *et al.* [4] used HPLC too, but worked with radiolabelled benzene. This is not possible in most laboratory environments. In particular, it is not suitable for analyses in human monitoring (which is important for occupational medicine). This publication describes a procedure for the identification of *trans,trans*-muconic acid, catechol, hydroquinone and phenol from the urine of mice. Using solid-phase extraction the background of the chromatograms was reduced and therefore the analysis time was considerable shorter.

MATERIALS AND METHODS

Chemicals

Methanol was supplied by LAB-SCAN and *trans,trans*-muconic acid by Fluka. All other chemicals were from Merck. All chemicals were of the highest purity available. For hydrolysation, three enzymes were used: β-glucuronidase/arylsulphatase (Merck, 4114), arylsulphatase (Sigma, S 1629) and β-glucuronidase (Boehringer, 127051).

Phosphate solutions were prepared from an aqueous 5 mmol/l K_3PO_4 solution which was treated with concentrated phosphoric acid to pH 3.4 and pH 7.

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Urine source

Groups of 16 female BDF-1 mice weighing 20–25 g were housed in metabolism cages designed for urine collection. Animal rooms were maintained at 24°C with a relative humidity of 50%. Rooms were on a 12-h light–dark cycle. Water was provided *ad libitum*. During the inhalation period no food was present in the cages. The mice were exposed to 300 ppm benzene for 6 h a day, 5 days a week. The urine (300 ppm urine) was collected for 24 h in phycoerythrin (PE)-coated vials containing 30 mg of ascorbic acid to avoid oxidation of urine. Urine was stored at −20°C until analysis.

Determination of urinary benzene metabolites

Hydrolysation. A 0.125-ml aliquot of mice urine was diluted with water to 4 ml and adjusted to pH 4.5 with ascorbic acid. The diluted urine was treated with three enzymes:

Sample 1: 0.0125 ml of β-glucuronidase/arylsulphatase (Merck, 4114).

Sample 2: 0.0125 ml of arylsulphatase (Sigma, S 1629).

Sample 3: 0.0125 ml of β-glucuronidase, prediluted with water (1:6, v/v) (Boehringer, 127051).

Hydrolysation was performed at 37°C for 48 h. This hydrolysation procedure is not necessary for unconjugated metabolites.

Sample 4: urine was thawed at room temperature, diluted, acidified and applied to the anion exchanger.

Separation by anion exchanger

After hydrolysation of urine, 4 ml of the samples were applied to an anion exchanger. The anion exchanger was a Bond Elut extraction cartridge filled with 500 mg of SAX sorbent (Analytichem International) previously conditioned with 3 ml of methanol and 3 ml of distilled water. After application of the urinary samples, the cartridge was washed with 3 ml phosphate solution (pH 7) to elute the fraction of phenols (I). The next eluent was 3 ml of 0.5 *M* aqueous sulphuric acid for the *trans,trans*-muconic acid fraction (II).

Ether extraction

Ether extraction was performed at a pH less than 3 after acidification of the phenol fraction (I) with concentrated hydrochloric acid. Each fraction was treated separately. The fractions were vortexed

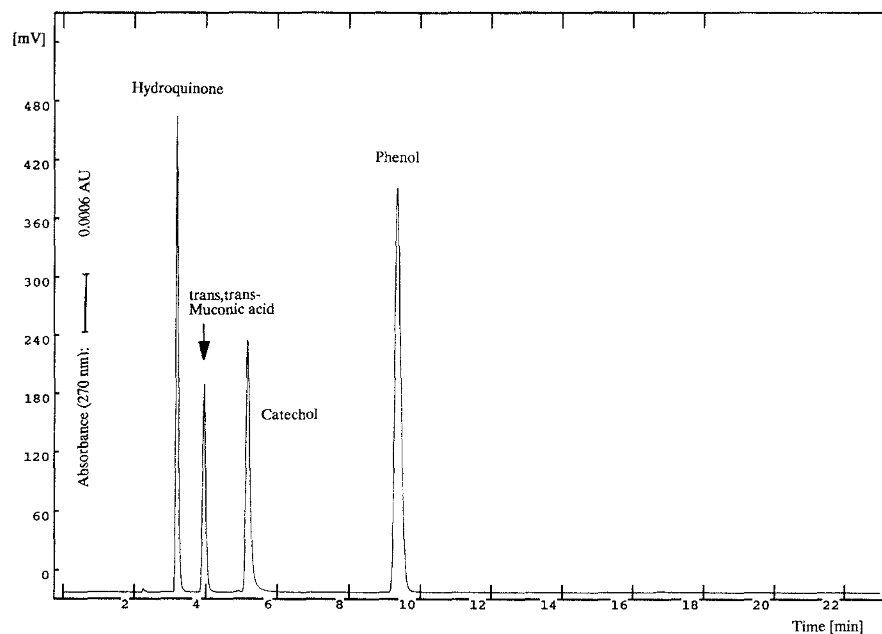

Fig. 1. Chromatogram of a standard solution of catechol (90 mg/l), hydroquinone (186 mg/l), phenol (300 mg/l) and muconic acid (7.5 mg/l).

three times with 5 ml of diethyl ether. The ether layers were removed, combined and evaporated to dryness at 30°C and 400 hPa and the residue dissolved in 1 ml of 1% aqueous phosphoric acid. A 20-μl volume was injected for HPLC analysis.

Chromatographic conditions

Analysis was carried out by HPLC with an ODS column (250 × 4.6 mm I.D.) filled with Nucleosil ODS, 5 μm particle size (Bischoff). The detector (SM 4000, LDC Analytical) was set at 270 nm and

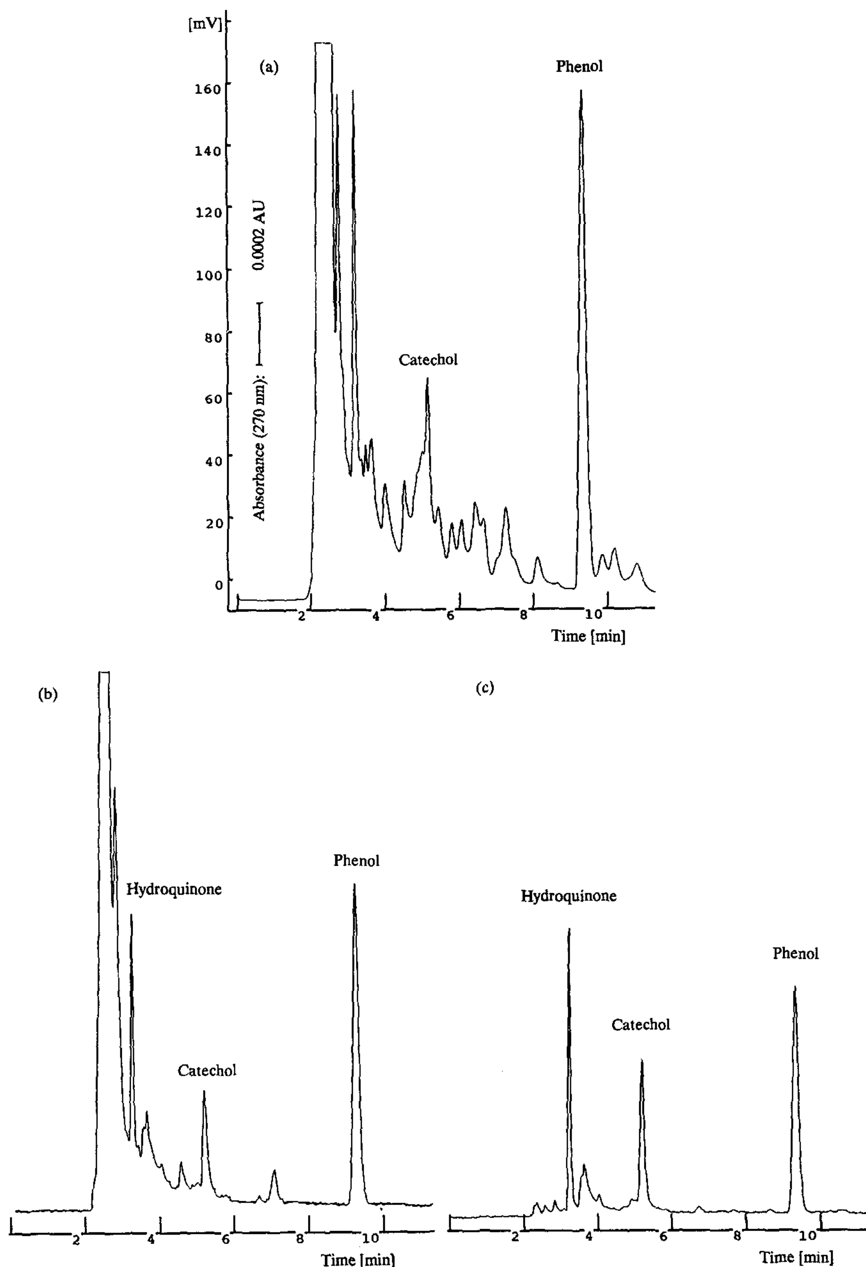

Fig. 2. Chromatograms of 300 ppm urine (a) after hydrolization, (b) after solid-phase extraction (fraction I), and (c) after evaporation (fraction I).

0.01 AUFS. The eluent was a solution of pH 3.4 phosphate solution–methanol (70:30, v/v). The detector was connected to AXXIOM integrating software.

With a flow-rate of 1 ml/min the retention time of hydroquinone was 3.2 min, of *trans,trans*-muconic acid 3.9 min, of catechol 5.2 min, and of phenol 9.3 min. The duration of an analytical cycle was 45 min.

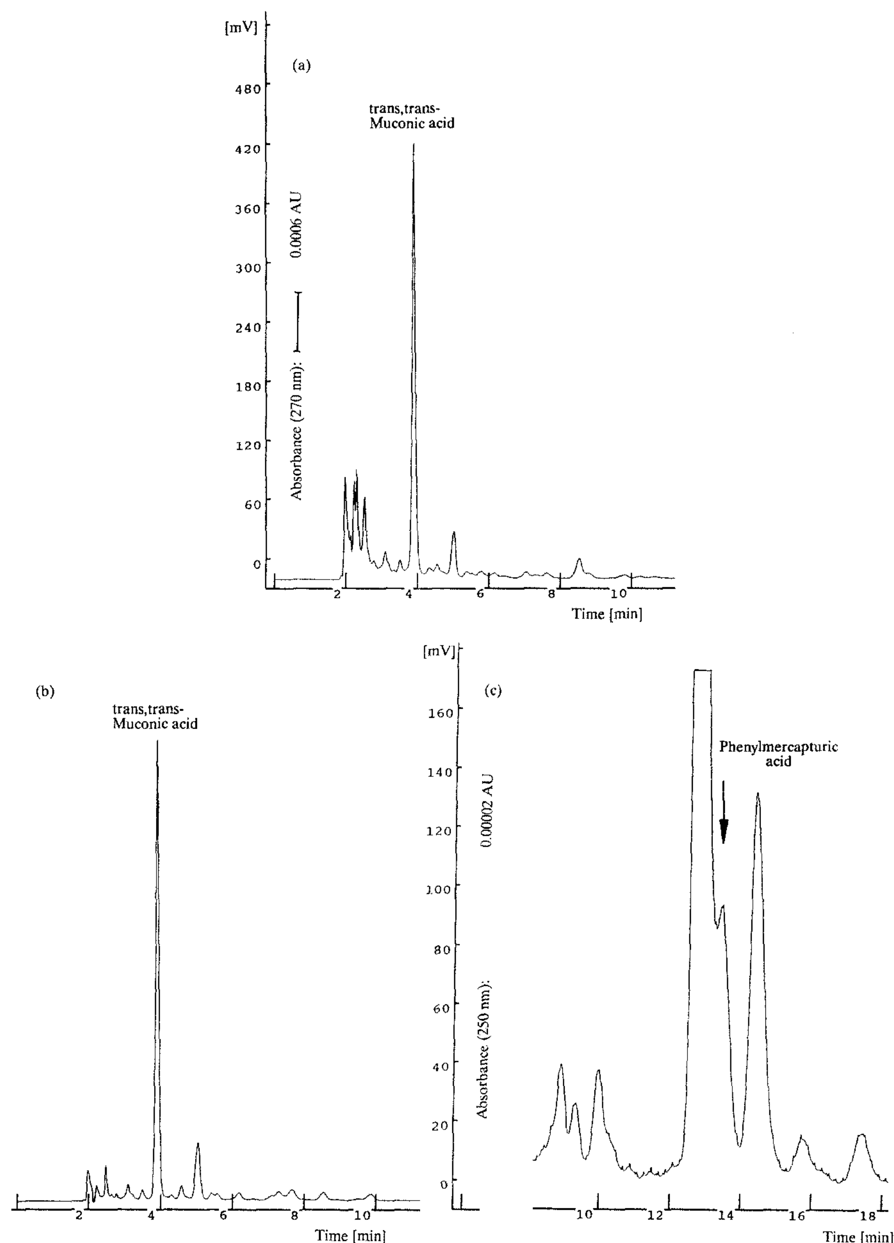

Fig. 3. Chromatograms of 300 ppm urine (a) after solid-phase extraction (fraction II), (b) after evaporation (fraction II) and (c) after evaporation at a wavelength of 250 nm for detection of phenylmercapturic acid.

RESULTS AND DISCUSSION

We tried to establish an HPLC method which enables the simultaneous identification of four benzene metabolites in urine. We found the optimum HPLC conditions to be an ODS column and an isocratic solvent of methanol and phosphate solution. The retention time of *trans,trans*-muconic acid could be influenced by changing the pH of the solvent. An increase in the pH from 1 to 7 reduces the retention time from 7 to 2 min. Under the conditions mentioned in the Materials and Methods section separation of the four metabolites was optimized (Fig. 1). The advantage was that the retention time of phenol, the last metabolite in the chromatogram, was around 10 min and this therefore guaranteed a short analysis time [3].

For the isolation of benzene metabolites from urine, a clean-up procedure was necessary to discriminate sufficiently between natural background and metabolite signals (Fig. 2a). The purification embraced a solid-phase extraction (anion exchanger) and a liquid–liquid extraction with diethyl ether. The SAX column separated *trans,trans*-muconic acid from phenols. The *trans,trans*-muconic acid fraction could be injected directly into the HPLC system, because ether extraction does not significantly improve the chromatogram (Fig. 3a and b). However extraction with diethyl ether had the advantage that the *trans,trans*-muconic acid fraction could be concentrated if necessary. For the phenol fraction (I) the ether extraction was indispensable, because there was an important background reduction, especially in the neighbourhood of the hydroquinone signal (Fig. 2b and c).

The detection limits (3 × signal-to-noise ratio) of the benzene metabolites are: catechol 7 mg/l, hydroquinone 60 mg/l, phenol 36 mg/l and *trans,trans*-muconic acid 3 mg/l.

By using enzymes it was possible to differentiate between conjugated (glucuronides and sulphate esters) and unconjugated metabolites. Nevertheless, the *trans,trans*-muconic acid peak was depressed when using enzymes and the rate of recovery was reduced below 50%. Since *trans,trans*-muconic acid in the urine of mice is not conjugated [3], the acid could be detected using a non-enzymatic method. Overloading of the 500-mg SAX column occurred when using 1 ml of 300 ppm urine, because *trans,trans*-muconic acid was then also found in the phenol fraction (I).

Using different amounts of native mice urine (1, 0.5, 0.25 ml) the recovery rates for benzene metabolites showed considerable variations. This problem needs further investigation.

In addition to the four benzene metabolites, phenylmercapturic acid has a remarkable role in benzene metabolism. This acid from the glutathione pathway could be isolated using the same clean-up procedure as for *trans,trans*-muconic acid. However, the HPLC method used used was not sensitive enough, because there was an unknown signal in the chromatogram which interfered with the phenylmercapturic acid signal (Fig. 3c). There will be a further publication dealing with this problem.

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