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Determination of phenol, *m*-, *o*- and *p*-cresol, *p*-aminophenol and *p*-nitrophenol in urine by high-performance liquid chromatography

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ABSTRACT

A method for the biological monitoring of human exposure to aromatic hydrocarbons, nitrocompounds, amines and phenols has been developed. Phenol, cresols, *p*-aminophenol, *p*-nitrophenol and their glucorono- or sulpho-conjugates, were quantified by HPLC; 4-chlorphenol was added as internal standard. After enzymatic hydrolysis, the free compounds were extracted with an organic solvent and analyzed by an isocratic HPLC Perkin Elmer system at ambient temperature and at a flow-rate of 1 ml/min. The column was a reversed-phase Pecosphere 3 × 3 C_{18} Perkin Elmer; the mobile phase was a 30:70:0.1 (v/v/v) methanol–water–orthophosphoric acid mixture and the chromatogram was monitored at 215 nm. Identification was based on retention time and quantification was performed by automatic peak height determination, corrected for the internal standard.

The recovery was *ca.* 95% for phenol and cresols; 90% for *p*-nitrophenol; 85% for *p*-aminophenol; the coefficients of variance were $<6\%$ within analysis ($n=20$) and $<10\%$ between analysis ($n=20$). The detection limits, at a signal/noise ratio of 2, were 0.5 mg/l for phenol and cresols and 1 mg/l for *p*-aminophenol and *p*-nitrophenol.

INTRODUCTION

Urinary phenol, cresols, *p*-nitrophenol and *p*-aminophenol are biological indicators of human exposure to aromatic hydrocarbons, nitro compounds, amines and phenols. Phenol, cresols and their glucurono or sulpho conjugates are excreted in urine by workers exposed to phenol and cresols. About 30% of retained benzene also gives rise to phenol *in vivo*. Therefore, the measurement of excreted urinary phenol has applications in the evaluation of exposure [1].

The determination of urinary cresols (particularly *o*-cresol) has been proposed [2,3] as a biological monitoring method for toluene. However, only a small fraction of the inhaled vapour is oxidized at the aromatic ring with the production of cresols. The

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urinary concentration of *p*-nitrophenol has been proposed for assessing the exposure to nitrobenzene [4], which is used in many industrial processes, mainly handled in solution, the skin being the principal route of exposure [5]. *p*-Aminophenol excretion in urine is a biological indicator of exposure to aniline [6], which is readily absorbed through the skin as either a liquid or vapour.

Several methods have been developed for the determination of free and conjugated phenolic biological indicators, especially spectrophotometry and gas chromatography [7,8]. Gas chromatographic methods are specific and precise when used with an internal standard [9]. Many workers have developed gas chromatographic methods for the determination of urinary phenol, cresols, *p*-nitrophenol and *p*-aminophenol [10–18]. However, published gas chromatographic methods suffer from a number of problems.

Pretreatment of samples is necessary to hydrolyse the conjugated compounds and then extract the compounds of interest. Better results can be obtained by distillation of the specimen after acidification and direct analysis of the aqueous distillate by gas chromatography [19] high-performance liquid chromatography (HPLC), but this is time consuming and laborious. Enzymatic hydrolysis seems to be the method of choice, taking into consideration the relative volatility of phenols, but the addition of a protein matrix introduces the need for another extraction.

The purpose of this study was to determine urinary phenol, cresols, *p*-nitrophenol and *p*-aminophenol by using a reversed-phase HPLC system with an isocratic mobile phase. Enzymatic hydrolysis and extraction of the compounds of interest with an organic solvent are followed by return to the alkaline aqueous phase and HPLC.

EXPERIMENTAL

Materials

The following standard solutions were used: 4-aminophenol, *o*-cresol, *p*-cresol, *m*-cresol, 4-methoxyphenol and phenol from Merck (Darmstadt, F.R.G.), 4-nitrophenol from BDH (Poole, U.K.) and *p*-chlorophenol from Fluka (Buchs, Switzerland). Acetic acid, ethyl acetate, hydrochloric acid, orthophosphoric acid, sodium hydroxide pellets, dichloromethane, methyl ethyl ketone from Merck, HPLC-grade methanol from Fluka and β-glucuronidase–arylsulphatase (*Helix pomatia*) from Boehringer (Mannheim, F.R.G.) were used. The urine control Lyphochek level 1 was obtained from Bio-Rad Labs. (Anaheim, CA, U.S.A.).

Apparatus

Chromatographic separation and peak detection of urinary phenol, cresols, *p*-nitrophenol and *p*-aminophenol were carried out on a Pecosphere 3 × 3 C_{18} reversed-phase column, 3 μm packing, 3.3 cm × 4.6 mm (Perkin-Elmer, Norwalk, CT, U.S.A.) with a Perkin-Elmer HPLC system with a Series 10 LC pump and an LC 90 UV ultraviolet spectrophotometric detector coupled to an integrator developed by SPE Sistemi e Progetti Elettronici (Brescia, Italy). The mobile phase was methanol–water–orthophosphoric acid (30:70:0.1, v/v/v) at flow-rate of 1.0 ml/min.

Methods

The urine specimens were obtained from healthy men and from workers exposed

in a workshop and were kept in dark, well closed containers. Stock standard solutions were prepared by dissolving known amounts of phenol and cresols in methanol, *p*-nitrophenol and *p*-aminophenol in water to obtain a 1 g/l concentration and kept in dark, well stoppered glass bottles. Whereas the stock solutions of phenol and cresols remain stable for 30 day at 4°C, those of *p*-aminophenol and *p*-nitrophenol were prepared immediately before use. Working standard solutions were prepared by diluting the stock standard solutions with water.

The enzymatic hydrolysis of each sample or standard (2 ml in test-tubes with screw caps at pH 5 in 1 *M* acetate buffer) was carried out with β-glucuronidase–arylsulphatase for 12 h at 37°C. The internal standard, *p*-chlorophenol, was added to all samples at a final concentration of 50 mg/l. After hydrolysis, phenol, cresols and *p*-nitrophenol were extracted by vortex mixing the samples (acidified to pH 2 with hydrochloric acid) with 4 ml of dichloromethane, and *p*-aminophenol at pH 5 with 4 ml of methyl ethyl ketone.

After centrifugation at 2400 *g* for 15 min, 2.5 ml of each organic extract was transferred into another test-tube, 0.5 ml of 0.2 *M* sodium hydroxide solution were added and, by vortex mixing, the compounds returned to the aqueous phase. An aliquot (0.3 ml) of the alkaline phase was transferred to the final test-tube and brought to pH 7.0 with hydrochloric acid; 10 μl of this solution were injected at room temperature into the HPLC system; the mobile phase was methanol–water–orthophosphoric acid (30:70:0.1, v/v/v) and the chromatogram was monitored at 215 nm. Identification was based on retention times, and quantification was performed by automatic peak-height determination, corrected for the internal standard.

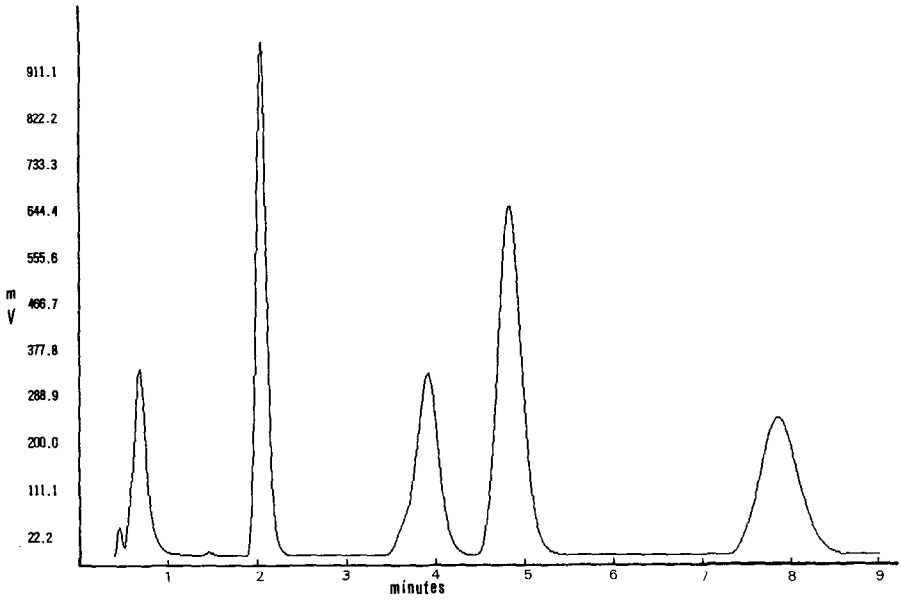

Fig. 1. Chromatographic elution (from left to right) of *p*-aminophenol, phenol, *p*-nitrophenol, cresols and internal standard (*p*-chlorophenol). Column, Pecosphere 3 × 3 C_{18} reversed-phase column (Perkin-Elmer); mobile phase, methanol–water–orthophosphoric acid (30:70:0.1, v/v/v); room temperature; injection volume, 10 μl; detection at 210 nm.

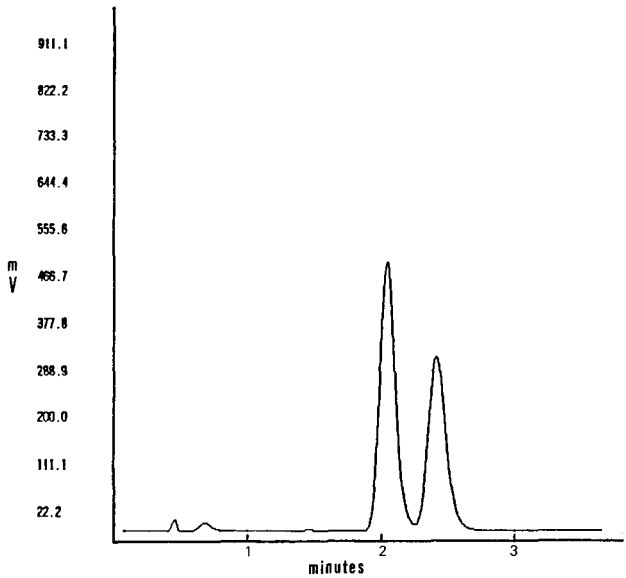

Fig. 2. Chromatographic elution of phenol and *p*-methoxyphenol, with retention times of 2.02 and 2.39 min respectively. Detector signals, 509.3 and 330.1 mV.

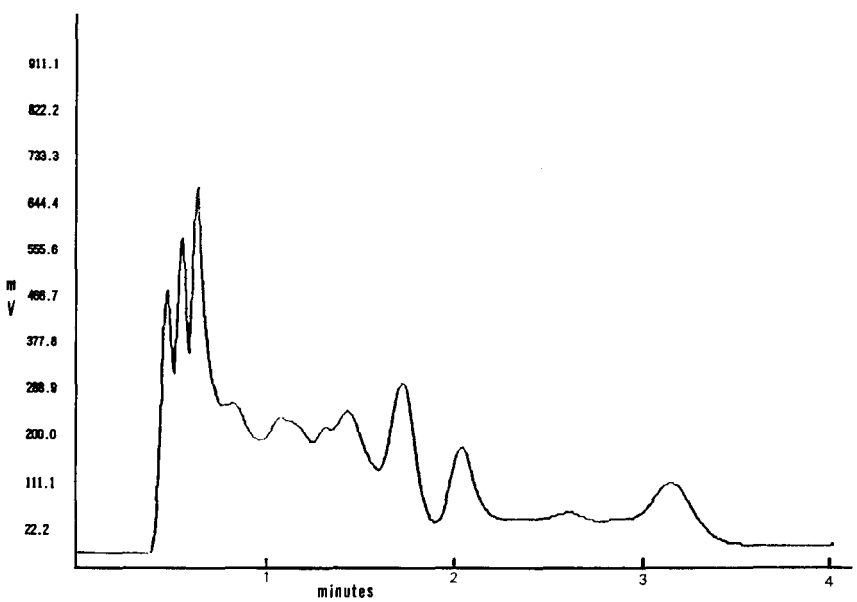

Fig. 3. Determination of phenol in urine control Lyphochek level 1. The retention time is 2.02 min; detector signal, 193.8 mV.

RESULTS AND DISCUSSION

The retention times of *p*-aminophenol, phenol, *p*-nitrophenol and cresols were 0.40, 2.02, 3.54, 4.49 and 7.40 min, respectively (Fig. 1). *p*-Methoxyphenol, used in pharmaceutical preparations, does not interfere with phenol (Fig. 2). *o*-Cresol, *m*-cresol and *p*-cresol have identical retention times.

Background absorptions in urine specimens obtained from healthy, unexposed men contained negligible amounts of *p*-amino and *p*-nitrophenol. However, for phenol, we obtained concentration values < 10 mg/l (40 samples), in good agreement with the data from gas chromatographic methods [9,11].

The calibration graphs for phenol, cresols, *p*-nitrophenol and *p*-aminophenol in water were linear up to at least 50 mg/l. The precision of the method was satisfactory, with relative standard deviations of $< 6\%$ within analyses ($n = 20$) and $< 10\%$ between analyses ($n = 20$). The recovery was *ca.* 95% for phenol and cresols, 90% for *p*-nitrophenol and 85% for *p*-aminophenol. Solutions of phenol, cresols, *p*-nitrophenol and *p*-aminophenol were diluted to give concentrations of 0.5 mg/l in water (phenol and cresols) and 1 mg/l in normal urine samples (*p*-amino- and *p*-nitrophenol), hydrolysed and extracted as usual. We calculated the detection limits, at a signal-to-noise ratio of 2, to be 0.5 mg/l for phenol and cresols and 1 mg/l for *p*-amino and a*p*-nitrophenol.

We examined urine samples from workers exposed to phenol and benzene; the results obtained in at least 500 samples examined were less than biological limits of exposure adopted by ACGHI 1987/88 [20].

Urine control Lyphochek level 1 (Fig. 3) is used in assessing the accuracy and precision of assay procedures; the experimental concentrations obtained for phenol ($n = 230$) were in the range 10.8–17.2 mg/l, which compared well with the target values for phenol by HPLC of 10.4–17.4 mg/l.

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