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Short Communication

Simultaneous high-performance liquid chromatographic determination of urinary metabolites of benzene, nitrobenzene, toluene, xylene and styrene

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ABSTRACT

A high-performance liquid chromatographic method is described for the simultaneous determination of six urinary metabolites of several aromatic chemicals: phenol (from benzene), hippuric acid (from toluene), 3-methylhippuric acid (from xylene), mandelic and phenylglyoxylic acid (from styrene) and 4-nitrophenol (from nitrobenzene). Reversed-phase liquid chromatography was performed in an isocratic mode at 1 ml/min on a 5-μm C_{18} column using two mobile phases: (A) acetonitrile–1% phosphoric acid (10:90); (B) acetonitrile–1% phosphoric acid (30:70). Phase A separates the six metabolites well, but phase B allows to a more rapid and reproducible simultaneous determination of phenolic compounds than phase A. For these compounds a prior enzymic hydrolysis step using *Helix pomatia* juice is performed to hydrolyse their sulphate and glucuronate conjugates. The reproducibility and the specificity are both excellent. Furthermore, the method is rapid, economical and easily automated. The proposed method appears very suitable for the routine monitoring of workers exposed to these chemicals on the basis of the biological threshold limit values.

INTRODUCTION

Measurements of industrial chemicals or their metabolites in biological materials (expired air, blood, urine) allow the estimation of the degree of exposure to these chemicals [1–3]. The definition of permissible levels of exposure to chemical agents can be expressed in terms of allowable atmospheric concentrations [3] or in terms of permissible biological levels for these chemicals or their metabolites expressed as the biological "threshold limit values" (TLV), representing a biological marker of the environmental conditions under which it is considered that nearly all workers may be exposed during 8 h a day, without significant adverse effect [4].

In occupational laboratory medicine, if the problem is the routine monitoring of workers exposed to chemicals, the analytical performance of a suitable method must address the following question: are the workers being exposed to higher than acceptable levels on the basis of their biological TLVs? Since the biological TLVs of the studied chemicals are relatively high, sensitivity is not a real problem, but an acceptable specificity, a low cost and a rapid and possibly automated method are required. Indeed, a high specificity is less critical for routine monitoring than for pharmacokinetic or forensic studies, since a more detailed and specific analysis can be performed if the concentration of a metabolite is found to be above its biological TLV.

Urinary determination of metabolites of aromatic solvents offers important and decisive ad-

vantages over other monitoring methods: (1) it takes into account absorption by all possible routes (skin, lung, etc.); (2) it can allow for individual variations in the toxicokinetics and biotransformations of the solvent; (3) it may reflect the total exposure (amounts excreted during a given period); (4) it is easily performed for routine purposes. Consequently, a screening method for the simultaneous determination of the urinary metabolites of major aromatic compounds, such as benzene, nitrobenzene, toluene, xylene and styrene, could be very useful in occupational toxicology.

Thin-layer chromatography has been used to separate urinary hippuric, mandelic and phenylglyoxylic acids after exposure to toluene and xylene [5,6]. In addition to our previously published method [7], high-performance liquid chromatographic (HPLC) determination of hippuric acid and of *o*-cresol levels after toluene exposure has been proposed [8,9]. Phenolic compounds are currently determined in urine using a separation step by steam distillation and a colorimetric method [10], or by gas chromatography [11], but these methods are time-consuming, complex and not suitable for routine analysis.

This paper describes a method for the simultaneous HPLC determination of urinary metabolites of five aromatic chemicals: (1) benzene (phenol) and nitrobenzene (4-nitrophenol); (2) toluene (hippuric acid), xylene (3-methylhippuric acid) and styrene (phenylglyoxylic and mandelic acids).

EXPERIMENTAL

Chemicals

3-Methylbenzoic acid, hippuric acid and acetonitrile (LiChrosolv grade) were purchased from Merck (Darmstadt, Germany). Phenylglyoxylic acid (benzoyl formic acid), mandelic acid, phenol (recrystallized before use) and 4-nitrophenol were obtained from Sigma (St. Louis, MO, USA). *Helix pomatia* juice (containing 100 000 Fishman Units of β-glucuronidase and 1 000 000 Roy Units of sulphatase) was obtained from IBF (Villeneuve la Garenne, France). 4-Methylhippuric acid was synthesized in our laboratory using a method previously described for the synthesis of hippuric acid [12]. Briefly, 4 g of 3-methylbenzoyl chloride (obtained from 3-methylbenzoic acid using the thionyl chloride method) were refluxed with 5 g of glycine in 50 ml of 10% sodium hydroxide solution for 1 h. After acidification with concentrated hydrochloric acid, the crystalline precipitate was washed with water and recrystallized in boiling water. All other chemicals used were of analytical grade. Reference compounds (except phenol) were desiccated overnight under vaccum before use. Stock solutions were made in distilled water (10 g/l for hippuric and mandelic acids, 1 g/l for phenylglyoxylic acid and phenol), in water–methanol (50:50, v/v, 3-methylhippuric acid; 10 g/l) or in methanol (4-nitrophenol; 1 g/l) and stored at 4°C. A stock solution of phenol was prepared weekly, and its exact concentration was determined using the USP bromuration method [13].

Chromatography

The HPLC system consisted of a pump (Jasco 880 PU, Tokyo, Japan) and a 200-μl loop injector (Rheodyne 7025, Berkeley, CA, USA). A 25 cm × 0.46 cm I.D. reversed-phase column packed with 5-μm ODS 2 (Sup-Rs Classic, Prolabo, Paris, France) and a 10-μm ODS 2 precolumn (1.5 cm × 0.46 cm I.D.) were used for separation.

Detection and quantification were performed using a variable-wavelength UV detector operating at 265 or 280 nm (Jasco UV-875) and an integrator (Chromatopac C-R6A, Shimadzu, Kyoto, Japan) or a recorder (Linear). The mobile phases used were mixtures of acetonitrile and 1% phosphoric acid: (A) (screening procedure) 10:90, v/v; (B) (simultaneous specific determination of phenol and 4-nitrophenol) 30:70, v/v. The mobile phases were filtered through a 5-μm glass filter and degassed by ultrasonification. The flow-rate was 1 ml/min (linear velocity $u = 0.16$ cm/s). The polarity parameters P' and the solvent strength parameter S were calculated according to Snyder and Kirkland [14].

Urine samples

Normal 24-h urine specimens were obtained from out-patients not exposed to solvents. Test specimens were obtained from laboratory personnel and from workers followed by the Occu-

pational Medicine Department of our hospital. The samples were filtered (0.22 μm) and stored at −30°C if not immediately analysed.

Urinary determination

For the assay of samples not containing hydrolysed phenol conjugates, 100 μl of 24-h urine specimens were diluted with 900 μl of mobile phase and 200 μl were injected into the chromatograph.

For the assay of hydrolysed samples, 1 ml of 24-h urine specimens was mixed with 1 ml of acetate buffer (pH 4.8, 0.2 *M*) and 200 μl of *Helix pomatia* juice. The mixture was incubated overnight at 37°C in a sealed tube. Other samples of urine were spiked with 20 or 50 μl of a 1 g/l stock solution of phenol and processed as described above. The incubation mixture was then diluted ten-fold with the mobile phase and centrifuged at 10 000 *g* for 5 min, and 200 μl were injected into the loop. Enzymic hydrolysis of sulphated conjugates, currently used for the determination of several urinary steroids [15,16], using the juice of *Helix pomatia*, which contains sulphatase and β-glucuronidase, was preferred to acid hydrolysis in order to prevent a possible loss of the volatile phenol under the heating conditions required by the acid treatment, and a partial hydrolysis of hippuric and 3-methylhippuric acids, which are glycine conjugates. To validate this method, urinary samples were hydrolysed for 48 h under the above conditions and the free phenol level was determined at various times. Overnight incubation at 37°C was found to be sufficient since the phenol peak was at a maximum after 6 h, suggesting complete hydrolysis of conjugates.

Calculations

Two "limit" standard chromatograms were obtained daily by direct injection of two standard mixtures, M1 and M2, obtained by appropriate dilutions of the corresponding stock solutions in the respective mobile phases A and B to obtain the following final concentrations: M1, 200 mg/l for mandelic acid, 250 mg/l for hippuric and 3-methylhippuric acids and 25 mg/l for phenylglyoxylic acid; M2, 2.5 mg/l for phenol and 0.5 mg/l for 4-nitrophenol. These values correspond to the published biological TLVs of each metabolite [15], and allow for the ten-fold dilution step (the biological TLV for hippuric acid for example is 2 g/l) [1,3,17]. Elution was performed using the respective mobile phase: phase A for the mixture M1 and phase B for the mixture M2. If the concentration of a particular metabolite in a urine specimen exceeded the corresponding value in the limit chromatograms (simple screening determination) or if needed for a specific purpose, a calibration curve was then constructed by spiking the sample with appropriate amounts of this metabolite. The exact concentration of the metabolite was then determined as described above.

Concentrations were determined from the standard curves (peak area or height *versus* added concentration) by linear regression (or polynomial regression needed) according to the classical "standard addition" method.

RESULTS AND DISCUSSION

Several mixtures of acetonitrile and 1% phosphoric acid wered tested as mobile phases. Fig. 1 shows the variation of the capacity factor k' for the four acid metabolites and phenol as a function of the composition of the mobile phase. Since these metabolites are relatively strong acids (pK_a <4.0 for hippuric, 3-methylhippuric, mandelic and phenylglyoxylic acids), slightly acidic (pK_a = 7.15 for 4-nitrophenol) or very slightly acidic (pK_a = 9.89 for phenol), the use of an ap-

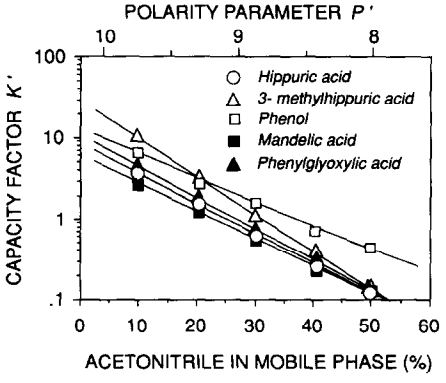

Fig. 1. Variation of the capacity factor k' for hippuric, 3-methylhippuric, mandelic and phenylglyoxylic acids and for phenol as a function of the percentage of acetonitrile in the mobile phase. Each point represents the mean of four determinations (C.V. < 1%).

propriately acidic mobile phase renders these molecules in a non-ionized lipophilic state, leading to sufficient retention of the C_{18} stationary phase.

A 10:90 mixture of acetonitrile and 1% phosphoric acid (phase A; polarity parameter $P' = 9.76$, solvent strength parameter $S = 0.31$) was found to give an excellent separation of the metabolites of benzene, toluene, xylene and styrene (resolution factor $R_s \geq 1$ between all metabolites) using an isocratic mode. For acetonitrile concentrations up to 30%, there was a good separation between hippuric acid and its 3-methyl derivative but mandelic acid coeluted with hippuric acid ($k' = 1.37$ for hippuric acid and 1.46 for mandelic acid). With 10% acetonitrile, the k' values were 3.67 and 3.03, respectively, allowing an acceptable separation between these two compounds. Using mobile phase A, the retention times were 10.9 min for mandelic acid, 12.6 min for hippuric acid, 16.2 min for phenylglyoxylic acid and 31.6 min for 3-methylhippuric acid. Phenol eluted at 20.1 min. Since there was no significant peak after 45 min, the mean interval between two consecutive injections can be *ca.* 50 min. Fig. 2 shows a typical chromatogram obtained from an urine specimen from a laboratory worker exposed to benzene (containing endogenous hippuric acid).

The precision of the method was determined by repeated analyses of the standard mixture M1 ($n = 10$). The coefficients of variation (C.V.) for intra- and inter-day analyses were less than 5% for the four metabolites in M1. The accuracy was determined using urine samples spiked with the same amounts of metabolites as in M1. The reproducibility was 2.7% for mandelic and hippuric acids, 3.6% for phenylglyoxylic acid and 4.8% for 3-methylhippuric acid. The bias (using the confidence limit) was not significant for all compounds at $P = 0.975$.

However, for the determination of 4-nitrophenol, despite a good separation pattern using mobile phase A, it was considered that a mixture containing 30% acetonitrile (phase B; $P' = 8.88$, $S = 0.93$) gave more reproducible and rapid results. However, if monitoring of exposure to nitrobenzene is not required, mobile phase A can give an excellent and rapid separation of the other metabolites and appears to be very suitable for routine monitoring. With phase A, the retention time for 4-nitrophenol is too long (> 45 min); with phase B, the retention times were 7.05 ± 0.05 min for phenol and 9.3 ± 0.02 min for 4-nitrophenol, and did not vary over the concentration range tested (1–100 mg/l). Hippuric acid was well separated from the phenolic compounds (retention time 4.33 ± 0.03 min). 3-Methylhippuric acid was eluted after phenol with phase A, but before it with phase B (retention time 6.1 ± 0.1 min).

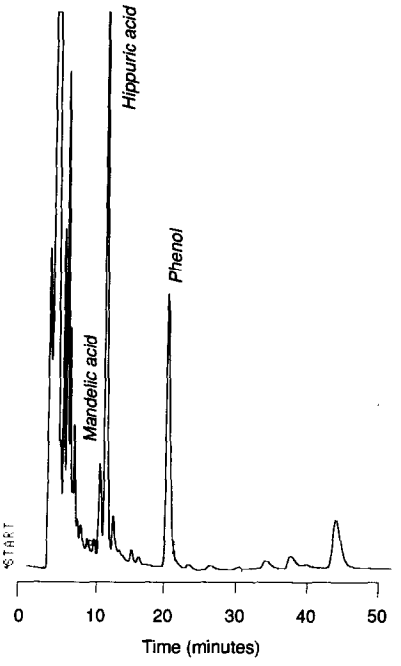

Fig. 2. Typical chromatogram obtained from a 24-h urine specimen of a laboratory worker exposed to benzene. Conjugates were hydrolysed overnight using *Helix pomatia* juice (pH 4.8; 37°C). The metabolite levels were 1.27 g/l for hippuric acid, 207 mg/l for mandelic acid and 32.7 mg/l for phenol. Conditions: mobile phase, acetonitrile–1% phosphoric acid (10:90, v/v); flow-rate, 1 ml/min; detection wavelength, 280 nm; 0.08 a.u.f.s.

A good linearity between the peak areas and the concentrations was observed in the range 0–25 mg/l ($r = 0.998$, $n = 4$) but, for concentrations greater than 25 mg/l, the relationship was best fitted with a second-order polynomial model. If needed, an additional sample dilution may be performed. Using the same methodology as above described, the reproducibility was also excellent (C.V. = 4.2% for phenol and 3.1% for 4-nitrophenol, mixture M2 or spiked urine, $n =$

10). Since no pure phenolic conjugates were available, the accuracy of the assay was not determined.

Hippuric acid is a normal constituent of human urine (from canned foods, fruits and endogenous protein metabolism), but amounts of hippuric acid in the urine vary tremendously between patients [7,17] and its maximum physiological rate of excretion has been reported to be 33 mg/h (*ca.* 1.5 g/l). Since the biological TLVs for phenol and 4-nitrophenol are only 25 and 5 mg/l, respectively [1,5], a good separation between hippuric acid and these compounds is essential for an accurate determination. Under our experimental conditions, hippuric acid did not interfere, as demonstrated on the typical chromatogram of an urine specimen from a laboratory worker exposed to benzene (containing 1.25 g/l hippuric acid, 207 mg/l mandelic acid and 32.7 mg/l phenol; Fig. 2).

Since urine is a complex matrix containing a number of endogenous or exogenous compounds of variable composition, it is difficult to establish definitely the specificity of the method. The criteria used here were the retention time compared with a standard mixture and coelution with an authentic sample if the peak exceeded the limit value. Furthermore, in this case, repeated analysis may be performed with a spectral analysis of the peak in order to confirm its identity. In our experience from several hundred determinations, we had no real problem of specificity.

Finally, the cost per determination may be also an important concern. The proposed method takes into account this requirement, since the cost of reagents per determination is very low and the apparatus is relatively economical and sufficiently versatile to be used for several other laboratory determinations. Furthermore, our method can be easily automated.

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