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Short communication

Simultaneous determination of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone in urine by capillary gas chromatography

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Abstract

A method for the simultaneous determination of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone in urine has been described. The metabolites were analyzed after enzymatic hydrolysis and extraction on octyl (C8) cartridges by using gas chromatography with flame ionization detection and a 5/95% copolymer of diphenyl–poly(dimethylsiloxane) capillary column. Methoxyphenols were well separated within 12 min. Recovery was over 90% in the range from 0.5 to 20 μg/ml; the detection limit was varying in the range of 0.05–0.11 μg/ml. The relative standard deviations and the accuracy were in the range of 3.1–15.5 and 2.4–16.0%, respectively.

Keywords: 2-Methoxyphenol; 2-Methoxy-4-methylphenol; 2,6-Dimethoxyphenol; 4′-Hydroxy-3′-methoxyacetophenone

1. Introduction

The analysis of urinary metabolites of different chemicals is usually carried out for the biological monitoring of workers exposed occupationally to toxic compounds. Phenolic compounds, e.g. phenol, cresol isomers and xylenol isomers, have been found to be the main metabolites in the urine of coke industry workers [1]. Detection of urinary methoxyphenols and its use for biological monitoring of workers exposed to wood smoke was previously described by Dills et al. [2]. Methoxyphenols found in wood smoke include homologues of guaiacol (2-methoxyphenol) and syringol (2,6-dimethoxyphenol). According to Dills et al. [2], methoxyphenols in urine were extracted after acidic hydrolysis using XAD resin in a solid-phase extraction cartridge, eluted with ethyl acetate and analyzed by GC–MS. Methoxyphenols in other matrices were assessed by GC–MS [3–7] for separation and identification. Methoxyphenols were also identified in urine of the coke plant workers exposed occupationally to aromatic hydrocarbons and phenols.

In the present paper, a new analytical method for the separation and quantitative determination of

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methoxyphenols in urine and particularly the development of solid-phase extraction is presented.

2. Experimental

2.1. Reagents

All chemicals were of reagent grade quality or better and were used as received without further purification. 2-Methoxyphenol (98%), (CAS registry no. 90-05-1), 2-methoxy-4-methylphenol (99%) (CAS registry no. 93-51-6), 2,6-dimethoxyphenol (99%) (CAS registry no. 92-10-1) and 4′-hydroxy-3′-methoxyacetophenone (98%) (CAS registry no. 498-02-2) were obtained from Aldrich (Milwaukee, WI, USA). β-Glucuronidase/aryl sulfatase from Helix Pomatia containing about 30 U/ml of β-glucuronidase (EC 3.2.1.31) and about 60 U/ml of sulfatase (EC 3.1.6.1) was obtained from Merck (Darmstadt, Germany). Methanol of HPLC grade was obtained from Riedel-de Haen (Seelze, Germany). Concentrated hydrochloric acid, 0.1 M (pH 5) acetate buffer were obtained from POCH (Gliwice, Poland). Bakerbond SPE columns packed with reversed phase octylsilane (C8) bonded to silica gel (3 ml, 200 mg; 40 μm particle size, 60 Å pore size); silica gel normal phase (3 ml, 200 mg) and columns packed with styrene–divinylbenzene copolymer (3 ml, 200 mg) (7619-02) were from Baker B.V. (Deventer, The Netherlands). The Baker spe-12G SPE column processing system was from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA).

2.2. Standard solutions

Stock solution of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone was prepared in methanol at a concentration of 200 μg/ml. Calibration standards were prepared by diluting enzymatically-hydrolyzed urine obtained from non-exposed and non-smoker subjects, so that the concentration of each of them was 0.5, 1.0, 2.0, 4.0, 8.0, 10.0, and 20 μg/ml. The calibration standards were kept at 4 °C until use.

Upper range quality controls (10 and 20 μg/ml) were prepared by spiking stock solution to hydrolyzed urine obtained from non-exposed and non-smoker subjects. These quality controls were then serially diluted with hydrolyzed urine to obtain the remaining quality controls (0.5, 1.0, 2.0, and 5.0 μg/ml). Then, the quality controls were frozen at −20 °C until use.

2.3. Sample preparation

Urine was stored at −20 °C until analysis. Then, each sample was thawed, and a 10 ml aliquot was transferred to an Erlenmayer flask. One mole hydrochloric acid solution was used to bring the sample to pH 5, and 5 ml of 0.1 M pH 5 acetate buffer was added. Thirty microliter of β-glucuronidase/arylsulfatase were added to each urine sample in order to hydrolyze conjugated methoxyphenols. The solutions were incubated for 20 h at 37 °C. After cooling to room temperature, samples were loaded onto SPE columns.

2.4. Procedures for SPE

The extraction was performed by the use of column processing system. The SPE columns were pre-activated with 6 ml of methanol and 8 ml of distilled water. Then, the hydrolyzed urine sample was added to the column and slowly aspirated. The column was washed with 6 ml of distilled water and gently dried by aspiration for 30 min. The methoxyphenols were eluted with 2 × 0.5 ml of methanol into vials. The eluate was evaporated to dryness under a stream of nitrogen, and then redissolved in 1 ml of methanol. The vials were capped and kept at 0 °C before being analyzed to avoid evaporation. A 1 μl aliquot of the sample was injected for GC analysis.

2.5. Chromatographic conditions

Extracts were analyzed using a Hewlett-Packard gas chromatograph 5890 II (Palo Alto, CA, USA) equipped with a flame ionization detector, an autosampler–autoinjector (HP 7673) and an integrator (HP 3396 II).

The methoxyphenols were separated using a capillary column Ultra 2 (cross-linked 5/95% diphenyl–poly(dimethylsiloxane)) 25 m × 0.32 mm i.d., 0.52 μm film thickness. GC conditions were as follows: injector temperature 220 °C and detector

temperature 240 °C; oven temperature, 40 °C held for 1 min, increase by 15 °C/min to 200 °C held for 4 min; carrier gas, helium at a flow rate of 2.5 ml/min; injection volume, 1 μl; splitless time, 1 min.

3. Results and discussion

3.1. Sample preparation

Urinary methoxyphenols are usually analyzed after enzymatic hydrolysis of conjugates and extraction with C8-solid phase extraction cartridges. In order to optimize the procedure, different SPE cartridge packings, e.g. octyl, silica gel and styrene–divinylbenzene copolymer, were tested. The chromatogram obtained on styrene–divinylbenzene copolymer shows less contaminants than the chromatogram on octyl material, as it is shown in Fig. 1. Moreover, the recoveries of the extraction of methoxyphenols on octyl are significantly higher than on the styrene–divinylbenzene copolymer. Table 1 presents recoveries of the methoxyphenols for different extraction cartridges. It supports the statement, that the C8 material is better suited for the extraction procedure of urine phenols than the styrene–divinylbenzene copolymer.

Table 1
Recoveries of analytes from different extraction cartridges at concentration 5 μg/ml ($n = 6$)

Metabolite	Recovery (%)	
	Styrene–divinylbenzene copolymer	Octyl material
2-Methoxyphenol	84.7 ± 11	94.1 ± 6
2-Methoxy-4-methylphenol	82.6 ± 9	96.7 ± 10
2,6-Dimethoxyphenol	78.4 ± 13	89.4 ± 9
4′-Hydroxy-3′-methoxyacetophenone	70.3 ± 15	97.8 ± 11

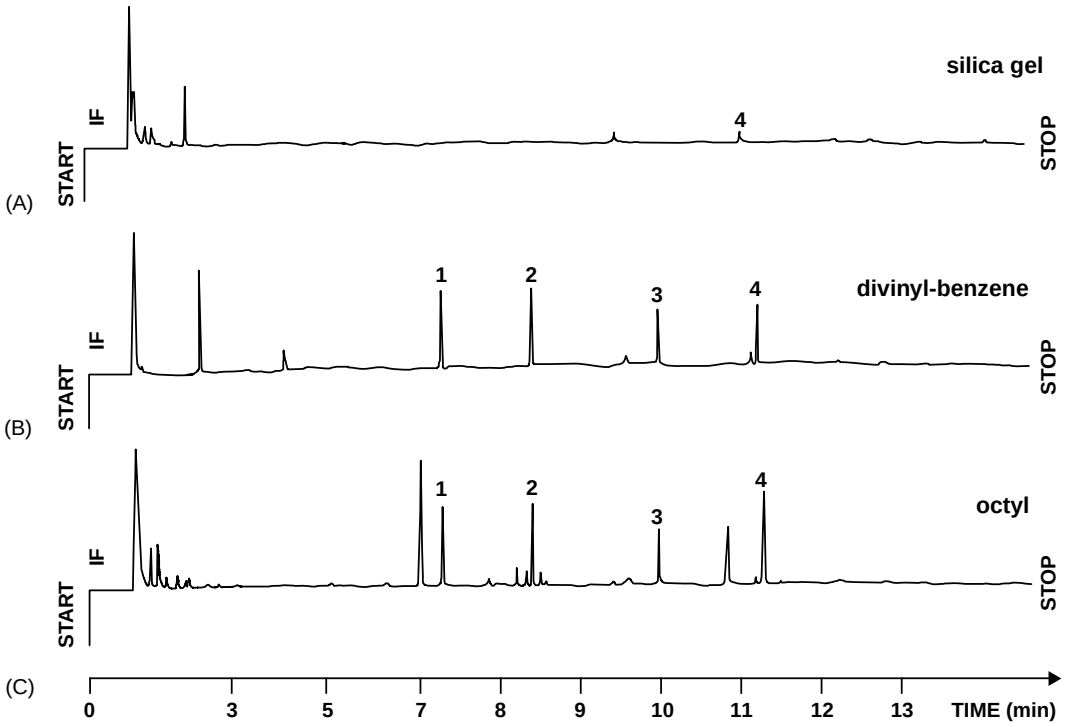

Fig. 1. Chromatograms of methoxyphenols in urine of non-exposed subjects spiked with 25 μl stock solution after: (A) silica gel normal phase; (B) styrene–divinylbenzene copolymer; (C) octyl material SPE clean-up. Peaks: (1) 2-methoxyphenol; (2) 2-methoxy-4-methylphenol; (3) 2,6-dimethoxyphenol; (4) 4′-hydroxy-3′-methoxyacetophenone.

3.2. Chromatography

It has been found that the use of the slightly polar stationary phase is the most efficient method for the GC separation of the methoxyphenols. Chromatograms obtained from blank and spiked urine are shown in Fig. 2. Fig. 2(A) was obtained by the analysis of a urine sample collected from workers exposed occupationally to the mixture of aromatic hydrocarbons and phenolic compounds. Fig. 2(B) shows the analysis of a urine sample collected from a non-exposed subjects. Fig. 2(C) shows the analysis of a urine sample collected from a non-exposed subjects and enriched with 4 μg/ml of each of the four methoxyphenols. The separation of methoxyphenols was completed within 12 min. External standards were used in the quantitation of the analytes. It has been found, that the 2-chloro-4-methoxyphenol previously used by Dills et al. [2], is not convenient as an internal standard in analyses described in the paper. Many peaks, assigned to the different metabolites present simultaneously in urine from occupationally exposed workers have obscured the standard peak. Earlier investigations have shown, that it is possible to determine simultaneously the presence of the phenol, cresol isomers, xylenol isomers in urine [1].

3.3. Linearity

A linear relationship between the peak area and the mass of the compound was found for each measurement consisting of six samples spiked at the levels given in Section 2. The parameters of the calibration lines (peak area versus mass (ng) of each compound per 1 μl of the injected sample) were determined with the following values of the mean calibration curve slope CV of 8.4, 6.3, 5.9 and 7.2% ($n = 12$), over a period of 2 weeks.

3.4. Recovery

The recoveries of the standards spiked in water at 1 μg/ml and carried through the entire procedure ranged from 90.5 to 95.7%. Recoveries from urine samples spiked at 1 μg/ml ranged from 97.2 to 112.4% ($n = 5$), when calculated from external standard calibration (Table 2). Free and conjugated methoxyphenols were also present in urine of non-exposed subjects [2].

3.5. Precision and limits of detection

The intra- and inter-day precisions of the method were evaluated over 12 days in 13 batches, using urine samples spiked at the levels given in Section 2. Each concentration was calculated on the basis of peak area using calibration curves. An integrator program (external standard) was employed. The recoveries of the phenols at the investigated concentration levels were between 95 and 112% and the CVs ranged from 3 to 13%. The detection limit was determined at a signal-to-noise ratio of 3. The detection limits of methoxyphenols were found to vary from 0.05 to 0.11 μg/ml.

3.6. Matrix stability

Calibration standards were prepared daily and compared to standard solutions of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone in the urine. Methoxyphenols were found to be stable in urine for at least 2 weeks when stored at −20 °C.

3.7. Application

It has been found, that the temperature program described in this paper, can be used in the determination

Table 2
Recoveries (mean ± S.D.) of spiked samples at the concentration 1 μg/ml ($n = 5$)

Sample	Recovery (%)			
	2-Methoxyphenol	2-Methoxy-4-methylphenol	2,6-Dimethoxyphenol	4′-Hydroxy-3′-methoxyacetophenone
Spiked in water	93.2 ± 5	95.7 ± 9	92.4 ± 7	90.5 ± 6
Spiked in urine	112.4 ± 23	98.4 ± 13	103.8 ± 17	97.2 ± 9

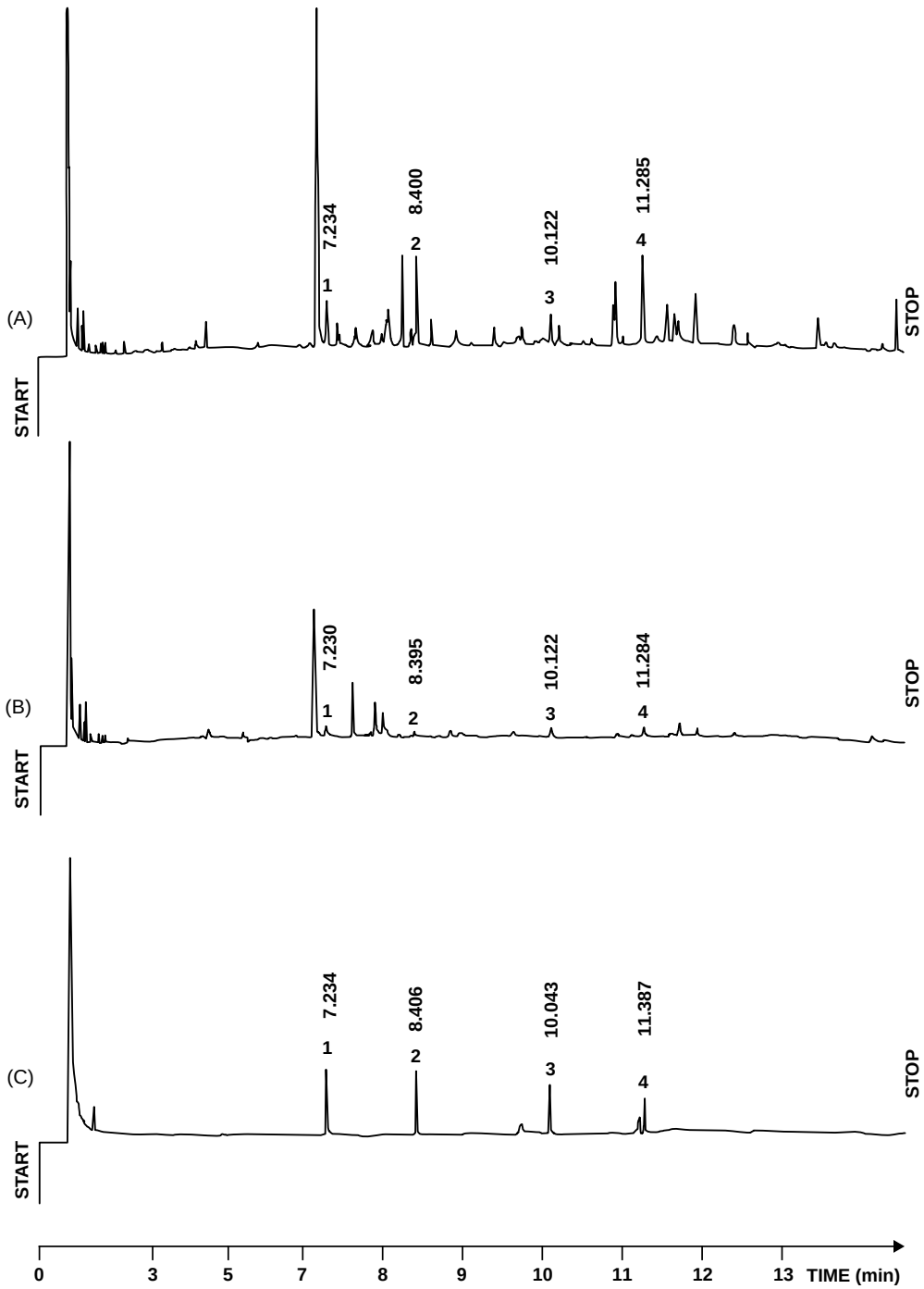

Fig. 2. Representative chromatograms of the methoxyphenols in urine: (A) urine of coke plant workers exposed to aromatic hydrocarbons and phenolic compounds; (B) blank urine of non-exposed subjects; (C) urine of non-exposed subjects enriched with 4 μg/ml of standards. Peaks: (1) 2-methoxyphenol; (2) 2-methoxy-4-methylphenol; (3) 2,6-dimethoxyphenol; (4) 4′-hydroxy-3′-methoxyacetophenone.

Table 3
Concentrations of methoxyphenols in urine of the exposed coke plant workers ($n = 32$) and non-exposed subjects ($n = 19$)

Metabolite	Concentration (μg/ml) in exposed			Concentration (μg/ml) in non-exposed		
	Median	5th percentile	95th percentile	Median	5th percentile	95th percentile
2-Methoxyphenol	4.83 (93.7%)	0.83	7.12	0.72 (84.2%)	0.54	0.89
2-Methoxy-4-methylphenol	1.80 (78.1%)	0.74	3.04	0.58 (47.4%)	0.46	0.64
2,6-Dimethoxyphenol	1.22 (53.1%)	0.55	4.26	0.48 (31.5%)	0.49	0.59
4′-Hydroxy-3′-methoxyacetophenone	1.13 (68.8%)	0.62	2.13	0.54 (52.6%)	0.51	0.67

Percentage of subjects with the methoxyphenols above the analytical detection limits are given in parenthesis.

of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone in the extracts of both urine samples and aqueous solutions. To demonstrate the application of our method, concentrations of the four methoxyphenols in urine of coke plant workers and non-exposed subjects have been determined, and results are summarized in Table 3. Concentrations of the methoxyphenols above the analytical detection limits, were found to be distributed log-normally. Parameters of the urinary metabolites were characterized as median, 5 and 95th percentiles (measures of spread). Significant differences ($P < 0.05$) can be seen between the average concentrations of urinary methoxyphenols of exposed coke plant workers engaged as operators in the tar distillation department and non-exposed subjects.

4. Conclusions

A method for the simultaneous determination of 2-methoxyphenol, 2-methoxy-4-methylphenol, 2,6-dimethoxyphenol and 4′-hydroxy-3′-methoxyacetophenone based on solid-phase extraction with an octyl material (C8) and analysis by GC–FID has been described. The proposed method can be useful for environmental and toxicological studies of methoxyphenols in urine as well as in aqueous solutions.

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