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Simultaneous determination of hydroquinone, catechol and phenol in urine using high-performance liquid chromatography with fluorimetric detection

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ABSTRACT

A method was developed for simultaneous determination of urinary hydroquinone, catechol and phenol using high-performance liquid chromatography (HPLC) with variable-wavelength fluorimetric detection. Urine samples, after acid hydrolysis, were saturated with sodium sulphate and extracted by diethyl ether. The two buffers used for gradient elution were (A) 10 m*M* sodium acetate containing 0.5% (v/v) acetic acid and (B) the same as buffer A but containing an additional 20% (v/v) acetonitrile. Hydroquinone, catechol and phenol were separated in a C_{18} column and detected at 2.9, 6.8 and 13.6 min, respectively. The recovery and reproducibility were generally over 90%. Over 300 extracted samples were analysed and no change in column efficiency was noted. Comparisons were also made with HPLC using ultraviolet (UV) detection and with gas chromatography (GC). The proposed method appears to be more sensitive and reliable than other existing methods. This new method was also validated with urine samples collected from cigarette smokers and from refinery workers exposed to low concentrations of benzene.

INTRODUCTION

Benzene is classified as a group I carcinogen [1]. It is also a constituent of engine emissions and tobacco smoke [2]. Owing to its potential carcinogenic effect and widespread industrial use, biological monitoring of benzene exposure is important for disease prevention. The current occupational exposure limit for benzene is 1 ppm, and the American Conference of Governmental Industrial Hygienists (ACGIH) has recently proposed that it be reduced to 0.1 ppm [3].

The most commonly used method for biological monitoring of benzene exposure is measurement of phenol in urine. However, because of the high background level of phenol resulting from its presence in many foodstuffs, urinary phenol measurement has been reported to be an unreliable parameter, especially for low levels of benzene exposure [4,5]. Other benzene metabolites such as hydroquinone, catechol, *trans,trans*-muconic acid and *S*-phenylmercapturic acid have been proposed as more sensitive biological markers [6–13]. A sensitive method for urinary analysis of *trans,trans*-muconic acid has recently been developed in our laboratory [14]. In this paper we report a method for the simultaneous determination of hydroquinone, catechol and phenol by using HPLC with variable-wavelength fluorimetric detection.

Owing to technical difficulty, until recently very few reports on the analysis of catechol and hydroquinone in biological fluids have been published [11–13]. A recent HPLC method described by Inoue *et al.* [12] using UV detection has been

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found to be non-specific and insensitive for low-level detection. An improved method has been developed by Schad *et al.* [13] to assay the urine of mice exposed to 300 ppm benzene. However, this method is impractical for human risk assessment, as the current occupational exposure limit for benzene exposure is much lower. Here, we propose a method that is more specific and reliable. Urinary hydroquinone, catechol and phenol at levels as low as 0.03, 0.2 and 0.3 mg/l, respectively, can be detected. This method was evaluated on 36 workers exposed to low concentrations of benzene as well as on 48 cigarette smokers and 42 non-smokers.

EXPERIMENTAL

Reagents and chemicals

Standards of the analytes of interest were purchased from three different chemical companies: hydroquinone from Fluka (Buchs, Switzerland), catechol from TCI (Tokyo, Japan), and phenol from BDH (Poole, UK). Sodium acetate, acetic acid, sodium sulphate, HPLC-grade methanol, diethyl ether and acetonitrile were obtained from Merck (Darmstadt, Germany). Distilled and deionized water was used for the preparation of all solutions.

Apparatus

Gradient elution HPLC was performed at ambient temperature using a Hewlett-Packard Model 1050 quaternary pumping system (Palo Alto, CA, USA) connected to a Gilson Model 231-401 autoinjector (Villiers-le-Bel, France). The analytical column used was a 110 mm × 4.7 mm I.D. Partisphere 5 C_{18} replaceable cartridge protected by a guard cartridge (C_{18}, 5 μm) (Whatman, Clifton, NJ, USA). Hydroquinone, catechol and phenol were detected with a Shimadzu Model RF-551 spectrofluorimetric detector (Kyoto, Japan) at different excitation (λ_{ex}) and emission (λ_{em}) wavelengths programmed for analysis as follows: 0–5 min, λ_{ex} 304 nm with λ_{em} 338 nm; 5–10 min, λ_{ex} 284 nm with λ_{em} 313 nm; and 10–15 min, λ_{ex} 274 nm with λ_{em} 298 nm. The slit width of the detector was set at 15 nm. For comparison, a UV–vis spectrophotometer (Hewlett-Packard Model 1050) was used for the detection of analytes at 280 nm and GC (HP Model 5890A) was used for phenol analysis. Analytes were characterized by retention time and quantitated by peak height with a Shimadzu Model CR-5A integrator.

Preparation of standards

Standards were usually prepared with methanol or diethyl ether by using a Hamilton syringe (Reno, NV, USA). Individual stock standards of 1, 5 and 10 mg/l hydroquinone, catechol and phenol, respectively, were prepared in methanol. A second stock solution containing 10 mg/l hydroquinone, 50 mg/l catechol and 100 mg/l phenol was prepared in diethyl ether. Standards for calibration, with concentrations ranging from 0.05 to 5 mg/l hydroquinone, 0.25 to 25 mg/l catechol and 0.5 to 50 mg/l phenol, were obtained by diluting the second stock solution 2- to 200-fold with diethyl ether (Table I). In order to have the same dilution factor as in sample preparation,

TABLE I

LINEARITY AND DAY-TO-DAY VARIATION

y = concentration (mg/l); x = peak height (mV); r = coefficient of correlation; C.V. = coefficient of variation; n = 5.

Compound	Concentration range (mg/l)	Mean linear regression	r	C.V. of slope (%)
Hydroquinone	0.05–5	$y = 4.922x - 0.03$	0.99	8.7
Catechol	0.25–25	$y = 2.286x - 0.07$	0.99	12.2
Phenol	0.50–50	$y = 8.726x - 0.02$	0.99	15.1

these standard solutions were further diluted four times with diethyl ether.

Urine sample collection and storage

Urine samples were collected from 90 male subjects. These included 36 refinery workers who were exposed to benzene concentrations ranging from less than 0.01 to 0.63 ppm. In addition, 34 smokers, smoking fewer than 30 cigarettes per day, and 20 non-smokers, with no known history of benzene exposure, also participated in this study. For the occupationally exposed group, urine samples before and after exposure were collected. A 10-ml aliquot of the collected urine sample was preserved with 100 μl of 6 *M* hydrochloric acid and stored frozen at −20°C until analysis.

Sample preparation and chromatographic conditions

A 500-μl urine sample was hydrolysed with 100 μl of concentrated hydrochloric acid at 95°C for 90 min. Following cooling to room temperature, approximately 150 mg of sodium sulphate was added. After vortex-mixing for 30 s, the sample was extracted with 1 ml of diethyl ether by vortex-mixing for 1 min, followed by centrifugation at 2000 *g* for 5 min at 4°C. The upper ether layer was transferred and capped in a test tube. The organic extraction was repeated with 1 ml of fresh ether. The two organic extracts were then combined and filtered. The extracted sample was kept in a crimp-capped amber sampling vial and 5 μl were used for HPLC analysis with fluorimetric detection.

The two buffer solutions used for gradient separation were 10 m*M* sodium acetate containing 0.5% (v/v) acetic acid. The pH was 3.4 for buffer A and 3.8 for buffer B, which contained an additional 20% (v/v) acetonitrile. The analysis started with 70% buffer A and increased to 95% from 2 to 7 min. It was gradually reduced to 75% at 14 min and changed to 95% buffer B from 15 to 18 min for column cleaning. The column was then re-equilibrated with 70% buffer A for 3 min before the next injection. The flow-rate was set at 1.0 ml/min from 0 to 9 min, 1.5 ml/min from 10 to 18 min and then back to 1.0 ml/min for column re-equilibration.

As for UV detection, the organic extraction procedure was the same as for fluorimetric detection. A 1-ml aliquot of ether extract was transferred to a test tube covered with aluminium foil. A small hole was left for sample drying at room temperature. After drying, the sample was reconstituted with 250μl of 50% (v/v) methanol–water and 5μl were used for HPLC analysis. The two mobile phase buffers were 15 m*M* sodium acetate containing 1% (v/v) acetic acid with pH adjusted to 4.3 for buffer 1 and 4.4 for buffer 2, which also contained 10% (v/v) acetonitrile. The analysis started with 95% buffer 1 for the first 9 min and changed to 95% of buffer 2 from 10 to 20 min. This was then followed by a 3-min post-run for column re-equilibration. The flow-rate was set at 1.0 ml/min.

For GC determination of urinary phenol, sample preparation and GC conditions were according to the method described by Ong *et al.* [15].

RESULTS AND DISCUSSION

Chromatographic separation

Simultaneous measurement of hydroquinone, catechol and phenol is generally performed using HPLC with UV detection [11,13]. In fact, because of the high boiling points of hydroquinone (286°C) and catechol (245°C), only phenol is suitable for analysis using the GC method [15–18]. However, the HPLC determination of these urinary phenolic compounds was found to be complex with UV detection, one of the major setbacks being matrix interferences. Fig. 1 shows the chromatogram of an aqueous standard and urine sample of a refinery worker exposed to low concentration of benzene detected at UV 280 nm. Despite thorough investigations for optimal chromatographic conditions and further dilution of sample, it was still impossible to separate catechol from other interfering matrix components (Fig. 1b). In contrast, using the proposed method with variable-wavelength fluorimetric detection, reliable determination of the analytes could be achieved within 15 min without interferences.

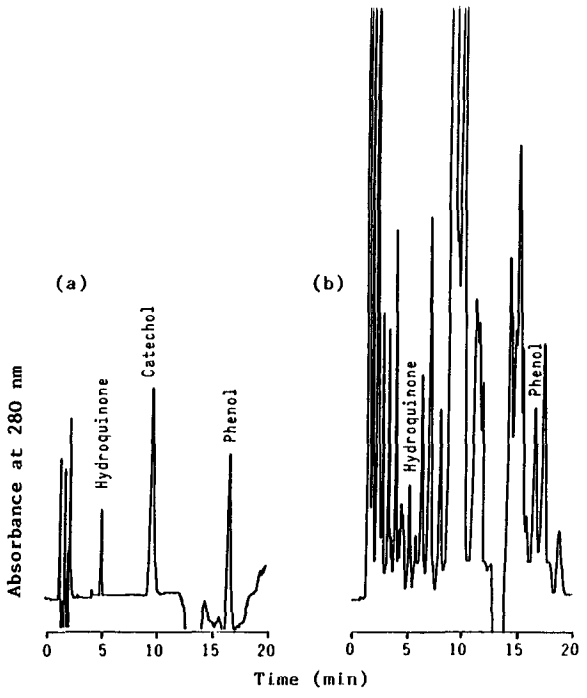

Fig. 1. Chromatograms of (a) a four times diluted pure standards solution and (b) a urine sample pretreated with the procedures described in the text, analysed by UV detection. The original concentration of standards (a) were 5, 25 and 50 mg/l hydroquinone, catechol and phenol, respectively. Chromatograms are presented with integrator attenuation set at 2 mV.

The excitation and emission wavelengths set for each component were the best detection conditions studied by Risner and Cash [19]. As shown in Fig. 2a, aqueous standards of hydroquinone (2.5 mg/l), catechol (12.5 mg/l) and phenol (25 mg/l) were detected at 2.9, 6.8 and 13.6 min, respectively. The retention times of the analytes in a blank and spiked urine samples in Fig. 2b and c were identical to those of known standards (Fig. 2a). Fig. 2d shows a chromatogram of the same urine sample as in Fig. 1b, which was analysed with the present method. The latter result was obviously very promising and reliable. The two chromatograms also demonstrate that fluorimetric detection is much more sensitive than UV detection. The lowest detection limit for fluorimetric detection at a signal-to-noise ratio of 3 was 40 pg of hydroquinone, 200 pg of catechol and 300 pg of phenol and, accordingly, urinary hydroquinone, catechol and phenol levels as low as 0.03, 0.2 and 0.3 mg/l, respectively, could be detected.

A comparison of fluorimetric and UV detection for hydroquinone analysis was made using 70 sets of urine samples (Fig. 3). The results showed good agreement between the two methods with a correlation coefficient (r) of 0.95. Fig. 4 also shows good correlation between the HPLC and GC methods for urinary phenol analysis. These results suggest that the present method is reliable and is convenient for simultaneous determination of benzene metabolites in the urine.

Optimum conditions for sample preparation

Using the present method, the sample preparation time was about 4 h for 40 samples, which included 90 min incubation time for acid hydrolysis. The hydrolysis conditions described here were the optimal conditions determined experimentally by Ong *et al.* [15]. The use of acid hydrolysis offers a convenient and reliable technique as compared with lengthy enzymatic digestion [13,16]. Traditionally, sodium chloride has been used for sample saturation before organic separation [15–18]. In the present study, urine samples were treated with sodium chloride for urinary phenol analysis by the GC method [15], but sodium sulphate was used for the proposed procedure. It is interesting to note that the same samples, treated with two different salts and analysed under the same HPLC conditions, gave similar results of hydroquinone and phenol, but different results for catechol (Fig. 5). Samples treated with sodium sulphate were found to have significantly higher catechol levels than those treated with sodium chloride ($p < 0.001$). A lower recovery was observed when sodium chloride was used. On the other hand, it was also noted that the recovery of catechol and phenol was seriously affected by the drying time and drying temperature. Therefore, the proposed method avoided the tedious procedure of drying and reconstitution of analytes. A 5-μl volume of the diethyl ether extract was directly used for HPLC analysis. So far, over 300 extracted samples have been analysed and no adverse effects on column efficiency have been noted.

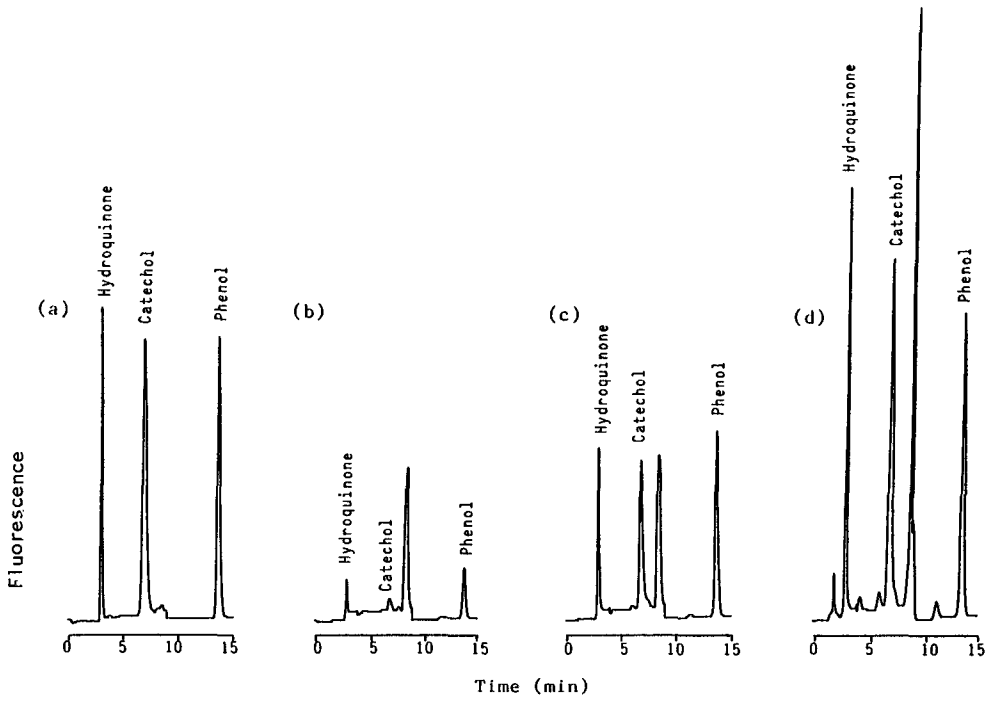

Fig. 2. Chromatograms of standards and samples with the same dilution factor (four-fold), analysed by fluorimetric detection: (a) an aqueous standard containing hydroquinone, catechol and phenol with original concentrations of 2.5, 12.5 and 25 mg/l, respectively; (b) a blank urine sample; (c) the same sample spiked with 1 mg/l hydroquinone, 5 mg/l catechol and 10 mg/l phenol; (d) the same urine sample as in b. Chromatograms were presented with integrator attenuation programmed as follows: at 0 min, 32 mV; 4 min, 64 mV; 9 min, 512 mV.

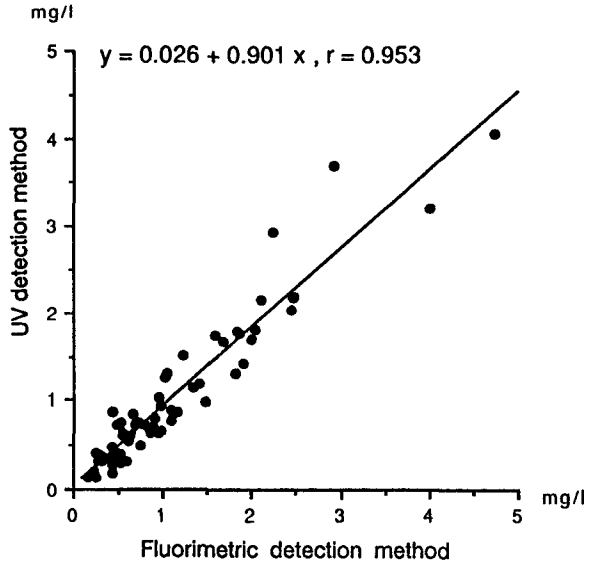

Fig. 3. Comparison of two detection methods for urinary hydroquinone analysis.

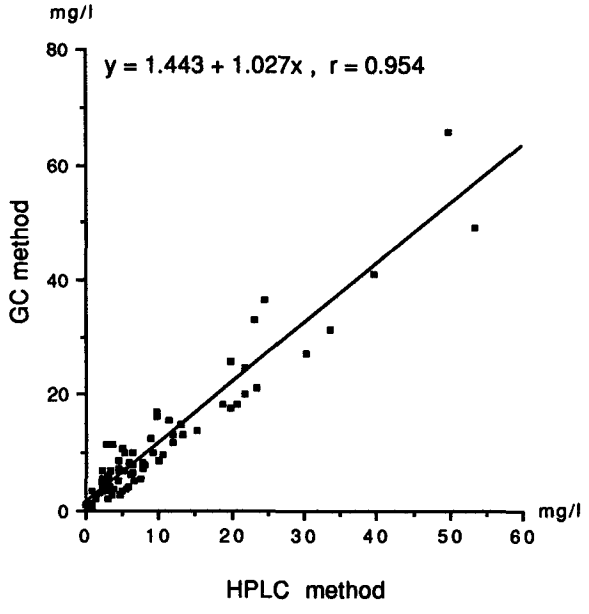

Fig. 4. Comparison of two analytical methods for urinary phenol analysis.

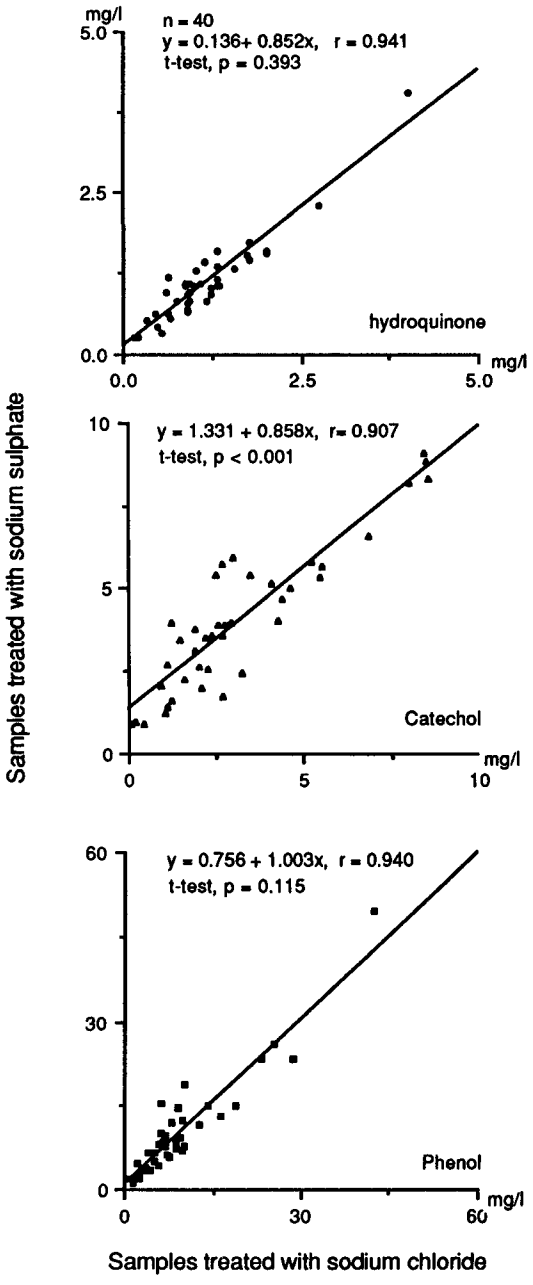

Fig. 5. Comparison of results obtained from samples treated with sodium sulphate and treated with sodium chloride.

Calibration, recovery and reproducibility

The amount of the analytes was quantified by using external standards. The calibration curves were linear from 0.05 to 5 mg/l for hydroquinone, 0.25 to 25 mg/l for catechol and 0.5 to 50 mg/l for phenol. Slopes of the calibration curves and correlation coefficients of five daily calibrations are presented in Table I. The day-to-day variation of calibration was less than 15%. For recovery and reproducibility studies, pooled urine samples were spiked with various concentrations of three analytes (Table II). The pooled and spiked samples were processed and analysed four times within a day as well as on three consecutive days. The individual recovery of analytes, spiked with various concentrations in urine, was generally over 90% (Table II). The results of reproducibility are also presented, with within-day and between-day coefficients of variation (C.V.) of 3.1 and 5.6% for hydroquinone, 3.8 and 7.6% for catechol, and 3.6 and 8.1% for phenol, respectively.

Phenolic metabolites and exposure to low concentrations of benzene

A total of 36 urine samples collected from refinery workers exposed to benzene concentrations of 0.01–0.63 ppm for 8 h were analysed for hydroquinone, catechol and phenol. The mean values obtained before and after exposure are presented in Table III. Although smoking workers were noted to have higher concentrations of catechol and hydroquinone, no significant increase in phenolic compounds was observed after exposure to benzene for both smoking and non-smoking workers. This finding is in agreement with several other reports that urinary phenolic compounds are not useful biological markers for exposure to benzene below 1 ppm [7–10,14].

Measurements of urinary hydroquinone and catechol in cigarette smokers

It has been observed that cigarette smoke contains several phenolic compounds such as hydroquinone, catechol and phenol in significant amounts, in addition to benzene [10,20]. A comparative study of urinary excretion of these compounds was carried out on 48 smokers and 42 non-smokers. The results are summarised in Table IV. The mean values of urinary hydroquinone and catechol obtained for non-smokers were 0.58 and 2.64 mg/l (0.56 and 2.12 mg/g creatinine, re-

TABLE II

PRECISION AND RECOVERY OF ANALYSIS (n = 4)

Sample	Mean (mg/l)	Mean recovery (%)	Coefficient of variation (%)	
			Within-day	Between-day
Hydroquinone				
Blank	0.25	–	5.9	6.8
+ 0.1 mg/l	0.35	100	2.8	4.0
+ 1.0 mg/l	1.21	96	2.7	7.7
+ 5.0 mg/l	4.71	89	0.9	3.8
Mean		95	3.1	5.6
Catechol				
Blank	0.76	–	6.4	9.2
+ 0.5 mg/l	1.24	96	4.5	8.1
+ 5.0 mg/l	5.54	96	3.4	8.6
+25.0 mg/l	24.16	94	0.8	4.4
Mean		95	3.8	7.6
Phenol				
Blank	4.10	–	1.9	9.5
+ 1.0 mg/l	5.13	103	4.8	12.6
+10.0 mg/l	13.01	89	4.3	6.7
+50.0 mg/l	45.84	83	3.3	3.7
Mean		92	3.6	8.1

spectively). These values are about half those of cigarette smokers, which were 1.18 and 4.75 mg/l (0.84 and 3.53 mg/g creatinine), respectively. The differences were significant with p values less than 0.05, as shown in Table IV. On the other hand, it was noted that urinary phenol concentrations did not appear to be different between the two groups. This may result from the high back-

TABLE III

MEAN VALUES OF URINARY RESULTS FOR 36 REFINERY WORKERS BEFORE AND AFTER EXPOSURE TO BENZENE CONCENTRATIONS RANGING FROM <0.01 TO 0.63 ppm

Compound	Mean ± S.D. (mg/l)		t-Test p value	Mean ± S.D. (mg/g creatinine)		t-Test p value
	Before exposure	After exposure		Before exposure	After exposure	
Non-smokers (n = 22)						
Hydroquinone	0.60 ± 0.43	0.57 ± 0.37	0.762	0.57 ± 1.01	0.43 ± 0.17	0.501
Catechol	2.77 ± 1.64	2.31 ± 1.34	0.238	2.17 ± 2.21	1.76 ± 0.75	0.361
Phenol	10.96 ± 12.45	8.33 ± 7.33	0.330	6.54 ± 5.19	5.82 ± 3.98	0.338
Smokers (n = 14)						
Hydroquinone	1.37 ± 1.09	1.46 ± 1.24	0.805	1.03 ± 0.55	0.87 ± 0.34	0.355
Catechol	5.86 ± 5.33	4.46 ± 3.28	0.231	4.25 ± 2.49	3.21 ± 1.98	0.118
Phenol	8.91 ± 12.25	9.01 ± 11.03	0.975	5.53 ± 5.06	4.70 ± 3.07	0.430

TABLE IV

MEAN VALUES OF URINARY RESULTS FOR 42 NON-SMOKERS AND 48 SMOKERS

Compound	Mean ± S.D. (mg/l)		*t*-Test *p* value	Mean ± S.D. (mg/g creatinine)		*t*-Test *p* value
	Non-smokers	Smokers		Non-smokers	Smokers	
Hydroquinone	0.58 ± 0.42	1.18 ± 0.90	<0.001	0.56 ± 0.81	0.84 ± 0.45	0.046
Catechol	2.64 ± 1.61	4.75 ± 3.93	0.002	2.12 ± 1.85	3.53 ± 2.54	0.004
Phenol	10.54 ± 11.65	6.73 ± 8.21	0.074	8.59 ± 12.24	4.49 ± 3.96	0.031

ground of phenol present in many foodstuffs and from the metabolism of amino acids [5–10,14].

CONCLUSION

In conclusion, the use of acid hydrolysis, sodium sulphate and diethyl ether extraction provides a convenient and efficient procedure of sample preparation for urinary analysis of hydroquinone, catechol and phenol using HPLC. Further purification steps such as solid-phase extraction described in a recent report [13] were unnecessary for the analysis with fluorimetric detection. The proposed method can be considered specific, reliable and time- and cost-effective for simultaneous determination of these phenolic compounds in urine. It is therefore useful for risk assessment for exposure to phenolic compounds.

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