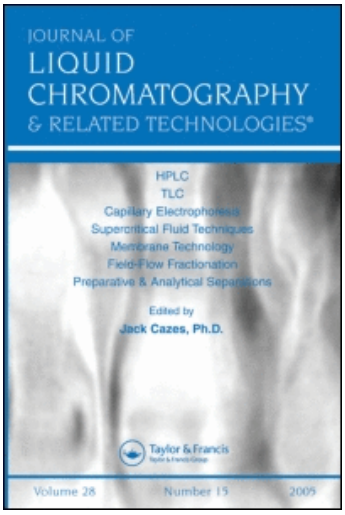

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OFF LINE EXTRACTION OF PHENOL FROM HUMAN URINE SAMPLE WITH ISOAMYL ALCOHOL AND DETERMINATION BY HPLC

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ABSTRACT

This method has been developed for extraction and determination of phenol in a urine sample by high performance liquid chromatography.

After acid hydrolysis, the free phenol was extracted with isoamyl alcohol solvent, followed by back extraction with 0.5 $mol \cdot L^{-1}$ sodium hydroxide solution and analyzed by an isocratic HPLC Varian System, equipped with reverse-phase column (MicroPak-C_{18}). The mobile phase was acetonitrile in 0.01 $mol \cdot L^{-1}$ hydrochloric acid solution, (20:80 v/v), and at a flow-rate of 1.0 mL·min-1. The chromatogram was monitored at 220 nm in room temperature. The identification was based on retention time and the quantification was performed by automatic peak-area determination, corrected for the external standards method.

The recovery was higher than 99.5 % for phenol and reproducibility of method was shown to be 2.3% standard deviation and 5.6% coefficient of variance (n=20). The limit detection was 0.05 mgL^{-1} and a range of 0.05 to 20.0 mgL^{-1} of phenol for linearity.

INTRODUCTION

Monitoring of phenolic compounds in biological fluid is important in clinical chemistry, occupational medicine, and clinical toxicology as a biological indicator of human exposure to aromatic hydrocarbons. The benzene exposure is not the only source of urinary phenol. Several drugs, such as phenyl-salicylate, zinc phenyl-sulphate, phenobarbiturate, nitro compounds, and phenols are metabolized by the liver and their sulphates of glucuronates are excreted in urine as phenol conjugates. It has been known that long time inhalations of benzene in the atmospheric air, can cause toxic symptoms, while much less exposure by inhalation or ingestion can induce mucous irritations, convulsions, depression, excitation, etc.[1]

The great number of respiratory system cancers have been associated with phenol in Finnish woodworkers.[2] Benzene, its phenol metabolite, is considered as part of a carcinogen group.[3] Likewise phenols and dihydroxybenzenes are well known components of cigarette smoke condensate. They are formed from the pyrolysis of tobacco during the smoking process, contributing to the amounts of phenols excreted in urine. The human diet also contributes to the rise of phenol in urine. This is an individual characteristic and varies with nutrition, smoking habit, etc.[4,5]

Several methods have been developed for the determination of free phenol and phenol conjugates, especially spectrophotometry, based on the indophenol reaction to determinate phenol in water or biological fluids,[6] gas chromatography methods,[7] and high performance liquid chromatography methods.[8,9]

Sample preliminary treatment is usually carried out with steam distillation,[10] solid-liquid extraction,[11-13] or on line liquid chromatographic precolumn-based column-switching techniques,[14] for extraction phenolic compounds from feces and urine samples.

After hydrolysis, an additional clean-up step of the sample is frequently made through liquid-liquid partition with solvents such as dichloromethane,[1] diethyl ether,[15] and hexane,[16] to extract the phenolic compounds, from the urine sample.

This work describes the analysis of phenol in urine samples. After hydrolysis the phenol was extracted from the human urine sample with isoamyl alcohol followed for back extraction with 0.5 mol·L^{-1} sodium hydroxide solution and analyzed by HPLC system.

EXPERIMENTAL

Chemical and Solvents

Acetonitrile was obtained from Carlo Erba (Milan, Italy), isoamyl alcohol , sodium hydroxide pellets, phenol.and hydrochloric acid, were obtained from Merck(E. Merck RgaA, Darmstadt, Germany), all of than were HPLC grade. The pure water was obtained with Milli-Q unit (Millipore, Bedford,MA,USA).

The urine samples were obtained from healthy men and woman exposed or unexposed. To the stock standards, solutions were prepared by dissolving known amounts of phenol in water to obtain 0.25 to 2.0 μg·L^{-1} concentration.

Sample and Phenol Standard Preparation

The acid hydrolysis of each sample was done with the addition of 5.0 mL of sample in test-tubes followed by 1.0 mL of 12.0 mol·L^{-1} hydrochloric acid and the mixture were submerged in a water at 100°C for 30 minutes. The same process was used on the standard solutions. After hydrolysis, the standard solutions were diluted 20 fold with water to obtain: 0.25, 0.50, 1.00, 1.50 and 2.00 μg·L^{-1} phenol standard solutions. After hydrolysis, the urine samples were centrifuged at 2500 rpm for 5 minutes and diluted 40 fold with water. All the solutions were submitted to extraction, transferring 1.0 mL of the solution to test-tubes, followed by the addition of the 1.0 mL of isoamyl alcohol, previously saturated with 6.0 mol·L^{-1} hydrochloric acid solution. The tubes were shook by vortex mixing for 2 minutes. Aliquots of 0.5 mL of each organic phase was transferred into another test-tube, followed by the addition of 0.5 mL 0.5 mol·L^{-1} sodium hydroxide solution, and the mixture was shook by vortex mixing for 2 minutes. The phenol returned to the aqueous phase. Then 10 μL of this alkaline solution was injected into the HPLC at room temperature.

Instrumentation

Chromatographic experiments were performed using a Varian Model 2510 Liquid Chromatograph reciprocating pump, equipped with a Varian Model 2550 UV-visible variable wavelength detector set at 220 nm, and a SP 4400 chromajet

Table 1

Phenol Recovery Study using Extraction Technique with Isoamyl Alcohol

Phenol Standard ($mg \cdot L^{-1}$)	Recovery (%) N=9
0.250	104.5±5.0
0.500	106.9±4.6
1.000	102.4±4.3
1.500	99.5±1.3
2.00	103.3±1.0

integrator system, was obtained from Varian Associates, Inc. (Sunnyvale, CA, USA). The samples and standards solutions were injected on MicroPak RP-C_{18} column, with a manual injector (Rheodyne 7125, Cotati, CA, USA), fitted with a *10 μL loop*.

Chromatographic Conditions

The column used was a MicroPak RP-18 (300 mm x ID 4.6 mm), and was obtained from Varian Associates, Inc. (Sunnyvale, CA, USA). The particle size of the spherical packing material of the column was 10 μm.

Separation was conducted at room temperature using flow-rate of 1.0 $mL \cdot min^{-1}$. The mobile phase used was composed by acetonitrile in 0.01 $mol \cdot L^{-1}$ hydrochloric acid solution, (20:80 v/v).

RESULTS AND DISCUSSION

The first aim in this work was to develop a method to simplify the clean-up and efficient recovery of phenol based on liquid extraction. All previous methods published are more complicated, using hydrolysis and extraction, because they require sophisticate apparatus and complex reagents. This novel method does not require complex equipment or chemical reagents.

The extraction with isoamyl alcohol was explored using the different acid-base properties and solubility of the phenol. The salts of phenol are insoluble in organic solvents, so it was possible to do the extraction employing the process known as back extraction.

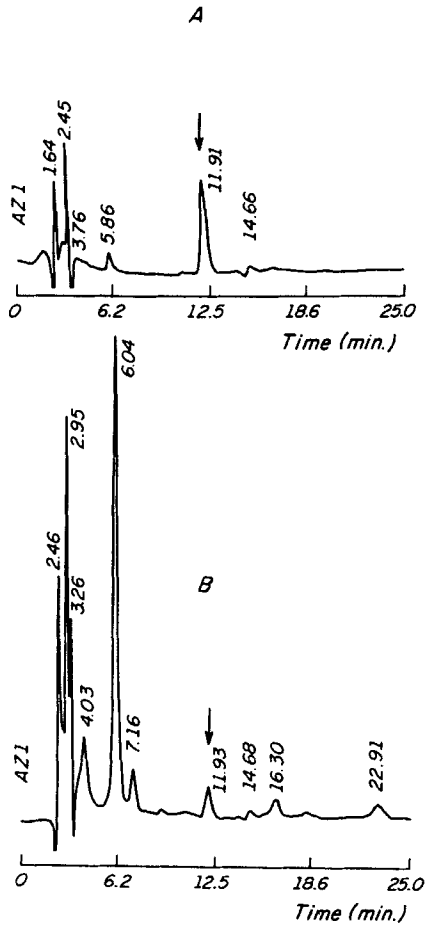

Figure 1. Chromatograms: (A) 2.0 $mg \cdot L^{-1}$ of phenol standard. (B) of a urine sample. Both were injected after back extraction with 0.5 $mol \cdot L^{-1}$ NaOH. The arrow indicates the peak and retention time of phenol. Chromatographic conditions: RP - C_{18} (300 mm x 4.6 mm) column, mobile phase: acetonitrile in 0.01 $mol \cdot L^{-1}$ hydrochloric acid (20:80 v/v); flow-rate 1.0 $mL.min^{-1}$, room temperature , detection UV at 220 nm, injection volume: 10 μL.

It consisted of two steps. In the first, the phenol is transferred to organic phase (isoamyl alcohol), and in the second, with addition of 0.5 $mol \cdot L^{-1}$ sodium hydroxide solution, the phenol salt returned to the aqueous phase in the sodium phenolate form.

The recoveries obtained in the extraction method was checked, by doing three extractions of different phenol concentration (0.25, 0.50, 1.00, 1.50, and 2.00 $\mu g \cdot L^{-1}$), followed by nine injections for each extraction, as can be seen in Table 1. The results obtained by this method yielded good recoveries; higher than 99.5% in all assayed extractions, indicating good precision in the extraction method.

The reproducibility of this method was performed by a statistical method [standard deviation, (Sd %) and coefficient of variance, (CV %)], where the obtained results were satisfactory. The values found for the standard deviation 2.3% and coefficient variance 5.6% (n=20) indicated the method resulted in good precision.

The detection limit was determined by measuring the minimum amount which was injected to provide a peak signal approximately twice the noise. Thus, we found a minimum value of 0.05 $mg \cdot L^{-1}$ of phenol. The linearity was determined in the range 0.05 to 20 $mg \cdot L^{-1}$. In order to determine the phenol in real samples, we studied the dilution of the sample. There are many metabolites in urine samples after the hydrolysis, so is necessary to do a dilution followed by extraction of metabolites from the sample with isoamyl alcohol. The best dilution of urine samples were obtained in 1/40. When isoamyl alcohol was used, an impurity solvent peak appeared at the 14.66 ± 0.02 minutes retention time, but it did not cause any interference with the phenol analyzed as can be seen in Figure1 (A and B chromatograms). The applicability of this procedure was checked with the analysis of real samples. Figure 1B, shows a urine sample chromatogram of a patient, analyzed on the RP-C_{18} column, after isoamyl alcohol off line extraction. Twenty urine samples from workers exposed and unexposed to phenol and benzene were examined; the results obtained, as can be seen in the Table 2. The values found were less than biological limits of exposure adopted by American Conference of Governmental Industrial Hygienists (ACGIH) (30 $mg \cdot L^{-1}$).[17]

The phenol can be determined without interference, with retention time 11.93 ±0.02 minutes, as can be seen Figure 1, A and B chromatograms.

As is seen in the B chromatogram (Figure 1), there are many metabolic peaks in urine samples, but our main purpose was in the determination of phenol.

In all the samples examined, many unknown peaks were detected. These peaks are not the same in all patients, because of their diet and metabolism. Over all, this is a very simple method, yielding excellent results with a small urine sample and simple reagents.

Table 2

Validation Data for Phenol Determination in Twenty Real Urine Samples, using Liquid Extraction Technique with Isoamyl Alcohol

Patient Number	$C_{phenol}=C_a x F_d$* (mg.L^{-1})	CV (%)
001	19.4±0.01	0.06
002	4.5±0.12	2.70
003	11.1±0.16	1.40
004	9.6±0.24	2.50
005	6.9±0.20	2.80
006	4.6±0.01	0.30
007	0.000	
008	2.8±0.03	1.00
009	3.8±0.04	1.00
010	5.9±0.12	2.00
011	12.7±0.02	0.15
012	0.000	
013	3.5±0.08	2.20
014	10.6±0.12	1.10
015	15.2±0.04	0.30
016	9.2±0.03	0.30
017	22.8±0.16	0.70
018	6.9±0.16	2.30
019	8.1±0.12	1.40
020	9.4±0.03	0.30

* C_{phenol}- phenol concentration, C_a-analytical concentration, F_a-dilution factor (1/48).

CONCLUSIONS

This novel performed method has shown good recoveries, of higher than 99.5% and detection limit 0.05 mgL^{-1} for phenol.

The proposed method is adequate by determination and to quantify the phenol in real urine samples with great precision without interference of either compound present in the urine samples.

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