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Short communication

Gas chromatographic–mass spectrometric identification and quantitation of urinary phenols after derivatization with 4-carbethoxyhexafluorobutyryl chloride, a novel derivative

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Abstract

Urinary phenol is analyzed widely to determine benzene exposure in humans. Most methods utilize direct measurements of phenols after extraction from urine using gas chromatography or high-performance liquid chromatography. We describe a novel derivatization of urinary phenols using 4-carbethoxyhexafluorobutyryl chloride after extraction from urine and subsequent analysis by gas chromatography–mass spectrometry. The derivative elutes at significantly higher temperature than phenol and the method is free from interferences from more volatile components in urine. We also observed excellent chromatographic properties of these derivatives. In addition, we observed strong molecular ions for the 4-carbethoxyhexafluoro butyryl derivative of phenol (*m/z* 344), *p*-cresol (*m/z* 358) and the internal standard 3,4-dimethylphenol (*m/z* 372) and other characteristic ions in the electron ionization, thus aiding in unambiguous identification of these compounds. The protonated molecular ions (*m/z* 373 for derivatized phenol, *m/z* 359 for derivatized *p*-cresol and *m/z* 373 for the internal standard) were the base peaks (relative abundance 100%) in the chemical ionization, although other secondary peaks were less abundant. The assay is linear for phenol concentration of 1–100 mg/l. The within-run and between-run precisions were 4.8% ($\bar{X}$=52.4, S.D.=2.5 mg/l) and 8.1% ($\bar{X}$=53.0, S.D.=4.3 mg/l) respectively, and the detection limit was 0.5 mg/l.

Keywords: Phenols; 4-Carbethoxyhexafluorobutyryl chloride

1. Introduction

Benzene has been classified as a group I carcinogen by the International Agency for Cancer Research [1]. Benzene is an important constituent of gasoline as well as tobacco smoke [2]. Benzene is also widely used in chemical industry, especially in the paint and dye industry [3]. Absorption of benzene occurs through inhalation of vapors and secondarily through skin contact. A fraction of benzene is excreted unchanged in the exhaled air and the remaining fraction is metabolized in the liver and excreted in the urine. Bone marrow depression leading to leukopenia (depressed white blood cell count) is one of the most serious side effects of prolonged benzene exposure. Benzene can be detected in exhaled air, blood and urine [4–6]. Measuring the amount of benzene exposure through air exhalation does not provide accurate results because it is dependent on the sampling technique and time of collection. Blood sampling is not a preferred method due to it's invasive nature. Therefore, urinary

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metabolites of benzene are commonly measured to evaluate benzene exposure. The primary metabolite of benzene is phenol. Phenol and its oxidation products are excreted as glucuronide or sulfate conjugates in urine. The most commonly used methods for determination of urinary phenol are high-performance liquid chromatography (HPLC) and gas chromatography (GC) [7–13]. HPLC methods using electrochemical detection are generally less suitable for routine use than GC methods [14]. However, the polarity and volatility of phenol may cause reproducibility problem in the GC methods [10,15]. Moreover, in the GC method, the identification of phenol is solely based on the retention time. Other urinary phenols like *p*-cresol have similar retention times as phenol, therefore misidentification problems exist. Moreover, phenol is a small molecule and is not ideal for gas chromatography–mass spectrometric (GC–MS) analysis. A new derivatization technique of phenol which could lead to a less volatile derivative and produce strong peaks at a much higher mass range, could be useful for unambiguous confirmation and quantitation of phenol in human urine. In this paper we report identification and quantitation of urinary phenol as its 4-carbethoxyhexafluorobutyryl derivative which produced a strong molecular ion at *m/z* 344 (molecular mass of underivatized phenol: 94) in the electron ionization mode.

2. Materials and methods

Phenol, phenyl glucuronide, *p*-cresol and the internal standard 3,4-dimethylphenol were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). The derivatizing reagent 4-carbethoxyhexafluorobutyryl chloride was obtained from PCR Chemicals (Gainsville, FL, USA). The GC–MS analysis was performed by a Model 5890 gas chromatograph coupled with a 5970 series mass selective detector for electron ionization and a 5890 series II gas chromatograph coupled to a 5972 series mass selective detector for chemical ionization spectra (Hewlett-Packard, Palo Alto, CA, USA). An Ultra 1 column also obtained from Hewlett-Packard was used in the gas chromatograph. The length of the column was 25 m while the diameter was 0.2 mm. The inside of the column was coated with methyl silicone with a film thickness of 0.33 μm. The initial oven temperature of the gas chromatograph was 120°C. After maintaining that temperature for 7 min, the oven temperature was increased at a rate of 7°C/min to reach an oven temperature of 160°C. Then, the oven temperature was increased at a rate of 15°C/min to reach a final oven temperature of 290°C. The final oven temperature was maintained for an additional 2 min. The injector port temperature was 250°C and the run time was 23.38 min. We used split less injection and the mass spectrometer was operated either in the scan mode (*m/z* 50–500) or selected ion monitoring mode (ions scanned: *m/z* 77, 94, 108, 119, 122, 195, 223, 344, 358 and 372). The carrier gas was helium with a column flow-rate of 0.29 ml/min and a linear velocity of 21 cm/s. For chemical ionization mass spectrometric analysis, we used methane as a reagent gas.

We analyzed urine samples from volunteers with no known exposure to benzene with and without adding either phenol or phenyl glucuronide. The internal standard, 3,4-dimethylphenol (500 mg/l stock solution) was added (200 μl) to a 2-ml aliquot of urine supplemented with phenol. Then phenol, along with the internal standard, was extracted in 10 ml of chloroform. When urine was supplemented with phenyl glucuronide, we added 0.5 ml concentrated hydrochloric acid to a 2-ml aliquot of urine. The acid hydrolysis was performed by heating the mixture at 95°C for 90 min as described by Ong et al. [16]. After cooling the sample, we added the internal standard (200 μl) and then sodium chloride. Urinary phenol along with the internal standard were again extracted in 10 ml of chloroform. We also performed enzymatic hydrolysis using glucuronidase (Sigma Chemical Company). We added urine specimen along with the internal standard into the ready-to-use vial containing the enzyme. The specimen was incubated at 56°C for 3 h, followed by extraction of phenol along with the internal standard in chloroform.

For derivatization, the organic phase was evaporated under nitrogen at room temperature. Then 50 μl of 4-carbethoxyhexafluorobutyryl chloride was added to the dry extract followed by incubation at 80°C for 20 min. After derivatization, the excess derivatizing agent was evaporated and the residue

was reconstituted with 50 μl of ethyl acetate. We injected 1–2 μl into the GC–MS system for further analysis. The quantitation of the phenol peak was done by comparing the area under the curve with the area under the curve for the internal standard which eluted after the derivatized phenol peak.

3. Results and discussion

3.1. Mass spectral characteristics of derivatized phenol

We observed baseline separation between phenol,

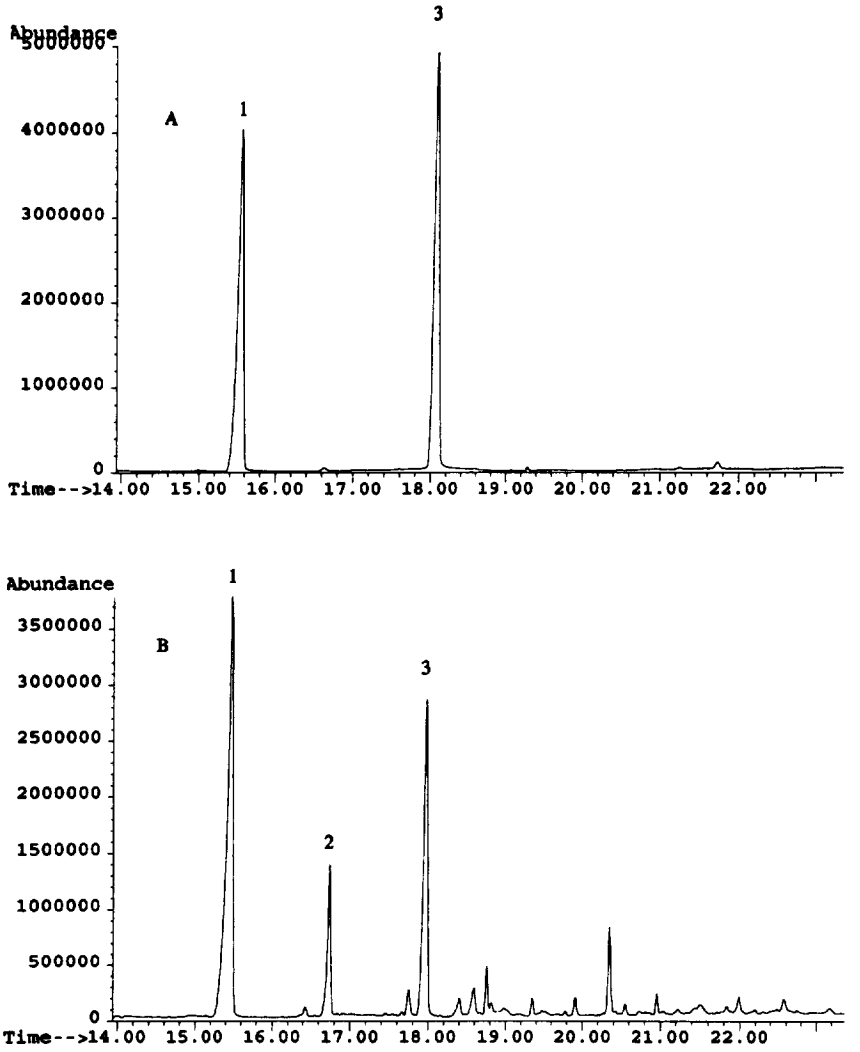

Fig. 1. Total ion chromatograms of typical urinary extracts. (A) Urine supplemented with 40 mg/l of phenol; peak 1, derivatized phenol; peak 3, derivatized internal standard. (B) Urine supplemented with phenyl glucuronide. The urinary phenol was extracted after acid hydrolysis; peak 1, derivatized phenol, peak 2; derivatized cresol which was endogenously present in the urine, peak 3; derivatized internal standard. The cresol peak was absent in the chromatogram (A) because no acid hydrolysis was performed. Both chromatograms were obtained by using mass spectrometry in the selected ion monitoring mode. Time was expressed in min.

p-cresol and the internal standard after derivatization with 4-carbethoxyhexafluorobutyryl chloride. Phenol eluted first from the column followed by *p*-cresol and internal standard. We observed excellent chromatographic properties of these peaks. Since these derivatized phenols elute at a much higher temperature than the underivatized phenol, our analysis is free from interferences from volatile components of the urinary matrix (Fig. 1).

Phenol is a small molecule with a molecular mass of 94. In our novel derivatization protocol, the molecular mass of the 4-carbethoxyhexafluorobutyryl derivative of phenol was 344. We observed a strong molecular ion at *m/z* 344 (relative abundance 57.9%) using electron ionization. The base peak was observed at *m/z* 94 [$M+H-COCF_2CF_2CF_2$-$COOC_2H_5$]. We also observed another strong characteristic peak at *m/z* 77 [$M-OCOCF_2CF_2CF_2$-$COOC_2H_5$]. The peaks at *m/z* 251, 223 and 195 were probably derived from the 4-carbethoxyhexafluorobutyryl moiety. In the chemical ionization mode, using methane as a reagent gas, we observed a protonated molecular ion at *m/z* 345 as the base peak. Another characteristic peak at *m/z* 94 was also

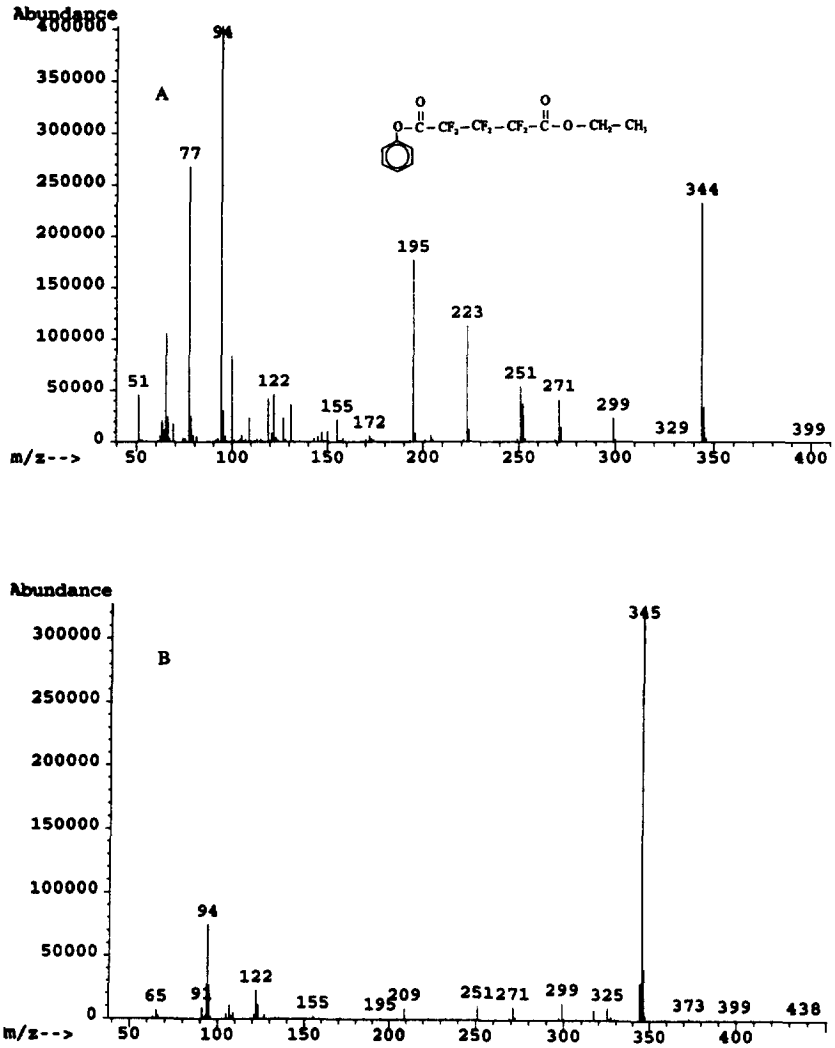

Fig. 2. Mass spectra of the 4-carbethoxyhexafluorobutyryl derivative of phenol. (A) Electron ionization, (B) chemical ionization.

observed (Fig. 2). In the electron ionization, the 4-carbethoxyhexafluorobutyryl derivative of *p*-cresol showed a strong molecular ion at *m/z* 358 (relative abundance 58.8%). The base peak was shifted to *m/z* 108 due to the addition of one methyl group to the benzene ring. Again, we observed strong peaks at *m/z* 223 and 195. In the chemical ionization, we observed the protonated molecular ion as the base peak at *m/z* 359. Another strong peak was also present at *m/z* 108 (Fig. 3). The 4-carbethoxyhexafluorobutyryl derivative of internal standard, 3,4-dimethylphenol also showed a strong molecular ion at *m/z* 372 (relative abundance 41.0%), as expected. The base peak was observed at *m/z* 121. We also observed other strong peaks at *m/z* 223, 195, 122 and 77. In the chemical ionization, we observed a base peak at *m/z* 373, which is the protonated molecular ion. We also observed another strong peak at *m/z* 123 (Fig. 4).

Because we observed strong molecular ions for derivatized phenol, *p*-cresol and the internal standard, the further experiments to determine precision and accuracy of the assay was carried out using electron ionization mode of operation of the mass spectrometer.

3.2. Acid vs. enzymatic hydrolysis

Ong et al. demonstrated that acid hydrolysis at 95°C for 90 min is sufficient for complete release of phenol from its conjugates [16]. Ahmad et al. reported multiple late-eluting peaks on the chromatograph following acid hydrolysis, but a much cleaner chromatogram using enzymatic hydrolysis [14]. Our

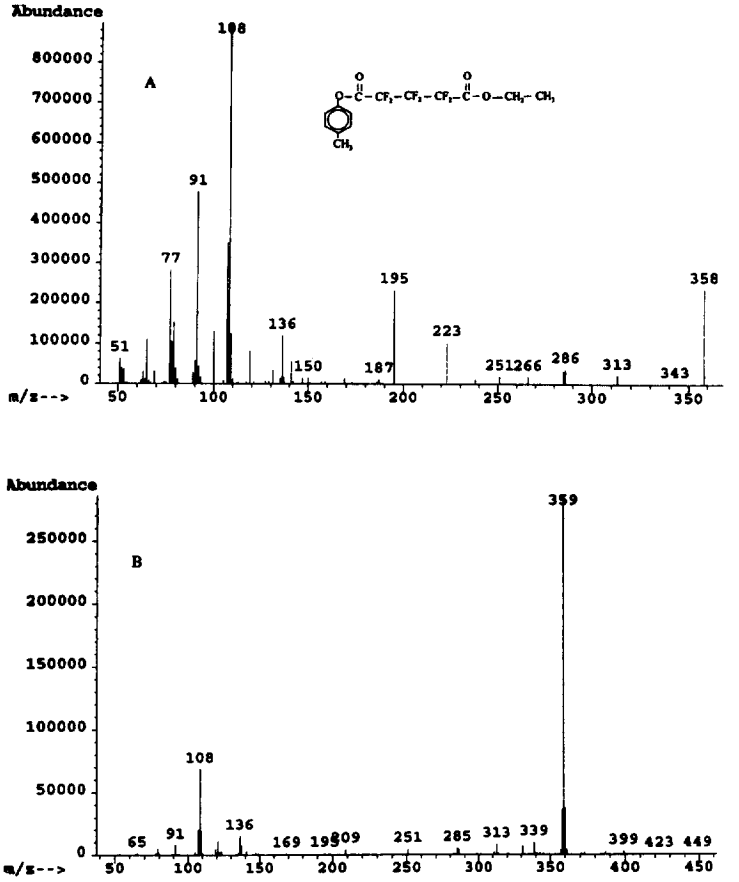

Fig. 3. Mass spectra of the 4-carbethoxyhexafluorobutyryl derivative of *p*-cresol. (A) Electron ionization, (B) chemical ionization.

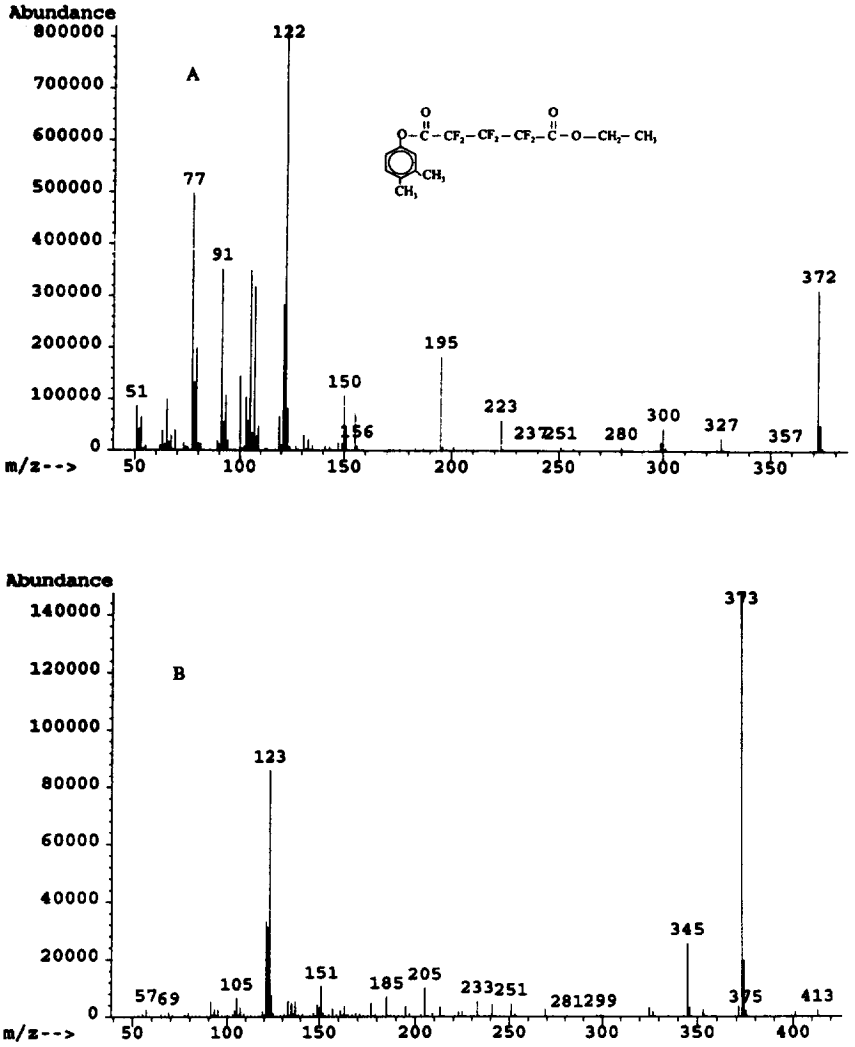

Fig. 4. Mass spectra of 4-carbethoxyhexafluorobutyryl derivative of 3,4-dimethylphenol, the internal standard. (A) Electron ionization, (B) chemical ionization.

results are in agreement with those of Ahmad et al. and we also observed several late-eluting peaks in the chromatograph, even using our novel derivatization technique. Therefore, the column needs to be conditioned for an additional 10 min at 290°C after completion of the run and prior to the next injection when we used the acid hydrolysis technique to release phenol from its conjugates. However, we observed a much cleaner chromatogram using the enzymatic hydrolysis protocol and additional conditioning of the column after completion of the run was not necessary.

3.3. Precision, linearity and detection limit

The precision of the assay was determined by using an aqueous standard of phenol (50 mg/l). Ahmad et al. also used aqueous phenol standard for the determination of precision and linearity of their assay for urinary phenol using capillary gas chromatography [16]. The C.V. of within-run precision was 4.8% ($\bar{X}$=52.4 mg/l, S.D.=2.5 mg/l, n=6), while the C.V. of between-run precision was 8.1% ($\bar{X}$=53.0 mg/l, S.D.=4.3 mg/l, n=5). The linearity and detection limits were also determined using aqueous

standards of phenol with various concentrations. Again, Ahmad et al. also used aqueous phenol standards for determining the linearity and detection limit of their assay [16]. The assay was linear from 1–100 mg/l of phenol concentrations. Using x-axis as the target concentration and the y-axis as the observed concentration in the linearity study, we obtained the following regression equation: $y = 1.02x + 1.4$ ($r = 0.99$). The detection limit was 0.5 mg/l of aqueous phenol concentration using scan mode of operation of the mass spectrometer.

3.4. Application of the assay

Exposure to benzene vapor is a serious health hazard in industry. The European Community Benzene Directives calls for an action level of 1 ppm benzene and a limit value of 5 ppm time-weighted average [17]. With new evidence of the risks from benzene exposure associated with neoplasia, the American Conference of Government Industrial Hygienists has proposed to lower the threshold limit for 8-h exposure to 0.1 ppm [18]. Although, other markers of benzene exposure had been proposed for example, *trans-trans* muconic acid, measurement of urinary phenol is most commonly used to monitor exposure to benzene. The currently employed GC techniques without derivatization uses only retention time to identify phenol. Moreover, the peaks elute at a relatively lower temperature due to high volatility. In our novel derivatization technique, urinary phenols elute at much higher temperature with baseline resolution and are free from interferences from volatile components of the urinary matrix. Moreover, due to the presence of strong molecular ion and other peaks at the higher mass range for 4-carbethoxyhexafluorobutyryl derivative of phenol, unambiguous identification is also possible. Our derivatization protocol is simple and can be easily adopted in a clinical laboratory.

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