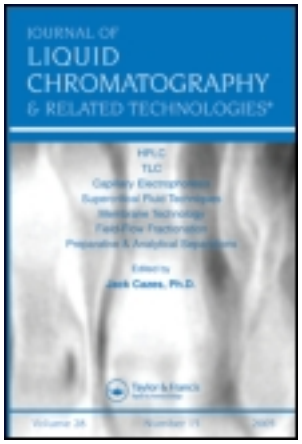

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AN IMPROVED METHOD FOR THE DETERMINATION OF URINARY 1-HYDROXYPYRENE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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□ *An improved method based on solid-phase extraction and high-performance liquid chromatography with fluorescence detection was developed for the determination of urinary 1-hydroxypyrene (1-OHP). The solid-phase extraction procedure, the excitation and emission wavelengths for 1-OHP, and the pH of the mobile phase were optimized. Excitation and emission wavelengths of 239 and 392 nm, respectively, afforded much higher fluorescence response than the wavelengths reported in previous studies. Lowering the pH of the mobile phase significantly decreased the fluorescence response of 1-OHP but increased data reproducibility for urine samples. Under optimized conditions, the average relative standard deviation (*n *=5) of intra-day precision for 11 urine samples was 2.7% (range 0.8–4.0%). Because of the excellent data reproducibility, we expect the method to be useful for the determination of urinary 1-OHP, especially in biomonitoring projects involving high-volume screening.*

Keywords 1-hydroxypyrene, high-performance liquid chromatography, metabolite, polycyclic aromatic hydrocarbon, solid phase extraction, urine

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are produced during pyrolysis and during incomplete combustion of organic materials such as coal, gasoline, and tobacco smoke. PAHs are ubiquitous organic contaminants in environmental media such as air, water, soil, and sediment.[1–4]

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Owing to their potential human carcinogenicity and mutagenicity,[5] PAHs have received much attention during the last two decades.

Human exposure to PAHs generally occurs by three routes: ingestion, inhalation, and dermal contact.[6–8] After entering the body, PAHs undergo hydroxylation reactions to form monohydroxy, dihydroxy, and tetrahydroxy metabolites.[9,10] These hydroxylated metabolites are excreted in human urine mainly as glucuronide and sulfate conjugates,[11] which are classified as the final metabolites of PAHs. Therefore, urinary levels of hydroxylated PAHs (OH-PAHs) might directly reflect human internal exposure to PAHs from various sources.

In the last few years, methods for simultaneous determination of OH-PAHs have been developed. For example, Li et al.[11] developed a method for the simultaneous determination of more than 20 OH-PAHs in urine by gas chromatography–mass spectrometry. Onyemauwa et al.[12] reported a method for determination of 16 urinary OH-PAHs using liquid chromatography–tandem mass spectrometry. Although both these methods are highly sensitive, the former requires derivatization reactions and is time consuming; and matrix effects can significantly affect the results obtained by the latter. Therefore, high-performance liquid chromatography with fluorescence detection (HPLC-FLD) has been the preferred method for detecting OH-PAHs.

Since Jongeneelen et al.[13] developed a method for the determination of urinary 1-OHP using HPLC-FLD, this method has been widely employed in the assessment of individual exposure to PAHs. In recent years, several methods have been reported for the simultaneous determination of multiple urinary OH-PAHs. However, 1-OHP has been the most widely used biomarker for assessing human exposure to PAHs, especially for occupational exposure and in epidemiological research.[14–17] It has also been used to investigate internal exposure to PAHs in general population, especially in children.[18,19]

When we used the reported methods[12,15] for determination of urinary 1-OHP, the data we obtained in an intra-day test (1-OHP standards were injected for 5 consecutive d) exhibited large variation. The relative standard deviation (RSD) at 1-OHP concentrations of 5, 20, and 100 μg/L ranged from 5.7% to 19.7%. This large variation might significantly affect the results when biomonitoring data are correlated with medical parameters for evaluation of the effects of PAHs on human health. Therefore, it is necessary to develop an improved HPLC method for the determination of urinary 1-OHP with good sensitivity and reproducibility. In this study, solid-phase extraction (SPE) was used for sample cleanup. We optimized the SPE conditions and also investigated various HPLC mobile phases and excitation and emission wavelengths for FLD. Finally, the optimized method was used for the determination of 1-OHP in urine samples from high school students in the city of Guangzhou, China.

EXPERIMENTAL

Chemicals and Materials

1-OHP and β-glucuronidase/arylsulfatase (124,400 β-glucuronidase units/mL, 36,010 sulfatase units/mL) were purchased from Sigma (St. Louis, MO, USA). Creatinine was purchased from Fluka (Buchs, Switzerland). Formic acid, acetonitrile, and methanol were purchased from Merck (Darmstadt, Germany). All other reagents employed were of analytical grade and were used without further purification. SPE cartridges (C_{18} ENVI, 500 mg/3 mL) were purchased from Supelco (Bellefonte, PA, USA).

Sample Collection and Cleanup

Urine samples were collected randomly from students of a high school in Guangzhou city, Guangdong Province, China. Before sample collection, students were asked to complete a questionnaire regarding age, sex, weight, height, dietary habits, vehicle use, and cigarette smoking habits. Urine samples were then collected in polyethylene bottles that had been cleaned with deionized water and 0.1 M hydrochloric acid. To minimize the variation of the 1-OHP concentration in the urine due to variations in fluid intake, body temperature, physical exercise, and ambient temperature, urinary creatinine was also determined according to the Jaffe method (colorimetric method).[20]

A previously described procedure for sample cleanup was used with slight modifications.[21] In brief, 10 mL of urine sample was transferred to a glass flask. The pH was adjusted to 5.0 with 0.1 M hydrochloric acid, and then 2.5 mL of 0.5 M acetate buffer (pH 5.0) was added. After the addition of 20 μL of β-glucuronidase/arylsulphatase, the flask was placed in a shaker at 37°C for 16 hr (overnight) to hydrolyze the hydroxylated PAH conjugates. Then the hydrolyzed sample was centrifuged at 540 g for 10 min, and the supernatant was subjected to SPE cleanup.

A C_{18} SPE cartridge was pretreated with 5 mL of methanol and 10 mL of deionized water. The hydrolyzed urine sample was then loaded onto the cartridge at a flow rate less than 1 mL/min. After sample loading, the cartridge was washed sequentially with 10 mL of deionized water, 10 mL of 30% methanol, and 5 mL of 50% methanol to remove interfering matrix components. After the cartridge was dried completely, 1-OHP was eluted with 6 mL of methanol, and the eluate was concentrated to a volume of 1 mL with a gentle stream of nitrogen gas. The final solution was filtered through a 0.2-μm filter, and stored at −20°C until HPLC determination.

TABLE 1 Gradient Program for 1-OHP Determination

	Mobile Phase (v/v)	
Time (min)	Methanol	Water (10 mM Acetic Acid/ Sodium Acetate, pH 3.5)
0.00	80	20
15.00	80	20
17.00	100	0
20.00	100	0
22.00	80	20
26.00	80	20

HPLC Analysis

An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, and a fluorescence detector was used. The separation of 1-OHP was performed on a Phenomenex C_{18} column (4.6 mm × 250 mm, 5 μm). Methanol (mobile phase A) and 10 mM acetic acid/sodium acetate buffer (pH 3.5; mobile phase B) were used as mobile phases with the gradient program shown in Table 1. The flow rate was 0.6 mL/min, and the column temperature was 25°C. The excitation and emission wavelengths for 1-OHP were 239 and 392 nm, respectively.

Calibration Curves

Determination was based on the peak area of the target analyte with reference to calibration curves. Linearity was determined with 1-OHP standards in methanol over a concentration range of 1–200 μg/L. The calibration curves were linear, with correlation coefficients (R^2) ranging from 0.9983 to 0.9990 over 5 consecutive days.

RESULTS AND DISCUSSION

Optimization of Sample Cleanup

Sample pretreatment for urinary 1-OHP determination consisted mainly of two steps: enzymatic hydrolysis and SPE cleanup. Many previous studies had been performed to optimize enzyme hydrolysis,[11,21,22] so in this study, we optimized the SPE cleanup procedure. Several studies of various SPE procedures have been reported. For example, Jongeneelen et al.[13] used 10 mL of water, and Romanoff et al.[23] used 40% methanol in sodium acetate without affecting recovery. Here, we removed the biological matrix and

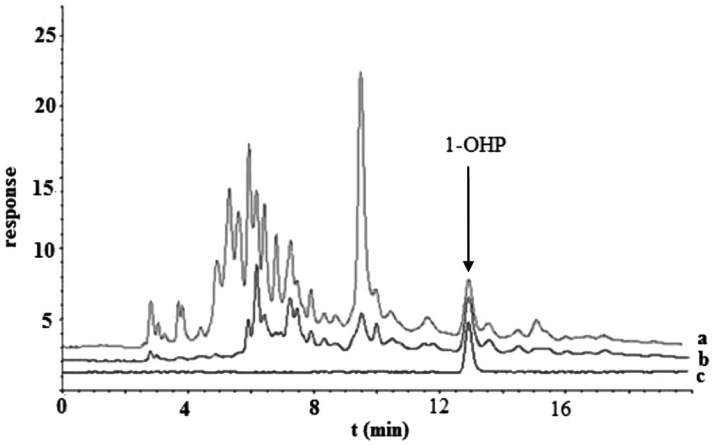

FIGURE 1 HPLC chromatograms of 1-OHP standard and urine samples. (Color figure available online.)

other chemicals by washing the sample-loaded SPE cartridges sequentially with deionized water and 30% methanol or with deionized water, 30% methanol, and 50% methanol. Figure 1 shows typical HPLC chromatograms obtained after the two cleanup procedures. When the column was washed with 10 mL of deionized water, 10 mL of 30% methanol, and 5 mL of 50% methanol (Figure 1, curve b), the baseline of the chromatogram was clearly lower than the baseline obtained when the column was washed only with deionized water and 30% methanol (using the method described by Wang et al.[21]). Thus, our results indicated that the additional wash with 50% methanol was clearly helpful for removing the biological matrix.

Optimization of Fluorescence Wavelengths

Since Jongeneelen et al.[13] developed an HPLC-FLD method for the determination of urinary 1-OHP, many studies have been performed to optimize the excitation and emission wavelengths (the literature data are summarized in Table 2). The most commonly used excitation and emission wavelengths for 1-OHP are 242 and 388 nm, 345 and 388 nm, and 270 and 388 nm, respectively. In study, we scaned at various wavelengths using 1-OHP standard, and found that 239 and 392 nm were more sensitive than any of the previously reported wavelengths. Then we measured the fluorescence responses at various wavelengths and at mobile phase pH values of 2.8 (0.1% formic acid), 3.5 (10 mM acetic acid/sodium acetate buffer), 4.5 (10 mM acetic acid/sodium acetate buffer), and 5.5 (deionized water) (Figure 2). At all the wavelengths, the fluorescence response clearly decreased with decreasing pH. At all the pH values, the fluorescence

TABLE 2 Literature Parameters for Determination of Urinary 1-OHP Using HPLC-FLD

λ_{ex} and λ_{em} (nm)	Mobile Phase	Reference
242/388	methanol, water	[15,21,24]
242/388	acetonitrile, water	[25]
346/387	acetonitrile, 0.5% aqueous acetic acid	[17]
346/384	acetonitrile, water	[22]
345/390	methanol, water	[26]
348/388	acetonitrile, water	[27]
345/388	not mentioned	[28]
270/387	acetonitrile, water	[29,30,31]
270/393	acetonitrile, water containing 5 mM citric acid and 2.83 mM ascorbic acid	[32]

λ_{ex}: excitation wavelength; λ_{em}: emission wavelength.

response decreased in the following order: 239/392 nm > 242/388 nm > 270/388 nm > 345/388 nm. At all the pH values, the fluorescence responses at 239 and 392 nm (excitation and emission) were significantly higher than the responses at the other wavelengths. At pH 4.5, the response at 239/392 nm was at least 8.3% higher than the response at 242/388 nm, whereas at pH 3.5, the response at 239/392 nm was 39.0% higher. Therefore, we chose 239 and 392 nm as the optimum FLD wavelengths.

Effect of Mobile Phase pH on Chromatographic Sensitivity

HPLC mobile phase parameters such as solvent polarity, buffer constituents, and solvent gradient program can affect peak resolution and

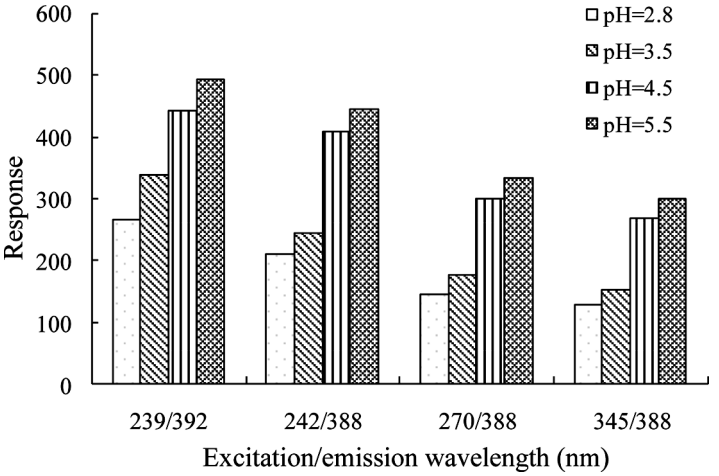

FIGURE 2 Fluorescence responses at different excitation and emission wavelengths and mobile phase pH values.

indirectly affect the sensitivity of the instrument and the reproducibility of the data for target analytes. As shown in Table 2, methanol, acetonitrile, and water were the most commonly used mobile phases in previous studies. Two studies used acetic acid or ascorbic acid buffer instead of water. When we used the reported methods to quantify urinary 1-OHP, the data obtained for an intra-day test (a standard was injected for 5 consecutive days) exhibited large variation. The RSDs at 1-OHP concentrations of 5, 20, and 100 μg/L ranged from 5.7% to 19.7%. Therefore, in this study, we used buffers with various pH values, such as 0.1% formic acid (pH 2.8), 10 mM acetic acid/sodium acetate buffer (pH 3.5), and 10 mM acetic acid/sodium acetate buffer (pH 4.5), deionized water (5.5) to investigate the effect of pH on data reproducibility. Inter- and intra-day tests at three 1-OHP concentrations (5, 20, and 100 μg/L) were conducted by injection of each standard solution six times in one day and once every day for 5 consecutive days, respectively. The data reproducibility is expressed as an RSD based on the peak area of 1-OHP. The RSD decreased as the pH of the mobile phase decreased, ranging from 0.5% to 1.0% for the inter-day test and from 0.7% to 1.6% for the intra-day test. Our results agreed with those of Bouchard and Viau,[33] who added a low concentration of ascorbic acid (1 mg/L) to the mobile phases to improve the data reproducibility and instrumental sensitivity. Ascorbic acid might mask specific adsorption sites—probably uncapped silica groups that constitute the solid support for the hydrocarbon chains—that could retain 1-OHP. However, we also found that the response of 1-OHP decreased as pH decreased. Therefore, considering both response sensitivity and data reproducibility, we chose 10 mM acetic acid/sodium acetate buffer (pH 3.5) and methanol as the mobile phases.

The mobile phase gradient program was also evaluated. After comparing the results of previously reported tests with different mobile phases, we developed the gradient program described in Table 1. This program efficiently separated 1-OHP from the matrix components. After 1-OHP was eluted from the column, additional 100% methanol was used to remove weakly polar solutes. The whole gradient program took only 26 min, so we expect this method to be useful for high-volume screening studies.

Evaluation and Application of the Method

To validate the method, we performed recovery experiments as follows. First, 1-OHP standard solutions in different levels (5, 25, and 50 ng) were added to 10 mL of urine sample obtained from non-smokers before enzyme hydrolysis. Then, the spiked urine samples were enzyme hydrolyzed, pretreated and analyzed by HPLC according to the procedure described in the experimental part. The mean recoveries of 1-OHP at 5, 25, and 50 ng

were 92 ± 16%, 98 ± 10% and 105 ± 10% ($n = 6$), respectively. The limit of detection of the column was 0.44 μg/L. Even though the sensitivity was lower at the lower mobile phase pH, the sensitivity was nevertheless comparable to the sensitivities reported in previous studies, owing to the optimization of the excitation and emission wavelengths.

Inter- and intra-day reproducibility were also assessed using urine samples collected from 11 high school students in the city of Guangzhou, China. None of the students were occupationally exposed to PAHs. The mean concentration of 1-OHP was 0.50 μmol/mol creatinine (range 0.15–1.10 μmol/mol creatinine). The average intra-day RSD ($n = 5$) for 11 samples was 2.7% (range 0.8–4.0%). The data reproducibility was excellent. The measured 1-OHP levels were comparable to those of the background population in China. For example, Zhang et al.[15] reported that urinary 1-OHP concentrations for smokers and nonsmokers were 0.39 and 0.15 μmol/mol creatinine, respectively, in the background population in Beijing. However, the levels measured in our study were much higher than those reported in other countries. The overall geometric mean urinary 1-OHP concentrations for non-occupationally exposed population in Japan and Korea were 0.045 and 0.040 μmol/mol creatinine,[25,34] respectively.

CONCLUSIONS

The sample pretreatment process was optimized. Washing the sample-loaded SPE column with 10 mL of water, 10 mL of 30% methanol and 5 mL of 50% methanol was the best way to eliminate matrix interference without affecting recovery. By comparing the fluorescence signals at different wavelengths, we determined 239 and 392 nm to be the most sensitive wavelengths for the determination of 1-OHP. By comparing HPLC mobile phases with various pH values, we found that sensitivity decreased with decreasing pH. Adding a buffer to the mobile phases enhanced data reproducibility. Thus, methanol and 10 mM acetic acid/sodium acetate buffer (pH 3.5) were chosen as the mobile phases.

The reported method was reliable. Good linearity, recovery, and inter- and intra-day precisions were obtained. The method was successfully used for the biomonitoring of urinary 1-OHP in 11 high school students in Guangzhou, China.

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