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PAPER

Development of a sensitive method for the quantification of urinary 3-hydroxybenzo[a]pyrene by solid phase extraction, dansyl chloride derivatization and liquid chromatography–tandem mass spectrometry detection

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Analysis of urinary metabolite of benzo[a]pyrene (BaP), 3-hydroxybenzo[a]pyrene (3-OHBP), is useful for the biomonitoring of carcinogenic polycyclic aromatic hydrocarbons (PAHs) exposure. Determination of 3-OHBP by liquid chromatography tandem mass spectrometry (LC–MS/MS) suffers from the poor sensitivity of detection in commonly used electrospray ionization (ESI) source. In this work, a sensitive and selective LC–MS/MS method for the determination of urinary 3-OHBP was developed. Following enzymatic hydrolysis of the glucuronide and sulfate conjugates, the metabolite was enriched and cleaned up by solid-phase extraction and then derivatized with dansyl chloride. The derivative was analyzed by LC–MS/MS with ESI in the positive ion mode. The derivatization of 3-OHBP introducing a dansyl group into the molecule greatly enhanced the detection sensitivity by improving both the efficiency of ESI in the positive ion mode and collision-induced dissociation in the collision cell. Good linearity was obtained in the range of 0.25–40.0 pg/mL with a correlation coefficient (r^2) of 0.9924. The limit of detection (LOD) and lower limit of quantification (LLOQ) were 0.1 pg/mL and 0.25 pg/mL, respectively. Accuracy ranged from 87.7% to 107.5%. Intra- and inter-day relative standard deviations varied from 4.6% to 8.4% and 7.2% to 10.6%, respectively. Finally, this developed method was successfully applied for the analysis of urine samples from smokers and non-smokers to measure human exposure to PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic compounds containing two or more fused aromatic rings and ubiquitously present in the environment. Generally produced during the incomplete combustion of organic matter containing carbon and hydrogen, PAHs originate from motor vehicles, tobacco smoke, broiled and roasted food, and various industrial sources^{1,2}. Humans are exposed to PAHs from air, water and food through inhalation, ingestion and dermal

absorption³. Because a number of PAHs are carcinogenic and exposure to PAHs may increase the risk of lung, skin and bladder cancer in humans^{4,5}, monitoring human exposure to PAHs is of great environmental and toxicological importance.

To monitor human exposure to PAHs, many studies have been carried out to investigate metabolites of PAHs in human urine. Urinary 1-hydroxypyrene (1-OHP), the primary metabolite of pyrene, is a widely used biomarker in assessment of PAHs exposure since pyrene is one of the most abundant components of PAHs mixture^{1,6,7}. However, pyrene is not carcinogenic. Therefore, 1-OHP may be not a suitable indicator of the exposure to carcinogenic PAHs^{5,8}. There have been increasing studies of urinary biomarkers of PAHs exposure other than 1-OHP during the past few years wherein direct evidence may be provided to show the exposure to PAHs of greater toxicological relevance than pyrene. Among them, 3-hydroxybenzo[a]pyrene (3-OHBP) present in human urine, the main monohydroxy metabolite of carcinogenic BaP, has

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received particular attention for the more accurate assessment of the exposure and metabolism of carcinogenic PAHs^{5, 8, 9}. However, in vivo, BaP is metabolized and transformed into phenols, diols, quinones, tetraols or epoxydes^{10, 11}, and these metabolites are mainly eliminated in feces which leads to the low levels of urinary 3-OHBP (in the range of pg/mL). Therefore, measuring such low concentration level of 3-OHBP in the complex urine matrix presents a considerable analytical challenge.

Current methods for measuring 3-OHBP have involved high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD)^{5, 12, 13}, gas chromatography/mass spectrometry (GC-MS) after conversion to volatile derivatives^{14, 15} and HPLC coupled with tandem mass spectrometry (HPLC-MS/MS)^{16, 17}. The HPLC-FLD methods suffer with low sensitivity, long run time, poor separation and specificity. The isotope-dilution gas chromatography-high-resolution mass spectrometry (GC-HRMS) is sensitive, specific, and accurate, which is capable of quantifying urinary PAHs metabolites^{15, 18}. But the promising technique requires expensive instruments which are not widely available, so it is currently available only in research laboratories¹⁹. Recently, liquid chromatography coupled with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry instruments have become available in many laboratories and have been widely applied for the quantitative analysis of 3-OHBP. LC-MS/MS has been demonstrated to be a rapid, specific and high-throughput analytical technique for the determination of 3-OHBP in biological samples. However, the main drawback to LC-MS/MS analysis is the poor detection sensitivity of 3-OHBP in commonly used ESI or APCI sources. Although the authors reported pg/mL detection limit, the sensitivity was adequate only for analysis of urine samples from persons with relatively high levels of BaP exposure¹⁶.

The purpose of the present study was to develop a sensitive and accurate method that could be used to analyze trace concentration of 3-OHBP in human urine. To achieve sufficient sensitivity, a simple chemical derivatization procedure with dansyl chloride was employed to increase the detection sensitivity of 3-OHBP in LC-MS/MS. The sample processing procedure included enzymatic hydrolysis of the glucuronide and sulfate conjugates, solid-phase extraction and derivatization before LC-MS/MS detection. Advantages of this method include determination of 3-OHBP with good accuracy and precision, short instrument run time, and the substantial improvement of the detection sensitivity of 3-OHBP relative to non-derivatized 3-OHBP. Finally, the proposed analytical method was applied to analyze 3-OHBP in human urine from smokers and nonsmokers for biomonitoring the exposure to carcinogenic PAHs.

2. Experimental

2.1. Chemicals and materials

3-Hydroxybenzo[a]pyrene and the internal standard 3-hydroxybenzo[a]pyrene-d₁₁ were obtained from Toronto Research Chemicals (Toronto, ON, Canada). β -glucuronidase/arylsulfatase was purchased from Merck (Darmstadt, Germany). Dansyl chloride was purchased from J&K (Shanghai, China). HPLC-grade methanol was provided by Tedia (Fairfield, OH, USA). Analytical-grade acetone, hexane, sodium acetate, acetic acid, hydrochloric acid and sodium bicarbonate were obtained from Guoyao (Shanghai, China). Formic acid was obtained from Guangfu (Tianjin, China). Ammonium hydroxide was obtained from Zhenxing (Shanghai, China). High-purity water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used for all solutions. Cleanert PAX SPE cartridges (60 mg) were purchased from Agela (Wilmington, DE, USA). Supelclean ENVI-18 C₁₈ solid-phase extraction (SPE) cartridges (500 mg) were supplied by Supelco (Bellefonte, PA, USA). Oasis HLB SPE cartridges (60 mg) were purchased from Waters Corporation (Milford, MA, USA).

Urine samples used in this study were obtained from four smokers and four non-smokers among volunteers. These subjects are all non-occupationally exposed smokers and non-smokers. The study protocol was approved by the Institutional Review Board at the University of Science and Technology of China. Written informed consent was obtained from all volunteers. All urine samples were stored at -20 °C until further use for biomarker analysis.

2.2. LC-MS/MS conditions

Analysis of the standards and prepared samples was accomplished using an Agilent QQQ 6460 tandem mass spectrometer coupled with the Agilent 1200 series HPLC (Wilmington, DE, USA). The Agilent QQQ 6460 tandem mass spectrometer was operated in the electrospray ionization (ESI) mode. The capillary voltage was set at 4000 V and the ion source gas temperature was 300 °C with a flow rate of 10 L/min. The HPLC method was performed on a reversed-phase Agilent Eclipse Plus-C₁₈ column (100 mm × 4.6 mm i.d., 3.5 μ m). The column temperature was maintained at 35 °C and the flow rate was 0.40 mL/min. The total run time was 10.0 min. For the non-derivatization method, an isocratic mobile phase of 15% water and 85% methanol was used. Negative ESI in multiple reaction monitoring (MRM) mode with collision energy of 45 eV was used by monitoring the transitions of m/z 267.1 → 239.2 (3-OHBP) and m/z 278.1 → 250.2 (3-OHBP-d₁₁). For the derivatization method, the mobile phase consisted of 15% water with 0.1% formic acid and 85% methanol. The mass spectrometer was operated under MRM positive mode with collision energy of 20 eV for the ion transitions m/z 502.4 → 268.3 (3-OHBP-Dansyl) and m/z 513.3 → 279.4 (3-OHBP-d₁₁-Dansyl). The dwell time for each MRM transition was 0.25 s. All optimal mass spectrometric parameters were chosen automatically using the Mass Hunter Optimizer program (Santa Clara, CA) by a series of injections of pure standards under different conditions.

2.3. Sample preparation procedure

The standard stock solution of 100 $\mu\text{g/mL}$ of 3-OHBP was prepared in methanol and stored at $-20\text{ }^\circ\text{C}$. Standard working solutions of 50 ng/mL, 20 ng/mL, 2 ng/mL and 0.5 ng/mL were prepared by proper dilution of the stock solution with water. Since PAHs are ubiquitous environmental pollutants, the urine samples from non-smokers containing the concentration of 3-OHBP below the lower limit of quantification were diluted with water (20-fold) for the preparation of calibration standards. Appropriate volumes of the working solutions of 3-OHBP were spiked into the diluted urine to obtain calibration standards spanning the concentration range of 0.25–40 pg/mL with internal standard 3-OHBP- d_{11} added to each for a concentration of 4 pg/mL.

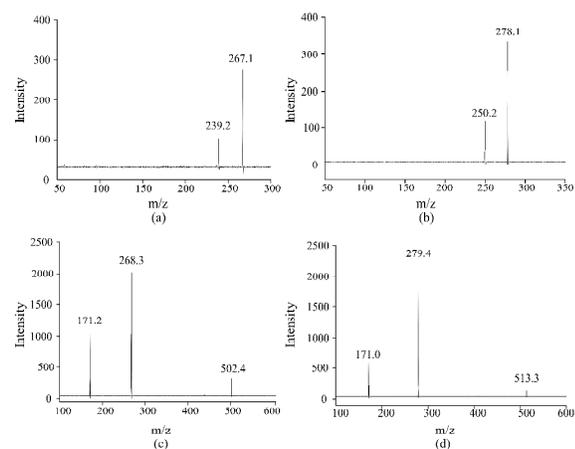


Fig. 1 Product ion spectra of underivatized 3-OHBP (a), 3-OHBP- d_{11} (b) and derivatized 3-OHBP (c), 3-OHBP- d_{11} (d).

50.0 mL of urine sample was adjusted to pH 5–6 with 0.1 M HCl and the solution was diluted with 10.0 mL sodium acetate buffer (100 mM, pH 5.0), then 10 μL of internal standard working solution (20 ng/mL 3-OHBP- d_{11}) was added. After spiking with 75 μL of β -glucuronidase/arylsulfatase (30 and 20 U/mL, respectively), the mixture was incubated overnight at $37\text{ }^\circ\text{C}$. The enzymatically hydrolyzed sample was loaded onto a C_{18} cartridge preconditioned with 5.0 mL of methanol and 5.0 mL of water. After loading, the cartridge was washed with 3.0 mL of water and 2.0 mL of 10% methanol in water. Finally, the analytes were eluted from the column using 8.0 mL of 97:3 (v/v) methanol–ammonium hydroxide. The eluants were evaporated to dryness at $40\text{ }^\circ\text{C}$ under a gentle nitrogen flow then re-dissolved in 1.0 mL of 3% ammonium hydroxide in water. The extract was applied to a PAX SPE cartridge, which was preconditioned with 3.0 mL of methanol followed by 3.0 mL of 3% ammonium hydroxide in water. The PAX SPE cartridge was then washed with 1.0 mL of 3% ammonium hydroxide in water, 1.0 mL of water, 2.0 mL of 10% methanol in water and 1.0 mL of methanol. The elution step was performed using 4.0 mL of 3% acetic acid in methanol. The eluate was evaporated to dryness at $40\text{ }^\circ\text{C}$ under a gentle nitrogen flow. The residue was re-dissolved in 50 μL of sodium bicarbonate buffer (100 mM, pH 9.5) followed by

vortex-mixing for 1 min. To each sample, 120 μL of acetone solution containing 0.8 mg/mL of dansyl chloride was added followed by vortex-mixing for another 1 min. The mixture was incubated at $60\text{ }^\circ\text{C}$ for 7 min to facilitate derivatization, and then cooled to room temperature. The derivatives were extracted using 3.0 mL of hexane. The organic extract was evaporated to dryness then reconstituted in 100 μL of methanol, and a 10 μL aliquot was injected for LC–MS/MS analysis.

3. Results and discussion

3.1. Mass spectrometry

Initially, mass spectrometric detection of underivatized 3-OHBP and 3-OHBP- d_{11} was investigated using ESI source on an Agilent QQQ 6460 instrument. The ESI of 3-OHBP and 3-OHBP- d_{11} was examined in both positive and negative ion modes. Both 3-OHBP and 3-OHBP- d_{11} produced signals only in the negative ion mode, probably because the hydroxy group of 3-OHBP and 3-OHBP- d_{11} tend to lose the phenolic hydrogen and generate $[\text{M}-\text{H}]^-$ ion. Under the optimized collision conditions, the product ion mass spectra of the $[\text{M}-\text{H}]^-$ ion of 3-OHBP and 3-OHBP- d_{11} are plotted in Fig. 1(a) and (b). The ion fragmentation pattern, loss of 28 Da from the precursor ions $[\text{M}-\text{H}]^-$, can be observed for 3-OHBP and 3-OHBP- d_{11} . However, the direct LC-ESI-MS/MS analysis of compounds with low polarity has shown ionization to be generally inefficient wherein the reason was attributed to the poor efficiency of generating gas-phase ions from liquid droplets during the ESI process²⁰. Moreover, due to the highly conjugated and rigid chemical structure that was difficult to fragment in the collision cell, a considerable fraction of precursor ions (m/z 267.1 for 3-OHBP and m/z 278.1 for 3-OHBP- d_{11}) still remained unchanged even at the optimized collision energy (45 eV). The levels of 3-OHBP routinely should be in the picograms per milliliter range. Thus, use of direct LC–MS/MS detection method resulted in poor sensitivity for the quantification of 3-OHBP. To solve this problem, modification of the physicochemical properties of 3-OHBP by chemical derivatization was considered. Among the different derivatization reagents that were reported in the literature, dansyl chloride has been used widely to facilitate the ionization and fragmentation of phenols^{21–25}. And the derivatization of phenolic group with dansyl chloride is rapid and simple. Therefore, dansylation of 3-OHBP was employed in this study in order to enhance the detection sensitivity in LC–MS/MS. By introducing a dansyl functional group that contains basic nitrogen, an intense molecular ion $[\text{M}+\text{H}]^+$ peak was possible and thus much higher ionization efficiency and yield. Moreover, under MS/MS conditions, much higher collision-induced dissociation efficiency in the collision cell was also observed. The precursor ion of dansylated 3-OHBP produced an intense fragment ion at m/z 268.3. Both product ion spectra of dansylated 3-OHBP and 3-OHBP- d_{11} had a fragment ion at m/z 171, which was formed through the cleavage of sulfonyl function group from the derivatization

reagent^{21, 26}. The product ion spectra of dansylated 3-OHBP and 3-OHBP-d₁₁ in the positive ESI mode are shown in Fig. 1(c) and (d). The collision-induced dissociation pathways of both 3-OHBP and 3-OHBP-Dansyl are proposed in Fig. 2. Injecting the same concentration of 3-OHBP-Dansyl and 3-OHBP (10 ng/mL, 10 μ L), an increase in the response (peak height) of approximately 30-fold over the underivatized 3-OHBP was observed. Fig. 3 shows the direct comparison of chromatograms of non-derivatized 3-OHBP and 3-OHBP-Dansyl.

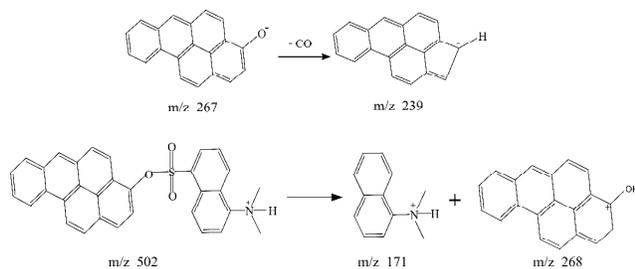


Fig. 2 Collision-induced dissociation of 3-OHBP and 3-OHBP-Dansyl.

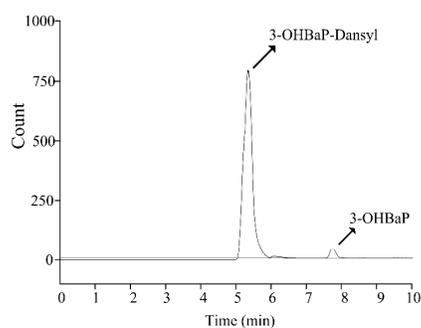


Fig. 3 Comparison of chromatograms of underivatized 3-OHBP (Retention time: 7.8 min) and 3-OHBP-Dansyl (Retention time: 5.1 min). For 3-OHBP, ESI (-) mode, isocratic elution condition: 15% water and 85% methanol. For 3-OHBP-Dansyl, ESI (+) mode, isocratic elution condition: 15% water with 0.1% formic acid and 85% methanol.

3.2. Optimization of the SPE procedure

Due to the large volume of urine sample used and the complex composition of urine matrix, suitable sample pretreatment is required before instrumental analysis for the enrichment of the analytes and the removal of the interfering components. Solid-phase extraction (SPE) is the most commonly used sample preparation technique for the extraction and preconcentration of analytes in complex matrix with the advantages of low consumption of organic solvent and processing time, simplicity, ease of automation, high reproducibility and recovery²⁷⁻³⁰. Initially, PAX anion-exchange cartridges, HLB cartridges and C₁₈ cartridges were evaluated to obtain optimal recovery for 3-OHBP. For the PAX and C₁₈ SPE procedure, the loading pH, washing and

eluting conditions were described in Section 2.3. For the HLB SPE procedure, 50.0 mL of spiked water was adjusted to pH 5.0 with sodium acetate buffer. The aqueous solution was loaded onto the HLB cartridge preconditioned with 3.0 mL of methanol and 3.0 mL of water. After loading, the cartridge was washed with 3.0 mL of water and 2.0 mL of 10% methanol in water. Finally, the analytes were eluted from the column using 4.0 mL of methanol. Because of excessive retention of 3-OHBP in the HLB 60 mg cartridge, HLB sorbent was found to give the lowest extraction recovery (45.8 \pm 2.2%, n=3) compared with the PAX (97.3 \pm 3.5%, n=3) and C₁₈ (82.7 \pm 3.0%, n=3) sorbents for 40 μ g/mL of 3-OHBP.

In this study, C₁₈ cartridge was used for the preliminary clean-up and enrichment of the analytes from the large volume of urine sample. Though the C₁₈ sorbent can be applied for the isolation of non-polar or neutral analytes from complex matrices based on the reverse-phase retention mechanism, the selectivity is poor. Therefore, an additional clean-up step was introduced to remove nontargeted matrix components from the urine extract. The mixed mode anionic sorbent PAX combines retention based on lipophilicity with anionic interactions and can be used for the isolation of acids²¹. The wash sequence was 1.0 mL of 3% ammonium hydroxide in water and 1.0 mL of water to lock the acidic compounds to the column packing and remove inorganic salts, followed by 2.0 mL of 10% methanol in water and 1.0 mL of methanol to remove nonionized hydrophobic contaminants. With this method, nearly 100% recovery was obtained with loading pH in the range of 7-8. Therefore, PAX cartridge was used for further clean-up after the C₁₈ SPE procedure.

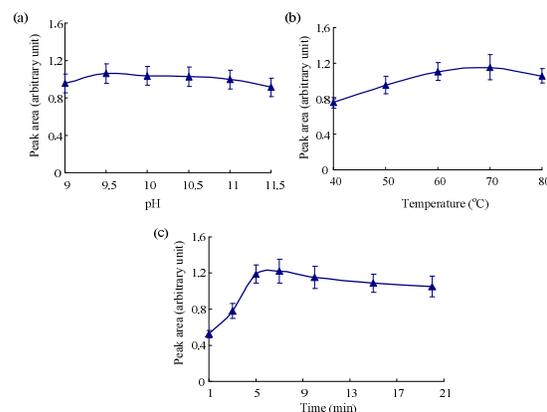


Fig. 4 pH (a), temperature (b) and time (c) optimum for 3-OHBP derivatization.

3.3. Optimization of dansyl chloride derivatization conditions

As illustrated in Section 2.3, after SPE and evaporation of the eluants, the obtained extract was derivatized with dansyl chloride in base solution. In order to obtain the optimized derivatization conditions, the parameters of the reaction affecting the dansyl chloride derivatization yield, including pH of sodium

bicarbonate buffer, derivatization temperature and time were investigated using 2000 pg of 3-OHBAp standard. As indicated in Fig. 4 (a), no significant change in the efficiency of dansylation for 3-OHBAp at pH 9.0-11.5 was observed. When other conditions were the same, heating sample at 60 °C for 7 min gave the best yield of dansylation for 3-OHBAp (Fig. 4 (b) and 4 (c)). The derivatization efficiency was also validated by monitoring non-derivatized 3-OHBAp after derivation using the non-derivatization method, and no signals were observed. After the derivatization procedure, a back-extraction step using hexane was performed to remove water soluble components from the reaction mixture and obtain cleaner extract.

3.4. Validation of the method and application

3.4.1 Quality assurance and quality control

Linearity of the method was obtained by spiking aliquots of 50.0 mL of the diluted urine with 12.5, 50, 100, 200, 500, and 2000 pg of 3-OHBAp standard and 200 pg of the internal standard. The spiked urine samples were then subjected to the optimized procedure described above. Good linear relationship was obtained over the concentration range of 0.25–40.0 pg/mL with a correlation coefficient (r^2) of 0.9924. The limit of detection (LOD) and lower limit of quantification (LLOQ) values, based on S/N ratio of 3.0 and 10.0, were 0.1 pg/mL and 0.25 pg/mL, respectively.

Quality control samples representing low (LQC), medium (MQC) and high (HQC) quality controls were prepared at 0.6, 8, and 30 pg/mL, respectively. Accuracy and precision were determined by analysis of spiked blank urine (diluted urine) samples at LQC, MQC and HQC. For accuracy, the concentrations of the QC samples were calculated from the calibration curve. For intra-day precision, each sample was analyzed five times. For inter-day precision, samples were analyzed once a day on five different days within two weeks. As summarized in Table 1, accuracy ranged from 87.7% to 107.5%. Intra- and inter-day relative standard deviations varied from 4.6% to 8.4% and 7.2% to 10.6%, respectively. To investigate recovery, standard spiked blank urine samples and blank urine SPE extracts spiked 3-OHBAp standard samples were prepared at three QC concentration levels in three replicates. All the samples were further derivatized with dansyl chloride. The overall recovery was determined by comparing the peak area of spiked blank urine sample to the peak area of blank urine SPE extracts spiked with equivalent 3-OHBAp standard prior to the derivatization procedure. The mean recoveries of 3-OHBAp from human urine determined at LQC, MQC and HQC concentration levels were 32.2%, 35.4% and 42.2%, respectively.

The matrix effect was investigated by comparing the peak area of real urine SPE extracts spiked with 3-OHBAp standard (with the background area subtracted) to the peak area of 3-OHBAp standard solution with equivalent concentration after the derivatization procedure. Negative suppression of the response by approximately 57±5% was observed for 3-OHBAp-Dansyl which was due to co-extracted constituents in

the urine matrix. The internal standard method was used for quantitative analysis of 3-OHBAp. Since the isotopically labeled internal standard showed the same ion suppression as the target analyte, the ion suppression effect was normalized by this method^{20,24}. Sample carryover was assessed by injecting urine extract with high analyte concentration five times, followed by blank (methanol) injection. The solvent run was checked for the analyte peak. In the current assay, the mobile phase methanol was used as the autosampler needle wash solution with rinse volume of 10 µL. No detectable carryover in the LC-MS/MS system used was observed.

Table 1 Accuracy and precision data of the analytical method for 3-OHBAp in human urine

Nominal concentration (pg/mL)	Intra-day (n=5)		Inter-day (n=5)	
	Accuracy	RSD	Accuracy	RSD
0.6	104.7%	8.4%	107.5%	10.6%
8	90.6%	5.3%	87.7%	8.3%
30	97.7%	4.6%	93.2%	7.2%

3.4.2 Application of the method

The proposed method was applied to analyze 3-OHBAp in urine samples collected from non-occupationally exposed smokers and non-smokers. The results are summarized in Table 2. The measured concentrations for four non-smokers' urine samples ranged from below the LLOQ to 0.35 pg/mL and for four smokers' urine samples ranged from 0.32 to 0.67 pg/mL. This outcome is comparable to values reported in literature^{5,12,31}. Chromatograms of urine extracts (extracted blank urine (diluted urine) spiked with 100 pg 3-OHBAp and 200 pg 3-OHBAp-d₁₁, extracted smoker's urine sample spiked with 200 pg 3-OHBAp-d₁₁, extracted non-smoker's urine sample spiked with 200 pg 3-OHBAp-d₁₁) are presented in Fig. 5. Though the exposure to PAHs might be from tobacco smoke, exhaust gas from automobiles, consumption of broiled food or contaminants from various industrial sources, biomonitoring of urinary metabolite of PAHs is useful for assessing of environmental and occupational exposure and the results of the study show that the proposed sensitive analytical method can be used in various circumstances.

Table 2 3-OHBAp measured in urine samples of smokers and non-smokers.

	Subject	Gender	Age (years)	Cigarettes per day	3-OHBAp (pg/mL)
Smokers	1	Male	56	20-25	0.39
	2	Male	45	15-20	0.42
	3	Male	37	15-20	0.67
	4	Male	52	10-15	0.32
Non-smokers	5	Male	26	N.R.	0.27
	6	Male	30	N.R.	- ^a
	7	Female	52	N.R.	0.35
	8	Female	33	N.R.	0.31

^a -: Not found

N.R.: not relevant

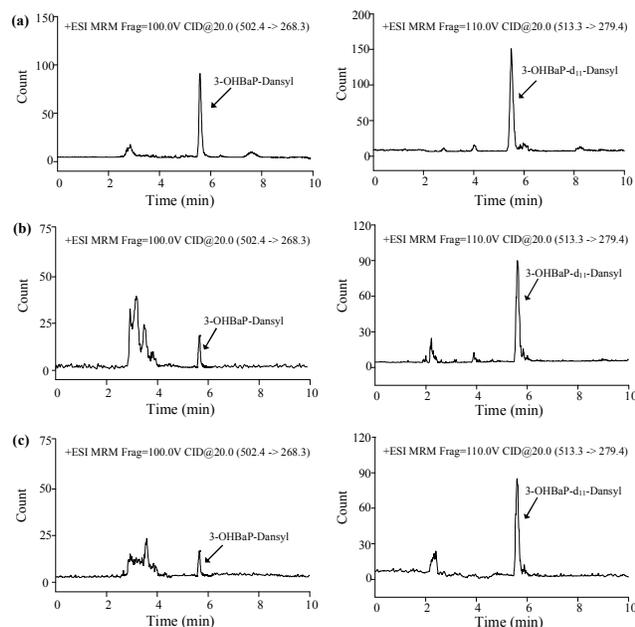


Fig. 5 Chromatograms of 3-OHBA-P-Dansyl and 3-OHBA-P-d₁₁-Dansyl, (a) blank urine sample spiked with 100 pg 3-OHBA-P and 200 pg 3-OHBA-P-d₁₁, (b) smoker's urine sample spiked with 200 pg 3-OHBA-P-d₁₁, (c) non-smoker's urine sample spiked with 200 pg 3-OHBA-P-d₁₁.

4. Conclusions

A sensitive bioanalytical method was developed and validated for the determination of trace 3-OHBA-P in human urine by solid phase extraction cleanup, dansyl chloride derivatization and liquid chromatography–positive electrospray ionization–tandem mass spectrometry detection. The use of chemical derivatization of 3-OHBA-P with dansyl chloride significantly enhanced the mass spectrometric detection sensitivity of the analyte. The method has been used to monitor human exposure to PAHs among non-occupationally exposed smokers and non-smokers. Therefore, this new method should be useful in toxicology and epidemiologic evaluation of carcinogenic PAHs exposure in environmental and occupational sources.

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