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# Sensitive determination of trace urinary 3-hydroxybenzo[*a*]pyrene using ionic liquids-based dispersive liquid–liquid microextraction followed by chemical derivatization and high performance liquid chromatography–high resolution tandem mass spectrometry

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ABSTRACT

3-Hydroxybenzo[*a*]pyrene (3-OHBaP) is widely used as a biomarker for assessing carcinogenic benzo[*a*]pyrene exposure risks. However, monitoring urinary 3-OHBaP suffers from an insufficient sensitivity due to the pg/mL level in urine excretion. In this study, a sensitive method for determination trace urinary 3-OHBaP was developed, involving enzymatic hydrolysis of the glucuronide and sulfate conjugates, ionic liquids dispersive liquid–liquid microextraction (IL-DLLME) enrichment, derivatization with dansyl chloride and HPLC-HRMS/MS analysis in the positive ion mode. Using IL-DLLME makes the enrichment of trace 3-OHBaP very simple, time-saving, efficiency and environmentally-friendly. To enhanced HPLC-HRMS/MS response, an MS-friendly dansyl group was introduced to increase the ionization and fragmentation efficiency. The optimal IL-DLLME extraction parameters and derivatization reaction conditions were investigated. Good linearity was obtained over a concentration range of 0.6–50.0 pg/mL with correlation coefficients ($r^2$) of 0.9918. The limit of detection (LOD) and limit of quantification (LOQ) values were 0.2 pg/mL and 0.58 pg/mL, respectively. The recoveries were 92.0 ± 4.2% with the intra-day and inter-day RSD values ranged from 2.2% to 3.8% and from 3.3% to 6.8%, respectively. The proposed IL-DLLME-Dansylation-HPLC-HRMS/MS method was successfully applied to determine urinary 3-OHBaP of non-occupational exposed smokers and nonsmokers.



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of ubiquitous environmental contaminants containing at least two fused aromatic rings. PAHs are generated during pyrolysis and incomplete combustion of organic matter. Human may be exposed to them by inhalation of polluted air, tobacco smoke, consumption of broiled or fried food and so on [1,2]. Because many PAHs are carcinogens and/or cocarcinogens, human exposure to them involves a potential health risk. It has been reported that people under occupational PAH exposure have higher possibility to suffer from lung, skin, and bladder cancers. The reason maybe that the PAH DNA adducts would cause errors in DNA replication and mutations [3,4]. Among PAHs, benzo[*a*]pyrene (BaP) is classified as group 1 (carcinogenic for humans) by the International Agency for Research on Cancer [5]. The carcinogenicity of BaP is initiated with the aid of cytochrome P450 (CYP) enzymes and epoxide hydrolase to form 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE), the ultimate carcinogens. Other complex metabolisms include rearrange or hydrolyze reaction to form phenols or dihydrodiols. These hydroxyl BaP metabolites were excreted as glucuronide or sulfate conjugates through urine and faeces [4,6].

As the monohydroxy metabolite of BaP, urinary 3-hydroxybenzo[*a*]pyrene (3-OHBaP) has been proved to be more suitable as a biomarker of carcinogenic PAH exposure compared to 1-hydroxypyrene (1-OHP). However, the level of 3-OHBaP in urine excretion are of the order of pg/mL, that is three to four orders of magnitude lower than those of 1-OHP [1,7,8]. This is a challenge for biomonitoring of 3-OHBaP exposure. Current preconcentration methods for 3-OHBaP involve liquid–liquid extraction (LLE) [9], solid-phase extraction (SPE) [10–12], solid-phase microextraction (SPME) [2] and so on [13]. In comparison, SPE uses less volume of volatile toxic organic solvents than LLE, but the

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method is time-consuming and relatively expensive. SPME realizes solvent-free extraction but the fiber is fragile and has relatively limited lifetime [14]. So it is of great importance to adopt a proper effective preconcentration method for the determination of such low-concentration 3-OHBaP from the complex urine matrix prior to chromatography analysis.

Dispersive liquid–liquid microextraction (DLLME) has been proved to be an simple, time-saving, efficiency method during low-concentration sample preconcentration [15,16]. In this method, an appropriate mixture of an organic extraction solvent and a water miscible disperser is rapidly injected into the aqueous sample to form a cloudy solution. Because analytes experience enrichment from large volume sample solution into the small volume sediment, the enrichment of the trace analytes can be very effective. Traditional DLLME procedures employ chlorinated extractants, such as chlorobenzene, chloroform ($CHCl_3$), carbon tetrachloride ($CCl_4$), which are volatile, toxic and environmentally hazardous. Under the call of green chemistry, ionic liquids (ILs) have been used as environmentally friendly alternative extractants in DLLME recently. ILs own many outstanding physicochemical properties, such as high polarity, low volatilities, high thermal stabilities, adjustable miscibilities, good chromatographic behavior and good abilities to extract organic compounds [17,18]. All these properties enable ILs to be a desirable extraction media for DLLME. As an effective pretreatment and preconcentration technique, ionic liquid dispersive liquid–liquid microextraction (IL-DLLME) has successfully been used for the determination of plasticizer [19], bioactive compound [20], antihypertensive drugs [21] from urine samples. However, to the best of our knowledge, there are no articles that use IL-DLLME in the determination of 3-OHBaP.

Recently, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has been widely used for the determination of trace urinary 3-OHBaP on account of its short analysis time, ability to analyze complex biological matrices and high selectivity [10,22,23]. However, the determination of 3-OHBaP suffers from low MS detection sensitivity because its highly conjugated and rigid chemical structure is difficult to ionization and fragmentization [24]. Chemical derivatization to enhance MS detection has been used as common approaches to improve sensitivity [25–27]. Among various derivatization reagents, dansyl chloride is widely used to reaction with phenolic compounds. The introduction of dansyl group could increase both ESI source ionization efficiency and collision cell collision-activated dissociation (CAD) [24,28].

Therefore, a novel IL-DLLME-Dansylation-HPLC-HRMS/MS method to determine human urinary 3-OHBaP is presented here. Using IL-DLLME makes the enrichment of trace 3-OHBaP very simple, time-saving, efficiency and environmently-friendly. Chemical derivatization using dansyl chloride was performed to enhance MS response, which endows the analysis very sensitive. The proposed method was applied to analyze urinary 3-OHBaP of non-occupationally exposed smokers and nonsmokers to assess the exposure to carcinogenic PAHs.

## 2. Experimental

### 2.1. Chemicals and materials

1-Butyl-3-methylimidazolium hexafluorophosphate ($[C_4MIM][PF_6]$, purity, 99.0%), 1-hexyl-3-methylimidazolium hexafluorophosphate ($[C_6MIM][PF_6]$, purity, 99.0%), and 1-octyl-3-methylimidazolium hexafluorophosphate ($[C_8MIM][PF_6]$, purity, 99.0%), were obtained from LanZhou Institute of Chemistry Physics (Lanzhou, China). Dansyl chloride (DNS) was supplied by J&K (Shanghai, China). Acetonitrile, methanol, acetone, sodium acetate, acetic acid, hydrochloric acid, ascorbic acid, sodium bicarbonate, sodium carbonate, chlorobenzene, chloroform, carbon tetrachloride were purchased from Guoyao (Shanghai, China). 3-Hydroxybenzo[*a*]pyrene and 3-hydroxybenzo[*a*]pyrene-$d_{11}$ was supplied by Toronto Research Chemicals (Toronto, ON, Canada). β-Glucuronidase/arylsulfatase (30 and 20 units/mL, respectively) was obtained from Merck (Darmstadt, Germany). Highpurity water was supplied by a Milli-Q water system (Millipore, Bedford, MA, USA).

### 2.2. Preparation of stock solution and urine samples

Urine samples were collected from non-occupationally exposed smokers and nonsmokers by their own voluntary and were stored in polyethylene bottles at −20 °C for further use. The standard stock solution of 3-OHBaP (100 μg/mL) was prepared in methanol and calibration standard solutions were prepared in high purity water by diluting with the stock solution.

### 2.3. Enzymatic hydrolysis of the urine sample

10 mL urine was filtered with 0.45 μm filter membrane and then transferred to a conical flask with stopper and pH of the sample was adjusted to 5 by adding 1 M HCl dropwise. Then 500 μL 1 M acetate buffer (pH 5) and 10 μL of internal standard solution (10 μg/L, 3-OHBaP-$d_{11}$) were added. After the addition of 20 μL β-glucoronidase/arylsulfatase (30 and 20 units/mL, respectively), the mixture was incubated in a shaker (at 37 °C) for 4 h to completely hydrolyze the 3-OHBaP conjugates.

### 2.4. IL-DLLME procedure

The enzymatically hydrolyzed urine sample was transferred into a screw cap glass centrifuge tube with conical bottom. The sample pH was adjusted to 9 and 10% NaCl was added. Then 60 μL of $[C_8MIM][PF_6]$ pre-mixed with 1 mL of acetone was injected into the solution rapidly by syringe. After gently shaking, a cloudy solution formed. Then the tube was placed in ultrasonic bath for 2 min followed by an ice-water bath for 2 min to enhance mass-transfer processes. Finally, the mixture was centrifuged at 4000 rpm for 4 min. Accordingly, the emulsion was disrupted and the dispersed fine particles of ionic liquid were sedimented at the bottom of centrifuge tube.

### 2.5. Dansyl chloride derivatization

The upper supernatant was removed and then 50 μL sodium bicarbonate buffer (200 mM, pH 11) and 10 μL 5.0 mg/mL DNS acetone solution was added to the IL phase followed by ultrasonic bath for 20 min at 60 °C. The mixture was then centrifuging 5 min at 3000 rpm. After removing the upper supernatant, the sedimented phase was diluted with methanol to 50 μL and 20 μL was injected into the HPLC-HRMS/MS system for analysis. The reaction was shown in Fig. 1.

### 2.6. HPLC-HRMS/MS conditions

HPLC-HRMS/MS analyses were carried out with LTQ Orbitrap XL hybrid mass spectrometer interfaced to a HPLC system equipped with an Accela 600 pump and Accela autosamper (Thermo Fisher Scientific, Bremen, Germany). A Hypersil GOLD column (Thermo Electron Corp., 150 mm × 2.1 mm, 3 μm) was used for the chromatography. The capillary voltage was set at 4000 V and the ion source gas was set at 300 °C, 10 L/min. The column was maintained at 35 °C. The analytes were separated using 85% methanol and 15% water with 0.1% formic acid. The flow rate of the mobile phase was 0.4 mL/min and injection volume was 20 μL. Positive ESI