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# Measurement of phenolic environmental estrogens in human urine samples by HPLC–MS/MS and primary discussion the possible linkage with uterine leiomyoma

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ABSTRACT

A method was established for the determination of three phenolic environmental estrogens, namely bisphenol A (BPA), nonylphenol (NP) and octylphenol (OP), in urine from women of uterine leiomyoma group ($n = 49$) and control group ($n = 29$), by using solid-phase extraction (SPE) coupled with liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). Urine samples were spiked with 2,4,6-tribromophenyl-terminated tetrabromobisphenol-A carbonate oligomer (TBBPA) and nonylphenol D8 (NP-D8) as internal standard (I.S.) and de-conjugated by adding β-glucuronidase and sulfatase before the SPE. The extraction recoveries of BPA, NP and OP were more than 73.3%; the standard curve was linear over the validated concentrations in the range of 1.0–100.0 ng/mL and the limits of detection (LOD) of BPA, NP and OP were 0.32 ng/mL, 0.18 ng/mL and 0.15 ng/mL, respectively. Moreover, by analysing quality control urine samples in 5 days, the results showed that the method was precise and accurate, for the intra- and inter-day CV% within 15.2%. Except that OP was not found (<LOQ) in any of the control urine samples, the three phenolic environmental estrogens were detected in all urine samples. For the uterine leiomyoma women, the mean concentrations of BPA, NP and OP were 13.9 ± 12.7 ng/mL, 2.77 ± 2.22 ng/mL and 4.09 ± 5.51 ng/mL (mean ± SD), respectively. For the control group, the mean concentrations of BPA and NP were 8.50 ± 12.2 ng/mL and 3.84 ± 3.90 ng/mL (mean ± SD), respectively. The Wilcoxon rank sum test was employed for the comparison of BPA and NP between and control in 2 subgroups defined by the number of gravidity (≤3 and >3). NP levels were significantly higher in uterine leiomyoma patients than control group in low gravidity subgroup. Though BPA levels in experimental and control groups were not significantly different, the mean levels and concentration distribution were different. The study suggested that there is certain relationship between exposure concentrations of phenolic environmental estrogens and uterine leiomyoma disease.



## 1. 1 Introduction

Bisphenol A (BPA), nonylphenol (NP) and octylphenol (OP) are man-made alkyl phenols (APs) and are well known in the scientific research as environmental endocrine disruptors [1]. BPA, a primary raw material, is widely used in the industry as an important intermediate in the production of epoxy resins and polycarbonates plastic [2,3]. NP and OP are extensively used in the production of elasticizers, technical grade abstergents and pesticide emulsifiers [4]. The exposure of the non-occupational exposure of humans to BPA, NP and OP has been considered to be ubiquitous and persistent, which causes serious harm to human health consequently [5,6].

In recent years, some studies have suggested the existence of a relationship between gynaecology tumour and the exposure of APs. BPA and BPB exposure levels have been demonstrated to have a relationship with endometriosis in human [7]. Takeuchi reported BPA might cause ovarian dysfunction [8]. Uterine leiomyoma is the

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most common tumour in the female reproductive system, which often causes infertility and pain. There is no specific therapy besides surgical operation at present [9–11]. BPA has been demonstrated to partly account for the growth of uterine leiomyoma cells [12]. Moreover, in animal studies, the BPA can induce the increase of uterine wet weight [13]. With uterotropic assay, BPA and NP joint effect was synergistic or additive [14], and OP could stimulate the growth and maturing of uterus [15]. It is reasonable to assume that there may be a correlation between the exposure levels of the non-occupational humans to the three APs and uterine leiomyoma in female patients. However, to our knowledge, no relevant reports have appeared up to now.

Biomonitoring is playing an important role in exposure assessment and can provide integrated and reliable information compared to indirect assessments [16]. When the APs enter into the human blood circulation, the compounds are subject to glucuronidation and sulfation, and then, the conjugates are cleared away from blood and end up in urine for excretion in several hours [17,18]. Therefore, for the purpose of detecting the total concentration of APs in human body, urine samples are widely used matrix for biomonitoring.

In this study, solid-phase extraction coupled with liquid chromatography mass spectrometry (SPE–HPLC–MS/MS) is established and evaluated to detect BPA, NP and OP in human urine. The method was used to determinate the concentration of the three APs in the urine of women affected by uterine leiomyoma and the control group in order to evaluate possible linkage between APs exposure levels in human body and uterine leiomyoma. In the present paper, the uterine leiomyoma women were diagnosed in the medical field, and the control group was non-uterine leiomyoma and hormone independent gynecopathy women.

## 2. Experimental

### 2.1. Reagents and materials

BPA, NP and OP were purchased from Aldrich–Sigma (purity > 99.0%, St. Louis, MO, USA). NP-D8 and TBBPA (purity > 99.0%, HuaErBo Technology Co., Beijing, China), HPLC grade dichlormethane, methanol and ammonium acetate were purchased from TEDIA (Ohio, USA) and keMiO (Tianjing China). Analytical grade acetic acid and sodium acetate were obtained from Shanghai National Medicine Chemicals Corporation. β-Glucuronidase/sulfatase (β-glucuronidase 18 U/mg, arylsulfatase 16 U/mg) was purchased from Aldrich–Sigma, USA. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the study. C18 SPE cartridges (500 mg, 3 mL) were purchased from Dikma Inc., China.

### 2.2. Standards and QC solutions

For standards, a stock solution for analytes was prepared at the concentration of 0.1 mg/mL. Duplicate stocks were prepared and compared to verify accuracy. For QCs, a stock solution of analytes was prepared at 10.0 μg/mL. The I.S. stock solutions (NP-D8 and TBBPA) were both prepared at 100.0 μg/mL. All stock solutions were prepared in methanol monthly and stored at 4 °C to ensure the analyte stock solution stability within the usage interval.

Working solutions to obtain the standard points of the calibration curve and the working solutions to prepare the urine QC samples (low, medium and high concentration), were prepared daily by serial dilutions different amounts of the stock solutions with methanol to obtain three APs compounds solutions at the final concentrations of 40.0 ng/mL, 80.0 ng/mL, 200.0 ng/mL, 400.0 ng/mL, 800.0 ng/mL, 2000.0 ng/mL, and 4000.0 ng/mL, respectively.

The I.S. working solutions of NP-D8 and TBBPA were prepared at 1.0 μg/mL daily by serially diluting the stock solution with methanol.

### 2.3. Blank controls

Due to the ubiquitous presence of the three target analytes in the environment and urine sample matrices, the analysis of these compounds was complicated by the lack of appropriate blanks. Therefore, special precaution was taken regarding the experimental control: laboratory glassware was consecutively rinsed with ethyl acetate, iso-octane, and purified water twice each before use. "Blank control human urine" was prepared with mixing urines from 8 individual matrix. Prior to the validation study, the three target analytes content levels in the blank control human urine were measured and used as the baseline values for blank controls. In other words, the blank control human urine was used to prepare calibration standards and QC samples of the final concentrations allowing for the baseline values (with the baseline values deducted).

### 2.4. Preparation of standards and QC samples

Blank control human urine aliquots (1.95 mL) were spiked with 50 μL of each working solution to obtain a final dilution of 1:40, giving six calibration standards in the analysis at the final concentrations of 1.0 ng/mL, 2.0 ng/mL, 5.0 ng/mL, 10.0 ng/mL, 20.0 ng/mL, 50.0 ng/mL and 100.0 ng/mL, respectively. The calibration standards prepared fresh daily.

To prepare QC samples, three fractions of urine were added with an appropriate amount of QC solutions, obtaining QC samples at the final analysis concentrations of 1.0, 5.0 and 50.0 ng/mL (for low, medium and high concentrations, respectively). For the validation of intra-day and inter-day precision, extraction recoveries, matrix effect and benchtop stability, QC samples were prepared fresh daily. While for the validation of stability of all analytes in frozen matrix, QC samples should be prepared in advance and frozen at −20 °C in glass tubes with a plug until relevant tests.

### 2.5. Sample

Urine samples were collected from seventy-eight women volunteers. We established two different groups for the operative procedure: uterine leiomyoma patients ($n = 49$; age 37.9 ± 4.64 years, mean ± SD; age range 30–50) and a control group ($n = 29$; age 36.4 ± 4.47 years, mean ± SD; age range 30–50).

### 2.6. Sample preparation

Urine samples (2 mL) were mixed with 50 μL (50 ng) TBBPA and 20 μL (20 ng) NP-D8 of corresponding I.S. working solution, 200 μL of sodium acetate (pH = 5.5) and 20 μL of β-glucuronidase/sulfatase by vortex for 30 s. And then the mixture was diluted with water to 4 mL, sealed and hydrolyzed to release free APs in a water bath at 37 °C for 3 h and followed by centrifugation at 4500 rpm for 10 min. The supernatant was separated and passed through the pre-conditioned C18 SPE cartridges (with 1 mL dichlormethane, 3 mL methanol and 3 mL water) at a rate of 1.0 mL/min by vacuum pumping. After washed with 1 mL water and 1 mL water/methanol (3:7), the target analytes retained on the C18 SPE cartridges were eluted with 3 mL methanol and 1 mL dichloromethane into the collecting tubes and the eluate was evaporated to dry at 40 °C under a stream of nitrogen. The dried sample was reconstituted with 200 μL of mobile phase and vortex-mixed, and centrifuged at 12 000 rpm for 5 min. Then 10 μL of the supernatant was injected into a chromatographic system.