Original Article

A Study on Bisphenol A, Nonylphenol, and Octylphenol in Human Urine amples Detected by SPE-UPLC-MS*

XIAO Jing[1], SHAO Bing[2,#], WU XiaoYan[2], SUN XiaoJie[2], and WU YongNing[1,#]

1.National Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, Beijing 100021, China; 2. Beijing Municipal Centre for Disease Control and Prevention, Beijing 100013, China

Abstract

Objective To establish a comprehensive analytical method based on SPE-UPLC-MS for the simultaneous determination of bisphenol A (BPA), nonylphenol (NP), and octylphenol (OP) in urine samples.

Methods Sixty urine samples collected from healthy subjects were analyzed for BPA, NP, and OP concentrations. The samples were de-conjugated by adding β-glucuronidase and sulfatase. After the enzymatic treatment, the samples were subjected to the OASIS HLB column solid phase extraction cartridges so as to be cleaned and concentrated. The UPLC separation was performed on a Acquity UPLCTM BEH C18 column (2.1×100 mm, 1.7 μm) with a gradient elution system of methanol-water as the mobile phase. Triple-quadrupole mass spectrometry analyzer was used for the qualitative and quantitative analysis of UPLC-MS/MS system.

Results The limit of detection of BPA, NP, and OP was 0.10, 0.10, and 0.15 ng/mL, respectively. The recoveries of BPA, NP and OP were 80.1%-108%, 81.3%-109%, and 81.5%-98.7%, respectively. Among the 60 urine samples, BPA was detected in 8 samples at the level of 0.297-32.7ng/mL, NP was detected in 29 samples at the level of 1.69-27.8 ng/mL, and OP was detected in 17 samples at the level of 0.407-11.1 ng/mL.

Conclusion The method is simple with high sensitivity and selectivity, and is suitable for the determination of BPA, NP, and OP in urine. As shown by our analysis , BPA, NP, and OP appear to be prevalent in human urine. This is particularly true for NP. The results from our study is therefore valuable for future studies to assess the exposure to BPA, NP, and OP in the general population.

Key words: Bisphenol A; Nonylphenol; Octylphenol; Urine; Solid phase extraction; Ultra high performance liquid chromatography -mass/mass

Biomed Environ Sci, 2011; 24(1):40-46 *doi:10.3967/0895-3988.2011.01.005* *ISSN:0895-3988*

www.besjournal.com(full text) *CN11-2816/Q*

INTRODUCTION

Endocrine disrupting compounds (EDCs) are exogenous environment tal chemicals that can interfere with human or animal's normal hormone function, and pose a potential threat to environment and human health. Specifically, the environmental estrogen triggers or interferes with the endogenesis of estrogen by binding to the estrogenic receptor or influencing signal conduction or other cellular activities that induce estrogen-like action. Bisphenol A (BPA), nonylphenol (NP), and octylphenol (OP) are such endocrine disruptors that are being industrially manufactured and widely used[1-3].

BPA is a raw material for producing epoxide resins, polycarbonates and polystyrene resins, which has been used to stabilize phenol formaldehyde

*This research was supported by the National Science and Technology Fundation as par of the Key Technologies of Food Safety Project. Chemical pollutants exposure assessment technology research(2006BAK02A01).

#Correspondence should be addressed to SHAO Bing, Tel: 86-10-64407191. Fax: 86-10-64407310. E-mail: shaobingch@sina.com; WU YongNing, Tel: 86-10-67776790. Fax: 86-10-67711813. E-mail: wuyn@public.bta.net.cn

Biographical note of the first author: XIAO Jing, female, born in 1972, associate professor, majoring in food and medicinal chemistry.

Received: June 23, 2010; Accepted: December 23, 2010

resins, bactericidal agents, antioxidants, polyvinyl chloride, and dyes. NP and OP are used for the production of elasticizers, technical grade abstergents, pesticide emulsifiers, as well as trimming in spinning and weaving. BPA, NP, and OP have been widely used in the production of food package materials, food containers, composites and other personal care or consumer products. Therefore，BPA, NP, and OP could migrate to the food, through which enters the human body and endangers human health. Many researchers have stated that such EDCs as BPA, NP, and OP present serious threat to human health and environment[4-5].

Various analytical methods have recently been developed to analyze BPA, NP, and OP in different samples. The common method is gas chromatography coupled with mass spectrometry (GC-MS)[6-8]. In 2004, a stir bar sorptive extraction and thermal desorption-gas chromatography- mass spectrometry (SBSE-TD-GC-MS) method was develop ped for the simultaneous measurement of the trace amounts of phenolic xenoestrogens in water, inclu ding OP, NP, and BPA[9]. This method was further used for the analysis of human urine samples[10]. In the following year, a liquid phase microextraction and gas chromatography- mass spectrometry (LPME-GC-MS) method was developed for the determination of the trace amounts of BPA in the water sample from rivers[11]. Because of the low volatility of these compounds, derivatization steps had to be used to improve the sensitivity of the analysis before the GC-MS method. The derivatization reagents of GC-MS were highly toxic, containing toxins such as trifluoroacetic anhydride (TFAA) and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and others. In addition, these procedures are very tedious and time-consuming.

Moreover, other analytical methods, such as liquid chromatography equipped with fluorescence (LC-FD)[12], mass spectrometry (LC-MS)[13-14], electrochemical detection (LC-ED)[15], and uterotropic assay[16] have also been used. LC-FD method is frequently affected by the sample matrix. LC-ED method requires the strict maintenance to keep the system running. The uterotropic assay is not accurate in detecting the trace amounts of BPA, NP, and OP due to the non-specific binding of the interferences to the antibody. LC-MS/MS method may be a more sensitive and accurate technique when used to detect these compounds. It has been used for the measurement of BPA, NP, and OP in water, beverages[17], animal tissues[18], milk, and egg samples[19]. BPA, OP, and especially NP, widely exist in these samples. Therefore, it is necessary to develop a method to measure BPA, NP and OP in biological samples for the assessment of human exposure to these chemicals. Some papers have reported that BPA, NP and OP are measured in biological samples including human urine[11,20], serum[21], follicular fluid[22], and breast milk[23]. BPA, NP, and OP in human urine[11] are detected by the SBSE-TD-GC-MS method, but need to be derivatizated. Only BPA is detected in human urine by LC-MS[20]. Hence, analytical methods for the simultaneous measurement of BPA, NP, and OP in biological samples are lacking.

In this paper, solid-phase extraction and liquid chromatography equipped with mass spectrometry (SPE-LC-MS) is used for the measurement of BPA, NP, and OP in human urine. The method is simple with high sensitivity and selectivity, and is suitable for the analysis of a large number of urine samples.

MATERIALS AND METHODS

Materials

Chemicals and reagents Bisphenol A (purity>99.0%, Kanto Chemical Industries, Ltd.Tokyo, Japan); nonylphenol (purity >99.0%, Kanto Chemical Industries, Ltd.Tokyo, Japan); octylphenol (purity >99.0%, Dr. Ehrenstorfer GmbH, Germany).

Water was purified by a Milli Q.system (Millipore,Bedford,MA,USA). HPLC-grade methanol was purchased from Fisher Scientific Inc. HPLC-grade dichlormethane was purchased from J.T.Baker Company. Analytical grade hydrochloric acid, acetic acid and sodium acetate were obtained from Beijing Chemicals Corporation. β-glucuronidase/ sulfatase (β-glucuronidase 30 U/mL, arylsulfatase 60 U/mL ,liquid, 2 mL)was purchased from Roche Diagnotics GmbH Mannheim, Germany.

Stock standard solutions of BPA, NP, and OP (1 mg/mL)were prepared in methanol. Standard working solutions were prepared by dilution of the stock standard solutions with methanol.

Samples Urine samples were collected from sixty healthy volunteers aged from 20 to 70 years. All samples were stored at 2 ℃ until analysis.

Methods

Sample preparation One milliliter of urine sample was buffered with 100 μL of sodium acetate (pH 5.5). After β-glucuronidase/sulfatase (10 μL) was added, the sample was sealed in a glass tube, gently

mixed and incubated at 37 ℃ for 3h. The mixture was centrifuged at 3 000 rpm for 15 min.

After the enzymatic treatment, the samples were subjected to the solid phase extraction cartridge for clean-up and concentration. Firstly, the solution was acidified with 0.1 mol/L hydrochloric acid to a pH of 3.0-3.5. The enrichment and desalting were performed on OASIS HLB SPE cartridges (200mg/3cc, Waters, USA). Before use, the SPE cartridges were conditioned successively with 5 mL dichlormethane –methanol (9:1), 5 mL methanol, 5 mL water (pH=3.0-3.5 by HCl). After the sample was loaded on the cartridge and subsequently washed with 5 mL 20% methanol, the cartridges were dried on the vacuum system. The analytes were eluted from the SPE cartridges with 5mL dichlormethane–methanol (9:1), and the eluent was concentrated to dryness under a gentle stream of nitrogen. The eluent was reconstituted with methanol to a final volume of 0.5 mL for UPLC analysis.

Instrument conditions UPLC-MS/MS analysis was performed using a Waters system, consisting of a Waters Acquity™ Ultra Performance LC system connected to a Waters Quattro Ultima™ Pt Micromass spectrometer system. The UPLC-MS operation control and the data processing were achieved by a Masslynx software. The chromatographic system was equipped with an automatic injector, a vacuum membrane degasser, a quaternary pump and a column oven.

UPLC conditions The analytical column was Acquity UPLC™ BEH, C_{18}, (1.7 μm,2.1×100 mm). Mobile phases A and B were methanol and water, respectively. The system was run with a gradient program: 35%A-90%A (2 min)/90%A-96%A (0.5 min)/96%A-97%A(2.5 min)/97%A-100%A(0.1 min)/ 100%A held for 0.9 min/100%A-35%A(3 min). The flow rate was 0.3 mL/min, the column temperature was 40 ℃, the sample temper ature was 10 ℃, the injection volume was 10 μL, and the whole analysis lasted for 9 min.

MS conditions Mass spectrometry was carried out on a triple quadrupole mass spectrometer using the electrospray ionization (ESI), and with MRM scan mode. The selected parameters were as follows: capillary voltage:3.0 kV; cone voltage:40 kV; RF Lens 1:13.5; RF Lens 2:0.5, source temperature:120 ℃; desolvation temperature: 350 ℃; capillary gradient: 3.5; desolvation gas: 462 (L/h).The optimized collision energy and precursor and product ions for each analyte are listed in Table 1.

RESULTS

Method Validation

BPA, NP, and OP were well separated under the above conditions. The desirable linearity was obtained at a range from 1.00 to 500 ng/mL with the correlation coefficient of no less than 0.999. The LOD of BPA, NP and OP in urine samples were 0.10, 0.10 and 0.15 ng/mL based on a signal-to-noise ratio (*S/N*) of 3, respectively (Table 2). The recovery and precision of the method were assessed by relicate

Table 1. MS/MS Parameters of the Target Compounds

Compounds	Qualitative Transition	Quantitative Transition	Collision Energy (eV)
BPA	227>212	227>212	20
	227>211		25
NP	219>133	219>133	25
	219>161		25
OP	205>106	205>106	20
	205>133		20

Table 2. List of Linear Range, Calibration Curve, and LOD of Method

Compounds	Linear Range (ng/mL)	Calibration Curve	Correlation Coefficient(*r*)	LOD (ng/mL)
BPA	1.0-500	y=0.0223x+0.186	0.9992	0.10
NP	1.0-500	y=0.0127x+0.182	0.9995	0.10
OP-	1.0-500	y=0.00908x+0.0608	0.9995	0.15

analysis (n=6) of human urine samples spiked with standards at the 1, 10, and 50 ng/mL levels. The average recovery was higher than 85% (*RSD* %< 10%) for human urine samples. The results are shown in Table 3. The CID of standard sample and the extracted ion Chromatogram of BPA, NP, and OP in standard sample were shown in Figure 1-2.

Analysis of BPA, NP, and OP in Urine Samples

The method was applied to the analysis of BPA, NP and OP in urine samples from the healthy volunteers aged from 20 to 70 years. The results are shown in Table 4-5. The CID of urine sample and the extracted ion chromatogram of BPA, NP, and OP in urine samples were shown in Figure 3-4. Among 26 male samples, BPA at the level of 5.05 ng /mL was detected in 1 sample, NP ranging from 1.69 to 20.8 ng /mL was detected in 12 samples, and OP was detected at the level of 0.554-5.96 ng/mL. Among 34 female samples, BPA were detected in 7 samples at the level of 0.297-32.7 ng/mL, 17 samples were found to contain NP at the level of 1.86-27.8 ng/mL, and OP was detected in 10 samples at the level of 0.407-11.1 ng/mL. The concentrations of BPA, NP, and OP in females were higher than those in males. There were no significant differences in the detection ratios among different age groups.

Table 3. Recoveries of BPA, NP, and OP in Spiked Human Urine Samples

Compounds	Amount Spiked					
	1 ng/mL (n=6)		10 ng/mL (n=6)		50 ng/mL (n=6)	
	Average (Range) Recovery (%)	*RSD* (%)	Average (Range)Recovery (%)	*RSD* (%)	Average (Range)Recovery (%)	*RSD* (%)
BPA	90.7 (80.1-109)	9.11	98.2 (89.8-106)	7.56	87.4 (80.2-90.7)	8.82
NP	87.1 (81.3-105)	8.05	92.4 (85.1-109)	8.82	85.4 (82.1-98.5)	7.08
OP	89.3 (83.5-107)	8.54	90.4 (86.0-98.7)	8.15	87.5 (81.5-96.3)	8.9

Table 4. The Concentrations of BPA, NP, and OP in Urine Samples of the Different Gender Groups

Gender	No.of Samples	BPA		NP		OP	
		No.of Positive Samples	Range (ng/mL)	No.of Positive Samples	Range (ng/mL)	No.of Positive Samples	Range (ng/mL)
Male	26	1	5.05	12	1.69-20.8	7	0.554-5.96
Female	34	7	0.297-32.7	17	1.86-27.8	6	0.407-11.1

Table 5. Concentrations of BPA, NP, and OP in Urine Samples of the Different Age Groups

Age (year)	No.of Samples	BPA		NP		OP	
		No.of Positive Samples	Range(ng/mL)	No.of Positive Samples	Range (ng/mL)	No.of Positive Samples	Range (ng/mL)
21-30	14	0	-	5	2.05-12.6	2	1.14-2.53
31-40	17	1	5.05	10	1.86-27.8	6	0.407-11.1
41-50	11	1	0.297	4	5.10-20.8	3	0.849-5.96
51-60	8	4	1.42-5.03	5	3.03-5.68	4	1.09-2.64
60-	10	2	2.67-32.7	5	2.98-26.1	2	1.39-10.0

Note. - not found (less than LOQ).

DISCUSSION

The impact of environment on humans and animals has become an international issue. BPA, NP, and OP as estrogen substances have caused wide public concern. Environmental exposure to these phenolic compounds has been associated with the adverse reproductive and developmental effects. The European legislation set the specific migration limit (SML) for BPA at 3 mg/kg (Directive n°90/128/EEC and its amendments). Influenced by an opinion issued by the EFSA and published in 2002, SML for BPA was lowered to 0.6 mg/kg (directive n°2004/19/EC, first amendment of directive n°2002/72/EC). The European Union set SML for NP and OP at 50 mg/kg and 50 mg/kg, respectively.

Highly reliable methods are needed for the detection of these trace compounds in biological

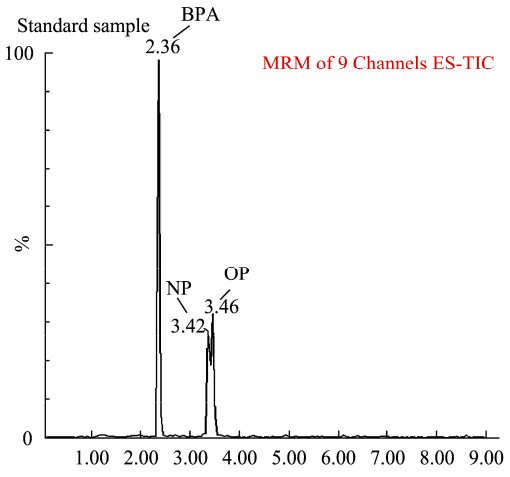

Figure 1. The CID of standard sample.

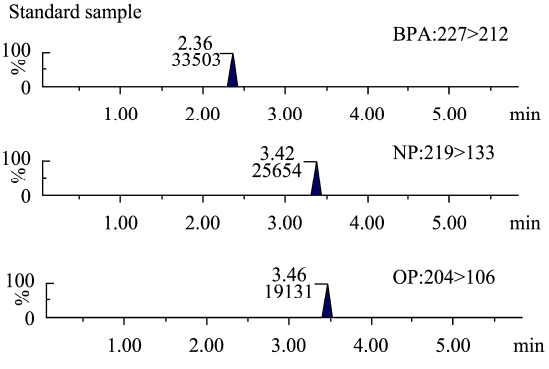

Figure 2. The extracted ion chromatogram of BPA, NP and OP in the standard sample.

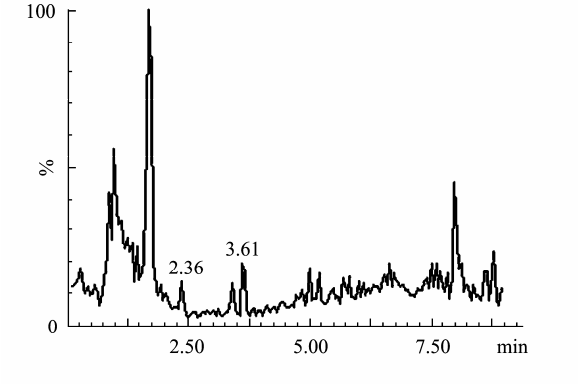

Figure 3. The CID of urine sampl.

samples. Urine sample collection is more widespread and easier than other biological sample collection. The determi nation of urine samples can be used to assess the relevance of human exposure to BPA, NP and OP in large-scale studies. What is lacking at the

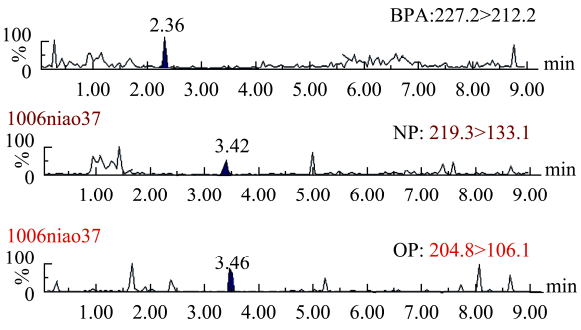

Figure 4. The extracted ion chromatogram of BPA, NP and OP in urine sample.

moment is analytical method for the simultaneous determination of BPA, NP, and OP in human urine samples. Previously, Inoue K, et al.[20] reported an approach for assessing human exposure to BPA. The sample solution was separated and detected by LC-MS. LOD was 0.1 ng/mL. The BPA level was investigated in human urine upon ingestion of canned food. The average urinary creatinine level was 13.1±6.1 mg/dL. Other sources of human exposure to BPA may also exist, including drinking water, indoor air, medical treatment and work environment. Kuklenyik Z, et al.[8] developed a sensitive method for measuring BPA and six APs in urine. The method was based on the use of automated SPE coupled with isotope dilution-GC/MS. The LOD was 0.1 ng/mL. Thirty urine samples collected from people who used painting products were analyzed. The frequency of detection was 96% (0.4-22.1 ng/mL) for BPA. Later, Kawaguchi M, et al.[11] measured trace amounts of phenolic xenoestrogens (PXs) in human urine samples by SBSE-TD-GC-MS. The LOD of OP, NP, and BPA were 10, 50, and 20 pg/ mL, respectively. Five samples were detected by this method, BPA at the level range of 0.93 to 5.41ng/mL was detected in 4 samples, NP at the level range of 1.04 to 2.00 ng/mL was detected in all samples, OP at the level of 0.05 ng/mL was detected only in one sample. Both investigated objects as described above were defined as occupational-risk populations.

To further develop the method, we have studied the levels of BPA, NP, and OP in urine of the general population by SPE-UPLC-MS. The analytical method based on previous works was improved and optimized. The metabolic products of BPA, NP, and OP in urine are mainly glucoside conjugate and sulphate conjugate[24]. In this study, we quantified both free and glucuronidated forms of BPA, NP, and OP by hydrolyzing glucuronide metabolites to

improve the measurement of the BPA, NP, and OP level in the human body.

The SPE technique has been shown to be a useful means for analyzing urine samplessince it can be easily performed and requires only a small amount of urine sample (about 1 mL) and organic solvent. In order to achieve a more sensitive quantification of these compounds, t OASIS HLB cartridge was used in this study as an extraction-preconcentration step prior to chromatogra phic determination. As BPA, NP, and OP are often used for the production of polycarb onates and polystyrene resins, it is ubiquitous for the plastic products that contain these compounds. In the current study, we found that the SPE cartridge was contaminated by NP. Therefore, the SPE cartridge was washed before use by CH_2C1_2–CH_3OH, CH_3OH and H_2O (pH=3.0-3.5), which not only activated the cartridge but also removed impure materials. Using methanol or dichlormethane only cannot completely elute these target compounds. The ratio of methanol or dichlormethane as eluting solvent in the solid-phase extraction was optimized. Methanol-dichlormethane (9:1) was chosen as the eluting solvent with the desirable recoveries.

In short, an analytical method has been developed based on enzymolysis– SPE-UPLC-MS/MS for the quantitative measurement of BPA, NP, and OP in urine samples. This method is free from tedious and toxic derivation procedures. UPLC-MS/MS spectrometry is faster with higher sensitivity than LC-MS/MS. It is accurate and suitable for the analysis of a large number of samples. The result of 60 urine samples collected from healthy people showed that BPA, NP, and OP appear to be prevalent in human urine. This is especially true for NP. The method is therefore valuable for future studies assessing exposure to BPA, NP, and OP in the general population.

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