Environ. Eng. Res. Vol. 12, No. 3, pp. 93~100, 2007
Korean Society of Environmental Engineers

SENSITIVE DETERMINATION OF ELEVEN PHENOLIC ENDOCRINE-DISRUPTING CHEMICALS IN HUMAN URINE USING GAS CHROMATOGRAPHY/MASS SPECTROMETRY-SELECTED ION MONITORING

Hyub Kim†, Cheol-Hyeon Jang[1]

Department of Chemistry, Pohang University of Science and Technology, Pohang 790-784, Korea
[1]Department of Environmental Engineering, Hanbat National University, Daejeon 305-719, Korea
(received , accepted)

Abstract : I improved an analytical method for determining trace amounts of eleven phenolic endocrine-disrupting chemicals (11 phenolic EDCs) in human urine. The 11 phenolic EDCs were subjected to hydrolysis and then to solid phase extraction with a XAD-4 column. Alkylphenols, chlorophenols, and bisphenol A in XAD-4 column were eluted with acetonitrile, and the eluate was concentrated under a nitrogen stream, and then *tert*-butyldimethylsilylation. Separation and determination were done by gas chromatography, using mass spectrometry operating in the selective ion monitoring mode for quantitation. For *tert*-butyldimethylsily (TBDMS) derivatization the recoveries were 91.2~125.9%, the limits of quantitation (LOQ) for the 11 phenolic EDCs in the nanogram-per-milliliter range (0.025~1.000 ng/mL) were thus achieved by using 1 mL of urine, and the SIM responses were linear with the correlation coefficient varying by 0.9300~0.9943. Based on the results for urine samples from unexposed individuals, 4-*tert*-octylphenol and pentachlorophenol were detected in hydrolysed urine sample. Other alkylphenols, chlorophenols and bisphenol A were not detected.

Key Words : Endocrine disrupting chemicals, TBDMS, GC/MS-SIM, LOQ, Phenol

INTRODUCTION

Phenolic compounds were common by-products of many industrial processes (manufacture of dyes, plastics, drugs, antioxidants, paper and the petroleum industry), and the degradation of some pesticides.

They can be absorbed into the human body, whether dermally, orally or via the airways.[1,2)]

Concern has recently been increased about the endocrine disrupting effects of these compounds.

Due to their toxicological potentials and ubiquitous environmental occurrence, alkylphenols, chlorophenols, and bisphenol A were classified as “endocrine disrupting chemical” (EDC) by the Japan Environmental Health and Safety Division, Environmental Health Department, Environment Agency.

In Japan, the maximum admissible concentration of 11 phenolic EDCs in biological sample should be 1 μg/kg (nonylphenol 10 μg/kg) for each compound,[3)] but human urine limitation was not established.

However, chlorophenols and bisphenol A were frequently monitored in human urine and other biological samples to obtain an indication of occupational exposure or exposure to environmental contamination.[2,4)] Thus, the potential effects of 11 phenolic EDCs on human health and human

† Corresponding author
E-mail: khlyj@postech.ac.kr
Tel: 054-279-8158, Fax: 054-279-3399

exposure to the 11 phenolic EDCs must be examined for risk assessment.

The 11 EDCs were released in urine, both as such as sulfate or glucoronide conjugates, the amount of conjugation depending on the particular 11 EDCs and their concentrations in the urine.[5,6)]

When information was lacking about sources of exposure, internal does measurements must be from the basis of human exposure assessment.[7)] Therefore, a rapid, accurate and sensitive analytical means of human urine samples needs to be developed.

In general, the gas chromatography/mass spectrometry (GC/MS) method was the most commonly employed technique for the human urine analysis of 11 phenolic EDCs.[8-10)] However, due to the low volatility of some compounds including a hydroxyl group, derivatization steps aimed at producing more the 11 phenolic EDCs were required to improved technique for the environmental and biological analysis of the subsequent GC/MS analysis. To overcome these problems, acetylation, benzylation, benzoylation, alkylation, and silylation have been employed.[11-18)] Acetylation and benzylation, however, require phenols to be reacted as phenolate anions in alkaline solution, and are thus not suitable for phenols such as pentachlorophenol that are prone to oxidative degradation at pH above 8.[11)]

Moreover the EI mass spectra of the derivatives should be characteristic allowing trace level determination of the each phenol in human urine samples by GC/MS detection.

This study was undertaken to develop and validate a single gas chromatographic-mass spectrometric method for simultaneous quantitation of 11 phenolic EDCs in human urine samples. It was based on an acidic deconjugation, solid phase extraction and TBDMS derivatization. The resulting TBDMS derivatives were then subjected to GC/MS-SIM quantitation.

MATERIALS AND METHODS

Materials

The nine phenol standards, phenanthrene-d_{10}, and bisphenol-d_{16} were purchased from Sigma-Aldrich (Milwaukee, WI, USA); 4-*n*-hexylphenol and 4-n-heptylphenol from TCI (Tokyo, Japan) and Acros (Belgium).

N-methyl-N-(*tert*-butyl-dimethylsilyl)-trifluouoa cetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA).

Triethylamine (TEA), sulfuric acid, and anhydrous sodium sulfate were obtained from Junsei (Tokyo, Japan).

Acetonitrile, ethylacetate and n-hexane were purchased from J.T. Baker Analytical (Phillipsbug, NJ, USA).

All other chemicals were analytical grades and used as received.

20-60 mesh of AMBERLITE XAD-4 was purchased from Sigma (St. Louis, MO, USA). A luer-tipped glass tube (10 mm I.D.) packed with 0.5 g of XAD-4 was washed successively with dichloromethane, *n*-hexane, ethylacetate, acetonitrile, and deionized water followed by activation pH 2 water prior to being used as a solid-phase extraction (SPE) tube. The pH 2 water was acidified to pH 2 with H_2SO_4.

Phenol and Internal Standard Solutions

Each stock solution of the phenols was made up at 1 mg/mL in acetonitrile and stored frozen.

Working solutions were made by combining aliquots of each stock solution and diluting with acetonitrile and stored in a refrigerator. Two separate internal standard (I.S.) solutions were prepared by dissolving phenanthrene-d_{10}, and bisphenol A-d_{16} at 0.1 mg/mL and 0.05 mg/mL in acetonitrile respectively.

Sample Preparation

100 mL of Urine samples from unexposed individuals were collected in sterilized containers and frozen immediately (-20°C) until analysis. For the recovery experiments, sample solutions containing 2 μg/mL of each 11 phenolic EDCs and bisphenol A-d_{16} as I.S. at 1 μg/mL, in acetonitrile per milliliter of urine.

The 11 phenolic EDCs were hydrolysed by heating 1 mL of urine sample plus 1 mL of 6

M HCl in a screw-capped glass tube that was immersed in a DRY THERMO BATH (EYELA, JAPAN) at 100°C for 30 min. After cooling to room temperature, the hydrolysed urine was adjusted to pH 1 with 2 M NaOH (about 5 mL). The hydrolysed urine passed through a preactivated XAD-4 column, using a solid-phase extractor (IST, UK). The column was eluted with 20 mL of hexane and then the eluate was discarded. Next, the 11 phenolic EDCs were eluted twice with 4 mL of acetonitrile allowing the solvent to react with the adsorbent for 5 min before elution. The eluate was collected in 50 µL of TEA by evaporation (N_2 steam, 60°C).

O-*tert*-butyldimethylsilylation

50 µL of the eluate obtained from SPE, were derivatized at 100°C using 40 µL of MTBSTFA. The derivatization was carried out in sampling with a Teflon-lined sampler cap and were placed in a heating module for 1 hr. The solution containing the derivatives was added 1 µg of phenanthrene-d_{10}. All the samples were individually prepared in three replicate and directly examined by GC/MS-SIM.

Gas Chromatography/mass Spectrometry

To obtain mass spectra, an Agilent 6890 plus gas chromatograph with an DB-5ms (SE-54 bonded phase) capillary column (30 m x 0.25 mm I.D., 0.25 µm film thickness), interfaced to an Agilent 5973N mass selective detector (70 eV, electron impact mode) and on-line to an HP G1701 DA MS Chemstation program was used. The 1.0 µL volume samples were injected in splitless mode with a purge delay time of 0.7 min. The oven temperature was initially 60°C for 1 min and then raised to 280°C at 10°C/min, and held for 20 min. The injector and interface temperatures were 260 and 200°C, respectively.

The GC/MS measurement was performed by monitoring the ion mode. The quantitation ions for SIM are shown in Table 2. The time for solvent delay was set to 5 min. For selected ion monitoring (SIM), two or three (nonylphenol) characteristic ions were selected for each compound and scanned using corresponding time windows, with dwell times of the range 150 ms per ion. The insert liner were exchanged after a maximum of 50 injections.

Calculations

All the quantitative calculations for the recoveries and linearity tests were based on the peak area ratios relative to the I.S. The SIM response curves used for quantitation were generated from derivatized phenols standards at five concentration levels ranging from 0.05 to 4 ng/µL. Least-squares regression analysis was performed on the measured peak area ratios against increasing weight ratios of phenols to I.S., in order to test linearity of the whole procedure and to plot calibration curves.

Limits of Quantitation (LOQ)

The LOQ for the GC/MS-SIM method was evaluated by spiking three replicates of human urine samples with alkyl-, chlorophenols and bisphenol A at a concentration the estimated signal to noise ratio 3~5.

RESULTS AND DISCUSSION

The simultaneous detection and identification of 11 phenolic EDCs in single analysis was frequently monitored in human urine and other biological samples to obtained an indication of occupational exposure or exposure to environmental contamination.

In this study, the 11 phenolic EDCs in urine samples were hydrolysed and then, solid-phase extraction with XAD-4 and subsequent conversion to TBDMS derivatives for sensitive analysis with the GC/MS-SIM.

GC/MS Characteristics of 11 Phenolic EDCs TBDMS Derivatization

On the whole, all the 11 phenolic EDCs included in our study have been silylated successfully, applying derivatization with MTBSTFA.

Table 1 compiles the most important masses and their corresponding relative abundances in the EI mass spectra of the TBDMS derivatives of all 11

Table 1. Relative retention times (RRT) and mass spectral characteristic ions of phenol *tert*-butyldimethylsilyl derivatives

Compound	RT	RRT	$[M]^+$	$[M-15]^+$	$[M-57]^+$	Other characteristic ion, m/z (%)
4-*t*-Butylphenol	13.79	0.855	276 (3)	261 (1)	219 (100)	221 (74) 201 (36) 183 (28)
2,4-Dichlorophenol	14.26	0.885	264 (62)	249 (27)	207 (4)	193 (20) 169 (66) 151 (100)
4-*n*-Butylphenol	14.65	0.909	264 (36)	249 (2)	207 (7)	225 (100) 208 (10) 169 (12)
4-*n*-Pentylphenol	15.75	0.977	278 (40)	263 (2)	263 (2)	221 (9) 239 (100) 169 (20)
Phenanthrene-d10	16.12	1.000	188 (100)			
4-*n*-Hexylphenol	16.83	1.044	292 (37)	277 (1)	235 (6)	253 (100) 183 (11) 165 (28)
4-*t*-Octylphenol	17.07	1.059	320 (7)	305 (1)	263 (1)	249 (100) 73 (12)
4-*n*-Heptylphenol	17.85	1.107	306 (43)	291 (3)	291 (3)	249 (7) 267 (100) 165 (41)
Nonylphenol	18.04	1.118	334 (8)	319 (0)	277 (96)	306 (51) 267 (100) 235 (75)
	18.12	1.124	334 (18)	319 (0)	277 (3)	305 (37) 263 (53) 249 (100)
	18.17	1.127	334 (23)	319 (2)	277 (5)	305 (33) 263 (74) 249 (100)
	18.31	1.136	334 (15)	319 (2)	277 (10)	249 (100) 73 (14)
	18.47	1.146	334 (9)	319 (1)	277 (100)	305 (18) 291 (29) 235 (49)
	18.55	1.151	334 (2)	319 (6)	277 (100)	235 (34) 221 (26)
4-*n*-Octylphenol	18.84	1.169	320 (55)	305 (3)	263 (8)	281 (100) 264 (21) 165 (74)
Pentachlorophenol	23.80	1.476	378 (1)	363 (0)	321 (1)	376 (29) 375 (100) 357 (77)
Bisphenol A-d_{16}	24.40	1.514	470 (37)	455 (4)	413 (0)	453 (100) 217 (39)
Bisphenol A	24.51	1.520	456 (32)	441 (100)	399 (0)	207 (35)

Relative retention times (RRT) : RT of analyte / RT of phenanthrene-d_{10}

phenol EDCs that were investigated in our study.

In almost all EI mass spectra, the ion $[M-57]^+$ resulting from the cleavage of the *tert*-butyl moiety (M^+-$C(CH_3)_3$) or the ion $[M-15]^+$ resulting from the cleavage of the methyl moiety (-CH_3) from the molecule was the base peak.

Selected Ion Monitoring (SIM)

Since GC/MS in the full-scan mode does not often provide the sensitivity necessary in trace-level analysis, SIM was applied as a routine method to achieve lower detection limits. Two characteristic ions from the mass spectrum for each compound were selected and recorded in corresponding retention time windows (Table 2).

For all TBDMS derivatives, the ion $[M-57]^+$ and one other indicative ions were recorded as qualifiers, respectively (Table 1).

Table 2. Selected ion groups of 11 phenolic EDCs for GC/MS-SIM mode analysis

Compound	Time window (min)	Selected ion (m/z)	Dwell time (ms per ion)
4-*t*-Butylphenol	13.50-15.50	264, 151	150
2,4-Dichlorophenol	13.50-15.50	221, 219	150
4-*n*-Butylphenol	13.50-15.50	264, 225	150
Phenanthrene-d_{10}	15.50-17.50	188	150
4-*n*-Pentylphenol	15.50-17.50	278, 239	150
4-*n*-Hexylphenol	15.50-17.50	292, 253	150
4-*t*-Octylphenol	15.50-17.50	320, 249	150
4-*n*-Heptylphenol	18.00-20.00	306, 249	150
Nonylphenol	18.00-20.00	334, 277, 249	150
4-*n*-Octylphenol	18.00-20.00	320, 263	150
			150
Pentachlorophenol	23.00-25.00	375, 357	150
Bisphenol A-d_{16}	23.00-25.00	470, 453	150
Bisphenol A	23.00-25.00	456, 441	150

Recovery, Precision and Limits of Quantitation

A spike and recovery study was performed to determine the efficiency and reproducibility for the target analyte. Three human urine blank samples (man, age=4) replicates were spiked with all target analytes, hydrolysed, extracted and analyzed. The spiked samples for GC/MS-SIM analysis were derivatized prior to analysis. The experimental results showed that the 11 phenolic EDCs had average recoveries falling between 91.2~125.9%. The relative standard deviation (RSD) for replicates recovery analyses ranged in 3.44~9.66 % (Table 3).

The combined method of SPE and TBDMS derivatization was examined to test the linear relation between detector response (expressed as peak area ratio) and amounts of phenols. As listed in Table 4, linear response were obtained for the 11 phenolic EDCs in range of 50~400 ng with correlation coefficients varying 0.9300~ 0.9983. The linearity of the TBDMS derivatizations for GC/MS-SIM separation of 11 phenolic EDCs appear to be satisfactory for their quantitative measurements in unknown samples (Table 4).

A list of LOQ for alkyl-, chlorophenols and bisphenol A were given in Table 3. Results shown that limit of quntitation at 0.025~1.000 ng/mL for the 11 phenolic EDCs. An example for the performance of the method applied to human urine samples containing a high load of matrix is presented in Figure 1, showing the SIM- chromatogram of a spiked human urine sample. This shows that chromatograms can be evaluated easily, along with the identification and quantitation of the origin of the sample and its matrix content.

Table 3. Recoveries and limits of quantitation for the 11 phenolic EDCs extracted from human urine samples using GC/MS-SIM

Compound	Recovery[a] (% rsd)	Limits of quantitation[b] (ng/mL)
4-*t*-Butylphenol	92.0 (6.57)	0.025
2,4-Dichlorophenol	92.8 (5.04)	0.250
4-*n*-Butylphenol	105.3 (6.85)	0.025
4-*n*-Pentylphenol	103.4 (7.46)	0.025
4-*n*-Hexylphenol	105.6 (9.66)	0.050
4-*t*-Octylphenol	112.4 (3.48)	0.025
4-*n*-Heptylphenol	101.4 (5.89)	0.250
Nonylphenol	91.2 (9.21)	1.000
4-*n*-Octylphenol	97.1 (3.44)	0.250
Pentachlorophenol	123.9 (7.89)	0.500
Bisphenol A	125.9 (7.47)	0.025

[a] A human urine sample spiked with alkylphenols, chlorophenols, and bisphenol A (2 ug/mL, n=3)
[a,b] I.S. : phenanthrene-d_{10}

Table 4. Linear regression analysis of relative response vs. relative quantities of phenols as TBDMS derivatives

Compound	Regression line		Correlation coefficient
	m[a]	b	r
4-*t*-Butylphenol	0.0006	0.0167	0.9772
2,4-Dichlorophenol	0.0004	0.0046	0.9798
4-*n*-Butylphenol	0.0017	0.0016	0.9983
4-*n*-Pentylphenol	0.003	0.0835	0.9802
4-*n*-Hexylphenol	0.0027	0.0812	0.9875
4-*t*-Octylphenol	0.0031	1.2171	0.9889
4-*n*-Heptylphenol	0.0029	0.1493	0.9682
Nonylphenol	0.0046	0.1352	0.9943
4-*n*-Octylphenol	0.0031	0.1503	0.9635
Pentachlorophenol	7E-06	0.0009	0.9531
Bisphenol A	0.0025	0.1421	0.9300

[a]m = Slope=relative mass response=mean peak area ratio of phenol x mass of I.S./mass of phenol ;
b=y-intercept; r=correlation coefficient
Calibration range; 50~400 ng

Application to Real Samples

The proposed method was applied to human urine samples. 4-tert-octylphenol and pentachlorophenol were detected in human urine sample (man, age=40) for the value 130.5 and 109.6 ng/mL, respectively (Table 5). In Figure 2, showed a SIM chromatogram of the human urine sample.

Table 5. Concentration of target compounds from human urine sample

RT (min)	Compound Identity	Value[a] (ng/mL)	Recovery[b] (%)
17.07	4-*t*-Octylphenol	130.5	112.8
23.80	Pentachlorophenol	109.6	

[a]I.S. : phenanthrene-d_{10}
[b]I.S. : bisphenol A-d_{16}

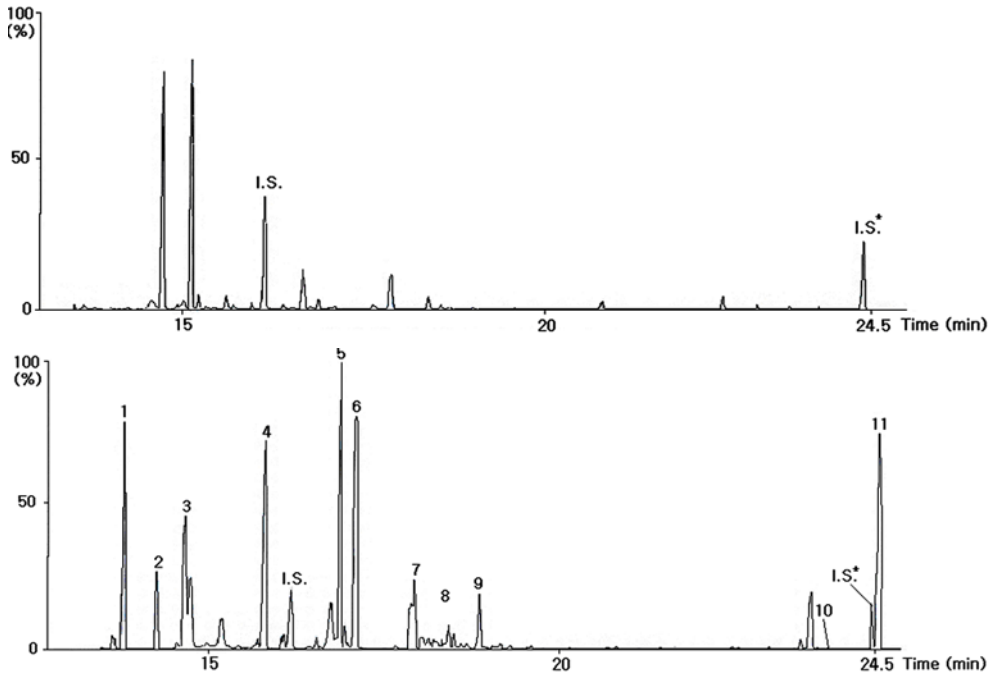

Figure 1. SIM chromatograms obtained from human urine blank sample (man, age=4) after TBDMS derivatization (upper trace), and spiked human urine blank sample at 2 ug/mL of 11 phenolic EDCs after TBDMS derivatization. Peaks; 1=4-*t*-butylphenol; 2=2,4-dichlorophenol; 3=4-*n*-butylphenol; 4=4-*n*-pentylphenol; I.S.=phenanthrene-d_{10}; 5=4-*n*-hexylphenol; 6=4-*t*-octylphenol; 7=4-*n*-heptyl-phenol; 8=nonylphenol; 9=4-*n*-octylphenol; 10=pentachlorophenol; I.S.*=bisphenol A-d_{16}; 11=bisphenol A.

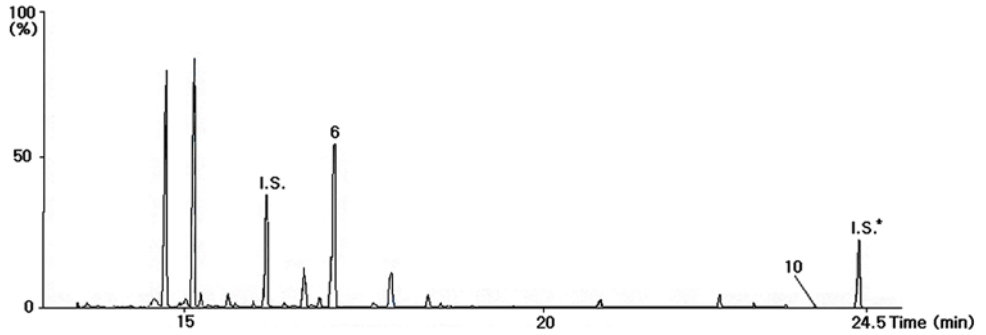

Figure 2. SIM chromatograms obtained from human urine sample (man, age=40) after TBDMS derivatization. Peaks; I.S.=phenanthrene-d_{10}; 6=4-*t*-octylphenol; 10=pentachloro-phenol; I.S.*=bisphenol A-d_{16}.

CONCLUSIONS

The proposed method allowed the multivariate determination of 11 phenol EDCs in a urinary matrix. The method developed offers good precision and accuracy without the need of standard additions or previous computational estimation of the shape of the spectrum of the interference components.

An extension of the present method for the rapid profiling and screening of human urine and biological samples for toxic phenols and their quantitative measurements is in progress.

ACKNOWLEDGEMENTS

The authors thank the Department of Ministry of Commerce, Industry and Energy and Sangju National University, for supporting this research.

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