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Measurement of 4-nonylphenol and 4-*tert*-octylphenol in human urine by column-switching liquid chromatography–mass spectrometry

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Abstract

We report a method for determining 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) levels in human urine samples by column-switching liquid chromatography–electrospray mass spectrometry after enzymatic deglucuronidation. The method involves enzymatic deconjugation by β-glucuronidase and correction by the stable isotopically labeled internal standard, 4-(1-methyl)octylphenol-d_5. The compounds were separated by reversed-phase chromatography with a C_{18} column, and detected by selected ion monitoring in the negative mode. After adding an internal standard to urine samples, a direct analysis was carried out. The average recoveries of OP and NP were above 85.0% with correction using the added internal standard. The quantitation limit in the urine samples was 0.3 ng ml^{-1}. The method enables the precise determination of standards and may be applied to the detection of trace amounts of OP and NP in human urine samples.

Keywords: Nonylphenol; Octylphenol; Column-switching LC–MS; Solid-phase extraction; Urine

1. Introduction

The alkylphenols 4-nonylphenol (NP) and 4-*tert*-octylphenol (OP) exist mainly as intermediates in the manufacturing industry; NP and OP are also degradation products of non-ionic surfactants alkylphenols ethoxylate used in industrial and institutional formulations. Alkylphenols, OP and NP, have been shown to be exist in the environment such as river water and sewage sludge, and in fish tissue [1–5]. In addition, the estrogenic activity of OP and NP has been extensively evaluated in a variety of assays [6–8].

Recently, many novel analytical techniques have been used for the determination of OP and NP in environmental and water samples. The reliable methods for NP analysis are direct solid-phase microextraction (SPME)–gas chromatography–mass spectrometry (GC–MS) [1,9], liquid chromatography (LC) with on-line solid-phase extraction (SPE) [10], and LC with coulometric-array detection [11]. In addition, there are

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many reports on the use of LC with mass spectrometry (LC–MS) for the determination of NP in environmental samples [12–15]. LC–MS is very useful for the determination of trace levels of alkylphenols such as OP and NP because the MS detector has higher sensitivity, selectivity and reliability than other commonly used detectors. In contrast, few studies are available regarding the use of the high-throughput and on-line sample preparation LC–MS method for the identification and quantitative analysis of OP and NP in human biological samples.

The importance of knowing the sources of human exposure is poorly understood. Recently, leaching and contamination of NP from food wrapping films, food-contacting plastics, toys and foods have been reported [16–20]. Therefore, it is possible that healthy humans are exposed to OP and NP via a variety of daily activities. However, to our knowledge, there have been no studies on OP and NP exposure in humans except for our study of human blood levels [21]. In at study, trace levels of OP and NP ranging from ND (<0.5) to 1.0 ng ml^{-1} were found in human blood samples [21]. However, blood is thought to be unsuitable for exposure assessment of healthy people. Additionally, compared to urine collection, blood collection is thought to be unsuitable for exposure assessment of healthy people because of its invasiveness. Equally important in measuring OP and NP in human biological samples are the following: their contamination in the laboratory and during pretreatment, their accurate and sensitive measurement, and the monitoring of their free forms and metabolites. In the present study, we took necessary precautions to prevent contamination during sampling and pretreatment. Moreover, an accurate, sensitive, selective and high-throughput analytical method was developed for the quantification of OP and NP by using internal standards. The use of β-glucuronidase to hydrolyze glucuronide metabolites enables the quantification of both free and glucuronidated forms of OP and NP. We evaluated the method for screening these compounds in human urine samples with creatinine correction.

In the present study, we employed column-switching LC with an electrospray MS detector coupled with on-line extraction, enzymatic deconjugation, and creatinine correction for the sensitive, selective and accurate determination of OP and NP. This novel method was successfully used to determinate OP and NP in urine samples from healthy humans, and the main source of human exposure was clarify.

2. Experimental

2.1. Materials and reagents

4-Nonylphenol (mixture) (NP), [2,3,5,6-$^2H_4$]4-*n*-nonylphenol (NP-d_4) and 4-*tert*-octylphenol (OP) of environmental analytical grade were purchased from Kanto Chemical Inc., Tokyo, Japan. [2,3,5,6-$^2H_4$]4-*tert*-Octylphenol (OP-d_4) and 4-(1-methyl)octylphenol-d_5 (*m*-OP-d_5) were from Hayashi, Osaka, Japan. The structures of these stable isotopically labeled internal standards are shown in Fig. 1. *E. coli* β-glucuronidase (15 μl, 89 U ml^{-1}) was used for enzymatic deconjugation (Fluka, Buchs, Switzerland). Other reagents and solvents were of pesticide or HPLC grade and purchased from Wako, Osaka, Japan. The distilled water purification system was Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). Creatinine determination was carried out using creatinine test wako (Law of Jaffé) from Wako.

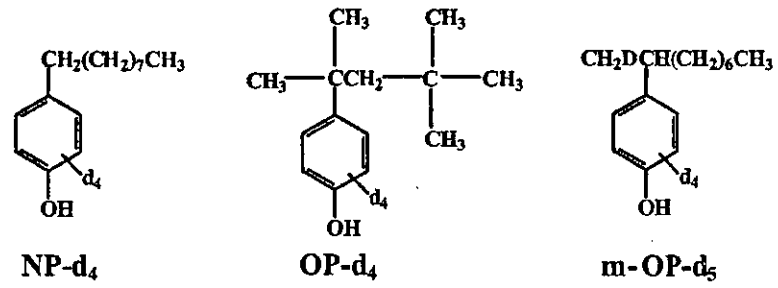

Fig. 1. Structures of stable isotopically labeled internal standards: NP-d_4, [2,3,5,6-$^2H_4$]4-*n*-nonylphenol; OP-d_4, [2,3,5,6-$^2H_4$]4-*tert*-octylphenol; *m*-OP-d_5, 4-(1-methyl)octylphenol-d_5.

2.2. Standards

Concentrated solutions (1.0 mg ml^{-1}) of NP and OP were prepared as required by the addition of methanol with specific amounts of internal standard. The MS signals were monitored in the selected ion mode (SIM) at *m/z* 219 (NP), 205 (OP), 223 (NP-d_4), 209 (OP-d_4) and 224 (*m*-OP-d_5).

2.3. Instrumentation and analytical conditions

Liquid chromatography with electrospray mass spectrometry (LC–ESI–MS) was performed using an Agilent 1100 MSD-SL system (Agilent Technologies, Palo Alto, USA). The direct injection volume was 100 μl in the needle washing mode. The column used was Mightysil RP-18 (2.0 mm × 100 mm: 5 μm) with a Mightysil RP-18 GP pre-column (2.0 mm × 5 mm: 5 μm). The column oven was maintained at 40 °C.

The working conditions for ESI–MS were as follows: the drying nitrogen gas temperature was set at 350 °C and was introduced into the capillary region at a flow rate of 12 l min^{-1}; the capillary was held at a potential of 3500 V relative to the counter electrode in the negative-ion mode. The fragmentor voltage was 130 V during the chromatographic run. The chromatographic monitoring mode was gain 1.0. When working in the SIM mode, each *m/z* ion was designated as the $[M - H]^-$ of each compound.

The column-switching LC–MS coupled with an on-line extraction system consists of this LC–MS combined with an LC pump (Shimadzu LC-10AS pump: Shimadzu, Kyoto, Japan) and an extraction column (TSK-Precolumn BSA-ODS/S 4.6 mm × 10 mm: Tosoh, Tokyo, Japan). The column-switching LC–MS system, as depicted in Fig. 2, was used for the direct injection of urine sample. In addition, the system program was shown in Table 1. After a urine sample was injected by an auto-sampler, it was loaded onto the extraction column by flowing pure water at a rate of 0.5 ml min^{-1} using pump A (Shimadzu LC-10AS) for 5 min. While the extraction column was directed to drain during the 5 min run, the sample was extracted and purified on the on-line extraction column. After on-line extraction for 5 min, the position of the switching valve was changed (see Fig. 2: configuration B). This configuration connected the back-flashing extraction column to the analytical column and the MS detector in the flow path of pump B (Agilent 1100 system). Separation was carried out using a gradient mobile phase of 0.1 mM ammonium acetate in water-acetonitrile at a flow rate of 0.2 ml min^{-1}. The gradient mode was as follows: 0 to 10 min using 40% acetonitrile based solution, then 10 to 20 min using a linear increase from 40 to 80% (v/v) acetonitrile solution, and holding at 80%. After detection of these compounds, the switching valve was returned to its original position (see Fig. 2: configuration A). The run time for the assay of the sample mixture was 30 min.

Table 1
Time program of column-switching LC–MS coupled with an on-line extraction condition

Time (min)	Event	Column position (configrations are shown in Fig. 2)	Mobile phase (A–B, v/v)[a]
0.0	Sample injection	Configuration A	60:40
5.0	Valve changed	Configuration B	60:40
10.0		Configuration B	60:40
	Gradient		↓
20.0		Configuration B	20:80
30.0		Configuration A	20:80

[a] Pump B solvent: (A) 0.1 mM ammonium acetate in water; (B) acetonitrile.

In the quantitative procedure, standard solutions of OP and NP were prepared in aqueous solution to cover the calibration range. Quantitative analysis was performed in the SIM mode in order to maximize sensitivity. OP and NP concentrations in each sample were calculated relative to the stable isotopically labeled internal standards added to the sample prior to direct analysis, and gave a final detecting concentration of 50 ng ml^{-1}. Six-point calibrations (0.2–12.5 ng ml^{-1}) were performed daily for all samples with internal standards.

2.4. Human urine samples

Urine samples were obtained from 10 healthy volunteers aged 21–28. All samples were stored at −80 °C prior to use.

2.5. Enzymatic deconjugation of glucuronidated forms of OP and NP in urine

One milliliter of human urine sample was buffered with ammonium acetate (200 μl, 1.0 M, pH 6.8).

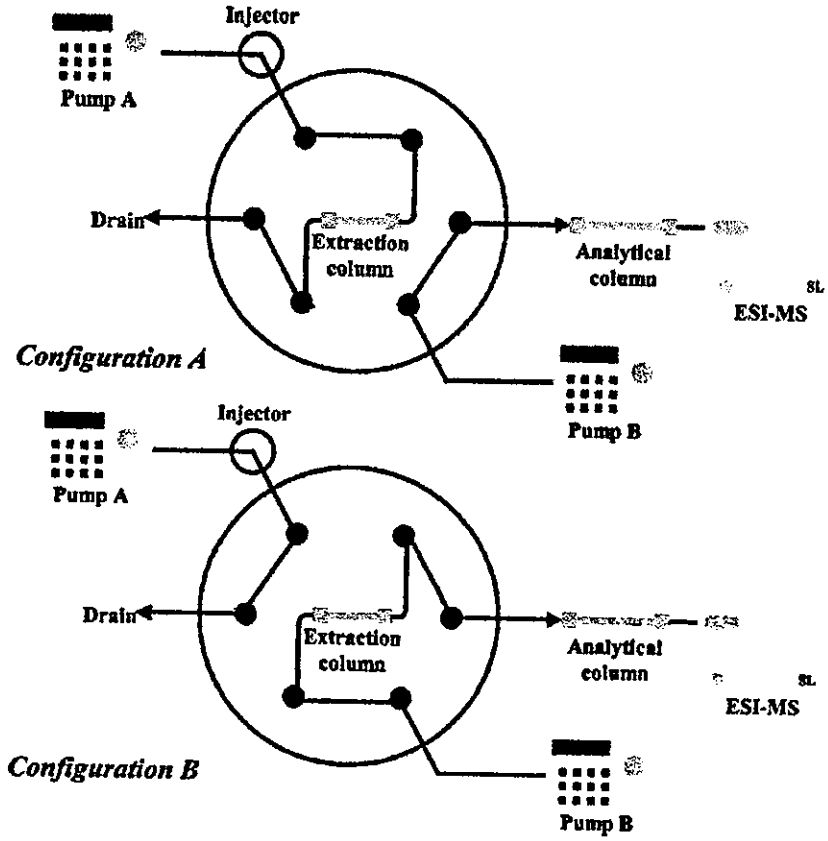

Fig. 2. Schematic representation of the column-switching LC–MS system. LC–ESI–MS was performed using an Agilent 1100 MSD-SL system (pump B + ESI–MS). Sample preparation system was performed using a Shimadzu LC-10AS pump (pump A).

After *E. coli* β-glucuronidase (15 μl, 89 U ml^{-1}; Fluka) was added, the sample was sealed in a glass tube and gently mixed. Quantitative glucuronidase hydrolysis to release the free OP and NP was accomplished by incubating at 37 °C for 3 h. After enzymatic deconjugation, the sample were filled with water to a total volume of 1.5 ml. This treatment is sufficient to deconjugate the glucuronidase of glucuronidated bisphenol A and phthalate monoesters [22–24].

3. Results and discussion

3.1. Optimization of column-switching LC–MS detection and conditions

In the mass spectral analysis using the ESI–MS detector with flow-through injection analysis of the standard solutions (1.0 μg ml^{-1}), the molecular ions that were designated as the $[M - H]^-$ ions were observed as the main peaks (Table 2). Then, we examined the optimal ionization for detecting these compounds. The parameters affecting the determination of compounds by LC–MS are the fragmentor voltage and the mobile phase. In order to determine the optimum fragmentor voltage for the detection of OP and NP, the *m/z*

Table 2
Ions monitored (*m/z*) for the determination of standards and stable isotopically labeled internal standards

Compound	Quantitation ion (*m/z*)
4-*tert*-Octylphenol (OP)	$[M - H]^-$ 205
4-Nonylphenol (NP)	$[M - H]^-$ 219
[2,3,5,6-$^2H_4$]4-*tert*-Octylphenol (OP-d_4)	$[M - H]^-$ 209
[2,3,5,6-$^2H_4$]4-*n*-Nonylphenol (NP-d_4)	$[M - H]^-$ 223
4-(1-Methyl)octylphenol-d_5 (*m*-OP-d_5)	$[M - H]^-$ 224

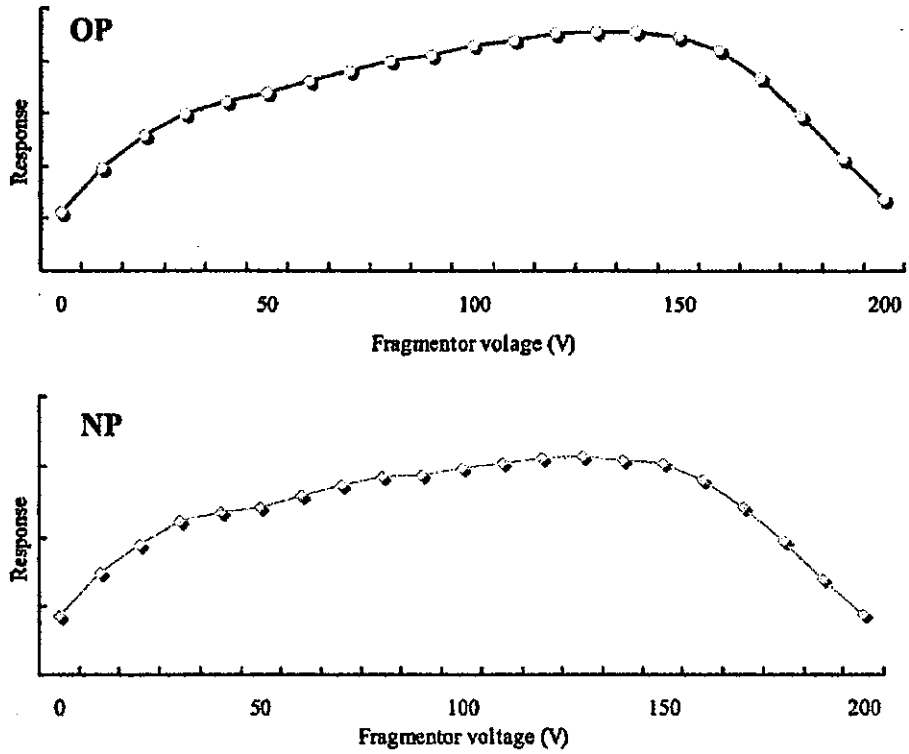

Fig. 3. Effect of fragmentor voltage on the peak responses of OP (*m/z* 205) and NP (*m/z* 219). Analytical conditions: electrospray MS (drying nitrogen gas temperature set at 350 °C; capillary region flow rate, 12 l min^{-1}; capillary potential 3500 V relative to the counter electrode; negative-ion mode; fragmentor voltage range 0–200 V; and SIM mode). Carrier solution: 0.1 mM ammonium acetate in water-acetonitrile (40:60, v/v) at a flow rate of 0.2 ml min^{-1}.

signals for OP and NP were plotted against the fragmentor voltage (Fig. 3). The effect of the mobile phase was also investigated (Fig. 4). The main *m/z* signals showed a maximum in 0.1 mM ammonium acetate as the mobile phase at 130 V for OP and NP.

The calculated detection limits of OP and NP were 0.05 and 0.1 ng ml^{-1}, respectively, for column-switching LC–MS detection with the ratio of the compound's signal to the background signal (S/N) = 3 (Table 3). In addition, the quantitation limit calculated when S/N = 5 was 0.3 ng ml^{-1} in urine (Table 3). Peak area ratios with respect to each internal standard were plotted, and the response was found to be linear over the validated range with correlation coefficients (r) higher of 0.999 (Table 3). Therefore, the method enables the precise determination of standards and may be applied to the detection of trace amounts of OP and NP in human urine samples.

3.2. Recovery from the coupled on-line solid-phase extraction with internal standards

A previously reported method for the determination of alkylphenol ethoxylate metabolites in aquatic environmental samples was used for the correction by the stable isotopically labeled internal standards [25]. Recovery was calculated relative to the stable isotopically labeled *n*-nonylphenol internal standard that was added to the sample prior to extraction. By LC on a reversed-phase column, 4-nonylphenol (mixed type) and 4-*n*-nonylphenol compounds were well separated on the basis of the branched alkyl chain [25]. Therefore, we examined whether 4-*n*-nonylphenol internal standard may be used for correcting the recovery of OP and NP because these compounds are different in terms of chemical and physical behaviors. The absolute recovery using this method is

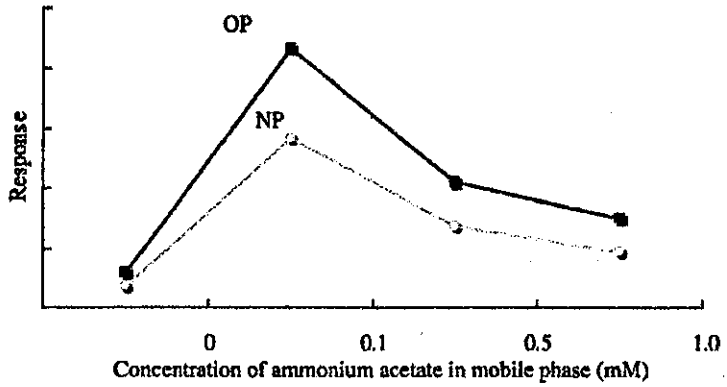

Fig. 4. Effect of mobile phase on the peak responses of OP (*m/z* 205) and NP (*m/z* 219). Analytical conditions: electrospray MS (drying nitrogen gas temperature set at 350 °C; capillary region flow rate, 12 l min^{-1}; capillary potential 3500 V relative to the counter electrode; negative-ion mode, fragmentor voltage 130 V; and SIM mode). Carrier solution: 0–1.0 mM ammonium acetate in water-acetonitrile (40:60, v/v) at a flow rate of 0.2 ml min^{-1}.

shown in Table 4. Average recoveries of NP were 51.7 and 51.9% (relative standard deviation (R.S.D.) 3.9 and 3.4%, respectively) by using NP-d_4 of the straight alkyl chain type. On the other hand, the average recoveries of NP from urine sample spiked with *m*-OP-d_5, which is a stable isotopically labeled nonylphenol of the branched alkyl chain type, were 97.0 and 85.7% (R.S.D. 4.1 and 1.7%, respectively) (Table 4). In addition, *m*-OP-d_5 internal standard was useful for correcting the recovery of OP in urine samples (Table 4). In other words, OP-d_4 and *m*-OP-d_5 are similar in terms of correction of the recovery of OP from urine sample. For this reason, the accuracy of the method was confirmed for both recoveries of OP and NP relative to only the stable isotopically labeled *m*-OP-d_5 internal standard. The method was successfully applied to pretreated urine samples. Thus, we decided to use this method for the simple and selective on-line pretreatment and quantitative determination of OP and NP in urine samples.

3.3. Background OP and NP levels in urine sample by experiments

The analysis of trace levels of OP and NP in biological samples is complicated by contamination, particularly by leaching from plastics. Thus, care must be taken to control contamination during experiments and where possible, to eliminate the source of contamination. For example, for the collection of human urine use of a plastic cup may contaminate the sample. We therefore investigated this potential source of contamination using control urine for the determination of leaching additives in plastic, and found that OP and NP were not detected (<0.1 ng ml^{-1}); therefore, plastic cup was not a source of contamination in these experiments. Investigations of OP and NP contamination of the Milli-Q water and LC system also gave negative results. However, trace levels of NP (15.6 ng ml^{-1}) in blank sample were detected after injecting pure water into a glass vial. This proves that the screw cap septum is a potential source of contamination. Based on

Table 3
Validation data of column-switching LC–MS system

Compound	Retention time (min)	Detection limit (S/N = 3) (ng ml^{-1})	Quantitation limit in urine (ng ml^{-1})	Calibration curve[a] (*r*) (0.2–100 ng ml^{-1})
4-*tert*-Octylphenol (OP)	23.3	0.05	0.3	0.999
4-Nonylphenol (NP)	25.0	0.1	0.3	0.999

[a] Peak area ratios with respect to standard and *m*-OP-d_5 internal standard (retention time: 26.5 min) were plotted.

Table 4
Recovery levels of OP and NP levels in human urine sample spike the stable isotopically labeled internal standard (IS)

Spike amount (ng ml^{-1})	NP: average[a] (R.S.D.)		OP: average[a] (R.S.D.)	
	IS NP-d_4	*m*-OP-d_5	OP-d_4	*m*-OP-d_5
1.0	51.7 (3.9)	97.0 (4.1)	101.4 (2.4)	96.2 (3.3)
5.0	51.9 (3.4)	85.5 (1.7)	99.1 (3.3)	99.6 (1.1)

[a] Background levels in the unspiked urine can be neglected (S/N < 3), $N = 6$.

this finding, we used a non-contaminated septum for our study.

In addition, OP and NP contamination in reagents used for measuring these compounds in urine was determined by this method. Trace amounts of OP (<0.1 ng ml^{-1}) in the *E. coli* β-glucuronidase solution were detected. If we take into consideration the quantitation limit calculated for S/N > 5 (0.3 ng ml^{-1}), this OP contamination may be disregarded in monitoring OP in human urine samples.

3.4. Measurement of NP and OP in human urine samples

Healthy volunteers were enrolled in this study. As expected, the average urinary creatinine level was 11.4 mg dl^{-1} (Table 5). All urine sample analyzed by this column-switching LC–MS system demonstrated no detectable OP and NP before deglucuronidation. After deglucuronidation, trace amounts of urinary NP in healthy volunteer G were detected. The urinary NP levels in the healthy volunteers varied from n.d. < 0.3 to 0.96 ng ml^{-1} (Table 5). On the other hand, urinary OP even was not detected after deglucuronidation (n.d. < 0.3 ng ml^{-1}).

We used this method to assess OP and NP levels in human urine samples to provide a reference range. In addition, variations in human metabolism were also be examined by monitoring both free and glucuronidated OP and NP in urine.

3.5. Risk estimations based on the daily intake and the main source of human exposure

In a previous study where the pharmacokinetic behavior OP and NP was investigated, the elimination

Table 5
Concentrations of OP and NP in urine samples from volunteers

Volunteer	Case	Old	Creatinine (mg dl^{-1})	OP[a] (ng ml^{-1})		NP[b] (ng ml^{-1})	
				None	β-Glucuronidase	None	β-Glucuronidase
A		20	9.14	n.d.	n.d.	n.d.	n.d.
B		23	11.7	n.d.	n.d.	n.d.	n.d.
C		23	10.3	n.d.	n.d.	n.d.	n.d.
D		21	30.4	n.d.	n.d.	n.d.	n.d.
F		24	10.1	n.d.	n.d.	nd.	n.d.
F		22	2.69	n.d.	n.d.	n.d.	n.d.
G		22	12.7	n.d.	n.d.	n.d.	0.96
H	1	23	5.48	n.d.	n.d.	n.d.	n.d.
	2[c]		13.9	n.d.	n.d.	n.d.	109.7
I	1	28	13.5	n.d.	n.d.	n.d.	n.d.
	2[c]		9.02	n.d.	n.d.	n.d.	15.9
J	1	26	10.4	n.d.	n.d.	n.d.	n.d.
	2[c]		8.99	n.d.	n.d.	n.d.	110.9

[a] n.d. indicates OP concentrations lower than 0.3 ng ml^{-1}.
[b] n.d. indicates NP concentrations lower than 0.3 ng ml^{-1}.
[c] These volunteers had taken a meal of rice or meat wrapped in PVC film.

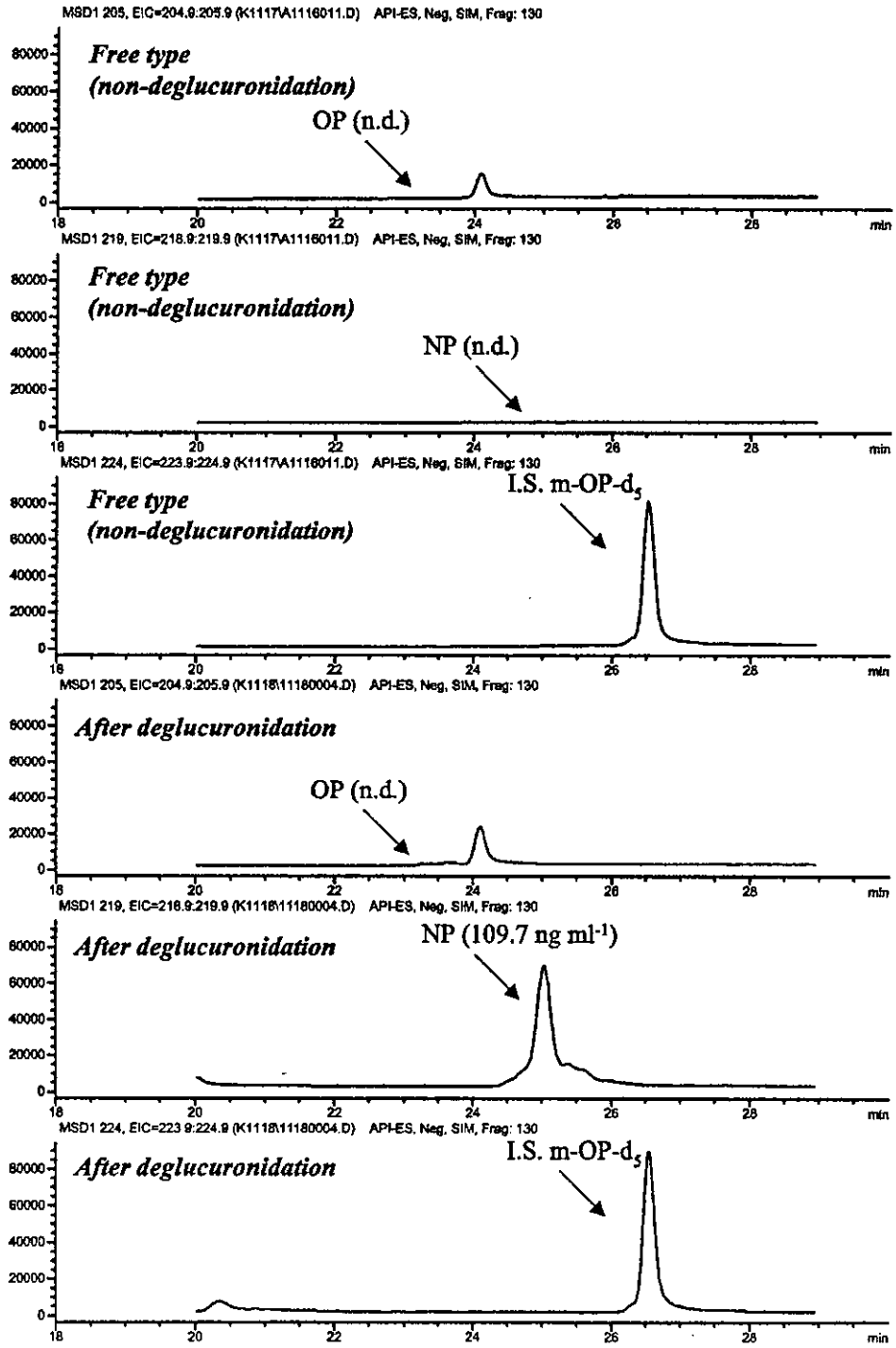

Fig. 5. Chromatograms of OP, NP and internal standard [4-(1-methyl)octylphenol-d_5: *m*-OP-d_5] in human urine sample (volunteer H, case 2).

half-life from blood and the bioavailability (determined from oral and intravenous AUCs) were found to be 2–3 h and 20%, respectively [26]. The low bioavailability was due to extensive metabolism during the first passage in the liver and excretion via the urine. To evaluate the potential estrogenic effects of these compounds on human, the levels of human exposure must be investigated. In an estimation of the daily intake of NP, the oral intake of NP by non-occupationally exposed human was found to be less than 0.16 mg per day [27]. In an other study, NP in fish products available commercially was detected at the level of 9–800 ng g^{-1} [28]. That study suggested that most of the NP detected in the fish products was due to migration from food wrapping film.

We investigated the main source of human exposure by the levels of NP in human urine samples. The volunteers had taken a meal of rice or meat wrapped in polyvinyl chloride (PVC) film. The experiment plan is following: PVC film was most contamination of NP than other in our study [16], these foods wrapped using PVC film were re-heated in a microwave oven for 1 min, volunteer H and J had taken rice and meat wrapped in PVC film, volunteer I had taken rice wrapped in PVC film, and rough estimations of intake amount of NP in this meal were above 35 μg (volunteer H and J) and below 35 μg (volunteer I) using our report [16]. Than, three urine samples on first time after meal were used that we examined the NP exposure using the present method.

The urinary glucuronidated NP was detected at the levels of 109.7 (volunteer H), 15.9 (volunteer I), and 110.9 ng ml^{-1} (volunteer J) (Table 5, case 2). Fig. 5 shows the chromatograms obtained from volunteer H's urine sample. It is therefore reasonable to surmise that the main source of human exposure is the ingestion of food contaminated by NP that leached from the PVC film. Recently, there have been on the contamination and leaching of NP from PVC films [17–19]. In addition, the potential exposure to NP due to the use of tris(nonylphenyl) phosphate in food-contacting materials was calculated [29]. In our previous study, we demonstrated that NP may be leached from food wrapping film into foods in varying degrees [17]. Moreover, daily exposure to NP in healthy human may take place by taking a meal of NP-contaminated foods from the food wrapping film. Certain populations may be exposed to NP; in addition, infants and the developing fetus may be exposed at critical points of their development. However, little research has been conducted on the effects of NP on the human health including epidemiological, analytical, and toxicological studies. We confirmed that there is a need for developing non-NP-containing or contamination-free plastic food containers for use in our daily lives.

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ANALYTICAL SCIENCES JANUARY 2003, VOL. 19

Notes

Determination of Trace Amounts of Bisphenol A in Urine by Negative-Ion Chemical-Ionization-Gas Chromatography/Mass Spectrometry

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We improved an analytical method for determining trace amounts of bisphenol A (BPA) in urine. BPA was subjected to enzymolysis and then to solid phase extraction with a C_{18} cartridge. The extract was eluted with methanol, and the eluate was concentrated under a nitrogen stream, and then pentafluorobenzylized in an alkali solution. The obtained pentafluorobenzylized compound was purifed using a florisil cartridge, followed by a determination using NCI-GC/MS. This method exhibited an excellent selectivity and reproducibility with a determination limit of 0.1 ng/ml.

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Introduction

BPA, a suspected endocrine disruptor, has been widely used as a plastic material for polycarbonate and epoxy resins. There has been great concern about the release of bisphenol A (BPA) into the environment, migration into food stuffs, exposure to humans, and potential endocrine disrupting effects on humans. There has been no report on the daily intake of BPA by humans. We improved an analytical method for determining trace amounts of BPA in urine so that the intake of BPA by humans could be estimated.

There have been reports on the determination of trace amounts of BPA using HPLC,[1,2] LC/MS,[3-7] and GC/MS.[8-13] Analyses using HPLC and LC/MS have an advantage in that they do not require derivatization, but analyses using HPLC exhibit poor selectivity, separability and sensitivity, and analyses using LC/MS exhibit poor sensitivity. Analyses using GC/MS enable a direct anaylsis, but require derivatization for determination at low concentrations. Thus, the Japanese Ministry of the Environment proposed a method[13] in which BPA is trimethylsilylized (TMS), and then subjected to GC/MS-SIM using electron ionization (EI). Analyses using negative-ion chemical ionization (NCI), which makes use of an electron-capture reaction, can achieve a highly sensitive and selective detection of chemicals having a high electron affinity. Nakamura *et al.*[8] determined BPA in river water at pg/ml levels using NCI-GC/MS after pentafluorobenzylization of the BPA. Brock *et al.*[9] determined BPA in urine and Yoshimura *et al.*[10] determined it in serum using NCI-GC/MS after pentafluorobenzylization of the BPA. A highly sensitive analysis is required for biological samples, since only small amounts of biological samples can be obtained because of the existence of a large number of coexisting substances. We investigated in detail the methods of Brock *et al.*[9] and Yoshimura *et al.*,[10] while improving the method of derivatizing into a pentafluorobenzylized compound, followed by determining by means of NCI-GC/MS with a determination limit of 0.1 ng/ml in a 2 ml urine sample. This method has excellent selectivity, determination limit, and precision, and is applicable to real samples.

Experimental

Reagents

Sodium hydroxide was purchased from Mallinckrodt and tetrabutylammoniumhydrogensulfate was obtained from Kodak. Dichloromethane, ethyl acetate, 2,2,2-trimethylpentane, methanol, and purified water were purchased from Caledon. Pentafluorobenzylbromide was a product of Superuco and bisphenol A and formic acid were products of Aldrich. ^{13}C-BPA was purchased from Cambridge Isotope. C_{18} cartridges and florisil cartridges were obtained from J. T. Baker, and C-cartridges were purchased from Superuco. Ammonium acetate was a product of Sigma, and β-glucuronidase solution (200 U/ml) was purchased from Roche Biochemical.

BPA free-water was obtained by using a C-cartridge. Ammonium acetate solution containing 1% β-glucuronidase was prepared by dissolving β-glucuronidase in a 1 M ammonium acetate solution that had been adjusted to have a pH of 6.5 by glacial acetic acid so that the concentration of β-glucuronidase would be 1%.

Urine samples were collected in glass bottles in an amount of about 200 ml after informed consent.

Method

Two milliliters of a sample were put in a 15 ml test tube, to which 20 µl of a 0.076 µg/ml ^{13}C-BPA solution was added as a surrogate compound. To the mixture, 200 µl of ammonium

acetate solution containing 1% β-glucuronidase was added, followed by enzymolysis at 37°C for 90 min. To the resultant mixture, 1 ml of 32% formic acid was added, followed by irradiation with ultrasonic waves for 5 min. The thus-obtained mixture was loaded into a C_{18} column that had been prewashed with 10 ml of methanol and 5 ml of purified water. After washing with 5 ml of a 10% methanol solution, the mixture was eluted into a 15 ml centrifuging tube with 3 ml of methanol; to the resultant eluate, 0.5 ml of a 0.2 M sodium hydroxide solution was added, followed by blowing nitrogen gas, to thereby concentrate the eluate to approximately 0.2 ml. To the concentrated eluate, 2 ml of dichloromethane, 0.5 ml of a 0.2 M sodium hydroxide solution, 0.5 ml of a 0.1 M tetrabutylammoniumhydrogensulfate solution, and 20 µl of pentafluorobenzylbromide were added; the tube was sealed with an aluminium closure, followed by irradiation with ultrasonic waves for 20 min using an ultrasonic washer. Then, the resultant eluate was subjected to centrifugation at 3000 rpm for 5 min, and the lower layer was collected with a pipet into a 10 ml centrifuging tube, followed by blowing nitrogen gas, to thereby allow dichloromethane to evaporate. After the residue was dissolved in 0.5 ml of isooctane, the mixture was loaded into a florisil cartridge that had been prewashed with 10 ml of isooctane, followed by washing with 10 ml of isooctane containing 1% ethyl acetate. Subsequently, BPA was eluted with 5 ml of isooctane containing 10% ethyl acetate, and the eluted BPA was concentrated to dryness with nitrogen gas, and then dissolved in 0.5 ml of isooctane. The resultant mixture was subjected to GC/MS.

GC/MS conditions

GC/MS conditions were as follows: GC, Agilent 6800; column, HP-5MS, 30 m × 0.25 mm × 0.25 mm; column temperatures, 60°C - 15°C/min - 215°C (7 min) - 20°C/min - 300°C (5 min); inlet temperature, 245°C; carrier gas, He, flow velocity 1 ml/min; MS, Agilent 5973; reaction gas, methane, flow velocity, 2.5 ml/min; ion-source temperature, 230°C; monitor ions (*m/z*): 407 (BPA), 419 (^{13}C-BPA).

Results and Discussion

Hydrolysis of a glucuronide conjugate

BPA is considered to be excreted in urine as a glucuronide conjugate and a sulfate conjugate.[14] Because Brock *et al.*[9] did not investigate in detail the hydrolysis of the glucuronide conjugate of BPA in urine, we investigated the amount of β-glucuronidase and the time required for the hydrolysis of a glucuronide conjugate of BPA. Figure 1(A) shows the BPA concentrations when 1 - 5 µl of β-glucuronidase was added to 2 ml of urine, followed by enzymolysis at 37°C for 90 min. A glucuronide conjugate of BPA was found to be hydrolyzed by almost 100% by the addition of 1 µl of the enzyme to 2 ml of urine. We chose to add 2 µl of the enzyme. Figure 1(B) shows the relationship between the BPA concentration and the hydrolysis time when 2 µl of β-glucuronidase was added to 2 ml of urine, followed by enzymolysis at 37°C. The result shows that hydrolysis was almost completed in 60 min. In this method, we chose to perform enzymolysis for 90 min.

Extraction, washing, and elution using a C_{18} cartridge

The extraction of phenols using a C_{18} cartridge is generally performed with a pH of 3, or lower. In this method, 1 ml of 32% formic acid was added to denature protein in urine, resulting in a pH of 3 or lower. Since a variety of substances

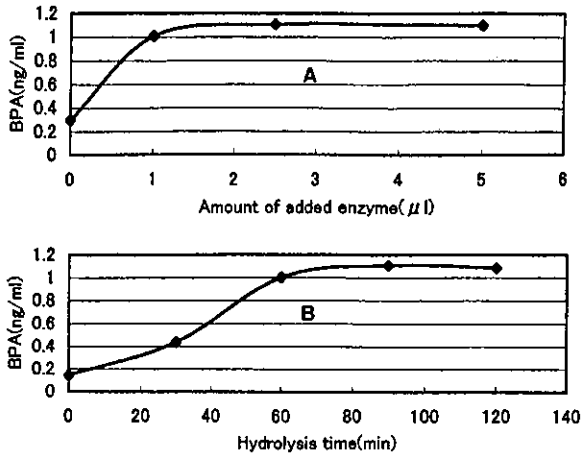

Fig. 1 Hydrolysis of glucuronide conjugate of BPA. (A) amount of added enzyme and BPA concentration; (B) hydrolysis time and BPA concentration.

exist in urine, the removal of as many substances as possible, except for the target substance, with a washing solvent and derivatization is required. In this method, the use of 10 ml of a 10% methanol solution as a washing solvent was found to cause the no elution of the target substance, and the use of 3 ml of methanol was found to cause the elution of the target substance by almost 100%.

Reaction with pentafluorobenzylbromide

Brock *et al.*[9] and Yoshimura *et al.*[10] used a circulator for pentafluorobenzylization. However, this method has a drawback in that data may not be obtained due to leakage from the stopper. We studied the use of ultrasonic waves for pentafluorobenzylization, and found that the production ratio obtained after irradiation with ultrasonic waves for 20 min was comparable to the production ratio obtained by using a circulator. In the NCI mode, the predominant ion (*m/z* = 407) is formed by the loss of one of the pentafluorobenzyl moieties. Based on the obtained NCI mass spectrum, we chose *m/z* = 407 (BPA) and *m/z* = 419 (^{13}C-BPA) as fragment ions for the determination.

Clean-up using a florisil cartridge

NCI is generally believed to have relatively few interfering peaks. In reality, however, without any clean-up, interfering substances and peaks would overlap, producing errors. We compared the clean-up using a florisil and a silicagel cartridge, and found that a florisil cartridge achieved a greater clean-up effect. Accordingly, we chose a florisil cartridge for this method. Pentafluorobenzylized compounds of BPA were found not to elute unless the polarity of the elution solvent was increased. Thus, in this method, after washing with 10 ml of isooctane containing 1% ethyl acetate, BPA was eluted with 5 ml of isooctane containing 10% ethyl acetate. Washing with isooctane containing 1% ethyl acetate caused pentafluorobenzylized compounds, such as nonylphenol and octylphenol, to elute. When the concentration of ethyl acetate was 20% or higher, interfering substances were found to elute and overlap with the BPA peaks, causing positive interference. Accordingly, we chose the concentration to be 10%.

Preparation of a calibration curve and a recovery test

A calibration curve was prepared in the following manner.

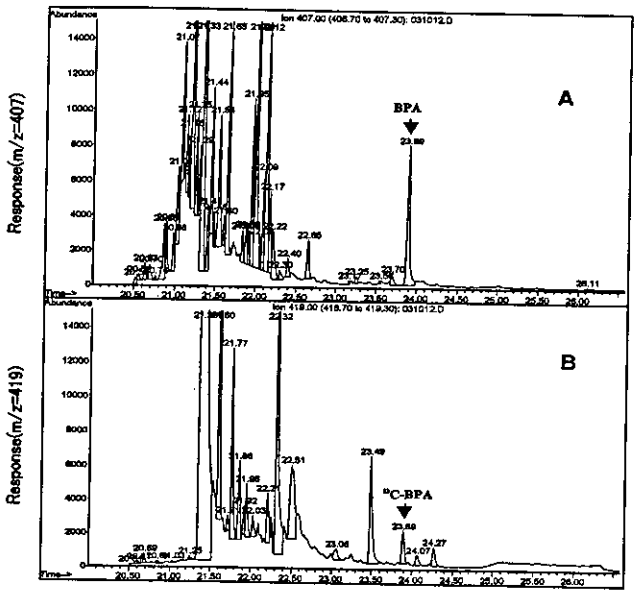

Fig. 2 SIM chromatograms of urine sample. (A) BPA; (B) ^{13}C-BPA.

Standard solutions having concentrations in the range of 0.05 μg/ml to 1.0 μg/ml were prepared, and 20 μl of the solution was put in a test tube, to which a surrogate compound was added. Then, the reaction and its succeeding procedures described in *Method* were performed and a calibration curve was prepared based on the area ratio in relation to the surrogate compound. The calibration curve remained linear up to concentrations of 2 μg/ml for the GC/MS inlet. One nanogram of BPA was added to 2 ml of urine to perform a recovery test, and the obtained recovery was found to be 83% with a standard deviation of 7.4% ($n = 5$).

Application to real samples

The proposed method was applied to urine samples (n = 6). BPA was detected in all of the urine samples in the range of 0.2 to 3.8 ng/ml with the average being 1.6 ng/ml. Figure 2 shows an SIM chromatogram of the urine samples.

Conclusion

We improved an analytical method for determining trace amounts of BPA in urine so that the exposure of humans to BPA could be estimated. BPA in urine was subjected to enzymolysis, and then to solid phase extraction with a C_{18} cartridge. The extract was eluted with methanol, and the eluate was concentrated by blowing nitrogen gas, and then pentafluorobenzylized in an alkali solution. The obtained pentafluorobenzylized compound was cleaned up using a florisil cartridge, followed by determination by NCI-GC/MS. This method exhibited an excellent selectivity and reproducibility with a determination limit of 0.1 ng/ml. When this method was applied to real samples, BPA was detected in all of them.

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Female Reproductive Tract and Mammary Disorders Caused by Endocrine Disruptors

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Abstract: Several possible endocrine disruptors, including bisphenol A, chlorobenzenes, benzo (A) pyrene, phthalate, PCB, and chlordane, were assayed in cord blood, maternal venous blood, breast milk and ascitic fluid to investigate the mechanisms of endocrine disrupting action in human subjects. The data indicate that, once reliable techniques for quantitation are established, it may be possible to elucidate endocrine disrupting action through comprehensive studies of the real levels of endocrine disruptors in human samples and the *in vivo* presence of the receptors and their metabolisms.

Key words: Endocrine disruptors; Female reproductive tracts; Cord bloods; Ascitic fluids

Introduction

It has been some time now since endocrine-disrupting chemical substances caught the attention of society, and as reports on environmental exposure and biogenesis come out sporadically, it is only recent that the directions we should be taking to study this field are becoming clarified. The initial social confusion over endocrine-disrupting chemical substances had started in reports on abnormal biogenetic phenomena and their fleeting detection in such natural environments as water, soil and fishes, where they were treated as "somewhat scary substances."

The comprehensive and basic research into these substances that followed—in health sciences, for example—suggested how to proceed with research and operations. Additionally, the basic facts concerning the connection between endocrine-disrupting chemical substances and the reproductive functions have gradually been established.[1,2]

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The Japanese text is a transcript of a lecture originally aired on October 9, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

Establishment of Endocrine-Disrupting Chemical Substance Assays

The first fundamental of endocrine-disrupting chemical substance research is the establishment of endocrine-disrupting chemical substance assays that are satisfactory in sensitivity and specificity. This is because, without the establishment of an assay, there can be no discussion of the effect of endocrine-disrupting chemical substances on reproductive functions.

The handling of samples must not be overlooked among the elements of an assay. All the substances are present *in vivo* only in minuscule amounts. It is especially important to establish the methodology for each step of the process, from taking biological samples to preventing background interference and admixture of contaminants during the separation and storage processes and through obtaining reliable absolute values.

We have already studied this field in cooperation with the Health Sciences Research report "Establishment of analysis of endocrine-disrupting chemical substances derived from consumer goods comprised of polymer materials" (Lead researcher: Prof. Hiroyuki Nakazawa, Hoshi University).

Biological Exposure to Endocrine-Disrupting Chemical Substances

Over 70 kinds of so-called endocrine-disrupting chemical substances are present in natural environments, but in a discussion of their effect on human health and reproductive functions, the second important issue is the exact level of biological exposure, i.e. analysis of *in vivo* concentrations of these substances.

In the Health Sciences Research report "Development of Biological Sample (Cord Blood, etc.) Analytical Methods Relating to Endocrine-Disrupting Chemical Substances and Research into Their Effects on Human Health Based on the Results of Sample Analysis" (Lead researcher: Tsunehisa Makino), working in concert with the Nakazawa team mentioned above, assays were established that enabled routine obtaining of stable results, and the report identified the following substances as candidate endocrine-disrupting chemical substances that cannot be neglected in the past and current volume of Japanese industrial production.

1) Bisphenol A
2) Chlorobenzenes
3) Parabens
4) Phthalate
5) Benzo (A) pyrene
6) PCB
7) Chlordane
8) Butyl tin compounds

The human biological samples subjected to assay were primarily reproductive system samples, (a) cord blood, (b) maternal venous blood, (c) breast milk, and (d) ascites. To the extent possible, samples (a) through (d) were taken simultaneously from each case subject, and we also examined the concentration gradients among *in vivo* internal organs of individual subjects.

Our results, as previously reported in detail at several opportunities,[1,2] were that **bisphenol A**, still produced in volumes of 300,000 tons annually as a raw material of plastics, was detected in all such samples as (a) through (d) and was found to have *in vivo* concentrations in the range 0.21–0.79 ppb.

Among **chlorobenzenes**, we assayed hexachlorobenzene and detected it in 100% of general peripheral blood and maternal peripheral blood samples and in 88% of cord blood samples, and found it to have concentrations in range of 0.03–0.10 ppb. Hexachlorobenzene concentrations in samples taken from individual subjects showed a significant positive correlation (coefficient of rank correlation = 0.722, $n = 12$, $p = 0.017$) between human peripheral bloods and ascites.

Among **parabens,** we detected methyl paraben in cord blood and maternal milk, and

inferred that parabens to which the pregnant women were exposed migrated via their blood to maternal milk and cord blood.

Phthalate, which is used as a plasticizer in plastics and the like, was detected in peripheral blood and ascites in concentrations averaging 1–5 ng/m*l* in the forms MBP (monobutyl phthalate), MBzP (monobenzyl phthalate), and MEHP (mono-2-ethylhexyl phthalate).

Benzo (A) pyrene, released into the atmosphere through the incomplete combustion of fossil fuels, was detected in male urines in the form OH-BaP, and we plan to go on to study the status of exposure to it in maternal milks, cord bloods, maternal peripheral bloods, and ascites.

The production of **PCB** (polychlorinated biphenyl), used as an incombustible and insulator, has been suspended since 1972, but it was detected as 35 different isomers in maternal milk, maternal peripheral blood, and cord blood in concentrations in the range, on a fat basis, of 60–99 ng/g.

The production of **chlordane**, used in the extermination of termites and other pests, has been suspended since 1986, but trans-nonachlor was detected in 63% (0.06–0.17 ppb) and cis-nonachlor in 17% (0.03–0.05 ppb) of samples, whereas none of heptachlor epoxide, oxychlordane, trans-chlordane or cis-chlordane was detected in any of the samples at all.

For **butyl tins**, used as ship's bottom paints and fishing-net anti-contaminants and use of the open systems of which has now been partially suspended, results varied in different assays performed. They were detected (5–45 ppb) in 33% to 77% of hair samples. We reported in 1999 cases of high concentrations (41–45 ppb) detected within a single family.

Such volatile organic compounds as **toluene**, **benzene**, **xylene**, and **styrene** were detected in peripheral blood and ascites in concentrations of 0.6–4.0 ppb. 80% of samples and they were positive for toluene, 49% for P-dichloro benzene, 29% for O-xylene, and 26% for styrene. Naphthalene was not detected at all.

In Vivo Action and Expression

The third important task in the study of the effects of endocrine-disrupting chemical compounds on human health and reproductive functions is the investigation of their mechanisms of action *in vivo* in human beings. Specifically, this entails the investigation of (1) whether there are **receptors** for these substances in the human body, (2) whether they display **action** and **expression** as hormones, and (3) what the mechanisms of **metabolism** and **detoxification** of these substances are in the human body.

Receptors for endocrine-disrupting chemical substances have been identified in the human body similar to such *in vivo* estrogens as human adrenocortical-derived cells (H295R) and human mammary cells (T47D). In detailed studies of receptors with human endometrial cells (HHUA) and human mammary-derived cells (MCF-7), it has been confirmed that they bound with estrogen alpha and beta receptors. In addition to known receptors, we have also decided to investigate the existence of so-called "orphan receptors" hitherto unknown.

With respect to the *in vivo* **action** and **expression** of endocrine-disrupting chemical substances, we established that they regulated the cortisol production of human adrenocortical cells. We also confirmed that they stimulated the multiplication of mammary cells and endometrial cells. We found that in mice butyl tins were active in the immune system and affect the induction of oral tolerances, and that in rats benzo (A) pyrene affected the process of differentiation of trophoblast stem (TS) cell lines.

Work that remains to be done in the study of the action and expression of these substances is a study to find out what actions, if at all, are expressed within the range of exposure in which these substances are actually present *in vivo*.

Much scope remains for further research into the **metabolism** and **detoxification** of

endocrine-disrupting chemical substances. To take bisphenol A as an example, we found that in rats the bulk of the substance was glucuronidated in the gastrointestinal tract and the liver. On the other hand, it was surmised that it was not metabolized in the kidneys, but only filtered and excreted. We identified the presence of an enzyme (beta-glucuronidase) that broke down glucuronate conjugates into the original endocrine-disrupting chemical substance, and we are planning to study it in the human body in the future.

Conclusion

We have thus assayed exact levels of biological exposure on the basis of the establishment of assays for several substances derived from polymers. Through investigation of *in vivo* receptors, action and expression, and metabolism and detoxification of endocrine-disrupting chemical substances, we continue further research towards our primary objective of working towards a conclusion on their effects on human health and reproductive functions.

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GLUCURONIDATION AND EXCRETION OF NONYLPHENOL IN PERFUSED RAT LIVER

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ABSTRACT:

Nonylphenol, an environmental estrogenic chemical, is reported to have adverse effects on the reproductive organs of animals. In this study, the metabolism of nonylphenol and that of other alkylphenols in the rat liver was investigated using liver perfusion. Alkylphenols (nonylphenol, hexylphenol, butylphenol, and ethylphenol) were glucuronidated by rat liver microsomes. Nonylphenol was found to be conjugated with glucuronic acid by an isoform of UDP-glucuronosyltransferase, UGT2B1, expressed in yeast AH22 cells. However, when nonylphenol was perfused into rat liver in situ, it was difficult for free nonylphenol and conjugated metabolite to be excreted into the bile or vein, and most of the perfused nonylphenol remained free and as a glucuronide conjugate in the liver tissue, even after 1 h of perfusion. After 1 h of perfusion of the other alkylphenols, most of them were excreted into the bile as glucuronides. Ethylphenol, which has the shortest alkyl chain, was excreted rapidly into both the bile and vein; however, the excretion rates of alkylphenols having longer alkyl chains tended to be slow. MRP-2-deficient Eisai hyperbilirubinemic rats could not secrete alkylphenol-glucuronides into the bile, indicating that alkylphenol-glucuronides are transported by MRP-2 to the bile in normal Sprague-Dawley rats. The results indicate that the kinetics of excretion of alkylphenol-glucuronides into the bile or vein depends on the length of alkyl chain and suggest that nonylphenol-glucuronide formed in the liver cannot be transported by MRP-2.

Environmental estrogenic chemicals such as bisphenol A and nonylphenol, which are contained in many industrial products, can be detected in foods, tap water, and many environmental materials. Nonylphenol is used in a wide variety of detergents and plastics and has been reported to be environmentally persistent (White et al., 1994). The mean daily oral intake of nonylphenol by humans is estimated to be 0.16 mg/day (Muller et al., 1998). Nonylphenol has been shown to be a possible endocrine disrupter due to its estrogenic effects in MCF7 cell proliferation assays (Soto et al., 1991), binding assays to the estrogen receptor (White et al., 1994) and uterotropic assays in mice (Shelby et al., 1996). Exposure of male rainbow trout (*Oncorhynchus mykiss*) to four different alkylphenolic chemicals, including nonylphenol, resulted in synthesis of vitellogenin, a process normally dependent on endogenous estrogens, and a concomitant inhibition of testicular growth (Jobling et al., 1996). Male and female ratios of Japanese medaka (*Oryzias latipes*) in a control group (2:1) and a 100 μg/l nonylphenol treatment group (1:2) were reported to be significantly different (Gray and Metcalfe, 1997). Early neonatal exposure to nonylphenol has been reported to cause dysfunction of postpubertal reproductive function in female rats as well as disrupted development of gonads in male and female rats (Nagao et al., 2000). It has also been reported that nonylphenol administered orally at a dose of 50 mg/kg of body weight induced a significant increase in uterine weight of prepubertal rats and advanced the age of vaginal opening (Laws et al., 2000). Elucidation of the metabolism and fate of nonylphenol is important for estimating the risks of the chemical for animals. Xenoestrogens such as bisphenol A and diethylstilbestrol have been shown to be conjugated with glucuronic acid by a UDP-glucuronosyltransferase isoform, UGT2B1, in the rat liver (Yokota et al., 1999). Nonylphenol has also been reported to be glucuronidated extensively in the liver of animals such as rainbow trout (Lewis and Lech, 1996; Coldham et al., 1998; Thibaut et al., 1998a,b) and rats (Moffat et al., 2001; Yokota et al., 2002). Since the liver is the main barrier against drugs in the body, identifying or tracing the metabolites of nonylphenol is important for elucidation of the disruptive effects of the compound on the reproductive system.

In this study, we investigated the metabolism and kinetics of nonylphenol and those of other alkylphenols by means of liver perfusion, and we found that nonylphenol was delayed in excretion from the liver and that the length of the alkyl chain is a critical factor for the excretion rate and route out of the liver.

Materials and Methods

Chemicals. 4-Nonylphenol and other alkylphenols were purchased from Kanto Chemical Co. (Tokyo, Japan). High-performance liquid chromatography (HPLC[1])-grade acetonitrile was obtained from Labscan Ltd. (Dublin, Ireland). β-Glucuronidase was obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Male young adult Sprague-Dawley (SD) rats (8–13 weeks old) and MRP-2-deficient Eisai hyperbilirubinemic rats (EHBR) were used in all experiments. The rats were housed under standard conditions and given food and water ad libitum before use. The rats were handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University based on

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[1] Abbreviations used are: HPLC, high-performance liquid chromatography; SD, Sprague-Dawley; EHBR, Eisai hyperbilirubinemic rats; MRP-2, multidrug resistance-related protein-2; NP-G, nonylphenol-β-D-glucuronide; NP, nonylphenol; ER, estrogen receptor.

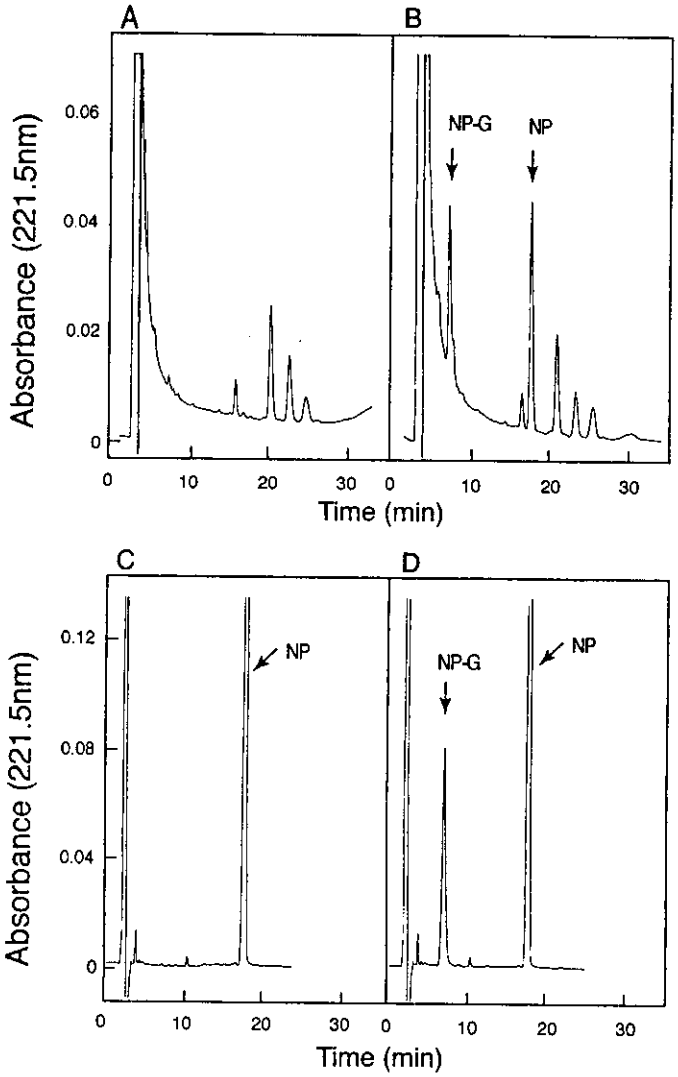

FIG. 1. *HPLC profiles of nonylphenol metabolites after liver perfusion and glucuronide conjugate of nonylphenol produced by UDP-glucuronosyltransferase UGT2B1.*

The liver was perfused for 60 min with Krebs' solution as a control (A). Nonylphenol perfusion was performed with the same buffer containing 0.050 mM nonylphenol for 5 min and then perfused with Krebs buffer for 55 min (B). Nonylphenol and its metabolites in the liver were extracted with acetonitrile and then analyzed by HPLC after the perfusion as described under *Materials and Methods*. The UDP-glucuronosyltransferase isoform UGT2B1 was expressed in yeast AH22 cells, and nonylphenol was glucuronidated by microsomes prepared from the yeast transformant as described under *Materials and Methods*. The enzyme reactions were performed for 0 min (C) and 60 min (D), and the resultant glucuronide produced by UGT2B1 was analyzed by HPLC. The arrows indicate NP-G and unconjugated nonylphenol (NP).

the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Expression of UDP-Glucuronosyltransferase 2B1. cDNA of rat UDP-glucuronosyltransferase isoform UGT2B1 was obtained by reverse transcriptase-polymerase chain reaction and expressed in yeast AH22 cells as previously described (Yokota et al., 1999). Nonylphenol glucuronidation was performed using yeast microsome-expressed UGT2B1, and the resultant glucuronide conjugate was analyzed by HPLC as described previously (Yokota et al., 1999).

Surgical Procedure. The rats were anesthetized by intraperitoneal injection of 60% urethane (0.3 ml/100 g of body weight). Whole liver perfusion was performed according to the method reported by Sugano et al. (1978) with slight modifications (Inoue et al., 2001). Briefly, after anesthesia, the abdomen was opened and the liver, portal vein, bile duct, and inferior vena cava were

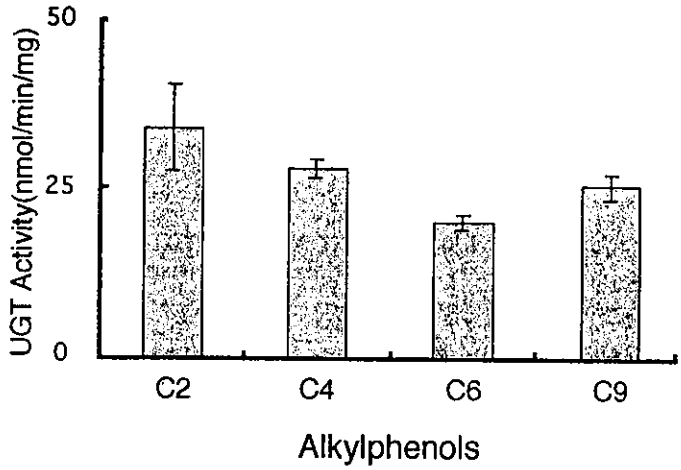

FIG. 2. *UDP-glucuronosyltransferse activities in rat liver microsomes toward various alkylphenols.*

The alkylphenols, ethylphenol (C2), butylphenol (C4), hexylphenol (C6), and nonylphenol (C9), were incubated with rat liver microsomes, which was activated by sodium cholate in the presence of UDP-glucuronic acid. UDP-glucuronosyltransferase activities were determined by HPLC analysis as described under *Materials and Methods*. Parameters are shown as means ± S.E. (n = 3 animals).

exposed. The common bile duct and the portal vein were cannulated with PE-10 and PE-50 polyethylene tubes (BD Biosciences, San Jose, CA), respectively, and oxygenated Krebs-Ringer buffer, described below, was pumped through the liver via the portal vein. The abdominal vena cava was incised immediately after perfusion, and a dripping polyethylene tube (2 mm i.d., 3 mm o.d.) was inserted. The thorax was then opened and the thoracic vena cava was ligated. The liver was not excised. All experiments were performed in situ. While the animals were still under anesthesia, euthanasia was performed by exsanguination.

Liver Perfusion. Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) was used in all experiments. The buffer solution was aerated by 95% O_2 + 5% CO_2, and the pH was adjusted to 7.4. Ethanol containing alkylphenol was dispersed into the buffer solution, and final concentration of ethanol was 1%. The substrate buffer solution contained alkylphenol in a final concentration of 0.025 or 0.05 mM. These buffer solutions were maintained in a water bath at 37°C. The perfusion system consisted of a peristaltic pump (MP-32N; EYELA, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) and silicone tubes, as described in our previous paper (Inoue et al., 2001). The buffer solution was pumped at a constant rate of 30 ml/min, and the liver perfusion was carried out in a flow-through mode. Preliminary perfusion was done for 15 min, and then the substrate buffer solution was perfused for 5 min, followed by reperfusion of the Krebs-Ringer solution. After perfusion of the substrate buffer, the excreted bile and perfusate in the vein were collected at 5-min or 10-min intervals over a 1-h period.

HPLC Analysis of Reaction Products. The bile was dissolved in 40-fold 50% acetonitrile, and perfusate was diluted with an equal volume of acetonitrile; then, the perfusate mixtures were centrifuged for 10 min at 10,000g. The supernatant fractions were analyzed by HPLC (Shimadzu, Tokyo, Japan) according to the method described previously (Yokota et al., 1999; Inoue et al., 2001). Alkylphenols and its metabolites were eluted from HPLC by 75% acetonitorile solution (acetonitrile/water/acetic acid, 75:25:0.1) for nonylphenol, 60% solution for hexylphenol, 50% solution for butylphenol, and 35% solution for ethylphenol. Recordings were made using a C-R8A integrator (Shimadzu, Tokyo, Japan). Alkylphenols and its metabolites retained in the liver after the perfusion were extracted with acetonitrile, the extractions were centrifuged for 30 min at 25,000g, and the supernatants were analyzed by HPLC with the same procedure described above. Alkylphenol-glucuronides were quantitated by the amount of the deconjugated free alkylphenols after β-glucuronidase treatment of the reaction products as previously described (Shibata et al., 2002).

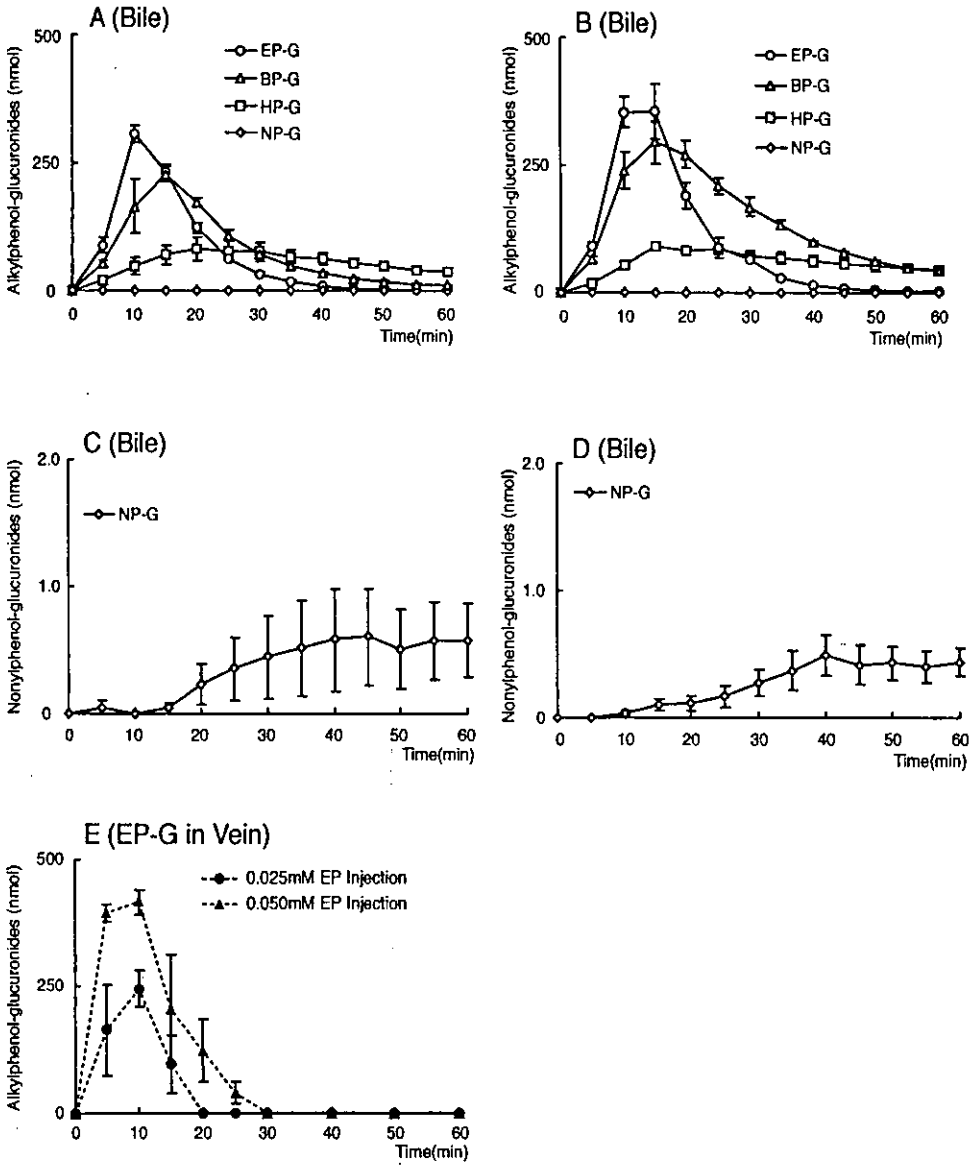

FIG. 3. *Excretion of alkylphenol metabolites during liver perfusion.*

Rat livers were perfused for 5 min with Krebs' solution containing various alkylphenols and then perfused with normal Krebs buffer. Bile was collected every 5 min and analyzed by HPLC. Glucuronide conjugates of alkylphenols (0.025 mM, A; and 0.050 mM, B) are: ethylphenol (EP-G; ○), butylphenol (BP-G; △), hexylphenol (HP-G; □) and nonylphenol (NP-G; ◇). The profiles of nonylphenol-glucuronide excretion are shown at high scales in C (0.025 mM) and D (0.050 mM). Only ethylphenol metabolites appeared in the perfusate vein, and the profiles are shown in E [0.025 mM (●) and 0.050 mM (▲) ethylphenol]. Parameters are shown as means ± S.E. (n = 4 animals or n = 3 animals in ethylphenol analysis).

Results

The HPLC profiles of the metabolites of nonylphenol produced in the liver, after the perfusion had been performed as described under *Materials and Methods*, are shown in Fig. 1. The nonylphenol metabolite extracted from the perfused liver was eluted at an early retention time as a single peak [nonylphenol-β-D-glucuronide (NP-G); 7 min] with slight shoulder peaks (Fig. 1B). The main peak produced by liver microsomes had the same retention time and was reduced by treatment with β-glucuronidase as was previously reported in rainbow trout (Lewis and Lech, 1996; Coldham et al., 1998; Thibaut et al., 1998a,b) (data not shown). A glucuronide conjugate of nonylphenol was produced by UGT2B1 (Fig. 1D), indicating that the main metabolite in the liver perfusion is NP-G. The peak of NP glucuronide was confirmed to be not produced by AH22 yeast microsomes, expressing no UGT2B1, and in the enzyme reactions where the substrate was omitted. The K_m value of the microsomal UDP-glucuronosyltransferase was estimated to be 0.25 mM nonylphenol. UDP-glucuronosyltransferase activities (V_{max}) toward nonylphenol and other alkylphenols in rat liver microsomes were assayed, and the estimated enzymatic activities are shown in Fig. 2. UDP-glucuronosyltransferase activity toward nonylphenol showed about the same value as that of ethylphenol. These alkylphenols were injected into the portal veins of rats, and then the bile and perfusate in the veins were sampled and analyzed by HPLC as previously described (Inoue et al., 2001). The resultant metabolites of alkylphenols were mainly glucuronide conjugates (data not shown), as was the case for nonylphenol. The concentrations of the glucuronide conjugates of alkylphenols in the bile and vein during liver perfusion are shown in Fig. 3. After the liver perfusion, alkylphenol-glucuronides were excreted only into the bile (Fig. 3, A–D), and only ethylphenol-glucuronide was excreted into