we also developed miniaturized HF-LPME for BP analysis.[32] In that study,[32] five kinds of BPs (BP, BP-OH, 2OH-BP, BP-3, and BP-10) could be analyzed without derivatization. However, an even wider variety of BP-related compounds should be analyzed to reveal the extent of exposure to BPs. Using our proposed improvement method with *in situ* derivatization, BP-1, 3OH-BP, and 4OH-BP were detected, although they could not be detected when *in situ* derivatization was not performed. Moreover, previous GC conditions should be modified to improve the separation of BPs.

This work is the first to determine trace amounts of BPs in human urine samples using HF-LPME with *in situ* derivatization and GC-MS. The proposed method has many practical advantages, including simplicity of the extraction method, the use of a small volume of organic solvent for extraction, and high sensitivity, and is sufficiently applicable to analyses of human urine samples. The proposed method is expected to have potential applications in human urine samples.

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Note

Effect of gamma-ray irradiation on degradation of di(2-ethylhexyl)phthalate in polyvinyl chloride sheet

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ABSTRACT

The risk assessment of di(2-ethylhexyl)phthalate (DEHP) migration from polyvinyl chloride (PVC) medical devices is an important issue for patients. The aim of this study was to determine DEHP degradation and migration from PVC sheets. To this end, the method for the simultaneous determination of DEHP and its breakdown products (mono(2-ethylhexyl)phthalate (MEHP) and phthalic acid (PA)) was improved. Their migration levels from 0 to 50 kGy gamma-ray irradiated PVC sheets were determined. DEHP migration level decreased in proportion to the dose of gamma-ray irradiation, while MEHP and PA migration levels increased. The hardness and the elastic modulus of PVC sheets were examined, but no clear relationship between DEHP migration and these parameters was observed.

1. Introduction

Phthalate esters are widely used as industrial plasticizers. In particular, di(2-ethylhexyl)phthalate (DEHP) is used in the production of polyvinyl chloride (PVC) and other plastics to increase flexibility, softness, and stability for specific applications. PVC is one of the most widely used plastic polymers in such medical products as blood containers, blood tubing, and catheters. However, it has been reported that DEHP is easily released from PVC products into food, drugs, and body fluids [Earls et al., 2003; Inoue et al., 2003; Takatori et al., 2004; Ito et al., 2005]. DEHP is considered to exhibit reproductive and developmental toxicity [Lovekamp-Swan and Davis, 2003], carcinogenicity, and testicular toxicity [Tickner et al., 2001; Yakubovich and Vienken, 2000; Hill et al., 2001]. Some phthalates including DEHP are said to exhibit toxic effects, including antiandrogenic effects during reproductive system development and normal sperm production in male rat [Poon et al., 1997; Lamb et al., 1987; Tyl et al., 1988], and the decrease in blood 17β-estradiol level in female rat [Davis et al., 1994]. In addition, recent studies have shown that certain phthalate exposure levels in pregnant women are associated with the reproductive health of male infants [Latini et al., 2003; Swan et al., 2005; Marsee et al., 2006]. In Japan, The Ministry of Health, Labour and Welfare (2000) has set the tolerable daily intake (TDI) of DEHP at 40–140 $\mu g\,kg^{-1}\,day^{-1}$ and has regulated the use of DEHP as plasticizer in the manufacture of infant toys.

In our previous studies, we observed that not only DEHP but also mono(2-ethylhexyl)phthalate (MEHP) and phthalic acid (PA) migrated from PVC medical devices into simulated pharmaceuticals even without enzymatic hydrolysis [Ito et al., 2005, 2006, 2008]. DEHP migration was suppressed by the sterilization process, particularly gamma-ray sterilization [Ito et al., 2006]. In contrast, MEHP migration from gamma-ray sterilized PVC medical device was increased dramatically [Ito et al., 2006, 2008]. Since MEHP is thought to be even more toxic than DEHP, the formation of MEHP as a breakdown product of DEHP is a critical problem.

In this study, DEHP, MEHP, and PA migration levels were determined to confirm the effect of gamma-ray irradiation on the degradation of DEHP. Commercially available PVC medical devices are generally subjected to 20–25 kGy gamma-ray sterilization. Therefore, PVC sheets used in the manufacture of blood bags were irradiated with 1–50 kGy gamma rays. No sterilization process

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was performed on the control sample (non-irradiated gamma ray: 0 kGy). DEHP, MEHP, and PA migration levels were examined in relation to the dose of gamma rays. Moreover, the hardness and the elastic modulus of irradiated PVC sheet were examined because surface processing, an example of which is polyethylene glycol grafting, is known to suppress DEHP migration [Lakshmi and Jayakrishnan, 1998]. Then, the effect of gamma-ray irradiation on the PVC surface was studied to understand the relationship between DEHP, MEHP, and PA migration levels and the hardness and the elastic modulus of irradiated PVC sheets.

2. Materials and methods

2.1. Chemicals and materials

Environmental analytical grade DEHP and DEHP-d_4 were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). MEHP and MEHP-d_4 were purchased from Hayashi Pure Chemical Industries (Osaka, Japan). PA and PA-d_4 were purchased from CDN Isotope Central Chemicals Co. Inc. (Tokyo, Japan). Phthalic acid esters, analytical grade acetonitrile, and acetone were used in the experiments. Analytical grade formic acid was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA).

The test material was PVC sheet subjected to gamma-ray irradiation (^{60}Co; 1, 5, 10, 25, 50 kGy). Commercial medical devices were irradiated with approximately 25 kGy gamma rays for sterilization. The control sample was not irradiated with gamma rays. The PVC sheets were kindly supplied by the manufacturer.

The extraction solvents were 5% glucose solution for injection (Otsuka Pharmaceuticals Co., Tokyo, Japan), polyoxyethylated hydrogenated castor oil 60 (HCO-60) (Wako Pure Chemical Industries Ltd., Osaka, Japan), and purified water.

2.2. Instrumentation and LC–MS/MS conditions

A Series 1100 liquid chromatograph from Agilent Technologies (USA) was coupled to an API 4000™ (Applied Biosystems Japan, Tokyo, Japan) equipped with a Turbo Ionspray™ ionization source. Mass spectrometry data were processed with Analyst 1.3.2 software. An Inertsil-Ph3 column (50 mm × 2.1 mm, 5 μm particle size) from GL Sciences was used for separation.

After 5 μL of the sample was injected with an auto-sampler, it was loaded onto the analytical column by introducing the mobile phase at the flow rate of 0.2 ml min^{-1}. The auto-sampler was maintained at 4 °C to keep the sample cool. Acetonitrile (mobile phase A) and 0.05% formic acid in water (mobile phase B) were used. Separation was carried out with the following profile: mobile phase A/B was 15/85 (0–4 min) → 90/10 (4.01–15 min for elution) → 15/85 (15.01–25 min for equilibration) (v/v). The column oven was maintained at 40 °C for LC.

The working parameters for turbo ionspray ionization MS/MS were as follows: curtain gas, 20 psi (DEHP and DEHP-d_4 for the positive ion mode) and 20 psi (MEHP, PA, and their internal standards for the negative ion mode); nebulizer gas (N_2) pressure, 80 psi for the positive ion mode and 80 psi for the negative ion mode; and turbo ionspray gas (N_2) pressure, 60 psi for the positive ion mode and 60 psi for the negative ion mode. Ion source temperature was maintained at 650 °C and turbo ionspray voltages for the positive ion mode (DEHP, DEHP-d_4) and the negative ion mode (MEHP, PA, and their internal standards) were 5000 and −4500 V, respectively. Declustering potentials of DEHP, MEHP, and PA were 61 V, −60 V, and −35 V, respectively. DEHP and DEHP-d_4 were monitored in the positive ion mode, whereas MEHP, PA, and their internal standards were monitored in the negative ion mode. The combinations of precursor ion and product ions were as follows: DEHP (precursor ion → product ion, m/z 391 → 149), DEHP-d_4 (m/z 395 → 153), MEHP (m/z 277 → 134), MEHP-d_4 (m/z 281 → 138), PA (m/z 165 → 121), and PA-d_4 (m/z 169 → 125). The collision gas (N_2) pressure was set at 4 units for both positive and negative ion modes. These conditions were modified from those of our previous paper [Ito et al., 2008].

2.3. Effect of gamma-ray irradiation on migration test

The migration of DEHP, MEHP, and PA from PVC sheet (1 cm × 3 cm) into 5 ml of each extraction solvent was examined. 5% glucose solution, HCO-60, and purified water were used as extraction solvent. They also served as simulated pharmaceuticals. HCO-60 is a surfactant that is involved in the migration of DEHP into such drugs as Prograf®. The extent of DEHP migration was dependent on the concentration of HCO-60 [Hanawa et al., 2003]. In this study, 2 mg ml^{-1} HCO-60 was prepared for the migration test. The concentration of HCO-60 was set with reference to its content in commercial pharmaceuticals as surfactant. The samples were kept in test tubes and extraction was carried out by shaking at 37 °C for 1 h. An aliquot (1 ml) of the extract was pipetted into another test tube and DEHP-d_4, MEHP-d_4, and PA-d_4 were added. Then, the sample solution was appropriately diluted prior to LC–MS/MS analysis.

2.4. Instrumentation and conditions for indentation measurement

The hardness and the elastic modulus of non-irradiated (0 kGy) and irradiated PVC sheets (25 and 50 kGy) were measured by a depth-sensing nanoindentation technique with Nano indenter XP (MTS Systems Co., Oak Ridge, TN, USA). PVC sheets were indented with a Berkovich diamond tip to a maximum depth of 55 μm at room temperature (23 ± 1 °C). The indentation load–displacement behavior of gamma-ray irradiated PVC sheets was tested in the continuous stiffness measurement mode. As shown in Fig. 1, load–displacement curves were obtained to determine the

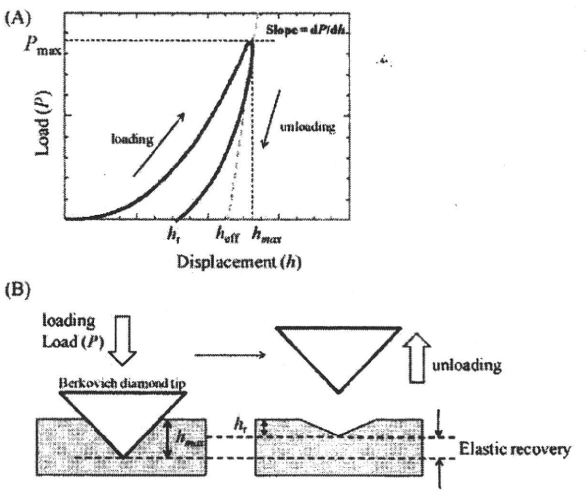

Fig. 1. Determination of hardness and the elastic modulus. (A) Typical load versus displacement curve. (B) Schematic diagram of indentation measurement.

Table 1
Figures of merit of LC–MS/MS method for determination of DEHP, MEHP, and PA.

	Range (ng ml^{-1})	Spiked conc. (ng ml^{-1})	Purified water		5% glucose		HCO-60	
	(r)		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
DEHP	20–1000	100	97.5	6.2	97.2	4.4	98.5	1.8
	(0.998)	500	97.5	1.8	99.9	0.9	94.5	1.2
MEHP	2–1000	100	102	2.4	105	5.2	105	2.5
	(0.999)	500	98.2	3.1	101	1.8	104	3.9
PA	5–1000	100	97.0	7.8	90.4	7.0	107	3.4
	(0.999)	500	102	1.8	102	2.1	104	1.4

r: correlation coefficient; RSD: relative standard deviation ($n = 3$).

hardness (H) and the elastic modulus (E) of the sheets. Theoretical elastic modulus and theoretical hardness were calculated as follows:

$$E \approx \frac{\sqrt{\pi}}{2\beta}\frac{1}{\sqrt{kh_{eff}^2}}\frac{dP}{dh} \quad \text{and} \quad H = \frac{P_{max}}{kh_{eff}^2}$$

where β and k are constants. When the Berkovich diamond tip was used, $k = 24.56$ and $\beta = 1.034$.

Hardness and elastic modulus were calculated by multiplying modification coefficient (η) by the theoretical hardness and the theoretical elastic modulus. In addition, modification coefficient was confirmed with a load–displacement curve obtained from a calibration experiment using silica.

3. Results and discussion

3.1. Optimization of the LC–MS/MS method

In the scan mode, DEHP, MEHP, and PA were monitored at m/z 391, 277, and 165, which were assigned to $[M+H]^+$, $[M-H]^-$, and $[M-H]^-$, respectively. Moreover, in the product ion MS/MS measurement, selective reaction monitoring ions (SRMs) of DEHP, DEHP-d_4, MEHP, MEHP-d_4, PA, and PA-d_4 were set depending on their precursor ions. When the auto-sampler was maintained at room temperature, the peak shape was not good. However, when the auto-sampler was maintained at 4 °C to keep the sample cool, a reproducible peak area was obtained. In addition, the sample solution was acidified (1%) to improve separation. No interference from peaks of the other compounds present in the extraction solvents was noted.

3.2. Figures of merit of LC–MS/MS analysis for determination of DEHP, MEHP, and PA

In the proposed method, determination was achieved by stable isotope dilution analyses. The limits of detection (LODs) of DEHP, MEHP, and PA subjected to LC–MS/MS analysis were 5, 0.5, and 1 ng ml^{-1}, respectively, and the signal-to-noise (S/N) ratio was 3. In addition, the limits of quantification (LOQs) of DEHP, MEHP, and PA when S/N > 10 were 20, 2, and 5 ng ml^{-1}, respectively. The method showed good linearity and the correlation coefficients (r) were higher than 0.998 for all the analytes. The figures of merit of the present method are summarized in Table 1. Sensitivity and accuracy were sufficient for the determination of DEHP, MEHP, and PA migration levels from PVC sheets.

The recovery and precision of the method were assessed by replicate analyses ($n = 3$) of each solvent spiked at 100 and 500 ng ml^{-1} levels. Non-spiked and spiked samples were subjected to LC–MS/MS analysis. Recovery was calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using calibration curves acquired from standard solutions containing the surrogate compounds. The recovery and precision were 97.0–102% (relative standard deviation—RSD: 1.8–7.8%), 90.4–105% (RSD: 0.9–7.0%), and 94.5–107% (RSD: 1.2–3.9%) for purified water, 5% glucose solution, and HCO-60, respectively (Table 1). Therefore, the method enables the precise determination of standards and may be applicable to the determination of DEHP, MEHP, and PA in pharmaceutical solutions containing HCO-60 as surfactant.

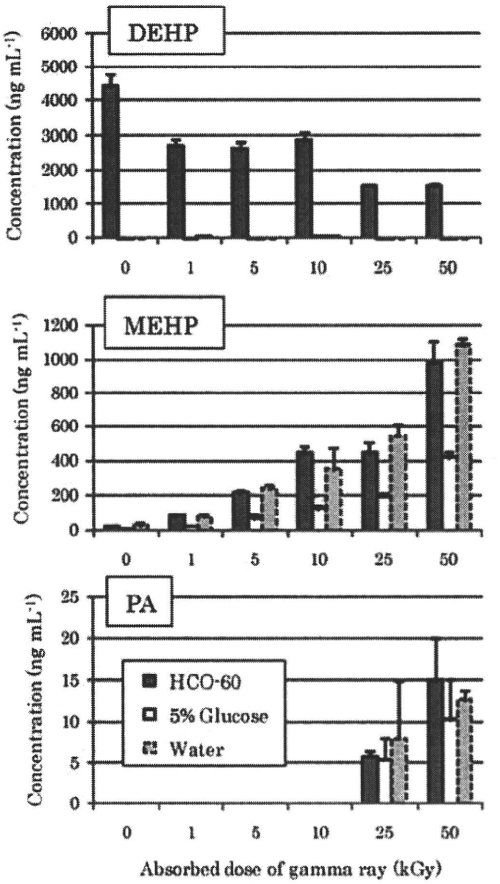

Fig. 2. Concentrations of DEHP, MEHP, and PA that migrated from gamma-ray irradiated PVC sheet. Extraction solvents HCO-60, 5% glucose solution, and water are represented by dark columns with a solid line, white columns with a solid line, and gray columns with a dotted line, respectively.

3.3. Effect of gamma-ray irradiation on DEHP degradation

DEHP, MEHP, and PA migration levels from gamma-ray irradiated PVC sheets are shown in Fig. 2. Similar to that reported in another paper [Ito et al., 2006], DEHP migration was marked when HCO-60 was used as the extraction solvent. DEHP, MEHP, and PA migration from PVC sheets was influenced by gamma-ray irradiation. The higher the dose of irradiated gamma rays, the lower DEHP migration level from the PVC sheets. In contrast, MEHP migration from the irradiated PVC sheets was dependent on the dose of gamma-ray irradiation. In the case of PVC sheets subjected to 1 and 5 kGy gamma-ray irradiation and non-irradiated (0 kGy), no PA was detected (below LOD). In PVC sheets exposed to 10 kGy gamma-ray irradiation, trace level of PA was detected (between LOD and LOQ). PA was clearly detected when the gamma-ray irradiation exceeded 25 kGy. The amount of PA that migrated from the 50 kGy gamma-ray irradiated PVC sheet was higher than that from the 25 kGy irradiated one. Therefore, PA migration from the irradiated PVC sheet was thought to be dependent on the gamma-ray irradiation dose applied to the PVC sheet.

It should be noted that the molar concentration of DEHP that was decreased upon gamma-ray irradiation was not equal to the total molar concentration of MEHP and PA that was increased upon gamma-ray irradiation; however, taking into consideration the fact that MEHP migration level was well correlated with the dose of gamma-ray irradiation, we can say that MEHP and PA were formed from the breakdown of DEHP by gamma-ray irradiation. Moreover, there is a possibility that other factors were involved in the migration of these compounds.

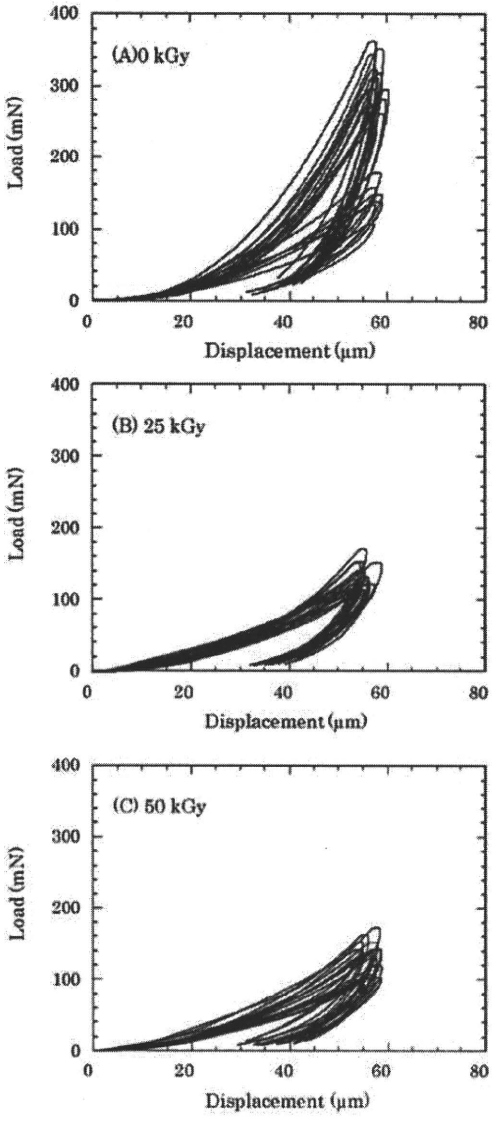

Fig. 3. Load–displacement curves for 0, 25, and 50 kGy gamma-ray irradiated PVC sheets. These load–displacement curves were obtained from (A) 0 kGy gamma-ray irradiated PVC sheet, (B) 25 kGy gamma-ray irradiated PVC sheet, and (C) 50 kGy gamma-ray irradiated PVC sheet.

3.4. Indentation measurement of gamma-ray irradiated PVC sheets

The load–displacement curves for non-irradiated (0 kGy) and irradiated (25 and 50 kGy gamma ray) PVC sheets are shown in Fig. 3. Although the load–displacement curves were scattered widely, replicate studies ($n = 20$) were conducted to determine the average behavior. It was thought that the contact state between the Berkovich diamond tip and the PVC sheet could not be kept in the same state because of the rough surface of the PVC sheet (Fig. 4). From the load–displacement curves, Young's modulus versus depth plots (Fig. 5A) and hardness versus depth plots (Fig. 5B) were obtained. As shown in Fig. 5, the correlation between hardness and depth or between elastic modulus and depth was observed. In particular, below 20 μm depth, a difference was observed between non-irradiated sample (0 kGy) and irradiated samples (25 and 50 kGy). However, it is thought that the data were influenced by the rough surface of the PVC sheet when monitoring was carried out at small depths. Similarly, when monitoring was carried out at large depths (≥40 μm), the data were influenced by the rough back surface as the PVC sheet itself was approximately 400 μm thick. The elastic modulus and the hardness at 1, 5, 10, and 20 μm depth from the surface are shown in Table 2. In calculating the elastic modulus, the Poisson ratio was empirically assumed to be 0.3. As a result, the elastic modulus of gamma-ray irradiated PVC sheet was higher than that of the non-irradiated sample. Surface PVC chains might be cross-linked or broken by gamma-ray irradiation [Mendizabal et al., 1996; Baccaro et al., 2003; Silva et al., 2008] and might be influenced by the migration of DEHP, MEHP, and PA.

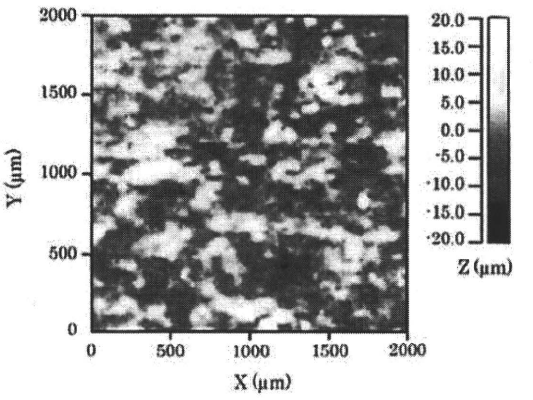

Fig. 4. 3D surface topography of non-irradiated PVC sheet. KLA-Tencor P-15 contact stylus profiler was used to measure the surface roughness of a sample PVC sheet.

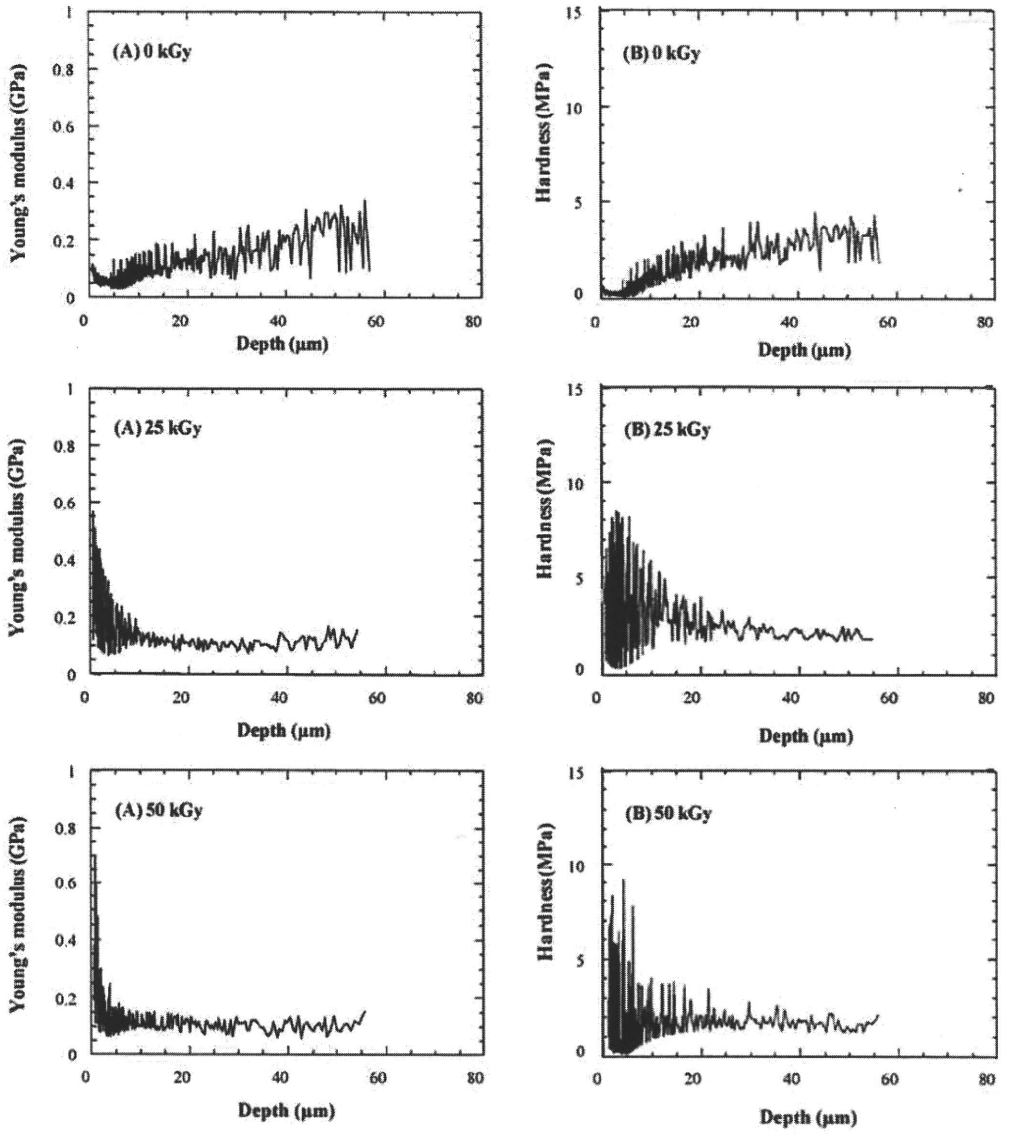

Fig. 5. Young's modulus versus depth (A) and hardness versus depth (B) diagrams. Top panels show data for non-irradiated PVC sheet (0 kGy). Middle panels show data for 25 kGy irradiated PVC sheet. Bottom panels show data for 50 kGy irradiated PVC sheet.

Table 2
Elastic modulus and hardness.

Depth (μm)	Control		25 kGy		50 kGy	
	Elastic modulus (MPa)	Hardness (MPa)	Elastic modulus (MPa)	Hardness (MPa)	Elastic modulus (MPa)	Hardness (MPa)
1	80 ± 17	0.42 ± 0.11	330 ± 120	2.9 ± 1.9	240 ± 140	2.0 ± 2.3
5	59 ± 22	0.37 ± 0.22	180 ± 50	4.3 ± 2.3	130 ± 37	2.3 ± 2.3
10	93 ± 35	1.1 ± 0.45	130 ± 21	3.6 ± 1.5	120 ± 19	2.2 ± 1.2
20	130 ± 32	2.0 ± 0.56	100 ± 14	2.5 ± 0.7	100 ± 19	2.1 ± 0.6

Poisson ratio of sample was assumed to be 0.3 when elastic modulus was calculated.

4. Conclusion

MEHP was formed from DEHP degradation depending on the dose of gamma-ray irradiation. Moreover, DEHP migration depended on its decomposition to MEHP or PA. The cross-linking or scission of surface PVC chains might be influenced by DEHP, MEHP, and PA migration from the PVC sheet. However, it is an undeniable fact that MEHP migration level increased with increasing dose of irradiated gamma rays. MEHP is thought to be more toxic than DEHP. Therefore, MEHP exposure should be taken into consideration in the assessment of DEHP exposure in high-risk patients. The gamma-ray sterilization process uses at least 20–25 kGy gamma rays as sterilization dose, and it is possible that even higher doses are used to sterilize medical devices. In the present paper,

apparent high correlations between hardness or elastic modulus of PVC surface and migration levels of DEHP, MEHP, or PA were not observed. However, any relationship between plasticizer migration and gamma-ray effect on the PVC surface is undeniable possibility. We should further study in greater detail the relationship between plasticizer migration and gamma-ray effect on the PVC surface.

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Table 1. List of chemicals detected in serum of pregnant mothers and cord blood

Group	Chemical	mothers' serum			cord blood's serum			exposure	Reference
		mean±SD (ppb)	detection range (ppb)	detection (%)	mean±SD (ppb)	detection range (ppb)	detection (%)	to cells (ppb) (×1, ×10)[e]	
A	3-PBA	0.3	0.3	1/22 (4.5)	<0.2[b]	<0.2	0/22 (0)	0.1, 1.0	–
(pesticides)	TCP	0.9	0.9	1/22 (4.5)	<0.2[b]	<0.2	0/22 (0)	0.1, 1.0	–
	DMP	8.6±4.2	1.9–18.0	14/22 (63.6)	4.3±3.9	0.9–14.0	11/22(50)	0.1, 1.0	–
	DEP	0.3±0.1	0.2–0.5	9/22 (40.9)	0.28±0.1	0.1–0.4	3/22(13.6)	0.1, 1.0	–
	DMTP	16.2±4.5	10.6–22.2	9/22 (40.9)	0.9±0.8	0.4–1.9	3/22(13.6)	0.1, 1.0	–
	DETP	7.9±3.0	3.2–14.5	21/22 (95.5)	2.8±1.8	1.0–6.8	15/22(68.2)	0.1, 1.0	–
	DMDTP	0.3[a]	0.3	1/19 (0.05)		ND		0.1, 1.0	[37]
	DEDTP	<0.05[a,b]	<0.05	0/19 (0)		ND		0.1, 1.0	[37]
	S-421	10.3[a,c]	0.7-231	58/58 (100)		ND		0.01, 0.1	–
B	nicotine	1.6±2.6	1.0–12.4	33/51 (64.7)	1.4±0.57	1.0–1.8	2/11 (18.2)	100, 100	[3]
(tobacco)	cotinine	43.7±55.8	0.5–177.2	14/51 (27.5)	8.7	8.7	1/11 (9.1)	100, 1000	[34]
C	PFOA	1.5±0.6	0.8–3.7	40/40 (100)	1.4±0.5	0.7–3.1	40/40 (100)	10, 100	[35]
(PFCs)	PFOS	3.9±1.4	1.7–9.3	40/40 (100)	1.4±0.6	0.5–3.0	39/40 (97.5)	10, 100	[35]
D	penta BDE	0.017±0.01[a,d]	0.006–0.04	10/10 (100)		ND		0.01, 0.1	[36]
(PBDEs)	deca BDE	9.2[a,c]	1.3-31.0	102/156 (65.4)		ND		0.01, 0.1	[38]
E	Sn	1.02±0.51[a]	0.34–2.38	36/36 (100)		ND		1.0, 10	–
(metals)	Se	110±18	75.0–147	84/84 (100)		ND		100, 1000	–
	Cd	0.038±0.016	0.012–0.096	84/84 (100)	0.042±0.003	0.005–0.072	27/30 (90.0)	0.1, 1.0	–
	Hg	0.6±0.34	0.2–2.0	84/84 (100)		ND		1.0, 10	–
	Pb	0.3±0.12	0.13–0.83	84/84 (100)	0.30±0.05	0.031–1.004	26/30 (87.0)	1.0, 10	–
F	2-EHA	<1.0[b]	<1.0–3.0	7/38 (18.0)		ND		1.0, 10	–
(VOCs)	2-EH	58.3±48.3	10.0–182	38/38 (100)		ND		10, 100	–
	DCB	5.7±8.8	0.9–53.0	38/38 (100)		ND		10, 100	–
G	DEHP	5.3±0.8	4.1–6.0	5/5 (100)	4.0±1.1	2.6-5.4	5/5 (100)	1.2, 12	[4]
(phthalate)	MEHP	4.3±1.5	3.3–6.9	5/5 (100)	6.3±5.1	3.1–15.4	5/5 (100)	5.0, 50	[4]

ND; not determined. –, chemical level in samples was determined in this study. a, concentrations determined in the previous studies using nonserum samples. b, detection limit level. c, ng/g-lipid. d, Sum of TrBDE-28/33, TeBDE-47, PeBDE-100, Pe-BDE-99, HxBDE-153, and HxBDE-154. e, based on the serum level (×1) and tenfold higher level than serum concentrations (×10).

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Hollow-fiber-supported liquid phase microextraction with in situ derivatization and gas chromatography–mass spectrometry for determination of chlorophenols in human urine samples

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ABSTRACT

A simple and highly sensitive method that involves hollow-fiber-supported liquid phase microextraction (HF-LPME) with in situ derivatization and gas chromatography–mass spectrometry (GC–MS) was developed for the determination of chlorophenols (CPs) such as 2,4-dichlorophenol (DCP), 2,4,6-trichlorophenol (TrCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) in human urine samples. Human urine samples were enzymatically de-conjugated with β-glucuronidase and sulfatase. After de-conjugation, HF-LPME with in situ derivatization was performed. After extraction, 2 μl of extract was carefully withdrawn into a syringe and injected into the GC–MS system. The limits of detection (S/N = 3) and quantification (S/N > 10) of CPs in the human urine samples are 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. The calibration curve for CPs is linear with a correlation coefficient of >0.99 in the range of 0.5–500 ng ml^{-1} for DCP and TrCP, and of 1–500 ng ml^{-1} for TeCP and PCP, respectively. The average recoveries of CPs (n = 6) in human urine samples are 81.0–104.0% (R.S.D.: 1.9–6.6%) with correction using added surrogate standards. When the proposed method was applied to human urine samples, CPs were detected at sub-ng ml^{-1} level.

1. Introduction

Chlorophenols (CPs) are used extensively as preservatives, fungicides, pesticides, disinfectants, and intermediates in many industries. CPs are generated from phenols during treatment of tap water with chlorine [1] and are considered to be one of the most obnoxious contaminants [2,3] because they deteriorate taste and produce an unfavorable smell. Moreover, they are thought to be serious health hazards because they accumulate in moderate amounts and show high toxicity [4,5]. CPs are usually detected in human urine because of the intake of food and water containing CPs and other chlorinated substances as metabolites present in the environment [6]. In order to assess human exposure to CPs, a reliable and sensitive analytical method is required. Many analytical methods, including capillary electrophoresis [7], high-performance liquid chromatography (HPLC) [8,9], and gas chromatography (GC) [10,11], are available for the determination of CPs in human urine samples. HPLC is a simple method as it does not require any derivatization steps; however, because of the relatively low concentrations of CPs in human urine samples, preconcentration is required. Solid phase extraction (SPE) is usually used for preconcentration. However, although SPE requires a small volume of organic solvent, the manual version is tedious and time-consuming. Moreover, a certain kind of SPE cartridge is expensive. Recently, solvent-free and solvent-minimized polymer sorption techniques, such as stir bar sorptive extraction (SBSE), were developed [12]. Kawaguchi et al. reported SBSE-thermal desorption (TD)-gas chromatography–mass spectrometry (GC–MS) for the determination of CPs in human urine sample [13]. In their method, the SBSE tool could be used repeatedly. In addition, the method had high sensitivity and selectivity. However, one shortcoming is that the method requires the use of an instrument for TD-GC–MS. Another method, liquid phase microextraction (LPME), requires a microsyringe and only single drop of organic solvent [14–16]. The extract (organic) solvent in the microsyringe is injected into the GC–MS system. The main advantages of LPME are simplicity of preparation and high-cost

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performance. LPME has been applied to environmental samples [17–21] and food samples [22,23]. Moreover, it has been applied successfully to the determination of alkylphenols (APs), chlorophenols (CPs), and bisphenol A (BPA) in water samples [24]. We considered applying LPME to the determination of CPs in human urine samples; however, because of interfacial activity of urine samples, it is too difficult to retain a single droplet on the microsyringe needle tip. In this study, hollow-fiber-supported (HF) LPME was developed to improve retention of a single droplet of extract on the needle tip, and used to determine CPs in human urine samples.

2. Experimental

2.1. Materials and reagents

2,4-Dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TrCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), and pentachlorophenol (PCP) of environmental analytical grade and acetic acid anhydride for trace analysis were purchased from Kanto Chemical Inc. (Tokyo, Japan). 2,4-Dichlorophenol-d_4 (2,4-DCP-d_4), 2,4,6-trichlorophenol-$^{13}C_6$ (2,4,6-TrCP-$^{13}C_6$), 2,3,4,6-tetrachlorophenol-$^{13}C_6$ (2,3,4,6-TeCP-$^{13}C_6$), and pentachlorophenol-$^{13}C_6$ (PCP-$^{13}C_6$) were used as surrogate standards and were purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). *E. coli* β-glucuronidase (25,000 U 0.4 ml^{-1}) and *H. pomatia* sulfatase (3650 U ml^{-1}) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Prior to use, β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 U ml^{-1}. Other reagents and solvents of pesticide or analytical grade were purchased from Wako Pure Chemical Inc. (Osaka, Japan). The water purification system was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA). Concentrated solutions (1.0 mg ml^{-1} in methanol) of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP were prepared independently. Then, mixture-standard solution (10 μg ml^{-1}) was obtained by mixing the four concentrated solutions. Urine samples were collected from five healthy volunteers and sample preparation was performed immediately. All the healthy volunteers did not eat anything after 10 p.m. the night before sampling the urine. In addition, the volunteers skipped breakfast, and the urine sample was collected before lunch. The volunteers were able to drink water freely.

2.2. Instrumentation

A 10-μl microsyringe for LPME was purchased from SGE Japan (Kanagawa, Japan). The microsyringe needle had a cone tip of 50 mm length and 0.63 mm o.d. Accurel Q 3/2 polypropylene hollow-fiber membrane of 600 μm i.d., 200 μm wall thickness, and 0.2 μm pore size was purchased from Membrana (Wuppertal, Germany). The hollow-fiber membrane was cut manually and carefully into 1.1 cm lengths. Then, the hollow-fiber segments were cleaned in acetone prior to use. For the extraction, 2 ml sample vials from Agilent Technologies (Palo Alto, CA, USA) were used.

2.3. GC–MS instrument and analytical conditions

GC–MS was performed with an Agilent 6890N gas chromatograph equipped with a 5973N mass-selective detector (Agilent Technologies). Injection was performed in the pulsed splitless mode, and injection volume was 2 μl. The splitless time was set to 1 min. The temperature of the inlet was 250 °C. Separation was conducted on a DB-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). Oven temperature was programmed to increase from 100 °C (held for 1 min) to 220 °C at 5 °C min^{-1}, and then increased to 280 C (held for 3 min) at 15 °C min^{-1}. Helium was used as carrier gas at the flow rate of 1.2 ml min^{-1}. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron ionization (EI) (ionization voltage: 70 eV). The monitoring ions were as follows: *m/z* 162 and 164 for the acyl derivative of 2,4-DCP, *m/z* 196 and 198 for the acyl derivative of 2,4,6-TrCP, *m/z* 230 and 232 for the acyl derivative of 2,3,4,6-TeCP, and *m/z* 266 and 268 for the acyl derivative of PCP. The underlined numbers are the *m/z* of the ion used for quantification. The monitoring ions for the acyl derivatives of 2,4-DCP-d_4, 2,4,6-TrCP-$^{13}C_6$, 2,3,4,6-TeCP-$^{13}C_6$, and PCP-$^{13}C_6$ were *m/z* 169, 206, 240, and 276, respectively.

2.4. Human urine sample preparation by LPME

Human urine sample (1 ml) spiked with surrogate standards was buffered with 1 M ammonium acetate solution (100 μl). After adding β-glucuronidase (10 μl; 10,000 units ml^{-1}) and sulfatase (10 μl; 3650 units ml^{-1}), the sample was sealed in a glass tube and gently mixed. Enzymatic de-conjugation to release free CPs was performed by incubating at 37 °C for 3 h [13]. 1 M sodium hydroxide solution (NaOH; 20 μl) for pH adjustment and acetic acid anhydride (20 μl) as the derivatization reagent were added. Then, the sample was agitated. Finally, the sample was subjected to HF-LPME using a 10 μl microsyringe. Before extraction, the microsyringe was rinsed 10 times each with acetone and toluene to avoid carryover and air bubble formation. Three microliters of toluene was withdrawn into the microsyringe. Then, the microsyringe needle tip was inserted into the hollow fiber and the assembly was immersed in toluene for about 20 s to impregnate the pores of the hollow fiber. After impregnation, the fiber was removed from toluene, and air in the syringe was injected to flush the hollow fiber, removing excess organic solvent from inside the fiber. The prepared fiber assembly was set below the surface of the sample solution to prevent desiccation of the hollow fiber. LPME was performed at room temperature for 0–24 min while stirring at 500 rpm. After extraction, 2 μl of the extract was carefully withdrawn into the microsyringe and injected into the GC–MS system.

3. Results and discussion

3.1. Optimization of in situ derivatization and GC–MS conditions

The volumes of NaOH (0–200 μl) and acetic acid anhydride (0–50 μl) in the in situ derivatization step were optimized. As shown in Figs. 1 and 2, when 20 μl of NaOH and 20 μl of acetic acid anhydride were used for the in situ derivatization of CPs, relatively high responses were obtained.

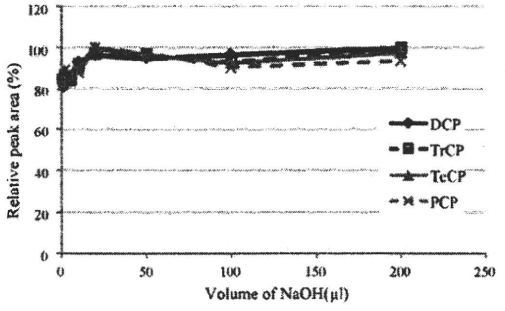

Fig. 1. Optimum volume of NaOH for in situ derivatization. In situ derivatization step was optimized. Profile of the optimum volume of NaOH for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}).

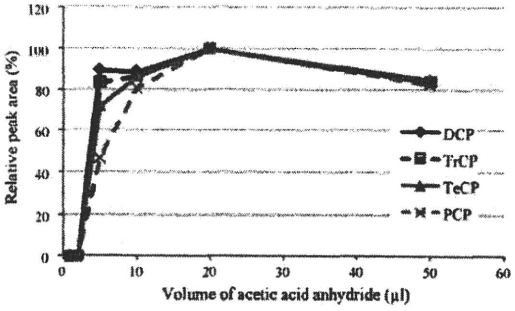

Fig. 2. Optimum volume of acetic acid anhydride for in situ derivatization. Optimum volume of acetic acid anhydride for in situ derivatization of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

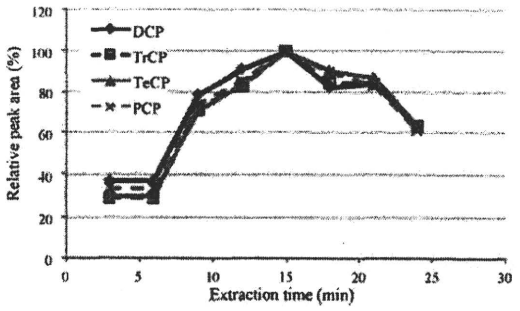

Fig. 4. Extraction time profiles of CPs by LPME. Optimum extraction time of acyl derivatives of CPs in 1 ml standard solutions (5 ng ml^{-1}) using LPME with in situ derivatization and GC–MS.

In EI-MS analysis of the standard solutions of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP in the scan mode, the analytes were observed as acyl derivatives, and *m/z* 162, 196, 230, and 266 were obtained as the major signals, respectively, while *m/z* 164, 198, 232, and 268 were observed as the minor signals, respectively. Mass spectra of the acyl derivatives of CPs are shown in Fig. 3.

3.2. Extraction time

One of the most important parameters affecting LPME was the extraction time. To optimize the extraction time, 5 ng ml^{-1} standard solutions of CPs were used. The extraction time profiles of 1 ml standard solutions of the acyl derivatives of CPs using LPME with in situ derivatization and GC–MS are shown in Fig. 4. The highest responses were obtained when the extraction time was 15 min. One possible reason for the decrease in relative peak area was the reduced volume of toluene used as extraction solvent after extraction for 15 min. This condition was therefore used for the determination of CPs in human urine samples.

3.3. Analytical figures of merit

The limits of detection (LODs) (signal–noise ratio: S/N = 3) and the limits of quantification (LOQs) (S/N > 10) of CPs in human urine samples subjected to in situ derivatization were 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. For CPs determination, calibration curves were obtained by plotting the peak area ratio (CPs/corresponding surrogate standards) versus CPs concentrations. For example, a calibration curve of PCP was obtained by plotting the peak area ratio (PCP/PCP-$^{13}C_6$) versus PCP concentration. The calibration curve for CPs was linear with a correlation coefficient of >0.99 in the range of 0.5–500 ng ml^{-1} for 2,4-DCP and 2,4,6-TrCP, and 1–500 ng ml^{-1} for 2,3,4,6-TeCP and PCP (Table 1). The relative recovery and precision of the method were assessed by replicate analyses ($n = 6$) of human urine samples spiked with 50 and 200 ng ml^{-1} surrogate standards. Non-spiked and spiked samples were subjected to LPME with in situ derivatization and GC–MS. The relative recoveries were calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using calibration curves of the standard solutions with surrogate standards. The average recoveries of CPs ($n = 6$) in human urine samples spiked with 50 and

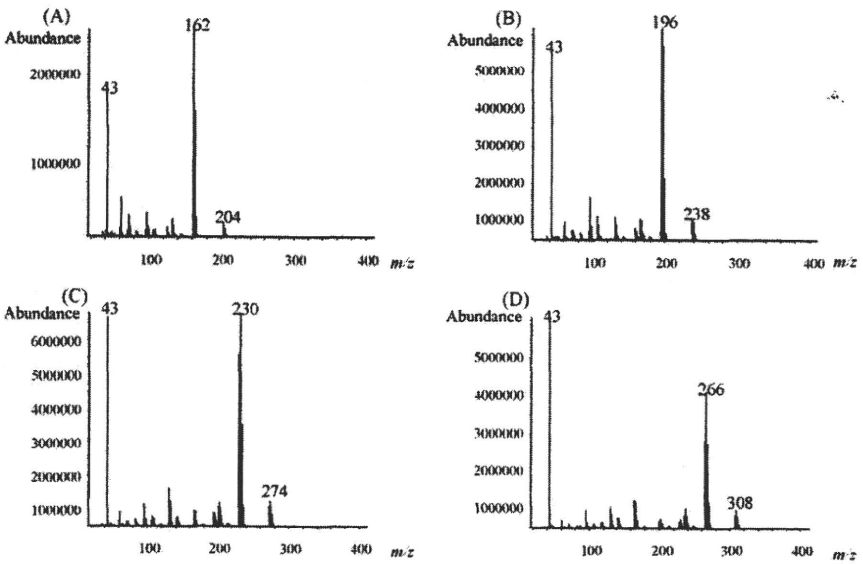

Fig. 3. Mass spectra of acyl derivatives of CPs. Mass spectra of acyl derivatives of 2,4-DCP (A), 2,4,6-TrCP (B), 2,3,4,6-TeCP (C), and PCP (D).

Table 1
Figures of merit of HF-LPME with in situ derivatizaion GC–MS

Analyte	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Linear range (ng ml^{-1})	Correlation coefficient (r)
2,4-DCP	0.1	0.5	0.5–500	0.99
2,4,6-TrCP	0.1	0.5	0.5–500	0.99
2,3,4,6-TeCP	0.2	1	1–500	0.99
PCP	0.2	1	1–500	0.99

LOD: limit of detection (S/N = 3); LOQ: limit of quantification (S/N > 10).

Table 2
Recoveries of CPs in human urine samples

Analyte	50 ng ml^{-1} spiked		200 ng ml^{-1} spiked	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
2,4-DCP	85.9	4.7	81.0	2.8
2,4,6-TrCP	91.4	1.9	104.0	6.6
2,3,4,6-TeCP	91.7	2.3	102.8	5.6
PCP	91.0	2.3	102.2	5.6

Recoveries and R.S.D. were also examined by replicate analyses (n = 6) of human urine samples. Recoveries of CPs were calculated as follows. Recovery (%) = (spiked sample − blank sample)/50 or 200 ng ml^{-1} CPs standard sample × 100.

200 ng ml^{-1} CPs were 85.9–91.7% (relative standard deviation, RSD; <4.7%) and 81.0–104.0 ng ml^{-1} (R.S.D. < 6.6%), respectively, with correction using the added surrogate standards (Table 2). Therefore, the method enables the precise determination of standards and can be applied to the determination of CPs in human urine samples.

3.4. Determination of CPs in human urine samples

Urine samples from five healthy volunteers were analyzed using the present method. As is obvious from the typical chromatograms shown in Fig. 5, CPs could be hardly detected in the samples before enzymatic de-conjugation. However, CPs of sub-ng ml^{-1} level were detected in the samples after enzymatic de-conjugation. When de-conjugation was performed, 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP were detected at 5.8–81.1 ng ml^{-1}, 1.09–5.16 ng ml^{-1}, ND to trace, and trace to 1.12 ng ml^{-1}, respectively (Table 3). Of note was that high levels of 2,4-DCP were detected in all the urine samples. It has been reported that the levels of 2,4-DCP, 2,4,6-TrCP, and PCP in human urine samples subjected to SPE and GC–MS were ND (<1.0) to 50 ng ml^{-1}, ND (<2.0 ng ml^{-1}), and ND (<2 ng ml^{-1}), respectively [25]. There is also another report stating that the levels of 2,4-DCP, 2,4,6-TrCP, 2,3,4,6-TeCP, and PCP in human urine samples subjected to SBSE-TD-GC–MS were 17.24–43.46 ng ml^{-1}, 0.38–2.42 ng ml^{-1}, 0.09–1.31 ng ml^{-1}, and 0.1–0.43 ng ml^{-1}, respectively [13]. The detected level of 2,4-DCP was almost the same as that determined by our method. The spot urine was used to confirm the potential of proposed method in this time, however, 24-h-urine or creatinine correction was worthy of consideration for exposure assessment.

Table 3
Concentrations of CPs in human urine samples

Analyte	A	B	C	D	E
2,4-DCP	10.3	81.1	17.9	5.8	44.2
2,4,6-TrCP	1.6	2.4	1.1	1.2	5.2
2,3,4,6-TeCP	Trace	ND	Trace	Trace	Trace
PCP	Trace	Trace	Trace	Trace	1.1

Urine samples were collected from healthy volunteers (A–E). Concentration is expressed in ng ml^{-1}. ND: below LOD level; Trace: between LOD level and LOQ level.

HF-LPME was compared with SPE and SBSE. SBSE had the highest sensitivity, followed by HF-LPME and SPE. HF-LPME was able to detect low concentrations of CPs sufficiently. In terms of cost performance, HF-LPME was the best. SBSE required a TD-GC–MS

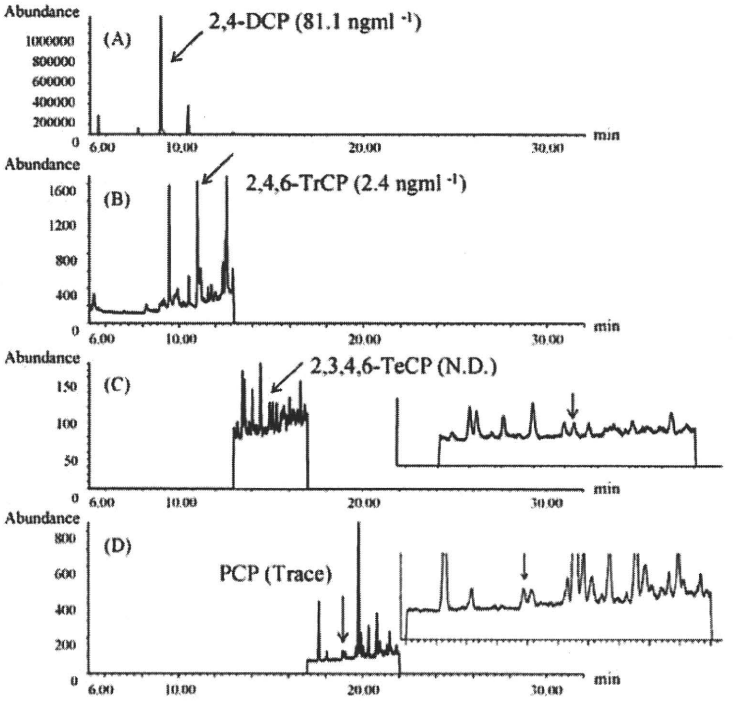

Fig. 5. SIM chromatograms of CPs in human urine sample. SIM chromatograms of CPs in urine sampled from a healthy human volunteer (Volunteer B). The chromatograms of 2,4-DCP (A), 2,4,6-TrCP (B), 2,3,4,6-TeCP (C), and PCP (D) were monitored at m/z 162, 196, 230, and 266 as acyl derivatives, respectively.

system, and the TD system had high running cost because liquid nitrogen was used. By contrast, HF-LPME did not require any special instrument for analysis; it required only a microsyringe and conventional GC–MS. Therefore, the present method may be useful for the assessment of human exposure to CPs.

4. Conclusions

This is a first paper to determine trace amounts of CPs in human urine samples using HF-LPME with in situ derivatization and GC–MS. The proposed method has many practical advantages, including simplicity of the extraction method, use of a small volume of organic solvent for extraction, and high sensitivity. The method was sufficiently applicable to the analyses of human urine samples. The limits of detection (S/N = 3) and quantification (S/N > 10) of CPs in human urine samples are 0.1–0.2 ng ml^{-1} and 0.5–1 ng ml^{-1}, respectively. The present method showed good linearity and high correlation coefficients using surrogate standards. In addition, the average recoveries of CPs (n = 6) in human urine samples were 81.0–104.0% with good precision (R.S.D.: 1.9–6.6%). This simple, accurate, and highly sensitive method is expected to have potential applications in human urine samples.

Acknowledgement

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Short communication

Determination of urinary triclosan by stir bar sorptive extraction and thermal desorption–gas chromatography–mass spectrometry

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ABSTRACT

We have developed an analytical method for the determination of urinary 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan), which utilizes stir bar sorptive extraction (SBSE) and thermal desorption (TD)–gas chromatography–mass spectrometry (GC–MS). Human urine sample is deconjugated by treatment with β-glucuronidase and sulfatase. A stir bar coated with polydimethylsiloxane (PDMS) is added to the urine sample in a vial and the sample is stirred for 60 min at room temperature (25 °C). Then, the PDMS stir bar is subjected to TD–GC–MS. The detection limit of triclosan is 0.05 ng mL^{-1}. The method shows linearity over the calibration range (0.1–10 ng mL^{-1}) and the correlation coefficient (r) is higher than 0.993 for triclosan standard solution. The average recoveries of triclosan in human urine sample are 102.8–113.1% (RSD: 2.4–6.7%). This simple, sensitive, and selective analytical method may be used in the determination of trace amounts of triclosan in human urine samples.

1. Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy)phenol, is widely used as a broad-spectrum antimicrobial agent. As an additive in plastics, it prevents growth of microorganisms and suppresses the formation of stain and odor, thereby extending the polymer's shelf life [1]. For this reason, it has been considered that triclosan in polymeric materials come in contact with food [2,3]. As healthy humans may be exposed to triclosan via a variety of daily activities, exposure assessment of triclosan in human is an important issue.

The urinary excretion of orally ingested triclosan as free triclosan and glucuronide and sulfate conjugates has been reported [4]. Thus, it is thought that human exposure can be evaluated by measuring triclosan in urine sample. To this end, high-sensitivity and high-accuracy analytical methods are required.

Many analytical methods for the determination of triclosan in biological samples have been reported, including liquid chromatography (LC) with diode array detection (DAD) [5], electrochemical detection (ECD) [6] or tandem mass spectrometry (MS–MS) [7]. On the other hand, gas chromatography (GC) with MS or MS–MS was initially used for the determination of triclosan in environmental analysis [8–11].

Liquid–liquid extraction (LLE) [6] and online solid-phase extraction (SPE) [7] have been developed for the determination of triclosan in urine sample. Recently, a new sorptive extraction technique that uses a stir bar coated with polydimethylsiloxane (PDMS) was developed [12] and is known as stir bar sorptive extraction (SBSE). Its main advantage is its wide application range that includes volatile aromatics, halogenated solvents, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, endocrine disruptors, preservatives, odor compounds, and organotin compounds [13–15]. Silva and Nogueira determined triclosan in biological and environmental matrices with the SBSE-liquid desorption (LD)-LC-DAD method [5]. In LC analysis, LD was used to desorb the analyte from the PDMS stir bar. On the other hand, thermal desorption (TD) is often used to desorb the analyte from the PDMS stir bar. Because TD enables injection of the entire quantity of desorbed analyte into an analytical instrument such as GC–MS, high-sensitivity analysis is expected.

The aim of this study was to determine trace amounts of triclosan in human urine samples by SBSE and TD–GC–MS.

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2. Experimental

2.1. Materials and reagents

Triclosan was purchased from Wako Pure Chemical, Inc. (Osaka, Japan). Benzophenone-d_{10} (BP-d_{10}, used as internal standard) was purchased from Kanto Chemical Inc. (Tokyo, Japan). *E. coli* β-glucuronidase (25,000 units/0.4 mL, 62,500 units mL^{-1}) and *H. pomatia* sulfatase (3540 units mL^{-1}) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Prior to use, β-glucuronidase was added to 0.1 M ammonium acetate to make a total concentration of 10,000 units mL^{-1}. Other reagents were purchased from Wako Pure Chemical, Inc. The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA).

2.2. Standard solution

A concentrated solution (1.0 mg mL^{-1}) of triclosan was prepared by dissolving in methanol. Seven-point calibration (0.1, 0.2, 0.5, 1, 2, 5, and 10 ng mL^{-1}) was performed daily in duplicate analysis for all samples containing the internal standard using the SBSE method.

2.3. Instrumentation

TD was performed with a Gerstel TDS 2 thermodesorption system equipped with a Gerstel TDS A autosampler and a Gerstel Cooled Injection System (CIS) 4 programmable temperature vaporization (PTV) inlet. GC–MS was performed with an Agilent 6890N gas chromatograph equipped with a 5973N mass-selective detector (Agilent Technologies).

Stir bars coated with a 0.5-mm thick PDMS layer (24 μL; Twister™: a magnetic stirring rod is placed inside a glass jacket and coated with PDMS) were obtained from Gerstel (Mülheim an der Ruhr, Germany). The stir bars were conditioned for 1 h at 300 °C in a flow of helium. Then, the PDMS stir bars were kept in new 2 mL vials until immediately prior to use. The stir bars could be used more than 50 times with appropriate re-conditioning. For the extraction, a 10 mL headspace vial from Agilent Technologies (Palo Alto, CA, USA) was used.

2.4. TD–GC–MS conditions

TDS 2 temperature was programmed to increase from 20 °C (held for 1 min) to 240 °C (held for 5 min) at 60 °C min^{-1}. The desorbed compounds were cryofocused in CIS 4 at −150 °C. After the desorption, CIS 4 temperature was programmed to increase from −150 °C to 300 °C (held for 10 min) at 12 °C s^{-1} to inject the trapped compounds into the analytical column. CIS 4 is a type of PTV device. Once an analyte is trapped by means of temperature control, the entire quantity is subjected to GC–MS. Injection was performed in the solvent vent mode. Separations were conducted on a DB-5ms fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). Oven temperature was programmed to increase from 40 °C (held for 1 min) to 190 °C at 5 °C min^{-1} and from 190 °C to 280 °C (held for 3 min) at 15 °C min^{-1}. Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron ionization (ionization voltage: 70 eV). Monitoring ions are shown in Table 1.

2.5. Human urine samples

Urine was collected from six healthy volunteers (I, II, III, IV, V, and VI). All samples were stored at 4 °C prior to use.

2.6. Sample preparation

One milliliter of urine sample spiked with BP-d_{10} was pipetted into a 10 mL vial. Then, 1.0 M ammonium acetate (100 μL, pH 6.8) was added. After β-glucuronidase (10 μL, 10,000 units mL^{-1}) and sulfatase (10 μl, 3540 units mL^{-1}) were added, the sample was gently mixed. Glucuronidase and sulfatase hydrolysis to release free triclosan was accomplished by incubating at 37 °C for 3 h. After enzymatic de-conjugation, 1 mL of purified water was added to the vial. A PDMS stir bar was added and the vial was crimped with a Teflon-coated silicone septum cap. SBSE was performed at room temperature for 60 min while stirring at 500 rpm. After the extraction, the stir bar was easily removed, rinsed with purified water, dried with lint-free tissue, and placed inside a glass TD tube. The TD tube was placed inside the TD system where the stir bar was thermally desorbed and the desorbed analyte was subjected to GC–MS thereafter.

3. Results and discussion

3.1. Optimization of GC–MS conditions

In the mass analysis of standard solutions using electron impact ionization (EI)-MS, *m/z* 288 was observed as the main peak of triclosan. The mass spectrometer was operated in the SIM mode. Three ions were monitored (*m/z* 288, 218 for triclosan; and *m/z* 192 for BP-d_{10}. The underlined and none underlined number are the *m/z* of the ion used for quantitation and qualitative analysis, respectively).

3.2. Optimization of SBSE conditions

One important parameter affecting SBSE was the extraction time. To determine the optimum extraction time, 1 mL of human urine sample (5 ng mL^{-1} triclosan standard solution) and 1 mL of purified water were mixed and used. The extraction time profile (0–180 min) of triclosan in the human urine sample that was subjected to SBSE and TD–GC–MS is shown in Fig. 1. Triclosan reached equilibrium after approximately 60 min. Therefore, this condition was used for the determination of triclosan in human urine samples.

Table 1
Figures of merit of SBSE and TD–GC–MS.

Compound	SIM[a] (*m/z*)	LOD[b] (ng mL^{-1})	LOQ[c] (ng mL^{-1})	Range (ng mL^{-1})	Correlation coefficient (*r*)	Amount spiked			
						0.5 ng mL^{-1}		5 ng mL^{-1}	
						Recovery (%)	RSD (%)[d]	Recovery (%)	RSD (%)[d]
Triclosan	288, 218	0.05	0.1	0.1–10	0.993	102.8	2.4	113.1	6.7

[a] The underlined number is the *m/z* of the ion used for quantification.
[b] LOD: limit of detection (S/N = 3).
[c] LOQ: limit of quantification (S/N > 10).
[d] Recoveries and precision were also examined by replicate analysis ($n = 6$) of human urine samples.

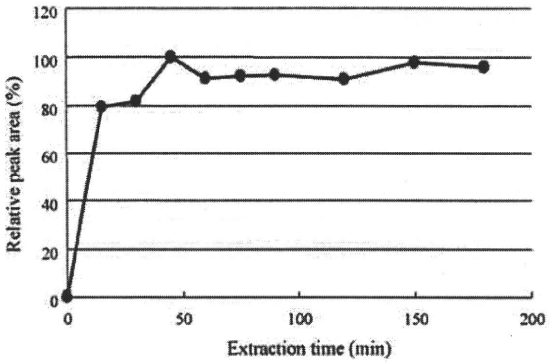

Fig. 1. Optimization of extraction time.

3.3. Figures of merit of SBSE and TD–GC–MS for determination of triclosan

The calculated detection limit (LOD) is 0.05 ng mL^{-1} for SBSE and TD–GC–MS, with the ratio of the compound's signal to the background signal (S/N) being 3. In addition, the limit of quantification (LOQ) when S/N > 10 is 0.1 ng mL^{-1} for triclosan. Some different lots of human urine were used for calculation of LOD and LOQ. The blank and internal standard only samples were checked. The interference was not observed. The method shows linearity over the calibration range (0.1–10 ng mL^{-1}) and the correlation coefficient (r) is higher than 0.993 for triclosan standard solution. The figures of merit of the present method are summarized in Table 1. The LOD of triclosan in urine sample previously determined by LLE-LC-ECD [6] and the online SPE–LC–MS–MS method [7] was 1.0 and 2 ng mL^{-1}, respectively. Compared to those studies, approximately 10–20 times higher sensitivity was achieved in this study.

The recovery and within-day precision of the method were assessed by replicate analysis (n = 6) of human urine samples fortified at 0.5 and 5 ng mL^{-1} levels. Non-spiked and spiked samples were subjected to SBSE and TD–GC–MS. Recovery was calculated by subtracting the results for the non-spiked samples from those for the spiked samples. The results were obtained by using calibration curves obtained from standard solutions with internal standard. The recovery and precision were 102.8–113.1% (RSD: 2.4–6.7%) for human urine samples (Table 1). Therefore, the method enables the precise determination of standards and may be applicable to the

Table 2
Concentrations of triclosan in human urine samples.

Compound	De-conjugation	Human urine					
		I	II	III	IV	V	VI
Triclosan (ng mL^{-1})	Without de-conjugation	0.17	0.15	0.15	0.15	0.52	0.15
	De-conjugation	2.62	1.35	1.13	4.30	13.98	1.26
Ratio of conjugate (%)[a]		93.5	89.2	86.8	96.4	96.3	87.9

[a] Ratio of conjugate was calculated by (Total-Free)/Total*100.

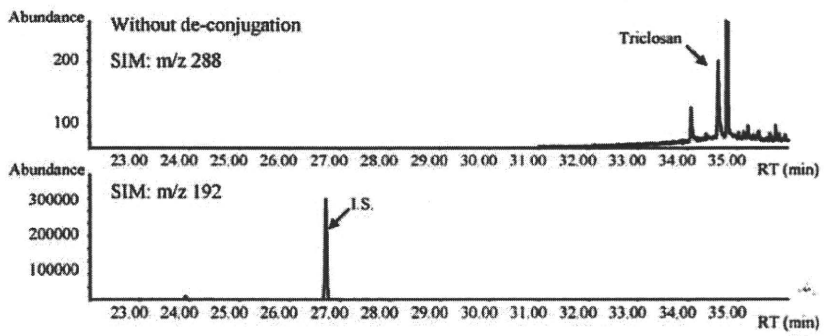

Fig. 2. Typical SIM chromatogram of triclosan in human urine sample (I) by SBSE–TD–GC–MS without de-conjugation. Triclosan: 0.17 ng mL^{-1}.

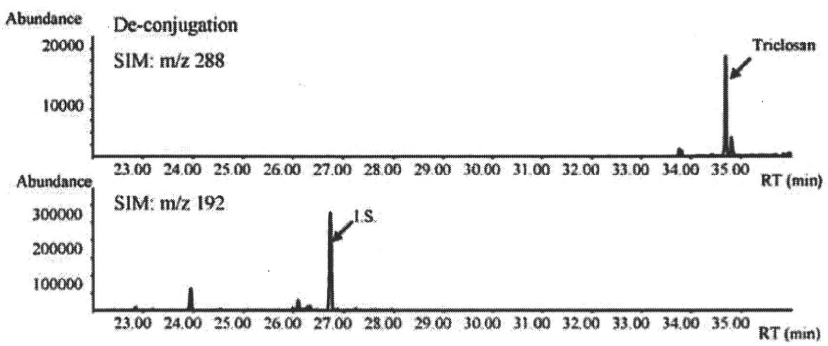

Fig. 3. Typical SIM chromatogram of triclosan in human urine sample (I) by SBSE–TD–GC–MS with de-conjugation. Triclosan: 2.62 ng mL^{-1}.

determination of trace amounts of triclosan in human urine samples.

3.4. Determination of triclosan in human urine samples

A total of six human urine samples were analyzed for triclosan using the present method and the results are shown in Table 2. For quality control, the urine samples spiked with standard were analyzed between the measurements of six human urine samples. In the human urine samples without de-conjugation, 0.15–0.52 ng mL^{-1} triclosan was detected. On the other hand, in the human urine sample treated with β-glucuronidase and sulfatase, 1.13–13.98 ng mL^{-1} triclosan was detected by the present method. Typical chromatograms of human urine sample (Volunteer I) by SBSE–TD–GC–MS without de-conjugation and with de-conjugation are shown in Figs. 2 and 3, respectively. The ratio of conjugate in human urine sample was 86.8–96.4%. Most triclosan existed in human urine sample as conjugate. The comparatively high concentration of triclosan was determined in the sample. It was thought that the volunteers may be exposed to triclosan via a variety of daily activities.

SBSE and TD–GC–MS enabled the successful determination of trace amounts of triclosan in human urine sample. Because the previous methods [6,7] were low sensitivity, it was considered that triclosan in human urine sample might not be determined. The proposed method has many practical advantages, including small sample volume (1 mL) and simplicity of extraction; it is also solvent-free and has high sensitivity.

4. Conclusions

The determination of trace amounts of triclosan in human urine samples using SBSE and TD–GC–MS was performed. The detection limit for triclosan was 0.05 ng mL^{-1}. In addition, the present method showed good linearity and high correlation coefficient using the internal standard. The recovery was high (102.8–113.1%) and the precision was good (RSD: 2.4–6.7%) for human urine samples fortified at 0.5 and 5 ng mL^{-1} levels. This simple and highly sensitive method is expected to have potential applications in various aqueous samples.

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Simultaneous Determination of Di(2-ethylhexyl)phthalate, Mono(2-ethylhexyl)phthalate, and Phthalic Acid Migrating from Gamma-Ray Irradiated Polyvinyl Chloride Sheet by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: The aim of the present study was to establish a liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for the simultaneous determination of di(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl)phthalate (MEHP), and phthalic acid (PA). In the proposed method, the limits of detection of DEHP, MEHP, and PA were 5, 0.5, and 1 ng/mL, respectively, and the limits of quantification with standard solutions were 20, 2, and 5 ng/mL, respectively. Intra- and interday assays showed good accuracy and repeatability. The recoveries of DEHP, MEHP, and PA from respective extraction solvents ranged from 98.9 to 104.2% (relative standard deviation was below 10.3%). DEHP and its breakdown products migrating from gamma-ray irradiated polyvinyl chloride (PVC) sheets were determined simultaneously since DEHP is easily eluted from PVC medical devices. DEHP migration was noted from

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both gamma-ray irradiated and control PVC sheets. Compared with the migration from the control PVC sheet, MEHP showed significant migration from the gamma-ray irradiated PVC sheet. In contrast, PA migration was noted only from the gamma-ray irradiated PVC sheet.

Keywords: LC-MS/MS, Di(2-ethylhexyl)phthalate, Mono(2-ethylhexyl)phthalate, Phthalic acid, Gamma-ray sterilization

INTRODUCTION

Phthalate esters, particularly di(2-ethylhexyl)phthalate (DEHP), are extensively used as plasticizers to increase the flexibility of polyvinyl chloride (PVC) products. PVC is one of the most widely used plastic polymers in such medical products as blood containers, blood tubing, and catheters. However, it has been reported that DEHP was easily eluted from PVC products into food, drugs, and body fluids.[1-4] DEHP is considered to exhibit reproductive and developmental toxicity,[5,6] carcinogenicity, and testicular toxicity.[7-9] It was also found to affect the reproductive organs and fertility.[10] It has been reported that DEHP is hydrolyzed enzymatically into mono(2-ethylhexyl)phthalate (MEHP),[11-13] and that MEHP may be even more toxic than the parent compound. In vitro studies have shown that MEHP inhibits FSH stimulated cAMP accumulation in cultured Sertoli cells,[14-18] in addition to reducing 17β-estradiol production and aromatase mRNA expression.[19,20] DEHP was determined to be the common plasticizer migrating from PVC medical devices into the blood.[2,3] MEHP was also determined to be the metabolite of DEHP. PA was not determined because it is not as toxic as DEHP or MEHP and the amount of PA in blood is negligible, although it is also produced by the enzymatic hydrolysis of DEHP.[21]

In our previous studies, we observed that not only DEHP but also MEHP migrated from PVC medical devices into simulated pharmaceuticals even without enzymatic hydrolysis.[4,22] In addition, we found that hydrolysis may occur during the sterilization process, particularly gamma-ray sterilization.[22] Therefore, a method for the simultaneous analysis of DEHP, MEHP, and PA was developed to confirm that MEHP and PA are produced from DEHP even without enzymatic hydrolysis. In addition, the method was used to determine DEHP, MEHP, and PA migrating from gamma-ray irradiated PVC sheets into purified water, 5% glucose solution, and polyoxyethylated hydrogenated castor oil 60 (HCO-60). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was proven to be a suitable method for the determination of DEHP, MEHP, and PA with high sensitivity, precision, and selectivity.

EXPERIMENTAL

Chemicals and Materials

Environmental analytical grade DEHP and DEHP-d_4 were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). MEHP and MEHP-d_4 were purchased from Hayashi Pure Chemical Industries (Osaka, Japan). PA and PA-d_4 were purchased from CDN Isotope Central Chemicals Co., Inc. (Tokyo, Japan). Phthalic acid esters, analytical grade acetonitrile, and acetone were used in the experiments. Analytical grade formic acid was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The water purification system used was a Milli-Q gradient A 10 with an EDS polisher (Millipore, Bedford, MA, USA).

The test material was PVC sheets subjected to gamma-ray (^{60}Co; 24.2 kGy). The gamma-ray doses were set with reference to sterilization conditions used by commercial medical devices. The control sample was not irradiated gamma-ray. These PVC sheets were kindly supplied by the manufacturer.

The extraction solvents were 5% glucose solution for injection (Otsuka Pharmaceuticals Co., Tokyo, Japan), polyoxyethylated hydrogenated castor oil 60 (HCO-60) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and purified water.

Instrumentation and Chromatographic Conditions

A Series 1100 liquid chromatograph from Agilent Technologies (USA) was coupled to an API 4000™ (Applied Biosystems Japan, Tokyo, Japan) equipped with a Turbo Ionspray™ ionization source. Mass spectrometry data were processed with Analyst 1.3.2 software. An Inertsil-Ph3 column (50 mm × 2.1 mm, 5 μm particle size) from GL Sciences was used for the separation.

After 5 μL of the sample was injected with an autosampler, it was loaded onto the analytical column by flowing mobile phase at the flow rate of 0.2 mL/min. Acetonitrile (mobile phase A) and 0.05% formic acid in water (mobile phase B) were used. Separation was carried out with the following profile: mobile phase A/B was 15/85 (0–4 min) → 90/10 (4.01–15 min for elution) → 15/85 (15.01–25 min for equilibration) (v/v). The column oven was maintained at 40°C for LC.

MS/MS Conditions

The working parameters for turbo ion spray ionization MS/MS were as follows: curtain gas flow rates, 10 psi (DEHP and DEHP-d_4 for positive ion mode) and