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Simulated neonatal exposure to DEHP and MEHP from PVC enteral nutrition products

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Abstract

The leaching of di(2-ethylhexyl)phthalate (DEHP) and mono(2-ethylhexyl)phthalate (MEHP) from medical products made of polyvinyl-chloride (PVC) to enteral nutrition (EN) for neonatal patients was determined in a simulated study. The study simulated a typical case of EN administration to a neonatal patient (body weight, 3 kg) in a neonatal care unit (temperature, 25 °C); the medical products used were an irrigator and catheter containing DEHP (9.1–31.8%, w/w) as a plasticizer. The worst-case daily exposures of the neonatal patient to DEHP and MEHP by the administration of EN were estimated to be 148 and 3.72 μg/(kg day), respectively, as assessed from the levels of these compounds leaching from the medical products to the EN. The use of DEHP-free medical products reduced the exposure of DEHP and MEHP to the minimum levels contained in the EN at preparation. A transition to DEHP-free medical products for neonatal patients would be effective in reducing the exposure of neonatal patients to DEHP via EN administration.

Keywords: Di(2-ethylhexyl)phthalate; Mono(2-ethylhexyl)phthalate; Polyvinyl-chloride; Enteral nutrition; Medical product; Neonate

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a common plasticizer used to impart flexibility to polyvinyl-chloride (PVC). It readily leaches from PVC into the environment and transfers to other materials attached to the PVC or via the atmosphere. PVC is used in a variety of medical products for its excellent physical characteristics such as flexibility, strength, and transparency. Patients undergoing medical procedures such as intravenous therapy, nutritional support, blood transfusion, haemodialysis, cardiopulmonary bypass, or extracorporeal membrane oxygenation (EMO) are potentially exposed to DEHP released from medical products. Previous studies have shown detectable amounts of DEHP in blood products, intravenous solutions, and intravenous fat emulsions stored in PVC bags (Mazur et al., 1989; Dine et al., 1991; Waugh et al., 1991; Faouzi et al., 1999; Hanawa et al., 2000). DEHP is hydrolyzed to mono(2-ethylhexyl)phthalate (MEHP) *in vivo* and in blood products by esterase activities (Albro and Thomas, 1973; Lake et al., 1977). DEHP and MEHP have also been detected in the blood of haemodialysed patients (Flaminio et al., 1988a). Orally administrated DEHP in enteral nutrition (EN) can be hydrolyzed to MEHP by lipase, which is then absorbed via the intestine as well as within food and water (Albro and Thomas, 1973).

In animal studies, DEHP and/or MEHP are toxicants to the reproductive and developmental systems (Gray and Gangolli, 1986; Sjöberg et al., 1986; Moss et al., 1988; Davis et al., 1994). The toxicities of DEHP and/or MEHP are dose-, time-, and age-dependent for the target organ and tissue deposition (Latini, 2000). Recent epidemiologic studies report that certain phthalates exposure levels of pregnant women were significantly associated with duration of pregnancy and reproductive health of male infants (Latini et al., 2003; Swan et al., 2005; Marsee et al., 2006), and the possible effects of phthalates on the reproductive systems of male infants are well documented

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(Lottrup et al., 2006). Male neonates and infants are considered a sensitive population to toxicity because their reproductive systems are still developing.

Calafat et al. (2004) reported that the geometric mean of MEHP and its glucuronide concentrations in the urine of ill neonates who spent time in neonatal intensive care units was 30 times higher than that in healthy children; the neonatal patients were exposed to DEHP via medical procedures. The Center for Devices and Radiological Health in U.S. Food and Drug Administration (FDA/CDRH) has reviewed the potential health risks of DEHP leaching from medical devices (FDA/CDRH, 2001, 2002) and recommended considering alternatives when high-risk procedures such as transfusion, haemodialysis, total parenteral nutrition (TPN), EMO, or EN are to be performed on male neonatal patients, pregnant women carrying a male fetus, or peripubertal males (FDA/CDRH, 2002). The FDA report estimated the daily exposure of neonatal patients to DEHP from EN administration to be 140 μg/(kg day) (FDA/CDRH, 2001), while the exposure of infants to DEHP from infant formula and breast milk was estimated to be 8–21 μg/(kg day) (Latini et al., 2004). The difference in exposure level between EN and infant formula or breast milk is deriving from the DEHP leached from medical products; however, information regarding the exposure of neonatal patients to DEHP and MEHP via EN administration is limited. We previously developed a method for determining DEHP and MEHP levels in human sera without severe contamination of DEHP, using a liquid chromatography with tandem mass spectrometry (LC/MS/MS) (Takatori et al., 2004). In the present study, we use this method to investigate the exposure of neonatal patients to DEHP and MEHP via EN administration.

2. Materials and methods

2.1. Materials

DEHP (99.6%), MEHP (99.3%), DEHP-d_4 (99.0%), and MEHP-d_4 (99.8%) were purchased from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan), while analytical-grade acetone, hexane, acetonitrile, ethanol, and tetrahydrofuran were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Water for HPLC was purified using the Milli-Q system (Milli-Q, Millipore, Bedford, MA). The water used in this study (except for eluent of LC/MS/MS) was prepared by washing the Milli-Q water with hexane. To eliminate contamination by DEHP and MEHP, glassware was washed twice with acetone and hexane and then baked at 200 °C for 2 h in a clean oven.

In the present study, we used two types of irrigators and catheters that are commercially available in Japan and are commonly used for neonatal or infant patients: PVC (standard-type) containing DEHP as a plasticizer, and poly-butadiene (DEHP-free type) that contains no DEHP. Both types of irrigators consist of four components (bag, tube, drip tube, and adaptor) and a clamp for flow control. The catheters consist of a connector and a tube. The sizes of the two types of irrigators and catheters are shown in Table 1.

Table 1
Sizes of irrigators and catheters

Component	Size	
	Standard	DEHP-free
Irrigator		
Bag[a]	500	500
Tube[b]	890, 3.5	1010, 3.5
Drip tube[b]	55, 16	50, 18
Adaptor[b]	35, 3.5	40, 3.5
Catheter		
Tube[b]	420, 1.7	420, 1.7
Adaptor[b]	25, 3.5	25, 3.5

[a] Volume (ml).
[b] Total length and internal diameter (mm).

2.2. LC/MS/MS conditions

LC/MS/MS was used to determine DEHP and MEHP. LC/MS/MS analysis was performed using an API3000 mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electro-spray ionization (ESI) interface and an Agilent 1100 series high performance liquid chromatography (HPLC, Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler, and a G1379A degasser. We used a reverse-phase HPLC column (Wakosil3C18, 2.0 mm × 150 mm, 3 μm; Wako Pure Chemical Co. Ltd.). The mobile phases consisted of 100% acetonitrile (A) and 5×10^{-4}% aqueous acetic acid (B). Elution was performed using an isocratic mode (A/B: 80/20, v/v) at 0.2 ml/min, and the ESI interface was controlled by Analyst Software (v.1.3.2). The MS/MS was operated in negative or positive ion mode. The heated capillary and voltage were maintained at 500 °C and ±4.0 kV (negative/positive mode), respectively. MEHP and MEHP-d_4 were detected in the negative mode. The declustering potential (DP), focussing potential (FP), and collision energy (CE) for MEHP and MEHP-d_4 measurements were −31, −110 V, and −22 eV, respectively. DEHP and DEHP-d_4 were detected in the positive mode. The DP, FP, and CE for DEHP and DEHP-d_4 measurements were 26, 100 V, and 27 eV, respectively. The respective combinations of precursor ions and product ions were as follows: MEHP (−277, −134), MEHP-d_4 (−281, −138), DEHP (391, 149), and DEHP-d_4 (395, 153).

2.3. DEHP and MEHP content in irrigators and catheters

To determine the DEHP content in the components of the standard-type irrigator and catheter, sections of each were cut into 2 mm × 2 mm squares. To dissolve the pieces, 5.0 ml tetrahydrofuran was added to a 50-ml volumetric flask containing 0.2 g of the cut squares. PVC polymer was precipitated by adding ethanol up to the volumetric line of the flask. After removing the precipitant by centrifugation, the aliquot of supernatant was diluted with acetonitrile before being analyzed using LC/MS/MS.

Table 2
DEHP content of the standard-type irrigator and catheter ($n = 3$)

Component	Content (%, w/w)
Irrigator	
Bag	26.1 ± 0.8
Tube	22.0 ± 1.4
Drip tube	15.2 ± 1.6
Adaptor	9.1 ± 1.3
Catheter	
Tube	31.8 ± 0.6
Adaptor	31.3 ± 0.6

2.4. Preparation of EN

Four commercially available brands of EN (EN-A, -B, -C, and -D) were used in the present study. Both EN-A and -B are used for neonatal and infant patients, while EN-C and -D are used for adult patients. EN-A, -B, and -C are supplied as powders that were prepared immediately prior to the experiments with warmed hexane-washed Milli-Q water (40 °C) to minimize contamination with DEHP and MEHP. EN-A, -B, and -C were prepared at standard concentrations (EN-A: 20%, w/v; EN-B: 17%, w/v; EN-C: 27%, w/v) according to the manufacturer's directions. EN-D is a canned liquid that was opened just before the experiments and used without further preparation.

2.5. Leaching of DEHP and MEHP from medical products to EN

Simulated studies were performed assuming that EN-A is administrated to a neonatal patient. Details of the case are as follows. A neonatal patient (body weight, 3 kg) was cared for in a neonatal patient care unit at 25 °C. Daily nutrition (312 kcal/day) was supplied by EN-A (20%; 392 ml) with an irrigator and catheter that contained DEHP. The daily EN-A was divided into seven portions (56 ml/portion), administered seven times a day. Each portion was administered over 15 min. The simulated studies were performed in our laboratory in an incubator regulated at 25 °C. The glass flask of prepared EN-A was equilibrated to 25 °C in the incubator for 30 min. The EN-A was then poured into the irrigator before flowing into clean glass tubes via the catheter. The flow time of 56 ml of EN-A from the irrigator to the glass tubes was regulated using a clamp to 15 min. The flow rate was approximately 3.5–4 ml/min. To compare leaching of DEHP and MEHP between the ENs, the same volume (56 ml) of EN-A (10–15%), -B, -C, -D, and water was run through the irrigator and catheter set as described above. The collected EN solutions were frozen at −40 °C until analysis.

2.6. Determination of DEHP and MEHP in EN

DEHP and MEHP in the EN were determined using the previously described procedure for determining these compounds in human serum (Takatori et al., 2004). Briefly, 50 ng DEHP-d_4 and MEHP-d_4 were added to a glass tube containing 0.5 ml EN. DEHP and MEHP in the EN were initially extracted with acetone. The acetone extraction was dried under an N_2 stream and then dissolved with 0.5 ml hexane-washed Milli-Q water containing 4 μl acetic acid. Next, DEHP and MEHP in the acetic acid aqueous solution were extracted three times with hexane. The extract was dried as described above and then dissolved in 0.5 ml acetonitrile before being analyzed using LC/MS/MS.

2.7. Raman microspectroscopic analysis of the medical products

A Raman microspectrometer, NRS-3100 (JASCO, Tokyo, Japan), was used to measure the Raman spectra of the internal surface of irrigator and catheter. The Raman microspectrometer was coupled with thermoelectrically cooled charge-coupled device detector. The holographic grating was 1800 g/mm. The exciting wavelength was 532 nm from a green laser with a power of 9.2 mW. The laser was focused on the surface of the sample by using a 5× microscope objective. The spatial resolution was 120 μm in the X–Y plane. The spectral resolution was 1 cm^{-1}. Raman spectrum was collected over the range from 1879 to 541 cm^{-1} and the integration time was 30 s. The measurements were repeated five times to normalize data.

3. Results

The content of DEHP and MEHP was determined for each part of the irrigator and catheter made of PVC (standard-type) without surrogates. DEHP and MEHP were recovered from the extraction solution to satisfactory levels (95–105%). All parts

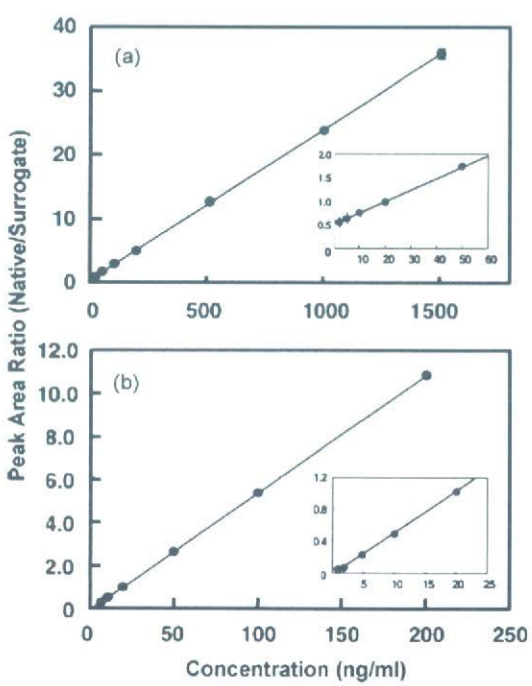

Fig. 1. Calibration curves for DEHP and MEHP. Points and bars represent the averages and standard deviations of five independent experiments. (a) Vertical axis corresponds to the peak-area ratio (DEHP/DEHP-d_4). Inset indicates the low range (2–60 ng/ml) of the curve. (b) Vertical axis corresponds to the peak-area ratio (MEHP/MEHP-d_4). Inset indicates the low range (1–25 ng/ml) of the curve.

Table 3
DEHP and MEHP concentrations in the EN at preparation and after flowing through the irrigator and catheter ($n = 6$)

EN	Concentration (%, w/v; kcal/ml)	Fat (mg/ml)	DEHP (ng/ml)		MEHP (ng/ml)	
			Before	After	Before	After
A	20; 0.8	7.2	49.6 ± 8.4	1130 ± 190	<LOQ[b]	28.5 ± 4.8
A	15; 0.6	5.4	32.8 ± 4.1	1080 ± 40	<LOQ[b]	30.5 ± 1.5
A	10; 0.4	3.6	21.6 ± 4.1	1000 ± 65	<LOQ[b]	28.6 ± 1.6
B	17; 0.6	4.3	58.6 ± 3.0	203 ± 43	6.0 ± 0.3	23.5 ± 1.4
C	27; 1.0	1.7	64.3 ± 2.4	513 ± 60	6.9 ± 0.7	26.1 ± 0.1
D	–; 1.0	33.2	158 ± 7.7	721 ± 93	<LOQ[b]	24.9 ± 1.9
Water	–; 0	0	<LOQ[a]	54 ± 17	<LOQ[b]	24.4 ± 0.9

[a] 15 ng/ml.
[b] 5 ng/ml.

contained 9.1–31.8% (w/w) of DEHP (Table 2), while the content of MEHP in the same parts was less than 0.1%.

For DEHP measurement in EN, the calibration curve was obtained for the peak-area ratio (DEHP/DEHP-d_4) versus DEHP concentration (Fig. 1(a)). The curve was linear over the range of 2.0–1500 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0237x + 0.566$ ($r = 0.999$), with mean values for the slope and intercept of 0.0237 ± 0.0003 and 0.566 ± 0.129, respectively (y, peak-area ratio; x, DEHP concentration, ng/ml). For MEHP measurements, the calibration curve was obtained for the peak-area ratio (MEHP/MEHP-d_4) versus the MEHP concentration (Fig. 1(b)). The curve was linear over the range of 1.0–200 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0541x - 0.0530$ ($r = 0.999$) with mean values for the slope and intercept of 0.0541 ± 0.0012 (mean ± S.D. (standard deviation)) and −0.0530 ± 0.0186, respectively (y, peak-area ratio; x, MEHP concentration, ng/ml). The recoveries of 20 ng/ml DEHP and MEHP fortified into EN-A (20%) were 91 ± 3.0 and 99 ± 3.0%, respectively ($n = 6$). The average and S.D. of background levels of DEHP and MEHP of the preparation of test solution were 5.5 ± 1.8 and less than 1.0 ng/ml, respectively ($n = 6$). The limits of quantification (LOQ) of DEHP and MEHP determined by the equation LOQ = background + S.D. × 5 were 15 and 5 ng/ml, respectively.

At preparation, the concentrations of DEHP and MEHP in the EN were 21.6–158 and <5–6.9 ng/ml, respectively (Table 3). After flowing the EN through the irrigator and catheter, we observed that DEHP and MEHP had leached from the medical products. Typical chromatograms of EN-A are shown in Fig. 2. The degree of DEHP leaching was dependant on the brand of EN; in contrast, MEHP leaching was approximately 20 ng/ml for all EN. The leaching of DEHP was not affected by the concentration of EN-A.

We calculated the exposure of a neonatal patient to DEHP from EN administration using the DEHP concentration in EN-A

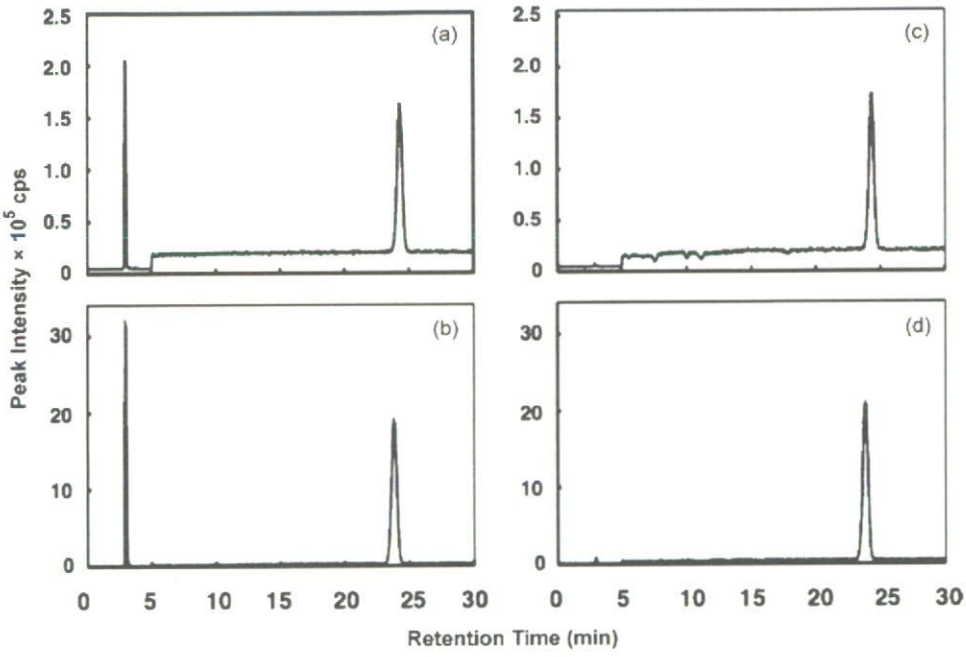

Fig. 2. DEHP and MEHP chromatograms. Retention times of MEHP and DEHP were 3.0 and 23.8 min, respectively. (a) Standard mixture 50 ng/ml; (b) standard mixture 1000 ng/ml; (c) EN-A (20%) at preparation; (d) EN-A (20%) after flowing through the irrigator and catheter.

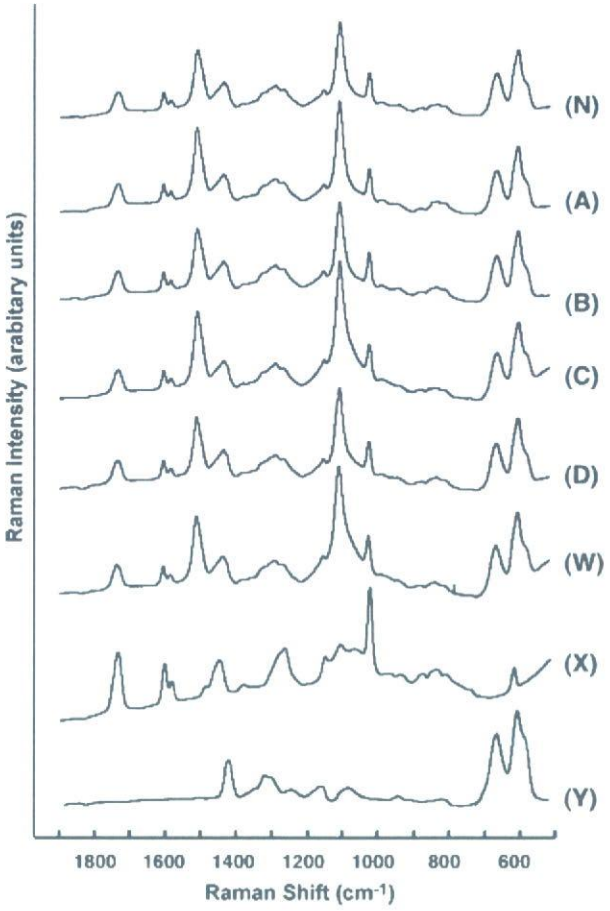

Fig. 3. Raman spectra of the internal surface of irrigator bags used for administration of EN under the conditions as described. (N) unused; (A) used for EN-A administration; (B) used for EN-B administration; (C) used for EN-C administration; (D) used for EN-D administration; (W) used for water administration; (X) DEHP standard; (Y) Pure PVC, which did not contain plasticizers including DEHP.

(20%) and the following equation:

$$Y\,(\mu g/(kg\,day)) = \frac{X\,(ng/ml) \times 392\,(ml/day)}{3\,(kg) \times 1000}$$

Where Y is the daily exposure per kg body weight and X is the concentration of DEHP in EN-A (20%).

In this case, the exposures to DEHP and MEHP from EN-A administration were calculated as 148 and 3.72 μg/(kg day), respectively. For the case of EN-B (17%), supplying the same calories (312 kcal/day) via EN administration, exposure to DEHP and MEHP was estimated to be 35.4 and 4.09 μg/(kg day), respectively.

When a standard-type irrigator and DEHP-free type catheter set were used, the leaching of DEHP and MEHP to EN-A (20%) was reduced to 795 ± 44 and 24.0 ± 3.1 ng/ml, respectively ($n = 3$). When a DEHP-free type irrigator and a standard-type catheter set were used, the leaching of DEHP and MEHP was reduced to 439 ± 19 and 17.3 ± 4.2 ng/ml, respectively ($n = 3$). When a DEHP-free type irrigator and DEHP-free type catheter set were used, leaching of DEHP and MEHP was not observed. Thus, in this case the exposures of neonatal patients to DEHP and MEHP can be reduced to a minimum level that can be calculated from the concentration at preparation. The exposure to DEHP and MEHP from EN-A (20%) administration was 6.48 and <0.653 μg/(kg day), respectively.

Raman spectroscopic analysis was conducted to examine the loss of DEHP at the internal surface of medical products. Fig. 3 shows the Raman spectra of the internal surface of the irrigator bags. The spectra consist of peaks derived from PVC (1429, 692 and 632 cm^{-1}), DEHP (1725, 1597, 1585, 1454, 1273 and 1037 cm^{-1}) and unknown components of the part (1505 and 1118 cm^{-1}). Significant differences were not found between the spectra of unused and used irrigators. The Raman intensity ratio (RIR) between 1725 and 692 cm^{-1} ($I_{peak\ 1725}/I_{peak\ 692}$) was calculated as an index of content ratio of DEHP to PVC at the internal surface. RIR of the irrigators were as follows: unused, 0.54; used for EN-A, 0.55; used for EN-B, 0.56; used for EN-C, 0.55; used for water, 0.55. Significant differences were also not found between the spectra of unused and used catheters (data not shown).

4. Discussion

To prevent adverse effects such as diarrhea, EN can be prepared in a range of concentrations appropriate to the physical condition of the patient. We observed that the lipid content of the EN was not a factor in promoting the leaching of DEHP; interestingly, leaching of DEHP from the irrigator and catheter was not affected by the concentration of EN-A. Some EN products incorporate an emulsifier to dissolve lipophilic components uniformly; lecithin and polysorbate 80 (PS80) are added to EN-A and EN-C, while lecithin alone is added to EN-D. The lecithin and PS80 exert emulsification in the ranges of appropriate concentration. Neither lecithin nor PS80 is added to EN-B, which recorded the lowest levels of DEHP leaching. Thus, we consider that the presence of an emulsifier in EN is an important factor that promotes leaching of DEHP from EN medical products. Hanawa et al. (2000) reported that the leaching of DEHP from medical products to saline reached a plateau with the addition of PS80 at around its critical micelle concentration; this finding corresponds with the observations of the present study. The leaching of DEHP from medical products to intravenous solutions is also promoted by the addition of lipophilic components and emulsifiers (Haishima et al., 2005). In these cases, emulsifiers would be potentially one of the most important factors in determining the level of leaching.

We observed that a small amount of MEHP leached from the medical products into all the ENs and Milli-Q water. MEHP is produced from DEHP in medical products by gamma ray irradiation or ethylene oxide gas treatment for sterilizing. MEHP detected in the present study would have been produced from DEHP in the medical products after sterilization with ethylene oxide gas (Ito et al., 2006). MEHP is more soluble in water than DEHP; thus, the components of the EN would not have affected the leaching of MEHP.

The daily exposure of a neonatal patient to DEHP by EN-A administration with the irrigator and catheter set was 148 μg/(kg day), which is close to the amount of 140 μg/(kg day) estimated by the FDA (FDA/CDRH, 2001). This exposure

level corresponds to the upper acceptable daily intake of DEHP (140 μg/(kg day)). Leaching of DEHP and MEHP from DEHP-free medical products was not observed; thus, by using DEHP-free type medical products, exposure to DEHP via EN administration can be reduced to the minimum level that is present in the EN at initial preparation. Recently, DEHP-free medical products have become widely used. It would seem reasonable that such products should be used for the population sensitive to the toxicity of DEHP and MEHP, including pregnant women, male neonatal patients, and infants; alternatively, medical products with substitute plasticizers such as tri(2-ethylhexyl)trimellitate (TOTM) and di(2-ethylhexyl)adipate (DEHA) should be used. The leachability of TOTM from haemodialysis tubes to blood is lower than that of DEHP (Flaminio et al., 1988b). The hepatic toxicity of TOTM is lower than that of DEHP (Hodgson, 1987; Kambia et al., 2004). The testicular toxicity of DEHA has not been observed in animal studies (Kang et al., 2006); however, there is much less information regarding the toxicities of these plasticizers compared to DEHP, and further investigation into these potential plasticizers is required to guarantee pregnant women and neonatal and infant patients against the risk associated with usage.

A Raman spectrometer is useful to determine the content of components in resin (Kikuchi et al., 2004; Norbygaard and Berg, 2004). Norbygaad and Berg successfully determined the content of phthalate diesters in PVC by a Raman spectrometer (Norbygaard and Berg, 2004). We utilized a Raman microspectrometer to examine the loss of DEHP at the internal surface of medical products. No obvious differences between the unused products and used products were found on the Raman spectra. Norbygaad and Berg determined the content of DEHP in PVC by the RIR at 1726 and 696 nm, which are independent and characteristic peaks of DEHP and PVC, respectively. In this study, the corresponding RIR of the internal surface of irrigator bags remained at approximately 0.5, whether they were unused or used. The main parts of the irrigator and catheter have a total content of DEHP corresponding to around 25% of their total weight of 47 g. Their total DEHP content is thus estimated to be 11.8 g. From the results described above, the leached DEHP to EN was estimated to be at most 59 μg, which is only 5.0×10^{-4}% of the total DEHP contained in those medical products. These observations suggest that the loss of DEHP would be too low to detect it by Raman spectroscopic changes even though at the surface, and/or DEHP corresponding to the loss by leaching to EN may be promptly supplied to the surface from the inside of the resin. Our results imply that used medical products have the potential to leach similar amounts of DEHP to EN to new products. Further studies to determine correlations of DEHP leaching from medical products with times of their use may be informative and may provide a basis for avoiding excessive exposure of patients to DEHP.

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報　文

高速液体クロマトグラフィー/タンデム型質量分析法によるヒト母乳中のフタル酸モノエステル類の分析

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フタル酸モノエステル類（PMEs）には，精巣毒性を有するものがあり，当毒性に感受性の高い胎児及び乳幼児への暴露が懸念されている．しかし，母乳中のPMEsに関する報告は少ない．そこで，高速液体クロマトグラフィー/タンデム型質量分析器を用いて母乳中のPMEs｛フタル酸モノエチル，フタル酸モノブチル，フタル酸モノベンジル，フタル酸モノ(2-エチルヘキシル)，フタル酸モノ(2-エチル-5-ヒドロキシヘキシル)及びフタル酸イソノニル｝の分析法を構築した．試料に内部標準を添加後，酢酸エチル/シクロヘキサン混液で抽出し，得られた有機相を乾固した．酢酸アンモニウム緩衝液に再溶解後，脱抱合反応を施し，固相抽出法により精製して試験液とした．PMEs（5.0 ng/mL添加）の回収率は，96.3～103%であった．本分析法を活用し，日本人の母乳について11検体を分析した結果，フタル酸モノ(2-エチル-5-ヒドロキシヘキシル)を除く5種類のPMEsを検出した．

1　緒　　言

ポリ塩化ビニル（PVC）は，優れた耐久性，絶縁性，加工性，透明性及び可塑性を有しており，シート，絶縁性品，建材，医療器具並びに履物等の生活雑貨に多く用いられている．PVCには，用途に応じた柔軟性を付与するために，可塑剤が添加されており，最も使用される可塑剤としてフタル酸ジエステル類（PDEs）が挙げられる[1,2]．PVC等の樹脂中に配合されたPDEsは，樹脂と共有結合していないため，樹脂中から容易に溶出又は気化し，樹脂と接触している飲食物，医薬品や空気中に移行する[3)–5)]．我が国で多用されているPDEsは，フタル酸ジ(2-エチルヘキシル)（DEHP），フタル酸ジイソノニル（DiNP），フタル酸ジソデシル及びフタル酸ジブチル（DBP）であり[2]，日本人は，日常的にこれらを中心としたPDEsに暴露されていると考えられる．

生体内に取り込まれたPDEsは，リパーゼ等の酵素により速やかに加水分解を受け，対応するフタル酸モノエステル類（PMEs）に変換されて血流中に移行する[6,7]．そのPMEsの一部は，側鎖の水酸化を受けた後，そのままPMEsとして，もしくはグルクロン酸抱合体として主に尿中に排泄される[6,7]．米国では尿中又は血清中のグルクロン酸抱合体を含むPMEsをPDEsの暴露指標として，ヒトに対する暴露評価が大規模に実施された[8,9]．その結果，血清及び尿中からPMEsが高頻度に検出され，ヒトが日常的にPDEsあるいはPMEsに暴露されていることが示唆された．著者らも健康な日本人の血清を分析し，数ng/mLのPMEsを検出している[10,11]．

DBP，DEHP及びそれらの代謝物であるフタル酸モノブチル（MBP），フタル酸モノ(2-エチルヘキシル)（MEHP）について，げっ歯類を用いた動物実験により，発生又は発育期にある精巣に対して毒性を示すことが明らかにされている[12)–14)]．ヒトに対する研究では，胎盤を用いたモデル実験により，妊娠期の母親がPDEsに暴露されたときに血流中へ移行したPMEsは，臍帯を介して胎児にも移行することが示唆されている[15]．更に，ヒトの臍帯血清中からMEHPが検出されており，ヒトは，胎児期からMEHPに暴露されていることが示唆されている[16]．このため，ヒトについても胎児及び乳幼児への影響が懸念されている．実際にヒトへの影響を示唆する報告もある．妊娠後期における母親の尿中のフタル酸モノエチル（MEP），MBP，フタル酸モノベンジル（MBzP）及びフタル酸モノイソブチルの濃度と，その母親から生まれた男児の肛門性器間距離の短縮（男性生殖器の発育不全の指標）との間には相関があると認められている[17]．これらのことから，発生又は発育期にある精巣への毒性を考慮し，妊婦及び胎児並びに男児

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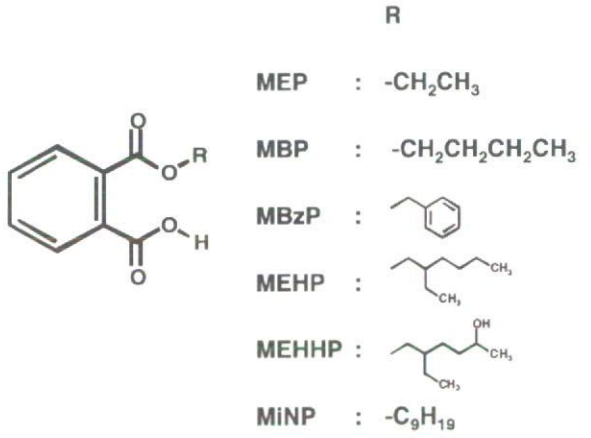

Fig. 1 Structures of phthalate monoesters examined in this study

Table 1 HPLC Conditions

Column	Symmetry C18, 100 × 2 mm, 3.5 μm (Waters)
Elution	Gradient mode
Flow rate	200 μL/min
Column temperature	40 ℃
Injection volume	5.0 μL

Gradient Program

Time/min	A, %	B, %	Profile
0	60	40	Linear
5	35	65	Step
5	0	100	Hold
10	0	100	

A: 2×10^{-4}% formic acid aqueous solution; B: 2×10^{-4}% formic acid containing acetonitrile

乳幼児は，PDEs及びPMEsの毒性に対する高感受性群と見なされており[18]，その暴露評価が求められている．

母乳は，乳幼児の主要な栄養源であり，その中に含まれるPMEsの量を明らかにすることは，乳幼児のPMEsの暴露量を把握するうえで重要である．最近，Mainらのグループは，ヒト母乳中のPMEsを分析し，高頻度でPMEsが，検出されることを報告している[19]．しかしながら，日本人の母乳中のPMEs濃度を報告した例は見当たらない．そこで，高速液体クロマトグラフィー/タンデム型質量分析器（LC/MS/MS）を用いて，母乳中の主要なPMEs（グルクロン酸抱合体を含む）の分析法を構築し，日本人の母乳11検体中の当該化学物質の濃度を測定したので報告する．

2 実　　験

2・1 試料及び試薬

試料：母乳は，1999年に11名の協力者から東海大学医学部産婦人科において，分娩後，4～5日経過した時点で採取された．当該母乳は，東海大学医学部の倫理規定に従って採取された．試料は，冷凍下で大阪府立公衆衛生研究所に移送後，分析時まで－40℃で保存された．

標準品：6種類のPMEs，すなわち，MEP，MBP，MBzP，MEHP，フタル酸モノ(2-エチル-5-ヒドロキシヘキシル)（MEHHP）及びフタル酸モノイソノニル（MiNP）を分析対象とした（Fig. 1）．MEP，MEP-$^{13}C_4$，MBP，MBP-$^{13}C_4$，MBzP，MBzP-$^{13}C_4$，MEHP，MEHP-$^{13}C_4$，MEHHP，MEHHP-$^{13}C_4$，MiNP及びMiNP-$^{13}C_4$の各100 μg/mL標準溶液は，Cambridge Isotope Laboratoriesより購入した．

標準溶液：標準混合原液（5 μg/mL）及び内部標準混合原液（5 μg/mL）は，上記標準溶液を，それぞれ20 mLのメスフラスコ中で混和し，アセトニトリルで定容して調製した．これら標準混合原液及び内部標準混合原液を混合し，25%アセトニトリル水溶液で希釈して標準溶液とした．各標準溶液には，内部標準10 ng/mLが等しく含まれる．

試薬：リン酸（HPLC用），ギ酸（HPLC用），リン酸二水素ナトリウム二水和物（特級），無水硫酸ナトリウム（残留農薬分析用），酢酸アンモニウム（特級），25%アンモニア水（精密分析用），酢酸エチル（残留農薬分析用），シクロヘキサン（残留農薬分析用），抽出用アセトニトリル（環境分析用），移動相用アセトニトリル（LC/MS用）及びβ-グルクロニダーゼ（*E. coli*由来；8.5 U/mL）は，和光純薬製を使用した．また，4-methylumbelliferone（4-MU）及び4-methylumbelliferyl glucuronide（4-MU-Glu）は，SIGMA製を用いた．超純水は，ミリポア製のMilli-Q SP. TOC. により精製したもの（Milli-Q水）をそのまま用いた．酸性リン酸塩緩衝液（2.5 mol/L）は，リン酸二水素ナトリウム二水和物2.18 g及びリン酸1 mLをMilli-Q水で溶解後，100 mLに定容して調製した．

器具等：PMEsのコンタミネーションを排除するため，加熱可能なガラス器具及び無水硫酸ナトリウムは，アセトン及びヘキサンで洗浄した後，ステンレス製容器中に移して200℃で2時間以上加熱し，ステンレス製容器中で冷却して用いた．

2・2 装置及び測定条件

HPLCは，Agilent 1100 seriesを用いた．HPLCの条件をTable 1に示した．MS/MSは，Applied Biosystems API3000を用いた．インターフェイスには，electrospray ionization法を用いた．キャピラリー電圧及び温度は，－4500 V及び450℃とした．測定は，multiple reaction monitoring（MRM）で行った．MRM条件は，100 ng/mLの各標準溶液及び内部標準溶液をシリンジポンプで10 μL/minでMS/MSに導入し，Analyst 1.3.2で最適化した．

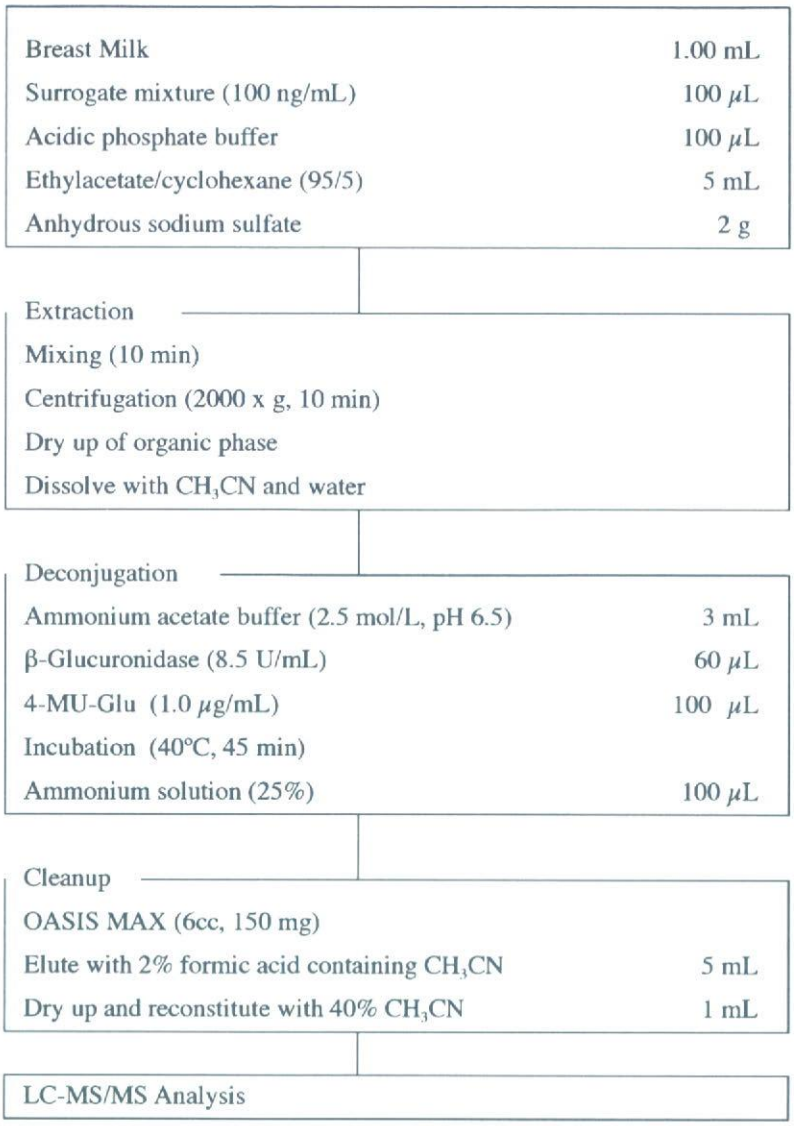

Fig. 2　Sample preparation for determination of phthalate monoesters in human breast milk

2・3　前処理法

概要を Fig. 2 に示した．解凍後，直ちに母乳 1.00 mL に 100 ng/mL 内部標準混合溶液 100 μL，2.5 mol/L 酸性リン酸塩緩衝液 100 μL，酢酸エチル/シクロヘキサン（v/v: 95/5）5 mL 及び無水硫酸ナトリウム 2 g を添加して 10 分間撹拌した[20]．遠心分離（2000 × g，10 分間）後，有機相を回収し，窒素気流下で乾固した．これにアセトニトリル 50 μL，Milli-Q 水 1 mL の順で添加して溶解した．次に 2.5 mol/L 酢酸アンモニウム緩衝液（pH 6.5）3 mL，8.5 U/mL β-グルクロニダーゼ 60 μL 及び 1.0 μg/mL 4-MU-Glu 100 μL を添加して撹拌後，40℃ で 45 分間インキュベートした．インキュベート後，氷上に移し，25% アンモニア水溶液を添加した．全量をあらかじめアセトニトリル 15 mL 及び Milli-Q 水 5 mL でコンディショニングした OASIS MAX カートリッジカラム（6 cc，150 mg; Waters）に負荷した．カラムを Milli-Q 水 5 mL 及びアセトニトリル 10 mL で洗浄し，2% ギ酸含有アセトニトリル 5 mL で溶出した．溶出液は，窒素気流下，40℃ で乾固した後，40% アセトニトリル含有水 1 mL に再溶解して試験液とした．なお，操作過程での PMEs のコンタミネーションを把握するため，あらかじめ PMEs が検出されないことを確認したヘキサン洗浄水を空試験試料として併行処理し，試料と同様に分析した．

3　結果及び考察

3・1　分析条件の最適化

分析対象とした PMEs について，0.001% ギ酸存在下，ネガティブモードで良好なイオン化が認められた．また，4-MU 及び 4-MU-Glu については，ポジティブモードで良好なイオン化が認められた．代表例として，MiNP の MRM スペクトラムを示した（Fig. 3）．MiNP の構造を反映したスペクトルが認められ，ベースピーク $[M-H]^-$

($m/z = 291$) に対して，ピーク強度比が最も大きいフラグメント ($m/z = 141$) をモニタリングイオンとした．同様に他の PMEs についてもモニタリングイオンを定め，その諸条件を Table 2 に示した．

移動相に添加するギ酸の量を最適化するため，50% アセトニトリル水溶液にギ酸を添加したものを移動相として，各 20 ng/mL PMEs を含む標準溶液を MS/MS にフローインジェクション方式で導入した．Fig. 4 に移動相に含まれるギ酸濃度とピーク面積の関係を示した．その結果，2.0×10^{-4}% ギ酸添加時に感度が最も良好になることが分かった．また，相対検量線は，シグナル/ノイズ比が 10 を越える 0.2 から 50 ng/mL まで直線性が認められた．それら検量線は，以下のとおりであった {x: 標準品濃度 (ng/mL)，y: ピーク面積比 (標準品/内部標準)}．MEP: $y = 0.129x - 0.0001$ (r^2, 1.000)；MBP: $y = 0.0814x + 0.0083$ (r^2, 0.999)；MBzP: $y = 0.0341x + 0.0008$ (r^2, 1.000)；MEHP: $y = 0.0658x + 0.0040$ (r^2, 1.000)；MEHHP: $y = 0.0650x + 0.0020$ (r^2, 1.000)；MiNP: $y = 0.1640x - 0.0107$ (r^2, 0.998)．

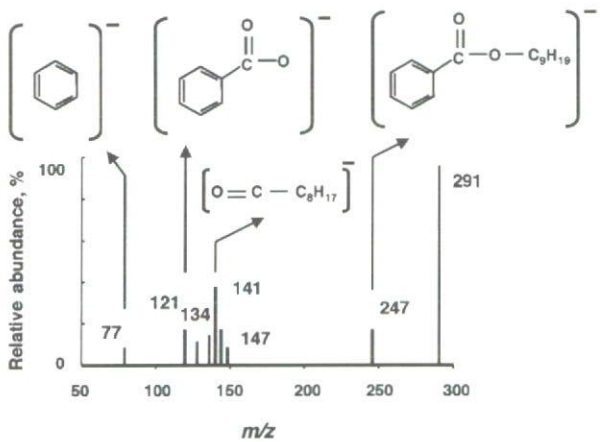

Fig. 3 MRM spectrum of MiNP

3・2 前処理法

母乳は，酸性条件下で有機溶媒を作用させて脂肪球を破壊し，同時にタンパク質を変性除去した．次に酢酸アンモニウム緩衝液中で β-グルクロニダーゼを作用させた．酵素の作用時間については，PMEs のグルクロン酸抱合体標品の入手，並びに検討に使用できる十分量の母乳の確保が困難なため，4-MU-Glu の脱抱合を指標として検討した．その結果，酵素の作用時間は，4-MU-Glu が完全に消失して 4-MU の生成がプラトーに達する 45 分間とした．また，クリーンアップには，逆相と陰イオン交換能を有する OASIS MAX を用いた．試料を塩基性条件下でカラムに負荷し，Milli-Q 水及びアセトニトリルで洗浄後，PMEs，4-MU-Glu 及び 4-MU が溶出する 2% ギ酸含有アセトニトリル 5 mL で溶出することとした．

3・3 定量下限

PMEs についても PDEs と同様に実験環境から試料中へのコンタミネーションが危惧される．このため，試薬及び分析操作過程から混入する PMEs 濃度 (バックグラウンド値) を把握することが不可欠である．このため母乳に替えて，あらかじめ PMEs が含まれないことを確認したヘキサン洗浄水を試料として同様の操作を行い，調製した試験液中から検出される PMEs 濃度をバックグラウンド値とした．MEP，MBzP，MEHHP 及び MiNP のバックグラウンド値は，0.2 ng/mL 未満であった．MBP 及び MEHP のバックグラウンドは，それぞれ，0.25 ± 0.09 及び 2.33 ± 0.06 ng/mL であった．MBP 及び MEHP の定量下限をバックグラウンド値＋標準偏差×5 に定め，ぞれぞれ 0.7 及び 2.7 ng/mL とした．

Table 2 MRM conditions

Compounds	MRM transition/m/z	CE[a]/eV	RT[b]/min
MEP	−193 > −121	−16	1.6
MEP-$^{13}C_4$	−197 > −79	−26	1.6
MBP	−221 > −77	−26	2.8
MBP-$^{13}C_4$	−225 > −79	−24	2.8
MBzP	−255 > −77	−32	2.9
MBzP-$^{13}C_4$	−259 > −107	−20	2.9
MEHP	−277 > −134	−24	7.1
MEHP-$^{13}C_4$	−281 > −137	−24	7.1
MEHHP	−293 > −121	−30	2.5
MEHHP-$^{13}C_4$	−297 > −145	−20	2.5
MiNP	−291 > −141	−26	7.6
MiNP-$^{13}C_4$	−295 > −79	−36	7.6
4-MU	+177 > +77	+49	1.3
4-MU-Glu	+353 > +177	+23	1.0

a) collision energy; b) retention time

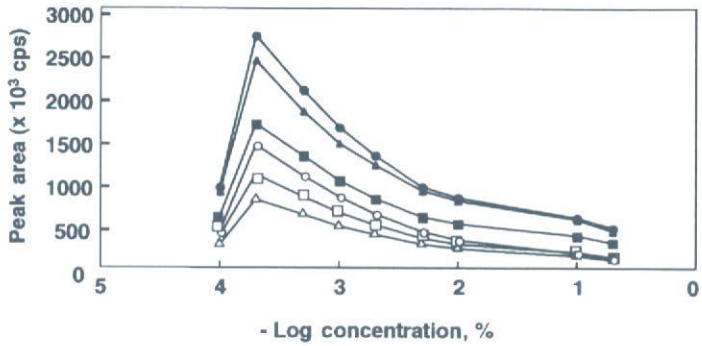

Fig. 4　Effects of formic acid in eluent on peak area of MPEs
○: MEP; □: MBP; △: MBzP; ●: MEHP; ▲: MEHHP; ■: MiNP. Points represent the average of three experiments

Table 3　Means of recoveries and relative standard deviations (RSD) of PMEs from consumer milk

PMEs	Fortified concentration			
	5.0 ng/mL		50 ng/mL	
	Recovery, %	RSD	Recovery, %	RSD
MEP	103	(1.3)	103	(0.1)
MBP	96.3	(4.8)	103	(2.4)
MBzP	101	(3.0)	105	(1.0)
MEHP	99.8	(3.0)	106	(0.4)
MEHHP	101	(1.7)	106	(3.2)
MiNP	102	(3.1)	104	(0.2)

$n = 5$

3・4　添加回収試験

市販の牛乳に PMEs を終濃度が 5.0 及び 50 ng/mL になるように添加したものを試料として添加回収試験を行った．試行数は 5 とした．5.0 及び 50 ng/mL 添加の双方において，良好な回収率（96.3～106%）及び相対標準偏差（5% 未満）を示した（Table 3）．

3・5　日本人の母乳中の PMEs 濃度

分析した日本人の母乳（11 検体）のすべてから，MEP，MBP，MBzP，MEHP 及び MiNP を検出した．MEHP の側鎖が水酸化された代謝物である MEHHP については，1 検体のみから検出された．代表的なクロマトグラムを Fig. 5 に示した．本研究での母乳の分析結果と Main らによるデンマーク及びフィンランドでの分析結果[19]を Table 4 において比較した．MEP 及び MBzP については，日本とデンマーク及びフィンランドの三者で，その中央値は類似した値であった．一方，MBP 及び MEHP の中央値は，日本人の母乳のほうが同程度もしくはやや高い傾向にあると推察された．一方，MiNP の濃度については，日本人の母乳では中央値が 1.2 ng/mL であるのに対して，デンマーク及びフィンランドでは，それぞれ，101 及び 89 ng/mL であり，日本人の母乳と比較して高値を示した．このような差違が認められたことは，PDEs 及び PMEs の動態を研究するうえで興味深い知見である．デンマーク及びフィンランドを含む欧州連合（EU）では，MiNP の親化合物である DiNP の安全性が示されている[21]が，DBP 及び DEHP は，

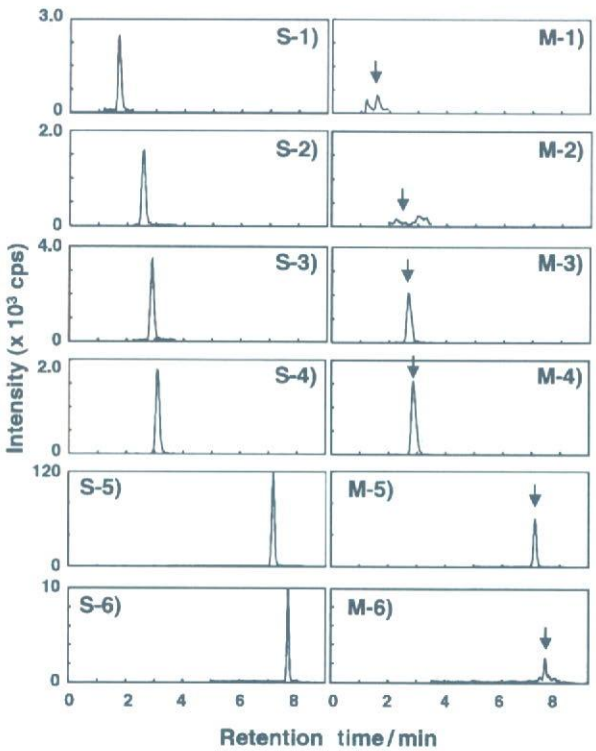

Fig. 5　Typical MRM chromatograms of standard solution (S) and test solution prepared from human breast milk sample (M)
S-1) MEP, 1 ng/mL; M-1) MEP in human breast milk; S-2) MEHHP, 1 ng/mL; M-2) MEHHP in human breast milk (not detected); S-3) MBP, 5 ng/mL; M-3) MBP in human breast milk; S-4) MBzP, 1 ng/mL; M-4) MBzP in human breast milk; S-5) MEHP, 50 ng/mL; M-5) MEHP in human breast milk; S-6) MiNP, 5 ng/mL; M-6) MiNP in human breast milk

Table 4 PMEs in human breast milk (ng/mL)

PMEs	Japan (n = 11; This study)		Denmark (n = 65)[19]		Finland (n = 65)[19]	
	Median	Range	Median	Range	Median	Range
MEP	0.5	0.3 ~ 1.3	0.93	0.07 ~ 33.6	0.97	0.25 ~ 41.4
MBP	26.0	1.8 ~ 156	4.3	0.60 ~ 10900	12	2.4 ~ 123
MBzP	1.0	0.7 ~ 74.3	0.9	0.2 ~ 14.0	1.3	0.4 ~ 26
MEHP	27.9	4.4 ~ 129	9.5	1.5 ~ 191	13	4.0 ~ 1410
MEHHP	<0.2	<0.2 ~ 0.2	ND[a]		ND[a]	
MiNP	1.2	0.7 ~ 8.7	101	27 ~ 469	89	28 ~ 230

a) not detected

生殖毒性を有する化学物質として流通に制限が設けられている[22][23]．EUでのDiNPとDEHPに対する安全性評価の差が，これらの国の母乳中でMiNPがMEHPよりも高濃度で検出される理由のひとつとして推察される．

本分析結果に基づいて，乳児の母乳中からのPMEsの摂取量を見積もった．乳児の体重は4 kgとし，中央値の濃度の各PMEsを含む母乳を1日当たり600 mL摂取したケースを想定して，PMEsの体重1 kg当たりの1日摂取量を以下のとおり算出した．MEP: 0.075; MBP: 3.9; MBzP: 0.15; MEHP: 4.2; MEHHP: <0.030; MiNP: 0.18 (μg/kg/day)．DEHPの1日耐容摂取量（TDI）は，37～140 μg/kg/dayである．DEHPの毒性とMEHPの毒性が等しいとの仮想のもと，これを分子量に基づき，MEHPのTDIとして換算すると，26～100 μg/kg/dayとなる．当該母乳からのMEHPの摂取量（4.2 μg/kg/day）は，この仮想のTDIを下回る．また，最大検出濃度を想定した場合についても，MEHPの摂取量は19.4 μg/kg/dayであり，仮想のTDIを下回る．

4 結　　言

著者らは，母乳中のPMEsの分析法を構築した．また，本法を用いて日本人の母乳のPMEs濃度とその特徴を明らかにした．母乳は，乳幼児の主要な栄養源であることから，本分析法は乳幼児のPMEsの暴露量の解析に役立つものと考えられる．今後，分析例を増やすとともに，母体血清中のPMEsの濃度を併せて分析し，母乳中のPMEsの濃度との関係を明らかにすることも必要であろう．現在，母体血清中のPMEs分析法を構築し，実試料の分析を進めている．

本研究は，厚生労働科学研究費による助成を受けて実施された．母乳を提供していただいた協力者の方々に深謝します．

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Determination of Phthalate Monoesters in Human Breast Milk by High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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Phthalate diesters (PDEs) are ubiquitous contaminants in our life. Phthalate monoesters (PMEs) and their glucuronide conjugates are common metabolites of PDEs, which are detectable in sera and urine of humans exposed to PDEs. In animal studies, some PDEs and PMEs are toxicants to the reproductive and developmental systems. Thus, studies about exposure to PDEs and PMEs in human fetus and infants are required. Breast milk is one of the most important resources of nutrition to infants. However, there is significant information about the concentrations of PMEs in human breast milk. We developed a method to determine PMEs in human breast milk by using a liquid chromatograph-tandem mass spectrometer. Breast milk was extracted with ethylacetate/cyclohexane under an acidic condition. The organic phase was dried under an N_2 stream and dissolved in an acetic ammonium buffer. The extract was processed using enzymatic deconjugation of the glucuronidase, followed by solid-phase purification to obtain a test solution. The recoveries and relative standard deviations of PMEs (5.0 ng/mL) fortified in consumer milk were satisfactory, which were 96.3～103% and less than 5%, respectively. Eleven human milk samples obtained from Japanese women were examined. Monoethylphthalate (median; range, 0.5; 0.3～1.3 ng/mL), monobutylphthalate (26.0; 1.8～156), monobenzylphthalate (1.0; 0.7～74.3), monoethylhexylphthalate (27.9; 4.4～129) and monoisononylphthalate (1.2; 0.7～8.7) were detected from all of the samples. This method would be useful for the determination of PMEs in human breast milk.

Keywords: phthalate monoester; breast milk; LC/MS/MS.

DIETARY INTAKE ESTIMATIONS OF POLYBROMINATED DIPHENYL ETHERS BASED ON A TOTAL DIET STUDY IN OSAKA, JAPAN

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Abstract

This study presents the results of a total diet study (TDS) performed for estimating the dietary intake of polybrominated diphenyl ethers (PBDEs) in Osaka, Japan. The concentrations of 36 PBDEs were measured in 14 TDS food group samples (groups I–XIV). PBDEs were detected only in groups IV (oils and fats), V (legumes and their products), X (fish, shellfish, and their products), and XI (meat and eggs) at the concentrations of 1.8, 0.03, 0.48, and 0.01 $ng \cdot g^{-1}$, respectively. For an average adult, we estimated the lower bound dietary intake of penta- and decaBDEs (sum of tri- and nona- through hexabrominated and decabrominated congeners, respectively) to be 46 and 21 $ng \cdot d^{-1}$, respectively (assuming ND = 0). PentaBDE constituents were dominant in groups V, X, and XI. In contrast, we observed a high proportion of DeBDE-209 in group IV. To confirm the presence of DeBDE-209 in vegetable oils, we performed an additional analysis using 18 vegetable oil samples; of these, 7 contained DeBDE-209 at the ppb level. Further studies are required to reveal the pathways of oil contamination and to examine the formation of toxic polybrominated dibenzo-*p*-dioxins/dibenzofurans (PBDDs/PBDFs) from PBDEs under specific cooking conditions.

Introduction

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in a variety of consumer products. PBDEs are persistent and lipophilic in nature, and thus they accumulate in the food chain. Fish and other fatty foods have been recognized as sources of human contamination with PBDEs. We have reported the PBDE levels detected in marine fish[1] and dietary supplements[2] from samples collected in Japan. Other researchers have also reported the PBDE levels in a variety of Japanese food items[3–5]. However, human exposure to PBDEs has not been sufficiently documented through a total diet study (TDS) in Japan. This study presents the results of a local-scale TDS performed for estimating the dietary intake of PBDEs. Further, the results of an additional analysis of 18 vegetable oil samples has been discussed.

Materials and Methods

Sample collection: A total of 125 food samples were purchased from 2 supermarkets in Osaka in 2006. TDS samples were prepared based on the official food classification and consumption data obtained from the National Nutrition Survey, which was conducted by the Ministry of Health and Welfare of Japan from 2001 to 2003. These food samples were cooked or prepared for consumption in a typical manner and blended to form 14 food group composites. These food groups were designated as groups I–XIV as shown in Table 1. In addition, 18 bottled vegetable oil samples obtained from rapeseed, corn, safflower, sesame, olive, and soybean were purchased from 2 supermarkets in Osaka in 2006 (Table 2).

Chemicals: Standard solutions of PBDEs were purchased from AccuStandard (New Haven, CT, USA) and Wellington Laboratories (Ontario, Canada). In this study, 36 PBDE congeners having 3–10 bromine atoms were monitored. PBDE numbers were assigned according to the IUPAC PCB nomenclature. Acetone, acetonitrile, and *n*-hexane of pesticide analysis grade and 44% sulfuric acid-impregnated silica gel and *n*-nonane of dioxin analysis grade were purchased from Wako Pure Chemicals (Osaka, Japan).

PBDE measurements: The TDS sample was digested with 1 $mol \cdot L^{-1}$ aqueous KOH for 2 h at room temperature after adding $^{13}C_{12}$-labeled surrogate standards. Subsequently, alkaline hydrolysate was extracted

twice with *n*-hexane. The extract was purified with sulfuric acid-impregnated silica gel by using *n*-hexane as an eluent. The eluate was concentrated and then spiked with a $^{13}C_{12}$-labeled injection standard. The additional vegetable oil samples were diluted with *n*-hexane after adding $^{13}C_{12}$-labeled surrogate standards and partitioned 3 times with *n*-hexane-saturated acetonitrile. The acetonitrile phase was combined and evaporated to dryness. The residue was treated with the sulfuric acid-impregnated silica gel and then spiked with the $^{13}C_{12}$-labeled injection standard. The cleaned extract was assayed with a gas chromatography/mass spectrometry (GC/MS) system. Quantitation was based on an isotope dilution method by using $^{13}C_{12}$-labeled internal standards. The mean percent recovery of most PBDEs ranged from 80% to 110%. The limit of detection (LOD) for all the PBDE congeners ranged from 0.01 to 0.1 ng·g^{-1}.

Results and Discussion

Example chromatograms of the standard solution and TDS samples are shown in Fig. 1. The PBDE concentrations in the TDS samples are shown in Fig. 2A. PBDEs were detected only in food groups IV (oils and fats), V (legumes and their products), X (fish, shellfish, and their products), and XI (meat and eggs) at the concentrations of 1.8, 0.03, 0.48, and 0.01 ng·g^{-1}, respectively. Fig. 2B reveals the lower bound intake of PBDEs (assuming ND = 0). For an average adult, we estimated the lower bound dietary intakes of pentaBDE (sum of 26 PBDEs: #17, #25, #28, #30, #32, #33, #35, #37, #47, #49, #66, #71, #75, #77, #85, #99, #100, #116, #118, #119, #126, #138, #153, #154, #155, and #166) and decaBDE (sum of 4 PBDEs: #206, #207, #208, and #209) to be 46 and 21 ng·d^{-1}, respectively, and the middle- (ND = 1/2 LOD) and upper bound intakes (ND = LOD) to be 330 and 610 ng·d^{-1} for pentaBDE and 310 and 600 ng·d^{-1} for decaBDE, respectively. Comparison data of the estimated dietary intake of PBDEs in different countries are shown in Table. 3. The lower bound PBDE dietary intake values estimated in this study were almost comparable to

Table 1. Information of 14 food groups of the total diet study in Osaka, 2006

Group No.	Food composition	Food variety (No. of food items)	Lipid content (%)	Daily intake per capita (g·d^{-1})
I	Rice and rice products	2	0.28	334
II	Grains, seeds, and tubers	15	2.9	179
III	Sugar and confectioneries	6	20	34.2
IV	Oils and fats	4	93	11.4
V	Legumes and their products	6	8.2	53.8
VI	Fruits	11	0.16	123
VII	Brightly colored vegetables	13	0.29	100
VIII	Other vegetables, mushrooms, and seaweeds	13	0.28	184
IX	Beverages	7	0.01	577
X	Fish, shellfish, and their products	23	7.7	89.4
XI	Meat and eggs	8	18	124
XII	Milk and dairy products	5	6.7	157
XIII	Seasonings and other processed foods	11	8.8	91.3
XIV	Drinking water	1	0	250

Daily intake was estimated for an average adult consumer in Osaka based on the reports of National Nutrition Survey, 2001 to 2003.

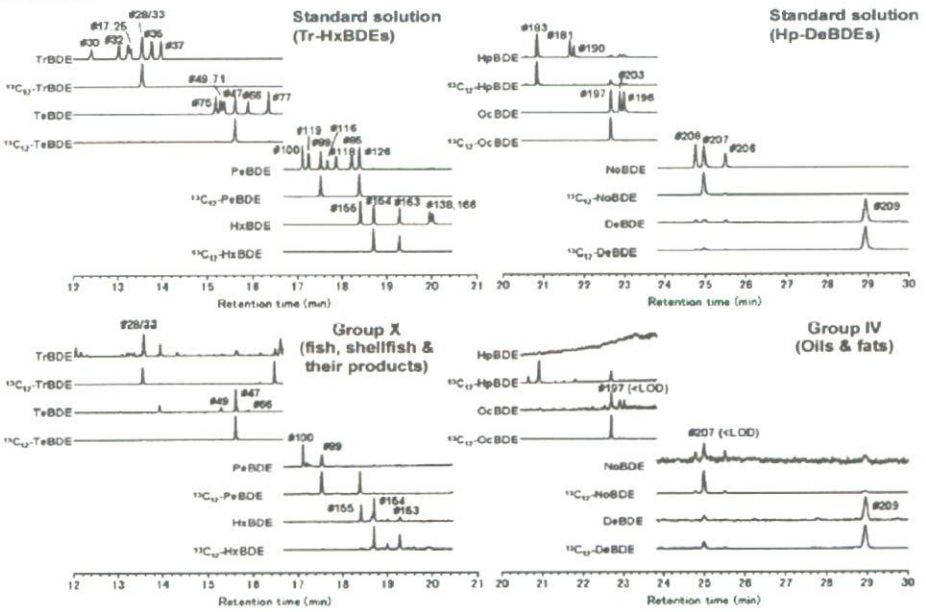

Figure 1. Example chromatograms of standard solution and food samples

those reported in the UK (91 ng·d^{-1})[6], USA (88 ng·d^{-1})[7], Spain (82 ng·d^{-1})[8], Japan (68 ng·d^{-1})[4], Sweden (51 ng·d^{-1}) (middle bound)[9], and Belgium (23 ng·d^{-1})[10]. Assuming that a typical Japanese adult weighs merely 50 kg, the lower, middle, and upper bound intakes were estimated to be 0.0009, 0.007, and 0.012 μg·kg^{-1}·d^{-1} for pentaBDE and 0.0004, 0.006, and 0.012 μg·kg^{-1}·d^{-1} for decaBDE, respectively. These values were 2–5 orders of magnitude lower than the reference doses of penta- and decaBDE (2 and 10 μg·kg^{-1}·d^{-1}, respectively), both of which were

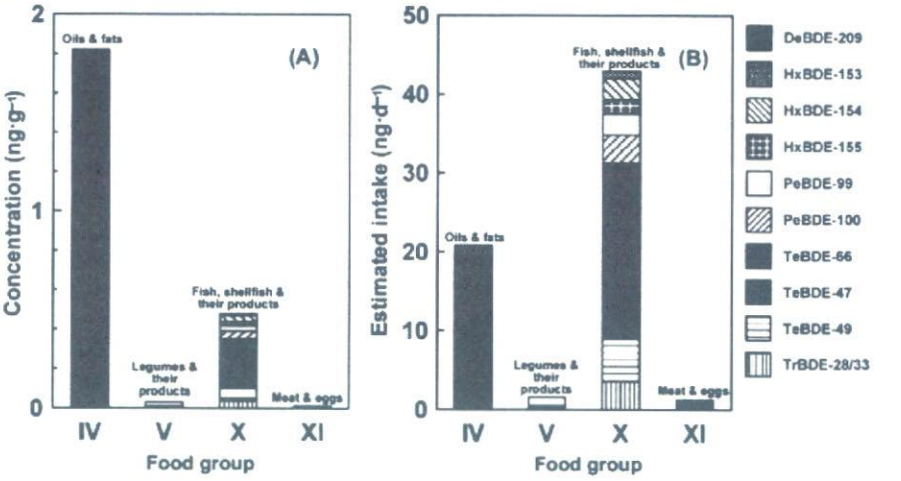

Fig. 2 Concentrations of PBDEs in foods (A) and estimated daily intakes of PBDEs from foods (B)

Table 2. Information of vegetable oil samples

No.	Oil composition	Country of origin	Bottle material	JAS certified
1	Rapeseed oil	NA	PET	No
2	Rapeseed oil	NA	PET	Yes
3	Rapeseed oil	NA	PE, EVOH	Yes
4	Rapeseed oil	Japan	PET	No
5	Rapeseed oil	Japan	Glass	No
6	Rapeseed oil	Japan	Glass	No
7	Brended oil (rapeseed, soybean)	NA	PE, EVOH	Yes
8	Brended oil (rapeseed, soybean)	NA	PE, EVOH	Yes
9	Corn oil	NA	PET	Yes
10	Corn oil	NA	PET	No
11	Corn oil	NA	PE, EVOH	No
12	Safflower oil	NA	PET	Yes
13	Safflower oil	NA	PET	Yes
14	Sesame oil	NA	Glass	No
15	Sesame oil	NA	Glass	Yes
16	Brended oil (perilla, sesame)	NA	Glass	No
17	Olive oil	Spain	Glass	No
18	Brended oil (unspecified)	NA	PET	No

Abbreviation: NA, not available; PET, polyethylene terephthalate; PE, polyethylene; EVOH, ethylene-vinyl alchol copolymer; JAS, Japanese Agricultural Standard

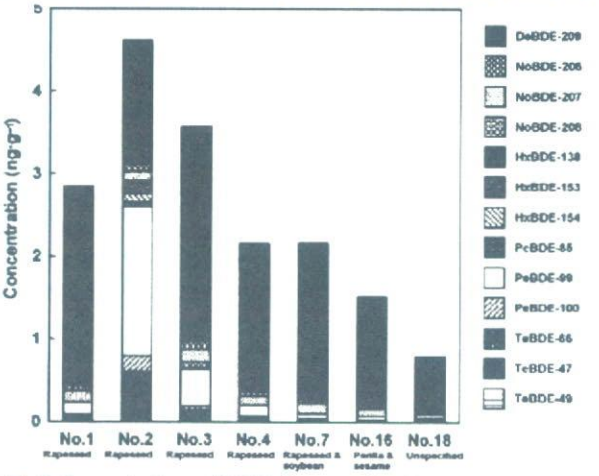

Fig 3. Concentrations of PBDEs in vegetable oil samples

Table 3. Comparison of estimated dietary intake of PBDEs in defferent countries

Country	Daily intake per capita (ng·d^{-1})*			Sampling year	Target congeners	Reference
	Lowerbound	Middlebound	Upperbound			
UK	91	–	–	1999–2000	47, 99, 100, 153, 154	Harrad et al., 2004
USA	88**	–	–	2003–2004	17, 28, 47, 66, 77, 85, 99, 100, 138, 153, 154, 183, and 209	Schecter et al., 2006
Spain	82	97		2000	Tetra- through octabrominated congeners	Bocio et al., 2003
Japan	–	94	–	1995	47, 99, 100, and 153	Wada et al., 2005
Japan	68	–	–	–	47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, and 183	Ashizuka et al., 2004
Sweden		51		1999	47, 99, 100, 153, 154	Darnerud et al., 2001
Belgium	23	35	48	2005	28, 47, 99, 100, 153, 154, and 183	Voorspoels et al., 2007
Japan	46	330	610	2006	PentaBDE (17, 25, 28, 30, 32, 33, 35, 37, 47, 49, 66, 71, 75, 77, 85, 99, 100, 116, 118, 119, 126, 138, 153, 154, 155, and 166)	This study
Japan	21	310	600	2006	DecaBDE (206, 207, 208, and 209)	This study

*Lower, middle, and upper bound intakes were estimated by assuming the nondetect values as zero, one half of the detection limit, and the detection limit, respectively. **The intakes were estimated for 70 kg males aged 20–39 years.

proposed by the US Environmental Protection Agency. These results suggested that the dietary exposure to PBDEs was not serious in Japan as well as in the other reported countries.

PentaBDE constituents such as TeBDE-47 and PeBDE-99 were dominant in food groups V, X, and XI. In contrast, a high proportion of DeBDE-209, a major constituent of decaBDE, was observed in the group IV food samples, which mainly consisted of vegetable oils (Fig. 2A). To confirm the presence of DeBDE-209 in vegetable oils, we performed an additional analysis using individual oil samples obtained from rapeseed, corn, safflower, sesame, olive, and soybean. We observed that 7 out of the 18 oil samples contained DeBDE-209 as a major or secondary dominant congener at approximately the ppb level (0.7–2.6 $ng \cdot g^{-1}$, Fig. 3). These results partially explained the reason for the high proportion of DeBDE-209 found in the group IV food samples. Sample No. 2 was the most contaminated rapeseed oil, and it contained TeBDE-47, PeBDE-99, and DeBDE-209 at the concentrations of 0.59, 1.8, and 1.5 $ng \cdot g^{-1}$, respectively. The results indicated that this vegetable oil sample was contaminated with both decaBDE and pentaBDE. The contamination may have occurred during the oil manufacturing processes. Another possible pathway of contamination involved the absorption and adsorption of PBDEs by the original farm plants during their growth processes. Mueller et al. reported that both radish (*Raphanus sativus L.*) and summer squash (*Cucurbita pepo L.*) absorbed pentaBDE from contaminated soil in a model experiment[11]. Thus, farm plants probably absorb a part of the PBDEs from contaminated soil. Hale et al. reported that 11 biosolid fertilizer (recycled sewage sludge) samples that were collected from different regions in the US all contained high concentrations of penta- and decaBDE (1100–2290 and 84.8–4890 $ng \cdot g^{-1}$ dry weight, respectively)[12]. The land application of biosolids may increase PBDE levels in farm plants and their products. However, the relationship between PBDE levels in plants and those in soils has not been sufficiently documented. In addition, it is known that considerable amounts of polybrominated dibenzo-p-dioxins/dibenzofurans (PBDDs/PBDFs) can be formed from PBDEs under thermal stress conditions[13]. Further studies are required to reveal the pathways of oil contamination and to examine the formation of toxic PBDDs/PBDFs from PBDEs in heated vegetable oils under specific cooking conditions.

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POLYBROMINATED DIPHENYL ETHERS IN HUMAN SERUM AND SPERM QUALITY

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Abstract

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in different types of consumer products. PBDEs are now ubiquitous environmental contaminants. Several studies have indicated that PBDEs may affect male fertility. We present the results of a pilot study on the relationship between human serum PBDEs and sperm quality. Serum and sperm samples from 10 healthy Japanese males aged 18–22 years were obtained in St. Marianna University. The PBDE concentrations in the serum samples were determined using gas chromatography/mass spectrometry. Four PBDE congeners (2,2′,4,4′-tetrabromodiphenyl ether (TeBDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (PeBDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (PeBDE-100), and 2,2′,4,4′,5,5′-hexabromodiphenyl ether (HxBDE-153)) were quantified in all serum samples. The median levels of the individual PBDE congeners were 1.4 ng·g^{-1} lipid weight (lw), TeBDE-47; 0.21 ng·g^{-1} lw, PeBDE-99; 0.24 ng·g^{-1} lw, PeBDE-100; and 0.72 ng·g^{-1} lw, HxBDE-153. These levels are comparable to those found in European countries. Clear inverse correlations were observed between the serum HxBDE-153 concentration and sperm concentration (r = –0.838, p = 0.002) and testis size (r = –0.764, p = 0.01). However, the serum concentrations of the other 3 congeners did not correlate with sperm concentration or testis size. Extensive studies on the relationship between PBDEs and sperm quality are required.

Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in the production of common consumer products. PBDEs are now ubiquitous and persistent environmental contaminants, and they have been detected in human tissues. Because PBDEs have some structural similarity to thyroid hormones such as thyroxine (T4), it was speculated that PBDEs may mimic thyroid hormones and may disrupt thyroid homeostasis. Several studies indicate that exposure to PBDEs can reduce circulating levels of T4 in laboratory animals[1] and can cause permanent neurological effects similar to those associated with thyroid hormone deficiencies[2]. In addition, several PBDEs possess weak estrogenic/antiestrogenic activities[3]. The proliferation and differentiation of Sertoli cells and sperm production are regulated by thyroid and sexual hormones. Thus, PBDEs may affect male reproductive health by interfering with thyroid and sexual hormone function. Kuriyama et al. have reported that developmental exposure to a single low dose (60 μg·kg^{-1} body weight) of 2,2′,4,4′,5-pentabromodiphenyl ether (PeBDE-99) decreased sperm counts in male Wistar rats[4]. However, no previous studies have examined the relationship between human PBDE levels and sperm quality. We participated in the international project examining the sperm quality of fertile men and found that the sperm concentration of Japanese men was lower than that of European men[5]. The examination of sperm quality and the estimation of the concentration of chemicals in the serum would be required to reveal the correlation between the sperm quality of Japanese men and their exposure to chemicals. The aim of this pilot study was to measure PBDEs in serum samples from Japanese young males and to examine the relationship between serum PBDE levels and sperm quality.

Materials and Methods

Sample collection: Blood serum and sperm samples were collected monthly from 45 young Japanese males at the Department of Urology, St. Marianna University School of Medicine, in 2003. The men were asked to remain abstinent for at least 48 h before sperm collection. The blood samples were collected in vacuum tubes, and the serum fractions were separated by centrifugation. Serum samples were stored at –80°C until analysis. Of the 45 sample sets, 10 sample sets were randomly chosen for this study. For PBDE analysis, 10 pooled serum