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Determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in human serum using liquid chromatography-tandem mass spectrometry

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Abstract

Concentrations of mono(2-ethylhexyl)phthalate (MEHP), and di(2-ethylhexyl)phthalate (DEHP), in serum of healthy volunteers were determined by high performance liquid chromatography (HPLC) with tandem mass spectrometry (LC/MS/MS). The serum was extracted with acetone, followed by hexane extraction under acidic conditions, and then applied to the LC/MS/MS. Recoveries of 20 ng/ml of MEHP and DEHP were 101 ± 5.7 ($n = 6$) and $102 \pm 6.5\%$ ($n = 6$), respectively. The limits of quantification (LOQ) of MEHP and DEHP in the method were 5.0 and 14.0 ng/ml, respectively. The concentration of MEHP in the serum was at or less than the LOQ. The concentration of DEHP in the serum was less than the LOQ. Contaminations of MEHP and DEHP from experimental reagents, apparatus and air during the procedure were less than the LOQ and were estimated to be <1.0 and 2.2 ± 0.6 ng/ml, respectively. After subtraction of the contamination, the net concentrations of MEHP and DEHP in the serum were estimated at or <5 and <2 ng/ml, respectively. To decrease contamination by DEHP, the cleanup steps and the apparatus and solvent usage were minimized in the sample preparation procedures. The high selectivity of LC/MS/MS is the key for obtaining reliable experimental data from in the matrix-rich analytical samples and for maintaining a low level contamination of MEHP and DEHP in this experimental system. This method would be a useful tool for the detection of MEHP and DEHP in serum.
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Keywords: Di(2-ethylhexyl)phthalate; Mono(2-ethylhexyl)phthalate

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a common plasticizer used to impart flexibility to polyvinylchloride (PVC). It leaches readily from PVC into the environment and transfers to other materials attached to the PVC or via the atmosphere. Patients undergoing medical procedures, such as intravenous therapy, nutritional support, blood transfusion, hemodialysis, cardiopulmonary bypass or extracorporeal membrane oxygenation (EMO) can be exposed to DEHP. Previous studies have shown detectable amounts of DEHP in blood products, in intravenous solutions, and in intravenous fat emulsions stored in PVC bags [1–5]. In animal studies, DEHP and/or MEHP are toxicants to the reproductive and developmental systems [6–10]. DEHP is hydrolyzed to MEHP in vivo and in blood products by esterase activities [11,12]. DEHP and MEHP have been detected in the blood of hemodialysed patients [13,14]. The FDA Center for Devices and Radiological Health (FDA/CDRH) has reviewed the potential health risks of DEHP leaching from medical devices [15]. Furthermore, the FDA/CDRH has recommended considering alternatives when high-risk procedures including transfusion, hemodialysis, total parenteral nutrition, EMO, or enteral nutrition are to be performed on male neonates, pregnant women who are carrying a male fetus, and peripubertal males [16].

To assess patient risk of DEHP and MEHP intake via medical procedures, the concentration of DEHP and MEHP in drugs, blood products and patients' serum should be determined accurately. However, the widespread usage and stability of DEHP in the experimental environment have led to DEHP being present as a ubiquitous contaminant. For this reason, the contamination of DEHP arising from

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the environment often injures the reliability of experimental data. There are documented cases of high levels of DEHP contamination in experimental environments and/or including reagents in DEHP measurements [17,18]. To decrease contamination by DEHP, it is reasonable to minimize the cleanup steps, and the apparatus and solvent usage. However, omission of the cleanup steps increases the potential for enough matrices remaining in the analytical samples to interfere with the accurate determination of analytes. To overcome this problem, we have adopted a high performance liquid chromatography with tandem mass spectrometry (LC/MS/MS) system for its high selectivity of the analytes. High performance liquid chromatography (HPLC) systems can measure MEHP without esterification at the carboxylic group of MEHP. Furthermore, elution performed in an isocratic mode is free from detection of MEHP and DEHP from in the LC systems including pump, lines, ferrules and eluents. These are advantages of HPLC systems over gas chromatography systems. Here, we describe a simple and sensitive method for determination of the concentrations of MEHP and DEHP in human serum by using LC/MS/MS.

2. Experimental

2.1. Materials

DEHP (99.6%), MEHP (99.3%), DEHP-d4 (99.0%) and MEHP-d4 (99.8%) were purchased from Hayashi Pure

Chemical Industries Ltd. (Osaka, Japan). Environment analytical grade acetone, hexane and acetonitrile were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HPLC grade acetonitrile and acetic acid were also obtained from Wako Pure Chemical Industries Ltd. The water for HPLC was purified by the Milli-Q system (Milli-Q, Millipore, Saint-Quentin Yvelines, France). The water for extraction was prepared by washing the Milli-Q water with hexane.

To eliminate contamination of DEHP and MEHP from glassware, the glassware was washed twice with acetone and hexane and then baked at 200 °C for 2 h in a clean oven.

2.2. Preparation of standard solutions and human serum

The stock solutions of DEHP, MEHP, and their isotopes were prepared in acetonitrile at 1.0 mg/ml using DEHP and MEHP-free glassware. They were mixed at the desired ratio and serially diluted for calibration curves. Human blood was obtained from four healthy volunteers with syringes made of glass through metal needles. To prepare serum samples, the blood was allowed to stand at 20 °C for 30 min in glass tubes with aluminum foil caps and then centrifuged at 3000 rpm for 10 min. The serum was stored at −40 °C until analysis. To avoid the contamination of DEHP and MEHP, all glassware and metal needles were washed and baked as mentioned above. The gender, age, body weight and nutrition of the volunteers are shown in Table 1.

Table 1
The gender, age, body weight and nutrition of four volunteers (A, B, C and D)

	Gender	Age (years)	Body weight (kg)	Meal[a]	Nutrition[b]
A	F	30	56	M1	Bread (40 g), butter (3 g), apple (40 g), coffee (400 ml)
				M2	Rice (80 g), grilled horse mackerel, deep-fried vegetables (pumpkin, onion, asparagus, eggplant; 20 g each), soup (miso 20 g, sweet potato, radish, 20 g each)
				M3	Pasta (200 g), source (200g; ground meat, tomato, onion, potato, cheese)
				M4	Bread (80 g), apple (40 g), coffee (200 ml), yogurt (50 g)
B	M	28	63	M1	Rice balls (200g)
				M2	Beef stew (250 g; beef, onion, carrot, potato, source), deep-fried chicken (100 g), beer (350 ml)
				M3	Pasta (100 g), mushrooms (30 g), thick white noodles made of wheat flour, salt and water (200 g)
				M4	Bread (90 g), coffee (180 ml)
C	M	29	62	M1	Cereal (30 g), milk (100 ml), coffee (200 ml), banana (90 g)
				M2	Rice (200g), boiled chicken (150 g), lettuce (100g), spinach (50 g), soybean pulp (50 g), soup (miso 20 g, potato, onion, 10 g each)
				M3	Burger put deep-fried chicken (200 g), french fries (50 g), deep-fried chicken (50 g), orange juice (200ml)
				M4	Bread (80 g), blueberry jam (10 g), milk (100 ml)
D	M	34	58	M1	Rice (80 g), pancake (200 g; wheat flour, pork, cabbage, egg), soup (miso 20 g, onion 20 g), omelet (20 g)
				M2	Rice (200 g), chinese-style dumpling (300 g; wheat flour, ground meat, chinese cabbage, onion, spring onion), boiled crab (50 g)
				M3	Doughnuts (150 g), Corn snack foods (75 g)
				M4	Rice balls wrapped with deep-fried soybean curds (250 g)

Blood sampling was performed at 10 a.m. (set at zero time). The nutrition of the volunteers taken prior to the blood sampling for 26 h was presented.
[a] M1, taken at 3–4 h; M2, taken at 13–16 h; M3, taken at 20–22 h; M4, 24–26 h.
[b] The weight of nutrition was at served. Italicized: nutrition served in a plastic dish or a package.

2.3. Sample preparation procedures

To a tube containing 0.50 g of serum, 10 ng of MEHP-d4 and DEHP-d4 was added at 4 °C. Then, 4.0 ml acetone was added and the sample was sonicated for 2 min and vigorously shaken for 5 min. The serum was centrifuged at 3×10^3 g and the supernatant was collected. To the precipitant, 1.0 ml acetone was added and extracted as described above. The supernatants were combined together and dried under an N_2 stream. To this tube 0.50 ml hexane-washed water and 4.0 μl acetic acid were added. After a 2 min sonication, MEHP and DEHP were extracted four times with 1.0 ml hexane. After drying under an N_2 stream, the extract was resolved in 0.50 ml acetonitrile. The analytical samples were placed in inactivated insert vials capped with aluminum foil and 5.0 μl of these samples were injected into an LC/MS/MS system.

2.4. LC/MS/MS conditions

LC/MS/MS analysis was performed on an API3000 (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. A reverse phase HPLC column (Wakosil3C18, 2.0 × 100 mm, 3 μm; Wako Pure Chemical Industries Ltd.) was used. The mobile phases consisted of 100% acetonitrile (A) and 0.05% aqueous acetic acid (B). Elution was performed using an isocratic mode (A/B: 15/85, v/v) at 0.2 ml/min. The ESI interface was controlled by the Analyst software (v.1.3.2).

ESI-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-d4 were detected in the negative mode. DEHP and DEHP-d4 were detected in the positive mode. Daughter ion mass spectra of MEHP, MEHP-d4, DEHP and DEHP-d4 obtained by the LC/MS/MS system are shown in Fig. 1. The combinations of parent ions and daughter ions were as follows; MEHP (parent ion/daughter ion, 277/134), MEHP-d4 (281/138), DEHP (391/149), DEHP-d4 (395/153). The daughter ions were formed in the collision cell using N_2 gas as the collision gas. The optimum collision energies for MEHP (MEHP-d4) and DEHP (DEHP-d4) were −22.0 and 27.0 V, respectively.

3. Results

The retention times of MEHP, MEHP-d4, DEHP and DEHP-d4 were 3.0, 3.0, 25.6 and 25.3 min, respectively. The relative standard deviations of the retention times were <0.03%. The signal to noise ratios of the MRM (multiple reaction monitoring) of 1 ng/ml MEHP and DEHP were 4.0 and 3.5, respectively. For MEHP measurement, the calibration curve was obtained for the peak-area ratio (MEHP/MEHP-d4) versus the MEHP concentration. It was linear over the range of 2.0–500 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0581x - 0.097$ ($r = 0.999$) with mean values for slope and intercept of 0.0581 ± 0.0012 (mean ± S.D.; S.D., standard deviation) and -0.097 ± 0.017, respectively (y, peak-area
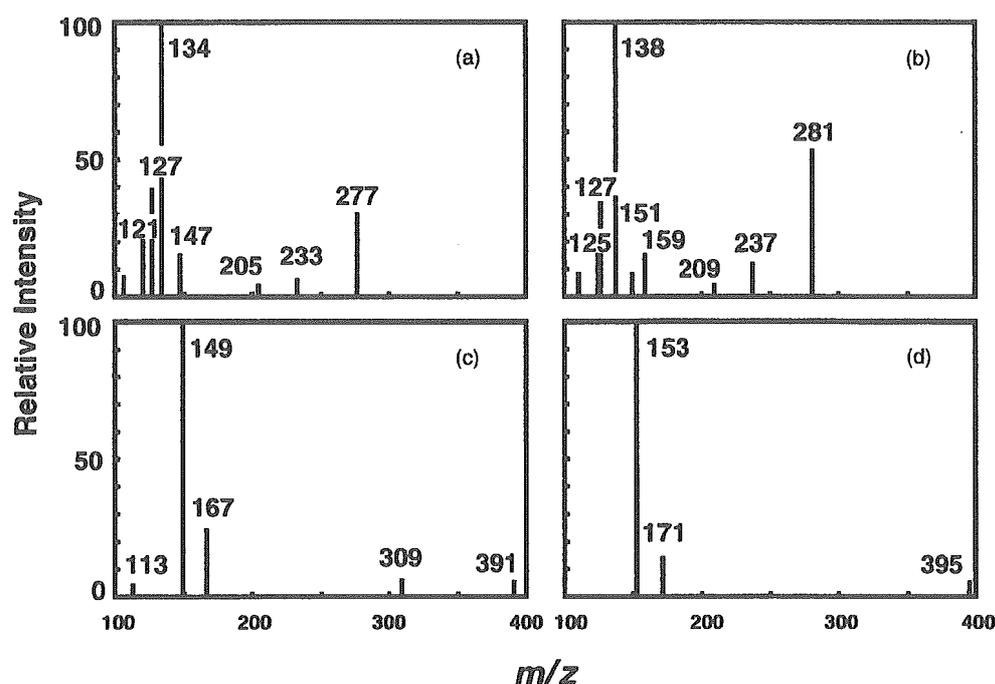

Fig. 1. Daughter ion spectra of MEHP (a), MEHP-d4 (b), DEHP (c), and DEHP-d4 (d).

Table 2
Concentrations of MEHP and DEHP in human sera

Serum from volunteers	Concentration (ng/ml)	
	MEHP[a]	DEHP[b]
A	5.7 ± 2.7	N.D. (3.8 ± 1.3)
B	N.D. (4.1 ± 1.5)	N.D. (3.7 ± 0.8)
C	N.D. (3.3 ± 0.6)	N.D. (2.9 ± 0.6)
D	N.D. (3.4 ± 0.6)	N.D. (3.9 ± 1.0)
Blank	N.D. (<1.0)	N.D. (2.2 ± 0.6)

The blank was the result of measurements of MEHP and DEHP in hexane washed water which contained >1 ng/ml MEHP and 1 ng/ml DEHP. Values in parentheses represent averages of the five independent measurements and SDs.

[a] N.D.; MEHP concentrations lower than 5 ng/ml.
[b] N.D.; DEHP concentrations lower than 14.0 ng/ml.

ratio; x, MEHP concentration ng/ml). For DEHP measurement, the calibration curve was obtained for the peak-area ratio (DEHP/DEHP-d4) versus DEHP concentration. It was linear over the range of 1.0–4000 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0318x + 0.337$ ($r = 0.999$) with mean values for slope and intercept of 0.0318 ± 0.0012 and 0.337 ± 0.035, respectively (y, peak-area ratio; x, DEHP concentration ng/ml). The recovery tests were performed using MEHP-d4 and DEHP-d4 to avoid the effects of possible contamination by MEHP and DEHP. The recoveries of 20 ng/ml of MEHP-d4 and DEHP-d4 from human serum were 101 ± 5.7 ($n = 6$) and $102 \pm 6.5\%$ ($n = 6$), respectively. The recoveries of 100 ng/ml of MEHP-d4 and DEHP-d4 from human serum were 93.8 ± 6.8 ($n = 6$) and $102 \pm 6.2\%$ ($n = 6$), respectively.

To determine the contamination of DEHP and MEHP generated by this extraction method, a blank test was performed using hexane-washed water instead of human serum. The concentrations of MEHP and DEHP in sera of healthy volunteers and the blank are shown in Table 2. The typical MRM chromatogram of the human serum is shown in Fig. 2. The
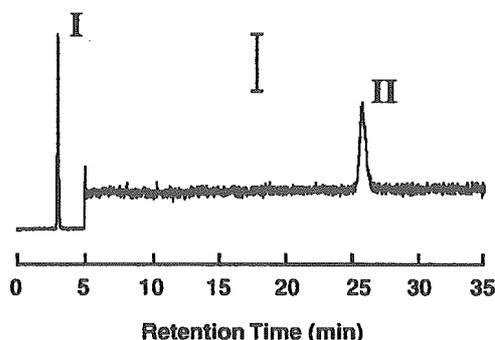

Fig. 2. The MRM chromatogram of human serum. From 0 to 5 min, monitoring of the daughter ion (m/z 134) of the parent ion (m/z 277), is in the negative mode. From 5 to 35 min, monitoring of the daughter ion (m/z 149) of the parent ion (m/z 391), is in the positive mode. The bar corresponds to 5.0×10^3 counts per second. The concentrations of MEHP (I) and DEHP (II) were estimated at 3.2 and 3.7 ng/ml, respectively.

blank of MEHP and DEHP were <1.0 and 2.2 ± 0.6 ng/ml, respectively. The limits of quantification (LOQ) of MEHP and DEHP in this method were determined by the formula, LOQ = 5 x (the blank + S.D.), and were 5 and 14.0 ng/ml, respectively. The concentrations of MEHP and DEHP in sera of healthy volunteers were at or below the LOQ. The concentrations of MEHP and DEHP under the LOQ are shown in parentheses. The concentrations include the blank levels of MEHP and DEHP. Thus, the net concentrations of MEHP and DEHP in the human serum were estimated at or <5 and <2 ng/ml, respectively.

4. Discussion

Severe contamination of MEHP and DEHP make it difficult to know the accurate concentrations of MEHP and DEHP in normal serum. In our trial to determine the MEHP in the serum by using gas chromatography with mass spectrometry, the contaminations of MEHP and DEHP derived from the esterification with 2,3,4,5,6-pentafluorobenzylbromide and subsequent clean up were 120 and 420 ng/ml, respectively (data not shown). By using an LC/MS/MS system, we developed a method to measure the concentrations of MEHP and DEHP with a low level contamination, and demonstrated that in serum of healthy volunteers these concentrations were at or less than the LOQ (5 and 14.0 ng/ml, respectively). Kessler et al. concluded that MEHP and DEHP in blood obtained from rats actually presented minute amounts, because there was no difference between the concentrations obtained from rat blood and water [17]. Inoue et al. have developed a method using LC/MS with column-switching systems for measurement of MEHP and DEHP in human blood samples and demonstrated that the concentrations of MEHP and DEHP in serum of healthy volunteers were <5 and <25 ng/ml, respectively [19]. Our results confirm their findings.

The direct injection methods using a column switching LC/MS system [19] and a solid-phase microextraction/HPLC [20] were effective in minimizing the contaminations of MEHP and DEHP during experimental procedures. However, these methods would have the potential for loading matrices into the LC/MS system or HPLC, which interfere with the accurate determination of analytes. The reliability of experimental data supported by MS/MS is one of the advantages of this method. Especially in the case of shortened cleanup steps, this advantage would be important. In our procedure, a large part of the contamination came from the solvents. Adopting the column switching system instead of the extraction steps in our procedures to decrease solvent usage would be possibly minimize the contamination and set the LOQ lower.

EU and IARC estimated that the human daily oral DEHP intake would be in the range of 5–21 µg/kg per day [21,22]. However, the concentrations of MEHP and DEHP in the serum of human that were orally administrated DEHP in

that range, have not yet been determined. After oral administration of DEHP, the concentration of DEHP in serum is lower than that of MEHP since a large part of orally administrated DEHP is absorbed after hydrolyzing to MEHP in the intestine [23,24]. The ratio of the concentration of MEHP and that of DEHP (MEHP/DEHP) in serum was 6–12:1 in rats [23,24]. In this study, MEHP/DEHP in human serum was calculated to be 2.0–4.7:1. There is difference in DEHP hydrolysis activities to MEHP among several species [12]. The DEHP hydrolysis activity of human intestine was conceived to be lower than that of the rat [12]. Assuming that the DEHP hydrolysis activity in the intestine reflect the MEHP/DEHP in serum, the large part of MEHP and DEHP detected in the serum should be sourced from the volunteers' nutrition. The concentrations of MEHP and DEHP in volunteers' nutrition did not determined in this study. There are few studies of human about relationship between the dose of orally administrated DEHP and the concentrations of MEHP and DEHP in serum [25]. To assess the daily exposure level of DEHP, determination of the concentrations of MEHP and DEHP in human serum would be informative.

The leaching of DEHP from medical devices into solutions was affected by the lipid content, the flow rate of solutions [26,27], and the concentration of the surface-active agent [28]. The exposure of DEHP to infants via TPN was estimated to be non-negligible from model studies [27]. To minimize the exposure of MEHP and DEHP to patients, improvement of medical devices using PVC, and determination of the checkpoints for handling of the medical devices would be important. Furthermore, model studies of the leaching of DEHP from medical devices, as well as investigations of the relationship between contamination and storage conditions of materials;, such as time, temperature and light would be informative to improve the medical devices. Changing DEHP in the medical device to an alternative would be effective in decreasing the exposure of MEHP and DEHP to patients. As a candidate of an alternative plasticizer for DEHP, trioctyltrimellitate (TOTM) is being used in medical devices for its minimal leaching and low hepatic toxicity [29–31]. For the safety of patients, more knowledge of the toxicities and application of TOTM in medical devices will be required. Thus, the risk assessment of medical usage of DEHP and the improvement of medical devices using DEHP should be continued. To achieve these goals, reliable methods for the measurement of MEHP and DEHP in blood is required. The method reported here would be applicable towards this end.

Acknowledgements

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Determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in human serum using liquid chromatography-tandem mass spectrometry

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Abstract

Concentrations of mono(2-ethylhexyl)phthalate (MEHP), and di(2-ethylhexyl)phthalate (DEHP), in serum of healthy volunteers were determined by high performance liquid chromatography (HPLC) with tandem mass spectrometry (LC/MS/MS). The serum was extracted with acetone, followed by hexane extraction under acidic conditions, and then applied to the LC/MS/MS. Recoveries of 20 ng/ml of MEHP and DEHP were 101 ± 5.7 ($n = 6$) and $102 \pm 6.5\%$ ($n = 6$), respectively. The limits of quantification (LOQ) of MEHP and DEHP in the method were 5.0 and 14.0 ng/ml, respectively. The concentration of MEHP in the serum was at or less than the LOQ. The concentration of DEHP in the serum was less than the LOQ. Contaminations of MEHP and DEHP from experimental reagents, apparatus and air during the procedure were less than the LOQ and were estimated to be <1.0 and 2.2 ± 0.6 ng/ml, respectively. After subtraction of the contamination, the net concentrations of MEHP and DEHP in the serum were estimated at or <5 and <2 ng/ml, respectively. To decrease contamination by DEHP, the cleanup steps and the apparatus and solvent usage were minimized in the sample preparation procedures. The high selectivity of LC/MS/MS is the key for obtaining reliable experimental data from in the matrix-rich analytical samples and for maintaining a low level contamination of MEHP and DEHP in this experimental system. This method would be a useful tool for the detection of MEHP and DEHP in serum.
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Keywords: Di(2-ethylhexyl)phthalate; Mono(2-ethylhexyl)phthalate

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a common plasticizer used to impart flexibility to polyvinylchloride (PVC). It leaches readily from PVC into the environment and transfers to other materials attached to the PVC or via the atmosphere. Patients undergoing medical procedures, such as intravenous therapy, nutritional support, blood transfusion, hemodialysis, cardiopulmonary bypass or extracorporeal membrane oxygenation (EMO) can be exposed to DEHP. Previous studies have shown detectable amounts of DEHP in blood products, in intravenous solutions, and in intravenous fat emulsions stored in PVC bags [1–5]. In animal studies, DEHP and/or MEHP are toxicants to the reproductive and developmental

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systems [6–10]. DEHP is hydrolyzed to MEHP in vivo and in blood products by esterase activities [11,12]. DEHP and MEHP have been detected in the blood of hemodialysed patients [13,14]. The FDA Center for Devices and Radiological Health (FDA/CDRH) has reviewed the potential health risks of DEHP leaching from medical devices [15]. Furthermore, the FDA/CDRH has recommended considering alternatives when high-risk procedures including transfusion, hemodialysis, total parenteral nutrition, EMO, or enteral nutrition are to be performed on male neonates, pregnant women who are carrying a male fetus, and peripubertal males [16].

To assess patient risk of DEHP and MEHP intake via medical procedures, the concentration of DEHP and MEHP in drugs, blood products and patients' serum should be determined accurately. However, the widespread usage and stability of DEHP in the experimental environment have led to DEHP being present as a ubiquitous contaminant. For this reason, the contamination of DEHP arising from

the environment often injures the reliability of experimental data. There are documented cases of high levels of DEHP contamination in experimental environments and/or including reagents in DEHP measurements [17,18]. To decrease contamination by DEHP, it is reasonable to minimize the cleanup steps, and the apparatus and solvent usage. However, omission of the cleanup steps increases the potential for enough matrices remaining in the analytical samples to interfere with the accurate determination of analytes. To overcome this problem, we have adopted a high performance liquid chromatography with tandem mass spectrometry (LC/MS/MS) system for its high selectivity of the analytes. High performance liquid chromatography (HPLC) systems can measure MEHP without esterification at the carboxylic group of MEHP. Furthermore, elution performed in an isocratic mode is free from detection of MEHP and DEHP from in the LC systems including pump, lines, ferrules and eluents. These are advantages of HPLC systems over gas chromatography systems. Here, we describe a simple and sensitive method for determination of the concentrations of MEHP and DEHP in human serum by using LC/MS/MS.

2. Experimental

2.1. Materials

DEHP (99.6%), MEHP (99.3%), DEHP-d4 (99.0%) and MEHP-d4 (99.8%) were purchased from Hayashi Pure

Chemical Industries Ltd. (Osaka, Japan). Environment analytical grade acetone, hexane and acetonitrile were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HPLC grade acetonitrile and acetic acid were also obtained from Wako Pure Chemical Industries Ltd. The water for HPLC was purified by the Milli-Q system (Milli-Q, Millipore, Saint-Quentin Yvelines, France). The water for extraction was prepared by washing the Milli-Q water with hexane.

To eliminate contamination of DEHP and MEHP from glassware, the glassware was washed twice with acetone and hexane and then baked at 200 °C for 2 h in a clean oven.

2.2. Preparation of standard solutions and human serum

The stock solutions of DEHP, MEHP, and their isotopes were prepared in acetonitrile at 1.0 mg/ml using DEHP and MEHP-free glassware. They were mixed at the desired ratio and serially diluted for calibration curves. Human blood was obtained from four healthy volunteers with syringes made of glass through metal needles. To prepare serum samples, the blood was allowed to stand at 20 °C for 30 min in glass tubes with aluminum foil caps and then centrifuged at 3000 rpm for 10 min. The serum was stored at −40 °C until analysis. To avoid the contamination of DEHP and MEHP, all glassware and metal needles were washed and baked as mentioned above. The gender, age, body weight and nutrition of the volunteers are shown in Table 1.

Table 1
The gender, age, body weight and nutrition of four volunteers (A, B, C and D)

	Gender	Age (years)	Body weight (kg)	Meal[a]	Nutrition[b]
A	F	30	56	M1	*Bread* (40 g), *butter* (3 g), apple (40 g), coffee (400 ml)
				M2	*Rice* (80 g), grilled horse mackerel, deep-fried vegetables (pumpkin, onion, asparagus, eggplant; 20 g each), soup (miso 20 g, sweet potato, radish, 20 g each)
				M3	Pasta (200 g), source (200g; ground meat, tomato, onion, potato, cheese)
				M4	*Bread* (80 g), apple (40 g), coffee (200 ml), *yogurt* (50 g)
B	M	28	63	M1	Rice balls (200g)
				M2	Beef stew (250 g: beef, onion, carrot, potato, source), *deep-fried chicken* (100 g), beer (350 ml)
				M3	Pasta (100 g), mushrooms (30 g), thick white noodles made of wheat flour, salt and water (200 g)
				M4	Bread (90 g), coffee (180 ml)
C	M	29	62	M1	Cereal (30 g), milk (100 ml), coffee (200 ml), banana (90 g)
				M2	Rice (200g), boiled chicken (150 g), *lettuce* (100g), spinach (50 g), *soybean pulp* (50 g), soup (miso 20 g, potato, onion, 10 g each)
				M3	*Burger put deep-fried chicken* (200 g), french fries (50 g), *deep-fried chicken* (50 g), orange juice (200ml)
				M4	*Bread* (80 g), blueberry jam (10 g), milk (100 ml)
D	M	34	58	M1	Rice (80 g), *pancake* (200 g: wheat flour, pork, cabbage, egg), soup (miso 20 g, onion 20 g), omelet (20 g)
				M2	Rice (200 g), chinese-style dumpling (300 g: wheat flour, ground meat, chinese cabbage, onion, spring onion), boiled crab (50 g)
				M3	Doughnuts (150 g), *Corn snack foods* (75 g)
				M4	Rice balls wrapped with deep-fried soybean curds (250 g)

Blood sampling was performed at 10 a.m. (set at zero time). The nutrition of the volunteers taken prior to the blood sampling for 26 h was presented.
  [a] M1, taken at 3–4 h; M2, taken at 13–16 h; M3, taken at 20–22 h; M4, 24–26 h.
  [b] The weight of nutrition was at served. Italicized: nutrition served in a plastic dish or a package.

2.3. Sample preparation procedures

To a tube containing 0.50 g of serum, 10 ng of MEHP-d4 and DEHP-d4 was added at 4 °C. Then, 4.0 ml acetone was added and the sample was sonicated for 2 min and vigorously shaken for 5 min. The serum was centrifuged at 3×10^3 g and the supernatant was collected. To the precipitant, 1.0 ml acetone was added and extracted as described above. The supernatants were combined together and dried under an N_2 stream. To this tube 0.50 ml hexane-washed water and 4.0 μl acetic acid were added. After a 2 min sonication, MEHP and DEHP were extracted four times with 1.0 ml hexane. After drying under an N_2 stream, the extract was resolved in 0.50 ml acetonitrile. The analytical samples were placed in inactivated insert vials capped with aluminum foil and 5.0 μl of these samples were injected into an LC/MS/MS system.

2.4. LC/MS/MS conditions

LC/MS/MS analysis was performed on an API3000 (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. A reverse phase HPLC column (Wakosil3C18, 2.0 × 100 mm, 3 μm; Wako Pure Chemical Industries Ltd.) was used. The mobile phases consisted of 100% acetonitrile (A) and 0.05% aqueous acetic acid (B). Elution was performed using an isocratic mode (A/B: 15/85, v/v) at 0.2 ml/min. The ESI interface was controlled by the Analyst software (v.1.3.2).

ESI-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-d4 were detected in the negative mode. DEHP and DEHP-d4 were detected in the positive mode. Daughter ion mass spectra of MEHP, MEHP-d4, DEHP and DEHP-d4 obtained by the LC/MS/MS system are shown in Fig. 1. The combinations of parent ions and daughter ions were as follows; MEHP (parent ion/daughter ion, 277/134), MEHP-d4 (281/138), DEHP (391/149), DEHP-d4 (395/153). The daughter ions were formed in the collision cell using N_2 gas as the collision gas. The optimum collision energies for MEHP (MEHP-d4) and DEHP (DEHP-d4) were −22.0 and 27.0 V, respectively.

3. Results

The retention times of MEHP, MEHP-d4, DEHP and DEHP-d4 were 3.0, 3.0, 25.6 and 25.3 min, respectively. The relative standard deviations of the retention times were <0.03%. The signal to noise ratios of the MRM (multiple reaction monitoring) of 1 ng/ml MEHP and DEHP were 4.0 and 3.5, respectively. For MEHP measurement, the calibration curve was obtained for the peak-area ratio (MEHP/MEHP-d4) versus the MEHP concentration. It was linear over the range of 2.0–500 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0581x - 0.097$ ($r = 0.999$) with mean values for slope and intercept of 0.0581 ± 0.0012 (mean ± S.D.; S.D., standard deviation) and -0.097 ± 0.017, respectively (y, peak-area
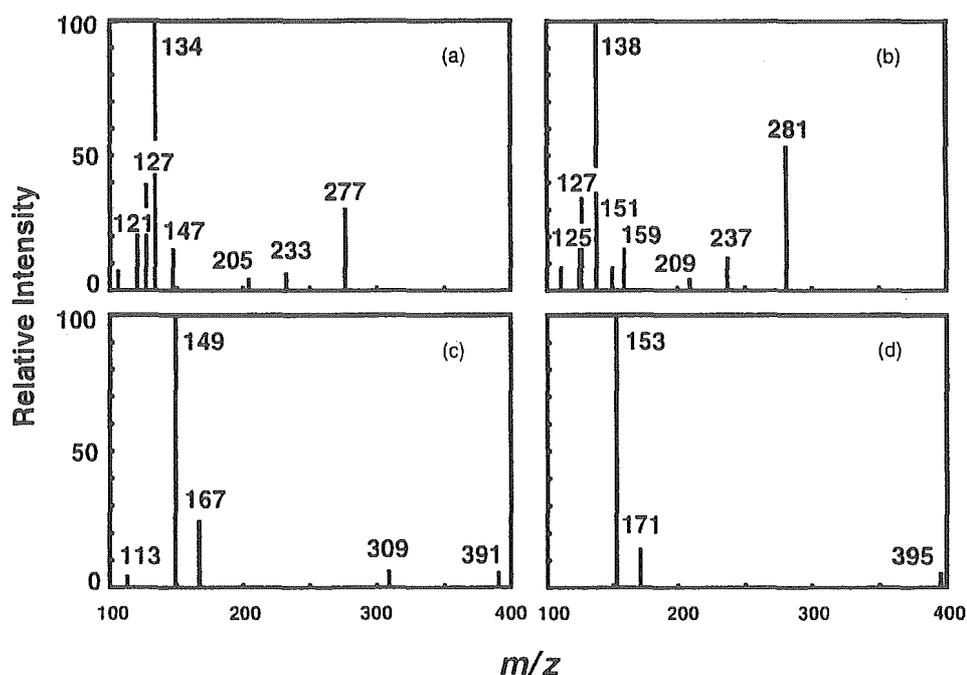

Fig. 1. Daughter ion spectra of MEHP (a), MEHP-d4 (b), DEHP (c), and DEHP-d4 (d).

Table 2
Concentrations of MEHP and DEHP in human sera

Serum from volunteers	Concentration (ng/ml)	
	MEHP[a]	DEHP[b]
A	5.7 ± 2.7	N.D. (3.8 ± 1.3)
B	N.D. (4.1 ± 1.5)	N.D. (3.7 ± 0.8)
C	N.D. (3.3 ± 0.6)	N.D. (2.9 ± 0.6)
D	N.D. (3.4 ± 0.6)	N.D. (3.9 ± 1.0)
Blank	N.D. (<1.0)	N.D. (2.2 ± 0.6)

The blank was the result of measurements of MEHP and DEHP in hexane washed water which contained >1 ng/ml MEHP and 1 ng/ml DEHP. Values in parentheses represent averages of the five independent measurements and SDs.

[a] N.D.; MEHP concentrations lower than 5 ng/ml.
[b] N.D.; DEHP concentrations lower than 14.0 ng/ml.

ratio; x, MEHP concentration ng/ml). For DEHP measurement, the calibration curve was obtained for the peak-area ratio (DEHP/DEHP-d4) versus DEHP concentration. It was linear over the range of 1.0–4000 ng/ml. The mean linear regression equations obtained from five replicates were $y = 0.0318x + 0.337$ ($r = 0.999$) with mean values for slope and intercept of 0.0318 ± 0.0012 and 0.337 ± 0.035, respectively (y, peak-area ratio; x, DEHP concentration ng/ml). The recovery tests were performed using MEHP-d4 and DEHP-d4 to avoid the effects of possible contamination by MEHP and DEHP. The recoveries of 20 ng/ml of MEHP-d4 and DEHP-d4 from human serum were 101 ± 5.7 ($n = 6$) and $102 \pm 6.5\%$ ($n = 6$), respectively. The recoveries of 100 ng/ml of MEHP-d4 and DEHP-d4 from human serum were 93.8 ± 6.8 ($n = 6$) and $102 \pm 6.2\%$ ($n = 6$), respectively.

To determine the contamination of DEHP and MEHP generated by this extraction method, a blank test was performed using hexane-washed water instead of human serum. The concentrations of MEHP and DEHP in sera of healthy volunteers and the blank are shown in Table 2. The typical MRM chromatogram of the human serum is shown in Fig. 2. The
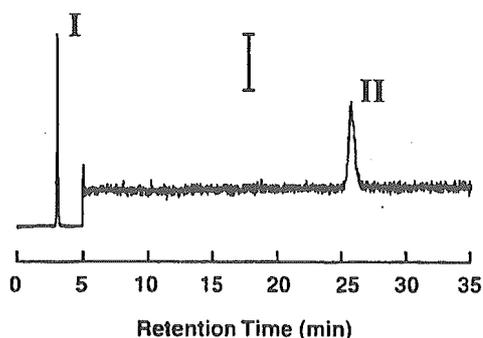

Fig. 2. The MRM chromatogram of human serum. From 0 to 5 min, monitoring of the daughter ion (m/z 134) of the parent ion (m/z 277), is in the negative mode. From 5 to 35 min, monitoring of the daughter ion (m/z 149) of the parent ion (m/z 391), is in the positive mode. The bar corresponds to 5.0×10^3 counts per second. The concentrations of MEHP (I) and DEHP (II) were estimated at 3.2 and 3.7 ng/ml, respectively.

blank of MEHP and DEHP were <1.0 and 2.2 ± 0.6 ng/ml, respectively. The limits of quantification (LOQ) of MEHP and DEHP in this method were determined by the formula, $LOQ = 5 x$ (the blank + S.D.), and were 5 and 14.0 ng/ml, respectively. The concentrations of MEHP and DEHP in sera of healthy volunteers were at or below the LOQ. The concentrations of MEHP and DEHP under the LOQ are shown in parentheses. The concentrations include the blank levels of MEHP and DEHP. Thus, the net concentrations of MEHP and DEHP in the human serum were estimated at or <5 and <2 ng/ml, respectively.

4. Discussion

Severe contamination of MEHP and DEHP make it difficult to know the accurate concentrations of MEHP and DEHP in normal serum. In our trial to determine the MEHP in the serum by using gas chromatography with mass spectrometry, the contaminations of MEHP and DEHP derived from the esterification with 2,3,4,5,6-pentafluorobenzylbromide and subsequent clean up were 120 and 420 ng/ml, respectively (data not shown). By using an LC/MS/MS system, we developed a method to measure the concentrations of MEHP and DEHP with a low level contamination, and demonstrated that in serum of healthy volunteers these concentrations were at or less than the LOQ (5 and 14.0 ng/ml, respectively). Kessler et al. concluded that MEHP and DEHP in blood obtained from rats actually presented minute amounts, because there was no difference between the concentrations obtained from rat blood and water [17]. Inoue et al. have developed a method using LC/MS with column-switching systems for measurement of MEHP and DEHP in human blood samples and demonstrated that the concentrations of MEHP and DEHP in serum of healthy volunteers were <5 and <25 ng/ml, respectively [19]. Our results confirm their findings.

The direct injection methods using a column switching LC/MS system [19] and a solid-phase microextraction/HPLC [20] were effective in minimizing the contaminations of MEHP and DEHP during experimental procedures. However, these methods would have the potential for loading matrices into the LC/MS system or HPLC, which interfere with the accurate determination of analytes. The reliability of experimental data supported by MS/MS is one of the advantages of this method. Especially in the case of shortened cleanup steps, this advantage would be important. In our procedure, a large part of the contamination came from the solvents. Adopting the column switching system instead of the extraction steps in our procedures to decrease solvent usage would be possibly minimize the contamination and set the LOQ lower.

EU and IARC estimated that the human daily oral DEHP intake would be in the range of 5–21 μg/kg per day [21,22]. However, the concentrations of MEHP and DEHP in the serum of human that were orally administrated DEHP in

that range, have not yet been determined. After oral admin-istration of DEHP, the concentration of DEHP in serum is lower than that of MEHP since a large part of orally ad-ministrated DEHP is absorbed after hydrolyzing to MEHP in the intestine [23,24]. The ratio of the concentration of MEHP and that of DEHP (MEHP/DEHP) in serum was 6–12:1 in rats [23,24]. In this study, MEHP/DEHP in hu-man serum was calculated to be 2.0–4.7:1. There is differ-ence in DEHP hydrolysis activities to MEHP among several species [12]. The DEHP hydrolysis activity of human in-testine was conceived to be lower than that of the rat [12]. Assuming that the DEHP hydrolysis activity in the intestine reflect the MEHP/DEHP in serum, the large part of MEHP and DEHP detected in the serum should be sourced from the volunteers' nutrition. The concentrations of MEHP and DEHP in volunteers' nutrition did not determined in this study. There are few studies of human about relationship be-tween the dose of orally administrated DEHP and the con-centrations of MEHP and DEHP in serum [25]. To assess the daily exposure level of DEHP, determination of the con-centrations of MEHP and DEHP in human serum would be informative.

The leaching of DEHP from medical devices into solu-tions was affected by the lipid content, the flow rate of so-lutions [26,27], and the concentration of the surface-active agent [28]. The exposure of DEHP to infants via TPN was estimated to be non-negligible from model studies [27]. To minimize the exposure of MEHP and DEHP to patients, improvement of medical devices using PVC, and deter-mination of the checkpoints for handling of the medical devices would be important. Furthermore, model studies of the leaching of DEHP from medical devices, as well as investigations of the relationship between contamination and storage conditions of materials;, such as time, tem-perature and light would be informative to improve the medical devices. Changing DEHP in the medical device to an alternative would be effective in decreasing the ex-posure of MEHP and DEHP to patients. As a candidate of an alternative plasticizer for DEHP, trioctyltrimellitate (TOTM) is being used in medical devices for its minimal leaching and low hepatic toxicity [29–31]. For the safety of patients, more knowledge of the toxicities and applica-tion of TOTM in medical devices will be required. Thus, the risk assessment of medical usage of DEHP and the improvement of medical devices using DEHP should be continued. To achieve these goals, reliable methods for the measurement of MEHP and DEHP in blood is required. The method reported here would be applicable towards this end.

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Development of a simple method for predicting the levels of di(2-ethylhexyl) phthalate migrated from PVC medical devices into pharmaceutical solutions

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Abstract

This study deals with the development of a simple method for predicting the elution levels of di-2-ethylhexyl phthalate (DEHP) from medical devices made of polyvinyl chloride (PVC) by using the physicochemical properties of pharmaceutical injections as a marker. GC-MS analysis showed that the release of DEHP from medical grade PVC product was concentration-dependently increased by extraction with two kinds of lipophilic injections (Sandimmun® and Prograf®) and three kinds of surfactants (HCO-60, Tween® 80, and SDS). The solubility of lipophilic pigments such as Sudan III, methyl yellow, and 1,4-diamino-anthraquinone against these solutions were also increased in a concentration-dependent manner, in which methyl yellow showed the highest response regarding the increase of optical density (O.D.). Further, electrical conductivity and static contact angle to the PVC sheet of the solutions were also increased or decreased in the same manner. As a result of the comparative study, significant correlation was found between DEHP release levels and these three physicochemical properties. particularly methyl yellow solubility, of the solutions tested. To evaluate the relationship in detail, DEHP release levels from PVC tubing and methyl yellow solubility of 53 injections used in gynecologic and obstetric fields were determined. None of the hydrophilic medicines showed any significant release of DEHP, and all showed low solubility of methyl yellow. On the other hand, the lipophilic medicines releasing a large amount of DEHP showed high solubility of methyl yellow (greater than O.D. 0.8). These

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results indicate that a significant proportional relationship exists between DEHP release potency and methyl yellow solubility of pharmaceutical solutions, and the risk of DEHP exposure to the patients administered pharmaceuticals through transfusion set could be easily predicted by the solubility test without complicated elution tests of DEHP using GC-MS or LC-MS.

1. Introduction

Phthalate esters, and DEHP in particular, have been extensively used as plasticizers due to the increased flexibility of PVC a plastic polymer used in a wide array of products including medical devices such as tubings, intravenous bags, blood containers, and catheters. DEHP is easily eluted from PVC products into not only foods but also pharmaceuticals and body fluids that come in contact with the plastic, and the migrated DEHP is directly and/or indirectly introduced into the human body (Allwod, 1986; Loff et al., 2000; Tickner et al., 2001). Some phthalates including DEHP are considered to be a toxic compound exhibiting effects similar to those of endocrine disruptors in rodents; they have antiandrogenic effects in male rats during the development of the male reproductive system and the production of normal sperm (Poon et al., 1997; Lamb et al., 1987; Tyl et al., 1988), and decrease the 17β-estradiol level in blood in female rats (Davis et al., 1994). General toxicity of DEHP has been well evaluated, and so far the result of risk assessment to human health indicates that this compound is relatively safe to humans. However, because the reproductive and developmental toxicity of DEHP to the human body is not well understood, it has recently been suggested that precautions be taken to limit the exposure of humans, particularly that of high risk patient groups such as male neonates, male fetuses, and peripubertal males, to DEHP. The concern is that DEHP's potency might have adverse effects on humans similar to those demonstrated on young rodents.

Taking the above into consideration, several agencies and official organizations in the world individually evaluated the safety of DEHP released from PVC products (Center for Devices and Radiological Health, 2001; Health Canada, 2002), and the Japanese Ministry of Health, Labor and Welfare (JMHLW) restricted the oral tolerable daily intake (TDI) value to 40–140 μg/kg/day.

It is very important that the exposure amount be exactly determined to conduct a risk assessment of the effect of DEHP on human health. Although some studies on the elution of DEHP from PVC medical devices have been performed as one of the JMHLW projects (Haishima et al., 2004; Inoue et al., 2003a,b; Takatori et al., 2004), it is not easy to identify the release behavior of DEHP from the variety of PVC products used in Japan by elution test under conditions that are the same as or similar to those of medical use. In addition, analytical methods having high sensitivity, precision, selectivity of quantitative ions, and low background, such as tandem LC-MS, high resolution GC-MS, and column-switching LC-MS methods, are required to determine DEHP for clinical assessment. Thus, regardless whether an investigation is in vivo or in vitro, the release test of DEHP is at present time-consuming and labor-intensive.

Jenke (2001) reported that the chemical compatibility assessment considers two distinct yet complementary mechanisms by which a device and its contacted solution can interact. These mechanisms include the migration of a chemical component out of the device and into the contacted solution (leaching) and the sorption of contained solution components by the device (binding). Alternatively, the product/device interaction can be modeled based on a rigorous scientific assessment of the physicochemical processes. Such models are based on the linear correlation of polymer/solution interaction constants with solvent/water partition coefficients (Nasim et al., 1972; Pitt et al., 1988; Hayward et al., 1990; Kenley and Jenke, 1990; Jenke, 1991; Jenke et al., 1991; Atkinson and Duffull, 1991; Roberts et al., 1991; Jenke et al., 1992). In addition, it is known that extraction occurs either by leaching or after an extracting material such as blood and pharmaceutical solutions diffuses into the PVC matrix and dissolves the plasticizer, which is relatively lipophilic. In consideration of these issues, we suspected that the release behavior of DEHP from PVC medical devices may be predicted from the physicochemical properties of

pharmaceutical injections applied to the devices, without a complicated elution test.

In the present study, to develop a simple method for predicting the release level of DEHP from PVC medical devices, we examined the relationship between the release potency of DEHP from PVC product and physicochemical properties such as the solubility of lipophilic pigments, electrical conductivity, and the static contact angle to PVC sheet, using two kinds of lipophilic injections and three kinds of surfactants as test solutions. Further, to evaluate the relationship in detail, DEHP release levels from PVC tubing and the physicochemical properties of 53 injections used in gynecologic and obstetric fields were determined.

2. Materials and methods

2.1. Chemicals and utensils

Medical grade PVC sheet for blood container and PVC tubing for transfusion set were provided by Terumo Co. (Tokyo, Japan).

Sandimmun® (50 mg/ml cyclosporine) and Prograf® (5 mg/ml tacrolimus) were provided by Novartis Pharma K.K. (Tokyo, Japan) and Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan). The other 51 injections listed in Table 1 were purchased from commercial companies. Polyoxyethene hydrogenated castor oil 60 (HCO-60) provided by Nikko Chemicals Co. (Tokyo, Japan), polysorbate 80 (Tween® 80, ICN Biomedicals Inc., Ohio, USA), and sodium lauryl sulfate (SDS, Sigma Aldrich Japan, Tokyo, Japan) were used as surfactants. In these materials, Sandimmun®, Prograf®, HCO-60, Tween® 80, and SDS were used as pretest solutions for evaluating the relationship between release potency of DEHP and physicochemical properties of pharmaceuticals.

Methyl yellow (Wako Pure Chemical Industries, Ltd., Osaka, Japan), Sudan III (Sigma Aldrich Japan, Tokyo, Japan), and 1,4-diamino-anthraquinone (Tokyo Kasei Co., Tokyo, Japan) were used as lipophilic pigments. DEHP and DEHP-d_4 were purchased from Kanto Chemical Co. (Tokyo, Japan). Hexane, anhydrous sodium sulfate, sodium chloride of phthalate esters of analytical grade, diethyl ether of dioxin of analytical grade, and distilled water of HPLC grade were used in this study.

All utensils were made of glass, metal, or teflon, and were heated at 250 °C for more than 16 h before use.

2.2. Classification of pharmaceuticals

As shown in Table 1, based on the properties of principal drugs and additives contained in each pharmaceutical, 53 injections used in this study were divided into five groups. Expression rule on solubility of the drugs has been established in general notices in the Japanese Pharmacopoeia IX edition regarding the relationship between descriptive term and the degree of dissolution. Pharmaceuticals such as Sandimmun® and Prograf® containing principal drugs that are expressed as practically insoluble or insoluble to water in the instruction manuals were assigned to group 1 as lipophilic injections. Most of pharmaceuticals in this group were contained various additives such as surfactants, oils, glycerin, ethanol, benzyl alcohol, and so on. The principal drugs of pharmaceuticals classified into group 2 are also insoluble or very slightly soluble to water, but these drugs can be dissolved in acidic or basic solutions. Gaster®, Droleptan®, Elaspol®, Aleviatin®, Methotrexate® Parenteral, Serenace®, and Bosmin® were assigned to this group, and pH of each pharmaceutical is expressed in the instruction manuals as 4.7–5.7, 2.5–4.5, 7.5–8.5, approximately 12, 7.0–9.0, 3.5–4.2, and 2.3–5.0, respectively. Pharmaceuticals consisted of drugs that are slightly soluble or sparingly soluble to water were classified into group 3. Solubility of principal drugs contained in the pharmaceuticals assigned to groups 4 and 5 was expressed as very soluble, freely soluble, or soluble to water in each instruction manual. Pharmaceuticals of group 5 are hydrophilic injections as negative control regarding DEHP migration. Although pharmaceuticals assigned to group 4 are also hydrophilic injections, these pharmaceuticals were suspected to induce DEHP migration, because some of them are human serum products or containing chlorobutanol, phenol, and benzyl alcohol as additives.

2.3. Solubility test of lipophilic pigments

One millilitre of each surfactant solution and pharmaceutical injection was added to each lipophilic pigment (5 mg) followed by sonication for 10 min at room temperature and centrifugation at 3000 rpm for 10 min. The supernatant was passed through a membrane filter (pore size 0.2 μm) and the filtrate (100 μl) was

Table 1
List of phamaceutical injections used in this study

Product name	Principal drug	Concentration for medical use	Additives	Medication	Color
Group 1[a]					
Sandimmun®	Cyclosporin	500 µg/mL	Polyoxyethene castor oil, ethanol	Instillation	Clear
Prograf® injection 5 mg	Tacrolimus hydrate	10 µg/mL	Absolute ethanol, HCO-60	Instillation	Clear
1% Diprivan® injection	Propofol	10 mg/mL	Soybean oil, concentrated glycerin, pure egg-yolk lecithin, edetate sodium pH adjuster	Intravenous injection	White emulsion
Ropion®	Flurbiprofen axetil	10 mg/mL	Pure soybean oil, pure egg-yolk lecithin, concentrated glycerin	Intravenous injection	White emulsion
Sohvita®	Vitamins including fat-soluble vitamin	Whole amount of Sobita was mixed with PN-Twin No.2 (2.2 L)	Sodium citrate, pH adjuster, sodium pyrosulfite, sodium thioglycollate, HCO-60, benzyl alcohol, polysorbate 80	Instillation	Yellow (clear)
Kaytwo® N	Menatetrenone	5 mg/mL	Aminoethylsulfonic acid, sesame oil, pure soybean lecithin, D-sorbitol, concentrated glycerin, pH adjuster	Intravenous injection	Buff yellow (translucence)
Humulin® R	Insulin human	40 units/mL	Concentrated glycerin, m-cresol, pH adjuster	Intravenous injection	Clear
Prostarmon®-F	Dinoprost	2 mg/mL		Instillation	Clear
Florid®-F	Miconazole	1 mg/mL	HCO-60	Instillation	Clear
Horizon®	Diazepam	5 mg/mL	Propylene glycol, ethanol, benzyl alcohol, sodium benzoate, benzoic acid	Intravenous injection	Buff yellow (clear)
Predonine®	Prednisolone sodium succinate	① 10 mg/mL, ② 1 mg/mL	Dried sodium carbonate, sodium hydrogenphosphate, sodium dihydrogenphosphate crystal	① Intravenous injection, ② instillation	Clear
Group 2[a]					
Gaster®	Famotidine	20 mg/mL	L-Aspartic acid, D-mannitol	Instillation	Clear
Droleptan®	Droperidol	① 2.5 mg/mL, ② 50 µg/mL	p-Oxymethyl benzoate, p-oxypropyl benzoate pH adjuster (acidic)	① Intravenous injection, ② instillation	Clear
Elaspol®	Sivelestat sodium hydrate	1 mg/mL	D-Mannitol, pH adjuster	Intravenous injection	Clear
Aleviatin®	Phenytoin	50 mg/mL	Sodium hydroxide, propylene glycol, ethanol	Intravenous injection	Clear
Methotrexate® parenteral	Methotrexate	0.2 mg/mL	Sodium chloride, sodium hydroxide	Instillation	Clear
Serenace®	Haloperidol	5 mg/mL	Glucose, lactic acid, sodium hydroxide	Instillation	Clear
Bosmin® injection	Epinephrine	0.25 mg/mL	Chlorobutanol, sodium hydrogen sulfite, hydrochloric acid, sodium chloride, pH adjuster	Intravenous injection	Clear
Group 3[a]					
Partan M injection	Methylergometrine maleate	0.2 mg/mL		Intravenous injection	Clear
Musculax® intravenous	Vecuronium bromide	2 mg/mL	D-Mannitol	Intravenous injection	Clear
Carbenin® for intravenous drip infusion	Panipenem Betamipron	5 mg/mL	pH Adjuster	Instillation	Achroma yellow (clear)

Table 1 (Continued)

Product name	Principal drug	Concentration for medical use	Additives	Medication	Color
Minomycin® intravenous for drip use	Minocycline Hydrochloride	1 mg/mL		Instillation	Clear
Perdipine®	Nicardipine Hydrochloride	0.1 mg/mL	D-Sorbitol, pH adjuster	Instillation	Clear
Bisolvon® injection	Bromhexine Hydrochloride	2 mg/mL	Glucose	Intravenous injection	Clear
Modacin® injection	Ceftazidime	10 mg/mL	Sodium carbonate	Instillation	Clear
Diflucan® intravenous solution	Fluconazole	1 mg/mL		Instillation	Clear
Doyle® for injection	Aspoxicillin	50 mg/mL	Sodium chloride	Instillation	Clear
Adona® (AC-17) injection	Carbazochrome sodium sulfonate	0.05 mg/mL	Sodium hydrogensulfite, D-sorbitol, propylene glycol	Instillation	Clear
Group 4[a]					
Atonin®-O	Oxytocin	0.01 units/mL	Chlorobutanol	Instillation	Clear
Atarax®-P Parenteral solution	Hydroxyzine Hydrochloride	0.05 mg/mL	Benzyl alcohol, pH adjuster	Instillation	Clear
Zantac® injection	Ranitidine hydrochloride	0.1 mg/mL	pH adjuster, phenol	Instillation	Achroma yellow (clear)
Kenketsu venoglobulin®-IH YOSHITOMI	Human immunoglobulin G	50 mg/mL	D-Sorbitol, pH adjuster	Intravenous injection	Clear
Pantol® injection	Panthenol	250 mg/mL	Benzyl alcohol	Intravenous injection	Clear
Buminate® 25%	Human serum albumin	250 mg/mL	Sodium N-acetyl tryptophan, sodium caprylate, sodium hydrogen carbonate	Intravenous injection	Clear
Neuart®	Human antithrombin III	25 units/mL	Sodium chloride, sodium citrate, D-mannitol	Instillation	Achroma yellow (barely opacity)
Millisrol® injection	Nitroglycerin	0.5 mg/mL	D-Mannitol, pH adjuster	Instillation	Clear
Metilon®	Sulpyrine	2.5 mg/mL	Benzyl alcohol	Instillation	Clear
Erythrocin®	Erythromycin Lactobionate	2.5 mg/mL	Benzyl alcohol	Instillation	Clear
Dalacin® S injection	Clindamycin phosphate	3 mg/mL	Benzyl alcohol	Instillation	Clear
Group 5[a]					
Tienam® for intravenous drip infusion	Imipenem Cilastatin sodium	5 mg/mL	Sodium hydrogencarbonate	Instillation	Achroma yellow (clear)
Glucose® injection	5% glucose			Instillation	Clear
Fesin®	Ferric oxide, saccharated	0.4 mg/mL		Instillation	Clear
Actit® injection	Maltose, sodium chloride, potassium chloride, magnesium chloride, potassium dihydrogen phosphate, sodium acetate			Instillation	Clear
Atropine sulfate injection	Atropine sulfate	0.5 mg/mL		Intravenous injection	Clear
Viccillin® for injection	Ampicillin sodium	10 mg/mL		Instillation	Clear
Neophyllin®	Aminophyline	0.5 mg/mL	Ethylenediamine	Instillation	Clear
Fosmisin®-S Bag 2g for intravenous drip infusion	Fosfomycin sodium	20 mg/mL	Glucose solution	Instillation	Clear
Calcicol®	Calcium gluconate	85 mg/mL		Instillation	Clear
Cefamezin® α	Cefazolin sodium hydrate	10 mg/mL		Instillation	Clear
PN-Twin® No.2	Amino acids, electrolytes		Sodium hydrogen sulfite	Instillation	Clear
Succin®	Suxamethonium chloride	2 mg/mL		Instillation	Clear
Optiray®	Ioversol	320 mg/ml as iodine		Intravenous injection	Clear
Proternol®-L injection	l-Isoprenaline hydrochloride	1 μg/mL	Sodium hydrogen sulfite, L-cysteine hydrochloride	Instillation	Clear

[a] A detailed information on this classification was described in the part of Section 2.

transferred to a 96-well plate, and absorbance of the sample was measured by μQuant (BIO-TEK Instruments, Inc., Vermont, USA) at 450 nm for methyl yellow, 530 nm for Sudan III, and 590 nm for 1,4-diaminoanthrazuinone.

2.4. Measurement of static contact angle and electrical conductivity

Ten microlitre of each surfactant solution and pharmaceutical injection was dropped on PVC sheets. After 120 s, the width and height of the drops were measured with a G-1-1000 instrument (ERMA, Tokyo, Japan). The static contact angle was computed by the following formulas

$$r^2 = (w/2)^2 + (r - h)^2, \qquad \sin \delta = (w/2)/r$$

where, r is the radius of drop (mm), w the width of drop (mm), h the height of drop (mm), δ the static angle of contact.

Electrical conductivity of each test solution was measured by COS conductivity analyzer (CEH-12, Horiba, Tokyo, Tokyo).

2.5. Elution test of DEHP and determination of DEHP content

PVC sheet (1 cm × 3 cm, thickness: 0.4 mm) was put in a screw-capped glass tube, and 5 ml of pretest solutions (Sandimmun®, Prograf®, HCO-60, Tween® 80, and SDS) were added to the respective tubes. After shaking for 2 h at room temperature, an aliquot (0.1 ml) of the solution was taken into another glass tube, and distilled water (2 ml), sodium chloride (10 mg), and 5 ml of diethyl ether containing 50 ng/ml DEHP-d_4 were added to the tube. After shaking for 30 min followed by centrifugation at 3000 rpm for 10 min at room temperature, the organic phase was collected and dehydrated with anhydrous sodium sulfate followed by GC-MS analysis described below.

Pharmaceutical injections including Sandimmun® and Prograf® adjusted to the concentration used for medical treatment were enclosed in PVC tubing (inner diameter, 2.13 mm) cut to 10 cm length. The length and volume of the enclosed injection were 8 cm and 0.285 ml, respectively, and the surface area in contact with the enclosed injection was 5.35 cm². After shaking the tube for 1 h at room temperature, the enclosed test solution was transferred to a screw-capped glass tube,

and the sample for GC-MS analysis was prepared by the same method as that described above.

To determine DEHP content, PVC sheet and tubing (20 mg) were dissolved in 20 ml of THF by soaking overnight at room temperature. An aliquot (0.1 ml) of the solution was diluted 10,000 times with diethyl ether containing 50 ng/ml DEHP-d_4, and then analyzed by GC-MS. DEHP contents of the PVC sheet and tubing used in this study were 36.2 and 32.9% (w/w), respectively.

2.6. GC-MS analysis

A JMS700 instrument (JEOL, Tokyo, Japan) equipped with a Hewlett-Packard HP6890 series GC system and an auto-injector (Agilent Technologies, Palo Alto, CA) were used for GC-MS analysis (resolution = 5000). Chromatographic separation was performed with BPX-5 fused silica capillary column (25 m × 0.22 mm I.D., film thickness: 0.25 μm, SGE, Melbourne, Australia).

The sample (2 μl) was injected in the pulsed splitless mode. The injector temperature was 260 °C. Flow rate of helium carrier gas was 1 ml/min. Column temperature was programmed as initial temperature to 120 °C for 2 min then increasing to 300 °C at 10 °C/min. Electron impact (EI)-mass spectrum was recorded at 70 eV, and the ions of m/z 149.024 for DEHP and 153.049 for DEHP-d_4 were selected as the quantitative ions in the selective ion mode (SIM) analysis using the lock and check method of calibrating standard ions (m/z 168.989 of PFK). Quantitative analysis of each sample was repeated five times for calibration lines and three times for the other samples. Preparation of calibration curves and calculation of quantitative data were performed by the computer software TOCO (Total Optimization of Chemical Operations), Version 2.0, practicing the function of mutual information (FUMI) theory (Hayashi and Matsuda, 1994; Hayashi et al., 1996, 2002; Haishima et al., 2001, 2004).

3. Results and discussion

3.1. Precision of quantitative GC-MS analysis and release profile of DEHP from PVC sheet

Background analyses of DEHP originating from each reagent and GC-MS instrument showed that
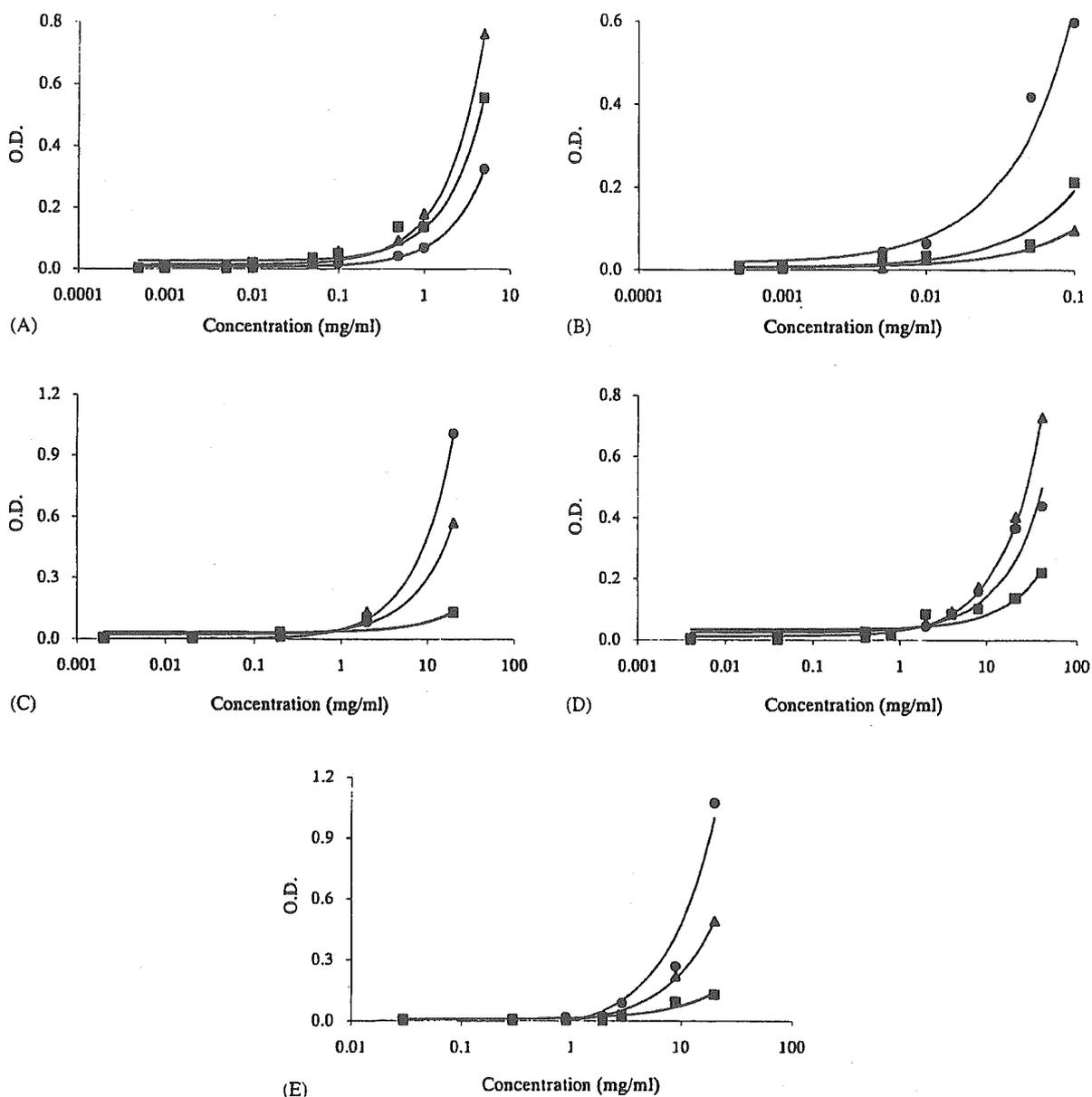
Fig. 1. Lipophilic pigment solubility against various concentrations of (A) Sandimmun®. (B) Prograf®, (C) HCO-60, (D) Tween® 80, and (E) SDS. Methyl yellow(●), Sudan III (■), and 1,4-diamino-anthraquinone (▲) were used as the pigments. Absorbance of methyl yellow dissolved in Sandimmun® and Tween® 80 was measured after five times dilution with distilled water.

0.93 ± 0.31 ng/ml DEHP ($n = 5$) was detected as background contamination when 50 ng of the internal standard (DEHP-d_4) was used in the quantitative analyses. On the basis of the background value, the experimental LOD and LOQ were calculated as 1.85 and 4.01 ppb, respectively. A calibration curve was obtained for the peak ratio of DEHP to DEHP-d_4 versus

DEHP concentration level. The response was found to be linear in the validated range (5–200 ppb) with correlation coefficient (r) exceeding 0.999. Further, the 95% confidence interval calculated by TOCO was sufficiently narrow, indicating that the present GC-MS method could be used for DEHP analysis with high accuracy.

Table 2
DEHP release capacity and physicochemical properties of lipophilic injections and surfactants

Solution (mg/ml)	Release amount of DEHP		Lipophilic pigments' solubility[a]						Electrical conductivity		Contact angle to PVC sheet	
			Methyl yellow[b]		Sudan III		1,4-diamino anthraquinone					
	ppm	S.D.	O.D. at 450 nm	S.D.	O.D. at 530 nm	S.D.	O.D. at 590 nm	S.D.	μS/cm	S.D.	°	S.D.
Sandimmun®												
0.0005	0.22	0.003	0.001	0.002	0.001	0.001	0.001	0.001	12.13	0.56	84.69	1.35
0.001	0.35	0.01	0.003	0.003	0.009	0.001	0.001	0.001	11.93	0.82	nt	nt
0.005	0.77	0.01	0.003	0.001	0.006	0.002	0.003	0.001	12.55	0.46	78.17	1.77
0.01	1.16	0.01	0.004	0.001	0.020	0.001	0.005	0.001	12.02	0.61	72.36	0.21
0.05	2.84	0.01	0.019	0.001	0.036	0.000	0.019	0.001	12.46	0.31	64.72	0.55
0.1	4.22	0.03	0.018	0.001	0.051	0.001	0.059	0.001	11.66	0.55	60.39	0.97
0.5	9.01	0.05	0.042	0.001	0.137	0.001	0.094	0.001	18.91	0.36	50.47	1.48
1	10.90	0.15	0.069	0.001	0.136	0.001	0.180	0.004	26.90	0.78	46.65	1.98
5	22.19	0.26	0.325	0.001	0.555	0.002	0.762	0.005	104.80	1.32	42.05	1.62
Prograf®												
0.0005	0.25	0.01	0.006	0.001	0.009	0.001	0.002	0.002	8.11	0.26	81.07	0.26
0.001	0.34	0.02	0.010	0.001	0.009	0.005	0.004	0.001	8.09	0.32	79.38	1.01
0.005	0.99	0.01	0.043	0.001	0.022	0.002	0.006	0.001	8.53	0.15	75.06	0.66
0.01	1.71	0.003	0.063	0.001	0.033	0.005	0.025	0.001	8.61	0.22	74.66	1.52
0.05	5.31	0.05	0.418	0.005	0.062	0.002	0.057	0.001	10.52	0.45	67.54	0.88
0.1	8.95	0.04	0.597	0.004	0.211	0.005	0.097	0.001	11.51	0.38	65.07	0.87
0.5	42.26	1.64	nt	nt	nt	nt	nt	nt	nt	nt	55.67	0.83
HCO-60												
0.002	0.09	0.01	0.003	0.003	0.005	0.001	0.001	0.001	13.27	0.52	84.22	1.92
0.02	0.28	0.01	0.003	0.003	0.010	0.001	0.001	0.001	16.07	0.66	80.79	1.39
0.2	1.15	0.01	0.011	0.001	0.033	0.001	0.012	0.001	16.51	0.43	76.54	2.48
2	5.72	0.04	0.083	0.001	0.106	0.002	0.135	0.001	16.39	0.59	66.23	0.34
20	22.32	0.25	1.006	0.005	0.130	0.013	0.571	0.007	18.36	0.64	63.31	5.18
40	28.90	0.22	nt	nt	nt	nt	nt	nt	26.80	0.80	61.02	0.70
Tween® 80												
0.004	0.38	0.01	0.001	0.001	0.005	0.001	0.002	0.002	15.93	0.38	84.01	1.28
0.04	0.49	0.01	0.001	0.001	0.009	0.001	0.002	0.003	14.82	0.29	77.91	0.40
0.4	2.77	0.02	0.011	0.001	0.027	0.001	0.010	0.003	15.60	0.41	70.28	0.87
0.8	4.30	0.03	0.015	0.002	0.018	0.001	0.017	0.001	16.49	0.35	68.78	1.23
2	6.58	0.03	0.045	0.001	0.083	0.002	0.055	0.001	15.20	0.47	64.43	6.80
4	9.26	0.15	0.083	0.001	0.083	0.004	0.094	0.003	13.49	0.33	58.70	1.03
8	13.17	0.17	0.159	0.002	0.101	0.001	0.175	0.003	18.50	0.50	56.05	0.33
20	20.07	0.32	0.365	0.007	0.136	0.001	0.403	0.002	31.40	0.82	54.21	0.53
40	25.56	0.20	0.438	0.004	0.219	0.002	0.728	0.004	57.70	0.91	51.89	0.61
SDS												
0.03	0.44	0.005	0.001	0.001	0.009	0.001	0.001	0.001	20.90	0.59	82.48	1.29
0.3	1.10	0.02	0.002	0.001	0.006	0.002	0.001	0.001	41.90	0.72	77.65	0.57
0.9	2.25	0.01	0.021	0.019	0.007	0.001	0.001	0.001	102.20	1.33	63.15	0.93
2	3.70	0.01	0.022	0.001	0.018	0.001	0.002	0.001	373.00	1.56	41.51	0.63
3	6.67	0.03	0.088	0.001	0.027	0.001	0.024	0.001	533.00	1.96	40.03	1.21
9	14.75	0.09	0.268	0.003	0.094	0.001	0.220	0.003	1120.00	2.42	40.23	0.64
20	18.05	0.18	1.071	0.014	0.129	0.003	0.491	0.004	3220.00	2.68	33.94	3.09

nt, not tested.
[a] Values after substracting blank value.
[b] O.D. of Sandimmun and Tween 80 was measured after five times dilution with distilled water.

Release test of DEHP from medical grade PVC sheet was performed using GC-MS analysis. Two kinds of pharmaceuticals and three kinds of surfactants were used as the test solutions for DEHP extraction. Qualitative analysis of DEHP was performed by scan mode EI-MS (Haishima et al., 2004), and the release profile of DEHP from the sheet is shown in Table 2. Sandimmun® and Prograf®, typical lipophilic injections containing polyoxyethene castor oil or HCO-60, and ethanol as additives, were found to release DEHP from the sheet concentration-dependently. Significant release of DEHP was observed at concentrations higher than 0.05 mg/ml, and the released amounts reached 22.19 ± 0.26 ppm by Sandimmun® (5 mg/ml) and 42.26 ± 1.64 ppm by Prograf® (0.5 mg/ml). Three kinds of surfactant, including HCO-60, Tween® 80, and SDS, were also found to release DEHP from the PVC sheet in a concentration-dependent manner. In particular, the release was significantly increased more than the concentration of approximately 1 mg/ml that is critical micelle concentration (CMC) of each surfactant, and the released amounts reached 28.90 ± 0.22, 25.56 ± 0.20, and 18.05 ± 0.18 ppm by the extraction with 40 mg/ml of HCO-60, Tween® 80, and 20 mg/ml of SDS, respectively (Table 2).

3.2. Determination of physicochemical property of test solution

Three kinds of physicochemical properties of Sandimmun®, Prograf®, HCO-60, Tween® 80, and SDS were measured to determine whether the properties could be used as markers to predict the level of DEHP released by these solutions from medical grade PVC sheet as described above. As shown in Fig. 1 and Table 2, the absorbance of each lipophilic pigment, including methyl yellow, Sudan III, and 1,4-diaminoanthraquinone, which have different absorption maximums, dissolved in each solution was increased in proportion to the rise of the solution concentration. Of the three kinds of lipophilic pigment, methyl yellow exhibited the highest response regarding the increase of absorbance, and the response of Sudan III was the lowest.

In order to evaluate the affinity of the test solutions against PVC sheet, static contact angle to the surface of PVC sheet was measured. As shown in Table 2, the angle of each solution was decreased in a concentration-

dependent manner, indicating that the affinity was increased according to the rise of solution concentration. The electrical conductivity of each test solution was also measured as a marker predicting DEHP release level. As shown in Table 2, electrical conductivity of all the solutions except Prograf® was increased in a concentration-dependent manner. In particular, the value of SDS, an ionic surfactant, was remarkably increased according to the increase of concentration. On the other hand, no significant change was observed in the electrical conductivity of Prograf®.

As shown in Figs. 2–4, the profiles of these physicochemical properties appear to significantly relate to the release behaviors of DEHP from medical grade PVC sheet by the extraction with the solutions. However, some pharmaceuticals may exhibit very low electrical conductivity, similar to that of Prograf® (Fig. 4 and Table. 2), and the value is greatly influenced by the amounts of electrolytes present in solution rather than by the lipotropy of the solution, which is not the case for other two physicochemical properties. Taking the above findings into consideration, electrical conductivity may be not useful as a marker to predict the level of DEHP released from PVC medical devices. On the other hand, no such disadvantage was recognized in the lipophilic pigment solubility test, in which good correlation to the release behavior of DEHP was observed (Fig. 2), indicating that the DEHP release level from PVC medical devices could be predicted by the test. Although static contact angle value appears to change linearly according to the concentration of the test solution, the value suggests that this property may also be useful as a marker (Fig. 3).

3.3. Detailed evaluation of the relationship between release potency of DEHP and physicochemical properties of pharmaceuticals

A detailed investigation was performed to evaluate the relationship between release behavior of DEHP from medical grade PVC tubing used as a transfusion set and the physicochemical properties, namely lipophilic pigment solubility and static contact angle, of pharmaceuticals. For this investigation, 53 pharmaceutical injections including Sandimmun® and Prograf® as positive control were scientifically selected from 180 injections used in the department of Obstetrics