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	Cefazolun 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 cm^2. 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

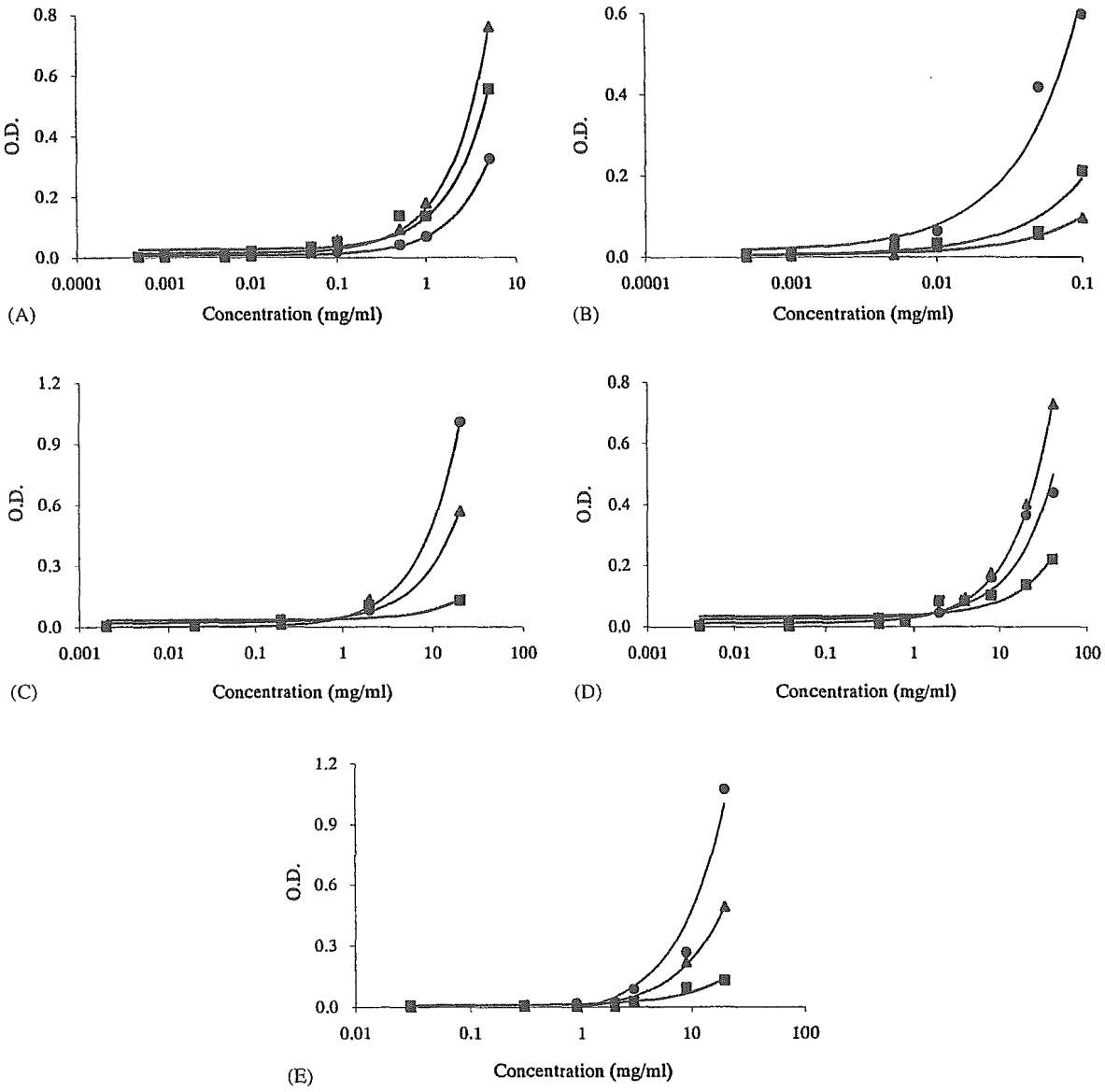

Fig. 1. Lipophilic pigment solubility against various concentrations of (A) Sandimmun®, (B) Prograf®, (C) HCO-60, (D) Tween® 80, and (E) SDS. Methyl yellow(●), Sudan III (■), and 1,4-diamino-anthraquinone (▲) were used as the pigments. Absorbance of methyl yellow dissolved in Sandimmun® and Tween® 80 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

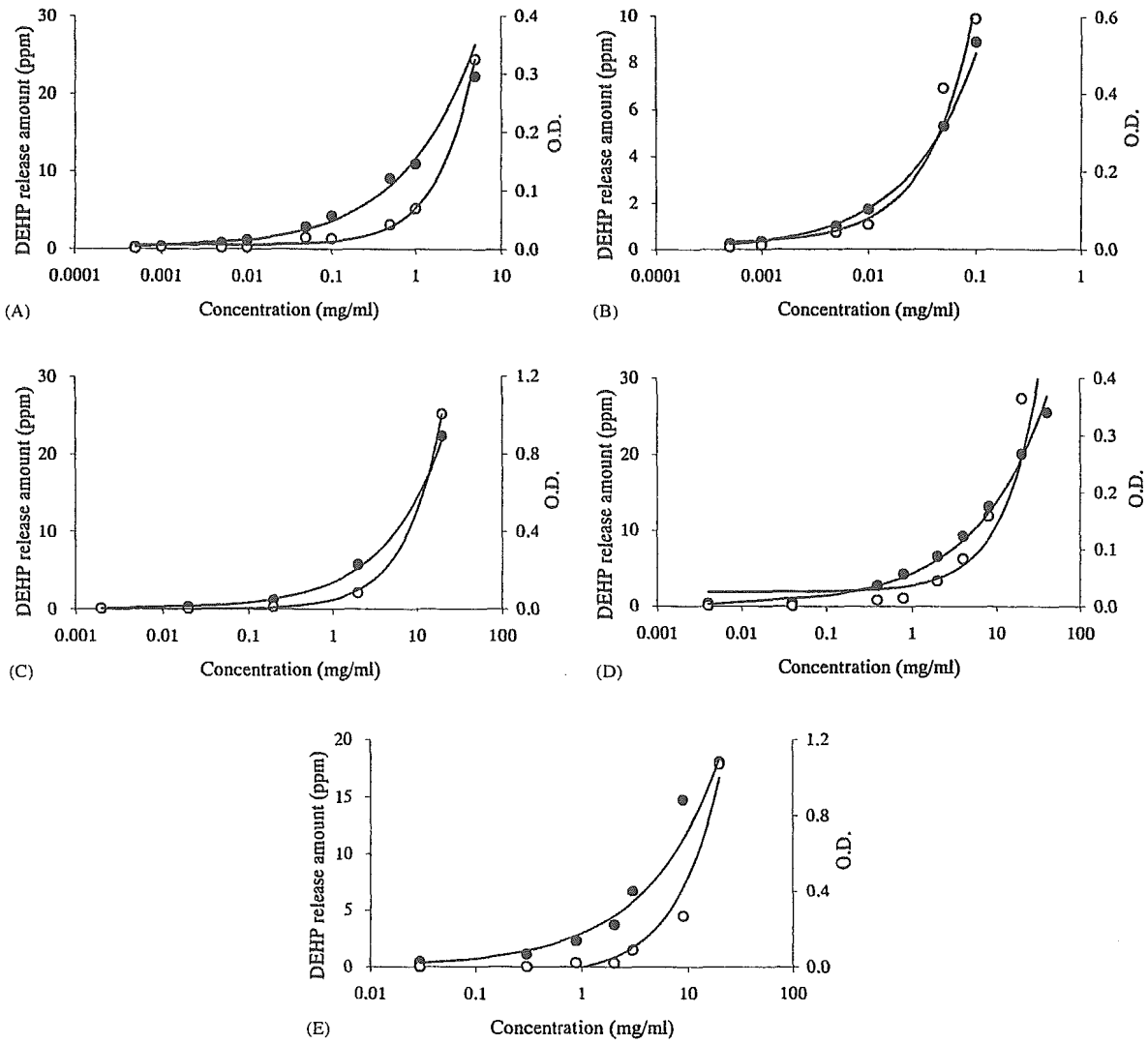

Fig. 2. Relationship between DEHP release potency (●) and methyl yellow solubility (○) of various concentrations of (A) Sandimmun®, (B) Prograf®, (C) HCO-60, (D) Tween® 80, and (E) SDS. Absorbance of Sandimmun® and Tween® 80 was measured after five times dilution with distilled water.

and Gynecology, School of Medicine, Tokai University (Kanagawa, Japan). Based on the properties of drugs and additives contained in each pharmaceutical, these injections were divided into five groups, as follows: lipophilic injections (group 1), pH-dependent pharmaceuticals for solubilization (group 2), low solubility pharmaceuticals (group 3), pharmaceuticals suspected to induce DEHP migration (group 4), and hydrophilic injections as negative control (group 5), as shown in Table 1.

The release potency of DEHP from the PVC tubing was estimated by using 53 injections adjusted to the concentration used for medical treatment (Table 1). As shown in Table 3, Sandimmun®, Diprivan®, Ropion®, and Florid®-F, assigned to group 1, released large amounts of DEHP, and significant release was also observed by Prograf®, Sohvita®, Kaytwo® N, and Horizon®. In the other injections assigned to group 1, Predonine® (10 mg/ml) showed relatively low release of DEHP, and no remarkable release was recognized by

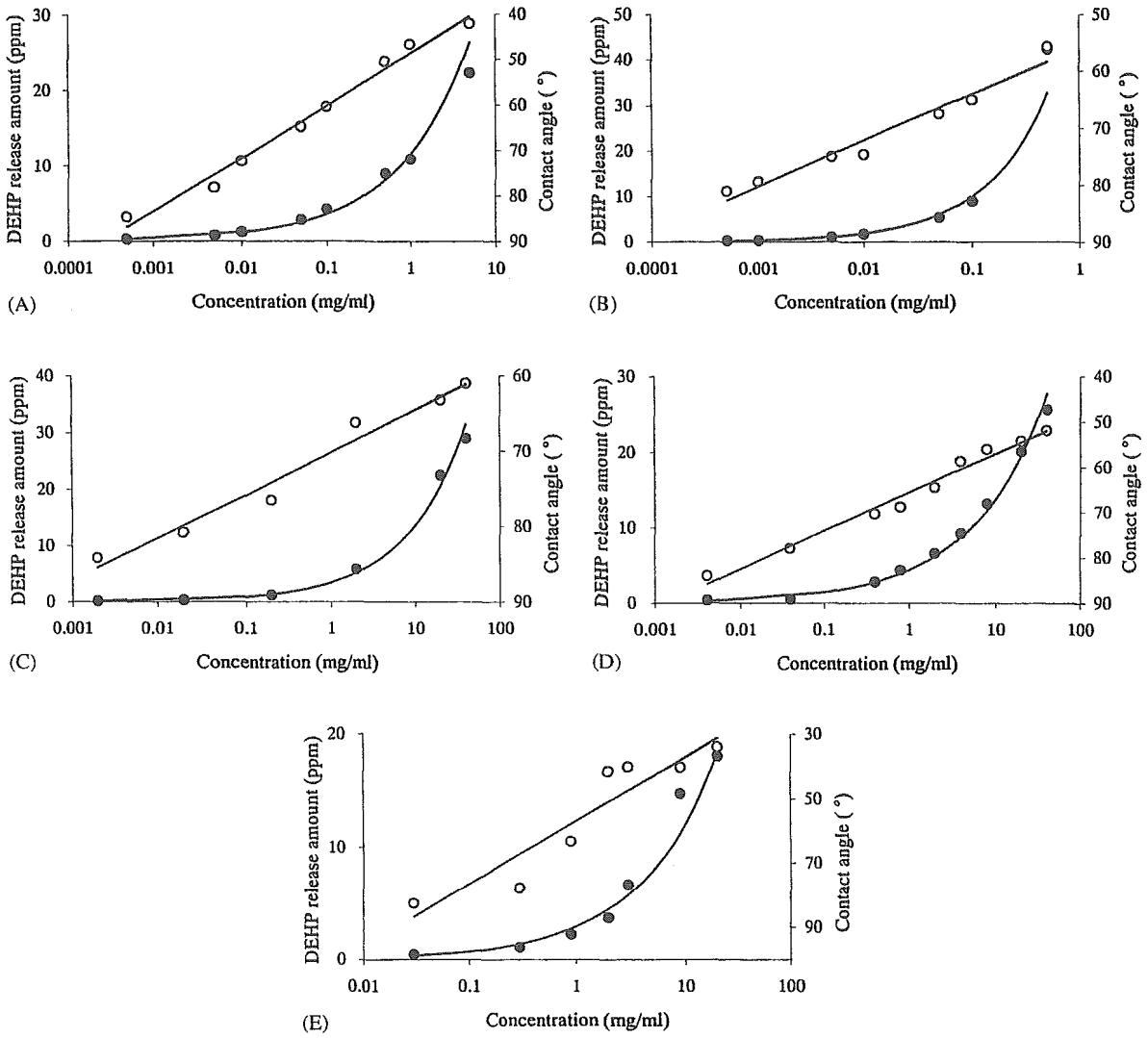

Fig. 3. Relationship between DEHP release potency (●) and static contact angle to PVC sheet (○) of various concentration of (A) Sandimmun®, (B) Prograf®, (C) HCO-60, (D) Tween® 80, and (E) SDS.

Humulin® R, Prostamon®, or Predonine® (1 mg/ml). On the other hand, no significant DEHP migration was observed by most of the other injections assigned to groups 2 through 5, and the concentration range of DEHP released into each injection was approximately 100–400 ppb. Exceptionally, Aleviatin® containing propylene glycol and ethanol (group 2) and Buminate® and Neuart®, which are human serum preparations (group 4), released relatively high amounts of DEHP, and Elaspol® (group 2) released a relatively low amount of DEHP.

The amount of methyl yellow, which exhibited the highest response regarding the increase of absorbance described above, dissolved in each pharmaceutical is listed in Table 3 as the absorbance at 450 nm. In this solubility test using lipophilic pigment, Sandimmun®, Buminate®, Florid®-F, Aleviatin®, Horizon®, Kaytwo® N, Diprivan®, and Ropion®, all of which showed potent DEHP release, showed high absorbance (over 0.8). However, absorbance of Prograf®, Neuart®, Sohvita®, and Elaspol® were lower than approximately 0.05. On the other hand, the

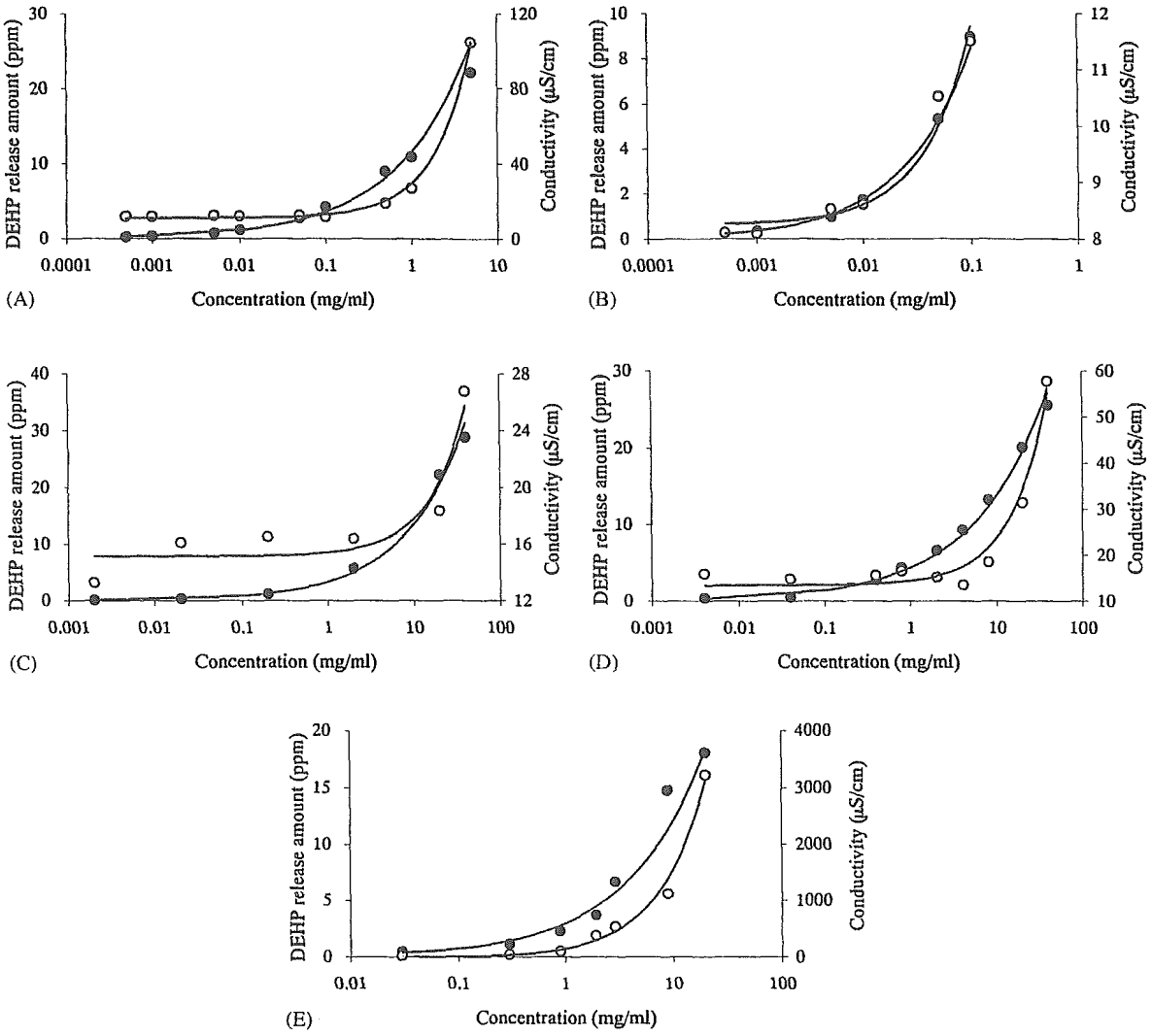

Fig. 4. Relationship between DEHP release potency (●) and electrical conductivity (○) of various concentrations of (A) Sandimmun®, (B) Prograf®, (C) HCO-60, (D) Tween® 80, and (E) SDS.

values of other injections that demonstrated low potency of DEHP release were lower than 0.026. Exceptionally, absorbance of Optiray® and of Pantol® was approximately 0.1.

Static contact angle values of 53 pharmaceuticals to PVC sheet are listed in Table 3. All pharmaceuticals that did not exhibit remarkable release of DEHP from medical grade PVC tubing showed relatively large contact angles ranging from approximately 70°–90°. On the other hand, among the injections showing high potency of DEHP release, Florid®-F, Horizon®, Sandimmun®, and Aleviatin® exhibited low contact angles of $36.68° \pm 2.81°$, $48.74° \pm 2.66°$, $52.73° \pm 0.93°$, and $58.30° \pm 2.53°$, respectively. However, static contact angle of Predonine® (10 mg/ml), Diprivan®, Prograf®, Sohvita®, Ropion®, Buminate®, Kaytwo® N, Elaspol®, and Neuart®, all of which also released DEHP from PVC sheet, were relatively high, with values ranging from 72.83° to 88.61°.

The relationship between the released amount of DEHP and the value of the physicochemical properties

Table 3
DEHP release capacity and physicochemical properties of pharmaceutical injections used in this study

Product name	DEHP amount migrated into injections		Contact angle to PVC sheet		Solubility of methyl yellow[a]	
	ppb	S.D.	°	S.D.	O.D. at 450 nm	S.D.
Group 1						
Sandimmun®	27363.9	384.8	52.73	0.925	0.989	0.000
Prograf®	4091.9	31.9	78.11	1.418	0.041	0.001
Diprivan®	19451.2	852.5	78.17	0.961	5.983[b]	0.103
Ropion®	17838.5	821.6	81.31	1.778	19.500[b]	0.007
Sohvita®	1157.1	5.1	81.32	1.362	0.008	0.001
Kaytwo® N	8457.5	62.9	82.20	1.102	4.105[c]	0.007
Humulin® R	281.6	6.0	76.11	2.338	0.003	0.001
Prostarmon®-F	185.8	17.3	88.41	0.451	0.001	0.000
Florid®-F	30098.3	423.3	38.68	2.810	1.366	0.028
Horizon®	2008.8	257.6	48.74	2.656	2.596	0.150
Predonine® 10 mg/ml	915.6	182.3	72.83	2.122	0.022	0.001
Predonine® 1 mg/ml	407.1	2.4	87.46	0.445	0.002	0.000
Group 2						
Gaster®	166.0	0.9	87.83	0.445	0.003	0.001
Droleptan® 2.5 mg/ml	171.0	0.6	77.74	0.880	0.008	0.001
Droleptan® 50 μg/ml	167.4	24.6	89.55	0.521	0.002	0.001
Elaspol®	885.7	10.6	86.59	1.871	0.002	0.000
Aleviatin®	5009.0	288.1	58.30	2.534	1.872	0.015
Methotrexate®	372.8	6.8	88.64	0.926	0.001	0.001
Serenace®	50.6	2.5	77.59	1.881	0.005	0.000
Bosmin®	290.3	24.6	86.63	0.819	0.006	0.000
Group 3						
Partan M	462.7	4.2	88.52	0.898	0.007	0.000
Musculax®	192.7	1.5	87.60	2.737	0.001	0.001
Carbenin®	237.0	1.2	87.14	1.205	0.001	0.001
Minomycin®	150.0	8.9	88.65	0.900	0.012	0.001
Perdipine®	211.6	24.0	87.28	1.961	0.002	0.001
Bisolvon®	174.9	23.7	85.38	0.629	0.017	0.000
Modacin®	301.0	0.5	88.86	0.870	0.002	0.001
Diflucan®	210.5	1.2	88.08	0.610	0.002	0.001
Doyle®	296.7	2.6	86.16	1.814	0.002	0.001
Adona®	246.1	3.0	88.00	2.189	0.001	0.001
Group 4						
Atonin®-O	423.1	0.8	87.48	1.170	0.002	0.001
Atarax®-P	430.8	144.4	88.53	1.242	0.002	0.001
Zantac®	197.9	29.5	88.85	0.468	0.002	0.001
Kenketsu Venoglobulin®-IH	243.9	14.3	83.98	1.888	0.018	0.001
Pantol®	412.1	18.2	69.78	1.093	0.087	0.000
Buminate®	10080.8	84.1	81.68	1.915	1.130	0.057
Neuart®	2008.2	21.8	88.61	0.930	0.003	0.001
Millisrol®	267.6	8.9	87.74	0.630	0.002	0.000
Metilon®	302.8	3.8	86.80	1.745	0.001	0.001
Erythrocin®	92.2	0.7	81.49	3.162	0.003	0.000
Dalacin® S	274.9	4.0	84.56	1.232	0.002	0.001
Group 5						
Tienam®	205.1	1.6	88.64	0.909	0.002	0.000
Glucose®	284.6	4.8	87.38	1.333	0.002	0.001
Fesin®	244.5	5.5	87.97	1.859	0.026	0.011

Table 3 (*Continued*)

Product name	DEHP amount migrated into injections		Contact angle to PVC sheet		Solubility of methyl yellow[a]	
	ppb	S.D.	°	S.D.	O.D. at 450 nm	S.D.
Actit®	262.8	5.0	86.88	2.117	0.002	0.001
Atropine sulfate	200.7	5.1	87.99	1.065	0.001	0.001
Viccillin® for injection	262.3	6.8	88.85	0.886	0.003	0.000
Neophyllin®	301.1	4.0	89.77	0.466	0.001	0.005
Fosmisin®-S	289.6	6.7	88.39	0.462	0.001	0.000
Calcicol®	179.4	4.3	88.20	1.259	0.001	0.001
Cefamezin® α	215.1	0.9	87.93	1.171	0.003	0.001
PN-Twin® No.2	328.5	5.0	88.37	0.941	0.001	0.000
Succin®	228.6	2.1	89.20	0.226	0.002	0.001
Optiray®	404.0	79.5	85.49	0.761	0.162	0.002
Proternol®-L	326.3	8.6	87.75	1.425	0.002	0.001

[a] Values after substracting blank value.
[b] Measured after 50 times dilution.
[c] Measured after five times dilution.

is shown in Figs. 5 and 6. The released amount of DEHP was calculated as the absolute value when 3 m of PVC tubing (inner diameter, 2.13 mm) is used for medical treatment (one time per day), and the times required for intravenous injection and instillation through transfusion set was assumed to be 5 min and 1 h, respectively. Although it is known that the released amount of DEHP from PVC tubing is influenced by drip rate (Hanawa et al., 2000; Hanawa et al., 2003), this factor was not considered in this risk assessment. When body weights of adult and neonate patients were assumed to be 50 and 3 kg, respectively, the absolute amounts of DEHP corresponding to the lower limit (40 μg/kg/day) of TDI value restricted by JMHLW represented 2000 and 120 μg per day, respectively. As shown in Fig. 5, a good proportional correlation was recognized between the DEHP release potency and methyl yellow solubility of each pharmaceutical. The response was found to be linear with correlation coefficient exceeding 0.707 for the pharmaceuticals administered by instillation and

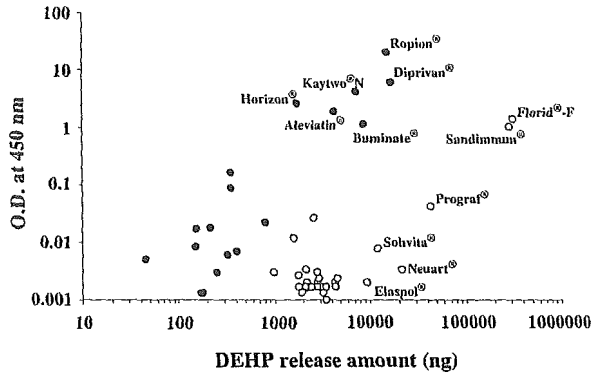

Fig. 5. Relationship between the released amount of DEHP and methyl yellow solubility of the medical use concentration of 53 pharmaceuticals. The released amount of DEHP was calculated as the absolute value when 3 m of PVC tubing (inner diameter, 2.13 mm) is used for medical treatment (one time per day), and the times required for intravenous injection (●) and instillation (○) through transfusion set were assumed to be 5 min and 1 h, respectively.

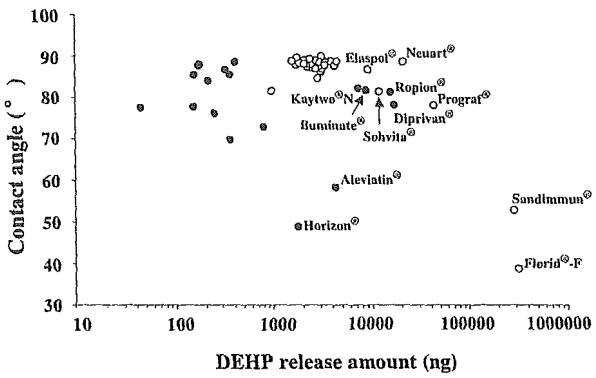

Fig. 6. Relationship between the released amount of DEHP and static contact angle of the medical use concentration of 53 pharmaceuticals. The released amount of DEHP was calculated as the absolute value when 3 m of PVC tubing (inner diameter, 2.13 mm) is used for medical treatment (one time per day), and the times required for intravenous injection (●) and instillation (○) through transfusion set were assumed to be 5 min and 1 h, respectively.

0.819 for the pharmaceuticals by intravenous injection. Most of the pharmaceuticals administered by instillation did not cause DEHP exposure to patients over the lower limit of the TDI value. It was noted, however, that Sandimmun® and Florid®-F exhibited release of DEHP over the lower limit (120 μg) for neonates. When the threshold of DEHP exposure in medical treatment using transfusion set to neonate patients was set at 0.8 as absorbance of methyl yellow, only Sandimmun® and Florid®-F of all the pharmaceuticals administered by instillation showed high absorbance (i.e., over the threshold). Although Prograf®, Neuart®, Sohvita®, and Elaspol® could release relatively large amounts of DEHP, the exposure amounts to neonate patients were under the lower limit of TDI value and the absorbance of each pharmaceutical was lower than 0.8 in methyl yellow solubility test. On the other hand, none of the pharmaceuticals demonstrating significant release potency of DEHP from PVC tubing (Table 3) when administered to the patients by intravenous injection through transfusion set, including Diprivan®, Ropion®, Buminate®, Kaytwo® N, Aleviatin®, and Horizon®, caused DEHP exposure over the lower limit of TDI value, largely because of the short time required for administration. It was demonstrated, however, that methyl yellow solubility test could reflect the real potency of DEHP release, by which Diprivan®, Ropion®, Buminate®, Kaytwo® N, Aleviatin®, and Horizon® showed high absorbance (more than 0.8). These results clearly indicate that the risk of DEHP exposure to the patients could be predicted by methyl yellow solubility test.

Similar risk assessment was performed with static contact angle to PVC sheet of pharmaceuticals as a marker, the results of which are shown in Fig. 6. The risk of DEHP release caused by Sandimmun® and Florid®-F could be predicted by creating a borderline at an angle of 60°. All other injections, with the exception of Horizon® and Aleviatin®, exhibited a large angle more than the set value. It was suggested that the pairing of propylene glycol and ethanol, contained only in Horizon® and Aleviatin® as additives, may be responsible for DEHP release and low value of static contact angle, and that the angle was not influenced by the concentrations of soy bean oil, glycerin, and lecithin contained in Kaytwo® N, Ropion®, and Diprivan®. The concentration of HCO-60 must be very significant regarding DEHP release and low contact angle, because although Prograf® contains the same or similar surfactant as Florid®-F and Sandimmun®, the medical use concentration of Prograf® is relatively low compared to those of Sandimmun® and Florid®-F; hence, Prograf® shows a high contact angle on this test. From these results, it was suggested that static contact angle to PVC sheet of pharmaceuticals could be a useful marker to predict the risk of DEHP exposure to neonate patients. It seems, however, that in contrast with the results of the methyl yellow solubility test, the contact angle to PVC sheet of pharmaceuticals does not always reflect the real potency of DEHP release, based on the findings that Kaytwo® N, Ropion®, Buminate®, and Diprivan® showed relatively high contact angles despite their high potency of DEHP release (Table 3).

4. Conclusions

In the present study, the DEHP release behavior of pharmaceutical injections was compared with the potency of physicochemical properties of the injections in order to develop a simple method for predicting the level of DEHP migrating from PVC medical devices into the injections. It was shown that although some pharmaceuticals had high release potency of DEHP from PVC products, most of the pharmaceuticals tested did not cause significant DEHP exposure to patients in the form applied for medical use. However, neonate patients may be exposed to DEHP over the lower limit of TDI value when Sandimmun® and Florid®-F are administered by instillation through transfusion set. The risk could be predicted by methyl yellow solubility test, the results of which were closely related to DEHP release potency of pharmaceuticals. Some pharmaceuticals possess their own color characteristic, and the measurement of absorbance of methyl yellow may be inhibited by a color having a λ_{max} similar to that of methyl yellow. In this case, however, it appears that Sudan III and 1,4-diamino-anthraquinone, which have different λ_{max}, can be used instead of methyl yellow as marker pigments. Thus, the solubility test of lipophilic pigments is very simple and rapid in comparison with the typical and complicated elution tests of DEHP using GC-MS and LC-MS, and it may be applicable in the medical field, particularly in hospital, as one of the methods for the safety and risk assessment of DEHP exposure originating from the use of PVC products.

Acknowledgement

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In vitro induction of polyploidy and chromatid exchanges by culture medium extracts of natural rubbers compounded with 2-mercaptobenzothiazole as a positive control candidate for genotoxicity tests

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Abstract: We tested extracts of custom-made natural rubber samples for cytotoxicity using V79 cells and for chromosome aberration (CA) induction using CHL cells in compliance with the Japanese guidelines for basic biological tests of medical materials and devices. The samples were formulated with a high level of 2-mercaptobenzothiazole (MBT) (A); a low level of MBT (B); or zinc dibutyldithiocarbamate (ZDBC) (C). In the CA test, MBT induced mainly polyploidy, including endoreduplication, and ZDBC induced structural CAs. In the cytotoxicity test, culture medium extracts of A, B, and C suppressed colony formation to 50% of the control value at 53.1%, 94.3%, and >100%, respectively. Culture medium extracts of sample A induced polyploidy and structural CAs in the absence of an exogenous metabolic activation system (S9 mix), but at lower concentrations in its presence, indicating the existence of other leachable promutagens. The extracts of sample B induced structural CAs at the highest concentration and only with S9 mix. Sample C was negative. The facts suggest that sample A may be a candidate for a positive control for genotoxicity tests. The high frequency of polyploidy induced by sample A was not predicted by MBT, suggesting the usefulness of the test for safety evaluation of medical devices. Numerical CAs induced by MBT and sample A are discussed. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 439–444, 2005

Key words: cytotoxicity; chromosome aberrations; natural rubber; zinc dibutyldithiocarbamate; endoreduplication

INTRODUCTION

Safety evaluation of medical materials is an important step in the production and marketing of medical devices. The Japanese guidelines for basic biological tests of medical materials and devices[1] cover nine assay systems for the initial evaluation. Tests for medical materials are different from those for a single chemical substance in that the samples are extracts of the test material and contain a mixture of chemicals, thus, additive and/or compound effects are expected. We have been testing model materials to search for a positive control for genotoxicity tests because there are no standard positive materials for them.

In the present study, we investigated the culture medium extracts of natural rubber–based materials in the cytotoxicity test using V79 cells and in the *in vitro* chromosomal aberration test using CHL cells in compliance with the Japanese guidelines mentioned above. Rubber materials are widely used for surgical and household gloves and for urinary catheters, although they have induced strong cytotoxicity,[2] severe allergic reactions,[3–5] and urethral strictures.[6–10] The model rubber materials used in this study were originally prepared for sensitization tests.[11] They were custom made with low allergenicity. The only allergenic components are 2-mercaptobenzothiazole and zinc dibutyldithiocarbamate.

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MATERIALS AND METHODS

Cells

We obtained Chinese hamster fibroblast V79 cells (established by Elkind and Sutton[12]) from Japanese Collection of Research Bioresources (JCRB0603, Tokyo) and grew them in

TABLE I
Recipe for Model Materials

Component	Sample A	Sample B	Sample C
ıtural rubber	100	100	100
ıc oxide	5	5	5
ɛaric acid	1	1	1
ler	62	62	62
ıck factice	5	5	5
lected microcrystalline wax	1.3	1.3	1.3
ılfur	2.0	2.0	2.0
BT	2.0	0.2	0
DBC	0	0	0.5
ım	178.3	176.5	176.8

Values: dry parts per hundred parts of rubber.

agle's minimum essential medium (MEM) (GIBCO 61100-61) supplemented with 10% heat-inactivated fetal calf se-um (FCS) in a 37°C humidified atmosphere of 5% CO_2 in ir.

We used CHL cells originally established from the lung of ı female newborn Chinese hamster by Koyama and col-eagues[13] and cloned by Ishidate and Odashima.[14] They vere maintained in Eagle's MEM (GIBCO 11095-080) sup-ɔlemented with 10% heat-inactivated FCS. The doubling ime was around 13 h, and the modal chromosome number was 25.

Chemicals and model materials

2-Mercaptobenzothiazole (MBT, CAS No. 149-30-4) from Ouchi Shinko Chemical Industrial Co., Ltd. (Tokyo, Japan) and zinc dibutyldithiocarbamate (ZDBC, CAS No. 136-23-2) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) were dissolved in dimethyl sulfoxide.

Model materials of three rubber sheets (samples A, B, and C; thickness, 1 mm) were prepared with the components shown in Table I by Atom Co., Ltd. (Tokyo) and sterilized with ethylene oxide. Zinc oxide and stearic acid were compounded as vulcanizing accelerator activators. Black factice, selected microcrystalline wax, and sulfur were compounded as a softener, an antioxidant/antiozonant, and a crosslinking agent, respectively. MBT and ZDBC are vulcanizing accelerators. Sample A contained a high level of MBT and the level of MBT in sample B was lower than one tenth of that in sample A. Sample C contained ZDBC instead of MBT.

Cytotoxicity test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL MEM supplemented with 5% FCS, nonessential amino acids, and 1 m*M* sodium pyruvate (5% FCS-GMNP) was added. After incubation at 37°C in a humidified atmosphere for 24 h, the extract, designated 100%, was decanted and serially diluted with 5% FCS-GMNP to give 80%, 64%, 51%, and 41% extracts.

V79 cells were seeded at 50/well in 24-well plates. After 24-h incubation, the medium was exchanged for 0.5 mL of the serially diluted medium extract or the medium without the extract (for control), and the cells were cultured for 6 days. The colonies formed were fixed with 10% formalin and stained with 5% Giemsa solution. The number of colonies on each well was counted, and the relative plating efficiency was calculated as the ratio of the number of colonies in the treated sample to the number in the control. The cytotoxic potential of the extracts was expressed as the concentration at which the relative plating efficiency was 50% of control (IC_{50}). The IC_{50} value was calculated by the probit method.

Chromosome aberration (CA) test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL culture medium for CHL cells was added. After incubation at 37°C in a humidified atmosphere for 48 h, the extract, designated 100%, was decanted and diluted with the culture medium.

CHL cells were seeded at 1.5 × 10^5/plate (60 mm in diameter) and incubated for 17 h. They were then treated with extracts for 6 h in the presence or absence of S9 mix followed by expression cultivation with fresh medium for another 18 h. S9 mix was purchased from Kikkoman (Noda, Japan). The S9 fraction[15] was prepared from the livers of Sprague Dawley rats pretreated with phenobarbital and 5,6-benzoflavone. The final concentration of S9 was 5 v/v%. Colcemid (0.2 μg/mL) was added for the last 2 h. Chromosome preparations were made as follows: Cells were trypsinized and incubated in hypotonic KCl solution for 15 min and fixed three times with ice-cold fixative (glacial acetic acid:methanol, 1:3). Two drops of the fixed cell suspension were spread on a clean glass slide, air dried, and stained with Giemsa solution. All slides were coded, and the number of cells with structural or numerical CAs was counted on 100 well-spread metaphases with a modal chromosome number of 25 ± 2. The number of mitotic cells was counted on 1000 live cells and the mitotic index (MI) was used to express the cytotoxic potential of the treatment. The structural CAs were classified into 6 groups: chromatid and chromosome gap (ctg), chromatid break (ctb), chromatid exchange (cte), fragmentation (f), chromosome break (csb), and chromosome exchange (cte, mainly dicentrics and ring chromosomes). The mean and standard deviation (SD) for our historical negative controls of CHL cells are 1.03 ± 1.11 (without S9 mix) and 1.25 ± 1.16 (with S9 mix) for structural aberrations, 0.60 ± 0.93 (without S9 mix) and 0.84 ± 1.02 (with S9 mix) for polyploidy, and 0 for endoreduplication. The experimental groups were judged[16] as negative if the total CA frequency was less than 5.0%, inconclusive if it was 5.0 to up to 10.0%, and positive if it was 10.0% or more. Solvent-treated cells served as the negative control. Experiments were performed at least twice. A representative data from a single experiment are shown, unless otherwise stated.

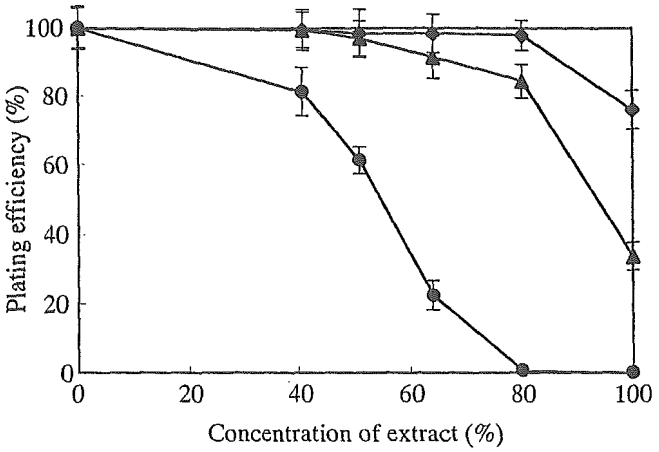

Figure 1. Plating efficiencies of V79 cells treated with medium extracts of samples A (●), B (▲), and C (◆). The samples were extracted with 5% FCS-GMNP for 24 h and the extracts were tested in the colony assay. Values are expressed as means ± SD for eight wells.

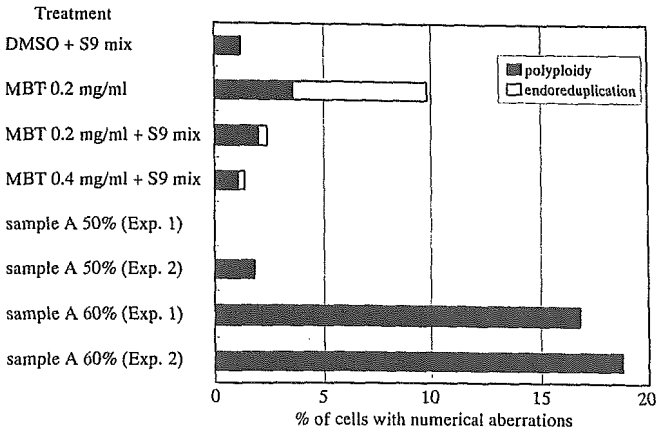

Figure 2. Numerical chromosome aberrations induced by MBT and sample A. Five hundred cells were examined.

RESULTS

In the cytotoxicity test performed with V79 cells, sample A showed the strongest response (Fig. 1). IC_{50} was 53.1%, 94.3%, and more than 100% for samples A, B, and C, respectively.

In the CA test performed with CHL cells, MBT induced polyploidy, including endoreduplication, in the absence and presence of S9 mix (Table II). The number of polyploid cells and endoreduplications was counted on another 500 metaphases for confirmation (Fig. 2). Frequency of polyploid cells and endoreduplications was 3.6% and 6.2% without S9 mix, and 2% and 0.4% with S9 mix, respectively. MBT showed inconclusive response in structural CA induction at 0.4 mg/mL with S9 mix. ZDBC induced structural CAs, mainly cte, in the presence of S9 mix and at lower concentrations in its absence.

Culture medium extracts of sample A induced numerical and structural CAs in the absence of S9 mix and structural CAs in its presence (Table III). The extracts were toxic at the higher concentrations. Structural CAs were induced at lower concentrations in the presence of S9 mix than in its absence. Interestingly, MI of the 40% extract remained high in the presence of S9 mix, although almost all the cells were dead. The numerical CAs were counted on another 500 metaphases for confirmation. The 60% extract induced 16.8% polyploidy and no endoreduplication without S9 mix (Fig. 2, Exp. 1). The 100% of extract of sample B induced 7% CAs in the presence of S9 mix. Sample C did not induce any CAs either with or without S9 mix.

TABLE II
Chromosome Aberration Test of MBT and ZDBC

Chemical	S9 mix	Conc.[a] (mg/mL)	Poly.[b] (%)	Cells with structural aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	Total	
MBT	−	0	0	2	0	0	0	0	0	2	100
		0.2	8(3)	0	3	1	0	0	0	4	110
		0.4								Tox	12
	+	0	1	0	0	1	0	0	0	1	100
		0.2	5(3)	0	2	0	0	0	0	2	158
		0.4	2	1	2	4	0	0	0	7	87
		0.6								Tox	9
ZDBC	−	0	0	2	0	0	0	0	0	2	100
		0.002	1	0	0	0	0	0	0	0	99
		0.004	0	1	4	11	0	1	0	16	116
		0.006								Tox	10
	+	0	1	0	0	1	0	0	0	1	100
		0.006	0	1	0	2	0	0	0	3	120
		0.008	1	0	2	16	0	0	0	18	104
		0.010	2	2	3	12	0	0	0	16	55

[a]Concentration.
[b]Frequency of polyploidy. Figures in parentheses indicate the number of endoreduplication included.
[c]Mitotic index.

TABLE I
Recipe for Model Materials

Component	Sample A	Sample B	Sample C
Natural rubber	100	100	100
Zinc oxide	5	5	5
Stearic acid	1	1	1
Filler	62	62	62
Black factice	5	5	5
Selected microcrystalline wax	1.3	1.3	1.3
Sulfur	2.0	2.0	2.0
MBT	2.0	0.2	0
ZDBC	0	0	0.5
Sum	178.3	176.5	176.8

Values: dry parts per hundred parts of rubber.

Eagle's minimum essential medium (MEM) (GIBCO 61100-061) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 37°C humidified atmosphere of 5% CO_2 in air.

We used CHL cells originally established from the lung of a female newborn Chinese hamster by Koyama and colleagues[13] and cloned by Ishidate and Odashima.[14] They were maintained in Eagle's MEM (GIBCO 11095-080) supplemented with 10% heat-inactivated FCS. The doubling time was around 13 h, and the modal chromosome number was 25.

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TABLE III
Chromosome Aberration Test of Culture Medium Extracts of Samples

Sample	S9 mix	Conc.[a] (%)	Poly.[b] (%)	Cells with Structural Aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	total	
A	−	0	1	0	0	1	0	0	0	1	100
		50	1	1	0	8	0	0	0	9	107
		60	20	1	0	10	0	0	0	11	146
		70	26	1	3	7	0	0	0	11	20
	+	0	4	0	0	0	0	1	0	1	100
		10	0	0	0	0	0	0	0	0	84
		20	3	0	0	7	0	0	0	7	114
		30	3	1	3	12	0	0	0	14	139[d]
		40	0	2	6	10	0	0	0	16	93[e]
B	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	0	0	0	0	0	0	92
		80	0	1	0	0	0	0	0	1	91
		100	0	0	0	0	0	0	0	0	101
	+	0	4	0	0	0	0	1	0	1	100
		60	2	1	0	1	0	1	0	3	86
		80	1	1	0	0	0	0	0	1	86
		100	2	0	0	7	0	0	0	7	85
C	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	1	0	0	0	0	1	103
		80	1	0	0	0	0	0	0	0	107
		100	1	1	0	0	0	0	1	2	110
	+	0	4	0	0	0	0	1	0	1	100
		60	1	2	0	0	0	0	0	2	93
		80	1	0	0	0	0	0	1	1	99
		100	3	0	0	0	0	0	0	0	106

[a,b, and c] See the footnote in Table II.
[d]Around 50% cells on the preparation were dead.
[e]Around 90% cells on the preparation were dead.

DISCUSSION

In an earlier study, the IC_{50} for MBT and ZDBC in the cytotoxicity test was 49 and 5.4 μg/mL, respectively.[17] These values are compared with samples A to C in the present study and the lowest toxicity concentration in the CA test (Table IV). Similar cytotoxicity was shown in both tests.

ZDBC, strongly cytotoxic, is weakly positive in the CA test using human lymphocytes[18] and negative in the bacterial reverse mutation assay,[18–20] mouse lymphoma cell mutation assay,[18] and the *in vivo* micronucleus test.[18] In the present study, ZDBC induced structural CAs at relatively low concentrations both in the absence and presence of S9 mix, but at lower concentrations in its absence, indicating that ZDBC does not require S9 mix for activity. Sample C, which was compounded with ZDBC, showed a slight cytotoxicity and was negative in the CA test, perhaps due to its low extraction efficiency by culture medium.[11,18]

TABLE IV
Comparison of Cytotoxicity

	IC_{50}*	LTC**
MBT	49 μg/mL***	400 μg/mL
ZDBC	5.4 μg/mL***	6 μg/mL
Sample A	53.1%	70%
Sample B	94.3%	>100%
Sample C	>100%	>100%

*Inhibition concentration at which the relative plating efficiency is inhibited to 50% of the control value in the cytotoxicity test.
**Lowest toxic concentration in the CA test.
***Nakamura et al.[17]

On the contrary, sample A, which was compounded with MBT, showed strong cytotoxicity and induced structural CAs at lower concentrations in the presence of S9 mix than in its absence, suggesting the existence of promutagenic leachates other than MBT. In the present study, MBT yielded an inconclusive response in structural CA induction in the presence of S9 mix, while it yielded a positive response at similar concentrations in another Chinese hamster cell line, CHO.[21] Four percent cte-type structural CAs, however, suggest, based on our historical database, that MBT may show a biologically positive response in the presence of S9 mix.

Sample A showed an interesting phenomenon in that so many dead cells coincided with well living cells in the presence of S9 mix. In the present study the number of mitotic cells were counted on 1000 live cells. MI was 139% and 93% of control at 30% and 40%