

# Screening study on hemolysis suppression effect of an alternative plasticizer for the development of a novel blood container made of polyvinyl chloride

Yuji Haishima,<sup>1\*</sup> Tsuyoshi Kawakami,<sup>2</sup> Chie Hasegawa,<sup>1</sup> Akito Tanoue,<sup>3</sup> Toshiyasu Yuba,<sup>4</sup> Kazuo Isama,<sup>2</sup> Atsuko Matsuoka,<sup>1</sup> Shingo Niimi<sup>1</sup>

<sup>1</sup>Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

<sup>2</sup>Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

<sup>3</sup>Department of Pharmacology, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

<sup>4</sup>Corporate Research and Development Division, Kawasumi Laboratories, INC., Shinagawa Intercity Tower B, 9th Floor 2-15-2, Konan, Minato-ku, Tokyo 108-6109, Japan

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**Abstract:** The aim of this study is to identify a plasticizer that is effective in the suppression of the autohemolysis of the stored blood and can be used to replace di(2-ethylhexyl) phthalate (DEHP) in blood containers. The results of hemolysis test using mannitol-adenine-phosphate/red cell concentrates (MAP/RCC) spiked with plasticizers included phthalate, phthalate-like, trimellate, citrate, and adipate derivatives revealed that di-isononyl-cyclohexane-1,2-dicarboxylate (Hexamol<sup>®</sup> DINCH), di(2-ethylhexyl)-1,2,3,6-tetrahydro-phthalate (DOTP), and diisodecyl phthalate (DIDP) exhibited a hemolysis suppression effect almost equal to that of DEHP, but not other plasticizers. This finding suggested that the presence of 2 carboxy-ester groups at the *ortho* position on a 6-membered ring of carbon atoms may be required to exhibit such an effect. The hemolytic ratios of MAP/RCC-soaked polyvinyl chloride (PVC) sheets containing DEHP or different

amounts of DINCH or DOTP were reduced to 10.9%, 9.2–12.4%, and 5.2–7.8%, respectively (MAP/RCC alone, 28.2%) after 10 weeks of incubation. The amount of plasticizer eluted from the PVC sheet was 53.1, 26.1–36.5, and 78.4–150 µg/mL for DEHP, DINCH, and DOTP, respectively. PVC sheets spiked with DIDP did not suppress the hemolysis induced by MAP/RCC because of low leachability (4.8–6.0 µg/mL). These results suggested that a specific structure of the plasticizer and the concentrations of least more than ~10 µg/mL were required to suppress hemolysis due to MAP/RCC. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2013.

**Key Words:** DEHP, alternative plasticizer, hemolysis, blood container, PVC medical device

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## INTRODUCTION

Phthalate esters, particularly di(2-ethylhexyl) phthalate (DEHP), have been extensively used as plasticizers due to their ability to increase the flexibility of polyvinyl chloride (PVC), a plastic polymer used in a wide array of products, including medical devices such as tubing, intravenous bags, blood containers, and catheters. DEHP is easily eluted from PVC products into food, pharmaceuticals, and body fluids that touch the plastic, causing the migration of DEHP directly and/or indirectly into the human body.<sup>1,2</sup>

Some phthalates, including DEHP, are considered toxic because they exhibit effects in young rodents that are similar to the antiandrogenic effects of endocrine disruptors in

male rats, wherein alterations in the development of the male reproductive system and production of normal sperm are observed.<sup>3–5</sup> Mono(2-ethylhexyl) phthalate (MEHP) is an active metabolite of DEHP and suggested that the toxic effects of orally ingested DEHP are likely caused by the corresponding monoester, and not by the intact DEHP.<sup>6–9</sup> Although the *in vivo* reproductive and developmental toxicity of DEHP in the human body are not yet well understood, recent *in vitro* toxicological studies using human cells have reported that MEHP causes adverse effects such as reduction in the number of germ cells by increasing their apoptosis without altering their proliferation.<sup>10–12</sup> Therefore, precautions should be taken to limit human exposure to

**Correspondence to:** Y. Haishima (e-mail: haishima@nihs.go.jp)

MEHP, particularly in the case of high-risk patients such as male neonates, male fetuses, and peripubertal male individuals.

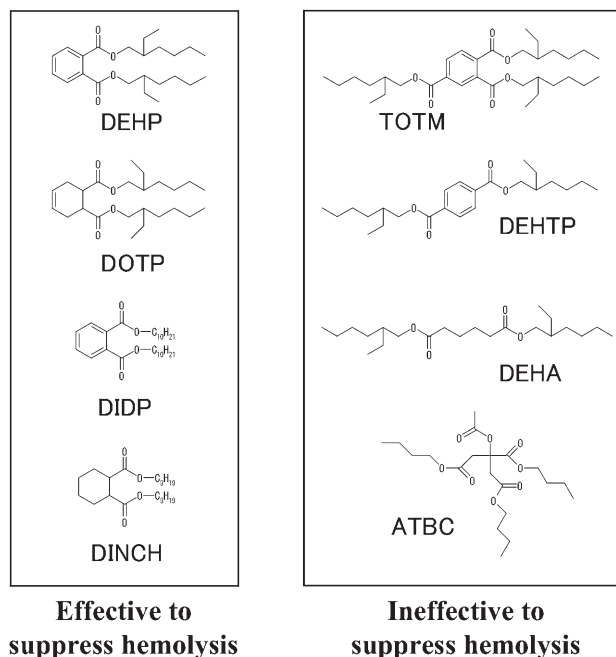
The use of plasticizers other than DEHP is an option for developing safer PVC products for human use. PVC medical devices that use trioctyl trimellitate (TOTM), di-isononyl-cyclohexane-1,2-dicarboxylate (Hexamoll® DINCH),<sup>13,14</sup> or acetyl tributyl citrate (ATBC) instead of DEHP have already been developed and are commercially available. Several agencies and official organizations worldwide have individually evaluated the safety of DEHP released from PVC products. Recently, regulation of the use of DEHP has been tightened worldwide, particularly in Europe, not only for toys, childcare products, food apparatus, containers, and packages but also for medical devices. In many countries, including USA, Canada, and Japan, the use of alternative plasticizers to develop safer PVC products and a switch to other plastic products have been recommended for the medical treatment of high-risk patients. However, the use of PVC blood bags containing DEHP has been permitted in Japan without any regulation other than storage at low temperatures because DEHP has been found to be effective in preserving stored red blood cells (RBCs).<sup>15</sup>

We recently developed a prototype of a novel and biologically safer blood container consisting of UV-irradiated PVC sheets<sup>16</sup> from which the elution of DEHP was almost suppressed. On evaluating the safety of the prototype, we found that the hemolytic ratio of the heparinized bovine blood stocked in the container was lower than that of the blood stored in normal PVC blood bags (data not shown). It has also been reported that under periodic mixing conditions, DINCH-PVC bags exhibit protective effects against RBC hemolysis almost identical to that of normal DEHP-PVC containers.<sup>17</sup> These findings suggested that the ability of DEHP to prevent hemolysis must be reviewed and that some plasticizers other than DEHP may also suppress hemolysis. In the present study, we estimated the ability of multiple plasticizers to suppress hemolysis and examined the relationship between the degree of the prevention effect and the amount of plasticizer eluted from the PVC sheets as a preliminary screening in order to identify a candidate for the replacement of DEHP in RBC storage bags.

## MATERIALS AND METHODS

### Materials, chemicals, and utensils

Eight kinds of plasticizers were used in this study (Figure 1). TOTM, ATBC, diisodecyl phthalate (DIDP), di(2-ethylhexyl)-1,2,3,6-tetrahydro-phthalate (DOTP), bis(2-ethylhexyl) terephthalate (DEHTP), di(2-ethylhexyl) adipate (DEHA), and epoxidized soybean oil (ESBO) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Hexamoll® DINCH was provided by BASF (Ludwigshafen, Germany). DEHP, DEHP-*d*<sub>4</sub>, diethyl ether of pesticide residue and PCB analysis grade, and phthalate-analytical-grade hexane were purchased from Kanto Chemical (Tokyo, Japan). Sodium chloride of pesticide residue and PCB analysis grade and phthalate-analytical-grade anhydrous sodium sulfate were purchased from Wako Pure Chemical Industries, (Tokyo,



**FIGURE 1.** Chemical structures of the plasticizers used in this study. The iso-nonyl side-chain of DINCH consists of ~10% n-nonyl, 35–40% methyloctyl, 40–45% dimethylheptyl, and 5–10% methylethylhexyl isoforms. DIDP contains an isometric mixture of phthalates with primary C10 branched dialkyl chains.

Japan) and ultrapure water obtained using Milli-Q Synthesis A10 (Millipore, Tokyo, Japan) were used to prepare the sample for gas chromatography/tandem mass spectroscopy (GC-MS/MS) analysis. Heparin sodium was purchased from the Society of Japanese Pharmacopoeia (Tokyo, Japan), and other chemicals were purchased from Wako Pure Chemical Industries. All the utensils made of glass, metal, or Teflon® were heated at 250°C for more than 16 h prior to use.

### Preparation of heparinized blood and MAP/RCC

Human blood (total of 200 mL) was obtained from a volunteer at our own laboratory. The procedure was performed in accordance with the ethical guidelines of the National Institute of Health Sciences (approval number 188). The blood was immediately mixed at 4°C with heparin (2000 units) or citrate-phosphate-dextrose (CPD) solution (28 mL) consisting of sodium citrate hydrate (26.3 g/L), citric acid hydrate (3.27 g/L), glucose (23.2 g/L), and sodium dihydrogen phosphate (2.51 g/L). The heparinized blood (Htc, 43%) was stocked at 4°C until use. The blood mixed with the CPD solution was centrifuged at 3000*g* for 10 min at 4°C followed by removal of the upper layer. Mannitol-adenine-phosphate (MAP) solution (46 mL) consisting of D-mannitol (14.57 g/L), adenine (0.14 g/L), sodium dihydrogen phosphate (0.94 g/L), sodium citrate hydrate (1.5 g/L), citric acid hydrate (0.2 g/L), glucose (7.21 g/L), and sodium chloride (4.97 g/L) was added to the remaining RCC layer to prepare MAP/RCC (Htc, 59%), and the solution was stocked at 4°C until use.

### Preparation of PVC sheets

The PVC powder (100 g) was gradually added to the mixture of DEHP (55 g) and ESBO (8 g) while stirring with a spatula. The mixed powder was gently heated from room temperature to 100°C in an oven and then stirred well. The powder was stirred a second time after heating at 100°C for 5 min to completely plasticize PVC. The plasticized powder was heat-pressed at 180°C to prepare the PVC sheets (thickness = 0.4 mm; final DEHP concentration = 33.7 w/w%). The PVC powder was also plasticized in the presence of TOTM (85 g; final concentration = 44.0 w/w%), different amounts of DINCH, DIDP, or DOTP (35, 60, 85, and 110 g, respectively; final concentration = 24.5, 35.7, 44.0, and 50.5 w/w%, respectively), instead of DEHP, and then heat-pressed using the same method. Each sheet was cut into small pieces (3.2 × 1 cm).

### Hemolysis test

Each plasticizer was dissolved in suitable amounts of diethyl ether, which was placed into a screw-capped glass bottle. After drying on a clean bench overnight without the bottle cap, 5 mL of heparinized blood or MAP/RCC freshly prepared was added to the bottle (final concentration: 1 or 100 µg/mL, respectively). Additionally, each PVC sheet (3.2 × 1 cm, thickness = 0.4 mm) containing different kinds and amounts of plasticizers was placed into a screw-capped glass bottle, and 5 mL of freshly prepared MAP/RCC was added to the bottle.

During incubation at 4°C for 35 days in the case of heparinized blood or 10 weeks in the case of MAP/RCC under continuous gentle shaking, an aliquot (50 µL) of the blood sample was collected into Eppendorf tubes every week. PBS (1 mL) was added to each sample and gently mixed, followed by centrifugation at 500 × *g* for 2 min at 4°C, and then the absorbance of the supernatant (100 µL) was measured at 415 nm with a SH-9000 Lab microplate reader (Corona Electric, Ibaraki, Japan). Heparinized blood or MAP/RCC alone was also tested under the same conditions as the negative control, while the positive control was prepared by adding distilled water instead of PBS. This test was repeated in triplicate, and the significant difference was calculated by two-way analysis of variance (ANOVA). The hemolytic ratio was calculated in accordance with the following formula.

$$\% \text{ Hemolysis} = (A_T - A_N) / (A_P - A_N) \times 100$$

$A_T$ : Test sample absorbance,  $A_N$ : Average negative control mean absorbance,  $A_P$ : Average positive control mean absorbance

### Elution test for the plasticizer

The quantity of plasticizer in each PVC sheet soaked with the blood samples was measured according to a previously reported method.<sup>18–20</sup> Briefly, an aliquot (50 µL) of MAP/RCC sample for the hemolysis test was collected into a screw-capped glass tube every week, and sodium chloride (1 mL, 1 w/v%), DEHP-*d*<sub>4</sub> (0.1 µg), and hexane (1 mL) were added. After shaking vigorously for 15 min and centrifuging at 3000 rpm for 10 min at room temperature, the organic phase was collected and dehydrated with anhydrous sodium sulfate and this was followed by GC-MS/MS analysis, as described below. This test was repeated in triplicate, concomitantly with the hemolysis test. The significant difference was calculated by two-way ANOVA.

### GC-MS/MS analysis

The plasticizers in each sample were measured by GC-MS/MS, using a Trace GC with a Quantum XLS (Thermo Fisher Scientific, Waltham, MA) equipped with DB-5MS fused silica capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 µm; Agilent Santa Clara, CA). The carrier gas was He with a flow rate of 1.0 mL/min. The temperature of the injector, transfer line, and ion source were 250°C. The sample (1 µL) was injected in the splitless mode. The GC oven temperature was initially maintained at 60°C for 2 min, and it was increased to 310°C at a rate of 20°C/min. The MS/MS system was operated under the multiple reaction-monitoring mode (MRM) with electron impact ionization (EI: 70 eV). Ar gas was used as the collision gas (0.13 Pa). The retention times, the precursor (*Q*<sub>1</sub>) and product (*Q*<sub>2</sub>) ions of the plasticizers, and the collision energies of each plasticizer were listed in Table I.

The product ions of all the plasticizers were used for quantification that was performed using DEHP-*d*<sub>4</sub> as the internal standard. The concentrations of DINCH and DIDP were determined using the sum of the total peak area of their isomers, similar to a previous study.<sup>21</sup> The limits of detection and quantification (LOD and LOQ, respectively)

**TABLE I. Retention Times, Precursor Ions (*Q*<sub>1</sub>), Product Ions (*Q*<sub>2</sub>), Collision Energies, LODs, LOQs, Recoveries, and its Coefficients of Variation (CV) of the Target Chemicals.**

Chemicals	Retention Time (min)	<i>Q</i> <sub>1</sub> [m/z]	<i>Q</i> <sub>2</sub> [m/z]	Collision Energy (V)	LOD <sup>a</sup> (ng/mL)	LOQ <sup>a</sup> (ng/mL)	Recovery <sup>b</sup> (%)	CV (%)
DOTP	14.34	170	124	7	0.12	0.39	101	2.8
DEHP	14.54	167	149	4	0.051	0.17	99	1.5
DINCH	14.60–16.60	155	109	5	0.45	1.5	101	1.8
DIDP	15.60–17.60	307	149	13	0.64	2.1	109	5.5
TOTM	20.97	305	193	16	0.040	0.13	123	7.1
DEHP- <i>d</i> <sub>4</sub> <sup>c</sup>	14.53	171	153	4				

<sup>a</sup> Calculated by TOCO version 2.0 (FUMI theory). The values correspond to the concentration in the injected solution.

<sup>b</sup> Adding every compound to the blood sample (10 µg/mL, *n* = 5).

<sup>c</sup> Internal standard.

were calculated using total optimization of chemical operations (TOCO) software version 2.0 and the function of mutual information (FUMI) theory.<sup>22</sup> The concentrations obtained using the relative standard deviations of 33% and 10% on the basis of the mass chromatograms of the standard and blank solutions, respectively, were used as the instrumental LOD and LOQ. The recovery test was conducted by adding every compound to the blood sample (10 µg/mL,  $n=5$ ). The blank test was conducted using the blood sample without PVC sheets. Only DEHP was detected under the LOQ level.

## RESULTS

### Identification of an alternative plasticizer effective in suppressing hemolysis

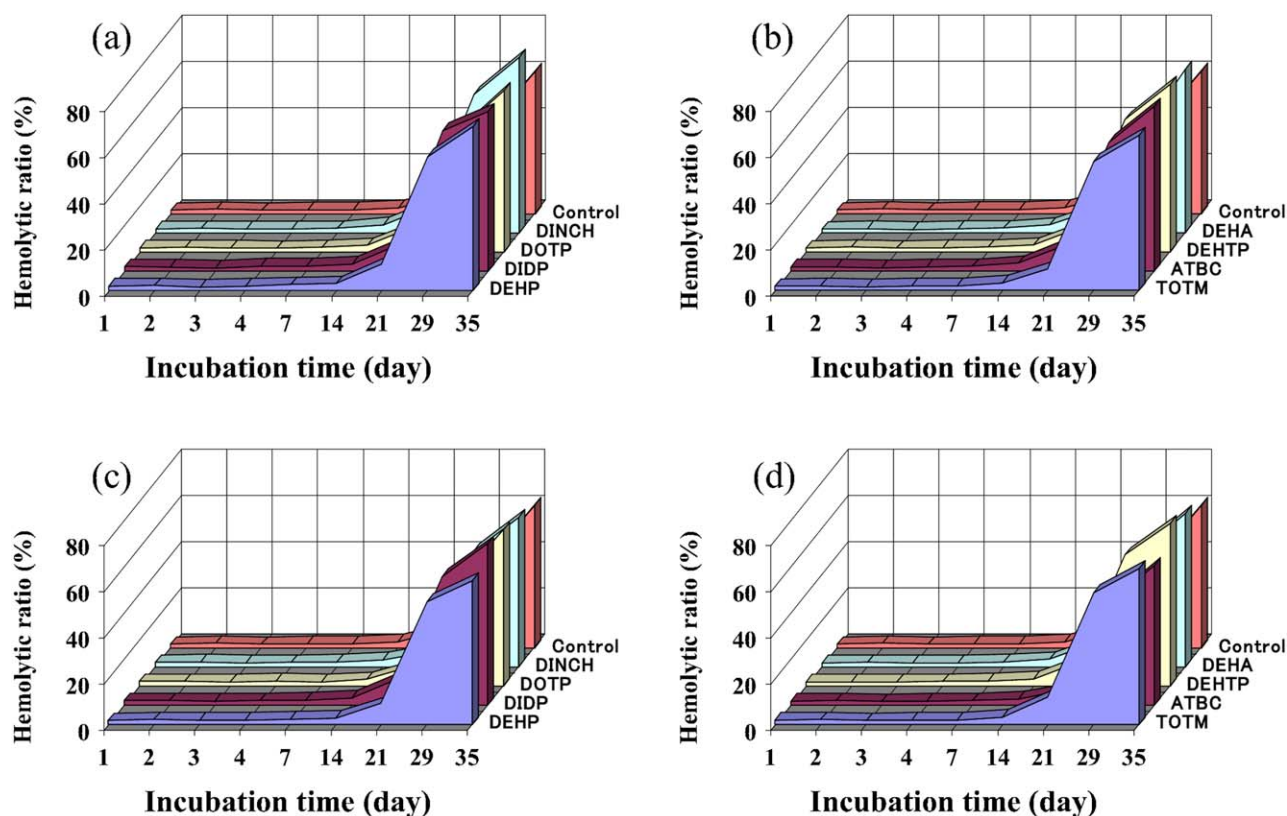
First, we estimated whether the plasticizer itself exhibited the suppression effect on hemolysis. As shown in Figure 2, the hemolytic ratio of the heparinized blood in the absence of plasticizers increased in a time-dependent manner and reached 61.6% after 35 days of incubation (control). The hemolysis behavior of the heparinized blood spiked with each plasticizer was similar to that of the control or was slightly higher, indicating that all the plasticizers were ineffective in suppressing hemolysis irrespective of the type and amount spiked. Although all the plasticizers did not exhibit hemolysis suppression against MAP/RCC at the concentration of 1 µg/mL [Figures 3(a,b)], the hemolytic ratio of

MAP/RCC spiked with DINCH, DIDP, or DOTP at the dose of 100 µg/mL significantly decreased to 10.3%, 12.1%, and 9.5%, respectively, in comparison with the ratio of MAP/RCC containing no additives, which reached 18.9% after 10 weeks of incubation [Figure 3(c)]. The degree of the effect of the plasticizers was almost identical to that of DEHP (hemolysis ratio = 10.9% after 10 weeks of incubation). Additionally, TOTM, ATBC, DEHTP, and DEHA did not suppress the hemolysis of MAP/RCC even at the concentration of 100 µg/mL [Figure 3(d)].

### Hemolysis suppression by the PVC sheet containing plasticizer

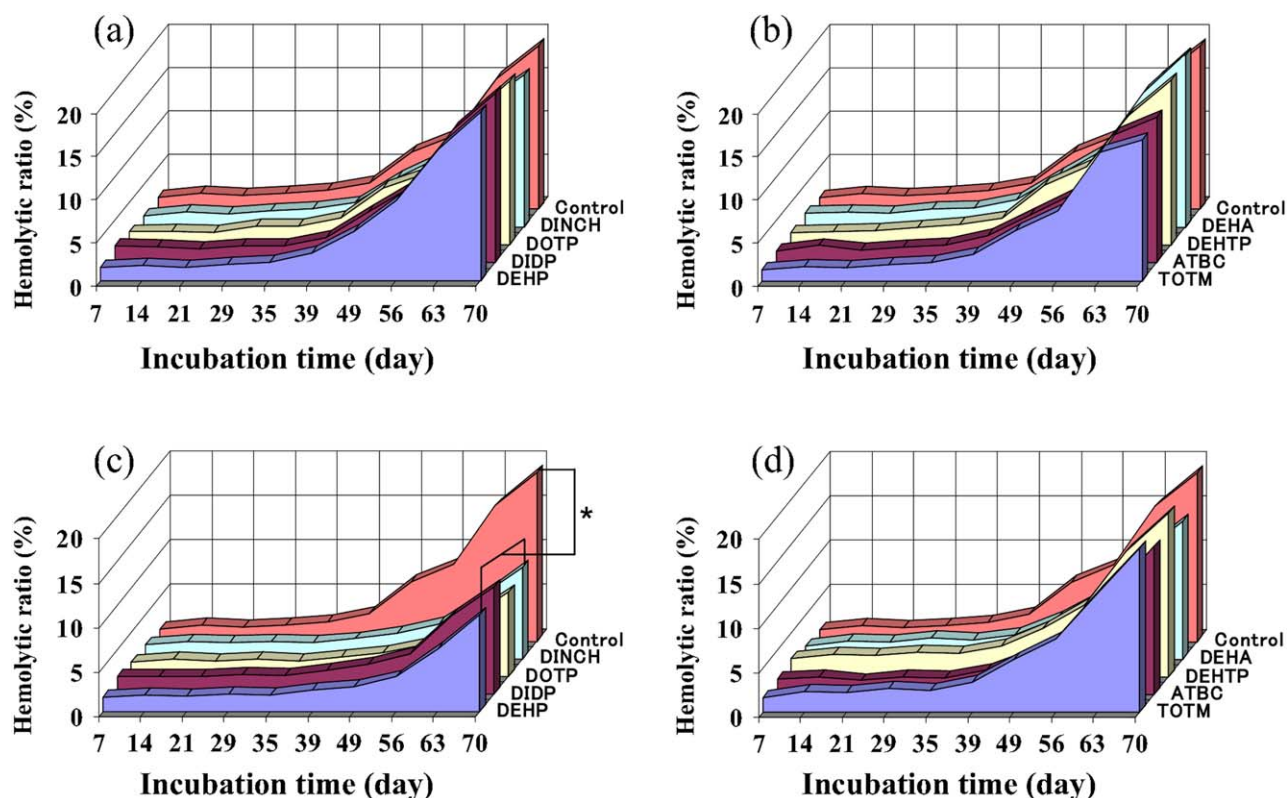
Similar to the result of the first screening, PVC sheets spiked with different amounts of DINCH, DIDP, or DOTP were prepared, and their hemolysis suppression effect against MAP/RCC was estimated. The size of the PVC sheet and the amount of MAP/RCC used in this study were decided based on the ratio between the inner surface area and the blood volume of the typical RBC storage bags (KARMI Blood Bag KBQ-200AML, Kawasumi Laboratories, INC., Tokyo, Japan). The PVC sheets spiked with DEHP or TOTM were used as the positive or negative control, respectively.

As shown in Figure 4(a), the hemolytic ratio of MAP/RCC in the absence of the PVC sheets increased in a time-dependent manner and reached 29.4% after 10 weeks of



**FIGURE 2.** Hemolytic behavior of heparinized blood containing plasticizers at the concentration of 1 µg/mL (a, b) or 100 µg/mL (c, d). No significant difference was detected between the control and each plasticizer irrespective of the type and amount spiked. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]





**FIGURE 3.** Hemolytic behavior of MAP/RCC containing plasticizers at the concentration of 1  $\mu\text{g/mL}$  (a, b) or 100  $\mu\text{g/mL}$  (c, d). \*Significant difference ( $p < 0.05$ ) was detected between the control and plasticizers, such as DEHP, DIDP, DOTP, and DINCH, effective in suppressing hemolysis at the concentration of 100  $\mu\text{g/mL}$  (c), but not among these four plasticizers. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

incubation. The hemolytic behavior of MAP/RCC in the presence of the PVC sheet containing TOTM (50.5%) was almost identical, and the ratio reached 28.2% after incubation [Figure 4(a)]. The PVC sheets spiked with DEHP (33.7 w/w%), DINCH (24.5–50.5 w/w%), or DOTP (24.5–50.5 w/w%) considerably decreased the hemolytic ratio to 10.9%, 9.2–12.4%, and 5.2–7.8%, respectively [Figure 4(a–c)]. The degree of hemolysis suppression effect of the PVC sheets containing DINCH or DOTP was not greatly influenced by the difference in the plasticizer content. Although DIDP itself had the ability to suppress hemolysis of MAP/RCC, the PVC sheet containing DIDP (24.5–50.5 w/w%) did not exhibit a significant hemolysis suppression effect, irrespective of the amounts spiked [Figure 4(d)].

#### Plasticizer elution test

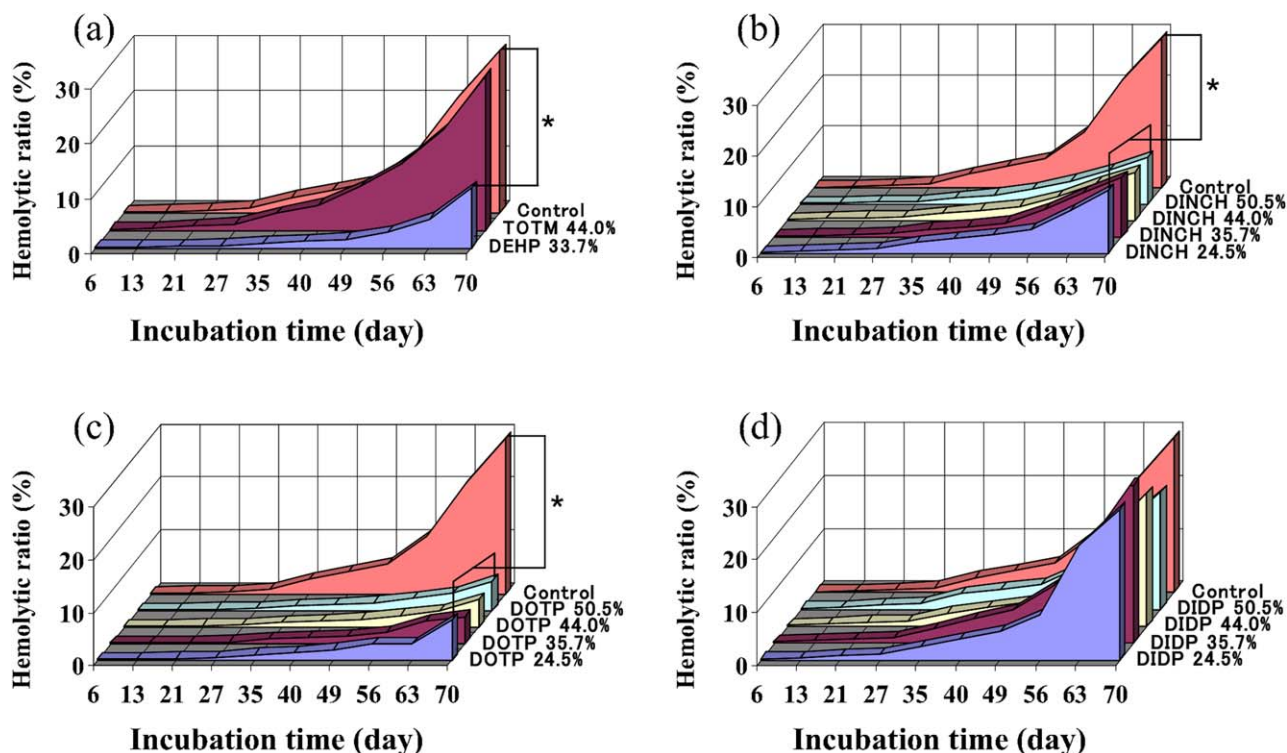
The elution of the plasticizers from the PVC sheets containing DEHP, TOTM, DINCH, DIDP, or DOTP was determined to evaluate the relationship between the degree of the hemolysis suppression effect against MAP/RCC and the amount of plasticizer released from each PVC sheet. These plasticizers were quantified by GC-MS/MS analysis by using DEHP- $d_4$  as the internal standard. The LOD, LOQ, and recovery values are listed in Table I.

As shown in Table II, the amount of DEHP released from the PVC sheet spiked with DEHP (33.7 w/w%) increased in

a time-dependent manner and reached  $53.1 \pm 6.07 \mu\text{g/mL}$  after 10-week incubation in MAP/RCC. The amount of plasticizer eluted from the PVC sheet containing TOTM (50.5%) was significantly low, corresponding to only  $0.27 \pm 0.09 \mu\text{g/mL}$  even after the 10 week incubation. The amount of plasticizer eluted from the PVC sheet spiked with DOTP (24.5, 35.7, 44.0, and 50.5 w/w%) increased in a time- and dose-dependent manner. The amount was approximately 2–3 times greater than that of DEHP and reached  $78.4 \pm 13.7$ ,  $117 \pm 8.0$ ,  $143 \pm 16.8$ , and  $150 \pm 26.0 \mu\text{g/mL}$ , respectively, after 10 weeks of incubation. The amounts of DINCH and DIDP released from the PVC sheets containing each plasticizer (24.5, 35.7, 44.0, and 50.5 w/w%) also increased in a time-dependent manner and reached 26.1–36.5 and 4.80–5.96  $\mu\text{g/mL}$ , respectively. However, a significant difference was not clearly detected in the amount of elution in response to the plasticizer content. The degree of elution of DIDP was  $\sim 10$  times lower than that of DEHP, irrespective of the amounts spiked. The amount of elution of DINCH was also lower than that of DEHP.

#### DISCUSSION

This screening study investigated alternative plasticizers exhibiting a hemolysis suppression effect against RBCs and evaluated the relationship between the degree of the suppression effect and the amount of the plasticizer eluted



**FIGURE 4.** Hemolytic behavior of MAP/RCC in the presence of PVC sheets spiked with several plasticizers. (a) No plasticizer (control); DEHP (33.7 w/w%); and TOTM (44.0 w/w%). (b) DINCH (24.5, 35.7, 44.0, and 50.5 w/w%). (c) DOTP (24.5, 35.7, 44.0, and 50.5 w/w%). (d) DIDP (24.5, 35.7, 44.0, and 50.5 w/w%). \*Significant difference ( $p < 0.01$ ) was detected between the control and DEHP (a), and control and DINCH (b) or DOTP (c), while there was no significant difference between the control and DIDP. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

from the PVC sheet. The chemical structures of the plasticizers used in this study are shown in Figure 1. The results of the hemolysis test, in which 8 kinds of plasticizers structurally categorized into five groups were used, suggested that the presence of 2 carboxy-ester groups at the *ortho* position on a 6-membered ring of carbon atoms may be needed for the plasticizer to exhibit the effect. DEHP, DIDP, DINCH, and DOTP, all of which have the ability to suppress the hemolysis of MAP/RCC, have such a structure. TOTM possesses this basic structure as a part of the molecule, but its 6-membered ring is substituted by an additional carboxy-ester group, and this might be the reason why TOTM seems to be ineffective in suppressing hemolysis. The same basic structure is not present in DEHP, DEHA, and ATBC, which did not exhibit any hemolysis suppression effect on MAP/RCC. The structure of the 6-membered ring does not seem to be restricted to aromatic compounds because the suppression effect is also seen in the case of partially unsaturated or saturated rings. The mechanism underlying hemolysis suppression by DEHP, DINCH, DIDP, and DOTP is unknown, but it could be speculated that these plasticizers may be incorporated into and stabilize the lipid bilayer of the RBC membrane, because there is a possibility that plasticizer molecules having the two carboxy-ester groups oriented to the same direction of the *ortho* position may form a pair at the inner and outer sides of the RBC membrane and act as one component of the lipid bilayer. On the other hand, in

case the orientation of the acyl groups is different from each other, the plasticizer molecules may be also incorporated into the lipid bilayer, but not form such a pair. The results suggested that the degree of hemolysis suppression effect is due in part to the concentration of the plasticizer eluted, but also to the structurally intrinsic ability of the plasticizer molecule. However, since the degree of the effect of PVC sheets containing DINCH or DOTP was not greatly influenced by the differences in the plasticizer content, the effect is saturated at a certain concentration. As shown in Figure 4, the hemolysis suppression effect induced by the PVC sheet spiked with DEHP, DINCH, or DOTP appeared after 35 days of incubation. The minimum amounts of these plasticizers eluted from each PVC sheet during the incubation period were 34.7 (DEHP), 13.5 (DINCH), and 61.3 (DOTP)  $\mu\text{g/mL}$ , respectively, while the maximum elution amount of DIDP was only 2.94  $\mu\text{g/mL}$  (Table II). Taking these results into consideration, the use of plasticizer at a concentration of least more than  $\sim 10 \mu\text{g/mL}$  seems to be necessary to protect RBCs from hemolysis because the PVC sheet spiked with DIDP did not significantly prevent the hemolysis of MAP/RCC. It has been reported that the leachability of TOTM, DINCH, and diisononyl phthalate (DINP) from PVC products is significantly lower than that of DEHP.<sup>23,24</sup> The reason for this phenomenon is still unknown, but it could be speculated that the degree of intermolecular interactions and/or steric hindrance between the PVC and

TABLE II. Amount of Plasticizer Eluted from PVC Sheet Incubated in MAP/RCC at 37°C During 10 Weeks Under the Conditions of Continuously Gentle Shaking.

Plasticizer	Final content (w/w %)	Elution amount (μg/ml)									
		6 days	13 days	21 days	27 days	35 days	40 days	49 days	56 days	63 days	70 days <sup>a</sup>
TOTM	44.0	0.04±0.01	0.04±0.01	0.05±0.01	0.06±0.01	0.11±0.07	0.25±0.19	0.35±0.34	0.12±0.05	0.09±0.04	0.27±0.09
DEHP	33.7	15.0±2.60	16.2±3.59	28.3±6.78	28.5±6.06	34.7±4.54	38.8±5.57	42.4±6.22	40.1±11.5	45.0±7.86	53.1±6.07
DINCH	50.5	3.73±0.73	4.34±1.32	11.3±1.20	12.3±2.38	16.3±4.91	20.4±2.00	22.5±4.24	25.0±3.77	27.8±5.33	36.5±4.34
	44.0	3.55±0.42	5.27±1.15	9.85±1.34	11.9±2.81	15.7±3.91	19.5±2.11	21.3±3.65	24.8±3.93	29.2±2.98	32.5±4.64
	35.7	3.31±0.66	5.03±1.59	10.2±2.17	10.7±3.19	13.5±2.31	18.3±4.87	18.2±2.02	22.0±2.82	25.2±3.52	33.3±2.39
	24.5	2.88±0.51	4.40±1.13	9.34±1.95	9.92±3.55	14.2±2.91	17.3±3.20	15.5±2.67	18.9±2.48	21.3±2.09	26.1±4.79
DIDP	50.5	0.62±0.08	0.92±0.34	1.87±0.23	1.81±0.45	2.94±0.73	3.70±0.51	3.74±0.65	4.00±0.72	5.02±0.54	5.96±0.66
	44.0	0.54±0.08	1.17±0.31	1.43±0.39	1.63±0.60	2.37±0.31	3.60±0.84	4.25±0.46	4.33±0.89	4.53±1.45	5.51±0.56
	35.7	0.53±0.05	0.86±0.20	1.57±0.20	1.67±0.55	2.41±0.63	3.39±0.52	4.05±0.48	3.75±0.34	4.40±0.88	5.35±1.39
	24.5	0.45±0.05	0.92±0.31	1.40±0.22	1.59±0.45	2.32±0.40	3.34±0.60	3.71±0.60	3.31±0.25	4.57±0.83	4.80±0.75
DOTP	50.5	40.8±4.67	45.6±14.7	58.4±21.1	83.0±21.9	92.5±23.6	110±15.1	116±12.8	123±12.3	128±26.0	150±26.0
	44.0	42.3±5.80	52.9±11.8	62.1±22.6	86.2±15.7	91.9±13.9	91.0±11.1	129±21.7	122±18.9	128±16.8	143±16.8
	35.7	28.8±7.55	36.0±12.1	53.4±15.7	71.3±18.7	73.2±12.9	97.0±23.1	112±21.2	101±11.6	94.6±16.6	117±8.00
	24.5	24.1±3.63	29.8±1.70	44.2±2.14	56.1±4.48	61.3±9.35	70.2±12.5	75.9±6.08	71.6±17.4	97.0±36.8	78.4±13.7
Control (DEHP)	–	tr <sup>c</sup>	tr	tr	tr	tr	tr	tr	tr	tr	tr

<sup>a</sup>tr, trace amount less than LOQ.<sup>a</sup>Significant difference ( $p < 0.01$ ) was detected between the elution amounts of DEHP and other plasticizers.

the plasticizer molecules may be related to differences in the elution behavior, depending on the type and number of the carboxy-ester groups present in the plasticizer molecule.

The type of anticoagulant and the viability of RBCs may be closely correlated with the expression of the hemolysis suppression effect of the plasticizer to RBCs.<sup>25</sup> The biochemical property of heparinized blood is very different from that of MAP/RCC, and hence, it is meaningful that the effect of the plasticizer on these two blood types is compared to evaluate the difference in the anticoagulant activity and the viability of RBCs as a typical case. DEHP, DIDP, DINCH, and DOTP exhibited a hemolysis suppression effect against MAP/RCC, but not against heparinized blood. Heparin inhibits the coagulation system by activating antithrombin, and the citrate present in CPD and MAP solutions inhibits the coagulation cascade by binding to  $\text{Ca}^{2+}$  ions (Factor IV). MAP solution has higher ability than heparin with respect to preserving the viability of RBCs because of the presence of glucose and mannitol in the solution. In fact, the lifetime of RBCs in heparinized blood is approximately half that of MAP/RCC, suggesting that the decrease in RBC viability may dominate over the stabilization of the cell membrane by these plasticizers. Probably, the ATP present in the heparinized blood was nearly depleted after 21 days, corresponding to the allowed limit of storage for human blood collected in heparin.

Recently, Hirata-Koizumi et al. reviewed the toxicity of alternative plasticizers such as DEHTP, DINCH, ATBC, diisononyl adipate (DINA), 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB), and tri-n-butyl citrate (TBC).<sup>26</sup> The overall toxicity of DOTP is still unknown. However, the results of the repeated dose toxicity, reproduction/development toxicity, bacterial reverse mutation, and chromosomal aberration tests conducted by BOZO Research Center (Shizuoka, Japan) as commissioned projects of the Japanese Ministry of Health, Labor, and Welfare showed that there is a high possibility that the plasticizer could be used to produce medical-grade PVC products because of minimal toxicity. These data are uploaded in the websites ([http://dra4.nihs.go.jp/mhlw\\_data/home/pdf/PDF2915-49-3d.pdf](http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3d.pdf), [http://dra4.nihs.go.jp/mhlw\\_data/home/pdf/PDF2915-49-3e.pdf](http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3e.pdf), and [http://dra4.nihs.go.jp/mhlw\\_data/home/pdf/PDF2915-49-3f.pdf](http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3f.pdf)). DOTP is a cold-resistant plasticizer and has been used to produce a power-transmission line. Cold resistance of DOTP higher than that of DEHP may provide an advantage with respect to preventing cracking, which rarely occurs during cryopreservation of DEHP-PVC containers for blood products such as human plasma. The amount of DOTP eluted from the PVC sheet is relatively higher than that of DEHP, but that amount could be artificially adjusted to the minimal amount to suppress hemolysis (~10 μg/mL as described above) by decreasing the mixing ratio of DOTP to PVC and mixing precise doses of plasticizers with low leachability, such as DINCH or TOTM, to reduce patient exposure to these substances during medical treatments. The amount of plasticizers eluted from heat-pressed PVC sheets seems to be higher than that from T-die molded PVC sheets used to manufacture commercial PVC products. A possible reason



for this behavior could be the different orientation and density of the PVC molecules present in the sheets. In addition, the conditions used in this study are different from typical RBC storage because the blood was not leukocyte-reduced and was continuously in contact with the air present in the bottle head. On the basis of these results, preparation of a novel blood container prototype made from PVC sheets containing DOTP instead of DEHP and studies on the chemical, physicochemical, biological, and dynamical characteristics of the prototype are now in progress in our laboratory.

## CONCLUSIONS

DINCH and DOTP are possible candidates as a replacement for DEHP in RBC storage bags because of their hemolysis suppression abilities. Although DIDP itself was effective in suppressing hemolysis, it could not be used as an alternative plasticizer because the amount of DIDP eluted from the PVC sheet did not reach the concentration essential for exhibiting its suppression effect.

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