示唆される医薬品添加剤を用いて，検討を行った．実際の医薬品に使用されている添加剤である，PEG 400, Tween 80, Ethylenediamine, PG, 大豆油の 5 種類の添加剤を使用した．

(3) 分子量の検討

平均分子量 23000, 26000, 27000 の 3 種類の PC 試験片を用い，分子量の違いによる破損への影響を検討した．試験には，Tween 80 を用い，撓み値を 1, 2, 3, 4 mm に設定し，PC 試験片の分子量ごとに試験に供した．

(4) 試験片の GPC 分析による分子量測定

それぞれの分子量レベルのブランク試料各 1 本と，Polyoxyethylene sorbitan monoolaeate (Tween 80)に浸漬し，応力をかけることで破損させた試料各 2 本，計 9 本を供試した．

測定にあたって，試験片の長手方向で分子量に差違があることが懸念されたため，サンプリングは各供試体とも端部と中央部(破損部)の 2 箇所とし，とくに破損品群については割れが発生している近傍からサンプリングし，測定した．測定条件は，B-2-①と同様である．

B-3. 論理面への配慮

本研究では，ヒト及び動物由来の組織，臓器，細胞などを実験に使用していないため，倫理面への特別な配慮は行っていない．

C. 研究結果

C-1-① 三方活栓を用いた検討

(1) GPC 分析

表1に平均分子量測定結果をまとめた．この結果より，試料の分子量を重量平均分子量(*M*w)で比較すると，試料1は試料2,3より低いが，2と3の間には有意差はない．ただし，各分子量は単分散ポリスチレン分子量基準の相対値で示されているので，絶対値とは隔たりがあることに注意を要する．

(2) NMR, GC/MS, HPLC分析

図1に，3試料のNMR測定チャートを示した．この図では，ポリマーの末端基や微量成分を比較し，より詳細に内容分析するために，縦軸を拡大し，PC樹脂の主鎖のピークを振り切らせている．

解析の結果，樹脂の末端として，フェノール末端が3試料に共通して観測されているのに加え，試料1,2ではt-ブチル末端が存在することが明らかとなった．このt-ブチル末端は試料3にはほとんど存在しない．

PC樹脂(ビスフェノールA型)の工業的製造法には，エステル交換法と，ホスゲン法の2方法がある．t-ブチル末端が観測されるのは，t-ブチルフェノールを停止剤として使用するホスゲン法の場合と考えられる[6]．

以上の結果より，試料3検体ではPCの製造方法が異なっており，試料1,2はホスゲン法，試料3はエステル交換法で製造されていることが推定された．

また，NMRの解析より，試料3に高分子の配合剤としてポリプロピレングリコール(PPG)が使用されていることが明らかとなった．NMRピークの積分強度よりPPGの配合量は，試料中0.5%と算出された．

表2に，GC/MSとHPLCにより定性・定量された添加剤その他について，前述のPPGを含めてまとめた．

試料1,2からは，酸化防止剤(加工安定剤)としてPC樹脂に一般的に使用されるIrgafos168 (CIBA Specialty Chemicals商品名)の酸化物，モノグリセライド，溶剤類が共通して検出された．各々の定量値はやや異なっているものの，この結果から，試料1,2はきわめてよく似たプロセスで製造された樹脂と考えられる．これに対し，試料3にはIrgafos168が使用された形跡はなく，モノグリセライドも検出されなかったことから，試料1,2とは異なるプロセスで製造された樹脂と判断できる．

C-1-② PC 試験片を用いたモデル実験

(1) 撓み値及び薬剤の検討

撓み値を変化させ，PC 試験片の破損頻度を検討したところ，撓み値が大きくなるにつれて，PC 試験片の破損頻度が増加した．これは，三方活栓の締め付け強度が破損に影響するという既報[4]と同様の結果であり，本試験系の有用性が確証された．

5 種類の医薬品添加剤を使用した試験結果を表 3 に示す．撓み値の検討と同様に，撓み

値が大きくなるほど，破損頻度が増加した．しかしながら，PEG 400，Ethylenediamine，Tween 80 で激しい破損が生じた一方で，PG，大豆油では破損が全く確認されなかった．これにより，PEG 400，Ethylenediamine，Tween 80 を添加剤として使用する医薬品では，三方活栓の破損が起こりやすいものと考えられる．

(2) 分子量の検討

Tween 80 を試験溶液として用い，分子量の異なる PC 試験片を試験に供した結果を表 4 に示す．撓み値を 1，2，3，4 mm に設定した際，撓み値の検討時と同様に，撓み値が大きくなるほど，破損頻度が増加した．更に，同じ力を与えた(撓み値が同じ)場合，分子量が小さいほど，破損頻度が増加した．他の薬剤を用いた場合もほぼ同様の結果が得られた．

(3) 試験片の GPC 分析による分子量測定

表 5 に分子量水準ごとの各試料の平均分子量測定結果をまとめた．重量平均分子量(Mw)で比較すると，今回測定した範囲では，ブランク品と破損品とも，端部と中央部で分子量に有意な差は認められない．またブランク品と各破損品で同じサンプリング位置での結果を比較しても有意な差はなく，同等の重量平均分子量である．なお，各分子量は単分散ポリスチレン分子量基準の相対値で示されているので，絶対値とは隔たりがあることに注意を要する．

各測定試料とも，分子量は約 400～約 30 万の範囲に分布しており，サンプリング位置による違いは見られない．また，ブランク品と破損品の中央部での分子量分布を比較すると，分子量の大，中，小ともに，分子量が 300 程度までの低分子量域の分布に微妙な違いが見られ，破損品の方がわずかに低分子量成分が多い傾向が見られるが，その差は小さく，有意な差とは断定できないレベルであった．

D. 考察

D-① 三方活栓を用いた検討

各測定の結果より，使用されている PC 樹脂や添加剤の内容と，破損しやすさの関係について考察する．

まず，分子量の観点から考えると，一般的に，ポリマーの分子量が低いと溶剤に溶解しやすい(耐薬品性が低い)，という関係があることと，試料 1(破損しやすい試料)のみ，やや平均分子量が低いことは，傾向としては一致している．ただし，PC 樹脂はもともと耐薬品性が低く，有機溶剤にはかなり溶解しやすいポリマーであるため，平均分子量の 1 割程度の試料間差が，どの程度影響するか定量的には判断できない．

なお，PC は，成形に伴う残留応力が発生するためソルベントクラックが起こしやすいとされている．明確なことはわからないが，残留応力の大きさにも，分子量が影響する可能性が考えられる．

樹脂・添加剤の内容を比較してみると，試料 1,2 が類似しており，試料 3 のみ，樹脂の製造プロセスも添加剤内容も異なっていることがわかる．試料 1 は破損しやすい試料，試料 2 も程度の差はあるものの破損する可能性のある試料であるのに対し，試料 3 は全く破損の起きない試料であることから，製造プロセスや添加剤が PC 製三方活栓の破損しやすさに関係していることが十分考えられる．

樹脂・添加剤内容が，どのように破損しやすさと関係しているのかは不明であるが，樹脂に別の高分子材料を配合することで強度特性が向上する場合があることを考慮すると，試料 3 にのみ，ポリプロピレングリコールが配合されていることが試料 3 の破損しにくさと関係している可能性がある．

D-② PC 試験片を用いたモデル実験

PC 試験片の分子量が小さいほど，PC 試験片の破損頻度が増すことから，分子量の大きさが PC 破損に影響を及ぼすことを確認した．今回使用した平均分子量 23000，26000，27000 の PC 樹脂は，実際に医療用具に使用されている分子量であり，破損発生頻度の違いは，分子量の違いが影響を与えていることが示唆された．一般的に分子量が大きいほど ESC に耐性があると言われており，今回の試験系においても，同様の結果が得られた．

また，5 種類の医薬品添加剤を用いて PC 試

験片の破損度合いを比較したところ，顕著な差が見られた．Ethylenediamine は化学構造中のアミンが化学反応性を有することから，PC を破損させやすいと考えられる．

GPC 分析により，ブランク品と破損品の中央部での分子量分布を比較すると，分子量の大，中，小ともに，分子量が 300 程度までの低分子量域の分布に違いが見られ，破損品の方がわずかに低分子量成分が多い傾向が見られる．算出の定義から，低分子量域の分布挙動を色濃く反映する数平均分子量(Mn)は，破損品の方が 10%程度ブランク品よりも小さいことがこのことを物語っているが，当該手法の測定精度などを考慮すると，この差は有意な差とは断定できないレベルであった．これは，破損部位からのサンプリング時に，PC 鎖が切断されていない樹脂部分も必然的にサンプリングしてしまうために，分子量水準は平均化され，顕著な差とならないことが考えられた．

E. 結論

三方活栓による検討では，試料 1 では，試料 2,3 と比較して，重量平均分子量が約 10%低いことが明らかとなった．この分子量低下は，三方活栓の破損しやすさと関係している可能性が示唆された．また，PC 試験片を使用したモデル実験においても，分子量の低い試験片ほど破損発生頻度が高いこと，及びブランクと破損部での分子量分布を比較すると，分子量が 300 程度までの低分子量域の分布に微妙な違いが見られ，破損品の方がわずかに低分子量成分が多い傾向が見られたことを考え合わせると，PC 樹脂の分子量が破損発生頻度に影響を与えることが強く示唆される．

また，試験に適用した 3 種の三方活栓は，樹脂の製造方法や添加剤が異なっていることが明らかとなり，PC 樹脂の製造方法や高分子配合剤の存在は，三方活栓の破損しやすさと関係していると考えられた．

PC 試験片を用いたモデル実験においては，使用する医薬品(添加剤)の違いにより破損頻度に影響を与えることが示唆された．

これらにより，PC 製三方活栓に使用する PC 樹脂の分子量を増加することで，破損頻度を抑えることができることが考えられた．また，添加剤だけでなく，医薬品への適用も考慮し，三方活栓の使用時に破損頻度が高い医薬品(及び添加剤とその化学構造)を更に広範囲に精査していく必要がある．

E. 健康危険情報

特になし

F. 研究成果

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G. 知的財産権の出願・登録状況

なし

H. 参考文献

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研究目的

6) (社)日本分析化学会編:「新版高分子分　　析ハンドブック」722(1995)

表 1 平均分子量測定結果

試料名	数平均分子量 (*Mn*)	重量平均分子量 (*Mw*)	z平均分子量 (*Mz*)	多分散度 (*Mw*/*Mn*)	多分散度 (*Mz*/*Mw*)
1	17400	45900	69900	2.64	1.52
2	19200	50400	77400	2.62	1.54
3	17500	50300	77100	2.87	1.53

ポリスチレン分子量基準の相対値

表 2 添加剤類分析結果

分類	化合物名	試料 1	試料 2	試料 3
高分子配合剤	ポリプロピレングリコール(PPG)	−	−	5000
酸化防止剤分解物	Irgafos168(商品名)の酸化物(＊)	160	110	−
モノグリセライド	モノステアリン	170	230	−
	モノパルミチン	61	93	−
溶剤類	ブチルカルビトールアセテート	27	8	33
	ブチルカルビトール	19	2	16
	N-メチルピロリドン(NMP)	7	2	8

単位：μg/g (ppm)

＊：Irgafos168(CIBA Specialty Chemicals 商品名)の酸化物

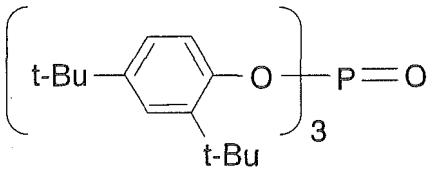

表 3　　薬剤の種類による破損度合いの変化

	撓み値(mm)			
Solvent	1	2	3	4
PEG 400	0 / 3	3 / 3	3 / 3	3 / 3
PG	0 / 3	0 / 3	0 / 3	0 / 3
Tween 80	0 / 3	2 / 3	3 / 3	3 / 3
Ethylenediamine	0 / 3	3 / 3	3 / 3	3 / 3
大豆油	0 / 3	0 / 3	0 / 3	0 / 3

破損発生頻度, (n=3), 試験片分子量: 23000

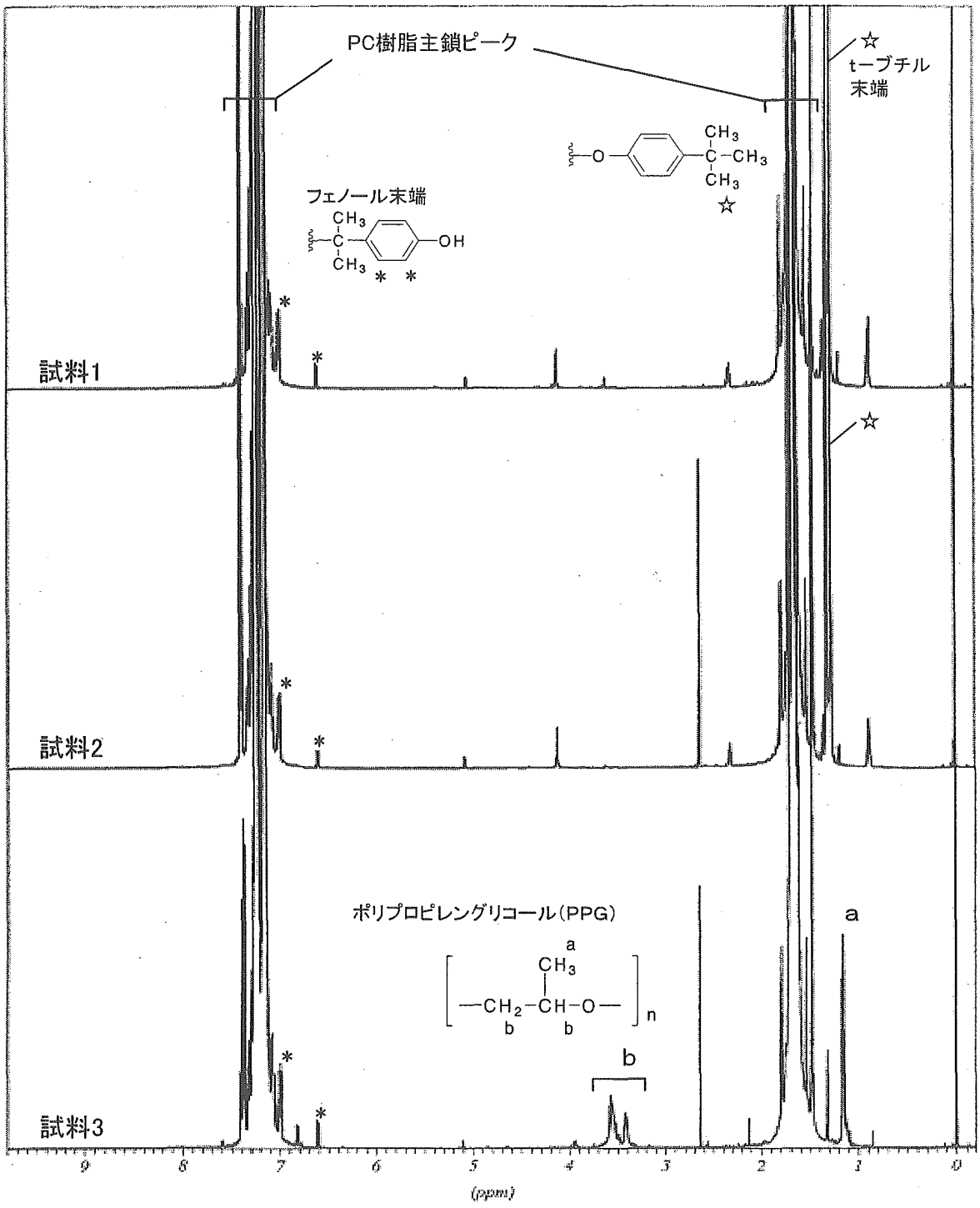

図 1. ^{1}H-NMR スペクトル分析結果

表 4　　分子量の大きさによる破損度合いの変化

撓み値 (mm)	PC 試験片分子量		
	23000	26000	27000
1	0 / 3	0 / 3	0 / 3
2	0 / 3	0 / 3	0 / 3
3	3 / 3	2 / 3	2 / 3
4	3 / 3	3 / 3	2 / 3

破損発生頻度, (n=3), 薬剤: Tween 80

表 5 GPC 法による各分子量水準の平均分子量測定

分子量	試　料	場所	数平均 分子量 (*Mn*)	重量平均 分子量 (*Mw*)	z 平均 分子量 (*Mz*)	多分散度 (*Mw*/*Mn*)	多分散度 (*Mz*/*Mw*)
小	ブランク	端	18100	43000	65700	2.38	1.53
		中央	18100	42700	66100	2.36	1.55
	破　損 Run 1	端	16900	42300	65500	2.5	1.55
		中央	17400	42400	65500	2.44	1.54
	破　損 Run 2	端	17600	42400	65400	2.41	1.54
		中央	17000	42100	65300	2.48	1.55
中	ブランク	端	18700	47400	73000	2.53	1.54
		中央	20200	47700	72900	2.36	1.53
	破　損 Run 1	端	18600	46900	72000	2.52	1.54
		中央	17300	46200	71200	2.67	1.54
	破　損 Run 2	端	19700	46800	71500	2.38	1.53
		中央	19000	47100	72300	2.48	1.54
大	ブランク	端	19500	48400	73800	2.48	1.52
		中央	20100	48200	73400	2.4	1.52
	破　損 Run 1	端	18000	47400	72500	2.63	1.53
		中央	18000	47600	72800	2.64	1.53
	破　損 Run 2	端	17100	46400	71500	2.71	1.54
		中央	18300	46500	70700	2.54	1.52

Ⅳ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表雑誌	巻号	ページ	出版年
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Ⅴ. 研究成果の刊行物・別刷り

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International Journal of Pharmaceutics 274 (2004) 119–129
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Risk assessment of di(2-ethylhexyl)phthalate released from PVC blood circuits during hemodialysis and pump–oxygenation therapy

Yuji Haishima [a,*], Rieko Matsuda [b], Yuzuru Hayashi [c], Chie Hasegawa [a], Takeshi Yagami [a], Toshie Tsuchiya [a]

[a] *Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*
[b] *Division of Foods, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*
[c] *Division of Safety Information on Drug, Food and Chemicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*

Received 20 October 2003; received in revised form 14 December 2003; accepted 10 January 2004

Abstract

This study deals with in vitro investigation of the release of di(2-ethylhexyl)phthalate (DEHP) during hemodialysis and pump–oxygenation therapy using medical grade PVC tubing. High resolution GC–MS analysis showed that the release of DEHP was time-dependently increased by circulation of bovine blood into a major system for the hemodialysis that is used in Japan, and the amount of DEHP released into the blood had reached 7.3 mg by 4 h of circulation. No significant difference was observed in the release patterns of DEHP under the conditions with and without fluid removal treatment during hemodialysis, indicating that the treatment seems not to be effective for eliminating DEHP from the blood through the hemodialysis membrane. Mono(2-ethylhexyl)phthalate (MEHP) analysis revealed that a small amount of DEHP (3–4%) was converted to MEHP by hydrolysis during the circulation of blood. A considerable amount of DEHP was also released from the PVC circuit mimicking the pump–oxygenation system, and 7.5–12.1 mg of DEHP had migrated into bovine blood from the circuit by 6 h. It was noticed, however, that the release was obviously suppressed by covalently coating the inner surface of the PVC tubing with heparin, though this effect was not observed with ionic bond type-heparin coating. Covalent bond type-heparin coating of PVC tubing seems to offer the advantage of decreasing the amount of DEHP exposure to patients during treatment using a PVC circuit.

Keywords: DEHP; MEHP; Hemodialysis; Pump–oxygenator; PVC tubing; Blood circuit

1. Introduction

Many kinds of phthalate esters have been reported to have weak estrogenic activity in vitro, though there has been no evidence of uterotrophic activity by esters such as di(2-ethylhexyl)phthalate (DEHP) and di-*n*-butyl phthalate (Zacharewski et al., 1998). Phthalate esters do not have a structure likely to bind to estrogen receptors, and they are therefore not considered estrogenic compounds in vivo (Koizumi et al., 2000). However, some of them are considered to be toxic compounds exhibiting effects similar to those of endocrine disruptors in rodents, characterized in male rats by antiandrogenic effects on the development of the reproductive system and production of normal sperm (Poon et al., 1997; Lamb et al., 1987; Tyl et al., 1988), and in female rats by decrease of 17β-estradiol level in the blood (Davis et al., 1994).

* Corresponding author. Tel.: +81-3-3700-4842; fax: +81-3-3707-6950.
E-mail address: haishima@nihs.go.jp (Y. Haishima).

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doi:10.1016/j.ijpharm.2004.01.009

It has been reported that orally administrated DEHP may be absorbed from the gut as a monoester derivative, mono(2-ethylhexyl)phthalate (MEHP), after enzymatic hydrolysis in the intestine (Lake et al., 1977). Recent in vitro studies found that MEHP inhibits FSH stimulated c-AMP accumulation in cultured Sertoli cells and induced apoptosis of germ cells in coculture with Sertoli cells (Heindel and Chapin, 1989; Grasso et al., 1993; Richburg and Boekelheide, 1996; Lee et al., 1999; Richburg et al., 2000), in addition to reducing 17β-estradiol production and aromatase mRNA expression (Davis et al., 1994; Lovekamp and Davis, 2001). These results indicate that MEHP is an active metabolite of DEHP, and suggest that any toxic effects of orally ingested DEHP are more likely governed by the properties of the corresponding monoester rather than by intact DEHP.

Phthalate esters, and DEHP in particular, have been extensively used as plasticizers as a result of the increased flexibility of polyvinyl chloride (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 released from PVC products not only into foods but also into pharmaceuticals and body fluids that come in contact with the plastic, and the migrated DEHP is directly and/or indirectly introduced into human body (Allwod, 1986; Loff et al., 2000; Tickner et al., 2001). The general toxicity of DEHP was well evaluated in the latter half of the 20th century, and so far the results of risk assessment to human health had indicated that this compound is relatively safe to humans. It has recently been considered, however, that precautions should be taken to limit human exposure to DEHP, particularly that of high risk patients such as male neonates, male fetuses, and peripubertal males, based on findings that DEHP has the potency to cause adverse effects in young rodents.

In consideration of these issues, several agencies and official organizations around the world, including the Japanese Ministry of Health, Labor and Welfare (JMHLW), individually evaluated the safety of DEHP released from PVC products. The US Food and Drug Administration has calculated the ratio of tolerable daily intake (TDI) value to the exposure amount of DEHP in medical treatments with various PVC medical devices utilizing data on toxicity and the release profile of DEHP obtained in reports on various devices (Center for Devices and Radiological Health, 2001). Such data may be very useful for evaluating the safety of these devices for patients.

It is essential that the exposure amount be precisely determined in order to evaluate its significance as an integral part of the assessment of the risk of DEHP to human health. Although several studies on the release of DEHP from PVC medical devices have been reported in Japan (Muramatsu et al., 2000; Hanawa et al., 2000; Tanaka et al., 2001), the JMHLW recently decided to re-estimate the exact amount of DEHP exposure to patients during medical treatments with the major PVC devices used in Japan. In the present study, which was one of the JMHLW projects, in order to clarify safety and evaluate risk assessment, we examined the release level of DEHP from hemodialysis and pump–oxygenation systems using a PVC blood circuit, and also estimated the exposure amount to MEHP that is an active metabolite of DEHP.

Analytical method having high sensitivity, precision, selectivity of quantitative ions, and low background are required to determine DEHP and MEHP for clinical assessment, and hence most of the conventional methods developed up to the present are not available in this point. Column-switching LC–MS method recently developed must be very useful for direct analysis of DEHP released from PVC medical devices because of the high-throughput and low-contamination (Inoue et al., 2003a,b). In addition, LC–MS/MS and high resolution GC–MS analytical techniques having high sensitivity and selectivity of target ions may be also available for the analysis. From these potential methods, we chose high resolution GC–MS technique that has the highest resolution and selectivity of quantitative ions, as the method for determination of the phthalates.

2. Materials and methods

2.1. Chemicals and utensils

The standards, including DEHP, DEHP-d_4, MEHP, MEHP-d_4 and fluoranthene-d_{10} (F-d_{10}), were purchased from Kanto Chemical Co. (Tokyo, Japan) or Hayashi Chemical Co. (Osaka, Japan). Hexane, methanol, anhydrous sodium sulfate, sodium chloride

of DEHP analytical grade, dioxin analytical grade diethyl ether, and HPLC analytical grade distilled water were used in this study. Fresh bovine blood containing heparin (10,000 U/l) purchased from DARD Co. (Tokyo, Japan) was used as a solvent to be circulated into the hemodialysis and pump–oxygenation systems. All utensils were made of glass, metal, or Teflon, and were heated at 250 °C for more than 16 h before use.

2.2. Blood circuits

The hemodialysis system consisted of medical grade PVC tubing (i.d. 3.5 mm), a major product in Japan, provided by company A, and a hemodialyzer composed of a combination of polycarbonate casing and polyethersulfone hollow-fiber provided by company B. The inner volume of the blood circuit and the total area of the inner surface of PVC products including connectors were approximately 140 ml and 950 cm^2, respectively. Pooled bovine blood (5000 ml, Htc. 30%, TP 5.7–5.9 g/dl) containing heparin was circulated into the circuit via a thermoregulator (37 °C) for 4 h at 200 ml/min employing a widely used pump system (JMS GC100), under the respective conditions with and without fluid removal treatment (15 ml/min) during hemodialysis. Physiological saline was used as a dialysate at a flow rate of 500 ml/min, and saline was added to the pooled blood at the same ratio (15 ml/min) for adjusting the Htc. value under the condition of fluid removal treatment. During blood circulation, the blood samples were collected in increments of 10 ml at 10, 30, 60, 120, and 240 min, and stored at −30 °C.

Four kinds of medical grade PVC tubings were used to construct blood circuits mimicking the pump–oxygenation system. Two identical tubings (i.d. 6 mm, length 3 m) were provided by company C, and the inner surface of one was covalently coated with heparin. The remaining two identical tubings (i.d. 9 mm, length 3 m) were provided by company D, and one was coated with an ionic bond type-heparin. A thermoregulator (37 °C) was set in the middle portion of each tubing, and pooled bovine blood (500 ml, Htc. 36 ± 3%) was circulated into each PVC circuit at a flow rate of 1.5 l/min by a pump system (Sarns 8000) typically used for pump–oxygenation treatment. During blood circulation, blood samples were collected in 10 ml increments at 0, 1, 3, and 6 h, and stored at −30 °C.

All investigations of extraction of DEHP from these circuits were repeated in triplicate.

2.3. Extraction of phthalate esters from bovine blood

For DEHP analysis, samples of bovine blood circulated into hemodialysis system (100 μl) and pump–oxygenation system (20 μl) were transferred into screw-capped glass tubes, which were filled up to the level of 1 ml by distilled water. DEHP-d_4 (50 ng) and sodium chloride (10 mg) were added to the sample, which was then mixed well and incubated for 30 min at room temperature. Hexane (2 ml) was added to the sample, which was then shacked for 20 min at room temperature. After centrifugation, the organic phase was collected and dehydrated with anhydrous sodium sulfate followed by GC–MS analysis described below.

For MEHP analysis, 0.01 M HCl (800 μl), MEHP-d_4 (50 ng), and sodium chloride (10 mg) were added to the blood sample (200 μl). After incubation, MEHP was extracted with diethyl ether (2 ml) followed by dehydration, carboxyl-methylation with diazomethane, and GC–MS analysis.

Recovery of DEHP and MEHP from bovine blood was estimated using F-d_{10} as a spike substance and the blood containing DEHP-d_4 or MEHP-d_4, according to the methods described above.

2.4. Measurement of phthalate esters

DEHP and the carboxyl methylated MEHP (MEHP-Me) contents in each sample were measured by GC–MS analysis using a JEOL JMS-700 instrument equipped with a BPX-5 fused silica capillary column (0.22 mm × 25 m, SGE) under the temperature conditions of initial temperature to 120 °C for 2 min and then increasing to 300 °C at 10 °C/min. The electron impact (EI)-mass spectrum was recorded at 70 eV for qualitative analysis, and the ions of *m/z* 149.0240 (DEHP), 153.0492 (DEHP-d_4), 163.0395 (MEHP-Me), 167.0647 (MEHP-d_4-Me), and 212.1410 (F-d_{10}) were selected as the quantitative ions in selected-ion mode (SIM) analysis (resolution = 5000) using the lock and check method of calibrating standard ions (*m/z* 168.9888 of PFK).

Quantitative analysis of each sample was repeated six 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, Version 2.0 (Total Optimization of Chemical Operations), practicing the function of mutual information (FUMI) theory (Hayashi and Matsuda, 1994; Hayashi et al., 1996, 2002; Haishima et al., 2001).

3. Results and discussion

3.1. Precision of quantitative analysis and recovery of phthalate ester

The precision of the quantitative analysis, which is described as the R.S.D. or S.D. of the measurements, is very important to evaluation of other analytical characteristics such as specificity, linearity, range, accuracy LOD (limit of detection), LOQ (limit of quantitation), and robustness, which parameters are proposed by the ICH guidelines (ICH Guidelines, 1996). Although the exact precision is not easy to estimate in practice, FUMI theory can provide the measured S.D. of every calibration sample without repeated measurements (Hayashi and Matsuda, 1994).

Each calibration line to quantify the concentration and recovery of DEHP and MEHP was prepared by using DEHP-d_4, MEHP-d_4, and F-d_{10} as internal standards. All calibration lines had good linearity ($r = 0.999$) in the low (0–25 ppb) and high (25–200 ppb) concentration ranges tested in GC–MS analysis. The 95% confidence intervals of the calibration lines, which represent the error between the calibration lines obtained under the same experimental conditions, were very narrow, indicating that the precision was sufficiently high. Instrumental LOD and LOQ predicted by FUMI theory from data of DEHP-d_4/F-d_{10} and MEHP-d_4/F-d_{10} standard curves were 0.0204 and 0.6748 ppb for DEHP, and 0.0380 and 0.1266 ppb for MEHP, respectively. Background analyses of DEHP and MEHP originating from each reagent and GC–MS instrument showed that 1.2 ± 0.27 ppb of DEHP and 0.08 ± 0.023 ppb of MEHP were detected as background contamination when 50 ng each of the internal standards (DEHP-d_4 and MEHP-d_4) were used in the quantitative analyses. From these results, the experimental LOD and LOQ were calculated as 2.01 and 3.90 ppb for DEHP, and 0.149 and 0.31 ppb for MEHP, respectively, and the quantitative data described below were corrected by these background values.

Recovery rates of DEHP and MEHP extracted from bovine blood in this investigation were 90.1 ± 6.8 and 72.4 ± 2.47%, respectively.

3.2. Identification of DEHP and MEHP

SIM chromatograms in DEHP and MEHP analyses of bovine blood circulated into the pump–oxygenation systems described below are shown in Fig. 1. A peak detected at 16.7 min in DEHP analysis (Fig. 1A) was identified as DEHP by scan-mode EI-mass spectrometry in which characteristic fragment ions were observed at *m/z* 70, 83, 104, 112, 149, 167, and 279. In MEHP analysis (Fig. 1B), MEHP-Me was detected at 12.4 min, and typical fragment ions such as *m/z* 70, 83, 104, 112, 149, 164, and 181 were observed in the EI-mass spectrometry. The retention times and EI-mass spectra were the same as those of the authentic DEHP and MEHP standards.

3.3. DEHP release from hemodialysis system

Release test of DEHP from the hemodialysis circuit was performed by using the major system in current use in Japan. The release profile of DEHP from the system is shown in Fig. 2. Bovine blood used in this experiment contained 248.9 ± 123.6 ppb of DEHP as the background. Under the condition of fluid removal treatment, the concentration of DEHP in the blood time-dependently increased by circulating the blood through the hemodialysis circuit. The concentration of DEHP after blood circulation for 30, 60, 120, and 240 min was shown in Table 1. A similar amount of DEHP was released from the hemodialysis system under the condition without fluid removal treatment. In this test, the concentration of DEHP had reached 1741.8 ± 65.1 ppb at 4 h of circulation (Fig. 2 and Table 1).

Hemodialyzers are very utile devices often employed in the medical field in treatment for renal failure. Precise evaluation of DEHP exposure is very

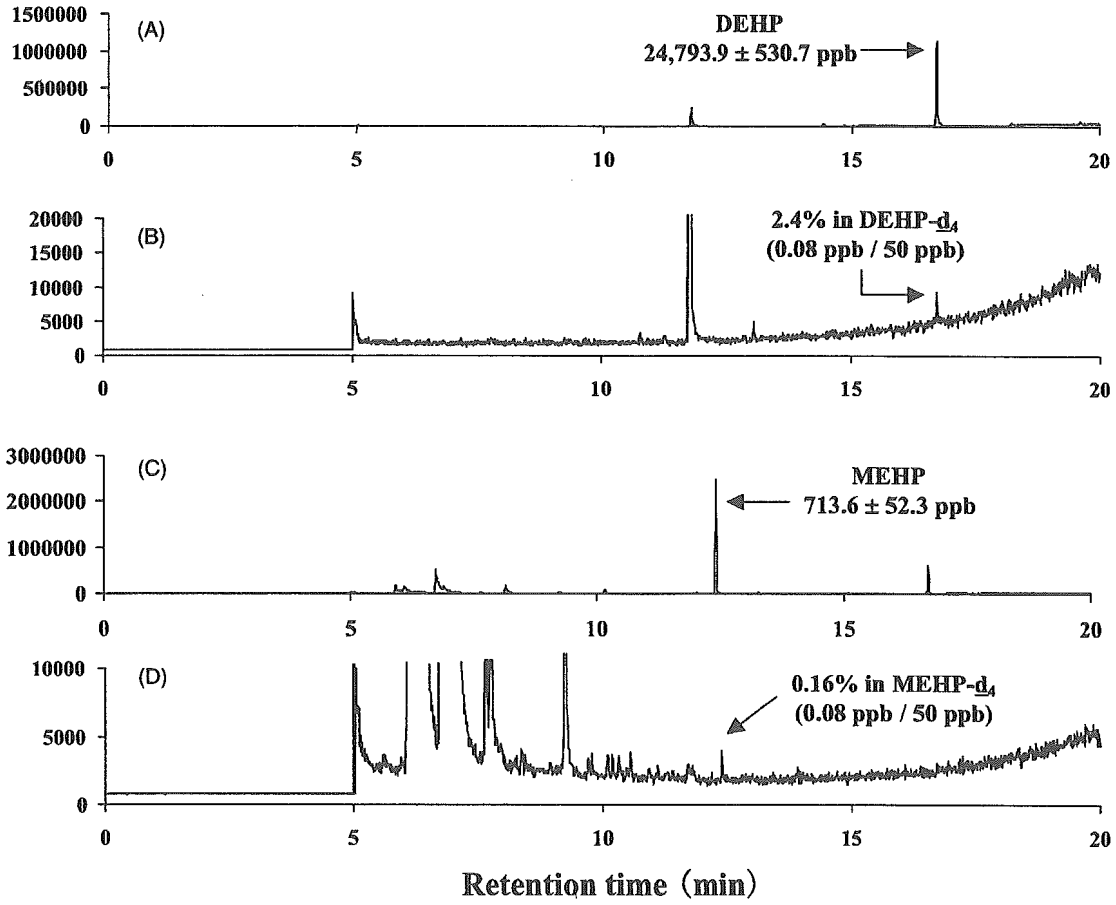

Fig. 1. SIM chromatograms in GC–MS analysis of DEHP and MEHP extracted from bovine blood circulated for 6 h through a pump–oxygenator system consisting of non-coated tubing produced by company D: (A) DEHP analysis; (B) background in DEHP analysis; (C) MEHP analysis; (D) background in MEHP analysis.

important for hemodialysis patients due to the frequent necessity of long-term therapy. In vivo and in vitro studies have reported on the release of DEHP into circulated blood during hemodialysis; DEHP in a range of 3.23–360 mg was extracted from the hemodialysis circuits during a single 4 h dialysis session (Kambia et al., 2001; Faouzi et al., 1999; Flaminio et al., 1988; Pollack et al., 1985; Lewis et al., 1978). US-FDA

Table 1
Amounts of DEHP and MEHP detected from bovine blood circulated into hemodialysis system

Circulation time (min)	Concentration (ppb)			
	DEHP		MEHP	
	With fluid removal treatment	Without fluid removal treatment	With fluid removal treatment	Without fluid removal treatment
0	248.9 ± 123.6	261.6 ± 147.6	13.3 ± 6.9	14.0 ± 6.4
30	441.4 ± 55.5	473.4 ± 124.9	35.6 ± 4.0	30.3 ± 4.6
60	606.2 ± 28.4	657.2 ± 91.8	48.1 ± 5.8	42.9 ± 7.0
120	949.9 ± 85.3	979.6 ± 42.7	57.2 ± 7.5	54.6 ± 2.5
240	1717.8 ± 147.4	1741.8 ± 65.1	78.1 ± 9.2	81.5 ± 6.0

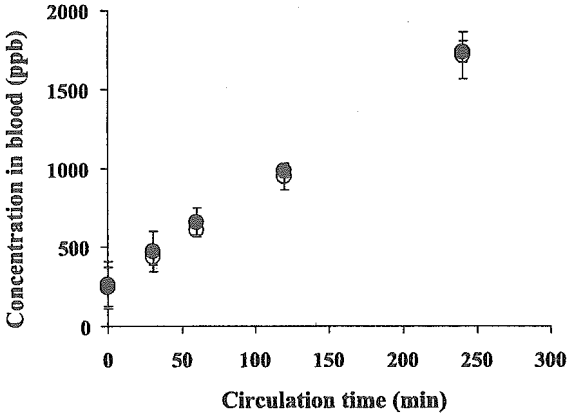

Fig. 2. Release profile of DEHP in a hemodialysis system under condition (○) with and condition (●) without fluid removal treatment.

calculated DEHP exposure amounts of hemodialysis patients as 0.02–0.36 mg/kg per day (4 h dialysis, three times a week; body weight 70 kg) (Center for Devices and Radiological Health, 2001). Our in vitro study revealed that 7.3 mg of DEHP was released from the hemodialysis system over 4 h of blood circulation, and the total amount of DEHP was corrected as 7.8 mg by adding the amount converted to MEHP from DEHP during the 4 h period. The amount of DEHP exposure was calculated from the corrected value as 0.067 mg/kg per day (4 h dialysis, three times a week; body weight 50 kg) if the total amount of DEHP released from the circuit was absorbed into the body. This value was remarkably lower than the TDI value (0.6 mg/kg per day) for intravenous injection to humans proposed by the FDA (Center for Devices and Radiological Health, 2001), but slightly higher than the lower limit of TDI value (0.04–0.14 mg/kg per day) for oral administration to humans estimated by the JMHLW.

In this investigation, fluid removal treatment during hemodialysis session seemed not to be effective for removal of DEHP and MEHP from the circulated blood. However, since DEHP introduced into the body is rapidly excreted as gluconide and other metabolites (Rhodes et al., 1986; Woodward, 1988), a portion of the hydrophilic metabolites may be eliminated during in vivo blood circulation through the dialyzer if sufficient fluid removal treatment is performed during the session.

3.4. DEHP release from the pump–oxygenation system

The blood circuit mimicking the pump–oxygenation system consisted of a pump, a thermoregulator, and four kinds of PVC tubing in medical treatment in Japan. The amounts of DEHP released from these circuits (i.e. the same setup with the four different kinds of tubing) were evaluated. The background content of DEHP in bovine blood used as a circulation solvent was 503.3 ± 69.2 ppb. In the release test using non-coated PVC tubing provided by company C, a significant amount of DEHP was time-dependently released from the circuit as shown Fig. 3, and the concentration of DEHP at each time of circulation is shown in Table 2. On the other hand, DEHP release from the circuit employing covalently bond type of heparin-coated PVC tubing produced by the same company was obviously suppressed; DEHP content in the blood after 6 h circulation was 7480.3 ± 376.2 ppb (Fig. 3 and Table 2). A relatively large amount of DEHP was released from the circuit using non-coated tubing provided by company D, and the concentration of DEHP in the circulation blood reached 24792.9 ± 530.7 ppb after 6 h circulation (Fig. 3 and Table 2). It was noticed that the ionic bond type heparin coating on the inner surface of the PVC tubing (company D), in comparison with the covalent bond type heparin coating, did not greatly effect the release of DEHP.

Several in vivo investigations have been reported on the release of DEHP during extracorporeal membrane oxygenation (ECMO) therapy, which is used mainly for neonates in respiratory failure (Karle et al., 1997). In this investigation, a considerable amount of DEHP was also released from blood circuits mimicking pump–oxygenation therapy for pediatric patients. The total amounts of DEHP, including the amount of MEHP converted from DEHP, during 6 h of circulation were calculated to be 7.8 mg (company C non-coat tubing), 3.7 mg (company C heparin-coat tubing), 12.6 mg (company D non-coat tubing), and 10.6 mg (company D heparin-coat tubing), respectively. The DEHP exposure amount calculated from these value was 0.708–0.721 mg/kg per day when non-coated tubings were used and 0.334–0.606 mg/kg per day for the circuits using heparin-coated tubings (6 h circulation, one time; body weight 11 kg). Exposure amounts of DEHP for the adult patient (body weight 50 kg)

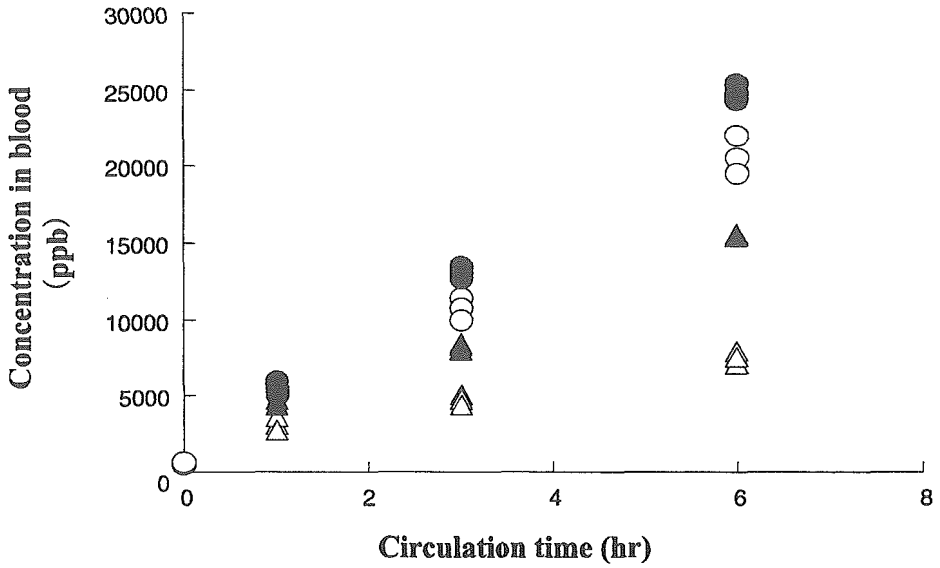

Fig. 3. Release profile of DEHP in PVC blood circuits of the pump–oxygenator. Non-coated tubing produced by companies C (▲) and D (●), and covalent bond (△) and ionic bond (○) types of heparin-coated tubing provided by companies C and D, respectively.

predicted by hypothetically adjusting the size of the tubing (i.d. 10 mm, length 4 m) were calculated as 0.346–0.352 mg/kg per day (non-coated tubings) and 0.163–0.296 mg/kg per day (heparin-coated tubings). All of these values were higher than the upper limit of TDI value estimated by the JMHLW.

It has been reported that heparin coating of the inner surface of PVC tubing is very effective for suppressing the release of DEHP from the tubing (Karle et al., 1997; Mejak et al., 2000; Lamba et al., 2000). It was shown in this study that DEHP release was decreased to approximately 50% that of the control tubing by the use of covalent bond-type heparin coating, indicating that this coating may be useful to suppress patients' exposure to DEHP.

3.5. MEHP analysis

MEHP contents in bovine blood circulated into hemodialysis and pump–oxygenation systems were measured in order to determine the conversion ratio of DEHP to MEHP in the blood. As shown in Fig. 4, similar profiles of MEHP detected from the blood circulated through a hemodialysis system were obtained irrespective of the condition of fluid removal treatment (i.e. with or without). Amounts of MEHP in circulated blood originally containing 13.3 ± 6.9 ppb of MEHP as a background were time-dependently increased; after circulation for 240 min, the contents in the blood had reached 78.1 ± 9.2 ppb under the condition of fluid removal treatment and 81.5 ± 6.0 ppb without the treatment (Fig. 4 and Table 1).

MEHP was also detected in the blood circulated into circuits mimicking a pump–oxygenation system, as shown in Fig. 5. The blood used in this

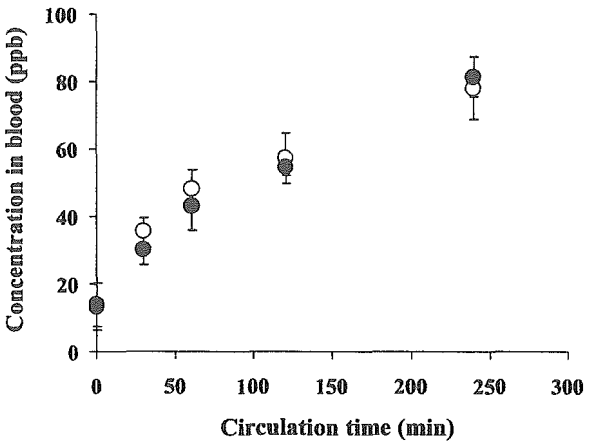

Fig. 4. Amounts of MEHP detected in bovine blood circulated through the hemodialysis system under condition (○) with and condition (●) without fluid removal treatment during hemodialysis session.

Table 2
Amounts of DEHP and MEHP detected from bovine blood circulated into pump–oxygenation system

Circulation time (h)	Concentration (ppb)							
	DEHP				MEHP			
	Company C		Company D		Company C		Company D	
	Non-coat	Heparin-coat	Non-coat	Heparin-coat	Non-coat	Heparin-coat	Non-coat	Heparin-coat
1	4528.4 ± 194.0	3140.4 ± 429.7	5626.2 ± 266.3	5216.0 ± 185.3	221.3 ± 31.4	142.8 ± 19.0	303.4 ± 27.9	479.6 ± 5.2
3	8240.1 ± 193.0	4734.0 ± 365.1	13062.6 ± 335.2	10676.1 ± 687.0	302.0 ± 22.6	199.6 ± 13.1	497.7 ± 37.6	626.1 ± 10.7
6	15503.0 ± 88.5	7480.3 ± 376.2	24792.9 ± 530.7	20683.2 ± 1212.2	416.1 ± 26.3	258.5 ± 24.3	713.6 ± 52.3	696.5 ± 21.7

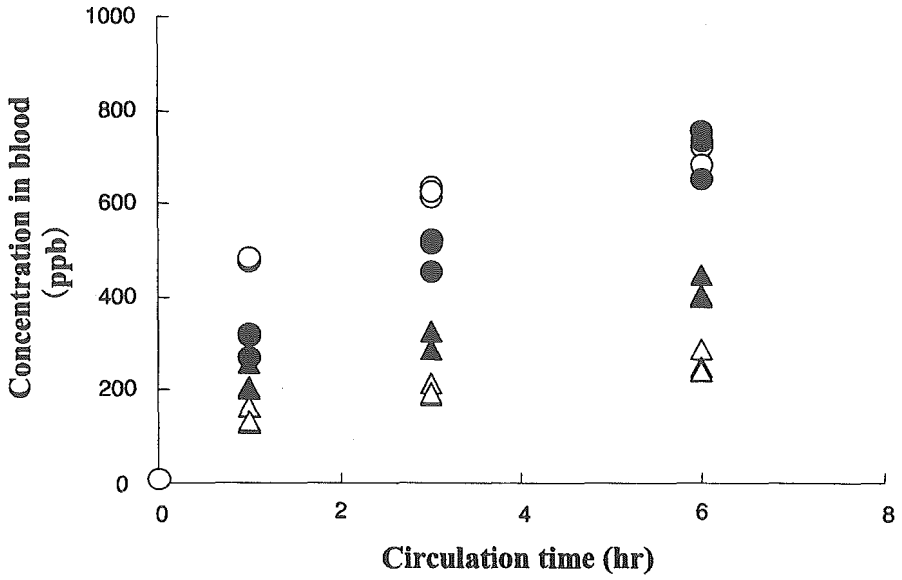

Fig. 5. Amounts of MEHP detected in bovine blood circulated through PVC circuits of the pump–oxygenator. Non-coated tubing produced by companies C (▲) and D (●), and covalent bond (△) and ionic bond (○) types of heparin-coated tubing provided by companies C and D, respectively.

investigation contained 9.1 ± 1.0 ppb of MEHP as a background. As shown in Table 2, 221.3 ± 31.4, 302.0 ± 22.6, and 416.1 ± 26.3 ppb of MEHP were detected in blood circulated for 1, 3, and 6 h in the circuit using the non-coated PVC tubing produced by company C. However, the detectable amount was obviously decreased by use of the heparin-coated PVC tubing (Fig. 5 and Table 2). On the other hand, no significant difference with regard to the profile of MEHP detection was observed between the PVC circuits with non-coated tubing and those with ionic bond type heparin-coated tubing produced by company D. MEHP of 713.6 ± 52.3 and 696.5 ± 21.7 ppb was detected in the blood circulated for 6 h through the non-coated-tubing circuit and the coated-tubing circuit, respectively (Fig. 5 and Table 2).

MEHP is an active metabolite of DEHP, and therefore, given that a portion of DEHP is converted to MEHP in stocked blood, plasma, and transfusion blood, evaluation of patient exposure to MEHP is very important. In this experiment, it was shown that 3–4% of DEHP is also converted to the monoester during blood circulation for hemodialysis and in pump–oxygenator circuits at 37 °C, probably as a result of esterase in the blood.

Thus, present study showed the risk that patients are exposed to considerable amount of DEHP during hemodialysis and pump–oxygenation treatments. However, benefit of medical devices used for the treatments is obviously over than the risk factor, because these devices are essential to save patients' life. Although David et al. (1999) demonstrated that DEHP promoted the proliferation and hepatomegaly associated with hepatocellular tumorigenesis, it has been clearly shown that the toxic mechanism is characteristic in rodents and no tumorigenesis activity is observed to human (Doull et al., 1999). Pharmacokinetics assay showed that metabolic rate of DEHP is relatively fast, and 62–76% of DEHP taken into body is excreted by 24 h after orally administration to marmosets (Rhodes et al., 1986). Furthermore, Japan Plasticizer Industry Association recently reported the results on risk assessment of DEHP against primates, on their web site (http://www.kasozai.gr.jp/) in January 2003, that DEHP administrated to marmosets was not accumulated in testis and did not exert any testicular toxicity such as testicular atrophy different from the case of rodents, suggesting that species specificity regarding appearance of the toxicity may exist between rodents and primates. In fact, no clin-

ical adverse events originated from DEHP exposure to human have been reported up to the present. In consideration of these issues, PVC medical devices used for hemodialysis and pump–oxygenation treatments seem to be relatively safe to patients, in addition to the great benefit factor to patients. However, since the influence of DEHP on humans is not fully understood, precautions should be taken to limit human exposure to DEHP, at least that of high risk patients, originating from use of PVC medical devices.

4. Conclusion

We evaluated the release of DEHP from hemodialysis and pump–oxygenator circuits comprised of PVC tubings. The amount of DEHP exposure for adult patients in hemodialysis therapy did not appear to be remarkably high, though use of normal PVC tubing perhaps should be reconsidered if this treatment is to be applied to patient groups having a high sensitivity to DEHP and/or facing the likelihood of long term therapy. A considerable amount of DEHP (well over the TDI value) was released from the PVC circuits for the pump–oxygenator currently in wide used in surgery for heart and/or lung failure patients in Japan. Although the oxygenator is mainly used for adult patients receiving therapy different from ECMO therapy, and for whom the incidence of use is relatively low in the life of a patient, non-coated PVC tubing for the circuit may also be exchanged for alternative (i.e. coated) tubing if the treatment is to be applied to a high risk patient group even if no significant adverse events have been associated with therapy, based on the finding that the amount of exposure to DEHP by the therapy is over the upper limit of TDI value as estimated by the JMHLW. One current alternative, covalent bond type heparin-coated PVC tubing, may be useful for suppressing the release of DEHP from PVC tubing.

Regardless whether an investigation is in vivo or in vitro, the release test of DEHP is time-consuming and labor-intensive. Consequently, the development of a simple method for predicting the amount of DEHP released from PVC medical devices is now in progress in our laboratory.

Acknowledgements

We greatly appreciate the Japan Medical Devices Manufacturers Association skillful assistance and the provision of medical devices used in this study. This work was supported by grant H13-Iyaku-004 from the Ministry of Health, Labor, and Welfare of Japan.

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