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DETERMINATION OF PHTHALIC ACID, MONO-(2-ETHYLHEXYL) PHTHALATE AND DI-(2-ETHYLHEXYL) PHTHALATE IN HUMAN PLASMA AND IN BLOOD PRODUCTS

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SUMMARY

Pretreatment for the determination of phthalic acid, mono-(2-ethylhexyl) phthalate (MEHP) and di-(2-ethylhexyl) phthalate (DEHP) in human serum or plasma, and the determination of these compounds in blood products by high-performance liquid chromatography was studied. The amount of phthalic acid, MEHP and DEHP, migrated into blood products from a flexible bag, was studied.

About 0.1% of DEHP in a flexible bag was found to have migrated into human platelet plasma. Most of the MEHP and phthalic acid detected in human platelet plasma was not derived from the flexible bag but was produced by enzymatic hydrolysis of the migrated DEHP. The amount of DEHP eluted into blood products from the flexible bag differed, depending upon storage time, storage temperature, etc.

INTRODUCTION

The most common material for medical devices such as flexible bags and tubing is polyvinyl chloride (PVC) with di-(2-ethylhexyl) phthalate (DEHP) as the main plasticizer. Recently the toxicity, mutagenicity and carcinogenicity of DEHP have been questioned [1], and it has been pointed out that DEHP is changed by heat and enzymes into mono-(2-ethylhexyl) phthalate (MEHP) and further to phthalic acid (PA) [2]. Therefore the safety of these compounds has to be considered, particularly as MEHP is considered to be more toxic than DEHP [3].

We have studied the analytical conditions for high-performance liquid chromatography (HPLC) by which these compounds can be determined in human serum or plasma and have established a quantitative method of excellent reproducibility with an internal standard. Poor recovery of these compounds

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with the pretreatment of serum reported in literature [4—6] has, after various attempts, led us to find a method of pretreating serum ensuring nearly 100% recovery. In particular, we have employed, for the first time, deproteinization with an acetonitrile—sodium hydroxide mixture and ultrafiltration for the pretreatment of serum. In order to examine the elution of PA, MEHP and DEHP from a flexible bag into blood products, we determined these compounds in blood products. The interesting results we obtained are reported here.

EXPERIMENTAL

Reagents

Commercially available and chemically pure reagents were used except for MEHP and monomethyl phthalate (MMP), which were synthesized by the method described in the literature [7].

Materials

Materials used were fresh-frozen human plasma (Japan Red Cross, JRC, Tokyo, Japan; this was stored at —30°C for one week in a flexible bag), concentrated human platelet plasma (JRC; this was stored at 22°C for two days in a flexible bag; during one day, centrifugation was carried out), human serum (JRC; this was stored in a glass bottle), human plasma (JRC; this was kept frozen for 21 days in a flexible bag), Plasmanate (Green Cross, Osaka, Japan),

TABLE I

CONDITIONS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRETREATMENT OF SERUM

HPLC			
Compound	PA	MEHP	DEHP
Injection volume (μl)	10	10	10
Column	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)
Temperature	Ambient	Ambient	Ambient
Flow-rate (ml/min)	0.8	0.8	1.2
Detection	UV (254 nm)	UV (254 nm)	UV (254 nm)
Retention time (min)	4.6	10.6	6.0
Mobile phase*	A	B	C
Internal standard	MMP	MBP	DnOP

Pretreatment	
Serum PA:	Serum (1 ml), water (3 ml) and 50% phosphoric acid (0.1 ml) were mixed and after ultrafiltration by Centrifree MPS-3, the ultrafiltrate was injected for HPLC.
Serum MEHP:	Serum (1 ml), 50% phosphoric acid (50 μl) and the mixing solution of diethyl ether—methanol, 2:1 (3 ml) were mixed, kept one day at 4°C, again mixed and after centrifugation the diethyl ether layer of the supernatant was injected for HPLC.
Serum DEHP:	Serum (1 ml), 1 *M* sodium hydroxide (1 ml) and acetonitrile (3 ml) were mixed and after centrifugation the upper layer was injected for HPLC.

*A: 5 m*M* Monosodium phosphate aqueous solution—acetonitrile (80:20), pH 2.8 adjusted with phosphoric acid. B: 5 m*M* Monosodium phosphate aqueous solution—acetonitrile (50:50), pH 2.8 adjusted with phosphoric acid. C: Water—acetonitrile (10:90).

dried human plasma (Nippon Pharmaceutical, Tokyo, Japan), human and equine plasma (Japan Biological Materials Center, JBMC, Tokyo, Japan; the human plasma was stored at 22°C for 25 days in a flexible bag and the equine plasma was stored in a glass bottle), equine serum (Flow Labs., VA, U.S.A.; this was kept in a glass bottle), and a flexible bag (Terumo, Tokyo, Japan; this was made of PVC).

Equipment

The high-performance liquid chromatograph (Shimadzu LC-3A) was equipped with an ultraviolet (UV) detector (Shimadzu SPD 2A), data processing equipment (Shimadzu Chromatopac C-RIA) and ultrafilter (Amicon MPS-3).

Procedures

Table I summarizes the HPLC and pretreatment conditions for serum and plasma that we established. Fig. 1 shows representative chromatograms for PA, MEHP and DEHP in human serum. Almost the same chromatograms were obtained for PA, MEHP and DEHP in human plasma.

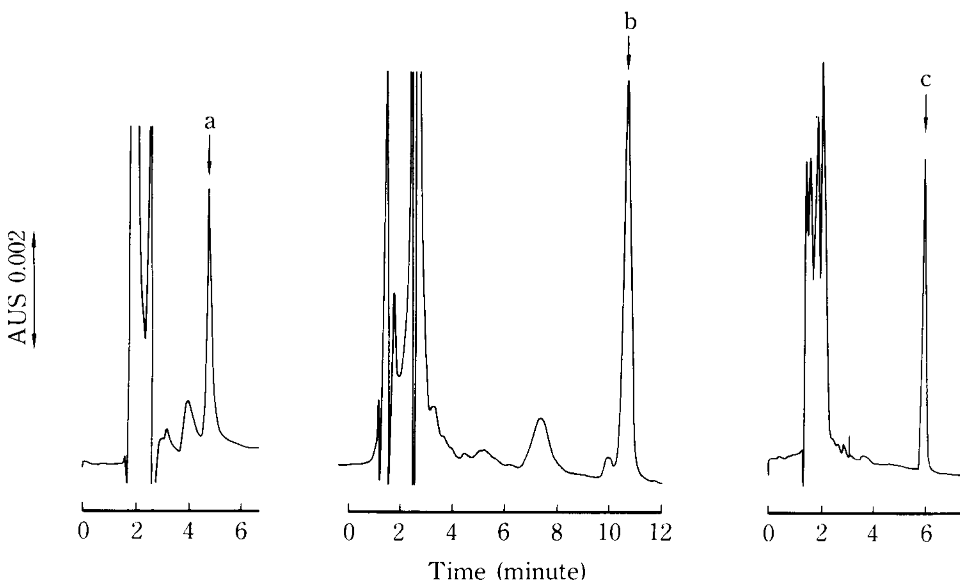

Fig. 1. Typical chromatograms of phthalic acid, mono-(2-ethylhexyl) phthalate and di-(2-ethylhexyl) phthalate in human serum. a, b and c indicate peaks of PA, MEHP and DEHP, respectively. Concentrations of PA, MEHP and DEHP were 6.1, 39.4 and 26.0 μg/ml, respectively; 10 μl were injected. Conditions for serum pretreatment and for HPLC are given in the text. Almost identical chromatograms were obtained for PA, MEHP and DEHP in human plasma.

RESULTS AND DISCUSSION

Identification of synthesized MMP and MEHP

These compounds were identified by elementary analysis, infrared, nuclear magnetic resonance (Fig. 2) and mass spectra (Fig. 3). The values obtained for

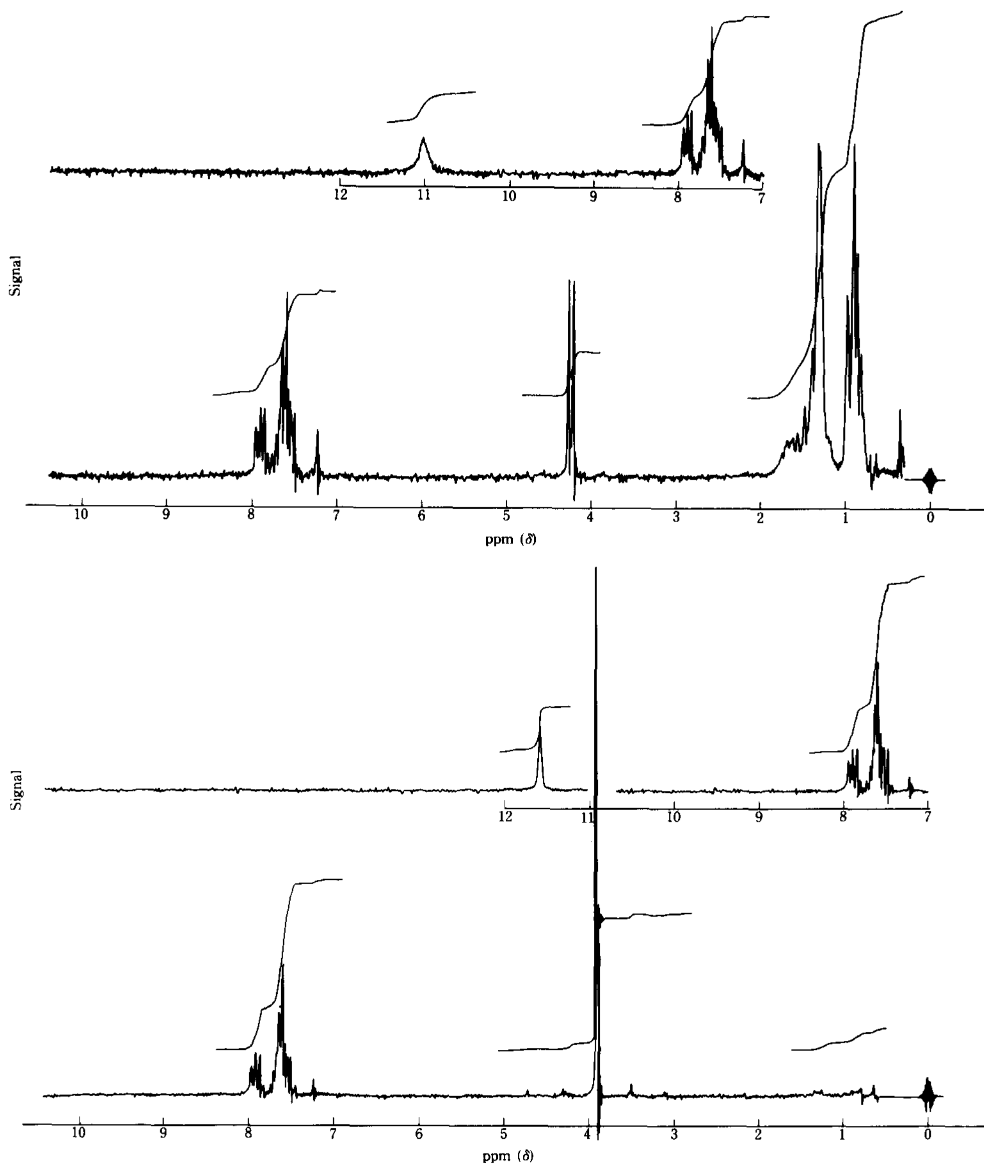

Fig. 2. Nuclear magnetic resonance spectra of mono-(2-ethylhexyl) phthalate (upper two traces) and of monomethyl phthalate (lower two traces). Nucleus ^{1}H, zero reference trimethylsilane, sample temperature ambient, solvent $C^{2}HCl_{3}$.

elementary analysis of MEHP and MMP were: MEHP, carbon 67.58%, hydrogen 7.45%, m.p. 4°C; and MMP, carbon 60.88%, hydrogen 5.15%, m.p. 82–83.5°C. There was a linear relation between the number of carbons in the alkyl group of the monoalkyl phthalates and the logarithm of the retention time (t_R).

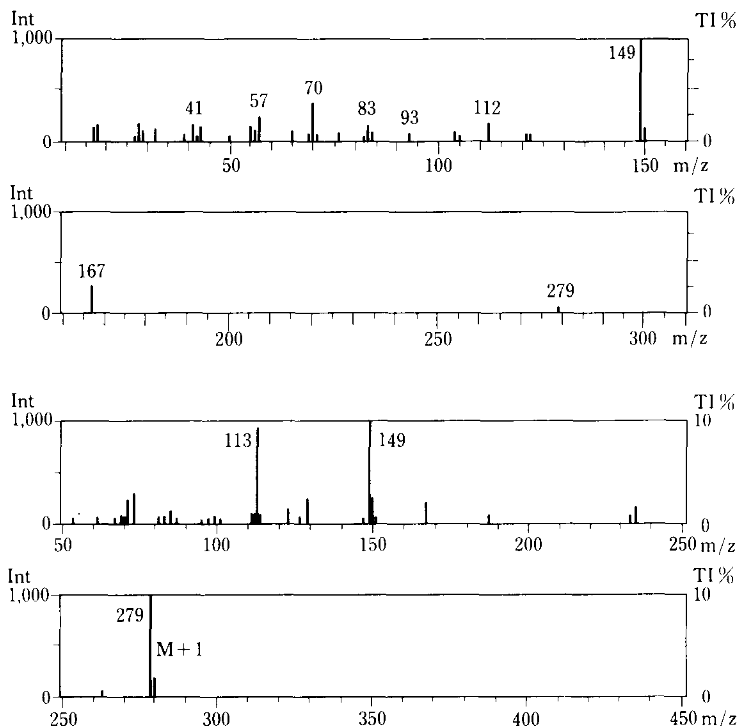

Fig. 3. Electron-impact (EI) mass spectrum (upper two traces) and chemical-ionization (CI) mass spectrum (lower two traces) of mono-(2-ethylhexyl) phthalate. Analytical conditions for EI and CI mass spectra:

Mass spectrum	EI	CI
Equipment	JMA-D 300	JMA-D 300
Data processing	JMA-200 S	JMA-200 S
Ionization voltage	70 V	200 V
Reaction gas	—	Isobutane
Ionization current	300 μA	300 μA
Accelerating voltage	3 kV	3 kV
Ion multiplier voltage	1.25 kV	1.25 kV
Chamber temperature	150°C	130°C

Effect of acetonitrile concentration on the analysis of PA and MEHP in human serum and plasma

Using a mixture of phosphate buffer, which contained 5 m*M* sodium phosphate, and acetonitrile as the mobile phase adjusted with phosphoric acid to pH 2.8, the effect of acetonitrile concentration on the t_R of PA and MEHP was studied (Fig. 4). With 13—20% acetonitrile a good separation of PA from serum admixtures was obtained. The acetonitrile concentration was set at 20% with regard to the t_R of the internal standard, MMP.

With 60% acetonitrile, separation of MEHP from serum admixtures was unsatisfactory, but 50% acetonitrile afforded a good separation of monobutyl phthalate (MBP) as internal standard from MEHP and of these compounds from serum admixtures; 40% acetonitrile failed to elute MEHP from the column.

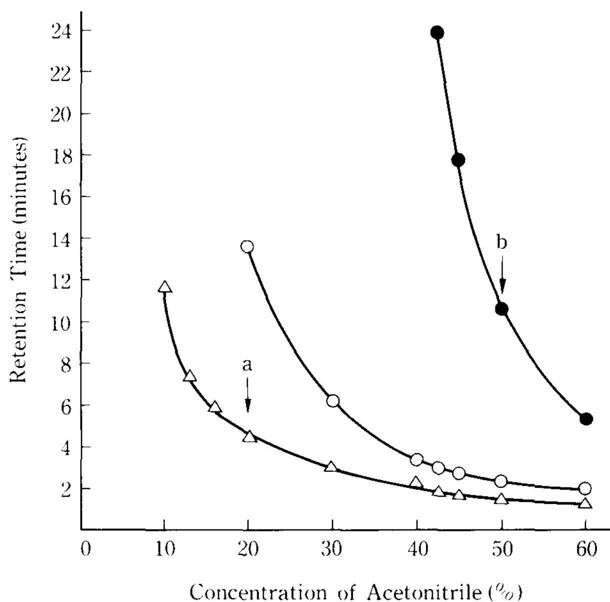

Fig. 4. Relation between retention time and concentration of acetonitrile. a and b indicate the recommended mobile phase for the determination of phthalic acid (▵) and mono-(2-ethylhexyl) phthalate (•), respectively, in human serum. With the mobile phase used for the determination of MEHP, PA could not be satisfactorily separated from serum admixtures.

Under the analytical conditions for MEHP, PA was eluted in the vicinity of the void volume, resulting in insufficient separation from serum admixtures. Therefore, the simultaneous determination of PA and MEHP in serum or in plasma under isocratic conditions by the ion-suppression method was impossible. To make it possible to perform the simultaneous determination of PA and MEHP in serum and in plasma under isocratic conditions, paired-ion chromatography was attempted with various mixtures of 1/15 *M* phosphate buffer (pH 7.38) containing 5 m*M* *n*-butyltetraammonium phosphate and acetonitrile (from 50:50 to 80:20) as the mobile phase. However, we failed to find a condition under which satisfactory simultaneous separation of PA from MEHP and of these compounds from serum admixtures is attained.

The gradient elution method, being somewhat unsatisfactory in its reproducibility, was not employed as the quantitative condition in the present study.

The effect of acetonitrile concentration on the analysis of DEHP in human serum and plasma

The use of 100% acetonitrile resulted in poor separation of DEHP and the internal standard di-(*n*-octyl) phthalate (DnOP) from serum admixtures; 80% acetonitrile failed to elute DEHP from the column; at 90% the separation of DEHP from DnOP and of these compounds from serum admixtures was excellent.

Linearity of the calibration curve and the detection limits

Data are presented in Table II. The coefficient of variation (C.V.) of

TABLE II

LINEARITY AND DETECTION LIMIT

Signal-to-noise ratio = 2. Application volume = 10 µl. Sensitivity = 0.02 a.u.f.s. (254 nm).

Compound	Linear range (µg/ml)	Detection limit (ng)
PA	2—250	2.5
MEHP	5—500	10.5
DEHP	5—400	8.8

repeated injections of PA, MEHP and DEHP was within 1% (n = 5) in all cases. Serum that was not stored in a flexible bag and did not contain PA, MEHP or DEHP was treated as blank.

Pretreatment of PA, MEHP and DEHP in human serum and plasma

Deproteinization for the recovery of MEHP and DEHP in human serum and plasma. We failed to find a suitable deproteinization method with satisfactory recovery rates for PA, MEHP and DEHP in the literature [4—6]. Therefore, we tried to find a method of pretreatment which gives a good recovery rate (Table III). To human serum samples of between 5 and 8 ml in which PA, MEHP and DEHP were not detected, were added 20 and 10 µl, respectively, of acetonitrile—water (60:40) solutions of PA and MEHP (10.0 and 39.4 mg/ml) and 10 µl of a hexane solution of DEHP (39.08 mg/ml), shaken well, allowed to stand overnight at 4°C and used for the study of the deproteinization as described below.

At first, the effect of deproteinizing eluate on the elution of DEHP from serum was studied. Although direct injection of a two-fold dilution of serum with water gave good recovery of MEHP and DEHP, deterioration of the column with serum protein occurred (Table III). Addition of 1 *M* sodium hydroxide with acetonitrile to serum for deproteinization resulted in nearly 100% recovery of DEHP. The possibility of DEHP and DnOP being hydrolysed with sodium hydroxide was discounted. Deproteinization with acetonitrile alone did not give a satisfactory recovery of DEHP, and an acid added in place of 1 *M* sodium hydroxide also gave a low recovery.

We then studied the effect of deproteinizing eluents on the elution of MEHP in serum. Although the same pretreatment of serum as with DEHP resulted in about 90% recovery (Table III), a variety of attempts were made for a higher recovery. To suppress ionization of MEHP, phosphoric acid was added to serum and further a mixture of diethyl ether—methanol (2:1) was added followed by stirring, centrifugation and injection of the diethyl ether layer of the supernatant into the HPLC system. The recovery obtained was about 86%, but a recovery of 100% was attained when the mixture was allowed to stand overnight at 4°C after stirring without centrifugation. The mixture was stirred, centrifuged and the diethyl ether layer of the supernatant injected into the HPLC system. Recovery of MEHP and DEHP in human plasma was also about 100% using the above deproteinization method.

An attempt to find an efficient deproteinizing eluent for the recovery of PA

TABLE III

DEPROTEINIZATION PRETREATMENT OF HUMAN SERUM FOR PA, MEHP AND DEHP DETERMINATION

Twenty microlitres of 10 mg/ml PA, 10 µl of 39.4 mg/ml MEHP in acetonitrile—water (3:2) and 10 µl of 39.08 mg/ml DEHP in hexane were added to 5—8 ml of human serum and mixed. It was kept for 24 h at 4°C and was used as the sample named as serum in the column of methods of deproteinization. The recovery ratio in parentheses was that of the sample centrifuged after being stirred overnight.

Method of deproteinization	Recovery ratio (%)		
	PA	MEHP	DEHP
Serum 1 ml + acetonitrile 3 ml	23.0	54.5 (85.6)	72.5 (92.8)
Serum 1 ml + 1 *M* sodium hydroxide 1 ml + acetonitrile 3 ml	15.6	90.4 (87.9)	99.9 (101)
Serum 1 ml + 6 *M* perchloric acid 0.1 ml	76.0	1.3	None
Serum 1 ml + 6 *M* perchloric acid 0.1 ml + acetonitrile 3 ml	45.2	35.0	66.3 (84.8)
Serum 1 ml + 1 *M* sodium hydroxide 1 ml + tetrahydrofuran 3 ml	—	—	99.8
Serum 1 ml + 50% phosphoric acid 50 µl + chloroform 2 ml	4.1	10.1	2.8
Serum 1 ml + 50% phosphoric acid 50 µl + diethyl ether 2 ml	72.2	45.6	9.4
Serum 1 ml + 50% phosphoric acid 50 µl + hexane 2 ml	N.D.	59.0	1.9
Serum 1 ml + 50% phosphoric acid 50 µl + chloroform—methanol (2:1) 3 ml	38.7	10.4	37.7 (37.6)
Serum 1 ml + chloroform—methanol (2:1) 6 ml	—	—	85.7 (82.0)
Serum 1 ml + 50% phosphoric acid 50 µl + diethyl ether—methanol (2:1) 3 ml	78.0 (78.5)	85.6 (100)	75.5 (92.0)
Serum 1 ml + diethyl ether—methanol (2:1) 6 ml	—	—	75.4 (89.4)
Serum 1 ml + 50% phosphoric acid 50 µl + heptane—isopropanol (1:4) 5 ml	58.4	34.6	77.8 (98.0)
Serum 1 ml + heptane—isopropanol (1:4) 6 ml	—	—	78.8 (98.6)
Serum 1 ml + 50% phosphoric acid 50 µl + chloroform—diethyl ether (1:1) 4 ml	66.0	18.6	3.9
Serum 1 ml + water 1 ml	48.0	103.0	101.0
Serum 1 ml + water 3 ml	—	102.0	—

in serum failed within the range of this study (Table III); therefore we investigated the ultrafiltration method described below.

Ultrafiltration method for the recovery of PA in human serum and plasma. With the same materials as used for deproteinization, ultrafiltration was studied as a pretreatment method (Table IV). Of the PA, MEHP and DEHP in human

TABLE IV

ULTRAFILTRATION PRETREATMENT OF HUMAN SERUM FOR PA, MEHP AND DEHP DETERMINATION

The same sample as described in the legend to Table III was used.

Method of ultrafiltration	Recovery ratio (%)		
	PA	MEHP	DEHP
Serum 1 ml	76.8	3.3	None
Serum 1 ml + 1 *M* sodium hydroxide 1 ml	12.6	23.4	None
Serum 1 ml + 50% phosphoric acid 50 μl	80.6	1.8	None
Serum 1 ml + 50% phosphoric acid 200 μl	81.2	1.5	None
Serum 1 ml + water 1 ml + 50% phosphoric acid 100 μl	92.5	1.8	None
Serum 1 ml + water 3 ml + 50% phosphoric acid 100 μl	99.9	6.8	5.6

serum, about 77%, 3% and 0%, respectively, were found to be present in the ultrafiltrate. Addition of acid to the serum increased the amount of PA in the ultrafiltrate to about 80%, but it caused little change in amounts of MEHP and DEHP. An increased amount of acid did not alter the amounts of PA, MEHP and DEHP in the ultrafiltrate, but dilution of serum with water increased the recovery; in particular, PA was found for the most part in the ultrafiltrate after the addition of acid to a four-fold dilution of serum with water. The fact that this method gave 100% recovery for MMP suggests that it is suitable for the pretreatment of hydrophilic compounds. PA in human plasma was about 100% recovered by this ultrafiltration method.

Pretreatment using Sep-Pak C_{18}. To 1 ml of human serum as used for deproteinization, 3 ml of water and 0.1 ml of 50% phosphoric acid were added and shaken vigorously. Then 1 ml of the mixture was applied to Sep-Pak C_{18} and eluted with 8 ml of methanol or acetonitrile. The solvent was evaporated and the residue was dissolved again in 1 ml of the mobile phase for MEHP analysis, with a recovery of 86.6% for PA and 87.0% for MEHP. No difference was found in the recovery ratio between acetonitrile and methanol. Experiments under various conditions revealed that the Sep-Pak C_{18} method for the recovery of PA and MEHP in human serum was inferior to the two pretreatment methods previously described, in terms of the low recovery ratio and the troublesome handling of the evaporation of the solvent.

Determination of PA, MEHP and DEHP in a flexible bag and their migration into blood products

About 1 g of that part of a flexible bag which comes in contact with blood (about 15 g) was cut into fine pieces, to which 100 ml each of diethyl ether and phosphoric acid—diethyl ether (pH 2) were added. After shaking for four days at room temperature, the solvent was evaporated and PA, MEHP and DEHP were determined after re-dissolution in the mobile phase (Table V). The extraction by phosphoric acid—diethyl ether produced a similar result to that obtained with diethyl ether extraction. Table V summarizes total amounts of PA, MEHP and DEHP having migrated into human platelet plasma. About 0.13% of DEHP from the flexible bag migrated into the human platelet plasma.

TABLE V

AMOUNTS OF PA, MEHP AND DEHP EXTRACTED WITH DIETHYL ETHER FROM A FLEXIBLE BAG AND THE AMOUNTS OF THESE COMPOUNDS ELUTED INTO CONCENTRATED HUMAN PLASMA PLATELETS

One gram of flexible bag was cut into small pieces, 100 ml of either diethyl ether or acid—diethyl ether solution were added and the whole was kept constantly agitated for four days at room temperature.

	Amount extracted* (n = 3)	Amount eluted** (n = 3)
DEHP (mg)	3128.1 ± 179.4	4.0 ± 0.5
MEHP (μg)	130.8 ± 14.3	589.5 ± 101.3
PA (μg)	0.0	9.0 ± 1.2

*Amount was that contained in 15 g of flexible bag.
**Amount was that contained in 15 ml of concentrated human plasma platelets.

The fact that PA, which was not detected in the flexible bag, was detected in human platelet plasma suggests that it was produced by enzymatic hydrolysis from the eluted DEHP. It is likely that the speculation will hold true concerning MEHP. This fact will be supported by the findings that in spite of the DEHP/MEHP ratio being about 24,000:1 in the flexible bag, the ratio in human platelet plasma was about 7:1 and that the amount of MEHP in human platelet plasma was greater than in the flexible bag.

MEHP and PA were not detected as impurities in 10,000 ppm of industrial DEHP (Ishikawa Product, Tokyo, Japan) that is added to PVC. This suggests a possibility that MEHP detected in the flexible bag (about $4 \cdot 10^{-3}$% of DEHP) was produced from DEHP by heating during the PVC moulding procedures [8]. During moulding procedures and also during the acid—diethylether extraction procedure PA was not produced from DEHP or from MEHP.

Quantitative determination of PA, MEHP and DEHP in blood products

Using the following blood products — fresh-frozen human plasma, concentrated human platelet plasma, heated human plasma protein of Plasmanate, dried human plasma, human plasma and equine plasma — the amounts of PA, MEHP and DEHP eluted from the flexible bag into these blood products were determined (Table VI). The results indicated that contact time with the flexible bag with or without centrifugation, storage temperature, etc. were responsible for the differences in the amount of DEHP eluted. With high storage temperature (22°C) and vigorous shaking with centrifugation for one day, DEHP was markedly eluted into the concentrated human platelet plasma and MEHP was also present in a large amount. Of importance is the fact that highly toxic MEHP was detected in fresh-frozen human plasma and concentrated human platelet plasma in amounts of about 1/7 to 1/17 that of DEHP. In Plasmanate manufactured after heating for 10 h at 60°C most of the eluted DEHP was found to be hydrolysed to PA by heat and enzymes. PA, MEHP and DEHP were contained in a significantly large amount in human plasma (JBMC), a product that had been imported from the U.S.A. and which had been stored at room temperature of 22°C for the long period of 25 days. Therefore, DEHP was thought to have been eluted in a larger amount during the storage than was

TABLE VI

CONCENTRATION OF PA, MEHP AND DEHP IN SEVERAL BLOOD PRODUCTS AND IN HUMAN PLASMA

Sample	Concentration (μg/ml)		
	PA	MEHP	DEHP
Fresh frozen human plasma (JRC) (n = 3)	0.3 ± 0.1	1.54 ± 0.3	26.7 ± 0.3
Concentrated human plasma platelet (JRC) (n = 3)	0.6 ± 0.1	39.3 ± 6.8	267.0 ± 30.5
Plasmanate (n = 3)	12.0 ± 1.0	0.9 ± 0.1	2.0 ± 0.2
Dried human plasma (n = 3)	0.7 ± 0.1	N.D.	1.0 ± 0.1
Human plasma (JRC) (n = 3)	3.0 ± 0.4	5.6 ± 1.1	72.5 ± 2.9
Human serum (JRC) (n = 3)	N.D.*	N.D.	N.D.
Human plasma (JBMC, imported from U.S.A.) (n = 2)	4.9 ± 0.0	54.4 ± 2.5	172.6 ± 5.6
Horse plasma (n = 1)	0.6	0.4	2.2
Horse serum (n = 3)	N.D.	N.D.	N.D.

*N.D., not detected.

the case for human plasma from JRC. Furthermore, storage at room temperature (22°C) may have caused the eluted DEHP to change into MEHP and PA.

CONCLUSION

For the pretreatment of serum for the determination of PA, serum was diluted four-fold with water followed by the addition of acid and ultrafiltration. The pretreatment of serum for the determination of MEHP was the addition of acid and a mixture of diethyl ether—methanol (2:1), overnight standing and centrifugation to obtain the supernatant. The pretreatment of serum for the determination of DEHP was the addition of sodium hydroxide followed by deproteinization with acetonitrile.

Under the isocratic HPLC conditions it was impossible to separate and determine PA, MEHP and DEHP simultaneously in serum. Therefore, determination of PA and MEHP in serum was carried out by reversed-phase chromatography employing ion suppression, and the determination of DEHP in serum was carried out by reversed-phase chromatography without ion suppression.

The amount of DEHP eluted into blood products from the flexible bag differed, depending upon the period of storage with or without centrifugation, the storage temperature, etc. A large amount of DEHP was detected in human platelet plasma. The amount of MEHP was about 1/7 that of DEHP. Detection of PA and MEHP in addition to DEHP suggests the involvement of hydrolytic enzymes.

About 0.1% of the DEHP in a flexible bag was found to have migrated into concentrated human platelet plasma. Most of the PA and MEHP detected in

human platelet plasma was not derived from the flexible bag but was produced by enzymatic hydrolysis of DEHP, thus causing the migration.

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