Pharmaceutica Analytica Acta
ISSN: 2153-2435

Shintani, Pharm Anal Acta 2011, S11
DOI: 10.4172/2153-2435.S11-005

Research Article Open Access

Pretreatment and Chromatographic Analysis of Phthalate Esters, and their Biochemical Behavior in Blood Products

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Summary

A method combining pretreatment by automated solid-phase extraction (SPE) with high-performance liquid chromatography (HPLC) has been developed for analysis of the endocrine disrupters mono-(2-ethylhexyl) phthalate (MEHP) and di-(2-ethylhexyl) phthalate (DEHP) in blood products. For successful analysis it was necessary to use acidified blood and acidified SPE eluent to suppress the ionization of MEHP and phthalic acid (PA), the amounts of PA, MEHP, and DEHP migrating into blood products from flexible blood bags made from poly (vinyl chloride) (PVC) was determined. By addition of inhibitors of endogenous blood lipase and esterase to blood it was shown that most of the MEHP detected in human plasma is not derived directly from flexible PVC bags but is produced by the action of these enzymes on DEHP. Esterase hydrolytic activity was greater than lipase activi1y.

Keywords: Column liquid chromatography; Solid-phase extraction; Plasticizer; Endocrine disrupter

Introduction

The major material used to manufacture flexible blood bags and catheter tubing is polyvinyl chloride (PVC) containing ca 20-50% di-(2-ethylhexyl) phthalate (DEHP) as plasticizer. It has been reported that DEHP and MEHP are toxic, mutagenic, teratogenic, and carcinogenic, and also affect the reproductive organs and fertility [1] and that DEHP is changed by heating and by hydrolytic enzymes to mono-(2-ethylhexyl) phthalate (MEHP) and to phthalic acid (PA) [2]. When the binding of these compounds to human plasma proteins was studied, PA was not discussed because it is not an endocrine disrupter and the amount of PA in blood was negligible.

To confirm that MEHP and PA are produced by enzymatic hydrolysis of DEHP, a study has been conducted to confirm that levels of MEHP and PA in blood increase as levels of DEHP decrease. There have been no reports of the use of enzyme inhibitors to confirm enzymatic hydrolysis of DEHP.

The safety of these compounds must be considered because it has been reported that MEHP is more toxic than DEHP [3].

A method is described for simultaneous analysis of PA, MEHP, and DEHP in blood by HPLC with linear gradient elution after automated solid-phase extraction (SPE); the first report of such a method. The method has been used for quantitative determination of the amounts of DEHP, MEHP, and PA in blood products.

Experimental

Reagents

Commercially available chemically pure reagents were used, except for MEHP, which was synthesized by the author in accordance with a method reported in the literature [4].

In addition to the use of endogenous lipase and esterase in human blood, commercially available lipase (Behlinger; lipase (1), EC3.I.l.3, from *Rhizopus nigricans*, specific activity 14901 Units/mg) and esterase (Behlinger; EC3.l.l, from porcine liver homogenate, specific activity 100 Units/mg) were used.

Commercially available lipase and esterase kits (Dainihon Pharmaceutical, Tokyo, Japan) were used for determination of enzyme activity.

Materials

Flexible PVC blood bags (Terumo, Tokyo, Japan) were materials for medical use. Fresh-frozen human plasma (Japan Red Cross; JRC, Tokyo) was stored at -30°C for one week in flexible PVC bags. Concentrated human platelet plasma (JRC) was stored at 22°C for two days in flexible PVC bags; it was centrifuged on the first day. Human serum (JRC) was stored in a glass bottle. Human plasma (JRC) was kept frozen for 21 days in a flexible PVC bag. Plasmanate (Green Cross, Osaka, Japan) was stored in a glass bottle at 60 °C for 10 h after fractionation. Dried human plasma was from (Nihon Pharmaceutical, Tokyo, Japan). Human plasma (Japan Biological Materials Center, JBMC, Tokyo, Japan) was stored at 22°C for 25 days in a flexible PVC. Equine plasma (JBMC) and serum (Flow Labs, VA, USA) were stored in glass bottles.

Equipment

HPLC was performed with a SpectraPhysics SP-8750 instrument equipped with a Shimadzu (Kyoto, Japan) SPD 2A ultraviolet (UV) detector. Compounds were separated on a Shiseido (Tokyo, Japan) 250 x 4.6 mm i.d. Capcel Pak C-18 AG-120 analytical column. Automated SPE was performed with BenchMate (Zymark, MA, USA) equipment in combination with Varian (CA, USA) BondElut C-18 columns containing 100 mg resin (void volume 120 µL).

HPLC conditions

The mobile phase was a mixture of acetate buffer (pH 3, 10 mM)

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Received September 11, 2011; **Accepted** December 10, 2011; **Published** December 10, 2011

Citation: Shintani H (2011) Pretreatment and Chromatographic Analysis of Phthalate Esters, and their Biochemical Behavior in Blood Products. Pharm Anal Acta S11:005. doi:10.4172/2153-2435.S11-005

and acetonitrile. The ratio of acetate buffer to acetonitrile was changed linearly from 9: 1 to 1:9 in 17 min, then changed to 100% acetonitrile in another 6 min to clean the column, after which the initial mobile phase was delivered for 5 min before the start of the next analysis. The total analysis time was, therefore, 28 min. The flow rate was 1 mL/min; detection was at 235 nm; and the injection volume was 20 μL. Acetate was added to suppress the formation of carboxylate ions by MEHP and PA.

Conditions for SPE of MEHP and DEHP from blood

Suppression of the ionization of the carboxylate group in MEHP was essential if the compound was to be retained on the C-18 column. Thus, 1:1 blood and 10mM acetate buffer, pH 3, were well mixed before application (1 mL) to an SPE column previously conditioned with acetonitrile (2 mL) then acetate buffer (10 mM, pH 3; 2 mL). The sample was then passed through the column, by application of vacuum, the column was rinsed with acetate buffer (10 mM, pH 3; 0.5 mL), and the compounds of interest were eluted with 1 mL acetonitrile acidified with acetic acid at pH 2. The eluate was collected and 20 μL were analyzed by HPLC. Conditioning, rinsing, and elution of the column were performed by using a computer-controlled vacuum system in conjunction with the BenchMate equipment.

Results and Discussion

Preparation of MEHP

MEHP was synthesized by the author in accordance with a published method [4]. Although, ideally, 1mol PA should be esterified to MEHP by 1 mol 2-ethylhexanol, several reaction products were obtained. These were identified, by HPLC-mass spectrometry (MS) with atmospheric-pressure chemical ionization (APCI), as DEHP and MEHP (major products) and the mono-and dimethyl esters of PA (minor products). The starting compounds were also present in the mixtures (confirmed by MS, data not shown).

MEHP was isolated from among these compounds and purified. The melting point of the MEHP obtained was 82-83 °C, in good agreement with that reported elsewhere [4]. The infrared, nuclear magnetic resonance and mass spectra of MEHP were identical with those reported in the literature.

HPLC and SPE

Because it has been reported that conventional liquid-liquid extraction of DEHP, MEHP, and PA results in poor recovery [5-7], an attempt was made to develop an alternative method for satisfactory and reproducible recovery of these compounds from blood. Initially the author studied deproteinization with a mixture of acetonitrile and sodium hydroxide, and then ultrafiltration, for pretreatment of serum [8]. The upper layer was concentrated by evaporation. Because this conventional liquid-liquid extraction procedure was complicated and tedious, and the rate of recovery was relatively low (< 80%, n =5) [8], automated SPE was investigated.

This procedure was found to be simpler, less time-consuming, and consumed less organic solvent, which is of advantage both to the analyst and to the environment. The greatest benefit of using automated SPE was satisfactory recovery reproducibility, because of the availability of constant pressure control. The recovery by automated SPE was 98-102% (average 99%, n =5). Manual SPE is, however, not recommended because the reproducibility of recovery was, in the author's experience, poor, between 40 and 105%; this indicates that accurate pressure control is essential when performing SPE.

This experiment was performed on human serum spiked with MEHP and DEHP. The human serum used did not contain DEHP and MEHP because it was stored in a glass bottle. Linear gradient elution was used for HPLC analysis after SPE. The retention times of PA, MEHP, and DEHP were 4.8, 9.6, and 15.7min, respectively. There was no interference with these peaks from other compounds present in the blood, indicating SPE pretreatment was satisfactory (Figure 1). The recovery of PA, MEHP, and DEHP from blood was satisfactory, as already mentioned. If the blood samples and the solution used to condition the SPE column were not acidified, recovery of PA and MEHP from the column was poor. They were not retained successfully by the C-18 column when ionization of the carboxylate group was not completely suppressed. (This is an ion-suppression effect.) A procedure which did not use ion suppression has been reported, but the recovery of the carboxylate compounds was not satisfactory [9]. The success of the experiment reported herein was a direct result of acidification and the consequent ion-suppression effect and common ion effect for the acidic compounds of MEHP and PA [10-15]. The common ion effect must also be significant. When the SPE procedure was compared with conventional liquid-liquid extraction, SPE afforded superior recovery, because the lack of need to concentrate eliminated evaporative loss [8,12,13,15]. Liquid-liquid extraction requires troublesome concentrative evaporation, consumption of large volumes of organic solvent, and complicated handling, and often results in lower recovery because of loss during evaporation [8], especially of lower boiling-point compounds. It has been reported elsewhere [8] that concentration of dioxin extracts by use of a jet of nitrogen gas resulted in lower recovery, because of blowing out during the concentration procedure.

Binding ratio of DEHP and MEHP to blood serum and plasma

Solutions of DEHP and MEHP in acetonitrile (39.08 and 39.4 mg/mL, respectively; 10 μL) were added to DEHP-free serum (5 mL) and the sample was well mixed. Plasma samples contaminated with DEHP and MEHP (69.6 and 6.7 μg/mL, respectively) were also used. The samples were centrifuged at 10000 g for 40 min using 5000 Dalton cellulose cut off membrane from Amicon Co. DEHP and MEHP in the centrifuged liquid were determined by HPLC. The amounts of DEHP and MEHP in the centrifuged liquids were divided by the amount spiked, and the ratio of free DEHP and MEHP in serum and plasma were calculated to be 0 and 1.0%, and 0 and 3.3%, respectively. In both types of sample almost all DEHP and MEHP was bound to protein.

Determination of MEHP and DEHP in Flexible PVC Bags for Medical Use, and Amounts Migrating into Blood Products from the PVC Bag

Approximately 1 g of a flexible PVC bag (total weight 15 g; Table 1) in contact with blood was accurately weighed, cut into fine pieces, and mixed with diethyl ether acidified to pH 2 with phosphoric acid (100 mL). After shaking for four days at room temperature, the solvent was evaporated and the amounts of PA, MEHP, and DEHP in the residue were determined after re-dissolution in the HPLC mobile phase (1: 1 acetate buffer (pH 3, 10 mM) : acetonitrile). As shown in Table 1, the PVC contained ca 20% DEHP. Table 1 also summarizes the total amounts of MEHP and DEHP migrating from the PVC bag to human platelet plasma. According to Table 1, ca 0.13% of the DEHP present in the PVC bag migrated into human platelet plasma.

PA, which was not detected in flexible PVC bags, was detected in human platelet plasma, suggesting it was produced by enzymatic hydrolysis of the DEHP. The DEHP/MEHP ratio in the flexible PVC

bag was ca 24000: 1 whereas that in human platelet plasma was ca 7: 1, i.e. a relatively larger amount of MEHP was present in human platelet plasma than in the original flexible PVC bag. This might be because of enzymatic hydrolysis of DEHP from the PVC bag. Formation of MEHP upon heating and/or by chemical hydrolysis is also possible, however (although it is shown below that chemical hydrolysis did not significantly increase MEHP production). Heating at ca 37°C will be effective for enzyme function. Heating above 37°C would denature the enzymes and so would not be very effective for MEHP production.

The possibility of acid hydrolysis during extraction with acidified diethyl ether was discounted because MEHP was not produced on contact with acidified diethyl ether. Thus production of MEHP in blood products must be a result of enzymatic hydrolysis of DEHP.

Activity and Michaelis-Menten Constants (Km) of Lipase and Esterase in Human Plasma. By use of a commercially available kit (see Experimental) the activity of lipase and esterase in human plasma was determined to be 10.73±0.44 and 47.04±5.15 μ mol/min/mL (n= 3), respectively. Different amounts of DEHP lipase or esterase (1 mg) were added to human plasma (20 mL) to generate the Lineweaver-Burk plot from which Km for lipase and esterase were calculated to be 1.47 and 0.46 μmol/mL, respectively. Thus esterase was more active than lipase toward DEHP (the smaller the value of Km, the more reactive the enzyme).

Time Course of the Amount of DEHP, MEHP, and PA with and without Addition of Endogenous Lipase or Esterase or Commercial Enzymes. When human plasma contaminated with DEHP (100 mL) was stirred on a water bath at 37°C and sampled at one-hour intervals for 8 h (Figure 2) the amount of DEHP decreased linearly with time whereas those of MEHP and PA linearly increased with time. This was because of endogenous enzymes in the plasma.

Commercially available lipase (3.6 mg/mL, specific activity of 54185 Units/mL; 100 μL) and esterase (10.0 mg/mL, specific activity of 1000 Unires/mL; 100 μL) were added separately to human plasma (25 mL) containing DEHP and the mixtures were stirred at 37°C. Levels of DEHP, MEHP, and PA were determined by HPLC. Addition of commercial lipase or esterase to human plasma resulted in a significant increase in hydrolysis of DEHP (Figure 2). The decrease of DEHP and increase of MEHP and PA were greater than for endogenous enzymes only. The increases and decrease were linear and first-order. Comparison of the effect of lipase and esterase on DEHP indicated that esterase contributed more than lipase to DEHP hydrolysis; although the specific activity of esterase was ca 54-150 times lower than that of lipase. Because the increases in amounts of PA were much less than those of MEHP, the step MEHP-PA was thought to be the rate-limiting step in the pathway from DEHP to PA through MEHP (DEHP →MEHP→PA); this contradicts previous results implying that the rate-limiting step was from DEHP to MEHP [16].

In enzymatic hydrolysis with or without addition of enzymes to blood, chemical stoichiometry was consistent; the decrease in the amount of DEHP in 8 h was equal to the sum of the increases in the amounts of MEHP and PA, indicating that other alkyl esters were not produced during the 8-h reaction period. Increased production of MEHP and PA was confirmed by addition of esterase and lipase. This does not, however, prove that production of MEHP and PA was promoted only by addition of the enzyme. It is possible that addition of the enzymes triggers the production of other enzymes which increase levels of MEHP and PA and reduce those of DEHP. Addition of inhibitors of esterase and lipase is needed to confirm these enzymes are the major contributors to the degradation of DEHP.

Time Course of Amount of DEHP, MEHP and PA after Addition of Lipase and Esterase Inhibitors

To study further whether lipase or esterase contributes more to the decrease of DEHP and the increase of MEHP and PA separate additional experiments were performed using lipase or esterase inhibitors.

The esterase inhibitor phenylsulfonylfluoride (3.48 or 13.92 mg/mL; 2mL) was added to human plasma (20 mL) contaminated with DEHP and the time course of the amount of DEHP was followed (Figure 3). It was found that the amount of DEHP in blood was unchanged by esterase inhibition. A slight increase in the amount of MEHP was observed, probably because of endogenous lipase in blood. When the lipase inhibitor di-isopropylfluorophosphate was also added to the blood, MEHP production was completely terminated (Figure 3). No difference between the amount of MEHP produced was observed

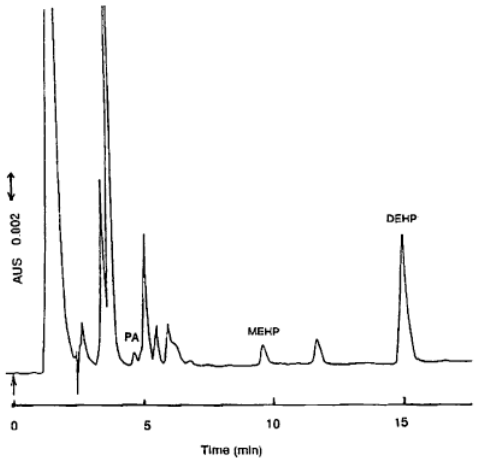

Figure 1: HPLC chromatogram obtained after SPE of fresh frozen human plasma from the Japanese Red Cross. Recovery of PA from plasma was somewhat unsatisfactory, because of the hydrophilicity of the compound; recovery was, however. Sufficient for quantitative work, as mentioned in the text.

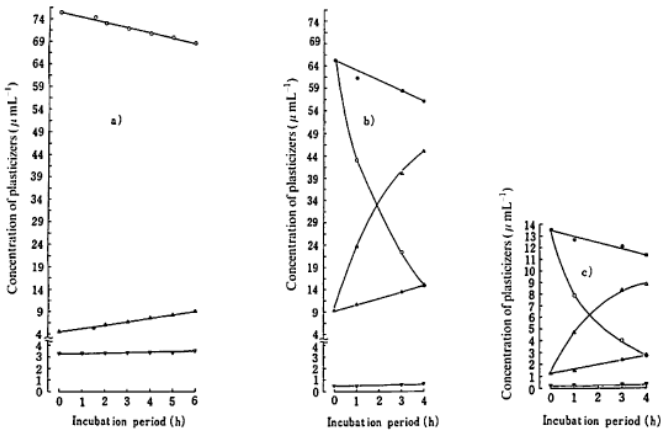

Figure 2: Changes of the concentrations of PA, MEHP, and DEHP when incubated at 37 °C: (a) without addition of enzymes to plasma (○=DEHP, △=MEHP, △=PA); (b) with addition of lipase (3.6mg/ mL, 100 μL) or esterase (10 mg/mL, 100 μL) ti okasna (○=DEHP, △=MEHP, ▽=PA); (c) with addition of lipase (3.6mg/mL, 278 μL; (○=DEHP, △=MEHP, △=PA) or esterase (10 mg/mL, 100 μL; ●=DEHP, ▲=MEHP, ▼PA) to plasma.

when the concentration of esterase inhibitor was changed from 3.48 to 13.92 mg/mL, thus 3.48 mg/mL inhibitor was sufficient.

Hydrolysis of DEHP as a result of heating or non-enzymatic effects such as chemical hydrolysis was not significant, as already described above; it was therefore not necessary to check whether these were factors affecting the production of MEHP from DEHP in this experiment. That enzymatic hydrolysis is a valid means of accounting for the production of MEHP from DEHP can be tested by heating blood to the optimum temperature for enzyme activity, ca 37°C.

The time course of migration of DEHP from to human plasma, with or without enzyme inhibitor, followed a linear first order equation.

When lipase inhibitor di-isopropylfluorophosphate (2-4 mM), rather than the Esterase inhibitor was added to human plasma contaminated with DEHP (Figure 3), MEHP production was greater than with lipase inhibitor added. From this it was speculated that the activity of esterase in DEHP hydrolysis is greater than that of lipase. When inhibitors of lipase and esterase were both added to blood products in flexible PVC bags, no production of MEHP was observed, because of inhibition of both enzymes; this indicates that addition of both inhibitors effectively prevents MEHP production, although the effects of residues of the toxic inhibitors must also be considered.

Quantitative Determination of PA, MEHP and DEHP in Blood Products

The amounts of PA, MEHP, and DEHP migrating into blood products from flexible PVC bags for medical use are listed in Table II. It was found that contact time with the bag affected the amount of DEHP in the blood product more than other factors (centrifugal treatment, storage temperature, total storage period, etc.). High storage temperature (22°C) and vigorous shaking with centrifugation for one day resulted in marked migration of DEHP into concentrated human platelet plasma, and the amount of MEHP was also higher. It

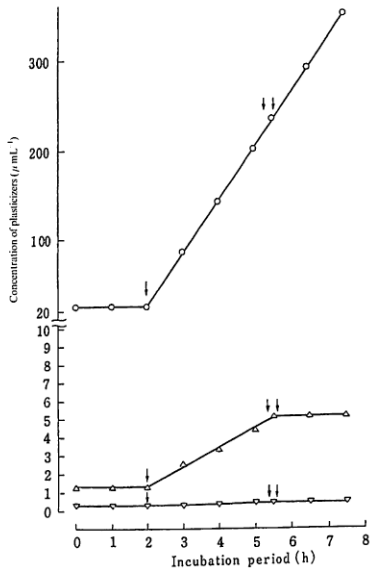

Figure 3: Changes of the concentrations of PA, MEHP, and DEHP when incubated at 37 °C: (a) without addition of enzymes to plasma (○=DEHP, Δ=MEHP, Δ=PA); (b) with addition of lipase (3.6mg/ mL, 100 μL) or esterase (10 mg/mL, 100 μL) ti okasna (○=DEHP, Δ=MEHP, ∇=PA); (c) with addition of lipase (3.6mg/mL, 278 μL; (○=DEHP, Δ=MEHP, Δ=PA) or esterase (10 mg/mL, 100 μL; ●=DEHP, ▲=MEHP, ▼PA) to plasma.

	Amount extracted From PVC bag (n-3)*	Amount migrating into plasma (n=3) **
DEHP (mg)	3128.1 ± 179.4	4.0 ± 0.5
MEHP (μg)	130.8 ± 14.3	589.5 ± 101.3
PA (μg)	N.D	9.0 ± 1.2

A flexible PVC bag for medical use (1 g) was cut into small pieces, acidified diethyl ether (100 ml) was added, and the mixture was kept constantly agitated for four days at room temperature.
* Amount contained in 15 g of flexible PVC bag for medical use.
** Amount contained in 15L concentrated human platelet plasma.
N. D. = not detected.

Table 1: Amounts of PA, MEHP and DEHP extracted with diethyl ether from flexible PVC bags for medical use, and the amounts of these compounds migrating into concentrated human platelet plasma.

is important to note that the more toxic MEHP was detected in fresh-frozen human plasma and concentrated human platelet plasma at levels between ca 1/7 and 1/17 that of DEHP. In Plasmanate® manufactured after heating for 10 h at 60°C (treatment for deactivation of HIV), most of the DEHP was hydrolyzed to PA by enzymes; thus PA was the most abundant compound and MEHP, fortunately, the least abundant. PA, MEHP, and DEHP were present in significantly larger amount in human plasma from JBMC, a product that was stored at 22°C for a long period (25 days). It is thought, therefore, that DEHP migrates to a greater extent because the storage period is longer than that of human plasma from JRC. During storage at room temperature (22°C), migrating DEHP can be hydrolyzed to MEHP and PA.

Little or no DEHP, MEHP, and PA were detected in blood products stored in the glass bottles (Table 2) because there was no migration of DEHP.

Limit of Determination (LOD) of PA, MEHP and DEHP

A concentration > the limit of detection (S/N = 2), within 20% of the reliability of determination accuracy and within 20% of precision can be regarded as a satisfactory LOD. The determination of LOD of PA, MEHP and DEHP in this experiment was 2 ng/mL, 5 ng/mL and 6 ng/mL, respectively (n=5) in serum and plasma. These LOD was satisfactory in this experiment (Table 2).

Conclusion

Automated SPE on a C-I8 column, with acidified blood and acidified eluent, enables satisfactory recovery of PA, MEHP, and DEHP from blood. If acidified solvent was not used, recovery of MEHP and PA from blood by SPE was unsatisfactory. Linear gradient elution of a C-18 column, with ion suppression and UV detection, enabled satisfactory separation of PA, MEHP, and DEHP from blood (Figure 1).

The amount of DEHP migrating into blood products from the flexible PVC bags depended more on the storage period than on other treatment (e.g. centrifugation, the storage temperature, etc), as shown in Table 2. A large amount of DEHP was detected in human platelet plasma. The amount of MEHP was ca 1/7 that of DEHP (Table I). Detection of PA and MEHP in addition to DEHP suggests the involvement of hydrolysis enzymes; this was confirmed by use of commercially available lipase and esterase and indicates that endogenous hydrolysis enzyme can reduce the amount of DEHP and increase of MEHP and PA in blood linearly with time. Esterase activity in DEHP hydrolysis was greater than that of lipase. In the reaction pathway from DEHP to PA through MEHP, the reaction from MEHP to PA was found to be rate-limiting step. The LOD of PA, MEHP and

Sample	Concentration (μg/mL) in:			
	PA	MEHP	DEHP	
Fresh frozen human plasma (JRC; n= 3)	0.3± 0.1		1.54 ± 0.3	26.7 ± 0.3
Concentrated human platelet plasma (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; n =2)	4.9± 0.0		54.4 ± 2.5	172.6 ± 5.6
Equine plasma (n=1)	0.6	0.4	2.2	
Equine serum (n= 3)	N.D.	N.D.	N.D.	

Table 2: Concentration of PA, MEHP and DEHP in several blood products and in human plasma.

DEHP in this experiment was satisfactory. That of PA, MEHP and DEHP were 2, 5, 6 ng/mL. PVC was found to contain ca 20% DEHP (Table 1). Migration of DEHP from flexible PVC bags was linear with time and approximately 0.1 % of the DEHP in a flexible PVC bag was found to migrate into concentrated human platelet plasma (Table 1). Most of the PA and MEHP detected in human platelet plasma was not derived directly from the bag, but was produced by enzymatic hydrolysis of migrating DEHP. The amount of DEHP, MEHP, and PA bound to blood protein was, 100, 97-99, and 25%, respectively.

When human plasma contaminated with DEHP was stirred at 37°C the amount of DEHP decreased linearly with time whereas levels of MEHP and PA increased linearly. Consistent chemical stoichiometry was measured for these compounds.

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This article was originally published in a special issue, **Pharamacokinetics: Validation, Metabolism, etc.** handled by Editor(s). Dr. Hideharu Shintani, Chuo University, Japan