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DETERMINATION OF DI(ETHYLHEXYL) PHTHALATE IN HUMAN PLASMA AND PLASMA PROTEINS BY ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A rapid method for the determination of di(ethylhexyl) phthalate (DEHP) in plasma and plasma proteins by electron capture gas chromatography is presented. Plasma samples are evaporated to dryness after addition of methanol. The residue is soaked with methanol, followed by toluene and finally water is added, and the DEHP content of the toluene layer is determined quantitatively with "dinonyl" phthalate as internal standard. The relative recoveries at the 20 μg/ml level are 100 ± 3.6%. The DEHP content of plasma stored in plastic bags ranged from 16 to 120 μg/ml (corresponding to 0.24–0.7 mg/g of protein). Plasma in poly(vinyl chloride) bags may be stored at −20° without increase in the DEHP content; if stored at 4°, however, storage time should be as short as possible. Completely filled bags are preferred as the degree of extraction (per ml) is then lower. In comparison with that of blood plasma, the DEHP content of fractionated plasma proteins was rather small, *i.e.*, fibrinogen 3–18 μg/g, albumin 5–25 μg/g and immunoglobulin G 3–160 μg/g. The risks of loading patients with DEHP are therefore less with fractionated proteins than with transfused blood.

INTRODUCTION

The presence of plasticizers, especially di(ethylhexyl) phthalate (DEHP), as contaminants in solvents[1–3], chemical equipment[4], filter paper[5], body tissues[6] and so on has been repeatedly reported; they can also be regarded as environmental contaminants[7–8]. Interference or disturbance caused by some phthalates in the use of the highly sensitive electron capture detector has also been noticed[4–9].

In 1970, two reports[10,11] appeared showing that the extent of contamination, by phthalates, of blood stored in plastic bags increased with time, and a recent study[12] confirmed that plasma from blood stored in poly(vinyl chloride) bags had high levels of DEHP. Transfusion of blood will transfer substantial amounts of DEHP to the patient. Jaeger and Rubin[11] noticed that most of the DEHP in blood stored in plastic bags was present in the protein fraction; it was therefore of interest to investigate the distribution of this phthalate contamination among the proteins after fractionation.

A review of the findings of Jaeger and Rubin on plasticizers and their extraction into blood has been published recently[13].

A method has been developed for the rapid determination of DEHP in plasma proteins after fractionation as described by Björling[14]. This method, which comprises extraction and determination with electron capture detection, is presented, together with some results; a preliminary communication of this work has been given[15].

EXPERIMENTAL

Apparatus

A Varian 1400 gas chromatograph with an electron capture detector (ECD) (^{3}H source) was used, with a 1.5 m × 0.18 cm glass column filled with 2% of Carbowax–terephthalic acid on Gas-Chrom P (100–120 mesh, acid-washed and silanized). The column was conditioned overnight at 250° and then used at about 215–235°. The detector temperature was 240°, giving a temperature of about 220° at the foil[16], and the injector was kept at 250°. The nitrogen flow-rate was 30 ml/min.

The injection membranes were purchased from Varian Aerograph and had a PTFE connection towards the column.

All glassware in continuous use was shaken with a chromic acid reagent for 10 min, washed with water and then rinsed with ethanol.

Chemicals and reagents

Toluene and methanol, each distilled in all-glass apparatus, were obtained from Burdick & Jackson Labs. (Muscegon, Mich., U.S.A.). DEHP was purchased from K & K Labs. (Plainview, N.Y., U.S.A.), and di-(3,5,5-trimethylhexyl) phthalate ("dinonyl" phthalate) was obtained from Fluka (Buchs, Switzerland). Chromic acid reagent, a solution of chromium trioxide in acetic acid, was prepared according to Vessman *et al.*[17].

Standard solutions. The solution of DEHP used contained 200 μg of solute per ml; the stock solution of internal standard contained 800 μg of "dinonyl" phthalate per ml. Both these solutions were prepared in methanol.

Procedures

Proteins in aqueous solution. For such samples, proceed as follows. By pipette, transfer about 2 ml of sample to a 30-ml centrifuge tube. Add a suitable amount of the internal standard solution, so concentrated that only a few μl (usually 50–100 μl) can be used. Then add 6 ml of methanol and evaporate almost to dryness on a boiling water-bath. To the residue add 3 ml of methanol, and bring the sample on the walls of the tube to the bottom by means of a Pasteur pipette. Centrifuge (Sorvall GLC-1, 2000 rpm), and filter through a plug of silanized glass-wool into small extraction tubes with a tapered base of volume 200 μl. Add sufficient toluene to give a concentration of the internal standard in the final solution of about 8 μg/ml, mix well, add 2 ml of water, and shake three times. Centrifuge, remove the aqueous phase with a Pasteur pipette, and inject about 2 μl of the organic phase into the gas chromatograph. Load the syringe with 1 μl of toluene before and after the sample volume.

Solid samples. Weigh about 0.5 g of sample in a 30-ml centrifuge tube, and add a suitable amount of the internal standard in a few μl of methanol. Add 5 ml of meth-

anol, evaporate on a boiling water-bath till about 3 ml remain, and continue as described under *Proteins in aqueous solution*.

Solutions for the calibration curve are prepared in 10-ml volumetric flasks in which, in order, are placed 0.1, 0.2, 0.3, 0.4 and 0.5 ml of standard DEHP solution; to each is added 0.1 ml of the internal standard solution, and the mixtures are diluted to volume with toluene. Portions (2 μl) are injected into the gas chromatograph, and a calibration curve is constructed from peak-area ratios *vs.* weight ratios. For samples having unknown concentrations of DEHP, a preliminary determination is made to get an approximation of the level of DEHP and to ascertain that no interfering phthalates are present.

RESULTS AND DISCUSSION

There has been considerable interest in the presence of phthalates, especially DEHP, in blood stored in plastic bags since the first report of Marcel and Noel[10]. The work of Jaeger and Rubin[11] confirmed that the contamination increased with the storage time. The presence of DEHP in certain body tissues could also be correlated with the administration of blood, and it was suggested that the ester came both from the bag and the tubings used[18].

The presence of DEHP has been studied by gas chromatography and flame ionization detection as the levels have been rather high. The phthalate esters are good electrophors and can be quantified with an ECD in small amounts[19]. The general contamination of solvents[1–3], laboratories[3], equipment and accessories[1,4] with some phthalates has frequently been noticed. In order to follow the expected low levels of phthalates in fractionated plasma proteins, a method with the high sensitivity and good selectivity offered by the ECD was deemed necessary.

Plasticizers in blood bags

The plasticizers found in such plastic materials as poly(vinyl chloride) have been investigated by Lerche and Morch[20], who found that DEHP predominated. This ester has also been the major phthalate identified in plasma in contact with plastic materials[11]. Acetyltributyl citrate has also been mentioned[20,21], but this compound could not be found in the blood bags used in our company when the plastic material was analyzed according to Lerche and Morch[20]. The search for phthalates in plasma proteins was therefore concentrated on DEHP.

Choice of detector and sensitivity

The Varian 1400 has a ^{3}H-source ECD operating in the direct current (d.c.) mode. As phthalates capture electrons according to a resonance mechanism[22], they can in principle be detected with lower sensitivity at higher temperatures. As has been shown for some equivalent benzophenones, and for dibutyl phthalate, the Varian d.c. mode concentric detector does not exhibit the decrease in sensitivity until a temperature over 200° has been attained[22]; this is advantageous in the determination of DEHP, as a column temperature of more than 215° is required. When the detector is operated in the pulsed mode the sensitivity will be inferior[22]. The sensitivity in this d.c. mode detector is quite good, permitting the detection of picogram amounts. The minimum detectable quantity (MDQ) was determined for the internal standard,

where no blank interferes, and was found to be 5.2×10^{-14} moles/sec; this is somewhat less than that of benzophenone[16,22]. Quantitative electron capture detection was first reported by Bunting and Walker[19], who used a Varian d.c. mode detector; the ECD has also found some use in environmental-pollution studies[8,23].

The flame ionization detector (FID) had about the same sensitivity (MDQ $\approx 5 \times 10^{-14}$ moles/sec), and could from the point of view of sensitivity have been used; however, the selectivity of the FID renders it unsuitable, due to extraneous material in the samples (*cf.* Fig. 2). Hall has discussed the advantage of using the FID over the ECD in studies of phthalates in polluted water[24].

Stationary phase

Several stationary phases were investigated. The non-polar ones (OV 1 and OV 17) showed adsorption phenomena when used in the range 1 to 5%. Higher loading (10%) reduced the adsorption, but required the use of a high column temperature, which resulted in bleeding and detector malfunction. The more polar phases (Carbowax 20M and cyclohexane dimethanol succinate) gave more symmetrical peaks, but showed a tendency to adsorb and desorb the ester in repeated injections. The choice of Carbowax–terephthalic acid eliminated these problems. The lifetime of this column was not too long, and the useful life was indicated by a reduction (from 240 to 215°) in the column temperature required. An old column could be "refreshed" by removing a small part of the column filling facing the injector and moving the rest of the filling towards the injector by suction; this could be repeated twice before the column had to be replaced.

Injection problems

The presence of phthalates in the injector membranes has been noted, and it has been suggested that the membranes should be baked at high temperature before use to remove the plasticizers[4,25]. The lower phthalates are reduced in amount by this treatment, but DEHP was still detectable when ordinary single-type membranes were used. An improvement was achieved with PTFE-lined membranes. In order to thermally save the membranes, an injector extender was tried in the later part of this work; some improvement resulted, *i.e.*, a membrane lasted for 5 days instead of 1 day.

As will be discussed below, the solvents used were never free from DEHP; the injection of fixed volumes of sample was therefore important. To facilitate this, the sandwich technique described by Änggård *et al.*[26] was used, so that solvent evaporation from the needle in the injector would not cause too much variation in results.

Solvents and extraction of DEHP

No suitable solvent was completely free from DEHP upon direct injection into the gas chromatograph (see below under *Limit of detection*). The best ones were toluene and hexane, but hexane was inferior to toluene in the extraction procedure. Various attempts, including distillation, treatment with acid, alkali or reducing agents, distillation over sodium and preparative gas chromatography, to purify some solvents were unsuccessful; usually, the quality was impaired, as other compounds were introduced. Chloroform or dichloromethane could be used as such, but their electron

capture properties made evaporation necessary and this increased the magnitude of the blank. It was therefore decided that treatment of the sample up to the gas chromatographic stage should be performed with as few steps as possible and with no evaporation step. Direct extraction of DEHP from plasma into hexane or toluene was not possible (*cf.* ref. 18).

Lyophilization followed by soaking of the residue with methanol (2 ml) and toluene (0.2 ml) and then addition of water (2 ml) released most of the plasticizer. This process was straightforward, but time-consuming; the procedure was shortened and simplified by adding methanol to the sample and evaporating the mixture to dryness. Thus, sample-preparation time was reduced from 18 h to 15 min. The proportions of methanol, toluene and water (2:0.2:2) were worked out for samples of about 0.5 g. Experiments with different amounts of toluene showed that 0.2 ml was sufficient to ensure quantitative extraction of DEHP from a protein-free sample. When plasma proteins were present, as when fresh human plasma was spiked with DEHP, the absolute recovery was around 60%.

The method of Piechocki and Purdy[12] with a column extraction procedure was run simultaneously on some samples spiked with 8 μg of DEHP/ml. At this low level, the blank was approx. 3.5 μg/ml in that method, compared with approx. 0.8 μg/ml for the proposed method. The difference has to do with the concentration step, which accentuates the blank at this low level. Samples containing about 200 μg/ml of DEHP gave the same result by either method. The batch extraction method is, however, more rapid in performance.

It was noticed that, with time, the laboratory glassware built up a level of DEHP that could not be removed by ordinary washing or by alcoholic alkali treatment. An oxidation reagent[17] containing chromium trioxide in sulphuric acid–acetic acid was successfully used in removing this contamination from, *e.g.*, centrifuge tubes.

Internal standard

"Dinonyl" phthalate, a homologue of DEHP, was chosen as internal standard and added to the sample before the extraction procedure. As the properties of these compounds are closely similar, they behave in the same way in the extraction and are quantitatively detected by the ECD. The addition of the internal standard as well as that of DEHP has to be made in μl amounts in methanol to the sample. If the internal standard solution was evaporated to dryness in an empty glass tube, subsequent addition of the sample would not dissolve the standard.

Limit of detection

The sensitivity of the procedure is limited by the presence of DEHP in the reagents, *viz.*, methanol and toluene. In separate experiments with the internal standard added in known amounts it was found, by comparison and with the assumption that the sensitivity of DEHP is the same, that the DEHP contents of hexane, toluene and methanol were 0.45, 0.47 and 0.5 μg/ml, respectively. Somewhat higher values were obtained for reagents other than those used here. The DEHP in methanol will concentrate in the toluene layer and is the major source of disturbance. The reagent blank corresponds to a concentration of 0.3 μg/ml of DEHP of sample; this means that quantitative determinations with acceptable precision can be made at levels down to 0.5 μg/ml of DEHP.

When freshly drawn plasma (Vacutainer®) was analyzed, the content of DEHP did not differ from that of the blank, indicating that DEHP is normally present only in very minute amounts, if at all, in human blood.

Precision and recovery

A plasma sample containing 20 μg/ml of DEHP was analyzed 10 times with a relative standard deviation of 3.6%. Relative recoveries were 100% due to the use of an internal standard with properties closely similar to those of DEHP. As mentioned above, the absolute recovery was of the order of 60%.

Selectivity

Most proteins, supernatants and fractions containing more than 5 to 10 μg/g of DEHP gave gas chromatograms on which the phthalate was easily distinguishable. Sometimes, however, one fraction contained more peaks after one step in the fractionation process than before. The sensitivity of ECD is high and the selectivity is good but interference is still quite common. As it was unlikely that several phthalates were present (see Fig. 1A) it was necessary to confirm or disprove these results. The technique of mass fragmentography as described by Hammar and Hessling[27] and further developed in this laboratory by Hammar and his collaborators[28] served this purpose

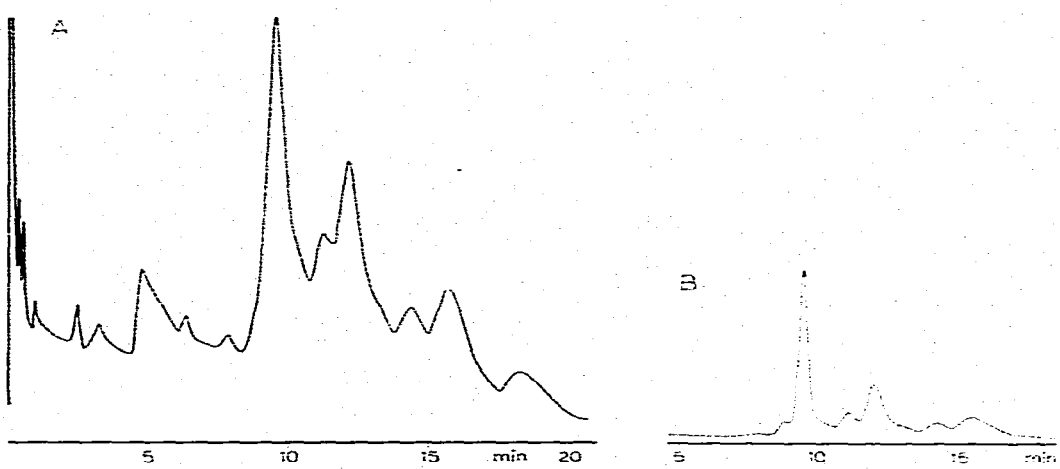

Fig. 1. Extract from a freeze-dried plasma protein. A, gas chromatogram with ECD; B, mass fragmentogram with monitoring of the fragment m/e = 149.

excellently. Phthalates higher than the methyl ester exhibit one large fragment (m/e = 149) in common, and this fragment was used for tracing the compounds in the extract shown in Fig. 1A. As seen in Fig. 1B, the pattern is almost the same with the two techniques. The appearance of these phthalates could be traced to a special brand of plasticized sheet used in the freeze-drying process; this plastic cover was shown by direct analysis to contain the same esters.

For most samples of fibrinogen, the first fraction in the fractionation process, there were several other peaks of non-phthalate origin. Only by mass fragmentography was it possible to determine DEHP in fibrinogen at levels below 10 μg/g.

In the present study, the FID could not be used, owing to the presence of interfering compounds (see Fig. 2A). The problems discussed in this study demonstrate the usefulness of the ECD and also the need to verify the results by mass fragmentography. The use of mass spectrometry to verify ECD tracings of phthalates in air samples was recently described by Thomas[8]; similarly, mass chromatography has also been used[23] for this purpose.

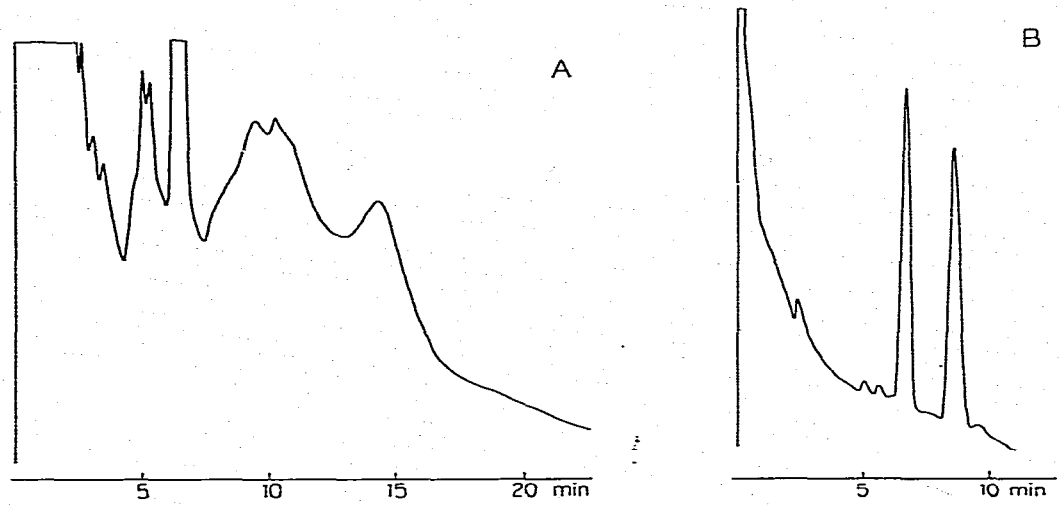

Fig. 2. Extract from a plasma sample. Gas chromatogram with A, FID; B, ECD.

APPLICATIONS

DEHP in plasma

When plasma delivered in 10 different blood bags was analyzed, the DEHP concentration ranged from 16 to 120 μg/ml, corresponding to 0.24 to 1.7 mg/g of protein. This range is in agreement with published values of 70 μg/ml for blood[11] and 115 μg/ml for plasma[10]. There are probably larger differences between individual bags than those noticed here. Piechocki and Purdy reported somewhat lower values[12].

Some plasma samples were stored in polyethylene vessels, which contain no phthalate plasticizer. Yet there were high levels of DEHP originating from the tapping of the blood into a plastic bag and the time it was stored therein. The increase in DEHP content with storage time was reported by Jaeger and Rubin[18] as well as by Marcel and Noel[10]; the former authors found a linear increase over 21 days.

In a study with freshly drawn plasma stored for 7 days both at 4° and −20°, we found an increase that seemed to level off (see Fig. 3); this agrees with the results of Marcel and Noel for plasma[10]. These results were from bags only partly filled with plasma (about 50 ml) from the beginning. In another similar study over 5 weeks, the levelling-off effect was not pronounced; Fig. 4 shows that the concentration of DEHP in plasma stored at −20° for 5 weeks did not exceed 20 μg/ml. This indicates that the major extraction into these plasma samples must occur during the thawing

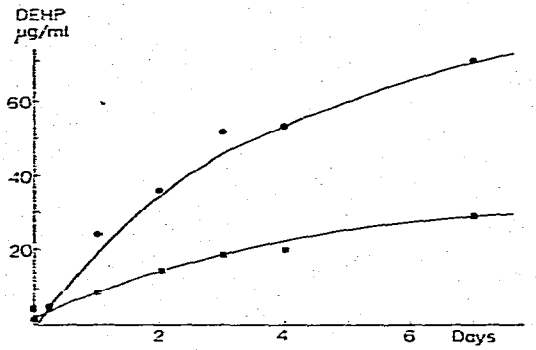

Fig. 3. Extraction of DEHP into plasma during 7 days (bags partly filled, *ca.* 50 ml): ■, −20°; ●, +4°.

period before sampling for analysis. The sample stored at 4° (*cf.* Fig. 4) was analyzed after 10 weeks (when its volume was 10 ml) and contained 890 µg/ml of DEHP.

The irregularities observed in some of the curves may be attributable to a possible inhomogeneity in the rather viscous sample, as well as to the exposure to the plasma of fresh areas of the plastic material due to the small volume of plasma used.

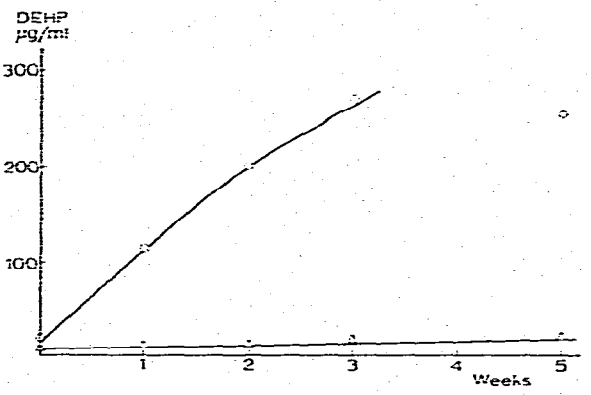

Fig. 4. Extraction of DEHP into 50 ml of plasma during 5 weeks: ○, +4°; △, −20°.

In Fig. 5 are shown some results from bags almost completely filled (400 ml). The concentration of DEHP does not reach the same level as those found in the previous study. The storage time is, of course, unrealistic, but the capacity of the plasma to "extract" DEHP is high. After 7 weeks at 4°, about 56 mg had been transferred from the bag material into the plasma proteins. Analysis after 18 weeks indicated some kind of equilibrium, as the concentration of DEHP levelled off; about 78 mg had been extracted of the 87 mg theoretically possible in that type of bag. The conclusions from these experiments are that plasma samples in poly(vinyl chloride) bags might be stored at −20° without much increase in the DEHP content. The bags should be completely filled in order to give the lowest possible degree of extraction (per ml). If stored at 4° or above, the storage time should be as short as possible.

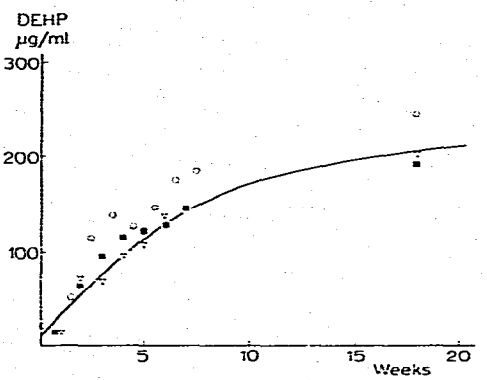

Fig. 5. Extraction of DEHP into plasma during 18 weeks from completely filled bags: at 4° ▽ and ■ ordinary blood bags filled with donor plasma containing *ca.* 1 μg/ml of DEHP; ○, transfer bag filled with donor plasma with *ca.* 20 μg/ml of DEHP from the original tapping bag. Volume at start 400 ml; at each sampling point, 1 ml has been removed.

DEHP in fractions and supernatants during a fractionation

The distribution of DEHP in some plasma-protein fractions after fractionation according to Björling[14] is shown in Table I. As can be seen, most of the DEHP was found in the lipoprotein fraction. The higher the concentration of DEHP in the starting material, the more goes into fraction II + III.

TABLE I

DISTRIBUTION OF DEHP IN SOME PLASMA PROTEIN FRACTIONS

The values are given as a percentage of the amount of DEHP in the starting material. Because of the large volumes processed in the fractionation and the small samples taken for analysis, there may be discrepancies when results from some steps are compared.

Type of protein	*Plasma pool (μg/ml)*	*76.6 (= 100)*	*16.0 (= 100)*	*61.6 (= 100)*
Fibrinogen	Fraction I	—	—	0.5
	Supernatant I	—	—	88
	Fraction II + III	68	48	54
Plasminogen	Fraction III	—	—	53
Protrombin				
β-Lipoprotein etc.				
	Supernatant III	—	—	5
Immunoglobulins	Fraction II	—	—	0.3
	Supernatant II	—	—	4
	Supernatant II + III	20	48	16
Ceruloplasmin	Fraction IV	0.7	18	16
β-Lipoprotein				
Transferrin				
Haptoglobulin				
	Supernatant IV	9	27	4
Albumin	Fraction V	2	5	2
	Supernatant V	4	5	—

DEHP in fractionated plasma proteins

The contents of DEHP in the proteins of major interest from a fractionation are shown in Table II. The content in fibrinogen and albumin was low. The higher levels found in immunoglobulin G are in line with our findings and those of Jaeger and Rubin[18] in that the DEHP concentration is highest in the lipoprotein part (from which immunoglobulins are fractionated).

TABLE II

CONTENT OF DEHP IN SOME FRACTIONATED PLASMA PROTEINS

Ten different batches were investigated

Protein	*DEHP (μg/g)*
Albumin	5– 25
Fibrinogen	3– 18
Immunoglobulin G	3–160

Most of the DEHP does not remain with the proteins of interest after fractionation. In comparison with the DEHP content of stored blood plasma (in this study 240–1700 μg/g of protein in plasma), the fractionated plasma proteins contain small amounts of DEHP (3–160 μg/g of protein). The risk of loading patients with DEHP is therefore less with fractionated proteins than with blood transfusions.

The toxicity of DEHP in three species was reported by Carpenter *et al.*[29]; the "no-effect" level in the rat was about 60 mg per kg per day. Schaffer and his co-workers[30] concluded that DEHP was a chemical of low toxicity. The metabolism of DEHP in the rat was studied by Albro *et al.*[31], who found that mono-(2-ethylhexyl) phthalate was the major metabolite, only 3% of phthalic acid being excreted: the same major metabolite was found in fish[23]. There are indirect data on the metabolism of DEHP in man. Jaeger and Rubin[18] reported increased excretion of a phthalic acid-forming substance from patients who had received large volumes of blood stored in plastic bags. This may in part be the mono-ester, which seems to be resistant to metabolism. The levels of DEHP in fractionated plasma proteins, however, do not approach the amounts of phthalate mentioned by Jaeger and Rubin[13,18].

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