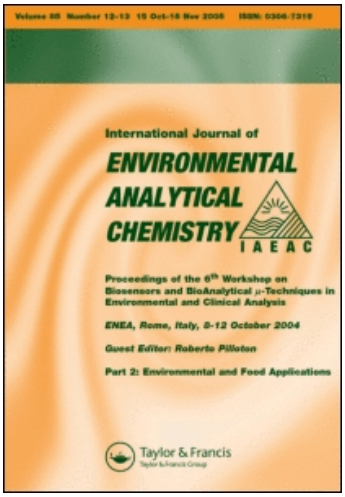

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Differential Contribution of Albumin and Lipids to the Hydrophobic Extraction of Bis-(2-ethylhexyl) Phthalate from Hemodialysis Tubing by Human Serum: Analysis by Gas Chromatography - Stable Isotope Dilution Mass Spectrometry

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DIFFERENTIAL CONTRIBUTION OF ALBUMIN AND LIPIDS TO THE HYDROPHOBIC EXTRACTION OF BIS-(2-ETHYLHEXYL) PHTHALATE FROM HEMODIALYSIS TUBING BY HUMAN SERUM: ANALYSIS BY GAS CHROMATOGRAPHY – STABLE ISOTOPE DILUTION MASS SPECTROMETRY

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A method is presented which allows quantitative assignment of hydrophobic human serum components to the extraction of bis-(2-ethylhexyl) phthalate (DEHP) from medical tubing. Under optimized conditions (sample pH 5.5; fluid-fluid extraction with ethyl acetate + *tert*-butyl methyl ether 1 + 1 v/v; DEHP-ring-D4 as internal standard with ratios of endogenous (m/z = 149) and added deuterated DEHP (m/z = 153) adjusted to around 1.0; equilibration of added internal standard with the hydrophobic sample for 24 hours), a high precision can be achieved with an intra-assay coefficient of variation of 1.5 % (n = 7) for sample DEHP quantification. Phthalate migration from hemodialysis tubing was quantified by use of a peristaltic pump and recirculation (200 minutes) of serum with different degrees of hypertriglyceridemia (range from 2.26 to 14.5 g/L) or solutions of human albumin (10 to 50 g/L). Only DEHP, but no other phthalates are detected in the extracts. There exist linear relations between DEHP extraction and triglyceride content (increase by 1.01 μg DEHP / g tubing material per g triglyceride / L serum) as well as between DEHP extraction and albumin content (0.59 μg DEHP / g tubing material per g albumin / L). Under physiological conditions, the total amount of albumin extracts 17.7-fold more DEHP than the total triglyceride amount in human serum. The suitability of the proposed method as a candidate reference method as well as consequences for dyslipidemic and hypalbuminemic patients on hemodialysis schemes are discussed.

Keywords: Isotope dilution mass spectrometry; hemodialysis; diabetes mellitus; phthalates; albumin; triglycerides

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INTRODUCTION

Phthalic acid esters are widely used as plasticizers in the industrial production of synthetic materials such as polyvinyl chloride (PVC) and are accumulating as environmentally persistent hydrophobic chemicals. In medical devices made from plasticized PVC, bis-(2-ethylhexyl) phthalate (DEHP), which is metabolized to 2-ethylhexyl phthalate and 2-ethylhexanoic acid in most organisms[1], is the most common of these additives, but other compounds such as di-n-butyl phthalate and diethyl phthalate have likewise been detected by thermodesorption gas chromatography – mass spectrometry[2].

The general interest in qualitative and quantitative phthalate analysis is based on the suspicion that these plasticizers have to be accused to act – at least in rodents – as xenoendocrine disrupters, causing, for instance, thyroid imbalance[3] and exhibiting reproductive toxicity. The latter may be induced by functional zinc deficiency during pregnancy[4] or by antiandrogenic activity[5], whereas estrogenic effects of phthalates are established only in vitro but not in vivo[6]. In contrast to numerous previous reports, it has recently been concluded that DEHP should be classified an unlikely carcinogen in humans. The discrepancy in genotoxic risk assessment can most probably be explained by the clear difference between humans and rodents regarding the susceptibility to hepatic carcinogenesis by DEHP. This appears to be due to the fact that expression levels of the responsible nuclear transcription factor, peroxisome proliferator-activated receptor (PPAR) α, are 10- to 100-fold higher in rodents than in humans[7]. However, DEHP metabolites have to be considered as ligands for PPAR γ[8], which is expressed in human adipose tissue (where hydrophobic phthalates might accumulate) and is possibly involved in the regulation of adipogenesis. Furthermore, nephrotoxic effects have been described in vitro[1].

Normal rates of DEHP uptake in humans are low, amounting to about 2 mg/day, but occupational exposure may be up to 20-fold higher[9]. A particular problem is the uptake from biomedical devices in patients, e.g. during hemodialysis or extracorporeal membrane oxygenation (ECMO). In a recent study, the balance of DEHP extraction during a 4-hour hemodialysis was calculated from dialyzer input versus output concentrations and plasma flow rates and found to range from 4 to 59 mg DEHP which agrees with other reports[10]. Extraction of DEHP from ECMO tubing in vitro under normal application conditions was as high as 500 ng / (mL • hour), whereas limited accumulation of DEHP (2 mg / kg) in children during ECMO was ascribed to rapid clearance of this substance[11].

Though blood is undoubtedly able to extract phthalates from biomedical materials, the role of individual blood compartments in this process is still unknown. Due to the hydrophobic nature of phthalate esters, cell (erythrocyte, leukocyte)

membranes, albumin and lipoproteins are presumably the dominant components[12]. Up to now, no differentiation as to the specific extraction capacities has been made, though it can be proposed that phthalate uptake might considerably vary with changing concentration ratios of those components which may occur in certain clinical situations.

One such condition is diabetic nephropathy which is one of the most frequent causes of hemodialysis and also of kidney transplantation[13]. Especially in end-stage renal failure, severe loss of albumin occurs despite a compensatory up-regulation of hepatic albumin synthesis. Since, in addition, nearly all patients with diabetes mellitus undergoing chronic hemodialysis present with dyslipidemia[14], there may be a severe shift of hydrophobic serum components that determine phthalate extraction and distribution. Thus, it is important to quantify the individual contributions of serum albumin and lipids in order to predict eventually altered phthalate uptake rates by diabetic patients under dialysis regimes.

For this purpose, we first established a gas chromatographic – mass spectrometric method including prequantification in order to increase accuracy and precision[15]by addition of an optimal amount of deuterated DEHP as the internal standard, and then determined the role of serum albumin and lipids in order to allow, for the first time, a quantitative prediction of DEHP extraction at any known concentration of both variables.

EXPERIMENTAL

Pool sera as well as hyperlipidemic sera were collected from inpatient and outpatient specimens of the German Diabetes Research Institute and stored at −40°C prior to analysis. They were analyzed for protein (Biuret method), cholesterol (cholesterol esterase / cholesterol oxidase / peroxidase method), and triglyceride concentrations (lipoprotein lipase / glycerokinase / glycerol phosphate oxidase / peroxidase method) on a Hitachi 912E using reagents from Roche Diagnostics, Mannheim, FRG. Stock solutions (50 g/L) of human albumin (Behring, Marburg, FRG) were prepared in phosphate-buffered saline (Oxoid, Wesel, FRG), and the albumin content was controlled by immunonephelometry on an Array 360 (Beckman, München, FRG). All weighings were made on a Mettler (Greifensee, Switzerland) AT 261 DeltaRange balance with an imprecision of 0.03 mg.

To investigate a possible pH-dependence of DEHP recovery, 1 mL aliquots of pool serum were spiked with 10 µL of a methanolic (purity > 99.9 %; Merck, Darmstadt, FRG) standard solution of DEHP (5 mg / mL; purity > 98 %; Merck,

Darmstadt, FRG) giving a final concentration of 50 μg / mL, internal standard was added (see below), and pH was adjusted to values ranging from 2.0 to 8.0 by addition of diluted HCl, HCl / citrate, or HCl / borate buffers (Merck, Darmstadt, FRG). As a consequence of the results (see below), all subsequent samples were adjusted to pH 5.5 with HCl / citrate prior to extraction and analysis. A DEHP recovery study was performed on the basis of a 25 g / L solution of human albumin. For this purpose, 1 mL aliquots were spiked with 10 μL of DEHP standard solutions yielding concentrations ranging from 2.5 to 50 μg / mL. For the quantitative analysis of phthalate extraction from medical tubing in vitro, arterial or venous ports of hemodialysis systems supplied by different manufacturers were cut into 60 cm pieces, and 30 mL of serum or albumin solution were pumped via recirculation for 200 minutes (simulating the duration of a typical dialysis regime) through a peristaltic pump at a flow rate of 15 mL / min. As a negative control, a 10 cm piece of a caval catheter made of polyurethane was immersed into 10 mL serum for 16 hr.

The method of internal standardization was used throughout the study. Prior to pH adjustment and extraction, 10 μL of a 5.0 mg / mL methanolic solution of bis-(2-ethylhexyl) phthalate-ring-D4 (DEHP-D4; 98.0 atom % D; Cambridge Isotope Laboratories, Andover, MA, USA) was added to 1 mL aliquots of the respective samples in order to allow a preliminary estimation of DEHP concentrations. In some initial experiments, butylbenzyl phthalate (purity > 97 %; Merck, Darmstadt, FRG) was used as an alternative internal standard at the same concentration. Due to systematic deviations of results from expected values especially at low DEHP / DEHP-D4 or DEHP / butylbenzyl phthalate concentration ratios it was then necessary to repeat analyses in a second aliquot of the same sample with ratios of labelled to nonlabelled DEHP (alternatively, ratios of butylbenzyl phthalate to DEHP) adjusted to around 1.0 (range from 0.8 to 1.2). In the experiments proving DEHP leaching from tubes, internal standard was also allowed to equilibrate with endogenous DEHP in the mixtures for variable periods (up to 72 hrs).

A 1+1 (v/v) mixture of ethyl acetate and *tert*-butyl methyl ether (purity of both solvents > 99.8 %; Sigma, Deisenhofen, FRG) was used for fluid-fluid extraction of phthalates from serum samples or protein solutions. Five mL of solvent was added to 1 mL of serum or albumin solution in a glass-stoppered glass tube, mixed thoroughly for 30 s, and centrifuged for 5 min at 3000 g. Four mL of the organic phase were evaporated to dryness at 60°C in a vial under a stream of nitrogen and the residual redissolved in 0.5 mL methanol.

For qualitative and quantitative analysis of phthalates, 1 μL of the respective extracts was injected (split 1:10) into a Varian (Wallnut Creek, CA, USA) model 3400 gas chromatograph equipped with a 15 m × 0.32 mm (i.d.) DB-1 dimethyl-

polysiloxan column (0.25 μm film thickness; J & W Scientific, Folsom, CA, USA). The injector temperature was 250°C, and the column temperature was programmed from 80 to 280°C at a rate of 20°C / min. The mass spectrometric system was the Finnigan MAT (San José, CA, USA) model SSQ7000 equipped with a quadrupol mass filter and an electron (70 eV) impact source; the interface temperature was 280°C and the ion source temperature was 220°C. Data acquisition, selected ion monitoring, and peak area calculations were done under control of the Alpha AXP DEC 3000 Data System (Digital Equipment, Maynard, MA, USA).

Whereas qualitative measurements were performed in the total ion current mode covering the range of 40 to 400 amu (resolution 0.2 amu peak half-width), the results of the conventional or isotope dilution mass spectrometry of DEHP were calculated from the peak area ratios measured either by single ion monitoring (m/z = 149) using butylbenzyl phthalate as the internal standard or by multiple ion monitoring (m/z = 149 for DEHP and m/z = 153 for DEHP-D4) with DEHP-D4 as the internal standard, and mean peak ratios and coefficients of variation were obtained from n = 4 to 7 injections.

RESULTS

In the first series of experiments, a qualitative and quantitative screening of phthalate recovery from samples and of phthalate extraction was performed. If a blank serum was spiked with 50 μg / mL DEHP (final concentration), recoveries after addition of either 25 or 50 μg / mL butylbenzyl phthalate, which could clearly be separated (elution after 9 m 25 s) from DEHP (10 m 41 s), were 116 % (intra-assay coefficient of variation 3.8 %; n=7) and 102 % (coefficient of variation 2.5 %), respectively, at pH = 5.5. Recoveries of DEHP were similar with DEHP-D4 as the internal standard, which could be used since it was assured beforehand that no other potentially interfering substance with m/z 153 was present. Imprecision was considerably lower with both 25 μg / mL (recovery 106 %; coefficient of variation 3.1 %) and 50 μg / mL DEHP-D4 (recovery 98 %; coefficient of variation 1.5 %) than with butylbenzyl phthalate. Relative recoveries (pH 5.0 = 100 %) from DEHP-spiked pool serum samples were 35 %, 99 %, 46 %, and 11 % at pH values of 8.0, 6.0, 4.0, and 3.0, respectively.

The mass chromatogram (Figure 1A) obtained from a 30 mL aliquot of pool serum containing 63.7 g protein / L, 2.26 g triglycerides / L, and 1.42 g cholesterol / L, that was continuously pumped through hemodialysis tubing (sample no. 2 in table I) for 200 minutes, revealed two peaks: a major one at 14 m 02 s, which

was identified as cholesterol (m/z of the molecule ion = 386) and a minor one at 10 m 41 s, which was identified as DEHP (Figure 1B; m/z of the molecule ion = 390) in accordance with the literature spectrum. The limit for DEHP detection (signal to noise ratio = 3) under the conditions employed was 0.3 μg / mL serum. There was no indication of significant extraction of other phthalates such as diethyl phthalate (expected retention time 4 m 37 s on the basis of own measurements), diisobutyl phthalate (6 m 34 s), dibutyl phthalate (7 m 09 s), or butylbenzyl phthalate (9 m 25 s; Figure 1A).

Aliquots of the above-mentioned pool serum, which contained a low endogenous DEHP level (sample 0), were tested for phthalate extraction from hemodialysis tubing (Table I). Considerable extraction of DEHP (over 20 μg / g tubing material) but not of other phthalates could be detected in all but one (no. 1) samples after the 200-minutes recirculation period. There was no dependence on the manufacturer or on the production date. A caval catheter was used as a negative control; in this case, no extraction but rather an adsorption of DEHP was observed (Table I).

TABLE I Calculation of bis-(2-ethylhexyl) phthalate (DEHP) extraction from different medical tubings by human pool serum

Sample	*Date of production*	*DEHP-d4 standard added (μg/mL)*	*DEHP concentration in serum (μg/mL)*	*Coefficient of variation (%)*	*Net total DEHP amount extracted by 30 mL serum (μg)*	*Net relative DEHP amount extracted from tubing (μg/g material)*
0 = blank	----	5.0	1.67	5.7	±0	±0
1 = I-V	VIII-1998	5.0	1.80	4.4	+ 3.90	+ 0.24
2 = I-A	XI-1997	15.0	14.5	1.1	+ 384	+ 23.7
3 = II-V	XII-1998	15.0	10.3	1.8	+ 259	+ 25.4
4 = II-A	XII-1998	15.0	10.4	2.0	+ 262	+ 25.2
5 = III-C	nd	5.0	1.36	2.9	na	na

Abbreviations used: I, II, III, manufacturer; V, venous port; A, arterial port; C, cava catheter; nd, not documented; na, not applicable. All values are means from n=7 determinations.

In order to obtain information on the serum components which might be responsible for phthalate extraction from medical tubing, simulation experiments were performed using albumin solutions in phosphate-buffered saline as well as patient sera with three different degrees of hypertriglyceridemia. In these investigations, particular attention was paid to the equilibration of internal standards with sample phthalate prior to extraction. With both moderately and highly

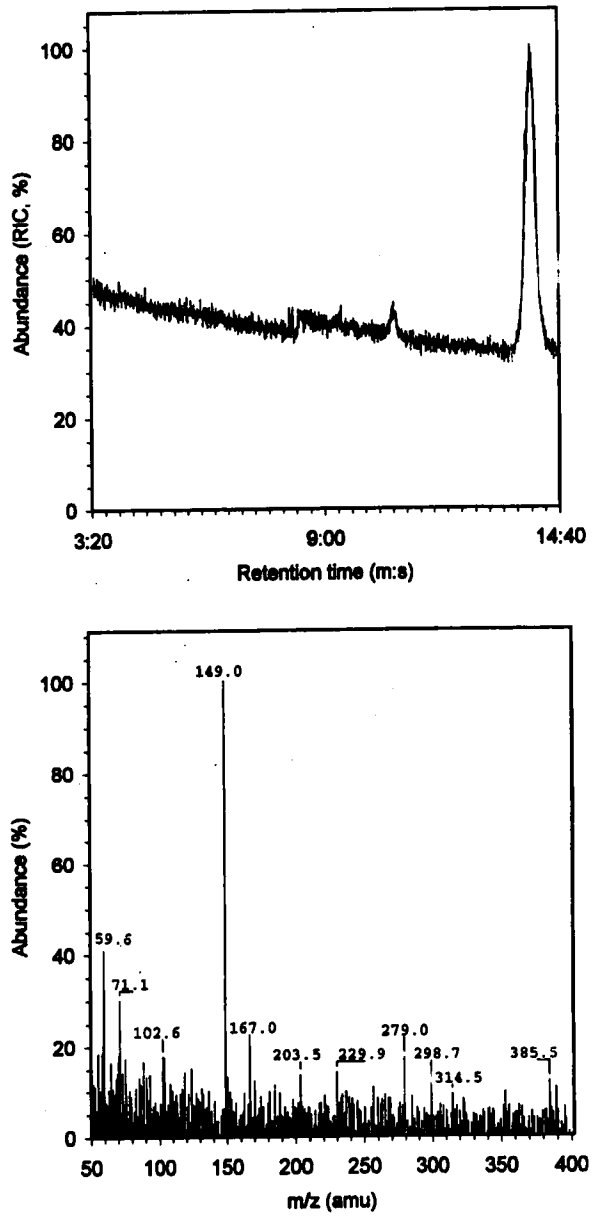

FIGURE 1 Mass chromatogram (total ion current mode; 100% = 5.37 × 10^6 ions) of a pool serum sample after recirculation for 200 minutes through hemodialysis tubing (sample no. 4 in Table I) by means of a peristaltic pump (upper panel). The mass spectrum (100% = 1.04 x 10^5 ions) of the compound with a retention time of 10 m 41 s, which is identified as bis-(ethylhexyl) phthalate, is shown in the lower panel

hyperlipidemic sera, there was a continuous apparent decrease of detected DEHP with increasing standard equilibration time (Figure 2). Only after a period of 24 hours, ligand exchange appeared to be completed with small variations (92 % versus 106 % of the 24 hours value at 48 and 72 hours, respectively) later on, indicating the reliability of an 24 hour equilibration. This phenomenon resulted most probably from unequal extraction of bound versus unbound test compounds, leading to an artificial over-estimation of added standard by the peak-area-ratio method prior to its complete binding to serum components such as albumin (see below) and lipoproteins. Independent of these constraints, it was demonstrated that hyperlipidemic sera extract considerably more DEHP from hemodialysis tubing than do normolipidemic samples (Figure 2), a preliminary regression estimation yielding an increase by 1.01 μg DEHP / g material per g triglyceride / L serum (n=4; r=0.532) at constant protein and albumin concentrations of 60 and 39 g / L, respectively.

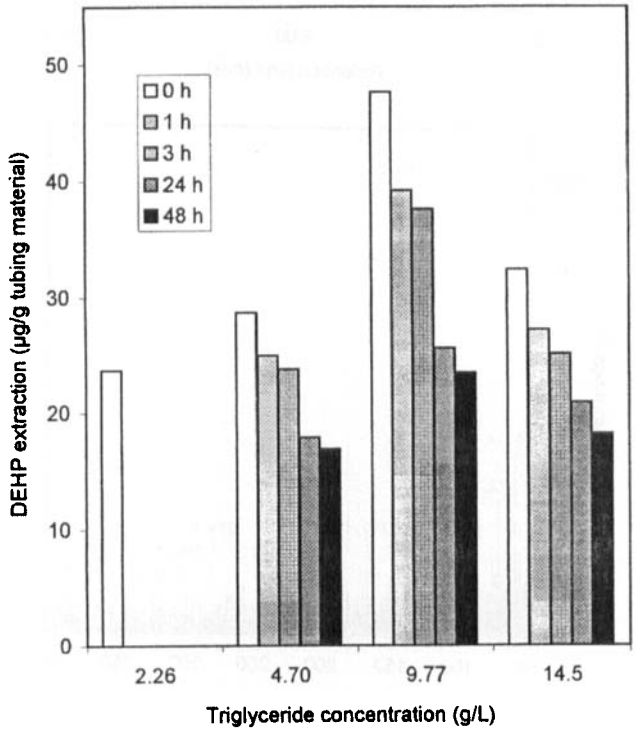

FIGURE 2 Dependence of bis-(2-ethylhexyl) phthalate (DEHP) extraction from hemodialysis tubing (sample no. 2 in Table I) on concentration of triglycerides in human serum samples. Effects of varying periods of equilibration with added internal standard (DEHP-D4) are depicted. All values are means from triplicate determinations after correction for endogenous DEHP concentrations

The apparent dependence of measurable DEHP concentrations on sample equilibration with the added internal standard was also evident for buffered albumin solutions (Figure 3), and there was no systematic difference between human sera and albumin solutions regarding the equilibration rate (measured levels of DEHP after 72 hours of standard equilibration were 99 % of the 24-hour values). Irrespective of this result, there was a linear dependency of DEHP extraction on albumin concentrations within the 10 to 50 g/L concentration range which covered normal serum content as well as hypoalbuminemic states associated with renal failure. Linear regression analysis (n=5) yielded slopes of 0.59 (r=0.94) and 0.29 (r=0.93) μg DEHP / g material per g albumin / L and ordinate intercepts of 2.4 and 1.2 μg DEHP / g material for the 0 and 24 hour standard equilibration periods, respectively. Though phthalate extraction by albumin-free buffer could not be analyzed due to limiting solubility of the internal phthalate standard, extrapolation to zero albumin concentration allows the conclusion that there was no significant phthalate extraction in the absence of protein. Accordingly, linear regression lines fixed at the origin of axes had slopes of 0.65 (r=0.99) and 0.33 (r=0.99) with the 0 and 24 hour equilibration periods, respectively (Figure 3).

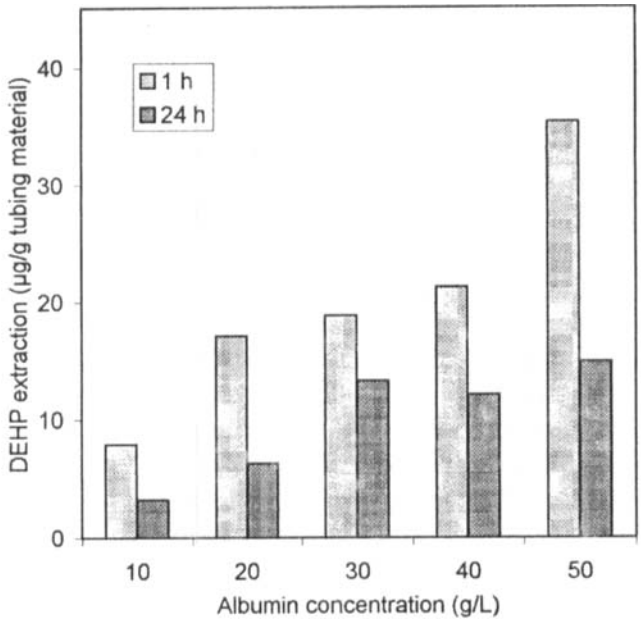

FIGURE 3 Dependence of bis-(2-ethylhexyl) phthalate (DEHP) extraction from hemodialysis tubing (sample no. 2 in Table I) on concentrations of human albumin in phosphate-buffered solutions. Effects of equilibration with added internal standard (DEHP-D4) are also depicted. All values are means from quadruplicate determinations after correction of background DEHP concentrations

The observation that DEHP extraction from hemodialysis tubing by human serum after extrapolation to zero triglyceride concentration was comparable to that by pure albumin solutions of equal protein concentration allowed the conclusion that serum albumin and lipids, especially triglycerides, are the only serum components that contribute significantly to phthalate extraction. A comparison between albumin and triglycerides regarding extraction efficiency demonstrates that, on a mass basis, the hydrophobic attraction of DEHP by serum triglycerides is by a factor of 1.71 higher than by albumin, whereas the total amount of albumin in human serum (45 g / L) extracts 17.7-fold more DEHP than the total amount of triglycerides (1.5 g / L) under physiological conditions.

DISCUSSION

Even if it would be confirmed in the future that DEHP does not act as a human carcinogen, there remain proven adverse effects of this compound that initiated search for possible alternative plasticizers, for instance tri-(2-ethylhexyl) trimellitate. Recent attempts have been made also to reduce DEHP surface concentration and migration[16]. Nevertheless, DEHP and its metabolites as well as (to a lesser degree) other phthalates are still ubiquitously present in the environment, and their quantitative analysis in human serum remains an important element in all strategies to minimize occupational and environmental incorporation.

In this report, we describe the application of the stable isotope dilution mass spectrometric technique for the quantification of DEHP migration. This method, which uses a highly selective detection step and excludes sample-related (so-called matrix-) effects on analytical results, thus fulfills one of the genuine requirements of a candidate reference method[15,17]. The potential of this approach is reflected by the good precision, which is up to 4-fold higher than with less selective detection systems[10], and recoveries could be optimized as well. It should be mentioned, however, that less sophisticated methods, i.e. gas chromatography with flame ionization detection, may also yield acceptable results as demonstrated in our experiments using BBP as an internal standard. The combination of solvents chosen for fluid-fluid extraction in this study was favorized since it had three advantages, i.e. effective extraction due to the polarization force arising from the dipole moment of ethyl acetate, high evaporation rate due to the low (55°C) boiling point of *tert*-butyl methyl ether, and avoidance of formation of stable emulsions as was observed with n-hexane. An important novel aspect of the analytical procedure appears to be the consideration of hydrophic sample equilibration with added internal standard. Though not investi-

gated in detail, the exchange rate of phthalates already bound to hydrophobic serum components such as albumin appears to be rather low, and the extraction of bound phthalate is obviously incomplete even under optimized conditions.

To our knowledge, this is the first report differentiating the relative contributions of hydrophobic serum constituents on DEHP extraction from medical tubings. On a mass basis, the extraction potential of albumin and triglycerides is in the same order of magnitude, and this finding is consistent with recent objective quantification of albumin and lipoprotein hydrophobicities[12]. It is realized that more complex models, including membranes from blood cells (erythrocytes, leukocytes) as DEHP vehicle, are necessary for a better simulation of the true in-vivo situation. Since our results, however, suggest already that serum albumin and lipoproteins are the only significant phthalate-extracting serum components, extraction rates by serum become now predictable at any known concentration of those two parameters, defined compositions of tubing material provided. It is proposed that a quantitative description of this differential attraction of hydrophobic environmental ligands such as phthalate esters is important whenever it is intended to compare and to normalize contamination data in heterogenous populations (normo-versus hyperlipidemic or normo- versus hypoalbuminemic individuals).

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