High-performance liquid chromatographic method for the determination of di(2-ethylhexyl) phthalate in total parenteral nutrition and in plasma

K. Kambia[a], T. Dine[a,*], B. Gressier[a], A.-F. Germe[b], M. Luyckx[a], C. Brunet[a], L. Michaud[c], F. Gottrand[c]

[a]*Laboratoire de Pharmacologie, Pharmacocinétique et Pharmacie Clinique, Faculté des Sciences Pharmaceutiques et Biologiques, 3 Rue du Professeur Laguesse, B.P. 83, 59006 Lille, France*
[b]*Pharmacie Centrale du C.H.R.U., 59037 Lille, France*
[c]*Unité de Gastro-Entérologie, Hépatologie et Nutrition Pédiatriques, Hôpital Jeanne de Flandre, 59037 Lille, France*

Abstract

A simple, rapid and sensitive reversed-phase high-performance liquid chromatographic method with UV detection was developed for the quantification of di(2-ethylhexyl) phthalate (DEHP) in parenteral nutrition admixtures containing fat emulsion and in plasma samples of children daily treated by total parenteral nutrition. The analyte and the internal standard, di-*n*-heptyl phthalate, were extracted twice using hexane and the organic layer separated and dried under nitrogen. The residues were reconstituted with acetonitrile and 20 μl was injected into a Waters Spherisorb C_{18} column, the UV detector was set at 202 nm. The mobile phase was acetonitrile–aqueous buffer (triethylamine 0.08% adjusted to pH 2.8 with 1 *M* phosphoric acid) mixture (88:12, v/v) and it was pumped at 1 ml/min. Average recoveries were 97% or greater. This method was successfully used to investigate the amounts of DEHP which can leach from bags and tubing into fat emulsion and which could contaminate children under long-term parenteral nutrition. On the other hand, the circulating DEHP concentrations were estimated in four children under regular long-term parenteral nutrition. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plastics materials require the addition of a certain amount of plasticizer to obtain specific physico-chemical and mechanical properties required for practical applications. Di(2-ethylhexyl) phthalate (DEHP) is a common plasticizer used to impart flexibility to polyvinylchloride (PVC). It is readily released into the environment through volatilization and leaching from plastics and other sources. Its widespread usage coupled with its stability has led to DEHP being present as an ubiquitous environmental contaminant [1,2]. Intravenous fat emulsion is used for delivering total parenteral nutrition (TPN). It is recommended to use non-PVC containers to administer these solutions because it is likely that fat

emulsions may extract plasticizer added. However, fat emulsions are administered through PVC-containing administration sets and it is to be considered that plasticizer extraction could occur during parenteral nutrition session. Previous studies have shown detectable amounts of DEHP in blood products [3–5], in intravenous solutions [6–9] and in intravenous fat emulsions [10] stored in PVC bags. The DEHP concentration reaches a final level depending on the duration, the temperature of PVC-solution contact and also on the lipid content.

The DEHP presence in biological fluids or various solutions led to the development of various chromatographic methods including gas chromatography with flame ionization [11] or electron-capture detection [12] and high-performance liquid chromatography (HPLC) [13]. These procedures have all obvious advantages and limitations in terms of specificity, sensitivity and time. The aim of this study was to develop a HPLC method to make the measurement of low levels of phthalate easier and to demonstrate that, children under maintenance parenteral nutrition of fat emulsion are regularly exposed to non negligible amounts of DEHP. This paper describes a simple and sensitive method for the determination of DEHP by HPLC using rapid liquid–liquid phase extraction. This method has been successfully applied to investigate the DEHP presence in fat emulsion as well as in human plasma.

2. Experimental

2.1. Chemicals

DEHP and di-*n*-heptyl phthalate (DNHP), used as internal standard, were obtained from commercial sources purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France) and were used as analytical standards without further purification. HPLC-grade acetonitrile and hexane were purchased, respectively, from SDS (ZI de Valdome Peypin, France) and from Sigma–Aldrich and were each assayed for the presence of DEHP. Analytical-grade sodium hydroxide, phosphoric acid and triethylamine were obtained from Prolabo (Paris, France). The water used to prepare aqueous buffers was deionized and purified by distillation (Milli-Q; Millipore, Saint-Quentin

Yvelines, France). To minimize the risk of contamination with DEHP during sample handling and analysis, all glassware used in the study was washed previously first with tetrahydrofuran–methanol mixture then rinsed with hexane. All the other reagents used were analytical grade or better.

2.2. Preparation of standard solutions and samples

The stock solutions of DEHP and DNHP were prepared in acetonitrile at 1 mg/ml in the glass tubes previously washed as described above and stored at 4°C. The working solutions for sample spiking (DEHP at 1.25, 2.5, 10, 40, 80, 160 μg/ml and DNHP at 5 μg/ml) were freshly prepared. The standard samples for DEHP were then prepared by spiking both fat emulsion (1 ml) stored in glass bottles and blank plasma (1 ml) with 50 μl of the working solutions. Calibration graphs were obtained by analyzing 1-ml plasma samples spiked with 62.5, 125, 500, 2000, 4000, 8000 ng of DEHP and 250 ng of internal standard. As for plasma samples, calibration graphs were obtained by analyzing 1-ml fat emulsion samples spiked with 125, 250, 500, 1000, 2500, 5000 ng of DEHP and 250 ng of internal standard.

2.3. Chromatographic conditions and instrumentation

Chromatographic analyses were performed using a HP 1090 high-performance liquid chromatograph (Hewlett-Packard, Orsay, France) equipped with a variable-volume injector, an automatic sampling system and a Hewlett-Packard Model 79994A diode-array UV detector operating at 202 nm. The output from the detector was connected to a Hewlett-Packard 9000 Model 300 integrator and the data were recorded on a HP Thinkjet printer. Separation was achieved using a 5 μm Waters Spherisorb C_{18} column (150×4.6 mm) (Waters, Milford, MA, USA) operating at 20±2°C. DEHP and the internal standard were eluted isocratically with a mobile phase consisting of acetonitrile–aqueous buffer (0.08% triethylamine adjusted to pH 2.8 with 1 *M* phosphoric acid) mixture (88:12, v/v) at a flow-rate of 1.0 ml/min with a system back-pressure averaging about 230 kPa. The mobile phase was filtered through a

0.45-μm membrane and degassed under a helium stream before use. The run time was 10 min.

2.4. Samples extraction procedure and precautions

DEHP quantification needs extraction procedure before chromatographic analysis. However, it is an ubiquitous environmental contaminant and precautions must be taken to avoid false positive identification.

All glassware used in the study were washed previously in first with tetrahydrofuran–methanol mixture then rinsed with hexane. Fat emulsion blank was made using TPN provided in glass bottles to avoid DEHP contamination from parenteral nutrition bags.

Plasma blank was made using blood samples collected from randomly selected healthy subjects. Venous blood (5 ml) was drawn into heparinized glass tubes and centrifuged to obtain plasma. Both blank samples were each assayed for the presence of DEHP.

2.4.1. Emulsion samples extraction

Fat emulsion samples (1 ml aliquot) were directly withdrawn into infusion glass bottles and were spiked with 50 μl of DNHP (250 ng) as an internal standard, followed by 1 M sodium hydroxide (1 ml) and hexane (2 ml). The mixture was vortexed (2 min), centrifuged (1620 g for 5 min) and the separated organic layer (fraction 1) was transferred into a clean conical glass tube. The aqueous phase was extracted again with 2 ml of hexane and the mixture was treated as above. The separated organic phase (fraction 2) was combined with fraction 1 and the total organic phase was evaporated to dryness in a water bath at 40°C under nitrogen. The residue was dissolved in 100 μl of acetonitrile and after centrifugation, 20 μl of the supernatant was finally injected into the chromatograph.

2.4.2. Plasma samples extraction

Each sample (1 ml aliquot) was treated with 50 μl of the internal standard (250 ng) in the glass tubes and extracted twice as above with emulsion samples. However, 2 ml of acetonitrile was added to the mixture before hexane to precipitate plasma proteins.

In both cases, a double extraction procedure with hexane allowed one to obtain a good recovery.

2.5. Quantitative determination

For DEHP quantification, the peak ratio (DEHP peak area/DNHP peak area) (y) was calculated for each fat emulsion sample and plasma sample and the amount of DEHP (x) was determined using the calibration curves obtained, respectively during the validation of methods.

3. Results and discussion

3.1. Chromatography

Typical chromatograms obtained from extracted plasma samples and fat emulsion samples are shown in Fig. 1 and Fig. 2. Figs. 1A and 2A show representative chromatograms of processed blank, indicating that no co-extracted endogenous compound from plasma or from fat emulsion exist at the retention time of DEHP. Figs. 1B and 2B were obtained from a plasma sample and fat emulsion sample containing 500 ng/ml and 400 ng/ml of DEHP, respectively, and 250 ng/ml of the internal standard. Identification of DEHP and its internal standard in plasma and fat emulsion samples was achieved by comparison with authentic specimens obtained from Sigma–Aldrich. The retention times were 8.60 min for DEHP and 6.60 min for DNHP. The capacity factor k' values were 7.6 for DEHP and 5.6 for DNHP, showing a satisfactory separation and a relatively short analysis time.

3.2. Linearity, precision and accuracy

Analytical reproducibility from plasma and fat emulsion containing 62.5, 125, 500, 2000, 4000, 8000 ng and 125, 250, 500, 1000, 2500, 5000 ng added DEHP, respectively, were assessed in five replicates samples (Table 1). The calibration curves were obtained for peak-area ratio (DEHP/DNHP) versus DEHP concentration. They were linear over the range 62.5–8000 ng/ml for plasma samples extraction and 125–5000 ng/ml for fat emulsion samples extraction. Mean linear regression equations
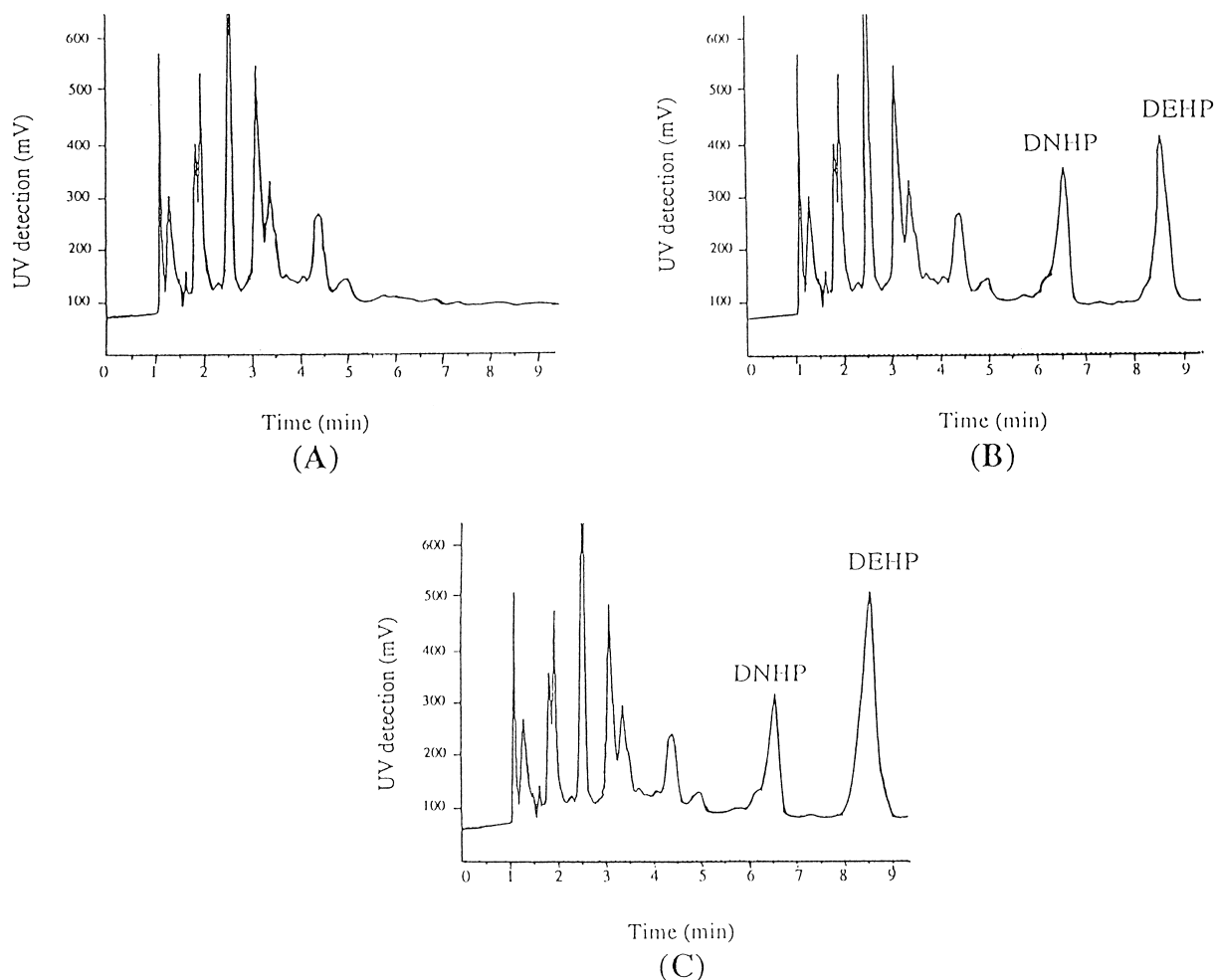
Fig. 1. Chromatograms obtained from extracted TPN samples: (A) blank TPN, (B) blank TPN spiked with 400 ng of DEHP and 250 ng of internal standard DNHP, (C) extracted TPN sample at the outlet tubing. DEHP concentration was estimated at 600 ng/ml.

obtained from five replicates were $y=0.0046x+0.202$ ($r=0.999$) for plasma samples extraction and $y=0.0015x+0.426$ ($r=0.999$) for the fat emulsion samples extraction (y, peak-area ratio; x, analyte concentration ng/ml) with mean values for the slope and intercept ($\pm$standard deviations) of 0.0046 ± 0.0008 and 0.0015 ± 0.0007, respectively, and 0.202 ± 0.018 and 0.426 ± 0.029, respectively. The correlation coefficients of the regression lines were better than 0.999. No significant differences were observed between the equation parameters. The calibration graphs were not forced through the origin.

The precision of the assay was estimated by performing simultaneously the analysis of replicates ($5\times$) of spiked fat emulsion and plasma samples. The precision of both methods were good, as shown by the low values of the intra-assay and inter-assay relative standard deviations (Table 1). Intra-assay reproducibility and accuracy were determined by simultaneously assaying replicates of plasma or fat emulsion spiked with DEHP. The intra-assay reproducibility was within 2.73% for plasma samples extraction and 3.16% for fat emulsion samples extraction and the accuracy, which was defined as [(measured concentration−theoretical concentra-
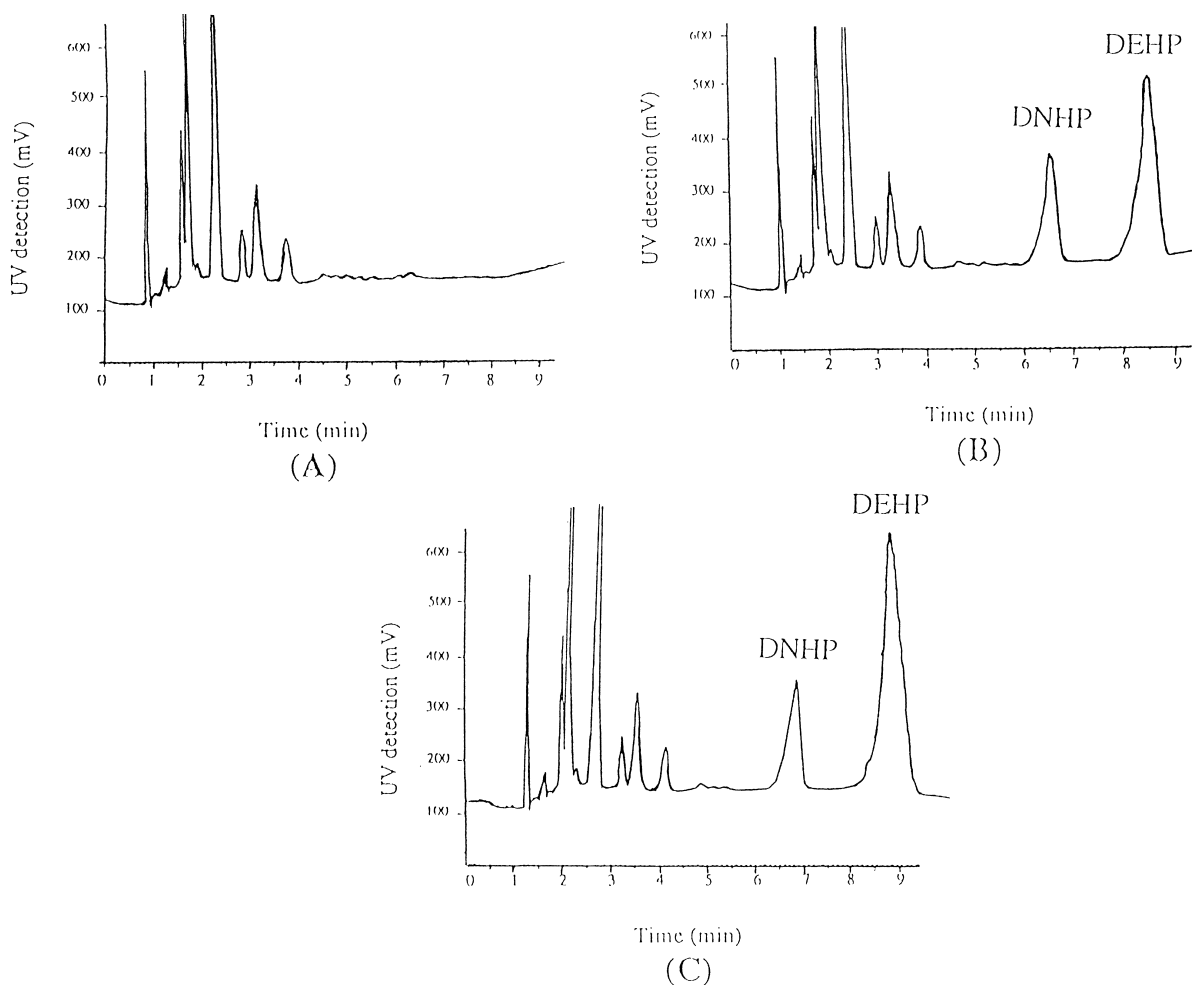
Fig. 2. Chromatograms obtained from extracted plasma samples: (A) blank plasma, (B) blank plasma spiked with 500 ng of DEHP and 250 ng of internal standard DNHP, (C) extracted plasma sample from a newborn infant after parenternal nutrition. DEHP concentration was estimated at 975 ng/ml.

tion)/theoretical concentration] was 4.2% and 3.7% or better, respectively. Inter-assay reproducibility and accuracy were measured using spiked DEHP plasma or fat emulsion with different calibration graphs. The inter-assay reproducibility (Table 1) was within 9.54% for plasma samples extraction and 6.07% for fat emulsion samples extraction. The absolute accuracy was 10.72% and 7.04%, respectively. Extraction recoveries of DEHP and DNHP from plasma or fat emulsion were on average better than 97% when a double extraction procedure was used. The limit of quantification observed was 20 ng/ml.

3.3. Clinical applications

The HPLC method developed in this study was successfully implemented for the analysis of DEHP in both TPN intended to children and plasma. Figs. 1C and 2C show chromatograms, respectively obtained from the outlet tubing during simulated infusion of home parenteral nutrition and from the plasma of children under parenteral nutrition.

All preparations (infusion bags) studied were prepared by Fasonut Laboratory (Montpellier, France) and were provided by Lille General Hospital

Table 1
Reproducibility and accuracy of DEHP determinations ($n=5$)

Sample	Amount added (ng/ml)	Amount found (ng/ml)±SD	Inter-assay	
			RSD (%)	Accuracy (%)
Plasma	62.5	69.2±6.60	9.54	10.72
	125	128.4±1.60	1.25	2.72
	500	495.6±4.32	0.87	0.88
	2000	2001.05±0.97	0.05	0.05
	4000	3990.75±1.27	0.03	0.23
	8000	7999.56±1.93	0.02	0.005
Fat emulsion	125	116.2±5.91	5.08	7.04
	250	244.4±9.76	4.00	2.24
	500	496.02±30.10	6.07	0.80
	1000	1008.6±34.90	3.46	0.86
	2500	2514.05±42.60	1.70	0.56
	5000	4983±81.11	1.63	0.34

RSD, Relative standard deviation.

Pharmacy (France). The infusion bags were made of ethylvinyl acetate (EVA) but they contained PVC sites. The administration kits were also made of PVC. The infusates were magistral preparations containing amino acids, glucose, electrolytes, vitamins, trace elements in the higher bag part and lipids in the separate lower bag compartment. The all-in-one mixture was prepared just before simulated infusion, by opening a connection between the two compartments of the bag. Two formulae have been investigated:

Formula 1: 2200 ml containing 3.85% lipid.
Formula 2: 650 ml containing 1.85% lipid.

The two different formulae were stored at 4°C. Just before simulated administration, each bag containing total nutrient solutions was shaken to mix lipid source with the rest of the preparation. Each bag containing homogenous preparation was then attached to an administration set connected to the infusion pump (IMED Gemini PC-1 or AVI 270 Infusion pumps) (VitalAire Nord, France) that allowed the emulsion to flow through at a constant rate. The rate was then increased progressively until a defined value. Fat emulsion samples were aseptically drawn from each infusion bag and from outlet tubing at times 0, 0.25, 1, 2, 4, 8 and 11 h during simulated infusion. Each sample aliquot was placed in a glass tube and stored at 4°C until HPLC analysis.

All the material used and procedures of infusion were similar to the clinical practice of home parenteral program.

As shown in our study, TPN extracts DEHP from bags and tubing. Extraction depends on the lipids rate of each preparation and the flow-rate used. This process is time-dependent: DEHP leachability is higher when lipid content increased, inversely when the flow-rate is low, the preparation-tubing contact time increased the leachability of DEHP in preparations. In the bag samples, DEHP was detectable immediately after mixing and remained stable during infusion. The outlet samples also demonstrated detectable levels of DEHP during infusion time, but the concentrations of DEHP in these samples increased throughout the 11 h study period (Fig. 3A and B). This finding is consistent with earlier reports that significant amounts of DEHP are leached in the TPN regimens containing lipid [6,10]. In the present study, quantities of DEHP released into TPN were estimated to be 0.2±0.008 to 0.7±0.02 mg from bags and 0.8±0.09 to 2±0.07 mg from the outlet tubing. So, one can presume that children under maintenance parenteral nutrition of fat emulsion are regularly exposed to non negligible amounts of DEHP. To confirm our findings, four randomized children under parenteral nutrition were studied. Blood samples were collected before and after parenteral nutrition session into glass tubes intended
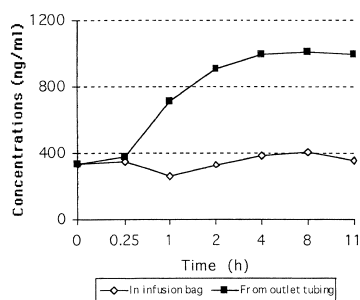
A - Kinetics of DEHP leachability during simulated infusion of TPN 24 hours after reconstitution of the preparation (n = 2 bags).

Formula 1: infusion volume of 2200 mL (flow rate 177 mL/h, lipid concentration 3.85 %)
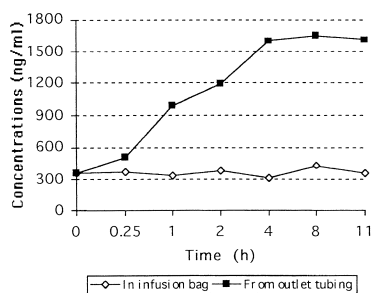
B - Kinetics of DEHP leachability during simulated infusion of TPN 24 hours after reconstitution of the preparation (n = 2 bags).

Formula 2 : infusion volume of 650 mL (flow rate 46 mL/h, lipid concentration 1.85 %)

Fig. 3. Comparative kinetics of DEHP leachability during simulated infusion of TPN.

to biological analysis. After centrifugation, a plasma aliquot of each sample was immediately frozen at $-20°C$ and stored until HPLC analysis. DEHP plasma concentrations in these four children before and after parenteral nutrition session widely ranged from 0.3 to 6.9 μg/ml. To our knowledge, this is the first study of the DEHP determination in plasma from children under parenteral nutrition. Further studies including more patients are needed to confirm our findings.

However, it would appear in the light of the present study, advisable to use non-phthalate-containing sets or tubing to administer TPN containing lipid source to children, especially neonates.

Acknowledgements

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