Journal of Chromatography, 311 (1984) 101—108
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2252

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF DI-(2-ETHYLHEXYL)PHTHALATE IN HUMAN BLOOD SPECIMENS

PROBLEMS OF VARIABLE-EXTRACTION YIELD AND THE USE OF STANDARD ADDITION FOR CALIBRATION

GARY M. POLLACK*, RICHARD L. SLAUGHTER, JAMES F. BUCHANAN and DANNY D. SHEN*,**

Departments of Pharmacy and Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Amherst, NY 14260 (U.S.A.)

(First received March 28th, 1984; revised manuscript received June 28th, 1984)

SUMMARY

A high-performance liquid chromatographic procedure was developed for the determination of di-(2-ethylhexyl)phthalate (DEHP) concentrations in human whole blood samples. The solvent extraction of DEHP was found to be highly variable between samples obtained from different subjects (coefficient of variation of 30.4%). The recovery of DEHP following extraction with ethyl acetate was negatively correlated with serum lipid content, as expressed by the sum of serum cholesterol and triglyceride concentrations ($r = -0.864$). The technique of standard addition of DEHP allowed a single-point calibration of DEHP extractability in individual blood samples, and provided an accurate estimation of DEHP concentration (coefficient of variation of approximately 6% in replicate samples). The potential for intersample variability in the solvent extraction of other highly lipid-soluble compounds should be considered.

INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) is a plasticizer commonly used in the production of polyvinyl chloride plastics, comprising up to 40% by weight of

*Present address: Division of Pharmaceutics, School of Pharmacy, Beard Hall 200-H, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, U.S.A.
**Present address: Department of Pharmaceutics, BG-20, School of Pharmacy, University of Washington, Seattle, WA 98195, U.S.A.

0378-4347/84/$03.00

some medical plastic products such as blood storage bags and flexible tubings [1]. Animal studies have indicated that this compound can induce hepatomegaly and affect a variety of biochemical functions of the liver [2]. A recent long-term toxicity study has also revealed its capacity for producing hepatic tumors in rodents [3]. Concerns over the potential toxicity of DEHP and other plasticizers have led to the problem of assessing the degree of exposure to plasticizers in various hospital patient populations. Several investigations have been directed towards identifying the presence of plasticizers in blood and tissues of those patients likely to be at risk of exposure. For example, significant concentrations of DEHP have been reported in patients with end-stage renal failure requiring maintenance hemodialysis [4—6] and surgical patients who have received blood transfusions [6] or undergone cardiac bypass [7].

In order to further characterize the extent of exposure and the disposition kinetics of DEHP, an accurate and reliable method for the measurement of the phthalate ester in various biological fluids or tissue specimens is required. Several chromatographic procedures have been reported for the quantitation of DEHP in blood or plasma [4, 6, 8, 9]. During our attempt to adopt the published assays for DEHP in human blood samples, we encountered a high degree of intersubject variability in the solvent extraction of the plasticizer from whole blood. Although in principal the variability problem can be overcome by preparing calibration standards using each subject's own blank blood, in practice it poses a serious dilemma as completely DEHP-free blood cannot be obtained.

The present study was undertaken to determine whether any specific biochemical factors could be identified which may be responsible for the observed variability in the chemical extraction of DEHP. Furthermore, an assay methodology involving the technique of standard addition was developed for the determination of DEHP concentrations in individual whole blood samples.

EXPERIMENTAL

Chemicals

All solvents were of HPLC grade and were purchased from a commercial source (J.T. Baker, Phillipsburg, NJ, U.S.A.). DEHP (Aldrich, Milwaukee, WI, U.S.A.) and di-*n*-octylphthalate (DOP, Eastman-Kodak, Rochester, NY, U.S.A.) were used without further purification.

Subjects

Blood samples (20 ml) were obtained from thirteen normal volunteers for the purposes of determining the individual variation in DEHP calibration curves and relating the extraction of DEHP from blood to serum lipid and α_1-acid glycoprotein (AAG) concentrations. Blood was drawn through a metal needle into glass syringes. A 10-ml aliquot was set aside and stored in glass vials containing heparin (20 U/ml) at −20° C. The serum fraction was separated from the remaining blood and stored in glass vials at −20° C.

DEHP extraction

Ethyl acetate (5 ml) containing 1 μg/ml of the internal standard DOP was

added to 0.5 ml of heparinized whole blood samples in acid-washed glass culture tubes (16 × 125 mm with PTFE-lined caps, American Scientific Products, McGaw Park, IL, U.S.A.). Preliminary experiments indicated no difference in the extraction of DOP added with the extraction solvent as opposed to added prior to extraction. DOP was subsequently added with the ethyl acetate to facilitate the processing of samples. The tubes were vortexed vigorously (15 sec), shaken (15 min), and centrifuged (1800 *g*, 15 min). The organic layer was transferred to a fresh glass tube, evaporated to dryness at 50°C under a stream of dry nitrogen, and reconstituted with 100 μl methanol.

Chromatographic analysis

Aliquots (10—80 μl) of the reconstituted extracts were analyzed by high-performance liquid chromatography (HPLC). The chromatograph consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A constant flow-rate pump, a U6K variable-volume injector, and a Model 440 UV absorbance detector. Separation was achieved with a Partisil 5 ODS Rapid Analysis Column, particle size 5 μm (Whatman, Clifton, NJ, U.S.A.). The mobile phase consisted of methanol—water (93:7). The high methanol content was required due to the extremely lipophilic nature of DEHP. The flow-rate was set at 3.0 ml/min with a column back pressure of 7 MPa. Absorbance of the eluent was monitored at 254 nm, and peak area ratios (DEHP to DOP) were determined with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator. Interday variability in instrument response, as assessed by daily injections of a standard methanolic solution containing DEHP and DOP, was 1.8%.

Linearity of DEHP calibration curves

Blood from ten of the thirteen normal volunteers was spiked with DEHP to yield a series of blood standards containing 0, 0.5, 1.0, 2.5, and 5.0 μg/ml plasticizer. A 25-μl aliquot of a methanolic solution of DEHP was added to acid-washed glass centrifuge tubes (16 × 125 mm) and the methanol was gently evaporated under a stream of dry nitrogen. Lysed whole blood was added to each tube, followed by brief vortexing. No DEHP was lost during this preparation. The blood samples were extracted and analyzed by HPLC as described in the preceding sections. Peak area ratios were plotted versus DEHP concentration for each individual set of blood standards, and linear calibration plots were fitted with least-squares regression lines.

Correlation of ethyl acetate extractability with serum biochemistry

The extraction yield of DEHP from blood was assessed with 0.5-ml samples from thirteen normal volunteers to which a known amount (2.5 μg) of DEHP had been added. Since blood from all volunteers contained a measurable amount of DEHP, the peak area ratio due to the spiked DEHP, i.e. $AR(s)$, was calculated:

$$AR(s) = AR(2) - AR(1) \quad (1)$$

where $AR(1)$ and $AR(2)$ are the peak area ratios before and after the addition of 2.5 μg (corresponding to 5 μg/ml) of DEHP. The increase in peak area ratio after the standard addition represents an assay response factor. The only require-

ment with this type of single-point calibration procedure is strict adherence to linearity in response. Extraction yield was calculated by referencing $AR(s)$ to the peak area ratio obtained by direct injection of a methanolic solution containing 5 μg DEHP and 10 μg DOP per ml.

Serum concentrations of triglycerides, total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were determined in the same samples using standard clinical chemistry procedures [10, 11]. Serum concentrations of AAG were measured with a commercial radial immunodiffusion kit (M-Partigen, Calbiochem-Behring, San Diego, CA, U.S.A.).

Validation of the standard addition method as a calibration procedure

Aliquots of pooled whole blood were spiked with DEHP to yield concentrations of 0, 0.5, 1.0, 2.5, and 5.0 μg/ml. Five 1.0-ml blood samples were prepared at each concentration. Each sample was divided into two 0.5-ml portions. The first portion was extracted directly, as described previously, while the remaining portion was added to a glass tube containing 2.5 μg DEHP (i.e. equivalent to an addition in concentration of 5 μg/ml). The split samples were analyzed as described in the preceding sections. The blood concentration of DEHP was calculated as follows:

$$\text{Sample DEHP concentration } (\mu\text{g/ml}) = AR(1) \times \frac{5\ \mu\text{g/ml}}{AR(2) - AR(1)} \qquad (2)$$

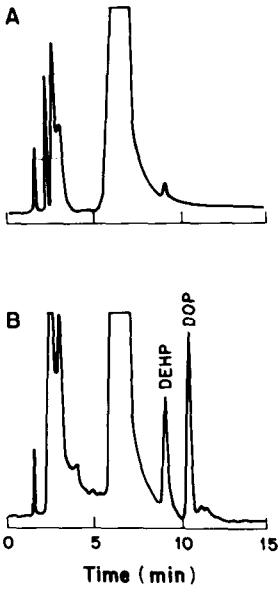
To determine the concentration of added DEHP, a mean peak area ratio was determined in five blank samples of pooled blood. This mean ratio was subtracted from $AR(1)$ and $AR(2)$ in order to eliminate the contribution of endogenous DEHP. The reproducibility of the procedure was assessed with five replicate samples at each concentration of DEHP.

RESULTS

Several organic solvents and solvent mixtures were tested to identify the optimum solvent for recovery of DEHP from pooled human whole blood samples. Hexane, diethyl ether, chloroform, chloroform—methanol (2:1, v/v), ethylene dichloride, methylene chloride, and methylene chloride—pentane (1:1, v/v) were all associated with unacceptably low extraction yields (i.e. < 50%). Only ethyl acetate appeared to be a practical extraction solvent for this compound (mean recovery from pooled blood of 65.2%).

Representative chromatograms of extracts of blank and spiked blood samples are displayed in Fig. 1. DEHP and DOP eluted at 8.8 and 10.4 min, respectively. It should be noted that low concentrations of DEHP were present even in supposedly blank blood samples.

The calibration plots obtained with blood from ten subjects are presented in Fig. 2. Linear response was observed over the entire concentration range in all cases. Correlation coefficients of the regression lines ranged from 0.990 to 0.999. However, significant intersubject differences in the slope of the calibration plots were observed. The slope estimates varied over a two-fold range (0.0200—0.0475) with a coefficient of variation of 30.4%. The large variation in slope value suggested that the extractability of DEHP from whole blood was



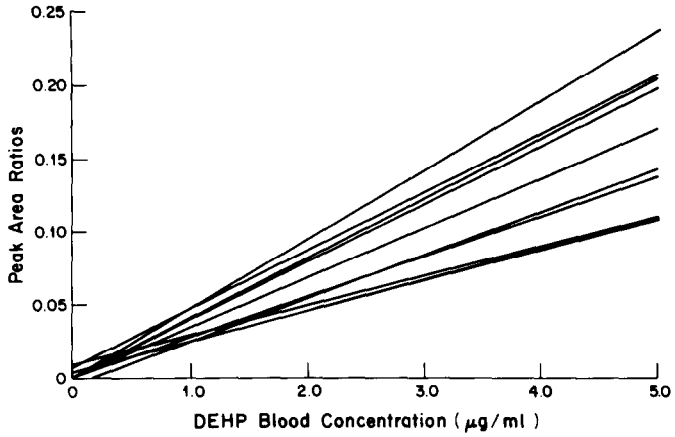


Fig. 1. Representative chromatograms following extraction of a whole blood sample obtained from a normal volunteer (A) and blood spiked with 5 μg/ml DEHP and 10 μg/ml DOP (B). The concentration of DEHP in the unspiked sample was 0.12 μg/ml.

Fig. 2. DEHP calibration plots generated in blood obtained from ten normal volunteers. The lines indicate the least-squares regression of the peak area ratio versus concentration data. Individual points have been omitted for clarity.

highly variable between subjects, while the apparent linearity of the individual calibration curves indicated that the extractability of DEHP was constant for a given sample within the range of DEHP concentrations studied.

A summary of DEHP extraction yield and relevant biochemical data for each subject is presented in Table I. The results of the correlation analyses between DEHP extraction yield and individual biochemical factors and combinations thereof are presented in Table II. Extractability of DEHP was negatively correlated with the concentration of each of the four indices of serum lipid content (viz. LDL, HDL, cholesterol, triglycerides), indicating that increases in serum lipid content result in a decrease in the extractability of DEHP. The strongest correlation was observed between DEHP extraction yield and the sum of serum cholesterol and triglycerides ($r = -0.864$, $p < 0.001$). This relationship is displayed in Fig. 3.

Although DEHP has been found to bind to AAG [12], the serum concentration of this protein did not correlate with the extraction yield of the plasticizer from blood. It is doubtful that intersubject differences in serum albumin concentration would contribute to the intersubject variation in extraction since DEHP does not appear to bind to this protein [13]. It is interesting to note that DEHP extraction appeared to increase with increasing hematocrit, suggesting that the compound is removed from the cellular blood fraction more readily than from the serum fraction. Considering that DEHP is concentrated in red blood cells relative to serum in a ratio of approximately 2:1 [14], extraction of DEHP from whole blood should allow a lower detection limit than extraction from the corresponding serum sample.

TABLE I

DEHP EXTRACTION YIELD AND SERUM BIOCHEMICAL CORRELATES

Subject	Extraction* (%)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	AAG (mg/dl)	Hematocrit (%)
1	50.4	82	187	58	113	96	42.5
2	69.7	71	165	46	105	100	48.0
3	50.9	115	167	96	48	84	42.8
4	39.3	154	193	56	107	94	42.0
5	60.5	84	211	68	126	92	46.4
6	56.0	82	233	42	175	102	46.4
7	67.8	97	164	49	96	ND**	43.7
8	58.8	66	183	60	110	98	47.3
9	89.0	63	138	38	87	ND	46.3
10	51.3	85	189	36	136	98	44.2
11	68.9	51	167	67	90	86	40.7
12	70.6	78	153	51	86	ND	47.2
13	74.6	66	150	71	66	ND	42.0

*Extraction from whole blood with ethyl acetate.
**ND = not determined.

TABLE II

CORRELATIONS OF EXTRACTION YIELD WITH SERUM BIOCHEMICAL FACTORS

Serum factor	Correlation coefficient	Statistical significance
Cholesterol (mg/dl)	—0.671	<0.02
Triglycerides (mg/dl)	—0.712	<0.01
HDL (mg/dl)	—0.322	>0.2
LDL (mg/dl)	—0.219	>0.4
Hematocrit (%)	+0.471	>0.1
AAG (mg/dl)	+0.547	>0.05
HDL + LDL (mg/dl)	—0.550	>0.05
Cholesterol + triglycerides (mg/dl)	—0.864	<0.001

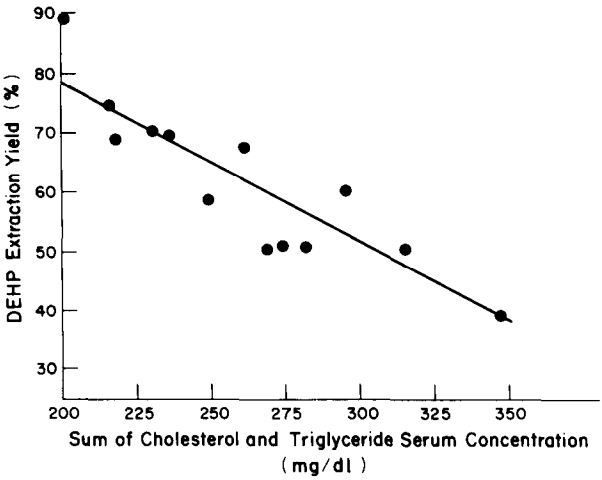


Fig. 3. Relationship between DEHP extraction recovery and the sum serum cholesterol and triglyceride concentrations in blood obtained from thirteen normal volunteers. The line indicates the least-squares regression of the data ($r = -0.864, p < 0.001$).

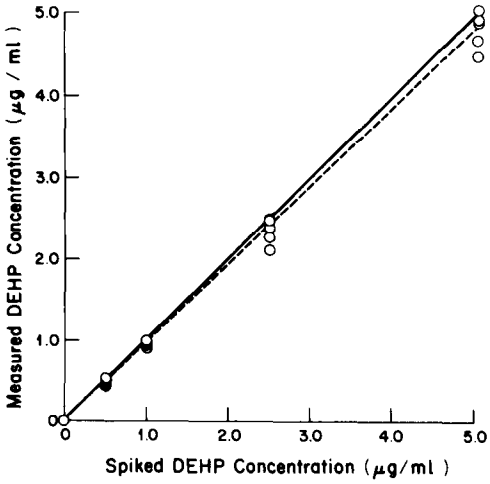


Fig. 4. Comparison of DEHP concentrations estimated using the standard addition procedure with known DEHP concentrations in pooled blood from ten volunteers. Five samples were analyzed at each concentration. The broken line indicates the least-squares regression of the data. The solid line represents a perfect correspondence between the estimated and known concentrations.

Estimates of DEHP concentration in the whole blood standards as determined by the standard addition procedure are compared to the actual concentrations in Fig. 4. The slope of the least-squares regression line between the measured and actual DEHP concentrations was 0.963, which is not significantly different from unity. The regression line also had a negligible intercept. The concentration estimates had an acceptable degree of reproducibility, with a coefficient of variation (n = 5) ranging from 5.16% (5 μg/ml) to 6.78% (2.5 μg/ml). Thus, the method of standard addition should be a reasonable approach to estimating whole blood concentrations of DEHP.

DISCUSSION

Differences in the extractability of a given compound from different biological matrices (e.g. serum, urine, and tissues) or from the same type of specimens from different animal species are well known. However, the potential problem of intersubject variation in extraction from biological samples is often neglected. The usual approach to quantitating a compound in blood, for example, is to develop a calibration curve, usually in pooled blood from several donors. Measurements in unknown samples are subsequently referenced to this standard curve. This traditional calibration procedure becomes invalid given the degree of intersample variability in DEHP extraction observed in the present study. It thus becomes necessary to determine the extraction yield in each sample analyzed.

The physicochemical basis for the intersubject differences in the extractability of DEHP is nor known. The data presented here suggest that increases in serum lipids, particularly as expressed by the sum of serum cholesterol and triglyceride concentrations, decreased the extraction yield. Albro and Corbett [13] have shown that more than 80% of the DEHP present in human plasma

after storage in plastic bags is associated with the lipoprotein fraction. It would appear that a portion of DEHP present in whole blood is tightly associated with the lipoprotein fraction, and that as the lipid content of blood increases the amount of DEHP available for extraction by ethyl acetate decreases. An alternative explanation may be that a fraction of DEHP in blood is trapped in precipitated lipoproteins following the addition of ethyl acetate, leading to incomplete and variable recovery. The latter hypothesis is supported by our observation that no DEHP is detectable in the supernatant after proteins in a serum sample have been precipitated with trichloroacetic acid or ice-cold acetonitrile.

The unusual extraction problem with DEHP is likely due to the high lipid solubility of the plasticizer. It would be anticipated that similar problems may be encountered with other highly lipophilic compounds. The solvent extraction of the plasticizer tris(butoxyethyl)phosphate (TBEP), a constituent of rubber stoppers used in many evacuated blood collection systems, has been found to be highly variable between serum samples obtained from different subjects [15]. The reported variation in the extraction yield of this compound was even greater than that associated with DEHP (i.e. a four-fold variation in calibration slope between subjects). As observed in the present study, the extraction yield of TBEP was found to correlate well ($r = 0.88$) with the serum concentration of cholesterol and triglycerides.

The phenomenon of significant intersample variation in extraction recovery is not restricted to extraction with ethyl acetate. Significant variability in the recovery of DEHP from serum or blood was also observed when using hexane, ethylene dichloride, and chloroform as extraction solvents. Thus, it is likely that the dependence of extraction yield on serum lipid content is a general analytical problem for highly lipid-soluble compounds, and is not restricted to either a single class of compounds or to a specific extraction solvent. The standard addition procedure described herein provides an efficient and acceptably accurate means of estimating blood concentrations of compounds subject to a high degree of intersample variability in extraction.

REFERENCES

1 B. Gulbring, Vox Sang., 9 (1964) 513.
2 W.H. Lawrence, Clin. Toxicol., 13 (1978) 89.
3 W.M. Kluwe, J.K. Haseman, J.F. Douglas and J.E. Huff, J. Toxicol. Environ. Health, 10 (1982) 797.
4 T.P. Gibson, W.A. Briggs and B.J. Boone, J. Lab. Clin. Med., 87 (1976) 519.
5 L.M. Lewis, T.W. Flechtner, J. Kerkay, K.H. Pearson, W.J. Chen, K.L. Popowniak and S. Nakamoto, Trans. Amer. Soc. Art. Intern. Organs, 23 (1977) 566.
6 N.P.H. Ching, G.N. Jhan, C. Subbarayan, D.V. Bowen, A.L.C. Smith, Jr., C.E. Grossi, R.G. Hicks, F.H. Field and T.F. Nealson, Jr., J. Chromatogr., 222 (1981) 171.
7 E.J. Dravian, K.H. Pearson and J. Kerkay, Anal. Lett., 15 (1982) 1729.
8 J.Y. Piechocki and W.C. Purdy, Clin. Chim. Acta, 48 (1973) 385.
9 J. Vessman and G. Rietz, J. Chromatogr., 100 (1974) 153.
10 G.R. Warnick and J.J. Albers, J. Lipid Res., 19 (1978) 65.
11 G.E. Mott and M.L. Rogers, Clin. Chem., 24 (1978) 354.
12 E. Pike, B. Skuterud, P. Kierkulf, D. Fremstad, S.M. Abdel-Sayed and P.K.M. Lundle, Clin. Pharmacokin., 6 (1981) 367.
13 P.W. Albro and J.T. Corbett, Transfusion, 18 (1978) 750.
14 G.M. Pollack and D.D. Shen, unpublished results.
15 G.D. Anderson, A.T. Elvin and D. Lalka, J. Pharm. Sci., submitted for publication.