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Research Article

Liquid Chromatographic Analysis of Di(2-ethylhexyl) Phthalate: Application to Pharmacokinetic Studies in the Mongrel Dog

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A high-performance liquid chromatographic (HPLC) assay was developed for the determination of di(2-ethylhexyl) phthalate (DEHP) in serum or plasma. Plasma DEHP concentrations that were measured by HPLC in specimens obtained from hemodialysis patients were in good agreement with corresponding concentrations that were measured by gas chromatography with selected ion monitoring (GC-SIM) (r^2 = 0.996). Plasma DEHP concentrations were measured after intravenous DEHP administration (1.2–4.4 mg DEHP/kg body weight) to determine the effect of bilateral ureteral ligation on DEHP elimination in the mongrel dog. DEHP plasma clearance (~6.3 ml/min/kg), steady-state distribution volume (~0.21/kg), and terminal half-life (~50 min) were unchanged in two dogs following bilateral ureteral ligation. DEHP terminal half-life and steady-state distribution volume were substantially smaller (25- to 70-fold) than reported previously in the rat or dog.

KEY WORDS: di(2-ethylhexyl) phthalate; liquid chromatographic (LC) analysis; pharmacokinetics; renal failure; mongrel dog.

INTRODUCTION

Di(2-ethylhexyl) phthalate (DEHP) is a common plasticizer used in the production of medical-grade polyvinyl chloride (PVC) products (1). Human exposure to small quantities of DEHP (~1–5 mg DEHP/kg body weight) leached from PVC blood storage containers or on contact of blood with PVC hemodialysis tubing is documented (2–5). Moreover, animal studies demonstrate that DEHP is carcinogenic, teratogenic, and potentially hepatotoxic (6–8). The relevance of such high-dose animal studies of DEHP toxicity or studies performed to characterize the elimination of DEHP is uncertain since concentration-dependent DEHP distribution and elimination are reported (9). A high-performance liquid chromatographic (HPLC) method was developed for the analysis of DEHP in serum or plasma with increased sensitivity and less variable extraction from plasma than that reported previously (10). The assay was then used to determine the effect of bilateral ureteral ligation on the elimination of small doses of DEHP (~1–5 mg DEHP/kg body weight) in the dog to approximate more closely typical human DEHP exposure such as that from hemodialysis therapy or from blood transfusion.

MATERIALS AND METHODS

Reagents

Methyl alcohol, hexane (UV grade), acetonitrile (UV grade), and chloroform (with 1% ethanol) were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). DEHP was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and di-*n*-ocytl phthalate (DOP) was obtained from Supelco Inc. (Bellefonte, Pa.). Di(2-ethylhexyl) phthalate (*carbonyl*-^{14}C labeled; sp act, 32.6 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and was repurified by HPLC. The radiochemical purity of an aliquot of HPLC-purified DEHP was determined to be 98% by thin-layer chromatography.

Chromatography

The HPLC system consisted of a Model 6000A HPLC pump, a Model U6K injector, a Model 440 UV detector set at 254 nm, and a μ-Bondapak-C18, 3.9 × 30-cm (10-μm-particle size) HPLC column purchased from Waters Associates (Milford, Mass.). Peak heights were recorded on a Hewlett Packard Model 3390A integrating recorder (Palo Alto, Calif.). The mobile phase was prepared by adding

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100 ml distilled deionized water to 900 ml methanol and the mobile phase flow rate was maintained at 1.8 ml/min.

Extraction

Screw-capped test tubes (15 ml) were soaked in Dichrol (American Scientific, McGraw Park, Ill.) and then carefully washed with Contrad (Curtis Matheson Scientific Inc., Houston, Tex.), rinsed three times with tap water, rinsed three times with distilled water, and dried for 1 hr in a convection oven. The capped tubes were then rinsed twice with chloroform. These rinsing procedures were necessary to eliminate DEHP contamination of glassware and spuriously high blank values. Plasma standards were prepared by adding 0–20,400 ng DEHP (in methanol) and 2000 ng DOP (in methanol) to 15-ml glass Teflon-capped centrifuge tubes and taking the solution to dryness under a gentle stream of nitrogen. Dog plasma (1 ml) was added to each standard tube and the solutions were vortex mixed. Plasma specimens (1 ml) submitted for DEHP analysis were added to glass centrifuge tubes containing 2000 ng DOP. Water (4 ml), acetonitrile (5 ml), and hexane (5 ml) were added to each tube and the mixture was shaken on a reciprocating shaker for 60 min. The hexane containing layer was harvested and the remaining mixture was reextracted with a second 5-ml aliquot of hexane for 30 min. The hexane-containing layers were combined and evaporated to dryness under nitrogen in a 15-ml conical test tube. The residue was reconstituted in 0.2 ml methanol and any remaining residue carefully dispersed from the walls of the conical tube with a glass Pasteur pipette. The mixture was sonicated for 10 sec, then centrifuged at 750*g* for 10 min, and 75 μl of the supernatant was injected onto the HPLC column.

ANIMAL STUDIES

A mixture of ^{14}C-labeled and unlabeled DEHP was solubilized in dog plasma by the method of Albro and Corbett (11), and the DEHP concentration was determined by HPLC. Five mongrel dogs received a rapid intravenous injection of a plasma-solubilized preparation containing 1.2–4.4 mg DEHP/kg body weight. Blood samples were obtained prior to DEHP administration and at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, and 300 min postinjection through an 18-gauge Teflon catheter (Critikon Inc., Tampa, Fla.) located in a jugular vein. The dogs received a 0.9% sodium chloride infusion and thiopental anesthesia throughout the experiment. Plasticizer-containing tubings and bags were avoided. Dogs were housed in metabolic cages, and urine and feces were collected until the radioactivity (dpm/ml) in urine was two to three times background. Two dogs then underwent bilateral ureteral ligation with thiopental anesthesia and were allowed to recover. After the dogs became uremic (1–2 days), a second intravenous DEHP dose was administered and the experiment was repeated. The uremic dogs were sacrificed 5 hr (dog 5) or 29 hr (dog 2) after DEHP administration. An autopsy was then performed to obtain tissue samples for scintillation counting. A correction for quenching in plasma, urine, bile, and tissue digests was performed using [^{14}C]toluene (New England Nuclear, Boston) and an internal standardization technique. The blood/plasma DEHP partition ratio was determined by incubating whole dog blood at 37°C with ^{14}C-labeled DEHP. After equilibrium was achieved aliquots of blood (0.1 ml) were digested and counted. A correction for quenching was performed using an external standard ratio technique with a quench curve prepared from blood digests. The blood-to-plasma DEHP partition ratio was determined from the disintegrations per milliliter whole blood to disintegrations per milliliter plasma quotient.

PHARMACOKINETIC ANALYSIS

The area under the plasma DEHP concentration–time curve (AUC) was calculated by trapezoidal integration with extrapolation of the area from the last measured concentration (C_n) to time infinity from the C_n/λ_n quotient. The terminal rate constant (λ_n) was obtained by fitting the terminal DEHP plasma concentration–time data to a monoexponential equation by nonlinear regression using an extended least-squares weighting technique (12). The area under the first moment of the plasma concentration–time curve (AUMC) was estimated by trapezoidal integration with extrapolation of the last plasma concentration–time product to time infinity (13). The mean residence time in the body was calculated as the AUMC/AUC quotient. The steady-state volume of distribution (V_{ss}) and plasma clearance (Cl) were calculated from the intravenous dose, AUC, and AUMC as described previously (14). The half-life was calculated from the quotient $0.693/\lambda_n$.

RESULTS

Serum was pooled according to triglyceride content and spiked with Intralipid (Cutter Medical, Miles Laboratories, Berkeley, Calif.) to achieve a wide range of cholesterol (104–284 mg/dl), triglyceride (29–185 mg/dl), high-density lipoprotein (26–75 mg/dl), total lipid (450–1000 mg/dl), albumin (3.7–4.7 g/dl), and total protein (6.2–7.8 g/dl) concentrations. The extraction recovery of DEHP and DOP from pooled human serum was approximately 95% and did not vary with the lipid content of serum from six normal volunteers.

DEHP and DOP were resolved (R = 1.06) at retention times of ~6 and ~7 min, respectively (Fig. 1). Assay response (peak height ratio) was linearly related to DEHP concentration ranging from 64 to 20,400 ng/ml. The between-day precision of the assay was determined using a point delete technique. Each plasma standard was deleted from the standard curve and the concentration was then calculated from the respective peak height ratios and the linear regression equation obtained with the remaining standards (Table I). Assay sensitivity criteria were calculated according to the procedure described by Oppenheimer *et al.* (15). The critical level (the assay response above which a level is reliably recognized as detected) was 23 ng/ml. The detection limit (response which would a priori be expected to be detected) was 46 ng/ml, and the determination limit (response which would be detected with a corresponding relative standard deviation of 10%) was 81 ng/ml. Plasma DEHP concentrations were measured by HPLC in 23 specimens obtained from patients exposed to DEHP during hemodialysis therapy and were in good agreement with corresponding concentrations measured by gas chromatograph–

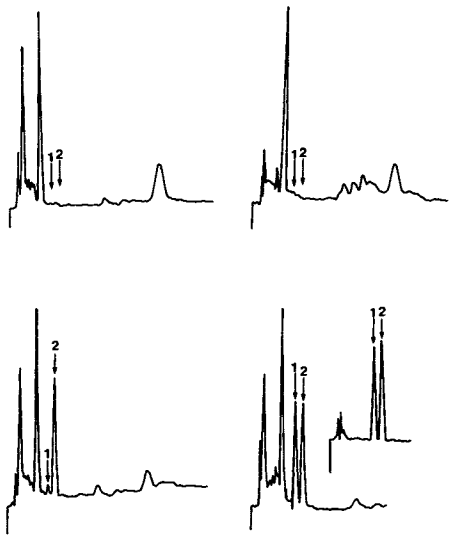

Fig. 1. Top: Chromatogram of blank dog plasma (left) and human plasma (right). DEHP (1) and DOP (2) were eluted at ~6 min and ~7 min, respectively. Bottom: Chromatogram of dog plasma containing 64 ng/ml DEHP and 2000 ng/ml DOP (left) and dog plasma spiked with 1633 ng/ml DEHP and 2000 ng/ml DOP (right). The inset (right) is a chromatogram obtained after injecting a methanolic mixture containing 1633 ng DEHP and 2000 ng DOP onto the HPLC column.

selected ion monitoring (GC-SIM) (Fig. 2). Interfering peaks were not detected in human plasma, but an interfering peak (retention time, ~6.5 min) was detected in plasma from dog 5. Plasma specimens from dog 5 were then reanalyzed by GC-SIM. Radioactivity from the HPLC column effluent which corresponded to the retention time for DEHP was harvested and was used to calculate plasma DEHP concentrations (dog 5) from the specific activity of the administered DEHP dose. Plasma DEHP concentrations measured by GC-SIM (dog 5) were in good agreement with those concentrations calculated from radioactivity data (Fig. 3). Plasma DEHP concentrations declined in a biexponential fashion following DEHP administration and were below the assay determination limit within 5 hr (Fig. 3). The HPLC column effluent radioactivity corresponding to the retention time for DEHP (dpm/ml plasma) was subtracted from the total dpm/ml plasma to determine the concentration of radioactivity (dpm/ml) that was attributed to ^{14}C-labeled compounds other than DEHP (Fig. 4). Considerable background "bleed" of radioactivity occurred in the HPLC analysis of plasma specimens from dogs 1 and 2 (Fig. 4). This resulted in erratic and elevated measured ^{14}C-DEHP plasma concentrations in the terminal portion of the ^{14}C-DEHP plasma concentration–time curve and was subsequently avoided by analyzing plasma specimens in order of increasing concentration of radioactivity and by analyzing the DEHP dosage preparation only *after* all the plasma extracts were injected onto the HPLC column (dogs 4 and 5).

Table I. Between-Day Precision: Analysis of DEHP Serum Standards and Quality-Control Specimens

Nominal DEHP concentration (ng/ml)	$\bar{X}$ (ng/ml)	SD (ng/ml)	RSD (%)[a]	N[b]
Serum standards				
63.8	59.9	8.5	14.2	15
127.4	129.6	8.9	6.9	16
255.0	260.1	29.3	11.3	16
510.0	522.8	54.6	10.4	16
735.0	741.0	21.8	2.9	16
1,020.0	1,015.0	27.9	2.7	16
2,040.0	2,033.3	38.7	1.9	16
5,100.0	5,020.8	273.0	5.4	6
10,204.0	10,284.9	721.9	7.0	7
20,408.0	20,863.7	2,555.6	12.2	7
Quality-control specimens				
408.2	410.6	24.7	6.0	16
1,632.7	1,633.9	46.0	2.8	16

[a] Relative standard deviation.
[b] Number of samples.

At the time of autopsy (dogs 2B and 5B) the disintegrations per minute per gram of tissue were barely above background levels compared to "blank" tissue digests of lung, right ventricle, kidney, spleen, ovary, cerebrum, liver, bone, muscle, and fat. Approximately 71% (dog 2B) and 39% (dog 5B) of the administered radioactivity was recovered in bile collected at the time of autopsy. The mean cumulative urinary and fecal excretion of radioactivity (dogs 2A, 4, and 5A) accounted for 33% (urine) and 36% (feces) of the administered amount of radioactivity. Less than quantitative recovery of the administered amount of radioactivity was likely due to incomplete collection of urine or feces, as the total recovery of radioactivity ranged from 57 to 91%.

Plasma creatinine concentrations were ~1.0 mg/dl, and blood urea nitrogen concentrations were ~11 mg/dl in dogs 2 and 5 just prior to bilateral ureteral ligation. Plasma creatinine and blood urea nitrogen concentrations were ~8.5 and 106 mg/dl in dogs 2 and 5 just prior to the start of the second experiment. A small increase in plasma alkaline phosphatase concentration from a mean value of 32 to 85 IU/liter and an increase in plasma potassium concentration from a mean value of 3.5 to 5.9 mEq/liter were noted at the time of the second experiment (dogs 2 and 5).

The mean DEHP plasma clearance, steady-state distribution volume, and terminal half-life were ~6.3 ml/min/kg, ~0.24 liter/kg, and ~50 min in the five normal dogs and were similar to those values measured in dogs 2 and 5 after bilateral ureteral ligation (Table II). The blood to plasma DEHP concentration ratio was ~0.77 and 0.82 in two dogs with blood hematocrit values of 32 and 26%, respectively.

DISCUSSION

The proposed HPLC method was suitable for single-dose pharmacokinetic studies of DEHP. The amounts of DEHP administered (1.2–4.4 mg DEHP/kg body weight) were nearly two orders of magnitude lower than in previous pharmacokinetic or toxicity studies (16,17) and were chosen to simulate human exposure to DEHP such as that from hemodialysis therapy (4) or blood transfusion (5). DEHP was rapidly eliminated in the anesthetized dog, and estimates of

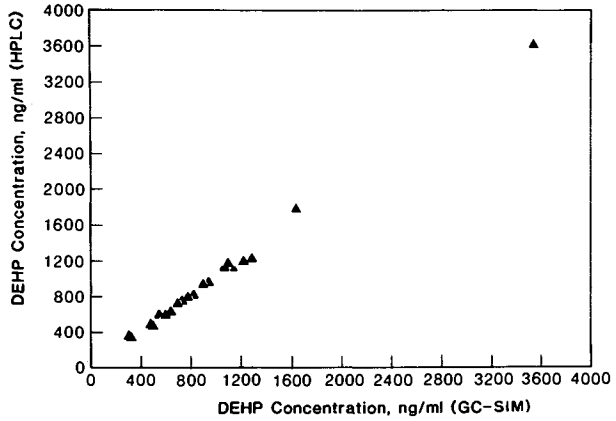

Fig. 2. Comparison of DEHP concentrations measured by HPLC and GC-selected ion monitoring (GC-SIM) in 23 serum specimens obtained from patients exposed to DEHP during hemodialysis therapy ($Y = 1.03X - 4.52$; $r^2 = 0.998$).

the DEHP steady-state distribution volume, plasma clearance, and half-life in two dogs with experimentally induced renal failure were quite similar to corresponding values obtained in dogs with normal renal function. However, these values were substantially lower than the estimated DEHP plasma half-life (approximately 72 hr) and distribution volume (approximately 160 liters/kg) calculated from previously published plasma DEHP concentration–time data in the dog reported by Chen and co-workers (16). Moreover, the authors state that a prolonged increase in serum DEHP concentrations in nephrectomized dogs as compared to control or sham-operated dogs suggests an apparent inability of nephrectomized dogs to eliminate an intravenous dose of 225 mg/kg (16). The results of the present investigation, which were obtained after the administration of much lower and probably more relevant DEHP doses (in terms of typical human exposure to DEHP), demonstrated that DEHP was rapidly eliminated in dogs with experimentally induced renal failure.

Several explanations for these discrepancies can be postulated. The aqueous solubility of DEHP (estimated from light-scattering techniques) is reported to be less than 100 ng/ml (18). Variation in the terminal plasma half-life and variability in tissue distribution of DEHP in the rat are related to subtle differences in the techniques used to solubilize or disperse DEHP in the intravenous dosage preparation (18). For example, plasma DEHP terminal half-life values (approximately 30 min) are substantially shorter in the rat when ^{14}C-DEHP is administered as a "diffusion complex" compared to half-life values (approximately 263 min) measured after the administration of a solubilized suspension of DEHP prepared from a 1% ethanolic solution (18). There are

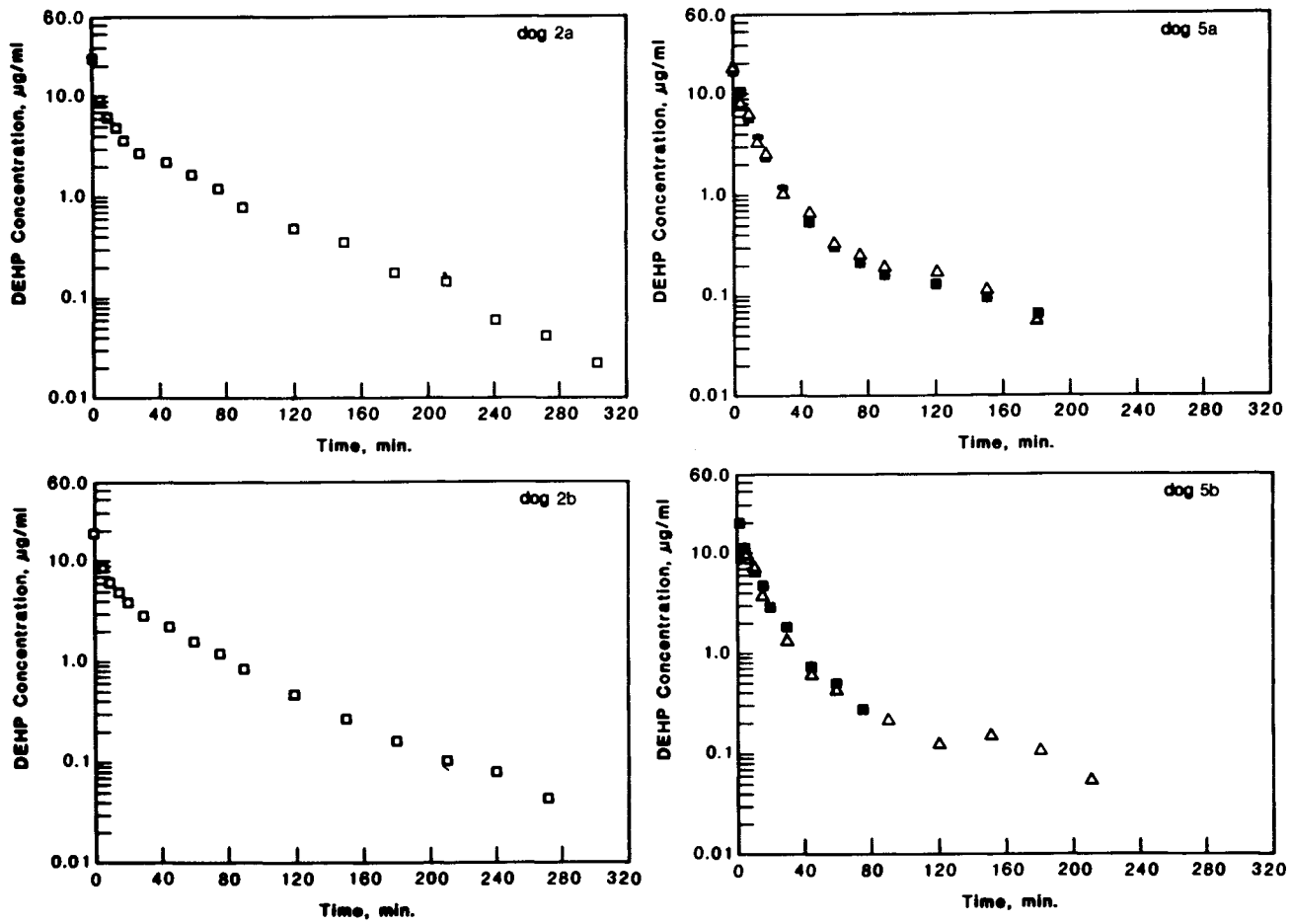

Fig. 3. Serum DEHP concentrations following intravenous DEHP administration measured by HPLC (□) or GC-SIM (■) or calculated from radioactivity data (△) (see text).

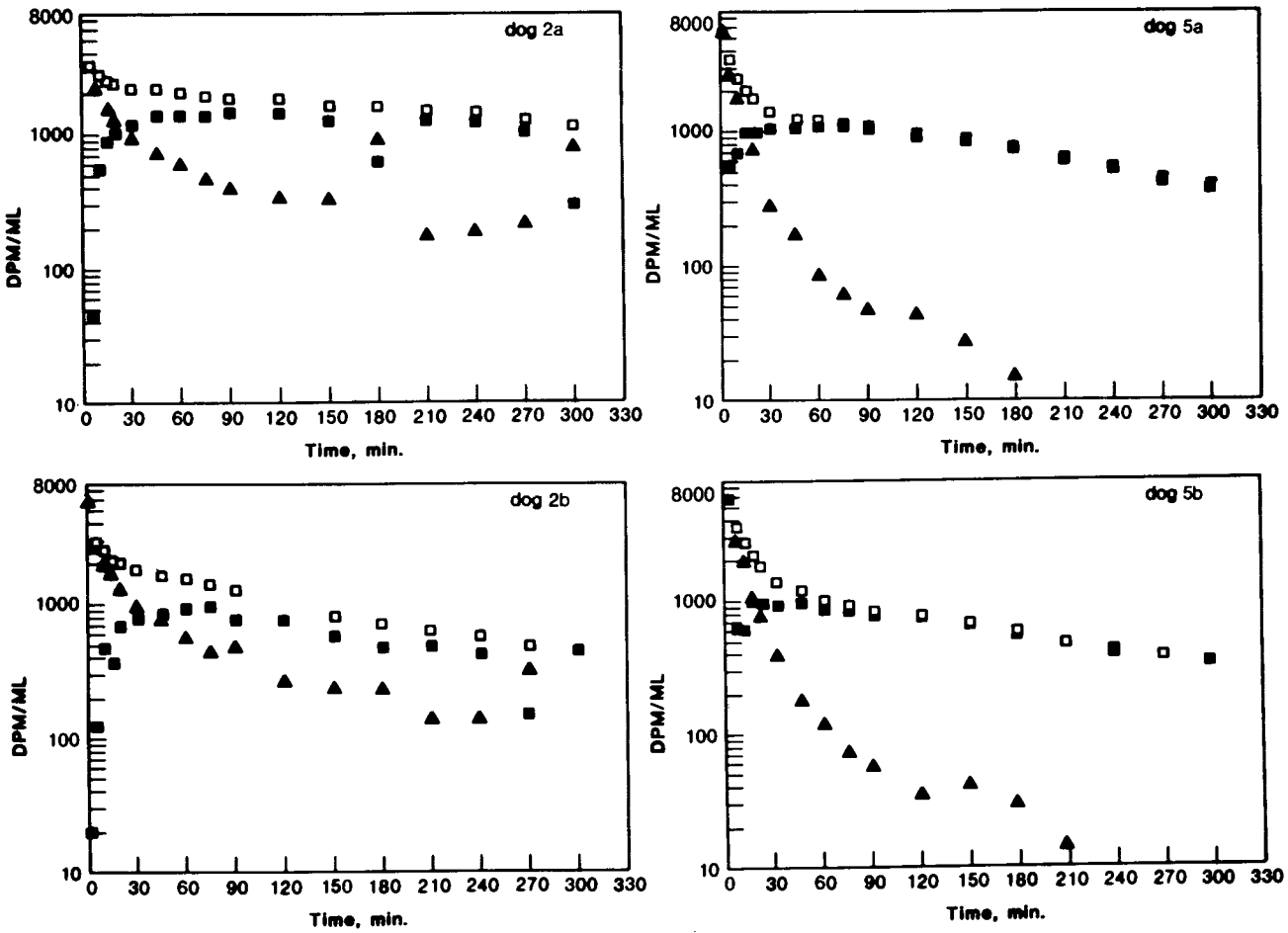

Fig. 4. Serum concentrations of total radioactivity (dpm/ml; □), radioactivity from the HPLC column effluent with a retention time the same as DEHP (^{14}C-DEHP, dpm/ml; ▲), and total radioactivity minus ^{14}C-DEHP (dpm/ml; ■).

also pronounced interspecies differences in DEHP metabolism. Albro and colleagues demonstrated that rodents do not form glucuronide conjugates of DEHP metabolites and that the urinary metabolite excretion profile following DEHP administration differs substantially from that of higher primates and humans (19,20). Dose-dependent changes in DEHP plasma clearance, steady-state distribution volume, and plasma terminal half-life are reported in a recent study of DEHP elimination in rats (9). Furthermore, estimates of DEHP terminal half-life based on measured plasma DEHP concentrations were 10-fold lower in one study (9) than estimates of the terminal half-life based on measured whole blood concentrations in the rat (17).

An appropriate animal model for the study of DEHP

Table II. Summary of DEHP Pharmacokinetic Parameters

Dog No.	Dose (mg/kg)	Sp act (dpm/mg)	AUC (min · mg/liter)	AUMC (min^2 · μg/ml)	Apparent MRT (min)	Apparent VD_{ss} (liters/kg)	Apparent Cl (liters/min/kg)	Apparent half-life (min)
1	4.36	1.76×10^5	543.7	23571.4	43	0.348	0.008019	47
2A	1.80	2.73×10^5	350.0	14401.2	41	0.212	0.005143	39
2B[a]	1.72	2.76×10^5	335.6	13695.0	41	0.209	0.005125	34
3	2.47	2.10×10^5	348.0	14569.9	42	0.297	0.007098	56
4	2.44	2.25×10^5	472.6	17170.4	36	0.188	0.005163	50
5A	1.30	2.77×10^5	207.6	5283.1	25	0.159	0.006262	56
5B[a]	1.20	2.86×10^5	243.0	7222.0	30	0.147	0.004938	70
$\bar{X}$	2.18	2.46×10^5	357.2	13701.9	37	0.223	0.005964	50
SD	1.08	4.24×10^4	118.4	6103.9	7	0.074	0.001202	12
RSD (%)[b]	49.5	17.2	33.1	44.5	19	33.2	20.2	24

[a] Experiment performed 1–2 days after bilateral ureteral ligation.
[b] Relative standard deviation.

metabolism and DEHP toxicity has not been identified. The results of the present investigation indicate that further studies are justified (i) to characterize the metabolism of DEHP in the mongrel dog, (ii) to examine the dose-dependent metabolism of DEHP in normal and renally impaired dogs, and (iii) to determine the utility of the proposed renal failure dog model for chronic DEHP toxicity studies. Pronounced variability in the values of DEHP half-life and distribution volume reported in the literature may be related to concentration-dependent DEHP distribution or elimination (9), variability in distribution or elimination of DEHP associated with the physical form of the intravenous dosage preparation (18), or interspecies differences in DEHP distribution and metabolism (19). These observations may have important implications with respect to the design and interpretation of DEHP toxicity studies.

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REFERENCES

1. L. Fishbein. In J. Jarvisalo, P. Pfaffli, and H. Vainio (eds.), *Industrial Hazards of Plastics and Synthetic Elastomers,* Alan R. Liss, New York, 1984, pp. 19–42.
2. R. J. Jaeger and R. J. Rubin. *N. Engl. J. Med.* 287:1114–1118 (1972).
3. R. J. Jaeger and R. J. Rubin. *Science* 170:460–461 (1970).
4. L. M. Lewis, T. W. Flechtner, J. Kerkay, K. H. Pearson, and S. Nakamoto. *Clin. Chem.* 24:741–746 (1978).
5. P. O. J. Sjoberg, U. G. Bondesson, E. G. Sedin, and J. P. Gustafsson. *Transfusion* 25:424–428 (1985).
6. S. Kevy and M. Jacobson. *Contr. Nephrol.* 36:82–89 (1983).
7. W. M. Kluwe, J. E. Huff, H. B. Matthews, R. Irwin, and J. K. Haseman. *Carcinogenesis* 6:1577–1583 (1985).
8. W. M. Kluwe, E. E. McConnell, J. E. Huff, J. K. Haseman, J. F. Douglas, and W. V. Hartwell. *Environ. Health Perspect.* 45:129–133 (1982).
9. P. Sjoberg, U. Bondesson, and M. Hammarlund. *Arch. Toxicol.* 58:72–77 (1985).
10. G. M. Pollack, R. L. Slaughter, J. F. Buchanan, and D. D. Shen. *J. Chromatogr.* 311:101–108 (1984).
11. P. W. Albro and J. T. Corbett. *Transfusion* 18:750–755 (1978).
12. L. B. Sheiner. *ELSFIT Users Manual,* Technical Report of the Division of Clinical Pharmacology, University of California, San Francisco.
13. M. Gibaldi and D. Perrier. In *Pharmacokinetics,* Marcel Dekker, New York, 1982 pp. 409–417.
14. L. Z. Benet and R. L. Galeazzi. *J. Pharm. Sci.* 68:1071–1074 (1979).
15. L. Oppenheimer, T. P. Capizzi, R. M. Weppelman, and H. Mehta. *Anal. Chem.* 55:638–643 (1983).
16. W. Chen, J. Kerkay, K. H. Pearson, E. Paganini, and S. Nakamoto. *Proc. Dial. Transplant Forum* 8:113–116 (1978).
17. G. M. Pollack, R. C. K. Li, J. C. Ermer, and D. D. Shen. *Toxicol. Appl. Pharm.* 79:246–256 (1985).
18. I. J. Stern, J. E. Miripol, R. S. Izzo, and J. D. Lueck. *Toxicol. Appl. Pharm.* 41:507–522 (1977).
19. P. W. Albro, J. T. Corbett, J. L. Schroeder, S. Jordan, and H. B. Matthews. *Environ. Health Perspect.* 45:19–25 (1982).
20. P. W. Albro, S. T. Jordan, J. L. Schroeder, and J. T. Corbett. *J. Chromatogr.* 244:65–79 (1982).