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Estimation of Tris(2-butoxyethyl) Phosphate in Biological Fluids: Novel Intersubject Variability in Recovery from Human Serum

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Abstract □ Tris(2-butoxyethyl) phosphate (I), a plasticizer commonly found in evacuated blood collection tubes, displaces many basic drugs from their binding sites on serum proteins and causes them to redistribute from serum into red blood cells (*i.e.*, artificially lowering serum or plasma drug concentration). Thus, the ability to quantitate I in serum or plasma may be helpful in establishing the suitability of various lots of evacuated blood collection tubes for use in drug level monitoring and pharmacokinetic studies. In the process of establishing a minor modification of an assay which has been reported, remarkable and reproducible interindividual variability ($n = 10$) in the slope of standard curves was observed (range, 0.0143–0.0486). This variability appeared to be caused by differences in the recovery of I from the serum of these individuals. The source of this difference seemed to be related to serum lipoprotein concentration since the slope of standard curves was highly correlated with serum triglyceride concentration ($r = -0.800$) as well as with the sum of serum triglyceride and cholesterol concentrations ($r = -0.881$). These observations suggest that the examination of interindividual differences in the recovery of drugs and related compounds from serum should be a routine part of assay development.

Keyphrases □ Tris(2-butoxyethyl) phosphate—recovery variability, human serum, correlation to lipoprotein, triglyceride and cholesterol levels

Tris(2-butoxyethyl) phosphate (I), a plasticizer found in the rubber stoppers of many evacuated blood collection tubes and in some catheter materials, has been shown to significantly displace basic drugs such as propranolol (1), quinidine (2), lidocaine (3), imipramine (4), and alprenolol (4) from their binding sites on α_1-acid glycoprotein (the principal base binding protein in human serum). Spuriously low (typically 25%) plasma or serum concentrations accompany this displacement due to increased partitioning of the drugs into red blood cells (1, 3). Artifacts related to this phenomenon may pervade much of the published data describing the pharmacokinetics of this type of compound. Furthermore, this artifact may adversely affect therapeutic drug monitoring. Specifically, it is likely that different lots of evacuated blood collection tubes cause varying amounts of "displacing activity" (3) and that different lots of tubes may be used to collect blood during a hospital admission. Thus, the capacity to approximate the concentration of I in plasma or serum samples is important.

A second reason to examine the analysis of I in biological fluids was that preliminary studies in this laboratory suggested remarkable intersubject variability in the slopes of standard curves for I in human serum. It is well known that the recovery of some compounds is dependent upon the biological fluid (urine, blood, serum, *etc.*) being extracted. For example the lidocaine metabolites, monoethyl-glycinexylidide and glycinexylidide, have a significantly lower recovery from serum than from urine (5). The present text describes the evidence for large (approximately threefold) interindividual differences in the recovery of I from serum, provides preliminary observations regarding the plasma constituents which exhibit covariance with these interindividual differences, and presents a description of analytical methods which allow the estimation of I in serum samples.

EXPERIMENTAL SECTION

Tris(2-butoxyethyl) phosphate[1] and dioctyl phthalate[1] were used as received. All solvents were spectral or HPLC grade.

Standard Assay of I in Serum—The concentration of I in serum samples was determined using a minor modification of a previously published procedure (4). The original extraction method was modified by increasing the organic to aqueous phase volume ratio from 2:1 to 10:1. To a 0.50-mL serum sample was added 5 mL of dichloromethane containing 1.0 μg/mL of dioctyl phthalate as an internal standard. The mixture was agitated on a fixed-angle rotary mixer (20 rpm) for 10 min and centrifuged (1000×*g*). The organic phase was transferred to a clean tube and evaporated to dryness. The residue was redissolved in 25 μL of ethyl acetate, and 1 μL was injected into a gas chromatograph equipped with flame-ionization detectors[2]. This instrument contained glass columns (122 × 0.2 cm) packed with 3% OV-101 on 80/100-mesh chromosorb W(HP)[3]. Helium was used as a carrier gas at a flow rate of 30 mL/min. The analysis was run isothermally at a column temperature of 220°C with injector and detector temperatures of 250°C. The flow rates of hydrogen and air were 30 and 300 mL/min, respectively.

[1] Aldrich Chemical Company, Inc., Milwaukee, Wis.
[2] Model 5730A; Hewlett Packard.
[3] Alltech Associates, Arlington Heights, Ill.

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Table I—Serum Biochemistry and Characteristics of the Tris(2-butoxyethyl) Phosphate Standard Curves Observed in Normal Volunteers

Subject	Standard Curve Slope	Standard Curve r	Serum α_1-Acid Glycoprotein Conc., mg/dL	Serum Albumin Conc., g/dL	Serum Triglyceride Conc., mg/dL	High-Density Lipoprotein Cholesterol Conc., mg/dL	Serum Cholesterol Conc., mg/dL	Sum of Serum Triglyceride and Cholesterol Conc., mg/dL
1	0.04855	0.970	57	4.6	32	52	155	187
2	0.03290	0.996	58	4.7	55	93	187	242
3	0.03225	0.997	63	—	30	69	192	222
4	0.02985	0.989	86	4.6	65	49	198	263
5	0.02290	0.991	88	5.0	166	52	212	378
6	0.02280	0.980	74	4.7	181	49	169	350
7	0.02035	0.966	80	5.0	95	48	193	288
8	0.01865	0.972	61	4.7	291	56	139	430
9	0.01655	0.993	71	4.9	193	56	258	451
10	0.01425	0.984	110	4.5	275	50	204	479

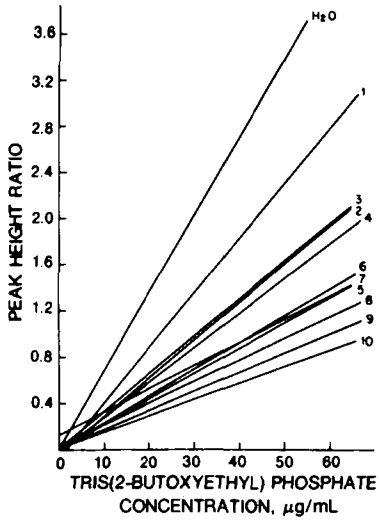

Figure 1—*The intersubject variability of peak height ratio [tris(2-butoxyethyl) phosphate/dioctyl phthalate] as a function of the concentration of tris(2-butoxyethyl) phosphate added to human serum with different normal volunteers. The identification number of each volunteer is given at the end of each standard curve.*

Preparation of Standard Curves for I—Serum samples for use in the preparation of standard curves were drawn into glass syringes. Ten normal adult volunteers (eight males and two females) ranging in age from 23 to 37 years served as study subjects. Each volunteer gave 50 mL of blood to allow the construction of standard curves for I (0, 10, 20, 30, 40, and 50 μg/mL) as well as to perform the battery of biochemical tests intended to identify the basis for the interindividual differences in standard curve slope (see below). Compound I was added to serum samples as a methanol solution[4]. Peak height ratios were plotted *versus* concentrations of I. Linear least-square regression analysis was used to determine the slopes, y-intercepts, and correlation coefficients of the lines of best fit. Repeated standard curves and single-concentration quality-control checks performed on numerous occasions were consistent with the interindividual slope differences observed in this study.

External Standard Experiments—In an effort to determine if the intersubject differences in the standard curves were due to variable recovery of the internal standard (dioctyl phthalate)[1], this compound was added as an external standard and as an internal standard to separate aliquots of serum from subjects 2 and 9 (concentration of I was 30 μg/mL). One set of the samples was extracted and assayed as described above with the exception of the transfer of an exact volume (3.0 mL) of organic phase to a clean tube. The other set of serum samples from the same subjects was extracted with 5 mL of blank dichloromethane (without internal standard) and 3.0 mL of this organic phase was transferred to a clean tube. Five micrograms of dioctyl phthalate in 1.0 mL of dichloromethane was added as an external standard. Blank dichloromethane (1.0 mL) was added to the control samples. All samples were then assayed as described above.

Effect of Alteration of Extraction Conditions—*Overview*—The primary objective of the following experiments was to establish a method which minimized or eliminated the intersubject differences in the extraction of I from serum. Pooled serum samples from individuals known to exhibit high recovery (subjects 1-3) or low recovery (subjects 8-10) of I were used for this series of studies.

Multiple Extraction Experiment—The assay procedure for I was modified to include repeated extractions of 0.5 mL of serum with dichloromethane (5.0 mL × 3). The first organic phase was transferred to a clean test tube leaving the aqueous phase in the original test tube. An additional 5 mL of dichloromethane (without internal standard) was added to the aqueous phase. This sample was then rotary mixed for 10 min, centrifuged, and transferred as described above. This procedure was repeated a third time and the pooled dichloromethane extracts containing I and internal standard were evaporated to dryness and chromatographed as described[5].

Ethyl Acetate Experiment—Serum samples (0.25 mL) were extracted as described above except that 5 mL of ethyl acetate containing 0.5 μg/mL of dioctyl phthalate was used instead of dichloromethane. Evaporation of the organic phase and GC was then performed as described above.

Assay of Serum Triglycerides, Total Cholesterol, High-Density Lipoprotein Cholesterol, Albumin, and α_1-Acid Glycoprotein—Serum concentrations of cholesterol, high-density lipoprotein cholesterol, and triglycerides were determined by previously described methods (7, 8). Serum albumin concentrations were determined using an auto analyzer[6] and the concentration of α_1-acid glycoprotein in serum was determined using a radial immunodiffusion assay[7]. This last procedure was performed in a manner to yield a coefficient of variation of 9.5%.

Statistical Analysis—All data are presented as population mean ± *SD* unless indicated otherwise. The quality of the various standard curves was estimated using linear regression analysis.

RESULTS AND DISCUSSION

Using the standard assay procedure described above, the lower limit of detection for I was 1 μg/mL and standard curves were linear to at least 50 μg/mL (standard curves were performed using single determinations at six concentrations; see above). A linear relationship between peak height ratio and serum concentrations of I was observed in all subjects. The range of correlation coefficients was 0.965-0.999 (mean = 0.984). A greater than threefold intersubject variability in slopes of standard curves was observed with a range of 0.0142-0.0486 (Table I, Fig. 1).

Addition of dioctyl phthalate as an external standard confirmed that the intersubject variability was related to the extraction of I and not the internal standard. The data presented in Table II demonstrate that a threefold range of slopes exists between "high" and "low" extraction sera independent of when the dioctyl phthalate is introduced. The diminished peak height ratio observed

[4] Preliminary studies indicated that initial evaporation of the methanol using either a stream of nitrogen or a 50°C water bath caused a loss >50% of I (*i.e.*, as estimated from reduction in peak height ratio). Later studies comparing samples dried at room temperature (18 h) to those prepared by the addition of I as a methanol solution yielded essentially identical results (*i.e.*, standard curves). The data from this series of studies were all obtained with serum samples containing <5% methanol (v/v).

[5] A number of other alterations in the extraction procedure, including protein precipitation with trichloroacetic acid and dilution of serum with distilled water, were also evaluated as methods to reduce the intersubject variability in the recovery of I. These approaches were all unsuccessful.

[6] Model SMA-2; Technicon Corporation.

[7] M-partigen; Calbiochem-Behring Corp., La Jolla, Calif.

Table II—Influence of Selected Treatments on the Peak Height Ratio of Tris(2-butoxyethyl) Phosphate to Dioctyl Phthalate

	Peak Height Ratio from High-Extraction Serum	Peak Height Ratio from Low-Extraction Serum	Peak Height Ratio from High-Extraction Serum/ Peak Height Ratio from Low-Extraction Serum
		External Standard Experiment[a]	
Internal standard (control)	1.17 ± 0.0600	0.396 ± 0.0295	2.96
External standard	0.614 ± 0.0483	0.225 ± 0.0177	2.73
		Multiple Extraction Experiment[b]	
	0.871 ± 0.152	0.685 ± 0.118	1.27

[a] The serum used in this study was from subject 2 (high extraction) and subject 9 (low extraction). [b] The serum used in these studies were prepared by pooling serum from subjects 1, 2, and 3 (high extraction) and 8, 9, and 10 (low extraction).

Table III—Intersubject Variability in Peak Height Ratio When Ethyl Acetate is Used in Place of Dichloromethane[a]

Subject	Peak Height Ratio
2	1.1232 ± 0.1435
3	1.7668 ± 0.1319
5	1.5404 ± 0.0680
8	1.8942 ± 0.0400
9	1.1761 ± 0.0162
10	1.6229 ± 0.0235

[a] All serum samples contained I at a concentration of 30 μg/mL.

in the external standard component of this study is presumably related, at least in part, to the incomplete recovery of dioctyl phthalate when it is used as an internal standard.

The results of the experiments utilizing repeated extraction to eliminate the intersubject difference in the recovery of I are also presented. Multiple extractions with dichloromethane substantially reduced the intersubject variability in the peak height ratio of I/dioctyl phthalate (Table II). However, this procedure was substantially more laborious than the standard procedure. Replacing dichloromethane with ethyl acetate (Table III) did result in elimination of much of the variability. Though this assay still has limitations, it appears to be the best procedure presently available (*i.e.*, assuming that blank serum which could be used to prepare an individual's own standard curve is unavailable). The use of ethyl acetate as the organic phase for the extraction of I from serum samples eliminates much of the intersubject variability and enables investigators to ensure the absence of I from study samples.

The chemical basis for this variability has not been established. However, highly significant correlations were found between the slope of the individual standard curves and serum concentrations of triglycerides ($r = -0.80$, $p < 0.005$) and triglycerides plus cholesterol ($r = -0.881$, $p < 0.0025$). A much weaker correlation was also found between the standard curve slope and serum concentration of α_1-acid glycoprotein ($r = -0.578$, $p < 0.05$). However, significant covariance existed between α_1-acid glycoprotein concentration and the sum of triglycerides and cholesterol serum concentrations ($r = -0.563$, $p < 0.05$)[8]. There was no significant correlation between the standard curve slope and albumin ($r = -0.292$), total cholesterol ($r = -0.403$), or high-density lipoprotein cholesterol concentrations ($r = -0.289$). Based on the observation that the addition of α_1-acid glycoprotein to serum did not affect the extraction of I[8], it is presumed that the correlation between concentration of this protein and the slope of the individual standard curves is fortuitous (*i.e.*, it was observed because α_1-acid glycoprotein exhibited covariance with triglyceride concentrations, an indicator of the concentration of very low-density lipoproteins). The precise nature of the chemical basis for the intersubject variability in serum extraction of I cannot be established on the basis of the present series of experiments. However, given the lipophilic characteristics of I, it is reasonable to assume that association with or dissolution in lipoproteins plays a major role.

Part of the overall significance of these results lies in the widespread use of pooled serum by research investigators and analytical laboratories. The use of pooled serum in the preparation of standard curves for I could have resulted in a greater than threefold error in estimates of the concentration of I in serum of certain individuals. This variability in serum extraction observed with tris(2-butoxyethyl) phosphate may occur with other highly lipophilic compounds. Indeed, it has been observed recently that bis(2-ethylhexyl) phthalate behaves in a very similar manner (9). Therefore, it is necessary to rule out possible serum-dependent extractability during the early stages of assay development for at least certain classes of compounds.

Based upon the results of the present study, the use of ethyl acetate would appear to be the best approach to minimizing assay error due to intersubject variability in the extraction of I if a relatively simple assay is to be utilized.

[8] When α_1-acid glycoprotein (the gift of Dr. Kenneth Piafsky of the University of Toronto) was added to serum, the recovery of I was not altered.

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