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Research Article

Effect of the Plasticizer DEHP in Blood Collection Bags on Human Plasma Fraction Unbound Determination for Alpha-1-Acid Glycoprotein (AAG) Binding Drugs

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Abstract. Fraction unbound (f_u) is a critical drug distribution parameter commonly utilized for modeling efficacious dosage and safety margin predictions. An over-estimation of f_u for 13 chemically diverse small molecule drugs primarily bound to alpha-1-acid glycoprotein (AAG) in human plasma was discovered when *in vitro* results from our screening lab were compared to literature values. Di-(2-ethylhexyl) phthalate (DEHP), a plasticizer known to be used in the manufacture of blood collection bags, was extracted from plasma obtained through three common techniques that allowed contact with DEHP, and drug f_u values in plasma from each collection method were estimated using the HTDialysis protein binding methodology. Additionally, f_u of test compounds in plasma spiked with varying concentrations of DEHP (0–800 μM) was determined, and DEHP extractions were performed from plasma stored in Terumo bags over 7 days. Blood stored in Terumo bags, blood collected in Terumo bags, but immediately transferred to conical vials, and vacutainer-collected blood yielded DEHP concentrations of 300–1000 μM, 1–10 μM, and 0.1–2 μM, respectively. This finding corresponded with the f_u of tested drugs in DEHP-spiked plasma increasing between 2- and 5-fold. Additionally, DEHP was discovered to leach from the Terumo bag, with concentrations increasing 10-fold over a 7-day test period. In summary, the presence of DEHP in commercially available blood collection bags confounds *in vitro* f_u estimation for drugs that bind primarily to AAG. It is recommended that vacutainer-collected human plasma, which contains negligible DEHP, be used for the most accurate estimation of f_u in human plasma.

KEY WORDS: alpha-1-acid glycoprotein; equilibrium dialysis; plasma protein binding; plasticizer; di-(2-ethylhexyl) phthalate (DEHP).

INTRODUCTION

In vitro assays for estimating fraction unbound (f_u) in plasma for drug candidates are essential for prediction of free drug concentrations, efficacious dose, and safety margins. These results can be applied directly to correct total plasma concentration data or be used as an input parameter for physiologically based pharmacokinetic (PBPK) modeling (e.g., GastroPlus or SimCYP). As with any *in vitro* system utilized for predicting the disposition of drug candidates, it is necessary to properly identify and mitigate sources of contamination, impurities, or other confounding factors that may influence the results in order to provide the most accurate *in vitro* estimation. Human plasma, in which an estimate of f_u is commonly performed early in drug discovery programs, contains several major components, including lipoproteins, glycoproteins, albumin, and globulins. Due to the relative abundance of each protein, drug binding occurs predominantly to either human serum albumin (HSA), composing 50–60% of plasma proteins (35–50 g/L), or alpha-1-acid glycoprotein (AAG), which represents only 1–3% of total plasma proteins (0.4–1.0 g/L) (1,2).

The binding affinity to each type of protein is dependent on structural characteristics and physicochemical properties such as pKa of the drug of interest. Most basic drugs bind preferentially to AAG, and in most disease states, levels of AAG increase in the body (3), which when coupled to the relatively low concentration of this protein in the plasma, results in alterations and variability in the distribution of basic drugs in patients. In addition, competitively binding contaminants that may be introduced during plasma collection and processing may particularly compromise f_u determination for

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basic compounds *in vitro*. The use of phthalates to soften PVC-based products is widespread in numerous medical devices, such as blood collection bags, dialyzers, and catheters (4). This family of chemicals serves to maintain flexibility of collection bags and help to protect the integrity of the red blood cells in the collection samples (5). Phthalate plasticizers such as DEHP, its metabolite mono-(2-ethylhexyl) phthalate (MEHP), tris-(2-butoxyethyl)-phosphate (TBEP), and others have been widely reported as endocrine disruptors (6) and have been shown to displace chemicals from binding sites on AAG and mononuclear leukocytes (7). Although negative impacts on health and development have been reported leading to DEHP being banned in many European countries (8), the impact of the presence of plasticizers such as DEHP in plasma is not well studied in regard to protein binding (9). It is plausible that plasticizers such as DEHP can propagate through the collection devices into biological materials such as blood or plasma, representing a potential source of contamination to *in vitro* studies conducted with these matrices.

To the best of our knowledge, while it has been reported that phthalate plasticizers present in human plasma can displace drugs from AAG-binding sites (2), there has been minimal focused effort on quantifying the amount of plasticizer present in human plasma from various plasma collection methods, while also directly comparing the effect of DEHP concentration on *in vitro* protein binding assay results. Thus, a series of *in vitro* experiments were conducted in our screening lab using 13 drugs with reported human fraction unbound values ranging from 0.01 to 0.3, to investigate if a direct relationship could be observed between DEHP concentration and f_u values determined utilizing equilibrium dialysis (HTDialysis) as the methodology for protein binding determination (10,11).

MATERIALS AND METHODS

Chemicals and Reagents

Verapamil, haloperidol, propranolol, quinidine, erythromycin, vismodegib, lidocaine, disopyramide, alprenolol, imatinib, diclofenac, labetalol, imipramine, DEHP, and d4-DEHP were purchased as powders from Sigma (St. Louis, MO). Fentanyl, methadone, and alprazolam were purchased as methanol (MeOH) solutions (1 mg/mL) from Cerilliant (Round Rock, TX). Human plasma following various blood collection methods was obtained from either BioreclamationIVT (Baltimore, MD) or Zen-Bio Inc. (Research Triangle Park, NC). Pooled plasma from preclinical species (male CD1-mouse, Sprague-Dawley rat, Beagle dog, and Cynomolgus monkey) was also obtained from BioreclamationIVT (Baltimore, MD). The equilibrium dialysis protein binding apparatus and accompanying dialysis membranes (molecular weight cut-off of 12–14 kDa) were purchased from HTDialysis (Gales Ferry, CT). Liquid chromatography mass spectrometry grade methanol, water, ammonium bicarbonate, and formic acid were purchased from Fisher Scientific. Ultra-pure water was prepared in-house using a MilliQ Advantage 10 production unit (Millipore, Billerica, MA). All other reagents and chemicals were of the highest purity available.

Biological Material

Two different methods of plasma collection from Terumo bags were used: three lots that had been stored under routine conditions according to the vendor (i.e., stored bag lot), and two lots that were collected into Terumo bags, but immediately processed, transferred to conical vials, and shipped to our site for testing. DEHP exposure was limited as much as possible for the latter samples but were still collected in Terumo blood bags (i.e., "fresh lot"). According to the FDA, in clinical settings and in neonatal care, contact between DEHP and the patient should be minimized "by using the freshest, coldest blood products available, or by using heparin-coated blood tubing" (12). Thus, plasma collected using heparin-coated vacutainers (i.e., vacutainer lot, not exposed to blood bags) was also purchased as a third collection method for comparison studies.

During drug discovery, multiple pharmacokinetic (PK) species are commonly selected for use in predicting drug disposition in human. Thus, the collection of blood from the most common PK species became an area of concern and was also worth investigation. Plasma from these preclinical species (CD-1 mouse, Sprague-Dawley rat, Beagle dog, and Cynomolgus monkey) was also obtained to assess DEHP exposure in non-blood bag collection methods. Lastly, an additional Terumo and vacutainer collection of plasma was purchased from Zen-Bio, Inc. and was used to investigate the possibility of DEHP leaching over time.

Bioanalysis

All data acquired for DEHP and d4-DEHP (13,14) was obtained on an AB Sciex API4000 triple quadrupole mass spectrometer (MS) in positive ionization mode, coupled with a LEAP PAL HTS-xt autosampler and Shimadzu 30AD UPLC (ultra performance liquid chromatograph) pumps. DEHP was monitored at a mass to charge ratio (m/z) of 391.4/112.8, with a collision energy (CE) of 15. Mobile phases consisted of 0.1% formic acid in ultra-pure water (A) and 100% methanol (B). A Waters Isolator Column (2.1 × 50 mm) was installed before the injection port to separate plasticizer contaminants present in the mobile phases. An Acquity UPLC BEH C18 1.7 μm, 2.1 × 50 mm column performed all DEHP separation from the samples. The LC gradient was held at 40% organic for 30 s, then ramped to 85% over the next 30 s. Over 4 min, organic was ramped from 85 to 95%, then stepped to 99% to be held for 1 additional minute, and returned to starting conditions for 30 s post-acquisition. Figure 1 is a representative LC-MS/MS chromatogram of DEHP, with mobile phase contaminants displayed post-elution of the analyte peak. Analysis of all samples was performed against a linearly-fit calibration curve ranging from 6.86 to 15,000 nM with $1/x^2$ weighting.

Analysis for all drugs assayed in the fraction unbound experiments was performed by tandem LC-MS/MS on an AB Sciex API5000 triple quadrupole MS in positive ionization mode, coupled with a Thermo Transcend autosampler system using Dionex Ultimate 3500 UPLC pumps. The sample injection size was 2 μL across a Supelco Ascentis Express C_{18} column (5 μm × 2.1 mm ID) equipped with a Phenomenex Security Guard HPLC C_{18} (5 μm × 2.1 mm ID). The aqueous mobile phase was composed of 98.5% Milli-Q ultra-pure water, 1% methanol, and 0.5% 1 M ammonium bicarbonate,

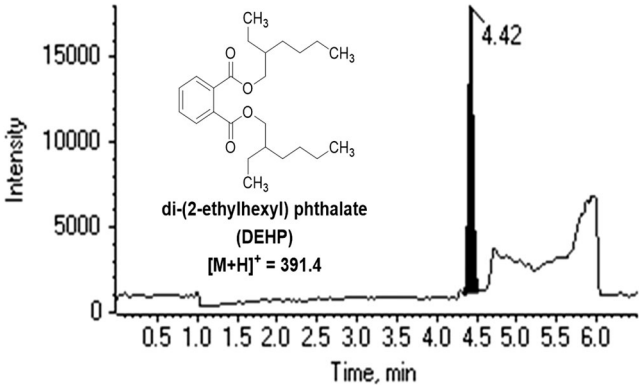

Fig. 1. Representative LC-MS/MS chromatogram of the plasticizer DEHP (78.125 nM standard) in plasma

and the organic mobile phase consisted of 100% methanol. All test compounds were chromatographically optimized by ramping from 1% organic at 0.25 min to 99% at 0.75 min, with a return to starting conditions at 1.08 min. Run time per injection was approximately 1.2 min. Standard curves for each test compound ranged from 2.5 to 5000 nM, with quadratic $1/x^2$ weighting, and all data were acquired using Analyst v1.6.1. The MS parameters for each tested drug are summarized in Table I.

DEHP Extraction Method

The extraction method and efficiency for DEHP were assessed by spiking d4-DEHP into vacutainer lot plasma, neat acetonitrile, and phosphate buffer to 4.5 μM. Extractions were performed by adding 3:1:1 acetonitrile/5 N sodium hydroxide/plasma, mixing thoroughly, and centrifuging at 4000 rpm for 10 min at room temperature. The supernatants were then matrix matched into an internal standard solution containing imipramine, phosphate buffer, and neat acetonitrile. The extraction method for d4-DEHP performed from neat acetonitrile was considered to be the actual concentration of the spiked samples, and plasma extractions were compared relative to these neat samples.

Extractions of DEHP were performed following this method on all plasma samples containing an unknown quantity of DEHP. Supernatants were then further diluted with acetonitrile (1:10 for "Fresh Lots", "Vacutainer Lots", and preclinical species or 1:100 for "Stored Bag Lots") to minimize matrix suppression. An aliquot (25 μL) of the final sample was then plated into 75 μL of methanol quench containing internal standard (imipramine), pH 7.4 phosphate buffer (25 μL), and acetonitrile (25 μL). A DEHP calibration curve ranging from 6.86 to 15,000 nM was generated in acetonitrile and plated to equivalency.

Table I. Summary of Mass Spectrometer Parameters Used for Bioanalysis of AAG-Binding Drugs

Compound	Q1	Q3	DP	CE
Verapamil	455	165	180	55
Methadone	310	265	44	20
Haloperidol	376	165	100	38
Alprazolam	309	281	90	36
Propranolol	260	116	144	27
Quinidine	325	160	192	41
Erythromycin	734	158	91	42
Vismodegib	421	139	90	53
Lidocaine	235	86.0	90	24
Disopyramide	340	239	90	12
Alprenolol	250	173	90	24
Imatinib	494	394	90	40
Fentanyl	337	188	187	50
Diclofenac[a]	296	214	40	45
Labetalol[a]	329	294	64	27
Imipramine[a]	281	58.1	45	55

[a] Internal standards

Fraction Unbound Reagent Preparation

The fraction unbound determination assay was conducted using an HTDialysis® 96-well plate methodology, as described previously (10,11). Incubation buffer (1 L of 100 mM sodium phosphate, pH 7.4) was prepared by adding approximately 11.93 g of NaH_2PO_4 (anhydrous) and approximately 2.22 g of NaH_2PO_4 (monohydrate) to 1 L of Milli-Q water, followed by adjustment to pH 7.4 with phosphoric acid.

Dialysis membranes were prepared according to manufacturer specifications. Each dry membrane strip consists of a pair of dialysis membranes that separate upon hydration. The membrane strips were separated by soaking in distilled water for 60 min, followed by addition of 20% ethanol by volume, and the strips were soaked for an additional 20 min. Prior to use, the membranes were rinsed twice in distilled water.

The HTDialysis Teflon block was prepared according to manufacturer specifications. A single dialysis membrane was placed between each of the eight Teflon bars below the top edge of the Teflon bar, with the membrane covering the bottom of all wells. The Teflon block was placed into the base and the assembly tightened using the cam levers. Phosphate buffer (pH 7.4) was added as quickly as possible after block assembly to prevent the strips from drying.

Upon determining a range of concentrations present in the collected plasma samples, DEHP was spiked into vacutainer plasma, bringing DEHP concentrations to 0, 40, 50, 100, 200, 400, and 800 μM DEHP in plasma to simulate Terumo bag collections. A 30-mL aliquot of plasma was thawed on the days of use and pH adjusted to 7.4 ± 0.05 using 4:1 H_2O/25% phosphoric acid. A 250 μM dimethylsulfoxide (DMSO) stock dose of test compounds was generated by adding 7.5 μL of 10 mM DMSO stock into a final volume of 300 μL DMSO.

Fraction Unbound Assay

Each test compound (2 μL of 250 μM stock) was directly spiked into 500 μL of plasma for an incubation concentration of 1 μM. Triplicates were generated in a secondary plate from the pool of each dosed plasma sample. Dosed plasma of 100 μL was plated into the HTDialysis apparatus on the opposite chamber from the buffer. A 5-μL aliquot of plasma at T0 was plated from the triplicate plate into 125 μL of methanol quench containing imipramine, labetalol, and diclofenac internal standard mixture, 5 μL DMSO, and 50 μL of phosphate buffer. The dialyzer was then covered with a breathable adhesive cover and placed in an incubator for 4.5 h at 37°C and was subjected to orbital shaking

at 167 rpm. Upon completion of the incubation, 50 μL of the dialysate chamber matrix was plated into 125 μL of methanol quench containing imipramine, labetalol, and diclofenac internal standard mixture, 5 μL DMSO, and 5 μL plasma. Similarly, 5 μL from the plasma chamber matrix was plated into 125 μL of methanol quench containing imipramine, labetalol, and diclofenac internal standard mixture, 5 μL DMSO, and 50 μL of phosphate buffer (i.e., matrix matching).

Calibration curves were generated for all test articles in DMSO at the following concentrations: 5, 2, 0.2, 0.02, 0.005, 0.0025 μM and were plated to sample equivalency to the assay samples. All final plates were sealed and centrifuged at 4000 rpm for 30 min and submitted for bioanalysis.

DEHP Leaching

To assess DEHP leaching into plasma samples, a series of collections were conducted from a single donor using both a Terumo blood bag and a vacutainer, collected in a single session, performed by Zen-Bio, Inc. An aliquot of the Terumo collection was immediately frozen for processing (day 0), while a second aliquot was stored at 4°C in the collection bags for 7 days (day 7), in accordance with typical storage procedures communicated by the plasma vendor. The vacutainer was used as a DEHP-free control for the donor. Extractions were performed as previously described, DEHP was quantified, and fraction unbound was assessed for the 13 test compounds.

Data Processing

Extraction efficiency of DEHP was estimated using Eq. 1:

$$Extraction\ Efficiency\ (\%) = \frac{Average[DEHP\text{-}d4]_{Spiked\ Plasma}}{Average[DEHP\text{-}d4]_{Neat\ ACN}} * 100$$

DEHP extractions from unknown samples were calculated by plotting the peak area ratios of the extracted samples against the calibration curve ranging from 6.86 to 15,000 nM. Linear curve fitting was used with a $(1/x^2)$ weighting. Because extractions from plasma were performed in a 3:1 ratio of acetonitrile to plasma, a dilution factor of 3 was applied to all unknown samples. For theoretically high samples that required additional acetonitrile dilutions, as stated in the "MATERIALS AND METHODS" section, a dilution factor of 30 or 300 was used to account for curve non-linearity above 15,000 nM.

Quadratic curve fitting with $(1/x^2)$ weighting was used to calculate test compound concentrations in unknown samples.

Fraction unbound data processing was performed using Eq. 2:

$$\%Fraction\ unbound = \frac{[Compound\ in\ buffer]}{[Compound\ in\ plasma]} * 100$$

Relative recovery (data not shown) was calculated with the following equation:

$$\%Recovery = ([Compound\ in\ buffer] + [Compound\ in\ plasma]) * 100$$

As all compounds tested were commercial and well characterized, the recovery calculation was used only to ensure proper dialyzer and assay performance.

RESULTS

Bioanalytical Method and Extraction for DEHP

A bioanalytical method to quantify DEHP was developed using liquid chromatography tandem mass spectrometry (LC-MS/MS) on an AB Sciex API4000 triple quadrupole mass spectrometer. During method development, it was discovered that plasticizer was present in the system before samples were introduced (i.e., low-level interference), which initially made analysis of DEHP via LC-MS/MS challenging. Using bottle-fresh mobile phases and Teflon-coated and stainless steel tubing where possible helped minimize the interference. In addition, the use of an isolator column (purchased from Waters) upstream of the autosampler was essential to filter out existing DEHP from the mobile phase, as shown in the representative chromatogram in Fig. 1. Over time, contamination levels would nonetheless continue to increase, eventually interfering with the analyte peak. Two control blanks were injected in between every unknown sample, before, and after the standard curve to monitor and assess data quality. LLOQ was maintained at 6.86 nM, and flushing the entire LC system with pure isopropyl alcohol each day was found to be ideal in order to maintain an appropriate peak shape and LLOQ.

Once a suitable bioanalytical method was developed, the extraction efficiency for DEHP was assessed by spiking d4-DEHP (4.5 μM) into vacutainer lot plasma, phosphate buffer, or neat acetonitrile, followed by extracting the samples by the addition of 3:1:1 acetonitrile/5 N sodium hydroxide/plasma. The results of the extraction procedure are shown in Table II. With a target concentration of 4.5 μM, the percent (%) accuracy of extracted and measured concentration was 81.4 to 87.0 (±3)% from plasma, 90.5 to 91.8 (±0.6)% from buffer, and ~100 (±6)% from neat acetonitrile. Using Eq. 1, the extraction efficiency from plasma was estimated to be 78.1% (Table II).

Employing the DEHP bioanalytical and extraction methods previously described, DEHP was measured and found to be present at high concentrations in plasma stored under normal conditions in Terumo blood collection bags,

Table II. Extraction Efficiency Analysis of d4-DEHP from Plasma and Buffer. The Target Concentration of DEHP was 4.5 μM

Sample name	Calculated concentration (μM)	% Accuracy
Spiked plasma extraction (replicate 1)	3.86	85.8
Spiked plasma extraction (replicate 2)	3.91	87.0
Spiked plasma extraction (replicate 3)	3.66	81.4
Spiked buffer extraction (replicate 1)	4.07	90.5
Spiked buffer extraction (replicate 2)	4.10	91.1
Spiked buffer extraction (replicate 3)	4.13	91.8
Neat ACN (replicate 1)	4.86	108
Neat ACN (replicate 2)	5.16	115
Neat ACN (replicate 3)	4.63	103
Efficiency (spiked plasma to neat acetonitrile, Eq. 1)		78.10%

with ~ 380, ~ 240, and 1165 μM DEHP measured in three separate lots of plasma (Table III). This data not only suggests high concentrations, but also substantial lot-to-lot variability in levels of DEHP contamination. In contrast, the "Fresh Lots" of plasma contained 7.8 and 0.6 μM, while the vacutainer lots contained only 0.1, 0.3, and 2.6 μM DEHP. It was also investigated and discovered that DEHP was not detectable in plasma from preclinical species cynomolgus monkey, Sprague-Dawley rat, beagle dog, and CD1 mouse (Table III). Communications with the vendor confirmed a difference in blood collection procedure; specifically, blood is collected in glass bottles in preclinical species and subsequently stored in conical vials.

Fraction Unbound in Plasma Spiked with DEHP

The direct effect of a range of spiked concentrations of DEHP into vacutainer-collected plasma (the most devoid of DEHP) was clearly shown in the behavior of the tested drugs (Fig. 2), with fraction unbound increasing dramatically as DEHP concentration increased. For example, the protein binding of lidocaine was observed to be greatly affected by the presence of DEHP. Literature values report that lidocaine is roughly 30% unbound, although with the addition of 800 μM DEHP, the unbound percentage increased to 75.7%. Similar trends were observed for most tested compounds, ranging from 1.1 to 3.64-fold increase in fraction unbound. Vismodegib is worth special mention, as we found the f_u to be lower than the reported literature value, and increased from 0.003 to 0.029 as DEHP was introduced, an almost 10-fold increase. Meanwhile, little to no effect of DEHP was consistently observed with fentanyl, a drug reported to predominantly bind to HSA, not AAG (15).

DEHP Leaching

In the single-donor DEHP-leaching assessment, a substantial increase (~ 10-fold) in measurable DEHP concentration from ~ 33 to ~ 300 μM was observed over 7 days. The vacutainer collection from the same donor was below the lower limit of quantitation of 6.8 nM. The amount of free drug measured *versus* accepted literature values again became more disparate in day 7 due to the presence of DEHP, but more closely aligned at day 0 (Fig. 3).

Table III. DEHP Extraction Concentrations in Plasma Samples (In Singlet)

Plasma sample	Concentration in plasma (μM)
Stored bag lot 1	381
Stored bag lot 2	238
Stored bag lot 3	1165
Fresh lot 1	7.8
Fresh lot 2	0.6
Vacutainer lot 1	0.1
Vacutainer lot 2	0.3
Vacutainer lot 3	2.6
Cyno monkey	< 0.0068
Sprague-Dawley rat	< 0.0068
Beagle dog	< 0.0068
CD1 mouse	< 0.0068

DISCUSSION

In vitro screening assays to estimate the ADME properties of drug candidates are routinely performed in drug metabolism and pharmacokinetic (DMPK) laboratories across the industry (16,17), including assessment of fraction unbound in plasma. Since only "free" drug can distribute out of the central blood compartment into tissues to interact with therapeutic targets or drug-metabolizing enzymes, f_u in plasma data is utilized for prediction of free drug concentrations, a critical parameter for efficacious dose, drug-drug interaction (DDI) risk, and safety pharmacology margin assessments (18–20). This data may also be utilized as an input parameter for more sophisticated physiologically based pharmacokinetic (PBPK) modeling (21,22). Thus, the accuracy of this, and all other *in vitro* ADME data used in this capacity, is critical for reliable prediction of drug disposition properties *in vivo*.

High throughput ADME screening involves conducting *in vitro* studies for numerous compounds on a routine basis, which in turn drives the need for large volumes of various matrices, such as plasma for protein-binding experiments. Typically, human plasma is obtained from vendors, whose standard practice is to collect and store until time of purchase. To enable sufficient inventory for expedited shipment upon purchase, most vendors routinely collect blood using blood bags such as Terumo bags, and plasma is processed within 30 min (personal communication with vendor). The plasma bags may then be stored at 4°C for up to 30 days, and if the material is not requested and shipped in that time, they are moved to − 20°C for long-term storage of up to 2 years (personal communication with vendor). A major component of the Terumo bags is di-(2-ethylhexyl) phthalate (DEHP), a known plasticizer that has gained wide attention over the years as a potentially toxic chemical (6,8). Thus, the objectives of this investigation included testing the possible DEHP contamination levels in stored plasma following various collection techniques and determine the impact to fraction unbound determination in plasma from human and preclinical species for a subset of compounds that primarily bind to AAG. As AAG levels are relatively lower in abundance in the blood compared to albumin (1,2), it may become saturated by dosed drugs and/or contaminants in collected plasma, allowing for a potential indicator of a contaminant effect to be observed with AAG-binding compounds (Table I) (1). Factors that may affect AAG saturation include high drug (or contaminating plasticizer) concentrations, frequency of dose, affinity (Kd) of drug to AAG, and affinity of drug to albumin. Drugs with a selectively high affinity to AAG and a low affinity to albumin are more likely to become saturated and exhibit concentration-dependent increase in f_u, as opposed to drugs that have high affinities to both AAG and albumin, the latter of which can act a sink if AAG becomes saturated.

DEHP Extractions

DEHP extractions were performed on various lots of plasma samples in singlet to assess absolute limits of DEHP concentrations to be used in the f_u assessment *versus* DEHP influence (Table III). Upon analysis of the DEHP extractions against a calibration curve, high concentrations of DEHP

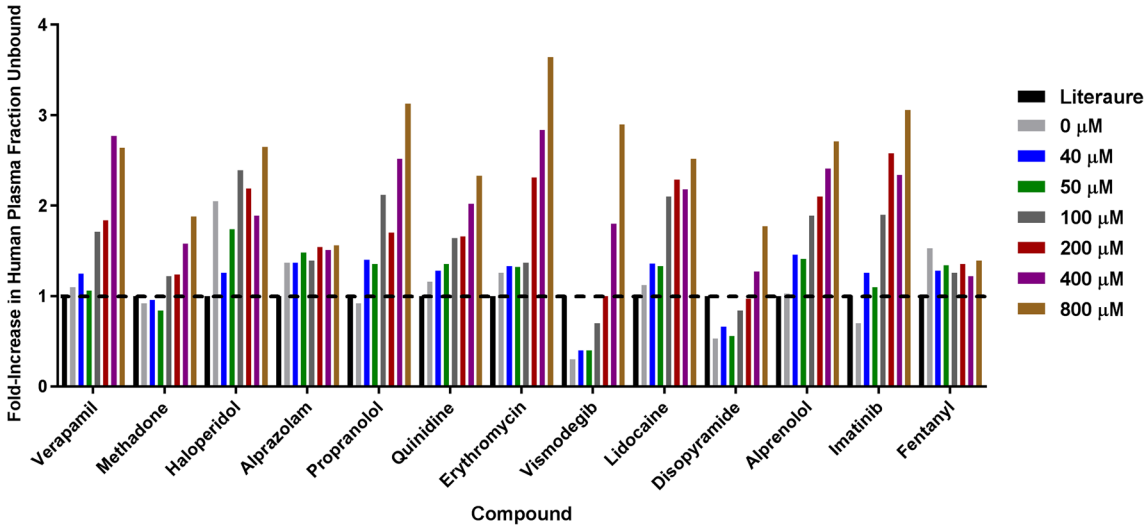

Fig. 2. Fold-increase in fraction unbound against literature values of 13 drugs in human plasma spiked with various concentrations of DEHP up to 800 μM. The dotted line indicates the literature value against which all human fraction unbound data is compared

were measured in commercially available and stored human plasma. Variability within the collections using this method was high, ranging from ~240 to over 1 mM. Because the plasma storage time for this method is likely also highly variable (but not clearly recorded by the vendor), the roughly 5-fold range in DEHP concentration between tested plasma lots in our studies was of particular interest.

In the collection method that minimized the time plasma was present in the Terumo bags (e.g., fresh lot), levels were expectedly low, ranging from 0.6 to 7.8 μM, implying that there is a time component influencing the DEHP concentration present in the human plasma samples. DEHP levels in plasma collected in vacutainers were also low, although it was noted that the concentrations could reach as high as 2.6 μM. Other sources of DEHP or similar plasticizer contact are possible even in this collection method which would require further investigation, but the contamination was not nearly as significant as in the Terumo bags themselves.

Preclinical species tested negative for DEHP, or tested well below the lowest calibration curve point of 6.86 nM, again signifying that contamination is inherent to the collection and storage process of human plasma. According to the plasma vendor as stated in personal communications, preclinical animal samples are typically collected in glass bottles and do not come in contact with collection bags during any stage of the process.

Fraction Unbound Assessment

The influence of DEHP on the fraction unbound of AAG-binding drugs was readily apparent across the

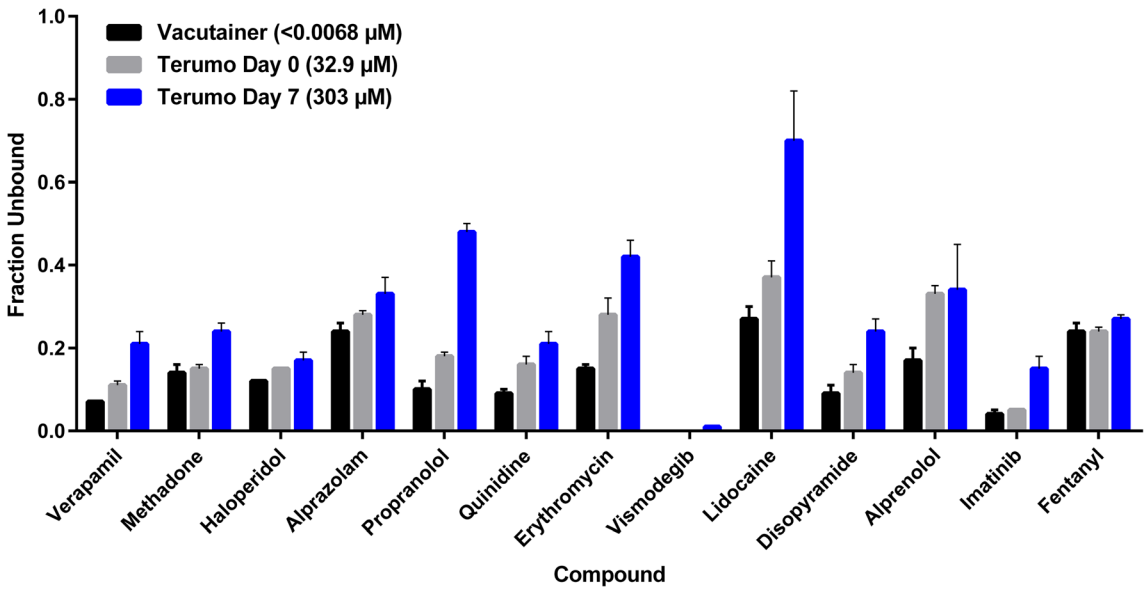

Fig. 3. Fraction unbound of 13 drugs in vacutainer lot of human plasma and in plasma obtained from blood collected in Terumo bags following 0 and 7 days of Terumo bag exposure. The increase in DEHP concentration during 7-day test period suggests that DEHP may leach out of the bag into plasma over time

concentrations tested (Fig. 2). The quantity of DEHP required to influence the free drug percentage appeared to be somewhat compound-dependent, although for most test compounds, the effect on binding became apparent by 50–100 μM DEHP, a concentration that is below that measured in the three separate stored plasma lots (Table III). Plasma subjected to routine storage conditions all contained DEHP concentrations at least 2-fold higher than this benchmark. Compounds such as imatinib, propranolol, and lidocaine were affected at much lower levels and displayed marked increases to f_u at only 40 μM DEHP. At 800 μM DEHP, all drugs tested displayed a significant increase of f_u ranging from 2- to 5-fold that of accepted literature values. As DEHP levels in routinely stored plasma were shown to range from ~ 240 μM to > 1 mM, it is clear that this plasticizer is a major contributing factor to errant *in vitro* f_u results. At this time though, we cannot discount that there may be some effect to albumin-binding drugs, but the effect appears to be more profound for AAG-binding drugs. For example, alprazolam is more highly bound to albumin than to AAG (23), but the AAG-binding affinity is enough to show a ~ 5–10% effect of DEHP. That being said, there appears to be a correlation between the AAG-binding affinity and the DEHP-effect, where the more AAG-dependent the compound is, the more pronounced the observed effect.

DEHP Leaching

Because routine storage time of plasma is highly variable and unlikely well controlled, a targeted experiment was performed to estimate contamination over the course of 7 days from plasma drawn from a single donor. A 10-fold increase of DEHP was detected between the day 0 and day 7 aliquot in the Terumo blood bags, from ~ 33 to ~ 300 μM. Meanwhile, from the same donor and same collection session, only trace amounts of DEHP were detected from the vacutainer method. The tested drugs were again assessed for f_u (Fig. 3), and as expected, a similar over-estimation of f_u was observed with similar trends as the results detailed in Fig. 2, increasing from vacutainer to day 0 Terumo bag to day 7 Terumo bag. With the rapid rate of DEHP leaching, expediently processing blood collected in bags becomes essential when using the collections for *in vitro* assays, as the fraction unbound values of many AAG-binding drugs may be influenced after only a day of storage in Terumo bags, and are likely to become compromised even further within 1 week of storage.

Maintaining a large supply of biological material that is as devoid of contaminants allows for *in vitro* assays to be performed at a high rate and yield results that closely simulate *in vivo* behavior. Closely examining how biological material is collected and stored enables labs to identify and mitigate any confounding variables. Because plasticizers such as DEHP are known chemicals present in many collection methods, it would be ideal to determine and implement a process to collect and store large volumes of biological material while minimizing contact with plasticizers.

CONCLUSION

The dangers of plasticizers in blood collections have been examined in an *in vivo* setting (8), but to our knowledge, there has been limited investigation into how plasticizer contamination may confound *in vitro* plasma protein-binding results. As *in vitro* studies are performed on new drug entities to predict their drug disposition properties, obtaining reliable predictive results is essential in the drug discovery process. AAG-binding drugs may be especially prone to inaccurate *in vitro* binding data, as AAG protein levels in human plasma is relatively low and thus may become saturated under certain conditions, leading to a higher possibility of competitive binding or displacement effects from unexpected contaminant chemicals. To this end, an assessment of DEHP levels was measured in plasma derived from common collection methods, and a range of DEHP concentrations was co-incubated with AAG-binding drugs in plasma to assess its impact. For all tested compounds, between a 2- and 5-fold increase of f_u was observed when compared to accepted literature values. The impact of such drastic over-estimations of f_u can lead to misinterpretation of data and incorrect dosage and safety margin predictions, as the *in vitro* result implies that there would much more active drug available in an *in vivo* setting than actual. Currently, there are DEHP-free collection bags on the market, but further testing would be required to ensure that other variables from these do not have the same effect on *in vitro* results as DEHP. It is recommended that for screening studies where a large volume of plasma is required, plasma with minimum exposure to blood bags would be ideal, and for definitive studies, vacutainer-collected plasma with minimal DEHP present is clearly preferred. Lastly, although only speculative at this point, the findings reported here suggest that possible displacement of drugs bound to AAG or other plasma components due to DEHP exposure via tranfusions from blood bags is at least plausible and may warrant additional directed research efforts to assess this risk.

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REFERENCES

1. Brown K, Hingorani G, Franklin R. An effective screening approach to assess the impact of α-1 acid glycoprotein binding on the fraction unbound of a drug. Poster session presented at: 17th Annual ISSX Meeting; 2011. Oct 16–20; Atlanta GA.
2. Routledge PA. The plasma protein binding of basic drugs. Br J Clin Pharmacol. 1986;22(5):499–506.
3. Al-Dewik N, Jewell A, Yassin M, Morsi H. Studying the impact of presence of alpha acid glycoprotein and protein glycoprotein in chronic myeloid leukemia patients treated with Imatinib Mesylate in the State of Qatar. Biomark Cancer. 2015;7:63–7.
4. Pollack GM, Buchanan JF, Slaughter RL, Kohli RK, Shen DD. Circulating concentrations of di(2-ethylhexyl) phthalate and its de-esterified phthalic acid products following plasticizer exposure in patients receiving hemodialysis. Toxicol Appl Pharmacol. 1985;79(2):257–67.
5. Bicalho B, Serrano K, dos Santos Pereira A, Devine DV, Acker JP. Blood bag plasticizers influence red blood cell vesiculation rate without altering the lipid composition of the vesicles.

Transfus Med Hemother. 2016;43:19–26. https://doi.org/10.1159/000441639.
6. Yue Y, Lui K, Liu R, Sun Y, Li X, Fan J. The binding affinity of phthalate plasticizers-protein revealed by spectroscopic techniques and molecular modeling. Food Chem Toxicol. 2014;71:244–53. https://doi.org/10.1016/j.fct.2014.06.022.
7. Sager G, Little C. The effect of the plasticizers TBEP (tris-(2-butoxyethyl)-phosphate) and DEHP (di-(2-ethylhexyl)-phthalate) on beta-adrenergic ligand binding to alpha 1-acid glycoprotein and mononuclear leukocytes. Biochem Pharmacol. 1989;28(15):2551–7.
8. Leetz A. Frace first EU country to ban the use of DEHP in paediatrics, neonatology maternity wards in Hospitals Health Care Without Harm Europe. 2012; Dec 13.
9. Griffiths WC, Camara PD, Sarietlli A, Gentile J. The in vitro serum protein-binding characteristics of bis-(2-ethylhexyl) phthalate and its principal metabolite, mono-(2-ethylhexyl) phthalate. Environ Health Perspect. 1988;77:151–6.
10. Banker M, Clark T, Williams J. Development and validation of 96-well equilibrium dialysis apparatus for measuring plasma protein binding. J Pharm Sci. 2003;92(5):967–74.
11. Zamek-Gliszczynski M, Ruterbories J, Ajamie R, Wikremsinhe E, Pothuri L, Rao MVS, et al. Validation of 96-well equilibrium dialysis with non-radiolabeled drug for definitive measurement of protein binding and application to clinical development of highly-bound drugs. J Pharm Sci. 2011;100(6):2498–507.
12. D. Feigal. PVC devices containing the Plasticzier DEHP. In: FDA Public Health Notification. U.S. Food and Drug Administration. 2002. http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm062182.htm. Accessed 25 Aug 2016.
13. Shah D, Burgess J. A simple, fast, and reliable LC-MS/MS method for determination and quantification of phthalates in distilled beverages. In: Application Notes. Waters Corporation. 2015. http://www.waters.com/webassets/cms/library/docs/720005403en.pdf. Accessed 29 Apr 2016.
14. Dine T, Luyckx M, Cazin M, Brunet C, Cazin JC, Goudaliez F. Rapid determination by high performance liquid chromatography of di-2-ethylhexyl phthalate in plasma stored in plastic bags. Biomed Chromatogr. 1991;5:94–7.
15. Bista SR, Haywood A, Hardy J, Lobb M, Tapuni A, Norris R. Protein binding of fentanyl and its metabolite nor-fentanyl in human plasma, albumin and α-1 acid glycoprotein. Xenobiotica. 2015;45(3):207–12.
16. Di L, Kerns EH. Application of pharmaceutical profiling assays for optimization of drug-like properites. Curr Opin Drug Discov Dev. 2005;8(4):495–504.
17. Di L, Kerns EH, Carter GT. Drug-like property concepts in pharmaceutical design. Curr Pharm Des. 2009;15(19):2184–94.
18. McGinnity DF, Collington J, Austin RP, Riley RJ. Evaluation of human pharmacokinetics, therapeutic dose and exposure predictions using marketed oral drugs. Curr Drug Metab. 2007;8(5):463–79.
19. Obach RS, Walsky R, Venkatakrishnan K, Gaman E, Houstan JB, Termain L. The utility of in vitro cytochrome P450 inhibiation data in the prediction of drug-drug interactions. JPET. 2006;316(1):336–48.
20. Harmer AR, Valentin J-P, Pollard CE. On the relationship between block of the cardiac Na^+ channel and drug-induced prolongation of the QRS complex. Br J Pharmacol. 2011;164:260–73.
21. Jones H, Gardner I, Collard W, Stanley P, Oxley P, Hosea N, et al. Simulation of human intravenous and oral pharmacokinetics of 21 diverse compounds using physiologically based pharmacokinetic modelling. Clin Pharmacokinet. 2001;50(5):331–47.
22. Sinha VK, Snoeys J, Osselaer NV, Peer AV, Mackie C, Heald D. From preclinical to human—prediction of oral absorption and drug-drug interaction potential using physiologically based pharmacokinetic (PBPK) modeling approach in an industrial setting: a workflow by using case example. Biopharm Drug Dispos. 2012;33(2):111–21. https://doi.org/10.1002/bdd.1782.
23. Drugs@FDA: FDA Approved Drug Products [Internet]. Silver Spring: U.S. Food and Drug Administration; c1996. Xanax; (revised 2011 Mar; cited 2018 Apr 16; 44 p.). Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/018276s044,021434s006lbl.pdf.