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Influence of storage vial material on measurement of organophosphate flame retardant metabolites in urine

Courtney C. Carignan[1,*], **Craig M. Butt**[2], **Heather M. Stapleton**[2], **John D. Meeker**[3], **Lidia Minguez-Alarcón**[1], **Paige L. Williams**[4], and **Russ Hauser**[1,5,6]

[1]Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[2]Nicholas School of the Environment, Duke University, Durham, North Carolina, USA

[3]Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, Michigan, USA

[4]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[5]Department of Obstetrics and Gynecology, Harvard Medical School/Massachusetts General Hospital Fertility Center, Boston, Massachusetts, USA

[6]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

Abstract

Use of organophosphate flame retardants (PFRs) has increased over the past decade with the phase out of polybrominated diphenyl ethers. Urinary metabolites of PFRs are used as biomarkers of exposure in epidemiologic research, which typically uses samples collected and stored in polypropylene plastic cryovials. However, a small study suggested that the storage vial material may influence reported concentrations. Therefore, we aimed to examine the influence of the storage vial material on analytical measurement of PFR urinary metabolites. Using urine samples collected from participants in the Environment and Reproductive Health (EARTH) Study, we analyzed the PFR metabolites in duplicate aliquots that were stored in glass and plastic vials (n=31 pairs). Bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), diphenyl phosphate (DPHP) and isopropyl-phenyl phenyl phosphate (ip-PPP) were detected in 97%, 97% and 78% of duplicates. We observed high correlations between glass-plastic duplicates for BDCIPP (r_s=0.95), DPHP (r_s =0.79) and ip-PPP (r_s =0.82) (p<0.0001). Urinary ip-PPP was an average of 0.04 ng/ml (p=0.04) higher among samples stored in glass, with a mean relative difference of 14%. While this difference is statistically significant, it is small in magnitude. No differences were observed for

* carignan@hsph.harvard.edu; Current address: Harvard T.H. Chan School of Public Health, 665 Huntington Ave, Building 1, 14th Floor, Boston, Massachusetts 02115 USA.

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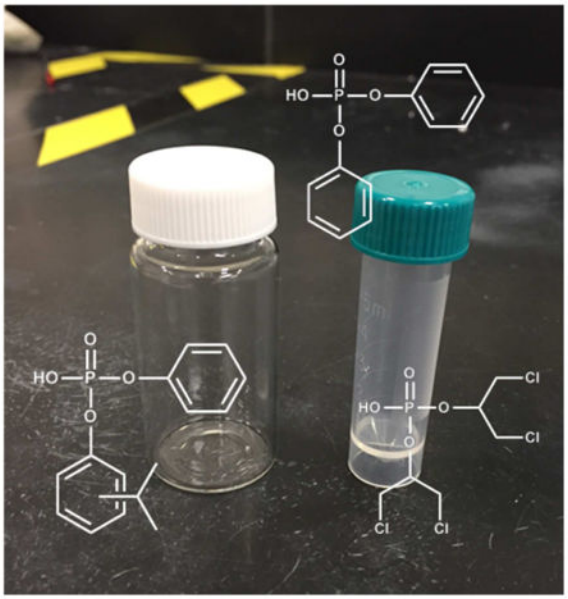
BDCIPP or DPHP, however future research should seek to reduce the potential for type II error (false negatives). We conclude that storing urine samples in polypropylene plastic cryovials may result in slightly reduced concentrations of urinary ip-PPP relative to storage in glass vials and future research should seek to increase the sample size, reduce background variability and consider the material of the urine collection cup.

Graphical Abstract



Keywords

urinary biomarkers; organophosphate flame retardants; human; sample storage; quality control

Introduction

Organophosphate flame retardants (PFRs) have been used in the polyurethane foam of upholstered furniture [1] with increasing prevalence over the past decade following the phase out polybrominated diphenyl ethers (PBDEs) [2]. Flame retardants used in polyurethane foam are additives that are not chemically bound, and therefore migrate into the air and dust of indoor environments [3] and lead to human exposure. Two commonly used PFRs are triphenyl phosphate (TPHP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP), which after intake are excreted within hours primarily as the urinary metabolites diphenyl phosphate (DPHP) and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), respectively [4–6]. TPHP is often used in the Firemaster® 550 mixture in combination with two brominated flame retardants as well as various mono-substituted triphenyl phosphate isomers (Figure 1). In addition to being used as a flame retardant, TPHP is also used as a plasticizer and is found in nail polish, hydraulic fluids and polyvinyl chloride (PVC) [7, 3]. Additionally, the primary metabolite of TPHP, diphenyl phosphate (DPHP), is also sold and used as a plasticizer, although production volumes of DPHP are significantly lower than

TPHP. TCIPP in contrast, is primarily used as a flame retardant, primarily in rigid polyurethane foam for insulation and construction (80% of use) as well in flexible polyurethane foam (e.g., furniture cushions) [8].

While population-wide data are limited, studies suggest that exposure to these PFRs is likely widespread in the U.S. as DPHP and BDCIPP have been detected in over 90% of adult urine samples [9–12]. This is of concern because TDCIPP and TPHP are suspected endocrine disrupting chemicals that have been shown to disrupt thyroid hormone and estrogen signaling as well as to reduce reproductive performance in zebrafish and chickens [13–15]. TPHP is a suspected obesogen that can initiate adipocyte differentiation and antagonize osteogenesis [16, 17]. TCIPP is considered a carcinogen under Proposition 65 regulated by the State of California [18] and is a potential developmental neurotoxicant [19]. TCIPP can also disrupt the endocrine system, with in vitro evidence of antiandrogenic and antiestrogenic activity [20]. In vivo studies report morphological changes in the thyroid and adverse effects on reproduction including changes to the estrous cycle, increased uterine weights, low birth weight, and delayed hatching [21, 22, 13, 23]. Little is known regarding toxicity of the mono-substituted triphenyl phosphate isomers. Few epidemiologic studies have investigated PFRs, however an exploratory analysis of 33 men found that urinary BDCIPP and DPHP were associated with reductions in sperm motility and increased total T_3 [24].

Epidemiologic studies investigating PFRs are needed and may utilize urinary metabolites as biomarkers of exposure. Typically, urine samples are collected in plastic (polypropylene) specimen cups and frozen in plastic cryovials. However, preliminary results have suggested that PFR metabolites may adhere to these collection and storage containers [25]. Therefore, our objective was to determine whether the material of the storage vial material biases analytical determination of PFR urinary metabolites using a subset of aliquots from a U.S. preconception cohort that were stored both in glass and plastic vials.

Materials and Methods

Participants

Study participants were women recruited into the Environment and Reproductive Health (EARTH) study between November 2005 and October 2015 from patients undergoing assisted reproductive technologies at the Massachusetts General Hospital Fertility Center. Female participants must be between the ages of 18 and 46 to enroll in the study. The EARTH study was approved by the Human Studies Institutional Review Boards of Massachusetts General Hospital and Harvard T.H. Chan School of Public Health. Participants signed an informed consent after the study procedures were explained by a research nurse and any questions were answered.

Urine Samples

Urine was collected in a sterile polypropylene cup and specific gravity (SG) was measured using a handheld refractometer (National Instrument Company, Inc.). Each sample was divided into aliquots (2.5 to 5 mL) and stored at −80°C. We randomly selected duplicate

samples collected between 2008–2009 that were stored in glass vials (Shorty Vials®, Borosilicate Glass, PTFE lined Screw Cap, Wheaton) and plastic cryovials (Nalgene® Cryogenic Vials, Polypropylene, Sterile, External Thread with Screw Cap, Thermo Scientific) (glass-plastic duplicates). While the main objective of our analysis was to compare analytical results from duplicates stored in glass and plastic storage vials, we also evaluated analytical variability from duplicates stored only in plastic storage vials. To do so, we selected duplicate samples collected between 2005–2015 that were stored in plastic cryovials (plastic-plastic duplicates). The glass-plastic (n=31 pairs) and plastic-plastic (n=30 pairs) duplicates were shipped on dry ice overnight to Duke University (Durham, NC, USA) for quantification of urinary metabolites used as biomarkers of exposure to PFRs.

Extraction and Instrumental Analysis

Extraction and analysis methods for BCIPP, BDCIPP, DPHP, ip-PPP and tb-PPP followed methods previously developed by our laboratory [9]. Briefly, urine samples were thawed and a 2.5 to 5 ml aliquot was transferred to a clean glass test tube and spiked with mass-labeled internal standards (d_{10}-BDCIPP = 80 ng, d_{10}-DPHP = 60 ng). After acidifying to pH <6.5 with formic acid, samples were diluted 1:1 with water and, concentrated and cleaned using solid-phase extraction techniques (SPE). The SPE eluent was blown to dryness under a gentle nitrogen stream, reconstituted in 500 µl of 1:1 H_2O:MeOH and spiked with the recovery standard ($^{13}C_2$-DPHP = 81.5 ng). Extracts were analyzed by negative electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described [9]. Chromatography was achieved under gradient conditions using a Luna C18(2) column (50 x 2.0 mm, 2.5 µm particle size, Phenomenex, Torrance, CA) preceded by a SecurityGuard Polar-RP (4 x 2.0 mm) guard cartridge. The mobile phases were methanol and water (modified with 0.8 mM ammonium acetate), flow rate was 300 µl/min, the injection volume was 5 µl and the column oven was 45°C. Data were acquired under multiple reaction monitoring conditions using optimized parameters. Analyte responses were normalized to internal standard responses. BCIPP and BDCIPP were normalized using d_{10}-BDCIPP, while DPHP, ip-PPP and tb-PPP were normalized using d_{10}-DPHP. Urinary specific gravity ranged from 1.002 to 1.028 with a mean of 1.016.

Quality assurance/quality control

In the urine samples, the mean recovery of the mass-labeled standards was 103% (standard error = 1.7%) for d_{10}-DPHP and 146% (5.7%) for d_{10}-BDCIPP. The apparent high recovery for d_{10}-BDCIPP was presumably due to matrix effects in the urine samples since the blanks (clean water only) showed d_{10}-BDCIPP mean recovery of 112%. This issue has been observed in our previous studies [9] and is related to the fact that $^{13}C_2$-DPHP was used as the recovery standard. One laboratory blank (5 ml Milli-Q water only) sample was extracted with every batch (n=5). Two of the individual sub-samples were analyzed in duplicate to assess method precision and were generally within 10% with the exception of BDCIPP which was within 30%. Very low levels of DPHP (mean = 0.71 ng) and ip-PPP (mean = 0.28 ng) were routinely detected in the laboratory blanks and analyte values were blank corrected using the mean laboratory blank values. Method detection limits (MDLs) were calculated as three times the standard deviation of laboratory blanks normalized to the volume of water

extracted (5 ml). MDLs were 68 pg/ml for BCIPP, 56 pg/ml for BDCIPP, 183 pg/ml for DPHP, 59 pg/ml for ip-PPP, 154 pg/ml for tb-PPP, respectively.

Data Analysis

Results below the method detection limit (MDL) were imputed using MDL/v2. Because aliquots from the same sample would have the same specific gravity, all statistical analyses were performed on urinary metabolite data that was not normalized to urinary specific gravity. As urinary metabolite concentrations were log-normally distributed, geometric mean (GM) urinary metabolite concentrations were calculated for each duplicate pair. Spearman correlation coefficients between duplicates were calculated separately for glass-plastic and plastic-plastic duplicates, and agreement was presented graphically via scatterplots. Agreement between duplicate samples was assessed using Bland-Altman plots. Pairwise differences were calculated as glass minus plastic. As pairwise differences appeared to follow an approximate normal distribution, a one-sample t-test was conducted to test whether the mean difference was zero. Detection frequencies between glass and plastic duplicates were compared using Fisher's exact test. To compare urinary metabolite concentrations to those measured in other populations, we accounted for urinary dilution by normalizing to urinary specific gravity (SG) using the approach described by Pearson et al. [26]: $C_{SG}=(SG_m-1)/(SG_i-1)$ where C_{SG}=SG normalized urinary metabolite concentration, SG_m=population mean SG and SG_i= SG for an individual sample. Correlations and Bland-Altman plots were visualized using JMP® Pro (version 12.0.1, SAS Institute Inc., Carey, NC). Relative difference was calculated as the concentration of urinary metabolite measured in the sample stored in a plastic vial divided by the concentration measured in the sample stored in a glass vial, minus 1 and multiplied by 100. Statistical analyses were performed using SAS® (version 9.3; SAS Institute Inc., Cary, NC) with statistical significance defined as $\alpha = 0.05$.

Results and Discussion

Among all samples, detection frequencies were high for BDCIPP (98%), DPHP (97%) and ip-PPP (87%) but low for BCIPP (0%) and tb-PPP (13%) (Table 1).

Similar detection frequencies and GM concentrations were observed between subsamples of glass-plastic and plastic-plastic duplicate pairs (Supplemental Table S2).

BDCIPP, DPHP and ip-PPP were detected among all of the 31 (100%) glass aliquots but only 29 (94%) of the plastic duplicates; this difference was not statistically significant (p=0.49, Fisher's exact test). Detection frequencies and GM concentrations were similar to other adult populations in the U.S. and Norway [9, 10, 27, 28] and lower than measured in pooled samples from an Australian study [29] (Supplemental Table S2).

For BDCIPP and ip-PPP, duplicate samples were highly correlated for both the glass-plastic ($r_s > 0.8$) and plastic-plastic ($r_s > 0.9$) duplicates (Figure 2). For DPHP, the plastic-plastic duplicates were more highly correlated ($r_s > 0.97$) than the glass-plastic duplicates ($r_s > 0.79$). Pearson correlations using log transformed data were similar.

We used a one-sample t-test to determine whether the difference between glass-plastic duplicates differed from zero and found that urinary ip-PPP was an average of 0.04 ng/ml (p=0.04) higher in glass duplicates, with a mean relative difference of 14% (Figure 3). There was no significant difference for BDCIPP (−0.02 ng/ml, p=0.80) or DPHP (−0.02 ng/ml, p=0.80) between glass plastic duplicates. Sample volume, the length of storage time, and urine SG were not associated with absolute or relative differences between duplicate samples (data not shown).

This analysis suggests that storage of urine samples in plastic cryovials as compared to glass vials may result in a systematic bias of reduced ip-PPP. The finding of lower measured concentrations of ip-PPP among urine samples stored in plastic cryovials is consistent with our hypothesis that the concentrations in aliquots stored in plastic cryovials would be lower than duplicates stored in glass, which was based on a very limited study that reported lower concentrations of urinary DPHP and BDCIPP in 2 out of 3 samples collected and stored both in glass and polypropylene containers [25]. Note that while the observed difference is statistically significant, it is very small in magnitude.

We hypothesize two possible explanations for the absence of a difference between glass-plastic aliquots for BDCIPP and DPHP. First, this may be related to the higher hydrophobicity (Log Kow) of ip-PPP (4.79) compared to BDCIPP (2.18) and DPHP (2.88), which could lead to increased absorption to polypropylene [30]. Second, this may be related to potential type II error (false negative) for BDCIPP (27%) and DPHP (56%) due to the higher variability in the difference between duplicates for these PFRs. Some of this variability may be from analytical noise or from sorption to the polypropylene specimen cup during urine sample collection. While collection using a polypropylene specimen cup is common for collection of biological samples, including in our study, samples used by Cooper et al. [25] were collected using glass. Therefore, future work should seek to control for potential differences introduced by the material of the specimen collection cup as well as other variables that may influence the degree of sorption such as sample volume, urine properties, storage time and storage temperature.

Conclusion

We conclude that storing urine samples in polypropylene plastic cryovials may result in reduced concentrations of urinary ip-PPP relative to storage in glass vials, although this difference is small compared to other sources of variability. Future research should seek to increase the sample size, reduce background variability and consider the material of the urine collection cup.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BDCIPP	Bis(1,3-dichloro-2-propyl) phosphate
BCIPP	bis(1-chloro-2-propyl) phosphate
DPHP	diphenyl phosphate
EARTH	Environment and Reproductive Health
GM	Geometric mean
ip-PPP	isopropyl-phenyl phenyl phosphate
MDL	Method detection limit
PFRs	Organophosphate flame retardants
SG	Specific gravity
tb-PPP	tert-butyl-phenyl phenyl phosphate

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Highlights

- We measured PFR metabolites in duplicate urine samples stored in glass and plastic vials
- Concentrations were highly correlated between duplicates
- ip-PPP was slightly but significantly higher using glass compared to plastic vials for storage
- DPHP and BDCIPP showed no storage difference between the glass and plastic vials

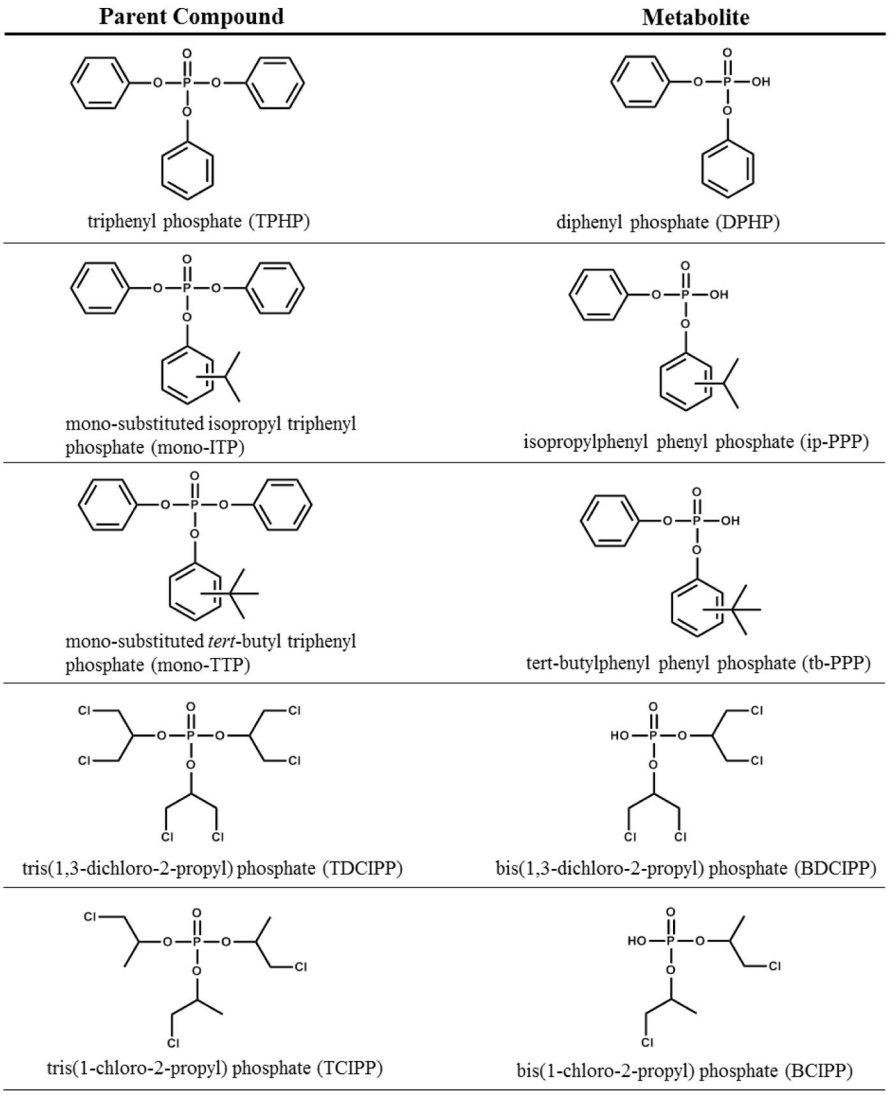

Figure 1.
Organophosphate flame retardant parent compound and primary urinary metabolite.

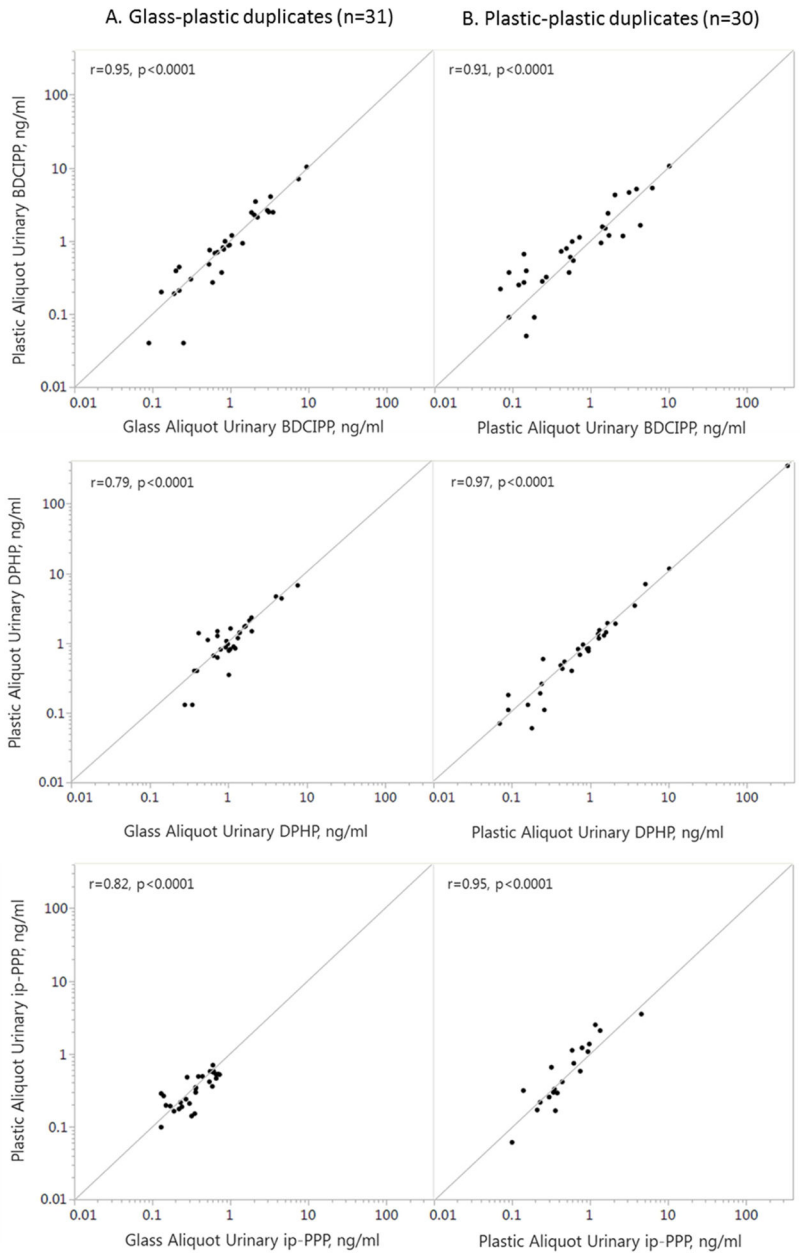

Figure 2.

Comparison between duplicate glass-plastic (n=31) and duplicate plastic-plastic (n=30) aliquots with infinity line and Spearman correlation coefficients (r).

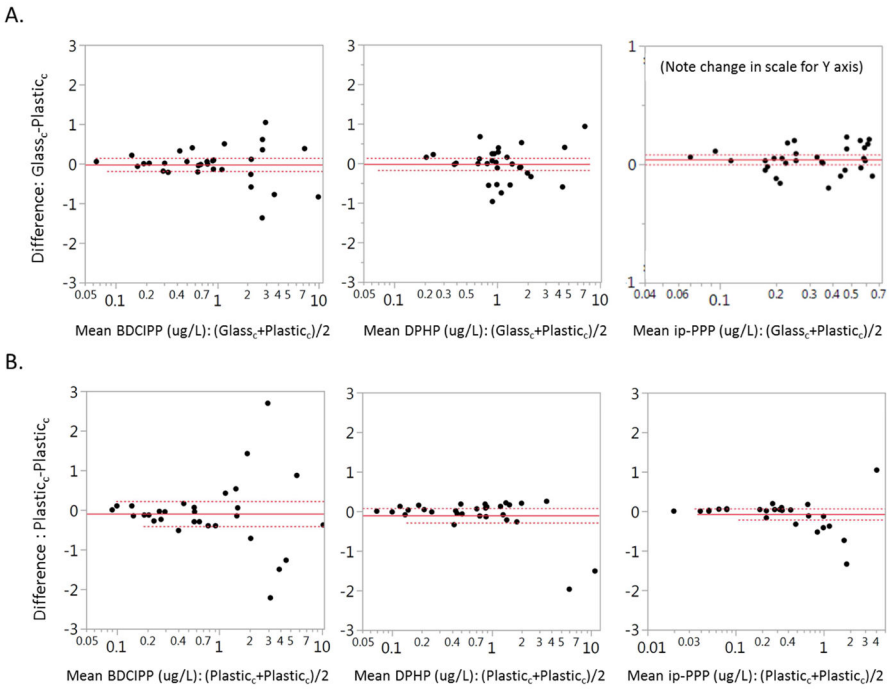

Figure 3.

Bland-Altman plot of relative difference between duplicate aliquots: A) Glass-plastic, B) Plastic-plastic. Solid line indicates the average for all samples and dotted lines delineate the 1.96 standard deviation.

Table 1

Distribution of urinary metabolites (ng/ml) from all 61 duplicate pairs.

	N > MDL (%)[a]	GM (95% CI)	Minimum	25th Pctl	50th Pctl	75th Pctl	Maximum
BCIPP	0 (0)	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
BDCIPP	60 (98)	0.76 (0.55, 1.05)	<MDL	0.30	0.76	2.14	10.38
DPHP	59 (97)	0.92 (0.66, 1.28)	<MDL	0.48	0.90	1.41	340.36
ip-PPP	53 (87)	0.26 (0.20, 0.35)	<MDL	0.18	0.28	0.56	4.00
tb-PPP	8 (13)	<MDL	<MDL	<MDL	<MDL	<MDL	0.47

[a]Each pair of duplicates was considered >MDL when one or more duplicate was >MDL.

Summary statistics calculated using geometric mean concentrations from each pair of duplicates.