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Effect of storage time and temperature on levels of phthalate metabolites and bisphenol A in urine

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Abstract. Urine is a widely used matrix in biomonitoring studies on the assessment of human exposure to environmental chemicals such as phthalate esters and bisphenol A (BPA). In addition to the need to apply valid analytical techniques, assurance of specimen integrity during collection and storage is an important prerequisite for the presentation of accurate and precise analytical data. One of the common issues encountered in the analysis of non-persistent contaminants is whether shipping and storage temperature and time since collection have an effect on sample integrity. In this study, we investigated the stability of phthalate metabolites and BPA in spiked and unspiked urine samples stored at room temperature (20°C) or at -80°C for up to 8 weeks. Concentrations of phthalate metabolites declined, on average, by 3% to 15%, depending on the compounds, and BPA declined by ~30% after 4 weeks of storage of spiked urine samples at 20°C. In a test of 30 unspiked urine samples stored at 20°C and at -80°C for 8 weeks, the concentrations of phthalate metabolites and BPA decreased by up to 15% to 44%, depending on the compound and on the samples. It was found that the small reduction in phthalate concentrations observed in urine, varied depending on the samples. In a few urine samples, concentrations of phthalate metabolites and BPA did not decline even after storage at 20°C for 8 weeks. We found a significant relationship between concentrations of target analytes in urine stored at 20°C and at -80°C for 8 weeks. We estimated the half-lives of phthalate metabolites and BPA in urine stored at 20°C. The estimated half-life of monoethyl phthalate (mEP) and mono (2-ethyl-5-carboxyphentyl) phthalate (mECPP) in urine stored at 20°C was over two years, of mono (2-ethyl-5-oxohexyl) phthalate (mEOHP) and monobenzyl phthalate (mBzP) was approximately one year, and of other phthalate metabolites was approximately 6 months. The estimated half-life of BPA in urine stored at 20°C was approximately 3 months, which is much longer than that reported for aquatic ecosystems.

Keywords: storage; urine; biomonitoring; phthalate; BPA; half-life

1. Introduction

Urine is a widely used matrix in biomonitoring studies for the assessment of human exposure to non-persistent organic pollutants such as phthalates (Itoh *et al*. 2005, Silva *et al*. 2004) and

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bisphenol A (BPA) (Ye *et al*. 2008, Zhang *et al*. 2011). Phthalates and BPA have short half-lives in the human body and are excreted into urine, as free and conjugated forms (Koch *et al*. 2004, Koch *et al*. 2005, Koch *et al*. 2003), on the order of a few hours (Koch *et al*. 2004, Lorber *et al*. 2010). Measurement of environmental chemicals in urine can be used in the assessment of daily exposure doses of chemicals and in epidemiological studies that concern the associations between environmental chemical exposures and health outcomes in people (Weuve *et al*. 2010, Wolff *et al*. 2007). The outcome of biomonitoring studies and data quality depends greatly on the representativeness and integrity of samples as well as on analytical and data interpretation methods. Choices made with regard to collection, shipment, and storage of urine samples can influence the validity and reliability of analytical data.

After sampling (in locations such as hospitals or homes), urine samples are generally shipped and then stored in a laboratory until analysis. To prevent microbial activity and degradation of contaminants, urine samples are usually stored at temperatures below 0°C in laboratories. However, during sampling and shipment, cold storage facilities may not be available. Further, unexpected delays in the delivery of shipment due to unforeseen circumstances or accidents (e.g., power failure due to natural calamities) in laboratory storage facilities can compromise sample integrity. Therefore, an understanding of the effect of temperature and storage time on the reliability and reproducibility of analytical results is essential in the determination of data quality assurance.

An earlier study showed that total phthalate metabolite concentrations were stable in urine stored at -70°C (Silva *et al*. 2008) for over a year; nevertheless, concentrations of phthalate metabolites in urine decreased following storage at 4°C and at 25°C for several months, while glucuronide conjugates of phthalates decreased following storage at 4°C and 25°C for several days (Samandar *et al*. 2009). Similarly, total concentrations of BPA were relatively stable for 30 days in urine samples stored at room temperature (Ye *et al*. 2007). Another study indicated that storage of urine at room temperature for 3 days did not affect concentrations of BPA and phthalate metabolites (Hoppin *et al*. 2006).

The earlier studies on the stability of BPA and phthalate metabolites in urine stored at different conditions were based on samples that were spiked with target contaminants and/or stored at room temperature for a short period of time. In the present study, two sets of experiments were conducted to evaluate the effect of storage temperature and time on the urinary concentrations of phthalate metabolites and BPA. The first set of experiments involved urine samples, collected from 10 volunteers that were spiked with native phthalates and BPA and stored at 20°C for up to 4 weeks. The second set of experiment involved unspiked urine samples (n = 30), collected and stored at 20°C and/or at -80°C for 8 weeks, for the assessment of temporal stability in the concentrations of the target chemicals.

2. Materials and methods

2.1 Samples

Two sets of urine samples were collected. The first set contained 10 samples, provided by 5 male and 5 female volunteers (ranging in age from 5 to 47 years) in March 2012. The samples were kept in 15 mL glass tubes and 15 mL polypropylene (PP) tubes for phthalate metabolites and BPA analysis, respectively. The urine samples were fortified with 25 ng/mL native phthalate metabolites and 20 ng/mL BPA and stored at room temperature (20°C) for 4 weeks. An aliquot (0.5

mL) was drawn from the tubes at weekly intervals and analyzed for BPA and phthalate metabolites. The initial concentrations of BPA and phthalate metabolites were determined immediately after collection and fortification (time 0). The second set of samples encompassed 30 paired urine samples from anonymous volunteers that were taken in 2 mL PP tubes; one group of PP tubes was stored at -80°C, and another group was stored at 20°C for 8 weeks. At the end of 8 weeks, both groups were analyzed for BPA and phthalate metabolites. Institutional Review Board approvals were obtained from the New York State Department of Health (NYSDOH) for the analysis of urine samples.

2.2 Analytical methods

Eight phthalate metabolites that are commonly found in human urine (Silva *et al*. 2006, Wolff *et al*. 2007) were analyzed. The analytical method has been described in detail elsewhere (Guo *et al*. 2011). Briefly, 0.5 mL of urine samples were enzymatically deconjugated overnight and extracted by solid phase extraction (SPE). Eight phthalate metabolites, monomethyl phthalate (mMP), monoethyl phthalate (mEP), mono(2-isobutyl) phthalate (miBP), mono-n-butyl phthalate (mBP), mono(2-ethyl-5-carboxyphentyl) phthalate (mECPP), mono(2-ethyl-5-oxohexyl) phthalate (mEOHP), monobenzyl phthalate (mBzP), and mono(2-ethylhexyl) phthalate (mEHP), were analyzed. Seven isotopically-labeled phthalate metabolites ($^{13}C_4$-mMP, $^{13}C_4$-mEP, $^{13}C_4$-mBP, $^{13}C_4$-mECPP, $^{13}C_4$-mEHP, $^{13}C_4$-mBzP, and d_4-miBP) were used as internal standards. mBP, mMP, mEP, mBzP, and mEHP (purity > 99%) were purchased from AccuStandard (New Haven, CT, USA), and the other phthalates were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Isotopically-labeled phthalate metabolites (purity > 99%, $^{13}C_4$-mMP, $^{13}C_4$-mEP, $^{13}C_4$-mBP, $^{13}C_4$-mECPP, $^{13}C_4$-mBzP, and $^{13}C_4$-mEHP) and D_4-miBP were purchased from Cambridge Isotope Laboratories and CDN Isotopes (Quebec, Canada), respectively.

The method for the analysis of total BPA has been described in detail previously (Zhang *et al*. 2011). Briefly, 0.5 mL of urine was spiked with ^{13}C-labeled BPA as an internal standard, enzymatically deconjugated overnight, and extracted by SPE. The native and ^{13}C-labeled BPA (purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Cambridge Isotope Laboratories, respectively.

An API 2000 electrospray triple quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA), equipped with an Agilent 1100 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA), was used for the determination of phthalate metabolites and BPA. Chromatographic separation was achieved using a Betasil C18 column (Thermo Electron, Bellefonte, PA, USA; 100 mm × 2.1 mm, 5 μm). Target compounds were determined by multiple reaction monitoring (MRM) in the negative ionization mode. The limits of quantification (LOQ) of mMP/mEHP and other phthalate metabolites were 0.5 ng/mL and 0.1 ng/mL, respectively; LOQ of BPA was 0.1 ng/mL. The concentrations of target analytes at time 0 (from the sample that was analyzed immediately after collection) were used as the reference value. To assess the relative impact of time and temperature, we estimated percent change from time 0.

2.3 Quality assurance/quality control

For every batch of samples, three method blanks, a spiked blank, and a pair of matrix spiked sample/duplicates were processed. Low concentrations of mBP, miBP, and mEHP were detected in method blanks, at mean concentrations of 0.76, 0.39, and 6.9 ng/mL, respectively. Concentrations

in samples were subtracted from blank values for these compounds. BPA was not detected in method blanks. The recoveries of internal standards of phthalate metabolites spiked in samples and in method blanks were 49% to 73% and 74% to 93%, respectively. The recoveries of ^{13}C-BPA spiked in samples ranged from 65% to 82%, and the mean recovery of ^{13}C-BPA spiked in blanks was 92%. Concentrations below the LOQ were assigned a value of zero for data analysis. Data were not creatinine adjusted, as this was deemed unnecessary for the proposed goals of this study. Data analysis was conducted using SPSS, Version 17.0. Comparison among different sample types was conducted using non-parametric tests (2-related samples, Wilcoxon test). Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1 Stability of BPA and phthalates in spiked urine samples for 4 weeks at 20°C

Native standards of phthalate metabolites and BPA were fortified at 25 and 20 ng/mL, respectively, into 10 urine samples. The average concentrations of phthalate metabolites and BPA in urine samples at time 0 and at weekly intervals following storage at 20°C for 4 weeks are shown in Fig. 1. Among individual samples, residue concentrations of phthalate metabolites in urine declined by 3% (mEP, average) to 15% (mBzP) during the 4-week period, except for mEHP, which increased slightly (27% at the end of 4-week). Residue concentrations of total BPA declined, on average, 30% during the 4-week period. The reduction in total BPA concentration was pronounced during the first week, and then no further reduction was observed. The coefficient of

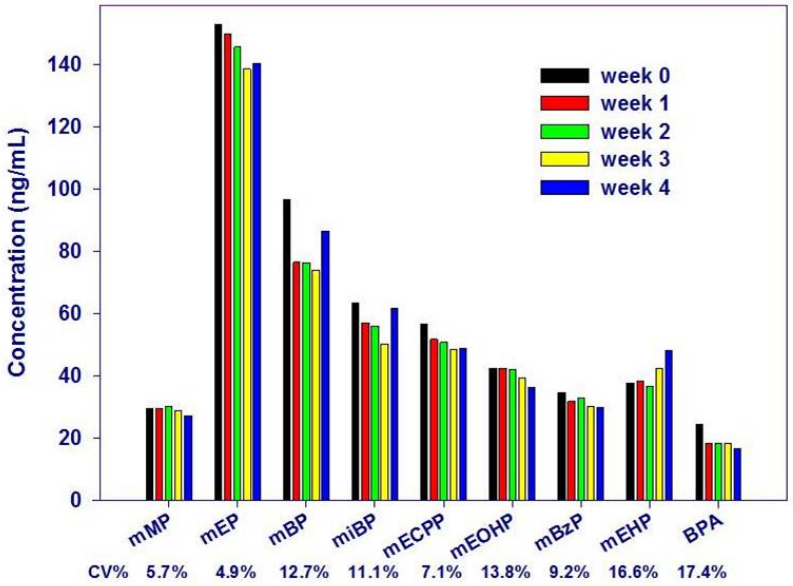

Fig. 1 Mean (ng/mL; n=10) and coefficient of variation (CV %) in concentrations of phthalate metabolites and BPA in 'spiked' urine samples stored at room temperature (20°C) for 4 weeks

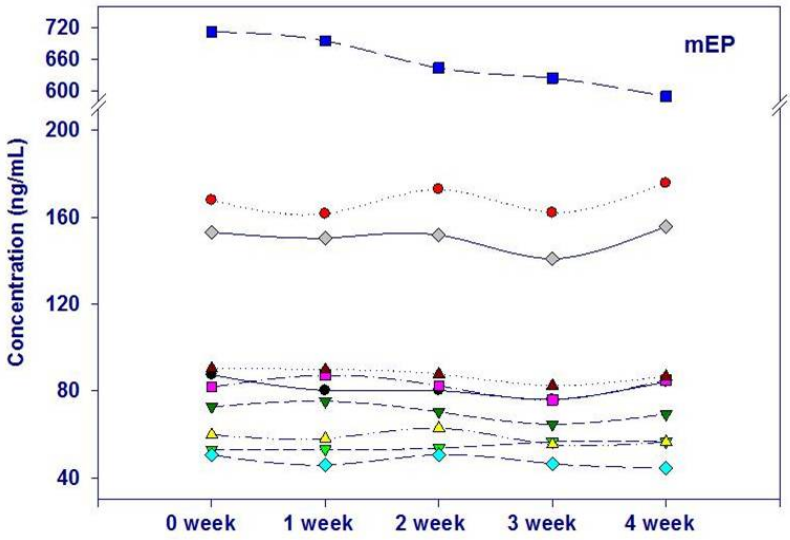

Fig. 2 Concentrations of mono-ethyl phthalate (mEP; ng/mL) in spiked urine samples (n = 10) stored at room temperature (20°C) for 4 weeks and analyzed at weekly intervals

variation (CV in %) in concentrations was calculated as the ratio of the standard deviation of the average concentrations to the average concentrations measured during the 4-week period. The average CV for urinary phthalate metabolite concentrations ranged from 4.9% (for mEP) to 16.6% (for mEHP) and 17.4% for BPA (Fig. 1). The CV of individual phthalate metabolites and BPA in urine samples ranged from 1% to 43%, and 5% to 32%, respectively. For example, the CV of concentrations of mEP determined at 5 different time intervals was less than 8% during the 4-week period for all samples (Fig. 2). Thus, irrespective of the concentrations found in samples, the observed decline in concentrations of phthalate metabolites and total BPA was in the range of analytical uncertainty for these compounds (approximately 20% to 30%). The results indicate that the total concentrations of phthalates and BPA decreased by up to 30% when urine samples were stored at 20°C for 4 weeks. Although this reduction appears to be gradual, it varied, depending on the compound. mEHP increased with time, while several phthalate metabolites decreased from week 1, while a slight increase in concentrations was found at the end of the study period (4 weeks). BPA concentrations decreased in a week, but no further reduction was observed after week 1. The slight increase of mEHP in samples stored at 20°C may partly be explained by the high background level of its parent compound, diethylhexyl phthalate (DEHP), in the laboratory environment (Guo and Kannan 2012). In addition to potential degradation, evaporation loss from the tubes cannot be ruled out.

3.2 Stability of phthalate metabolites and BPA in unspiked urine stored for 8 weeks

A set of 30 paired urine samples was stored at 20°C and at -80°C for 8 weeks. Except for the low detection frequency of mMP (< 15%), the other six phthalate metabolites and BPA were found in all samples (Table 1). Generally, concentrations of phthalate metabolites in these samples were similar to those reported in the U.S. National Health and Nutrition Examination Survey (NHANES) of 1999-2000; the mean concentrations of mEP, mBP, and mBzP reported in the NHANES survey

were 179, 24.6, and 15.3 ng/mL, respectively (Silva *et al*. 2004). As shown in Table 1, among the 30 paired samples, concentrations of some compounds remained stable in 9% to 39% of the samples. This suggests that the degradation or reduction of target analytes in urine depends on the sample. The concentrations of phthalate metabolites and BPA in other urine samples decreased (15% to 44% depending on samples) following storage at 20°C for 8 weeks. For instance, the mean concentration of mECPP in 30 urine samples was 213 ng/mL. After storage at 20°C for 8 weeks, the mean mECPP concentration was189 ng/mL. The concentrations of mECPP decreased from 0.7% to 72% (among individual samples), with a mean of 21% for all samples (Table 1). Concentrations of several phthalate metabolites and BPA were significantly higher in samples stored at -80°C for 8 weeks than for those stored at 20°C ($p < 0.05$), except for mMP and mEOHP ($p > 0.05$). Nevertheless, a significant linear relationship in concentrations of target analytes was found between the samples that were stored at 20°C and at -80°C, with correlation coefficients ranging from 0.88 to 0.99 (Table 2). The correlation between the concentrations of mEP in 30 urine samples stored at 20°C and at -80°C is shown (Fig. 3). Theoretically, the slopes of regression lines should be one (unity) if there were no changes in the concentrations of target analytes with storage temperature. However, the slopes declined to 0.7 to 0.96, indicating loss of target analytes

Table 1 Average concentrations of phthalate metabolites and bisphenol A in urine (n=30) stored at -80°C and 20°C for 8 weeks (unspiked urine), percentage of samples that showed no reduction in concentrations, and average percentage reduction from baseline concentrations (for those samples that showed reduction)

Analyte	Concentration (mean, ng/mL)		% sample with no reduction	% reduction
	-80°C	20°C		
mMP	0.93	0.85	63	-44
mEP	189	179	12	-15
mBP	23.4	18.7	18	-35
miBP	9.67	7.77	21	-31
mECPP	213	189	12	-21
mEOHP	58.5	51.4	39	-31
mBzP	16.2	15.2	9	-33
mEHP	23.5	17.1	33	-35
BPA	1.0	0.76	12	-40

Table 2 Relationship between concentrations and trends of phthalate metabolites and BPA in urine samples (n=30; unspiked) stored at 20°C and -80°C for 8 weeks

Compound	R^2	Slope	*P* value (2-related)	Trend
mMP	0.774	0.96	0.782	Stable
mEP	0.987	0.89	< 0.000	Decreased
mBP	0.908	0.88	< 0.000	Decreased
miBP	0.946	0.70	< 0.000	Decreased
mECPP	0.995	0.91	< 0.000	Decreased
mEOHP	0.963	0.94	0.078	Stable
mBzP	0.873	0.78	< 0.000	Decreased
mEHP	0.969	0.73	0.019	Decreased
BPA	0.911	0.73	0.004	Decreased

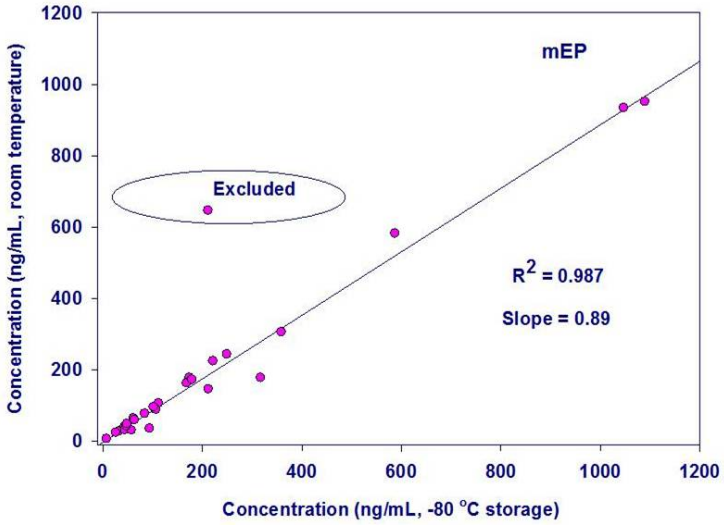

Fig. 3 Relationship of mEP concentrations in urine samples (n = 30; unspiked) stored at -80°C and 20°C for 8 weeks

during storage at 20°C for 8 weeks. Many target analytes showed a loss of < 10%, whereas miBP, mEHP, and BPA showed a loss of up to 30% in 8 weeks.

3.3 Estimation of half-life in urine stored at room temperature

The temporal stability study of phthalates and BPA in urine also allowed for the estimation of half-life of target compounds stored at 20°C (room temperature) for 8 weeks. In this exercise, it can be assumed that urine is a surrogate for environmental waters. Analysis of half-life of phthalate metabolites and BPA stored at 20°C provides some insight into the degradation of these compounds in aquatic medium. We assumed that the degradation of contaminants in urine followed first-order kinetics. The half-lives of phthalate metabolites and BPA were estimated only for those samples that showed decreasing concentrations after 8 weeks of storage at 20°C (Table 3). The estimated half-life was > 2 years for mEP and mECPP, 1 year for mEOHP and mBzP, and approximately 6 months for other phthalate metabolites. However, as shown in Table 1, the

Table 3 Estimated half-life of phthalate metabolites and BPA in urine samples (unspiked) stored at 20°C ($t_{1/2}$, months) for 8 weeks*

	mEP	mBP	miBP	mECPP	mEOHP	mBzP	mEHP	BPA
$t_{1/2}$ mean	24	8	7	26	12	10	7	4
$t_{1/2}$ median	13	4	4	8	7	4	3	3
$t_{1/2}$ range (95% CI)	10-37	1-7	4-10	8-44	3-21	3-17	4-11	3-6

*Of the 30 samples analyzed, only those samples that showed reduction in concentrations were used in the estimation of half-life

concentrations of phthalate metabolites and BPA were stable in 9% to 39% of the samples stored actual values. No earlier studies have measured the half-life of phthalate metabolites in water. Thus, our estimates may provide some information for the half-life of phthalate metabolites in aquatic medium. The estimated half-life of BPA was around 3 months. Our estimate is much higher than the half-life of BPA reported for aquatic ecosystems, which was less than a week (Kang and Kondo 2002, Klecka *et al*. 2001). The half-life of target analytes in urine can be longer than in environmental waters because urine is a sterile matrix in comparison to environmental waters.

4. Conclusions

Overall, concentrations of phthalate metabolites and BPA in urine stored at 20°C for over 8 weeks decreased by up to 30%, and this reduction was in the range of analytical uncertainty encountered in the analysis of the target chemicals. Our results indicated that, for the determination of total concentrations of phthalate metabolites and BPA in urine, the samples can withstand storage at room temperatures for up to a couple of weeks during shipment. Nevertheless, we recommend storage at frozen conditions immediately after collection and transport (if such facilities exist). Our results are consistent with what was reported earlier for the storage and time for analysis for phthalate metabolites and BPA in urine (Hoppin *et al*. 2006). However, when speciation studies of free and conjugated forms are needed, storage at room temperature may not be appropriate. Further studies are needed in this regard.

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