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Metabolite Profiles of Di-*n*-butyl Phthalate in Humans and Rats

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Di-*n*-butyl phthalate (DBP) is widely used in consumer products. In humans and in rats, DBP is metabolized to mono-*n*-butyl phthalate (MBP). MBP may also further oxidize to other metabolites of DBP. We studied the metabolic profiles of DBP in rats and humans to evaluate the similarities between the two species and between different exposure scenarios. In rats administered DBP by oral gavage, we identified MBP and three urinary oxidative metabolites of DBP: mono-3-oxo-*n*-butyl phthalate, mono-3-hydroxy-*n*-butyl phthalate (MHBP), and mono-3-carboxypropyl phthalate (MCPP). MBP, MHBP, and MCPP were also present in serum, albeit at lower levels than in urine. Statistically significant correlations ($p < 0.01$) existed between the concentrations of MBP and the concentrations of MHBP (Pearson correlation coefficient $r = 0.82$ [urine] and $r = 0.96$ [serum]) and MCPP ($r = 0.77$ [urine] and $r = 0.97$ [serum]). However, the concentrations of these metabolites in urine collected 6 h after dosing and in serum 24 h after dosing were not correlated, suggesting continuous metabolism of DBP and/or individual differences among rats. Serum DBP metabolite concentrations increased with the dose, whereas urinary concentrations did not. We also identified MBP, MHBP, and MCPP in the urine of four men exposed to DBP by taking a prescription medication containing DBP, and MBP and MCPP in 94 adults with no documented exposure to DBP. In the human samples, we observed statistically significant correlations ($p < 0.01$) among the urinary concentrations of MBP and MCPP, although the correlation was stronger for the four exposed men ($r = 0.99$) than for the adults without a documented exposure to DBP ($r = 0.70$). Our results suggest that regardless of species and exposure scenario, MBP, the major DBP metabolite, is an optimal biomarker of exposure to DBP. In addition to MBP, MCPP and MHBP may be adequate biomarkers of exposure to DBP in occupational settings or in potential high-exposure scenarios.

Introduction

Di-*n*-butyl phthalate (DBP) is widely used in consumer products, such as cosmetics, toys, flooring, wallpaper, and furniture (*1*). DBP is also used extensively in the adhesive industry to plasticize polyvinyl acetate emulsions and nitrocellulose lacquers and is often used in conjunction with higher molecular weight phthalates to improve the flexibility of plasticizer combinations (*1*). The general population is exposed to DBP through food, water, air, and the use of DBP-containing consumer products. Specific activities may result in an increased risk of exposure to DBP, including consumption of medications containing DBP in the enteric coatings (*2*, *3*).

Both in humans and in rodents, DBP is rapidly metabolized to its hydrolytic mono-ester mono-butyl phthalate (MBP), which is excreted in urine in its free or glucuronide conjugated forms as the major DBP metabolite (*1*, *4*, *5*). MBP is relatively hydrophilic and amenable to urinary excretion; therefore, further oxidation of MBP is likely to be less relevant for the metabolism of DBP than for other high molecular weight phthalates (*6*−*10*). However, mono-3-oxo-*n*-butyl phthalate (MOBP), mono-3-hydroxy-*n*-butyl phthalate (MHBP), and mono-3-carboxypropyl phthalate (MCPP), three oxidative metabolites of MBP, also have been identified in urine of rats dosed with DBP (*11*−*13*).

MBP has been used as a biomarker of exposure to DBP in epidemiological studies for exposure assessment purposes (*14*−*17*). MBP was detected in more than 99% of the urine specimens analyzed from representative samples of the U.S. general population (*14*, *18*). These data suggest that exposure to DBP in the general population is widespread, although some differences in exposure patterns may exist based on demographic and lifestyle factors. In particular, in some populations such as patients treated with medications containing DBP in the enteric coatings, the urinary concentrations of MBP were several-fold higher than in the general population (*2*, *3*).

Exposure to DBP is known to cause adverse developmental and reproductive health effects in rats (*19*−*21*). Limited data are available on the health effects of DBP in humans (*22*, *23*). The translation of animal research to assess the potential human health effects of DBP requires an understanding of the elimination metabolic profile of DBP in animals and in humans. In this study, we used mass spectrometry to confirm the presence of MBP and to identify three DBP oxidative metabolic products (Figure 1) in human urine and in the urine and serum of rats administered DBP to compare the metabolite profile of DBP in humans and rats. We also examined the usefulness of MBP as the sole biomarker of exposure to DBP in different human exposure scenarios, for example, environmental versus occupational or other high-exposure situations.

Materials and Methods

The sources of reagents and most analytical standards were reported previously (*24*). MOBP and MHBP were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Timed-pregnant Sprague−Dawley rats (Charles River Laboratories, Inc., Raleigh, NC) were administered DBP (0, 100, or 250 mg/kg/day) in 2.5 mL/kg corn oil by gavage on gestational day (GD) 12−18 (six rats per dose and two control rats). The dosing procedure is described in detail elsewhere (*5*). Urine was collected 6 h after dosing on GDs 15 and 17. The rats were killed and necropsied on GD 18, 24 h after the last DBP administration. Amniotic fluid and serum samples were collected at necropsy (*5*) using DBP free tubing and syringes. Potential contamination of urine samples was avoided by use of metabolism cages designed to avoid excess food to contaminate excreta and to allow the separate

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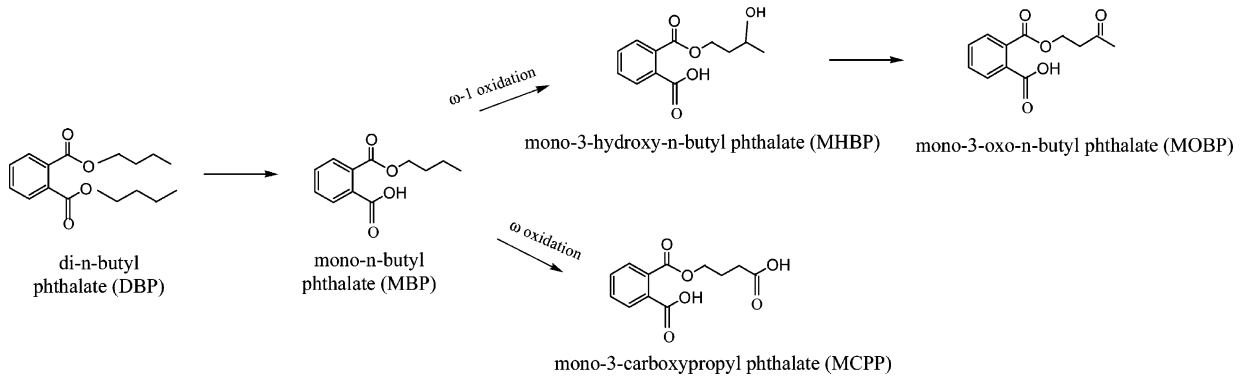

FIGURE 1. Proposed metabolism of di-*n*-butyl phthalate in rats and humans.

collection of feces and urine. The urinary (GD 17) and amniotic fluid concentrations of MBP in these rats have been reported (*5*). The urine collected from five rats on GD 17 was reanalyzed for MBP, MHBP, and MCPP for the current study. The animal treatment protocol was reviewed and approved by the EPA Internal Animal Care and Use Committee.

Urine samples were collected anonymously from a demographically diverse group of 94 male and female adults with no documented exposure to DBP. Urine samples were also collected from four men exposed to DBP via medications containing DBP. No personal information from any of these subjects was available. Study protocols were reviewed and approved by the Centers for Disease Control and Prevention's Human Subjects Institutional Review Board.

Collection materials (e.g., cryovials and urine cups) were pre-screened for MBP. The samples were shipped in cryovials to the Centers for Disease Control and Prevention for analysis of DBP metabolites and were stored at −70 °C until analysis. The analytical methods used to extract the DBP metabolites from urine and serum are described elsewhere (*24*, *25*). For structural identification, the metabolites were extracted from the samples by automated solid-phase extraction (SPE), using a commercial SPE system (Zymark Corporation, Hopkinton, MA) after enzymatic hydrolysis of the metabolite conjugates with β-glucuronidase at 37 °C for 90 min. The DBP metabolites in the extract were chromatographically resolved by high-performance liquid chromatography (HPLC), using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA) equipped with a Betasil phenyl HPLC column (3 μm, 100 mm × 2.1 mm, ThermoHypersil-Keystone, Bellefonte, PA) and by use of a nonlinear water/acetonitrile solvent gradient. The metabolites were detected by negative ion electrospray ionization tandem mass spectrometry, using a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer operated in the full scan mode.

For quantification, urine and serum samples were spiked with an internal standard solution containing $^{13}C_4$-mono-3-carboxypropyl phthalate ($^{13}C_4$-MCPP), $^{13}C_4$-mono(2-ethyl-5-oxohexyl) phthalate ($^{13}C_4$-MEOHP), $^{13}C_4$-mono(2-ethyl-5-hydroxyhexyl) phthalate ($^{13}C_4$-MEHHP), $^{13}C_4$-MBP, and $^{13}C_4$-4-methylumbelliferone ($^{13}C_4$-MeUmb) and a standard solution of 4-MeUmb-glucuronide. The DBP metabolite conjugates were hydrolyzed with β-glucuronidase and extracted by online or offline SPE (*24*, *25*). 4-MeUmb was measured to evaluate the completion of the deglucuronidation reaction. The DBP metabolites in the extract were chromatographically resolved on a Betasil phenyl HPLC column (3 μm, 150 mm × 2.1 mm) by use of a nonlinear water/acetonitrile solvent gradient and analyzed by negative ion electrospray ionization tandem mass spectrometry (*24*). For quantification of MBP and MCPP, calibration curves were generated using the peak area ratio of each analyte to its isotopicaly labeled analogue versus concentration. For MHBP, for which an isotope labeled analogue was not available, we used $^{13}C_4$-MEHHP as the internal standard to measure the concentration. Only MBP and MCPP were quantitatively measured in 94 humans with no documented exposure to DBP.

Results and Discussion

We relied on mass spectrometric data for the structural identification of the DBP metabolites. In the urine extracts of rats administered with DBP, the precursor scan for the $m/z = 121$ product ion $[C_6H_5COO]^-$ showed intense precursor peaks corresponding to the following ions (m/z): 221, 237, and 251; a signal with $m/z = 235$ was also present. On the basis of matching mass spectral fragmentation patterns of the relevant precursor ions and authentic standards eluting at the same retention time, we identified four DBP metabolites: MBP ($m/z = 221$), MHBP ($m/z = 237$), MCPP ($m/z = 251$), and MOBP ($m/z = 235$) (Figure 2). MBP, MCPP, and MBP were also positively identified in the urine of four men highly exposed to DBP. MOBP concentrations were too low to achieve full scan mass spectral confirmation, although the MOBP precursor/product ion scan (235/221) was present (Figure 2). The urinary concentrations of MOBP were not determined because of the large dilution of urine that would have been necessary to prevent carryover of MBP during analysis. Additional oxidation products of DBP were not detected in either rat or in human urine.

Interestingly, in the rat urine collected 6 h after the last dosing, the average MBP levels did not increase with dose, in agreement with our previous findings on the same group of rats (*5*). Similarly, the mean concentrations of MCPP and MHOP in serum, but not in urine (Table 1), increased with the DBP dose. On the other hand, MBP was also present in the rat serum collected 24 h after the last dosing. The mean serum concentrations were 39.8 ± 29.3 μg/mL (100 mg/kg/day DBP dose) and 122.1 ± 49.7 μg/mL (250 mg/kg/day DBP dose) (Table 1). The mean MBP concentrations in fetal amniotic fluid, reported previously (*5*), were 1.4 μg/mL (100 mg/kg/day DBP dose) and 13.4 μg/mL (250 mg/kg/day DBP dose) and increased with the serum levels (Table 1). These data suggest that DBP or MBP can cross the placenta in a dose-related manner. However, saturation of the enzymes involved in the hydrolysis of DBP, limited urinary clearance of the DBP metabolites, or a combination of these factors may occur at these high DBP doses. A saturation of the glucuronidation of MBP at increasing doses of DBP in pregnant rats has been reported before (*12*).

In rats dosed with DBP, both urinary and serum concentrations of MBP were correlated significantly with MCPP ($p < 0.01$, $r = 0.77$ [urine] and $r = 0.97$ [serum]) and MHBP ($p < 0.01$, $r = 0.82$ [urine] and $r = 0.96$ [serum]). Similar correlations were observed for the urinary DBP metabolites in the four men highly exposed to DBP ($r = 0.99$, Figure 3 and Table 2). However, there was no correlation of MBP,

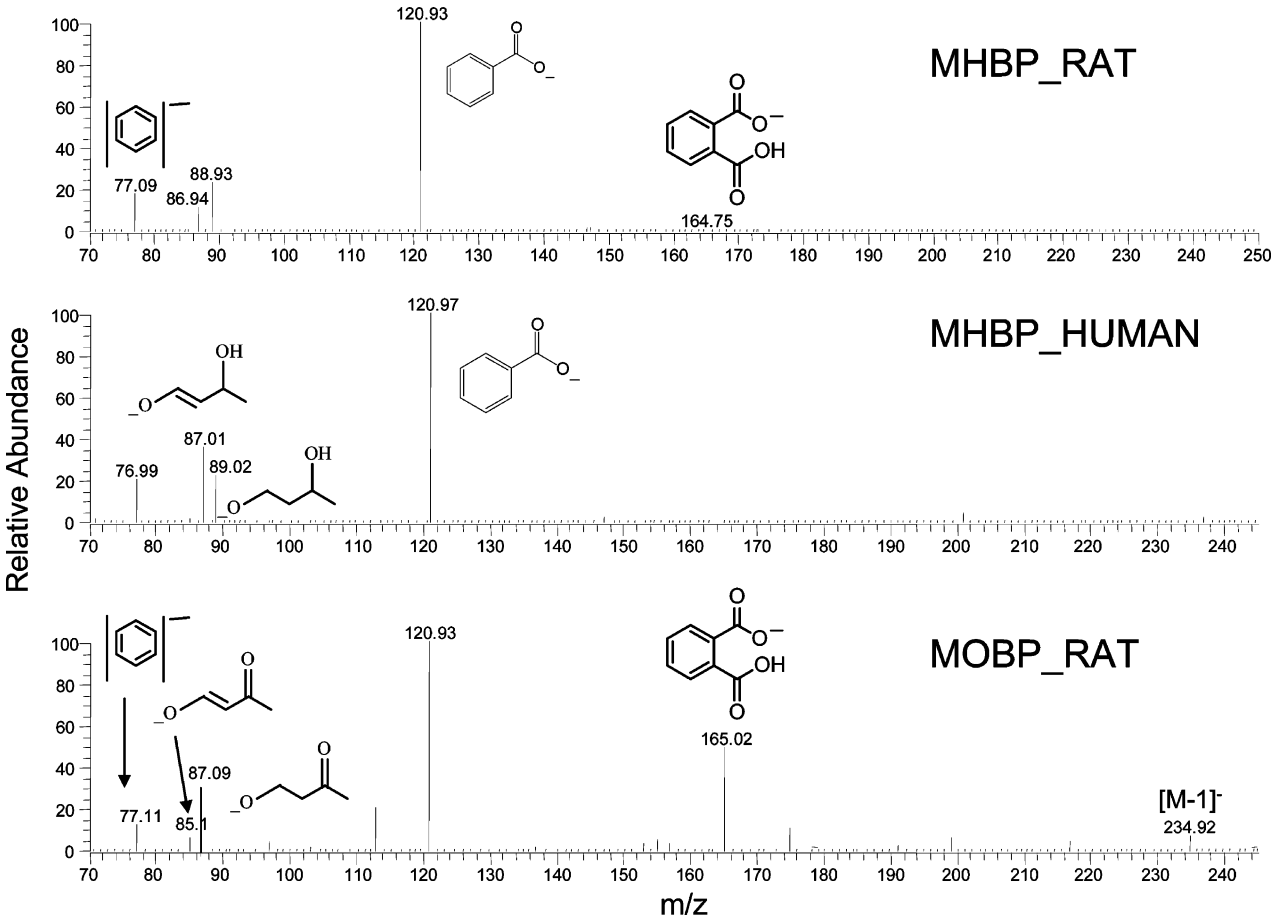

FIGURE 2. Mass spectral fragmentation pattern for MHBP in rat and human urine and for MOBP in rat urine.

TABLE 1. Mean Urine, Serum, and Fetal Amniotic Fluid DBP Metabolite Concentrations in Pregnant Sprague–Dawley Rats after Oral Administration of DBP

DBP dose (mg/kg/day)	DBP metabolite	urinary levels[a] ±SD (μg/mL) GD 15 (*N* = 6)	urinary levels[a] ±SD (μg/mL) GD 17 (*N* = 5)	serum levels[b] ±SD (*N* = 5) (μg/mL) GD 18	fetal amniotic fluid levels[c] (μg/mL) GD 18
0	MBP	<LOQ	<LOQ	<LOQ	<LOQ
	MHBP	<LOQ	<LOQ	<LOQ	nd
	MCPP	<LOQ	<LOQ	<LOQ	nd
100	MBP	2750 ± 1782	3389 ± 2337	39.8 ± 29.3	1.4
	MHBP	127.0 ± 117.8	147.1 ± 128.2	0.171 ± 0.139	nd
	MCPP	65.5 ± 69.0	95.5 ± 96.4	0.403 ± 0.311	nd
250	MBP	2113 ± 1196	2961 ± 2270	122.1 ± 49.7	13.4
	MHBP	177.0 ± 98.2	281.9 ± 129.7	0.629 ± 0.327	nd
	MCPP	47.0 ± 27.9	90.7 ± 47.6	1.336 ± 0.675	nd

[a] Urine was collected for 6 h after dosing. [b] Serum and fetal amniotic fluid samples were collected after 24 h of dosing. [c] Reference *5*; nd: not determined.

MHBP,or MCPP urinary (GD 15 and 17) and serum (GD 18) levels. This may be due to the continuous hydrolysis of DBP absorbed and circulated in the blood after the collection of the urine, although the contribution from individual differences may not be completely eliminated. At environmental levels, DBP is most likely absorbed as MBP because of high lipase enzyme activity from lipases in the gut. However, at the levels used for dosing the rats, direct absorption of DBP may result due to enzyme saturation. Because MBP is known to be the bioactive species, depending on the dose, the toxicological significance may differ. Furthermore, the detection of MBP in amniotic fluid does not provide definitive evidence of DBP metabolism in the fetus or of MBP crossing the placenta. Future studies are warranted to further investigate the metabolism of DBP at low and high doses.

The correlation between the concentrations of MCPP and MBP in the adults ($n = 94$) with MBP concentrations within environmental reference range levels was statistically significant ($p < 0.01$, $r = 0.70$, Figure 4 and Table 2). Although statistically significant ($p < 0.01$), this correlation was weaker than for the four exposed men ($r = 0.99$). Furthermore, the mean percentage ratio of MCPP to MBP concentrations was 26 ± 26%, considerably higher than among the exposed persons (1 ± 0.1%). These data suggest sources other than DBP for urinary MCPP or dose-related differences in metabolism at environmental versus high exposure levels. MCPP has been previously identified as a major metabolite of di-*n*-octyl phthalate in rats (*11*) and humans (*26*). Therefore, phthalates other than DBP may contribute to the presence of MCPP in the urine, and thus, MCPP may not be suitable

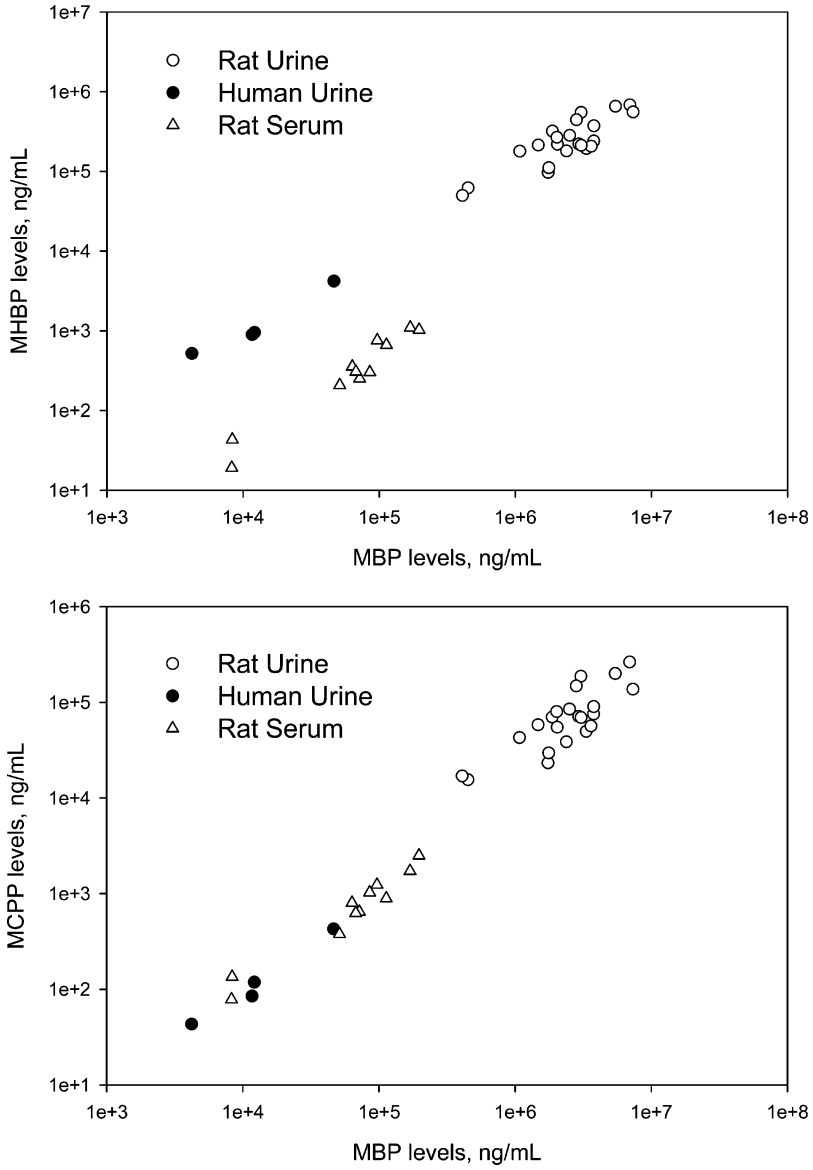

FIGURE 3. Correlation analysis of DBP metabolites in four persons exposed to DBP through consumption of DBP-containing medications and in rats administered with DBP (100 or 250 mg/kg) by gavage.

TABLE 2. Correlation Analysis of DBP Metabolites

	rats				exposed men		environmentally exposed adults	
	urine		serum					
	p	*r*	*p*	*r*	*p*	*r*	*p*	*r*
MBP/MCPP	<0.01	0.82	<0.01	0.96	<0.01	0.99	<0.01	0.70
MBP/MHBP	<0.01	0.77	<0.01	0.97	<0.01	0.99	nd	nd

as a sole biomarker for DBP exposure assessment, especially at environmental exposure levels.

Our data suggest that both rats and humans excrete MBP as the major urinary DBP metabolite. In agreement with previous data for MBP and MCPP (*4*, *5*), we found that glucuronidated species were the most predominant in urine for MBP and also MHBP, whereas the free form was most prevalent for MCPP.

In the rats administered DBP, the mean percentage elimination ratios of oxidative metabolite to MBP were 7.0 ± 2.4% (MHBP) and 2.4 ± 0.9% (MCPP); in the four men exposed to DBP from the consumption of a medication containing DBP, these ratios were 9.2 + 2.2% (MHBP) and

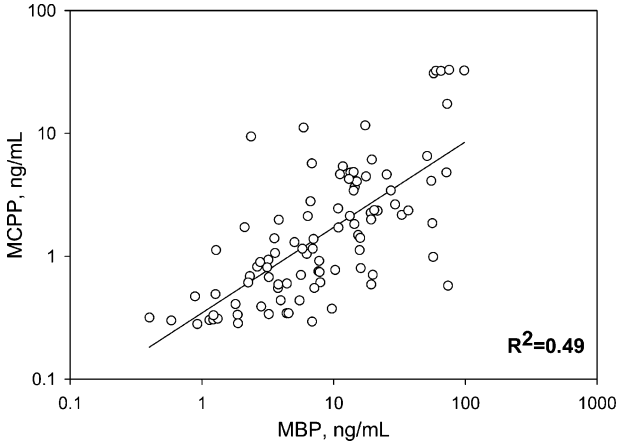

FIGURE 4. Correlation analysis of urinary MBP and MCPP in an adult population ($N = 94$) with no documented exposure to DBP.

0.9% ± 0.1% (MCPP). Because of the limited sample size, these comparison data in DBP metabolite elimination among species are not conclusive. Similar to urine, MBP was the

major metabolite in serum. However, in serum, MCPP levels were higher than for MHBP. The differences in urine and serum may be associated with excretion toxicokinetics of these three DBP metabolites and may be different at environmental exposure levels. Further studies are needed to assess excretion toxicokinetics, species differences, and patterns of metabolite excretion.

In conclusion, in both rats and humans, DBP is rapidly metabolized to form its hydrolytic mono-ester, MBP. Degradation of DBP to form MBP (*27*) is possible if DBP contamination is present during sampling, collection, and storage, especially when using lipase-containing matrices (e.g., serum, amniotic fluid, milk, and meconium) (*28*). Because MHBP and MCPP cannot be formed as a result of contamination, these oxidative metabolites may be adequate biomarkers both in occupational settings and when using matrices with a high lipase activity for biomonitoring purposes. Nevertheless, our results suggest that regardless of exposure scenario, urinary concentrations of MBP can be used to accurately classify human exposure to DBP.

Acknowledgments

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