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Glucuronidation patterns of common urinary and serum monoester phthalate metabolites

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Abstract Metabolism of most diesters of phthalic acid in humans occurs by an initial phase I biotransformation in which phthalate monoesters are formed, followed by a phase II biotransformation in which phthalate monoesters react with glucuronic acid to form their respective glucuronide conjugates. The phase II conjugation increases water solubility and facilitates urinary excretion of phthalate, and reduces the potential biological activity because the putative biologically active species is the monoester metabolite. In this study, we report percentages of glucuronidation of four common phthalate monoesters, monoethyl (mEP), monobutyl (mBP), monobenzyl (mBzP), and mono-2-ethylhexyl phthalate (mEHP) in a subset of urine (mEP $n=262$, mBP $n=283$, mBzP $n=328$, mEHP $n=119$) and serum (mEP $n=93$, mBP $n=149$, mEHP $n=141$) samples from the general US population. The percentages of free and conjugated monoester excreted in urine differed for the various phthalates. For the more lipophilic monoesters (i.e., mBP, mBzP, and mEHP), the geometric mean of free monoester excretion ranged from 6 to 16%. The contrary was true for the most hydrophilic monoester, mEP, for which about 71% was excreted in urine as its free monoester. Furthermore, percentages of free and conjugated monoesters were similar for mEP, mBP and mEHP among serum and urine samples. Serum mBzP

was largely below the method limit of detection. Interestingly, the serum mEP and mBP levels were less than 3% and 47%, respectively, of their urinary levels, whereas the level of mEHP was similar both in urine and serum.

Keywords Serum Phthalates · Urinary phthalates · Monoethyl phthalate · Monobutyl phthalate · Monobenzylphthalate · Mono-2-ethylhexyl phthalate · Phthalate metabolism · Phthalate glucuronidation

Introduction

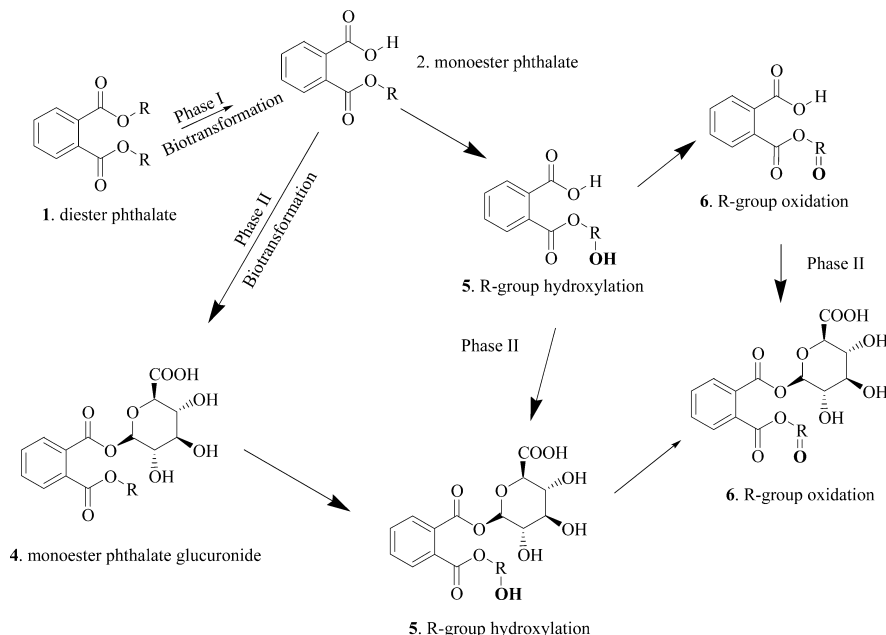
Public concern has increased in recent years about the potential health risks associated with exposure to phthalic acid diesters, commonly known as phthalates. Phthalates are a group of ubiquitous industrial chemicals used in a variety of consumer products such as solvents, additives and plasticizers (Nassberger et al. 1987; ATSDR 1995, 1997, 1999, 2000). Some phthalates and their monoester metabolites are known to cause carcinogenic, toxic or endocrine-modulating effects in animals (Autian 1973, 1982; Douglas et al. 1986; Doull et al. 1999; Banerjee et al. 2002). Phthalate exposure of humans can occur through oral, dermal or inhalation routes. Dermal exposure is considered the major route of exposure to diethyl phthalate (DEP) that is found in hygiene products such as soap, shampoo, and conditioners. In contrast, for phthalates that are used mainly as plasticizers, such as di-2-ethylhexyl phthalate (DEHP), oral or inhalation exposures predominate. In addition, for dialysis and blood transfusion patients, direct exposure (Mettang et al. 1996) to DEHP can occur through intravenous injection.

Understanding the metabolism of the various phthalates is important from a risk-assessment standpoint. In general, biotransformation or metabolism of xenobiotics most frequently results in detoxification of

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Fig. 1 General metabolism pathways for phthalates



the chemical and facilitates excretion from the body. However, this may not be the case for phthalates. For example, dibutyl phthalate (DBP) and DEHP are known to be metabolized to the bioactive monoesters monobutyl phthalate (mBP) and mono-2-ethylhexyl phthalate (mEHP), upon phase I biotransformation, and are excreted in urine (Heindel and Powell 1992; Fig. 1). The phthalate monoesters, formed during the phase I biotransformation, can be excreted unchanged or they can undergo phase II biotransformation, which is catalyzed by the enzyme uridine 5'-diphosphoglucuronyl transferase (UGT) to produce glucuronide-conjugated monoesters. Alternatively, the phthalate monoesters may be further metabolized to produce more hydrophilic oxidative products (Albro et al. 1973; Albro and Thomas 1973; Albro and Moore 1974; ATSDR 1997, 2000; Barr et al. 2003; Silva et al. 2003) and their glucuronide conjugates (Fig. 2). The oxidative products and their glucuronide conjugates were not measured in this study.

Phthalate monoesters are valuable biomarkers for exposure to phthalate diesters, and have been measured in previous exposure assessment studies (Blount et al.

2000b; Brock et al. 2002; CDC 2001, 2003; Duty et al. 2002). In most of these studies, total phthalate monoester concentrations (i.e., free plus glucuronide-bound) were measured. However, from a toxicological standpoint, it may be important to note the relative degree of the bioactive free monoester metabolite to its respective conjugated, less bioactive, metabolite.

In this study, we report the relative degree of free monoester metabolite (phase 1) with respect to its glucuronide conjugated metabolite (phase 2) for four phthalate diesters, DEP, benzylbutylphthalate (BzBP), DBP, and DEHP in a subset of human urine and serum samples from the general US population.

Materials and methods

Subjects

The monoester metabolites of phthalates have been routinely measured since 1999 as a part of the ongoing National Health and Nutrition Examination Survey (NHANES-1999) conducted by the US National Center for Health Statistics (NCHS) at the Centers for Disease Control and Prevention (CDC). NHANES was designed to provide information from the civilian, non-institutionalized population that is representative of the general US population. Total phthalate monoesters, which include both free and glucuronidated forms, were measured in a subset of samples collected during 1999 and 2000. The distribution of the results from 1999 were reported in CDC's National Report on Human Exposure to Environmental Chemicals (CDC 2001, 2003), and the composite

Fig. 2 Glucuronidation (phase II biotransformation) of monoester phthalates to their glucuronides. The reaction is catalyzed by the enzyme uridine 5'-diphosphoglucuronyl transferase to produce glucuronide-conjugated monoesters (*UDPGA* uridine 5'-diphospho-glucuronic acid, *UDP* uridine 5'-diphosphate)

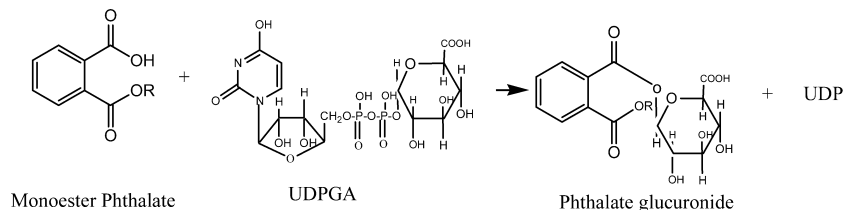


Table 1 Total, free and percentages of free (free/total) urinary phthalate monoester concentrations (*mEP* monoethyl phthalate, *mBP* monobutyl phthalate, *mBzP* monobenzyl phthalate, *mEHP* mono-2-ethylhexyl phthalate, each in nanograms per milliliter) in a nonrepresentative subset of samples from the National Health and

Nutrition Examination Survey (NHANES 1999–2000). Results from samples in which the total phthalate concentrations were below the limit of detection ($< LOD$) were eliminated from the calculations

Urinary phthalate monoester	<i>n</i>	Form	Minimum	Percentile					Maximum	Geometric mean
				5th	25th	50th	75th	95th		
<i>mEP</i>	262	Total	9.2	23.6	75.9	184.95	494.8	1677.7	23810.0	199.21
		Free	5.2	16.4	54.7	145.1	449.0	1397.4	14972.0	153.07
		% Free	8.78	30.75	62.88	78.98	94.36	100	100	70.72
<i>mBP</i>	283	Total	1.3	5.4	14.5	29.7	55.5	149.3	412.1	28.95
		Free	< LOD	< LOD	0.3	1.40	3.50	8.8	32.9	1.76
		% Free	< LOD	< LOD	1.25	3.93	9.60	48.67	100	5.60
<i>mBzP</i>	328	Total	0.8	2.5	9.35	20.05	43.9	108.0	1921.0	19.22
		Free	< LOD	< LOD	< LOD	0.95	2.40	8.2	303.3	1.61
		% Free	< LOD	< LOD	2.4	4.30	9.33	32.38	64.57	7.07
<i>mEHP</i>	119	Total	1.2	1.3	2.4	3.80	6.3	14.9	26.0	3.88
		Free	< LOD	< LOD	< LOD	< LOD	0.90	2.2	10.0	0.74
		% Free	< LOD	< LOD	< LOD	3.00	18.42	86.67	100	15.65

1999–2000 data will be reported elsewhere. From approximately 2300 samples analyzed during 1999 and 2000, 328 samples were randomly selected for analysis of the free monoester only. Although NHANES was designed to provide representative information, our random sampling for free monoester analysis was a convenience sampling and the urine and serum samples were not necessarily matched for the same person. However, the participants from whom samples were obtained and analyzed represented a diverse population comprising persons from both sexes and all races, ethnicities, age groups (age 6 years and above) and geographic regions in the United States.

Analytical methods

The analytical methods used to quantify both urinary and serum phthalate metabolites have been reported (Blount et al. 2000a; Kato et al. 2003; Silva et al. 2003). Briefly, the urine and serum samples were spiked with $^{13}C_4$ -labeled monoester internal standards, 4-methylumbelliferone (quantified as a surrogate to evaluate the completion of the deglucuronidation) and its labeled internal standard, were subjected to an enzyme hydrolysis with β -glucuronidase enzyme (*Escherichia coli* K12; Roche Biomedical, Mannheim, Germany) and extracted by solid-phase extraction. The extracts were concentrated and resuspended in water. The extractions were repeated, eliminating the deglucuronidation step for analysis of the free monoester. The urinary phthalate monoesters were resolved (Silva et al. 2003) by a high-performance liquid chromatography (HPLC; Waters 2690, Milford, MA, USA) system equipped with a Betasil phenyl column (Keystone Scientific, Bellefonte, PA, USA) using a nonlinear water/acetonitrile solvent gradient and analyzed by atmospheric pressure chemical ionization-tandem mass spectrometry (APCI-MS/MS; ThermoFinnigan TSQ 7000; San Jose, CA, USA). The serum phthalate monoesters (Kato et al. 2003) were chromatographically resolved by an Agilent 1100 HPLC (Wilmington, DE, USA), using the same water/acetonitrile solvent gradient, and were analyzed by electrospray ionization (ESI) tandem mass spectrometry (PE Sciex API 3000; Applied Biosystems, Foster City, CA, USA). Concentrations were calculated using linear regression lines from isotope dilution calibration plots. Bound concentrations were determined by subtracting the free monoester concentrations from the total monoester concentrations for a given sample. Data points for total analyte concentrations below detection limits were eliminated from the study, and urinary data were not creatinine-corrected. Reagent blanks, quality control samples and standards were analyzed along with the unknown human samples. The serum samples were acid-treated upon

receipt before extractions to avoid hydrolysis of diester phthalates to monoesters by serum enzymes. We assumed the contribution of *mEHP* from blood products contaminated with plastics containing DEHP was minimal.

Results and discussion

In the samples tested, we observed significant interindividual variations in the ratios of free monoester to their respective bound monoester (Table 1), which were not consistent among analytes (Fig. 3). Our data strongly suggest phase II biotransformation by glucuronidation as the major route of detoxification for DBP, BzBP, and DEHP, but not for DEP. In the population studied the urinary *mEP* concentrations ranged over three orders of

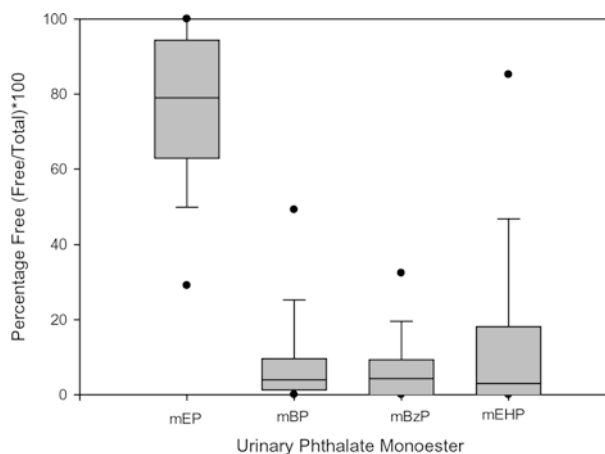


Fig. 3 Percentage of the free form of urinary phthalate monoesters (*mEP* monoethyl phthalate, *mBP* monobutyl phthalate, *mBzP* monobenzyl phthalate, *mEHP* mono-2-ethylhexyl phthalate) in a subset of a human population from National Health and Nutrition Examination Survey-1999. (solid points indicate 5th and 95th percentiles, horizontal bars indicate the medians, boxes enclose the 25th and 75th percentiles, and error bars are standard deviations)

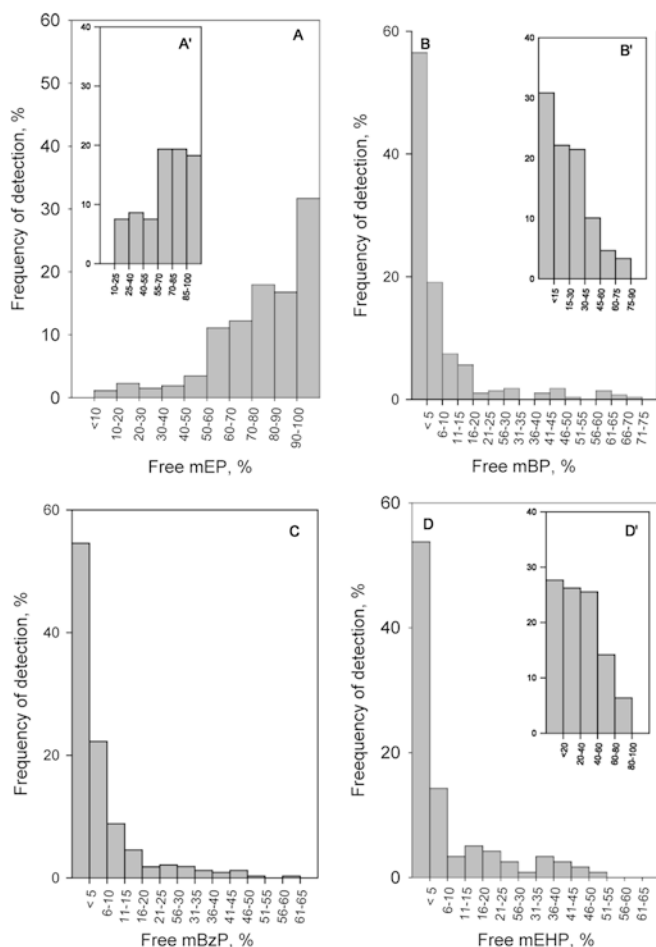


Fig. 4A–D Frequency of detection of free urinary and serum monoester phthalates. **A** Urinary mEP with inset **A'** presenting serum mEP. **B** Urinary mBP with inset **B'** presenting serum mBP. **C** Urinary mBzP; serum levels of mBzP were not detectable. **D** Urinary mEHP with inset **D'** presenting serum mEHP (which may show falsely elevated levels due to DEHP hydrolysis by serum enzymes)

magnitude (Table 1). We observed urinary mEP predominantly in its free form (Fig. 4A) with a geometric mean of 71% of total monoester, whereas urinary mBP, mBzP, and mEHP were present predominantly in their glucuronide-bound form (Fig. 4B–D) with geometric means of 6, 7 and 16%, respectively (Table 1). In more than half of the samples analyzed, less than 5% of total

urinary mBP, mBzP and mEHP were present as the free monoesters (Fig. 4). Furthermore, more than three-quarters of persons excreted 10% or less of mBP and mBzP as the free monoester (Fig. 4B,C). A statistical correlation analysis of the study population for free mBP and mBzP levels indicated similar distributions ($P < 0.001$). This suggests relatively similar metabolic pathways for DBP and BzBP, or may reflect metabolism of BzBP to produce both mBP and mBzP.

The high propensity of mBP, mBzP, and mEHP toward glucuronidation reduces the effective concentration of the free bioactive species for target organs, thus potentially minimizing the adverse effects of exposure to these chemicals. The differences in glucuronidation patterns of mEP, when compared with those of mEHP, mBP, and mBzP, demonstrated obvious differences in phase II metabolism of these phthalates. Perhaps these differences can be attributed to the relative solubility of these monoesters in urine. Because glucuronidation tends to make xenobiotic chemicals and their reaction products more soluble in urine, and thus more easily eliminated, the more nonpolar or lipophilic phthalates such as mBP, mBzP, and mEHP may require more polar moieties added to them to make them appreciably soluble in urine. Conversely, the more hydrophilic compounds, such as mEP, may be more easily or rapidly eliminated in the urine, making them less available for phase II metabolism.

In serum, we found only three phthalate metabolites, mEP, mBP and mEHP, at concentrations high enough for quantification (Table 2). Interestingly, glucuronide distribution patterns of these metabolites in serum closely resembled those of urine (Fig. 4). Similar to the pattern in urine, mBP and mEHP were found predominantly glucuronidated in serum, whereas mEP was found mostly free. We observed markedly lower serum mEP levels compared with the urinary levels (Fig. 5), likely indicating that mEP is more readily excreted in urine. The pattern remained unchanged irrespective of whether levels were compared with matched urine and serum samples or unmatched samples (Fig. 5A,B). The much higher levels of mEP in urine than in serum suggest that DEP is absorbed unchanged into the bloodstream, and that the kidneys may be the primary organ for phase I biotransformation of DEP to produce the monoester mEP. If formed in the kidneys, mEP may

Table 2 Total (combined free and glucuronidated) serum phthalate monoester concentrations (mEP monoethyl phthalate, mBP monobutyl phthalate, mEHP mono-2-ethylhexyl phthalate, each in nanograms per milliliter) in a nonrepresentative subset of samples from the National Health and Nutrition Examination Survey

Serum phthalate	<i>n</i>	Minimum	Percentile					Maximum	Geometric mean
			5th	25th	50th	75th	95th		
mEP	93	1.2	1.3	2.3	4.1	7.3	30.8	73.3	4.49
mBP	149	0.6	3.7	7.9	14.4	26.7	38.2	139.0	13.54
mEHP	141	0.7	0.9	3.4	5.4	8.9	15.4	34.8	4.96

(NHANES 1999–2000). Results from samples in which the concentrations were below the limit of detection were eliminated from the calculations. Serum levels of monobenzyl phthalate (mBzP) were not detectable

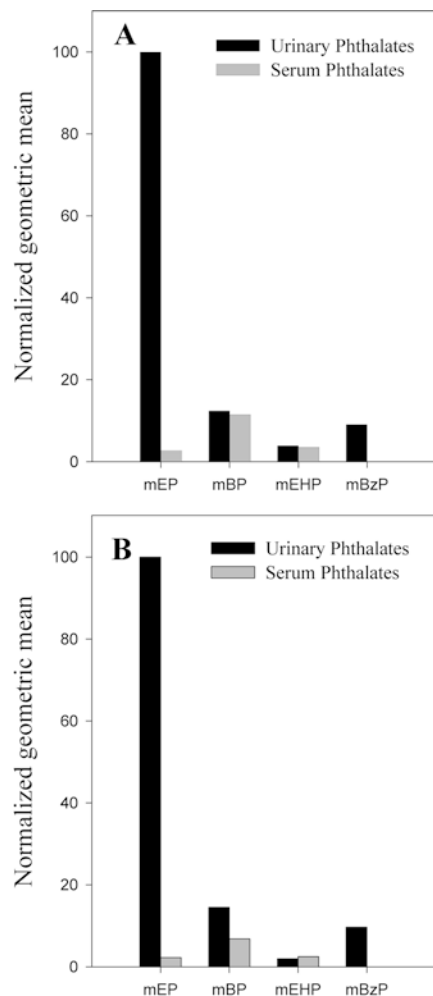


Fig. 5A, B Geometric mean concentrations (in nanograms per milliliter, ppb) of total monoethyl phthalate (*mEP*), monobutyl phthalate (*mBP*), mono-2-ethylhexyl phthalate (*mEHP*) and monobenzyl phthalate (*mBzP*) in urine and serum. **A** Urine and serum samples not necessarily from the same individuals. **B** Urine and serum samples from the same individuals

pass directly into the urine without reaching the liver for the phase II biotransformation, thus explaining the significantly low urinary and serum levels of glucuronidated *mEP*. This mechanism of *DEP* metabolism increases the effective concentration of *DEP* for systemic circulation. Our preliminary data suggest presence of high levels of *DEP* in serum, supporting our theory. However, further research is needed to confirm this proposed mechanism. Furthermore, *mEP* is smaller and more hydrophilic than the other three monoester phthalates; therefore, glucuronidation may not be needed to facilitate excretion. The comparable levels of *mBP* and *mEHP* in urine and serum suggest that for *DBP* and *DEHP* both hydrolysis and glucuronidation occur, probably in the liver, so producing mostly the glucuronidated form of the monoester metabolites.

In plots of the percentage of free monoester versus the log total monoester concentration, we observed no concentration-dependent changes in the relative free and

glucuronide-bound concentrations among the analytes (Fig. 6), thus indicating no apparent saturation of glucuronyl transferase enzyme at exposed concentrations. The percentage of free *mEP*, *mBP*, *mBzP* and *mEHP* found in urine remained scattered with geometric means, 71, 6, 7 and 16%, respectively, and showed no apparent decrease with increased concentration of total phthalate as should occur if enzyme saturation occurred. We plotted the concentrations of the free phthalate monoesters in the urine against the total phthalate concentration for each monoester (Fig. 7). The free urinary phthalate concentrations of *mEP*, *mBP* and *mBzP* increased ($P < 0.0001$) with increasing concentrations of total combined free and glucuronidated forms. The correlation was not significant for *mEHP*. Because the concentrations of *mEHP* found in urine were low with a significant number of samples having concentrations of free *mEHP* below or close to the limit of detection, the analytical procedure may have introduced some errors in the detection making it difficult to interpret correlation. The high correlation of free *mEP* with total combined *mEP* may also indicate less interindividual variability in metabolism of *DEP* than in the other phthalates. Differences in metabolism have been documented with age, sex, race, ethnicity, diseases, pregnancy, and other factors. Because of a limited sample size, we were not able to assess differences in glucuronidation efficiencies among different demographic groups.

Our data show higher systemic circulation of glucuronides of *mBP* and *mEHP* than of their free metabolites, potentially reducing the possible toxic effects associated with the free monoesters upon exposure to their parent compounds *DBP*, and *DEHP*. For *DEP*, the free monoester is the predominant excretion product, despite significantly low serum *mEP* concentration. In our previous studies, the total urinary phthalate concentration, consisting of free and conjugated forms, was used for exposure assessment. Although glucuronide may serve as the transport form of the free phthalates, because the free form of the phthalate metabolite may be more bioactive than its parent compound (e.g., *mBP*, *mEHP*) the

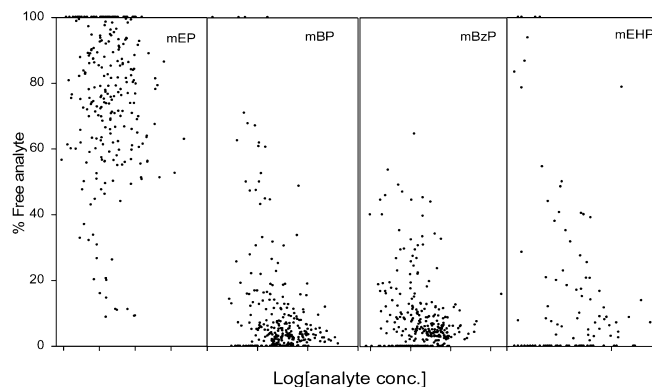


Fig. 6 Percentage of free urinary monoethyl phthalate (*mEP*), monobutyl phthalate (*mBP*), monobenzyl phthalate (*mBzP*), and mono-2-ethylhexyl phthalate (*mEHP*) versus log concentration of total analytes

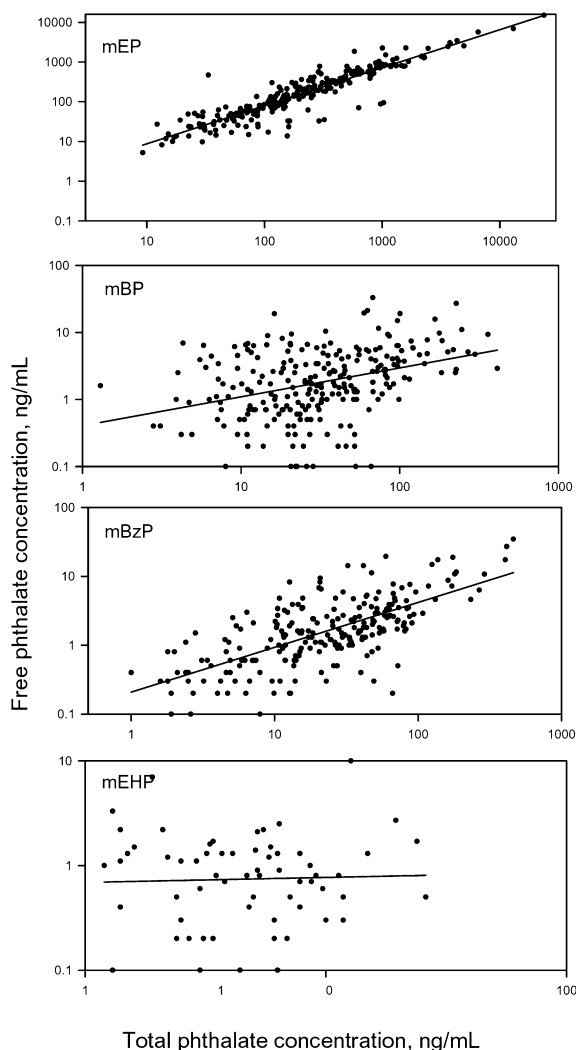


Fig. 7 Dependency of the concentration of free phthalate monoesters monoethyl phthalate (*mEP*), monobutyl phthalate (*mBP*), monobenzyl phthalate (*mBzP*) and mono-2-ethylhexyl phthalate (*mEHP*) on the total exposure as measured by the concentration of combined free and glucuronidated forms

amount of the free form provides valuable information regarding the biologically effective dose of the active toxicant and the risks associated with the exposure.

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