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TOXICOKINETICS AND METABOLISM

Metabolism and elimination of methyl, *iso*- and *n*-butyl paraben in human urine after single oral dosage

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Abstract Parabens are used as preservatives in personal care and consumer products, food and pharmaceuticals. Their use is controversial because of possible endocrine disrupting properties. In this study, we investigated metabolism and urinary excretion of methyl paraben (MeP), *iso*-butyl paraben (*iso*-BuP) and *n*-butyl paraben (*n*-BuP) after oral dosage of deuterium-labeled analogs (10 mg). Each volunteer received one dosage per investigated paraben separately and at least 2 weeks apart. Consecutive urine samples were collected over 48 h. In addition to the parent parabens (free and conjugated) which are already used as biomarkers of internal exposure and the known but non-specific metabolites, *p*-hydroxybenzoic acid (PHBA) and *p*-hydroxyhippuric acid (PHHA), we identified new, oxidized metabolites with hydroxy groups on the alkyl side chain (3OH-*n*-BuP and 2OH-*iso*-BuP) and species with oxidative modifications on the aromatic ring. MeP represented 17.4 % of the dose excreted in urine, while *iso*-BuP represented only 6.8 % and *n*-BuP 5.6 %. Additionally, for *iso*-BuP, about 16 % was excreted as 2OH-*iso*-BuP and for *n*-BuP about 6 % as 3OH-*n*-BuP. Less than 1 % was excreted as ring-hydroxylated metabolites. In all cases, PHHA was identified as the major but non-specific metabolite (57.2–63.8 %). PHBA represented 3.0–7.2 %. For all parabens, the majority of the oral dose captured by the above metabolites was excreted in the first 24 h (80.5–85.3 %). Complementary to the parent parabens excreted in urine, alkyl-chain-oxidized metabolites of the butyl parabens are introduced as valuable and contamination-free biomarkers of exposure.

Keywords Parabens · Human metabolism · Urinary metabolites · Human biomonitoring

Introduction

Alkyl or aryl esters of p-hydroxybenzoic acid (parabens) have been widely used, individually or in combination, as antimicrobial preservatives in cosmetics, pharmaceuticals and food for more than 50 years (Guo and Kannan 2013; Soni et al. 2005). Worldwide, the use of parabens as preservatives has been debated, because of possible endocrine disrupting activities. In the last decades, several in vitro and in vivo (in rodents) studies were published suggesting estrogenic activity (Blair et al. 2000; Routledge et al. 1998; Okubo et al. 2001; Byford et al. 2002), antiandrogenic effects (Satoh et al. 2005; Chen et al. 2007; Kjærstad et al. 2010), uterotrophic effects (Lemini et al. 2003), effects on sperm count and testosterone levels after dietary exposure (Oishi 2001, 2002a, b) and carcinogenic potential for some parabens (Darbre et al. 2004). However, a comprehensive overview of all studies reveals a rather heterogeneous picture of toxicological findings, and several effects described above could not be reproduced or confirmed in follow-up studies, although very high doses were applied (e.g., Hoberman et al. 2008). In 2014, the maximum allowed concentrations of propyl and butyl paraben as preservatives in cosmetics have been reduced in the European Union from 0.4 to 0.14 % when used individually. The

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maximum concentration of 0.8 % for the sum of all parabens contained in a cosmetic product has been maintained (European Parliament 2014a). Furthermore, the use of *iso*-propyl, *iso*-butyl, benzyl and pentyl paraben in cosmetic products is banned in the European Union (European Parliament 2014b).

The widespread use of parabens in personal care products, in foodstuff and in pharmaceuticals results in omnipresent paraben exposures through a variety of exposure routes, i.e., dermal absorption, ingestion and inhalation (Błędzka et al. 2014). After absorption, all parabens are rather rapidly hydrolyzed by unspecific esterases to *p*-hydroxybenzoic acid (PHBA), which is conjugated with sulfate, glucuronic acid or glycine (*p*-hydroxyhippuric acid, PHHA) prior to being excreted in urine (Abbas et al. 2010; Janjua et al. 2008; Ye et al. 2005). Thus, the main urinary metabolite of all parabens is unspecific PHBA and its conjugates. Only relatively minor amounts of the parabens are excreted as the parent parabens (after conjugation with sulfate and glucuronic acid) in urine (Boberg et al. 2010). The metabolic efficiency and pattern of hydrolysis of parabens have been described to depend on exposure routes (Aubert et al. 2012) and on alkyl chain length (Boberg et al. 2010); however, specific conversion factors are generally lacking. For human biomonitoring purposes, only the parent parabens (after hydrolysis) in urine are currently used as (specific) biomarkers of internal exposure. A large number of biomonitoring studies, including studies from our group, have shown that parabens are ubiquitously present in urine samples from the general population and various subpopulations (Ma et al. 2013; Asimakopoulos et al. 2014; Frederiksen 2010, 2013, 2014; CDC 2015; Calafat et al. 2010; Casas et al. 2011; Philippat et al. 2012; Dewalque et al. 2014; Wang et al. 2013; Shirai et al. 2013; Kang et al. 2013, Meeker et al. 2013; Guidry et al. 2015; Moos et al. 2014, 2015; Koch et al. 2014).

Currently, the interpretation of urinary paraben data is impeded to some extent by the limited quantitative knowledge on human paraben metabolism and excretion. Furthermore, urinary analysis of the parent parabens has to be embedded into rigorous quality assurance measures to minimize both pre-analytical (e.g., sample collection and sample preservation) and intra-laboratory contamination due to their omnipresence in consumer products (Ye et al. 2006; Guidry et al. 2015). The above issues related to paraben analysis and interpretation resemble in many ways issues encountered for phthalates (Barr et al. 2003; Koch et al. 2003; Koch and Calafat 2009). Our previous study on the short-chain phthalates di-*iso*- and di-*n*-butyl phthalate (DiBP and DnBP) has identified metabolites with oxidative modifications at their alkyl side chain as valuable biomarkers of exposure in addition to the simple phthalate monoesters without being prone to external contamination (Koch et al. 2012). Similar side-chain-oxidized metabolites can be postulated for *iso*-butyl (*iso*-BuP) and *n*-butyl paraben (*n*-BuP). Also, metabolites with oxidative modifications at the aromatic ring have recently been mentioned (Wang and Kannan 2013).

In this human metabolism study with oral dosage, we therefore intended to establish urinary excretion factors for the classical biomarkers of paraben exposure, the parent parabens (free and conjugated), and additional, specific biomarkers with oxidative modifications. In order to avoid influences by omnipresent paraben exposure, we dosed deuterium-labeled (D4-ring-labeled) analogs. We selected three parabens (methyl paraben (MeP), *iso*-BuP and *n*-BuP) with the aim to investigate possible differences in metabolism depending on the length and isomeric structure of their alkyl side chain. Next to the parent parabens and the unspecific metabolites PHBA and PHHA, we investigated specific metabolites with oxidative modifications at the alkyl side chain (3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH-*n*-BuP) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH-*iso*-BuP)) and at the aromatic ring (esters of protocatechuic acid: methyl 3,4-dihydroxybenzoate (rOH-MeP), *n*-butyl (rOH-*n*-BuP) and *iso*-butyl 3,4-dihydroxybenzoate (rOH-*iso*-BuP)) using authentic standards. A comparative metabolism and elimination scheme for the three parabens investigated is depicted in Fig. 1. The results of this study will allow extrapolating from urinary paraben (metabolite) levels to actual paraben doses taken up, to understand differences between the parabens and thus to fine-tune exposure and risk assessments based upon human biomonitoring data.

Materials and methods

Experimental design

Due to the known urinary background exposure to parabens of the general population, isotope-labeled analogs of the individual parabens were used for dosage. Three healthy volunteers (31 years old, one woman and two men, 52–82 kg body weight, born and living in Germany) were orally dosed with three individual doses of the deuterated (D4-ring-labeled) parabens MeP, *iso*- and *n*-BuP. To avoid interferences resulting from shared metabolites, administration of the different parabens was carried out at least 2 weeks apart (Soni et al. 2005; Aubert et al. 2012; Koch et al. 2014). Approximately 50 mg deuterated (D4-ring-labeled) paraben (either MeP, *iso*- or *n*-BuP) was dissolved in 5 mL ethanol, and 1 mL of this solution was spiked to coffee or tea provided to each volunteer in an edible waffle cup with a chocolate surface for breakfast. Each volunteer ingested approximately 10 mg paraben (D4-MeP: 10.07 mg;

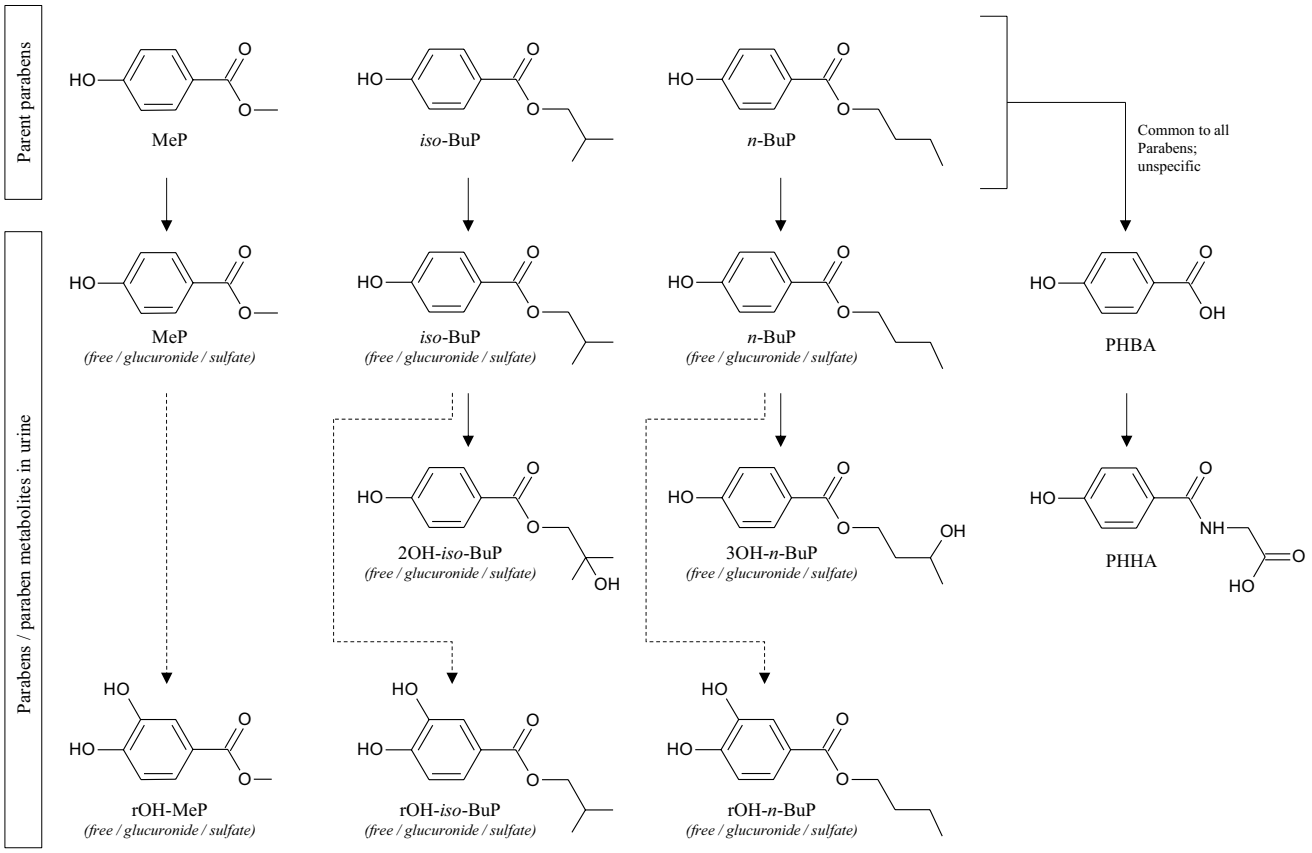

Fig. 1 Postulated human metabolism of the parabens. *Dashed lines* show the minor metabolites

D4-*n*-BuP: 10.03 mg; D4-*iso*-BuP: 9.48 mg), resulting in respective doses for the three individuals between 0.12 and 0.19 mg/kg body weight. The individual doses were below the group acceptable daily intake (ADI) of 10 mg/kg bw/day for the sum of methyl and ethyl paraben and their sodium salts. The first urine samples (T_0) were collected prior to dosage followed by consecutive and complete urine samples collected over 48 h. The volunteers recorded the time of the void of each sample. The urine volume of each individual sample was determined as the difference between the weight of the filled and the empty container. In the event a volunteer provided more than one container per void, the total volume was calculated, combined and mixed. Aliquots of the voids were stored in 15-mL polypropylene/polyethylene vessels and frozen at −18 °C within 12 h after collection, the latest. Over the whole study, we collected and analyzed a total of 251 urine samples.

The study was performed in accordance with the ethical standards of the Declaration of Helsinki (1964) and was approved by the Ethics Commission of the Ruhr University Bochum (Reg. No.: 4332-12). The participants were informed about the study design and provided written informed consent, prior to the study.

Chemicals

The ring-deuterated standards methyl 4-hydroxybenzoate-2,3,5,6-d_4 (D4-MeP), *iso*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 (D4-*iso*-BuP), *n*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 (D4-*n*-BuP) and 4-hydroxybenzoic-2,3,5,6-d_4 acid (D4-PHBA) were purchased from C/D/N Isotopes (Dr. Ehrenstorfer GmbH, Augsburg, Germany). The $^{13}C_6$-ring-labeled standards methyl 4-hydroxybenzoate, *n*-butyl 4-hydroxybenzoate and 4-hydroxybenzoic acid were purchased from Cambridge Isotope Laboratories (Wesel, Germany). Methyl 3,4-dihydroxybenzoate (rOH-MeP) and ethyl 3,4-dihydroxybenzoate (rOH-EtP) were purchased from Sigma-Aldrich (Steinheim, Germany). The unlabeled standards *n*-butyl 3,4-dihydroxybenzoate (rOH-*n*-BuP), 3-hydroxy *n*-butyl 4-hydroxybenzoate (3OH-*n*-BuP) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate (2OH-*iso*-BuP) and the labeled standards *p*-hydroxyhippuric-2,3,5,6-d_4 acid (D4-PHHA), 3-hydroxy *n*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 (D4-3OH-*n*-BuP) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 (D4-2OH-*iso*-BuP) and $^{13}C_6$-ring-labeled *p*-hydroxyhippuric acid were synthesized from Dr. Belov, Max Planck Institute for Biophysical

Chemistry, Germany. All standards had a purity ≥95 %. Deionized water was obtained using a Millipore Advantage A10 with a Quantum®-cartridge. Acetonitrile (LC/MS grade) and acetic acid (glacial, extra pure) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). ß-Glucuronidase/sulfatase enzyme type HP-2 (from Helix pomatia), ammonium acetate p.a. and ammonium bicarbonate p.a. were purchased from Sigma-Aldrich (Steinheim, Germany). ß-glucuronidase enzyme type K-12 (from Escherichia coli) was purchased from Roche Applied Science (Penzberg, Germany).

Analytical procedure

The analytical method for the determination of urinary paraben metabolites was based upon the method previously published by Moos et al. 2014 for parent parabens applying the principle of online sample cleanup and enrichment. In short, to each sample aliquot of 300, 600 µL 0.5 M ammonium acetate buffer at pH 5.0, 25 µL internal standard solution and 6 µL β-glucuronidase/arylsulfatase solution (≥100,000 units/mL) were added. After incubation at 37 °C for 3.5 h, samples were frozen overnight, subsequently thawed and centrifuged. The supernatant was injected into the HPLC system (Agilent Technologies LC1200) coupled with a tandem mass spectrometer (AB Sciex QTrap 5500). The method was modified to fit the requirements of the current study. To capture both the parent parabens and the more polar paraben metabolites (acids from breakdown and oxidatively modified parabens) in one analytical run, we had to adjust chromatography. For online cleanup and enrichment, a Cyclone™ MAX TurboFlow column (0.5 × 50 mm, Thermo Scientific) with reversed-phase (uncharged metabolites) and anion-exchange (acidic metabolites) characteristics was used in back-flush mode. Chromatographic separation was realized on an Accucore® Phenyl-X column (3 mm × 150 mm; 2.6 µm, Thermo Scientific). The gradient for transfer and chromatographic separation is given in supplementary materials table 1. Detection and quantification were performed on an AB Sciex QTrap 5500 tandem mass spectrometer in negative ionization mode (ESI−).

Furthermore, target analytes of this study carried isotope labels derived from the dosage of the D4-ring-labeled parabens. Therefore, we calibrated with respective deuterium-labeled standards and used $^{13}C_6$-labeled analogs for internal standardization, wherever possible. Calibration standards were prepared in water, and linear calibration curves were obtained with a 1/*x* weighting by plotting the quotient of peak area of each analytical standard and the peak area of the specific internal standard as a function of the concentration. All analytical standards for the target analytes, together with their respective internal standards, including mass transitions and other analyte specific parameters are given in supplementary materials table 2. For the ring-hydroxylated metabolites rOH-MeP, rOH-*n*-BuP and rOH-*iso*-BuP, the non-labeled standards were used for recording the calibration curves, because no D3-labeled standards were available. For internal standardization, we used the structurally related rOH-EtP. For the side-chain-hydroxylated metabolites D4-3OH-*n*-BuP and D4-2OH-*iso*-BuP, we used the non-labeled metabolite standards as internal standards. Product ion scans of the side-chain-oxidized metabolites 3OH-*n*-BuP and 2OH-*iso*-BuP and their deuterated analogs are shown in supplementary materials figure 1. The limits of quantification (LOQ) defined as a signal-to-noise ratio of nine were estimated to be 0.1 µg/L for PHHA, *iso*-BuP, 2OH-*iso*-BuP and 3OH-*n*-BuP and 0.25 µg/L for PHBA, MeP and *n*-BuP and 1.0 µg/L for rOH-MeP and rOH-*n*-BuP (see supplementary table 3). Relative standard deviations determined from prepared quality control material (pooled native urine) were below 7 % for all analytes for intraday precision and below 10 % for inter-day precision (see supplementary table 3). The mean relative recoveries determined from eight spiked individual urine samples (creatinine concentrations between 0.2 and 2.6 g/L) were between 91 and 116 %, with an imprecision of <8 %. Only for rOH-MeP, relative standard deviations of recovered spiked concentrations were between 16 and 28 % (see supplementary table 3). Preparation of standard stock solutions, calibration standards and quality control material were carried out as described in Moos et al. (2014).

In principle, all analytical results were generated after enzymatic hydrolyses with β-glucuronidase/sulfatase (Helix pomatia HP-2). Thus, these results represent the total concentration of the respective metabolite in urine consisting of the free form, the glucuronic acid conjugate and the sulfate conjugate ($PB_{free+glu+sul}$). To further investigate the conjugation status of the individual metabolites (parabens and oxidized metabolites) in representative 48-h pooled urine samples (prepared separately for each participant and each dosage), sample preparation was also performed without addition of enzyme to determine the concentrations of the free paraben species (PB_{free}). An additional workup with β-glucuronidase K-12 (no arylsulfatase side activity) was used to deconjugate only glucuronide metabolites and thus determine the sum of free and glucuronidated species ($PB_{free+glu}$). Via this approach, we could calculate the concentration of the glucuronide species as the difference between $PB_{free+glu}$ and PB_{free} and the concentration of sulfate species as the difference between $PB_{free+glu+sul}$ and $PB_{free+glu}$.

Statistics

For statistical analysis, we used Excel 2010 (Microsoft Corporation, Redmond, USA). The maximum concentration of the paraben metabolites in urine, the time of maximum concentration and elimination half times were determined on the creatinine-adjusted values. Elimination half times were determined from the rate constant k (half time = ln(2)/k), obtained from a first-order regression model including all urine events after exposure, up to the end of sample collection, or to the point where metabolite levels fell below the LOQ. The fraction excreted in urine (F_{ue}) was calculated using the urine collected over 48 h following exposure. The F_{ue} represents the percentages of excreted metabolites on a molar basis in relation to the orally applied dose.

Results and discussion

Using isotope-labeled parabens for the dosage study, we were able to circumvent omnipresent paraben exposure and paraben (metabolite) excretion. We were able to unambiguously identify and quantify each of the above-postulated, labeled paraben metabolites in post-dose urine samples of the metabolism study.

In Fig. 2, exemplary chromatograms are presented for each of the three separate dosing experiments, depicted (a) for D4-MeP, (b) for D4-*iso*-BuP and (c) for D4-*n*-BuP. The left column shows chromatograms of a processed standard sample with analytical standards of relevance for each paraben investigated (about 80 µg/L for each standard). The chromatograms of the middle column are from representative urine samples taken before the dose of the respective paraben. In these urine samples, none of the labeled paraben metabolites could be detected at quantifiable concentrations. Small peaks visible in the ion traces of D4-MeP, D4-*n*-BuP and D4-PHBA were introduced only after internal standard addition and thus stem from some trace level contamination of the $^{13}C_6$-labeled internal standards with $^{13}C_4$-labeled species. Concentrations, however, were distinctly below 0.1 µg/L and thus did not interfere with the quantification of the metabolites generated post-dose. Pre-dose urine samples measured without internal standards revealed no background contamination for the labeled species (chromatograms not shown). The column to the right shows chromatograms of representative urine samples taken approximately 2–3 h after oral dosage. All of the postulated paraben metabolites emerged post-dose at concentration levels well above the limit of quantification. The peaks of the side-chain-hydroxylated metabolites D4-2OH-*iso*-BuP and D4-3OH-*n*-BuP showed some small shoulders in the post-dose samples contrary to the analytical standards, indicating oxidative modifications in addition to the ω-1 position (e.g., at the terminal carbon of the alkyl side chain). Similar, additional but minor oxidative modifications of the *iso*- and *n*-butyl side chain have previously been described for the oxidative monoester metabolites of di-*n*-butyl and di-*iso*-butyl phthalate (Koch et al. 2012). Because we were not able to achieve a sufficient chromatographic separation of these shoulders from the main standard peaks, we quantified the sum (each peak was integrated over the whole elution time) of the side-chain-hydroxylated metabolites of *iso*-BuP based on the specific standard 2OH-*iso*-BuP and the sum of the side-chain-hydroxylated metabolites of *n*-BuP based on the specific standard 3OH-*n*-BuP.

The time course of elimination of the parabens after the single oral dose (on the example of one volunteer; profiles were similar for the other two volunteers) is shown in Fig. 3, separately for the three dosing experiments (A: D4-MeP, B: D4-*iso*-BuP and C: D4-*n*-BuP). For simplification, in all further data presentation and discussion, we omit specifically referring to the D4 isotope label of the parabens and their respective metabolites. The non-specific metabolites PHHA and PHBA are depicted with white markers, and all paraben specific biomarkers are depicted in black. Elimination curves are plotted on semilogarithmic scale and represent creatinine-adjusted concentration values in µg/g creatinine. In all dosing experiments, the parabens (and their metabolites, respectively) reached their maximum concentration in urine (mean of the three individuals) within the first two hours after dosing. While for MeP metabolite concentrations immediately decreased after 2 h, for *iso*-BuP and *n*-BuP metabolite concentrations remained at higher levels for a longer time (approximately 6–10 h post-dose). Thereafter, for all parabens, metabolite concentrations declined rapidly over the remaining time of the study. Elimination characteristics (maximum urinary concentration (c_{max}), time of maximum concentration (t_{max}) and estimated elimination halftimes ($t_{1/2}$)) with mean values and ranges over all three volunteers are summarized in Table 1.

Elimination halftimes were determined mathematically from the creatinine-adjusted concentrations over time (Fig. 3) via the rate constant k (halftime = ln(2)/k). Metabolites were excreted generally in at least two different phases. For the first elimination phase (see Table 1), we roughly estimated halftimes below 1 h for the MeP metabolites, between 1.3 and 2.2 h for the *n*-BuP metabolites and between 0.7 and 1.2 h for the *iso*-BuP metabolites. In the second phase, elimination halftimes are considerably longer for all metabolites. In detail, MeP was excreted with a halftime of 6.9 h, while PHBA (5.8 h), PHHA (5.7 h) and rOH-MeP (2.5 h) were excreted slightly faster. For *iso*-BuP, the elimination halftime was 3.7 h, followed by 2OH-*iso*-BuP (3.9 h), PHBA (4.4 h), rOH-*iso*-BuP (4.7 h) and PHHA (6.2 h). For *n*-BuP, the shortest elimination halftime

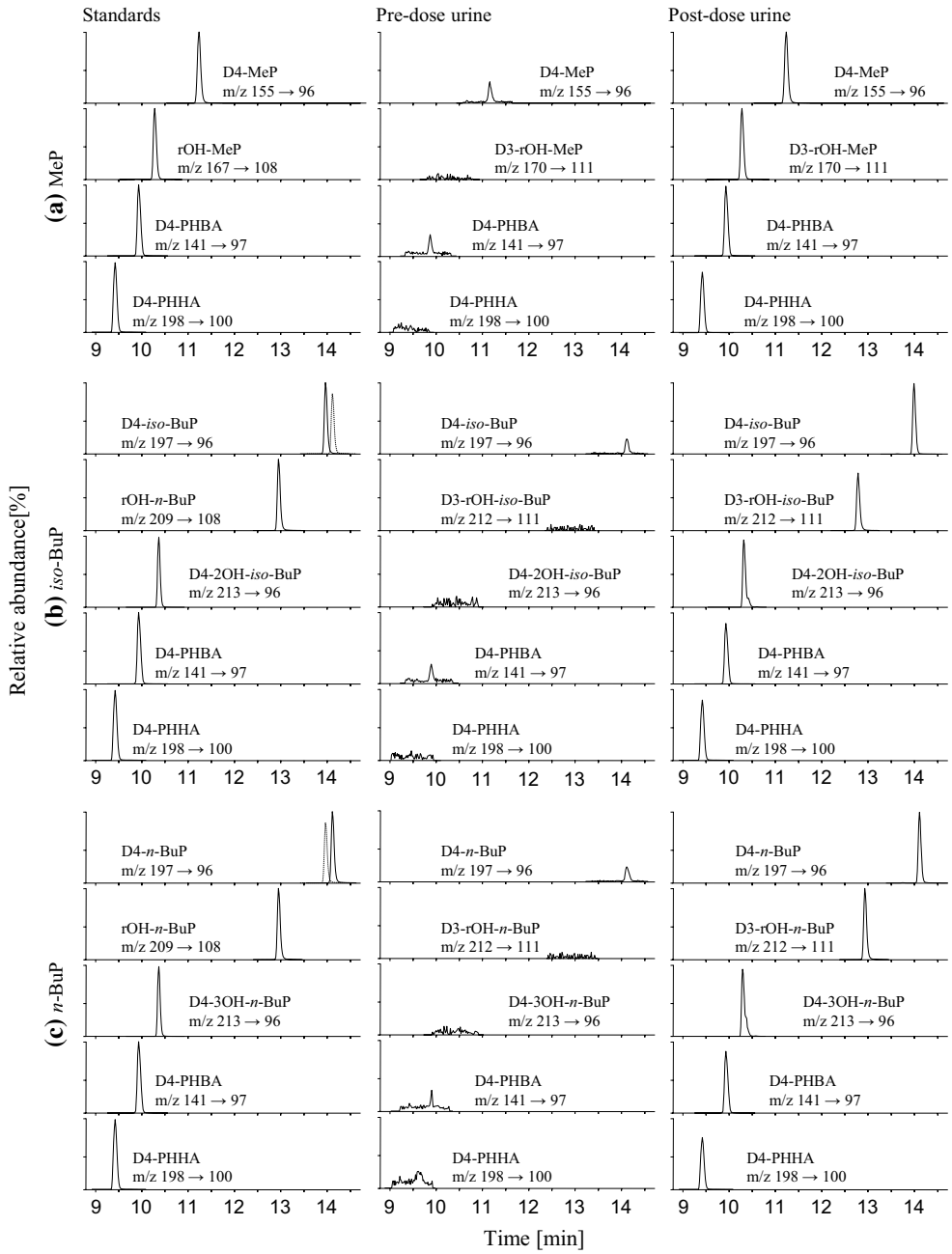

Fig. 2 Chromatograms of different urine samples from the human metabolism study of MeP, *iso*-BuP and *n*-BuP, representing the quantifier trace (*bold line*) of scheduled multiple reaction monitoring (MRM). *Left column* represents a processed calibration standard (about 80 µg/L for each analyte). The *dashed lines* represent the chromatographically separated isomers of *iso*- and *n*-BuP. *Middle column* shows urine samples taken before the dose of the respective paraben. *Right column* shows chromatograms of representative urine samples taken approximately 2–3 h after oral dosage of MeP, *iso*-BuP and *n*-BuP

was determined for 3OH-*n*-BuP with 3.3 h, followed by *n*-BuP (3.6 h), PHBA (3.7 h), PHHA (4.6 h) and rOH-*n*-BuP (4.9 h). Comparing the *n*- and *iso*-BuP metabolites, great similarities in the elimination halftime were observed.

Percentages of the urinary paraben metabolites in relation to the applied dose are summarized in Table 2 for the three dosing experiments over all three volunteers. For all three parabens investigated, we recovered more than 80 % of the dose via the above biomarkers in urine within the first 24 h. On day two post-dose, considerably smaller amounts (representing one percent or less of the dose) were excreted. Overall, within two days, 84.4 % of the MeP dose, 86.0 % of the *iso*-BuP dose and 80.8 % of the *n*-BuP dose were recovered in urine.

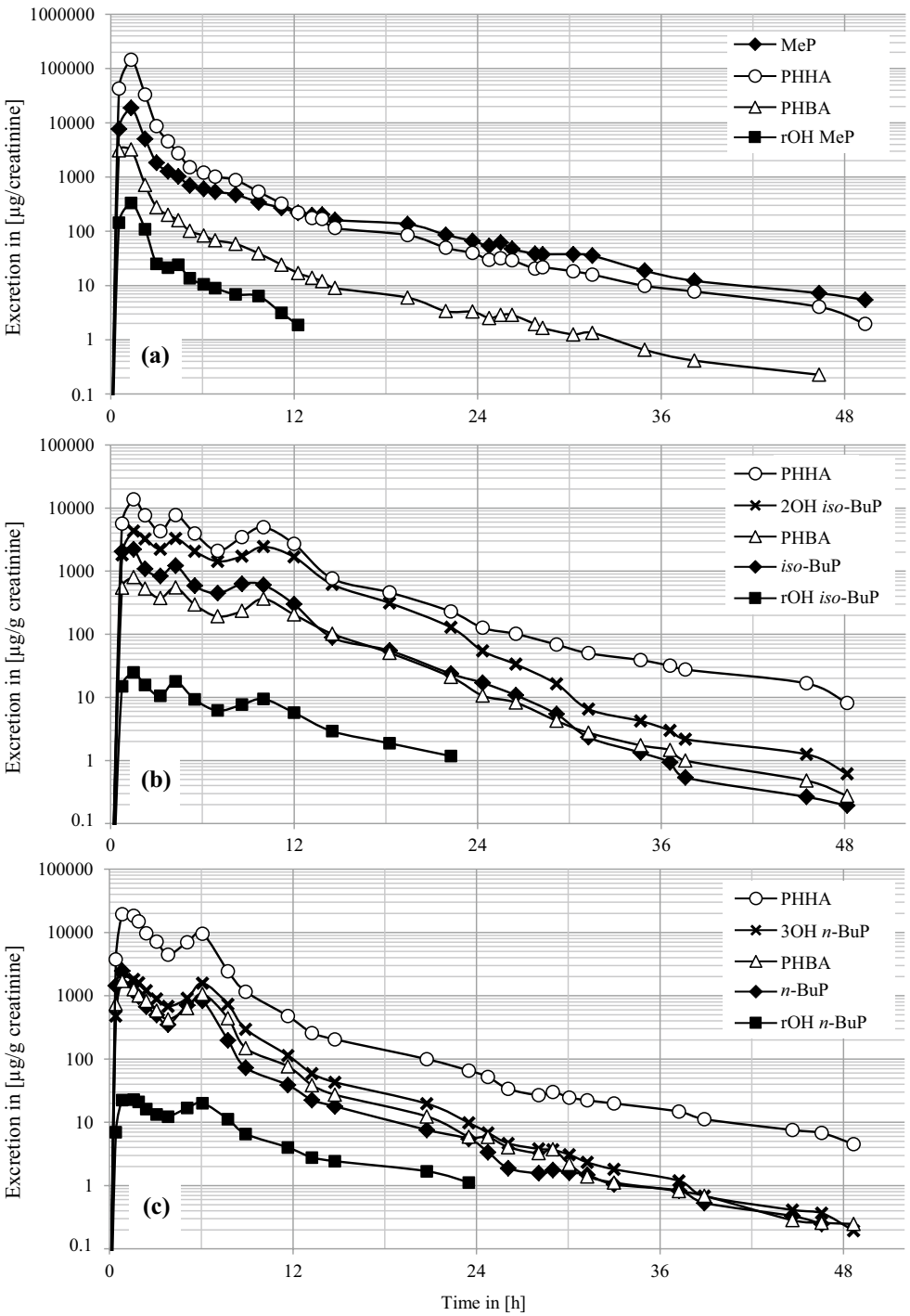

Fig. 3 Creatinine-corrected metabolite concentrations in urine after oral dosage, shown in semilogarithmic scale (continuous data from one volunteer; profiles were similar for the other two volunteers). The non-specific metabolites PHHA and PHBA are depicted with white markers, and all paraben specific biomarkers are depicted in *black*. **a** MeP metabolites, **b** *iso*-BuP and **c** *n*-BuP

The predominant metabolite for all parabens was the unspecific PHHA with a share of approximately 60 %. Between 3.0 and 7.2 % of the doses were excreted as the other unspecific metabolite PHBA. The parent paraben (after hydrolysis, thereby representing the sum of free and conjugated paraben) made up 17.4 % of the dose for MeP, followed by 6.8 % for *iso*-BuP and 5.6 % for *n*-BuP. These results indicate that the share of parent paraben (free plus conjugated) excreted in urine considerably decreases with increasing chain length of the alkyl moiety of the paraben. One reason for this effect might be that MeP, due to better water solubility, can be excreted much easier in urine than the more lipophilic butyl parabens. Apart from conjugation, further metabolic (oxidative) modifications might be needed to increase the water solubility of the longer chain parabens. As a logical consequence, we found significant amounts of the side-chain-oxidized metabolites of *iso*- and *n*-BuP. These

Table 1 Maximum urinary concentration (c_{max}), time of maximum concentration (t_{max}) and estimated elimination half times ($t_{1/2}$) for MeP, *iso*-BuP and *n*-BuP metabolites, as the mean of the three volunteers (range in brackets)

Dosage	Biomarker	Maximum urinary concentration c_{max} (mg/L)	Time of maximum t_{max} (h)	Estimated elimination half-time $t_{1/2}$ phase 1 (h)	Estimated elimination half-time $t_{1/2}$ phase 2 (h)
MeP	MeP	12.6 (5.7–18.8)	1.3 (0.8–1.7)	0.8 (0.6-1.2)	6.9 (6.1–7.3)
	rOH-MeP	0.2 (0.1–0.3)	1.3 (0.8–1.7)	0.9 (0.8–0.9)	2.5 (2.3–2.7)
	PHHA	101.8 (56.8-144.4)	1.0 (0.8–1.3)	0.6 (0.5–0.7)	5.7 (4.8–6.5)
	PHBA	2.6 (1.9–3.2)	1.0 (0.8–1.3)	0.7 (0.6–0.8)	5.8 (4.4–6.8)
iso-BuP	*iso*-BuP	4.7 (2.2–9.7)	1.0 (0.8–1.5)	0.7 (0.4–1.1)	3.7 (3.3–4.2)
	2OH-*iso*-BuP	7.3 (4.6–12.1)	1.2 (0.8–1.5)	1.5 (1.1–1.9)	3.9 (3.4–4.4)
	rOH-*iso*-BuP	0.1 (0.02–0.2)	1.2 (0.8–1.5)	1.1 (1.1–1.1)	4.7 (4.1–5.1)
	PHHA	43.2 (13.6–92.2)	1.2 (0.8–1.5)	1.1 (0.8–1.3)	6.2 (4.4–8.9)
	PHBA	2.5 (0.8–5.3)	1.2 (0.8–1.5)	1.2 (0.8–1.6)	4.4 (3.9–5.4)
n-BuP	*n*-BuP	3.2 (2.5–3.7)	0.8 (0.7–0.8)	1.3 (1.1–1.6)	3.6 (2.6–4.4)
	3OH-*n*-BuP	2.5 (1.8–2.9)	1.3 (0.8–1.7)	1.5 (1.3–1.9)	3.3 (2.6–3.9)
	rOH-*n*-BuP	0.1 (0.02–0.3)	1.5 (1.3–1.7)	2.2 (2.0–2.4)	4.5 (3.1–5.7)
	PHHA	33.6 (19.4–51.3)	1.3 (0.8–1.7)	1.3 (1.0–1.5)	4.6 (2.8–5.7)
	PHBA	2.1 (1.7–2.6)	1.3 (0.8–1.7)	1.6 (1.5–2.0)	3.7 (2.9–4.3)

Table 2 Mean values and ranges of urinary excretion factors of the three volunteers (in % of the dose, on a molar basis) of MeP, *iso*-BuP and *n*-BuP metabolites

Dosage	Biomarker	Percentage of applied dose between 0 and 24 h (%)	Percentage of applied dose between 24 and 48 h (%)	Percentage of applied dose between 0 and 48 h (%)
MeP	MeP	16.8 (15.3–18.3)	0.6 (0.3–0.9)	17.4 (15.5–19.2)
	rOH-MeP	0.1 (0.1–0.25)	0.0 (–)	0.1 (0.1–0.25)
	PHHA	63.5 (59.8–68.1)	0.3 (0.1–0.5)	63.8 (60.3–68.2)
	PHBA	3.0 (2.7–3.2)	0.0 (–)	3.0 (2.7–3.2)
	Over all Σ	83.4 (81.2–86.8)	0.9 (0.4–1.4)	84.4 (82.6–87.2)
iso-BuP	*iso*-BuP	6.7 (5.7–8.3)	0.0 (–)	6.8 (5.7–8.4)
	2OH-*iso*-BuP	15.8 (9.9–21.3)	0.1 (0.0–0.1)	15.8 (9.9–21.5)
	rOH-*iso*-BuP	0.2 (0.1–0.3)	0.0 (–)	0.2 (0.1–0.4)
	PHHA	56.7 (48.3–65.1)	0.5 (0.2–0.7)	57.2 (49.0–65.3)
	PHBA	6.0 (5.2–6.5)	0.0 (0.0–0.1)	6.0 (5.3–6.6)
	Over all Σ	85.3 (83.3–88.0)	0.6 (0.3–1.0)	86.0 (84.3–88.3)
n-BuP	*n*-BuP	5.6 (5.2–6.4)	0.0 (–)	5.6 (5.2–6.4)
	3OH-*n*-BuP	5.8 (4.5–7.1)	0.0 (–)	5.8 (4.5–7.1)
	rOH-*n*-BuP	0.3 (0.1–0.8)	0.0 (–)	0.3 (0.1–0.8)
	PHHA	61.6 (54.7–72.1)	0.2 (0.0–0.3)	61.8 (55.0–72.1)
	PHBA	7.2 (6.9–7.5)	0.0 (–)	7.2 (7.0–7.5)
	Over all Σ	80.5 (74.6–89.7)	0.2 (0.1–0.4)	80.8 (75.1–89.8)

specific, side-chain-oxidized metabolites 2OH-*iso*-BuP and 3OH-*n*-BuP represent 15.8 % and 5.8 % of the dose, respectively, and are surpassing the shares of the parent parabens excreted in urine. Therefore, these side-chain-oxidized metabolites represent important, novel and specific biomarkers for butyl paraben exposure that are—contrary to the parent parabens—not prone to external contamination. The extent of oxidative modification is considerably higher for *iso*-BuP than for *n*-BuP (factor of 2.7). We have made a similar observation in our previous study comparing di-*iso*-butyl and di-*n*-butyl phthalate metabolism. For these two phthalates, the extent of oxidative modification of the *iso*form was 2.5 times higher than of the *n*-form (Koch et al. 2012). We also detected the postulated ring-oxidized metabolites for all three parabens (rOH-MeP, rOH-*iso*-BuP and rOH-*n*-BuP) (Wang

Table 3 Mean values and ranges of urinary concentrations of the three volunteers (in %; total concentration of the respective metabolites was set to 100 %) of the free, glucuronidated and sulfated conjugates in 48-h pooled urine samples

	Percentage of total amount* of respective metabolites mean (range)
MeP, free	7.1 (2.6–12.6)
MeP, glucuronide	30.4 (22.7–34.7)
MeP, sulfate	63.4 (61.5–67.8)
iso-BuP, free	0.8 (0.4–1.1)
iso-BuP, glucuronide	89.2 (77.3–97.5)
iso-BuP, sulfate	12.2 (6.1–22.1)
n-BuP, free	1.0 (0.4–2.0)
n–BuP, glucuronide	87.2 (83.0-90.3)
n-BuP, sulfate	13.5 (9.6–19.6)
2OH-*iso*-BuP, free	1.7 (1.2–2.1)
2OH-*iso*-BuP, glucuronide	46.0 (25.5–60.4)
2OH-*iso*-BuP, sulfate	53.3 (39.3–73.2)
3OH-*n*-BuP, free	2.8 (0.4–6.9)
3OH-*n*-BuP, glucuronide	63.7 (51.4–77.3)
3OH-*n*-BuP, sulfate	34.2 (22.1–42.9)

* Determined after hydrolyses with glucuronidase/arylsulfatase HP2, set to 100 %

and Kannan 2013). However, their peak concentrations and their urinary excretion fractions were negligibly low (below 1 %), which suggests that these are of limited use as biomarkers to detect exposures to these specific parabens. Furthermore, analogous structures to these ring-oxidized metabolites have been reported to be occurring naturally, so-called alkyl protocatechuates, e.g., in wine (Baderschneider and Winterhalter 2001) and peanut seed coat (Huang et al. 2003).

In addition to elimination kinetics and urinary metabolite excretion fractions, we examined the conjugation status and distribution of glucuronide, sulfate and free paraben species (see Table 3). Interestingly, the glucuronide of MeP represented only 30 % of total urinary MeP, while the glucuronide was dominant for the butyl parabens (89 % for *iso*-BuP and 87 % for *n*-BuP). In reverse order, the sulfate conjugate represented 64 % of total MeP and only between 12 and 13 % for the butyl parabens, with some variation between the three individuals. These findings point out the necessity of using enzymes with deconjugation properties for both glucuronides and sulfates (preferably from Helix pomatia) in order to correctly capture the total amount of parabens excreted in urine. For all three parabens investigated, only small percentages were excreted as the free paraben species (7.1 % for MeP, 0.8 % for *iso*-BuP and 1.0 % for *n*-BuP). These findings from our controlled dosage study confirm previous population studies in regard to the distribution of individual conjugates and in regard to the decreasing share of free paraben excreted in urine with increasing chain length (Ye et al. 2006; Guidry et al. 2015). The proportions of free paraben in general population samples or individual samples with high total paraben levels could be used to identify possible external contamination either in the pre-analytical or analytical phase. The share of free paraben (in combination with total paraben) should therefore generally be checked in all samples to exclude external paraben contamination during sample collection or storage (Moos et al. 2015; Guidry et al. 2015; Ye et al. 2013; Longnecker et al. 2013).

Conclusions

This is the first study to investigate metabolism and elimination kinetics of parabens in humans after oral dosage. The urinary metabolite excretion factors for the individual parabens are essential to evaluate and quantify exposure based upon human biomonitoring measurements. The most immediate benefit of the data will be the estimation of daily intakes of the individual parent parabens based upon urinary biomarker concentrations. Currently, biomonitoring-based internal exposure data are mainly restricted to the analyses of parent parabens (after hydrolyses) in urine. For the parabens MeP, *iso*-BuP and *n*-BuP, we provide valuable metabolic conversion factors to extrapolate from urinary levels to daily intake. Our study shows that urinary excretion factors for the butyl parabens (5.6–6.8 %) are considerably smaller than for MeP (17.4 %). In consequence, this means that similar urinary concentrations of MeP and the butyl parabens extrapolate to daily intakes that are approximately three times higher for the butyl parabens. In other words, a mere comparison of urinary paraben levels considerably underestimates butyl paraben exposure compared with MeP exposure.

Furthermore, the results of our study indicate that the fraction of parent paraben excreted in urine generally decreases with increasing molecular weight (increasing length of the alkyl side chain). We have investigated parabens with one (MeP) and four (butyl parabens) carbon atoms in their alkyl chain. Assuming that parent paraben excretion of the other parabens (like ethyl paraben and propyl paraben) follows a similar pattern, we would roughly and preliminarily postulate a urinary excretion fraction of 13–14 % for ethyl paraben (two carbon atoms) and 9–10 % for propyl paraben (three carbon atoms). Whether unchanged parabens with higher molecular weights (like benzyl, hexyl and heptyl paraben) are excreted in urine at all and, in consequence, whether these (unchanged) parabens in urine are valid biomarkers of exposure for these parabens remain to be investigated.

In addition to the parent parabens, we identified new and specific oxidized metabolites. While the use of the ring-oxidized metabolites as specific biomarkers of paraben exposure is questionable (low share in the metabolite spectrum and possible natural sources), the oxidized side-chain metabolites 2OH-*iso*-BuP and 3OH-*n*-BuP represent an important and not negligible part of the exposure excreted in urine, even surpassing the share of the parent parabens excreted in urine. The fact that these oxidized metabolites represent a sufficiently high share of the paraben dose and that they are not prone to pre-analytical and analytical contaminations make these metabolites important and valid biomarkers that might be used in future human biomonitoring studies investigating paraben exposure.

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