Human-Biomonitoring von Parabenen: Entwicklung und Anwendung analytischer Methoden zur Untersuchung des Humanmetabolismus und zum Expositionsmonitoring

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Rebecca Katharina Moos

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- Koch HM, Aylward LL, Hays SM, Smolders R, Moos RK, Cocker J, Jones K, Warren N, Levy L, Bevan R. (2014) Inter- and intra-individual variation in urinary biomarker concentrations over a 6-day sampling period. Part 2: personal care product ingredients. Toxicology Letters 231(2):261-9. doi: 10.1016/j.toxlet.2014.06.023.

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- International Society of Exposure Science (22th annual Meeting) Human Biomonitoring of Parabens and other Phenols in the General German Population (Poster). Moos, Rebecca K.; Modick, Hendrik; Angerer, Jürgen; Brüning, Thomas; Koch, Holger M. Seattle 2012.

- Environment and Health (Conference of ISEE, ISES and ISIAQ) Parabens and other phenols in urine samples of the German Environmental Specimen Bank (ESBHum) from 1995 to 2012: a retrospective exposure assessment. R.K. Moos, J. Angerer, M. Kolossa-Gehring, A. Conrad, T. Brüning, H.M. Koch. Basel 2013.

- Gordon Research Conference: Environmental Endocrine Disruptors. Differences in human metabolism of parabens: implications for urinary parabens used as biomarkers of exposure. Rebecca K. Moos, Jürgen Angerer, Thomas Brüning, Holger M. Koch. Lucca 2014.

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Inhaltsverzeichnis

Einleitung

Human-Biomonitoring

Unter Human-Biomonitoring (HBM) versteht man die quantitative Bestimmung von Fremdstoffen und/oder deren Metaboliten in biologischen Materialien (Belastungsmonitoring) oder von biochemischen bzw. biologischen Veränderungen (Effektmonitoring). Ziel des Belastungsmonitorings ist die Erfassung der individuellen inneren Belastung; diese erlaubt neben der Beschreibung und dem Vergleich der unterschiedlichen Belastungen auch die Ableitung und Bewertung der daraus resultierenden Risiken oder Gefährdungen für die menschliche Gesundheit. Im Gegensatz zum Umgebungsmonitoring bzw. Ambient-Monitoring (z.B. Messung der Stoffkonzentration in der Luft am Arbeitsplatz oder in der Umwelt) erfasst das HBM die tatsächliche innere, kumulative Belastung, die gleichzeitig aus verschiedenen Expositionsquellen stammen und über verschiedene Wege (dermal, inhalativ und oral) aufgenommen worden sein kann. Hinsichtlich der Beurteilung der individuellen aber auch der kollektiven Belastungen hat das HBM eine erheblich größere Aussagekraft gegenüber der rein quellenbezogenen Erfassung der äußeren Belastung und hat sich in den vergangenen Jahrzehnten als ein wichtiges und zusätzliches Instrument zur Risikoabschätzung und zum Risikomanagement etabliert. Einflussfaktoren auf das HBM ergeben sich zwangsläufig aus den biologischen Halbwertszeiten der untersuchten Stoffe (Eliminationskinetik) und vorhandener inter- und intraindividueller Variabilitäten. Ebenfalls erlauben die Untersuchungsergebnisse zunächst keine Rückschlüsse auf die jeweilig relevanten Expositionsquellen oder Aufnahmewege (Triebig 2012).

In arbeits- und umweltmedizinischen Fragestellungen werden für die quantitative Bestimmung der inneren Belastung überwiegend Blut- oder Urinproben als Analysenmaterial verwendet. Daneben können auch andere biologische Matrices wie Humanmilch, Haare, Nägel, Atemkondensat, Gewebeproben etc. für die Messung einer inneren Belastung herangezogen werden. Dabei ist die Wahl des geeigneten biologischen Materials unter anderem abhängig von der zur Verfügung stehenden Untersuchungsmethode, den untersuchten Stoffen und deren pharmakokinetischen Eigenschaften (Triebig 2012). Für persistente Chemikalien mit langen biologische Halbwertszeiten und geringer Ausscheidung (häufig mit einer Anreicherung im Fettgewebe verbunden) stellt Blut das klassische Analysenmaterial dar. Stoffe, die eine relativ kurze biologische Halbwertszeit im

menschlichen Organismus aufweisen (nicht-persistente Chemikalien) und schnell über den Urin ausgeschieden werden, liegen oft nur in geringen Konzentrationen im Blut vor.

Für diese Stoffe ist Urin häufig das Analysenmaterial der Wahl. Darüber hinaus bietet Urin den Vorteil, dass er nicht-invasiv zugänglich ist und in ausreichender Menge zur Verfügung steht (Needham et al. 2007).

Die Verwendung von Urin als Analysenmaterial erfordert bei der Interpretation der Analysenergebnisse die Berücksichtigung des individuellen Hydratationsstatus, d.h. die diuresebedingte Verdünnung der Probe. In den meisten HBM-Studien wird hierfür der Kreatiningehalt des Urins herangezogen. Kreatinin ist ein Stoffwechselprodukt, welches mit einer relativ konstanten Rate über die Nieren an den Urin abgegeben wird. Durch den Bezug der renal ausgeschiedenen Stoff- und Metabolitkonzentrationen auf den Kreatiningehalt können diuresebedingte Schwankungen kompensiert werden und ermöglichen den Vergleich einzelner Urinproben innerhalb einer Studienpopulation. Dabei unterliegt die Kreatinin-ausscheidung ebenfalls individuellen Schwankungen (unter anderem abhängig von Alter, Geschlecht, Gewicht, Muskelmasse etc.), deshalb sollten für die Bewertung von HBM-Daten sowohl die gemessenen Konzentrationen wie auch die Kreatinin-normierten Werte berücksichtig werden. Neben dem Kreatiningehalt als Korrekturfaktor, kann auch das spezifische Gewicht des Urins (auch Urindichte) als Bezugsgröße herangezogen werden. Das spezifische Uringewicht ist, wie der Kreatiningehalt, ein Maß für den Verdünnungsgrad einer Urinprobe und unter anderem abhängig von der Flüssigkeitsaufnahme (Triebig 2012).

Für umweltmedizinische Fragestellungen ist das HBM ein wichtiges und leistungsstarkes Werkzeug zur Bestimmung innerer Belastungen gegenüber Fremdstoffen. Die Interpretation und Bewertung einer Fremdstoffbelastung erfordert das Vorhandensein einheitlicher Beurteilungswerte. Der sogenannte Referenzwert eines Stoffes (RV95) ist ein rein statistisch abgeleiteter Wert, ohne toxikologische Aussagekraft. Er wird definiert als das innerhalb des 95%-Konfidenzintervall gerundete 95. Perzentil der Messwerte einer Stoffkonzentration in einem bestimmten Körpermedium, einer möglichst repräsentativen Bevölkerungsstichprobe (HBM-UBA 1996). Referenzwerte dienen der Beschreibung der aktuellen Hintergrund-belastung einer Bevölkerungsgruppe und erlauben die Bewertung individueller Belastungen einzelner Personen im Vergleich zur Normalbevölkerung, sowie die Identifizierung besonders belasteter Bevölkerungsgruppen.

Die Belastung der Allgemeinbevölkerung unterliegt zeitlichen Veränderungen, z.B. durch Änderungen gesetzlicher Höchstmengen oder ein generelles Verbot bestimmter Stoffe. Dementsprechend gilt der Referenzwert nur für den untersuchten Zeitraum und sollte regelmäßig überprüft und gegebenenfalls angepasst werden (Triebig 2012; Angerer et al. 2011). Im Gegensatz zu den Referenzwerten ist durch die Verwendung der HBM-Werte (HBM-I- und HBM-II-Wert) eine gesundheits- bzw. risikobezogene Bewertung individueller Belastungen möglich (HBM-UBA 2014). Diese Werte (ausgedrückt als Biomarker-Konzentration in Blut oder Urin) werden basierend auf epidemiologischen und toxikologischen Untersuchungen abgeleitet. Bei Substanzen, für die keine Studien zu relevanten biologischen Wirkungen am Menschen verfügbar sind, erfolgt die Ableitung anhand gesundheitsbezogener Grenzwerte (z.B. Acceptable daily intake, ADI oder Tolerable daily intake, TDI, bzw. direkt aus Tierversuchsdaten anhand des NO(A)EL-Wertes (No Observed (Adverse) Effect Level, Dosis ohne beobachtete (schädigende) Wirkung). Der HBM-I-Wert entspricht der Konzentration eines Stoffes bei der nicht von einem gesundheitlichen Risiko auszugehen ist (kein Handlungsbedarf). Der Bereich zwischen HBM-I- und HBM-II-Wert entspricht einem Prüf- oder Kontrollbereich, in dem positive Befunde zu prüfen sind (dauerhafte oder zufällige Erhöhung) und der Grund für die Erhöhung zu untersuchen ist. Bei Messwerten oberhalb des HBM-II-Werts ist von einer als relevant anzusehenden gesundheitlichen Beeinträchtigung des Betroffenen auszugehen (Interventions- und Maßnahmenwert). Referenz- und HBM-Werte werden von der Kommission „Human-Biomonitoring“ des Umweltbundesamtes abgeleitet und veröffentlicht (Triebig 2012; Angerer 2007, 2011).

Die Untersuchung von Stoffen in menschlichen Körperflüssigkeiten wurde erstmals in der Arbeitsmedizin verwendet. Die Bestimmung von Blei (Kehoe et al. 1933) und Benzol (Yant et al. 1936) in Blut und Urin sind frühe Beispiele für die Verwendung des HBMs für die Untersuchung von Belastungen am Arbeitsplatz (Triebig 2012; Angerer et al. 2007). Mit zunehmender Produktvielfalt der letzten Jahrzehnte und den stetig wachsenden Anforderungen an die Produkte steigt auch die Zahl der Fremdstoffe, mit denen der Mensch täglich in Berührung kommt. Immer häufiger stehen diese Umweltchemikalien wegen ihrer potentiell schädlichen Wirkung auf die menschliche Gesundheit im Mittelpunkt der Diskussionen. Es gibt eine Vielzahl standardisierter Analysenvorschriften (z.B. Arbeitgruppe „Analysen im biologischen Material“ (AibM) der Kommission zur Prüfung gesundheits-

schädlicher Arbeitsstoffe der Deutschen Forschungsgemeinschaft, DFG) und leistungsstarker Analysemethoden, die ständig aktualisiert und den Anforderungen angepasst werden. Die Untersuchung und Bewertung von Umweltchemikalien mit kurzen biologischen Halbwertszeiten stellt dabei eine besondere Herausforderung dar. Neben verschiedenen Umweltchemikalien wie Weichmacher (Phthalate, 1,2-Cyclohexandicarbonsäurediisononylester und Diethylhexylterephthalat), Schmerzmitteln, Pestiziden etc. sind auch Konservierungsstoffe inzwischen fester Bestandteil von HBM-Studien.

Ester der p-Hydroxybenzoesäure - Parabene

Konservierungsstoffe sind antimikrobiell wirksame Substanzen, die verschiedensten Produkten zugesetzt werden, um deren Haltbarkeit zu verlängern. Sie hemmen das Wachstum von Mikroorganismen und schützen dadurch vor dem Verderb. Die Ester der *p*-Hydroxybenzoesäure (Parabene) und deren Natriumsalze werden seit vielen Jahrzehnten als Konservierungsstoffe in kosmetischen Produkten, Konsumgütern, in der pharmazeutischen Industrie, sowie in bestimmten Lebensmitteln (E214 bis E219) eingesetzt. Dabei unterscheiden sich die einzelnen Parabene lediglich in der Länge und dem Verzweigungsgrad der Seitenkette am Alkoholrest des Esters (**Abbildung 1)**.

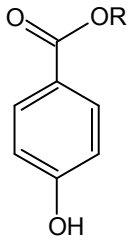

Abbildung 1 Grundstruktur der Parabene (Ester der *p*-Hydroxybenzoesäure). R: Alkyl- oder Arylrest.

Die am häufigsten verwendeten Parabene sind Methyl- (MeP), Ethyl- (EtP), *n*-Propyl (*n*-PrP) und *n*-Butylparaben (*n*-BuP). Die verzweigten Isomere von Propyl- (*iso*-PrP) und Butylparaben (*iso*-BuP), sowie Benzylparaben (BeP), werden seltener eingesetzt (Jewell et al. 2007; Soni et al. 2005). Oft werden Parabene auch in Kombination verwendet, um ihre antimikrobielle Wirksamkeit zu verstärken (Soni et al. 2005). Parabene besitzen ein breites Wirkungsspektrum (gegen Schimmelpilze, Hefen und Bakterien) und sind schon in geringen Konzentrationen antimikrobiell wirksam. Ihre Wirksamkeit nimmt mit der Anzahl der Kohlenstoffatome und dem Verzweigungsgrad der Seitenkette zu (Boberg et al. 2010). Parabene sind zudem über einen weiten pH- und Temperaturbereich chemische stabil und wirksam (Belitz 2008).

Überdies besitzen sie eine geringe systemische Toxizität und im Vergleich mit anderen Konservierungsstoffe ein deutlich geringeres allergenes Potenzial (BfR 2011, EFSA 2004). Neben diesen Eigenschaften haben auch geringe Produktionskosten zu der Popularität der Parabene beigetragen (Błędzka et al. 2014; Soni et al. 2005). In den USA waren zwischen 2005 und 2014 vier Parabene unter den fünf meist verwendeten Konservierungsstoffe (basierend auf den bei der FDA (U.S. Food and Drug Administration) registrierten kosmetischen Produkten)(Steinberg 2008, 2010, 2016). Für den europäischen Markt gibt es nur begrenzte Informationen über Produktionszahlen und/oder Verbrauchsmengen. Es ist jedoch davon auszugehen, dass der Einsatz in Europa vergleichbar mit dem in den USA ist. Laut ECHA (Europäische Chemikalienagentur) lagen die Produktions- und Importzahlen im Jahr 2015 für MeP zwischen 1000-10.000 Tonnen und für EtP und *n*-PrP im Bereich von 100-1000 Tonnen (ECHA 2016).

Seit dem Aufkommen wissenschaftlicher Studien zur potentiellen endokrinen Wirksamkeit von Parabenen, wird auch deren Einsatz als Konservierungsstoffe kontrovers diskutiert. Während die *p*-Hydroxybenzoesäure und kurzkettige Parabene inzwischen als relativ sicher in ihrer Verwendung gelten, stehen besonders die längerkettigen Parabene im Mittelpunkt der Diskussionen. *In vitro* und *in vivo* Studien zeigten, dass Parabene eine schwache estrogene Wirkungen besitzen, wobei diese Wirkung mit steigender Seitenkettenlänge (Blair et al. 2000; Routledge et al. 1998; Byford et al. 2002) und dem Verzweigungsgrad (Okubo et al. 2001; Darbre et al. 2002; Vo et al. 2010) in der Alkylkette zunimmt. Neben der estrogenen Wirkung wurden auch antiandrogene Effekte (Satoh et al. 2005; Chen et al. 2007; Kjærstad et al. 2010) und uterotrophe Effekte (Lemini et al. 2003) nachgewiesen. Studien zur Reproduktionstoxizität bei Nagetieren zeigten Auswirkungen auf die Spermienzahl und den Testosteronspiegel (Oishi 2001, 2002a, 2002b, 2004). Darüber hinaus befassen sich aktuelle Diskussionen auch vermehrt mit der kumulativen Toxizität der verschiedenen Parabene untereinander und in Kombination mit anderen endokrinen Disruptoren (Boberg et al. 2010; Ma et al. 2014; Kjærstad et al. 2010; Christiansen et al. 2012). Die zur Verfügung stehenden Studien zeigen ein eher heterogenes Bild der toxikologischen Befunde. Einige der oben beschriebenen Effekte konnten in anderen Studien nicht reproduziert oder bestätigt werden (z.B. Hobermann et al. 2008; Shaw and deCatanzaro 2009; Übersichtsartikel von Błędzka et al. 2014; Boberg et al. 2010).

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Seit 2005 werden, unter Einbezug aktueller Studien, vom Wissenschaftlichen Ausschuss für Verbrauchersicherheit der Europäischen Kommission (SCCS, Scientific Committee on Consumer Safety früher SCCP, Scientific Committee on Consumer Products), regelmäßig Stellungnahmen zur toxikologischen Bewertung von Parabenen veröffentlicht (SCCP 2005a, 2005b, 2006, 2008, 2011a, 2011b, 2013). Unter anderem wurde durch den SCCS die Datenlage für einen Zusammenhang zwischen der Anwendung parabenhaltiger kosmetischer Produkte und dem Auftreten von Brustkrebs als nicht ausreichend erachtet (SCCS 2005b).

In einer Stellungnahme aus dem Jahr 2011 erklärte der SCCS den Einsatz von MeP und EtP innerhalb der gesetzlich erlaubten Höchstmengen von 0,4% (als einzelner Ester) und 0,8% als Estergemische als sicher. Für die längerkettigen Parabene wie *n*-PrP und *n*-BuP konnte aufgrund erheblicher Mängel bei der Durchführung reproduktionstoxischer Studien an Nagetieren bislang kein NO(A)EL-Wert abgeleitet werden. Da ordnungsgemäß durchgeführte dermale- und/oder toxikokinetische Studien am Menschen fehlen, wurde seitens des SCCS eine konservative Risikobewertung durchgeführt. In der entsprechenden Stellungnahme empfahl der SCCS die Senkung der zugelassenen Höchstmengen von 0,4% auf 0,19% (als einzelner Ester) für die Verwendung von *n*-PrP und *n*-BuP in kosmetischen Mitteln, unter Verwendung eines NOEL (No observed effect level, Dosis ohne Anzeichen einer Wirkung) von 2 mg/kg KG/Tag (Milligramm pro Kilogramm Körpergewicht pro Tag) für *n*-BuP, basierend auf Reproduktionstoxizitätsstudien bei Nagetieren (SCCS 2011a). Für die Risikobewertung von *iso*-PrP, *iso*-BuP, Phenylparaben (PhP), BeP- und Pentylparaben (PeP) lagen nur begrenzte oder gar keine Informationen vor, so dass ein von diesen Verbindungen ausgehendes Risiko für den Menschen nicht bewertet werden konnte. Im selben Jahr verbot die dänische Regierung im Zuge ihrer nationalen Gesetzgebung *n*-PrP und *n*-BuP und deren verzweigte Isomere in Körperpflegeprodukten für Kinder bis zu drei Jahren (SCCS 2011b). Im Jahr 2014 wurden die Empfehlungen des SCCS auch von der Europäischen Union umgesetzt, indem die zulässigen Höchstmengen von *n*-PrP und *n*-BuP in kosmetischen Mitteln von 0,4% auf 0,19% gesenkt und deren Einsatz in kosmetischen Mitteln für die Anwendung im Windelbereich von Kindern unter drei Jahren verboten wurde. Die Höchstkonzentration für Estergemische von 0,8% wurde beibehalten (Europäisches Parlament 2014a). Darüber hinaus wurde der Einsatz von *iso*-PrP, *iso*-BuP, PhP, PeP und BeP sowie derer Salze in der EU verboten (Europäisches Parlament 2014b). Bereits 2006 wurde aufgrund einer Stellungnahme der Europäischen Behörde für Lebensmittelsicherheit (EFSA, European Food Safety Authority) *n*-PrP aus der Liste der für Lebensmittel

zugelassenen Zusatzstoffe gestrichen. Der bis dahin geltende Gruppen-ADI-Wert von 0-10 mg/kg KG/Tag, für die Summe aus MeP, EtP und *n*-PrP und ihrer Natriumsalze, wurde für *n*-PrP ausgesetzt. Grund hierfür war, dass *n*-PrP bei geringen Dosen Auswirkungen auf die Spermienproduktion bei männlichen Jungratten hatte (EFSA 2004; Europäisches Parlament 2006). Die Europäische Arzneimittel-Agentur (EMA, European Medicines Agency) veröffentlichte 2015 einen PDE (Permitted Daily Exposure, Erlaubte tägliche Exposition)-Wert von 2 mg/kg KG/Tag für die Verwendung von PrP in Arzneimitteln bei Erwachsenen und pädiatrischen Patienten, basierend auf einem NOEL von 100 mg/kg KG/Tag. Der von der EFSA festgelegte ADI für MeP von 10 mg/kg KG/Tag ist auch für den Einsatz in Arzneimitteln anwendbar (EMA 2015).

Parabene werden nach der Aufnahme schnell durch unspezifische Esterasen hydrolisiert. Die entstandene *p*-Hydroxybenzoesäure wird dann an Sulfat, Glucuronsäure oder Glycin (Reaktionsprodukt: *p*-Hydroxyhippursäure) gebunden. Sowohl die *p*-Hydroxybenzoesäure wie auch die hieraus gebildete *p*-Hydroxyhippursäure (Hauptmetabolit) sind gemeinsame und demzufolge unspezifische Metaboliten der Parabene (Boberg et al. 2010; Soni et al. 2005; Abbas et al. 2010; Janjua et al. 2008). Nur relativ geringe Mengen werden als Mutterparabene über den Urin ausgeschieden, wobei der überwiegende Teil (über 90%) in konjugierter Form, d.h. gebunden an Sulfat oder Glucuronsäure, vorliegt (Aubert et al. 2012; Ye et al. 2006; Boberg et al. 2010).

HBM-Studien haben gezeigt, dass die Allgemeinbevölkerung diesen Stoffen permanent ausgesetzt und die Belastung gegenüber Parabenen ein weltweit zu beobachtendes Phänomen ist (Asimakopoulos et al. 2014, 2015; Buttke et al. 2012; Calafat et al. 2010; Casas et al. 2011; CDC 2015; Dewalque et al. 2014, 2015; Frederiksen et al. 2010, 2013, 2014; Guidry et al. 2015; Hines et al. 2015; Kang, et al. 2013; Kang et al. 2016; Larsson et al. 2014; Ma et al. 2013; Meeker et al. 2013; Mortensen et al. 2014; Myridakis et al. 2015, 2016; Philippat et al. 2015; Shirai et al. 2013; Tefre de Renzy-Martin et al. 2014; Wang et al. 2013, 2015; Xue et al. 2015; Ye et al. 2006, 2012). Bisher dienen in HBM-Studien die jeweiligen Mutterparabene (nach Konjugat-Hydrolyse) als Biomarker für die Erfassung einer Parabenexposition. Diese Biomarker sind jedoch aufgrund der ubiquitären Verbreitung der Parabene kontaminationsanfällig (Guidry et al. 2015; Ye et al. 2013). Darüber hinaus fehlen Studien, aus denen quantitative Informationen zum Metabolismus und zur Ausscheidungs-kinetik beim Menschen hervorgehen.

Ziel der vorliegenden Arbeit ist die Entwicklung einer validen, empfindlichen und selektiven Analysenmethode zur Bestimmung von neun Parabenen (MeP, EtP, *iso*- und *n*-PrP, *iso*- und *n*-BuP, PeP HeP und BeP) und sieben umweltrelevanten Phenolen (Triclosan, Triclocarban, Bisphenol-A, Benzophenon-1, Benzophenon-3, Benzophenon-8, o-Phenylphenol) in Urin. Im Rahmen erster HBM-Studien soll anschließend das Ausmaß der Belastung der deutschen Allgemeinbevölkerung gegenüber Parabenen bestimmt werden. Um von den in Urin gefundenen Biomarker-Konzentrationen auf die tatsächlich aufgenommene Menge hochrechnen zu können (Berechnung der Daily Intakes), soll der Human-Metabolismus von Parabenen und deren Eliminationskinetik nach oraler Dosierung untersucht werden. Überdies sollen neue, spezifische Metaboliten identifiziert werden, die in ausreichend hohen Konzentrationen im Urin vorliegen und im Gegensatz zu den klassischen Biomarkern (unmodifizierte Parabene) nicht anfällig gegenüber Kontaminationen sind. Mit den ermittelten Daily Intakes können dann erste Risikobewertungen durch den Vergleich mit gesundheitsbezogenen Grenzwerten durchgeführt werden.

Methodenentwicklung

Kapitel I beschreibt neben der Entwicklung und Validierung einer Analysenmethode zur Bestimmung der Hintergrundbelastung gegenüber Parabenen und einer Reihe weiterer umweltrelevanter Phenole auch die anschließende Durchführung einer ersten Populationsstudie.

Die entwickelte Multianalytmethode ermöglichte die Quantifizierung der zuvor genannten Analyten mittels vollautomatischer online-Festphasenextraktion und anschließender Hochleistungs-Flüssigkeitschromatographie mit Tandem-Massenspektrometrie-Kopplung (online-SPE HPLC-MS/MS). Die Verwendung eines online-SPE (Solid Phase Extraction)-Verfahrens ermöglicht neben der Abtrennung der Probenmatrix gleichzeitige die Extraktion und Anreicherung der Analyten. Hierfür wurde der klassische Aufbau einer HPLC-Anlage um ein 6-Wegeventil und eine Anreicherungspumpe erweitert (siehe **Abbildung 2**). Hierdurch konnten die einzelnen (anfällig gegenüber labor- und aufarbeitungsbedingten Kontaminationen) und zeitaufwendigen Verfahrensschritte der offline-SPE (Konditionierung, Probenaufgabe, Anreicherung und Elution) automatisch durchgeführt werden. Darüber hinaus konnte auch der personelle und materielle Aufwand der Probenvorbereitung reduziert werden.

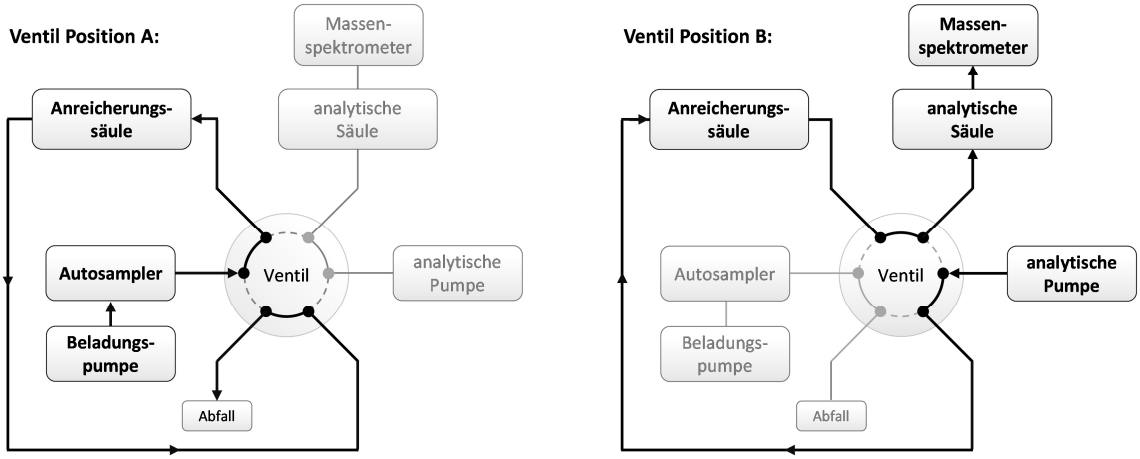

Abbildung 2 Aufbau der Zwei-Säulen-HPLC mit 6-Wegeventil. Position A: Aufreinigung und Anreicherung, Position B: Transfer der Analyten von der Anreicherungssäule auf die analytische Säule. Position A: Chromatographische Trennung und Detektion (in Anlehnung an Moos et al. 2014).

Die Urinproben werden nach vorhergehender enzymatischer Hydrolyse und grober Aufreinigung (Fällung und Abtrennung schwerlöslicher Matrixbestandteile durch Einfrieren und anschließender Zentrifugation) in das HPLC-System injiziert. Im ersten Schritt (Ventil Position A) erfolgt die Aufgabe der Probe auf eine Anreicherungssäule. Das hierfür verwendete Restricted Access Material (RAM) besitzt zwei chromatographische Trennmechanismen, die Größenausschluss- und die Reversed-Phase-Chromatographie. Die RAM-Säule besteht aus einen porösen Trägermaterial (Kieselgel) mit einem mittleren Porendurchmesser von 60 Å (6 nm). Dieses Material zeigt ein Ausschluss-Molekulargewicht für höhermolekulare Probenbestandteile von etwa 15 kDa. Während Matrixbestandteile (z.B. Proteine und Enyzm) von den Poren ausgeschlossen und von der Säule nicht retardiert werden, gelangen niedermolekulare Analyten (<15 kDA) in das Porensystem und werden dort an der inneren Oberfläche an den entsprechenden funktionellen Gruppen (Reversed-Phase-Oberfläche, hier C8) retardiert (Kromidas 2006). Dies ermöglicht die Anreicherung der Analyten und das gleichzeitige Abtrennen unerwünschter Matrixbestandteile. Im anschließenden Schritt (Ventil Position B), werden die Analyten in entgegengesetzter Flussrichtung von der Anreicherungssäule auf die analytische Säule eluiert. Um eine möglichst hohe Peakkapazität und chromatographische Effizienz zu erreichen, muss die Elutionskraft der mobilen Phase so gewählt werden, dass neben der vollständigen Elution der Analyten von der Anreicherungssäule noch keine Elution der Analyten am Säulenkopf der analytischen Säule stattfindet und somit eine maximale Fokussierung erreicht wird (Kromidas 2006).

Der letzte Schritt (Ventil Position A) umfasst neben der chromatographischen Trennung der Analyten, das gleichzeitige Spülen und Rekonditionieren der Anreicherungssäule. Aufgrund des unterschiedlichen toxikologischen Potentials, lag der Fokus während der Methodenentwicklung unter anderem auf der Trennung der verzweigten Isomere von PrP und BuP. Mit der verwendeten Reversed-Phase-Säule (C18) wurde eine ausreichende Trennung erreicht, wodurch die Quantifizierung der einzelnen Isomere trotz gleicher Molekulargewichte und Fragmentierungsmuster realisiert werden konnte. Die anschließende Detektion und quantitative Bestimmung der Analyten erfolgt mittels Elektrospray-Ionisation-(ESI)-Tandem-Massenspektrometrie (MS/MS) und Isotopenverdünnungsanalyse unter der Verwendung deuterierter, bzw. ^{13}C-markierter, strukturanaloger Verbindungen.

Methodenvalidierung

Im Rahmen der Validierung wurden Präzision (Präzision in Serie und Präzision von Tag zu Tag), Richtigkeit (relative Wiederfindung), Robustheit (Matrixabhängigkeit und Einfluss der Inkubationszeit bei der enzymatischen Hydrolyse), Verfahrensstabilität, sowie Nachweis- und Bestimmungsgrenzen der Methode ermittelt. Für die Methodenvalidierung und die fortlaufende Qualitätssicherung der Methode wurden Qualitätskontrollproben in zwei verschiedenen Konzentrationsbereichen hergestellt. Zu diesem Zweck wurden Urinproben mit bekannten (nativen) Konzentrationen der Analyten gemischt, wobei einzelne Analyten mit den entsprechenden analytischen Standards dotiert wurden, um die gewünschten Konzentrationen zu erreichen.

Die Untersuchung der Präzision beinhaltet sowohl die Wiederholpräzision innerhalb einer Serie, als auch die Wiederholbarkeit der Ergebnisse in verschiedenen Serien und an unterschiedlichen Tagen. Für die Präzision in Serie (beide Qualitätskontrollproben wurden achtmal aufgearbeitet und innerhalb einer Serie gemessen) lagen die relativen Standardabweichungen für alle Analyten und in beiden Konzentrationsbereichen zwischen 1,2 und 8,2%. Für die Präzision von Tag zu Tag (beide Qualitätskontrollproben wurden an acht verschiedenen Tagen aufgearbeitet und gemessen) lagen die relativen Standardabweichungen für alle Analyten und in beiden Konzentrationsbereichen zwischen 2,2 und 12,1%. Die relative Wiederfindung lag zwischen 72,0 und 121,2% (für PeP lag die relative Wiederfindung zwischen 135 und 161% und für HeP zwischen 176 und 219%). Die relativen Standardabweichungen lagen für alle Analyten und Konzentrationsniveaus zwischen 1,4 und 20,5%.

Keiner der untersuchten Analyten zeigte eine Kreatinin-, bzw. Matrixabhängigkeit. Die Verfahrensstabilität wurde geprüft, indem dieselben Qualitäts-kontrollproben wiederholt (zu Beginn, in der Mitte und am Ende) innerhalb einer Analysenserie gemessen wurden. Die relativen Standardabweichungen waren für beide Konzentrationsbereiche vergleichbar mit denen der Präzision in Serie.

Die Kalibrierung erfolgte aufgrund der ubiquitären Anwesenheit der Parabene in den Urinen der Allgemeinbevölkerung und dem daraus resultierenden Mangel an unbelasteten Urinproben (d.h. frei von den untersuchten Analyten) in Wasser. Einflüsse der Matrix auf die Eigenschaften der Kalibrierfunktion, wie z.B. die Steigung oder den Arbeitsbereich, konnten durch den Vergleich zweier Kalibrierungen in Wasser bzw. Urin ausgeschlossen werden. Bei den hier untersuchten Analyten hatte die Kalibrierung in Wasser keinerlei Nachteile gegenüber der Kalibrierung in Matrix. Darüber hinaus ist das Arbeiten mit Wasser als Kalibriermatrix im Vergleich zu biologischen Materialien wie Urin deutlich praktikabler und weniger zeit- und arbeitsintensiv.

Während der Methodenentwicklung wurden verschiedene Quellen von äußeren Kontaminationen identifiziert und minimiert. Hierzu wurden Laborreagenzien und Lösungsmittel untersucht und gegebenenfalls ersetzt und Glasgeräte vor Verwendung mehrfach mit Aceton bzw. Acetonitril gespült. Darüber hinaus wurden im gesamten Laborbereich keine Pflegeprodukte, Seifen oder Desinfektionsmittel verwendet, die Parabene oder einen der anderen Analyten enthielten. Trotz der beschriebenen Maßnahmen ließen sich Blindwerte von ungefähr 0,1 µg/L für einige Analyten (MeP, EtP und *n*-PrP) nicht vermeiden, was auf zusätzliche diffuse Quellen, wie z.B. Stäube, Raumluft etc. (Rudel et al. 2003; Wang et al. 2012; Fan et al. 2010) hindeutet. Aus diesem Grund wurde die Bestimmungsgrenze für alle Parabene konservativ auf 0,5 µg/L festgelegt. Für die übrigen Analyten ohne Blindwertproblematik wurden die Nachweisgrenze als Dreifaches und die Bestimmungsgrenze als Neunfaches des Signal-Rausch-Verhältnisses festgelegt.

Die in der vorliegenden Arbeit beschriebenen und für die Auswertung verwendeten Kreatiningehalte, wurde als Auftragsanalyse durch die L.u.P. GmbH, Bochum mittels Jaffé-Reaktion ermittelt.

Ermittlung der Hintergrundbelastung in Proben der deutschen Allgemeinbevölkerung

Die entwickelte Methode ermöglichte die Bestimmung der inneren Belastung/Exposition gegenüber Parabenen und anderen umweltrelevanten Phenolen in Urin. Zur Erfassung der Hintergrundbelastung in der deutschen Allgemeinbevölkerung wurden 157 Spontanurinproben (einzelne Probe zu einem beliebigen Zeitpunkt) von 59 Frauen, 39 Männern und 59 Kindern untersucht. Ein Teil der Studienpopulation wurde im Rahmen der Duisburg–Kohorte gesammelt (Kasper-Sonnenberg et al. 2012; Wilhelm et al. 2008). Die höchsten Detektionsraten (n > Bestimmungsgrenze; in %) wurden für MeP (97%), EtP (82%), *n*-PrP (77%) und *n*-BuP (36%) beobachtet. Für *iso*-PrP (3%), *iso*-BuP (17%) und BeP (3%) waren die Detektionsraten deutlich geringer. In keiner der Proben konnte PeP oder HeP nachgewiesen werden. Die höchsten Urinkonzentrationen fanden sich für MeP, mit einem Median von 24,5 µg/L, gefolgt von EtP und *n*-PrP mit 1,4 und 1,2 µg/L. Die Maximalkonzentrationen von MeP (3,2 mg/L) und *n*-PrP (1,5 mg/L) lagen 100- bzw. 1000-fach über den entsprechenden Medianen und veranschaulichen den großen Konzentrationsbereich, in dem Parabene gemessen werden können. Die verzweigten Isomere von PrP und BuP wurden weniger häufig und in geringeren Konzentrationen gefunden (siehe **Tabelle 1**).

Diese Studie umfasste neben den Parabenen ebenfalls die Untersuchung und Bewertung einer Reihe weiterer umweltrelevanter Phenole (siehe oben). Diese Ergebnisse werden an dieser Stelle nicht näher erläutert und sind **Kapitel I** zu entnehmen.

Tabelle 1 Mediane, 95. Perzentile und Maximalkonzentrationen in µg/L, der HBM-Studie mit 157 Teilnehmern (in Anlehnung an Moos et al. 2014).

Analyt	Detektions-rate (%)	Männer			Frauen			Kinder		
		P.50	P.95	Max.	P.50	P.95	Max.	P.50	P.95	Max.
MeP	97	20.7	231	234	51.4	609	1058	14.5	511	3230
EtP	82	1.3	47.1	85.5	4.2	44.0	238	0.8	9.2	33.8
iso-PrP	3	< LOQ	< LOQ	0.7	< LOQ	2.5	175	< LOQ	< LOQ	< LOQ
n-PrP	77	0.8	25.8	77.3	3.9	79.4	531	0.9	13.9	1530
iso-BuP	17	< LOQ	2.2	12.2	< LOQ	3.8	15.3	< LOQ	0.8	1.7
n-BuP	36	< LOQ	17.7	51.8	0.6	16.9	73.4	< LOQ	2.3	5.9
BeP	3	< LOQ	< LOQ	0.6	< LOQ	< LOQ	1.3	< LOQ	< LOQ	0.9
PeP	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
HeP	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Der Vergleich der einzelnen Subpopulationen zeigte bei den meisten untersuchten Parabenen eine deutlich höhere Belastung von Frauen. Diese lässt sich durch eine erhöhte und vielfältigere Verwendung von mit Parabenen konservierten Körperpflegeprodukten erklären (Biesterbos et al. 2012). Die Mediankonzentrationen der untersuchten Kinder waren generell geringer als die der erwachsenen Bevölkerung. Dennoch wurden die Maximalwerte für MeP und *n*-PrP in der Subpopulation der Kinder gemessen. Dieser Effekt wurde bereits bei anderen Umweltchemikalien beobachtet und kann unter anderem mit Kind-spezifischen Expositionspfaden (z.B. Hand-Mund-Kontakt) zusammenhängen (UBA 2014).

Zusammenfassend wurde in diesem Studienteil eine schnelle, valide und leistungsstarke Methode für die Extraktion, Trennung und Quantifizierung von Parabenen und eine Reihe weiterer umweltrelevanter Phenole in Urin entwickelt. Durch eine strenge Qualitätskontrolle der einzelnen Arbeitsschritte und eine fortlaufende Qualitätssicherung der analytischen Methode konnten laborinterne und/oder durch verwendete Materialien und Chemikalien verursachte Kontaminationen identifiziert und minimiert werden. Erstmals wurde im Rahmen einer HBM-Studie die Belastung der deutschen Allgemeinbevölkerung gegenüber Parabenen gemessen, inklusive der verzweigten Isomere von PrP und BuP. Die Ergebnisse zeigen, dass eine ubiquitäre Belastung gegenüber diesen Stoffen vorliegt. Bei fast 99% der untersuchten Personen wurde mindestens ein Paraben nachgewiesen, wobei der überwiegende Teil (etwa 90%) der Studienpopulation gegenüber mehreren Parabenen gleichzeitig exponiert war.

Kapitel I wurde als Peer-Review Publikation veröffentlicht:

Moos RK, Angerer J, Wittsiepe J, Wilhelm M, Brüning T, Koch HM. Rapid determination of nine parabens and seven other environmental phenols in urine samples of German children and adults. International Journal of Hygiene and Environmental Health. 2014; 217(8): 845-853.

Die Beiträge des Autors dieser Dissertation umfassten folgende in **Kapitel I** dargestellten wissenschaftlichen Arbeiten: Entwicklung und Validierung der Analysenmethode; Probenaufarbeitung und Analyse; Datenauswertung und Interpretation; Abfassen der Publikation.

Entwicklung der Parabenexposition in Deutschland zwischen 1995 und 2012

Kapitel II beschreibt die Untersuchung von 660 Urinproben (24-Stunden-Urin) der Umweltprobenbank (UPB) aus den Jahren zwischen 1995 und 2012. Für jedes Jahr bestand die Studienpopulation aus 30 männlichen und 30 weiblichen Probanden (vorwiegend Studenten, aus vier Standorten in der Bundesrepublik) im Alter von 20 bis 30 Jahren. Diese Proben erlaubten neben der Beurteilung der aktuellen Belastung (wie in **Kapitel I**), auch die Betrachtung der zeitlichen Entwicklung der Parabenbelastung der deutschen Bevölkerung. Die untersuchten Urinproben wurden durch die UPB gesammelt und zur Verfügung gestellt. Die UPB wird vom Umweltbundesamt (UBA) im Auftrag des Bundesministeriums für Umwelt, Naturschutz, Bau und Reaktorsicherheit (BMUB) koordiniert.

In dieser Studie wurden über den gesamten Untersuchungszeitraum betrachtet die höchsten Detektionsraten für MeP (99%), *n*-PrP (81%) und EtP (79%), gefolgt von *n*-BuP (40%) bestimmt. Die Detektionsraten für *iso*-PrP (4%), *iso*-BuP (24%) und BeP (1,4%) waren deutlich geringer. In keiner der 660 Urinproben wurde PeP oder HeP nachgewiesen. Die höchsten Urinkonzentrationen fanden sich für MeP, mit einem Median von 39,8 µg/L (48,8 µg/g Kreatinin), gefolgt von *n*-PrP und EtP mit 4,8 (5,5 µg/g Kreatinin) und 2,1 µg/L (2,4 µg/g Kreatinin). Die verzweigten Isomere von PrP und BuP wurden in deutlich geringeren Konzentrationen gefunden. Auch in dieser Studie zeigte die Subpopulation der Frauen eine deutlich stärkere Belastung gegenüber Parabenen als die der Männer.

Die Untersuchung der zeitlichen Entwicklung wurde aufgrund einer deutlichen Zunahme des 24-Stunden-Urinvolumes in den Proben der UPB (konstante Zunahme der Flüssigkeitsaufnahme) der Studienpopulation innerhalb des betrachteten Zeitraums, ausschließlich mit Kreatinin-normierten Konzentrationen durchgeführt. Trotz der andauernden öffentlichen Diskussionen über den Einsatz von Parabenen als Konservierungsstoffe und dem steigenden Angebot von parabenfreien Produkten, wurden für fast alle Parabene im Laufe der Jahre konstante Konzentrationen gefunden. Entgegen der Erwartung konnte für die Belastung gegenüber MeP sogar ein signifikanter Anstieg beobachtet werden, welcher sich ebenfalls in den einzelnen Subpopulationen widerspiegelte. In der Subpopulation der Männer konnte zusätzlich ein signifikanter Anstieg der Belastung gegenüber *n*-BuP beobachtet werden. Der Anstieg in den Urinkonzentrationen für *iso*-PrP und *iso*-BuP (siehe **Abbildung 3**), erwies sich als statistisch nicht signifikant, was sich möglicherweise auch durch die geringeren Detektionsraten erklären lässt.

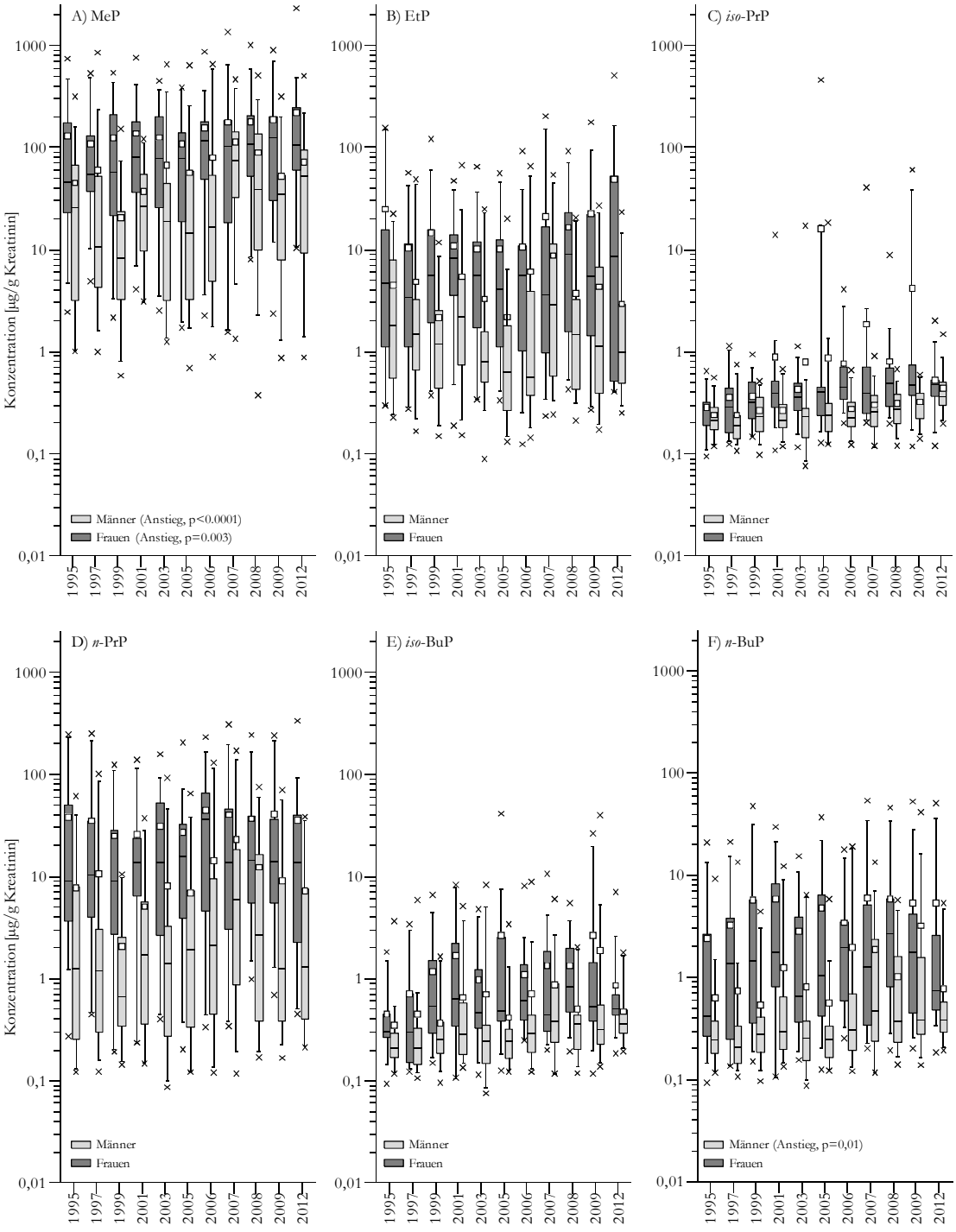

Abbildung 3 Chronologische Darstellung der Kreatinin-normierten Parabenkonzentrationen (µg/g Kreatinin) zwischen 1995-2012 getrennt für Männer und Frauen. Untere und obere Begrenzung der Box entsprechen dem ersten und dritten Quartil. Der Strich innerhalb der Box zeigt den Median. Das kleine Quadrat entspricht dem Mittelwert. Die Whisker stellen das 5. und das 95. Perzentil dar und minimale und maximale Werte werden durch ein x dargestellt (in Anlehnung an Moos et al. 2015).

Die Ergebnisse dieser Studie zeigten eine allgegenwärtige und überwiegend konstante Belastung des untersuchten Kollektivs gegenüber Parabenen zwischen 1995 und 2012. Obwohl diese Studienpopulation nicht repräsentativ für die deutsche Gesamtbevölkerung ist, haben frühere Studien mit anderen Umweltchemikalien gezeigt, dass die gemessenen Belastungen der UPB-Population sowohl die Urinkonzentrationen als auch die zeitliche Entwicklung der Expositionssituation in Deutschland wiedergeben (Wittassek et al. 2007; Göen et al. 2011; Schütze et al. 2014, 2015; Koch et al. 2012a).

Die UPB-Studienpopulation, wie auch die 157 Proben aus der Allgemeinbevölkerung (**Kapitel I**), zeigen sehr ähnliche Ergebnisse bezüglich der am häufigsten in Urin gefundenen Parabene (MeP, EtP und *n*-PrP), sowie im Hinblick darauf, dass Frauen gegenüber den meisten untersuchten Parabenen stärker belastet sind als Männer.

Auch im Vergleich mit internationalen Studien zeigt sich ein sehr einheitliches Bild mit ähnlichen Konzentrationsbereichen, Detektionsraten und Verteilungsmustern (Asimakopoulos et al. 2014, 2015; Buttke et al. 2012; Calafat et al. 2010; Casas et al. 2011; CDC 2015; Dewalque et al. 2014, 2015; Frederiksen et al. 2010, 2013, 2014; Guidry et al. 2015; Hines et al. 2015; Kang, et al. 2013; Kang et al. 2016; Larsson et al. 2014; Ma et al. 2013; Meeker et al. 2013; Mortensen et al. 2014; Myridakis et al. 2015, 2016; Philippat et al. 2015; Shirai et al. 2013; Tefre de Renzy-Martin et al. 2014; Wang et al. 2013, 2015; Xue et al. 2015; Ye et al. 2006, 2012). Unterschiede zwischen den Mediankonzentrationen der hier beschriebenen Studien sowie der anderen HBM-Studien, können unter anderem durch die Unterschiede im jeweiligen Studiendesign (Art der Probensammlung) und/oder die Zusammensetzung der Studienpopulation (Geschlecht, Alter etc.), aber auch durch die individuelle Verwendung von Körperpflegeprodukten und der Produktzusammensetzung erklärt werden. Ein Vergleich der Mediankonzentrationen und 95. Perzentile mit internationalen Studien (USA und Dänemark) ist in **Kapitel I Tabelle 4** (**Appendix I**) wiedergegeben.

Ableitung von Referenzwerten

Aus dem erhaltenen UPB-Datensatz wurden von der Kommission Human-Biomonitoring des Umweltbundesamtes erstmals für Deutschland vorläufige Referenzwerte für fünf der hier untersuchten Parabene abgeleitet (UBA 2014). Aufgrund des unterschiedlichen Gebrauchs von Körperpflegeprodukten und der daraus resultierenden unterschiedlichen Belastung, wurden einzelne Referenzwerte für Frauen und Männer abgeleitet. Die vorliegenden Daten für Kinder (**Kapitel I**) wurden in der Stellungnahme des UBAs berücksichtigt, aufgrund der relativ geringen Größe der Studienpopulation wurde auf die Ableitung eines Referenzwertes verzichtet.

Tabelle 2 Vorläufige Referenzwerte für fünf Parabene (µg/L), getrennt für Männer und Frauen (UBA 2014).

	vorläufiger* Referenzwert (µg/L)	
	Frauen	Männer
Methylparaben	400	240
Ethylparaben	50	25
Propylparaben	100	50
iso-Butylparaben	10	3
n-Butylparaben	20	10

* da die Repräsentativität bezüglich der Auswahl der Probanden und einer Schichtung nach regionalen und demografischen Merkmalen nicht gegeben ist

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Kapitel II wurde als Peer-Review Publikation veröffentlicht:

Moos RK, Koch HM, Angerer J, Apel P, Schröter-Kermani C, Brüning T, Kolossa-Gehring M. Parabens in 24 h urine samples of the German Environmental Specimen Bank from 1995 to 2012. International Journal of Hygiene and Environmental Health. 2015; 218(7): 666-674.

Im Auftrag des Umweltbundesamtes, Forschungskennzahl (UFOPLAN) FKZ 3711 62 229 1: Anwendung von neuen HBM-Analyse-Methoden an ausgewählten Kollektiven zur Bestimmung der Belastung der Allgemeinbevölkerung und damit zur Unterstützung von REACH - Human Biomonitoring von „neuen" Schadstoffen; Teilprojekt 1 - Parabene.

Die Beiträge des Autors dieser Dissertation umfassten folgende in **Kapitel II** dargestellten wissenschaftlichen Arbeiten: Probenaufarbeitung und Analyse; Datenauswertung und Interpretation; Abfassen der Publikation.

Inter- und intraindividuelle Variation der Biomarker-Konzentrationen in Urin

Die Interpretation und Bewertung der in Urin gemessenen Biomarker-Konzentrationen ist ohne das Wissen über den Metabolismus und die Ausscheidungskinetik beim Menschen nur begrenzt möglich. Besonders bei der Untersuchung von Stoffen mit relativ kurzen biologischen Halbwertszeiten (nach Exposition rasche Zu- und Abnahme der Biomarker-Konzentrationen) ist es schwierig zu erkennen, ob die gemessene Biomarker-Konzentration repräsentativ für eine langfristige durchschnittliche Exposition ist, oder ob es sich um eine einmalige Belastung handelt. Bedingt durch den vielfältigen Einsatz der Parabene, der individuellen Verwendung und der daraus resultierenden Variabilität der Exposition, können die in Urin gemessenen Biomarker-Konzentrationen starken Schwankungen unterliegen (2-3 Größenordnungen, **Kapitel I/II**). Zusätzlich können das Studiendesign (Art der Probensammlung, z.B. 24-Stunden-Urine oder Spontanurine und die Zusammensetzung der Studienpopulation) und der Hydratationsstatus einen Einfluss auf die aus HBM-Studien erhaltenen Daten haben.

In **Kapitel III** wurde der Zusammenhang zwischen der Verwendung von mit Parabenen konservierten Körperpflegeprodukten und den intra- und inter-individuellen Schwankungen der Biomarker-Konzentrationen in Urin untersucht. Ebenfalls sollte der Einfluss der Probensammlung (Spontanurine, Morgenurin und 24-Stunden-Urine) auf die in Urin gefundenen Konzentrationen untersucht werden. Hierfür wurden von acht Personen (vier Frauen und vier Männer im Alter zwischen 31 und 66 Jahren) über einen Zeitraum von sechs Tagen alle Urinproben in Einzelproben gesammelt. An zwei der sechs Tagen, wurden die üblicherweise verwendeten Körperpflegeprodukte gegen Ersatzprodukte, die keinen der untersuchten Analyten beinhalteten, ausgetauscht (siehe **Abbildung 4**).

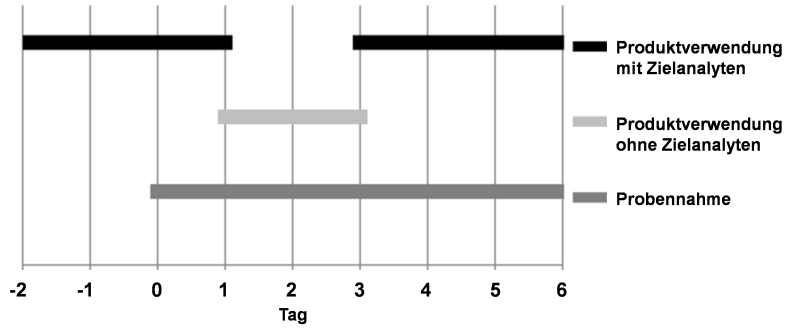


Abbildung 4 Zeitlicher Verlauf der Produktnutzung und Urinsammlung (in Anlehnung an Koch et al. 2014).

Jeder Teilnehmer dokumentierte über den gesamten Untersuchungszeitraum, beginnend zwei Tage vor Versuchsbeginn/Urinsammlung, den Verzehr von Speisen und Getränken, Rauchen und die Verwendung von Körperpflegeprodukten, in halbstündigen Intervallen.

Für die nachfolgende statistische Auswertung wurden die Tage mit Basisprodukten (4 Tage) getrennt von den Tagen mit Ersatzprodukten (2 Tage) betrachtet. Sie beschränkt sich lediglich auf Analyten mit Detektionsraten von oder über 70% (MeP, EtP und *n*-PrP). Die Ergebnisse für die neben den Parabenen analysierten umweltrelevanten Phenole werden an dieser Stelle nicht näher erläutert und sind **Kapitel III** zu entnehmen.

Die einzelnen Parabene zeigten starke intra- und inter-individuellen Schwankungen in den Urinkonzentrationen der Spontanurine, wobei die einzelnen Detektionsraten gut mit dem Vorhandensein der Inhaltsstoffe auf den Produktetiketten und der dokumentierten Produktverwendung übereinstimmen. MeP konnte in jeder der untersuchten Proben nachgewiesen werden, auch in Proben, die während der Verwendung von Ersatzprodukten gesammelt wurden. Dies deutet neben den Körperpflegeprodukten auf zusätzliche Expositionsquellen hin, wie z.B. Konservierungsstoffe in Lebensmitteln. Hohe Konzentrationen der einzelnen Parabene konnten immer auf die dokumentierte Verwendung parabenhaltiger Körperpflegeprodukte zurückgeführt werden.

Abbildung 5 zeigt am Beispiel von *n*-PrP (ein Teilnehmer) die gute Übereinstimmung der gefundenen Urinkonzentrationen mit der Anwesenheit der Inhaltsstoffe, bzw. mit den einzelnen Anwendungen. Die gleichzeitige Verwendung von zwei Produkten, die *n*-PrP enthielten (Tag 1, 22:00 Uhr), führte zu einem deutlichen Anstieg der Konzentration im Urin. An den folgenden Tagen (Tag 4; Tag 6), an denen nur ein Produkt verwendet wurde, zeigen sich deutlich geringere Zu- und Abnahmen. Der ausgeprägte Anstieg und die schnelle Abnahme der Konzentration an Tag eins wurden für die Schätzung der biologischen Eliminations-Halbwertszeit herangezogen. Für *n*-PrP wurde eine Eliminations-Halbwertszeit von etwa 4 Stunden abgeschätzt, welche der nicht-persistenten Natur dieser Verbindung entspricht und die im Tierversuch gefundene kurze Eliminationszeit bestätigt (**Soni et al. 2005**).

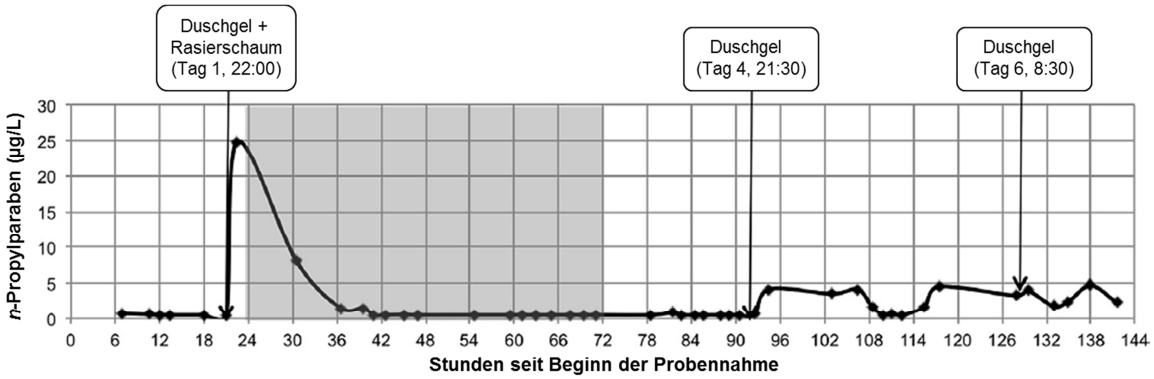


Abbildung 5 Auftragung der Urinkonzentration von *n*-PrP gegen die Zeit, am Beispiel eines Teilnehmers. Der schraffierte Bereich markiert die beiden Tage, an denen Ersatzprodukte verwendet wurden (in Anlehnung an Koch et al. 2014).

Die Auftragung der Urinkonzentrationen für die einzelnen Parabene getrennt nach Art der Probensammlung und Teilnehmern (siehe **Abbildung 6**) zeigt erhebliche intra-individuelle Schwankungen (bis zu zwei Größenordnungen) innerhalb der Urinkonzentrationen der einzelnen Spontanurine, während die 24-Stunden-Urine deutlich geringere Schwankungen aufweisen. Auch die statistische Auswertung (Intra-Klassen-Korrelation, Intra-Class-Correlation, ICC; Verhältnis zwischen individueller Varianz mit der Gesamtvarianz) zeigt eine deutlich höhere Korrelation innerhalb der 24-Stunden-Urine (ICC: 0,7-0,9) im Vergleich zu den Spontanurinen (ICC: 0,4-0,8). Dies lässt sich dadurch erklären, dass 24-Stunden-Urine die durchschnittliche Exposition und Ausscheidung eines ganzen Tages widerspiegeln, während kontinuierlich im Verlauf eines Tages gesammelter Spontanurine, die gesamten oft raschen Zu- und Abnahme der Biomarker-Konzentrationen widerspiegeln. Die relativ hohen Korrelationen dieser Studie beruhen dabei eher auf den großen Unterschieden innerhalb der Exposition der verschiedenen Teilnehmer und weniger auf den niedrigen individuellen Unterschieden im Metabolismus. Die Untersuchung der Korrelationen zwischen den Urinkonzentrationen der jeweiligen Morgenurine und der 24-Stunden-Urine (alle Versuchstage), ergab mäßig bis starke Korrelationen (R^2=0,8 für MeP, R^2=0,8 für EtP und R^2=0,7 für *n*-PrP).

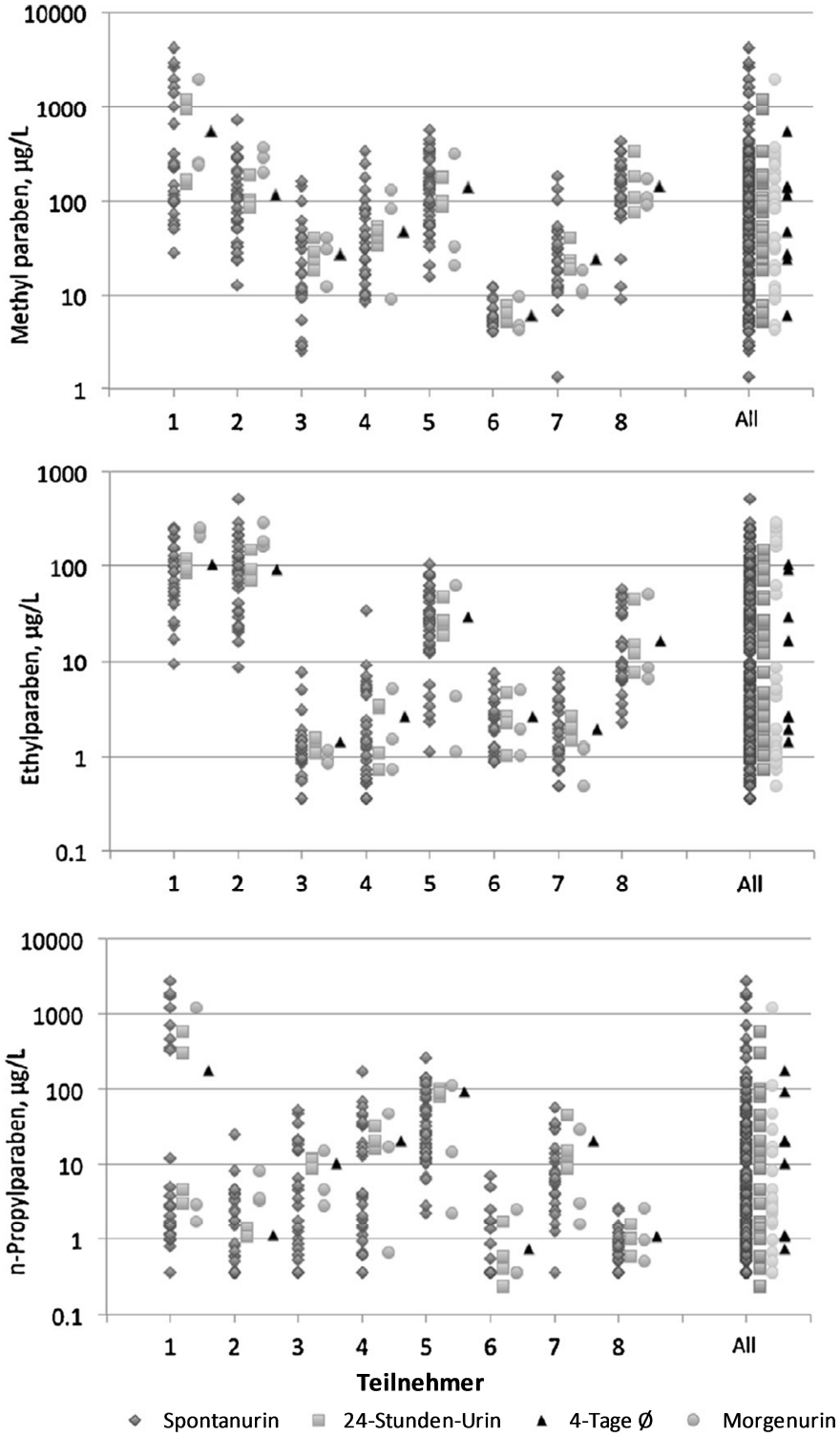


Abbildung 6 Auftragung der Urinkonzentrationen getrennt nach Art der Probensammlung (Spontanurin, 24-Stunden-Urin, Morgenurin und durchschnittliche Urinkonzentrationen für die 4 Tage (4-Tage Ø) an denen die Basisprodukte verwendet wurden), getrennt nach Teilnehmern und für alle Teilnehmer zusammen (in Anlehnung an Koch et al. 2014).

Zusammenfassend zeigen die in dieser Studie untersuchten Analyten deutliche intra-individuelle Schwankungen (bis zu zwei Größenordnungen) in den Biomarker-Konzentrationen der Spontanurine, wobei die einzelnen Detektionsraten immer mit dem Vorhandensein der Inhaltsstoffe auf den Produktetiketten und der Produktverwendung übereinstimmten. Es konnte gezeigt werden, dass die Art der Probensammlung einen Einfluss auf die erhaltenen Ergebnisse und somit auch auf die anschließende Interpretation und Bewertung der Expositionen hat. Bei Stoffen mit kurzen biologischen Halbwertszeiten zeigen die Urinkonzentration aus Spontanurinproben nur Expositionen innerhalb eines relativ kurzen Zeitraums vor der Probennahme. Dies kann zu Über- oder Unterschätzungen der tatsächlichen Exposition führen. Im Gegensatz dazu zeigen 24-Stunden-Urine die gesamte tägliche Exposition, erlauben jedoch nicht die Betrachtung individueller Schwankungen innerhalb eines Tages.

Kapitel III wurde veröffentlicht als Peer-Review Publikation:

Koch HM, Aylward LL, Hays SM, Smolders R, Moos RK, Cocker J, Jones K, Warren N, Levy L, Bevan R. Inter- and intra-individual variation in urinary biomarker concentrations over a 6-day sampling period. Part 2: personal care product ingredients. Toxicology Letters. 2014; 231(2): 261-269.

Im Auftrag der Long-Range Research Initiative (LRI) des Verband der Europäischen chemischen Industrie (European Chemical Industry Council - CEFIC). Forschungskennzahl CEFIC-LRI HBM4: Understanding inter- and intra-individual variability in human biomonitoring spot samples.

Die Beiträge des Autors dieser Dissertation umfassten folgende in **Kapitel III** dargestellten wissenschaftlichen Arbeiten: Probenaufarbeitung und Analyse; Beteiligung an Datenauswertung und Interpretation; Beteiligung am Abfassen der Publikation.

Untersuchung des Humanmetabolismus von Parabenen nach oraler Dosierung

Die bisher aus HBM-Studien erhaltenen Datensätze geben wertvolle Informationen über das Ausmaß der Belastung gegenüber Parabenen und können zur Identifizierung besonders belasteter Bevölkerungsgruppen herangezogen werden. Jedoch ist die Interpretation und Bewertung der erhaltenen HBM-Daten aufgrund unzureichender Kenntnisse über den Humanmetabolismus der Parabene nur begrenzt möglich. Hier werden vor allem spezifische metabolische Konversionsfaktoren zur quantitativen Abschätzung der tatsächlich aufgenommen Menge ausgehend von Konzentrationen im Urin dringend benötigt. Diese Dosen (Daily Intakes) können dann mit gesundheitsbezogenen Grenzwerten bzw. Tierversuchsdaten verglichen werden.

Ziel der in **Kapitel IV** beschriebenen Studie war die quantitative Untersuchung des menschlichen Metabolismus von Parabenen nach oraler Aufnahme und die Etablierung spezifischer metabolischer Konversionsfaktoren der Ausscheidung im Urin. Es wurden drei Parabene (MeP, *iso*- und *n*-BuP) ausgewählt, um mögliche Unterschiede im Metabolismus, abhängig von Länge und Verzweigungsgrad der Seitenkette zu untersuchen. Aufgrund seiner besseren Wasserlöslichkeit und dem niedrigeren Molekulargewicht ist davon auszugehen, dass MeP leichter über den Urin ausgeschieden werden kann als die höhermolekularen Butylparabene. Das bedeutet gleichzeitig, dass neben der Konjugation auch weitere metabolische Modifikationen wahrscheinlich sind, um die Wasserlöslichkeit der höhermolekularen Parabene zu erhöhen. Diese Phase I-modifizierten Metaboliten sollen auf ihre Eignung als spezifische Biomarker untersucht werden. Zudem sind diese funktionalisierten Metaboliten im Gegensatz zu den klassischen Biomarkern nicht anfällig gegenüber Kontaminationen. Bei den strukturverwanden kurzkettigen Phthalaten (gleiche Alkylseitenketten) haben sich Metaboliten mit oxidativen Modifikationen an der Alkylseitenkette als wertvolle zusätzliche Biomarker erwiesen (Koch et al. 2012b). Darüber hinaus wurden für die Parabene Stoffwechselprodukte mit oxidativen Modifikationen am aromatischen Ring in der Literatur postuliert (Wang und Kannan 2013).

Neben den klassischen Mutterparabenen (inkl. deren Phase II Konjugaten) und den unspezifischen Metaboliten *p*-Hydroxybenzoesäure und *p*-Hydroxyhippursäure wurden daher spezifische Metaboliten mit oxidativen Modifikationen an der Alkylseitenkette (3-Hydroxy-*n*-Butyl-4-hydroxybenzoat (3OH-*n*-BuP) sowie 2-Hydroxy-*iso*-butyl-4-hydroxy-benzoat (2OH-*iso*-BuP)) und am aromatischen Ring (Ester der Protocatechusäure:

Methyl-3,4-dihydroxybenzoat (rOH-MeP), *iso*-Butyl- (rOH-*iso*-BuP) und *n*-Butyl-3,4-dihydroxy-benzoat (rOH-*n*-BuP)) untersucht. **Abbildung 7** zeigt den postulierten Metabolismus der untersuchten Parabene und die daraus resultierenden Stoffwechselprodukte.

Aufgrund der bekannten Hintergrundbelastung der Allgemeinbevölkerung gegenüber Parabenen (**Kapitel I/II/III**), wurden für die Studie ring-deuterierte Strukturanaloga der jeweiligen Parabene verwendet. Die in dieser Studie als interne Standards verwendeten $^{13}C_6$-markierten Strukturanaloga wurden von Dr. Vladimir Belov des Max-Planck-Instituts für biophysikalische Chemie in Göttingen synthetisiert. Zur Vereinfachung wird in diesem Kapitel in allen weiteren Abbildungen, Tabellen und Diskussionen auf die Erwähnung der D4-Isotopenmarkierung der Parabene und deren Metaboliten verzichtet.

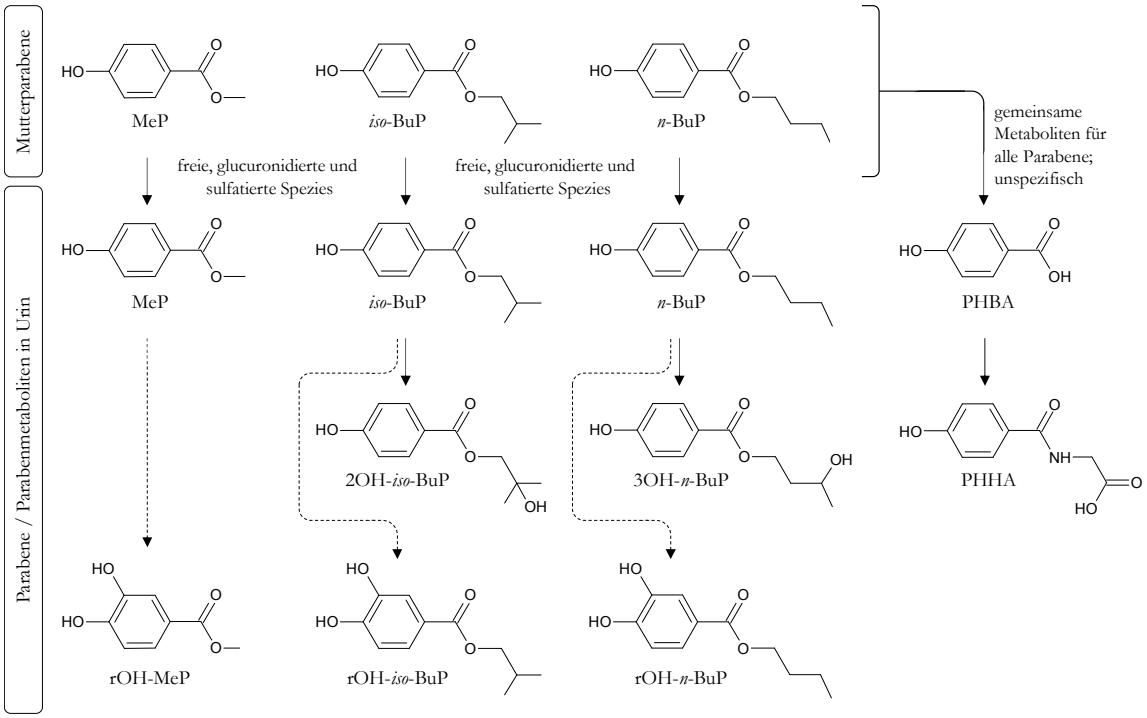


Abbildung 7 Postulierter Humanmetabolismus von MeP, *iso*- und *n*-BuP (in Anlehnung an Moos et al. 2016a).

Die gleichzeitige Untersuchung der polareren Parabenmetabolite (Säuren und oxidativ modifizierte Parabene) neben den unmodifizierten Parabenen erforderte die Entwicklung einer neuen Analysenmethode Die Entwicklung und Validierung der Online-SPE-HPLC-MS/MS-Methode erfolgte analog zu der in **Kapitel I** beschriebenen Methode. Die für die online-Anreicherung verwendete TurboFlow Cyclone™MAX Turbulent Flow-Chromatographie-Säule besteht aus einer Mixed-Mode-Phase, die neben Reversed-Phase-

Eigenschaften (Anreicherung ungeladener Metaboliten) auch die Funktionalität eines Anionentauschers besitzt (Anreicherung saurer Metaboliten). Die chromatographische Trennung wurde auf einer Accucore® Phenyl-X-Säule realisiert. Alle weiteren Details zur Methodenentwicklung, Validierung und Probenaufarbeitung sind **Kapitel IV** zu entnehmen.

Die Studie wurde mit drei gesunden Probanden (jeweils 31 Jahre alt, eine Frau und zwei Männer, 52 bis 82 kg Körpergewicht) durchgeführt. Jeder der Teilnehmer erhielt drei einzelne Dosierungen von etwa 10 mg (genau eingewogen) der deuterierten Parabene. Die jeweiligen Dosierungen wurden in einem zeitlichen Abstand von mindestens zwei Wochen durchgeführt, um eventuelle Störungen durch gemeinsame Metabolite (*p*-Hydroxybenzoesäure und *p*-Hydroxyhippursäure) ausschließen zu können. Die erste Urinprobe (T_0) wurde vor der Dosierung abgegeben. Alle weiteren Urinproben wurden kontinuierlich über einen Zeitraum von 48 Stunden in Einzelproben gesammelt. Die Studie wurde in Übereinstimmung mit den ethischen Standards der Deklaration von Helsinki (1964) durchgeführt und von der Ethikkommission der Ruhr-Universität Bochum (Reg. Nr.: 4332-12) genehmigt. Alle Teilnehmer wurden über das Studiendesign informiert und gaben vor Studienbeginn eine schriftliche Einverständniserklärung ab.

Abbildung 8 zeigt die Eliminationskinetik der Parabene (am Beispiel eines Probanden) nach oraler Dosierung, getrennt für die drei Dosierungsexperimente (A: MeP, B: *iso*-BuP und C: *n*-BuP). In allen Dosierungsexperimenten erreichten die Parabene und ihre Stoffwechselprodukte die maximale Konzentration im Urin innerhalb der ersten zwei Stunden nach Aufnahme. Während für MeP die Metabolitkonzentrationen unmittelbar nach Erreichen des Maximums abnahmen, blieben die Konzentrationen für *iso*- und *n*-BuP über einen längeren Zeitraum (ca. 6 - 10 Stunden nach der Dosierung) auf einem höheren Niveau. Alle untersuchten Metaboliten wurden in mindestens zwei verschiedenen Phasen ausgeschieden.

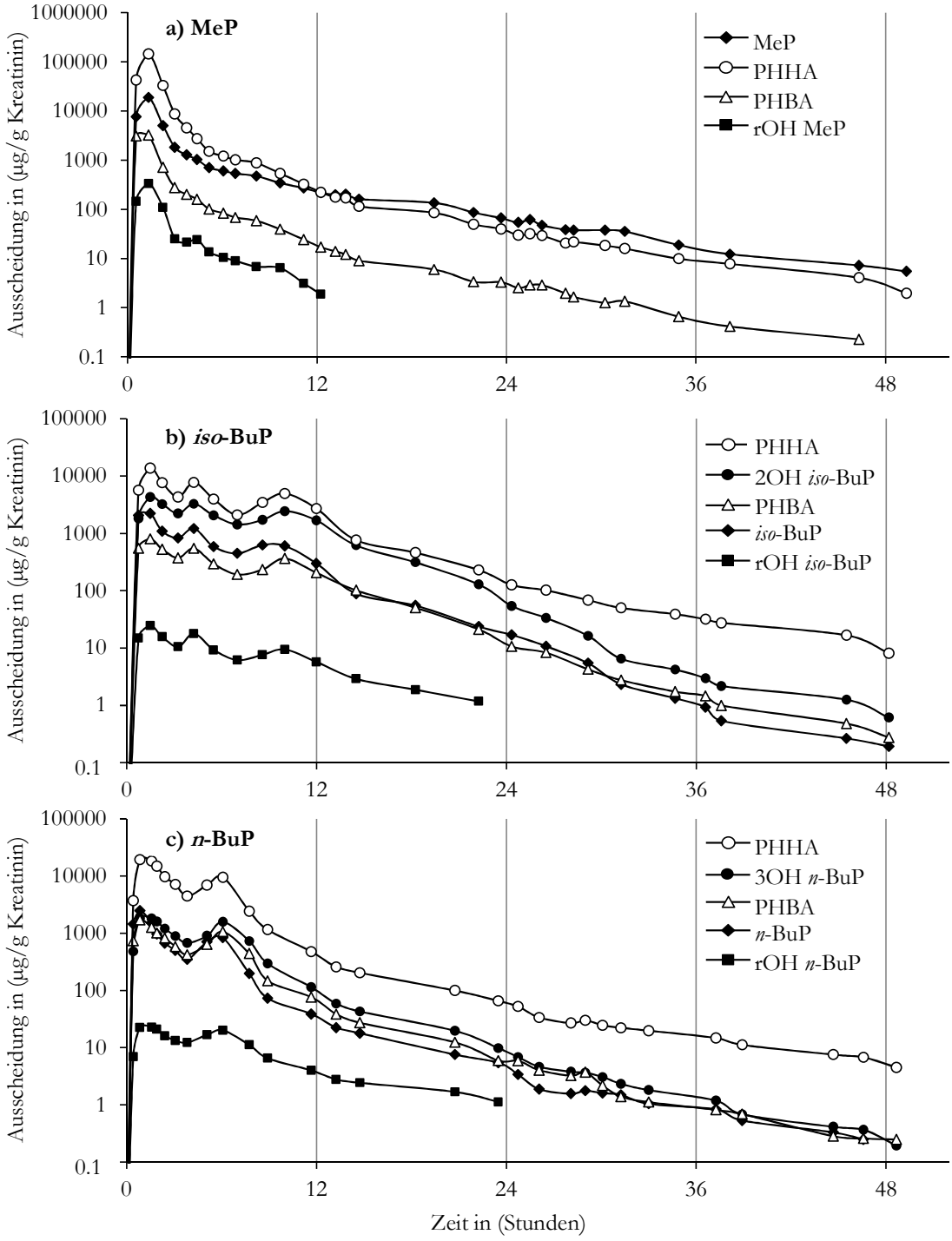


Abbildung 8 Kreatinin-normierte Metabolitkonzentrationen in Urin nach oraler Dosierung von A) MeP B) *iso*-BuP und C) *n*-BuP, auf halblogarithmischer Skala. Die unspezifischen Metaboliten *p*-Hydroxybenzoesäure (PHBA) und *p*-Hydroxyhippursäure (PHHA) sind mit weißen Markierungen dargestellt, alle spezifischen Biomarker sind schwarz dargestellt (in Anlehnung an Moos et al. 2016a).

MeP war über den gesamten Zeitraum der Studie messbar und lag am Ende bei allen Probanden etwa 50-fach über der Nachweisgrenze (0,25 µg/L). Im Gegensatz dazu lagen die Konzentrationen von *n*- und *iso*-BuP und deren seitenketten-hydroxylierter Metaboliten am Ende der Studie nahe der Nachweisgrenzen. Die Urinkonzentrationen der ring-hydroxylierten Metaboliten sanken bereits innerhalb der ersten 24 Stunden der Studie unter die Nachweisgrenzen. Die Kenngrößen der Elimination sind in **Tabelle 3** zusammengefasst. Aus der Auftragung der Kreatinin-normierten Konzentrationen gegen die Zeit wurde graphisch die metabolitspezifische Geschwindigkeitskonstante k ermittelt (**Abbildung 8**) und die Eliminations-Halbwertszeit $t_{1/2}$ über die mathematische Beziehung $t_{1/2} = \ln(2)/k$ bestimmt.

Tabelle 3 Kenngrößen der Elimination für MeP, *iso*-BuP und *n*-BuP und den entsprechenden Metaboliten. Mittelwert aus drei Probanden (Bereich) (in Anlehnung an Moos et al. 2016a).

	Biomarker	maximale Urinkonzentration c_{max} (mg/L)	Zeitpunkt der Maximalkonzentration t_{max} (h)	Eliminations-Halbwertszeit $t_{1/2}$ Phase1 (h)	Eliminations-Halbwertszeit $t_{1/2}$ Phase2 (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)

Für alle drei untersuchten Parabene wurden mehr als 80% der Dosis innerhalb der ersten 24 Stunden nach Aufnahme über den Urin ausgeschieden. An Tag 2 wurden wesentlich kleinere Mengen (≤ 1%) ausgeschieden. Insgesamt wurden nach Ende der Studie (48 Stunden) im Mittel 84,4% der MeP Dosis, 86,0% der *iso*-BuP Dosis und 80,8% der *n*-BuP Dosis in Form der untersuchten Metaboliten im Urin wiedergefunden.

Tabelle 4 Mittelwerte und Bereiche der metabolischen Konversionsfaktoren (in %) von MeP, *iso*-BuP und *n*-BuP und deren Metaboliten (in Anlehnung an Moos et al. 2016a).

	Biomarker	Dosisanteil 0-24 h (%)	Dosisanteil 24-48 h (%)	Dosisanteil 0-48 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)
	Gesamt 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)
	Gesamt Σ	85.3 (83.3-88.0)	0.6 (0.3-1.0)	86.0 (84.3-88.3)
***n*-BuP**	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)
	Gesamt Σ	80.5 (74.6-89.7)	0.2 (0.1-0.4)	80.8 (75.1-89.8)

Die Konversionsfaktoren der einzelnen Parabene und der entsprechenden Metaboliten sind in **Tabelle 4** zusammengefasst. Der gemeinsame Hauptmetabolit aller Parabene war die unspezifische *p*-Hydroxyhippursäure mit einem Anteil von etwa 60%. Zwischen 3,0 und 7,2% der Dosierung wurden als *p*-Hydroxybenzoesäure ausgeschieden. Diese Biomarker sind als Biomarker für eine Parabenexposition ungeeignet, tragen jedoch maßgeblich zum quantitativen Verständnis des Humanmetabolismus der Parabene bei.

Die Anteile der bisher für die Bestimmung eine Exposition herangezogenen Mutterparabene (nach Hydrolyse) lagen für MeP bei 17,4%, für *iso*-BuP bei 6,8% und für *n*-BuP bei 5,6%. Diese Ergebnisse zeigen, dass der Anteil der Mutterparabene (frei und konjugiert), der über den Urin ausgeschieden wird, mit zunehmender Seitenkettelänge deutlich sinkt. Wie zuvor vermutet sind neben der Bindung an Sulfat oder Glucuronsäure für die längerkettigen Butylparabene weitere Modifikationen erforderlich, um die Wasserlöslichkeit zu erhöhen und somit die Ausscheidung über den Urin zu erleichtern.

Diese Annahme wurde zusätzlich durch die Identifizierung neuer, spezifischer seitenketten-hydroxylierter Metaboliten von *iso*- und *n*-BuP bestätig. Diese spezifischen Metaboliten 2OH-*iso*-BuP und 3OH-*n*-BuP repräsentieren 15,8% und 5,8% der Dosis und lagen so über den in Urin gefundenen Konzentrationen der Mutterparabene. Die Tatsache, dass diese oxidierten Metaboliten in ausreichend hohen Konzentrationen im Urin vorliegen, und dass sie Kontaminationen gegenüber nicht anfällig sind, machen sie zu wichtigen Biomarkern für zukünftige HBM-Studien.

Weiterhin konnten für alle drei Parabene auch die postulierten ring-hydroxylierten Metaboliten (rOH-MeP, rOH-*iso*-BuP und rOH-*n*-BuP) im Urin nachgewiesen werden. Der Anteil dieser ring-hydroxylierten Parabene war jedoch mit weniger als 1% vernachlässigbar klein. Diese Metaboliten sind aufgrund der geringen Mengen, die im Urin ausgeschieden wurden und der Tatsache, dass diese Strukturen auch natürlich in der Umwelt vorkommen (z.B. in Wein, Baderschneider and Winterhalter 2001) eher ungeeignet als Biomarker für eine Exposition gegenüber Parabenen.

Neben der Eliminationskinetik und den metabolischen Konversionsfaktoren wurde zusätzlich der Konjugationstatus bzw. die Verteilung der einzelnen Parabenspezies (glucuronidiert, sulfatiert und frei) untersucht. Die Ergebnisse zeigen, dass auch die Bindung an Sulfat oder Glucuronsäure von der Länge und dem Verzweigungsgrad der Seitenkette abhängig ist. Während für MeP nur etwa 30% der Gesamtmenge als glucuronidierte Spezies im Urin vorlagen, wurden 89% bzw. 87% von *iso*- und *n*-BuP als Glucuronid ausgeschieden. Im Gegensatz dazu lagen etwa 64% der ausgeschiedenen Gesamtmenge von MeP und nur 12% bzw. 13% von *iso*- und *n*-BuP als sulfatierte Spezies vor. Demzufolge müssen für die vollständige Erfassung der über den Urin ausgeschiedenen Parabene Enzyme für die Hydrolyse gewählt werden, die sowohl eine Glucuronidase- sowie eine Sulfatase-Aktivität besitzen. Der Anteil, der als freie Spezies ausgeschieden wurde, war für alle Parabene relativ gering (zwischen 0,8% für *iso*-BuP und 7,1% für MeP) und nahm mit zunehmender Kettenlänge ab. Demzufolge kann die Bestimmung hoher Konzentrationen (über 10%, bezogen auf die gefundene Gesamtkonzentration) freier Parabene zur Identifizierung mögliche Kontaminationen während der präanalytischen Phase (Probenahme, Lagerung und Verarbeitungsbedingungen) genutzt werden (Guidry et al. 2015; Ye et al. 2013).

Zusammenfassend wurde in diesem Studienteil erstmals der Human-Metabolismus von Parabenen (MeP, *iso*- und *n*-BuP) nach oraler Dosierung untersucht. Durch die Verwendung deuterierter Standards konnte der Metabolismus ohne Störung durch die allgemeine Hintergrundbelastung und somit spezifisch und kontaminationsfrei für alle untersuchten Metabolite durchgeführt werden. Die erhaltenen Konversionsfaktoren der einzelnen Parabene ermöglichen, basierend auf den in HBM-Studien ermittelten inneren Belastungen, eine verlässliche Hochrechnung der tatsächlich aufgenommenen Mengen. Die Ergebnisse können in Zukunft zur Expositions- und Risikoabschätzung im arbeits- und umwelt-medizinischen Bereich dienen.

Kapitel IV wurde veröffentlicht als Peer-Review Publikation:

Moos RK, Angerer J, Dierkes G, Brüning T, Koch HM. Metabolism and elimination of methyl, *iso*- and *n*-butyl paraben in human urine after single oral dosage. Archives of Toxicology. 2016; 90(11), 2699-2709.

Die Beiträge des Autors dieser Dissertation umfassten folgende in **Kapitel IV** dargestellten wissenschaftlichen Arbeiten: Entwicklung und Validierung der Analysenmethode; Schreiben des Ethikantrages; Design, Organisation und Durchführung der Studie; Probensammlung, Probenaufarbeitung und Analyse; Datenauswertung, Evaluation und Interpretation; Abfassen der Publikation.

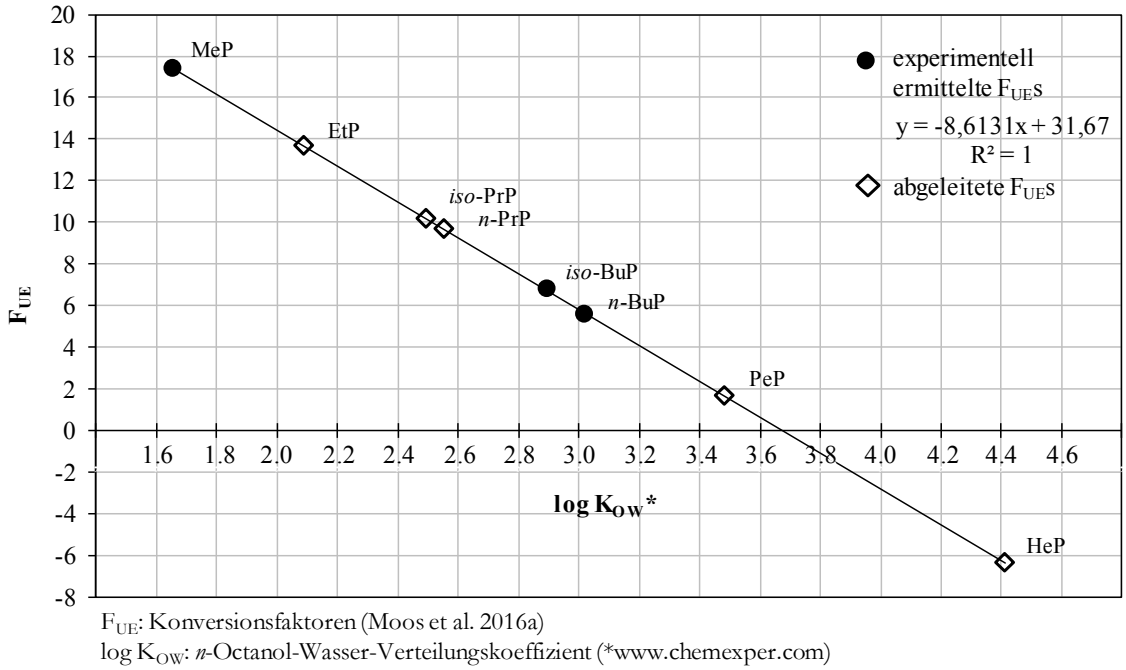
Berechnung der Daily Intakes

Kapitel V beschreibt die Berechnung der aufgenommenen Paraben-Mengen (Daily Intake, DI) anhand gemessener Urinkonzentrationen in Verbindung mit robusten metabolischen Konversionsfaktoren (F_{UE}). Die berechneten DIs können zur Risikobewertung durch den Vergleich mit gesundheitsbezogenen Grenzwerten herangezogen werden. Zusätzlich können die Wirksamkeit von Regulierungsmaßnahmen überprüft und gegebenenfalls Empfehlungen für weitere Maßnahmen zur Expositions-/Risikoreduzierung gegeben werden. Für die Berechnung der DIs wurden die zuvor ermittelten Urinkonzentrationen der UPB-Studie herangezogen (**Kapitel II**). Die dort untersuchten 24-Stunden-Urinproben erlauben, im Gegensatz zu Spontanurinproben (nur ein Ausschnitt der täglichen Expositionen wird erfasst), die direkte Berechnung des Daily Intakes, ohne den Einbezug eines durchschnittlichen Tagesurinvolumens bzw. anderer Annahmen.

Der DI (µg/kg KG/Tag) berechnet sich aus dem Produkt der Urinkonzentration (uc) und dem 24-Stunden-Urinvolumen (uv_{24h}), geteilt durch das Produkt des Konversionsfaktors (F_{UE}) und dem Körpergewicht (KG):

$$DI\ (\mu g/kg\ KG/Tag) = \frac{uc\ (\mu g/L) \times uv_{24h}\ (L/Tag)}{F_{ue} \times KG\ (kg)}$$

Basierend auf den experimentell ermittelten Konversionsfaktoren für MeP, *iso*-BuP und *n*-BuP (**Kapitel IV**), wurden die fehlenden F_{UE}s der übrigen Parabene (EtP, *iso*-PrP, *n*-PrP, PeP und HeP) mathematisch abgeleitet. Die Untersuchung des menschlichen Metabolismus zeigte, dass die Anteile der Parabene die über den Urin ausgeschieden werden, mit zunehmender Seitenkettenlänge abnehmen. Im Gegensatz dazu steigt der Octanol-Wasser-Verteilungskoeffizient (K_{OW}) mit zunehmender Länge der Seitenkette (Golden et al 2005; Błędzka et al 2014). Die unbekannten F_{UE}s wurden durch lineare Regression der Auftragung von F_{UE}s gegen Octanol-Wasser-Verteilungskoeffizient abgeschätzt (siehe **Abbildung 9**).



F_{UE}: Konversionsfaktoren (Moos et al. 2016a)
log K_{OW}: *n*-Octanol-Wasser-Verteilungskoeffizient (*www.chemexper.com)

Abbildung 9 Auftragung der Konversionsfaktoren gegen die Octanol-Wasser-Verteilungskoeffizienten der Parabene (in Anlehnung an Moos et al. 2016b).

Die Berechnung des F_{UE}s für HeP führte zu einem negativen Wert. Dies deutet darauf hin, dass HeP nicht in unveränderter oder einfacher konjugierter Form (gebunden an Sulfat oder Glucuronsäure) über den Urin ausgeschieden wird bzw. dass weitere Modifikationsschritte erforderlich sind, um die Wasserlöslichkeit zu erhöhen. Dieses Ergebnis stützt sich durch die Tatsache, dass bislang weder HeP (mit einem negativen F_{UE}) noch PeP (mit einem relativ kleinen F_{UE}) in einer der untersuchten Proben (weder in den 660 Proben des UPB, noch in einer anderen bislang am IPA untersuchten Studienpopulation) detektiert wurden. Allerdings muss erwähnt werden, dass PeP und HeP weder in Lebensmitteln, noch in kosmetischen Mitteln in der Europäischen Union zugelassen sind (Europäisches Parlament 2009). Aufgrund der strukturellen Unterschiede zu den Alkylestern und den daraus resultierenden Unterschieden im Metabolismus und der Eliminationskinetik, wurde für BeP kein F_{UE} abgeleitet. Alle abgeschätzten und empirisch ermittelten F_{UE}s sind in **Tabelle 5** angegeben.

Tabelle 5 Octanol-Wasser-Verteilungskoeffizienten (K_{OW}), Molekulargewicht und die Konversionsfaktoren (F_{UE}) der Parabene (in Anlehnung an Moos et al. 2016b).

	Octanol-Wasser-Verteilungskoeffizient* (K_{OW})	Molekulargewicht (g/mol)	Konversionsfaktor (F_{ue})
MeP	1.656	152.15	17.4 °
EtP	2.091	166.18	13.7
iso-PrP	2.495	180.20	10.2
n-PrP	2.556	180.20	9.7
iso-BuP	2.896	194.23	6.8 °
n-BuP	3.019	194.23	5.6 °
PeP	3.484	208.26	1.7
HeP	4.412	236.31	-6.3

* www.chemexper.com (abgerufen am 10. März 2016); ° F_{UES} (Moos et al. 2016a)

Für die Berechnung der DIs, wurden die zuvor ermittelten Urinkonzentrationen der UPB-Studie herangezogen (**Kapitel II**). Für vier Personen der Studienpopulation konnte kein DI berechnet werden, da das Körpergewicht im Datensatz fehlte. Die höchsten DIs wurden für MeP abgeleitet mit einem Median von 5,8 µg/kg KG/Tag und einem 95. Perzentil von 47,5 µg/kg KG/Tag. Die Mediane der DIs für die anderen Parabene waren deutlich niedriger mit 1,2 µg/kg KG/Tag für *n*-PrP, gefolgt von 0,4 µg/kg KG/Tag für EtP und 0,2 µg/kg KG/Tag für *n*-BuP. Für die verzweigten Isomere wurden maximale DIs von 13,4 µg/kg KG/Tag für *iso*-BuP und 32,3 µg/kg KG/Tag für *iso*-PrP bestimmt.

Die Betrachtung der zeitlichen Entwicklung zeigte für fast alle Parabene relativ konstante DIs im Laufe der Jahre 1995 bis 2012. Im Vergleich zur vorangegangenen Untersuchung der Urinkonzentrationen (**Kapitel II**) konnte lediglich für MeP ein signifikanter Anstieg des DI beobachtet werden, wobei dieser vor allem durch die Subpopulation der Männer verursacht wird. Der scheinbare Anstieg in der Subpopulation der Frauen erwies sich als nicht signifikant (siehe **Abbildung 10**).

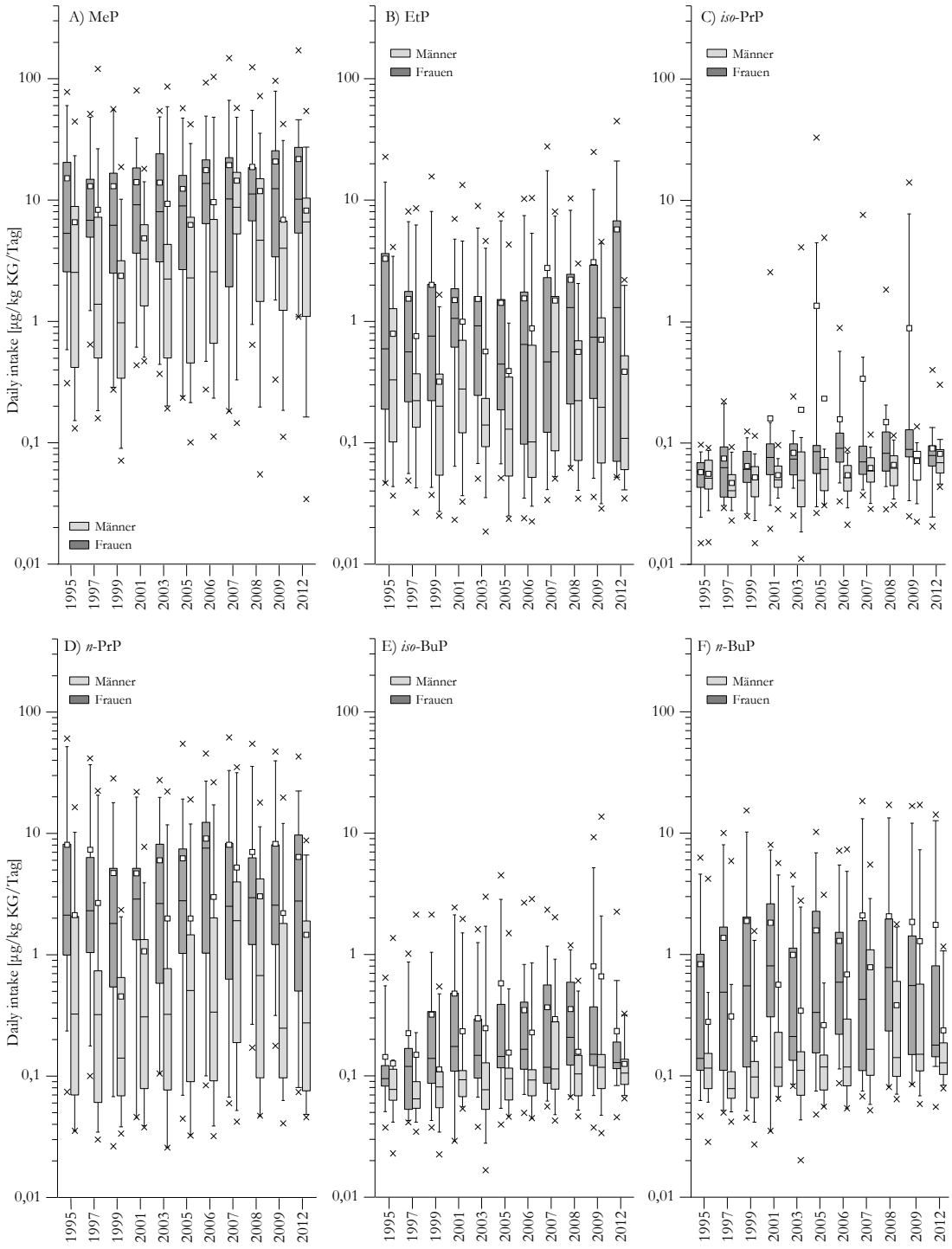


Abbildung 10 Chronologische Darstellung der DIs (µg/kg KG/Tag) zwischen 1995-2012 getrennt für Männer und Frauen. Untere und obere Begrenzung der Box entsprechen dem ersten und dritten Quantil. Der Strich innerhalb der Box zeigt den Median. Das kleine Quadrat entspricht dem Mittelwert. Die Whisker stellen das 5. und das 95. Perzentil dar und minimale und maximale Werte werden durch ein x dargestellt (in Anlehnung an Moos et al. 2016b).

Der direkte Vergleich der in dieser Studie berechneten DIs (basierend auf 24-Stunden-Urinproben und metabolischen Konversionsfaktoren) mit anderen HBM-basierten Abschätzungen (Ma et al. 2013; Asimakopoulos et al. 2014; Myridakis et al. 2015, 2016; Cowan-Ellsberry and Robinson 2009) ist nur bedingt möglich. Bislang veröffentlichte Studien zur täglichen Aufnahme von Parabenen beruhen auf abgeschätzten Konversionsfaktoren für die einzelnen Parabene und/oder Konversionsfaktoren die anhand von Metaboliten abgeleitet wurden, die weder für einzelne Parabene noch für Parabene generell, spezifisch sind (*p*-Hydroxybenzoesäure). Darüber hinaus gibt es weitere Berechnungsansätze, wie dermale Expositionsmodelle (SCCS 2011a) oder Hochrechnungen aus externen Expositionsquellen (Liao et al. 2013a, 2013b; Wang et al. 2012). Bei der Untersuchung der aus externer Expositionsquellen resultierenden DIs konnte gezeigt werden, dass die Verwendung von Körperpflegeprodukten den überwiegenden Anteil der Parabenbelastung ausmacht (Guo et al. 2013). Andere Quellen, wie Nahrung oder Hausstaub, spielen dagegen nur eine untergeordnete Rolle (Liao et al. 2013a, 2013b; Wang et al. 2012). Eine ausführliche Tabelle mit den DIs aus verschiedenen Berechnungsmodellen ist in **Kapitel V Tabelle 4** wiedergegeben.

Kumulative Risikobetrachtung

Die ermittelten DIs erlauben durch den Vergleich mit bestehenden gesundheitsbezogenen Grenzwerten eine erste Risikobewertung. Die DIs dieser Studie lagen um ein Vielfaches unter dem Gruppen-ADI-Wert von 10 mg/kg KG/Tag für die Summe von MeP und EtP. Selbst der höchste in dieser Studienpopulation berechnete DI für die Summe von MeP und EtP (204 µg/kg KG/Tag) lag um den Faktor 50 unter dem geltenden ADI. Für die anderen Parabene wurden bislang keine offiziellen gesundheitsbezogenen Grenzwerte abgeleitet.

Für eine kumulative Risikobewertung der Exposition gegenüber Parabenen wurde der Hazard-Index (HI) herangezogen, definiert als die Summe der einzelnen Hazard-Quotienten (HQ) (Teuschler et al. 1995; Kortenkamp et al. 2010; Søeborg et al. 2012). Diese Quotienten ergeben sich aus dem Verhältnis der tatsächlichen Belastung (z.B. in Form des DIs) und einer als unbedenklich geltenden Dosis in Form gesundheitsbezogener Grenz- oder Beurteilungswerte. Somit zeigt ein HQ (oder HI) von über 1, dass die tatsächliche Belastung die akzeptable Exposition für ein einzelnes Paraben (oder für die Mischung von Parabenen) überschreitet.

In der praktischen Anwendung werden gesundheitsbezogene Grenzwerte (z.B. ADI) in die Berechnung der HQs einbezogen. Für die Parabene ohne offizielle Grenz- oder Referenzwerte wurde, basierend auf einer Risikobewertung des SCCS (SCCS 2011a), für die Ableitung der HQs/HIs für *n*-PrP und *n*-BuP und deren verzweigte Isomere ein konservativer NOEL von 2 mg/kg KG/Tag für *n*-BuP (Fisher et al. 1999) verwendet. Der NOEL wurde hierfür mit einem Sicherheitsfaktor von 100 (ähnlich wie bei der Ableitung eines ADI aus dem entsprechenden NO(A)EL) kombiniert (US CPSC 2014; Lioy et al. 2015). Dies könnte zu einer tendenziell zu konservativen Einschätzung der kurzkettigen Parabene (*iso*- und *n*-PrP) führen. Die Tatsache, dass eine vierwöchige Verabreichung von *n*-PrP bei der niedrigsten Dosierung von 10 mg/kg KG/Tag (Lowest Observed Adverse Effect Level, LOAEL) bei männlichen Jungratten zu einer Reduktion der täglichen Spermaproduktion führte (Oishi 2002; EFSA 2004), bestätigt jedoch die Verwendung eines relativ niedrigen NOEL für die Berechnung des HQ/HI von *n*-PrP.

Die HIs wurden für jeden Probanden aus der Summe der jeweiligen HQs der einzelnen Parabene berechnet (siehe **Tabelle 6**). Zur Berechnung der HQs von MeP und EtP wurde der Gruppen-ADI-Wert für die Summe aus MeP und EtP herangezogen. Für die Summe beider Parabene lag der HQ bei der maximalen Belastung mit 0,02 deutlich unter 1. Im Gegensatz zu MeP und EtP wurden für die anderen Parabene höhere HQs bestimmt. Der höchste HQ wurde für *n*-PrP bestimmt, mit einem maximalen HQ von über 3. Bei etwa 34 (5%) der 656 Probanden kam es für *n*-PrP zu einer Überschreitung des HQ von 1. Neben *n*-PrP wurde nur für *iso*-PrP eine weite Überschreitung des HQ-Werts von 1 (1,6) bestimmt. Für *iso*- und *n*-BuP lagen die maximalen HQs zwischen 0,7 und 0,9. Die Berechnung der HIs ergab, dass 55 (8,4%) Personen der untersuchten Studienpopulationen einen HI von 1 überschritten (bis zu einem HI von 4,4). Dies bedeutet, dass 8,4% der Studienpopulation Parabenen in einem Umfang ausgesetzt waren, bei dem ein Gesundheitsrisiko nicht mehr ausgeschlossen werden kann, wobei etwa 90% (7,6% der gesamten Studienpopulation) dieser Personen Frauen waren.

Tabelle 6 Berechnete HQs/HIs der Parabene (in Anlehnung an Moos et al. 2016b).

	MeP + EtP	*iso*-PrP	*n*-PrP	*iso*-BuP	*n*-BuP	HI
	Gruppen-ADI: 10 mg/kg KG/Tag	abgeleiteter Grenzwert*: 0,02 mg/kg KG/Tag				
P.50	0,001	< 0,001	0,060	< 0,001	< 0,001	0,093
P.95	0,005	< 0,001	1,027	0,052	0,230	1,304
Max	0,020	1,616	3,015	0,671	0,906	4,368
n > 1 (%)	0	0,2	5,2	0	0	8,4

* NOEL von 2 mg/kg KG/Tag SCCS (2011a) basierend auf Fisher et al. (1999)

Zusammenfassend wurden in dieser Studie wurden erstmals verlässliche DIs für Parabene, basierend auf Urinkonzentrationen in Kombination mit menschlichen Konversionsfaktoren, berechnet. Die Ergebnisse zeigen, dass obwohl die höchsten DIs für MeP bestimmt wurden, die kurzkettigen Parabene nur eine untergeordnete Rolle bezüglich ihres Risikobeitrages in der kumulativen HI-Betrachtung spielen. Hier hat *n*-PrP den stärksten Einfluss, gefolgt von *n*-BuP. Die untersuchten Urinproben wurden zwischen 1995 und 2012 gesammelt. Erst im Jahr 2014 wurden die maximal zulässigen Konzentrationen von *n*-PrP und *n*-BuP in kosmetischen Produkten reduziert und die Verwendung von *iso*-PrP, *iso*-BuP, BeP und PeP innerhalb der EU verboten. Weitere HBM Untersuchungen an aktuellen Studienpopulationen sollten nun klären, ob diese regulatorischen Maßnahmen ausreichen, um beständige HI Werte unter 1 in der Allgemeinbevölkerung zu erreichen.

Kapitel V wurde veröffentlicht als Peer-Review Publikation:

Moos RK, Apel P, Schröter-Kermani C, Kolossa-Gehring M, Brüning T, Koch HM. Daily intake and hazard index of parabens based upon 24 h urine samples of the German Environmental Specimen Bank from 1995 to 2012. Journal of Exposure Science and Environmental. 2016. doi:10.1038/jes.2016.65

Die Beiträge des Autors dieser Dissertation umfassten folgende in **Kapitel V** dargestellten wissenschaftlichen Arbeiten: Datenauswertung, Evaluation und Interpretation; Abfassen der Publikation.

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Kapitel I

Rapid determination of nine parabens and seven other environmental phenols in urine samples of German children and adults

Rebecca K. Moos, Jürgen Angerer, Jürgen Wittsiepe,
Michael Wilhelm, Thomas Brüning, Holger M. Koch
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Abstract

We developed a fast, selective and sensitive on-line LC/LC–MS/MS method for the simultaneous determination of nine parabens and seven environmental phenols in urine. Parabens are widely used as antimicrobial preservatives. Bisphenol A, triclosan, triclocarban, 2-phenylphenol, and benzophenones are used inter alia in disinfectants, sunscreens and in polymers. Some of these substances are suspected endocrine disruptors.

Limits of quantification and analytical quality criteria fully met the needs for determining exposure levels occurring in the general population. We analyzed 157 spot urine samples from the general German population (59 females, 39 males and 59 children). For the parabens, we found methyl, ethyl and *n*-propyl paraben with high detection rates (77–98%), followed by *n*-butyl (36%), *iso*-butyl (17%), *iso*-propyl (3%) and benzyl paraben (3%). We detected no pentyl and heptyl paraben. Urinary concentrations were highest for methyl paraben (median 24.5 µg/L; 95th percentile 379 µg/L) followed by ethyl (1.4 µg/L; 35.2 µg/L) and *n*-propyl paraben (1.2 µg/L; 68.1 µg/L). Other environmental phenols with high detection rates were BPA (95%), triclosan (45%) and benzophenone 1 and 3 (26%). For most of the parabens/environmental phenols we found higher urinary levels in females than in males or children, probably due to differences in (personal care) product use. However, high levels (in the mg/L range) were also observed in children.

Exposure to the above substances is occurring worldwide. Differences between countries do seem to exist and might be caused by different product compositions or different use habits. Human metabolism data is urgently needed to extrapolate from urinary biomarker levels to doses actually taken up.

Introduction

Because of the ubiquitous presence and the extensive use of environmental phenols and parabens in personal care and consumer products, food and beverage processing, pharmaceuticals and disinfectants humans are constantly exposed to these substances. Esters of *p*-hydroxybenzoic acid (parabens) are widely used as antimicrobial preservatives in cosmetics, pharmaceuticals and food for more than 50 years (Guo and Kannan 2013; Soni et al. 2005; SCCS 2010). Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; TCS) is a broad spectrum antibacterial agent used in personal care products, including toothpaste, mouthwash, deodorants (Calafat et al. 2008a; Ye et al. 2008; Dann and Hontela 2011; SCCP 2009). Triclocarban (1-(4-chlorophenyl)-3-(3,4-dichlorophenyl)urea; TCC) is employed as an antimicrobial agent in personal care products including bar soap, detergents, toothpaste, deodorant, and cleansing lotions (Ye et al. 2011; SCCP 2005). 2-phenylphenole (2-PP) is used as active ingredient in broad spectrum fungicides used in wood preservation and as a surface biocide. Furthermore, it is used for inhibition of mold growth on citrus fruits (Bomhard et al. 2002; US EPA 2006). Bisphenol A (4,4'-(propane-2,2-diyl)diphenol); BPA) is used to manufacture polycarbonate plastics and epoxy resins. Polycarbonate is a plastic widely used in articles we are in contact trough everyday life, e.g., such as tableware, storage containers, returnable bottles and dental sealants and composites, and thermal receipt paper (Frederiksen et al. 2013a; EFSA 2006). Benzophenone-3 (2-hydroxy-4-methoxybenzo-phenone; BP-3) and its metabolites (Wang and Kannan 2013b) Benzophenone-1 (2,4-dihydroxybenzophenone; BP-1) and benzophenone-8 (2,2'-dihydroxy-4-methoxybenzo- phenone; BP-8) are sunscreen agents used in cosmetics, including sun creams and anti-aging products, and also in plastic surface coating for food packaging (Calafat et al. 2008b; SCCP 2006). BP-1 and BP-8 are not authorized as additives in Europe.

The above phenolic substances are currently under intensive scrutiny because of their possible endocrine disrupting potency both alone and in combination (Schlumpf et al. 2001, Krause et al. 2012; Christiansen et al. 2012; Isling et al. 2013). In regard to parabens, in vitro studies have shown the potential for endocrine modifying effects with estrogenic activity increasing with length of the alkyl side chain (Darbre and Harvey 2008; Boberg et al. 2010). All parabens can be regarded as estrogenic in vivo due to their uterotrophic effects, with LOELs for butyl- and propylparaben of 7 and 20 mg/kg bw/day (Lemini et al. 2003), respectively.

Additionally, reproductive toxicity studies in rodents have shown effects on sperm count and testosterone levels after dietary exposure to butyl- and propyl paraben (Oishi 2001, 2002a, 2002b). Overall, the data basis for a toxicological evaluation of the parabens is still insufficient. BPA and TCS have also been reported to have weak estrogenic effects (Dann and Hontela 2011; Clayton et al. 2011; Henry and Fair 2013; Jung et al. 2012; Richter et al. 2007). Several studies also report low dose effects of BPA on sexual development (Christiansen et al. 2013; Vandenberg et al. 2010). Additionally, effects of both chemicals on thyroid function have been reported (Axelstad et al. 2013; Chevrier et al. 2013). BP-3 and phenylphenols are regarded to have weak estrogenic or anti-androgenic properties (Calafat et al. 2008b; Krause et al. 2012; Li et al. 2010). TCC is suspected to have a weak androgen effect (Ahn et al. 2008; Chen et al. 2008; Duleba et al. 2011).

Because of the widespread use of these phenolic compounds, their presence in personal care products, in foodstuff (intended or unintended) and in other applications, exposures will occur in an aggregate manner and via multiple routes of uptake, i.e., through dermal absorption, ingestion, and inhalation. To determine the exposure to such chemicals via classical means of exposure assessment (measurements in environmental media, personal care products and foodstuff; collection of questionnaire data on personal lifestyle, product use and food consumption; estimations of contact times and incorporated quantities) can be cumbersome and prone to errors (due to lack of knowledge, e.g., on relevant sources and pathways). Human biomonitoring, the determination of chemicals or their metabolites in human tissues like urine as an integral measure of exposure, allows assessing exposures even when the quantity and quality of external exposures are unknown or ambiguous. Urinary concentrations of several of the above compounds (or their metabolites) have already been successfully used as biomarkers of internal exposure (Ye et al. 2006). Various studies have shown that the general population is exposed to parabens and other environmental phenols (Frederiksen et al. 2013b, 2014; Asimakopoulos et al. 2014a, 2014b; CDC 2013; Calafat et al. 2010; Casas et al. 2011; Li et al. 2013; Pirard et al. 2012; Philippat et al. 2012; Ye et al. 2011; Wang et al. 2013).

In this work we present a fast, robust and reliable LC/LC–MS/MS method with isotope dilution for the determination of nine parabens (methyl- (MeP), ethyl- (EtP), *n*-propyl- (*n*-PrP), *n*-butyl- (*n*-BuP), benzyl- (BeP), pentyl- (PeP) and heptyl paraben (HeP); including *iso*-butyl- (*iso*-BuP) and *iso*-propyl paraben (*iso*-PrP)) and seven environmental phenols (BPA, 2-PP, BP-1, BP-3, BP-8, TCS and TCC) in urine covering a wide concentration range.

With this method we can thus assess exposures resulting from environmental background expo-sures, personal care product use and occupational exposures. We applied this method on urine samples of children and adults from the general German population and for the first time provide internal exposure data for this set of chemicals in Germany.

Experimental

Materials

Methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *iso*-butyl- and benzyl paraben (esters of *p*-hydroxybenzoic acid), triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol; TCS), triclocarban (1-(4-chlorophenyl)-3-(3,4-dichlorophenyl)urea; TCC), 2-phenylphenol, bisphenol A (4,4'-(propane-2,2-diyl)diphenol, BPA), benzophenone-1 (2,4-dihydroxybenzophenone, BP-1), benzophenone-3 (2-hydroxy-4-methoxybenzophenone, BP-3), benzophenone-8 (2,2'-dihydroxy-4-methoxybenzophenone, BP-8) were purchased from Sigma–Aldrich (Steinheim, Germany). *Iso*-propyl- and heptyl paraben were purchased from TCI Europe (Eschborn, Germany). Pentyl paraben was purchased from VWR (Darmstadt, Germany). The deuterated standards d4-methyl-, d4-ethyl-, d4-propyl-, d4-butyl- and d4-benzyl paraben, d3-triclosan, d4-triclocarban, d16-bisphenol A and d5-benzophenone-3 were purchased from C/D/N Isotopes (Dr. Ehrenstorfer GmbH, Augsburg, Germany). 13C6-2-phenylphenol was purchased from Cambridge Isotope Laboratories (Wesel, Germany). All standards had a purity ≥95%. The deuterated compounds had no detectable impurities of the unlabeled or partially labeled compounds. Water and acetonitrile (LC/MS grade) and acetic acid (glacial, extra pure) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). β-Glucuronidase enzyme Type HP-2 and ammonium acetate p.a. were purchased from Sigma–Aldrich (Steinheim, Germany).

Analytical method

Preparation of stock solutions

Standard stock solutions were prepared by dissolving 10 mg of the standards in 10 ml acetonitrile (1 g/L). Stock solutions were stored at -20 °C until further use. For further analysis, eight calibration standards were prepared by diluting the stock solutions to final concentrations in a range between 0.5 µg/L and 300 µg/L water.

The stock solutions for the internal standards were prepared by dissolving 5 mg of each labeled standard in 5 mL acetonitrile (1 g/L). These stock solutions were used to prepare an internal standard mixture with a concentration of 0.275 mg/L acetonitrile.

Sample preparation

Urine samples were stored in 250 mL polyethylene containers at −20 °C. Before analysis all samples were equilibrated to room temperature and homogenized by shaking thoroughly. Aliquots of 300 µL urine were transferred into 1.8 mL glass screw-cap vials. Each sample was spiked with 300 µL 1 M ammonium acetate buffer at pH 5.0, 25 µL internal standard solution and 6 µL β-glucuronidase/arylsulfatase solution (≥100,000 U/mL) for the hydrolysis of the conjugated species. Thus, the total concentration (free and conjugated species) of each target analyte was measured. After incubation in a water bath at 37 °C for 3.5 h, all samples were frozen overnight to precipitate cryophobic proteins, subsequently thawed and centrifuged (3500×g for 10 min). The supernatant was transferred into a second 1.8 mL screw-cap vial. A volume of 100 µL was injected into the HPLC-system.

Calibration procedure and quality control

Calibration standards (prepared in water), quality controls and blanks (Millipore water) were treated equally to the urine samples (described in Section 'Sample preparation'). Quadratic calibration curves were obtained with a 1/x weighting by plotting the quotients of peak areas of each analyte and the peak areas of the specific internal standard as a function of the concentration. Quality control material was prepared from two different pooled urine mixtures. The composition of the urine mixtures was chosen based upon the native urinary concentrations of the target analytes (free and conjugated species) in the individual urine samples to build a low and a high concentration quality control (Q low and Q high). For some analytes we had to spike the quality controls with the respective analytical standard material to achieve the desired concentrations. The quality control samples were frozen, thawed and filtered three times before final use. Repeatability and precision of the method was determined by analyzing the quality control standards eight times in a row (intra-day precision) and at eight different days (inter-day precision) using newly obtained calibration graphs for the calculation of the concentrations of the quality control samples. Accuracy (and precision) of the method and the possible influence of the urinary matrix were investigated by analyzing eight urine samples with different creatinine concentrations between 0.2 g/L and 2.5 g/L.

These urine samples were analyzed without any deviation from the standard protocol as described above and additionally spiked at two respectively three concentration levels. From these spiking experiments the individual recoveries could be calculated which then could be used to determine accuracy and precision.

LC–MS/MS analysis

High-performance liquid chromatography

The high-performance liquid chromatography system consisted of several Agilent 1200 modules (Agilent autosampler G1329A, quaternary pump G1311A, binary pump G1312A and vacuum degasser G1379B and G1322A) and one column compartment with a 6-port switching valve. For the procedure of extracting the analytes from the matrix we used a column-switching system (see **supplementary materials Fig. 1; Appendix I**) with two columns, previously described by Koch et al. (2003) and Ye et al. (2005). In short, extraction from urinary matrix and enrichment was achieved using a RAM (Restricted Access Material) phase (LiChrospher® RP-8 ADS (25 µm) 25 mm × 4 mm RAM from Merck, Darmstadt, Germany), chromatographic separation was realized on a reversed phase C18 column (Atlantis dC18 30 mm × 150 mm; 3 µm, Waters, Ireland). Two different solvent mixtures were used for analysis: Solvent A 99.98% water and 0.02% acetic acid and solvent B 99.98% acetonitrile and 0.02% acetic acid. The process of extracting the analytes from the matrix is a three step approach. In the first step (0–7 min, valve position A) 100 µL of the sample are injected onto the RAM phase with a constant flow of 1 mL/min with 98% solvent A by the loading pump (quaternary pump). In step 2 the analytes are transferred onto the reversed phase C18 column in backflush mode (7–10 min, valve position B) with a constant flow of 0.4 mL/min by the analytical pump (binary pump). In step 3 the switching valve is set back to position A. In this step the analytes are chromatographically separated with a constant flow of 0.4 mL/min by the analytical pump (10–26 min) and a solvent gradient increasing to 95% solvent B. At the same time the RAM phase is regenerated and equilibrated by the loading pump. The method has a total running time of 26 min. The gradient for transfer, chromatographic separation and flushing is given in supplementary materials **Table 1** (**Appendix I**).

Mass spectrometry

Detection and quantification was performed on an AB Sciex 5500 QTrap tandem mass spectrometer, used in negative ionization mode (ion spray voltage (IS): −4250 V). Nitrogen was used as nebulizer gas and turbo heater gas (ion source gas GS1 and GS2) with a pressure of 50 psi. The pressure of the curtain gas (CUR) was set to 30 psi. Nitrogen was also used as collision gas (CAD) at concentration level LOW. The entrance potential was set on -10 V. High resolution was used for both quadrupoles (Q1 and Q3).

Ionization parameters and collision cell parameters were optimized individually for each analyte. For each analyte at least two specific parent and daughter ion combinations were selected. The fragment with the highest intensity was used for quantification, (quantifier) the other for verification (qualifier). The MS/MS was operated in scheduled multiple reaction monitoring (MRM) mode. The fragment ions and other analyte specific parameters are shown in **Table 1**.

Table 1 MRM-parameters for mass spectrometric detection.

Analyte	RT [min]	Q1 mass → Q3 mass quantifier (qualifier) [Da]	
		Native standard	Internal standard
MeP	14.2	151 → 92 (136)	155 → 96
EtP	15.1	165 → 92 (136)	169 → 96
iso-PrP	16.2	179 → 136 (92)	183 → 96
n-PrP	16.4	179 → 92 (136)	183 → 96 [a]
iso-BuP	17.9	193 → 92 (136)	197 → 96
n-BuP	18.1	193 → 92 (136)	197 → 96 [b]
BeP	18.1	227 → 92 (136)	231 → 96
PeP	19.4	207 → 92 (136)	231 → 96 [c]
HeP	21.0	235 → 92 (136)	290 → 35 [d]
BPA	16.1	227 → 133 (93)	241 → 142
TCS	21.0	287 → 35 (289 → 35)	290 → 35
TCC	21.1	313 → 160 (315 → 162)	317 → 160
2-PP	18.6	169 → 115 (141)	175 → 121
BP-1	17.3	213 → 91 (135)	232 → 215 [e]
BP-3	20.3	227 → 211 (183)	232 → 215
BP-8	18.8	243 → 93 (123)	232 → 215 [e]

[a] d4-propylparabene is used as the internal standard of *iso*-propylparaben
[b] d4-butylparabene is used as the internal standard of *iso*-butylparaben
[c] d4-benzylparabene is used as the internal standard of pentyl paraben
[d] d3-triclosan is used as the internal standard of Heptylparaben
[e] d5-benzophenone-3 is used as the internal standard of benzophenone-1 and benzophenone-8

Study subjects

For a human biomonitoring study we investigated 157 spot urine samples from the general German population. The females and children investigated were mother–child pairs and random subset of a larger cohort study, the Duisburg-cohort study. Urine samples of the mother–child pairs were first morning urine samples (central stream urine) collected between October 2007 and February 2009. Details of the Duisburg-Cohort study design were published elsewhere (Kasper-Sonnenberg et al. 2012; Wilhelm et al. 2008). At the time of urine collection, the age of the mothers was 29–48 years (median 40 years); the age of the children was 6–8 years (median 6.5 years). This study population was complemented with spot urine samples from 39 male volunteers (18–64 years old, median 37) that were part of a control population collected at the Institute for Prevention and Occupational Medicine (IPA) in 2010 and 2011. All individuals were not known to be occupationally exposed to the parabens and other phenols investigated. Creatinine levels were in the range from 0.2 to 3.0 g/L. Both the Duisburg-cohort study (registry no. 1478; 3220-08) and the sampling of the male volunteers (registry no. 3867-10) were reviewed and approved by the ethical commission of the medical facilities at the Ruhr University Bochum, Germany.

Statistics

For each biomarker, descriptive statistics (median, 95th percentile, standard deviations) were calculated with Excel 2010 (Microsoft Corporation, Redmond, USA). Metabolite concentrations below the LOQ were substituted with LOQ/2 by convention (Hornung and Reed 1990). Boxplots were generated with OriginPro 9.1 (OriginLab Corporation, Northampton, USA).

Results and discussion

Analytical approach – general considerations

The method was designed for the purpose of human biomonitoring of parabens and other environmental phenols. Special focus during method development was laid upon the inclusion of the iso-forms of propyl und butyl paraben: *iso*-PrP and *iso*-BuP. We achieved a sufficient chromatographic separation of these isomers (see **Fig. 1**) enabling us to quantify each isomer individually despite identical molecular weights and fragmentation patterns of the n- and the iso-forms (see **Table 1**).

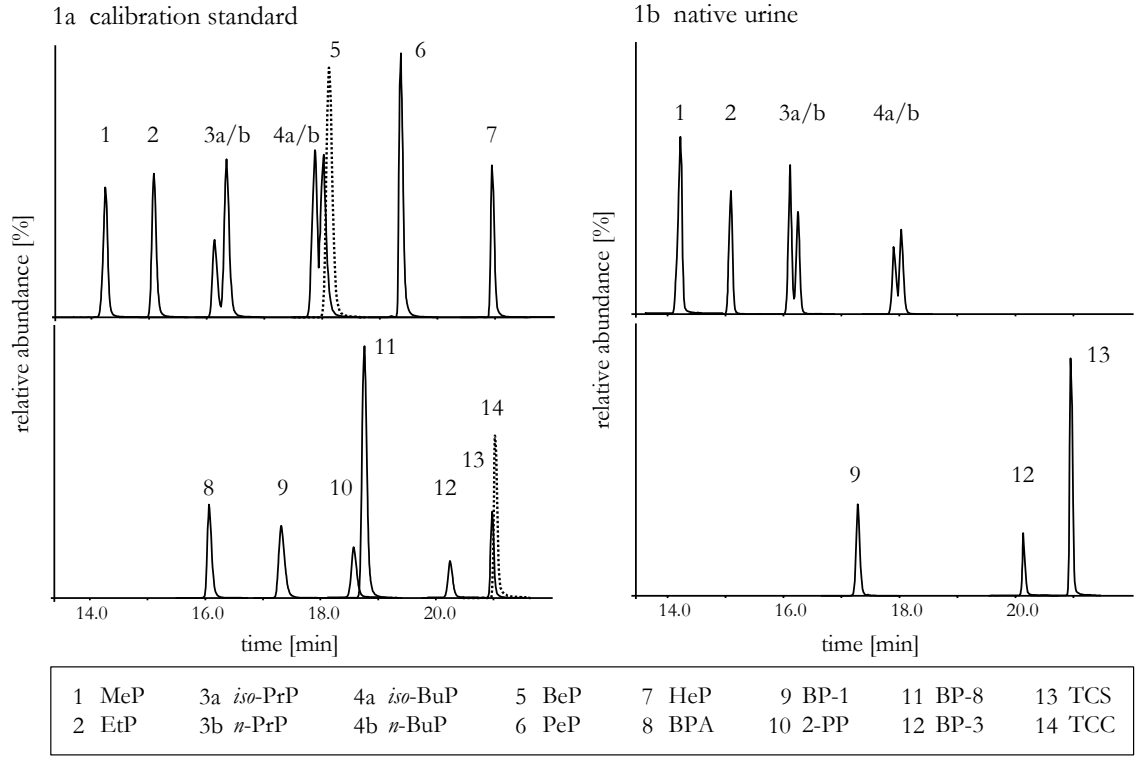

Figure 1 a) Chromatogram of a processed calibration standard. Only the quantifier parent daughter ion combinations for the parabens and other phenols are shown. Concentrations were 80 µg/L for each analyte. b) Chromatogram of a processed urine sample. Only the quantifier parent daughter ion combinations for the analytes are shown. Concentrations were as follows: MeP: 250 µg/L, EtP: 58.8 µg/L, *iso*-PrP: 175 µg/L, *n*-PrP: 54.0 µg/L, *iso*-BuP: 15.3, µg/L *n*-BuP: 29.0 µg/L, BPA: 4.1 µg/L, TCS: 1630 µg/L, BP-1: 8.0 µg/L and BP-3: 59.8 µg/L. Creatinine content was 1.45 g/L.

The limits of quantification (LOQ), generally defined as a signal-to-noise ratio of nine, are shown in **Table 2**. Due to the omnipresent nature of some parabens, mainly MeP, EtP, *n*-PrP and *n*-BuP we had to overcome issues of laboratory blank values. Therefore, several sources of external contamination were identified and minimized; all laboratory reagents were screened and reagents with trace levels of parabens were replaced by paraben-free reagents; all laboratory equipment was pre-washed with acetone and acetonitrile and all lab-technicians involved in any analytical step strictly refrained from the use of paraben (or other target analyte) containing personal care products. In spite of all precautions, trace level contaminations could still be observed for some target analytes, possibly due to various and diffuse (indoor dust) sources (Wang et al. 2012; Fan et al. 2010). For the parabens (MeP, EtP and *n*-PrP) we estimated blank values at or below 0.1 µg/L, interfering with a proper determination of the LOD/LOQ.

Thus we conservatively set the LOQ for all parabens to be 0.5 µg/L. Peak responses for the other target analytes were generally weaker than for the parabens, resulting in LOQs of 1.0 µg/L for TCS and TCC and 2.0 µg/L for 2-PP and BP-3. These LOQs proved to be sufficiently low to detect the environmental background exposure of the general population.

Table 2 Intra-day and inter-day precision of the method calculated by analysis of self preöpared quality control materials with two different concentration levels and quantification limits of this method.

Analyte	Intra-day series (n=8)				Inter-day series (n=8)				LOQ (µg/L)	Calibration range (µg/L)
	Q low		Q high		Q low		Q high			
	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)		
MeP	19.3	2.6	73.0	1.2	20.2	2.8	80.6	2.2	0.5	0.5 – 300
EtP	4.5	4.6	44.1	3.9	4.2	4.5	43.8	4.0	0.5	0.5 – 150
iso-PrP	3.4	2.0	17.6	2.9	3.2	4.1	17.0	4.0	0.5	0.5 – 80
n-PrP	7.4	3.6	60.2	3.4	6.7	5.1	59.8	4.7	0.5	0.5 – 150
iso-BuP	2.9	3.3	12.9	6.2	3.4	4.6	15.8	8.2	0.5	0.5 – 80
n-BuP	3.5	4.5	49.3	4.9	3.3	6.9	44.7	7.9	0.5	0.5 – 150
BeP	1.9	5.1	17.7	3.4	1.9	7.0	18.6	4.8	0.5	0.5 – 80
PeP	2.6	2.7	25.0	2.4	2.5	6.8	26.1	7.4	0.5	0.5 – 25
HeP	2.3	3.0	16.7	6.0	2.3	8.1	16.6	7.3	0.5	0.5 – 25
BPA	2.8	3.6	19.3	2.9	2.4	8.6	18.2	2.8	0.5	0.5 – 80
TCS	4.7	6.2	33.0	4.0	4.7	3.4	32.7	4.3	1.0	0.5 – 80
TCC	1.5	4.4	17.2	6.2	1.4	8.2	16.4	10.9	1.0	0.5 – 25
2-PP	4.0	6.8	35.5	4.6	4.1	8.2	38.4	6.9	2.0	0.5 – 100
BP-1	1.5	7.3	14.2	6.5	1.4	8.3	12.8	12.1	0.5	0.5 – 25
BP-3	7.5	8.2	42.4	7.0	7.6	6.8	43.0	5.0	2.0	0.5 – 80
BP-8	1.4	4.9	14.4	3.9	1.4	7.8	14.4	9.1	0.5	0.5 – 25

RSD: relative standard deviation

The calibration range varied considerably for the various parameters (see **Table 2**), possibly due to different ionization yields and ionization effects of the electrospray ionization. To widen the calibration range we chose to calibrate via a quadratic calibration curve instead of a linear regression calibration curve. Several of the analytes (e.g., MeP, EtP, *n*-PrP, TCS and BP-3), are present in a wide concentration range in population samples (see 'Results of bio-logical monitoring') as a special characteristic of chemicals used in personal care products, with non-users and users. Urine samples with analyte concentrations outside the linear calibration range were diluted with water and reanalyzed for the respective analytes. Within the calibration range, no relevant carry-over was observed (checked with water blanks after the highest calibration standard).

For native samples with concentrations above the calibra-tion range, the sample was diluted and re-analyzed. In addition, the sample immediately following such "high-concentration" samples in the analytical batch was also re-analyzed to exclude false values due to a possible carry-over. Taking all the necessary precautions into account, the intra-day precisions for all analytes at two concentration levels (analyses of the quality control standards eight times in a row) were highly acceptable. For the Q low, relative standard deviations for the various parameters were 2.0–8.2%, for the Q high the relative standard deviations were 1.2–7.0%. Inter-day precision (analyzing the quality control stand-ards on eight different days) were 2.8–8.6% for Q low and 2.2–12.1% for Q high. The complete validation data is given in **Table 2**.

The accuracy (relative recovery) of the method was determined by analyzing eight urine samples with different creatinine concentrations between 0.2 g/L and 2.5 g/L. These urine samples were analyzed with and without spiking known concentrations of the target analytes at different concentration levels. In case the non-spiked urine already contained native background levels of the respective analytes, these had to be subtracted from the results of the spiked urine samples to correctly derive the recovery of the spiked analyte. Relative recoveries are depicted in supplementary materials **Table 2** (**Appendix I**). For all analytes with labeled internal standards (MeP, EtP, *n*-PrP, *n*-BuP and BeP, BPA, TCS, TCC, 2-PP and BP-3), relative recoveries were in the range from 95.0% to 121.2%. For the iso-parabens and the BP-3 metabolites with structurally closely related internal standards relative recoveries were between 72.0% and 108.1%. For PeP and HeP relative recoveries were between 135% and 219%. The relative standard deviations of recovered spiked concentrations were 1.4–15.8% (18.2% for HeP and 20.5% for PeP) and thus only slightly higher than standard deviations derived from intra- and inter-day precision measurements.

Results of biological monitoring

The results of the biomonitoring study on 157 German children and adults, not occupationally exposed to parabens and other environmental phenols, are summarized in **Table 3**. We found MeP, EtP and *n*-PrP with high detection rates (77–98%), followed by *n*-BuP (36%). This is in agreement with the production volume/consumption volume being highest for these parabens, led by MeP and followed by EtP, *n*-PrP and *n*-BuP (DHI Water and Environment 2007). Additionally, we detected the isomers *iso*-PrP and *iso*-BuP in 3 respectively 17% of the samples, next to BeP (3%). We detected no PeP and HeP.

Table 3 Results of the human biomonitoring study with 157 volunteers. Median, 95th P and maximum concentration (g/L) of paraben and environmental phenols.

Analyte	Frequency of detection (%)	Concentration in µg/L			Concentration in µg/g creatinine		
		Median	95th P.	Max.	Median	95th P.	Max.
MeP	97	24.5	379	3230	23.3	385	2555
EtP	82	1.4	35.2	238	1.5	30.7	201
iso-PrP	3	< LOQ	< LOQ	175	< LOQ	< LOQ	121
n-PrP	77	1.2	68.1	1530	1.5	52.6	962
iso-BuP	17	< LOQ	2.2	15.3	< LOQ	2.8	11.3
n-BuP	36	< LOQ	9.2	73.4	< LOQ	8.1	97.4
BeP	3	< LOQ	< LOQ	1.3	< LOQ	< LOQ	1.4
PeP	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
HeP	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
BPA	95	2.2	6.5	59.6	1.9	6.2	78.6
TCS	45	< LOQ	269	1630	< LOQ	300	1123
TCC	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
2-PP	9	< LOQ	4.5	76.6	< LOQ	5,0	65.1
BP-1	26	< LOQ	4.1	10.2	< LOQ	3.2	13.9
BP-3	26	< LOQ	23.4	96.8	< LOQ	18.7	94.0
BP-8	0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

LOQ: limit of quantification

The paraben occurring in highest concentrations in urine was MeP with a median of 24.5 µg/L, followed by EtP and *n*-PrP. Based upon detection rates, reliable 95th percentiles could be calculated for five parabens, ranging from 2.2 µg/L for *iso*-BuP to 379 µg/L for MeP. We observed maximum values for MeP and *n*-PrP well in the mg/L range. These maximum values were 100 (MeP) to 1000 (*n*-PrP) times higher than the respective median values, impressively illustrating the range of exposures possible for these parabens. The isomeric forms of both PrP and BuP were generally found at lower concentrations than their classical straight chain analogs; still, the maximum urinary concentrations in this study were 15.3 µg/L for *iso*-BuP and 175 µg/l for *iso*-PrP. Thus, these isomeric parabens also contribute to the general paraben body burden in the general population.

Next to the parabens we found the German population broadly exposed to TCS with 45% of the urine samples above the LOQ of 1 µg/L. Urinary levels of 269 µg/L (95th percentile) and a maximum value of 1630 µg/L indicate to relevant sources of exposure possibly through body care and consumer products. Similarly, roughly a quarter of the study population (26%) excreted BP-3 (95th percentile 23.4 µg/L), substance used in body care products mainly as sunscreen agents. BP-1 was found in 26% (95th percentile 4.1 µg/L) of samples. BP-8 was not found in any of the samples.

The latter benzophenones are not allowed to be used in Europe; however, BP-1 is a known metabolite of BP-3. While TCC was not detected in any of the samples, 2-PP was detected in 9% of the samples. 2-PP is no longer allowed in the EU as a food additive but still used as a surface disinfectant a fungicide for waxing citrus fruits post-harvest. BPA was detected in 95% of the samples. Urinary levels (median 2.2; 95th percentile 6.5 µg/L) are in agreement with values previously reported for Germany (Kasper-Sonnenberg et al. 2012; Koch et al. 2012).

The composition of our study population, albeit with a limited sample size (59 women, 39 men, 59 children), enables us to roughly compare urinary levels of men with women and children (see **supplementary materials Table 3; Appendix I**). **Fig. 2** depicts the boxplots of urinary concentrations (in µg/L) of each biomarker separately for men, women and children. Values below the LOQ were set to LOQ/2. PeP, HeP, BP-8 and TCC are not included in **Fig. 2**, because all values were below the LOQ. For all of the parabens, women generally seem to excrete higher levels than men and children.

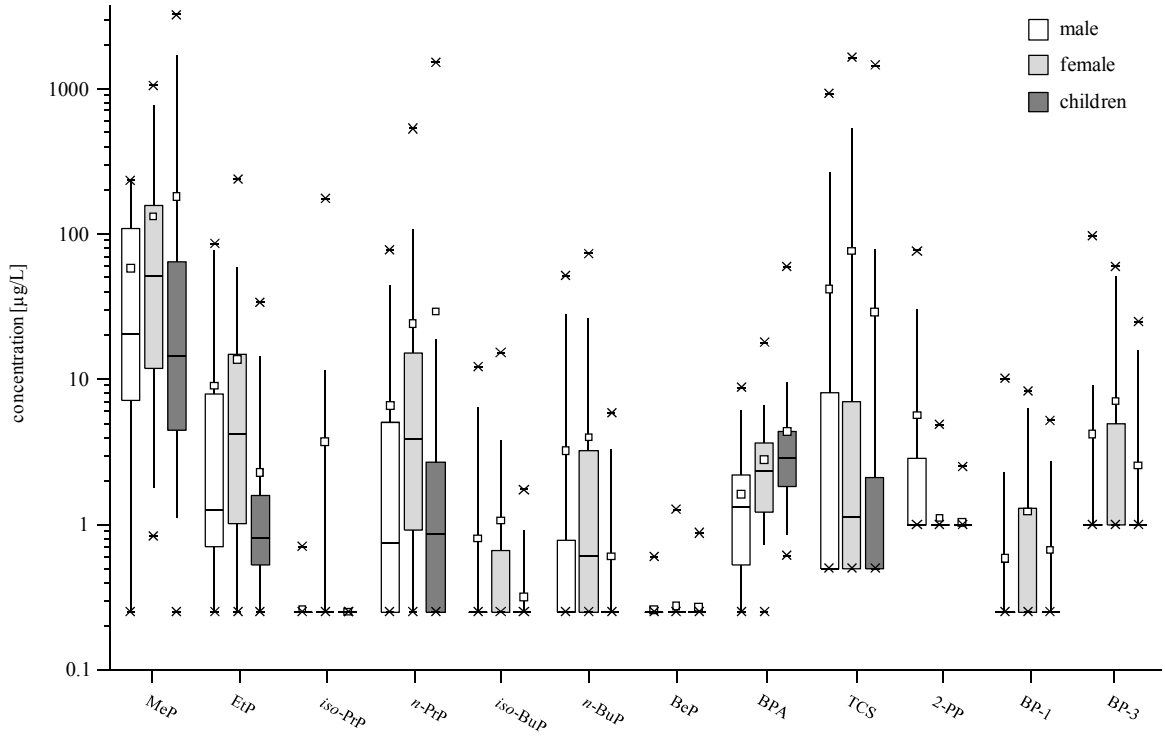

Figure 2 Boxplots of the results of the human biomonitoring study with 157 volunteers. For each substance, data is shown separately for males (left), females (middle) and children (right). Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile. The minimum and maximum values are represented by an x.

The median concentration of MeP is 51.4 µg/L for women, 20.7 µg/L for men and 14.5 µg/L for children; for EtP it is 4.2 µg/L for women, 1.3 µg/L for men and 0.8 µg/L for children; for *n*-PrP it is 3.9 µg/L for women, 0.8 µg/L for men and 0.9 µg/L for children. Also, for the parabens with lower detection rates (*n*-BuP, *iso*-PrP, *iso*-BuP), higher urinary levels are generally observed for women. Still, as is the case for MeP and *n*-PrP, maximum urinary levels measured belonged to children, showing the wide spread of urinary levels with high maxima also in this susceptible population. The generally higher paraben levels in women probably arise from a heavier and/or more diverse use of personal care products preserved with parabens. Similar to the parabens, benzophenone levels seem to be generally higher in women compared to men and children. In regard to TCS women and men excrete comparable levels, while children seem to have lower levels. One explanation for this observation might be that triclosan is used amongst others in adult toothpastes, while its use in toothpastes for children seems limited. But, again, as with the parabens, the maximum value observed in the children of our study population is comparable with the adults in the mg/L range. Concerning BPA, differences between the three subpopulations are rather small. The highest BPA levels in children might be related to foodstuff as the predominant route of BPA exposure with children having a higher food intake related to their body weight. We have no hint why most of the 2-PP detections were observed in the men.

In **Table 4** we put the biomonitoring data from our study (Germany) into perspective with selected data available from Denmark and the US, separately for children, males and females, depicted as the 95th percentiles (for median values see **supplementary materials Table 4; Appendix I**). In addition, there are a variety of other studies from China, Japan, Greece and Spain reporting on urinary parabens and other environmental phenols (Ma et al. 2013; Asimakopoulos et al. 2014a; Casas et al. 2011; Shirai et al. 2013; Zhang et al. 2011; Wang et al. 2013; Wang and Kannan 2013b). Due to its heterogeneity, this data was not included in **Table 4**.

Among the nine parabens analyzed, MeP was the predominant compound in urine, followed by EtP and *n*-PrP. We observe a similar distribution pattern of exposures in other studies. Also, high detection rates and high urinary concentrations of parabens have already been described in other studies (Calafat et al. 2010; Frederiksen et al. 2010, 2014; Ma et al. 2013; Asimakopoulos et al. 2014a; Casas et al. 2011; Shirai et al. 2013).

Table 4 Biomonitoring data from our study compared with data from Denmark and the US, separately for children, males and females, depicted as the 95th percentiles.

Analyte	children 95th P. (µg/L)			female 95th P. (µg/L)			male 95th P. (µg/L)		
	Germany	Denmark[a]	USA[b]	Germany	Denmark[a]	USA[b]	Germany	Denmark[c]	USA[b]
MeP	511	62.0	873	609	275	1230	231	-	727
EtP	9.2	3.7	11.5	44,0	44.0	138	47.1	-	36.4
iso-PrP	< LOQ	< LOQ	-	2.5	3,8	-	< LOQ	-	-
n-PrP	13.9	14.0	114	79.4	33.0	361	25.8	-	134
iso-BuP	0.8	< LOQ	-	3.8	< LOQ	-	2.2	-	-
n-BuP	2.3	1.4	2.2	17.0	9.3	31.8	17.7	-	2.7
BeP	< LOQ	< LOQ	-	< LOQ	< LOQ	-	< LOQ	-	-
PeP	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-
HeP	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-
BPA	7.7	7.9	9.2	5.8	11.0	9.2	4.5	14.9	10.9
TCS	14,8	271	200	348	581	488	179	377	455
TCC	< LOQ	0.6	-	< LOQ	0.5	-	< LOQ	0.2	-
2-PP	< LOQ	0.5	0.6	< LOQ	0.5	0.5	23.5	1.0	0.5
BP-1	2.6	-	-	4.9	-	-	1.0	-	-
BP-3	10.6	40.0	1570	37.0	312	3200	8.5	80.4	610
BP-8	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-

LOQ: limit of quantification

[a] Frederiksen et al. (2013b), children n=145 (age 6–11), female n=145 (age 31–52)

[b] CDC (2013), children n=415 (age 6–11), female n=1350 including children (age 6–20 years and older), male n=1399 including children (age 6–20 years and older)

[c] Frederiksen et al. (2014), male n=309

We confirm the presence of *iso*-PrP and *iso*-BuP in urinary samples previously measured in Denmark (by now not included in measurements in other studies). For most parabens and irrespective if measured in children, females or males, exposures in Germany seem higher than those in Denmark and China, but lower than those in the US. This might point to a different extent of paraben preserved personal care products or product uses in these countries. In detail, compared with other countries, the median values of MeP in urine of children in Germany seem higher than those in Denmark and China, but lower than those in the US. The median values of EtP and *n*-PrP in Germany were similar to those reported for children in the US, Denmark and China. Similar distributions could be observed for women, except for *n*-PrP in US women with 5 times higher levels compared to German women. Similar or higher values than in the US were only observed in pregnant women (Shirai et al. 2013; Meeker et al. 2013; Casas et al. 2011). For men, the exposure to parabens seems to be very similar in all countries, except for MeP in China. Urinary concentrations of MeP in China were significantly lower (factor of 5) than those in Germany, Denmark and the US.

As in women and children, in men MeP and *n*-PrP were the most commonly detected parabens, followed by EtP. PeP and HeP have not been measured before. Based upon these biomarkers, exposures to these parabens seem negligible in all subpopulations.

The observation for Germany, that females are a population with highest paraben exposures seems to be similar in the US and China. In Denmark, no significant difference in urinary paraben concentration between males and females could be observed.

For BPA we again confirm the omnipresent and rather uniform exposure to this chemical worldwide. While TCC and 2-PP exposures seem to be rather low and restricted to some individuals, substantial urinary TCS levels can be found in all three countries. As already pointed out above for Germany, children seem to be somewhat lower exposed than adults, with no differences between females and males. Concerning the benzophenones, we determined BP-1 and BP-8 in addition to BP-3. Urinary levels of BP-1 that can both be an active ingredient (not allowed in Europe) and a metabolite of BP-3 were generally lower but confirmed the measurements of BP-3. Strikingly, at least at the 95th percentiles, urinary BP-3 levels in the US were approximately 100 times above those in Germany and 10 times above those in Denmark. Similarly, median TCS levels in the US were approximately a factor of 5–10 higher in the US than in Germany and Denmark. These substantial differences probably again originate from differences in use or composition of personal care products in these countries.

Conclusions

We have developed a fast, robust sensitive and selective method to determine 16 phenolic substances in urine samples in a wide concentration range. The method was built on existing on-line column-switching HPLC–MS/MS methodologies previously described by Koch et al. (2003) and Ye et al. (2005) with the special focus on the chromatographic separation of the n- and the iso-forms of propyl and butyl paraben.

We could show that the general German population, including children, is simultaneously exposed to several parabens and other substances predominantly used in personal care or consumer products as preservatives, antimicrobials, or sunscreen agents. Exposure to these substances is ubiquitous and occurring worldwide, possibly with some differences due to different product compositions or different product uses.

Several of the substances included in this method are under scrutiny because of their potential endocrine potency. With this method we can thus provide valuable data on the extent of body burden to these chemicals, to identify potentially highly exposed subpopulations and to be used in epidemiological studies to investigate possible exposure-health affect associations.

For many of the phenolic substances included in this method, there is sparse information on human metabolism and human elimination kinetics. This information is urgently needed to evaluate the validity of the analytes measured as (quantitative) biomarkers of exposure. From previous studies it is already known that several parabens are hydrolyzed to a common but nonspecific metabolite, 4-hydroxybenzoic acid (Abbas et al. 2010; Aubert et al. 2012). Also, metabolites with an additional hydroxy group at the aromatic ring (protocatechuates acid, alkyl protocatechuates) have been described as possible metabolites of parabens (Wang and Kannan 2013a).

Due to its good water solubility, MeP, the lowest molecular weight paraben, can be excreted much easier in urine than *n*-BuP or even HeP. Apart from conjugation, further metabolic modifications might be needed to increase the water solubility of BuP and HeP. In consequence, the share of the intact or conjugated (parent) paraben will probably decrease with increasing molecular weight. Thus, similar uptakes of MeP and BuP will most probably lead to much lower levels of (unchanged) BuP in urine. Whether unchanged HeP is excreted in urine at all, and in consequence, whether HeP in urine is a valid biomarker of HeP exposure remains to be seen.

Thus, to reliably extrapolate from urinary biomarker levels to doses actually taken up, metabolite conversion factors are needed (Wittassek et al. 2011; Koch and Calafat 2009). Currently we are performing human metabolism studies to investigate human paraben metabolism and derive key toxikokinetic data and urinary metabolite conversion factors for several parabens. Furthermore, metabolites and metabolism data from animals (both qualitatively and quantitatively) might not always be transferable to humans, as has recently been shown for TCC (Ye et al. 2011) or some phthalates (Koch et al. 2005).

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Kapitel II

Parabens in 24 h urine samples of the German Environmental Specimen Bank from 1995 to 2012

Rebecca K. Moos, Holger M. Koch, Jürgen Angerer, Petra Apel,
Christa Schröter-Kermani, Thomas Brüning, Marike Kolossa-Gehring
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Abstract

Parabens are widely used as antimicrobial preservatives in personal care and consumer products, food and pharmaceuticals. Due to their ubiquity, humans are constantly exposed to these chemicals. We assessed exposure to nine parabens (methyl-, ethyl-, *n*- and *iso*-propyl-, *n*- and *iso*-butyl-, benzyl-, pentyl- and heptyl paraben) in the German population from 1995 to 2012 based on 660 24 h urine samples from the German Environmental Specimen Bank (ESB) using on-line HPLC coupled to isotope dilution tandem mass spectrometry. The limit of quantification (LOQ) was 0.5 µg/L for all parabens. We detected methyl-, ethyl- and *n*-propyl paraben in 79–99% of samples, followed by *n*-butyl paraben in 40% of samples. We infrequently detected *iso*-butyl-, *iso*-propyl- and benzyl paraben in 24%, 4% and 1.4% of samples, respectively. Urinary concentrations were highest for methyl paraben (median 39.8 µg/L; 95th percentile 319 µg/L) followed by *n*-propyl paraben (4.8 µg/L; 95th percentile 74.0 µg/L) and ethyl paraben (2.1 µg/L; 95th percentile 39.1 µg/L). Women had significantly higher urinary levels for all parabens than men, except for benzyl paraben. Samples from the ESB revealed that over the investigation period of nearly 20 years urinary paraben levels remained surprisingly constant; only methyl paraben had a significant increase, for both men and women. We found strong correlations between methyl- and *n*-propyl paraben and between *n*- and *iso*-butyl paraben. These results indicate that parabens are used in combination and arise from common sources of exposure. Urinary excretion factors are needed to extrapolate from individual urinary concentrations to actual doses.

Background

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). The antimicrobial activity of parabens increases with increasing molecular weight and length of the alkyl side chain (Golden et al. 2005; Soni et al. 2005). The most frequently used parabens are methyl- (MeP), ethyl- (EtP), *n*-propyl- (*n*-PrP), and *n*-butyl paraben (*n*-BuP). The isomeric forms of PrP and BuP, and BeP are less commonly used (Jewell et al. 2007). The widespread use of parabens, their presence in personal care products, in foodstuff and in other niche applications, results in their ubiquitous occurrence in the environment. Parabens were detected in surface waters, sediments, as well as in indoor air and dust (Kasprzyk-Hordern et al. 2008; González-Mariño et al. 2009; Viglino et al. 2011; Pérez et al. 2012; Rudel et al. 2003; Wang et al. 2012; Fan et al. 2010). This results in a variety of exposure routes, i.e., dermal absorption, ingestion, and inhalation. Nevertheless, the main sources of human exposure to parabens are personal care products (Błędzka et al. 2014).

In recent decades, the use of parabens as a preservative has been highly controversial, because of possible endocrine activity. The findings of parabens in human breast tumour tissue contribute to this discussion (Darbre et al. 2004). Several in vitro studies have shown a weak estrogenic effect of some parabens (Routledge et al. 1998; Blair et al. 2000; Okubo et al. 2001; Byford et al. 2002). The potential of endocrine modifying effects and estrogenic activity increases with the length of the alkyl side chain (Darbre and Harvey 2008; Boberg et al. 2010). Furthermore, studies have shown an antiandrogenic effect of some parabens (Satoh et al. 2005; Chen et al. 2007; Kjærstad et al. 2010). All parabens can be regarded as estrogenic in vivo due to their uterotrophic effects, at doses similar to those corresponding to the Acceptable Daily Intake (ADI) of 10 mg/kg bw/day for of MeP and EtP (Lemini et al. 2003). Additionally, reproductive toxicity studies have shown effects on sperm count and testosterone levels after dietary exposure to BuP and PrP in rodents (Oishi, 2001, 2002, 2002a) although these observations of Oishi could not be confirmed or repeated (Hoberman et al. 2008).

In 2014, the European Parliament established that the maximum concentration of PrP and BuP as preservatives in cosmetics should be reduced from 0.4% to 0.14% when used individually. Moreover, the maximum concentration of 0.8% for the sum of all parabens contained in a cosmetic product should be maintained (European Parliament 2014b). Due to lack of data, no safety assessment for human health could be carried out for *iso*-propyl- (*iso*-PrP), *iso*-butyl- (*iso*-BuP), benzyl- (BeP) and pentyl paraben (PeP), therefore, their use in cosmetic products is no longer permitted (European Parliament 2014a).

Once taken up by the body, parabens are rapidly metabolized by hydrolysis to *p*-hydroxybenzoic acid, which is conjugated with sulfate, glucuronide or glycine (*p*-hydroxyhippuric acid) prior to being excreted in urine. Only minor amounts are excreted as the parent parabens (after conjugation with sulfate and glucuronide or unconjugated) in urine, depending on the paraben (Boberg et al. 2010). Because *p*-hydroxybenzoic acid is a non-specific metabolite common to all parabens, human biomonitoring studies have focussed on analyzing the specific parabens (after hydrolysis) in urine as biomarkers of internal exposure (Ye et al. 2006; Moos et al. 2014). Recent biomonitoring studies have shown that these parabens are ubiquitously present in urine samples from the general population and various sub-populations (Ma et al. 2013; Asimakopoulos et al. 2014; Frederiksen et al. 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).

The current analytical method includes the determination of the iso-forms of propyl- and butyl paraben: *iso*-PrP and *iso*-BuP. For a comprehensive exposure assessment detection of the iso-forms is essential, because the iso-form of BuP has been shown to exhibit a higher estrogenic activity than the straight chain homologue (Vo et al. 2010). Furthermore, the analysis of the iso-forms of parabens and other chemicals, e.g. phthalates, has long been neglected. For the phthalates we know today, that exposure to the iso-form di-*iso*-butyl phthalate is increasing and even surpassing exposure to the straight chain di-*n*-butyl phthalate (Zota et al. 2014; Lorber and Koch 2013; Koch et al. 2012a).

The urine samples investigated in this study were collected by the German Environmental Specimen Bank (ESB). The ESB is a major component of the German health related environmental monitoring from the Federal Environment Agency (UBA) and continuously collects human specimens since 1985.

It provides a scientific basis for decisions of the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB). The main tasks of the German ESB are not only monitoring and documenting, but also assessing the changes in the chemical burden of humans and the environment over time. Thus, a retrospective an sensitive analysis of the full set of parabens (including the iso-forms) in these 24 h urine samples is possible and allows for the assessment of both past and current exposures including the investigation of exposure time trends, as has been done previously with phthalates (Wittassek et al. 2007; Göen et al. 2011; Schütze et al. 2015), DINCH (Schütze et al. 2014) and bisphenol A (Koch et al. 2012).

Methods

Subjects and urine specimens

In the frame of the German Environmental Specimen Bank (ESB) 24 h urine samples have continuously been collected since 1985 according to standard operating procedures (http://www.umweltprobenbank.de). The Federal Environment Agency (UBA) is responsible for the scientific steering, the central data maintenance and data assessment as well as the administration and coordination of the ESB. The concept and sampling criteria of the ESB are described in detail by Kolossa-Gehring et al. (2012, 2012a). All urine specimens are collected as 24 h urine samples. The 24 h urine is aliquoted into polypropylene tubes and stored at temperatures below −150 °C (Lermen et al. 2014). The study protocol of sampling human specimens has been reviewed and approved by the ethics committees of the Medical Associations Saarland and Westfalen-Lippe and the Medical Faculty of the Westphalian Wilhelms-University Muenster. All participants gave written informed consent on standardized forms approved by the same ethics committees.

In this study we analyzed 660 24 h urine samples from the years 1995, 1997, 1999, 2001, 2003, 2005, 2006, 2007, 2008, 2009 and 2012. All samples were mainly from students (age range 20–30 years) located at the University of Muenster (Germany). For each year, the study population consisted of 30 male and 30 female volunteers. Further sample information and anthropometric data are given in **Table 1**. All urine samples were blinded by the ESB before shipment to the analyzing laboratories at the IPA, Bochum, Germany.

Table 1 Description of the ESB study population.

Sampling year	Subjects (f/m)	Age mean (range)	24h urine volume mean (range)	Creatinine mean (range)
1995	60 (30/30)	24.0 (20-29)	1579 (450-2600)	1.164 (0.400-2.748)
1997	60 (30/30)	24.8 (20-29)	1492 (550-2650)	1.208 (0.347-2.414)
1999	60 (30/30)	24.3 (21-29)	1624 (550-4000)	1.027 (0.275-2.656)
2001	60 (30/30)	23.6 (20-28)	1740 (350-2800)	1.002 (0.197-2.403)
2003	60 (30/30)	22.9 (20-28)	1754 (410-3500)	1.108 (0.230-3.410)
2005	60 (30/30)	23.5 (20-28)	1821 (691-3330)	1.010 (0.189-2.088)
2006	60 (30/30)	24.1 (20-29)	1880 (719-4250)	0.909 (0.290-2.108)
2007	60 (30/30)	24.2 (20-29)	1877 (927-3758)	0.889 (0.235-2.174)
2008	60 (30/30)	23.4 (20-29)	2076 (855-5460)	0.819 (0.150-2.150)
2009	60 (30/30)	23.1 (20-28)	2081 (540-3660)	0.827 (0.271-2.118)
2012	60 (30/30)	24.2 (20-30)	2108 (574-3027)	0.703 (0.205-2.181)
total	660	24.0 (20-30)	1819 (350-5460)	0.971 (0.150-3.410)
male	330	24.0 (20-30)	1830 (410-4000)	1.118 (0.189-3.410)
female	330	24.0 (20-30)	1809 (350-5460)	0.824 (0.150-2.748)

Chemical standards

Methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *iso*-butyl- and benzyl paraben (esters of *p*-hydroxybenzoic acid) were purchased from Sigma–Aldrich (Steinheim, Germany). *Iso*-propyl- and heptyl paraben were purchased from TCI Europe (Eschborn, Germany). Pentyl paraben was purchased from VWR (Darmstadt, Germany). The deuterated standards d4-methyl-, d4-ethyl-, d4-propyl-, d4-butyl- and d4-benzyl paraben were purchased from C/D/N Isotopes (Dr. Ehrenstorfer GmbH, Augsburg, Germany). All standards had a purity ≥95%. Water and acetonitrile (LC/MS grade) and acetic acid (glacial, extra pure) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). β-Glucuronidase/arylsulfatase enzyme Type HP-2 and ammonium acetate p.a. were purchased from Sigma–Aldrich (Steinheim, Germany).

Analytical method

The on-line HPLC–MS/MS method used has been described in detail by Moos et al. (2014). In short, urine samples were equilibrated to room temperature and homogenized. Aliquots of 300 µL urine were transferred into 1.8 mL glass screw-cap vials. 300 µL 1 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 (3500 g for 10 min). The supernatant was injected into the HPLC-system (Agilent Technology LC1200) coupled with a tandem mass spectrometer (ABSciex QTrap 5500). For online clean-up and enrichment a RAM (Restricted Access Material) phase (LiChrospher® RP-8 ADS (25 µm) 25 × 4 mm RAM from Merck, Darmstadt, Germany) was used in back flush mode and chromatographic separation was performed on a reversed phase C18 column (Atlantis dC18 30 × 150 mm; 3 µm, Waters, Ireland). The method has a total run time of 26 min.

Calibration curves were obtained with a 1/x weighting by plotting the quotients of peak areas of each paraben and the peak areas of the specific internal standard as a function of the concentration. Several parabens (MeP, EtP and *n*-PrP) were observed to be present in laboratory blanks, originating from the ubiquitous presence of these parabens in the environment. Applying rigorous cleaning and pre-testing procedures we were able to keep the within-laboratory blank value constantly below 0.1 µg/L. To exclude contamination that might arise during sample preparation (through the vessels or chemicals used), and the subsequent analysis (by the solvent used), for each series blank values were processed. Furthermore, to investigate and to exclude a possible pre-analytical sample contamination (the urine samples of the ESB were collected without a special protocol to minimize external paraben contamination) we investigated the conjugation status of the parabens in a random subset of the ESB samples (n = 30) prior to analyzing the full set of samples as previously done for BPA (see Koch et al. 2012). The conjugation status in this subset of samples was comparable to the conjugation status of the various parabens in recently collected urine samples (data not shown). Higher shares of free parabens would have indicated a likelihood of external contamination during sample collection. Yet, to take account of any pre-analytical trace level contamination we conservatively set the LOQ for all parabens to 0.5 µg/L. Furthermore, samples with high paraben concentrations were re-analyzed without enzyme to check and verify their conjugation status (data not shown).

All quality criteria of the method are described in detail by Moos et al. (2014). For all parabens, the relative standard deviations of the laboratory control material (measured within each analytical batch) were below 10%, except for PeP and HeP (20%). However, PeP and HeP were determined without analogous labelled internal standards; d4-*n*-BuP was used instead. For all parabens with labelled internal standards, relative recoveries were between 95.0 and 116%.

For the iso-parabens with structural closely related internal standards relative recoveries were between 77.0 and 108%. For PeP and HeP relative recoveries were between 135 and 219%.

Statistical analysis

For statistical analysis we used IBM SPSS Statistics 22. Boxplots were generated with OriginPro 9.1 (OriginLab Corporation). Urinary metabolite concentrations were calculated in µg/L and creatinine adjusted values (µg/g creatinine). Metabolite concentrations below the LOQ were substituted with LOQ/2 (Hornung and Reed 1990). The estimation of descriptive values can be influenced by left censored values (Lotz et al. 2013). If the distribution is known or can be adopted, an estimation of summary statistics is possible. In this study only summary statistics which were not influenced by values below the LOQ were given; otherwise "not applicable" (N.A.) was reported. To assess gender differences we applied the Mann–Whitney-U-test. We investigated chronological trends in paraben exposure by the Jonckheere-Terpstra test and for analysis of associations the bivariate correlation Spearman's rho were used. The Jonckheere-Terpstra test is similar to the Kruskal–Wallis test for independent samples. It is a nonparametric test to verify whether the central tendencies of more than two different samples differ significantly from each other. In contrast to the Kruskal–Wallis test, the Jonckheere-Terpstra test verifies the existence of a trend among groups. The statistical analysis of association and chronological trend was performed for all analytes (MeP, EtP, *n*-PrP, *n*-BuP and *iso*-BuP), except for analytes with detection rates below 5% (*iso*-PrP and BeP). For these analytes, the detection rates were considered to be insufficient for statistical evaluation. Detection rates are given in supplementary materials **Table 1** (**Appendix II**).

Results and discussion

The results of the biomonitoring study on parabens in the 660 24 h urine samples are presented in **Table 2a** (values in µg/L) and **Table 2b** (values in µg/g creatinine) for each sampling year, as the total of all sampling years and separately for males and females. Over all samples, we found MeP, EtP and *n*-PrP with high detection rates between 79 and 99%, followed by *n*-BuP with 40%. Further, we detected the isomers *iso*-PrP and *iso*-BuP in 4 respectively 24% and BeP in 1.4% of the samples. In none of the 660 urine samples PeP or HeP were detected (see **supplementary materials Table 1; Appendix II**).

Table 2a Urinary paraben concentrations (µg/L) measured in 60 24 h urine samples.

Year	MeP			EtP			*iso*-PrP			*n*-PrP			*iso*-BuP			*n*-BuP			BeP		
	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max
1995	33.6	321	593	3.1	58.3	118	<LOQ	<LOQ	<LOQ	7.2	131	325	<LOQ	1.8	4.1	<LOQ	10.3	17.1	<LOQ	<LOQ	<LOQ
1997	38.4	301	1410	2.0	26.1	98.7	<LOQ	<LOQ	2.0	3.9	92.6	206	<LOQ	1.9	9.7	<LOQ	9.5	32.0	<LOQ	<LOQ	1.1
1999	22.3	280	428	1.9	42.4	67.8	<LOQ	<LOQ	0.5	2.5	40.4	127	<LOQ	2.9	6.4	<LOQ	11.6	43.9	<LOQ	<LOQ	<LOQ
2001	37.9	191	1760	3.3	23.5	78.8	<LOQ	<LOQ	14.8	4.8	41.3	267	<LOQ	4.8	8.6	0,6	11.0	20.6	<LOQ	<LOQ	<LOQ
2003	35.4	323	402	1.6	32.7	79.6	<LOQ	<LOQ	29.4	3.7	77.0	133	<LOQ	3.0	14.3	<LOQ	8.4	20.2	<LOQ	<LOQ	0.9
2005	37.4	206	374	1.3	19.4	27.8	<LOQ	1.5	99.5	4.6	51.7	117	<LOQ	3.2	9.0	<LOQ	8.2	12.7	<LOQ	<LOQ	<LOQ
2006	45.8	255	742	1.6	30.2	65.8	<LOQ	1.3	4.3	6.2	73.5	102	<LOQ	2.0	9.0	<LOQ	7.9	19.0	<LOQ	<LOQ	0.5
2007	65.5	394	1230	2.3	36.1	181	<LOQ	<LOQ	36.8	8.7	128	284	<LOQ	3.1	4.3	<LOQ	9.0	49.3	<LOQ	<LOQ	<LOQ
2008	50.8	501	928	2.2	33.2	97.6	<LOQ	<LOQ	7.4	5.7	65.8	226	<LOQ	2.1	3.9	<LOQ	6.5	39.1	<LOQ	<LOQ	1.2
2009	32.1	324	988	1.8	39.5	77.6	<LOQ	6.0	44.2	4.8	69.9	270	<LOQ	8.2	23.0	<LOQ	14.7	28.0	<LOQ	<LOQ	<LOQ
2012	42.6	252	808	1.0	67.3	175	<LOQ	<LOQ	1.2	2.2	41.9	118	<LOQ	1.1	4.1	<LOQ	10.3	25.7	<LOQ	<LOQ	0.6
total	39.8	319	1760	2.1	39.1	181	<LOQ	<LOQ	99.5	4.8	74.0	325	<LOQ	3.1	23.0	<LOQ	10.7	49.3	<LOQ	<LOQ	1.2
male	23.2	224	1410	1.2	17.7	78.8	<LOQ	<LOQ	29.4	1.5	46.0	164	<LOQ	2.2	23.0	<LOQ	5.4	23.7	<LOQ	<LOQ	1.2
female	57.2	388	1760	4.1	51.5	181	<LOQ	1.3	99.5	9.5	122	325	<LOQ	3.9	19.4	0.9	12.7	49.3	<LOQ	<LOQ	0.6

P.50: 50th percentile, P.95: 95th percentile, LOQ: limit of quantification (LOQ: 0.5µg/L).

Table 2a Urinary paraben concentrations (µg/L) measured in 60 24 h urine samples.

Year	MeP			EtP			*iso*-PrP			*n*-PrP			*iso*-BuP			*n*-BuP			BeP		
	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max
1995	34.0	369	722	3.1	91.0	149	N.A.	N.A.	N.A.	5.0	94.8	244	N.A.	N.A.	3.6	N.A.	8.3	20.5	N.A.	N.A.	N.A.
1997	37.1	342	831	2.2	41.6	54.2	N.A.	N.A.	1.1	4.1	105	246	N.A.	2.7	5.7	N.A.	8.3	20.8	N.A.	N.A.	3.1
1999	23.4	314	525	2.0	38.4	115	N.A.	N.A.	0.4	2.6	80.2	122	N.A.	3.0	6.5	N.A.	13.4	46.8	N.A.	N.A.	N.A.
2001	45.8	282	737	3.7	24.8	64.0	N.A.	N.A.	13.5	6.0	52.2	137	N.A.	5.1	8.2	N.A.	17.5	29.1	N.A.	N.A.	N.A.
2003	33.4	359	640	1.6	24.1	62.0	N.A.	N.A.	16.6	3.0	85.1	156	N.A.	4.0	8.1	N.A.	9.1	15.1	N.A.	N.A.	0.4
2005	30.1	363	625	1.4	28.1	53.8	N.A.	N.A.	443	5.2	59.4	202	N.A.	3.7	40.2	N.A.	12.4	36.4	N.A.	N.A.	N.A.
2006	53.8	385	848	N.A.	38.5	88.4	N.A.	N.A.	3.9	6.9	128	230	N.A.	2.3	8.6	N.A.	14.7	18.9	N.A.	N.A.	0.4
2007	79.2	455	1319	3.0	53.4	194	N.A.	N.A.	39.5	10.3	142	305	N.A.	3.6	10.4	N.A.	13.8	52.9	N.A.	N.A.	N.A.
2008	88.0	477	986	2.6	35.8	87.8	N.A.	N.A.	8.6	8.0	89.5	240	N.A.	N.A.	5.3	N.A.	10.3	45.2	N.A.	N.A.	0.8
2009	45.4	548	873	2.4	61.7	169	N.A.	5.5	58.5	7.5	122	239	N.A.	9.5	39.2	N.A.	17.1	51.4	N.A.	N.A.	N.A.
2012	80.1	369	2249	N.A.	123	487	N.A.	N.A.	1.9	5.0	78.9	328	N.A.	N.A.	6.9	N.A.	15.5	50.0	N.A.	N.A.	1.9
total	48.8	398	2249	2.4	50.6	487	N.A.	N.A.	443	5.5	101	328	N.A.	3.5	40.2	N.A.	13.2	52.9	N.A.	N.A.	3.1
male	22.8	250	831	N.A.	19.5	64.0	N.A.	N.A.	17.6	N.A	49.4	168	N.A.	N.A.	39.2	N.A.	5.5	40.3	N.A.	N.A.	3.1
female	86.9	476	2249	5.4	78.1	487	N.A.	N.A.	443	12.8	152	328	N.A.	4.2	40.2	N.A.	20.6	52.9	N.A.	N.A.	1.7

P.50: 50th percentile, P.95: 95th percentile, N.A.: not applicable.

We determined highest urinary concentrations for MeP with a median of 39.8 µg/L (48.8 µg/g creatinine) over all samples, in individual samples we observed maximum values of up to 1.7 mg/L (2.2 mg/g creatinine). The overall median of *n*-PrP was 4.8 µg/L (5.5 µg/g creatinine) and of EtP 2.1 µg/L (2.4 µg/g creatinine). Reliable 95th percentiles could be calculated for five parabens, ranging from 3.1 µg/L (3.5 µg/g creatinine) for *iso*-BuP to 319 µg/L (398 µg/g creatinine) for MeP. The isomeric forms of PrP and BuP were generally found at lower concentrations than their straight chain analogues; maximum urinary concentrations were 23.0 µg/L (40.2 µg/g creatinine) for *iso*-BuP and 99.5 µg/L (443 µg/g creatinine) for *iso*-PrP. The median levels for the young adults investigated in this study were about 2-fold higher than levels observed in our previous study (MeP: 24.5 µg/L, 23.3 µg/g creatinine; EtP: 1.4 µg/L, 1.5 µg/g creatinine and *n*-PrP 1.2 µg/L, 1.5 µg/g creatinine) (Moos et al. 2014). This may be due to the differences in study design. While the 24 h urines collected for the ESB represent exposure and excretion of a whole day the random spot urine samples investigated in the previous study cover only episodes of the daily exposure. Also, differences in the composition of the study population (previous study: children, women and men aged 6–64 years; present study: women and men between 20 and 30 years) might have had an influence on the levels found.

Fig. 1 depicts boxplots of urinary concentrations (in µg/L) of the parabens separately for men, women and total over all years. Obviously, urinary concentrations of all parabens with a sufficient number of positive detects were higher in women compared to men. On average, for women median concentrations of MeP and EtP were about 3-fold higher and for *n*-PrP 7-fold higher than for men. Also, for the parabens with lower detection rates (*n*-BuP, *iso*-PrP, *iso*-BuP), men had distinctly lower paraben concentrations than women. The above observations are consistent with the findings of our previous study (Moos et al. 2014). The United States National Health and Nutrition Examination Survey (NHANES) reported comparable gender specific differences like in this study. In the NHANES study women had 3-fold higher adjusted geometric mean levels of MeP and 7-times higher adjusted geometric mean levels of *n*-PrP compared to men (Calafat et al. 2010). In other international studies similar patterns were observed (Asimakopoulos et al. 2014; Wang et al. 2013; Ma et al. 2013; Dewalque et al. 2014).

The generally higher paraben levels in women are probably caused by a more frequent use of personal care products (Biesterbos et al. 2012) preserved with parabens. Median paraben concentrations for each year, depicted separately for men and women are given in **supplementary materials Table 2a** (values in µg/L) and **Table 2b** (values in µg/g creatinine)(**Appendix II**).

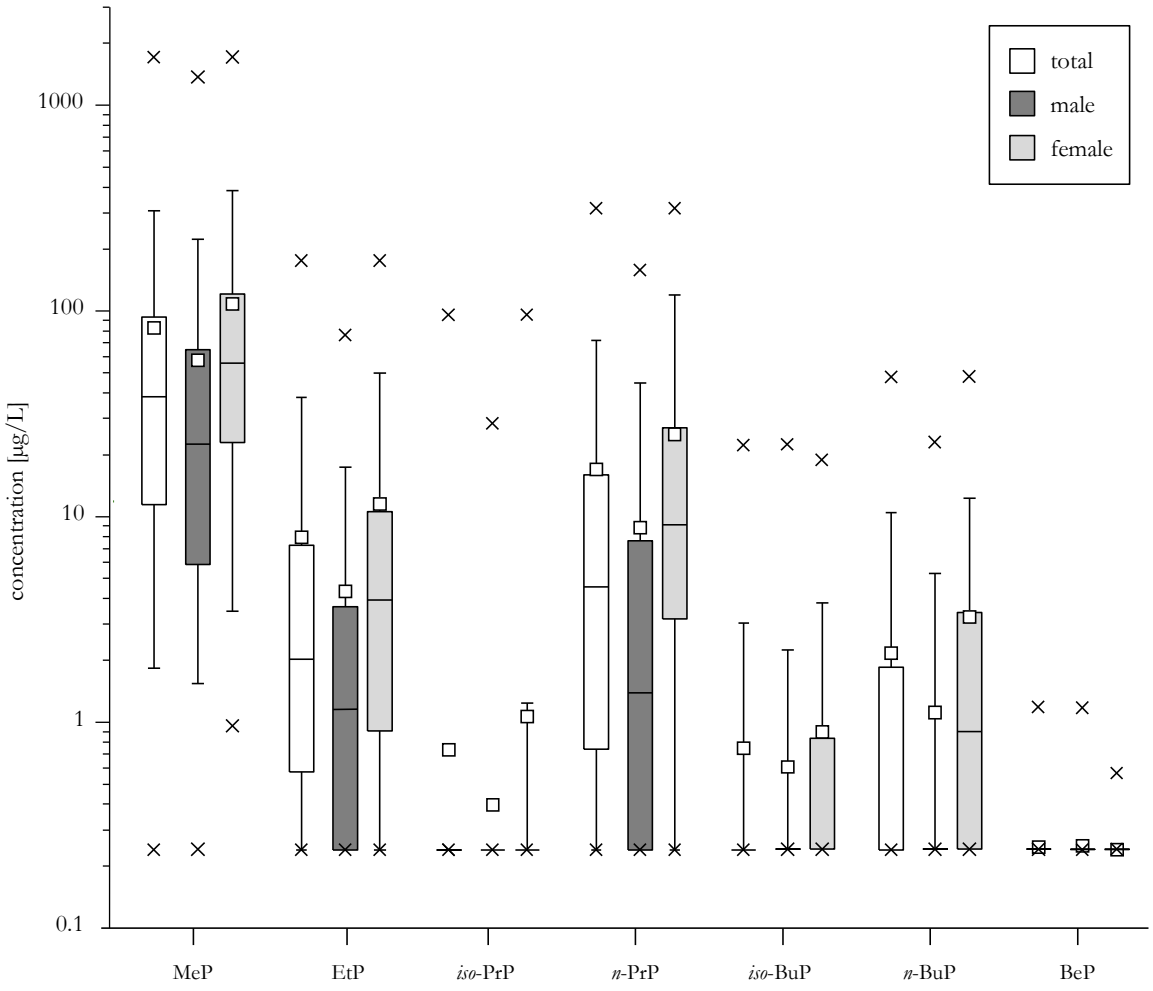

Figure 1 Boxplots of the results of the human biomonitoring study with 660 volunteers. For each paraben, data is shown separately for females, males and total. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95thpercentile. The minimum and maximum values are represented by an x.

Boxplots of the results of the human biomonitoring study with 660 volunteers. For each paraben, data is shown separately for females, males and total. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile. The minimum and maximum values are represented by an x.

As pointed out above, the ESB is designed for time trend analyses and thus the samples are eminently suitable for the investigation of temporal trends of paraben exposures over the last decades. However, to take account of considerable changes of the hydration status of the young adults donating urine for the ESB over the years (constantly increasing 24 h urine volumes from 1579 mL in 1995 to 2108 mL in 2012, see **Table 1**) we performed the time trend analyses with creatinine adjusted concentrations, only. An increase in 24 h urine volumes of the ESB may be due to various health campaigns aimed at providing the body with sufficient water intake (Koch et al. 2012). We found rather constant urinary paraben levels (men and women combined, 60 samples per year) over the years for nearly all parabens investigated (see **Fig. 2**) despite the public debate regarding the use of parabens and the increased number of (cosmetic) products offered as paraben free. Suggestive increases for the isoforms *iso*-PrP and *iso*-BuP according to **Fig. 2** proved not to be statistically significant, possibly also due to their lower detection rates. Only for MeP we found a significant ($p < 0.0001$) increase. The median concentration from 2012 (80.1 µg/g creatinine) was more than two times higher than the median from 1995 (34.0 µg/g creatinine). We observed this significant increase over time also when investigated separately for men ($p < 0.0001$) and women ($p = 0.003$) (**supplementary materials Fig. 1; Appendix II**). For both men and women the median MeP values were two times higher in 2012 (men: 52.0 µg/g creatinine; women 106.4 µg/g creatinine) than in 1995 (men: 26.1 µg/g creatinine; women 45.7 µg/g creatinine). Next to MeP we found indications of a chronological trend (increase, $p = 0.010$) for *n*-BuP in the male population. Nevertheless, male *n*-BuP levels in 2012 (95th percentile: 3.4 µg/g creatinine) were still considerably lower than female *n*-BuP levels (95th percentile: 27.8 µg/g creatinine) (**supplementary materials Fig. 1; Appendix II**).

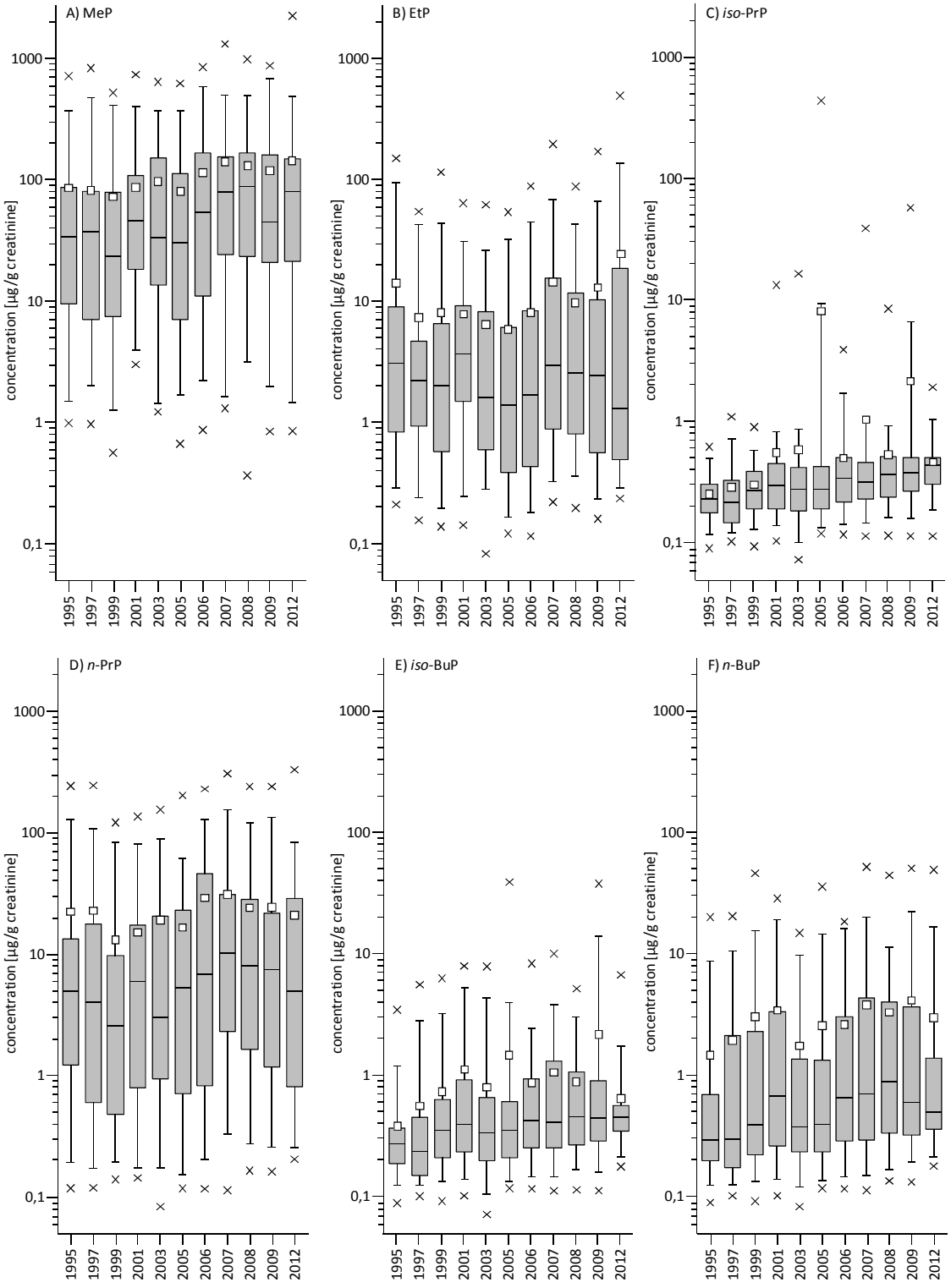

Figure 2 Chronological presentation of the creatinine adjusted values (µg/g creatinine) between 1995 and 2012. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile. The minimum and maximum values are represented by an x.

We also investigated correlations between the urinary paraben levels and observed positive correlations between all parabens. Correlations between the parabens MeP vs. EtP, MeP vs. *n*-PrP, EtP vs. *n*-BuP and *n*-BuP vs. *iso*-BuP are shown in **Fig. 3** and for the rest in **supplemental Fig. 2 (Appendix II)**. The urinary levels of MeP and *n*-PrP were most strongly correlated ($r = 0.795$, $p < 0.0001$). MeP and *n*-PrP are the most commonly used parabens and often used in combination in personal care products (Guo and Kannan 2013; Soni et al. 2005). We also observed a strong correlation for *n*- and *iso*-BuP ($r = 0.758$, $p < 0.0001$). A slightly weaker correlation was found between EtP and *n*-BuP ($r = 0.688$, $p < 0.0001$). The correlations of the other parabens were not as strong, but also statistically significant ($p < 0.0001$). Correlations between all parabens are indicating to simultaneous and concurrent exposures and reflect that parabens are often used in combination for a stronger antimicrobial activity (CIR 2008). These results fit very well with previously published values. In various studies, the highest correlations also were found between MeP and PrP (Ma et al. 2013; Asimakopoulos et al. 2014; Frederiksen et al. 2013; Calafat et al. 2010) followed by EtP and BuP (Wang et al. 2013). To our knowledge, *n*- and *iso*-butyl paraben have not been previously compared with each other.

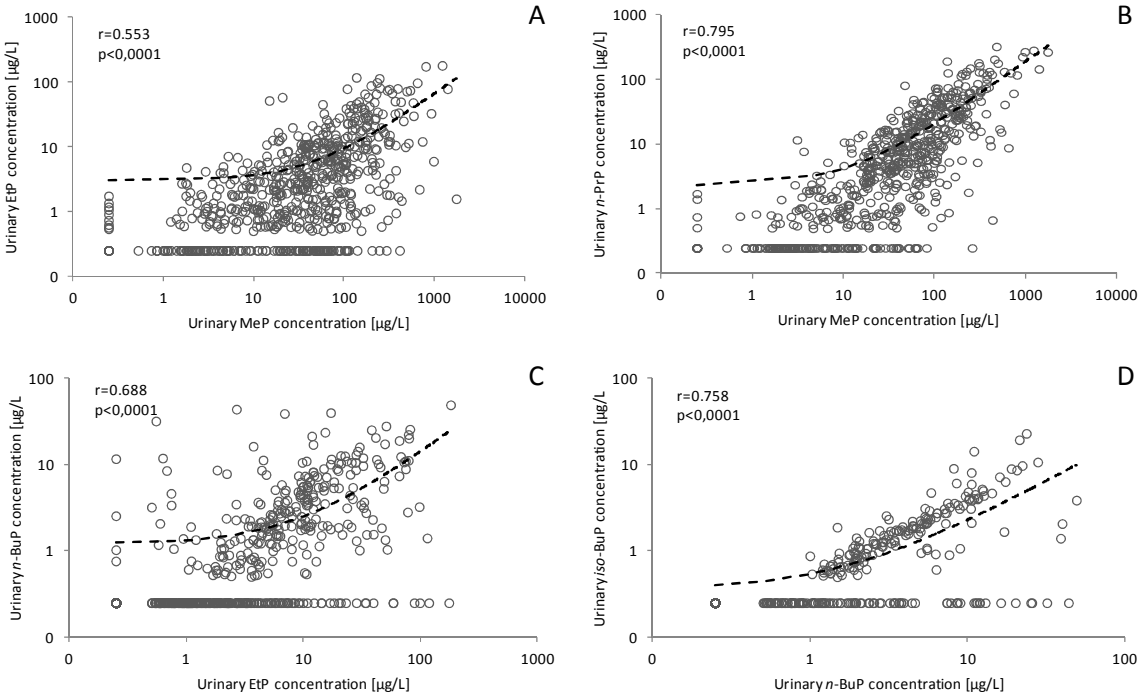

Figure 3 Correlation of urinary concentration of A: MeP and EtP, B: MeP and *n*-PrP, C: EtP and *n*-BuP, D: *n*-BuP and *iso*-BuP in µg/L on logarithmic scale.

Conclusions

The results of our study indicate a ubiquitous exposure of the German ESB population to nearly all parabens investigated during the last 20 years. The ESB population represents the non-occupationally exposed group of people aged 20 to 29 years. However, it cannot be regarded as fully representative for the German population. Although, previous investigations in regard to body burdens with other environmental chemicals such as bisphenol A, phthalates and phthalate substitutes (Wittassek et al. 2007; Göen et al. 2011; Schütze et al. 2014, 2015; Koch et al. 2012) have shown that both the urinary levels in itself and the time trends represent a good image of the exposure situation in Germany. Obviously, personal life style and product use seem to be the most influential factors in paraben exposure as expressed in considerable differences in urinary paraben concentrations between males and females. Although not investigated, but also likely due to changes in lifestyle und product use over ones individual lifetime, changes in exposure over age have to be expected. Thus, some differences between studies investigating paraben exposure can be explained by the composition of the study population. Exposure to parabens is a phenomenon which can be observed worldwide. Urinary levels we found are in the same order of magnitude as in studies from other countries and the distribution pattern both in terms of most prevalent paraben in urine (MeP followed by *n*-PrP and EtP) and in terms of women generally having higher exposures compared to men is similar to most other international studies (Calafat et al. 2010; Frederiksen et al. 2010, 2014; Ma et al. 2013; Asimakopoulos et al. 2014; Casas et al. 2011; Shirai et al. 2013; Dewalque et al. 2014). Urinary levels of the iso-forms of PrP and BuP were generally lower than their straight chain analoges. However, we have shown that these iso-forms are detectable in urine samples and also strongly correlate with their straight chain analoges. Currently, we can only speculate if these iso-forms are added to products intentionally or have to be regarded as unintentional (or accepted) contaminations.

Surprisingly, we found rather constant exposures to most of the parabens over the last 20 years investigated. Only for MeP we found an overall increasing trend. Furthermore, for the male subpopulation investigated separately, we found indications to increasing exposures to *n*-BuP. Some increases in the male subpopulation could possibly be explained by the growing importance of personal care products in this population.

The findings for the parabens are in strong contrast to previous findings for phthalates for which considerable changes in exposure over the last decades have been reported (Zota et al. 2014; Wittassek et al. 2007; Göen et al. 2011; Lioy et al. 2014). Obviously, substitutions that have been taking place in the field of phthalates because of regulative measures and public opinion have not (yet) taken place for parabens. It remains to be seen if the critical discussion of parabens in regard to their potential impact on human health, regulatory measures on parabens and the ongoing (voluntary) substitution of parabens in consumer products find their reflection in urinary levels of parabens.

With our data set we can provide valuable information on the extent of body burden to parabens, e.g. to be used in epidemiological studies to investigate possible exposure-health effect associations. However, we have to point out possible pitfalls in interpreting urinary paraben concentrations. Comparing urinary concentrations of the parabens with each other (e.g. the 95th percentiles of MeP, EtP and *n*-BuP) one might assume that exposure to MeP is ten-times higher than exposure to EtP and 30-times higher than to *n*-BuP. This assumption cannot be made. First of all, we have to be aware that only a minor share of the paraben taken up is excreted as parent paraben (unconjugated and conjugated) in urine. Secondly, the share of each paraben excreted as the parent paraben itself might change with chain length of the alkyl-moiety. Assuming analogy to the phthalates it is likely that at similar doses a higher share of MeP (short alkyl chain) is excreted in urine compared to BuP (longer alkyl chain). Conversely, this means that similar urinary levels of MeP and BuP would point to much higher actual intakes of BuP than of MeP. Thus, to reliably extrapolate from urinary paraben levels to doses actually taken up metabolite conversion factors are urgently needed (Wittassek et al. 2011; Koch and Calafat 2009; Koch et al. 2012a, 2013) making a comparison with health based benchmark levels possible. To meet these ends, we are currently performing human metabolism studies to derive key toxikokinetic data and urinary metabolite conversion factors for several parabens.

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Kapitel III

Inter- and intra-individual variation in urinary biomarker concentrations over a 6-day sampling period. Part 2: Personal care product ingredients

Holger M. Koch, Lesa L. Aylward, Sean M. Hays, Roel Smolders, Rebecca K. Moos, John Cocker, Kate Jones, Nicholas Warren, Len Levy, Ruth Bevan

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Abstract

An intensive study was conducted to provide data on intra- and inter-individual variation in urinary excretion of a series of ingredients in personal care products (parabens, triclosan, benzophenones) and bisphenol A (BPA, not expected to be an ingredient) in 8 volunteers over 6 days. Exposure diaries recorded use of personal care products with identified target analytes as ingredients. Participants' usual products were replaced with products without the target analytes for 2 of the 6 days. Urine void volumes and times were recorded. Methyl, ethyl, and *n*-propylparabens, triclosan, benzophenone-3, and BPA were frequently detected (≥70% of samples). Urinary concentrations of the parabens and triclosan were lower on product replacement days. First morning void concentrations correlated moderately to highly with 24-h composite concentrations for all analytes. Intraclass correlation coefficients (ICCs) for spot samples collected on days with usual product use were low for BPA (0.15), moderate for *n*-propylparaben and methylparaben (0.39 and 0.56, respectively), and high for ethylparaben, benzophenone-3, and triclosan (0.76, 0.81, and 0.934, respectively); ICCs were consistently higher on the basis of cr-adjusted concentrations. Hydration status adjustment methods were assessed by comparing unadjusted and adjusted concentrations to urinary excretion rates (ER, ng/kg-h) for all analytes and samples. Specific gravity-adjusted concentrations correlated slightly better with ER than creatinine-adjusted concentrations. Within-individual variation in biomarker concentrations was highest for methyl and ethylparabens (2 orders of magnitude variation in spot sample concentrations) and lower for the other analytes (1–1.5 orders of magnitude). This dataset provides insight into the design and interpretation of urinary biomonitoring studies for non-persistent chemicals.

Introduction

Human biomonitoring, the analysis of human biological media samples to measure concentrations of chemicals of interest (or their respective metabolites), has become a fundamental component of the evaluation and management of chemical exposures from sources in the environment, in foods, and in consumer products (Polkowska et al. 2004; Sexton et al. 2004). Large-scale biomonitoring studies on a national scale are now conducted in many countries (Porta et al. 2008). Biomonitoring has also become a central tool in addition to external exposure measures for exposure characterization in environmental epidemiology studies. While a variety of biological media can be assessed, the most common matrices are blood and urine. For non-persistent analytes, urine is most often the matrix of choice due to fast excretion of these substances or their metabolites in urine, which generally results in higher concentrations in urine than in blood, and to the ease of collection of urine samples and lack of invasive sampling required.

The interpretation of biomonitoring data in the context of exposure and risk assessment requires consideration of issues related to within- and between-individual variation in biomarker concentrations. In particular, biomonitoring data for non-persistent analytes (in the context of this discussion, those with elimination half-lives of less than a day) poses challenges due to the rapid rise and fall of biomarker concentrations that can occur between exposure events. Within-individual, within-day variations in biomarker concentrations of 2–3 orders of magnitude are possible (Aylward et al. 2012, 2014; Smolders et al. 2009, 2014). In addition to this temporal variability in excretion rate and resulting urinary concentrations, within- and between-individual variation in urinary flow rate (often called "hydration status") also contributes to the variability of biomarker concentrations in urine. Thus, information provided by biomonitoring data, and particularly by concentrations measured in single spot urine samples, may be difficult to interpret in the context of longer term average exposures of interest in risk and exposure assessment or in understanding potential health effects in studies of persons exposed in the general population. These issues are important in the consideration of the design and interpretation of biomonitoring studies, whether these studies are intended to survey general population levels or to investigate potential associations between biomarker concentrations and health outcomes.

This study provides an initial description of an intensive dataset that allows examination of the inter- and intra-individual variation in urinary analytes associated with ingredients used in personal care products over the course of multiple days. The analytes evaluated here include a series of parabens, triclosan, triclocarban, three benzophenone compounds, and bisphenol A. Parabens are often used as preservatives to prevent growth of bacteria and fungus in products and can also be used in some food products (USFDA 2014). Triclosan and triclocarban may be used in products for their disinfectant properties. Benzophenones are used as UV blockers but also as stabilizers on personal care products, plastics and coatings. BPA is not anticipated to be intentionally used in personal care products, but was included as an analyte of general interest.

This initial evaluation of the dataset provides descriptive statistics for frequently detected target analytes in the study participants with a focus on characterizing within- and between-individual variability in biomarker concentrations. A companion publication (Smolders et al. 2014) presents detailed data on urinary metals and in-depth data on urinary flow characteristics. A subsequent publication will examine mass balance for the personal care product ingredients analyzed in this study based on diary and product use information in conjunction with the urinary biomarker data.

Methods

This observational and interventional study consisted of a 6-day collection of all urine voids from 8 participants. In the autumn of 2012, four husband–wife couples were recruited in Flanders, the Northern part of Belgium, to participate in the current study. The study was approved by the Ethics Committee of the University of Antwerp, and informed consent was obtained from all study participants.

Personal care products

The participants identified their usual personal care products including shampoos, hair conditioners, skin lotions, hand soaps, shower gels, toothpastes, make-up, deodorants, sunscreens, and anti-bacterial products such as hand sanitizers. Participants provided photographs of the ingredient labels for each product. Target analytes (see Section 2.4) were identified on each product label. The researchers identified replacement products of the same type that did not list as ingredients any of the target analytes for use during the 2-day intervention period.

These were provided to the participants for use instead of their usual products on days 2 and 3 of the 6-day collection period. Thus, days 1, 4, 5, and 6 reflect use of baseline products and days 2 and 3 reflect use of the replacement products that did not contain the target analytes (**Fig. 1**). Exposure to the target analytes from other sources may also have occurred (e.g., to parabens used as preservatives in food products), but were not tracked in this effort.

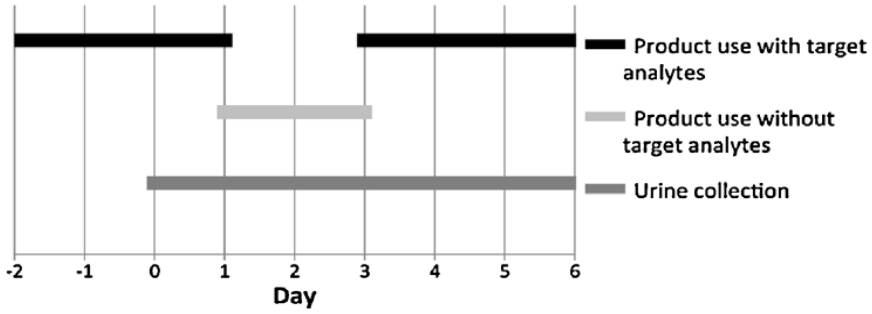

Figure 1 Time course of product usage, diary completion, and urine collection.

Urine collection

Participants collected each complete urine void for a period of 6 days in pre-weighed polyethylene 250 mL containers. Collections began with the first void after 00:00 on a Monday and continued through the following Saturday evening at 24:00. Participants recorded the time of each urine void to the nearest half hour and stored the samples as close to 4 °C as practically achievable. Each day, all samples were collected from the participants and returned to the lab. Upon entry in the lab, all samples were weighed. Volumes were calculated by dividing each sample weight by its measured specific gravity. Samples were subsequently aliquoted and stored at −20 °C until further analyses.

Participant diaries

Each participant maintained a diary beginning 2 days prior to urine collection. Consumption of food and drink, smoking, and use of personal care products were recorded at half-hour intervals.

Target analytes and analytical methods

The target analytes for this study included a series of parabens (methylparaben (MP), ethylparaben (EP), *iso*propylparaben (iPP), *n*-propylparaben (*n*PP), *iso*butylparaben (iBuP), *n*-butylparaben (*n*BuP), benzylparaben (BzP), pentylparaben (PenP), and heptylparaben (HpP)); a series of benzophenones (BP1, BP3, and BP8); triclocarban (TCC); triclosan (TCS); and bisphenol A (BPA). All of the above analytes were analyzed in the urine in a single analytical run via on-line LC/LC–MS/MS with quantification by isotope dilution, according to Moos et al. (2014). In short, aliquots of 300 µL urine were spiked with 300 µL 1 M ammonium acetate buffer at pH 5.0, 25 µL internal standard solution and 6 µL β-glucuronidase/arylsulfatase solution. After incubation at 37 °C for hydrolysis of the conjugated species, 100 µL were injected into the HPLC-system. In a two column assembly, extraction from urinary matrix was achieved using a RAM (restricted access material) phase (LiChrospher® RP-8 ADS 25 mm × 4 mm; 25 µm, Merck, Darmstadt, Germany), chromatographic separation was realized on a reversed phase column (Atlantis dC18 30 mm × 150 mm; 3 µm, Waters, Ireland) using a water–acetonitrile gradient in 0.02% acetic acid. Detection and quantification was performed on a tandem mass spectrometer (AB Sciex 5500 QTrap) in negative ionization mode. For each analyte and internal isotope standard specific mass transitions were recorded in scheduled multiple reaction monitoring (MRM) mode. The limits of quantification (LOQ), defined as a signal-to-noise ratio of nine, shown in **Table 2**, were between 0.5 and 2 µg/L, depending on the analyte.

Specific gravity (SG) was measured using a refractometer, and creatinine was determined through the alkaline picrate method (Jaffe 1886). Creatinine-adjusted concentrations were calculated as the quotient of the measured analyte concentration and the measured creatinine concentration in each void. Specific gravity adjusted concentrations were calculated as follows:

$$C_{SG-adj} = C_{vol} \times \left(\frac{SG_{ref} - 1}{SG_{measured} - 1}\right)$$

where C_{SG-adj} is the biomarker adjusted for specific gravity, C_{vol} is the volumetric concentration (in nanogram per milliliter), S_{Gref} is a reference specific gravity for the population (here, the arithmetic mean of all the collected spot samples), and $SG_{measured}$ is the specific gravity of the specific sample (Cone et al. 2009).

As detailed information was available on the total urinary volume of each sample (V), the time since the previous void (t) as well as each participant's body weight (BW) and the urinary concentration of each target analyte, the average excretion rate (ER) of the analyte over the time period covered by the collected urinary void can be calculated in ng/kg BW-h:

$$ER(ng/kgBW-h) = \frac{C(ng/mL) \times V(mL)}{T(h) \times BW(kg)}$$

The calculated analyte excretion rate directly accounts for hydration status variations by incorporating the actual urinary flow rate (volume per time), and represents the "true" excretion rate of the analyte for each spot sample.

Data analysis

Various summary statistics and analyses were conducted based on both unadjusted measured concentrations and on creatinine-adjusted and specific gravity-adjusted concentrations. Concentrations in the samples that were below the LOQ were imputed as LOQ/sqrt(2). Statistical analyses were conducted using Stata IC 12 (Stata Corp., College Station, Texas).

Intraclass correlation coefficients (ICCs) were calculated as the ratio of between-individual variance to the total variance based a simple random effects model for ln-transformed concentrations by participant using the urine data from the four baseline product use days only, in order to represent typical conditions. ICCs were calculated based on spot and 24-h composite concentrations and were also calculated based on both unadjusted and creatinine-adjusted concentrations.

Spearman correlation coefficients between spot sample analyte excretion rates and unadjusted, creatinine-adjusted, or specific gravity-adjusted urinary concentrations were assessed to evaluate the most accurate concentration surrogate when detailed information on urine flow rate is not available and analyte excretion rates cannot be directly calculated.

First morning voids (FMV) were identified based on the data on breakfast time, personal care product application, and consistency throughout the 6-day sampling period. Hence, FMVs were not always the first sample of the calendar day (which might have been at 01:30 at night), but the first sample that was closely followed by a normal morning ritual of breakfast and personal hygiene. FMVs were identified as belonging to the previous 24-h period.

Thus, exposure days were defined as running from post-FMV on 1 day through FMV on the following day. 24-h composite concentrations were calculated as the sum of mass of target analyte excreted (mass in each void calculated as the product of the measured concentrations and urine volumes) divided by the sum of urine volume or sum of excreted creatinine during the defined exposure day including the concluding FMV. The correlation between FMV and 24-h composite concentrations was evaluated.

Urinary concentrations of the target analytes on days with use of replacement products were compared to those on days with use of the baseline products. Variation in individual spot, 24-h composite, and multiday average concentrations on days with use of baseline products was also assessed visually.

Spearman rank correlation coefficients for the "true" excretion rate (ng/kg-h) and various concentration surrogates (unadjusted, cr-adjusted, and SG-adjusted) were assessed for each of the frequently detected analytes to identify the concentration surrogate that most accurately represents the true excretion rate.

Results

Basic characteristics of the 8 participants are presented in **Table 1**. The participants ranged in age from 31 to 68 years and were in good physical health, with no known acute or chronic diseases present. 1 participant was a frequent smoker, and 1 participant reported using dietary supplements. Urine collection over the 6 study days was complete – no voids were missed by any participant. In all, 352 individual urine samples were collected over the 6 day period with total urine volumes ranging from 7.5 to 21 L per participant. Other summary information on urine voids is presented in Smolders et al. (2014).

Table 1 Participant characteristics. Details regarding urine voids are presented in Smolders et al. (2014).

	Couple 1		Couple 2		Couple 3		Couple 4	
	Participant 1	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8
Gender	Female	Male	Female	Male	Female	Male	Female	Male
Age (years)	31	38	31	40	58	58	66	66
Height (cm)	170	190	167	184	172	189	163	178
Weight (kg)	58	92	57	85	66	95	63	78
#Urine samples	43	46	40	45	65	34	39	40

The target analytes, LOQs, and detection rates are presented in **Table 2**. Specific parabens were reported on the labels of numerous of the personal care products in use by the participants. Triclosan was listed on labels of some toothpastes and one deodorant. Triclocarban, benzophenones, and BPA were not identified on any label. The urinary detection rates were generally consistent with the presence of ingredients on the product labels and product use. MP, EP, and *n*PP were detected at or above 70% frequency, while *n*BuP was detected less frequently. Triclosan, BP3, and BPA were also detected at rates near or above 70%.

Table 2 Target analyte detection rates and limits of quantitation.

Parabens			Benzophenones			Others		
Analyte	% > LOQ	LOQ	Analyte	% > LOQ	LOQ	Analyte	% > LOQ	LOQ
Methyl	100.0	0.5	Benzophenone-1	13.3	2.0	Triclocarban	0.0	1.0
Ethyl	93.2	0.5	Benzophenone-3	69.9	2.0	Triclosan	79.8	1.0
n-Propyl	70.7	0.5	Benzophenone-8	0.0	0.5	BPA	71.9	0.5
n-Butyl	26,1	0.5						
Benzyl	4,5	0.5						
iso-Propyl	0.0	0.5						
iso-Butyl	0.0	0.5						
Pentyl	0.0	0.5						
Heptyl	0.0	0.5						

Methyl-, ethyl-, *n*-propyl-, and *n*-butylparabens were identified on the labels of shampoo, day cream, conditioner, body lotion, shower gel, shaving cream, some toothpastes, and make-up products. Triclosan was identified on the labels of some toothpastes and one deodorant. No products had benzophenones or BPA identified on the labels.

Exposure to BPA likely occurred via the food pathway or other unknown sources, consistent with other studies (Ye et al. 2011; Christensen et al. 2012). As benzophenones can be used in clear plastic packaging to inhibit product degradation by ultraviolet light, BP3 may have been present in packaging for food or beverage products.

Summary statistics for urinary concentrations (cr-adjusted) of those analytes with detection rates at or above 70% are presented in **Table 3**. The statistics are segregated by days with use of usual product and days with use of replacement products without target analytes. On days with use of usual products, average spot sample concentrations varied substantially by participant and within and between days (**Fig. 2**). Within-individual spot sample concentrations on those days varied over approximately 2 orders of magnitude for the parabens, and over about 1.5 orders of magnitude for triclosan, BP3, and BPA. Differences between individuals in usual exposure rates were clearly identifiable for most analytes except BPA, for which spot sample concentrations varied over a similar range for all 8 individuals. Variation in 24-h composite concentrations was lower than in spot samples for each individual.

Table 3 Average (SD) creatinine-adjusted concentrations (µg/g cr) by participant and day for six frequently-detected analytes on days with exposure to participants' usual personal care products and on days with replacement products without target analyt.

Participant	Usual product days					Replacement product days		
	1	4	5	6	4-d average	2	3	2-d average
	Methylparaben							
1	104.2 (86.1)	111.6 (82.1)	648.2 (863.8)	1259 (277.5)	495.1 (653.2)	78.9 (56.5)	79.1 (55)	79 (54)
2	96.2 (29.6)	90 (48.1)	155.2 (67.3)	44.6 (13.2)	99.8 (58.1)	32.8 (16)	8.6 (3.1)	19.9 (16.4)
3	35.9 (34.4)	27.7 (26.4)	33.1 (37)	44.6 (46.1)	35.4 (34.8)	6.9 (3.8)	3 (0.9)	5.1 (3.4)
4	73.3 (35.7)	61.8 (45.4)	83 (47.2)	106.4 (67.9)	83 (50.8)	22.4 (13.7)	5.8 (1.3)	13.5 (12.4)
5	324.6 (209.6)	301.2 (160.8)	286.2 (167.3)	388.8 (276.3)	321.4 (194)	130.6 (74.8)	64.7 (31.4)	100.6 (66.9)
6	4.2 (0.9)	3.5 (1.1)	5.4 (1.8)	3.9 (0.9)	4.3 (1.4)	7.8 (4.5)	9.8 (5.1)	9 (4.8)
7	41.6 (26.5)	40.3 (21.5)	49.4 (32.2)	66.7 (24.1)	47.9 (26.5)	10 (3.9)	3.2 (0.5)	7.1 (4.5)
8	141 (67.6)	145.2 (79.7)	122 (59.4)	297.5 (57.6)	156.9 (85.5)	29.2 (12.3)	8 (1.6)	20.4 (14.3)
All	130.7 (152)	121.2 (132)	182.7 (361.4)	270.4 (405.7)	168.2 (282.2)	48.3 (60)	26.2 (38)	37.6 (51.6)
	Ethylparaben							
1	50.1 (39.6)	58.6 (53.1)	92.5 (37.8)	101 (22)	75.2 (42)	18.1 (6.9)	7.8 (2)	13.3 (7.4)
2	76 (28.4)	62.6 (37.9)	124.2 (47.3)	48 (5.3)	78.6 (44.1)	34 (17.1)	8.4 (4.2)	20.4 (17.6)
3	1.3 (0.6)	1.4 (0.6)	1.9 (1.8)	4.6 (8)	2.3 (4)	0.6 (0.3)	2.8 (3.1)	1.7 (2.3)
4	5 (2.5)	5.6 (2.7)	1.7 (0.4)	2.6 (3.9)	4.1 (3.7)	1.6 (1.6)	10 (11.1)	6.1 (9)
5	91.5 (33)	46.9 (30.9)	29.5 (14.9)	112.9 (49.4)	69 (44.3)	40.4 (7.9)	20.4 (12.4)	31.3 (14.2)
6	1.8 (0.4)	0.7 (0.1)	1.8 (1)	2.5 (1.1)	1.7 (0.9)	1.6 (0.3)	1 (0.2)	1.3 (0.4)
7	3.5 (1.2)	3.3 (1.4)	3.6 (2.8)	3.4 (2.5)	3.6 (2)	1.7 (0.6)	6.1 (7.6)	3.6 (5.2)
8	9.4 (2.4)	11.4 (5.9)	24.3 (19.5)	39.1 (6.8)	19.1 (15.3)	3.6 (1.4)	11.2 (13.7)	6.8 (9.2)
All	37.6 (43.9)	29 (36.1)	36.8 (49.9)	40.2 (49.3)	35.5 (44.5)	15.1 (17.7)	9 (9.8)	12.2 (14.7)
	n-Propylparaben							
1	1.9 (2.3)	3.3 (2.6)	387 (599.3)	616 (294)	238.7 (423)	2.9 (2.9)	3.8 (3.1)	3.3 (3)
2	2 (2.5)	1 (0.6)	1.2 (0.2)	1.5 (0.5)	1.3 (1.3)	0.7 (0.3)	0.5 (0.4)	0.6 (0.3)
3	9.1 (12.1)	10.1 (12.3)	10.1 (15.5)	16.5 (22.7)	11.2 (15.4)	1.1 (1.1)	0.9 (0.6)	1 (0.9)
4	15 (18.9)	21 (28.2)	24.4 (30.3)	81.9 (52)	23.9 (30.5)	1.4 (0.9)	0.8 (0.5)	1.1 (0.7)
5	83.8 (64.1)	81.6 (56.5)	93.5 (66.3)	81.9 (52)	86.7 (58.8)	18.9 (14.1)	5.3 (1)	12.7 (12.3)
6	0.6 (0.6)	0.2 (0)	1.8 (1.8)	0.4 (0.3)	0.8 (1.1)	0.2 (0)	0.3 (0.2)	0.3 (0.1)
7	9.8 (5.1)	28.5 (31.3)	16.4 (19.7)	16.9 (8.6)	18.3 (20.2)	1.7 (0.8)	0.6 (0.3)	1.2 (0.8)
8	0.5 (0.2)	0.8 (0.7)	1.4 (0.7)	1 (0.2)	0.9 (0.6)	0.6 (0.3)	0.6 (0.6)	0.6 (0.4)
All	22.6 (44.7)	25 (42.8)	70 (240.9)	93.4 (123)	49.7 (161.1)	4.7 (9.2)	1.8 (2.2)	3.3 (6.9)
	Triclosan							
1	1392.3 (820.3)	556.1 (217.5)	1229.7 (336.4)	1245 (291.2)	1136.6 (553.5)	831.2 (361.4)	250.3 (110.3)	557.8 (399.9)
2	721.5 (298.3)	531.4 (312.3)	970.9 (349.4)	803.6 (268.9)	730.9 (341.1)	277.4 (134)	76.7 (21.2)	170.4 (136.6)
3	1.2 (0.9)	0.9 (0.3)	1 (0.7)	1.4 (0.6)	1.2 (0.7)	1 (0.6)	1.2 (0.7)	1.1 (0.6)
4	2.3 (1.7)	1.5 (0.9)	1.8 (1.1)	1.4 (0.6)	1.7 (1.1)	2.2 (1.2)	1.5 (1)	1.8 (1.1)
5	1194 (202.4)	756.4 (452.6)	1109 (161.3)	983.4 (89.8)	1005.2 (327)	1142 (542.3)	260.2 (70)	741.1 (598.4)
6	253.5 (38.8)	226.3 (66.2)	331.3 (69.1)	237.7 (101.7)	262.4 (76.9)	173.9 (58.5)	53.5 (20.7)	103.7 (72.9)
7	636.9 (137.2)	339.1 (176)	673.7 (372.6)	907.5 (359.2)	603.5 (336)	308.4 (183)	112.3 (33.2)	224.4 (169.1)
8	620.5 (168.1)	129.8 (53.3)	444 (141.1)	437.8 (154)	418.4 (220.5)	368.3 (208.2)	120.4 (62.4)	265 (203.4)
All	678.3 (579.2)	383.4 (374.3)	617.9 (504.5)	554.2 (497.4)	555 (501.1)	461.3 (504.1)	116.8 (113.3)	294.7 (407.7)
	Benzophenone-3							
1	9.9 (2.9)	19.1 (6.2)	15.9 (3)	15.6 (4.6)	14.7 (5)	13.1 (7.4)	14.7 (4.9)	13.8 (6.2)
2	5.2 (1.2)	5.1 (1)	4.9 (1)	3.1 (0.6)	4.8 (1.2)	9.4 (1.8)	8.1 (2)	8.7 (1.9)
3	721.9 (409.8)	508.6 (196)	624.2 (203.9)	202.7 (40.4)	510.9 (309.1)	835.3 (325.7)	734.8 (322.9)	788.4 (316.8)
4	9.8 (3.3)	9.1 (3.6)	9.7 (5.9)	8.4 (1.9)	9.4 (3.8)	8.3 (5.6)	8.7 (5.2)	8.5 (5.2)
5	4.3 (2.3)	7.2 (4.9)	12.3 (5.2)	3 (0.8)	8.9 (6.4)	4.5 (2.1)	7.4 (4.6)	5.8 (3.7)
6	1.1 (0.2)	0.9 (0.2)	1 (0.3)	17 (6.1)	1 (0.2)	1.9 (1.4)	1.4 (0.8)	1.6 (1.1)
7	4.1 (1)	3.9 (0.5)	3.5 (0.8)	0.9 (0.1)	3.6 (0.8)	4.8 (1.6)	3.6 (1.6)	4.3 (1.7)
8	4.2 (1.1)	4.8 (1.1)	4.4 (1.8)	6.1 (2.6)	4.8 (1.8)	5.3 (5.4)	4.2 (1.6)	4.9 (4.1)
All	78.4 (249.4)	42.7 (138.3)	87.2 (219.6)	35 (69.1)	62 (186.8)	112.1 (298.8)	93.6 (259.1)	103.2 (279.3)
	BPA							
1	1.6 (0.7)	1 (0.4)	5.3 (8.9)	4 (2.5)	3 (5.2)	2.1 (0.9)	1.2 (0.4)	1.7 (0.9)
2	1.3 (0.4)	1 (0.3)	1.2 (0.4)	1.6 (0.7)	1.2 (0.4)	0.8 (0.2)	0.8 (0.4)	0.8 (0.3)
3	1.6 (0.7)	3.6 (2.6)	3.1 (0.6)	8.1 (6.5)	4 (4)	1.1 (0.5)	1.3 (0.5)	1.2 (0.5)
4	1.4 (0.7)	2.2 (2)	1 (0.5)	2.5 (2.3)	1.7 (1.6)	1.2 (0.5)	1.1 (0.4)	1.1 (0.5)
5	2.1 (1.6)	3.4 (0.8)	4.1 (2.7)	2.6 (0.7)	2.9 (1.8)	2.5 (0.5)	3.5 (1.3)	3 (1)
6	0.5 (0.5)	0.4 (0.3)	1.6 (1.7)	1.2 (0.7)	0.9 (1.1)	0.3 (0.1)	0.7 (0.3)	0.5 (0.3)
7	2.2 (0.5)	2.4 (2.5)	2.3 (2.9)	8.7 (11)	3.7 (5.5)	2.3 (0.9)	1.9 (0.6)	2.1 (0.8)
8	1.9 (0.6)	1.3 (0.5)	3.8 (2.7)	2.7 (2.5)	2.5 (2)	1.8 (1.6)	1.9 (0.4)	1.8 (1.2)
All	1.7 (1)	1.9 (1.7)	2.9 (3.8)	3.9 (5)	2.5 (3.2)	1.6 (1)	1.6 (1.1)	1.6 (1.1)

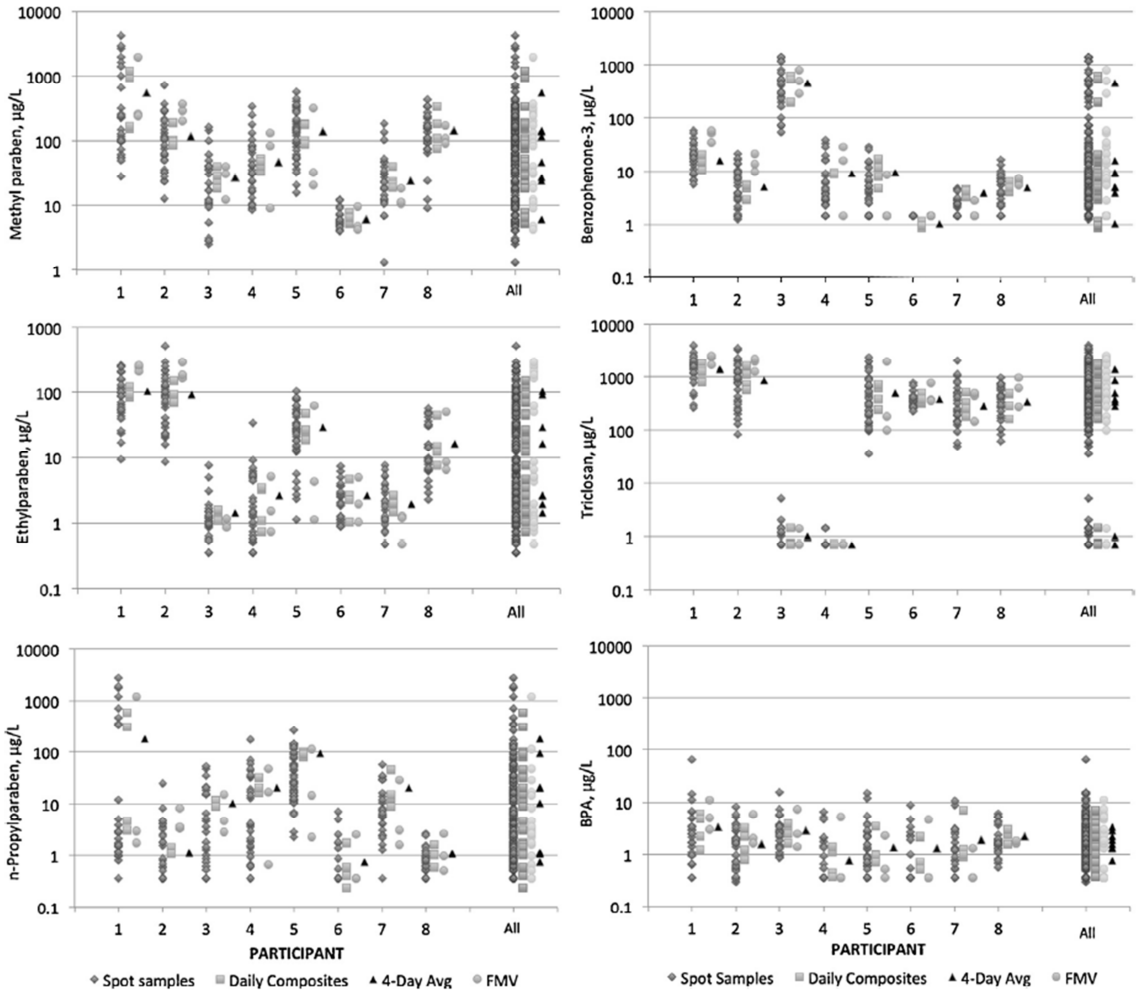

Figure 2 Participant spot, 24-h, first morning void (FMV) and 4-day average urine concentrations for frequently detected target analytes during regular use (not including intervention phase), by participant and for the 8 volunteers together.

Intraclass correlation coefficients (ICCs) for spot samples and composited 24-h samples for the 4 days with use of the usual products are presented in **Table 4** both on the basis of un-adjusted and cr-adjusted concentrations for the frequently detected analytes. Consistent with the visual inspection of **Fig. 2**, moderate to high ICCs were observed for the three parabens, triclosan, and BP3, while ICCs for BPA were low, consistent with previous data (Ye et al. 2011). ICCs were higher for the repeat 24-h composites than for spot samples, and were generally higher for cr-adjusted concentrations than unadjusted concentrations.

Table 4 ICCs for target analytes based on repeated spot samples and repeated 24-h composite samples for four baseline product days only.

Analyte	ICC-unadjusted concentrations		ICC-cr-adjusted concentrations	
	Spot	24-h	Spot	24-h
Methylparaben	0.557	0.843	0.710	0.871
Ethylparaben	0.756	0.923	0.821	0.916
n-Propylparaben	0.385	0.715	0.538	0.759
Triclosan	0.934	0.982	0.957	0.987
Benzophenone-3	0.812	0.962	0.919	0.942
Bisphenol A	0.151	0.147	0.264	0.280

Concentration differences between days with use of baseline products vs. days with use of replacement products without target analytes were clearly evident for the parabens and triclosan (**Fig. 3**). BP3 and BPA showed no differences between usual and replacement product days (**Table 3**), consistent with the lack of these compounds as identified ingredients on the personal care product labels.

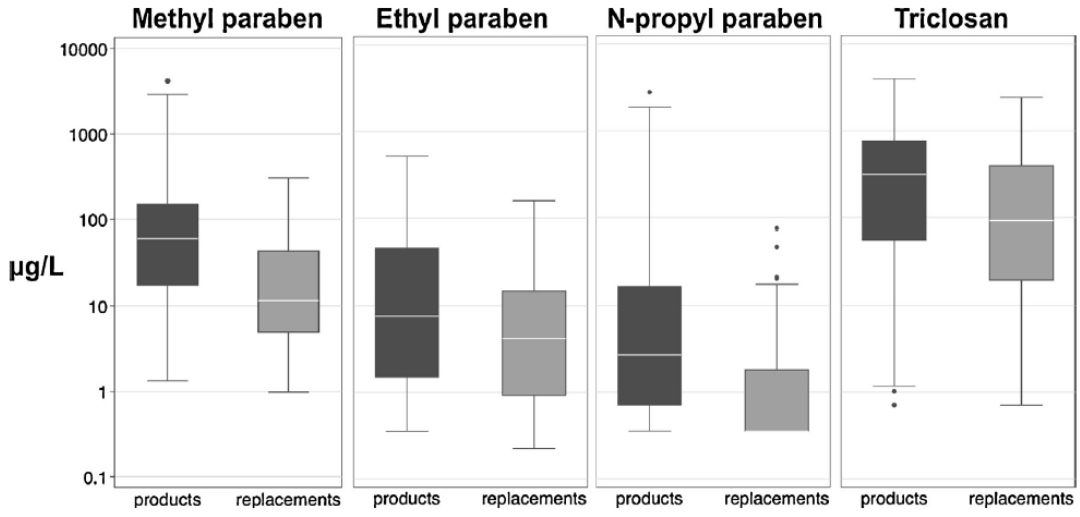

Figure 3 Spot sample urine concentrations, baseline product days vs. days with replacement products without target analytes. Boxes represent interquartile range, with median line. Whiskers extent to 1.5 times above and below the interquartile range; dots represent outlying datapoints. No differences between baseline and replacement product days were seen for BP3 and BPA (data not shown).

We examined the correlation between paired FMV and 24-h composite concentrations (for the day preceding and including the FMV) across all 6 days of the study (**Fig. 4**). In general, FMV concentrations were moderately to highly correlated with 24-h composite concentrations, with R2 value of 0.95 for triclosan, followed by EP, MP, BP3, nPP, and BPA.

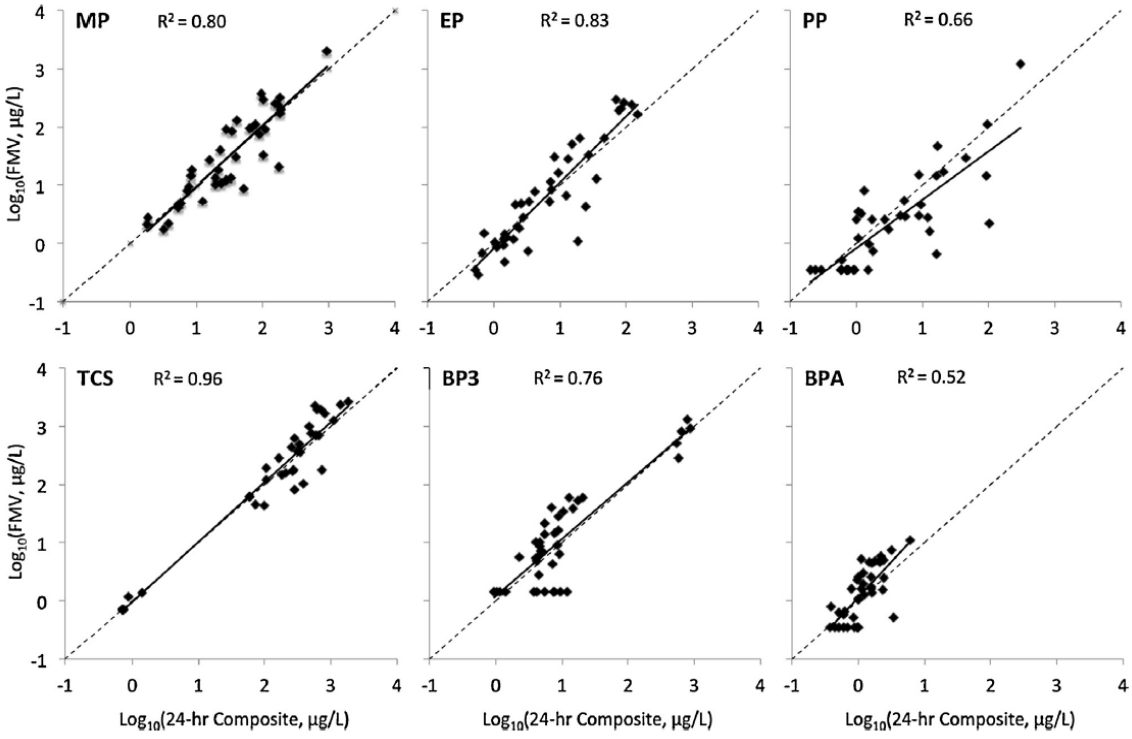

Figure 4 First morning void concentrations vs. 24-h composite concentrations (all days of the study). Dotted lines represent the 1:1 axis; solid lines show the best-fit regression line.

We also examined the correlations between the calculated excretion rate (ng/kg-h) of each analyte in each void and the various concentration measures available, either unadjusted or adjusted, for hydration status using either creatinine or specific gravity approaches as described above. All concentration measures were highly correlated with excretion rate (**Table 5**). For all analytes, SG-adjusted concentrations were most highly correlated, followed by cr-adjusted concentrations, with unadjusted concentrations least correlated. This suggests that, at least based on this dataset and for these analytes, specific gravity adjustment may provide the most accurate surrogate for true excretion rate in those situations in which urinary flow and void timing information is not available for calculation of analyte excretion rate. However, the differences in accuracy between specific gravity adjusted and creatinine-adjusted concentrations were small.

Table 5 Spearman rank correlation coefficients between analyte excretion rate and unadjusted, creatinine-adjusted, and specific gravity-adjusted concentrations for six frequently-detected analytes.

	Excretion rate (ng/kg-h)	Unadj. (ng/mL)	Cr-adj. (µg/g cr)	SG adj. (ng/mL)	Excretion rate (ng/kg-h)	Unadj. (ng/mL)	Cr-adj. (µg/g cr)	SG adj. (ng/mL)
	Methylparaben				*Triclosan*			
Excretion rate (ng/kg-h)	1				1			
Unadj. (ng/mL)	0.87	1			0.90	1		
Cr-adj. (µg/g cr)	0.91	0.86	1		0.92	0.85	1	
SG adj. (ng/mL)	0.93	0.93	0.96	1	0.94	0.94	0.95	1
	Ethylparaben				*Benzophenone-3*			
Excretion rate (ng/kg-h)	1				1			
Unadj. (ng/mL)	0.92	1			0.81	1		
Cr-adj. (µg/g cr)	0.94	0.91	1		0.85	0.74	1	
SG adj. (ng/mL)	0.95	0.95	0.98	1	0.90	0.88	0.92	1
	n-Propylparaben				*BPA*			
Excretion rate (ng/kg-h)	1				1			
Unadj. (ng/mL)	0.89	1			0.66	1		
Cr-adj. (µg/g cr)	0.95	0.88	1		0.73	0.61	1	
SG adj. (ng/mL)	0.95	0.92	0.98	1	0.78	0.83	0.86	1

The product use based on diary report was clearly detectable in urinary concentration profiles in many cases. A comprehensive analysis of the diary data and linkage to the urinary biomonitoring data will be conducted in a subsequent paper. However, two cases illustrate the value of the information.

n-Propylparaben was identified as an ingredient in a shower gel and shaving cream used by 1 participant. Diary information on the use of these products in conjunction with the measured urinary concentrations is presented in **Fig. 5A**. Use of the two products in conjunction with one another resulted in a clear urinary peak on day 1; uses on subsequent days of a single product were associated with less-pronounced peaks. Examination of the rapid rise in urinary concentration following product use on day 1 suggests that systemic exposure may have occurred either principally or substantially via inhalation, consistent with the moderate volatility of nPP and the use in an enclosed area (bathroom). The sharp peak and rapid decline also provide a basis for estimating urinary elimination half-life of this compound. Based on **Fig. 5A**, an elimination half-life of approximately 4 h can be estimated, confirming the non-persistent nature of this compound, consistent with animal data indicating rapid metabolism and elimination (Soni et al. 2005).

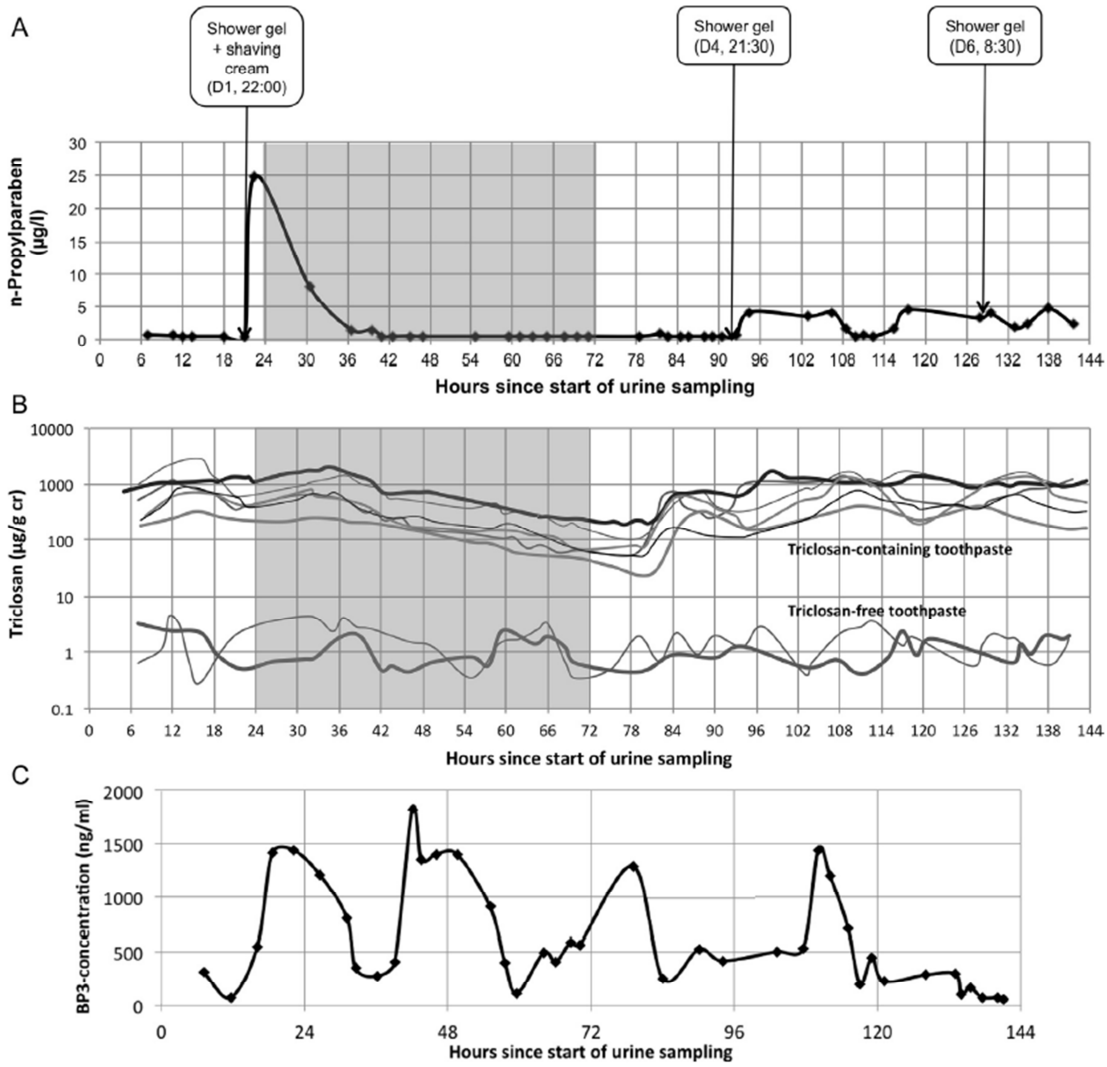

Figure 5 Example of participant urine concentration timelines. The shaded area of the graphs represents the time period of use of replacement personal care products that did not contain the target analytes. (A) Use of shower gel plus shaving cream on day 1 resulted in a clearly discernible peak in *n*-propylparaben excretion shortly after product use in 1 participant, while use only of shower gel on later occasions did not result in such pronounced peaks. (B) Triclosan urinary concentrations for 8 participants, 6 of whom used triclosan-containing toothpaste and 2 who did not. (C) Pattern of BP3 urine concentrations in individual linked to use of sanitizing hand soap in the workplace. BP3 was not identified in the product ingredient list.

Triclosan was listed as an ingredient in toothpastes used by 6 of the 8 participants, and the urinary profiles for the two groups are entirely distinct (**Fig. 5B**). Triclosan was rarely detected (with positive detects close to the LOQ) in the 2 participants who used toothpaste without triclosan. In the other participants, the urinary concentrations were generally 100–1000 times above the LOQ of 1 ng/mL and varied by approximately a factor of 10 during usage days.

The upper end of the detected concentrations (1000–3000 ng/mL) is similar to the upper percentiles of triclosan urinary concentrations observed in the US NHANES datasets (CDC 2013), suggesting that this use is a plausible source of these upper end concentrations in the US as well as in this group of volunteers. The urinary concentrations of triclosan in persons using a replacement toothpaste during the 2 day intervention period declined by approximately a factor of 10, consistent with an elimination half-life on the order of 12 h, as previously measured in a controlled dosing study by Sandborgh-Englund et al. (2006). The data demonstrate the value of intervention studies such as this one and one by Koch et al. (2013), which examined urinary concentrations of phthalate compounds over a fasting period in volunteers. Such studies can provide basic information on elimination half-life and exposure sources of commonly encountered substances without the ethical or experimental issues associated with controlled dosing studies.

As discussed above, BP3 was not identified on the labels of any of the personal care products, but was frequently detected at low levels, perhaps consistent with the use of BP3 as a stabilizer in plastic packaging. However, for the individual with highest BP3 levels, an additional source was identified based on the timing of urinary peaks (**Fig. 5C**). This participant was required in the workplace to frequently wash hands with an antibacterial soap, and the timing of soap use and non-use and the relatively high concentrations in the corresponding urine samples suggested that BP3 was an ingredient of the soap, although it was not listed on the label.

Discussion and conclusions

The study presented here provides an intensive dataset for evaluation of target analyte urinary biomarkers associated with personal care product use. A similar dataset was collected previously and analyzed for BPA and selected phthalate metabolites (Ye et al. 2011; Preau et al. 2010) and another research group has examined pyrethroids in a similar dataset (Wielgomas 2013). The current dataset is the first dataset to our knowledge that provides such intensive data for urinary parabens, triclosan, and benzophenone analytes. Based on the initial evaluations conducted here for the dataset, several observations and conclusions can be drawn.

Parabens exhibit substantial intra-individual variability and urinary concentrations can be tied to use of personal care products with these compounds identified on the labels. Some evidence of exposures via other sources (e.g., from preservatives in food) is present in this study, particularly with respect to methylparaben, which was detected in every urine sample. However, the personal care product uses appear to account for the samples with higher detected concentrations in this population.

Intraclass correlation coefficients among spot samples were moderate to high among the participants in this study for all of the analytes except BPA. In general, the ICCs observed here were higher than those observed in other studies (Aylward et al. 2012; Meeker et al. 2013; Smith et al. 2012). The between-individual variation observed here appears to be predominantly due to differences in product use patterns rather than inter-individual differences in metabolism rates or patterns; however, this issue has not been extensively evaluated to date.

In many cases the relatively high ICC values in the current study derived from large between-individual differences in exposure levels rather than from low within-individual variation (**Fig. 2**). Thus, while a single spot sample may relatively accurately rank a subject with respect to exposure to a compound compared to other subjects, it may still substantially over- or under-represent average exposure to that individual. This is particularly important with respect to understanding uncertainty in exposure reconstruction via reverse dosimetry approaches. In addition, the use of a simple imputation of LOQ/sqrt(2) for non-detected samples will bias the ICC values to a higher value for those analytes with higher rates of non-detects. BPA, BP3, and nPP all had approximately 30% non-detects in this dataset. Finally, the ICC values calculated are based on an unusual situation in which every urine concentration over a relatively condensed time period from a few individuals was included. Most studies that have calculated ICC values do so based on more widely spaced and isolated samples; so comparison of ICC values from this analysis to those in other studies may be misleading.

For this set of analytes and this volunteer population, FMVs were moderately to highly correlated with 24-h composite concentrations without consistent bias for all six of the frequently-detected analytes. This suggests that in study design, it may be worthwhile to attempt to collect FMVs when possible in order to more accurately represent the daily exposure rates for compounds of interest, at least among these analytes.

In this participant group, SG-adjusted urinary concentrations provided the best correlation to true urinary excretion rates. Creatinine adjustment has been used more frequently for adjustment for urinary dilution. However, these data suggest that SG-adjustment may provide a somewhat more accurate adjustment approach. The potential issues associated with identification of appropriate 'reference' SG values for the adjustment across different population elements remain to be assessed. The potential biases associated with differences in normal creatinine excretion rates have been identified for creatinine (Barr et al. 2005; Garde et al. 2004; Mage et al. 2008; Remer et al. 2002), but comparable evaluation of SG of normal urine and variations by age, gender, and other factors has not been done to our knowledge.

Further intensive analysis of the data collected in this project will be conducted to evaluate mass balance for target analytes, estimate half-lives of elimination for the frequently detected target analytes, and describe product use patterns in more detail. However, the data presented here clearly illustrate the insight that can be gained into the utility of urinary biomonitoring for the target analytes and the considerations that should be addressed in the design of further biomonitoring studies related to these analytes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would sincerely like to thank the 8 participants that voluntarily participated in the study. Without their effort, this study would not have been feasible. Francis Boonen and Stijn Van Uytsel processed and aliquoted the urine samples upon arrival in the lab. This study was performed as part of the project "Understanding inter- and intra-individual variability in HBM spot samples" funded by an LRI grant (LRI-HBM4) of CEFIC (the European Chemical Industry Council). The authors had complete freedom to design, carry out, analyze, interpret, and report the results of this study.

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Kapitel IV

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

Rebecca K. Moos, Jürgen Angerer, Georg Dierkes,
Thomas Brüning, Holger M. Koch

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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.

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, 2002b) 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 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.

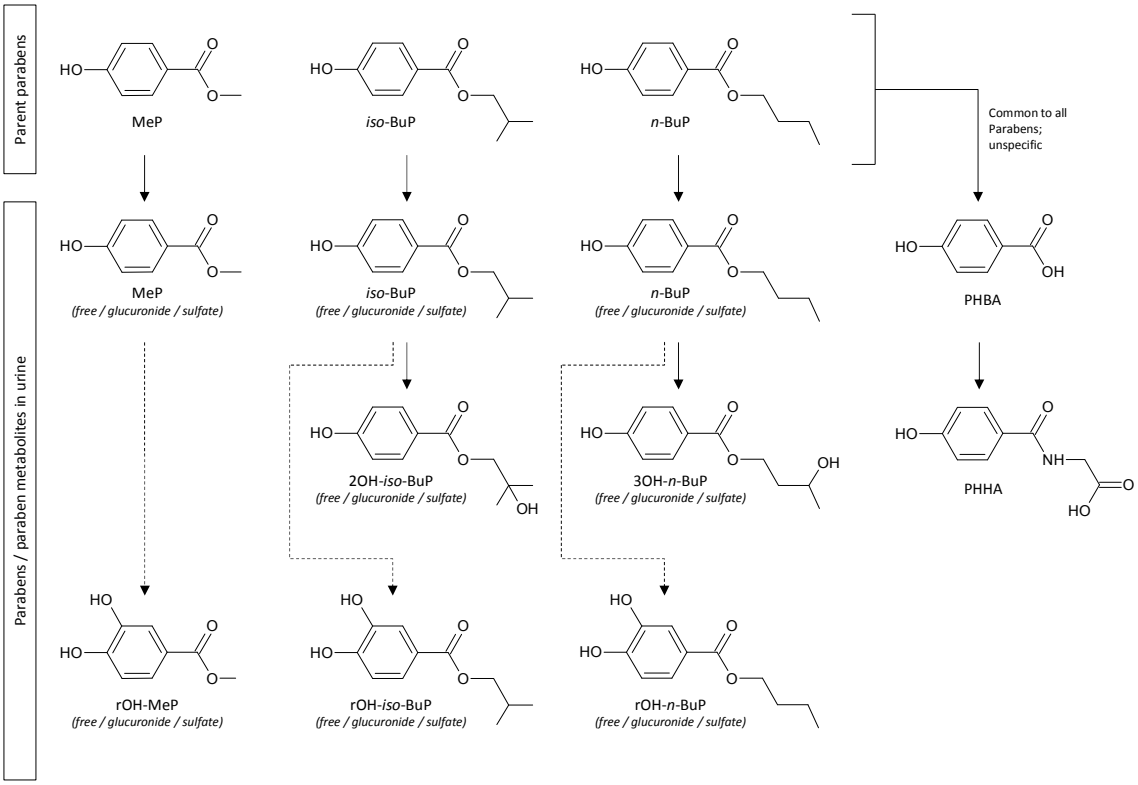

Figure1 Postulated human metabolism of the parabens. Dashed lines show the minor metabolites.

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; 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-d4 (D4-MeP), *iso*-butyl 4-hydroxybenzoate-2,3,5,6-d4 (D4-*iso*-BuP), *n*-butyl 4-hydroxybenzoate-2,3,5,6-d4 (D4-*n*-BuP) and 4-hydroxybenzoic-2,3,5,6-d4 acid (D4-PHBA) were purchased from C/D/N Isotopes (Dr. Ehrenstorfer GmbH, Augsburg, Germany). The 13C6-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-d4 acid (D4-PHHA), 3-hydroxy *n*-butyl 4-hydroxybenzoate-2,3,5,6-d4 (D4-3OH-*n*-BuP) and 2-hydroxy *iso*-butyl 4-hydroxybenzoate-2,3,5,6-d4 (D4-2OH-*iso*-BuP) and 13C6-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** (**Appendix III**). 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 13C6-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** (**Appendix III**).

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** (**Appendix III**). 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; Appendix III**). 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; Appendix III**).

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; Appendix III**). 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 13C6-labeled internal standards with 13C4-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.

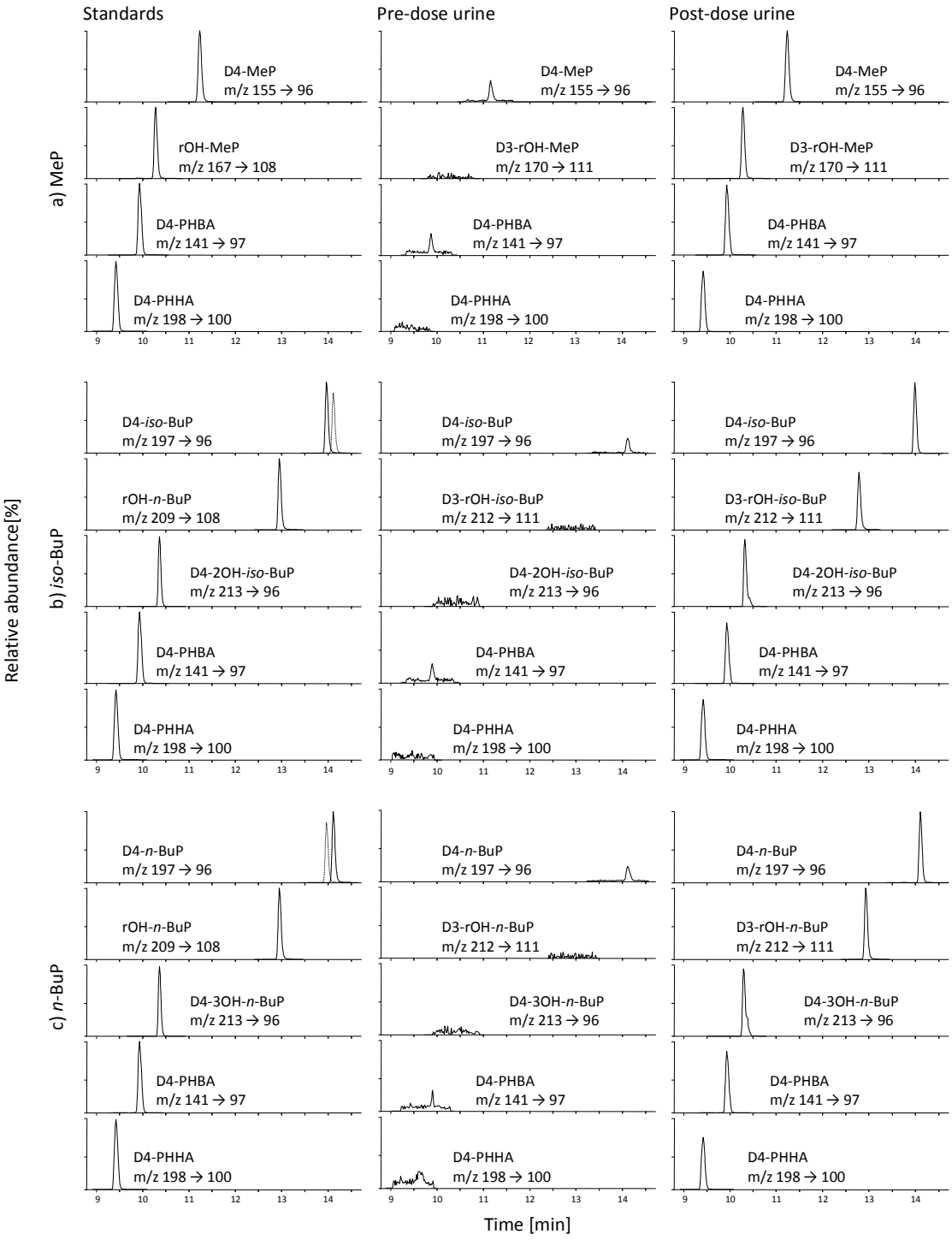

Figure 2 Chromatograms of different urine samples from the human metabolism study of MeP, *iso*-BuP and *n*-BuP, representing the quantifier trace (bold line) of scheduled multiple reaction monitoring (MRM). Left column represents a processed calibration standard (about 80 µg/L for each analyte). The 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.

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**.

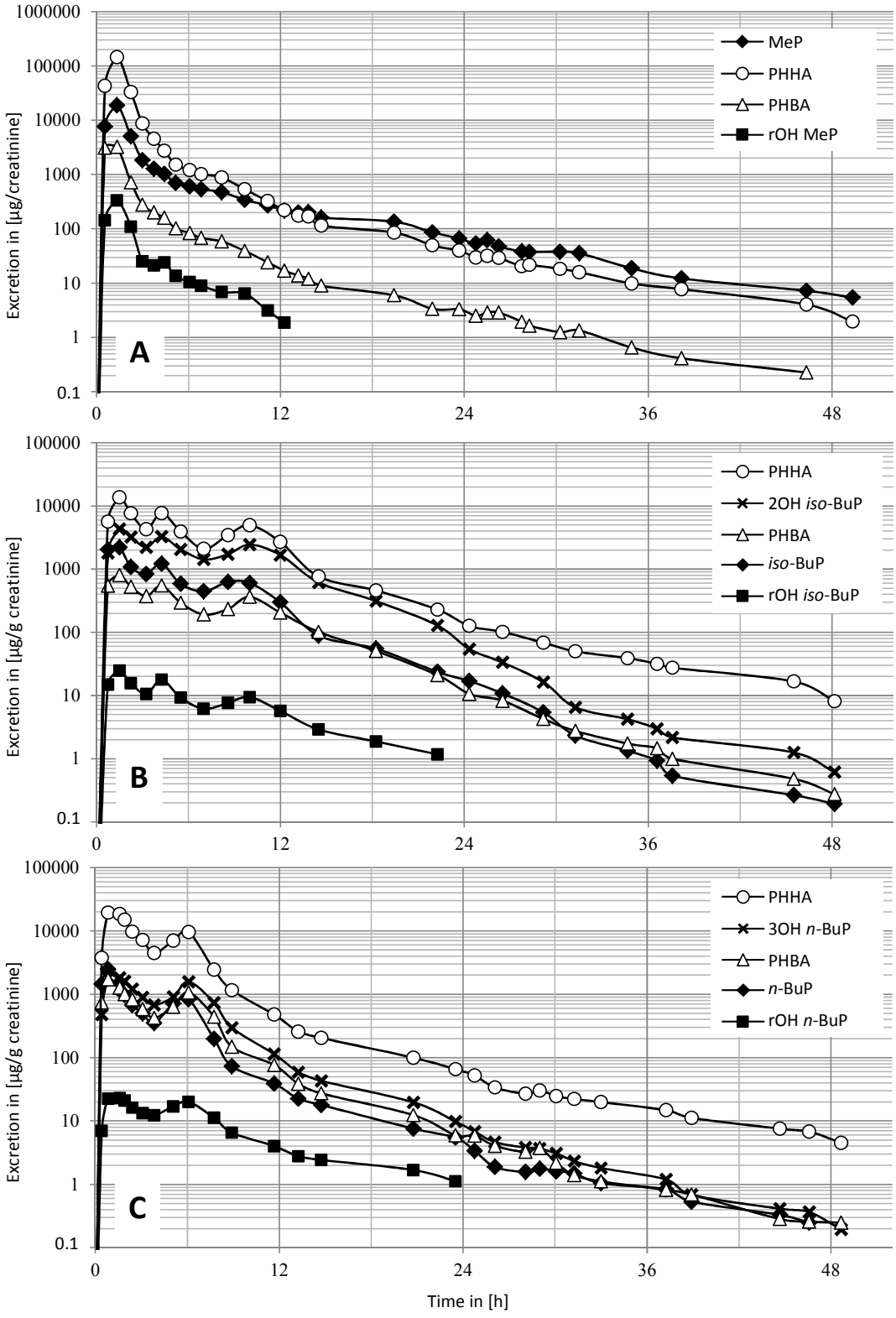

Figure 3 Creatinine-corrected metabolite concentrations in urine after oral dosage, shown in semilogarithmic scale (continuous data from one volunteer; profiles were similar for the other two volunteers). The non-specific metabolites PHHA and PHBA are depicted with white markers, and all paraben specific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

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)

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 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. 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 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 isoform 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 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).

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 0-24 h (%)	Percentage of applied dose between 24-48 h (%)	Percentage of applied dose between 0-48 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**	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)

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).

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 %.

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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Kapitel V

Daily intake and hazard index of parabens based upon 24 h urine samples of the German Environmental Specimen Bank from 1995 to 2012

Rebecca K. Moos, Petra Apel, Christa Schröter-Kermani,
Marike Kolossa-Gehring, Thomas Brüning, Holger M. Koch
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Abstract

In recent years, exposure to parabens has become more of a concern because of evidence of ubiquitous exposure in the general population, combined with evidence of their potency as endocrine disruptors. New human metabolism data from oral exposure experiments enable us to back calculate daily paraben intakes from urinary paraben levels. We report daily intakes (DIs) for six parabens based on 660 24 h urine samples from the German Environmental Specimen Bank collected between 1995 and 2012. Median DI values ranged between 1.1 μg/kg bw/day for *iso*-butyl paraben and 47.5 μg/kg bw/day for methyl paraben. The calculated DIs were compared with acceptable levels of exposure to evaluate the hazard quotients (HQs) that indicate that acceptable exposure is exceeded for values of >1. Approximately 5% of our study population exceeded this threshold for individual paraben exposure. The hazard index (HI) that takes into account the cumulative risk of adverse estrogenic effects was 1.3 at the 95th percentile and 4.4 at maximum intakes, mainly driven by n-propyl paraben exposure. HI values of >1 indicate some level of concern. However, we have to point out that we applied most conservative assumptions in the HQ/HI calculations. Also, major exposure reduction measures were enacted in the European Union after 2012.

Introduction

Parabens (alkyl or aryl esters of *p*-hydroxybenzoic acid) have been widely used, individually or in combination, as antimicrobial preservatives in cosmetics, pharmaceuticals and food since the 1920s.[1, 2] The widespread use of parabens results in their ubiquitous occurrence in the environment and in humans via a variety of exposure routes (dermal absorption, ingestion and inhalation). Biomonitoring studies have shown that parabens are omnipresent in urine samples from the general population and various subpopulations.[3-19] Several *in vitro* and *in vivo* studies were published suggesting weak estrogenic activity,[20-23] antiandrogenic effects,[24-26] uterotrophic effects[21, 27, 28] and effects on sperm count and testosterone levels after dietary exposure.[29-31] 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 high doses were applied.[32, 33] Since 2005, parabens have been assessed by the Scientific Committee on Consumer Safety (SCCS, then SCCP) of the European Union in a number of opinions.[34-40] In 2011, SCCS concluded that methyl (MeP) and ethyl paraben (EtP) were safe in cosmetics, when used at maximum concentrations of 0.4% for one ester or 0.8% in combination. However, SCCS recommended lowering of limits for *n*-propyl (*n*-PrP) and *n*-butyl paraben (*n*-BuP) to a maximum total concentration of 0.14%, based upon a conservative no-observed-effect level (NOEL) of 2 mg/kg bw/day for *n*-BuP derived from Fisher et al.[41] For *iso*-propyl (*iso*-PrP), *iso*-butyl (*iso*-BuP), benzyl (BeP) and pentyl paraben (PeP), the human risk could not be evaluated because of lack of data.[38] In the same year, the Danish government banned the use of *n*-PrP, *n*-BuP, and their isoforms in personal care products intended for children up to 3 years of age.[39] In 2014, the European Parliament responded to the ongoing controversial debate regarding parabens and lowered the permitted maximum concentration of the sum of *n*-PrP and *n*-BuP in cosmetics to 0.14%.[42] Because no definitive safety assessment for human health could be carried out for *iso*-PrP, *iso*-BuP, BeP and PeP, these parabens were banned for use in cosmetic products.[43]

Previously, we analyzed 660 24 h urine samples of the German Environmental Specimen Bank (ESB) collected between 1995 and 2012 for the presence of parabens.[19] In this subset of the German population the results indicate a ubiquitous exposure to nearly all parabens investigated for the past two decades. We found MeP, EtP and *n*-PrP with high detection rates between 79% and 99%, followed by *n*-BuP with 40%.

Furthermore, *iso*-PrP, *iso*-BuP and BeP were detected in 4%, 24% and 1.4% of the samples, respectively. Neither PeP nor heptyl paraben (HeP) were detected in any of the 660 urine samples. Urinary concentrations were highest for MeP (95th percentile 319 µg/l) followed by *n*-PrP (95th percentile 74.0 µg/l), EtP (95th percentile 39.1 µg/l) and *n*-BuP (95th percentile 10.7 µg/l). *Iso*-PrP, *iso*-BuP and BeP were generally found at lower concentrations.

Biomonitoring data represent a measure of exposure from multiple sources and routes of exposures, even when these routes and sources are not well characterized or understood. Because of lack of human metabolism data, up to now it was not possible to reliably extrapolate from urinary paraben levels to doses actually taken up. In our previous study we investigated human metabolism and urinary excretion of three parabens (MeP, *iso*- and *n*-BuP) after oral dosage[44] and obtained valuable metabolic conversion factors to back calculate from urinary paraben levels (µg/l) to daily intakes (µg/kg bw/day). Remarkably, the urinary excretion factor for MeP (17.4%) was found to be ~3 times higher than for the butyl parabens (*iso*-BuP: 6.8% and *n*-BuP: 5.6%). These results indicate that the share of parent paraben excreted via urine considerably decreases with increasing chain length of the alkyl-moiety (and lipophilicity) of the paraben. Therefore, a direct comparison of urinary paraben levels increasingly underestimates actual intakes of parabens as the alkyl chain length increases. The results of the retrospective study of parabens in 24 h urine samples[19] allowed the investigation of both past and current exposures including the investigation of exposure time trends, as it has been done previously with phthalates,[45-47] DINCH[48] and bisphenol A.[49] With the knowledge of the human metabolism of parabens and the fact that urine samples are collected as 24 h urine samples, we are now able to reliably estimate oral equivalent daily intake (DI) doses for the parabens investigated. These estimated DIs can be used for risk assessment purposes by comparison with health based limit values like tolerable/acceptable daily intakes (TDIs/ADIs). Furthermore, for a cumulative risk evaluation of the parabens we calculated the hazard index (HI) for each individual based upon the concurrent urinary paraben concentrations.[50] Both approaches provide valuable information on individual parabens and parabens as a substance group for risk management purposes to prove the effectiveness of regulatory measures or to advise further measures for exposure/risk reduction.

Materials and Method

Subjects and Urine Specimens

The 24 h urine samples were collected between 1995 and 2012 and provided by the ESB. For each year, the study population consisted of 30 male and 30 female volunteers (mainly students; age range 20–30 years; 24 h urine volume over the years was on average 1820 ml) located at the University of Muenster (Germany). The study protocol of sampling human specimens has been reviewed and approved by the ethics committees of the Medical Associations of Saarland and Westfalen-Lippe and the Medical Faculty of the Westphalian Wilhelms-University Muenster. For more details about the concept and sampling criteria of the ESB, see Kolossa-Gehring et al.[51, 52] and Lermen et al.[53] Further sample information and anthropometric data are given in detail by Moos et al.[19]

Analytical Method

The analytical procedure applied for the determination of parabens (MeP, EtP, *n*-PrP, *n*-BuP, BeP, PeP and HeP; including *iso*-BuP and *iso*-PrP) in human urine has been described in detail by Moos et al.[54] In short, the method comprises enzymatic hydrolysis (β-glucuronidase/arylsulfatase enzyme type HP-2 from *Helix pomatia*) of glucuronidated and sulfated conjugates followed by online extraction (LiChrospher RP-8 ADS (25 µm) 25 × 4 mm Restricted Access Material) of the analytes from the urinary matrix. After back-flush transfer onto the analytical column (Atlantis dC18 3 × 150 mm; 3 µm), analytes were detected by ESI–tandem mass spectrometry in negative ionization mode and quantified by isotope dilution.

Data Analysis

The statistical analysis (median, 95th percentile and range values) was performed using Excel 2010 (Microsoft, Redmond, WA, USA). For the evaluation of gender differences and chronological trends we used IBM SPSS Statistics 22.0. Boxplots were generated with OriginPro 9.1 (OriginLab). Metabolite concentrations below the limit of quantification (LOQ) were substituted by LOQ/2. The estimation of descriptive values can be influenced by left censored values.[55] Therefore, only summary statistics that were not influenced by values below LOQ were given; otherwise "not applicable" (NA) was reported.

To assess gender differences we applied the Mann–Whitney U-test. Chronological trends in paraben exposure were investigated by applying the Jonckheere–Terpstra test. The statistical analysis of association and time trends was performed for MeP, EtP, *n*-PrP and *n*-BuP. For the other analytes, detection rates were considered to be insufficient for statistical evaluation.

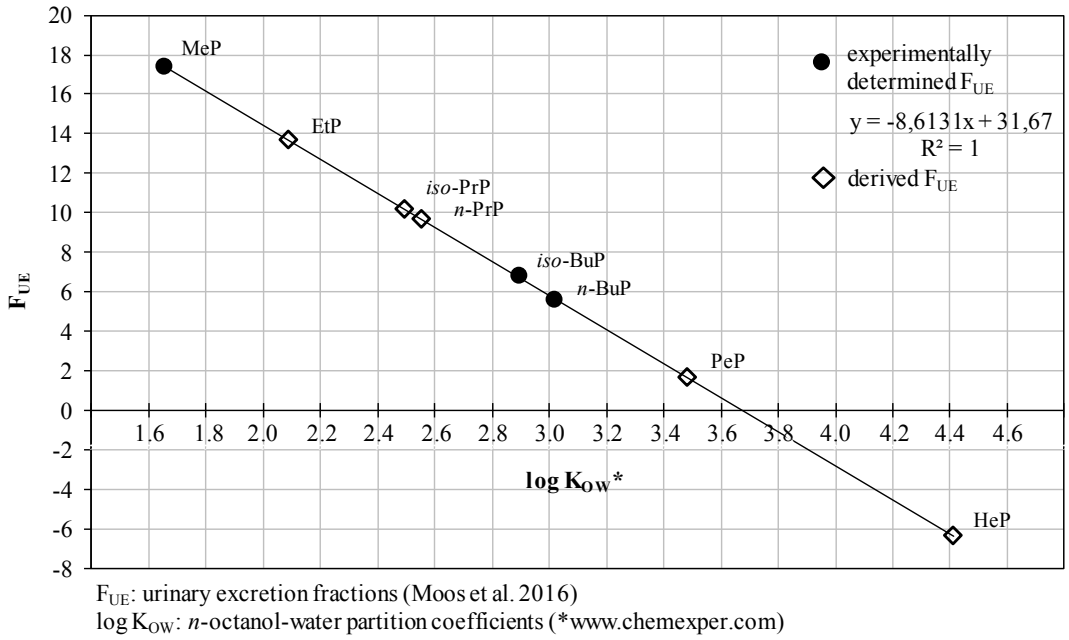
Based upon the urinary paraben level (total paraben determined after enzymatic hydrolysis) we calculated daily paraben intakes (DIs) separately for each individual.

$$\text{DI [µg/kg bw/day]} = \frac{\text{uc [µg/L]} \cdot \text{uv}_{24h} \text{ [L/day]}}{F_{ue} \cdot \text{bw [kg]}}$$

The paraben intake results from the product of the urinary concentration (uc) of the paraben and the 24 h urine volume (uv_{24h}) for each individual, divided by the product of the fraction excreted in urine (F_{ue}) and the body weight (bw). The F_{ue} describes the amount of paraben excreted via urine within 48 h after oral application relative to the applied dose. Whereas DI calculation via parent compounds can be performed directly, extrapolation based on metabolites requires a molar correction (not performed in this study). In our previous study we have determined F_{ue} values for three parabens (MeP, *iso*-BuP and *n*-BuP) in humans after oral dosage of isotope-labeled parabens.[44] The urinary excretion factors for the other parabens were derived by linear regression of log *n*-octanol/water partition coefficients (log K_{OW}) against the F_{ue}.

Results and Discussion

Based upon the experimentally determined oral F_{ue}s of MeP, *iso*-BuP and *n*-BuP,[44] we mathematically derived F_{ue} values for the remaining parabens (EtP, *iso*-PrP, *n*-PrP, PeP and HeP) used as biomarkers of paraben exposure in the study of Moos et al.[19] and other biomonitoring studies. According to Moos et al.,[44] the share of paraben excreted in urine decreases with increasing length of the alkyl side chain. Conversely, the log K_{OW} increases with increasing length of the alkyl chain (increasing molecular weight).[56, 57] We obtained log K_{OW} values for all parabens from chemexper (www.chemexper.com) and derived unknown F_{ue}s by linear regression of log K_{OW} against the known, experimentally determined F_{ue}s (see **Figure 1**).



F_{UE}: urinary excretion fractions (Moos et al. 2016)
log K_{OW}: *n*-octanol-water partition coefficients (*www.chemexper.com)

Figure 1 F_{ue}s derived by linear regression of log K_{OW}s against the known F_{ue}s of MeP, *iso*-BuP and *n*-BuP. The experimentally determined F_{ue}s are depicted with black markers, and the derived F_{ue}s for the other parabens are depicted in white.

We thus calculated a F_{ue} of 13.7% for EtP, followed by 10.2% for *iso*-PrP, 9.7% for *n*-PrP, 1.7% for PeP and −6.3% for HeP. All empirically derived and calculated F_{ue}s are given in **Table 1**. We chose to omit calculation of a F_{ue} for BeP because of structural differences of this paraben compared with the other parabens (aryl ester vs alkyl ester) presuming considerable differences also in regard to metabolism (metabolite profile) and elimination kinetics.

Table 1 Log *n*-octanol/water partition coefficient (log K_{OW}), molecular weight and urinary excretion factors (F_{ue}) after oral exposure for all relevant aliphatic parabens.

	Log *n*-octanol/water partition coefficient[a] (log K_{OW})	Molecular weight (g/mol)	urinary excretion factors (F_{ue})
MeP	1.656	152.15	17.4[b]
EtP	2.091	166.18	13.7
iso-PrP	2.495	180.20	10.2
n-PrP	2.556	180.20	9.7
iso-BuP	2.896	194.23	6.8[b]
n-BuP	3.019	194.23	5.6[b]
PeP	3.484	208.26	1.7
HeP	4.412	236.31	-6.3

[a]www.chemexper.com; [b]Moos et al. 2016

Interestingly, the calculated F_{ue} for HeP resulted in a negative value, implying that HeP is not excreted in its unchanged or conjugated form in urine, suggesting that further modification steps (e.g., oxidation of the alkyl side chain) are required to facilitate excretion in urine. This result is supported by the fact that neither HeP (with a negative F_{ue}) nor PeP (with a very small F_{ue}) has been detected in any of the investigated samples (neither in the 660 samples of the ESB nor in any other of our studies). Nevertheless, it must be mentioned that PeP and HeP are neither approved in food nor in cosmetics in the European Union.

The calculation of DIs (in µg/kg bw/day) was performed based upon the µg/l concentrations of the respective paraben according to Moos et al.,[19] the 24 h volume of the urine sample, the body weight of each volunteer and the F_{ue}s from oral exposure. Results for the DIs calculated from the analytical data of 656 (for 4 individuals, the DI could not be calculated because body weights were missing in the data set) samples of the ESB are presented in **Table 2**, for each sampling year, as the total of all sampling years and separately for males and females. As pointed out above, neither PeP and HeP (not detected in any of the samples investigated) nor BeP (with no reliable F_{ue} but also with a very low detection rate of 1.4%) have been included in the DI calculations.

We determined highest DI values for MeP with an overall median of 5.8 µg/kg bw/day and a 95th percentile of 47.5 µg/kg bw/day. In individual samples we observed maximum values up to 160 µg/kg bw/day. The overall median DIs for the other parabens were considerably lower, with 1.2 µg/kg bw/day (95th percentile of 20.6 µg/kg bw/day) for *n*-PrP, followed by 0.4 µg/kg bw/day (95th percentile of 7.4 µg/kg bw/day) for EtP and 0.2 µg/kg bw/day (95th percentile of 4.6 µg/kg bw/day) for *n*-BuP. For the isoforms of PrP and BuP, we generally found lower DIs; maximum DIs were 13.4 µg/kg bw/day for *iso*-BuP and 32.3 µg/kg bw/day for *iso*-PrP.

Table 2 Daily intakes (µg/kg bw/day) determined in 656 24 h urine samples.

Year	MeP			EtP			*iso*-PrP			*n*-PrP			*iso*-BuP			*n*-BuP		
	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max
	µg/kg bw/day																	
1995	5.2	44.1	76.0	0.5	11.8	22.3	N.A.	N.A.	N.A.	1.3	19.7	58.9	N.A.	0.3	1.3	0.1	2.5	6.2
1997	5.7	45.9	118	0.3	6.7	8.4	N.A.	N.A.	N.A.	0.8	25.5	40.6	N.A.	0.9	2.1	0.1	3.5	9.9
1999	3.2	32.4	55.2	0.3	4.9	15.4	N.A.	N.A.	N.A.	0.6	15.1	27.7	N.A.	0.8	2.1	0.1	4.2	15.2
2001	5.1	26.9	78.7	0.7	4.6	13.1	N.A.	N.A.	2.5	1.3	9.8	21.4	N.A.	1.6	2.4	0.3	5.6	7.9
2003	3.8	49.9	84.6	0.2	4.5	8.8	N.A.	N.A.	4.0	0.8	17.5	26.8	N.A.	1.3	2.9	0.2	3.2	4.5
2005	4.8	31.6	56.1	0.3	4.4	7.4	N.A.	N.A.	32.3	1.3	16.4	53.5	N.A.	1.5	4.4	0.2	4.5	10.1
2006	6.9	48.2	102	0.3	5.5	10.3	N.A.	N.A.	0.9	1.5	23.2	44.4	N.A.	0.8	2.8	0.2	4.9	7.2
2007	9.3	56.5	146	0.5	8.0	27.2	N.A.	N.A.	7.4	2.4	31.6	60.3	N.A.	1.2	2.3	0.2	5.5	18.1
2008	9.8	43.9	122	0.5	5.6	10.1	N.A.	N.A.	1.8	1.6	16.4	53.4	N.A.	0.7	1.2	0.3	3.4	16.8
2009	5.4	52.7	94.3	0.4	8.0	24.4	N.A.	0.9	13.8	1.5	21.2	46.2	N.A.	2.9	13.4	0.2	7.6	16.8
2012	8.6	43.2	160	0.2	17.4	44.0	N.A.	N.A.	0.4	0.7	14.9	41.9	N.A.	N.A.	2.2	0.2	5.9	14.0
total	5.8	47.5	160	0.4	7.4	44.0	N.A.	N.A.	32.3	1.2	20.6	60.3	N.A.	1.1	13.4	0.2	4.6	18.1
male	3.0	31.7	118	0.2	3.4	13.1	N.A.	N.A.	4.8	0.3	11.9	34.3	N.A.	0.6	13.4	0.1	2.2	16.8
female	9.0	52.7	160	0.7	10.1	44.0	N.A.	N.A.	32.3	2.5	26.9	60.3	N.A.	1.2	9.1	0.5	7.0	18.1

P.50: 50th percentile; P.95: 95th percentile; N.A.: not applicable.

Figure 2 shows boxplots of the DI values (in µg/kg bw/day) of the parabens separately for men, women and total, summarized over all years. Regarding gender, women had significantly (P<0.0001) higher DIs compared with men. On average for women, median DIs of MeP and EtP were approximately threefold higher, for *n*-PrP eightfold higher and for *n*-BuP fivefold higher compared with men (see **Table 3**). Similar differences with higher paraben levels in women were also found in our previous study[19] and are probably caused by women using personal care products preserved with parabens more frequently than men.[58]

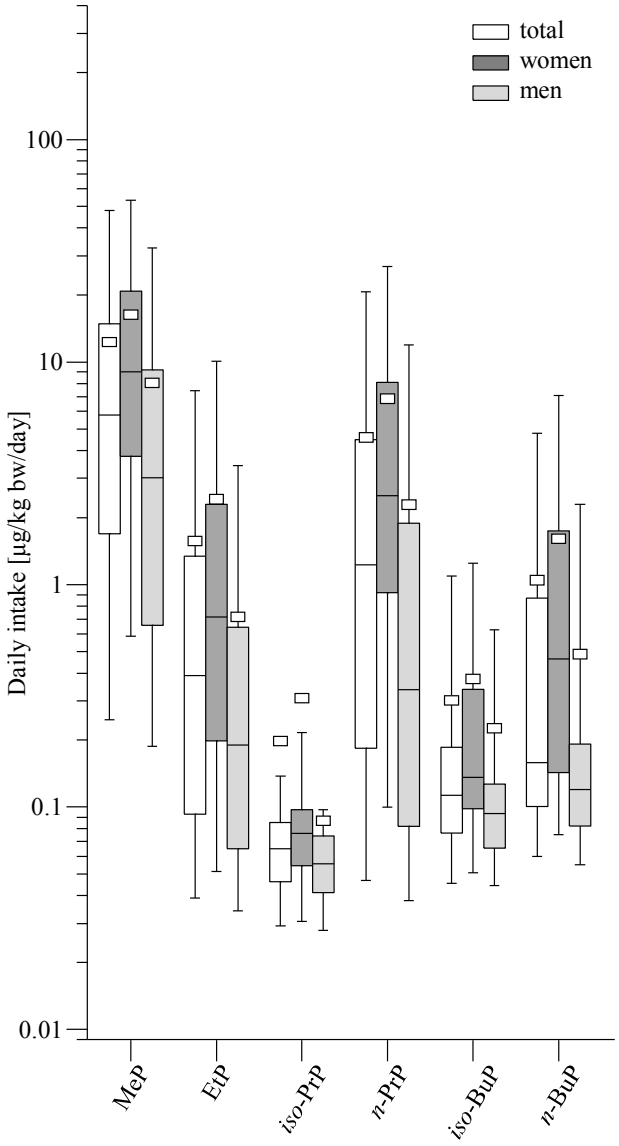


Figure 2 Boxplots of daily intakes (µg/kg bw/day) with 656 volunteers. For each paraben, data are shown separately for females, males and total. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile.

Table 3 Daily intakes depicted separately by men and women (P.50, P.95 and range values in µg/kg bw/day).

Year	MeP			EtP			*iso*-PrP			*n*-PrP			*iso*-BuP			*n*-BuP		
	P.50	P.95	range	P.50	P.95	range	P.50	P.95	range	P.50	P.95	range	P.50	P.95	range	P.50	P.95	range
									µg/kg bw/day									
female																		
1995	5.3	55.0	0.3-76.0	0.6	13.4	N.A.-22.3	N.A.	N.A.	N.A.	2.1	41.5	N.A.-58.9	N.A.	0.5	N.A.-0.6	0.1	3.6	<0.1-6.2
1997	6.8	47.0	0.6-50.5	0.6	6.7	N.A.-7.9	N.A.	N.A.	N.A.	2.3	31.9	N.A.-40.6	N.A.	0.9	N.A.-1.0	0.5	6.0	<0.1-9.9
1999	6.2	48.4	0.3-55.2	0.8	7.5	N.A.-15.4	N.A.	N.A.	N.A.	1.8	17.1	N.A.-27.7	N.A.	1.0	N.A.-2.1	0.6	8.8	<0.1-15.2
2001	9.2	32.5	0.4-78.7	1.1	3.9	N.A.-6.9	N.A.	N.A.	N.A.-2.5	2.9	18.2	0.1-21.4	N.A.	1.9	N.A.-2.4	0.8	6.9	<0.1-7.9
2003	8.0	41.0	0.4-53.5	0.9	5.4	N.A.-8.8	N.A.	N.A.	N.A.	2.6	18.7	N.A.-26.8	N.A.	1.1	N.A.-1.6	0.2	3.6	0.1-4.4
2005	9.0	40.1	0.2-56.1	0.4	6.1	0.1-7.4	N.A.	N.A.	N.A.-32.3	2.8	17.8	N.A.-53.5	N.A.	2.4	N.A.-4.4	0.3	6.1	<0.1-10.1
2006	13.7	46.7	0.3-91.5	0.6	6.6	N.A.-10.0	N.A.	0.6	N.A.-0.9	7.5	25.2	N.A.-44.4	N.A.	0.8	N.A.-2.6	0.6	5.0	0.1-7.1
2007	10.2	62.7	0.2-146	0.5	13.8	N.A.-27.2	N.A.	N.A.	N.A.-7.4	2.5	27.9	N.A.-60.3	N.A.	1.2	N.A.-2.3	0.4	10.4	0.1-18.1
2008	11.2	49.5	0.6-122	1.3	8.2	N.A.-10.1	N.A.	N.A.	N.A.-1.8	2.9	26.8	N.A.-53.4	N.A.	1.0	N.A.-1.2	0.8	9.0	0.1-16.8
2009	12.4	75.1	0.3-94.3	0.7	12.1	N.A.-24.4	N.A.	4.9	N.A.-13.8	2.6	35.3	0.2-46.2	N.A.	4.1	N.A.-9.1	0.6	8.7	0.1-16.5
2012	9.7	43.5	1.0-160	1.3	19.8	N.A.-44.0	N.A.	N.A.	N.A.-0.4	2.7	21.0	N.A.-41.9	N.A.	N.A.	N.A.-2.2	0.2	10.1	0.1-14.0
total	9.0	52.7	0.2-160	0.7	10.1	N.A.-44.0	N.A.	N.A.	N.A.-32.3	2.5	26.8	N.A.-60.3	N.A.	1.2	N.A.-9.1	0.5	7.0	<0.1-18.1
male																		
1995	2.5	22.6	0.1 – 43.6	0.3	2.9	N.A.- 4.0	N.A.	N.A.	N.A.	0.3	9.5	N.A.-16.0	N.A.	N.A.	N.A.- 1.3	0.1	0.4	<0.1 - 4.1
1997	1.4	22.1	0.2 – 118	0.2	3.9	N.A.- 8.4	N.A.	N.A.	N.A.	0.3	18.4	N.A.-22.0	N.A.	N.A.	N.A.- 2.1	0.1	0.5	<0.1 – 5.8
1999	1.0	8.3	0.1 – 18.4	0.2	1.2	N.A.- 1.6	N.A.	N.A.	N.A.	N.A.	1.9	<0.1 – 2.3	N.A.	N.A.	N.A.- 0.5	0.1	0.9	<0.1 – 1.5
2001	3.3	14.0	0.5 – 17.8	0.3	3.2	N.A.- 13.1	N.A.	N.A.	N.A.	0.3	3.6	N.A.- 7.6	N.A.	1.1	N.A.- 1.9	0.1	3.2	<0.1 – 5.6
2003	2.2	49.7	0.2 – 84.6	N.A.	3.2	N.A.- 4.5	N.A.	N.A.	N.A.- 4.0	0.3	10.8	N.A.- 21.7	N.A.	1.1	N.A.- 2.9	0.1	1.9	<0.1 – 2.7
2005	2.3	26.4	0.1 – 41.3	N.A.	1.0	N.A.- 4.2	N.A.	N.A.	N.A.- 4.8	0.5	9.8	N.A.- 18.6	N.A.	N.A.	N.A.- 1.5	0.1	0.6	0.1 – 3.1
2006	2.6	40.1	0.1 – 102	N.A.	3.9	N.A.- 10.3	N.A.	N.A.	N.A.	0.3	16.2	N.A.- 25.7	N.A.	0.7	N.A.- 2.8	0.1	3.8	0.1 – 7.2
2007	8.8	47.0	0.1 – 56.4	0.6	7.0	N.A.- 7.9	N.A.	N.A.	N.A.	1.9	26.9	N.A.- 34.3	N.A.	0.9	N.A.- 2.0	0.2	2.7	0.1 – 5.4
2008	4.7	34.3	N.A. –70.7	0.2	2.0	N.A.-2.9	N.A.	N.A.	N.A.	0.7	11.1	N.A.- 17.5	N.A.	0.5	N.A.- 0.6	0.1	1.4	0.1 – 1.7
2009	4.0	26.0	0.1 – 41.5	0.2	3.1	N.A.- 4.5	N.A.	N.A.	N.A.	0.2	9.8	N.A.- 19.3	N.A.	1.4	N.A.-13.4	0.2	6.2	0.1 – 16.8
2012	6.3	21.0	N.A. –50.9	N.A.	1.5	N.A.- 2.2	N.A.	N.A.	N.A.- 0.3	N.A.	6.5	N.A.- 8.6	N.A.	N.A.	N.A.	0.1	0.9	0.1 – 1.1
total	3.0	31.7	N.A. –118	0.2	3.4	N.A.- 13.1	N.A.	N.A.	N.A.- 4.8	0.3	11.9	N.A.- 34.3	N.A.	0.6	N.A.-13.4	0.1	2.2	<0.1 – 16.8

P.50: 50th percentile; P.95: 95th percentile; N.A.: not applicable.

Furthermore, we performed a chronological trend analysis for daily paraben intake over the past decades. For nearly all parabens, we found rather constant DI values over the years. Only for MeP we found a significant ($P<0.0001$) increase in DI values. The median DI from 2012 (8.6 µg/kg bw/day) was distinctly higher than the median from 1995 (5.2 µg/kg bw/day).

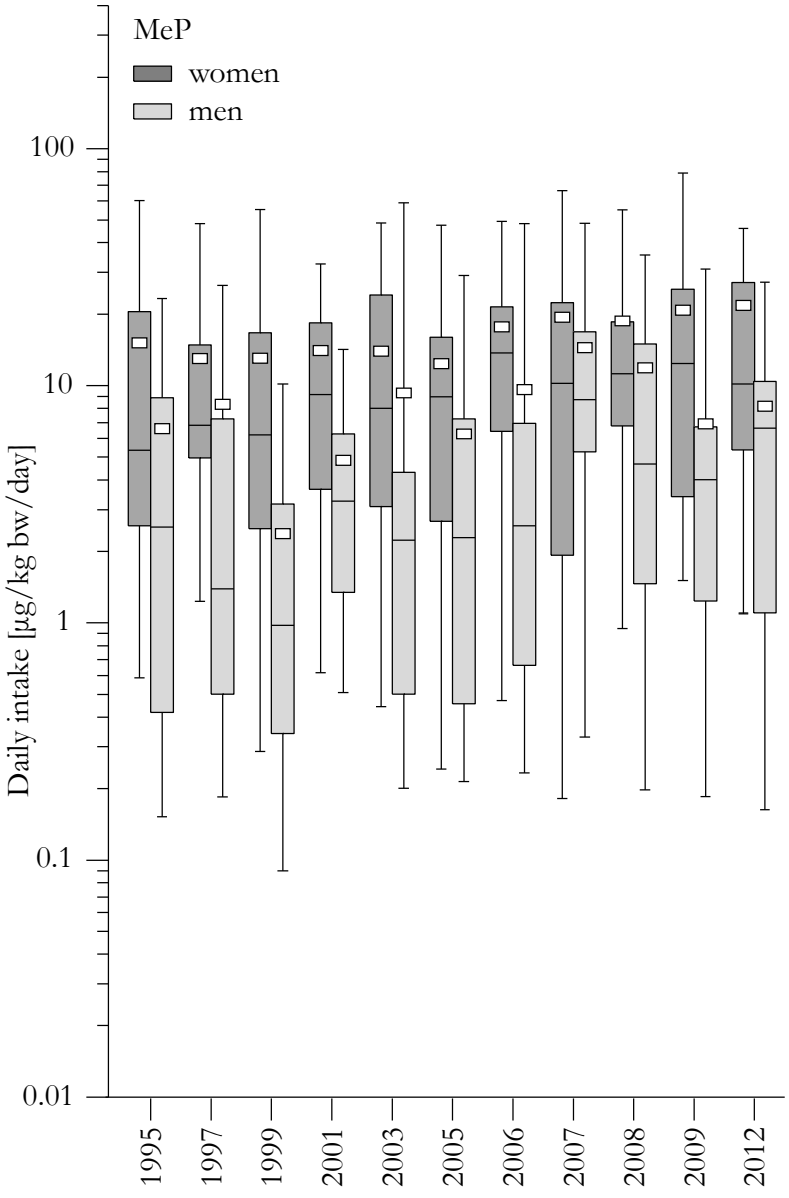


Figure 3 Chronological presentation of the daily intake (µg/kg bw/day) of MeP between 1995 and 2012 separately for men and women. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile.

Further data analysis revealed that this trend is mainly driven by the male subpopulation. The median DI for MeP was more than two times higher in 2012 (6.3 µg/kg bw/day) compared with 1995 (2.5 µg/kg bw/day). Suggestive increases for MeP in women (from 5.3 µg/kg bw/day in 1995 to 9.7 µg/kg bw/day in 2012) were of no statistical significance. The development of the MeP intake over the years, separately for males and females, is illustrated in **Figure 3**. Figures for the other parabens are presented in the **Supplementary Figure 1** (**Appendix IV**).

We can compare the DIs derived in our study with estimated DIs derived by other studies (**Table 4**). Available studies on daily paraben intakes can be categorized in dermal exposure models,[38] external source (dust, foodstuff, personal care product use) extrapolations[59-62] and DI calculations using human biomonitoring data.[3,4,63-65] External exposure models clearly identified personal care product use as the predominant source of paraben exposure, with food or dust only as subordinate sources. Of note, calculated DIs in our HBM-based approach agree rather well (within the same order of magnitude) with personal care product use-derived DI models, both at median and maximum levels. For example, for females and personal care product use, Guo et al.[62] calculated for MeP a median intake of 22.5 and a maximum intake of 150 µg/kg bw/day, whereas we derived a median intake of 9.0 and a maximum intake of 160 µg/kg bw/day. For *n*-PrP they calculated a median intake of 8.4 and a maximum intake of 105 µg/kg bw/day, whereas we derived a median intake of 2.5 and a maximum intake of 60.3 µg/kg bw/day. Interestingly, these intakes are above the intake of 43 µg/kg bw/day estimated by worst case dermal exposure calculations.[38]

Table 4 Daily intakes from our study compared with estimated daily intakes (dermal exposure models, external source extrapolations and calculations using human biomonitoring data) from other studies (P.50, P.95 and maximum DI in µg/kg bw/day).

				MeP			EtP			*iso*-PrP			*n*-PrP			*iso*-BuP			*n*-BuP		
				P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max	P.50	P.95	Max
		n	sampling year	µg/kg bw/day																	
dermal exposure calculation																					
SCCS			2011												43.0						43.0
personal care products exposure																					
US[62]	female (leave-on)	170	2012	22.5		150			43.8				8.4		105						46.7
	infants	170	2012	0.08		766			94.9				0.11		231				0.004		0.02
dietary exposure																					
China[59]	male	282	2012	0.70*	1.49		0.17*	0.89					0.12*	0.43					0.02*	0.09	
	female	282	2012	0.73*	1.56		0.17*	0.92					0.13*	0.45					0.02*	0.09	
US[60]	children	267	2008-2011	0.23*	0.58		0.16*	0.46					0.08*	0.21					0.001*	0.003	
	adults	267	2008-2011	0.13*	0.36		0.14*	0.40					0.04*	0.10					0.001*	0.001	
indoor dust exposure																					
East Asian	children	118	2010-2012	0.0039*	0.0193		0.0002*	0.0034					0.0018*	0.0079					0.0001*	0.0005	
Countries[61]°	adults	118	2010-2012	0.0009*	0.0042		<0.0001*	0.0007					0.0004*	0.0017					<0.0001*	0.0001	
US[61]	children	40	2006-2010	0.0014*	0.0063		<0.0001*	0.0001					0.0015*	0.0074					<0.0001*	<0.0001	
	adults	40	2006-2010	0.0002*	0.001		<0.0001*	<0.0001					0.0002*	0.0012					<0.0001*	<0.0001	
HMB data																					
China[3]	female	41	2010	15.5	242		2.5	74.9					10.7	125							
	male	69	2010	5.2	154		1.8	84.2					3.0	125							
Greece[63]	mothers	239	2007-2008	500	17076	388042	13.2	600	2388		4.0	281	73.3	3819	162106		8.1	183.5		82.6	781
	children	239	2007-2008	66.6	3675	26527	5.8	375	4353			42.0	3.4	435	5813		0.2	2.6		2.9	217
Greece[64]	children	500	2007-2008	25.8	590	8617	2.0	57.2	706			33.5	1.9	76.7	1082		0.3	113		1.5	218
US[65]	adults	100	2003-2005		19.9			1.4						8.2						0.9	
Germany	**female**	**330**	**1995-2012**	**9.0**	**52.7**	**160**	**0.7**	**10.1**	**44.0**			**32.3**	**2.5**	**26.8**	**60.3**		**1.2**	**9.1**	**0.5**	**7.0**	**18.1**
(this study)	**male**	**330**	**1995-2012**	**3.0**	**31.7**	**118**	**0.2**	**3.4**	**13.1**			**4.8**	**0.3**	**11.9**	**34.3**		**0.6**	**13.4**	**0.1**	**2.2**	**16.8**
	adults	**660**	**1995-2012**	**5.8**	**47.5**	**160**	**0.4**	**7.4**	**44.0**			**32.3**	**1.2**	**20.6**	**60.3**		**1.1**	**13.4**	**0.2**	**4.6**	**18.1**

°highest daily intake of the three countries; *mean; P.50: 50th percentile; P.95: 95th percentile; n: number of samples

For the comparison of our HBM-derived DIs with other HBM-derived DI data it has to be kept in mind that our calculations are based upon 24 h urine samples and robust human Fues from oral exposure experiments. Previous HBM-based DI estimates were based upon spot urine samples and unknown and therefore estimated Fues. In some cases dose extrapolations were also based upon metabolites that are not entirely specific to individual parabens or parabens in general.[3,63,64] In addition to the calculation model, differences in personal care product use habits, product compositions, demographic properties and study design might also explain why estimated DIs differ considerably between these studies. DI values for Greece[63,64] are by far the highest, surpassing DIs of other studies by several orders of magnitude. Compared with DI estimates reported for the Chinese population,[3] intakes of our study were a factor of 3 to 10 lower (depending on paraben and percentile), and compared with DI estimates for the US population,[65] intakes of our study were a factor of 2 to 5 higher.

DIs calculated in this study have to be regarded as oral equivalent DI doses because extrapolations from urinary metabolite levels are based upon metabolite F_{ue}s derived after oral exposure.[44] Moos et al.[44] have recovered between 81% and 86% of the orally applied dose of the parabens MeP, *iso*-BuP and *n*-BuP in urine via authentic standards (between 5.6% and 17% as unchanged parabens). Thus, we can assume that the major share of the orally applied paraben dose is resorbed systemically. Some minor share of the oral dose might be excreted via urine as additional (unknown) metabolites, or via other routes of excretion (e.g., feces). Janjua et al.[66] have found that 0.32% of *n*-BuP dermally applied to male human volunteers (cream containing 2% *n*-BuP) was excreted as *n*-BuP in urine. Compared with 5.6% of renally excreted *n*-BuP from the oral study by Moos et al.,[44] this would indicate that only 5% of the dermally applied amount is systemically absorbed. However, this rough comparison has several caveats as both metabolism (share of different metabolites) and distribution/elimination kinetics could differ to some extent between oral and dermal (or inhalation) exposure. In addition, some portion of dermally applied paraben in Janjua et al.[66] may have volatilized before being absorbed or lost by absorption by clothing or by rinsing off. Interestingly, Mathews et al.[67] reported for rats that ~80% of the oral radiolabeled *n*-BuP dose was excreted in urine, whereas 2.2% to 46% of the dermally applied dose (depending upon dose and timing) was excreted in urine, and this is in good accordance with both Moos et al.[44] and Janjua et al.[66] As pointed out above, real-life exposure to parabens encompasses multiple routes of exposure, with dermal exposure probably playing a dominant role.

Urinary metabolite levels measured in this study represent an integral measure of all possible exposure pathways. In terms of systemically absorbed (total) daily doses (in µg/kg bw/day) we are confident that DI extrapolations based upon orally derived metabolite conversion factors do present a good and robust approximation. However, differentiating between the different routes of exposure is not possible.

We can compare the oral equivalent daily paraben intakes calculated in our study with exposure limit values deduced by the Joint Expert Committee on Food Additives of the Food and Agriculture Organization of the United Nations and the World Health Organization (JECFA)[68] and the European Food Safety Authority (EFSA).[69] These authorities derived an ADI of 10 mg/kg bw/day for the sum of MeP and EtP, based upon the no-observed-adverse-effect levels (NOAELs) of 1000 mg/kg bw/day for each compound in long-term toxicity studies and studies on sex hormones and the male reproductive organs in juvenile rats.[69,70] The maximum DI of MeP+EtP (204 µg/kg bw/day; 160 µg/kg bw/day for MeP and 44 µg/kg bw/day for EtP) from our study is considerably below this ADI (factor 50). For the other parabens no official health-based threshold values have been derived so far.

For a cumulative risk assessment of exposure to all parabens we chose the hazard index (HI) defined as the sum of the hazard quotients (HQs). The HQ for each paraben is calculated as the ratio of exposure (e.g., the DI of a substance) to the dose of no concern.[50,71,72] Thus, an HQ (or HI) of >1 indicates that exposure exceeds the acceptable exposure for an individual paraben (or the mixture of parabens). In practical applications of the HI approach, regulatory health-based threshold values (e.g., the ADI, TDI or RfD) have been used as the denominator in HQs. However, as pointed out above, with the exception of MeP and EtP, no official health-based threshold values have been derived for parabens. For the parabens, with no official health-based threshold value, we used a conservative NOEL of 2 mg/kg bw/day for *n*-BuP,[38] based upon reproductive toxicity studies in rodents,[41] as a point of departure and combined it with an uncertainty factor (UF) of 100 to obtain input values analogous to the TDI or RfD (20 µg/kg bw/day) for the HQ/HI approach.[73,74] We consider this derivation a valuable approach as the end points can be regarded as of comparable outcome, namely endocrine disrupting properties, and therefore justify use for a cumulative risk evaluation. However, it has to be noted that these parabens (*iso*-PrP, *n*-PrP, *iso*-BuP and *n*-BuP) differ in their estrogenic activity[75] and using the same NOEL might be overly conservative.

The fact that administration of *n*-PrP to juvenile male rats for 4 weeks resulted in a reduction of the daily sperm production in the testes at the lowest dose level of 10 mg/kg body weight/day (lowest observed adverse effect level (LOAEL)) reinforces the use of a relatively low NOEL for the calculation of the HQ for n-PrP.[31,69]

For MeP and EtP we used the group ADI of 10 mg/kg bw/day for the calculation of the HQ. The HI was calculated for each individual participant as the sum of the respective HQs of each individual paraben. Calculated HQs and the cumulative HI for our study population are depicted in **Table 5**.

Table 5 Hazard quotients (HQs) and hazard indices (HIs) based upon EFSA group ADI for the sum of MeP and EtP and a derived threshold for *iso*-PrP, *n*-PrP, *iso*-BuP and *n*-BuP based upon NOEL of 2 mg/kg bw/day for *n*-BuP derived by SCCS[37] based upon Fisher et al.[41].

	MeP + EtP	*iso*-PrP	*n*-PrP	*iso*-BuP	*n*-BuP	HI
	group ADI: 10 mg/kg bw/day	derived benchmark[a]: 0.02 mg/kg bw/day				
P.50	0.001	< 0.001	0.060	< 0.001	< 0.001	0.093
P.95	0.005	< 0.001	1.027	0.052	0.230	1.304
Max	0.020	1.616	3.015	0.671	0.906	4.368
n > 1 [%]	0	0.2	5.2	0	0	8.4

P.50: 50th percentile; P.95: 95th percentile. [a]NOEL of 2 mg/kg bw/day derived by SCCS[37] based upon Fisher et al.[41]

The HQ for the sum of MeP and EtP of 0.020 at the maximum DI observed in our study (204 µg/kg bw/day for MeP+EtP) is considerably below 1 (2% of the ADI) and thus far below exposure levels that could be associated with an attributable risk. At the 95th percentile, daily MeP/EtP intakes represented only 0.5% and at the median only 0.1% of the ADI. Data broken up for MeP and EtP in addition to their sum are provided in the **Supplementary Table 1 (Appendix IV)**. However, contrary to MeP and EtP, we calculated HQ values of >1 for some of the other parabens. We calculated highest HQ values for *n*-PrP, with peak values of up to 3. Approximately 5% (n=34) of the 656 individuals of our study exceeded the HQ of 1. In addition to *n*-PrP we observed one HQ value of >1 (1.6) for *iso*-PrP. For *n*- and *iso*-BuP we did not observe HQ values of >1, with maximum HQ values of 0.9 for *n*-BuP and 0.7 for *iso*-BuP. For the HI, the sum of the respective HQs of each individual, we calculated a median HI of 0.1, but an HI at the 95th percentile of 1.3 and a maximum HI of 4.4. 55 individuals (8.4%) of the study population exceeded an HI of 1. Women accounted for 90% of these individuals with an HI of >1 (7.6% of the total study population).

n-PrP clearly is the most influential contributor to the HI, followed by the other longer chain parabens. At the 95th percentile the contribution of *n*-BuP to the HI is ~20%, and the contribution of *iso*-BuP is ~5%. The short-chain parabens (MeP and EtP) only play a subordinate role in the cumulative HI of the parabens. With regard to the years of the study (1995–2012), governed by rather steady exposure levels to the longer chain parabens,[19] HI values of >1 were rather evenly distributed over the years.

Conclusions

To our knowledge, this is the first study that provides daily paraben intakes based upon specific urinary paraben biomarkers in combination with robust human urinary metabolite conversion factors (F_{ue}s). Because the Fues were derived after oral exposure, intakes estimated in this study have to be regarded as oral equivalent DI doses representing a combined measure of exposures from all sources and routes of exposure. For several parabens it has to be assumed that dermal exposure can be a major contributor to total exposure. In our study population (as in most other studies) MeP remains the paraben with highest DIs (95th percentile: 47.5 µg/kg bw/day). However, other longer alkyl chain parabens such as *n*-PrP and *n*-BuP follow with DIs (95th percentile: 20.6 and 4.6 µg/kg bw/day) much closer than suggested by simple comparison of urinary metabolite levels. This effect is because of urinary metabolite conversion factors that are considerably smaller for the longer chain parabens (*n*-PrP, *iso*-BuP and *n*-BuP), than for the short-chain parabens (MeP and EtP). Over the years of the study (1995–2012) DIs are constant for all parabens (with the exception of rising intakes for MeP) and women generally have higher DIs than men (3–8-fold higher).

Our risk assessment by means of the HQ/HI approach revealed that exposures to MeP and EtP (even at maximum DIs) are far below levels that might pose a health risk. However, we were surprised to observe HQ >1 for *n*-PrP and *iso*-PrP, and HQs close to 1 for *iso*-BuP and *n*-BuP. In consequence, we determined a maximum HI value (for the sum of all parabens) of 4.4 and found 8.4% of the study population exceeding the HI of 1. From risk assessment perspective this observation would mean that 8.4% of the study population (predominately women) were concurrently exposed to parabens at levels at which the health risk can no longer be excluded.

As a caveat in these calculations we have to point out that because of the lack of existing health threshold values we chose the lowest and therefore most conservative point of departure of 2 mg/kg bw/day for *n*-BuP for all parabens (except for MeP and EtP) as the basis for our calculations. However, it must be stated that the NOEL used in our conservative approach is not that far from the LOAEL of 10 mg/kg bw/day for *n*-PrP, wherein toxicological effects could be observed, especially as *n*-PrP had the greatest impact on our HI calculation. New, more refined toxicological studies might come up with health threshold values that are higher than our tentative threshold value or with threshold values that differ between the parabens. In addition, we based the HI calculations on the assumption of a common mode of action of endocrine disruption and on the assumption of dose additivity. This clearly represents a precautionary approach (relying on the inherent endocrine properties of the parabens included) that might be confirmed or rebutted in future toxicological and epidemiological studies.

As a second caveat, we have to point out that the most recent samples in our study have been collected in 2012. As mentioned before, in 2014 the maximum permissible concentration of n-PrP and n-BuP in cosmetic products in Europe was reduced to 0.14% (sum of *n*-PrP and *n*-BuP) and the parabens *iso*-PrP and *iso*-BuP (together with BeP and PeP) were completely banned for use in cosmetic products. With personal care products playing the most important role in exposure to parabens, these regulations will most likely have an effect on overall paraben exposure and paraben intakes of the general population and users of respective products. It remains to be seen when and to what extent these regulatory measures lead to a reduction of exposure and if the reduction of exposure is sufficient to consistently reach HI values below 1 in the general population.

Conflict of interest

The authors declare no conflict of interest.

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Zusammenfassung und Ausblick

Der umfangreiche Einsatz von Parabenen als Konservierungsstoffe in kosmetischen Mitteln, Konsumgütern, pharmazeutischen Produkten, sowie in bestimmten Lebensmitteln wird aufgrund ihrer potenziell endokrinen Wirkung derzeit kontrovers diskutiert. Während kurzkettige Parabene als relativ sicher in ihrer Verwendung gelten, liegt der besondere Fokus bei der toxikologischen Betrachtung auf den längerkettigen Parabenen. Die Belastung gegenüber diesen Stoffen ist ein weltweit zu beobachtendes Phänomen. HBM-Studien haben gezeigt, dass die Allgemeinbevölkerung, inklusive Kindern, diesen Stoffen permanent ausgesetzt ist.

Die in der vorliegenden Arbeit entwickelten analytischen Methoden und die dargestellten Ergebnisse geben wertvolle Informationen über das Ausmaß der Belastung gegenüber Parabenen und können neben direkten Expositions-Risiko-Extrapolationen auch in epidemiologischen Studien eingesetzt werden, um einen Zusammenhang zwischen Exposition und daraus resultierenden Auswirkungen auf die menschliche Gesundheit zu untersuchen. Erstmals wurden im Rahmen von HBM-Studien die Belastungen gegenüber Parabenen der deutschen Allgemeinbevölkerung gemessen. Die Ergebnisse zeigten eine ubiquitäre und über die Jahre überwiegend konstante Belastung gegenüber diesen Stoffen. Basierend auf den Ergebnissen der vorliegenden Arbeit konnten erstmals für Deutschland von der Kommission Human-Biomonitoring des Umweltbundesamtes vorläufige Referenzwerte für fünf Parabene, getrennt nach Frauen und Männern, abgleitet werden.

Darüber hinaus wurde in dieser Arbeit der Human-Metabolismus von Parabenen nach oraler Dosierung untersucht. Die erhaltenen metabolischen Konversionsfaktoren der einzelnen Parabene ermöglichen eine verlässliche Hochrechnung von den in Urin gefundenen Konzentrationen auf die tatsächlich aufgenommene Menge. Die Ergebnisse können in Zukunft zur Expositions- und Risikoabschätzung im arbeits- und umweltmedizinischen Bereich herangezogen werden. Neben den unmodifizieren Parabenen als klassischen Biomarkern konnten neue, spezifische Metaboliten identifiziert werden. Die Tatsache, dass diese Metaboliten in ausreichend hohen Konzentrationen im Urin vorliegen und sie Kontaminationen gegenüber nicht anfällig sind, machen sie zu wichtigen Biomarkern für zukünftige HBM-Studien.

Weiter wurden in dieser Arbeit erstmals, basierend auf den ermittelten menschlichen Konversionsfaktoren, verlässliche Daily Intakes für Parabene berechnet. Anhand der ermittelten Daily Intakes wurde eine kumulative Risikobewertung (Hazard Quotient/Hazard Index) der Exposition gegenüber Parabenen durchgeführt. Die Berechnung ergab, dass 8,4% der untersuchten Studienpopulation Parabenen in einem Umfang ausgesetzt waren, bei dem ein Gesundheitsrisiko nicht mehr mit Sicherheit ausgeschlossen werden kann. Obwohl die höchsten Urinkonzentrationen und Daily Intakes für die kurzkettigen Parabene bestimmt wurden, zeigen die längerkettigen Parabene, aufgrund ihres höheren toxikologischen Potentials und durch den Einbezug „konservativer" gesundheitsbezogener Grenzwerte, einen deutlich stärkeren Einfluss auf die kumulative Risikobewertung.

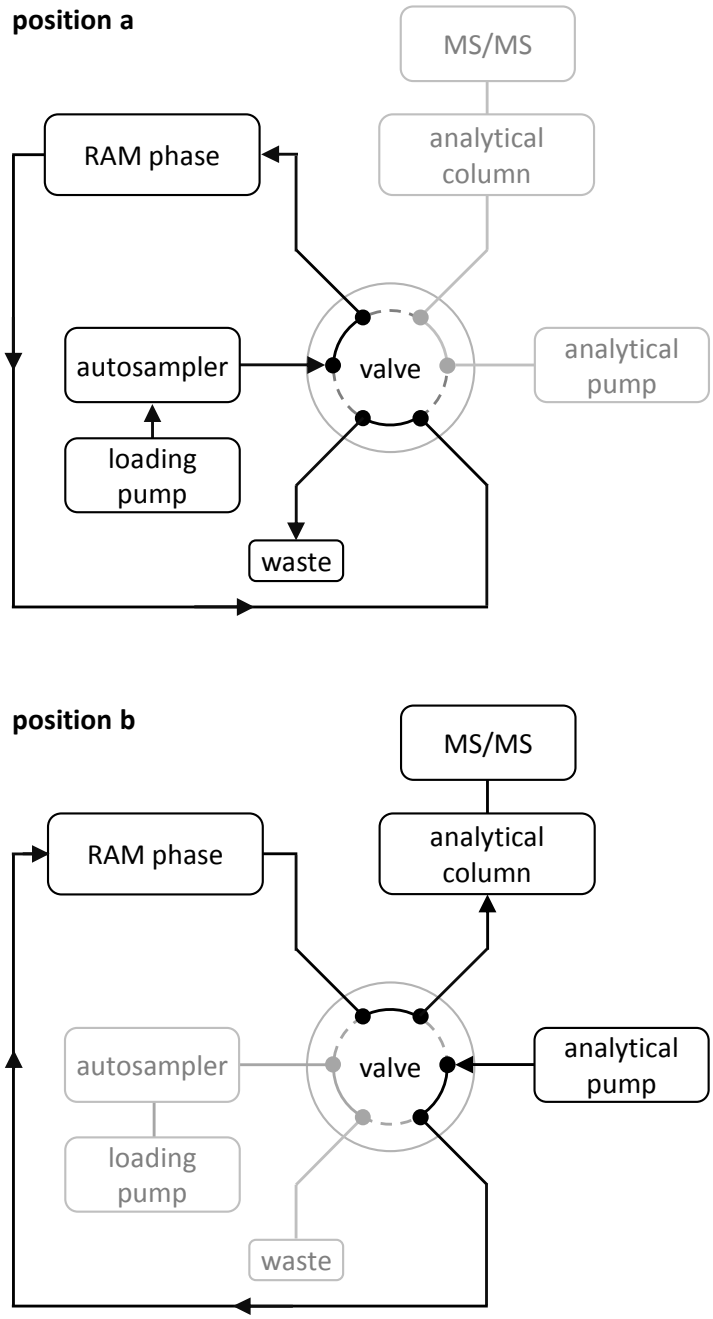
In der Europäischen Union wurden im Jahr 2014 die maximal zulässigen Konzentrationen von *n*-PrP und *n*-BuP in kosmetischen Produkten reduziert und die Verwendung von *iso*-PrP, *iso*-BuP, BeP und PeP verboten. Es bleibt abzuwarten, ob diese regulatorischen Maßnahmen einen Einfluss auf die generelle Parabenexposition haben. Mit der inzwischen gut etablierten HBM-Methode kann die Entwicklung der Belastung gegenüber Parabenen in den kommenden Jahren beobachtet und beurteilt werden.

Aufgrund der Tatsache, dass Parabene überwiegend in kosmetischen Produkten verwendet werden, gilt die dermale Exposition gegenüber diesen Stoffen als einer der Hauptexpositionswege. Bislang wurde erst eine Studie veröffentlicht, in welcher der menschliche Metabolismus nach dermaler Applikation untersucht wurde (Janjua et al. 2008). In dieser Studie wurden etwa 0,9% der applizierten Dosis von *n*-BuP (unmarkierter Standard) im Urin wiedergefunden, wobei hier allein die freie und glucuronidierte Spezies des Mutterparabens bestimmt wurden. Mit dem Wissen über den menschlichen Metabolismus nach oraler Aufnahme und den zur Verfügung stehenden analytischen Methoden soll in naher Zukunft diese Studie unter Einbeziehung mehrerer Parabene am IPA in Zusammenarbeit mit den Autoren der oben genannten Arbeit wiederholt werden.

Unter Berücksichtigung aller in der vorliegenden Arbeit beschriebenen Parabenmetaboliten (inklusive der identifizierten spezifischen Metaboliten) und –spezies (freie, sulfatierte und glucoronidierte Spezies) sollen analog zum Studiendesign zur Aufklärung des Humanmetabolismus nach oraler Aufnahme, valide Konversionsfaktoren für die dermale Aufnahme von Parabenen etabliert werden.

Diese Konversionsfaktoren können wertvolle Informationen über den Zusammenhang zwischen dermaler Aufnahme, der daraus resultierenden inneren Belastung und den in Urin gefundenen Konzentrationen geben und ermöglichen eine verbesserte Abschätzung des durch diese Stoffe für den Menschen ausgehenden Risikos.

Appendix I



Supplementary Figure 1
Two-column HPLC system with backflush arrangement. Valve position A: cleanup and enrichment of the analytes using a RAM. Valve position B: analyte transfer onto the C18-RP phase, chromatographic separation and mass spectrometric detection.

II | Appendix I

Supplementary Table 1

Gradient program for cleanup and enrichment and chromatographic separation, solvent A: 99.98% water and 0.02% acetic acid, solvent B: 99.98% acetonitrile and 0.02% acetic acid.

	Analytical pump				Loading pump		
Total time [min]	Flow rate [µL/min]	Solvent A (%)	Solvent B (%)	Valve postion	Flow rate [µL/min]	Solvent A (%)	Solvent B (%)
0.0	400	85	15	A	1000	98	2
7.0	400	85	15	A	1000	98	2
7.1	400	85	15	B	100	98	2
10.0	400	45	55	B	100	70	30
10.1	400	45	55	A	1000	70	30
12.0	400	43	57	A	1000	3	97
16.0	400	39	61	A	1000	3	97
16.1	400	10	90	A	1000	3	97
19.0	400	-	-	A	1000	3	97
19.1	400	-	-	A	1000	98	2
20.0	400	5	95	A	1000	98	2
24.0	400	5	95	A	1000	98	2
24.1	400	85	15	A	1000	98	2
26.0	400	85	15	A	1000	98	2

Supplementary Table 2

Precision and accuracy calculated from analysis of eight different urine samples with varying creatinine concentrations and two or three different spiking levels.

Analyte	Native conc. (µg/L)	low			medium			high		
		Spiked conc. (µg/L)	Accuracy (%)	RSD (%)	Spiked conc. (µg/L)	Accuracy (%)	RSD (%)	Spiked conc. (µg/L)	Accuracy (%)	RSD (%)
MeP	4.3 – 55.4	8.9	101	4.8	35.6	106	2.8	89.0	111	4.5
EtP	0.6 – 88.2	8.9	97	5.0	35.4	98	4.2	88.6	102	4.2
iso-PrP	<LOQ	8.8	79	7.7	35.4	77	4.3	88.4	83	5.8
n-PrP	<LOQ – 12.5	8.5	95	3.4	34.1	99	3.1	85.3	99	2.9
iso-BuP	<LOQ	8.5	98	2.7	33.8	105	5.1	84.6	108	3.9
n-BuP	<LOQ – 2.1	8.8	95	5.0	35.4	102	6.7	88.4	104	5.7
BeP	<LOQ	8.9	113	6.6	35.8	116	3.9	89.4	111	3.5
PeP	<LOQ	9.6	161	20.5	38.5	135	19.2	-	-	-
HeP	<LOQ	8.5	176	18.2	33.9	219	12.3	-	-	-
BPA	<LOQ – 20.7	8.4	105	6.5	33.5	107	5.3	83.7	113	6.6
TCS	<LOQ – 11.6	9.0	106	4.9	35.8	108	3.8	89.6	103	1.4
TCC	<LOQ	9.1	121	4.2	36.3	105	5.3	-	-	-
2-PP	<LOQ – 19.6	9.6	108	5.3	38.3	106	6.1	95.8	106	3.2
BP-1	<LOQ – 10.6	8.7	72	15.8	34.7	75	15.7	-		-
BP-3	<LOQ – 40.2	7.8	111	2.9	31.3	104	3.6	78.3	98	6.7
BP-8	<LOQ	8.3	87	14.9	33.0	86	12.9	-	-	-

RSD: relative standard deviation; LOQ: limit of quantification

Supplementary Table 3

Results of the HBM study depicted separately for men, women and children. Median, 95th P and maximum concentration (µg/L) of paraben and environmental phenol.

Analyte	Median [µg/L]			95th P. [µg/L]			Max. [µg/L]		
	male	female	children	male	female	children	male	female	children
MeP	20.7	51.4	14.5	231	609	511	234	1058	3230
EtP	1.3	4.2	0.8	47.1	44.0	9.2	85.5	238	33.8
iso-PrP	< LOQ	< LOQ	< LOQ	< LOQ	2.5	< LOQ	0.7	175	< LOQ
n-PrP	0.8	3.9	0.9	25.8	79.4	13.9	77.3	531	1530
iso-BuP	< LOQ	< LOQ	< LOQ	2.2	3.8	0.8	12.2	15.3	1.7
n-BuP	< LOQ	0.6	< LOQ	17.7	16.9	2.3	51.8	73.4	5.9
BeP	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.6	1.3	0.9
PeP	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
HeP	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
BPA	1.3	2.3	2.9	4.5	5.8	7.7	8.8	18.0	59.6
TCS	< LOQ	1.1	< LOQ	179	347.5	14.8	924.0	1630	1460
TCC	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
2-PP	< LOQ	< LOQ	< LOQ	23.5	< LOQ	< LOQ	76.6	4.9	2.5
BP-1	< LOQ	< LOQ	< LOQ	1.0	4.9	2.6	10.2	8.3	5.2
BP-3	< LOQ	< LOQ	< LOQ	8.5	37.0	10.6	96.8	59.8	24.8
BP-8	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

LOQ: limit of quantification

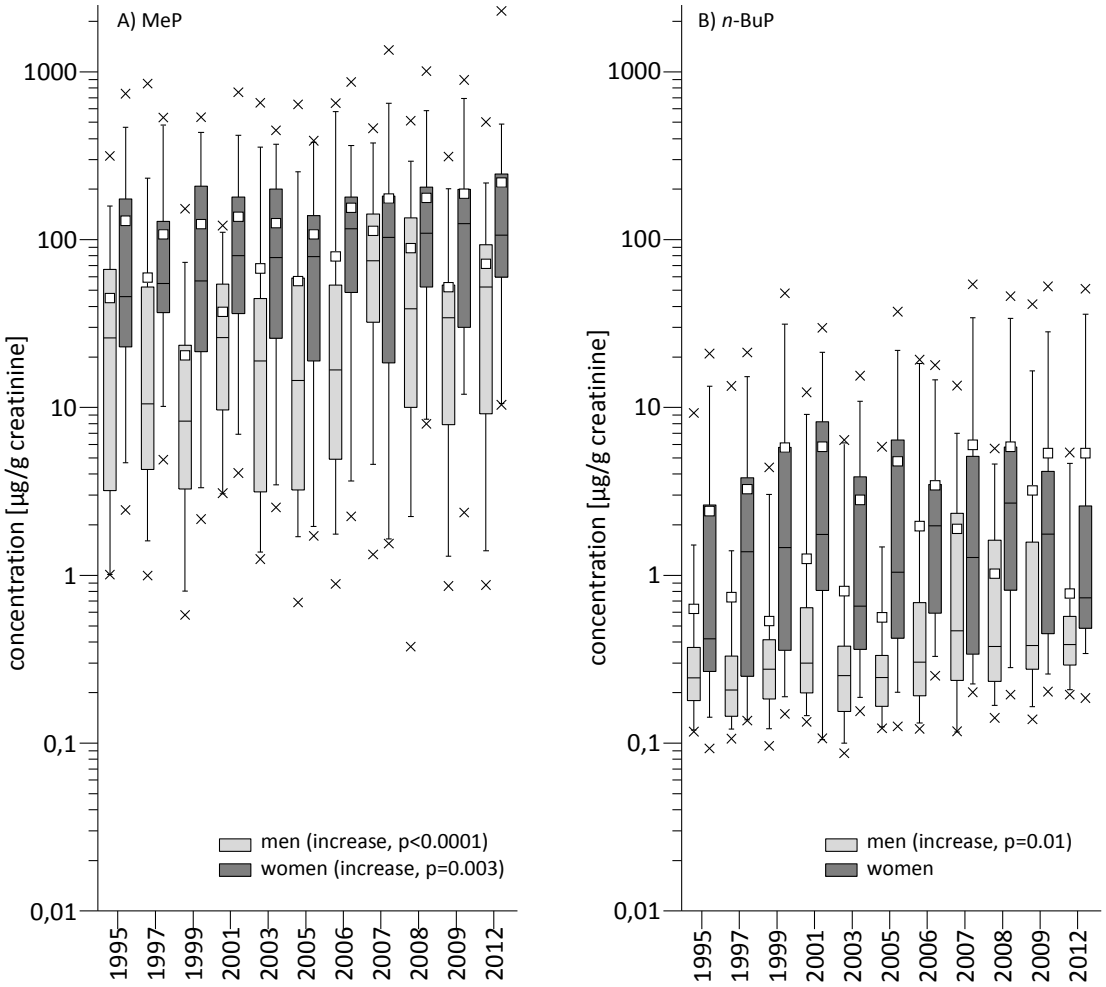
Supplementary Table 4

Biomonitoring data from our study compared with data from Denmark and the US, separately for children, males and females, depicted as the 50th percentiles.

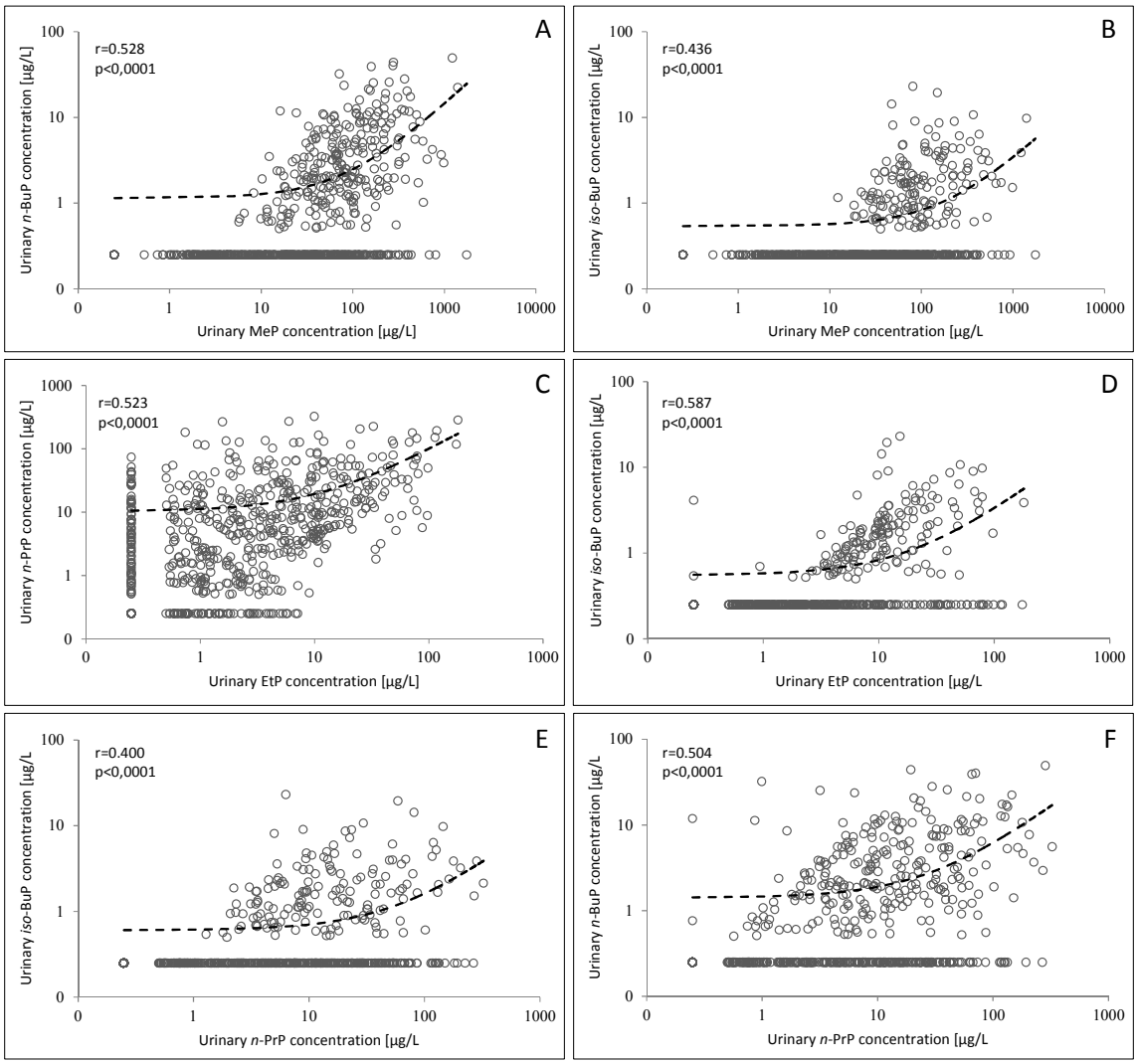
Analyte	children 50th P. [µg/L]			female 50th P. [µg/L]			male 50th P. [µg/L]		
	Germany	Denmark [1]	USA [2]	Germany	Denmark [1]	USA [2]	Germany	Denmark	USA [2]
MeP	14.5	3.0	26.5	51.4	14.0	106	20.7	17.7 [4]	25.3
EtP	0.8	0.4	< LOQ	4.2	0.9	2.0	1.3	2.0 [4]	< LOQ
iso-PrP	< LOQ	< LOQ	-	< LOQ	< LOQ	-	< LOQ	-	-
n-PrP	0.9	< LOQ	2.7	3.9	1.7	20.2	0.8	3.6 [4]	2.0
iso-BuP	< LOQ	< LOQ	-	< LOQ	< LOQ	-	< LOQ	-	-
n-BuP	< LOQ	< LOQ	< LOQ	0.6	< LOQ	0.3	< LOQ	0.2 [4]	< LOQ
BeP	< LOQ	< LOQ	-	< LOQ	< LOQ	-	< LOQ	< LOQ [4]	-
PeP	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-
HeP	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-
BPA	2.9	1.7	1.7	2.3	2.1	1.8	1.3	3.2 [3]	1.9
TCS	< LOQ	0.5	9.9	1.1	0.6	10.5	< LOQ	2.6 [3]	10.9
TCC	< LOQ	< LOQ	-	< LOQ	< LOQ	-	< LOQ	< LOQ [3]	-
2-PP	< LOQ	0.1	< LOQ	< LOQ	0.1	< LOQ	< LOQ	0.3 [3]	< LOQ
BP-1	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-
BP-3	< LOQ	1.8	14.6	< LOQ	3.7	23.0	< LOQ	3.0 [3]	12.2
BP-8	< LOQ	-	-	< LOQ	-	-	< LOQ	-	-

1. Frederiksen et al. 2013b, children n=145 (age 6-11), female n=145 (age 31-52)
2. Fourth National Report on Human Exposure to Environmental Chemicals, 2013, children n=415 (age 6-11), female n=1350 incl. children (age 6-20 years and older), male n=1399 incl. children (age 6-20 years and older)
3. Frederiksen et al., 2014, male n=309
4. Frederiksen et al., 2010, male n=60 (age 18-26)

Appendix II



Supplementary Figure 1
Chronological presentation of the creatinine adjusted values (µg/g creatinine) between 1995–2012 separately by men and women. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile. The minimum and maximum values are represented by an x.



Supplementary Figure 2

Correlation of urinary concentration of A: MeP and n-BuP, B: MeP and iso-BuP, C: EtP and n-PrP, D: EtP and iso-BuP, E: n-PrP and iso-BuP, F: n-PrP and n-BuP in µg/L on logarithmic scale.

Supplementary Table 1
Detection rates [%].

Year	MeP		EtP		*iso*-PrP		*n*-PrP		*iso*-BuP		*n*-BuP		BeP	
	n	DR %	n	DR %	n	DR %	n	DR %	n	DR %	n	DR %	n	DR %
1995	59	100	60	85	60	0	60	80	60	10	60	27	60	0
1997	57	100	60	92	60	3	59	83	60	17	60	32	60	2
1999	58	100	60	82	60	2	60	78	60	25	60	37	60	0
2001	58	100	60	87	60	3	60	83	60	33	60	53	60	0
2003	56	100	60	83	60	3	60	80	60	22	60	35	60	3
2005	60	100	60	77	60	7	60	80	60	27	60	37	60	0
2006	59	100	60	68	60	8	60	82	60	28	60	48	60	2
2007	59	100	60	78	60	5	60	82	60	30	60	48	60	0
2008	59	98	60	77	60	3	60	83	60	30	60	45	60	5
2009	59	100	60	78	60	8	60	83	60	27	60	48	60	0
2012	59	98	59	64	60	5	60	75	60	15	60	30	60	3
total	643	99	659	79	660	4	659	81	660	24	660	40	660	1
male	314	99	330	72	330	1	329	70	330	14	330	24	330	2
female	329	100	329	86	330	8	330	92	330	34	330	57	330	0

DR %: Detection rate %

Supplementary Table 2a

Results of the human biomonitoring study depicted separately by men and women (median values in µg/L).

Year	MeP		EtP		*iso*-PrP		*n*-PrP		*iso*-BuP		*n*-BuP		BeP	
	male	female	male	female	male	female	male	female	male	female	male	female	male	female
1995	20.7	39.6	2.2	4.5	<LOQ	<LOQ	1.6	10.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1997	11.4	61.6	1.6	2.8	<LOQ	<LOQ	1.5	8.0	<LOQ	<LOQ	<LOQ	0.9	<LOQ	<LOQ
1999	6.4	46.1	1.3	3.4	<LOQ	<LOQ	0.8	6.8	<LOQ	<LOQ	<LOQ	1.6	<LOQ	<LOQ
2001	23.0	52.2	2.3	4.8	<LOQ	<LOQ	1.3	9.1	<LOQ	0.4	<LOQ	1.7	<LOQ	<LOQ
2003	18.0	54.5	1.0	3.5	<LOQ	<LOQ	1.9	9.6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2005	16.7	55.8	0.8	3.2	<LOQ	<LOQ	1.8	12.6	<LOQ	<LOQ	<LOQ	0.9	<LOQ	<LOQ
2006	20.9	74.4	0.7	3.3	<LOQ	<LOQ	1.3	19.1	<LOQ	<LOQ	<LOQ	1.5	<LOQ	<LOQ
2007	65.5	67.4	3.6	2.2	<LOQ	<LOQ	8.7	9.4	<LOQ	<LOQ	<LOQ	0.8	<LOQ	<LOQ
2008	38.1	51.4	1.4	6.7	<LOQ	<LOQ	2.4	9.7	<LOQ	<LOQ	<LOQ	1.6	<LOQ	<LOQ
2009	28.1	69.8	1.1	2.8	<LOQ	<LOQ	1.0	7.4	<LOQ	<LOQ	<LOQ	1.0	<LOQ	<LOQ
2012	35.8	57.4	0.7	7.9	<LOQ	<LOQ	0.9	10.0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
total	23.2	57.2	2.1	4.1	<LOQ	<LOQ	1.5	9.5	<LOQ	<LOQ	<LOQ	0.9	<LOQ	<LOQ

LOQ: limit of quantification.

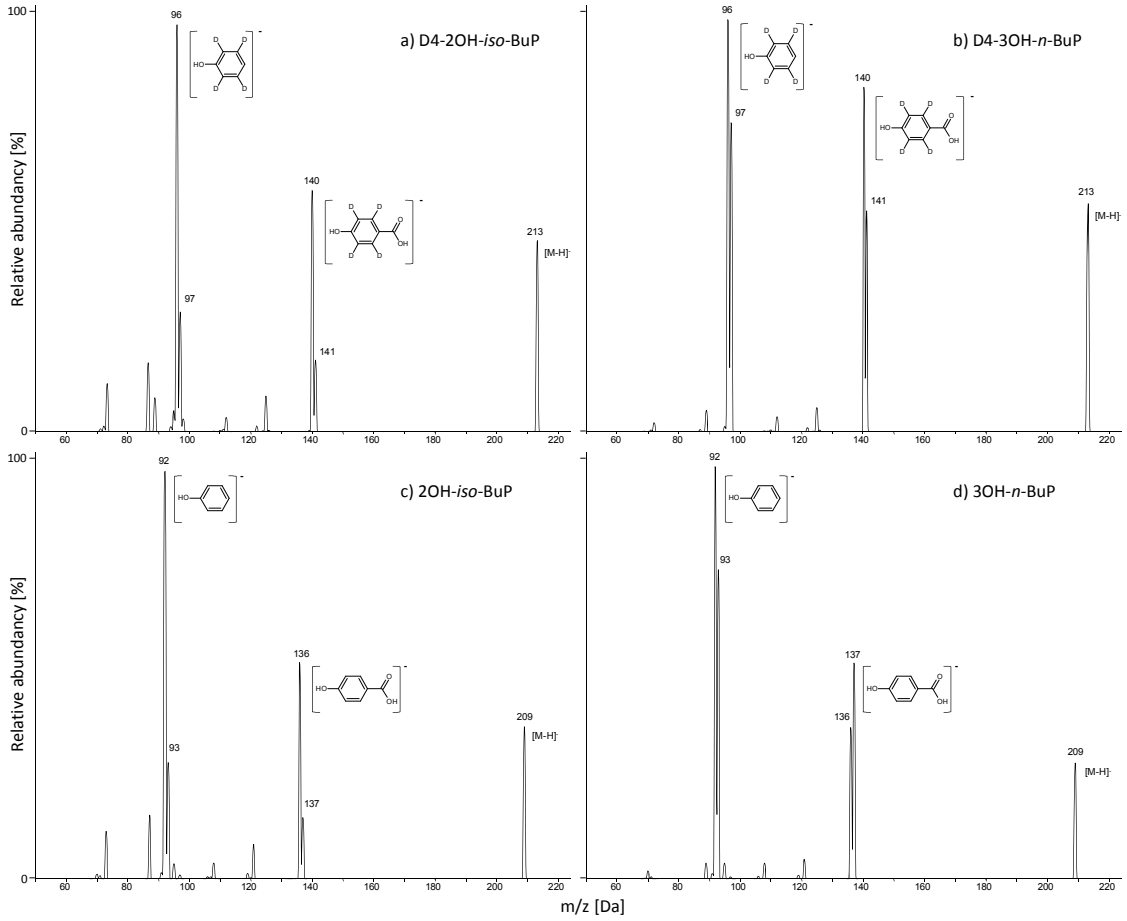
Supplementary Table 2b

Results of the human biomonitoring study depicted separately by men and women (median values in µg/g creatinine).

Year	MeP		EtP		*iso*-PrP		*n*-PrP		*iso*-BuP		*n*-BuP		BeP	
	male	female	male	female	male	female	male	female	male	female	male	female	male	female
1995	26.1	45.7	1.7	4.6	N.A.	N.A.	1.3	9.2	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
1997	10.5	54.9	1.5	3.3	N.A.	N.A.	N.A.	10.5	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
1999	8.3	56.6	1.2	5.4	N.A.	N.A.	N.A.	9.2	N.A.	N.A.	N.A.	1.5	N.A.	N.A.
2001	26.1	80.0	2.2	8.1	N.A.	N.A.	1.7	13.8	N.A.	N.A.	N.A.	1.8	N.A.	N.A.
2003	18.9	78.0	0.8	5.5	N.A.	N.A.	1.4	13.7	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2005	14.5	79.3	N.A.	4.0	N.A.	N.A.	1.9	15.8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2006	16.8	116	N.A.	5.4	N.A.	N.A.	2.1	37.0	N.A.	N.A.	N.A.	2.0	N.A.	N.A.
2007	75.0	104	2.8	3.5	N.A.	N.A.	5.9	13.8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2008	38.7	109	1.4	8.9	N.A.	N.A.	2.7	14.5	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2009	34.3	124	N.A.	5.4	N.A.	N.A.	1.3	14.1	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2012	52.0	106	N.A.	8.4	N.A.	N.A.	N.A.	13.8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
total	22.8	86.9	N.A.	5.4	N.A.	N.A.	N.A.	12.8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

N.A.: not applicable.

Appendix III



Supplementary Figure 1
ESI-negative Q3 mass spectra of D4-2OH-*iso*-BuP, D4-3OH-*n*-BuP, 2OH-*iso*-BuP and 3OH-*n*-BuP.

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Supplementary Table 1

Gradient program for cleanup and enrichment and chromatographic separation, solvent A: 99.95% water and 0.05% acetic acid, solvent B: 99.95% acetonitrile and 0.05% acetic acid, solvent C: ammonium bicarbonate buffer 3 mM.

Total time [min]	Analytical pump			Valve postion	Loading pump			
	Flow rate [µL/min]	Solvent A (%)	Solvent B (%)		Flow rate [µL/min]	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.0	300	95	5	A	2000	0	0	100
2.0	300	↓	↓	A	2000	↓	↓	↓
3.0	300	↓	↓	B	500	0	0	100
4.0	300	95	5	B	500	95	5	0
5.0	300	55	45	B	500	↓	↓	↓
6.0	300	↓	↓	A	500	↓	↓	↓
8.0	300	55	45	A	500	↓	↓	↓
8.5	300	45	55	A	500	↓	↓	↓
12.0	300	↓	↓	A	500	95	5	0
12.5	300	40	60	A	500	↓	↓	↓
13.0	300	↓	↓	A	500	5	95	0
13.5	300	5	95	A	500	↓	↓	↓
20.0	300	↓	↓	A	500	5	95	0
21.0	300	↓	↓	A	500	0	0	100
21.1	300	↓	↓	A	500	0	0	100
22.0	300	5	95	A	2000	↓	↓	↓
23.0	300	95	5	A	2000	↓	↓	↓
25.0	300	95	5	A	2000	0	0	100

Supplementary Table 2
MRM-parameters for mass spectrometric detection.

Analyte	RT [min]	Q1 mass → Q3 mass quantifier (qualifier) [Da]	Analyte	RT [min]	Q1 mass → Q3 mass quantifier [Da]
Analytical standard			Internal standard		
D4-PHHA	9.4	198 → 100 (97)	$^{13}C_6$-PHHA	9.4	200 → 99
D4-PHBA	9.9	141 → 97 (69)	$^{13}C_6$-PHBA	9.9	143 → 99
rOH-MeP	10.3	167 → 108 (152)	rOH-EtP	10.9	181 → 108
D3-rOH-MeP	10.3	170 → 111 (155)	rOH-EtP	10.9	181 → 108
D4-2OH-iso-BuP	10.4	213 → 96 (140)	3OH-*n*-BuP	10.4	209 → 92
D4-3OH-*n*-BuP	10.4	213 → 96 (140)	3OH-*n*-BuP	10.4	209 → 92
D4-MeP	11.2	155 → 96 (140)	$^{13}C_6$-MeP	11.2	157 → 98
D4-*iso*-BuP	14.0	197 → 96 (140)	$^{13}C_6$-*n*-BuP	14.2	199 → 98
D4-*n*-BuP	14.2	197 → 96 (140)	$^{13}C_6$-*n*-BuP	14.2	199 → 98
D3-rOH *iso*-BuP	12.8	212 → 111(155)	rOH-EtP	10.9	181 → 108
rOH-*n*-BuP	12.9	209 → 108 (152)	rOH-EtP	10.9	181 → 108
D3-rOH *n*-BuP	12.9	212 → 111(155)	rOH-EtP	10.9	181 → 108
D4-*iso*-BuP	14.0	197 → 96 (140)	$^{13}C_6$-*n*-BuP	14.2	199 → 98
D4-*n*-BuP	14.2	197 → 96 (140)	$^{13}C_6$-*n*-BuP	14.2	199 → 98

Supplementary Table 3

Intra-day precision of the method calculated by analysis of self-prepared quality control materials with three different concentration levels.

Analyte	Intra-day series (n=8)					
	Q low		Q medium		Q high	
	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)
D4-MeP	3.2	0.8	82.6	1.2	187	1.9
D3-rOH-MeP	4.4	2.7	55.1	1.6	-	-
D4-*iso*-BuP	3.3	3.0	54.2	3.2	142	1.9
D4-*n*-BuP	3.1	1.8	82.1	2.5	193	1.9
D3-rOH-*n*-BuP	5.7	1.9	54.4	1.8	-	-
D4-3OH-*n*-BuP*	5.7	2.0	16.5	1.3	59.3	6.9
D4-PHBA	5.9	1.4	74.4	1.8	227	2.3
D4-PHHA	5.4	1.7	95.8	3.1	469	1.0

RSD: relative standard deviation; *quantified as sum of 2OH-*iso*-BuP and 3OH-*n*-BuP, because it is pooled native urine from different dosages

Inter-day precision of the method calculated by analysis of self-prepared quality control materials with three different concentration levels.

Analyte	Inter-day series (n=8)					
	Q low		Q medium		Q high	
	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)	Mean (µg/L)	RSD (%)
D4-MeP	3.4	3.0	84.6	1.6	195	2.6
D3-rOH-MeP	4.3	4.8	50.9	5.9	-	-
D4-*iso*-BuP	3.5	6.8	58.8	5.1	152	4.4
D4-*n*-BuP	3.2	4.8	84.7	3.2	196	2.9
D3-rOH-*n*-BuP	6.1	9.2	54.1	6.8	-	-
D4-3OH-*n*-BuP*	5.7	2.7	17.1	3.2	62.4	3.0
D4-PHBA	6.1	7.4	77.1	4.8	234	6.5
D4-PHHA	5.5	4.3	96.9	2.6	474	1.7

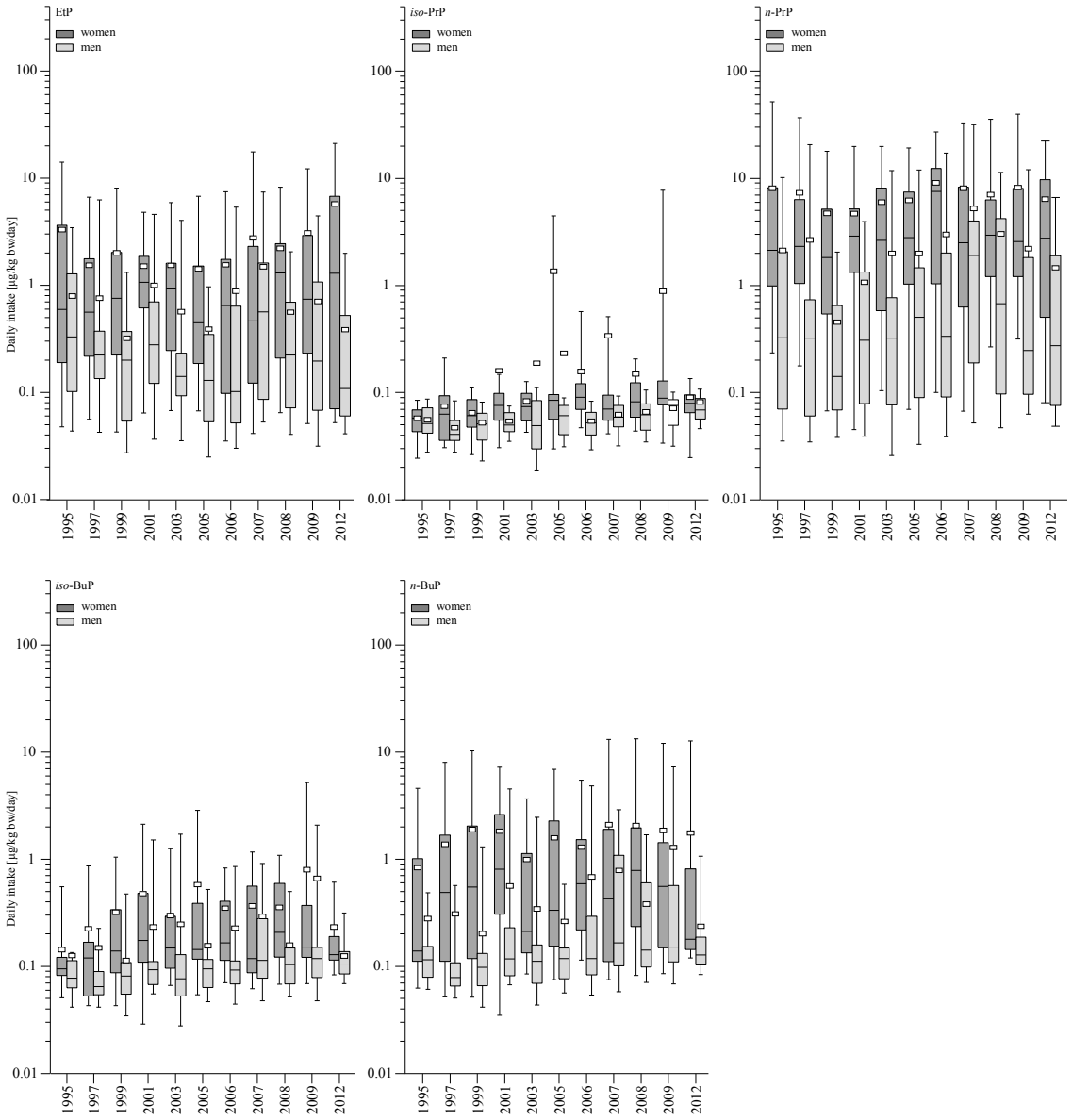
RSD: relative standard deviation; *quantified as sum of 2OH-*iso*-BuP and 3OH-*n*-BuP, because it is pooled native urine from different dosages

Precision and accuracy calculated from analysis of ten different urine samples with varying creatinine concentrations and two different spiking levels, calibration ranges and quantification limits of this method.

Analyte	low			med			high			LOQ (µg/L)	Calibration range (µg/L)
	Spiked conc. (µg/L)	Accuracy (%)	RSD (%)	Spiked conc. (µg/L)	Accuracy (%)	RSD (%)	Spiked conc. (µg/L)	Accuracy (%)	RSD (%)		
D4-MeP	6.6	100	3.1	66.4	104	3.1	249	101	2.8	0.25	0.25-300
D3-rOH-MeP	8.0	98	28.0	80.4	94	15.9	-	-	-	1.0	1.0-80
D4-*iso*-BuP	6.5	116	5.1	65.1	95	5.8	244	91	5.2	0.1	0.1-300
D4-*n*-BuP	7.0	105	1.5	70.3	109	1.8	264	104	2.0	0.25	0.25-300
D3-rOH-*n*-BuP	7.8	94	6.8	78.3	99	7.6	-	-	-	1.0	1.0-80
D4-3OH-*n*-BuP*	8.2	107	1.2	82.3	104	1.7	309	103	1.7	0.1	0.1-500
D4-PHBA	7.6	94	5.3	76.4	101	3.4	287	98	3.7	0.25	0.25-500
D4-PHHA	6.0	97	4-5	60.0	103	3.5	225	100	5.5	0.1	0.1-500

RSD: relative standard deviation; *quantified as sum of 2OH-*iso*-BuP and 3OH-*n*-BuP, because it is pooled native urine from different dosages

Appendix IV



Supplementary Figure 1

Chronological presentation of daily intakes (µg/kg bw/day) between 1995–2012 separately by men and women. Bottom and top of the box are the first and third quartiles. The band inside the box shows the median. The small square indicates the mean. The whiskers represent the 5th and the 95th percentile.

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Supplement Table 1
Hazard quotients (HQ) and hazard indexes (HI) based upon EFSA group ADI separately for MeP and EtP.

	MeP	EtP	MeP + EtP
		group ADI: 10 mg/kg bw/day	
P.50	0.001	< 0.001	0.001
P.95	0.005	0.001	0.005
Max	0.016	0.004	0.020
n > 1 [%]	0	0	0

P.50: 50th percentile; P.95: 95th percentile