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Impact of enzymatic hydrolysis on the quantification of total urinary concentrations of chemical biomarkers

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HIGHLIGHTS

- Finding one optimal condition for complete hydrolysis of multiple biomarkers was challenging.
- Enzyme type and amount used, reaction time, temperature critically affected hydrolysis extent.
- Only two/seven enzymes evaluated completely hydrolyzed O− and *N*-glucuronides simultaneously.
- ≥ 30 units/μL urine of solid β-glucuronidase (*Helix pomatia*, H-1) resulted in complete hydrolysis.
- ≥ 30 μL/100 μL urine of liquid β-glucuronidase/arylsulfatase (*Helix pomatia*) resulted in complete hydrolysis.

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ABSTRACT

Human exposure to consumer and personal care products chemicals such as phenols, including parabens and other antimicrobial agents, can be assessed through biomonitoring by quantifying urinary concentrations of the parent chemical or its metabolites, often after hydrolysis of phase II conjugates. Developing suitable analytical methods for the concurrent quantification of multiple exposure biomarkers is challenging because optimal conditions for the hydrolysis of such conjugates (e.g., O-glucuronides, *N*-glucuronides, sulfates) may differ depending on the biomarker. We evaluated the effectiveness of seven commercial hydrolytic enzymes to simultaneously hydrolyze *N*-glucuronides (using the antibacterial triclocarban as example compound) and other conjugates (using select phenols and parabens as examples) by using on-line solid phase extraction–high performance liquid chromatography-isotope dilution-tandem mass spectrometry. Incubation (30 min, 55 °C) with a genetically engineered *β*-glucuronidase (IMCS, ≥15 units/μL urine) hydrolyzed *N*-glucuronide triclocarban, but did not fully hydrolyze the conjugates of phenols and parabens. By contrast, incubation (4 h, 37 °C) with solid *β*-glucuronidase (*Helix pomatia*, Type H-1, ≥30 units/μL urine) or liquid β-glucuronidase/arylsulfatase (*Helix pomatia*, 30 units/μL urine [i.e., 30 μL/100 μL urine]) in the presence of 100 μL methanol for 100 μL urine completely hydrolyzed *N*-glucuronide triclocarban and the conjugates of several phenols and parabens, without cleaving the ester bond of the parabens to form *p*-hydroxybenzoic acid. These results highlight the relevance of method validation procedures that include optimizing the hydrolysis of phase II urinary conjugates (e.g., enzyme type and amount used, reaction time, temperature) to quantify accurately and concurrently multiple exposure biomarkers for biomonitoring purposes.

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1. Introduction

Exposure to environmental chemicals (e.g., preservatives, antimicrobial agents, UV filters) may occur through use of consumer and personal care products. Some of these xenobiotics are suspect endocrine disruptors (Calafat, 2012), and concerns exist about their potential adverse health effects in humans. Biomonitoring—the measurement of the chemicals or their metabolites in biological matrices—is a useful tool to estimate human exposure to these chemicals (Calafat et al., 2006).

Upon exposure, xenobiotics may undergo phase II biotransformation, a common detoxification pathway to facilitate urinary elimination (Tephly, 1990; Zhou and Miners, 2014). During phase II

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biotransformations, xenobiotics undergo reactions such as glucuronidation, sulfation, methylation, acetylation, or glutathione and amino acid conjugation to form hydrophilic conjugates (Kaivosaari et al., 2011; Kroemer and Klotz, 1992; Tephly and Burchell, 1990). For example, environmental phenolic compounds present in personal care products such as benzophenone-3 (ultraviolet filter), parabens (preservatives), and triclosan (antimicrobial), often form O-glucuronide and sulfate conjugates (Ye et al., 2011). Other personal care product chemicals such as the antimicrobial triclocarban (3,4,4′-trichlorocarbanilide) can form *N*-glucuronide conjugates (Howes and Black, 1976; Hawes, 1998; Schebb et al., 2012).

Human exposure can be assessed from the urinary concentrations of xenobiotics or their metabolites; these biomarker concentrations are often obtained after enzymatic hydrolysis of phase II conjugates (e.g., glucuronides, sulfates) and reported as total (free [unconjugated] plus conjugated) concentrations. Thus, optimizing the hydrolysis of urinary conjugates is critical for accurate quantification of the total concentration of these chemicals for exposure assessment. Biomonitoring is an expensive effort, and high-throughput multi-analyte methods that concurrently quantify multiple biomarkers are most cost effective. However, optimal hydrolysis conditions for deconjugation of some conjugates (e.g., O-glucuronides) may not be appropriate for others (e.g., *N*-glucuronides) when both O− and *N*-glucuronides of various biomarkers are included in the same analytical method. For instance, *β*-glucuronidases preferentially hydrolyze O-glucuronides over *N*-glucuronides (Zenser et al., 1999; Babu et al., 1996; Kowalczyk et al., 2000), and *N*-glucuronides may be labile under acidic conditions while O-glucuronides are labile under basic conditions. Furthermore, some enzymes also possess nonspecific lipase activity capable of hydrolyzing ester bonds such as those present in phthalates and parabens (Abbas et al., 2010; Blount et al., 2000a).

We investigated seven commercially available enzymes used before for biomonitoring applications (Morris et al., 2014; Frederiksen et al., 2013; Kato et al., 2005; Moos et al., 2014; Schmidt et al., 2013) to assess their effectiveness on the enzymatic deconjugation of urinary *N*-glucuronides (using triclocarban as example compound) and of other conjugates (using select phenols and parabens) for the concurrent quantification of these biomarkers by using a multi-analyte mass spectrometry-based analytical method.

2. Experimental section

2.1. Analytical materials and standards

We obtained HPLC-grade methanol (MeOH) from Fisher Scientific (Pittsburgh, PA, USA), analytical-grade formic acid (>98%) from EM Science (Gibbstown, NJ, USA), and 75 mM potassium phosphate (K_2HPO_4) buffer, pH 6.8 from Tedia (Fairfield, OH, USA). We used water (18.0 MΩ/cm resistance) from an ultrapure water system (AQUA Solutions, Jasper, GA, USA). We purchased ammonium acetate (>98%), 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, triclocarban, triclosan, bisphenol A (BPA), bisphenol F (BPF), bisphenol S (BPS), 2,4-dichlorophenol, 2,5-dichlorophenol, and methyl-, ethyl-, propyl-, and butyl parabens from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, USA); benzophenone-3 was provided by EMD Chemicals Inc. (Hawthorne, NY, USA). We obtained $^{13}C_4$-4-methylumbelliferone, $^{13}C_{12}$-BPA, $^{13}C_6$-2,4-dichlorophenol, $^{13}C_6$-2,5-dichlorophenol, and D_2-triclocarban from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); $^{13}C_{12}$-triclosan from Wellington laboratories Inc. (Ontario, Canada); D_3,^{13}C-benzophenone-3 from Los Alamos National Laboratory (Los Alamos, NM, USA); D_4-methyl paraben from CDN Isotopes (Quebec, Canada); and $^{13}C_{12}$-BPS, D_4-ethyl, D_4-propyl, and D_4-butyl parabens from CanSyn Chem Corp. (Toronto, Canada).

For the present study, we evaluated seven commercially available enzymes, used before for other similar biomonitoring applications (Morris et al., 2014; Frederiksen et al., 2013; Kato et al., 2005; Moos et al., 2014; Schmidt et al., 2013); their sources were:

1. H-1: β-Glucuronidase from Helix Pomatia, Type H-1 (Sigma Aldrich, Item #G0751, β-glucuronidase activity ≥300,000 units/g solid; sulfatase activity 10,000 units/g solid).
2. SH-1: Sulfatase from Helix pomatia, Type H-1 (Sigma Aldrich, Item #S9626, sulfatase activity ≥10,000 units/g solid, β-glucuronidase activity ≥300,000 units/g solid).
3. K-12: BGALA-RO β-Glucuronidase from E. coli-K12 liquid enzyme (Roche Biomedical through Sigma Aldrich, Item #03707601001, β-glucuronidase activity ≈140 units/mg protein).
4. BL-21: β-Glucuronidase from E. coli-BL21 (Sigma Aldrich, Item #G8420, β-Glucuronidase activity ≥20,000,000 units/g protein).
5. HP-2: β-Glucuronidase from Helix Pomatia, Type HP-2 (Sigma Aldrich, Item #G7017, β-glucuronidase activity ≥100,000 units/mL; sulfatase activity 7500 units/mL).
6. IMCS: Recombinant β-Glucuronidase from snail (Integrated Micro-Chromatography Systems Columbia, Item #04−E1F, β-glucuronidase activity >50,000 units/mL).
7. ALS: BGALA-RO β-Glucuronidase/Arylsulfatase liquid enzyme from Helix pomatia (Roche Life Science through Sigma Aldrich, Item #10127698001, β-glucuronidase activity ≈100,000 units/mL; sulfatase activity ≈47,500 units/mL).

For the experiments, unless specified, we evaluated the testing conditions (e.g., buffer, pH) recommended by the manufacturer of the enzyme. We used archived urine samples (stored at −70 °C) collected in Atlanta, GA, USA between 2010 and 2016 from convenience samplings of male and female adults. No personal information from the subjects was available. The Centers for Disease Control and Prevention's (CDC) Human Subjects Institutional Review Board reviewed and approved the study protocol. A waiver of informed consent was requested under 45 CFR 46.116(d).

2.2. Preparation of standard stock solutions and quality control (QC) materials

Stock solutions and QC materials were prepared as described before (Zhou et al., 2012, 2014; Ye et al., 2006). Initial stock solutions of analytical standards and stable isotope-labeled internal standards of BPA, BPS, BPF, methyl-, ethyl-, butyl-, and propyl parabens, 2,4-dichlorophenol, 2,5-dichlorophenol, benzophenone-3, triclosan, and triclocarban were prepared in MeOH. Ten working standard spiking solutions that contained all twelve analytes were generated by serial dilutions of the initial stock with MeOH. Final concentrations of the ten working standards were such that a 100-μL spike covered a concentration range from 0.1 to 100 ng/mL (three bisphenols, 2,4-dichlorophenol), 200 ng/mL (triclocarban), or 1000 ng/mL (parabens, 2,5-dichlorophenol, benzophenone-3, triclosan). The stable isotope-labeled internal standard (IS) working solution was prepared by diluting the internal standard stock solution using MeOH, so that 50-μL spike would result in a 25 ng/mL or 100 ng/mL concentration of internal standards. The IS working solution also contained 4-methylumbelliferyl sulfate, 4-methylumbelliferyl glucuronide, and $^{13}C_4$-4-methylumbelliferone, compounds used as deconjugation standards, as described in detail before (CDC, 2017; 2016; Ye et al., 2005a; Zhou et al., 2014). QC materials were prepared from pooled urine. The QC low (QCL) and the QC high (QCH) pools were enriched with different levels of the target analytes. All standard stock solutions, spiking solutions, and