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Alkyl protocatechuates as novel urinary biomarkers of exposure to *p*-hydroxybenzoic acid esters (parabens)

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ABSTRACT

Human exposure to *p*-hydroxybenzoic acid esters (parabens) is a concern, owing to adverse health effects of these compounds. Parabens are metabolized and eliminated from the human bodies within a few hours of exposure. In this study, for the first time, methyl- and ethyl-protocatechuates (OH-MeP and OH-EtP) and their parent compounds, methyl- (MeP) and ethyl-parabens (EtP), were determined in urine samples collected from U.S. children and adults. Alkyl protocatechuates were found in almost all urine samples, with median concentrations of 11.8 (OH-MeP) and 2.90 ng/mL (OH-EtP) in adults, and 5.43 (OH-MeP) and 0.85 ng/mL (OH-EtP) in children. In adults, the concentrations of urinary OH-MeP and OH-EtP were higher than the corresponding concentrations of MeP and EtP. Significant correlation between OH-MeP/OH-EtP and MeP/EtP was observed. This is the first report to document hydroxylation of parabens in humans, and to propose hydroxylated metabolites (i.e., alkyl protocatechuates) as alternative biomarkers of exposure to parabens in human biomonitoring studies. The rates of transformation of parabens between children and adults appeared to be different, as evidenced from the slopes of regression between alkyl protocatechuates and parabens. In addition to alkyl protocatechuates, hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (3,4-DHB) were found at considerable levels in the urine samples. The occurrence of a significant proportion of alkyl protocatechuates and 3,4-DHB suggests the need for inclusion of these derivatives in accurate estimation of human exposure to parabens and in epidemiological studies that associate paraben exposure to health outcomes in populations.

1. Introduction

The esters of *p*-hydroxybenzoic acid (i.e., parabens) are commonly used as broad-spectrum microbial preservatives in many personal care products. Occurrence of parabens in cosmetics, foodstuffs, and pharmaceuticals has been reported (Liao et al., 2013; SCCS, 2011; Soni et al., 2005). Concern over the safety of parabens has recently increased due to the potential health risks associated with exposure to these substances. Methyl- (MeP), ethyl- (EtP), propyl- (PrP), butyl- (BuP) and benzyl-parabens (BzP) have been reported to possess estrogenic properties (Ahn et al., 2012; Darbre et al., 2003; Golden et al., 2005; Hu et al., 2013; Okubo et al., 2001; Routledge et al., 1998). As the evidence for endocrine and reproductive toxicities of parabens is mounting, the reference doses recommended for these compounds are being revised. For example, the acceptable daily intake for PrP recommended by the Joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) Expert Committee on Food Additives was withdrawn in 2007 (JECFA, 2007). Consequently, PrP and BuP were banned for use in children's cosmetics in Denmark (SCCS, 2011).

Studies have reported the occurrence of parabens in human urine. In 2006, the occurrence of MeP, EtP, PrP, and BuP was reported in urine samples collected from U.S. adults (Ye et al., 2006). The U.S. National Health and Nutrition Examination Survey (NHANES) of 2005–2006 reported median urinary concentrations of 63.5 and 8.7 ng/mL for MeP and PrP, respectively (Calafat et al., 2010). MeP, EtP, and PrP were reported to occur in pooled sera of 3- to 11-year-old children frequently (60–100%) (Ye et al., 2012). Although parabens have been reported to be metabolized by animals (Aubert et al., 2012; Harville et al., 2007; Jewell et al., 2007; Tsukamoto and Terada, 1964) and humans (e.g., intestine/liver/skin microsomes) (Harville et al., 2007; Janjua et al., 2007; Jewell et al., 2007; Lakeram et al., 2007), studies on the analysis of metabolic products of parabens in urine are scarce (Shirai et al., 2013).

Parabens can be metabolized by esterase hydrolysis of the ester (Abbas et al., 2010). Both animal and human studies have indicated the transformation of several parabens to a common metabolite, *p*-hydroxybenzoic acid (4-HB) (Abbas et al., 2010; Aubert et al., 2012; Tsukamoto and Terada, 1964). However, 4-HB is not a specific marker

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of exposure to individual parabens. Because humans are exposed to several paraben compounds simultaneously, assessment of a specific marker for individual parabens will enable assessment of exposure sources and health risks. The pathways of human exposure to parabens can be through dermal exposure (through application of cosmetics) or oral ingestion (through diet) (Soni et al., 2005). The metabolic transformation rates and metabolic products can vary, depending on the pathways of exposure. For instance, oral exposures in rats appear to result in more efficient transformation of parabens to 4-HB than does the dermal route (Aubert et al., 2012). Other pathways of biotransformation, including hydroxylation of parabens, are not known.

Hydroxylation is a common mechanism of transformation of many xenobiotics (Guengerich, 2001). For example, hydroxylated derivatives of polycyclic aromatic hydrocarbons (OH-PAHs) have been widely used as biomarkers of exposure to PAHs (Buratti et al., 2007; Guo et al., 2013; Li et al., 2006). Formation of 3,4-dihydroxybenzoic acid (i.e., protocatechuic acid) by hydroxylation of 4-HB has been reported in laboratory animals (Liu et al., 2002; Ste-Marie et al., 1999). In addition, light-induced hydroxylation of methyl paraben to 3-hydroxy-methyl paraben (i.e., methyl protocatechuate) has been reported (Okamoto et al., 2008). For accurate assessment of total exposure and for epidemiological studies associating exposures to health outcomes, it is imperative to determine total concentrations of parabens including all of the metabolites in human matrices.

In this study, concentrations of methyl- and ethyl-protocatechuates were determined in 70 urine samples collected from U.S. children and adults, and the measured concentrations were compared with the concentrations of corresponding parent compounds (parabens) and protocatechuic acids. The objectives of this study were to determine the occurrence of methyl- and ethyl-parabens and their metabolites in the urine of children and adults from the U.S. and to elucidate the suitability of alkyl protocatechuates as novel biomarkers of paraben exposure.

2. Materials and methods

2.1. Reagents and standards

Standard solutions of MeP, EtP, and 4-HB were purchased from AccuStandard, Inc. (New Haven, CT). Methyl protocatechuate (OH-MeP), ethyl protocatechuate (OH-EtP), protocatechuic acid (3,4-dihydroxybenzoic acid; 3,4-DHB), and β-glucuronidase (from *Helix pomatia*, containing 145,700 U/mL β-glucuronidase and 887 U/mL sulfatase) were purchased from Sigma-Aldrich (St. Louis, MO). The structures and physiochemical properties of the target analytes are shown in Fig. 1 and Table 1. ^{13}C-labeled internal standards, $^{13}C_6$-MeP (99%) and $^{13}C_6$-4-HB (99%), were purchased from Cambridge Isotope Laboratories (Andover, MA). Milli-Q water was prepared using an ultrapure water system (Barnstead International, Dubuque, IA). The stock solutions of target analytes and internal standards were prepared at 1 mg/mL in methanol and stored at −20 °C.

2.2. Sample collection

Urine samples from adults were collected randomly from healthy volunteers in Albany, New York, from May to July 2011 ($n = 30$). Forty urine samples from children aged 3–8 years were collected randomly from March to September 2012. All urine samples were collected in polypropylene (PP) tubes and stored at −80 °C prior to analysis. Institutional Review Board approvals were obtained from the New York State Department of Health (NYSDOH) for the analysis of human specimens.

2.3. Sample preparation

Free and total forms of MeP, EtP, OH-MeP, OH-EtP, 4-HB, and 3,4-DHB were extracted as described earlier (Wang et al., 2013). Briefly, urine sample (500 μL) was transferred into a 15-mL PP tube, and 50 μL of methanol, which contained $^{13}C_6$-MeP and $^{13}C_6$-4-HB (5 ng each), were spiked. The samples were buffered with 300 μL of 1 M ammonium acetate and extracted three times with 3 mL aliquots of ethyl acetate. The mixture was shaken in an oscillator shaker for 60 min (Eberbach Corp., Ann Arbor, MI) and then centrifuged at 4500 ×*g* for 5 min (Eppendorf Centrifuge 5804, Hamburg, Germany). The extracts were combined and washed with 1 mL of Milli-Q water. The supernatant was transferred into a glass tube and concentrated to near-dryness under a gentle nitrogen stream. Finally, 0.5 mL of methanol was added and vortex mixed for analysis of the "free" form of analytes by HPLC-MS/MS. For the analysis of "total" (i.e., free plus

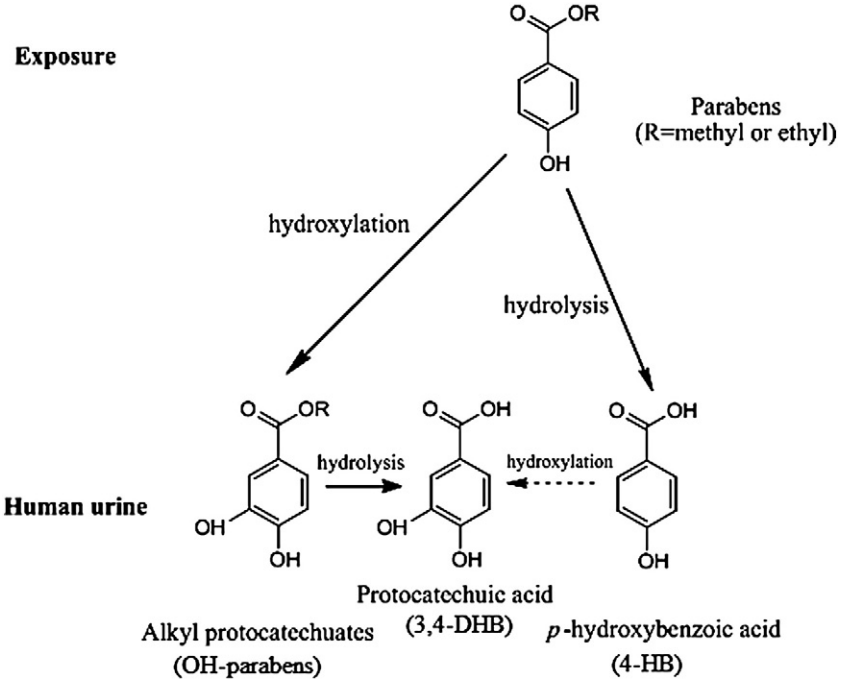

Fig. 1. Chemical structures and potential metabolic transformation routes of parabens in biological systems.