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Determination of glucuronide conjugates of hydroxyl triphenyl phosphate (OH-TPHP) metabolites in human urine and its use as a biomarker of TPHP exposure

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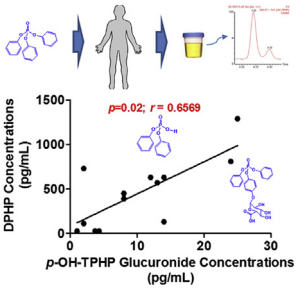
HIGHLIGHTS

- A method was developed for determination of *para*(*p*)- and *meta*(*m*)-OH TPHP glucuronides in urine.
- Both *p*- and *m*-OH-TPHP glucuronides were detected in urine samples.
- Concentration ranges of *p*- and *m*-OH-TPHP glucuronides were <MLOQ-25 and nd-4 pg/mL.
- *p*-OH-TPHP glucuronide and DPHP concentrations in urine were strongly and positively correlated.
- This is first known report demonstrating TPHP hydroxylation occurs in TPHP exposed humans.

GRAPHICAL ABSTRACT



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ABSTRACT

In vitro studies using avian hepatocytes or human liver microsomes suggest that hydroxylation is an important pathway in the metabolism of triphenyl phosphate (TPHP), a chemical used as a flame retardant and plasticizer. TPHP metabolism can lead to the formation of *para*(*p*)- and *meta*(*m*)-hydroxyl-(OH-)TPHP products as well as their glucuronide conjugates. To determine whether the TPHP hydroxylation and depuration pathway also occurs *in vivo* in humans, the present study developed a sensitive method for quantification of *p*- and *m*-OH-TPHP glucuronides in human urine samples. In n = 1 pooled urine sample and n = 12 individual urine samples collected from four human volunteers from Ottawa (ON, Canada), *p*- and *m*-OH-TPHP glucuronides were detectable in 13 and 9 of the 13 analyzed samples and at concentrations ranging from <MLOQ-25 pg/mL and nd-4 pg/mL, respectively. A strong, positive correlation ($p = 0.02$, $r = 0.6569$) was observed between *p*-OH-TPHP glucuronide and diphenyl phosphate concentrations (DPHP, a known dealkylated metabolite of TPHP). To our knowledge, this is the first

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Glucuronidation
Human urine
Biomarker

report demonstrating that TPHP hydroxylation and conjugation occurs *in vivo* in humans, and further suggests that *p*-OH-TPHP glucuronide can be used as a specific biomarker of TPHP exposure in humans.

1. Introduction

Triphenyl phosphate (TPHP) is widely used as a plasticizer, lubricant and in hydraulic fluids (Andresen et al., 2004; van der Veen and de Boer, 2012). TPHP is also used as a flame retardant (FR) chemical (Keller et al., 2014; Boor et al., 2015), e.g. the FR formulation Firemaster® 550 (FM550) is composed of approximately 40% of a mixture of bis-(2-ethylhexyl) tetrabromophthalate (TBPH) and tetrabromobenzoate (TBB), and the remaining 60% is a mixture of triaryl phosphates including TPHP and several isomers of mono-, di-, and tri-isopropylated triaryl phosphates (ITPs) (Stapleton et al., 2009; Pillai et al., 2014). According to a recent review on organophosphate (OP) FRs, the annual production of TPHP was 20000–30000 tonnes for Western Europe in 2000, and 4500–22700 tonnes for United States in 2006 (van der Veen and de Boer, 2012), and this production volume is greater than most other OPFRs in current use in the same places (van der Veen and de Boer, 2012).

TPHP is an additive FR that is not chemically-bonded to polymer products, and is thus likely to be released into the environment over the life time of these products (van der Veen and de Boer, 2012). TPHP is frequently detected in various environment matrices (Stapleton et al., 2009; Salamova et al., 2014; Su et al., 2014b). For instance, Stapleton et al. detected TPHP at concentrations up to 1.8 mg/g in 98% of house dust samples collected from homes in the Boston, MA, USA area between 2002 and 2007, and these concentrations were higher than those of most of other FRs in the same matrix (Stapleton et al., 2009). TPHP was also detected at concentrations ranging from 42 ± 9 to 200 ± 27 pg/m^3 in particle phase samples collected at five sites in the North American Great Lakes basin from March 2012 to December 2012 (Salamova et al., 2014).

There remain considerable knowledge gaps for OP triester FRs in the environment including their fate, bioaccumulation potential, and toxicokinetics including deposition and biotransformation processes, that determine the internal burden in exposed organisms (van der Veen and de Boer, 2012). Numerous *in vitro* studies have been conducted to examine the TPHP metabolic pathways (Sasaki et al., 1984; Cooper and Stapleton, 2012; Van den Eede et al., 2013a). For example, in rat liver microsomes, 91% and 66% of TPHP was metabolized with or without nicotinamide adenine dinucleotide phosphate, respectively, and one dealkylated metabolite of TPHP, diphenyl phosphate (DPHP), was reported as the major metabolite (Sasaki et al., 1984). Cooper et al. suggested that the primary metabolism of TPHP to DPHP occurs via cleavage of an ester bond between the phosphate group and benzene ring (Cooper and Stapleton, 2012). However, a recent study proposed a more diverse *in vitro* metabolic pathway profile for TPHP when incubated with human liver microsomes or human liver S9 fractions, suggesting that hydroxylation might be another pathway of metabolism of TPHP (Van den Eede et al., 2013a). Based on *in vitro* chicken embryonic hepatocytes (CEH), we also found that hydroxylation occurs at the *para* (*p*)- and *meta* (*m*)-positions of the phenyl ring of TPHP, and *p*- or *m*-OH-TPHPs are rapidly conjugated to form *p*- and *m*-OH-TPHP glucuronides (Su et al., 2014a, 2015b). To our knowledge, the *in vivo* formation of OH-TPHPs or their glucuronides has not been reported for any organism including humans.

The objectives of the present study were to 1) develop a sensitive method for the determination of *p*- and *m*-OH-TPHP and their glucuronide conjugates in human urine samples; and 2) to then apply the developed method for quantification of these TPHP metabolites in a human urine samples.

2. Experimental section

2.1. Chemicals and reagents

The standards, *p*- and *m*-OH-TPHP isomers, were synthesized at Duke University in the Duke Small Molecule Synthesis Facility and prepared by Dr. David M. Gooden. The protocol of the synthesis of these two isomers was provided in our previous study (Su et al., 2015b).

For method development and determination of TPHP glucuronide conjugates in human urine, we prepared a *p*-OH-TPHP glucuronide standard by incubating *p*-OH-TPHP with chicken embryonic hepatocytes (CEH). The detailed protocol for the preparation of CEH cultures can be found in our previous publications (Lorenzen et al., 1993; Head et al., 2006; Porter et al., 2013; Farhat et al., 2014; Su et al., 2014c). In brief, twenty fertilized, unincubated white leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, Canada) and incubated for 19 days (37.5 °C, 60% relative humidity). On incubation day 19, the embryos were euthanized by decapitation and livers were removed, pooled, and treated with Percoll (GE Healthcare, Little Chalfont, UK) and DNase I (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The resulting cell pellet was suspended in 32 mL of Medium 199 (Life Technologies, Burlington, ON, Canada) supplemented with 1 μg/mL insulin and 1 μg/mL thyroxine (Sigma Aldrich, Oakville, ON, Canada). Twenty-five μL of the cell suspension was added to 500 μL of fresh supplemented medium in 48-well plates. The plates were incubated (37.5 °C and 5% CO_2) for 24 h prior to chemical administration and then CEH were treated with the 2.5 μL *p*-OH-TPHP stock standard/well with a final concentration of 10 μM. During the incubation, *p*-OH-TPHP and *p*-OH-TPHP glucuronide concentrations were monitored at the time points of 0, 1 h, 2 h and 4 h (Fig. S1). At the time point of 4 h when >80% of *p*-OH-TPHP was depleted (Fig. S1), CEH medium (containing *p*-OH-TPHP glucuronide) was collected and used as the stock solution during the investigation the deconjugation efficiency of *p*-OH-TPHP glucuronide in human urine samples.

2.2. Urine samples

In total, 13 human urine samples were analyzed in the present study, including 12 individual urine samples and 1 urine pool. The 12 individual urine samples were kindly provided by 4 Canadian (Ottawa, ON) resident volunteers (male, non-smokers) for 3 consecutive days. These urine samples were collected each morning by the volunteers, and into 50 mL pre-cleaned (450 °C for overnight) glass bottles, which were immediately stored at −20 °C until analysis. The pooled urine was a Standard Reference Material (SRM 3673) obtained from the National Institute of Standards & Technology (Gaithersburg, MD) and which was used by our