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First insights in the metabolism of phosphate flame retardants and plasticizers using human liver fractions

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HIGHLIGHTS

- First qualitative metabolism study on PFRs using human liver microsomes.
- Rates of substrate depletion were lowest for tris(2-chloroethyl) phosphate.
- Tris(dichloropropyl)phosphate metabolism was similar to *in vivo* rat studies.
- Triphenyl phosphate was mostly transformed by hydroxylation.
- Tris(chloropropyl) phosphate was mostly metabolized by oxidative dehalogenation.

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ABSTRACT

Phosphate flame retardants and plasticizers (PFRs) are additives used in a wide range of polymers. Important representatives, such as tris(2-butoxyethyl) phosphate (TBOEP), triphenyl phosphate (TPHP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCIPP), tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), have been found in the indoor environment at high levels. Biotransformation of these PFRs needs to be investigated because it can be a major determinant of their bioavailability and toxicity in humans. TBOEP, TPHP, TCEP, TCIPP and TDCIPP were incubated with human liver S9 fraction and microsomes. Supernatants were analyzed using a liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer. Chromatograms were scanned for the presence of Phase-I and Phase-II metabolites and tentatively identified based on mass accuracy of the molecular formula, isotopic pattern, and MS/MS spectra. The two major metabolites of TBOEP were products of *O*-dealkylation and of hydroxylation, respectively. TPHP was mainly transformed to its diester metabolite by *O*-dearylation and to a hydroxylated metabolite. TCEP was poorly metabolized into its diester and a product of oxidative dehalogenation. The major metabolite of TCIPP was a product of oxidative dehalogenation. TDCIPP was mainly transformed into its diester and a glutathione S-conjugate. The metabolites identified in the present study are candidate biomarkers for future human biomonitoring studies.

1. Introduction

Phosphate-containing flame retardants and plasticizers (PFRs) are widely used in plastics, upholstery, paints, *etc.* PFRs have been detected in several environmental matrices, including in indoor air and dust, and their levels are of growing concern (van der Veen and de Boer, 2012). While PFRs were reported under diverse abbreviations, we use the abbreviations suggested by Bergman et al. (2012) for the sake of uniformity. Several PFRs, including triphenyl phosphate (TPHP), tris(2-butoxyethyl) phosphate (TBOEP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCIPP), and tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) were found in Belgian indoor dust up to 74 μg/g (Van den Eede et al., 2011). As daily intake of PFRs may be higher than those of polybrominated diphenyl ethers (PBDEs) (Stapleton et al., 2009; Van den Eede et al., 2011; Dirtu et al., 2012), and long term health effects are not fully known, there is a need for human biomonitoring of PFRs.

Human exposure to PFRs can be a health concern because of the toxicity exerted by TCEP, TDCIPP and TPHP in animal studies. TCEP and TDCIPP are suspected carcinogens based on animal exposure studies (WHO, 1998) and TPHP is suspected to affect male fertility (Fang et al., 2003; Meeker and Stapleton, 2010). Although TBOEP and TCIPP are the most abundant PFRs in Europe, they were only studied for subchronic health effects in rodents (WHO, 1998, 2000; van der Veen and de Boer, 2012). TCIPP is structurally related to TDCIPP and TCEP, and could therefore cause similar effects. Biotransformation can be an important determinant

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of the toxicological effects and bioaccumulation of xenobiotics. However, a limited number of studies investigating the (*in vivo* or *in vitro*) biotransformation of TPHP, TCEP and TDCIPP is presently available (WHO, 1998; Cooper and Stapleton, 2011). Diesters, *e.g.* diphenyl phosphate (DPHP) and bis(1,3-dichloropropyl) phosphate (BDCIPP), were recently reported as important metabolites of TPHP and TDCIPP, respectively, produced by human liver microsomes (Cooper and Stapleton, 2011). *In vivo* rodent studies reported the structure of the metabolites of TCEP and TDCIPP in urine (WHO, 1998).

Further characterization of the biotransformation of TPHP, TBOEP, TCEP and TCIPP by human liver preparations is needed to better estimate the *in vivo* bioavailability, and bioaccumulation of these compounds in humans. The aims of the present study were (i) to investigate the oxidative metabolism of TPHP, TBOEP, TCEP and TCIPP by human liver microsomes (HLM) and human liver S9 fraction by monitoring the formation of Phase-I metabolites, such as those typically produced by cytochrome P450 enzymes (CYPs) and of Phase-II metabolites, which are typically produced by glucuronyl transferases (UGTs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs) and (ii) to identify (major) oxidative metabolites of TPHP, TBOEP, TCEP and TCIPP produced by human liver preparations as candidate markers of human exposure for future biomonitoring studies.

2. Materials and methods

2.1. Chemicals and reagents

Pooled human liver microsomes (HLM) (50 donors, mixed gender) and pooled human liver S9 fractions (4 donors, mixed gender) were purchased from Celsis In Vitro Technologies (Baltimore, MD, USA). TBOEP (94% purity), tris(hydroxymethyl)aminomethane (TRIS), β-nicotinamide adenine dinucleotide 2′-phosphate sodium salt hydrate (NADP), glutathione (GSH), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), adenosine 3′-phosphate 5′-phosphosulfate lithium hydrate (PAPS), and TPHP-d15 (internal standard, IS) were purchased from Sigma–Aldrich (Bornem, Belgium). Standards of TPHP, TCEP, and TDCIPP (mixture of 2 isomers) were purchased from Chiron AS (Trondheim, Norway). The standard for TCIPP (mixture of 3 isomers) was purchased from Pfaltz & Bauer (Waterbury, CT, USA). Internal standards TBOEP-d6, TCEP-d12, TDCIPP-d15 (all with purity > 98%) were synthesized by Dr. Vladimir Belov (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Acetonitrile was purchased from Merck KgA Chemicals (Darmstadt, Germany). Ultrapure water (18.2 MΩ) was obtained from an Elga LabWater water purification instrument (Saint Maurice, France).

2.2. In vitro biotransformation assay

To investigate the formation of Phase-I and Phase-II metabolites of TBOEP, TCEP, TCIPP and TPHP, reaction mixture containing 50 mM TRIS buffer (pH adjusted to 7.4 at 37 °C), HLM or human liver S9 fraction (1 mg/mL, final protein concentration) and TBOEP, TCEP or TCIPP (50 μM, final concentration) or TPHP or TDCIPP (20 μM, final concentration) in a total volume of 0.99 mL were preincubated in a shaking water bath at 37 °C. Lower substrate concentrations were used for TPHP and TDCIPP to avoid precipitation of these PFRs as they have a lower aqueous solubility (WHO, 1998; UNEP, 2002). The reaction was initiated by addition of a NADPH regenerating system (0.6 mM NADP, final concentration) and only for the S9 fraction samples, the following co-factors were also added individually: UDPGA, GSH (2 mM, final concentration) or PAPS (0.1 mM, final concentration). Alamethicin (50 μg/mL, final concentration) was also added to the reaction mixture containing the S9 fraction to increase membrane porosity and therefore facilitate the diffusion of the substrate to the membrane bound UGTs (Fisher et al., 2000). The reaction was quenched after 1 h by adding 1 mL of acetonitrile and storing the tubes on ice. A fixed amount of IS (375 ng) was added to each tube. As internal standards, TBOEP-d6 and BBOEP-d4 were used for the measurement of TBOEP and its metabolites, respectively; TCEP-d12 and BCEP-d8 were used for the measurement of TCEP, TCIPP and their metabolites; TDCIPP-d15 and BDCIPP-d10 were used for the measurement of TDCIPP and its metabolites, respectively; and TPHP-d15 and DPHP-d10 were used for quantifying TPHP and its metabolites, respectively.

Tubes containing the liver fractions, parent compound, acetonitrile and IS were then centrifuged for 10 min at 3500 rpm. Then, the supernatant was transferred to new tubes, concentrated to 1 mL by evaporation using N_2. The solution was then filtered through a 0.45 μm mesh size nylon filter into an HPLC vial. Blank and negative control samples were included in each assay. Cofactor negative control samples were prepared as described above, but devoided of the cofactor(s) specific of the enzyme families of interest. Enzyme negative control samples were prepared as described above, but devoided of the HLM or S9 human liver fraction, which was replaced by an equivalent volume of buffer. Blank samples were prepared as described above, but without cofactors and substrate, which were replaced by an equivalent volume of buffer. Blank and negative control samples were processed as described above.

2.3. LC-QTOF method

Phase-I and Phase-II metabolites of TPHP, TBOEP, TCEP, TCIPP and TDCIPP were identified and quantified by liquid chromatography–mass spectrometry (LC–MS) using a HPLC 1290 series coupled to a 6530 quadrupole-time-of-flight mass spectrometer (Q-TOF-MS) (Agilent Technologies). Chromatographic separation of the metabolites formed was achieved using a Zorbax Extend C18 column (50 mm × 2.1 mm × 3.5 μm, Agilent Technologies) and following mobile phase composition: 5 mM ammonium acetate in water (A) and methanol (B). Gradient elution was as follows: 10% B from 0 to 0.5 min, linear increase of B to 95%, from 0.5 to 10.5 min followed by isocratic elution at 95% B from 10.5 to 15.5 min. At 15.6 min solvent B was decreased to 10% and maintained at 10% from 15.6 to 20 min to re-equilibrate the column. The flow rate was 0.3 mL/min and the injection volume 10 μL. Total run time was 20 min.

The Q-TOF-MS was run in ESI positive and negative mode scanning m/z from 100 to 1000 amu at a scan rate of 1.25 spectra/s. The following MS parameters were used: gas temperature 300 °C, gas flow 8 L/min, nebulizer pressure 35 psi, sheath gas temperature 325 °C, sheath gas flow 12 L/min. Nozzle, capillary, fragmentor, and skimmer voltages were set to 500 V, 3000 V, 130 V, and 65 V, respectively. The instrument was calibrated during run times by monitoring positive ions with m/z 59.0604 and 922.0098 and negative ions with m/z 112.9856 and 1033.9881.

2.4. Database of the chemical structures and data analysis

Considering the chemical structures of the individual substrates, the family enzymes present in the human liver preparation used and activated by the addition of the corresponding cofactors and the reactions that these families of enzymes are known to catalyze, a database containing the molecular structure of all metabolites that can be theoretically formed was prepared. The Phase-I biotransformation reactions considered included hydroxylation, *O*-dealkylation, oxidative dearylation and reductive and oxidative dehalogenation (Levi et al., 1988; Testa and Krämer, 2010a). For Phase-II reactions, metabolites from Phase-I were presumed to be substrates for glucuronidation and sulfation. Because GSTs react primarily with electrophilic functions or substituents in molecules (Testa and Krämer, 2010b), TDCIPP, TCEP and TCIPP were considered to be possible substrates of GSH conjugation.

When analyzing the data, several criteria were formulated to confirm the identification of any metabolite: (i) the measured m/z value should be within 10 ppm of its calculated value; (ii) the isotope pattern should be matched within 7.5% of the predicted abundances; (iii) if the m/z was fragmented, its fragmentation pattern should be explainable. These criteria were not set as strict as "the seven golden rules" for comparing generated molecular formulas for unknown entities (Kind and Fiehn, 2007), because searching chromatograms against a database of formulas yields less identification hits per m/z than using non-targeted approaches.

3. Results and discussion

The LC/Q-TOF-MS provides high mass accuracy (typically 2–10 ppm error) and resolution (>20,000) which is necessary to generate a formula for an unknown compound with a given m/z value. Another advantage of the Q-TOF-MS is its ability to fragment compounds providing high resolution mass spectra of the product ions to confirm the generated formula. Another feature is the isotope pattern, which can be distinguished in low mass resolution MS as well. These patterns reflect the presence of naturally occurring heavier isotopes of common atoms such as ^{13}C, ^{2}H, ^{18}O, ^{37}Cl, ^{15}N, and ^{34}S.

The present study is the first to investigate the full range of Phase-I and Phase-II metabolites formed incubating five major PFRs (namely TBOEP, TPHP, TCEP, TCIPP and TDCIPP) individually with HLM and human liver S9 fractions. Because only standards for the five substrates and for their diester metabolites, but not for any of their putative Phase-I and Phase-II metabolites, are presently commercially available, quantitative data are reported only for the depletion of the substrates and the formation of diester metabolites. The formation of Phase-II and Phase-I metabolites other than diesters is reported here only qualitatively (based on peak areas). Also, it must be acknowledged that the metabolic pathways described here are only tentative, since incubations containing only