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Article

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Does biotransformation of aryl phosphate flame retardants in blood cast a new perspective on their debated biomarkers?

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Short running title: Biotransformation of aryl-PFRs by serum hydrolases

Competing financial interests

The authors declare to have no competing financial interests.

Abstract

Aryl phosphate flame retardants (aryl-PFRs), such as triphenyl phosphate (TPHP) and 2-ethylhexyl diphenyl phosphate (EHDPHP), are emerging contaminants that can exhibit toxic properties, including severe aquatic toxicity and endocrine disruptive effects. Monitoring exposure to aryl-PFRs through specific biomarkers is necessary to assess the health risk associated with chronic exposure. Hydrolytic serum enzymes could play an important role in the formation of the hydrolysis product diphenyl phosphate (DPHP), the seemingly most abundant *in vivo* biomarker of TPHP in urine. Here, we assess whether serum enzymes have an impact on the toxicokinetics of TPHP and EHDPHP and on the contribution of both aryl-PFRs to *in vivo* DPHP levels. TPHP and EHDPHP were incubated separately with pooled human serum to measure the formation of hydrolysis products DPHP and 2-ethylhexyl phenyl phosphate (EHPHP) by liquid chromatography-tandem mass spectrometry. Clearance of TPHP and EHDPHP was 70 and 8.6 mL/min/L serum (as measured by formation of DPHP and EHPHP, respectively). No discernible amount of DPHP was produced from EHDPHP by serum hydrolases. Our results suggest that serum hydrolases can significantly contribute to the *in vivo* levels of DPHP formed from TPHP and can play an important role in the toxicokinetics, toxicity, and selection of biomarkers for aryl-PFRs.

Introduction

Aryl phosphorus flame retardants (aryl-PFRs) and plasticizers are used in a large variety of consumer products (plastics, textile, paints, etc.) and their use is increasing as replacements for the recently banned polybrominated diphenyl ethers (PBDEs).[1] Despite their well-intended use to delay the spreading of fire and/or to improve the physicochemical properties of polymers, aryl-PFRs are chemicals of concern since they easily leach into the environment and can exert toxic properties. Recent evidence has appeared regarding endocrine disruptive effects of triphenyl phosphate (TPHP);[2-4] one of the most commonly used aryl-PFR (see Figure 1), raising suspicion against its use in consumer goods, such as electronics, curtains, and building materials.[1] Exposure to this chemical may also cause hepatotoxicity and reduced fertility.[5-7] Another ubiquitous aryl-PFR is 2-ethylhexyl diphenyl phosphate (EHDPHP), which, differently from TPHP, has one aliphatic side chain attached to the phosphate group (Figure 1). EHDPHP is used similarly to TPHP as an additive in vinyl polymers and also as plasticizer in food packaging.[8-9] Chronic exposure to EHDPHP could be also worrisome due to its potential for developmental toxicity.[9] For this reason, the Californian government has listed EHDPHP as a priority chemical for biomonitoring.[10]

TPHP is abundantly present in the indoor environment, including dust and air.[1] Considering the likely daily exposure via inhalation, dermal uptake, and inadvertent ingestion, more information regarding the presence and behavior of TPHP in the human body is needed. Previous research suggested that TPHP was mostly transformed to diphenyl phosphate (DPHP) in the liver by hydrolytic and oxidative enzymes,[11-12] and thus this metabolite was considered its major biomarker *in vivo*. Efforts to monitor human exposure to TPHP have revealed urine levels of more than 100 ng/mL of DPHP.[13-15] In contrast, the biomonitoring of EHDPHP exposure has not received much attention yet, despite the ubiquity and potential toxicity of this PFR. So far, the correlation between the exposure to TPHP via dust and the

presence of DPHP in human urine was significant in only one study.[14] Since several other aryl-PFRs share a common structure with TPHP and have shown to produce DPHP as a metabolite or degradation product,[16-17] it remains unclear which aryl-PFR exposure is reflected in the biological levels of DPHP. Additionally, the possible existence of unexplored biotransformation pathways of TPHP and other aryl-PFRs leading also to DPHP could be a confounding factor in the interpretation of urine levels of DPHP. For instance, when only its formation from TPHP by liver enzymes is considered, the estimated exposure to TPHP would be much higher than when the sum of several substrates and/or pathways are taken into account.

More toxicokinetic research is needed to provide a better exposure assessment to aryl-PFRs and to improve the understanding of the formation of DPHP as suitable biomarker by exploring the possibility of other sites of PFR metabolism in the human body, and more specifically blood. Serum enzymes, such as phosphate triester hydrolases (including paraoxonases, E.C.3.1.8.1), may also be involved in the hydrolysis of aryl phosphates considering the structural similarity between these PFRs and organophosphate pesticides, e.g. paraoxon.[18] Paraoxonases were demonstrated to be the major factor of paraoxon clearance in blood compared to irreversible binding to circulating cholinesterases[19] and may therefore also play an important role in the toxicokinetics of aryl-PFRs in the human body. Furthermore, aryl esterases in serum have also shown hydrolytic activity towards phosphate esters such as paraoxon.[20]

In the present study we aim to evaluate the extent of DPHP formation from aryl-PFRs by serum hydrolases by investigating TPHP and EHDPHP as representative compounds. Formation rates of the identified hydrolysis products of these two PFRs are presented in this paper. Special attention is given to the formation of DPHP to elucidate new pathways for the

generation of this possibly major aryl-PFR biomarker and to clarify the potential role of this additional biotransformation route in the toxicity and biomonitoring of TPHP.

Experimental section

Materials

Tris(hydroxmethyl) aminomethane (TRIS), $CaCl_2$, paraoxon, 4-nitrophenol, DPHP, and DPHP-d_{10} were purchased from Sigma Aldrich (Bornem, Belgium). TPHP was purchased from Chiron AS (>98% purity, Trondheim, Norway). 2-ethylhexyl phenyl phosphate (EHPHP) was synthesized by dr. Vladimir Belov (Max Planck Institute, Göttingen, Germany) as an ammonium salt (98% purity). EHDPHP was bought from Sigma Aldrich at a purity of 91%. Serum was collected in the frame of an ongoing study (registered at http://clinicaltrials.gov/ with number NCT01778868). This study was approved by the Ethical Committee of the Antwerp University Hospital (Belgian Registry number B30020097009) and all participants provided their written informed consent. Serum from 10 lean volunteers was pooled and stored at -20°C until analysis. Acetonitrile (analytical grade) was obtained from Merck KgA Chemicals (Darmstadt, Germany) and ultrapure water (18.2 MΩ) from an Elga LabWater water purification instrument (Saint Maurice, France).

Incubation methods

Assessing the linearity of serum concentration and incubation time. Optimization experiments were conducted similarly to our previous study.[21] Incubation mixtures consisted of 100 mM TRIS buffer (adjusted to pH 8.5 at 37 °C), 2 mM $CaCl_2$, and serum in a range of 0.5 to 3% concentration (v/v), final volume of 500 µL. Reactions were initiated by adding 20 or 100 µM of TPHP (in 1% acetonitrile, total volume). All samples were prepared in duplicate. Reactions were stopped by adding 250 µL of acetonitrile containing 25 ng of DPHP-d_{10} as internal

113 standard. A negative control was similarly prepared without the addition of serum. Samples
114 were vortexed and centrifuged, after which supernatants were filtered using a 0.22 μm nylon
115 filter. At both concentration levels and with 10 min incubation time, DPHP formation was
116 linear up to 2% serum (v/v). Different incubation times (3-20 min) were then tested with 20 or
117 100 μM of TPHP. At both concentrations, DPHP formation was linear up to 10 min.
118 Similar experiments were conducted for EHDPHP at 50 μM. A linear increase in EHPHP
119 production was observed up to 1% (v/v) serum. Using this serum concentration, the optimal
120 incubation time was 15 min. DPHP formation was also observed, with similar optimal
121 conditions for incubation time.
122
123 *Substrate concentration dependent kinetics.* TPHP (1-200 μM) was incubated in 100 mM
124 TRIS buffer (adjusted to pH 8.5 at 37 °C) and 2 mM $CaCl_2$ in a final volume of 500 μL at 37
125 °C. 10 μL serum (2% v/v) was added to initiate the reaction. For every concentration,
126 replicates were prepared. After 10 min, reactions were stopped by addition of 250 μL
127 acetonitrile containing 25 ng DPHP-d_{10} as internal standard. Samples were vortexed and
128 filtered using a 0.22 μm nylon membrane filter before analysis. The same incubation
129 experiments were carried out for EHDPHP, with slightly different values of serum
130 concentration (1%, v/v) and incubation time (15 min).
131 Negative controls (same preparation procedure, three replicates per substrate level) were
132 prepared by adding acetonitrile to the samples before the reaction was initiated in order to
133 account for chemical hydrolysis or impurities present in the standards. For all incubating
134 conditions, paraoxon was included in separate replicates at concentrations of 1 to 100 μM,
135 monitoring 4-nitrophenol as the target end product. The samples containing paraoxon were
136 both included for the purpose of positive control, and to have a comparison between kinetics
137 of enzymatic hydrolysis for an organophosphate pesticide and these two aryl-PFRs.

Procedural blanks were treated the same as the samples, except for the addition of 5 μL of pure acetonitrile instead of substrate, and were included in every batch, to exclude possible interferences by contamination.

Serum hydrolase activity. The hydrolase activity of the pooled serum employed in this study was tested by assessing the formation of 4-nitrophenol. Paraoxon (1 mM final concentration) was incubated in triplicate in 100 mM TRIS buffer (adjusted to pH 8.5 at 37 °C) and 2 mM $CaCl_2$ in a final volume of 500 μL at 37 °C. 5 μL serum (1% v/v) was added to initiate the reaction. Since enzyme reaction rate is dependent on substrate concentration, the same incubation conditions and substrate concentration were used as described by Furlong et al.[20], so that specific activity of serum paraoxonase could be compared with literature. Negative controls containing the same mixture were prepared simultaneously, but with the addition of the internal standard DPHP-d_{10} (25 ng) in 250 μL acetonitrile before incubation (so that the reaction was not initiated). All samples except the negative controls were quenched after 5 min by the addition of the same amount of internal standard solution.

Analytical Quality Control. Matrix-matched calibrations were prepared to account for possible matrix effects on the signal response of EHPHP and 4-nitrophenol (for which isotopically labelled standards were not used, instead DPHP-d_{10} was employed). Calibration curves were prepared for all analytes in the same mixture of buffer, solvent and serum as the samples and the negative controls and were analyzed under the same conditions.

Instrumental analysis

DPHP, EHPHP, and 4-nitrophenol were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1290 LC coupled to a 6460 triple quadrupole

MS. A Kinetex Biphenyl column (50 x 2.1 mm, 1.7 µm, Phenomenex) was used for the separation of the extracts. For DPHP and EHPHP analysis, the aqueous mobile phase consisted of 5 mM ammonium acetate in water at pH 5.5 (A), while for 4-nitrophenol the aqueous mobile phase (of 5 mM ammonium acetate) was acidified with acetic acid until pH 4.5. Acetonitrile was used as organic solvent (B) for all analyses. The following gradient conditions were applied: 5% B (1.5 min hold), increase to 20% B in 0.5 min, increase to 60% B in 2 min, followed by a sharp increase to 95% B in 0.8 min (4 min hold), and equilibration at starting conditions for 3 min. Flow rate was 0.3 mL/min, temperature 40 °C, injection volume 1 µL. MS parameters for the analysis of serum extracts were: gas temperature 300 °C, sheath gas heater 250 °C, gas flow 5 L/min, sheath gas flow 11 L/min, nebulizer pressure 45 psi, capillary and nozzle voltage 3000 and 500 V, respectively. MRM transitions are provided in Table S1. Cell accelerator voltage was 4 V.

Data analysis

Regression analysis for each metabolite formation rate was applied using GraphPad Prism 5.0. The following models were tested for the calculation of metabolite formation kinetics: linear regression (on truncated datasets), Michaelis-Menten equation, and substrate inhibition model. No weights were applied for the regression analysis. An F-test was used to detect if one of the models fit significantly better, and the simplest model was retained if there was no significant difference in fit ($p > 0.05$).

$$v = \frac{V_{\max} \times [S]}{K_m + [S]} \qquad \text{(Michaelis-Menten model)}$$

$$v = \frac{V_{\max}}{1 + K_m/[S] + [S]/K_i} \qquad \text{(substrate inhibition model)}$$

Results

Selection of main hydrolysis products

4-nitrophenol was selected as target end product of the positive control based on literature,[20] while DPHP and EHPHP (Figure 1) were selected based on their detection in *in vitro* liver assays[12,22] and their resemblance to hydrolysis products of organophosphate pesticides. For TPHP and EHDPHP, we rather monitored the phosphate diester as end product, because phenol and 2-ethylhexanol are less specific for use as biomonitoring target in *in vivo* studies.

Serum hydrolase activity determination

The activity of serum hydrolases was determined by the formation of the metabolite 4-nitrophenol from paraoxon. In the procedural blanks, 4-nitrophenol was not detected, although a minor amount of 4-nitrophenol was found in the negative controls. This amount was subtracted from the measured concentration in the positive control samples. In every tested batch of TPHP or EHDPHP hydrolysis experiments, the formation of 4-nitrophenol from paraoxon was detectable and constant.

Following the conditions described in the literature by Furlong et al.[20] for paraoxonase, namely 5 min and 1% (v/v) serum, the activity of the serum employed in this study was calculated as 221 ± 35 U/L (1 unit = 1 μmol paraoxon hydrolyzed per min). We also tested optimal incubation conditions for paraoxon as we did for TPHP and EHDPHP, observing that net 4-nitrophenol formation increased linearly with longer incubation times or higher serum concentrations, up to 10 min and 2% (v/v) serum. Because of good repeatability, these conditions were used to calculate the rate of 4-nitrophenol formation from 1 to 100 μM of paraoxon. In contrast to the hydrolysis products of TPHP and EHDPHP, 4-nitrophenol showed a linear formation pattern using substrate levels of 1 to 100 μM.

Comparative hydrolysis rates of the three organophosphates

Hydrolysis rates of TPHP, EHDPHP, and paraoxon were compared at low substrate concentrations (in the linear range of 1 to 10 μM), since most realistic exposure scenarios to flame retardants involve chronic, low dose exposure. An F-test revealed that the slopes of the regression line for each dataset were significantly different ($p < 0.001$), with slopes (95% confidence interval) of 70 (58 - 82), 8.6 (7.3 - 9.9), and 485 (468 - 503) mL/(min L serum) for DPHP, EHPHP, and 4-nitrophenol, respectively. These slopes roughly indicate the extent of *in vivo* clearance in blood. The data of DPHP and EHPHP are shown in Figure S1.

Kinetic profile of TPHP hydrolysis by serum

Formation of DPHP, as hydrolysis product of TPHP, increased linearly with increasing time or serum concentration up to 10 min and 2% (v/v) serum. However, DPHP was also observed in the enzyme-deactivated negative controls, which may have been due to chemical hydrolysis.[23] Therefore, DPHP levels in enzyme-deactivated negative controls were subtracted from the levels measured in the samples to obtain net enzymatic hydrolysis rates. The highest concentration (200 μM) was omitted, because TPHP proved to be insoluble in the buffer at this level. Regression analysis indicated Michaelis-Menten kinetics were the most suitable model for DPHP formation (Figure 2), with an extrapolated K_m of 108 ± 30 μM and V_{max} of 8.16 ± 1.41 μmol/min/L serum. Clearance, calculated as V_{max}/K_m, was 75.6 mL/min/L serum, which is comparable to the value of 70 mL/min/L serum mentioned above.

Kinetic profile of EHDPHP hydrolysis by serum

EHPHP was identified as the main hydrolysis product of EHDPHP by serum enzymes, while formation of DPHP was in any case minor (see below). Optimization of the initial rate conditions showed a clear linear EHPHP formation rate up to 15 min at 1% (v/v) serum.

When plotting the rate of EHPHP formation versus EHDPHP concentration, a clear decline was observed at the highest tested concentration suggesting substrate inhibition kinetics (Figure 3). When applying non-linear regression to the data using both the substrate inhibition model and the Michaelis-Menten equation, the latter was preferred because more precise estimates of K_m, V_{max} were obtained based on the available data points. V_{max} was 0.273 (SD 0.030) µmol/min/L serum, while K_m was 13.1 (SD 4.7) µM. However, when the highest level was removed from the dataset, the V_{max} and K_m values increased up to to 0.41 µmol/min/L serum and 29 µM, respectively. SDs of these non-linear regression parameters increased to 0.12 µmol/min/L serum and 17 µM, indicating less precise estimates.

The formation of DPHP in EHDPHP preparations was corrected for its presence in the negative control samples, with rates ranging from 0.025 µmol/min/L serum at 2.4 µM EHDPHP to 0.28 µmol/min/L serum at 114 µM EHDPHP. However, since TPHP was present in the EHDPHP standard (3.3% impurity), DPHP could originate both from EHDPHP and from the TPHP impurity. To clarify if the formation of DPHP in the EHDPHP preparations was originating only from the TPHP impurity, we compared the slope of the regression of DPHP formation (32 mL/min/L serum, 95% confidence interval - 0.56 to 70 mL/min/L serum) versus the calculated level of TPHP present as impurity in the EHDPHP preparations (2.4-20 µM) with the slope obtained from the TPHP-DPHP regression data explained in section 3.2 (70, 95% confidence interval: 58.3 to 81.7 mL/min/L serum). As such, the slope of DPHP in EHDPHP samples was significantly lower than in TPHP samples (F-test, $p < 0.001$). The position of average DPHP formation in the lower tested levels of EHDPHP (2.4-20 µM) and TPHP (1-2.5 µM) are shown in Figure S2. Furthermore, we compared at the different tested levels of EHDPHP if the measured rate of DPHP was similar to its predicted range (calculated based on the concentration of TPHP as impurity). This was the case for all tested levels of EHDPHP starting from 20 µM on. Only at the lower level of 2.4 µM of EHDPHP, at

which the concentration of TPHP as impurity (0.1 μM) could be too low to detect its hydrolysis product, DPHP formation was higher, respectively, than the predicted range (10 versus 5.8 to 8.1 μmol/min/L serum). These data suggest possible additional production of DPHP from EHDPHP which would occur at a much lower rate than from TPHP. At lower levels than 2.4 μM of EHDPHP, the overall produced amount of DPHP was below the quantification limit (1 ng/mL).

Discussion

Serum hydrolase (including paraoxonase) activity

The results of our study indicate that hydrolysis by serum enzymes takes place for the two aryl-PFRs. While chemical hydrolysis was reported to occur for TPHP and EHDPHP at alkaline pH,[23] the levels of DPHP and EHPHP in the samples incubated with active serum enzyme exceeded those of the negative controls with deactivated serum enzymes. This means that the enzymatic hydrolysis rate exceeded the chemical hydrolysis rate. Lower clearance was observed for EHDPHP (8.6 mL/min/L serum) and TPHP (70 mL/min/L serum) compared to paraoxon (485 mL/min/L serum). Therefore *in vivo* serum enzymes could degrade aryl-PFRs albeit to different extents. Not only the structural differences between the compounds can play a role here, but also the presence of enzyme polymorphisms. For instance, the assay we used was optimized for measuring the activity of the enzyme paraoxonase, which is known to be dependent on the functional genotype.[24] In this study, we used pooled serum because of ethical reasons as a tool to investigate the general role of serum in biotransformation of aryl-PFRs in the human body and DPHP formation. Our data therefore represent the average biotransformation rate in serum from a group of volunteers.

Formation of DPHP from TPHP and EHDPHP

When considering the two potential hydrolysis products of EHDPHP, the discrepancy between the different tested levels in the likeliness of EHDPHP contributing to DPHP formation may have several reasons. Firstly, at 2.4 μM EHDPHP, the concentration of TPHP (~0.1 μM) was well below the lowest tested level (~1 μM) in the TPHP test samples. Extrapolation of the regression would thus lead to a larger uncertainty in the predicted formation of DPHP from the TPHP impurity. Secondly, since we observed indications of substrate inhibition effects for EHPHP formation, these effects might also influence DPHP formation from EHDPHP negatively. Taking this into account, it is difficult to estimate any significant contribution in DPHP formation from EHDPHP by serum enzymes. However, we emphasize that at levels of EHDPHP < 10 μM, where the amount of the TPHP impurity may be insignificant to contribute to DPHP formation, the observed minor amount of DPHP could come mainly from EHDPHP. This could suggest a considerably lower DPHP formation rate from EHDPHP than from TPHP that is masked by the impurity at higher levels. A similar observation was reported using oxidative liver enzymes.[22] Furthermore, the preferred hydrolysis product in serum appears to be EHPHP with phenol as leaving group, which is logical considering that in paraoxon hydrolysis by serum enzymes the preferred leaving group is also aromatic (4-nitrophenol). This is also in agreement with a recent study that investigated hydrolysis of EHDPHP in aqueous solutions at alkaline pH, where they reported EHPHP as degradation product and not DPHP.[23] One issue we did not account for was the possible contribution to EHPHP formation by another impurity, namely bis(2-ethylhexyl) phenyl phosphate, that was reported by Ballesteros-Gomez et al.[22] However, it should be noted that this impurity can only contribute by producing 2-ethylhexanol as leaving group, which is a less favorable reaction compared to the production of phenol.

Implications for the toxicokinetics, toxicity, and biomonitoring of aryl-PFRs

Given the observed activity of hydrolytic enzymes in serum towards TPHP and EHDPHP, the stability of these PFRs in the human body circulation may be limited, especially in the case of TPHP. This would have several consequences in the field of toxicokinetics, toxicity, and biomonitoring of TPHP and other aryl-PFRs:

1. Less TPHP is available for distribution (and accumulation) in tissues;

2. The amount of TPHP that reaches the liver (after uptake from the gastro-intestinal system, lungs, or skin) in its original form may be strongly reduced. For this reason, the *in vivo* metabolite profile of TPHP can differ from that observed in *in vitro* studies using liver cells or subcellular fractions. In other words, the metabolite 4-HO-TPHP (with its glucuronide and sulfate conjugates), that was found to be the major or second major TPHP metabolite,[12,25-26] could be much less abundant in biological matrices, such as blood and urine, than DPHP. In addition, there is no information whether 4-HO-TPHP would be more stable towards serum enzymes compared to TPHP. Recently, 4-HO-TPHP-(glucuronide) was evidenced in human urine from Canadians[27] at much lower concentrations than DPHP. As the authors suggested, the discrepancy with 4-HO-TPHP being the major metabolite formed by hepatic enzymes could be due to either *in vitro – in vivo* differences or to contributions to DPHP formation by other aryl-PFRs, such as EHDPHP (to a minor extent) and resorcinol bis(diphenyl phosphate). However, we recommend that also the role of serum enzymes should not be overlooked in the study of toxicokinetics of aryl-PFRs.

3. Our findings are also important for the interpretation of *in vitro* toxicity tests. On the one hand, serum enzymes could play a role in the detoxification of TPHP since interactions that were found between nuclear receptors and TPHP[1,28] could be occurring only to a very minor extent because of the limited stability of TPHP in the circulation. Furthermore, DPHP is, because of its high polarity, probably cleared faster from the human body and less capable of penetrating tissues by passive diffusion to cause harm. Next, Kojima et al.[29] demonstrated

that the 4-HO-TPHP metabolite expressed similar toxicity compared to its parent. Therefore, the reduced formation of 4-HO-TPHP in the liver due to earlier hydrolysis of TPHP to DPHP in the blood, suggests that this process could be considered as detoxification. On the other hand, we still do not have full knowledge how TPHP induces toxic effects in various organs. Moreover, Su et al.[6] discovered that in chicken embryonic hepatocytes, toxic effects were mostly correlated to DPHP. Therefore, bio-activation by serum enzymes cannot be ruled out.

4. All of the aforementioned points could also apply to EHDPHP and its hydrolysis product EHPHP. However, for this aryl-PFR the impact of serum enzymes on its toxicokinetic and toxicodynamic properties and biomonitoring targets would be less pronounced since the enzymatic activity was relatively low. We do emphasize however, that the metabolites of EHDPHP were only recently confirmed in human tissues and no assessment has yet been made of the toxicity of the transformation products, including EHPHP.

Implications for selecting biomarkers of exposure

DPHP has become heavily debated as biomarker for measuring TPHP exposure in e.g. human urine samples because it likely lacks specificity. Both results of our current and previous study could not evidence a significant contribution from EHDPHP to the presence of DPHP in biological tissues.[22] This indication of specificity is a welcome finding for the discussion of which TPHP metabolite should be monitored, since 4-HO-TPHP (sum of the free form and its glucuronide) was not detected in urine [15] or at very low levels.[27] Therefore, DPHP may be a useful biomarker of exposure to TPHP alongside with total 4-OH-TPHP so that low exposure can be monitored (using DPHP). In subjects with high urinary DPHP concentration, total 4-OH-TPHP levels might help to confirm high exposure to TPHP. However, since another study[17] demonstrated that DPHP is present as impurity in resorcinol bis(diphenyl)phosphate (RDP or PBDPP) formulations and it can be also formed by the spontaneous hydrolysis of

this compound at physiological pH and body temperature, DPHP is recommendable to be used as human biomarker for aryl-PFR exposure rather than for TPHP only. Additionally, our findings can be used as a recommendation for using EHPHP as an alternative biomarker of EHDPHP exposure, in addition to the oxidation products that were reported in hepatic metabolism.[22]

In conclusion, serum enzymes were shown to be involved in the biotransformation of TPHP and EHDPHP in humans which could affects the metabolite profile, toxicokinetics, and toxicity of both aryl-PFRs. The formation of DPHP from EHDPHP could not be estimated and was minor in any case, which implies that EHDPHP is not likely a confounding factor in the interpretation of DPHP levels in human body fluids and tissues.

Further research is needed to estimate inter-individual differences in the clearance of aryl-PFRs in blood and to determine the extent of the impact of serum hydrolases on the kinetics and toxicity of these PFRs and their metabolites in humans. Furthermore, the availability of an EHDPHP standard of higher purity to assess the possible formation of DPHP as a minor hydrolysis product would be highly desirable.

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Supporting information

Additional information including Table S1 and Figures S1 and S2 can be found in the supporting information. This information is available free of charge via the Internet at http://pubs.acs.org/.

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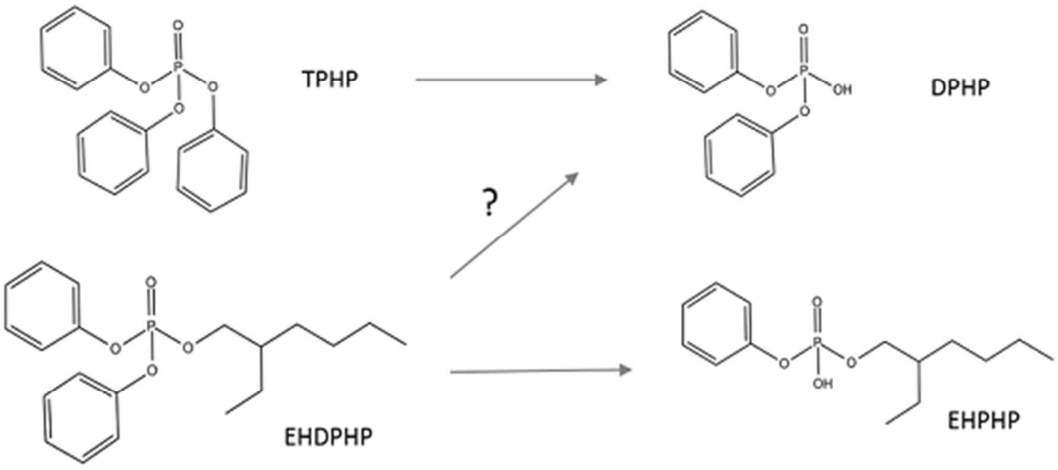
Figure legends

Figure 1. Structures of 2-ethylhexyl diphenyl phosphate (EHDPHP), triphenyl phosphate (TPHP) and their hydrolysis products, ethylhexyl phenyl phosphate (EHPHP) and diphenyl phosphate (DPHP)

Figure 2. Kinetics of triphenyl phosphate (TPHP) hydrolysis to its diester structure (diphenyl phosphate, DPHP) by serum enzymes.

Figure 3. Kinetic formation of 2-ethylhexyl phenyl phosphate (EHPHP) by serum enzymes in relation to concentration of 2-ethylhexyl diphenyl phosphate (EHDPHP).

Figures



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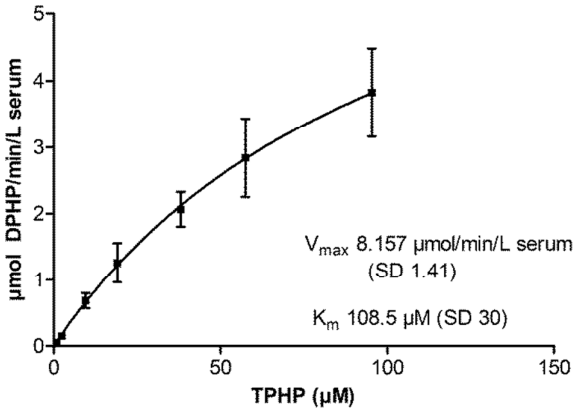

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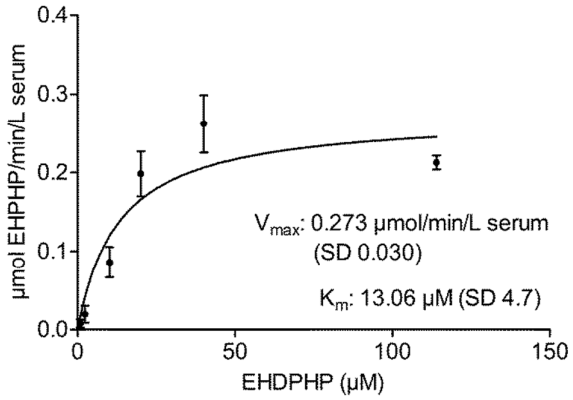

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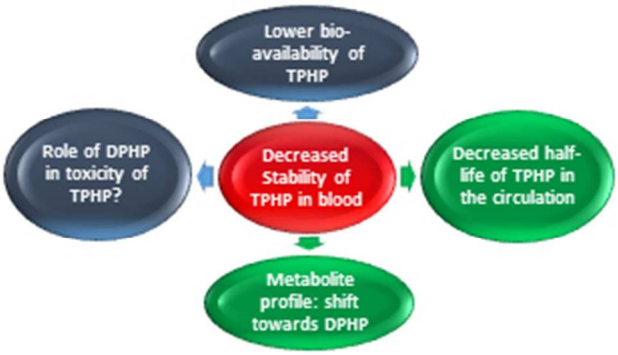

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