Optimizing human biomonitoring targets for environmental contaminants based on *in vitro* metabolism experiments- a case study of flame retardants

Nele Van den Eede[1], Hugo Neels[1,2], Adrian Covaci[1]
1 - University of Antwerp – Department of Pharmaceutical sciences - Toxicological Center
2 - Antwerp University Hospital (UZA) – Clinical Laboratory
Belgium

Scope of the lecture:

This lecture will demonstrate several approaches of *in vitro* metabolism (or biotransformation) experiments and how these can be applied to improve the biomonitoring strategy for emerging contaminants when few toxicokinetic data are available.

Learning objectives:

1. Human exposure to environmental contaminants can be monitored via measurements of possible sources or via measurements of biological matrices, such as urine. The latter, however, requires knowledge of toxicokinetics (absorption, distribution, elimination, excretion) and biotransformation pathways

2. Biotransformation pathways can be investigated *in vitro* or *in silico*. For the design of *in vitro* experiments a careful selection of the material should be made while keeping in mind the costs and required expertise versus the desired output (generalizability of data) and predicted pathways.

3. *In vitro* biotransformation data can be applied to adjust biomonitoring methods, yet in vitro results are not completely transferable. Other toxicokinetic processes may influence the *in vivo* situation.

Extended abstract:

Background

Human exposure to emerging environmental contaminants is often understudied and thus poorly understood. The safety of these chemicals regarding human health is not completely known either, despite their potential use in everyday consumer products. For example, organophosphate flame retardants (PFRs) were considered safe additives, and have been increasingly used over the last decades, leading to their ubiquitous presence in the indoor environment. Several possible routes of exposure in the indoor environment have been identified, but the relative contribution of each route remains uncertain which renders estimating daily exposure challenging. Characterizing human exposure to PFRs by biomonitoring is an alternative approach, though it proved to be difficult because of a lack of data on their biotransformation and toxicokinetic pathways in humans. Until a few years ago, hydrolysis products (phosphate diesters) were assumed to be the major biotransformation products[1]. In a few studies, only a minority of the PFRs had detectable levels of their diester product in human urine. As *in vivo* and *in vitro* evidence suggested good absorption[1], we aimed to re-evaluate the biomonitoring targets used for measuring PFR exposure. In this study, *in vitro* experiments were performed to identify biotransformation products and confirm these in a biomonitoring study. We focused on the cytochrome P450 enzymes in human liver microsomes and used human hepatocytes as a model simulating in vivo conditions more closely. Since phosphate triester structures are possible substrates of serum hydrolase enzymes we also performed experiments using human serum to detect such activity.

Methods

High grade purity standards of triphenyl phosphate, tris(2-butoxyethyl) phosphate, and tris(2-chloro-1-propyl) phosphate were incubated with human liver microsomes and cofactors for phase I and II reactions at pH 7.4 and 37°C for one hour[2]. The three PFRs were in later experiments incubated with primary human hepatocytes[3] or human serum at 37°C. Reactions were quenched by addition of organic solvent and mixtures were analyzed using liquid chromatography quadrupole-time-of-flight mass spectrometry.
Biotransformation products were identified using non-targeted and targeted approaches. The most abundant hypothetical structures for the biotransformation products were confirmed by analysis of custom synthesized standards.
As second part of the project, a method was optimized for analyzing these biotransformation products in urine using the custom synthesized standards[5]. The method consisted of enzymatic deconjugation (for glucuronide and sulfate conjugates), offline solid phase extraction and liquid chromatography-tandem mass spectrometry. A total of 95 pools of surplus pathology urine samples, which had been stratified and pooled by age and sex (3224 individuals aged 0 to 75 years), were subsequently analyzed.

Results

The information obtained from the biotransformation experiments varied in its implication for the most appropriate biomarker of exposure.
Triphenyl phosphate:
Several new biotransformation products were identified using human liver microsomes

(figure 1).[2] All products could be confirmed in human hepatocytes, except for TPHP-M7 and its respective glucuronyl and sulfate conjugates TPHP-M2 and M4.[3]
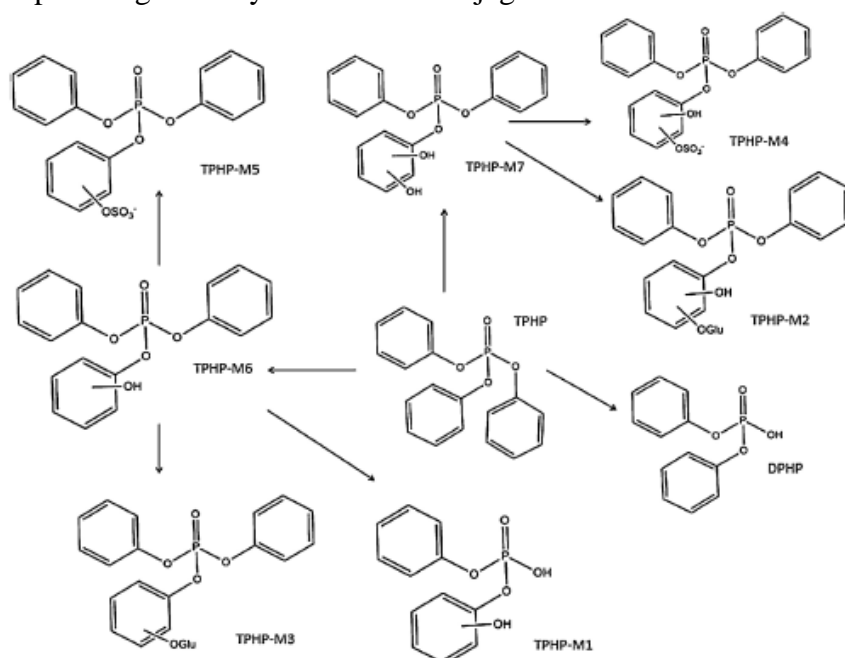

Figure 1. Biotransformation pathways of triphenyl phosphate (TPHP) in human liver microsomes. DPHP: diphenyl phosphate. TPHP-M3,M4,M6 and DPHP were included as biomonitoring targets.

After incubation with human serum, formation of DPHP, the conventional diester product was observed.[4] In urine samples, the only biotransformation product detected was DPHP, which was the conventional biomarker.[5] Possibly this is due to biotransformation by the serum enzymes.

In this case the results from the biotransformation experiments eventually did not impact the selection of the biomonitoring target. However, for this PFR the conventional biomonitoring target was also frequently detected in previous studies.[1]

Tris(2-butoxyethyl) phosphate:
In the incubations with human serum, no formation of the diester BBOEP could be detected. Two of the newly identified (hepatic) biotransformation products were produced *in vitro* to a similar or even greater extent than BBOEP in hepatocytes and were therefore included as new biomonitoring targets[2-3]. The most abundant hepatic biotransformation product, a result of an oxidative O-dealkylation, was detected in the majority of the urine extracts. A hydroxylation metabolite was found in all human liver enzyme experiments, but could not be detected in any urine sample.[5] Considering the larger number of detects for the O-dealkylation product compared to the conventional target BBOEP, with respectfully a detection frequency of 85 and 4%, the inclusion of this new biomarker can be beneficial to future biomonitoring studies.

Tris(2-chloro-1-propyl) phosphate :
In the incubations with human serum, no formation of the diester BCIPP could be detected.[6] For tris(2-chloro-1-propyl) phosphate one product of oxidative dehalogenation, was formed to a lower extent than the conventional diester product BCIPP (figure 2).[6] Due to technical issues, only the oxidative dehalogenation product, was included as biomonitoring target for urine analysis. Its detection frequency was 100%,[5] whereas that of the conventional target had been maximum 30% in previous studies. By targeting this new biomarker, more data on

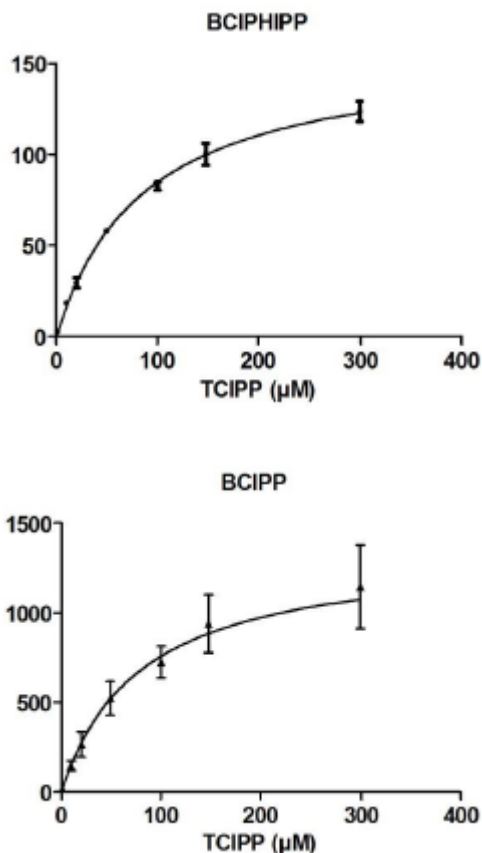human exposure could be generated.

Figure 2: enzymatic formation (pmol/min/mg microsomal protein) of the conventional (below) and the oxidative dehalogenation product (above) of tris(1-chloro-2-propyl) phosphate

Conclusions

Here, we demonstrated the benefit of *in vitro* screening for biotransformation products in cases when few toxicokinetic data are available. *In vitro* experiments can especially be useful for finding alternative options when biomonitoring targets are less frequently detected than expected, or when the current targets pose analytical challenges.
However, translation to the *in vivo* situation may still be unpredictable. Several toxicokinetic processes other than biotransformation should also be considered when selecting the optimal biomonitoring target.

References

1: Hou R, Xu Y, Wang Z. Review of OPFRs in animals and humans: Absorption, bioaccumulation, metabolism, and internal exposure research. Chemosphere. 2016;153:78-90
2: : Van den Eede N, Maho W, Erratico C, Neels H, Covaci A. First insights in the metabolism of phosphate flame retardants and plasticizers using human liver fractions. Toxicol Lett. 2013 ;223(1):9-15
3: Van den Eede N, de Meester I, Maho W, Neels H, Covaci A. Biotransformation of three phosphate flame retardants and plasticizers in primary human hepatocytes: untargeted metabolite screening and quantitative assessment. J Appl Toxicol. 2016;36(11):1401-8

4: Van den Eede N, Ballesteros-Gómez A, Neels H, Covaci A. Does Biotransformation of Aryl Phosphate Flame Retardants in Blood Cast a New Perspective on Their Debated Biomarkers? Environ Sci Technol. 2016 ;50(22):12439-12445

5: Van den Eede N, Heffernan AL, Aylward LL, Hobson P, Neels H, Mueller JF, Covaci A. Age as a determinant of phosphate flame retardant exposure of the Australian population and identification of novel urinary PFR metabolites. Environ Int. 2015 ;74:1-8

6: Van den Eede N, Tomy G, Tao F, Halldorson T, Harrad S, Neels H, Covaci A. Kinetics of tris (1-chloro-2-propyl) phosphate (TCIPP) metabolism in human liver microsomes and serum.Chemosphere. 2016;144:1299-305