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ASSESSING THE HUMAN INTERNAL EXPOSURE TO PFRS VIA URINE AND SERUM: METHOD VALIDATION STUDY

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Introduction

Flame retardants (FRs) are commonly added to construction materials and consumer products to meet fire safety criteria and regulations in each country[1,2]. Polybrominated diphenyl ethers (PBDEs) were the best known and widely used FRs, until they were gradually and globally phased out due to their persistency and toxicity[2,3]. In recent years, many emerging FRs, such as organophosphate esters (PFRs), have replaced the market-share of PBDEs. PFRs have different persistency, toxic and bioaccumulative properties compared to PBDEs[1]. They are commonly added in textiles, electronic items, lubricants, paintings, etc[1,2]. PFRs were frequently reported in indoor air, dust, hand wipes and other matrices[1]. Some PFRs were suspected to be toxic, including being carcinogenic, mutagenic and harmful on hormonal balance in humans [4]. PFRs have a less persistent character and have lower log Kow than PBDEs, being easier to be metabolized and further excreted via urine[1,5,6]. Several studies have reported the presence of PFR metabolites in urine and even found moderate correlation between those metabolites and their parent compounds in air, dust or handwipes[3,4,6]. In vivo studies suggested that human liver could metabolize PFRs into diesters and hydroxylated PFR metabolites (HO-PFRs) [7,8]. 2-ethylhexyl diphenyl phosphate (EHDPHP) is used in food packaging, which have been found in food in our parallel studies, have been reported to be very toxic to aquatic organisms and mammals and subjecting to phase I metabolism by human liver [8]. However, no method has been developed for analyzing PFR metabolites in serum so far, and several aspects of the previously developed method in urine needed improving. Hereby, we present a method for this purpose.

Materials and Methods

Materials

Bis(2-chloroethyl) phosphate (BCEP), bis(1-chloro-2-propyl) phosphate (BCIPP), BCEP-D8, 4-hydroxyphenyl diphenyl phosphate (4-HO-TPHP), 3-hydroxyphenyl diphenyl phosphate (3-HO-TPHP), bis(2-butoxyethyl)3'-hydroxy-2-butoxyethyl phosphate (HO-TBOEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), bis(2-butoxyethyl) phosphate (BBOEP), 4-hydroxyphenyl phenyl phosphate (HO-DPHP), 2-hydroxyethyl bis(2-butoxyethyl) phosphate (BBOEHEP), 1-hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP, 2 isomers), 2-ethyl-5-hydroxyhexyl diphenyl phosphate (HO-EHDPHP); ethylhexyl hydroxy phenyl phosphate (EHPHP); BBOEP-D4, BBOEHEP-D4, BDCIPP-D10, TCEP-D12 and TBOEP-D6 were custom synthesized by Dr. Vladimir Belov (all purity>95%; Max Planck Institute, Germany). TCEP was purchased from Chiron AS (Trondheim, Norway). Dibutyl phosphate (DNBP), diphenyl phosphate (DPHP), DPHP-D10, and TPHP-D15 were purchased from Sigma-Aldrich (Bornem, Belgium). Other materials were purchased from different suppliers, including ammonia acetate, sodium chlorine (NaCl), methanol (MeOH, analytical grade), hydrogen chloride acid (HCl, 37%) and formic acid (98%) from Merck; β-glucuronidase (from E.coli, >10 000 000 unit/g, lyophilized powder) and DSC-18 sorbent from Sigma-Aldrich; 3mL empty Bond Elut cartridges purchased from Agilent. Urine and serum samples were kindly donated by volunteers from University of Antwerp (Belgium). Horse serum was used as a surrogate matrix for validation.

Sample preparation

0.5 mL of urine or serum was mixed with 0.375 mL of phosphate buffer (1 M, pH=6), adding 25 µl of beta-glucuronidase enzyme (5 mg/mL in 50 mM phosphate buffer, pH=6.8) in a glass test tube. The mixture was vortexed, then incubated for 3 h at 45 °C. After that, internal standards were spiked (and standards for recovery test) in to all samples. During incubation, 200 mg DSC-18 sorbent was packed in cartridge, and cartridges were wash with 3 mL MeOH and 2 mL of MilliQ water.

Urine samples were spiked with 25 µL formic acid to adjust pH to 4-5 and to cease the incubation, vortexed and loaded to cartridges. 0.5 mL of water was added to the tube, vortexed, and loaded to the cartridge. Serum samples were added with 20% NaCl solution, vortexing. Then, 30 µL of HCl (37%) was spiked to stop the incubation and to precipitate proteins. After being placed for 2 min, the serum tube was slowly shaken or vortexed for homogenization, followed by 5-min-centrifugation at 3500 rmp.

Later, the supernatant was carefully transferred into the DSC-18 cartridge. The precipitated protein was further washed with 0.5 mL MilliQ water, vortexed, centrifuged. The supernatant was transferred to the same cartridge.
For both urine and serum samples, another 1.5 mL of MilliQ water was add to the cartridge to remove the matrix. Before loading 3 mL MeOH for elution, water was pushed out from cartridges. The eluent was further blow down to nearly dryness under gentle nitrogen stream and reconstitute in 100 μL H2O:MeOH (1:1). After filtration, the final extract was ready for instrumental analysis.
Instrumental analysis
Instrumental analysis was performed on an Agilent 1290 Infinity liquid chromatography system coupled to an Agilent 6460 Triple Quadrupole mass spectrometer (LC-MS/MS, Santa Clara, CA, USA), equipped with a Jetstream® electrospray ionization (ESI) ion source. A volume of 5 μL of extract was injected on a Phenomenex Kinetex Biphenyl reversed phase column (2.1 x 50 mm, 1.7 μm; Torrance, CA, USA), at a column oven temperature of 40 °C. The mobile phases were A: H_2O (2% MeOH, 5 mM ammonium acetate) and B: MeOH(2% H_2O, 5 mM ammonium acetate). The mobile phase (B) gradient started from 5% for 0.5 min and increased to 50% at 3.5 min, rose to 65% at 7.5 min, reached 97% at 9.5 min and holds for 3 min, then decreased to 5% at 13 min and conditioned for another 2.5 min. A constant flow of 0.35 mL/min was applied. The mass spectrometer was operated with Dynamic multiple reaction monitoring (dMRM) with positive/negative switching (+/-) mode (segment: 1 minute for each compound). The drying gas temperature was set at 325 °C, the gas flow at 10 L/min, the nebulizer at 30 psi, sheath gas temperature 275 °C, sheath gas flow 10 L/min, capillary voltage 3500 V and nozzle voltage 0 V.

Result and discussion
Instrumental optimization
The instrumental method was further developed and optimized based on our previous studies[6], which analyzed a several organophosphate flame retardant metabolites by LC-MS/MS using +/- switching with MRM mode for diesters and positive mode for HO-PFRs. In this study, more metabolites were validated, such as HO-DPHP, BBOEHEP, EHPHP and HO-EHDPHP. Table 1 shows some LC-MS/MS parameter for our target compounds. Different from previous studies [5,6], most of the HO-PFRs and isotope labelled PFRs (used as IS) were found to have much better sensitivity with positive mode, except 4-HO-TPHP, 3-HO-TPHP. In order to analyze all compounds in one injection, the +/- switching with dMRM mode was applied. Traditional MRM have weak performance conducting polarity switching in the same time segments. Frequent polarity switching in a segment reduces the scanning sensitivity of MS. Normally, this could be circumvented by separating the compounds with the same polarity in two groups by optimizing the LC gradient, yet full/complete separation could not be achieved here. To solve the problem, the dMRM mode was applied. dMRM provides shorter time scanning (set as 1 minute) of each compound with individual segment base on its retention time. Once a compound is about to be eluted, its monitoring starts; once being eluted, the monitoring of such compound stops. Compared with MRM that usually scans several compounds within entire segment, dMRM has much shorter (but varying) scanning cycle through out the entire run. It can significantly improve the sensitivity and reduce the background noise, especially during the +/- mode. Our LC gradient achieved a good separation of 23 compounds within 9.5 min (15 min run time, see table 1). Throughout the run only 2 short time windows (5.6-6.6 min; 7.8-8.8 min) were operated with +/- switching mode, minimized the sensitivity lost during the polarity alternation. For the rest of the time, only positive or negative mode was operated. Usually four to six, maximum ten, transitions were monitored at the same time, while MRM often scans more transitions in a segment. Injections of standards proved that dMRM has similar or better performance comparing to MRM. The selection of buffer and organic phase were also optimized, finding that MeOH is a better mobile phase than acetonitrile and ammonium acetate is a better buffer than ammonium formate for our method performance.
Sample preparation
Several sorbent have been tested, including aminopropyl silica (3 mL, 200 mg, Agilent), PSA (200 mg, 3 mL, home-packed; Supleco) and DSC-18. DSC-18 was found to have the best performance than others. Compared with our previous method[6], which used an ion exchange sorbent – StrataX-AW (50 mg, 1mL, Phenomenex), PFR metabolites could be sufficiently eluted from DSC-18 (reverse phase elution) by MeOH without adding triethyl amine. We performed a spiking test with urine to compare the new methods with our previous method[6]. Results showed that the new method have similar or better recovery and MLOQ for all compounds, especially for DNBP, 3-HO-TPHP and 4-HO-TPHP, BCIPHIPP and 5-HO-EHDPHP. A major reason it that StrataX-AW has less retention of TCEP-D12 and TPHP-D15 that were used as the IS of several HO-PFRs. Also, DSC-18 could strong retention of non-

polar compounds, such as lipids, which might help to remove the lipids from serum and reduce matrix effect. However, comparison also showed that the standard deviations for some diesters are slightly larger with DSC-18 than use with Stratax-AW. Probably, Stratax-AW, the ion-exchange sorbent, has better retention of diesters (strong acids) than a reversed phase sorbent.
Formic acid was used to stop the incubation of urine, but it is not feasible for serum. Since serum contain large amount of protein, a thorough protein precipitation needs to be conducted to prevent the blockage of SPE cartridges or LC column by suspending protein particle. However, since a large amount of buffer was added to serum before incubation, even adding 150 µl HCl (37%) could not achieve sufficient precipitation. As reversed phase sorbent is used, organic solvents, such as MeOH or acetonitrile, could not be used to precipitate protein, since they might influence the retention of our target on SPE cartridge. To solve this problem, 0.5 mL 20% NaCl solution was added to serum, then a sufficient precipitation could be achieved with adding only 30 µl of HCl (37%). Moreover, incubation time and temperature were also optimized. Both serum and urine were incubated for 3h at 45 °C.
Validation and method application
Human urine and horse serum were used for validation and the results were presented in Table 2. Nine pairs (Qlow=2ng, Qhigh=6ng) of spiked samples were analyzed in three days for both urine and serum. For serum, duplicate (or triplicate) matrix calibration is recommended, since a certain amount of compounds seems to remain in protein. Matrix calibration is not necessary for urine. For most metabolites, the recoveries ranged 70%-130% with RSD<20%. For urine, MLOQs ranged from 0.05 ng/mL (BBOEP) to 1.5 ng/mL (HO-DPHP) for diesters and 0.008 ng/mL(BBOEHEP) to 0.1 ng/mL (HO-TBOEP) for HO-PFR. Same with our previous study [5,6], BCEP and BCIPP had very high MLOQs (>6 ng/mL and >3 ng/mL, respectively for both urine and serum) and bad peak shapes due to the instrumental limitation; it is recommended to analyzed them with GC-MS/MS [5]. In general, HO-PFRs have lower MLOQs than diesters; while urine has lower MLOQ than serum for the same compounds. EHPHP had bad recoveries and RSDs for both urine and serum, an ideal IS (especially for serum) could not be found. The influence of +/- switching might also contribute. So EHPHP was only used for qualitatively screening at the moment. High STDVs were observed on 3-HO-TPHP and 4-HO-TPHP (also TPHP-D15, their IS) in serum, not for urine. Different with urine, the peaks of these compounds were barely quantifiable. Further experiments (spike standard before and after precipitation) showed that substantial proportion of 3-HO-TPHP, 4-HO-TPHP and TPHP-D15 were retained in protein, which was not observed for other TPHP metabolites, such DPHP and HO-DPHP. Vortex speed during precipitation could also influence their protein binding.
Our method was further applied to human urine (n=9) and serum (n=10). BDCIPP (nd-0.30 ng/g), DPHP (nd-0.67 ng/mL), BCIPHIPP (nd-1.93 ng/mL) and HO-EHDPHP (nd-0.06 ng/mL) and were detected in more than 4 samples in urine with low levels. In serum, DPHP (0.5-1.6 ng/mL) and BDCIPP (nd-1.9 ng/mL) were detected in more then 3 samples. HO-EHDPHP, DNBP and BBOEP were only detected in one or two serum samples. This is the first study reporting PFR metabolites in serum and it is the first study finding HO-EHDPHP in urine.

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Table 1 The Optimized retention time, polarity and transitions of each compound

	RT (min)	+/-	Precursor	Product		RT (min)	+/-	Precursor	Product
BCEP-D8	0.58	-	231	37	EHPHP	5.07	-	285	93
			229*	35				285*	79
BCEP	0.61	-	223	37	TCEP-D12	5.15	+	299	102
			221*	35				297*	102
BCIPP	1.73	-	251	35	TCEP	5.20	+	285	99
			249*	35				285*	63
HO-DPHP	2.01	-	265	108	BBOEHEP-D4	6.68	+	347	247
			265*	93				347*	45
DNBP	2.83	-	209*	79	BBOEHEP	6.70	+	343	243
			209	153				343*	45
BDCIPP-D10	3.10	-	329	35	TBOEP-OH	8.10	+	415	99
			327*	35				415*	45
BDCIPP	3.13	-	319	37	4-HO-TPHP	8.25	-	341	249
			317*	35				341*	93
DPHP-D10	3.16	-	259	159	3-HO-TPHP	8.45	-	341*	249
			259*	98				341	93
DPHP	3.20	-	249	155	EHDPHP-OH	8.90	+	379*	251
			249*	93				379	153
BBOEP-D4	4.08	-	301	199	TPHP-D15	9.20	+	342*	223
			301*	79				342	82
BBOEP	4.13	-	297*	79	TBOEP-D6	9.37	+	405	303
			297	197				405*	47
BCIPHIPP	4.85	-	309	175					
			309*	99					

*** the transastion used as quantifier**

Table 2 The result of method validation. The validation was conducted in 3 days. Three pairs of Qhigh-Qlow spiked samples analyzed for both urine (human) and serum (horse).

	Urine Validation (n=9)				Serum Validation (n=9)			
	Qlow (2ng)		QHigh (6ng)		Qlow (2ng)		QHigh (6ng)	
	Rec	RSD	Rec	RSD	Rec	RSD	Rec	RSD
BCEP	<MLOQ	<MLOQ	104%	11%	<MLOQ	<MLOQ	95%	5%
BCIPP	89%	15%	85%	18%	102%	10%	97%	7%
HO-DPHP	80%	9%	72%	16%	90%	16%	98%	15%
DNBP	97%	13%	84%	16%	92%	12%	85%	28%
BDCIPP	107%	10%	91%	14%	101%	11%	91%	10%
DPHP	102%	9%	93%	14%	95%	11%	89%	9%
BBOEP	104%	13%	92%	15%	81%	11%	90%	14%
BCIPHIPP	99%	12%	101%	15%	83%	9%	93%	5%
EHPHP	194%	11%	193%	15%	142%	39%	114%	62%
BBOEHEP	92%	10%	90%	15%	96%	9%	99%	6%
TCEP	106%	12%	102%	16%	95%	10%	98%	5%
TBOEP-OH	118%	11%	117%	15%	95%	10%	99%	7%
4-HO-TPTHP	118%	10%	111%	14%	63%	66%	68%	62%
3-HO-TPTHP	110%	9%	106%	14%	88%	26%	88%	37%
HO-EHDPHP	133%	14%	128%	15%	97%	10%	96%	9%