METABOLITES OF ORGANOPHOSPHATE FLAME RETARDANTS AND PLASTICISERS IN HUMAN URINE

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Introduction

Organophosphate esters (OPEs) are used as additive flame retardants (FRs) and plasticisers in polymers, paints, synthetic rubber and hydraulic fluids[1]. These compounds were detected abundantly in the indoor environment in dust (at the µg/g level)[1] and in air at the ng/m³ level[2] and recent publications demonstrated the presence of their hydrolysed metabolites in human urine[3-5]. We determined the concentrations of a selected number of metabolites in human urine of which the parent compounds were most abundant in Belgian house dust samples, namely tris(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCPP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tributyl phosphate (TiBP).[6] In this project, we aimed to screen the total level of exposure to OPEs through the level of metabolites present in urine from a group of volunteers. This study also describes the difficulties encountered for some of the metabolites during the analytical method development using LC/MS/MS.

Materials and methods

Materials

Diphenyl phosphate (DPP), dibutyl phosphate (DBP) and trimethylsilyl diazomethane (TMSDM) were purchased from Sigma-Aldrich (Bornem, Belgium). Bis(2-butoxyethyl) phosphate (BBEP), bis(2-chloroethyl) phosphate (BCEP), bis(1-chloro-2-propyl) phosphate (BCPP), bis(1,3-dichloro-2-propyl) phosphate (BDCPP), BBEP-d4, BCEP-d8, BDCPP-d10 and DPP-d10 were synthesised in the Max Planck Institute (Göttingen, Germany).

WAX 60 mg/3 mL was purchased from Waters (Millford, MA, USA), Synergi Polar-RP (100 x 2.0 mm x 2.1 µm) was purchased from Phenomenex Inc. (Torrance, CA, USA). Ammonium hydroxide (29%), ammonium acetate and methanol (of analytical grade) were purchased from Merck KgA Chemicals (Darmstadt, Germany). Ultrapure water (18.2 MΩ) was obtained from an Elga LabWater water purification instrument (Saint Maurice, France).

Samples

This study was part of a project investigating the detection of environmental contaminants in human urine. Spot urine samples were taken from a group of obese and lean volunteers in the morning and collected in glass tubes. The samples were stored at -20°C until further analysis.

Methods

Sample preparation. A volume of 2 mL urine was spiked with 15 ng of IS and adjusted to pH 5 using a 0.1 M sodium acetate buffer. Oasis WAX cartridges were pre-washed using 1 mL of 5% NH_4OH in methanol followed by 2 mL of sodium acetate buffer and 1 m of water. The urine was quantitatively transferred to the cartridges using twice 0.5 mL of diluted buffer solution. Interferences were removed using 30 % methanol at pH 5. DAPs were eluted with 2 mL of 5% NH_4OH in methanol. The methanol fraction was collected and evaporated to dryness. Extracts that would not be derivatised were reconstituted in 200 µl of 15 % MeOH.

Derivatisation. Extracts were evaporated until complete dryness and derivatised by adding 50 µl of MeOH and 150 µl of 0.2 M TMSDM in acetonitrile to the reaction tube with a screw cap. Tubes were vortexed for 2 min and placed in an oven at 100°C for 2 h. After derivatisation, the tubes were left to cool, vortexed, evaporated until complete dryness and resolubilised in 200 µl MeOH-water (50:50, *v/v*).

Accuracy and precision. A volume of 2 mL of a pool of 25 urine samples was spiked in triplo with three concentrations of DAPs over three days (Qlow: 3 ng/mL, Qmedium: 9 ng/mL, Qhigh: 37 ng/mL).

Matrix effects: a pool of 12 urine samples was extracted according to the sample preparation protocol and spiked afterwards with 15 ng of standards and IS before analysis by LC/MS/MS. The response of the post extraction spiked extract was compared with that of the blank pooled urine extract and the response of a solvent mixture containing the same amount of DAPs and IS.

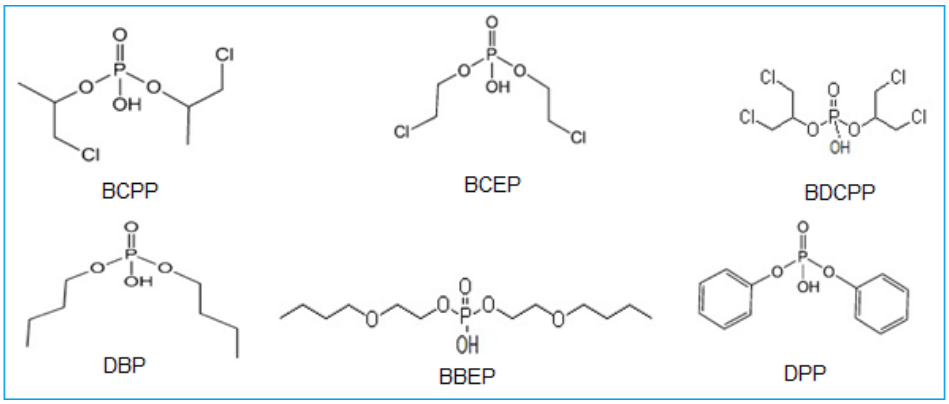

Figure 1. Chemical structures of the six selected organophosphate diesters.

LC conditions. Column : Synergi Polar-RP (100 x 2.1 mm x 2.0 µm), Mobile phase: 5 mM ammonium acetate in water (A), methanol (B) start: 15% B, hold 0.5 min, to 40% B in 2.5 min, hold 1.5 min, to 95% B in 3.5 min, hold 10 min, equilibrate to 15% B during 12 min, flow: 150 µL/min, temperature 35°C, injection volume 5 µL.

MS conditions. ESI positive and negative: N_2 drying gas, gas temperature 325°C, gas flow 10 L/min, nebuliser pressure 30 psi, ΔEMV 300 V, capillary voltage 3000 V, dwell 40 msec. Two multi-reaction monitoring (MRM) transitions were optimised for each standard (Table 1).

Data analysis was done using SPSS 16.0 (SPSS Inc).

Table 1. MRM transitions for DAPs.

Compound	Fragmentor Voltage (V)	Quantifier (Collision Energy)	Qualifier (Collision Energy)
BCEP	60	221.0 > 35.0(5)	223.0 > 35.0(5)
BCPP	60	249.0 > 35.0(5)	251.0 > 35.0(5)
BDCPP	80	318.9 > 35.0(5)	316.9 > 35.0(5)
DBP	120	209.1 > 79.2(22)	209.2 > 153.0(10)
DPP	130	249.1 > 93.2(27)	249.1 > 155.1(17)
BBEP	115	297.1 > 79.0(27)	297.1 > 197.3(15)
BCEP-d8	60	229.0 > 35.0(5)	231.0 > 35.0(5)
BDCPP-d10	80	329.0 > 35.0(5)	327.0 > 35.0(5)
DPP-d10	130	259.2 > 98.1(27)	259.2 > 159.1(17)
BBEP-d4	115	301.2 > 78.8(27)	301.2 > 198.9(15)

Results

Sample preparation

Both in standard solutions and post-spike extracts, it seemed that BCEP and BCPP suffered from low sensitivity because of a poor fragmentation and lower retention behaviour (figure 2). We tried to improve the latter by a derivatisation reaction with TMSDM, based on a publication regarding the analysis of bisphosphonates in plasma[7]. Sensitivity improved twofold for BCEP and fivefold for BCPP. To prove the applicability of this protocol in real samples we analysed three urine samples and a spiked urine pool sample (3 ng/mL DAP, in triplicate) once by SPE and LC-ESI(-)MS/MS, once by methylating the extracts with TMSDM reagent followed by LC-ESI(+)MS/MS. It appeared that the gain in instrumental sensitivity was counteracted by

the loss in selectivity, because a positive ionisation was mandatory for the methylated DAPs. The loss of selectivity resulted in a noisier baseline, which adversely affected the detection of DBP, BDCPP and BBEP. Moreover, the protocol using TMSDM did not allow for an accurate quantification of DPP in real samples or standard solutions, as the reaction was not complete for this compound. After these results, it was decided to abandon the methylation reaction.

Validation

Matrix effects, accuracy (Table 2), precision (Table 2) and sensitivity were assessed. Matrix effects were significant, with a signal suppression varying between 39 % (BBEP) and 93% (DPP). There was a similar signal suppression for DAPs and the deuterium labeled analogues. The recovery of BCEP was lower at all spiking levels, probably because of its polarity which caused it to elute close to the solvent front and the non-retained matrix components.

The method quantification limit (LOQm) was calculated based on the relative standard deviation (RSD) of the Qlow spiked urine samples. The low response and precision for BCEP and BCPP resulted in a high LOQm, respectively 1.2 and 3.7 ng/mL. For other DAPs, the method quantification limit ranged from 0.76 ng/mL (DBP) to 0.15 ng/mL (BBEP).

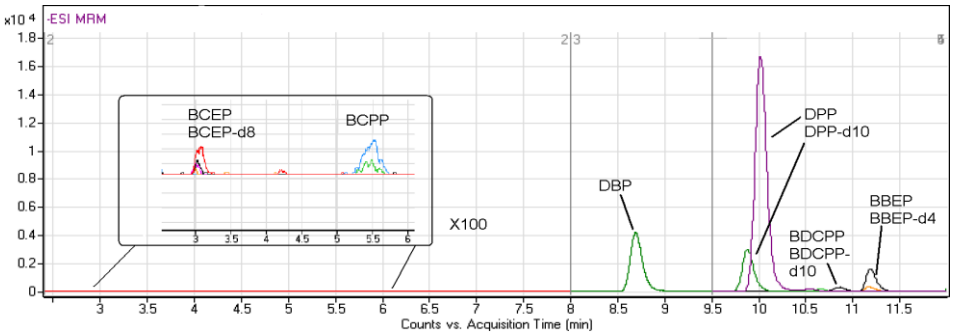

Figure 2. Chromatogram of a spiked sample (3 ng/mL DAPs in urine).

Table 2. Validation parameters using weak anion exchange SPE and analysis by LC-(-)ESIMS/MS.

	Q low (3 ng/mL)		**Q medium (9 ng/mL)**		**Q high** (37 **ng/mL)**	
Compound	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %
BCEP	69	18	72	21	60	19
BCPP	119	33	174	29	147	23
BDCPP	95	27	115	11	100	16
DBP	86	20	99	7	90	7
DPP	102	20	117	8	102	9
BBEP	95	33	113	10	107	13

Method application

The method was applied to a batch of spot urine samples from volunteers. DPP was detected in more than 90% of the samples, in contrast to the other DAPs which had a detection frequency of less than 50 %. The quartile concentrations for all DAPs are displayed in Figure 3. A logical explanation for the low detection frequencies would be the low sensitivity of this method for BCEP, BCPP, BDCPP in comparison to other published methods[3,4]. This certainly seemed to be the case for BCEP, which was detected in other studies at a median concentration below 0.1 ng/mL. Though for BDCPP, the low detection frequency could also be due to lower exposure through dust ingestion in Belgium, as median TDCPP levels were 0.57 µg/g compared to 1.89 µg/g in US dust [6].

For statistical analysis, the data were transformed using a natural logarithm. No significant differences for DPP between genders were found (independent samples t-test, $p > 0.05$). Pearson correlation could not reveal any relationship between age or BMI and DPP levels ($p > 0.05$). Correlations between DPP levels and smoking

behaviour and alcohol consumption were investigated using one way ANOVA. For the latter, there was no significant correlation ($p > 0.35$), in contrast to the smoking behaviour, for which a significant correlation was observed ($p = 0.02$). When calculating the corresponding mean concentrations, the DPP level in smokers' urine was 1.55 ng/mL versus 0.82 ng/mL of non-smokers and 0.69 ng/mL of ex-smokers.

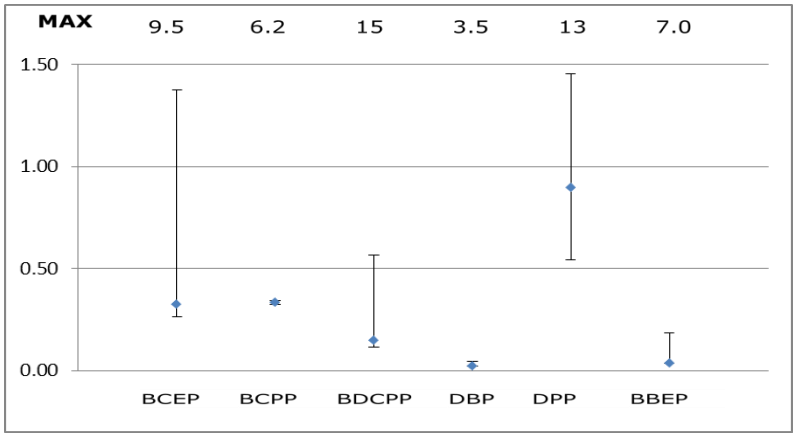

Figure 3. Quartile concentrations of DAPs in urine (ng/mL). Maximum concentrations are displayed above.

The higher levels of DPP in the smoking population could be a result of an induced metabolism of TPP, e.g. if the metabolising enzyme would be CYP1A1 or CYP1A2, of which activities are known to be induced by tobacco smoke[8].

To conclude: the current method should be improved to measure BCEP and BCPP at lower concentrations by using a derivatisation reagent which is suitable for selective detection by MS/MS, and which also reacts completely with DPP. Next, human hepatic metabolism of OPEs *in vitro* should be further investigated. This would help to include other metabolites which might be more abundant, and to identify the metabolising enzymes, so that influences of lifestyle, diet and genetics to the metabolism of OPEs may be estimated.

Acknowledgements
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