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Kinetics of tris (1-chloro-2-propyl) phosphate (TCIPP) metabolism in human liver microsomes and serum

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Abstract

Tris(1-chloro-2-propyl) phosphate (TCIPP) is an emerging contaminantwhich is ubiquitous in the indoor and outdoor environment. Moreover, its presence in human bodyfluids and biota has been evidenced. Since no quantitative data exist on the biotransformation or stability of TCIPP in the human body, we performed an in vitro incubation of TCIPP with human liver microsomes (HLM) and human serum (HS). Two metabolites, namely bis(2-chloro-isopropyl) phosphate (BCIPP) and bis(1-chloro-2-propyl) 1-hydroxy-2-propyl phosphate (BCIPHIPP), were quantified in a kinetic study using HLM or HS (only BCIPP, the hydrolysis product) and LC-MS. The Michaelis-Menten model fitted best the NADPH-dependent formation of BCIPHIPP and BCIPP in HLM, with respective V_{MAX} of 154 ± 4 and 1470 ± 110 pmol/min/mg protein and respective apparent K_m of 80.2 ± 4.4 and 96.1 ± 14.5 µM. Hydrolases, which are naturally present in HLM, were also involved in the production of BCIPP. A HS paraoxonase assay could not detect any BCIPP formation above 38.6 ± 10.8 pmol/min/µL serum. Our data indicate that BCIPP is the major metabolite of TCIPP formed in the liver. To our knowledge, this is the first quantitative assessment of the stability of TCIPP in tissues of humans or any other species.Further research is needed to confirm whether these biotransformation reactions are associated with a decrease or increase in toxicity.

Keywords

Organophosphate flame retardant; biotransformation; liver; serum; clearance

43 **Abbreviations**

44 BCIPHIPP: bis(1-chloroisopropyl) 1-hydroxyisopropyl phosphate

45 BCIPP: bis (1-chloroisopropyl) phosphate

46 HLM: human liver microsomes

47 PFR: phosphate flame retardant

48 QTOF: quadrupole-time-of-flight

49 TCIPP: tris(1-chloroisopropyl) phosphate

50 TRIS: tris (hydroxymethyl)aminomethane

51

52 **1. Introduction**

53 Tris(1-chloro-isopropyl) phosphate (TCIPP) is an additive flame retardant which is used in
54 polymers, such as polyurethane foams, and is used also as a replacement of tris(2-chloroethyl)
55 phosphate (EU 2008). TCIPP has been reported as one of the predominant phosphate flame
56 retardants (PFRs) in the indoor and outdoor environment (van der Veen and de Boer 2012),
57 and its uptake in living organisms has been demonstrated by several studies (Sundkvist et al.
58 2010, Greaves and Letcher 2014). In comparison to non-chlorinated PFRs, TCIPP could be
59 more resistant to abiotic degradation processes (Kawagoshi et al. 2002, Meyer and Bester
60 2004). In the benthic food web, bioaccumulation has been suggested by Brandsma et al.
61 (2015). Our group recently detected a suite of PFR metabolites including a hydroxylated
62 metabolite of TCIPP in human urine (Van den Eede et al. 2015a). While the detection of PFR
63 metabolites in humans does not necessarily imply that the parent compounds are
64 bioaccumulative, it does imply that human exposure occurs. This is concerning, as little is
65 known about the possible effects on human health of chronic exposure to PFRs like TCIPP.
66 Atopic dermatitis was recently correlated with increasing dust levels of TCIPP in an
67 epidemiological study (Araki et al. 2014). *In vitro*, agonistic activity towards the pregnane X
68 receptor and increases in 17-β-estradiol and testosterone were noted as effects on the
69 endocrine system (Kojima et al. 2013, Liu et al. 2012), yet no estrogenic effects were detected
70 (Zhang et al. 2014). In animals, a possible interference with thyroid hormone axis for growth
71 and development in chicken embryos by TCIPP was observed (Farhat et al. 2013), though in
72 zebrafish limited neurobehavioral changes and no teratogenic or hormonal effects were
73 reported for this flame retardant (Dishaw et al. 2014).

74 While biomonitoring can assist in correlating human health parameters with exposure to
75 TCIPP, urinary analysis of the TCIPP metabolite, bis(2-chloro-isopropyl) phosphate (BCIPP),
76 has had little success in contrast to other PFR metabolites which were targeted in

77 epidemiological studies (Schindler et al. 2009, Van den Eede et al. 2013a, Butt et al. 2014,
78 Dodson et al. 2014, Fromme et al. 2014). Based on the results of *in vitro* metabolism
79 screening (Van den Eede et al. 2013b) and a biomonitoring study (Van den Eede et al. 2015a)
80 we suggested that another metabolite, namely bis(2-chloro-isopropyl) hydroxy-isopropyl
81 phosphate (BCIPHIPP), might be a more suitable biomarker than BCIPP for monitoring
82 exposure to TCIPP. However, a comparison based on quantitative data for both BCIPP and
83 BCIPHIPP metabolites has not yet been made *in vitro,* nor *in vivo* to support this hypothesis.
84 Investigation of the production rates of these metabolites and their contribution to TCIPP
85 clearance, and insight in the involved metabolic processes is a first step towards obtaining
86 factors for the conversion of urinary concentrations to exposure estimates. Additionally, such
87 toxicokinetic measurements are helpful in estimating the degree of persistence and the rate of
88 (de)toxification of this flame retardant in the human body (depending on the role of the
89 metabolites in the toxicity mechanism).
90 Liver is the major site of metabolism for many xenobiotics, where the majority of
91 oxidative enzymes are expressed (Lipscomb and Poet 2008). Yet in the case of
92 organophosphate triesters, hydrolytic enzymes such as paraoxonases could also be involved in
93 biotransformation (Furlong et al. 2000). Paraoxonases, and more specifically PON1, have
94 been identified as the responsible enzymes for detoxification of the toxic "oxon" metabolites
95 of organophosphate pesticides, including diazoxon, chlorpyrifos-oxon, and paraoxon(Furlong
96 et al. 2000). PON1 is expressed both in liver and in serum (Furlong et al. 2000) and catalyzes
97 the hydrolysis reaction with formation of a dialkyl phosphate (diethyl phosphate for
98 paraoxon) and an alcohol (4-nitrophenol for paraoxon). For TCIPP only the hepatic
99 metabolism has been studied so far, either focusing on oxidative processes or not
100 distinguishing between oxidative and hydrolytic reactions (EU 2008, Van den Eede et al.
101 2013b, Abdallah et al. 2015). In order to provide a more complete dataset for integration in

102 pharmacokinetic models, it is necessary to investigate the stability of TCIPP in presence of
103 liver and serum enzymes, such as PON1, and to confirm the extent of contribution of
104 oxidative and hydrolytic enzymes to TCIPP degradation *in vitro*.

105 There were five aims to our study: (i) to apply μ-LC-QTOF technology to screening
106 TCIPP metabolites formed by human liver microsomes (HLM), (ii) to investigate metabolite
107 clearance in HLM by quantifying the production rates of BCIPP and BCIPHIPP, (iii) to
108 compare the intrinsic clearance of BCIPP to that of BCIPHIPP and relate both to *in vivo*
109 findings, (iv) to extrapolate the intrinsic *in vitro* hepatic clearance to *in vivo* hepatic clearance,
110 and (v) and to investigate the extent of hydrolysis of TCIPP to BCIPP by serum enzymes.

111

112 **2. Materials and Methods**

113 **2.1.Materials and Reagents**

114 Tris (hydroxmethyl) aminomethane (TRIS), β-Nicotinamide adenine dinucleotide 2′-
115 phosphate reduced tetrasodium salt hydrate (NADPH), $CaCl_2$, paraoxon, and diphenyl
116 phosphate-d10 (DPHP-d10) were purchased from Sigma Aldrich (Bornem, Belgium). TCIPP
117 standard was acquired from Pfalz & Bauer (Waterbury, USA). BCIPP, bis(2-chloroethyl)
118 phosphate-d8 (BCEP-D8), tris(2-chloroethyl) phosphate-d12 (TCEP-D12), and BCIPHIPP
119 were synthesized at the Max Planck Institute (Göttingen, Germany). TCIPP was a mixture of
120 three isomers, namely tris(1-chloro-2-propyl) phosphate, bis(1-chloro-2-propyl) 1-
121 chloropropyl phosphate, and bis(1-chloropropyl) 1-chloro-2-propyl phosphate. All other
122 reference and internal standards were of analytical grade, BCIPHIPP was a mixture of 2
123 isomers as described elsewhere (Van den Eede et al. 2015a). HLM pools were purchased from
124 Xenotech LLC, a 50-donor pool was used for the metabolite screening assay (lot 1210267)
125 and a 200-donor pool for the metabolism kinetics study (lot 1210347, appendix B). Surplus
126 serum samples collected in the frame of another study (registered at http://clinicaltrials.gov/

127 with number NCT01778868) were pooled for use. This study was approved by the Ethical
128 Committee of the Antwerp University Hospital (Belgian Registry number B30020097009)
129 and all participants provided their written informed consent. Serum from 15 patients was
130 pooled and stored at -20°C until analysis. Acetonitrile (analytical grade) was obtained from
131 Merck KgA Chemicals (Darmstadt, Germany) and ultrapure water (18.2 MΩ) from an
132 ElgaLabWater water purification instrument (Saint Maurice, France).

133 **2.2.Biotransformation assays**

134 2.2.1. HLM screening assay

135 Reaction mixtures contained 50 mM TRIS buffer adjusted to pH 7.4 at 37 °C and 100 µM
136 TCIPP in a total volume of 0.98 mL (final concentrations). 20 µl of NADPH (1 mM final
137 concentration) was repeatedly added (every 30 min) to ensure continued CYP activity. After
138 120 min, reactions were quenched using 1 mL of ice-cold acetonitrile and by storing the tubes
139 on ice. Either no HLM or no NADPH was added in the negative controls.

140 2.2.2. Preliminary experiments to establish steady state conditions

141 *2.2.2.1.HLM*

142 Preliminary experiments were run to establish the steady state conditions by monitoring the
143 formation of BCIPHIPP metabolite. Firstly, the optimal protein concentration was established
144 using concentrations of 0.05 mg/mL up to 1.0 mg/mL HLM (final concentration). Secondly,
145 the optimal incubation time was established starting from 0 to 15 minutes. Details on the
146 preliminary experiments can be found in supporting information.

147 2.2.2.2.*Serum*

148 Incubation mixtures consisted of 100 mM TRIS buffer (adjusted to pH 8.5 at 37 °C), 2 mM
149 $CaCl_2$, and serum in a 0.5 to 3% concentration (v/v), in a final volume of 500 µl. Reactions
150 were initiated by adding 100 µM of TCIPP or paraoxon (positive control) in acetonitrile (1%
151 of total volume). After 10 min, reactions were stopped by adding 110 µl of 20 mM Na_2EDTA

152 and 60 µl of 10% acetic acid in water. Diphenyl phosphate-d10 (20 ng) was added to
153 paraoxon samples, and 20 ng of BCEP-d8 was added to the TCIPP incubations. 200 µl of
154 methanol was added to paraoxon samples, after which samples were vortexed and
155 centrifuged. Supernatants were filtered before analysis. Serum samples incubated with TCIPP
156 were extracted on Oasis WAX SPE columns, to enrich the extracts for BCIPP and BCEP-d8
157 based on a urine extraction protocol (Van den Eede et al. 2013a). Cartridges were conditioned
158 with 2 mL methanol and 2 mL Milli Q water. After loading of the samples, cartridges were
159 washed with 1 mL Milli Q water and BCIPP and BCEP-d8 were eluted with 2 mL of 5%
160 NH_4OH in methanol. The eluate was collected and evaporated under a gentle N_2 stream until
161 dryness. Extracts were reconstituted in 100 µl 15% methanol in water.

162 2.2.3. Kinetic metabolism study (HLM)

163 Initial rate conditions were selected based on BCIPHIPP formation, which was linear up to
164 0.25 mg/mL protein (HLM) and 7.5 min. Therefore these conditions were selected for
165 conduct of our HLM kinetic metabolism study. Reaction mixtures contained 50 mM TRIS
166 buffer adjusted to pH 7.4 at 37 °C, HLM (0.25 mg/mL final protein concentration) and 1 to
167 300 µM TCIPP in a total volume of 0.98 mL (final concentrations). For each substrate
168 concentration, five replicates were prepared. All samples were pre-incubated in a shaking
169 water bath at 37 °C for 5 min. The reaction was started by adding 20 µl of NADPH stock
170 solution (1 mM, final concentration), and quenched after 5 min by 500 µl of ice-cold
171 acetonitrile and storing the tubes on ice. A fixed amount of IS (250 ng TCEP-d12 and BCEP-
172 d8) was added to each tube before centrifugation (10 min at 3,500 rpm). The supernatant was
173 transferred to a new set of tubes, evaporated to 1 mL using nitrogen gas and filtered through a
174 0.45 µm nylon filter into HPLC glass vials. Characterization of PON activity in HLM under
175 these conditions is described in supporting information

176 **2.3.Quality control (QC)**

177 For the HLM assay, NADPH negative control samples consisted of 50 mM TRIS buffer,
178 HLM (0.25 mg/mL final protein concentration) and 1 to 300 μM TCIPP (final concentrations)
179 in a total volume of 1 mL (in triplicate). Enzyme negative control samples consisted of 50
180 mM TRIS buffer, 1 mM NADPH and 1 to 300 μM TCIPP (final concentrations) in a total
181 volume of 1 mL (three replicates for each substrate level). Blank samples (three replicates)
182 consisted of 50 mM TRIS buffer and HLM (0.25 mg/mL final protein concentration) in a total
183 volume of 0.98 mL.
184 Recovery of BCIPP and BCIPHIPP in HLM incubation mixtures, and the recovery of BCIPP
185 in serum incubation mixtures were tested as described in the supporting information.

186 **2.4.Instrumental analysis**

187 2.4.1. Screening with μ-LC-QTOF

188 An Eksigent 200 μLC was coupled to an ABSciex Triple-ToF 5600 for screening of the
189 TCIPP metabolites in HLM samples. Analytes were separated on a Halo C18 column (50 x
190 0.5 mm, 2.7 μm) with a mobile phase of 5 mM ammonium acetate in water (A) and
191 acetonitrile (B). The following gradient was used: 10% B was held for 0.5 min, followed by a
192 linear increase to 30% B in 1 min (0.5 min hold), and then to 95% B in 1 min (0.6 min hold)
193 at a flow rate of 50 μL/min. After each analysis the column was re-equilibrated at 10% B
194 during 0.6 min. An electrospray ionization source was used with the following parameters:
195 gas 1, gas 2, and curtain gas were set at 15, 40, and 30 L/min, respectively. A source
196 temperature of 300 °C was used, and ion spray fragmentor voltage was set at 4500 (negative
197 mode) or 5000 (positive mode). TOF range was set to acquire masses between 100.0000 and
198 1000.0000 Da with an accumulation time of 250 msec. MS/MS spectra were recorded in IDA
199 (information dependent acquisition) mode, with fixed collision energy of 35 V.

200 2.4.2. Quantitative analysis

201 For the TCIPP kinetics studies in HLM and serum, extracts were analyzed on an Agilent 1290
202 LC coupled to a 6460 triple quadrupole MS. Phenylhexyl column (100 x 2.1 x 2.6 µm,
203 Phenomenex) was used for separation of the extracts. The mobile phase consisted of 5 mM
204 ammonium acetate in water (A) and acetonitrile (B), with the following gradient conditions
205 for HLM extracts: 10% B (0.5 min hold), increase to 30% B in 2.5 min (2 min hold), increase
206 to 40% B in 5 min, followed by a sharp increase to 95% B (3.5 min hold), and equilibration at
207 starting conditions for 3.5 min. A different gradient program was used for serum extracts: 5%
208 B (0-2 min), 20% B (at 2.5 min), 30% B (4 min), 40% B (6 min), 95% B (11-11.5 min), and
209 equilibration at starting conditions for 7 min.
210 Flow rate was 0.3 mL/min, temperature 40 °C, injection volume 1 µL. The following MS
211 parameters were used: gas temperature 325 °C, sheath gas heater 250 °C, gas flow 10 L/min,
212 sheath gas flow 11 L/min, nebulizer pressure 35 psi, capillary and nozzle voltage 3500 and 0
213 V, respectively. MRM transitions can be found in Table A1.

214 **2.5.Data analysis**

215 2.5.1. Statistics

216 Linear and non-linear regression of the HLM and serum data was done using Graphpad Prism
217 5 (GraphPad software, Inc). For HLM data, the following models were compared: Michaelis-
218 Menten (equation 1), Hill equation, and substrate inhibition (see SI). BCIPP concentrations in
219 cofactor and enzyme negative control samples were also analyzed by linear regression.
220 Selection of the most appropriate model was based on an F-test of the goodness of fit of each
221 model. If the difference in fit between models was not significant (p>0.05), the simplest
222 model was selected.More details can be found in Supplementary Information.

223

224 $$\boldsymbol{v} = \frac{\boldsymbol{V_{max}} \times [\boldsymbol{S}]}{\boldsymbol{K_m} + [\boldsymbol{S}]}$$ (Equation 1, Michaelis-Menten model)

225 For the analysis of serum data, an F-test was used after regression to determine if the slope
226 was significantly different from zero. To estimate the sensitivity of the serum assay, Cohen's
227 d for minimal effect size (equation 2), was considered as the minimal net formation of BCIPP
228 by serum enzymes.

229 $$\bar{x}_1 - \bar{x}_2 = d \times \sqrt{\frac{(n_1-1)\times s_1^2+(n_2-1)\times s_2^2}{n_1+n_2-2}} \qquad \text{(Equation 2)}$$

230 2.5.2. *In vitro-in vivo* extrapolation

231 Intrinsic clearance and extrapolation of *in vitro* data to *in vivo* data were calculated based on
232 the following equations (Lipscomb and Poet 2008):

233 $$CL_{int,LM} = \frac{V_{max}}{K_m} \qquad \text{(Equation 3)}$$

234 $$CL_{int} = CL_{int,LM} \times \frac{mgmicrosomalprotein}{gliver} \qquad \text{(Equation 4)}$$

235 $$CL_h = \frac{CL_{int} \times Q_H}{CL_{int} + Q_H} \qquad \text{(Equation 5)}$$

236 Equation 3 represents the intrinsic *in vitro* clearance $CL_{int,LM}$, equation 4 scales $CL_{int,LM}$ to the
237 full liver, while equation 5 assumes the direct scaling approach as representative of *in vivo*
238 hepatic clearance (Poulin et al. 2011), although this approach may overestimate the *in vivo*
239 clearance in case of extensive plasma protein binding. In these equations, the following
240 scaling factors were used: 34 mg/g microsomal proteins per g liver, 0.71 mL/min/g liver for
241 hepatic clearance (Q_H) and a relative liver mass of 2.6 g liver/kg bodyweight (Lipscomb and
242 Poet 2008).

243

244 **3. Results**

245 **3.1.Micro-LC-QTOF**

246 Four metabolites were found within 3 ppm mass error margin (table A3, figure 1): BCIPP (2
247 isomers), BCIPHIPP (2 isomers), a carboxyl-metabolite (2 isomers) and hydroxy-TCIPP (1

248 isomer), which were previously named BCIPP, TCIPP-M2, TCIPP-M1 and TCIPP-M3,
249 respectively (Van den Eede et al. 2013b).
250 Isomers were not completely separated (figure A1), because the μ-LC gradient program was
251 not fully optimized in this quick screening assay. MS/MS spectra (figure A2) confirmed the
252 general structure of the metabolites, by showing the protonated phosphate, mono-ester and
253 sometimes di-ester fragments in positive ionization mode. TCIPP-M1 indicated two additional
254 rearrangements, namely one to a mono-ester with additional loss of formic acid to an ethenyl
255 side chain (m/z 124.9978) and one to a lactone structure (m/z 152.9944).

256 **3.2.Performance of kinetic HLM and serumassay**

257 3.2.1. HLM

258 The method detection limit (MDL) in HLM samples was 10 ng/mL for BCIPP and 1.5 ng/mL
259 for BCIPHIPP. Using the optimized initial rate conditions for the experiments, TCIPP-M1
260 and TCIPP-M3 were undetectable, probably because of slower formation rates so that they
261 could only be detected in the 2h screening assay.

262 3.2.2. serum

263 BCIPP formation in the serum assay did not show any significant increase with increasing
264 enzyme concentrations in incubation mixtures. At 20 μM TCIPP, BCIPP levels in samples
265 were not higher than in method blanks. At 100 μM TCIPP, BCIPP levels in samples with
266 serum, buffer, and $CaCl_2$ (cofactor) were not higher than in samples without serum enzymes.
267 The slope of BCIPP formation versus enzyme concentration was not significantly different
268 from 0 ($p = 0.27$). A careful estimation of the sensitivity of the serum assay (equation 1)
269 indicated that BCIPP formation would be below 38.6 (SD 10.8) pmol/min/μL serum.
270 Compared to the positive control, 4-nitrophenol formation from 100 μM paraoxon was on
271 average 88.3 (SD 5.6) pmol/min/μL serum.

272 **3.3.Metabolism kinetics in HLM**

273 BCIPP was detected in reaction mixtures and also in our negative control samples. A large
274 variability in BCIPP concentrations in samples was observed due to its variable presence in
275 negative control samples (20 to 40% of total concentration). While BCIPP concentrations
276 versus substrate level followed a non-linear trend characterized by saturation in samples
277 (figure 2), this trend was linear in negative controls (figure 3). After subtraction of negative
278 control levels, Michaelis-Menten (equation 1) was the optimal model for non-linear
279 regression of NADPH-dependent BCIPP formation with an associated V_{MAX} of 1470 ± 110
280 pmol/min/mg protein, and K_m of 96.1 ± 14.5 μM.
281 Linear regression of BCIPP concentrations versus substrate concentration in enzyme and
282 cofactor negative controls indicated good fit with R^2 values of >0.95 for both curves. Both
283 slopes were significantly different from 0 and also significantly different from each other (p
284 values all <0.01, see Table A2 for more information), indicating involvement of hydrolases in
285 HLM, though their reaction rates were not quantifiable due to variable BCIPP formation in
286 enzyme negative control replicates. We tested paraoxonase activity in HLM to test the
287 possibility of hydrolases being involved in the production of BCIPP. Paraoxon hydrolysis by
288 HLM under the same conditions (using 100 μM substrate) was 64.5 pmol (4-
289 nitrophenol)/min/mg protein, which supports the possible involvement of hydrolases in HLM.
290 BCIPHIPP was formed solely by CYP- or other NAPDH-dependent enzymes. When plotting
291 reaction velocity versus substrate concentration, a typical Michaelis-Menten curve was
292 obtained with V_{max} of 153.5 ± 4.0 pmol/min/mg protein and K_m of 80.2 ± 4.4 μM (figure 2).
293 Production rate of BCIPHIPP was nearly tenfold lower than that of BCIPP, yet BCIPHIPP
294 concentrations showed less variability in reaction mixtures as this metabolite was not present
295 in negative control samples. As a result, the kinetics model that was predicted for BCIPHIPP
296 had a lower standard error for constants such as apparent K_m and V_{max}.

297 Based on the V_{max} and K_m parameters, *in vitro* intrinsic clearance $CL_{int,LM}$ (equation 3) was
298 estimated to be 15.3 ± 2.6 and 1.91 ± 0.12 µL/min/mg protein for BCIPP and BCIPHIPP,
299 respectively. As pointed out elsewhere (Pelkonen and Turpeinen 2007), $CL_{int,LM}$of different
300 pathways can be summed resulting in 17.2 ± 2.6 µL/min/mg protein. After application of
301 equation 4 and the scaling factor mentioned under the methods section, the intrinsic clearance
302 would be 585 µL/min/g liver for TCIPP. Using the direct scaling approach (equation 5) this
303 would be comparable to 0.32 mL/min/g liver or 0.83 mL/min/kg bodyweight..

304

305 **4. Discussion**

306 **4.1.Micro-LC-QTOF screening**

307 The qualitative profile of metabolites, namely BCIPHIPP as major metabolite, was
308 comparable both to our previously published work (Van den Eede et al. 2013b) and to the
309 findings of Abdallah et al. (2015). Although the latter did not report any presence of TCIPP-
310 M3, this could have been due to the lower substrate concentrations used as TCIPP-M3 gave
311 only a minor signal in our samples. Aside from demonstrating the reproducibility of our
312 previous findings, the major purpose of repeating the metabolite screening for TCIPP was to
313 test the applicability of µ-LC-QTOF in rapid analysis of suspect compounds. With a low
314 injection volume, and only a three minute gradient elution program we were able to detect the
315 same metabolites with a slightly better mass accuracy compared to the HPLC-QTOF
316 instrument conditions in our previous study (Van den Eede et al. 2013b). While Abdallah et
317 al. (2015) also achieved rapid separation with an ultra-high performance-LC coupled to an
318 Orbitrap instrument, a µ-LC-QTOF is less expensive in acquisition and maintenance. The
319 application of µ-LC-QTOF to this small sample set can serve as an indication of how similar
320 sensitivity and resolution can be achieved along with a reduction in analysis time and solvent
321 consumption in screening for metabolites of environmental contaminants.

322 **4.2.Hydrolysis by serum enzymes**

323 Although it did not seem likely that serum had a major influence on TCIPP metabolism, the
324 sensitivity of our assay could be limited. Because of the low number of replicates, a lower
325 standard deviation in the samples and negative controls were needed to distinguish a small
326 catalytic effect of serum enzymes. Hence the sensitivity threshold may have interfered with
327 the detection of any BCIPP formation and we cannot exclude any extra-hepatic degradation of
328 TCIPP in blood. On the other hand, paraoxon hydrolysis by serum enzymes was observed,
329 therefore the assay in itself was valid. This obvious difference between the catalytic activity
330 of the paraoxonase enzyme towards paraoxon and TCIPP may be explained by structure-
331 related differences, such as the absence of an aryl side chain, since paraoxonases are known to
332 hydrolyze mainly dialkyl aryl phosphate structures (Testa and Krämer 2010). In spite of this
333 lack of interaction between TCIPP and paraoxonases, we chose to test this enzyme family
334 since to our knowledge, no other enzymes have been characterized in humans that are capable
335 of hydrolyzing organophosphate triesters without being consumed in the reaction.

336 **4.3.Biotransformationof TCIPP in HLM**

337 The possible involvement of hydrolases in HLM in the formation of BCIPP is surprising
338 considering the non-detectable BCIPP formation by serum enzymes.These contradictory
339 results can have two explanations: either hydrolases with different characteristics or origin
340 than paraoxonases are involved in TCIPP metabolism, or the serum assay did not achieve
341 sufficient sensitivity to distinguish any effect of paraoxonases.As mentioned previously,
342 degradation of TCIPP by serum enzymes cannot be ruled out.

343 As for the extrapolation of the kinetics of TCIPP biotransformation in HLM to the whole
344 liver, the estimated intrinsic *in vivo* clearance of TCIPP (0.585 mL/min/g liver) is more than
345 80% of the average hepatic blood flow (section 2.5.2), suggesting that TCIPP is not a low
346 clearance chemical (meaning intrinsic clearance <20% of hepatic blood flow) and that its

347 hepatic clearance is not flow-limited either. As a consequence, TCIPP is not likely to exhibit
348 the same accumulating behavior in the human body as more apolarenvironmental
349 contaminants, such as polychlorinated biphenyls or polybrominated diphenyl ethers
350 (Darnerud et al. 2015).

351 **4.4.Comparison of findings to *in vivo*toxicokinetics in rat**

352 Exposure of rats to ^{14}C-labeled TCIPP revealed quick absorption and distribution of TCIPP in
353 the body, as after 5.7 h maximum concentrations of the radiolabel were found in the tissues
354 (Minegishi et al. 1988). TCIPP was mostly found in the liver and the kidneys, followed by the
355 lungs. The majority of the TCIPP radiolabel was excreted in urine (67% within one week)
356 (Minegishi et al. 1988). No metabolite structures were identified or measured for comparison
357 with clearance rates in this study, though its findings suggest that uptake of TCIPP in the liver
358 occurs, which is a prerequisite for hepatic clearance. Therefore hepatic clearance could play
359 an important role in elimination of TCIPP besides excretion.

360 **4.5.Comparison of findings to human biomonitoring data**

361 4.5.1. Human milk

362 The hepatic clearance rates measured in this study were comparable to those of TBOEP (Van
363 den Eede et al. 2015b). However, in pooled breast milk samples higher detection frequency
364 and median levels of TCIPP were reported in comparison to TBOEP (Sundkvist et al. 2010),
365 which could be explained by hightissue distribution, as observed in rats for TCIPP (Minegishi
366 et al. 1988). Investigation of toxicokinetic processes, such as renal and biliary clearance, and
367 tissue distribution in the human body, is required to confirm the accumulation potential of this
368 FR .

369 4.5.2. Human urine

370 BCIPP was detected only in a minority (≤30%) of urine samples in several studies as
371 mentioned in the introduction. While limited method sensitivity could have been a reason for

372 this observation in some cases, in others BCIPP was still rarely detected despite a lower
373 method detection limit (Schindler et al. 2009, Dodson et al. 2014, Fromme et al. 2014).
374 Another logical explanation could have been low exposure, which might be the case for the
375 United States (Stapleton et al. 2009, Dodson et al. 2012). However, in Europe this scenario
376 would be less likely since TCIPP has been reported as a predominant PFR in indoor dust and
377 air (Fromme et al. 2014, Cequier et al. 2015, Brandsma et al. 2014). We did find BCIPHIPP
378 recently in more than 90% of analyzed Australian urine samples at levels up to 9.4 ng/mL
379 (Van den Eede et al. 2015a) even though dust levels of TCIPP in Australia were similar to
380 those in Canada and the European mainland (Brommer, 2014). In light of the current findings,
381 it seems that BCIPP is the major metabolite formed by liver enzymes, though two factors
382 could explain the difficulty of detecting BCIPP in urine: (i) analytical difficulties, and (ii)
383 possible pharmacokinetic processes interfering with its excretion in urine, such as protein
384 binding, storage in tissues or other excretion pathways. Paired measurements of BCIPHIPP
385 and BCIPP levels in urine and serum are needed to confirm this hypothesis.

386 **4.6.Role of biotransformation in toxicity of TCIPP**

387 To date, the toxicity of TCIPP and BCIPP or BCIPHIPP cannot be compared due to lack of
388 data on these two metabolites.The biotransformation pathway to BCIPHIPP includes two
389 intermediate structures (Abdallah et al. 2015) which could also exert toxic effects at the site of
390 the liver. Consequently, we cannot state if the obtained clearance values represent a
391 detoxification or bioactivation. Furthermore, the hydrolytic formation of BCIPP could
392 produce 1-chloro-2-propanol as a byproduct. This chemical causedadverse effects on the liver
393 in rats at doses of 100 mg/kg/day and higher during a period of fourteen weeks (NTP 1998),
394 which is slightly higher than the low observed adverse effect level for TCIPP under similar
395 conditions and toxicity endpoint (EU 2008). As far as the potential byproduct is concerned,
396 hydrolysis of TCIPP would lead to a slight decrease in toxicity.

397 **4.7.Limitations**

398 The major limitations of this study as mentioned above, are the absence of plasma protein
399 binding data, which could result in an overestimation of the current value of hepatic
400 clearance; and the limited ability of the serum assay to distinguish any BCIPP formation.
401 Because not all four metabolites of TCIPP were detectable at the initial rate conditions in the
402 HLM assay, our calculations were based only on the two major metabolites. This exclusion of
403 the two other, but minor,metabolites may have impacted our estimation of the intrinsic
404 clearance of TCIPP, leading to an underestimation.

405

406 **5. Conclusions**

407 This study is the first to present quantitative data on TCIPP metabolism in humans (or any
408 other species). Our results indicated faster formation of the hydrolysis product BCIPP than the
409 dechlorinated metabolite BCIPHIPP by hepatic enzymes. The role of serum hydrolases in
410 TCIPP hydrolysis could not be confirmed, although hepatic hydrolases did contribute to
411 BCIPP formation. More information is needed onthe roles of the studied metabolites BCIPP
412 and BCIPHIPP in the toxicity of this flame retardant.

413

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421

Supplementary material

Information regarding preliminary experiments, non-linear regression, and μ-LC-QTOF chromatograms and spectra are presented in appendix A.

CYP isoform activities in the commercially obtained HLM are available from appendix B.

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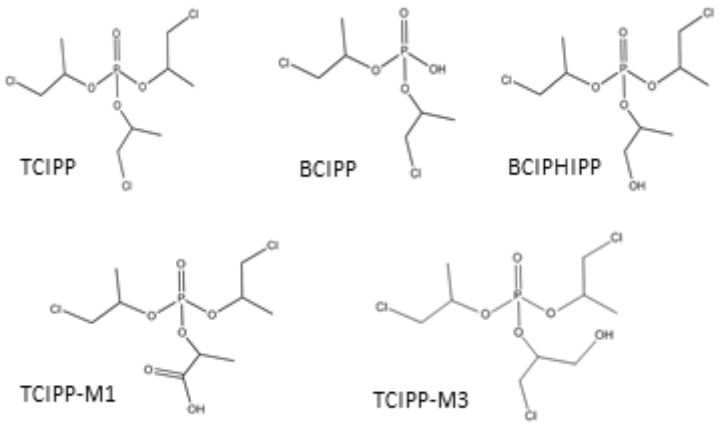
527

528 **Figures**

529

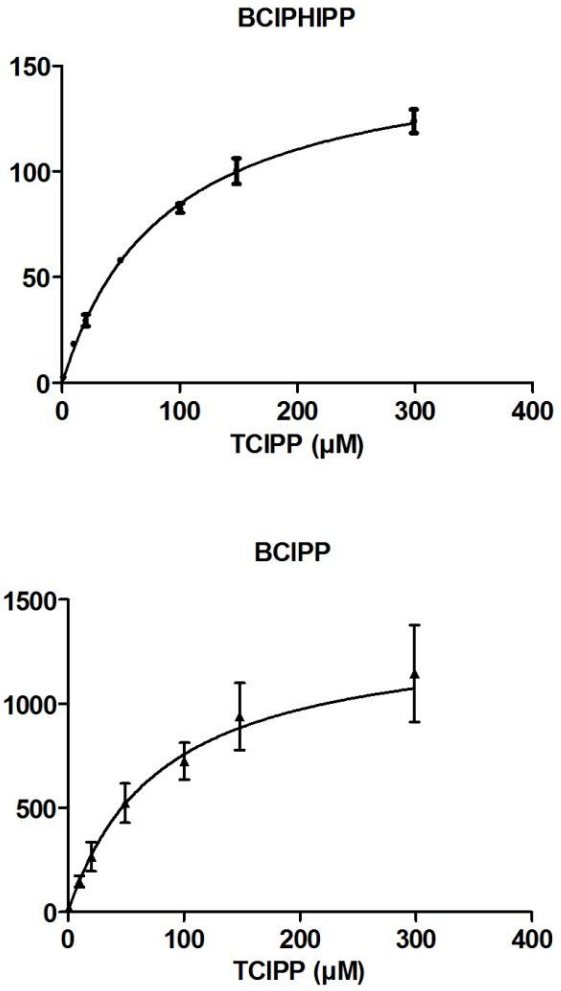


530

531 Figure 1. Proposed structures of tris(1-chloro-2-propyl) phosphate (TCIPP) metabolites.
532 BCIPP: bis(1-chloro-2-propyl) phosphate; BCIPHIPP: bis(1-chloro-2-propyl)1-hydroxy-2-
533 propyl phosphate; TCIPP-M1 and TCIPP-M3 were named according to the previous
534 publication (Van den Eede et al. 2013b). Only the structures of BCIPP and BCIPHIPP (2nd
535 isomer) were confirmed using nuclear magnetic resonance spectroscopy and authentic
536 standards.

537

538

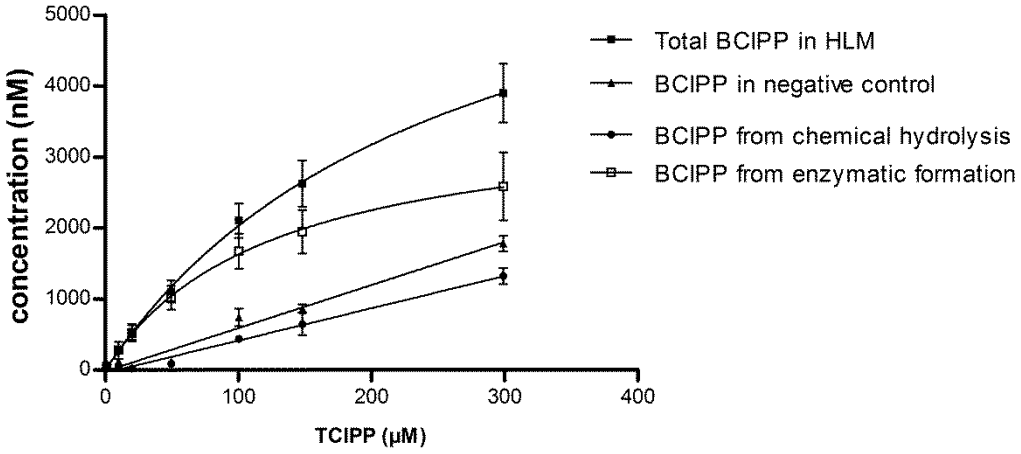


539

540 Figure 2. Formation of BCIPP (bis(1-chloro-2-propyl) phosphate; bottom panel) and
541 BCIPHIPP (bis(1-chloro-2-propyl) 1-hydroxy-2-propyl phosphate; top panel) by NADPH-
542 dependent enzymes. TCIPP (tris(1-chloro-2-propyl) phosphate was incubated in the presence
543 of 0.25 mg/ml human liver microsomes and 1 mM NADPH for 7.5 min at 37 °C. Y-axis:
544 Metabolite formation rate (pmol/(min * mg microsomal protein). Triangles indicate average
545 production rate (n = 5), while error bars indicate the standard deviation between replicas.

546

547



548

549 Figure 3. Comparison of BCIPP (bis(1-chloro-2-propyl) phosphate) levels in incubation
550 samples of TCIPP (tris1-chloro-2-propyl) phosphate with human liver microsomes and/or
551 buffer. Total BCIPP: BCIPP levels as a result of both enzymatic formation and chemical
552 hydrolysis; BCIPP from enzymatic formation: sum of BCIPP due to NADPH-catalyzed
553 reactions and due to enzymatic hydrolysis; BCIPP in negative control: chemical and
554 enzymatic hydrolysis of TCIPP in absence of NADPH; chemical hydrolysis: BCIPP
555 formation in presence of buffer alone. Symbols indicate average concentrations, error bars
556 represent the standard deviation..

appendix A

appendix B