In Vitro Biotransformation of Chlorinated Paraffins

Yiran Ji[1,*], Sicco H. Brandsma[1], Timo Hamers[1], Louise M. van Mourik[1], Jon E. Johansen[2], Huiling Liu[2], Pim E.G. Leonards[1]

[1] Section of Environmental and Health, Amsterdam Institute for Life and Environment, Vrije Universiteit Amsterdam, De Boelelaan 1108, 1081 HZ Amsterdam, the Netherlands
[2] Chiron AS, Stiklestadveien 1, 7041 Trondheim, Norway

1 Introduction

Chlorinated paraffins (CPs) are defined to be polychlorinated n-alkanes with a chain length of 10 – 30 carbon atoms and a degree of chlorination of 30 – 70% (w/w) (Glüge et al., 2016). They are extensively used as plasticizers, flame retardants, additives in paints and lubricants, and cutting fluids in metalworking (van Mourik et al., 2016). Based upon their carbon chain lengths, CPs are consensually classified into short-chain (SCCPs, $C_{10\text{-}13}$), medium-chain ($C_{14\text{-}17}$), long-chain CPs (LCCPs, $C_{>17}$). SCCPs have been listed to be persistent organic pollutants (POPs) under the Stockholm Convention since 2017 owing to their persistence, bioaccumulation and toxicity (PBT) towards environmental and human health in addition to their long-range transport potential (UNEP, 2016). Though prohibition on SCCP production has been announced by many countries, such as the US and the European Union, MCCPs and LCCPs are still being produced worldwide with an annual production of more than two million tons (Glüge et al., 2016; van Mourik et al., 2016).

Early *in vivo* studies on ^{14}C-labelled CPs-fed rats (Birtley et al., 1980; Serrone et al., 1987) or mice (Darnerud et al., 1982) reported a highest initial uptake of SCCPs and MCCPs in liver followed up by a long-lasting retention in fat. These results suggested a fast elimination of CPs from liver, which was then confirmed to be cytochrome P450-dependent (Darnerud, 1984). Cytochromes P450 (CYPs) enzymes abundant in liver are well known for executing a variety of biotransformation on diverse, mostly hydrophobic, substrates including drugs and other xenobiotics (Esteves et al., 2021; Sono et al., 1996).

More recent studies endeavored to identify the biotransformation products of CPs throughout plants (Chen et al., 2020), bacteria (Heeb et al., 2019; Knobloch et al., 2021) and human liver microsomes (He et al., 2021). Indicated biotransformation pathways included HCl elimination into olefins (Chen et al., 2020; Heeb et al., 2019), hydrolytic dechlorination into multi-alcohols (Chen et al., 2020; Knobloch et al., 2021), oxidation into ketones and carboxylic acids and chain shortening into very SCCPs (vSCCPs) (He et al., 2021). Nevertheless, metabolism studies on CPs are still very limited and to our knowledge, no metabolites have been structurally identified, due to the lack of certified reference materials (CRMs).

The goals of this study are 1) to investigate the relative extent of the *in vitro* phase I biotransformation of CPs by rat liver S9 induced by different CYP inducers, 2) to identify the chemical structures of the biotransformation products formed, and 3) to reveal the biotransformation pathways underneath CP metabolisms. The biotransformation information will finally be used to synthesize analytical standards of the main biotransformation products.

2 Materials and Methods

2.1 Chemicals and solvents

All solvents were obtained from Biosolve (Valkenswaard, the Netherlands) of the highest available quality (e.g., analytical grade). 1,2,4,5,8,9-Hexanchloroundecane ($C_{11}Cl_6$) and 1,2,5,6,9,10-hexachlorododecane ($C_{12}Cl_6$) were provided by Chiron (Trondheim, Norway). 3,3',5,5'-Tetrabromobisphenol A (TBBPA, purity 97%) was obtained from Sigma-Aldrich (St. Louis, USA) and ^{13}C-TBBPA was from Cambridge Isotope Laboratories (Andover, USA). Three rat liver S9 fractions (Aroclor 1254 induced, phenobarbital-5,6 and benzoflavone induced and uninduced) were bought from Trinova Biochem (Giessen, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH, purity 96%) tetrasodium salt hydrate was obtained from Fisher Scientific (Landsmeer, the Netherlands). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was acquired from Roche Diagnostics (Mannheim, Germany).

2.2 Incubation experiments

The reaction mixtures containing the S9 fractions (1 mg/mL) and the substrate ($C_{11}Cl_6$, 1 µM) in Tris-HCl buffer (100 mM, pH 7.4) were pre-incubated at 37 °C for 10 min. 10 µL NADPH solution (20 mM) was added to initiate the reaction, making up a total reaction volume of 200 µL. Additional aliquots (10 µL) of NADPH solution were added every 15 min during the reaction to avoid depletion. The reaction was stopped at 0, 5, 10, 20, 30, 45 and 60 min by adding an equal volume of ice-cold methanol. 20 µL CP internal standard ($C_{12}Cl_6$) was added to the samples before centrifuge at 5000

rpm for 15 min. The supernatant was then collected and stored at –20 °C until analysis. Each incubation experiment included three control groups: negative controls using heat-inactivated S9 fractions, positive controls using a reference chemical (TBBPA), and blank controls without substrates.

2.3 Instrumental analysis

Measurement of the CPs and biotransformation products was performed with a slightly adopted analytical method recently developed by Knobloch et al., (2021). Samples were analyzed by an Agilent 1290 Infinity HPLC system (Agilent Technologies, Amstelveen, the Netherlands) coupled to a quadrupole time-of-flight spectrometer (qTOF-HRMS) (Compact, Bruker, Bremen, Germany) operating with an atmospheric pressure chemical ionization (APCI) source. Separation of the parent compound and the metabolites was achieved by reversed phase HPLC using a ZORBAX SB-C18 RRHD column (50 mm × 3 mm, 1.8 µm). The column was maintained at 40 °C and the mobile phases composed of water (A) and methanol with 10% DCM (v/v) (B) was eluted in 11 min with a gradient of 40% B for 1 min, 40 – 98% B in 7 min, 98% B for 2 min, and 98 – 40% B in 1 min. The injection volume was 15 µL and the flow rate was 0.4 mL/min. 10% DCM solution was used for an enhanced formation of $[M+Cl]^-$ ions in the negative mode (Bogdal et al., 2015). Full scan MS data was recorded across m/z 90 – 1600. Each injection was performed with internal mass calibration using the Agilent APCI-L low concentration tuning mix (part no. G1969-85010). Data processing was operated with Bruker TASQ Client 1.4 (Bremen, Germany).

3 Results

The reversed phase C_{18} column performed good separation of the biotransformation products from each other and their precursor (chromatograms not shown). The average retentions times are given in Table 1. The more polar metabolites eluted before their parent compound.

Table 1: Average retention times of the CP biotransformation products and the CP parent compound ($C_{11}H_{16}Cl_6$) deduced from $[M+Cl]^-$ adducts.

	Retention time (min)	Functional group(s)
CP ($C_{11}H_{16}Cl_6$)	7.75	-
Metabolite 1	6.90	Mono-ketones
Metabolite 2	6.55, 6.32	Mono-alcohols
Metabolite 3	6.51	Mono-alcohols
Metabolite 4	5.90	Di-ketones
Metabolite 5	3.98	Di-alcohols
Metabolite 6	0.65	Hydroxyketones

The effect of the different CYP inducers on the biotransformation of CP after incubation was summarized in Table 2. The CP degradation was determined to follow the 1st-order kinetics and the corresponding rate constants were obtained from the linear portion of the 1st-order reaction plot (60 min for un- and Aroclor 1254-induced S9, and 30 min for phenobarbital and benzoflavone (PB/BF)-induced S9, data not shown). It can be seen that both inducers had a positive effect on the CP degradation, increased by 57.6% and 272% respectively by Aroclor 1254, and PB/BF inducers.

Table 2: Effects of cytochrome P450 inducers on the degradation of CP after incubation.

P450 inducer	Uninduced	Aroclor 1254	PB/BF
Rate constant (min^{-1})	0.0085	0.0134	0.0316
Half-lives (min)	82	52	22

Figure 1 shows the formation of the different biotransformation products of CPs versus time. The corrected peak areas of the metabolites were calculated from the ratio of the peak area of the metabolite from that of the internal standard. As there is no CRMs available for the metabolites of CPs, it is impossible to quantify the concentrations of them, nor to compare between the different metabolites. What can be obtained from Figure 1 were the relative concentrations of one metabolite over time and to compare the effect of the different inducers on the formation of the same metabolite.

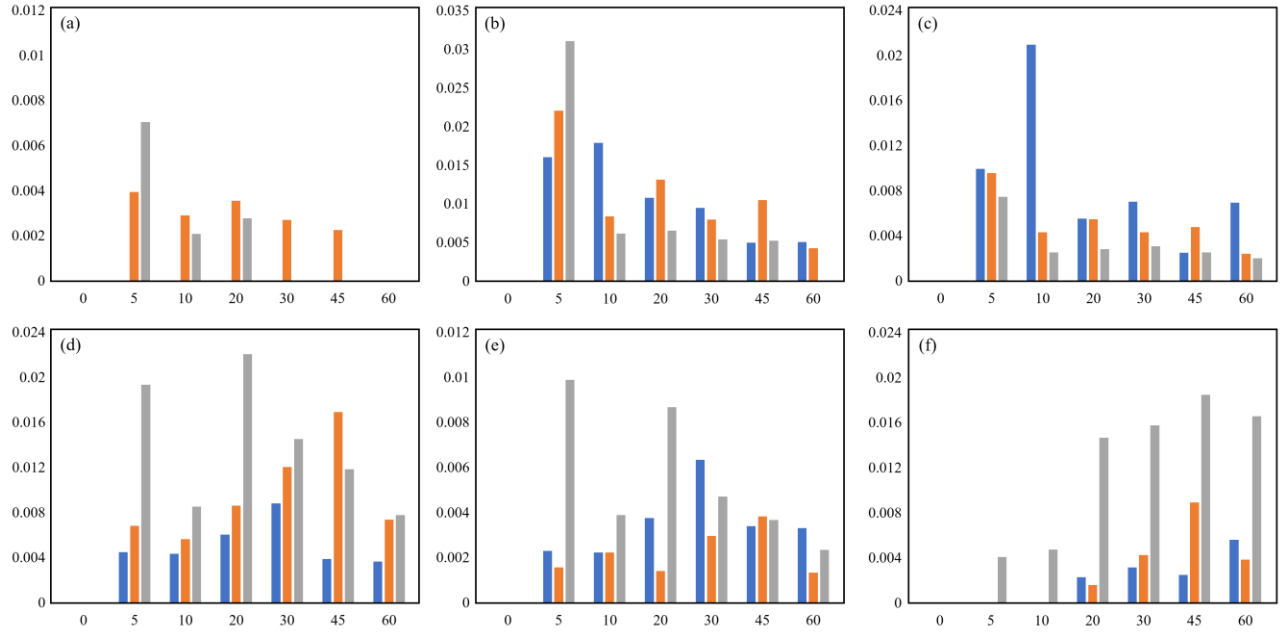

Figure 1: Internal standard corrected peak areas of the different metabolites, (a) Metabolite 1 (mono-ketones), (b) Metabolite 2 (mono-alcohols), (c) Metabolite 3 (mono-alcohols), (d) Metabolite 4 (di-ketones), (e) Metabolite 5 (di-alcohols) and (f) Metabolite 6 (hydroxyketones), after incubation with un- (blue), Aroclor 1254- (orange) and phenobarbital and benzoflavone- (grey) induced rat liver S9.

4 Discussion

The results of this present study give evidence for the liver microsomal P450-dependent *in vitro* biotransformation of SCCPs. Aroclor 1254-treated rat S9 moderately increased the elimination rate of SCCP by 57.6% (Table 2) while the PB/BF inducer significantly enhanced the SCCP degradation by 272%. This indicates the CYP enzymes, and most likely the Cyp2b1/2b2, play a role in the degradation of SCCPs in liver. This result is consistent with a previous *in vivo* study on the inhibitory effects of the CYP inhibitors on $^{14}CO_2$ exhalation from 1-^{14}C-chlorododecanes in mice (Darnerud, 1984). Although the same study showed limited effect of Aroclor 1254 and PB pretreatments on the formation of $^{14}CO_2$, possible explanation was several steps were involved in the ultimate formation of $^{14}CO_2$ from CPs whereof the CYP-containing step was not rate-determining.

The *in vitro* half-lives of CPs were determined to be 1.36 h in un-induced rat S9 in this study. The result clearly contradicts the half-lives (22 – 60 years) measured in rat microsomes by Dong et al., (2019). No other parallel experiments are available for comparison, but most reports demonstrated a much faster elimination of CPs in liver, e.g., Birtley et al., (1980) reported the retention of radioactivity of ^{36}Cl-Cereclor S52 in rat liver was below the detection limit within 1 week. It is worth noting that this present study only focuses on Phase I biotransformation and the resulting metabolites would stay in liver. They will then either get involved in Phase II conjugation, products of which can be excreted from liver, or be bioactivated into highly reactive and electrophilic metabolites that lead to toxicity (Esteves et al., 2021).

As shown in Table 1, six metabolites were chemically identified in this present work, including two mono-hydroxylated products, one di-hydroxylated product, one mono-ketone, one di-ketone and one hydroxyketone. The different molecular formulas of the two mono-hydroxylated products (Metabolite 2 and 3) indicated hydroxylation of CPs could take place at both sp^3 C-H and C-Cl bonds. The chromatograms of the carbon hydroxylated product (Metabolite 2, data not shown) showing two isolated peaks (eluted at 6.55 and 6.32 min, respectively) suggested two isomers may be formed during incubation with rat liver S9.

In Figure 2, it can be seen that the two CYP inducers did not enhance the hydrolytic dechlorination pathway (Figure 1c). Oxidative dechlorination (Figure 1a) was not observed in un-induced rat S9 while the corresponding mono-ketones were detected with CYP inducers. This indicates a CYP-dependent mechanism of CP biotransformation. Besides oxidative dechlorination, the two inducers also preferred the carbon hydroxylation (Figure 1b) and the formation of metabolites with a higher degree of oxygen (Figure 1d, 1e and 1f). Despite an accumulative formation of the hydroxyketones (Figure 1f) within 60 min, the overall trend for the formation of other SCCPs metabolites was found with a sharp increase at the initial stage (in 5 – 10 min) followed up by a much slower reduction over time. This possibly resulted from the further biotransformation of these metabolites into hydroxyketones and other un-defined products.

5 Conclusions

Our work confirmed the liver microsomal P450-dependent *in vitro* biotransformation of short chain chlorinated paraffins. To our knowledge, this is the first time demonstrating that both the sp^3 C-H and C-Cl bonds in SCCP can be hydroxylated, although previous findings have reported the formation of hydroxylated products from SCCPs (Knobloch et al., 2021). Formation of mono-ketones from oxidative dechlorination by rat liver CYP inducers also reflected the CYP-dependent mechanisms of SCCP biotransformation. Stepwise hydroxylation into diols and further oxidation into hydroxyketones was also observed. CRMs for these biotransformation products are needed to enable quantification as well as toxicity assessments. After full identification of the chemical structures of the biotransformation products analytical standards will be synthesized to finally prepare CRMs.

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