IN VITRO BIOTRANSFORMATION AND IDENTIFICATION OF POTENTIAL TRANSFORMATION PRODUCTS OF CHLORINATED PARAFFINS

He C[1], van Mourik L[2], Tang S[3], Thai P[1], Wang X[1], Leonards PEG[2], Thomas K[1], Mueller J[1]

[1] The University of Queensland, Brisbane, Australia 4102 (He C: c.he@uq.edu.au); [2] Vrije Universiteit, Amsterdam, The Netherlands, 1081; [3] Dongguan University of Technology, 511700, Dongguan, China

Introduction

Chlorinated paraffins (CPs) are a group of polychlorinated n-alkanes (PCAs) with different carbon chain lengths and number of chlorine atoms [1]. They are widely used as coolants and lubricants in metal working fluids, and as flame retardants and plasticizers in polymers, mainly in polyvinyl chloride (PVC) products [2]. Based on the carbon chain length, CPs can be divided into three main subgroups: short-chain chlorinated paraffins (SCCPs) with a carbon chain length shorter than C_{13}, medium-chain chlorinated paraffins (MCCPs) with a carbon chain length between C_{14} and C_{17}, and long-chain chlorinated paraffins (LCCPs) with a carbon chain length longer than C_{17} [3]. In 2017, SCCPs was listed in the Stockholm Convention of POPs, due to their long-range environmental transport and significant adverse effects on human health and the environment [4]. However, the occurrence and concentration of CPs, as well as their half-lives in humans were poorly understood. To the best of our knowledge, no published study on the occurrence of CPs in human urine is available yet.

The aims of this study are 1) to investigate the half-lives and biotransformation of CPs using human liver microsomes (HLM), which has been used to assess the metabolism for variety chemicals [5-7], 2) to assess the change of profile of CPs due to the biotransformation, and 3) to explore the possible transformation products using suspect screening strategy.

Materials and methods

In vitro assays

In this study, a two-tiered approach for investigating biotransformation of CPs with Phase I and Phase II enzymes was adopted from Lai et al. [7]. The purpose of tier I was to assess the persistence and potential transformation of CPs, and different transformation rate using different groups of enzymes. Tier II was designed to assess the effects from different conditions, including incubation time, enzyme concentrations, and concentrations of CPs.

Instrumental analysis

The instrumental method used for CP analysis was adopted from our previous study [8]. Briefly, 10 µL of the sample extract was directly injected, without using an analytical column, into a quadrupole time-of-flight high resolution mass spectrometer (QToF-MS, Triple TOF 5600+ Sciex, Concord, Ontario, Canada) using the negative Atmospheric Pressure Chemical Ionization (APCI) mode. ACN was used as eluent with a flow of 250 µL/min. To improve the response of CPs in APCI mode, DCM at a flow rate of 40 µL/min was used as a dopant and mixed with the eluent just prior to entering the ion source. The APCI-QToF-MS was set as follow: 200 °C for nebulizer temperature, −20 V for declustering potential (DP), and −10 V for collision energy (CE) and collision exit cell potential (CXP). The mass spectrometer was operated in ToF-MS mode accruing HRMS full scan spectra across the range of m/z 250−1100. The minimal resolving power of the detector was 22500. The window used for extracting the m/z values was set as ± 0.0025. Accurate mass spectra to 4 decimal places was used for tentative transformation product peak identification with an error threshold of 5 ppm.

Data analysis

Quantitative method was used to calculate the concentrations of SCCPs, MCCPs, and LCCPs, using six-point dilution series that prepared by SCCP (Cl: 55.5%), MCCP (Cl: 52%), and LCCP (Cl: 49%), respectively. Different with the analysis of CPs in environmental method, deconvolution was not needed in this study, because each sample have only one major source of CPs, which was from the spiking solution. For suspect screening, the potential biotransformation products were predicted based on authors' knowledge, including the molecular structure and the types of reactions that these families of enzymes are known to catalyse.

Results and discussion:

Biotransformation of CPs

All the groups of CPs were detected in experiment A, B and C, despite that only one single standard was added in each experiment, and this was caused by the co-existence of different CPs in the standards. The phase II clearance rates for different CPs were all higher than 60 % after 4 hours. More specifically, cytochrome P450

(CYPs) were the most efficient enzyme group on average, with the clearance rates of 81 %, 97 % and 73 % for SCCP, MCCP, LCCP, respectively. This was not surprising, because CYPs were a unique family of enzymes which were capable of catalysing oxidation of hydrocarbons [9]. The results in this study suggest short half-lives for CPs (Fig. 1); <2.0 h for all different CP groups. This could explain the low internal CP exposure that we recently reported [10]. In addition, compared with the parent chemicals, their transformation products might be more important for assessing the internal exposure of CPs.

Biotransformation profiling

The biotransformation of CPs at different incubation times was investigated in Tier II experiment to characterize the change in concentration of each CP group during enzymatic transformation (Fig. 1). Following biotransformation, the concentrations of the dominant group in each spiking solution decreased with time. On the other hand, concentrations of MCCPs and SCCPs in experiment C, and SCCPs in experiment B increased following enzymatic exposure, suggesting CPs are transformed into shorter carbon chain CPs by liver microsomes. However, concentrations of LCCPs, and MCCPs in experiment A, and LCCPs in experiment B decreased following biotransformation. This was likely caused by the degradation of these chemicals. Our findings are consistent with previous in vivo studies, where CPs were found to be partly degraded to CO2 by quails and mice [11, 12].

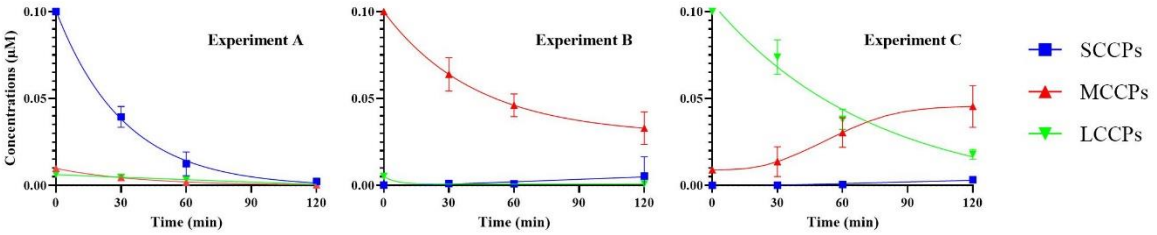

Figure 1. Concentrations of CPs (μM) detected following the biotransformation at different times, where 0.1 μM of SCCPs (experiment A), MCCPs (experiment B), and LCCPs (experiment C) were spiked separately.

A comparison of carbon chain length profile and chlorine content before and after biotransformation are shown in Fig. 3. The average carbon chain decreased after biotransformation in both experiment B and C. In experiment C, C_{18} (42 %) and C_{17} (22 %) were the predominant congeners in the mixture solution, but after transformation, their contributions decreased to 4 % and 10 %, respectively, while the contribution of C_{15} congeners increased from <1 % to 42 %. Similarly, in experiment B, the contribution of C_{14} and C_{15} decreased after the biotransformation, while the shorter chain length congeners, i.e. C_{12} and C_{13} increased significantly. In contrast, the average carbon chain length increased in experiment A. This we believe was due to the rapid degradation of SCCPs in experiment A, with relatively low concentrations of SCCPs and other groups of CPs after biotransformation, which can be transformed into even shorter congeners, i.e. vSCCPs [13, 14].

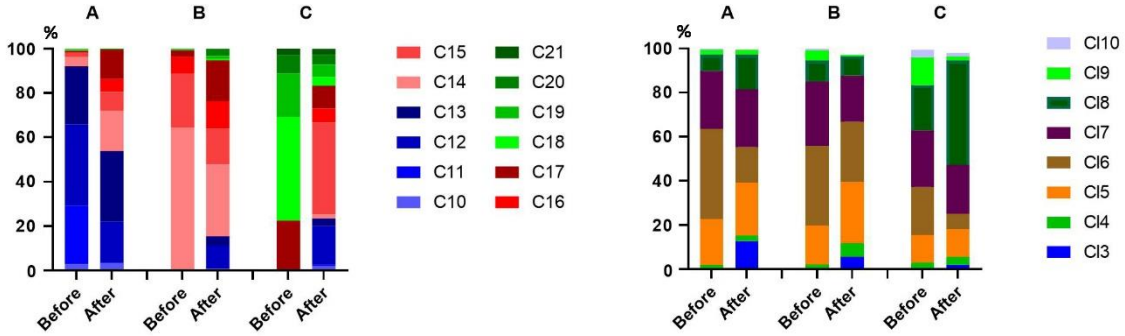

Figure 2. Change of relative abundance of carbon chain length (left panel) and chlorine contents (right panel) in the biotransformation. Contribution from Cl>10 were not shown due to the very low concentrations detected both before and after incubation (< 1% of the contribution)

The average chlorine content also decreased in most of the samples after biotransformation. More specifically, the relative concentrations of Cl_3 congeners increased in all the three experiment. However, it was possible that

the change in chlorine-based profile was caused by a change in carbon chain length, as the chlorine atoms could also leave the carbon chain together with the broken carbon atoms. This is similar to an oral administration study in which the distribution of SCCPs congener groups changed in rats' urine in 5 days [15].

Identification of potential transformation products using suspect screening

To assess the potential biotransformation products of CPs, we calculated the peak area of their oxidation products, including vSCCPs, hydroxylation products (OH-CPs, $C_nH_{2n-m+2}Cl_mO$), aldehyde products (CO-CPs, $C_nH_{2n-2m}Cl_mO$), and carboxylated products (COOH-CPs, $C_nH_{2n-2m}Cl_mO_2$), with carbon chain length between 6 to 21 and chlorine atoms between 3 to 10, using accurate mass. The pathway postulated for the formation of these potential products involved initial attack at the two adjacent non-halogenated sites by oxygenase, followed by chain shortening via β-oxidation [16]. In total, 351 potential metabolites were investigated, where 328 of them were detected in the samples after enzymatically transformation. The kinetics of these biotransformation products by HLM were shown in Fig S1, where all the groups of products increased with the concentrations of substrate. However, some of these oxidation products were also detected in the standards, and the kinetic results did not indicate any evidence on the transformation results by HLM. To investigate if these oxidative products were formed during the biotransformation, we compared the peak area of each product in the sample following transformation ($Area_{tran}$) and its peak area in negative control ($Area_{nc}$), where 33 % of vSCCPs, 39 % of OH-CPs, 50 % of CO-CPs, and 49 % of COOH-CP showed increased peak area in samples after transformation.

Table 1. Results of suspect screening for oxidative products and very short chain CPs

	No. of chemicals investigated	$\sum Area_{tran} > \sum Area_{nc}$ (%) [a]	$\sum Area_{nc}$	$\sum Area_{tran}$	Transformation rate (%) [b]
∑vSCCPs	20	33	791	6983	783
OH-vSCCPs	21	36	10014	40486	304
OH-SCCPs	31	31	544733	82125	-85
OH-MCCPs	32	35	199160	80244	-60
OH-LCCPs	32	53	56686	115607	104
∑OH-CPs	116	39	810593	318462	-61
CO-vSCCPs	21	42	2903	11724	304
CO-SCCPs	32	40	203128	34751	-83
CO-MCCPs	32	44	78630	142660	81
CO-LCCPs	32	71	24225	271752	1022
∑CO-CPs	117	50	199483	460887	131
COOH-vSCCPs	22	40	2080	8628	315
COOH-SCCPs	32	45	313975	177260	-44
COOH-MCCPs	32	42	340845	106861	-69
COOH-LCCPs	32	66	87545	100260	15
∑COOH-CPs	118	49	744445	393009	-47

a: $\sum Area_{tran} > \sum Area_{nc}$ (%): the percentage of chemicals with increased peak area after biotransformation.
b: Transformation rate = $\sum Area_{tran} / \sum Area_{nc}$, positive values suggested an increase after the biotransformation.

The peak area of ∑vSCCPs increased 7 fold after biotransformation, suggesting they might be formed during the enzymatically transformation. However, the concentrations of vSCCPs in both negative control and samples after biotransformation were relatively low, and the quantification of vSCCPs was still a huge. Nevertheless, the change of carbon chain length profile that was found above suggested that the cleavage of C-C bonds as an important metabolism pathway of CPs.

CO-CPs were another group of transformation products with the increased peak area (Average 131%) following biotransformation, especially for CO-LCCPs. In set C, where LCCPs were spiked into the reaction mixture, the peak area of all the subgroups of CO-CPs increased more than 10 times, suggesting CO-CPs might be an important group of metabolites of LCCPs.

OH-CPs were identified in 39 % of samples, with increased peak areas after biotransformation. However, the total peak area of OH-CPs decreased 61 %, suggesting OH-CPs were not a major group of transformation products, and they could also been metabolised into other products. Despite of the decrease of total OH-CPs,

peak area for vSCCPs, which had less interference from the spiking standards, increased 3 times, indicating hydroxylation also appeared in the biotransformation processes. Similarly, COOH-CPs were not a major group of transformation products of CPs, neither. Although the peak areas for some subgroups increased in experiment B and C, the total peak area decreased 47 % after the biotransformation.

One of the limitations of this study was that we were not able to identify all of the potential transformation products of CPs due to the complexity of the CP standards currently available, and challenges with their chromatographic separation. In the future, non-target analysis will be need to confirm our findings using individual CP congener standards, or less complicated standards.

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