Original Article

The Effect of the Structure of Polychlorinated Biphenyls on their Hydroxylation, Oxidation, and Glutathionyl Conjugation Reactions*

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Abstract

Objective To compare the nature of the metabolites formed from the phase I metabolism (hydroxylation and oxidation) and phase II metabolism (glutathionyl conjugation) of PCBs that have different chlorine substitution patterns. To discuss the structure-activity relationships and metabolic mechanisms of PCBs.

Methods 4-Cl-biphenyl (PCB3), 4,4'-Cl-biphenyl (PCB15), 3,4,3',4'-Cl-biphenyl (PCB77) were used for *in vitro* metabolic study. LC/MS and UV-Vis studies were performed for metabolites identification.

Results The cytochrome P-450 catalyzed hydroxylation rate decreased as the number of chlorine substitutions increased. In this reaction, PCB3 was fully metabolized, approximately half of the PCB15 was metabolized and PCB77 was not metabolized at all. The oxidation rate of PCB15-HQ was higher than that of PCB3-HQ under various oxidation conditions. The LC/MS and UV-Vis data suggest that in the conjugation reaction of PCB15-Q and GSH, the Michael addition reaction occurs preferentially over the displacement reaction.

Conclusion The metabolic profiles of polychlorinated biphenyls (PCBs) are dramatically affected by chlorine substitution patterns. It is suggested that the metabolic profiles of PCBs are related to their chlorine substitution patterns, which may have implications for the toxicity of PCB exposure.

Key words: PCB; metabolism; Cytochrome P450; Glutathione; Quinone; Hydroquinone

Biomed Environ Sci, 2013; 26(2):138-147 doi: 10.3967/0895-3988.2013.02.008 ISSN:0895-3988

www.besjournal.com(full text) CN: 11-2816/Q

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a family of persistent organic pollutants that are distributed widely throughout the environment[1]. Being highly stable, they can be metabolized through different pathways[2-3]. PCBs have a large number of congeners and isomers, which makes their downstream metabolites more diverse. In general, halogenated aromatic compounds, *i.e.,* PCBs, undergo phase I and phase II metabolism. In the initial oxygenation step, the PCB parents oxidize into their corresponding hydroxylated and dihydroxylated metabolites *via* arene oxide intermediates[4]. As shown by scientific researches, the aromatic hydroxylation of PCB can occur *via* a non-arene oxide mechanism[5]. This phase I metabolism occurs in the presence of the cytochrome P-450 (CYP) enzyme. In the second step, the products are metabolized through glutathione conjugation or the mercapturic acid pathway (MAP);

*This work was supported by the Program for New Century Excellent Talents in University (NCET-10-0660), the National Natural Science Foundation of China (NSFC-20907037, 21035005), The Natural Science Foundation of Chongqing (CSTC-2009BB5005), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, National Ministry of Education (2011[508]) and the 211 Project of Southwest University (the Third Term).

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Received: December 5, 2011; Accepted: April 30, 2012

for instance., 2,4',5-trichlorobiphenyl is metabolized by the mercapturic acid pathway[6]. In the final sulfoxidation step, the mercapto-, methylthio-, methylsulfinyl-, and methysulfonyl metabolites are generated[7]. Our previous work indicated that both the PCB parents and the PCB quinones could undergo glutathione conjugation without the involvement of an enzyme and this conjugation occurred by a direct Michael addition or a displacement reaction[8].

Metabolites are shown to be generally more reactive than their precursors[4]. It is suggested that the toxicities of the PCB metabolites may contribute to the overall toxicity of the PCB parents. PCB congeners can exhibit significantly different bioactivities. Therefore, the toxic effects of PCBs are difficult to evaluate because of the contributions from their various metabolites. In order to resolve this problem, the structure-toxicity relationships of individual PCBs and their metabolites were investigated[9-13] because an understanding of the metabolism of typical PCBs will help explain their toxicities.

In an ideal experiment, the PCB metabolites would be investigated by administering PCBs and then identifying the metabolites that appeared in excrement and in tissues. However, owing to a series of factors it would be difficult to obtain direct evidence using this method: (1) the transformation ratios of the products of interest are usually low because of the complexity of the metabolic pathways, and the presence of impurities and byproducts results in a low signal/noise ratio; and (2) the solubility of the products changes dramatically at each metabolic step. The PCB parents are soluble in organic solvents such as hexane; however, the PCB glutathionyl conjugate has a high polarity and is soluble in water. As a result, it is difficult to extract and concentrate the samples. Therefore, this *in vitro* study was performed and was divided into three separate parts: hydroxylation, oxidation and conjugation. In each step, synthetic standards were used as precursors to the next generation of metabolites. To be specific, we measured the potential of each PCB parent to form its corresponding PCB hydroquinone, quinone and glutathionyl conjugates.

MATERIALS AND METHODS

Chemicals

4-Cl-biphenyl (PCB3), 4,4'-Cl-biphenyl (PCB15), 3,4,3',4'-Cl-biphenyl (PCB77) and their monohydroxylated and dihydroxylated metabolites were synthesized using a Suzuki-coupling reaction[14-15]. The PCB quinones were synthesized as previously described[16]. 1,4-Dichlorobenzene and 2,5-dichlorophenol were purchased from Aladdin Reagent Database Inc, and 2,5-dichloro-p hydroquinone and 2,5-dichloro-p-quinone were purchased from Alfa Aesar. A stock solution of each PCB compound (100 mmol/L) was prepared by dissolving the compound in DMSO and storing it at 4 °C. The chemical structures and abbreviated names of the PCB compounds are shown in Figure 1. β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate, glucose-6-phosphate dehydrogenase and superoxide dismutase were purchased from Sigma-Aldrich. All of the other chemicals that were used in this study were of analytical grade.

Rat Liver Microsomal Preparation

Male Sprague-Dawley rats (200-220 g) were obtained from Tengxin-Bill animal Inc. (Chongqing, China). The rat liver samples were homogenized in a 100 μmol/L EDTA/250 mmol/L sucrose buffer (pH 7.3) at a ratio of 1 g of liver per 5 mL of buffer. After centrifugation at 10 000 g (4 °C) for 20 min, the supernatant was centrifuged at 100 000 g (4 °C) for 1 h. The microsomal pellet was resuspended in a 100 μmol/L EDTA/250 mmol/L sucrose buffer. The cytochrome P-450 content was measured using the method developed by Omura & Sato[17]. The microsomes were divided into aliquots and stored at -80 °C.

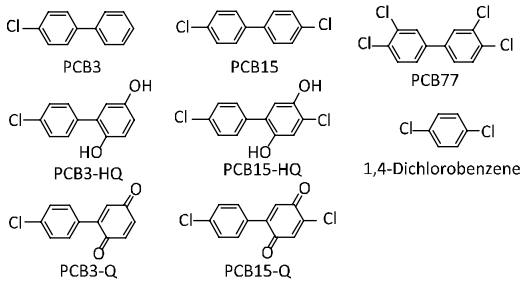


Figure 1. Chemical structures and nomenclatures of the PCBs and their derivatives.

Hydroxylation of the PCB Parents by Cytochrome P-450

A typical incubation mixture (with a total volume of 1.0 mL, 25 mmol/L Tris-HCl buffer, pH 8.0) contained the liver microsomal protein (2.0 mg/ mL), an NADPH-generating system (0.5 mmol/L $NADP^+$, 5.0 mmol/L glucose 6-phosphate, 0.75 unit/mL glucose

6-phosphate dehydrogenase and 2 mmol/L $MgCl_2$), and PCB (1.0 mmol/L)[4]. The solutions were incubated at 37 °C for 1 h, and the incubations were terminated by adding 0.03 mL of ice-cold HCl. The protein was removed with a 3 kD cut-off filter, and the filtrate was lyophilized and then reconstituted in 0.5 mL of acetonitrile. The mixture was analyzed using a HITACHI-2000 HPLC or a Shimadzu LC/MS system. The samples were quantified by comparing the relative areas of the liquid chromatograms with known standards. The recovery rate of PCBs in this procedure was nearly 100%.

Oxidation of the PCB Hydroquinones to Quinones

A typical incubation mixture (with a total volume of 1.0 mL) contained PCB hydroquinone (0.5 mmol/L) and horseradish peroxidase (HRP) (23 nmol/L)/H_2O_2 (5 mmol/L) in a 0.3 mol/L sodium citrate buffer (pH 7.4). To compare the oxidation efficiencies, parallel experiments were performed either in the absence of the NADPH regeneration system or incubated with a superoxide dismutase (SOD) (30 Unit). The solutions were incubated at 37 °C for 24 h. The protein was removed with a 3 kD cut-off filter, and the filtrate was lyophilized and reconstituted in 0.5 mL of acetonitrile. The mixture was analyzed using a HITACHI-2000 HPLC.

Formation of Glutathione Conjugates from PCB Quinones

A typical incubation mixture (with a total volume of 10.0 mL) contained a PCB quinone (4.5 mmol/L) in a 0.1 mol/L sodium acetate buffer (pH 9.0). Ten equivalents of a glutathione solution were slowly added by titration. After the titration, the solutions were incubated at 37 °C for 30 min. The mixture was analyzed using a Shimadzu LC/MS system. The absorbance was recorded with a UV-Visible spectrophotometer (UV2450, Shimadzu).

HPLC-DAD Detection

The liquid chromatography system consisted of a pump and a diode array detector (DAD) (HITACHI-2000) with an analytical C18 reversed-phase column (5 μm, 4.6 mm × 250 mm). The gradient mobile phase consisted of CH_3CN and 0.1% formic acid at a flow rate of 1.0 mL min^{-1}. The gradient was adapted to suit each analyte: from 0-10 min, the mobile phase was 20-25% CH_3CN (v/v) in a phosphate buffer; from 10-11 min, it was 25% CH_3CN (v/v); from 11-12 min, it was 80% CH_3CN (v/v); and from 12-18 min, it was 20% CH_3CN (v/v). The wavelength of the UV detector was set at 254 nm. The individual PCBs and their metabolites were identified and quantified by comparing their relative peak areas with internal and known standards.

LC-MS Analysis

The experiments were performed on a HPLC system that was coupled to a UV-detector and a single-quadrupole mass spectrometer, which had an ESI source (2010EV, Shimadzu, Japan). The measurements were performed in full scan mode. The data were acquired from 50-1500 Da. To identify the metabolites, the samples were loaded onto a C18-A column (Phenomenex, USA) using a gradient elution that consisted of H_2O (A) and CH_3CN (B). The elution gradient was as follows: the mobile phase initially contained 40% B; from 0-20 min, this percentage was linearly increased to 70% B(v/v); from 21-25 min, it was maintained at 70% B(v/v); from 26-40 min, it was linearly increased to 90% B(v/v); from 41-45 min, it was linearly decreased to 40% B(v/v); and from 46-50 min, it was maintained at 40% B(v/v).

RESULTS AND DISCUSSION

Previous scientific researchers have shown that PCBs metabolize stepwise into hydroxylated, quinone- and glutathionyl-conjugated compounds[4,11,18]. The ultimate goal of this study is to elucidate the metabolic profiles of PCBs that have different chlorine substitution patterns. Thus, the hydroxylation of the PCB parents, the oxidation of the PCB hydroquinones and the conjugation of the glutathione are discussed.

Microsomal Metabolism of the PCB Parents into Hydroxyl Metabolites

The LC/MS of the mixture of the PCB parents and the rat microsome/NADPH regeneration system confirms that the PCB parents are converted into monohydroxylated or dihydroxylated PCBs. The ion chromatogram shows the profiles of the hydroxylated PCB metabolites (Figure 2). As shown in Figure 2A, the monohydroxylated PCB3 (PCB3-OH, M1) (M^-, m/z 203), the dihydroxylated (hydroquinone) PCB 3 (PCB3-HQ, M2) (M^-, m/z 219) and the PCB3 parent were detected. PCB3-HQ was the major metabolite that appeared on the ion chromatogram. The hydroxyl groups that were formed via CYP-450 oxidation were usually in the meta-position[13,19]. We were unable to identify the exact positions of the hydroxyl groups; however, it was likely that 3-hydroxyl compounds were formed. Surprisingly, another ion peak appeared in the

chromatogram, indicating that the benzene might have been attached to the PCB3-HQ molecule (M3, m/z 295=219+76). Cytochrome P-450 could cleave C-C bonds[20-21], and breaking the C-C bond in PCB3 might generate an unstable benzene radical moiety that reacted with the adjacent PCB3-HQ. The calculations of the mass spectra isotope pattern also strongly support this hypothesis (Table 1). Theoretically, this reaction could occur on the aromatic ring or the hydroxyl group, which would generate two isomers. The proposed metabolite structures and metabolic pathways are shown in Table 2 and Figure 3.

Table 1. Mass Spectra Isotope Pattern of Proposed Metabolites Listed in Figure 2

	M/z=219		M/z=329		M/z=363	
	Found	Cal	Found	Cal	Found	Cal
M^-	100.0	100.0	100.0	100.0	100.0	100.0
M^-+1	19.4	19.7	24.7	19.7	37.1	19.6
M^-+2	30.5	34.2	58.4	66.2	79.0	98.1
M^-+3	7.4	6.5	7.9	12.8	30.5	19.0
M^-+4			9.9	11.7	28.6	32.8
M^-+5					3.8	6.2

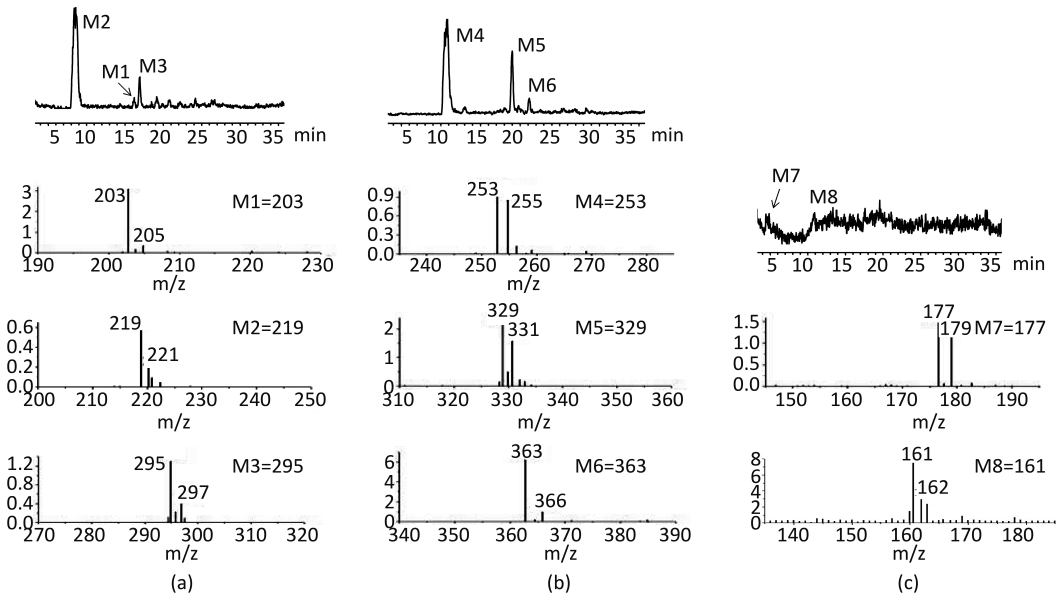
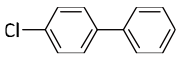
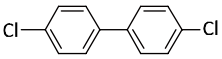
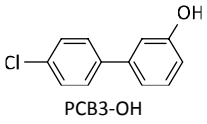
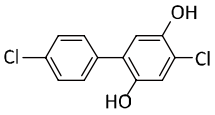
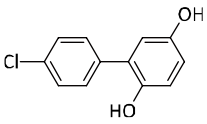
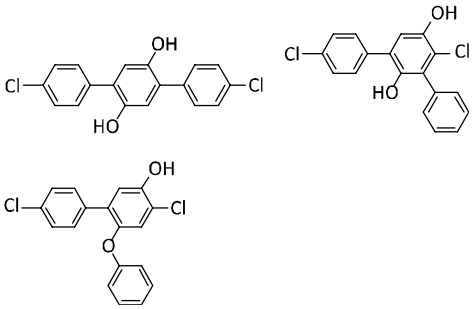
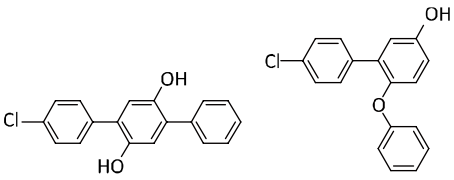
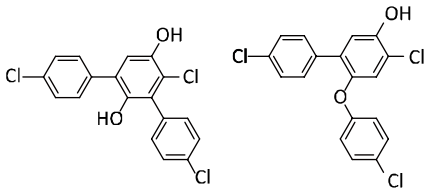


Figure 2. LC/MS ion chromatograms and mass spectra of the PCB metabolites. (A) PCB3, (B) PCB15 and (C) 1,4-dichlorobenzene (1.0 mmol/L) were incubated with liver microsomal protein (2.0 mg/mL) and an NADPH-generating system (0.5 mmol/L NADP+, 5.0 mmol/L glucose 6-phosphate, 0.75 unit/mL glucose 6-phosphate dehydrogenase and 2 mmol/L $MgCl_2$) at 37 °C for 1 h.

The ion chromatogram clearly demonstrated that dihydroxylated (hydroquinone) PCB15 (PCB15-HQ, M4) (M^-, m/z 253) was the major metabolite. Interestingly, no monohydroxylated PCB15 (PCB15-OH) was detected; it was possible that the binding of PCB15 to the CYP-450 enzyme was the rate-limiting step. Once this step occurred, the tandem addition of the two hydroxyl groups to the benzene ring rapidly followed. Similarly, there were two unidentified peaks in the ion chromatogram. It was likely that a chlorobenzene radical moiety was formed and reacted with the PCB15-HQ to generate the M5 (M^-, m/z 329) and M6 (M^-, m/z 363) conjugates (Figure 2 and Table 2). The presence of metabolite 5 indicated that a dechlorination reaction occurred, and either the chlorine on the PCB15-OH (the first M5 structure) or the chlorine on the chlorobenzene moiety (the second and third M5 structures) was ejected. Likewise, when 1,4-dichlorobenzene was incubated under the same conditions, 2,5-dichloro-p- ydroquinone (M7) (M^-, m/z 177) and 2,5-dichlorophenol (M8) (M^-, m/z 161) were detected in the ion chromatogram (Figure 2C). In the absence of the NADPH regeneration system, no metabolites were detected in any of the tested substrates (data not shown).

Table 2. Proposed Metabolite Structures Detected in PCB3 and PCB15

Substrate		
Proposed Metabolite Structures	M1  PCB3-OH	M4  PCB15-HQ
	M2  PCB3-HQ	M5 
	M3 	M6 

Note. Due to the possibility of multiple isomers formation, the substituent hydroxyl and benzene/ chlorobenzene groups are NOT necessarily located on the position shown in the table.

The loss of the PCBs parents and the formation of their metabolites were quantified by comparing their relative peak areas with internal and known standards, and the results are shown in Table 3. The HPLC spectra are shown in Figure 4, which can be found in the supplemental material. PCB3 was fully converted into its metabolites. The HPLC/UV chromatogram suggests that PCB3-HQ was the major metabolite (35.5%). When PCB15 was used as a substrate, PCB15 was partially metabolized, and no detectable metabolites were found. These data are consistent with a study that concluded that multiple halogen substituents were more resistant to metabolism by CYP-450[22]. The present study revealed that PCB3 was metabolized at the highest rate and the reactions occurred primarily on the non-substituted ring; PCB15 had one chlorine on each phenyl ring, and it was metabolized at a lower rate. However, PCB77 was resistant to be metabolized by the microsome/NADPH regeneration system.

Oxidation of PCB Hydroquinones to Quinones

Previous research has shown that PCB hydroquinones undergo further oxidation by peroxidase to form quinone metabolites[11]. It has been suggested that quinones lead to higher levels of toxicity and oxidative stress in cells than the PCB parents and hydroquinones[23]. Therefore, the oxidation profiles of PCB3-HQ and PCB15-HQ were investigated in this study. The amounts of quinone that were formed are listed in Table 4. All of the experiments were performed under aerobic conditions. The PCB3-Q yield of the autoxidation reaction was only 58.8%, and it increased to 66.8% and 73.6% in the presence of HRP/H_2O_2 and SOD, respectively. In the presence of both HRP/H_2O_2 and SOD, the PCB3-Q yield was 77.5%. These data suggested that the rate of the oxidation of hydroquinone to quinone increased in the presence of an enzyme, which was consistent with the results of previous work[11,18]. However, SOD and HRP/H_2O_2 accelerate the oxidation rate in different ways. The end products of the oxidation of hydroquinones are quinones and H_2O_2. SOD removes the superoxide radicals that are generated by H_2O_2, which accelerates the formation rate of the quinones[24]. In contrast, HRP/H_2O_2 acts as a direct oxidizing reagent.

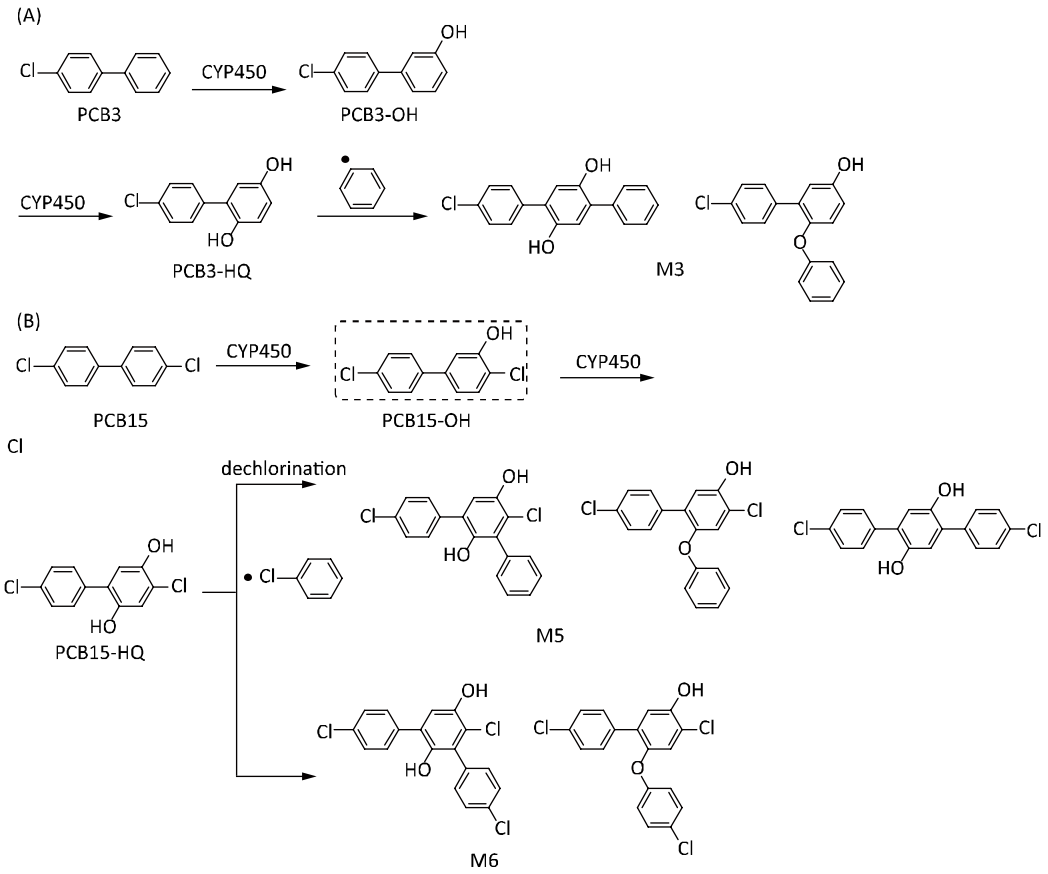


Figure 3. Proposed metabolite pathway of PCB3 and PCB15 with the catalysis of microsome/NADPH regenerating system. Due to the possible multiple isomers formation, the substituent hydroxyl and benzene/chlorobenzene groups are NOT necessarily located on the position as shown in the table.

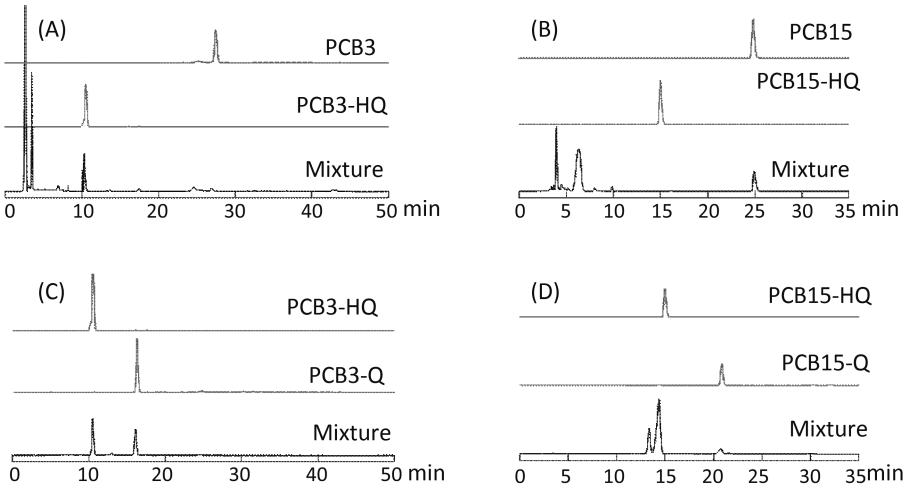


Figure 4. HPLC spectra of PCB parent and PCB hydroquinone metabolites. (A), PCB3 hydroxylation into PCB3-HQ, (B) PCB15 hydroxylation into PCB15-HQ, (C) PCB3-HQ oxidize into PCB3-Q, (D), PCB15-HQ oxidize into PCB15-Q.

Table 3. The Amount of the PCB Parent that Remained in the Solution and the Amount of Hydroquinone that Formed

Incubation	Remain PCB parent	Hydroquinone
	%	%
PCB3	0	35.5
PCB15	46.4	~0
PCB77	100	0

Note. The results were based of the initial number of moles of the PCB parent that were incubated. The standard deviation was normalized using the external standard method.

Table 4. The Quinone Yield under Different Conditions

Incubation	Quinone (%)			
	Autoxidation	HRP/ H_2O_2	SOD	HRP/H_2O_2 + SOD
PCB3-HQ	58.8	66.8	73.6	77.5
PCB15-HQ	60.9	74.8	81.1	83.9

Note. The standard deviation was normalized using the external standard method.

The autoxidation rate of PCB15-HQ was slightly higher than that of PCB3-HQ (60.9% *vs*. 58.8% was converted to quinone). Similarly, in the presence of HRP/H_2O_2, SOD and HRP/H_2O_2+SOD, the PCB-15Q yields were 74.8%, 81.1%, and 83.9%, respectively. This occurred because the redox potential of Q/Q^{2-} increased with the number of chlorine substituents. Electron-withdrawing substituents, e.g., chlorine, added to the benzoquinone ring, which made the quinone more electrophilic[25]. Our previous experiment on oxygen consumption also indicated that the rate of oxidation increased with the increasing chlorination degree of the hydroquinone ring[16].

Formation of Glutathione Conjugates from PCB Quinones

Quinones are known to behave as electrophiles in reactions with sulfhydryl groups[26]. The chemistry and toxicity of quinones have been extensively studied and reviewed[27]. Many of the mechanisms of quinone toxicity have been described herein. GSH is believed to contribute to the conjugation of quinones with cellular nucleophiles. The formation of the glutathionyl conjugate increases the solubility of quinone, which can then be easily excreted into bile and urine.

However, the conjugation between glutathione and quinones does not always result in detoxification[28]. First, the conjugation process depletes GSH; this depletion may disrupt the cellular redox potential and lead to oxidative stress. Second, the reduction that occurs in the conjugation process may produce hydroquinone, which is then available for comproportionation with quinone; the reaction then undergoes redox cycling, which also yields semiquinone radicals and ROS[18,29]. Third, the conjugates are effective substrates for certain metabolic enzymes, e.g., DT-diaphorase (DTD)[30] and GST[31-32]; therefore, they can block the active sites of these enzymes and eventually inhibit their activity.

Quinones can undergo enzymatic or nonenzymatic reactions with glutathione. GST catalyzes the conjugation of GSH with chlorinated compounds *via* the displacement of chlorine[33-34]. In the Michael addition reaction, quinone and GSH can automatically form the reduced glutathionyl conjugate without the involvement of the GST catalyst[26]. In a previous work, we reported the occurrence of a nonenzymatic dechlorination reaction between glutathione and chlorinated quinones[8]. This reaction is a nucleophilic substitution that displaces the chlorine on the quinone ring and yields conjugates that maintain the quinone moiety. Therefore, unsaturated, chlorine-substituted quinones, e.g., PCB15-Q**,** can follow two reaction pathways. However, it is still not clear whether the predominant reaction is the Michael addition or chlorine displacement. The answer will help predict the potential metabolites *in vivo*. To address this question, the LC/MS technique was used to detect the glutathionyl conjugates (Figure 5). When 10 equivalents of GSH were introduced to the PCB3-Q solution, monoglutathionyl (M9) (M^-, m/z 524) and diglutathionyl (M10) (M^-, m/z 829) adducts were detected. The proposed mechanism for the formation of M10 is shown in Scheme 1. When PCB15-Q and GSH were mixed, the Michael addition reaction product, M11 (M^-, m/z 558), was trapped, but M10 was not detected. In an earlier work, M11 and M10 were identified using different instruments; however, the intensity of M11 was still 10 times greater than that of M10[8]. A lack of M10 is observed because the liquid chromatography separation process disrupts the sensitivity of the mass spectrometer. These results indicate that the Michael addition occurs preferentially over the displacement reaction, but the displacement reaction does occur to some extent.

Further evidence was collected using a UV-Visible spectrophotometer. Our previous work indicates that the displacement reaction only occurs on the chlorine that is located on the quinone ring[8]. Therefore, PCB3-Q

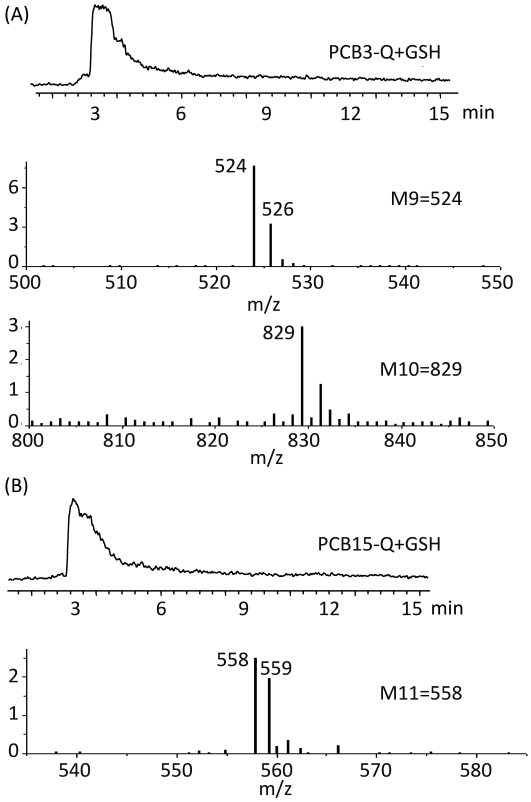


Figure 5. LC/MS ion chromatograms and mass spectra of the PCB metabolites. (A) PCB3-Q and (B) PCB15-Q (0.5 mmol/L) were titrated with 10 equivalents of glutathione, and the solutions were incubated at 37 °C for 30 min.

and GSH can only form the reduced hydroquinone-GSH conjugate *via* the Michael addition reaction. However, PCB15-Q could undergo a displacement reaction with GSH to form the GSH conjugate while maintaining the quinone moiety. Therefore, this concept was used to distinguish between the two types of conjugation reactions. A 60 nmol/L sample of PCB3-Q was titrated with up to 1.0 equivalents of GSH (which was divided equally into 6 aliquots), and the solution was monitored every 5 min for 30 min; the results are shown in Figure 6A. The quinone absorbance (284 nm and 362 nm) decreased, and a new peak (322 nm) appeared; the new peak arose from the hydroquinone-GSH conjugate. When a 60 nM sample of PCB15-HQ was titrated with an equal number of moles of GSH, no new peak appeared, but the absorbance at 320 nm decreased slightly; this decrease indicated that some non-covalent binding occurred (Figure 6B). Interestingly, the PCB15-Q solution that was titrated with GSH exhibited two phases (Figure 6C). The first phase occurred during the titration of up to 1:1 molar equivalents of GSH. In this phase, the peaks at 304 nm and 386 nm decreased, and the peak at 326 nm increased, indicating the loss of quinone and the formation of hydroquinone. The second phase was observed during the titration from 1:1 to 1:2 equivalents. In this phase, the peak at 326 nm decreased, indicating that there was binding between the hydroquinone and GSH. The detailed change in the absorbance during the titration is shown

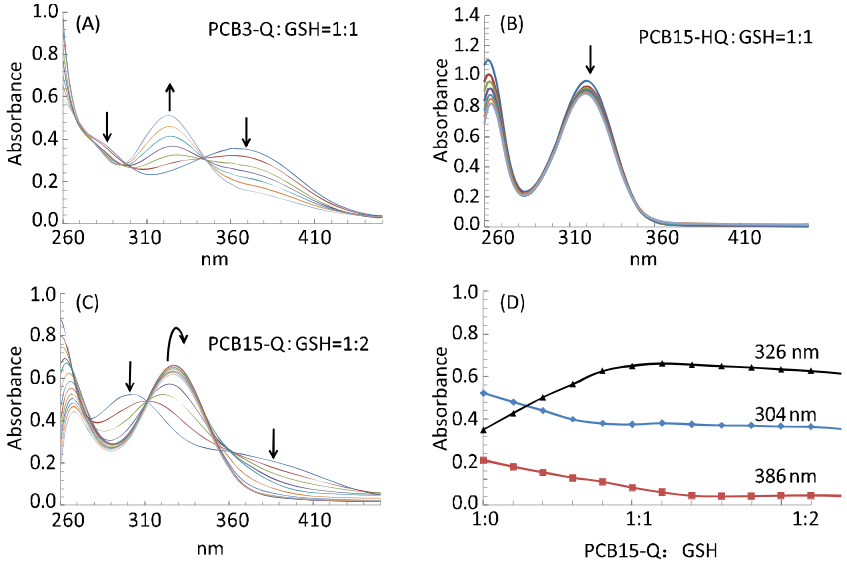
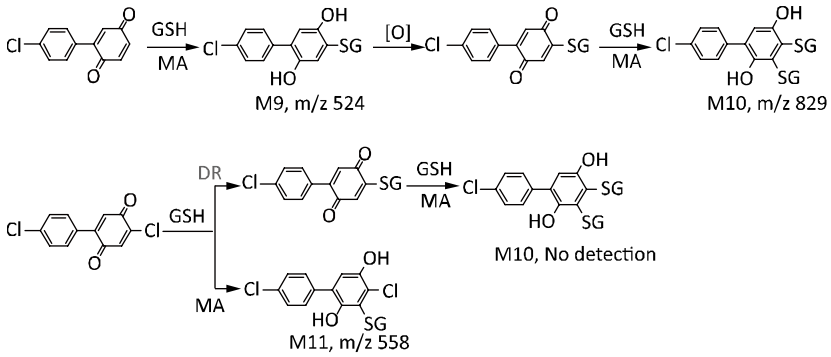


Figure 6. UV-Visible spectra depicting the changes in absorbance as the PCB quinones reacted with glutathione at pH 7.0. The concentration of the PCB quinone/hydroquinone was 60 nmol/L, and an aliquot of GSH was titrated every 5 min. The arrows indicate the wavelengths at which the greatest change in absorbance was observed. (A) PCB3-Q:GSH = 1:1. (B) PCB15-HQ:GSH = 1:1. (C) PCB15-Q:GSH = 1:2. (D) The change in the absorbance in. (C) at the selected wavelengths.



Scheme 1. The Michael addition (MA) and displacement reaction (DR) metabolic pathways that have been proposed for the formation of the PCB3-Q/PCB15-Q and the glutathione conjugates. Note that (a) the displacement reaction only occurs on the chlorine on the quinone ring, and (b) the position at which the Michael addition occurs is NOT necessarily the position that is shown in the scheme.

in Figure 6D. These data suggest that the 1:1 molar equivalent of GSH is primarily consumed by the Michael addition, which yields the hydroquinone-GSH conjugate. It also indicates that the Michael addition reaction occurs preferentially over the displacement reaction.

CONCLUSION

In summary, this study presents the hydroxylation, oxidation and GSH conjugation profiles of PCBs with different chlorine substitution patterns. The data confirm that the metabolic rates of PCBs are strongly dependent on their chlorine substitution patterns. Therefore,these findings may provide new insights that will spur more in-depth studies of the metabolism and toxicology of PCB.

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