TABLE 2

Levels of $MeSO_2$-PCBs in the livers of rats, guinea pigs, and hamsters dosed with Kanechlor 500

Each value represents the mean S.E. for three to four animals.

Abbreviation	Structure	Levels (g/g wet wt)		
		Rat	Guinea Pig	Hamster
2,5-Dichloro-substituted				
3 -49	3 -$MeSO_2$-2,4,2 ,5 -tetraCB	17 2	60 17	N.D.
4 -49	4 -$MeSO_2$-2,4,2 ,5 -tetraCB	8 2	N.D.	N.D.
3–70	3-$MeSO_2$-2,5,3 ,4 -tetraCB	50 11	6 2	6 1
4–70	4-$MeSO_2$-2,5,3 ,4 -tetraCB	41 6	N.D.	20 7
3 -87	3 -$MeSO_2$-2,3,4,2 ,5 -pentaCB	32 8	160 44	7 2
4 -87	4 -$MeSO_2$-2,3,4,2 ,5 -pentaCB	27 4	70 7	5 2
3 -101	3 -$MeSO_2$-2,4,5,2 ,5 -pentaCB	43 10	32 4	42 10
4 -101	4 -$MeSO_2$-2,4,5,2 ,5 -pentaCB	48 5	5 2	25 5
3 -141	3 -$MeSO_2$-2,3,4,5,2 ,5 -hexaCB	2 1	33 4	N.D.
4 -141	4 -$MeSO_2$-2,3,4,5,2 ,5 -hexaCB	3 1	N.D.	N.D.
	Total	271 44	366 72	105 16*
2,3,6-Trichloro-substituted				
5–64	5-$MeSO_2$-2,3,6,4 -tetraCB	5 1	130 26	N.D.
4–64	4-$MeSO_2$-2,3,6,4 -tetraCB	6 1	N.D.	N.D.
5–91	5-$MeSO_2$-2,3,6,2 4 -pentaCB	4 1	77 19	15 4
4–91	4-$MeSO_2$-2,3,6,2 ,4 -pentaCB	2 1	N.D.	9 2
5–110	5-$MeSO_2$-2,3,6,3 ,4 -pentaCB	N.D.	6 2	N.D.
4–110	4-$MeSO_2$-2,3,6,3 ,4 -pentaCB	42 9	N.D.	5 3
5 -132	5 -$MeSO_2$-2,3,4,2 ,3 ,6 -hexaCB	20 4	146 42	2 1
4 -132	4 -$MeSO_2$-2,3,4,2 ,3 ,6 -hexaCB	21 3	N.D.	N.D.
5 -149	5 -$MeSO_2$-2,4,5,2 ,3 ,6 -hexaCB	14 2	110 21	2 1
4 -149	4 -$MeSO_2$-2,4,5,2 ,3 ,6 -hexaCB	17 3	N.D.	3 1
	Total	131 20	469 82	36 8
	Sum *meta*-$MeSO_2$-CBs	187 50	760 93*	74 16
	Sum *para*-$MeSO_2$-CBs	215 71	75 13.3	67 11
	Total $MeSO_2$-CBs	402 90	835 184*	141 42*
Ratio				
	meta-/*para*-$MeSO_2$-CBs	0.87	10.1	1.10
	2,5-/2,3,6-chloro substituted	2.07	0.78	2.92

N.D., not detected (0.1 g/g).
* Significantly different from rats, *P* 0.01 (Student's *t* test).

resistant to metabolism in the order hamsters (34%) rats (21%) guinea pigs (15%). Nonpersistent PCBs with 2,5- or 2,3,6-chlorine substitution (group D, 33% of total PCBs) were eliminated more rapidly in rats (2.7%) and guinea pigs (3.5%) than in hamsters (8.6%).

$MeSO_2$-CBs in the Liver. Figure 1 shows the ECD gas chromatograms of the $MeSO_2$ fraction of liver extracts of the three species treated with Kanechlor 500. GC/MS analysis showed that most of the $MeSO_2$-CBs detected were originated from 2,5-di- or 2,3,6-trichloro-substituted congeners. The levels and *meta*/*para* substitution ratios of $MeSO_2$-CB congeners in the liver are shown in Table 2. Rats showed preferential production of *meta*- and *para*-substituted $MeSO_2$ metabolites of 2,5,3 ,4 -tetraCB, 2,3,4,2 ,5 -pentaCB, 2,4,5,2 ,5 -pentaCB, 2,3,6,3 ,4 -pentaCB, 2,3,4,2 ,3 ,6 -hexaCB, and 2,3,6,2 ,4 ,5 -hexaCB, with a similar 3-/4-substitution ratio. Hamsters produced minute amounts of *meta*- and *para*-substituted $MeSO_2$-CBs derived from 2,5,3 ,4 -tetraCB and 2,4,5,2 ,5 -pentaCB. In contrast, guinea pigs showed selective production of *meta*-substituted $MeSO_2$-CBs derived from 2,3,6,4 -tetraCB, 2,3,6,2 ,5 -pentaCB, 2,4,5,2 ,5 -pentaCB, 2,3,4,2 ,3 ,6 -hexaCB, 2,3,4,5,2 ,5 -hexaCB, and 2,3,6,2 ,4 ,5 -hexaCB, although 2,3,4,2 ,5 -pentaCB yielded 3 - and 4 -$MeSO_2$-2,3,4,2 ,5 -pentaCBs at a similar concentration ratio (Fig. 1). Total levels of $MeSO_2$-CBs were higher in the order guinea pigs rats hamsters. The concentration ratios of *meta*- and *para*-$MeSO_2$-CBs were the highest (10.1) for guinea pigs, whereas the ratios were 0.87 and 1.10 for rats and hamsters, respectively. On the other hand, marked and selective retention of 4 -$MeSO_2$-2,4,5,2 ,5 -pentaCB was observed in the lungs of rats (4 -/3 -$MeSO_2$ substitution ratio 11), although no selective retention of *para*-$MeSO_2$-CBs was observed in the lungs of guinea pigs or hamsters (data not shown).

OH-PCBs in Serum. Figure 2 shows the retention profiles of phenolic PCBs in serum of the three species after exposure to Kanechlor 500. The concentrations of major OH-PCBs and the metabolite/parent CB ratios are shown in Table 3. Rat serum showed specific retention of 4-OH-2,3,5,3 ,4 -pentaCB (89% contribution), most likely derived from 2,3,4,3 ,4 -pentaCB and/or 2,4,5,3 ,4 -pentaCB. On the other hand, guinea pig serum showed specific retention of 3-OH-2,4,5,2 ,4 -pentaCB (56%) and 3-OH-2,4,5,3 ,4 -pentaCB (20%). In contrast, hamster serum retained primarily 3 ,4 -$(OH)_2$-2,4,5,2 ,5 -pentaCB, 4,5-$(OH)_2$-2,3,6,3 ,4 -pentaCB, and 4,5-$(OH)_2$-2,3,6,2 ,4 ,5 -hexaCB, as well as 3- or 4-hydroxy metabolites derived from 2,4,5,2 ,4 -pentaCB and 2,4,5,3 ,4 -pentaCB (Fig. 2). Thus, phenolic PCB levels showed the following order: hamsters rats guinea pigs. The ratios of 3-OH-2,4,5,2 ,4 -pentaCB/2,4,5,2 ,4 -pentaCB were higher in hamsters (12.3) and guinea pigs (12.0) than in rats (0.3). The ratio of (4-OH-2,3,5,3 ,4 -pentaCB 3-OH-2,4,5,3 ,4 -pentaCB)/(2,3,4,3 ,4 -pentaCB 2,4,5,3 ,4 -pentaCB) was higher in rats (6.3) than in guinea pigs (0.6).

Metabolites in the Serum of Guinea Pigs. The phenolic fraction in the serum of guinea pigs contained several persistent metabolites with different molecular weights from OH-PCBs or $MeSO_2$-CBs, although no such metabolites were detected in rats and were formed at lower levels in hamsters. Figure 3 shows the GC/MS total ion chromatogram of phenolic fraction (M_a–M_d, at longer GC retention times than $MeSO_2$-CBs) in the serum of guinea pigs after exposure to Kanechlor 500. The electron impact mass spectra of M_a to M_d are shown in Fig. 4. By GC/ECD and GC/MS analyses, M_a was tentatively identified as methoxy-$MeSO_2$-tetraCB (M , *m*/*z* 398), and M_d as a methoxy-$MeSO_2$-pentaCB (M , *m*/*z* 432) due to a frag-

TABLE 3

Serum concentration of phenolic PCBs in rats, guinea pigs, and hamsters after dosing with Kanechlor 500 (100 mg/kg i.p.)

Each value represents the mean S.E. for three to five animals.

PCB and Metabolite Congeners	Serum Concentration (ng/g wet wt)		
	Rat	Guinea Pig	Hamster
2,4,5,2 ,4-pentaCB (CB99)	33 3	12 2	8 2
3-OH-2,4,5,2 ,4 -pentaCB	9 2	144 40	74 22
4-OH-2,3,5,2 ,4 -pentaCB	N.D.	N.D.	24 8
Ratio of OH-PCBs/CB99	0.27 0.22	12.0 2.1*	12.3 2.1*
2,3,4,3 ,4 -pentaCB (CB105)	15 3	20 3	14 2
2,4,5,3 ,4 -pentaCB (CB118)	56 6	87 14	50 14
3-OH-2,4,5,3 ,4 -pentaCB	12 3	51 10	37 6
4-OH-2,3,5,3 ,4 -pentaCB	342 75	N.D.	111 16
Ratio of OH-PCBs/(CB105 CB118)	6.3 0.9	0.59 0.1*	3.0 0.5*
2,3,4,2 ,4 ,5 -hexaCB (CB138)	93 8	38 7	37 3
3 -OH-2,3,4,2 ,4 ,5 -hexaCB	14 4	33 6	24 6
4 -OH-2,3,4,2 ,3 ,5 -hexaCB	N.D.	N.D.	11 3
Ratio of OH-PCBs/CB138	0.15 0.02	0.87 0.11*	0.95 0.14*
2,3,5,2 ,4 ,5 -hexaCB (CB146)	13 3	9.1 2.0	6.2 2.1
2,4,5,2 ,4 ,5 -hexaCB (CB153)	80 15	74	55
3-OH-2,4,5,2 ,4 ,5 -hexaCB	9 3	28 8	4 1
4-OH-2,3,5,2 ,4 ,5 -hexaCB	N.D.	N.D.	3 1
Ratio of OH-PCBs/(CB146 CB153)	0.11 0.02	0.38 0.06*	0.13 0.02
3 ,4 -dihydroxy-2,4,5,2 , 5 -pentaCB (diOH-CB101)	N.D.	9 3	42 6
4,5-dihydroxy-2,3,6,3 ,4 -pentaCB (diOH-CB110)	N.D.	N.D.	84 10
4,5-dihydroxy-2,3,6,2 ,4 ,5 -hexaCB (diOH-CB149)	N.D.	N.D.	61 9
Total phenolic PCBs	386 29	256 32*	475 49*

N.D., not detected (0.005 g/g).
* Significantly different from rats, *P* 0.01 (Student's *t* test).

ment ion, [M 81] (due to loss of oxygen and OCH_3Cl from M), and [M 144] (loss of OCH_3Cl-O-$SOCH_2$ from M). M_b showed a weak molecular ion (*m/z* 400) and an abundant fragment at [M 32] (loss of two oxygen atoms from M), which was characteristic of vicinal methoxy-methylthio-pentaCB. M_c showed a weak molecular ion (*m/z* 416), and fragment ion [M 16] (loss of oxygen) as a base peak that was characteristic of vicinal methoxy-methylsulfinyl pentaCBs. Thus, the PCB metabolites in the serum of guinea pigs were tentatively identified as hydroxy-$MeSO_2$-tetraCB for M_a, hydroxy-methylthio-pentaCB for M_b, hydroxy-methylsulfinyl-pentaCB for M_c, and hydroxy-$MeSO_2$-pentaCB for M_d.

Discussion

Although oxidative metabolism of PCBs by rats has been studied extensively (Kato et al., 1980; Kaminsky et al., 1981), there has been

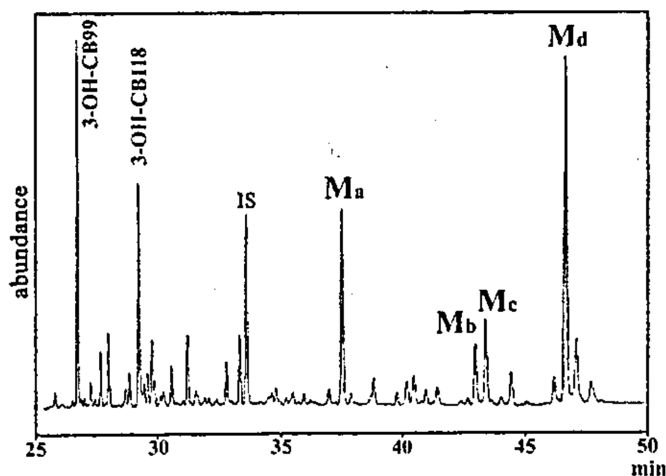

FIG. 3. Total ion chromatogram of GC/MS (electron impact mode) of phenolic PCBs (after methylation) in guinea pigs after exposure to Kanechlor 500 (100 mg/kg i.p.). The phenolic fraction was spiked with 2,3,4,5,6,3 ,4 ,5 -octaCB (internal standard) after separation of PCBs.

only limited comparative analysis of in vivo metabolism of PCBs between hamsters and guinea pigs (Koga and Yoshimura, 1996). The results of the present study indicated that the tissue distribution profiles of PCB residues and OH- and $MeSO_2$-CBs were largely dependent on the chlorination patterns classified into four groups. This may have been due to differences in substrate specificity of each species, especially between guinea pigs and hamsters, following administration of Kanechlor 500.

PCB Residues in the Liver. The percentage composition of residual PCBs in the liver indicated that guinea pigs had lower elimination ratios of coplanar PCBs with 4-, 3,4-, or 3,4,5-chlorine substitution (group A in Table 1) than rats or hamsters. For example, the composition of 3,3 ,4,4 -tetraCB, which originally accounted for 0.03% in Kanechlor 500, increased to 1.8% in the livers of guinea pigs 5 days after exposure, whereas the values were 0.02 and 0.06% in the livers of rats and hamsters, respectively (Table 1). These observations suggest that guinea pigs have lower ability to metabolize coplanar PCBs. This was supported by the in vitro observation that 3,3 ,4,4 -tetraCB can hardly be metabolized by liver microsomes from MC or phenobarbital-treated guinea pigs, whereas it is metabolized effectively by those of rats and hamsters treated with MC (Koga et al., 1995). The reduced elimination of coplanar PCBs may partially explain the higher sensitivity to the toxicity of coplanar PCBs in guinea pigs.

On the other hand, hamsters exhibited reduced elimination of the 2,4,5-trichloro-substituted CB congeners (group C) compared with rats or guinea pigs. This observation suggests that hamsters have a reduced capability to hydroxylate 2,4,5-trichloro-substituted CBs than the other two species. The results of a previous in vitro study indicated that 2,4,5,2 ,4 ,5 -hexaCB can hardly be metabolized by rats or hamsters, whereas it is metabolized effectively by guinea pigs (Ariyoshi et al., 1997). This may be due to the lower levels of induction of CYP2B1/2 in rats and hamsters compared with guinea pigs. CYP3A may also be related to the metabolism of these PCBs, depending on species. Furthermore, it is noted that nonpersistent CBs with 2,5- or

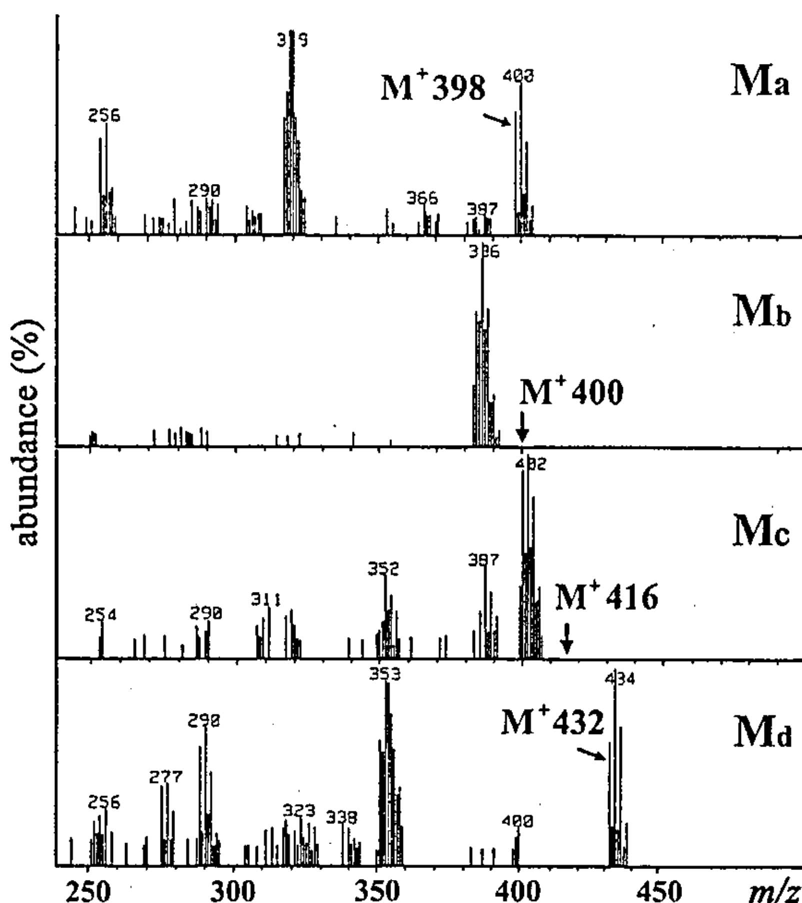

FIG. 4. Mass spectra of M_a through M_d in total ion chromatogram (Fig. 3) of phenolic metabolites (as methyl derivatives) from the serum of guinea pigs after exposure to Kanechlor 500.

2,3,6-chlorine substitution (group D) tend to be eliminated more slowly in hamsters than in rats or guinea pigs. The reduced elimination of these congeners is probably due to the lower catalytic activity of CYP2B in hamsters than in rats (Ishida et al., 1991; Koga et al., 1995), or to the lower abilities of phase II isozymes, such as glutathione *S*-transferase, to conjugate for subsequent excretion in hamsters.

$MeSO_2$-CBs in Liver. The $MeSO_2$ metabolites detected in the present study were consistent with the results of previous studies regarding metabolism of PCB congeners with 2,5- or 2,3,6-chlorine substitution (Haraguchi et al., 1997, 1999). However, the levels and metabolite profiles in guinea pigs and hamsters were quite different from those in rats. Hamsters formed minute amounts of *meta*- and *para*-$MeSO_2$-CBs from 2,5,3 ,4 -tetraCB and 2,4,5,2 ,5 -pentaCB, whereas guinea pigs produced much higher levels of $MeSO_2$-CBs and showed selective distribution as *meta*-substituted-$MeSO_2$-CBs derived from CBs with 2,3,6-chlorine substitution. These results indicate that the *meta*-position of the 2,3,6-trichlo-substituted phenyl ring in the molecule is the preferred site for methylsulfonylation and guinea pigs may have substrate-specific phase II isozymes that catalyze the nucleophilic reaction of arene oxide intermediates with sulfhydryl groups. Indeed, the selective retention of *meta*-$MeSO_2$-CBs has been observed in the livers of otters, minks, and seals in Canadian and Swedish water (Bergman et al., 1994b). Especially, 5 -$MeSO_2$-2,3,4,2 ,3 ,6 -hexaCB was the most abundant in human tissues (Chu et al., 2003). Kato et al. (1997) reported that only *meta*-substituted $MeSO_2$-CBs strongly induced CYP2B1/2 and reduced the serum thyroid hormone level in rats. Exposure of minks to a synthetic mixture of *meta*- and *para*-$MeSO_2$-CBs also resulted in the selective retention of *meta*-$MeSO_2$-CBs in the liver and increased induction of some hepatic CYP2B isozymes as well as alteration of thyroid hormone levels in blood (Lund et al., 1999). Higher levels of induction of CYP2B1/2 by $MeSO_2$-CBs as well as induction of CYP3A-like activity (Schuetz et al., 1986, 1998) may alter the metabolic pathways of individual PCBs in rats and contribute to the high level of toxicity of nonplanar PCBs.

Phenolic PCBs in Serum. For the three species examined in the present study, persistent OH-PCBs in serum were derived metabolically from 2,4,5-trichloro-substituted CBs (e.g., 2,4,5,2 ,4 -pentaCB, 2,4,5,3 ,4 -pentaCB, 2,3,4,2 ,4 ,5 -hexaCB, and 2,4,5,2 ,4 ,5 -hexaCB) via direct insertion of a hydroxyl group into the 3-position or via epoxidation and subsequent NIH chlorine shift at the 4-position. Indeed, all the OH-PCBs detected in the present study have been observed in gray seal and in human blood (Bergman et al., 1994a; Sandau et al., 2000); however, the levels of OH-PCBs and metabolite/parent CB ratios were species-specific since different species have different types of P450 enzymes (Koga et al., 1998).

Exposure of animals to Kanechlor 500 resulted in higher levels of elimination of 2,4,5,3 ,4 -pentaCB in rats than in guinea pigs or hamsters. For this metabolism, rats showed selective formation of 4-OH-2,3,5,3 ,4 -pentaCB (metabolite/parent CB ratio 6.3), whereas guinea pigs showed selective formation of 3-OH-2,4,5,3 ,4 -pentaCB (ratio 0.59). Hamsters showed formation of 3-OH-2,4,5,3 ,4 -pentaCB and 4-OH-2,3,5,3 ,4 -pentaCB in a ratio of 1:3 (ratio 3.0). Although serum does not necessarily have the same profile as liver, the differences in the levels of OH-PCBs and bio-

transformation ratios in serum may be explained by the species-dependent metabolic capacities. In fact, these results are consistent with the in vitro observation that 2,4,5,3 ,4 -pentaCB can be metabolized readily to 4-OH-2,3,5,3 ,4 -pentaCB (CYP1A1/2) by MC-inducible hepatic microsomes of rats, whereas it shows a lesser degree of metabolism to 2- or 3-OH-2,4,5,3 ,4 -pentaCB (CYP2B18) by guinea pig liver microsomal P450 isozymes (Koga et al., 2002).

For metabolism of 2,4,5,2 ,4 -pentaCB, both guinea pigs and hamsters showed selective formation of 3-OH-2,4,5,2 ,4 -pentaCB (transformation ratios 12.0 and 12.3, respectively), whereas this PCB was relatively resistant to metabolism by rats (ratio 0.27). On the other hand, the elimination of 2,4,5,2 ,4 ,5 -hexaCB and 2,3,4,2 ,4 ,5 -hexaCB was delayed in rats and hamsters, resulting in lower distribution of their metabolites in the liver. These findings were supported by those of previous in vitro studies indicating that 2,3,4,2 ,4 ,5 -hexaCB and 2,4,5,2 ,4 ,5 -hexaCB can be hydroxylated effectively by the guinea pig cytochrome P450 isoform CYP2B18 but are resistant to metabolism by rats or hamsters (Koga et al., 2001).

Other persistent phenolic PCBs were catechol metabolites derived from CBs with 2,5- or 2,3,6-chlorine substitution (e.g., 2,4,5,2 ,5 -pentaCB, 2,3,6,3 ,4 -pentaCB, and 2,3,6,2 ,4 ,5 -hexaCB), which were retained in the serum of hamsters at concentrations similar to those of monohydroxylated PCBs. The formation of catechol may involve a two-step direct hydroxylation process or a pathway via arene oxide and dihydrodiol intermediates. Thus, the tissue distribution ratios of catechols in hamsters and of $MeSO_2$-CBs in guinea pigs would reflect the extreme differences in metabolism of CBs with 2,5- or 2,3,6-chlorine substitution. Although their mechanisms of retention are still unknown, catechols may act as endocrine disruptors, similar to OH-PCBs, with affinity to the thyroid hormone transport protein transthyretin, concomitant with a reduction in circulating thyroid hormone level (Brouwer et al., 1998) or to estrogen receptor with estrogenicity (Garner et al., 1999).

The phenolic PCB fraction in the serum of Kanechlor 500-treated guinea pigs contained several additional metabolites that were observed in GC/MS total ion chromatograms at longer GC retention times than those of $MeSO_2$-CBs (Fig. 3). Two of these, M_a and M_d, were identified tentatively as hydroxy-$MeSO_2$-tetraCB and -hexaCBs, respectively. The other two, M_b and M_c, were presumably the precursors of M_d because when the fraction was oxidized chemically, the two peaks were converted to the corresponding methylsulfones (M_d). Mio and Sumino (1985) identified 3-hydroxy-4-methylthio-2,5,2 ,5 -tetraCB in the feces from rats, mice, and guinea pigs dosed with 2,5,2 ,5 -tetraCB, suggesting that the precursor of the metabolite was 3,4-epoxide. The epoxide may have been opened by nucleophile attack of the sulfhydryl group and subsequently converted to the hydroxylated metabolite by dehydrogenation (Bakke et al., 1982). Therefore, the metabolites retained in the serum of guinea pigs may be congeners containing a hydroxyl group in the position adjacent to the $MeSO_2$ group.

Conclusion

In Kanechlor 500 metabolism, guinea pigs showed reduced elimination of coplanar PCBs with 4-, 3,4-, or 3,4,5-chlorine substitution. In contrast, hamsters showed reduced elimination of 2,4,5-trichloro-substituted PCBs. Guinea pigs exhibited a higher ability for selective metabolism of CBs with 2,3,6-chlorine substitution to *meta*-$MeSO_2$-CBs, whereas hamsters showed selective metabolism to catechol metabolites. The reduced elimination rate for coplanar PCBs and the differences in distribution of *meta*-$MeSO_2$-CBs, and phenolic and catechol PCBs may have implications for the differences in sensitivity to PCB toxicity between guinea pigs and hamsters.

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Species differences in the tissue distribution of catechol and methylsulphonyl metabolites of 2,4,5,2′,5′-penta- and 2,3,4,2′,3′,6′-hexachlorobiphenyls in rats, mice, hamsters and guinea pigs

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Abstract
Polychlorinated biphenyls (PCBs) are metabolized to phenolic or methylsulphonyl PCBs ($MeSO_2$-CBs) in animal species. The study determined the species differences in the tissue distribution of persistent PCB metabolites in rats, mice, hamsters and guinea pigs 4 days after exposure to 2,4,5,2′,5′-pentachlorobiphenyl (CB101) or 2,3,4,2′,3′,6′-hexachlorobiphenyl (CB132). For CB101 metabolism, the hydroxylation in rats, mice and hamsters occurred primarily at the 3′-position in the 2′,5′-dichlorinated phenyl ring, whereas the hydroxylation in guinea pigs occurred preferentially at the 3-position. Metabolite profiles in tissues of hamsters were dominated by 3′,4′-catechol-CB101, whereas metabolite profiles in rats and mice were dominated by 3′- or 4′-$MeSO_2$-CBs. For CB132 metabolism, rats and mice produced 4′- and 5′-$MeSO_2$-CBs at similar concentration ratios, whereas guinea pigs produced $MeSO_2$-CBs at higher levels and selectively retained 5′-$MeSO_2$-CB in liver. In contrast, hamsters preferentially produced 4′,5′-catechol-CB132 that was retained in serum. Consequently, hamsters produced catechols, whereas guinea pigs produced *meta*-substituted $MeSO_2$-CBs, preferentially from CB132. These findings indicate that PCBs with 2,3,6-chlorine substitution are preferred substrates for the formation of catechols or $MeSO_2$-CBs and the differences in metabolite profiles are related to species-dependent metabolic capacities.

Keywords: *Species difference, tissue distribution, methylsulphonyl, catechol, polychlorinated biphenyl*

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Introduction

Non-planar polychlorinated biphenyls (PCBs) with *meta/para*-unsubstituted carbon atoms are major components of technical PCB mixtures and are detected frequently in the environment and biota (Brevik et al., 2002). These PCBs are readily metabolized by cytochrome P450 (CYP)-mediated enzymes to phenolic PCBs via arene oxide intermediates (Forgue et al., 1979) or by direct insertion of a hydroxyl group (Preston et al. 1983). The arene oxides are metabolized further to methylsulphonyl PCBs ($MeSO_2$-CBs) via the mercapturic acid pathway (Bakke et al., 1982).

$MeSO_2$-CBs have been detected in the liver and adipose tissues of both wildlife (Haraguchi et al., 1992, Bergman et al., 1994, Klasson-Wehler et al., 1998) and humans (Chu et al., 2003). These metabolites, particularly *meta*-substituted $MeSO_2$-CBs, have been shown to induce extensively CYP2B-related enzymes in rats (Kato et al., 1995) and to elicit hormone-disrupting effects (Letcher et al., 2002).

Catechols have been shown to be produced by *in vivo* and *in vitro* metabolism of selected PCBs in some species (McLean et al., 1996, Garner et al., 1999). More recently, several catechol PCBs were identified in uridine diphosphate glucuronosyltransferase (UGT)-deficient Gunn rats following administration of a PCB mixture, Kanechlor 500 (Haraguchi et al., 2004). The results indicated that CBs with 2,5- or 2,3,6-chlorine substitution could be preferred substrates for the formation of catechols. Several catechols from lower chlorinated biphenyls had potential oestrogenic activities *in vitro* (Garner et al., 1999) and might be related to perturbation of the endocrine systems (Garner et al., 2000).

An *in vitro* study with 2,5,2′,5′-tetraCB metabolism has demonstrated that there are marked differences in the catalytic activity of each P450 (phase I) in hydroxylation among animal species (Koga et al., 1998a); 3-hydroxylation is mediated by guinea pig liver microsomal CYP2B18 (Koga et al., 1998b), whereas 4-hydroxylation is mediated by hamster liver microsomal CYP2A8 (Koga et al., 1996). An *in vitro* metabolism study by Tampal et al. (2002) has demonstrated that some persistent phenolic PCBs in the body are poor substrates for UGTs (phase II) and resist conjugation, whereas the present *in vivo* study in Gunn rats indicated that catechol PCBs might be substrates for UGTs (Haraguchi et al. 2004). The findings suggest that the persistent PCB metabolite profiles in tissues vary largely depending on the activities of phase I or II enzymes in different species or strains.

The study was performed to gain insight into the species differences in the distribution of phenolic PCBs, catechols and $MeSO_2$-CBs in the liver and serum after *in vivo* exposure to 2,4,5,2′,5′-pentachlorobiphenyl (CB101) and 2,3,4,2′,3′,6′-hexachlorobiphenyl (CB132) in Wistar rats, ddy mice, Syrian hamsters and Hartley guinea pigs.

Materials and methods

Chemicals

CB101 and CB132 were synthesized by the Cadogan (1962) coupling reaction. The 3′-methoxy- and 4′-methoxy-2,4,5,2′,5′-pentaCBs, and 4′-methoxy- and 5′-methoxy-2,3,4,2′,3′,6′-hexaCBs were synthesized by the method of Bergman et al. (1995). 3′- and 4′-$MeSO_2$-2,4,5,2′,5′-penta-CBs, and 4′- and 5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCBs were synthesized as described (Haraguchi et al., 1987). 3′-Methoxy-2,4,5,2′,5′-pentaCB was synthesized from 2,5-dichloroaniline and 2,3,6-trichloroanisole. 3′,4′-Dimethoxy-2,4,5,2′,5′-pentaCB and 4′,5′-dimethoxy-2,3,4,2′,3′,6′-hexaCBs were synthesized from 4-aminoveratrole and chlorobenzene as described by Haraguchi et al. (2004). The purities of isomers were determined to be > 99% by gas chromatography. Phenolic metabolites were

methylated by diazomethane prepared from N-methyl-N′-nitro-N-nitroso-guanidine and 6 M sodium hydroxide solution. Note that diazomethane is potentially toxic by inhalation and therefore should be carefully handled in a well-ventilated place.

Animal treatments

Male Wistar rats (150–200 g), male ddy mice (27–35 g), male Syrian hamsters (95–120 g) and male Hartley guinea pigs (400–540 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Animals were housed three or four per cage with free access to commercial chow and tap water, and maintained under a 12-h dark/light cycle (lights on, 08.00–20.00 hours) in an air-controlled room (24.5 1 C, 55% 5% humidity). Animals received an intraperitoneal injection of CB101 (11 mg kg ^{1}) and CB132 (19 mg kg ^{1}) dissolved in Panacete 810 (5 ml kg ^{1}). Food and water were provided *ad libitum*. All animals were killed by decapitation on day 4 after dosing, and the liver, lung, adipose tissue and blood were removed, weighed and stored at 20 C before chemical analysis.

Sample clean-up procedure

Sample clean-up and quantification were carried out as described (Haraguchi et al., 1998). Briefly, tissue samples were homogenized with acetone/*n*-hexane (2:1, v/v). Three internal standards were added to each extract, which were then applied to a gel permeation column packed with Bio-Beads S-X3 (50 g, Bio-Rad Laboratories, Hercules, CA, USA). Dichloromethane/*n*-hexane (1:1) was used as a mobile phase at a flow rate of 4 ml min ^{1}. The metabolite fraction (120–200 ml) was collected and concentrated to dryness. The residue was methylated by diazomethane. The methylated fraction was subjected to a silica gel (1 g, Wako gel S-1, Wako Pure Chemical Industries, Osaka, Japan), eluted with dichloromethane (10 ml). The eluate was analysed without any further purification.

Instruments and quantification

Structural identification of PCBs and their metabolites was carried out on a GC/MS system (AOC-17, GC-17A, QP-5000, Shimadzu, Co., Ltd, Kyoto, Japan) with a DB-5MS capillary column (60 m 0.25 mm i.d.; J&W Scientific, Folsom, CA, USA) in electron ionization — scan mode. Temperature programme: 100 C (2 min), 100–250 C at 20 C min ^{1}, 250–280 C at 2 C min ^{1}. Quantification of PCBs and PCB metabolites was performed on a GC-14A (Shimadzu) instrument equipped with a ^{63}Ni electron capture detector (ECD) with column conditions analogous to those described for GC/MS. The three internal standards, 2,3,4,5,6,3′,4′,5′-octaCB, 4-OH-2,3,5,6,3′,4′,5′-heptaCB and 4-methyl-3-MeSO$_2$-5,2′,3′,4′,5′-pentaCB, were used for quantification. The phenolic metabolites were analysed as their methoxy derivatives by comparison with authentic standards. Recoveries of 4′-hydroxy-2,3,3′,4,5,5′-hexaCB spiked into control liver samples before extraction were 88–92% (n 4).

Results

CB101 metabolism

After exposure of four species to CB101, four phenolic PCB metabolites (as methylated derivatives) and two MeSO$_2$-CBs were isolated from the extracts of all tissues. By GC/MS

comparison with authentic compounds, these metabolites were identified as 3-OH-, 3′-OH-, 4′-OH- and 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCBs, and 3′-$MeSO_2$- and 4′-$MeSO_2$-2,4,5,2′,5′-pentaCBs. These metabolites were characterized by Haraguchi et al. (1999, 2004).

Figure 1 shows the concentrations of six PCB metabolites in liver and serum of the four species dosed with CB101. In rats and mice, 3′-$MeSO_2$- or 4′-$MeSO_2$-2,4,5,2′,5′-pentaCBs were predominant metabolites in the liver, followed by 3′-OH-2,4,5,2′,5′-pentaCB, whereas 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB was a predominant metabolite in serum, followed by 3′-OH-2,4,5,2′,5′-pentaCB. In guinea pigs, 3-OH-2,4,5,2′,5′-pentaCB was a predominant metabolite in the liver, followed by 3′-OH-2,4,5,2′,5′-pentaCB, and their concentrations were higher than $MeSO_2$-CBs. In hamsters, 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB was a predominant metabolite in both liver and serum.

The concentrations of unchanged CB101 and its metabolites in adipose tissue, liver, lung and serum are summarized in Table I. In rats and mice, $MeSO_2$-CBs were retained in adipose tissue, and a lesser extent in lung. In guinea pigs, $MeSO_2$-CBs were most abundant in adipose tissues. In hamsters, 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB constituted more than 90% of total phenolic PCBs in all tissues and the concentration was the highest in adipose tissues, followed by serum. The ratios of catechol to parent CB101 were the highest (1.75) in serum of hamsters.

CB132 metabolism

After treatment of four species with CB132, three phenolic PCBs (as methylated derivatives) and two $MeSO_2$-CBs were detected in all tissues. They were identified as 4′-OH-, 5′-OH- and 4′,5′-$(OH)_2$-2,3,4,2′,3′,6′-hexaCBs, and as 4′-$MeSO_2$- and 5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCBs. The characterization of catechol metabolite was described by Haraguchi et al. (2004).

Figure 2 shows the concentrations of five PCB metabolites in liver and serum of the four species dosed with CB132. In rats and mice, persistent metabolites in the liver were dominated by 4′- or 5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCBs, followed by 5′-OH-2,3,4,2′,3′,6′-hexaCB. In guinea pigs, on the other hand, 5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCB was selectively distributed in liver (5′-/4′-$MeSO_2$-hexaCB ratio 4.2). In hamsters, 4′,5′-$(OH)_2$-2,3,4,2′,3′,6′-hexaCB was a predominant metabolite in both liver and serum.

The tissue concentrations of unchanged CB132 and its metabolites are summarized in Table II. In rats, mice and guinea pigs, $MeSO_2$-CBs were predominantly distributed to adipose tissues with less accumulation in liver and lung, and the lowest concentration in serum. Particularly in the liver of guinea pigs, the sum of $MeSO_2$-CB levels was higher than the residual CB132 level (the ratio of $MeSO_2$-CBs/CB132 1.8). In contrast, hamsters retained 4′,5′-$(OH)_2$-2,3,4,2′,3′,6′-hexaCB in adipose tissues at higher levels than rats or mice. The concentration ratios of the catechol to parent CB132 were the highest (5.5) in serum, followed by lung.

Discussion

The study indicated that metabolism of CB101 and CB132 by the four species examined resulted in considerable variations in the tissue distribution profiles of catechols and $MeSO_2$-CBs. After treatment with each PCB, rats, mice and guinea pigs preferentially produced two $MeSO_2$-CBs, whereas hamsters preferentially produced a catechol metabolite. In addition, guinea pigs exhibited different metabolite profiles for CB101 and

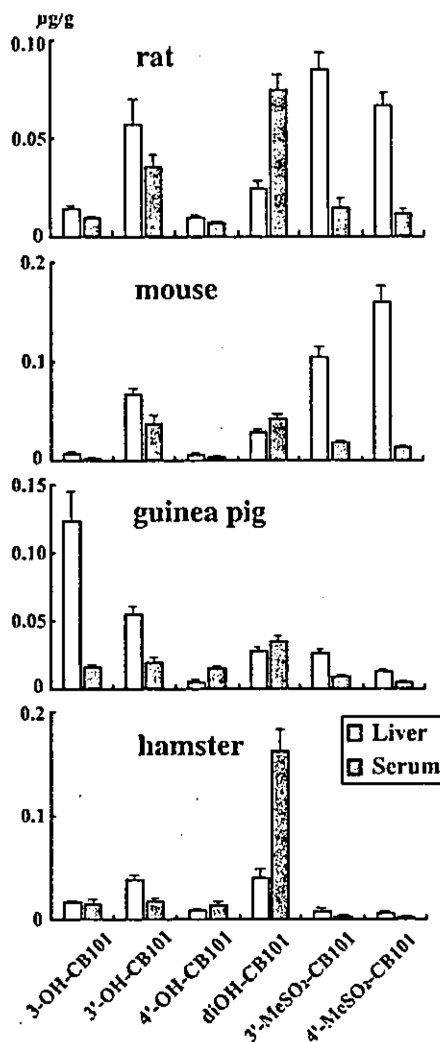

Figure 1. Concentrations of six PCB metabolites present in the liver and serum of four species after exposure to 2,4,5,2′,5′-pentachlorobiphenyl (CB101) (11 mg kg ^{1}, i.p.). Values are the mean standard error (vertical bars) for n 4; diOH-CB101 3′,4′-$(OH)_2$-2,4,5,2′,5′-pentaCB.

CB132. The structures of major PCB metabolites distributed in tissues of guinea pigs and hamsters are shown in Figure 3.

Metabolic pathways

The metabolism of each PCB involved hydroxylation, catechol formation and methylsulfonylation in all species. Hydroxylation proceeds mainly at the *meta*-position in the 2′,5′-dichlorinated phenyl ring for CB101, and in the 2′,3′,6′-trichlorinated phenyl ring for CB132, probably via direct insertion of a hydroxyl group (Preston et al., 1983). Methylsulfonylation proceeds mainly at the *meta*/*para* unsubstituted position of the phenyl

Table I. Concentrations and ratios of unchanged PCB, hydroxy- and methylsulfonyl metabolites in tissues of rats, mice, guinea pigs and hamsters 4 days after exposure to 2,4,5,2′,5′-pentachlorobiphenyl (CB101, 11 mg kg $^{-1}$, i.p.).

	Concentration (μg g $^{-1}$ wet weight)			
	Adipose tissue	Liver	Lung	Serum
Rat				
Unchanged CB101	58 21	0.87 0.19	0.23 0.10	0.18 0.03
OH-PCB	0.45 0.14	0.09 0.03	0.08 0.02	0.11 0.03
OH-PCBs/CB101	0.01	0.10	0.35	0.61
$MeSO_2$-CB	1.9 0.4	0.11 0.02	0.55 0.17	0.03 0.01
$MeSO_2$-CBs/CB101	0.03	0.13	2.39	0.17
Mouse				
Unchanged CB101	22 6.5	1.1 0.7	0.31 0.12	0.31 0.13
OH-PCB	0.20 0.10	0.09 0.03	0.04 0.01	0.09 0.02
OH-PCBs/CB101	0.01	0.08	0.13	0.29
$MeSO_2$-CB	2.3 0.9	0.22 0.04	0.99 0.22	0.03 0.01
$MeSO_2$-CBs/CB101	0.10	0.20	3.19	0.10
Guinea pig				
Unchanged CB101	8.3 0.3	0.38 0.09	0.08 0.03	0.05 0.02
OH-PCB	0.17 0.02	0.17 0.03	0.05 0.02	0.04 0.01
OH-PCBs/CB101	0.02	0.45	0.63	0.80
$MeSO_2$-CB	0.62 0.25	0.06 0.01	0.06 0.02	0.01 0.002
$MeSO_2$-CBs/CB101	0.07	0.16	0.75	0.20
Hamster				
Unchanged CB101	10 3.5	0.68 0.24	0.13 0.05	0.12 0.04
OH-PCB	0.52 0.12	0.08 0.02	0.08 0.03	0.21 0.03
OH-PCBs/CB101	0.05	0.12	0.62	1.75
$MeSO_2$-CB	0.06 0.02	0.01 0.003	0.002 0.001	0.002 0.001
$MeSO_2$-CBs/CB101	0.01	0.01	0.02	0.02

Values are means standard errors for $n = 4$; OH-PCB, sum of four phenolic metabolites including catechol; $MeSO_2$-CB, sum of two $MeSO_2$-CBs.

ring for both CBs via the mercapturic acid pathway from arene oxide intermediates (Bakke et al., 1982). Catecholation, on the other hand, involves two possible pathways. One possibility is further oxidation of phenolic CBs, in which the major metabolite 3′-OH-2,4,5,2′,5′-pentaCB for CB101 or 5′-OH-2,3,4,2′,3′,6′-hexaCB for CB132 could be a substrate for formation of the catechol. This hypothesis may be supported by the *in vitro* metabolism of 4-chlorobiphenyl by rat liver microsomes via a second hydroxylation step (McLean et al., 1996). The alternate pathway is hydrolysis of the arene oxide intermediate to dihydrodiol, followed by dehydrogenation to the catechol (McLean et al., 1996, Garner et al., 1999). As both 3′,4′-dihydrodiol- and 3′,4′-dihydroxy-2,4,5,2′,5′-pentaCB have been detected in rats dosed with CB101 (Chen et al., 1976), the dihydrodiol may be oxidized by dehydrogenase to the catechol (Penning et al., 1996).

Rats and mice

The metabolism of both CBs in rats and mice involved mainly 3′ (5′)-hydroxylation as well as 3′ (5′)- or 4′-methylsulfonylation. The *meta*-hydroxylation has been elucidated for the *in vitro* metabolism of 2,5,2′,5′- or 2,5,3′,4′-tetraCB in rats (Ishida et al., 1991, Koga et al., 1998a), where 3-hydrolyation was increased in liver microsomes from rats treated with

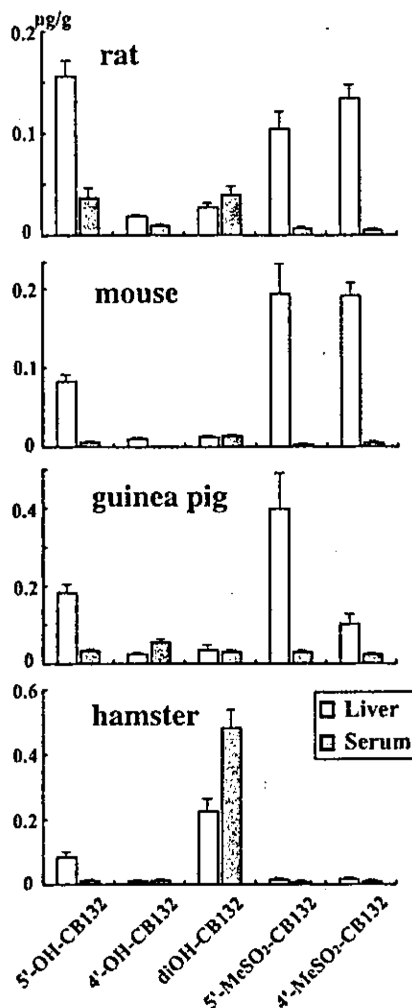

Figure 2. Concentrations of five PCB metabolites present in the liver and serum of four species after exposure to 2,3,4,2',3',6'-hexachlorobiphenyl (CB132) (19 mg kg $^{-1}$, i.p.). Values are the mean standard error (vertical bars) for *n* 4; diOH-CB132 4',5'-$(OH)_2$-2,3,4,2',3',6'-hexaCB.

phenobarbital. Previously, it was found that the *in vivo* formation rates of phenolic and $MeSO_2$-CBs from 2,5-chlorinated PCB congeners in rats were largely dependent on the degree of chlorination in the non-metabolized ring (Haraguchi et al. 1997). In the current study, metabolite profiles in rats and mice dosed with CB101 or CB132 were similar to each other, and dominated by $MeSO_2$-CBs. The selective retention of *para*-$MeSO_2$-CBs from CB101 or CB132 was observed in the lungs of rats and mice (Klasson-Wehler et al., 1996, Haraguchi et al., 1997), although there was no specificity for guinea pig and hamster lung.

Guinea pigs

The metabolite profile in guinea pigs differs from those in other species and between CB101 and CB132. For CB101 metabolism, guinea pigs preferentially produced 3-OH-2,4,5,2',5'-pentaCB rather than 3'-OH-2,4,5,2',5'-pentaCB (Figure 1). Such 3-hydroxylation was

Table II. Concentrations and ratios of unchanged PCB, hydroxy- and methylsulfonyl metabolites in tissues in rats, mice, guinea pigs and hamsters 4 days after exposure to 2,3,4,2′,3′,6′-hexachlorobiphenyl (CB132, 19 mg kg $^{-1}$, i.p.).

	Concentration (μg g $^{-1}$ wet weight)			
	Adipose tissue	Liver	Lung	Serum
Rat				
Unchanged CB132	87 27	0.37 0.12	0.29 0.09	0.04 0.01
OH-PCB	0.30 0.11	0.16 0.05	0.02 0.005	0.06 0.01
OH-PCBs/CB132	0.003	0.43	0.07	1.50
$MeSO_2$-CB	1.49 0.61	0.19 0.03	0.22 0.10	0.01 0.003
$MeSO_2$-CBs/CB132	0.02	0.51	0.76	0.25
Mouse				
Unchanged CB132	36 16	0.97 0.25	0.53 0.17	0.12 0.04
OH-PCB	0.12 0.01	0.16 0.04	0.05 0.01	0.03 0.01
OH-PCBs/CB132	0.003	0.16	0.09	0.25
$MeSO_2$-CB	3.1 1.1	0.58 0.15	0.24 0.11	0.02 0.003
$MeSO_2$-CBs/CB132	0.09	0.60	0.45	0.17
Guinea pig				
Unchanged CB132	11 4	0.34 0.13	0.20 0.12	0.07 0.02
OH-PCB	0.09 0.01	0.11 0.05	0.06 0.02	0.04 0.01
OH-PCBs/CB132	0.01	0.32	0.30	0.57
$MeSO_2$-CB	6.9 3.6	0.63 0.07	0.12 0.04	0.03 0.01
$MeSO_2$-CBs/CB132	0.63	1.85	0.60	0.43
Hamster				
Unchanged CB132	14 4	0.38 0.08	0.16 0.05	0.09 0.03
OH-PCB	1.14 0.25	0.36 0.15	0.36 0.05	0.50 0.09
OH-PCBs/CB132	0.08	0.95	2.25	5.56
$MeSO_2$-CB	0.13 0.03	0.03 0.01	0.004 0.001	0.02 0.005
$MeSO_2$-CBs/CB132	0.01	0.08	0.03	0.22

Values are means standard errors for $n=4$; OH-PCB, sum of three phenolic metabolites including catechol; $MeSO_2$-CB, sum of two $MeSO_2$-CBs.

a minor pathway in the other three species (Figure 1) and has been observed in CB101-treated minks (Klasson-Wehler et al., 1996). Ariyoshi et al. (1997) reported that guinea pig livers can metabolize 2,4,5,2′,4′,5′-hexaCB to 3-OH-2,4,5,2′,4′,5′-hexaCB. It is therefore likely that the 3-position of the 2,4,5-trichlorinated phenyl ring could be a preferred site of hydroxylation in guinea pigs. On the other hand, guinea pigs readily metabolize CB132 to the corresponding $MeSO_2$-CBs, but metabolize CB101 less readily. In addition, 5′-$MeSO_2$-2,3,4,2′,3′,6′-hexaCB formed from CB132 was selectively retained in liver (*meta*/*para*-substitution ratio 4.2) rather than other tissues (the ratio 1.6 for adipose tissues). The results indicate that guinea pigs may have substrate-selective phase II enzymes for 4,5-epoxide-2,3,6-trichlorinated congeners and/or have high affinity binding proteins for *meta*-substituted $MeSO_2$-CBs in liver. Specific retention of *meta*-$MeSO_2$-CBs has been observed in the livers of wild seals, otters and minks from Canada and Sweden (Bergman et al., 1994) as well as in human adipose tissues (Chu et al., 2003). According to Kato et al. (1995), only *meta*-substituted $MeSO_2$-CBs have been shown to induce CYP2B-related enzymes strongly in rats, indicating that the retention of 5-$MeSO_2$-2,3,6-trichlorinated PCBs may contribute significantly to the toxicity of the parent CBs in guinea pigs.

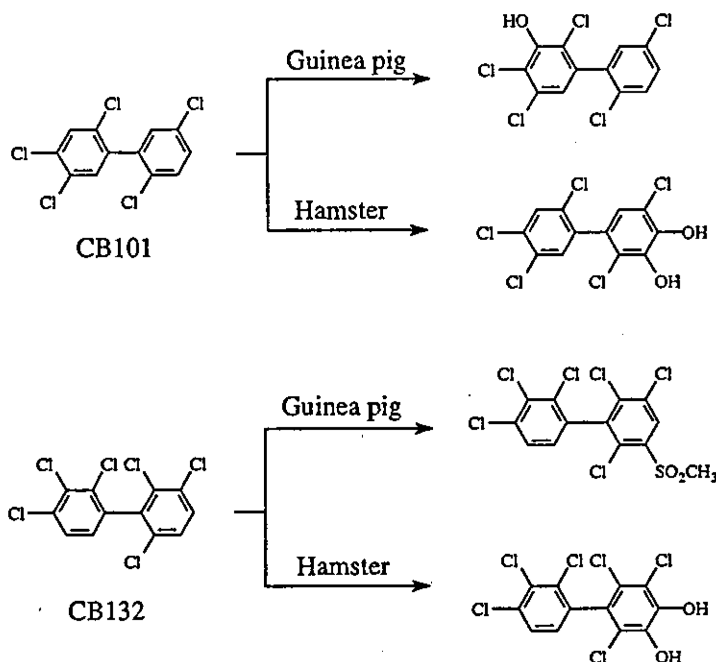

Figure 3. Chemical structures of major PCB metabolites distributed in tissues of guinea pigs and hamsters after exposure to 2,4,5,2′,5′-pentachlorobiphenyl (CB101) or 2,3,4,2′,3′,6′-hexachlorobiphenyl (CB132).

Hamsters

In contrast to guinea pigs, hamsters metabolize CB101 and CB132 primarily to their catechol PCBs rather than $MeSO_2$-CBs. Although the formation ratio of the catechol to parent CB for CB101-treated hamsters was comparable with the ratios for the other species, CB132-treated hamsters produced the highest amounts of a catechol, which was retained in tissues, especially in adipose tissues and serum. This suggests that CBs with 2,3,6-chlorine substitution are more preferred substrates than CBs with 2,5-chlorine substitution for the formation of catechols in hamsters. The ratios of 3′ (5′)- to 4′-hydroxylation for both CBs were about 5:1 in the liver of hamsters. This is in contrast to the ratio of 1:8 in the *in vitro* metabolism of 2,5,3′,4′-tetraCB by hamster liver microsomes (Ishida et al., 1991, Koga et al., 1998a).

Mechanism of catechol retention

In Haraguchi et al. (2004), UGT1A-deficient Gunn rats exposed to CB101 (112 $mg\,kg^{-1}$, i.p.) showed preferential distribution of a catechol PCB metabolite as compared with Wistar rats, which was present in serum at higher concentrations than parent CB101. The distribution profiles of catechol PCBs in Gunn rats may resemble those in hamsters in this study. The retention of catechols in Gunn rats was explained by poor conjugation ability due to UGT deficiency. However, hamsters exposed to PCBs potentially induce UGTs to a similar extent to Wistar rats (Kato et al., 2003), indicating that UGT activities may not be crucial factors for the increased amounts of catechol PCB metabolite. Another retention

mechanism for catechols, such as binding affinity to transthyretin (Lans et al., 1993) or to oestrogen receptors (Garner et al., 2000), cannot be excluded. Taken together, the formation and retention of catechols and $MeSO_2$-CBs may be associated with species-different phase II enzymes. The potential effects of the catechol metabolites in the blood are still unknown, even though the catechols from lower chlorinated congeners have been shown to exhibit oestrogenic activity (Garner et al., 1999) and could be precursors for quinone products that show potent cytotoxicity (Srinivasan et al., 2001).

Conclusion

Rats and mice preferentially produced $MeSO_2$-CBs rather than catechols from both CBs, whereas hamsters and guinea pigs showed different metabolite profiles to each other and between CB101 and CB132. Hamsters preferentially produced a catechol from CB132 rather than from CB101, which was retained in serum or adipose tissues at higher concentrations, whereas guinea pigs formed *meta*-$MeSO_2$-CBs selectively from CB132, but not much from CB101. The findings indicate that PCBs with 2,3,6-chlorine substitution could be the preferred substrates to yield catechols or $MeSO_2$-CBs, and the differences in the metabolite profiles are related to the species-dependent metabolic capacities in the liver.

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Evaluation of developmental toxicity of β-thujaplicin (hinokitiol) following oral administration during organogenesis in rats

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Abstract

The objective of this study was to evaluate the developmental toxicity of β-thujaplicin (TP) in rats. Pregnant rats were given TP by gastric intubation at 15, 45, or 135 mg/kg on days 6–15 of pregnancy. The maternal body weight gain during administration at 45 and 135 mg/kg and after administration at 136 mg/kg and adjusted weight gain at 45 and 135 mg/kg were significantly reduced. A significant decrease in food consumption during and after administration was found at 45 and 135 mg/kg. A significant increase in the incidence of postimplantation loss was found in pregnant rats given TP at 135 mg/kg. A significantly lower weight was found in female fetuses at 45 and 135 mg/kg and in male fetuses at 135 mg/kg. Although a significantly increased incidence of fetuses with skeletal variations and decreased degree of ossification were found at 135 mg/kg, no significant increase in external, skeletal and internal malformations was detected after administration of TP. The data demonstrated that TP had adverse effects on embryonic/fetal survival and growth only at maternal toxic doses. No adverse effects on morphological development were found in rats fetuses. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.
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Keywords: β-Thujaplicin; Hinokitiol; Developmental toxicity; Teratogenicity; Rat

1. Introduction

β-Thujaplicin (TP; CAS No. 499-44-5; Hinokitiol; 4-isopropyltropolone) is a phenolic component of essential oils extracted from cypress trees. TP has been found to act as an antibacterial agent (Saeki et al., 1989; Osawa et al., 1990; Tonari, 1998) and an antitumor agent (Yamato et al., 1984; Inamori et al., 1993). In addition, it possesses phytogrowth-inhibitory effects (Inamori et al., 1991). TP is used as a natural food preservative in Japan.

Several reports on the toxicity of TP are available. In mutagenicity screening tests of TP, positive results were obtained in a Rec-assay with S9 mix at 1.0 mg/disk and chromosome aberration test in vitro at 0.002–0.003 mg/ml, but not in the Ames test or a micronucleus test in mice (Sofuni et al., 1993). The DNA damaging activity of TP was weak in a spore Rec-assay (Ueno and Ishizaki, 1992). The values of LD50 have been reported to be 504 mg/kg in male ddy mice and 469 mg/kg in female ddy mice after oral gavage of TP (Shimizu et al., 1993). Recently, Ogata et al. (1999) reported a significant increase in the incidence of fetuses with malformations after oral administration of TP at 560 mg/kg and higher on day 9 of pregnancy in ICR mice and that TP induced dysmorphogenicity in cultured mouse embryos at concentrations of 6.25 and 12.5 μg/ml. However, there is no information on the developmental toxicity of TP in rats. Therefore, the present study was conducted to evaluate the potential teratogenicity of TP after administration throughout organogenesis in rats.

Abbreviations: TP, β-thujaplicin; LOAEL, lowest-observed-adverse-effect level; NOAEL, no-observed-adverse-effect level.

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2. Materials and methods

2.1. Animals

Wistar rats (Jcl: Wistar, Clea Co., Ltd., Tokyo, Japan) were used throughout this study. Animals were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad libitum and maintained in an air-conditioned room at 24±1 °C, with a relative humidity of 55±5%, under a controlled 12-h light/dark cycle. Virgin female rats, weighing 216–244 g, were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats were distributed on a random basis into four groups of 16–17 rats each and housed individually.

2.2. Chemicals and dosing

The female rats were dosed once daily by gastric intubation with TP (purity >98%, SEIWA Technological Laboratories Ltd., Tokyo, Japan) at a dose of 0 (control), 15, 45, or 135 mg/kg from day 6 through day 15 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of TP by gastric intubation on days 6–15 of pregnancy caused maternal deaths and decreased maternal body weight gain and caused an increase in postimplantation loss and decrease in fetal weight at 125 mg/kg and higher in rats. TP was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control rats received olive oil only. The formulations were kept in a cool and dark place for no more than 7 days.

2.3. Observations

The maternal body weight and food consumption were recorded daily. The pregnant rats were euthanized by ether overdose on day 20 of pregnancy. The peritoneal cavity and uterus were opened, and the numbers of live and dead fetuses and of resorptions were counted. The gravid uterus was removed and the dams weighed again. The adjusted weight gain, i.e. maternal weight gain throughout pregnancy corrected for gravid uterine weight, was calculated. To confirm the dam's pregnancy status, the uteri were immersed in 2% sodium hydroxide solution for over 1 h. The uteri were cleared and the implantation traces were seen to be stained yellowish-brown (Yamada et al., 1985). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Kawamura et al., 1990) and examined for skeletal malformations. The remaining live fetuses in each litter were fixed in Bouin's solution prior to dissection. To detect internal malformations, fetal heads were examined by the free-hand razor-blade sectioning method of Barrow and Taylor (1969) and the thoracic areas were examined by Nishimura's micro-dissecting method (1974), a modification of Barrow and Taylor's method.

2.4. Data analysis

The litter was considered the experimental unit. The initial body weight, body weight gain and food consumption of pregnant rats, numbers of implantations, postimplantation loss and live fetuses per litter and body weight of live fetuses were evaluated by analysis of variance, followed by Dunnett's multiple comparison test if differences were found. The incidences of post-implantation loss and fetal malformations per litter were analyzed by the Kruskal–Wallis test to assess the overall effects. Whenever a significant trend was noted, pairwise comparisons were made using the Mann–Whitney test. Fisher's exact test was used when the incidence in the control group was zero. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given TP during organogenesis. One pregnant rat was dead on day 20 of pregnancy at 135 mg/kg. The body weight gain on days 6–16 at 45 and 135 mg/kg and on days 16–20 at 135 mg/kg was reduced significantly. The adjusted weight gain, which indicates the net weight gain of pregnant rats, was significantly lower in the 45 and 135 mg/kg groups than in the control group. The food consumption on days 6–16 and days 16–20 was significantly lower in the 45 and 135 mg/kg groups than the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for pregnant rats are 45 and 15 mg/kg, respectively.

Pregnancy outcome in rats given TP during organogenesis are presented in Table 2. Litters totally resorbed were found in three of the 16 pregnant rats at 135 mg/kg. A significant increase in the number of resorptions per litter and incidence of postimplantation loss per litter and a significant decrease in the number of live fetuses per litter were also noted at 135 mg/kg. The weights of live fetuses were significantly decreased at 45 mg/kg and higher in females and at 135 mg/kg in males.

A summary of morphological findings in live fetuses of rats given TP during organogenesis is shown in Table 3. No fetus with external malformations was observed in any group. Skeletal examination revealed

Table 1
Maternal findings in rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. pregnant rats	16	16	16	17
No. of dead rats	0	0	0	1
Initial body weight	227±8	227±7	227±6	227±6
Body weight gain during pregnancy (g)[a]				
Days 0–6	17±5	17±4	16±2	17±3
Days 6–16	45±4	39±6	32±7*	13±9*
Days 16–20	48±6	48±5	42±6	21±12*
Adjusted weight gain during pregnancy (g)[a,b]	39±7	36±8	28±10*	24±5*
Food consumption during pregnancy (g)[a]				
Days 0–6	105±7	101±6	98±5*	101±5
Days 6–16	157±12	147±13	129±12*	103±11*
Days 16–20	72±5	70±4	63±7*	66±6*

[a] Values are given as mean±S.D.
[b] Adjusted weight gain refers to maternal body weight gain excluding the gravid uterus.
* Significantly different from the control, $P<0.05$.

Table 2
Reproductive findings in rats givenβ-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. litters	16	16	16	16
No. corpora lutea per litter[a]	16.3±1.3	16.3±1.3	15.7±1.4	16.3±0.9
No. implantations per litter[a]	15.4±1.4	15.5±1.2	14.8±1.7	15.6±1.3
No. of litters totally resorbed	0	0	0	3
No. resorptions per litter[a]	1.3±1.4	1.3±1.2	2.0±1.0	9.9±4.6*
No. dead fetuses per litter[a]	0.1±0.3	0	0	0
% Postimplantation loss per litter[b]	8.5	8.0	13.6	63.5*
No. live fetuses per litter[a]	14.1±1.4	14.3±1.5	12.8±1.8	5.7±4.6*
Sex ratio of live fetuses (male/female)	114/111	116/112	107/97	56/35
Body weight of live fetuses (g)[a]				
Male	3.39±0.19	3.26±0.19	3.25±0.18	2.71±0.21*
Female	3.19±0.18	3.13±0.18	3.02±0.19*	2.62±0.11*

[a] Values are given as mean±SD.
[b] (No. resorptions and dead fetuses/No. implantations)×100.
* Significantly different from the control, $P<0.05$.

one fetus with sternoschisis at 135 mg/kg. Skeletal variations in the vertebrae, ribs, and/or sternebrae were found in all groups. The incidences of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs were significantly higher in the 135 mg/kg group than the control group. The numbers of ossification centers of the caudal vertebrae and of the sternebrae were significantly decreased at 135 mg/kg. Hypoplasia of the spleen occurred in two fetuses in one dam at 135 mg/kg. A few fetuses with thymic remnant in the nick and/or left umbilical artery were found in the control group and TP-treated groups. However, there was no significant difference in the incidence of fetuses with internal malformations and variations between the TP-treated groups and the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for fetal rats are 45 and 15 mg/kg, respectively.

4. Discussion

This study was designed to screen for general developmental toxicity in rats. Doses of TP expected to induce maternal and developmental toxicity, such as a decrease in maternal body weight gain and food consumption and in fetal weight and an increase in postimplantation loss, were given to pregnant rats to characterize the effects of TP on embryonic/fetal development. Maternal toxicity, as evidenced by a significant decrease in body weight gain and food consumption