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Using the Scoparone O-Demethylase Assay for Categorizing Types of Hepatic Microsomal Monooxygenase Inducers

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Since 7-ethoxycoumarin was introduced by Ullrich and Weber (1972) as an interesting substrate for measuring mixed-function monooxygenase activity in hepatic microsomes, the O-dealkylation activities of several 7-alkoxycoumarins have been measured to characterize the different species of cytochrome P-450 (Kamataki et al. 1980; Komori et al. 1984). In studies with scoparone (6,7-dimethoxycoumarin) as a substrate, Müller-Enoch et al. (1981) have demonstrated that the regioselective O-demethylation of this compound to 6-hydroxy-7-methoxycoumarin (6-OH-7-OCH_3) and 7-hydroxy-6-methoxycoumarin (7-OH-6-OCH_3) varies significantly with hepatic microsomal monooxygenases induced by phenobarbital (PB) and 3-methylcholanthrene (MC). Therefore, the ratio of the two O-demethylations of scoparone at the positions 6 and 7 may be used as a probe to classify the effects of various inducers of hepatic microsomal monooxygenases.

Polychlorinated biphenyl (PCB) isomers and congeners which induce hepatic microsomal monooxygenases have been divided into three main groups, PB-type, MC-type and mixed (PB plus MC)-type inducers (Goldstein et al. 1977; Yoshimura et al. 1978; Parkinson et al. 1980). In the present study, the substrate scoparone was examined for its ability to divide various PCB isomers and congeners into these three groups of inducers. For purposes of comparison, 7,8-dimethoxycoumarin was also evaluated for its use as a substrate.

MATERIALS AND METHODS

Scoparone, 7,8-dimethoxycoumarin, 6-OH-7-OCH_3, 7-OH-6-OCH_3, 7-hydroxy-8-methoxycoumarin (7-OH-8-OCH_3) and 8-hydroxy-7-methoxycoumarin (8-OH-7-OCH_3) were prepared as previously described (Murray et al. 1982). The deuterated analogues of these four monomethyl ethers of dihydroxycoumarins were similarly prepared by methylation with [$^2H_6$]dimethyl sulphate. According to described methods (Hutzinger et al. 1971), 3,3'-dichlorobiphenyl

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(DCB), 4,4'-DCB, 2,4,2',4'-tetrachlorobiphenyl (TCB), 2,5,2',5'-TCB, 3,4,3',4'-TCB, 2,3,4,2',3',4'-hexachlorobiphenyl (HCB) and 3,4,5,3',4',5'-HCB were synthesized by Ullmann condensation of the appropriate chlorinated iodobenzenes; 2,4,5,2',4',5'-HCB was synthesized by Sandmeyer reaction of 2,5,2',5'-tetrachlorobenzidine. The PCB congeners were purified by column chromatography on silica gel and by recrystallization from ethanol and/or benzene.

Male Wistar rats weighing 160-200 g were used throughout this study. PB (80 mg/kg/d) dissolved in isotonic saline, MC (40 mg/kg/d) dissolved in olive oil and PB plus MC (80 + 40 mg/kg/d) were injected ip individually once a day for 3 d, and the animals were killed 24 hr after the last injection. PCB congeners dissolved in olive oil were given ip once a day for 3 d at the designated doses shown in Tables 2 and 3, and the animals were killed 48 hr after the last dosing. Control rats received olive oil. All rats were fasted over the last 24 hr to lower liver glycogen levels. Liver microsomes were prepared as previously described (Tajima et al. 1985), and microsomal protein was determined by the method of Lowry et al. (1951). A typical incubation mixture for the assay of O-demethylase activity consisted of microsomes (about 3 mg), Na, K-phosphate (pH 7.4, 0.1 M), EDTA (0.1 mM), an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 3 units of glucose 6-phosphate dehydrogenase, 5 mM $MgCl_2$) and either scoparone or 7,8-dimethoxycoumarin (1.5 μmol) in a final volume of 3 mL. Incubation was carried out aerobically at 37°C for 20 min and terminated by the addition of 0.1 mL of 10% HCl.

Microsomal O-demethylation activities were estimated by determining the monodemethylated metabolites of dimethoxycoumarin isomers as follows: To an incubation mixture were added 3 μg each of deuterated 6-OH-7-OCH_3 and 7-OH-6-OCH_3 or 3 μg each of deuterated 7-OH-8-OCH_3 and 8-OH-7-OCH_3 as internal standards. The mixture was extracted with diethyl ether and the extract was subjected to gas chromatography/mass spectrometry/selected-ion monitoring analysis as previously described (Yamamoto et al. 1986). Statistical differences were evaluated at the 5% ($p < 0.05$) level of significance using the Student's t test.

RESULTS AND DISCUSSION

Before examining the effects of PCB isomers and congeners, the effects of pretreatment with PB, MC and PB plus MC on the scoparone O-demethylase activity in hepatic microsomes were examined for comparison (Table 1). Compared to the control animals, PB pretreatment enhanced the O-demethylation of scoparone at position 7 much more than at position 6 and then greatly lowered the ratio of the two metabolites 6-OH-7-OCH_3 to 7-OH-6-OCH_3 to about 0.8. MC pretreatment, in contrast, enhanced the O-demethylation at position 6 slightly more than at position 7, and moderately elevated the ratio to 3.7. The

Table 1. Effects of Pretreatment with PB, MC and PB plus MC on Scoparone O-Demethylase Activity in Rat Liver Microsomes

Treatment[a]	Metabolite formed (nmol/mg protein/min)[b]		Ratio of 6-OH-7-OCH_3 to 7-OH-6-OCH_3
	6-OH-7-OCH_3	7-OH-6-OCH_3	
Control	0.195 ± 0.010	0.069 ± 0.004	2.9 ± 0.1
PB	0.336 ± 0.011*	0.410 ± 0.015*	0.82 ± 0.03*
MC	0.882 ± 0.019*	0.238 ± 0.008*	3.7 ± 0.2*
PB + MC	0.947 ± 0.031*	0.433 ± 0.025*	2.2 ± 0.1*

[a]Rats were injected ip with PB (80 mg/kg/d), MC (40 mg/kg/d) or PB plus MC (80 + 40 mg/kg/d) once a day for 3 d. Control rats received olive oil.
[b]Values are means ± SE for 4 rats.
*Significantly different from the control at $p < 0.05$.

difference between the effects of PB and MC pretreatment on the regioselectivity of scoparone O-demethylation was comparable to that observed in the previous studies (Müller-Enoch et al. 1981). Simultaneous pretreatment with PB and MC caused the 6-OH-7-OCH_3 to 7-OH-6-OCH_3 ratio to be similar to the average of those in PB- and MC-induced microsomes.

In the case of 7,8-dimethoxycoumarin as a substrate, the ratio of 7-OH-8-OCH_3 to 8-OH-7-OCH_3 formed in control microsomes was 1.8 ± 0.2. Neither PB nor MC pretreatment had significant effect on this ratio although each enhanced the total O-demethylation activity for 7,8-dimethoxycoumarin. Therefore, the effects of pretreatment with PCB congeners were assessed by determining the ratio of the two O-demethylations of scoparone used as a substrate.

Table 2 shows the effects of pretreatment with each PCB isomer and congener on scoparone O-demethylation activity in hepatic microsomes. All PCB pretreatment significantly enhanced the two O-demethylations of scoparone. At high doses (100 and 150 mg/kg/d), 4,4'-DCB caused practically the same ratio of 6-OH-7-OCH_3 to 7-OH-6-OCH_3 as that in PB plus MC-induced microsomes (Table 1), whereas 3,3'-DCB brought about no significant change in the ratio. Both 2,4,2',4'- and 2,5,2',5'-TCB caused the ratio to be similar to that in PB-induced microsomes (Table 1). 3,4,3',4'-TCB and 3,4,5,3',4',5'-HCB caused the 6-OH-7-OCH_3 to 7-OH-6-OCH_3 ratio to be greater than that in MC-induced microsomes (Table 1). The last two congeners, moreover, enhanced total O-demethylation activity for scoparone more than the other congeners examined.

Table 2. Comparative Effects of Pretreatment with PCB Congeners on Scoparone O-Demethylase Activity in Rat Liver Microsomes

Treatment[a]	Dose (mg/kg/d)	Metabolite formed (nmol/mg protein/min)[b]		Ratio of 6-OH-7-OCH_3 to 7-OH-6-OCH_3
		6-OH-7-OCH_3	7-OH-6-OCH_3	
Control		0.194 ± 0.010	0.070 ± 0.004	2.8 ± 0.1
3,3'-DCB	100	0.244 ± 0.017*	0.095 ± 0.003*	2.6 ± 0.1
4,4'-DCB	100	0.418 ± 0.020*	0.183 ± 0.012*	2.3 ± 0.1*
	150	0.506 ± 0.011*	0.237 ± 0.012*	2.1 ± 0.1*
2,4,2',4'-TCB	100	0.433 ± 0.013*	0.523 ± 0.025*	0.83 ± 0.04*
2,5,2',5'-TCB	100	0.520 ± 0.015*	0.452 ± 0.010*	1.1 ± 0.1*
3,4,3',4'-TCB	50	2.36 ± 0.07*	0.481 ± 0.012*	4.9 ± 0.1*
3,4,5,3',4',5'-HCB	1	1.68 ± 0.08*	0.378 ± 0.017*	4.5 ± 0.1*

[a]Rats were injected ip with PCB congeners once a day for 3 d at the designated doses. Control rats received olive oil.
[b]Values are means ± SE for 4 rats.
*Significantly different from the control at $p < 0.05$.

Table 3. Dose-Effect Relationship of 2,4,5,2',4',5'-HCB vs 2,3,4,2',3',4'-HCB Pretreatment on Scoparone O-Demethylase Activity in Rat Liver Microsomes

Treatment[a] Dose (mg/kg/d)	Metabolite formed (nmol/mg protein/min)[b] 6-OH-7-OCH_3	7-OH-6-OCH_3	Ratio of 6-OH-7-OCH_3 to 7-OH-6-OCH_3
Control	0.200 ± 0.010	0.070 ± 0.003	2.9 ± 0.2
2,4,5,2',4',5'-HCB			
10	0.259 ± 0.014*	0.228 ± 0.009*	1.1 ± 0.1*
50	0.469 ± 0.017*	0.531 ± 0.043*	0.90 ± 0.09*
100	0.388 ± 0.022*	0.482 ± 0.028*	0.81 ± 0.05*
2,3,4,2',3',4'-HCB			
10	0.200 ± 0.008	0.090 ± 0.002*	2.3 ± 0.1*
50	0.401 ± 0.028*	0.214 ± 0.027*	1.9 ± 0.2*
100	0.529 ± 0.028*	0.344 ± 0.015*	1.5 ± 0.1*

[a]Rats were injected ip with 2,4,5,2',4',5'-HCB or 2,3,4,2',3',4'-HCB once a day for 3 d at the designated doses. Control rats received olive oil.
[b]Values are means ± SE for 4 rats.
*Significantly different from the control at $p < 0.05$.

The effects of different doses of 2,4,5,2',4',5'- and 2,3,4,2',3',4'-HCB on the scoparone O-demethylase activity are shown in Table 3. At low and high doses (10, 50 and 100 mg/kg/d), 2,4,5,2',4',5'-HCB caused the 6-OH-7-OCH_3 to 7-OH-6-OCH_3 ratio to be similar to that in PB-induced microsomes (Table 1). 2,3,4,2',3',4'-HCB, in contrast, caused the ratios to be close to that in PB plus MC-induced microsomes (Table 1) at low doses (10 and 50 mg/kg/d) but not at a high dose (100 mg/kg/d).

Based on the ratio of the two O-demethylations of scoparone used as a substrate after pretreatment with each PCB congener (Tables 2, 3), we divided the congeners into two main groups of inducers: 2,4,2',4'-TCB, 2,5,2',5'-TCB and 2,4,5,2',4',5'-HCB are classified as PB-type inducers, and 3,4,3',4'-TCB and 3,4,5,3',4',5'-HCB as MC-type inducers. This classification is consistent with the previous classification based on their effects on the spectral properties and various catalytic profiles of hepatic microsomal monooxygenases (Goldstein et al. 1977; Yoshimura et al. 1978). At low doses of 10 and 50 mg/kg/d (Table 3), 2,3,4,2',3',4'-HCB caused ratios to be similar to that in PB plus MC-induced microsomes so that this congener was classified as a mixed-type inducer as previously described (Parkinson et al. 1980). In Table 2, of the two dichlorobiphenyls classified as

weak PB-type inducers (Goldstein et al. 1977; Yoshimura et al. 1978), 4,4'-DCB also caused a ratio similar to that in PB plus MC-induced microsomes. These results collectively suggest that the distinction between weak PB-type and mixed-type inducers is made difficult by the ratio of the two O-demethylations of scoparone. However, irrespective of the limits of its application, the use of scoparone as a single substrate can provide a convenient assay for distinguishing preliminarily between PB-type and MC-type inducers of hepatic microsomal monooxygenases.

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