Metabolism of four toxaphene congeners by rat hepatic microsomes: extrapolation of kinetic data to humans

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Introduction

Toxaphene has been used extensively as a pesticide. It is a complex mixture consisting of more than 670 polychlorinated constituents [1]. Nowadays its congeners are ubiquitously present in the environment, but not all congeners are of even persistence. High levels of especially three chlorinated bornane (CHB) congeners have been reported in several fish species used for human consumption, namely CHB26, 50, and 62 [2, 3]. These three CHBs can be used as indicator compounds for toxaphene exposure of humans, while a fourth congener CHB32 might serve as an indicator of recent toxaphene contamination [4]. In the present study, we assessed the metabolic rate of these four congeners by rat hepatic microsomes. The ultimate goal of the present study is to improve the extrapolation of the available toxicokinetic data of rat studies to humans. This is achieved by comparing *in vitro* metabolic rates by rat and human hepatic microsomes. Metabolism of these CHBs by human hepatic microsomes are currently under investigation, and will be presented at the conference.

Phase I metabolism is performed by microsomal cytochrome P450 enzymes (CYPs). To detect which members of the CYP family are involved, a correlation analysis is performed between the metabolic rate of CHB26, 32, 50, and 62, and specific CYP activities in 18 different microsomal samples. To increase the power of the correlation analysis in the present study, some rats are treated with selective CYP inducing compounds.

The regio- and stereospecific hydroxylation of testosterone by rat hepatic microsomes can be indicative for the presence of a certain CYPs (Table 1 [5-7]). The O-dealkylation rates of methoxy- (MROD), ethoxy- (EROD), and pentoxyresorufin (PROD) by rat liver microsomes are indicative for the presence of CYP1A2, CYP1A1/2, and CYP2B1, respectively [8].

Table 1: Formation of testosterone metabolites by rat hepatic microsomal CYPs. CYPs in bold letters are mainly responsible for the production in untreated rat microsomes [5-7].

OHT	CYP	OHT	CYP
15□	**3A1**, 3A2	16β	2B1, 2B2
6□	1A1, 1A2, 2A2, 2C13, 3A1, **3A2**	12β	
15□	**2A2**	2α	**2C11**
7□	2A1, 2A2	2β	3A1, 3A2
16□	**2C11**, 2B1, 2B2	17O	2B1, 2B2, 2C

Experimental: Animals, Material and Methods

Mature (16 weeks old) Wistar rats (CPB:uWu, SPF), obtained from the Central Animal Laboratory (Utrecht University), were i.p. injected daily for 4 days with inducers at doses as shown in Table 2. All animals were sacrificed 24 h after the last injection. Livers were immediately removed, and microsomes were prepared as described by Bouwman *et al.* [9]. The protein content was determined using the Bradford method with BSA as a standard.

Table 2: Treatment of rats with several inducers of hepatic CYP enzymes. Rats were injected i.p. daily for 4 days with the indicated dose, or received no treatment (CF and CM).

Female	Male	Inducer (main induced CYPs)	Daily dose (mg/kg BW)
BF	BM	□-naphtoflavone (CYP1A1/2)	40
CLF	CLM	Clofibric acid (CYP4A)	300
CF1, 2, 3	CM1, 2, 3	none	
DF	DM	Dexamethasone (CYP3A1/2)	75
IF	IM	Isosafrole (CYP1A1/2)	230
PF1, 2	PM1, 2	Phenobarbital (CYP2B1/2)	75

MROD, EROD and PROD activities were measured in triplicate according to Burke *et al.* [10] with some minor adjustments described by Rutten *et al.* [11]. Testosterone hydroxylase activities were determined in triplicate according to Sonderfan *et al.* [12] and adapted by Wortelboer *et al.* [13].

Microsomes (0.7 mg protein) were preincubated for 8 min in triplicate in an incubation volume of 0.95 ml of phosphate buffer (pH=7.4, 37 °C) with one of the toxaphene congeners (0.72 μM) and PCB153 (0.17 μM) as a (non-metabolized) internal standard. The incubation was started by adding 50 μl of a NADPH regenerating system. After 0, 20, 40, and 60 min of incubation at 37 °C, 0.2 ml aliquots were pipetted into 0.8 ml of phosphate buffer and 1 ml of methanol. The samples were extracted three times with 2 ml of hexane, with cis-chlordane (6.00 ng) as a volume standard and 0.1 ml iso-octane as a keeper. The extracts were allowed to evaporate to nearly dryness. The extracts (in 0.2 ml iso-octane) were analyzed on a Carlo Erba Mega 5360 GC-ECD equipped with a 15 m DB5 column (ID 0.32 mm, film thickness 0.25 μm). The samples were run using splitless injection (200 °C, splitless time 99 s). The temperature program was: 2 min 80 °C, 20 °C/min to 200 °C, 2 °C/min to 237 °C, 20 °C to 260 °C.

Results and discussion

The distribution of CYP activities between the 18 microsomal samples is depicted in Figure 1. As can be seen, the differences of enzyme activities are quite large for all measured CYP activities. The response of CHB relative to PCB153 was used to calculate CHB concentrations in samples. No loss of PCB153 during the incubation time was observed for any microsomal sample. The metabolism of the four toxaphene congeners can be described by first order elimination which means linear regression analysis can be applied after logarithmic transformation of the time dependence of the concentrations: $\ln C_t = -K_{el}t + \ln C_0$. The first order elimination rate constant (K_{el}) is set to zero if the model showed not to be significant at $p<0.05$. The K_{el}s of all microsomal samples are shown in Figure 2.

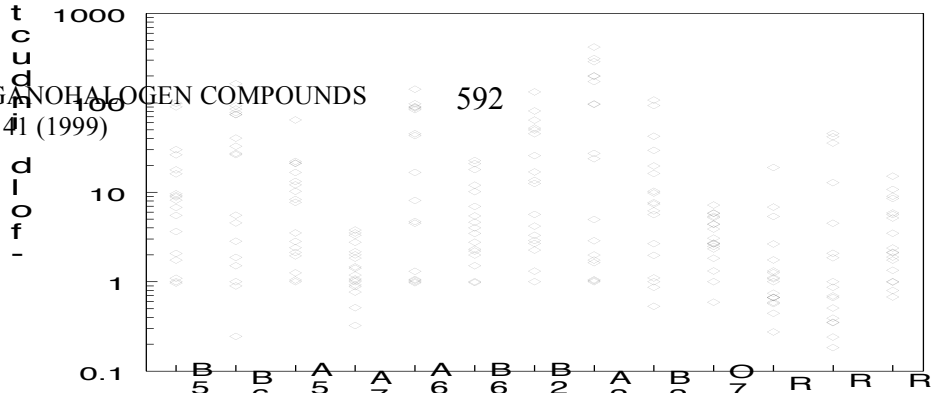
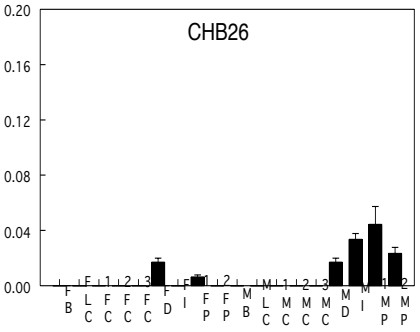


Figure 1. Measured CYP enzyme activities (OHT formation, Table 1, and AROD) of the microsomes relative to a control female sample.



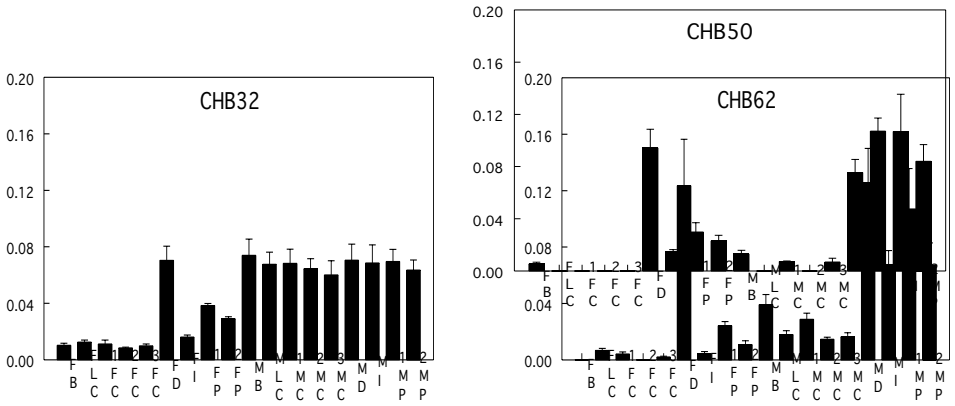


Figure 2. First order elimination rate constants of four CHBs by hepatic microsomes (for samples, see Table 2). Standard errors of the K_{el}s are given on top of the bars.

A correlation analysis between the CYP activities and the K_{el}s of the CHBs was performed using the non-parametric Spearman's method (SPSS for Windows). The correlation coefficients between CYP activities and toxaphene metabolism that significantly differ from zero at $p<0.05$ are given in Table 3.

Table 3: Correlation coefficients significant at $p<0.05$ between CYP enzyme activities and K_{el}s.

	Testosterone Hydroxylation (OH metabolites, 17O = androstenedione)										Alkoxyresorufin O-dealkylation		
CHB	**15□**	**16□**	**2□**	**7□**	**17O**	**12□**	**15□**	**16□**	**2□**	**6□**	**Mr**	**Er**	**Pr**
26						0.54	0.49	0.58	0.49	0.47			0.57
32	0.85	0.70	0.71		0.51	0.84	0.80	0.62	0.76	0.78			
50	0.60			0.58		0.65	0.63	0.72	0.66	0.62			0.56
62	0.78	0.62	0.60		0.52	0.83	0.80	0.74	0.78	0.76			

Mr = ethoxyresorufin, Er = methoxyresorufin, Pr = pentoxyresorufin.

CHB32 is metabolized at the highest rate of the four investigated CHBs by untreated rat microsomes. Hepatic microsomes from harbour seals, whitebeaked dolphin, harbour porpoise, and Laysan albatross also metabolized this CHB faster than the other three [14]. In biological samples concentration of CHB26, CHB50, and to a minor extend CHB62 are higher than of CHB32 [2,3], which could partially be the result of a difference in metabolism. Treatment of rats with dexamethasone and phenobarbital alters the metabolism of all four CHBs drastically (Figure 2). The CYPs that are mainly responsible for metabolism of the CHBs are (see Table 3): **CHB26**: 2B, 3A; **CHB32**: 2B, 2C11, 3A; **CHB50**: 2A, 2B, 3A; **CHB62**: 2A2, 2B, 2C11. For a reliable

extrapolation of kinetic data of CHBs from rat studies to humans, we are currently investigating the metabolic rate of the four CHBs by two human hepatic microsomal pools. To get insight in the intra-species differences, we use specific inhibitors to identify the CYPs that are responsible for metabolization of these CHBs.

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