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ORIGINAL PAPER

INVESTIGATION OF MECHANISM BASED INHIBITION OF CYP450 ENZYMES BY SELECTED ORGANOPHOSPHOROUS, PYRETHROIDS AND BENZOYL UREA PESTICIDES IN POOLED HUMAN LIVER MICROSOMES

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Abstract. The **goal** of this work was to investigate the mechanism based inhibition of selective model activities for some major xenobiotic-metabolizing enzymes, namely CYP1A1/2, 2A6, 2C8, 2C9, 2C19, 2D6 and 3A4 by certain pesticides. The inhibitory effects were evaluated for different CYP-selective model activities in pooled human hepatic microsomes. At the initial screening of 18 pesticides, chlorpyrifos, fenitrothion, malathion, phenthoate and profenofos displayed the smallest IC50 values towards CYP1A2 and were chosen for the investigation of mechanism based inhibition of CYP1A2, CYP2A6 and CYP2C8 (atrazine in addition for CYP2C8); fenitrothion, malathion, phenthoate, hexaflumuron, glyphosate, chlorfluazuron were chosen for investigating CYP2C9, chlorpyrifos, fenitrothion, phenthoate, abamectin, deltamethrin, lambda-cyhalothrin, fenvalerate for CYP2C19, chlorpyrifos, phenthoate, profenofos, lambda-cyhalothrin, fenvalerate, deltamethrin and carbendazim for CYP2D6 and chlorpyrifos, fenitrothion, phenthoate, lambda-cyhalothrin and atrazine for CYP3A4. The **results** showed that there was no considerable decrease in the IC50 values except for fenitrothion towards CYP2A6, the IC50 values being 23.99 μM for 2-minutes and 4.33 μM for 15-minutes preincubation indicating mechanism based inhibition. Some other pesticides showed decrease in the 15 minutes preincubation IC50 values like chlorpyrifos towards CYP2A6, phenthoate towards CYP2C19 and CYP2C9 and profenofos and carbendazim towards 2D6 however, values were more than 10 μM indicating time dependent inhibition but of weak potency. Otherwise IC50 values for 15 minutes preincubation were either the same as noticed with chlorpyrifos and fenitrothion towards CYP1A2, the IC50 values being 0.56 for both 2 and 15 minutes preincubation (potent inhibition) or even higher than the IC50 values for 2 minutes preincubation indicating reversible inhibition.
Keywords: CYP450, organophosphorous, benzoyl urea

Introduction

Cytochrome P450 enzymes are highly involved in xenobiotic biotransformation through a number of metabolic reactions. CYPs are found in high concentration in the liver, but are present in a variety of other tissues [1-4]. CYP-xenobiotic interactions involve either induction or inhibition of metabolising enzymes. Inhibition can take place in several ways including the destruction of pre-existing enzymes, inhibition of enzyme synthesis or by complexing and thus inactivating the metabolising enzyme.

In addition to direct reversible inhibition, these enzymes could be subjected to mechanism-based inhibition. Mechanism-based inhibition results from a metabolic product that binds irreversibly to the enzyme, rendering it inactive and synthesis of new enzyme is required before activity is restored. Mechanism-based inhibition is differentiated from direct inhibition primarily by being time dependent and it involves catalytic steps [5]. The consequences of mechanism-based inhibition are auto-inhibition of the clearance of the inactivator itself and prolonged inhibition of the clearance of other drugs that share the same enzyme. There may also be

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serious immunotoxicological consequences if a reactive intermediate is covalently bound to the enzyme. Therefore, screening of new compounds for mechanism-based enzyme inhibition is now standard practice within the pharmaceutical industry. However, species variability and the specific compound in question must be considered regarding CYP interactions [6]. Furthermore, human interactions data are needed for human risk assessment. By comparing the effects of a new chemical entity on the CYP-specific-activities to the respective effects of diagnostic inhibitors, a tentative prediction of the in vivo situation can be made [7].

In our study, xenobiotics are represented by different types of pesticides including organophosphosphates (chlorpyrifos, fenitrothion, malathion, phenthoate and profenofos), pyrethroids (deltamethrin, lambda-cyhalothrin and fenvalerate), benzoylurea (hexaflumuron and chlorfluazuron), abamectin, atrazine, carbendazim and glyphosate (Figure 1).

Materials and methods

Chemicals

Pesticides were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and ChemService (West Chester, PA). Midazolam was a kind gift from F. Hoffmann La Roche (Basel, Switzerland) and omeprazole from Astra Zeneca (Mölndal, Sweden). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from the Sigma Chemical Company (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with the Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP grade (ultra pure, 18.2 M Ω).

Human hepatic microsomes

Human liver samples used in this study were obtained from the University Hospital of Oulu as surplus from organ donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All liver samples were of Caucasian race including four female and six male between the ages of 21 and 62. Intracerebral hemorrhage was the primary cause of death. A weight-balanced pool of ten individual microsomal samples was employed in this study. The livers were transferred to ice immediately after the surgical excision and cut into pieces, snap-frozen in liquid nitrogen and stored at -80^{0} C. All microsomes were prepared by standard differential ultracentrifugation [8]. The final microsomal pellet was suspended in 100 mM phosphate buffer, pH 7.4. Protein content was determined by the Bradford method [9].

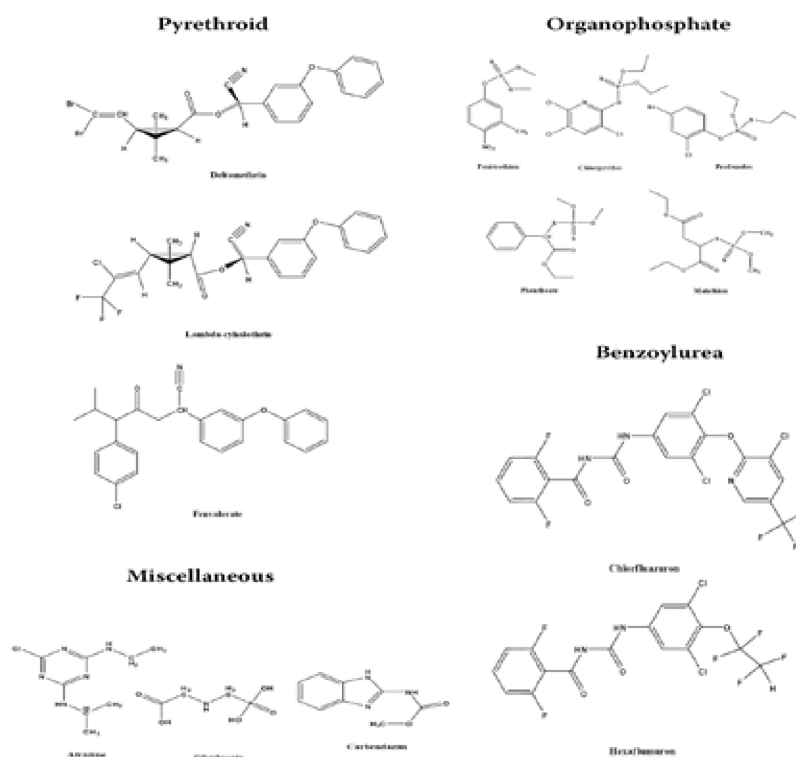

Figure 1. Chemical structures of pesticides tested for mechanism based inhibition in the current study

Inhibitory interactions assays

Each pesticide was added in different concentrations (final concentrations in the incubation mixture were 0.01–100 μM) to the incubation mixture with a small volume of dimethyl sulfoxide (DMSO) as solvent. All pesticides used in this study were soluble in DMSO. Fresh pesticide dilutions from a stock solution of DMSO were used for each assay. The final amount of DMSO was 1% in incubation mixtures.

The reaction mixture, in a final volume of 200 μL, was preincubated for 2 minutes at +37 c in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) before the reaction was initiated by the addition of nicotinamide adenine dinucleotide phosphate (NADPH). For investigation of mechanism-based inhibition, an inhibitor was further preincubated with NADPH for 15 min before the reaction was started by the addition of substrate. Each reaction was terminated by adding 200 μL of ice- cold acetonitrile. Samples were subsequently cooled in an ice bath to precipitate the proteins and stored at -20^{0} C until analyzed. Duplicate incubations and one control incubation (where the stopping reagent was added prior to the preincubation period) were performed for each sample. The incubation conditions to assess CYP enzyme activities are mentioned in detail in Abass et al, 2007 [10].

The IC50 values for inhibitors (concentration causing 50% reduction of control activity) were determined from three replicates by linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the activity remaining after inhibition using MicroCal Origin 6.0 (MicroCal Software, Inc., Northampton, MA).

Analytical methods

Ethoxyresorufin O-deethylation (EROD) (CYP1A1/2) and coumarin 7-hydroxylation (COH) (CYP2A6) assays were analyzed fluorometrically. EROD activity was determined with the method of Burke et al. [11] and COH activity was analyzed as described previously in detail in Raunio et al. [12]. The other CYP assays were analyzed by HPLC, and these included, amodiaquine de-ethylation (CYP2C8) [13], tolbutamide methylhydroxylation (CYP2C9) [14], dextromethorphan O-demethylation (CYP2D6) [15], midazolam1′-hydroxylation (CYP3A4) [16], and omeprazole 5- hydroxylation (CYP2C19) [17].

Metabolites of amodiaquine, tolbutamide, omeprazole, dextromethorphan and midazolam were analyzed by a Shimadzu VP series high performance liquid chromatographer (HPLC) with an auto injector (Shimadzu, Kyoto, Japan). The analytical column was a Waters Symmetry C18 (3.9 mm × 150 mm, particle size of 5 μm) together with a Lichospher 100 RP-18 4,0 mm x 4,0 mm pre-column (Merck, Darmstadt, Germany). The eluents used were 1) 75% 50 mM o-phosphoric acid buffer (pH 3.0): 25% acetonitrile for dextromethorphan, 2) 70% 50 mM o-phosphoric acid buffer (pH 3.0) : 30% acetonitrile for tolbutamide and amodiaquine, 3) 60% water : 40% acetonitrile for midazolam and 4) 25 mM o-phosphoric acid buffer (pH 3.0) (A) and acetonitrile (B) for omeprazole. Chromatographic methods were isocratic, except in the case of omeprazole when a linear gradient elution from 15% A to 35% A in 8 min was used. Mobile phases were pumped at a flow rate of 1.0 mL/min. The injection volume used was 20 μL. The concentrations of metabolites were calculated from peak height ratios of the UV-chromatograms on the basis of standard calibration curves of authentic metabolites. All the other analysis conditions are summarized in detail in our laboratory previous publication.

Results

After the initial screening of altogether 18 pesticides with respect to inhibition of CYP1A1/2 mediated ethoxyresorufin O-deethylation (EROD), CYP2A6 mediated coumarin 7-hydroxylation (COH), CYP2C8 mediated amodiaquine de-ethylation, CYP2C9 mediated tolbutamide methylhydroxylation, CYP2C19 omeprazole 5-hydroxylation, CYP2D6 mediated dextromethorphan O-demethylation, CYP3A4 mediated midazolam 1'-hydroxylation, pesticides which showed the smallest IC50 values were chosen for the investigation of mechanism based inhibition. The IC50 values of the studied pesticides for 2 and 15 minutes preincubations are listed in table 1.

CYP1A1/2:

Chlorpyrifos and fenitrothion showed relatively potent inhibition towards CYP1A1/2 with IC50 values of 0.56 μM for both 2 and 15 minutes preincubations. Profenofos was a relatively potent inhibitor with IC50s 2.06 and 3.82 μM for 2 and 15 minutes preincubations respectively. IC50 values of malathion and phenthoate were higher than 10 with no considerable differences between 2 and 15 minutes preincubations.

CYP2A6:

IC50 values of fenitrothion were 23.99 and 4.33 μM for 2 and 15 minutes preincubations respectively indicating a component of mechanism based inhibition. Although IC50 values of chlorpyrifos,

	1A1/2 2 min/15 min	2A6 2 /15min	2C8 2/15 min	2C9 2/15 min	2C19 2/15 min	2D6 2/15 min	3A4 2/15min
Fenitrothion	0.56/ 0.56	23.99/4.33	4.3/85,43	4.8 / 15.4	58.6 / 16.12	--	3.1 / >100
Chlorpyrifos	0.56/ 0.56	>100 /18.11	22.2/44.55	--	96.0 / 31.35	3.3 / >100	4.0 / 57.7
Malathion	30.87/ 32.38	>100 / 72.72	31.0/82.47	2.5 / >100	--	--	--
Phenthoate	15.8/ 13.26	>100 /44.65	10.3/42.64	35.0 / 25.24	36.2 / 13.89	3.0 / 77.9	3.0 / 48.7
Profenofos	2.06/ 3.82	>100 / 85	84.0 / >100	--	--	72.7 / 19.5	--
Lambda-cyhalothrin	--	--	--	--	>100/ >100	3.1 / 99.4	3.1 / >100
Deltamethrin	--	--	--	--	>100 / >100	3.3 / >100	--
Fenvalerate	--	--	--	--	>100/ >100	3.1 / >100	--
Chlorfluazuron	--	--	--	7.5 / >100	--	--	--
Hexaflumuron	--	--	--	6.0 / >100	--	--	--
Atrazine	--	--	31.3 / >100	--	--	--	2.8 / >100
Glyphosate	--	--	--	3.7 / >100	--	--	--
Abamectin	--	--	--	--	>100 / >100	--	--
Carbendazim	--	--	--	--	--	12.0 / 2.6	--
Imazaquin	>100 />100	>100/>100	>100 />100	>100 />100	>100 />100	>100/>100	>100 />100

Table I. IC50 values (μM) of the studied pesticides on different human P450 enzymes for 2 and 15 minutes preincubation using pooled human liver microsomes.

Legend: -- Not tested

malathion, phenthoate and profenofos were more than 100 μM for 2 minutes preincubations, these pesticides showed decrease in the IC50 values for 15 minutes preincubations. Chlopyrifos showed the most noticable decrease with IC50 value of 18.11 μM.

CYP2C19:

Among the organophosphorous pesticides, chlorpyrifos, fenitrothion and phenthoate showed decrease in the IC50 values when they were incubated with CYP2C19 for 15 minutes indicating mechanism based inhibition but of weak potency.

Abamectin and Pyrethroids including deltamethrin, lambda-cyhalothrin and fenvalerate showed IC50 values higher than 100 μM for both 2 and 15 minutes preincubations.

CYP2C9 CYP2C8 and CYP3A4:

Fenitrothion and malathion (organophosphorous pesticides), hexaflumuron and chlorfluazuron (benzoylurea), and glyphosate showed relatively potent inhibitions when they were preincubated with CYP2C9 for 2 minutes with IC50 values 4.8, 2.5, 6, 7.5 and 3.7 respectively however, the IC50 values representing 15 minutes preincubations were higher. IC50 values of phenthoate for 2 and 15 minutes preincubations were roughly the same.

All pesticides that were tested with CYP2C8 showed increase in the IC50 values for 15 minutes preincubations than those for 2 minutes preincubations. These include chlorpyrifos, fenitrothion, malathion, phenthoate, profenofos and atrazine.

The same observation was recorded with pesticides tested with CYP3A4. These pesticides include chlorpyrifos, fenitrothion, phenthoate, lambda-cyhalothrin and fenvalerate.

CYP2D6:

IC50 values of carbendazim were 12 and 2.6 for 2 and 15 minutes preincubation respectively indicating time dependent inhibition.

Among organophosphorous pesticides, profenofos showed decrease in the IC50 value when it was preincubated with CYP2D6 for 15 minutes. In contrast chlorpyrifos, phenthoate and pyrethroids (deltamethrin, lambda-cyhalothrin and fenvalerate) showed increase in the IC50 values.

In this study, imazaquin (an imidazolinone herbicide) was tested for its ability to inhibit the previously mentioned CYP450 enzymes and also was investigated for mechanism based inhibition. The results showed no inhibition at all for both 2 and 15 minutes preincubation.

Discussion

OPs are synaptic poisons, which bind and inhibit acetylcholinesterase. The interactions of these compounds with acetylcholinesterase have been well-characterized. However, these compounds display other mammalian effects which are often poorly understood and unrelated to acetylcholinesterase inhibition [18]. One such potentially significant effect of these compounds is that the reactive sulphur released during the bioactivation of OPs via CYP-mediated desulfuration to their phosphate oxon

metabolites binds irreversibly to the heme iron of CYP, inhibiting its activity through the mechanism of suicidal inhibition [19-21]. Furthermore, the variety of types of atoms (e.g, C, O, P, N, S, and Cl) and the variety of groups (e.g., acids, alcohols, esters, and ethers) present in OP insecticides enhance the opportunities for the phase I reactions [22]. As a consequence, a change in the CYP-mediated metabolism may occur especially if the same CYPs are involved in their biotransformation [23,24].

In the present study, when fenitrothion was preincubated with CYP2A6 for 2 and 15 minutes and further preincubated for 5 and 10 minutes, the IC50 values decreased with time (from 23.99 to 4.33 μM) indicating a definite component of mechanism based inhibition (figure 2). Fenitrothion also showed time dependent inhibition of CYP 2C19 and potent inhibition of CYP1A2 (IC50 values being 0.56 μM for both 2 and 15 minutes preincubations). It also showed relatively potent inhibitions of CYP2C8, CYP2C9, and CYP3A4 only after 2 minutes preincubations. A previous study suggested that fenitrothion is a mixed inhibitor of 2-hydroxylation of 17β-estradiol, serving both as a substrate and as an irreversible inhibitor of those P450s forming 2-hydroxyestradiol [25]. Berger also suggested that fenitrothion is oxidatively desulfurated by the same mouse hepatic P450s that hydroxylate 17-β estradiol at the 2 and 4 positions which were suggested to be CYP1A1/1A2 in the rat [26,27] and CYP1A2, 3A4 and 2C9 in humans [28].

Chlorpyrifos showed potent inhibition of CYP1A1/2 mediated ethoxyresorufin O-de-ethylation,

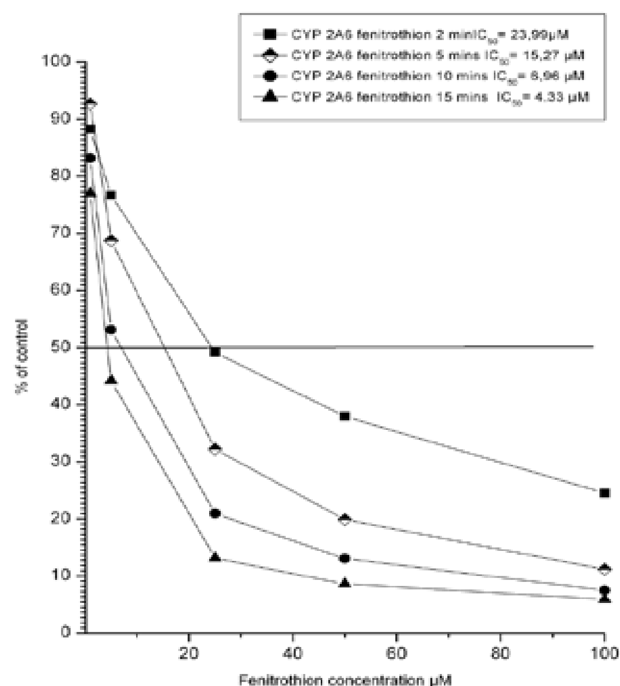

Figure 2. Effect of fenitrothion on CYP2A6 specific activities using pooled human liver microsomes

IC50 values for 2, 5, 10 and 15 minutes preincubations were all 0.56 μM (figure 3). According to some studies, CYP1A seems not to contribute to OP oxidative metabolism [29], whereas a more recent study demonstrates that CYP1A2 is involved in OP desulfuration at low concentrations [30]. Use of human CYP isoforms expressed in human lymphoblastoma cells demonstrated that CYP1A2, 2C19, and 3A4 are involved in CPS metabolism. [31] In the current study, chlorpyrifos showed relatively potent inhibition of CYP2D6 and CYP3A4 only after 2 min preincubation. A previous study established that chlorpyrifos significantly inhibited the CYP3A4 metabolism of 17β-estradiol in a concentration- and time-dependent manner [32]. This finding is in contrast with our finding that chlorpyrifos showed no time dependent inhibition when it was preincubated with CYP3A4 using midazolam as a substrate. These results are in agreement with Nomeir et al. that the response of human liver microsomal CYP3A4 to certain inhibitors can be affected by the substrate used [33]. Chlorpyrifos also showed time dependent inhibition of CYP2A6 and CYP2C19.

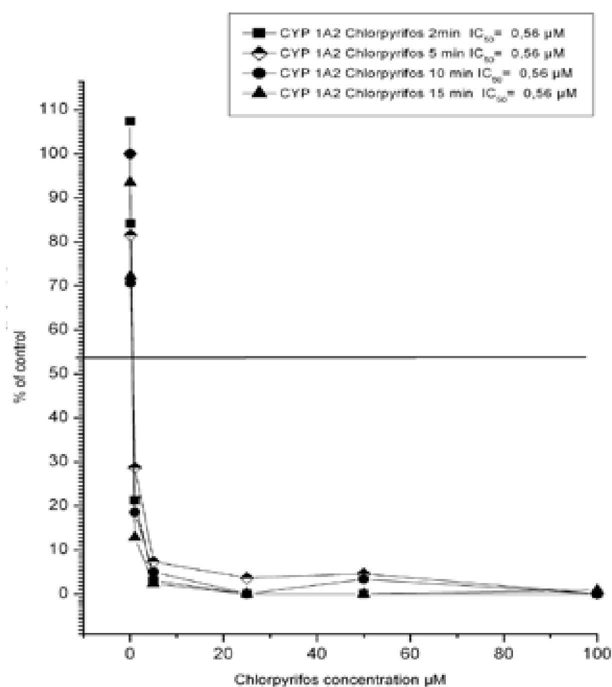

Figure 3. Effect of Chlorpyrifos on CYP 1A2 using pooled human liver microsomes

It is worth noting that IC50 values for 2 and 15 minutes preincubations of other organophosphorous pesticides tested to inhibit CYP1A1/2 (profenofos, phenthoate and malathion) were roughly the same (figure 4 and 5). In the same manner, other organophosphorous pesticides tested to inhibit CYP2A6 rather than fenitrothion and chlorpyrifos showed decrease in the IC50 values when they were preincubated for 15 minutes indicating time

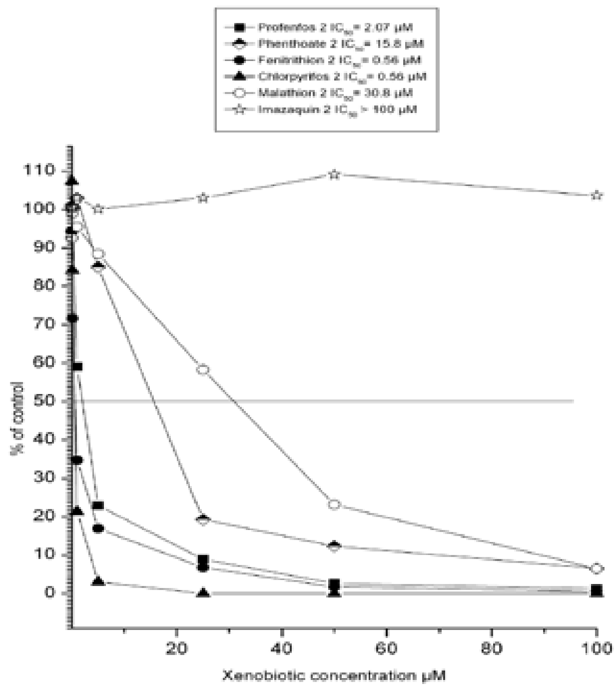

Figure 4. Effect of different xenobiotics on CYP 1A2 after 2 minutes preincubation using pooled human liver microsomes

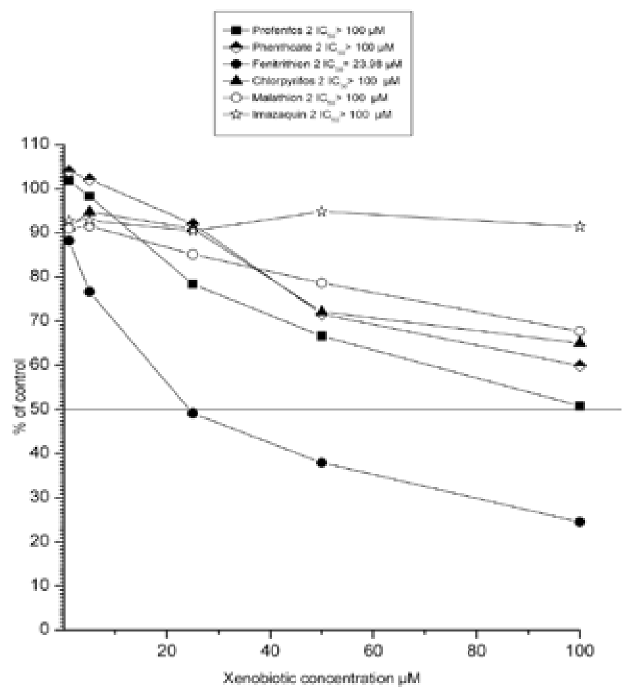

Figure 6. Effect of different xenobiotics on CYP 2A6 after 2 minutes preincubation using pooled human liver microsomes

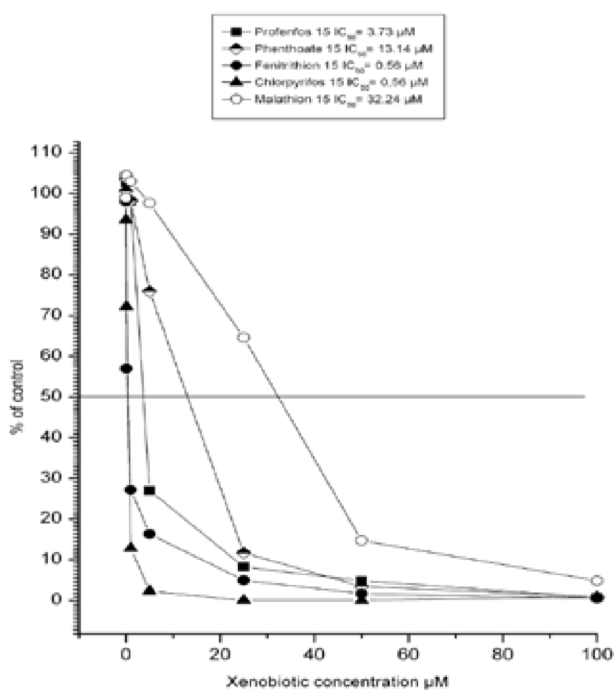

Figure 5. Effect of different xenobiotics on CYP 1A2 after 15 minutes preincubation using pooled human liver microsomes

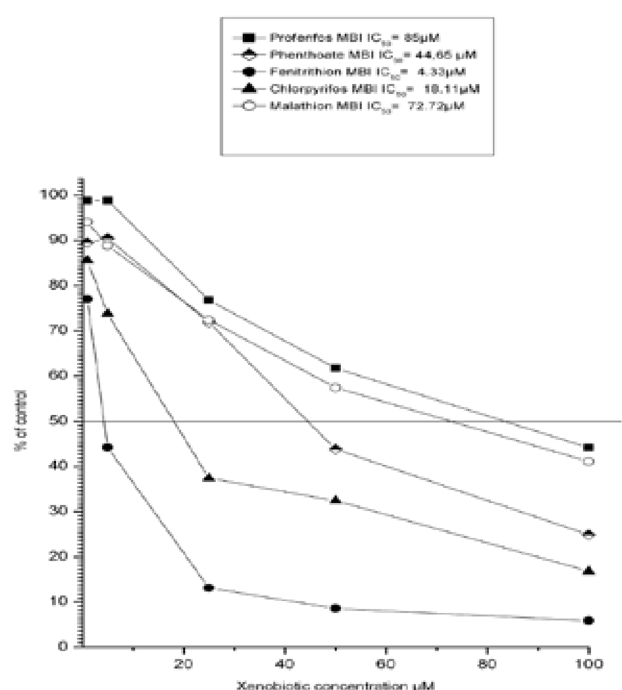

Figure 7. Effect of different xenobiotics on CYP 2A6 after 15 minutes preincubation using pooled human liver microsomes

dependent inhibition (figures 6 and 7). The same observation was recorded when chlorpyrifos, fenitrothion and phenthoate were preincubated with CYP2C19 for 2 and 15 minutes indicating cases of time dependent inhibition.

In contrast, all organophosphorous pesticides tested to inhibit CYP2C8 (chlorpyrifos, fenitrothion, malathion, phenthoate and profenofos) and CYP3A4 (chlorpyrifos, fenitrothion and phenthoate) showed increase in the 15 minutes preincubation IC50 values. The increase in IC50 value can be attributed to a rapid metabolism of the inhibitor into non-interacting metabolites and consequently less inhibition of the enzyme.

Fenitrothion and malathion showed increase in the 15 minutes preincubation IC50 when they were tested with CYP2C9 while phenthoate showed slight decrease in the 15 minutes preincubation IC50 value.

Chlorpyrifos and phenthoate showed increase in the 15 minutes preincubation IC50 value when they were tested to inhibit CYP2D6 while profenofos showed decrease in the IC50 (72.7 to 19.5 μM for 2 and 15 minutes respectively).

Pyrethroid insecticides e.g., deltamethrin, fenvalerate and lambda-cyhalothrin potently inhibited CYP2D6 (IC50 □3 μM). CYP3A4 activity was moderately inhibited by deltamethrin and fenvalerate and potently by lambda-cyhalothrin, suggesting that these pesticides might be metabolized by CYP2D6 and/or CYP3A4. These results were in agreement with the findings of Godin et al. [34] (2007) that two pyrethroid insecticides, deltamethrin and fenvalerate, were metabolized partially by human CYP3A4. In the current study, the 15 minutes preincubation IC50 values were many times higher than that for 2 minutes preincubation. This increase in IC50 may be explained by the efficient metabolism into non-interacting metabolites.

Pesticides with such variable chemical structures as OPs, benzoylurea, and glyphosate acid were found to be potent inhibitors of CYP2C9. CYP2C9 activity was potently inhibited by malathion, fenitrothion, chlorfluazuron, hexaflumuron, and glyphosate with IC50 values of 2.5 μM, 4.8 μM, 7.5 μM, 6.0 μM, and 3.7 μM, respectively. When these pesticides were tested for mechanism based inhibition towards CYP2C9, the IC50 values were higher, 15.43 μM for fenitrothion and more than 100 μM for the others. Atrazine, a triazine herbicide, inhibits CYP2C8 and CYP3A4 (IC50 values 31.3 and 2.8 μM, respectively). 15 minutes preincubation of atrazine with these 2 CYP enzymes resulted in IC50 values more than 100 μM.

Carbendazim showed time dependent inhibition when it was preincubated with CYP2D6 for 15 minutes (IC50 value decreased from 12 to 2.6 μM).

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