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Original Research

Toxicokinetic Interactions of α-Cypermethrin and Chlorpyrifos in Rats

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Abstract

Preparations containing both organophosphates and pyrethroids are commonly used in insect control. Toxicokinetic interactions between α-Cypermethrin (CM) and Chlorpyrifos (CPF) were studied in rats. The animals were given a solution of CM or CPF in rapeseed oil at a dose of 10 mg/kg and a mixture of CM and CPF at a dose of 5 mg/kg each by an intragastric tube once a day for 28 days. The concentrations of unchanged CM and CPF were determined in blood, liver and brain by GC-ECD. Also, the concentrations of CM and CPF were individually monitored in blood after administration of their single doses to calculate toxicokinetic parameters ($T_{max.}$, $C_{max.}$, AUC). In urine the main metabolites 3-(4'-hydroxyphenoxy)benzoic acid (4OH3PBA) and 3,5,6-trichloro-2-pyridinol (TCP) were determined by HPLC in the rats treated daily with CM, CPF or their mixture.

In the animals dosed with a single insecticide, the highest concentration of CM was found in blood and of CPF in liver. In the co-exposed rats, CPF decreased in all the tissues, while CM increased particularly in liver. The excretion of 4OH3PBA following CM administration increased significantly during the exposure period, whereas in the CPF-exposed rats, TCP was excreted at the same rate. Following the co-administration of both insecticides 4OH3PBA excretion decreased, but did not influence TCP excretion. In the co-exposed animals, $C_{max.}$ and AUC increased for CM, and decreased for CPF.

Keywords: Chlorpyrifos; α-Cypermethrin; 3,5,6-trichloro-2-pyridinol; 3-(4'-hydroxyphenoxy)benzoic acid; Interaction in toxicokinetic phase

Introduction

Organophosphates (OPs) and pyrethroids (PYRs) belong to the most often used groups of insecticides. These compounds are characterized by high insecticidal potency. Acute exposure can cause serious adverse effects in humans and other mammals due mainly to neurotoxic action, which in the case of OPs is linked with the inhibition of esterases and as a consequence, pathological retention of acetylcholine in the synaptic gaps [1-3]. The mechanism of PYR toxicity is mainly associated with the prolonged opening time of the sodium ion channels. The effects of toxicity therefore include choreoathetosis, hyperexcitability and salivation – signs specific to synthetic pyrethroids containing the α– cyano group in the molecule, e.g. cypermethrin (CM) and deltamethrin [4, 5].

OPs and PYRs are rapidly metabolized in mammals, the result of their chemical structure containing ester bonds easily hydrolyzed by animal esterases: phosphoric triester hydrolases, paraoxonase and carboxylesterases [1, 4, 6, 7]. OP, like chlorpyrifos-ethyl (CPF), is bioactivated mainly in the liver to its oxon by CYP450-dependent oxidative desulfuration, the product of this reaction is about a thousand times more potent an AChE inhibitor than the parent compound [8, 9]. Oxon in mammals is hydrolyzed by paraoxonase (PON) – an enzyme associated with high-

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density lipoproteins, to non-active metabolites excreted with urine after phase II metabolism [7, 10-13]. A schematic diagram of the most significant metabolic pathways for CPF is presented in Figure 1. Recently Bicker et al. [6] described some novel aspects of CPF metabolism in which 15 metabolites were identified. Out of many metabolites of CPF, only TCP (3,5,6-trichloro-2-pyridinol) is specific enough to be used as a biomarker of exposure [6, 7, 11, 14, 15].

CPF is the most studied OP compound, primarily because of its large usage in crop protection and also in wide nonagricultural aspects: veterinary medicine, households. Its metabolite – TCP – is the most frequently found pesticide degradation product in urine of the general population – suggesting that CPF is introduced to humans, not only with food but also from other sources [14].

Similarly, CM is one of the most commonly used synthetic pyrethroids nowadays, not only in crop protection but also in nonagricultural areas. In contrast to cypermethrin (mixture of *cis*:*trans* isomers), α-cypermethrin is a racemate comprising (S)-α-cyano-3-phenoxybenzyl-(1R)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate and (R)-α-cyano-3-phenoxybenzyl-(1S)-*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate. *Cis*- isomers of pyrethroids are significantly more potent than *trans*- isomers, which reflects the difference in DL50 value: cypermethrin (62.8: 37.2, *trans*: *cis*) – 1800 mg/kg vs. α-cypermethrin – 145 mg/kg [16]. In this work, the abbreviation CM refers to α – cypermethrin.

Concurrent exposure to CPF and CM leads to the inhibition of esterases responsible for hydrolysis of CM and consequently to slowing down its metabolism. Such interaction enables the use of smaller doses of CM for pest control. The blockage of serine esterases by OPs can inhibit the hydrolysis of other xenobiotics, like some drugs (e.g. aspirin) [1, 17-19]. In addition, lipases sensitive to OPs, which play a critical role in cell regulation, nutrition and disease, are the action sites for these inhibitors [20].

The effects of single compounds are well known [2-5, 15, 21-23]. Recently attention has been drawn not only to the effects of particularly toxic xenobiotics, but also to their interactions with one another [17, 24, 25]. Since co-exposure to two or more xenobiotics can result in antagonistic or synergistic effects, studies of these interactions represent one of the most important areas of recent pesticide toxicology.

The interaction between organosphosphates and pyrethroids is relatively well known, since formulations containing both classes of insecticides are available on the market. The nonreversible inhibition of esterases by OP leads to slowing down in the activity of enzymes responsible for cleavage of the ester bond in the PYR molecule [17, 19]. In normal conditions the ester bond is quickly metabolized in mammals, the products of hydrolysis are non-active and rapidly excreted from the body mainly through urine. Blocking the hydrolysis of PYR significantly reduces the metabolism of these pesticides and

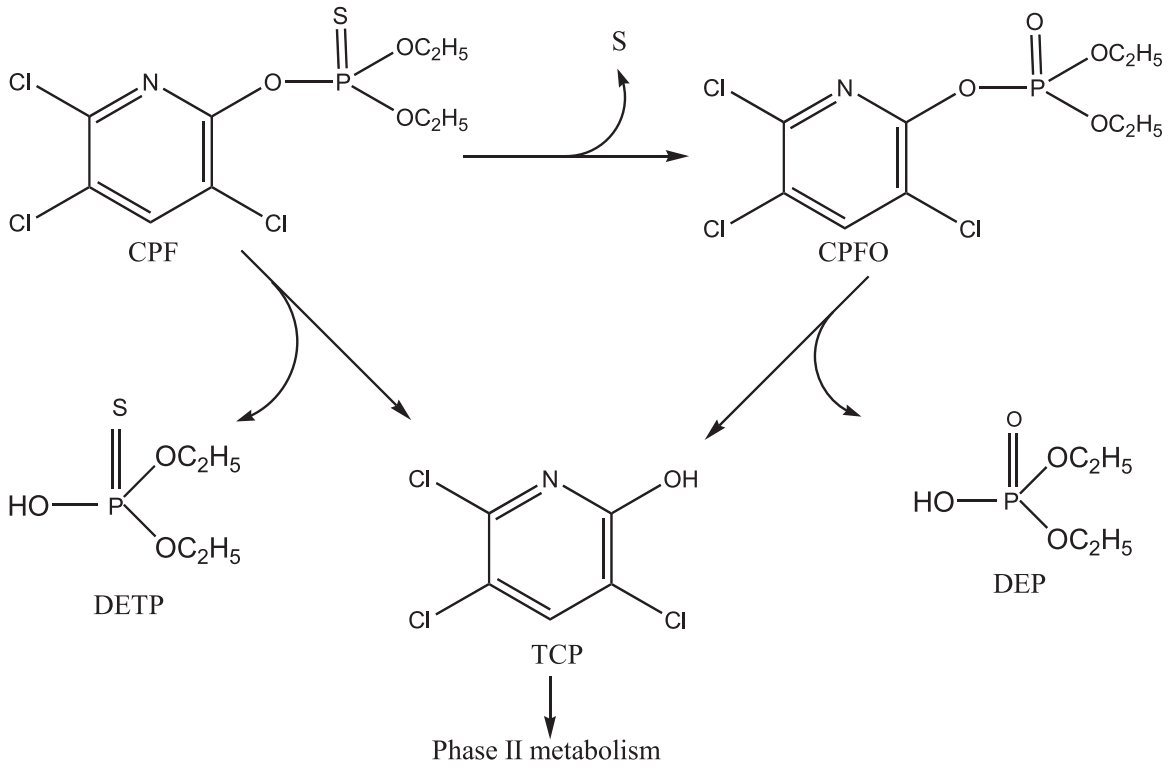

Fig. 1. Metabolic pathway of CPF. CPFO – Chlorpyrifos-oxon; DEP – Diethylphosphate; DETP – Diethylthiophosphate; TCP – 3,5,6-trichloro-2-pyridinol.

for that reason, a stronger insecticidal effect of mixture OP/PYR is observed. From an economical and ecological point of view, such a combination enables reduction in the amount of PYR, keeping insecticidal efficiency at the same level.

The aim of that study was to investigate the effect of exposure to CM or CPF individually or in combination for 28 days on their individual metabolism in male Wistar rats.

Experimental Procedures

Chemicals

Standards for chromatography: α-cypermethrin (99.1%), chlorpyrifos-ethyl (99.3%), chlorpyrifos-methyl (99.6%) were obtained from the Institute of Organic Industry (Warsaw, Poland). Deltamethrin (>99%), 4-hydroxy-3-phenoxybenzoic acid were from Roussell-Uclaf (Paris, France), 3,5,6-trichloro-2-pyridinol (99.0%) from Supelco (Bellefonte, USA). Technical grade α-cypermethrin and chlorpyrifos were from the Institute of Organic Industry (Warsaw, Poland). n-Hexane, acetone, diethyl ether, all for residue analysis were from J.T.Baker (Holland). Florisil 60-100 mesh was obtained from Fluka AG (Switzerland). For SPE procedure, Bakerbond 12-G system was employed with glass columns self-filled in the laboratory with 300 mg of Bakerbond C_{18} PolarPlus filling from J.T.Baker (Deventer, Holland). Acetonitrile HPLC gradient grade was purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland). All other chemicals used in this experiment were analytical grade and were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Animals and Experimental Design

Male Wistar rats weighing approximately 300 g were kept in standard conditions with free access to tap water and rat chow (Labofeed B, Wytwórnia Pasz, mgr inż. A. Morawski, Kcynia, Poland) unless otherwise stated. All the procedures were approved by the Local Bioethics Committee of the Medical University of Gdańsk. After 2 weeks of acclimatization, the animals were randomly allocated to three groups of twelve rats each:

1. CM – Animals receiving 10 mg CM/kg in rapeseed oil,
2. CPF – Animals receiving 10 mg CPF/kg in rapeseed oil,
3. CMCPF – Animals receiving both 5 mg CM and 5 mg CPF/kg in rapeseed oil.

The rats were given daily the oil solution of pesticides with an intragastric tube, seven days a week, for 28 days. The volume of the dosed oil solution of pesticide was 0.5 ml. The animals were observed daily for overt signs of toxicity. No mortality was observed during the study. 24 hrs before sacrifice, the animals were placed individually in metabolic cages for 24 hours of urine collection. Rats were deprived of food but had free access to tap water. On the next day they were dosed with pesticides for the last time and placed in the housing cages. 4 hours later the animals were anesthetized with ethyl ether and exsanguinated by cardiac puncture. Their blood was collected in heparinized tubes and stored on ice before analysis. Brain and liver were dissected, washed with ice-cold saline to remove traces of blood and then frozen at -20°C until unchanged insecticides were determined. All analyses of blood samples were conducted within a few hours after sampling.

Toxicokinetics of CM and CPF

3 animals from each experimental group were given a single dose of CM, CPF or both compounds and placed individually in metabolic cages for 24 hours to collect urine and whole blood at 0.5, 1, 2, 4, 6, 8, 12 and 24 hours after dosing. Blood was collected from the tail vein and analyzed by GC-ECD for CM and CPF.

Blood Analysis

0.2 ml of whole blood (or 0.1 ml in kinetics study) were placed in Eppendorf tubes and 0.6 ml (or 0.3 ml) of n-hexane were added. The samples were extracted by shaking for 10 minutes in a laboratory shaker followed by centrifugation at 13,000 rpm. 2 µl of the hexane layer were analyzed by GC-ECD. Blood from untreated rats served as a blank matrix to prepare the calibration curve. The appropriate volumes of n-hexane solutions of standards were placed in the Eppendorf tubes and gently evaporated in a stream of air at room temperature. 1 ml of blood was then pipetted to the tubes and shaken for 30 minutes. The aliquots of spiked blood were processed as unknowns and the calibration curve was based on the peak area of the analytes.

Tissue Analysis

The samples of 1 g liver or 250 mg brain were spiked with internal standards (chlorpyrifos-methyl for CPF and deltamethrin for CM) and then homogenized with 10 ml of acetone:n-hexane mixture (1:1, v/v) in the presence of anhydrous sodium sulphate. The resulting supernatant was washed twice with water (3 ml) to remove acetone and water soluble interferences. The remaining hexane extracts were concentrated to a volume less than 1 ml and subjected to florisil cleanup. Florisil was heated overnight at 140°C, then cooled to room temperature and deactivated by adding 2% (v/w) of water. After 2 hour-equilibration, florisil was used to fill the glass microcolumns (φ 3mm). At the bottom and top of the column, a layer of sodium sulphate was placed with a layer of 1 g of florisil between them. 5 ml of n-hexane was used to wet the column, and then the concentrated extract was

transferred to the top of the column, and the pesticides were eluted with 18 ml of diethyl ether:n-hexane mixture (1:9, v/v). The eluate was concentrated under a stream of air at room temperature and 2 µl of the final extract was analyzed by GC-ECD.

GC-ECD Conditions

The analyses were performed using a Varian 3400 gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD). The chromatographic separation was achieved on a medium polarity 25 m x 0.530 mm CP-Sil 13 CB capillary column (14% phenyl, 86% dimethylpolysiloxane), 1 µm film thickness (Varian Inc., Walnut Creek, CA, USA). High purity nitrogen was used as a carrier and make-up gas with flow rates: 7 and 23 ml min^{-1}. The on-column injector was operated at 270°C and the detector at 300°C. The temperature program for the column oven was as follows: 140°C to 270°C at 25°C min^{-1} and then ramped at 30°C to 300°C with 8 minutes hold.

A Galaxie Workstation v. 1.9.3.2 was used for data acquisition and chromatograms management. A manual injection was made using a 10 µl Hamilton 701 microsyringe with 26s needle and a bevel tip – (Style 2). The calculations were based on internal standardization.

The method was revalidated. Six replications of the samples at two concentration levels were done to assess the precision of the method. For CPF in blood and tissues, the precision was 3.6 and 2.8%, while for CM 5.1 and 3.3% respectively.

Urine Analysis

A modification of the method of Abu-Quare et al. was used in this experiment [26].

Thawed 24-hour urine samples were mixed, pipetted to glass vials and concentrated hydrochloric acid was added (0.2 ml HCl/1.0 ml urine) to each vial. The vials were sealed and placed in a laboratory dryer at 95°C for 90 minutes to complete hydrolysis. The samples were cooled and diluted 1:1 with water before solid phase extraction. The glass columns for SPE were filled with 300 mg of C_{18} PolarPlus Bakerbond, then conditioned with 4 ml of acetonitrile, followed by 4 ml of water (pH 3 with phosphoric acid). 3 ml of the diluted sample were loaded into a preconditioned column. The column was washed by passing 5 ml of 20% acetonitrile in acidified water (pH 3 with phosphoric acid). Afterwards a strong vacuum was applied to dry out the SPE filling. Two portions of 1 ml acetonitrile were used to elute the adsorbed analytes. In all cases, the eluates were diluted to 10 ml before HPLC analysis and 20 µl were injected into the HPLC system. The calibration curves were constructed by analyzing blank urine samples spiked with known amounts of 4OHPBA and TCP.

A P580 gradient pump, a UVD 340 S diode array detector, a UCI-100 universal chromatography interface and a STH 585 column oven, all from Dionex were used in the experiment. A Chromeleon Data Management System version 6.20 was used to control HPLC conditions, data acquisition and chromatograms integration. The separations were performed at 40°C. A TSKgel SuperODS column (10 cm, 4.6 mm ID, particle size 2 µm) from Toso-Haas (Montgomeryville, USA) with a mobile phase flow rate of 1.2 ml min^{-1} was employed. The mobile phase consisted of: A – water acidified with phosphoric acid to pH 3, B – acetonitrile. The compounds were separated using a fast gradient: 0-1 min. – 25% B, 1-8 min. – 25-45% B, 8-10 min. – 45% B, and 10-12 min. – 25% B. The method was revalidated, for both analytes good linearity was observed ($r>0.999$) in the calibration range, and the precision was below 9% in both cases.

Statistical Analysis

The data were analyzed by the analysis of variance (ANOVA) followed by a Student t-test. The value of $p<0.005$ was considered significant.

Results

Concentrations of unchanged CM and CPF in blood, liver and brain are given in Table 1. The highest concentration of CM when administered alone was found in blood, the lowest in brain. In the investigated tissues CM concentration increased in an exposure-time dependent manner, but the increase in blood was not significant. CPF when dosed as a single compound was found in the highest concentration in liver and the lowest in blood. In blood and liver, CPF concentration did not increase significantly during the period of exposure. In the rats co-exposed to CM and CPF, the concentration of CM in blood decreased, but in liver and brain increased in an exposure time-dependent manner. The concentration of CPF in blood, liver and brain did not change significantly.

In the rats given CM and CPF, the concentration of CM increased significantly in the liver, but decreased in the brain when compared to the animals treated only with CM.

In the animals co-exposed to CM and CPF, the concentration of CPF decreased in all investigated tissues when compared to the rats exposed only to CPF, the decrease was not significant in blood and brain after 28 days of exposure.

The excretion of CM and CPF metabolites in urine is shown in Table 2.

The excretion of 4OH3PBA following the CM administration almost doubled between the 13th and 27th day of exposure. The calculated recovery of the parent compound based on the metabolite excretion increased from 46.5 to 88%. The co-administration of CPF decreased to about 30% excretion of 4OH3PBA when compared to dosing with CM only.

Table 1. Concentration of CM and CPF in blood and tissues.

Group		Blood (ng/ml)		Liver (ng/g)		Brain (ng/g)	
		CM	CPF	CM	CPF	CM	CPF
CM	14 days	168.2±84.4	-	144.5±73.0	-	109.1±37.2	-
	28 days	287.3±142.6	-	273.0±99.7	-	198.0±64.5	-
CPF	14 days	-	68.9±12.5	-	1053.6±736.0	-	345.7±275.2
	28 days	-	59.8±60.5	-	1044.8±932.7	-	272.6±277.9
CM/CPF	14 days	250.2±92.3	18.2±7.5	342.8±143.6	250.7±48.1	53.2±15.1	78.5±30.7
	28 days	154.9±31.4	14.4±7.6	631.9±283.3	195.8±110.3	128.3±29.2	75.3±69.0

Values are presented as means ± S.D. of 6 determinations.

Table 2. Excretion of 4OH3PBA and TCP in urine of animals exposed to CM and CPF alone or in combination.

Group	Doses	Urine samples (hrs)	Excretion (concentration) and (total amount)				Dose of parent compound recovered in urine (%)	
			4OH3PBA		TCP		CM	CPF
			(μg/ml)	(μg)	(μg/ml)	(μg)		
CM	1	0-12	31.94	187.22	-	-	24.39	-
		12-24	22.29	233.34	-	-	30.40	-
	13	0-24	28.09	416.57	-	-	46.52	-
	27	0-24	53.14	788.06	-	-	88.01	-
CPF	1	0-12	-	-	113.44	499.15	-	29.34
		12-24	-	-	59.88	467.09	-	27.46
	13	0-24	-	-	70.20	1041.07	-	52.45
	27	0-24	-	-	81.40	1207.16	-	60.82
CMCPF	1	0-12	2.20	9.85	36.17	161.70	1.28	19.00
		12-24	4.06	46.67	32.44	373.01	6.08	43.85
	13	0-24	5.00	74.15	27.60	409.31	8.28	41.25
	27	0-24	7.35	109.00	41.70	618.41	12.17	62.32

Values are presented as means of 3 determinations.
Abbreviations: CM – α – cypermethrin; CPF – chlorpyrifos; 4OH3PBA – 3-(4'-hydroxyphenoxy)benzoic acid; TCP – 3.5.6-trichloro-2-pyridinol

Table 3. Kinetic parameters in blood for CM, CPF and their mixture.

Kinetic parameter	CM 10 mg/kg	CPF 10 mg/kg	CMCPF 5 mg/kg each	
			CM	*CPF*
$T_{max.}$ (h)	3	4	6	6
$C_{max.}$ (ng ml^{-1})	123.9±8.92	164.9±9.34	347.1±43.3	17.3±3.3
AUC_{total} (h ng ml^{-1})	671.9±42.1	1164.1±84.7	1553.5±184.2	163.6±26.9

Values are presented as means ± S.D.

CPF when administered alone is excreted as TCP during the whole exposure period (about 57% of the daily administered dose of the parent compound). The co-administration with CM did not influence TCP excretion. No unchanged insecticides CM and CPF were detected in the investigated urine samples.

The toxicokinetic parameters were based on the data obtained from whole blood pesticide concentration following the single oral dose of chemicals using the noncompartmental analysis. The basic parameters, including $C_{max.}$, $T_{max.}$, and AUC, are presented in Table 3. When single compounds were administered, maximal concentrations were attained after 3 and 4 hours for CM and CPF, respectively. The co-administration of both insecticides resulted in an increase in $T_{max.}$ for CM and CPF to 6 hours. The simultaneous administration of halved doses of CM and CPF resulted in a 10-fold decrease in $C_{max.}$ and 7-fold decrease in AUC values for CPF, whereas a significant increase in both $C_{max.}$ and AUC values for CM was found when compared to the data obtained when single compounds were dosed.

Discussion

The results of this study indicate that unchanged CM and CPF were found in all investigated tissues and their concentration increased in an exposure time-dependent manner, except for CPF in brain.

On account of lipophilic properties of CPF and even more of CM they are preferably deposited in tissues rich in lipids like the adipose tissue, brain, and liver, and easily absorbed in the gastrointestinal tract from oil solutions [15, 22]. In the studies performed with radiolabelled insecticides traces of CM and CPF in the adipose tissue were detected even a long time after the end of exposure [27]. However, chronic animal feeding studies have revealed that there is little storage or accumulation of these agents in the body burden and, perhaps, an efficient detoxification of these chemicals exists [15, 23, 24]. The co-exposure to CM and CPF resulted in an exposure-time dependent increase in CM in liver and brain, whereas the concentration of CPF did not change during the exposure period. In the co-exposed animals, the concentration of CM in liver significantly increased and in brain it roughly corresponded to

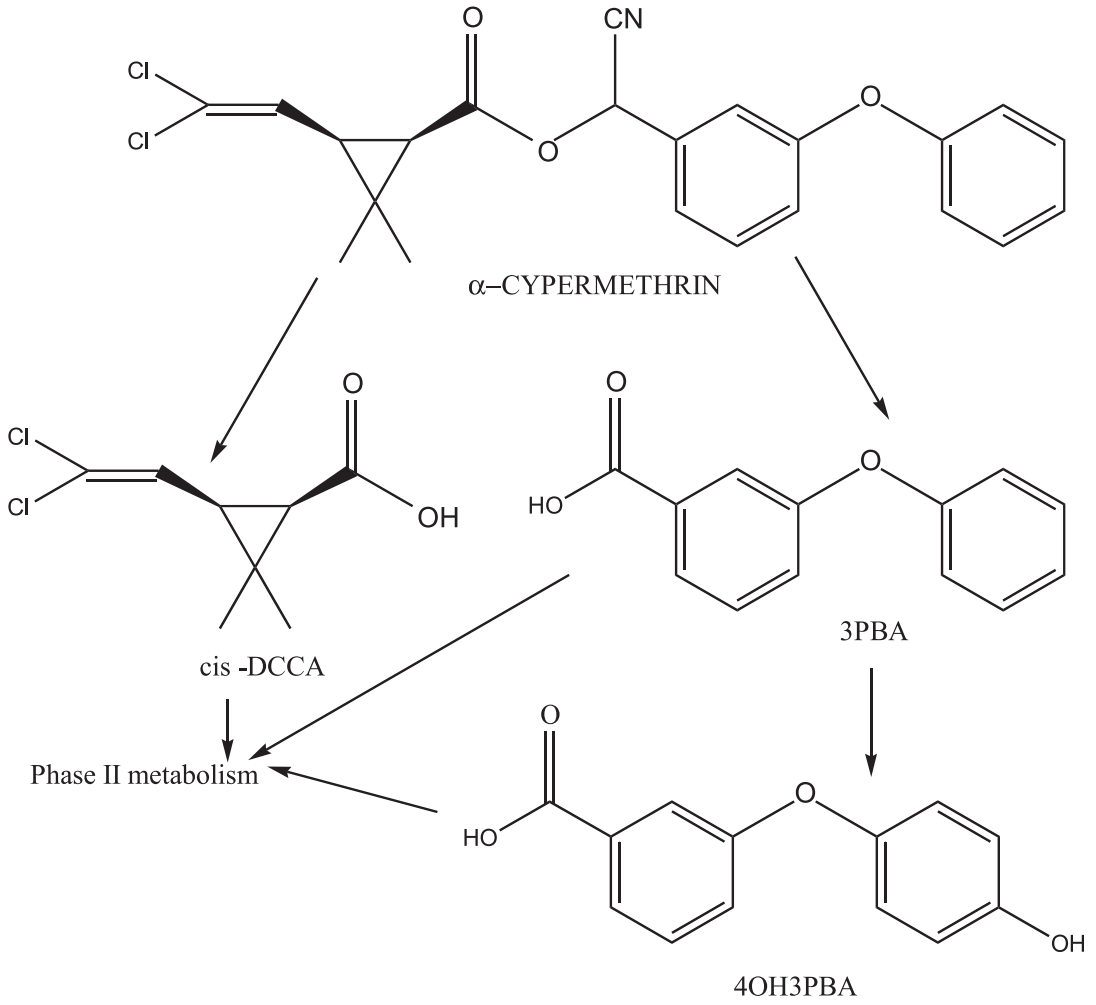

Fig. 2. Main metabolic pathways of α-cypermethrin in mammals. *cis*-DCCA – *cis*-3-(2,2-dichlorethenyl)-2,2-dimethylcyclopropanecarboxylic acid; 3PBA – 3-phenoxybenzoic acid; 4OH3PBA – 3-(4'-hydroxyphenoxy)benzoic acid.

the lower dose, when compared to the rats given only CM. The CPF concentration in the co-exposed rats very distinctly decreased in all investigated tissues when compared to the animals treated only with CPF. Both insecticides are easily metabolized in rats (Figs. 1 and 2). CM, as for all pyrethroid esters, is susceptible to degradation by hydrolytic enzymes; possible nonspecific carboxylesterases associated with the microsomal fraction of tissue homogenates in various species were found [1]. The hydrolytic products of CM are DCCA and 3-phenoxybenzoic acid (3PBA). 3PBA undergoes hydroxylation of the phenoxybenzoic moiety under cytochrome P-450 enzymes to 4OH3PBA, which is the main metabolite of CM in the rat (Fig. 2). 4OH3PBA in rats is further conjugated mainly with sulfuric acid and excreted with urine. In other species, 4OH3PBA is conjugated with glucuronic acid. To a much lesser degree, it is excreted in feces [28].

Chlorpyrifos firstly undergoes CYP 450-dependent oxidative desulfuration to chlorpyrifos-oxon, which is an active anticholinesterase compound and then is hydrolyzed by A-esterase, the paraoxonase (PON) to the inactive metabolite TCP (3,5,6-trichloro-2-pyridinol), which is the main metabolite of CPF, serving as a biomarker of exposure, preferentially excreted with urine [6, 9, 10, 13]. A schematic diagram of the most significant metabolic pathways of CPF is presented in Fig. 1. In our study, the excretion of 4OH3PBA when CM was administered alone increased in an exposure time-dependent manner. By the co-administration of CPF the excretion of the metabolite decreased significantly. This is most probably caused by organophosphate esters' (CPF) capability of inhibiting esterases tissues (B-carboxylesterases), which block CM biotranformation and hereby potentiate pyrethroid ester toxicity in binary combinations of insecticides. The findings are consistent with the significant increase in both $C_{max.}$ and AUC values and CM concentration in liver for CM administered with CPF, when compared to the data obtained when only CM was dosed. CPF is excreted at the same rate during the whole exposure period. In addition, co-administration of CM did not influence TCP excretion. The decrease in CPF tissue concentrations, in $C_{max.}$ and AUC values may be caused by increased biotransformation of CPF to metabolites other than TCP.

The results obtained in this study indicate that co-exposure to CM and CPF inhibits the hydrolysis of CM, which in turn causes an increase in CM content in the tissues and a decrease in 4OH3PBA excretion in urine.

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