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In vitro metabolism of the new insecticide flupyrazofos by rat liver microsomes

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1. The *in vitro* metabolism of the new insecticide flupyrazofos was studied using rat liver microsomes. Two metabolites were produced and identified as *O*,*O*-diethyl *O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl) phosphoric acid ester (flupyrazofos oxon) and 1-phenyl-3-trifluoromethyl-5-hydroxypyrazole (PTMHP) based on UV and mass spectral analysis.

2. Cytochrome P450 oxidatively converted flupyrazofos to flupyrazofos oxon, a major metabolite and phenobarbital-induced microsomes increased this desulphuration by 8-fold.

3. Flupyrazofos oxon was converted to PTMHP with a half-life of 47·8 min by chemical hydrolysis and this conversion also proceeded non-enzymatically under our microsomal incubation conditions.

Introduction

Flupyrazofos [*O*,*O*-diethyl *O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl) thiophosphoric acid ester] (figure 1) was developed by the Korea Research Institute of Chemical Technology (KRICT, Taejeon, Korea) as a new insecticide in 1987. This organophosphorothionate was found to be very effective against the diamond-back moth (*Plutella xylostella*) acting as a contact and stomach poison inhibiting acetylcholinesterase as other organophosphorous pesticides do. No phototoxicity was observed and a low acute toxicity (rat, oral) of 372–605 mg/kg was reported (SungBo Chemical's report, unpublished data). In an aquatic toxicity test, the EC_{50} for carp in 48 h was 0·9 ppm, and mutagenicity, teratogenicity and delayed neurotoxicity were not observed (unpublished data).

Good thermostability (Cho and Han 1992) and photostability (Cho *et al*. 1993) were reported with small amounts of degradation products including *O*,*O*-diethyl *O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl) phosphoric acid ester (flupyrazofos oxon), *O*,*S*-diethyl *O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl) phosphorothiolate and 1-phenyl-3-trifluoro-methyl-5-hydroxypyrazole (PTMHP).

The purpose of the present study is to understand *in vitro* metabolism of flupyrazofos using rat liver microsomes and to characterize the major cytochrome P450 isozymes involved in flupyrazofos metabolism.

Materials and methods

Chemicals

Flupyrazofos (99%), flupyrazofos oxon (99%) and PTMHP (99%) were kindly donated from SungBo Chemicals Co. (Ansan, Korea). ^{14}C-flupyrazofos (specific activity 28 mCi/mmol, > 99%) was synthesized by GSF (Germany) in collaboration with KRICT. $NADP^+$, glucose 6-phosphate, glucose 6-

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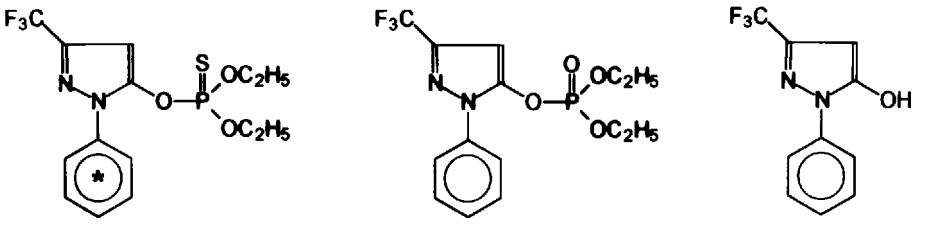

Figure 1. Structures of flupyrazofos and its metabolites. *Site of the ^{14}C label.

phosphate dehydrogenase and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Phenobarbital sodium (PB) were obtained from Daehan Pharmaceutical Co (Seoul, Korea) and the protein assay kit was from Bio-Rad Lab. (Richmond, CA, USA). Scintillation cocktail (Ready Flow II) for flowthrough radioisotope detector was obtained from Beckman (USA). All other chemicals were the highest grade commercially available.

Instruments

Hplc analysis was conducted with P4000 (Thermo Separation Production, Fremont, CA, USA) equipped with a Focus scanning detector (TSP) using an Inertsil ODS-2 column (0·46 × 15 cm, 5 μm, GL Science, Tokyo, Japan). A β-RAM radioactivity flow-through detector (IN/US Corp., FL, USA) was used for the determination of radioactivity. The chromatograms were obtained from an IBM-compatible computer using PC 1000 software package (TSP), or in part, Winflow software package (IN/US Corp.).

Mass spectra were recorded on GC/MS (Finnigan MAT GCQ) with electron impact mode using an MS-Select column (0·25 mm i.d. × 30 m).

Preparation of liver microsomes

Specific pathogen-free male Sprague-Dawley rats (160–180 g) were pretreated with either PB (80 mg/kg, i.p. for 3 days) in saline, ethanol (5 ml/kg, p.o. for 3 days) or TCDD (10 μg/kg, i.p. 1 day) in corn oil to enrich specific types of cytochrome P450 isozymes. The rats were sacrificed at 48 h after the last injection by cervical dislocation before the livers were perfused with saline to remove excess blood and homogenized with 4 vol ice-cold 1·15% potassium chloride solution (pH 7·4). The liver homogenates were centrifuged at 9000*g* for 10 min at 4 °C and the resulting post-mitochondrial supernatants were centrifuged again at 105 000*g* for 60 min at 4 °C. The microsomal pellets were resuspended in 50 mM potassium phosphate buffer (pH 7·4) containing 0·1 mM EDTA. Aliquots of liver microsomes were stored at −70 °C until use. The content of microsomal protein was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Incubations and extraction

Control or induced rat liver microsomes (0·1 mg) were preincubated in 1·0 ml 50 mM potassium phosphate buffer (pH 7·4) in the presence of 50 μM flupyrazofos with or without 0·1 μCi ^{14}C-flupyrazofos or 50 μM parathion for 3 min at 37 °C in shaking water bath. The reactions were initiated by the addition of a NADPH-generating system containing 0·8 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase into the reaction mixture (final volume 1·1 ml). The reactions were stopped after a 10-min incubation by the addition of 50 μl 43% phosphoric acid and 2 ml methylene chloride. After mixing and centrifugation, the organic layer was removed and dried under a stream of nitrogen gas. The residue samples were stored at −20 °C until analysis.

Control rat liver microsomes (0·1 mg/ml) were incubated for 10 min with increasing flupyrazofos concentrations ranging between 2 and 100 μM in the presence of the NADPH-generating system and the formation of flupyrazofos oxon and PTMHP were monitored.

The recoveries of flupyrazofos, flupyrazofos oxon and PTMHP in the concentration range of 1–50 nmol/ml were 96·5 ± 4·5, 97·6 ± 3·8 and 94·5 ± 3·9% respectively.

Hydrolysis of flupyrazofos oxon

PTMHP could be produced either by enzymatic hydrolysis of flupyrazofos and flupyrazofos oxon or by non-enzymatic hydrolysis of flupyrazofos oxon. To address the mechanism for the formation of PTMHP, flupyrazofos oxon (10 or 50 μM) was incubated with control rat liver microsomes as described

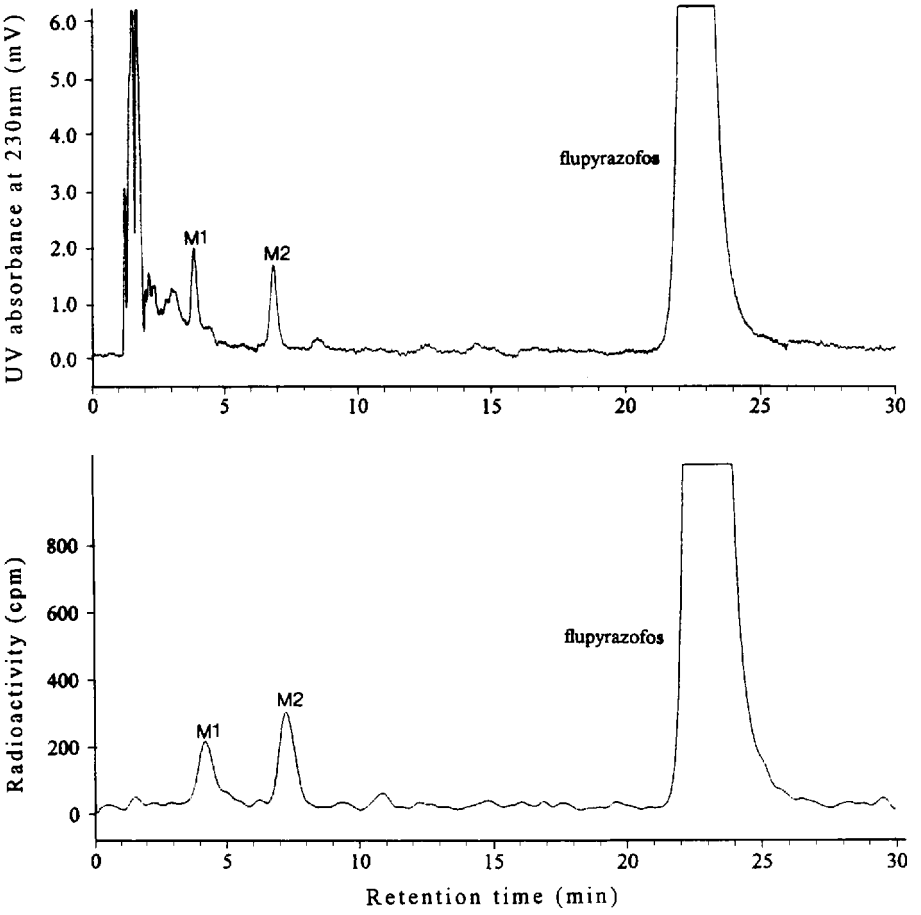

Figure 2. Hplc chromatograms of flupyrazofos and its metabolites obtained from incubations of flupyrazofos with rat liver microsomes in the presence of an NADPH-generating system. Peaks: M1, PTMHP; M2, flupyrazofos oxon.

for flupyrazofos. The hydrolysis of flupyrazofos oxon to PTMHP was measured in 50 mM phosphate buffer (pH 7·4) at 37 °C. Aliquots were removed from the incubations at different time points and the concentrations of flupyrazofos oxon and PTMHP were determined using reversed-phase hplc.

Analysis of metabolites

The samples were dissolved in 100 μl of the mobile phase for the analysis. An aliquot (20 μl) was analysed by hplc using acetonitrile in 10 mM potassium phosphate (pH 3·0) (60 : 40, v/v) as a mobile phase with a flow rate of 1·0 ml/min. The effluent was monitored using a Focus scanning detector at 230 nm and a β-RAM radioactivity flow-through detector with 2 ml/min scintillation cocktail when ^{14}C-flupyrazofos was used.

For the identification of metabolites, the hplc fractions of non-radioactive extraction were evaporated under reduced pressure and the residues analysed by GC/MS.

Results

From the incubation studies of rat liver microsomes with flupyrazofos in the presence of NADPH-generating system, the formation of its two possible metabolites, flupyrazofos oxon and PTMHP (figure 1) were observed and they were well

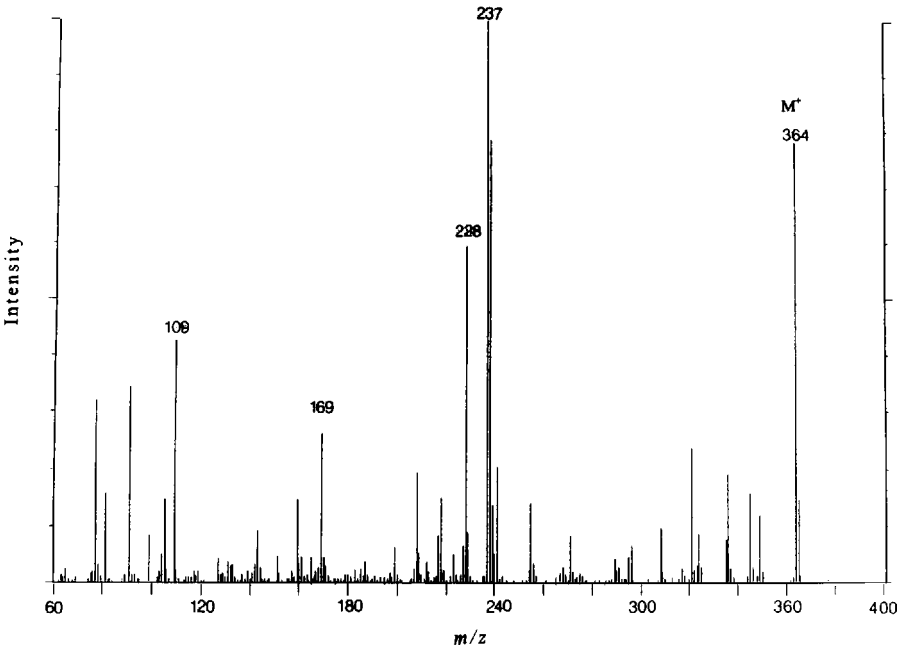

Figure 3. Electron impact mass spectrum of the flupyrazofos oxon metabolite.

separated by reversed-phase hplc (figure 2). These metabolites were identified on the basis of the mass spectral data, UV spectra and hplc elution profile comparing with the authentic compounds (figures 3 and 4).

The apparent kinetic constants for the formation of flupyrazofos oxon (including PTMHP) were 10·1 ± 3·59 μM and 729 ± 73 pmol/mg protein/min for K_m and V_{max} respectively.

In the absence of NADPH-generating system there were no metabolites formed, thus, inferring, but not absolutely proving, the participation of cytochrome P450 and not microsomal esterases.

To investigate the effects of different cytochrome P450 isoforms on flupyrazofos and parathion metabolism, the products formed during the incubation of flupyrazofos or parathion with control, PB-, EtOH- and TCDD-induced rat liver microsomes are shown in table 1. PB-induced microsomes increased the desulphuration of flupyrazofos 8-fold when compared with the control microsomes. EtOH and TCDD-induced microsomes had no significant effect on the desulphuration of flupyrazofos in comparison with the control microsomes. However, the desulphuration of parathion to paraoxon was increased four times by PB- and TCDD-induced microsomes in comparison with the control microsomes.

The hydrolysis kinetics of flupyrazofos oxon were investigated for the possibility of PTMHP formation by chemical hydrolysis. The hydrolysis of flupyrazofos oxon to PTMHP was rapid and approximately followed first-order kinetics with a half-life of 47·8 min at 37 °C and pH 7·4.

To examine the possible enzymatic hydrolysis of flupyrazofos oxon to PTMHP by esterases, flupyrazofos oxon was incubated with control rat liver microsomes in the absence of the presence of an NADPH-generating system. The production of PTMHP from flupyrazofos oxon (10 μM) was the same in both the

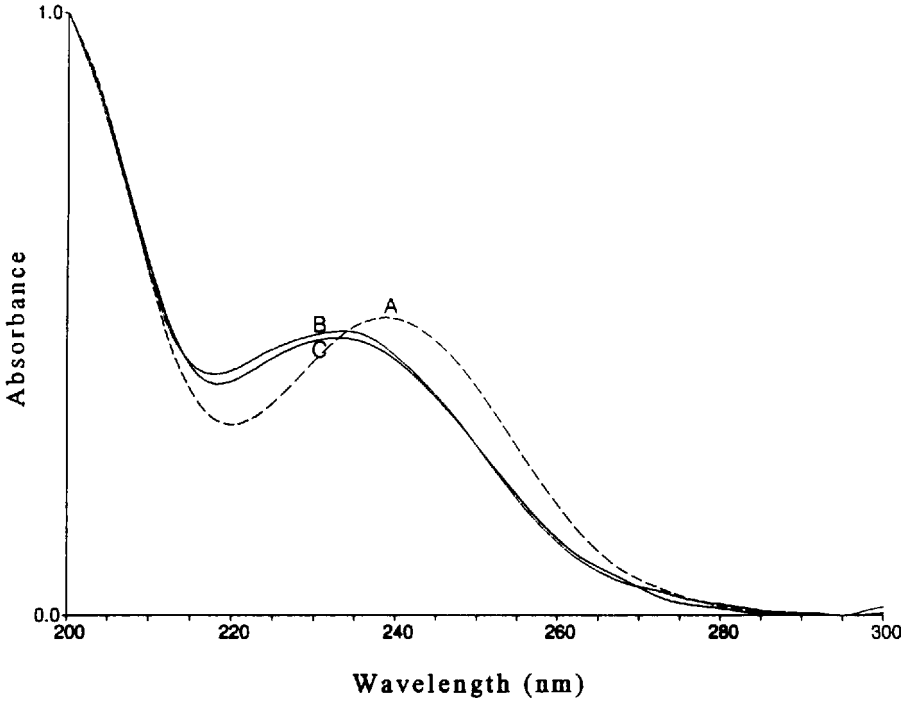

Figure 4. UV spectra of rat liver microsomal flupyrazofos metabolites. (A) PTMHP; (B) M2, flupyrazofos oxon; (C) flupyrazofos.

Table 1. Metabolism of flupyrazofos and parathion by rat liver microsomes. Flupyrazofos (50 μM) or parathion (50 μM) and rat liver microsomes (0·1 mg/ml) were incubated in the presence of NADPH-generating system for 10 min at 37 °C as described in the Materials and methods. Results are expressed as mean ± SD from separate microsomal preparations (n = 4).

	Metabolite production (pmol/mg protein/min)			
Metabolites	Control	EtOH-induced	TCDD-induced	PB-induced
Flupyrazofos				
Flupyrazofos	417 ± 178	390 ± 76	503 ± 145	3198 ± 417**
PTMHP	197 ± 109	206 ± 84	159 ± 58	288 ± 106
Parathion				
Paraoxon	1080 ± 295	1303 ± 219	3811 ± 415**	3750 ± 554**
p-Nitrophenol	355 ± 104	404 ± 142	763 ± 236**	1203 ± 402**

** $p < 0{\cdot}01$ (compared with control).

absence and the presence of the NADPH-generating system (1·18 ± 0·08 and 1·24 ± 0·10 μmol/incubation) and was not significantly different from that (1·34 ± 0·03 μmol/incubation) in buffer-only incubations. As shown in figure 5, flupyrazofos oxon decreased but PTMHP increased with duration of incubation time. These results suggest that PTMHP formation could be formed non-enzymatically from flupyrazofos oxon and not by microsomal esterase and/or dearylase activity.

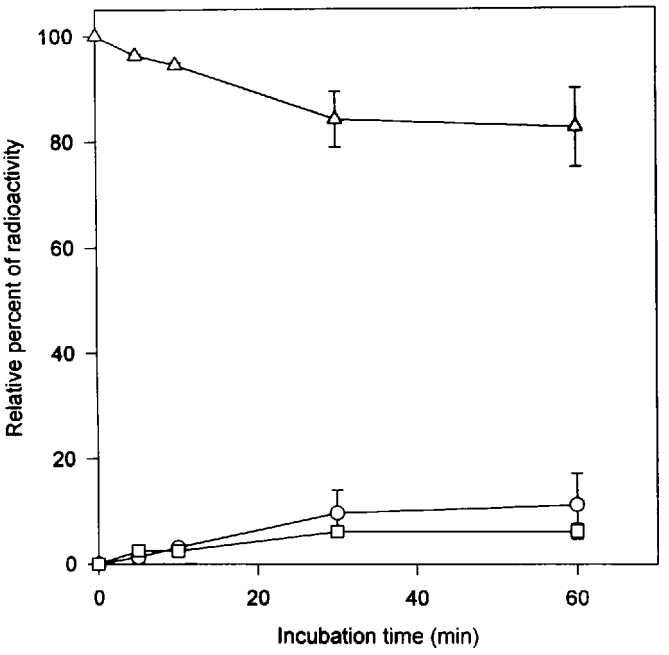

Figure 5. Time-course for the metabolism of flupyrazofos by control liver microsomes. Incubations contained ^{14}C-flupyrazofos (50 μM), control liver microsome (0·1 mg/ml), and an NADPH-generating system. Flupyrazofos (△), flupyrazofos oxon (□), PTMHP (○). Each point represents the mean and SEM of four experiments.

Discussion

In the present study, we investigated the metabolic profile of flupyrazofos by rat liver microsomes and the contribution of cytochrome P450 to the metabolism of flupyrazofos. Two metabolites were produced from flupyrazofos by rat liver microsomes and this reaction was NADPH-dependent, suggesting that cytochrome P450 is involved in the desulphuration of flupyrazofos. Those metabolites were identified as flupyrazofos oxon and PTMHP on the basis of UV and EI mass spectra. Similar results were reported from microsomal oxidation of parathion and fenitrothion which were metabolized to the corresponding oxon and phenol (Neal and Halpert 1982, Neal 1985, Levi *et al*. 1988). This result demonstrated that the initial metabolic reaction was oxidative desulphuration which is common for organophosphorothionates (Kulkarni and Hodgson 1980).

The biotransformation of flupyrazofos or flupyrazofos oxon was particularly enhanced by the pretreatment of PB, which is an inducer of cytochrome P4502B1/2 isozymes amongst others (Okey 1990). The biotransformation of parathion and fenitrothion (Levi *et al*. 1988) to their oxon forms was particularly enhanced by the pretreatment of phenobarbital and TCDD. These results indicate that cytochrome P4502B1/2 isozymes are responsible for the desulphuration process of flupyrazofos, but does not exclude the participation of additional, PB-inducible cytochrome P450s. The different effect of TCDD pretreatment on parathion (or fenitrothion) and flupyrazofos could be rationalized by their chemical properties and reactivities.

During the incubation of flupyrazofos with rat liver microsomes, the amount of flupyrazofos oxon decreased with a corresponding increase of PTMHP. From these studies, PTMHP was produced from flupyrazofos oxon to a similar extent,

regardless of incubation conditions, suggesting that PTMHP can be formed from chemical hydrolysis rather than from an enzyme-mediated process. This observation was interesting because *p*-nitrophenol and *m*-cresol were formed from parathion and fenitrothion by a monoxygenase-catalyzed reaction (Kamataki *et al*. 1976, Levi *et al*. 1988).

In conclusion, the new organophosphorous insecticide flupyrazofos was desulphurated *in vitro* to flupyrazofos oxon by cytochrome P450, and the hydrolytic transformation of flupyrazofos oxon to another metabolite PTMHP was catalysed non-enzymatically.

Acknowledgement

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