J Vet Sci. 2022 Nov;23(6):e82
https://doi.org/10.4142/jvs.22178
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Rapid Communication
Toxicology

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Comparative *in vitro* biotransformation of fipronil in domestic poultry using liver microsome

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Received: Jun 29, 2022
Revised: Aug 21, 2022
Accepted: Sep 1, 2022
Published online: Sep 15, 2022

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ABSTRACT

Domestic poultry are among the non-target species of exposure to fipronil, but limited information is available on the metabolic effects of fipronil exposure in avian. We investigated the comparative capacity of *in vitro* biotransformation of fipronil among chicken, duck, quail, goose, and rat. Interspecies differences in kinetic parameters were observed; the clearance rate calculations (Vmax/Km) indicated that chicken and duck are more efficient in the cytochrome P450-mediated metabolism of fipronil to sulfone than quail, goose and rat. The lower hepatic clearance of fipronil in quail, goose and rat, suggested that fipronil sulfone may serve as a biomarker to indicate fipronil exposure in these species.

Keywords: Biotransformation; cytochrome P450; sulfone; microsome; poultry

INTRODUCTION

Fipronil is known as a broad-spectrum insecticide that belongs to the class of phenylpyrazole chemicals used to control of a variety of insects in growing crops, livestock and veterinary medicine. Disruption of the gamma-aminobutyric acid (GABA)-gated chloride channels by fipronil can lead to excess neuronal excitation and the death of target insects [1]. Since it has a high affinity for insect compared to mammalian GABA receptors, fipronil is much more toxic to insects than to mammal [2]. However, there are some exceptions; the selective toxicity of some insecticides is due predominantly to differences in the detoxifying enzymes between mammals and insects [3]. Therefore, the exposure, metabolism, and toxicity of fipronil have been of concern not only in invertebrates but also in non-target vertebrates such as human and wildlife [4,5].

The primary metabolite formed during the metabolism of fipronil by hepatic cytochrome P450 (CYP) is fipronil sulfone [6]. Studies have suggested that fipronil sulfone is among the metabolites of interest that are more toxic and persistent than the parent compound [1]. In mice and rats, fipronil sulfone affected emotional and cognitive behaviors and induced massive changes in the dopamine and serotonin systems in the brain [5,7]. The results of *in vitro* exposure to human neuroblastoma cell indicated that fipronil induces neurotoxicity via an oxidative stress mechanism and fipronil sulfone might be responsible for the fipronil-induced toxicity rather than the parent fipronil [8].

Inappropriate use of fipronil can lead to drastic contamination in poultry farms, especially its detection in chicken eggs in poultry farms located in the Belgium and Netherland in 2017 [9]. Dermal and oral contaminations of fipronil linked to the presence of fipronil sulfone in chicken feathers and eggs have been suggested in a recent biomonitoring study [9]. In addition, several studies revealed that the level of fipronil sulfone detected in eggs was consistently higher than that of the parent fipronil compound [9,10]. Although the accumulation and transfer of fipronil sulfone in avian species have been increasingly considered, the kinetics and CYP involved in the fipronil metabolism, the main pathway of sulfone formation, are still unknown. Therefore, the aim of this study was to preliminarily elucidate the differences in the *in vitro* metabolism of fipronil using microsome of domestic poultry (chicken, duck, quail, and goose) and rat.

MATERIALS AND METHODS

Animals and microsome preparation

All experimental procedures were performed according to the Guidelines for Animal Experiments and approved by the Animal Ethics Research Committee of the Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand (Approval No. UI-00469-2558). Avian species consisting of laying chickens, laying ducks, geese, and Japanese quails were obtained from domestic farms in Nakhon Pathom province, Thailand. Sprague-Dawley rats were purchased from Nomura Siam International (Bangkok, Thailand). Information on species, sex, age and weight of animals is summarized in **Table 1**. All animals were euthanized using carbon dioxide gas. All tissues were collected and stored at −80°C until further analyses.

A 5 g sample of liver was homogenized with 0.1 M potassium phosphate buffer (KPB, pH 7.4) using a homogenizer. The homogenate was centrifuged at 9,000×*g* at 4°C for 20 min. The supernatant was then transferred and centrifuged twice at 105,000×*g* at 4°C for 60 min. The microsomal pellet was suspended and homogenized with 0.1 M KPB. The protein concentrations of microsomal fractions were measured using the BCA protein assay kit (Thermo Fisher Scientific, USA) and total CYP concentrations were determined using a previously reported method [11] before being frozen using liquid nitrogen and stored at −80°C until analysis.

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Conflict of Interest
The authors declare no conflicts of interest.

Funding
The authors all thank the financial support of the Smart Start up Research Fund: SRF from the Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand.

Table 1. Characterization of animals used for liver microsomes

Species	Scientific name	Sex	Age	Weight (g)	Number of samples	Total CYPs (nmol/mg protein, mean ± SD)
Chicken	*Gallus domesticus*	Male	12 mon	1,500–2,000	5	0.59 ± 0.1
Duck	*Anas platyrhynchos*	Female	18 mon	1,200–1,500	4	0.74 ± 0.1
Quail	*Coturnix coturnix*	Male	8 mon	150–200	5	0.82 ± 0.2
Goose	*Anser domesticus*	Male	1.5 yr	3,500–4,000	3	0.80 ± 0.1
Rat	*Rattus norvegicus*	Male	8 wk	290–300	3	0.73 ± 0.2

In vitro metabolism of fipronil

Fipronil biotransformation assay using liver microsomes was modified using the methods described by Suzuki et al. [5]. A mixture of fipronil substrate (final concentrations: 1.25, 2.5, 5, 10, 25, 50, 100, 200, 400, and 800 μM in 0.1 M KPB), 0.1 M KPB, $MgCl_2$ (final concentration 3 mM), glucose 6-phosphate (G6P, final concentration 5 mM), and pooled hepatic microsome (final protein concentration 1 mg/mL) of each species was pre-incubated for 5 min in a thermo shaker. Then, the reaction was started by adding a mixture of glucose-6-phosphate dehydrogenase (G6PDH, final concentration 2 IU/mL) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH, final concentration 0.5 mM) and continuously incubating for 30 min. The temperatures for pre-incubation and incubation were set depending on the body temperatures of the animal species (40–42°C for avian species and at 37°C for rat). After 30 min, 1% formic acid in acetonitrile was added to stop the reaction. Then, reaction samples were placed on ice for 15 min before centrifugation at 15,000×*g* for 10 min. The collected supernatant was evaporated using nitrogen gas and dissolved with n-hexane before analysis. All tests were performed in triplicate with negative control for each sample.

Analyses of fipronil sulfone

Gas chromatography using a micro-electron capture detector (GC-μECD, GC 7890B; Agilent Technologies, USA) equipped with an HP-5 column (30 m × 320 μm × 0.25 μm fused silica capillary; Agilent Technologies) was used to analyze the fipronil sulfone. The carrier gas was set at a constant flow rate of 20 mL/min for helium and 60 mL/min for nitrogen. The column temperature was operated as: initial temperature of 220°C (for 2 min), increased to 240°C at a rate of 20°C/min (for 2 min), and further increased to 265°C at a rate of 20°C/min, then held for 3 min. The total run time was 22.6 min per sample. The injection temperature was set at 260°C in splitless mode and the detector temperature was set at 300°C. Fipronil sulfone was identified and quantified by comparing the retention time and peak area of samples with that of the analytical standard. The calibration curve was created using seven calibration levels (R^2: 0.998–0.999). The limit of detection and limit of quantification were 0.01 μg/L and 0.04 μg/L, respectively. The recovery, precision in terms of repeatability, and intermediate precision for fipronil sulfone are shown in **Table 2**.

Data and statistical analysis

All results were presented as the mean±SD. Calculations of kinetic parameters including maximum velocity (Vmax) and Michaelis-Menten constants (Km), and statistical analyses were performed using the GraphPad Prism version 9.3.1 software for macOS (GraphPad Software, USA). Tukey's multiple comparisons test was analyzed to compare the values of kinetic parameters among species. A p value < 0.05 was considered statistically significant in all analyses.

Table 2. The accuracy and precision for fipronil sulfone

Spike level (ug/L)	Repeatability		Intermediated precision	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
0.025	92.9	2.8	99.3	6.3
0.1	94.5	8.1	94.6	11.6
1	89.5	7.7	92.3	6.7
10	106.7	8.2	101.6	7.8
20	102.1	10.1	100.3	7.7

RSD, relative standard deviation.

RESULTS

Fig. 1 and **Table 3** present the comparison of Michaelis-Menten plots and kinetic parameters of the fipronil sulfone among studied animals. The reaction mixture using microsome of duck had the significantly highest maximum reaction rate (Vmax = 2,195 ± 562 pmol/min/mg protein) compared to those of other avian species (chicken: 373 ± 26 pmol/min/mg protein, quail: 271 ± 107 pmol/min/mg protein, and goose: 200 ± 35 pmol/min/mg protein) and rat (Vmax = 221 ± 49 pmol/min/mg protein). The affinities of fipronil for CYP enzymes involved in the oxidation to sulfone were significantly less in duck (Km = 568 ± 185 μM), and goose (Km = 500 ± 197 μM) than in chicken (Km = 60 ± 14 μM). Notably, there were significant differences in the efficiency of hepatic intrinsic clearance (Vmax/Km) for fipronil among the avian species; the rate of oxidation of fipronil sulfone was the most rapid for chicken liver microsomes (6.4 ± 1.1 μL/min/mg protein), followed by duck (4.0 ± 0.5 μL/min/mg protein), quail (1.5 ± 0.3 μL/min/mg protein), goose (0.42 ± 0.1 μL/min/mg protein) and rat (0.38 ± 0.03 μL/min/mg protein) microsomes. On the other hand, we did not detect the differences of Vmax, Km and Vmax/Km values in the reactions between using quail and goose microsomes.

DISCUSSION

This study focused on the comparative analyses of fipronil's metabolizing ability among avian species. Our results indicated that chicken and duck had higher ability of CYP-mediated fipronil metabolism compared to quail, goose and rat. In bird species, fipronil sulfone is

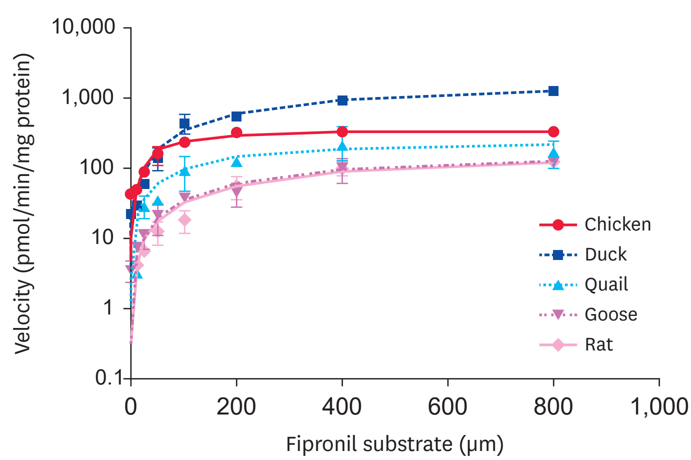

Fig. 1. Michaelis-Menten plots of chickens, ducks, quails, and rats obtained from the reactions for metabolic activity using their microsomal fractions (mean ± SD).

Table 3. Kinetic parameters for CYP biotransformation of fipronil. Values (mean ± SD) followed by different lowercase superscripts (a, b, and c) indicate statistically significant differences of Vmax/Km (Tukey's multiple comparisons test, $p < 0.05$)

Species	Michaelis-Menten kinetic parameters		
	Vmax (pmol/min/mg protein)	Km (μM)	Vmax/Km (μL/min/mg protein)
Chicken	373 ± 26[b]	60 ± 14[c]	6.4 ± 1.1[a]
Duck	2,195 ± 562[a]	568 ± 185[a]	4.0 ± 0.5[b]
Quail	271 ± 107[b]	180 ± 70[bc]	1.5 ± 0.3[c]
Goose	200 ± 35[b]	500 ± 197[ab]	0.42 ± 0.1[c]
Rat	221 ± 49[b]	598 ± 176[a]	0.38 + 0.03[c]

https://doi.org/10.4142/jvs.22178

the major metabolite found in many organs [9,12]. The study of oral and dermal exposure in laying hens and roosters found that sulfone metabolite persists for much longer duration than fipronil in feathers, eggs, liver, kidney and other organs [9]. The high rate of fipronil conversion to sulfone in chicken and duck in our study may result in the higher accumulation of fipronil sulfone in the bodies that consequently have been transferred to eggs and other such products consumed daily concerning the purity of animal products, consumer's safety, and animal health. However, the studies are needed on the specific CYP isoform of fipronil biotransformation, phase II metabolism, excretion, and the toxicity of sulfone metabolite in poultry to ensure animal welfare and food safety.

The rat microsomes in this study were not significantly different regarding the values of Vmax/Km compared with those using microsomes of quail and goose. Since fipronil sulfone persists much longer in the organism than fipronil, sulfone metabolite is used as a critical biomarker of fipronil exposure in human and rat [13,14]. The lower internal clearance of fipronil to sulfone in rat, quail, and goose, suggested that fipronil sulfone may serve as a marker to indicate environmental exposure to fipronil in these species. However, interspecies differences in excretion of fipronil involved in the enzymes in phase II metabolism should be considered because these could affect species variation in the residue levels of fipronil sulfone in the blood.

Kinetic parameters of CYP-mediated fipronil metabolism using rat microsome in our study differed from the other studies; a comparison of the activity of fipronil metabolizing enzymes among rat microsome (Vmax/Km value = 18 ± 0.4 mM/min/mg protein), mouse, dog, and cat microsomes [5], and the *in vitro* fipronil metabolism using rat liver microsomes showed that the Km and Vmax values were 19.9 µM and 0.39 nmol/min/mg protein, respectively [15]. Although the hepatic microsome of Sprague-Dawley rats was similarly used in the other studies and in current study, differences of Vmax, Km, and Vmax/Km values for rat microsome were noted. Therefore, the differences in age diversity and individual factors of Sprague-Dawley rats, as well as the materials and methods (concentrations of microsome and fipronil, time of incubation, chemical extraction, and detector machine) used in each study, may have caused the differences in kinetic parameters of fipronil sulfone formation in rats between this study and other reports.

In summary, we found the interspecies differences of Michaelis-Menten kinetic parameters among avian species; the intrinsic hepatic clearance for fipronil was the most efficient in chicken, followed by duck, quail, goose and rat. The lower hepatic clearance of fipronil in rat, quail, and goose indicated that fipronil sulfone may serve as a biomarker to indicate environmental exposure to fipronil in these avian, in a similar manner as in rat.

REFERENCES

1. Singh NS, Sharma R, Singh SK, Singh DK. A comprehensive review of environmental fate and degradation of fipronil and its toxic metabolites. Environ Res. 2021;199:111316.
PUBMED | CROSSREF
2. Mohamed F, Senarathna L, Percy A, Abeyewardene M, Eaglesham G, Cheng R, et al. Acute human self-poisoning with the N-phenylpyrazole insecticide fipronil--a GABAA-gated chloride channel blocker. J Toxicol Clin Toxicol. 2004;42(7):955-963.
PUBMED | CROSSREF

3. Narahashi T, Zhao X, Ikeda T, Nagata K, Yeh JZ. Differential actions of insecticides on target sites: basis for selective toxicity. Hum Exp Toxicol. 2007;26(4):361-366.
PUBMED | CROSSREF
4. Gibbons D, Morrissey C, Mineau P. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environ Sci Pollut Res Int. 2015;22(1):103-118.
PUBMED | CROSSREF
5. Suzuki T, Hirai A, Khidkhan K, Nimako C, Ichise T, Takeda K, et al. The effects of fipronil on emotional and cognitive behaviors in mammals. Pestic Biochem Physiol. 2021;175:104847.
PUBMED | CROSSREF
6. Roques BB, Lacroix MZ, Puel S, Gayrard V, Picard-Hagen N, Jouanin I, et al. CYP450-dependent biotransformation of the insecticide fipronil into fipronil sulfone can mediate fipronil-induced thyroid disruption in rats. Toxicol Sci. 2012;127(1):29-41.
PUBMED | CROSSREF
7. Bharatiya R, Chagraoui A, De Deurwaerdere S, Argiolas A, Melis MR, Sanna F, et al. Chronic administration of fipronil heterogeneously alters the neurochemistry of monoaminergic systems in the rat brain. Int J Mol Sci. 2020;21(16):E5711.
PUBMED | CROSSREF
8. Romero A, Ramos E, Ares I, Castellano V, Martínez M, Martínez-Larrañaga MR, et al. Fipronil sulfone induced higher cytotoxicity than fipronil in SH-SY5Y cells: Protection by antioxidants. Toxicol Lett. 2016;252:42-49.
PUBMED | CROSSREF
9. Corrias F, Atzei A, Taddeo R, Arru N, Casula M, Salghi R, et al. Fipronil and fipronil sulfone distribution in chicken feathers and eggs after oral and dermal exposure. Foods. 2021;10(12):3077.
PUBMED | CROSSREF
10. Lyu B, Chen DW, Li JG, Zhou S, Zhao YF. Analysis of fipronil and metabolites residues distrubution in eggs. Chin J Prev Med 2017;51(10):949-953.
PUBMED
11. Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. J Biol Chem. 1964;239(7):2370-2378.
PUBMED | CROSSREF
12. Kitulagodage M, Isanhart J, Buttemer WA, Hooper MJ, Astheimer LB. Fipronil toxicity in northern bobwhite quail Colinus virginianus: reduced feeding behaviour and sulfone metabolite formation. Chemosphere. 2011;83(4):524-530.
PUBMED | CROSSREF
13. Vasylieva N, Barnych B, Wan D, El-Sheikh EA, Nguyen HM, Wulff H, et al. Hydroxy-fipronil is a new urinary biomarker of exposure to fipronil. Environ Int. 2017;103:91-98.
PUBMED | CROSSREF
14. McMahen RL, Strynar MJ, Dagnino S, Herr DW, Moser VC, Garantziotis S, et al. Identification of fipronil metabolites by time-of-flight mass spectrometry for application in a human exposure study. Environ Int. 2015;78:16-23.
PUBMED | CROSSREF
15. Tang J, Amin Usmani K, Hodgson E, Rose RL. In vitro metabolism of fipronil by human and rat cytochrome P450 and its interactions with testosterone and diazepam. Chem Biol Interact. 2004;147(3):319-329.
PUBMED | CROSSREF