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Evaluation of the enantioselective *in vitro* metabolism of the chiral pesticide fipronil employing a human model: Risk assessment through *in vitro-in vivo* correlation and prediction of toxicokinetic parameters

Daniel Blascke Carrão[a], Isabel Cristina dos Reis Gomes[a], Fernando Barbosa Junior[b], Anderson Rodrigo Moraes de Oliveira[a,*]

[a] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901, Ribeirão Preto, SP, Brazil
[b] Laboratório de Toxicologia e Essencialidade de Metais, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14049-903, Ribeirão Preto, SP, Brazil

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ABSTRACT

The chiral pesticide fipronil is employed as a racemic mixture to control pests. Although there are no enantioselective differences in the fipronil enantiomer activities toward target organisms, fipronil enantiomers may exhibit enantioselective differences in their bioaccumulation, toxicity, and metabolism toward non-target organisms, including humans. The present work aims to provide significant reliable enantioselective information concerning fipronil risk assessment in humans. For that, the *in vitro* metabolism of *rac*-fipronil, S-fipronil, and R-fipronil by human liver microsomes was evaluated, the *in vivo* enantioselective toxicokinetic parameters were predicted and the main CYP450 isoforms involved in the enantioselective metabolism were determined. The obtained results demonstrated that fipronil may undergo a clearance by the liver and it is exclusively metabolized by the CYP3A4 isoform. Although no significative stereoselective differences were observed, the results provide reliable information on fipronil risk assessment for humans.

1. Introduction

Fipronil (Fig. 1A, (R,S)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl) phenyl]-4-(trifluoromethylsulfinyl)pyrazole-3-carbonitrile) is a highly effective broad-spectrum phenylpyrazole insecticide (Tingle et al., 2003). It is employed worldwide for several crops (barley, corn, cotton, potato, rice, sugar cane, sorghum, tomato and wheat) (Qu et al., 2014), public hygiene and veterinary pest control (Tingle et al., 2003). Its insecticidal activity is associated with the disruption of the central nervous system via interference with the passage of ions through the gamma-aminobutyric acid (GABA) regulated chloride channel, which causes the death of the insect (Tingle et al., 2003). Fipronil is among the most widely employed pesticides in the world due to its high activity, its long persistence, its systemic nature, and its flexibility in application to the target crops (Bonmatin et al., 2015). However, all these properties also increase the possibility of environmental contamination and, consequently, the exposure risk to non-target organisms (Bonmatin et al., 2015). Fipronil is a chiral due the presence of an asymmetric sulfur atom (Qu et al., 2014) (Fig. 1A). Since the fipronil enantiomers activities toward the target organism do not display a significant enantioselective difference, it is commercialized as a racemic mixture (Teicher et al., 2003). The main reported fipronil metabolites are fipronil sulfone (Fig. 1B), fipronil desulfinyl (Fig. 1C) and fipronil sulfide (Fig. 1D), which are formed by oxidation, reduction and photolysis reactions, respectively (Kumar et al., 2013).

The presence of fipronil in food (vegetables, eggs, and seafood) (Guo et al., 2018; Kaur et al., 2015; Zhang et al., 2018), along with the occupational (Hamsan et al., 2017) and urban exposure risks (Gan et al., 2012), may be indicative of its potential contamination risks for humans. In 2017, Belgium's Federal Agency for the Safety of the Food Chain (FASFC) discovered the illegal use of fipronil for the treatment of red lice in poultry farms (FASFC, 2017). Although the Belgium agency has reported that pesticide levels found in eggs pose no health problems, millions of contaminated eggs were recalled from the market in 16 countries, including Germany, England, Sweden, Switzerland and France (FASFC, 2017). In addition to food contamination, studies have also reported that fipronil levels in personal paddy farm air samples (Hamsan et al., 2017) and in urban residential runoff (Gan et al., 2012)

* Corresponding author. Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP, Av. dos Bandeirantes, 3900, Ribeirão Preto, São Paulo, 14040-901, Brazil.
E-mail address: deoliveira@usp.br (A.R.M. de Oliveira).

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Abbreviations

CL_{INT}	intrinsic clearance
CL'_{INT}	*in vivo* intrinsic clearance
CYP450	cytochrome P450
EMA	European Medicines Agency
GABA	gamma-aminobutyric acid
HLMs	human liver microsomes
IS	internal standard
K_M	substrate concentration at which half-maximal velocity is achieved
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate hydrate
NR	normalized rate
rCYP450	recombinant CYP450 isoforms
v	enzymatic reaction velocity
V_0	initial velocity condition
V_{MAX}	maximal velocity
% I	percentage of inhibition
% TNR	total normalized ratio

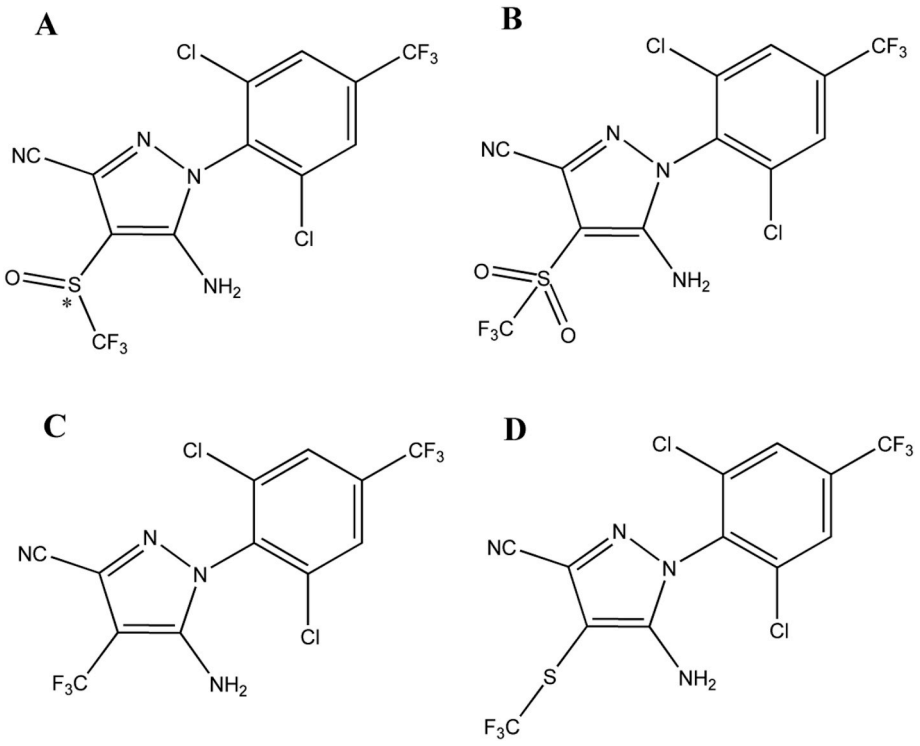

Fig. 1. Chemical structures of fipronil and its metabolites. (A) fipronil, (B) fipronil sulfone, (C) fipronil desulfinyl, and (D) fipronil sulfide. *chiral center.

may increase the contamination risks to humans. McMahen et al. investigated the human environmental exposure to fipronil (McMahen et al., 2015). Although the presence of fipronil was not observed, the presence of the metabolite fipronil sulfone in 25% of the human plasma samples was reported (McMahen et al., 2015). The contamination by fipronil sulfone observed in this study suggests that environmental exposure to fipronil has occurred (McMahen et al., 2015). A case report of intoxication through dermal and inhalation exposure to fipronil has been reported by Chodorowski and Anand (2004). A patient was admitted in the hospital 5 h after spraying his field with a fipronil solution and presented symptoms such as headache, nausea, vertigo and weakness (Chodorowski and Anand, 2004). Although all the symptoms disappeared spontaneously after 5 h, the fipronil risk assessment for humans should be conducted in order to act more accurately during cases of exposure (Chodorowski and Anand, 2004). Taking this into account, the risk of ingestion or contamination makes the fipronil risk assessment an urgent matter (de Albuquerque et al., 2016, 2018; Abass, 2013). The pesticide's risk assessment is an important and necessary tool to provide reliable scientific information to protect human health (Abass, 2013).

Since fipronil is a chiral pesticide, its interaction with non-target organisms may exhibit an enantioselective behavior (Drăghici et al., 2012). Therefore, the enantioselective evaluation of fipronil bioaccumulation, toxicity, and metabolism in non-target organisms, mainly humans, is important for its risk assessment (Drăghici et al., 2012). Enantioselective studies in non-target organisms have already demonstrated differences in the toxicity of fipronil enantiomers (Qu et al., 2014; Qin et al., 2015; Qian et al., 2017). Qu et al. reported that fipronil enantiomers acute toxicity to freshwater algae *Scenedesmus obliguus* suspensions are substantially different with effective concentrations of 0.54, 1.50 and 0.29 mg L^{-1} for the *rac*-fipronil, S-fipronil and R-fipronil, respectively (Qu et al., 2014). The subchronic exposure of the earthworm *Eisenia foetida* to fipronil showed that R-fipronil and *rac*-fipronil are more toxic than S-fipronil (Qin et al., 2015). The enantioselective evaluation of the fipronil toxicity in zebrafish embryo-larvae demonstrated that S-fipronil causes severe developmental toxicity in embryos due to changes in DNA methylation (Qian et al., 2017). These studies highlighted enantioselective differences in fipronil

toxicity in non-target organisms, even though fipronil enantiomers lack stereoselective activity toward the target organism.

In this context, the enantioselective evaluation of fipronil bioaccumulation, toxicity and metabolism in non-target organisms, mainly humans, is important for its risk assessment (Kaur et al., 2015; Gan et al., 2012). Investigations into the *in vitro* enantioselective metabolism of this pesticide by human liver microsomes (HLMs) are an important tool to predict the fipronil enantioselective risk assessment in humans (de Albuquerque et al., 2018; Abass, 2013). *In vitro* nonchiral fipronil metabolism by rat and human liver microsomes have already been described by Tang et al. (2004). The enzymatic kinetic parameters of fipronil metabolism have been determined and the major isoforms involved in fipronil nonchiral metabolism have been identified (Tang et al., 2004). However, the investigation of the enantioselective metabolism of fipronil is important to assess the metabolic behavior of each fipronil enantiomer (de Albuquerque et al., 2018). Therefore, the risk assessment of the fipronil racemic mixture and its enantiomers in humans may be individually evaluated using the characterization of its enantioselective metabolism and toxicokinetics (Abass, 2013).

The present work reports, for the first time, the evaluation of fipronil *in vitro* enantioselective metabolism by HLMs. The enantioselective enzymatic kinetic parameters of fipronil metabolism were determined, the *in vivo* toxicokinetic parameters were predicted and the main CYP450 isoforms involved in the metabolism of fipronil enantiomers were characterized.

2. Materials and methods

2.1. Chemicals and reagents

Rac-fipronil (≥99%), fipronil sulfone (≥99%), fipronil sulfide (≥98%) and fipronil desulfinyl (≥98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The standard stock solutions of fipronil and its metabolites were prepared at 8000 μmol L^{-1} and 1200 μmol L^{-1}, respectively, in ethanol (HPLC grade, Panreac, Barcelona, Spain). Carbamazepine (≥99%) (Sigma-Aldrich, St. Louis, MO, USA), which was employed as the internal standard (IS), was prepared at 500 μg mL^{-1} in methanol (HPLC grade, Panreac, Barcelona, Spain). The selective chemical inhibitors used in CYP450 reaction phenotyping (diethyldithiocarbamate (≥99%), ketoconazole (≥98%), montelukast (≥98%), pilocarpine (≥98%), quinidine (≥98%), sulfaphenazole (≥98%), ticlopidine (≥99%), and α-naphtoflavone (≥98%)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard stock solutions of the selective inhibitors were prepared at 8000 μmol L^{-1} for diethyldithiocarbamate, 40 μmol L^{-1} for ketoconazole, 80 μmol L^{-1} for montelukast, 400 μmol L^{-1} for pilocarpine, 80 μmol L^{-1} for quinidine, 800 μmol L^{-1} for sulfaphenazole, 240 and 800 μmol L^{-1} for ticlopidine, and 80 μmol L^{-1} for α-naphtoflavone, all in acetonitrile (HPLC grade, Panreac, Barcelona, Spain). All the solutions were stored in amber tubes at −20 °C. The human liver microsomes (HLMs), the 150-donor pool, and the recombinant CYP450 isoforms (Supersomes®) were obtained from Corning Life Science (Phoenix, AZ, USA) and stored at −80 °C. The components of the NADPH cofactor system (β-nicotinamide adenine dinucleotide phosphate hydrate ($NADP^+$), glucose-6-phosphate sodium salt, and glucose-6-phosphate dehydrogenase) were acquired from Sigma Aldrich (St. Louis, MO, USA). The solutions were prepared in a tris-KCl buffer (tris (hydroxymethyl)aminomethane 0.05 mol L^{-1} and KCl 0.15 mol L^{-1}, pH 7.4) at the following concentrations: $NADP^+$ at 2.5 mmol L^{-1}, glucose-6-phosphate at 50 mmol L^{-1}, and glucose-6-phosphate dehydrogenase at 8.0 units mL^{-1}. All the solutions were stored at −20 °C. Ultrapure water was obtained from a Direct-Q3 system (Millipore, Billerica, MA, USA). The HPLC grade solvents of methanol, ethanol, acetonitrile, and chloroform were purchased from Panreac (Barcelona, Spain) and isopropanol and hexane were acquired from Honeywell Riedel-de Häenm (Seelze, Germany). All the other reagents were of analytical grade. Sodium phosphate monobasic, sodium phosphate dibasic, formic acid, hydrochloric acid and diethylamine and were purchased from Synth (Sao Paulo, Brazil), tris(hydroxymethyl)aminomethane was acquired from J. T. Baker (Phillisburg, NJ, USA), trifluoroacetic acid was obtained from Vetec (Rio de Janeiro, Brazil), potassium chloride was purchased from Mallinckrodt Chemicals (Xalostoc, Mexico), and poloxamer 407 (2-methyloxiraneoxirane polymer) was acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation of fipronil enantiomers

A Shimadzu HPLC system (Kyoto, Japan) consisting of two LC-6AD solvent pump units, an SCL-10AVP system controller, and a UV/VIS SPD-10AV detector was employed to isolate the fipronil enantiomers. The enantioselective analytical method used for this isolation was adapted from the method described by Tan et al. (2008). The isolation was carried out at ambient temperature (20 °C) on a Chiralcel OD-H® column (150 × 4.6 mm, 5 μm) acquired from Daicel Chemical Industries (Tokyo, Japan) employing hexane: isopropanol (95:5, v/v) as the mobile phase at a flow rate of 1.5 mL min^{-1}. Manual injections of 100 μL of the fipronil standard stock solution were sequentially performed. The compounds were detected at 280 nm, and the data were analyzed with the Class-VP software (Shimadzu, Kyoto, Japan). The fipronil enantiomers were individually collected and the mobile phase was evaporated under a gentle compressed air stream. After that, the isolated enantiomers were identified by comparison with the method described by Tan et al. (2008) without any modification [Chiralcel OD-H® column (250 × 4.6 mm, 5 μm) and hexane: isopropanol (90:10, v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1}]. Finally, the isolated enantiomers were stored in amber tubes at −20 °C.

2.3. Fipronil enantioselective analysis and method validation

A Shimadzu HPLC system (Kyoto, Japan), which comprised a LC-20AT solvent pump unit, a CTO-20A column oven, a DGU-20A5 online degasser, a CBM-20A system controller, and an SPD-M20A (190–800 nm) diode array detector, was employed in the development of the enantioselective method. Injections were automatically performed by using a SIL-10AF loop and data were collected with the aid of the LC solution software 1.25 SP1 (Shimadzu, Kyoto, Japan). The new enantioselective HPLC method for analysis of fipronil and its metabolites was developed by employing screening procedures (Supplementary Material). The analysis was performed by employing a Chiralcel OD-H® column (150 × 4.6 mm, 5 μm) and the mobile phase consisted of hexane: isopropanol (gradient elution (v/v): 0.01 min–10% isopropanol, 12.00 min–30% isopropanol, 12.50 min–10% isopropanol, and 15.00 min–10% isopropanol) at a flow rate of 1.5 mL min^{-1}. The analyses were performed at 30 °C, the injection volume was 50 μL, and the analytes were detected at 280 nm. The elution order of the fipronil enantiomers, in the developed enantioselective method, was determined by individual injections of the identified fipronil isolated enantiomers. The first eluted enantiomer was S-fipronil and the second was R-fipronil. The enantioselective HPLC analytical method was validated according to the Guideline on Bioanalytical Method Validation of the European Medicines Agency (EMA) (EMA (European Medicines Agency), 2012). The evaluated parameters were linearity, selectivity, carryover, precision and accuracy within and between days, lower limit of quantification (LLOQ) and stability. In addition, the racemization evaluation was also carried out (Supplementary Material).

2.4. In vitro metabolism incubations

The microsomal medium for the *in vitro* metabolism studies consisted of the substrate (*rac*-fipronil, S-fipronil or R-fipronil), the NADPH cofactor system, HLMs, and phosphate buffer (0.1 mmol L^{-1}, pH 7,4) with 0.05% (m/v) of poloxamer 407 with a final volume of 200 µL. The microsomal medium samples were prewarmed for 5 min at 37 °C in a shaking water bath, and the reaction was started by the addition of the NADPH cofactor. After the incubation time, the reaction was stopped with 500 µL of chloroform and 20 µL of IS that were added to the samples. After that, the samples were shaken for 5 min at 1500 rpm in a Vibrax VXR® agitator (IKA, Staufen, Germany) and centrifuged at 1800 × *g* for 10 min at 4 °C in a HIMAC CF15D2 centrifuge (Hitachi, Tokyo, Japan). Then, the organic phase was collected and evaporated in a Concentrator Plus speed vacuum (Eppendorf, Hamburg, Germany). Finally, the residue was solubilized in 120 µL hexane: isopropanol (80:20, v/v) and analyzed by the developed HPLC enantioselective method.

2.5. Enzymatic kinetics

To determine the initial velocity conditions (V_0) of the fipronil metabolism reaction, the linear range of fipronil sulfone formation was determined with respect to the microsomal protein content and incubation time at fipronil concentrations of 6.00, 25.00 and 60.00 µmol L^{-1}. The enantioselective enzymatic kinetics of fipronil metabolism by HLMs were determined at V_0 conditions by employing a microsomal protein content of 0.50 mg mL^{-1} and incubation for 40 min. The metabolism enzymatic kinetics were individually performed for *rac*-fipronil, S-fipronil and R-fipronil. The substrate concentrations that were used were as follows: 2.00, 4.00, 6.00, 8.00, 10.00, 15.00, 20.00, 30.00, 40.00, 50.00, and 60.00 µmol L^{-1} for *rac*-fipronil; 3.51, 5.08, 7.05, 8.83, 13.86, 17.64, 21.72, 25.48, 33.74, 42.18, 46.84, and 58.08 µmol L^{-1} for S-fipronil; and 2.97, 5.16, 7.74, 11.84, 15.56, 19.24, 23.39, 31.56, 40.74, 46.89, and 58.14 µmol L^{-1} for R-fipronil. After the incubation, the metabolism reactions were stopped by the addition of chloroform and by beginning the sample preparation procedure. Next, the samples were analyzed by the validated HPLC enantioselective method. The metabolism samples were quantified using analytical curves that were prepared on the same day. The enzymatic reaction velocity (v) was determined by normalizing the concentration of the metabolite by the microsomal protein content and the incubation time. The results were plotted in graphs of v *versus* the substrate concentration. Finally, the enantioselective kinetic parameters (K_M and V_{MAX}) were obtained by nonlinear regression analysis with the GraphPad Prism 5 software (San Diego, CA, USA) and the intrinsic clearance (CL_{INT}) was calculated by the ratio between the V_{MAX} and K_M (Seibert et al., 2014).

2.6. In vitro-in vivo correlation and prediction of in vivo toxicokinetic parameters

To predict the *in vivo* toxicokinetic parameters, the binding percentages of *rac*-fipronil, S-fipronil and R-fipronil to plasmatic and microsomal proteins were determined. The substrate concentrations that were used were below the obtained K_M values (20 µmol L^{-1}). To determine the pesticide binding to microsomal proteins, the substrate was incubated with HLMs (microsomal protein content of 0.50 mg mL^{-1}) in the absence of the NADPH cofactor solution. The pesticide binding to plasma proteins was determined after the substrate incubation with human plasma (plasma protein content of 42 mg mL^{-1}) (Chang et al., 2010) in a phosphate buffer (pH 7.4, 0.1 mol L^{-1}) with 0.05% (m/v) poloxamer 407. Control samples without protein were simultaneously prepared. All the samples were incubated at 37 °C for 10 min. After that, the samples were transferred to Amicon Ultra ultrafiltration device (0.5 µm regenerate cellulose membrane 30 kDa, Millipore Corporation, Bedford, MA, USA) and centrifuged at 10000 g for 20 min (HIMAC CF16RXII, Hitachi). Next, the filtrates were collected, submitted to the sample preparation procedure and analyzed by the developed HPLC enantioselective method. The unbounded fraction (f_u) was determined using the ratio between the analyte concentrations in the samples and in the control samples (Chang et al., 2010). The CL_{INT} was scaled to *in vivo* intrinsic clearance (CL'_{INT}) using as scaling factor 40 mg microsomal protein/g liver and 21.4 g liver/kg bodyweight (Bowman and Benet, 2018). The hepatic clearance (CL_H) and hepatic extraction rate (E_H) were calculated according to Equations (1) and (2) (Bowman and Benet, 2018; Damre et al., 2012).

$$CL_H = \frac{Q\ x\ (\frac{f_{u,plasm}}{f_{u,mic}})\ x\ CL'_{INT}}{Q + (\frac{f_{u,plasm}}{f_{u,mic}})\ x\ CL'_{INT}} \quad (1)$$

where CL_H is the hepatic clearance, $f_{u,plasm}$ is the unbounded fraction of the substrate to the plasmatic proteins, $f_{u,mic}$ is the unbounded fraction of the substrate to the microsomal proteins, CL'_{INT} is the *in vivo* intrinsic clearance and Q is the hepatic blood flow (20 mL min^{-1} kg^{-1}) (Damre et al., 2012).

$$E = \frac{\frac{f_{u,plasm}}{f_{u,mic}}\ x\ CL'_{INT}}{Q + (\frac{f_{u,plasm}}{f_{u,mic}})\ x\ CL'_{INT}} x\ 100\% \quad (2)$$

where E is the hepatic extraction rate, $f_{u,plasm}$ is the unbounded fraction of the substrate to the plasmatic proteins, $f_{u,mic}$ is the unbounded fraction of the substrate to the microsomal proteins, CL'_{INT} is the *in vivo* intrinsic clearance and Q is the hepatic blood flow (20 mL min^{-1} kg^{-1}) (Damre et al., 2012).

2.7. CYP450 reaction phenotyping

To evaluate the main CYP450 isoform(s) that is (are) involved in the fipronil metabolism, two different methods were employed: metabolism with HLMs using selective chemical inhibitors and metabolism with recombinant CYP450 isoforms (rCYP450).

In the first method, the substrate was incubated with HLMs in the presence of each selective chemical inhibitor (n = 3). The samples consisted of *rac*-fipronil, S-fipronil or R-fipronil (20 µmol L^{-1}), a selective chemical inhibitor (α-naphtoflavone (1.00 µmol L^{-1}, CYP1A2 (Carrão et al., 2017)), ticlopidine (3.00 µmol L^{-1}, CYP2B6 (Walsky and Obach, 2007)), montelukast (1.00 µmol L^{-1}, CYP2C8 (Nirogi et al., 2015)), sulfaphenazole (10.00 µmol L^{-1}, CYP2C9 (Carrão et al., 2017)), ticlopidine (10.00 µmol L^{-1}, CYP2C19 (Carrão et al., 2017)), quinidine (1.00 µmol L^{-1}, CYP2D6 (Zhao et al., 2012)) diethyldithiocarbamate (100.00 µmol L^{-1}, CYP2E1 (Hua et al., 2014)) or ketoconazole (0.50 µmol L^{-1}, CYP3A4/5 (Carrão et al., 2017))), HLMs (0.50 mg mL^{-1}), the NADPH cofactor solution and phosphate buffer (0.1 mol L^{-1}; pH 7.4) with 0.05% (m/v) poloxamer 407 in a final volume of 200 µL. Control samples without the selective chemical inhibitor were also prepared. The incubation time was 40 min. The metabolite concentration in the control samples and in the samples containing one of the selective chemical inhibitors was used to determine the percentage of inhibition (% I) according to Equation (3). The results were analyzed with the GraphPad Prism 5 software (San Diego, CA, USA).

$$\%I = \frac{v_0 - v_1}{v_0} \quad (3)$$

where v_0 and v_i are the reaction velocities in the absence and presence of the selective chemical inhibitor, respectively.

In the second method, the substrate was incubated with a recombinant CYP450 isoform (rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP2E1, rCYP3A4 or rCYP3A5) (n = 3). The samples consisted of *rac*-fipronil, S-fipronil or R-fipronil (20 μmol L^{-1}), a rCYP450 (50 pmol mL^{-1}), the NADPH cofactor solution and phosphate buffer (0.1 mol L^{-1}; pH 7.4) with 0.05% (m/v) poloxamer 407 in a final volume of 200 μL. The control samples contained insect cells instead of rCYP450. The incubation time was 40 min. The samples were quantified using analytical curves that were prepared on the same day. The rate of metabolite formation was multiplied by the abundance of each CYP450 in the native HLMs, which gave a normalized rate (NR). The NRs for each CYP were summed to provide a total normalized rate (TNR) according to Equation (4). The NR of each CYP was expressed as a percentage of the TNR (Rodrigues, 1999). The results were analyzed with the GraphPad Prism 5 software (San Diego, CA, USA).

$$\%TNR = \frac{NR\ x\ 100}{TNR} \quad (4)$$

where %TNR is the total normalized ratio percentage, NR is the hepatic normalized ratio, and TNR is the total normalized ratio.

3. Results and discussion

3.1. Enzymatic kinetics

The risk of intoxication by fipronil through ingestion of contaminated food (Guo et al., 2018; Kaur et al., 2015; Zhang et al., 2018; FASFC, 2017), through occupational exposure during its application (Hamsan et al., 2017; Chodorowski and Anand, 2004), and through environmental exposure (Gan et al., 2012; McMahen et al., 2015) makes risk assessment for humans extremely important (Abass, 2013). To accomplish that, the enantioselective enzymatic kinetics after fipronil metabolism by the HLMs was determined. However, before that, the fipronil solubility in the human microsomal medium should be determined. Our results showed that its solubility in the microsomal medium was approximately 25 μmol L^{-1} (Figure S1), which indicated that the use of higher fipronil concentrations in this medium could give misleading results (Seibert et al., 2014). To enhance the solubility of fipronil in the human microsomal medium without a significant influence on the CYP450 enzymatic activity (Randall et al., 2011), a solubilizing agent was employed. The addition of a 0.05% (m/v) poloxamer 407 (a hydrophilic nonionic solubilizing agent and a protein stabilizing agent (Naik et al., 2014)) enhanced the fipronil solubility in the human microsomal medium to approximately 60 μmol L^{-1} (Figure S2). Furthermore, the influence of poloxamer 407 in the CYP450 enzymatic activity on the fipronil metabolism was not significant (Figure S3). Therefore, the use of this solubilizing agent allowed the evaluation of the fipronil metabolism at concentrations up to 60 μmol L^{-1} and ensured that the *in vitro* metabolism results were reliable.

As previously reported, the *in vitro* metabolism of fipronil by HLMs yielded only the achiral metabolite fipronil sulfone (oxidation reaction) (Tang et al., 2004). Therefore, all the *in vitro* metabolism studies were evaluated by monitoring the fipronil sulfone formation velocity. The V_0 conditions for the fipronil *in vitro* metabolism into fipronil sulfone were evaluated. The enzymatic kinetics for *rac*-fipronil, S-fipronil and R-fipronil were determined by employing a microsomal protein content of 0.50 mg mL^{-1} and an incubation time of 40 min. No racemization of fipronil enantiomers was observed during all *in vitro* metabolism studies.

The *in vitro* enzymatic kinetics by HLMs were investigated for the fipronil racemic mixture and its isolated enantiomers, S-fipronil and R-fipronil (enantiomeric purities of 99.1% and 98.7, respectively). All the evaluated enzymatic kinetics have demonstrated Michaelis-Menten kinetic profiles, as confirmed by the Eadie-Hofstee linear plots (Fig. 2). The enzymatic kinetic parameters (K_M and V_{MAX}) for *rac*-fipronil, S-fipronil and R-fipronil were determined (Table 1). Both the K_M and V_{MAX} values for *rac*-fipronil and R-fipronil were very similar. However, both kinetic parameters for S-fipronil were approximately 1.6-fold higher, which could be an indication of the enantioselective differences in the fipronil metabolism by CYP450 enzymes. From the obtained enzymatic parameters for the fipronil racemic mixture and its isolated enantiomers, the CL_{INT} values, which express the maximal substrate clearance capacity of the liver without considering the substrate binding to proteins and the blood flow (Chang et al., 2010), were calculated. Although enantioselective differences in the K_M and V_{MAX} values of S-fipronil metabolism were observed by comparing both parameter values for *rac*-fipronil and S-fipronil, the obtained CL_{INT} results indicated that the S-fipronil and R-fipronil metabolism CL_{INT} were 0.80- and 0.96-fold, respectively, of the *rac*-fipronil metabolism CL_{INT} (Table 1). These results demonstrate slight differences in the liver capacity to clear the fipronil racemic mixture and its individual isolate enantiomers, which may be an indication of low enantioselective differences in the fipronil metabolism by HLMs.

The nonchiral fipronil *in vitro* metabolism by HLMs have already been described by Tang et al. (2004). The enzymatic kinetic parameters of *rac*-fipronil for *in vitro* metabolism that were obtained in the present work demonstrate some significant differences when compared to those obtained by the previously reported work. Although the K_M values obtained in both works did not show very significant differences (22.4 [in this work] *versus* 27.2 μmol L^{-1} (Tang et al., 2004)), the V_{MAX} value obtained in the present work was 2.5-fold higher than the V_{MAX} value obtained in the previous work (110 pmol min^{-1} mg^{-1}) (Tang et al.,

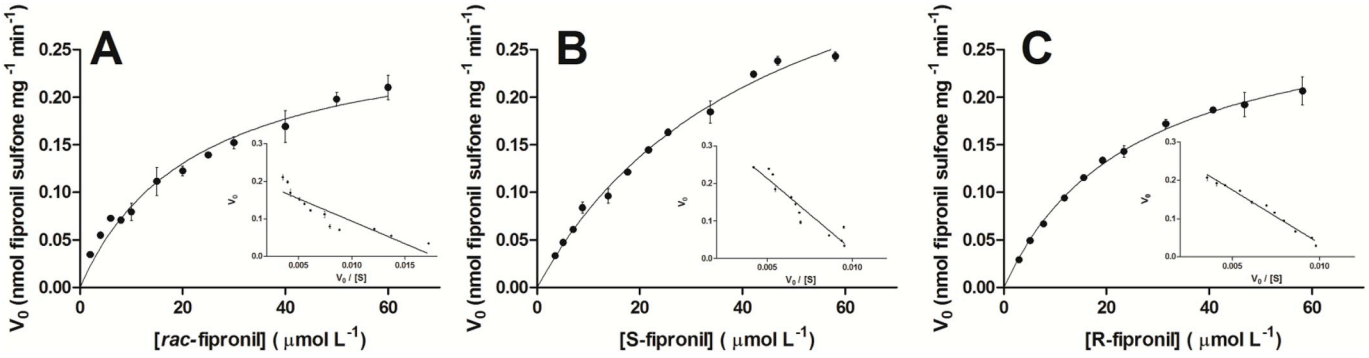

Fig. 2. Michaelis-Menten kinetic plots after (A) *rac*-fipronil, (B) S-fipronil and (C) R-fipronil metabolism by HLMs incubated for 40 min with 0.50 mg mL^{-1} of microsomal protein content (Inside: Eadie-Hofstee plot) (n = 3).

Table 1
Enantioselective enzymatic kinetic parameters.

Metabolism Reaction	K_M (μmol L^{-1})	V_{MAX} (pmol $min^{-1} mg^{-1}$)	CL_{INT} (mL $min^{-1} mg^{-1}$)
rac-fipronil → fipronil sulfone	22.4 ± 2.3	276.1 ± 12.5	12.3 ± 1.4
S-fipronil → fipronil sulfone	45.0 ± 3.4	446.8 ± 19.3	10.0 ± 0.9
R-fipronil → fipronil sulfone	25.3 ± 1.5	299.9 ± 8.2	11.9 ± 0.8

2004). These differences in the V_{MAX} may be associated with fipronil solubility in the human microsomal medium. In the previously published work, the nonchiral fipronil enzymatic kinetic was evaluated by employing fipronil concentrations up to 80 μmol L^{-1} without considering the fipronil solubility in the human microsomal medium. This may lead to misleading results in the fipronil metabolism reaction velocity determination, which directly results in the underestimation of the V_{MAX} value (Seibert et al., 2014).

3.2. Prediction of in vivo toxicokinetic parameters

The enzymatic kinetic parameters obtained from fipronil *in vitro* enantioselective metabolism by HLMs can be used to predict the *in vivo* toxicokinetic parameters of hepatic clearance (CL_H) and hepatic extraction rate (E_H). Both *in vivo* toxicokinetic parameters can be predicted using the obtained CL_{INT} values, the rate of the substrate binding to plasmatic and microsomal proteins and the human hepatic flow (20 mL min^{-1} kg^{-1}) (Chang et al., 2010). The CL_H is associated with the efficiency of the substrate elimination process by the liver and the E_H is the fraction of the substrate eliminated during the first pass through the liver considering oral ingestion (Chang et al., 2010; Wienkers and Heath, 2005). Since the predicted *in vivo* toxicokinetic parameters are obtained from the extrapolation of *in vitro* data, the determinations of both the enzymatic kinetic parameters and the substrate binding to proteins are critical to obtaining reliable results (Chang et al., 2010). The results obtained from the ultrafiltration assay showed that the fipronil racemic mixture and its individual enantiomers bind both to microsomal and plasmatic proteins, apparently without any significant enantioselectivity. The unbounded fraction to microsomal proteins is 18.4, 15.0 and 19.9% for the *rac*-fipronil, S-fipronil and R-fipronil incubations, respectively. The unbounded fraction to plasmatic proteins is less than 2.0% for all the incubations. Table 2 presents the predicted enantioselective *in vivo* toxicokinetic parameters, CL_H and E, obtained from the extrapolated CL_{INT} values of the enzymatic kinetic studies and the substrate unbounded fractions to proteins. The results from both predicted *in vivo* toxicokinetic parameters indicated no significant enantioselectivity in fipronil metabolism by CYP450 enzymes. Since CL_H and E_H are determined by employing the hepatic blood flow, CL_{INT} and the substrate binding to proteins (Equations (1) and (2)), and neither these results nor the *in vitro* determinations have shown significant enantioselective results, the lack of enantioselectivity in the predicted *in vivo* toxicokinetic parameters is justified. The CL_H values close to the human hepatic blood flow (20 mL min^{-1} kg^{-1}) may suggest that a xenobiotic is highly cleared by the liver (Chiba et al., 2009). The obtained CL_H values close to 1 suggest that fipronil is metabolized by the liver. The E_H values close to 100% may suggest that a xenobiotic is practically eliminated during the first pass through the liver (Wienkers and Heath, 2005). The obtained E_H values close to 5% may indicate that fipronil is not significantly metabolized by the first pass effect. So, if fipronil is ingested, the first pass effect will not be significant in decreasing its concentration from the blood. The predicted enantioselective *in vivo* toxicokinetic parameters of fipronil provide important information concerning the pesticide risk assessment for humans. Although no significant enantioselectivity is observed, the results may indicate that any damage to the liver functions or the inhibition of CYP450 enzymes by other compounds may result in fipronil accumulation in the body, which may lead to human intoxication.

3.3. CYP450 reaction phenotyping

To determine the human CYP450 isoforms involved in the metabolism of a substrate, at least two independent approaches are recommended (Zientek and Youdim, 2015). Therefore, the determination of the CYP450 isoforms responsible for fipronil enantioselective metabolism into fipronil sulfone was carried out by employing selective chemical inhibitors (Nirogi et al., 2015) and rCYP450 isoforms (Rodrigues, 1999). The method employing selective chemical inhibitors depends on the selectivity of the chemical inhibitor and the concentrations in the incubation medium (Nirogi et al., 2015). The CYP450 isoforms evaluated by this method were CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5. The obtained results indicated that CYP3A4/5 was the only isoform involved in the metabolism of *rac*-fipronil, S-fipronil and R-fipronil into fipronil sulfone (Fig. 3A). However, due to the lack of specificity in the chemical inhibitors to differentiate between CYP3A4 and CYP3A5 isoforms, it is not possible to determine which of these isoforms is responsible for the metabolism of fipronil enantiomers by this method (Nirogi et al., 2015). The method employing rCYP450 isoforms allows us to individually evaluate which CYP450 isoforms are responsible for the substrate metabolism. The rCYP450 isoforms can be more active than each CYP450 isoform that is naturally present in HLMs. However, the normalization of the contribution of each isoform to the metabolism of a substrate by employing the abundance of the isoforms in native HLMs can avoid that limitation (Rodrigues, 1999). The evaluated isoforms were as follows: rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP2E1, rCYP3A4 and rCYP3A5. The results obtained from the rCYP450 phenotyping method suggested that fipronil is metabolized only by CYP3A4 and not by CYP3A5 (Fig. 3B). Furthermore, no enantioselective differences were observed in the human CYP450 isoforms involved in the metabolism of *rac*-fipronil, S-fipronil and R-fipronil into fipronil sulfone. In the nonchiral *in vitro* metabolism performed by Tang et al. (2004), CYP3A4 was reported to be the major isoform responsible for fipronil metabolism and CYP2C19 was considered less active (Tang et al., 2004). However, the CYP450 reaction phenotyping was

Table 2
In vivo predicted toxicokinetic parameters.

Substrate	CL_H (mL $min^{-1} mg^{-1}$)	E_H (%)
rac-fipronil	1.02	5.1
S-fipronil	0.83	4.1
R-fipronil	0.98	4.9

CL_H – hepatic clearance.
E_H – hepatic extraction rate.
Human hepatic blood flow of 20 mL min^{-1} kg^{-1} (Damre et al., 2012).

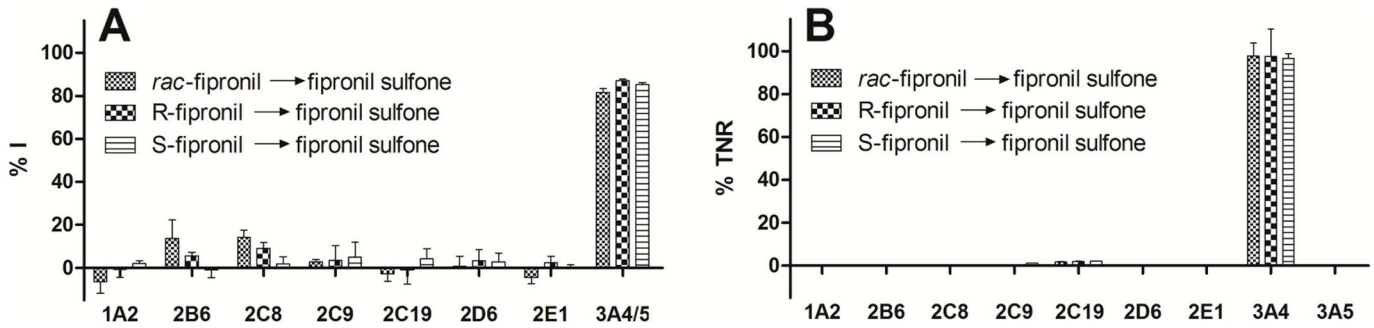

Fig. 3. Determination of human CYP450 isoforms involved in the *in vitro* metabolism of *rac*-fipronil, S-fipronil and R-fipronil by employing (A) CYP450 chemical inhibitors and (B) recombinant CYP450 isoforms (n = 3).

performed by employing only rCYP450 and the abundance of each CYP450 in native HLMs were not considered, which might lead to considering that CYP2C19 is also responsible for fipronil metabolism. From the results obtained in the present work, CYP3A4 is the only human CYP450 isoform responsible for fipronil metabolism and no enantioselectivity was observed. CYP3A4 is reported to be the human CYP450 isoform responsible by the metabolism of most pesticides (24%) (Abass et al., 2012) and drugs (60%) (Zanger and Schwab, 2013). The fact that fipronil is metabolized only by the CYP3A4 isoform increases the risk of human intoxication by this pesticide since only this enzyme is involved in its metabolism and any interference in the hepatic metabolic route, such as its inhibition by another xenobiotic, may cause fipronil accumulation in the human body (Chiba et al., 2009). In the case reported regarding human intoxication by fipronil through dermal and inhalation exposure, the administration of benzodiazepines, B1 agonists and steroids was performed (Chodorowski and Anand, 2004). If any of these indicated drugs were able to inhibit the CYP3A4 activity, it could result in fipronil accumulation in the human body and increase the toxic effects (Chiba et al., 2009). Therefore, although no enantioselectivity was observed, the identification of CYP3A4 as the only CYP450 isoform responsible for fipronil hepatic metabolism presents essential information for its risk assessment in humans and provides important knowledge to avoid possible pesticide-drug interactions in the case of human intoxication.

4. Conclusion

For the first time, the human enantioselective *in vitro* metabolism of fipronil was investigated and its risk was assessed. The enantioselective kinetic parameters were determined by considering the fipronil solubility in the microsomal medium, which is a crucial parameter for accurate enzymatic parameter measurements. In addition, the main CYP450 isoform involved in the metabolism of the enantiomers was determined by using two different approaches and by normalization that considers the abundance of the isoforms in native HLMs. Although no enantioselective differences were observed, the obtained results provide reliable information concerning the fipronil risk assessment for humans. They indicated that fipronil may be cleared by the liver (exclusively by the CYP3A4 isoform). E_H values showed that if fipronil is ingested, the first pass effect will not be significant in decreasing its concentration from the blood. These results indicate that any damage to the liver function or the inhibition of the CYP3A4 isoform may result in fipronil accumulation in the body, which may lead to human intoxication. These results provide important information concerning the fipronil risk assessment for humans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.060.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.060.

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