Environment International 103 (2017) 91–98

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Hydroxy-fipronil is a new urinary biomarker of exposure to fipronil

Natalia Vasylieva [a], Bogdan Barnych [a], Debin Wan [a], El-Sayed A. El-Sheikh [b], Hai M. Nguyen [c], Heike Wulff [c], Rebecca McMahen [d], Mark Strynar [d], Shirley J. Gee [a], Bruce D. Hammock [a,*]

[a] Department of Entomology and Nematology, and UCD Comprehensive Cancer Center, University of California Davis, Davis, CA 95616, United States
[b] Faculty of Agriculture, Plant Protection Department, Zagazig University, Egypt
[c] Department of Pharmacology, University of California Davis, Davis, CA 95616, United States
[d] United States Environmental Protection Agency, National Exposure Research Laboratory, Research Triangle Park, NC, United States

ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form 15 March 2017
Accepted 16 March 2017
Available online 23 March 2017

Keywords:
Insecticide
GABA receptor bioassay
Metabolite
LC-MS/MS
Antibody
Immunoassay
Screening
Sensor

ABSTRACT

Occupational medical surveillance is highly desirable in manufacturing facilities where exposure to chemicals is significant. The insecticide fipronil is generally considered safe for humans but with increasing use, exposure to fipronil is of concern. Identification of urinary metabolites of fipronil may allow development of affordable, cheap and rapid procedures for human exposure evaluation. In this study we developed a fast and easy approach for synthesis of hydroxy-fipronil, a potential urinary metabolite of fipronil. This standard was used to develop a sensitive analytical LC-MS/MS method with a limit of quantification (LOQ) of 0.4 ng/mL. Fipronil sulfone, a known metabolite, and hydroxy-fipronil were quantified in urine samples from rats treated with a fipronil containing diet. Fipronil sulfone concentration centered around 20 ng/mL, while the concentration of hydroxy-fipronil was dose-dependent ranging in 10–10,000 ng/mL and thus being a more sensitive marker of fipronil exposure. A fipronil immunoassay with cross-reactivity to hydroxy-fipronil showed a good correlation in signal intensity with LC-MS data. It was also used to demonstrate the applicability of the method for sample screening in the evaluation of exposure levels.

1. Introduction

Fipronil is a broad spectrum insecticide from the phenylpyrazole family. It is often used in four major domains: pest control in a wide variety of field crops, urban pest management, veterinary applications (especially in topical pet care products), and to control rice water weevil infestation in rice-crawfish rotation (EPA, 2011; Jackson et al., 2009; Simon-Delso et al., 2015; Watts, 2012). Fipronil is a neurotoxic compound acting on invertebrate GABA-gated chloride channels in the central nervous system and thus has found a wide application mainly because the target insects have not developed resistance to the pesticide. Fipronil is also relatively persistent in the environment providing long-term protection, with half-life ($t_{1/2}$) over 100 days in certain conditions (Gunasekara et al., 2007). A recent report by the U.S. Environmental Protection Agency (EPA) indicated a significant increase in the amount of fipronil used between 1998 and 2008 (Simon-Delso et al., 2015).

With increasing amounts of insecticide use, human exposure to fipronil is of greater concern, in particular in the case of people directly working with the chemical at manufacturing plants and those applying it in the fields and other corresponding sites. In addition to occupational exposure, the general population is also exposed to fipronil through household uses of fipronil. In particular, human exposure may occur through application of fipronil to animals and interaction with pets. Animals may spread fipronil residues by rubbing against carpet and furniture, suggesting sub-chronic or chronic exposure in the general population (Cochran et al., 2015). Although, fipronil is accepted to be relatively safe for humans because of its lower binding affinity to vertebrate GABA-gated chloride channels (Hainzl et al., 1998), it has been recently demonstrated that exposure to micromolar concentrations of fipronil induced cell death (Vidau et al., 2009; Vidau et al., 2011). Interestingly, cytotoxic effects of fipronil were shown to be stronger with lower micromolar concentrations and less pronounced with higher concentrations (Das et al., 2006), indicating that continuous exposure to low levels of fipronil as in the case of occupational exposure may have a pronounced impact on human health. A number of studies with animals showed that continuous exposure to fipronil leads to significant hepatic effects (e.g. periacinar hypertrophy) in mice and rats even at low doses. Mice showed reduced bodyweight gains at the high doses tested (4.5 mg/kg body weight (bw)/day for 13 weeks) (Hamernik, 1998). Rats, in particular females, showed a significant increase in liver weight following treatment with fipronil even with low doses (3.4 mg/kg bw) (Hamernik, 1998). Dogs treated with a diet containing

* Corresponding author.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

http://dx.doi.org/10.1016/j.envint.2017.03.012
0160-4120/

low fipronil doses (up to 3 mg/kg bw/day) did not experience abnormal weight gain or severe neurological effects (usually 6–30 mg/kg bw for flea and tick control). However, other signs of toxicity were observed including convulsions, head nodding and muscles twitching (Hamernik, 1998; Woodward, 2012). Herin et al. (2011) assessed serum concentrations of thyroid-stimulating hormone (TSH), fipronil and fipronil sulfone in 159 workers in a factory manufacturing fipronil-containing veterinary drugs. They found a significant positive correlation between exposure and fipronil/fipronil sulfone concentration in the blood. In addition, fipronil sulfone concentration correlated negatively with serum TSH. Authors suggested the possibility that fipronil has a central inhibitory effect on TSH secretion in humans that potentially leads to adverse health consequences, the major one being loss of bone density and osteoporosis (Clark et al., 2010; Ozkaya et al., 2015). Thereby they emphasized that close occupational medical surveillance is highly desired to monitor the exposure of factory workers to fipronil in order to maintain health and well-being.

A number of studies were conducted to determine fipronil distribution and metabolism in mammals, as well as to identify its metabolites as potential biomarkers of exposure to fipronil (Cravedi et al., 2013; FAO, 2001; McMahen et al., 2015). Fipronil sulfone is a commonly accepted and proven major metabolite in all tissues, excrement, and blood (Cravedi et al., 2013; FAO, 2001; McMahen et al., 2015). Patients with signs of distress indicating potential poisoning with fipronil are usually subjected to blood analysis to identify the parent compound or fipronil sulfone (Mohamed et al., 2004). However, blood screening is an invasive method causing discomfort, pain and carries a risk of developing infection. Therefore, urine is a much more convenient biospecimen for screening tests. Two recent studies performed a detailed analysis of urine samples obtained from rats treated with a fipronil-containing diet (Cravedi et al., 2013; McMahen et al., 2015). Based on mass fragmentation of the compound both teams assigned a structure of hydroxy-fipronil but it has not been proven by analytical methods. Nevertheless, based on signal ratios in chromatograms McMahen et al. (2015) provided an estimate of concentration of metabolites present in the urine. From these data, hydroxy-fipronil appears to be a dominant metabolite among the other identified metabolites of fipronil.

The specific objectives of this study were to develop an approach for the synthesis of hydroxy-fipronil and use it as a standard to verify the identity of the discovered metabolite. A synthetic standard of hydroxy-fipronil was tested on mammalian $GABA_A$ receptors to characterize the biological activity of the new compound. It was also used to assess its toxicity to insects. Finally, hydroxy-fipronil was used to develop a high performance liquid chromatography method (HPLC) coupled with tandem mass spectrometry detection (MS/MS) to provide a quantitative estimate of the biomarker in the urine of treated animals.

2. Materials and methods

Information concerning chemicals, instruments, buffers, reagents and synthesis is detailed in the Supporting Information (SI) or in the sections below.

2.1. Urine samples

Urine samples were generated as part of a study reported by Freeborn et al. (2015). Briefly, rats were treated by oral gavage with a fipronil-containing corn oil using an 18 Ga feeding needle with a blunt tip. Animals were treated daily at 5 or 10 mg/kg either for two weeks (repeated) or on a single day only. Urine was collected in a syringe either from voids on a clean table or via bladder puncture and transferred to a micro-centrifuge tube, immediately frozen on dry ice, and stored at −80 °C.

2.2. Enzymatic hydrolysis of urine

Urine aliquots of 250 μL were mixed with 150 μL of methanol and hydrolyzed using a 100 μL solution containing 33 μL (85,000/7500 U/mL) of β-glucuronidase/sulfatase and 1.1 mL of 1 M ammonium acetate buffer at pH 5.5. The reaction was left overnight at 37 °C with gentle mixing.

2.3. Hydroxy-fipronil identification

Analysis was carried out with a method reported by McMahen et al. (2015). Briefly, an Agilent 1100 HPLC interfaced with an Agilent 6210 (TOF) mass spectrometer fitted with an electrospray ionization (ESI) source was used. The HPLC was performed with a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 3.5 μm, Agilent Technologies) fitted with a Phenomenex guard column (Torrance, CA). The method consisted of the following: 0.2 mL/min flow rate; at 30 °C; mobile phases: A: ammonium formate buffer (0.4 mM) and DI water:methanol (95:5 v/v), and B: ammonium formate (0.4 mM) and methanol:DI water (95:5 v/v); gradient: 0–5 min a linear gradient from 50:50 A:B to 100% B; 5–15 min, 100% B; 15–18 min re-equilibration to 50% A and 50% B.

2.4. Method 1 (the Hammock group). For fipronil sulfone and hydroxy-fipronil

Rat urine samples (40 μL) were mixed with 10 μL of 1 μM 12-(3-cyclohexyl-ureido)-dodecanoic acid (CUDA) methanol solution. We used CUDA as an external standard since it showed a retention time close to fipronil sulfone, as well as spike-recovery studies with blank urine matrix showed CUDA to be an appropriate standard to account for ion suppression. The samples were cleared with a centrifugal filter device under 20,000*g* for 5 min. The resulting solutions were transferred to vials with 100 μL volume inserts, and stored at −20 °C prior to analysis. Separation of the target compounds was performed on an UPLC system (Waters Corp., Milford, MA). Samples were stored in an autosampler at 4 °C, and 10 μL were injected by a partial loop with needle overfill. The UPLC column Kinetex C18 (1.7 μm, 2.1 × 100 mm, 1.7 μm particle size, Kinetex, Phenomenex) was kept at 40 °C. Mobile phases were composed of water with 0.1% acetic acid (phase A) and acetonitrile with 0.1% acetic acid (phase B). The following gradient was applied: 0–3 min 20% B, 3.1–6 min, a linear gradient from 20 to 80% B, 6.1–8 min, a linear gradient from 80 to 100% B, 8.1–9.1 min, 100% B, 9.1–10 min re-equilibration to 20%. The flow rate remained constant at 0.4 mL/min. The run event was designed to pre-purify samples online, 0–3 min to waste; 3–8 min to MS; 8–10 min to waste. The UPLC system was interfaced with the Quattro Premier MS equipped with an ESI source operated in positive ionization mode. MS operating parameters and compound specific information are provided in the SI, Tables S1 and S2. The data were acquired and processed using Masslynx 4.1 software with instrument and Masslynx 4.1 with TargetLynx.

2.5. LC/MS/MS analysis. Method 2 (the EPA group). For fipronil sulfone only

Method 2 is an alternative method for fipronil sulfone quantification and for independent evaluation of the accuracy of method 1. Analysis was carried out with a method reported by McMahen et al. (2015). Briefly, rat urine (100 μL) was precipitated with 900 μL of cold acetonitrile and centrifuged for 8 min at 12,500 ×*g*. An aliquot of the supernatant was extracted and mixed 50:50 with 10 mM ammonium acetate buffer before LC/MS analysis. Quantification analysis (LC/triple-quad) was carried out using an Agilent 1100 HPLC interfaced with a Sciex 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) fitted with an ESI operated in the negative ionization mode. Fipronil sulfone specific transitions used for quantification were 451.1/415, 451.1/281.9, 451.1/243.9. The HPLC system consisted of a Phenomenex Luna C18 column (50 × 3 mm, 5 μm; Torrance, CA, USA)