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The tissue distribution, metabolism and hepatotoxicity of benzoylurea pesticides in male *Eremias argus* after a single oral administration

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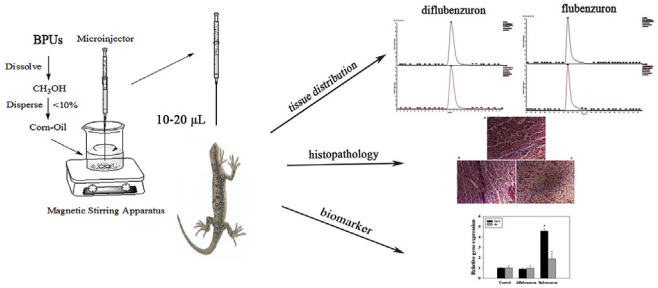
HIGHLIGHTS

- Diflubenzuron preferred to accumulate in the fat and brain.
- The flufenoxuron levels in all tissues were greater than 1.0 mg kg^{-1}.
- The excretion of flufenoxuron in the faeces was 1.5 fold higher than diflubenzuron.
- The *Cyp1a* and *Ahr* genes can serve as biomarkers to assess the liver toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

Benzoylurea pesticides (BPUs) are widely used to control the locust, but the toxicokinetics and hepatotoxicity of BPUs in lizards have not been investigated. In this study, the tissue distribution, metabolism and liver toxicity of diflubenzuron and flufenoxuron were assessed in the *Eremias argus* following a single oral exposure. Diflubenzuron preferred to accumulate in the fat and brain (>1.0 mg kg^{-1}) and was rapidly eliminate in other tissues. In the liver, 4-chloroaniline was one of diflubenzuron metabolites, although with a concentration less than 0.05% of the accumulated diflubenzuron. No significant difference was observed in the liver histopathology between the control and diflubenzuron exposure group. The expressions of *Cyp1a* and *Ahr* gene which control the cell apoptosis were also equal to the control level. After flufenoxuron exposure, biomodal phenomenon was observed in the liver, skin, brain, gonad, kidney, heart and blood circulation was an important route for the flufenoxuron penetration. The concentrations of flufenoxuron in all tissues were greater than 1.0 mg kg^{-1} at 168 h. The excretion of flufenoxuron in the faeces was 1.5 fold higher than diflubenzuron. The hepatocytes in the flufenoxuron treated group showed vacuolation of cytoplasm and decreased nucleus. In addition, the *Cyp1a* and *Ahr* genes were significantly up-regulated in the flufenoxuron exposure group. These results suggested that the higher hepatotoxicity of flufenoxuron may be attributed to the higher residual level in the lizard tissues and the *Cyp1a* and *Ahr* genes can serve as biomarkers to assess the liver toxicity.

1. Introduction

The lizards are the largest family of reptiles, accounted for 71% of the reptile species (Gibbons et al., 2000). It has been regarded as an

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ideal model to assess the toxic effect of contaminants on the reptiles (Talent et al., 2002). The Mongolian racerunner (*Eremias argus*) is a small lizard species belonging to family Lacertidae, Reptilia. *Eremias argus* primarily inhabits grasslands near mountains, sand dunes formed along coastlines in the Korean peninsula, Mongolia, and certain areas of Russia and China (Kim et al., 2010).

In the grasslands, benzoylurea pesticides (BPUs) are widely used to control locust through inhibiting the chitin synthesis (Coppen and Jepson, 1996). Diflubenzuron is one of the early used BPUs, which could persistent in the grassland for two months (Symmons, 1992). The aerial application of diflubenzuron resulted in deposition levels ranging from 867.5 to 1824.4 ng g^{-1} (Rodriguez et al., 2001). Diflubenzuron has limited impact on the non-target invertebrate populations (Soltanimazouni, 1994) and low toxicity to birds (Whitmore et al., 1993).

Several other BPUs, such as flufenoxuron and teflubenzuron are now available as candidates in locust control for their enhanced toxicity compared with diflubenzuron (Clarke and Jewess, 1990). After leaching for 70 d in the soil, more than 99% of diflubenzuron was eliminated while flufenoxuron and novaluron remained at >30% and 50%, respectively (Hsiao et al., 2013). Lizards can contact with the pesticides through both oral and dermal routes. However, the toxic effect of BPUs to lizards has not been investigated.

The degradation of BPUs is mainly through cleavage of the urea bridge. 4-chloroanilian and 2,6-difluorobenzamide are two of the possible metabolites of BPUs (Fig. S1), which have been detected in the water (Rodriguez et al., 1998). 2,6-difluorobenzamide is detected on the foliage due to the photodegradation of diflubenzuron (Rodriguez et al., 2001). 4-chloroanilian is not the major metabolite in the fish and rat, but it is considered to be mutagenic, and possibly a human carcinogen (Olsvik et al., 2013).

The liver is one of the most sensitive organs to show alteration in biochemistry, physiological and structure following exposure to various types of environmental pollutants (Giari et al., 2007). The enzymes, especially cytochrome P450 (CYP) in the liver play an important role for the detoxification of contaminants. However, there is paucity of information regarding the impact of BPUs on the metabolic enzymes.

The aim of this study is to investigate the bioaccumulation, metabolism and hepatotoxicity of BPUs (diflubenzuron and flufenoxuron) in the Chinese native lizard (*Eremias argus*) after a single dose treatment. Uptake kinetics in the lizard tissues was examined. The hepatic index, liver histopathology and metabolism-related genes were measured to explore the toxic effect of BPUs in the lizard liver.

2. Methods and materials

2.1. Chemicals

Diflubenzuron, 4-chloroaniline and flufenoxuron (Fig. S1) were (purity 98%) purchased from J&K Scientific Ltd. (Beijing, China). The solvents including methanol, acetonitrile, and *n*-hexane (HPLC grade) were obtained from Dikma (Beijing, China).

2.2. Animal husbandry

We collected juvenile *Eremias argus* from the wild in the Inner Mongolia Province. We have maintained them in our laboratory for more than 4 years and established the reproduction method of *Eremias argus* (Wang et al., 2014). The 2–3-year old mature *Eremias argus* (3.5–4 g) were obtained from our breeding colony in Changping district, Beijing, China on 20th May. Due to the potential variability in dosage associated with vitellogenesis and egg production, females were not included in the exposure experiment. The lizards were kept in 5.0 × 1.2 × 0.4 m solid bottom indoor aquariums. Ultraviolet lamps were set on 12 h:12 h light/dark cycles to provide enough light and maintain the needed temperature. The temperature and humidity were maintained at 25–30 °C and 30–50%, respectively. The lizards were fed with live mealworms (*Tenebrio molitor*) twice a day and the lizard excreta were removed every other day.

2.3. Exposure experiment and sample collection

The data regarding the exposure and toxic effects of BPUs on lizards are lack. Birds have been used as a surrogate for reptiles to assess the risk of pollutants (Weir et al., 2010). The LD_{50} of diflubenzuron and flufenoxuron is greater than 2000 mg kg^{-1}. In this study, 1% of LD_{50} value (20 mg kg^{-1}) was selected as the exposure concentration for lizards.

Before experiment, the lizards were allowed to acclimate in the experimental glass cages (60 × 60 × 40 cm) for one week, and then separated randomly into the control and exposure groups (total 3 groups, control group n = 6, exposure groups n = 48). The lizards in the exposure groups were orally administered (10–20 μL) with 20 mg kg^{-1} diflubenzuron or flufenoxuron (diluted by corn oil) once. In the control group, only methanol diluted with corn oil was dosed. In the exposure group, the lizards were killed at 1, 3, 6, 12, 24, 72, 120, 168 h after dosing. In the control group, the lizards were only sacrificed at 168 h because of its non distinctiveness among different time points (Chang et al., 2016). The body weights of lizards were measured. The blood was immediately centrifuged at 2500 × g for 10 min, and upper phase (plasma) was collected. The liver, brain, kidney, heart, skin, fat, and gonad were weighted and then frozen at −20 °C. A part of liver was stored in 4% paraformaldehyde or RNA store. Two lizards were selected randomly from each group, and three replicates were prepared. The faeces of the lizards were also collected during 7 d exposure and stored at −20 °C before analysis.

2.4. Chemical analysis

The plasma (50–100 μL) or homogenized tissue matrix (0.05–1.32 g) was put into a 50 mL polypropylene centrifuge tube with 15 mL acetonitrile as extracting solution. The tube was stirred for 2 min on a vortex mixer, ultrasound extracted for 10 min and centrifuged at 8000 rpm for 5 min. The extraction was repeated again, the upper phase was collected and combined. The extracts were mixed with 2 × 30 mL *n*-hexane for liquid-liquid partition to remove most of lipids (only for liver and fat extraction). The upper layer was collected and filtered through 10 g of anhydrous sodium sulfate for dehydration and evaporated to dryness on a vacuumed rotary at 40 °C. The extract was diluted with 1 mL methanol and passed through a 0.22 μm filter membrane before measuring on HPLC/MS/MS.

Diflubenzuron, flufenoxuron and 4-chloroaniline were detected by HPLC/MS/MS. HPLC/MS/MS analyses were performed on a TSQ QUANTUM ACCESS MAX triple quadrupole MS and an Accela 600 pump/auto sampler HPLC (Thermo Electron, Hopkinson, MA). The data were collected and analyzed by the Thermo Fisher LC Quan software package (v. 2.7). A C_{18} column (2.1 mmΦ × 100 mm × 5 μm, Thermo) was used and the flow rate in the mobile phase was 200 μL min^{-1}. For diflubenzuron and flufenoxuron analysis, the mobile phase was combined with methanol and 5 mmol/L ammonium acetate water (90:10, v/v) while for 4-chloroaniline analysis, methanol and 0.1% formic acid water (80:20, v/v) were used. The analytes were detected by multiple reaction monitoring (MRM) mode using electronic spray ionization mass spectrometry (ESI-MS). The *m/z* 308.99 → 93.18 (negative ion mode), 487.07 → 329.13