Stereoselective metabolism, distribution, and bioaccumulation of triadimefon and triadimenol in lizards

Yinghuan Wang, Dingyi Yu, Peng Xu, Baoyuan Guo, Yanfeng Zhang, Jianzhong Li, Huili Wang*

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Haidian District, Beijing 100085, PR China

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ABSTRACT

In this research, Chinese lizards (*Eremias argus*) were chosen as laboratory animal to evaluate the stereoselectivity in the processes of metabolism, distribution, and bioaccumulation of triadimefon. A validated chiral high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC–MS/MS) method was developed for determining enantiomers' residues of parent compound triadimefon and its metabolite triadimenol in lizard blood and tissues. Pharmacokinetic results of single-dose exposure suggested that S-(+)-triadimefon was metabolized easier than R-(−)-triadimefon, and RR-(+)-triadimenol was the main metabolic product of triadimefon. During the continuous exposure of two dose (40 mg/kg^{bw}·d and 200 mg/kg^{bw}·d), enantiomers of triadimefon and triadimenol were detected in all body compartments, with the highest triadimefon concentrations in brain. However, the triadimenol concentrations were not significantly different among the compartments. The concentrations of RS-(+)-triadimenol were negative correlated with concentrations of RR-(+)-triadimenol both in blood ($r = -0.775$, $p = 0.024$) and liver ($r = -0.834$, $p = 0.02$) in 200 mg/kg^{bw}·d group, which indicates that chiral conversion between enantiomers of triadimenol might exist in the metabolic process of triadimefon. In all the processes, the enantiomer fractions (EFs) of R-(−)-triadimefon and RR-(+)-triadimenol were significantly different from their natural ratios, 0.5 and 0.1, respectively, which proved that metabolism, bioaccumulation, and distribution of triadimefon and triadimenol in lizards were enantioselective. These results help enrich and supplement the knowledge of the stereoselective behaviour of triadimefon and triadimenol in reptile.

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1. Introduction

Pesticides are widely adopted in agriculture to control pest damage to crops and raise production. However, there is proof that the residues of some pesticides and their metabolites cause environment problems (Randhawa et al., 2014). Triadimefon and triadimenol are two registered broad-spectrum systemic fungicides, and extensively used in the field (Crowell et al., 2011). Triadimefon (TF) [(RS)-1-(4-chlorophenoxy)-3, 3-dimethyl-1-(1H-1, 2, 4-triazol-1-yl) butan-2-one, CAS NO: 43121-43-3] has been commonly used for the control of mildews and fungi on food crops, turf grasses, shrubs, and trees (Roberts and Hutson, 1999). Triadimenol (TN) [(1RS, 2RS; 1RS, 2SR)-1-(4-chlorophenoxy)-3, 3-dimethyl-1-(1H-1, 2, 4-triazol-1-yl) butan-2-ol, CAS NO: 55219-65-3], a metabolite of triadimefon, has greater fungicidal activity than TF (Liang et al., 2013). TF and TN enter into the soil ecosystem because of direct spraying on the soil surface during agriculture

application, which poses potential danger to the animals living in the soil. Previous studies showed that TF and TN have clear teratogenic effects on the bronchial arches and cranial nerves of rat embryos (Menegola et al., 2000). TF and TN are also harmful to mammalian central nervous system and are neurotoxins in rats, mice, and rabbits (U.S. Environmental Protection Agency (U.S. EPA), 2006; Walker et al., 1990). These two fungicides have been categorized as “possible human carcinogens” (U.S. Environmental Protection Agency (U.S. EPA), 2006).

Pesticides have been considered as one of the major factors contributing in the global decline of reptiles (Gibbon et al., 2000). Past attention was focused on measuring body burdens of various pollutants to wild reptiles which gave an understanding of historical exposure of given populations (Buono et al., 2007; Holem et al., 2008; Mann et al., 2007; Moss et al., 2009; Trinchella et al., 2006; De Falco et al., 2007; Keller et al., 2006; Simoniello et al., 2010; van de Merwe et al., 2010). However, the population-level effects and actual risks of pollutants on reptiles are still generally understudied (Weir et al., 2010). In soil, TF is easily decomposed and converted to TN with a half-life of 6–30 days (Petrovic et al., 1993; Singh, 2005). As a comparison, TN exhibits a much longer persistence (soil half-life > 240 days)

* Corresponding author. Fax: +86 1 62849790.

E-mail address: huiliwang@rcees.ac.cn (H. Wang).

(Bromilow et al., 1999). Because the abuse of triazole pesticides in China (Lin et al., 2008), these two triazole pesticides may be transported from cultivated soils in significant amounts especially TN, which pose threats to the life in soils. However, to our knowledge, there is no known research on the toxicity of these two compounds to reptiles. Reptiles are remaining as the least studied vertebrates in ecotoxicology (Hopkins, 2000).

In order to fill the gap in this research area, Chinese lizards (*Eremias argus*) were used in this study to evaluate the acute and chronic effects of TF and TN on reptiles. Lizards are regarded as one of the significant ecological animals in agriculture systems because they prey on crop pests. *E. argus* are widely distributed in the north of Yangtze River including North China Plain and Northeast China Region, the main agricultural areas in China. Excessive usage of TF and TN in these areas (Lin et al., 2008) has been a direct threat to the *E. argus*.

About 30 percent of the organic agrochemicals are chiral compounds and consist of two or more enantiomers, which have identical physico-chemical properties (Ulrich et al., 2012). Nevertheless, the bioactivity, toxicity, metabolism, excretion, distribution, and bioaccumulation of these enantiomers may be entirely different (Williams, 1996). The enantiomers of chiral pesticides could be different when binding to structure-sensitive biological receptors and naturally occurring chiral biomolecules because of their different molecular configurations (Dong et al., 2013). Stereoselectivity in the processes of absorption, distribution, metabolism, and bioaccumulation in organisms may result in ecotoxicological effects that are beyond the traditional knowledge that treat them as single compounds in risk assessment (Buser et al., 2002; Dong et al., 2013; Wong, 2006). TF has one chiral center and consists of an equimolecular mixture of the two enantiomers while TN has two chiral centers and consists of four stereoisomers including two pairs of diastereomers. The biotransformation of TF into TN is stereoselective and the biological response of each TN stereoisomer is different (Deas et al., 1986; Garrison et al., 2011; Spindler and Fruh, 1998) (Fig. 1). The stereoselective formation of TF and TN is an important issue in ecological risk assessment when considering the difference in fungicidal activity and toxicity among the enantiomers (Li et al., 2014). Stereoselective metabolism of TF to TN has been observed in soil microorganisms and rainbow trout (Garrison et al., 2011;

Kenneke et al., 2010; Li et al., 2011). Unfortunately, the stereoselectivity of TF and TN in reptiles have never been studied.

To describe actual environmental fate and ecological risks from a complete view, and understand environmental impact of these pesticides, it is crucial to study the stereoselective behaviors of chiral pesticides. In this study, the experiments were conducted to obtain a better understanding of the biological fate of the TF and TN enantiomers in lizard blood on a single acute exposure and assess the stereoselective metabolism, distribution and bioaccumulation of TF and TN in different tissues in lizards on the continuously exposure. The results complement the knowledge of the stereoselectivity of chiral pesticides in reptiles, and are instructive for future TF and TN toxicity study on reptiles.

2. Materials and methods

2.1. Chemicals and reagents

Racemic TF (99.55 percent) and TN (99.1 percent), analytical standards of TN-A (racemate of RS- and SR-enantiomer, 99.9 percent purity) and TN-B (racemate of RR- and SS-enantiomer, 99.9 percent purity) were kindly provided by College of Science, China Agricultural University (Beijing, China) (Liang et al., 2012). Stock solutions of TF and TN were prepared in methanol (HPLC grade, Dikma, USA) at 1000 mg/L and kept in dark at -20°C .

2.2. Dosing

Dosing was prepared according to those described previously with modifications (Di Renzo et al., 2009; McFarland et al., 2011, 2009; Suski et al., 2008). TF was first dissolved in the ethanol (analytical grade, Beijing Chemical Reagent Co. Ltd, China) then dispersed in corn oil (ethanol:corn oil was 1:9, v/v). Test preparations were stored in dark at 4°C until use to prevent compound degradation. The corn oil–ethanol lactescence were warmed to room temperature (25°C) and continually mixed by magnetic stirring apparatus (90-1, Shanghai ZhenRong Scientific Instrument Co. Ltd, China) before dosing. The micro injector was used to oral inject a volume of 20–50 μL corn oil or corn oil–ethanol lactescence.

2.3. Test lizard and culture conditions

The juvenile *E. argus* were collected in Abag Banner, Inner Mongolia (China) and maintained in laboratory since July 2009. Lizards were kept in $5 \times 1.2 \times 0.4 \text{ m}$ solid bottom indoor aquarium covered with 10 cm mollisol and fallen leaves. The

Fig. 1. The metabolic transformation of TF to TN and the enantiomers of TF and TN.

temperature and humidity were maintained at 25–30 °C and 30–60 percent. Daylight lamps (100 W) were set to a 14:10 h light:dark photoperiod to provide enough light and maintain the needed temperature. Lizards were fed with mealworms (*Tenebrio molitor*) twice a day. The water was sprayed every other day and the excreta and residues were cleaned twice a week.

The sexually mature lizards (3–3.5 g, 2–3 years old) used in the experiment were randomly divided into several groups of 10 lizards (sex ratio 1:1). Each group was housed in 30 × 30 × 20 cm glass cage with a water dish. The experimental conditions were the same as the rearing condition. Each lizard was fed two mealworms (about 1.0 cm each) per day.

Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). The animal care and use procedures were approved by Research Center for Eco-Environmental Sciences, CAS.

2.4. Preliminary acute and subacute experiments

Preliminary acute study was conducted according to previous research to find out the safe concentration to carry out the subacute experiment (Feder et al., 1991; U.S. Environmental Protection Agency (U.S. EPA), 1996a, 1996b; Organization for Economic Cooperation and Development (OECD), 1984). Control group, vehicle control group and four treatment groups (Total 6 groups, each group $n=10$) were used in the acute study. In each group, the dose was delivered orally to each individual. Each lizard was independent and it is appropriate to consider these as replicates (same in further experiments). Four experimental dose ranges (2, 20, 200, 2000 mg/kg^{bw}) were selected. Lizards were administered orally once and responses were recorded during a 7-day observation period. The 1/10 of the maximum non-lethal dose in acute study was chosen as the highest dose in subacute experiment.

A subacute experiment was conducted to find out the safe concentration to carry out further experiments. The control group, vehicle control group and three treatment groups (Total 5 groups, each group $n=10$) were used in 14-day subacute experiments. Three experimental dose ranges (40, 90, 200 mg/kg^{bw}·d) were selected based on results from the acute study. Doses were administered daily for 14 consecutive days with dose adjusted the body weight of each lizard. All signs of intoxication, other abnormal behaviour, and mortality were recorded. The maximum non-lethal dose in subacute study was chosen as the highest dose in metabolism, distribution, and bioaccumulation exposure.

2.5. Metabolism, distribution, bioaccumulation, and restoring experiment

Two experimental dose ranges (40, 200 mg/kg^{bw}) were selected based on results from the subacute study for the further experiments.

Metabolism experiment was conducted using 54 new lizards (27 lizards for each dose). Each lizard was administered orally once. Blood samples were collected at 2, 6, 8, 12, 24, 48, 72, 120 and 168 h after lizards were dosed. Three lizards were euthanized with carbon dioxide randomly for each dose group at once.

Considering the possible death of lizards during the exposure period, a distribution experiment was conducted using 10 new lizards (5 lizards for each dose). These lizards were continuously administered of TF daily for 14 days with dose adjusted according to the body weight. All lizards were euthanized with carbon dioxide after 14 days of exposure. Each lizard's blood, brain, heart, lungs, liver, kidneys, and intestines were collected for concentration analysis of enantiomers of TF and TN.

A chronic bioaccumulation experiment was conducted using 80 new lizards (40 lizards for each dose). New lizards were continuously administered of TF daily for 35 days with dose adjusted according to the body weight. Lizards were euthanized with carbon dioxide at 1, 3, 5, 7, 10, 14, 21, 28, 35 days. Blood and liver samples were collected for concentration analysis of enantiomers of TF and TN. Three lizards were euthanized randomly from each group at once.

A restoring experiment was conducted using 50 lizards (25 lizards for each dose). These lizards were first administered of TF daily for 14 days with dose adjusted the body weight. Then these lizards were stopped exposing under TF and fed normally for 14 days. Lizards were euthanized with carbon dioxide at 1, 2, 3, 5, 7, 10, 14 days in normal feeding. Blood and liver samples were collected for concentration analysis of enantiomers of TF and TN. Three lizards were selected at randomly from each group and were simultaneously euthanized.

2.6. HPLC–MS/MS conditions

HPLC was performed using Thermo ACCELA series (Thermo Electron Corporation, Hopkinson, MA) equipped with ACCELA Autosampler, ACCELA 600 pump, a 20 µL injection loop, and a 2 µL flow cell. Enantiomers were separated on a Phenomenex Lux Cellulose-1 column (250 × 4.6 mm id, 5 µm particles), packed with CSP of CDMPC and obtained from Guangzhou FLM Scientific Instrument (Guangzhou, China). The mobile phase was a mixture of 73 percent methanol, 27 percent water with a flow rate of 0.5 mL/min. Chromatographic separation was conducted at 20 °C, and the injection volume was 10 µL. TSQ QUANTUM ACCESS

MAX was used for LC–MS/MS analysis (Thermo Electron Corporation, Hopkinson, MA). Quantification was achieved in positive-ion mode (ESI+). The signals were received and processed with Thermo Xcalibur 2.2 SP1.48 software. The optimized major working parameters were as follows: Spray Voltage 3200 V, Vaporizer Temperature 250 °C, Sheath Gas Pressure 30 psi, Aux Gas Pressure 10 arbitrary units, Capillary Temperature 350 °C, Capillary Offset 35 V, Q2 Collision Gas Pressure 1.5 mTorr. Multiple reactions monitoring mode was used; the precursor and product ions of TF and TN with corresponding declustering potentials and collision energies are summarized in Supporting Information Table S1.

2.7. Extraction procedure

Whole blood (50–100 µL) or homogenized tissue matrix (0.01–0.03 g) was transferred into a 2-mL polypropylene centrifuge tube and added with 2 mL acetonitrile (analytical grade, Beijing Chemical Reagent Co. Ltd, China). The mixture was vortex-mixed for 3 min, exposed to ultrasonic vibration for 20 min and then centrifuged at 10,000 r/min for 5 min. The supernatants were transferred to a separator funnel. The sample was re-extracted in the same way and the supernatants were combined. Five mL *n*-hexane (HPLC grade, Dikma, USA) was added for liquid–liquid partition to extract most of lipid. The upper layer of *n*-hexane was discarded, and the layer of acetonitrile was collected and evaporated to near dryness with a vacuum rotary evaporator at 30 °C under a gentle nitrogen stream. The residue was re-dissolved in 1 mL of methanol and filtered through a 0.22-mm filter into a sample vial for HPLC–MS/MS analysis.

2.8. Data and statistical analysis

The enantiomer fraction (EF) was used to measure the enantioselectivity of the bioaccumulation of TF enantiomers (Eq. (1)).

$$EF = \text{concentration of } \frac{(-)}{(-) + (+)} \quad (1)$$

The EF values defined range from 0 to 1, with EF=0.5 representing the racemic mixture.

Each diastereomer of TN is a racemic mixture. TN-A is 80 percent of the total triadimenol, while TN-B is about 20 percent (Detail shown in Part 3.1). The EF measured for each of the four triadimenol stereoisomers are calculated according to Eq. (2) (Kenneke et al., 2010).

$$EF = \text{concentration of } \frac{\text{enantiomer } x}{\text{sum of 4 enantiomers}} \quad (2)$$

Pharmacokinetic parameters were calculated in one dose experiment. The concentrations of TF enantiomers of blood sample would decline after the concentrations reached their maximum value. The TF release pattern was in accord with the first order kinetics. The relation between concentrations of TF enantiomers (C) and the sampling time (t) was calculated according to Eq. (3).

$$\lg C = -\frac{kt}{2.303 + b} \quad (3)$$

The half-life period $t_{1/2}$ of TF enantiomers was calculated according to Eq. (4)

$$t_{1/2} = \frac{0.693}{k} \quad (4)$$

Area under curve (AUC_{0-t}) was calculated by trapezoidal rule. Injected dose (D) was considered as total drug concentration. Blood clearance (CL) was expressed on Eq. (5).

$$CL = \frac{D}{AUC_{0-t}} \quad (5)$$

SPSS 16.0 was used for statistics analysis. All the other values in the text were presented as mean ± SD. A *t*-test was used to show the difference between the means of EF (TF1) or EF (TN4) and its nature ratio. A paired-samples *t*-test was used to determine the difference of the means of EF (TF1) or EF (TN4) between two exposure groups or any two of the tissues (including blood). One-way ANOVA were performed to analyze the significant differences of enantiomer concentrations among the tissues (including blood). Significant difference existed while $p < 0.05$.

3. Results and discussion

3.1. Method validation

Enantiomers of TN and TF were baseline-resolved under the optimized condition. Fig. 2 shows the pattern of a commercial mixture of triadimenol chromatographic separation of TN-A and TN-B, each of which shows two enantiomer peaks in equal amounts (1 and 2, 3 and 4). Each diastereomer is a racemic mixture. TN-A is 80 percent of the

total triadimenol, while TN-B is about 20 percent. Liang et al. (2012, 2013) confirmed elution order. Enantiomer peaks were shown in Fig. 2. (1) SR-(–)-TN (TN1); (2) RS-(+)-TN (TN2); (3) SS-(–)-TN (TN3); (4) RR-(+)-TN (TN4); (5) R-(–)-TF (TF1); (6) S-(+)-TF (TF2). Linear calibration curves were obtained over the concentration range of 0.001–1 mg/L for TN1 ($y=1.7222300x+68,045$, $r=0.9994$), TN2 ($y=16934700x+77,529$, $r=0.9994$), TN3 ($y=1757710x+12,802$, $r=0.9997$), TN4 ($y=2251540x+16,282$, $r=0.9987$), TF1 ($y=15752600x+44,686$, $r=0.9997$), TF2 ($y=14791300x+31,392$, $r=0.9998$). Recovery evaluation was carried out with six replicates at three fortified concentration levels. The concentration levels were 0.01, 0.1, and 1 mg/kg (mg/L for blood) for TF enantiomers; 0.008, 0.08, and 0.8 mg/kg (mg/L for blood) for TN-A stereoisomers, 0.002, 0.02, and 0.2 mg/kg (mg/L for blood) for TN-B stereoisomers. Recoveries of each enantiomer ranged from 88 to 107 percent. The precision of the assay for each enantiomer ranged from 2.5 to 12.8 percent (RSD). LODs were 0.003 mg/kg in lizard tissues and 0.002 mg/L in lizard bloods for the 2 enantiomers of TF. The LODs were 0.002 mg/kg in lizard tissues and 0.002 mg/L in lizard bloods for the 2 enantiomers of TN-A. The LODs were 0.0007 mg/kg in lizard tissues and 0.0006 mg/L in lizard bloods for the 2 enantiomers of TN-B. The LOQs were established as being 0.01 mg/kg (mg/L) for TF enantiomers, 0.008 mg/kg (mg/L) for TN-A stereoisomers, 0.002 mg/kg (mg/L) for TN-B stereoisomers based on the minimum fortified concentration level in lizard tissues and bloods. The stereoselective HPLC–MS/MS method described in this investigation was validated for simultaneous separation and determination of TF and TN stereoisomers in lizards.

3.2. Preliminary acute and subacute experiments

Systemic signs of toxicity were observed in the acute experiments. Anorexia, lethargy, and drumble occurred in 2000 mg/kg^{bw} group. Signs of recovery were showed at day 5 after dosed. TF can produce hyperactivity in both mice and rats, repeated treatment with TF can lead to behavioral sensitization (Reeves et al., 2004, 2003), but those signs weren't observed in this study. No death occurred in all four groups. According to the avian oral toxicity test guidelines of U.S. Environmental Protection Agency (U.S. EPA) (1996a, 1996b), Organization for Economic Cooperation and Development (OECD) (1984), a chemical could be recognized as low acute oral toxicity while there is no mortality occurred at the limit test of 2000 mg/kg^{bw}. TF could be recognized as low acute

oral toxicity to *E. argus*. Dosage of 200 mg/kg^{bw} (1/10 of 2000 mg/kg^{bw}) was chosen as the highest dose in subacute experiment.

Three groups 40, 90 and 200 mg/kg^{bw}·d were relatively safe feeding concentrations in the subacute experiment according to the result of acute experiment. Anorexia and lethargy occurred at the first 3 days, movement and food consumed were recovered at approximately day 5. Only one lizard died in 90 mg/kg^{bw}·d group at day 7, therefore, 200 mg/kg^{bw}·d (maximum no-death dosage) was chosen as the highest dose to carried out the metabolism, distribution and bioaccumulation exposure.

3.3. Metabolism in lizard blood

Changes in the concentrations of racemic parent compound TF and its metabolic product TN in single oral injection of rac-TF were studied (Fig. 3). Concentrations of TF1 and TF2 reached their maximum value at 6 h after administration of rac-TF in 40 mg/kg^{bw} group. However, the time reduced to 2 h in 200 mg/kg^{bw} group. Concentrations of metabolic products TN1, TN3, TN4 reached their maximum value at the 4th hour; concentrations of TN2 reached their maximum value at the 6–8th hour in both groups. These indicated that rac-TF might be absorbed and metabolized rapidly. When the dosage exceeded a level beyond metabolic ability, concentrations of TF1 and TF2 reach their maximum value immediately.

Pharmacokinetic results were shown in Table 1. The $t_{1/2}$ of TF1 and TF2 was 2.33 and 1.98 h in low dose group, 2.84 and 2.71 h in high dose group, respectively. The clearance rate of TF1 and TF2 was 2.30 and 3.13 L/h in low dose group, 7.39 and 10.04 L/h in high dose group, respectively. The concentrations of TF1 were little higher than its antipode in the blood, a *t*-test was carried out to compare the means of the EF values of TF1 in blood samples with 0.5. The average EF(TF1) was 0.568 ($p=0.007$) in low dose group and the average EF(TF1) was 0.568 ($p=0.009$) in high dose group, respectively. These suggested that TF2 was easier metabolized and eliminated than TF1 in lizard blood.

A difference was observed that the concentrations of TN4 were much higher than other enantiomers. As shown in Fig. 2, the content of TN4 was only half of TN-B and 10 percent of the provided mixture of TN. A *t*-test was carried out to compare the means of the EF values of TN4 in blood samples with 0.1. The average EF(TN4) was 0.804 ($p<0.001$) in low dose group and the average EF(TN4) was 0.739 ($p<0.001$) in high dose group. These indicated that the metabolism of TF was stereoselective and TN4 was the main metabolic product.

It is reported that (–)-TF (TF1) was 2-fold more toxic than the racemate, while the (+)-TF (TF2) LC₅₀ fell in between the racemate and TF1 values (Kellock et al., 2009). TN-A is about 10 times more acutely toxic to rats than triadimenol-B (World Health Organization, 2006). The toxicity of TF and TN enantiomers is different. Because of metabolizing to TN rapidly, the ecotoxicology effects of TF we discussed in the paper were actually the combination of all the enantiomers of these two compounds. Due to our experimental set ups, we could not differentiate the effects of individual enantiomers. These effects are worthy of future study.

3.4. Tissue distribution

The final residues of all enantiomers of TF and TN were detected in tissue and blood samples at day 14 in both groups (Supporting Information, Fig. S2). *T*-test was carried out to compare the means of the EF(TF1) and EF(TN4) values in different tissues with EF(TF1)=0.500 and EF(TN4)=0.100 (Supporting Information Table S2). The means of the EF(TF1) values in different tissues are extremely significant difference with 0.5, which indicated that stereoselective distribution of TF enantiomers and its

Fig. 2. Simultaneous stereoselective separation of TF and TN. 0.5 mg/L for each racemate of TF and TN: (1) SR-(–)-TN (TN1); (2) RS-(+)-TN (TN2); (3) SS-(–)-TN (TN3); (4) RR-(+)-TN (TN4); (5) R-(–)-TF (TF1); (6) S-(+)-TF (TF2).

Fig. 3. Blood concentration–time curves and EFs (TF1 and TN4) in Chinese lizard (*Eremias argus*). (a) 40 mg/kg^{bw} · d, TF enantiomers blood concentration–time curves and EFs (TF1). (b) 40 mg/kg^{bw} · d, TN enantiomers blood concentration–time curves and EFs (TN4). (c) 200 mg/kg^{bw} · d, TF enantiomers blood concentration–time curves and EFs (TF1). (d) 200 mg/kg^{bw} · d, TN enantiomers blood concentration–time curves and EFs (TN4). TF1 represents the R-(–)-TF, TN4 represents the RR-(+)-TN.

Table 1
Pharmacokinetic parameters of enantiomers after administration with rac-TF in lizard blood.

Pharmacokinetic parameters	40 mg/kg ^{bw}		200 mg/kg ^{bw}	
	TF1	TF2	TF1	TF2
First-order kinetics equation	$y = -0.1287x + 0.6293$	$y = -0.1518x + 0.6672$	$y = -0.1059x + 0.05567$	$y = -0.1112x + 0.04545$
R^2	0.8478	0.9083	0.9679	0.9932
k	0.2964	0.3496	0.2439	0.2561
$t_{1/2}$ (h)	2.338	1.982	2.841	2.706
$AUC_{(0-24)}$ (mg/L h)	5.656	4.200	12.847	9.780
$AUC_{(0-t)}$ (mg/L h)	17.42	12.80	27.08	19.92
CL (L/h)	2.296	3.125	7.386	10.04

metabolic product TN enantiomers were occurred in different tissues of lizard. In the four enantiomers of TN, the proportion of TN4 was from 53.5 to 79.6 percent in different tissues. In addition, the means of the EF(TN4) values are extremely significant difference with 0.1. These results meant that the concentrations of TN4 were much higher than other three enantiomers in all tissues. TN4 was also the main metabolic product of TF in lizard tissues.

A one-way ANOVA test was carried out to compare the difference of TF and TN concentrations among lizard tissues. During the exposure of 40 mg/kg^{bw} · d, both TF1 and TF2 concentrations in organs were in the following order: brain > intestine > heart > kidney > lungs > liver, while under the exposure of 200 mg/kg^{bw} · d, the order of TF enantiomers concentrations changed to: brain > liver > kidney > heart > lungs > intestine. Both TF1 and TF2 were easier accumulated in brain ($p < 0.05$ in 40 and 200 mg/kg^{bw} · d).

It was reported that TF could induce neurobehavioural effects in rodents. The possible reason might be that TF could act as a dopamine transporter inhibitor and could act on the serotonergic system (Gagnaire and Micillino, 2006; Perkins et al., 1991). The concentrations of TF1 and TF2 in absorptive organ (intestine) were higher than concentrations in metabolic organ (liver and kidney) in 40 mg/kg^{bw} · d group. However, opposite appearance were observed in 200 mg/kg^{bw} · d group ($p < 0.05$). These results showed TF could be metabolized rapidly in low dose group. When the daily dosage exceeded a level beyond metabolic ability, accumulation would also occur in metabolic organs, indicating a substantial stereoselectivity on distribution of TF enantiomers in lizard. The concentrations of TN enantiomers in lizard were depended on the metabolic rate of TF and the degradation rate of themselves. All the four TN enantiomers were detected in all tissues while the residue of TN1, TN2 and TN3

were very low. However, the TN4 concentrations did not show significant difference among the comparisons.

3.5. Enantiomers bioaccumulation

During bioaccumulation experiment, concentrations of TF1 and TF2 stayed low concentrations before day 10 but increased after then until day 35 sustainably both in blood and liver in low dose group (Fig. 4). The increases slowed down after 28 days. Concentrations of TF1 and TF2 first declined before day 3 but increased rapidly to the highest level at about 7–14 days in both the blood and liver in the high dose group. After 14 days of exposure, concentrations reached steady state for two enantiomers. Bioaccumulation of TF and TN enantiomers levels were time-dependent.

One-tailed correlation analysis was carried out to test the correlation of the means of TF concentrations in liver and blood. The results indicated that TF1 concentrations in liver had correlation with TF1 concentrations in blood ($r=0.824$ and $r=0.831$ in 40 and 200 mg/kg^{bw}·d, respectively, $p < 0.01$), TF2 concentrations in liver had correlation with TF2 concentrations in blood ($r=0.905$ and $r=0.900$ in 40 and 200 mg/kg^{bw}·d, respectively, $p < 0.01$). The concentrations of TF enantiomers in liver were consistent with variation of the concentrations in blood. One-way ANOVA test showed that concentrations of TF enantiomers in blood were significant higher than the concentrations in liver at first 14 days in both groups ($p < 0.05$). No significant differences were observed after 14 days ($p > 0.05$). TF enantiomers first absorbed by stomach and intestines then entered into blood. The enantiomers were transported to liver through blood circulation. TF enantiomers were slowly accumulated in liver. TF concentrations in blood were

higher than TF concentrations in the liver at first. However, with the bioaccumulation of TF in liver, there were no significant differences between TF concentrations in liver and blood. The concentration of TF1 in liver was even higher than concentration in blood in 40 mg/kg^{bw}·d ($p=0.036$) group at the end of exposure.

A *t*-test was carried out to compare the means of the EF values in lizard bloods and livers with EF(TF1)=0.5 and EF(TN4)=0.1. Concentrations of TF1 were higher than TF2 in the blood and the liver in both groups. The average EF(TF1) was 0.549 ($p < 0.001$) in blood and average EF(TF1) was 0.590 ($p < 0.001$) in liver in low dose group. The average EF(TF1) was 0.544 ($p < 0.001$) in blood and average EF(TF1) was 0.622 ($p=0.004$) in liver in high dose group. The bioaccumulation behavior of TF was enantioselective. The EF(TF1)s in liver was higher than EF(TF1)s in blood, this result indicated that liver might be the main organ where enantioselective behavior occurred. Concentrations of metabolic product TN4 were much higher than other three enantiomers. The average EF(TN4) was 0.791 ($p < 0.001$) in blood and average EF(TN4) was 0.779 ($p < 0.001$) in liver in low dose group. The average EF(TN4) was 0.678 ($p < 0.001$) in blood and average EF(TN4) was 0.712 ($p < 0.001$) in liver in high dose group. The metabolic processes of TF and bioaccumulation behavior of TN were enantioselective. The EF(TN4)s in low dose group were higher than EF(TN4)s in high dose group in blood (a paired-samples *t*-test, $p=0.009$) and liver (a paired-samples *t*-test, $p=0.015$), respectively. The possible cause of decline of EF(TN4)s might be the bioaccumulation of TN2 in high dose group. On the contrary, concentrations of TN2 showed negative correlation with concentrations of TN4 both in blood ($r=-0.775$, $p=0.024$) and liver ($r=-0.834$, $p=0.02$) in high dose group after day 5. TN2 and TN4 were pair metabolites of

Fig. 4. Accumulation curves for enantiomers of TF and TN in liver and blood of Chinese lizard (*Eremias argus*). (a) blood samples in 40 mg/kg^{bw}·d, (b) blood samples in 200 mg/kg^{bw}·d, (c) liver samples in 40 mg/kg^{bw}·d, (d) liver samples in 200 mg/kg^{bw}·d.

TF2. The chiral conversion between enantiomers of TN, especially chiral conversion between TN2 and TN4 might be the possible reason caused the negative correlation between TN2 and TN4 and the decline of EF(TN4)s in high dose group.

3.6. Chronic effects on lizard

Signs of toxicity in chronic bioaccumulation experiment were similar to the subacute study, though more subtle, and were best characterized as anorexia and generalized, progressive lethargy.

Movement and food consumed were recovered at first week but reduced again after about 2 weeks. Only one lizard died in 40 mg/kg^{bw}·d group, but 17.5 percent mortality was observed in 200 mg/kg^{bw}·d group. Changes in body weight (m) and wet ratio of liver (r) over exposure time were studied (Supporting Information, Fig. S3). Significant losses of body weight in both dose groups were observed (a paired-samples *t*-test, *p*=0.030 and *p*=0.018 in 40 and 200 mg/kg^{bw}·d, respectively). Lizards lost an average of 22.32 and 31.75 percent body weight in 40 and 200 mg/kg^{bw}·d group, respectively. The wet ratio of liver was significantly increased compared to the controls (a paired-samples *t*-test, *p*=0.043 and *p*=0.019 in 40 and 200 mg/kg^{bw}·d, respectively) and showed a clear response to dose (*p*=0.029). The wet ratio of liver increased by 49.0 and 72.1 percent in 40 and 200 mg/kg^{bw}·d, respectively. Hepatic histological lesions might be caused during the exposure. No differences in wet ratio of other organs were found (*p* > 0.05).

3.7. Restoring experiment

Lizards were continuously dosed in the first 14 days and then fed normally for another 14 days in restoring experiment. Concentrations of TF1, TF2, and TN4 declined sharply in the first 3 days during the normal feeding. Concentrations of TN1, TN2, and TN3 stayed low values. The concentrations level of all enantiomers reduced to very low values (<0.005 mg/kg) after 7 days. These results suggested that concentrations of TF and TN could be metabolized rapidly in lizard blood and liver.

Lizards lost an average of 12.47 percent body weight at day 7 but recovered to 11.33 percent at day 14 in 40 mg/kg^{bw}·d group. Lizards lost an average of 15.75 percent body weight at day 7 and 28.32 percent at day 14 in 200 mg/kg^{bw}·d group. Foods consumption was recovered in low dose group. However, anorexia and lethargy were still observed in high dose group. No lizard died in low dose group but 12 percent (3/25) mortality was observed in high dose group. These results indicated that concentrations level of all enantiomers could be reduced rapidly in both group, but the damages to lizards were difficult to restore in high dose group.

4. Conclusions

According to the findings of this study, both the parent compound TF and its metabolic product TN could be metabolized rapidly in lizard. TF had been tested as low acute oral toxicity to *E. argus* in our study. The bioaccumulation of TF and TN enantiomers can still be observed even under the continuously low-dose exposure. During the chronic exposure, the wet ratio of liver was significantly increased and showed a clear response to dose which meant hepatic histological lesions might be caused. These indicated that the low residual of TF and TN in the environment could also be a threat to the lizard and other animals living in the soil. According to the results in restoring experiment, concentrations level of all enantiomers could be reduced rapidly, but the signs of intoxication and foods consumption still could not be recovered in a short period.

In this study, we also observed stereoselectivity of TF and TN enantiomers occurred in all the processes of metabolism, distribution, and bioaccumulation of TF and TN in lizard. To achieve the complete understanding of the effects of TF on Chinese lizard, efforts should focus on analyzing the effects of individual enantiomer of these two compounds.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2014.06.021>.

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