JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Gender-Related Differences in Stereoselective Degradation of Flutriafol in Rabbits

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ABSTRACT: The stereoselective pharmacokinetics of flutriafol were investigated in male and female adult Japanese white rabbits. Following intravenous administration of rac-flutriafol to rabbits at 5 mg/kg (bd wt), the concentrations of the enantiomers in plasma were determined by a HLPC-UV method using a CDMPC-CSP chiral column. *R*-Flutriafol exhibited a shorter distribution half-life but a longer elimination half-life than the *S*-isomer. In female rabbits, the distribution half-lives of *R*- and *S*-flutriafol were found to be 0.09 and 0.18 h, respectively, significantly shorter than those in male rabbits, but the volume of distribution and elimination half-life for flutriafol enantiomers in both sexes of rabbit showed no significant differences. Female rabbits had a higher clearance for both flutriafol enantiomers. The protein binding value was high for both isomers, with enantioselectivity, but no gender difference. It was an important factor in modulating the disposition of flutriafol. Flutriafol concentrations in kidney, liver, fat, and lung were higher than in other tissues at 10 h after administration, and the concentrations of *R*-flutriafol were higher in all tissues than those of its antipode. However, gender difference in flutriafol residues in tissues was not observed. It is concluded that the stereoselectivity of flutriafol on distribution and elimination in rabbits mainly depends upon gender.

KEYWORDS: flutriafol, gender-related differences, stereoselective degradation, plasma protein binding

INTRODUCTION

Flutriafol [(*RS*)-2,4-difluoro-α-(1*H*-1,2,4-triazol-1-ylmethyl)-benzhydryl alcohol, Figure 1] attained an important position in the global fungicide market because of its proven effectiveness in controlling several diseases affecting a wide range of crops. However, this fungicide is an extremely persistent soil triazole, presenting high mobility potential, and likely to be a groundwater contaminant,[1] so there may be an increased risk of secondary poisoning to animals. Furthermore, animals are sensitive to the influence of pesticides because they are able to take up and retain xenobiotics via active or passive processes. Therefore, adverse effects on animals are possible. Despite its low acute toxicity, some works have indicated that exposure to low doses of flutriafol can cause adverse effects in both rats and rabbits.[2,3]

Gender-related pharmacokinetic differences of numerous drugs tested in animal models have received greater attention in recent years since it was first reported that adult male rats remain asleep for a shorter period than adult female rats when administered the same dose of hexobarbital.[4,5] Since then, gender-based differences have been noted for a large number of species including rats, mice, beagle dogs, cats, rabbits, dwarf goats, cattle, and humans,[6−8] but very little attention has been paid to the gender-based chiral dimorphism. Therefore, an understanding of gender differences in metabolism and toxicity enhances the utility of physiologically based pharmacokinetic modeling, an increasingly important tool for chemical risk assessment. Most importantly, gender differences of animals in safety evaluations of chemical entities may provide evidence for biological behavior based on gender in humans.

Flutriafol has one stereogenic center, consisting of a pair of enantiomers. Because the stereogenic center of flutriafol is located close to the 1,2,4-triazole ring, a key template in the binding of flutriafol to their target sites, chirality is expected to play a crucial role in the bioactivities of flutriafol. This has actually been proven by the fact that the (+)-isomer of flutriafol has greater antimicrobial activity than the (−)-isomer against both *Alternaria solani* and *Alternaria mali*.[9] Investigations treating racemates as single entities may produce inaccurate and misleading results in that the enantiomers of chiral compounds often have different biological and physiological properties.[10,11] There is an insufficient understanding of flutriafol in vertebrates and whether potential differences in flutriafol metabolism between males and females confer greater vulnerability. Thus, it is extremely essential to fully understand the biological behavior of flutriafol.

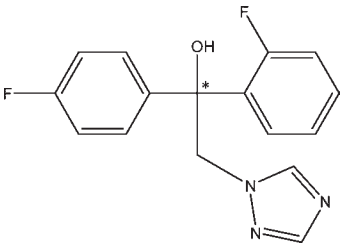

Figure 1. Structure of flutriafol (*, chiral center).

Gender-based chiral differences in the response to a compound may be due to the agent's pharmacodynamic action or its pharmacokinetic properties. It is clear that the fundamental pharmacokinetic parameters can demonstrate biological behavior in a wide variety of animal species. To address the gender-based chiral dimorphism in rabbits, we investigated the pharmacokinetics of flutriafol enantiomers. Plasma protein binding had an important effect on the pharmacology and toxicology of

Received: May 6, 2011
Accepted: July 8, 2011
Revised: July 4, 2011
Published: July 08, 2011

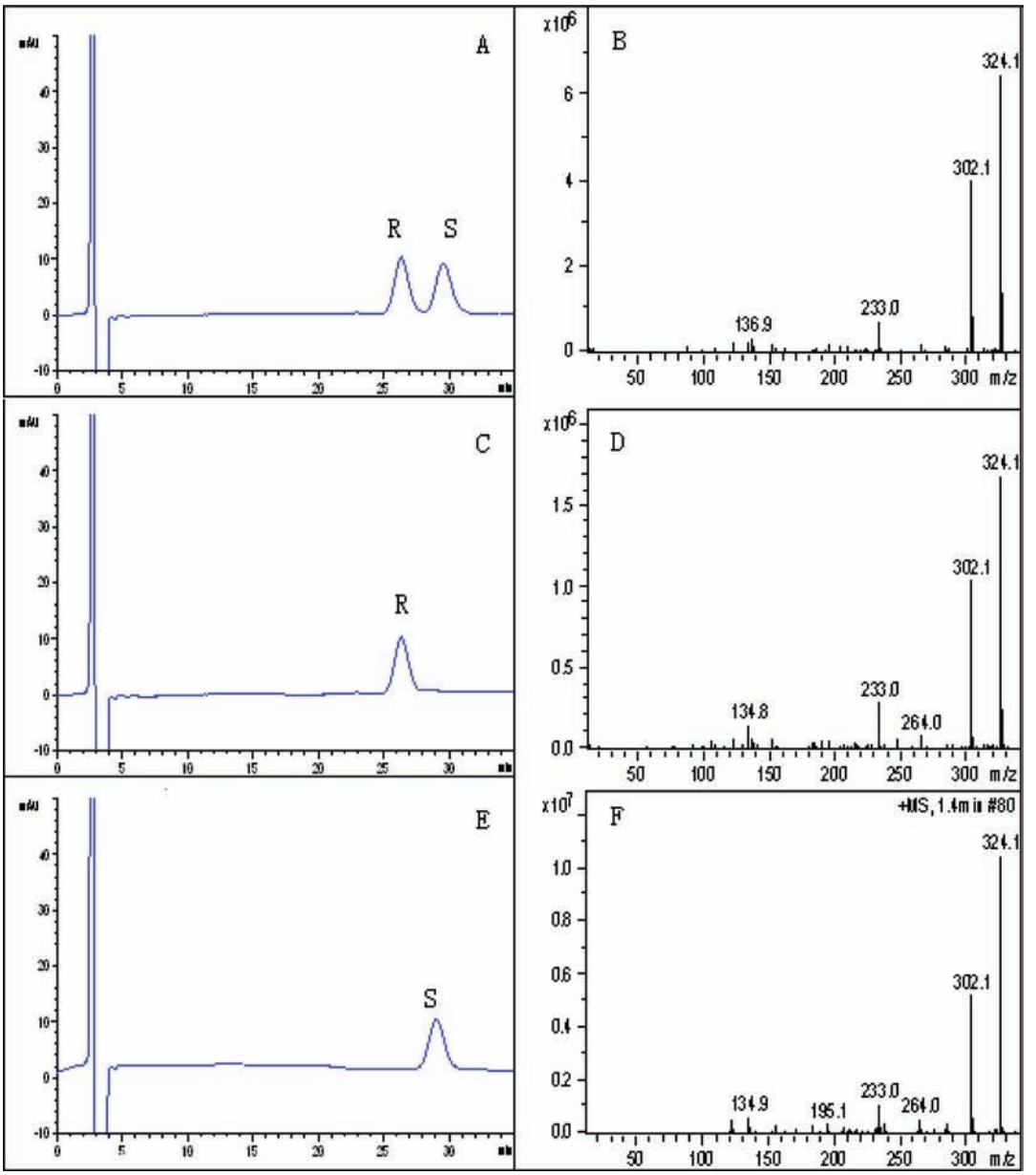

Figure 2. Chromatograms (*n*-hexane/2-propanol = 90:10, CDMPC-CSP column) and mass chromatogram of standards: (A) chromatograms of *rac*-flutriafol; (B) mass chromatogram of *rac*-flutriafol; (C) chromatograms of *R*-flutriafol; (D) mass chromatogram of *R*-flutriafol; (E) chromatograms of *S*-flutriafol; (F) mass chromatogram of *S*-flutriafol.

drugs; for the determination of plasma protein binding an ultracentrifugation method had been employed. The main purpose of this study was to address the sexual dimorphism in the toxicity and metabolism of flutriafol isomers in animals.

MATERIALS AND METHODS

Chemicals and Reagents. *rac*-Flutriafol (>99% purity) was obtained from the China Ministry of Agriculture's Institute for Control of Agrochemicals. *R*- and *S*-flutriafol were prepared on an Agilent HPLC system with a preparative chiral column (CDMPC-CSP, provided by the Department of Applied Chemistry, China Agricultural University, Beijing), and the purities of *R*- and *S*-flutriafol were >99%. The spectrograms of standards are shown in Figure 2. Chang et al. have confirmed the absolute configuration of flutriafol on a Chiralcel AD-H column.[12] We used the optically pure isomer on the same column with the same mobile phase to determine the absolute configuration. HPLC grade solvents were puchased from Fisher Chemicals (Fair Lawn, NJ). All other chemicals and solvents were of analytical reagent grrade and purchased from common commercial suppliers. Stock solutions of *rac*-flutriafol, *R*-flutriafol, and *S*-flutriafol were prepared in ethanol and stored at −20 °C.

Animals. Male and female adult Japanese white rabbits (weighing 2−2.25 kg), purchased from the Experimental Animal Research Institute of China Agriculture University (Beijing, China), were housed in an air-conditioned room with a 12/12 h light/dark cycle and given free access to commercial food pellets and tap water. Animal experiments were carried out in compliance with the standard ethical guidelines.

Degradation Studies. Eighteen healthy adult rabbits, nine males and nine females, were administered *rac*-flutriafol at 5 mg/kg by intravenous

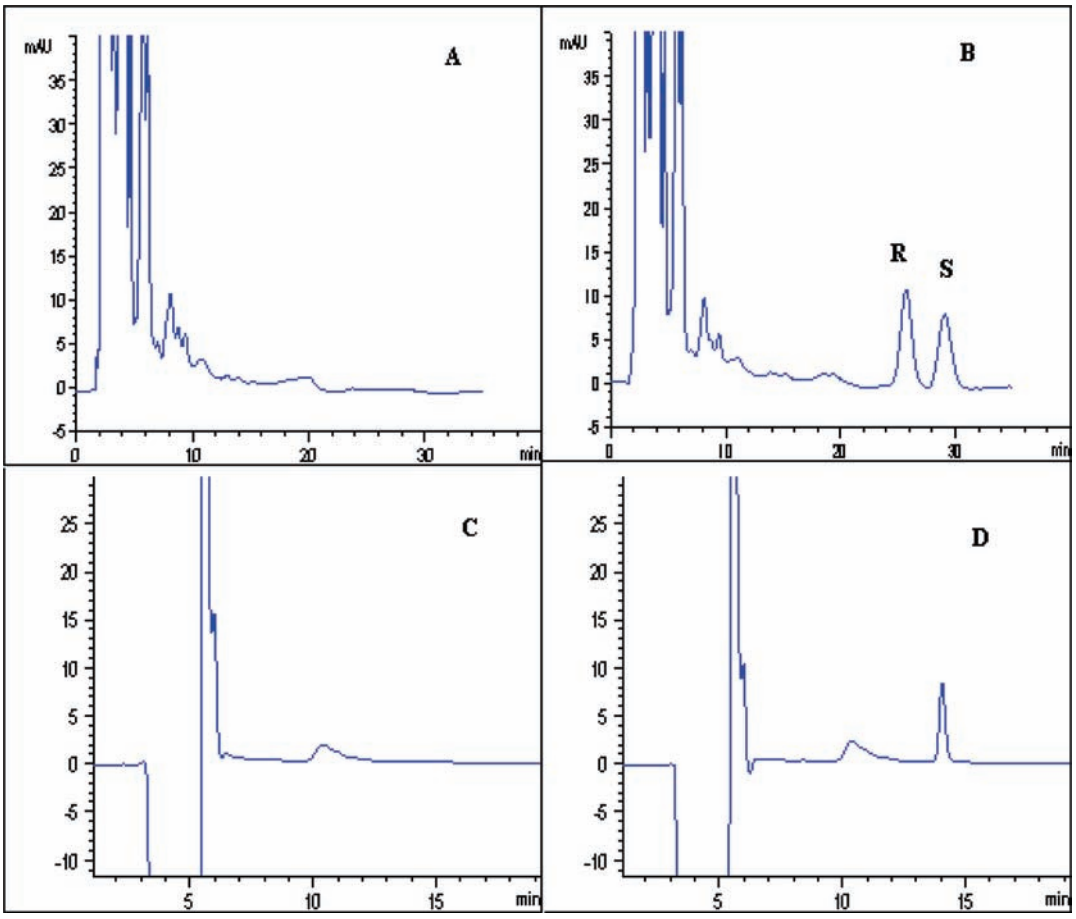

Figure 3. Representative HPLC chromatograms of (A) extract from untreated rabbit plasma, (B) extract from a plasma sample collected from a rabbit at 6 h (*n*-hexane/2-propanol = 90:10, CDMPC-CSP column), (C) filtrate of untreated plasma, and (D) filtrate of *R*-flutriafol added plasma (methanol/water = 65:35, HC-C18 column).

injection in the left ear vein. Blood samples were collected in heparinized tubes at 0.05, 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h after treatment. At 10 h, the rabbits were euthanized, and the heart, kidney, liver, lung, fat, muscle, spleen, and brain of each rabbit were excised and weighed separately. Blank blood samples were collected before drug administration. Blood samples were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant serum was separated and stored at −20 °C until analysis.

Samples were prepared as the follows: 1.0 mL of plasma samples or 1 g of homogenized tissue matrix was transferred to a 15 mL polypropylene tube, and then 5 mL of acetic ether was added followed by vortex mixing (5 min). After centrifugation at 3000 rpm for 5 min, the supernatant was decanted into another tube, and the remains were reextracted with another 5 mL of ethyl acetate. Subsequently, the organic phase was combined and evaporated to dryness under a gentle stream of nitrogen at 40 °C. To remove the adipose tissue sample, the residue was reconstituted in 1 mL of acetonitrile and then washed with *n*-hexane (2 mL) twice. The acetonitrile layer was redried under a gentle stream of nitrogen. Then the residue was diluted to 200 μL with 2-propanol and filtered through a 0.22 μm filter prior to HPLC analysis.

Analysis of flutriafol in the sample was performed on an Agilent 1200 series HPLC (Agilent Technology) as previously reported.[13] After filtration, 20 μL of the sample solution was injected into a CDMPC-CSP column. The column temperature used was 20 °C. The mobile phase used was *n*-hexane/*n*-propanol (90:10, v/v), and the flow rate was 1.0 mL/min. The eluent was monitored by a UV detecor at 210 nm.

Plasma Protein-Binding Analysis. The protein binding of isomers was measured by adding known amounts of solus isomer to drug-free plasma and measuring the free drug using an ultrafiltration/centrifugation technique.

The concentrations studied were 2.50, 1.75, and 1.00 μg/mL. The stock solution of test compounds was added to a tube and evaporated to dryness under a stream of nitrogen. Then drug-free plasma (1.5 mL) or phosphate-buffered saline (PBS, pH 7.4) was then added to it and vortexed for 30 s. After incubation of plasma at 37 °C during 1 h in a shaking bath, to achieve binding equilibrium, 1 mL of plasma or PBS buffer was transferred to an ultracentrifuge tube (Ultra-4; 10 000 MW exclusion limit; Millipore) and centrifuged for 5 min at 6000*g*. All samples were made in triplicate. The filtrate of the plasma contained only the plasma free drug concentration, and the PBS filtrate was used to estimate the adsorption of the drug to the ultracentrifuge tube.

After centrifugation, 10 μL of the sample was analyzed using an Agilent 1200 HPLC system. An HC-C18 column (4.6 × 250 mm; 5 μm; Agilent Technology, Palo Alto, CA) was used at 20 °C, and a combination of methanol/water (65:35, v/v) was used as the mobile phase. The flow rate was 0.5 mL/min, and UV detection was at 210 nm.

Validation of Method. Six standards of various concentrations (0.1−5 μg/mL) were used for initial calibration curves.

For determination of the precision and accuracy of the method, native plasma and tissue samples were fortified at three fortification levels with *R*-flutriafol and *S*-flutriafol. The data were calculated as a ratio of the found concentration of each enantiomer spiked in a blank sample to the predicted concentration.

Both intra- and interday precisions are presented as relative standard deviation [RSD, (SD/mean) × 100%].

Data Analysis and Statistics. The pharmacokinetic parameters were calculated by a DAS (Drug and Statistic, version 2.0) program and compared by statistical analysis.

The enantiomer fraction (EF) was used as a measure of the enantioselectivity of the flutriafol enantiomers in rabbits. EF is defined by eq 1:

$$\text{EF} = \text{peak areas of the } \frac{R}{R+S} \quad (1)$$

A racemic EF = 0.50, whereas preferential degradation of the *R*- or *S*-isomer yields EF <0.500 and >0.500, respectively.

The nonspecific binding of the compounds to the filtration membrane was expressed as the ratio of the compounds in PBS buffer before and after ultrafiltration.[14] Plasma protein binding at various concentrations was calculated according to the equations

$$\text{NSB} = 1 - C_f^b/C_{uf}^b \quad (2)$$

$$\text{PB} = 1 - (C_f^p/C_{uf}^p)(1 + \text{NSB}) \quad (3)$$

where PB and NSB are percent protein binding and nonspecific binding. C_f^b, C_{uf}^b, C_f^p, and C_{uf}^p represent filtered and unfiltered drug concentrations in plasma and buffer, respectively.

The data were expressed as the mean ± SD. The significance of difference between groups was evaluated by using Dunnett's *t* test. Statistical differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

The optically pure enantiomer was prepared on CDMPC-CSP, and Chang et al. have confirmed the absolute configuration of flutriafol on a Chiralcel AD-H column.[12] We used the optically pure isomer on the same column with the same mobile phase to determine the absolute configuration; the first eluted enantiomer on CDMPC-CSP was *R*-flutriafol and the second was *S*-flutriafol in our study.

Typical chromatograms of extracts of *R*- and *S*-flutriafol from plasma samples are shown in Figure 3. As shown, *R*- and *S*-flutriafol were baseline separated, and no endogenous interference peaks eluted at retention times in blank samples. The standard curve of flutriafol in plasma was linear between 0.1 and 5.0 μg/mL and exhibited excellent linearity through the coefficient of correlation: $r^2 = 0.998$. Table 1 shows the intraday and interday precisions and accuracies of the method. The results showed acceptable RSD values (<10%) for both intra- and interday ($n = 5$) studies. Method recovery data for fortified samples are presented in Table 2. Each recovery was acceptable for the determination. The limit of detection (LOD) was 0.1 μg/mL for plasma and tissue samples and 0.01 μg/mL for filtrate samples.

Various methods have been employed to determination plasma protein binding, among which equilibrium dialysis and ultracentrifugation play a prominent role.[15] For our experimental approach we used ultracentrifugation to measure protein binding of flutriafol in the plasma. Protein-binding rates for the enantiomers in plasma for both male and female rabbits were found to be stereoselective. As shown in Table 3, the mean free fractions for the *R*-isomer were more than for *S*-flutriafol across the concentration range from 1.0 to 2.5 μg/mL. No significant differences in enantioselective binding from different genders were observed. A low protein binding generally enabled rapid and extensive distribution into the intra- and extracellular space. On the basis of this result, protein binding is likely to be important in modulating the disposition of flutriafol.

The no-effect level of flutriafol in the rabbit study was considered to be 7.5 mg/kg bw/day, although its acute toxicity was relatively low,[2] so the dose of intravenous administration was 5 mg/kg to rabbits in this study. The concentration−time curves of both flutriafol enantiomers (Figure 4) were best described by a two-compartment model in male and female rabbits. Relevant pharmacokinetic parameters of *R*- and *S*-flutriafol were computed and are summarized in Table 4. Flutriafol serum concentrations rapidly decreased during the first-point postadministration in male and female rabbits. As shown, in both sexes of rabbits, *R*-flutriafol exhibited a longer elimination half-life, a longer mean residence time, and a larger AUC than the *S*-isomer, whereas the total body clearance and the distribution half-life of the *R*-enantiomer were

Table 1. Precision and Accuracy Data of Assay Method for Determination of Flutriafol Enantiomers

	intraday ($n = 5$)		interday ($n = 5$)	
concn added (μg/mL)	concn found (μg/mL)	RSD (precision, %)	concn found (μg/mL)	RSD (precision, %)
R-flutriafol				
0.1	0.09 ± 0.01	8.70	0.09 ± 0.01	9.43
2.5	2.38 ± 0.08	3.40	2.39 ± 0.08	3.50
5.0	4.82 ± 0.14	3.00	4.89 ± 0.02	0.47
S-flutriafol				
0.1	0.09 ± 0.01	6.52	0.09 ± 0.01	6.62
2.5	2.38 ± 0.07	3.25	2.38 ± 0.08	3.42
5.0	4.81 ± 0.14	3.07	4.85 ± 0.05	1.00

Table 2. Recovery of the HPLC Method for Determination of Flutriafol ($n = 5$)

concn added (μg/mL)	recovery (%)	RSD (%)
R-flutriafol		
0.1	91.86 ± 6.23	6.78
2.5	94.98 ± 3.54	3.49
5.0	97.33 ± 1.09	1.12
S-flutriafol		
0.1	94.04 ± 6.40	6.80
2.5	96.79 ± 2.50	2.58
5.0	97.17 ± 0.95	0.97

Table 3. Plasma Protein Binding of *R*- and *S*-Enantiomers of Flutriafol in Rabbit Plasma at 37 °C As Determined by Ultrafiltration and RP-HPLC (Mean ± SD, $n = 3$)

	plasma protein bound[a] (%)			
	male		female	
concn (μg/mL)	*R*-flutriafol	*S*-flutriafol	*R*-flutriafol	*S*-flutriafol
1.00	83.29 ± 4.15*	91.59 ± 3.15	83.10 ± 1.94*	87.90 ± 1.60
1.75	83.65 ± 0.96*	90.08 ± 1.05	80.85 ± 1.91*	89.12 ± 2.35
2.50	74.90 ± 0.84*	82.38 ± 1.95	70.66 ± 3.45*	83.78 ± 0.79

[a] *, significantly different from the (*S*)-isomer value at $p < 0.05$.

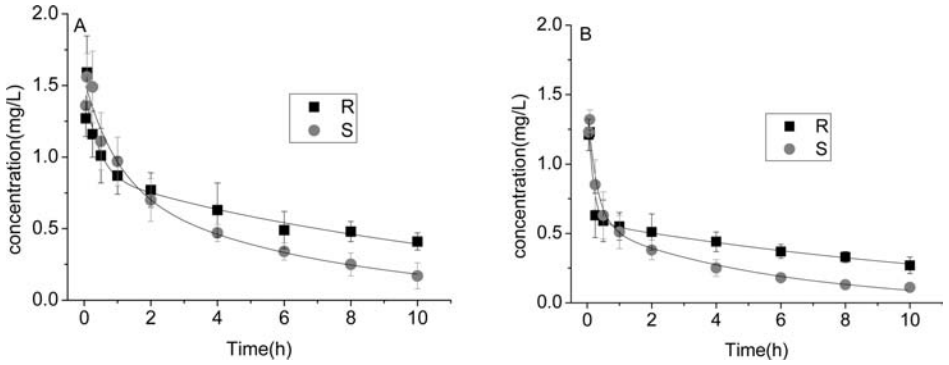

Figure 4. Mean ± SD flutriafol plasma concentration−time profile obtained after a single intravenous administration at a dose of 5 mg/kg to rabbits (n = 9): (A) adult male; (B) adult female.

Table 4. Mean ± SD Plasma Pharmacokinetic Parameters of Flutriafol in Japanese White Rabbits (n = 9) following Intravenous Administration at a Dose Rate of 5 mg/kg bd wt

		male[a]			female[a]		
parameter[b]	units	*R*-flutriafol	*S*-flutriafol	*R*/*S*[c]	*R*-flutriafol	*S*-flutriafol	*R*/*S*[c]
AUC_{0-10}	mg L^{-1} h	6.26 ± 0.69 ab	4.97 ± 0.46 b	1.26 ± 0.09	4.30 ± 0.59 a	2.82 ± 0.42	1.54 ± 0.15
$AUC_{0-\infty}$	mg L^{-1} h	11.53 ± 1.71 ab	5.92 ± 0.43 b	1.95 ± 0.32	8.96 ± 2.96 a	3.50 ± 0.33	2.23 ± 0.47
MRT	h	4.10 ± 0.18 a	3.27 ± 0.17	1.26 ± 0.11	4.21 ± 0.21 a	3.25 ± 0.15	1.30 ± 0.03
$t_{1/2\alpha}$	h	0.28 ± 0.20 ab	0.68 ± 0.20 b	0.45 ± 0.07	0.09 ± 0.03 a	0.18 ± 0.09	0.50 ± 0.21
$t_{1/2\beta}$	h	8.63 ± 0.63 a	4.58 ± 0.66	2.31 ± 0.40	9.51 ± 1.47 a	3.82 ± 0.76	2.34 ± 0.91
V	L kg^{-1}	1.66 ± 0.11	1.62 ± 0.07	1.00 ± 0.08	1.53 ± 0.14	1.69 ± 0.14	0.91 ± 0.07
Cl	L kg^{-1} h^{-1}	0.22 ± 0.03 ab	0.42 ± 0.03 b	0.52 ± 0.08	0.30 ± 0.08 a	0.72 ± 0.08	0.41 ± 0.09

[a] Data are expressed as arithmetic mean ± SD: (a) value significantly (p < 0.05) different from the concentration of *S*-flutriafol; (b) value significantly (p < 0.05) different from the concentration of female. [b] $AUC_{(0-10)}$, area under the curve from 0 to 10 h; $AUC_{0-\infty}$, area under the curve from 0 to infinity by the trapezoidal integral; MRT, mean residence time from; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, elimination half-life; V, apparent volume of distribution; Cl, total body clearance. [c] *R* and *S* indicate *R*- and *S*-flutriafol enantiomers, respectively.

smaller than those of its antipode. However, there was no statistical difference in volumes of distribution between these two enantiomers. Additionally, the total body clearance in the male was smaller than that in the female, indicating a lower metabolic or excretion capacity in the male. Thus, there was a significant gender difference in degradation. It was interesting to note that at the initial time the EF of *rac*-flutriafol was smaller than 0.5 and then exceeded 0.50, increasing with time in the plasma (Figure 5).

In the present study, stereoselective distribution of *rac*-flutriafol enantiomers in tissues was observed. Because of its strong first-pass effect, flutriafol was easily stockpiled by the lung after intravenous administration.[16] In all tissues, *R*-flutriafol had a higher concentration than its antipode, but gender differences based on statistics were not observed. Thus, it would appear that the stereoselective degradation in rabbits resulted from metabolism and excretion differences.

Many chiral pesticides degrade enantioselectively in animals, and the pharmacokinetic parameters showed differences. (+)-Hexaconazole decreased more rapidly than (−)-hexaconazole in plasma, liver, and kidney after intravenous administration of racemic hexaconazole in the rabbit, and the total plasma clearance value of (+)-enantiomer was >1.3-fold higher than that of the (−)-isomer.[17] Stereoselective degradation kinetics of tebuconazole and diniconazole were also observed in rabbits.[18,19] The differences in the bioavailability, distribution, metabolism, and elimination between the sexes result in sex-related discrepancies in the pharmacokinetics of the drugs. In this study, we noted that

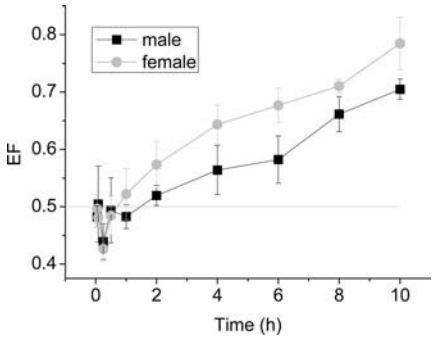

Figure 5. EF of flutriafol enantiomers in rabbits following *rac*-flutriafol administration at 5 mg/kg bd wt.

flutriafol had a similar pattern in male and female rabbits. Pharmacokinetics was described as the time course and dose affected by the absorption, distribution, metabolism, and excretion of xenobiotics, so differences in any of these four processes could lead to differences in the pharmacokinetics of the administered compound. In this study, intravenous administration was used so the absorption effect can be excluded. Plasma protein binding is normally recognized as an important factor in assessing drug disposition,[20] so it may contribute to the chiral dimorphism. The optically pure enantiomer was used to evaluate the in vitro plasma protein binding because the levels of the separate enantiomers

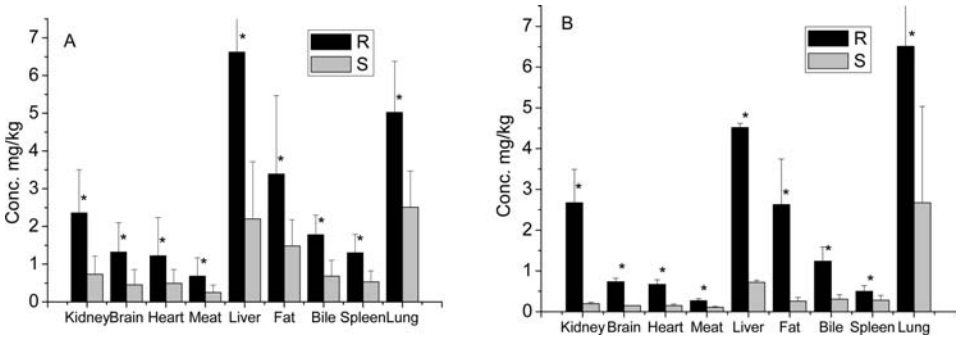

Figure 6. Flutriafol concentration in tissues at 10 h after intravenous administration at 5 mg/kg bd: (A) adult male; (B) adult female. ∗, value significantly ($p < 0.05$) different from the concentration of *S*-flutriafol.

in plasma were variant with time and it was impossible to simulate the real situation in vivo. The data indicated that *S*-flutriafol had lower free fractions across the concentration range from 2.5 to 1.0 mg/kg, but no statistically significant differences were found between males and females. Clearance (CL) was considered to be the theoretical volume of plasma or tissue from which the compound was totally removed per unit time.[21] *S*-Flutriafol exhibited a higher CL than its antipode in females and males. Both enantiomers were excluded from female rabbits much more quickly. Thus, there was a significant gender difference in the degradation of flutriafol. In a sulfamethazine degradation study, male rabbits displayed lower CL than female rabbits. This gender difference was not based on metabolism differences because there were no differences in metabolite profiles.[22] After intravenous administration, *R*-flutriafol in rabbits was distributed more quickly but more slowly eliminated than the *S*-isomer because a low protein binding generally enables rapid distribution (Figure 6). The male rabbits had a longer $t_{1/2\alpha}$ but a similar elimination half-life, which strongly suggested that the gender difference was mainly caused by the difference in flutriafol distribution.

Because *R*-flutriafol has a higher free fraction, it distributes more rapidly after intravenous administration, but the CL of the *S*-isomer was much higher. It could be removed more quickly than *R*-flutriafol. On the basis of these findings, we could explain that at the initial time the EF of *rac*-flutriafol was <0.50 and then exceeded 0.50 and increased with time in the plasma, which was similar to the metabolism of tebuconazole in rabbits.[18] The data are direct evidence to prove that the protein binding plays an important role in chiral degradation.

Flutriafol concentrations in kidney, liver, fat, and lung were higher than those in other tissues. The drug was easily stockpiled by the lung (lung first-pass effect), so stereoselective distribution may easily occur. Stereoselective metabolism and excretion would also result in stereoselective behavior of the chiral chemicals in animals.[23] In this study, the deviation from EF = 0.5 in the liver suggested that stereoselective metabolism of the two enantiomers might exist. The renal excretion of *rac*-flutriafol may be also stereoselective.

Healthy male and female rabbits were used as experiment subjects. We estimated the pharmacokinetics of *rac*-flutriafol and protein binding of optically pure flutriafol. An HPLC method was used to assay the concentration of flutriafol. Pharmacokinetic parameters were estimated by the DAS 2.0 program.

In this study, the degradation of the two enantiomers of racemic flutriafol occurred at different rates and showed gender-based chiral difference. The distribution process was mainly affected by protein binding. These results revealed that the elimination of flutriafol was different between males and females; it is possible that complex hepatic and gastrointestinal metabolism could mask greater differences due to sex.

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Funding Sources

This study was supported by both the National Science Foundation of China (20877100) and the Chinese Universities Scientific Fund and Specialized Research Fund for the Doctoral program of Higher Education (Project 20090008120016).

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