Stereoselective Toxicokinetics and Tissue Distribution of Ethofumesate in Rabbits

WENTAO ZHU, ZIHENG DANG, JING QIU, CHUNGUANG LV, GUIFANG JIA, LI LI, AND ZHIQIANG ZHOU*
Department of Applied Chemistry, China Agricultural University, Beijing 100094, People’s Republic of China

ABSTRACT The stereoselective toxicokinetics of ethofumesate enantiomers following a single intravenous (i.v.) administration at doses of 30 mg/kg were investigated in rabbits. Plasma concentrations of (+)- and (−)-ethofumesate were analyzed by a validated chiral HPLC method that involved extraction of plasma with organic solvent followed by separation on a cellulose-Tris-(3,5-dimethylphenylcarbamate)-based chiral column and quantification by UV absorbance at 230 nm. Plasma concentration-time curves after i.v. administration were best described by an open two-compartment model. The concentration of the (−)-enantiomer decreased more rapidly than that of the (+)-enantiomer. Significant differences in toxicokinetic parameters between the two enantiomers indicated that stereoselective behavior occurred with the (−)-enantiomer being preferentially metabolized and eliminated. Chirality 19:632–637, 2007.

KEY WORDS: stereoselectivity; toxicokinetics; ethofumesate; chiral separation

INTRODUCTION Many of the most widely used pesticides have optical isomers. At present, there are approximately 650 pesticides in market, among which about a quarter have optical enantiomers. Most of them are released into the environment as racemic mixtures. It is noteworthy that stereochemistry strongly influences not only biological activity but also metabolic processes in organisms and in the environment. Thus growing concern about the side effects of chiral agrochemicals on nontarget organisms and natural resources has promoted the use of enantiomerically pure or stereochemically enriched compounds. Different environmental bodies, including animals, would also exert influences on the stereoselective degradation of enantiomers. So the studies on stereoselective kinetic of chiral chemicals either in the environment or in the organisms have been an important research topic during the last few years.

Ethofumesate [(±)-2-ethoxy-3,3-dimethyl-2,3-dihydrobenzofuran-5-yl methanesulfonate] (Fig. 1), was first reported by Pfeiffer in 1969. It can be used both as a pre- and post-emergence herbicide in sugarbeet and other root crops as well as turf, rye grass, and other pasture grasses to control a wide range of important grasses and broad-leaved weeds. Ethofumesate is absorbed by the emerging shoots of grasses and by the roots of dicotyledons and translocated to the foliage. It inhibits the growth of meristems, retards cell division and limits cuticle formation. Ethofumesate is metabolized in plants to its 2-hydroxy and 2-oxo derivatives, methanesulfonic acid, and CO₂, while major metabolite in animals is the lactone or free acid form of the respective 2-oxo compound. Foliar injury and growth inhibition caused by ethofumesate vapor was observed in 14 wild higher plant species, which showed inhibition potential of ethofumesate on nontarget organisms.

Since there is a chiral center in the furan moiety (Fig. 1), two enantiomers of ethofumesate exist. Desiderio et al. reported the enantiomeric resolution of racemic ethofumesate by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as chiral selector. The resolution method of ethofumesate enantiomers was built up by high performance liquid chromatography-chiral stationary phase (HPLC-CSP) technology in previous work. To date, toxicokinetic data have not been reported for ethofumesate in mammals. Because the information regarding kinetic profile improves the scientific basis for risk decisions, the objective of the study reported here was to determine tissue and plasma concentrations of ethofumesate enantiomers in rabbit after intravenous administration and obtain a better understanding of the biological fate of each enantiomer in animals.

MATERIALS AND METHODS

Chemicals and Reagents

Racemic ethofumesate standard (98%) was obtained from Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China. Stock solution of racemic standard was prepared in 2-propanol and was stored at −20 °C. Working standard solutions were obtained by dilutions of the stock solution in 2-propanol. All the mobile
phase reagents were HPLC grade. Other solvents were analytical grade. Water was purified by a Milli-Q system.

**Preliminary Toxicokinetic Studies**

Male Japanese white rabbits weighing 2-2.25 kg (provided by the Experimental Animal Research Institute of China Agriculture University) were housed under a 12-h light/12-h dark cycle at 22°C. The rabbits were kept on fast for 12 h before administration with free access to water. Racemic ethofumesate was dissolved in 10% (w/v) alcohol and then diluted to final concentrations with normal saline.

Racemic ethofumesate was administered at 30 mg/kg body weight by intravenous (i.v.) injection in the ear vein. Plasma samples were collected and stored immediately in heparinized tubes at 5, 15, 30, 60, 90, 120, and 240 min after administration, each data point is the mean of six replicates. Blank blood samples were collected before drug administration. After blood sample collection, the animals were killed at 5, 15, 30, 60, 90, 120, and 240 min after being anesthetized. The heart, kidney, liver, lung, fat, muscle, spleen, and brain of each rabbit were excised and weighed separately. Plasma and tissue samples were stored at –80°C for later analysis.

**Sample Preparations**

Analysis of ethofumesate was performed using the methods as follows: (1 ml) of the rabbit plasma or 1 g of homogenized tissue matrix was weighed into a 15 ml polypropylene centrifuge tube. About 5 ml of ethyl acetate was added, and the sample was vortexed for 5 min. After centrifugation at 4000 rpm for 5 min, the clear solution was decanted into a test tube. The extraction and centrifuge steps were repeated with another 5 ml of ethyl acetate. The organic phase was combined and evaporated to dryness under a stream of nitrogen, the residue of the organic phase was dissolved in 4 ml acetonitrile, then partitioned with 5% 2-propanol and hexane. The hexane was evaporated to dryness under a stream of nitrogen. All the remaining residue was redisolved in 0.2 ml 2-propanol, and this solution was vortexed, A 20-μl aliquot was injected into the HPLC.

**Toxicokinetic Analysis**

Individual toxicokinetic parameters of ethofumesate enantiomers were determined using standard compartmental analysis methods and calculated with the Drug and Statistics computer program (Section of Quantitative Pharmacology, Chinese Pharmacological Society). An open two-compartment model best described the plasma concentration versus time data for both enantiomers after i.v. administration based on the Akaike’s Information Criterion (AIC).

For the estimation of toxicokinetic parameters, distribution and elimination half-lives \( t_{1/2a} \) and \( t_{1/2b} \), the plasma clearance (CL) was calculated according to standard pharmacokinetic equations. The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule. Mean residence time (MRT) was calculated by dividing the area under first-moment curve AUMC by AUC. The enantiomeric ratio (EF) was used as a measure of the enantioselectivity of the two isomers in animal kinetic analysis. The EF is defined by eq. 1.

\[
EF = \frac{(+)}{(-)} \quad \text{(1)}
\]

where \((+)\) and \((-)\) are the concentrations of the first eluted \((+)\)- and the second eluted \((-)\)-enantiomer according to the determination result of polarimeter in previous work. The EF for racemate is 0.5 \([(+) = (-)]\). A paired \(t\)-test was used to test the significance of stereoselective differences in toxicokinetic parameters.

**Apparatus and HPLC Conditions**

The HPLC system used in this study consisted of an Agilent 1100 HPLC equipped with a G1311A pump, G1322A degasser, G1328A injector, a 20-μl sample loop, and G1314 VWD detector. The signal was received and processed by an HP1100 Chemstation. Enantiomers were separated on CDMPC-CSP (provided by the Department of Applied Chemistry, China Agricultural University, Beijing). The chromatographic separation was conducted at room temperature. The mobile phase was made up of 95% hexane and 5% 2-propanol with a flow rate of 1.0 ml/min. The eluates were monitored at 230 nm.

**Calibration Curves and Method Validation**

Plasma (1 ml) obtained from untreated rabbits was spiked with working standard rac-ethofumesate solutions to generate calibration samples ranging from 0.20 to 25 μg/ml for both \((+)\)- and \((-)\)-ethofumesate. Calibration samples were prepared as described earlier. Calibration curves were generated by plotting the concentration of each enantiomer in the spiked samples versus the peak area of each enantiomer. Linear regression analysis was performed using Microsoft Excel. The precision and accuracy of the assay were obtained by comparing the predicted concentration (obtained from the calibration curve) to the actual concentration of each enantiomer fortified in drug free plasma. The standard deviation (SD) and the coefficient of variation (CV = SD/mean) were calculated over the entire calibration range. The within-day precision was determined in six replicates at concentration of 2.5, 5.0, and 10.0 μg/ml on the same day. The inter-day precision was evaluated in six replicates at the earlier concentrations on six different days. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curve with acceptable precision and accuracy (The acceptance criteria for the LOQ were that the precision and accuracy for extracted samples were under 20%
variability). The limit of detection (LOD) for each enantiomer was considered to be the concentration that produced a signal-to-noise (S/N) ratio of 3.

Drug-free plasma samples fortified with racemic ethofumesate at different levels were extracted and determined as described in sample preparation chapter. Recovery was estimated by comparing the peak area ratio of the extracted analytes with the peak area of an equivalent amount of the standard solution in pure solvents.

RESULTS

Calibration and Method Validation

There were no endogenous interference peaks eluted at retention times in plasma and in tissue samples. Representative HPLC chromatograms of extracts from untreated rabbit plasma spiked with rac-ethofumesate (10 μg/ml), treated plasma, liver, kidney, and fat sample are shown in Figure 2. As shown, (+) and (−)-ethofumesate were baseline separated.

Fig. 2. Representative HPLC chromatograms of (A) extract from untreated rabbit plasma, (B) extract from untreated rabbit plasma spiked with rac-ethofumesate (10 μg/ml), (C) extract from a plasma sample collected from a rabbit 5 min, (D) extract from a liver sample collected from a rabbit 5 min, (E) extract from a kidney sample collected from a rabbit 5 min, (F) extract from a fat sample collected from a rabbit 15 min after i.v. treatment with rac-ethofumesate at 30 mg/kg bd wt, [n-hexane/2-propanol = 95:5, flow rate = 1.0 ml/min].

Chirality DOI 10.1002/chir
The ratio was considerably varied from 0.5 of the racemate. EF at each time point is presented in Figure 3B (right).

The stereoselective degradation of ethofumesate enantiomers in these three tissues was higher than its antipode. In the liver, especially the concentration of the (+)-enantiomer in these three tissues was higher than its antipode. In the brain, the concentration of the (-)-enantiomer was much higher at earlier times but was undetectable at 90 min. There is no obvious stereoselective degradation in lung, heart and spleen. All data are shown in Figure 4.

Linear calibration curves were obtained over the concentration range of 0.20–25 μg/ml for both (+)-ethofumesate ($y = 125.01x + 14.5950, R^2 = 0.9983$) and (-)-ethofumesate ($y = 124.96x + 8.9545, R^2 = 0.9991$). The accuracy and precision of the assay for both enantiomers are suitable with the coefficients of variation (CV) from 2.7% to 11.2% (Table 1). Recoveries of each enantiomer fortified at 0.25, 2.5, and 10.0 μg/ml ranged from (89.1 ± 2.8)% to (98.1 ± 6.8%). The LOD was 0.025 μg/ml and the LOQ was 0.1 μg/ml plasma.

**Toxicokinetics in Rabbit Plasma**

Plasma concentration-time curves of (+)- and (-)-ethofumesate after i.v. administration of 30 mg/kg of racemate to the rabbits are shown in Figure 3A (left). The mean plasma concentrations of two enantiomers were different from each other at each time point. The plasma concentration of the (-)-ethofumesate was significantly lower than that of its antipode. The preferential disappearance of the (-)-enantiomer from plasma was evident from the chromatograms of plasma extracts at 5 min after administration (Fig. 2C).

Compartmental toxicokinetic analysis and t-test results showed significant differences between the principal toxicokinetic parameters of two enantiomers (Table 2). The EF at each time point is presented in Figure 3B (right). The ratio was considerably varied from 0.5 of the racemate.

### Table 1. Precision and accuracy for measurement of enantiomers in rabbit plasma

<table>
<thead>
<tr>
<th></th>
<th>(+)-Ethofumesate</th>
<th>(-)-Ethofumesate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated (%)</td>
<td>Calculated (%)</td>
</tr>
<tr>
<td>Within-day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n = 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.39</td>
<td>2.31</td>
</tr>
<tr>
<td>5.0</td>
<td>4.68</td>
<td>4.58</td>
</tr>
<tr>
<td>10.0</td>
<td>8.86</td>
<td>8.84</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n = 6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.35</td>
<td>2.27</td>
</tr>
<tr>
<td>5.0</td>
<td>4.60</td>
<td>4.49</td>
</tr>
<tr>
<td>10.0</td>
<td>8.79</td>
<td>8.76</td>
</tr>
</tbody>
</table>

CV (%) = 100 × (SD/Mean).

**Discussion**

According to the previous studies, racemic ethofumesate was ideally separated on a CDMPC chiral stationary phase under normal condition. In the present work, this method was successfully applied to analysis of the two enantiomers extracted from biological samples. The sample preparation procedure for plasma samples was proved to be reproducible and precise with mean recovery above 85%. The extraction procedure did not cause epimerization of ethofumesate enantiomers.

Enantioselective behavior of chiral pesticides has been observed in various environmental compartments. Mecoprop and dichloprop were reported with preferential degradation of the (S) enantiomer over (R) enantiomer by a flavobacterium strain. Studies on degradation of five acetamide herbicides and fungicides in soils and sewage sludge showed different stereoselectivity in different environmental media. Stereoselective degradation of organochlorine pesticide was observed in marine microorganisms, in cetaceans, in seabirds, and also in soils. The stereoselective degradation of ethofumesate enantiomers in turfgrass and soils was proved in a previous report. Wang et al. reported the enantioselective degradation and chiral conversion of β-cypermethrin (TCYM) in rats after i.v. injection. The degradation of the (+)-TCYM was much faster than that of the (-)-TCYM in plasma, heart, liver, kidney, and fat after administration of racemic TCYM. Zhu et al. built a stereoselective analytical...
method of tebuconazole enantiomers in vivo, the degradation rate of tebuconazole enantiomers was different. The major novel finding of this study is that acute administration of rac-ethofumesate resulted in the stereoselective disposition of the (+)- and (−)-enantiomers of ethofumesate. The concentration of the (−)-enantiomer in plasma declined more rapidly than that of the (+)-enantiomer. This preferential disappearance of the (−)-enantiomer resulted in the significant differences in its toxicokinetic parameters (Table 2).

In this context, it is noteworthy that these in vivo studies showed rapid degradation in the liver (Fig. 4) and the EFs of the two enantiomers were above 0.75 in all liver

<table>
<thead>
<tr>
<th></th>
<th>(+)-ethofumesate</th>
<th>(−)-ethofumesate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1/2\alpha}$ (min)</td>
<td>13.53</td>
<td>13.44</td>
</tr>
<tr>
<td>$T_{1/2\beta}$ (min)</td>
<td>69.32</td>
<td>69.31</td>
</tr>
<tr>
<td>Vd (l/kg)</td>
<td>2.87</td>
<td>4.43</td>
</tr>
<tr>
<td>CL (l/min/kg)</td>
<td>0.09</td>
<td>0.14</td>
</tr>
<tr>
<td>AUC₀–₁₂₀ (mg/l min)</td>
<td>273.15</td>
<td>172.27</td>
</tr>
<tr>
<td>AUC₀–∞ (mg/l min)</td>
<td>333.49</td>
<td>210.16</td>
</tr>
<tr>
<td>MRT₀–₁₂₀ (min)</td>
<td>25.22</td>
<td>24.26</td>
</tr>
<tr>
<td>MRT₀–∞ (min)</td>
<td>32.25</td>
<td>30.16</td>
</tr>
</tbody>
</table>

Fig. 4. Tissues distribution of ethofumesate enantiomers in rabbits following rac-ethofumesate administration at 30 mg/kg bd wt.
samples (data not shown). These results suggested that the (−)-enantiomer was metabolized faster than the (+)-enantiomer in rabbit liver. Stereoselective xenobiotic disposition could also be attributed to excretion, especially in the kidneys. In this study, the concentration of (−)-ethofumesate in rabbit kidney was lower than that of (+)-ethofumesate (Fig. 4). Thus renal excretion of rac-ethofumesate may have been stereoselective, with (−)-ethofumesate excreted more rapidly than its antipode. Several reasons mentioned above may have contributed to the observed stereoselective toxicokinetics of ethofumesate.

The detection of two enantiomers in brain tissue revealed that both enantiomers could penetrate the blood-brain barrier and the EF ratio in rabbit brain tissue was contrary to that in plasma. The preferential distribution of (−)-enantiomer from plasma might be at least part of the explanation. Higher concentrations of both enantiomers were found in fat showing the lipophilic nature of ethofumesate. This accumulation also stereoselective, and the (−)-enantiomer was more prevalent in fat.

In this study, the symptoms of toxicity of ethofumesate were not investigated but there were no obvious signs of toxicity. Ethofumesate has low toxicity and is a low residue herbicide to animals. In mammalian species, the stereoselective metabolism of ethofumesate is still not fully elucidated. Further work is necessary to clarify the possible mechanisms.

CONCLUSION

The chiral HPLC method described in this investigation was validated for the study of the stereoselective behavior of ethofumesate enantiomers in rabbits. Toxicokinetics analysis showed evidence of stereoselective disposition of two enantiomers in rabbit. (−)-Enantiomer was preferentially eliminated from plasma compared with its antipode. A future study will be focused on the vitro stereoselective metabolism of the two enantiomers and the enzymes that contribute to its stereoselectivity.

LITERATURE CITED


Chirality DOI 10.1002/chir