Stereoselective Pharmacokinetics of Diniconazole Enantiomers in Rabbits

QIUXIA WANG,1* JING QIU,2 ZHIQIANG ZHOU,3 AOCHENG CAO,1 XINQUAN WANG,4 WENTAO ZHU,3 AND ZIHENG DANG3

1Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China
2Institute of Quality Standards and Testing Technology for Agro-Products, Chinese Academy of Agricultural Sciences, Beijing, China
3Department of Applied Chemistry, China Agricultural University, Beijing, China
4Institute of Quality and Standard for Agro-Products, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

ABSTRACT Diniconazole [(E)-(RS)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazole-1-yl)pent-1-en-3-ol] is a potent triazole fungicide. The enantioselective pharmacokinetics of diniconazole enantiomers in rabbits was studied via intravenous (i.v.) injection. The pharmacokinetics and the enantiomer fraction (EF) were determined using normal high-performance liquid chromatography with diode array detection and a cellulose-tris-(3,5-dimethylphenylcarbamate)-based chiral stationary phase (CDMPC-CSP). The time–concentration curves in plasma were fitted by a two-compartment open mode. The results showed that the concentration of S-diniconazole in plasma decreased faster than that of R-diniconazole, and EFs increased with time after administration of racemic diniconazole (rac-diniconazole). The R/S-enantiomer ratio of the area under the time–plasma concentration curve (AUC0–∞) after administration was 1.52. The total plasma clearance value of S-enantiomer was 1.57-fold higher than that of the R-diniconazole. These results indicate substantial stereoselectivity in the kinetics of diniconazole enantiomers in rabbit. Chirality 21:699–703, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: stereoselectivity; diniconazole; pharmacokinetics; rabbit

INTRODUCTION

Diniconazole was reported by Takano et al.1 in 1983 and then introduced as a triazole fungicide by Sumitomo Chemical Co., Ltd. Diniconazole is a steroid demethylation (ergosterol biosynthesis) inhibitor that can be used to control leaf and ear diseases in cereals, powdery mildew in vines, Sigatoka disease in bananas, etc. It has two enantiomers because of the presence of an asymmetric carbon in molecular structure (see Fig. 1). Previous studies showed that its fungicidal activity mainly originates from (−)-(R)-enantiomer with diniconazole-M as the common name.2 For example, (−)-(R)-enantiomer strongly inhibited lanosterol 14α-demethylation catalyzed by a yeast cytochrome P450/14DM but (+)-(S)-enantiomer was a weaker inhibitor for that.3

In our previous papers, the enantioselective degradation/metabolism of some triazole pesticides in animals was studied. After intravenous administration of racemic pesticides to rabbits, the degradation of (+)-(S)-tebuconazole was much faster than that of (−)-(R)-tebuconazole in plasma,4 (+)-enantiomer of hexaconazole in plasma, liver, and kidney decreased more rapidly than its (−)-enantiomer.5 As for diniconazole, the experiment in rabbits following oral administration suggested it was rapidly metabolized by hydroxylation of the tert-butyl methyl groups, 52–87% and 13–46%, respectively, of dose were excreted in the feces and the urine within 7 days.6 However, to our knowledge, kinetic stereoselectivity of two diniconazole enantiomers in animals has not been reported. This study was conducted to understand their different degradation behavior in animals.

MATERIALS AND METHODS

Chemicals and Reagents

Rac-diniconazole (purity >98%) was provided by Qizhou Agro-chemical (Jiangsu Province, China). Water was purified by a Milli-Q system. n-Hexane and ethanol (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Ethyl acetate (analytical grade) was obtained from Beijing Yili Reagent Group (China).

HPLC-DAD Analysis

Chromatography was performed using an Agilent 1100 series HPLC (Agilent Technology, USA) equipped with a G1311A pump, G1322A degasser, G1328A injector, a 20-μl sample loop, and G1315A diode array detection (DAD). The signal was received and processed by an Agilent Chemstation for 3D LC.

Enantiomers were separated on CDMPC-CSP (provided by Department of Applied Chemistry, China Agricultural University, Beijing) using a mobile phase of 98% n-hexane

*Correspondence to: Qiuxia Wang, Key Laboratory of Pesticide Chemistry and Application, MOA, Beijing 100094, China.
E-mail: wxqcasy@163.com
Received for publication 10 April 2008; Accepted 2 September 2008
DOI: 10.1002/chir.20667
Published online 30 October 2008 in Wiley InterScience (www.interscience.wiley.com).
and 2% ethanol with a flow rate of 1 ml/min. Capacity factor \( k \), selectivity \( \alpha \), and resolution \( R_s \) were calculated from the formulas:

\[
k = \frac{(t - t_0)}{t_0}, \quad \alpha = \frac{k_2}{k_1}, \quad R_s = \frac{2(t_2 - t_1)}{(W_1 + W_2)},
\]

where \( t \) was the retention time, \( t_0 \) was the void time at given conditions, and \( W \) was the base line peak width. The CSP was prepared according to the procedure described in the literature,\(^7,8\) and then packed into a 250-mm \( \times \) 4.6-mm (i.d.) stainless steel column. Chromatographic separation was conducted at 25°C, and DAD at 254 nm.

**Sample Preparations**

Aliquots (1 ml) of the rabbit plasma were transferred into a 15-ml polypropylene centrifuge tube. About 5 ml of ethyl acetate was added, and then the sample was extracted with a shaker for 5 min. After centrifugation at 4000 rpm for 5 min, the clear solution was transferred into a 10-ml glass tube. The extraction and centrifuge steps were repeated with another 5 ml of ethyl acetate. The combined extracts were then evaporated to dryness under reduced pressure at 40°C and reconstituted in 0.2 ml of \( n \)-hexane. A 20-\( \mu l \) aliquot was injected into the HPLC.

**Calibration Curves and Assay Validation**

Plasma (1 ml) obtained from untreated rabbits was spiked with working standard rac-diniconazole solutions to generate calibration samples ranging from 0.05 to 25 \( \mu g/\) ml for both \( R \)- and \( S \)-diniconazole. Calibration curves were generated by plotting peak area of each enantiomer versus the concentration of the enantiomer in the spiked samples. Linear regression analysis was performed using Microsoft Excel. The precision and accuracy of the method were obtained by comparing the predicted concentration (obtained from the calibration curve) to the found concentration of each enantiomer spiked in blank 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 0.05, 0.25, 1, and 5 \( \mu g/\) ml on the same day. The between-day precision was evaluated in six replicates at the aforementioned concentrations on 6 different days. The recoveries of each enantiomer of diniconazole in plasma were determined by analyzing quality control samples at three different concentrations. The peak-area ratios of six extracted samples at each spiked concentration were compared with those of six injections of standard solutions to derive a percent recovery. The plasma sample (1 \( \mu g/ml \)) and standard solution (5 \( \mu g/ml \)) were stored in a refrigerator at \(-20\)°C, and the recoveries of these samples were determined at 0, 7, and 10 days after freezing. The racemization of diniconazole enantiomers was determined by comparing the ER values (peak areas ratio of the \( R/S \)-enantiomer), and the stability was determined by comparing the recovery values at different time. Limit of detection (LOD) and limit of quantification (LOQ) for each enantiomer, respectively, were considered to be the concentration that produced a signal-to-noise (S/N) ratio of 3 and 10.

**Pharmacokinetics Studies**

Male rabbits weighing 2 to 2.25 kg (provided by the Experimental Animal Research Institute of China Agricultural
University) were housed under a 12-h light/dark cycle at 22°C. Twelve hours before experiments, the rabbits were fasted but had free access to water. Sixteen rabbits were divided into four groups of 4 each, diniconazole was dissolved in ethanol and then diluted to 20 mg/ml with normal saline, and the percentage of ethanol in the whole system was 55%. Rac-diniconazole was administered at 20 mg/kg body weight (bd wt) by i.v. injection in the ear vein. The blood samples were collected from the first group at 5 and 30 min, from the second group at 15 and 60 min, from the third group at 120, 180, and 240 min, from the fourth group at 300, 360, 420, and 480 min after treatment. Blood (4 ml) was collected and centrifuged at 4000 rpm for 5 min, and the plasma was transferred to a new tube. The pharmacokinetics experiments were all performed in duplicate on two occasions.

Pharmacokinetics Analysis

The time–plasma concentration curves were fitted by a two-compartment open model in the Drug and Statistics computer program (Section of Quantitative Pharmacology, Chinese Pharmacological Society). Data were weighted with the reciprocal of the individual plasma concentration. Concentration in the central compartment (C) after a single intravenous dose was expressed as eq. 1:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$  (1)

where \(t\) is time; \(A\) and \(B\) are \(D(\alpha - k_{21})/V_1(\alpha - \beta)\) and \(D(k_{21} - \beta)/V_1(\alpha - \beta)\), respectively (\(D\) is the dose, \(V_1\) is the apparent volume of the central compartment, and \(k_{21}\) is the apparent first-order distribution rate from the peripheral to the central compartment); \(\alpha\) and \(\beta\) denote slopes of \(\alpha\)-phase and \(\beta\)-phase of log C, respectively (\(\alpha > \beta\)).

The area under the time–plasma concentration curve (AUC\(_{0\rightarrow\infty}\)) was calculated by the trapezoidal rule from time 0 to the time of the last measured plasma concentration divided by the terminal-phase elimination rate constant (\(\beta\)). \(\beta\) was determined by log-linear regression analysis of the terminal data points. The distribution and elimination phase half-life (\(t_{1/2\alpha}, t_{1/2\beta}\)) were calculated by 0.693/\(\alpha\) and 0.693/\(\beta\). The total apparent volumes of distribution (\(V_d\)) was calculated by \(D/(\beta AUC_{0\rightarrow\infty})\), and the total body clearance (CL) was calculated by \(D/AUC_{0\rightarrow\infty}\).

The enantiomer fraction (EF), which was considered superior to the enantiomeric ratio,

$$EF = \text{peak areas of the } (R)/[(R) + (S)]$$  (2)


EF of rac-diniconazole = 0.500, whereas preferential degradation of the (R) or (S) yields EF <0.500 or >0.500, respectively. The peak area corresponding with the limit of detection will be used if the enantiomer is not found.

Data Analysis

The data of racemization and stability of diniconazole enantiomers in plasma sample and standard solution were statistically analyzed according to Duncan’s multiple range

| TABLE 1. Recovery of assay method for determination of diniconazole enantiomers in rabbit plasma \((n = 6)\) |

<table>
<thead>
<tr>
<th>Fortification ((\mu g/ml))</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-Diniconazole</td>
</tr>
<tr>
<td>0.25</td>
<td>106.42 ± 9.07</td>
</tr>
<tr>
<td>1</td>
<td>90.34 ± 4.70</td>
</tr>
<tr>
<td>5</td>
<td>93.92 ± 6.86</td>
</tr>
</tbody>
</table>

*Values represent the means ± SD.

| TABLE 2. Accuracy and precision (CV) of assay method for determination of diniconazole enantiomers in rabbit plasma \((n = 6)\) |

<table>
<thead>
<tr>
<th>Theoretical conc. ((\mu g/ml))</th>
<th>Conc. found</th>
<th>Accuracy %</th>
<th>CV %</th>
<th>Conc. found</th>
<th>Accuracy %</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-to-day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.048 ± 0.002</td>
<td>95.87</td>
<td>3.81</td>
<td>0.049 ± 0.002</td>
<td>97.83</td>
<td>3.10</td>
</tr>
<tr>
<td>0.25</td>
<td>0.27 ± 0.02</td>
<td>106.41</td>
<td>6.62</td>
<td>0.26 ± 0.02</td>
<td>102.78</td>
<td>9.68</td>
</tr>
<tr>
<td>1</td>
<td>0.91 ± 0.04</td>
<td>91.11</td>
<td>4.29</td>
<td>0.91 ± 0.05</td>
<td>90.52</td>
<td>5.26</td>
</tr>
<tr>
<td>5</td>
<td>4.69 ± 0.23</td>
<td>93.83</td>
<td>5.00</td>
<td>4.73 ± 0.29</td>
<td>94.55</td>
<td>6.04</td>
</tr>
<tr>
<td>0.05</td>
<td>0.049 ± 0.002</td>
<td>98.01</td>
<td>3.78</td>
<td>0.049 ± 0.001</td>
<td>97.79</td>
<td>4.42</td>
</tr>
<tr>
<td>0.25</td>
<td>0.23 ± 0.01</td>
<td>92.74</td>
<td>5.79</td>
<td>0.23 ± 0.01</td>
<td>91.29</td>
<td>5.06</td>
</tr>
<tr>
<td>1</td>
<td>0.90 ± 0.06</td>
<td>90.16</td>
<td>6.92</td>
<td>0.90 ± 0.05</td>
<td>94.05</td>
<td>5.32</td>
</tr>
<tr>
<td>5</td>
<td>4.96 ± 0.44</td>
<td>99.15</td>
<td>8.89</td>
<td>5.07 ± 0.50</td>
<td>101.47</td>
<td>9.90</td>
</tr>
</tbody>
</table>

Pharmacokinetics Analysis

The Pharmacological Society). Data were weighted with the reciprocal of the individual plasma concentration. Concentration in the central compartment \((C)\) after a single intravenous dose was expressed as eq. 1:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$  (1)

where \(t\) is time; \(A\) and \(B\) are \(D(\alpha - k_{21})/V_1(\alpha - \beta)\) and \(D(k_{21} - \beta)/V_1(\alpha - \beta)\), respectively (\(D\) is the dose, \(V_1\) is the apparent volume of the central compartment, and \(k_{21}\) is the apparent first-order distribution rate from the peripheral to the central compartment); \(\alpha\) and \(\beta\) denote slopes of \(\alpha\)-phase and \(\beta\)-phase of log C, respectively (\(\alpha > \beta\)).

The area under the time–plasma concentration curve (AUC\(_{0\rightarrow\infty}\)) was calculated by the trapezoidal rule from time 0 to the time of the last measured plasma concentration divided by the terminal-phase elimination rate constant (\(\beta\)). \(\beta\) was determined by log-linear regression analysis of the terminal data points. The distribution and elimination phase half-life (\(t_{1/2\alpha}, t_{1/2\beta}\)) were calculated by 0.693/\(\alpha\) and 0.693/\(\beta\). The total apparent volumes of distribution (\(V_d\)) was calculated by \(D/(\beta AUC_{0\rightarrow\infty})\), and the total body clearance (CL) was calculated by \(D/AUC_{0\rightarrow\infty}\).

The enantiomer fraction (EF), which was considered superior to the enantiomeric ratio,

$$EF = \text{peak areas of the } (R)/[(R) + (S)]$$  (2)

EF of rac-diniconazole = 0.500, whereas preferential degradation of the (R) or (S) yields EF <0.500 or >0.500, respectively. The peak area corresponding with the limit of detection will be used if the enantiomer is not found.

Data Analysis

The data of racemization and stability of diniconazole enantiomers in plasma sample and standard solution were statistically analyzed according to Duncan’s multiple range

| TABLE 3. Racemization and stability of diniconazole enantiomers in plasma sample and standard solution |

<table>
<thead>
<tr>
<th>Fortification ((\mu g/ml))</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-Diniconazole</td>
</tr>
<tr>
<td>0.25</td>
<td>106.42 ± 9.07</td>
</tr>
<tr>
<td>1</td>
<td>90.34 ± 4.70</td>
</tr>
<tr>
<td>5</td>
<td>93.92 ± 6.86</td>
</tr>
</tbody>
</table>

*Values represent the means ± SD.
test \((P = 0.05)\). The differences in pharmacokinetic parameters were evaluated by using Student’s \(t\)-test with significant level of \(P < 0.05\).

**RESULTS AND DISCUSSION**

**Calibration and Method Validation**

On CDMPC, the \(R\)-diniconazole elutes first in \(n\)-hexane/2-propanol mobile phase.\(^{12}\) In this study, it was found that the elution order of the two enantiomers in \(n\)-hexane/2-propanol was the same as that in \(n\)-hexane/ethanol mobile phase. Thus, the first eluted stereoisomer in \(n\)-hexane/ethanol mobile phase was confirmed as \(R\)-diniconazole, while the second one was \(S\)-diniconazole.

There were no endogenous interference peaks eluted at retention times equal to \(R\)- and \(S\)-diniconazole in blank plasma. The \(R_s\) of two enantiomers was 1.08. Representative HPLC chromatograms of extracts from untreated rabbit plasma, untreated rabbit plasma spiked with \(r\)-diniconazole (1 \(\mu\)g/ml), and treated plasma are shown in Figure 2.

Linear calibration curves were obtained over the concentration range of 0.05–25 \(\mu\)g/ml in plasma for both \(R\)-diniconazole \((y = 57.734x + 6.3417, R^2 = 0.9994)\) and \(S\)-diniconazole \((y = 57.463x + 5.9267, R^2 = 0.9993)\). The accuracy and precision of the assay, for both enantiomers, ranged from 4 to 10% (CV) and 90 to 107% (accuracy), respectively, over the entire calibration range (Table 1). The fortified recovery is presented in Table 2. The lowest recovery was over 80%. Table 3 shows that there were no significant difference in ERs and recoveries of diniconazole in plasma samples and standard solutions, which had been stored for different time. The LOD was 0.02 \(\mu\)g/ml and the LOQ was 0.05 \(\mu\)g/ml for both enantiomers in plasma.

**Pharmacokinetics**

The time–plasma concentration curves of \(R\) and \(S\)-diniconazole after i.v. treatment of rabbits with \(r\)-diniconazole at 20 mg/kg bd wt are shown in Figure 3A. There was no significant difference between concentrations of two enantiomers in rabbit plasma from 5 to 15 min after treatment. But with time increasing, the concentration of \(S\)-diniconazole decreased rapidly. The \(R\)-diniconazole was prevailing in plasma from 15 min to the last time point.

Plasma EFs–time curve of two enantiomers after treatment is shown in Figure 3B. The EFs were considerably varied from 0.5 of the racemate at 30 to 480 min after treatment and increased with time increasing. Average EFs ranged from 0.49 (5 min) to 0.68 (480 min).

Compartmental pharmacokinetic analysis showed some statistical differences between the enantiomers in principal pharmacokinetic parameters (Table 4). The \(R/S\)-diniconazole ratio of the distribution half-life \((t_{1/2a})\) was 1.50, whereas the elimination half-life \((t_{1/2b})\) of \(R\)-diniconazole was same as that of \(S\)-enantiomers. The result indicated that the \(S\)-diniconazole distributed quickly than its antipode in rabbit. The total plasma clearance (CL) of \(S\)-diniconazole was higher than that of its antipode, the CL ratio

![Fig. 3. Time–plasma concentration curves (A) and EFs (B) of diniconazole enantiomers in rabbits following \(r\)-diniconazole administration at 20 mg/kg bd wt (● = \(R\)-diniconazole, □ = \(S\)-diniconazole; values represent the means ± SD).](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(R)-Diniconazole</th>
<th>(S)-Diniconazole</th>
<th>(P^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(t_{1/2a}, t_{1/2b}) ((\text{min}))</td>
<td>69.315 ± 2.411</td>
<td>46.131 ± 1.811</td>
<td>0.0165</td>
</tr>
<tr>
<td>(t_{1/2b}) ((\text{min}))</td>
<td>69.315 ± 1.756</td>
<td>69.315 ± 3.456</td>
<td>1</td>
</tr>
<tr>
<td>(V_f) ((l/kg))</td>
<td>2.363 ± 0.117</td>
<td>2.378 ± 0.237</td>
<td>0.9208</td>
</tr>
<tr>
<td>CL ((l/(min kg)))</td>
<td>0.007 ± 0.0001</td>
<td>0.011 ± 0.0003</td>
<td>0.0318</td>
</tr>
<tr>
<td>AUCO–480 ([\text{mg/(l min)}])</td>
<td>957.595 ± 12.378</td>
<td>711.777 ± 5.876</td>
<td>0.0168</td>
</tr>
<tr>
<td>AUCO–(\infty) ([\text{mg/(l min)}])</td>
<td>1443.145 ± 59.785</td>
<td>946.825 ± 48.978</td>
<td>0.0139</td>
</tr>
</tbody>
</table>

*\(P < 0.05\) was considered statistical significance.

Chirality DOI 10.1002/chir
between two enantiomers was 1.50. The $R$/S-diniconazole ratios of the AUC$_{0–480}$ min and AUC$_{0–\infty}$ were 1.35 and 1.52, respectively.

These results indicated substantial stereoselectivity on pharmacokinetics of diniconazole enantiomers in rabbits. The most likely reason involved in these results was stereopreferable distribution of $R$-diniconazole in rabbit plasma.

**CONCLUSION**

This study showed that an acute administration of rac-diniconazole to rabbit resulted in stereoselective pharmacokinetics of $R$- and $S$-enantiomer. Plasma concentration of the $S$-enantiomer decreased more quickly than that of the $R$-enantiomer and resulted in higher concentration of $R$-enantiomer in plasma. These may have some application for the environmental and ecological risks assessment of chiral pesticides.

**LITERATURE CITED**

1. Takano H, Oguri Y, Kato T. Mode of action of (E)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (s-3308) in ustilago-maydis. J Pestic Sci 1983;8:575–582.
2. Takano H, Oguri Y, Kato T. Antifungal and plant-growth regulating activities of enantiomers (e)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (s-3308). J Pestic Sci 1986;11:373–378.