Simultaneous Determination of Silibinin A and Silibinin B in Rat Plasma and Pharmacokinetic Study

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Abstract: **Objective** To investigate the bioavailability and pharmacokinetics of silibinin A and silibinin B in rats, respectively. **Methods** Following iv and ig administration of silibinin to 20 Wistar rats, the plasma samples were collected at different time points up to 12 h. Sample pretreatment was involved in one-step protein precipitation with acetonitrile. Silibinin A and silibinin B were simultaneously determined by LC-MS/MS. **Results** After ig dosing silibinin 28, 56, and 112 mg/kg to rats, the $t_{1/2\beta}$ values were 5.48, 5.08, and 5.73 h for silibinin A, and 4.56, 4.12, and 5.53 h for silibinin B; The C_{max} were 674.3, 1349.4, and 2042.5 ng/mL for silibinin A, and 671.0, 1365.4, and 2066.2 ng/mL for silibinin B; The T_{max} were 0.20, 0.23, and 0.20 h for silibinin A, and 0.20, 0.23, and 0.20 h for silibinin B; The AUC were 454.4, 845.9, and 1219.5 h·ng/mL for silibinin A, and 432.0, 817.1, and 1153.6 h·ng/mL for silibinin B. The absolute bioavailabilities of silibinin A and silibinin B were 2.86% and 1.93%, respectively. **Conclusion** Silibinin A and silibinin B have very low bioavailability after ig administration, and there is no significant difference in the pharmacokinetic parameters between silibinin A and silibinin B, which indicates that the two diastereoisomers have similar pharmacokinetic behavior in rats.

Key words: bioavailability; LC-MS/MS; pharmacokinetics; silibinin A; silibinin B
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Introduction

Silibinin is the main isomer of a group of flavanoids extracted from the fruit of *Silybum marianum* (L). Gaertn., which has been widely used to maintain liver health and for the treatment of liver disorder as a common herb (Flora *et al*, 1998; Kidd and Head, 2005; Kvasnicka *et al*, 2003). Recent literatures have demonstrated that silibinin also has anti-inflammatory effect (Trappoliere *et al*, 2009; Gu *et al*, 2007) and high antitumor activity in *in vitro* and *in vivo* studies (Lin, Sukarieh, and Pelletier, 2009; Kim *et al*, 2004; 2009; Singh *et al*, 2008; Raina *et al*, 2007; Chen *et al*, 2006; Gallo *et al*, 2003). Silibinin, a mixture of diastereo-isomers, is composed of silibinin A and silibinin B.

Several papers have been published on the determination of silibinin in biosamples after administration of the drug to human and various animals (Li *et al*, 2006; Gatti and Perucca, 1994; Skottova *et al*, 2001; Filburn, Kettenacker, and Griffin, 2007; Wu *et al*, 2007). However, in these methods, silibinin was determined as a single entity due to the absence of reference substances of silibinin A and silibinin B. Although Rickling *et al* (1995) reported an HPLC-ECD method for the quantification of silibinin A and silibinin B in human plasma, this method made sensitivity low and sample preparation complicated. Li *et al* (2008) determined silibinin A and silibinin B in Chinese subjects by LC-MS/MS following a single oral dose of silybin-phosphatidylcholine complex, but there were no pharmacokinetic data after an iv administration in the experiment, so the absolute bioavailability values of silibinin A and silibinin B were not obtained. To the best of our knowledge, the bioavailability and pharma- cokinetic characteristics of silibinin A and silibinin B have not been researched completely so far, therefore, an intensive investigation on the bioavailability and pharmacokinetics of the two diastereoisomers is expected.

In the present study, a simple, rapid, and sensitive LC-MS/MS method was developed and validated for

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the determination of silibinin A and silibinin B in rat plasma after iv and ig administrations, and the method was successfully applied to investigate the bio-availability and pharmacokinetics of silibinin A and silibinin B in rats, respectively.

Materials and methods

Chemical reagents

Silibinin A and silibinin B (Fig. 1) reference substances were separated and purified by Tianjin Tasly Institute (Tianjin, China) in our previous work (Li *et al*, 2008). Their purity was above 98.5% via HPLC analysis. Naringin, which was used as the internal standard (IS), was purchased from the National Institute for Food and Drug Control (Beijing, China). Methanol (HPLC grade) was obtained from Fisher Scientific (Pittsburgh, USA), while all other chemicals were of analytical grade and used without further purification. The distilled water, prepared from demineralized water, was used throughout the study.

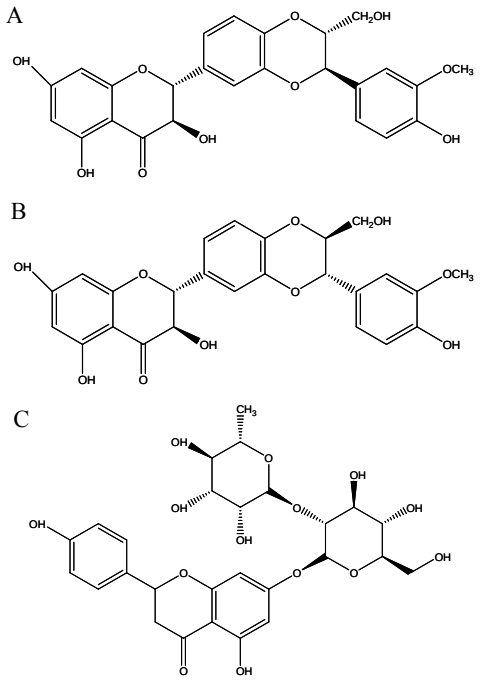

Fig. 1 Chemical structures of silibinin A (A), silibinin B (B), and naringin (C)

LC-MS/MS condition

An LC-MS/MS system utilized a Surveyor MS pump, a Surveyor autosampler, and a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer equipped with an ESI source. Data acquisition was performed with Xcalibur 1.3 software (Thermo Finnigan, USA). The column was Agilent Zorbax Eclipse XDB-C_{18} column (150 mm × 2.1 mm, 5 μm). The mobile phase was mixed with methanol-water-formic acid (48:52:0.1). The flow rate was 0.25 mL/min for an isocratic elution. The column temperature was maintained at 30 ℃. The mass spectrometer was operated in the negative mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of *m/z* 481→301 for silibinin A and silibinin B, and *m/z* 579→271 for IS, respectively (Fig. 2). The optimized parameters were as follows: electrospay voltage 3.8 kV, capillary temperature 390 ℃, nitrogen (N_2) was used as sheath gas and auxiliary gas at the pressures of 1.72 × 10^5 and 2.07 × 10^4 Pa, respectively, and the collision energy of 20 eV was used for the analytes and 34 eV for IS.

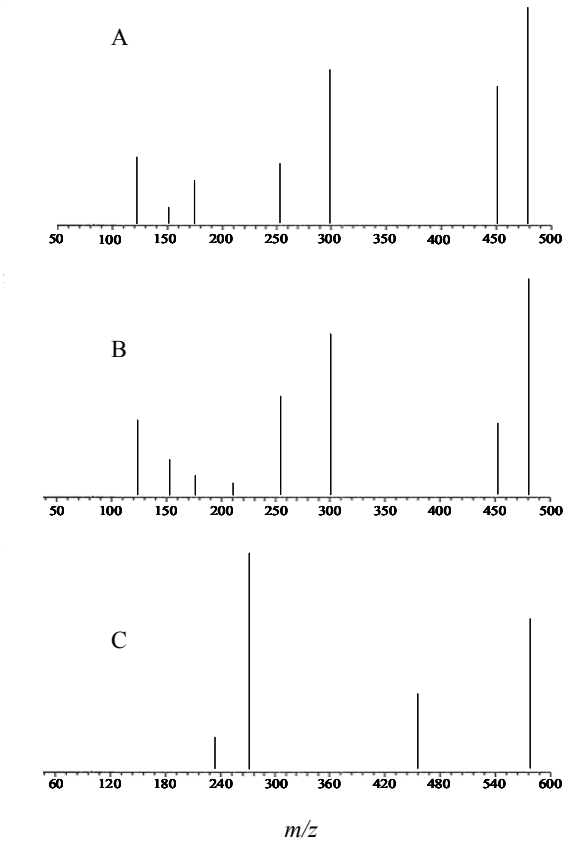

Fig. 2 Mass spectra of silibinin A (A), silibinin B (B), and naringin (C)

Animal experiment

Male Wistar rats, weighing 240－260 g, were provided by Vital River Lab Animal Technology Co., Ltd. (Beijing, China) and housed with a 12 h light/12 h night cycle at ambient temperature (about 25 ℃) and 45% relative humidity. All the animal experiment

procedure was approved by the Animal Ethics Committee of Tianjin Tasly Institute. Silibinin was dissolved in ethanol-PEG200 (1:1) solution. Free access to food and water was allowed at all time except for fasting 12 h before the experiment. Fifteen rats were ig administered with silibinin 28, 56, and 112 mg/kg (equivalent to silibinin A 13.3, 26.6, and 53.2 mg/kg; silibinin B 14.7, 29.4, and 58.8 mg/kg), respectively. For the absolute bioavailability study, another five Wistar rats were iv administered with silibinin 28 mg/kg (equivalent to silibinin A 13.3 mg/kg, silibinin B 14.7 mg/kg). Blood samples were collected into heparinized tubes from each rat by the puncture of the retroorbital sinus at 0, 0.17, 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, and 12 h after administration, and centrifuged at 3000 × *g* for 8 min to obtain plasma samples, which were stored at –20 ℃ and analyzed within one week.

Sample preparation

Thawed plasma samples were vortexed thoroughly at room temperature and employed as follows: In a 1.5 mL Eppendorf tube, 100 μL IS solution (200 ng/mL naringin prepared by acetonitrile) and 100 μL acetonitrile were added to 100 μL plasma sample. After vortexing for 30 s, the sample was centrifuged at 12 000 × *g* for 5 min, and 10 μL of the supernatant was injected into the LC-MS/MS system for analysis.

Method validation

This LC-MS/MS assay was validated thoroughly in terms of specificity, matrix effect, linearity, precision, extraction recovery, and stability according to the guiding principles issued by China SFDA. The quality control (QC) samples containing appropriate amount of silibinin A and silibinin B, prepared based on the procedures described above, at low, middle, and high concentrations on three sequential days, were used for validation.

Data analysis

Pharmacokinetic parameters of silibinin A and silibinin B were calculated using the Topfit 2.0 program by the non-compartmental method. The elimination rate constant (λ_z) was obtained as the slope of the linear regression of the log-transformed concentration values versus time date in the terminal phase. The elimination half-life ($t_{1/2\beta}$) was calculated as $0.693/\lambda_z$. The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the curve to the last measurable concentration ($AUC_{0\text{-}t}$) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0\text{-}\infty}$) was calculated as $AUC_{0\text{-}\infty} = AUC_{0\text{-}t} + C_t/\lambda_z$, where C_t is the last measurable concentration.

Results

Specificity and matrix effect

A better resolution for silibinin A and silibinin B and a shorter run time were achieved by the combination of Agilent Zorbax Eclipse XDB-C_{18} column (150 mm × 2.1 mm, 5 μm) and the mobile phase of methanol-water (48:52) containing 0.1% formic acid. The chromatograms of blank plasma, blank plasma spiked with silibinin A, silibinin B, and IS, as well as the rat plasma sample were presented in Fig. 3, and no interference was observed at the retention time (t_R) of the analytes and IS due to endogenous substances in rat plasma. Matrix effects for silibinin A, silibinin B, and IS were evaluated by comparing the peak areas of analytes in extracted samples of blank plasma with the corresponding areas obtained by direct injection of reference solutions. Matrix effects for silibinin A, silibinin B, and IS were minimal based on 90.2%—107.8% of nominal concentrations.

Calibration curves and linearity

Calibration curves were constructed by performing weighed linear regression analysis ($w_i = 1/C^2$) of peak area ratios (R) of respective analyte to IS versus the corresponding concentrations (C, ng/mL). The obtained calibration curves showed a good linearity over the range of 2.0—50 000 ng/mL for silibinin A and silibinin B. The regression equations and their correlation coefficient (r) were calculated as follows: silibinin A, R = 0.001 25 C + 0.000 63 (r = 0.9981); silibinin B, R = 0.001 22 C + 0.000 96 (r = 0.9959). The LOD (S/N = 3) and LOQ (S/N = 60) were determined as 0.1 and 2.0 ng/mL for silibinin A and silibinin B, respectively.

Precision and accuracy

The precision and accuracy of the method were presented in Table 1. The intra- and inter-batch precisions, expressed as RSD, were obtained by analyzing QC samples containing silibinin A and silibinin B at low, middle, and high concentrations in six replicates of each concentration for each batch and calculated by SD

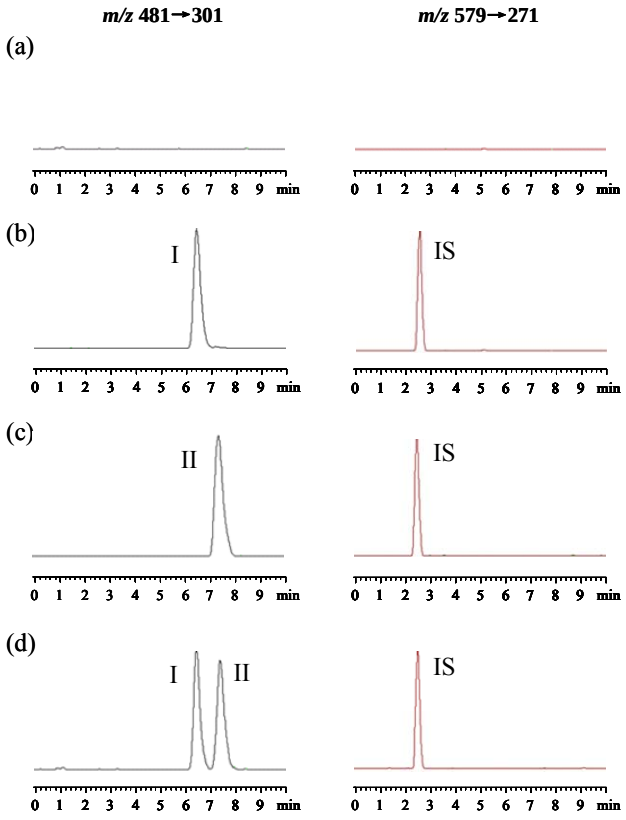

Fig. 3 Representative chromatograms of analytes (left) and IS (right) in rat plasma

I: Silibinin A (t_R 6.45 min) II: Silibinin B (t_R 7.34 min) IS: Naringin (t_R 2.55 min) a: blank plasma b: blank plasma spiked with silibinin A and IS c: Blank plasma spiked with silibinin B and IS d: rat plasma sample

of measured concentrations from one batch (within one day) and from three batches (on three consecutive days) divided by the mean measured concentration. The RSD was found to be 1.9%—9.1% for intra-batch precision and 0.8%—5.7% for inter-batch precision, indicating that this method had a good reproducibility. The accuracy (relative error, RE) was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentration (C_{obs}) as follows: RE = $[(C_{obs}-C_{nom})/(C_{nom})] \times 100\%$. The values were estimated from –5.8% to 5.6% (intra-batch) and from –2.1% to 5.7% (inter-batch), indicating high accuracy of this method. In addition, the precision and accuracy of LOQ were also investigated, and the values were all less than 12%.

Recovery and stability

The mean recoveries of silibinin A, silibinin B, and IS were more than 90%. In processed samples, the analytes were stable for a period of 24 h at room temperature (RE < ± 4.8%), plasma sample showed no sign of degradation at –20 ℃ within 60 d (RE < ± 7.4%), and no instability of analytes in spiked samples was observed over three freeze/thaw cycles (RE < ± 6.2%).

Bioavailability and pharmacokinetics of silibinin A and silibinin B

The mean silibinin A and silibinin B plasma concentrations versus time curves in rat by iv and ig administration were shown in Fig. 4, and the main pharmacokinetic parameters of silibinin A and silibinin B using non-compartmental analysis with Topfit 2.0 software were summarized in Tables 2 and 3. The mean absolute bioavailability values of silibinin A and silibinin B were 2.86% and 1.93%, respectively, which were calculated by the formula: Absolute bioavailability = $(AUC_{ig} \times Dose_{iv}/ AUC_{iv} \times Dose_{ig}) \times 100\%$.

Table 1 Precision and accuracy of the method

Compound	Concentration / ($ng \cdot mL^{-1}$)	Inter-batch		Intra-batch	
		RSD / %	RE / %	RSD / %	RE / %
silibinin A	2.0	4.3	–7.1	11.9	–6.0
	20.0	5.7	3.7	8.3	5.6
	500.0	2.7	–1.6	1.9	–3.4
	20 000.0	2.1	–1.3	2.1	–2.4
silibinin B	2.0	7.6	–2.5	10.0	–4.1
	20.0	4.8	5.7	9.1	–4.7
	500.0	2.1	2.4	5.0	–5.8
	20 000.0	0.8	–2.1	2.2	1.1

Discussion

In this paper, a simple, rapid, and sensitive LC-MS/MS method was developed and validated for the determination of silibinin A and silibinin B in rat plasma. The LOQ of the current assay was only 2.0 ng/mL for both silibinin A and silibinin B, and it was demonstrated to be useful for the study of silibinin A and silibinin B disposition in rats for up to 12 h after iv

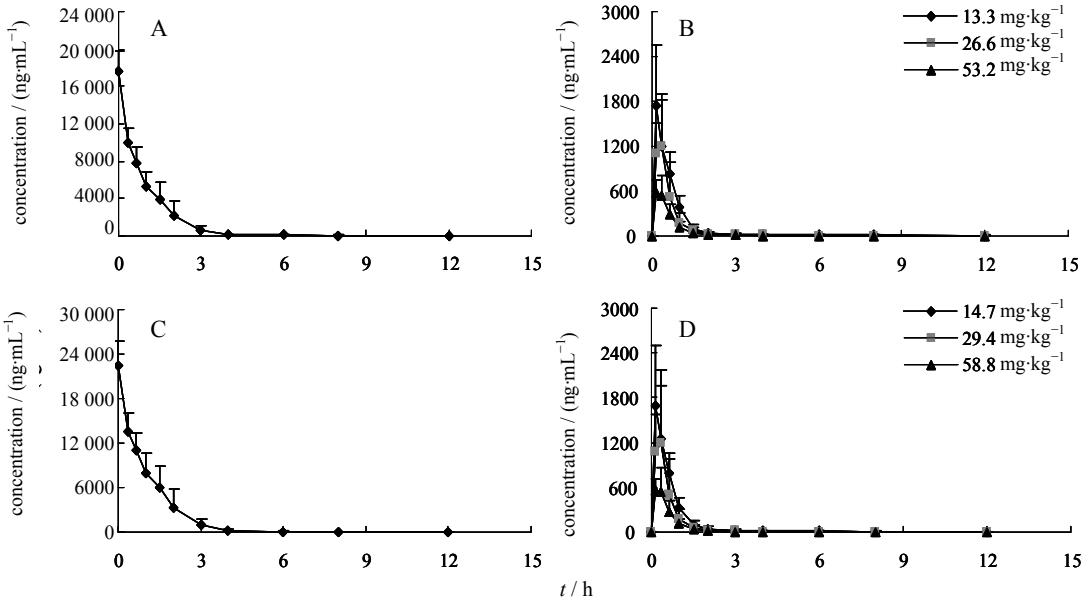

Fig. 4 Mean plasma concentration-time curves of silibinin A and silibinin B in rats (*n* = 5)

A: silibinin A, iv administration of 13.3 mg/kg B: silibinin A, ig administration of 13.3, 26.6, and 53.2 mg/kg C: silibinin B, iv administration of 14.7mg/kg D: silibinin B, ig administration of 14.7, 29.4, and 58.8 mg/kg

Table 2 Main pharmacokinetic parameters of silibinin A in rats (*n* = 5)

Parameters	Units	iv	ig		
		13.3 mg·kg^{-1}	13.3 mg·kg^{-1}	26.6 mg·kg^{-1}	53.2 mg·kg^{-1}
$t_{1/2}$	h	4.11 ± 0.98	5.48 ± 2.23	5.08 ± 0.65	5.73 ± 2.82
C_{max}	ng·mL^{-1}	—	674.3 ± 223.7	1349.4 ± 591.1	2042.5 ± 714.2
T_{max}	h	—	0.20 ± 0.07	0.23 ± 0.09	0.20 ± 0.07
AUC_{0-t}	ng·h·mL^{-1}	15 907.8 ± 3897.9	454.4 ± 152.3	845.9 ± 392.3	1219.5 ± 139.3
$AUC_{0-\infty}$	ng·h·mL^{-1}	16 096.4 ± 3941.1	474.4 ± 169.5	871.9 ± 385.1	1272.4 ± 166.6
MRT	h	1.05 ± 0.21	1.15 ± 0.27	1.05 ± 0.33	1.11 ± 0.32
CL	mL·min^{-1}·kg^{-1}	14.6 ± 4.1	516.8 ± 178.2	574.0 ± 193.6	708.0 ± 107.0
V_d	L·kg^{-1}	5.19 ± 1.82	224.0 ± 68.3	259.6 ± 113.0	334.6 ± 141.8

Table 3 Main pharmacokinetic parameters of silibinin B in rats (*n* = 5)

Parameters	Units	iv	ig		
		14.7 mg·kg^{-1}	14.7 mg·kg^{-1}	29.4 mg·kg^{-1}	58.8 mg·kg^{-1}
$t_{1/2}$	h	2.89 ± 0.44	4.56 ± 2.26	4.12 ± 0.58	5.53 ± 4.28
C_{max}	ng·mL^{-1}	—	671.0 ± 274.9	1365.4 ± 733.5	2066.2 ± 848.1
T_{max}	h	—	0.20 ± 0.07	0.23 ± 0.09	0.20 ± 0.07
AUC_{0-t}	ng·h·mL^{-1}	22 432.9 ± 5812.1	432.0 ± 158.2	817.1 ± 454.7	1153.6 ± 205.4
$AUC_{0-\infty}$	ng·h·mL^{-1}	22 527.8 ± 5832.8	442.2 ± 165.3	829.4 ± 453.0	1179.4 ± 214.5
MRT	h	1.03 ± 0.23	1.02 ± 0.28	0.94 ± 0.28	0.99 ± 0.30
CL	mL·min^{-1}·kg^{-1}	11.6 ± 3.5	616.0 ± 214.5	718.4 ± 314.1	855.2 ± 165.8
V_d	L·kg^{-1}	2.92 ± 1.04	225.8 ± 87.1	258.8 ± 124.0	393.0 ± 306.1

and ig administration. The plasma volumes required in our assay was only 100 μL. This lower volume permitted the use of serial blood sampling in studies involving a small animal species, such as rats. Furthermore, the high sample throughput was achieved by simple sample preparation and short chromatographic run time (within 10 min). In conclusion, the high sensitivity, small sample volume requirement, and relatively short analytical time of the assay make it suitable for preclinical pharmacokinetic studies of silibinin A and silibinin B. An exhaustive investigation on the bioavailability and pharmacokinetics of the two diastereoisomers was first reported. It provides useful information for the further research of silibinin and its preparation.

In the reported literature (Li *et al*, 2008), it was

found that silibinin A had better absorption and faster elimination than silibinin B, and the velocity of absorption was similar between the two diastereo-isomers in human. The pharmacokinetic difference between silibinin A and silibinin B might be due to stereoselective absorption, distribution, and metabolism. However, in this study, we not only found that silibinin A and silibinin B had very low bioavailability after ig administration, but also there was no significant difference in the pharmacokinetic parameters between silibinin A and silibinin B, which indicated that the two diastereoisomers had similar pharmacokinetic behavior in rats. Compared to ig administration, iv administration of silibinin A and silibinin B showed a shorter $t_{1/2\beta}$, suggesting that silibinin A and silibinin B were eliminated more rapidly from rat plasma after iv administration. Following ig administration of silibinin at 28 and 56 mg/kg, the dose and AUC/C_{max} for silibinin A and silibinin B were positively correlated. In addition, there was no significant difference in systemic clearance at the two dose levels. It was demonstrated that silibinin A and silibinin B might have linear pharmacokinetic characteristics in rats within the two dose ranges tested. However, the AUC and C_{max} of the two diastereoisomers did not increase proportionally at the dose of 112 mg/kg. We estimated that the nonlinearity might be due to absorption saturation at high dosage.

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