

College of Pharmacy¹, Yanbian University, Jilin, China; College of Natural Science², BK21 Project team,³ College of Pharmacy, Chosun University, Gwangju, Republic of Korea

Effects of silybinin, CYP3A4 and P-glycoprotein inhibitor *in vitro*, on the bioavailability of loratadine in rats

C. LI^{1,3}, M. Y. LEE², J. S. CHOI³

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Prof. Jun-Shik Choi, College of Pharmacy, Chosun University, Dong-Gu, Gwangju 501-759, Republic of Korea
jsachoi@chosun.ac.kr

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The effect of silybinin on the pharmacokinetics of orally and intravenously administered loratadine in rats was investigated. Pharmacokinetic parameters of loratadine were determined in rats following oral ($4 \text{ mg} \cdot \text{kg}^{-1}$) and intravenous ($1 \text{ mg} \cdot \text{kg}^{-1}$) administration to rats in the presence and absence of silybinin (0.3 , 1.5 and $6 \text{ mg} \cdot \text{kg}^{-1}$). Compared to those animals in an oral control group (given loratadine alone), the area under the plasma concentration-time curve (AUC) and the peak plasma concentration (C_{max}) of loratadine were increased significantly ($P < 0.05$ for $1.5 \text{ mg} \cdot \text{kg}^{-1}$, $P < 0.01$ for $6 \text{ mg} \cdot \text{kg}^{-1}$) by 50.0 – 76.7% and 65.4 – 90.1% , respectively, by silybinin. Consequently, the absolute bioavailability of loratadine in the presence of silybinin (1.5 and $6 \text{ mg} \cdot \text{kg}^{-1}$) was 8.6 – 10.2% , which was significantly ($1.5 \text{ mg} \cdot \text{kg}^{-1}$, $P < 0.05$; $6 \text{ mg} \cdot \text{kg}^{-1}$, $P < 0.01$) enhanced compared to that in oral control group (5.8%). Moreover, the relative bioavailability of loratadine was 1.50 - to 1.77 -fold greater than that in the control group. In contrast, silybinin had no effect on any pharmacokinetic parameters of loratadine given intravenously, implying that co-administration of silybinin could inhibit the cytochrome P450 (CYP) 3A4-mediated metabolism of loratadine, resulting in reducing gastrointestinal and hepatic first-pass metabolism, and the P-glycoprotein (P-gp) efflux pump in the small intestine. Silybinin significantly enhanced the oral bioavailability of loratadine, suggesting that concurrent use of silybinin and loratadine should be monitored closely for potential drug interactions.

1. Introduction

Loratadine is a widely prescribed, non-sedating, antihistamine, with selective peripheral histamine H₁-receptor antagonist activity that is not associated with performance impairment and has an excellent safety record (Bradley and Nicholson 1987; Ramaekers et al. 1992; Kay et al. 1997; Philpot 2000; Prenner et al. 2000). Loratadine is orally administered and is used to treat the symptoms of allergies, skin hives and itching in people with chronic skin reactions (Clissold et al. 1989).

Loratadine undergoes extensive first-pass metabolism in the liver (Hilbert et al. 1987) and CYP3A4 enzymes are responsible for the metabolism of loratadine (Yumibe et al. 1996). Loratadine is also a substrate of P-gp (Wang et al. 2001). Since P-gp is co-localized with CYP3A4 in the small intestine, P-gp and CYP3A4 may act synergistically in absorption and first-pass metabolism of drugs, respectively (Wacher et al. 1998; Ito et al. 1999; Pichard et al. 1990).

Flavonoids are phytochemicals that are produced in high quantities by various plants (Dixon and Steel 1999). Flavonoids have been referred to as “nature’s biological response modifiers” because of strong experimental evidence of their inherent ability to modify the body’s reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobial and anti-cancer activity (Yamamoto and Gaynor 2001; Cushnie and Lamb 2005). Silymarin, a flavonoid complex, is extracted from seeds of the milk thistle (*Silybum marianum* L.), which is a medicinal plant widely used in traditional European medicine (Morazzoni and Bombardelli 1995). Silymarin

has strong antioxidant activity (Zhao et al. 2000) and exhibits cytoprotective, anti-inflammatory and anticarcinogenic effects (Katiyar et al. 1997). Silybinin is the major and most active component in silymarin, comprising 60 – 70% of silymarin (Saller et al. 2001). Kosina et al. (2005) reported that silybinin inhibits human CYP1A2 and 3A4 while Zuber et al. (2002) found that silybinin inhibits human CYP2D6 and 3A4. Thus, the inhibition effects of silybinin against human CYP enzymes remain somewhat controversial. Silybinin is an inhibitor of P-gp in the KB/MDR cell line (Dzubák et al. 2006), but the inhibitory effect of silybinin against P-gp is ambiguous. Therefore, we re-evaluated the inhibition of CYP enzyme activity and P-gp activity by silybinin using CYP inhibition assay and rhodamine-123 retention assay in P-gp-overexpressing MCF-7/ADR cells. Silybinin and loratadine are sometimes prescribed for the treatment or prevention of dermatological or allergic diseases as a combination therapy. However, the possible effects of silybinin on the bioavailability and pharmacokinetics of loratadine have not been reported *in vivo*. Therefore, the aim of this study was to investigate the effect of silybinin on the bioavailability and pharmacokinetics of loratadine in rats.

2. Investigations and results

2.1. Inhibition of CYP3A4

The inhibitory effect of silybinin on CYP3A4 activity is shown in Fig. 1. The IC_{50} values of silybinin on CYP3A4 is $1.97 \mu\text{M}$.

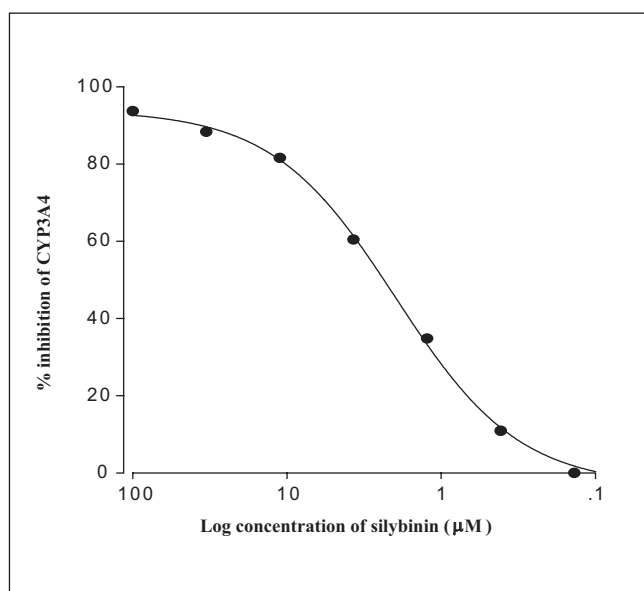


Fig. 1: Inhibitory effect of silybinin on CYP3A4 activity. All experiments were done in duplicate, and the result is expressed as the percent of inhibition (IC_{50} : 1.97 μ M)

Silybinin inhibited CYP3A4 enzyme activity in a concentration-dependent manner.

2.2. Rhodamine-123 retention assay

In this study, the cell-based P-gp activity test using rhodamine-123 also showed that silybinin (100 μ M, $P < 0.01$) significantly inhibited P-gp activity (Fig. 2).

2.3. Effect of silybinin on the pharmacokinetics of loratadine after oral administration

The mean plasma concentration-time profiles of loratadine after oral administration (4 $\text{mg} \cdot \text{kg}^{-1}$) in the presence or absence of

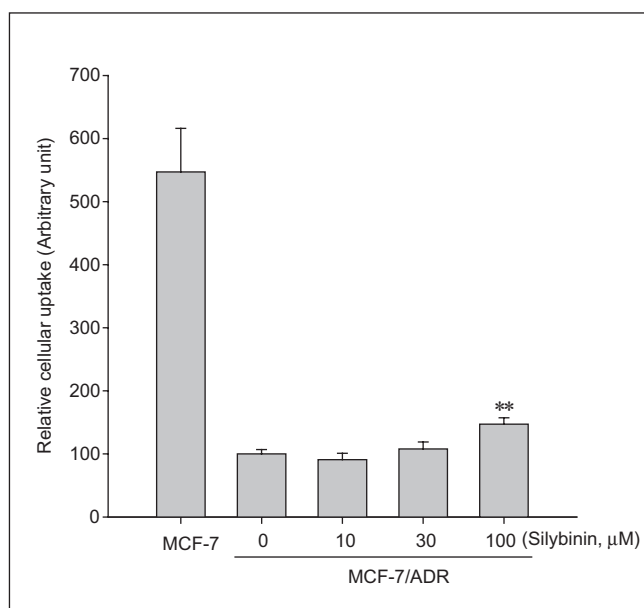


Fig. 2: Rhodamine-123 retention. MCF-7/ADR cells were preincubated with silybinin for 30 min. After incubation of MCF-7/ADR cells with 20 μ M R-123 for 90 min, the R-123 fluorescence values in cell lysates were measured using the excitation and emission wavelengths of 480 and 540 nm, respectively. The values were divided by the total protein content of each sample. Data represents mean \pm SD of 6 separate samples (significant versus control MCF-7 cells, $**P < 0.01$)

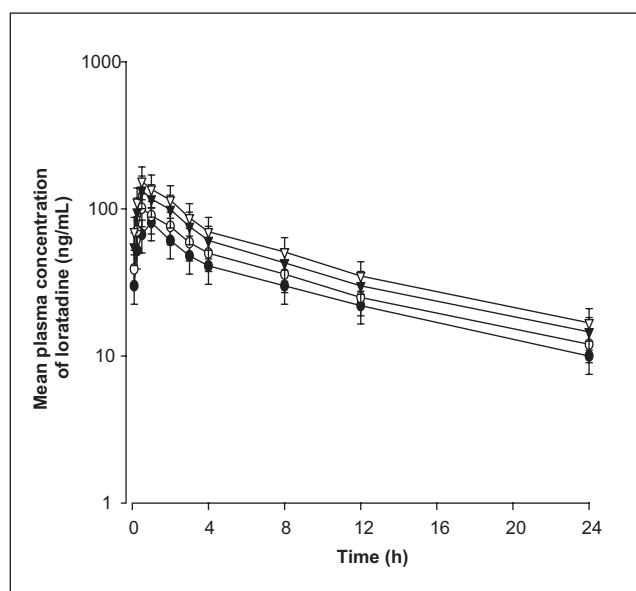


Fig. 3: Mean plasma concentration-time profiles of loratadine after oral administration (4 $\text{mg} \cdot \text{kg}^{-1}$) of loratadine to rats in the presence or absence of silybinin (0.3, 1.5 and 6 $\text{mg} \cdot \text{kg}^{-1}$) ($n = 6$, each). Bars represent the standard deviation: (●) Oral administration of loratadine (4 $\text{mg} \cdot \text{kg}^{-1}$); (○) with 0.3 $\text{mg} \cdot \text{kg}^{-1}$ of Silybinin; (▼) with 1.5 $\text{mg} \cdot \text{kg}^{-1}$ of Silybinin; (▽) with 6 $\text{mg} \cdot \text{kg}^{-1}$ of Silybinin

silybinin (0.3, 1.5 or 6 $\text{mg} \cdot \text{kg}^{-1}$) were characterized in rats and illustrated in Fig. 3. The pharmacokinetic parameters of loratadine were also summarized in Table 1. Compared to animals in the oral control group, the AUC was significantly (1.5 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.05$; 6 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.01$) increased by 50.0–76.7% in the presence of silybinin. The C_{max} was significantly (1.5 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.05$; 6 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.01$) increased by 65.4–90.1% in the presence of silybinin. The absolute bioavailability (A.B.) of loratadine was 8.6–10.2%, which was significantly enhanced (1.5 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.05$; 6 $\text{mg} \cdot \text{kg}^{-1}$, $P < 0.01$) compared to that in oral control group (5.8%). Consequently, the relative bioavailability (R.B.) of loratadine was increased by 1.54 to 1.96-fold. However, there were no significant changes in the T_{max} and the $t_{1/2}$ of loratadine in the presence of silybinin.

2.4. Effect of silybinin on the pharmacokinetics of loratadine after intravenous administration

The pharmacokinetic profiles of loratadine were also evaluated after its intravenous administration (1 $\text{mg} \cdot \text{kg}^{-1}$) in the presence or absence of silybinin (0.3, 1.5 or 6 $\text{mg} \cdot \text{kg}^{-1}$) and illustrated in Fig. 4. As summarized in Table 2, silybinin had no effect on the pharmacokinetic parameters of intravenous loratadine although it exhibited a significant effect on the bioavailability of loratadine given orally, suggesting that silybinin may improve the oral bioavailability of loratadine by increasing the absorption in the intestine or reducing gastrointestinal and hepatic first-pass metabolism in rats.

3. Discussion

Based on the broad overlap in substrate specificities as well as co-localization in the small intestine, the primary site of absorption for orally administered drugs, CYP3A4 and P-gp, are recognized as a concerted barrier to drug absorption (Cummins et al. 2002; Wolozin et al. 2000). CYPs enzymes contribute significantly to first-pass metabolism and oral bioavailability of many drugs. The first-pass metabolism of compounds in the intestine limits the absorption of toxic xenobiotics and

Table 1: Mean (\pm S.D.) pharmacokinetic parameters of loratadine after oral administration of loratadine ($4 \text{ mg} \cdot \text{kg}^{-1}$) to rats in the presence or absence of silybinin ($0.3, 1.5$ and $6 \text{ mg} \cdot \text{kg}^{-1}$) ($n = 6$, each)

Parameter	Control	Loratadine + Silybinin		
		$0.3 \text{ mg} \cdot \text{kg}^{-1}$	$1.5 \text{ mg} \cdot \text{kg}^{-1}$	$6 \text{ mg} \cdot \text{kg}^{-1}$
AUC ($\text{ng} \cdot \text{h} \cdot \text{mL}^{-1}$)	810 ± 162	970 ± 194	$1215 \pm 243^*$	$1431 \pm 286^{**}$
C_{\max} ($\text{ng} \cdot \text{mL}^{-1}$)	81.0 ± 14.6	101.1 ± 20.2	$134.3 \pm 26.9^*$	$154.1 \pm 30.8^{**}$
T_{\max} (h)	0.9 ± 0.2	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.2
$t_{1/2}$ (h)	9.8 ± 1.9	9.8 ± 2.0	9.9 ± 2.0	9.9 ± 2.1
A.B. (%)	5.8 ± 1.2	6.9 ± 1.4	$8.6 \pm 1.7^*$	$10.2 \pm 2.0^{**}$
R.B. (%)	100	120	150	177

AUC: area under the plasma concentration-time curve; C_{\max} : peak plasma concentration; T_{\max} : time to reach C_{\max} ; $t_{1/2}$: terminal plasma half-life; A.B.: absolute bioavailability; R.B.: relative bioavailability
 $^* p < 0.05$, $^{**} p < 0.01$, significant difference compared to control

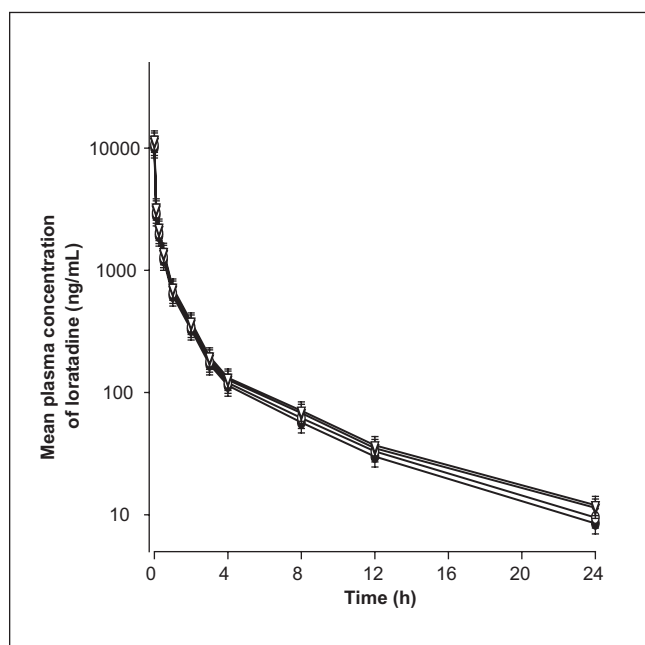


Fig. 4: Mean plasma concentration-time profiles of loratadine after intravenous administration ($1 \text{ mg} \cdot \text{kg}^{-1}$) of loratadine to rats in the presence or absence of silybinin ($0.3, 1.5$ and $6 \text{ mg} \cdot \text{kg}^{-1}$) ($n = 6$, each). Bars represent the standard deviation: (●) Oral administration of loratadine ($1 \text{ mg} \cdot \text{kg}^{-1}$); (○) with $0.3 \text{ mg} \cdot \text{kg}^{-1}$ of silybinin; (▼) with $1.5 \text{ mg} \cdot \text{kg}^{-1}$ of silybinin; (▽) with $6 \text{ mg} \cdot \text{kg}^{-1}$ of silybinin

may ameliorate side effects. Moreover, induction or inhibition of intestinal CYPs may be responsible for significant drug-drug interactions when one agent decreases or increases the bioavailability and absorption rate constant of a concurrently administered drug (Kaminsky and Fasco 1991).

Loratadine has low bioavailability because of poor solubility and the first-pass metabolism in the liver and epithelial cells of the small intestine. It is reported that loratadine is a substrate of P-gp

(Wang et al. 2001). Now that P-gp is co-localized with CYP3A4 in the small intestine or in liver, P-gp and CYP3A4 can act synergistically during the absorption and first-pass metabolism of drugs (Wacher et al. 1998; Ito et al. 1999; Pichard et al. 1990). Silymarin is a popular herbal product marketed to treat liver disorders. Despite the popularity of silybinin, limited information is available on its safety, interactions with other drugs, or the mechanisms of such interactions. Silymarin inhibits both phase I and phase II enzymes (Wu et al. 2009). As shown in Fig. 1, silybinin inhibited human CYP3A4 with an IC_{50} value of $1.97 \mu\text{M}$. A cell-based P-gp activity test using rhodamine-123 also showed that silybinin ($100 \mu\text{M}$, $P < 0.01$) significantly inhibited P-gp activity (Fig. 2). Phase I and phase II metabolizing enzymes are expressed along with P-gp in the liver, kidney and intestine (Sutherland et al. 1993; Turgeon et al. 2001), regulating the bioavailability of many orally ingested compounds. Therefore, inhibitors against both the metabolizing enzyme CYP3A4 and P-gp should have a large impact on the bioavailability of those compounds. Since silybinin competitively inhibited P-gp and the metabolizing enzymes CYP3A4, this study examined the effect of silybinin on the bioavailability of loratadine.

Silybinin (1.5 and $6 \text{ mg} \cdot \text{kg}^{-1}$) significantly increased the AUC and C_{\max} of loratadine (Table 1). Since orally administered loratadine is a substrate for CYP3A-mediated metabolism and P-gp-mediated efflux in the intestine and liver, silybinin might obstruct this metabolic pathway. This result was consistent with previous studies showing that a single oral administration of cimetidine, clarithromycin and ketoconazole significantly increased the AUC and C_{\max} of loratadine in rats by inhibition of CYP3A4 (Carr et al. 1998; Kosoglou et al. 2000).

Silybinin did not increase the AUC, CL_t , $t_{1/2}$ of loratadine (Table 2), meaning that the intravenous pharmacokinetics of loratadine were not affected by the concurrent use of silybinin in contrast to the oral administration of loratadine. These results were consistent with the results reported by Li et al. (2008), where roxithromycin did not significantly increase the AUC of intravenous loratadine, a substrate for P-gp and CYP 3A4 in rats. Accordingly, the enhanced oral bioavailability in the presence

Table 2: Mean (\pm S.D.) pharmacokinetic parameters of loratadine after its intravenous administration ($1 \text{ mg} \cdot \text{kg}^{-1}$) to rats in the presence and absence of silybinin ($0.3, 1.5$ and $6 \text{ mg} \cdot \text{kg}^{-1}$) ($n = 6$, each)

Parameter	Control	Loratadine + Silybinin		
		$0.3 \text{ mg} \cdot \text{kg}^{-1}$	$1.5 \text{ mg} \cdot \text{kg}^{-1}$	$6 \text{ mg} \cdot \text{kg}^{-1}$
AUC ($\text{ng} \cdot \text{h} \cdot \text{mL}^{-1}$)	3535 ± 636	3743 ± 674	3998 ± 720	4168 ± 750
CL_t ($\text{mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	282.8 ± 50.9	267.1 ± 48.1	250.0 ± 45.0	239.9 ± 43.2
$t_{1/2}$ (h)	5.6 ± 1.0	5.6 ± 1.1	5.8 ± 1.2	5.8 ± 1.2

AUC: area under the plasma concentration-time curve; CL_t : total body clearance; $t_{1/2}$: terminal plasma half-life

of silybinin, while there was no significant change in the intravenous pharmacokinetics of loratadine, could be mainly due to the increased intestinal absorption via P-gp inhibition by silybinin rather than the reduced elimination of loratadine. These results suggest that the increase in the oral bioavailability of loratadine might be mainly attributed to enhanced absorption in the gastrointestinal tract via the inhibition of P-gp and the reduced first-pass metabolism of loratadine due to inhibition of CYP3A4 in the small intestine and/or in the liver by silybinin. In conclusion, the presence of silybinin significantly enhanced the bioavailability of loratadine in rats. The enhanced bioavailability of loratadine may be due to inhibition of both CYP3A4-mediated metabolism and P-gp in the intestine and/or liver by the presence of silybinin. In further clinical studies, the dosage of loratadine might be readjusted when used concomitantly with silybinin for the treatment or prevention of dermatological or allergic diseases as a combination therapy.

4. Experimental

4.1. Chemicals and apparatus

Loratadine, silybinin and propranolol (an internal standard for HPLC analysis of loratadine) were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile were acquired from the Merck Co. (Darmstadt, Germany). All other chemicals for this study were of reagent grade and were used without further purification. An HPLC equipped with a Waters 1515 isocratic HPLC Pump, a Waters 717 plus autosampler and a WatersTM 474 scanning fluorescence detector (Waters Co., Milford, MA, USA), an HPLC column temperature controller (Phenomenex Inc., Torrance, CA, USA), a Branson[®] Ultrasonic Cleaner (Branson Ultrasonic Co., Danbury, CT, USA), a vortex-mixer (Scientific Industries Co., Bohemia, NY, USA), and a high-speed micro centrifuge (Hitachi Co., Tokyo, Japan) were used in this study.

4.2. Animal experiments

Male Sprague-Dawley rats (weighing 270–300 g) were purchased from the Dae Han Laboratory Animal Research Co. (Choongbuk, Korea), and were given access to a commercial rat chow diet (No. 322-7-1, Superfeed Co., Gangwon, Korea) and tap water. The animals were housed, two per cage, and maintained at $22 \pm 2^\circ\text{C}$ and 50–60% relative humidity under a 12:12 h light-dark cycle. The experiments were initiated after acclimation under these conditions for at least 1 week. The Animal Care Committee of Chosun University (Gwangju, Korea) approved the design and the conduct of this study. The rats were fasted for at least 24 h prior to the experiments and each animal was lightly anaesthetized with ether. The left femoral artery and vein were cannulated using polyethylene tubing (SP45, i.d. 0.58 mm, o.d. 0.96 mm; Natsume Seisakusho Co. LTD., Tokyo, Japan) for blood sampling and i.v. injection, respectively.

4.3. Oral and intravenous administration of loratadine

The rats were divided into six groups ($n=6$ in each group): the oral group ($4\text{ mg} \cdot \text{kg}^{-1}$ of loratadine dissolved in water, homogenized at 36°C for 30 min) without (control) or with 0.3, 1.5 and $6\text{ mg} \cdot \text{kg}^{-1}$ of silybinin (mixed in distilled water, $3.0\text{ mL} \cdot \text{kg}^{-1}$), as well as the intravenous group ($1\text{ mg} \cdot \text{kg}^{-1}$ of loratadine, dissolved in 0.9% NaCl solution, homogenized at 36°C for 30 min) without (control) or with 0.3, 1.5 and $6\text{ mg} \cdot \text{kg}^{-1}$ of silybinin (mixed in distilled water, $3.0\text{ mL} \cdot \text{kg}^{-1}$). Oral loratadine was administered intragastrically using a feeding tube, and silybinin was intragastrically administered 30 min before oral administration of loratadine. Loratadine for intravenous administration was injected through the femoral vein within 0.5 min. Blood samples (0.35 mL) were collected into heparinized tubes via the femoral artery at 0 (as a control), 0.017 (at the end of infusion), 0.1, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h after intravenous administration, and 0.1, 0.25, 0.5, 1, 2, 3, 4, 8, 12 and 24 h for the oral study. Blood samples were immediately centrifuged for 5 min at 13,000 rpm and a 0.2-mL aliquot of plasma was stored in a -40°C freezer until HPLC analysis of loratadine. Approximately 0.9 mL of whole blood collected from untreated rats was infused via the femoral artery at 0.25, 1, 3 and 8 h to replace the blood loss due to blood sampling.

4.4. HPLC Assay

The plasma concentrations of loratadine were determined by the HPLC assay modified from the method of Yin et al. (2003) and Amini et al. (2004).

Briefly, $50\text{ }\mu\text{L}$ of propranolol ($0.21\text{ }\mu\text{g} \cdot \text{mL}^{-1}$, as the internal standard), $50\text{ }\mu\text{L}$ 2 N sodium hydroxide solution and 1.1 mL diethyl ether were added to 0.2 mL of the plasma samples. The mixture was then stirred for 3 min and centrifuged at 13000 rpm for 10 min. 1.0 mL of the organic layer was transferred to a clean test tube and evaporated at 35°C under a stream of nitrogen. The residue was dissolved in $150\text{ }\mu\text{L}$ of the mobile phase and centrifuged at 13,000 rpm for 5 min. A $70\text{ }\mu\text{L}$ aliquot of the supernatant was injected into the HPLC system. Fluorescence detection was performed at excitation and emission wavelengths of 290 and 460 nm. The stationary phase was a Kromasil KR 100-5C₈ column ($150 \times 4.60\text{ mm}$, $5\text{ }\mu\text{m}$, EKA chemicals, Sweden) and the mobile phase was methanol : acetonitrile : $0.05\text{ M KH}_2\text{PO}_4$ (3: 30: 67, v/v/v, pH 2.0 adjusted with phosphoric acid). The retention times at a flow rate of $1.2\text{ mL} \cdot \text{min}^{-1}$ were as follows: internal standard at 4.49-min and loratadine at 11.86-min. The calibration curves of loratadine were linear within the range of $10\text{--}500\text{ ng} \cdot \text{mL}^{-1}$. The lower limit of quantitation for loratadine in rat plasma was $10\text{ ng} \cdot \text{mL}^{-1}$. The coefficients of variation were less than 13.3% for loratadine.

4.5. Pharmacokinetic analysis

The plasma concentration data were analyzed by the non-compartmental method using WinNonlin software version 4.1 (Pharsight Co., Mountain View, CA, USA). The elimination rate constant (K_{el}) was calculated by log-linear regression of loratadine concentration data during the elimination phase. The terminal half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. The peak plasma concentration (C_{max}) and time to reach peak plasma concentration (t_{max}) of loratadine in plasma were obtained by visual inspection of the data from the concentration-time curve. The area under the plasma concentration-time curve (AUC_{0-t}) from time zero to the time of last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The AUC zero to infinite ($AUC_{0-\infty}$) was obtained by the addition of AUC_{0-t} and the extrapolated area determined by C_{last}/K_{el} . The absolute bioavailability (A.B.) was calculated by $(AUC_{oral}/AUC_{IV} \times \text{Dose}_{IV}/\text{Dose}_{oral}) \times 100$, and the relative bioavailability (R.B.) was calculated by $(AUC_{with\text{ silybinin}}/AUC_{control}) \times 100$.

4.6. CYP inhibition assay

The assays of inhibition on human CYP3A4 enzyme activities were performed in a multiwell plate using the CYP inhibition assay kit (GENTEST, Woburn, MA) as described previously (Crespi et al. 1997). Briefly, human CYP enzymes were obtained from baculovirus-infected insect cells. CYP substrates (7-BFC for CYP3A4) were incubated with or without test compounds in the enzyme substrate contained buffer consisting of 1 pmol of P450 enzyme and NADPH generating system (1.3 mM NADP , 3.54 mM glucose 6-phosphate, $0.4\text{ U} \cdot \text{mL}^{-1}$ glucose 6-phosphate dehydrogenase and 3.3 mM MgCl_2) in potassium phosphate buffer (pH 7.4). Reactions were terminated by adding stop solution after 45 min incubation. Metabolite concentrations were measured with a spectrofluorometer (Molecular Device, Sunnyvale, CA) set at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive controls ($1\text{ }\mu\text{M}$ ketoconazole for CYP3A4) were run on the same plate and produced 99% inhibition. All experiments were performed in duplicate, and results are expressed as the percent of inhibition.

4.7. Rhodamine-123 retention assay

MCF-7/ADR cells were seeded on 24-well plates. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed to Hanks' balanced salt solution and the cells were incubated at 37°C for 30 min. After incubation of the cells with $20\text{ }\mu\text{M}$ rhodamine-123 for 90 min, the medium was completely removed. The cells were then washed three times with ice-cold phosphate buffer (pH 7.0) and lysed in EBC lysis buffer. The rhodamine-123 fluorescence in the cell lysates was measured using excitation and emission wavelengths of 480 and 540 nm, respectively. Fluorescence values were normalized to the total protein content of each sample and presented as the ratio to controls.

4.8. Statistical analysis

Statistical analysis was conducted using one-way ANOVA followed by *a posteriori* testing with the Dunnett correction. Differences were considered significant at the level of $P < 0.05$. All mean values are presented with their standard deviation (Mean \pm S.D.).

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