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Effects of Morin Pretreatment on the Pharmacokinetics of Diltiazem and Its Major Metabolite, Desacetyldiltiazem in Rats

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The purpose of this study was to investigate the effect of morin, a flavonoid, on the pharmacokinetics of diltiazem and one of its metabolites, desacetyldiltiazem in rats. Pharmacokinetic parameters of diltiazem and desacetyldiltiazem were determined after oral administration of diltiazem (15 mg/kg) in rats pretreated with morin (1.5, 7.5, and 15 mg/kg). Compared with the control group (given diltiazem alone), pretreatment of morin significantly increased the absorption rate constant (K_a) and peak concentration (C_{max}) of diltiazem ($p<0.05$, $p<0.01$). Area under the plasma concentration-time curve (AUC) of diltiazem in rats pretreated with morin were significantly higher than that in the control group ($p<0.05$, $p<0.01$), hence the absolute bioavailability (AB%) of diltiazem was significantly higher than that of the control group ($p<0.05$, $p<0.01$). Relative bioavailability (RB%) of diltiazem in rats pretreated with morin was increased by 1.36- to 2.03-fold. The terminal half-life ($t_{1/2}$) and time to reach the peak concentration (T_{max}) of diltiazem were not altered significantly with morin pretreatment. AUC of desacetyldiltiazem was increased significantly ($p<0.05$) in rats pretreated with morin at doses of 7.5 and 15 mg/kg, but metabolite-parent ratio (MR) of desacetyldiltiazem was decreased significantly ($p<0.05$), implying that pretreatment of morin could be effective to inhibit the CYP 3A4-mediated metabolism of diltiazem. There were no apparent changes of T_{max} and $t_{1/2}$ of desacetyldiltiazem with morin pretreatment. Collectively, the pretreatment of morin significantly altered pharmacokinetics of diltiazem, which can be attributed to increased intestinal absorption as well as reduced first-pass metabolism. Based on these results, dose modification should be taken into consideration when diltiazem is used concomitantly with morin or morin-containing dietary supplements in clinical setting.

Key words: Diltiazem, Desacetyldiltiazem, Pharmacokinetics, Morin, CYP3A4, Rat

INTRODUCTION

Diltiazem is a calcium channel antagonist that is widely used in the treatment of angina, supraventricular arrhythmias and hypertension (Chaffman and Brogden, 1985; Pool, 1996; Weir, 1995). Diltiazem undergoes an extensive presystemic metabolism (Buckley *et al.*, 1990), and the absolute bioavailability is approximately 40%, with a large inter individual variation. It was reported that in humans and dogs, *N*-demethyldiltiazem was the most abundant metabolite in plasma. In contrast, desacetyldiltiazem (DAD) and *O*-deacetyl-*N*-monodemethyl diltiazem were most predominant in the rabbits and rats, respectively (Yeung *et al.*, 1998). CYP3A4 is the main human isoform of the *N*-demethylation of diltiazem in liver microsomes (Pichard *et al.*, 1990). CYP 3A4 is mainly located in the liver, but it is also found in the intestine (Watkins *et al.*, 1987; Kolars *et al.*, 1992). It was reported that diltiazem is metabolized in small intestine (Lefebvre *et al.*, 1996; Homsy *et al.*, 1995a, 1995b).

The reduced oral bioavailability of diltiazem might not only be due to the metabolizing enzyme CYP 3A4 but also to the P-glycoprotein (P-gp) efflux transporter in the small intestine. Yusa and Tsuruo (1989) reported that the calcium channel blockers verapamil, nicardipine, and diltiazem competitively inhibit the multi-drug resistance of P-gp. Saeki *et al.* (1993) reported that diltiazem is not only a multi-drug resistance (MDR) modulator but also a substrate for the efflux of P-gp. *In vitro* study using rat intestine suggested that diltiazem is a P-gp substrate (Molden *et al.*, 2000b). In the small intestine, P-gp is co-

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localized at the apical membrane of the epithelial cells with CYP 3A4 (Gottesman and Pastan, 1993). Wacher et al. (2001) reported that diltiazem is both CYP 3A4 and P-gp substrate. P-gp and CYP3A4 might act synergistically to the presystemic drug metabolism by circulating the substrates of P-gp between the lumen and epithelial cells, leading to prolonged exposure to CYP 3A4, resulting in a reduced absorption of the drug.

Flavonoids are the most abundant polyphenolic compounds present in fluits, vegetables, and plant-derived beverages such as grape-fruit juice, tea, and red wine (Dixon *et al.*, 1999).

Among flavonoids, morin (3,5,7,2',4'-pentahydroxyflavone) is found in the fig and other Moraceae that are used as herbal medicines and exhibits various biological activities including antioxidation (Hanasaki *et al.*, 1994; Kok *et al.*, 2000 and Ramanathan *et al.*, 1994), anti-mutagenesis (Bhattacharya and Firozi, 1988; Francis *et al.*, 1989) and anti-inflammation (Kim *et al.*, 1999; Raso *et al.*, 2001; Fang *et al.*, 2003). Furthermore, morin inhibits P-gp mediated cellular efflux of various agents (Zhang and Morris, 2003). Previous studies have reported that morin could modulate the activities of the metabolic enzymes including cytochrome p-450 (CYPs, Hodek at al., 2002). In human liver microsomes, the formation of paclitaxel metabolite, 6α-hydroxypaclitaxel (formed by CYP2C8), C3'-hydroxypaclitaxel, and C2-hydroxypaclitaxel (formed by CYP3A4) were inhibited by morin, the formation of 6α-hydroxypaclitaxel was generally more inhibited than that of C3'-hydroxypaclitaxel (Vaclavikova *et al.*, 2003). Panc-1 is a human pancreatic adenocarcinoma cell line, which expresses multidrug resistance-associated protein1 (MRP1), and morin significantly increased the accumulation of both daunomycin and vinblastine in Panc-1 cells (Nguyen *et al.*, 2003). So, it seems that morin might acts as dual inhibitors of CYP 3A4, P-gp, and MRP1 to modulate pharmacokinetic behavior of many drugs.

The pharmacokinetics of oral diltiazem is mainly affected by CYP 3A4 and P-gp at the first-pass metabolism. Morin, as a dual inhibitor of CYP 3A4 and P-gp, might influence the pharmacokinetics of diltiazem. Therefore, the purpose of this study was to investigate the effect of morin, a flavonoid, on the pharmacokinetics of diltiazem and one of its metabolites, desacetyldiltiazem in rats.

MATERIALS AND METHODS

Meterials

Diltiazem hydrochloride, desacetyldiltiazem, imipramine hydrochloride, and morin hydrate (3,5,7,2',4'-pentahydroxyflavone) was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Saline (0.9% NaCl injectable solution) was obtained from Choongwae Co. (Seoul, Korea). Acetonitrile, methanol, *tert*-butylmethyl ether were acquired from Merck Co. (Darmstadt, Germany). All other chemicals in this study were of reagent grade and used without further purification. The apparatus used in this study were a high performance liquid chromatograph (LC-10AD liquid chromatograph pump, SIL-10A autoinjector, SPD-10A UV-Vis detector, CBM-10A communications bus module, Shimadzu, Kyoto, Japan), a microcentrifuge (National Labnet, U.S.A.) and a sonicator (Daihan Co., Korea).

Animal studies

Male Sprague-Dawley rats (270-300 g) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea), and had free access to normal standard chow diet (Jae Il Chow, Korea) and tap water. Throughout the experiment, the animals were housed, four or five per cage, in laminar flow cages maintained at 22 ± 2°C, 50-60% relative humidity, under a 12 h light-dark cycle. The animals were kept under this condition for at least one week before the experiment. This experiment was carried out in accordance with the "Guiding Principles in the Use of Animals in Toxicology" adopted by the Society of Toxicology (U.S.A.) in July 1989 and revised in March 1999. The animal care committee in our institution (Chosun University) approved the present protocol.

Rats were divided in five groups of six each: a control group, given an oral single dose of diltiazem 15 mg/kg; three pretreated group, pretreated orally with morin (1.5 mg/kg, 7.5 mg/kg or 15 mg/kg) 30 min prior to diltiazem administration; i.v. group, injected 5 mg/kg of diltiazem through the femoral vein within 0.5 min.

Oral diltiazem solution was prepared by adding diltiazem (15 mg/kg) to distilled water (1.2 mL). Morin solution was prepared by adding morin (1.5, 7.5, and 15 mg/kg) to distilled water (1.2 mL). The right femoral artery was cannulated with polyethylene tubing (PE-50, Intramedic, Clay Adams, NJ, U.S.A.) for blood sampling. Blood samples (0.6 mL) were withdrawn from the femoral artery at 0.1, 0.25, 0.5, 1, 2, 3, 4, 8, 12, and 24 h after the administration of diltiazem, and centrifuged at 5,000 rpm for 5 min to gain plasma samples (0.2 mL). The plasma samples were stored at -40°C until the HPLC analysis.

HPLC Assay

The plasma concentrations of diltiazem were determined by an HPLC assay by modification of the method reported by Goebel and Kolle (1985). Briefly, 50 μL of imipramine (2 μg/mL), as the internal standard, and 1.2 mL of *tert*-butylmethyl ether were added to 0.2 mL of the plasma sample. It was then mixed for 2 min using a vortex-mixer and centrifuged at 13,000 rpm for 10 min. One mL of the organic layer were transferred to another clean test tube,

0.2 mL of 0.01N hydrochloride was added and mixed for 2 min. Fifty μL of the water layer were injected into the HPLC system.

The detector wavelength was set to 237 nm; and the column, a μ-bondapack C_{18} (3.9 × 300 mm, 10 μm, Waters Co., Ireland) was used at room temperature. Mixtures of methanol: acetonitrile: 0.04 M ammonium bromide: triethylamine (24: 31: 45: 0.1, v/v/v, pH 7.4, adjusted with acetic acid) was used as the mobile phase at a flow rate of 1.5 mL/min. Chromatograms obtained from rat's blank plasma and the plasma spiked with diltiazem, desacetyldiltiazem and imipramine are showed in Fig. 1. The retention times are as follows: internal standard at 11.1 min, diltiazem at 9.6 min and desacetyldiltiazem at 7.6 min. The calibration curves of diltiazem and desacetyldiltiazem were linear within the concentration range 5-400 ng/mL (r=0.9999). Detection limit of diltiazem and desacetyldiltiazem were defined as 5 ng/mL. The coefficients of intra-day (n=5) and inter-day (n=5) variation were less than 5% for diltiazem and desacetyldiltiazem, 1.5% for imipramine. Recovery (%) was assessed from replicate analysis (n=5) for five days by adding 20 ng/mL and 200 ng/mL of diltiazem to rat's plasma shown 106 ± 5.7 and 101 ± 4.9, respectively.

Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed using the LAGRAN computer program (Rocci *et al.*, 1983), which uses the LARGAN method to calculate the AUC of the plasma concentration (C_p) as a function of time (t). The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were determined by a visual inspection of experimental data. The elimination rate constant (K_{el}) was obtained from the terminal slope by regression analysis, and the half-life ($t_{1/2}$) of the drug was calculated by $0.693/K_{el}$. The absolute bioavailability of diltiazem was calculated as follows:

$$\text{Absolute bioavailability (A.B\%)} = \frac{AUC_{oral}}{AUC_{IV}} \times \frac{\text{IV dose}}{\text{Oral dose}} \times 100$$

The relative bioavailability of diltiazem was calculated as follows:

$$\text{Relative bioavailability (R.B\%)} = \frac{AUC_{\text{Pret reated}}}{AUC_{control}} \times 100$$

Statistical analysis

An unpaired Student's t-test was used to determine the statistical significance of difference between the control and experimental group. P values less than 0.05 was considered significant.

RESULTS AND DISCUSSION

The plasma concentration-time profiles of diltiazem

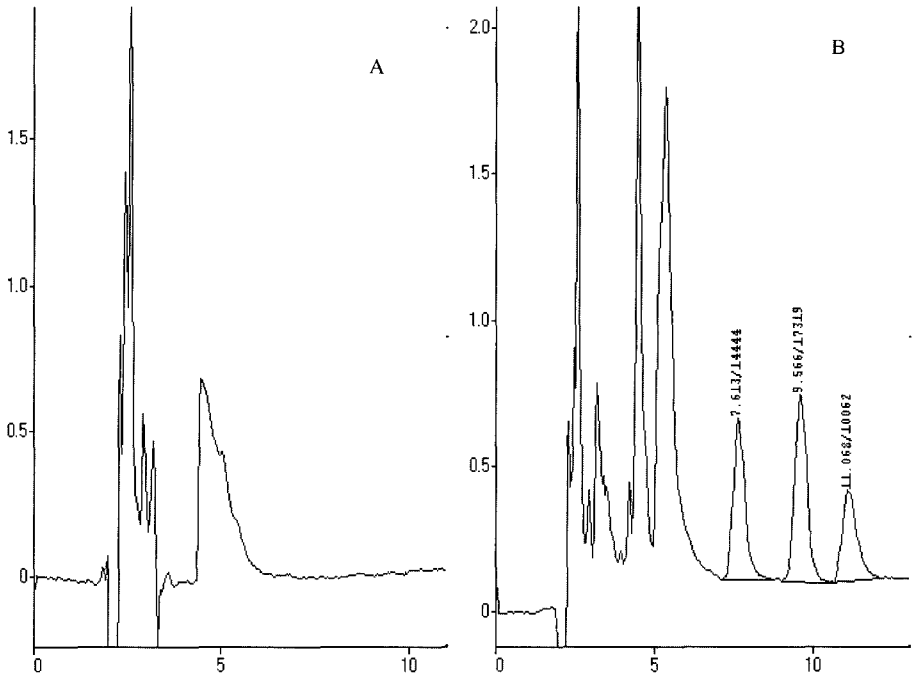

Fig. 1. Chromatogram of rat's blank plasma (A) and the plasma (B) spiked with desacetyldiltiazem (7.6 min), diltiazem (9.6 min) and the internal stand, imipramine (11.1 min)

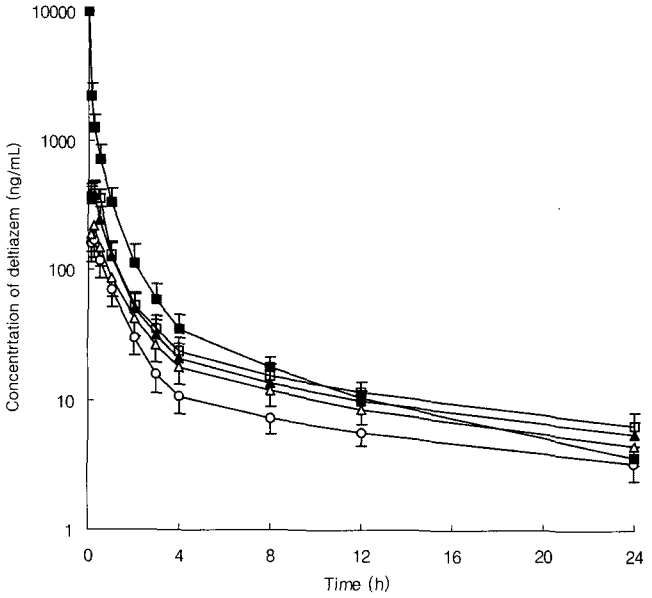

Fig. 2. Mean plasma concentration-time profiles of diltiazem following an intravenous (5 mg/kg) or oral (15 mg/kg) dose to rats pretreated with morin. Values are the mean ± s.d., n = 6. (○) Control (diltiazem alone); (△) Pretreated with morin 1.5 mg/kg, (▲) 7.5 mg/kg, (□) 15 mg/kg; (■) intravenous administration of diltiazem 5 mg/kg.

pretreated with morin (1.5, 7.5, and 15 mg/kg) were shown in Fig. 2. The pharmacokinetic parameters of diltiazem were summarized in Table I. As shown in Table I, the absorption rate constant (K_a) and peak concentration (C_{max}) were significantly higher in rats pretreated with morin ($p<0.05$ or $p<0.01$). The area under the plasma concentration-time curve (AUC) of diltiazem were significantly ($p<0.05$ or $p<0.01$) higher than those of control group. The absolute bioavailability (AB%) of diltiazem in rats pretreated with morin was increased by up to 12.2% ($p<0.05$ or $p<0.01$) compared to the control group (6.6%). Relative bioavailability (RB%) of diltiazem was increased by 1.36-2.03 fold in rats pretreated with morin. But the terminal half-life ($t_{1/2}$) and the time to reach the peak concentration (T_{max}) of diltiazem were not altered significantly in rats pretreated with morin.

The plasma concentration-time profiles of desacetyldiatiazem were shown in Fig. 3. The pharmacokinetic parameters of desacetyldiatiazem were summerized in Table II. AUCs of desacetyldiltiazem were increased significantly ($p<0.05$) in rats pretreated with morin. (7.5, 15 mg/kg), but metabolite-parent ratio (MR) of desacetyldiltiazem were decreased significantly ($p<0.05$) in rats pretreated with morin, implying that pretreatment of morin could be effective to inhibit the CYP 3A4-mediated metabolism of diltiazem. There were no apparent changes of T_{max} and $t_{1/2}$ of desacetyldiltiazem in rats pretreated with morin.

Collectively, pretreatment of morin significantly altered pharmacokinetic parameters of diltiazem by increasing intestinal absorption.

The oral bioavailability of diltiazem is approximately 40% due to extensive presystemic metabolism through CYP3A4 mediated *N*-demethylation, desacetylation, and CYP2D6 mediated *O*-demethylation (*Buckley et al., 1990*; Pichard *et al.*, 1990; Fraile *et al.*, 1996; Molden *et al.*, 2000a). CYP 3A4 is mainly located in the liver, and it is also found in the small intestine (Watkins *et al.*, 1987; Kolars *et al., 1992*). The other important factor interfering oral diltiazem absorption is P-gp, MDR efflux transporter. Diltiazem is not only inhibitor but also substrate for P-gp (Saeki *et al.*, 1993; Molden *et al.*, 2000b).

In this study, as summarized in Table I, morin pretreatment significantly increased K_a, AUC, and C_{max} of diltiazem. It might be due to promoted oral absorption of diltiazem in the small intestine by morin, a dual inhibitor of P-gp and CYP 3A4. These results are similar to observations reported by Choi *et al.* (2004) and Li *et al.* (2005), in that quercetin, other natural flavonoid, increased the bioavaila-

Table I. Mean pharmacokinetic parameters of diltiazem following an intravenous (5 mg/kg) or oral (15 mg/kg) administration of diltiazem in the presence or absence of morin in rats

Parameters	Diltiazem (Control)	Morin pretreatment			Diltiazem (i.v.)
		1.5 mg/kg	7.5 mg/kg	15mg/kg	
C_{max} (ng/mL)	172.4 ± 41.3	219.7 ± 74.7*	369.0 ± 90.4**	380.1 ± 96.8**	
AUC (ng/mL · h)	338 ± 76.8	461 ± 82.6*	638 ± 126.9**	690 ± 131.6**	1702 ± 446
T_{max} (h)	0.25	0.25	0.25	0.25	
K_a	2.9 ± 0.83	4.2 ± 0.89*	5.0 ± 1.50**	5.6 ± 1.50**	
$t_{1/2}$ (h)	11.7 ± 2.91	11.9 ± 3.06	12.2 ± 3.21	12.5 ± 3.31	6.0 ± 1.56
AB (%)	6.6 ± 1.24	8.3 ± 2.34*	11.3 ± 2.53**	12.2 ± 3.02**	100
RB (%)	100	136	189	203	–

Mean ± S.D. (n = 6), *p<0.05, **p<0.01, significant difference compared to control; C_{max}: peak concentration, AUC: area under the plasma concentration-time curve from 0 h to infinity, T_{max}: time to reach peak concentration, K_a: absorption rate constant, $t_{1/2}$: terminal half-life, AB(%): absolute bioavailability, RB(%): relative bioavailability, compared $AUC_{pretreated}$ to $AUC_{control}$.

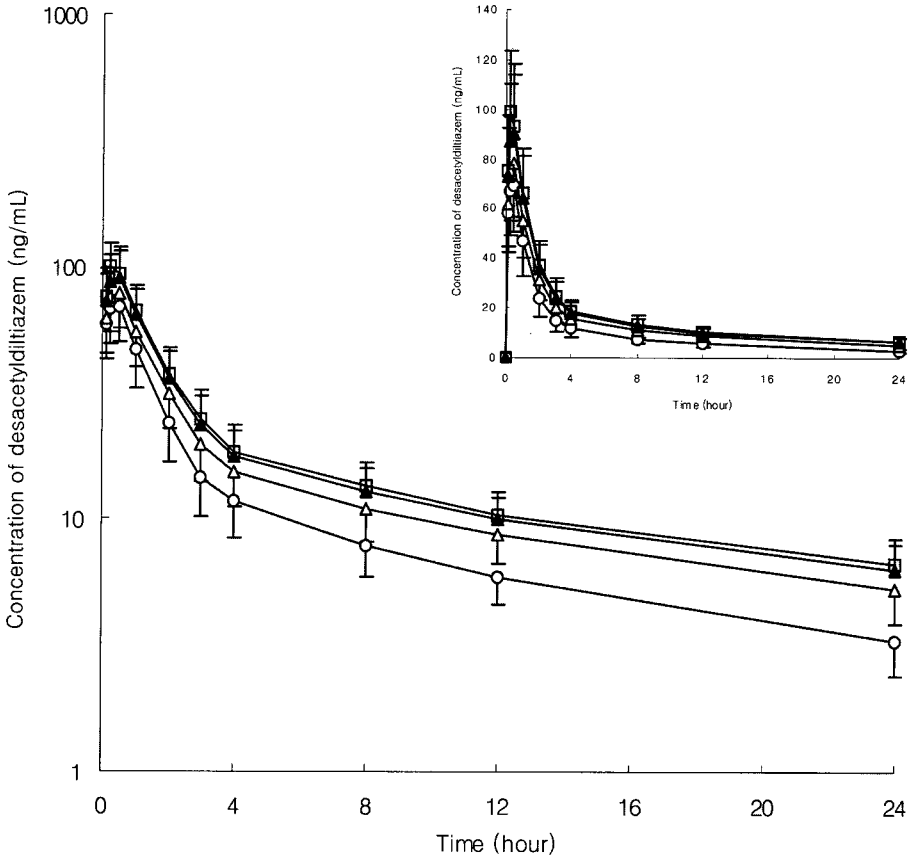

Fig. 3. Mean plasma concentration-time profiles of desacetyldiltiazem after an oral administration of diltiazem (15 mg/kg) to rats pretreated with morin. Values are the mean ± s.d., n = 6. (○) Control (diltiazem alone); (△) Pretreated with morin 1.5 mg/kg, (▲) 7.5 mg/kg, (□) 15 mg/kg.

Table II. Mean pharmacokinetic parameters of desacetyldiltiazem, a major metabolite of diltiazem following an oral administration of diltiazem (15 mg/kg) in the presence or absence of morin in rats

Parameters	Diltiazem (Control)	Morin pretreatment		
		1.5 mg/kg	7.5 mg/kg	15 mg/kg
C_{max} (ng/mL)	68.9 ± 15.6	78.0 ± 17.4	89.5 ± 19.0	93.0 ± 23.4*
AUC (ng/mL · h)	319 ± 85.4	368 ± 95.9	429 ± 115.8*	473 ± 121.8*
T_{max} (h)	0.5	0.5	0.5	0.5
$T_{1/2}$ (h)	14.5 ± 1.38	13.8 ± 1.71	13.5 ± 1.53	14.4 ± 1.81
MR	0.97 ± 0.34	0.79 ± 0.26	0.67 ± 0.16*	0.68 ± 0.15*

Mean ± S.D. (n = 6), *p<0.05, significant difference compared to control; C_{max}: peak concentration, AUC: area under the plasma concentration-time curve from 0 h to infinity, T_{max}: time to reach peak concentration, $t_{1/2}$: terminal half-life, MR: metabolite ratio, compared $AUC_{desacetyldilitiazem}$ to $AUC_{diltiazem}$.

bility of paclitaxel, cyclosporine, verapamil, and diltiazem by inhibiting either the P-gp efflux pump or CYP3A4. These results was also consistent with the results reported by Choi *et al.* (2005), Na *et al.* (2005), Kim *et al.* (2005), and Kim *et al.* (2005), in that naringin, another natural flavonoid, increased the bioavailability of paclitaxel, nifedipine, tamoxifen, and diltiazem.

The pharmacokinetic parameters of desacetyldiltiazem were evaluated in the presence of morin (Table II). The AUC of desacetyldiltiazem significantly increased in rats pretreated with morin 7.5 and 15 mg/kg; but the metabolite-parent AUC ratio of desacetyldiltiazem was decreased significantly ($p<0.05$) at the dose of 7.5 and 15 mg/kg. The dose of 1.5 mg/kg of morin might be too low to inhibit P-gp or CYP 3A4 sufficiently, because the morin pretreatment did not altered pharmacokinetic parameters of both diltiazem and desacetyldiltiazem. These results are also consistent with the results reported by Choi *et al.* (2004)

and Han *et al.* (2005), in that naringin another natural flavonoid, decreased the metabolite-parent AUC ratio of desacetyldiltiazem.

On the whole, the bioavailability of diltiazem was enhanced significantly in rats pretreated with morin, a dual inhibitor of CYP 3A4 and P-gp. The importance of these findings requires further investigation in clinical trials.

CONCLUSION

In rats pretreated with morin, the oral bioavailability of diltiazem was significantly enhanced, which can attributed to the increased intestinal absorption as well as reduced first-pass metabolism of diltiazem. The present results obtained from the rat model need to be confirmed in a clinical setting in order to determine whether the diltiazem dose should be adjusted when diltiazem is given concomitantly with morin or morin-containing dietary supplements.

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