Effects of Ticlopidine on the Pharmacokinetics of Diltiazem and Its Main Metabolite, Desacetyldiltiazem, in Rats

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Abstract
The purpose of this study was to investigate the effect of ticlopidine on the pharmacokinetics of diltiazem and its active metabolite, desacetyldiltiazem, in rats. Pharmacokinetic parameters of diltiazem and desacetyldiltiazem were determined in rats after oral administration of diltiazem (15 mg · kg⁻¹) with ticlopidine (3 or 9 mg · kg⁻¹). The effects of ticlopidine on P-glycoprotein (P-gp) and cytochrome P450 (CYP) 3A4 activities were also evaluated. Ticlopidine inhibited CYP3A4 enzyme activity in a concentration-dependent manner with a 50% inhibition concentration (IC₅₀) of 35 μM. In addition, ticlopidine did not significantly enhance the cellular accumulation of rhodamine-123 in NCI/ADR-RES cells overexpressing P-gp. Compared with the control (given diltiazem alone), ticlopidine significantly altered the pharmacokinetic parameters of diltiazem. The peak concentration (C max) and the area under the plasma concentration-time curve (AUC) of diltiazem were significantly (9 mg · kg⁻¹, p<0.05) increased in the presence of ticlopidine. The AUC of diltiazem was increased by 1.44-fold in rats in the presence of ticlopidine (9 mg · kg⁻¹). Consequently, the absolute bioavailability (A.B.) of diltiazem in the presence of ticlopidine (9.3-11.5%) was significantly higher (9 mg · kg⁻¹, p<0.05) than that in the control group (8.0%). Although ticlopidine significantly (p<0.05) increased the AUC of desacetyldiltiazem, the metabolite-parent AUC ratio (M.R.) in the presence of ticlopidine (9 mg · kg⁻¹) was significantly decreased compared to that in the control group, implying that ticlopidine could effectively inhibit the metabolism of diltiazem. In conclusion, the concomitant use of ticlopidine significantly enhanced the oral bioavailability of diltiazem in rats by inhibiting CYP3A4-mediated metabolism in the intestine and/or liver rather than by inhibiting intestinal P-gp activity or renal elimination of diltiazem.

Key Words: Diltiazem, Desacetyldiltiazem, Ticlopidine, Pharmacokinetics, CYP3A4, Rat

INTRODUCTION
Diltiazem is a calcium channel blocker that is widely used for the treatment of angina, supraventricular arrhythmias and hypertension (Chaffman and Brogden, 1985; Yeung et al., 1993; Weir, 1995). Diltiazem undergoes extensive and complex phase I metabolism including desacetylation, N-demethylation, and O-demethylation. The absolute bioavailability of diltiazem is approximately 40%, with large inter-subject variability (Buckley et al., 1990; Yeung et al., 1993). In preclinical studies, the estimated hypotensive potency of desacetyldiltiazem appeared to be about one-half to equivalent to that of diltiazem, whereas the potencies of N-demethyldiltiazem and N-demethyl desacetyldiltiazem were about one-third the potency of diltiazem (Narita et al., 1986; Yeung et al., 1998). Considering the potential contribution of active metabolites to the therapeutic outcome of diltiazem treatment, it may be important to monitor the active metabolites as well as the parent drug in pharmacokinetic studies of diltiazem. CYP3A4, a key enzyme in the metabolism of diltiazem is mainly located in liver, but it is also expressed in the small intestine (Watkins et al., 1987; Pichard et al., 1990; Kolars et al., 1992). Thus, diltiazem could be metabolized in both the small intestine and the liver (Homsy et al., 1995a; Homsy et al., 1995b; Lefebvre et al., 1996). Lee et al. (1991) reported that the extraction ratios of diltiazem in the small intestine and the liver after oral administration to rats were about 85% and 63%, respectively, indicating that diltiazem is highly extracted in the small intestine and the liver. In addition to the extensive metabolism, P-glycoprotein (P-gp) may also lower the bioavailability of diltiazem. Yusa and Tsuruo (1989) reported that calcium channel blockers such as verapamil and diltiazem competitively restrained the multidrug resistance of P-gp. Wacher et al. (2001) also suggested that diltiazem is a substrate of both CYP3A4 and P-gp. Since P-gp is co-localized with CYP3A4 in the small intestine, P-gp and CYP3A4 may act synergistically in the presystemic drug
metabolism, resulting in limited absorption of drugs (Gan et al., 1996; Wacher et al., 1998; Ito et al., 1999; Wacher et al., 2001).

Ticlopidine is extensively metabolized in the liver and is a potent inhibitor of platelet aggregation induced by adenosine diphosphate (ADP), whereas its ability to inhibit aggregation caused by thrombin, collagen, arachidonic acid, adrenaline, and platelet-activating factor varies (Saltiel and Ward, 1987). It has been tried in a variety of platelet-dependent disease states (Gent et al., 1989; Hass et al., 1989; Janzon et al., 1990). Indeed, several recent reviews recommend ticlopidine as a valuable alternative when patients cannot tolerate aspirin (Haynes et al., 1998; Verhaeghe, 1991; Ito et al., 1992; Solomon and Hart, 1994; Buur et al., 1997; Ko et al., 2000).

Ticlopidine is a novel antiplatelet drug reported to cause significant inhibition of several drugs metabolized by the hepatic cytochrome P-450 enzyme system, including antipyrine and theophylline. For example, ticlopidine co-medication results in a significant increase in mean warfarin concentrations (Gidal et al., 1995). There is also report that the oral bioavailability of ticlopidine administered with a meal was increased by 20% and the absorption of ticlopidine administered with antacid was approximately 20% lower than those under fasting conditions (Shah et al., 1990). We therefore evaluated the inhibition of CYP enzyme activity and P-gp activity by ticlopidine using the CYP inhibition assays and rhodamine-123 retention assays in P-gp-overexpressing NCI/ADR-RES cells.

Clinically, diltiazem and ticlopidine can be prescribed for treatment and prevention of cardiovascular disease. However, pharmacokinetic interaction between ticlopidine and diltiazem has not been reported in vivo. Therefore, the present study aims to investigate the effect of ticlopidine on the bioavailability and pharmacokinetics of diltiazem and its active metabolite, desacetyldiltiazem, after oral and intravenous administration of diltiazem in rats.

MATERIALS AND METHODS

Materials

Diltiazem hydrochloride, desacetyldiltiazem, imipramine hydrochloride and ticlopidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methanol, and tert-butylmethylether were obtained from Merck Co. (Darmstadt, Germany). All other chemicals were reagent grade and all solvents were HPLC grade.

Animal studies

Male Sprague-Dawley rats (270-300 g) were purchased from Dae Han Laboratory Animal Research and Co. (Choongbuk, Korea) and were given a normal standard chow diet (No. 322-7-1; Superfeed Co., Gangwon, Korea) and tap water ad libitum. 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 in these facilities 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 (USA) in July 1989 and revised in March 1999. The Animal Care Committee of Chosun University approved the present study.

The rats were divided into six groups (n=6, each): an oral control group (15 mg · kg⁻¹ of diltiazem dissolved in distilled water, 3.0 ml/kg) without or with 3 or 9 mg · kg⁻¹ of ticlopidine (mixed in distilled water, 3.0 ml/kg), and an i.v. group (5 mg · kg⁻¹ of diltiazem, dissolved in 0.9% NaCl solution, 1.5 ml/kg) without or with 3 or 9 mg · kg⁻¹ of oral ticlopidine (mixed in distilled water, 3.0 ml/kg). Sprague-Dawley rats were fasted for at least 24 h prior to the experiment and were given water freely. Each rat was anaesthetized with ether and the right femoral artery was cannulated with polyethylene tubing for blood sampling. Blood was collected from the femoral artery at 0.1, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hr post-dose. Blood samples were centrifuged and the plasma was removed and stored at −80°C until analyzed by HPLC.

HPLC assay

Plasma concentrations of diltiazem were determined by an HPLC assay modified from the method of Goebel and Kolle (Goebel and Kolle, 1985). Briefly, 50 μl of imipramine (2 μg · ml⁻¹), as the internal standard, and 1.2 ml of tert-butylmethylether were added to 0.2 ml of the plasma samples. The mixture was then stirred for 2 min and centrifuged for 10 min. 1 ml of the organic layer was transferred to a clean test tube and 0.2 ml of 0.01 N hydrochloride was added and mixed for 2 min. 50 μl of the water layer was injected into the HPLC system. The HPLC system consisted of two solvent delivery pumps (Model LC-10AD, Shimadzu Co., Japan), a UV-Vis detector (Model SPD-10A), a system controller (Model SCL-10A), degasser (Model DGU-12A) and an autoinjector (SIL-10AD). The UV detector was set to 237 nm. The stationary phase was a μ-bondapack C18 column (3.9×300 mm, 10 μm, Waters Co., Ireland) and the mobile phase was methanol/acetonitrile/0.04 M ammonium bromide/triethylamine (24:31:45:0.1, v/v/v/v, pH 7.4, adjusted with acetic acid). The retention times at a flow rate of 1.5 ml/min were as follows: internal standard at 10.5 min, diltiazem at 8.0 min and desacetyldiltiazem at 6.5 min. The calibration curves of diltiazem and desacetyldiltiazem were linear within the range of 10-400 ng · ml⁻¹. The intra-day (n=5) and inter-day (n=5) coefficients of variation were less than 5% for diltiazem and desacetyldiltiazem, and 1.5% for imipramine. Recovery (%) assessed by replicate analysis (n=5) for five days after adding 20 ng · ml⁻¹ and 200 ng · ml⁻¹ of diltiazem to rat plasma was 106 ± 5.7% and 101 ± 4.9%, respectively. The detection limit of diltiazem and desacetyldiltiazem was 10 ng · ml⁻¹.

CYP inhibition assay

The inhibition assays of the human CYP3A4 activities were performed in multiwell plates 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 [50 mM 7-Benzoxo-4-(trifluoromethyl) coumarin (7-BFC) for CYP3A4] were incubated with or without test compounds in a reaction mixture containing 1 pmol of P450 enzyme and the NADPH generating system (1.3 mM NADP, 3.54 mM glucose 6-phosphate, 0.4 μM glucose 6-phosphate dehydrogenase and 3.3 mM MgCl₂) in potassium phosphate buffer (pH 7.4). Reactions were terminated by addition of stop solution after 45 min. Metabolite concentrations were measured with a spectrophotometer (Molecular Device, Sunnyvale, CA) at an excitation wavelength of 409 nm and an emission wavelength of 530 nm. Positive controls (1 μM 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.

Rhodamine-123 retention assay
NCI/ADR-RES cells were seeded on 24-well plates at a seeding density of 105 cells. At 80% confluence, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was exchanged to Hanks’ balanced salt solution and the cells were incubated at 37°C for 30 min. After incubation of the cells with 20 μM rhodamine-123 in the presence of ticlopidine (0, 1, 3, 10 and 30 μM) 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 lysis buffer. 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 are presented as the ratio to control values.

Pharmacokinetic analysis
The total area under the plasma concentration-time curve from time zero to time infinity (AUC) was calculated using the trapezoidal rule-extrapolation method (Chiou, 1978). The extrapolated area is given by C_{\text{max}}/\text{terminal rate constant}. The terminal half-life was calculated as 0.693/terminal-phase rate constant. The relative bioavailability (R.B.) was estimated by AUC_{\text{ticlopidine}}/AUC_{\text{control}} (without ticlopidine). The metabolite ratio (M.R.) was calculated by AUC_{\text{desacetyldiltiazem}}/AUC_{\text{diltiazem}}. The maximum plasma concentration (C_{\text{max}}) and the time to reach C_{\text{max}} (T_{\text{max}}) were directly read from the experimental data.

Statistical analysis
All means are presented with their standard deviation. The pharmacokinetic parameters were compared by one-way ANOVA, followed by a posteriori testing with the use of the Dunnett correction. A p-value <0.05 was considered statistically significant.

RESULTS

Inhibition of CYP3A4
The inhibitory effects of ticlopidine on CYP3A4 activities are shown in Fig. 1. Ticlopidine inhibited CYP3A4 enzyme activities with an IC_{50} value of 35 μM.

Rhodamine-123 retention assay
In this study, the cell-based P-gp activity test using rhodamine-123 showed that ticlopidine (1-30 μM) did not inhibit P-gp activity (Fig. 2).

Effect of ticlopidine on the pharmacokinetics of oral diltiazem
The mean plasma concentration-time profiles of diltiazem in the presence and absence of ticlopidine were characterized in rats and illustrated in Fig. 3. The mean pharmacokinetic parameters of diltiazem were also summarized in Table 1. As shown in Table 1, pretreatment with ticlopidine (9 mg · kg^{-1}) significantly altered the pharmacokinetic parameters of diltiazem compared to the control (given diltiazem alone). The C_{\text{max}} and AUC of diltiazem were significantly increased in rats pretreated with ticlopidine (9 mg · kg^{-1}, p<0.05), while there was no significant change in T_{\text{max}} and t_{1/2} (Table 1). Consequently, the absolute bioavailability (A.B.) of diltiazem in rats pretreated with ticlopidine was significantly higher (9 mg · kg^{-1}, p<0.05) than that in the control group.

Effect of ticlopidine on the pharmacokinetics of i.v. diltiazem
Mean arterial plasma concentration-time profiles of diltiazem following intravenous administration of diltiazem (5 mg · kg^{-1}) to rats in the presence or absence of ticlopidine (3 and 9 mg · kg^{-1}) are shown in Fig. 4; the corresponding pharmacokinetic parameters are shown in Table 2.

The AUC of diltiazem was increased, but was not statistically significant compared to that in the control. The t_{1/2} of diltiazem was...
zem was also prolonged, but this increase was not significant. In contrast to those of oral diltiazem, the pharmacokinetics of intravenous diltiazem were not affected by the concurrent use of ticlopidine. Accordingly, the enhanced oral bioavailability in the presence of ticlopidine, while there was no significant change in the pharmacokinetics of intravenous diltiazem, may be mainly due to inhibition of the CYP3A-mediated metabolism of diltiazem in the small intestine and/or liver by ticlopidine rather than renal elimination of diltiazem.

**Effect of ticlopidine on the pharmacokinetics of desacetyldiltiazem**

The pharmacokinetic profiles of desacetyldiltiazem were also evaluated in the presence and absence of ticlopidine. The plasma concentration time profiles of desacetyldiltiazem are shown in Fig. 5. As summarized in Table 3, the oral AUC of desacetyldiltiazem was significantly (p<0.05) increased in rats pretreated with 9 mg/kg of ticlopidine. However, the metabolite-parent AUC ratio (M/R) in rats pretreated with 9 mg·kg⁻¹ of ticlopidine was significantly (p<0.05) decreased compared to that in the control group. This result suggests that ticlopidine could inhibit presystemic metabolism of diltiazem, resulting in enhanced oral bioavailability of diltiazem.

**DISCUSSION**

Lee *et al.* (1991) reported that the extraction ratios of diltiazem in the small intestine and the liver after oral administration to rats were about 85% and 63%, respectively, suggesting that diltiazem is highly extracted in the small intestine as well as in the liver. In addition to the extensive metabolism, P-glycoprotein (P-gp) may also lower the bioavailability of diltiazem. Wacher *et al.* (2001) also suggested that diltiazem is a substrate of both CYP3A4 and P-gp. Since P-gp is co-localized with CYP3A4 in the small intestine, P-gp and CYP3A4 may act synergistically in the presystemic drug metabolism, resulting in limited absorption of drugs (Gan *et al.*, 1996; Wacher *et al.* 2001).
be mainly due to inhibition of the CYP3A-mediated metabo-
change in the pharmacokinetics of intravenous diltiazem, may
Bars represent the standard deviation (n=6).

Fig. 5. Mean plasma concentration-time profile of desacetyl-dil-
tiazem after oral administration of diltiazem (15 mg · kg⁻¹) without (●) or with 3 mg · kg⁻¹ (□) and 9 mg · kg⁻¹ (▼) of ticlopidine to rats. This result was consistent with a previous report by Gidal et al. (1995) in which ticlopidine co-medication resulted in a significant increase in warfarin concentrations. And this result was also consistent with a previous report in which oral admin-
istration of flavusvat or atorvastatin, inhibitors of CYP3A4, significantly increased the AUC and C_{max} of diltiazem in rats (Choi et al., 2006; Hong et al., 2007).

The increased bioavailability of diltiazem with ticlopidine suggests that CYP3A4 is inhibited by ticlopidine, which may reduce the first-pass metabolism of diltiazem in the intestine and/or liver. We therefore investigated the cell-based P-gp ac-
tivity using rhodamine-123, because diltiazem is also a sub-
strate of P-gp, and the result showed that ticlopidine did not affect P-gp activity. The inhibitory effect of ticlopidine against CYP3A4-mediated metabolism was confirmed with recombi-
nant CYP3A4 enzyme. As shown in Fig. 1, ticlopidine exhib-
ited inhibitory activity against CYP3A4-mediated metabolism with an IC₅₀ of 35 μM. Therefore, the pharmacokinetic char-
acteristics of diltiazem were evaluated in the absence and
the presence of ticlopidine in rats. As CYP3A9 expressed in
rat is the ortholog of CYP3A4 in human, (Kelly et al., 1999) rat CYP3A9 is similar to human CYP3A4 (Guengerich et al.,
1986; Bogaards et al., 2000). Human CYP2C9 and 3A4 and
rat CYP2C11 and 3A1 have 77 and 73% protein homology,
respectively (Lewis, 1996). Rats were selected as an animal
model in this study to evaluate the potential pharmacokinetic
interactions mediated by CYP3A4, although there may be
some difference in enzyme activity between rat and human
(Cao et al., 2006). The enhanced bioavailability of diltiazem
in rats might mainly be due to the inhibition of CYP3A4 rather
than to the inhibition of P-gp activity by ticlopidine.

The enhanced bioavailability of diltiazem in rats might be
due to the inhibition of CYP3A4-mediated metabolism in
the small intestine and/or liver rather than the inhibition of
P-gp activity or renal elimination of diltiazem by ticlopidine.
Therefore, concomitant use of ticlopidine with diltiazem will
require close monitoring of potential drug interactions for
the safe therapy of cardiovascular diseases. The clinical impor-
tance of these findings should be further investigated in clini-
cal trials.

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Table 3. Mean (± S.D.) pharmacokinetic parameters of desacetyl-dil-
tiazem following oral administration of diltiazem (15 mg · kg⁻¹) to rats pre-
treated with ticlopidine

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diltiazem (Control)</th>
<th>Diltiazem+Ticlopidine 3 mg · kg⁻¹</th>
<th>9 mg · kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (ng · h · ml⁻¹)</td>
<td>296 ± 72</td>
<td>322 ± 89</td>
<td>358 ± 96a</td>
</tr>
<tr>
<td>C_{max} (ng · ml⁻¹)</td>
<td>66 ± 16</td>
<td>68 ± 18</td>
<td>71 ± 18</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>T_{1/2} (h)</td>
<td>12.8 ± 3.0</td>
<td>13.2 ± 3.3</td>
<td>14.5 ± 3.5</td>
</tr>
<tr>
<td>M.R.</td>
<td>0.85 ± 0.13</td>
<td>0.78 ± 0.12</td>
<td>0.71 ± 0.11*</td>
</tr>
</tbody>
</table>

Mean ± S.D. (n=6), p<0.05, significant difference compared to the control (given diltiazem alone orally). AUC: area under the plasma concentration-time curve, C_{max}: peak plasma concentration, T_{max}: time to reach peak plasma concentration, T_{1/2}: terminal half-life, M.R.: metabolite-Parent AUC ratio.

Therefore, the present study evaluated the effect of ticlo-
pidine on the bioavailability and pharmacokinetics of diltiazem
in rats to examine a potential drug interaction between ticlopi-
dine and diltiazem. As shown in Table 1, ticlopidine significan-
tly enhanced the C_{max} and AUC of diltiazem, which might be
due to the inhibition of CYP3A4 by ticlopidine. Subsequently,
the relative bioavailability (R.B.) of diltiazem was increased by
117% to 144% in rats treated with ticlopidine (9 mg · kg⁻¹).

In contrast to those of oral diltiazem, the pharmacokinetics
of intravenous diltiazem were not affected by the concurrent
use of ticlopidine. Accordingly, the enhanced oral bioavail-
ability in the presence of ticlopidine, while there was no significant
change in the pharmacokinetics of intravenous diltiazem, may
be mainly due to inhibition of the CYP3A4-mediated metabo-
lism of diltiazem in the small intestine and/or liver by ticlopidine
rather than renal elimination of diltiazem.

In this study, the pharmacokinetic profiles of desacytildil-
tiazem were also evaluated in the presence and absence of
ticlopidine (Table 3). The metabolite-parent ratio in the rats
treated with ticlopidine (9 mg · kg⁻¹) was significantly (p<0.05)
decreased compared to the control. This result suggests that
ticlopidine inhibited presystemic metabolism of diltiazem, re-
sulting in the enhanced oral bioavailability of diltiazem.

This result was consistent with a previous report by Gidal et al.
(1995) in which ticlopidine co-medication resulted in a
significant increase in warfarin concentrations. And this result
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