INHIBITORY EFFECT OF DILTIAZEM ON DIAZEPAM METABOLISM IN THE MOUSE HEPATIC MICROSOMES

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In order to elucidate the drug interaction between diltiazem and diazepam, the effect of diltiazem on the *N*-demethylation of diazepam in the mouse hepatic microsomes was investigated. Kinetic study showed that diltiazem noncompetitively inhibited the *N*-demethylation of diazepam with inhibition constant(K_i) value of 247.8 μ M, indicating that diltiazem exhibits an inhibitory effect on the hepatic oxidative metabolism of diazepam. It was therefore suggested that diltiazem may impair the metabolism of diazepam *in vivo*.

KEYWORDS cytochrome P-450; enzyme inhibition; diltiazem; drug interaction; diazepam

Diltiazem, a calcium antagonist, is extensively used in the treatment of hypertension and angina. It has been reported by *in vitro* experiment that diltiazem inhibits the cytochrome P-450-dependent monooxygenase system in the mouse hepatic microsomes.¹⁾ Furthermore many investigators revealed that diltiazem elevated plasma concentrations of various coadministered drugs metabolized by the hepatic P-450-dependent drug oxidation system.²⁻⁵⁾ Diazepam, an antianxiety drug, is often used in combination with diltiazem for the treatment of hypertension. It has also been well known that diazepam is metabolized by the microsomal drug oxidation system in man and animals,⁶⁾ and that the metabolism of diazepam is affected by the drugs having an inhibitory effect on hepatic microsomal cytochrome P-450.⁷⁾ However, little is known about the influence of diltiazem on the metabolism of diazepam. Thus, in order to elucidate the drug interaction between diltiazem and diazepam, we investigated the effect of diltiazem on the *N*-demethylation of diazepam in the mouse hepatic microsomes.

MATERIALS AND METHODS

Chemicals Diltiazem, diazepam, nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate(G 6-P), glucose 6-phosphate dehydrogenase(G-6-PDH) were purchased from Sigma(St, Louis, MO, U.S.A.). *N*-Desmethyldiazepam was a gift from Takeda Pharmaceutical Co., Ltd., Osaka, Japan. All other chemicals and solvents were of analytical grade.

Preparation of Microsomes Male ddY strain mice(6 weeks old) were used. Mice were killed by cervical dislocation. Their livers were perfused with ice-cold 0.01 M sodium-potassium phosphate buffer(pH 7.4) containing 1.15% KCl, and homogenized in the same ice-cold solution(1:4, w/v). The homogenate was centrifuged at 10,000 $\times g$ for 20 min, and the resultant supernatant was centrifuged at 105,000 $\times g$ for 60 min to obtain a microsomal pellet. The microsomes were suspended in 0.1 M sodium-potassium phosphate buffer(pH 7.4) containing 1.15% KCl at the protein concentration of 30 mg/ml and stored at -80°C until used. Microsomal protein concentrations were determined by the method of Markwell *et al.*⁸⁾

Enzyme Assay The incubation mixture(3 ml) contained 50 mM KCl, 0.5 mM NADP, 5 mM G 6-P, 5 mM MgCl_2 , 2 units of G-6-PDH, 22–160 μ M diazepam and microsomal protein(0.6mg/ml) in 0.1 M sodium-potassium phosphate buffer(pH 7.4). Diazepam dissolved in ethanol was added to the incubation mixture, and the concentration of

ethanol in the mixture was finally adjusted to 1%(v/v). The mixture without microsomes was preincubated for 5 min at 37°C, and the reaction was started by the addition of microsomes. After incubation under air at 37°C with shaking for 15 min, 1 ml of the incubation mixture was withdrawn and mixed with 1 ml of ice-cold 10% trichloroacetic acid to stop the enzyme reaction.

Determination of Diazepam and N-Desmethyldiazepam The concentrations of diazepam and *N*-desmethyldiazepam in the sample mixture described above were determined by high-performance liquid chromatography(HPLC). The samples were centrifuged for 20 min at 3,000 r.p.m., and 20 μ l of the supernatant fraction was injected onto the chromatography column. The system consisted of a LC-6A liquid delivery module(Shimadzu), SPD-6A UV-detector, and a reversed-phase column(Zolbax-ODS, 4.6 x 150 mm i.d.). The mobile phase was a mixture of 0.01 M sodium-potassium phosphate buffer(pH 6.0), methanol and acetonitrile(45:35:20, v/v) operated at a flow-rate of 1.0 ml/min, and the oven temperature was 50°C. The column eluate was monitored at 230 nm, and the area under each peak was calibrated by a Shimadzu CR-3A chromatopack. The diazepam and *N*-desmethyldiazepam were eluted at retention times of 10.1 min and 7.5 min, respectively.

RESULTS

It has been generally accepted that the major routes for diazepam metabolism are oxidative *N*-demethylation and 3-hydroxylation.⁶⁾ In our prepared mouse hepatic microsomes, *N*-desmethyldiazepam accounted for about 75% of the metabolized diazepam, and the formation of *N*-desmethyldiazepam from diazepam was linear for 20 min. There was no appearance of *N*-desmethyldiazepam in the absence of NADPH-generating system.

Then the effect of addition of diltiazem to the above mixture on *N*-desmethyldiazepam formation was examined with 100 μ M diazepam. The *N*-demethylation of diazepam was inhibited by diltiazem concentration-dependently (data not shown).

Fig.1. Lineweaver-Burk Plots of Diazepam N-Demethylation in the Absence(■) and Presence of 1mM Diltiazem(●)
Each value is the mean of three experiments.

As shown in Fig. 1, kinetic analysis using Lineweaver-Burk plot of inhibition experiments revealed that diltiazem was a noncompetitive inhibitor for *N*-demethylation with inhibition constant(K_i) value of 247.8 μ M. In this case, the calculated maximum velocity(V_{max}) value for *N*-demethylation of diazepam was decreased to about 20 % in the presence of 1 mM diltiazem, while the Michaelis constant(K_m) value was not affected(Table I). This observation indicated that diltiazem binds at a site on the P-450 other than the diazepam binding site, altering the conformation of the enzyme molecule so that reversible inactivation of the catalytic site results.

Table I. Kinetic Constants for the Inhibition of Diazepam
N-Demethylase by Diltiazem

Treatment	K_m (μM)	V_{max} (nmol/mg protein/h)
None	112.8 $\pm$ 9.8	297.6 $\pm$ 31.6
Diltiazem(1mM)	113.6 $\pm$ 1.8	59.1 $\pm$ 3.1

Each value is the mean $\pm$ S.D. of three experiments.

DISCUSSION

It has been reported that diltiazem undergoes microsomal P-450-dependent N- and O-demethylations and hydroxylation of aromatic ring.⁹⁾ Moreover, using mouse hepatic microsomes, Renton has demonstrated that diltiazem competitively inhibits the N-demethylation of aminopyrine.¹⁾ From these findings, we considered that the N-demethylation of diazepam would also be competitively inhibited by diltiazem. However, the present study clearly showed that diltiazem noncompetitively inhibited the N-demethylation of diazepam. This finding indicated that the inhibitory effects of diltiazem on the diazepam and aminopyrine N-demethylases are essentially different: diltiazem interacts with a different site from the substrate binding site on the diazepam N-demethylase. On the other hand, by means of human hepatic microsomes, Brockmöller *et al.* showed that diltiazem noncompetitively inhibited the hydroxylation of cyclosporin.⁴⁾ Furthermore, using rat hepatic microsomes, Egan *et al.* reported that the hydroxylation of antipyrine was competitively inhibited by diltiazem.¹⁰⁾ From these results and the present finding, it was considered that diltiazem has some specificity for the inhibition of the hepatic cytochrome P-450 isozymes. The present finding suggests that diltiazem may impair the metabolism of diazepam *in vivo*. Further studies are necessary to elucidate the clinical importance of the interaction between diltiazem and diazepam.

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REFERENCES

- 1) K. W. Renton, *Biochem. Pharmacol.*, **34**, 2549(1985).
- 2) M. J. Brodie and G. J. A. Macphee, *Br. Med. J.*, **292**, 1170(1986).
- 3) D. R. Abernethy, J. M. Egan, T. H. Dickinson and G. Carrum, *J. Pharmacol. Exp. Ther.*, **244**, 994(1988).
- 4) J. Brockmöller, H. H. Neumayer, K. Wagner, W. Weber, G. Heinermeyer, H. Kewitz and I. Roots, *Eur. J. Clin. Pharmacol.*, **38**, 237(1990).
- 5) D. R. Abernethy, L. S. Kaminsky and T. H. Dickinson, *J. Pharmacol. Exp. Ther.*, **257**, 411(1991).
- 6) T. Seddon, I. Michelle and R. J. Chenery, *Biochem. Pharmacol.*, **38**, 1657(1989).
- 7) R. Gugler and J. C. Jensen, *Gastroenterology*, **89**, 1235(1985).
- 8) M. A. K. Markwell, S. M. Haas, L. L. Bieber and N. E. Tolbert, *Anal. Biochem.*, **87**, 206(1978).
- 9) Y. Sugawara, M. Ohashi, S. Nakamura, S. Usuki, T. Suzuki, Y. Ito, T. Kume, S. Harigaya, A. Nakao, M. Gaino and H. Inoue, *J. Pharmacobio-Dyn.*, **11**, 211(1988).
- 10) J. M. Egan, H. Hughes, E. B. Nelson and D. R. Abernethy, *Pharmacologist*, **28**, 215(1986).

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