AUTOINDUCTION OF 450191-S, A NEW SLEEP INDUCER OF 1H-1,2,4-TRIAZOLYL BENZOPHENONE DERIVATIVE, IN DOGS

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Alterations of plasma metabolite profiles were studied following single or multiple oral administrations of 5-[(2-aminooacetamido) methyl]-1-[p-chloro-2-(6-chlorobenzoyl)phenyl]-N, N-dimethyl-1H-1,2,4-triazole-3-carboxamide hydrochloride dihydrate (450191-S) in Beagle dogs. In plasma, unchanged 450191-S was not detected, but active metabolite, 8-chloro-6-(2-chlorophenyl)-2-(N, N-dimethylcarbamoyl)-4H-1,2,4-triazolo [1, 5-a] [1, 4] benzodiazepine (M-1) appeared first, followed by four active metabolites that were hydroxylated or demethylated in the N, N-dimethylcarbamoyl side chain of M-1. At single doses of 5 to 50 mg/kg, the areas under the plasma concentration-time curves (AUCs) of the metabolites were linearly increased, showing that there was no saturable process in the steps of absorption, distribution, metabolism and excretion. After multiple administrations (50 mg/kg/d for 15 d), the same metabolites appeared in the plasma but the patterns of the plasma metabolite profiles were considerably different from those after single administrations. The peak plasma levels of M-1 and its hydroxylated metabolites in the carbamoyl side chain were attained more rapidly in the multiple administrations, demonstrating higher peak values compared to those in the single administrations, and the eliminations of these metabolites from plasma were also rapid. However, no difference in the values of the AUCs were observed between single and multiple administrations. With the other active metabolites, the peak plasma levels after multiple administrations were considerably lowered by rapid elimination, resulting in a marked decrease in AUCs. Cytochrome P-450 content and oxidative O-dealkylase activities in dog liver homogenates increased two to five times after a 15-d administration of 450191-S. We concluded from these results that hepatic drug-metabolizing enzymes were induced during multiple administrations of 450191-S in dogs, resulting in decreased plasma levels of its metabolites.

Keywords — ring-opened 1, 4-benzodiazepine derivative; sleep inducer; multiple administration; plasma metabolite; oxidative dealkylase; autoinduction; liver; dog

INTRODUCTION

5-[(2-Aminooacetamido)methyl]-1-[p-chloro-2-(o-chlorobenzoyl)phenyl]-N, N-dimethyl-1H-1,2,4-triazole-3-carboxamide hydrochloride dihydrate (450191-S) is a newly synthesized sleep inducer\(^1\) which dose not have a 1, 4-benzodiazepine structure but demonstrates a pharmacological action through enzymatic activation.\(^2\)\(^,\)\(^3\)\) The metabolic activation mechanisms have been studied extensively and the main metabolic pathway has been postulated in rats,\(^4\) monkeys,\(^5\) mice\(^6\) and humans\(^7\) as shown in Fig. 1. 450191-S is desglycylated by intestinal aminopeptidase(s) during a single passage through the intestinal wall to form 191DG,\(^*\) which is spontaneously converted to M-1,\(^**,\) the primary active metabolite. The N, N-dimethylcarbamoyl side chain of M-1 is successively oxidized to form M-D,\(^***\) M-2,\(^*^4\) M-A\(^*^5\) and M-3.\(^*^6\) Another reaction, the hydrolysis of the carbamoyl side chain by carboxy esterase(s), also occurs independently to form M-4.\(^*^7\)

In the metabolism of 450191-S in dogs, there is no information except that the urine did not
contain M-1, M-D, M-2, M-A and M-3 but several unknown metabolites following intraduodenal administration of \(^{14}\text{C}-450191\)-S. The urinary metabolites were assumed to be further oxidized products (unpublished data).

A chronic toxicity study of 450191-S was carried out with dogs at doses of 1, 5, 25 and 50 mg/kg/d for 12 months, and the plasma metabolites were determined at months 1, 3, 6, 9 and 12 of the dosing regimen. No metabolites were detected in the plasma 24 h after the administration of all doses at 1 month of the dosing regi-
men. Furthermore, even 3 h after administration of the doses, only slight levels of metabolites could be detected in the plasma at months 3, 6, 9 and 12 of the dosing regimen. Additionally, dose-corresponding increase in plasma levels diminished (data not shown). These phenomena alerted us to the fact that 450191-S at the higher doses might be incompletely absorbed or that it might facilitate elimination because of induced drug-metabolizing enzymes. To clarify the diminished plasma metabolite levels in the chronic toxicity study of 450191-S, we examined the dose-dependency in plasma levels of the metabolites and induction of the drug-metabolizing enzymes by multiple administrations of 450191-S, using dogs.

MATERIALS AND METHODS

Materials — Male Beagle dogs weighing 7—10 kg were used. During the experiments, the animals were fed with a standard dog chow (Oriental Yeast, Saitama, Japan) amounting to 180 g/d at 10:00 a.m. except for the day on which blood specimens were collected. During the collection of blood specimens, the animals were kept being fasted but allowed to have free access to water. Authentic metabolites (M-1, M-2, M-A and M-3) used to obtain calibration curves were gifts from Dr. I. Kikkawa of the Chemistry Division of this laboratory.

Animal Experiments — The dogs were subjected to single-administration experiments (5, 25 and 50 mg/kg, p.o.) and multiple-administration experiments (50 mg/kg/d for 15 d, p.o.). After oral administration of capsulated 450191-S at 09:00 a.m., blood was withdrawn via the median veins of the forelegs and immediately centrifuged for 30 s to obtain plasma (centrifuge 5414S, Eppendorf, Hamburg, W. Germany). This plasma (0.5 ml) was added to a tube containing 1.0 ml of pH 5 acetate buffer (1 M) and stored at -20°C until assays were performed. In the multiple-administration experiment, the plasma metabolites were determined after 3-, 8- and 15-d administrations of the drug. At 24 h after the last administration of the drug, the dogs were sacrificed by exsanguination from the carotid artery and their liver samples were obtained.

Metabolite Determination by High Performance Liquid Chromatography (HPLC) — The stored plasma sample in the acetate buffer was thawed at room temperature, then extracted with 8 ml of ethyl acetate. The organic solvent was then evaporated to dryness under reduced pressure, and the residue was subjected to HPLC after being dissolved in 100 μl of methanol. Nucleosil 5C18 (4.6 mm i.d. × 200 mm in length, Macherey-Nagel, Düren, W. Germany) was used as the analytical column attached to a pre-column packed with Nucleosil 10 C18 (4.0 mm i.d. × 50 mm in length). A solvent mixture of water and acetonitrile (65 : 35) containing PIC® B7 (Waters Associates, Mass., USA) was passed through the column using a pump (Type LC-3A, Shimadzu, Kyoto, Japan) equipped with an injector (Type 7125, Rheodyne, Calif., USA) at a rate of 1.5 ml/min, and monitored using an ultraviolet detector (Type SPD-2A, Shimadzu, Kyoto, Japan) at 254 nm. Calibration curves, ranging from 0.1 to 20 μg/ml, were prepared by addition of authentic M-1, M-2, M-A and M-3 to control plasma of dogs. Since authentic M-D was not available because of its instability, its plasma levels were calculated using the calibration curve for M-2.

Determinations of in Vitro Dealkylation Activities and Cytochrome Content — O-Dealkylase activities in the liver homogenate were determined using 7-alkoxy coumarins as substrates as described previously.80 Cytochrome P-450 in the liver homogenate was detected59 and calculated using the molar extinction difference of 104 mm-1 cm-1 for the absorption difference between the peak position (about 450 nm) and 490 nm.10 Cytochrome b5 content was determined according to a method of Kajihara and Hagihara.11

RESULTS AND DISCUSSION

Under the analytical conditions employed, we detected the metabolites, M-1, M-D, M-2, M-A and M-3, in 450191-S treated dog plasma, but unchanged 450191-S was not detected in the systemic plasma as in the other animal species4–6 and humans.7 Since the desglycylated activity for 450191-S in dog intestinal mucosa was 2-fold higher than that in dog liver and the value was almost the same as that in rat intestine (data not shown), the desglycylation of 450191-S seemed to occur in the intestinal wall during the absorption process when it was orally administered to dogs. Although M-D is labile and easily converted to M-2, we succeeded in
determining it by a method using rapid centrifugation and maintenance of the plasma samples at acidic pH. Interestingly, M-4 was not detected in dog plasma, unlike in other species.\textsuperscript{4–6} We then determined the five metabolites involving M-D. Figure 2 shows the plasma metabolite profiles following a single administration of 450191-S (50 mg/kg, p.o.). The levels of both M-1 and M-D peaked within 2 h, then gradually declined and disappeared by 24 h. The peak plasma level of M-2 at 4 h was the highest compared with other metabolites and the M-2 level was detected for 30 h after the administration. The appearance of M-A and M-3 was delayed and their peaks were found at 10 and 16 h, respectively. Their plasma levels persisted for more than 30 h after the administration.

Rat intestinal aminopeptidase(s) have been reported to contribute to 450191-S uptake by small intestine\textsuperscript{12} and its absorption rate from the small intestine was demonstrated to be saturable (data not shown). The absence of the dose-corresponding increase observed in the chronic toxicity study of 450191-S was at first thought to be caused by incomplete absorption at higher doses. Since there is little information on urinary or biliary metabolites in dogs, AUC values of plasma metabolites were compared to determine the extent of absorption. Figure 3 shows the relationship between the dose and the AUC of each metabolite after single administration. The AUCs of the metabolites increased linearly as the dose was increased to 50 mg/kg, indicating, probably, that there was no saturable process in the absorption and metabolism, and furthermore that the absorption of 450191-S from the intestinal tract occurred completely even at the higher dose in dogs. Since it is difficult to obtain direct evidence for complete absorption of 450191-S during multiple-administration experiments, this information was deduced from the results of single-administration experiments. Based on the facts that the AUCs of M-1 and M-D remained constant during the multiple administrations and moreover that the multiple administration of 450191-S raised no histological and pathological alterations in the gastrointestinal tract in dogs (data not shown), this drug was assumed to be absorbed completely even during the multiple-administration experiments. Therefore, we concluded that incomplete absorption was not involved in the decreased plasma levels of the metabolites observed in the chronic toxicity tests.

Next, 450191-S was repeatedly administered to dogs at a dose of 50 mg/kg/d for 15 d and
plasma levels of the metabolites were monitored following the 3-, 8- and 15-d administrations. The plasma levels are shown in Fig. 4 and their AUC values are presented in Table I. The figure and table also contain the results of single-administration experiments shown in Fig. 1. Peak plasma levels of M-1 (panel A) and M-D (panel B) increased following multiple administrations, but the AUC values remained unchanged, even following 15-d administration. The factors influencing the AUC values are considered to be the amount of formation and the

![Figure 4](image)

**FIG. 4.** Effect of Multiple Administration of 450191-S on Its Plasma Metabolites, M-1 (A), M-D (B), M-2 (C), M-A (D) and M-3 (E), in Dogs

*Key: single administration (●), 3-d administration (○), 8-d administration (△), 15-d administration (▲).*

**TABLE I.** Effect of Multiple Administration of 450191-S on AUCs of Its Metabolites in Dogs

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Single (n = 3)</th>
<th>3-d (n = 3)</th>
<th>8-d (n = 4)</th>
<th>15-d (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>39.6 ± 20.5</td>
<td>21.4 ± 7.5</td>
<td>34.8 ± 11.0</td>
<td>27.1 ± 3.9</td>
</tr>
<tr>
<td>M-D</td>
<td>6.5 ± 3.3</td>
<td>7.0 ± 2.6</td>
<td>8.7 ± 0.5</td>
<td>8.0 ± 1.1</td>
</tr>
<tr>
<td>M-2</td>
<td>156.1 ± 33.9</td>
<td>42.4 ± 9.7</td>
<td>39.2 ± 5.1</td>
<td>34.7 ± 4.9</td>
</tr>
<tr>
<td>M-A</td>
<td>123.8 ± 8.0</td>
<td>62.1 ± 14.1</td>
<td>52.4 ± 8.2</td>
<td>48.5 ± 6.2</td>
</tr>
<tr>
<td>M-3</td>
<td>50.0 ± 16.1</td>
<td>12.0 ± 2.3</td>
<td>7.8 ± 3.5</td>
<td>6.8 ± 2.6</td>
</tr>
</tbody>
</table>

*a) Calculated by the trapezoidal rule over 24 h (µg · h/ml, mean ± S.D.). b) Dose: 50 mg/kg/d, p.o. c) Significant at p < 0.05. d) Significant at p < 0.01.*
velocity of elimination under the condition of constant distribution volume. Table II shows the elimination half-lives for plasma metabolites calculated by linear regression analysis using log values of plasma levels of terminal linear portion. Elimination of all the metabolites was accelerated by multiple administrations. Although the elimination half-lives of M-1 and M-D decreased to 20–21% of the single-administration value, their AUC values remained constant. This phenomenon could be accounted for by the increased formation rate demonstrated by the increased peak concentration (Fig. 4). Reduced AUC of M-2 could be accounted for mostly by the increased elimination rate because the degree of reduction of AUC was comparable to that of accelerated elimination. In the case of M-A and M-3, although their elimination half-lives were decreased to 63 and 55%, respectively, of single-administration value, the degree of reduction of AUC exceeded that of elimination half-lives, suggesting that the formation amount of M-A and M-3 decreased additionally, in other words, the other pathway, such as ring hydroxylation of M-2 and M-A, was accelerated by multiple administrations.

In order to determine the activities of the drug-metabolizing enzymes with multiple administrations of 450191-S, we also determined the cytochrome contents and cytochrome P-450-dependent dealkylase activities in the liver 24 h after the last repeated administrations of 50 mg/kg dose of 450191-S. As shown in Table III, cytochrome P-450 content increased markedly (peak shift was not observed), and demethylation activities increased by 49-fold, deethylolation by 5.4-fold and depropylation by 2.4-fold. Thus, the oxidative enzyme activity related to cytochrome P-450s increased several times as much as the control through the multiple administration of 450191-S. Although direct evidence for the induction of 450191-S metabolizing enzymes in dogs was absent, rat liver microsomes obtained after the multiple administration of 450191-S showed increased activities for 450191-S metabolism as well as increased

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### TABLE II. Elimination Half-lives of Plasma Metabolites During Multiple Administration of 450191-S in Dogs

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Half-life (h) following 450191-S administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
</tr>
<tr>
<td>M-1</td>
<td>2.8</td>
</tr>
<tr>
<td>M-D</td>
<td>4.6</td>
</tr>
<tr>
<td>M-2</td>
<td>2.7</td>
</tr>
<tr>
<td>M-A</td>
<td>4.6</td>
</tr>
<tr>
<td>M-3</td>
<td>5.1</td>
</tr>
</tbody>
</table>

*a) Calculated by a regression analysis using log values of mean plasma levels of the terminal linear phase.

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### TABLE III. Effect of Multiple Administrations of 450191-S on Hepatic 7-Alkoxycoumarin O-Dealkylase Activity and Cytochrome Content in Dog

<table>
<thead>
<tr>
<th>Dealkylase and cytochrome</th>
<th>Control (n = 4)</th>
<th>450191-S treatment* (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demethylase</td>
<td>33.4±4.9</td>
<td>163.9±12.1</td>
</tr>
<tr>
<td>Deethylase</td>
<td>70.2±7.2</td>
<td>379.4±14.7</td>
</tr>
<tr>
<td>Depropylase</td>
<td>180.0±3.5</td>
<td>43.6±2.3</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>21.2±4.9</td>
<td>60.0±13.9</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>16.1±1.4</td>
<td>25.3±1.7</td>
</tr>
</tbody>
</table>

*Each value represents the mean ± S.D. a) 50 mg/kg/d for 15 d. b) 7-Methoxyoumarin used as a substrate (nmol/g liver/min). c) 7-Ethoxycoumarin used as a substrate (nmol/g liver/min). d) 7-Propoxyoumarin used as a substrate (nmol/g liver/min). e) nmol/g liver. f) Significant at p < 0.01.
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coumarin O-dealkylase activities (data not shown). Induction of hepatic drug-metabolizing enzymes with multiple administration was demonstrated to be closely related to the plasma level of metabolites of 450191-S in rats,13,14 and this was also the case in dogs as shown in this report. The decreased plasma levels and $AUC$ values of M-2, M-A and M-3 are attributed to the induction of the 450191-S-metabolizing enzyme system, which produced unidentified polar metabolites, perhaps hydroxylated products of the 1,4-benzodiazepine structure. Such decreased plasma levels following multiple administrations were observed with promethazine,15 carbamazepine16-18 and phenobarbital,19 which have been reported to account for induction of the drug-metabolizing enzymes.

Our findings indicate the autoinduction of 450191-S in dog livers, but we can not explain the increased plasma peak levels of M-1 and M-D (panel A and B in Fig. 4). One of the possible explanations is an enhanced absorption rate of 450191-S from the intestinal tract based on increased gastric emptying rate or increased uptake rate by the small intestine. The uptake of 450191-S by rat small intestine was demonstrated to correlate with the activities of aminopeptidase(s) in the intestinal mucosa.20 It is thus interesting to obtain the relationship between the intestinal aminopeptidase activities and plasma level of the metabolites, M-1 and M-D, in animals after multiple administrations of 450191-S, and it is the subject for further study.

We concluded that 450191-S is a potent inducer for drug-metabolizing enzymes even in dogs, which results in reduced plasma levels and $AUC$s of active metabolites, except for M-1 and M-D, following multiple administrations.

Acknowledgement The authors thank Mr. M. Sawai, Mr. N. Nakagaki and their coworkers for technical assistance in the administration to and sampling in dogs.

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