Original Research

Evaluation of hypothermia on the *in vitro* metabolism and binding and *in vivo* disposition of midazolam in rats

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Abstract

We evaluated the effect of hypothermia on the *in vivo* pharmacokinetics of midazolam (MDZ), with a focus on altered metabolism in the liver and binding to serum proteins. Rat primary hepatocytes were incubated with MDZ (which is metabolized mainly by CYP3A2) at 37, 32, or 28°C. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of MDZ were estimated using the Michaelis–Menten equation. The K_m of CYP3A2 MDZ remained unchanged, but the V_{max} decreased at 28°C. In rats, whose temperature was maintained at 37, 32, or 28°C by a heat lamp or ice pack, plasma concentrations of MDZ were higher, whereas those in the brain and liver were unchanged at 28°C. Tissue/plasma concentration ratios were, however, increased significantly. The unbound fraction of MDZ in serum at 28°C was half that at 37°C. These pharmacokinetic changes associated with hypothermic conditions were due to reductions in CYP3A2 activity and protein binding.

INTRODUCTION

"Therapeutic hypothermia" (TH) is the recommended regimen for adult subjects after cardiac arrest and in neonates with hypoxic ischemic encephalopathy [1, 2]. Several clinical studies have reported the benefits of TH (e.g., neuroprotection [3]). However, side effects found to be associated with TH include arrhythmia, dysfunction of blood coagulation, and impaired immune functions [4]. Remedies are needed to negate these side effects or provide sedation. However, changes in the pharmacokinetics of drugs such as midazolam (MDZ) [5, 6], phenytoin [7], or vecuronium [8] have been reported under hypothermic conditions. The mechanisms responsible for changes in the pharmacokinetics of drugs under hypothermic conditions have not been clarified fully.

MDZ was selected for the present study because it is used frequently for sedation during TH [5, 6] and is metabolized by cytochrome P450 (CYP)3A4 in the human liver. CYP3A4 is known to metabolite ≈50% of drugs in use today [9]. In the present study, we evaluated CYP3A2 activity under hypothermia because it is a major component of the CYP3A family, and because its sequence is ≈90% identical and functionally equivalent to that of human CYP 3A4 [10–12]. CYP3A1 is present at low levels in normal untreated rats but CYP3A2 accounts for ≈25% of total CYP450 in rat livers [11]. We evaluated factors affecting the pharmacokinetics of MDZ to clarify the effects of hypothermia

(defined as a change in temperature from 37°C to 32°C and 28°C in the present study) on drugs metabolized by CYP3A in rats. Typically, TH is carried out at 32–34°C in clinical settings, so determination of alternations in the hepatic disposition of drugs at 32°C is needed. We also induced hypothermia at 28°C to more thoroughly examine unexpected conditions (e.g., excessive cooling and the dependency of pharmacokinetic changes in MDZ) on body temperature in rats at three temperatures.

We examined the effects of low temperatures on CYP3A2 activity using rat hepatocytes. We also evaluated MDZ disposition in rats to determine the factors affecting its pharmacokinetics under hypothermic conditions, and speculated if these were identical to those in humans.

MATERIALS AND METHODS

Materials

MDZ (Dormicum®) used for *in vivo* and protein binding studies was purchased from Astellas Pharma Inc. (Tokyo, Japan). MDZ (for *in vitro* study), diazepam, collagenase, soybean trypsin inhibitor, ethylene glycol tetraacetic acid, and Trypan Blue were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-Aminobenzotriazole (ABT) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Hanks solution was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

Animals

Male Wistar rats (180–210 g or 240–270 g) were housed in cages in an air-conditioned room and maintained on a standard laboratory diet (MF; Oriental Yeast, Co., Ltd., Tokyo, Japan) and water *ad libitum*. All animal experiments conformed to the Guidelines for Animal Experimentation of Nagasaki University (Nagasaki, Japan) and were approved by the Committee of Animal Experimentation of Nagasaki University (approval number: 0506280443).

Preparation of rat hepatocytes and incubation of drugs with hepatocytes

Isolated rat hepatocytes were prepared from the livers of male Wistar rats by collagenase perfusion using a method based on that of Seglen *et al*. [13].

To investigate the effects of temperature on MDZ uptake into rat livers, isolated rat hepatocytes were diluted with Krebs–Henseleit buffer containing 5 mM ABT to inhibit MDZ metabolism by CYP3A2 (2.0×10^6 cells/mL) [14]. ABT has been reported to be a "suicide substrate" of CYP, and that inactivation of CYP is caused by heme alkylation, which requires enzyme catalysis. [15–17] MDZ was incubated with rat hepatocytes at 37, 32, or 28°C while CYP3A activity was inhibited. Incubation was carried out 20, 60, 180, or 300 s after MDZ addition and the incubation sample centrifuged immediately at 15,000 × *g* for 1 min at 4°C. MDZ concentration in the supernatant was determined.

To investigate the effects of temperature on CYP3A2 activity, MDZ (0.25, 0.5, 1, 1.5, 2.5, 5, 7.5, 10 μg/mL) was incubated with rat hepatocytes diluted with Krebs–Henseleit buffer (1.0×10^6 cells/mL) at 37, 32, or 28°C for 10 min. Preliminary experiments showed that the MDZ concentration decreased linearly for 10 min for each temperature (data not shown). After incubation, the sample was mixed with acetonitrile, centrifuged at 17,863 × *g* at room temperature for 5 min, and the MDZ concentration in supernatants determined. The rate of metabolism (v) of MDZ in the incubation sample was calculated using the

following equation:

$$v = \frac{(C_0 - C_{10}) \times V}{10},$$

where C_0 and C_{10} are the concentration of MDZ at 0 min or 10 min, and V is the volume of incubation sample, respectively.

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were obtained by fitting the data to the Michaelis–Menten equation:

$$v = \frac{V_{max} \cdot C}{K_m + C}.$$

Evaluation of MDZ pharmacokinetics

Male Wistar rats (240–270 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The left femoral artery was cannulated with a polyethylene tube (i.d. 0.25 mm; o.d. 0.61 mm; Dual Plastics, Dural, NSW, Australia).

Rats were divided into two groups: (i) a control group in which the rectal temperature was maintained at 37°C by a heat lamp throughout the procedure; (ii) a hypothermic group maintained at 32 or 28°C, and hypothermia was induced by external cooling with ice packs before drug administration.

MDZ (5 mg/kg) was injected into the jugular vein. For determination of the plasma concentration of MDZ, blood was collected at 2, 5, 10, 20, 30, 45, and 60 min from the heparinized cannula inserted into the femoral artery. Samples were centrifuged at 17,863 × *g* at room temperature for 5 min. To determine the tissue concentration of MDZ, the liver and brain were excised at 1, 5, 15, 30, and 60 min. Excised tissues were weighed and homogenized in twofold volumes of their weight in pH 7.4 phosphate buffer.

ABT (50 mg/kg) was pre-administered to rats through the femoral vein 1 h before MDZ administration to determine the effects of changes in CYP3A2 activity on MDZ pharmacokinetics in rats [18, 19]. MDZ (0.5 mg/kg) was administered to rats.

Pharmacokinetic analyses

The area under the plasma concentration–time curves (AUC_p) and mean resistant time (MRT_p) were calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a mono-exponential equation [20].

Total body clearance (CL_{tot}) and distribution volume at steady state (V_{ss}) were calculated *via* dose/AUC_p and CL_{tot}/MRT_p, respectively.

Protein-binding study

The protein-binding ratio of MDZ with rat serum was evaluated using the equilibrium dialysis method. The molecular weight cutoff of the dialysis membrane was 12,000–14,000. Serum was collected from normal rats and stored at −80°C until experimentation. MDZ was mixed with rat serum (final concentration of MDZ: 100 μg/mL) and 1 mL of sample was added into the dialysis membrane bag. These bags were suspended in a beaker containing 300 mL of phosphate-buffered saline at 37, 32, or 28°C using a water bath with shaking. Equilibrium was attained in 18 h, and then MDZ concentrations inside and outside the dialysis membrane bag were determined. The unbound fraction ratio (f_u) of MDZ in rat serum was calculated using the following equation:

$$f_u = \frac{C_{buffer}}{C_{total}},$$

where C_{buffer} and C_{total} are MDZ concentrations outside and inside of the dialysis membrane bag, respectively.

Assay

The MDZ concentration was determined by high-performance liquid chromatography with ultraviolet detection using an established method [21]. The plasma sample or tissue

homogenate was mixed with 0.1 M NaOH and 20 μg/mL diazepam (internal standard) and then extracted by diethyl ether. Diethyl ether was evaporated under N_2 gas at 49°C and the dried sample dissolved with the mobile phase (pH 4.7; acetic buffer:acetonitrile = 55:45 (v/v)).

High-performance liquid chromatography was carried out under the following conditions: column, 5C_{18}-MS-II; column temperature, 25°C; mobile phase, pH 4.7, acetic buffer:acetonitrile = 55:45 (v/v); flow rate, 1 mL/min; detector, SPD-20Av, 220 nm (Shimadzu, Kyoto, Japan).

Statistical analyses

Statistical comparisons were made using the Tukey test following an analysis of variance (ANOVA) or repeat-measure ANOVA. $p < 0.05$ compared with the control group (37°C) was considered significant. Data are the mean ± S.E. or S.D.

RESULTS

Effect of temperature on MDZ metabolism in rat hepatocytes

To determine the effects of temperature on CYP3A2 activity using rat hepatocytes, we evaluated MDZ uptake in hepatocytes at different temperatures. The MDZ concentration in supernatants decreased for each temperature grouping for each 20 s, and remained unchanged between a 20-s lapse in duration but at 300 s in the presence of a CYP3A2 inhibitor.

Figure 1 shows the Michaelis–Menten plot for MDZ elimination from rat hepatocytes. K_m and V_{max} at 37, 32, or 28°C were obtained from the Michaelis–Menten equation. No significant changes were observed in the K_m at 37, 32, or 28°C (Table I). The V_{max} of MDZ was ≈25% lower at 32°C and 40% lower at 28°C than that at 37°C.

Evaluation of MDZ pharmacokinetics under hypothermic conditions

Figure 2 shows the plasma concentration–time curves of MDZ after its intravenous administration to rats at different body temperatures. The plasma concentration of MDZ at 28°C was significantly higher than that at 37°C. The AUC_p, MRT_p, and CL_{tot} of MDZ at each temperature are listed in Table II. The AUC_p was 1.4- (32°C) and 2.3-fold (28°C) higher than that at 37°C, and a significant difference was observed between AUC_p values

at 37°C and 28°C. The MRT_p was not altered under hypothermic conditions. The CL_{tot} was significantly lower at 32 and 28°C than that at 37°C and the distribution volume at steady state (V_{ss}) was decreased significantly in a temperature-dependent manner. However, the *in vivo* CL_{int} did not decrease according to body temperature (37°C: 4267 mL/min/kg; 32°C: 5573 mL/min/kg; 28°C: 3987 mL/min/kg). Moreover, these values were almost twofold higher than the *in vitro* CL_{int} (37°C: 2039 mL/min/kg, 32°C: 1912 mL/min/kg; 28°C: 1598 mL/min/kg).

Changes in MDZ pharmacokinetics when CYP3A2 was inhibited by ABT

The plasma concentration–time curves of MDZ after its intravenous administration to rats at different body temperatures, with or without ABT, are shown in Figure 3. The plasma concentration of MDZ was higher at 28°C than at 37°C without ABT, and this change was also observed in the ABT treatment group. The AUC_p of MDZ at 28°C without ABT was significantly higher than that at 37°C (Table III). The AUC_p of MDZ at 28°C with ABT was 1.7-times higher than that at 37°C, though this was not significantly different. The *in vivo* CL_{int} with ABT was 1368 mL/min/kg at 37°C, 2514 mL/min/kg at 32°C, and 1945 mL/min/kg at 28°C, and these values decreased compared with those without ABT.

Effect of temperature on MDZ distribution in the brain and liver

Tissue concentration–time profiles of MDZ after its intravenous administration to rats at different body temperatures are shown in Figure 4. MDZ concentrations in the brain and liver remained unchanged under hypothermic conditions. Figure 5 shows the tissue-to-plasma (T/P) ratios of MDZ after its intravenous administration to rats at different body temperatures. The T/P ratio of MDZ at 1 min after intravenous administration was decreased significantly in the brain at 32 and 28°C (37°C: 1.31 ± 0.24; 32°C: 0.75 ± 0.10; 28°C: 0.68 ± 0.06) and this tendency was observed for 60 min.

Alteration in the unbound fraction ratio of MDZ in rat serum at low temperatures

The f_u of MDZ in rat serum at 37, 32, or 28°C was determined using the equilibrium dialysis method. The f_u of MDZ at 37°C was 2.4%, and was decreased significantly at 32 and 28°C (32°C: 1.2%; 28°C: 1.0%).

DISCUSSION

MDZ is used as a sedative and its excessive use has been associated with respiratory depression or death. The plasma concentration of MDZ has been shown to increase during hypothermia in humans [5, 6]. Therefore, MDZ administration to patients needs to be optimized during TH to negate these side effects. Hence, we examined the effects of temperature on MDZ metabolism in rat hepatocytes, and identified the factors responsible for alteration of its pharmacokinetics under hypothermic conditions.

Rat hepatocytes were used to evaluate MDZ metabolism in the liver because its uptake, efflux, and metabolism can be evaluated in these cells using enzymes such as CYP [22–24]. Initially, we evaluated the effects of temperature on MDZ uptake into rat hepatocytes because drugs are known to be metabolized after their uptake into rat hepatocytes [25]. The concentration of MDZ in the supernatant of the hepatocyte suspension decreased to ≈0.3 μg/mL from 0.5 μg/mL within 20 s, and there were no significant differences between values at 37, 32, or 28°C. Studies have shown that transporters such as P-glycoprotein or organic anion transporters are not involved in MDZ uptake into rat hepatocytes [26–28], and that MDZ can enter rat hepatocytes by passive diffusion.

MDZ metabolism by rat hepatocytes was examined at 37, 32, or 28°C and the K_m and V_{max} of MDZ calculated from the Michaelis–Menten equation. The K_m of MDZ was not

altered at each temperature, but the V_{max} of MDZ at 28°C was 40% lower than that at 37°C (Table I). MDZ is metabolized by CYP3A2 in rat hepatocytes, therefore our results suggest that CYP3A2 activity is reduced at low temperatures. Empey *et al.* also evaluated CYP3A2 activity at 33°C using microsomes obtained from a model of cardiac arrest in rats [29]. In their report, the K_m of MDZ at 33°C was not significantly different from that at 37°C, whereas the V_{max} was decreased significantly; those findings were consistent with the results obtained in the present study. Therefore, low temperatures could cause a reduction in the V_{max} of MDZ without altering the affinity between CYP3A2 and MDZ. NADPH, NADPH-CYP reductase, and lipids are known to be required for metabolism by CYP [30]. Further study is needed to clarify the mechanisms of the change in V_{max} under hypothermic conditions.

We also examined the pharmacokinetics of MDZ under hypothermic conditions in rats to ascertain if alterations in CYP3A2 activity could influence the pharmacokinetics of MDZ. The plasma concentration of MDZ increased slightly in a body temperature-dependent manner (Figure 2), and the AUC_p of MDZ was significantly higher at 28°C than at 37°C (Table II). The CL_{tot} of MDZ was significantly lower at 32 and 28°C than at 37°C. The CL_{tot} of MDZ is similar to hepatic blood flow because MDZ is a highly cleared compound. It has been reported that the blood flow is decreased under hypothermia [31].

Hence, alterations in blood flow owing to temperature could also affect the CL_{tot}. Moreover, the concentration of MDZ at 0 min estimated by analyses of a two-compartment model was increased slightly under hypothermia (Figure 2) and the distribution volume of central compartment (V_c) was significantly lower at 28°C than at 37°C (data not shown). In our previous study, the initial concentrations of several drugs (phenolsulfonphthalein, indocyanine green, fluorescein isothiocyanate-dextran, 4-nitrophenol) after intravenous injection were also increased according to body temperature [31, 32]. Tveita *et al.* reported that blood volume and plasma volume were decreased post-hypothermia [33]. These alterations might affect the V_c of drugs and increase the initial concentration.

To determine the effect of CYP3A2 activity on MDZ pharmacokinetics under hypothermia, we evaluated the pharmacokinetics of MDZ in rats when the activity of CYP3A2 was inhibited by ABT. ABT administration to rats has been shown to cause reductions in the content of CYP in the liver, and this effect is maintained for 1 h [15, 19]. MDZ (0.5 mg/kg) could negate the side effects of MDZ caused by high plasma concentration. Despite inhibition of CYP3A2 activity by ABT, the AUC_p of MDZ at 28°C was ≈1.7-times greater than that at 37°C (Table III). In addition, the *in vivo* CL_{int} with ABT was 1368 mL/min/kg at 37°C, 2514 mL/min/kg at 32°C, and 1945 mL/min/kg at

28°C. Furthermore, these values decreased compared with those observed without ABT. These results suggest that the pharmacokinetics of MDZ under hypothermic conditions could differ not only by reductions in the activity of CYP3A2, but also by decreases in V_{ss}.

The decrease in the V_{ss} of MDZ under hypothermic conditions may have been caused by a reduction in tissue distribution, therefore we evaluated the concentration of MDZ in the brain and liver. Although the plasma concentration of MDZ was increased under hypothermic conditions (Figure 2), MDZ concentrations in the brain and liver remained unchanged (Figure 4). We also calculated the T/P of MDZ to evaluate the tissue distribution of MDZ under hypothermic conditions. The T/P was decreased significantly in the brain under hypothermic conditions. These results suggest that MDZ distribution in the brain was inhibited under hypothermic conditions, and this may have caused the reduction in V_{ss}. In addition, reduced tissue binding of drug could cause the reduction of V_{ss} since $V_{ss} = V_p +$ summed $V_t \times f_B/f_t$ (where the V_t is distribution volume of tissue and f_t is the unbound fraction in tissue). Further study is needed to determine effect of temperature on f_t. Drugs reach organs by blood flow within organs and diffuse to organ-only unbound fractions. Hence, the tissue distribution of drugs can be determined by organ blood flow, the protein-binding ratio, and cellular uptake [34–36]. Organ blood

flow is known to be lower under hypothermic conditions than at normal body temperature [37]. Our previous report also showed that hepatic blood flow was decreased ≈50% at 32°C and 80% at 28°C, respectively, in rats [31]. Here, we showed that uptake of MDZ into rat hepatocytes was not altered under low temperatures, and that the f_u of MDZ in rat serum was significantly lower at 32 and 28°C than at 37°C. In the present study, MDZ binding to proteins in the serum may not have become saturated because the protein binding of benzodiazepines has been shown not to be dependent on the drug concentration [38]. These results suggest that the reduction in blood flow and the f_u of MDZ in rat serum could inhibit the tissue distribution of MDZ under hypothermic conditions. The effect of blood flow and protein binding on the tissue distribution of drugs on an individual level needs to be studied. In addition, an identical study using human hepatocytes and human albumin is needed to ascertain if these results are applicable for humans.

Conclusion

We demonstrated that a reduction in CYP3A2 activity and the unbound fraction ratio of MDZ in rat serum could cause changes in the pharmacokinetics of MDZ under hypothermic conditions. These results provide an insight into the optimization of drug administration under hypothermic conditions.

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Figure legends

Fig. 1 Michaelis–Menten plot for the elimination rate of MDZ in rat hepatocytes at 37, 32, and 28°C. Each symbol is the mean ± S.E. of at least four experiments. Solid lines represent the result of the best fit by the least-square method; 37°C (○), 32°C (▲), and 28°C (□).

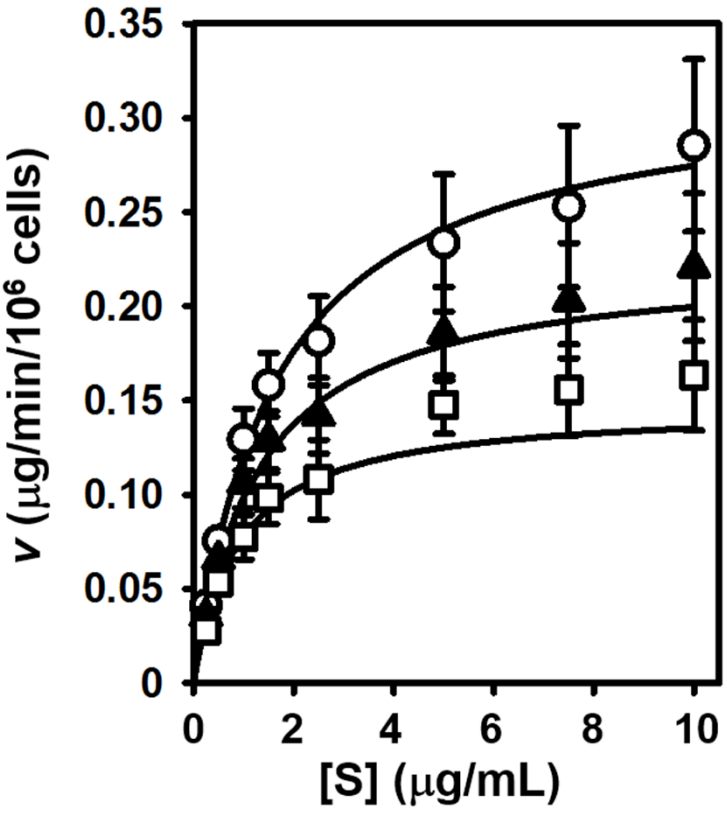


Fig. 2 Plasma concentration–time profiles of MDZ (5 mg/kg) after its intravenous administration to rats at different body temperatures. Each symbol is the mean ± S.E. of four experiments; 37°C (○), 32°C (▲), and 28°C (□).

$^{**}p < 0.01$, significantly different from value at 37°C.

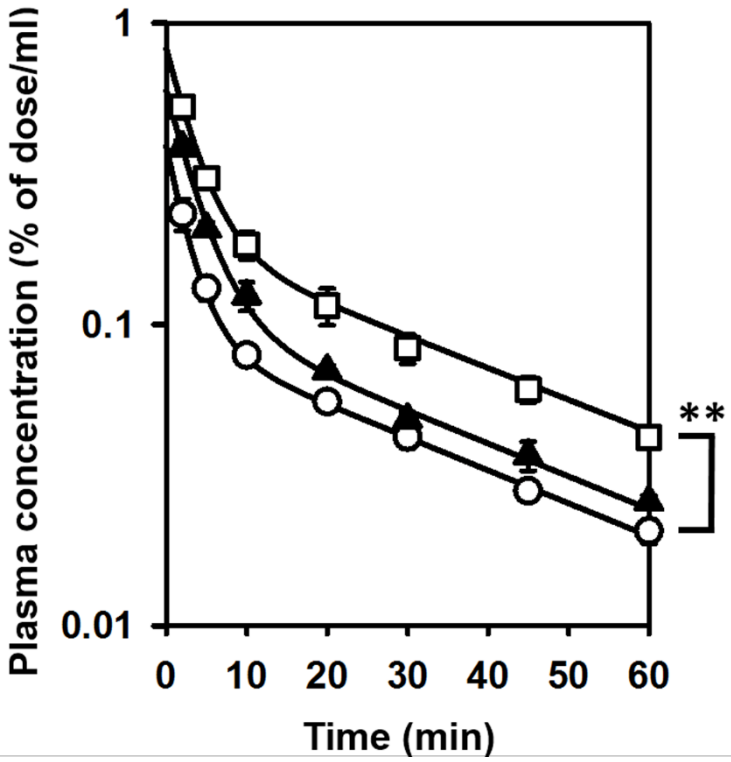


Fig. 3 Plasma concentration–time profiles of MDZ (0.5 mg/kg) without ABT (A) or with ABT (B) after its intravenous administration to rats at different body temperatures. Each symbol is the mean ± S.E. of five experiments; 37°C (○), 32°C (▲), and 28°C (□).

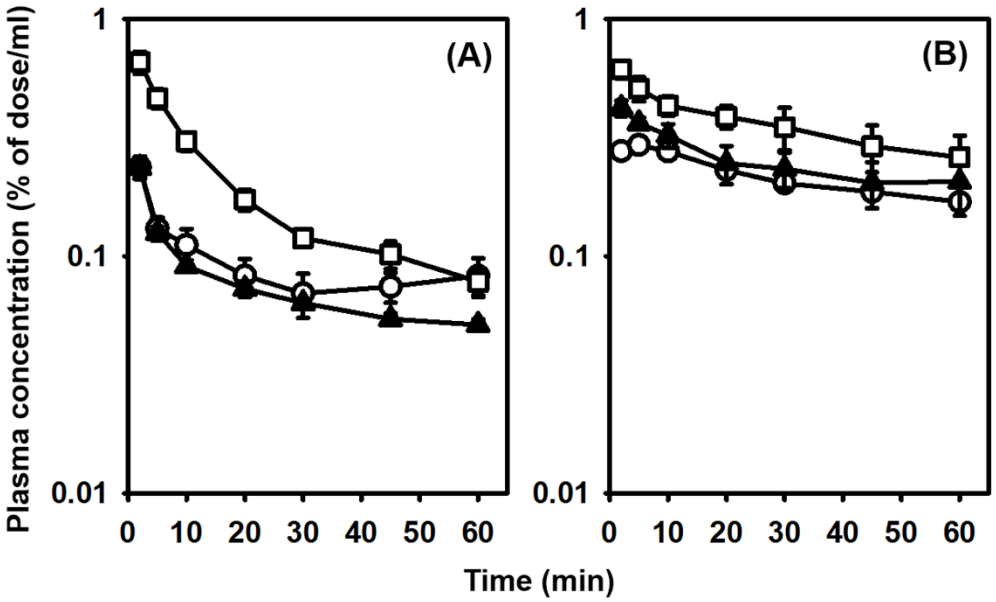


Fig. 4 MDZ concentrations in the brain (A) and liver (B) after its intravenous administration to rats at 5 mg/kg at different body temperatures. Each point is the mean ± S.E. of at least four experiments; 37°C (○), 32°C (▲), and 28°C (□).

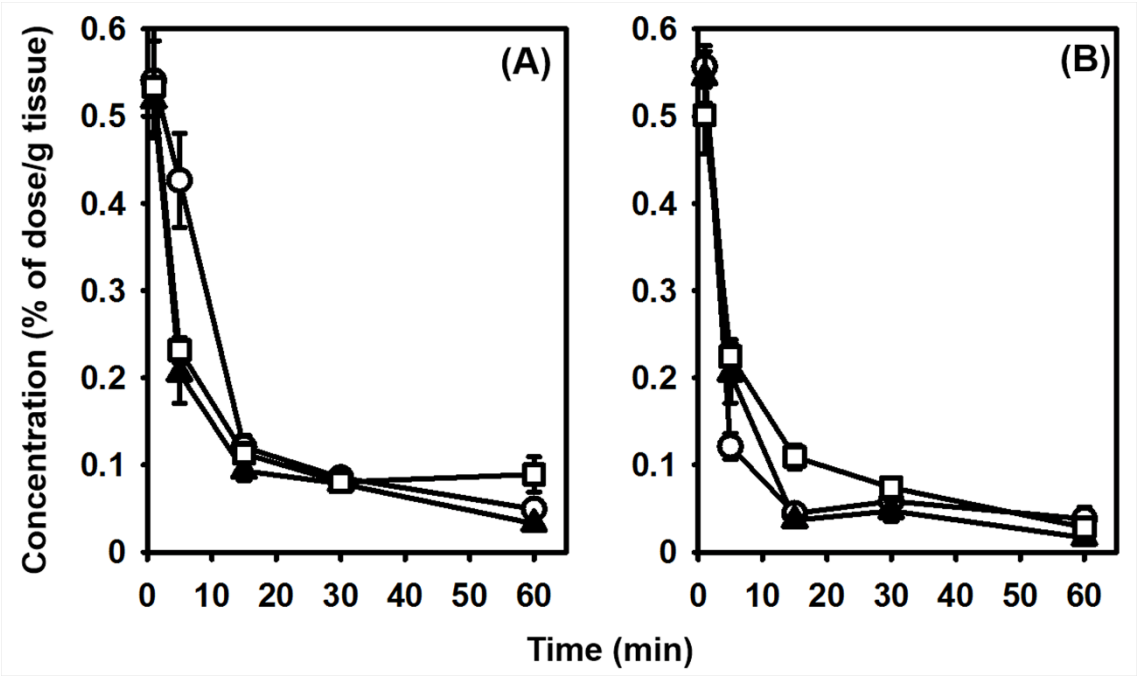
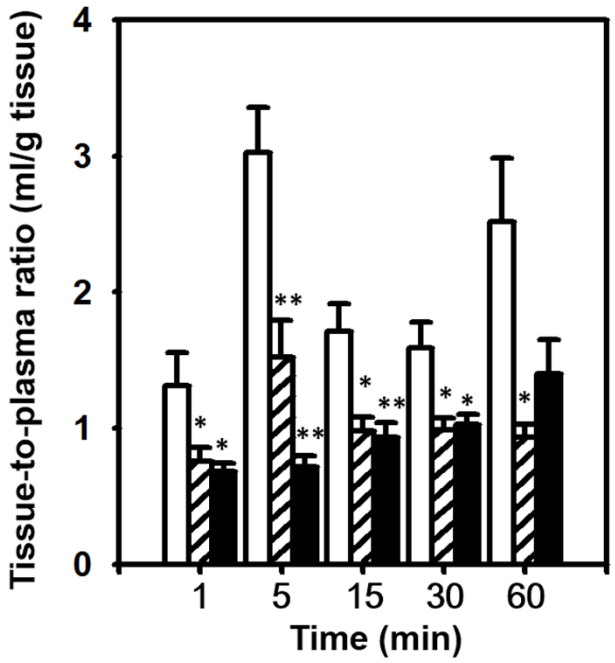


Fig 5. Tissue-to-plasma ratio in the brain of MDZ after its i.v. administration to rats at a dose of 5 mg/kg under different body temperatures. Each bar represents the mean + S.E. of at least four experiments. 37°C (open column), 32°C (slashed column) and 28°C (closed column). *: $p < 0.05$, **: $p < 0.01$, significantly different from value at 37 °C. †: $p < 0.05$, significantly different from value at 32°C.



Tables

Table I Michaelis–Menten enzyme kinetic parameters in an MDZ-estimated *in vitro* metabolism study using rat hepatocytes at 37, 32, and 28°C

	37°C	32°C	28°C
K_m (μg/mL)	1.66 ± 0.33	1.42 ± 0.31	1.54 ± 0.34
V_{max} (μg/min/10^6 cells)	0.32 ± 0.06	0.25 ± 0.05	0.19 ± 0.03

Each value is the mean ± S.E. of at least four experiments.

Table II Moment parameters calculated by moment analyses for the plasma concentration–time profiles of MDZ and pharmacokinetics parameters of MDZ at 5 mg/kg after its intravenous administration to rats at different body temperatures

	37°C	32°C	28°C
AUC_p (μg • min/mL)	59.4 ± 2.54	84.7 ± 2.68*	134.5 ± 10.10**,††
MRT_p (min)	34.9 ± 2.3	33.5 ± 3.5	36.7 ± 4.9
CL_{tot} (mL/min/kg)	84.7 ± 3.5	59.2 ± 1.8**	37.8 ± 3.0**,††
V_{ss} (L/kg)	2.96 ± 0.22	1.97 ± 0.15*	1.39 ± 0.21**

Each value is the mean ± S.E. of four experiments.
$^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the value at 37°C. $^{\dagger\dagger}p < 0.01$, significantly different from the value at 32°C.

Table III Area under the plasma–concentration time curves of MDZ after its intravenous administration to rats at 0.5 mg/kg with or without ABT at different body temperatures

	37°C	32°C	28°C
Without ABT (% of dose•min/mL)	5.39 ± 0.67	4.50 ± 0.26	11.13 ± 0.95**,††
With ABT (% of dose•min/mL)	12.87 ± 0.43	14.93 ± 2.38	21.40 ± 3.36

Each value is the mean ± S.E. of five experiments.

$^{**}p < 0.01$, significantly different from the value at 37°C. $^{\dagger\dagger}p < 0.01$, significantly different from the value at 32°C.