Effect of renal ischaemia/reperfusion-induced acute kidney injury on pharmacokinetics of midazolam in rats

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19 **Abstract**

20 **Objectives**

21 This study aimed to investigate the effects of renal ischaemia/reperfusion (I/R)-
22 induced acute kidney injury (AKI) on the distribution of midazolam (MDZ), a probe
23 drug for cytochrome P450 3A (CYP3A) activities.

24 **Methods**

25 We established an AKI model inducing ischaemia of both renal pedicles for 60 min
26 followed by 24 h reperfusion. MDZ was administrated intravenously (i.v.) to the rats via
27 the jugular vein and then blood samples were collected to determine the plasma
28 concentration of MDZ.

29 **Key findings**

30 While the plasma concentration of MDZ after i.v. administration was decreased in the
31 I/R rats, the tissue concentration was not altered. In addition, the tissue-to-plasma (T/P)
32 ratio of MDZ was increased in the I/R rats. The unbound fraction of MDZ and the level
33 of indoxyl sulphate (IS) in plasma was elevated in the I/R rats. Furthermore, the
34 unbound fraction of MDZ was significantly increased by the addition of IS.

35 **Conclusions**

36 These results indicated that the displacement of albumin-bound MDZ by IS changed

the unbound fraction of MDZ and elevated the T/P ratio of MDZ in I/R rats.

Keywords: Acute kidney injury, midazolam, protein binding, indoxyl sulphate, distribution.

Introduction

Acute kidney injury (AKI) is a common complication occurring among hospitalised patients. The incidence of AKI is increasing [1], and some studies have reported the incidence as more than 20% among inpatients [2,3]. In some cases, AKI is induced by drugs such as aminoglycosides, non-steroidal anti-inflammatory drugs, methotrexate, cisplatin, and cyclosporine [4].

The renal clearance of drugs and toxins is altered in patients with AKI because it affects glomerular filtration, tubular secretion, and renal drug metabolism [5]. Furthermore, AKI can affect the non-renal clearance of drugs and toxins by affecting hepatic clearance [6]. The pharmacokinetics (PK) of drugs is determined not only by metabolism but also by various other phenomena such as absorption and distribution.

Medication strategy in AKI patients is usually based on experimental rules or extrapolated from that used in patients with chronic kidney disease (CKD) [7]. Several studies have shown that CKD may alter the activities of transporters and cytochrome P450 (CYP) [8]. Apart from evaluating the efficacy of drugs, the evaluation of PK in renal disease is limited to assessment of the plasma concentration of drugs. Furthermore, it is difficult to identify the factors affecting drug disposition because various disorders are involved in AKI, which also varies in magnitude.

For optimising medications in AKI patients, factors affecting the PK of drugs should be elucidated. Recently, various enzyme-specific probe drugs have been used for the determination of enzyme activity *in vivo* [9]. Midazolam (MDZ) is often used as a probe drug for CYP3A activities [10]. CYP3A is the most abundant phase I enzyme present in the liver and intestine that metabolises approximately 50% of marketed drugs [11]. Therefore, in this study, we evaluated the PK of MDZ and assessed the factors affecting its disposition in renal ischaemia/reperfusion (I/R)-induced AKI in a rat model.

Materials and methods

Chemicals

MDZ (Dormicum®) was purchased from Astellas Pharma Inc. (Tokyo, Japan). Diazepam, Transaminase CII-test Wako, LabAssay™ Creatinine, Evans Blue (EB), and methyl *p*-hydroxybenzoate were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bromocresol green was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Bovine serum albumin (BSA) and indoxyl sulphate (IS) potassium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest available purity.

Animals

78 Male Wistar rats (8-week-old) were obtained from CLEA Japan, Inc. (Tokyo, Japan)
79 and maintained on a standard laboratory diet (MF; Oriental Yeast, Co., Ltd, Tokyo,
80 Japan) and water *ad libitum*. All animal experiments conformed to the Guidelines for
81 Animal Experimentation of Nagasaki University (Nagasaki, Japan) and were approved
82 by the Committee on Animal Experimentation of Nagasaki University (approval no.
83 1607081322-2).
84 The abdominal cavity was incised, opened under sodium pentobarbital (50 mg/kg)
85 anaesthesia, and renal I/R was induced by placing vascular clamps over both renal
86 pedicles for 60 min followed by 24 h reperfusion. After the clamps were released, the
87 abdomens were closed using 4-0 sutures. Sham-operated rats underwent identical
88 surgical procedures, but both renal pedicles were not clamped. All animals received
89 saline (3 mL) instilled into the abdominal cavity during the surgical procedure. Serum
90 creatinine concentration, aspartate aminotransferase (AST), and alanine
91 aminotransferase (ALT) activities were estimated using the LabAssay^{TM} creatinine or
92 transaminase CII-test Wako, respectively.
93 **Evaluation of MDZ PK**
94 The rats were anaesthetised with sodium pentobarbital (50 mg/kg, intraperitoneally
95 [i.p.]) and placed under a heat lamp to maintain the body temperature at 37℃. The

96 femoral artery was cannulated using a polyethylene tube (i.d. 0.5 mm, o.d. 0.8 mm,
97 Natsume Seisakusho, Co., Ltd., Tokyo, Japan).
98 MDZ (5 mg/kg) was administrated intravenously (i.v.) to the rats via the jugular vein
99 with anaesthesia maintained by pentobarbital. To determine the MDZ plasma
100 concentration, blood samples were collected 2, 5, 10, 20, 30, 45, and 60 min after
101 heparinised cannulas were inserted into the femoral arteries. The plasma samples were
102 obtained by centrifugation (17,860 × *g*) at 25℃. To evaluate the tissue concentration of
103 MDZ, brain, liver, kidney, lung, and heart tissue samples were excised after 30 min,
104 weighed, and homogenised in 2-fold volumes of their weight in cold phosphate buffer at
105 pH 7.4.
106 The MDZ concentration was determined using high-performance liquid
107 chromatography (HPLC) including an ultraviolet detector following an established
108 method [12]. The plasma sample or tissue homogenate was mixed with 0.1 M NaOH
109 and 10 μg/mL diazepam (internal standard) and then extracted using diethyl ether,
110 which was evaporated under nitrogen gas at 49℃ and the dried sample was dissolved
111 in the mobile phase [acetate buffer, pH 4.7: acetonitrile 55:45 (v/v)].
112 The HPLC conditions were as follows: column, 5C_{18}-MS-II (Nacalai Tesque Inc.,
113 Kyoto, Japan); column temperature, 25℃; mobile phase, acetate buffer, pH 4.7:

acetonitrile 55:45 (v/v); flow rate, 1 mL/min; and detector, SPD-20Av, 220 nm (Shimadzu, Kyoto, Japan). For 100 μL samples, the limit of quantitation (LOQ) of MDZ was 62.5 ng/mL. The intra and inter-assay precision defined by the percentage of relative standard deviation (RSD) were less than 10% across four concentration (312.5-2500 ng/mL). The sample size for intra and inter-assay precision was three and eight, respectively. The accuracies ranged from 83% to 105% across three concentration (312.5-2500 ng/mL) in plasma, and other tissue homogenates showed similar trends.

PK analysis

The area under the plasma concentration-time curve (AUC_p) and mean residence time (MRT_p) were analyzed by the non-compartment model. This analysis was performed by numerical integration using a linear trapezoidal formula and extrapolating the data to infinity time based on a mono-exponential equation. The total body clearance (CL_{tot}) and volume of distribution at steady state (V_{ss}) were calculated by dose/AUC_p and CL_{tot}・MRT_p, respectively.

Preparation of rat hepatocytes and incubation of drugs with hepatocytes

Isolated rat hepatocytes were prepared from the livers of sham or I/R rats by collagenase perfusion using an established method [12].

To investigate the effects of I/R on CYP3A 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℃ for 10 min. MDZ concentration linearly decreased for 10 min in the rat hepatocytes (data not shown). After the incubation, the sample was mixed with acetonitrile, centrifuged at 17,860 × g for 5 min, and the concentration of MDZ in the supernatants was determined. The elimination velocity of MDZ from the incubation sample was calculated using the following equation.

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

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

The Michaelis constant (K_m) and maximum elimination velocity (V_{max}) were obtained by plotting the reciprocal of the velocity versus the reciprocal of the concentration (Lineweaver-Burk plot). Apparent K_m and V_{max} values were calculated based on the intercept on the X-axis and Y-axis of the Lineweaver-Burk plot, respectively,

$$\frac{1}{v} = \frac{K_m + C}{V_{max} \cdot C}$$

where v is the elimination velocity, C is the concentration of MDZ, K_m is the Michaelis constant, and V_{max} is the maximum elimination velocity. Hepatic intrinsic clearance (CL_{int}) was calculated by V_{max}/K_m.

Plasma volume determination

150 The plasma volumes were estimated using the EB dye technique. EB binds almost
151 exclusively to plasma albumin and is used to determine plasma volume [13,14]. EB-
152 BSA was prepared using the following method: EB and BSA were dissolved in saline,
153 and the resultant EB-BSA (40 mg/mL) was purified using gel filtration.
154 A baseline blood sample (400 μL) was collected by inserting a heparinised cannula
155 into the femoral artery prior to plasma volume determination and a comparable volume
156 of sterile saline was infused. Then, a bolus dose of EB-BSA solution (1 mL/kg) was
157 injected into the femoral artery. After 5 min, a second blood sample (400 μL) was
158 withdrawn. The plasma was diluted 20-fold with saline, and the absorbance was
159 determined at 605 nm (UV-1600, Shimadzu, Kyoto, Japan). The plasma volume was
160 calculated using the following equation:

161 $$\text{Plasma volume (mL/kg)} = \frac{\text{Injected EB-BSA (μg) / EB in plasma (μg/mL)}}{\text{body weight (kg)}}$$

162 **Evaluation of serum albumin concentration**

163 According to a previously described procedure [15], 1.0 mL bromocresol green (50
164 μM) in citrate buffer (pH 4.0) was added to 10 μL serum and kept at room temperature
165 for 10 min. The absorbance of these solutions was determined (UV-1600) at 628 nm.
166 The standard curves were prepared with BSA in saline.

167 **Determination of MDZ unbound fraction in plasma**

168 The unbound fraction of MDZ in the plasma was determined using the erythrocyte
169 versus buffer or plasma partitioning method [16,17]. Briefly, sham and I/R rats were
170 anaesthetised with sodium pentobarbital (50 mg/kg, i.p.), blood samples were collected
171 from the inferior vena cava, and centrifuged (2,500 × g) at room temperature for 10
172 min. After removing the plasma and buffy-coat layers, the blood cells were gently
173 washed three times in 500 μL phosphate-buffered saline (PBS, pH 7.4). Then, either
174 PBS or plasma (diluted 10-fold with PBS) was added to yield a haematocrit (HCT) of
175 0.3. The MDZ solution was then added to each suspension to final concentrations of 500
176 or 5000 ng/mL. The mixtures were incubated in a water bath for 1 h at 37℃. After
177 centrifugation at 9,000 × g for 10 min, the MDZ concentration in the supernatant was
178 determined using HPLC, as described above.
179 The unbound fraction (f_u) was calculate as described below [16,17]. The erythrocyte
180 concentration of MDZ in the plasma diluted 10 times with PBS (C_E) was determined
181 using the following equation:

182 $$C_E = \frac{C_B - C_P \times (1 - \mathrm{HCT})}{\mathrm{HCT}}$$

183 where C_B and C_P are the total concentration of MDZ in the blood suspension and
184 supernatant, respectively.
185 Likewise, to estimate the erythrocyte concentration of MDZ in PBS (C_E^*), the total

MDZ concentrations in the suspension (C_B^*) and supernatant (C_P^*) were substituted for C_P and C_B, respectively. The f_u values were determined as:

$$f_u{}' = \frac{P_{E/P}}{P_{E^*/P^*}}$$

$$f_u = 100 \times \frac{d \times f_u{}'}{1 - f_u{}' \times (1 - d)}$$

where f_u is the unbound fraction in the diluted plasma and d is the dilution factor (e.g., d = 0.1 for a 10-fold plasma dilution). The partition coefficients of erythrocytes to diluted plasma or PBS are represented by $P_{E/P}$ (C_E/C_P) and P_{E^*/P^*} (C_E^*/C_P^*), respectively.

Determination of IS in plasma

The IS concentration was estimated using HPLC fluorescence detection [18]. The plasma samples were stored at –80℃ and thawed at room temperature before processing. Plasma samples were mixed with 1 mg/mL methyl *p*-hydroxybenzoate dissolved in methanol (internal standard), kept at room temperature for 10 min, centrifuged (17,860 × *g*) at 25℃ for 5 min, and the supernatants were injected into the chromatographic system.

The HPLC conditions were as follows: column, InertSustain® C18 (GL Sciences Inc., Tokyo, Japan); column temperature, 40℃; mobile phase, acetate buffer, pH 4.7: acetonitrile 80:20 (v/v); flow rate, 1.3 mL/min; detector, RF-20A (Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were 280 and 375 nm, respectively.

For 20 μL samples, the intra and inter-assay precision defined by the percentage of RSD was less than 10% across four concentration (3.125-25 μM). The sample size for intra and inter-assay precision was three and eight, respectively. The accuracies ranged from 93% to 106% across three concentration (3.125-25 μM) in plasma.

Effects of IS on MDZ protein binding

To further investigate possible competitive interactions with IS in I/R rats, the unbound fraction of MDZ to IS was determined using the erythrocyte versus buffer or plasma partitioning method after adding IS at a physiologically achievable concentration. Briefly, untreated rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.), and blood samples were collected from the inferior vena cava. Blood cells were collected as described in the method of "Determination of MDZ unbound fraction in plasma". Blood samples were centrifuged (2,500 × g) at room temperature for 10 min, the plasma and buffy-coat layers were removed, and blood cells were gently washed three times in 500 μL PBS. PBS or plasma (diluted 10-times with PBS or PBS plus IS [500 μM]) was added to the blood cells to yield an HCT of 0.3. The MDZ solution was then added to each suspension to final concentrations of 5000 ng/mL. The f_u of MDZ in each sample was determined as described in the method of "Determination of MDZ unbound fraction in plasma".

222 **Statistical analyses**

223 We performed Student's *t*-test using JMP 14 (SAS Institute Inc., Cary, NC, USA) and

224 $p < 0.05$ was considered statistically significant.

225

226 **Results**

227 **Biochemical assays**

228 Serum creatinine concentration was used to assess the degree of AKI induced by

229 renal I/R. As shown in Table 1, 24 h after surgery, the serum creatinine concentration

230 was significantly increased in the I/R rats compared to that in the sham rats. Moreover,

231 renal I/R led to a rise in serum AST and ALT activities relative to the sham rats.

232 **Effects of renal I/R on MDZ PK**

233 Figure 1 shows the plasma concentration-time curves of MDZ after i.v.

234 administration to sham or I/R rats. The plasma concentration of MDZ in the I/R rats was

235 markedly lower than that of the sham rats. The AUC_p, MRT_p, CL_{tot}, and V_{ss} of MDZ are

236 listed in Table 2. The AUC_p of I/R rats was decreased by approximately 40% compared

237 to that of the sham rats. On the other hand, the MRT_p was not altered between the sham

238 and I/R rats. The CL_{tot} and V_{ss} of MDZ were significantly increased in the I/R rats

239 compared to the sham rats.

[**Insert Figure 1 here**]

Effects of I/R on MDZ metabolism in rat hepatocytes

Figure 2 shows the Lineweaver-Burk plot for MDZ elimination from rat hepatocytes. No significant changes were observed in the CL_{int} of MDZ between the sham (0.18 ± 0.0049, n=5) and I/R (0.17 ± 0.0074, n=4) rats.

[**Insert Figure 2 here**]

Effects of renal I/R on MDZ tissue distribution

The tissue concentrations of MDZ 30 min after i.v. administration to the rats are shown in Figure 3. The MDZ concentration in the analysed tissues was not affected by renal I/R. Figure 4 shows the tissue-to-plasma (T/P) ratios of MDZ 30 min after i.v. administration, which was significantly increased in the I/R rats.

[**Insert Figures 3 and 4 here**]

Plasma volume determination and evaluation of serum albumin concentration

We estimated the plasma volume and serum albumin concentration of sham and I/R rats to investigate the effects of I/R on V_{ss}. The plasma volume was not significantly

different between the sham and I/R rats (38.6 ± 0.73 [n=3] and 43.4 ± 2.00 [n=5] mL/kg, respectively). In addition, the serum albumin concentrations did not change between sham and I/R rats (3.34 ± 0.80 [n=3] and 4.35 ± 0.22 [n=3] g/dL, respectively).

Alteration of unbound MDZ fraction in I/R rats

The plasma unbound fraction of MDZ in the sham and I/R rats at a concentration of 500 or 5000 ng/mL is shown in Table 3. The MDZ concentration range corresponded to the MDZ plasma concentration profiles in the PK study. The plasma unbound fractions of each I/R rat samples were significantly higher than those of the sham rats were.

Determination of IS concentration in plasma

Various uremic toxins that are normally excreted in the urine are accumulated in the circulatory system in renal dysfunction. Among these uremic toxins, IS is markedly increased in the serum in renal disease and it binds strongly to albumin [19]. In the present study, the plasma IS concentration was elevated by more than 10 times in the I/R rats than that in the sham rats (Figure 5).

[**Insert Figure 5 here**]

Effect of IS on protein binding of MDZ

To further investigate whether IS participates in increasing the unbound fraction of MDZ, we evaluated the unbound MDZ ratio in the presence of 500 μM IS. This IS concentration corresponded to the approximate value observed in the plasma in I/R rats. The unbound fraction of MDZ was significantly increased in the presence of IS (Figure 6).

[**Insert Figure 6 here**]

Discussion

Several diseases such as CKD or AKI can change the disposition of various drugs [20]. Many studies have evaluated the effects of CKD on the PK of drugs, focusing on hepatic metabolism [21] because the liver is mainly responsible for drug metabolism. In contrast, there are few reports on the alteration of drug distribution in kidney injury despite the fact that the tissue concentrations of drugs have an impact on their effects. Therefore, we aimed to elucidate the PK behaviour of MDZ, which undergoes hepatic metabolism and evaluated its disposition in a rat model of AKI. Specifically, we focused on the effect of AKI on MDZ distribution, protein binding, and tissue concentration.

In the present study, we used renal I/R rats as an experimental AKI model. A

294 significant increase in serum creatinine concentration (Table 1) was observed, indicating
295 that AKI was established by renal I/R. Furthermore, the marked increase in AST and
296 ALT activities (Table 1) suggested that hepatic dysfunction also occurred with AKI, and
297 the findings were similar to those of a previous study using an AKI rat model [22].
298 The plasma concentration of MDZ after i.v. administration was markedly decreased
299 in the I/R rats (Figure 1). Furthermore, the CL_{tot} and V_{ss} were increased in the I/R rats
300 (Table 2).
301 The metabolism of MDZ is mediated primarily by CYP3A and MDZ has intermediate
302 hepatic extraction [23]. Therefore, based on the well-stirred model of hepatic clearance,
303 MDZ clearance depends on hepatic blood flow, CL_{int} and unbound fraction. Indocyanine
304 green (ICG) clearance depends on hepatic blood flow and is used for quantifying
305 hepatic blood flow [24]. Although we evaluated the effect of ICG clearance on I/R-
306 induced AKI, ICG clearance remained unchanged in I/R rats (data not shown).
307 Moreover, to investigate the effects of I/R on CL_{int}, MDZ was incubated with rat
308 hepatocytes to calculate the CL_{int} by Lineweaver-Burk plot (Figure 2). No significant
309 changes were observed in the CL_{int} between the sham and I/R rats. Therefore, the
310 alteration in clearance of MDZ depends on the change in MDZ unbound fraction. In
311 particular, because MDZ is highly bound in plasma (approximately 95%), its

312 pharmacokinetics is susceptible to unbound fraction.

313 To evaluate the effects of I/R on MDZ distribution in tissues, the MDZ concentration
314 in the tissues was determined. While the plasma concentration of MDZ was decreased
315 in the I/R rats, the tissue concentration was not altered except in the kidneys (Figure 3).
316 The pathogenic mechanism underlying renal I/R injury involves tubular necrosis and
317 apoptosis, inflammation, and oxidative stress [25]. These damages provoke increasing
318 vascular permeability, interstitial edema, and compromise in renal blood flow [26].
319 Since the kidney is rich in blood flow, a decrease in renal blood flow might have led to a
320 decrease in renal concentration of MDZ. In addition, the T/P of MDZ was increased in
321 the I/R rats (Figure 4). The V_{ss} is captured in the equation

322 $$V_{ss} = V_{plasma} + V_{tissue} \times K_p$$

323 where the V_{plasma} and V_{tissue} are the volumes of plasma and tissue, and K_p is the T/P
324 concentration ratio which in turn is influenced by the unbound ratio of plasma and tissue,
325 respectively. Plasma volume was not changed between sham and I/R rats. Therefore, as
326 when K_p increases, V_{ss} is expected to increase.

327 Further, protein binding was evaluated to investigate the factors affecting the T/P
328 ratio of MDZ. In this study, we determined the unbound fraction of MDZ using the
329 erythrocyte versus buffer or plasma partitioning method. We found that the plasma

unbound fraction obtained by this method was approximately 5% in sham rats, which is similar to that previously reported in humans using equilibrium dialysis [27,28]. The unbound fraction of MDZ in the plasma was increased in the I/R rats compared to the sham rats (Table 3). Serum albumin concentration did not alter between sham and I/R rats. These results indicated that the unbound fraction of MDZ increased because of protein displacement, and not a reduction of plasma albumin concentration.

Uremic syndrome is attributed to the cumulation of various compounds, which are normally eliminated by healthy kidneys. These compounds are called uremic toxins when they exert negative influences on the human body. Approximately 100 uremic toxins have been reported [29]. Among these uremic toxins, protein-bound solutes such as IS, 3-carboxy-4-methyl-5-propyl-2- furanpropanoic acid (CMPF), *p*-cresyl sulphate (PCS), and indoleacetic acid (IA) bind strongly to plasma protein, mostly albumin. IS, PCS, and IA mainly bind to site II of albumin where MDZ binds. On the other hand, CMPF mainly binds to site I of albumin [30,31]. The plasma IS level was increased by more than 10 times in the I/R rats (Figure 5) compared to levels in the sham rats. The plasma concentrations of IA and PCS were also evaluated, but the increase was not as great as that which was observed for IS (data not shown). Furthermore, the unbound fraction of MDZ was significantly increased after the addition of IS (Figure 6), similarly

to that of I/R rats. Thus, the change in the unbound fraction of MDZ in plasma could be explained by the displacement of albumin-bound MDZ by IS.

Our results showed that the plasma concentration of MDZ decreased in the I/R-induced AKI, and resulted from displacement of binding between MDZ and albumin by IS. However, Kirwan CJ *et al* reported that the plasma concentration of MDZ increased in critically ill patients, and concluded that increasing severity and duration of AKI were associated with decreased MDZ elimination[32]. These critically ill patients with AKI had various complications including CKD, and took other medicines such as alfentanil, which were also metabolized by CYP3A. Additionally, there was a lack of hepatic blood flow and unbound fraction of MDZ in critically ill patients. Each of these aspects complicates our understanding of PK in AKI. Further studies are needed in order to fill in the gaps between human studies and animal studies.

Conclusions

In this study, we found that the plasma concentration of MDZ decreased in the I/R rats, while the tissue concentration did not change. The accumulated IS in the I/R rats inhibited the binding of MDZ to albumin, which increased the unbound fraction of MDZ. These results could provide new insights into drug administration in AKI.

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midazolam in critically ill patients. *Intensive Care Med* 2011; 38: 76-84.

Tables

Table 1. Biochemical parameters of serum from sham and ischaemia/reperfusion (I/R) rats

	Sham	I/R
Creatinine (mg/dL)	0.63 ± 0.08	3.70** ± 0.52
AST (IU/L)	42.7 ± 2.7	163.5 ± 76.7
ALT (IU/L)	12.7 ± 1.0	22.0* ± 2.8

Each value represents mean ± standard error (S.E.) of three experiments (*$p < 0.05$ and **$p < 0.01$ vs. sham rats).

446 **Table 2.** Moment parameters after intravenous (i.v.) administration of midazolam (5

447 mg/kg) to sham and ischaemia/reperfusion (I/R) rats

	Sham	**I/R**
AUC_p (ng · min/mL)	67880 ± 4555	38891** ± 3135
MRT_p (min)	36.3 ± 1.0	36.0 ± 0.9
CL_{tot} (mL/min)	18.7 ± 1.0	33.9** ± 1.6
V_{ss} (mL)	679.5 ± 47.4	1217.4** ± 32.7

448 Each value represents mean ± standard error (S.E.) of three (sham) or four (I/R)

449 experiments (**$p < 0.01$ vs. sham rats).

450

Table 3. Plasma unbound fraction of midazolam in sham and ischaemia/reperfusion (I/R) rats

MDZ concentration (ng/mL)	Sham	I/R
500	5.05 ± 0.43	8.20** ± 0.60
5000	5.19 ± 0.36	6.94** ± 0.31

Each value represents mean ± standard error (S.E.). n=5 for sham experiment, n=4 for 500 ng/mL of I/R and n=5 for 5000 ng/mL of I/R (**$p < 0.01$ vs. sham rats).

456 **Figure legends**

457 **Figure 1**. Plasma concentration-time profiles of midazolam (5 mg/kg) after intravenous

458 (i.v.) administration to sham (○) and ischaemia/reperfusion (I/R) (■) rats. Each

459 symbol represents mean ± standard error (S.E.) of three (sham) or four (I/R)

460 experiments.

461 **Figure 2.** Lineweaver-Burk plot for the elimination rate of midazolam in rat hepatocytes

462 from sham (○) and ischaemia/reperfusion (I/R) (■) rats. Each symbol represents mean

463 ± standard error (S.E.) of five (sham) or four (I/R) experiments.

464 **Figure 3.** Midazolam (5 mg/kg) concentration in tissues after intravenous (i.v.)

465 administration to sham (□) and ischaemia/reperfusion (I/R) (■) rats. The tissue samples

466 were collected 30 min after administration. Each bar represents mean + standard error

467 (S.E.) of six (sham) or five (I/R) experiments ($^{**}p < 0.01$ vs. sham rats).

468 **Figure 4.** Tissue-to-plasma ratio (T/P) of midazolam (5 mg/kg) after intravenous (i.v.)

469 administration to sham (□) and ischaemia/perfusion (I/R) (■) rats. The plasma and tissue

470 samples were collected 30 min after administration. Each bar represents mean +

471 standard error (S.E.) of six (sham) or five (I/R) experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$

472 vs. sham rats).

Figure 5. Plasma concentration of indoxyl sulphate (IS) in sham or ischaemia/reperfusion (I/R) rats. Each bar represents mean + standard error (S.E.) of eight (sham) or nine (I/R) experiments ($^{**}p < 0.01$ vs. sham rats).

Figure 6. Effects of indoxyl sulphate (IS) on midazolam protein binding. Each bar represents mean + standard error (S.E.) of four experiments ($^{**}p < 0.01$ vs. normal).

Figure 1.

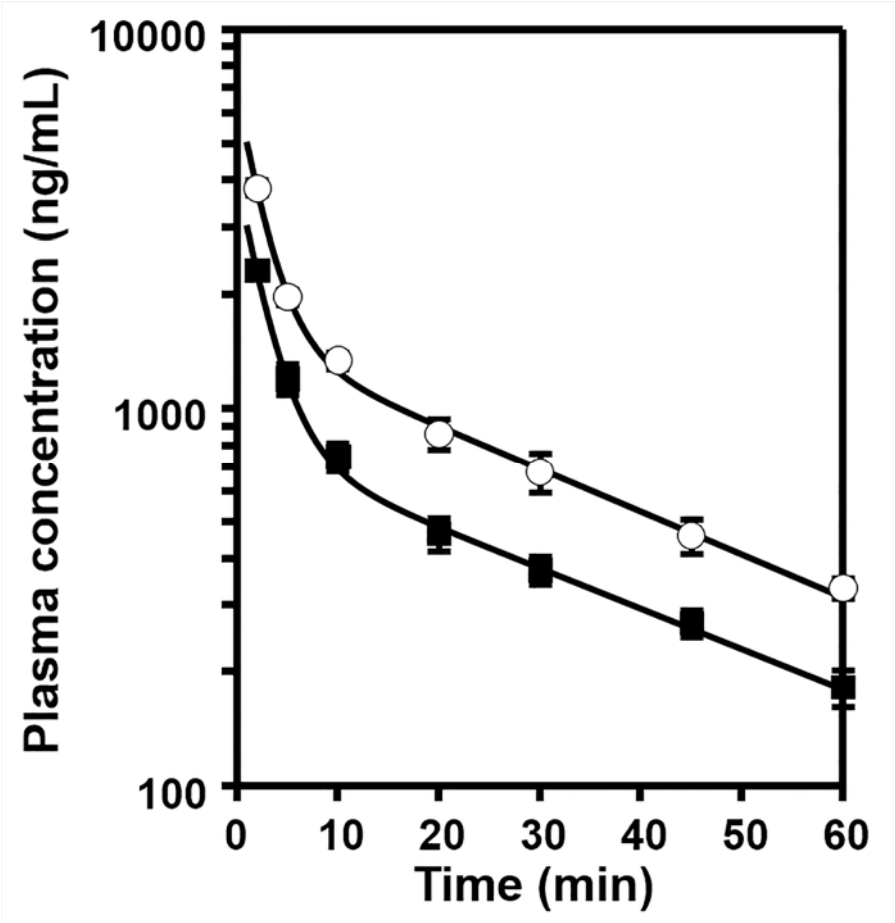
10000
1000
100
Plasma concentration (ng/mL)
0
10
20
30
40
50
60
Time (min)

Figure 2.

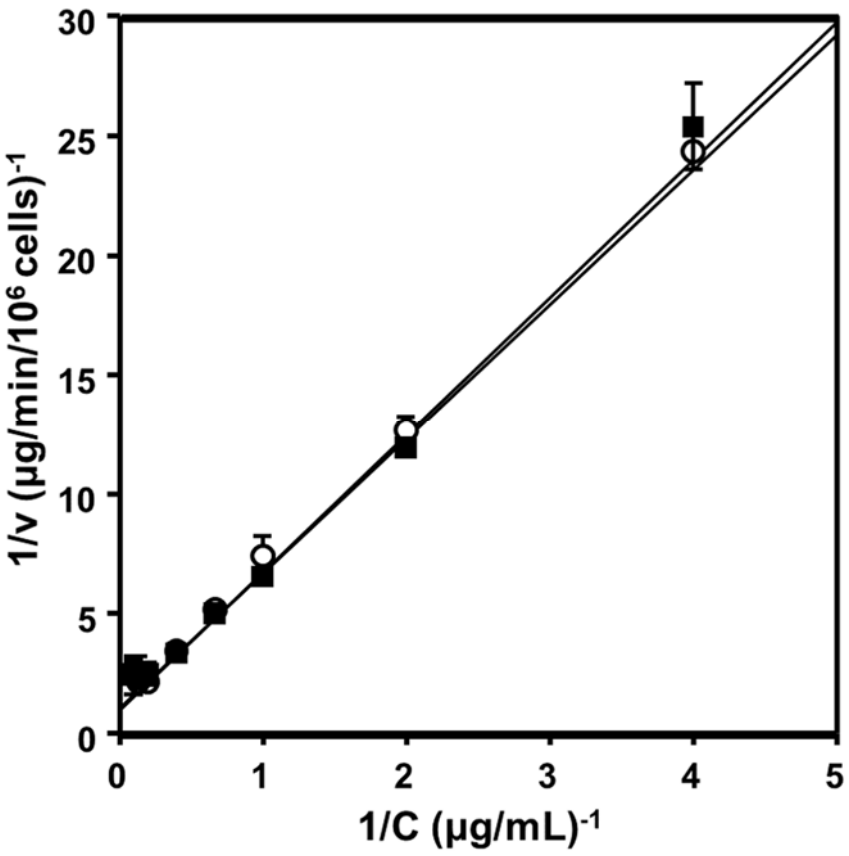

Figure 3.

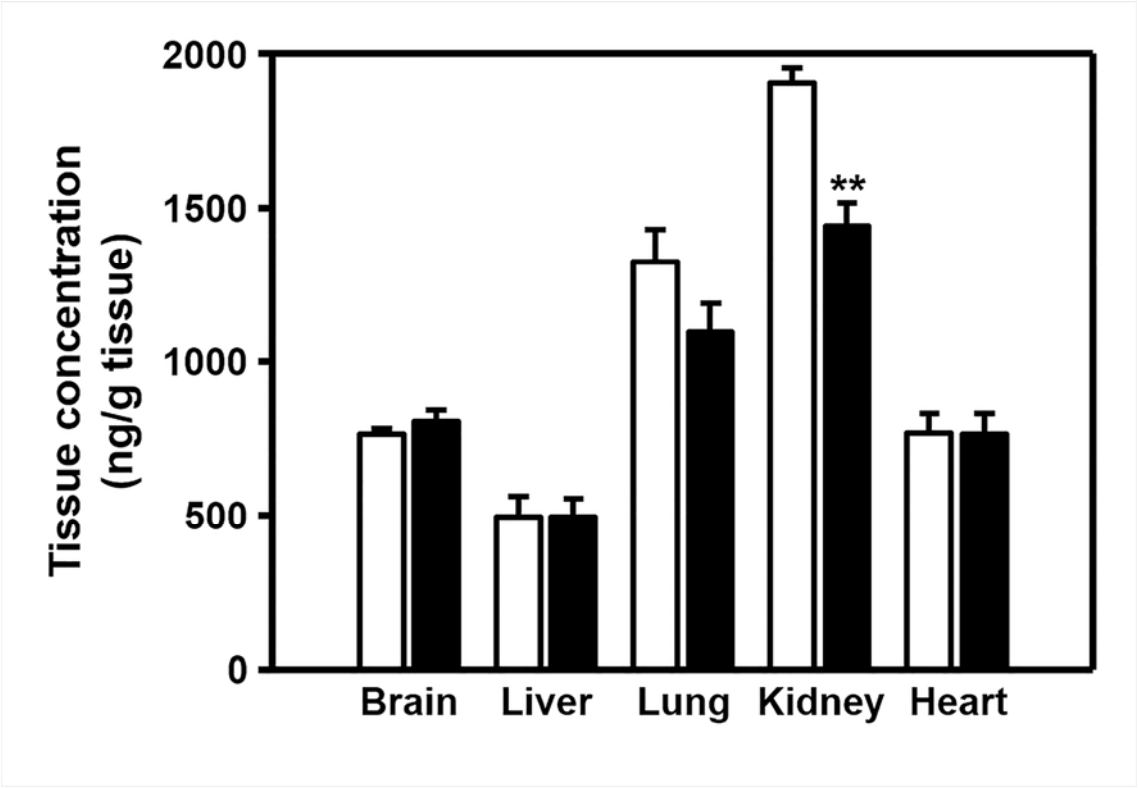
Tissue concentration
(ng/g tissue)
2000
1500
1000
500
0
**
Brain
Liver
Lung
Kidney
Heart

Figure 4.

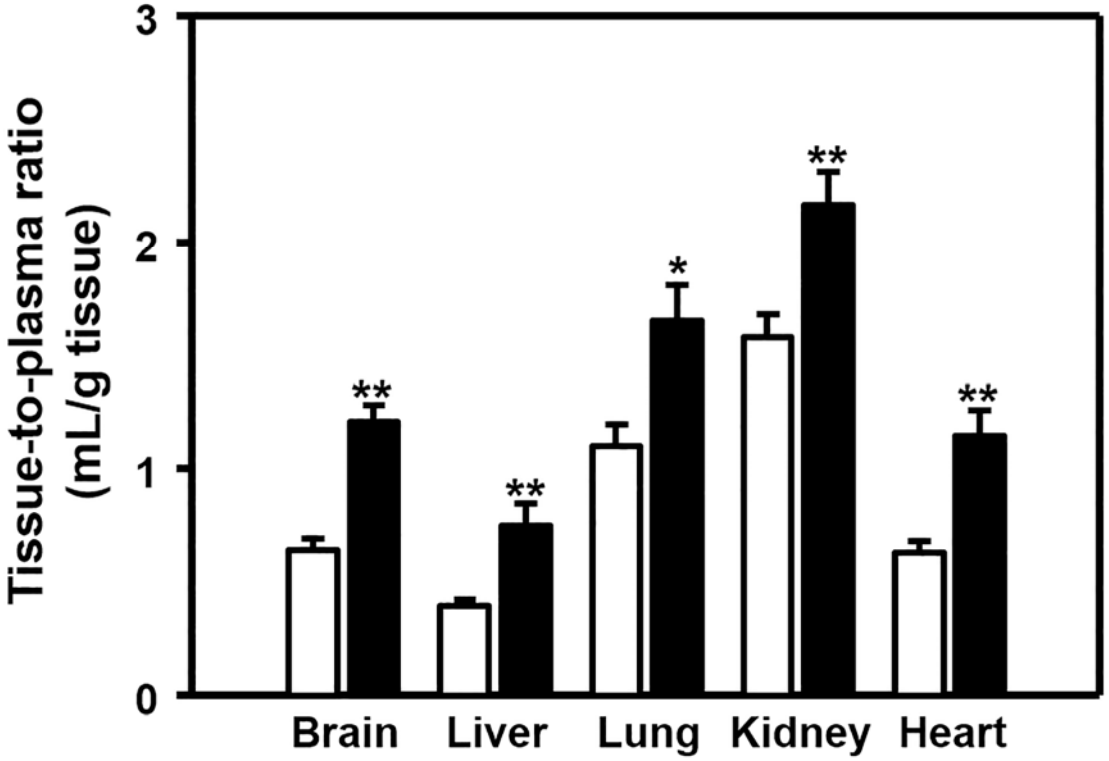
Tissue-to-plasma ratio
(mL/g tissue)
3
2
1
0
**
**
*
**
**
Brain
Liver
Lung
Kidney
Heart

Figure 5.