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In-vivo and In-vitro Metabolic Clearance of Midazolam, a Cytochrome P450 3A Substrate, by the Liver under Normal and Increased Enzyme Activity in Rats

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Abstract

The metabolic clearance of midazolam, a cytochrome P450 (CYP) 3A substrate, by the liver under normal and increased enzyme activity in rats was determined in-vivo and in-vitro to elucidate the reproducibility of the in-vivo hepatic extraction ratio of midazolam from the in-vitro study. The hepatic enzyme activity was modified by pretreating rats with a CYP inducer such as dexamethasone and clotrimazole.

The in-vivo hepatic extraction ratio ($ER_{h,obs}$) of midazolam under a steady-state plasma concentration (approx. 3 nmol mL^{-1}) in untreated (control) rats was 0·864. This value increased to 0·984 in dexamethasone-pretreated rats and to 0·964 in clotrimazole-pretreated rats. The in-vitro hepatic intrinsic clearance ($CL_{int,in\text{-}vitro}$), expressed as mL min^{-1} (mg microsomal protein)$^{-1}$, of midazolam was estimated as V_{max} $(K_m)^{-1}$ by in-vitro metabolism studies using liver microsomes. The $CL_{int,in\text{-}vitro}$ value was converted to the $CL_{int,cal}$ value, expressed as mL min^{-1} kg^{-1}, by considering the microsomal protein content (g liver)$^{-1}$ and the microsomal protein content (g liver)$^{-1}$ kg^{-1}. The estimated $CL_{int,cal}$ value was then converted to the ER_h value ($ER_{h,cal}$) according to the well-stirred, the parallel-tube and the dispersion models. The $ER_{h,cal}$ values obtained by the parallel-tube model were in good agreement with corresponding in-vivo $ER_{h,obs}$ values.

In conclusion, it was demonstrated that high hepatic clearances of midazolam under normal and increased CYP3A activity were reasonably predicted from in-vitro metabolism studies using liver microsomes.

The cytochrome P450 (CYP) 3A subfamilies, especially CYP3A4 in man, metabolize many clinically important pharmaceutical and potentially toxic environmental chemicals such as some immunosuppressing agents, antibiotics, calcium-channel blockers, anticancer drugs, mycotoxins and aryl hydrocarbons (Porter & Coon 1991; Guengerich 1992; Lown et al 1994). Midazolam is a commonly used short-acting 1,4-benzodiazepine that is extensively metabolized to 1′-hydroxy-midazolam and 4-hydroxymidazolam by CYP3A4 in man and by CYP3A1 or 3A2, or both, in rats (Kronbach et al 1989; Gorski et al 1994; Ghosal et al 1996). The use of midazolam as a CYP3A probe for characterization of inter- and intra-individual hepatic CYP3A variability has also been reported (Thummel et al 1994a, b). The CYP3A enzymes present in hepatocytes and small bowel enterocytes reduce the oral bioavailability of midazolam by first-pass metabolism (Paine et al 1996; Thummel et al 1996). In-vivo and in cultured cells these CYP3A enzymes are highly inducible by dexamethasone, clotrimazole, phenobarbital, pregnenolone-16α-carbonitrile, rifampin and erythromycin and the oral bioavailability of midazolam is further reduced by the induction (Kolars et al 1992; Schmiedlin-Ren et al 1993; Lee & Werlin 1995; Backman et al 1996; Mahnke et al 1996; Higashikawa et al 1999). CYP3A-mediated midazolam metabolism is also readily inhibited by other CYP3A substrates and inhibitors such as erythromycin, ketoconazole, itraconazole, cimetidine and grapefruit juice, resulting in increased oral bioavailability and changes in the pharmacodynamics of midazolam (Olkkola et al 1994; Wrighton & Ring 1994; Kupferschmidt et al 1995; Higashikawa et al 1999). Recently, such first-pass

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metabolism and drug–drug interactions arising from modulated CYP3A activities have received considerable attention in clinical pharmacotherapeutics.

Prediction of in-vivo clearance of such drugs, or drug–drug interactions, from data obtained in-vitro would provide clinically important information. The recent development of physiological pharmacokinetics and clearance concepts have made it possible to predict the in-vivo clearances and drug–drug interactions from in-vitro data for a number of drugs (reviewed by Iwatsubo et al (1996, 1997) and Ito et al (1998)).

In this study we have estimated the in-vivo and in-vitro metabolic clearances of midazolam by rat liver under normal and increased enzyme activity to elucidate the reproducibility of in-vivo clearance of midazolam from in-vitro study. For this purpose, hepatic CYP3A activity was modulated by pretreating the rats with the enzyme inducers dexamethasone and clotrimazole, as reported previously (Higashikawa et al 1999).

Materials and Methods

Midazolam was kindly supplied by Nippon Roche (Tokyo, Japan). Other reagents used were of the highest grade available.

Pretreatment of rats with dexamethasone or clotrimazole

Dexamethasone (50 mg) or clotrimazole (30 mg) was suspended in water (0·5 mL) containing 2% Tween 20. Male Sprague–Dawley rats, 240–280 g, were given dexamethasone (50 mg) daily for two days or clotrimazole (30 mg) daily for three days by intraperitoneal injection. Untreated (control) rats received an equal volume of 2% Tween 20. These rats were subjected to in-vivo and in-vitro studies 24 h after the last dose of inducer. Experiments with animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University School of Medicine.

Intravenous administration of midazolam

Untreated and dexamethasone-pretreated rats were anaesthetized by intraperitoneal administration of sodium pentobarbital (30 mg kg^{-1}), and the femoral artery and vein were cannulated with polyethylene tubing (PE-50; Clay Adamus, USA). Midazolam (10 μmol) was dissolved in a mixture (1 mL) of 20% ethanol, 20% propylene glycol, and 60% water. The drug solution (1 mL kg^{-1}) was administered intravenously via a cannula inserted at the femoral vein. Blood samples (0·25 mL) were collected from the femoral artery via the cannula at designated time intervals for analysis of midazolam concentrations in plasma or blood.

In-vivo hepatic extraction ratio ($ER_{h,obs}$) of midazolam

Untreated rats and rats pretreated with an inducer were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.p.), and the femoral artery and vein were cannulated with polyethylene tubing (PE-50; Clay Adamus, USA). Midazolam was administered intravenously at a dose of 1·25 μmol, then by constant-rate infusion of 50 nmol min^{-1}, via a cannula inserted in the femoral vein, to give a steady-state arterial plasma concentration of approximately 3 nmol mL^{-1}. Blood samples were collected from the femoral artery, the hepatic vein, and the portal vein 50–60 min after the initiation of constant-rate infusion for the analysis of plasma midazolam concentrations in each specimen. In-vivo hepatic extraction ratio ($ER_{h,obs}$) was estimated from the difference between midazolam plasma concentrations in the femoral artery and the hepatic vein according to the equation $ER_{h,obs} = (C_a - C_h)/C_a$, where C_a and C_h denote the arterial plasma and the hepatic vein plasma concentrations, respectively. Similarly, plasma concentrations of midazolam in the femoral artery and portal vein were used for estimation of the in-vivo intestinal extraction ratio ($ER_{g,obs}$).

Protein binding and blood-to-plasma concentration ratio (R_B) of midazolam

Blood samples were taken at the end of the animal experiments described above and the plasma protein binding and R_B value of midazolam were measured. A protein-binding study to estimate the unbound fraction of midazolam in plasma (f_p) was performed by equilibrium dialysis. The R_B value of midazolam was estimated by determining the concentration in both blood and plasma specimens, and the unbound fraction of midazolam in blood (f_B) was calculated by dividing f_p by R_B.

In-vitro hepatic metabolic clearance ($CL_{int,in\text{-}vitro}$) of midazolam

The livers of anaesthetized rats were excised after infusion of a sufficient amount of cold saline through the portal vein. The excised livers from differently treated rats were weighed and homogenized in a fourfold volume of pH 7·4, 10 mM Tris–HCl buffer containing 150 mM KCl, by means of a glass-Teflon Potter homogenizer (1000 rev min^{-1}, 10 strokes). Microsomes were prepared by differential centrifugation. The 105 000 *g* pellet

obtained was weighed and resuspended in the same buffer at a microsomal protein concentration of 5 or 0·4 mg mL^{-1}. Midazolam was dissolved in the same buffer in the concentration range 12·5–250 μM. $MgCl_2$ (100 mM, 100 μL) was added to the microsomal protein suspension (1 mL), and the mixture was pre-incubated at 37°C in a shaking water-bath for 10 min. NADPH (20 mM, 100 μL) and drug solution (0·8 mL) prewarmed at 37°C were then added to initiate the enzyme reactions. Thus, the final concentrations of microsomal protein in the reaction mixture were 0·2 mg mL^{-1} for dexamethasone-pretreated microsomal samples and 2·5 mg mL^{-1} for the others, and the concentration range of midazolam was from 5 to 100 μM. The enzyme reaction was performed for 30 s, and the reaction was terminated by adding cold acetonitrile (2 mL) to the sample. The solvent was evaporated to dryness under reduced pressure for determination of the concentration of unchanged midazolam.

In a separate experiment, the binding of midazolam (5, 20 or 100 μM) to microsomal protein (0·2 or 2·5 mg mL^{-1}) at 4°C was determined in the absence of NADPH by an equilibrium dialysis method.

Overall kinetic data for midazolam metabolism in liver microsomes prepared from differently treated rats were fitted to equation 1 with a single component for K_m and V_{max}.

$$\text{Overall metabolic rate (v)} = V_{max} \cdot S(K_m + S)^{-1} \quad (1)$$

where S denotes the unbound concentration of midazolam in the reaction mixture. The unbound fractions of midazolam (5, 20 or 100 μM) in microsomal suspensions were 42·5 ± 1·1% at 2·5 mg microsomal protein mL^{-1} and 80·2 ± 6·2% at 0·2 mg microsomal protein mL^{-1}, respectively. The in-vitro hepatic intrinsic clearance ($CL_{int,in\text{-}vitro}$) under linear conditions, expressed as mL min^{-1} (mg microsomal protein)$^{-1}$, was calculated using equation 2.

$$CL_{int,in\text{-}vitro} = v(S)^{-1} = V_{max}(K_m)^{-1} \quad (2)$$

Calculation of hepatic intrinsic clearance ($CL_{int,cal}$) of midazolam

$CL_{int,in\text{-}vitro}$ was further converted to $CL_{int,cal}$, expressed as mL min^{-1} kg^{-1}, according to equation 3 by taking the values of microsomal protein content (g liver)$^{-1}$ and g liver kg^{-1} of differently treated rats into consideration.

$$CL_{int,cal} = V_{max}(K_m)^{-1} \times \text{microsomal protein} \quad (3)$$

The microsomal protein content and liver wet weight of differently treated rats measured in this study were 20·7 ± 0·5 mg microsomal protein (g liver)$^{-1}$ and 46·5 ± 2·1 g liver kg^{-1} for untreated rats, 24·6 ± 0·7 and 61·9 ± 2·3 for dexamethasone-pretreated rats, and 23·5 ± 1·8 and 50·9 ± 1·9 for clotrimazole-pretreated rats.

Calculation of hepatic extraction ratio ($ER_{h,cal}$) of midazolam

The $ER_{h,cal}$ value of midazolam was calculated from the $CL_{int,cal}$ value according to the well-stirred (equation 4), parallel-tube (equation 5) and dispersion (equation 6) models (Roberts & Rowland 1986; Iwatsubo et al 1997).

$$ER_{h,cal} = f_B.CL_{int,cal}(Q_{h,B} + f_B CL_{int,cal})^{-1} \quad (4)$$

$$ER_{h,cal} = 1 - \exp(-f_B.CL_{int,cal}(Q_{h,B})^{-1}) \quad (5)$$

$$ER_{h,cal} = 1 - 4a\{(1+a)^2\exp[(a-1)(2D_N)^{-1}] - (1-a)^2\exp[-(a+1)(2D_N)^{-1}]\}^{-1} \quad (6)$$

where $a = (1 + 4R_N.D_N)^{½}$ and $R_N = f_B.CL_{int,cal}(Q_{h,B})^{-1}$.

In calculation, the unbound fraction of midazolam in blood (f_B) and the hepatic blood flow rate ($Q_{h,B}$) used were 0·1134 (average, n = 10) and 0·95 mL (g liver)$^{-1}$ × g liver kg^{-1}, respectively (Dedrick et al 1973; Montandon et al 1975; Iwatsubo et al 1997). The value of f_B was estimated by dividing the plasma free fraction of midazolam (f_p = 0·0832, average, n = 10) by the blood-to-plasma concentration ratio of midazolam (R_B = 0·7332, average, n = 4). A D_N of 0·17 (Roberts & Rowland 1986; Iwatsubo et al 1997) was used for the dispersion model.

Analysis

Concentrations of midazolam in plasma, blood and microsomal preparations were analysed by high-performance liquid chromatography equipped with UV detection and a reversed-phase TSK gel ODS-80TM column (Tosoh, Tokyo, Japan). Blood samples were haemolysed in a fivefold volume of water. Briefly, saturated disodium phosphate (100 μL) was mixed with each biological sample (100 μL) and midazolam was extracted with ethyl acetate (6 mL). The organic solvent was evaporated under reduced pressure and the residues were dissolved in methanol (100 μL). The mobile phase was 60:29:10:1 (v/v) methanol–water–0·1 M phosphate

buffer (pH 7·4)–acetonitrile. Midazolam was detected at 229 nm.

Results

Estimation of in-vivo $ER_{h,obs}$ of midazolam

Midazolam (10 μmol kg^{-1}) administered intravenously to untreated and dexamethasone-pretreated rats disappeared biexponentially from the plasma of both groups, and there was no significant difference between plasma-time profiles. The total plasma clearances of midazolam for untreated (control) and dexamethasone-pretreated rats were 71·6 ± 5·5 (mean ± standard error of the mean (s.e.m.) of results from six trials) and 87·0 ± 13·0 (six trials) mL min^{-1} kg^{-1}, respectively.

The $ER_{h,obs}$ of midazolam was determined at a steady-state arterial plasma concentration (Table 1). In a preliminary study we determined the biliary and urinary excretion of midazolam after intravenous administration to untreated rats. Biliary and urinary excretion of midazolam were both less than 0·1% of dose. Thus, hepatic extraction of midazolam is a result of metabolism in the liver. In Table 1, the in-vivo intestinal extraction ratios ($ER_{g,obs}$) of midazolam are also listed. The $ER_{g,obs}$ value of midazolam was negligible even in dexamethasone-pretreated rats. The high $ER_{h,obs}$ value of midazolam in untreated rats, 0·864, indicated that the hepatic clearance of midazolam is essentially limited by the hepatic blood flow rate. The $ER_{h,obs}$ value of midazolam was further increased in dexamethasone- and clotrimazole-pretreated rats.

Table 1. In-vivo hepatic and intestinal extraction ratios of midazolam at steady-state plasma concentration in differently treated rats.

Treatment	Extraction ratio	
	Hepatic	Intestinal
Untreated	0·864 ± 0·019	0·044 ± 0·060
Dexamethazone-pretreated	0·984 ± 0·009*	0·135 ± 0·125
Clotrimazole-pretreated	0·964 ± 0·005*	0·161 ± 0·252

The concentration of midazolam in plasma was approximately 3 nmol mL^{-1}. Each value is the mean ± s.e.m. of results from three to five trials. *$P < 0·01$ compared with untreated.

Prediction of in-vivo $ER_{h,obs}$ of midazolam from in-vitro *study*

Overall kinetic data for midazolam metabolism in liver microsomes prepared from differently treated rats were analysed by a single component for K_m and V_{max}. The K_m, V_{max}, $CL_{int,in\text{-}vitro}$ (V_{max} $(K_m)^{-1}$), and $CL_{int,cal}$ values are listed in Table 2. The $CL_{int,cal}$ value for dexamethasone-pretreated rats was approximately 6·9 that for untreated rats and 1·7-fold that for clotrimazole-pretreated rats. Using these $CL_{int,cal}$, f_B and $Q_{h,B}$ values for differently treated rats, the $ER_{h,cal}$ value of midazolam was estimated according to the well-stirred, parallel-tube, and dispersion models (Table 3). The $ER_{h,cal}$ values estimated according to the parallel-tube model were in good agreement with the corresponding $ER_{h,obs}$ value, whereas the well-stirred model gave significantly lower $ER_{h,cal}$ values. The values from the dispersion model were intermediate between those from the other two models.

Discussion

It has been reported that the oral bioavailability of midazolam in man is less than 50% of the dose. The remainder is eliminated by gastrointestinal and hepatic CYP3A-mediated metabolism, with the extraction ratios at both sites being comparable (Paine et al 1996; Thummel et al 1996). We recently reported that the oral bioavailability of midazolam was 11·5%, irrespective of the almost complete absorption of midazolam from the intestinal lumen in rats. Pretreatment with either dexamethasone or clotrimazole further reduced the bioavailability to 2·1% and 6·5%, respectively

Table 2. In-vitro kinetic data of overall midazolam metabolism by liver microsomes prepared from differently treated rats.

	Untreated	Dexamethasone-pretreated	Clotrimazole-pretreated
K_m (μm)	5·00 ± 1·38	9·97 ± 1·22	5·33 ± 1·30
V_{max} (nmol min^{-1} (mg microsomal protein)$^{-1}$)	2·67 ± 0·52	23·95 ± 1·87*	3·73 ± 0·35
$CL_{int,in\text{-}vitro}$ (mL min^{-1} (mg microsomal protein)$^{-1}$)	0·584 ± 0·065	2·539 ± 0·213*	0·781 ± 0·131
$CL_{int,cal}$ (mL min^{-1} kg^{-1})	562 ± 62	3868 ± 324*	933 ± 156

The in-vitro hepatic intrinsic clearance, $CL_{int,in\text{-}vitro} = V_{max} \times K_m^{-1}$, $CL_{int,cal} = CL_{int,in\text{-}vitro} \times$ microsomal protein content (g liver)$^{-1}$ × g liver kg^{-1}. Microsomal protein content (g liver)$^{-1}$ was 20·7 ± 0·5, 24·6 ± 0·7, and 23·5 ± 1·8 mg, respectively, for untreated, dexamethasone-pretreated and clotrimazole-pretreated rats. The weights of the livers (kg^{-1}) were 46·5 ± 2·1, 61·9 ± 2·3 and 50·9 ± 1·9 g, respectively, for untreated, dexamethasone-pretreated and clotrimazole-pretreated rats. Each value is the mean ± s.e.m. of results from four to seven trials. *$P < 0·01$ compared with untreated.

Table 3. Prediction of in-vivo hepatic extraction ratio of midazolam from in-vitro study.

	In-vivo hepatic extraction ratio		
	Well-stirred model	Parallel-tube model	Dispersion model
Untreated	0·586 ± 0·025**	0·756 ± 0·035	0·700 ± 0·033*
Dexamethasone-pretreated	0·878 ± 0·009**	>0·999 ± 0·001	0·986 ± 0·003
Clotrimazole-pretreated	0·673 ± 0·037**	0·865 ± 0·044	0·807 ± 0·044*

Each value is the mean ± s.e.m. of results from four to seven trials. *$P < 0.05$, **$P < 0.01$ compared with observed in-vivo hepatic extraction ratio (listed in Table 1).

(Higashikawa et al 1999). In this study we attempted precise estimation of the hepatic extraction ratio of midazolam under conditions of normal and increased CYP3A activity in rats, because such information is not available. We also examined the reproducibility of the in-vivo midazolam hepatic extraction ratio from in-vitro study.

Total blood clearance was extremely high for midazolam administered intravenously to untreated rats—109·8 ± 9·0 mL min^{-1} kg^{-1}, mean, three trials at a physiological hepatic blood flow rate of approximately 45 mL min^{-1} kg^{-1}. Thus the total clearance of midazolam in rats could not be accounted for by hepatic clearance alone. It has been reported that CYP3A is present in the lung and the kidney as well as the liver and the small intestine (Debri et al 1995). Also, CYP3A-inducers such as dexamethasone and clotrimazole increase CYP3A activity in rat leucocytes, although the induced activity is much lower than that observed in the liver (Mahnke et al 1996). However, the site and metabolism for extrahepatic elimination of midazolam in rats are not known.

The high $ER_{h,obs}$ of midazolam in rats was further increased by pretreatment with dexamethasone and clotrimazole (Table 1). Thus the reduced oral bioavailability of midazolam in treated rats as described above could be accounted for by the increase in $ER_{h,obs}$ values, although the contribution of intestinal first-pass metabolism in such animals should be taken into consideration (Higashikawa et al 1999).

It has been reported that rat liver microsomes metabolize midazolam predominantly to 4-hydroxymidazolam and to a lesser extent to 1′-hydroxymidazolam. Further, microsomes prepared from rats pretreated with dexamethasone give a minor metabolite, 1′,4-dihydroxymidazolam (Ghosal et al 1996). The reported K_m (V_{max}) values for the formation of 4-hydroxymidazolam in untreated and dexamethasone-pretreated rats are, respectively, 24·5 μM (5·9 nmol min^{-1} (mg microsomal protein)$^{-1}$) and 43·1 (28·9), and those for the formation of 1′-hydroxymidazolam are 32·3 (2·0) and 48·8 (14·1), respectively (Ghosal et al 1996). Similar tendencies were observed in the current study, that is, a small variation in K_m value and a significantly increased V_{max} value for rats pretreated with dexamethasone compared with values for untreated rats (Table 2). To estimate $CL_{int,cal}$ from $CL_{int,in\text{-}vitro}$ the microsomal protein content (g liver)$^{-1}$ and liver wet weight were measured in differently treated rats. The microsomal protein content (g liver)$^{-1}$ in untreated rats measured in the current study was in good agreement with the value reported by Tata (1970), although others have reported higher values (Lin et al 1996; Carlile et al 1997; Iwatsubo et al 1997). The rat liver weight after treatment with dexamethasone was significantly increased, as reported for dogs by Jayyoshi et al (1996). Estimation of $ER_{h,cal}$ values of midazolam from corresponding $CL_{int,cal}$ values was performed according to the well-stirred, parallel-tube, and dispersion models. It has been reported that when the extraction ratio of the drug is low small differences are obtained between values predicted by different models from in-vitro metabolic kinetics. On the other hand, the parallel-tube and dispersion models, especially the dispersion model, are reported to give a better fit for values observed for drugs with high extraction ratios (Iwatsubo et al 1996). In the current study the $ER_{h,cal}$ values estimated for midazolam by use of the parallel-tube model were comparable with in-vivo $ER_{h,obs}$ values whereas the well-stirred model gave significantly different values in all cases, as would be expected (Table 3). The dispersion model gave values intermediate between those of the other two models. The reason prediction by the parallel-tube model was better than that by the dispersion model is not clear, but might be partly because of the microsomal protein content used in this study.

In conclusion, prediction of hepatic clearance, or hepatic extraction ratio, from in-vitro studies would provide important information for the prediction of oral bioavailability of midazolam, especially hepatic first-pass metabolism under differently modulated enzyme activity. High hepatic clearance

of midazolam under conditions of normal and increased CYP3A activity was well predicted by results from in-vitro metabolism studies using liver microsomes.

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