In Vitro, Pharmacokinetic, and Pharmacodynamic Interactions of Ketoconazole and Midazolam in the Rat

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ABSTRACT

Interactions of midazolam and ketoconazole were studied in vivo and in vitro in rats. Ketoconazole (total dose of 15 mg/kg intraperitoneally) reduced clearance of intravenous midazolam (5 mg/kg) from 79 to 55 ml/min/kg (p < 0.05) and clearance of intragastric midazolam (15 mg/kg) from 1051 to 237 ml/min/kg (p < 0.05), increasing absolute bioavailability from 0.11 to 0.36 (p < 0.05). Presystemic extraction occurred mainly across the liver as opposed to the gastrointestinal tract mucosa. Midazolam increased electroencephalographic (EEG) amplitude in the β-frequency range. Ketoconazole shifted the concentration-EEG effect relationship rightward (increase in EC50), probably because ketoconazole is a neutral benzodiazepine receptor ligand. Ketoconazole competitively inhibited midazolam hydroxylation by rat liver and intestinal microsomes in vitro, with nanomolar Ki values. At a total serum ketoconazole of 2 μg/ml (3.76 μM) in vivo, the predicted reduction in clearance of intragastric midazolam by ketoconazole (to 6% of control) was slightly greater than the observed reduction in vivo (to 15% of control). However, unbound serum ketoconazole greatly underpredicted the observed clearance reduction. Although the in vitro and in vivo characteristics of midazolam in rats incompletely parallel those in humans, the experimental model can be used to assess aspects of drug interactions having potential clinical importance.

Theazole antifungal agent ketoconazole impairs clearance and enhances the central nervous system effects of several benzodiazepines metabolized by CYP3A isozymes, including triazolam, midazolam, and alprazolam (von Moltke et al., 1996a; Greenblatt et al., 1998b; Schmider et al., 1999; Tsunoda et al., 1999; Yuan et al., 1999; Venkatakrishnan et al., 2000, 2001). For orally administered benzodiazepine derivatives, such as triazolam and midazolam, that ordinarily undergo extensive hepatic and gastrointestinal presystemic extraction, coadministration of usual therapeutic doses of ketoconazole may increase area under the plasma concentration curve by 10-fold or more (von Moltke et al., 1996b; Greenblatt et al., 1998b; Schmider et al., 1999; Yuan et al., 1999; Venkatakrishnan et al., 2000). For low extraction benzodiazepines such as alprazolam, ketoconazole also reduces apparent oral clearance, although the magnitude of the interaction is less dramatic (Greenblatt et al., 1998b; Schmider et al., 1999). The capacity of ketoconazole to impair biotransformation of these and other CYP3A substrates in vitro is also well established (Thummel and Wilkinson, 1998; Venkatakrishnan et al., 2000, 2001; von Moltke et al., 1994, 1996a,b). Ketoconazole is a highly potent CYP3A inhibitor, with inhibition constant (Ki) values generally falling in the nanomolar range.

A clinical drug interaction study involving triazolam and ketoconazole indicated that the enhanced benzodiazepine agonist effects of triazolam due to coadministration of ketoconazole were less than would be anticipated based on the elevation of plasma triazolam levels (von Moltke et al., 1996a). Experimental observations indicate that ketoconazole binds to the benzodiazepine receptor and may act as a functional antagonist (Fahey et al., 1998). This unique property of ketoconazole indicates that ketoconazole influences not only pharmacokinetics but also pharmacodynamics of benzodiazepines.

The present study evaluated an experimental paradigm to assess the potential applicability of an experimental pharmacokinetic-pharmacodynamic model for study of complex
drug interactions of this type. We also characterized the P450 isoforms contributing to midazolam biotransformation in the rat, and evaluated the extent to which in vitro data on ketoconazole inhibition of midazolam biotransformation in vitro is predictive of actual pharmacokinetic interactions observed in vivo.

Materials and Methods

Animals. Male Sprague-Dawley rats (n = 4) weighing 300 to 350 g were housed individually under a 12-h light/dark cycle (lights on at 6:00 AM). Food and water were provided ad libitum, except on the trial days described below. Details of animal housing and maintenance, surgical procedures, and general study methodology have been described previously (Kotegawa et al., 1998, 1999, Laurijssens and Greenblatt, 2002).

Experimental Design. A four-way crossover design was used. The four treatment conditions were as follows: 1) 5 mg/kg midazolam intravenously, with intraperitoneal ketoconazole vehicle administered as described below; 2) 5 mg/kg midazolam intravenously, with intraperitoneal ketoconazole; 3) 15 mg/kg midazolam intragastrically, with intraperitoneal ketoconazole vehicle; and 4) 15 mg/kg midazolam intragastrically, with intraperitoneal ketoconazole. The sequence of these four trials was randomized. The washout period was 8 days if ketoconazole was administered in the previous trial, and 4 days if vehicle was administered in the previous trial. Seven days after completion of the four trials, rats were sacrificed and the liver and intestine were removed.

Surgery. Five days before the first trial, the surgery was performed under ketamine/xylazine anesthesia (Kotegawa et al., 1998, 1999; Laurijssens and Greenblatt, 2002). Two silicon rubber cannulas (Silastic; Dow Corning Co., Lansing, MI) were implanted. One cannula (length, 20 cm; internal diameter, 0.5 mm; and external diameter, 0.94 mm) was implanted in the right jugular vein, and the other, which was made by combining two different sizes of silicon tube (length, 15 and 5 cm; internal diameter, 0.5 and 0.3 mm; and external diameter, 0.94 and 0.63 mm), was implanted in the left carotid artery. The distal ends of both cannulas were tunneled subcutaneously, and exited between the ears. The venous cannula, used for drug administration, was positioned in the vena cava, and the arterial cannula, used for blood sampling, was positioned in the descending aorta. Part of a plastic tube with a cap was fixed on the skull using cranioplastic cement and the tunneled cannulas were fixed in the tube. Five EEG electrodes were also implanted onto the skull as described previously (von Moltke et al., 1996b; Cotreau et al., 2000). The electrode consisted of a stainless steel screw connected to a male amphenol pin with a 1 cm of insulated wire. The five electrodes placed on the skull were fixed using cranioplastic cement. The ends of the electrodes were connected to a miniature connector with EEG cables on the trial day. The electrodes were protected using an identical miniature connector without EEG cables during washout periods.

Pharmacokinetic and Pharmacodynamic Study in Vivo. Midazolam solution was prepared as 5 mg/ml midazolam base for intravenous administration, or 25 mg/ml midazolam base for intragastric administration, in 0.9% saline, pH 3.5. The ketoconazole solution was 10 mg/ml dissolved in polyethylene glycol 400. On the trial day, rats received an intravenous bolus dose of 5 mg/kg midazolam and an intragastric vehicle, or an intragastric dose of 15 mg/kg midazolam and an intravenous vehicle according to the predetermined schedule. Ketoconazole dosage was an intraperitoneal dose of 10 mg/kg 30 min before midazolam dosage, which was followed by 5 mg/kg given 180 min after the initial dose. This schedule maintains ketoconazole concentrations at 2 μg/ml or higher until at least 500 min after administration (Kotegawa et al., 1999). Blood samples for midazolam quantitation were collected from the arterial cannula up to 480 min after midazolam administration. Blood samples were centrifuged and the separated serum was stored at −20°C until the time of assay. Serum midazolam concentrations were determined by gas chromatography with electron capture detection (Arendt et al., 1984).

Bipolar EEG leads were continuously monitored during the experiment (Kotegawa et al., 1999; Laurijssens and Greenblatt, 2002). The 60-s EEG segments were recorded before midazolam dosage and at multiple time points up to 480 min after midazolam administration. EEG signals were subjected to fast Fourier analysis using RHYTHM 9.0 (Stellate Systems, Westmount, QC, Canada), and the average amplitudes (microwatts) of 1.5 to 3.5 Hz (δ), 4 to 7.5 Hz (θ), 8 to 12.5 Hz (α), and 13 to 31 Hz (β) were calculated. Percentage changes in the 3-amplitudes over the predose baseline value were used to quantitate pharmacodynamic benzodiazepine agonist effects (Mandema and Danhof, 1992; Laurijssens and Greenblatt, 1996, 2002; von Moltke et al., 1996a; Greenblatt et al., 1998a,b, 2000).

Liver and Intestinal Microsome Preparation. Rat liver microsomes were prepared as described previously (von Moltke et al., 1994, 1996a,b; Cotreau et al., 2000; Perloff et al., 2000). Small intestinal epithelial cell isolation and subsequent microsomal preparation were described in detail previously (Cotreau et al., 2000). Prepared liver and intestinal microsomes were stored at −80°C until use.

Heterologously expressed individual rat P450 isoforms (CYP1A1, 1A2, 2B1, 2C6, 2C11, 2C12, 2C13, 3A1, and 3A2), contained in microsomes from a cDNA-transfected insect cell line (Hi S), were obtained from BD Gentest (Woburn, MA), along with a vector control. Rat CYP2E1, contained in microsomes from cDNA-transfected human lymphoblastoid cells, was also obtained along with its corresponding vector control.

Incubation Procedure and Midazolam Metabolite Assay. The microsomal incubation procedure was similar to that described previously (von Moltke et al., 1996b; Cotreau et al., 2000; Perloff et al., 2000). The protein content and reaction time were predetermined based on linearity between microsomal protein concentration (up to 0.5 mg/ml) and the reaction time (up to 13 min) versus metabolite formation rate. Incubation mixtures contained 50 mM phosphate buffer, 5 mM MgCl2, 0.5 mM NADP+, and an isocitrate/isocitric dehydrogenase-regenerating system. Incubations were performed at 37°C with 0 to 300 μM midazolam and 0, 1, or 2 μM ketoconazole. Reactions were initiated by addition of microsomal protein (approximately 0.1 mg/tube; final volume, 250 μl). The incubation time was 7 min for liver microsomes or 10 min for intestinal microsomes. The reactions were stopped by cooling on ice and adding 100 μl of acetonitrile. Phenoxybenzamine was added as the internal standard. The incubation mixture was centrifuged, and the supernatant was subjected to HPLC analysis. The HPLC assay for α-hydroxy-midazolam (α-OH-midazolam) and 4-hydroxy-midazolam (4-OH-midazolam) was performed as described previously (von Moltke et al., 1996b; Cotreau et al., 2000; Perloff et al., 2000; Warrington et al., 2000).

For studies of heterologously expressed P450s, 50 μM midazolam was incubated as described above with individual rat P450s (50 pmol of P450/ml, insect cell vector control (1 mg/ml), rat CYP2E1 (1 mg/ml), and human vector control (1 mg/ml). Positive control incubations were performed using human liver microsomes (0.4 mg/ml) or rat liver microsomes (0.4 mg/ml) along with negative control incubations from which either cofactor, liver microsomal protein, or substrate was omitted. Incubation mixtures were processed and analyzed by HPLC.

Protein Binding. An equilibrium dialysis technique was used to examine the effect of ketoconazole on midazolam binding to serum proteins (Moschitto and Greenblatt, 1983; Laurijssens and Greenblatt, 2002). A 0.45-ml aliquot of rat serum containing 1 μg/ml midazolam and 0 to 10 μg/ml ketoconazole was placed in cellulose dialysis tubing that was sealed and submerged in 1.2 ml of phosphate buffer, pH 7.4. Samples were incubated at 37°C for 20 h. Midazolam concentrations in the dialyzed serum and dialysate were measured by...
gas chromatography (Arendt et al., 1984). Free fraction was calculated as the ratio of these concentrations.

**Data Analysis.** After intravenous midazolam, data points were fitted to a linear sum of two exponential terms. The fitted function was used to calculate elimination half-life, volume of distribution using the area method, total area under the serum concentration curve (AUC), and total clearance. After oral midazolam, the slope of the terminal elimination phase was used to calculate the elimination half-life. Area under the curve up to the final detectable concentration was measured using the linear trapezoidal method and extrapolated to infinity, yielding total AUC. Apparent clearance after intragastric administration was calculated as dose divided by total AUC. Systemic bioavailability of intragastric midazolam (F) was calculated as total AUC after intragastric administration (with or without ketoconazole) divided by total AUC for the corresponding intravenous trial, after normalization for differences in dosage (Tsunoda et al., 1999).

Relative contributions of the liver and intestine to observed F were based on the following scheme:

\[ F = F_G \cdot F_H \]  

(1)

under the assumption that midazolam is not lost in feces, decomposed in the intestinal lumen, or metabolized at sites other than the liver or enteric mucosa. \( F_G \) is the bioavailability across the intestinal mucosa, and \( F_H \) is bioavailability across the liver (Gorski et al., 1998; Thummel and Wilkinson, 1998; Tsunoda et al., 1999). \( F_H \) was estimated as follows:

\[ F_H = 1 - E_H = 1 - \frac{C_{1/2}}{Q_{H}} \]  

(2)

where \( E_H \) is the hepatic extraction ratio, \( C_{1/2} \) is total clearance of intravenously administered midazolam, and \( Q_H \) is hepatic blood flow. \( Q_{H} \) was assumed to be at least 80 ml/min/kg, and the influence of \( Q_{H} \) on estimates of \( F_H \) and \( F_G \) was subsequently analyzed.

For kinetic-dynamic modeling, an effect compartment model was linked to a sigmoid \( E_{\text{max}} \) model (Laurijssens and Greenblatt, 1998, 2002; Greenblatt et al., 2000) based on the assumption that EEG effects of midazolam are related to midazolam concentrations in a hypothetical effect site from where the drug disappears with a first order rate constant (\( K_{EO} \)). This rate constant was used to calculate the apparent half-life of equilibration (\( t_{1/2EO} \)). The relation between midazolam concentration in the hypothetical effect compartment (\( C_e \)) and the EEG effect (\( E \)) was evaluated using a sigmoid \( E_{\text{max}} \) model:

\[ E = 100 + E_{\text{max}} \gamma \left( \frac{C_e}{EC_{50} + C_e} \right) \]  

(3)

where \( E_{\text{max}} \) is the maximal effect, \( EC_{50} \) is the midazolam concentration at the effect site that produces 50% of \( E_{\text{max}} \), and \( \gamma \) is an exponent of uncertain biological significance describing the sigmoidicity of the curve. EEG data after intravenous and intragastric midazolam administration were simultaneously analyzed using the same \( E_{\text{max}} \) values for each animal, under the assumption that the route of administration does not influence \( E_{\text{max}} \).

Formation of \( \alpha \)-OH-midazolam and 4-OH-midazolam in liver microsomal samples was consistent with Michaelis-Menten kinetics with uncompetitive substrate inhibition (Venkatakrishnan et al., 2001), as described in previous in vitro studies of midazolam (Kronbach et al., 1989; von Moltke et al., 1996b; Perloff et al., 2000). Reaction velocities with coaddition of ketoconazole indicated competitive inhibition when evaluated using double reciprocal (Lineweaver-Burk) plots. Fitting of data points to a Michaelis-Menten model with uncompetitive substrate inhibition and mixed competitive-noncompetitive exogenous inhibition also yielded very large values of \( \alpha \), consistent with predominantly competitive exogenous inhibition. Therefore, inhibition constant (\( K_i \)) values for ketoconazole versus \( \alpha \)-OH-midazolam or 4-OH-midazolam formation represent competitive inhibition constants.

**Results**

**Body Weight Change.** Body weight (a general index of the animals’ health) increased throughout the study, except for the day of surgery or experimental trials (Fig. 1). One rat showed a weight reduction 2 days after the last trial. Pharmacokinetic and pharmacodynamic data obtained from this animal were used in the analysis, but liver and intestinal microsomes were not prepared from this animal. Additional microsomes were obtained from another rat.

**Pharmacokinetics of Midazolam in Vivo.** Coadministration of ketoconazole caused a significant increase in AUC and decrease in clearance of intravenous midazolam (Table 1; Fig. 2). The mean (±S.E.) ratio (value with ketoconazole divided by control value) was 1.45 (±0.56) for AUC and 0.69 (±0.26) for clearance. The effect of ketoconazole was greater when midazolam was administered intragastrically. Ketoconazole caused a significant increase in \( C_{\text{max}} \) and total AUC and a significant decrease in apparent oral clearance (Table 1; Fig. 2). Mean ratios were 6.5 (±2.2) for AUC and 0.15 (±0.05) for oral clearance. Midazolam oral bioavailability (\( F \)) increased from 0.11 in the control condition to 0.36 with ketoconazole (Table 1). Ketoconazole produced no significant change in midazolam elimination half-life either after intragastric or intravenous midazolam administration (Table 1), although half-life tended to be longer after intragastric as opposed to intravenous midazolam dosage.

Midazolam was extensively bound to serum protein. The free fraction was approximately 4% and was not influenced by ketoconazole (Fig. 3).

Estimation of relative contributions of enteric and hepatic sites (\( F_G \) and \( F_H \)) to net bioavailability of intragastric midazolam (\( F \)) depends on calculation of \( E_H \) (eq. 2), which in turn is critically dependent on an accurate estimate of \( Q_{H} \). Use of a customary value of \( Q_{H} \) in the rat (80 ml/min/kg) together
with intravenous midazolam clearance in the control condition (79 ml/min/kg) yields an estimate of 0.988 for $E_H$ and 0.012 for $F_H$ (eq. 2). Thus, even with $F_G$ set at 1.0, this value of $Q_H$ is inconsistent with the observed $F_H$ value of 0.11, under the described model assumptions. A $Q_H$ value of at least 89 ml/min/kg is needed to explain the observed data; with this value of $Q_H$, $F = F_H = 0.11$ and $F_G = 1.0$. At $Q_H$ values exceeding 89 ml/min/kg, estimates of $F_H$ increase and estimates of $F_G$ decrease (Fig. 4). With ketoconazole coadministration, $F_H$ and $F_G$ are also related to $Q_H$, but to a lesser degree (Fig. 4). Thus, with $Q_H$ in the range of 90 to 95 ml/min/kg, it is anticipated that hepatic rather than gastrointestinal metabolism is the major determinant of the low oral bioavailability of midazolam administered intragastrically, both with and without ketoconazole.

**Pharmacodynamics of Midazolam in Vivo.** Administration of midazolam produced characteristic increases in $\beta$-amplitude on the EEG (Fig. 5). After i.v. dosage, ketoconazole had no detectable influence on $\beta$-amplitude; mean area under the EEG effect versus time curve was essentially identical in the control condition and with ketoconazole. After intragastric midazolam, EEG $\beta$-amplitude was enhanced by an average of 11% (Fig. 5), but the difference did not reach significance.

The midazolam concentration-effect relationship was well described by eq. 3, incorporating an effect site equilibration delay. Mean values of $t_{1/2KEO}$ did not differ among the treatment conditions (Table 2). $E_{max}$ values also did not differ between control and ketoconazole conditions. However, ketoconazole produced a rightward shift in the relationship of

### TABLE 1

In vivo pharmacokinetics of midazolam in rats

Values represent the mean (±S.E.), n = 4. Because of an outlying value in one animal in the intragastric control condition, values of $C_{max}$, AUC, and clearance were subjected to logarithmic transformation for purposes of averaging.

<table>
<thead>
<tr>
<th></th>
<th>Intravenous (5 mg/kg)</th>
<th>Intragastric (15 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With Ketoconazole</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>With Ketoconazole</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>0.49 (±0.05)</td>
<td>0.61 (±0.02)</td>
</tr>
<tr>
<td>Elimination half-life (h)</td>
<td>0.49 (±0.05)</td>
<td>0.61 (±0.02)</td>
</tr>
<tr>
<td>Total AUC (ng/ml × h)</td>
<td>1051 (±14)*</td>
<td>1528 (±29)*</td>
</tr>
<tr>
<td>Volume of distribution (l/kg)</td>
<td>3.1 (±0.6)</td>
<td>2.7 (±0.2)</td>
</tr>
<tr>
<td>Clearance (ml/min/kg)</td>
<td>79 (±2)*</td>
<td>55 (±2)*</td>
</tr>
<tr>
<td>Net $F$</td>
<td>1054 (±97)*</td>
<td>163 (±4)*</td>
</tr>
</tbody>
</table>

* Indicates significant difference ($p < 0.05$) between control and ketoconazole conditions based on paired t test on ranks.

![Fig. 2](image-url)  
**Fig. 2.** Mean (± S.E.) serum midazolam concentrations after an intravenous bolus dose of 5 mg/kg midazolam (left) or an intragastric dose of 15 mg/kg (right), with and without coadministration of ketoconazole.

![Fig. 3](image-url)  
**Fig. 3.** Effect of ketoconazole on midazolam binding to rat serum protein (mean ± S.E.; n = 4).
effect site midazolam concentration versus EEG effect (Fig. 6). After i.v. dosage, the mean EC$_{50}$ value was shifted from 70 ng/ml in the control condition to 142 ng/ml with ketoconazole ($p < 0.01$); after intragastric dosage, values were 55 and 94 ng/ml ($p < 0.08$) (Table 2).

**Midazolam Metabolism in Vitro.** Midazolam was bio-transformed to $\alpha$-OH-midazolam and 4-OH-midazolam by liver microsomes (Table 3; Fig. 7) and intestinal microsomes (Table 3; Fig. 8). In liver microsomes, $K_m$ values for both pathways were similar, and the 4-OH pathway accounted for the majority of intrinsic clearance. Ketoconazole inhibited midazolam biotransformation via both pathways by an apparently competitive mechanism, with mean $K_i$ values of 0.65 $\mu$M for the $\alpha$-OH pathway and 0.14 for the 4-OH pathway. In intestinal microsomes, $K_m$ values differed from those in liver, with the $\alpha$-OH pathway having a lower $K_m$ value (mean 6 $\mu$M) than that for the 4-OH pathway (55 $\mu$M). The $\alpha$-OH pathway accounted for the majority of intrinsic clearance in
Kᵢ values for ketoconazole inhibition were in the nanomolar range. Studies of heterologously expressed individual rat P450s indicated that 4-OH-midazolam formation was mediated almost exclusively by CYP3A1 and CYP3A2 (Fig. 9). However formation of α-OH-midazolam was mediated by these two CYP3A isoforms along with four isoforms of the CYP2C subfamily, as well as a possible additional contribution of CYP2E1.

For purposes of in vitro-in vivo scaling, it was assumed that presystemic extraction of intragastric midazolam occurs mainly by hepatic extraction (Fig. 4). Using the mean ketoconazole Kᵢ values for hepatic microsomes (Table 3) and the further assumption that midazolam serum concentrations were considerably below the reaction Kᵢ, we calculated the ratio of net in vitro midazolam hydroxylation rate in the presence of ketoconazole divided by the control rate without ketoconazole (von Moltke et al., 1995, 1998; Venkatakrishnan et al., 2000, 2001). The concentration of the inhibitor (ketoconazole) in this relationship was taken either as the minimum total serum ketoconazole concentration (2 g/ml; 3.76 M) as determined in a previous study using this dosing regimen (Kotegawa et al., 1999), or the unbound serum concentration, calculated as the total concentration (3.76 M) multiplied by the free fraction. Two estimates of ketoconazole free fraction in rat serum or plasma available in the literature were 0.037 (Matthew et al., 1993) and 0.0095 (Higashikawa et al., 1999c; Yamano et al., 1999b), yielding 0.14 and 0.04 M, respectively, as unbound serum concentrations. The resulting in vitro velocity ratios were compared with the mean in vivo clearance ratios (clearance with coadministra-

**TABLE 2**
Effect of ketoconazole on EEG pharmacodynamics of midazolam
Values are mean (±S.E.), n = 4.

<table>
<thead>
<tr>
<th></th>
<th>Intravenous</th>
<th>Intragastric</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eₘₚₘ (%) in excess of 100%</td>
<td>Control</td>
<td>With Ketoconazole</td>
</tr>
<tr>
<td></td>
<td>86 (±10)</td>
<td>79 (±13)</td>
</tr>
<tr>
<td>EC₅₀ (mg/ml)</td>
<td>69.9 (±17.4)*</td>
<td>142.4 (±38.4)*</td>
</tr>
<tr>
<td>Exponent</td>
<td>1.43 (±0.22)</td>
<td>1.37 (±0.33)</td>
</tr>
<tr>
<td>t₁/₂(Kₑ₀) (min)</td>
<td>7.2 (±3.5)</td>
<td>7.2 (±3.5)</td>
</tr>
</tbody>
</table>

* p < 0.01. ** p < 0.05, outcome of comparison of control versus ketoconazole conditions based on t test on ranks.

**Fig. 6.** Relationship between midazolam concentrations at the hypothetical effect site and EEG effect in a representative animal after an intravenous bolus dose of 5 mg/kg midazolam (left) or an intragastric dose of 15 mg/kg midazolam (right), with and without coadministration of ketoconazole. The lines represent fitted functions consistent with a sigmoid Eₘₚₘ model (eq. 3).

**TABLE 3**
Kinetics of midazolam metabolite formation by rat hepatic and intestinal microsomes in vitro

<table>
<thead>
<tr>
<th></th>
<th>Mean (±S.E.), n = 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-OH-Midazolam 4-OH-Midazolam</td>
</tr>
<tr>
<td>Hepatic microsomes</td>
<td></td>
</tr>
<tr>
<td>Vₘₚₘ (nmol/min/mg of protein)</td>
<td>0.48 (±0.06) 2.39 (±0.21)</td>
</tr>
<tr>
<td>Kₘₚₘ (µM)</td>
<td>24 (±2) 22 (±2)</td>
</tr>
<tr>
<td>Kᵢ (µM)</td>
<td>0.65 (±0.66) 0.14 (±0.02)</td>
</tr>
<tr>
<td>Vₘₚₘ/Kᵢ ratio (µl/min/mg of protein)</td>
<td>21 (±2) 110 (±2)</td>
</tr>
<tr>
<td>Ketoconazole Kᵢ (µM)</td>
<td>0.05 (±0.005) 0.23 (±0.03)</td>
</tr>
</tbody>
</table>

* Mean value calculated after logarithmic transformation due to one high outlying value.

Studies of heterologously expressed individual rat P450s indicated that 4-OH-midazolam formation was mediated almost exclusively by CYP3A1 and CYP3A2 (Fig. 9). However formation of α-OH-midazolam was mediated by these two CYP3A isoforms along with four isoforms of the CYP2C subfamily, as well as a possible additional contribution of CYP2E1.
tion of ketoconazole divided by clearance in the control condition) after intragastric midazolam administration.

The actual in vivo clearance ratio was 0.15, corresponding to a predicted enzyme-available ketoconazole concentration of 1.13 μM (0.6 μg/ml). This is represented as the closed circle in Fig. 10. The predicted clearance decrement corresponding to the total serum ketoconazole concentration was 0.06 (Fig. 10, open circle), whereas the predicted clearance ratios based on unbound ketoconazole concentrations were 0.563 and 0.827 (Fig. 10, open squares). Thus, total serum ketoconazole concentrations slightly overpredicted the actual in vivo midazolam clearance decrement, whereas unbound serum ketoconazole levels grossly underpredicted the midazolam clearance decrement.

**Discussion**

Consistent with previous studies (Mandema and Danhof, 1992; Lau et al., 1996; Higashikawa et al., 1999a,b,c; Laurijssens and Greenblatt, 2002), clearance of intravenously administered midazolam in rats was high, with a mean control clearance value (without inhibitor) of 79 ml/min/kg. Intragastric midazolam had a mean net bioavailability of only 11%. Partitioning the net F value into hepatic and enteric components requires an assumed value of hepatic flow, and the specific estimates of $F_H$ and $F_G$ are highly dependent on the $Q_H$ value entered into the model. Small changes in $Q_H$ within the range of 90 to 110 ml/min/kg yield large changes in $F_H$ and $F_G$ (Fig. 4). Nonetheless, the general conclusions are consistent with previous studies in the rat in that clearance of intravenous midazolam is largely flow-dependent, and incomplete bioavailability of oral midazolam is principally determined by hepatic as opposed to enteric presystemic extraction. These relationships are only partly consistent with the pattern of midazolam kinetics in humans, in whom typical values of intravenous clearance (5–12 ml/min/kg) are less than 50% of hepatic blood flow, oral bioavailability averages 25 to 40%, and intestinal metabolism contributes in a major
way to incomplete oral bioavailability (Greenblatt et al., 1984; Gorski et al., 1998; Thummel and Wilkinson, 1998; Tsunoda et al., 1999).

The dosage regimen of ketoconazole used in the present study has been verified to produce serum ketoconazole concentrations consistently in excess of 2 µM (Kotegawa et al., 1999), which is similar to the range encountered with usual therapeutic doses of ketoconazole in humans (Venkatakrishnan et al., 2000). Clearance of intravenous midazolam was reduced by ketoconazole to 69% of control values, whereas apparent clearance of intragastric midazolam was reduced to 15% of control. Again, this is consistent with the high clearance of midazolam in the rat, such that ketoconazole would inhibit the component of intravenous clearance that is not flow-dependent, whereas ketoconazole would have a much greater effect on oral clearance. In humans, clearance of intravenous midazolam is 35 to 40% of hepatic blood flow, and ketoconazole reduced intravenous clearance to 20% of control values (Tsunoda et al., 1999). Clearance of oral midazolam in humans was reduced to about 6% of control values. Thus, species differences in the pharmacokinetics of midazolam, the relation of clearance to hepatic blood flow, and the relative contributions of hepatic and enteric sites to net presystemic extraction, are evident as predictable differences in the response to chemical inhibition by ketoconazole.

In agreement with previous studies from our laboratory and elsewhere (Mandema and Danhof, 1992; Laurijssens and Greenblatt, 1996, 2002), administration of midazolam to the rat produced increases in EEG -amplitude consistent with its benzodiazepine agonist properties. Kinetic-dynamic modeling of the data required inclusion of a hypothetical effect site, with apparent equilibration half-life values estimated in the range of 5 to 15 min, as described with intravenous midazolam administration in humans (Mandema and Danhof, 1992; Laurijssens and Greenblatt, 1996). We observed that coadministration of ketoconazole had no significant effect on the estimated \( E_{\text{max}} \) for the EEG effect parameter, but caused a significant rightward shift in the relationship between midazolam hypothetical effect site concentration and EEG effect. This was evident as an increase in the estimated \( EC_{50} \) values. A similar phenomenon was reported in a clinical study of triazolam and ketoconazole in humans (von Moltke et al., 1996a) and in an experimental model (Farley et al., 1998). This may be explained by the property of ketoconazole as a neutral ligand at the benzodiazepine receptor, acting functionally as an antagonist and shifting the concentration-response relationship (Fahey et al., 1998). It should be noted that ketoconazole alone has no detectable effect on the EEG, either in this experimental model (Kotegawa et al., 1999) or in humans (von Moltke et al., 1996a; Greenblatt et al., 1998b).

Midazolam was biotransformed to two principal metabolites, \( \alpha \)-OH-midazolam and 4-OH-midazolam, by rat liver
and intestinal microsomes. In liver microsomes, $K_{in}$ values for both pathways were in the range of 20 to 25 $\mu$M, and the 4-OH-midazolam dominated in terms of contribution to net intrinsic clearance. These results are similar to previous studies of rat liver microsomes (Ghosal et al., 1996), but differ from kinetic parameters in human liver microsomes (Kronbach et al., 1989; Gorski et al., 1994; Ghosal et al., 1996; von Moltke et al., 1996b; Perloff et al., 2000). Likewise, rat liver was less susceptible to inhibition of midazolam biotransformation by ketoconazole compared with human liver microsomes (Ghosal et al., 1996). Testosterone hydroxylation also was less susceptible to ketoconazole inhibition in rat as opposed to human liver microsomes (Eagling et al., 1998). Midazolam is presumably metabolized almost exclusively by CYP3A4 and 3A5 in humans. In rats, however, 4-OH-midazolam formation is largely mediated by CYP3A1 and 3A2, but other P450 isoforms of the CYP2C subfamily contribute substantially to formation of α-OH-midazolam (Fig. 9). This is consistent with the findings of Kobayashi et al. (2002). As in many previous studies (von Moltke et al., 1994, 1996a,b; Yamano et al., 1999a; Perloff et al., 2000), we combined in vitro ketoconazole $i$ values for rat and human microsomes together with anticipated in vivo total serum concentrations (2 $\mu$g/ml) based on a previous study of similar design (Kotegawa et al., 1999) to evaluate the actual reduction in clearance of intragastric midazolam caused by ketoconazole in relation to what would be predicted from the in vitro data. Assuming an in vitro inhibitor concentration equal to the in vivo total serum concentration (2 $\mu$g/ml; 3.76 $\mu$M), the in vitro reaction velocity would be predicted to be reduced to 6% of control. In the actual in vivo study, clearance of intragastric midazolam was reduced to 15% of control. In contrast, anticipated in vivo unbound serum midazolam concentrations (based on two literature estimates of ketoconazole free fraction in the rat) yielded predicted in vivo reaction velocity ratios of 56 and 83% of control, respectively. Therefore, use of total serum ketoconazole concentrations for purposes of scaling yields predictions that slightly overestimate the observed extent of interaction. However, use of unbound serum ketoconazole levels grossly underestimates the actual interaction, apparently attributable to underestimation of the in vivo enzyme-available ketoconazole concentration by a factor falling between 8- and 32-fold. In clinical and in vitro studies of the interaction of zolpidem and ketoconazole in humans, unbound plasma ketoconazole concentrations likewise greatly underesti-
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