Prediction of in Vivo Hepatic Metabolic Clearance of YM796 from in Vitro Data by Use of Human Liver Microsomes and Recombinant P-450 Isozymes

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ABSTRACT

The metabolic rate of (S)-(-)-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro [4,5] decane-L-tartarate monohydrate (YM796), an antidementia agent, was determined by use of 12 different human liver microsomal samples. The metabolism of YM796 was shown to consist of three components; one high-affinity (Km1 = 1.67 μM), one low-affinity (Km2 = 654 μM) and a nonsaturable component. Good correlations were observed between the individual CYP3A4 content in 12 different human liver microsomal samples and kinetic parameters such as CLint, all, the high-affinity component clearance (Vmax1/Km1) and the low-affinity component clearance (Vmax2/Km2). Anti-human CYP3A4/S antibodies inhibited the metabolism of YM796 at 1 μM by up to 75%. In addition, ketoconazole, an inhibitor of CYP3A4, inhibited YM796 metabolism by >90%. The metabolic clearance of YM796 in each of the 12 human liver microsomal samples was successfully predicted from the kinetic parameters obtained with the recombinant microsomes by taking into consideration the CYP3A4 content in each microsomal sample. Based on the CLint, all estimated from the in vitro experiments, the area under the plasma concentration-time curve after oral administration (AUCoral) of YM796 was also predicted by taking into account the hepatic blood flow rate (Qh), the unbound fraction of YM796 in human plasma (fup) and the fraction absorbed from the gut. In addition, AUCoral was determined in six healthy male volunteers. The predicted AUCoral was similar to the observed value in vivo, which suggests that the in vitro metabolism data obtained with human liver microsomes are useful for quantitatively predicting human liver metabolism in vivo and that recombinant microsomes are also available when the particular isozyme is almost completely responsible for the metabolism of the drug, the variation in P-450 content of human liver is known and the experimental conditions such as the amount of CYP reductase and cytochrome b5 are carefully optimized to mimic the activity found in native microsomes, as for YM796.

It is of clinical importance to predict hepatic and renal clearances in humans because many drugs are eliminated from the body predominantly by these pathways. There have been many successful attempts to predict renal clearance (CLR) in humans by applying the method for animal scaling based on data derived from animal experiments (Dedrick, 1974; Boxenbaum, 1982; Sawada et al., 1984). On the other hand, the application of the animal scaling method to the prediction of hepatic clearance (CLH) is limited because of large interspecies differences in the metabolic clearances (Boxenbaum, 1980).

An alternative method has been proposed by Rane et al. (1977) and Wilkinson (1987) to predict hepatic metabolic clearance from in vitro metabolism data in rats by use of liver microsomes or isolated hepatocytes by taking into account parameters such as Qh and the unbound fraction of drug in the blood.

ABBREVIATIONS: AUCoral, area under the plasma concentration-time curve after oral administration; CLH, hepatic clearance; CLint, all, overall intrinsic metabolic clearance (intrinsic hepatic clearance); CLint, nis, intrinsic metabolic clearance for the nonsaturable component; CLoral, oral clearance (= dose/AUCoral); CLR, renal clearance; CYP, cytochrome P-450; Dn, dispersion number; Fup, hepatic availability; fup, unbound fraction in human plasma; Km, r, Michaelis-Menten constant for the r-th component of the metabolic reaction; MS, microsomal; Qin, hepatic blood flow rate; Rsp, blood-to-plasma concentration ratio; Vmax, r, maximal metabolic rate for the r-th component of the metabolic reaction; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS-MS, tandem mass spectrometry.
blood (fu). We have also successfully predicted the in vivo metabolic clearances in rats for 14 drugs reported to be metabolized by CYP (Sugiyama et al., 1988; Sugiyama and Iwatsubo, 1994). Houston (1994) compared the intrinsic metabolic clearances (CLint) for many drugs estimated from Iwatsubo, 1994). Houston (1994) compared the intrinsic metabolic clearances in rats for 14 drugs reported to be human CYP isozyme (recombinant system) to predict an alternative to human liver microsomes, it is possible to use recombinant microsomes prepared from cells expressing the different recombinant human CYP isozymes were similar to those used for human liver microsomes, except for the quantity of microsomes. In the metabolism studies with each of the recombinant human CYP isozymes, the quantity of MS protein was adjusted to the amount of CYP isozyme similar to that reported for human liver microsomes. Enzyme reactions were initiated by adding 25 µl of the NADPH-generating system as mentioned above. After incubation at 37°C in a shaking water bath for 2 min, the reaction was terminated by adding 250 µl methanol, and then the reaction mixture was centrifuged at 10,000 x g for 5 min and an aliquot of supernatant was spotted onto silica-gel plates (E. Merck, Darmstadt, Germany) to separate metabolites from the parent drug by TLC with use of chloroform/methanol/27% ammonia (100:10:1) as a mobile phase. Experiments were performed in triplicate. YM796 concentrations to estimate the kinetic parameters were from 1 to 1000 µM. The quantification of metabolites was performed with BAS-2000 equipment (Fuji-film, Tokyo, Japan).

**Immunoinhibition study.** Human liver microsomes (H-35) at a final concentration of 0.1 mg/ml were preincubated for 30 min at room temperature with increasing amounts of antibodies (from 1 to 4 mg/mg MS protein) for human CYP3A4/5 or preimmunoglobulin G obtained from rabbits. The final YM796 concentration was 1 µM.

**Inhibition study.** As an inhibitor of human CYP3A4, ketoconazole was used to assess if it would have an inhibitory effect on YM796 metabolism. Assays were performed with human liver microsomes (H-35) under the optimal conditions above. Final YM796 concentrations were set at 1 and 1000 µM, whereas ketoconazole concentrations ranged from 0.01 to 10 µM.

**Purification of NADPH-cytochrome P-450 reductase and cytochrome b5.** NADPH-cytochrome P-450 reductase was purified from rat liver microsomes to a specific activity of 23 U/mg protein by the method of Yasukochi and Masters (1976) with minor modifications. Cytochrome b5 was purified from rat liver microsomes to a specific content of 28 nmol/mg protein by the method reported previously (Kamataki et al., 1981).

**Effects of NADPH-cytochrome P-450 reductase and cytochrome b5 on YM796 metabolism in recombinant microsomes for human CYP3A4.** Under the conditions described above, the effects of NADPH-cytochrome P-450 reductase and cytochrome b5 on YM796 metabolism in recombinant microsomes expressing human CYP 3A4 were estimated by use of increasing amounts of NADPH-cytochrome P-450 reductase (5–40 U/nmol P-450) or cytochrome b5 (0.5–8.0 nmol/nmol P-450). Before the addition of the substrate and the NADPH-generating system, the recombinant microsomes were preincubated with NADPH-cytochrome P-450 reductase or cytochrome b5 at 37°C for 10 min. The final YM796 concentration was 1 µM. As a positive control, the effects of NADPH-cytochrome P-450 reductase and cytochrome b5 on testosterone-6β-hydroxylase activity were also examined. Incubation conditions were essentially the same as those used for YM796 metabolism as described previously, except that the time used was 10 min. The final testosterone concentration was 250 µM. The 6β-hydroxysteroidase was determined by an HPLC-UV absorbance method as reported elsewhere (Yoshimoto et al., 1995). Nizatapen was used as an internal standard. The HPLC column used was a CAPCELL PAK C18 SG 120 column (250 x 4.6 mm internal diameter, Shiseido Co., Ltd., Tokyo, Japan). The mobile phase for the 6β-hydroxytestosterone assay was a 60:40 (v/v) mixture of methanol and 0.05 M potassium phosphate buffer (pH 3.4) and delivered at a flow rate of 1.0 ml/min.

**YM796 metabolism in human liver microsomes or recombinant human CYP isoforms.** YM796 and [14C]YM796 (1 µM; specific activity, 40 mCi/mmol) were incubated with a reaction mixture (0.25 ml) consisting of 25 µg human liver MS protein and an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 U/ml glucose-6-phosphate dehydrogenase, 6 mM MgCl2) in 100 mM potassium phosphate buffer (pH 7.4). Incubation conditions used for microsomes from B lymphoblastoid cells expressing the different recombinant human CYP isozymes were similar to those used for human liver microsomes, except for the quantity of microsomes. In the metabolism studies with each of the recombinant human CYP isoforms, the quantity of MS protein was adjusted to the amount of CYP isozyme similar to that reported for human liver microsomes. Enzyme reactions were initiated by adding 25 µl of the NADPH-generating system as mentioned above. After incubation at 37°C in a shaking water bath for 2 min, the reaction was terminated by adding 250 µl methanol, and then the reaction mixture was centrifuged at 10,000 x g for 5 min and an aliquot of supernatant was spotted onto silica-gel plates (E. Merck, Darmstadt, Germany) to separate metabolites from the parent drug by TLC with use of chloroform/methanol/27% ammonia (100:10:1) as a mobile phase. Experiments were performed in triplicate. YM796 concentrations to estimate the kinetic parameters were from 1 to 1000 µM. The quantification of metabolites was performed with BAS-2000 equipment (Fuji-film, Tokyo, Japan).

**Materials and Methods**

**Chemicals and reagents.** YM796 and [14C]YM796 were synthesized by Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan) and by Amersham International (Buckinghamshire, UK), respectively. 6β-Hydroxytestosterone and ketoconazole were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol and other reagents of analytical grade were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). NADP, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (Mannheim, Germany). Microsomal preparations of recombinant human CYP enzymes expressed by the human B lymphoblastoid cell line, AHH-1 (recombinant microsomes), were purchased from Gentest Corp. (Woburn, MA). Twelve human liver microsomes (H-19, H-35, H-36, H-38, H-50, H-51, H-56, H-57, H-62, H-66, H-67 and H-84) with large variations in the CYP3A4 content were selected and generous gifts for the in vitro metabolism experiments among 26 different microsomes prepared from human livers stored in the human liver bank of SRI International (Menlo Park, CA). Antibodies to human CYP3A4/5 were also a generous gift from International Medical Center of Japan (Tokyo, Japan).
Protein binding of YM796 in human plasma. To 2-ml aliquots of human plasma, 20 µl of phosphate-buffered isotonic solution containing [14C]YM796 were added to give concentrations of 0.5, 50 and 2500 µM. After incubation for 30 min at 37°C, a 50-µl aliquot was taken from each plasma sample to measure the total plasma concentration and the remainder was transferred to a ultrafiltration tube (Ultrafree CL, Millipore Corp., Bedford, MA). The tubes were centrifuged for 15 min (1,000 × g at 37°C), and then a 50-µl aliquot of filtrate was removed to measure the unbound plasma concentration. Aliquots of plasma and filtrated samples were subjected to liquid scintillation counting with 10 ml of liquid scintillator.

Blood-to-plasma concentration ratio (RB) of YM796 in humans. RB of YM796 was determined with heparinized whole blood (Lin et al., 1982). To 1-ml aliquots of human blood preincubated at 37°C, 20-µl aliquots of phosphate-buffered isotonic solution containing [14C]YM796 were added to give concentrations of 0.5, 50 and 2500 µM. After incubation for 5 min at 37°C, the blood samples were centrifuged for 5 min at 1,500 × g, and then aliquots of plasma were subjected to liquid scintillation counting with 10 ml of liquid scintillator.

Prediction of AUC or Fh of YM796 under linear conditions in humans from in vitro metabolic data. CLh under linear conditions was calculated by use of the CLint,all values obtained from in vitro studies. The following equations based on the dispersion model (Roberts and Rowland, 1986a; Sugiyama et al., 1988) were used:

\[
CLh = Qh(1 - Fh)
\]

\[
Fh = \frac{4a}{(1 + a + \exp((a - 1)2Dh)) - (1 - a + \exp(-(a + 1)2Dh))}
\]

where

\[
a = (1 + 4Rc \cdot D3)^{1/2}
\]

in which

\[
R_N = (f_p/R_h) \cdot CL_{int,all}/Q_h
\]

CLint,all is calculated from the \(K_m\) and \(V_{max}\) values obtained in vitro as follows:

\[
CL_{int,all} = \sum_{i} \frac{V_{max,i}}{K_{m,i}}
\]

The CLint,all values estimated from the in vitro experiments with human liver microsomes were expressed per gram of liver by taking into account the mass recovery of CYP (Iwatsubo et al., 1996, 1997). A \(Q_h\) value of 0.95 ml/min/g liver (Bischoff et al., 1971; Dedrick et al., 1973; Montandon et al., 1975) and a dispersion number (D3) of 0.17 (Roberts and Rowland, 1986b; Iwatsubo et al., 1996, 1997) were used for all calculations of CLh. The \(f_p\) and \(R_h\) values of YM796 used for equation 4 were 0.700 (±0.002) and 1.11 (±0.07) obtained at YM796 concentrations ranging from 0.5 to 2500 µM, respectively. Thus, the oral clearance (CLoral) was calculated from equation 6 by use of the CLh and Fh values under linear conditions as estimated above, together with the CLh value (72.7 ml/min) obtained from the urinary excretion data for the parent drug in humans.

\[
CL_{oral} = (CL_h + CL_a + F_h \cdot CL_2)/(F_h \cdot F_g \cdot F_h)
\]

where \(F_h\), \(F_g\) and \(CL_2\) represent the fraction absorbed from the intestinal tract, intestinal availability and clearance for intestinal metabolism, respectively. Taking into consideration the results obtained from the experiments in rats showing that the fraction of unchanged YM796 absorbed from the intestinal tract, estimated from the difference in plasma concentrations between the circulating arterial blood and portal vein blood after administration of YM796 into the intestinal loop, was close to unity and the fact that YM796 was not metabolized by microsomes from the small intestine, we assumed that \(F_h \cdot F_g\) was unity and CLg was negligible (close to 0) for all calculations in the present study.

AUC of YM796 after oral administration in humans. Six healthy male volunteers were enrolled in the study and admitted to the Kitasato University School of Medicine. The protocol had been approved by the Institutional Review Board and written consent was obtained from each of the subjects before the study. All subjects were given YM796 orally in a capsule form (lactose triturated powder) at a dose of 5 mg (14.3 µmol). Blood samples were collected from the antecubital vein with a heparinized syringe before dosing and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h postdose. After centrifugation, plasma was separated and stored at −20°C until assay. An aliquot of plasma (2.5 ml) was buffered with 0.5 ml saturated sodium bicarbonate solution after addition of 0.1 ml internal standard aqueous solution, and the resulting mixture was stirred and applied to a disposable column (Chem Elute, Analytichem International, Harbor City, CA) for liquid-liquid extraction. YM796 was extracted by passing 4 ml dichlorehane through the column twice. The extract was evaporated to dryness under reduced pressure, then the residue was dissolved in 0.5 ml of 0.1 N hydrochloric acid and washed with 8 ml diethyleneether. After stirring and centrifugation, the upper layer (ether) was discarded. To the aqueous layer, 1 ml saturated sodium bicarbonate solution was added and YM796 was extracted from the resulting mixture by use of 7 ml dichlorehane. After stirring and centrifugation, the aqueous layer was discarded and the organic layer was evaporated to dryness. The residue was dissolved in chloroform, and a small aliquot (25 µl) was injected into the GC-MS/MS system that was performed on a Finnigan MAT (San Jose, CA) TSQ70 triple quadrupole mass spectrometer connected to the gas chromatograph (Varian 3400). Gas chromatography was performed on a phenylmethyl silicone capillary column (DB-17, 15 m × 0.25 mm internal diameter, 0.25 µm, J&W Scientific, Folsom, CA). The column temperature was raised from 50°C to 242°C at a rate of 32°C/min. The sheath (nebulizing) gas pressure and auxiliary nitrogen flow were set at 70 p.s.i. (approximately 4.8 × 105 Pa) and 20 ml/min, respectively. Chemical ionization was performed in the reaction gas (methane) at an ionization voltage of 100 V. The mass spectrometer was set to admit positively charged protonated molecules [M+H]⁺ at m/z 182 (YM796) and m/z 196 (internal standard) via the first quadrupole filter (Q1) with collision-induced fragmentation in Q2 (collision gas argon, −25 eV, 1.5 mTorr (approximately 0.20 Pa)) and monitoring, via Q3, the production of fragments m/z 96 and m/z 110 for YM796 and its internal standard, respectively. Each selected reaction was monitored with a dwell time of 0.2 s.

Data analysis. The kinetic data for YM796 metabolism obtained in human liver microsomes and recombinant microsomes for human CYP3A4, respectively, were fitted to the following equations with use of MULTI (Yamaoka et al., 1981).

\[
v = V_{\text{max1}} \cdot S/(K_{\text{m1}} + S) + V_{\text{max2}} \cdot S/(K_{\text{m2}} + S) + CL_{\text{int}} \cdot S
\]

\[
v = V_{\text{max1}} \cdot S/(K_{\text{m1}} + S) + V_{\text{max2}} \cdot S/(K_{\text{m2}} + S)
\]

Fitting evaluation was carried out based on the Akaike’s information criterion value (Akaike, 1969).

In addition, the metabolic clearance in human liver microsomes was predicted from parameters obtained in recombinant microsomes by taking into consideration the CYP3A4 content in both microsomal preparations and by converting \(V_{\text{max1}}\) and \(V_{\text{max2}}\) given as per milligram recombinant MS protein in equation 8 to \(V_{\text{max1}}\) and \(V_{\text{max2}}\) given as per milligram human liver MS protein with the following equations, respectively:

\[
V_{\text{max1}'} = \frac{\text{CYP3A4 content/mg human liver MS protein}}{\text{CYP3A4 content/mg recombinant MS protein}} \cdot V_{\text{max1}}
\]

\[
V_{\text{max2}'} = \frac{\text{CYP3A4 content/mg human liver MS protein}}{\text{CYP3A4 content/mg recombinant MS protein}} \cdot V_{\text{max2}}
\]
Results

YM796 metabolism in human liver microsomes. Eadie-Hofstee plots for the formation of total metabolites of YM796 in three representative human liver microsomal samples which contain high (H-62; 148 pmol/mg MS protein), moderate (H-51; 51.0 pmol/mg MS protein) and low (H-57; 19.7 pmol/mg MS protein) amounts of CYP3A4 are shown in figure 1. For all microsomes, the formation of YM796 metabolites could be described by three components: high-affinity with low-capacity, low-affinity with high-capacity and nonsaturable components. Table 1 summarizes each kinetic parameter obtained by fitting analysis for all of 12 microsomes used in the present study. The mean $K_m$ and $V_{max}$ values for the high- and low-affinity components, respectively, were as follows: $K_m$ = 1.67 μM and $V_{max}$ = 0.0239 nmol/min/mg MS protein; and $K_m$ = 654 μM and $V_{max}$ = 1.51 nmol/min/mg MS protein (table 1). The clearance of the nonsaturable component (CL ns) was 0.00123 ml/min/mg MS protein. Under linear conditions where the YM796 concentration was much less than $K_m$, the fractional clearance of each component to CLint, all was 80.4, 13.0 and 6.6%, respectively. When CLint, all was expressed per nanomole of CYP3A4 instead of per milligram of MS protein, the CLint, all values estimated in the 12 human liver microsomal samples under linear conditions showed smaller interindividual variabilities irrespective of more than a 7-fold interindividual difference in CYP3A4 content (table 1).

At least four different metabolites of YM796 were detectable by TLC for each microsomal sample. The $R_f$ values of YM796 and each metabolite (M1–M4) were 0.52 and 0.12, 0.19, 0.39 and 0.45, respectively. Two of them (M1 and M2) were major and accounted for approximately 50 and 30% of the total metabolite formation, respectively (fig. 2). Eadie-Hofstee plots for the formation of M1 and M2 in the same three human liver microsomal samples as mentioned above are also shown in figure 1. For both metabolites, the metabolic pattern could be described by three components as well as the total metabolites, and the high-affinity component accounted for 80% of the CLint, all under linear conditions. The $K_m$ values estimated by fitting analysis for total metabolites, M1 and M2 were 1.94, 2.13 and 2.80 μM for H-51, 1.88, 1.13 and 2.09 μM for H-57 and 1.70, 1.03 and 2.16 μM for H-62, respectively, showing no marked difference in the $K_m$ values among metabolites in each microsomal sample. The contribution of each component to the CLint, all under the linear conditions was 71.6 to 89.2%, 9.9 to 15.0% and 0.8 to 13.7% for total metabolites, 78.8 to 90.7%, 5.8 to 11.3% and 3.0 to 10.5% for M1 and 69.6 to 90.0%, 3.7 to 14.9% and 1.5 to 15.5% for M2, showing no pronounced differences among the metabolites.

Identification of CYP isozyme(s) responsible for YM796 metabolism. Significant correlations were obtained between the CYP3A4 content and the CLint, all, the high-affinity component clearance ($V_{max1}/K_m$) or the low-affinity component clearance ($V_{max2}/K_m$) for the 12 human liver microsomal samples as shown in figure 3 (r = 0.917, 0.851 or 0.928, respectively). Furthermore, a significant correlation with $V_{max1}$ or $V_{max2}$ was obtained (table 2). Figure 4 shows the formation clearance for total metabolites over a wide range of YM796 concentrations in the recombinant human CYP isozymes (i.e., CYP1A2, 2C9, 2D6, 2E1 and 3A4). A high metabolic activity was observed only with the recombinant CYP3A4. In addition, antibodies to human CYP3A4/5 inhibited the formation of total metabolites of YM796 by approximately 75% (fig. 5). Similar inhibitory effects were observed for the formation of M1 and M2 as well as total metabolites. Ketoconazole, an inhibitor of CYP3A4, also inhibited YM796 metabolism in a concentration-dependent manner, and the inhibition was almost complete at 10 μM (fig. 6). The formation of M1 and M2 was also inhibited in a concentration-dependent manner by ketoconazole with a complete inhibition at 10 μM.

YM796 metabolism by recombinant human CYP3A4. Eadie-Hofstee plots for the formation of total metabolites of YM796 in the recombinant human CYP3A4 are shown in figure 7. They could be described by two components, one with high affinity and another with a very low affinity. The respective $K_m$ and $V_{max}$ values were 1.10 μM and 0.0160 nmol/min/mg protein, and 10.9 mM and 8.98 nmol/min/mg protein. The high-affinity component was more important under the linear conditions where YM796 concentrations were much lower than $K_m$. The $K_m$ value for the high-affinity component was similar to that obtained with human liver microsomal samples. With use of the recombinant human CYP3A4, the testosterone-6β-hydroxylation activity increased by about 2- to 3-fold after the addition of CYP reductase or cytochrome b5, whereas YM796 metabolism was unaffected (fig. 8).

Prediction of CLint, all in human liver microsomes from the recombinant data. The intrinsic metabolic clearances were calculated from the kinetic parameters obtained by use of the recombinant human CYP3A4. To predict the intrinsic metabolic clearance for each human liver microsomal sample, the CYP3A4 content of both the recombinant microsomes and individual human liver microsomal samples were taken into consideration. Shown as the dotted lines in figure 1, the predicted values were similar to the observed values despite the fact that there was more than a 7-fold interindividual difference in the CYP3A4 content of the human liver microsomal samples, which suggests that the intrinsic clearance in liver microsomes could be predicted with a reasonable accuracy from the correspondent recombinant data.

Prediction of AUC or $F_h$ of YM796 under the linear conditions in humans from in vitro metabolic data. To correlate the metabolic clearance determined in vitro with that in vivo, $f_p$ and $R_B$ of YM796 were determined. The $f_p$ values were almost constant despite the concentrations, being 69.8, 70.2 and 70.0% at 0.5, 50 and 2500 μM YM796, respectively. The $R_B$ values were 1.10, 1.18 and 1.05 at 0.5, 50 and 2500 μM YM796, respectively, which showed no concentration dependence. The individual data are summarized in table 1. The CLint, all values obtained for the 12 human liver microsomal samples were 0.94 ± 0.52 ml/min/g liver (mean ± S.D.). The predicted values of the AUCoral of YM796 corresponding to the aforementioned intrinsic clearances were 19.0 ± 14.6 nmol · min/ml (n = 12), which were similar to the observed values (20.2 ± 7.1 nmol · min/ml, n = 6) (tables 1 and 3). The hepatic availabilities were also predicted to be 0.647 ± 0.152. The coefficient of variation was smaller for CLint, all per nanomole of CYP3A4 than that for CLint, all per gram of liver (table 1).
Discussion

Eadie-Hofstee plots for the total metabolite formation of YM796 derived from each of the 12 different human liver microsomal samples showed that multiple metabolic components were responsible for the YM796 metabolism. Thus, the following three models were considered for the data fitting: i) one saturable and one nonsaturable component (equation 11), ii) two saturable components (equation 12) and iii) two saturable components and one nonsaturable component (equation 13).

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} + \text{CL}_{\text{n}} \cdot S \]  

\( (11) \)
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<td>0.30</td>
<td>1.10</td>
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<td>2.26</td>
<td>1.51 ± 1.09 (72.2)%</td>
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<td>0.00043</td>
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<td>0.0209</td>
<td>0.0194 ± 0.0107 (55.2)%</td>
<td></td>
</tr>
<tr>
<td>CYTP3A4 content (nmol/mg)</td>
<td>0.036</td>
<td>0.071</td>
<td>0.041</td>
<td>0.032</td>
<td>0.051</td>
<td>0.051</td>
<td>0.140</td>
<td>0.020</td>
<td>0.148</td>
<td>0.145</td>
<td>0.114</td>
<td>0.051</td>
<td>0.075 ± 0.048 (54.0)%</td>
</tr>
<tr>
<td>CLint,all/CYP3A4 (ml/min/nmol CYTP3A4)</td>
<td>0.489</td>
<td>0.296</td>
<td>0.210</td>
<td>0.239</td>
<td>0.240</td>
<td>0.302</td>
<td>0.231</td>
<td>0.204</td>
<td>0.272</td>
<td>0.199</td>
<td>0.203</td>
<td>0.410</td>
<td>0.275 ± 0.090 (32.7)%</td>
</tr>
<tr>
<td>VM1/Km1/CLint,all</td>
<td>0.906</td>
<td>0.792</td>
<td>0.900</td>
<td>0.697</td>
<td>0.860</td>
<td>0.892</td>
<td>0.817</td>
<td>0.716</td>
<td>0.805</td>
<td>0.702</td>
<td>0.714</td>
<td>0.844</td>
<td>0.804 ± 0.080 (10.0)%</td>
</tr>
<tr>
<td>VM2/Km2/CLint,all</td>
<td>0.044</td>
<td>0.117</td>
<td>0.050</td>
<td>0.238</td>
<td>0.098</td>
<td>0.099</td>
<td>0.161</td>
<td>0.147</td>
<td>0.150</td>
<td>0.145</td>
<td>0.169</td>
<td>0.143</td>
<td>0.130 ± 0.053 (40.8)%</td>
</tr>
<tr>
<td>CLint,all/CLint,all (ml/min/g liver)</td>
<td>0.051</td>
<td>0.090</td>
<td>0.050</td>
<td>0.065</td>
<td>0.041</td>
<td>0.008</td>
<td>0.022</td>
<td>0.137</td>
<td>0.045</td>
<td>0.153</td>
<td>0.117</td>
<td>0.013</td>
<td>0.066 ± 0.048 (72.7)%</td>
</tr>
<tr>
<td>CLint,all/CYP3A4 (ml/min/g liver)</td>
<td>0.86</td>
<td>1.03</td>
<td>0.42</td>
<td>0.38</td>
<td>0.59</td>
<td>0.75</td>
<td>1.58</td>
<td>0.20</td>
<td>1.96</td>
<td>1.41</td>
<td>1.13</td>
<td>1.02</td>
<td>0.94 ± 0.52 (72.7)%</td>
</tr>
<tr>
<td>AUCoral (predicted) (nmol · min/ml)</td>
<td>14.6</td>
<td>12.1</td>
<td>30.0</td>
<td>33.0</td>
<td>21.3</td>
<td>16.9</td>
<td>7.3</td>
<td>56.5</td>
<td>5.5</td>
<td>8.3</td>
<td>10.8</td>
<td>12.1</td>
<td>19.0 ± 14.6 (55.3)%</td>
</tr>
<tr>
<td>Bioavailability (predicted) (predicted)</td>
<td>0.650</td>
<td>0.603</td>
<td>0.807</td>
<td>0.824</td>
<td>0.738</td>
<td>0.685</td>
<td>0.472</td>
<td>0.903</td>
<td>0.398</td>
<td>0.506</td>
<td>0.574</td>
<td>0.604</td>
<td>0.647 ± 0.152 (23.5)%</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the coefficient of variation as a percentage.
* The CLint,all values per g liver were calculated assuming 48.8 mg microsomal protein per g liver.
* The bioavailability was predicted assuming that the fraction absorbed was unity, and no first-pass metabolism occurred in the gut.
caused by smoking or other environmental factors and an extrinsic component caused by a reduction in the metabolic activity during storage or the time to remove the liver from the body. It is very important to discriminate between these two variability factors. If the variability is accounted for by the intrinsic rather than extrinsic nature, the enzyme activity should be expected to correlate well with the amount of antigen in a series of liver specimens. In this study, a good correlation was observed between the CL_int, all of YM796 and the CYP3A4 content, which indicates that the variability in the metabolism of YM796 observed among the 12 liver microsomal samples used might have been predominantly intrinsic in nature. Indeed, good correlations have been observed previously between the CYP3A4 content and metabolic activity in human liver microsomes for typical substrates of the enzyme such as nifedipine, testosterone and lidocaine (Sesardic et al., 1988; Imaoka et al., 1990). Thummel et al. (1994) have reported that both the in vitro metabolic clearance of midazolam estimated by use of S-13 samples prepared from liver biopsies and the in vivo clearance of the same drug correlate well with the CYP3A content of the individual livers, independently of any large interindividual variability in the metabolic clearance. Furthermore, the absolute values of both in vitro and in vivo clearances were also similar to each other. All of these results suggest that it is possible to predict the in vivo clearance if the amount of CYP isozyme responsible for the metabolism of a drug is known, and if the interindividual variability in the metabolic clearance is caused predominantly by intrinsic factors.

During development of a new drug, it is important to predict its bioavailability in humans. This is particularly true for drugs exhibiting a nonlinear bioavailability, such as propranolol (Suzuki et al., 1974). It is also essential to predict nonlinearity at an early stage during the development of a drug because a nonlinear kinetic behavior can generally cause a large interindividual variation in its plasma concentrations. As a method for achieving this end, we proposed a method to predict in vivo clearance from in vitro kinetic parameters ($K_m, V_{max}$) (Iwatsubo et al., 1997). In the present study, we attempted to estimate AUC_oral of YM796 by predicting the in vivo CL_oral from the in vitro metabolism data. In predicting the in vivo CL_oral, it is necessary to use a mathematical model to describe drug concentrations in the liver in vivo. The most frequently used are the well-stirred model, parallel-tube model and dispersion model. It has been reported that there is little difference in the predicted values of $F_p$ and CL_oral among the models as far as low-clearance drugs are concerned, whereas pronounced differences are seen among the models for high-clearance drugs, especially in $F_p$ (Rane et al., 1977; Iwatsubo et al., 1997). Although YM796 generally is a relatively low-clearance drug in humans, a large interindividual variability in the metabolic clearance is observed among the liver samples. Therefore, in our study, we used the dispersion model which has been reported to predict the hepatic availability and clearance accurately from in vitro data for many drugs, despite the extent of clearance in rats (Roberts and Rowland, 1986a; a; Sugiyama et al., 1988). Although the most appropriate value of $D_N$ will not always be the same for all drugs and between rats and humans, a $D_N$ of 0.17 was assumed in the present study because the in vivo intrinsic clearance of various types of drug known to be metabolized by cytochrome P-450, which

\[
v = V_{max1} \cdot S/(K_{m1} + S) + V_{max2} \cdot S/(K_{m2} + S) + CL_{ns} \cdot S \tag{12}
\]

\[
v = V_{max1} \cdot S/(K_{m1} + S) + V_{max2} \cdot S/(K_{m2} + S) + CL_{ns} \cdot S \tag{13}
\]

When the data obtained with an arbitrary 3 of the 12 human liver microsomal samples (H-35, H-38 and H-62) were fitted to the three models above, the mean and the standard deviation of the calculated Akaike’s information criterion were $-8.86 \pm 1.83$, $-20.9 \pm 2.6$ and $-23.0 \pm 4.2$, respectively, which indicates that equation 13 gave the best fit of the data. Thus, the metabolism data on YM796 were all analyzed based on the three-component model (equation 13) for each microsomal sample. The contribution of each component under the linear conditions was 80.4, 13.0 and 6.6%, respectively, with the high-affinity component being the most important (table 1). Even if the data analysis was performed for individual metabolites (M1 and M2), the contribution of each component was similar to that found in the total metabolites. The contribution of the high-affinity component was the most important in all cases, and there were no marked differences in the $K_m$ values among the metabolites. In addition, the inhibition pattern of M1 and M2 formation by antibodies to human CYP3A4/5 or by ketoconazole was also very similar to that for the total metabolites, which suggests that the formation of the major YM796 metabolites is mediated predominantly by CYP3A4 as the metabolic reaction with almost the same $K_m$ values.

As shown in figure 3, a good correlation was observed between the CL_int, all and the CYP3A4 content of each of the 12 liver microsomal samples, even though there was a greater than 7-fold difference in the interindividual variability in CYP3A4 content. Thus, a large interindividual variability in the capacity of drug metabolism was suggested in humans. Factors which produce such interindividual variability can be classified into an intrinsic component caused by genetic polymorphism, disease or enzyme-induction.
have been calculated from the literature data involving \textit{in vivo} pharmacokinetics based on the dispersion model assuming this D\textsubscript{N} value, was similar to those calculated from \textit{in vitro} metabolism data reported previously (Iwatsubo \textit{et al.}, 1997). The predicted values of AUC\textsubscript{oral} at a dose of 0.24 \textmu mol/kg were 19.0 \pm 14.6 \text{ nmol} \cdot \text{min} / \text{ml} (n = 12), which were similar to the observed values (20.2 \pm 7.1 \text{ nmol} \cdot \text{min} / \text{ml}, n = 6) (tables 1 and 3). There were interindividual differences (35.1\% variation) in the observed values of AUC\textsubscript{oral} among the subjects \textit{in vivo}. Such interindividual differences have also been shown for the AUC\textsubscript{oral} values (76.8\% variation) predicted from individual \textit{in vitro} metabolic clearances (tables 1 and 3). Also, for CL\textsubscript{int, all} per gram liver and the CYP3A4 content of each microsomal sample, similar variations (55.3\% and 64.0\%, respectively) were observed (table 1). When the CL\textsubscript{int, all} was expressed per nanomole of CYP3A4 by taking into account the CYP3A4 content of each liver microsomal sample, the interindividual difference was greatly reduced (table 1), which indicates that the interindividual difference in the predicted AUC\textsubscript{oral} values would be attributable to a large interindividual variation in the CYP3A4 content of the liver used. Because 12 human livers with wide interindividual differences in CYP3A4 contents were selected in the present study for examining the correlation between CYP3A4 contents and the metabolic activi-
ties, the results from only 12 livers may not be appropriate to discuss the interindividual variability. We therefore examined the CYP3A4 contents and the variabilities with use of the randomly selected 26 livers which had been stored at SRI. The mean ± S.D. of CYP3A4 contents (n = 26) were 0.072 ± 0.038 nmol/mg MS protein (table 3), and the coefficient of variation (52.8%) was smaller than that from 12 livers (table 1). These contents and variations were similar to those (0.096 ± 0.051 nmol/mg MS protein and 53.1%) reported by Shimada et al. (1994) for 60 livers. We then attempted to predict the mean ± S.D. of the AUCoral value based on the CYP3A4 contents thus obtained and the variation of 26 livers (table 3). In this prediction, the CLint, all (0.275 ml/min/nmol CYP3A4) value obtained from 12 livers was used. The predicted AUCoral value (16.8 ± 9.8) was similar to that (20.2 ± 7.1) obtained from the in vivo human study and the predicted variation (58.3%) became closer to the variation in vivo (35.1%) (table 3). These analyses indicate that the interindividual variation in CYP3A4 can cause such large interindividual differences in the plasma concentrations or AUC of YM796.

The present work demonstrates that it may be possible to predict the in vivo metabolic clearance from in vitro human liver microsomal samples if the CYP isozyme(s) responsible for the metabolism of a drug is identified and its concentration in liver samples is determined. In the same manner, for drugs which are substrates toward CYP isozyme(s) other than CYP3A4, previous reports suggest that the metabolic activity of human liver microsomes correlates well with the liver concentration of the CYP isozyme involved (Sesardic et al., 1988; Shimada et al., 1994; Goldstein et al., 1994). Hence, the method for predicting in vivo clearance used in this study may also be applicable to isozyme(s) other than CYP3A4 that are involved in the metabolic pathway(s) of a drug. Furthermore, it may also be possible to estimate the degree of any intersubject differences in the plasma concentrations or AUC of a drug in vivo based on the range of interindividual variation in the intrinsic metabolic clearance or the liver concentration of metabolic enzymes.

As shown in figure 7, biphasic metabolite formation kinetic values were observed for YM796 in the recombinant microsomes. One possible explanation for this phenomenon is that in the expression process of CYP3A4, after the correspondent gene was transfected into the donor cells, two kinds of conformation were possible where the distance of the binding site for the drug from the surface of the membrane of recombinant microsomes was different, resulting in multiplicity in the affinity of the enzyme for the drug. Considering that the high-affinity component was more important under linear conditions where YM796 concentrations were much lower than Kmax, and that the Kmax value for the high-affinity component was similar to that obtained with human liver microsomal samples, the prediction of in vivo metabolic clearance from in vitro recombinant human CYP isozymes as an alternative to human liver microsomes may be also possible in some cases. As shown in figure 1, the predicted values for the metabolic clearance in human liver microsomes calculated from kinetic data (Km, Vmax) in the recombinant CYP3A4 by reconciling the CYP3A4 content per gram liver were similar to the observed values, regardless of a large difference in the absolute CYP3A4 content, which thus suggests the usefulness of the recombinant system for predicting metabolic clearance in human liver microsomes. This approach for predicting in vivo metabolic clearance from in vitro metabolism data with human liver microsomes, therefore, may also be applicable to the prediction of in vivo clearance with recombinant human CYP isozymes if the metabolism of the drug is almost completely caused by the particular isozyme, the variation in P-450 content of human liver is known and the experimental conditions such as the amount of CYP reductase and cytochrome b5 are carefully optimized to mimic the activity found in native microsomes, as for YM796.

Attention should be paid to the following points, however, if the recombinant CYP3A4 is used for in vitro metabolism experiments. In the recombinant system, the amounts of enzymes such as CYP reductase and cytochrome b5 differ from those found in human livers. In most cases, the amounts of these enzymes are less in the recombinant system. Therefore, it may be necessary to add these proteins to the recom
binant system to obtain the sufficient metabolic activity. For nifedipine and testosterone, which was used as a positive control in this study, metabolic activity is markedly increased by the addition of CYP reductase and cytochrome b5 (Nagata et al., 1990; Renaud et al., 1990). In contrast, as shown in figure 8, the metabolism of YM796 was unaffected by external P-450 reductase and cytochrome b5, although the testosterone metabolism was influenced to a great extent. Thus, attention should be paid to whether the metabolic activity is affected or not by these added enzymes depending on the substrate drugs used. Recently, a recombinant system expressing sufficient amounts of both CYP reductase and cytochrome b5, as well as CYP isozyme, has been developed. This recombinant system is expected to be helpful in predicting not only metabolic clearance in human liver microsomes but also in vivo CL\textsubscript{h}. However, YM796 used in the present study is metabolized mostly by CYP3A4 in humans and similar experiments are expected to be carried out soon on sev-

![Fig. 8. Effects of cytochrome P-450 reductase or b5 on testosterone 6β-hydroxylation (A and C) and YM796 metabolism (B and D) in the recombinant system. In (A), the amount of NADPH-cytochrome P-450 reductase used was 5–40 U/nmol P-450. The incubation time was 10 min, and the final testosterone concentration was set at 250 μM. In (B), the amount of NADPH-cytochrome P-450 reductase used was 5–40 U/nmol P-450. The incubation time was 2 min and, the final YM796 concentration was set at 1 μM. In (C), the amount of cytochrome b5 used was 0.5–8.0 nmol/nmol P-450. The incubation time was 10 min, and the final testosterone concentration was set at 250 μM. In (D), the amount of cytochrome b5 used was 0.5–8.0 nmol/nmol P-450. The incubation time was 2 min, and the final YM796 concentration was set at 1 μM.](image)

| TABLE 3 | In vivo observed AUC\textsubscript{oral}, predicted AUC\textsubscript{oral} and bioavailability based on the in vitro metabolism data |
|-----------------|-----------------|-----------------|-----------------|
| Human in vivo observed value (n = 6) | AUC\textsubscript{oral} (observed) (nmol ⋅ min/ml) | 20.2 | 7.1 | 35.1 |
| Prediction from human livers (n = 26) | CYP3A4 content (nmol/mg protein) | 0.072 | 0.038 | 52.8 |
| | CL\textsubscript{int,all} (ml/min/g liver) | 0.97 | 0.51 | 52.6 |
| | Bioavailability | 0.639 | 0.124 | 19.4 |
| | AUC\textsubscript{oral} (nmol ⋅ min/ml) | 16.8 | 9.8 | 58.3 |
eral drugs to examine whether the prediction of in vivo metabolic clearance from in vitro data obtained by using recombinant human P-450 isozymes is also possible when the object drug is metabolized by P-450 isozymes other than CYP3A4 or when the drug is metabolized by multiple P-450 isozymes, which is a very common situation.

In conclusion, the present study with YM796 as a model drug suggests that it may be possible to predict quantitatively the in vivo metabolic clearance of a target drug from in vitro metabolism experiments with use of human liver microsomes. In addition, for some drugs whose metabolism is mediated mainly by a particular human P-450 isozyme, like YM796, a recombinant human CYP isozyme system may also be applicable for predicting in vivo metabolic clearance by taking into account the isozyme content of each liver sample after the responsible isozyme is identified.

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References


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