CORRELATION BETWEEN IN VIVO AND IN VITRO HEPATIC UPTAKE OF METABOLIC INHIBITORS OF CYTOCHROME P-450 IN RATS

KATSUHIRO YAMANO, KOJIROU YAMAMOTO, HAJIME KOTAKI, SAYURI TAKEDOMI, HIROTAMI MATSUO, YASUFUMI SAWADA, AND TATSUJI IGA

Department of Pharmacy, University of Tokyo Hospital, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan (K.a.Y., T.I.); Biopharmaceutical and Pharmacokinetic Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Kashima, Yodogawa-ku, Osaka, Japan (K.a.Y.); Department of Clinical Pharmacology School of Medicine, Gunma University, Showa-machi, Maebashi, Japan (Ko.Y.); Department of Pharmacy, The Research Hospital, The Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan (H.K.); Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan (S.T., H.M., Y.S.)

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ABSTRACT:

To predict the degree of accumulation of hepatic metabolic inhibitors in the liver from the in vitro data, we investigated the relationship between cell/medium concentration ratios (C/M ratios) in isolated rat hepatocytes and liver/blood unbound concentration (K_{Bu}) after i.v. administration of various metabolic inhibitors such as itraconazole, ketoconazole, verapamil, diltiazem, enoxacin, ciprofloxacin, clarithromycin, cimetidine, and nizatidine. The C/M ratios of itraconazole were ~6000 and 200 at the concentrations of 0.1 and 10 μg/ml, respectively, and the uptake of ketoconazole and verapamil into the hepatocytes also showed a concentration dependence, although the degree was smaller than that of itraconazole. The uptake of diltiazem, enoxacin, ciprofloxacin, and clarithromycin into the hepatocytes showed linear profiles on concentration dependence. There was an excellent correlation between C/M ratios and K_{Bu} values of all nine drugs with a slope of 1. This finding suggested the possibility of predicting drug concentrations in the liver (C_{H}) from C/M ratios, the blood concentrations of drugs (C_{B}) and unbound fraction in blood (f_{B}), which was expressed by C_{H} = (C/M) · C_{B} · f_{B}. It may be possible to predict the drug concentrations in human liver from K_{Bu} values estimated with isolated human hepatocytes and concentrations in the blood in a similar manner as in rats.

In clinical cases, many adverse effects on drug-drug interactions have been reported. The metabolic inhibition of one drug by another in the liver and/or gut is one of the most important events among pharmacokinetic drug-drug interactions (Fee et al., 1987; Olkkola et al., 1993, 1994, 1996; Backman et al., 1994; Ahonen et al., 1995; Baldwin et al., 1995). We can estimate the degree of interactions quantitatively to some extent if the metabolic inhibition constants are obtained by using liver microsomes, primary cultured hepatocytes, and/or CYP-expressed cells (Richard et al., 1990; Gascon and Dayer, 1991; Wrighton and Ring, 1994; Ghosal et al., 1996). However, the predicted increase ratios of area under the concentration-time curve (AUC)\(^1\) of the interacting drugs were sometimes much underestimated by using the unbound concentration in plasma as the concentration of inhibitors in vivo. We could predict the increase ratio of the concentration (or AUC) of midazolam (MDZ) in the plasma by the metabolic inhibition from in vitro experiments quantitatively, when itraconazole (ITZ) and ketoconazole (KTZ),azole antifungal agents, and cimetidine (CIM) and nizatidine (NIZ), histamine H\(_2\) receptor antagonists, were concomitantly administered as inhibitors (Takedomi et al., 1998; Yamano et al., 1999). The predicted values were considerably underestimated by using unbound concentrations in the plasma as concentrations of inhibitors near the metabolic enzymes, whereas the predicted values with unbound concentrations in the liver were very close to the observed values, suggesting the necessity to take account of the concentrative uptake of inhibitors into liver. In a clinical situation, the concentrations of the inhibitors in human liver are required to predict the increase ratio of the concentration; however, it is difficult to measure drug concentration in the liver directly. It is thus necessary to develop a methodology to estimate the concentration in the liver from the concentration in the plasma, which can be measured actually. In this study, we tried to predict the concentration in the liver with in vitro uptake data into isolated hepatocytes in rats. We examined the correlation between cell/medium concentration ratios (C/M ratios) and the liver/blood unbound concentration (K_{Bu}) values of liver in rats.

**Experimental Procedures**

Materials. ITZ and KTZ were supplied by Janssen-Kyowa Co. (Tokyo, Japan). Enoxacin (ENX) and ciprofloxacin (CPFX) were supplied by Dainippon Pharmaceutical Corp. (Osaka, Japan). Clarithromycin (CAM) was sup-
Applied by Taisho Pharmaceutical Co. (Tokyo, Japan). Verapamil (VER) hydrochloride and diltiazem (DLZ) hydrochloride were purchased from Wako Pure Pharmaceutical Co. (Osaka, Japan). All other chemicals used as reagents were of reagent grade and reagents for HPLC.

Animals. Sprague-Dawley male rats (230–260 g) were purchased from Nippon Bio-Supply Center (Tokyo, Japan). The rats were allowed access to water and food pellets ad libitum.

Preparation of Drug Solutions. ENX and CPFX were dissolved in a small volume of 1 N NaOH, neutralized with a small volume of 0.5 N HCl, and then diluted with saline to prepare 5 mg/ml ENX or CPFX solutions. VER and DLZ were dissolved in saline to prepare 5 and 20 mg/ml VER or DLZ solutions. CAM was dissolved in equimolar HCl, neutralized with a small volume of 0.1 N NaOH, and then diluted with saline to prepare 5 mg/ml of solution.

Concentrations in Liver and Plasma (Blood) and Liver/Blood (Unbound) Concentration Ratios. Under light ether anesthesia, rats were cannu- lated through the femoral vein and artery. After recovery from the anesthesia, ENX (10 mg/kg), CPFX (10 mg/kg), CAM (10 mg/kg), VER (5 mg/kg), or DLZ (5 mg/kg) was administrated by bolus injection through the femoral vein. At 2, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min after the administration of each drug, blood samples were collected from the femoral artery and were centrifuged at 12,000 rpm for 2 min to obtain the plasma. The liver was then removed at 180 min after the administration and the plasma and liver were stored at −20°C until analyzed. The liver was homogenated with 4 volumes of ice-cold distilled water. The concentrations of each drug in the plasma and liver were determined by the methods described later, and the liver/plasma concentration ratios at 180 min after the administration were regarded as the apparent liver/plasma concentration ratio ($K_{Pl}$) values.

Plasma concentration profiles were analyzed by fitting the following biexponential equation with the nonlinear least-squares method (MULTI) (Yamaoka et al., 1981):

$$C_p = A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t)$$  

(1)

Pharmacokinetics parameters were calculated according to the following equations:

$$AUC_{0-\infty} = A/\alpha + B/\beta$$  

(2)

$$CL_{int} = \text{Dose/AUC}$$  

(3)

$$T_{1/2b} = 0.693/\beta$$  

(4)

where $AUC_{0-\infty}$, $CL_{int}$, and $T_{1/2b}$ are AUC from zero to infinity, total body clearance, and half-life in β phase, respectively.

Additionally, for VER, DLZ, ENX, CPFX, and CAM, the real $K_P$ values were calculated by correcting the apparent $K_P$ values as follows. Rats were cannu- lated in the femoral artery and portal vein under light ether anesthesia. After recovery from the anesthesia, ENX (10 mg/kg), CPFX (10 mg/kg), CAM (10 mg/kg), VER (20 mg/kg), or DLZ (20 mg/kg) was administrated by bolus injection through the portal vein. Blood samples were collected at 2, 5, 10, 20, 30, 45, 60, 90, 120, and 180 min after the administration of each drug and drug concentrations in the plasma and liver were determined as described above. The pharmacokinetic parameters were calculated by the nonlinear least-squares method (MULTI) (Yamaoka et al., 1981). The hepatic extraction ratios (E) were calculated from AUC after i.v. administration ($AUC_{i.v.}$) and AUC after intra-portal administration ($AUC_{i.p.}$) regarding as eq. 5:

$$E = 1 - (Dose_{i.v} \cdot AUC_{i.v.})/(Dose_{i.p} \cdot AUC_{i.p.})$$  

(5)

The hepatic intrinsic clearances ($CL_{int}$) were calculated from eq. 6:

$$CL_{int} = Q_H \cdot E/\beta (1 - E)$$  

(6)

where $Dose_{i.v}$ and $Dose_{i.p}$ represent i.v. and portal injection dose, respectively. The real liver-to-blood concentration ratios ($K_{Pl}$) values were calculated according to eq. 7 (Lin et al., 1982):

$$K_{Pl, real} = (Q_H + f_B \cdot CL_{int}) K_{Pl, app}/(Q_H + V_H \cdot B \cdot K_{Pl, app})$$  

(7)

where $K_{Pl, real}$, $K_{Pl, app}$, and $B$ represent real liver/blood concentration, apparent liver/blood concentration, and elimination rate constant in the β phase, respectively. $Q_H$ and $V_H$ represent hepatic flow rate (55.2 ml/min/kg) and volume of liver (78.4 ml/kg) (Davies and Morris, 1993), respectively.

The apparent $K_p$ ($K_{P, app}$) values of ITZ, KTZ, CIM, and NIZ were cited from our reports (Takedomi et al., 1998; Yamano et al., 1999). Hepatic clearances of ITZ and KTZ were much smaller than the hepatic blood flow rate. For KTZ, CIM, and NIZ, the $K_{P, app}$ values when the concentrations in plasma after infusion of drugs became at the steady state ($\beta = 0$), were used as $K_{P, app}$ values (Takedomi et al., 1998; Yamano et al., 1999). Therefore, the $K_{P, app}$ values of ITZ, CIM, NIZ, and KTZ were regarded as the real $K_P$ values.

The real concentration in liver/unbound concentration in blood ratios ($K_{Pl}$) of various drugs were calculated according to eq. 8:

$$K_{Pl} = K_{f,b}/K_{f,P} = (K_{P,b} f_P (C_B/C_P))$$  

(8)

where $K_{f,b}$, $f_P$, $f_P$, and $C_B$ represent liver/blood concentration, unbound fraction in the blood, unbound fraction in the plasma, concentration in the blood, and concentration in the plasma, respectively. The $f_P$ and the $C_B/C_P$ ratios were measured as follows.

The $f_P$ of VER, DLZ, ENX, CPFX, and CAM were evaluated using the equilibrium dialysis method. Dialysis was performed with an apparatus made of clear acrylic resin and consisted of two 1.5-ml chambers separated by a cellulose dialysis membrane (SC-101-M10H; Diachema, Zurich, Switzerland). Each drug was added to the rat fresh plasma at a concentration of 5 and 20 µg/ml and applied to one chamber and isotonic phosphate buffer (pH 7.4) was applied to the other. After incubation at 37°C for 6 h, 0.1 ml of sample was collected from both chambers for assay. The $f_P$ of various drugs was calculated according to eq. 9. The $f_P$ of ITZ, CIM, and NIZ was cited from our reports (Takedomi et al., 1998; Yamano et al., 1999).

$$f_P = C_B/C_P$$  

(9)

where $C_B$ and $C_P$ represent the concentration in the buffer and in the plasma after equilibrium dialysis, respectively.

The $C_B/C_P$ ratios of various drugs were measured as follows. Each drug was added to the rat fresh blood at a concentration of 0.5, 2, or 10 µg/ml and 1 ml of blood sample was incubated at 37°C for 15 min. Then, 0.2 ml of sample was taken, and the plasma was obtained by centrifugation and $C_B/C_P$ ratios were calculated. From the preliminary experiment, we confirmed that $C_B/C_P$ ratios were substantially constant after incubation at 37°C for 15 min and each drug was stable during incubation.

Uptake Kinetics by Isolated Rat Hepatocytes. Rat hepatocytes were isolated according to the procedure of Baur et al. (1975). Cell viability for each experiment was checked by the trypan blue exclusion test and was in the range of 85 to 95%. Protein concentration was determined by the colorimetric method of Lowry et al. (1951). All experiments were completed within 2 h after cell preparation, at which time the viability had not changed appreciably.

The time courses of the uptake of various drugs into isolated rat hepatocytes were investigated as follows. Isolated rat hepatocytes (protein concentration, 20 mg/ml) were suspended in Krebs-Henseleit buffer. Then, 0.3 ml of a hepatocyte suspension and 2.7 ml of Krebs-Henseleit buffer (albumin-free) were mixed and preincubated at 37°C for 5 min. Thirty microliters of a standard solution of various drugs was added to each hepatocyte suspension at a concentration of 1 µg/ml and incubated at 37°C. At 20, 40, 60, 120, or 300 s after addition of drugs, 400 µl of the cell suspensions was removed. For ITZ, a sample also was taken at 600 s. The cell suspensions were placed in 1.5-ml polyethylene tubes previously layered with 500 µl of silicone-oil (specific gravity, 1.050) and 200 µl of 3 N KOH. The samples were then centrifuged for 10 s in a table-top microfuge capable of extremely rapid acceleration to separate the cells from the medium. The samples were frozen at −20°C, and then the sample tubes were cut at the middle of the oil layer. The concentrations in the upper layers (medium) and lower layers (hepatocytes) were measured to investigate the time courses of the uptake into isolated rat hepatocytes. The uptake of drugs was corrected for the adherent fluid volume and then converted to true intracellular concentration. The values of adherent fluid (2.2 µl/mg protein) and intracellular space (5.2 µl/mg protein) were obtained from the literature (Miyayuchi et al., 1993).

Concentration dependence of uptake into isolated rat hepatocytes was investigated as follows. The concentrations of drugs were 0.1, 0.2, 0.5, 1, 2, 5, and 10 µg/ml for ITZ, KTZ, VER, and DLZ and 0.1, 1, and 10 µg/ml for
ENX, CPFX, and CAM. Uptake experiments into isolated rat hepatocytes were performed as described above. Incubation times for each drug were enough to reach equilibrium (5 min for KTZ, VER, DLZ, ENX, CPFX, and CAM; 10 min for ITZ).

Measurement of the Concentrations of Various Drugs. The concentrations of ITZ and KTZ in the plasma and blood were measured according to the methods reported previously (Yamano et al., 1999).

For the determination of VER and DLZ concentrations in the plasma, blood, and liver, 0.1 ml of plasma or blood, or 0.5 ml of 20% liver homogenate were mixed with 0.1 ml of methanol, 0.5 ml of 1 N NaOH, and 2.5 ml of isopropllylether and shaken for 5 min, followed by centrifugation at 3000 rpm for 5 min. Two milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 75 μl was injected into HPLC. The chromatographic system consisted of a pump LC-10AD and an SPD-10A variable-wavelength UV detector (Shimadzu Corp., Kyoto, Japan) operating at 229 nm and 237 nm for VER and DLZ, respectively. The mobile phase was acetonitrile-10 mM phosphate buffer (pH 6.5, 50:50, v/v) and was pumped isocratically at a flow rate of 1 ml/min. The lower limit of quantification was 50 ng/ml for both plasma and blood and 1000 ng/g for liver.

For the verification of VER and DLZ in separated cells or medium, 0.5 ml of 1 N NaOH and 5 ml of isopropllylether were mixed and shaken for 5 min and then centrifuged at 3000 rpm for 5 min. Four milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 75 μl was injected into HPLC. The HPLC condition was the same as that of the plasma concentration of VER and DLZ.

The concentrations of CIM and NIZ in the plasma and blood were measured by a modification of the method of Takedomi et al. (1998). In brief, 0.1 ml of plasma or blood, 0.1 ml of methanol, 0.5 ml of 1 N NaOH, and 5 ml of dichloromethane were mixed and shaken for 5 min, and then centrifuged at 3000 rpm for 5 min. Four milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 40 μl was injected into HPLC. For detection, a wavelength of 228 nm was used. The column was a reversed-phase YMC-Pack Pro C18, 3.0 mm × 150 mm (YMC, Kyoto, Japan) and was maintained at 40°C. The mobile phases were acetonitrile-10 mM phosphate buffer (pH 6.5) (80:20, v/v) for VER and acetonitrile-10 mM phosphate buffer (pH 3.0) (35:65, v/v) for DLZ and were pumped isocratically at a flow rate of 1 ml/min. The lower limit of quantification was 50 ng/ml for plasma and blood and 500 ng/g for liver.

For the determination of VER and DLZ in separated cells or medium, 0.5 ml of 1 N NaOH and 5 ml of isopropllylether were mixed and shaken for 5 min and then centrifuged at 3000 rpm for 5 min. Four milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 75 μl was injected into HPLC. The HPLC condition was the same as that of the plasma concentration of VER and DLZ.

The concentrations of CITZ and NIZ in the plasma and blood were measured by a modification of the method of Takedomi et al. (1998). In brief, 0.1 ml of plasma or blood, 0.1 ml of methanol, 0.5 ml of 1 N NaOH, and 5 ml of dichloromethane were mixed and shaken for 5 min, and then centrifuged at 3000 rpm for 5 min. Four milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 40 μl was injected into HPLC. For detection, a wavelength of 228 nm was used. The column was a reversed-phase YMC-Pack Pro C18, 3.0 mm × 150 mm (YMC, Kyoto, Japan) and was maintained at 40°C. The mobile phases were acetonitrile-10 mM phosphate buffer (pH 6.5) (80:20, v/v) for VER and acetonitrile-10 mM phosphate buffer (pH 3.0) (35:65, v/v) for DLZ and were pumped isocratically at a flow rate of 1 ml/min. The lower limit of quantification was 50 ng/ml for plasma and blood and 500 ng/g for liver.

For the determination of ITZ concentrations in the plasma, blood, and liver, 0.1 ml of plasma or blood, 0.5 ml of 20% liver homogenate were mixed with 0.1 ml of methanol, 0.5 ml of 1 N NaOH, and 2.5 ml of isopropllylether and shaken for 5 min, followed by centrifugation at 3000 rpm for 5 min. Two milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 75 μl was injected into HPLC. The HPLC condition was the same as that of the plasma concentration of VER and DLZ.

For the determination of CITZ concentrations in the plasma, blood, and liver, 0.1 ml of plasma or blood, 0.5 ml of 20% liver homogenate were mixed with 0.1 ml of methanol, 0.5 ml of 1 N NaOH, and 2.5 ml of isopropllylether and shaken for 5 min, followed by centrifugation at 3000 rpm for 5 min. Two milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 0.2 ml of the mobile phase and 75 μl was injected into HPLC. The HPLC condition was the same as that of the plasma concentration of VER and DLZ.

For the determination of CAM concentrations in the plasma and liver, 0.1 ml of plasma or 0.5 ml of 20% liver homogenate, 0.5 ml of 0.5 N NaOH, and 3 ml of tert-butyl methylether were mixed and shaken for 5 min and then centrifuged at 3000 rpm for 5 min. Two milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 50 μl of the mobile phase and 20 μl was injected into HPLC. The chromatographic system consisted of a pump LC-10AD and an ECD-10A electron chemical detector (Shimadzu Corp.). The detector cell potential for the oxidation was 1300 mV. The column was a reversed-phase TSKgel 80TM ODS, 4.6 mm × 150 mm (Toso, Tokyo, Japan) and was maintained at 30°C. The mobile phases were acetonitrile-100 mM phosphate buffer (pH 6.4, 50:50, v/v) and were pumped isocratically at a flow rate of 1 ml/min. The lower limit of quantification was 300 ng/ml for both plasma and blood and 1000 ng/g for liver.

For the determination of CAM in separated cells or medium, 0.5 ml of 0.5 N NaOH and 5 ml of tert-butyl methylether were mixed and shaken for 5 min and then centrifuged at 3000 rpm for 5 min. Four milliliters of the organic phase was then transferred to another tube and back-extracted with 3 ml of 100 mM phosphate buffer (pH 4.0). To 2 ml of the aqueous phase, 0.5 ml of 1 N NaOH was added and extracted with 2.5 ml of tert-butyl methylether. Two milliliters of the organic phase was then transferred to another tube and evaporated under nitrogen gas. The residue was dissolved in 50 μl of the mobile phase and 20 μl was injected into HPLC. The HPLC condition was the same as that of the plasma concentration of CAM.

In all measurements, coefficients of variation were <10% and within-run accuracies were ±10%. When the concentrations in the samples were below the limit of quantification, levels were determined by increasing the amount of sample.

Statistical Analysis. Statistical analysis was performed using Student’s t test. Differences were regarded as statistically significant when p values were <.05.

Results

Plasma Concentration Profiles and Hepatic Extraction Ratios of Various Drugs. Figure 1 and Table 1 show plasma concentration profiles and the pharmacokinetic parameters of VER, DLZ, ENX, CPFX, and CAM after intraportal and i.v. administration. The hepatic extraction ratios of VER, DLZ, ENX, CPFX, and CAM were 0.797, 0.782, 0.228, 0.529, and 0.088, respectively.

Cp/Cp Ratios, Kapp Values, and Kf Values of Various Drugs. Table 2 shows the Cp/Cp ratios of various drugs. In all drugs Cp/Cp ratios were within the range of 0.68 to 1.1 and were constant within the concentration range of 0.5 to 10 μg/ml. Kapp values of various drugs were calculated by eq. 8 and are listed in Table 3. The average Kapp values at the steady state obtained previously were used for ITZ, CIM, and NIZ (Takedomi et al., 1998; Yamano et al., 1999). Because the Kapp values of ENX, CPFX, CAM, VER, and DLZ reached a pseudosteady state (at β phase) at 3 h after i.v. administration in a preliminary experiment, the Kapp values at 3 h after i.v. administration were used for these drugs.

Table 3 shows the Kf values of various drugs. The Kf values of various drugs were varied from 0.0034 to 0.096 for NIZ. The maximum of apparent Kapp (Kapp/Kf) and the minimum were 6100 for ITZ and 3.0 for NIZ, respectively.

Because the Kapp values of VER and DLZ may be much larger than the Kapp values due to their large hepatic extraction ratio, hepatic extraction ratios were determined to calculate the Kapp values. The real Kapp values of VER and DLZ were 600 and 73 and were 4.9- and 4.6-fold compared with the apparent Kapp values, respectively. The real Kapp values of ENX, CPFX, and CAM were 9.3, 11, and 36, respectively, and were very close to the apparent Kapp values.

Uptake of Various Drugs into Isolated Rat Hepatocytes. Figure 2 shows the time course of uptake of various drugs into isolated rat
hepatocytes. The uptake of KTZ, VER, DLZ, ENX, CPFX, and CAM reached equilibrium in 5 min, whereas it took 10 min to reach equilibrium for ITZ. In the concentration-dependence study, incubation times were set to reach equilibrium for each drug. Figure 3 shows the concentration dependence on uptake of various drugs into isolated rat hepatocytes. The uptake of ITZ showed marked concentration dependence. The C/M ratios of ITZ were 200 and 6000 at the concentrations of 10 and 0.1 mg/ml, respectively. The concentration-dependence degree of uptake of KTZ and VER was smaller than that of ITZ, whereas the concentration dependence for DLZ, ENX, CPFX, and CAM was negligible.

Correlation between In Vivo Distribution to Liver and In Vitro Uptake by Isolated Rat Hepatocytes. The correlation between C/M ratios of drugs on the uptake into isolated rat hepatocytes and $K_{Bf}$ values in rat liver was investigated for the evaluation of the usefulness of the in vitro experiments for predicting the concentrations in the liver after administration of the inhibitors. C/M ratios at concentrations nearly equal to the unbound concentrations in the plasma were used to investigate the correlation between C/M ratios and the $K_{Bf}$ values of each drug except for ITZ. Because the unbound concentrations of CIM and NIZ after infusion at the rate of 5.7 and 11.4 mg/h/body to rats were 0.5 to 5 and 1 to 5 mg/ml, respectively, C/M ratios at the added concentrations of 3 mM (0.76 μg/ml) and 3 μM (0.99 μg/ml) were quoted for CIM and NIZ (Nakamura et al., 1994), respectively. The unbound concentrations of ITZ in the plasma after i.v. administration at the dose of 20 mg/kg to rats were within the range of 5 to 20 ng/ml, but it was difficult to determine C/M ratio of ITZ at such a low concentration. In this study, C/M ratio at an added concentration of 100 ng/ml was used because there was no significant difference between C/M ratios at the added concentrations of 0.1 and 0.2 μg/ml, suggesting constant C/M ratios at the lower concentration. Figure 4 shows the correlation between C/M ratios and $K_{Bf}$ values of the nine drugs. There was an excellent correlation with a slope of unity between logarithm of C/M ratios and $K_{Bf}$ values ($r = 0.981$).

**Discussion**

To develop a methodology to predict the risk of drug-drug interactions quantitatively, it is necessary to solve four problems: 1) prediction of the disposition of inhibitors in the liver is very important because many drugs are transported into the liver by carrier-mediated
hepatic uptake systems (Meijer et al., 1990; Yamazaki et al., 1996) and unbound concentrations in the liver are higher than those in the plasma; 2) prediction of the concentrations of inhibitors in the portal vein or the hepatic vein is necessary because in the clinical field, most drugs are orally administered and the drug concentration in the portal vein is higher than that in the systemic circulation (Hoffman et al., 1995; Tabata et al., 1995; Fujieda et al., 1996); 3) prediction of drug-drug interactions on the metabolic process in the intestine (jejunal, ileum) as well as in the liver is necessary because CYP3A4 activity in the intestine is half as much as that in the liver. It was reported that MDZ and cyclosporin were metabolized by CYP3A4 in the intestine (Paine et al., 1996; Thummel et al., 1996); and 4) prediction of the drug-drug interactions on the absorption process is necessary because P-glycoprotein exists in the intestine and takes part in the secretion of drugs.

However, we suggest that it is difficult to solve all above-mentioned problems at the same time. To evaluate the extent of drug-drug interactions concerning metabolic inhibition in the liver quantitatively, we tried to predict the plasma concentration increasing ratio (R) of MDZ in rats by using MDZ with concomitant administration of ITZ, KTZ, CIM, and NIZ as inhibitors (Takedomi et al., 1998; Yamano et al., 1999). Assuming that the interaction of drug metabolism is of a competitive inhibition type, the increasing ratio of plasma concentration can be estimated with the following equation:

$$R = 1 + \frac{1}{K_i}$$  \hspace{1cm} (10)

where I and $K_i$ represent the concentration of inhibitor and inhibition constant, respectively. The increasing ratios predicted with the unbound concentration in the plasma were underestimated, whereas the increase ratios predicted with the unbound concentration in the liver were very close to the observed increase value. The liver unbound concentration to the plasma unbound concentration ratios of the inhibitors were $>1$, suggesting a concentrative uptake of these drugs into the liver. Because the metabolic enzymes are localized on the endoplasmic reticulum in the hepatocytes and are physically separated

### TABLE 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Blood/Plasma Concentration Ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Blood concentration, µg/ml</td>
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<tr>
<td></td>
<td>0.5</td>
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<tr>
<td>ITZ</td>
<td>0.793 ± 0.015</td>
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<tr>
<td>KTZ</td>
<td>0.714 ± 0.031</td>
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<tr>
<td>VER</td>
<td>0.951 ± 0.021</td>
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<tr>
<td>CAM</td>
<td>1.097 ± 0.032</td>
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<tr>
<td>DLZ</td>
<td>0.927 ± 0.035</td>
</tr>
<tr>
<td>ENX</td>
<td>0.827 ± 0.015</td>
</tr>
<tr>
<td>CPFX</td>
<td>0.879 ± 0.035</td>
</tr>
<tr>
<td>CIM</td>
<td>0.974 ± 0.014</td>
</tr>
<tr>
<td>NIZ</td>
<td>0.941 ± 0.032</td>
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</tbody>
</table>

Means ± S.D. ($n = 3$).

### TABLE 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>$k_{u,app}^a$</th>
<th>$t_p$</th>
<th>$k_{u,app}^b$</th>
<th>$k_{u,real}^c$</th>
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<tr>
<td>ITZ</td>
<td>20.9 ± 4.69</td>
<td>0.0034$^a$</td>
<td>6100</td>
<td>6100</td>
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<tr>
<td>KTZ</td>
<td>4.36 ± 0.87</td>
<td>0.0095$^a$</td>
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<td>460</td>
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<tr>
<td>VER</td>
<td>7.67 ± 1.96</td>
<td>0.083</td>
<td>120</td>
<td>600</td>
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<tr>
<td>CAM</td>
<td>23.3 ± 7.82</td>
<td>0.069</td>
<td>34</td>
<td>36</td>
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<tr>
<td>DLZ</td>
<td>2.57 ± 0.95</td>
<td>0.16</td>
<td>16</td>
<td>73</td>
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<tr>
<td>ENX</td>
<td>5.41 ± 0.34</td>
<td>0.67</td>
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<tr>
<td>CPFX</td>
<td>4.88 ± 0.76</td>
<td>0.65</td>
<td>7.5</td>
<td>11</td>
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<tr>
<td>CIM</td>
<td>3.92 ± 0.68</td>
<td>0.80</td>
<td>4.9</td>
<td>4.9</td>
</tr>
<tr>
<td>NIZ</td>
<td>2.84 ± 0.85</td>
<td>0.96$^a$</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Values are means ± S.D.

$^a$ Calculated from the apparent $k_p$ values and $C_{B/P}$ ratios.

$^b$ Calculated according to eq. 8.

$^c$ Calculated according to eqs. 7 and 8.

$^d$ Cited from our article concerning the interaction between azole antifungal agents and MDZ (Yamano et al., 1999).

$^e$ Cited from our article concerning the interaction between histamine H$_2$ receptor antagonists and MDZ (Takedomi et al., 1998).

Fig. 2. Time courses of ITZ (A), KTZ (B), DLZ (C), VER (D), ENX (E), CPFX (F), and CAM (G) uptake by isolated rat hepatocytes.

The concentrations of drugs in the incubation medium were 1 µg/ml. Each point and vertical bar represent the mean ± S.D. ($n = 3$).
from the blood by the plasma membrane, the unbound concentrations in the liver may be more appropriate for predicting the increase rate of plasma concentration quantitatively. In humans, the liver unbound concentrations of the inhibitors are required. However, it is difficult to measure directly the liver unbound concentrations of the inhibitors; therefore, a methodology to predict the concentrations in the liver after administration of the inhibitors is necessary. We tried to predict the concentrations in the liver after administration of drugs to rats. First, we investigated the correlation between the $K_{\text{Bf}}$ values in rats and C/M ratios of the uptake into isolated rat hepatocytes. Second, we examined the possibility of the prediction of the concentration in the liver with C/M ratios and the concentrations in the plasma or the blood.

As C/M ratios of the uptake into isolated rat hepatocytes were the values when the uptake of drugs reached equilibrium, the $K_{\text{Bf}}$ values in rats when the liver concentrations were in parallel with the concentrations in the plasma or the blood. As for VER and DLZ, the liver is the main disappearance organ and the majority of the total clearance is the hepatic clearance. The total clearances after i.v. administration of VER and DLZ were $43.4 \pm 4.2$ and $63.9 \pm 6.3$ ml/min/kg (mean $\pm$ S.D., $n = 4$), respectively, and were similar to the hepatic blood flow rates. The $K_{\text{B,real}}$ values of VER and DLZ may be much larger than the $K_{\text{B,app}}$ values because of their large hepatic extraction ratio. Therefore, their hepatic extraction ratios were calculated from AUC after intraportal and i.v. administration and then the $K_{\text{B,real}}$ values were calculated according to eq. 7. The total clearances after i.v. administration of ENX and CPFX were $38.7 \pm 2.0$ and $51.9 \pm 14.4$ ml/min/kg (mean $\pm$ S.D., $n = 4$), respectively, but the elimination routes were both via hepatic metabolism and renal excretion and the hepatic clearances of ENX and CPFX were 12 and 25 ml/min/kg, respectively (Davis et al., 1995) and were much less than the hepatic blood flow rate. Therefore, the $K_{\text{Bf}}$ values calculated from the hepatic clearance and the hepatic blood flow rate were close to the real $K_{\text{Bf}}$ values.

The concentration-dependent uptake of ITZ, KTZ, and VER into isolated rat hepatocytes was observed and suggested saturable carrier-mediated uptake, whereas the uptakes of other drugs into isolated rat hepatocytes were not dependent on drug concentrations. Figure 4 shows the good correlation between C/M ratios of drugs and real $K_{\text{Bf}}$ values in rats. Therefore, drug concentrations in the liver ($C_H$) after administration to rats can be predicted from C/M on the uptake into rat isolated hepatocytes and drug concentrations in blood, as in eq. 11:

$$C_H = (\text{C/M}) \cdot C_B \cdot f_B$$

Cryopreservation of human hepatocytes has been established and human hepatocytes with high viability can be used (Adams et al., 1995). Lave et al. (1997) evaluated the use of human hepatocytes to classify compounds into low, intermediate, or high hepatic extraction ratio in humans, and reported that in vitro clearances in human hepatocytes were predictive for the hepatic ratios in vivo in humans and that human hepatocytes seemed to be a valuable way for screening compounds with respect to liver first-pass metabolism. Sun et al. (1996) investigated the biotransformation of lifibrol, a lipid-lowering drug, by using human hepatocytes in primary culture. Human hepatocytes offer certain advantages for predicting the degree of drug metabolism and interaction in humans at the biotransformation level (Fischer et al., 1997). Olinga et al. (1998) investigated the mechanisms and specificity of the uptake of the cardiac glycoside digoxin.
and the organic cation rocuronium into human hepatocytes. The hepatic extraction ratio was then calculated from the measured uptake rates of the compounds into human hepatocytes and compared with published in vivo data. The initial hepatic extraction ratio, calculated from the in vitro uptake data for digoxin and rocuronium, very well reflected the initial extraction ratio for distribution in the liver in vivo in humans. In a similar manner as rat hepatocytes, we can possibly predict the concentrations in human liver from the concentrations in the blood and $K_{H1}$ values estimated with human isolated hepatocytes. Moreover, it may be possible to predict drug-drug interactions based on the inhibition of hepatic metabolism in humans quantitatively from inhibition constant ($K_i$ value) obtained by in vitro metabolic inhibition experiments and the concentration in the liver predicted from in vitro data. We suggest that this is one of the best solutions of point 1 mentioned above.

References


