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Simultaneous Assessment of the In Vivo Amount of CYP1A2 and CYP3A2 by the PKCYP-test Using Theophylline in Rats

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Summary: Recently, we developed a method for assessing in vivo drug metabolism capacity by pharmacokinetic estimation of the quantity of cytochrome P450 (CYP) in vivo (PKCYP-test), in which an apparent liver-to-blood free concentration gradient in vivo (qg) is introduced. The qg value can be alternatively defined as the ratio of the in vivo-in vitro clearance by a single CYP isoform. In this study, we examined the application of the PKCYP-test to drugs metabolized by multiple CYP isoforms in a rat model with fluctuating CYP1A2 levels using theophylline as a model drug.

In control rats, the estimated qg values for each CYP1A2 and CYP3A2 based on the in vivo hepatic intrinsic clearance, in vitro Michaelis constant (K_m) and maximal rate of metabolism (V_max) values for liver slices agreed well. Moreover, the qg value for CYP1A2 determined by the K_m and V_max values for recombinant CYP1A2 was compatible with that based on liver slices. These qg values also agreed with that of rats pretreated with 3-methylcholanthrene. The time-course of theophylline concentrations in serum simulated by a physiologically-based pharmacokinetic model incorporating the hepatic clearance determined by the PKCYP-test agreed with the observed values.

These results demonstrate that the qg value in the PKCYP-test is applicable to drugs metabolized by multiple CYP isoforms.

Key words: PKCYP-test; theophylline; CYP1A2; CYP3A2; physiologically-based pharmacokinetic model

Introduction

In order to select the most appropriate drug dosage regimens, it is important to predict the drug clearance rate for individual patients. Since a number of isoforms of cytochrome P450 (CYP) exist in human liver for each individual CYP isoform, a drug mainly metabolized by a single isoform is used as a probe for “metabolic diagnosis”1. Clinically, it is necessary to estimate the drug clearance rate over a several-fold range, but the use of probe-based assays for measuring the in vivo activity of CYP is often limited to the identification of patients who are poor or extensive metabolizers due to a genetic polymorphism.

Recent advances in molecular cloning and expression technology have enabled us to use the commercially available cDNA-expressed CYP isoforms, and these heterologously expressed enzymes are being increasingly used as in vitro models of human liver microsomes in quantitative phenotyping. Several approaches have been used to predict in vivo drug metabolisms, such as scaling of in vivo metabolic clearance from in vitro data obtained from human liver microsomes or a recombinant system of human CYP isoforms. In order to improve the correlation between in vitro and in vivo metabolic clearance, some new methods have been described. For example, the ratio of in vivo to in vitro intrinsic clearance has been introduced as a scaling factor and the use of the unbound fraction in liver microsomes has also been examined. However, the reported methods may not adequately predict in vivo drug disposition from in vitro metabolic parameters, and the detailed significance of the scaling factor remains to be clarified.

From a pharmacokinetic point of view, one possible
explanation for a scaling factor is that the free concentration in the hepatic vein is used in place of the actual concentration of drug in the liver when the \textit{in vivo} clearance is estimated using \textit{in vitro} parameters. In previous work, we described a new method for assessing drug metabolism capacity based on a pharmacokinetic estimation of the quantity of CYP \textit{in vivo} (PKCYP-test). In setting up the PKCYP-test, an apparent liver-to-blood free concentration gradient \textit{in vivo} (qg) was introduced. Our hypothesis is that the \textit{in vivo} metabolic clearance of many drugs in patients may be predicted by measuring the quantity of metabolizing enzyme in the liver of individual patients, taking account of the qg (see Appendix). We have already demonstrated that in rats with fluctuating CYP levels, the amount of CYP1A2 or CYP2C11 could be predicted by the PKCYP-test using the qg values of age-matched control rats. Since several CYP isoforms are involved in \textit{in vivo} drug metabolism, it is necessary to evaluate the usefulness of the PKCYP-test in the case of multiple CYP isoforms. In order to apply the PKCYP-test to those drugs, it is important to determine the difference in qg values: 1) of each CYP isoform, 2) based on the \textit{in vitro} intrinsic clearance from liver slices and recombinant CYP, and 3) for a fluctuating CYP protein content \textit{in vivo}.

It has been reported that theophylline is metabolized by multiple CYP isoforms, and theophylline metabolism is known to be increased by 3-methylcholanthrene (MC) treatment of rats, whose CYP1A2 level was elevated. In the present study, using the PKCYP-test as a model drug, we examined the application of the PKCYP-test to a drug that is metabolized by multiple CYP isoforms, including CYP1A2. We used a rat model with fluctuating CYP1A2 levels.

Moreover, previously, we constructed a physiologically-based pharmacokinetic model which involved the PKCYP-test. By using the qg and the observed amount of CYP2C11 as an indicator of hepatic metabolism capacity, the time-course of tolbutamide concentrations in rat models with fluctuating CYP2C11 levels could be predicted. In this study, we also tried to predict the time-course of theophylline concentrations in rats using qg and the observed amount of CYP1A2.

**Methods**

**Materials:** Chemicals were obtained from the following sources: theophylline was from Wako Pure Chemicals (Osaka, Japan); MC, 1-methylxanthine (1-MC), 3-methylxanthine (3-MC), 1, 3-dimethyluric acid (1, 3-DMU), 1, 3, 7-trimethyluric acid (1, 3, 7-TMU), \( \beta \)-hydroxyethyltheophylline, goat serum, and rabbit serum were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); \([8-14\text{C}]\) theophylline was from America Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.); NADPH was from Oriental Yeast Co. (Tokyo, Japan); and microsomal preparations of recombinant rat CYP1A2 expressed by baculovirus-infected insect cells (recombinant rat CYP1A2), Anti-Rat CYP1A1 (anti-rat CYP1A1 antibody), Anti-Rat CYP1A2 (anti-rat CYP1A antibody), and Anti-Rat CYP3A2 (anti-rat CYP3A2 antibody) were from Daiichi Pure Chemicals Co. (Tokyo, Japan). Anti-rat CYP1A antibody inhibited both CYP1A1 and CYP1A2 activity. All other reagents were of reagent grade or HPLC grade and were used without further purification.

**Animals:** Male Sprague-Dawley rats, 6 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were pretreated with MC (25 mg/kg, dissolved in olive oil, i.p. daily for 3 days) (MC-treated rats). Control animals were used without pretreatment. The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoritsu College of Pharmacy.

**In vivo pharmacokinetic studies:** The control and MC-treated rats were given theophylline (1 mg/kg, dissolved in saline) by intravenous injection. Blood was collected from the jugular vein, and the liver was resected. Blood was centrifuged at 15000 \( \times \)g (MX-150, Tomy Seiko Co., Ltd., Tokyo, Japan) for 10 min at 4°C to obtain serum. Serum and liver were stored at \(-20\) and \(-80°C\), respectively, until assayed. To determine the urinary elimination of theophylline, the urine was collected at 24 and 48 hr following theophylline injection. After each collection interval, the volume was measured and the urine centrifuged at 700 \( \times \)g (RLX-105, Tomy Seiko Co., Ltd., Tokyo, Japan) for 10 min at 4°C. The supernatant was stored at \(-20°C\) until assayed.

**Determination of serum protein binding of theophylline:** Theophylline solution was added to the serum to give concentrations of 0, 1, 2, 4, 6, 8, or 10 \( \mu \text{g/mL} \) (in a total volume of 800 \( \mu \text{L} \)). The serum samples were incubated for 30 min at 37°C. Then, to separate the free fraction from the protein-bound fraction, 700 \( \mu \text{L} \) of each serum sample was transferred to a centrifuge tube (Centrifree, Amicon, Inc., Beverly, MA, U.S.A.) and centrifuged at 1000 \( \times \)g for 10 min at 37°C. Following this, 100 \( \mu \text{L} \) of the free fraction was obtained and stored at \(-20°C\) until analysis; 100 \( \mu \text{L} \) aliquots of serum samples containing protein-bound and free drug were also stored at \(-20°C\) until assayed.

**Quantitation of CYP1A2 and CYP3A2 in liver microsomes:** Liver microsomes were prepared as previously described. Quantitation of CYP1A2 and CYP3A2 in liver microsomes was performed by Western blotting. The liver microsomes of the control (0.2–0.6 \( \mu \text{g protein/lane} \)) rats were separated by SDS-polyacrylamide gel electrophoresis using 10% gels. Microsomes from male rats with known amounts of CYP3A2 were also applied (0.018–0.064 pmol/lane) as a standard. The separated proteins were transferred to a
nitrocellulose membrane which was then incubated with antibodies. Anti-rat CYP3A2 from goat (Daiichi Pure Chemicals Co., Tokyo, Japan) was used as the first antibody. The second antibody was anti-goat IgG peroxidase conjugate, developed in rabbit (Sigma Chemical Co., St. Louis, MO, U.S.A.). The peroxidase activity of the immunoblots was monitored using an enhanced chemiluminecenc kit (Amersham, Aylesbury, UK), and the nitrocellulose membrane was exposed to photographic film (Hyper ECL, Amersham, Aylesbury, UK) for 20 sec. The CYP3A2 protein band on the film was quantified densitometrically with an electrophoresis documentation and analysis system (Eastman Kodak, Rochester, NY, U.S.A.), and the CYP3A2 content was determined from a standard curve generated using microsomes from rats treated with phenobarbital. A standard curve was obtained by plotting the intensity of the band on a film on the ordinate axis versus the respective quantity of rat CYP3A2 on the abscissa. The conversion of the amount of CYP3A2 per kg body weight was achieved using a value of 1.09 ± 0.02 g microsomal protein/kg body weight. The conversion of the amount of CYP1A2 per kg body weight in control and MC-treated rats was achieved using values 1.09 ± 0.02 and 1.22 ± 0.06 g microsomal protein/kg body weight, respectively.

Inhibitory effect of anti-rat CYP1A1, CYP1A, and CYP3A2 antibodies on metabolism of theophylline in liver microsomes: The assay mixture for theophylline metabolism contained 13.5 μM [8-14C] theophylline, 0.4 mM NADPH, 0.67 mg/mL MgCl₂·6H₂O, and 25 mM Tris buffer (pH 8.0) in a final volume of 0.5 mL. The specific activity of [8-14C] theophylline was adjusted to 1887 Bq/nmol by dilution with unlabeled theophylline. The assay mixture without [8-14C] theophylline was preincubated in a shaking bath for 10 min at 37°C. The reaction was started by the addition of [8-14C] theophylline was preincubated in a shaking bath for 10 min at 37°C. The reaction was started by the addition of [8-14C] theophylline, and incubation was carried out in a shaking bath for 20 min at 37°C. The reaction was halted by the addition of 100 μL 20% perchloric acid, and the radioactivity due to each of the theophylline metabolites was measured. Assay of theophylline and its metabolites: Theophylline in serum, liver, and urine was assayed by HPLC, using a modification of the reported procedures. Briefly, 10 μL 20 μg/mL 1, 3, 7-TMU (internal standard), 100 μL 1 N perchloric acid were added to 100 μL serum followed by vortexing and centrifugation at 15000 × g for 10 min at 4°C. The supernatant was passed through a 0.45-μm filter (4 mm Millex-LH, Nihon Millipore Ltd., Tokyo, Japan), and 60 μL was subjected to HPLC. The liver was homogenized in an equal weight of distilled water. Then, 10 μL 20 μg/mL 1, 3, 7-TMU (internal standard) and 200 μL 1 N perchloric acid were added to 200 μL homogenate. The sample was then treated in the same way as serum. 200 μL 1 M sodium acetate buffer (pH 5.0), 10 μL 80 μg/mL β-hydroxyethyltheophylline (internal standard), and 9 mL methylene chloride were added to 200 μL urine followed by vortexing and centrifugation at 700 × g for 30 min at room temperature. The methylene chloride phase was evaporated. Then, the organic layer was removed and transferred to a fresh tube and dried at 40°C under nitrogen. Each sample was resuspended in 200 μL HPLC mobile phase, passed through a 0.45-μm filter, and 30 μL was subjected to HPLC. A calibration curve was constructed by spiking drug-free rat serum, liver homogenate, and urine with concentrated theophylline solution to give solutions of 0, 0.25, 0.5, 1, 2, and 4 μg/mL (for serum and liver homogenate) or 0, 0.25, 0.5, 1, 2, 4, and 8 μg/mL (for urine). Samples were submitted to the extraction procedure described above and a calibration curve was ob-
tained by plotting the peak-area on the ordinate axis versus the respective drug concentrations on the abscissa. The HPLC system consisted of a pump (Programmable pump Model 307, Gilson, Middleton, WI), an autoinjector (Model 234, Gilson, Middleton, WI), a variable wavelength UV detector (Programmable detector Model 118 (UV/VIS), Gilson, Middleton, WI), a recorder (Chromatocorder 21, System Instruments, Tokyo, Japan), and a column oven (655A-52 column oven, Hitachi, Tokyo, Japan). Separation was achieved on a C18 reversed-phase column (250 mm × 4.6 mm i.d., particle size 5 μm, Capcell Pak C18 UG120 S-5 μm, Shiseido, Tokyo, Japan) at a column temperature of 40°C. The mobile phase consisted of 22 mL: 1.25 mL: 175 μL: 463 mL acetonitrile: tetrahydrofuran: acetic acid: water, at a flow rate of 0.8 mL/min, and the column eluate was monitored at 280 nm. The retention times for theophylline, β-hydroxyethyltheophylline, and 1, 3, 7-TMU were 17.6, 24.1, and 25.0 min, respectively. The lower limit of quantification was 0.25 μg/mL.

To measure the radioactivity of theophylline metabolites in the samples from the in vitro theophylline metabolism and the inhibition studies of in vitro theophylline metabolism, the metabolites were first separated by HPLC. 10 μL 50 μg/mL 3-MC, 1-MC, and 1, 3-DMU (internal standard) were added to each sample, followed by vortexing and centrifugation at 15000 × g (M200-IVD, Sakuma, Tokyo, Japan) for 5 min at 4°C. The supernatant was filtered through a 0.45-μm filter and 400 μL filtrate was subjected to HPLC. The HPLC conditions were as follows: instruments, a Shimadzu LC-9A liquid chromatograph equipped with a SPD-6A spectrophotometric detector, a SCL-6B system controller, a data processor (Chromatopac C-R4A), and a CTO-6A column oven; the HPLC column used was the same as that for the analysis of the theophylline in serum, liver, and urine; column temperature, 40°C; the mobile phase was consisted of 5:95 (v/v) acetonitrile: water, at a flow rate of 0.8 mL/min, and the absorbance wavelength was 280 nm. The retention times for theophylline, β-hydroxyethyltheophylline, and 1, 3, 7-TMU were 17.6, 24.1, and 25.0 min, respectively. The lower limit of quantification was 0.25 μg/mL.

Estimation of the qg values of theophylline for CYP1A2 and CYP3A2 based on the K_m and V_max values for recombinant CYP1A2 in control and MC-treated rats: The qg values of theophylline for CYP1A2 and CYP3A2 in control and MC-treated rats were estimated according to Eq. (A6) as follows:

\[
q_g = \frac{f_{CYP} \cdot CL_{int}}{CL_{int,vitro}}
\]

(2)

Then, the qg value of theophylline based on the in vitro intrinsic clearance determined using liver slices was estimated according to Eq. (A5) as follows:

\[
q_g = \frac{CL_{int} \cdot 1}{Q_h - CL_{int,f} \cdot f_p}
\]

(1)

where \(CL_{int,vitro}\) is the in vitro intrinsic clearance.

In addition, for each CYP isoform, the intrinsic clearance can be obtained by multiplying the \(f_{CYP}\) by the apparent \(CL_{int}\). Therefore, the qg value of theophylline for CYP1A2 and CYP3A2 in control rats was estimated according to Eq. (A6) as follows:

\[
q_g = \frac{f_{CYP} \cdot CL_{int}}{CL_{int,vitro}}
\]

(2)

Analysis using a physiologically-based pharmacokinetic model: The physiologically-based pharmacokinetic model which introduced the PKCYP-test was used. In this model, Eq. (3) was introduced as the hepatic intrinsic clearance (\(CL_{int,h}\)).

\[
CL_{int,h} = \frac{V_{max,vitro}}{q_g} \cdot A-CYP_{vivo} + C_{L,f}
\]

(3)

Where A-CYP_{vivo} is the in vivo amount of CYP and C_{L,f} is the free concentration (not bound to macromolecules) of drug within the hepatic vein. \(K_m,vitro\) is the in vitro Michaelis constant for the drug-enzyme interaction and \(V_{max,vitro}\) is the in vitro maximal rate of metabolism.

The mean of the physiological data from the literature\(^{15-17}\) was used for the blood flow rate (Q) and tissue volume (V) parameters of each organ in the model. The reported values of the blood-to-plasma concentration ratio (R_h) and the tissue-to-plasma partition coefficient (K_v) for theophylline were also used.\(^{18}\) The mass balance equation for the concentration in each organ compartment was solved simultaneously by the Runge-Kutta-Gill method as described in the literature,\(^{19}\) and the concentration in serum after a single intravenous administration was calculated.

Data analysis: Serum concentration versus time data were analyzed by model-independent moment analysis. The last determined serum concentration was extrapolated to infinity using the terminal slope of the
last two points of the log serum concentration-time curve. The volume of distribution at steady-state ($V_{d,ss}$) and the total body clearance ($CL_{tot}$) were estimated as described by Yamaoka et al. The apparent tissue-to-plasma concentration ratio of free drug ($K_{pf,app}$) for theophylline was obtained by dividing the liver concentration by the free concentration in serum. Data were analyzed using Student's t test for comparison of unpaired means of two sets of data. Analysis of variance was used to compare more than two sets of data. The number of determinations (n) is noted in the table. A p value of 0.05 or less was used to indicate a significant difference between sets of data.

Results

Comparison of the pharmacokinetics of theophylline in control and MC-treated rats: In order to estimate the qg values, the theophylline clearance in control and MC-treated rats was investigated. The serum concentration versus time profiles of theophylline in control and MC-treated rats are shown in Fig. 1. Since the theophylline profile suggests linear pharmacokinetics, the $CL_{tot}$ was determined under linear conditions. The results of linear model-independent moment analysis are given in Table 1. The $CL_{tot}$ of theophylline in MC-treated rats was approximately 7-fold larger than that of control rats. Since in control and MC-treated rats, approximately 30 and 6% unchanged theophylline is eliminated in the urine, respectively, the $CL_h$ was corrected by using the fraction of the dose excreted unchanged in urine.

| Table 1. Pharmacokinetic and biochemical parameters of theophylline in control and MC-treated rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MC-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-CYP&lt;sub&gt;vivo&lt;/sub&gt; of CYP1A2 (pmol/mg of microsomal protein)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(nmol/kg)</td>
<td>4.24 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>85.4 ± 4.2&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>A-CYP&lt;sub&gt;vivo&lt;/sub&gt; of CYP3A2 (pmol/mg of microsomal protein)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;f&lt;/sup&gt;</td>
<td>229 ± 44&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>(nmol/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>223 ± 6</td>
<td>203 ± 10&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{tot}$ (mL/min/kg)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>2.25 ± 0.03</td>
<td>16.1 ± 0.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_h$ (mL/min/kg)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1.59 ± 0.02</td>
<td>15.2 ± 0.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$f_p$&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.37 ± 0.08</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>$K_{pf,app}$&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1.69 ± 0.41</td>
<td>1.30 ± 0.32</td>
</tr>
<tr>
<td>Urinary excretion (% of dose)</td>
<td>29.3 ± 3.8</td>
<td>5.8 ± 1.0&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$V_{d,ss}$ (mL/kg)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>548 ± 20</td>
<td>729 ± 56&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are means ± S.D. (n = 3–4). <sup>b</sup>In vivo amount of CYP. <sup>c</sup>Total body clearance. <sup>d</sup>Unbound fraction of drug in serum. <sup>e</sup>Apparent tissue-to-plasma concentration gradient of free drug in liver. <sup>f</sup>Volume of distribution at steady-state. <sup>i</sup>Ref. 6. The conversion of A-CYP<sub>vivo</sub> per kg body weight was achieved as described in Methods. <sup>j</sup>Not determined. <sup>k</sup>p < 0.05 **p < 0.01

Then, the $CL_h$ of theophylline in MC-treated rats was about 10-fold larger than that of control rats (Table 1). The physiological data for the theophylline-treated rats used in this study is also summarized in Table 1. There were some differences in body weight between control and MC-treated rats. There were no significant differences in the $f_p$ and the $K_{pf,app}$. The $V_{d,ss}$ of MC-treated rats was slightly larger than that of control rats.

Estimation of the contribution of CYP1A2 and CYP3A2 to the liver microsomal metabolic activity for theophylline in control and MC-treated rats: The contribution of CYP1A2 and CYP3A2 to liver microsomal theophylline metabolism in control and MC-treated rats was estimated using anti-rat CYP1A1, CYP1A, and CYP3A2 antibodies. The effects of these antibodies on theophylline liver microsomal metabolism in control and MC-treated rats are shown in Table 2. Control activity in the presence of preimmune serum in control and MC-treated rats was 0.68 ± 0.23 and 5.16 ± 0.97 pmol/min/mg, respectively. As shown in Table 2, the contribution of CYP1A2 to liver microsomal theophylline metabolism in MC-treated rats was slightly greater than that of control rats. In control rats, liver microsomal theophylline metabolism was mainly catalyzed by CYP3A2.

Estimation of the qg values of theophylline for CYP1A2 and CYP3A2 based on the $K_m$ and $V_{max}$ values for liver slices from control rats: The qg value of theophylline based on the in vitro intrinsic clearance determined using liver slices was estimated as 7.80 using parameters listed in Tables 1 and 3.

In addition, the qg values of theophylline for
K

In order to estimate the qg value for each CYP isoform, recombinant CYP1A2 in control and MC-treated rats:

\[ CL_{int,vitro}(\text{mL/min/kg}) = \text{V}_{max} \times 1.67 \times \text{kg/min} \]

Furthermore, there was no difference between the qg value for CYP1A2 in control and MC-treated rats.

Prediction of time-courses of theophylline concentrations in control and MC-treated rats: The theophylline concentration-time profiles in serum, following intravenous administration of theophylline to control and MC-treated rats at a dose of 1 mg/kg, were estimated using the physiologically-based pharmacokinetic model which involved the PKCYP-test. As shown in Fig. 3, by introducing the qg of control rats based on the \( K_m \) and \( V_{max} \) values for recombinant CYP1A2 and the observed amount of CYP1A2 as an indicator of hepatic metabolism capacity, the theophylline concentration-time profiles in serum from control and MC-treated rats could be simulated. In control and MC-treated rats, the predicted theophylline concentrations were within the 95% confidence interval of the observed theophylline concentrations.

Discussion

In the present study, we examined the application of the PKCYP-test to drugs metabolized by multiple CYP isoforms. The contribution of CYP1A2 and CYP3A2 to the liver microsomal metabolic activity for theophylline in control and MC-treated rats was estimated as 9.35 and 7.14, respectively using the parameters listed in Tables 1 and 4. Hence, they were almost identical. Furthermore, there was no difference between the qg value for CYP1A2 in control and MC-treated rats.

CYP1A2 and CYP3A2 in control rats were estimated as 7.77 and 7.80, respectively using parameters listed in Tables 1 and 3. Hence, the qg value of theophylline estimated from the total hepatic intrinsic clearance was comparable with both qg values estimated from the hepatic intrinsic clearance mediated by CYP1A2 and CYP3A2.

Estimation of the qg values of theophylline for CYP1A2 based on the \( K_m \) and \( V_{max} \) values for recombinant CYP1A2 in control and MC-treated rats: In order to estimate the qg value for each CYP isoform, the \( K_m \) and \( V_{max} \) values for theophylline metabolism by recombinant CYP1A2 were determined. Conversion of theophylline to 1-MC, 3-MC, and 1, 3-DMU by recombinant CYP1A2 exhibited Michaelis-Menten kinetics. The Lineweaver-Burk plot shown in Fig. 2 was used to derive the \( K_m \) and \( V_{max} \) values for recombinant CYP1A2 (Table 4). The qg values of theophylline for CYP1A2 based on the \( K_m \) and \( V_{max} \) values for recombinant CYP1A2 in control and MC-treated rats were estimated as 9.35 and 7.14, respectively using the parameters listed in Tables 1 and 4. Hence, they were almost identical. Furthermore, there was no difference between the qg value for CYP1A2 in control and MC-treated rats.

Table 2. The contribution of CYP1A2 and CYP3A2 to the liver microsomal metabolic activity for theophylline in control and MC-treated rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MC-treated</th>
</tr>
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<tbody>
<tr>
<td>% of control activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ anti-rat CYP1A1</td>
<td>125 ± 6</td>
<td>69 ± 18</td>
</tr>
<tr>
<td>+ anti-rat CYP1A</td>
<td>75 ± 7</td>
<td>31 ± 18</td>
</tr>
<tr>
<td>+ anti-rat CYP3A2</td>
<td>20 ± 9</td>
<td>—</td>
</tr>
<tr>
<td>Contribution of CYP1A2</td>
<td>0.25 ± 0.07</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Contribution of CYP3A2</td>
<td>0.80 ± 0.09</td>
<td>—</td>
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</table>

Values are means ± S.D. (n = 3). *Not determined.

Table 3. Kinetic parameters of theophylline biotransformation by liver slices in control rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>CYP3A2</td>
</tr>
<tr>
<td>( CL_{int,vitro}(\text{mL/min/kg}) )</td>
<td>0.566</td>
</tr>
<tr>
<td>( K_m,vitro (\mu M) )</td>
<td>67.5</td>
</tr>
<tr>
<td>( V_{max,vitro} (\text{nmol/min/kg}) )</td>
<td>52.1</td>
</tr>
</tbody>
</table>

*In vitro intrinsic clearance. *In vitro Michaelis constant. *In vitro maximal rate of metabolism. *The conversion of \( CL_{int,vitro} \) per kg body weight was achieved using 11.0 g liver/250 g body weight. *Ref. 26.

Table 4. Kinetic parameters of theophylline biotransformation by recombinant CYP1A2 in control and MC-treated rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MC-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>( CL_{int,vitro}(\text{mL/min/kg}) )</td>
<td>0.118 ± 0.002</td>
<td>2.37 ± 0.12</td>
</tr>
<tr>
<td>( K_m,vitro (\mu M) )</td>
<td>216 ± 66</td>
<td>—</td>
</tr>
<tr>
<td>( V_{max,vitro} (\text{nmol/min/nmol CYP1A2}) )</td>
<td>5.99 ± 1.61</td>
<td>—</td>
</tr>
</tbody>
</table>

*Values are means ± S.D. (n = 3). *In vitro intrinsic clearance. *In vitro Michaelis constant. *In vitro maximal rate of metabolism. *The conversion of \( CL_{int,vitro} \) per kg body weight was achieved using the \( V_{max} \) amount of CYP1A2 listed in Table 1.
ophylline in control and MC-treated rats. The PKCYP-test was constructed a physiologically-based pharmacokinetic model in control and MC-treated rats, respectively.

We estimated the $q_g$ values for CYP1A2 and CYP3A2 based on the $K_m$ and $V_{max}$ values for liver slices in control rats. Notably, the $q_g$ values for CYP1A2 and CYP3A2 were almost equal to the $q_g$ value which was estimated using the total hepatic intrinsic clearance. These results suggest that the $q_g$ values for each CYP isoform are equal in the case of theophylline. Thus, the drug concentration at the enzyme site for each CYP isoform seems to be equal indicating that the assumption for $q_g$, the apparent liver-to-blood free concentration gradient in vivo, is appropriate.

Thirdly, we compared the $q_g$ value for CYP1A2 based on the $K_m$ and $V_{max}$ values by recombinant CYP1A2 in control and MC-treated rats. It has been reported that the application of the relative activity factor (RAF) for each CYP isoform is needed in mathematically reconstruct the biotransformation rates in liver microsomes using recombinant CYP isoforms. There is a possibility that the $q_g$ values estimated using in vitro intrinsic clearance from liver slices and recombinant CYP isoforms are different for each CYP isoform. In control rats, the $q_g$ value for CYP1A2 based on the $K_m$ and $V_{max}$ values by recombinant CYP1A2 was similar to that by liver slices. Therefore, in case of theophylline, it seems that there is no significant difference between the $q_g$ values estimated using the in vitro intrinsic clearance from liver slices and recombinant CYP isoforms. Moreover, since the $q_g$ value in control and MC-treated rats were also almost identical, it seems that the $q_g$ value remains unaltered when CYP levels fluctuate. These results are consistent with the observation of a similar $K_{pf,app}$ of theophylline in liver for control and MC-treated rats (Table 1). All the theophylline $q_g$ values estimated in this study were greater than unity. This means that the free concentration of theophylline near CYP may be larger than that in the hepatic vein. However, the $K_{pf,app}$ of theophylline in both control and MC-treated rats was almost unity (Table 1). Hence, another reason for this incompatibility may be underestimation of the in vitro intrinsic clearance of theophylline. It has been reported that a correction for free fraction differences between microsomes and plasma improves the predictive utility

isoforms using theophylline as a model drug. We focused attention on the difference in $q_g$ values: 1) of each CYP isoform, 2) based on the in vitro intrinsic clearance from liver slices and recombinant CYP, and 3) for a fluctuating CYP protein content in vivo. We constructed a physiologically-based pharmacokinetic model which introduced the PKCYP-test.

Firstly, we examined the pharmacokinetics of theophylline in control and MC-treated rats. The $CL_{in}$ and $CL_{hep}$ of theophylline in MC-treated rats were about 7-fold and 10-fold larger than that of control rats (Table 1). The $CL_{hep}$ of theophylline in control rats was linearly related to dose and agreed well with the previously reported value. The $CL_{hep}$ of theophylline in control rats agreed well with the previously reported value.

Secondly, we estimated the contribution of CYP1A2 and CYP3A2 to liver microsomal theophylline clearance in control and MC-treated rats, and examined the difference in the $q_g$ value of each CYP isoform. Theophylline metabolism is reported to be catalyzed by CYP1A2 in rats. Moreover, it has been reported that another CYP isoform in rats, such as CYP3A2, a male-specific CYP isoform, is also involved in the metabolism of theophylline. We also examined the effect of anti-rat CYP2C11 antibody on theophylline liver microsomal metabolism in control rats, which is also a male-specific CYP isoform in rats, although no inhibitory effect was observed in control rats (data not shown). The $f_{CYP}$ for CYP1A2 and CYP3A2 with respect to liver microsomal theophylline metabolism in control rats was 0.25 and 0.80, respectively (Table 2). On the other hand, in MC-treated rats, the $f_{CYP}$ for CYP1A1 and CYP1A2 with respect to liver microsomal theophylline metabolism was 0.31 (data not shown) and 0.39 (Table 2), respectively. Therefore, liver microsomal theophylline metabolism in MC-treated rats seems to be mediated by more than three CYP isoforms including CYP1A1, CYP1A2, and putatively, CYP3A2.

We estimated the $q_g$ values for CYP1A2 and CYP3A2 based on the $K_m$ and $V_{max}$ values for liver slices in control rats. Notably, the $q_g$ values for CYP1A2 and CYP3A2 were almost equal to the $q_g$ value which was estimated using the total hepatic intrinsic clearance. These results suggest that the $q_g$ values for each CYP isoform are equal in the case of theophylline. Thus, the drug concentration at the enzyme site for each CYP isoform seems to be equal indicating that the assumption for $q_g$, the apparent liver-to-blood free concentration gradient in vivo, is appropriate.
of microsomal data. In the case of theophylline, there may be free fraction differences between microsomes and plasma, although accurate predictions of hepatic clearance have also been made without the need for such a correction. Thus, further studies are needed to clarify this point.

Fourthly, we evaluated the effects of qg and the amount of CYP as an indicator of hepatic metabolism capacity on the time-course of theophylline concentrations in serum using a physiologically-based pharmacokinetic model which introduced the PKCYP-test. The V_{d,ss} in MC-treated rats was greater than that of control rats (Table 1). Since there were no differences in the f_p and the K_{p,l,app} between control and MC-treated rats (Table 1), the reason for this remains unknown. However, one possible explanation is increased distribution into the eliminating organs in MC-treated rats. In both control and MC-treated rats, the simulated values agreed with the observed values (Fig. 3). Therefore, introduction of the qg and the amount of CYP also seems to be useful in the case of CYP1A2-mediated theophylline metabolism.

In conclusion, we have been able to show that in the case of theophylline, the PKCYP-test was applicable to drugs which are metabolized by multiple CYP isoforms using the qg value and considering the CL_{int} as the sum of the CL_{int} of each CYP isoform. We have also demonstrated that the qg value and the amount of CYP are useful parameters in the PKCYP-test using a physiologically-based pharmacokinetic model which was introduced by the PKCYP-test. However, it is necessary to discover the reasons for the relatively high qg values for the PKCYP-test. If they can be successfully identified, this will help clarify the meanings of the scaling factor, and confirm the qg as a useful parameter for the PKCYP-test.

Appendix

Theoretical basis for determination of qg^{\text{\textregistered}}: We used a well-stirred model to describe the hepatic metabolism of theophylline. As described by J. B. Houston, the intrinsic metabolic clearance (CL_{int}) is purely a measure of enzyme activity towards a drug and is not influenced by other physiological factors associated with liver clearance, such as hepatic blood flow or drug binding within the blood matrix.

The rate of metabolism in vivo (v) can be expressed as follows:

\[
v = CL_{int} \cdot C_{L,f}
\]

where C_{L,f} is the free concentration (not bound to macromolecules) of drug within the hepatic vein.

Let us assume that the drug is metabolized by a single CYP isoform. From a biochemical viewpoint, under linear conditions, when the drug concentration at the enzyme site (C_{E}) is less than 20% of the Michaelis constant (K_m), the CL_{int} can be considered in terms of the enzyme parameters of the Michaelis-Menten relationship shown in Eq. (A2).

\[
y = \frac{V_{max,vitro} \cdot A-CYP_{vivo} \cdot C_{E}}{K_{m,vitro}} \quad \text{(A2)}
\]

Where A-CYP_{vivo} is the in vivo amount of CYP. K_{m,vitro} is the in vitro Michaelis constant for the drug-enzyme interaction and V_{max,vitro} is the in vitro maximal rate of metabolism. From this, a relationship between CL_{int} and A-CYP_{vivo} can be derived as follows:

\[
CL_{int} = \frac{V_{max,vitro} \cdot A-CYP_{vivo} \cdot C_{E}}{K_{m,vitro} \cdot C_{L,f}} \quad \text{(A3)}
\]

Generally, the free concentration of drug within the hepatic vein is assumed to be C_{E}. However, if there is a concentration gradient in vivo (qg) between C_{E} and C_{L,f}, this equation can be rearranged to determine the A-CYP_{vivo}.

\[
A-CYP_{vivo} = \frac{CL_{int} \cdot K_{m,vitro}}{V_{max,vitro} \cdot qg} \quad \text{(A4)}
\]

Although the above equation allows estimation of the individual A-CYP_{vivo} in patients, a preliminary evaluation of qg is necessary. The qg value may be defined as follows, using a standard procedure:

\[
qg = \frac{CL_{int}}{A-CYP_{vivo}} \cdot \frac{K_{m,vitro}}{V_{max,vitro}} \quad \text{(A5)}
\]

Since the qg value cannot be determined practically in vivo, the measured values of CL_{int}, K_{m,vitro}, V_{max,vitro}, and A-CYP_{vivo} may be used to define it.

Moreover, the CL_{int} can be expressed as the sum of the CL_{int} of each CYP isoform. Therefore, the qg of each CYP isoform can be expressed as follows:

\[
qg = \frac{f_{CYP} \cdot CL_{int} \cdot K_{m,vitro}}{A-CYP_{vivo} \cdot V_{max,vitro}} \quad \text{(A6)}
\]

Where f_{CYP} is the contribution of each CYP isoform.

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References


