Application of the PKCYP-test to Predict the Amount of \textit{in Vivo} CYP2C11 Using Tolbutamide as a Probe

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Previous reports have shown that the determination of drug metabolism capacity can be made by the pharmacokinetic estimation of the quantity of cytochrome P450 (CYP) \textit{in vivo} (PKCYP-test), in which an apparent liver-to-blood free concentration gradient \textit{in vivo} (qg) is introduced, which is useful for evaluating fluctuations of CYP1A2 in rats. The aim of the present study was to examine the application of the PKCYP-test to evaluate the amount of \textit{in vivo} CYP2C11 by using tolbutamide as a probe, to confirm its validity using a physiologically-based pharmacokinetic rat model.

Rats treated with carbon tetrachloride (CCl\textsubscript{4}-treated) were used as a model for low levels of CYP2C11 in the liver. In CCl\textsubscript{4}-treated rats, the total body clearance (CL\textsubscript{tot}) of tolbutamide and the amount of CYP2C11 fell to about a quarter and a third of that in control rats, respectively. The time-course of tolbutamide concentrations in serum in control rats could be simulated by a physiologically-based pharmacokinetic model. In CCl\textsubscript{4}-treated rats, the amount of CYP2C11 was accurately predicted by the PKCYP-test, and the time-course of tolbutamide concentrations in serum could be predicted by the same physiologically-based pharmacokinetic model.

In conclusion, we have shown that the PKCYP-test can be used to predict levels of CYP2C11. It was also demonstrated that the qg and amount of CYP are useful parameters in the PKCYP-test by constructing a physiologically-based pharmacokinetic model which was applied to the PKCYP-test.

**Key words** PKCYP-test; probe; CYP2C11; carbon tetrachloride-treated; physiologically-based pharmacokinetic model

It is important to be able to predict the drug clearance rate for individual patients in order to select the most appropriate drug dosage regimen. Many attempts have been made to evaluate the drug metabolism capacity of individual patients by using specific probe drugs for cytochrome P450 (CYP) isozymes. In addition, several other approaches have been used to predict \textit{in vivo} drug metabolism, scaling of \textit{in vivo} metabolic clearance from \textit{in vitro} data obtained using human liver microsomes or a recombinant system of human CYP isozymes.

In our previous work, we described a new method for assessing drug metabolism capacity based on a pharmacokinetic estimation of the quantity of cytochrome P450 \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. In rats with fluctuating CYP1A2 levels, the amount of CYP could be predicted by the PKCYP-test using the clearance of acetanilide, as a probe drug, mediated by CYP1A2. Moreover, caffeine clearance mediated by CYP1A2 could also be predicted by using the predicted amount of CYP1A2. Several CYP isozymes are involved in \textit{in vivo} drug metabolism and it is important to determine how useful the PKCYP-test is in the case of other CYP isozymes.

CYP2C11 is the most abundant isozyme in liver microsomes from untreated male rats. Although CYP1A2 is inducible, CYP2C11 is a constitutive CYP isozyme. Therefore, it is of interest to examine the application of the PKCYP-test to CYP2C11. It is necessary to choose a probe drug and an animal model which has fluctuating CYP2C11 levels in order to examine the application of the PKCYP-test to CYP2C11. Tolbutamide is reported to undergo a single pathway of primary metabolism which is believed to be catalyzed by CYP2C11 in rats. Moreover, it is well known that carbon tetrachloride (CCl\textsubscript{4}) can be used to induce acute liver damage in rats. It has also been reported that the amount of CYP2C11 is reduced in rats treated with CCl\textsubscript{4}. In the present study, we examined the amount of CYP2C11 in rats treated with CCl\textsubscript{4} to see if this could be predicted by using tolbutamide as a probe.

To date, a physiologically-based pharmacokinetic model has been used as a method for analyzing the pharmacokinetics of drugs in a number of disease states. For example, serum and tissue concentrations of a drug in the presence of drug–drug interactions have been predicted by this model. One of the advantages of this model is that the effects of fluctuations in the pharmacokinetic or physiological parameters on drug disposition can be simulated. Therefore, we constructed a physiologically-based pharmacokinetic model which involved the PKCYP-test and evaluated the effects of qg and the amount of CYP used as an indicator of hepatic metabolism capacity on drug disposition.

**MATERIALS AND METHODS**

**Materials** Chemicals were obtained from the following sources: tolbutamide, chloropropamide, and goat serum were from Sigma Chemical Co. (St Louis, MO, U.S.A.); carbon tetrachloride was from Wako Pure Chemical Ind., Ltd. (Osaka, Japan); NADPH was from Oriental Yeast Co. (Tokyo, Japan); and hydroxytolbutamide and Anti-Rat CYP2C11 (anti-rat CYP2C11 antibody) were from Daiichi Pure Chemicals Co. (Tokyo, Japan). All other reagents were of reagent grade.

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grade or HPLC grade and were used without purification.

**Animals** Male Sprague–Dawley rats, 6 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats were pretreated with CCl₄ (after an 8-h fast, 2.5 ml/kg of 20% CCl₄, dissolved olive oil, was administered i.p.) to reduce CYP2C11 levels (CCl₄-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.

**Characterization of CCl₄-induced Acute Liver Damage** Analysis of serum samples from control and CCl₄-treated rats were carried out to determine the following: total protein (TP), albumin (Alb), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), cholinesterase (ChE), direct and indirect bilirubin, free fatty acid (FFA), and total cholesterol (TC) by SRL Communication for Health (Tokyo, Japan). The hematocrit (Hct) of blood samples from control and CCl₄-treated rats was determined after centrifugation at 10600×g (Kubota Hematocrit KH-120A Auto Speed Control, Kubota, Tokyo, Japan) for 15 min at room temperature.

**In Vivo Pharmacokinetic Studies** The control and CCl₄-treated rats were given tolbutamide (10 mg/kg, dissolved in saline) by intravenous injection. Blood was collected from the jugular vein and then centrifuged at 15000×g (MX-150, Tomy Seiko Co., Ltd., Tokyo, Japan) for 3 min at 20 °C until analysis; 100 μl of the free fraction was obtained and stored at −20 °C until assayed.

**Determination of Serum Protein Binding of Tolbutamide** Tolbutamide (dissolved in ethanol) was added to serum to give concentrations of 0, 10, 30, 50, 80, or 100 μg/ml (in a total volume of 800 μl). The serum samples were incubated for 30 min at 37 °C. Then, to separate the free and protein-bound fractions, 700 μl of each serum sample was transferred to a centrifuge tube (Centrifree, Amicon, Inc., Beverly, MA, U.S.A.) and centrifuged at 1000×g for 10 min at 37 °C. 100 μl of the free fraction was obtained and stored at −20 °C until analysis; 100 μl of serum samples containing protein-bound and free drug were also stored at −20 °C until assayed.

**Quantitation of CYP2C11 in Hepatic Microsomes** Hepatic microsomes were prepared as previously described. Quantitation of CYP2C11 in hepatic microsomes was performed by Western blotting. The hepatic microsomes of the control (0.3—0.6 μg protein/lane) and CCl₄-treated (0.5—3.0 μg protein/lane) rats were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 10% gels. Microsomes from male rats with known amount of CYP2C11 were also applied (26.2—52.3 pmol/lane) as a standard. The separated proteins were transferred to a nitrocellulose membrane which was then incubated with antibodies. Anti-rat CYP2C11 from goat (Daichi Pure Chemicals Co., Tokyo, Japan) was used as the first antibody. The second antibody was anti-goat IgG peroxidase conjugate, developed in rabbits (Sigma Chemical Co., St. Louis, MO, U.S.A.). The peroxidase activity of the immunoblots was monitored using an enhanced chemiluminescence kit (Amersham, Aylesbury, U.K.), and the nitrocellulose membrane exposed to photographic film (Hyper ECL, Amersham, Aylesbury, U.K.) for 20 s. The CYP2C11 protein band on the film was quantified densitometrically with an electrophoresis documentation and analysis system (Eastman Kodak, Rochester, NY, U.S.A.), and the CYP2C11 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 CYP2C11 on the abscissa.

**Inhibitory Effect of Anti-rat CYP2C11 Antibody on Tolbutamide Metabolism in Hepatic Microsomes** The assay mixture for tolbutamide metabolism contained 100 μl tolbutamide, 0.67 mg/ml MgCl₂·6H₂O, and 25 mM Tris buffer (pH 7.4) in a final volume of 2.0 ml. 0.4 μg/ml control and CCl₄-treated rat hepatic microsomes were preincubated with 100 μl anti-rat CYP2C11 antibody, which was diluted with 25 mM Tris buffer (pH 7.4) 2.5-fold, for 30 min at room temperature prior to the measurement of tolbutamide metabolite formation. Under these conditions, the CYP2C11 activity of all microsomes, irrespective of their origin, was maximally inhibited. Control incubations contained an equivalent amount of preimmune goat serum proteins. Then, the assay mixture without tolbutamide was added and preincubation was carried out in a shaking bath for 5 min at 37 °C. The reaction was started by the addition of tolbutamide and incubation carried out in a shaking bath for 30 min at 37 °C. The reaction was halted by the addition of 2 ml 2 N HCl, and the tolbutamide metabolite in the samples was assayed.

**Assay of Tolbutamide and Its Metabolite** Tolbutamide in serum was assayed by HPLC using a modification of the reported procedure. Tolbutamide and its metabolite were also assayed by HPLC. In this procedure, 2 ml 2 N HCl, 100 μl 12.5 μg/ml chlorpropamide in ethanol (internal standard) and 5 ml diethyl ether were added to 100 μl serum and then the serum sample was vortexed for 10 s and centrifuged at 450×g (RLX-105, Tomy Seiko Co., Ltd., Tokyo, Japan) for 3 min at 20 °C. Then, the organic layer was removed and transferred to a fresh tube and dried at 40 °C under nitrogen. The extraction with 5 ml diethyl ether was performed twice. Each sample was suspended in 200 μl 10% N,N-dimethylformamide and passed through a 0.45-μm filter (4 mm Milllex-LH, Nihon Millipore Ltd., Tokyo, Japan), and 30 μl was subjected to HPLC. A calibration curve was constructed by spiking drug-free rat serum with concentrated tolbutamide solution (dissolved in ethanol) to give solutions of 0, 10, 30, 50, 80, and 100 μg/ml. Samples were submitted to the extraction procedure described above and a calibration curve was obtained by plotting the peak area on the ordinate axis versus the respective drug concentrations on the abscissa.

**Characterization of CYP2C11 levels** (CCl₄-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.

**Characterization of CYP2C11 levels** (CCl₄-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.
The HPLC system consisted of a pump (L-7100, Hitachi, Tokyo, Japan), an autoinjector (L-7200, Hitachi, Tokyo, Japan), a UV detector (L-7400, Hitachi, Tokyo, Japan), an integrator (D-7500, Hitachi, Tokyo, Japan), and a column oven (TU-100, Japan Spectroscopic Co., Ltd., Tokyo, Japan). Separation was achieved using C18 reversed-phase column (150 mm × 46 mm i.d., Wakopak Handy ODS, Wako Pure Chemical Ind., Ltd., Osaka, Japan) operated at 40 °C. The mobile phase consisted of 47.5% methanol, 2.5% acetonitrile, 0.03% acetic acid and 50% water (for tolbutamide in serum) and 33.0% methanol, 2.5% acetonitrile, 0.03% acetic acid and 64.5% water (for hydroxytolbutamide in the samples for inhibition studies of in vitro tolbutamide metabolism), at a flow rate of 1.0 ml/min. The column eluate was monitored at 240 nm. The retention times for chlorpropamide and tolbutamide were 7.9 and 9.6 min, respectively (for hydroxytolbutamide in serum). The retention times of hydroxytolbutamide were 9.1 and 30.0 min, respectively (for hydroxytolbutamide in serum). The retention times of chlorpropamide and hydroxytolbutamide were 9.1 and 30.0 min, respectively (for hydroxytolbutamide in the samples for inhibition studies of in vitro tolbutamide metabolism). The lower limit of quantification was 10 μg/ml for tolbutamide and 3.125 ng/ml for hydroxytolbutamide.

**Theoretical Basis for Determination of qg**

We used a well-stirred model to describe the hepatic metabolism of tolbutamide. As described by 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 isozyme. From a biochemical viewpoint, under linear conditions, when the drug concentration at the enzyme site ($C_L$) is less than 20% of the $K_m$, the $CL_{int}$ can be considered in terms of the enzyme parameters of the Michaelis–Menten relationship shown in Eq. 2.

$$V_{max, in vitro} \cdot A\cdot CYP_{in vitro} \cdot C_E \quad K_{m, in vitro}$$

Where $V_{max, in vitro}$ is the in vitro maximal rate of metabolism and $K_{m, in vitro}$ is the in vitro Michaelis constant for the drug–enzyme interaction. $A\cdot CYP_{in vitro}$ is the in vivo amount of CYP.

From this, a relationship between $CL_{int}$ and $A\cdot CYP_{in vitro}$ can be derived as follows:

$$CL_{int} = \frac{V_{max, in vitro} \cdot A\cdot CYP_{in vitro} \cdot C_E \quad K_{m, in vitro}}{C_L \cdot f}$$

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 $C_L$, this equation can be rearranged to determine the $A\cdot CYP_{in vitro}$.

$$A\cdot CYP_{in vitro} = \frac{CL_{int} \cdot K_{m, in vitro}}{qg \cdot V_{max, in vitro}}$$

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

$$qg = \frac{CL_{int}}{A\cdot CYP_{in vitro}} \cdot \frac{K_{m, in vitro}}{V_{max, in vitro}}$$

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

Moreover, the $CL_{int}$ can be expressed as the sum of the $CL_{int}$ of each CYP isozyme. Therefore, qg and $A\cdot CYP_{in vitro}$ of each CYP isozyme can be expressed as follows:

$$qg = \frac{CL_{int} \cdot f_{CYP} \quad V_{max, in vitro}}{A\cdot CYP_{in vitro} \cdot K_{m, in vitro}}$$

$$A\cdot CYP_{in vitro} = \frac{CL_{int} \cdot f_{CYP} \quad V_{max, in vitro}}{qg \cdot K_{m, in vitro}}$$

Where $f_{CYP}$ is the contribution of each CYP isozyme.

**Analysis Using a Physiologically-Based Pharmacokinetic Model**

The physiologically-based pharmacokinetic model which introduced the PKCYP-test was applied. In this model, equation 8 was introduced to describe the hepatic intrinsic clearance ($CL_{int, h}$).

$$CL_{int, h} = \frac{V_{max, in vitro} \cdot A\cdot CYP_{in vitro} \cdot C_E \quad K_{m, in vitro}}{qg \cdot C_L \cdot f}$$

The mean of the physiological data for each animal taken from the literature was used for the blood flow rate ($Q$) and tissue volume ($V_d$) parameters of each organ in the model. The reported values of the blood-to-plasma concentration ratio ($K_P$) and the tissue-to-plasma concentration ratio of free drug ($K_{pl}$) for tolbutamide were also used. The tissue-to-plasma partition coefficient ($K_d$) values were calculated by multiplying the $K_{pl}$ values by the measured unbound fraction of drug in plasma ($f_u$) value. 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, and the concentration in serum after a single intravenous administration was calculated.

**Data Analysis**

Serum concentration versus time data were analyzed according to model-independent moment analysis. The area under the serum concentration versus time was estimated by means of the trapezoidal rule. 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 steady-state distribution volume and the total body clearance were estimated as described by Yamaoka et al. 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 given noted in the table. A $p$ value of 0.05 or less was used to indicate a significant difference between sets of data.
RESULTS

Effect of CCl4 Treatment Table 1 shows the characteristics of CCl4-induced acute liver damage in Sprague–Dawley rats. In the CCl4-treated rats, the levels of AST, ALT, ChE, ALP and bilirubin were higher than those in control rats. The serum concentration of FFA in the CCl4-treated rats was about 2-fold higher than that in control rats. CCl4-treated rats had a reduced TP and Hct level, and there were no changes in Alb and TC. The level of γ-glutamyl transpeptidase (γ-GTP) was below the limit of detection in both control and CCl4-treated rats (data not shown). Figure 1 shows the effect of CCl4 administration on hepatic microsomal CYP2C11 immunoreactivity in male rats. The amount of CYP2C11 per kilogram body weight of control and CCl4-treated rats is listed in Table 2. The amount of CYP2C11 in CCl4-treated rats was reduced to about a quarter of that in control rats.

Comparison of the Pharmacokinetics of Tolbutamide in Control and CCl4-treated Rats The blood concentration versus time profiles of tolbutamide in control and CCl4-treated rats are shown in Fig. 2. The tolbutamide profile suggests linear pharmacokinetics and, so, the total body clearance (CL tot) was determined under linear conditions. The results of linear model-independent moment analysis are given in Table 2. The CL tot of tolbutamide in control rats was linearly related to dose and agreed well with the previously reported value. Therefore, the CL tot of tolbutamide in CCl4-treated rats was about one third of that in control rats. There were no significant differences in f p and the volume of distribution at steady-state (Vd,ss).

The physiological data for the tolbutamide-treated rats used in this study is also summarized in Table 2. There were no significant differences in body weight and microsome content.

Estimation of the Contribution of CYP2C11 to the Hepatic Microsomal Metabolic Activity for Tolbutamide in Control and CCl4-treated Rats Tolbutamide is reported to be mainly catalyzed by CYP2C11 in rats. However, there are contradictory reports that another CYP isozyme is also involved in the metabolism of tolbutamide. Therefore, the contribution of CYP2C11 to hepatic microsomal tolbutamide metabolism in control and CCl4-treated rats was estimated using anti-rat CYP2C11 antibody. The effects of anti-rat CYP2C11 antibody on tolbutamide hepatic microsomal metabolism in control and CCl4-treated rats are shown in Table 3. We also examined the effect of anti-rat CYP2B1 antibody, which inhibits both CYP2B1 and CYP2B2 activities, on the hepatic microsomal metabolic activities for tolbutamide in control and CCl4-treated rats. However, we found that there were no inhibitory effects in either type of rats (data not shown). As shown in Table 3, the contribution of CYP2C11 to hepatic microsomal tolbutamide metabolism in control and CCl4-treated rats was almost equal.

Prediction of Hepatic CYP2C11 Content in CCl4-treated Rats The CL int in control rats was used to estimate the qg value of tolbutamide. Since the metabolism of tolbutamide essentially involves only the liver, CL int can be expressed by CL int, f p, and the hepatic blood flow rate (Qh), as follows:

Table 1. Characterization of CCl4-induced Acute Liver Damage in Sprague–Dawley Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl4-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g/dl)</td>
<td>5.7±0.2</td>
<td>5.4±0.2 a)</td>
</tr>
<tr>
<td>Alb (g/dl)</td>
<td>4.2±0.2</td>
<td>4.1±0.1</td>
</tr>
<tr>
<td>Hct (5%)</td>
<td>40.6±0.3</td>
<td>44.7±0.4 b)</td>
</tr>
<tr>
<td>AST (IU/L/37 °C)</td>
<td>210±35</td>
<td>2142±690 b)</td>
</tr>
<tr>
<td>ALT (IU/L/37 °C)</td>
<td>43.5±3.8</td>
<td>1377±388 b)</td>
</tr>
<tr>
<td>LDH (IU/L/37 °C)</td>
<td>5466±1173</td>
<td>9065±3441 c)</td>
</tr>
<tr>
<td>ALP (IU/L/37 °C)</td>
<td>115±169</td>
<td>1725±546 c)</td>
</tr>
<tr>
<td>ChE (IU/L/37 °C)</td>
<td>14.0±2.6</td>
<td>24.4±4.1 d)</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>0.0±0.04</td>
<td>0.29±0.14 g)</td>
</tr>
<tr>
<td>Indirect bilirubin (mg/dl)</td>
<td>0.02±0.04</td>
<td>0.16±0.11 h)</td>
</tr>
<tr>
<td>FFA (mEQ/L)</td>
<td>0.7±0.2</td>
<td>1.2±0.3)</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>79.8±9.2</td>
<td>64.4±23.0</td>
</tr>
</tbody>
</table>

a) Values are means±S.D (n=6—8). b) p<0.01. c) p<0.05.

Table 2. Pharmacokinetic and Biochemical Parameters of Tolbutamide in Control and CCl4-treated Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CCl4-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-CYP int (nmol/mg of microsomal protein)</td>
<td>0.162±0.047</td>
<td>0.043±0.011 a)</td>
</tr>
<tr>
<td>(nmol/kg)</td>
<td>181±53 b)</td>
<td>42±11 c)</td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>197±6</td>
<td>212±6</td>
</tr>
<tr>
<td>CL eq (ml/min/kg)</td>
<td>0.376±0.013</td>
<td>0.124±0.003 b)</td>
</tr>
<tr>
<td>F p (%)</td>
<td>0.045±0.004</td>
<td>0.047±0.016</td>
</tr>
<tr>
<td>Microsome content (g/kg)</td>
<td>1.12±0.12</td>
<td>0.97±0.10</td>
</tr>
<tr>
<td>Qh (ml/kg/37 °C)</td>
<td>59.7±4.0 b)</td>
<td>52±4.0 b)</td>
</tr>
<tr>
<td>V p (ml/kg/37 °C)</td>
<td>139±9</td>
<td>150±9</td>
</tr>
<tr>
<td>Km 0.05 (μM)</td>
<td>710 b)</td>
<td>—</td>
</tr>
<tr>
<td>V max int (nmol/min/mg of microsomal protein)</td>
<td>4.2 b)</td>
<td>—</td>
</tr>
<tr>
<td>(nmol/min/mmol CYP2C11)</td>
<td>25 b)</td>
<td>25 b)</td>
</tr>
<tr>
<td>A-CYP pred (nmol/kg)</td>
<td>—</td>
<td>64</td>
</tr>
</tbody>
</table>

a) Values are means±S.D (n=3—5). b) In vivo amount of CYP. c) Total body clearance. d) Unbound fraction of drug in plasma. e) Hepatic blood flow rate. f) Volume of distribution at steady-state. g) In vitro Michaelis constant. h) In vitro maximal rate of metabolism. i) Predicted in vivo amount of CYP. j) The conversion of A-CYP int per kilogram body weight was achieved by using values of 0.162±0.047 nmol/mg of microsomal protein and 1.12 g microsomal protein/kg body weight. k) Ref. 23. l) Ref. 12. m) The conversion of V max int per mmol CYP2C11 was achieved by using values of 4.2 nmol/min/mg of microsomal protein and 0.162 nmol CYP2C11/mg of microsomal protein. n) The conversion of A-CYP pred per kilogram body weight was achieved by using values of 0.043±0.011 nmol/mg of microsomal protein and 0.97 g microsomal protein/kg body weight. o) p<0.01.
For each CYP isozyme, the intrinsic clearance can be obtained by multiplying the fCYP by the apparent CL\text{int}. Therefore, the qg value of tolbutamide for CYP2C11 in control rats can be estimated according to Eq. 6 as follows:

\[
\text{CL}_{\text{int}} = \frac{Q_h \cdot CL_{\text{tot}}}{Q_h - CL_{\text{tot}}} \cdot \frac{1}{f_p} 
\]

For each CYP isozyme, the intrinsic clearance can be obtained by multiplying the fCYP by the apparent CL\text{int}. Therefore, the qg value of tolbutamide for CYP2C11 in control rats can be estimated according to Eq. 6 as follows:

\[
qg = \frac{Q_h \cdot CL_{\text{tot}}}{Q_h - CL_{\text{tot}}} \cdot \frac{1}{f_p} = \frac{Q_h \cdot CL_{\text{tot}}}{Q_h - CL_{\text{tot}}} \cdot \frac{1}{f_p} \cdot \frac{K_{m,\text{ vitro}}}{V_{\text{max, vitro}}} 
\]

\[
= \frac{59.7 \cdot 0.376 - 0.045}{181} 
\]

\[
= 0.76 \quad 710 
\]

\[
= 0.97 
\]

Assuming that the qg value is similar in control and CCl4-treated rats, when the value CL\text{tot} is obtainable, the A-CYP\text{ vitro} in CCl4-treated rats may be predicted from Eq. 7 as follows:

\[
\text{A-CYP}_\text{ vitro} = \frac{Q_h \cdot CL_{\text{tot}}}{Q_h - CL_{\text{tot}}} \cdot \frac{1}{f_p} \cdot \frac{f_{CYP}}{\text{qg}} \cdot \frac{K_{m,\text{ vitro}}}{V_{\text{max, vitro}}} 
\]

\[
= \frac{52.4 \cdot 0.124 - 0.047}{0.97} 
\]

\[
= 0.85 \quad 710 
\]

\[
= 25.9 
\]

\[
= 64 \text{ nmol/kg} 
\]

Hence, the predicted A-CYP\text{ vitro} was within the 95% confidence interval (CI) of the observed A-CYP\text{ vitro} (Table 2).

**Prediction of Time Course of Tolbutamide Concentration in Control and CCl4-treated Rats**

The tolbutamide concentration–time profiles in serum, following intravenous administration of tolbutamide to control rats at a dose of 10 mg/kg, were estimated using the physiologically based pharmacokinetic model involving the PKCYP-test. As shown in Fig. 3, by introducing the qg and the observed amount of CYP2C11 as an indicator of hepatic metabolism capacity, the tolbutamide concentration–time profiles in serum were also estimated using the physiologically-based pharmacokinetic model which involved the PKCYP-test following introduction of the qg value of control rats and the observed amount of CYP2C11. The error in predicting the tolbutamide concentration is less than 2-fold (Fig. 4).
DISCUSSION

In the present study, firstly, we examined the application of the PKCYP-test to CYP2C11. Since the amount of CYP2C11 in CCl₄-treated rats was reduced to about a quarter of that in control rats (Table 2), CCl₄-treated rats can be used as a model of reduced CYP. The increase in the level of AST, ALT, ChE, ALP, and bilirubin in CCl₄-treated rats confirms that CCl₄ treatment causes acute liver damage (Table 1). Moreover, it is believed that the increased FFA level in CCl₄-treated rats is due to stress caused by the fasting state (Table 1). The contribution of CYP2C11 to hepatic microsomal tolbutamide metabolism in both control and CCl₄-treated rats was about 80% (Table 3). Hence, qg and A-CYP\textsubscript{vivo} in CCl₄-treated rats were estimated by considering $f_{\text{CYP}} (=0.80)$. The qg value for tolbutamide estimated in control rats was found to be almost unity. Therefore, the tolbutamide concentration at the enzyme site seems to be fairly similar to its free concentration within the hepatic vein. In CCl₄-treated rats, the predicted A-CYP\textsubscript{vivo} was within the 95% confidence interval of the observed A-CYP\textsubscript{vivo}. It was demonstrated that, in the case of CYP2C11, fluctuations in the amount of CYP could be predicted by using the qg value which had been estimated in control rats.

Secondly, we constructed a physiologically-based pharmacokinetic model which was introduced in the PKCYP-test. Then, we evaluated the effects of the qg and the amount of CYP used as an indicator of hepatic metabolism capacity on the time course of tolbutamide concentrations in serum. Following application of the physiologically-based pharmacokinetic model which involved the qg and the observed amount of CYP, the simulated values agreed with the observed values in control rats (Fig. 3). Therefore, it seems that introduction of the qg and the amount of CYP is useful. In CCl₄-treated rats, the simulated values tended to be higher than the observed ones. This was probably caused by underestimation of the $f_{\text{d,ext}}$, and the reason for this underestimation is as yet unknown. However, the error in predicting the tolbutamide concentration is less than 2-fold when the observed amount of CYP2C11 was used. The observed values were within 100—80% of the lower 95% CI level, indicating that a good prediction had been obtained. Therefore, the use of the amount of CYP as an indicator of hepatic metabolism capacity seems to be useful when predicting in vivo pharmacokinetic parameters in disease states.

In conclusion, we have demonstrated that the PKCYP-test can be used in rats to predict the level of CYP2C11. We were also able to show that the qg and the amount of CYP are useful parameters in the PKCYP-test by constructing a physiologically-based pharmacokinetic model in conjunction with the PKCYP-test. In future, as far as individual patients are concerned, it will be possible to use the amount of CYP as an indicator of hepatic metabolism capacity and simulate the time course of the serum concentrations.

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