Application of the PKCYP-test in Cases of Altered CYP1A2 for Multiple CYP Systems in Rat Models of Disease

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Previously, we established a method to assess drug metabolism capacity based on a pharmacokinetic estimation of the quantity of cytochrome P450 (CYP) in vivo (PKCYP-test) by introducing an apparent liver-to-blood free concentration gradient in vivo (qg). The qg values were determined as the ratio of in vivo–in vitro clearance. In this study, we examined the application of the PKCYP-test to the clearance of acetanilide and caffeine mediated by CYP1A2 using rat models in which the levels of CYP enzymes were reduced. Rats fed a choline-deficient diet (CD-fed) and aged rats were used as models for a low level of CYP in the liver. In both rat models, the contribution (fCYP) of CYP1A2 to the in vivo intrinsic clearance values (CLint) of acetanilide and caffeine metabolism was less than unity, suggesting that other metabolic pathways are involved in the CLint. The in vivo clearance for CYP1A2 was estimated by multiplying fCYP by CLint, then the value of qg was determined as the ratio of in vivo–in vitro clearance. We predicted the level of CYP1A2 in CD-fed and aged rats, based on the clearance of acetanilide mediated by CYP1A2, using the qg value of control rats. The clearance of caffeine mediated by CYP1A2 in CD-fed and aged rats, as estimated from the predicted level of CYP1A2, correlated with the observed values.

In conclusion, we have demonstrated that the PKCYP-test can be applied to CYP1A2 for drugs metabolized by multiple CYP isozymes, and/or to models involving reduced CYP.

Key words PKCYP-test; probe; CYP1A2; choline-deficient; aged

In order to evaluate the drug metabolism capacity of individual patients, selective substrate probes have been used in vivo to identify the cytochrome P450 (CYP) isozymes involved in a variety of drug metabolism processes.1,2 It has been suggested that the in vivo intrinsic clearance by hepatic metabolism can be predicted from in vitro metabolism data by the use of either liver microsomes or a recombinant system of human CYP isozymes.2,3

Previously, we developed a novel method for determining drug metabolism capacity based on a pharmacokinetic estimation of the quantity of CYP in vivo (PKCYP-test). By using a specific probe, the drug metabolism capacity of each CYP isoform can be estimated from the PKCYP-test incorporating the apparent liver-to-blood free concentration gradient in vivo (qg).4 In rats whose CYP1A2 level has been induced by the administration of 3-methylcholanthrene (MC-treated rats), the amount of CYP1A2 could be predicted by the PKCYP-test using acetanilide as the probe. Furthermore, caffeine clearance could also be predicted by using the predicted amount of CYP1A2. However, there were some differences between observed and predicted values as far as the amount of CYP1A2 and caffeine clearance were concerned.

Both acetanilide and caffeine have been reported to be metabolized mainly by CYP1A2 in rats.5,6 However, there is a contradictory report that another CYP isozyme is also involved in the metabolism of caffeine.7 Since CYP1A2 participates mainly in the metabolism of both acetanilide and caffeine in MC-treated rats, the error in predicting the amount of CYP1A2 and caffeine clearance may be negligible. It is anticipated that the role of other CYP isozymes in their metabolism might have little effect on the prediction of the amount of CYP1A2 and caffeine clearance in other models in which the CYP1A2 enzyme level is reduced. Since there are many patients with reduced CYP levels, there is a need to investigate the application of the PKCYP-test to the reduced CYP model. Moreover, it is still unclear if the PKCYP-test can be applied to drugs that are metabolized by multiple CYP isozymes.

It has been reported that the amount of CYP is reduced both in rats fed a choline-deficient diet and in aged rats, and the amount of CYP in these rat models is regulated differently by each CYP isozone.8–11 In order to examine the application of the PKCYP-test to drugs that are metabolized by multiple CYP isozymes and/or in models with reduced CYP levels, we examined the application of the PKCYP-test to the clearance of acetanilide and caffeine mediated by CYP1A2 using rats fed a choline-deficient diet and aged rats.

MATERIALS AND METHODS

Materials Chemicals were obtained from the following sources: acetanilide, p-hydroxyacetanilide, caffeine, theobromine, and theophylline were from Wako Pure Chemicals (Osaka, Japan); 3-methylcholanthrene (MC), 1,7-dimethylxanthine and 1,3,7-trimethyluric acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); [8-14C] caffeine was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.); NADPH and a choline-deficient diet (CD) were from Oriental Yeast Co. (Tokyo, Japan); and anti-rat CYP1A1 serum for inhibition studies (anti-rat CYP1A1 antibody) and anti-rat CYP1A2 serum for inhibition studies (anti-rat CYP1A2 antibody) were obtained from Daiichi Pure Chemicals Co. (Tokyo, Japan). Anti-rat CYP1A1 antibody inhibited both CYP1A1 and CYP1A2 activity. All other chemicals used were of reagent grade or HPLC grade.

Animals Male Sprague-Dawley rats, 5, 6, and 11 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The rats 5 weeks of age were fed the CD for 5 weeks...
to induce a fatty liver (CD-fed rats). The rats 11 weeks of age were fed a normal diet for 24 weeks (aged rats). The rats 6 weeks of age were pretreated with MC, as described previously. Control animals, 6 weeks of age, 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, CD-fed, and aged rats were given acetanilide (10 or 20 mg/kg, dissolved in ethanol) or caffeine (10 or 20 mg/kg, dissolved in saline) by intravenous injection. Blood was collected from the jugular vein, and the liver was resected. The blood was centrifuged at 15000×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 they were assayed. The clearances of acetanilide and caffeine were obtained by moment analysis.

**Determination of Serum Protein Binding of Acetanilide and Caffeine** The serum protein binding of acetanilide and caffeine was determined by ultrafiltration using a Centrifree device (Amicon, Inc., Beverly, MA, U.S.A.), as described previously.

**Determination of Hepatic Blood Flow Rate** The hepatic blood flow rate was estimated using a modification of the reported procedure. CD-fed rats had a cannula inserted in the femoral artery and hepatic vein under light ether anesthesia, and indocyanine green (ICG) (2.5 mg/kg loading dose and 2.5 mg/h/kg maintenance dose) was given by injection into the femoral vein. Plasma was obtained from blood samples taken from the femoral artery at 30, 40, 50, and 60 min, and from the hepatic vein at 35, 45, 55, and 65 min after the administration of ICG. The ICG concentration in plasma was measured with a U-3210 Spectrophotometer (Hitachi, Tokyo, Japan) at a wavelength at 800 nm after an 11-fold dilution with distilled water. The hepatic blood flow rate was calculated using the equation developed by Bradley et al. The value of the hepatic blood flow rate in CD-fed rats was high enough not to affect the estimation of intrinsic metabolic clearance (\(CL_{int}\)).

**Quantitation of CYP1A2 in Hepatic Microsomes** Hepatic microsomes were prepared as previously described. The CYP1A2 level in hepatic microsomes was quantified by Western blotting, as described previously. Briefly, hepatic microsomes derived from the control (2—6 μg protein/lane) and CD-fed (2—32 μg protein/lane) rats were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Rat and CD-fed (2—32 μg protein/lane) microsomes derived from the control and aged rats) 11 weeks of age were pretreated with MC, as described previously. A polyacrylamide gel was cast in a mini electrophoresis documentation and analysis system (Eastman Kodak, Rochester, NY, U.S.A.), and the CYP1A2 protein content was determined from a standard curve generated using purified rat CYP1A2. In CD-fed rats, no CYP1A2 protein bands could be detected.

**Inhibitory Effect of Anti-rat CYP1A1 and CYP1A Antibodies on the Metabolism of Acetanilide and Caffeine in Hepatic Microsomes** The assay mixture for acetanilide metabolism contained 0.25 mM acetanilide, 0.67 mg/ml MgCl₂, 6H₂O, and 25 mM Tris buffer (pH 8.0) in a final volume of 0.5 ml (for MC-treated rats) or 1.0 ml (for control, CD-fed, and aged rats). Control, CD-fed, aged, and MC-treated rat hepatic microsomes (0.5, 1.0, 0.5, and 0.1 mg, respectively) were preincubated with 50 μl (for MC-treated rats) or 100 μl (for control, CD-fed, and aged rats) anti-rat CYP1A1 or CYP1A antibody for 30 min at room temperature prior to the measurement of acetanilide metabolite formation. Under these conditions, the CYP1A1 or CYP1A activity of all microsomes, irrespective of their origin, was maximally inhibited. Control incubations contained an equivalent amount of preimmune goat (for CYP1A1) or rabbit (for CYP1A) serum proteins. Then, the assay mixture without acetanilide was added, and preincubation was carried out in a shaking bath for 10 min (for control, CD-fed, and aged rats) or 20 min (for MC-treated rats) at 37 °C. The reaction was started by the addition of acetanilide, and incubation was carried out in a shaking bath for 10 min (for control, aged, and MC-treated rats) or 20 min (for CD-fed rats) at 37 °C. The reaction was halted by the addition of 5 ml ethyl acetate, and the acetanilide metabolites in the samples were assayed.

The assay mixture for caffeine metabolism contained 100 μM [8,14C] caffeine, 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] caffeine was adjusted to 1924 Bq/nmol by dilution with unlabeled caffeine. Control, CD-fed, aged, and MC-treated rat hepatic microsomes (0.125, 0.25, 0.0625, and 0.025 mg, respectively) were preincubated with 50 μl anti-rat CYP1A1 or CYP1A antibody for 30 min at room temperature prior to the measurement of caffeine metabolite formation. Under these conditions, the CYP1A1 or CYP1A activity of all microsomes, irrespective of their origin, was maximally inhibited. Control incubations contained an equivalent amount of preimmune goat (for CYP1A1) or rabbit (for CYP1A) serum proteins. Then, the assay mixture without caffeine was added, and preincubation was carried out in a shaking bath for 10 min (for control and CD-fed rats) or 20 min (for aged and MC-treated rats) at 37 °C. The reaction was started by the addition of caffeine, and incubation was carried out in a shaking bath for 10 min (for control, aged, and MC-treated rats) or 20 min (for CD-fed rats) at 37 °C. The reaction was halted by the addition of 500 μl 20% perchloric acid, and the radioactivity due to each of the caffeine metabolites was measured.

**Assay of Acetanilide, Caffeine and Their Metabolites** Acetanilide and caffeine in serum and liver were assayed by HPLC, as described previously. Acetanilide metabolites in the samples for inhibition studies of in vitro acetanilide metabolism were assayed by HPLC. In this procedure, 50 μl (for MC-treated rats) or 100 μl (for control, CD-fed, and aged rats) 1 μg/ml phenacetin (internal standard) and 2.5 ml 1 M sodium phosphate buffer (pH 7.0)
were added to each sample, followed by vortexing and centrifugation at 700×g (RLX-105, Tomy Seiko Co., Ltd., Tokyo, Japan) for 5 min at room temperature. The ethyl acetate phase was evaporated, and the residue was redissolved in 250 μl HPLC mobile phase and filtered through a 0.45 μm filter (4 mm Millex-LH, Nihon Millipore, Ltd., Tokyo, Japan). Following this, 30 μl of filtrate was subjected to HPLC. A calibration curve was constructed using 0.67 mg/ml MgCl2·6 H2O, 25 mM Tris buffer (pH 8.0) with concentrated p-hydroxyacetanilide solution to give standard solutions of 0.025, 0.05, 0.1, 0.2, and 0.4 μg/ml in a final volume of 0.5 ml (for MC-treated rats) or 1.0 ml (for control, CD-fed, and aged rats). Standards were then submitted to the extraction procedure described above. A calibration curve was obtained 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, U.S.A.), an autoinjector (Model 234, Gilson, Middleton, WI, U.S.A.), a variable wavelength UV detector (Programmable detector Model 118 (UV/VIS), Gilson, Middleton, WI, U.S.A.), a recorder (Chromatocorder 21, System Instruments, Tokyo, Japan), and a column oven (655A-52 column oven, Hitachi, Tokyo, Japan). A calibration curve was constructed using 0.67 mg/ml in a final volume of 0.5 ml (for MC-treated rats) or 1.0 ml (for control, CD-fed, and aged rats). Standards were then submitted to the extraction procedure described above. A calibration curve was obtained by plotting the peak-area on the ordinate axis versus the respective drug concentrations on the abscissa.

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To measure the radioactivity of caffeine metabolites in the samples from the inhibition studies of in vitro caffeine metabolism, they were separated by HPLC. Theobromine, theophylline, 1,7-dimethylxanthine, and 1,3,7-trimethyluric acid were separated by HPLC. Theobromine, theophylline, 1,7-dimethylxanthine, and 1,3,7-trimethyluric acid were separated by HPLC. Theobromine, theophylline, 1,7-dimethylxanthine, and 1,3,7-trimethyluric acid were separated by HPLC. Theobromine, theophylline, 1,7-dimethylxanthine, and 1,3,7-trimethyluric acid were separated by HPLC.

The retention times for theobromine, 1,7-dimethylxanthine, theophylline, and 1,3,7-trimethyluric acid, and caffeine were 11.6, 14.0, 14.8, 16.0, and 17.7 min, respectively. Each metabolite fraction was mixed with 5 ml Clear-sol I (Nacalai Tesque, Kyoto, Japan) and counted using a liquid scintillation counter (Beckman LS 5801, Beckman Instruments, U.S.A.).

**Theoretical Basis for Determination of qg** We used a well-stirred model to describe the hepatic metabolism of acetanilide and caffeine, as described in a previous report. Briefly, as described elsewhere, the CLint 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 \( CL_{int} \) 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, the \( CL_{int} \) can be considered in terms of the enzyme parameters of the Michaelis–Menten relationship shown in Eq. 2.

\[ v = \frac{V_{max, in vitro} \cdot A \cdot CYP_{vivo} \cdot C_{E}}{K_{M, in vitro}} \]  

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. \( A \cdot CYP_{vivo} \) is the in vivo amount of CYP and \( C_{E} \) is the drug concentration at the enzyme site.

From this, a relationship between \( CL_{int} \) and \( A \cdot CYP_{vivo} \) can be derived as follows:

\[ CL_{int} = \frac{V_{max, in vitro} \cdot A \cdot CYP_{vivo} \cdot C_{E}}{K_{M, in vitro}} \]  

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-CYPvivo:

\[ A \cdot CYP_{vivo} = \frac{CL_{int}}{K_{M, in vitro}} \cdot \frac{K_{M, in vitro}}{V_{max, in vitro}} \]  

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

\[ qg = \frac{CL_{int}}{A \cdot CYP_{vivo}} \cdot \frac{K_{M, in vitro}}{V_{max, in vitro}} \]  

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-CYPvivo may be used to define it.

If the probe or drug is metabolized solely by the liver, the total body clearance (CLtot) can be expressed as follows:

\[ CL_{tot} = \frac{Q_{h} \cdot f_{p} \cdot qg \cdot V_{max, in vitro} \cdot A \cdot CYP_{vivo}}{Q_{h} + f_{p} \cdot qg \cdot V_{max, in vitro} \cdot A \cdot CYP_{vivo}} \]  

where \( Q_{h} \) is the hepatic blood flow rate and \( f_{p} \) is the unbound fraction of drug in plasma.

Moreover, the \( CL_{int} \) can be expressed as the sum of the \( CL_{int} \) of each CYP isozyme. Therefore, qg and A-CYPvivo, and \( CL_{int} \) of each CYP isozyme can be expressed as follows:
ministration of acetanilide and caffeine in control, CD-fed, and aged rats are shown in Figs. 1 and 2, respectively. Since the body weight differed significantly among these rat models (Table 1), the total doses of acetanilide and caffeine were determined over a 5-fold range. The acetanilide and caffeine profiles suggest linear pharmacokinetics, so the $CL_{tot}$ was determined under linear conditions. The results of linear model-independent moment analysis are given in Tables 1 and 2. The $CL_{tot}$ of acetanilide in CD-fed and aged rats fell to about 20 and 30% of that in control rats, respectively, and the differences were statistically significant. The $CL_{tot}$ of caffeine in CD-fed and aged rats also fell to about 20 and 30% of that in control rats, respectively. A reduction in the $CL_{tot}$ of caffeine in aged rats agreed with a previous report.\(^{(18)}\) Although no distribution phase was observed following the administration of acetanilide and caffeine in control rats, it took 30 min for distribution in CD-fed and aged rats.

The physiological data for the acetanilide- and caffeine-treated rats used in this study are also summarized in Tables 1 and 2, respectively. There were no significant differences in $f_o$ or the volume of distribution at a steady-state ($V_{d,ss}$) for acetanilide and caffeine between the control and CD-fed or aged rats.

**Comparison of the Pharmacokinetics of Acetanilide and Caffeine in Control, CD-fed, and Aged Rats**

The blood concentration versus time profiles following i.v. administration of acetanilide and caffeine in control, CD-fed, and aged rats are shown in Figs. 1 and 2, respectively. Since the body weight differed significantly among these rat models (Table 1), the total doses of acetanilide and caffeine were determined over a 5-fold range. The acetanilide and caffeine profiles suggest linear pharmacokinetics, so the $CL_{tot}$ was determined under linear conditions. The results of linear model-independent moment analysis are given in Tables 1 and 2. The $CL_{tot}$ of acetanilide in CD-fed and aged rats fell to about 20 and 30% of that in control rats, respectively. A reduction in the $CL_{tot}$ of caffeine in aged rats agreed with a previous report.\(^{(18)}\) Although no distribution phase was observed following the administration of acetanilide and caffeine in control rats, it took 30 min for distribution in CD-fed and aged rats.

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**Estimation of the Contribution of CYP1A2 to the Hepatic Microsomal Metabolic Activity for Acetanilide and Caffeine in Control, CD-fed, Aged, and MC-treated Rats**

The contribution of CYP1A2 to hepatic microsomal ac
tanilide and caffeine metabolism in control, CD-fed, aged, and MC-treated rats was estimated using anti-rat CYP1A1 and CYP1A2 antibodies. The effects of anti-rat CYP1A1 and CYP1A2 antibodies on acetanilide and caffeine hepatic microsomal metabolism in control, CD-fed, aged, and MC-treated

\[
q_g = \frac{CL_{tot} \cdot f_{CYP}}{A \cdot CYP_{vivo}} \cdot \frac{V_{max,sterol}}{V_{max,sterol}}
\]

\[
A \cdot CYP_{vivo} = CL_{tot} \cdot f_{CYP} \cdot \frac{K_{R,sterol}}{V_{max,sterol}}
\]

\[
CL_{tot} = \frac{V_{max,sterol}}{K_{R,sterol}} \cdot A \cdot CYP_{vivo} \cdot q_g
\]

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

**RESULTS**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CD-fed</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-CYP$_{vivo}$ (pmol/mg of microsomal protein)(^{\text{a}})</td>
<td>3.89</td>
<td>ND(^{\text{a}})</td>
<td>—(^{\text{a}})</td>
</tr>
<tr>
<td>(nmol/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>186 ± 8</td>
<td>223 ± 29</td>
<td>622 ± 15(^{\text{f}})</td>
</tr>
<tr>
<td>$CL_{tot}$ (ml/min/kg)(^{\text{f}})</td>
<td>8.64 ± 0.44</td>
<td>1.95 ± 0.22(^{\text{h}})</td>
<td>2.90 ± 0.19(^{\text{h}})</td>
</tr>
<tr>
<td>$f_o$(^{\text{d}})</td>
<td>0.63 ± 0.04</td>
<td>0.68 ± 0.02</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>Microsome content (g/kg)(^{\text{g}})</td>
<td>1.09 ± 0.02</td>
<td>0.60 ± 0.06(^{\text{h}})</td>
<td>0.65 ± 0.06(^{\text{h}})</td>
</tr>
<tr>
<td>$V_{d,ss}$ (ml/kg)(^{\text{h}})</td>
<td>700 ± 95</td>
<td>728 ± 206</td>
<td>813 ± 137</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are means ± S.D. (n = 3—6). \(^{b}\) In vivo amount of CYP. \(^{c}\) Total body clearance. \(^{d}\) Unbound fraction of drug in plasma. \(^{e}\) Volume of distribution at steady-state. \(^{f}\) The conversion of A-CYP$_{vivo}$ per kg body weight was achieved by using values of 3.89 pmol/mg of microsomal protein and 1.09 g microsomal protein/kg body weight. \(^{g}\) Not detectable. \(^{h}\) p<0.01. \(^{i}\) Not determined. \(^{j}\) p<0.05.

**Table 2. Pharmacokinetic Parameters of Caffeine in Control, CD-fed, and Aged Rats\(^{\text{e}}\)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CD-fed</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>199 ± 10</td>
<td>219 ± 18</td>
<td>577 ± 25(^{\text{g}})</td>
</tr>
<tr>
<td>$CL_{tot}$ (ml/min/kg)(^{\text{g}})</td>
<td>3.26 ± 0.09</td>
<td>0.77 ± 0.18(^{\text{f}})</td>
<td>1.03 ± 0.07(^{\text{f}})</td>
</tr>
<tr>
<td>$f_o$(^{\text{d}})</td>
<td>0.85 ± 0.03</td>
<td>0.81 ± 0.06</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td>$V_{d,ss}$ (ml/kg)(^{\text{h}})</td>
<td>723 ± 45</td>
<td>636 ± 347</td>
<td>606 ± 103</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are means ± S.D. (n = 3—6). \(^{b}\) Total body clearance. \(^{c}\) Unbound fraction of drug in plasma. \(^{d}\) Volume of distribution at steady-state. \(^{e}\) p<0.01.
where qg is the value when the drug is assumed to be metabolized only by this CYP isozyme, and fb is assumed to be unity. The reported values of the K_{m,vivo} and V_{max,vivo} of acetanilide were used in this estimation.21)

Assuming that the qg value is similar in control, CD-fed, aged, and MC-treated rats, the A-CYP_{vivo} in CD-fed, aged, and MC-treated rats may be predicted from Eq. 8 as follows:

\[
\text{CYP1A2 in CD-f} = 0.90 \\
\text{CYP1A2 in aged} = 0.90 \\
\text{CYP1A2 in MC} = 0.90 \\
\]

Since the standard deviation of the f_{CYP} was the largest among the parameters used in the prediction, we took account of this when making any predictions. As far as MC-treated rats are concerned, values of CL_{rat} and f_{p} have been previously reported.4) The predicted A-CYP_{vivo} in MC-treated rats agreed with the observed value of 85.4 nmol/kg.4)

### Prediction of CYP1A2 Content in CD-fed, Aged, and MC-treated Rats

Since the contribution of CYP1A2 to the metabolism of caffeine was fluctuated in CD-fed, aged, and MC-treated rats, we tried to predict the clearance of caffeine mediated by CYP1A2 by applying the qg value. In a similar manner to acetanilide, the clearance of caffeine mediated by CYP1A2 in control rats was used to determine the qg value of caffeine for CYP1A2.

\[
\text{qg} = \frac{59.7 - 3.26}{4.24} \times \frac{1}{0.82} = \frac{200}{267} = 0.74 \\
\]

The reported values of the K_{m,vivo} and V_{max,vivo} of caffeine were used.21)

Assuming that the qg value is similar in control, CD-fed, aged, and MC-treated rats, caffeine clearance mediated by CYP1A2 (CL_{CYP1A2}) in CD-fed, aged, and MC-treated rats can be predicted by using A-CYP_{vivo} which was predicted from the acetanilide clearance mediated by CYP1A2 according to Eq. 9 as follows:

\[
\text{CL}_{\text{CYP1A2}} = \frac{V_{max,\text{vivo}}}{K_{m,\text{vivo}}} \times \text{A-CYP}_{\text{vivo}} \times \text{qg} \\
\]

\[
\text{CL}_{\text{CYP1A2}} \text{ in CD-fed} = \frac{267}{200} \times (0.44 \pm 0.23) \times 0.59 \\
= 0.35 \pm 0.18 \text{ ml/min/kg} \\
\]

Hence, the predicted CL_{CYP1A2} was smaller than the observed value (Table 4).
Table 4. Comparison of Observed and Predicted Clearance of Caffeine Mediated by CYP1A2 in Control, CD-fed, Aged, and MC-treated Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CD-fed</th>
<th>Aged</th>
<th>MC-treated</th>
</tr>
</thead>
</table>
| CL_{obs} (ml/min/kg)
| 3.33±0.24 | 0.68±0.06 | 0.66±0.15 | 80.3±2.6 |
| CL_{pred} (ml/min/kg)
| — | 0.35±0.18 | 0.49±0.13 | 76.3±2.4 |

a) Values are means±S.D. (n=3). b) Observed clearance mediated by CYP1A2. c) Predicted clearance mediated by CYP1A2. d) Reported value corrected by the present method.

Hence, the predicted CL_{CYP1A2} agreed with the observed value (Table 4).

\[ CL_{CYP1A2} \text{ in aged rats} = \frac{267}{200} \cdot (0.62±0.16) \cdot 0.59 = 0.49±0.13 \text{ ml/min/kg} \]

Hence, the predicted CL_{CYP1A2} in MC-treated rats agreed well with the measured values (Table 4).

\[ CL_{CYP1A2} \text{ in MC-treated rats} = \frac{267}{200} \cdot (96.9±3.0) \cdot 0.59 = 76.3±2.4 \text{ ml/min/kg} \]

Hence, the predicted CL_{CYP1A2} agreed with the observed value (Table 4).

DISCUSSION

In the case of drugs which are metabolized by more than two CYP isozymes, the intrinsic metabolic clearance is expressed as the sum of the intrinsic metabolic clearance of each CYP isozyme. It is important to confirm that the PKCYP-test can be applied to each CYP isozyme. Initially, we examined the PKCYP-test with regard to CYP1A2.

In the present study, we examined the application of the PKCYP-test to the clearance of acetanilide and caffeine mediated by CYP1A2 by using rat models in which the level of the CYP enzyme is reduced. In CD-fed and aged rats, the contribution of CYP1A2 to acetanilide and caffeine metabolism was less than that in the control rats (Table 3). It has been reported that the amount of CYP is reduced in CD-fed and aged rats, and the degree of reduction in each CYP is regulated in a different way.8—11) Therefore, it seems that the content of each CYP isozyme in CD-fed and aged rats might be different from that in control rats, and the contribution of CYP isozymes, except for CYP1A2, to acetanilide and caffeine metabolism could not be neglected in these rat models.

The predicted A-CYP_{vivo} corresponding to CYP1A2 from the clearance of acetanilide, according to Eq. 8 in CD-fed and aged rats, was 10 and 15% of the control rats, respectively. In CD-fed rats, because of the detection limit of the assay method, the observed value of A-CYP_{vivo} could not be obtained by Western blotting. Consequently, it was impossible to confirm whether the predicted and observed A-CYP_{vivo} values agreed or not. However, the clearance of caffeine mediated by CYP1A2 could be predicted by the PKCYP-test using the predicted A-CYP_{vivo}.

In aged rats, the predicted clearance of caffeine mediated by CYP1A2, which was estimated from the predicted A-CYP_{vivo}, agreed with the observed value (Table 4). On the other hand, in CD-fed rats, the predicted clearance of caffeine mediated by CYP1A2 was smaller than the observed value (Table 4). The excretion of unchanged acetanilide and caffeine into the urine in CD-fed rats was negligible (data not shown). Because of the detection limit of the assay method, the effect of anti-rat CYP1A1 and CYP1A2 antibodies on acetanilide and caffeine metabolism in CD-fed rat liver microsomes could not be estimated under linear conditions. Therefore, this difference may be due to an error in the estimated contribution of CYP1A2 to acetanilide and caffeine metabolism.

In MC-treated rats, the error in the predicted A-CYP_{vivo} and clearance of caffeine was 1.5- and 1.2-fold greater than the measured values, respectively, assuming that acetanilide and caffeine are metabolized only by CYP1A2.4) On the other hand, the predicted values both of A-CYP_{vivo} and the clearance of caffeine using the present method in MC-treated rats agreed well with the measured values (Table 4).

In conclusion, we were able to show that the PKCYP-test can be applied to the CYP1A2 for drugs metabolized by multiple CYP isozymes. By using the different qg values for acetanilide and caffeine estimated in the control rats, the A-CYP_{vivo} in CD-fed, aged, and MC-treated rats could be predicted. If there are specific probes for each CYP isozyme, the CL_{tot} of drugs which are metabolized by multiple CYP isozymes can be predicted by the PKCYP-test. In the future, it will be possible to measure the fCYP in a search for each individual enzymatic activity following the coadministration of each specific probe, as is the case in a “cocktail” study.

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