Characterization of cytochrome P450-mediated drug metabolism in cats

S. S. SHAH
S. SANDA
N. L. REGMI
K. SASAKI &
M. SHIMODA

Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan

In this study we examined activities of cytochrome P450 (CYP)1A, 2C, 2D and 3A using hepatic microsomes from five male and five female cats. CYP1A, 2C, 2D and 3A activities were referred by ethoxyresorufin O-deethylation (EROD), tolbutamide hydroxylation (TBH), bufuralol 1'-hydroxylation (BLH) and midazolam 1'- and 4-hydroxylation respectively. The anti-rat CYP1A2 and CYP3A2 serum significantly inhibited EROD and midazolam 1'- and 4-hydroxylation, suggesting that EROD and midazolam 1'- and 4-hydroxylation were catalysed by CYP1A and 3A in cats respectively. Quinidine inhibited BLH in cats microsomes at quite low concentrations, suggesting that BLH was catalysed by CYP2D in cats. Tolbutamide hydroxylation activities were negligible in hepatic microsomes from both male and female cats, suggesting CYP2C activities of cats are extremely low. This suggests that CYP2C substrates should be carefully administered to cats. Although there is no sexual difference in CYP1A activities, there are differences in CYP2D and 3A activities of cats. CYP2D activities were higher (3-fold), but CYP3A activities were lower (one-fifth) in female cats. These results might suggest that CYP2D and 3A substrates should be prescribed for male and female cats using different dosage regimen.

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Minoru Shimoda, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho 3-5-8, Fuchu, Tokyo 183-8509, Japan. E-mail: ms@cc.tuat.ac.jp

INTRODUCTION

Drug metabolism is one of the determinant factors for drug disposition. This is due to the fact that many drugs are lipophilic and mainly excreted from the body after they are metabolized to polar metabolites in the liver. Of the enzymes catalysing biotransformation of drugs, cytochrome P450 (CYP) subfamilies are well recognized to be important in phase I metabolism of drugs.

Of the CYP subfamilies, CYP1A, 2C, 2D and 3A subfamilies mainly relate to oxidative metabolism of many drugs in animal species. The activities of these CYPs, therefore, have been well characterized in many animal species, including rats (Kobayashi et al., 2002), mice (Perloff et al., 2000), dogs (Kuroha et al., 2002a), chickens (Khalil et al., 2001), pigs (Sztókóvá et al., 2004), cattle (Sztókóvá et al., 2004), goats (Sztókóvá et al., 2004), sheep (Sztókóvá et al., 2004) and humans (Shimada et al., 1997). Many drugs, which are metabolized by CYPs, have been used for feline diseases. In addition, prescription of off-label drugs is not rare in cats, because approved drugs are limited for cats. The information on the CYP activities is, therefore, quite important in feline clinics. The CYP activities, however, have not been characterized well in cats.

In the present study, therefore, we examined Michaelis–Menten kinetics of the metabolic reactions catalysed by CYP1A, CYP2C, CYP2D and CYP3A in male and female cats using hepatic microsomes to characterize the activities of the enzymes in this animal species. The reactions that were described in earlier studies for these CYP activities in humans and some other animal species (Chauret et al., 1997 and Sharer et al., 1995) were used after confirming by using antibodies or specific inhibitors. These reactions included ethoxyresorufin O-deethylation (EROD), tolbutamide hydroxylation (TBH), bufuralol 1'-hydroxylation (BLH), and 1'- and 4-hydroxylation of midazolam (MDZ1'H and MDZ4H) for CYP1A, 2C, 2D and 3A activities respectively.

MATERIALS AND METHODS

Animals

Five male and five female cats (short hair, 1 year old, 2.2–3.0 kg) were obtained from Iffa Credo (France). The cats were housed in stainless-steel cages individually with a 12-h light/dark cycle. Stable temperature and relative humidity were maintained at 19–22 °C and 40–70% respectively. The cats
were given food (Science diet, feline maintenance, Hill’s pet Nutrition, Topeca, KS, USA) once a day and allowed access to water ad libitum. The animals were subjected to the experiment after 24 h fast. This experiment was conducted in accordance with the guidelines for the care and use of laboratory animals, Faculty of Agriculture, Tokyo University of Agriculture and Technology.

Drugs and chemicals

Resorufin, ethoxyresorufin, d-glucose-6-phosphate, glucose 6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tolbutamide, hydroxytolbutamide, midazolam, 1'-hydroxymidazolam, 4-hydroxymidazolam, polyclonal antibody to rat CYP1A2, CYP3A2 were obtained from Daichi Chemical (Tokyo, Japan). Bufuralol hydrochloride and 1'-hydroxybufuralol were obtained from Gentest (Woburn, MA, USA). Quinidine sulphate (QN) was purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals used as reagents were of highest purity analytical or HPLC grade.

Preparation of feline liver microsomes

Cats were deeply anaesthetized by intravenous injection of sodium pentobarbital (25 mg/kg bw), and exsanguinated from a cannula inserted into the carotid and then the liver was immediately isolated. The liver was instantly perfused with ice-cold homogenated buffer (1.15% KCl, 0.2 mM EDTA-2Na, 0.1 mM dithiothreitol, 0.1 mM phenyl methyl sulphonyl fluoride and 20% glycerol) from the caudal vena cava until the efflux perfusion buffer was blood free, and then stored at −80 °C until preparation of microsomes.

Microsomal fractions were prepared from the liver specimens as described by van der Hoeven and Coon (1974). The obtained microsomal suspension was stored at −80 °C until used. The protein concentration and CYP content were determined as described by Bradford (1976) and Omura and Sato (1964) respectively.

Enzyme-specific assays

Enzyme kinetics of the CYP1A, CYP2C, CYP2D and CYP3A isoenzymes were used for assaying cat CYP activities. The reaction proceeded at 37 °C in a reaction mixture that contained a system of NADPH generation [50 mM phosphate buffer (pH 7.4), 0.5 mM β-NADP⁺, 5 mM glucose-6-phosphate, 1.5 U/mL glucose 6-phosphate dehydrogenase and 5 mM MgCl₂], liver microsomes (approx. 0.03 mg/mL for EROD and approx. 0.4 mg/mL for other reactions), and a substrate at various concentrations in a total volume of 0.25 mL, except for EROD which was conducted in a total volume of 1 mL. A 5-min preincubation step at 37 °C was performed before the reaction was started by the addition of substrate.

Specificities of substrates

We examined the inhibitory effects of antibodies against CYP1A and 3A on EROD and midazolam hydroxylolation (MDZ1'H and MDZ4H) respectively. The anti-serum was added into the assay system at 0, 10, 20 and 50 μL. The serum from immunized rabbits was also added into the assay system to keep 50 μL volume of added serum (anti-sera + normal sera). Ethoxyresorufin and midazolam concentrations were 0.5 and 122 μM respectively. The enzyme reaction was initiated after 30 min incubation at room temperature. As a positive control, the inhibitory effects were also examined in the assay system containing rat microsomes.

As anti-rat CYP2D serum was not available, the inhibitory effect of quinidine, specific CYP2D inhibitor (Newton et al., 1995), on BLH was examined in this study. Quinidine sulphate was dissolved in ethanol, and the solution was added into the assay system at 10 μL. The quinidine concentration in the assay system ranged from 0.03 to 0.1 μM. The enzyme reaction was initiated just after the addition of quinidine solution.

Determination of CYP1A activity

The metabolite of ethoxyresorufin, resorufin, was measured by use of fluorometric method described by Burke et al. (1977). Concentrations of ethoxyresorufin in the assay system ranged from 0.065 to 2.07 μM. Reactions were terminated by addition of 3 mL of methanol 15 min after the substrate was added, followed by placement on ice for 5 min. After centrifugation at 2000 g for 5 min, 1 mL of the resulting supernatant was transferred to a clean test tube, diluted with 4 mL of methanol, and then applied to a spectrophuorometer (RF-1500; Shimadzu Corporation, Kyoto, Japan). Excitation and emission wavelengths were set at 550 and 586 nm respectively. The detection limit was 0.05 nm at a signal-to-noise ratio of 3. The recovery of resorufin was 103 ± 6% (CV, 5.9%) at 20 nm (n = 4). The intraday CV values were 3.7% and 5.1% at 20 and 400 nm (n = 4) respectively. The inter-day CV values ranged from 3.4% to 6.4% and 1.6% to 5.1% at 20 and 400 nm respectively (3 days, 4 determinations/day).

Determination of CYP2C activity

The metabolite of tolbutamide, hydroxytolbutamide, was measured by use of HPLC as reported by Miners et al. (1988). Tolbutamide concentrations in the assay system ranged from 0.5 to 10 μM. After the addition of tolbutamide, the assay system was incubated for 30 min, and the reactions were quenched by 0.15 mM phosphoric acid (0.25 mL). Thirty microlitres of chlorproamid solution (10 μg/mL) were added as an internal standard. Samples were mixed with 3 mL of diethylether. After centrifugation at 2000 g for 5 min, the upper organic layer was transferred to a clean pear-shaped flask. The solvent was evaporated to dryness under reduced pressure. The residue was reconstituted with 500 μL of mobile phase, and then 50 μL of the solution was injected to a reversed-phase column (TSK-gel
ODS-120, 4.6 × 250 mm; TOSOH Co., Tokyo, Japan). The column effluent was monitored by UV absorbance at 230 nm. The gradient-elution programme was used to detect hydroxytolbutamide and chloropropamide. The mobile phase consisted of solvent A (50 mm phosphate buffer, pH 4.3) and solvent B (acetonitrile). The solvent composition was held at 75% solvent A for 8 min and then changed linearly to 80% solvent B for 15 min. The flow rate was 1 mL/min. The recoveries of hydroxytolbutamide and chloropropamide were 91.2 ± 1.2% (CV = 1.3%) and 96.2 ± 3.7% (CV = 3.9%) at 1 and 10 μg/mL respectively (n = 4). The intra-day CV values were 1% and 4% at 0.1 and 1 μg/mL respectively (n = 4). The inter-day CV values ranged from 1.9% to 8.2% and 1.3% to 6.3% at 0.1 and 1 μg/mL respectively (3 days, 4 determinations/day), with the limit of quantification of 20 ng/mL at a signal-to-noise ratio of 10.

Determination of CYP2D activity

The metabolite of bufuralol, 1′-hydroxybufuralol, was analysed by HPLC as described by Kronbach et al. (1987). The concentrations of bufuralol in the assay system ranged from 3.125 to 200 μM. After the addition ofbufuralol, the assay system was incubated for 10 min, and reaction was stopped by the addition of 0.03 mL of 60% perchloric acid. The denatured protein was precipitated by centrifugation at 10 000 g for 2 min, and then 50 μL of the supernatant was injected to reversed-phase column (TSK-gel ODS-120). The mobile phase consisted of 1 mm perchloric acid and acetonitrile (65:35, v/v). The flow rate was 1.0 mL/min. Excitation and emission wavelengths for fluorometric determination of 1′-hydroxybufuralol were 352 and 302 nm respectively. Recovery of 1′-hydroxybufuralol was 91.3 ± 3.0% (CV = 3.3%) at 0.1 μg/mL (n = 4). The intra-day CV values were 1.8% and 2.1% at 0.1 and 1 μg/mL respectively (n = 4). The inter-day CV values ranged from 1.9% to 7.9% and 1.9% to 3.1% at 0.1 and 1 μg/mL respectively (3 days, 4 determinations/day), with the limit of quantification of 20 ng/mL at a signal-to-noise ratio of 10.

Determination of CYP3A activity

The metabolites of midazolam, 1′-hydroxymidazolam and 4-hydroxymidazolam were determined using HPLC, as described by the method reported by Kuroha et al. (2002b). Midazolam concentrations in the assay system ranged from 3.8 to 307 μM. After the addition of midazolam, the mixture was incubated for 10 min, and the reaction was stopped by the addition of 0.25 mL of acetonitrile and placed on ice for 3 min. After centrifugation at 10 000 g for 2 min, the resulting supernatant was applied to a reversed phase column (TSK-gel ODS-120). The mobile phase consisted of 100 mm of acetate buffer (pH 4.7), acetonitrile and methanol (59.4:35.5:5.6, v/v/v). Column effluent was monitored by UV absorbance at 254 nm. The flow rate of the mobile phase was 1 mL/min. The detection limits of 1′- and 4-hydroxymidazolam were 3.6 and 2.5 ng/mL respectively, at a signal-to-noise ratio of 3. The recovery of 1′-hydroxymidazolam was 101 ± 1.4% (CV = 1.2%) at 1 μg/mL (n = 4). The intra-day CV values were 1.4% and 5.4% at 0.1 and 1 μg/mL respectively (n = 4). The inter-day CV values ranged from 1.2% to 2.4% and 2.1% to 5.7% at 0.1 and 1 μg/mL respectively (3 days, 4 determinations/day). The recovery of 4-hydroxymidazolam was 102 ± 3.4% (CV = 2.4%) at 1 μg/mL (n = 4). The intra-day CV values were 1% and 3.9% at 0.1 and 1 μg/mL respectively (n = 4). The inter-day CV values ranged from 0.2% to 0.5% and 2.6% to 8.5% at 0.1 and 1 μg/mL respectively (3 days, 4 determinations/day).

Michaelis–Menten kinetic analysis

The following equation was applied to the relation between reaction velocities (V) and substrate concentrations (S) to analyse the enzyme kinetics of EROD and MDZ4H:

\[ V = \frac{V_{\text{max}} S}{K_m + S} \]  

In Eqn 1, \( V_{\text{max}} \) and \( K_m \) represent maximal reaction velocity and Michaelis–Menten constant. To analyse kinetics of BLH, the following equation was applied to the relation between reaction velocities (V) and substrate concentrations (S):

\[ V = \frac{V_{\text{max}} S}{K_m + S} + AS \]

where A is proportionality constant in nonspecific reaction.

The kinetic profile of MDZ1′H was expressed by the following equation:

\[ V = \frac{V_{\text{max}} S}{K_m + S(1 + \frac{S}{K_i})} \]

In the above equation \( K_i \) is the substrate inhibition constant. Kinetic parameters were calculated, using a nonlinear curve-fitting program MULTI created by Yamaoka et al. (1981).

Intrinsic clearances (\( Cl_{\text{int}} \)) of each reaction were calculated by dividing \( V_{\text{max}} \) by \( K_m \) except for BLH. Those of BLH were calculated by the sum of A and \( V_{\text{max}}/K_m \).

Statistical analysis

Test for homogeneity of variance was conducted by F-test. If the test revealed homogeneity of variance, two-tailed Student’s t-test was used for comparison of enzyme kinetic parameters between male and female. In case of heterogeneity of variance, Aspin–Welch t-test was used. The difference was considered significant at \( P < 0.05 \).

RESULTS

Substrate specificity for cat CYPs

The anti-rat CYP1A2 and CYP3A2 polyclonal antibodies inhibited EROD and midazolam hydroxylation (MDZ1′H and MDZ4H), as shown in Fig. 1. The extent of inhibition was similar
between rats and cats, suggesting that EROD and MDZ1’H were catalysed by CYP1A and 3A, respectively, in cats. In the case of MDZ4H inhibitory effect was smaller in cats, suggesting that MDZ4H may be partially catalysed by a CYP other than CYP3A.

In Fig. 2, quinidine inhibited BLH in cat microsomes at quite low concentrations, suggesting that BLH was catalysed by CYP2D in cats.

In vitro CYP-mediated activities

Figure 3 shows Michaelis–Menten kinetics of EROD, BLH, MDZ1’H and MDZ4H in male and female cats. The kinetics of EROD and MDZ4H were consistent with single Michaelis–Menten kinetics. Kinetics of BLH was consistent with single Michaelis–Menten kinetics with first-order kinetics. MDZ1’H obeyed Michaelis–Menten kinetics with uncompetitive substrate inhibition (Kuroha et al., 2002b).

Although EROD and MDZ4H activities were not significantly different between males and females, BLH activities were significantly higher in females. On the other hand, MDZ1’H activities were significantly higher in males. TBH activities were negligible in both sexes as shown in Fig. 4.

Table 1 shows Michaelis–Menten kinetic parameters of the CYP-dependent reactions from male and female cats. The $V_{\text{max}}$
values of BLH were significantly higher in females. On the other hand, those of MDZ1\(\cdot\)H were significantly higher in males. In Km values of midazolam hydroxylation there were significant sexual differences. Based on intrinsic clearance (Cl\text{int}), BLH and MDZ4H activities of female cats were approximately 3-fold and 4-fold higher respectively. On the other hand, MDZ1\(\cdot\)H activities of male cats were similar to those of dogs. On the other hand, those of female cats were lower than dogs (less than one-fifth) but similar to humans.

The CYP contents were 0.20 ± 0.11 and 0.27 ± 0.07 nmol/mg protein in male and female cats respectively.

**DISCUSSION**

In this study, activities of CYP1A, 2C, 2D and 3A subfamilies were examined in male and female cats using hepatic microsomes. As a result, we demonstrated that cats have negligible TBH activities, suggesting that CYP2C activities are extremely low in cats. In addition, we demonstrated that there are sexual differences in CYP2D and 3A activities.

As there has been no information in cats that EROD, TBH, BLH and midazolam hydroxylation are mainly catalysed by CYP subfamilies in hepatic microsomes from male and female cats

<table>
<thead>
<tr>
<th>Reaction</th>
<th>(V_{max}) (nmol/min/kg)</th>
<th>(K_m) ((\mu)M)</th>
<th>(A) ((\mu)L/min/mg protein)</th>
<th>(K_s) ((\mu)M)</th>
<th>Cl\text{int} ((\mu)L/min/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>EROD</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>0.21 ± 0.07</td>
<td>0.075 ± 0.003</td>
<td>2800 ± 880</td>
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<tr>
<td>Female</td>
<td>0.17 ± 0.09</td>
<td>0.13 ± 0.05</td>
<td>1500 ± 1200</td>
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<tr>
<td>BLH</td>
<td></td>
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<tr>
<td>Male</td>
<td>0.023 ± 0.01(^a)</td>
<td>5.9 ± 3.1</td>
<td>0.07 ± 0.03</td>
<td>4.9 ± 2.7(^c)</td>
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</tr>
<tr>
<td>Female</td>
<td>0.036 ± 0.007(^a)</td>
<td>3.5 ± 2</td>
<td>0.059 ± 0.034</td>
<td>13 ± 7(^c)</td>
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<tr>
<td>MDZ1(\cdot)H</td>
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</tr>
<tr>
<td>Male</td>
<td>0.24 ± 0.06</td>
<td>1.9 ± 1.7</td>
<td>570 ± 240</td>
<td>200 ± 120</td>
<td></td>
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<tr>
<td>Female</td>
<td>0.15 ± 0.06</td>
<td>7.7 ± 4.5</td>
<td>400 ± 210</td>
<td>38 ± 50</td>
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<tr>
<td>MDZ4H</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.22 ± 0.02</td>
<td>43 ± 22(^b)</td>
<td>6.1 ± 3.0(^d)</td>
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<tr>
<td>Female</td>
<td>0.18 ± 0.06</td>
<td>8.1 ± 1.9(^b)</td>
<td>22 ± 6(^d)</td>
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</table>

Each value is represented by mean ± SD (\(n = 5\)). \(V_{max}\), \(K_m\) and \(K_s\) were estimated using a nonlinear least square fitting program. Cl\text{int} was calculated by dividing \(V_{max}\) by \(K_m\). Superscripts indicate significant differences between values with the same superscript (\(P < 0.05\)).
were referred from Umehara et al. Humans were referred from Perloff. Data of dogs were referred from Kuroha et al. 

phenobarbital, which is also a CYP2C substrate becomes much faster after repeated doses because of CYP2C induction in dogs (Hojo et al., 2002). In cats, however, the elimination is not affected by repeated doses even for 3 weeks (Cochrane et al., 1990). The nonalteration in phenobarbital elimination may suggest that the inducing effect of the drug was negligible in cats. This may be due to an extremely low activities of CYP2C in cats.

In contrast, the cats have S-mephénytoin 4’-hydroxylase activity, compared with other species (Chauret et al., 1997). As the reaction is catalysed by CYP2C19 in humans, the following conclusions may be drawn; cat has extremely low activities of CYP2C isoforms corresponding to CYP2C9 in humans, but they might have substantial activities of CYP2C isoforms corresponding to CYP2C19 in humans.

There were sexual differences in the activities of CYP2D and 3A of cats, when \( Cl_{int} (V_{max}/K_m) \) values were compared (Table 1). The difference in CYP2D activities were dependent on the values of \( V_{max} \) but not \( K_m \), suggesting that female cats have larger amount of this enzyme in their livers. In CYP3A activities, the difference was due to both \( V_{max} \) and \( K_m \). Although net activities of midazolam hydroxylation were higher in male, MDZ4H activities were higher in female because of lower \( K_m \) values. This observation may suggest that some CYP3A substrates show slower elimination in male cats, due to lower affinity to the enzyme, because higher \( K_m \) values indicate lower affinity.

Our data about CYP1A activities did not show variations due to sexual difference. This is in accordance with the result of Chauret et al. (1997), who have reported no differences in phenacetin O-deethylotation activity, another catalytic marker for human P450 1A1/2 (between male and female cats).

Prescription of off-label drugs is not rare in small animal including cats. We, therefore, compared the CYP activities with dogs and humans (Fig. 5). Based on intrinsic clearance of reactions used in this study, CYP1A activities of cats were quite higher than dogs and humans, suggesting that elimination rate of CYP1A substrates like theophylline may be higher in cats, compared with dogs and humans. Gregus et al. (1983) also observed high CYP1A activities of cats, compared with those of dogs. CYP2D activities of female cats were similar to dogs, whereas those of male cats were lower than dogs but similar to humans. These results may suggest that the elimination rate of CYP2D substrates in female cats may be similar to dogs and that in male cats may be similar to humans. On the other hand, CYP3A activities of female cats were similar to humans and those of male were similar to dogs. These results may suggest that the elimination rate of CYP3A substrates in female cats may be similar to humans and that male cats possess an activity in the same order of magnitude as that in dogs. Drugs recognized as a CYP2C substrate are widely used in humans and dogs. However, we should pay much attention to the administration of CYP2C substrates to cats, because cats have extremely low activities of CYP2C isoforms corresponding to CYP2C9 in humans.

Fig. 5. Comparison of intrinsic clearances (\( Cl_{int} \)) of ethoxyresorufin O-deethylolation (EROD), bufuralol 1’-hydroxylation (BLH), and midazolam 1’- and 4-hydroxylation (MDZ1’H and MDZ4H) among cats, dogs and humans. \( Cl_{int} \) values were calculated by dividing \( V_{max} \) by \( K_m \). Data of dogs were referred from Kuroha et al. (2001a,b). CYP 3A data of humans were referred from Perloff et al. (1999). Other data of humans were referred from Umehara et al. (2002).

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