

## Species Differences in the Pharmacokinetic Parameters of Cytochrome P450 Probe Substrates between Experimental Animals, such as Mice, Rats, Dogs, Monkeys, and Microminipigs, and Humans

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### Abstract

To clarify species differences in the pharmacokinetic parameters of cytochrome P450 (CYP) activities between humans and experimental animals, we assessed several CYP activities in mice, rats, dogs, monkeys, and microminipigs, using the simultaneous administration of typical human CYP substrates, such as caffeine (human CYP1A2 substrate), losartan (CYP2C9), omeprazole (CYP2C19), dextromethorphan (CYP2D6), and midazolam (CYP3A), to these animals. The intrinsic clearance ( $CL_{int}$ ) of these 5 substrates was also examined using the liver microsomes of humans and the experimental animals. The high bioavailabilities (BAs) and low  $CL_{int}$  values of caffeine in the experimental animals were similar to those in humans. Mice and monkeys had lower BAs and higher  $CL_{int}$  values of losartan than those in humans. Mice, rats, and monkeys had lower BAs and higher  $CL_{int}$  values of omeprazole than those in humans. The lowest BAs of dextromethorphan were observed in monkeys and microminipigs, and only the  $CL_{int}$  in dogs was similar to that in humans. The BA of midazolam in microminipigs only was similar to that in humans, while the  $CL_{int}$  values in the other animals were similar to that in humans. These results indicated that *in vitro* and *in vivo* experimental data obtained using multiple animals including microminipigs are useful for predicting human pharmacokinetics.

**Keywords:** Cytochrome P450 (CYP); Species differences; Drug metabolism; Microminipig

### Introduction

The cytochrome P450s (CYPs) are involved in the metabolism of many endogenous compounds and xenobiotics [1,2]. CYP3A is most commonly described as a contributor to drug clearance in humans (approximately 50% of the drug metabolized by P450s), followed by CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (5-15%, respectively) [3]. All families of CYPs possess highly conserved regions of amino acid residues and relatively small differences have been reported in the primary amino acid sequences of CYPs across species. However, even small changes in amino acid sequences can give rise to marked differences in substrate specificity and catalytic activity. Thus, species differences in CYP isoforms are a major cause of the differences reported in drug metabolism.

Experimental animals, such as mice, rats, dogs, monkeys, and minipigs, are commonly used in nonclinical studies for new drug candidates to predict pharmacokinetic profiles in humans. Therefore, species differences in CYP activities, which have been reported in a number of studies, need to be clarified between experimental animals and humans. Humans and rodents, such as mice and rats, generally exhibit greater species differences in CYP activities; however, rodents have frequently been used as animal models in drug discovery [4-11]. On the other hand, Bogaards et al. [12] reported that CYP1A activity in mouse microsomes and CYP3A activity in mouse and rat microsomes were similar to those in human microsomes [12]. Although dogs are frequently used as non-rodent species in nonclinical studies, information concerning the canine CYP system is more limited. The hepatic metabolism of specific human CYP2C substrates, such as tolbutamide, warfarin, and (S)-mephentoin, is known to be impaired more in dogs than in humans [13], whereas metabolism

studies using liver microsomes demonstrated that the metabolism of human CYP2D substrates, such as bufuralol and dextromethorphan, was similar in humans and dogs [10,11,14]. Monkeys were originally considered to have pharmacokinetic properties similar to those of humans due to genetic similarities, however, previous studies showed that the bioavailability (BA) of some drugs including human CYP3A substrates was markedly lower in monkeys than in humans [7,15-20]. Monkeys have also been shown to exhibit higher CYP activities for human CYP2D6 substrates in their liver microsomes than humans [10,12,16,21,22]. Pigs and minipigs are increasingly being used in pharmacokinetic and toxicity studies, because their metabolism has been subjected to further research and has recently attracted considerable attention [23]. Drug metabolism mediated by human CYP1A and CYP3A in their liver microsomes of these animals was reported to be similar to that of humans [12,24,25], whereas their CYP activities for CYP2D substrates were higher than human activities [12,26]. The microminipig (a small minipig registered as a novel variety of swine with the Japanese Ministry of Agriculture, Forestry,

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and Fisheries) has emerged as a possible experimental animal for *in vivo* nonclinical studies because of their more suitable body weight than pigs and minipigs [27,28]. We previously showed that CYP2D activity in microminipig was higher than that in humans, as well as pigs and minipigs [29,30]. However, species differences in *in vivo* CYP activities have not yet been examined simultaneously using the same CYP substrates in humans and experimental animals, such as mice, rats, dogs, monkeys, and microminipigs.

A cocktail approach is used to assess *in vivo* CYP activity and can concurrently detect the *in vivo* activities of multiple CYPs following a single simultaneous administration of multiple CYP-specific substrates. Many cocktail studies have been conducted using humans, such as the Cooperstown 5+1 cocktail [31], Karolinska cocktail [32], Inje cocktail [33], and Basal cocktail [34]. Of these cocktail methods, the Inje cocktail includes all commercially available drugs such as caffeine (human CYP1A2 substrate), losartan (human CYP2C9 substrate), omeprazole (human CYP2C19 substrate), dextromethorphan (human CYP2D6 substrate), and midazolam (human CYP3A substrate) for which the human pharmacokinetic properties are known [33]. Furthermore, no evidence has been found for drug-drug interactions in the Inje cocktail in humans [33].

To clarify species differences in the pharmacokinetic parameters of CYP activities between humans and experimental animals, we assessed multiple CYP activities in mice, rats, dogs, monkeys, and microminipigs, using simultaneous dosing with the Inje cocktail to these experimental animals. In addition, the intrinsic clearance ( $CL_{in}$ ) of the 5 CYP substrates using the liver microsomes of humans and these experimental animals were investigated.

## Materials and Methods

### Chemicals

Caffeine, omeprazole, 5-hydroxyomeprazole, dextromethorphan hydrobromide monohydrate, midazolam, and diazepam were purchased from Wako Pure Chemical (Osaka, Japan). Losartan potassium was purchased from LKT Laboratories (St. Paul, MN, USA). Paraxanthine, dextropropranolol tartrate, nadolol, and propranolol hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). EXP3174 and 1'-hydroxymidazolam were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co. (Tokyo, Japan). Liver microsomes from three individual microminipigs (8 months old, male) were prepared as described previously [29]. Human (mixed gender), ICR mouse (male), SD rat (male), beagle dog (male), and cynomolgus monkey (male) liver microsomes were purchased from Xenotech (Lenexa, KA, USA). All other chemicals and solvents were of the highest grade commercially available.

### Animal experiments

Crlj:CD1(ICR) mice (7 weeks old, male) and Crl:CD(SD) rats (7 weeks old, male) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). microminipigs (6 months old, male) were purchased from Fuji Micra Inc. (Shizuoka, Japan). The animal experiments (preparation of the dosing solution, administration, and blood collection) using beagle dogs (6 months old, male) and cynomolgus monkeys (3-4 years old, male) were contracted to Kamakura Techno-Science, Inc. (Kanagawa, Japan) and CMIC BIORESERCH CENTER Co., Ltd. (Yamanashi, Japan), respectively. 24 mice, 6 rats, 3 dogs, 3 monkeys, and 3 microminipigs were used for each animal experiment

and the plasma concentrations and the pharmacokinetic parameters were determined as the mean of three animals. All animals used in this study were deprived of food for 16 h before the experiments. They were intravenously and orally administered the Inje cocktail, in which caffeine (1.6 mg/kg), losartan (0.83 mg/kg), omeprazole (0.33 mg/kg), dextromethorphan (0.50 mg/kg), and midazolam (0.033 mg/kg) were dissolved in a 60% polyethylene glycol solution in-house. The intravenous administration was performed using the same dogs, monkeys, and microminipigs 7 days after the oral administration. Blood samples were collected 5, 15, and 30 min and 1, 2, 4, 8, and 24 h after the intravenous administration, and 15, 30, and 45 min and 1, 2, 4, 8, and 24 h after the oral administration to mice, rats, dogs, monkeys, and microminipigs. Blood samples were centrifuged at  $1200 \times g$  for 10 min at 4 °C. Isolated plasma was stored at -20 °C until later analysis. Animal experiments using mice, rats, and microminipigs were conducted according to the Guideline for the Animal Experiments, Research and Development Division, Toray Industries, Inc. Animal experiments using dogs were conducted according to the Guidelines for Animal Experiments, Kamakura Techno-Science, Inc. Animal experiments using monkeys were conducted according to the Guidelines for Animal Experiments, CMIC Bioreserch Center Co.

### Measurement of chemical concentrations in plasma by LC-MS/MS

Samples containing 50  $\mu$ l of plasma, 150  $\mu$ l of methanol, and 5  $\mu$ l of an internal standard solution (nadolol, propranolol, and diazepam) in 50% methanol (1000, 10, and 1000 ng/ml respectively) were centrifuged at  $1200 \times g$  for 10 min at 4 °C. Fifteen microliters of the supernatants were diluted in 135  $\mu$ l of 0.1% formic acid. Fifty-microliter aliquots were analyzed by LC-MS/MS. The analytical column was an Ascentis Express C18 (2.7  $\mu$ m, 2.1  $\times$  100 mm) column (Sigma-Aldrich, St. Louis, MO, USA). The LC system was an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). The flow rate was set at 0.2 ml/min. Separation was performed at 40°C with a gradient system generated from 0.1% formic acid (A) and 0.1% formic acid in methanol (B): B was held at 5% for 1 min, increased linearly to 100% in 16 min, held at 100% for another 4 min, and then returned back to 5% in 0.1 min, followed by reequilibration for 3 min. The total cycle time for one injection was 24.1 min. Mass spectrometry experiments were conducted on an API-5000 LC-MS/MS system (Applied Biosystems, Forster City, CA, USA) with positive ionization electrospray. Mass transitions were as follows: m/z 195 $\rightarrow$ 137 for caffeine, m/z 181 $\rightarrow$ 124 for paraxanthine, m/z 423 $\rightarrow$ 207 for losartan, m/z 437 $\rightarrow$ 235 for EXP3174, m/z 346 $\rightarrow$ 198 for omeprazole, m/z 362 $\rightarrow$ 214 for 5-hydroxyomeprazole, m/z 272 $\rightarrow$ 171 for dextromethorphan, m/z 258 $\rightarrow$ 157 for dextropropranolol, m/z 326 $\rightarrow$ 291 for midazolam, m/z 342 $\rightarrow$ 324 for 1'-hydroxymidazolam, m/z 310 $\rightarrow$ 254 for nadolol, m/z 261 $\rightarrow$ 116 for propranolol, and m/z 285 $\rightarrow$ 154 for diazepam. The relative scan times were as follows: 0.00 to 9.00 min for caffeine, paraxanthine, dextropropranolol, and nadolol as an internal standard, 9.01 to 13.5 min for omeprazole, 5-hydroxyomeprazole, dextromethorphan, midazolam, 1'-hydroxymidazolam, and propranolol as an internal standard, and 13.51 to 24.1 min for losartan, EXP3174, and diazepam as an internal standard. The lower limits of qualification were 2 ng/ml for caffeine and paraxanthine, 1 ng/ml for losartan and EXP3174, 0.2 ng/ml for omeprazole, 5-hydroxyomeprazole, dextromethorphan, and 1'-hydroxymidazolam, and 0.1 ng/ml for dextropropranolol and midazolam. The standard curves were linear from 2.0 to 1000 ng/ml for caffeine and paraxanthine, from 1.0 to 400 ng/ml for losartan and EXP3174, from 0.2 to 80 ng/ml for omeprazole, 5-hydroxyomeprazole, dextromethorphan, and 1'-hydroxymidazolam, and from 0.1 to 40 ng/ml for dextropropranolol and midazolam. Precision indicated as a coefficient validation was less

than 10.9 % for all chemicals. Accuracy ranged from 89.0% to 108% for all drugs in the determination.

### Pharmacokinetic data analysis

Plasma concentrations vs. time profiles were analyzed using WinNonlin software (Version 6.2, Pharsight Corporation, Mountain View, CA, USA). The non-compartmental model was employed to estimate the following pharmacokinetic parameters: terminal elimination half-life ( $t_{1/2}$ ), area under the plasma concentration vs. time curve from 0 h to 24 h ( $AUC_{0-24h}$ ), volume of distribution ( $V_{d,ss}$ ), and total body clearance ( $CL_{tot}$ ). The peak plasma concentration ( $C_{max}$ ) and time to reach  $C_{max}$  ( $T_{max}$ ) were directly recorded from experimental observations. BA was determined using the following equation: BA (%) =  $\{AUC_{0-24h}(p.o.)/AUC_{0-24h}(i.v.)\} \times \{Dose_{i.v.}/Dose_{p.o.}\} \times 100$ , where  $AUC_{0-24h}(i.v.)$  and  $AUC_{0-24h}(p.o.)$  are  $AUC_{0-24h}$  after the intravenous and oral administrations, respectively. The  $AUC_{0-24h}$  ratio of an unchanged drug/metabolite as a phenotypic measure of CYP activity was determined using the following equation:  $AUC_{0-24h}$  ratio of an unchanged drug/metabolite =  $\{AUC_{0-24h}(p.o.) \text{ of metabolite}/AUC_{0-24h}(p.o.) \text{ of an unchanged drug}\} \times (MW \text{ of an unchanged drug}/MW \text{ of a metabolite})$ , where MW is the molecular weight.

### In vitro metabolism using liver microsomes

The time courses of caffeine, losartan, omeprazole, dextromethorphan, and midazolam were obtained in the liver microsomes of humans, mice, rats, dogs, monkeys, and microminipigs. Incubation conditions were set based on previous studies [8,35]. Each drug was incubated with a reaction mixture (250  $\mu$ l) consisting of a liver microsomal protein and NADPH-generating system (2 mM NADPH, 10 mM glucose-6 phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 5 mM  $MgCl_2$ ) in the presence of 100 mM potassium phosphate buffer (pH 7.4). After being preincubated at 37°C for 10 min, enzyme reactions were initiated by the addition of 50  $\mu$ l of NADPH solution in water. The final concentration of each drug used was 1  $\mu$ M. Microsomal concentrations were 0.2 mg/ml (dextromethorphan and midazolam), 0.5 mg/ml (losartan and omeprazole), and 1.0 mg/ml (caffeine). After being incubated at 37°C for various time periods, reactions were terminated by the addition of 250  $\mu$ l of acetonitrile. After stopping the metabolic reactions, the reaction mixtures were centrifuged at 1200  $\times g$  for 10 min. Fifteen microliters of the supernatants were diluted in 135  $\mu$ l of 0.1% formic acid. Ten-microliter aliquots were analyzed by LC-MS/MS.

In this experiment, the unchanged concentrations of all drugs were determined using LC-MS/MS analysis. The analytical column was an Ascentis Express C18 (2.7  $\mu$ m, 2.1  $\times$  50 mm) column (Sigma-Aldrich, St. Louis, MO, USA). The LC system was an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). The flow rate was set at 0.6 ml/min. Separation was performed at 40°C with a gradient system generated from 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B): B was held at 1% for 0.5 min, increased linearly to 100% in 3.5 min, held at 100% for another 1 min, and then returned back to 1% in 0.1 min, followed by reequilibration for 1.5 min. The total cycle time for one injection was 6.6 min. Mass spectrometry experiments were conducted on an API-5000 LC-MS/MS system (Applied Biosystems, Forster City, CA, USA) with positive ionization electrospray. Mass transitions were as follows: m/z 195 $\rightarrow$ 137 for caffeine, m/z 423 $\rightarrow$ 207 for losartan, m/z 346 $\rightarrow$ 198 for omeprazole, m/z 272 $\rightarrow$ 171 for dextromethorphan, and m/z 326 $\rightarrow$ 291 for midazolam.

All assay methods had a concentration range of 0.1 to 2  $\mu$ M.

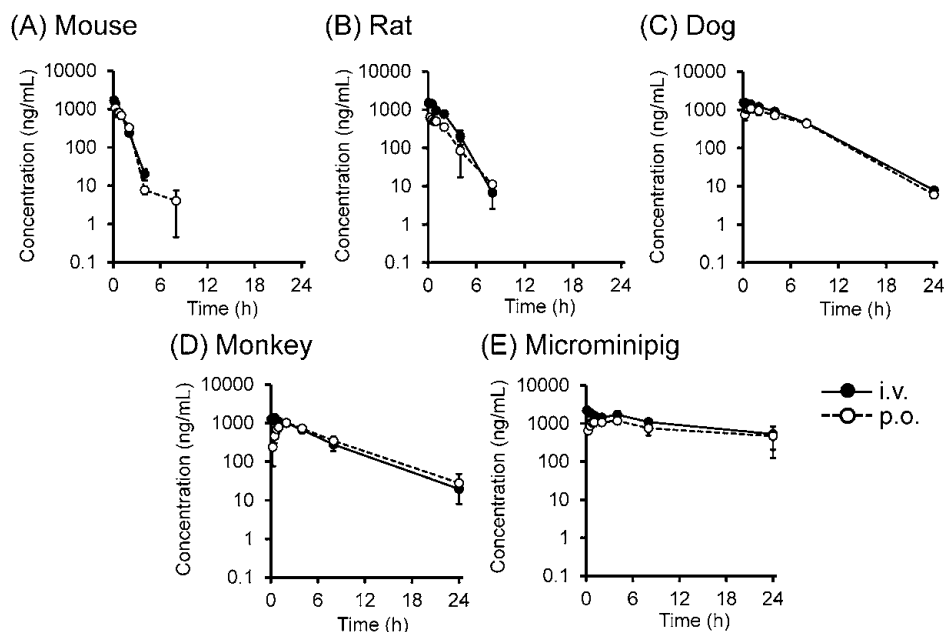
Reproducibility was evaluated by performing triplicate analyses of microsomal samples containing 0.1, 0.5, and 1  $\mu$ M of drug, respectively. The coefficient of validation was less than 15%, and the actual concentration of the drugs ranged between 91.3 and 110%.

$CL_{int}$  was calculated using the following equation based on the time course of the residual ratio of unchanged drugs as determined using least-squares linear regression [28]  $CL_{int}$  (ml/min/mg protein) =  $k_e$ /microsomal protein concentration, where  $k_e$  is the disappearance rate constant.

In the liver microsome study, the units of  $CL_{int}$  values were converted to per kilogram of body weight using the following equation [16]:  $CL_{int}$  (ml/min/kg) =  $CL_{int}$  (ml/min/mg protein)  $\times SF1$  (mg protein/g liver)  $\times SF2$  (g liver/kg body weight), where  $SF1$  is the microsomal protein content per gram of liver (45 was used for all species [9]) and  $SF2$  is the liver weight per kilogram of body weight (25.7, 87.5, 40.0, 32.0, and 32.0 were used for humans, mice, rats, dogs, and monkeys, respectively [36]). The value obtained in our laboratory, 17.3, was used as the  $SF2$  for microminipigs).

### Results

The plasma concentrations of caffeine, losartan, omeprazole, dextromethorphan, and midazolam as well as their metabolites were simultaneously measured after their intravenous and oral administration to mice, rats, dogs, monkeys, and microminipigs. The plasma concentrations of caffeine and pharmacokinetic parameters of caffeine and paraxanthine are shown in (Figure 1 and Table 1), respectively. After its intravenous and oral administration, the plasma concentrations of caffeine in mice and rats decreased rapidly with elimination  $t_{1/2}$  of 0.573 to 2.75 and 0.828 to 1.14 h, respectively (Figure 1A and 1B, Table 1). The plasma concentrations of caffeine after its intravenous and oral administration to dogs and monkeys decreased moderately with  $t_{1/2}$  of 2.81 to 2.82 and 3.94 to 4.21, respectively (Figure 1C and 1D, Table 1). A slow decline was found in the plasma concentrations of caffeine after its intravenous and oral administration to microminipigs with  $t_{1/2}$  of 12.9 to 19.9 h (Figure 1e, Table 1). Mice, rats, dogs, monkeys, and microminipigs showed high BAs of caffeine (89.3%, 44.7%, 83.0%, 101%, and 69.2%, respectively) (Table 1). The  $AUC_{0-24h}$  ratio of paraxanthine/caffeine was the highest in mice and monkeys (1.03 and 1.21, respectively), followed by rats (0.892) > dogs (0.374) > microminipigs (0.0731) (Table 1). The plasma concentrations of losartan and pharmacokinetic parameters of losartan and EXP3174 are shown in (Figure 2 and Table 2), respectively. After its intravenous and oral administration, the plasma concentrations of losartan in mice decreased rapidly with  $t_{1/2}$  of 0.0860 to 1.24 (Figure 2A, Table 2). The plasma concentrations of losartan after its intravenous and oral administration to rats and microminipigs decreased moderately with  $t_{1/2}$  of 1.87 to 2.70 and 1.82 to 3.59 (Figure 2B, 2E, Table 2). Slow declines were found in the plasma concentrations of losartan after its intravenous and oral administration to dogs and monkeys with  $t_{1/2}$  of 8.07 to 13.1 and 4.13 to 4.52, respectively (Figure 2C, 2D, Table 2). Mice and monkeys showed lower BAs for losartan (1.92% and 13.0%, respectively) than rats, dogs, and microminipigs (49.5%, 38.4%, and 28.7%, respectively) (Table 2). The  $AUC_{0-24h}$  ratio of EXP3174/losartan was markedly higher in mice (87.3), followed by rats and microminipigs (1.31 and 1.57, respectively) > monkeys (0.489) > dogs (0.133) (Table 2). The plasma concentrations of omeprazole and pharmacokinetic parameters of omeprazole and 5-hydroxyomeprazole are shown in (Figure 3, Table 3), respectively. After its intravenous and oral administration, the plasma concentrations of omeprazole in mice, rats, dogs, monkeys, and microminipigs decreased rapidly



**Figure 1:** Plasma concentration of caffeine in mice (A), rats (B), dogs (C), monkeys (D), and microminipigs (E) after the single intravenous and oral administration of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam. Each point and bar is represented as the mean  $\pm$  S.D. of three animals.

Pharmacokinetic parameters	Mouse		Rat		Dog		Monkey		Microminipig	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Caffeine										
C <sub>max</sub> (ng/ml)		1066		629 $\pm$ 166		1100 $\pm$ 200		1020 $\pm$ 105		1203 $\pm$ 216
T <sub>max</sub> (h)		0.250		0.417 $\pm$ 0.289		0.750 $\pm$ 0.250		1.67 $\pm$ 0.58		1.92 $\pm$ 1.81
t <sub>1/2</sub> (h)	0.573	2.75	0.828 $\pm$ 0.072	1.14 $\pm$ 0.51	2.82 $\pm$ 0.18	2.81 $\pm$ 0.22	3.94 $\pm$ 0.61	4.21 $\pm$ 0.74	12.9 $\pm$ 5.1	19.9 $\pm$ 13.8
AUC <sub>0-24h</sub> (h.ng/ml)	1850	1651	3629 $\pm$ 647	1622 $\pm$ 470	4.82 $\pm$ 0.03	9375 $\pm$ 1163	8233 $\pm$ 1237	8232 $\pm$ 1201	25137 $\pm$ 6209	17692 $\pm$ 6263
V <sub>d,ss</sub> (ml/kg)	794		749 $\pm$ 88		11282 $\pm$ 123		948 $\pm$ 80		797 $\pm$ 36	
CL <sub>tot</sub> (ml/h/kg)	876		456 $\pm$ 85		682 $\pm$ 8		195 $\pm$ 32		49.8 $\pm$ 18.9	
BA (%)		89.3		44.7	141 $\pm$ 2	83.0 $\pm$ 9.4		101 $\pm$ 13		69.2 $\pm$ 8.1
Paraxanthine		1066								
C <sub>max</sub> (ng/ml)		626		218 $\pm$ 24		243 $\pm$ 75		503 $\pm$ 41		60.8 $\pm$ 18.0
T <sub>max</sub> (h)		1.00		1.67 $\pm$ 0.58		4.00 $\pm$ 0.00		6.67 $\pm$ 2.31		12.0 $\pm$ 10.6
t <sub>1/2</sub> (h)		1.49		2.20 $\pm$ 1.30		7.05 $\pm$ 0.78		26.9 $\pm$ 16.6		25.4
AUC <sub>0-24h</sub> (h.ng/ml)		1577		1343 $\pm$ 328		3216 $\pm$ 1071		9145 $\pm$ 652		1075 $\pm$ 299
AUC <sub>0-24h</sub> ratio of paraxanthine/caffeine		1.03		0.89		0.374 $\pm$ 0.139		1.21 $\pm$ 0.19		0.0731 $\pm$ 0.0336

Each data represents the mean  $\pm$  S.D. of three determinations, except for data in mice and BA and AUC<sub>0-24h</sub> of paraxanthine/caffeine in rats.

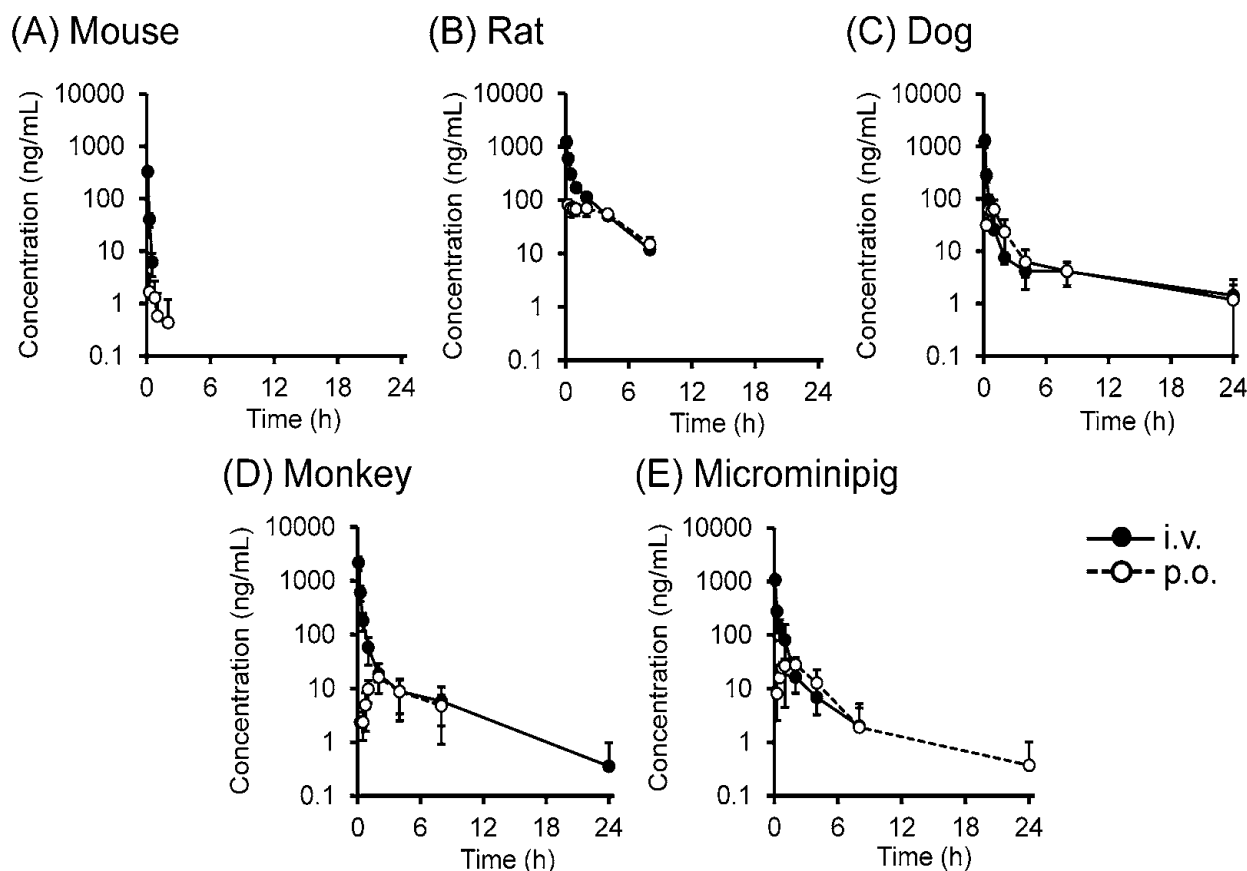
Data in mice are calculated from the average plasma concentration of the three animals.

BA and AUC<sub>0-24h</sub> of paraxanthine/caffeine in rats are calculated using the average AUC<sub>0-24h</sub> of the three animals.

i.v., intravenous; p.o., peroral; C<sub>max</sub>, the peak plasma concentration; T<sub>max</sub>, the time to reach C<sub>max</sub>; t<sub>1/2</sub>, terminal elimination half-life; AUC<sub>0-24h</sub>, area under the plasma concentration vs. time curve from 0 h to 24 h; V<sub>d,ss</sub>, volume of distribution; CL<sub>tot</sub>, total body clearance; BA, bioavailability; N.C., not calculated.

**Table 1:** Pharmacokinetic parameters of caffeine and paraxanthine in mice, rats, dogs, monkeys, and microminipigs after the intravenous and oral administrations of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam





**Figure 2:** Plasma concentration of losartan in mice (A), rats (B), dogs (C), monkeys (D), and microminipigs (E) after the single intravenous and oral administration of 5 mg losartan. Each point and bar is represented as the mean  $\pm$  S.D. of three animals.

Pharmacokinetic parameters	Mouse		Rat		Dog		Monkey		Microminipig	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
<b>Losartan</b>										
$C_{max}$ (ng/ml)		1.66		83.4 $\pm$ 20.6		73.9 $\pm$ 21.5		16.5 $\pm$ 1.2		28.7 $\pm$ 8.4
$T_{max}$ (h)		0.250		1.08 $\pm$ 0.88		0.750 $\pm$ 0.250		2.67 $\pm$ 1.15		1.25 $\pm$ 0.66
$t_{1/2}$ (h)	0.0860	1.24	1.87 $\pm$ 0.07	2.70 $\pm$ 1.12	13.1 $\pm$ 7.7	8.07 $\pm$ 3.40	4.13 $\pm$ 1.33	4.52 $\pm$ 1.34	1.82 $\pm$ 0.87	3.59 $\pm$ 3.02
$AUC_{0-24h}$ (h·ng/ml)	90.9	1.75	1044 $\pm$ 69	517 $\pm$ 101	464 $\pm$ 127	182 $\pm$ 76	798 $\pm$ 286	107 $\pm$ 42	458 $\pm$ 160	131 $\pm$ 64
$V_{d,ss}$ (ml/kg)	653		1506 $\pm$ 273		8584 $\pm$ 5560		1196 $\pm$ 585		1222 $\pm$ 384	
$CL_{tot}$ (ml/h/kg)	9206		849 $\pm$ 62		1771 $\pm$ 528		1146 $\pm$ 432		2015 $\pm$ 747	
BA (%)		1.92		49.5		38.4 $\pm$ 10.2		13.0 $\pm$ 3.8		28.7 $\pm$ 11.9
<b>EXP3174</b>										
$C_{max}$ (ng/ml)		62.3		57.6 $\pm$ 14.9		4.33 $\pm$ 2.92		8.73 $\pm$ 3.85		18.6 $\pm$ 15.1
$T_{max}$ (h)		0.750		5.33 $\pm$ 2.31		1.25 $\pm$ 0.66		2.67 $\pm$ 1.15		4.00 $\pm$ 0.00
$t_{1/2}$ (h)		2.21		6.11 $\pm$ 0.65		11.2 $\pm$ 7.7		2.19 $\pm$ 0.19		8.01 $\pm$ 3.75
$AUC_{0-24h}$ (h·ng/ml)		158		700 $\pm$ 89		22.3 $\pm$ 18.4		46.1 $\pm$ 20.6		173 $\pm$ 155
$AUC_{0-24h}$ ratio of EXP3174/ losartan		87.3		1.31		0.133 $\pm$ 0.092		0.489 $\pm$ 0.288		1.57 $\pm$ 1.57

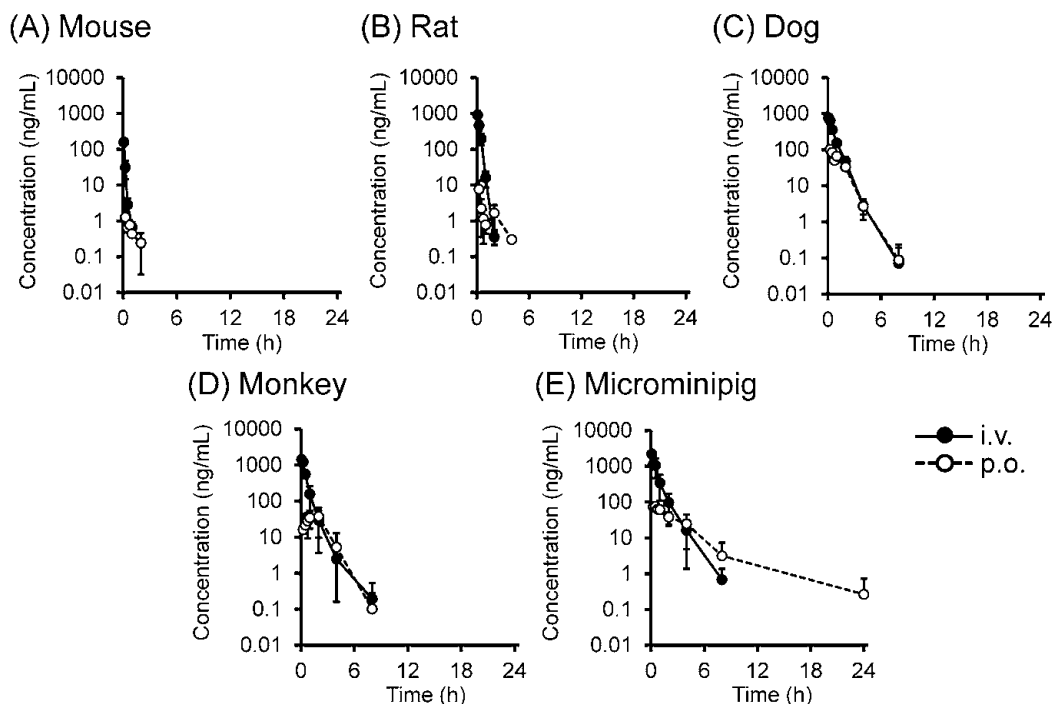
Each data represents the mean  $\pm$  S.D. of three determinations, except for data in mice and BA and  $AUC_{0-24h}$  of EXP3174/losartan in rats.

Data in mice are calculated from the average plasma concentration of the three animals.

BA and  $AUC_{0-24h}$  of EXP3174/losartan in rats are calculated using the average  $AUC_{0-24h}$  of the three animals.

i.v., intravenous; p.o., peroral;  $C_{max}$ , the peak plasma concentration;  $T_{max}$ , the time to reach  $C_{max}$ ;  $t_{1/2}$ , terminal elimination half-life;  $AUC_{0-24h}$ , area under the plasma concentration vs. time curve from 0 h to 24 h;  $V_{d,ss}$ , volume of distribution;  $CL_{tot}$ , total body clearance; BA, bioavailability; N.C., not calculated.

**Table 2:** Pharmacokinetic parameters of losartan and EXP3174 in mice, rats, dogs, monkeys, and microminipigs after the intravenous and oral administrations of 5 mg substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam.



**Figure 3:** Plasma concentration of omeprazole in mice (A), rats (B), dogs (C), monkeys (D), and microminipigs (E) after the single intravenous and oral administration of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam. Each point and bar is represented as the mean  $\pm$  S.D. of three animals.

with  $t_{1/2}$  of 0.200 to 0.973, 0.179, 0.666 to 0.741, 0.572 to 0.745, and 0.951 to 2.15, respectively (Figure 3, Table 3). Mice and rats showed low BAs of omeprazole (3.05% and 1.89%, respectively) (Table 3). The BAs of omeprazole in monkeys and microminipigs were slightly lower (12.3% and 17.7%, respectively) than that in dogs (26.7%) (Table 3). The  $AUC_{0-24h}$  ratio of 5-hydroxyomeprazole/omeprazole was the highest in mice (3.94), followed by rats (1.81) > dogs, monkeys, and microminipigs (0.276, 0.387, and 0.231, respectively) (Table 3). The plasma concentrations of dextromethorphan and pharmacokinetic parameters of dextromethorphan and dextropran are shown in (Figure 4 and Table 4), respectively. After its intravenous and oral administration, the plasma concentrations of omeprazole in mice, rats, dogs, monkeys, and microminipigs decreased rapidly with  $t_{1/2}$  of 0.722, 1.68, 1.78 to 2.96, 2.66, and 3.20, respectively (Figure 4, Table 4). All plasma concentrations of dextromethorphan after its oral administration to monkeys were below the quantitative detection limit (Figure 4D). Mice, rats, dogs, monkeys, and microminipigs showed low BAs of dextromethorphan (1.06%, 2.15%, 1.45%, 0.00%, and 0.676%, respectively) (Table 4). The  $AUC_{0-24h}$  ratio of dextropran/dextromethorphan was the highest in microminipigs (7.45), followed by mice (3.94) > rats (0.713) > dogs (0.00) (Table 4). The ratio of  $AUC_{0-24h}$  of dextropran/dextromethorphan could not be calculated in monkeys, because the  $AUC_{0-24h}$  of dextromethorphan was 0.00 (Table 4). The plasma concentrations of midazolam and pharmacokinetic parameters of midazolam and 1'-hydroxymidazolam are shown in (Figure 5 and Table 5), respectively. After its intravenous and oral administration, the plasma concentrations of midazolam in mice, rats, dogs, monkeys, and microminipigs decreased rapidly (Figure 5) with  $t_{1/2}$  of 0.275, 0.327, 0.555 to 0.960, 0.904, and 1.12 to 1.29, respectively (Table 5). Mice, rats, dogs, and monkeys showed low BAs of midazolam (7.26%, 1.21%, 7.94%, and 1.66%, respectively),

whereas its BA in microminipigs was 34.3% (Table 5). The  $AUC_{0-24h}$  ratio of 1'-hydroxymidazolam/midazolam was markedly higher in mice (23.9), followed by dogs (3.07) > rats (1.90) > monkeys (0.879) > microminipigs (0.108) (Table 5).

(Table 6) shows the  $CL_{int}$  calculated from the time courses of caffeine, losartan, omeprazole, dextromethorphan, and midazolam in the liver microsomes of humans and the experimental animals. The  $CL_{int}$  values of caffeine in the liver microsomes of the experimental animals were within  $\pm 3$ -fold that of humans (Table 6). The  $CL_{int}$  values of losartan in the liver microsomes of mice and monkeys were approximately 8- and 4-fold higher than that of humans, respectively, whereas the  $CL_{int}$  values in rats, dogs, and microminipigs were within  $\pm 3$ -fold that in humans (Table 6). The  $CL_{int}$  values of omeprazole in the liver microsomes of mice, rats, and monkeys were approximately 6-, 6-, and 4-fold higher than that of humans, respectively, whereas the  $CL_{int}$  values in dogs and microminipigs were within  $\pm 3$ -fold that in humans (Table 6). The  $CL_{int}$  values of dextromethorphan in the liver microsomes of mice, rats, monkeys, and microminipigs were approximately 17-, 23-, 24-, and 12-fold higher than that of humans, respectively, whereas the  $CL_{int}$  in dogs only was within  $\pm 3$ -fold that in humans (Table 6). The  $CL_{int}$  values of midazolam in the liver microsomes of the experimental animals were within approximately  $\pm 3$ -fold that of humans (Table 6).

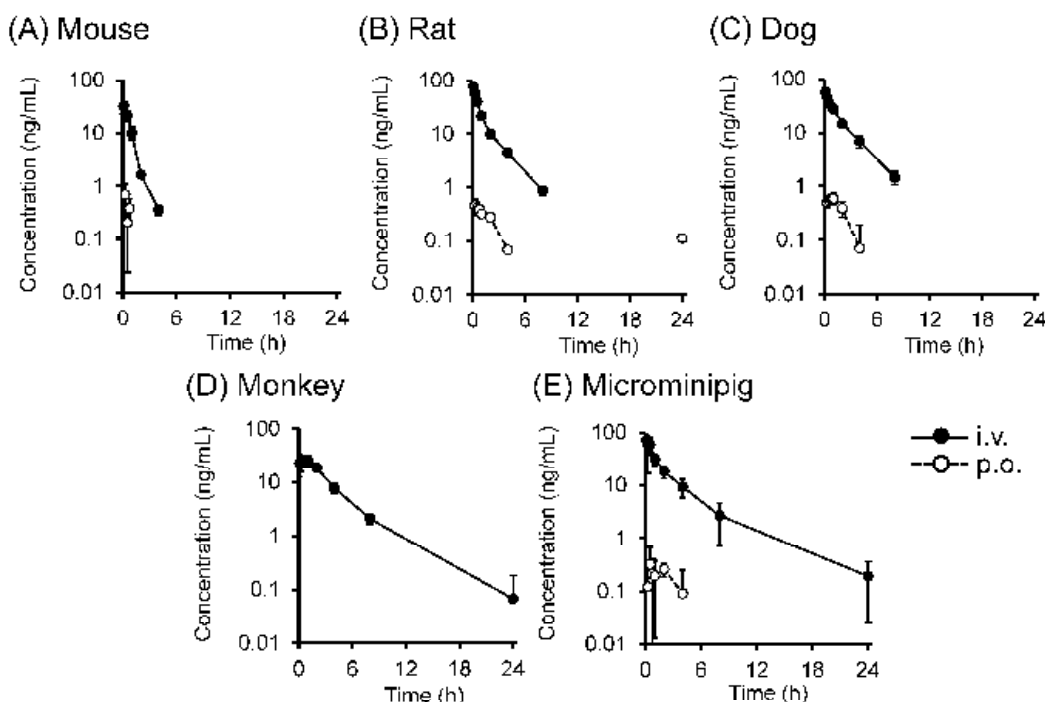
## Discussion

To clarify species differences in the pharmacokinetic parameters of CYP activities between humans and experimental animals, we assessed several CYP activities in mice, rats, dogs, monkeys, and microminipigs, using simultaneous dosing with Inje cocktail (typical human CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam) to these experimental animals. The  $CL_{int}$  of the 5 CYP

Pharmacokinetic Parameters	Mouse		Rat		Dog		Monkey		Microminipig	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Omeprazole										
C <sub>max</sub> (ng/ml)		1.27		7.61 ± 5.98		102 ± 21		53.8 ± 12.8		82.1 ± 6.9
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		0.250 ± 0.00		1.25 ± 0.66		0.583 ± 0.382
t <sub>1/2</sub> (h)	0.200	0.973	0.179 ± 0.007	N.C.	0.741 ± 0.392	0.666 ± 0.238	0.745 ± 0.383	0.572 ± 0.154	0.951 ± 0.247	2.15 ± 1.81
AUC <sub>0-24h</sub> (h·ng/ml)	42.7	1.30	349 ± 110	6.61 ± 5.01	598 ± 23	160 ± 19	879 ± 96	111 ± 47	1487 ± 648	255 ± 128
V <sub>d,ss</sub> (ml/kg)	770		243 ± 87		369 ± 31		173 ± 49		144 ± 19	
CL <sub>tot</sub> (ml/h/kg)	7754		1028 ± 395		555 ± 21		379 ± 41		268 ± 155	
BA (%)		3.05		1.89		26.7 ± 2.7		12.3 ± 4.0		17.7 ± 5.27
5-Hydroxyomeprazole										
C <sub>max</sub> (ng/ml)		6.92		10.9 ± 5.3		20.0 ± 6.6		18.5 ± 2.4		14.6 ± 1.3
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		0.750 ± 0.250		1.25 ± 0.66		1.08 ± 0.80
t <sub>1/2</sub> (h)		1.27		1.60 ± 0.12		0.869 ± 0.187		0.703 ± 0.192		1.57 ± 0.38
AUC <sub>0-24h</sub> (h·ng/ml)		5.36		12.6 ± 7.4		46.1 ± 8.3		41.3 ± 3.4		57.5 ± 22.1
AUC <sub>0-24h</sub> ratio of 5-hydroxyomeprazole/omeprazole		3.94		1.81		0.276 ± 0.047		0.387 ± 0.109		0.231 ± 0.070

Each data represents the mean ± S.D. of three determinations, except for data in mice and BA and AUC<sub>0-24h</sub> of 5-hydroxyomeprazole/omeprazole in rats. Data in mice are calculated from the average plasma concentration of the three animals. BA and AUC<sub>0-24h</sub> of 5-hydroxyomeprazole/omeprazole in rats are calculated using the average AUC<sub>0-24h</sub> of the three animals. i.v., intravenous; p.o., peroral; C<sub>max</sub>, the peak plasma concentration; T<sub>max</sub>, the time to reach C<sub>max</sub>; t<sub>1/2</sub>, terminal elimination half-life; AUC<sub>0-24h</sub>, area under the plasma concentration vs. time curve from 0 h to 24 h; V<sub>d,ss</sub>, volume of distribution; CL<sub>tot</sub>, total body clearance; BA, bioavailability; N.C., not calculated.

**Table 3:** Pharmacokinetic parameters of omeprazole and 5-hydroxyomeprazole in mice, rats, dogs, monkeys, and microminipigs after the intravenous and oral administrations of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam.



**Figure 4:** Plasma concentration of dextromethorphan in mice (A), rats (B), dogs (C), monkeys (D), and microminipigs (E) after the single intravenous and oral administration of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam. Each point and bar is represented as the mean ± S.D. of three animals.

substrates was investigated using the liver microsomes of humans and the experimental animals.

In the present study, the rapid elimination of the 5 CYP substrates in mice and 4 substrates, except for losartan, in rats was investigated *in vivo* or in their liver microsomes and compared to that in humans and non-rodent animals. Previous studies reported that mice and

rats appear to eliminate CYP substrates more rapidly than humans and non-rodent experimental animals because the relative amount of hepatic enzymes, such as CYP/gram body weight, is higher in small animals than in humans and non-rodent experimental animals [4-11]. The results of the present study are consistent with these findings.

The activity of CYP1A2 was examined using caffeine and

Pharmacokinetic Parameters	Mouse		Rat		Dog		Monkey		Microminipig	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
Dextromethorphan										
C <sub>max</sub> (ng/ml)		0.685		0.455 ± 0.126		0.610 ± 0.134		0.00 ± 0.00		0.389 ± 0.300
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		0.833 ± 0.144		N.C.		1.50 ± 0.87
t <sub>1/2</sub> (h)	0.722	N.C.	1.68 ± 0.02	N.C.	1.78 ± 0.14	2.96 ± 3.05	2.66 ± 1.33	N.C.	3.20 ± 1.64	N.C.
AUC <sub>0-24h</sub> (h·ng/ml)	29.8	0.317	93.6 ± 13.2	2.01 ± 1.40	110 ± 9	1.56 ± 0.62	110 ± 1	0.00 ± 0.00	140 ± 16	0.945 ± 0.869
V <sub>d,ss</sub> (ml/kg)	12309		9388 ± 1306		10424 ± 721		14637 ± 3426		11448 ± 6222	
CL <sub>tot</sub> (ml/h/kg)	16954		5706 ± 867		4887 ± 343		4818 ± 32		3620 ± 448	
BA (%)		1.06		2.15		1.45 ± 0.70		0.00 ± 0.00		0.676 ± 0.70
Dextrorphan										
C <sub>max</sub> (ng/ml)		0.720		0.363 ± 0.180		0.00 ± 0.00		0.219 ± 0.019		0.612 ± 0.211
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		N.C.		3.33 ± 1.15		2.00 ± 0.00
t <sub>1/2</sub> (h)		5.30		N.C.		N.C.		N.C.		4.15 ± 3.06
AUC <sub>0-24h</sub> (h·ng/ml)		1.18		1.36 ± 0.68		0.00 ± 0.00		1.65 ± 0.65		3.95 ± 0.22
AUC <sub>0-24h</sub> ratio of dextrorphan /dextromethorphan		3.94		0.713		0.00 ± 0.00		N.C.		7.45 ± 5.58

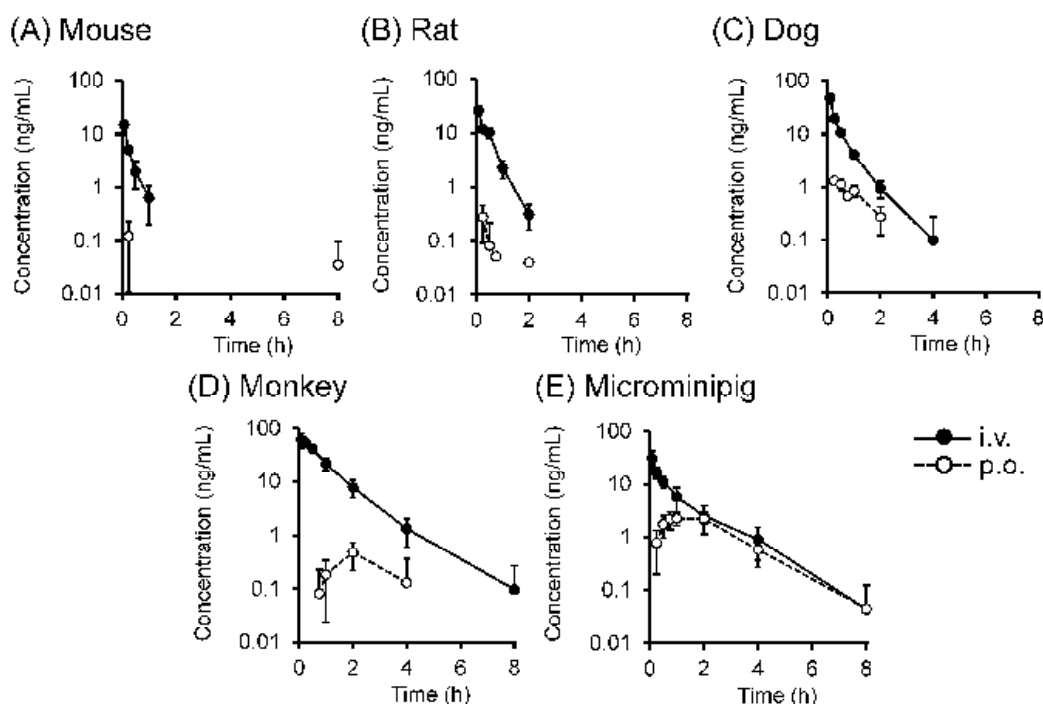
Each data represents the mean ± S.D. of three determinations, except for data in mice and BA and AUC<sub>0-24h</sub> of dextrorphan/dextromethorphan in rats.

Data in mice are calculated from the average plasma concentration of the three animals.

BA and AUC<sub>0-24h</sub> of dextrorphan/dextromethorphan in rats are calculated using the average AUC<sub>0-24h</sub> of the three animals.

i.v., intravenous; p.o., peroral; C<sub>max</sub>, the peak plasma concentration; T<sub>max</sub>, the time to reach C<sub>max</sub>; t<sub>1/2</sub>, terminal elimination half-life; AUC<sub>0-24h</sub>, area under the plasma concentration vs. time curve from 0 h to 24 h; V<sub>d,ss</sub>, volume of distribution; CL<sub>tot</sub>, total body clearance; BA, bioavailability; N.C., not calculated.

**Table 4:** Pharmacokinetic parameters of dextromethorphan and dextrorphan in mice, rats, dogs, monkeys, and microminipigs after the intravenous and oral administrations of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam.



**Figure 5:** Plasma concentration of midazolam in mice (A), rats (B), dogs (C), monkeys (D), and microminipigs (E) after the single intravenous and oral administration of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam. Each point and bar is represented as the mean ± S.D. of three animals.

paraxanthine because CYP1A2 is the primary enzyme responsible for caffeine metabolism in human, mouse, rat, dog, monkey, and pig livers [37-42]. The BA of caffeine in humans was previously reported to be 108% [43]. We found that the experimental animals had high oral BAs and low CL<sub>int</sub> values of caffeine, similar to those of humans. A significant first-pass effect was not detected after the oral administration of caffeine and its levels in the microsomes of humans and some experimental

animals decreased slowly [30, 42-45]. Furthermore, the AUC<sub>0-24h</sub> ratios of paraxanthine/caffeine in the 5 experimental animals were within ± 3-fold that in humans [33]. CYP1A2 has been detected in mouse, rat, dog, monkey, and pig liver as well as in human liver, and showed a strong conversion with an identity to human > 80% in these 5 animal species [4,42]. These results suggested that CYP1A2 activities in the 5 experimental animals were similar to that in humans.



Pharmacokinetic Parameters	Mouse		Rat		Dog		Monkey		Microminipig	
	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.v.	p.o.
<b>Midazolam</b>										
C <sub>max</sub> (ng/ml)		0.121		0.277 ± 0.187		1.32 ± 0.10		0.484 ± 0.238		2.32 ± 0.66
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		0.333 ± 0.144		1.67 ± 0.58		1.25 ± 0.66
t <sub>1/2</sub> (h)	0.275	N.C.	0.327 ± 0.032	N.C.	0.555 ± 0.276	0.960 ± 0.662	0.904 ± 0.340	N.C.	1.12 ± 0.28	1.29 ± 0.34
AUC <sub>0-24h</sub> (h·ng/ml)	5.24	0.380	13.3 ± 2.4	0.160 ± 0.205	21.7 ± 2.1	1.72 ± 0.16	70.5 ± 10.6	1.24 ± 0.91	24.4 ± 4.9	8.04 ± 0.85
V <sub>d,ss</sub> (ml/kg)	1624		1002 ± 135		765 ± 301		458 ± 85		1509 ± 636	
CL <sub>tot</sub> (ml/h/kg)	6379		2581 ± 517		1552 ± 141		483 ± 73		1411 ± 281	
BA (%)		7.26		1.21		7.94 ± 0.75		1.66 ± 1.01		34.3 ± 10.3
<b>1'-Hydroxymidazolam</b>										
C <sub>max</sub> (ng/ml)		11.1		0.493 ± 0.407		4.75 ± 1.35		0.469 ± 0.226		0.383 ± 0.071
T <sub>max</sub> (h)		0.250		0.250 ± 0.00		0.417 ± 0.144		2.00 ± 0.00		0.917 ± 0.144
t <sub>1/2</sub> (h)		2.31		N.C.		0.577 ± 0.404		N.C.		20.7 ± 27.2
AUC <sub>0-24h</sub> (h·ng/ml)		9.52		0.320 ± 0.442		5.56 ± 1.05		1.12 ± 0.77		0.917 ± 0.212
AUC <sub>0-24h</sub> ratio of 1'-hydroxymidazolam/midazolam		23.9		1.90		3.07 ± 0.31		0.879 ± 0.389		0.108 ± 0.016

Each data represents the mean ± S.D. of three determinations, except for data in mice and BA and AUC<sub>0-24h</sub> of 1'-hydroxymidazolam/midazolam in rats. Data in mice are calculated from the average of three plasma concentration of the three animals.

BA and AUC<sub>0-24h</sub> of 1'-hydroxymidazolam/midazolam in rats are calculated using the average AUC<sub>0-24h</sub> of the three animals.

i.v., intravenous; p.o., peroral; C<sub>max</sub>, the peak plasma concentration; T<sub>max</sub>, the time to reach C<sub>max</sub>; t<sub>1/2</sub>, terminal elimination half-life; AUC<sub>0-24h</sub>, area under the plasma concentration vs. time curve from 0 h to 24 h; V<sub>d,ss</sub>, volume of distribution; CL<sub>tot</sub>, total body clearance; BA, bioavailability; N.C., not calculated.

**Table 5:** Pharmacokinetic parameters of dextromethorphan and dextrorphan in mice, rats, dogs, monkeys, and microminipigs after the intravenous and oral administrations of 5 CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam.

Compounds	Human	Mouse	Rat	Dog	Monkey	Microminipig
<b>Caffeine</b>						
CL <sub>int</sub> (ml/min/mg protein)	0.00788 ± 0.00012	0.00627 ± 0.00074	0.00312 ± 0.00034	0.00359 ± 0.00065	0.00398 ± 0.00012	0.00667 ± 0.00055
CL <sub>int</sub> (ml/min/kg) <sup>a</sup>	9.11 ± 0.14	24.7 ± 2.9	5.61 ± 0.61	5.16 ± 0.93	5.37 ± 0.16	5.19 ± 0.43
<b>Losartan</b>						
CL <sub>int</sub> (ml/min/mg protein)	0.0379 ± 0.0062	0.0876 ± 0.0080	0.0149 ± 0.0020	0.0146 ± 0.0027	0.122 ± 0.007	0.0277 ± 0.0030
CL <sub>int</sub> (ml/min/kg) <sup>a</sup>	43.8 ± 7.2	345 ± 32	26.8 ± 3.5	21.1 ± 3.8	165 ± 9	21.6 ± 2.3
<b>Omeprazole</b>						
CL <sub>int</sub> (ml/min/mg protein)	0.0354 ± 0.0042	0.0590 ± 0.0050	0.136 ± 0.008	0.0112 ± 0.0012	0.117 ± 0.006	0.0260 ± 0.0070
CL <sub>int</sub> (ml/min/kg) <sup>a</sup>	41.0 ± 4.9	232 ± 20	245 ± 15	16.2 ± 1.7	158 ± 8	20.2 ± 5.5
<b>Dextromethorphan</b>						
CL <sub>int</sub> (ml/min/mg protein)	0.0327 ± 0.0066	0.167 ± 0.007	0.489 ± 0.001	0.0585 ± 0.007	0.669 ± 0.008	0.582 ± 0.011
CL <sub>int</sub> (ml/min/kg) <sup>a</sup>	37.8 ± 7.6	658 ± 27	880 ± 1	84.3 ± 9.8	903 ± 11	453 ± 8
<b>Midazolam</b>						
CL <sub>int</sub> (ml/min/mg protein)	0.569 ± 0.018	0.460 ± 0.016	0.472 ± 0.028	0.528 ± 0.020	0.973 ± 0.058	0.246 ± 0.043
CL <sub>int</sub> (ml/min/kg) <sup>a</sup>	658 ± 21	1811 ± 62	850 ± 50	736 ± 70	1314 ± 78	192 ± 34

Each in vitro intrinsic clearance value represents mean ± SD for triplicate determinations.

<sup>a</sup>Calculated using standard values of 45 mg of microsomal protein/g liver [29] for all species and the following values for g liver/kg body weight: human, 25.7; mouse, 87.5; rat, 40.0; dog, 32.0; monkey, 32.0 [30]; microminipig, 17.3.

CL<sub>int</sub><sup>a</sup>, in vitro intrinsic clearance value.

**Table 6:** In vitro intrinsic clearance values for caffeine, losartan, omeprazole, dextromethorphan, and midazolam in liver microsomes of various species.

CYP2C is the largest and most complicated CYP subfamily in several species including humans and the substrate specificities are largely different between human and animal isoforms [4]. Thus, we assessed the activity of CYP2C using both losartan and omeprazole, which are metabolized to EXP3174 and 5-hydroxyomeprazole by CYP2C9 and CYP2C19 mainly, respectively [46,47]. The oxidative conversion of losartan to EXP3174 is also observed in rats, dogs, monkeys, and pigs [46,48-50]. The BA of losartan in humans was previously reported to be 35.8% [51]. Mice and monkeys had lower BAs and higher CL<sub>int</sub> values of losartan than humans, while the BAs and CL<sub>int</sub> values in rats, dogs, and microminipigs were similar to those in humans. These results suggested that low BAs in mice and monkeys may be due to hepatic availability (Fh). In addition, the AUC<sub>0-24h</sub> ratio of EXP-3174/losartan in mice was approximately 9-fold higher than that in humans [33],

suggesting high metabolic CYP2C activity for losartan to EXP-3174 in mice. On the other hand, the AUC<sub>0-24h</sub> ratios of EXP-3174/losartan in dogs and monkeys were approximately 23- and 6-fold lower than that in humans, respectively, while monkeys had lower BA and higher CL<sub>int</sub> values and dogs had similar values to those in humans. CYP2C-mediated metabolisms for some substrates in dog and monkey are different from those in human, although monkey CYP2C subfamily has high identities to human CYP2C subfamily (> 90%) [4,52]. It was reported that the major metabolites of losartan in monkey liver are the tetrazole N2-gluconide and hydroxymethyl-O-β-glucuronide of losartan [46]. These findings suggest that the major metabolism pathway for losartan in dogs as well as in monkeys may differ from that in humans.

It was reported that omeprazole is metabolized by CYP2C mainly in rats, dogs, monkeys, and pigs, as well as in humans [11,13,22,47,53]. Although the BA of omeprazole in humans was previously reported to be 41.0% [47], the BAs of omeprazole were slightly lower in dogs, monkeys, and microminipigs and markedly lower in mice and rats than that in humans. Mice, rats, and monkeys had higher  $CL_{int}$  values than those in humans, dogs, and microminipigs. These results are consistent with previous findings [8,11,22,30]. In addition, the CYP2C family is the most abundant group of CYP isoforms in rat liver, different from human liver [4]. Our results suggested that the low BAs of omeprazole in mice, rats, and monkeys may have been due to Fh values. The  $AUC_{0-24h}$  ratios of 5-hydroxyomeprazole/omeprazole in dogs and microminipigs were approximately 4-fold lower than that in humans. 5-Hydroxyomeprazole is transformed into 5-hydroxyomeprazole sulfone in part by CYP3A4 in humans [54]. Our results indicated the major metabolism of omeprazole in dogs and microminipigs may be 5-hydroxyomeprazole sulfone or the other metabolites except for 5-hydroxyomeprazole.

Dextromethorphan is metabolized to dextrorphan by CYP2D mainly in mouse, rat, dog, monkey and pig liver as well as in human liver [14,21,26,55,56]. Therefore we used dextromethorphan and its major metabolite, dextrorphan, is often used as probe drugs to evaluate the activity of the CYP2D isoform. However, the BA of dextromethorphan has yet to be determined in humans. We found that the 5 experimental animals had low BAs of dextromethorphan; the BAs in monkeys and microminipigs did not exceed 1%. In addition, only the  $CL_{int}$  of dextromethorphan in dogs was similar to that in humans. Previous studies reported that hepatic metabolic activities for some human CYP2D6 substrates in mice, rats, monkeys, and microminipigs were higher than those in humans, although rat, monkey, and pig CYP2D isoforms in the livers showed a high sequence identity to human (70-94%) [4,10-12,21,22,29,30,52]. CYP2D15 is the major CYP2D in dog liver, with enzymatic activities to human CYP2D6 [4]. On the other hand, the  $AUC_{0-24h}$  ratio of dextrorphan/dextromethorphan in dogs is 0.00, different from humans [57]. Dextrorphan are transformed into 3-hydroxymorphinan in part by CYP3A4 in humans [54]. Our results suggest that the major metabolite of dextromethorphan in dogs may be 3-hydroxymorphinan and the other metabolites except for dextrorphan.

It is well-known that midazolam is metabolized to 1'-hydroxymidazolam by CYP3A mainly in humans, mice, rats, dogs, monkeys, pigs [21,39,58-60]. The BA of midazolam in humans was previously reported to be 30.0% [61]. The BA of midazolam in microminipigs was similar to that in humans, while the  $CL_{int}$  values in the other animals were within approximately  $\pm 3$ -fold that in humans. Hepatic metabolic activities for midazolam in mice, rats, dogs, and monkeys are known to be similar to that in humans, whereas higher intestinal metabolic activities were observed in mice, rats, and monkeys than in humans [11,15,60,62]. CYP3A isoform in mouse, monkey, and pig livers are similar to CYP3A4 in humans with identities (76-95%), and its content of total CYP in dog and monkey livers were similar to that in human liver [4,42,52,63]. Therefore, the lower BAs of midazolam in mice, rats, dogs, and monkeys than in humans and microminipigs appear to depend on the fraction absorbed multiplied by intestinal availability (FaFg) other than Fh. Few studies have examined CYP3A activities in the small intestines of dogs and pigs; therefore, further investigations are needed in order to assess species differences in intestinal CYP activities between humans and experimental animals. Furthermore, the  $AUC_{0-24h}$  ratio of 1'-hydroxymidazolam/midazolam

in microminipigs was similar to that in humans [64]. Previous studies showed that drug metabolism mediated by human CYP3A was similar in the liver microsomes of pigs, minipigs, and humans [12,24,25]. We found that CYP3A activity in microminipigs was similar to that in humans. This result suggests that microminipigs may be a good experimental animal for predicting the pharmacokinetics of CYP3A substrates in humans. However, Mogi et al. [30]. Reported that the BA of midazolam was low in microminipigs (3%). Thus, further studies are needed to investigate species differences in CYP3A activity between humans and microminipigs using human CYP3A substrates other than midazolam.

In this study,  $CL_{int}$  values in liver microsomes of human and experimental animals were used to assess species differences in the CYP activity for each substrate in their liver. However, the prediction of hepatic clearance values from  $CL_{int}$  values were generally performed by careful extrapolation *in vitro* metabolism data in liver microsomes to *in vivo* pharmacokinetic data [9,15,35]. Therefore, further studies may be needed to obtain additional data such as each unbound fraction in plasma and blood-to-plasma concentration ratio in each species, and evaluation the correlation between *in vivo* and *in vitro* results.

## Conclusion

We assessed multiple *in vivo* CYP activities in mice, rats, dogs, monkeys, and microminipigs by simultaneous dosing with typical human CYP substrates such as caffeine, losartan, omeprazole, dextromethorphan, and midazolam. In addition, the *in vitro*  $CL_{int}$  of the 5 CYP substrates was investigated using the liver microsomes of humans and the experimental animals. We showed that CYP3A activities in microminipigs were more similar to that in humans than the other animals.

These results indicated that *in vitro* and *in vivo* experimental data obtained using multiple animals including microminipigs are useful for predicting human pharmacokinetics.

## Conflict of Interest

The authors have declared that there is no conflict of interest.

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