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NONLINEAR PHARMACOKINETICS OF PROPAFENONE IN RATS AND HUMANS: APPLICATION OF A SUBSTRATE DEPLETION ASSAY USING HEPATOCYTES FOR ASSESSMENT OF NONLINEARITY

Hiroshi Komura and Masahiro Iwaki

Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka, 577-8502, Japan.

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Corresponding author: Masahiro Iwaki, Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka, 577-8502, Japan.
Tel: 81-6-6721-2332 Ext. 3819; Fax: 81-6-6730-1394.
E-mail: iwaki@phar.kindai.ac.jp

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[1]Abbreviations: NCE, new chemical entity; CYP, cytochrome P-450; C_{max}, maximum plasma concentration; AUC, area under the plasma concentration curve; HSA, human serum albumin; AGP, α_1-acid glycoprotein; LC/MS, liquid chromatography/mass spectrometry; KHB, Krebs-Henseleit buffer; $t_{1/2}$, elimination half-life; T_{max}, time to maximum concentration; CL, plasma clearance; V_{ss}, distribution volume at steady state; AUMC, area under the first moment curve; MRT, mean residence time.

ABSTRACT

Linear pharmacokinetic profiles of propafenone in female Wistar rats were found after oral administrations of up to 20 mg/kg. These profiles differed from nonlinear pharmacokinetics in a dose-dependent manner with increasing plasma concentrations in humans (Arzneimittelforschung 33: 763-770, 1983). We investigated the species differences in pharmacokinetics of propafenone between rats and humans. In rats, after intravenous administration, clearance was constant at all doses examined (0.2-10 mg/kg), whereas the distribution volume at a steady state increased and the resultant elimination half-life was prolonged with increasing doses. In a substrate depletion assay without plasma, rat and human hepatocytes showed a concentration-dependent elimination of propafenone with low K_m values (< 0.4 μM). However, in the depletion assay with plasma incubation, the profiles were altered to a concentration-independent profile in rat but not human hepatocytes. The differing effect of adding plasma in rat and human hepatocytes can be explained by species differences in plasma binding (unbound fraction: 0.0071 *vs*. 0.0754 for rats and humans, respectively, at 0.1 μg/ml). In rat plasma, the unbound fraction increased with concentrations of 0.1 to 1.0 μg/ml, whereas it was constant in human plasma. Accordingly, the *in vivo* nonlinear disposition in humans can be ascribed to the saturation of hepatic metabolism due to the low K_m values. In contrast, the influence of saturable metabolism is cancelled out with nonlinear plasma binding in rats leading to the apparent linear pharmacokinetic behavior. The newly developed depletion assay with plasma incubation gave insights into the nonlinear pharmacokinetics of propafenone.

Recently, pharmacokinetic and metabolic profiles of new chemical entities (NCEs) have become recognized as critical parameters of developability (Hodgson, 2001). Nonlinear pharmacokinetics is also an issue, and this lies on the fact that for drugs with high increase of AUC relative to the dose, designing appropriate dose regimens is difficult. Robust screening methods for evaluating membrane permeability, metabolic stability and cytochrome P-450 (CYP) inhibition have been developed to identify NCEs with desirable pharmacokinetic profiles (Ansede and Thakker, 2004; Roberts, 2001; Kariv et al., 2002). However, very few attempts are made to predict nonlinear pharmacokinetics in humans prior to nomination of NCEs in preclinical studies or clinical trials. Linearity is evaluated solely in dose escalation studies using rats and dogs.

Nonlinear pharmacokinetics is associated with absorption, hepatic metabolism, liver uptake or plasma protein binding (Ludden, 1991; Takeuchi et al, 2001; Han et al., 1999). Of these, saturation of hepatic metabolism and plasma protein binding are particularly important sources of *in vivo* nonlinearity. Metabolism-related nonlinearity basically depends on the balance of plasma concentrations and Michaelis constant (K_m) values for hepatic metabolism (Ludden, 1991). Although assessment of nonlinearity with *in vitro* kinetic data has been conducted retrospectively (Iwatsubo et al., 1998), there are difficulties in evaluating K_m values even in late phases of drug discovery due to the unidentified chemical structure of primary metabolites and unavailable reference compounds. In fact, *in vivo* human clearances are frequently predicted from the elimination rate constant of a substrate at 0.5-1 μM (Clarke and Jeffrey, 2001; Shibata et al,

2002; Naritomi et al., 2003) assuming that K_m values are at least higher than 5-10 μM.

Obach and Reed-Hagen (2002) developed a substrate depletion assay in which K_m values can be estimated from depletion rate constants of unchanged compounds at various initial concentrations. Estimated K_m values for substrates metabolized possibly via a predominant pathway by a CYP isoform, are comparable to those from metabolite formation assays in recombinant CYP isoform studies. We also demonstrated that depletion assays are applicable for estimating K_m values of propranolol metabolism with multiple pathways in rat liver microsome systems (Komura and Iwaki, 2005). It has been reported that human hepatocytes in the presence of serum rather than incubation buffer (serum incubation method) provide the intrinsic clearance of substrates with the effects of plasma protein binding (Shibata et al., 2002). Therefore, the substrate depletion assay combined with the serum incubation method might yield kinetic parameters such as K_m and intrinsic clearance accompanied by the impacts of plasma protein binding, and might be applicable for evaluating *in vivo* nonlinearity with regard to not only saturation of hepatic metabolism but also protein binding. This newly developed method might play an important role in selection of NCEs as a valuable tool of drug discovery.

Propafenone is an antiarrhythmic drug with sodium channel blocking and β-adrenergic antagonist properties (Funck-Brentano et al., 1990) that generally undergoes CYP2D6-dependent biotransformation to 5-hydroxypropafenone in humans (Botsch et al., 1993; Kroemer et al., 1989, 1991). Oral administrations to healthy volunteers provided the maximum

plasma concentration (C_{max}) and area under the plasma concentration curve (AUC) with a supraproportional increment against the change in dose (Hollmann et al., 1983). This is thought to involve saturation of hepatic metabolism, but there are no reports detailing this mechanism based on *in vitro* kinetic parameters in humans.

In our preclinical investigation, the linear pharmacokinetics of propafenone was exhibited in rats after oral administrations of up to 20 mg/kg. In the present study, the mechanisms involved in the species differences in pharmacokinetic profiles between rats and humans are examined using a substrate depletion assay with and without plasma in incubation (plasma incubation), in addition to a study on pharmacokinetics in rats after intravenous injections. This paper aims to clarify the different mechanisms involved in nonlinearity of propafenone in rats and humans, and to demonstrate the applicability of the newly developed method as a preferable tool for anticipating nonlinear pharmacokinetics.

Materials and Methods

Materials. Propafenone, recombinant human α_1-acid glycoprotein (AGP), human serum albumin (HSA, faction V) and collagenase type IV (prepared from *Clostridium histolyticum*), and trypsin inhibitor (prepared from soybean type II) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and pentobarbital (Nembutal™) was purchased from Dainippon Seiyaku Ltd. (Osaka, Japan). Cryopreserved human hepatocytes and pooled human liver microsomes were obtained from In Vitro Technologies Inc. (Baltimore, MD, USA) and XenoTech (Kansas City, KS, USA), respectively. Pooled human plasma was obtained from KAC Co. (Kyoto, Japan), and the dialysis membrane (Spectra/Por ®) and Centrifree® were obtained from Spectrum Laboratories Inc. (Rancho Domingnez, CA, USA) and Millipore Co. (Bedford, MA, USA), respectively. All other chemicals were reagent grade products obtained commercially.

Animal Studies. Female Wistar rats (6-7 weeks old) were purchased from Charles River Japan (Yokohama, Japan). Animals were housed in a temperature- and humidity-controlled room with free access to water and a standard diet, and were fasted for 16 hr prior to experimentation. Propafenone was dissolved in 10% cremophor EL in saline at 0.2 to 10 mg/ml, and intravenously or orally administered to 5 or 6 Wistar rats. At specified times, 0.25 to 0.5-ml blood samples were taken from three animals via the jugular vein by a syringe and placed into heparinized tubes under anesthesia by ether. To avoid a reduction in blood in the body, the total blood volume collected from each rat was less than 1.25 ml. Plasma was obtained after centrifugation at 3,000 rpm at 4°C.

Protein Binding and Blood/Plasma Concentration Ratio. The plasma protein binding study was conducted using either an equilibrium dialysis method or filtration method. Propafenone dissolved in dimethylsulfoxide was added into rat, human plasma, 0.098% AGP and 4% HSA, and the initial concentration of propafenone was 0.1 to 500 μg/ml (the final concentration of dimethylsulfoxide was less than 1%). With the dialysis method, one-ml samples were transferred to the dialysis membrane after incubation for 15 min at 37°C then both ends of the membrane were clipped. The membrane was placed in 3 ml of isotonic phosphate buffer (pH 7.4) or Krebs-Henseleit buffer (pH 7.4) with 10 mM HEPES (KHB), and incubated for 6 hr at 37°C with a shaking speed at 30 cycles/min. We confirmed preliminarily that the equilibrium was reached within this incubation period. With the filtration method, preincubated samples were applied to Centrifree®, and centrifuged at 3,000 rpm for 15 min to obtain the filtrate. The concentrations of propafenone in the biological fluids and buffer were determined by liquid chromatography/mass spectrometry (LC/MS) to estimate the bound concentrations (C_b), unbound concentrations (C_u) and unbound fraction (f_u). Blood/plasma concentration ratio of propafenone (R_B) was determined by comparing the plasma concentration after addition of propafenone into the blood with the initial concentration. The incubation was conducted for 15min at 37°C.

Preparation of Rat Hepatocytes. Rat hepatocytes were isolated using a two-step collagenase perfusion method according to the method of Kern et al. (1997). Rats were anesthetized with pentobarbital (Nembutal®, 150 mg/kg) and heparinized (500 U/kg). The liver was then perfused *in situ*

with calcium-free Hanks' buffer containing EGTA (0.5 mM) for 10 min at 37°C followed by perfusion of Hank's buffer with collagenase type IV (0.7 U/ml) and calcium chloride (4 mM) for 20 min. The hepatocytes were released from the digested liver, and the suspension was filtered through a nylon mesh with 100-μm pores then centrifuged. The obtained cell pellet was resuspended in KHB. Hepatocytes were counted using a hemocytometer in the presence of 0.04% trypan blue.

Metabolic Incubation during the Substrate Depletion Assay. Rat liver microsomes were prepared according to a previously reported method (Komura and Iwaki, 2005). Microsomal incubation was performed under the following conditions: the incubation mixture (2 ml final volume) contained liver microsomes of rats and humans (0.2 mg/ml), 1 mM EDTA, and an NADPH generating system consisting of 5 mM glucose-6-phosphate, 1.0 mM $NADP^+$, and 1 IU/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer (pH 7.4). After preincubation for 3 min at 37°C, the reaction was initiated by addition of propafenone dissolved in acetonitrile (less than 1%, v/v). At specified times, an aliquot of the reaction mixture was placed into a glass test tube with 0.1 ml of 1 M NaOH.

Cryopreserved human hepatocytes were thawed according to the vendor's instructions. Briefly, they were thawed at 37°C and gradually diluted with KHB. After centrifugation at 50 × g for 5 min, the pellet was resuspended with the same buffer, and viability was assessed by trypan blue exclusion. Incubation with rat and human hepatocytes at a cell density of 1.0×10^6 cells/ml in the presence or absence of rat and human plasma,

0.098% AGP or 4% HSA was performed with a shaking speed of 100 cycles/min at 37°C. Initiation and termination of the reactions were carried out in the same manner as with microsomal incubation. Propafenone was dissolved in methanol, and the final volume of organic solvent in the incubation mixture was less than 1%.

Determination of Propafenone. Propafenone was extracted from the biological fluids with 7 ml of ether under an alkaline condition, and the organic layer was evaporated under a nitrogen stream at 40°C. The residues were dissolved in acetonitrile: 10 mM ammonium acetate at pH 6.8 (20: 80, v/v) and injected into a LC/MS equipped with an electrospray probe (Agilent Technologies, Palo Alto, CA, USA). Chromatography was conducted on a 3.5 μm Symmetry C_{18}® column, 4.6 × 100 mm, eluted at a column temperature of 40°C and a flow rate of 1 ml/min under mobile phase gradient conditions (acetonitrile: 10 mM ammonium acetate pH 6.8, 50/50 at 2 min to 80/20 at 5 min, v/v). Eluted propafenone was detected at m/z of 342.2 in a positive ion mode. The limit of quantification was 1 ng/ml when 0.1 ml biological fluid was used. The calibration curve up to 500 ng/ml showed a good correlation ($r>0.997$). When the plasma concentration of more than 500 ng/ml was anticipated, the sample was diluted by blank plasma.

Data Analysis. Enzyme kinetic parameters in liver microsomes and hepatocytes were estimated using a substrate depletion assay previously reported (Obach and Reed-Hangen, 2002; Komura and Iwaki, 2005). Briefly, the time course of the remaining percentage was fitted to the first elimination function to determine the initial substrate depletion rate

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constant (k_{dep}). K_m was estimated by fitting the k_{dep} at various initial concentrations to the following equation using nonlinear least-squares regression analysis combined with MULTI (Yamaoka et al., 1981).

$$k_{dep} = k_{dep[S]=0} \cdot \left(1 - \frac{S}{S + K_m}\right) \qquad \text{Eq. (1)}$$

where S is the substrate concentration and $k_{dep([S]=0)}$ represents the theoretical maximum elimination rate constant at an infinitesimally low substrate concentration. The intrinsic clearances at an infinitesimally low substrate concentration ($CL_{int,app[S]=0}$) were calculated by dividing $k_{dep([S]=0)}$ by the concentrations of microsomal protein and hepatocytes used.

To predict *in vivo* hepatic clearance (CL_H), the CL_{int} was expressed per kilogram of body weight by taking the number of hepatocytes per gram liver and liver weight per kilogram of body weight shown in Table 1. In general, the CL_H can be estimated based on the well-stirred model (Pang and Rowland, 1977):

$$CL_H = \frac{Q_H \cdot f_b \cdot CL_{int}}{Q_H + f_b \cdot CL_{int}} \qquad \text{Eq.(2)}$$

where Q_H is the hepatic blood flow and the f_b is the fraction unbound in the blood calculated by f_u/R_B. Since the CL_{int} obtained from substrate depletion assay with plasma incubation includes any effect of plasma protein binding, Eq. (2) can be transferred to the following equation (Eq.3):

$$CL_H = \frac{Q_H \cdot CL_{int} / R_B}{Q_H + CL_{int} / R_B} \qquad \text{Eq. (3)}$$

The R_B used was 0.96 at 1μg/ml for rats because there was no significant difference in the values at 0.1 and 1 μg/ml. On the other hand, the R_B for humans was assumed to be 1 as no data was available.

To estimate the parameters of plasma protein binding of propafenone, the C_b and C_u values determined were fitted to a standard saturable binding

model with two binding components using MULTI:

$$C_b = \frac{B_{max1} \cdot C_u}{K_{d1} + C_u} + \frac{B_{max2} \cdot C_u}{K_{d2} + C_u} \qquad \text{Eq. (4)}$$

where B_{max1} and B_{max2} are the maximum drug concentration bound for the primary and secondary components, respectively, and K_{d1} and K_{d2} are the dissociation constants for each component.

In the *in vivo* experiments, the elimination half-lives ($t_{1/2}$) of different plasma concentrations were calculated from $t_{1/2} = 0.693/\beta$, where β is the elimination rate constant determined by least square regression analysis of plasma concentration *vs*. the time curve. The AUC from time 0 to infinity was calculated by the trapezoidal rule using the determined values, which were then extrapolated to infinity by dividing the last measured plasma concentration (C_{last}) by β. The area under the first moment curve (AUMC) was calculated by the trapezoidal rule to the last measured time (t_{last}) and with the addition of correction for infinity; that is, $(t_{last} \times C_{last})/ \beta + C_{last}/\beta^2$. Total plasma clearance (CL or CL/F) was estimated by dose/AUC, and the distribution volume at a steady-state (V_{ss}) was calculated from the following equation: $V_{ss} = (\text{Dose} \times \text{AUMC})/(\text{AUC})^2$. The mean residence time (MRT) was obtained by AUMC/AUC.

Statistical Analysis. Statistical analysis was performed by an unpaired students's t-test using Statistica™ (StatSoft, Inc., Tokyo) and the significance level adopted was $P < 0.05$.

Results

The plasma concentrations and pharmacokinetic parameters of propafenone after oral administrations (3, 10, 20, and 45 mg/kg) to female Wistar rats are shown in Fig. 1 and Table 2, respectively. AUC proportionally increased up to a dose of 20 mg/kg, and the resultant CL/F was almost constant. However, oral applications of 45 mg/kg appeared to yield an AUC that was disproportionately high relative to the dosage change. After intravenous administration, the CL was moderate and almost the same at all doses investigated (Fig. 1 and Table 3). However, V_{ss} remarkably increased from 1.32 to 6.82 l/kg with increasing dose. As a result, the elimination half-life from the plasma was prolonged (0.36 to 1.61 hr).

To estimate the enzyme kinetic parameters of propafenone, k_{dep} values at various initial concentrations were determined using a substrate depletion assay with liver microsomes and hepatocytes from rats and humans. The average elimination profiles of human hepatocytes prepared from three different donors and the relationship between the concentration and k_{dep} are shown in Fig. 2. Substrate concentration-dependent depletion was observed in the hepatocytes and liver microsomes of both rats and humans. The estimated kinetic parameters are summarized in Table 4. The K_m values in the liver microsomes and hepatocytes were almost identical (0.37–0.38 and 0.092–0.11 μM, respectively). The K_m values for humans were significantly lower than those for rats (P<0.01). Furthermore, the *in vitro* system of humans showed the significantly lower $CL_{int,app[S]=0}$ values than the corresponding system of rats (P<0.01). There was possibility that drug

metabolizing activities may be slightly underestimated due to the inactivation of the enzyme because the microsomes were exposed to NADPH before the addition of substrates.

The effects of plasma or plasma proteins on the depletion of propafenone in rat and human hepatocytes are shown in Tables 5 and 6, respectively. In rat hepatocytes, the k_{dep} values in the depletion assay with plasma incubation were constant at all concentrations tested, which differ from those in the control condition without plasma incubation that showed concentration-dependent elimination. Especially, there was a pronounced difference in k_{dep} values at the initial low concentration (0.05 μM) between with and without plasma (0.00565 vs. 0.601 min^{-1}). In contrast, regardless of whether plasma was utilized as the incubation medium or not, propafenone was eliminated in human hepatocytes in a concentration-dependent manner (Table 6), and the obtained K_m values were 0.57 and 0.23 μM, respectively. Such a dependency was also found in the presence of HSA or AGP at physiological concentrations instead of human plasma, however the addition of AGP provided relatively high K_m value (1.58 μM) unlike HSA.

The CL_H was estimated from the k_{dep} values at the initial concentrations of 0.05 and 5 μM obtained in the substrate depletion assay with plasma incubation. The estimated CL_H was not changed (1.3 l/hr/kg) for rats, but decreased from 0.78 to 0.18 l/hr/kg for humans with increasing the concentration.

Unbound fractions of propafenone in the plasma and other plasma proteins at 0.1 μg/ml were determined using a filtration assay (data not

shown). There was a large species difference in the unbound fraction in plasma; the fraction was 0.0071 for rats and 0.0754 for humans, and the corresponding values in HSA and human AGP were 0.384 and 0.121, respectively. In addition, using different lots of rat and human plasma, the unbound fraction of propafenone was determined at relevant *in vivo* concentrations ranging from 0.1 to 1 μg/ml; the unbound fraction is listed in Table 7. The unbound fractions in rat plasma were enhanced with increasing concentration, while human plasma provided similar values.

Using a wide range of propafenone concentrations (0.1 to 500 μg/ml), we estimated the binding parameters in rat and human plasma by Scatchard analysis. Since propafenone recovery was dramatically reduced at initial concentrations of more than 10 μg/ml in the filtration assay possibly due to adhesion to the device, an equilibrium assay was utilized in this in-depth assay. The parameters obtained from Scatchard analysis with two binding components are summarized in Table 8. The B_{max1} for the primary component were 1.43 μM for rat plasma and 8.63 μM for human plasma, and respective B_{max2} for the secondary component were 762 and 1084 μM. The B_{max1} in human plasma was 6-fold higher than that in rat plasma, and the K_{d1} in rat plasma was extremely lower than that in human plasma.

Discussion

The pharmacokinetic profile of propafenone was investigated after oral administration to female Wistar rats at doses varying from 3 to 45 mg/kg to compare the *in vivo* nonlinear kinetic data in humans reported by Hollmann et al. (1983). In rats, the oral plasma concentrations increased dose-dependently up to a dose of 20 mg/kg even in the concentration range (about 20 to 150 ng/ml) where nonlinearity was observed after oral administration in humans. It is possible that poor absorption at higher doses masked the suprapropportional increment of AUC relative to the dosage changes, although oral formulation of propafenone was a solution for all doses and propafenone was membrane permeable through the Caco-2 monolayer (Komura and Iwaki, unpublished data). Pharmacokinetics after intravenous administration was evaluated to characterize disposition by excluding the absorption and intestinal first-pass metabolism. The CL was almost constant over the doses examined, however, V_{ss} increased with increasing dose leading to prolonged elimination from the plasma.

It has been reported that nonlinear pharmacokinetics is mainly attributed to the saturation of either plasma protein binding or hepatic metabolism (Aoyama et al., 1990; Kiriyama et al, 1999; Niazi et al., 1996; Evans et al., 1990; Wong et al, 1999). To define the factors that affect the species differences in nonlinearity of propafenone, metabolism of propafenone in rats and human hepatocytes were kinetically evaluated using a substrate depletion assay; the K_m values in rat and human hepatocytes were within the low range (K_m < 0.4 μM). In the present study, the metabolic data for rats were obtained from freshly isolated hepatocytes.

In contrast, the corresponding data for humans were derived from cryopreserved hepatocytes. There is a question as to whether or not cryopreserved hepatocytes retain drug-metabolizing activities during the storage. However, the intrinsic clearances in cryopreserved and fresh human hepatocytes are reportedly similar (Lau et al., 2002; Naritomi et al., 2003). Furthermore, Griffin and Houston (2004) indicated that a cryopreserved rat hepatocyte suspension gave reliable K_m values only with compounds showing a typical Michaelis-Menten kinetic profile. The relationship between substrate concentration and k_{dep} in the present study was adapted to the normal Michaelis-Menten equation (Houston and Kenworthy, 2000), indicating that the kinetic data in cryopreserved hepatocytes can be compared with those in freshly isolated hepatocytes. Thus, propafenone appears to show a similar *in vitro* metabolic profile in both species.

Plasma protein binding is, in general, one determinant affecting the *in vivo* pharmacokinetics of drugs, and dose-dependent plasma clearance of some drugs arises from concentration-dependent protein binding (Ludden, 1991; Wong et al, 1999). Shibata et al. (2002) reported that the serum incubation method, in which test compounds are incubated with hepatocytes in the presence of serum, gives *in vitro* intrinsic clearances that include the impact of plasma protein binding, and that the obtained clearance is the most relevant parameter for predicting *in vivo* clearance. Since rat hepatocyte incubations in the presence of plasma and serum exhibited almost the same k_{dep} values (data not shown), we employed a substrate depletion assay combined with plasma incubation. The use of

plasma in rat hepatocyte incubation altered the concentration-dependent depletion of propafenone into the independent manners at least within the concentrations employed. On the contrary, the k_{dep} values of propafenone in human hepatocytes with plasma incubation remained concentration-dependent, and the estimated preliminary K_m value was also low, as observed in the control incubation. Large species differences in unbound fractions of propafenone were pronounced between rat and human plasma (0.0071 vs. 0.0754). The differentiated depletion profiles of rat and human hepatocytes with plasma incubation resulted mainly from species differences in plasma protein binding, indicating that protein binding plays a key role in *in vivo* nonlinearity in rats.

Furthermore, in the relevant *in vivo* concentration range of 0.1 to 1 μg/ml, conflicting results of plasma protein binding were found between rats and humans. The unbound fraction increased with increasing propafenone concentration in rat plasma although it remained constant in human plasma. When the binding capacities estimated based on a model of two binding components were compared with the reported concentrations of AGP and/or albumin in rat and human plasma (Komori et al., 2001; Israili and Dayton, 2001), the primary and secondary sites were identified as AGP and plasma albumin, respectively. Noticeably, the capacity of the primary component of rat plasma is about 6-fold lower than that of human plasma, which probably leads to the nonlinear plasma binding in rats at relevant *in vivo* concentrations. Therefore, the metabolic data in our newly developed method, together with the protein binding profiles, prove that *in vivo* nonlinear pharmacokinetics in rats is at least associated with

saturation of plasma protein binding with the limited capacity of AGP, whereas the saturation of propafenone metabolism connected to low K_m values is a dominant factor in *in vivo* nonlinearity in humans.

One characteristic of nonlinear plasma concentration profiles caused by saturation of plasma protein binding are the dose-independent elimination half-lives attributable to dose-dependent changes in V_{ss} and clearance. Propafenone apparently showed similar CL in rats at the doses examined. Shimizu et al. (2002) reported that due to hepatic blood flow dependent clearance, aprinidine with nonlinear plasma protein binding exhibits plasma concentration profiles with a convex elimination curve, as observed in propafenone after intravenous administration in rats. However, propafenone can be categorized as a moderate clearance compound because the blood-plasma partition ratio of propafenone was preliminarily estimated as 0.96. Additionally, this classification was supported by the data that the *in vivo* clearance estimated from the *in vitro* CL_{int} according to the well-stirred model was moderate. Hence, the *in vivo* disposition profile in rats cannot be explained only by nonlinear protein binding indicating that saturable metabolism, as an additional factor, is, at least in part, involved in the *in vivo* profiles in rats.

The k_{dep} values were independent of the initial concentration in rat hepatocytes with plasma incubation. However, taking the nonlinear protein binding in rat plasma into consideration, it is postulated that the independent k_{dep} values in the presence of plasma can be attributed to the fact that reduced k_{dep} based on low K_m values would be canceled out by the increased k_{dep} associated with the unbound fraction increment at the higher

concentration range. Therefore, propafenone apparently shows linear pharmacokinetic behavior with the counterbalance of the two saturable factors.

Furthermore, to quantitatively investigate the *in vitro* and *in vivo* relationship on the clearance of propafenone, the CL_H values were calculated from the k_{dep} values obtained in the substrate depletion assay with plasma incubation using rat and human hepatocytes. The CL_H value for humans decreased to ca. one-fourth with increasing the initial concentration from 0.05 to 5 μM, and the tendency was in accordance with the nonlinearity reported by Hollmann et al. (1983). Although the CL_H values for rats were relatively lower than the corresponding data after intravenous dosing, the values were similar between the two concentrations, being consistent to the dose-independent CL *in vivo*. To predict the plasma concentration profiles after intravenous and oral administrations, further investigation on a complex physiological model with the two saturable factors is needed. However, our newly developed method is valuable to anticipate dose-dependent change of *in vivo* clearance for drugs with one or two saturable factors.

In conclusion, the dose normalized AUC after oral administration of various doses of propafenone to female Wistar rats was constant at all doses examined, unlike in humans. Both rat and human hepatocytes without plasma or plasma protein showed a concentration-dependent depletion and low K_m values. However, in the assay with rat or human plasma incubation, the profile was altered into an independent profile in rat but not human hepatocytes. This difference was related to species differences in plasma

protein binding. Thus, low K_m values and protein binding of propafenone are key parameters for understanding species differences in its nonlinearity. These findings suggest that the substrate depletion assay with plasma incubation is a useful tool for evaluating the nonlinearity of NCEs with no identification of metabolic pathways.

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Legends

Fig. 1. Plasma concentrations (A, C) and their dose-normalized (B, D) profiles of propafenone after intravenous (A, B) and oral (C, D) administrations at various doses to female Wistar rats. Each point in A and C represents the mean ± SD (n = 3).

Fig. 2. Disappearance of propafenone in cryopreserved human hepatocytes at various initial concentrations (A). Propafenone at concentrations of 0.05, 0.2, 1 and 5 μM was incubated with 1×10^6 cells/ml of human hepatocytes individually prepared from three donors. Each point represents the mean ± SD of the remaining % of substrate to the initial concentration in hepatocytes from three donors. Relationship between initial substrate concentration and depletion rate constant of propafenone in cryopreserved human hepatocytes (B). Each point represents the mean ± SD of depletion rate constants of three donors. The line represents curve predicted from eq. (1).

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TABLE 1

Physiological parameters for calculation of intrinsic clearance in rats and humans

	Rat	Human
Number of hepatocytes/g liver (cells/g liver)	125×10^6	120×10^6
Liver weight/kg body (g liver/kg)	40.4	24.2
Liver blood flow (ml/min/kg)	69.1	25.4

Data from Boxenbaum (1980) and Iwatsubo et al., (1996).

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TABLE 2

Pharmacokinetic parameters of propafenone after oral administrations at 3, 10, 20 and 45 mg/kg to female rats

		Dose (mg/kg)			
		3	10	20	45
C_{max}	(ng/ml)	55.3[a]	134	231	706
T_{max}	(hr)	0.5	0.5	0.25	0.25
$t_{1/2}$	(hr)	0.584	0.584	0.780	0.859
MRT	(hr)	1.17	1.33	1.74	1.92
AUC	(ng x hr/ml)	62.8	214	377	1502
CL/F	(l/hr/kg)	47.8	46.7	53.1	30.0

[a] derived from the average of plasma concentrations of three animals.

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TABLE 3

Pharmacokinetic parameters of propafenone after intravenous administrations at 0.2, 1 and 10 mg/kg to female rats

		Dose (mg/kg)		
		0.2	1	10
$t_{1/2}$	(hr)	0.358[a]	0.602	1.61
V_{ss}	(l/kg)	1.32	2.12	6.82
AUC	(ng x hr/ml)	86.0	461	3518
MRT	(hr)	0.567	0.973	2.40
CL	(l/hr/kg)	2.33	2.17	2.84

[a] derived from the average of plasma concentrations of three animals.

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TABLE 4

Kinetic parameters for propafenone metabolism in liver microsomes and hepatocytes of rats and humans without plasma

	Rats		Humans	
	Microsomes	Hepatocytes	Microsomes	Hepatocytes[b]
K_m (μM)	0.37 ± 0.08[a]	0.38 ± 0.07	0.11 ± 0.02[c]	0.092 ± 0.030[c]
$CL_{int,app[S]=0}$ (ml/min/mg or ml/min/10^6cells)	3.72 ± 0.09	1.15 ± 0.17	1.76 ± 0.21[c]	0.239 ± 0.065[c]

[a] the mean ± SD for kinetic parameters estimated from individual incubation (n=3-4).
[b] hepatocytes from three donors (lot no: 83, 86 and 133) were used.
[c] significant difference from rats (P<0.01).

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TABLE 5

Depletion rate constant of propafenone in rat hepatocytes with and without plasma incubation

Initial concentration (μM)	k_{dep} (min^{-1})	
	Buffer[a]	Plasma
0.05	0.601 ± 0.166[b]	0.00565 ± 0.00011
0.2	0.333 ± 0.030	0.00788 ± 0.00321
1	0.172 ± 0.017	0.00331 ± 0.00137
5	0.0703 ± 0.0157	0.00572 ± 0.0103

[a] without plasma.
[b] the mean ± SD of three experiments.

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TABLE 6

Depletion rate constant of propafenone in human hepatocytes with or without plasma, HSA and AGP

Initial concentration (μM)	k_{dep} (min^{-1})			
	Buffer[a]	Plasma	HSA	AGP
0.05	0.0892 ± 0.0523[b]	0.00910 ± 0.00803 [b]	0.0201[c]	0.00336 [c]
0.2	0.0522 ± 0.0338	0.00999 ± 0.00783	0.0119	0.00621
1	0.0222 ± 0.0093	0.00258 ± 0.00111	0.00427	0.00289
5	0.00774 ± 0.00516	0.00120 ± 0.00022	0.000773	0.00257

[a] without plasma.
[b] the mean ± SD of hepatocytes from three donors (lot no: 83, 86 and RNG).
[c] the mean of hepatocytes from two donors (lot no: 86 and RNG).

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TABLE 7

Unbound fraction of propafenone in rat and human at the relevant concentrations *in vivo*

Initial concentrations (μg/ml)	Unbound fraction	
	Rats	Humans
0.1	0.0053 ± 0.0021[a]	0.0552 ± 0.0085
0.3	0.0111 ± 0.0004	0.0557 ± 0.0137
1	0.0495 ± 0.0059	0.0646 ± 0.0154

[a] the mean ± SD of three experiments.

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TABLE 8

Protein binding parameters for propafenone in rat and human plasma

		Estimated values ± SD[a]	
		Rats	Humans
B_{max1}	(μM)	1.43 ± 0.35	8.63 ± 2.49
K_{d1}	(μM)	0.00759 ± 0.00295	0.664 ± 0.253
B_{max2}	(μM)	762 ± 339	1084 ± 147
K_{d2}	(μM)	83.9 ± 43.5	322 ± 84.7

[a] derived from fitting the mean values of three experiments to eq (4).

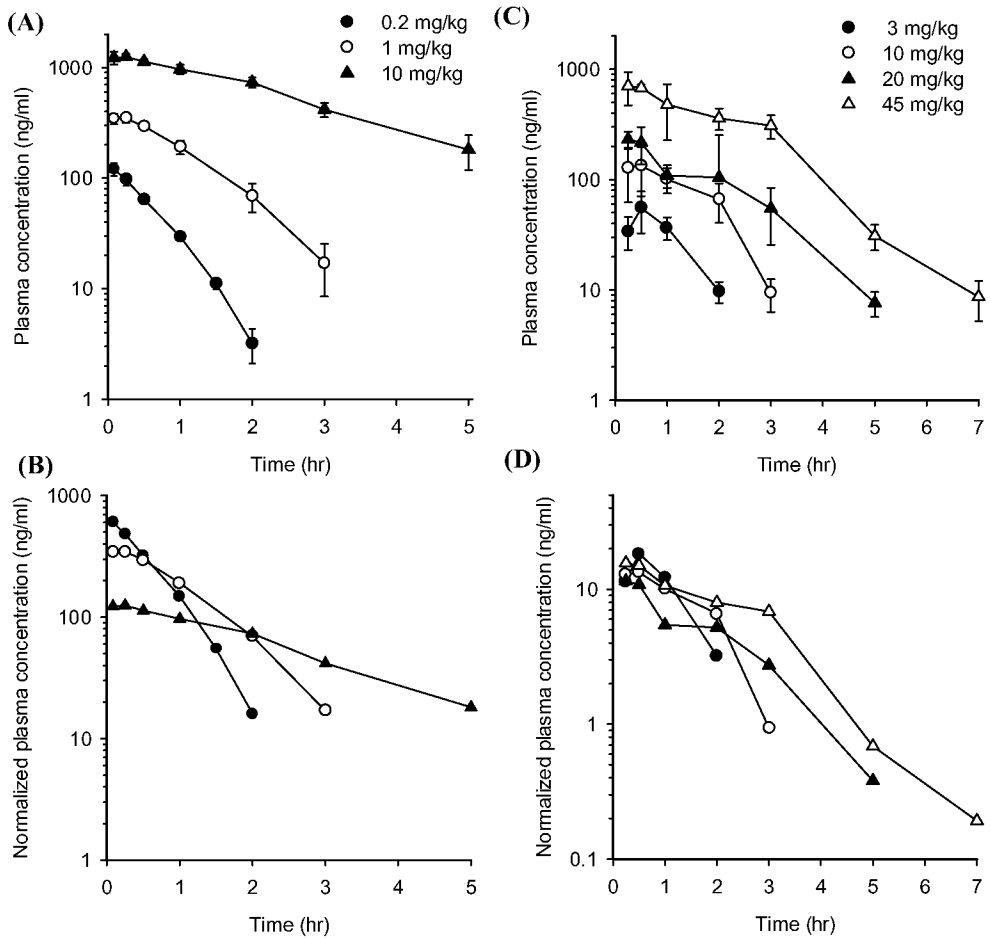

Figure 1

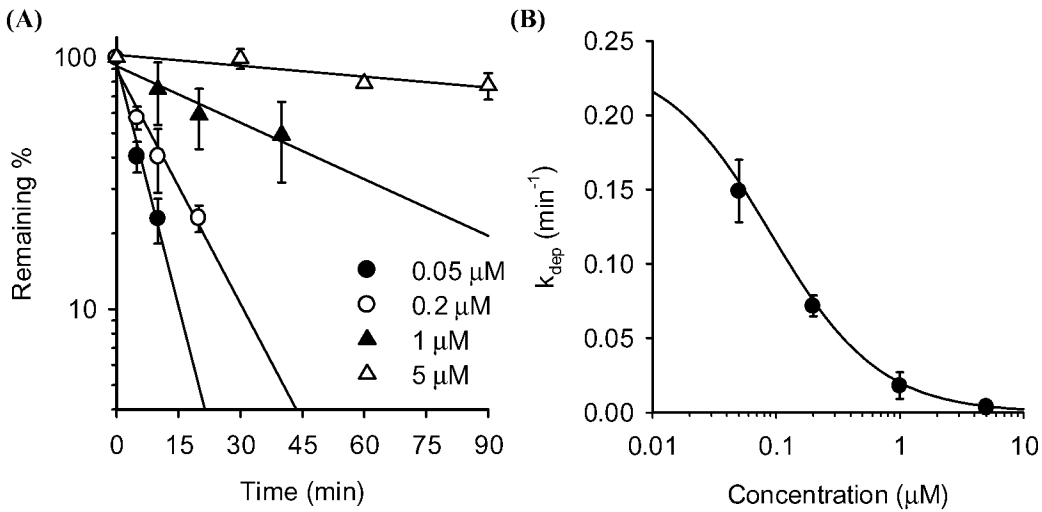

Figure 2