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, Osaka, Japan

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ABSTRACT:

Linear pharmacokinetic profiles of propafenone in female Wistar rats were found after oral administration of up to 20 mg/kg. These profiles differed from nonlinear pharmacokinetics in a dose-dependent manner with increasing plasma concentrations in humans (Hollmann M, Brode E, Hotz D, Kaumeier S, and Kehrnhahn OH (1983) Arzneim-Forsch 33:763–770). 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 ($<0.4 \mu M$). 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 versus 0.0754 for rats and humans, respectively, at 0.1 $\mu g/ml$). In rat plasma, the unbound fraction increased with concentrations of 0.1 to 1.0 $\mu g/ml$, 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 canceled 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 in the fact that for drugs with a high increase of area under the plasma concentration curve (AUC) relative to the dose, designing appropriate dose regimens is difficult. Robust screening methods for evaluating membrane permeability, metabolic stability, and cytochrome P450 (P450) inhibition have been developed to identify NCEs with desirable pharmacokinetic profiles (Roberts, 2001; Kariv et al., 2002; Ansede and Thakker, 2004). 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; Han et al., 1999; Takeuchi et al., 2001). 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 Michaealis 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 to 1 $\mu M$ (Clarke and Jeffrey, 2001; Shibata et al., 2002; Naritomi et al., 2003) assuming that $K_m$ values are at least higher than 5 to 10 $\mu M$.

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 P450 isofrom are comparable to those from metabolite formation assays in recombinant P450 isofrom studies. We also demonstrated that depletion assays are applicable for estimating $K_m$ values of propanololol 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 param-
ers 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 $\beta$-adrenergic-antagonist properties (Funck-Brentano et al., 1990) that generally undergoes CYP2D6-dependent biotransformation to 5-hydroxypropafenone in humans (Kroemer et al., 1989, 1991; Botsch et al., 1993). Oral administration to healthy volunteers provided the maximum plasma concentration ($C_{\text{max}}$) and 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 administration 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 $\alpha_1$-acid glycoprotein (AGP), human serum albumin (HSA, fraction V) and collagenase type IV (prepared from Clostridium histolyticum), and trypsin inhibitor (prepared from soybean type II) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan), 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) and Xenotech (Lexena, KS), respectively. Pooled human plasma was obtained from KAC Co. (Kyoto, Japan), and the dialysis membrane (Spectra/Per) and Centrifree (Lenexa, KY), respectively. Pooled human plasma was obtained from KAC Co. (Kyoto, Japan), and the dialysis membrane (SpectraPor) and Centrifree were obtained from Spectrum Laboratories Inc. (San Diego, CA) and Millipore Corporation (Bedford, MA), 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 h before experimentation. Propafenone was dissolved in 10% Cremophor EL in saline at 0.2 to 10 mg/ml, and intravenously or orally administered to five or six 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 3000 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 a filtration method. Propafenone dissolved in dimethyl sulfoxide was added into rat, human plasma, 0.098% AGP, and 4% HSA, and the initial concentration of propafenone was 0.1 to 500 $\mu$g/ml (the final concentration of dimethyl sulfoxide was less than 1%). With the dialysis method, 1-ml samples were transferred to the dialysis membrane after incubation for 15 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 50g 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 $\times$ 106 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 liquid chromatography/mass spectrometry apparatus equipped with an electrospray probe (Agilent Technologies, Palo Alto, CA). Chromatography was conducted on a 3.5-$\mu$m Symmetry C18 column, 4.6 $\times$ 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 to 80:20 at 5 min, v/v). Eluted propafenone was detected at m/z 342.2 in a positive ion mode. The limit of quantification was 1 ng/ml when 0.1 ml of 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-Hagen, 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 constant ($k_{\text{dep}}$). $K_m$ was estimated by fitting the $k_{\text{dep}}$ at various initial concentrations to the following equation using nonlinear least-squares regression analysis combined with MULTI (Yamaoka et al., 1981),

$$k_{\text{dep}} = k_{\text{dep}(0)} \left(1 - \frac{S}{S + K_m}\right)$$

where $S$ is the substrate concentration and $k_{\text{dep}(0)} - \alpha$ represents the theoretical maximum elimination rate constant at an infinitesimally low substrate concen-
TABLE 1

<table>
<thead>
<tr>
<th>Rat</th>
<th>Human</th>
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</thead>
<tbody>
<tr>
<td>Number of hepatocytes/g liver (cells/g liver)</td>
<td>125 × 10^6</td>
</tr>
<tr>
<td>Liver weight/kg body (g liver/kg)</td>
<td>40.4</td>
</tr>
<tr>
<td>Liver blood flow (ml/min/kg)</td>
<td>69.1</td>
</tr>
</tbody>
</table>

Data are from Bosenbaum (1980) and Iwatsubo et al. (1996).

Results

The plasma concentrations and pharmacokinetic parameters of propafenone after oral administration (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; Table 3). However, Vss 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 (0.36–1.61 h).

To estimate the enzyme kinetic parameters of propafenone, kdep 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 kdep 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 Km values in the liver microsomes and hepatocytes were almost identical (0.37–0.38 and 0.092–0.11 μM, respectively). The Km values for humans were significantly lower than those for rats (P < 0.01). Furthermore, the in vitro system of humans showed significantly lower CLint values than did the corresponding system of rats (P < 0.01). There was a 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 kdep values in the depletion assay with plasma incubation were constant at all concentrations tested, differing from those in the control condition without plasma incubation, which showed concentration-dependent elimination. Especially, there was a pronounced difference in kdep values at the initial low concentration (0.05 μM) between incubations with and without plasma (0.00565 versus 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 Km values were 0.57 and 0.23 μM, respectively. Such a dependence was also found in the presence of HSA or AGP at physiological concentrations instead of human plasma; however, the addition of AGP provided a relatively high Km value (1.58 μM), unlike HSA.

The CLH was estimated from the kdep values at the initial concentrations of 0.05 and 5 μM obtained in the substrate depletion assay with plasma incubation. The estimated CLH was not changed (1.3 l/h/kg) for rats, but decreased from 0.78 to 0.18 l/h/kg for humans with increasing 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, whereas human plasma provided similar values.

Using a wide range of propafenone concentrations (0.1–500 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 Bmax1 values for the primary component were
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 20–150 ng/ml) where nonlinearity was

1.43 μM for rat plasma and 8.63 μM for human plasma, and the respective $B_{\text{max2}}$ values for the secondary component were 762 and 1084 μM. The $B_{\text{max1}}$ 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 20–150 ng/ml) where nonlinearity was
observed after oral administration in humans. It is possible that poor absorption at higher doses masked the supraproportional increment of AUC relative to the dosage changes, although the oral formulation of propafenone was a solution for all doses and propafenone was membrane-permeable through the Caco-2 monolayer (H. Komura and M. 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, Vss 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; Evans et al., 1990; Niazi et al., 1996; Kiriyama et al., 1999; 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 Km values in rat and human hepatocytes were within the low range (Km < 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 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 Km values only with compounds showing a typical Michaelis-Menten kinetic profile. The relationship between substrate concentration and kdep 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 kdep values (data not shown), we used 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 mode at least within the concentrations used. On the contrary, the kdep values of propafenone in human hepatocytes with plasma incubation remained concentration-dependent, and the estimated preliminary Km 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 versus 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; Israel 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 Km 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 is the dose-independent elimination half-lives attributable to dose-dependent changes in Vss 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, aprindine 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 CLint according to the well

### Table 4

<table>
<thead>
<tr>
<th>Species</th>
<th>Microsomes</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>0.37 ± 0.08*</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>Humans</td>
<td>0.11 ± 0.02*</td>
<td>0.092 ± 0.030*</td>
</tr>
</tbody>
</table>

* Hepatocytes from three donors (lot no. 83, 86, and 133) were used.
* The mean ± S.D. for kinetic parameters estimated from individual incubation (n = 3–4).
* Significant difference from rats (P < 0.01).

### Table 5

<table>
<thead>
<tr>
<th>Initial Concentration</th>
<th>km (μM)</th>
<th>kdep (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>0.05 μM</td>
<td>0.601 ± 0.166b</td>
</tr>
<tr>
<td></td>
<td>0.2 μM</td>
<td>0.333 ± 0.030</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>0.172 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>5 μM</td>
<td>0.070 ± 0.0157</td>
</tr>
</tbody>
</table>

* Without plasma.
* Mean ± S.D. of three experiments.
ASSESSMENT OF NONLINEAR PHARMACOKINETICS OF PROPAFENONE

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

<table>
<thead>
<tr>
<th>Initial Concentration</th>
<th>Buffer*</th>
<th>Plasma</th>
<th>HSA</th>
<th>AGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 µM</td>
<td>0.0892 ± 0.0523b</td>
<td>0.00910 ± 0.00803b</td>
<td>0.0201c</td>
<td>0.00336c</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>0.0522 ± 0.0338</td>
<td>0.00999 ± 0.00783</td>
<td>0.0119</td>
<td>0.00621</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.0222 ± 0.0093</td>
<td>0.00258 ± 0.00111</td>
<td>0.00427</td>
<td>0.00289</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.00774 ± 0.00516</td>
<td>0.00120 ± 0.00022</td>
<td>0.000773</td>
<td>0.00257</td>
</tr>
</tbody>
</table>

* Without plasma.

The independent protein binding in rat plasma into consideration, it is postulated that hepatocytes with plasma incubation. However, taking the nonlinear propafenone apparently shows linear pharmacokinetic behavior with fraction increment at the higher concentration range. Therefore, calculated from the plex 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.

References


Address correspondence to: Masahiro Iwaki, Faculty of Pharmaceutical Sciences, Kinki University, 3–4–1 Kowakae, Higashi-Osaka, Osaka, 577-8502, Japan. E-mail: iwaki@phar.kindai.ac.jp