DMD Fast Forward. Published on February 23, 2009 as DOI: 10.1124/dmd.108.025478
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DMD Fast Forward. Published on February 23, 2009 as doi:10.1124/dmd.108.025478

Title: Application of Stable Isotope Methodology in the Evaluation of the Pharmacokinetics of (S,S)-3-[3-(Methylsulfonyl)phenyl]-1-Propylpiperidine Hydrochloride in Rats

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Running title: *Stable isotope coadministration approach for PNU96391*

Number of Text Pages: 34

Number of Tables: 4

Number of Figures: 7

Number of References: 28 (<=40)

Number of Words in the Abstract: 247 (<=250)

Number of Words in the Introduction: 743(<=750)

Number of Words in the Discussion: 1487 (<=1500)

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List of abbreviations:

ADME, absorption, disposition, metabolism and excretion; A_e, amount of unchanged drug in urine; AUC, area under the plasma concentration-time curve; C_{last}, quantifiable plasma concentration at the last time point; CL, plasma clearance; CL_{renal}, renal clearance; C_{max}, maximum plasma concentration; F_a, fraction absorbed; $F_a \cdot F_g$, bioavailability in the portal vein; F_g, gastrointestinal tract availability; F_h, hepatic availability; *fm*, fraction metabolized from PNU96391 to M1 in vivo; F_{oral}, oral bioavailability; GI, gastrointestinal tract; *k*, rate constant; IV, intravenous dose administration; LC-MS/MS , liquid-chromatography tandem mass spectrometry; M1, N-despropyl metabolite of PNU96391; PNU96391, (S,S)-3-[3-(methylsulfonyl)phenyl]-1-propylpiperidine hydrochloride; PO, oral dose administration; SI, stable isotope; $t_{1/2,z}$, apparent terminal half-life; t_{max}, time to reach maximum plasma concentration; λ_z, apparent elimination rate constant;

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Abstract

The primary objective of this study was to demonstrate the use of stable isotopes (SI) as an approach for pharmacokinetic analysis such as fraction absorbed, hepatic extraction ratio and fraction metabolized from parent drug to a metabolite. (S,S)-3-[3-(methylsulfonyl)phenyl]-1-propylpiperidine hydrochloride (PNU96391) was selected as the model compound because of its simple biotransformation pathway, i.e., the predominant metabolic pathway to N-despropyl metabolite (M1), which makes it a suitable candidate. The second objective was to fully characterize pharmacokinetics of PNU96391 in rats using the SI coadministration approach with quantitative analysis by liquid-chromatography tandem mass spectrometry. Overall the present study showed that 1) absorption of PNU96391 from the gastrointestinal tract was near-complete (>90% of the dose), 2) PNU96391 was predominantly metabolized to M1 (approximately 70% of the dose) and 3) M1 was exclusively eliminated into urine with negligible biotransformation (the ratio of renal clearance to plasma clearance ≈ 0.9). Therefore, the present study demonstrated the utility of the SI methodology for characterizing the pharmacokinetics of a compound within the drug discovery and development process. Furthermore, the compartmental pharmacokinetic modeling provided insights into the disposition and biotransformation rates of PNU96391 and M1, suggesting that the modeling could add further advantages to the SI coadministration approach. Despite the greater availability of SI-labeled compounds, ADME scientists have yet to take full advantage of the potential use of these analogues for mechanistic ADME studies. These SI-labeled compounds can be used more widely to gain a better understanding of ADME properties in drug discovery and development.

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Introduction

Therapeutic drugs are most frequently administered orally, and the majority of these are intended to act systemically. A number of important factors limit systemic availability of orally administered drugs; therefore, an early estimation of oral bioavailability of new chemical entities is often desired to provide guidance to the iterative chemistry effort. Furthermore, since the oral bioavailability is primarily limited by either high first-pass hepatic extraction or low delivery to the portal circulation (due to low solubility, poor absorption and/or intestinal extraction), it is often of interest to determine the relative importance of these two factors (Kwan 1997). The contribution of each factor is assessed indirectly by comparing exposure levels obtained by administration through different routes (Rowland 1972; Gibaldi 1971) and/or from different blood sampling sites (Ward, el al, 2001; Murakami et al, 2003). Such information is essential as guides to chemical modifications aimed towards optimizing the oral bioavailability. Furthermore, pharmacokinetic evaluations of metabolites are often important in drug discovery and development, particularly when drug effects are mediated by pharmacologically active metabolites.

Stable isotope (SI)-labeled compounds have been employed in a number of biomedical fields including absorption, distribution, metabolism and excretion (ADME) studies since the combination of SI-labeling techniques with mass-spectrometry promoted greater use of these SI-labeled compounds (Baillie, 1978; Murphy and Sullivan, 1980; Wolen and Gruber, 1980; Baillie, 1981; Haskins, 1982). Major examples of the application of SI methodology are in vitro and in vivo metabolism studies for elucidating metabolic pathways and structures of metabolites (Hawkins, 1980; Baillie and Rettenmeier, 1986; Mutlib, 2008). The SI methodology has also been applied to preclinical and clinical pharmacokinetic studies (Browne *et al.,* 1984; Shinohara

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and Baba, 1990; Theis et al., 1994; Preston et al., 1999). The use of SI methodology in pharmacokinetic studies involves the simultaneous administration, to the same individual, of the non-labeled drug with the SI-labeled drug, e.g., an intravenous (IV) and oral (PO) dose administration, respectively. The method offers considerable advantages over traditional crossover or parallel design studies because only intra-individual differences in pharmacokinetics are taken into account. Despite the greater availability of SI-labeled compounds, especially synthesized to be used as internal standards for quantitative analysis, ADME scientists have yet to take full advantage of the potential use of these analogues for mechanistic ADME studies. These SI-labeled compounds can be used more widely to gain a better understanding of ADME properties in drug discovery and development.

The primary objective of this work was to demonstrate the use of SI-labeled compounds as an approach for pharmacokinetic analysis such as fraction absorbed, hepatic extraction ratio, fraction metabolized from parent drug to a metabolite, etc. (S,S)-3-[3-(methylsulfonyl)phenyl]-1-propylpiperidine hydrochloride (PNU96391) was selected as the model compound because of its simple biotransformation pathway, i.e., the predominant metabolic pathway to N-despropyl metabolite (M1), which makes it a suitable candidate (Figure 1) (Wienkers and Wynalda, 2002). PNU96391 is a substituted (S)-3-phenylpiperidine derivative which exhibits some affinity to the dopamine D_2 receptor family (Tedroff *et al.* 1998). The second objective was to fully characterize the pharmacokinetic profile of PNU96391 in rats using the SI coadministration approach with quantitative analysis by liquid-chromatography tandem mass spectrometry (LC-MS/MS). To investigate the extent of absorption and the first-pass hepatic extraction of PNU96391 in rats, SI-labeled [^{13}C,$^{2}H_3$]PNU96391 was orally coadministered to animals with non-labeled PNU96391 intravenously. Plasma samples were simultaneously collected from

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jugular and portal veins, and urine samples were collected to estimate renal excretion. In addition to the parent drug, its main metabolite, N-despropyl metabolite (M1), derived from both SI-labeled [^{13}C,$^{2}H_3$]PNU96391 (PO) and non-labeled PNU96391 (IV), was quantitatively determined by LC-MS/MS. In a separate study, [^{13}C,$^{2}H_3$]PNU96391 was orally coadministered to rats with non-labeled M1 intravenously to estimate the fraction metabolized from PNU96391 to M1 in vivo. In addition to non-compartmental pharmacokinetic analysis, the pharmacokinetic model that describes the parent drug and M1 in portal and jugular veins was developed to evaluate pharmacokinetic parameters using the non-linear mixed effect model (NONMEM) (Beal and Sheiner, 1992). NONMEM technique has been widely used and applied to preclinical and clinical pharmacokinetic and pharmacokinetic-pharmacodynamic analyses of many xenobiotics (Yukawa, 1999; Bauer et al, 2005; Dartois et al, 2007; Yamazaki et al, 2008). In the present study, the compartmental pharmacokinetic modeling provided insights into the disposition and biotransformation rates of PNU96391 and M1, suggesting that the modeling could add further advantages to the SI coadministration approach. Overall these results demonstrated the utility of the SI methodology for pharmacokinetic studies in drug discovery and development by fully characterizing in vivo pharmacokinetics of PNU96391.

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Materials and Methods

Chemicals

PNU96391 (chemical purity >99%), its main metabolite (N-despropyl metabolite, M1, >99%) and [^{13}C,$^{2}H_3$]PNU96391 (>99%) were obtained from Pfizer Global Research & Development (Kalamazoo, MI). Other reagents were of reagent grade.

Animals

All experiments with rats were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, as well as with internal company policies and guidelines. Male Sprague-Dawley rats, weighing 280 to 320 g, implanted with either a jugular vein cannula or both jugular and portal vein cannulas were obtained from Charles River Laboratories (Portage, MI, or Hollister, CA). Each animal was housed in stainless cages or metabolic cages (urine collection) under controlled conditions (20 - 26°C, 30-70% relative humidity and 12 h light/dark cycle). The animals were fasted overnight with free access to water.

In Vivo Pharmacokinetic Studies

PNU96391 (hydrochloride salt), [^{13}C,$^{2}H_3$]PNU96391 (hydrochloride salt) and non-labeled M1 (hydrochloride salt) were dissolved in saline for the dosing formulation. The concentration of the formulation was 2.5 mg/mL. Dose levels of PNU96391, [^{13}C,$^{2}H_3$]PNU96391 and M1 were expressed as free base equivalents.

A total of 3 in vivo pharmacokinetics studies of PNU96391 were completed in the present work. The study outlines are summarized in Table 1. For the preliminary study investigating SI effects on pharmacokinetics of PNU96391 in jugular vein-cannulated rats (study #1), a mixture of PNU96391 and [^{13}C,$^{2}H_3$]PNU96391 in the solution (2.5 mg/mL each) was

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orally administered to animals at 10 mg/kg (n = 2 animals). Blood samples (approximately 0.1 mL) were collected from the jugular vein at 0 (pre-dose), 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h post-dose. Urine samples were collected at 0-6, 6-12 and 12-24 h post-dose with a minimal cage rinse. To fully characterize pharmacokinetics of PNU96391 and M1 in rats (study #2), animals implanted with both jugular and portal vein cannulas (n = 4 animals) were coadministered PNU96391 intravenously (5 mg/kg IV) with [^{13}C,$^{2}H_3$]PNU96391 orally (10 mg/kg PO). Blood samples (approximately 0.1 mL) were collected from the jugular and portal veins at 0 (pre-dose), 2, 10, 30 min, 1, 2, 4, 6, 8 and 24 h post-dose. To investigate the fraction metabolized from PNU96391 to M1 in vivo (*fm*) (study #3), animals implanted with jugular vein cannula (n = 4 animals) were coadministered M1 intravenously (5 mg/kg IV) with [^{13}C,$^{2}H_3$]PNU96391 orally (10 mg/kg PO). Blood samples (approximately 0.1 mL) were collected from the jugular veins at 0 (pre-dose), 2, 10, 30 min, 1, 2, 4, 6, 8 and 24 h post-dose. In studies #2 and #3, urine samples were also collected at 0-4, 4-8, and 8-24 h post-dose with a minimal cage rinse. All blood samples were collected with K_2EDTA as the anticoagulant and were centrifugated immediately after the collection. The resulting plasma samples were stored at approximately -20°C until analysis.

Assay of PNU96391 and Its N-Despropyl Metabolite (M1)

Concentrations of PNU96391, [^{13}C,$^{2}H_3$]PNU96391, M1 and labeled M1 (free base equivalents) in biological samples were quantitatively determined by LC-MS/MS after deproteinization with a 10% aqueous trichloroacetic acid solution. The separation of analyzed substances was achieved with a Waters Alliance 2790 Chromatography system (Waters Corporation, Milford, MA) with an ACE 5 Phenyl, 5 cm × 2.1 mm id column (Advanced Chromatography Technologies, Aberdeen, Scotland). Mass spectrometric analyses were

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performed on a Micromass Quattro Ultima (Waters Corporation) using electrospray ionization. A gradient mobile phase of 10 mM ammonium acetate buffer (pH 4) and acetonitrile was maintained at a constant flow rate of 0.25 mL/min for a total run time of 6 min. The gradient started with 90% buffer for 0.5 min and then changed to 80% acetonitrile over a 1-min period. After 0.5 min of flow, the gradient proceeded back to 90% buffer over a 0.1-min time period and was held for the remaining run time. The injection volume was 5 µL. The retention times of PNU96391 and M1 were approximately 3.3 and 2.6 min, respectively. Product ions with m/z 169 (non-labeled parent and M1) and m/z 173 (labeled parent and M1) from collisionally induced dissociation of the respective protonated molecules of PNU96391 (MH^+, m/z 282), M1 (MH^+, m/z 240), [^{13}C,$^2H_3$]PNU96391 (MH^+, m/z 286) and labeled M1 (MH^+, m/z 244) were monitored. The pilot study demonstrated that the precision and accuracy of the biological assay method were consistent with and without an internal standard correction. Therefore all analyses were subsequently performed without an internal standard. The calibration curves of non-labeled M1 were used for the quantifications of the labeled M1. The LC-MS/MS method exhibited a calibration range of 0.0045 to 3.6 µM for the parent drug and 0.0050 to 4.1 µM for M1. The precision and accuracy of the bioanalytical method were estimated by analysis of quality control samples, all of which were within 15%. All reported data are expressed as free base equivalents.

Pharmacokinetic Analysis

Non-compartmental pharmacokinetic parameters were determined using WinNonlin Version 5.2 (Pharsight, Mountain View, CA). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained from individual plasma concentration versus time data. The apparent elimination rate constant (λ_z) was determined from the linear regression

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slope of the terminal portion (last three to four quantifiable points) of the log plasma concentration-time curve. Apparent terminal half-life ($t_{1/2,z}$) was obtained from $0.693/\lambda_z$. The area under the plasma concentration-time curve ($AUC_{0\text{-}last}$) from time zero to the last time point with a quantifiable plasma concentration (C_{last}) was calculated using the linear trapezoidal rule. The area under the plasma concentration-time curve from time zero to infinity (AUC) was calculated by adding C_{last}/λ_z to $AUC_{0\text{-}last}$. Plasma clearance (CL) and renal clearance (CL_{renal}) were calculated by Dose/AUC and A_e/AUC, respectively, where A_e was the amount of unchanged drug in urine over 24 h post-dose. The volume of distribution at steady state (V_{ss}) was calculated by CL × (AUMC/AUC), where AUMC is the area under the first moment of the plasma concentration-time curve from time zero to infinity. An oral bioavailability (F_{oral}) was estimated as follows:

$$(AUC_{po,systemic} / AUC_{iv,systemic}) \times (Dose_{iv} / Dose_{po})$$

where $AUC_{iv,systemic}$ and $AUC_{po,systemic}$ are AUC estimates in the jugular vein after the intravenous and oral administration, respectively.

Additionally the bioavailability in the portal vein ($F_a \cdot F_g$) and the hepatic availability (F_h) were estimated by the following equations (Murakami *et al.*, 2003):

$$F_a \cdot F_g = (AUC_{po,portal} / AUC_{iv,portal}) \times (Dose_{iv} / Dose_{po})$$

$$F_h = (AUC_{po,systemic} / AUC_{po,portal}) \times (AUC_{iv,portal} / AUC_{iv,systemic})$$

where F_a and F_g are the fraction absorbed and bioavailability in the gastrointestinal tract (GI), respectively, and $AUC_{iv,portal}$ and $AUC_{po,portal}$ are AUC estimates in the portal veins after the intravenous and oral administration, respectively.

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The fraction metabolized from PNU96391 to M1 in vivo (*fm*) was estimated based on the method reported by Pang *et al.* (1979). Briefly, the plasma clearance estimate for M1 after the intravenous administration of M1 ($CL_{M1,IV}$) was calculated from the following equation:

$$CL_{M1,IV} = Dose_{M1,IV} / AUC_{M1,IV}$$

where $Dose_{M1,IV}$ is the intravenous dose of M1 and $AUC_{M1,IV}$ is the AUC estimate for M1 in the jugular vein after the intravenous administration of M1.

Assuming that 1) the oral dose of PNU96391 ($Dose_{PNU96391,po}$) was completely absorbed (i.e., $F_a = 1$ for PNU96391), 2) the first-pass metabolism of PNU96391 in the GI tracts was negligible (i.e., $F_g = 1$ for PNU96391) and 3) the sequential first-pass metabolism of M1 in the liver was negligible (the hepatic availability of the preformed M1 was complete, i.e., $F_h = 1$ for M1), the plasma clearance estimate for M1 after the oral administration of PNU96391 ($CL_{M1,PO}$) could be expressed by the following equation:

$$CL_{M1,PO} = fm \cdot Dose_{PNU96391,po} / AUC_{M1,PO}$$

where *fm* is the fraction metabolized from PNU96391 to M1 in vivo and $AUC_{M1,PO}$ is the AUC estimate for M1 in the jugular vein after the oral administration of PNU96391.

Assuming that in vivo clearance of M1 after the intravenous administration of M1 was equal to that after the oral administration of PNU96391, i.e., $CL_{M1,IV} = CL_{M1,PO}$, the fraction metabolized from PNU96391 to M1 in vivo (*fm*) was given by the following equation:

$$fm = (Dose_{M1,IV} / Dose_{PNU96391,po}) \times (AUC_{M1,PO} / AUC_{M1,IV})$$

Model Description of Compartmental Pharmacokinetic Analysis

In the coadministration study of PNU96391 intravenously (5 mg/kg IV) with [^{13}C,$^{2}H_3$]PNU96391 orally (10 mg/kg PO) to rats implanted with both jugular and portal vein cannulas (study #2), the compartmental model of drug disposition was developed based on

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pseudo multiple compartments of the parent drug and its main metabolite (M1). The final pharmacokinetic model contained 6 compartments: the GI tract, portal vein, central and peripheral compartments for the parent drug; portal vein and central compartments for M1 (Figure 2). Parameter predictions by a naïve-pooled pharmacokinetic analysis were accomplished with NONMEM version V program (University of California at San Francisco, San Francisco, CA) (Beal and Sheiner, 1992). The rates of change in each compartment can be expressed as follows:

$dA(1)/dt = -k12 \cdot A(1)$

$dA(2)/dt = k12 \cdot A(1) + k32 \cdot A(3) - k20 \cdot A(2) - k23 \cdot A(2) - k25 \cdot A(2)$

$dA(3)/dt = k23 \cdot A(2) + k43 \cdot A(4) - k30 \cdot A(3) - k32 \cdot A(3) - k34 \cdot A(3)$

$dA(4)/dt = k34 \cdot A(3) - k43 \cdot A(4)$

$dA(5)/dt = k25 \cdot A(2) + k65 \cdot A(6) - k50 \cdot A(5) - k56 \cdot A(5)$

$dA(6)/dt = k56 \cdot A(5) - k60 \cdot A(6) - k65 \cdot A(6)$

The subroutine ADVAN 8 was used for the NONMEM analysis with the first order estimation method. Random effects caused by factors (i.e., unknown pathophysiology, immeasurable differences in biochemistry and/or physiology, analytical variations) were estimated by the proportional error model. Model selection was based on a number of criteria such as the objective function value, estimates, standard errors and scientific plausibility as well as exploratory analysis of the goodness-of-fit plots. The difference in the objective function between two nested models was compared with a χ^2 distribution in which a difference of 6.63 was significant at the 1% level.

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Results

Stable Isotope Effects on Pharmacokinetics of PNU96391 and M1 in Rats – Study 1

As illustrated in Figure 3 on linear plots, the plasma concentrations of the SI-labeled parent drug and M1 were super-imposable to those of the non-labeled parent drug and M1. The differences in the plasma concentrations at each time point were less than ±13%. Mean C_{max} values were estimated to be 3.1 and 3.2 µM for the non-labeled and labeled PNU96391, respectively, and 5.4 and 5.5 µM for the non-labeled and labeled M1, respectively. Mean AUC values were estimated to be 5.5 and 5.7 µM·h for the non-labeled and labeled PNU96391, respectively, and 15 and 16 µM·h for the non-labeled and labeled M1, respectively. The urinary excretion of the SI-labeled parent drug and M1 were also super-imposable to those of the non-labeled parent drug and M1 (Figure 4). Mean urinary excretion of the parent drug over 24 h post-dose was 21% of the dose for the non-labeled compound and 23% of the dose for the labeled compound. Mean urinary excretion of M1 over 24 h post-dose was 72% of the dose for the non-labeled compound and 73% of the dose for the labeled compound. Collectively there was little difference in the plasma concentrations and urinary excretion of the parent drug and M1 between the labeled versus non-labeled compounds in rats after a single oral coadministration of PNU96391 and [$^{13}C,^{2}H_3$]PNU96391, demonstrating negligible SI effects on the in vivo pharmacokinetics of [$^{13}C,^{2}H_3$]PNU96391.

Pharmacokinetics of PNU96391 and M1 in Rats – Study 2

Plasma concentrations of PNU96391 and M1 in the portal and jugular veins are graphically presented in Figure 5 on semilog plots. Pharmacokinetic parameters of PNU96391 and M1 obtained from non-compartment pharmacokinetic analysis are summarized in Table 2. Following the coadministration, plasma concentrations of the non-labeled PNU96391 in the

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jugular vein (5 mg/kg IV) bi-exponentially declined with a $t_{1/2,z}$ of 1.5 h. The estimates of CL and V_{ss} for PNU96391 in the jugular vein were 2.4 L/h/kg and 2.7 L/kg, respectively. The CL_{renal} for PNU96391 was estimated to be 0.58 L/h/kg, which was approximately 25% of plasma clearance. The non-labeled M1 in the jugular vein showed the C_{max} value of 4.1 µM at 0.5 h post-dose. The AUC values for PNU96391 were 8.3 and 7.7 µM·h in the portal and jugular veins, respectively, whereas the AUC values for M1 were 12 and 10 µM·h, respectively. The mean AUC ratio of M1 to PNU96391 in the portal and jugular veins was approximately 1.5. There was little difference in the plasma concentrations of PNU96391 and M1 between the jugular and portal veins of rats after a single intravenous administration of PNU96391 at 5 mg/kg IV. For the oral administration of [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO), [$^{13}C,^{2}H_3$]PNU96391 was rapidly absorbed with a C_{max} of 6.7 µM in the portal vein at 0.5 h post-dose. The C_{max} value of [$^{13}C,^{2}H_3$]PNU96391 in the jugular vein was 2.8 µM, which was observed at 0.5 to 1 h post-dose. Thus the mean C_{max} value of [$^{13}C,^{2}H_3$]PNU96391 was 2 to 3-fold higher in the portal vein than the jugular vein. The plasma concentrations of [$^{13}C,^{2}H_3$]PNU96391 thereafter declined with $t_{1/2,z}$ of 1.2 to 1.4 h in both the portal and jugular veins. The C_{max} values of the labeled M1 in the portal and jugular veins were 5.6 µM and 5.3 µM, respectively, which were observed at 0.5 to 1 h post-dose. The mean AUC ratios of M1 to PNU96391 were 1.3 and 3.6 in the portal and jugular vein, respectively. The bioavailabilities in the portal vein ($F_a \cdot F_g$) and jugular vein (F_{oral}) of PNU96391 were estimated to be 95% and 40%, respectively. The hepatic availability (F_h) calculated from the AUC values in the portal and jugular veins was 43 ± 14%.

After a single coadministration of PNU96391 (5 mg/kg IV) with [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO) to male Sprague-Dawley rats, urinary excretion of the non-labeled PNU96391 over 24 h post-dose (5 mg/kg IV) was 25% of the dose whereas that of the non-labeled M1 was

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69% of the dose. The sum of PNU96391 and M1 excretions into the urine for non-labeled PNU96391 (5 mg/kg IV) accounted for >90% of dose. Urinary excretion of the labeled [^{13}C,$^{2}H_3$]PNU96391 (10 mg/kg PO) over 24 h post-dose was 23% of the dose whereas that of labeled M1 was 70% of the dose. The sum of PNU96391 and M1 excretions into the urine for the labeled [^{13}C,$^{2}H_3$]PNU96391 (10 mg/kg PO) accounted for >90% of the dose. Majority of urinary excretion of the parent drug and M1 was recovered up to 8 h post-dose after both the intravenous and oral administration.

Estimation of the Fraction Metabolized from PNU96391 to M1 in Rats – Study 3

Plasma concentrations of PNU96391 and M1 in the jugular vein are graphically presented in Figure 6 on semilog plots and the pharmacokinetic parameters are summarized in Table 3. Following the coadministration, plasma concentrations of the non-labeled M1 (5 mg/kg IV) bi-exponentially declined with an apparent terminal half-life of 1.9 h. Estimates of CL and V_{ss} for M1 were 1.4 L/h/kg and 2.2 L/kg, respectively. The CL_{renal} was estimated to be 1.2 L/h/kg, which was approximately 90% of plasma clearance. The estimate of $t_{1/2,z}$ for M1 after the intravenous administration (1.9 h) was nearly identical to that after the intravenous and oral administration of PNU96391 (2.0 to 2.4 h). The fraction metabolized from PNU96391 to M1 in vivo (*fm*) was estimated to be 0.73. The urinary excretion of M1 over 24 h post-dose was >90% of the intravenously administered dose. Majority of urinary excretion of M1 was recovered up to 4 h post-dose after the intravenous administration. Pharmacokinetic parameters and urinary excretion of the labeled parent drug and M1 for the oral administration of [^{13}C,$^{2}H_3$]PNU96391 (10 mg/kg PO) were comparable to those obtained from the study #2.

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Compartmental Pharmacokinetic Analysis of PNU96391 and M1 in Rats

The compartmental pharmacokinetic model analysis was performed based on the plasma concentrations of PNU96391 and M1 in the portal and jugular veins of rats after a single coadministration of PNU96391 intravenously at 5 mg/kg with [^{13}C,$^{2}H_3$]PNU96391 orally at 10 mg/kg. The pharmacokinetic parameters obtained from the 6 pseudo-compartmental analysis are tabulated in Table 4 and the observed and model-fitted plasma concentration-time profiles of PNU96391 and M1 in all animals are graphically presented in Figure 7. The variability in each pharmacokinetic parameter was reasonably acceptable (CV <40%). Intra-animal variability (proportional error model) was estimated to be 18%. The objective function value (OFV) was -136. The vast majority of the weighted residuals versus time after dosing and the model-predicted plasma concentrations of PNU96391 and M1 was symmetrically distributed around the zero ordinate and lay within 2 units of perfect agreement. Overall the fitness of prediction to observed data were satisfactory for both PNU96391 and M1 in the portal and jugular veins of rats.

The estimates of plasma clearance of the parent drug in the portal vein compartment (compartment 2) and the jugular vein compartment (compartment 3) were 0.63 and 1.6 L/h/kg, respectively. The estimate of CL for M1 in the jugular vein (compartment 5) was 0.42 L/h/kg which was 4-fold lower than that of the parent drug. The V_{ss} of the parent drug in the jugular vein (compartment 3) was estimated to be 1.8 L/kg. The biotransformation rate constant of the parent drug to M1 (k_{25}) was estimated to be 19 h^{-1} which was approximately 10-fold higher than the absorption rate constant (k_{12} = 1.7 h^{-1}). The estimates of the distribution rate constant of the parent drug and M1 from the portal to jugular veins (k_{23} = 61 $h^{-1}$and k_{65} = 77 h^{-1}) were relatively higher than those from the jugular to portal veins (k_{32} = 1.9 $h^{-}1$ and k_{56} = 1.6 h^{-1}). The estimates

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of the distribution rate constant of the parent drug between the central compartment and the peripheral compartment were comparable (k_{34} = 0.56 $h^{-1}$and k_{43} = 0.58 h^{-1}).

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Discussion

In the SI coadministration approach, it is important to first investigate the possibility of SI effects on pharmacokinetics of a test compound. In general, the SI-labeled position of test compound should be metabolically stable. The in vivo and in vitro biotransformation of PNU96391 has been reported with the particular emphasis placed on characterizing the importance of the *N*-depropylation pathway to M1, which is principally mediated by CYP2D6 in humans (Wienkers and Wynalda, 2002). The SI-labeled position of [^{13}C,$^{2}H_3$]PNU96391 is not at the piperidine ring having *N*-propyl group but at the methyl group of sulfonylphenyl ring (Figure 1), and the metabolism of the labeled position has not been observed in our laboratories. The present study demonstrated that oral pharmacokinetics of [^{13}C,$^{2}H_3$]PNU96391 in rats were super-imposable to those of the non-labeled PNU96391 (Figures 3 and 4), suggesting negligible SI effects on in vivo pharmacokinetics of [^{13}C,$^{2}H_3$]PNU96391. We therefore concluded that the SI-labeled PNU96391, i.e., [^{13}C,$^{2}H_3$]PNU96391, was appropriate for the use of SI methodology. Additionally we used Sprague-Dawley rats implanted with both jugular and portal vein cannulas. The estimates of CL and V_{ss} of PNU96391 in the jugular vein were 2.4 L/h/kg and 2.7 L/kg, respectively, in the present study, which were well consistent with the values reported previously (Shobe et al., 2000; Yamazaki et al., 2004). Thus, the effects of the cannula-implantation into both jugular and portal veins on the pharmacokinetics of PNU96391 appear to be negligible. Murakami et al. (2003) also reported that the gastrointestinal movement and the hepatic blood flow rate seemed to be little affected by the cannulation into both jugular and portal veins.

In the coadministration study of PNU96391 (IV) with [^{13}C,$^{2}H_3$]PNU96391 (PO), the bioavailability of PNU96391 in the portal vein ($F_a \cdot F_g$) and jugular vein (F_{oral}) were estimated in each animal by determining plasma concentrations of PNU96391 in the respective veins

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(Table 2). The bioavailability of PNU96391 in the portal vein ($F_a \cdot F_g$) was estimated to be 95%, suggesting that PNU96391 was almost completely absorbed from the GI tract. Near-complete absorption of PNU96391 was also suggested by the urinary excretion results showing that >90% of the oral dose was excreted into urine as PNU96391 and M1. In contrast, the oral bioavailability of PNU96391 in the jugular vein (F_{oral}) was estimated to be 40% (Table 2), suggesting that approximately 60% of the oral dose was eliminated by the first-pass metabolism. The hepatic availability (F_h) was estimated to be 43%, which was consistent with the oral bioavailability in jugular vein (F_{oral} = 40%) since the bioavailability in the portal vein ($F_a \cdot F_g$) was near-complete (>90%). The blood-to-plasma concentration ratio of PNU96391 has been reported to be approximately unity (Yamazaki, et al, 2004). Accordingly, the blood clearance of PNU96391 was calculated to be 2.4 L/h/kg which accounted for approximately 60% of hepatic blood flow in rats (4.2 L/h/kg) (Lin, et al, 1982). Thus, the oral bioavailability of PNU96391 (F_{oral} = 40%) is consistent with the estimated hepatic extraction ratio (ER = 60%), suggesting that the liver is likely to be the primary organ for elimination of PNU96391 in rats. Concerning the inter-animal variability on the oral bioavailability of PNU96391 determined in each animal by the SI methodology, the estimated coefficient of variation (CV) was 25%. In comparison, when utilizing a parallel study design to estimate the oral bioavailability by the individual oral AUC values divided by the mean intravenous AUC value, the oral bioavailability of PNU96391 was estimated to be 41 ± 18% with a CV of 43%. Thus the present SI coadministration approach appears to provide less variability by taking only the intra-animal variability into consideration. This advantage could be particularly important for bioequivalent studies, e.g., solution versus suspension, immediate-release tablet versus control-release tablet, etc.

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By the coadministration of M1 (IV) with [$^{13}C,^{2}H_3$]PNU96391 (PO), the fraction metabolized from PNU96391 to M1 (*fm*) was estimated to be 0.73 (Table 3). The *fm* value was estimated under the assumptions that 1) the oral dose of PNU96391 ($Dose_{PNU96391,po}$) was completely absorbed, 2) the first-pass metabolism of PNU96391 in the GI tracts was negligible and 3) the sequential first-pass metabolism of M1 in the liver was negligible. As mentioned above, the present results demonstrated that the absorption of PNU96391 was near-complete after the oral administration of PNU96391 (i.e., $F_a \approx 1$). Since the bioavailability of PNU96391 in the portal vein is near-complete and the liver is likely to be the primary organ for elimination of PNU96391 (i.e., $F_a \cdot F_g \approx 1$), the first-pass metabolism of PNU96391 in the GI tracts seems to be negligible. Regarding the third assumption, the urinary recovery of M1 was >90% of the administered dose after the intravenous administration of M1, suggesting that M1 was predominantly eliminated into urine with negligible hepatic metabolism. Therefore, this assumption was also supported by the present observation. Alternatively, taking into consideration the high urinary excretion of M1 (>90% of the dose) after the intravenous administration of M1, the urinary recovery of M1 (approximately 70% of the dose) after the oral administration of PNU96391 could also account for the in vivo f_m value, i.e., $f_m = 0.7$. Therefore the calculated f_m value (0.73) based on the plasma concentrations is consistent with the urinary excretion results. The comparison between plasma-based pharmacokinetics and urinary excretion is one of the advantages of PNU96391 as a model compound because of 1) its predominant biotransformation pathway from PNU96391 to M1 and 2) negligible biotransformation of M1 to further metabolites.

The compartmental pharmacokinetic model was constructed based on the assumption that PNU9639 was completely absorbed from the GI tract. As mentioned above, this assumption was

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supported by the present results. PNU96391 was rapidly absorbed with a large absorption rate constant (k_{12}= 1.7 h^{-1}), which was equivalent to absorption half-life of 0.4 h. This was consistent with the observation that PNU96391 was rapidly absorbed with t_{max} of <1 h. The absorption rate constant (k_{12} = 1.7 h^{-1}) was approximately 3-fold larger than the portal vein elimination rate constant (k_{20} = 0.63 h^{-1}) but >10-fold smaller than the distribution rate from the portal vein to the jugular vein (k_{23} = 61 h^{-1}) and the biotransformation rate constant (k_{25} = 19 h^{-1}), suggesting that the disposition of PNU96391 from the portal vein was faster than the absorption in the GI tract. It should be noted that real k_{23} and k_{25} values could be larger than the estimated values since the fraction metabolized to M1 and the fraction distributed into the central compartment were not incorporated into the compartmental pharmacokinetic analysis. The biotransformation rate constant of the parent drug to M1 (k_{25} = 19 h^{-1}) was equivalent to the biotransformation half-life of 0.04 h, which was consistent with the present observation that plasma concentrations of M1 were rapidly increased with t_{max} of <1 h after the intravenous and oral administrations of PNU96391. The CL estimate of PNU96391 in the central compartment (CL3 = 1.6 L/h/kg) was 30% to 40% lower than that from the non-compartment pharmacokinetic analysis (2.4 L/h/kg). Although the difference seemed to be acceptable (<2-fold), this might be, in part, due to the naïve-pooled pharmacokinetic analysis. Population pharmacokinetic analyses were also performed based on the individual plasma concentrations. However all attempts including step-by-step approaches have not been completed successfully. This could be largely due to a limited data set (n = 4 animals) compared to the number of parameters estimated. To the best of our knowledge, the current study represents the first attempt to apply pseudo multiple-compartmental modeling with NONMEM to pharmacokinetic analysis of both the parent drug and its metabolite as determined by the SI methodology.

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In conclusion, the present study showed that 1) PNU96391 was near-completely absorbed from the GI tract ($F_a \cdot F_g > 0.9$), 2) PNU96391 was predominantly metabolized to M1 ($fm \approx 0.7$) and 3) M1 was exclusively eliminated into urine with negligible biotransformation ($CL_{renal}/CL_{plasma} \approx 0.9$). Furthermore, the compartmental pharmacokinetic modeling provided insights into the disposition and biotransformation rates, suggesting that the modeling could add further advantages to the SI coadministration approach. Overall the present study demonstrates the utility of the SI methodology for pharmacokinetic studies in drug discovery and development. The approach is applicable to clinical studies as well and can potentially overcome the difficulties of a large inter-subject variance on different occasions that are found in the conventional parallel and crossover designs. The approach also reduces the number of animals (versus parallel design), the period of animal experiment (versus crossover design), and/or the number of in vivo samples required for analysis (versus parallel and crossover designs), thereby decreasing overall costs accrued from in vivo experiments and analysis of in vivo samples and resulting in less variability in pharmacokinetic evaluation. Despite the greater availability of SI-labeled compounds, especially synthesized to be used as internal standards for quantitative analysis, ADME scientists have yet to take full advantage of the potential use of these analogues for mechanistic ADME studies. These SI-labeled compounds can be used more widely to gain a better understanding of ADME properties in drug discovery and development.

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Acknowledgment

We greatly acknowledge the expert technical assistance of members of Preclinical Resource Laboratory Group (Pharmacia, Kalamazoo, MI), Brian W Jones, Jeri L Nederhoed, Lori R Norris, Renae J Ouding, Kenneth E Rousch and Terry L VandeGiessen, and In Vivo ADME Group (Pfizer, San Diego, CA), Melissa Cook and Robert Hunter, in the PNU96391 animal experiments described here. We also thank John A Easter and Ashok G Chaundhary for supplying [^{13}C,$^{2}H_3$]PNU96391 (Pharmacia, Kalamazoo, MI). We acknowledge with many thanks the excellent inputs on the draft manuscript from Bill J Smith and Zhongzhou (Andrea) Shen (Pfizer, San Diego, CA).

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Legends for figures

FIG. 1. Structures of PNU96391 (free base form) and its N-despropyl metabolite (M1).

Asterisk indicates the stable isotope labeled position of [^{13}C,$^{2}H_3$]PNU96391 and its N-despropyl metabolite (M1). Hydrochloride salt forms of PNU96391 and [^{13}C,$^{2}H_3$]PNU96391 were used for the dosing formulations.

FIG. 2. Six pseudo-compartmental pharmacokinetic model for PNU96391 and its N-despropyl metabolite, M1, in rats after a single coadministration of PNU96391 intravenously with [^{13}C,$^{2}H_3$]PNU96391 orally.

A, V and *k* are amount, volume of distribution and rate constant in each compartment, respectively. Clearance in each compartment was expressed as $CL_2=k_{20}\cdot V_2$, $CL_3=k_{30}\cdot V_3$, $CL_5=k_{50}\cdot V_5$ and $CL_6=k_{60}\cdot V_6$.

FIG. 3. Linear plasma concentration-time plots of PNU96391 and its N-despropyl metabolite (M1) in the male Sprague-Dawley rats #1 (A) and # 2 (B) after a single oral coadministration of PNU96391 with [^{13}C,$^{2}H_3$]PNU96391.

Each animal (n = 2) was orally coadministered PNU96391 (10 mg/kg PO) with [^{13}C,$^{2}H_3$]PNU96391 (10 mg/kg PO).

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FIG. 4. Urinary excretion of PNU96391 and its N-despropyl metabolite (M1) in the male Sprague-Dawley rats #1 (A) and # 2 (B) after a single oral coadministration of PNU96391 with [^{13}C,^{2}H$_3$]PNU96391.

Each animal (n = 2) was orally coadministered PNU96391 (10 mg/kg PO) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO).

FIG. 5. Semilog plasma concentration-time plots of PNU96391 and its N-despropyl metabolite (M1) in the male Sprague-Dawley rat after a single coadministration of PNU96391 IV (A) with [^{13}C,^{2}H$_3$]PNU96391 PO (B).

Each animal (n= 4) was coadministered PNU96391 (5 mg/kg IV) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO). Plasma concentrations of the labeled and non-labeled PNU96391 and M1 were determined in the portal and jugular veins by LC-MS/MS analysis. Values are expressed as mean ± SD.

FIG. 6. Semilog plasma concentration-time plots of PNU96391 and its N-despropyl metabolite (M1) in the male Sprague-Dawley rat after a single coadministration of M1 (IV) with [^{13}C,^{2}H$_3$]PNU96391 (PO).

Each animal (n= 4) was coadministered M1 (5 mg/kg IV) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO). Plasma concentrations of [^{13}C,^{2}H$_3$]PNU96391 and the labeled and non-labeled M1 were determined in the jugular veins by LC-MS/MS analysis. Values are expressed as mean ± SD.

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FIG. 7. Observed and model-fitted plasma concentrations of PNU96391 and its N-despropyl metabolite (M1) in the male Sprague-Dawley rats after a single coadministration of PNU96391 (IV) with [^{13}C,$^{2}H_3$]PNU96391 (PO).

Each animal (n = 4) was coadministered PNU96391 (5 mg/kg IV) with [^{13}C,$^{2}H_3$]PNU96391 (10 mg/kg PO). Plasma concentrations of the labeled and non-labeled PNU96391 and M1 were determined in the portal and jugular veins by LC-MS/MS analysis. OBS, Observed plasma concentrations; PRED, Model-fitted plasma concentrations.

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TABLE 1

Outlines of stable isotope coadministration studies of PNU96391 in rats

Study #	Compound dosed	Dosing Route	Dose (mg/kg)	Biological Samples Analyzed
1[a]	PNU96391	PO	10	Blood plasma (jugular vein) and urine
	[^{13}C,^{2}H$_3$]PNU96391	PO	10	
2[b]	PNU96391	IV	5	Blood plasma (portal and jugular veins) and urine
	[^{13}C,^{2}H$_3$]PNU96391	PO	10	
3[c]	M1	IV	5	Blood plasma (jugular vein) and urine
	[^{13}C,^{2}H$_3$]PNU96391	PO	10	

[a] Each animal (n=2) was coadministered PNU96391 (10 mg/kg PO) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO).

[b] Each animal (n=4) was coadministered PNU96391 (5 mg/kg IV) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO).

[c] Each animal (n=4) was coadministered M1 (5 mg/kg IV) with [^{13}C,^{2}H$_3$]PNU96391 (10 mg/kg PO).

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TABLE 2

Pharmacokinetic parameters of PNU96391 and its N-despropyl metabolite (M1) in male Sprague-Dawley rats after a single coadministration of PNU96391 (5 mg/kg IV) with [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO)

Dosing route	Substance analyzed	Sampling site	CL (L/h/kg)	V_{ss} (L/kg)	C_{max} (μM)	t_{max} (h)	$AUC_{0-\infty}$ (μM·h)	$t_{1/2,z}$ (h)	F[b] (%)
IV[a]	Parent	Portal	2.2 ± 0.6	2.6 ± 0.9	–	–	8.3 ± 2.2	1.3 ± 0.6	–
		Jugular	2.4 ± 0.6	2.7 ± 1.0	–	–	7.7 ± 1.8	1.5 ± 0.4	–
	M1	Portal	–	–	4.2 ± 1.2	0.5 ± 0	12 ± 3	2.4 ± 0.7	–
		Jugular	–	–	4.1 ± 1.2	0.5 ± 0	10 ± 2	2.1 ± 0.5	–
PO[a]	Parent	Portal	–	–	6.7 ± 1.9	0.5 ± 0	16 ± 3	1.4 ± 0.4	95 ± 18
		Jugular	–	–	2.8 ± 1.5	0.6 ± 0.3	6.4 ± 2.7	1.2 ± 0.6	40 ± 10
	M1	Portal	–	–	5.6 ± 1.6	0.9 ± 0.3	20 ± 3	2.2 ± 0.4	–
		Jugular	–	–	5.3 ± 1.2	0.8 ± 0.3	18 ± 3	2.0 ± 0.3	–

Values are expressed as mean ± SD.

[a] Each animal (n= 4) was coadministered PNU96391 (5 mg/kg IV) with [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO).

[b] Bioavailability in the portal vein ($F_a \cdot F_g$) and the jugular vein (F_{oral})

–: Not applicable

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TABLE 3

Pharmacokinetic parameters of PNU96391 and its N-despropyl metabolite (M1) in male Sprague-Dawley rats after a single coadministration of M1 (5 mg/kg IV) with [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO)

Dosing route	Substance analyzed	CL (L/h/kg)	V_{ss} (L/kg)	C_{max} (μM)	t_{max} (h)	$AUC_{0-\infty}$ (μM·h)	$t_{1/2,z}$ (h)	*fm*[b]
IV[a]	M1	1.4 ± 0.3	2.2 ± 0.7	–	–	16 ± 4	1.9 ± 0.4	–
PO[a]	Parent	–	–	4.4 ± 1.7	0.5 ± 0	6.6 ± 1.6	1.0 ± 0.3	–
	M1	–	–	7.9 ± 3.6	0.5 ± 0	19 ± 5	2.3 ± 0.6	0.73 ± 0.14

Values are expressed as mean ± SD.

[a] Each animal (n= 4) was coadministered M1 (5 mg/kg IV) with [$^{13}C,^{2}H_3$]PNU96391 (10 mg/kg PO).

[b] Fraction metabolized from PNU96391 to M1 (*fm*) was calculated by ($AUC_{M1,PO}$ / $Dose_{PNU96391}$) / ($AUC_{M1,IV}$ / $Dose_{M1,IV}$).

–: Not applicable

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TABLE 4

Pharmacokinetic parameter estimates of PNU96391 and its N-despropyl metabolite (M1) by the compartmental pharmacokinetic modeling

Parameters		Estimate	%RSE
CL_2	L/h/kg	0.63 (0.17)	27
V_2	L/kg	0.031 (0.0052)	17
CL_3	L/h/kg	1.6 (0.29)	18
V_3	L/kg	1.8 (0.16)	8
CL_5	L/h/kg	0.42 (0.14)	32
V_5	L/kg	0.46 (0.053)	11
CL_6	L/h/kg	0.056 (0.010)	18
V_6	L/kg	0.0081 (0.0030)	37
k_{12}	h^{-1}	1.7 (0.14)	8
k_{23}	h^{-1}	61 (14)	23
k_{32}	h^{-1}	1.9 (0.22)	12
k_{25}	h^{-1}	19 (3.5)	19
k_{34}	h^{-1}	0.56 (0.14)	25
k_{43}	h^{-1}	0.58 (0.10)	18
k_{56}	h^{-1}	1.6 (0.55)	33
k_{65}	h^{-1}	77 (28)	36
Variability		0.18 (0.015)	9

Precision of the estimates is expressed as standard error in parentheses.

Relative standard error (%RSE) was calculated by standard error/estimate $\times$ 100%.

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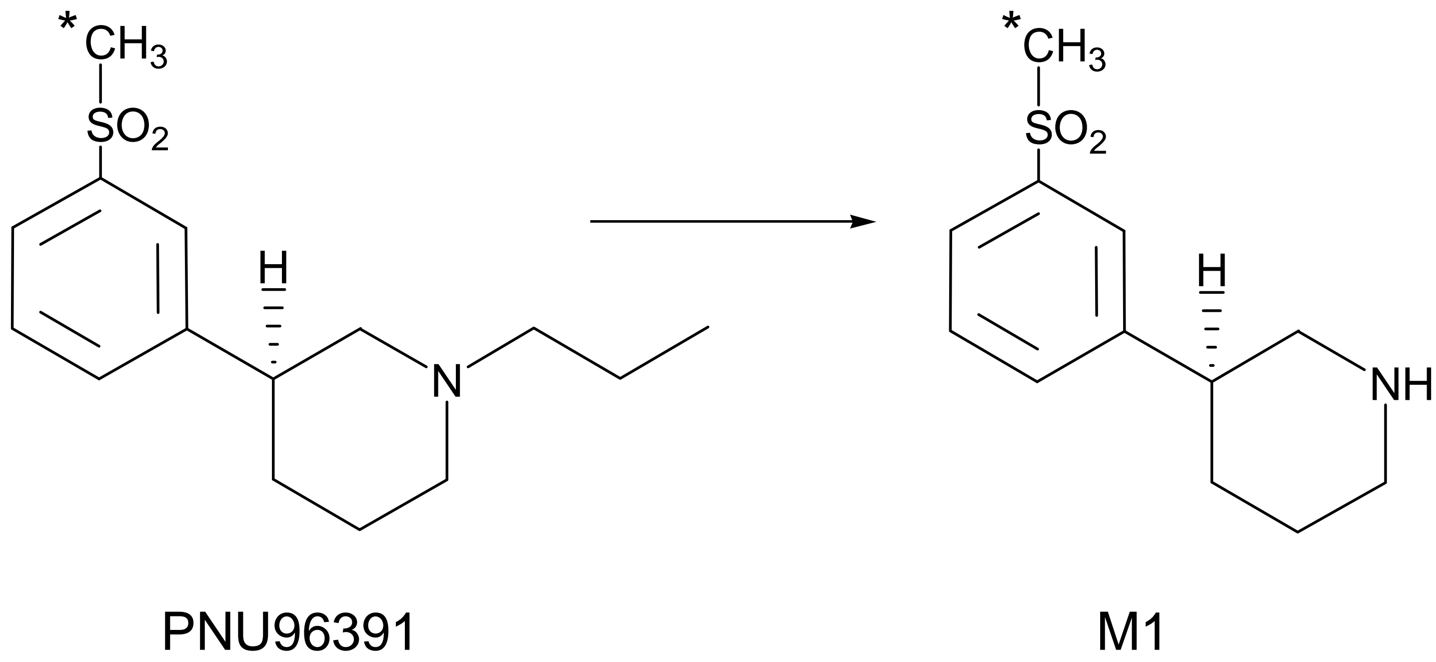

Figure 1

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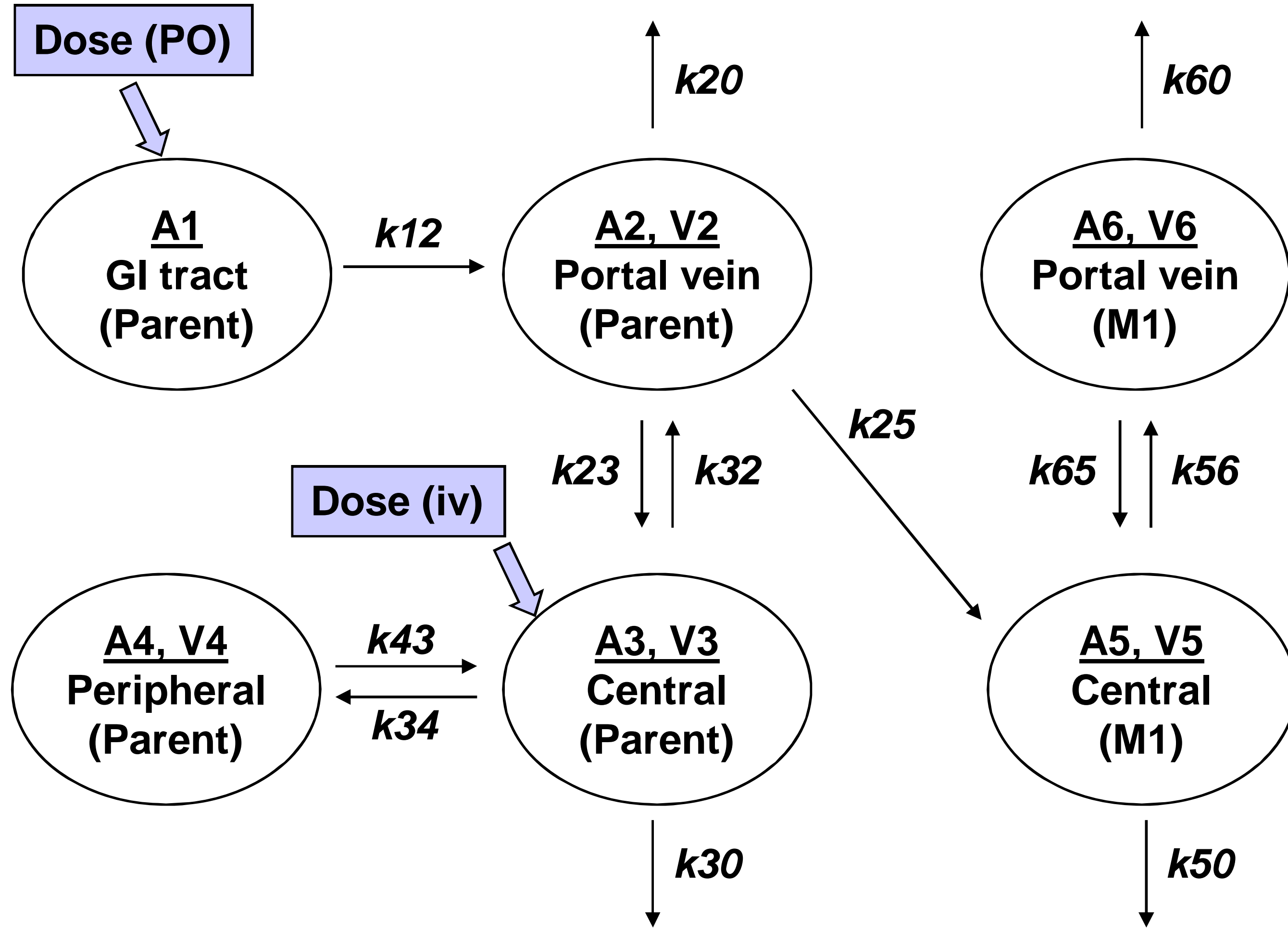

Figure 2

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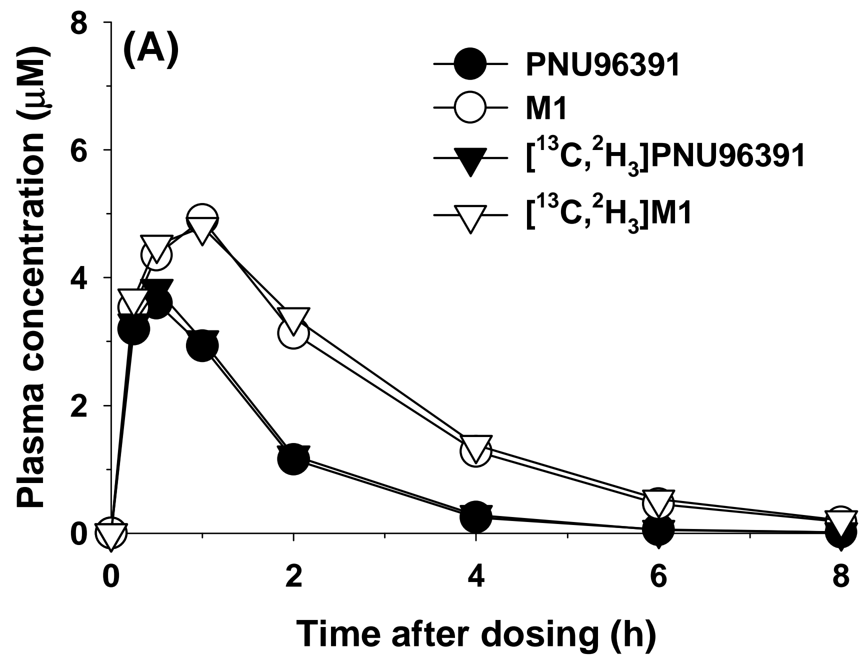

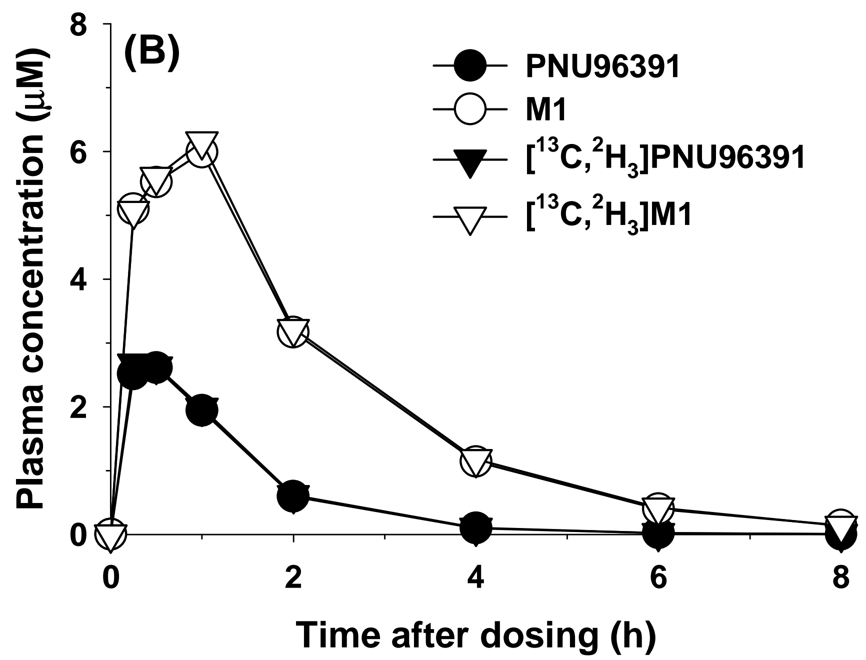

Figure 3

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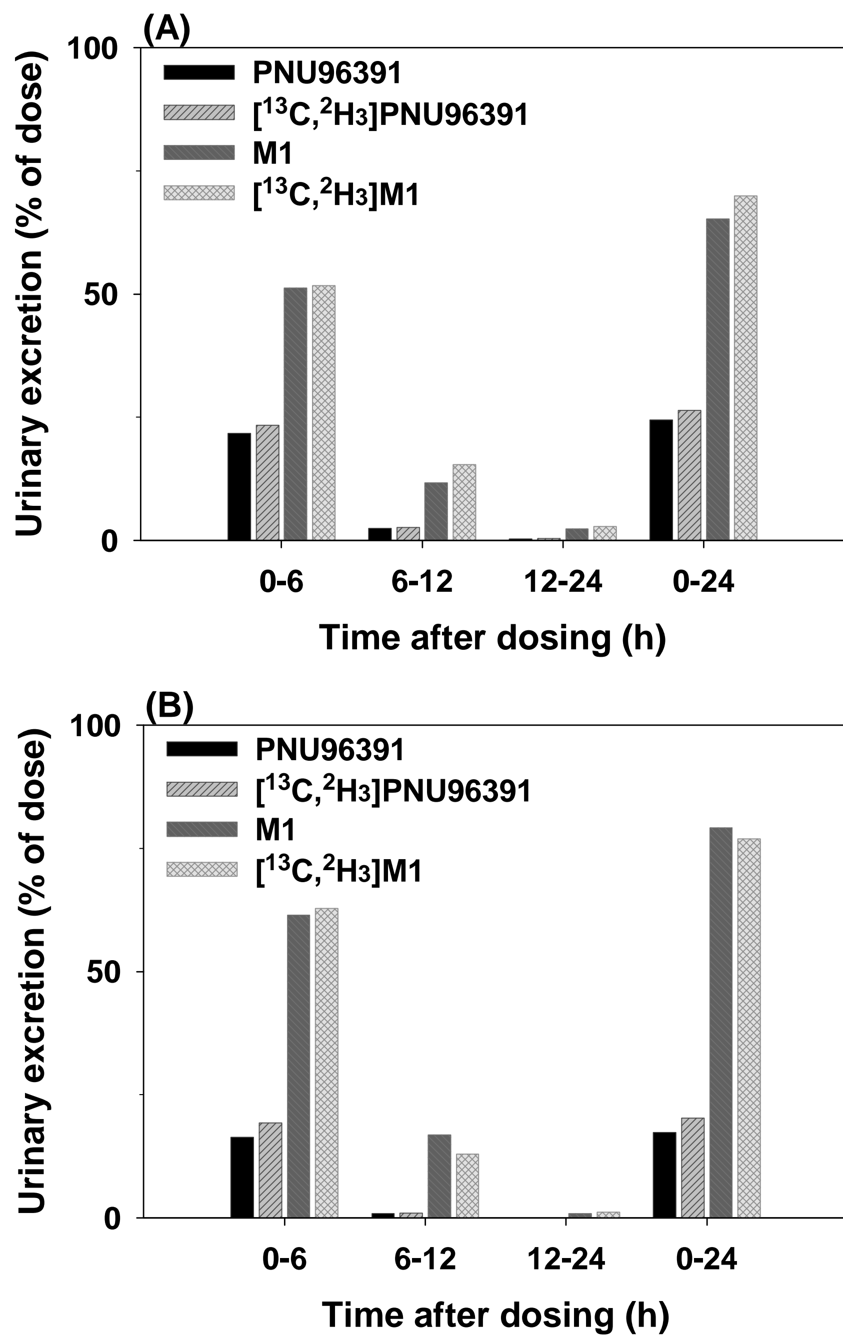

Figure 4

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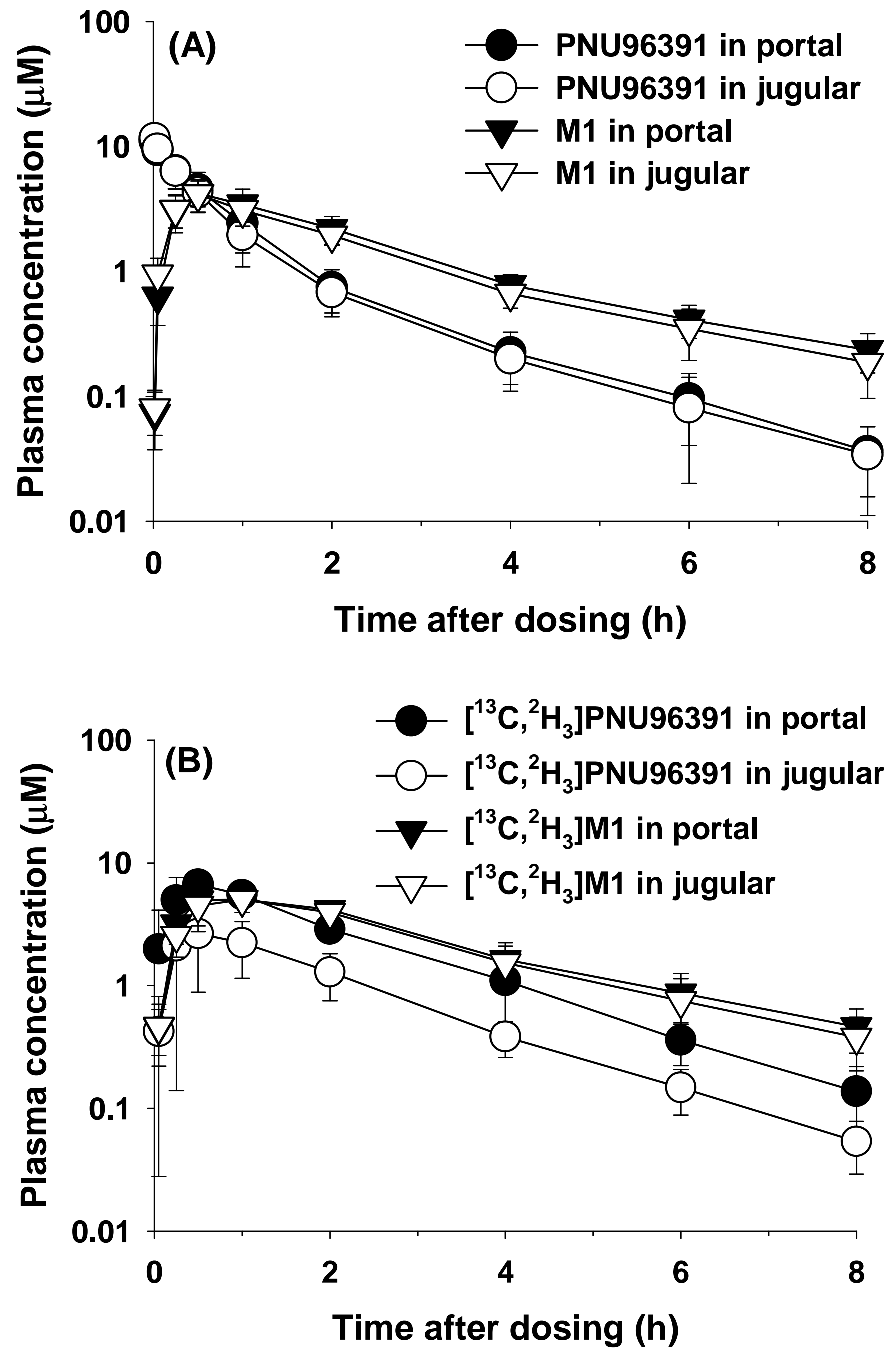

Figure 5

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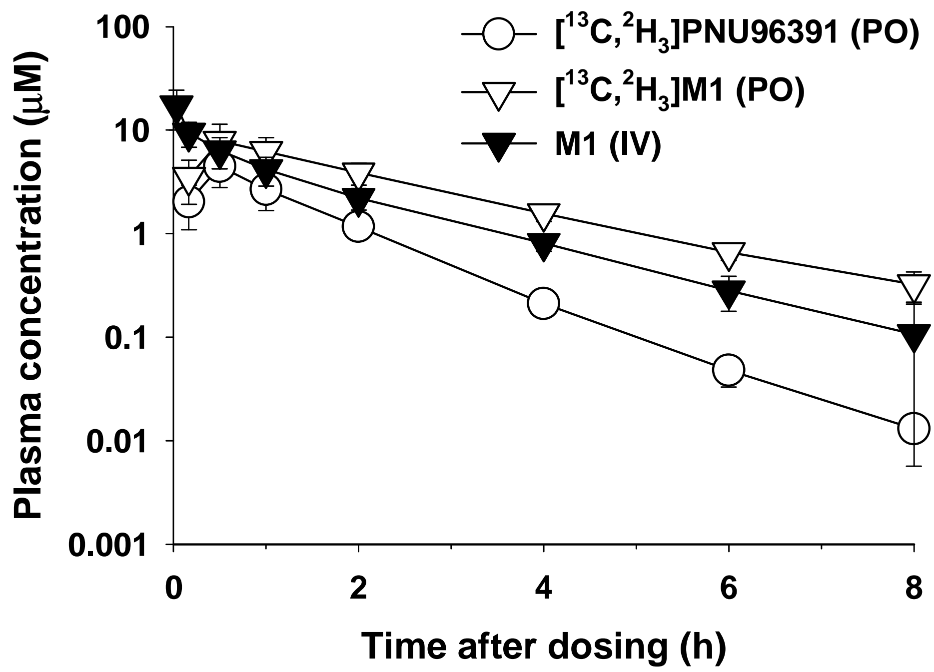

Figure 6

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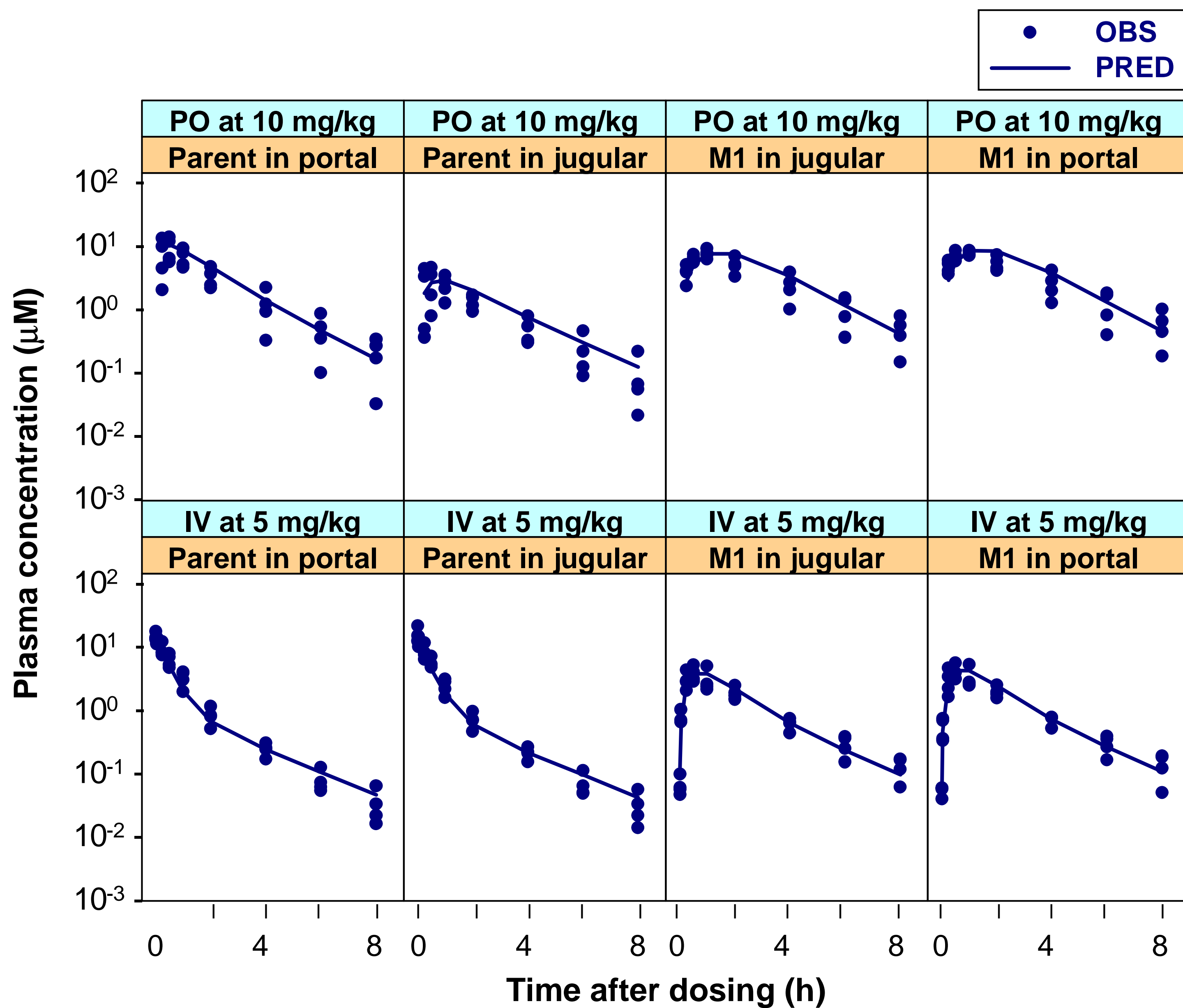

Figure 7

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