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ENANTIOSELECTIVE DISTRIBUTION OF VERAPAMIL AND NORVERAPAMIL INTO HUMAN AND RAT ERYTHROCYTES: THE ROLE OF PLASMA PROTEIN BINDING

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ABSTRACT

In this *in vitro* study, the distribution of the enantiomers of verapamil (VER) and its active metabolite, norverapamil (NOR), into the red blood cells (RBCs) of humans and rats was investigated using a chiral liquid chromatographic assay. When plasma was replaced with buffer, the distribution of VER and NOR enantiomers into both human and rat RBCs was substantial (RBC:blood concentration ratios, 1·39–1·79), non-stereoselective, concentration (125–1000 ng mL^{-1}) linear, and species independent. However, in the presence of plasma, the RBC distribution of VER and NOR was stereoselective, with opposite stereoselectivity for human (S > R) and rat (R > S) blood. Additionally, the presence of plasma caused a reduction in the extent of RBC distribution for both VER and NOR enantiomers and in some cases resulted in nonlinearity in the RBC distribution of the enantiomers. Plasma protein binding studies revealed opposite stereoselectivity in the free fractions in human (S > R) and rat (R > S) plasma for both VER and NOR. These data suggest that the stereoselective protein binding is responsible for the apparent stereoselectivity in the RBC distribution of VER and NOR. The data are also in agreement with the opposite stereoselectivity in the plasma concentrations of VER observed *in vivo* in rats and humans.

KEY WORDS: stereoselective pharmacokinetics; stereoselective erythrocyte uptake; stereoselective plasma protein binding; species-dependent pharmacokinetics

INTRODUCTION

In most pharmacokinetic studies, plasma (or serum) is used for the measurement of the concentrations of drugs and their metabolites, neglecting

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the importance of the partitioning of drugs and/or their metabolites into red blood cells (RBCs). This practice has been based on the findings that for most drugs the equilibrium between RBCs and plasma occurs very rapidly. However, recently Chiou and colleagues[1–3] showed that the drug in RBCs may not be easily available for extraction by the eliminating organs such as the kidneys and the liver. This was true for drugs with both slow (doxorubicin[1] and hydrochlorothiazide[3]) and rapid (propranolol)[2] equilibrium between RBCs and plasma; RBCs acted as almost total and partial barriers for the elimination of the former and latter drugs, respectively. Although not studied directly, the RBC distribution of drugs may also have significant impact on the other kinetic and dynamic processes such as the distribution into the non-eliminating organs and expression of the pharmacologic activity. Therefore, characterization of the RBC uptake of drugs is necessary for complete understanding of their kinetics and dynamics.

Verapamil (VER) is a racemic calcium channel blocker with stereoselective pharmacologic activities (S > R).[4] Several studies in humans[5–8] showed that the drug also exhibits stereoselective pharmacokinetics, resulting in substantially lower plasma concentrations of the S-enantiomer. Additionally, a recent study[9] indicated stereoselective pharmacokinetics for the drug in rats. However, the stereoselectivity in the plasma concentrations of VER in rats (S > R)[9] was opposite to that reported in humans (R > S).[5,7,8] The purpose of this *in vitro* study was to evaluate the species differences in the distribution of the enantiomers of VER and its active metabolite, norverapamil (NOR), into human and rat RBCs. Additionally, the role of stereoselective plasma protein binding on the apparent stereoselectivity in the RBC uptake of the drug and its metabolite was investigated.

MATERIALS AND METHODS

Materials

Racemic VER HCl and NOR HCl were obtained from Sigma Chemical Co. (St. Louis, MO) and G. D. Searle and Co. (Skokie, IL), respectively. Solvents used for chromatographic analysis of VER and NOR enantiomers were HPLC grade and purchased from Baxter Healthcare Co. (McGaw Park, IL). All other chemicals and reagents were analytical grade and obtained from commercial sources.

Fresh human blood was collected from consenting healthy volunteers after an overnight fast. Rat blood was obtained by cardiac puncture from anesthetized (pentobarbital; 50 mg kg^{-1}) male Sprague–Dawley rats (250–300 g). Both human and rat blood samples were collected in syringes containing 4 units heparin/mL blood as a 400 IU mL^{-1} solution.

Verapamil and NOR solutions were prepared by dissolving the powder in isotonic phosphate buffer (pH 7·4) before the addition to the blood samples.

Red blood cell uptake studies

Similar methods were used for human and rat blood. Additionally, the RBC uptake of racemic VER and NOR was studied in separate but similar experiments detailed below. Unless stated otherwise, the number of samples in each experiment was six.

Freshly obtained blood samples were transferred to microcentrifuge tubes, spiked with solutions of racemic VER or NOR, and gently mixed. The samples were then incubated at 37 °C for 15 min (except for the initial time dependency studies). Immediately afterward, the tubes were centrifuged in a microcentrifuge for 3 min, and the supernatant was stored at −20 °C until analysis for VER or NOR enantiomers. The hematocrit was measured in all blood samples using microhematocrit capillary tubes.

Initial experiments were carried out to determine the time dependence of the RBC uptake of VER and NOR at an enantiomeric concentration of 250 ng mL^{-1} and incubation times of 5, 15, and 30 min. The effects of blood concentration of racemic VER or NOR on their RBC uptake, on the other hand, were determined using enantiomeric blood concentrations of 125, 250, 500, and 1000 ng mL^{-1} at a fixed incubation time of 15 min. The concentration dependence studies were carried out both in blood samples in which the plasma was replaced by isotonic phosphate buffer and also in intact blood in the presence of plasma. In the former experiments, the blood was first centrifuged at 3000 rev min^{-1} for 10 min, and the plasma and buffy layers were removed. After twice washing the remaining RBC layer with isotonic phosphate buffer, the cells were resuspended in a volume of buffer similar to that of the original plasma.

Plasma protein binding studies

The binding of VER and NOR enantiomers to plasma proteins of humans and rats was determined by equilibrium dialysis as reported before.[10] These studies were carried out with racemic VER or NOR at enantiomeric concentrations of 125 and 1000 ng mL^{-1}, representing the lowest and highest blood concentrations used in our concentration dependence studies of the RBC uptake.

Analytical method

The concentrations of VER and NOR enantiomers in the plasma or buffer samples were measured by using the assay of Shibauka and Wainer[11] after minor modifications reported before.[10]

Data analysis

The concentrations of VER and NOR enantiomers in RBC (C_{rbc}) were estimated from the spiked blood concentrations (C_{blood}), the measured concentrations of the

enantiomers in the supernatant plasma or buffer ($C_{plasma/buffer}$), and the blood hematocrit according to the following equation:

$$C_{rbc} = \frac{C_{blood} - [C_{plasma/buffer}(1 - \text{hematocrit})]}{\text{hematocrit}} \quad (1)$$

The free fractions of VER or NOR enantiomers in plasma were estimated by dividing the concentration of each enantiomer in the buffer side (free) to that in the plasma side (total) of the dialysis cells.

The effects of incubation time on the plasma and RBC concentrations of VER and NOR enantiomers were analyzed using a one-factor ANOVA. For concentration dependence studies, the RBC:blood concentration ratios of the S- and R-enantiomers for different blood concentrations of VER or NOR were subjected to a two-factor (concentrations and enantiomers) repeated measure ANOVA. In protein binding studies, the differences between the free fractions of the two enantiomers were determined by using a two-tailed paired *t*-test; the effect of low and high concentrations on the free fractions of each enantiomer, however, was analyzed by a two-tailed unpaired *t*-test. All the statistical tests were conducted at a significance level of 0·05. Data are presented as mean ± SD.

RESULTS

Effects of incubation time

Initial studies using both human and rat blood at a fixed enantiomeric concentration of 250 ng mL^{-1} of VER or NOR enantiomers revealed that the plasma and RBC concentrations of the enantiomers were not significantly different after incubation times of 5, 15, and 30 min (data not shown). Therefore, an incubation time of 15 min was used for all the subsequent studies.

Distribution of enantiomers into RBCs from buffer

The concentration of VER and NOR enantiomers in the buffer and RBCs of humans and rats are depicted in Figure 1 for different concentrations of VER or NOR added to the blood. In the absence of plasma proteins, the uptake of both VER and NOR enantiomers into human and rat erythrocytes was extensive and similar for both enantiomers. For VER enantiomers, the human RBC concentrations corrected for the added blood concentrations were relatively constant over the concentration range of 125–1000 ng mL^{-1} (S-VER, 1·58–1·65; R-VER, 1·58–1·66). Comparable RBC:blood concentration ratios were found for rat RBCs (S-VER, 1·39–1·61; R-VER, 1·39–1·64). The RBC:blood concentration ratios for NOR enantiomers were also similar to those of the parent drug for both human (S-NOR, 1·61–1·70; R-NOR,

1·70–1·73) and rat (S-NOR, 1·67–1·78; R-NOR, 1·69–1·79) RBCs. In some cases, the minor differences among the RBC:blood concentration ratios for the different concentrations and/or the two enantiomers were statistically significant. However, overall, the distribution of VER and NOR into RBCs in the absence of proteins appeared to be concentration independent (linear) and non-stereoselective (Figure 1).

Distribution of enantiomers into RBCs from plasma

The plasma and RBC concentrations of VER enantiomers as a function of enantiomeric concentrations of VER added to blood are presented in Tables 1 and 2 for human and rat blood, respectively. The distribution of VER into RBCs was stereoselective for both human and rat blood. However, the direction of stereoselectivity in human blood (S > R, Table 1) was opposite to that in rat blood (R > S, Table 2). In both species, the RBC concentrations were less than or equal to the added blood concentrations (Tables 1 and 2). Additionally, the RBC distribution of VER enantiomers in rat blood was concentration dependent: an increase in the added blood concentration of VER enantiomers from 125 to 1000 ng mL^{-1} resulted in an increase in the RBC:blood ratios (Table 2).

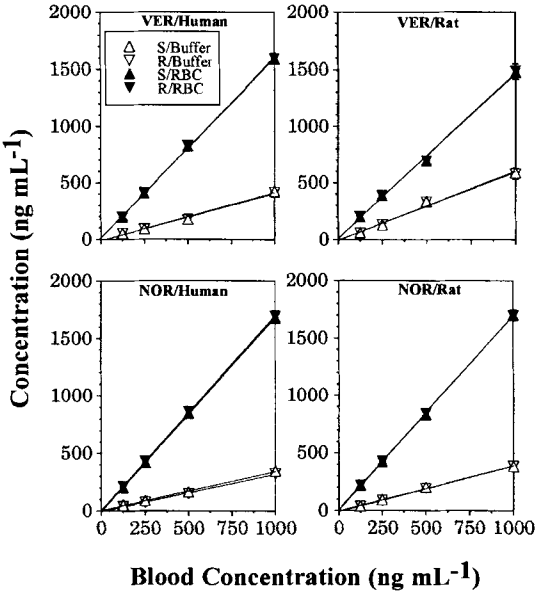

Figure 1. Average enantiomeric concentrations of VER (top) and NOR (bottom) in the buffer and RBCs of human (left) and rat (right) blood as a function of the concentration of each enantiomer added to the blood ($n = 4$–6). SD values are presented as error bars and are within the symbols for majority of the points. The lines represent the linear regression analysis of the experimental data (symbols)

Table 1. Average (±SD) concentrations of VER enantiomers in human plasma and RBCs at different blood concentrations of racemic VER. $n=6$ for each concentration group except for 1000 ng mL^{-1} ($n=5$)

Blood[a] (ng mL^{-1})	Plasma (ng mL^{-1})			RBCs (ng mL^{-1})			RBCs:blood[b]	
	S-VER	R-VER	S:R	S-VER	R-VER	S:R	S-VER	R-VER
125	175	201	0·873	73·8	47·8	1·74	0·591	0·382
	(14)	(17)	(0·017)	(14·0)	(17·6)	(0·69)	(0·112)	(0·141)
250	317	362	0·875	182	135	1·36	0·727	0·541
	(23)	(28)	(0·005)	(24)	(29)	(0·12)	(0·095)	(0·115)
500	669	767	0·871	328	227	1·53	0·656	0·455
	(83)	(86)	(0·014)	(84)	(87)	(0·28)	(0·169)	(0·175)
1000	1280	1480	0·864	719	513	1·46	0·719	0·513
	(108)	(136)	(0·031)	(110)	(139)	(0·29)	(0·110)	(0·139)

[a]Concentration for each enantiomer.
[b]$p=0·26$ for the differences among different concentrations and $p=0·0001$ for the differences between the two enantiomers (two-factor, repeated measure ANOVA).

Similar data for NOR enantiomers are presented in Tables 3 and 4 for human and rat blood, respectively. Similar to the parent drug, the RBC distribution of NOR was stereoselective with opposite stereoselectivity for human (S>R, Table 3) and rat (R>S, Table 4) blood. However, in each species, the direction of stereoselectivity for VER (Tables 1 and 2) and NOR (Tables 3 and 4) was the same. A general trend towards an increase in the RBC:blood concentration ratios of NOR enantiomers as a result of an increase in the added blood concentration of the metabolite was observed for both

Table 2. Average (±SD) concentrations of VER enantiomers in rat plasma and RBCs at different blood concentrations of racemic VER. $n=6$ for all the concentrations

Blood[a] (ng mL^{-1})	Plasma (ng mL^{-1})			RBCs (ng mL^{-1})			RBCs:blood[b]	
	S-VER	R-VER	S:R	S-VER	R-VER	S:R	S-VER	R-VER
125	169	138	1·22	72·7	109	0·663	0·582	0·875
	(6)	(4)	(0·02)	(6·8)	(5)	(0·042)	(0·055)	(0·041)
250	341	274	1·24	141	221	0·637	0·566	0·886
	(14)	(14)	(0·02)	(17)	(17)	(0·031)	(0·068)	(0·067)
500	626	500	1·25	349	500	0·696	0·699	1·00
	(42)	(34)	(0·01)	(51)	(41)	(0·046)	(0·102)	(0·08)
1000	1143	950	1·20	829	1060	0·781	0·829	1·06
	(58)	(53)	(0·01)	(69)	(63)	(0·020)	(0·069)	(0·063)

[a]Concentration for each enantiomer.
[b]$p=0·0001$ for the differences both among different concentrations and between the two enantiomers (two-factor, repeated measure ANOVA).

Table 3. Average (±SD) concentrations of NOR enantiomers in human plasma and RBCs at different blood concentrations of racemic NOR. $n=5$ (125 ng mL^{-1}), 3 (250 ng mL^{-1}), 6 (500 ng mL^{-1}), or 4 (1000 ng mL^{-1})

Blood[a] (ng mL^{-1})	Plasma (ng mL^{-1})			RBCs (ng mL^{-1})			RBCs:blood[b]	
	S-NOR	R-NOR	S:R	S-NOR	R-NOR	S:R	S-NOR	R-NOR
125	184	202	0·909	64·0	44·8	1·63	0·512	0·359
	(24)	(23)	(0·039)	(24·6)	(24·3)	(0·55)	(0·197)	(0·195)
250	364	440	0·828	131	52·2	2·19	0·525	0·209
	(23)	(35)	(0·018)	(24)	(36·5)	(0·69)	(0·094)	(0·146)
500	665	823	0·808	328	164	2·17	0·657	0·328
	(53)	(59)	(0·007)	(55)	(61)	(0·55)	(0·110)	(0·123)
1000	1230	1510	0·824	760	469	1·82	0·760	0·469
	(78)	(220)	(0·089)	(81)	(228)	(0·54)	(0·081)	(0·229)

[a]Concentration for each enantiomer.
[b]$p=0·1931$ for the differences among different concentrations and $p=0·0001$ for the differences between the two enantiomers (two-factor, repeated measure ANOVA).

human (Table 3) and rat (Table 4) blood. However, this trend was not statistically significant ($p>0·05$).

Protein binding

The free fractions of VER and NOR enantiomers in human and rat plasma are presented in Table 5. In human plasma, the free fractions of the S-enantiomers of both VER and NOR were significantly ($p<0·05$) higher than

Table 4. Average (±SD) concentrations of NOR enantiomers in rat plasma and RBCs at different blood concentrations of racemic NOR. $n=6$ for each concentration group except for 1000 ng mL^{-1} ($n=5$)

Blood[a] (ng mL^{-1})	Plasma (ng mL^{-1})			RBCs (ng mL^{-1})			RBCs:blood[b]	
	S-NOR	R-NOR	S:R	S-NOR	R-NOR	S:R	S-NOR	R-NOR
125	126	98·4	1·28	124	165	0·749	0·994	1·32
	(20)	(16·0)	(0·16)	(30)	(24)	(0·114)	(0·239)	(0·19)
250	248	216	1·15	253	301	0·833	1·01	1·20
	(37)	(32)	(0·03)	(55)	(48)	(0·058)	(0·221)	(0·19)
500	458	396	1·16	563	656	0·857	1·13	1·31
	(48)	(46)	(0·02)	(72)	(69)	(0·026)	(0·14)	(0·14)
1000	784	689	1·14	1320	1470	0·903	1·32	1·47
	(60)	(62)	(0·02)	(90)	(93)	(0·013)	(0·09)	(0·09)

[a]Concentration for each enantiomer.
[b]$p=0·062$ for the differences among different concentrations and $p=0·0001$ for the differences between the two enantiomers (two-factor, repeated measure ANOVA).

Table 5. Average (±SD) free fractions of VER and NOR enantiomers in human and rat plasma at different plasma concentrations of racemic VER or NOR. $n = 3$–5

Concentration[a] ($ng\,mL^{-1}$)	VER			NOR		
	S	R	S:R	S	R	S:R
Human plasma						
125	0·0791*†	0·0427*†	1·86	0·0593*‡	0·0246*‡	2·47
	(0·0104)	(0·0073)	(0·08)	(0·0117)	(0·0080)	(0·26)
1000	0·0844*†	0·0490*†	1·72	0·103*‡	0·0495*‡	2·08
	(0·0054)	(0·0031)	(0·02)	(0·014)	(0·0069)	(0·07)
Rat plasma						
125	0·0616*†	0·104*†	0·606	0·0683*‡	0·0871*‡	0·794
	(0·0161)	(0·036)	(0·082)	(0·0072)	(0·0177)	(0·077)
1000	0·0817*†	0·134*†	0·610	0·151*‡	0·189*‡	0·800
	(0·0105)	(0·022)	(0·031)	(0·037)	(0·048)	(0·034)

[a]Concentration for each enantiomer.
*Significant difference between the two enantiomers (two-tailed paired *t*-test).
†No significant difference between the low and high concentrations (two-tailed unpaired *t*-test).
‡Significant difference between the low and high concentrations (two-tailed unpaired *t*-test).

those of their antipode. The degree of stereoselectivity was higher for NOR, compared to VER (Table 5). On the other hand, in rat plasma, the free fractions of the S-enantiomers of VER and NOR were lower ($p < 0·05$) than their respective enantiomers and the stereoselectivity was higher for the parent drug, compared with its metabolite (Table 5). A concentration-dependent small increase in the free fractions of VER enantiomers in both human and rat plasma was not statistically significant ($p > 0·05$). However, substantial and significant ($p < 0·05$) increases in the free fractions of both enantiomers of NOR were observed when the human or rat plasma concentrations of NOR enantiomers were increased from 125 to 1000 $ng\,mL^{-1}$ (Table 5).

DISCUSSION

For racemic drugs, stereoselectivity in the plasma pharmacokinetics has generally been attributed to the pharmacokinetic processes of absorption, distribution, and/or elimination.[12] However, a stereoselective uptake of the drug into RBCs could influence the appearance of stereoselectivity if the plasma is measured instead of the whole blood.[13,14] Previous *in vitro* data from our laboratory[13] indicated that the stereoselective erythrocyte uptake of propafenone, a racemic drug, was consistent with, and possibly contributed to, the opposite stereoselectivity in the plasma concentrations of the drug observed *in vivo*: the (−)- and (+)-enantiomers of propafenone were preferentially

distributed into human and rat RBCs, respectively.[13] This was in agreement with the higher plasma concentrations of (+)- and (−)-propafenone observed *in vivo* in humans[15] and rats,[16] respectively. Similar to propafenone, the opposite stereoselective distribution of VER and NOR into the RBCs of humans (S>R) (Tables 1 and 3) and rats (R>S) (Tables 2 and 4) was consistent with the *in vivo* stereoselectivity in the plasma concentrations of VER and NOR in humans (R>S)[5,7,8] and of VER in rats (S>R).[9] However, the magnitude of the *in vivo* stereoselectivity in the plasma concentrations of VER and NOR in humans[5,7,8] and rats[9] is several-fold higher than the observed stereoselectivity in their RBC uptake (Tables 1–4). Therefore, although a contributing factor, the stereoselective RBC uptake should not be regarded as the only mechanism underlying the apparent plasma stereoselectivity of VER and NOR observed *in vivo*.

Theoretically, stereoselective distribution of a racemic drug into erythrocytes, as observed here for VER and NOR, could be due to an active uptake process or a stereoselective protein binding in the plasma and/or RBCs. The concentration linearity of the RBC distribution and the lack of significant stereoselectivity of VER and NOR in human and rat RBCs in the absence of plasma proteins (Figure 1) strongly argue against a carrier-mediated process and stereoselective RBC binding. Therefore, we hypothesized that the stereoselective RBC distribution of VER and NOR in the intact blood (Tables 1–4) is due to a stereoselective plasma protein binding coupled with a passive diffusion process of distribution. The results of further studies on the plasma protein binding of VER and NOR in human and rat plasma (Table 5) confirmed this hypothesis; higher free fractions of the S-enantiomers of VER and NOR in human plasma resulted in an apparent stereoselectivity in the RBC distribution in favor of this enantiomer in human plasma (Tables 1 and 3). On the other hand, the apparent stereoselective distribution of the R-enantiomers of VER and NOR into the RBCs of rats (Tables 2 and 4) was associated with a higher free fraction of this enantiomer in rat plasma (Table 5). These data indicate that the differences in the apparent RBC distribution of VER and NOR between the human and rat blood is mainly, if not totally, due to the differences between the two species in their plasma protein binding of the enantiomers.

The *in vitro* plasma free fractions of the enantiomers of VER obtained in our study (Table 5) are in complete agreement with the data obtained in humans[8] and rats[9] using procedures similar to ours. However, the *in vitro* free fraction values of VER enantiomers in human plasma reported by Gross *et al.*[17] ($0{\cdot}120 \pm 0{\cdot}020$ and $0{\cdot}066 \pm 0{\cdot}011$ for S- and R-VER, respectively) appear to be higher than the corresponding values in our study (Table 5). This may be due to methodological differences between the two studies. For NOR enantiomers, the only study[7] available to us reported free fractions of $0{\cdot}170 \pm 0{\cdot}049$ and $0{\cdot}10 \pm 0{\cdot}03$ for the S- and R-enantiomers in human plasma after the oral administration of VER. The much lower values obtained in our study (Table 5)

may be due to the fact that in the above study,[7] the binding of NOR enantiomers was determined after the *in vivo* administration of VER and in the presence of VER and the other metabolites of the drug. This postulate is consistent with a previous study[17] showing that the free fractions of VER enantiomers are higher after the oral administration of the drug, compared with *in vitro* studies using spiked plasma.

In the absence of plasma proteins, the RBC concentrations of VER and NOR enantiomers were several-fold higher than the corresponding concentrations in the buffer (Figure 1), indicating substantial binding of VER and NOR to the constituents of human and rat RBCs. This is in sharp disagreement with the very low RBC:buffer concentration ratios reported for the racemic VER (0·10–0·30) and NOR (0·10–0·27) by Czejka *et al.*[18] However, a closer examination of the data of Czejka *et al.*[18] reveals the source of this discrepancy; instead of using the relationship expressed in our equation (1), these authors estimated the RBC concentrations by subtracting the added blood concentrations from the measured concentrations in the supernatant of RBCs (buffer). This is expected to result in substantial underestimation of the RBC concentrations of the drug. The same miscalculation appears to be responsible for their conclusion[18] of dose dependence of the RBC accumulation of racemic VER and NOR and dismissal of the importance of the RBC distribution of VER and NOR.

In conclusion, both VER and NOR enter into RBCs of human and rat blood to a relatively substantial degree and in a stereoselective manner, with opposite stereoselectivity for human and rat RBCs. Additionally, the RBC distribution of VER and NOR enantiomers appears to be taking place via a passive diffusion process, attaining rapid equilibrium between plasma and RBCs. It is suggested that the species-dependent apparent stereoselectivity in the RBC distribution of VER and NOR is due to the opposite stereoselectivity in the plasma protein binding of the enantiomers in humans and rats. These *in vitro* data on RBC distribution of VER and NOR are consistent with the *in vivo* stereoselectivity in the plasma concentrations of the drug and its metabolite observed in humans and rats.

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