Enantioselective Kinetics of Verapamil and Norverapamil in Isolated Perfused Rat Livers

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The kinetics of the individual enantiomers of verapamil (VER) and its metabolite, norverapamil (NOR), were studied in isolated perfused rat livers (IPRLs) after administration of racemic drug or the preformed metabolite. After constant infusion of 20 μg/min of racemic VER to single-pass IPRLs, the hepatic availabilities (F) of the enantiomers were low (S-VER, 0.069 ± 0.030; R-VER: 0.046 ± 0.025) and stereoselective (S:R ratio, 1.6 ± 0.2). After administration of similar doses, the F values of the preformed NOR enantiomers (S-NOR: 0.24 ± 0.04; R-NOR, 0.10 ± 0.02) were higher than those of the VER enantiomers. However, the stereoselectivity in F of NOR (S:R ratio, 2.2 ± 0.1), was in the same direction of that of VER. Further, the fractions of R enantiomers unbound to bovine serum albumin in the perfusate were higher than those of their antipodes for both VER (R:S ratio, 1.9 ± 0.1) and NOR (R:S ratio, 2.6 ± 0.2). Therefore, for unbound moieties, modest stereoselectivity in the metabolism of VER in favor of the S-isomer and no stereoselectivity in the metabolism of NOR were observed. Overall, our data suggest that the stereoselective protein binding is a primary determinant of stereoselectivity in the hepatic availability of VER and NOR in IPRLs.

KEY WORDS: verapamil; noverapamil; stereoselective metabolism; isolated perfused rat liver; metabolite kinetics, first-pass metabolism.

INTRODUCTION

The calcium channel blocking agent verapamil (VER) is used as a racemate consisting of S- and R-enantiomers which differ in their pharmacologic activities (S>R) (1). Racemic VER is extensively metabolized in the liver with relatively high hepatic extraction ratio (E) and clearance in both humans (2) and animals (3–7). Further, the kinetics of the individual enantiomers of VER were studied extensively in humans (8–11). However, there is a paucity of stereoselective studies of VER in animals (6,12) and animal preparations.

Laboratory animals and *in vitro* organ preparations are useful tools in pharmacokinetics. For instance, the isolated perfused rat liver (IPRL) has been used (13) for the study of hepatic drug metabolism because it permits control of various experimental factors such as liver blood flow, perfusate contents, and drug input rate.

Studies conducted on the kinetics of VER in intact rats (3,5,7), measuring racemic VER, indicated that, similar to man, VER undergoes extensive hepatic metabolism with a high E in this species. Additionally, studies using the rat liver microsomal fraction (14,15) or flavin-containing monooxygenase (16) demonstrated stereoselectivity in different metabolic pathways of VER. However, the kinetics of the enantiomers of VER and its N-demethylated metabolite, norverapamil (NOR), after administration of racemic VER in rats or IPRL are unknown. In the present study, we investigated the metabolism of VER and NOR enantiomers in IPRL after the infusion of either the parent drug or its preformed metabolite.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (Fraction V) and racemic VER (HCl salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Racemic NOR (HCl salt) and individual enantiomers of VER and NOR were provided by G. D. Searle & Co. (Skokie, IL). Recently expired human red blood cells (RBCs) were obtained from the Blood Center of Central Iowa (Des Moines, IA). Hexane and isopropanol used in chromatography were HPLC grade and purchased from Baxter Scientific Products (McGaw Park, IL). All other reagents were analytical grade and available through commercial sources.

Isolated Perfused Rat Liver Preparation

Livers were isolated from adult male Sprague-Dawley rats (300–330 g) as described before (17). The isolated livers were perfused using a commercial perfusion apparatus (MX Perfuser II; MX International; Aurora, CO) in a single-pass manner.

The perfusate consisted of a Krebs-Henseleit bicarbonate buffer (pH 7.4) containing glucose (1.2 g/liter), bovine serum albumin (2%, w/v), and washed RBC (10%, v/v), delivered at a flow rate of 15 mL/min. The perfusate was oxygenated with an O_2:CO_2 (95:5) mixture for at least 30 min before entering the liver.

The viability of the liver was confirmed through overall macroscopic appearance of the liver, perfusate flow, and inlet and outlet pH measurements. Also, the inlet and outlet oxygen levels were continuously monitored during the perfusion period using flow-through probes (Lazar Research Labs, Los Angeles, CA). The levels of oxygen extraction by the livers included in this study were 75 ± 8% and 82 ± 5% for the VER and NOR studies, respectively. Further, the oxygen extraction during the entire perfusion period remained relatively constant (<10% change in the outlet oxygen readings).

Experimental Protocol

The livers were perfused with drug-free perfusates for 20 min before infusion of VER or NOR. Racemic VER or NOR was dissolved in isotonic phosphate buffer (pH 7.4) and delivered to the liver at a constant rate of approximately 20 μg/min for 90 min. Inlet samples were drawn at 30, 60,

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and 90 min. Outlet samples were collected at 10- (0–60 min) or 5- (60–90) min intervals. Samples were centrifuged in a microcentrifuge and the RBC-free perfusate ("plasma") was stored at −60 °C until analysis by HPLC. Additionally, at the end of experiments, the livers were washed for 3 min with drug-free perfusate and stored for analysis of VER and/or NOR enantiomers.

Protein Binding Studies

The free fractions of VER and NOR enantiomers in the "plasma" of the perfusate were determined by an equilibrium dialysis method (11). The blank perfusate was spiked with racemic VER (n = 5) or NOR (n = 4) to produce enantiomeric concentrations of 600 ng/mL (close to inlet concentrations). The samples were then dialyzed (Spectra/Por Dialyzer; Spectrum, Houston, TX) against isotonic Sörensen buffer (pH 7.4) for 5 h at 37 °C while rotating the dialysis cells at 20 r.p.m. A membrane with a molecular weight cutoff of 6–8 kD was used to separate the two sides of the cells. After dialysis, samples from both sides of the cells were collected for analysis of their contents of VER and NOR enantiomers. No appreciable volume shift was found in these studies.

Analysis of Verapamil and Norverapamil Enantiomers

The concentrations of VER and NOR enantiomers in the inlet and outlet samples were determined according to the stereospecific HPLC assay of Shibukawa and Wainer (18) with some modifications. First, the analytes were extracted from the samples into the organic solvent by using a rotary mixer instead of vortex-mixing. Second, instead of the diol column, a silica column (5 cm × 4.6 mm; Supelco, Bellefonte, PA) was used in series with the chiral column for the separation of VER and NOR enantiomers.

For the analysis of the liver concentrations of VER and NOR, the livers were, first, homogenized in 3 volumes of isotonic phosphate buffer. Then, 100 μL of the homogenate was diluted with 900 μL of distilled water and the samples were subjected to the procedure described above. The recovery of spiked VER and NOR enantiomers from liver tissue was similar to that of "plasma" samples.

Data Analysis

The steady-state outlet concentrations of VER and NOR (C_{out}) and the time to reach C_{out} (t_{ss}) were estimated for individual livers using the following method. Starting with the last three samples, the average and CV of the outlet concentrations were determined. If CV was <10%, the procedure continued by adding the previous sample to the pool and calculating a new average and CV. This procedure continued by going backward in time until the CV of the average concentration exceeded 10%, resulting in rejection of this point and all the preceding points for estimation of C_{out}. Therefore, going backward in time, the last sampling time resulting in a CV of ≤ 10 in the average concentration was considered as t_{ss}. It was decided that to be included in the calculation, at least 3 steady-state concentrations (at 80, 85, and 90 min) were necessary. Therefore, steady-state concentrations were not calculated for VER or NOR enantiomers with t_{ss} of > 80 min. For the inlet concentrations, the average of inlet samples taken at 30, 60, and 90 min (C_{in}) was used.

The hepatic availability (F) of the enantiomers of VER or NOR was determined by dividing C_{out} by C_{in} for each enantiomer. E was subsequently estimated from the following equation:

$$E = 1 - F \quad (1)$$

The hepatic intrinsic clearance of the enantiomers (CL_{int}) was calculated by using the following equations based on the well-stirred hepatic metabolism model (19):

$$E = \frac{CL_{int}}{Q + CL_{int}} \quad (2)$$

$$CL_{int} = \frac{E \cdot Q}{F} \quad (3)$$

where Q is the perfusate flow rate (15 mL/min).

The differences between the kinetic parameters of the two enantiomers of VER or NOR were determined using a two-tailed, paired t-test at a significance level (α) of 0.05.

RESULTS

Verapamil Infusion

The kinetic parameters of VER and NOR enantiomers after infusion of racemic VER are listed in Table I. Additionally, the outlet concentration-time profiles of VER and NOR enantiomers in a representative liver are presented in Fig. 1. The t_{ss} values for S- and R-VER were 75 ± 4 (range: 70–80) and 76 ± 4 (range: 70–80) min, respectively. The t_{ss} values for the generated S- and R-NOR were 74 ± 6 (range: 65–80) and 76 ± 5 (range: 70–80) min. In all the livers, C_{out} of the S-enantiomers of both the parent drug (VER) and its metabolite (NOR) was higher than that of their antipode (Table I).

The stereoselectivity in the amounts of VER enantiomers recovered in the liver tissue at the end of 90-min infusion with VER was opposite to that observed in the outlet concentrations; 6.6 ± 0.9% and 7.8 ± 1.2% of the infused S- and R-VER ($P < 0.01$) were found in the liver, respectively, resulting in an S:R ratio of 0.85 ± 0.06. However, for the generated NOR enantiomers, the recovery was slightly higher ($P < 0.05$) for the S-enantiomer (S, 2.7 ± 0.6%; R, 2.5 ± 0.5%; S:R, 1.1 ± 0.1).

Norverapamil Infusion

The kinetic parameters of the preformed NOR in IPRLs are presented in Table II. Also, the outlet concentration-time profiles of the NOR enantiomers are presented in Fig. 2. The t_{ss} values for the preformed S- and R-NOR were 70 ± 5 (range: 65–75) and 73 ± 6 (range: 65–80) min, respectively. Similar to VER and generated NOR, the outlet concentrations of S-NOR were higher than those of its antipode for the preformed metabolite.

The amounts of NOR enantiomers recovered in the liver after the infusion of the preformed metabolite were 12 ± 4%

Table I. Steady-State Kinetic Parameters of Verapamil and Norverapamil Enantiomers in Isolated Perfused Rat Livers After Constant Infusion of Racemic Verapamil

	C_{out}, ng/mL				C_{in}, ng/mL		F			CL_{int}, mL/min		
	VER		NOR		VER		VER			VER		
Rat	S	R	S	R	S	R	S	R	S:R	S	R	S:R
1	36	16	29	8.7	580	530	0.062	0.031	2.0	230	470	0.48
2	17	9.9	19	—[a]	670	620	0.025	0.016	1.6	590	920	0.64
3	58	40	33	12	630	580	0.093	0.069	1.3	150	200	0.73
4	25	14	25	7.3	570	530	0.044	0.026	1.7	330	560	0.58
5	53	32	32	11	550	510	0.096	0.062	1.5	140	220	0.62
6	45	31	—[a]	—[a]	471	430	0.095	0.072	1.3	140	190	0.74
Mean	39	24	28	9.9	580	540	0.069	0.046	1.6	260	430	0.63
SD	16	12	6	2.3	68	65	0.030	0.025	0.2	180	290	0.10
CV	41	51	20	23	12	12	44	53	16	67	67	15
P^b	0.001		0.0001		0.0001		0.001			0.019		

[a] CV of the outlet concentrations was >10%. Therefore, the data were not included in the calculation (See text for exclusion criteria).
[b] Statistical differences between the two enantiomers based on a paired *t*-test.

and 15 ± 5% of the dose ($P < 0.01$) for the S- and R-enantiomers, respectively (S:R ratio, 0.78 ± 0.06).

Protein Binding

The free fractions of VER and NOR enantiomers in the "plasma" of the perfusate are depicted in Fig. 3. The S-enantiomers of both VER and NOR were more extensively bound to the albumin of the perfusate than their R-enantiomers.

DISCUSSION

After constant infusion of VER to isolated rat livers, perfused with a perfusate containing bovine albumin, the metabolism of racemic VER was stereoselective in favor of R-VER. Consequently, the steady-state outlet concentrations of S-VER were, on average, 1.6 times those of its antipode (Table I). This is in contrast to the *in vivo* human (8–11) and dog (12) data which indicate that the metabolism of S-VER is more rapid than R-VER. However, consistent with the human data (9), the stereoselectivity in the VER-generated NOR was in the same direction of that in the parent drug (Table I).

After constant infusion of similar doses, the preformed NOR enantiomers achieved much higher C_{out} than did VER enantiomers (Figs. 1 and 2), indicating that F of the metabolite is higher than that of the parent drug. The higher F for NOR enantiomers was associated with less interanimal variability (%CV) in the kinetics of the preformed metabolite (Table II), compared with the parent drug (Table I).

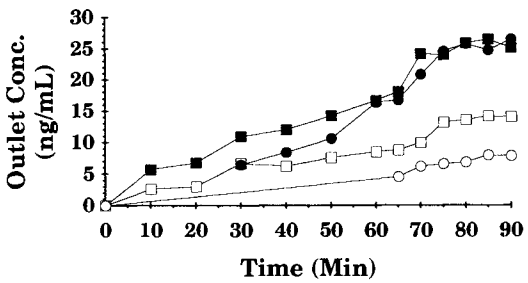

Fig. 1. The concentration-time courses of S-VER (■), R-VER (□), S-NOR (●) and R-NOR (○) in the outlet samples from a representative isolated perfused rat liver (Rat 4, Table I) after constant infusion of 20 μg/min racemic VER for 90 min.

Because hepatic blood flow is the same for both enantiomers of a racemic drug, the stereoselectivity in the *in vivo* metabolism of racemic drugs is dependent on the stereoselectivity in the intrinsic clearance of the total (free plus bound) enantiomers (CL_{int}). The latter parameter is, in turn, dependent on the intrinsic clearance of the free enantiomers ($CL_{int,free}$) and the free fraction of the enantiomers in the plasma (f_u), based on the following equation (19):

$$CL_{int} = f_u \cdot CL_{int,free} \quad (4)$$

Similarly, because the perfusate used in our study with IPRL contained 2% bovine serum albumin, the stereoselective metabolism observed for VER and NOR (Tables I and II) could be due to stereoselectivity in $CL_{int,free}$ and/or f_u. Therefore, to determine the source of stereoselective metabolism of VER and NOR in IPRL, the binding of the VER and NOR enantiomers to the perfusate albumin was determined. Subsequently, the average f_u (Fig. 3) and individual CL_{int} (Tables I and II) values were used to estimate the $CL_{int,free}$ values using Eq. (4). These values were 2200 ± 1500 and 1900 ± 1300 mL/min for the S- and R-enantiomers of VER (S:R ratio, 1.2 ± 0.2) and 760 ± 160 and 760 ± 130 mL/min for the S- and R-enantiomers of NOR (S:R ratio, 1.0 ± 0.1), respectively. The stereoselectivities in the estimated $CL_{int,free}$ values are different than those based on the total enantiomers; while the metabolism of the total enantiomers of VER is stereoselective for the R-enantiomer, the opposite is true when the free enantiomers are considered. Additionally, the stereoselectivity in the metabolism of NOR enantiomers completely disappears when the calculations are based on the free enantiomers. These studies suggest that the stereoselectivity in the metabolism of racemic drugs in IPRLs may be significantly affected by the perfusate contents and type and species of the proteins added to it. Therefore, caution

Table II. Steady-State Kinetic Parameters of Norverapamil Enantiomers in Isolated Perfused Rat Livers After Constant Infusion of Racemic Norverapamil

	C_{out}, ng/mL		C_{in}, ng/mL		F			CL_{int}, mL/min		
Rat	S	R	S	R	S	R	S:R	S	R	S:R
1	140	59	640	560	0.22	0.11	2.1	53	130	0.42
2	200	75	780	690	0.26	0.11	2.4	43	120	0.35
3	170	67	790	700	0.22	0.10	2.3	53	140	0.37
4	190	77	660	590	0.29	0.13	2.2	37	100	0.36
5	120	48	630	550	0.19	0.086	2.2	65	160	0.41
Mean	160	65	700	620	0.24	0.10	2.2	50	130	0.38
SD	36	12	78	74	0.04	0.02	0.1	11	22	0.03
CV	22	18	11	12	17	15	5.5	22	17	8.2
P^a	0.0007		0.0001			0.0003			0.0001	

[a] Statistical differences between the two enantiomers based on paired *t*-test.

must be exercised when extrapolating IPRL data to *in vivo* situations and human metabolism.

Because of the use of racemates for infusions, the inlet concentrations of the two enantiomers of VER or NOR are expected to be similar. However, examination of Table I and II indicates that for both compounds, the inlet "plasma" concentrations of the S-enantiomers were slightly (8 and 13% for VER and NOR, respectively), but consistently, higher than those of their R-enantiomers. Because our perfusate contained both RBC and albumin, a stereoselective binding to albumin in favor of the S-enantiomers (Fig. 3) could be the reason for the observed stereoselectivity in C_{in}. Therefore, the higher concentrations of S-enantiomers in the inlet "plasma" could be due to lower distribution of this enantiomer into the RBCs of the perfusate. Nevertheless, the issue of stereoselective RBC partitioning of VER and NOR in rat and human erythrocytes is currently under investigation in our laboratory.

Norverapamil is an important metabolite of VER possessing some pharmacologic activities (2). Therefore, it is of interest to determine the contribution of this metabolite to the overall metabolism of VER. The fraction of infused VER enantiomer converted into its respective enantiomer of NOR during one passage through the liver at steady-state (F_m) was determined using the following equation:

$$F_m = \frac{C^{VER}_{out(NOR)}}{C^{NOR}_{out(NOR)}} \times \frac{C^{NOR}_{in}}{C^{VER}_{in}} \quad (5)$$

where the superscripts refer to the administered moiety, and the subscripts determine the type of samples (inlet or outlet) and/or the measured moiety. The calculated average F_m values for S- and R-VER were 0.21 and 0.18, respectively. These values are the fractions of the administered enantiomers, and not the extracted enantiomers, which are converted to NOR in each passage through the liver. Correcting for the enantiomeric E, the fractions of the extracted enantiomers converted to NOR were 0.23 and 0.19 for the S- and R-enantiomers, respectively. These data indicate a relatively small stereoselectivity (S:R ratio, 1.2) in the formation of NOR from VER and are in excellent agreement with rat (15) and human (10) microsomal studies showing an S:R ratio of 1.20 and 1.25, respectively, for the formation of NOR. Nevertheless, because of assumptions inherent to this type of calculation (20), the F_m values obtained here should be considered only as estimates of the actual values.

In addition to being dependent on the C_{in} and binding to the liver tissue, the liver concentrations of VER and NOR enantiomers are dependent on the perfusate protein binding of the compounds. After administration of both VER and NOR, higher amounts of the less perfusate-bound R-enantiomers were recovered in the liver. This is consistent with higher metabolism of this enantiomer. Interestingly, however, for generated NOR, the stereoselectivity was opposite,

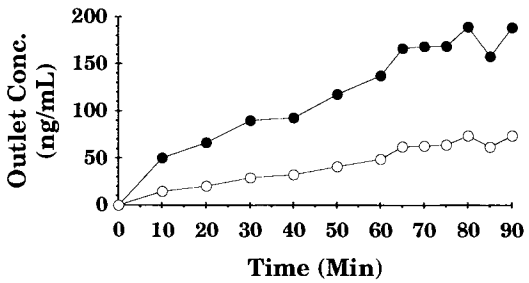

Fig. 2. The concentration-time courses of S-NOR (●) and R-NOR (○) in the outlet samples from a representative isolated perfused rat liver (Rat 3, Table II) after constant infusion of 20 μg/min racemic NOR for 90 min.

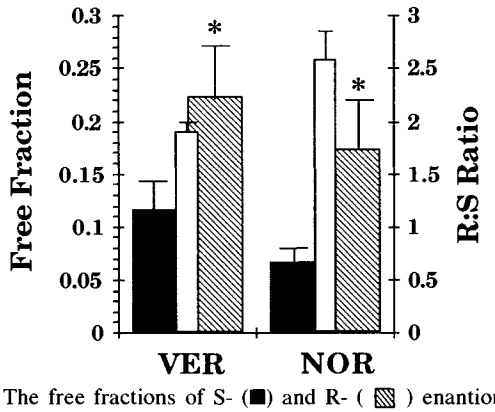

Fig. 3. The free fractions of S- (■) and R- (▨) enantiomers of VER and NOR along with R:S free fraction ratios (❑) in the red blood cell-free perfusate containing 2% bovine serum albumin. SD values are shown as error bars. * denotes significant differences ($P < 0.01$) between the two enantiomers.

as the amounts of S-NOR recovered in the liver tissue were slightly, but significantly, higher than those of R-NOR. This may be because of the fact that, in contrast to the preformed metabolite, the concentration of VER-generated NOR inside the hepatocytes is not a function of stereoselective protein binding in the perfusate. Additionally, F_m for the production of S-NOR is higher than that for the production of R-NOR.

Considering the short transit time of the perfusate in the liver (<60 sec), the time to reach steady-state for both VER and NOR enantiomers (60–80 min) is unusually long. In fact, even after 90 min of perfusion, it appeared that the outlet concentrations did not quite stabilize in some livers (Fig. 1). Theoretically, a long t_{ss} can be attributed to a binding of VER and NOR enantiomers to the liver cells and/or an inactivation of the enzymes responsible for the elimination of these compounds, resulting in a time-dependent reduction in the apparent E. Long t_{ss} and time-dependent kinetics have also been observed for other highly extracted drugs such as lidocaine (21) and diltiazem (22).

In conclusion, the enantioselective metabolism of VER and NOR by an isolated perfused rat liver preparation containing albumin and RBCs was demonstrated here. Additionally, the mechanisms of this stereoselectivity were discussed. These studies increase our understanding of the factors affecting stereoselectivity in the metabolism of racemic drugs, differences between *in vitro* and *in vivo* data, and species-dependent stereoselective kinetics.

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