Stereoselective Pharmacokinetics and Pharmacodynamics of Verapamil and Norverapamil in Rabbits

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We have estimated the pharmacokinetic and pharmacodynamic interactions of verapamil (VP) enantiomers and also the interaction between VP and its metabolite, norverapamil (NVP). ECGs of conscious rabbits were studied to determine the pharmacokinetics of VP enantiomers and racemic NVP in relation to their prolongation effect on PR intervals, which were used as an index of VP’s antiarrhythmic effect. Plasma free fractions of VP enantiomers showed constant values at concentrations ranging from 0.022 to 1.10 μM. There were no interactions between enantiomers or between VP and NVP. The pharmacological effect of the S-enantiomer (S-VP), which was determined by linear regression analysis, showed it was about 20 times more potent than that of the R-enantiomer (R-VP). The effect of racemic VP was the simple sum of those elicited by both enantiomers. These relationships were not significantly different between intravenous infusion and bolus injection. Simultaneous intravenous infusion of NVP had no influence on the PR intervals. In conclusion, we demonstrated that the relationship between plasma unbound concentration of VP enantiomers and their pharmacological effect was the simple sum of two enantiomers.

Key words verapamil; norverapamil; rabbit; interaction; pharmacodynamics; enantiomer

Verapamil (VP) is a calcium antagonist used clinically for the treatment of hypertension, and for prophylaxis of supraventricular and ventricular arrhythmias. VP has a relatively narrow therapeutic plasma concentration range and the variation in pharmacokinetics and pharmacodynamics between individuals is relatively large.

Although VP is commercially available as a racemic mixture, it has been reported that the pharmacokinetic properties of each enantiomer (such as hepatic metabolism, plasma protein binding and tissue distribution) are different in humans and animals. In humans, the bioavailability of S-VP is about 20%, whereas that of R-VP is about 50%.1–4) The plasma protein binding of R-VP is higher than that of S-VP (free fraction: 7% and 12%, respectively).5,14) We recently reported that the binding of VP to α1-acid glycoprotein and phosphatidylserine showed enantioselectivity and an interaction between enantiomers.6,7) A number of studies have also demonstrated that there is a stereoselective pharmacological effect in VP. Echizen et al. reported that the antiarrhythmic effect of S-VP is 10–1.5 times higher than that of R-VP in humans, after administration of each enantiomer.2,3–4) However, these studies have only compared the pharmacological effect between the VP enantiomers after their administration. It is not known whether racemic VP shows pharmacokinetic and further pharmacodynamic interactions between enantiomers. Furthermore, the effect of norverapamil (NVP), which is the main metabolite in human, on the pharmacokinetics and pharmacological effect of VP is also not well understood.

The purpose of this study was to clarify the interactions between VP enantiomers and between VP and NVP on the relationship between pharmacokinetics and pharmacological effect of PR intervals. We studied the PR intervals of rabbits as an index of VP’s antiarrhythmic effect.

In general, an unbound drug in plasma is only trans- 
portable thorough a biological membrane and binds to biological receptors. Therefore, the concentration of unbound 
drug in plasma should be estimated to study the relationship between pharmacokinetics and pharmacodynamics. The dosing rate of the drug and the presence of effect compartments should also be considered when relating the plasma concentration to the pharmacological effect. We therefore maintained the plasma VP concentration at a steady state, to equilibrate the concentration between the plasma and effect compartments.

MATERIALS AND METHODS

Materials Racemic VP hydrochloride and NVP hydrochloride were kindly provided by Eisai Co. (Tokyo, Japan). R-Propranolol hydrochloride was obtained from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). [N-Methyl-

3H]verapamil hydrochloride (specific activity, 2.2 TBq/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). The S- and R-enantiomers of VP were separated by HPLC8) and their stereochemical purities were as-certained by stereospecific HPLC resolution (the stereochemical purities of S-VP and R-VP were 98.6 and 99.3%, respectively). All other reagents used were of analytical grade, unless stated otherwise. VP was dissolved in physiological saline before use.

Protein Binding The protein binding of VP enantiomers was evaluated using equilibrium dialysis.5) Five microliters of 

3H-labeled R- or S-VP (2.5 GBq/mmol) was added to 0.5 ml freshly isolated serum. The solutions were dialyzed using a 5-cell equilibrium dialyzer (10 rpm, Spectrum Inc.) against phosphate buffer (0.113 M Na2HPO4 and 0.017 M KH2PO4, pH 7.4) for 2 h at 37°C. The membrane used was a Spectra/Por-2, MW cut-off 12000–14000 (Spectrum Inc.). After equilibrium had been reached, the radioactivities of the solutions (inside and outside the dialysis tube) were deter-mined using a liquid scintillation counter (Aloka). Sample volume alteration during dialysis was corrected according to the change in protein concentration. The concentration de-
pendency of VP binding to rabbit serum protein was also studied (the final drug concentration in serum ranged from 0.022 to 1.10 μM). The effect of NVP on the protein binding of VP was also studied.

**Animals** Male Japanese White rabbits (2.88—3.92 kg) were used in this study and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, 1996). Each rabbit had its own cage and was kept under stable conditions of humidity (60±5%) and temperature (22±2 °C). The rabbits were allowed free access to food and water for the duration of the experiment.

**Pharmacokinetic and Pharmacodynamic Study** Intra-venous Bolus Injection: Rabbits were placed on their back without anesthesia. Racemic VP, S-VP or R-VP was administered by intravenous bolus injection at a dose of 0.4, 0.2 or 4.0 mg/kg, respectively, via an auditory vein. Blood samples were withdrawn from another auditory vein at 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min and centrifuged at 2500×g for 3 min. The plasma was stored at −20 °C until analysis.

Intravenous Constant Infusion: Rabbits were placed on their back without anesthesia, and the following experiments were performed using a crossover design. Racemic, S- or R-VP was infused into a marginal ear vein using an infusion pump. The rate for the loading dose of racemic VP, S-VP and R-VP was 0.1, 0.2, 0.4 mg/kg/h, 0.05, 0.1, 0.2 mg/kg/h, and 0.5, 1.0, 2.0 mg/kg/h, respectively (4.6 ml/h). After 20 min, the maintenance dose was infused at a rate of 1.2 ml/h. Blood samples were withdrawn from another auditory vein at 20, 90, 120 and 180 min and centrifuged at 2500×g for 3 min. The plasma was stored at −20 °C until analysis. An ECG was recorded simultaneously as described below.

Effect of NVP on the Pharmacokinetics and Pharmacodynamics of VP: Racemic VP was infused intravenously at a rate of 0.2 mg/kg/h. After 120 min of VP infusion, racemic NVP was infused at a rate of 0.1 mg/kg/h (loading dose: 5.6 ml/h, maintenance dose: 1.1 ml/h). Blood samples were withdrawn at 20, 75, 90, 120, 135, 180, 195, 210, 240 and 300 min after start of VP infusion and centrifuged at 2500×g for 3 min. The plasma was stored at −20 °C until analysis. An ECG was recorded simultaneously as described below.

Pharmacokinetic Measurement: The PR interval reflects the conduction time of the node atrioventricularis. We used this interval to study the antiarrhythmic effect of VP. The polygraph system consisted of a Nihon Electrical Sanei apparatus (Tokyo, Japan), pen oscillograph recti-horiz-8K recorder, bioelectric amplifier, tachometer, head amplifier and surface electrode. The paper speed was 100 mm/s. The electrode was placed on precordial leads. After a stabilization period of 10 min, the ECG was recorded every 10 min for 30 min prior to administration of the drug (control value) and at blood sampling times after drug administration.

**Analytical Methods** The concentrations of VP and NVP enantiomers in plasma were determined using the enantioselective HPLC method.8,9 The HPLC system consisted of a Shimadzu HPLC apparatus (Kyoto, Japan), a LC-9A HPLC pump and a C-R6A Chromatopac integrator. VP was detected by an RF-535 fluorescence detector which was operated at excitation and emission wavelengths of 272 and 312 nm, respectively. Each enantiomer was separated with a ChiralPak AD column (250×4.6 mm I.D., Daicel, Tokyo, Japan) at 40 °C. The mobile phase comprised hexane–iso-propanol–diethylamine (94:6:0.1 (v/v)) at a constant flow rate of 1.2 ml/min. The drugs were extracted from biological fluids using an organic solvent (ethyl ether) under alkaline conditions. With regard to analytical accuracy, the within- and between-day coefficients of variation were less than 8.3% and the linearity of the calibration curve was higher than 0.998.

**Data Analysis** All data were represented as mean±S.D. The pharmacological effect (Epk) was represented as the percentage change in PR interval after VP administration. The pharmacokinetic and pharmacodynamic parameters were calculated by the nonlinear least squares program Win-Nonlin (Pharcyes Co., Chicago, IL, U.S.A.). The plasma concentration of VP (Cp) was fitted into a two exponential equation using the following equation:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

The total clearance (CLtot) and volume of distribution at a steady state (Vdss) were estimated by the following equations:

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$AUMC = \frac{A}{\alpha^2} + \frac{B}{\beta^2}$$

$$CLtot = \frac{\text{Dose}}{\text{AUC}}$$

$$Vdss = \frac{\text{Dose} \cdot \text{AUMC}}{\text{AUC}^2}$$

where AUC and AUMC are the area under the plasma concentration–time curve and the area under the moment curve, respectively.

The relationship between the plasma drug concentration and pharmacological effect (Epk) was analyzed following the linear model:

$$Epk = m \cdot Cp + 1$$

where m and l are the slope and intercept, respectively.

**Statistical Analysis** We used a linear regression analysis to assess the relationship between drug plasma concentration and effect variables over time. A Mann–Whitney rank sum test was used to compare the slopes and intercepts of the concentration–response curves for each parameter in each group. The differences between the means of the two groups were tested using Student’s paired or unpaired t-test. Differences at p<0.05 were considered to be significant.

**RESULTS**

We first determined the plasma protein binding of VP enantiomers, since the unbound drug in plasma generally induces both pharmacological and adverse effects. The free fractions of both enantiomers were almost constant against the concentration (Table 1), and no significant difference was observed between enantiomers over the concentration range of 0.010 to 1.10 μM (data not shown). Furthermore, the plasma protein binding did not change in the presence of excess amounts of the other enantiomer and NVP. This suggests that the affinity of VP for plasma protein is relatively low, and that the interaction of binding between enantiomers and between VP and NVP can be ignored.
Figure 1 shows the time-courses of S-VP and R-VP plasma concentrations and the percentage change in the PR intervals during their intravenous infusion into rabbits. No significant difference was observed in the PR intervals during the control experiment. Both the plasma concentration and pharmacological effect reached a steady state within 90 min of starting the infusion of each enantiomer. In human plasma, NVP is a major metabolite that shows activity, although this was not detected in rabbits. The duration of the PR interval after VP administration was significantly prolonged compared with that of saline administration. We therefore used the PR interval as a way of estimating the pharmacological effect of VP in conscious rabbits.

To estimate the effect of NVP on the pharmacokinetics and pharmacodynamics of VP, the rabbits were administered racemic NVP concomitantly with racemic VP (Fig. 2). NVP did not significantly affect the time-courses of either plasma VP concentration or the pharmacological effect. This sug-

<table>
<thead>
<tr>
<th>Free fraction (%)</th>
<th>S-verapamil</th>
<th>R-verapamil</th>
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<tbody>
<tr>
<td>Verapamil enantiomer (0.20 μM)</td>
<td>18.1±0.41</td>
<td>19.2±0.80</td>
</tr>
<tr>
<td>Verapamil racemate (0.40 μM)</td>
<td>18.2±2.55</td>
<td>18.9±1.98</td>
</tr>
<tr>
<td>Norverapamil racemate (0.42 μM)</td>
<td>18.8±0.98</td>
<td>18.1±0.72</td>
</tr>
</tbody>
</table>

Each of the data represents the mean±S.D. (n=3). The concentration of each verapamil enantiomer for protein binding was 0.01 μM.
gests that any pharmacokinetic or pharmacodynamic interaction between VP and NVP is negligible.

The relationship between VP plasma concentration and pharmacological effect in a steady state after intravenous infusion is shown in Fig. 3. Good linear correlations were observed between the plasma concentrations of VP and the pharmacological effect for each enantiomer. The slope observed for S-VP (0.296) was 20 times larger than that of R-VP (0.0145). We assumed that each enantiomer independently exerts a pharmacological effect and estimated this effect at each sampling time under the steady state condition after administration of racemic VP. The slope observed with racemic VP appeared to be the mean value of the two enantiomers. Also, the predicted values of the pharmacological effect of each enantiomer administered.

Table 2. Pharmacokinetic and Pharmacodynamic Parameters of Verapamil Enantiomers after Intravenous Bolus Administration of Racemic Verapamil to Rabbits

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S-verapamil</th>
<th>R-verapamil</th>
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<tbody>
<tr>
<td>$C_{Ltot}$ (l/h)</td>
<td>26.3±4.62</td>
<td>16.3±4.06</td>
</tr>
<tr>
<td>$V_{dss}$ (l)</td>
<td>50.5±2.95</td>
<td>43.5±16.6</td>
</tr>
<tr>
<td>$M$ (effect %, ml/ng)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolus injection</td>
<td>0.319±0.027</td>
<td>0.0146±0.002</td>
</tr>
<tr>
<td>Constant infusion</td>
<td>0.296±0.084</td>
<td>0.0145±0.0043</td>
</tr>
<tr>
<td>$I$ (effect %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolus injection</td>
<td>3.42±0.774</td>
<td>3.26±0.373</td>
</tr>
<tr>
<td>Constant infusion</td>
<td>3.93±1.28</td>
<td>3.45±0.477</td>
</tr>
</tbody>
</table>

Each of the data represents the mean±S.D. (n=4). $C_{Ltot}$: total clearance; $V_{dss}$: volume of distribution at steady state.

To estimate the contribution of the VP dosing rate to the relationship between pharmacokinetics and pharmacodynamics, we intravenously administered racemic VP to rabbits by bolus injection (Fig. 5). There was no significant difference in the pharmacokinetic parameters between S-VP and R-VP after racemic VP administration (Table 2). There was also no significant difference in the total clearance of VP enantiomers between bolus administration and constant infusion. However, the volume of distribution at a steady state after racemic VP administration was significantly decreased compared with that after enantiomer administration (Tables 2 and
There was no significant difference in the pharmacodynamic parameters, slope and intercept with the linear pharmacokinetics and pharmacodynamics in rabbits. The pharmacokinetic parameters of VP did not differ significantly between enantiomers, as has been reported previously. However, the Vdss obtained from each enantiomer were higher than that obtained from administration of the racemate. As VP has a relatively high Vd and the results in rabbits are approximately 20 times more potent than R-VP in rabbits using an ECG analysis of the prolongation effect of the PR interval. By administering each enantiomer, we demonstrated that the prolongation of this interval caused by administration of racemic VP was simply the sum of the pharmacological effects induced by the VP enantiomers. Racemic NVP did not affect the prolonged PR intervals induced by racemic VP after intravenous administration, as has been observed by Neugebauer. These results suggest that unbound VP concentration in plasma can be directly related to the pharmacological effect as estimated by PR intervals.

A dosing rate-dependent relationship between drug plasma concentration and pharmacological effect has been reported. Takahashi et al. reported that in humans the relationship between plasma propranolol concentration and beta-blockade is dependent on the dosing rate. Therefore, in this study we estimated the effect of the dosing rate of VP on the relationship between pharmacokinetics and pharmacodynamics. The pharmacodynamic parameters we obtained after intravenous bolus injection of racemic VP were not different from those after constant infusion, suggesting that there is equilibrium between the plasma compartment and the effect compartment in rabbits.

In conclusion, we have demonstrated that the relationship between the plasma concentration and pharmacological effect of racemic VP, after intravenous administration, was the sum of the effects induced by the two enantiomers. In addition, NVP, which is the main metabolite of VP, did not affect the prolongation effect on PR interval induced by VP.