Regular Article

Effects of Dipyridamole Coadministration on the Pharmacokinetics of Ribavirin in Healthy Volunteers

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Summary: Ribavirin (RBV), a guanosine analog for treatment of hepatitis C, is a substrate of a nucleoside transporter, solute carrier family 29 member 1 (SLC29A1). To clarify the impact of SLC29A1 on the pharmacokinetics of RBV, an open-label, crossover study of single-dose RBV (200 mg, p.o.) with and without coadministration of dipyridamole (DP), an inhibitor of SLC29A1, was performed. Plasma and erythrocyte concentrations of RBV in the control phase and DP phase (25 mg, 3 times daily for 4 days) were compared in 10 healthy volunteers. SLC29A1 mRNA expression in peripheral blood mononuclear cells was also determined. In the DP phase, area under the concentration-time curves (AUC) of RBV in plasma and erythrocytes showed reductions of 23% and 17%, respectively (p < 0.05), with increases in apparent oral clearance of 18% and 25%, respectively (p < 0.05). The reduction rate of the AUC of erythrocyte RBV in the DP phase was associated with SLC29A1 mRNA expression: higher mRNA expression showed greater AUC reduction. The elimination half-life of both plasma and erythrocyte RBV did not differ between the 2 phases. These results suggest that RBV/DP coadministration reduces the concentration of RBV in blood by inhibiting an important role of SLC29A1 in gastrointestinal absorption of RBV.

Keywords: ribavirin; pharmacokinetics; dipyridamole; SLC29A1; mRNA expression

Introduction

The current standardized treatment for hepatitis C virus infection is the combination therapy of pegylated interferon-α (IFN) and ribavirin (RBV), which cures the infection in 55% of patients with severe chronic hepatitis.¹² Sustained virological response (SVR) and occurrence of anemia, common side effects of RBV, in the course of IFN/RBV therapy have been associated with RBV exposure.³–⁸ The area under the concentration-time curve (AUC) of plasma RBV after the first dose and steady-state plasma RBV concentration have been reported to be factors in prediction of SVR.⁹ A steady-state erythrocyte RBV concentration greater than 1,000 µM induces severe reduction in hemoglobin, resulting in the occurrence of anemia.¹⁰ Although the pharmacokinetics of RBV plays an important role in IFN/RBV therapy, the cause of individual variability has not been fully identified except for the 4 covariates of age, sex, body weight, and renal function.⁵¹¹¹² RBV is a hydrophilic molecule that needs to be transported into cells via nucleoside transporters to produce its effectiveness.¹³ Nucleoside transporters comprise 2 families: equilibrative nucleoside transporters (solute carrier family 29 [SLC29]) and Na⁺-dependent concentrative nucleoside transporters (SLC28). Since RBV is a substrate of SLC29 and SLC28,¹⁴–¹⁶ both of which are expressed in the human intestine and erythrocytes,¹⁴,¹⁷ the pharmacokinetic features of RBV, such as intestinal absorption and erythrocyte disposition, may be altered according to changes in the activity of SLC29 and SLC28. A recent clinical study investigating a single nucleotide polymorphism (SNP) in SLC29A1 revealed that wild-type rs6932345 was a significant genetic factor associated with a higher blood RBV concentration and SVR rate in IFN/RBV therapy.¹⁸ Thus, we speculated that nucleoside transporter activity is involved in the individual differences in the pharmacokinetics of
Effect of Dipyridamole on the Pharmacokinetics of Ribavirin

To confirm this speculation, we investigated the effects of coadministration of RBV and dipyridamole (DP), an inhibitor of SLC29A1, on changes in the pharmacokinetics of RBV in healthy volunteers. The activity of SLC29A1 assessed by SNPs and its mRNA expression in peripheral blood mononuclear cells (PBMCs) were also examined in association with the pharmacokinetic alteration of RBV.

Materials and Methods

Volunteers: Ten healthy male volunteers participated in this study (Table 1). Physical examination and laboratory test findings were all normal. None of the volunteers had any history of anemia, virus infection, or of taking any medications during the 2 weeks before the study, and all provided written informed consent to participate in the study. The study was approved by the ethics committee of the University of Tsukuba Hospital and conducted according to the principles expressed in the Declaration of Helsinki, and the protocol was registered with the UMIN (University Hospital Medical Information Network) Clinical Trials Registry of Japan.

Study protocol and sample collection: The pharmacokinetics of RBV in plasma and erythrocytes after a single dose of RBV were investigated in an open-label, crossover study with a washout period of 3 months between treatments. In the control phase, the volunteers received a 200-mg dose of oral RBV (Rebetol Capsules; MSD, Tokyo, Japan) at 8:00 AM on the study day after overnight fasting (control phase). In the DP phase, the volunteers received 25 mg DP (Persantin Tablets; Boehringer Ingelheim Japan, Tokyo, Japan) 3 times daily for 3 days as pretreatment. On day 4 of the study, the volunteers took 25 mg DP 3 times daily (at 8:00 AM, 1 PM, and 7 PM) and 200 mg RBV at 8:00 AM. The doses for DP and RBV in the present study were fixed in terms of the safety for the healthy volunteers and clinical applicability. All volunteers completed the study without any adverse events.

Heparinized blood samples were collected before RBV dosing (0 h) and at 1, 2, 4, 8, 12, 24, 48, and 168 h after the dosing. Throughout the washout period, to confirm the complete disappearance of RBV, we periodically measured the RBV concentration in blood. Urine samples for determining the urinary excretion of RBV were also collected from 0 to 24 h after the dosing. A portion of the blood samples collected at 0 h was used for determination of mRNA expression and SNP genotyping. A portion of each blood sample was centrifuged at 1,000 g for 10 min at 4°C to separate the plasma. The remaining blood samples were used for determination of the RBV concentration in whole blood. These samples were stored at −20°C until analysis.

Determination of ribavirin concentration: RBV concentrations in plasma and whole blood were determined by high-performance liquid chromatography (HPLC) as previously described. Briefly, 20 µL of whole blood supplemented with 120 µL of ice-cold distilled water was subjected to acid phosphatase digestion (2 units; Sigma-Aldrich, St. Louis, MO) to convert phosphorylated RBV metabolites into free RBV. The resulting mixture, spiked with an internal standard, 3-methylcytidine phosphorolyase (Sigma-Aldrich), was treated by means of phenylboronic acid (PBA) column (Bond Elut PBA; Varian Medical Systems, Palo Alto, CA) extraction followed by reversed-phase HPLC. The dephosphorylation step was omitted when free RBV was determined in the plasma and urine. The concentration of erythrocyte RBV was calculated with the following formula:

\[ C_{\text{erythrocyte}} = \frac{C_{\text{whole}} - C_{\text{plasma}}(1 - Ht)}{Ht} \]

where \( C_{\text{erythrocyte}} \) is the concentration of RBV in erythrocytes, \( C_{\text{plasma}} \) is the concentration of RBV in whole blood, \( C_{\text{plasma}} \) is the concentration of RBV in plasma, and \( Ht \) is the study volunteer’s hematocrit.

The HPLC apparatus used in this study was the model 200 system ( Tosoh Corporation, Tokyo, Japan) equipped with a UV detector, an auto sampler, and a pump. A C18 reversed-phase column (TSK-Gel ODS-80Ts; Tosoh Corporation) was used for separation of RBV from other contaminants. The detection wavelength was set at 225 nm. The mobile phase solvent, 10 mM ammonium phosphate buffer (pH 2.5), was pumped out at a flow rate of 1.0 mL/min. All chemicals for the assay were of HPLC or reagent grade (Wako Pure Chemical Industries, Osaka, Japan or Sigma-Aldrich). The detection limit for plasma RBV was 0.02 and 0.24 µg/mL, respectively. Coefficients of variation for intra- and inter-day assays were 3–10% and 5–11%, respectively, for whole blood RBV (0.4–10 µg/mL), and 1–2% and 7–9%, respectively, for plasma RBV (0.1–5 µg/mL).

Pharmacokinetic analyses: The pharmacokinetic parameters of RBV such as maximum concentration (\( C_{\text{max}} \)), time to reach \( C_{\text{max}} \) (\( t_{\text{max}} \)), terminal half-life (\( t_{1/2} \)), and AUC of plasma (\( AUC_{\text{plasma}} \)) and erythrocytes (\( AUC_{\text{erythrocyte}} \)) were assessed by noncompartmental analysis using Phoenix WinNonlin version 6.1 software (Certara, St. Louis, MO). Apparent oral clearance (\( CL_{\text{oral/FO}} \)) was calculated as the dose/\( AUC_{0-24} \) in plasma and the dose/\( AUC_{0-168} \) in erythrocytes. Since the plasma RBV concentrations after 24 h were close to the detection limit for both control and DP coadministration, we did not extrapolate to 168 h after the administration when \( AUC \) was calculated for plasma RBV. Renal clearance (\( CL_{\text{R}} \)) was calculated as the amount excreted in the urine (\( AUC_{0-24} \)/\( AUC_{0-24} \) in plasma).

Determination of mRNA expression: SLC29A1 mRNA expression in PBMCs was determined by a fluorescence-based real-time detection method using an Mx3000P instrument (Agilent Technologies, Santa Clara, CA). Total RNA was isolated from peripheral blood using a PAXgene Blood RNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. cDNA was prepared from total RNA by use of a SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA).

The PCR reaction mixture consisted of 20 ng cDNA, 300 nM each primer, and 1× SYBR GreenER qPCR SuperMix (Life Technologies) containing a reference dye, to a final volume of 20 µL. PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 60 cycles at 95°C for 15 s and at 60°C for 1 min. The housekeeping gene, ubiquitin C (\( UBC \)), was also measured as a reference gene.

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Table 1. Demographic and baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male/female)</td>
<td>10 (10/0)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>67.7 ± 11.8</td>
</tr>
<tr>
<td>Age, y</td>
<td>30.1 ± 3.9</td>
</tr>
<tr>
<td>RBV dose, mg/kg</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>DP dose, mg/kg/d</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>SLC29A1 genotype</td>
<td>AA/AC/CC 9/1/0</td>
</tr>
<tr>
<td>rs6932345A&gt;C</td>
<td>9/1/0</td>
</tr>
<tr>
<td>Expression level of SLC29A1 mRNA</td>
<td>1.10 (0.25–2.77)</td>
</tr>
</tbody>
</table>

Data are presented as number, mean ± S.D., or median (range).
The primers sequences used were as follows: SLC29A1, 5′-AGACCAAGTTGACCTACTATGCA-3′ (forward) and 5′-CGGCTGAACATCCAAAT-3′ (reverse); UBC, 5′-ATTTGGGTCGCGGTCTTCTG-3′ (forward) and 5′-TGCCCTGACATTCTZCGATGGT-3′ (reverse). Threshold cycle (Ct) values were calculated using MxPro Software (Agilent Technologies). Ct values were determined in duplicate. The CtSLC29A1/CtUBC ratio was used to calculate the relative expression of SLC29A1 using the ΔΔCt method.\(^{21}\)

**Genotyping of SLC29A1:** Genomic DNA isolated from peripheral blood using the QIAamp DNA Mini Kit (QIAGEN) was subjected to genotyping of SLC29A1 rs6932345A>C. The impact of another SNP, rs747199G>C, was also examined because this SNP is located in the putative transcription factor binding site in the upstream region of the translation initiation site of SLC29A1 and is thought to be associated with an increase in SLC29A1 mRNA expression in PBMCs.\(^{22}\) The PCR reaction was performed with the following primers: rs6932345, 5′-GCAGTGGCACAAACACCA-3′ (forward) and 5′-GCAGTGGCACAAACACCA-3′ (reverse); rs747199, 5′-TGACTGAGGTCAAACCAGAGG-3′ (forward) and 5′-GAGTTGGGGAATGTGTCAGT-3′ (reverse), and consisted of 35 cycles at an annealing temperature of 63°C. Genotyping was performed by restriction fragment length polymorphism analysis with BspHI and AluI enzymes (New England Biolabs Japan, Tokyo, Japan). BspHI cut the variant rs6932345C allele into 2 fragments of 452 bp and 60 bp, and the AluI cut the variant rs6747199G allele into 3 fragments of 305 bp, 147 bp, and 60 bp.

**Statistical analyses:** Data were expressed as the mean ± S.D. Pharmacokinetic data from the control and DP phases in volunteers were determined in duplicate. The CtSLC29A1/CtUBC ratio was used to calculate the relative expression of SLC29A1 using the ΔΔCt method.\(^{21}\)

**Results**

The pharmacokinetic profiles of RBV in plasma and in erythrocytes and the erythrocyte/plasma ratio are shown in Figure 1. Plasma and erythrocyte RBV concentrations in the DP phase were significantly lower than those in the control phase (Figs. 1A and 1B). However, no significant difference between the control and DP phases was observed in the erythrocyte/plasma ratio (Fig. 1C).

In the DP phase, the mean C\(_{max}\) and AUC\(_{0-24}\) of plasma RBV were 36% and 23% lower than those in the control phase, respectively (p < 0.05) (Table 2). These changes corresponded to an increase in CL\(_{oral}/F\) in the DP phase, which was 18% higher than in the control phase (p < 0.05), but not to an increase in CL\(_{inj}/F\) (Table 2). No difference between the control and DP phases was observed in the t\(_{max}\) or t\(_{1/2}\) for plasma RBV (Table 2). A similar change was observed in the pharmacokinetic parameters of erythrocyte RBV. In the DP phase, the mean C\(_{max}\) and AUC\(_{0-16}\) of erythrocyte RBV were 17% lower than those in the control phase (p < 0.05), which corresponded to an increase in CL\(_{oral}/F\) that was 25% higher than that in the control phase (p < 0.05) (Table 2). No difference between the control and DP phases was observed in the t\(_{max}\) or t\(_{1/2}\) for erythrocyte RBV (Table 2). The

**Table 2. Pharmacokinetic data of RBV after a single dose of 200 mg RBV with or without DP in 10 healthy volunteers**

<table>
<thead>
<tr>
<th></th>
<th>RBV</th>
<th>RBV + DP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{max}), µg/mL</td>
<td>0.59 ± 0.28</td>
<td>0.38 ± 0.09*</td>
</tr>
<tr>
<td>t(_{max}), h</td>
<td>1.5 ± 0.5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>t(_{1/2}), h</td>
<td>27.3 ± 11.9</td>
<td>24.9 ± 6.1</td>
</tr>
<tr>
<td>AUC(_{0-24}), h·µg/mL</td>
<td>4.66 ± 1.81</td>
<td>3.57 ± 0.65*</td>
</tr>
<tr>
<td>CL(_{oral}/F), L/h</td>
<td>49.0 ± 18.5</td>
<td>58.0 ± 12.5*</td>
</tr>
<tr>
<td>CL(_{inj}/F), L/h</td>
<td>4.5 ± 1.0</td>
<td>5.0 ± 1.1</td>
</tr>
<tr>
<td><strong>Erythrocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{max}), µg/mL</td>
<td>7.33 ± 1.49</td>
<td>6.05 ± 1.92*</td>
</tr>
<tr>
<td>t(_{max}), h</td>
<td>43.2 ± 10.1</td>
<td>48.0 ± 0.0</td>
</tr>
<tr>
<td>t(_{1/2}), h</td>
<td>339.8 ± 89.8</td>
<td>326.6 ± 96.8</td>
</tr>
<tr>
<td>AUC(_{0-16}), h·µg/mL</td>
<td>1,033.0 ± 236.1</td>
<td>857.1 ± 253.8*</td>
</tr>
<tr>
<td>CL(_{oral}/F), L/h</td>
<td>0.20 ± 0.05</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td><strong>Ratio</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(<em>{erythrocyte}/AUC(</em>{plasma})</td>
<td>242.8 ± 84.2</td>
<td>246.5 ± 86.7</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ae(_{0-24}), mg</td>
<td>19.9 ± 5.1</td>
<td>18.0 ± 5.6*</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± S.D. C\(_{max}\), maximum concentration; t\(_{max}\), time to reach C\(_{max}\); t\(_{1/2}\), terminal half-life; AUC, area under the concentration-time curve; CL\(_{oral}/F\), apparent oral clearance; CL\(_{inj}/F\), renal clearance; Ae\(_{0-24}\), amount excreted in the urine.

*Significant difference was observed between the control and DP phases (p < .05).

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Fig. 1. Concentration-time profile of plasma and erythrocyte RBV and erythrocyte/plasma ratio of RBV after single administration of 200 mg RBV in healthy volunteers

A, plasma; B, erythrocytes; C, erythrocyte/plasma ratio. ○, RBV alone; ⫢, RBV administered with DP. Data are presented as mean ± S.D.

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AUC ratios (AUC erythrocyte/AUC plasma) of the control and DP phases were not different (Table 2). Urinary excretion of RBV after 24-h administration significantly decreased in the DP phase (p < 0.05) (Table 2).

Differences in individual change in AUC erythrocyte between the control and DP phases are shown in Figure 2. The reduction in AUC erythrocyte in the DP phase seemed to be associated with SLC29A1 mRNA expression. Three volunteers with lower SLC29A1 mRNA expression (0.25, 0.47, and 0.51) than those of the other volunteers (range, 0.75–2.77) showed little or no reduction in AUC erythrocyte in the DP phase (Fig. 2, open circle). The genotypes of the SLC29A1 rs6932345 and rs747199 SNPs for all the volunteers were the AA and GG genotypes, respectively, except for 1 volunteer (AC and GC, respectively) whose mRNA expression was the lowest (0.25) of the volunteers studied here (Table 1).

Discussion

The pharmacokinetics of oral RBV is characterized by moderate absorption (bioavailability: 64%), large volume of distribution (5.374 L at steady state), long elimination half-life (286 h at steady state), renal excretion (61% of dosed amount for 14 days after the administration) and no plasma protein binding. So far, 4 factors have been identified as covariates affecting RBV clearance: body weight, sex, age, and renal function. Other factors may be involved in the alteration of RBV pharmacokinetics, especially in the absorption stage, because dietary purines are known to affect RBV bioavailability via modification of SLC28 activity. Nucleoside transporters expressed in the intestinal tract and erythrocyte membrane can be factors affecting RBV absorption from the intestine and distribution in erythrocytes. Although RBV cellular transport via nucleoside transporters (SLC29A1, SLC29A2, SLC28A2, and SLC28A3) has been confirmed by in vitro experiments data on the effects of SLC29A1 inhibition on RBV pharmacokinetics have not been available.

The present study revealed that coadministration of DP altered the pharmacokinetics of RBV in healthy volunteers. The pharmacokinetic parameters of RBV, C max, and AUC were reduced by 23% to 36% in plasma and by 17% in erythrocytes when DP was coadministered (Figs. 1A, 1B and Table 2). A similar change was observed in the urinary excretion of RBV (Table 2). These alterations mean that DP coadministration reduced the bioavailability of oral RBV. The possible mechanism is due to the inhibitory effects of DP on gastrointestinal absorption of RBV via SLC29A1. Because the CL/ F for plasma RBV remained unchanged by DP coadministration, DP did not seem to alter the renal excretion of RBV. An extremely large amount of DP was found in the small intestine after oral administration in rat (6,000 times greater than in blood), suggesting that the DP concentration in epithelial cells of the intestinal tract was high enough (over 100 µM) to inhibit SLC29A1. It has been reported that DP does not act as an inhibitor for SLC28 expressing on the lumen of epithelial cells.

The C erythrocyte/C plasma and AUC erythrocyte/AUC plasma profiles remained unchanged when DP was coadministered (Fig. 1C, Table 2). These observations mean that DP did not inhibit erythrocyte SLC29A1, and consequently, the accumulation of RBV in erythrocytes did not change. It has been reported that for DP to produce inhibitory effects on erythrocyte uptake of RBV in vitro, a DP concentration of 25 µM was required, which is an extremely higher concentration than that of circulating DP (0.74 µM) under repeated administration of 25 mg DP. Since the percentage of unbound DP in plasma was 0.88%, the DP concentration that directly contacts erythrocyte SLC29A1 is estimated to be lower still (0.0065 µM). These speculations may explain why the erythrocyte disposition of RBV was not altered by coadministration of DP.

The impact of SLC29A1 on IFN/RBV therapy has been reported in a clinical study. Tsubota et al. found that patients with wild-type rs6932345, one of the SNPs in the SLC29A1 gene, had higher plasma RBV concentrations and SVR rates than did mutant carriers. Currently, we found differences in SLC29A1 mRNA expression in PBMCs between subjects with wild-type and mutant carriers on rs6932345 (1.20 vs. 0.70). We, therefore, used a cutoff value of 0.7 to discriminate between high and low mRNA expression in the present study’s subjects. Since individuals with wild-type rs6932345 provide higher SLC29A1 mRNA expression in PBMCs, DP might be enhanced in such individuals. On the other hand, 3 volunteers with lower mRNA expression (0.25–0.51), one of whom was a mutant carrier of rs6932345, produced no or little change in erythrocyte AUC when DP was coadministered (Fig. 2). These observations suggest that the inhibitory effects of DP on RBV absorption via SLC29A1 are minimal or null in individuals with lower SLC29A1 mRNA expression. Our present results also support Tsubota et al. ‘s findings in terms of the impact of rs6932345 for SLC29A1 on RBV concentration in blood.

In conclusion, our results showed that coadministration of DP 75 mg daily altered the pharmacokinetics of RBV in healthy volunteers. This drug interaction may occur as a result of inhibition of intestinal RBV absorption via SLC29A1. Since individuals with lower SLC29A1 mRNA in PBMCs were not susceptible to this drug interaction, SLC29A1 may be a factor associated with individual variation in the pharmacokinetics of RBV. The influence of SLC29A1 on the pharmacokinetics of RBV in terms of the SNP genotype and mRNA expression should be examined in future as well as additional covariates to those identified so far.

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References


