Liquid Chromatography Assay for Routine Monitoring of Cellular Ribavirin Levels in Blood

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Ribavirin-induced hemolytic anemia is one cause for cessation of combination therapy with alpha interferon 2b and ribavirin for hepatitis C infection. Determining cellular ribavirin levels in blood, including the levels of its phosphorylated metabolites, might be useful for predicting ribavirin-induced anemia, because the metabolites accumulate in erythrocytes. We simplified an assay method developed previously to make it suitable for routine monitoring of cellular ribavirin. Whole blood diluted with a sixfold volume of ice-cold distilled water was subjected to acid phosphatase digestion to convert phosphorylated ribavirin metabolites to free ribavirin. The resulting mixture, spiked with an internal standard, was treated by phenyl boronic acid column extraction, followed by reverse-phase high-performance liquid chromatography analysis. The calibration curve for ribavirin levels in whole blood was linear at concentrations of 5.3 to 1,024 μM ($r^2 = 0.9999$). Validation coefficients of variation for intra- and interday assays were 2.9 to 5.8% and 4.3 to 8.3%, respectively. We tested this method by monitoring blood ribavirin concentrations in two hepatitis C patients receiving alpha interferon 2b-plus-ribavirin combination therapy.

Ribavirin, a guanosine analog broad-spectrum antiviral agent, is used for hepatitis C virus (HCV) elimination in combination with alpha interferon 2b (2, 3, 13). Polyethyleneglycol-alpha interferon 2a combined with oral ribavirin also brings substantial benefit to HCV patients (12, 14). Despite the strong beneficial effects of these combination therapies in severe HCV infection, loss of hemoglobin occurs in a substantial population of HCV patients. Progressive loss of hemoglobin leads to anemia, which is counteracted by reducing the ribavirin dose or prematurely discontinuing the combination therapy (1, 5, 12). Current studies suggest that the excessive accumulation of ribavirin in erythrocytes is responsible for the anemia (8, 11). Once incorporated into erythrocytes, ribavirin is converted into phosphorylated metabolites by intracellular phosphorylation (9). The phosphorylated metabolites decrease intracellular ATP levels, resulting in the reduction of erythrocyte integrity, which is followed by extravascular hemolysis via the reticuloendothelial system (4). It should therefore be possible to predict the occurrence of ribavirin-induced anemia by determining the ribavirin concentrations in blood cells and plasma.

A high-performance liquid chromatography (HPLC) method was previously developed to determine ribavirin levels in order to assess the disposition of ribavirin in erythrocytes (7). Since phosphorylated metabolites are the main form of intracellular ribavirin (7, 8), whole-blood samples were treated with acid phosphatase prior to column extraction and analysis. However, the dephosphorylation procedure used in a previous

study (7) was too tedious to use for routine monitoring of cellular ribavirin. In the present study, we simplified the dephosphorylation procedure. The modified method was tested for therapeutic drug monitoring of cellular ribavirin levels in two patients during the first 8 weeks of HCV treatment by combination therapy with ribavirin and alpha interferon 2b.

MATERIALS AND METHODS

Chemicals and instruments. All chemicals were of HPLC or reagent grade and were obtained from Wako Pure Chemicals Industries (Osaka, Japan) or Sigma-Aldrich Corp. (St. Louis, Mo.). Ribavirin (1- β -D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), 3-methylcytidine methosulfate (internal standard), and acid phosphatase type 4 prepared from sweet potato were purchased from Sigma. Phenyl boronic acid (PBA) cartridges with a high specificity for *cis*-diol-containing compounds (100 mg; Bond Elute PBA), which were used for solid-phase extraction, were obtained from Varian (Palo Alto, Calif.).

The HPLC system used in this study was the Tosoh (Tokyo, Japan) model 8020 system equipped with a UV detector, an autosampler, and a pump. A C_{18} reversed-phase column (4.6 [inner diameter] by 150 mm; TSK-Gel ODS-80Ts, Tosoh) was used, and UV absorbance was monitored at 225 nm. The mobile-phase solvent, 10 mM ammonium phosphate buffer (pH 2.5), was pumped through at a flow rate of 1.0 ml min⁻¹.

Reference sample preparation. A stock ribavirin solution (4.1 mM) was prepared by dissolving ribavirin in distilled water. Reference samples were prepared by diluting the stock solution with a human plasma alternative (Twin-Consera H; Nissui, Tokyo, Japan) or with drug-free whole blood collected in heparinized tubes from a healthy volunteer. The concentrations of reference samples were 0.25, 0.5, 1, 5, 10, and 20 μM in the plasma alternative and 5.3, 21, 85, 341, 683, and 1,024 μM in whole blood. The concentration ranges of the reference samples were established in a previous study (8). Reference samples were stored at -30°C until use. An internal standard was prepared as a 250 μM solution of 3-methylcytidine methosulfate in distilled water, and the solution was stored at 4°C .

Enzyme digestion and column extraction. A 20- μl sample of whole blood was supplemented with 120 μl of ice-cold distilled water and vortexed intensely for 20 s to accomplish complete hemolysis. The hemolysate was treated with acid phosphatase to hydrolyze the phosphorylated metabolites, according to the method of Homma et al. (7). The reaction mixture, consisting of the hemolysate (140 μl), 30 μM Tris-HCl (200 μl), 1 M sodium acetate (20 μl), and 2 U of acid

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phosphatase, was incubated for 1 h at 37°C. The reaction was terminated by neutralizing the reaction mixture with 10 M KOH. That dephosphorylation of the phosphorylated metabolites of ribavirin went to completion was confirmed by monitoring the peak height ratio after various incubation times (30, 60, 90, and 120 min); a plateau was reached at 30 min. Furthermore, addition of another 2 U of enzyme preparation to the reaction mixture after 120 min of incubation did not lead to any change in peak height.

The dephosphorylated samples were supplemented with 20 µl of the internal standard and 500 µl of 250 mM ammonium phosphate buffer (pH 8.5). After being centrifuged at 1,500 × g for 5 min, the supernatants were loaded onto PBA cartridges that had been pretreated with 1 ml of 100 mM formic acid, followed by 5 ml of 250 mM ammonium phosphate buffer (pH 8.5). The cartridges were positioned in a 12-port manifold (Alltech, Lexington, Ky.) and washed with 5 ml of 250 mM ammonium phosphate buffer (pH 8.5). Ribavirin and the internal standard were subsequently eluted with 1 ml of 100 mM formic acid into glass tubes. The recovery rate was as almost the same as that described in a previous study (7). The effluents were evaporated to dryness at 40°C under a nitrogen gas flow. The samples were reconstituted with 200 µl of mobile-phase solution, and a 20-µl portion was injected into the HPLC system.

For quantification of plasma ribavirin, a plasma sample (200 µl) was supplemented with 20 µl of the internal standard and 500 µl of 250 mM ammonium phosphate buffer (pH 8.5) and subjected to PBA column extraction. Plasma samples were not treated with acid phosphatase, because phosphorylated ribavirin was not found in plasma in our preliminary study.

Assessment of cellular ribavirin disposition in blood. Our modified assay method was tested by assessing cellular ribavirin concentrations in two HCV patients treated with alpha interferon 2b plus ribavirin. The daily dose of ribavirin was 800 mg throughout the study, in accordance with the standard dosing instructions for HCV patients treated in Japan. The daily dose of alpha interferon 2b started at 10 million units intramuscularly for 14 days, followed by 6 million units three times weekly. Kidney function in both patients was normal throughout the treatment. A 10-ml sample of blood was collected in heparinized tubes at 0, 1, 2, 7, 14, 21, 28, 56, and 84 days after the start of the combination therapy. Blood samples (5 ml each) drawn from patients were immediately divided into two portions: one whole-blood portion to be treated by enzyme digestion, and the other portion to be used to obtain plasma by centrifugation.

The concentration of cellular ribavirin was calculated with the formula $C_c = [C_w - C_p(1 - Ht)]/Ht$, where C_c is the ribavirin concentration in blood cells, C_w is the concentration in whole blood, C_p is the concentration in plasma, and Ht is the hematocrit. Hematocrit includes many blood cell types such as erythrocytes, lymphocytes, and neutrophils. Most ribavirin in blood cells is located in erythrocytes, because ribavirin and its phosphorylated metabolites could not be detected in the fraction of white blood cells in our preliminary experiment (data not shown). In this study, therefore, C_c is essentially identical to ribavirin concentration in erythrocytes. The hematological parameters (hemoglobin and hematocrit values) were also measured on each sampling day. Informed consent was obtained from all patients, and the study was approved by the ethics committee of our university.

RESULTS

A typical HPLC chromatogram for determination of ribavirin levels in phosphatase-digested whole blood samples is shown in Fig. 1B. Peaks representing ribavirin and the internal standard were observed at retention times of 4.7 and 6.1 min, respectively. No interfering peaks generated from endogenous substances were observed on the chromatogram for blank (i.e., untreated) whole blood (Fig. 1A). Similar chromatograms were obtained from plasma samples (data not shown).

Calibration curves for ribavirin levels determined at concentrations of 0.25 to 20 µM for plasma and 5.3 to 1,024 µM for whole blood were linear. The equations for the lines calculated by regression analysis were $y = 0.0387x + 0.0037$ ($r^2 = 1.0000$) for plasma and $y = 0.0034x + 0.0026$ ($r^2 = 0.9999$) for whole blood, where y is the peak height ratio of ribavirin/internal standard and x the concentration of ribavirin.

The analytical precision of determination of ribavirin whole-blood levels was evaluated with whole-blood reference samples

FIG. 1. HPLC chromatograms of blank whole blood (A) and whole blood spiked with 1,024 µM ribavirin (RBV) and an internal standard (IS) (B).

containing ribavirin concentrations of 21, 341, and 1,024 µM. Validation coefficients of variation for intra- and interday assay precision was assessed five times in a single run and five times in separate runs, respectively (Table 1). Coefficients of variation for intra- and interday assays were 2.9 to 5.8% and 4.3 to 8.3%, respectively.

We tested our new method by using it to determine ribavirin cellular levels for two HCV patients. No interfering peaks were observed on the chromatograms despite various medications that were administered concomitantly, such as an analgesic (acetaminophen), an antacid (Sucralfate), and a cholagogue (ursodesoxycholic acid). A decrease in hemoglobin levels was observed for both patients within the first 3 weeks of combination treatment, as the concentration of cellular ribavirin increased (Fig. 2). Cellular ribavirin reached steady-state levels within 3 to 4 weeks after the combination therapy was begun. Steady-state levels of cellular ribavirin were 146 times higher than plasma ribavirin levels for patient 1 and 150 times higher than plasma ribavirin levels for patient 2 (Table 2). Around 90% of cellular ribavirin consisted of phosphorylated metabolites, which were not detected in plasma (Table 2). The difference in hemoglobin drop between the two patients appeared to

TABLE 1. Validation coefficients of variation for intra- and interday assay precision

Concn (µM)	Observed concn (µM)			
	Intraassay (n = 5)		Interassay (n = 5)	
	Mean ± SD	CV ^a	Mean ± SD	CV
21	22.8 ± 1.3	5.8	20.1 ± 1.7	8.3
341	344 ± 10	2.9	308 ± 16	5.3
1,024	1027 ± 34	3.4	1040 ± 44	4.3

^a CV, coefficient of variation.

FIG. 2. Changes in hemoglobin levels (○) and ribavirin concentrations in blood cells (●) during combination treatment of hepatitis C patients with alpha interferon 2b and ribavirin.

correspond to their cellular ribavirin levels. That is, patient 1, who showed marked hemoglobin reduction, had a higher cellular ribavirin concentration (1,662 μM) than did patient 2 (929 μM).

DISCUSSION

In this study, we succeeded in simplifying the quantification of ribavirin in whole-blood samples. The advantage of the present method is the elimination of the handling required for precipitation of whole blood and the tedious pH adjustment prior to enzyme digestion (both of which were required with the previous method). Since ribavirin concentrations in whole blood were extremely high ($>1,000 \mu\text{M}$ at steady state), we could decrease the sample volume, which enabled us to carry out hemolysis with ice-cold water instead of by acid precipitation. Another modification was the use of a TSK-Gel ODS-80Ts column (4.6 [inner diameter] by 150 mm) instead of a Novapak column (3.9 [inner diameter] by 300 mm) as the analytical C_{18} reverse-phase column. The TSK-Gel ODS-80Ts column achieved baseline separation of the ribavirin and internal standard peaks from those of other components more efficiently, even though the detection wavelength of 225 nm was lower and retention times were shorter than in a previously described method (4, 7). The limit of detection was also improved from 2.4 to 1.2 ng in terms of the amounts injected. This corresponds to ribavirin concentrations of 0.2 to 0.1 μM . And the signal-to noise ratio of >3 was higher than by the previous method.

We tested the revised method by determining ribavirin concentrations in plasma and blood cell samples of two HCV patients receiving alpha interferon 2b and ribavirin in combination. Cellular ribavirin concentrations reached steady-state levels 2 to 3 weeks after the patients started combination therapy, when hemoglobin reduction also bottomed out (Fig. 2). Interestingly, changes in cellular ribavirin concentration were inversely related to changes in the levels of hemoglobin. Patient 1, with 1,662 μM cellular ribavirin, showed a marked decrease in hemoglobin (-4.8 g dl^{-1}) compared with patient 2, whose cellular ribavirin level was only 929 μM . These obser-

vations are consistent with the hypothesis that marked accumulation of intracellular ribavirin in erythrocytes accelerates hemolysis by reducing erythrocyte life spans (5, 8). We should mention that interferon can also lead to a decrease in hemoglobin.

Cellular/plasma ratios of ribavirin were 146 and 150 in patients 1 and 2, respectively. The ratios were much higher than that reported for a previous study (i.e., 60) by Lertora et al. (11). This discrepancy is due to differences between the present HPLC assay method and the previous radioimmunoassay method. Since the radioimmunoassay procedure did not include a dephosphorylation step, it could not correctly determine the concentrations of phosphorylated metabolites, leading to underestimation of total cellular ribavirin levels.

Concerning the antiviral effects of ribavirin, normalization of the level of alanine aminotransferase and undetectable amounts of HCV RNA were observed with samples from patient 1 but not with samples from patient 2 (data not shown). Jen et al. (10) suggested that higher plasma ribavirin concentrations in treatment week 4 were associated with a higher virologic response rate. However, discussion of the antiviral effects of ribavirin in the present study would be inappropriate because of the small sample size of two patients. We intend to investigate this hypothesis further by studying a larger number of patients.

In conclusion, our HPLC method can be used for routine monitoring of cellular ribavirin disposition in blood. The impact of measuring cellular ribavirin levels to evaluate the effi-

TABLE 2. Steady-state average of plasma and cellular ribavirin concentrations and maximal drop in hemoglobin

Patient	ΔHb^b (g/dl)	Ribavirin concn (μM) ^a	
		Plasma	Cellular (metabolites)
1	4.8	11.4 ± 1.1	$1,662 \pm 50$ (1,430 $\pm$ 31)
2	1.7	6.2 ± 0.8	929 ± 55 (869 $\pm$ 76)

^a Values are means $\pm$ SD.

^b Maximum decrease of hemoglobin from baseline during combination treatment with alpha interferon-2b plus ribavirin.

cacy and toxicity of treatment needs to be clarified in future clinical studies with a large number of patients.

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