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RESEARCH ARTICLE

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Improved HPLC method for the determination of ribavirin concentration in red blood cells and its application in patients with COVID-19

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Abstract

Ribavirin is a synthetic, broad-spectrum antiviral drug. Ribavirin is recommended as an antiviral drug in the *Interim Guidance for Diagnosis and Treatment* (the seventh edition) of COVID-19. The ribavirin levels in red blood cells may be closely related to both its efficacy and adverse drug reactions. In this study, a simple and fast HPLC–UV method was established to determine the concentrations of total ribavirin in the red blood cells of 13 patients with COVID-19. Phosphorylated ribavirin was dephosphorylated by phosphatase incubation to obtain the total amount of ribavirin in red blood cells. The chromatographic column was an Atlantis C_{18}. The recoveries were 85.45–89.05% at three levels. A good linear response was from 1 to 200 μg/ml, with a correlation coefficient of $r^2 = 0.9991$. The concentration of total ribavirin in the red blood cells of the patients ranged from 30.83 to 133.34 μg/ml. The same samples without phosphatase incubation ranged from 4.07 to 20.84 μg/ml. About 85% of ribavirin was phosphorylated in red blood cells. In addition, we observed changes in these patients' hematological parameters and found that the erythrocyte, hemoglobin and hematocrit declined to the lowest levels on the fifth day after discontinuation of ribavirin ($p < 0.05$).

KEYWORDS
anemia, COVID-19, HPLC, red blood cells, ribavirin

1 | INTRODUCTION

Ribavirin (RBV) (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic nucleoside antiviral drug, which has a broad-spectrum antiviral effect by blocking the replication of both DNA and RNA viruses (Bosch et al., 2007; Loustaud-Ratti, 2016). Ribavirin is mainly used against hepatitis C virus or influenza virus in the clinic (Chan-Tack et al., 2009; Slavenburg et al., 2011). When ribavirin enters human cells, it can be phosphorylated to a variety of phosphorylated metabolites, such as ribavirin monophosphate, ribavirin diphosphate and ribavirin triphosphate. Their structures are shown in Figure 1 (Yeh et al., 2003). Owing to the lack of phosphatase in red blood cells, these metabolites cannot be converted back to ribavirin in red blood cells and thus accumulate in the cells (Yeh et al., 2003). Hemolytic anemia is a common adverse reaction of ribavirin. This is because the high levels of phosphorylated metabolites in erythrocyte may reduce erythrocyte integrity by consuming ATP, which would lead to hemolysis (de Franceschi et al., 2000). Several reports have shown that the occurrence of anemia is closely related to the concentration of ribavirin (Homma et al., 2004; Inoue et al., 2006). Therefore, some

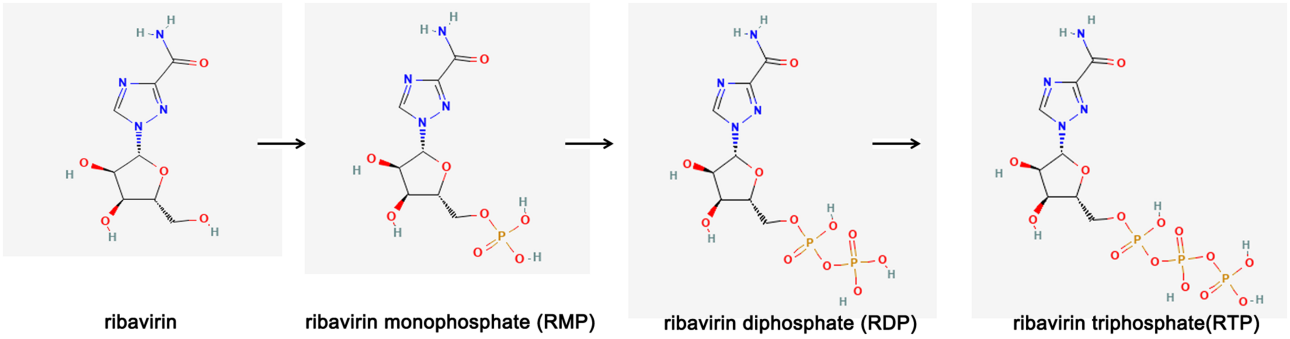

FIGURE 1 Structures of ribavirin and phosporylated metabolites

researchers have monitored the concentration of ribavirin to predict the antiviral effect and the occurrence of adverse reactions (Aguilar Marucco et al., 2008).

Coronavirus disease 2019 (COVID-19) is a global outbreak caused by a novel coronavirus. Currently, there is no effective drug available to treat COVID-19. As a broad-spectrum antiviral drug, ribavirin has been recommended for the treatment of SARS (Vonderen & Bos, 2003), and consequently it attracted the attention of researchers for trials to treat COVID-19. In the *Interim Guidance for Diagnosis and Treatment* (the seventh edition) for COVID-19, ribavirin was also recommended as an antiviral drug (National Health Commission, 2020). At present, there are few studies on ribavirin in the treatment of COVID-19 patients. Understanding ribavirin concentration in red blood cells in patients with COVID-19 is of great significance for treatment, especially in critical patients.

There have been few methods reported for the analysis of ribavirin in human red blood cells. Since ribavirin exists mainly in the form of phosphorylation in red blood cells, some researchers have successfully converted phosphorylated ribavirin back to ribavirin by phosphatase incubation *in vitro* to obtain total ribavirin (Agnesod et al., 2014; D'Avolio et al., 2012; Yeh et al., 2003). Li-Tain Yeh et al. established an LC–MS/MS method for the analysis of ribavirin in red blood cells. In this method, ribavirin was extracted directly by perchloric acid without lysing red blood cells. This may result in inadequate extraction owing to inadequate lysed red blood cells (Yeh et al., 2003). Antonio D'Avolio established a novel pretreatment method by adding a lysis solution, which can fully lyse red blood cells. However, this method was also complicated by the enrichment of ribavirin by vacuum drying (D'Avolio et al., 2012). Up until now, there has been no simple and economical method to determine the concentration of ribavirin in blood cells.

In this study, we first added a lysis solution to lyse the red blood cells. Second, we adopted a small amount of perchloric acid as a protein precipitant instead of acetonitrile, which could avoid the need for a complicated operation such as enrichment. Based on these improvements, a simple and fast pretreatment method coupled with an HPLC–UV system was established to determine the total ribavirin in human red blood cells. Also, we successfully determined the total ribavirin in the red blood cells of 13 patients with COVID-19 using this method. Furthermore, we observed the changes in hematological parameters after ribavirin treatment. These data are of great significance for further understanding the efficacy and adverse reactions of ribavirin in the treatment of patients with COVID-19.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Ribavirin was purchased from the China Food and Drug Research and Testing Institute. Phosphatase was obtained from Macklin. Phosphoric acid was purchased from Tianjin Tianli Chemical Reagent Co. Ltd. Tris–HCL was obtained from Dalian Meilun Biotechonology Co. Ltd. Acetonitrile was purchased from Merck. Potassium dihydrogen phosphate was provided by Sinopharm Chemical Reagent Co. Ltd. Ultrapure water was prepared on a Thermo Scientific Barnstead GenPure Pro Ultrapure water system. All reagents used in this study were analytical grade.

2.2 | Patient

Thirteen patients from February to March 2020 at the First Affiliated Hospital of Harbin Medical University diagnosed with COVID-19 were enrolled in our study. The diagnosis of these patients was based on the *Interim Guidance for Diagnosis and Treatment*. Patients' gender, age, combined medication and dosage were recorded by an experienced researcher. According to the severity of the disease, the course of ribavirin is 2–7 days (500 mg, twice daily). Blood samples were collected 12 h after the last dose of administration of ribavirin. Also, we observed the hematological parameters (RBC, hemoglobin and hematocrit) before and after administration of ribavirin. The detailed data is provided in Table 1. This study was approved by the First Affiliated Hospital of Harbin Medical University Ethical Committee for Medical Research. Informed consent was obtained from all of the patients in this study.

TABLE 1 Medication information and hematological parameters of 13 patients at the baseline

Number	Age	Gender	Dosage	Course (days)	Other antiviral drugs	Erythrocyte (10^{12}/L)	Hemoglobin (g/L)	Hematocrit (%)
Patient 1	66	Female	500 mg b.i.d.	4	Interferon, arbidol	3.25	114	32.9
Patient 2	67	Female	500 mg b.i.d.	2	Interferon, arbidol	3.42	104	30.8
Patient 3	76	Male	500 mg b.i.d.	4	Interferon, arbidol	3.97	120	36.2
Patient 4	62	Male	500 mg b.i.d.	4	Interferon, arbidol	4.68	147	42.6
Patient 5	57	Male	500 mg b.i.d.	4	Interferon, arbidol	4.34	133	39.6
Patient 6	46	Male	500 mg b.i.d.	3	Interferon, arbidol	3.49	118	32.8
Patient 7	60	Male	500 mg b.i.d.	4	Interferon, arbidol	4.45	137	40.8
Patient 8	52	Male	500 mg b.i.d.	2	Interferon, arbidol	3.03	102	34.1
Patient 9	71	Female	500 mg b.i.d.	4	Interferon, arbidol	4.02	115	34.3
Patient 10	23	Male	500 mg b.i.d.	4	Interferon, arbidol	3.65	118	34.4
Patient 11	54	Female	500 mg b.i.d.	3	Interferon, arbidol	3.36	109	30.9
Patient 12	65	Female	500 mg b.i.d.	7	Interferon, arbidol	4.04	137	36.4
Patient 13	61	Female	500 mg b.i.d.	5	Interferon, arbidol	2.97	92	29.4
Mean ± SD	58.46 ± 13.44			3.85 ± 1.28		3.74 ± 0.55	118.92 ± 15.85	35.02 ± 4.01

SD, Standard deviation.

2.3 | Calibration sample and quality control sample

Ribavirin stock solution was prepared in deionized water at a concentration of 2,000 μg/ml. Seven working standard solutions (10, 100, 200, 400, 800, 1,200 and 2,000 μg/ml) were further diluted with deionized water. Quality control (QC) solutions (low, medium and high levels) were prepared in the same way and the concentrations were 100, 1,000 and 1,600 μg/ml, respectively.

Blank blood samples were centrifuged at 4,000 rpm for 5 min at 4°C and the red blood cells were collected. A 45 μl aliquot of red blood cells and 5 μl of standard solution were added to 1.5 ml centrifuge tubes. After mixing, a series of calibration samples (1, 10, 20, 40, 80, 120 and 200 μg/ml) were obtained. The QC samples (10, 100 and 160 μg/ml) were prepared using the same method. All reagents and samples were stored in a freezer at −20°C.

2.4 | Sample preparation

Aliquots of 50 μl of QC solutions or patients' red blood cell samples were added to centrifuge tubes containing 5 mg phosphatase. After that, the following reagents were added to the centrifuge tube: 150 μl Tris–HCL (pH 7.9), 10 μl acetate buffer (pH 4.0) and 50 μl of deionized water. The mixture was vortex mixed for 1 min and then incubated in a 37°C water bath for 1 h. At the end of incubation, 50 μl of 20% perchloric acid was rapidly added to all samples to precipitate the protein. After vortex mixing for 1 min again, the samples were centrifuged at 13,000 rpm for 15 min. The supernatant was transferred to autosampler vials for analysis. During the experiment, the researchers strictly implemented three-level protection, including wearing personal protective clothing, gloves and masks. Also, a negative-pressure laboratory was used to ensure the safety of staff in the clean area.

2.5 | Chromatographic condition

The determination of ribavirin was performed using high-performance liquid chromatography (Thermo Ultimate 3,000) coupled with ultraviolet detection (HPLC–UV). The detection wavelength was 207 nm. The column was an Atlantis C_{18} column (150 × 4.6 mm, Waters). Pump A was a buffer containing 0.05 mol/L KH_2PO_4 (adjusted pH = 4.2 by phosphoric acid). Pump B was pure acetonitrile. The elution mode was adopted with a flow rate of 1 ml/min. The gradient was as follows: 0–5 min, A–B (100:0); 5.1–8 min, A–B (70:30); 8.1–12 min, A–B (100:0). The column oven was maintained at 40°C. The injection volume was 40 μl.

2.6 | Method validation

2.6.1 | Specificity

The specificity was accessed by analyzing six different sources of blank samples and the corresponding samples spiked with ribavirin at the lower limit of quantification (LLOQ). The resulting chromatograms of the samples were compared to observe whether there were interference peaks around the ribavirin peak.

2.6.2 | Standard curve and lower limit of quantification

Standard curves were constructed by the linear regression analysis of seven calibration samples by ploting the peak area of ribavirin (y) against the nominal concentration of ribavirin (x). The standards were run and curves ($n = 6$) were obtained for several days during the

experiment. The LLOQ was determined by five replicate samples as the lowest concentration of the standard curve with a signal-to-noise ratio of at least 10.

2.6.3 | Accuracy and precision

The inter-day and intra-day precision and accuracy were evaluated by the determination of five batches of QC samples of three levels (low, medium and high levels) for three consecutive days. The samples measured on 1 day were used to calculate intra-day precision and accuracy. The samples measured on three consecutive days were used to for calculate intra-day precision and accuracy. Values of the RSD for precision and bias for accuracy within 15% were considered acceptable.

2.6.4 | Recovery

The recovery was determined by comparing the peak areas of the extracts obtained from QC samples (low, medium and high levels) with those obtained by analyzing samples spiked post-extraction.

2.6.5 | Stability

The stability of ribavirin in red blood cells were evaluated by analyzing three levels (low, medium and high levels) of QC samples after being placed under the following conditions: during 8 h at room temperature for short-term stability; repeated freezing and thawing for three cycles for freeze–thaw stability; and stored at −80°C for 30 days for long-term stability. Ribavirin is considered stable under different conditions when the RSD is <15%.

3 | RESULTS

3.1 | Specificity

The typical chromatograms of different samples are shown in Figure 2. No interfering peaks were observed at the retention time of the ribavirin. This assay presents excellent specificity.

3.2 | Standard curve, lower limit of quantification

This method exhibited excellent linear response over the concentration range of 1–200 μg/ml by weighted ($1/x^2$) least-squares linear regression analysis. A typical equation of the calibration curve is: $y = 0.8192x + 17.785$ ($R^2 = 0.9991$), where y represents the peak area of ribavirin and x represents the concentration of ribavirin. The LLOQ was 1.0 μg/ml in this method.

3.3 | Accuracy and precision

The precision and accuracy data are shown in Table 2. The RSDs of precision for the low and high levels were <6.03 and 4.26%, respectively. All values of the RSD and the bias for accuracy were <15%. This method presents good accuracy and precision.

3.4 | Recovery

The mean recoveries of the assay for ribavirin in low, mediam and high QC samples were 89.05, 88.67 and 85.45%, respectively. The RSD of each level was ≤6.04%, indicating that there was no significant loss of ribavirin during the extraction and analysis procedure.

3.5 | Stability

There were no significant decreases in the three different conditions (room temperature for 8 h, stored at −40°C for 30 days and three freeze–thaw cycles). The stability data are shown in Table 3. The RSD % of each level was ≤7.56%. These data indicated that ribavirin is relatively stable under different conditions.

3.6 | Clinical application

We measured total ribavirin concentrations in the red blood cells of 13 patients with COVID-19. The total ribavirin concentrations in red blood cell were in the range from 30.83 to 133.34 μg/ml. The ribavirin concentrations in the same samples without phosphatase incubation were in the range from 4.07 to 20.84 μg/ml. About 85% of ribavirin was stored in the red blood cells as phosphorylated.

In the hematological parameter study, we found that the erythrocyte, hemoglobin and hematocrit declined to the lowest levels at day 5 after discontinuation of ribavirin ($p < 0.05$), as shown in Figure 3. On day 10 after discontinuation of ribavirin, the hematological parameters recovered somewhat, and they seemed to return to normal on day 20 after discontinuation. A typical patient's hematological parameter changes are shown in Figure 3(d–f).

4 | DISCUSSION

To better understand the distribution and concentration of total ribavirin in red blood cells of COVID-19 patients, a simple and fast HPLC–UV method was developed and validated to determine the ribavirin concentration in red blood cells. It has been reported that ribavirin exists mainly in the form of phosphorylation in red blood cells. The total ribavirin in erythrocytes includes the sum of phosphorylated ribavirin and unphosphorylated ribavirin in erythrocytes. In order to obtain the total ribavirin, we added phosphatases to the pretreatment

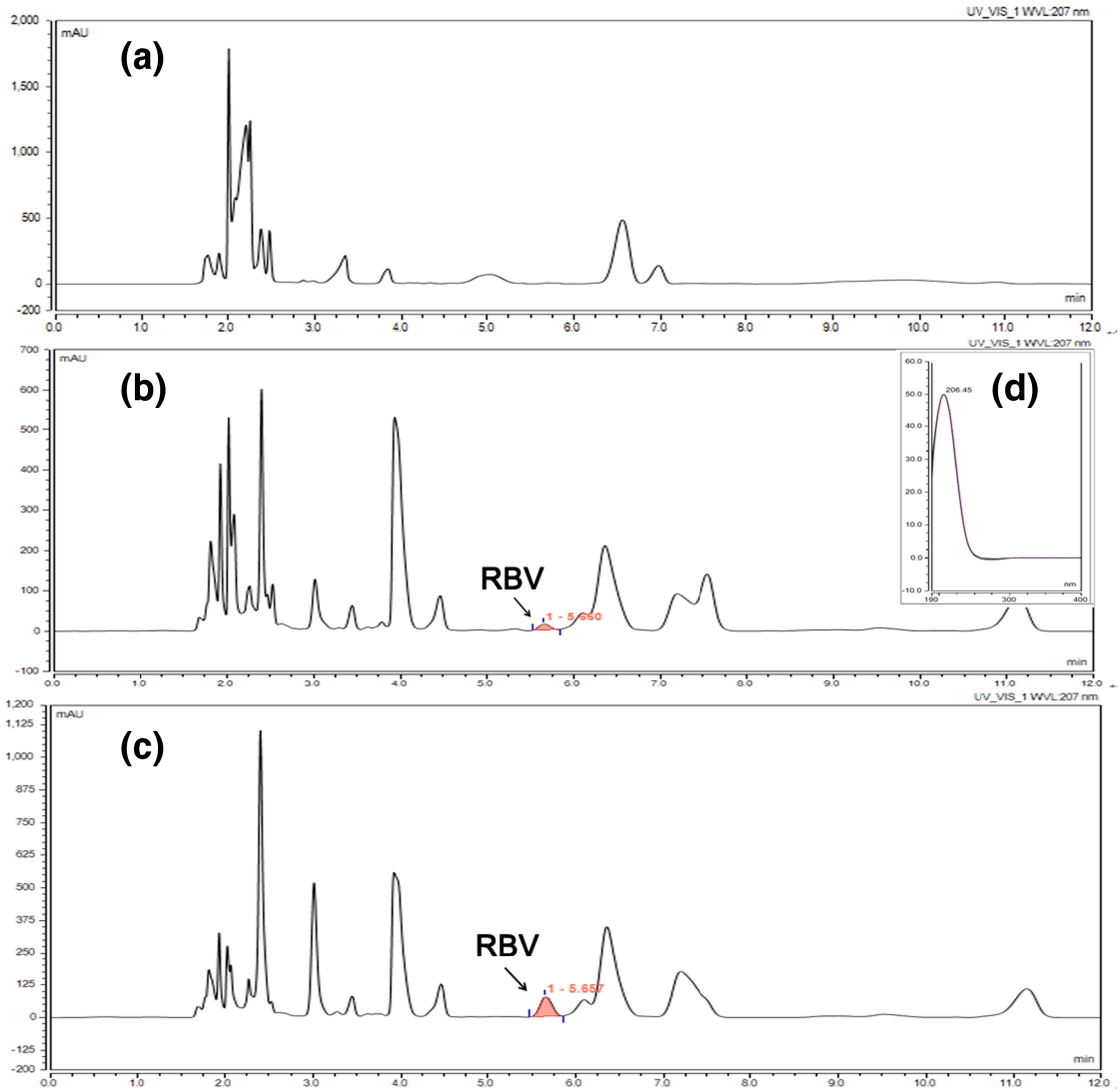

FIGURE 2 Representative chromatograms of ribavirin for (a) a blank red blood cell sample, (b) a blank red blood cell sample spiked with ribavirin at the lower limit of quantitation and (c) a red blood cell sample from a patient injected with ribavirin (RBV). (d) The ultraviolet absorption spectrum of ribavirin

TABLE 2 Intra-day and inter-day precision and accuracy of the approach for the determination of ribavirin in red blood cells

	Intra-day		Inter-day	
Norminal concentration (μg/ml)	RSD (%)	Accuracy bias (%)	RSD (%)	Accuracy bias (%)
1	7.47	6.37	9.26	−5.69
10	4.59	−4.51	6.03	6.89
100	3.36	−3.26	2.91	−4.21
160	4.26	2.69	3.84	5.38

RSD, Relative standard deviation. Bias (%) = [(concentration measured/concentration nominal) × 100%] − 100%.

system to convert the phosphorylated ribavirin back to ribavirin. In order to obtain the maximum conversion rate of phosphorylated ribavirin to ribavirin prototype, we randomly selected three patient samples and incubated each sample with different doses of phosphorylase (2, 5 and 10 mg). The data showed that there was no significant difference between the peak area of ribavirin incubated with 5 mg of phosphatases and the peak area of ribavirin incubated with 10 mg of phosphatases. However, the area of ribavirin incubated with 2 mg phosphatase was less than that of the samples incubated with 5 or 10 mg of phosphatase. This indicated that 5 mg of phosphorylase could convert most of the phosphorylated ribavirin to the ribavirin prototype. Therefore, we chose 5 mg of phosphorylase as the

TABLE 3 Stablilty of ribavirin under different conditions at three quality control levels

	Room temperature		Long-term		Freeze–thaw	
Norminal concentration (μg/ml)	RSD(%)	Accuracy bias(%)	RSD(%)	Accuracy bias(%)	RSD(%)	Accuracy bias(%)
10	7.56	−8.26	5.19	−5.92	5.98	4.89
100	6.35	−5.64	4.26	6.54	6.27	−3.65
160	7.27	2.36	5.16	−3.47	5.80	2.16

RSD, Relative standard deviation. Bias(%) = [(concentration measured/concentration nominal) × 100%] − 100%.

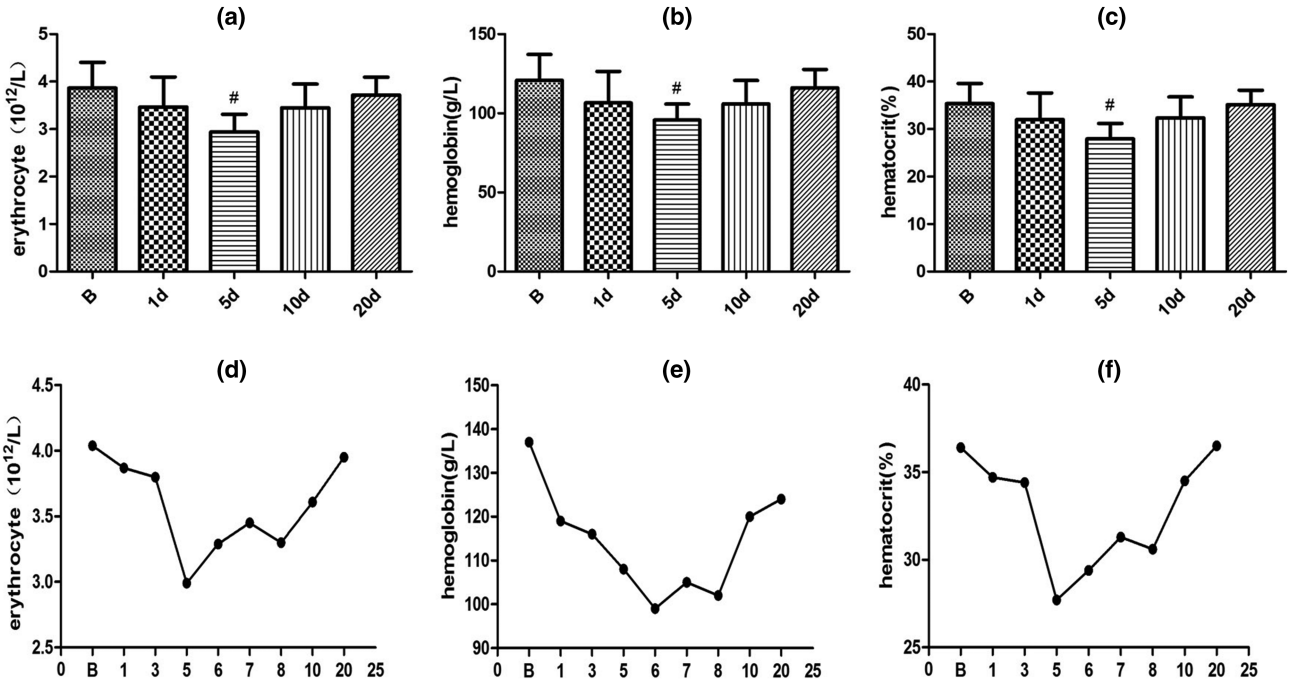

FIGURE 3 (a–c) The mean values of erythrocyte, hemoglobin and hematocrit of 13 patients with COVID-19 on different days after discontinuation of ribavirin ($n = 13$). (d–f) The changes in erythrocytes, hemoglobin and hematocrit of a typical patient with COVID-19. $^{\#}p < 0.05$ vs. B (baseline)

optimum incubation dose. Li-Tain Yeh et al. adopted two-step extraction with perchloric acid to improve the extraction rate of ribavirin (Yeh et al., 2003). However, we believed that this approach is complex and may result in inadequate ribavirin extraction owing to the incomplete lysis of red blood cells. Antonio D'Avolio et al. developed a method that can fully lyse red blood cells adequately by adding a lysis solution (such as Tris–HCL buffer; D'Avolio et al., 2012). In this method, a large amount of acetonitrile was used to precipitate protein, which resulted in a need for enrichment of ribavirin by vacuum drying. Based on that, we tried a small amount of perchloric acid instead of acetonitrile to precipitate proteins. This strategy avoided complex processes such as drying in a vacuum centrifuge. The specificity of this method was so good that there was no interference peak around ribavirin. The accuracy and precision of this improved method well satisfied the requirements of pharmaceutical analysis in biological samples.

We used this method to determine the total ribavirin concentration in red blood cells of 13 COVID-19 patients treated with ribavirin. The total ribavirin concentration ranged from 30.83 to 133.34 μg/ml. The unphosphorylated ribavirin concentration ranged from 4.07 to 20.84 μg/ml. Approximately 85% of ribavirin in red blood cells is phosphorylated. This proportion was consistent with previous reports in 2004 (Homma et al., 2004). At present, most studies of ribavirin concentrations have been in hepatitis C patients. It has been reported in the literature that the concentration of total ribavirin in red blood cells was 199–557 μg/ml (Yeh et al., 2003), which was higher than in our study. This may be caused by different courses and dosages of administration of ribavirin. Also, the ribavirin concentration in our study may not reach steady state (required for 3–4 weeks; Homma et al., 2004).

It has been reported that anemia appeared around 14 days after ribavirin administration (Agnesod et al., 2014). However, we found that the erythrocytes, hemoglobin and hematocrit declined to the lowest at day 5 after discontinuation of ribavirin. This may be because the time points in our study started after discontinuation of the ribavirin (did not include the period of medication). Also, the combination of other antiviral drugs (such as interferon) may accelerate the occurance of anemia. Unfortunately, we found no significant correlation

between ribavirin concentration and the decrease in these hematological parameters. The total ribavirin concentration ranged from 30.83 to 133.34 μg/ml. This could be explained by the low value of ribavirin concentration in our study. According to a report, a concentration of ribavirin >1,000 μmol/L (equal to 244 μg/ml) is associated with anemia (Homma et al., 2004).

It should be noted that since COVID-19 is a new disease, there was no conclusive evidence that ribavirin is effective for COVID-19. The patients enrolled in our study were critical patients with COVID-19 and the treatment was complicated. In our observation, there were no obvious effect after administration with ribavirin in these patients. This may also be due to the low concentration of ribavirin and short course of administration, which did not achieve the therapeutic blood concentration range. It should be noted that adverse reactions (such as anemia) are prone to occur during treatment, especially in critical patients. Supportive treatment should be given during hospitalization. These data in this study are of great significance for further understanding the efficacy and adverse reactions of ribavirin in the treatment of patients.

In summary, the contributions of this article are as follows: first, a very simple and fast HPLC–UV method was established for the determination of ribavirin in human red blood cells. Second, the range of total ribavirin concentrations in red blood cells after short-term intravenous administration (500 mg twice a day, 2–7 days) was indicated in our study. Third, changes in hematologic parameters after ribavirin injection in 13 patients were observed. These findings will further enhance our understanding of the antiviral drug ribavirin.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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