A Sensitive and Specific Method for the Determination of Total Ribavirin in Human Red Blood Cells by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

A sensitive and specific method using high-performance liquid chromatography (LC)–tandem mass spectrometry (MS) for the analysis of total ribavirin in human red blood cells (RBC) is developed and validated. The method involves the addition of an internal standard and perchloric acid, the conversion of ribavirin phosphorylated metabolites to ribavirin, purification with a solid-phase exchange cartridge, and LC–MS–MS analysis. The MS–MS is selected to monitor *m/z* 245–113 for ribavirin and *m/z* 250–113 for [^{13}C]ribavirin using positive electrospray ionization. The calibration curve is linear over a concentration of 100–10,000 ng/mL with a limit of quantitation of 100 ng/mL. Mean interassay accuracy for quality control (QC) at 100, 1000, and 10,000 ng/mL are 101.8%, 99.4%, and 98.8%, respectively. Mean interassay precision (%CV) for QC at 100, 1000, and 10,000 ng/mL are 5.0%, 5.0%, and 2.5%, respectively. Extractibility of total ribavirin from RBC is confirmed with RBC obtained from a [^{14}C]ribavirin-dosed monkey. The method is used to determine the free and total ribavirin concentration in human RBC obtained from hepatitis C patients treated with ribavirin.

Introduction

Ribavirin (1-β-D-ribofuransoyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analog first synthesized by Witkowski et al. in 1972 (1). It was reported to have broad-spectrum activity against a variety of DNA and RNA viruses in 1972 (2,3). Ribavirin/pegylated interferon-alfa combination has been widely used for the treatment of chronic hepatitis C disease (4). Intracellularly, ribavirin is phosphorylated to ribavirin monophosphate (RMP), ribavirin diphosphate (RDP), and ribavirin triphosphate (RTP) (Figure 1). This has been confirmed by in vitro studies in various cells using [^{3}H]ribavirin or [^{14}C]ribavirin (5–7). Red blood cells (RBCs) have the capacity to phosphorylate ribavirin to RMP, RDP, and RTP, but they are devoid of phosphatase activity to convert them back to ribavirin (4). As a result, high levels of phosphorylated ribavirin accumulate over time leading to hemolytic anemia (4,7). This RBC toxicity presents a significant challenge in clinical management of ribavirin dose levels. To better understand the correlation between total ribavirin (ribavirin, RMP, RDP, and RTP) concentration in RBC and the occurrence of hemolytic anemia, a method has been developed and validated to determine the total ribavirin concentration in human RBC.

Experimental

Chemicals and materials

Ribavirin was supplied by ICN Pharmaceuticals (Costa Mesa, CA). [^{13}C]Ribavirin (1-β-D-[1'-^{13}C, 2'-^{13}C, 3'-^{13}C, 4'-^{13}C, 5'-^{13}C]-ribofuransoyl-1,2,4-triazole-3-carboxamide), [^{14}C]ribavirin (54 mCi/mmole), and [^{3}H]ribavirin triphosphate (12 Ci/mmole) were synthesized by Moravek Biochemicals (Brea, CA). Blank human RBC (male/female pooled) with ethylenediame tetraacetic acid as the anticoagulant was purchased from Bioreclamation (East Meadow, NY) or harvested from freshly collected human blood. Acid phosphatase was purchased from Sigma (St. Louis, MO). The NH_2 solid-phase extraction (SPE) cartridges were purchased from Supelco (Bellefonte, PA). All other solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

High-performance liquid chromatography–radioactivity detector conditions

The high-performance liquid chromatography (HPLC) radioactivity detector used to study the conversion of RTP to ribavirin consisted of two Shimazdu LC-10AD pumps (Shimazdu,

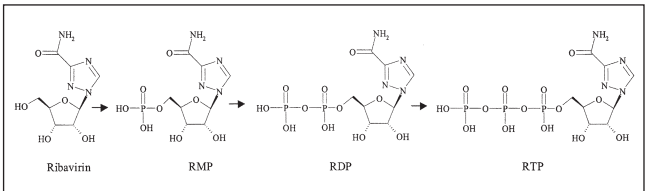

Figure 1. Structures of ribavirin and phosporylated metabolites.

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Columbia, MD), a Shimazdu SIL-10A autosampler, and an IN/US radioactivity detector (IN/US System, Tampa, FL). A 4.6- × 250-mm, 5-µm diethylaminoethyl (DEAE) column (TosoHaas, Montgomeryville, PA) was used to separate ribavirin, RMP, RDP, and RTP at a flow rate of 1 mL/min. The liquid chromatography (LC) conditions are summarized in Table I.

Under these conditions, good separation was obtained between ribavirin, RMP, RDP, and RTP. However, one metabolite, 1,2,4-triazole-3-carboxamide ($TCONH_2$), coeluted with ribavirin. To confirm that ribavirin was not further converted to $TCONH_2$ under the conditions of enzyme digestion, the sample was analyzed on a second HPLC column. This analysis was performed using a Devosil C30 column (4.6 × 250 mm, 5 µm) (Supelco). An isocratic run using 100mM ammonium phosphate (pH = 2.4) at a flow rate of 1.0 mL/min provided adequate separation between ribavirin and the metabolite.

LC–tandem mass spectrometry conditions

The LC–mass spectrometry (MS)–MS system used to validate the method consisted of two Shimazdu LC-10AD pumps, a PerkinElmer 200 autosampler (PerkinElmer, Norwalk, CT), and a SCIEX API3000 mass spectrometer (Applied Biosystems, Foster City, CA). A Zorbax SB-C18 column (4.6 × 150 mm, 3.5 µm, Agilent Technology, Palo Alto, CA) was used in the analysis. The LC and MS–MS conditions are summarized in Tables II and III. The product ion spectra of ribavirin and [^{13}C]ribavirin are presented in Figure 2.

Preparation of calibration and quality control samples

Calibration standards ranging from 100 to 10,000 ng/mL at six concentration levels were prepared by spiking ribavirin standards into human RBC blanks. The standards were prepared using the sample preparation procedure given below. Quality control (QC) samples at concentration levels of 100, 1000, and 10,000 ng/mL were prepared by spiking ribavirin standards into human RBC blanks. These QC samples were stored at –20°C until analysis. Aliquots of the QC samples (100 µL) were transferred into separate vials on the day of sample preparation for later use.

Table I. HPLC Gradient Conditions for Radioactivity Analysis

Time (min)	%A*	%B†
0	100	0
30	0	100
32	100	0
35	100	0

* A, 100mM ammonium phosphate–acetonitrile (80:20), pH = 3.0.
† B, 250mM ammonium phosphate–acetonitrile (80:20), pH = 3.5.

Table II. HPLC Gradient Conditions for LC–MS–MS Analysis

Time (min)	%A*	%B†
0.0	90	10
1.0	90	10
2.5	70	30
5.5	10	90
6.4	10	90
6.5	90	10
7.5	90	10

* A, 0.1% acetic acid in water.
† B, 0.1% acetic acid in methanol.

Sample preparation

A solution of [^{13}C]ribavirin internal standard (300 µL, 1.0 µg/mL in water) and perchloric acid (200 µL, 12.5% v/v) were added to 100 mL of human RBC. The sample was vortexed briefly

Table III. MS–MS Conditions for Ribavirin Analysis

Compounds	m/z Q1	m/z Q3	Time (ms)	Collision energy (V)
Ribavirin	245	113	150	15
[^{13}C]Ribavirin	250	113	150	15

Interface	Turbo ion spray
Polarity	Positive
Scan type	Multiple reaction monitoring
Resolution	Q1–unit, Q3–low
Curtain gas	12
Collision gas	3
Ionspray voltage (IS)	5500 V
Temperature	450°C
Ion source gas 1	10
Ion source gas 2	6.5 L/min
Solvent split ratio	240 µL into interface

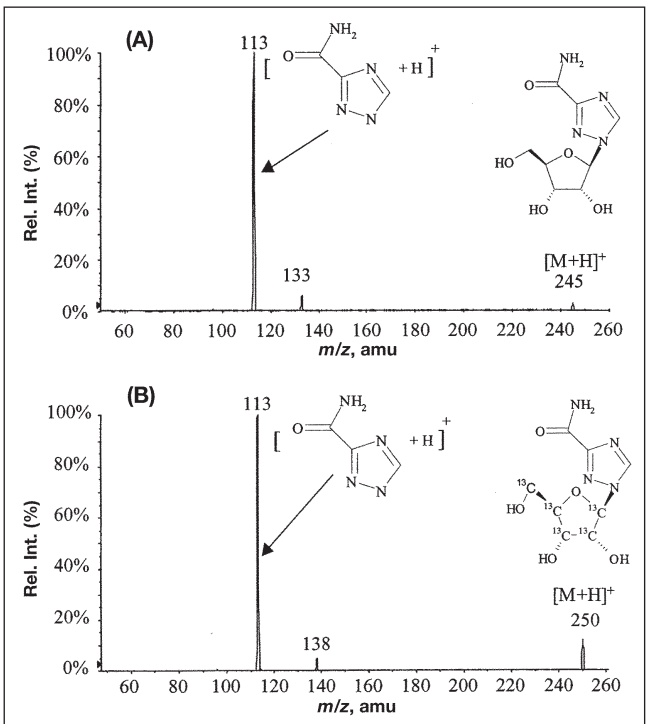

Figure 2. Product ion spectra of (A) ribavirin and (B) [^{13}C]ribavirin.

and centrifuged at 14,000 rpm for 10 min. The extract was transferred to a clean 13- × 100-mm tube. The pellet was extracted with additional water (150 µL) and perchloric acid solution (100 µL, 12.5% v/v) to improve the extraction efficiency. Duplicate extractions were combined, and a portion of the combined extract (450 µL) was transferred to a clean microcentrifuge tube. The pH of this solution was adjusted to approximately 4.8 by adding a solution of ammonium acetate–ammonium hydroxide. Enzyme digestion to convert all phosphorylated metabolites to ribavirin was accomplished by adding acid phosphatase (5 µL, 0.8 unit/µL) to the sample and incubating at 37°C for 1 h. After digestion, the resulting mixture was then purified using an NH_2 SPE cartridge. The final extract was analyzed by LC–MS–MS to quantitate the level of ribavirin in human RBC.

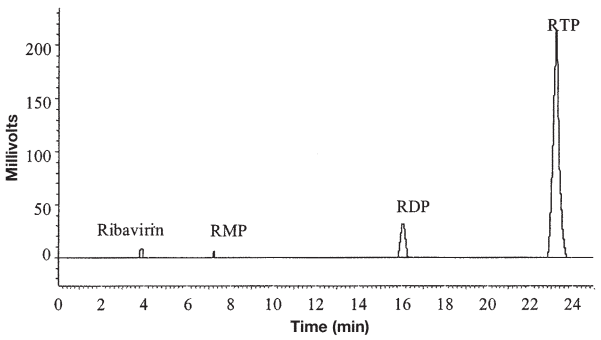

Figure 3. Radiochromatogram of [^{3}H]ribavirin triphosphate standard using a DEAE column.

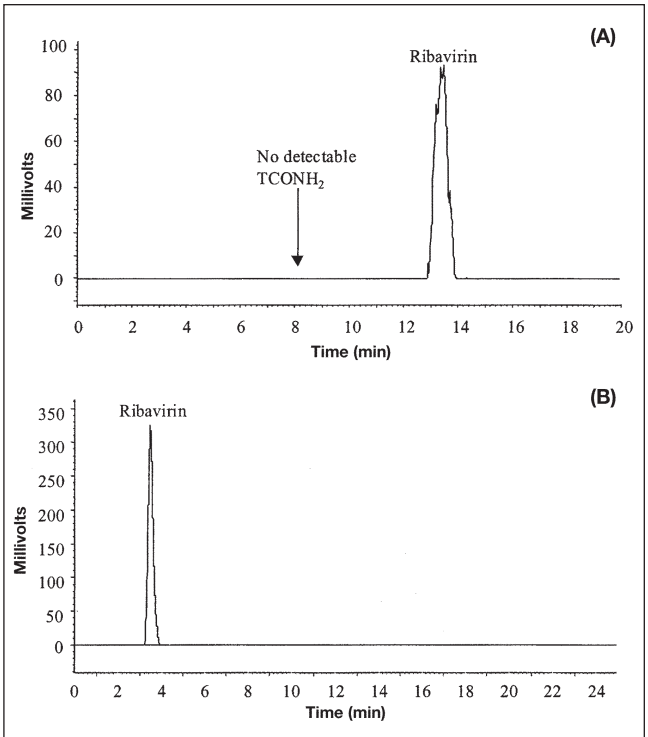

Figure 4. Radiochromatograms of [^{3}H]ribavirin triphosphate in human RBC incubated at 37°C for 1 h. (A) Devosil column analysis and (B) DEAE column analysis.

Results and Discussion

A method has been developed to determine the concentration of total ribavirin (ribavirin, RMP, RDP, and RTP) using LC–MS–MS. In this method, ribavirin and its phosphorylated metabolites are extracted from human RBC, and all phosphorylated metabolites are subsequently converted to ribavirin using

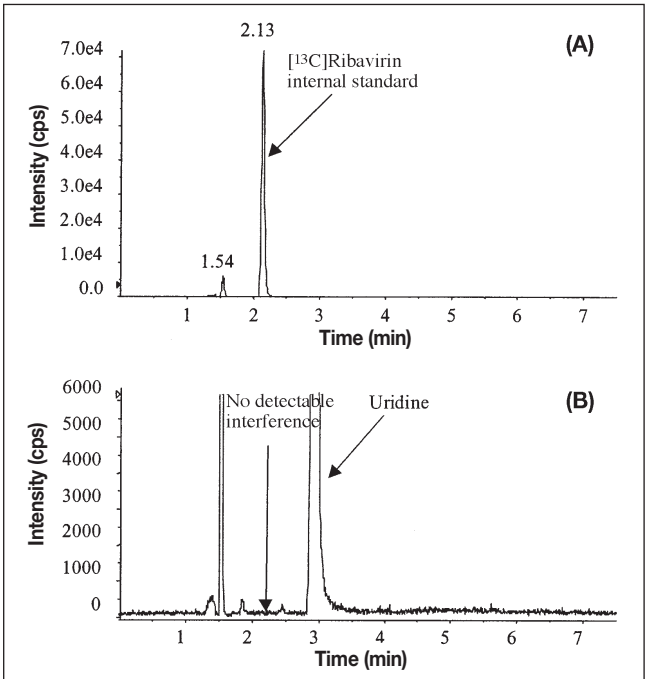

Figure 5. Typical extracted ion chromatograms of RBC blank extract (A) [^{13}C]ribavirin, *m/z* at 250–113 and (B) ribavirin, *m/z* at 245–113.

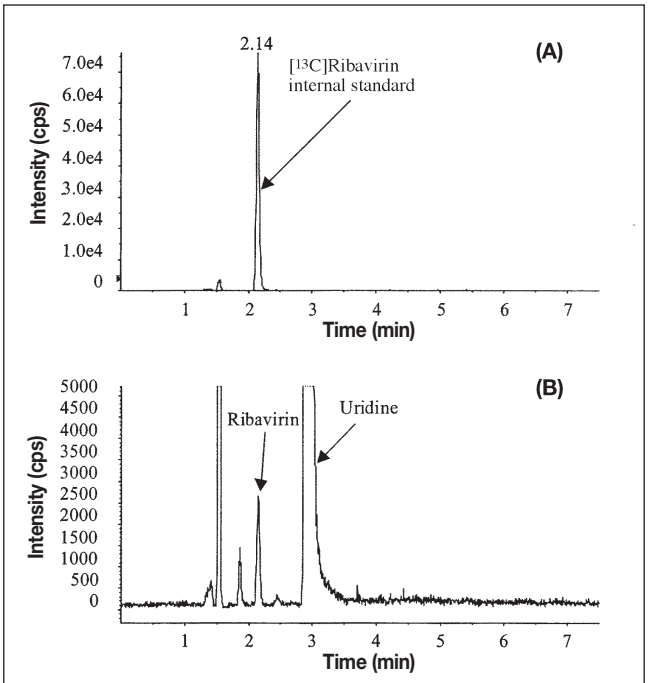

Figure 6. Typical extracted ion chromatograms of RBC limit of quantitation (100 ng/mL). The extracts were (A) [^{13}C]ribavirin, *m/z* at 250–113 and (B) ribavirin, *m/z* at 245–113.

acid phosphatase. The final extract is then purified using a NH_2 SPE cartridge and analyzed by LC–MS–MS. The extraction efficiency, enzyme conversion efficiency, selectivity, sensitivity, standard curve linearity, accuracy, precision, and stability of the method have been examined.

Extraction efficiency

Because there are no radioactive human RBC samples available, the extraction efficiency was determined by extracting RBC samples from a monkey dosed with [^{14}C]ribavirin. The total radioactivity extracted was compared with the value obtained from the digested value. The results indicate that more than 98% of the total radioactivity was extracted using the duplicate extraction method.

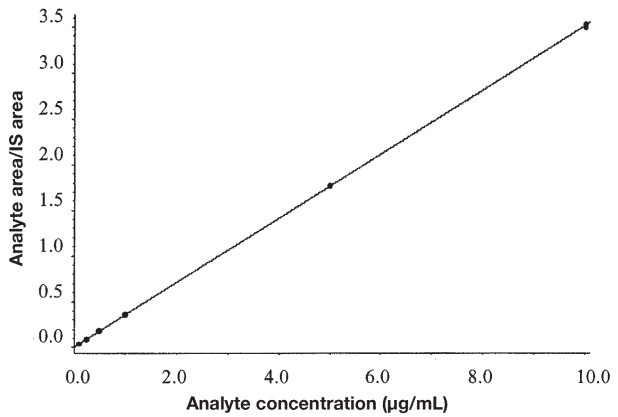

Figure 7. Typical calibration curve for the determination of ribavirin in human RBC. Linear equation, $Y = 0.00035X + 0.00332$; correlation coeffcient (r), 0.9999; and weighing, 1/X. IS = internal standard.

Table IV. Calibration Curve Analytical Results for Ribavirin in Human RBC

	Concentration (ng/mL)					
Curve ID	100	250	500	1000	5000	10,000
Day 1 curve 1	97.6	280	439	1000	5220	9810
Day 1 curve 2	111	259	475	910	4830	10,300
Day 1 curve 3	103	261	476	960	5060	9990
Day 1 curve 4	104	253	485	938	5320	9750
Day 1 curve 5	83.6	259	552	N/A	5280	9670
Day 2 curve 1	95.9	271	482	997	4940	10,100
Day 2 curve 2	104	247	491	995	4900	10,100
Day 2 curve 3	100	254	495	1000	4880	10,100
Day 2 curve 4	106	252	487	964	4900	10,100
Day 2 curve 5	98.9	256	491	1010	4900	10,100
Mean	100.4	259	487	974	5023	10,002
SD	7.4	9.7	27.7	34.1	183.9	196.5
%CV	7.4	3.8	5.7	3.5	3.7	2.0
n	10	10	10	9	10	10
%Bias	0.4	3.7	–2.5	–2.5	0.46	0.02

* SD, standard deviation and CV, coefficient of variation.

Enzyme conversion

Enzyme conversion efficiency was determined by spiking [^{3}H]ribavirin triphosphate in 10,000-ng/mL QC samples and converting it to ribavirin following the sample preparation procedure. The final extract was analyzed with an LC–radioactivity detector to confirm the total conversion of RTP to ribavirin. As presented in Figures 3 and 4, RTP was converted to ribavirin successfully after the incubation, and no additional metabolites were formed during the incubation process.

Separation, selectivity, and sensitivity

Analysis of RBC blanks showed no interference in the final extract, although there were several endogenous peaks exhibited the same mass-to-charge ratio transition at different retention times. Uridine was the major endogenous peak (retention time at ~ 3 min). The identities of the other peaks were not known. A

Table V. QC Analytical Results for Ribavirin in Human RBC

Set	Low QC (100 ng/mL)	Mid QC (1000 ng/mL)	High QC (10,000 ng/mL)
1	99	1090	9410
	107	947	10,100
	104	968	9860
Mean	103.3	1001.7	9790.0
SD	4.0	77.2	350.3
%CV	3.9	7.7	3.6
%Bias	3.3	0.17	–2.1
2	93.5	989	9850
	106	971	9940
	101	1000	10,100
Mean	100.2	986.7	9963.3
SD	6.3	14.6	126.6
%CV	6.3	1.5	1.3
%Bias	0.17	–1.3	–0.37
Overall			
Mean	101.8	994.2	9876.7
SD	5.0	50.4	254.0
%CV	5.0	5.0	2.5
%Bias	1.8	–0.58	–1.2

Table VI. Long-Term Storage Stability of Ribavirin in Human RBC

Set	Low QC (100 ng/mL)	Mid QC (1000 ng/mL)	High QC (10,000 ng/mL)
Day 1	97.1	1030	10,100
	101	1000	9980
Mean	99.1	1015	10,040
Day 1.5	96.4	940	9710
	104	914	9590
	103	926	9470
Mean	101	327	9590

typical chromatogram of a RBC blank extract is presented in Figure 5. The result indicates that the method provides adequate separation and selectivity through HPLC separation and LC–MS–MS detection. The method provides acceptable sensitivity for the compound of interest. Typical low QC has a signal-to-noise ratio greater than 10 in the validation. Typical chromatograms for the low-QC sample are presented in Figure 6.

Standard curve linearity

For the linear regression analysis, the correlation coefficient (*r*) was greater than 0.998 for the calibration curve determination during the method validation. This indicates linearity of the detector response as a function of the standard calibration curve. A representative calibration curve is presented in Figure 7. Mean back-calculated values from the fitted curve were within 5% of their nominal values between 100 and 10,000 ng/mL (Table IV).

Accuracy and precision

Accuracy and precision were determined based on low-, mid-, and high-QC samples. Mean intra-assay recoveries were within ± 4% of their nominal values and the percent of the mean interassay precision (%CV) varied between 1.3% and 7.7%. Mean interassay recoveries were within ± 2% of their nominal values, and mean interassay precision (%CV) varied between 2.5% and 5.0%. The recovery data and statistics are presented in Table V.

Overcurve dilution analysis

Overcurve dilution was determined by diluting 300-µg/mL QC samples by a factor of 33. The diluted samples were processed and analyzed as described in the method. The mean recovery and mean interassay precision (%CV) were 291.2 µg/mL and 2.2%, respectively. This result indicates that over the curve dilution can be achieved for high-concentration samples.

Long-term storage stability of ribavirin in RBC

Long-term storage stability of ribavirin in RBC was determined by analyzing the QC samples on the day of preparation and after 5 months storage at –20°C. Ribavirin appeared to be stable after being stored at –20°C for 5 months. Detailed results are presented in Table VI.

Overnight bench stability

Table VII. Short-Term Matrix Stability Analytical Results for Ribavirin in Human RBC at a Concentration of 100 ng/mL

	25°C for 1.5 days (ng/mL)	Three feeze/thaw cycles (ng/mL)
	83.8	93.8
	90.6	86.7
	107	87.2
Mean	93.8	89.2
SD	11.9	4.0
%CV	12.7	4.4
%Bias	–6.20	–10.8

Overnight bench stability was determined by leaving the low-QC samples (100 ng/mL) on the bench for 1.5 days before sample processing. After 1.5 days at room temperature, the mean recovery of the low QC was 93.8 ng/mL. The results indicate that ribavirin left at room temperature for 1.5 days showed no significant degradation. Detailed results are presented in Table VII.

Freeze and thaw stability

Freeze and thaw stability was determined by freezing and thawing the low-QC samples (100 ng/mL) for three cycles before sample processing. The mean recovery of the low QC was 89.2 ng/mL, indicating ribavirin was stable for at least three freeze and thaw cycles without significant degradation. Detailed results are presented in Table VII.

Bench stability of the final extract

Overnight bench stability of the final extract was determined by reanalyzing the QC samples from the day 1 method validation set with the day 2 validation set. The results indicate that ribavirin is stable in the final extract solution. Detailed results are presented in Table VIII.

Stability of RTP in RBC

The stability of ribavirin nucleotides, particularly RTP, in human RBC was not studied in this laboratory because of the lack of radioactive human RBC samples. However, RBC samples from monkeys dosed with [^{14}C]ribavirin showed that RTP was predominant in freshly collected RBC, and RMP was the most abundant component followed by RDP in RBC after 6 months storage at –70°C. Theodore et al. demonstrated that RMP was the dominant component in aging or damaged erythrocytes after incubating at 37°C for 48 h, and human erythrocytes showed no hydrolytic activity for the conversion of RMP to ribavirin (7). These findings indicate that the compositions of RMP, RDP, and RTP could vary based on extraction and storage conditions and present a challenge to metabolism profiling. However, the method described

Table VIII. Bench Stability of Final Extract

Time	Low QC (100 ng/mL)	Mid QC (1000 ng/mL)	High QC (10,000 ng/mL)
Day 1	99	1090	9410
	107	947	10,100
	104	968	9860
Mean	103.3	1001.7	9790.0
SD	4.0	77.2	350.3
%CV	3.9	7.7	3.6
%Bias	3.3	0.17	–2.1
Day 1.5	113	973	9560
	110	1000	9550
	100	1040	10,000
Mean	107.7	1004.3	9703.3
SD	6.8	33.7	257.0
%CV	6.3	3.4	2.7
%Bias	7.7	0.43	–3.0

here is designed to quantitate total ribavirin, thus was not affected by the relative composition of RMP, RDP, and RTP.

Patient sample analysis

Whole blood samples drawn from 10 patients receiving ribavirin at 600 mg/twice daily treatment were analyzed to determine total ribavirin concentration. In addition, the samples were also analyzed without the enzyme digestion steps to determine the concentration of free ribavirin in RBC. The results for the patient's samples and QC samples are presented in Tables IX and X. The level of total ribavirin in RBC ranged from approximately 649–1402µM. The levels of free ribavirin in RBC ranged from 8.0 to 25.6µM. This result indicates that more than 96% of ribavirin was present as phosphorylated metabolites inside RBC.

Conclusion

A method has been developed and validated for the determination of ribavirin in human RBC. The calibration curve was linear over a concentration of 100–10,000 ng/mL with an limit of quantitation of 100 ng/mL. Mean interassay accuracy for QC at 100, 1000, and 10,000 ng/mL were 101.8%, 99.4%, and 98.8%, respectively. Mean interassay precision (%CV) for QC at 100, 1000, and 10,000 ng/mL were 5.0%, 5.0%, and 2.5%, respectively. An over-curve dilution for QC at 300 µg/mL was validated to expand the analysis range. An enzyme digestion step has been added to convert all phosphorylated metabolites to ribavirin for final quantitation. This step proved to be critical when analyzing clinical samples. From the analytical results of ten patient samples, high concentrations of total ribavirin (ribavirin, RMP, RDP, and RTP) were observed (649–1402µM). However, only a small percentage of the ribavirin (8.0–25.6µM) was present as the free form, indicating that more than 96% of the ribavirin was present as phosphorylated metabolites.

Table IX. RBC Analysis for Ribavirin in Patient Samples

Patient ID	Free ribavirin (µM) (A)	Total ribavirin (µM) (B)	Ratio (%) (A:B)
A	25.6	818.0	3.1
B	8.0	1295.0	0.6
C	11.8	727.0	1.6
D	8.0	1401.6	0.6
E	20.6	1213.2	1.7
F	13.2	991.8	1.3
G	15.4	672.2	2.3
H	15.4	649.2	2.4
I	14.0	1237.8	1.1
J	12.2	777.4	1.6

Table X. QC Analytical Results for Patient Sample Analysis

	Low QC (100 ng/mL)	Mid QC (1000 ng/mL)	High QC (10,000 ng/mL)
	87.4	910	9120
	91.0	969	9030
	90.7	888	9100
Mean	89.7	922	9083
SD	2.0	41.9	47.3
%CV	2.2	4.5	0.52
%Bias	–10.3	–7.8	–9.2

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