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LC–MS/MS method for simultaneous determination of viramidine and ribavirin levels in monkey red blood cells

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Abstract

A high performance liquid chromatography-tandem mass spectrometry (LC–MS/MS) method has been developed for the simultaneous determinations of total viramidine (viramidine, viramidine monophosphate, viramidine diphosphate, and viramidine triphosphate) and total ribavirin (ribavirin, ribavirin monophosphate, ribavirin diphosphate, and ribavirin triphosphate) in monkey red blood cells (RBC). The method involves the addition of internal standards and perchloric acid, conversion of viramidine or ribavirin phosphorylated metabolites to viramidine or ribavirin, purification with an aminopropyl (NH_2) solid phase extraction (SPE) cartridge, and LC–MS/MS analysis. The MS/MS is selected to monitor *m/z* 245 → 113, 250 → 113, 244 → 112, and 249 → 112 for ribavirin, [^{13}C]ribavirin, viramidine, and [^{13}C]viramidine, respectively, using positive electrospray ionization. The calibration curves are linear over a concentration range of 100–10,000 ng/mL (0.412–41.2 μM) with a lower limit of quantification (LLOQ) of 100 ng/mL for both compounds. Mean inter-assay recoveries for ribavirin are 101%, 98.9%, and 96.0%, with coefficient of variance (%CV) values between 1.95 and 4.50% for 100, 1000, and 10,000 ng/mL quality control (QC) samples, respectively. Mean inter-assay recoveries for viramidine are 96.3%, 101%, and 102%, with coefficient of variation (%CV) values between 3.61 and 7.22%, for 100, 1000, and 10,000 ng/mL QC samples, respectively. Over-curve dilution QC at 400 μg/mL (1639 μM) for both viramidine and ribavirin are used to ensure the dilution accuracy (25 X dilutions) for monkey samples.

The method has been used to simultaneously determine the total concentrations of ribavirin and viramidine in monkey RBC following 5, 15, and 36 weeks dosing of viramidine or ribavirin (60 mg/kg). The concentrations of total ribavirin following ribavirin dosing are 1242 μM at week 5, 1257 μM at week 15, and 1146 μM at week 36. The concentrations of total ribavirin following viramidine dosing are 634 μM at week 5, 716 μM at week 15, and 683 μM at week 36. Only small amounts of viramidine are detected in RBC following viramidine dosing, 7.80 μM at week 5, 6.63 μM at week 15, and 10.4 μM at week 36. The results suggest that ribavirin levels in RBC were at steady state at week 5 of ribavirin or viramidine dosing. At steady state, ribavirin levels in RBC are approximately 2× after ribavirin dosing than viramidine dosing. The relatively small percentage of viramidine in RBC suggests that viramidine either poorly penetrated into RBC or was extensively converted to ribavirin following entry into RBC.

Keywords: Ribavirin; Viramidine; Prodrug; Nucleoside; Nucleotide; Hepatitis C; LC–MS/MS

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-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 [2,3]. Ribavirin/pegylated interferon-alfa combination has been widely used for the treatment of chronic hepatitis C disease. Intracellularly, ribavirin is phosphorylated to ribavirin monophosphate (RMP), ribavirin diphosphate (RDP), and ribavirin triphosphate (RTP) (Fig. 1). This has been confirmed by *in vitro* studies in various cells using [^{3}H]ribavirin or [^{14}C]ribavirin [4–6]. RBC has the capacity to phosphorylate ribavirin to RMP, RDP, and RTP, but are devoid of phosphatase activity to convert them back to ribavirin [7]. As a result, high levels of phosphorylated ribavirin accumulate over time, leading to hemolytic anemia [6,7].

Viramidine (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine) is a prodrug of ribavirin. Wu et al. demonstrated that viramidine is converted to ribavirin through adenosine

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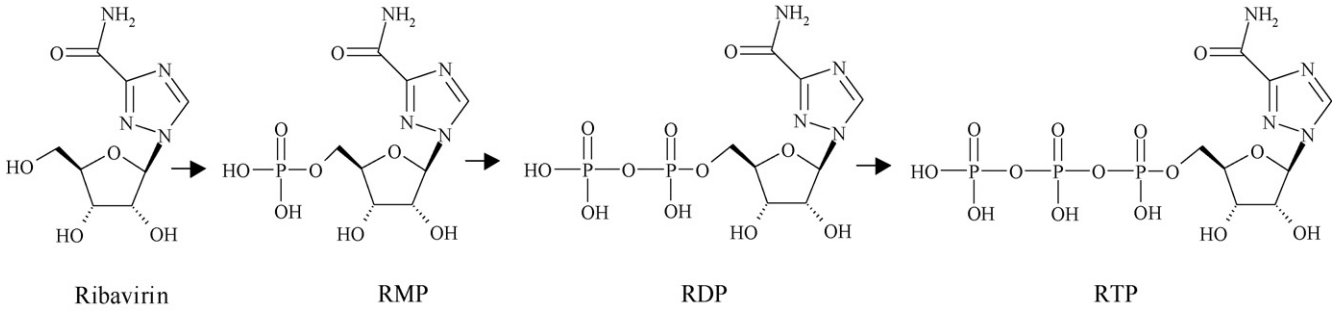
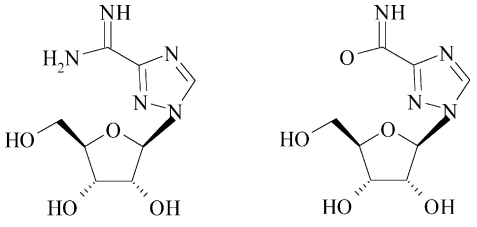


Fig. 1. Structures of ribavirin and phosphorylated metabolites.

deaminase [8]. The *in vitro* partition of viramidine and ribavirin between RBC and plasma has indicated that monkey shows similar distribution pattern to human [9]. Similar metabolic profiles in monkey RBC have been observed following single or 10-day dosing of [^{14}C]viramidine or [^{14}C]ribavirin [10]. However, viramidine dosing yielded 1/2 of the radioactivity in monkey RBC than following ribavirin dosing suggesting a potentially better safety profile for viramidine.

To further elucidate the potential safety advantage of viramidine over ribavirin, it is of interest to determine ribavirin levels in RBC from monkeys dosed with ribavirin or viramidine for an extended period (36 weeks). We have, therefore, developed and validated an LC–MS/MS method for simultaneous determination of ribavirin and viramidine in monkey RBC.



2. Materials and methods

2.1. Chemicals and materials

Viramidine and ribavirin were supplied by Valeant Research & Development (Costa Mesa, CA). [^{13}C]Viramidine (1-β-D-[1′-^{13}C, 2′-^{13}C, 3′-^{13}C, 4′-^{13}C, 5′-^{13}C]-ribofuranosyl-1,2,4-triazole-3-carboxamidine), [^{13}C]ribavirin (1-β-D-[1′-^{13}C, 2′-^{13}C, 3′-^{13}C, 4′-^{13}C, 5′-^{13}C]-ribofuranosyl-1,2,4-triazole-3-carboxamide), [^{3}H]ribavirin triphosphate (12 Ci/mmol) were synthesized by Moravek Biochemicals, Inc. (Brea, CA). Blank monkey RBC (male/female pooled), with EDTA as the anticoagulant, was purchased from Bioreclamation Inc. (East Meadow, NY). Acid phosphatase was purchased from Sigma (St. Louis, MO). Aminopropyl (NH_2, 100 mg/1 mL) SPE cartridges were purchased from Supelco (Supelco, Bellefonte, PA). All other solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. LC–MS/MS conditions

The LC–MS/MS system used to validate the method consisted of an Agilent 1100 binary pump (Agilent Technology, Palo Alto, CA), an Agilent 1100 autosampler, and a SCIEX API3000 mass spectrometer (Applied Biosystems, Foster City, CA). A Zorbax SB-C_{18} column (4.6 mm × 150 mm, 3.5 μm, Agilent Technology) was used in the analysis. The LC and MS/MS conditions are summarized in Tables 1 and 2.

Table 1
HPLC gradient conditions for LC–MS/MS analysis

Time (min)	*A* (%)	*B* (%)
0.0	99	1
2.5	99	1
5.5	5	95
6.4	5	95
6.5	99	1
9.5	99	1

A: 0.1% acetic acid in water; *B*: 0.1% acetic acid in methanol.

Table 2
MS/MS conditions for ribavirin analysis

Compound	Mass to charge ratio (*m*/*z*)		Time (ms)	Collision energy (V)
	Q1	Q3		
Ribavirin	245	113	150	15
[^{13}C]Ribavirin	250	113	150	15
Viramidine	244	112	150	12
[^{13}C]Viramidine	249	112	150	12

Interface	TurboIonSpray
Polarity	Positive
Scan type	MRM
Resolution	Q1 – unit, Q3 – low
Curtain gas (CUR)	12
Collision gas (CAD)	3
IonSpray voltage (IS)	5500 V
Temperature (TEM)	450 °C
Ion source gas 1 (GS1)	10
Ion source gas 2 (GS2)	6.5 L/min
Solvent split ratio	240 μL into interface

2.3. Preparation of calibration and quality control (QC) samples for LC–MS/MS analysis

Calibration standards ranging from 100 to 10,000 ng/mL at six concentration levels were prepared by spiking ribavirin and viramidine standards into monkey RBC blanks. The standards were prepared using the sample preparation procedure given below. QC samples at concentration levels of 100, 1000, and 10,000 ng/mL were prepared by spiking both ribavirin and viramidine standards into monkey RBC blanks. These QC samples were stored at −70 °C until analysis. Aliquots of the QC samples (100 μL) were transferred into separate vials on the day of sample preparation for later use.

2.4. Sample preparation for LC–MS/MS analysis

A solution of [^{13}C]viramidine and [^{13}C]Ribavirin internal standard (300 μL, 1.0 μg/mL each in water) and perchloric acid (200 μL, 8.8% (v/v)) were added to 100 μL of monkey RBC. The sample was vortexed briefly and centrifuged at 14,000 rpm for 10 min. The extract was transferred to a clean 13 mm × 100 mm tube. The pellet was extracted with additional water (150 μL) and perchloric acid solution (100 μL, 8.8% (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 micro-centrifuge 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 or viramidine was accomplished by adding acid phosphatase (5 μL, ∼0.7 U/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 quantify the levels of ribavirin and viramidine in monkey RBC.

3. Results

An LC–MS/MS method was developed to determine the concentrations of total ribavirin (ribavirin, RMP, RDP, and RTP) and total viramidine (viramidine, VMP, VDP, and VTP) in monkey RBC. In this method, ribavirin, viramidine, and their phosphorylated metabolites were extracted from monkey RBC, and all phosphorylated metabolites were subsequently converted to ribavirin or viramidine, respectively, using acid phosphatase. The final extract was then purified using an 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.

3.1. Extraction efficiency

The extraction efficiency was determined by extracting RBC samples from monkeys dosed with [^{14}C]viramidine or [^{14}C]ribavirin. The total radioactivity extracted was compared to the value obtained from the digested value. The results indicate that more than 98% of the total radioactivity was extracted using the current method.

3.2. Enzyme conversion efficiency

Enzyme conversion efficiency was determined by spiking [^{3}H]ribavirin triphosphate into 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. RTP was converted to ribavirin successfully after the incubation and no additional degradants were formed during the incubation process. Enzyme conversion of viramidine phosphates was not examined due to the lack of radioactive standards and *in vivo* samples. We assume that the conversion efficiency of viramidine phosphates is similar to that of ribavirin phosphates.

3.3. Separation, selectivity, and sensitivity

Analysis of RBC blanks showed no interference in the final extract, although there were several endogenous peaks that exhibited the same *m/z* transitions at different retention times for both viramidine and ribavirin. Uridine was the major endogenous peak (retention time at ∼6 min) for ribavirin, and cytidine was the major endogenous peak (retention time at ∼3 min) for viramidine. The identities of the other peaks were not known. Typical chromatograms of RBC blank extracts are presented in Figs. 2 and 3. The results indicate that the method provides adequate separation and selectivity through HPLC separation and LC–MS/MS detection. The method provides acceptable

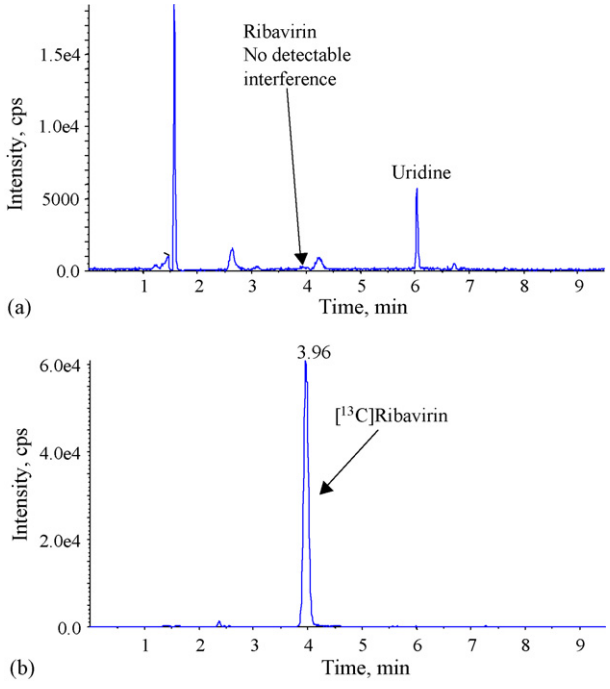


Fig. 2. Typical extracted ion chromatograms of RBC blank extract: (a) ribavirin *m/z* at 245 → 113 and (b) [^{13}C]ribavirin *m/z* at 250 → 113.

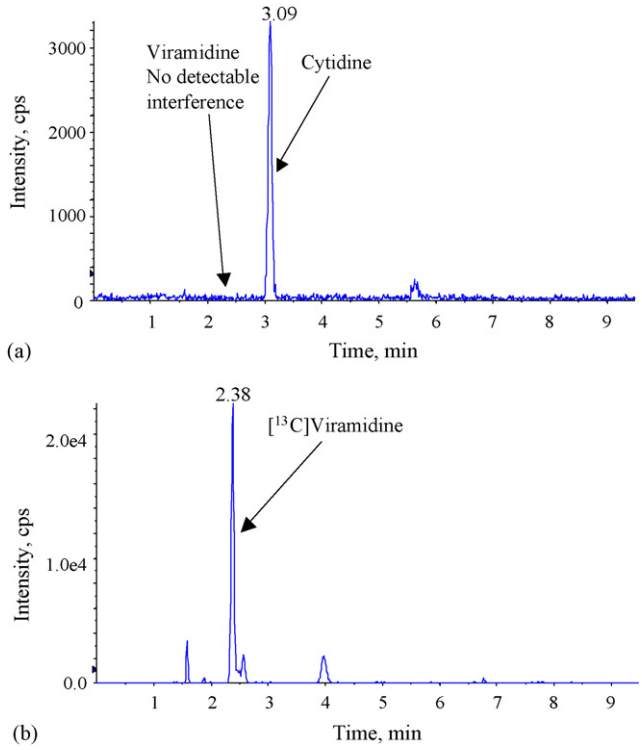


Fig. 3. Typical extracted ion chromatograms of RBC blank extract: (a) viramidine *m/z* at 244 → 112 and (b) [^{13}C]viramidine *m/z* at 249 → 112.

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 Figs. 4 and 5.

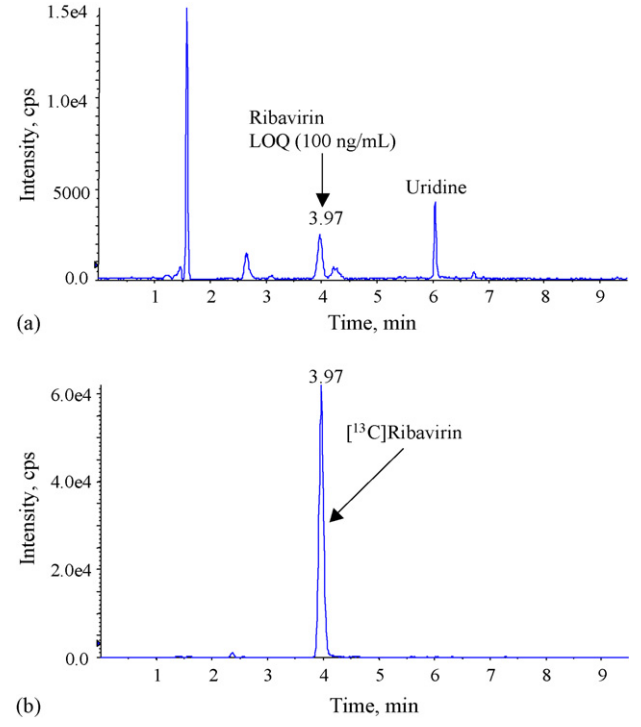


Fig. 4. Typical extracted ion chromatograms of RBC LLOQ (100 ng/mL) extract: (a) ribavirin *m/z* at 245 → 113 and (b) [^{13}C]ribavirin *m/z* at 250 → 113.

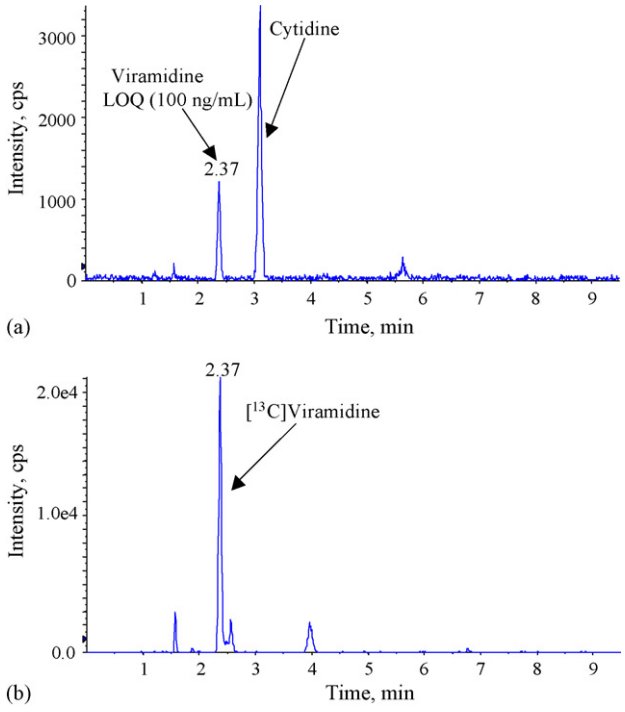


Fig. 5. Typical extracted ion chromatograms of RBC LLOQ (100 ng/mL) extract: (a) viramidine *m/z* at 243 → 112 and (b) [^{13}C]viramidine *m/z* at 249 → 112.

3.4. Standard curve linearity

For the linear regression analysis, the correlation coefficient (r) was greater than 0.9970 for ribavirin and 0.9986 for viramidine during the method validation. This indicates linearity of the detector response as a function of the standard calibration curve. Representative calibration curves for ribavirin and viramidine are presented in Fig. 6. For ribavirin, mean back-calculated values from the fitted curve were between −5.5 and 0.37% of their nominal values while %CV varied between 0.98 and 3.1%. For viramidine, mean back-calculated values from the fitted curve were between −3.8 and 4.1% of their nominal values while %CV varied between 2.07 and 6.07% (Tables 3 and 4).

3.5. Accuracy and precision

Accuracy and precision were determined based on low-, mid-, and hi-QC samples. Mean intra-assay recoveries were within ±7% of their nominal values while %CV varied between 0.548 and 3.47% for ribavirin and 0.559–11.0% for viramidine. Mean inter-assay recoveries were within ±4.0% of their nominal values for ribavirin and viramidine while %CV varied between 1.95–4.50% for ribavirin and 3.61–7.22% for viramidine. The recovery data and statistics are presented in Tables 5 and 6.

3.6. Over-curve dilution analysis

Over-curve dilution was determined by diluting 400 μg/mL QC samples by a factor of 40. The diluted samples were

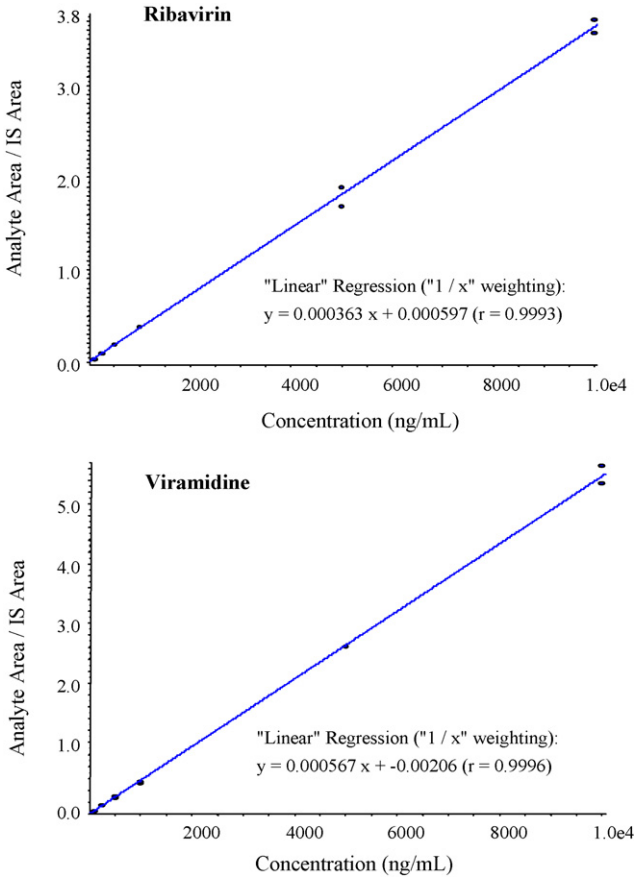


Fig. 6. Typical calibration curves for the determination of ribavirin (top) and viramidine (bottom) in monkey RBC.

processed and analyzed as described in the method. The mean recovery and %CV are 402 μg/mL and 0.779% for ribavirin and 416 μg/mL and 2.45% for viramidine, respectively. This result indicates that over the curve dilution can be achieved for high concentration samples.

3.7. Stability of ribavirin and viramidine in RBC and final extract

To determine the stabilities of ribavirin and viramidine in RBC, ribavirin and viramidine were spiked separately in monkey RBC blanks. The samples were analyzed to determine the 24-h bench stability, three freeze–thaw-cycle stability, and final extract stability. Ribavirin was stable on the bench for 24 h and was stable in three freeze–thaw cycles. The final extract of ribavirin was stable for at least 48 h in the autosampler. Viramidine was stable in three freeze–thaw cycles and its final extract was stable for at least 48 h in the autosampler. However, a noticeable amount of viramidine (∼30%) converted to ribavirin during the 24-h room temperature storage.

3.8. Monkey RBC analysis

Blood samples collected at 5, 15, and 36 weeks from monkeys receiving ribavirin or viramidine at 60 mg/kg daily were analyzed to determine total ribavirin or viramidine concentration (Table 7). The results indicate that ribavirin level in whole blood reached steady state by week 5 for ribavirin dosing. For viramidine dosing, both ribavirin and viramidine levels in whole blood reached steady state by week 15. At steady state, ribavirin level in whole blood following ribavirin dosing was approximately 2× of viramidine dosing (Fig. 7). The data shows only a small amount of viramidine present in the blood following multiple dosing, indicating an extensive conversion of viramidine to ribavirin.

4. Discussion

Glue et al. reported that following multiple oral dosing of ribavirin, ribavirin levels in RBC accumulated with time, leading to hemolytic anemia [7]. However, the bioanalytical methods for the determination of ribavirin concentration in RBC are limited. Austin et al. developed a radioimmunoassay (RIA)

Table 3
Calibration curve analytical results of ribavirin in monkey RBC

Curve ID	Concentration (ng/mL)					
	100	250	500	1000	5000	10,000
Day 1, #1	99.5	239	513	1020	5030	9,940
Day 1, #2	100	247	498	1040	4820	10,100
Day 2, #1	94.8	245	521	1060	4800	10,100
Day 2, #2	98.3	249	506	1010	5060	9,930
Day 3, #1	103	235	508	1010	5120	9,880
Day 3, #2	99.2	240	509	1020	5120	9,860
Day 3, #3	102	228	517	1040	4970	9,990
Parameters	Concentration (ng/mL)					
Mean	100	240	510	1029	4989	9971
S.D.	2.66	7.35	7.57	18.6	133	97.4
%CV	2.67	3.06	1.48	1.81	2.66	0.976
%Bias	−0.46	−5.5	−1.30	−1.1	0.37	−0.19
n	7	7	7	7	7	7

Table 4
Calibration curve analytical results of viramidine in monkey RBC

Curve ID	Concentration (ng/mL)					
	100	250	500	1000	5000	10,000
Day 1, #1	106	259	492	925	4890	10,200
Day 1, #2	93.4	256	528	982	5050	9,940
Day 2, #1	105	254	504	957	4650	10,400
Day 2, #2	102	274	446	954	5300	9,770
Day 3, #1	109	239	513	944	4840	10,200
Day 3, #2	102	253	488	986	5060	9,960
Day 3, #3	111	226	498	986	4980	10,100
Parameters	Concentration (ng/mL)					
Mean	104	252	496	962	4967	10,081
S.D.	5.76	15.3	25.7	23.6	204	209
%CV	5.54	6.07	5.18	2.45	4.10	2.07
%Bias	4.1	0.63	−0.89	−3.80	−0.66	0.81
n	7	7	7	7	7	7

method for the determination of ribavirin in urine or plasma [11]. Although RIA provides excellent sensitivity (10 pmol/mL or ∼2.5 ng/mL), the method was not validated in RBC and has the potential of cross-reacting with a major metabolite of ribavirin, 1,2,4-triazole-3-carboxamide. Moreover, the method involves the routine use of tritium-labeled compounds and has a lengthy and tedious sample preparation procedure, which makes it difficult to monitor pre-clinical and clinical studies with large numbers of samples. Homma et al. developed a HPLC–UV method for the determination of ribavirin in whole blood [12]. The HPLC–UV method has a calibration range between 1.67 and 40 μM (400–9760 ng/mL) with a LOQ at 400 ng/mL. This

Table 5
Quality control analytical results of ribavirin in monkey RBC

Se	Parameters	Low-QC 100 ng/mL	Mid-QC 1000 ng/mL	High-QC 10,000 ng/mL
1		98.9	1000	9880
		93.0	985	10,200
		97.1	997	9550
	Mean	96.3	994	9877
	S.D.	3.02	7.94	325
	%CV	3.14	0.799	3.29
	%Bias	−3.7	−0.60	−1.2
	n	3	3	3
2		106	973	9260
		105	1010	9300
		105	1010	9440
	Mean	105.3	998	9333
	S.D.	0.577	21.4	94.5
	%CV	0.548	2.14	1.01
	%Bias	5.3	−0.23	−6.7
	n	3	3	3
3		99.5	954	9660
		98.5	1000	9630
		105	972	9440
	Mean	101	975	9577
	S.D.	3.50	23.2	119
	%CV	3.47	2.38	1.25
	%Bias	1.0	−2.47	−4.2
	n	3	3	3
Overall	Mean	101	989	9596
	S.D.	4.54	19.3	296
	%CV	4.50	1.95	3.09
	%Bias	0.89	−1.1	−4.0
	n	9	9	9

Table 6
Quality control analytical results for viramidine in monkey RBC

Se	Parameters	Low-QC 100 ng/mL	Mid-QC 1000 ng/mL	High-QC 10,000 ng/mL
1		97.7	997	10,600
		95.9	997	9980
		89.5	1010	10,400
	Mean	94.4	1001	10,327
	S.D.	4.31	7.51	316
	%CV	4.57	0.750	3.06
	%Bias	−5.6	0.13	3.3
	n	3	3	3
2		100	1030	10,300
		105	1020	10,300
		91.1	1060	10,400
	Mean	98.6	1043	10,333
	S.D.	7.04	32.1	57.7
	%CV	7.11	3.08	0.559
	%Bias	−1.3	4.3	3.3
	n	3	3	3
3		107	986	10,100
		94.5	936	9930
		86.0	1030	9360
	Mean	95.8	984	9797
	S.D.	10.6	47.0	388
	%CV	11.0	4.78	3.96
	%Bias	−4.2	−1.6	−2.0
	n	3	3	3
Overall	Mean	96.3	1010	10,152
	S.D.	6.95	39.0	367
	%CV	7.22	3.87	3.61
	%Bias	−3.7	1.0	1.5
	n	9	9	9

Table 7
RBC analysis for ribavirin and viramidine in monkey samples

Time (week)	Ribavirin dosing		Viramidine dosing		
	Parameter	Concentration (μM)	Concentration (μM)		% of total
		Ribavirin	Ribavirin	Viramidine	Viramidine
5	Mean	1242	634	7.80	1.22
	%CV	12.2	20.9	31.9	32.2
	n	8	14	14	14
15	Mean	1257	716	6.63	0.92
	%CV	9.06	17.1	25.1	24.2
	n	8	15	15	15
36	Mean	1146	683	10.4	1.50
	%CV	8.81	14.9	15.7	18.8
	n	8	8	8	8

method has a less than desired calibration range and unacceptable recovery (accuracy) at the LOQ level (63.2%). Recently, ribavirin analysis using LC–MS/MS to obtain desired selectivity and sensitivity have been published by this laboratory [13–15] and by Shou [16] in different biological matrices using either normal or reverse phase column chromatography. In our laboratory, the reverse phase column has approved to be rugged to transfer to different contract laboratories and can be applied to different matrices. In addition, attempt of using normal phase column chromatography to analyze ribavirin in human RBC was unsuccessful in one of our contract laboratory; therefore, the reverse phase chromatography is chosen for the analysis of ribavirin and viramidine in human plasma [17].

Viramidine is a prodrug of ribavirin, which is converted to ribavirin. In monkeys following single and 10-day oral dosing, viramidine exhibited preferential liver uptake properties than ribavirin. On the other hand, viramidine gave 1/2 radioactivity in RBC than from ribavirin dosing. It is uncertain whether viramidine and ribavirin will yield similar RBC drug levels following an extended period of dosing; to evaluate this possibility, we

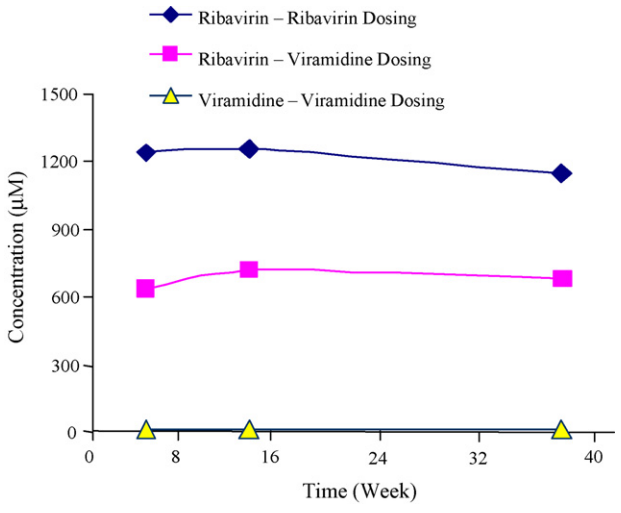
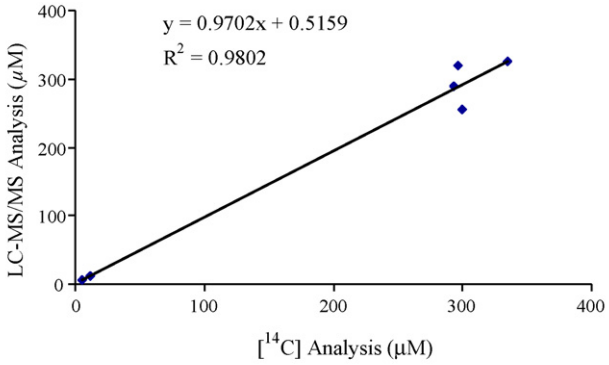


Fig. 7. Concentration of ribavirn and viramidine following 60 mg/kg ribavirin and viramidine dosing at 5, 15, and 36 weeks.



Monkey #	Time (hour)	Total Ribavirin Conc. (μM) [^{14}C]-detection	LC-MS/MS	Difference (%)
1002	2	5.84	5.87	0.467
1003	2	11.3	11.3	0.192
1001	228	335	326	-2.69
1001	240	300	256	-14.7
1002	240	293	289	-1.37
1003	240	296	319	7.77
Mean				4.52*

* Calculated from absolute value of the difference.

Fig. 8. Correlation of LC–MS/MS and radioactivity analysis of monkey RBC from [^{14}C]ribavirin dosing.

developed an LC–MS/MS method for the simultaneous quantitation of ribavirin and its prodrug, viramidine, in RBC. The method has broader linear range (100–10,000 ng/mL) and acceptable accuracy (±4% of nominal value for ribavirin and viramidine for bias) and precision (1.95–4.50% for ribavirin and 3.61–7.22% for viramidine for %CV). This validated method has been utilized to analyze total ribavirin concentration in six randomly chosen monkey RBC from [^{14}C]ribavirin single-dose study. Using ^{14}C-detection and LC–MS/MS analysis, the results shown in Fig. 8 indicate good correlation ($r^2 = 0.9802$) and a mean of 4.52% difference (−14.7 to 7.77%) between LC–MS/MS and radioactivity measurement. The method uses acid phosphatase to convert all nucleotides to their corresponding nucleosides for LC–MS/MS quantitation. If desired, the acid phosphatase step can be omitted and the amount of free ribavirin or viramidine will be determined. Therefore, the amount of phosphorylation can be indirectly detected and this approach has been used in the analysis of ribavirin in human RBC [15].

It is of great scientific interest and challenge to develop an LC–MS/MS method for the direct measurement of ribavirin, RMP, RDP, and RTP. Several researchers demonstrated that this is a viable approach to determine some of the nucleoside reverse transcriptase inhibitors (NRTIs) in peripheral blood mononuclear cells (PBMCs) [18–24]. However, the efforts to quantify RTP directly in RBC were hindered by the stability of RTP. RTP was predominant in freshly collected RBC from monkeys dosed with [^{14}C]ribavirin or [^{14}C]viramidine for 10 days (Figs. 9 and 10). However, the relative amounts of RMP, RDP, and RTP changed significantly based on extraction and storage conditions. Smee et al. demonstrated that the degree of phosphorylation was dependent on the extracellular concentration of ribavirin, and RTP degrades rapidly, having a short half-life (70–100 min) upon removal of ribavirin [4]. To obtain an

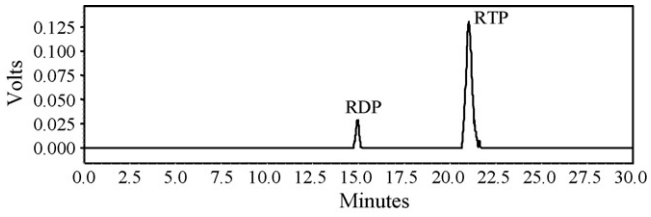


Fig. 9. Representative radiochromatograms at 240 h monkey RBC extract from 10 mg/kg *in vivo* ribavirin 10-day oral dosing experiment.

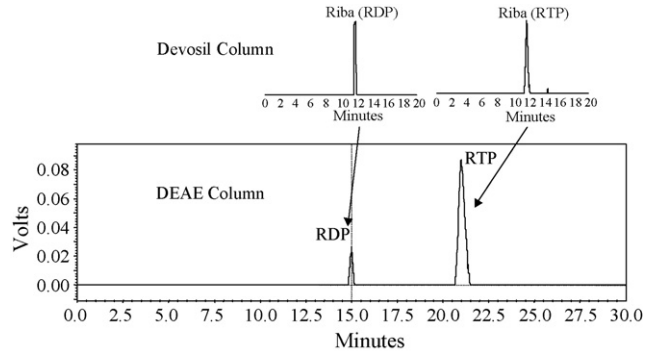


Fig. 10. Representative radiochromatogram at 240 h monkey RBC extract from 10 mg/kg *in vivo* viramidine 10-day oral dosing experiment.

accurate composition of phosphates, the samples need to be processed immediately following collection; this is impractical for long-term toxicology and clinical studies. In addition, the RTP standard continuously degraded, even when stored at −70 °C. Based on the stability of RTP and available metabolic profiles obtained from monkeys dosed with [^{14}C]viramidine or [^{14}C]ribavirin [10], we believe the quantification of total viramidine or ribavirin will be sufficient to provide safety evaluation of these two compounds.

5. Conclusion

An LC–MS/MS assay for the simultaneously measurement of total viramidine and ribavirin in monkey RBC has been established. The method is specific, sensitive, and accurate over a concentration range of 100–10,000 ng/mL. The method has been used to quantify levels of total viramidine and ribavirin in monkey RBC. Following long-term dosing (36 weeks) at 60 mg/kg, ribavirin dosing gives approximately 2× total ribavirin in RBC than viramidine dosing. This finding strongly suggests that viramidine has a potentially better safety profile than ribavirin in hepatitis C treatment.

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