

Lou Anne McKown, Lori Payne  
BASi Northwest Laboratory, McMinnville, OR

## Abstract

Ribavirin is an antiviral prodrug used for the treatment of Hepatitis C and respiratory syncytial virus (RSV) among other common viruses. A method for the analysis and quantification of ribavirin in rat plasma utilizing HPLC-MS/MS with updated HPLC column technology has been developed and validated according to the FDA guidance to the industry. The assay displays good linearity with correlation coefficient >0.99 over a range of 10 – 5000 ng/mL using a 25  $\mu$ L sample precipitated with 5mM ammonium acetate in 95% acetonitrile in water after the addition of a stable label internal standard. The supernatant is analyzed by HPLC utilizing a HILIC column with detection by MS/MS incorporating a turbo-ion spray interface in positive mode. The choice of HILIC column was essential for adequate retention and acceptable peak shape of ribavirin within 2 minutes of total analysis time. This method greatly facilitated the high throughput analysis of preclinical samples.

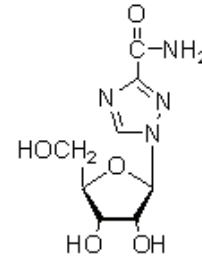


Figure 1. The molecular structure of ribavirin.

## Introduction

The synthetic compound with the IUPAC name 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide was first produced in 1972 [1] and reported to exhibit broad spectrum activity against DNA and RNA viruses [2]. The structure of ribavirin is shown in Figure 1 and the molecular weight is 244 with molecular formula of C<sub>8</sub>H<sub>12</sub>N<sub>4</sub>O<sub>5</sub>. The high abundance of heteroatoms in the structure of ribavirin and the number of atoms available for hydrogen bonding lends itself to be very polar and water soluble. These physical characteristics present a challenge for chromatographic resolution and the recent advances in column chemistries for HPLC separation provide ample new opportunities to develop a rapid method for the determination of ribavirin concentrations in biological samples. Numerous different column chemistries were evaluated and produced a new HPLC-MS/MS method for the rapid analysis of ribavirin. This method was validated to current GLP standards in rat plasma over a range of 10-5000 ng/mL and provided reliable results for the pharmacokinetic evaluation of preclinical samples.

[1] J. Med. Chem. 15 (1972) 1150. [2] Science 177 (1972) 705.

## Results and Discussion

Representative chromatograms of Ribavirin from traditional C-18 HPLC columns:

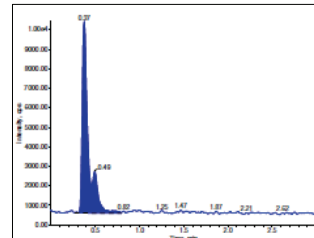


Figure 2. Chromatogram of Ribavirin from a Phenomenex Synergi 4 m Hydro-RP 80Å 2 x 50 mm eluted isocratic with 5% ACN in 0.1% formic acid in water.

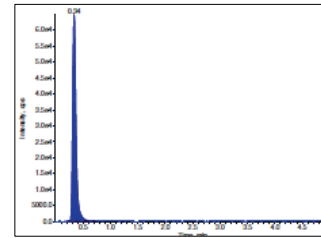


Figure 3. Chromatogram of Ribavirin from a GL sciences Inertsil ODS-EP 5 mm 2.1 x 33 mm eluted isocratic with 5% ACN in 0.1% formic acid in water.

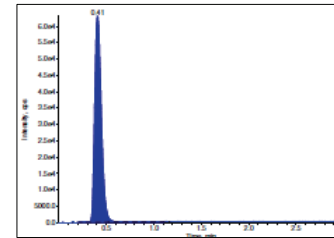


Figure 4. Chromatogram of Ribavirin from a Waters Xbridge Shield RP18 5 mm 2.1x 50 mm eluted isocratic with 5% ACN in 0.1% formic acid in water.

•Traditional C-18 HPLC columns or other common columns used for polar compounds did not exhibit suitable retention and/or peak shape was not obtained with published methods.

Representative chromatograms of Ribavirin from underivatized silica columns:

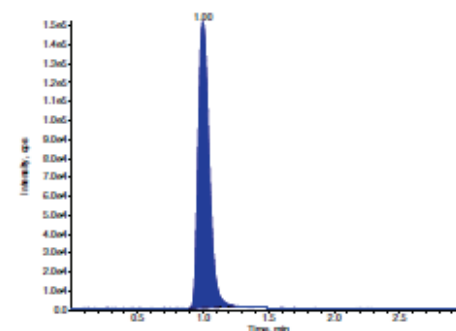


Figure 5. Chromatogram of Ribavirin from a Phenomenex Luna HILIC 3 mm 2 x 100 mm eluted isocratic with 95% ACN in water

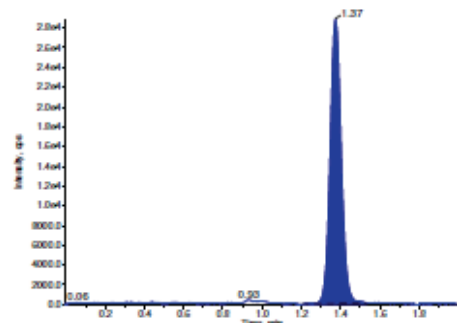


Figure 6. Chromatogram of Ribavirin from a Phenomenex Luna HILIC 3 mm 2 x 100 mm eluted isocratic with 5 mM ammonium acetate in 95% ACN

•A selection of underivatized silica columns were tested and suitable retention was achieved on a Phenomenex Luna HILIC column as seen in Figure 5.

•Modification of the mobile phase produced significant differences in the retention time of ribavirin and the peak shape on the Luna HILIC column.

•Buffer concentration- total overall buffer concentration of 5 mM ammonium acetate in 95% ACN in water at 0.5 mL/min exhibited symmetrical peak shape and increase the retention of ribavirin by 0.3 min over no buffer.

•Increase in the buffer concentration resulted in greater retention of ribavirin but peak shape suffered greatly.

•Decrease in the buffer concentration resulted in less retention, although the peak shape remained symmetrical.

•Injection volume - Injection volumes in excess of 5  $\mu$ L resulted in severe tailing and deteriorated peak shape.

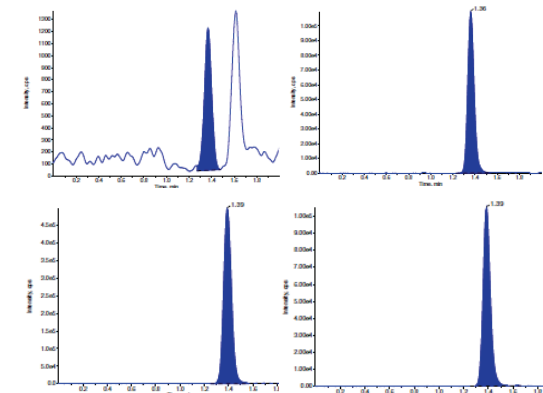


Figure 7. Representative chromatograms of plasma extracts of ribavirin. Upper left: 10 ng/mL ribavirin (LOQ), lower left: 5000 ng/mL ribavirin (ULQ), upper and lower right are ribavirin-<sup>13</sup>C<sub>5</sub> (internal standard).

Calibration Standards for Ribavirin (ng/mL)  
Regression Method = LINEAR - Weighting Factor = 1/X<sup>2</sup>  
Response = Slope \* Conc + Intercept  
Slope = 0.00193 Intercept = 0.00244 R-Squared = 0.9983

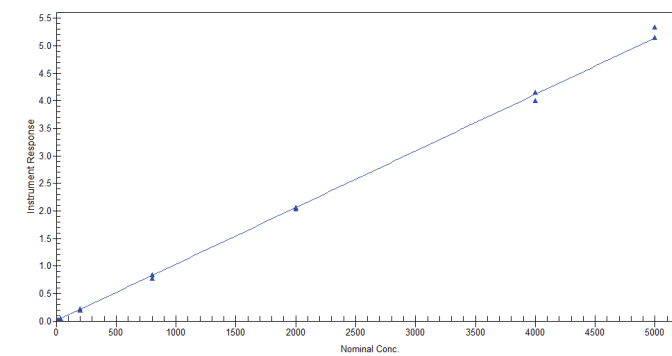


Figure 8. Example standard curve

Resolution and retention of ribavirin on the Phenomenex Luna HILIC column is very sensitive to changes in the constitution of injection solvent and injection volume so it dictated the treatment of the analyte in the preparation of samples. A simplified protein precipitation sample preparation follows:

- 25  $\mu$ L plasma sample size
- 96 well plate format
  - 50  $\mu$ L of a 500 ng/mL solution of ribavirin-<sup>13</sup>C<sub>5</sub> in mobile phase
  - 0.5 mL mobile phase to precipitate the proteins
  - Centrifugation at 4000 rpm for 5 min
  - 0.4 mL of the supernatant liquid was transferred to a clean 96 well plate.

•5  $\mu$ L of the supernatant liquid was injected directly into the HPLC-MS/MS system. Figure 7 shows representative extract chromatograms

•The example standard curve in Figure 8 shows the correlation over a range of 10-5000 ng/mL

## Experimental

Ribavirin and ribavirin-<sup>13</sup>C<sub>5</sub> were purchased from Toronto Research Chemicals, Inc. Na<sub>2</sub>EDTA rat plasma was purchased from Biochemed, inc. Ammonium acetate was purchased from Mallinckrodt. Methanol and acetonitrile were manufactured by Burdick and Jackson, and the water was deionized and filtered (0.2  $\mu$ m) using a Milli-Q Plus® system. The HPLC-MS/MS system consisted of a Shimadzu liquid chromatography system including 2 isocratic pumps, autosampler, a pre-column filter with replaceable frit (0.5  $\mu$ m), Phenomenex Luna HILIC column (3 mm, 2.0 x 100 mm), and a 6 port valve connected to a API Sciex 365 mass spectrometer with Ionics Interface upgrade and Analyst instrument control software (Applied Biosystems, Foster City, CA). The mass spectrometer was run in positive mode with multiple reaction monitoring of 245  $\rightarrow$  113 transition for ribavirin and 250  $\rightarrow$  113 transition for the internal standard.

## Conclusions

A new method for the quantification of ribavirin in rat plasma was successfully validated according to FDA guidance to the industry. Ribavirin was isolated from rat plasma by protein precipitation of 25  $\mu$ L of spiked plasma with 5 mM ammonium acetate in 95% acetonitrile in water (mobile phase) after the addition of 50  $\mu$ L of a solution of 500 ng/mL ribavirin-<sup>13</sup>C<sub>5</sub> in mobile phase. The method utilized a Phenomenex Luna HILIC column for HPLC purification which was very sensitive to the many factors such as buffer concentration and injection volume which influence the chromatography of an analyte.

Precision (% CV) and accuracy (% bias) of the quality control samples at concentrations of 10, 30, 1000, and 3800 ng/mL were 4.6-17.5 %CV and -6.4-3.3 % bias over 3 method performance batches (n=18). The accuracy and precision was within  $\pm$ 15% for validation samples independently subjected to the following conditions: 4 freeze-thaw cycles at -80°C, 24 h at room temperature (rt), 1 month of storage at -80°C, or extracts stored at rt for 4 days. No interference in the quantification of ribavirin was seen from 6 different lots of rat plasma and the method was not impacted by carryover of ribavirin or the internal standard in the system. This method was used to quantify ribavirin in preclinical samples.