Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 34–40
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A sensitive and specific method for the determination of total ribavirin in monkey liver by high-performance liquid chromatography with tandem mass spectrometry

Li-Tain Yeh*, Mai Nguyen, David Lourenco, Chin-chung Lin

Drug Development Department, Ribapharm Inc., 3300 Hyland Avenue, Costa Mesa, CA 92626, USA

Accepted 16 November 2004
Available online 19 January 2005

Abstract

A sensitive and specific method using high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the analysis of total ribavirin in monkey liver is developed and validated. In this method, ribavirin and its phosphorylated metabolites are extracted with perchloric acid. The metabolites are converted to ribavirin using acid phosphatase and further purified using a NH_2 solid-phase extraction (SPE) cartridge prior to LC–MS/MS analysis. [^{13}C]Ribavirin is added with the extraction solution as an internal standard to obtain better accuracy and precision of the analysis. The MS/MS was selected to monitor 245 → 113 and 250 → 113 transitions using positive electrospray ionization for ribavirin and [^{13}C]ribavirin. The calibration curve is linear over a concentration of 1.0–100 μg/g with a limit of quantitation (LOQ) of 1.0 μg/g. Mean inter-assay accuracy for QC at 1.0, 10 and 100 μg/g are 108, 99.7 and 99.7%, respectively. Mean inter-assay precision (CV) for QC at 1.0, 10 and 100 μg/g are 5.34, 5.24 and 4.59%, respectively. Extractability of total ribavirin from liver has been confirmed with liver obtained from monkey dosed with [^{14}C]ribavirin. The method has been proven to be useful in the determination of total ribavirin concentration in liver from monkeys in mass balance study (10 mg/kg) and in 28 days toxicology study (300 mg/kg/day). It is also used to determine the total ribavirin concentration in human livers from hepatitis C patients received dose of 600 mg ribavirin twice daily.

Keywords: Ribavirin; Nucleoside; Nucleotide; Hepatitis C; LC–MS/MS; Human liver biopsy

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 in 1972 [2,3]. Ribavirin/peglyated interferon-alfa combination has been recognized as the gold standard in the treatment of chronic hepatitis C. Intracellularly, ribavirin is phosphorylated to ribavirin mono-phosphate (RMP), ribavirin di-phosphate (RDP) and ribavirin tri-phosphate (RTP) Fig. 1 [4]. In vitro studies in various cells using [^{3}H]- or [^{14}C]ribavirin confirmed the formation of RMP, RDP and RTP [5–7]. Miller et al. [8] demonstrated that ribavirin entered liver and phosphorylated to RMP, RDP and RTP in rat following single oral dosing. Primarily results from our studies in monkey following single or 10 days oral dosing of [^{14}C]ribavirin confirmed the formation of RMP, RDP and RTP in liver [9]. However, it is impossible to carry out long-term study in animal or human using [^{14}C]ribavirin. As a liver-targeting drug, the evaluation of liver concentration of ribavirin following prolonged treatments has become increasingly important. To facilitate the monitoring of ribavirin concentration in liver, a quantitative method has been developed to determine the concentration of total ribavirin (ribavirin, RMP, RDP and RTP) in monkey liver and subsequently in human liver biopsy samples.

* Corresponding author. Tel.: +1 714 427 6236x4014; fax: +1 714 641 7201.
E-mail address: lyeh@valeant.com (L.-T. Yeh).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.12.006

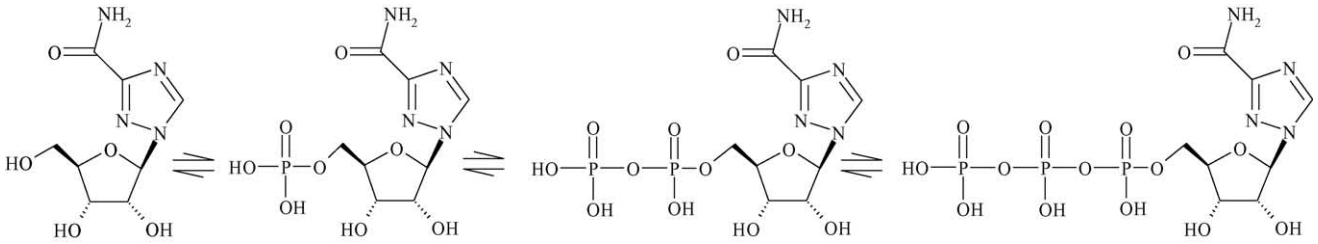

Fig. 1. Structures of ribavirin and its phosphorylated metabolites.

2. Materials and methods

2.1. Materials

Ribavirin was supplied by Ribapharm Inc. (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 5′-triphosphate (RTP, 12 Ci/mmole) were synthesized by Moravek Biochemicals Inc. (Brea, CA). Acid phosphatase was purchased from Sigma (St. Louis, MO). Acetic acid, ammonium hydroxide and methanol were purchased from Fisher Scientific (Pittsburgh, PA). The NH_2 solid-phase extraction (SPE) cartridges were purchased from Supelco (Supelco, Bellefonte, PA). All other solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. HPLC-radioactivity detector conditions

The HPLC-radioactive detector used to study the conversion of RTP to ribavirin consisted of two Shimazdu LC-10AD pumps (Shimazdu Corporation, Columbia, MD), a Shimazdu SIL-10A autosampler, and an IN/US radioactive detector (IN/US System Inc., Tampa, FL). A 4.6 mm × 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 LC conditions are summarized in Table 1. Under these conditions, good separation was obtained between ribavirin, RMP, RDP and RTP except the co-elution of a metabolite, $TCONH_2$, with ribavirin. To confirm that ribavirin was not further converted to $TCONH_2$ under the conditions of enzyme digestion, a second HPLC column was used for the analysis. The analysis was performed using a Devosil C_{30} column (4.6 mm × 250 mm, 5 μm, Supelco). An isocratic run using 100 mM ammonium phosphate (pH = 2.4) at a flow rate of 1.0 mL/min provided adequate separation between ribavirin and the metabolite.

Table 1
HPLC gradient conditions for radioactivity analysis

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

A: 100 mM ammonium phosphate/acetonitrile (80/20), pH = 3.0; B: 250 mM ammonium phosphate/acetonitrile (80/20), pH = 3.5.

2.3. LC–MS/MS conditions

The LC–MS/MS used to validate the method consisted of an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA), an Agilent 1100 autosampler, and a SCIEX API 3000 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 2 and 3. The product ion scan spectra of ribavirin and [^{13}C]ribavirin are presented in Fig. 2.

2.4. Preparation of calibration and quality control (QC) samples

Calibration standards ranged from 1.0 to 100 μg/g at six concentration levels were prepared by spiking ribavirin standards into monkey liver blanks using the sample preparation procedure given below. Because there are no blank human livers, monkey control livers were used to prepare calibration curves for both monkey liver and human liver analysis. QC samples at concentration levels of 1.0, 10 and 100 μg/g were prepared by spiking ribavirin standards into monkey liver on the same day of analysis.

2.5. Liver sample preparation

A solution of [^{13}C]ribavirin internal standard (1.0 μg/mL in water, 100 μL) and perchloric acid (900 μL, 5.0%, v/v) were added to monkey or human liver sample (≤20 mg).

Table 2
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.

Table 3
MS/MS conditions for ribavirin analysis

Compound	Mass to charge ratio (*m/z*)		Time (ms)	Collision energy (V)
	Q_1	Q_3		
Ribavirin	245	113	150	15
Ribavirin (ISTD)	250	113	150	15
Interface				TurboIonSpray
Polarity				Positive
Scan type				MRM
Resolution				Q_1—unit, Q_3—low
Curtain gas (CUR)				12.0
Collision gas (CAD)				3.0
IonSpray voltage (IS)				5500
Temperature (TEM, °C)				450
Ion source gas 1 (GS1)				10.0
Ion source gas 2 (GS2)				6500
Solvent split ratio				240 μL into interface

The mixtures were homogenized briefly and centrifuged at 2000 rpm for 10 min. A portion of the extract (450 μL) was transferred to a clean test 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 U/μL) to the sample and incubating the mixture at 37 °C for 1 h. After digestion, the resulting mixture was further purified using a NH_2 SPE cartridge. Final extract was analyzed by LC–MS/MS for the quantitation of ribavirin in liver.

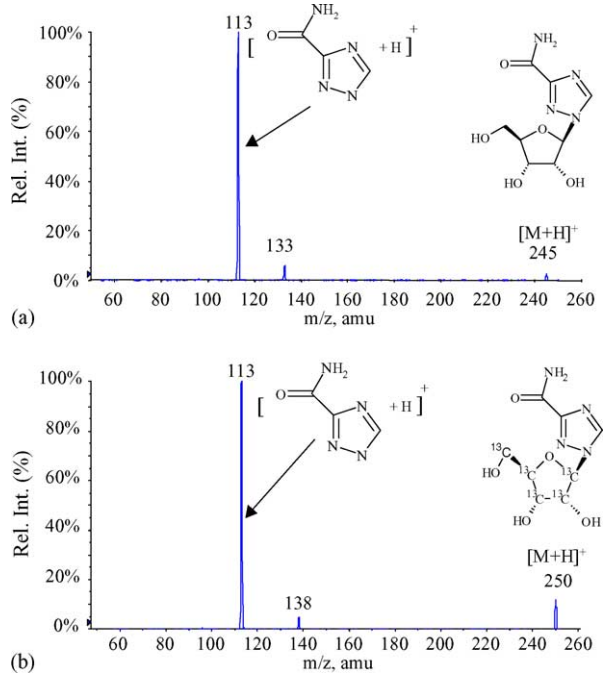

Fig. 2. Product ion scan spectra of (a) ribavirin and (b) [^{13}C]ribavirin.

3. Results

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 monkey liver and all phosphorylated metabolites are subsequently converted to ribavirin using 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.

3.1. Extraction efficiency

The extraction efficiency was determined by extracting a radioactive monkey liver sample from monkey dosed with [^{14}C]ribavirin. The total radioactivity extracted was compared to the value obtained from digested value. The results indicated that essentially the majority of radioactivity has been extracted (>95%).

3.2. Enzyme conversion efficiency

Enzyme conversion efficiency was determined by fortifying [^{3}H]RTP into blank monkey liver extract and converting it to ribavirin following the sample preparation procedure. The final extract was analyzed by LC-radioactive detector to confirm the total conversion of RTP to ribavirin. As presented in Figs. 3 and 4, RTP was converted to ribavirin successfully after the incubation and no other metabolites were formed during the incubation process.

3.3. Separation, selectivity and sensitivity

Analysis of blank liver extract shows no interference in the final extract, although there were several endogenous

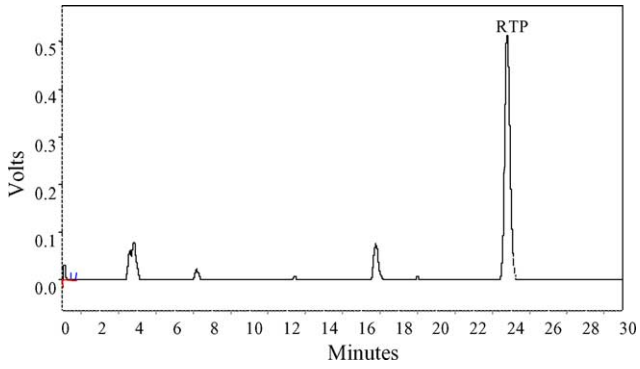

Fig. 3. Radiochromatogram of [^{3}H]RTP standard analyzing with a DEAD column.

peaks exhibited the same *m*/*z* transition at different retention times of ribavirin. Uridine was the major endogenous peak (retention time at approximately 3 min). A typical chromatogram of a blank liver extract is presented in Fig. 5. The result indicates that the method provides adequate separation and selectivity of ribavirin and endogenous components in liver through HPLC separation and MS/MS monitoring. The method provides acceptable sensitivity for the compound of interest. Typical low-QC has a signal-to-noise ratio greater than 5 in the validation. Typical chromatograms for the low-QC sample are presented in Fig. 6.

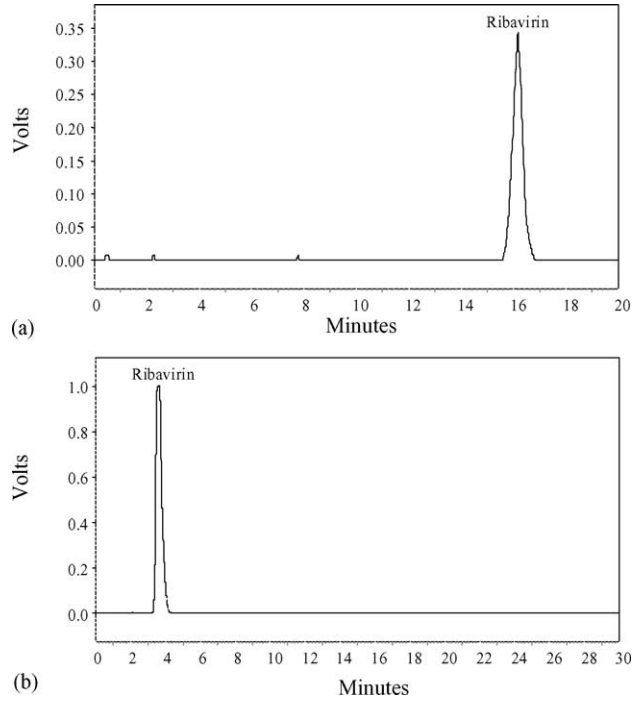

Fig. 4. Radiochromatograms of [^{3}H]ribavirin triphosphate in monkey liver incubated at 37 °C for 1 h: (a) Devosil column analysis; (b) DEAD column analysis.

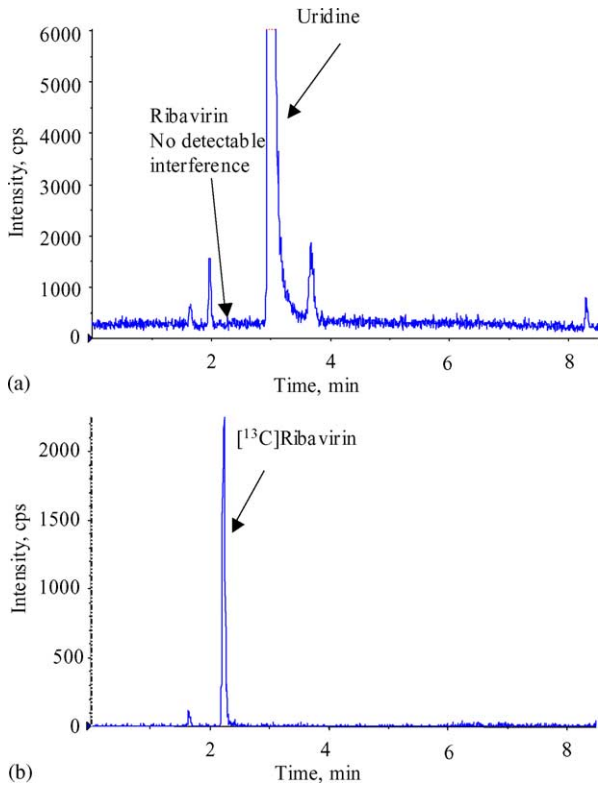

Fig. 5. Typical extracted ion chromatograms of liver control blank extract: (a) ribavirin; (b) [^{13}C]ribavirin.

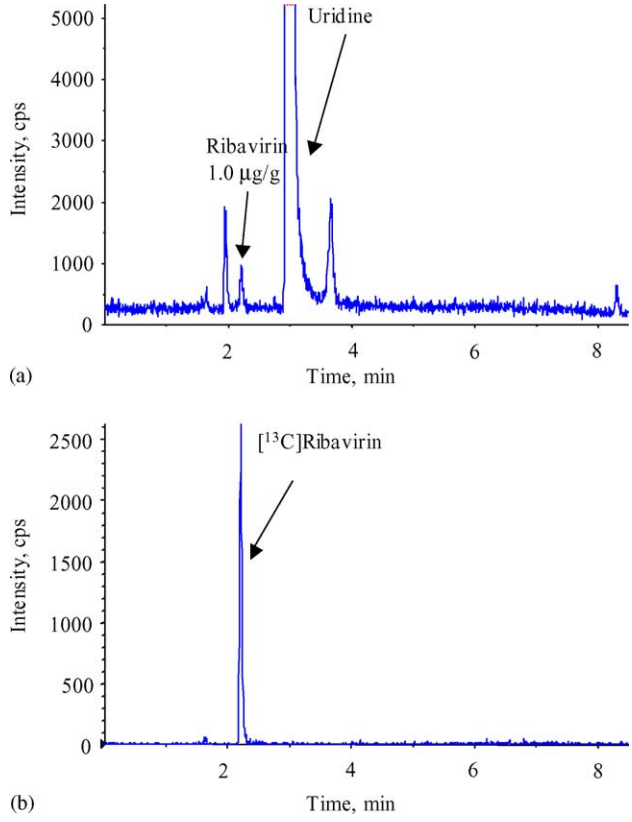

Fig. 6. Typical extracted ion chromatograms of liver LOQ (1.0 μg/g) extract: (a) ribavirin; (b) [^{13}C]ribavirin.

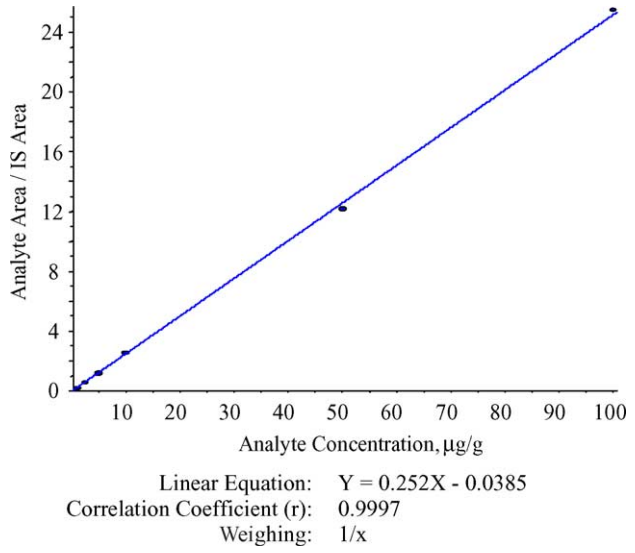

Fig. 7. Typical calibration curve for the determination of ribavirin in monkey liver.

3.4. Standard curve linearity

For the linear regression analysis, the correlation coefficients (r) were greater than 0.998 for each of all of the calibration curve determinations 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 Fig. 7. Mean back-calculated values from the fitted curve are within 3% of their nominal values between 1.0 and 100 μg/g (Table 4).

3.5. Accuracy and precision

Accuracy and precision are determined based on low-, mid- and hi-QC samples. Intra-assay shows mean accuracies are within −5.0 to 9.0% of their nominal values with CV varies between 0.55 and 6.42%. Inter-assay shows mean accuracies are within −0.35 to 8.3% of their nominal values with CV varies between 4.59 and 5.34%. The recovery data and statistics are presented in Table 5.

Table 4
Calibration curve analytical results for ribavirin in monkey liver

Curve ID	Concentration (μg/g)					
	1.00	2.50	5.00	10.0	50.0	100
1	1.01	2.45	4.93	10.1	52.0	98.1
2	1.07	2.41	4.77	9.79	53.2	97.3
3	0.989	2.44	5.14	9.82	52.1	98.0
4	1.01	2.47	4.86	10.5	48.5	101
5	0.827	2.46	5.76	10.7	47.5	101
Mean	0.98	2.45	5.09	10.2	50.7	99.1
S.D.	0.091	0.023	0.398	0.406	2.50	1.78
CV (%)	9.3	0.9	7.8	4.0	4.9	1.8
n	5	5	5	5	5	5
Bias (%)	−1.9	−2.2	1.8	1.8	1.2	−0.9

Table 5
Quality control analytical results for ribavirin in monkey liver

	Low-QC, (1.00 μg/g)	Mid-QC (10.0 μg/g)	High-QC (100 μg/g)
	1.11	10.5	99.5
	1.11	10.4	101
	1.01	10.4	94.1
Mean	1.08	10.4	98.2
S.D.	0.06	0.06	3.6
CV (%)	5.36	0.55	3.7
Bias (%)	7.7	4.3	−1.8
	1.04	9.62	101
	1.17	9.53	95.6
	1.06	9.34	107
Mean	1.09	9.50	101
S.D.	1.07	0.14	5.70
CV (%)	6.42	1.51	5.64
Bias (%)	9.0	−5.0	1.2
Overall			
Mean	1.08	9.97	99.7
S.D.	0.06	0.52	4.58
CV (%)	5.34	5.24	4.59
Bias (%)	8.3	−0.35	−0.30

3.6. Validation with monkey liver following oral dosing of [^{14}C]ribavirin

Liver sample from monkey dosed with [^{14}C]ribavirin at 10 mg/kg was analyzed to determine the amount of ribavirin. The results were then compared to the values obtained from radioactive detector analysis. The results are summarized in Table 6. Various amounts of liver were used in the analysis to demonstrate that the method can adapt to different sample size. This is critical because the method is intended to analyze biopsy samples from human patients which sample size will be variable and limited. Slightly higher CV (%) was observed in this analysis than typical plasma or RBC analysis. One major factor can attribute to the variation will be the homogeneity of the sample, especially when very small size of the liver sample was used for the analysis.

Table 6
Monkey liver analysis for [^{14}C]ribavirin treated monkey

Liver weight (mg)	Total ribavirin (μg/g)		Accuracy (%)
	^{14}C analysis	LC–MS analysis	
24.57	5.6	5.1	91.1
11.22	5.6	6.7	119.6
5.06	5.6	6.2	110.7
Mean			107.1
S.D.			14.6
CV (%)			13.6

Table 7
Hepatitis C patients liver biopsy analysis for total ribavirin

ID	Liver final concentration (μg/g)	Whole blood steady state (μg/mL)
1	0.0	8.10
2	26.8	94.6
3	112.2	65.8
4	282.3	No sample
5	367.0	189.6

3.7. Determination of total ribavirin in monkey liver

The method has been used to determine total ribavirin concentration from monkeys dosed at 300 mg/kg for 28 days. Liver samples from three monkeys show ribavirin levels at 259.4, 233.8 and 246.0 μg/g with mean value at 246.4 μg/g or 1 μmol/g (1 mM).

3.8. Determination of total ribavirin in human liver

Liver biopsy samples from hepatitis C patients under ribavirin treatment were analyzed by this method. The results are summarized in Table 7. The concurrent analysis of total ribavirin in whole blood is also presented in Table 7. Patient #1 withdrawn from the treatment several weeks prior to sampling shows no detectable level of ribavirin in liver and very low level of ribavirin in whole blood. The results indicated a correlation between liver and whole blood concentration ($R^2 = 0.81$, Fig. 8). It also reveals that significant high ribavirin concentration can be found in liver.

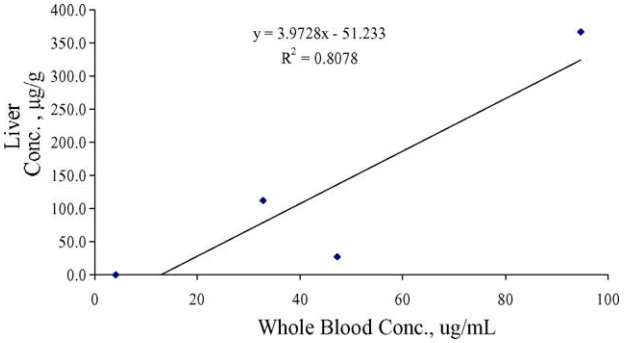

Fig. 8. Correlation of total ribavirin in human liver and whole blood.

4. Discussion

Glue reported that following multiple oral dosing of ribavirin, ribavirin levels in RBC accumulated with time leading to hemolytic anemia [4]. Recently, a LC–MS/MS method has been developed and applied to the analysis of RBC from ribavirin-treated hepatitis C patients. The results indicate that significant accumulation of phosphorylated ribavirin in RBC [10]. However, the information regarding the accumulation of ribavirin and its phosphorylated metabolites in liver, its target organ, is not available.

To evaluate the accumulation of ribavirin and its phosphorylated metabolites in animal and human, we developed a LC–MS/MS method for the quantitation of total ribavirin in liver. This method is similar to the RBC analysis with modification in the sample extraction. The method has a linear range from 1.0 to 100 μg/g and acceptable accuracy (−0.30 to 8.3% of nominal value for bias) and precision (4.59–5.34% for CV). This method has been validated by analyzing total ribavirin concentration in monkey liver from [^{14}C]ribavirin single dose study. Using ^{14}C radioactivity detection and LC–MS/MS analysis, the results shown in Table 6 indicate good agreement with a mean difference of 7.1% (−8.9 to 19.6%) between LC–MS/MS and radioactivity measurement. The method uses acid phosphatase to convert all ribavirin phosphates to corresponding ribavirin for LC–MS/MS quantitation. This is important because RMP was predominant in freshly collected liver from monkeys does with [^{14}C]ribavirin for 10 days (Fig. 9).

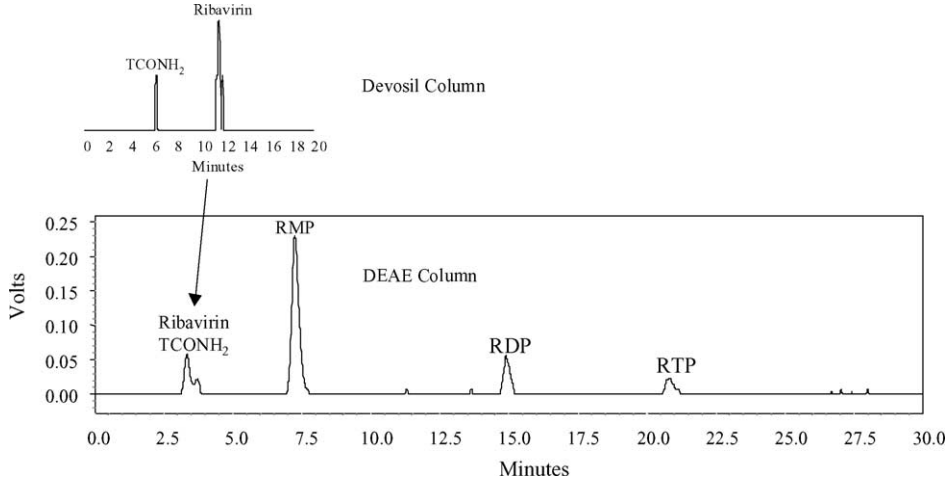

Fig. 9. Representative radiochromatogram of 240 h monkey liver extract from 10 mg/kg in vivo ribavirin, 10 days oral dosing experiment.

5. Conclusion

A method has been successfully validated to determine the concentration of total ribavirin in monkey liver. The method converts all phosphorylated metabolites to ribavirin and measures total ribavirin over the concentration range of 1.0–100 μg/g with a validated limit of quantitation (LOQ) of 1.0 μg/g. This method has been used to measure total ribavirin concentration in monkey liver samples from single oral dosing ^{14}C-metabolism study and 28-day toxicology study. Results indicate very high total ribavirin levels in liver after prolonged oral dosing of ribavirin. The analysis of liver biopsy samples from hepatitis C patients also indicates significant levels of total ribavirin accumulated in liver.

Acknowledgements

We would like to show our sincere gratitude to Dr. Nikolai and Dr. Haigh for providing the liver and whole blood samples in this study. The authors express their sincere appreciation to Dr. J. Lau for his support in this work.

References

[1] J. Witkowski, R. Robins, R. Sidwell, L. Simon, J. Med. Chem. 15 (1972) 1150–1154.
[2] R. Sidwell, J. Huffman, G. Khare, L. Allen, J. Witkowski, R.R. Virazole, Science 177 (1972) 705–706.
[3] R. Tam, J. Lau, Z. Hong, Antiviral Chem. Chemother. 172 (2002) 260–272.
[4] P. Glue, Seminars Liver Dis. 19 (1999) 17–24.
[5] D. Smee, T. Matthews, Antimicrob. Agents Chemother. 30 (1986) 117–121.
[6] T. Zimmerman, R. Deeprose, Biochem. Pharmacol. 27 (1978) 709–716.
[7] T. Page, J.D. Connor, Int. J. Biochem. 22 (1990) 379–383.
[8] J. Miller, L. Kigwana, D. Streeter, R. Robins, L. Simon, J. Roboz, Annu. N.Y. Acad. Sci. 284 (1977) 211–229.
[9] C. Lin, D. Lourenco, G. Xu, L. Yeh, Antimicrob. Agents Chemother. 48 (2004) 1872–1875.
[10] L. Yeh, M. Nguyen, C. Lin, J. Chromatogr. Sci. 41 (2003) 255–260.