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Specific, sensitive and accurate liquid chromatographic–tandem mass spectrometric method for the measurement of ribavirin in rat and monkey plasma

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Abstract

Ribavirin is a purine nucleoside analog with broad spectrum activity against a spectrum of DNA and RNA viruses. To facilitate pharmacokinetics studies, a LC–MS–MS method for the analysis of ribavirin in rat and monkey plasma was developed and validated. The method involved the addition of acyclovir as an internal standard and protein precipitation with acetonitrile followed by separation by an Intertsil Silica column and quantification by a MS–MS equipped with a positive electrospray ionization in the multiple reaction monitoring mode. The MS–MS reaction was selected to monitor the 245→113 and 226→152 transitions for ribavirin and internal standard, respectively. The calibration curve was linear over a concentration range of 10–5000 ng/ml. The lower limit of quantitation was 10 ng/ml, the coefficient of variation (CV) was 8–11%, and the bias was 1–3%. Intra-day and inter-day analysis of QC samples at 30, 1500 and 3500 ng/ml indicate that the method was precise (CV<18%) and accurate (bias<13%). Ribavirin in rat and monkey plasma was stable at 5 °C for at least 24 h, 0 °C for at least 4 h, and after three freeze–thaw cycles. This specific, accurate and precise assay is useful in the study of the pharmacokinetics of this compound.

Keywords: Ribavirin

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analog first synthesized by Drs Joseph Witkowski and Roland Robins in 1972 [1]. It was reported to have broad-spectrum activity against a variety of DNA and RNA viruses in 1972 [2]. It is approved as monotherapy for the treatment of severe respiratory syncytical virus (RSV) infection in children with respiratory distress and in combination with interferon alpha (IFNα) for the treatment of chronic hepatitis C virus (HCV) infection. It is widely accepted that ribavirin is a pleiotropic antiviral agent having multiple biologic activities which singly or collectively may contribute to its clinical efficacy in the treatment of viral infections. A number of studies have suggested that the in vivo activity of ribavirin may be ascribed to at least two distinct activities: direct versus indirect antiviral effects [3]. The direct antiviral

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activity of ribavirin is consistent with its intracellular metabolic profile in that ribavirin is converted to mono-, di- and tri-phosphorylated metabolites, which is characteristic of all known nucleoside analogues used clinically [4]. Ribavirin may also elicit indirect antiviral effects by: (1) promoting T-cell mediated immunity by inducing a T-helper subset 2 to T-helper subset 1 bias [5–7] and/or (2) by reducing the intracellular GTP concentration by inhibiting host enzyme inosine monophosphate dehydrogenase (IMPDH) [8].

To better understand the mechanism of action of ribavirin as well as its pharmacokinetic characteristics, an assay that can allow specific, sensitive and accurate measurement of ribavirin in biologic samples is critical. Austin et al. [9] described a radioimmunoassay for the measurement of ribavirin which is capable of determining plasma concentrations down to 0.01 μ*M* (2.45 ng/ml). However, the method is not very specific. The rabbit antibody used in the assay has been shown to cross-react with many of the major metabolites of ribavirin. Therefore, this radioimmunoassay is not an ideal method for measuring plasma ribavirin concentrations for the evaluation of pharmacokinetic parameters. Paroni et al. [10] described a HPLC with UV detection method for measuring ribavirin. This method has an assay sensitivity of 100 ng/ml and thus has limited usefulness in the pharmacokinetic evaluation of ribavirin in animals and man after oral administration.

We have developed a LC–MS–MS method for the specific, sensitive and accurate measurement of ribavirin in rat and Cynomolgus monkey plasma. This assay was validated and has been used in the study of pharmacokinetic parameters in rats and monkeys.

2. Experimental

2.1. Chemicals

Ribavirin (Fig. 1) was supplied by ICN Pharmaceuticals (Costa Mesa, CA, USA). Acyclovir (internal standard) (Fig. 1) was purchased from Sigma (St Louis, MO, USA), Acetonitrile (HPLC grade) and water (HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Trifluoroacetic acid was obtained from JT Baker (Phillipsburg, NJ, USA).

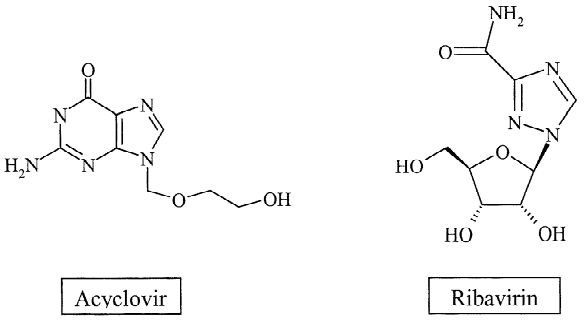

Fig. 1. Chemical structure of acyclovir (internal standard) and ribavirin.

2.2. Drug administration and plasma sample collection

Protocols for the following studies in rats and Cynomolgus monkeys were reviewed and approved by the Institutional Animal Care and Use Committee at Premedica (Worcester, MA, USA).

Male Sprague–Dawley rats ($n=3$) weighing approximately 300 g were used in this study. After an overnight fast, the rats received an intravenous (30 mg/kg) or an oral dose (30 mg/kg) of ribavirin. Serial blood samples at various time points were collected via cardiac puncture directly into heparinized vacutainer™ tubes following anesthesia with CO_2 and immediately centrifuged for 15 min to harvest the plasma. The plasma samples were stored at −20 °C until they were analyzed.

Male Cynomolgus monkeys ($n=4$) weighing about 5 kg were used in this study. After an overnight fast, the monkeys received an intravenous (10 mg/kg) or an oral dose (10 mg/kg) of ribavirin. Serial blood samples were collected from the indwelling venous cannula and vascular access port at various time points directly into heparinized vacutainer™ tubes and prepared in a similar fashion as rat plasma.

2.3. Sample preparation

Twenty microliters of internal standard (acyclovir, 2 μg/ml of acetonitrile/water in a 1:1 ratio) were

added to a 50-μl aliquot of rat or monkey plasma, 20 μl of water, and 0.425 ml of acetonitrile. The mixture was placed in a vortex mixer for 1 min and centrifuged for 5 min at 3500 rpm. The organic layer was transferred to another tube and evaporated to dryness at 35 °C. The residue was dissolved in 200 μl of HPLC mobile phase solution (see Section 2.4), and a 3-μl aliquot of the mixture was injected onto the LC–MS–MS system.

2.4. LC–MS–MS system

The HPLC system consisted of a Perkin-Elmer Series 200 Micro LC pump and a Perkin-Elmer Series 200 autosampler (Perkin-Elmer Instruments, Shekton, CT, USA) fitted with Peltier cooling trays (maintained at 5 °C). Separation was accomplished on an Intertsil Silica column, 50×3.0-mm, 5-μm (Keystone Scientific, Bellefonte, PA, USA), maintained at 40 °C. The mobile phase solution consisted of acetonitrile and 0.05% trifluoroacetic acid (TFA) in water (95:5, v/v) delivered at 0.5 ml/min. The effluent from the HPLC system was connected directly to a Perkin-Elmer Sciex API 3000 MS system (PE SCIEX, Foster City, CA, USA) equipped with a standard positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The operating conditions of mass spectrometry are summarized in Table 1. Spectra for product ion scan for acyclovir (internal standard) and ribavirin are shown in Fig. 2.

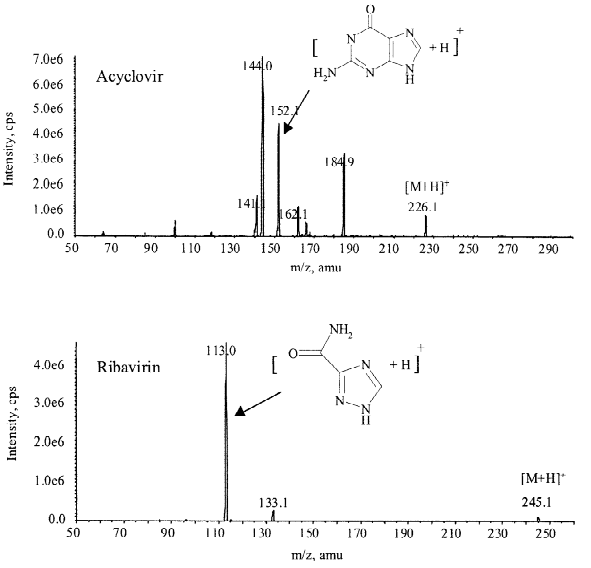

Fig. 2. Spectra of product ion scan for acyclovir and ribavirin.

2.5. Method of evaluation

Precision (%CV) and accuracy (%bias) were evaluated from the back-calculated concentrations of nine standard curves prepared in plasma. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the standard curve where the

Table 1
Mass spectrometry operating conditions

Interface	Turbo Ion Spray
Polarity	Positive
Scan type:	MRM
Resolution:	Q1—unit, Q3—low
Curtain gas (CUR):	12.0
Collision gas (CAD):	3.0
Ion spray voltage (IS):	5500
Temperature (TEM):	400 °C
Ion source gas 1 (GS1):	10.0
Ion source gas 2 (GS2):	6500

Compound	Mass to charge ratio (m/z)		Time (ms)	Collision energy (V)
	Q1	Q3		
Ribavirin	245	113	200	15
Acyclovir	226	152	25	17

%CV (nine replicates) and bias from the back-calculated concentrations were <20%. Specificity was established by the lack of interference peaks at the retention time for the internal standard and ribavirin. The recovery of the internal standard and ribavirin was determined generating a standard curve in a mixture of water–acetonitrile (50:50, v/v). The stability of ribavirin (30, 1500 and 3500 ng/ml) was evaluated for 24 h at room temperature, 4 h at 0 °C, and at the end of three freeze–thaw cycles.

2.6. Pharmacokinetic analysis

Plasma concentrations above the LLOQ were used for pharmacokinetic analysis using a model-independent method. Maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were the observed values. The area under the plasma concentration–time curve from time zero to the time of the final measurable sample [AUC (tf)] were calculated using the linear trapezoidal method. The elimination rate constant (K) was estimated as the negative slope of the regression of log concentration versus time. Half-life ($T_{1/2}$) was calculated by dividing 0.693 by K. Bioavailability was calculated by dividing the AUC (tf) obtained after oral dosing by the AUC (tf) obtained after intravenous dosing.

3. Results

Typical LC–MS–MS chromatograms for ribavirin and the internal standard extracted from plasma are shown in Figs. 3–8. The calibration curve was obtained by fitting the ratio of peak height of ribavirin to that of internal standard against a range of concentrations (10–5000 ng/ml) of added ribavirin using $1/X^2$ quadratic regression plots. Correlation coefficients were greater than or equal to 0.995 in both rat plasma and monkey plasma. The LLOQ was 10 ng/ml, and was characterized by a small coefficient of variation (10.8% in rat plasma and 8.2% in monkey plasma) and a small bias (1% in rat plasma and 3% in monkey plasma). Specificity based on endogenous interfering peaks was evaluated in six lots of rat and monkey plasma samples obtained from untreated animals. The results demonstrated a lack of analytically-significant interference at the same mass transitions and chromatographic retention as ribavirin in rat plasma (Fig. 3) and monkey plasma (Fig. 6), indicating that the LC–MS–MS analysis for ribavirin is specific.

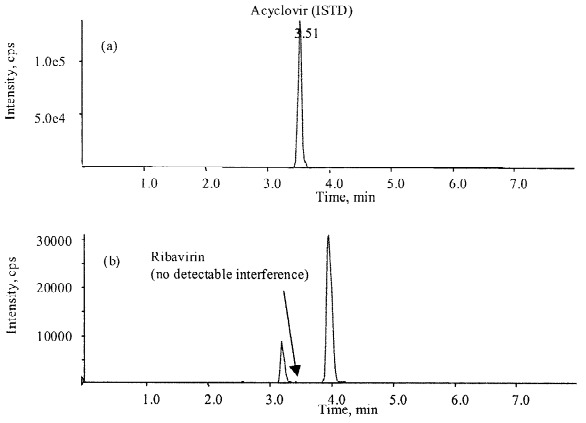

Fig. 3. Rat control plasma spiked with acyclovir. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

Intra-day and inter-day precision and accuracy of the method were evaluated as 30, 1500 and 3500 ng/ml. The results in rat plasma (Table 2) and monkey plasma (Table 3) demonstrated that LC–MS–MS method was accurate (bias<13%) and reproducible (CV<18%) in both species.

The recovery of ribavirin (10, 1000 and 5000

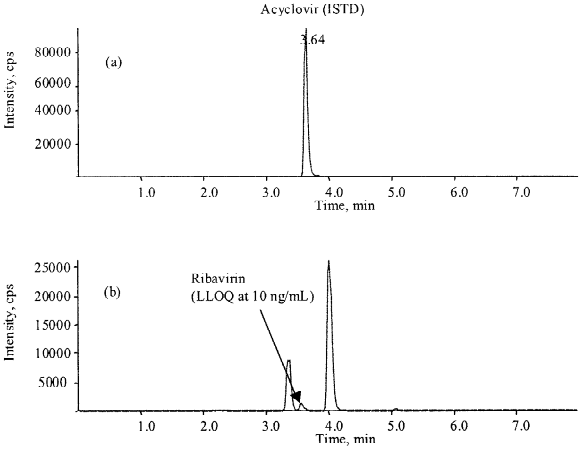

Fig. 4. Rat control plasma spiked with acyclovir and 10 ng/ml of ribavirin. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

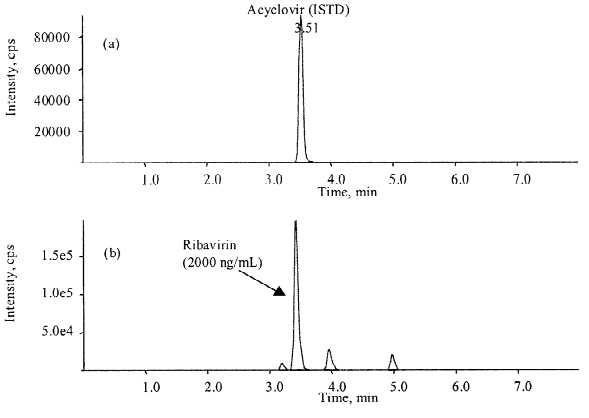

Fig. 5. Rat control plasma spiked with acyclovir and 2000 ng/ml of ribavirin. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

ng/ml) from rat and monkey plasma was determined by comparing the peak areas obtained from samples which were spiked with standard followed by extraction to the peaks areas obtained from samples which were extracted first followed by spiking with standard. Recovery was estimated to be >54%, with the CV ranging from 1 to 25% (Table 4). Ribavirin in rat and monkey plasma was found to be stable at 5 °C for at least 24 h and at 0 °C for at least 4 h. Ribavirin was also stable after three freeze–thaw cycles (Table 5).

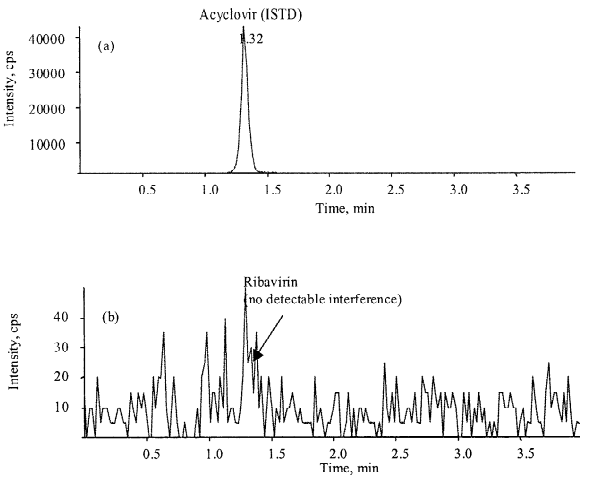

Fig. 6. Monkey control plasma spiked with acyclovir. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

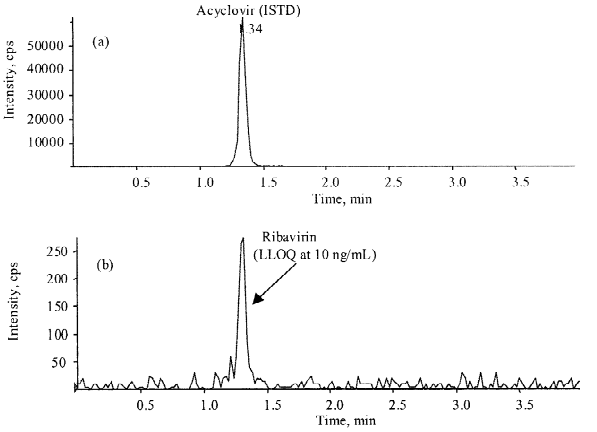

Fig. 7. Monkey control plasma spiked with acyclovir and 10 ng/ml of ribavirin. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

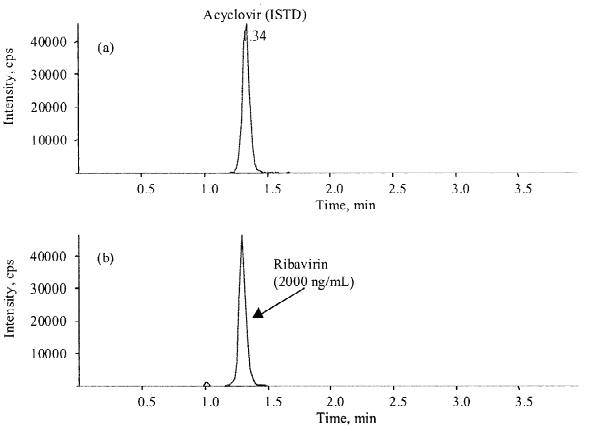

Fig. 8. Monkey control plasma spiked with acyclovir and 2000 ng/ml of ribavirin. (a) Extracted ion chromatogram for acyclovir (internal standard); (b) extracted ion chromatogram for ribavirin.

Table 2
Intra-day and inter-day variation of ribavirin analysis based on the LC–MS–MS method in rat plasma

	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Bias (%)
Intra-day	30	28.2	15.0	6.1
	1500	1692	14.0	12.8
	3500	3576	7.8	2.2
Inter-day	30	27.8	17.2	7.5
	1500	1506	15.0	0.4
	3500	3347	6.4	4.4

Table 3
Intra-day and inter-day variation of ribavirin analysis based on the LC–MS–MS method in monkey plasma

	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Bias (%)
Intra-day	30	30.7	6.9	2.3
	1500	1373	4.6	8.5
	3500	3372	7.3	3.7
Inter-day	30	30.7	6.5	2.3
	1500	1437	12.2	4.2
	3500	3417	6.1	2.4

Table 4
Recovery of ribavirin from rat and monkey plasma

	Concentration (ng/ml)	Recovery (%)	C.V. (%)
Rat	10	69.9	24.6
	1000	72.8	7.4
	5000	66.9	5.26
Monkey	10	53.8	20.4
	1000	69.7	7.1
	5000	67.3	0.7

This method was used to analyze plasma samples from rats (30 mg/kg) and Cynomolgus monkeys (10 mg/kg) after an oral and intravenous dosing. The mean pharmacokinetic parameters are summarized in Table 6. After intravenous administration, $T_{1/2}$ was 9.9 h in rats and 130 h in monkeys. Following oral administration, the maximum plasma concentration of ribavirin was 0.433 μg/ml in rats and 0.48 μg/ml in monkeys. Bioavailability was 27.1% in rats and 59.3% in monkeys.

4. Discussion

Austin et al. [9] developed a competitive binding radioimmunoassay for the measurement of ribavirin in serum and urine. The assay can detect ribavirin at concentrations as low as 0.25 ng/ml. This RIA, however, is not specific since anti-ribavirin serum also cross-reacts with the major metabolites of

Table 5
Stability of ribavirin in rat and monkey plasma

	Time and conditions	Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Bias (%)
Rat plasma	24 h at 5 °C	30	28.2	17.3	6.0
		1500	1423	1.6	5.2
		3500	3059	5.5	12.6
	End of three F/T cycles	200	214	3.6	7.0
		4000	3783	4.7	5.4
	4 h at 0 °C	200	214	5.5	7.0
		4000	4083	2.8	2.1
Monkey plasma	24 h at 5 °C	30	34.5	10.7	15.1
		1500	1457	7.0	2.8
		3500	3498	6.3	0.1
	End of three F/T cycles	200	210	4.9	5.0
		5000	5385	4.3	7.7
	4 h at 0 °C	200	219	4.5	9.5
		5000	5177	6.6	3.5

Table 6
Pharmacokinetic parameters of ribavirin in rats and monkeys

Species	Parameter	Unit	Intravenous	Oral
Rat	C_{max}	ng/ml	20 500	433
(30 mg/kg)	T_{max}	h	0.0833	1
	AUC	ng h/ml	11 200	3040
	$T_{1/2}$	h	9.9	8.1
	Bioavailability	%		27.1
Monkey	C_{max}	ng/ml	12 500	480
(10 mg/kg)	T_{max}	h	0.0833	2
	AUC	ng h/ml	45 500	27 000
	$T_{1/2}$	h	130	179
	Bioavailability	%		59.3

ribavirin. Moreover, this method involves the routine use of tritium-labeled compounds. Therefore, RIA is not an appropriate method for the evaluation of ribavirin pharmacokinetics parameters. Paroni et al. [10] developed an HPLC method with a LLOQ of 100 ng/ml for the measurement of ribavirin concentrations in human serum and urine. It thus appears that this HPLC method does not have sufficient sensitivity to properly evaluate the pharmacokinetic parameters for ribavirin in animals and man.

Until recently, most analytical methods for measuring drug concentrations in biological samples have been based on GC or HPLC methods. The introduction of the HPLC-API/MS–MS system (HPLC combined with atmospheric pressure ionization tandem mass spectrometry) has provided new opportunities for the development of more sensitive and specific methods. We report here the development of a LC–MS–MS method for the measuring ribavirin concentrations in rat and monkey plasma.

In the HPLC method, plasma samples are cleaned up with liquid/liquid phase extraction, followed by evaporation to dryness, reconstitution with HPLC mobile phase solution, and UV detection. The sample preparation is tedious and requires 0.2 ml of plasma. In contrast, the LC–MS–MS method reported here is much simpler than the HPLC method, does not require liquid/liquid phase extraction, and only requires 0.05 ml of plasma. We also show here that the LC–MS–MS method is both specific for ribavirin and is characterized by a LLOQ of 10 ng/ml. This sensitivity is sufficient to measure plasma concentrations after more than nine half-lives for C_{max} in rats (20.5 μg/ml) following the intravenous administration of 30 mg/kg, for C_{max} in monkeys (12.5 μg/ml) following the intravenous administration at 10 mg/kg and for C_{max} in man (0.782 μg/ml) following a single oral dose of 600 mg [11].

5. Conclusions

An LC–MS–MS assay for the measurement of ribavirin concentrations in rat and monkey plasma was established and validated, and was shown to be specific, sensitive, and accurate over a concentration range of 10–5000 ng/ml (CV<18%; bias<13%). Ribavirin was found to be stable at 5 °C for at least 24 h and after three freeze–thaw cycles. The method was found to be useful for determination of plasma concentrations of ribavirin in rats and monkeys following intravenous and oral administration.

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