Development and validation of a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of ribavirin in human plasma and serum

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Abstract

A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed for the analysis of antiviral agent ribavirin in human plasma and serum. The samples (0.1 ml) were extracted from the matrix using a simple protein precipitation procedure. The supernatants were evaporated to dryness, reconstituted and injected onto the LC/MS/MS system. The chromatography separation was achieved on a silica column operated with an aqueous–organic mobile phase. The use of a silica column not only provided adequate retention for the extremely polar compound of ribavirin, but also enhanced electrospray ionization sensitivity with the use of high percentage organic solvent in the mobile phase. The method has been validated over the concentration range of 10–10 000 ng/ml ribavirin in human plasma and serum. Bamethan was used as the internal standard. The protein precipitation extraction has been automated based on 96-well format with the use of robotic liquid handlers to improve the overall throughput of the analysis. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Ribavirin (Fig. 1) is a synthetic nucleoside with a broad spectrum of antiviral activity. It has been used in treating respiratory syncytial viral infections in infants and young children [1,2]. More recently, ribavirin, in combination with interferon alfa-2b, was approved as treatment for hepatitis C [3]. It also has been used in clinical trials for the treatment of acquired immunodeficiency syndrome (AIDS) and AIDS-related complex [4], as well as hepatitis A and B [5,6].

Several analytical methods for quantitating ribavirin in biological fluids have been reported [7–10]. Most of these are reversed-phase (RP) high performance liquid chromatography (HPLC)

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methods with ultraviolet (UV) detection. However, due to the extreme polar nature of the ribavirin molecule, a tedious solid phase extractions (SPE) using a phenyl boronic acid (PBA) phase had to be carried out to isolate ribavirin and the internal standard (IS) from the matrix. Furthermore, mobile phases with very low organic solvent (< 2%), or no organic at all had to be used in RP HPLC in order to obtain adequate retention for ribavirin and separate it from endogenous interference.

Liquid chromatography coupled with atmospheric pressure ionization/tandem mass spectrometry (LC/API/MS/MS) has become the method of choice for bioanalysis [11]. This paper reports the development and validation of an electrospray ionization (ESI) LC/MS/MS method for the determination of ribavirin in plasma and serum. A simple protein precipitation was used to extract ribavirin and IS bamethan from a 0.100-ml of plasma or serum sample. Chromatography separation was achieved on a silica column operated with an acetonitrile (ACN)–water mobile phase. The automation of the sample preparation step is also discussed.

2. Experimental

2.1. Chemicals and reagents

Ribavirin (purity 100%) was from USP (Rockville, MD, USA). Bamethan sulfate (purity > 99%) was from Sigma (St. Louis, MO, USA).

Trifluoroacetic acid (TFA, 97%) was from Fisher (St. Louis, MO, USA). HPLC grade water, ACN and methanol were also from Fisher. Control human serum and human plasma containing EDTA K3 as an anticoagulant were from Biochemed Pharmacologicals (Winchester, VA, USA).

2.2. Sample preparation

Samples were briefly vortex-mixed and centrifuged at 3000 rpm ($2560 \times g$) for 10 min on a Beckman Coulter J2-HS centrifuge (Beckman Coulter, Fullerton, CA, USA). Aliquots of 100 μl of sample were then transferred from vials into 12×75 mm glass test tubes (Fisher) by the Multi-PROBE II automated sample handling system (Packard Instrument Company, Meriden, CT, USA) controlled by the WinPrep software. IS solution (20.0 μl, 100 ng/ml of bamethan in water) was then added to all samples except blanks by the MultiPROBE II. ACN (500 μl) was added to each test tube manually and the samples were vortexed for 3 min on a multi-tube vortex mixer (Scientific Manufacturing Industries, Emerycille, CA, USA). The samples were then centrifuged at 3000 rpm ($2560 \times g$) for 10 min and the supernatants were transferred manually into another set of 12×75 mm glass test tubes. The extracts were evaporated to dryness under a stream of nitrogen in a TurboVap solvent evaporator (Zymark, Hopkinton, MA, USA) set at 45 °C and reconstituted in 500 μl of mobile phase. After brief mixing, the extracts were transferred into polypropylene injec-
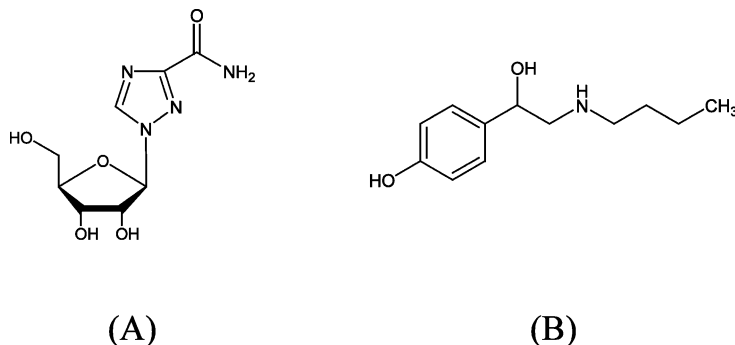

Fig. 1. The chemical structures of ribavirin (A) and IS bamethan (B).

tion vials (Waters, Milford, MA, USA) and were ready to be injected onto the LC/MS/MS system.

Later the extraction procedure was automated using 96-well format on a Tomtec Quadra 96-320 robot (Tomtec, Hamden, CT, USA). Samples were first transferred into a 1-ml 96-well deep well plate (Porvair Sciences, Shepperton, UK) by the MultiPROBE. The sample plate was then brought to a Tomtec Quadra 96-320 to carry out the semi-automated protein precipitation extraction. The Quadra was programmed to transfer 500 µl of ACN to each well of the sample plate. The plate was then covered with a polypropylene mat (Porvair Sciences) and vortexed for 3 min on a multi-tube vortex mixer. After centrifugation at 3000 rpm (1643 × g) on an Allegra™ 6 (Beckman Coulter) centrifuge, the sample plate was placed back on the Tomtec and the supernatants were transferred into a clean 1-ml deep well collection plate. The supernatants in the collection plate were dried under nitrogen using a TurboVap 96 concentrator (Zymark) and the residues were reconstituted with 500 µl of mobile phase using the Quadra. The collection plate was then heat-sealed with a Uniseal film (Whatman, Clifton, NJ, USA) and the extracts were ready to be injected onto the LC/MS/MS system.

2.3. LC/MS/MS method

The LC system used was a Shimadzu (Shimadzu, Columbia, MD, USA) series 10AD VP consisting of binary pumps, a degasser, a cooled autosampler (15 °C) and a system controller. Unless otherwise stated in the paper, a Betasil silica column (50 × 3 mm, 5 µm) from Keystone Scientific (Bellefonte, PA, USA) was used at a flow rate of 0.5 ml/min. The isocratic mobile phase was composed of 5:95 (v/v) A−B, where A was 0.05% TFA in water (v/v) and B was 0.05% TFA in ACN (v/v). Separation was performed at ambient temperature and the injection volume was 3 µl. The run time was less than 3 min.

Either an API 3000 mass spectrometer or an API III + mass spectrometer (both from Applied Biosystem, Foster City, CA, USA) equipped with its TurboIonspray interface was operated under positive mode for the development and validation work. The plasma samples were analyzed by the API 3000 while the serum samples were analyzed by the API III + . Nitrogen gas (99.999%) was used for all the gas supply. For the API 3000, the Ionspray needle was maintained at 5.5 kV. The turbo gas temperature was 350 °C and the auxiliary gas flow was 8.0 l/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 12, 8, and 5, respectively. The orifice (OR) and focusing ring (RNG) potentials were maintained at 26 and 120 V. The mass spectrometer was operated under selected reaction monitoring (SRM) mode with both quadrupoles maintained at unit resolution (0.7 mass unit at half height). For the API III + , the Ionspray needle was set at 5.5 kV. The nebulizing gas pressure was at 80 psi. The curtain gas flow rate was 1.0 l/min. The temperature for the turbo gas was set at 400 °C and the auxiliary gas flow was 8.0 l/min. The temperature for the interface heater was set at 60 °C. The orifice potential was maintained at 42 V. The collision gas thickness (CGT) was set at approximately 250×10^{13} atoms/cm^2. The mass spectrometer was operated under SRM mode with both quadrupoles maintained at unit resolution.

The SRM transitions monitored were $245 \rightarrow 113$ for ribavirin and $210 \rightarrow 192$ for bamethan with a dwell time of 400 ms for each pair. A Power Mac G3 workstation running Sample Control and MacQuan was used for data acquisition and processing for the API 3000 and a Quadra 800 Macintosh computer running Rad and MacQuan was used for the API III + . A weighted ($1/x^2$) linear least-squares regression was used to generate calibration curves from standards and calculate the concentrations of quality control (QC) samples.

Optimizations of the mass spectrometric conditions for ribavirin and bamethan were carried out by infusing 1.00 µg/ml solutions of ribavirin and bamethan dissolved in 1:1 mixture of ACN and water at 10 µl/min using a Harvard '22' syringe pump (Harvard Apparatus, South Natick, MA).

2.4. Preparations of standards and QC samples

Standards and QC samples were made from two separate ribavirin stock solutions (1.00 mg/ml

in methanol). Pooled calibration standards at concentrations of 10.0, 20.0, 50.0, 200, 800, 2000, 8000 and 10 000 ng/ml were prepared in blank matrix. QC samples at levels of 30.0, 500, 7500 and 40 000 ng/ml were prepared. All standards and QC samples were aliquoted into 2-ml polypropylene vials and stored frozen at −20 °C. Stock solutions were stored in polypropylene tubes in a refrigerator maintained at 2–8 °C.

2.5. Method validation

The method validations for plasma and serum matrix were carried out in similar fashions and included the following.

The method specificity was evaluated by screening six lots of blank matrix (plasma and serum) prior to the main validation batches. These lots were fortified with known concentrations of ribavirin, extracted and analyzed along with a calibration curve prepared in one of the six lots to confirm the absence of interference and lot-to-lot variation.

Three analytical batches were used to assess the precision and accuracy of the method. Each batch contained a single set of calibration standards and at least six replicates of regular QC samples at each level (30.0, 500 and 7500 ng/ml). One of the batches included six replicates of QC 40 000 and 7500 ng/ml after a fivefold dilution with blank matrix. In one of the batches six replicates of the low limit of quantitation (LLOQ) standards (10.0 ng/ml) were analyzed to assess the precision and accuracy at the LLOQ. One batch contained more than 100 samples to simulate the length of clinical sample analysis. QC samples and other test samples were interspersed among calibration standards. An extracted blank sample was always placed after the upper limit of quantitation (ULOQ) standard to determine the carry-over of the LC/MS/MS system.

Analyte stability through three freeze–thaw cycles and storage for 24 h at room temperature was tested. At least six replicates of QC 30.0, 500 and 7500 ng/ml were subjected to repeated freeze–thaw and room temperature storage, then extracted and analyzed. Stability study of ribavirin in human serum was carried out using only QC

30.0 and 7500 ng/ml. The obtained values for these QC samples were then compared to their theoretical concentrations. Stock solution stability was established by making a new stock solution of ribavirin and comparing the LC/MS/MS responses of secondary solutions (100 ng/ml in ACN) diluted from the freshly prepared and the stored stock solutions. The stock solution is considered stable if less than 5% difference in response is observed. Stability in reconstitution solvent was tested by re-injecting extracted samples (standard curve and six replicates of QC samples at each level) and comparing the results to those of freshly extracted samples.

Recovery of ribavirin using the protein precipitation procedure was evaluated by comparing the mean peak areas of the QC samples ($n = 6$) at 30.0, 500 and 7500 ng/ml extracted from matrix with those of matrix recovery samples prepared by adding compound to post-extraction matrix blanks at corresponding concentrations ($n = 6$). Matrix effect was determined by comparing the peak areas of the matrix recovery samples to those of neat ribavirin solutions ($n = 3$).

3. Results and discussion

3.1. Sample preparation

The extraction of ribavirin has been commonly achieved through SPE using a phenyl borate affinity gel column packed in-house [7,8] or a PBA cartridge bought commercially [9,10]. However, the gel extraction involved the tedious treatment of the extraction column while the SPE required gravity loading of the samples, or extensive pre-treatment of the cartridges. Thanks to the unparalleled specificity and high sensitivity of LC/MS/MS operated under SRM mode, we successfully simplified the extraction method by utilizing a protein precipitation procedure with ACN. Later this protein precipitation extraction step was further automated based on 96-well plate format by the use of robotic liquid handlers. The automation strategy will be discussed separately in the paper.

Table 1
Capacity factors (k') of ribavirin for tested columns at different mobile phase compositions

Column	B% in mobile phase			
	10	50	70	95
Inertsil ODS C18	0.30	0.10	[a]	0.21
Hypersil BDS C18	0.35	0.16	[a]	0.23
Hypersil HyPURITY C18	0.48	0.32	[a]	0.48
BioBasic C18	0.62	0.67	0.92	2.2
Betasil silica	[a]	0.58	0.78	2.3

The void volume for a 50×3 mm column was estimated as $V_0 = 0.487 \times d^2 \times L = 0.219$ ml.

[a] No data.

3.2. LC/MS/MS

In order to retain ribavirin on RP columns, mobile phase with extremely high water content had to be used. A related problem with the use of almost 100% aqueous mobile phase was the 'phase collapse' phenomenon encountered with RP columns [10]. Typically very long analysis time (> 15 min) was required with UV detection. The need to regenerate the analytical column using high organic mobile phase after each injection to elute the strongly retained hydrophobic interference under the aqueous mobile phase used during analysis further lengthened the cycle time for each sample. Granted, with the added mass resolution offered by LC/MS/MS, total run time can be shortened vastly compared to HPLC/UV. Nevertheless, matrix suppression caused by endogenous components in biological extracts has
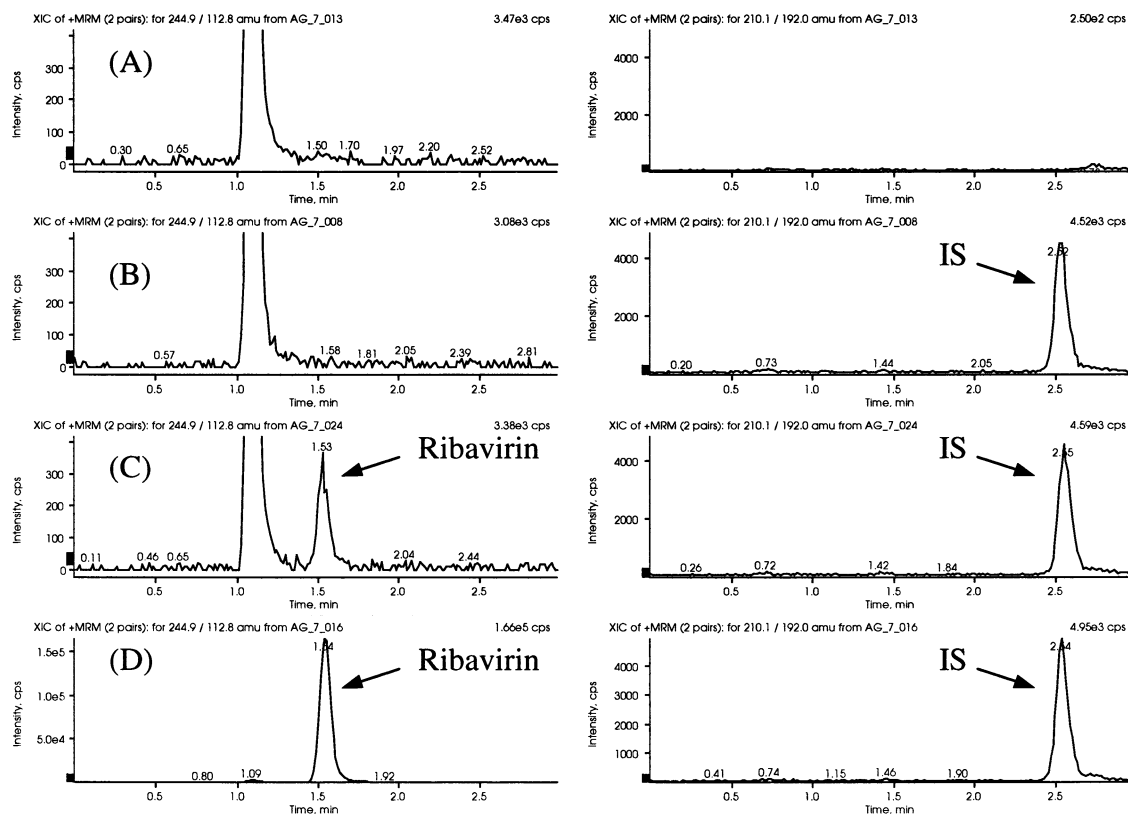

Fig. 2. Mass chromatograms of ribavirin (left column) and IS (right column) from: (A) a plasma blank; (B) a control zero (blank plasma fortified with IS only); (C) an LLOQ plasma standard (10.0 ng/ml); and (D) a high QC sample (7500 ng/ml).
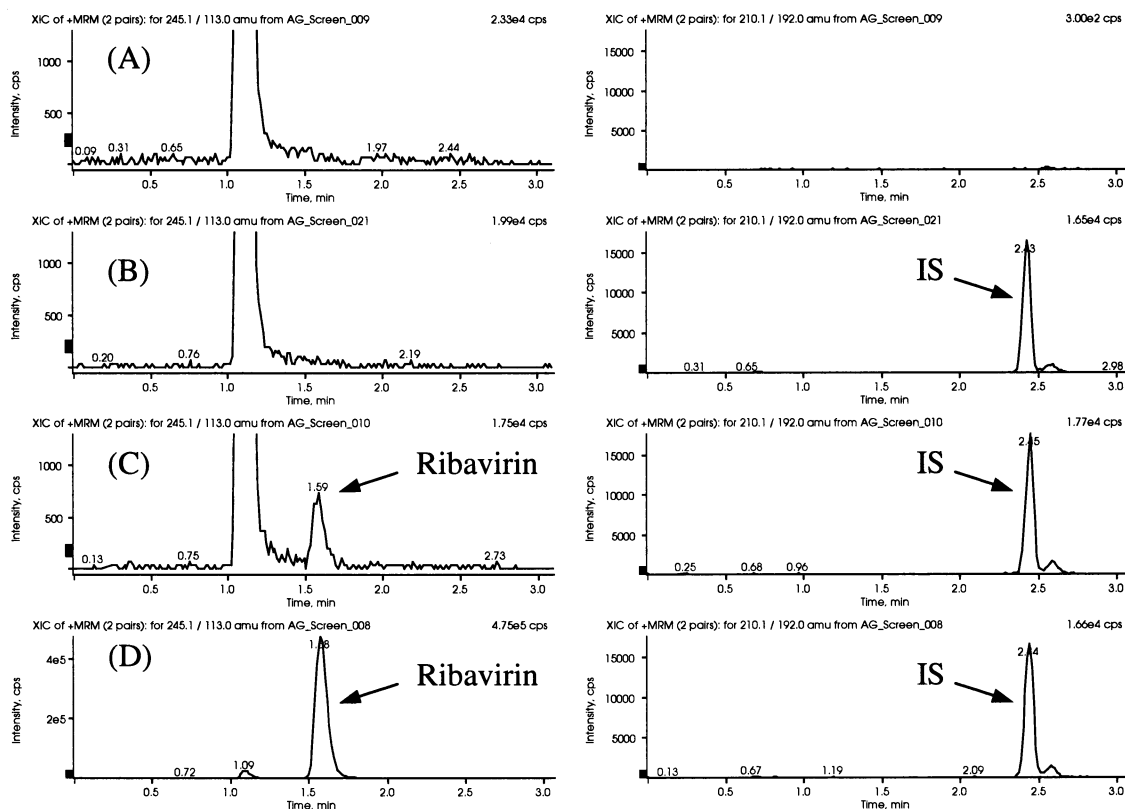
Fig. 3. Mass chromatograms of ribavirin (left column) and IS (right column) from: (A) a serum blank; (B) a control zero (blank serum fortified with IS only); (C) an LLOQ serum standard (10.0 ng/ml); and (D) a high QC sample (7500 ng/ml).

been reported to be the leading reason for assay failure for LC/ESI/MS/MS methods [12–14]. Therefore, adequate retention still needs to be maintained in order to avoid possible matrix effect. With typical RP chromatography, mobile phase with less than 5% organic had to be used in order to retain ribavirin. However, such a condition yields poor electrospray sensitivity: water is a much poorer solvent for electrospray than organic solvents such as methanol or ACN due to water's high surface tension and large heat of evaporation. This difficulty was solved by the use of a silica column with a mobile phase with high percentage of ACN. We have demonstrated [15–18] that this technique is a viable method for the LC/MS/MS analysis of polar compounds in biological fluids. The use of mobile phases with high organic content (usually from 70 to 95%) is naturally compatible with both ESI and atmospheric pressure chemical ionization

(APCI) techniques and can enhance the analytical sensitivity considerably.

To demonstrate the applicability of the silica columns for this assay, several different C18 columns, along with a silica column were screened for the analysis ribavirin. The C18 columns tested included Intertsil ODS C18, Hypersil BDS C18, Hypersil HyPURITY C18 and BioBasic C18 column. The silica column used was a Betasil silica column. All columns used were of 50×3 mm dimension with 5 μm particle size. The mobile phase consisted of different percentages of A and B, where A was 1% formic acid in water and B was 1% formic acid in ACN. The flow rate was maintained at 0.5 ml/min. A ribavirin neat solution (5 μl, 100 ng/ml) was injected onto each column with isocratic elution. The resulting k' values obtained for ribavirin on these columns are listed in Table 1. The data showed that for the

commonly used C18 columns such as Inertsil ODS C18 and Hypersil BDS C18, as well as for the newer HyPURITY C18 column, there was virtually no retention for ribavirin ($k' < 0.5$) even at 10% organic. This was due to the extremely polar nature of the ribavirin molecule. The only C18 column that exhibited favorable retention was the BioBasic C18 column at a 95% B mobile phase composition. However, if we examine the retention trend of ribavirin on this column, it is very clear that the behavior was not typical RP at all: the higher the organic, the longer the retention time. A closer examination of the retention trends exhibited by the other three C18 columns also revealed a dual retention mechanism: with the increase in the organic percentage in the mobile phase, the retention times first decreased, then increased. Therefore, caution must be taken in development to distinguish

which mechanism on the so-called 'reversed-phase' columns is involved in the specific LC/MS methods for polar, ionic compounds. Failure to recognize this could result in a mismatch of the injection and elution solvent, which in turn could cause deteriorated peak shapes, especially in high throughput LC/MS/MS applications where short columns are used. These implications have been discussed in details in our previous paper [19]. The normal phase-like retention behavior by ribavirin on the BioBasic column was also exhibited on the Betasil silica column, and adequate retention ($k' = 2.3$) was obtained at a 95% B mobile phase. The final condition for the method was 95% ACN and 5% water, both with 0.05% TFA additive to maintain good peak shape in biological extracts.

Because there is no stable isotope-labeled ribavirin available commercially, several similar nu-

Table 2
Blank screen data

Matrix lot	Theoretical concentration (ng/ml) in human plasma					
	0.00	RE (%)	10.0	RE (%)	800	RE (%)
1	0.00	N/A	10.4	4.0	820	2.5
2	0.00	N/A	11.0	10.0	813	1.6
3	0.00	N/A	10.7	7.0	743	−7.1
4	0.00	N/A	10.7	7.0	846	5.8
5	0.00	N/A	10.1	1.0	844	5.5
6	0.00	N/A	9.16	−8.4	846	5.8
Mean	0.00		10.3		819	
RSD (%)	N/A		6.3		4.9	
RE (%)	N/A		3.4		2.3	
	Theoretical concentration (ng/ml) in human serum					
	0.00	RE (%)	30.0	RE (%)	7500	RE (%)
1	0.00	N/A	31.8	6.0	7140	−4.8
2	0.00	N/A	30.3	1.0	7850	4.7
3	0.00	N/A	29.1	−3.0	7140	−4.8
4	0.00	N/A	31.0	3.3	7250	−3.3
5	0.00	N/A	31.5	5.0	7920	5.6
6	0.00	N/A	25.9	−13.7	6740	−10.1
Mean	0.00		29.9		7340	
RSD (%)	N/A		7.3		6.2	
RE (%)	N/A		−0.2		−2.1	

RSD (%), percent relative standard deviation; RE (%), percent relative error.

Table 3
QC sample data

	Theoretical concentration (ng/ml) in human plasma				
	30.0	500	7500	7500[a]	40 000[a]
Intra-batch ($n = 6$)					
Within-batch mean	30.5	475	7660	7860	39 700
RSD (%)	4.2	2.6	3.8	3.5	2.5
RE (%)	1.7	−5.1	2.2	4.8	−0.7
Inter-batch ($n = 18$)					
Overall mean	29.6	480	7640		
RSD (%)	7.3	5.7	5.5		
RE (%)	−1.3	−3.9	1.9		
	Theoretical concentration (ng/ml) in human serum				
	30.0	500	7500	7500[b]	40 000[b]
Intra-batch ($n = 6$)					
Within-batch mean	27.6	504	6950	7600	39 800
RSD (%)	8.4	2.9	6.5	11.3	10.4
RE (%)	−8.0	0.8	−7.3	1.3	−0.5
Inter-batch ($n = 18$)					
Overall mean	27.6	515	6750		
RSD (%)	8.8	4.1	7.2		
RE (%)	−8.0	3.0	−10.0		

[a] After $5 \times$ dilution with blank human plasma.
[b] After $5 \times$ dilution with blank human serum.

cleoside compounds, such as 3′-methylcytidine, were first evaluated for potential use as the IS, However, it was discovered that none of these compounds tracked ribavirin well in the protein precipitation extraction. Finally, bamethan was chosen because it worked well tracking ribavirin in the protein precipitation extraction despite their apparent difference in chemical structure. The use of bamethan as IS necessitated the careful examination of matrix effect ribavirin encountered in the LC/MS/MS analysis in development and validation. This was because the two compounds were well separated under the chromatography condition used; therefore it was impossible for bamethan to 'feel' the same effect caused by matrix compounds co-eluting with ribavirin. Provided that the matrix effects were carefully evaluated, rugged LC/MS/MS methods could still be developed using non-stable isotope ISs, as demonstrated by this paper.

3.3. Validation results

Blank matrix from six lots was tested for endogenous interference. Chromatograms representative of a blank matrix sample, and a control zero (blank matrix fortified with IS only) are shown in Fig. 2(A and B) for plasma, and Fig. 3(A and B) for serum. The ribavirin and IS regions were free from interference for both matrices. No carry-over of the LC/MS/MS system was observed. As shown in Table 2, The back-calculated values from all six plasma lots spiked at ribavirin concentrations of 10.0 and 800 ng/ml were within 91.6–110% of the theoretical. The back-calculated values from all six serum lots spiked at 30.0 and 7500 ng/ml were within 86.3–106% of the theoretical. These results indicated no significant lot-to-lot variation in matrix effect.

Calibration curves for ribavirin in both human plasma and serum were linear using weighted

(1/concentration^2) least-squares linear regression analysis in the concentration range from 10.0 to 10 000 ng/ml, with correlation coefficients equal to or greater than 0.995 for all curves. The LLOQ for ribavirin was set at 10.0 ng/ml. At the LLOQ,

Table 4
Stability data for plasma QC samples

	Theoretical concentration (ng/ml)		
	30.0	500	7500
(A) Reinjection after refrigeration for 24 h			
$n = 6$			
Mean	32.1	476	7410
RSD (%)	8.8	2.5	2.6
RE (%)	7.0	−4.8	−1.2
(B) Benchtop at room temperature for 24 h			
$n = 6$			
Mean	30.5	499	7650
RSD (%)	5.8	7.0	10.4
RE (%)	1.7	−0.2	2.0
(C) Three freeze–thaw cycles			
$n = 6$			
Mean	30.6	465	7890
RSD (%)	11.7	6.8	8.9
RE (%)	1.9	−7.0	5.2

Table 5
Stability data for serum QC samples

	Theoretical concentration (ng/ml)		
	30.0	500[a]	7500
(A) Reinjection after refrigeration for 24 h			
$n = 6$			
Mean	27.3	514	6860
RSD (%)	5.8	7.8	7.8
RE (%)	−9.0	2.8	−8.5
(B) Benchtop at room temperature for 24 h			
$n = 6$			
Mean	29.9		6520
RSD (%)	5.4		5.9
RE (%)	−0.3		−13.1
(C) Three freeze–thaw cycles			
$n = 6$			
Mean	30.6		6930
RSD (%)	8.7		2.0
RE (%)	2.0		−7.6

[a] $n = 5$.

the relative standard deviation (RSD) ($n = 6$) of the measured concentration was 11.2% and the relative error (RE) from the nominal value was +12.8% for the plasma matrix. For the serum matrix, the RSD ($n = 6$) was 7.6% and the RE was −13.7%. Representative chromatograms from an LLOQ calibration standard (10.0 ng/ml) in plasma and serum are shown in Fig. 2(C) and Fig. 3(C), respectively.

The precision and accuracy data for QC samples for the method validations are summarized in Table 3. For plasma samples, the overall precision for all QC samples ranged from 5.5 to 7.3% RSD. The within-batch RSD of all QC samples was no more than 9.3% for all the validation batches. The RE ranged from −6.9 to +4.3% for the within-batch means, and from −3.9 to +1.9% for the overall means for all QC samples. For serum samples, the overall precision for all QC samples ranged from 4.1 to 8.8% RSD. The within-batch RSD of all QC samples was no more than 11.3% for all the validation batches. The RE ranged from −12.9 to +4.8% for the within-batch means, and from −10.0 to +3.0% for the overall means for all QC samples. Representative chromatograms from a high QC sample (7500 ng/ml) in plasma and serum are shown in Fig. 2(D) and Fig. 3(D), respectively. Dilution integrity was established for both matrices, as demonstrated by the results obtained from QC 7500 and 40 000 ng/ml when they were diluted fivefold with blank human plasma or serum prior to extraction.

Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of matrix recovery samples prepared by adding compound to post-extraction matrix blanks at the corresponding QC concentrations. The mean recovery of ribavirin was at least 75.5% for all three QC concentrations (30.0, 500 and 7500 ng/ml) from human plasma. The overall mean recovery was 80.0% for ribavirin and 81.3% for the IS, bamethan from plasma. Matrix effect of ribavirin was assessed in this method by comparing the peak areas of neat ribavirin solutions to those of the matrix recovery samples. The peak areas of the matrix recovery samples at three QC levels ranged from 79.4 to 85.6% of those obtained from the corresponding neat ribavirin solu-
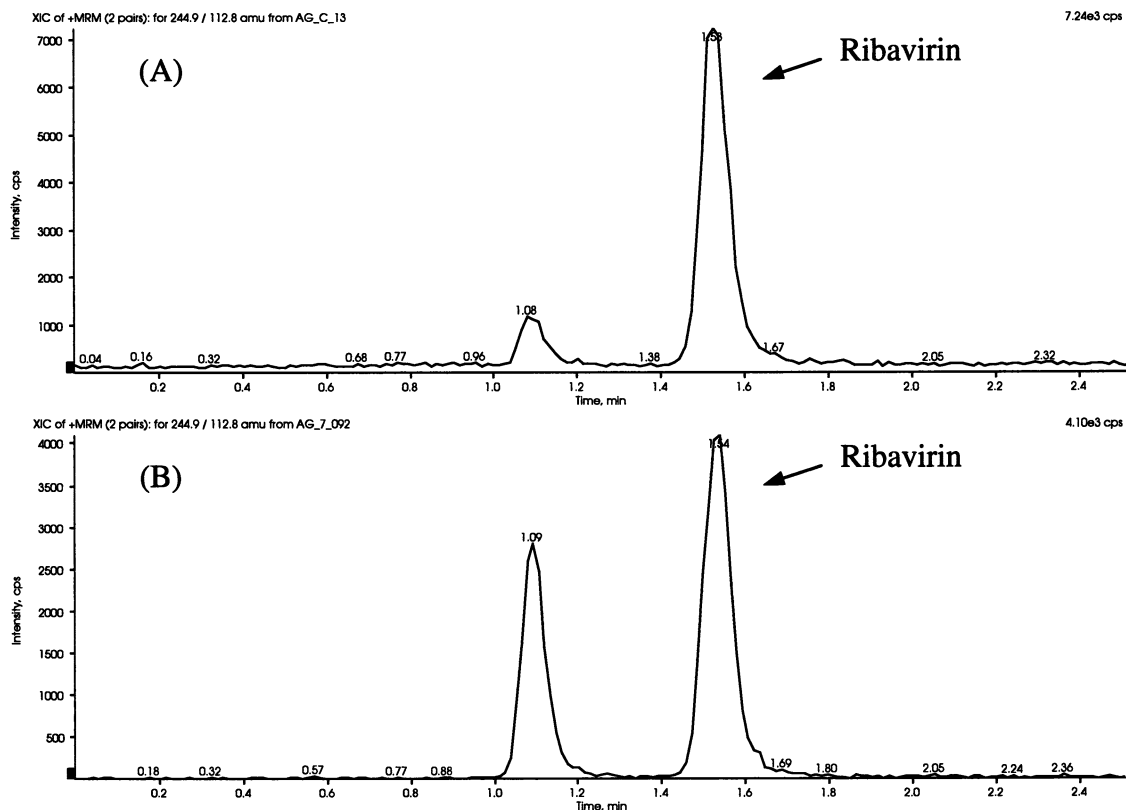
Fig. 4. Mass chromatograms from two extracted plasma samples injected onto the same silica column: (A) Injection #13; (B) Injection #355.

tions, indicating consistent matrix effect—approximately 20% suppression effect across all concentration levels of ribavirin caused by plasma matrix. Combined with the fact that there was no significant lot-to-lot variation (Table 2), it was demonstrated that matrix effect did not compromise the performance of the assay.

The stability tests were designed to cover the anticipated conditions clinical samples may experience. Stability during sample handling (repeated freeze–thaw and ambient thaw), and for processed samples (extracts) was tested and established. The results are summarized in Tables 4 and 5 for plasma and serum matrix, respectively. Three freeze–thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to extraction appeared to have little effect on the quantitation. QC samples stored in a freezer at −20 °C remained stable through the

course of the validation. Extracted calibration standards and QC samples were allowed to stand in a refrigerator set at 2–8 °C for 24 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. The

Table 6
QC sample data obtained by automated extraction

	Theoretical concentration (ng/ml) in human serum		
	30.0	500	7500
Intra-batch ($n = 6$)			
Within-batch mean	29.0	528	7060
RSD (%)	8.3	5.7	5.2
RE (%)	−3.3	5.6	−5.9

stock solution stability has also been established: the ribavirin stock solution was stable at 2–8 °C for at least 16 days.

The silica column exhibited excellent stability during the course of development, validation and sample analysis. No special treatment, except for equilibrating the columns with about 10 column volumes of mobile phase prior to injections, was needed. Representative mass chromatograms of ribavirin from Injections #13 and #355 are shown in Fig. 4(A and B), respectively. Good ribavirin peak shape and baseline separations from an endogenous interference peak were all well maintained.

3.4. Automated protein precipitation

In order to decrease analysts' time and labor spent in sample processing, the extraction procedure was further automated based on 96-well format by the combined use of the MultiPROBE II and the Tomtec. The flexibility of the Multi-PROBE II, with its liquid sensing and variable tip span capability, was fully utilized to transfer samples, standards and QC samples from tubes into 96-well deep well plates. The Tomtec, with its parallel processing ability enabled by its 96 tips, was then used to dispense reagent (ACN) and transfer the supernatant from each well of plate. It should be noted that, in order to prevent dripping of the organic solvent from the Tomtec tips, tips need to be wetted by the solvent using the 'mixing' function offered by the software before the actual transfer step. Another means to circumvent this problem involved the use of sequential aspirations of organic solvent followed by an air plug. As long as these precautions were taken, no dripping (and consequent cross contamination between wells) was observed. This automated extraction procedure was validated with one analytical batch that included the standard curve and six replicates of QC samples at of 30.0, 500 and 7500 ng/ml. The results are shown in Table 6. From the data it is clear that the automated extraction yielded just as good, if not better quality data as manual extraction.

4. Conclusion

A sensitive, fast and rugged LC/MS/MS method for ribavirin in human plasma and serum was developed using protein precipitation for sample clean-up and a silica column coupled with tandem mass spectrometry for detection. The sample preparation was successfully automated by the combined use of MultiPROBE II and Tomtec. The method has been successfully used for the analysis of clinical samples.

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