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Simultaneous determination of ribavirin and ribavirin base in monkey plasma by high performance liquid chromatography with tandem mass spectrometry

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Abstract

For the first time, a liquid chromatographic method with tandem mass spectrometric detection (LC–MS/MS) for the simultaneous determination of ribavirin and rabavirin base was developed and validated over the concentration range of 10–5000 ng/ml, respectively, using a 0.025 ml monkey plasma sample. Ribavirin, ribavirin base, and the internal standards were extracted from monkey plasma via protein precipitation. After evaporation of the supernatant, the extract was reconstituted with 5% methanol (containing 0.1% formic acid) and injected onto the LC–MS/MS system. Optimum chromatographic separation was achieved on a Waters Atlantis dc18 (150 mm × 2.1 mm, 5 μm) column with mobile phase run in gradient with 100% water containing 0.5% formic acid (**A**) and 90% acetonitrile (containing 0.5% formic acid (**B**). The flow rate was 0.4–0.6 ml/min with total cycle time of approximately 7.0 min. Post-column addition of acetonitrile (containing 0.1% formic acid) at 0.3 ml/min was used to increase the ionization efficiency in the MS source. The method was validated for sensitivity, linearity, reproducibility, stability and recovery. Lack of adverse matrix effect and carry-over was also demonstrated. The intra-day and inter-day precision and accuracy of the quality control (QC) samples were <9.0% relative standard deviation (R.S.D.) and 10.8% bias for ribavirin, and 10.3% R.S.D. and 11.3% bias for ribavirin base. The current specific, accurate and precise assay is useful in support of the toxicokinetic and pharmacokinetic studies of these compounds.

Keywords: Ribavirin; Ribavirin base; LC–MS/MS; Simultaneous analysis

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic purine nucleoside analog with a broad spectrum of antiviral activity [1,2]. It is activated by cellular adenosine kinase to ribavirin 5′-monophosphate and subsequently to ribavirin 5′-diphosphate and ribavirin 5′-triphosphate [3], which have been demonstrated to force the virus into 'error catastrophe' or directly inhibit virus replication by interfering with virus (e.g. hepatitis C virus, etc.) polymerase-mediated RNA synthesis [4–7]. Therapeutically, ribavirin inhalation has been a primary treatment of lower respiratory tract diseases (including bronchiolitis and pneumonia) caused by respiratory syncytial virus (RSV) in hospitalized infants and young children [8,9]. Orally administered ribavirin was used with some success for the treatment of various strains of influenza A and B [8,10]. Although oral therapy of ribavirin alone is not effective for the treatment of hepatitis C virus (HCV) infection [11], combination of the drug with either interferon α-2a/2b [12–16] or peginterferon α-2a/2b [17,18] was widely accepted for the treatment of chronic hepatitis C with an increased rate of sustained response by two- to three-fold and a decreased rate of relapse following discontinuance of the therapy. In addition, the use of ribavirin combined with other agents in patients triple-infected with human immunodeficiency virus (HIV), HCV and hepatitis B virus (HBV) has been evaluated [19,20].

Ribavirin undergoes two pathways of metabolism *in vivo* [10]. The first pathway is a reversible phosphorylation in nucleated cells [10,21,22], and the second is degradation involving deribosylation and/or amide hydrolysis [10,22]. As the result

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of deribosylation, ribavirin is biotransformed to ribavirin base (1,2,4-triazole-3-carboxamide) [10,22]. In healthy adults, it is estimated that approximately 30–55% of inhalated ribavirin was excreted as ribavirin base in urine within 72–80 h, and about 50% of orally administered ribavirin was excreted as ribavirin base within 24 h [10]. Unfortunately, no detailed plasma concentration data have been reported for this metabolite although the ratio of ribavirin base to ribavirin was estimated to be 1:1 in plasma [10,23]. In order to better understand the exposure of both ribavirin and ribavirin base, it is necessary to determine the plasma concentration of these two compounds in biological fluids.

Many high performance liquid chromatography (HPLC) based methods (HPLC-UV, LC–MS, etc.) have been reported for the determination of ribavirin in biological matrices [24–34]. However, separate methods using HPLC-UV or HPLC with radioactivity detection were used for the measurement of ribavirin base in these samples [22,31,34]. To our best knowledge, only a few HPLC-UV methods are available for the quantification of both ribavirin and ribavirin base in biological samples [35,36]. However, the reported sample preparation required many steps of manipulation, including dilution with water, filtration with 30,000 Da cut-off, extraction with dichloromethane, filtration through double-bed column, elution, dryness and reconstitution. The HPLC analysis cycle time was greater than 10 min with a LLOQ of 20 ng/ml when 1.0 ml of sample was used [35,36]. No liquid chromatographic method with tandem mass spectrometric detection (LC–MS/MS) is available to simultaneously measure the concentration of these two compounds in biological matrices. The present paper reports the development and validation of an eletrospray LC–MS/MS method for the simultaneous determination of ribavirin and ribavirin base in monkey plasma. Simple protein precipitation was used to extract ribavirin and ribavirin base and internal standards from 0.025 ml of plasma sample. Chromatographic separation was achieved on a reversed-phase column with an aqueous mobile phase.

2. Experimental

2.1. Chemicals and reagents

Ribavirin (chemical purity 100%, $C_8H_{12}N_4O_5$, MW = 244.20) and ribavirin base (chemical purity 98.9%, $C_3H_4N_4O$, MW = 112.09) were the products of Clariant Life Science Molecules (Italia) S. p. A. (Pogliano Milanese, Italy) and 3B Medical Systems, Inc. (Libertyville, IL, USA), respectively. The internal standards, $^{13}C_5$-ribavirin (isotopic purity >99.5%) and $^{13}C^{15}N$-ribavirin base (isotopic purity >99.5%) were synthesized in house. HPLC grade solvents, acetonitrile and methanol, and reagents, formic acid, ammonium hydroxide and ammonium acetate, were Fisher products (Fair Lawn, NJ, USA). Deionized water was produced in-house via an ELGA PureLab Ultra Water System (Lowell, MA, USA). Monkey plasma with Na-heparin as the anticoagulant was obtained from Bioreclamation (East Meadow, NY, USA).

2.2. Chromatographic condition

A Shimadzu liquid chromatograph model SCL10AvP integrated system consisting of a multi-channel mobile phase degasser (DGU-14A), a column heater (CTO10ASvp), three pumps (two LC10AD pumps, one LC10ADvp pump, Shimadzu, Columbia, MD, USA), and a Waters Atlantis dc-18 (150 mm × 2.1 mm, 5 μm particle size) column (Waters, Milford, MA, USA) was used for the chromatographic separation of ribavirin and ribavirin base and internal standards. The autosampler was HTS-PAL from Leap Technologies (Carrboro, NC, USA). The mobile phases were water containing 0.5% formic acid (**A**) and 90% acetonitrile containing 0.5% formic acid (**B**). The optimum separation of ribavirin and ribavirin base was achieved by running 100% **A** for 3.0 min at 0.4 ml/min isocratically and then 100% **B** at 0.6 ml/min isocratically for the next 2 min, followed by 100% **A** at 0.6 ml/min for 1 min and at 0.4 ml/min for another 1 min before the next injection. The column was maintained at 30 °C. The whole column effluent was post-column mixed via a "Tee" with acetonitrile (containing 0.1% FA) delivered by pump **C** at 0.3 ml/min before entering the switch valve and then the mass spectrometer interface from 1.0 to 3.0 min.

2.3. Mass spectrometric conditions

An API 4000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Concord, Ontario, Canada) with a Turboionspray (TIS) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. The mass spectrometric conditions were optimized for ribavirin, ribavirin base, $^{13}C_5$-ribavirin and $^{13}C^{15}N$-ribavirin base by infusing a 200–500 ng/ml standard solution in 50% methanol (containing 0.1% formic acid) at 10 μl/min using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA) directly connected to a "Tee", where the compounds were mixed with a mobile phase mixture [mobile phase **A** at 0.4 ml/min and acetonitrile (containing 0.1% formic acid) at 0.3 ml/min] before entering the mass spectrometer. The optimized instrument conditions were as follows: TIS source temperature, 550 °C; TIS voltage, 5000 V; curtain gas, 20; nebulizing (GS1), 70; TIS (GS2) gas, 80; CID gas, 10; collision energy (CE), 15 eV for ribavirin and $^{13}C_5$-ribavirin and 22 eV for ribavirin base and $^{13}C^{15}N$-ribavirin base, respectively. The following precursor → product ion transitions were used for multiple reaction monitoring: ribavirin, *m/z* 245 → 113; ribavirin base, *m/z* 113 → 96; $^{13}C_5$-ribavirin, *m/z* 250 → 113; and $^{13}C^{15}N$- ribavirin base, *m/z* 115 → 98, with dwell time of 150 ms for each mass transition of the analytes and internal standards. The mass spectrometer was operated at unit mass resolution (half-height peak width set at 0.7 Da) for both the first quadrupole and the third quadrupole.

2.4. Standards and quality control (QC) samples

Two primary stock solutions for ribavirin and ribavirin base were each prepared in 50% methanol (containing 20 mM ammo-

nium acetate and 0.1% formic acid) and pure DMSO, respectively, at concentration of 1.0 mg/ml in 20-ml glass vials. The stock solutions were kept refrigerated (2–8 °C). For validation purposes, the stock solutions from the two weighings must have less than a 5% difference in the LC–MS/MS responses. The stock solution was serially diluted with 50% methanol (containing 20 mM ammonium acetate and 0.1% formic acid) to prepare standard working solutions at the desired concentrations. The calibration standards were freshly prepared by spiking 25 μl of an appropriate amount of the standard working solution into 0.025 ml monkey plasma. Nine non-zero calibration standards were prepared at concentrations of 10.0, 20.0, 50.0, 100, 400, 800, 2000, 4000 and 5000 ng/ml. Pooled quality control samples were prepared by spiking appropriate amounts of standard working solutions into normal pooled monkey plasma with non-matrix composition less than 2% of the final volume. QC samples were prepared at concentrations of 10.0 (LLOQ), 30.0, 150, 500, 750 and 3760 ng/ml. Dilution QCs were prepared at concentrations of 25,000 and 3760 ng/ml, respectively. All the QCs were transferred into 2-ml polypropylene vials and stored at ≤−60 °C. An internal standard working solution containing 500 ng/ml of $^{13}C_5$-ribavirin and 1000 ng/ml $^{13}C^{15}N$-ribavirin base was prepared from IS stock solutions with 50% methanol.

2.5. Sample preparation

Samples were briefly vortex-mixed and 0.025 ml aliquots of samples were then transferred from the vials into corresponding wells of a 96 deep-well plate. A 25.0 μl aliquot of 50% methanol buffer was added to all samples except standards. Calibration standards were prepared by fortifying 25.0 μl of the appropriate standard working solutions to 0.025 ml of blank plasma as indicated above. Internal standard working solution (25.0 μl) was then added to all samples except blanks, which was followed by the addition of 25 μl of 10% ammonium hydroxide (10 times dilution from concentrated ammonium hydroxide, ∼29%). After the addition of 0.500 ml of 10% methanol in acetonitrile (v/v), the 96 deep-well plate was sealed with the sealing mat, followed by vortexing at high speed on a Gal-Col Vortexer (Terre Haute, IN, USA). The samples were centrifuged at approximately 3800 rpm for ∼8 min. Approximately 500 μl of the supernatant was transferred into a newly labeled 96-deep well plate using a TomTec Quadra 96-320 system (Hamden, CT, USA), which was followed by evaporation of the solvent using SPE-Dry 96® (Jones Chromatography, Lakewood, CO, USA) with upper and lower temperature set at 45–50 °C and nitrogen stream pressure at 45 psi. The resulting residues in the 96-deep well plate were reconstituted with 0.150 ml of 5% methanol containing 0.1% formic acid followed by sealing with the sealing mat, vortexing at high speed and centrifugation at approximately 3500 rpm. The samples (10 μl) were injected onto the HPLC column.

2.6. Data analysis

Data were processed using the AB/MDS-Sciex Analyst 1.4.1 software (Foster City, CA, USA). The calibration curves (analyte peak area/IS peak area *versus* analyte concentration) were constructed using the least square linear regression fit ($y = a + bx$), and a weighting factor of $1/x^2$ was applied to the data. Acceptance criteria were established to be >0.98 for the calibration curve coefficient of correlation (r^2), and within ±15% of the nominal concentration (accuracy) and ≤15% R.S.D. (precision) for QC samples except LLOQ samples in the intra-day and inter-day assay. For LLOQ samples, the intra-day and inter-day assay accuracy and precision should be within ±20% of the nominal concentration and ≤20% R.S.D.

2.7. Method validation

The method specificity was evaluated by screening at least six lots of blank plasma prior to the main validation batches. In this screening batch, six lots of plasma were fortified, individually, with ribavirin and ribavirin base at LLOQ level, extracted and analyzed along with six zero samples, to which only internal standards were fortified at working concentration, six blank plasma samples and a calibration curve prepared with one of the six lots of plasma. The reproducibility of these six blank matrices, zero samples and LLOQ samples is used to evaluate the presence or absence of interference, and the lot-to-lot variation.

Three validation batches were used to assess the precision and accuracy of the method. Each batch was processed on a separate day and had two set of calibration standards and six replicates of QC samples at 10.0, 30.0, 150, 500, 750 and 3760 ng/ml. One of the batches included six replicates of QC at 25,000, 25,000 and 3760 ng/ml after a 100, 10 and 5-fold dilution, respectively, with blank plasma. QC samples and other test samples were interspersed between two calibration curve samples. A blank sample was always placed right after the upper limit of quantification (ULOQ) standard to evaluate the carry-over of the LC–MS/MS system.

The short-term stability was included in one of the three validation batches, in which the QC samples at 30.0, 500 and 3760 ng/ml experiencing three cycles of freeze-thaw (free-thaw stability) or sitting on laboratory bench at room temperature for approximately 24 h (bench-top stability) were processed together with calibration standards and regular QC samples. One of the three validation batches had 96 injections in order to simulate a routine analysis run size. Among the three batches, one batch of extracted samples was stored in the auto-sampler for approximately 52 h before re-injection onto the LC–MS/MS system to determine the storage and re-injection reproducibility of the processed samples. The matrix effect of the method was determined by comparing the LC–MS/MS response of extracted blank plasma samples, to which the analyte neat solutions were spiked with the concentrations the same as low (30 ng/ml), medium (500 ng/ml) and high (3760 ng/ml) QC samples, with that obtained from QC neat solutions at the same concentrations as above, respectively. Recovery was determined by comparing the LC–MS/MS response of extracted normal QC samples at low (30 ng/ml), medium (500 ng/ml) and high (3760 ng/ml) concentration levels with that obtained from the extracted blank

samples post-fortified with the standard solutions at the same concentrations as the above.

3. Results and discussion

3.1. Method development

Positive ion electrospray MS/MS product-ion spectra of ribavirin and ribavirin base are shown in Fig. 1. The proposed product ions used in multiple reaction monitoring are inserted in the figure. For ribavirin, the most abundant product ion was observed at *m/z* 113 (Fig. 1A), which was due to the cleavage of the ribose moiety of the protonated molecule at *m/z* 245. In the product ion mass spectrum of ribavirin base (Fig. 1B), the predominant peak was seen at *m/z* 96 and was used in MRM in the current method. The formation of the ion at *m/z* 96 is through a characteristic neutral loss of the amine moiety (17 Da) from the protonated molecule (*m/z* 113).

Like previous reports [24,31,34], $^{13}C_5$-ribavirin was used as the internal standard to compensate for deviations caused during sample preparation and determination for ribavirin. For quantification of ribavirin base, $^{13}C_2{}^{15}N_3$-ribavirin base

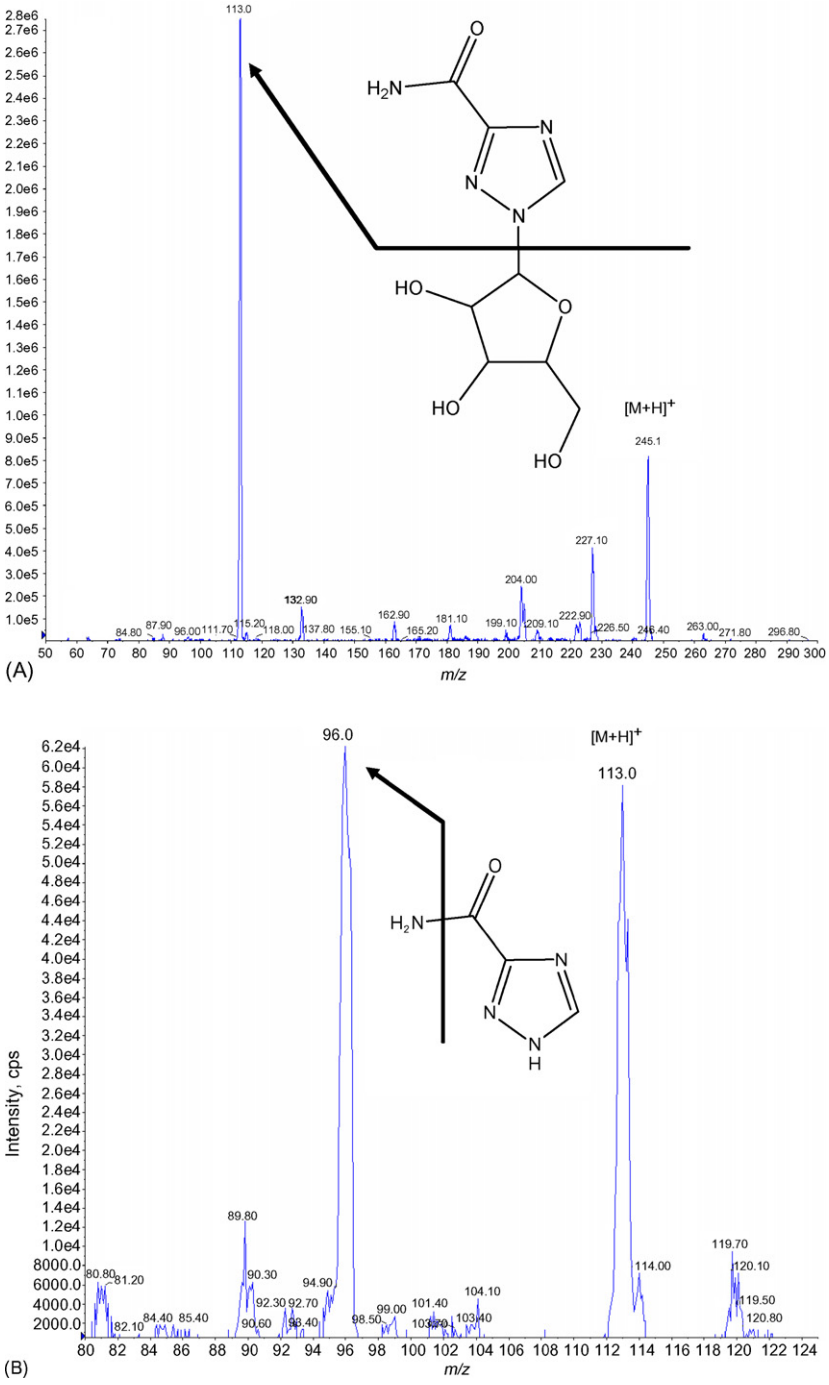

Fig. 1. Representative product ion mass spectra of ribavirin (**1A**) and ribavirin base (**1B**).

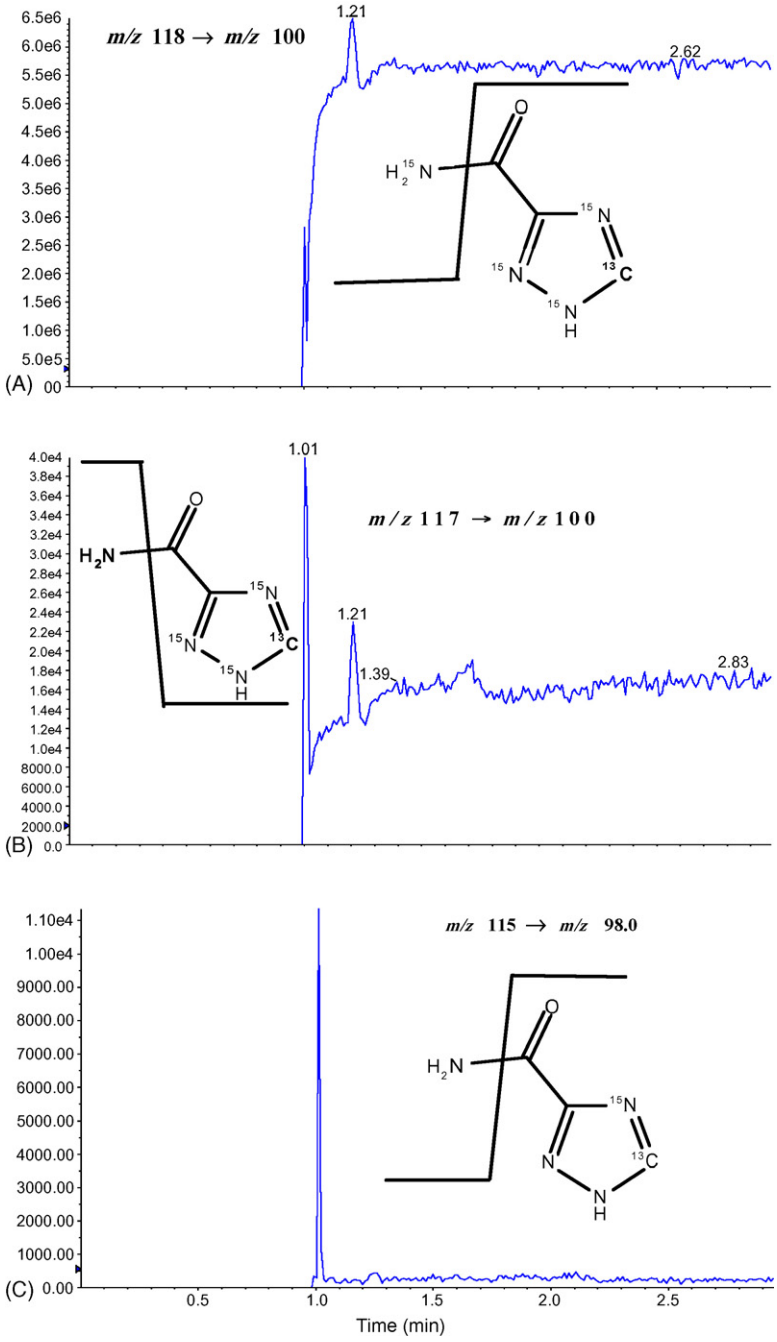

Fig. 2. Representative LC–MS/MS chromatograms of blank solvent injections (the inserts represent the proposed fragmentation and MRM mass transition for internal standard candidates): (**A**) *m/z* 118 → 110; (**B**) *m/z* 117 → 110; (**C**) *m/z* 115 → 98.

[M + 5] (*m/z* 118 → 110), $^{13}C^{15}N_3$-ribavirin base [M + 4] (*m/z* 117 → 110) and $^{13}C^{15}N$-ribavirin base [M + 2] (*m/z* 115 → 98) were evaluated as internal standard candidates. As expected, numerous solvent cluster ions were present in the low mass range (*m/z* 50–150) of the Q1 scan mass spectra of the above internal standard candidates (not shown). Unfortunately, one of the solvent cluster ions gave MRM mass transition at *m/z* 118 → 110, which is the same as that of $^{13}C_2{}^{15}N_3$-ribavirin base [M + 5]. As can be seen in Fig. 2A, a very high baseline (height >5,000,000 counts) was present in the LC–MS/MS chromatogram of a blank solvent injection when monitoring mass transition *m/z* 118 → 100, making $^{13}C_2{}^{15}N_3$-ribavirin base [M + 5] not suitable. Test on $^{13}C^{15}N_3$-ribavirin base [M + 4] was also not successful since it is impossible to distinguish the MRM mass transitions between *m/z* 117 → 100 for $^{13}C^{15}N_3$-ribavirin base [M + 4] and *m/z* 118 → 100 generated by solvent clusters under unit mass resolution (half-height peak width at 0.7 Da) for both the first quadrupole and the third quadrupole in mass spectrometer. As can be seen in Fig. 2B, a relative high noise level (height > 10,000 counts) was present in the LC–MS/MS chromatogram when *m/z* 117 → 100 was monitored for a blank solvent injection. Attempts of modifying

instrument parameters, including resolution, DP, CE, CXP, etc., changing the composition of the mobile phases, or using alternative product ions failed to minimize the interference of the solvent cluster ions with the detection of the above two internal standard candidates (data not shown). In contrast, the MRM mass transition of $^{13}C^{15}N$-ribavirin base [M + 2] (*m/z* 115 → 98) was less affected by the solvent cluster ions, which was demonstrated by the presence of a much lower noise level in the LC–MS/MS chromatogram for a blank solvent injection when *m/z* 115 → 98 was monitored (Fig. 2C). Thus, $^{13}C^{15}N$-ribavirin base [M + 2] was used in the current assay. It is well known that ESI is a soft ionization technique that accomplishes the transfer of ions from the liquid phase to the gas phase. However, despite its widespread application in the qualitative or quantitative analysis of small or large molecules, some anomalies of ESI have not been fully understood. The solvent cluster ions mentioned above, which are not present in the liquid phase, but in the gas phase, is a good example. The abundance of solvent cluster ions in the mass spectral response largely depends on their gas-phase proton affinities [37]. The solvent clusters might consist of two, three, four or more molecules of the same solvent or different solvents [37], etc. Attempts to examine all major solvent cluster ions observed in the low mass range (*m/z* 50–150) (not shown) during method development failed to identify the composition of the ion at *m/z* 118, although its mass transition, *m/z* 118 → 100, suggests that a molecule of water might be lost due to collision-induced dissociation.

Solid phase extraction using MCX cartridge (Waters Corp., Milford, MA, USA) was investigated with 0.1 N HCl or 20 mM potassium phosphate buffer (pH ~6.0) as the equilibration and loading solvents. Unfortunately, very low recovery was obtained for both analytes due to their extremely polar nature. Although a phenyl boronic acid (PBA) cartridge could be used to isolate ribavirin from biological matrix through an exclusive extraction of poly-ol species *via* interaction with surface-bound borate groups [27,28,32,33], ribavirin base was expected to be present in the wash waste during the loading step in SPE due to its lack of poly-ol groups in the molecule. Thus, protein precipitation with 10% methanol in acetonitrile was employed.

Initial LC–MS/MS experiments were carried out by using underivatized silica columns (Betasil and Hypersil silica columns from Thermo-electron, and Kromasil and Luna silica columns from Phenomenex) with the mobile phase consisting of high organic and low aqueous phase, and acetic acid and TFA [29,30]. However, significant matrix effect was observed for the analytes, especially for ribavirin base (data not shown) due to the presence of endogenous ingredients in the extracts after protein precipitation. Chromatographic separation could be performed on a C-18 column using aqueous or near aqueous (<5% organic solvent) mobile phase in order to retain the extremely polar analytes on the column, but the aqueous mobile phase can be detrimental to many conventional HPLC columns [28,30]. Thanks to the advance of HPLC column technology, several new columns have become available for chromatography of highly polar compounds under aqueous mobile phase condition. Among these columns, Waters Atlantis dc-18 column is a di-functionally bonded and silica-based reversed-phase column with super retention of polar compounds. It not only thrives 100% aqueous mobile phase, but also does not exhibit excessive retention of hydrophobic compounds [38]. Thus, a Waters Atlantis dc-18 column was used in the current assay with both ribavirin and ribavirin base eluted under 100% aqueous mobile phase. However, the use of aqueous mobile phase has been speculated to yield poor ionization efficiency or poor electrospray sensitivity since water possesses a high surface tension, needs large heat for evaporation, and is a much poorer solvent for electrospray than organic solvents such as acetonitrile or methanol in LC–MS/MS interface. In the current assay, such phenomenon was overcome by post-column addition of acetonitrile via a "Tee" before the column effluent enters the mass spectrometer, which yielded a 10–15% increase in signal intensity (figure not shown).

3.2. Specificity and selectivity

Under the current LC–MS/MS conditions, ribavirin and ribavirin base were well separated from interferences in the blank matrix. LC–MS/MS chromatograms of six lots of blank plasma were found to contain no endogenous peak co-eluted with any of the analytes and internal standards. Representative chromatograms of blank plasma samples without (blank) or with internal standards (zero sample) are shown in Figs. 3 and 4, respectively. Injection of ribavirin base at the highest concentration (5000 ng/ml) did not show significant interference (<2% of the internal standard response) at the $^{13}C^{15}N$- ribavirin base channel, even the $^{13}C^{15}N$-ribavirin base is only 2 Da different from ribavirin base.

3.3. Sensitivity

The current assay has a LLOQ of 10 ng/ml for ribavirin and ribavirin base, respectively, in monkey plasma based on a 0.025 ml plasma volume. Reliable precision (R.S.D.% <10.3%) and accuracy (bias% <10.8%) was obtained by analyzing three sets of six replicate LLOQ samples (Table 1) along with a standard curve and QCs at low (30 ng/ml), medium (150, 500, 750 ng/ml) and high (3760 ng/ml) levels in three validation runs. A typical LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 5. As can be seen in the figure, the noise levels in the LC–MS/MS chromatograms for both ribavirin base and $^{13}C^{15}N$-ribavirin base were relative high when compared with that of ribavirin and $^{13}C_5$-ribavirin. Besides solvent clusters as discussed above, matrix ingredients might make major contributions. During the validation, in order to consistently achieve the desired sensitivity. It is important to re-generate HPLC column using mobile phase **B** for at least 1 h and re-equilibrate the column with mobile phase **A** for another 1 h between runs. Vortexing the mixture after each addition step before the protein precipitation step is also critical.

3.4. Linearity

The standard curve range was 10–5000 ng/ml for ribavirin and ribavirin base when 0.025 ml plasma was used for the

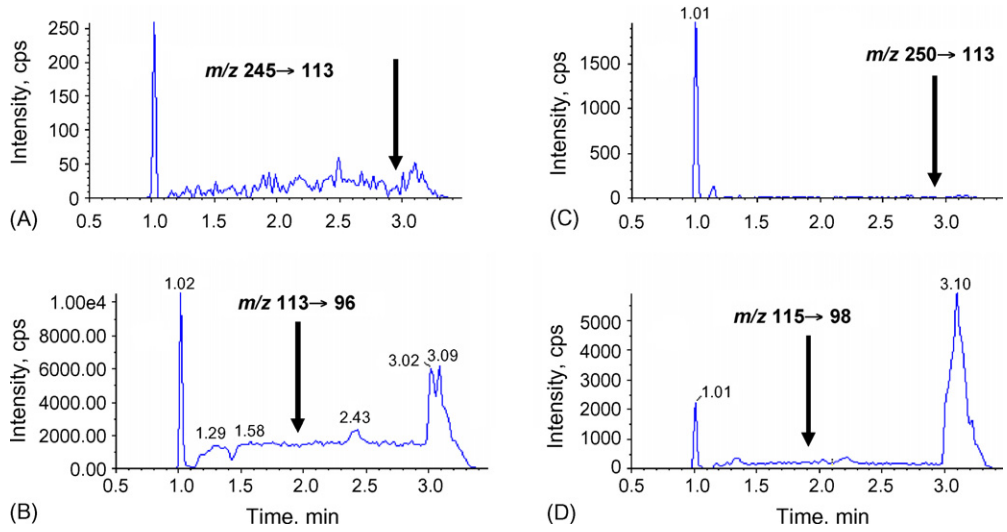

Fig. 3. Representative LC–MS/MS chromatograms of matrix blank samples (MBLK): (**A**) ribavirin (*m/z* 245 → 113), (**B**) ribavirin base (*m/z* 113 → 96), (**C**) $^{13}C_5$-ribavirin (*m/z* 250 → 113) and (**D**) $^{13}C^{15}N$-ribavirin base (*m/z* 115 → 98). The arrows indicate the retention time of the compounds.

assay. Nine none-zero standard calibration curves for the analytes were obtained by plotting the peak area ratio (y) of the analyte to corresponding internal standard against the concentrations (x) of the analyte in freshly prepared plasma calibrators. Representative calibration curves with linear regression (weighing of 1/concentration^2) for ribavirin and ribavirin base are $y = 0.00317 + 0.001265x$ ($r^2 = 0.9969$) and $0.04307 + 0.009671x$ ($r^2 = 0.9948$), respectively. The calibration curves obtained as described above were suitable for the quantification of ribavirin and ribavirin base in the samples during the intra- and inter-day validations and stability tests.

3.5. Precision and accuracy

The intra-assay accuracy and precision of the method were determined by analyzing six QC replicates at 30.0, 150, 500, 750 and 3760 ng/ml for ribavirin and ribavirin base, respectively, in each validation run. The accuracy of the method was determined by calculating bias (%) and the precision by calculating R.S.D. (%). Table 1 summarized the accuracy and precision on each of three assays for ribavirin and ribavirin base in monkey plasma with accuracy ranging from −8.0 to 5.3% and −10.4 to 11.3% bias, respectively, of nominated values and precision ranging from 1.2 to 6.3% and 1.0 to 5.4% R.S.D., respectively, over the five concentration levels evaluated for ribavirin and ribavirin base, respectively.

3.6. Dilution integrity

The dilution integrity was determined by 100, 10 and 5-fold diluting the dilution QCs with blank plasma prior to extraction and assayed in six replicates along with calibration curve and

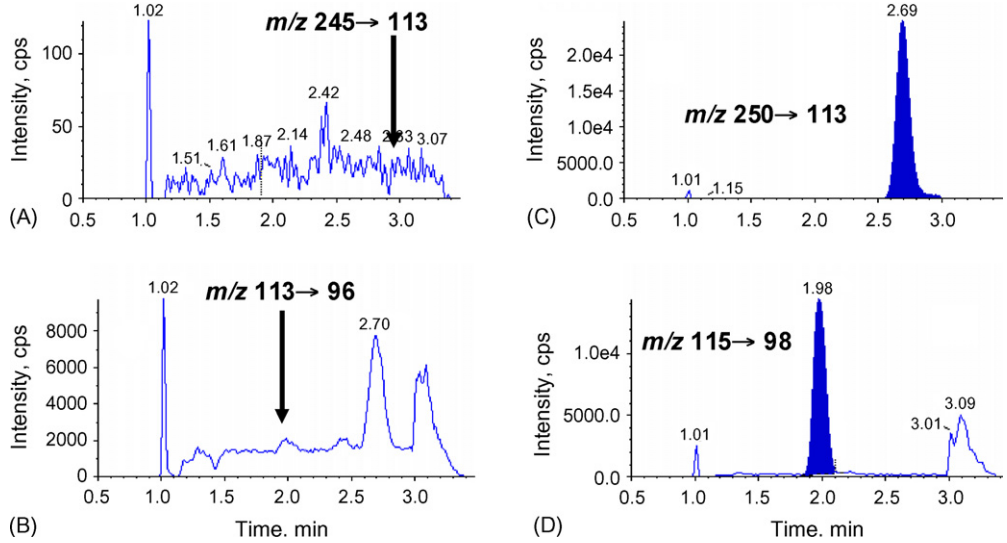

Fig. 4. Representative LC–MS/MS chromatograms of zero control samples (QC0): (**A**) ribavirin (*m/z* 245 → 113), (**B**) ribavirin base (*m/z* 113 → 96), (**C**) $^{13}C_5$-ribavirin (*m/z* 250 → 113) and (**D**) $^{13}C^{15}N$- ribavirin base (*m/z* 115 → 98). The arrows indicate the retention time of the analytes.

Table 1
Precision and accuracy of quality control samples

		LLOQ 10.0 ng/ml	LQC 30.0 ng/ml	MQC 150 ng/ml	MQC 500 ng/ml	MQC 750 ng/ml	HQC 3760 ng/ml
Ribavirin							
Day 1	*n*	6	6	6	6	6	6
	Mean	10.4	30.2	158	473	730	3700
	% R.S.D.	5.4	5.1	1.5	1.2	1.9	1.2
	% Bias	4.0	0.7	5.3	−5.4	−2.7	−1.6
Day 2	*n*	6	6	6	6	6	6
	Mean	8.92	27.6	154	462	695	3600
	% R.S.D.	4.3	6.3	3.1	3.0	1.4	2.1
	% Bias	−10.8	−8.0	2.7	−7.6	−7.3	−4.3
Day 3	*n*	6	6	6	6	6	6
	Mean	9.80	28.3	151	460	711	3600
	% R.S.D.	9.0	2.5	2.1	1.2	1.3	2.1
	% Bias	−2.0	−5.7	0.7	−8.0	−5.2	−4.3
Inter-day	*n*	18	18	18	18	18	18
	Overall mean	9.70	28.7	154	465	712	3630
	% R.S.D.	8.9	6.1	2.9	2.2	2.5	2.2
	% Bias	−3.0	−4.4	3.0	−7.0	−5.1	−3.5
Ribavirin base							
Day 1	*n*	6	6	6	6	6	6
	Mean	9.23	29.5	167	496	747	3470
	% R.S.D.	10.3	4.2	1.7	1.0	1.2	1.0
	% Bias	−7.7	−1.7	11.3	−0.8	−0.4	−7.7
Day 2	*n*	6	6	6	6	6	6
	Mean	10.3	29.9	164	497	730	3420
	% R.S.D.	5.4	3.4	1.8	1.3	1.9	1.5
	% Bias	3.0	−0.3	9.3	−0.6	−2.7	−9.0
Day 3	*n*	6	6	6	6	6	6
	Mean	10.1	29.5	160	493	741	3370
	% R.S.D.	7.2	5.4	1.7	1.8	1.3	1.7
	% Bias	1.0	−1.7	6.7	−1.4	−1.2	−10.4
Inter-day	*n*	18	18	18	18	18	18
	Overall mean	9.89	29.6	164	495	739	3420
	% R.S.D.	8.7	4.2	2.4	1.3	1.7	1.8
	% Bias	−1.2	−1.2	9.1	−1.0	−1.4	−9.0

normal QCs in a validation run. As can be seen in Table 2, the precision and accuracy of the dilution QCs ranged from 1.29 to 3.43% R.S.D. and from −10.6 to −5.20% bias for ribavirin and from 0.73 to 1.82% R.S.D. and from −10.6 to −4.00% bias for ribavirin base, demonstrating that samples with higher concentration can be analyzed to obtain acceptable data after dilution with blank plasma.

3.7. Stability of plasma sample during storage

The bench-top stability of ribavirin and ribavirin base in monkey plasma was evaluated at ambient temperature (∼22 °C) over 24 h using QC samples at 30.0, 500 and 3760 ng/ml. The measured concentration of ribavirin and ribavirin base in these QC samples sitting at room temperature for 24 h was compared to the

Table 2
Precision and accuracy of dilution QC samples ($n = 6$)

	Ribavirin			Ribavirin base		
Concentration (ng/ml)	25000	25000	3760	25000	25000	3760
Dilution factor	100	10	5	100	10	5
Mean (ng/ml)	22900	23700	3360	24000	24000	3360
S.D.	464	813	43.2	175	437	59.8
Precision (%)	2.02	3.43	1.29	0.730	1.82	1.78
Bias (%)	−8.40	−5.20	−10.6	−4.00	−4.00	−10.6

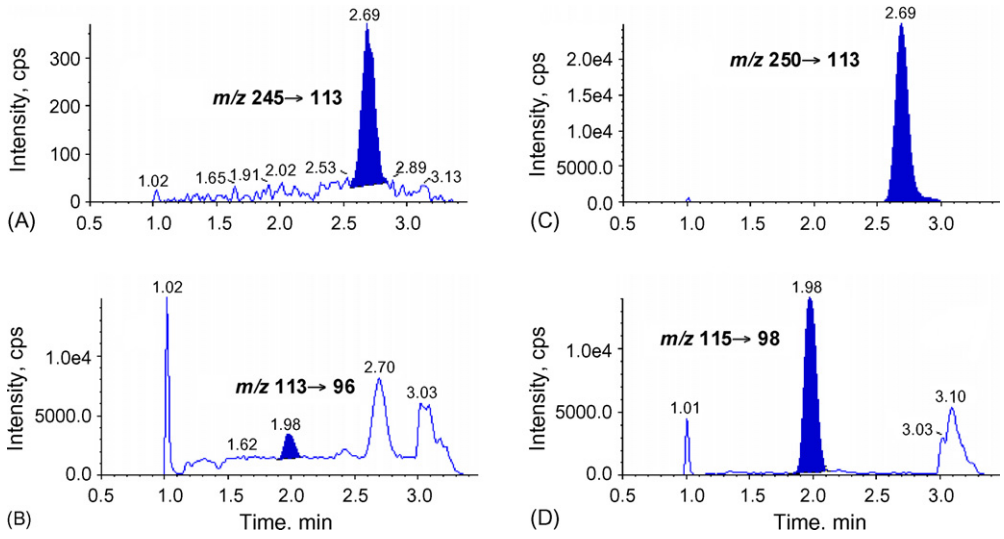

Fig. 5. Representative LC–MS/MS chromatograms of LLOQ samples: (**A**) ribavirin (*m/z* 245 → 113), (**B**) ribavirin base (*m/z* 113 → 96), (**C**) $^{13}C_5$-ribavirin (*m/z* 250 → 113) and (**D**) $^{13}C^{15}N$- ribavirin base (*m/z* 115 → 98).

nominal values, with bias (%) ranging from −6.2 to −2.9% for ribavirin and −8.2 to −0.6% for ribavirin base (Table 3), respectively, indicating that ribavirin and ribavirin base were stable for at least 24 h in monkey plasma when stored at ambient temperature. Freeze-thaw stability of QC samples at 30.0, 500 and 3760 ng/ml levels experiencing three cycles of freeze-thaw were analyzed together with one set of calibration standards and regular QC samples. The bias (%) was −9.7 to −4.0% for ribavirin and −8.8 to 2.3% for ribavirin base, respectively (Table 3).

3.8. Reinjection reproducibility

During the validation, one of the validation batches was stored in the HPLC autosampler for over 52 h and then re-analyzed and quantified. The precision (R.S.D.%) and accuracy (bias%) from these processed samples were from 1.0 to 3.9% R.S.D. and −8.0 to −4.0% bias for ribavirin, and from 1.1 to 5.0% R.S.D. and −8.8 to −1.8% bias for ribavirin base (Table 3), for the QC samples at 30.0, 500 and 3760 ng/ml levels, demonstrating that extracted samples could be analyzed after standing in the HPLC autosampler (∼15 °C) for at least 52 h with an acceptable precision and accuracy.

Table 3
Freeze/thaw and room temperature stability as well as re-injection reproducibility of ribavirin and ribavirin base ($n = 6$)

	Theoretical concentration (ng/ml)					
	Ribavirin			Ribavirin base		
	30.0	500	3760	30.0	500	3760
(A) Stability after three freeze-thaw cycles						
Mean	27.1	469	3610	30.7	497	3430
R.S.D.%	2.1	1.5	2.2	6.2	1.1	1.3
Bias%	−9.7	−6.2	−4.0	2.3	−0.6	−8.8
(B) Room temperature stability for 24 h						
Mean	29.0	469	3650	29.6	497	3450
R.S.D.%	3.8	1.7	1.1	5.2	2.0	1.0
Bias%	−3.3	−6.2	−2.9	−1.3	−0.6	−8.2
(C) Re-injection reproducibility after 52 h						
Mean	28.6	460	3610	28.9	485	3450
R.S.D.%	3.9	2.1	1.0	5.0	1.9	1.1
Bias%	−4.7	−8.0	−4.0	−3.7	−1.8	−8.8

3.9. Matrix effect and recovery

The matrix effect was estimated by spiking QC neat solutions into extracted blank plasma samples with the concentrations the same as normal QC samples (30.0, 500, 3760 ng/ml, $n = 6$). Results were calculated by comparing the mean peak areas of ribavirin and ribavirin base in these post-spiked samples with those in corresponding neat solutions. The overall matrix effect is +2% and +14% for ribavirin and ribavirin base, respectively. The recovery was estimated by comparing the mean peak areas of the analytes in the extracted QC samples (30, 500 and 3760 ng/ml, $n = 6$) with those obtained from the extracted blank plasma samples post-spiked with corresponding neat solutions in six replicates. The overall recovery is 101 and 98.8% for ribavirin and ribavirin base, respectively.

3.10. Batch size

The batch size was defined as a group of calibration standards, matrix blanks, reagent blanks, quality controls, and clinical samples that were processed through protein precipitation as described in Section 2, analyzed through sequential LC–MS/MS injections, and calculated based on the calibration curve. As a part of the validation, a total of 96 samples including calibration standards, quality controls, blanks and samples can be analyzed per run with acceptable precision and accuracy.

3.11. Carryover

In the current assay, 90% acetonitrile (containing 0.1% formic acid) and 5% methanol (containing 0.1% formic acid) were used

Table 4
Performance of quality control samples during the analysis of study samples

Run number	30.0 ng/ml	Bias%	150 ng/ml	Bias%	1500 ng/ml	Bias%	3760 ng/ml	Bias%	25000 ng/ml	Bias%
Ribavirin										
1	28.1	−6.3	146	−2.7	1380	−8.0	3650	−2.9		
	30.1	0.3	145	−3.3	1440	−4.0	3670	−2.4		
2	31.9	6.3	153	2.0	1440	−4.0	3590	−4.5		
	29.7	−1.0	151	0.7	1480	−1.3	3660	−2.7		
3	31.4	4.7	145	−3.3	1520	1.3	3630	−3.5		
	27.7	−7.7	149	−0.7	1450	−3.3	3730	−0.8		
							3500	−6.9		
							3520	−6.4		
							3480	−7.4		
							3480	−7.4		
4	29.3	−2.3	143	−4.7	1500	0.0	3510	−6.6		
	32.4	8.0	141	−6.0	1390	−7.3	3630	−3.5		
5	28.5	−5.0	150	0.0	1510	0.7	3710	−1.3	22300	−10.8
	29.3	−2.3	158	5.3	1460	−2.7	3630	−3.5	23500	−6.0
							3340	−11.2		
							3490	−7.2		
							3470	−7.7		
							3450	−8.2		
6	29.1	−3.0	157	4.7	1490	−0.7	3470	−7.7		
	30.5	1.7	144	−4.0	1470	−2.0	3490	−7.2		
							3500	−6.9		
							3440	−8.5		
							3590	−4.5		
							3510	−6.6		
7	29.3	−2.3	141	−6.0	1460	−2.7	3450	−8.2	22300	−10.8
	27.9	−7.0	142	−5.3	1370	−8.7	3230	−14.1	22900	−8.4
Mean	29.7		148		1450		3530		22800	
S.D.	1.46		5.64		47.2		114		574	
R.S.D.%	4.9		3.8		3.3		3.2		2.5	
Bias%		−1.0		−1.3		−3.3		−6.1		−8.8
Ribavirin base										
1	27.1	−9.7	157	4.7	1450	−3.3	3690	−1.9		
	26.5	−11.7	156	4.0	1500	0.0	3730	−0.8		
2	32.7	9.0	149	−0.7	1450	−3.3	3460	−8.0		
	30.2	0.7	156	4.0	1470	−2.0	3380	−10.1		
3	25.9	−13.7	140	−6.7	1380	−8.0	3470	−7.7		
	30.0	0.0	152	1.3	1360	−9.3	3450	−8.2		
							3930	4.5		
							3820	1.6		
							3720	−1.1		
							3700	−1.6		
4	31.6	5.3	150	0.0	1540	2.7	3380	−10.1		
	29.9	−0.3	152	1.3	1460	−2.7	3460	−8.0		
5	30.9	3.0	161	7.3	1530	2.0	3570	−5.1	23200	−7.2
	26.9	−10.3	167	11.3	1530	2.0	3570	−5.1	24500	−2.0
							3690	−1.9		
							3800	1.1		
							3770	0.3		
							3680	−2.1		
6	28.5	−5.0	162	8.0	1530	2.0	3440	−8.5		
	30.1	0.3	156	4.0	1490	−0.7	3400	−9.6		
							3640	−3.2		
							3640	−3.2		
							3760	0.0		
							3820	1.6		

Table 4 (*Continued*)

Run number	30.0 ng/ml	Bias%	150 ng/ml	Bias%	1500 ng/ml	Bias%	3760 ng/ml	Bias%	25000 ng/ml	Bias%
7	29.0	−3.3	148	−1.3	1470	−2.0	3340	−11.2	21300	−14.8
	27.6	−8.0	146	−2.7	1420	−5.3	3210	−14.6	23000	−8.0
Mean	29.1		154		1470		3600		23000	
S.D.	2.05		7.05		55.9		182		1310	
R.S.D.%	7.0		4.6		3.8		5.1		5.7	
Bias%		−3.0		2.7		−2.0		−4.3		−8.0

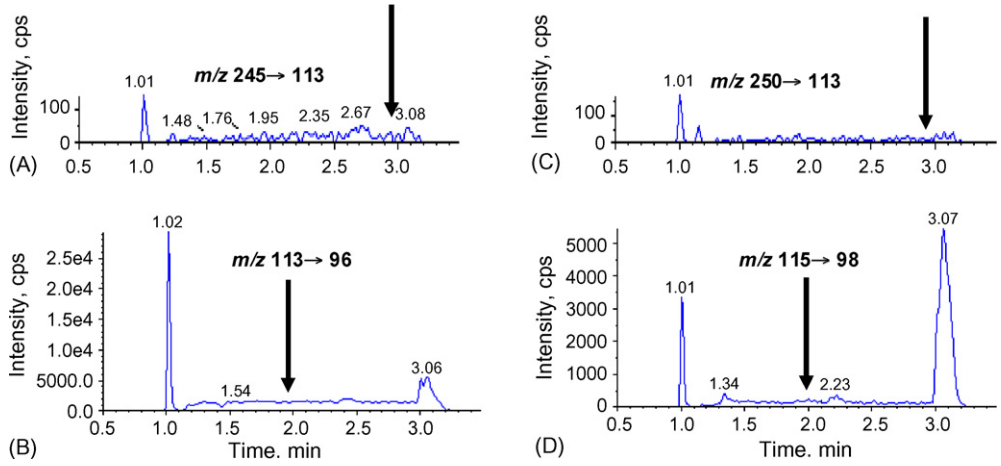

Fig. 6. Representative LC–MS/MS chromatograms of matrix blank samples (MBLK) injected right after the upper limit of quantification (ULOQ) samples: (**A**) ribavirin (*m/z* 245 → 113), (**B**) ribavirin base (*m/z* 113 → 96), (**C**) $^{13}C_5$-ribavirin (*m/z* 250 → 113) and (**D**) $^{13}C^{15}N$- ribavirin base (*m/z* 115 → 98). The arrows indicate the retention time of the compounds.

to wash syringe and injection port multiple times before and after each injection to make sure that carryover, if any, was minimized. As can be seen in Fig. 6, no significant carryover was observed.

4. Application

The present LC–MS/MS method was used to measure plasma concentrations of ribavirin and ribavirin base in monkeys following oral administration of ribavirin at 300 mg/kg/day for 28 days (data not shown). In order to demonstrate the suitability of the method, the performance of the quality control samples during the analysis of study samples are listed in Table 4. The mean bias (%) was found in the range of −8.8 to −1.0% for ribavirin and −8.0 to 2.7% for ribavirin base (Table 4).

5. Conclusions

For the first time, a bioanalytical method for the simultaneous determination of ribavirin and ribavirin base in monkey plasma was developed and validated using protein precipitation and LC–MS/MS over the concentration range of 10–5000 ng/ml, respectively, using a 0.025 ml monkey plasma sample. The current method is useful in support of the toxicokinetic and pharmacokinetic studies of these compounds.

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