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Determination of ribavirin in human serum using liquid chromatography tandem mass spectrometry

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ABSTRACT

A method has been developed for the determination of ribavirin in human serum for therapeutic drug monitoring purposes, using liquid chromatography electrospray ionization mass spectrometry. Separation was obtained with a mobile phase gradient starting and ending in 100% aqueous conditions using a Waters Atlantis® T3 column (100 × 2 mm, 3 μm). The entire sample preparation consisted of dilution, followed by ultrafiltration. From the clear ultrafiltrate 5 μL was injected on the LC–MS/MS system. The calibration curves were linear in the range of 0.2–10 mg/L with within-run and between-run precisions (CVs) in the range of 0–10%. The method was validated with respect to specificity, selectivity, linearity, accuracy, precision, recovery and stability and meets the requirements of the FDA. The method was extensively tested for matrix effects by determining the variation of the slopes of calibration curves in different sources of serum and plasma. This method is suitable for the determination of ribavirin in human serum for therapeutic drug monitoring.

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, see Fig. 1) is a synthetic guanosine analogue and has a high *in vitro* and *in vivo* activity against a broad spectrum of viruses [1]. It has mainly been used for treatment of chronic hepatitis C virus (HCV) infection in combination with pegylated interferon α [2]. The promotion of human T cell response by ribavirin is considered to be responsible for almost doubling the efficacy of this second antiviral agent [3].

Ribavirin is metabolized mainly by deribosylation to the active 1,2,4 triazole-3-carboxamide (ribavirin base), but it is also phosphorylated intracellularly to ribavirin monophosphate, diphosphate and triphosphate. Erythrocytes also have the ability to phosphorylate ribavirin, but lack the phosphatase activity to convert it back. As a consequence, the phosphorylated ribavirin becomes trapped and accumulates in these cells, which can eventually cause severe haemolytic anemia. Therefore, patients who are treated with ribavirin have to be closely monitored for these side effects.

Chan et al. concluded in 2009 that therapeutic drug monitoring (TDM) of ribavirin was not indicated [4], but Morello et al. stated that treatment failure in HIV patients treated for chronic HCV may be prevented by measuring plasma ribavirin trough concentrations at week 4, as these measurements were shown to predict relapse of HCV [5]. Ribavirin plasma target concentrations of at least 2–2.5 mg/L were proposed to maximize the achievement of sustained virologic response (SVR) in the treatment of HCV, whereas plasma concentrations >2.5 mg/L were associated with severe haemolytic anemia [6]. Furusyo et al. showed that a minimum concentration threshold of 1.5 mg/L at week 36 of the therapy was predictive of HCV relapse. They also concluded that a very early dosing adjustment of ribavirin is effective for achieving SVR [7]. Therefore TDM of ribavirin can be helpful to find a balance between the drug's efficacy and its toxic side effects.

Ribavirin is a very polar compound, due to the presence of multiple amino and hydroxyl groups within the molecule and is therefore very soluble in water. The logarithm of the partition coefficient n-octanol/water (logP) is −2.06. Ribavirin is a neutral molecule when it is kept within de pH working range of most reversed phase HPLC columns, which is between 2 and 8.

Because of its polarity, researchers have considered the possibility of using hydrophilic interaction chromatography (HILIC) for the separation of ribavirin. Shou et al. compared this technique with several reversed phase C18 sorbents and concluded that HILIC was the only technique that provided significant retention [8]. Lin et al. validated a method using HILIC for the determination of the compound in rat and monkey plasma [9].

Soon after the first HILIC publications, however, it was found that this type of separation suffered from matrix interferences [10].

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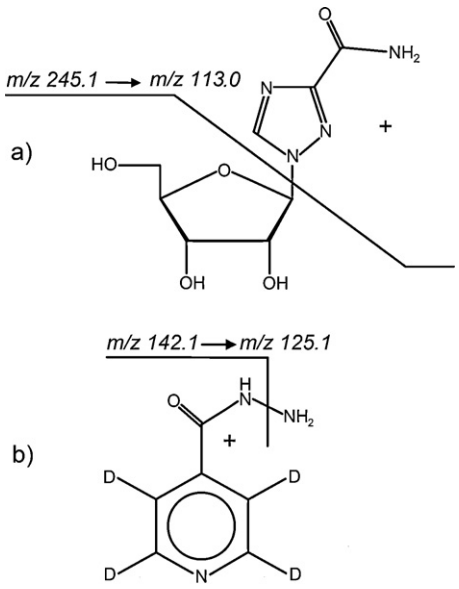

Fig. 1. Chemical structures and product ions of (a) ribavirin and (b) isoniazid-D4 (IS).

Since then, several reversed-phase methods have been developed to determine ribavirin in various matrices, ranging from red blood cells [11,12] to monkey livers [13]. In these methods, substantial capacity factors are obtained with second generation reversed-phase columns that can be operated with a purely aqueous mobile phase. However, when organic solvents are used for deproteinization of the samples [14,15], additional steps are required to change the phase of the sample extract to make it suitable for aqueous LC-conditions. Since ribavirin is a very polar compound, protein binding will be limited, which opens the possibility to apply ultrafiltration as a means for deproteinization of the serum samples. Ultrafiltration is commonly used for determining free unbound drug levels in serum [16]. Ultrafiltration has previously been applied as a means of sample preparation for other polar compounds such as acyclovir, ganciclovir and zidovudine [17–20]. The ultrafiltrate obtained is purely aqueous, and therefore suitable for direct injection on LC-systems that are equilibrated under the same conditions.

The determination of ribavirin in whole blood seems logical since ribavirin is irreversibly trapped within the erythrocytes. However, the therapeutic windows have been established in plasma and serum [6]. Therefore, the objective of this study was to develop a simple and fast method for the determination of ribavirin in human serum for TDM. The method is based on ultrafiltration as a means of sample preparation, and a reversed-phase separation, using an aqueous mobile phase at the start of the LC-gradient. The method was extensively tested for matrix effects according to the guidelines of Matuszewski [21,22].

2. Materials and methods

2.1. Chemicals, reagents and disposables

Ribavirin (purity 100%) was obtained from Roche (Woerden, The Netherlands). Isoniazid-D4 was produced by CDN Isotopes (Pointe-Claire, Canada) and imported by J.H. Ritmeester B.V. (Nieuwegein, The Netherlands). Water and acetonitrile (Ultra LC/MS-grade) were obtained from BioSolve (Valkenswaard, The Netherlands). Ammonium acetate and acetic acid (p.a. quality) from Merck were purchased from VWR (Amsterdam, The Netherlands). Pooled human serum samples were made available according to the guidelines of the University Medical Center Groningen.

Pall Nanosep 30 K Omega centrifugal devices (centrifuge filters) and Varian non-deactivated 2 mL screw top vials were purchased from VWR (Amsterdam, The Netherlands). The inserts were made from Schott Fiolax®-clear glass and purchased from Aluglas (Uithoorn, The Netherlands).

2.2. Preparation of standards, stocks and serum samples

2.2.1. Calibration standards and quality control samples for validation and routine analysis

Separate ribavirin stock solutions of 200 mg/L in water, were used for the preparation of calibration (reference) standards and quality control (QC) samples. For the preparation of calibration and QC samples appropriate amounts of stock solution were diluted with controlled blank human serum. The range of the calibration curve was based on the expected serum concentrations. The amounts of stock solution added to the serum did not exceed 5% of the total volume.

For determining the recovery special QC samples in water were prepared for comparison. Calibration standards and QC samples were freshly prepared on each validation day except for the QC samples for the bench top and freeze thaw stability, that were prepared beforehand. For determining the freeze/thaw stability separate low and high level QC samples in human serum were thawed, analyzed and frozen on three consecutive days. The samples for determining the bench top stability were freshly prepared and analyzed after being kept for 24 h at room temperature. Concentrations of the standards and QC samples are listed in Table 1. The internal standard solution was prepared by diluting a 2000 mg/L isoniazid-D4 stock solution to 3 mg/L with water.

2.2.2. Calibration standards for the assessment of matrix effects

The matrix-effect assessment was carried out using four different pools of human serum and one pool of human plasma, adding up to five different plasma/serum matrices. From each pool a calibration curve was prepared immediately before processing. For comparison, a calibration curve in water was freshly prepared with the same concentrations. A 60 mg/L isoniazid-D4 solution was used as a concentrated internal standard solution.

2.3. Sample preparation

2.3.1. Validation and routine analysis

Calibration standards, QC or human serum samples were homogenized and aliquots of 10 μL were directly transferred into the upper reservoir of the centrifuge filters and 200 μL of the internal standard solution was added. The centrifuge filters were closed and the samples were briefly homogenized, using a vortex mixer. Filtration was done by centrifugation for 10 min at 12,000 × *g* and the ultrafiltrate was transferred into a 100 μL glass insert, which was placed in a 2 mL screw top vial. From the insert, 5 μL of the ultrafiltrate was injected into the LC–MS/MS system.

2.3.2. Matrix-assessment tests

To assess matrix effects, three sets of five calibration lines were prepared. The first set (A) was prepared in water to evaluate the neat MS response of the compounds and the internal standard and it was further processed by diluting with water and by the addition of the internal standard solution according to Table 2. For the second set (B), five different calibration curves were prepared with the aqueous standards used for set (A), but here the standards were diluted with blank ultrafiltrate that was prepared beforehand, originating from the five batches of serum and plasma. The third set (C)

Table 1
Concentrations of calibration standards and QC samples.

Calibration standards (mg/L)	QC samples (mg/L)				
	LLQ	Low	Med	High	OTC
0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8,0 10	0.2	0.5	5.0	8.0	20

Table 2
Volumes used for the matrix-assessment tests.

Calibration curve set	A	B	C
Standard in water (μL)	10	10	–
Standard in serum (μL)	–	–	10
Water (μL)	190	–	190
Blank ultrafiltrate mixture(μL)	–	190	–
Conc. internal standard (μL)	10	10	10
Total volume	210	210	210

of calibration curves consisted of serum and plasma standards that were spiked before filtration.

From early tests it was noticed that the filtration efficiency of a centrifuge filter is influenced by the protein concentration of the sample; the efficiency is better when the samples are diluted. Since calibration standards of set C were diluted more than 20 times before filtration, care had to be taken to dilute the blank serum of set B in the same way as set C, without adding the internal standard. The blanks were diluted with water as much as possible before ultrafiltration and a small volume of a concentrated internal standard solution was added afterwards. Diluted blank ultrafiltrate was created by filtering 13.9 μL plasma and 250 μL water. After filtration, 10 μL of the standard solution and 10 μL of the concentrated internal standard solution was added to 190 μL of the diluted blank ultrafiltrate solution. Volumes used for this experiment are summarized in Table 2.

2.4. LC–MS/MS instrumentation and conditions

The LC–MS/MS system consisted of a Finnigan™ Surveyor® HPLC System along with a TSQ Quantum Discovery Max, triple quadrupole mass spectrometer (Thermo Fisher, San Jose, USA). The autosampler was set at a temperature of 20 °C. An Atlantis® T3 column 100 × 2 mm, 3 μm (Waters, Etten-Leur, The Netherlands) was used for separation at various flow rates. The mobile phase consisted of an aqueous 200 mM ammonium acetate buffer set at pH 5.0, water and acetonitrile. The mobile phase gradient program is given in Table 3.

Before each run, the system was conditioned by running at least five blanks using the full gradient loop. The mass spectrometer was operated in an electrospray positive ionization mode and performed selected reaction monitoring (SRM) at a scan width of 0.5 *m*/*z*. The mass parameters that were used are listed in Table 4.

The ion source spray voltage was set at 3500 V, the sheath and auxiliary gas flow rate at 35 and 5 arbitrary units respectively and the capillary temperature was set at 350 °C. Xcalibur® software version 1.4SR1 (Thermo Fisher, San Jose, USA) was applied for peak height integration for all components.

Table 3
Mobile phase gradient: A = buffer, pH 5.0, B = water and C = acetonitrile.

Step	Time (min)	Mobile phase % (v/v)			Flow
		A	B	C	μL/min
0	0	5	95	0	200
1	0.3	5	95	0	200
2	0.31	5	90	5	200
3	3	5	45	50	200
4	3.01	5	95	0	100
5	5	5	95	0	100
6	5.01	5	95	0	200
7	8	5	95	0	200

2.5. Method validation

The method was validated in accordance with the "Guidance for Industry–Bioanalytical Method Validation" of the FDA [23] over a period of 3 days On each day a calibration curve was obtained to quantify the QC samples that were being run during the same day using weighted ($1/X \times X$) linear regression. The daily QCs consisted of the LLQ, LOW, MED and HIGH samples for determination of accuracy and precision. To determine the autosampler stability the low and high level QC samples that were processed at day one were left for 24 h in the autosampler and re-injected on day two. All the QC samples were processed and run in fivefold.

The process efficiency, matrix effect and the recovery were calculated using the data of the matrix investigation. For determining the freeze/thaw stability, separate low and high level QC samples in human serum were thawed, analyzed and frozen on three consecutive days. The samples for determining the bench top stability were thawed and analyzed after being kept for 24 h at room temperature. Six independent blank matrices were processed and recorded to check for matrix interferences at the mass transitions that were monitored.

3. Results

3.1. Method development

3.1.1. Chromatography

When a reversed phase system is used for the separation of ribavirin, an almost 100% aqueous mobile phase has to be used. Under these conditions pore dewetting of the stationary phase may occur [24]. When the pressure is released from the column after a run, the polar mobile phase is repulsed from the pores of the reversed-phase sorbent. When the system is restarted, a loss of selectivity and retention of the components is then observed. We have tested a number of different columns with special sorbents that, according to the manufacturers, can be used under 100% aqueous conditions. We found, however, that not all of these columns work equally well for the analysis of ribavirin. The sorbents that were tested initially offered not enough retention, and some suffered from additional retention loss caused by pore dewetting, as was foreseen in previous literature [25]. In the literature the successful separation of ribavirin using the Waters Atlantis® dC18 column is described [14,15]. We have used its successor, the Atlantis® T3 sorbent, and no dewetting was observed in our experiments with this type of stationary phase.

As this method is also applied for the analysis of polar anti-tuberculosis drugs, the isotopically labeled internal standard isoniazid-D4 (Fig. 1) was chosen as the internal standard. Isoniazid is also a very polar compound with virtually no protein binding and therefore a suitable internal standard for the determination of ribavirin. As HCV-infected patients are often co-infected with tuberculosis and therefore treated with isoniazid, the isotopically labeled isoniazid-D4 was chosen to avoid interferences. Although the isotopically labeled ribavirin was available, it was considered

Table 4
Mass spectrometer settings for ribavirin.

Component	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	Tube lens potential (V)	Collision energy (V)
Ribavirin	245.1	113.0	157	15
Ribavirin base	113.0	96.0	100	18
Isoniazid-d4	142.1	125.1	72	14

to be too expensive for a method that is only used occasionally. In stead it was chosen to investigate the presence of any matrix-effect using the method described in Section 3.1, to compensate for the lack of an isotopically labeled internal standard.

Using the reported gradient, retention times averaged 2.7 min for ribavirin and 4.3 min for isoniazid-D4 (Fig. 2). At the mass transition of ribavirin a second non interfering endogenous compound is visible. As ribavirin decomposes to ribavirin base in the first quadrupole, it also causes a small peak at the mass transition *m*/*z* 113 to *m*/*z* 96 at the same retention time as ribavirin.

3.1.2. Calibration curve range

Therapeutic plasma concentrations range 2–2.5 mg/L when ribavirin is administered orally [6]. Calibration curve concentrations were based on these reported concentrations and ranged from 0.2 to 10 mg/L.

3.1.3. Sample preparation

When sample extracts or supernatants contain more organic solvents than the mobile phase at the start of the gradient, analyte peaks will be distorted. In our method the column was conditioned with an entirely aqueous mobile phase and hence organic solvents could not be used as a protein precipitant. Proteins can also be precipitated with zinc or ammonium sulphate solutions, but these salts are known to contaminate the LC–MS interface. A 10% trichloroacetic acid solution offers the best precipitation efficiencies of all inorganic acids being 91.4% for human serum [26]. Still the protein removal efficiencies are rather poor when compared to liquid–liquid or solid-phase extraction methods and supernatants obtained from protein precipitation remain comparatively dirty and contain relatively high concentrations of phospholipids. Since the polar ribavirin is expected to have low protein binding, we have tested ultrafiltration as a means of sample preparation. During ultrafiltration the serum is forced through a semi-permeable membrane usually by centrifugation. Proteins with a molecular size above the cut off value of the membrane are prevented from passing the membrane, and an ultrafiltrate is obtained which is virtually free from proteins. As membranes with small cut off values need longer filtration times, a balance must be found between cleanliness of the extracts and processing times. Human serum proteins consist predominantly of albumin and alpha, beta and gamma globulins. These proteins take of 99% of all the serum proteins. The molecular mass of human serum albumin ranges between 65 kDa and 69 kDa. Globulins are very divers in size and have a weight range between 50 and 1000 kDa. According to Blanchard 99.5% of all the serum proteins are removed when a 50 kDa filter is used and 99.8% when an 25 kDa is used [27]. These efficiencies are similar to or better than other deproteinization methods and when a 30 kDa filter membrane is used the ultrafiltrate should be clean enough to be directly injected into the LC–MS system.

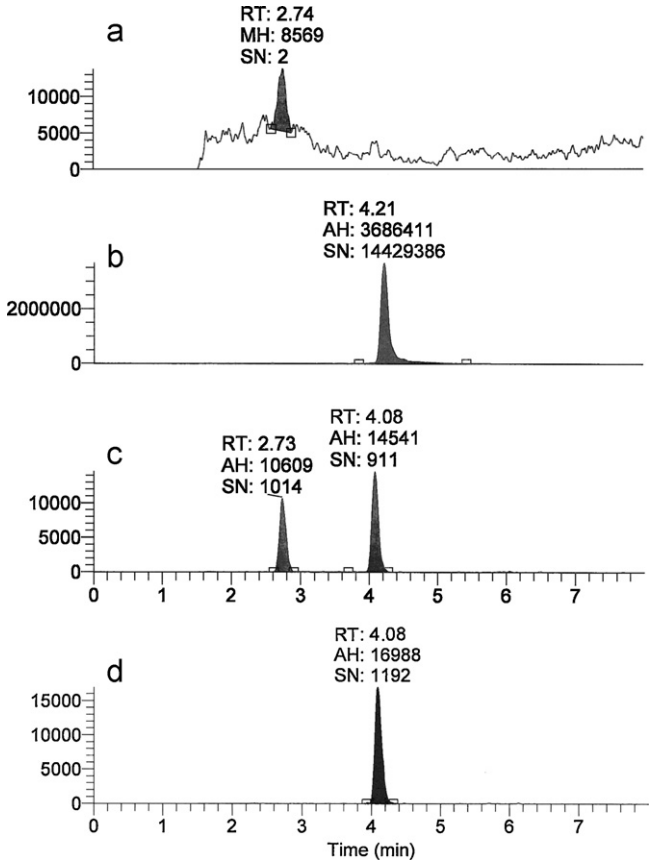

Fig. 2. Chromatogram of an LLQ level QC standard: (a) ribavirin base [*m*/*z* 113.0 → 96.0]; (b) isoniazid-D4 [*m*/*z* 142.1 → 125.1]; (c) ribavirin [245.1 → 113.0]; (d) Chromatogram of blank human serum at the mass transition of ribavirin. Retention times (RT), peak heights (MH and AH) and signal to noise ratios (SN) are indicated.

The Pall Nanosep 30 K centrifugal device was chosen because it could be easily fitted in the standard Microfuge. The "Omega" filtering membrane is made from modified polyethersulfone (PES). Preliminary tests revealed that the chromatograms of ultrafiltrated serum standards displayed a remarkable absence of phospholipids. The absence of phospholipids in the ultrafiltrate allowed for faster chromatography since only a modest amount of acetonitrile was needed in the mobile phase gradient. Parameters affecting ultrafiltration were set according to the guidelines of the manufacturer of the centrifugal devices. Samples were diluted to enhance filtration yields and to diminish protein binding. At these conditions protein filtering efficiencies were sufficient to allow for efficient protein removal.

3.2. Matrix effects, recovery and process efficiency

To assess recovery and to gain insight into absolute and relative matrix effects the strategies of Matuszewski and co-workers were applied [21,22].

Table 5
Calibration-curve parameters of the matrix assessment.

Calibration curve	A	B	C
Average slope	0.005811	0.006266	0.006588
CV	1.8%	1.5%	3.0%
Average regr. coff.	0.9985	0.9987	0.9987

Table 6
Results of the matrix assessment.

Parameters	Variables								Average
Concentrations (mg/L)	0.2	0.5	1.0	2.0	4.0	6.0	8.0	10	
Process efficiency (%)	139.2	140.0	121.4	116.2	117.9	114.2	110.3	111.8	121.4 ± 9.8%
Matrix effect (%)	126.9	125.9	126.4	117.3	113.6	113.4	110.6	110.0	118.0 ± 6.1%
Recovery (%)	109.7	111.1	96.0	99.1	103.7	100.7	99.7	101.7	102.7 ± 4.4%
Accuracy (%)	94.4	113.2	102.0	103.1	100.5	94.8	94.4	97.7	100.0
Precision CV (%)	8.6	3.6	8.7	3.2	5.1	2.7	5.7	3.7	5.2

3.2.1. Relative matrix effects

To assess the relative matrix effect, the variation coefficients (CV) of the slopes of the three sets of calibration curves were determined. According to Matuszewski's reports the CV of set C should be below 3–4%. The results for the three compounds are summarized in Table 5. The data in the tables show that the CV of the slopes for ribavirin is within 3%.

3.2.2. Process efficiency, absolute matrix effect and recovery

The overall process efficiency is calculated by comparing the peak heights of the serum standards spiked before sample preparation (set C) with the peak heights of the corresponding standards of the neat solution (set A). The absolute matrix effect is determined by comparing the peak heights of the standards that were spiked after sample preparation (set B) with the heights of the corresponding standards of set A. The recovery is determined by comparing the peak heights of set C with the peak heights of the corresponding standards of set B. Results are listed in Table 6. For calculating the matrix effect (ME), the recovery (RE) and the process efficiency (PE) the following formulas apply:

$$\text{PE}\ (\%) = \left(\frac{\text{C}}{\text{A}}\right) \times 100 \quad (1)$$

$$\text{ME}\,(\%) = \left(\frac{\text{B}}{\text{A}}\right) \times 100 \quad (2)$$

$$\text{RE}\,(\%) = \left(\frac{\text{C}}{\text{B}}\right) \times 100 \quad (3)$$

Although the average matrix effect that was measured is significant it remained constant between various pools of serum and plasma and therefore proposes no threat to the accuracy and precision of the method. From our own measurements we discovered that matrix effects associated with HILIC methods reduced the ribavirin signal to 50% of its original. Compared to this, the measured average matrix effect of 118% of this method is only modest and does not compromise the sensitivity of the method. To the authors best knowledge this method is the only method for the analysis of ribavirin where the existence of matrix effects is investigated and quantified. The average recovery did not differ significantly from 100% and the accuracy and precision levels for all components were well within the FDA's acceptance limits [23].

3.3. Analytical validation

3.3.1. Specificity and selectivity

Examination of the six independent blank human serum samples revealed no interfering components at the retention time of ribavirin.

3.3.2. Linearity

Calibration curves of ribavirin were evaluated during the matrix assessment and were obtained by fitting the ratio of peak height to that of the internal standard against the concentration (ranging 0.2–10 mg/L). The results are listed in Table 5. Although the calibration curves appeared to be slightly curved. Analysis of variance (ANOVA) after fitting the simplest available (linear) model showed that goodness of fit was significant en lack of fit was not, proving that this model described the curve adequately. Best results were obtained when weighted linear regression with a weighting factor of $1/X \times X$ was used.

Table 7
Validation results for accuracy and precision. n.s., not significant.

Parameter	LLQ	Low	Med	High
Concentration (mg/L)	0.2	0.5	4.0	8.0
Accuracy (% bias)	0.3	6.0	−2.2	−0.8
Within day precision (% CV)	7.7	7.1	6.2	5.5
Between day precision (% CV)	2.7	n.s.	2.8	1.4

Table 8
Stability testing results for ribavirin.

Test condition	Low	High
Autosampler, 24 h (% bias)	−1.0	−9.9
Bench top, 24 h (% bias)	3.0	−8.5
Freeze-thaw, 3 cycles (% bias)	−5.0	−9.1
After 5 months, −20 °C (% bias)	8.8	6.8

3.3.3. Recovery

The recoveries of all calibrators over the entire calibration-curve range were determined during the matrix-assessment tests. The results are shown in Table 6.

3.3.4. Accuracy and precision

The accuracy of the method was determined using the average outcome of the LLQ, LOW, MED and HIGH QC samples that were run on three consecutive validation days. These data were also used to determine the within-day and the between-day precision of the method, calculated as CVs. The results are listed in Table 7. All results obtained were within the acceptance criteria set by the FDA. These criteria are <20% bias and <20% CV for LLQ QC samples and <15% bias and <15% CV for the other QC samples.

3.3.5. Stability

Stability testing was performed on QC samples at low and high levels. The stability of the ribavirin was tested for 30 h in the autosampler (set at 20 °C), for 24 h at room temperature (bench top), after been subjected to three freeze-thaw cycles and after being stored for 5 months at −20 °C. Results are listed in Table 8. The data show that these circumstances did not affect the ribavirin concentration beyond the precision limit of 15%.

4. Conclusion

A simple LC–MS/MS assay for the routine analysis of ribavirin in human serum has been established. As the sample preparation is performed in 100% aqueous conditions no time consuming evaporation and reconstitution steps are needed for changing the phase of the extract. This is a major improvement compared to previous methods. The method meets all of the FDA's validation criteria over a concentration range (0.2–10 mg/L), making it suitable for TDM of

ribavirin in serum from patients who have received the compound orally in the treatment of HCV.

The matrix assessment shows that there is a significant matrix-effect for ribavirin, but it does not vary significantly between the tested matrices as the variation of the slopes of the calibration curves stayed within Matuszewski's limits [21,22].

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