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HPLC–MS/MS method for the intracellular determination of ribavirin monophosphate and ribavirin triphosphate in CEM_{SS} cells

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ABSTRACT

A sensitive and specific method using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) for the determination of ribavirin monophosphate (RBV-MP) and ribavirin triphosphate (RBV-TP) in cells has been developed and validated. In this method, ribavirin phosphorylated metabolites were extracted and separated by anion exchange solid phase extraction (SPE). The RBV-MP and RBV-TP fractions were dephosphorylated using acid phosphatase and further purified by phenyl boronate SPE prior to HPLC–MS/MS analysis. $^{13}C_5$-uridine was added as internal standard to obtain better accuracy and precision of the analysis. The MS/MS detector was optimized at multiple reaction monitoring (MRM) using positive electrospray ionization to detect 245 → 113 and 250 → 133 transitions for ribavirin and internal standard, respectively. The calibration curve was linear over a concentration range of 0.01–10 μg/mL with a limit of quantitation of 0.01 μg/mL. Mean inter-assay accuracy and precision for RBV-MP and RBV-TP quality control samples at 0.03, 0.3 and 8 μg/mL were 5% and 10%, respectively. This method was successfully used for the *in vitro* determination of RBV-MP and RBV-TP in CEM_{ss} cells cultured with RBV.

1. Introduction

Ribavirin (RBV) (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analog, structurally related to the endogenous ribonucleoside guanine (Fig. 1) and has demonstrated a broad-spectrum activity against a variety of DNA and RNA viruses [1]. RBV is rapidly and extensively transported into almost all cell types in the body via a nucleoside transporter [2].

Intracellularly, RBV is phosphorylated to mono-, di- and triphosphate moieties (RBV-MP, RBV-DP and RBV-TP, respectively), and dephosphorylation is required prior to exiting the cell. Erythrocytes lack dephosphorylation enzymes, thus RBV accumulates extensively and can only be released by cell disruption. The rate of accumulation in erythrocytes is dependent on extracellular RBV concentration; however, this mechanism does not apply to nucleated cells [3]. Consequently, the assumption that intracellular concentrations of RBV increase relative to extracellular concentrations is not correct and extracellular determination of RBV may not be indicative of the intracellular metabolism and antiviral activity. In order to better understand the intracellular pharmacokinetic characteristics, an assay that can allow specific, sensitive and accurate measurement of RBV metabolites is necessary.

2. Intracellular measurement of ribavirin metabolites

Homma et al. reported a RP HPLC–UV method for the determination of unchanged and phosphorylated RBV levels in whole blood [4]. This indirect methodology was based on the conversion of RBV metabolites to RBV with quantitation of the parent drug. The level of phosphorylated RBV was estimated by comparing the difference between the total and unchanged levels, which were measured in samples with and without dephosphorylation, respectively. In addition, concentrations of total (unchanged plus phosphorylated) RBV in erythrocytes was estimated from the corresponding plasma and whole blood levels, using the subject's hematocrit count. Major drawbacks of this methodology include a lower limit of quantitation (LLQ) of 400 ng/mL and mean recovery of 63.2% at the LLQ level. In addition, the extreme polar nature of RBV required highly aqueous composition of the HPLC mobile phase, in order to obtain reproducible retention time for RBV and adequate resolution from endogenous interferences.

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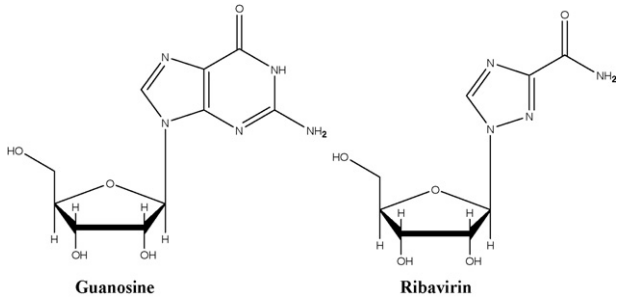

Fig. 1. Chemical structures of guanosine and its nucleoside analog ribavirin.

Various HPLC methods were developed with tandem mass spectrometers (MS/MS) to achieve better sensitivity and specificity for the RBV determination in biological fluids. Initial HPLC–MS/MS experiments were carried out by using underivatized silica columns with high organic mobile phases, acetic acid and trifluoroacetic acid (TFA) [5,6]. These methods presented significant matrix effect due to the presence of endogenous substances in the sample extracts after protein precipitation. Yeh et al. reported another HPLC–MS/MS method for the determination of total RBV in monkey liver samples (20 mg tissue) [7]. In this method, RBV and its phosphorylated metabolites were extracted from monkey liver samples using perchloric acid and all the metabolites were subsequently converted in RBV using acid phosphatase. The final extract was then purified using an aminopropyl (NH_2) solid phase extraction (SPE) cartridge and analyzed by HPLC–MS/MS. The MS/MS transition was m/z $245 \rightarrow 113$. The methodology has the disadvantage of a LLQ of 1000 ng/mL, which is not suitable for samples with small cell concentration ($<10 \times 10^6$ cells).

Lin et al. reported presented a HPLC–MS/MS method for the simultaneous determination of RBV and viramidine, a second generation analog and prodrug of RBV, in red blood cells. This methodology was applied to samples from rats and monkey plasma as well as human serum, with a LLQ of 10 ng/mL [8–10]. The same research group also reported a more sensitive HPLC–MS/MS assay for the simultaneous determination of RBV and viramidine in human plasma [11]. The LLQ was 1 ng/mL for both RBV and viramidine.

Although high sensitivity and specificity for RBV was achieved with the aforementioned methodologies, the measurements were performed in plasma samples and no information was presented regarding intracellular concentrations of key metabolites. Thus, there is a need for a sensitive and specific methodology, in which intracellular levels of RBV metabolites can been separated and determined individually, in order to evaluate the intracellular pharmacokinetic profile.

In this report, we describe a sensitive HPLC–MS/MS method for the intracellular determination of RBV-MP and RBV-TP with a LLD of 5 ng/mL and a LLQ of 10 ng/mL. This assay has been validated successfully used to determine the intracellular RBV-MP and RBV-TP levels of CEM_{ss} cells cultured with RBV.

3. Materials and methods

3.1. Chemicals

RBV (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was purchased from Sigma Chemical Company (St. Louis, MO). RBV-MP, RBV-TP, ^{3}[H]-RBV, ^{3}[H]-RBV-MP, and ^{3}[H]-RBV-TP were purchased from Moravek Biochemicals (La Brea, California). $^{13}C_5$-uridine (internal standard, IS) was obtained from Omicron Biochemicals (South Bend, IN). Ammonium acetate, ammonium hydroxide, formic acid, Tris–HCl, potassium chloride (KCl), glacial acetic acid, sodium acetate (NaOAc) and scintillation cocktail were purchased from Fisher Scientific Company (Fair Lawn, NJ). Acid phosphatase and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Company (St. Louis, MO). Culture media RPMI-1640 and L-glutamine were obtained from BioWhittaker (Baltimore, MD). Fetal bovine serum and penicillin/streptomycin solution were purchased from HyClone (Logan, UT). Methanol and acetonitrile were of MS grade (OPTIMA) quality obtained from Fisher Scientific Company (Fair Lawn, NJ). Nanopure water was produced by a Milli Q system.

3.2. Materials and equipment

CEM_{ss} cells were obtained from the NIH AIDS Research and Reference Reagent program. Anion exchange Sep-pak plus 360 mg QMA cartridges were purchased from Waters Company (Milford, Massachusetts). Bond-Elut PBA covalent cartridges were purchased from Varian Inc. (Palo Alto, California). The 24-port vacuum manifold into which the QMA and Bond-Elut PBA cartridges were adapted was purchased from Aldrich Scientific Company (Milwaukee, WI). The Phenomenex C_{18} reversed-phase column (100 mm × 1.1 mm, 3 μm particle size) was purchased from Phenomenex (Torrance, CA). A Labconco centrivap console was used to evaporate eluent solvent (Kansas City, MO). The LS9000 scintillation counter used for quantification of ^{3}[H]-RBV and its metabolites at the Physiology Department Core Lab was purchased from Beckman Instruments. Cells were counted using a Z_2 Series Coulter Counter purchased from Beckman Instruments (Fullerton, CA).

3.3. Instruments and settings

3.3.1. High performance liquid chromatography

HPLC sample analyses were performed using an Alliance 2965 system equipped with a refrigerated autosampler from Waters Company (Milford, MA). A reversed-phase chromatography was conducted using a Phenomenex Luna C_{18} (2) column (100 mm × 1.1 mm, 3 μm particle size). An isocratic elution was performed using an aqueous mobile phase consisting of 5% MeOH, 10% of 100 mM ammonium acetate (pH 5.0) at a flow rate of 0.03 mL/min and 30 °C temperature with a run time of 10 min.

3.3.2. Electrospray ionization tandem mass spectrometry

The HPLC was coupled to a Quattro Micro triple quadrupole mass spectrometer purchased from Waters (Milford, MA). For sample analysis, the instrument was operated and optimized in the positive electrospray ionization and multiple reaction monitoring (MRM) detection. Sample introduction was through an electrospray ionization source in the positive ion mode. The cone voltage was optimized for both RBV and IS (between 15 and 17 V), the desolvation temperature was 200 °C and the source temperature was 120 °C. Cone and desolvation gas flows were 70 and 500 L/h, respectively. Precursor ions were fragmented to product ions by collision at 4 eV, with a cell pressure of approximately 7×10^{-4} mbar argon. MRM data were acquired in a single function with two different transitions for the precursor ions (RBV: $245 \rightarrow 113$ and $^{13}C_5$-uridine: $250 \rightarrow 113$). Mass spectra for both RBV and $^{13}C_5$-uridine were obtained (Fig. 2). Total ion and MRM chromatograms were acquired and analyzed using the MassLynx software (version 4.1) supplied by the manufacturer.

3.4. Experimental procedures

3.4.1. Preparation of standard solutions

Stock solutions of RBV nucleosides and nucleotides as well as $^{13}C_5$-uridine were prepared at 1 mg/mL in Milli Q water from com-

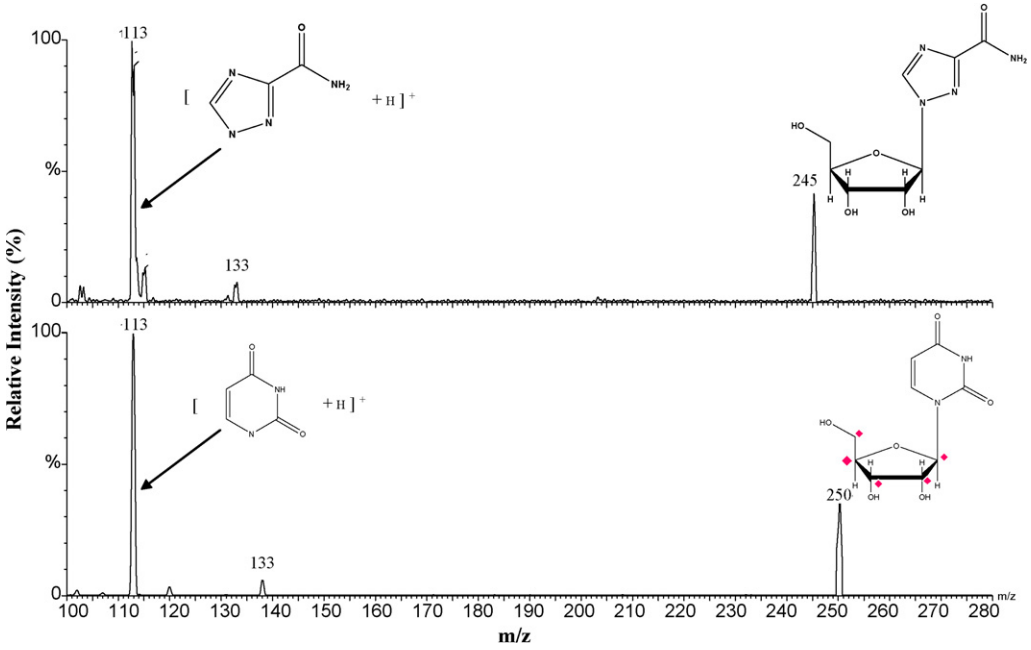

Fig. 2. Mass spectra of RBV (upper panel) and ^{13}C-uridine (lower panel).

mercially available reagents. Standard RBV nucleotides solutions for calibration curves were prepared from serial dilutions at concentrations ranging from 0.01 to 10.0 μg/mL and spiked into CEM_{ss} cells blanks. Quality control (QC) samples were prepared at concentrations of 0.03, 0.3 and 8.0 μg/mL by spiking RBV-MP and RBV-TP standards into CEM_{ss} cells blanks. Nucleosides standard solutions used in the optimization of MS parameters were also prepared from serial dilutions at concentration range from 0.005 to 10.0 μg/mL in HPLC mobile phase.

3.4.2. Sample preparation for LC–MS/MS analysis

Samples of 10×10^6 CEM_{ss} cells were harvested with 1 mL cold lysis buffer (70% MeOH, 15 mM Tris–HCl, pH 7.4). The extracts were centrifuged at $1700 \times g$ at 4 °C for 10 min. The supernatants were transferred to the anion exchange SPE column attached to a vacuum manifold. RBV-MP and RBV-TP fractions were separated and collected with a KCl gradient. Enzymatic digestion of RBV-MP and RBV-TP moieties was performed by addition of 2115 and 1615 μL of Milli Q water to the MP and TP solution, respectively. The pH of the sample was adjusted to 5.0 with NaOAc 1 M and 20 μL of acid phosphatase was added. The samples were incubated at room temperature for 24 h. After enzymatic digestion, 20 μL of $^{13}C_5$-uridine (0.5 μg/mL) was added as an internal standard to the resulting mixture. Following vortexing, 6 μL of ammonium hydroxide was added to adjust the pH to 8.5. This mixture was loaded onto the 500 mg phenyl boronic acid (PBA) cartridge, positioned on a 24-port vacuum elution manifold (Supelco). Before sample loading, PBA cartridges were pretreated with 6 mL of 3% formic acid in MeOH followed by 6 mL of 250 mM ammonium acetate buffer (pH 8.5). The samples at the cartridges were washed five times under reduced pressure with 6 mL aliquots of 100 mM ammonium acetate buffer (pH 8.5). Ribavirin and the internal standard were subsequently eluted with 4 mL of 3% formic acid in MeOH into glass tubes. The effluents were dried completely under vacuum at 35 °C and then reconstituted with 200 μL of HPLC mobile phase. Twenty microliters of the samples were injected onto the HPLC column for the analysis.

3.5. Method validation

3.5.1. Selectivity

Blank samples from the entire experimental process were analyzed to evaluate possible interferences from the experimental procedure. Possible interferences from endogenous components were also evaluated by the analysis of CEM_{ss} cells control samples. Chromatographic selectivity was evaluated by the presence or absence of interfering peaks at the analytes retention times. Mass spectral selectivity for possible interferences was also evaluated by the analysis of a mix of nucleoside standard solutions and their simultaneous detection at their ion transitions in the MRM detection mode.

Mass spectral selectivity was based on the ion selection used in the detection of the nucleosides. Electrospray mass spectra were obtained using 1 μg/mL RBV and internal standard solution prepared in HPLC mobile phase. Each solution was directly infused into the electrospray source of the mass spectrometer. Selection of the precursor and the daughter ions for each molecule were based on the mass spectra using previously optimized instrumental conditions. The optimized *m*/*z* values as well as cone voltages and collision energies were placed in the MRM function.

3.5.2. Linearity

Linearity was determined using calibration curves of standard solutions of RBV in the concentration range of 0.005–10.0 μg/mL. A regression plot was generated using peak area ratio versus RBV concentration. In addition, the linearity was evaluated using nucleotide solutions at concentrations from 0.01 to 10.0 μg/mL. Nucleotide standard solutions were processed trough the entire methodology including anion exchange solid phase extraction, enzymatic hydrolysis, XAD desalting, PBA solid phase extraction and HPLC–MS/MS analysis.

3.5.3. Lower limits of detection and quantitation

The lower limit of detection and quantitation (LLD and LLQ, respectively), were determined from calibration curves data. LLD was defined as the lowest concentration of RBV-MP with a signal to noise ratio of 3:1 or higher. The LLQ was defined as the lowest con-

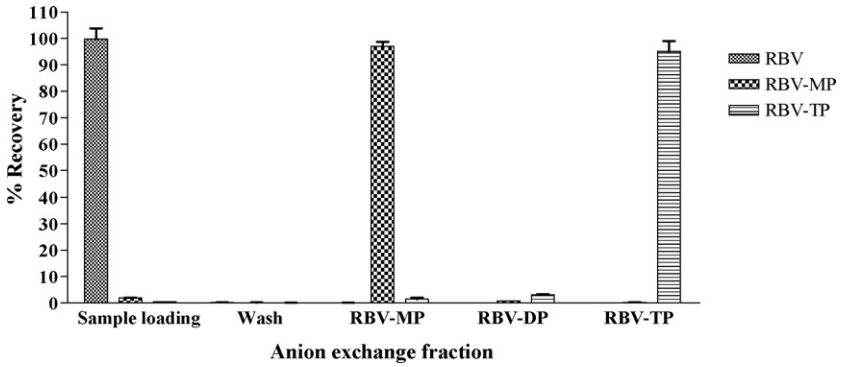

Fig. 3. Separation efficiency of RBV, RBV-MP and RBV-TP by anion exchange solid phase extraction. The results are the average of three independent experiments with their standard deviations.

centration of RBV-MP that can be identified and reproducible with a response at least 10 times the response of the blank chromatogram.

3.5.4. Accuracy and precision

Intra-assay accuracy and precision for the RBV-MP determination methodologies were evaluated using the QC samples. The QC samples (three replicates) were processed individually and analyzed by HPLC–MS/MS at the same run batch. For inter-assay accuracy and precision determinations QC samples were prepared at the same concentration ranges, but they were processed and analyzed by HPLC–MS/MS in different days using fresh prepared standard solutions.

3.5.5. Recovery

Recovery of the nucleotides RBV-MP and RBV-TP trough the SPE steps was evaluated by the use of ^{3}[H]-RBV-MP and ^{3}[H]-RBV-TP. Enzymatic hydrolysis efficiency was evaluated by HPLC–MS/MS.

3.5.6. Stability

Freeze and thaw stability was determined by freezing and thawing the low and high QC samples for three cycles before analysis with 24 h interval between each cycle. Short-term stability was measured using aliquots at low and high concentration (0.03 and 8.0 μg/mL, respectively) of RBV prepared in the corresponding mobile phase. Samples were kept at room temperature for 24 h and then analyzed by HPLC–MS/MS.

3.6. In vitro experiments

CEM_{SS} cells were cultured in RPMI-1640 medium supplemented with 10.0% of heat inactivated fetal bovine serum, 1.0% L-glutamine and 1.0% of 10,000 units of penicillin/streptomycin. The cell culture was maintained at 37 °C in an atmosphere of 5.0% carbon dioxide. After reaching logarithmic growth rate, the cell concentration was maintained at 0.5×10^{6} cells/mL for the subsequent experiments. Three biological replicates of four subcultures were prepared. Each biological replicate was composed of a Control and RBV subculture. Cells were incubated with 10 μM RBV during 14 days. Cell viability of the CEM_{SS} cells cultures were obtained with Trypan blue stain assay. A value above 95% was required for conducting the *in vitro* experiments. After day 14 of experiment, three aliquots of 10.0×10^{6} cells were removed from each subculture. Cells were pelleted by centrifugation at $1700 \times g$ at 4 °C for 10 min. The supernatant was discarded and pellet was rinsed with 1.0 mL of phosphate-buffered saline solution. Centrifugation was repeated and supernatant was discarded. Cells were harvested by addition of 1.0 mL of cold lysis buffer (70% methanol, 15 mM Tris–HCl, pH 7.4). The cell extracts were transferred to 1.5 mL cryovials and stored at −70 °C until analysis.

4. Results

A high performance liquid chromatography–tandem mass spectrometry method was developed for the simultaneous quantitation of intracellular RBV-MP and RBV-TP. Using this methodology, intracellular RBV-MP and RBV-TP were determined from *in vitro* experiments in CEM_{SS} cells exposed during 14 days to RBV combination. RBV metabolites were extracted from CEM_{SS} cells and separated by anion exchange SPE. The separated RBV-MP and RBV-TP fractions were dephosphorylated by enzymatic hydrolysis using acid phosphatase. After enzymatic hydrolysis, the RBV was then purified by PBA SPE cartridges the final extract was the reconstituted and analyzed by HPLC–MS/MS. The RBV metabolites isolation efficiency, enzyme digestion efficiency and purification efficiency in addition to the HPLC–MS/MS sensitivity, calibration curve linearity, accuracy, precision and stability have been examined.

4.1. Separation of ribavirin metabolites

In the first step of the sample preparation, the phosphorylated metabolites RBV-MP and RBV-TP were separated from RBV by anion exchange SPE. The anion-exchange SPE methodology was modified for the simultaneous isolation of RBV and ZDV metabolites. The percent recovery of RBV, RBV-MP and RBV-TP was determined using ^{3}[H]-RBV, ^{3}[H]-RBV-MP and ^{3}[H]-RBV-TP standards. The anion exchange was performed by increasing KCl concentration in order to elute the mono- and triphosphate moieties sequentially. Fig. 3 shows a representative experiment for the anion exchange recovery. Control samples were prepared by addition of 1 μCi RBV, RBV-MP or RBV-TP to 1.5 mL Milli Q water. Recovery experiments were performed in triplicate. The mean recovery percents were more than 90% for RBV, RBV-MP and RBV-TP. These results demonstrated the capacity of anion exchange solid phase extraction for the simultaneous separation of RBV and its metabolites. Anion exchange SPE is very simple and it has the advantage of processing multiple samples simultaneously, with the use of a 24-port vacuum manifold. In addition, it was possible to assess simultaneous isolation of RBV-MP and RBV-TP metabolites from one sample, which is an important factor to be considered if the method is applied to clinical protocols.

4.2. Enzyme conversion efficiency

The enzymatic hydrolysis efficiency was determined by HPLC–MS/MS using equimolar standard solutions of RBV, RBV-MP and RBV-TP. The solutions were exposed to an enzymatic digestion procedure with acid phosphatase enzyme. After digestion the hydrolyzed samples were purified and the final extracts were analyzed by HPLC–MS/MS. Both RBV-MP and RBV-TP were success-

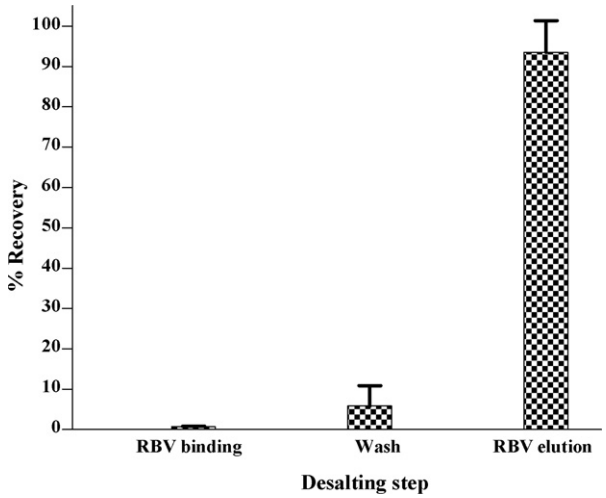

Fig. 4. Desalting efficiency of RBV using phenyl boronic acid SPE cartridges. The results are the average of three independent experiments with their standard deviations.

fully converted to RBV after the incubation with an efficiency of $95 \pm 2\%$.

4.3. RBV desalting efficiency

Since RBV is a highly polar compound, a covalent phase SPE cartridge containing PBA was used for the desalting of dephosphorylated samples after enzymatic hydrolysis. The efficiency of RBV desalting by PBA-SPE cartridges was evaluated using ^{3}[H]-RBV standard solutions. RBV was successfully desalted with a recovery percent of $(94 \pm 8)\%$. Fig. 4 shows a representative experiment for RBV desalting.

4.4. HPLC–MS/MS separation, selectivity and sensitivity

HPLC–MS/MS system blanks were obtained to assess any interference from the sample preparation procedure. Fig. 5 shows the typical chromatograms from system blanks at the *m*/*z* transitions of both RBV and internal standard. No interferences were detected at the system blanks, demonstrating the HPLC–MS/MS method selectivity at the selected *m*/*z* transitions.

In order to evaluate possible interferences from biological matrix, samples from blank CEM_{ss} cells extract were processed through the methodology and analyzed by HPLC–MS/MS. A typical chromatogram of CEM_{ss} blank extract is presented in Fig. 6. Only one peak exhibited the same RBV *m*/*z* transition (retention time ~6 min) and corresponds to the endogenous uridine phosphates. The results demonstrate that the method provides adequate separation of the endogenous interferences through HPLC and selectivity trough the MS/MS detection. Fig. 7 presents the LLQ for the HPLC–MS/MS method. It was determined to be 0.01 μg/mL and had a signal-to-noise ratio greater than 10 during the validation.

4.5. Standard curve linearity

Calibration curves for RBV were generated by analyzing 10×10^{6} cells extracts individually spiked with either RBV-MP or RBV-TP standard solutions at concentrations range between 0.01 and 10 μg/mL and processing trough the complete methodology. Linear regression analyses were determined for RBV with correlation coefficients (r^2) greater than 0.990 for the calibration curves from RBV-MP and RBV-TP standards. A representative calibration curve is presented in Fig. 8. The results indicated the linearity of the detector response as a function of the standard calibration curve. The mean back-calculated values from the fitted curves were between 6% and 10% of their nominal values for RBV-MP and RBV-TP standards, respectively (Table 1).

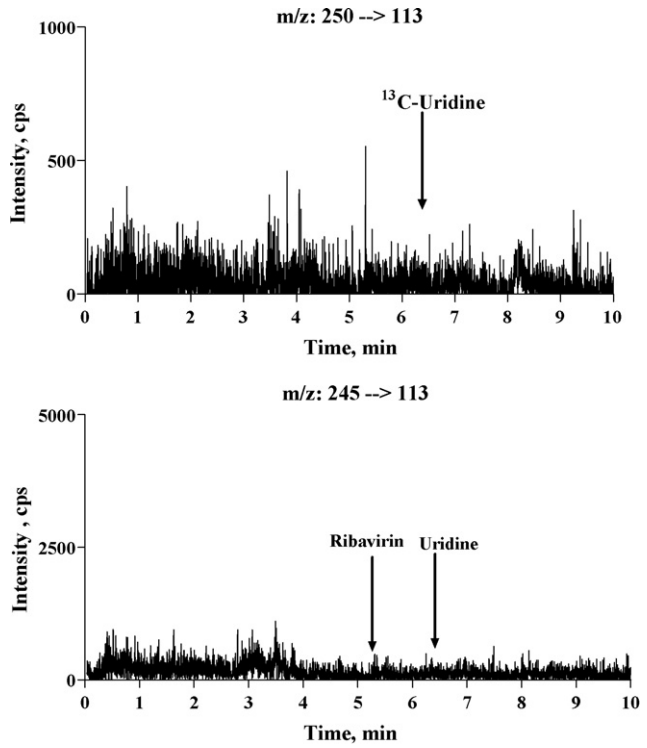

Fig. 5. Representative chromatogram of the HPLC–MS/MS system blank. Upper panel: *m*/*z* transition for ^{13}C-uridine (internal standard), lower panel: *m*/*z* transition for RBV.

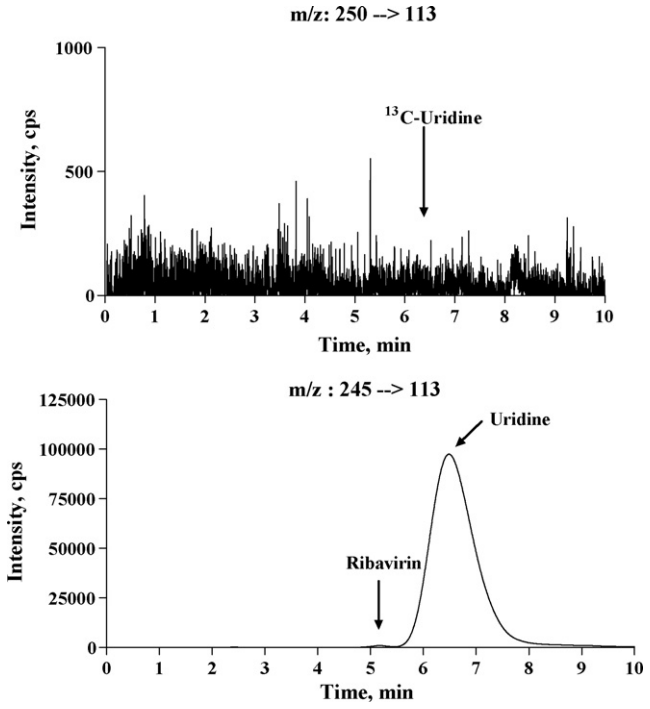

Fig. 6. Representative chromatograms of CEM_{ss} cell blank extract: ^{13}C-uridine (upper panel) and RBV *m*/*z* transition (lower panel).

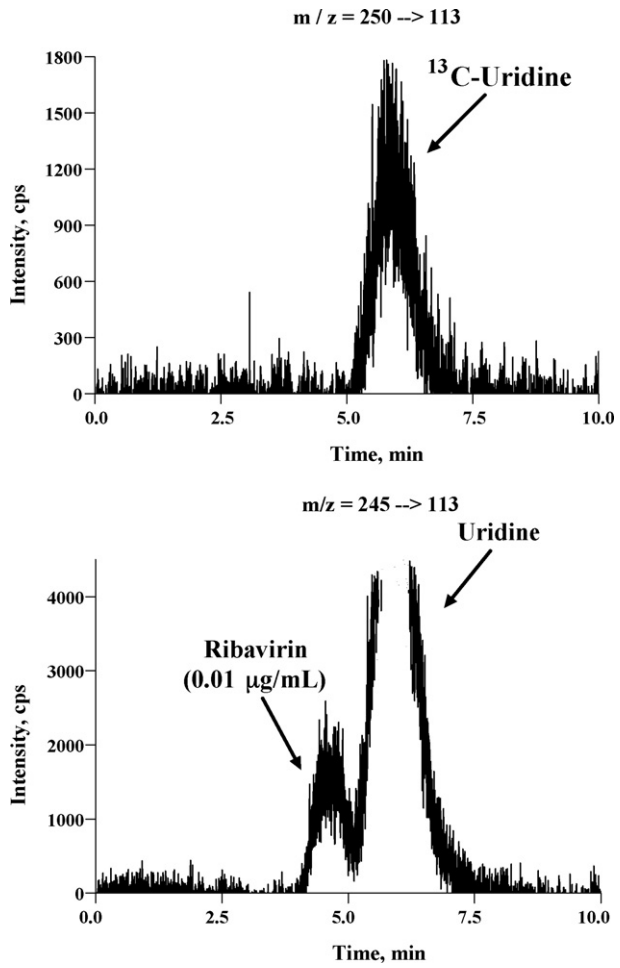

Fig. 7. Representative chromatograms of CEM_{ss} cells LLQ (0.01 μg/mL): ^{13}C-uridine (upper panel) and RBV *m*/*z* transition (lower panel).

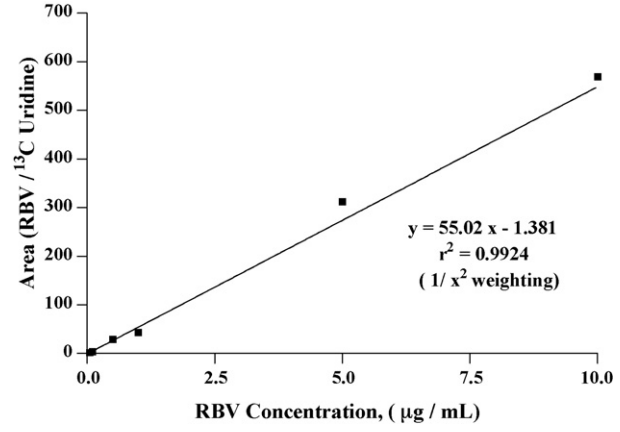

Fig. 8. Typical calibration curve for the determination of RBV in CEM_{ss} cells extracts.

4.6. Accuracy and precision

Accuracy and precision were determined based on low-, mid- and high-QC samples. For RBV-MP determination mean intra-assay accuracy (%Bias) was ±7.1% of their nominal values, while RBV-TP mean intra-assay accuracy was ±3.2%. Overall intra-assay precision (%CV) for RBV-MP and RBV-TP were between 6.6% and 17.3%, respectively. Inter-assay accuracy was also evaluated obtaining mean values of ±8.2% for RBV-MP and ±0.8% for RBV-TP. Inter-assay precision was between 8.4% and 14.8% for RBV-MP and between 3.7% and 11.9% for RBV-TP. Table 2 presents the results for RBV-MP and RBV-TP QC samples.

Table 1
Calibration curve analytical results for RBV-MP and RBV-TP in CEMss cells.

Curve ID	RBV-MP concentration (mg/mL)						
	0.01	0.05	0.10	0.50	1.00	5.00	10.00
1	0.01	0.05	0.09	0.56	1.00	4.56	10.30
2	0.01	0.05	0.10	0.50	0.93	5.00	11.10
3	0.01	0.04	0.08	0.52	1.01	6.10	10.00
4	0.01	0.05	0.10	0.45	0.94	5.10	9.60
5	0.01	0.05	0.11	0.50	1.00	5.60	9.80
TV	5	5	5	5	5	5	5
Mean	0.01	0.048	0.095	0.506	0.976	5.272	10.16
S.D.	0.00	0.00	0.01	0.04	0.04	0.59	0.59
CV (%)	0.00	9.32	13.93	7.86	3.87	11.24	5.76
Bias (%)	0.00	−4.00	−5.00	1.20	−2.40	5.44	1.60
Curve ID	RBV-TP concentration (mg/mL)						
	0.01	0.05	0.10	0.50	1.00	5.00	10.00
1	0.01	0.05	0.09	0.50	0.80	5.70	10.30
2	0.01	0.05	0.09	0.50	0.90	5.00	10.04
3	0.01	0.05	0.08	0.45	0.95	4.57	10.00
4	0.01	0.05	0.11	0.40	1.00	5.05	9.55
5	0.01	0.06	0.11	0.45	0.85	4.75	9.80
N	5	5	5	5	5	5	5
Mean	0.01	0.052	0.096	0.46	0.9	5.014	9.938
S.D.	0.00	0.00	0.01	0.04	0.08	0.43	0.28
CV (%)	0.00	8.60	13.98	9.09	8.78	8.57	2.82
Bias (%)	0.00	4.00	−4.00	−8.00	−10.00	0.28	−0.62

4.7. Over the curve dilution analysis

Over the curve dilution was determined by diluting 20 μg/mL of RBV-MP and RBV-TP QC samples by factors of 0.1, 0.2 and 0.02. The diluted samples were processed trough the complete methodology and analyzed by HPLC–MS/MS. Table 3 shows the results for the over the curve dilution analysis. The %CV values were between 3% and 12.5% for both RBV-MP and RBV-TP diluted samples at ranges from 0.4 to 4.0 μg/mL. These results indicate that over the curve dilution can be achieved for high concentration samples.

4.8. Stability of ribavirin metabolites in CEM_{ss} and final extracts

The stability of RBV-MP and RBV-TP in CEM_{ss} cells was evaluated using 10×10^6 cells individually spiked with RBV-MP and RBV-TP standard solutions of 0.03 and 8 μg/mL. After three freeze–thaw cycles, the samples were processed and analyzed by HPLC–MS/MS. The results were compared to freshly spiked samples processed simultaneously. Both RBV-MP and RBV-TP were stable in the three freeze–thaw cycles. In addition, RBV standard solutions were prepared at concentrations of 0.03 and 8 μg/mL to assess 24 h bench stability of the RBV final extract. RBV was stable on the bench for at least 24 h.

4.9. Determination of RBV-MP and RBV-TP in CEM_{ss} cells

CEM_{ss} cells cultured during 14 days with 10 μM RBV were analyzed by the aforementioned validated method. The results are presented in Fig. 9. Both intracellular RBV-MP and RBV-TP could be isolated and their levels could be measured from 10×10^6 cells extracts of the cultured samples. Neither RBV-MP nor RBV-TP was detected in control samples.

Table 2
Quality control anlytical results for RBV-MP and RBV-TP.

RBV-MP intra-assay	Low-QC (0.03 mg/mL)	Mid-QC (0.30 mg/mL)	High-QC (8.00 mg/mL)
	0.03	0.30	7.30
	0.04	0.35	8.00
	0.03	0.35	8.50
Mean	0.03	0.33	7.93
S.D.	0.01	0.03	0.60
CV (%)	17.3	8.7	7.6
Bias (%)	11.1	11.1	−0.8
RBV-MP inter-assay	Low-QC (0.03 mg/mL)	Mid-QC (0.30 mg/mL)	High-QC (8.00 mg/mL)
	0.03	0.35	7.93
	0.03	0.40	7.70
	0.04	0.30	6.75
Mean	0.03	0.35	7.46
S.D.	0.01	0.05	0.63
CV (%)	14.8	14.3	8.4
Bias (%)	14.8	16.7	−6.7
RBV-TP intra-assay	Low-QC (0.03 mg/mL)	Mid-QC (0.30 mg/mL)	High-QC (8.00 mg/mL)
	0.04	0.30	8.70
	0.03	0.30	7.70
	0.03	0.25	8.60
Mean	0.03	0.28	8.33
S.D.	0.01	0.03	0.55
CV (%)	17.3	10.2	6.6
Bias (%)	11.1	−5.6	4.2
RBV-TP inter-assay	Low-QC (0.03 mg/mL)	Mid-QC (0.30 mg/mL)	High-QC (8.00 mg/mL)
	0.04	0.28	8.33
	0.03	0.30	6.70
	0.03	0.28	7.60
Mean	0.03	0.29	7.54
S.D.	0.00	0.01	0.82
CV (%)	11.9	3.7	10.8
Bias (%)	7.4	−4.1	−5.7

5. Discussion

Several HPLC-based methods have been developed for the analysis of RBV metabolites in biological matrices, most of them using UV detection or ^{14}C compounds [4,12–14]. HPLC-UV methods have the disadvantage of interfering peaks at the same wavelengths for RBV. As a consequence, the methods detect significant matrix interferences, avoiding the high sensitivity needed for pharmacological assays. The introduction of HPLC–MS/MS systems allowed the development of more sensitive and specific methodologies for RBV determination. Various HPLC–MS/MS methods were developed for the determination of total RBV (RBV, RBV-MP and RBV-TP) from whole blood and red blood cells in animals and humans [7–10,15]. However, the methods developed only quantified the parent drug RBV and not the RBV metabolites. Since RBV is a nucleoside analog, its needs to be metabolized intracellularly to the RBV-MP moiety to exert antiviral functions in HCV infected patients [16]. In addition to the inhibition of inosine monophosphate dehydro-

Table 3
Partial volumes analytical results for RBV-MP and RBV-TP.

RBV-MP	Concentration		
	PV1 (4.0 mg/mL)	PV2 (2.0 mg/mL)	PV3 (0.4 mg/mL)
	3.80	2.10	0.47
	4.06	1.83	0.37
	4.50	1.93	0.40
Mean	4.12	1.95	0.41
S.D.	0.35	0.14	0.05
CV (%)	8.59	6.99	12.42
Bias (%)	2.21	1.71	3.21
RBV-TP	Concentration		
	PV1 (4.0 mg/mL)	PV2 (2.0 mg/mL)	PV3 (0.4 mg/mL)
	3.50	1.80	0.40
	4.00	1.70	0.32
	4.20	1.72	0.33
Mean	3.90	1.74	0.35
S.D.	0.36	0.05	0.04
CV (%)	9.25	3.04	12.45
Bias (%)	2.25	0.66	2.72

PV: partial volume dilution.

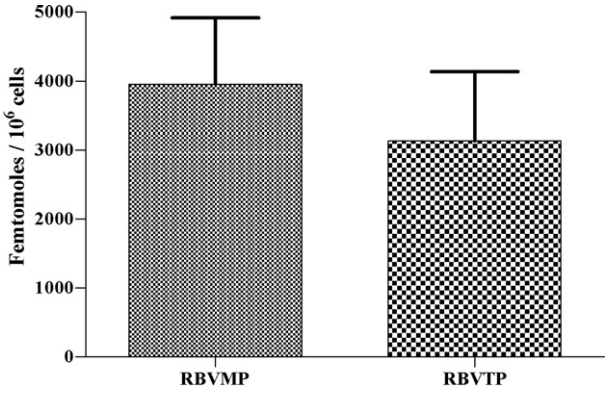

Fig. 9. Intracellular concentration of RBV-MP and RBV-TP in CEM_{ss} cells cultured with 10 μM RBV for 14 days. Results are the average of three independent experiments and error bars correspond to the standard error ($n = 9$).

genase by RBV-MP, the triphosphate metabolite (RBV-TP) has been suggested to interfere with viral capping enzymes and to inhibit the viral RNA synthesis [1]. Thus, it is of great interest to develop a method for the determination of RBV-MP and RBV-TP. Several researchers, including our laboratory, have demonstrated that it is possible to determine the intracellular metabolites of nucleoside reverse transcriptase inhibitors (NRTIs) in peripheral blood mononuclear cells (PBMCs) [17–24].

In the present study, we described the development and validation of a sensitive method, combining anion exchange SPE with HPLC–MS/MS for the intracellular determination of RBV-MP and RBV-TP with a LLQ of 10 ng/mL. Using anion exchange SPE, RBV metabolites were successfully separated for their individual determination. Dephosphorylation with acid phosphatase converted both RBV-MP and RBV-TP fractions into RBV. The use of PBA cartridges in the SPE provided the selective purification via covalent interaction of the cis-diol of RBV with the boronate group. The HPLC method allowed the chromatographic separation of the RBV from endogenous uridine, which has the same molecular weight and product ion. Finally, the MRM detection mode used in MS, allowed the double selectivity at the analysis. The method has a linear range (0.01–10 μg/mL for both RBV-MP and RBV-TP fractions), with acceptable accuracy (±7.1% of their nominal values for RBV-MP and ±3.2% for RBV-TP) and precision (8.4–14.8% for RBV-MP and 3.7–11.9% for RBV-TP). Although the method involves multiple steps during sample preparation, the validation with the quality control samples demonstrated high percent of recovery through all the process. Moreover, the stability of both RBV-MP and RBV-TP was evaluated during the validation, showing that degradation was absent.

This methodology was applied to *in vitro* experiments using CEM_{ss} cells cultured with RBV. Samples containing 10 million cells were sufficient to measure the intracellular RBV-MP and RBV-TP levels due to the high sensitivity of the methodology. This is an important aspect to be considered when applying to clinical protocols where small blood sample volumes are required. No statistically significant differences were observed for the intracellular levels between RBV-MP (3952 fmol/10^6 cells) and RBV-TP (3132 fmol/10^6 cells) when CEM_{ss} cells when exposed to RBV. Results from a previous study of RBV metabolism reported by Page et al. showed that a ratio of RBV-TP/RBV-MP was greater than 1 in erythrocytes and nucleated cells [3]. However, other studies revealed differences in the RBV metabolites ratios depending on the analyzed sample. Yeh et al. has reported that RBV-MP was the predominant moiety in monkey liver samples, while RBV-TP was the most abundant in monkey red blood cells using ^{14}C compounds [7,10]. Our method is the first one to determine directly concentrations of RBV metabolites inside the cells and not an indirect estimation of their concentrations.

6. Conclusion

An HPLC–MS/MS method for the intracellular measurement of RBV-MP and RBV-TP has been established. The method is specific, sensitive and accurate over a concentration range of 0.01–10 μg/mL. The combination of SPE and HPLC–MS/MS has provided the selectivity and sensitivity required for this type of analysis. The intracellular RBV-MP and RBV-TP determination was possible with samples of only 10 million cells, which is an important factor when applying to clinical protocols, in which sample volume is critical. A typical blood sample may contain between 30 and 40 million cells, thus they can be analyzed without any concern of sensitivity. Since there is not an estimate of individual RBV metabolite levels in human samples, the method provides a broad linear range for the measurement of both RBV-MP and RBV-TP. Moreover it was validated for sample dilutions; in case of the sample has a high concentration.

To the best of our knowledge, this is the first time that a HPLC–MS/MS method for the simultaneous determination of RBV-MP and RBV-TP has been reported. We already have a validated HPLC–MS/MS methodology for the intracellular determination of ZDV metabolites in HIV-HCV patients, therefore we now have the capacity to establish a correlation between the intracellular pharmacological parameters and drug interaction between ZDV and RBV.

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