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Short communication

Development of a rapid LC–MS/MS method for ribavirin determination in rat brain

Elisa Zironi, Teresa Gazzotti, Barbara Lugoboni, Andrea Barbarossa, Alessandra Scagliarini, Giampiero Pagliuca*

Department of Veterinary Public Health and Animal Pathology, Alma Mater Studiorum, University of Bologna, via Tolara di Sopra, 50 I 40064 Ozzano dell'Emilia, Bologna, Italy

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ABSTRACT

A rapid and specific liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the determination of ribavirin (RBV) in rat brain was developed.

Sample preparation required only two centrifuge steps before LC–MS/MS analysis and the chromatographic separation was achieved in isocratic conditions using an Atlantis T3 column with a nearly totally aqueous (95%) mobile phase. The method showed a good linearity over a concentration range of 5–1000 ppb and satisfactory results in terms of accuracy.

1. Introduction

Viral encephalitis represents an emerging health problem for humans and animals and has led to a growing interest in identifying new antiviral drugs. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (RBV) is a synthetic antiviral drug active against different DNA and RNA viruses.

In vitro experiments have shown that RBV is active against several RNA viruses responsible for encephalitis in animals and humans. Despite the encouraging laboratory results, the efficacy of the molecule against the cerebral viral load *in vivo* is limited by blood–brain interfaces preventing its cerebral distribution [1]. For this reason a number of attempts have been made to develop new formulations enhancing brain uptake using different routes of drug administration. To confirm brain RBV uptake after administration it is crucial to develop a reliable analytical method allowing accurate quantification of the molecule.

Several methods have been described for RBV determination in plasma [2–8] but few are available for other matrices [9,10]. Only Jeulin et al. measured the free RBV concentration in mice brain to demonstrate that RBV complexated with cyclodestrins and administered intraperitoneally passes through the blood–brain-barrier and reaches the brain [1]. The aim of the present work was to develop and validate a specific and reliable method for RBV determination in brain tissues by LC–MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

RBV standard (1 mg) was purchased from Sigma Chemical Company (St. Louis, MO, USA). The internal standard (IS) RBV $^{13}C_5$ (1 mg) was obtained from Campro Scientific GmbH (Berlin, Germany). Ammonium acetate, formic acid and acetonitrile were Fluka reagents of MS grade (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). Ultrapure water was produced in-house via a Human Power water purification system. The centrifugal filter devices (Amicon Ultra-0.5 3 kDa) were from Millipore Corporation (Billerica, MA, USA).

2.2. Standards and quality control (QC) samples

The two stock solutions of RBV and $^{13}C_5$RBV (IS) were prepared dissolving 1 mg of standard in 100 mL of methanol, and kept refrigerated at 4 °C. The working solutions of both standards were obtained diluting the stock solution in ammonium acetate aqueous buffer (20 mM, pH 6.9) to a final concentration of 1 μg/mL.

The calibration standard curve (0, 5, 10, 50, 100, 500 and 1000 ng/g) and the QC samples (30, 70, 300 and 700 ng/g) were prepared spiking 50 mg of blank rat brain with appropriate amounts of the RBV working solution; 25 μL of the internal standard $^{13}C_5$RBV working solution was also added to each sample. All the fortification levels and 8 blank samples were prepared in triplicate each validation day.

* Corresponding author.
E-mail address: giampiero.pagliuca@unibo.it (G. Pagliuca).

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2.3. Sample preparation

RBV was extracted from 50 mg of rat brain with 500 μL of ammonium acetate buffer 20 mM after the addition of 25 μL of IS working solution.

The sample was homogenized, vortexed for about 30 s and centrifuged at 14,000 rpm for 10 min. The supernatant was further centrifuged at 14,000 rpm for 20 min into the Eppendorf fitted with the Amicon-Ultra filter; 10 μL of the centrifuge-filtered solution was injected into the LC–MS/MS system.

2.4. Chromatographic conditions

The liquid chromatographic system was an Alliance 2695 Separations Module (Waters Corporation, Milford, MA, USA). Chromatographic separation was performed on a Waters Atlantis T3 column (3 μm 2.1 mm × 150 mm) equipped with a Waters Atlantis T3 guard column (3 μm 2.1 mm × 10 mm).

The column flow rate was 0.2 mL/min and the temperature was kept at 35 °C. The separation was carried out with isocratic mobile phase consisting of 90% water, 5% acetonitrile, 5% water containing 0.5% formic acid, for a total run time of 5 min.

2.5. Mass spectrometric conditions

The mass spectrometer was a Quattro Premiere XE, a triple quadrupole instrument equipped with an ESCI™ Multi-Mode Ionization Source (Waters Corporation, Milford, MA, USA). All the analyses were conducted in positive electrospray ionization mode (ESI+) using selected reaction monitoring (SRM); the transitions monitored were: RBV m/z 245 → 113, $^{13}C_5$RBV m/z 250 → 113.

Mass spectrometer conditions were: cone voltage 13 V, collision energy 22 eV, capillary voltage 1 kV, source temperature 140 °C, desolvation temperature 450 °C. Nitrogen was used as desolvation gas (890 L/h) and cone gas (250 L/h) while the collision gas was argon (flow rate of 0.35 mL/min). Data acquisition processing was performed using Mass Lynx 4.1 Software (Waters Corporation, Milford, MA, USA).

3. Results and discussion

3.1. Method development and validation

The first works on RBV used underivatized silica columns and highly organic mobile phases [2,3] but often showed strong matrix effects. Subsequent studies adopted reverse phase chromatography, using gradients with very low organic percentages [4,5,9]. Li et al. utilised an Atlantis dC18 with a 100% aqueous phase, but needed an acetonitrile post column addition to overcome the low ionization efficiency [11], while Zhou managed to obtain a good ionization using a different mobile phase with the same column [7].

The present work was developed on an Atlantis T3 and, after several trials, the parameters were optimised to obtain a good ionization without post column additions.

The sample was successfully purified using centrifugal filters instead of SPE. Method validation was in line with FDA Bioanalytical Method Validation guidelines [12].

3.2. Matrix effect

A standard RBV solution was continuously and directly infused into the mass spectrometer interface while a simultaneous LC flow

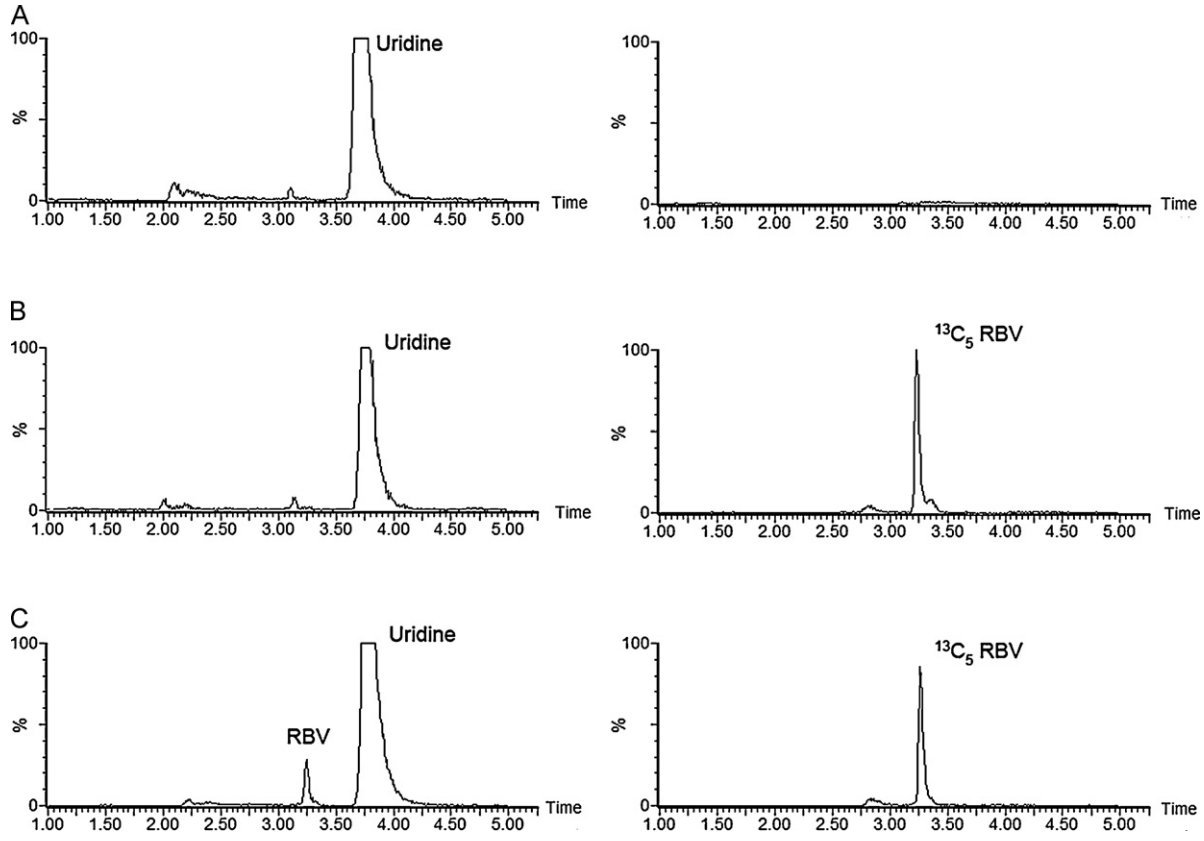

Fig. 1. Chromatogram (TIC mode) of a blank matrix sample (A), a control zero (B) (blank matrix fortified with IS only) and a matrix fortified at 100 ng/g (C).

of blank matrix was introduced [13]. The stability of the recorded ion current at the specific retention time proves the absence of ionization interferences.

3.3. Selectivity

The analyses of blank sample extracts always disclosed a peak, probably due to the presence of endogenous uridine that is the principal interferent in biological matrices [5,10]. Since uridine has the same molecular weight as RBV and shows the same transition at m/z 113, chromatographic separation was optimised (Fig. 1).

Fig. 2 shows the chromatograms of a blank rat brain sample (A), a control zero (B) (blank matrix fortified with IS only) and a matrix spiked at 100 ng/g (C) proving that no endogenous interferences were present at the specific elution times of RBV.

3.4. Stability

The stability of analytes was determined spiking two pools of 4 blank samples each with appropriate amounts of working solution to obtain final RBV concentrations of 100, 200, 300 and 400 ng/mL and a fixed IS concentration of 500 ng/g.

One pool of samples was kept at room temperature (about 26 °C) for 24 h and then analysed, while the other was analysed after three freeze–thaw cycles.

The comparison between these results and the fresh samples data showed a good stability, with response variations always below 8%, in agreement with other studies [6,9,10].

3.5. Linearity

Six points matrix calibration standard curves were freshly prepared each validation day and injected at least three times for each running batch.

The regression lines obtained were all satisfactory with a coefficient of determination (r^2) always higher than 0.991.

3.6. Accuracy

Accuracy (trueness and precision) was estimated analysing blank samples fortified at four different levels: 30, 70, 300, 700 ng/g (QC samples).

Table 1
Accuracy data.

	Day 1	Day 2	Day 3	Interday (n = 9)
30 ng/g				
Mean (n = 3)	32.6	32.9	29.1	31.5
Precision (CV %)	10.6	3.2	10.0	8.9
Trueness (Bias%)	8.5	9.5	−3.2	4.9
70 ng/g				
Mean (n = 3)	69.6	68.0	74.2	70.3
Precision (CV %)	0.8	1.8	9.3	5.6
Trueness (Bias%)	−0.6	−2.9	4.6	0.4
300 ng/g				
Mean (n = 3)	327.9	330.0	289.9	315.9
Precision (CV %)	3.4	5.0	9.3	8.0
Trueness (Bias%)	9.3	10.0	−3.4	5.3
700 ng/g				
Mean (n = 3)	708.5	694.0	668.4	690.3
Precision (CV %)	0.7	3.1	0.8	3.0
Trueness (Bias%)	1.2	−0.9	−4.5	−1.4

Trueness, determined as bias (%), was assessed correlating the estimated amounts with the nominal concentrations, while precision was expressed as coefficient of variation (CV%). The validation results are summarized in Table 1.

The obtained data not only fell always within the values suggested in FDA Bioanalytical Method Validation guidelines [12], but fitted also the more restrictive limits set by Commission Decision 2002/657/EC [14], which recommends a bias range from −20% to 10% for concentrations above 10 ng/g.

Referring to the same Commission Decision, the CV% in conditions of repeatability has to be lower than half of the value calculated with the Horwitz equation.

In particular, for concentrations lower than 100 ng/g the CV% value has to be as low as possible, while for 300 ng/g CV% < 9.5 and for 700 ng/g CV% < 8.5.

3.7. Recovery

For each QC recovery was calculated comparing the peak area of fortified matrix with that obtained injecting the standard solution at the same concentration. Average recoveries ranged from around 50% (for the highest QC points) to around 70% (for the lowest QC points). Moreover RBV quantification was evaluated using RBV $^{13}C_5$ (IS).

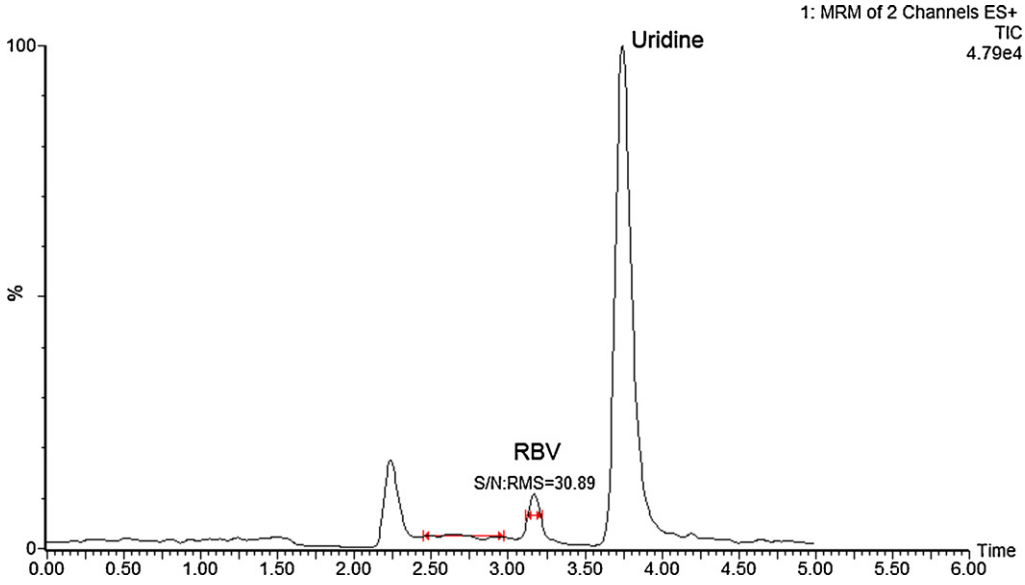

Fig. 2. Chromatogram of a blank rat brain sample fortified at the LOQ, corresponding to 5 ng/g.

3.8. Sensitivity

The limit of quantification (LOQ), defined as the lowest point of the calibration curve, is 5 ng/g; this concentration always yields a signal-to-noise ratio higher than 10 (Fig. 2). The LOQ in this study was based on experiments performed in matrix and so this value takes into account the extraction procedure.

The limit of detection (LOD) was calculated as the lowest RBV concentration that could be detected by the mass spectrometer with a signal-to-noise (S/N) ratio of at least 3 and was 1 ng/g in rat brain.

4. Conclusions

A rapid and selective method for ribavirin (RBV) determination in rat brain tissues has been developed. There are no other LC–MS/MS studies on RBV in this matrix. The advantages of the present method were: rapid sample preparation not requiring a solid phase extraction step; 95% aqueous chromatographic separation minimizing organic solvent consumption; isocratic conditions avoiding column re-equilibration. Performances were satisfactory in terms of selectivity, sensitivity and accuracy.

References

[1] H. Jeulin, V. Venard, D. Carapito, C. Finance, F. Kedzierewicz, Effective ribavirin concentration in mice brain using cyclodextrin as a drug carrier: evaluation in a measles encephalitis model, Antivir. Res. 81 (2008) 261–266.
[2] C.C. Lin, L.T. Yeh, J.Y.N. Lau, Specific, sensitive and accurate liquid chromatographic–tandem mass spectrometric method for the measurement of ribavirin in rat and monkey plasma, J. Chromatogr. B 779 (2002) 241–248.
[3] W.Z. Shou, H.Z. Bu, T. Addison, X. Jiang, W. Naidong, Development and validation of a liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the determination of ribavirin in human plasma and serum, J. Pharm. Biomed. Anal. 29 (2002) 83–94.
[4] Y. Liu, C. Xu, R. Yan, C. Lim, L.T. Yeh, C.C. Lin, Sensitive and specific LC–MS/MS method for the simultaneous measurements of viramidine and ribavirin in human plasma, J. Chromatogr. B 832 (2006) 17–23.
[5] L.T. Yeh, M. Nguyen, S. Dadgostari, W. Bu, C.C. Lin, LC–MS/MS method for simultaneous determination of viramidine and ribavirin levels in monkey red blood cells, J. Pharm. Biomed. Anal. 43 (2007) 1057–1064.
[6] W. Li, S. Luo, S. Li, L. Athill, A. Wu, T. Ray, W. Zhou, J. Ke, H.T. Smith, F.L.S. Tse, Simultaneous determination of ribavirin and ribavirin base in monkey plasma by high performance liquid chromatography with tandem mass spectrometry, J. Chromatogr. B 846 (2007) 57–68.
[7] Z.L. Zhou, X.Y. Yu, M. Yang, H.Y. Peng, T.F. Chen, Q.X. Lin, Z.X. Shan, X.Y. Liu, C. Deng, P. Zhu, X.Z. Huang, S.G. Lin, Rapid and sensitive HPLC–MS/MS method for pharmacokinetic assessment of ribavirin in healthy Chinese, Biomed. Chromatogr. 22 (2008) 1409–1415.
[8] A. Loregian, M.C. Scarpa, S. Pagni, S.G. Parisi, G. Palù, Measurement of ribavirin and evaluation of its stability in human plasma by high performance liquid chromatography with UV detection, J. Chromatogr. B 856 (2007) 358–364.
[9] L.T. Yeh, M. Nguyen, D. Lourenco, C.C. Lin, A sensitive and specific method for the determination of total ribavirin in monkey liver by high-performance liquid chromatography with tandem mass spectrometry, J. Pharm. Biomed. Anal. 38 (2005) 34–40.
[10] M. Meléndez, O. Rosario, B. Zayas, J.F. Rodríguez, HPLC–MS/MS method for the intracellular determination of ribavirin monophosphate and ribavirin triphosphate in CEMss cells, J. Pharm. Biomed. Anal. 49 (2009) 1233–1240.
[11] M. Espinosa Bosch, A.J. Ruiz Sánchez, F. Sánchez Rojas, C. Bosch Ojeda, Ribavirin: analytical determinations since the origin until today, J. Pharm. Biomed. Anal. 45 (2007) 185–193.
[12] FDA Bioanalytical Method Validation [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf].
[13] J.P. Antignac, K. de Wasch, F. Monteau, H. De Brabander, F. Andre, B. Le Bizec, The ion suppression phenomenon in liquid chromatography–mass spectrometry and its consequences in the field of residue analysis, Anal. Chim. Acta 529 (2005) 129–136.
[14] Commission Decision 2002/675/EC Commission decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of the analytical methods and the interpretation of results, Off. J. Eur. Commun. L 221 (August 17th 2002) 8–36.