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Short communication

Validation of liquid/liquid extraction method coupled with HPLC-UV for measurement of ribavirin plasma levels in HCV-positive patients

Antonio D'Avolio *, Allende Ibañez, Mauro Sciandra, Marco Siccardi, Daniel Gonzalez de Requena, Stefano Bonora, Giovanni Di Perri

Department of Infectious Diseases, University of Torino, Amedeo di Savoia Hospital, Corso Svizzera, 164-10149 Turin, Italy

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Abstract

Measurement of ribavirin plasma levels in HCV-positive patients have been shown to be useful in order to optimise individual ribavirin exposure. Efficacy and toxicity of this drug are shown to be concentration-dependant. A simple HPLC-UV method was developed and validated, which has an easy liquid/liquid extraction, sensitive limit of detection, without any interference peaks, reproducible and linear over the range of clinical relevant concentrations. The assay warrants further evaluation as a tool for ribavirin therapeutic drug monitoring in HCV-positive patients.

Keywords: Ribavirin; Liquid/liquid; HPLC; UV method

1. Introduction

The World Health Organization estimated that about 170 million peoples are infected by hepatitis C virus (HCV) worldwide [1]. HCV-associated chronic hepatitis is a major cause of morbidity and mortality [2].

Standard treatment for chronic HCV hepatitis is the association of pegylated alpha-interferon (PEG-IFN alpha 2a o PEG-IFN alpha 2b) and ribavirin [3,4]. Ribavirin is a synthetic purine analogue of guanosine (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). Several mechanisms of ribavirin action have been proposed, including inositol monophosphate dehydrogenase inhibition, mutagenesis, direct inhibition of the RNA dependent RNA polymerase, and immune-modulating action [5–10]. Daily dosage of ribavirin is based on body weight and HCV genotype [11]. Moreover, glomerular filtration rate has been recently proposed as an additional dosing parameter [12]. Usual dose ranges between 800 and 1200 mg/day, although further empirical dose adjustment is requested when the haemoglobin serum concentration falls below 10 mg/dl. Haemolytic anaemia, in fact, is the major ribavirin-related side effect, affecting up to 61% of the patients and being the most common reason for treatment discontinuation [13]. Several reports showed that ribavirin plasma concentration, rather than ribavirin weight-adjusted dose, drives the incidence and severity of haemolytic anaemia [14–17]. Similarly the rate of virological response has been shown to be concentration-dependant [18]. Therefore, therapeutic drug monitoring (TDM) of ribavirin could optimise both efficacy and tolerability of ribavirin-containing regimens. In this context, Lindahl et al. [16] recently reported that TDM-guided maintenance of ribavirin plasma concentration above 3000 ng/ml was associated with an unexpected high rate of virological response in HCV genotype 1 (90%) along with an unacceptable high incidence of haemolytic anemia. Rendon et al. [18] found that a ribavirin concentration higher than 2700 ng/ml was a predictor of both virological response and significant haemoglobin decrease in HIV/HCV co-infected subjects.

It is therefore important to investigate the therapeutic range of ribavirin plasma concentrations and clinical use of TDM of ribavirin.

Different methods have been developed for the analysis of ribavirin concentration in biological fluids [19–23]. As compared to these previously methods our assay provides some technical and cost advantages, like L/L extraction, avoidance of pheni-

* Corresponding author. Tel.: +39 011 4393996; fax: +39 011 4393882.
E-mail address: antodav@inwind.it (A. D'Avolio).

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boronic acid column utilization, use of 100% aqueous buffer and UV detection. The aim of this study was to validate a new, fast and low cost, HPLC-UV method for measurement of ribavirin plasma concentrations in HCV-positive patients, suitable for all TDM laboratories.

2. Materials and methods

2.1. Chemicals

Ribavirin, *ortho*-phosphoric acid and potassium dihydrogen phosphate were obtained from Sigma-Aldrich (Italy). Acetonitrile was obtained from J.T. Baker (Deventer, Holland). Plasma from healthy donors was kindly supplied by the Blood Bank of Maria Victoria Hospital (Turin, Italy).

2.2. Materials and chromatographic conditions

The HPLC system used to assay ribavirin was a Merck-Hitachi LaChrom (Tokyo, Japan). The system consisted of a pump model L-7100, an L-7200 autosampler, and L-7400 UV detector. The detector was operated at a wavelength of 235 nm. D-7000 interface and HPLC System Manager software (HSM, Version 4.1, Merck-Hitachi; Tokyo, Japan) were used for management of the HPLC system.

Chromatographic separation was performed by an Atlantis 3 μ dC18 column (150 mm × 4.6 mm I.D, Waters, Milan, Italy), protected by a SecurityGuard with C18 (4.0 mm × 3.0 mm I.D, Phenomenex, CA, USA), at 35 °C using a column thermostat L-7350 Merck-Hitachi LaChrom.

The run was performed with a gradient (Table 1), and the mobile phase was composed of Buffer A (KH_2PO_4 50 mM with *ortho*-phosphoric acid, final pH 3.23) and Buffer B (acetonitrile).

2.3. Stock solutions, standards and quality controls

A stock solution of ribavirin was made in methanol:water (90:10) with a final concentration of 1 mg/ml and stored at −20 °C until use. Standards (STDs) were prepared by addition of 10 μl of the stock solution in 1 ml of drug-free plasma. The highest concentration point of the calibration curve was 10,000 ng/ml (STD 8). Quality controls (QCs) were prepared in the same way, with concentrations of 8000 ng/ml (QC 1), 3000 ng/ml (QC 2) and 500 ng/ml (QC 3).

Table 1
Chromatographic condition (gradient)

TIME (min.)	Buffer A (%)	Acetonitrile (%)	Flow (ml/min)
0.0	100.0	0.0	1.0
1.5	100.0	0.0	1.0
2.5	70.0	30.0	1.0
4.0	70.0	30.0	1.0
4.1	100.0	0.0	1.0
10.0	100.0	0.0	1.0

Mobile phase: Buffer A (KH_2PO_4 50 mM with *ortho*-phosphoric acid, final pH 3.23) and acetonitrile.

STDs were prepared by serial dilution from 10,000 ng/ml (STD 8) to 78.125 ng/ml with drug-free plasma, to obtain eight different spiked concentrations plus a blank sample.

STDs, QCs and patient samples underwent heat inactivation for HIV (35 min at 58 °C) and then stored at −20 °C until analyses. Ribavirin was shown to be stable [21,23].

The calibration curve included a wide range of ribavirin concentrations, including the highest values reported in the clinical studies [16].

2.4. Sample extraction

Samples were obtained at the end of ribavirin dose interval in HCV-positive administered with this drug at different doses as a component of anti-HCV treatment.

The extraction of ribavirin from plasma was performed in a PTFE microfuge tube by addition of 250 μl of plasma (STD, QC or sample) followed by 1500 μl of acetonitrile. This solution was vortexed for 10 s and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was then transferred to glass tubes and dried in a vacuum centrifuge at 50 °C.

The dry extract was reconstituted with 125 μl of Buffer A and then 30 μl was injected into the column.

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by the analysis of five different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated. These included: nucleoside or nucleotide reverse transcriptase HIV inhibitors (zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir), non-nucleoside reverse transcriptase HIV inhibitors (nevirapine, efavirenz), fusion inhibitor (enfuvirtide) and protease inhibitors (saquinavir, nelfinavir and its active metabolite M-8, indinavir, amprenavir, atazanavir, ritonavir, lopinavir, tipranavir). Other concomitant drugs (see Table 2) were also investigated.

Table 2
Non-antiretroviral concomitant drugs administered to patients

Acetilsalicilic acid
Amoxicillin
Atorvastatin
Clavulanic acid
Enalapril meleate
Furesomide
Insuline
Interferon (PEG)
Levofloxacin
Methadone
Nimesulide
Omeprazole
Paracetamol
Pravastatin

2.6. Accuracy, precision, and limit of quantification

Intra-day and inter-day accuracy and precision of the method were determined by assaying 10 replicate plasma samples at three different concentrations (QCs). Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration.

The calibration curve was obtained using eight calibration points in duplicate, ranging from 78.125 to 10,000 ng/ml.

Limit of detection (LOD) in plasma was defined as the concentration that yields a signal-to-noise ratio of 3. Percent deviation from the nominal concentration (measure of accuracy) and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be <20%.

2.7. Recovery

Average recovery of ribavirin was determined by comparing the peak height of the extracts spiked samples (8000, 3000, and 500 ng/ml) with those obtained by direct injection of the same amount of drug.

3. Results

Retention time of ribavirin is 4.3 min (±0.1).

Representative chromatograms of the blank human plasma extract and STD 8 are shown in Figs. 1 and 2, respectively.

3.1. Specificity and selectivity

The assay did not show any significant interference by antiretroviral drugs as listed in methods section or by other concomitant drugs reported in Table 2.

Blank plasma did not show any interference peak at the ribavirin retention time (see Fig. 1).

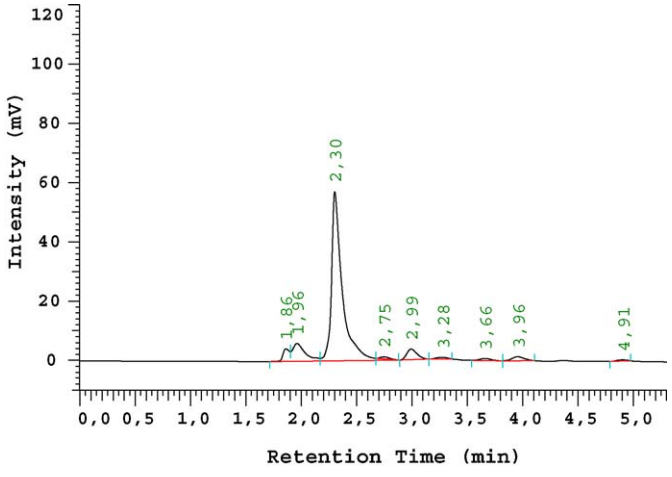

Fig. 1. Chromatogram of blank human plasma sample.

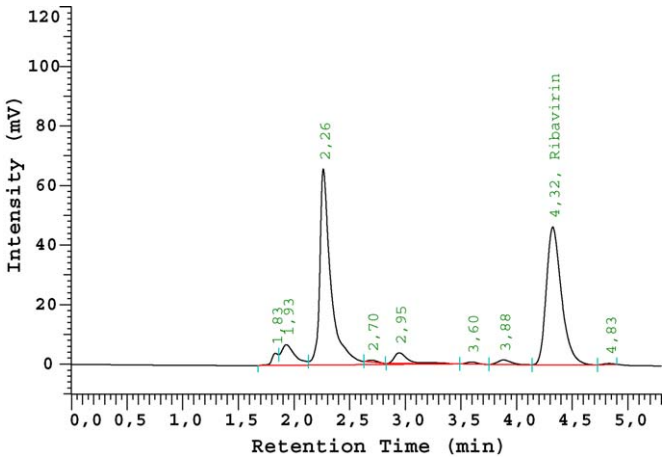

Fig. 2. Chromatogram of plasma sample spiked with 10,000 ng/ml (STD 8), showing a retention time of ribavirin at 4.32 min.

Table 3
Intra-day and inter-day precision for the analysis of ribavirin in plasma samples ($n = 10$) [mean R.S.D.%]

QC, spiked concentration (ng/ml)	Intra-day		Inter-day	
	Found	R.S.D.%	Found	R.S.D.%
QC 1, 500 ($n = 10$)	494	2.40	472	5.09
QC 2, 3000 ($n = 10$)	2993	1.50	2966	4.10
QC 3, 8000 ($n = 10$)	8084	1.48	8116	1.73

3.2. Accuracy, precision, and limit of quantification

Results of validation of the method are shown in Table 3.

The relative error at three concentrations ranged from −5.57 to +1.45%. Intra-day (R.S.D.%) and inter-day (R.S.D.%) precision ranged from 1.48 to 2.40% and from 1.73 to 5.09%, respectively.

LOQ and LOD were 78.125 and 19.5 ng/ml, respectively.

3.3. Recovery

Multiple aliquots ($n = 8$) at each different concentration were assayed. Mean recovery of ribavirin at 8000, 3000 and 500 ng/ml was 76.43% (R.S.D. = 4.59%).

3.4. Analysis of samples from patients

One hundred and seventy-two samples obtained from 65 patients were analysed in duplicate. Blood samples were obtained at a median of 12.15 h (IQR 11.75–12.9) after last drug intake. Mean ± S.D. ribavirin plasma concentration was 1699 ± 802 ng/ml. Maximum and minimum values were 4519 and 518 ng/ml, respectively.

4. Discussion and conclusion

We validated a new method to measure ribavirin plasma concentration. As compared to previously published methods [19–23], our assay provides some technical and cost advantages.

Firstly, some methods are based on solid phase extraction with phenilboronic acid columns which selectively binds ribose-containing structures. This procedure lead to a high selectivity of extraction, allowing removal of interferences and providing suitable detection levels, but it is known to be expensive and time-consuming [19–22].

Our liquid/liquid extraction procedure did not compromise the specificity and selectivity, as shown by the lack of neither endogenous nor exogenous interferences of ribavirin retention time. Specificity was enhanced by detecting at wavelength at 235 nm, the setting also avoid any interferences. Moreover, this extraction procedure provided an acceptable recovery from human plasma samples.

The use of a C-18 Atlantis column, allowed the use of 100% aqueous buffer to resolve the ribavirin peak from plasmatic interference, something usually encountered by other authors using protein precipitation procedure [22,23]. This contributed to the high rates of specificity and selectivity of our method. There are other RP-18 columns that allow the use of 100% aqueous buffer, but we have yet to test them.

Another advantage related to the validation of our method was the short analysis time run. In fact, ribavirin peak eluted in 4.3 min and the system was ready for a new analysis after 10 min, including wash and conditioning steps (see Table 1). Although the chromatogram was resolved in shorter time in a previously published method [19], the particular method has a complex initial conditioning step, aimed to avoid delay in ribavirin retention time and the collapse of the column on long set of analysis.

Moreover, the calibration curve of our method covered a wide range of ribavirin concentrations. It included highest ribavirin levels observed in patients and LOQ was adapted to correctly quantify lowest C_{trough} values reported in the clinical setting [16,17,24]. In our C_{trough} patient samples, observed mean ± S.D. ribavirin plasma concentration was 1699 ± 802 ng/ml, and maximum and minimum values were 4519 and 518 ng/ml, respectively, according with Glue et al. [24].

No IS was used to calculate the results, as opposed to that previously suggested by other authors [19–23]. The method produced good recovery (76.43%), low related R.S.D.% (4.59), low intra-day and inter-day R.S.D.% (1.48–2.40 and 1.73–5.09), and a good linearity response in the range of RBV quantification at standard dosing regimens ($r^2 = 0.999$) were observed. Moreover, in our procedure ribavirin measurements were performed in duplicate and results were validate with R.S.D.% < 15%. However, in the course of method optimisation two different IS, thymidine (THY) and 3-methyl-cytidine (3MTC), were evaluated. Addition of both IS did not improve neither linearity of the calibration curve nor R.S.D.% of QCs samples. Both THY and 3MTC required a longer chromatographic run to resolve the IS peak (14 min.), as compared to the one without them. Comparison of 3MTC with THY showed that the latter was slightly better in terms of linearity ($r^2 = 0.994$ for 3MTC versus $r^2 = 0.998$ for THY), intra and inter-day R.S.D.% (3.31–6.72 and 3.95–9.41 for 3MCT; 2.36–3.63 and 2.84–7.15 for THY). Therefore, THY could be used as an IS if necessary during the extraction procedure.

In conclusion, we validated a new liquid/liquid extraction method coupled with HPLC-UV detection for the measurement of ribavirin plasma concentrations in HCV-positive patients. It showed to be simple to perform, accurate, cheap and rapid, suggesting the need for its further evaluation as a tool for TDM of ribavirin in the clinical setting.

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