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Journal of Chromatography B, 856 (2007) 358–364
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Measurement of ribavirin and evaluation of its stability in human plasma by high-performance liquid chromatography with UV detection

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Received 12 April 2007; accepted 21 May 2007
Available online 2 June 2007

Abstract

A simple high-performance liquid chromatography method for the determination of the antiviral agent ribavirin in human plasma was developed and validated. The method involved solid-phase extraction on phenyl boronic acid cartridges, a reversed-phase liquid chromatography with a Waters Atlantis dC18 (150 mm × 3.9 mm, 5 μm) column and a mobile phase consisting of 10 mM potassium phosphate buffer (pH 4.0), and ultraviolet detection at 207 nm. This assay proved to be sensitive (lower limit of quantification of 0.05 μg/ml), linear (correlation coefficients ≥0.997), specific (no interference with various potentially co-administrated drugs), reproducible (both intra-day and inter-day coefficients of variation ≤4.3%), and accurate (deviations ranged from −5.6 to 2.2% and from −6.0 to 4.0% for intra-day and inter-day analysis, respectively). The method was applied to therapeutic monitoring of patients undergoing ribavirin treatment for hepatitis C and proved to be robust and reliable. Thus, this method provides a simple, sensitive, precise and reproducible assay for dosing ribavirin that can be readily adaptable to routine use by clinical laboratories with standard equipment. In addition, we evaluated the stability of ribavirin in plasma under various conditions, since no detailed study on thermal stability of ribavirin has been reported so far and discrepant data do exist on ribavirin stability upon conditions that clinical samples commonly experience. Ribavirin was stable in human plasma stored at room temperature for at least 24 h or at −20 °C for up to 1 month, after three freeze–thaw cycles, as well as in samples undergoing heat inactivation of infectious viruses for 60 min at 60 °C. The drug was also stable in processed samples stored at −20 °C for 3 days (as dried extracts) or at 20 °C for 4 days (as reconstituted samples).

Keywords: Ribavirin; Human plasma; Solid-phase extraction; High-performance liquid chromatography; UV method; Ribavirin stability; Therapeutic drug monitoring

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is a synthetic purine nucleoside analog that possesses broad-spectrum activity against a variety of DNA and RNA viruses *in vitro* [1,2]. It has been used in treating respiratory syncytial virus infections in seriously ill pediatric patients, and against influenza A and B virus and Lassa fever virus infections [3]. More recently, ribavirin has been found to be efficacious also in the treatment of hepatitis C virus (HCV) infections, in particular when combined with either interferon α-2a/2b or peginterferon α-2a/2b [4,5]. Chronic HCV infection is frequently associated with human immunodeficiency virus (HIV), particularly in patients with a past history of drug addiction, and liver disease has emerged as a major cause of morbidity and mortality in HCV–HIV-coinfected patients [6]. Thus, the combination therapy with ribavirin and interferon-α has also been suggested as an option for HCV–HIV-coinfected patients in combination with antiretroviral therapy [7].

A high level of interindividual variability of ribavirin plasma concentration has been observed both in healthy individuals and in patients with [8] and without [9] HCV infection, and it has therefore been estimated that dosage adjustment is required in ∼26% of patients [10]. Moreover, one of the most common complications associated with the use of ribavirin is haemolytic

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doi:10.1016/j.jchromb.2007.05.039

anemia, which is due to accumulation of ribavirin in erythrocytes [11]. Both the rate of virological response and the incidence and severity of haemolytic anemia have been shown to be concentration-dependent [12]. Therefore, therapeutic drug monitoring (TDM) of ribavirin is useful to determine the best dosage regimen adapted to each individual and to avoid drug toxicity.

Several analytical methods for quantitating ribavirin in serum or plasma have been developed; these include bioassay [13], radioimmunoassay [14], gas chromatography–mass spectroscopy [15], capillary electrophoresis (CE) [16], and high-performance liquid chromatography (HPLC) with UV [8,16–20] or mass spectrometric (MS) [21–24] detection. However, a number of these methods require employment of radioactive reagents, gradient elution runs, and CE or MS instrumentation, which is not always available in standard hospital laboratories. Although isocratic methods with UV detection have also been reported to determine ribavirin levels in plasma or serum [8,16–19], several of them show low sensitivity [8,16–18] and low precision [8,17]. Thus, simple, sensitive, and precise analytical methods that employ effective sample clean-up procedures, isocratic elution, and UV detection need to be developed.

In addition, only a few studies on stability of ribavirin in serum and plasma from human or other sources have been reported. To the best of our knowledge, no detailed investigation has been previously conducted on ribavirin stability during the thermisation procedure (56–60 °C for 30–60 min) that is recommended to heat-inactivate HCV and/or HIV particles in biological samples for safety issues [25,26]. Our paper reports the first detailed study on ribavirin stability upon such a heat treatment. Moreover, some debate exists about ribavirin stability under conditions that clinical samples commonly experience. Indeed, Larrat et al. [8] reported a gradual decrease in the ribavirin concentration up to 33% in samples undergoing three freeze–thaw cycles, while no degradation of ribavirin in plasma subjected to repeated freeze–thaw cycles was observed in other studies [21–24]. In addition, discrepant data have been reported on stability of ribavirin following extraction from plasma. In a recent study [8], storage of dried extracts at −20 °C for 3 days was shown to induce a significant (37%) loss of ribavirin, whereas other authors reported that the ribavirin concentration remains stable in processed samples kept at 2–15 °C up to 52 h [21,24]. These discrepancies put the stability of ribavirin in question, and demanded further investigation.

In this report, the development and validation of a simple, sensitive, and precise HPLC assay for the determination of ribavirin in plasma are described. The method employs standard equipment and is easy to set up, and thus its use is feasible in most hospital laboratories. In addition, we evaluated the stability of ribavirin in plasma. In particular, the tests were designed to investigate the stability of ribavirin to heat treatment (i.e., 60 °C for 60 min) and also to cover several common conditions to which TDM samples can be subjected in the clinical setting. The present HLPC method and the results of the stability experiments could be useful for dosing ribavirin and properly handling ribavirin-containing plasma samples both during routine clinical assays and in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Ribavirin was obtained from Roche (Basel, Switzerland). Acetonitrile for chromatography (Gradient Grade, LiChrosolv) was from Merck (Darmstadt, Germany), and methanol for HPLC was from Carlo Erba (Milan, Italy). All other chemicals were of analytical grade and purchased from J.T. Baker (Deventer, The Netherlands). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Bedford, MA, USA). Control human plasma (with K_3EDTA as anticoagulant) was purchased from Roche (Milan, Italy; COBAS AmpliScreen kit).

2.2. Equipment and chromatographic conditions

The chromatographic system consisted of an Alliance 2695 Separation Module equipped with an online degasser and an automatic injector thermostated at 20 °C, and a 996 Photodiode Array Detector coupled with the Empower data acquisition software (version 1.0) (Waters, Milford, MA, USA). Separations were performed on an Atlantis dC_{18} (3.9 mm × 150 mm, particle size 5 μm; Waters) analytical column equipped with an Atlantis dC_{18} (3.9 mm × 20 mm, particle size 5 μm; Waters) guard column. Both columns were maintained at 40 °C. The isocratic mobile phase consisted of potassium phosphate buffer (pH 4.0, 10 mM) and was filtered through a 0.2 μm nylon filter membrane (Millipore, Bedford, MA, USA) before use. The flow rate was maintained at 1.0 ml/min for 6 min, then it was brought to 2 ml/min in 1 min and maintained at 2 ml/min for 11 min to favour elution of plasma contaminants, and finally, the flow rate was brought again to 1 ml/min in 1 min at the 18th min. The total run-time was 20 min. Absorbance was measured at 207 nm. An Extraction Manifold (Waters) liquid handling system was used to perform the sample preparation. A DRI-BLOCK DB-3 evaporator (Techne, Stone, United Kingdom) was used for drying the solid-phase extraction (SPE) eluates.

2.3. Preparation of stock solutions, working solutions, calibration standards and quality control (QC) samples

A stock solution of 1 mg/ml ribavirin was prepared in water. The stock solution was diluted further with water to obtain working solutions with concentrations of 500, 250, 100, 50, 25, 12.5, 5 and 2.5 μg/ml. The stock and working solutions were stored at −20 °C. Plasma calibration standards at 10, 5, 2, 1, 0.5, 0.25, 0.1 and 0.05 μg/ml were prepared by 1:50 dilution of the respective working solution in control human plasma. QC samples at the lower limit of quantification (LLOQ = 0.05 μg/ml), low (0.2 μg/ml), medium (1 μg/ml), high (5 μg/ml) and the upper limit of quantification (ULOQ = 10 μg/ml) concentration levels were prepared by diluting the working solutions in plasma. The QC samples were prepared in batches at the same occasion, stored at −20 °C, and then thawed and thermised at 60 °C for 60 min on the day of analysis.

2.4. Sample pretreatment and preparation

Blood samples of patients were collected in tubes with K_3EDTA as anticoagulant. Plasma was immediately isolated by centrifugation and stored at −20 °C until analysis. On the day of analysis, plasma samples were thawed and heated at 60 °C for 60 min. The analysis of stability of ribavirin under these conditions is reported in the Results section. Sample cleanup was achieved by a modification of the procedure described by Breadmore et al. [16]. Briefly, aliquotes (0.5 ml) of heat-inactivated plasma samples were mixed with an equal volume of ammonium acetate buffer (250 mM, pH 8.5) and successively loaded onto Bond-Elut phenyl boronic acid (PBA) 1-ml (100 mg) cartridges (Varian, Turin, Italy) previously conditioned with 1 ml of methanol with 0.25% H_3PO_4 and then with 2 ml of ammonium acetate buffer. The cartridges were then washed with 1 ml of ammonium acetate buffer followed by 1 ml of methanol. The analytes were successively eluted with 1 ml of 2.5% (v/v) formic acid in methanol. The eluates were evaporated to dryness under a stream of nitrogen at 40 °C. The dried extracts were reconstituted with 200 μl of mobile phase. The reconstituted sample was centrifuged at 13,000 rpm for 5 min at room temperature and 40 μl of the supernatant was injected onto the HPLC system.

2.5. Recovery

The overall recovery of ribavirin from human plasma was determined at the LLOQ, low-, medium-, high-QC, and ULOQ level by comparing the peak area response of extracted plasma samples (four samples for each concentration level) with that obtained by direct injection of the same amount of drug diluted in mobile phase (four samples for each concentration level).

2.6. Analytical method validation

The validation of the assay was based on the FDA guidelines for bioanalytical method validation [27]. Assay validation involved linearity, specificity, accuracy, precision, limit of detection (LOD) and lower limit of quantification (LLOQ) determination. Intra-day and inter-day precision values were estimated by assaying plasma samples spiked with five different concentrations of ribavirin six times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of deviation between nominal and measured concentration (% bias). Analytic interferences from endogenous substances were investigated by testing 10 different lots of blank human plasma. The method specificity was also investigated by analysing both plasma spiked with possible co-administered drugs and patient samples. The absence of analytic interference both in spiked plasma and in patient samples was confirmed by the use of the peak purity testing system and the library matching of the Empower software.

2.7. Stability studies

The stability of ribavirin in plasma under the thermisation process (60 °C for 60 min) was assessed as follows: four series of calibration samples at the eight concentrations reported above (0.05–10.0 μg/ml) and of samples at the LLOQ, low-, medium-, high-QC, and ULOQ level were prepared. Two series were heated at 60 °C for 60 min, while the thermisation procedure was omitted in the other two. The four series were then subjected simultaneously to SPE and analysed. The slope of the calibrations curves was compared as well as the ribavirin levels in QC samples.

Further stability studies included:

(a) The short-term stability of ribavirin at room temperature (RT): six series of LLOQ, low-, medium-, high-QC, and ULOQ plasma samples were prepared. Three series were immediately analysed, while the three remaining series were kept at RT for 24 h. The ribavirin levels in samples were compared.
(b) The long-term stability of ribavirin in plasma samples kept frozen at −20 °C: six series of plasma spiked with ribavirin at the LLOQ, low-, medium-, high-QC, and ULOQ level were prepared. Three series were immediately analysed (i.e., without being frozen) while the three remaining series were stored for 30 days at −20 °C. The concentration of ribavirin in both groups was compared.
(c) The stability of ribavirin in plasma after multiple freeze–thaw cycles: plasma samples (in triplicate) spiked with ribavirin were compared with samples of the same batch that were frozen at −20 °C and thawed at ambient temperature. This freeze–thaw cycle was repeated three times. The ribavirin levels in samples collected at each of the freeze–thaw cycles were analysed and compared to the levels of samples not subjected to the freeze–thaw cycles.
(d) The stability of dried extracts and of extracts reconstituted in mobile phase: dried extracts (i.e., after SPE) containing ribavirin at LLOQ, low-, medium-, high-QC, and ULOQ concentration were analysed in triplicate either immediately after preparation, or after being stored at −20 °C for 72 h. Additionally, processed samples (i.e., reconstituted in mobile phase) were analysed in triplicate immediately after preparation and after being left for 96 h in the autosampler thermostated at 20 °C. The results were compared.

3. Results and discussion

3.1. Method development

To establish an efficient method for ribavirin extraction from plasma, several purification methods were compared. Liquid–liquid extraction methods with different solvents (i.e., acetonitrile, methanol, ethylacetate, ether, chloroform, dichloroethane, and dichloromethane) were initially tested, but they gave in general low recovery and/or low reproducibility (data not shown). In contrast, solid-phase extraction (SPE) with phenyl boronic acid columns, which selectively bind ribose-containing structures, provided a reproducible and efficient sample preparation. Our SPE procedure was based on an extraction protocol for a CE method reported by Breadmore et al.

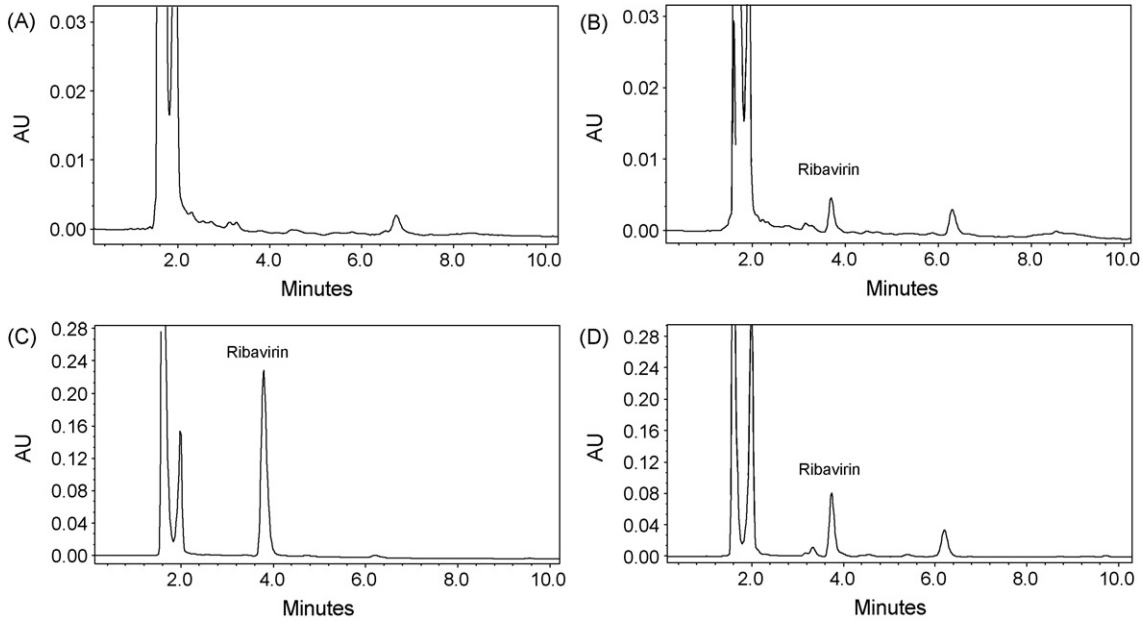

Fig. 1. Representative chromatograms of (A) blank plasma, (B) spiked plasma containing 0.05 μg/ml ribavirin (LLOQ), (C) spiked plasma containing 10 μg/ml ribavirin (ULOQ), and (D) a plasma sample from a patient with 3.23 μg/ml of ribavirin.

Table 1
Intra-day and inter-day accuracy and precision results

Nominal concentration (μg/ml)	Intra-day ($n=6$)			Inter-day ($n=3$)		
	Mean measured concentration (μg/ml)	Accuracy[a] (% bias)	Precision (% CV)	Mean measured concentration (μg/ml)	Accuracy (% bias)	Precision (% CV)
0.05 (LLOQ)	0.051	2.0	4.1	0.047	−6.0	4.3
0.2 (low QC)	0.196	−2.0	3.1	0.208	4.0	2.3
1.0 (medium QC)	0.992	−0.8	0.7	0.960	−4.0	0.5
5.0 (high QC)	5.112	2.2	0.2	4.980	−0.4	0.7
10.0 (ULOQ)	9.437	−5.6	1.3	10.210	2.1	0.4

[a] Accuracy = [(measured concentration − nominal concentration)/nominal concentration] × 100.

[16], which we improved by introducing several modifications. First, we used a methanolic solution containing 2.5% formic acid instead of 3% to elute ribavirin, as we found that such a concentration, among all those we tested, allowed the efficient removal of plasma interferences eluting in close proximity (at 3.2 min) to ribavirin peak (data not shown). Second, the dried extracts were reconstituted at a 2.5× instead of a 5× concentration, because we observed that such a lower sample concentration improved the solubility of ribavirin in mobile phase over time. Third, a centrifugation step was introduced before injection, which allowed the efficient elimination of unsoluble material in the reconstituted extracts that could interfere with the chromatographic analysis. This modified procedure provided a high recovery and high selectivity of extraction and led to very low quantification levels (see below). In addition, as previously reported [18], we found that the PBA columns could be re-used many times without any problem, with the advantage of making the SPE procedure much less expensive.

Table 2
Back-calculated values of both thermised (T) and non-thermised (NT) plasma samples spiked with ribavirin using calibration curves established with samples subjected or not to heat treatment (60 °C for 60 min)

Nominal concentration (μg/ml)	T vs. T (μg/ml)	Accuracy (%)	NT vs. T (μg/ml)	Accuracy (%)	T vs. NT (μg/ml)	Accuracy (%)
0.05 (LLOQ)	0.048	−4.0	0.051	2.0	0.047	−6.0
0.2 (low QC)	0.187	−6.5	0.189	−5.5	0.191	−4.5
1.0 (medium QC)	1.054	5.4	0.980	−2.0	1.032	3.2
5.0 (high QC)	4.966	−0.7	5.123	2.5	4.674	−6.5
10.0 (ULOQ)	10.364	3.6	9.794	−2.1	10.227	2.3

T vs. T: mean concentration of thermised LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with thermised samples; NT vs. T: mean concentration of non-thermised LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with thermised samples; T vs. NT: mean concentration of thermised LLOQ, QC, and ULOQ samples back-calculated with the calibration curves established with non-thermised samples.

Chromatographic separation was performed on a Waters Atlantis dC-18 column, a di-functionally bonded and silica-based reversed-phase column that allows very good retention of highly polar compounds. Moreover, the Atlantis dC-18 column allowed the use of 100% aqueous buffer (10 mM potassium phosphate, pH 4.0) to resolve the ribavirin peak from plasmatic interferences. Isocratic elution was preferred, as gradient elution requires control by a gradient HPLC pump system, re-equilibration time, perfect solvent mixing, etc. Different mobile phases have been previously proposed for eluting ribavirin from the Atlantis dC-18 column, but they either required a gradient run with mixed acetonitrile/potassium phosphate buffer [20] or consisted of a diethylamine solution with low pH (i.e., 2.5) [16], which can considerably shorten the column life and was found to be unstable and hence to lead to significant changes of ribavirin retention time through a set of analyses performed over a day (data not shown). The autosampler was thermostated at 20 °C, as sample precipitation was observed at lower temperatures. The stability of ribavirin under these conditions was investigated (see below). Moreover, column temperature was maintained at 40 °C, because slightly less reproducibility in ribavirin retention time was noted when the analytical and guard columns were not thermostated.

Representative chromatograms of a control human plasma sample, spiked plasma samples at the LLOQ and ULOQ level, and a plasma sample from a patient are shown in Fig. 1. The retention time of ribavirin was 3.7 min. At the selected detection wavelength of 207 nm, wherein ribavirin has UV-absorbance maximum, assays performed on 10 lots of drug-free human plasma (Fig. 1A is a representative chromatogram) did not show the presence of any interfering peak at the retention time of interest.

3.2. Method validation: linearity, recovery, sensitivity, accuracy and precision, selectivity and specificity

An 8-point calibration standard curve of ribavirin in plasma, ranging from 0.05 to 10 μg/ml, was prepared in triplicate in four independent runs. The calibration curves were linear over the tested concentration range, with correlation coefficients equal to or greater than 0.997 for all curves.

The recovery of ribavirin from plasma was determined by comparing the peak areas of extracted LLOQ, ULOQ, and QC samples with those of neat ribavirin solutions ($n = 4$). Mean recovery was 90.4%, with the CV ranging from 0.2 to 4.9%, indicating that the extraction procedure achieved a high degree of efficiency and reproducibility.

The LLOQ, defined as the lowest concentration in the standard curve that back-calculates with good accuracy and precision (bias from −6 to 2%, CV ≤ 4.3%, $n = 18$; see Table 1), was 0.050 μg/ml. A typical chromatogram of an LLOQ sample is shown in Fig. 1B. This LLOQ is lower compared with all the reported HPLC-UV assays for ribavirin [8,16–20]. Moreover, the LLOQ is well below the C_{trough} values observed in patients [12,28] and thus provides sufficient sensitivity for routine analysis of human plasma samples in the clinical setting. The LOD,

Table 3
Stability of ribavirin in human plasma after one, two, and three freeze–thaw cycles

Nominal concentration (μg/ml)	Mean measured concentration at $t = 0$ (μg/ml)	Number of freeze/thaw cycles							
		One cycle			Two cycles			Three cycles	
		Mean measured concentration (μg/ml)	Deviation (%)	CV (%)	Mean measured concentration (μg/ml)	Deviation (%)	CV (%)	Mean measured concentration (μg/ml)	Deviation (%)	CV (%)
0.05 (LLOQ)	0.048	0.046	−4.2	3.9	0.047	−2.1	3.7	0.049	2.1	4.0
0.2 (low QC)	0.194	0.189	−2.6	2.7	0.197	1.5	1.1	0.191	−1.5	2.2
1.0 (medium QC)	1.052	1.075	2.1	2.9	1.034	−1.7	1.5	1.008	−4.2	3.2
5.0 (high QC)	5.237	5.261	0.4	0.3	5.203	−0.6	0.6	5.373	2.6	1.1
10.0 (ULOQ)	9.484	9.445	−0.4	0.9	10.124	6.7	0.1	9.428	−0.6	0.4

defined as the concentration giving a signal-to-noise ratio of 3, was 0.025 μg/ml.

The analytical accuracy and precision were evaluated by assaying LLOQ, ULOQ and QC samples in six replicates on each of three different days. The accuracy (expressed as % bias) and precision (expressed as % CV) data are summarised in Table 1. The intra-day and inter-day deviations (% bias) from the nominal concentrations were always ≤5.6 and ≤6.0%, respectively. The CVs for intra-day and inter-day data ranged from 0.2 to 4.1% and from 0.4 to 4.3%, respectively. These values are similar to or much lower than previously reported values for HPLC-UV assays measuring ribavirin concentration in plasma or serum [8,16–20]. These results indicate that the method we developed achieves a high degree of reproducibility and accuracy.

Specificity based on endogenous interfering peaks was evaluated in 10 different lots of commercial control human plasma. The results demonstrated a lack of analytically significant interference at the retention time of ribavirin (see Fig. 1A). The nucleoside analogues abacavir, acyclovir, didanosine, ganciclovir, lamivudine, stavudine, zalcitabine, and zidovudine did not interfere with the analytical method. Other drugs which were tested included: ampicillin, amprenavir, atazanavir, carbenicillin, chloramphenicol, efavirenz, erythromycin, fluconazole, foscarnet, gentamicin, imipenem, indinavir, interferon, kanamycin, lopinavir, nevirapine, rifampicin, ritonavir, saquinavir, streptomycin, teicoplanin, tenofovir, tetracycline, vancomycin, voriconazole. None of these drugs was found to interfere with the assay. The absence of analytic interference in patient plasma samples was also confirmed by the use of the peak purity testing system and the library matching of the Empower software.

3.3. Stability of ribavirin

The slope of the calibration curves of ribavirin established with samples submitted to the thermisation procedure (60 °C for 60 min) was similar (mean variation of −2.6 ± 0.5%) to that of the calibration curves obtained with non-heated samples. In addition, Table 2 shows the back-calculated values of both thermised and non-thermised samples using calibration curves established with samples subjected or not to the same treatment. Considering the experimental variability (Table 1), these results indicated that such a procedure does not affect ribavirin concentrations within the considered concentration range.

The variations of ribavirin concentrations when submitting plasma samples to three successive freeze–thaw cycles are reported in Table 3. While several reasons (e.g., the accuracy and precision of the method) could be adduced for the instability of ribavirin observed previously [8], our studies show no significant loss of ribavirin after up to three freeze–thaw cycles (deviations from −4.2 to 6.7%). Moreover, no evidence of ribavirin decomposition was found during short-term (24 h) storage of plasma samples at room temperature and storage at −20 °C for 1 month (Table 4). This confirms the good stability of ribavirin in plasma both at room temperature and at −20 °C reported in previous studies [17,21,23,24].

Finally, the stability of processed samples was assessed. Three sets of extracted and reconstituted LLOQ, ULOQ and QC samples in mobile phase were analysed immediately after extraction and re-injected after standing 96 h in the HPLC autosampler. After 4 days in the autosampler at 20 °C, ribavirin levels in extracted samples were in the range 97.3–106.7% of their initial concentrations (Table 4), indicating that ribavirin is

Table 4
Stability of ribavirin in human plasma and in extracted samples under different storage conditions

	Nominal concentration (μg/ml)				
	0.05	0.2	1.0	5.0	10.0
(A) In plasma stored at room temperature for 24 h					
Mean measured concentration a $t = 0$ (μg/ml)	0.051	0.181	1.115	4.778	9.716
Mean recovered concentration (μg/ml)	0.048	0.187	1.047	4.979	9.815
Deviation (%)	−5.9	3.3	−6.1	4.2	1.0
CV (%)	3.3	2.3	3.0	2.9	0.8
(B) In plasma stored at −20 °C for 1 month					
Mean measured concentration a $t = 0$ (μg/ml)	0.048	0.207	0.956	4.993	9.794
Mean recovered concentration (μg/ml)	0.047	0.219	0.972	4.944	9.834
Deviation (%)	−2.1	5.8	1.7	−1.0	0.4
CV (%)	3.2	4.1	1.2	0.3	0.7
(C) In dried extracts stored at −20 °C for 72 h					
Mean measured concentration a $t = 0$ (μg/ml)	0.047	0.193	0.989	5.284	9.595
Mean recovered concentration (μg/ml)	0.049	0.202	0.978	5.188	9.788
Deviation (%)	4.2	4.7	−1.1	−1.8	2.0
CV (%)	3.7	3.3	0.8	1.3	0.9
(D) In reconstituted extracts stored at 20 °C for 96 h					
Mean measured concentration a $t = 0$ (μg/ml)	0.048	0.188	0.992	5.110	10.172
Mean recovered concentration (μg/ml)	0.046	0.194	0.965	5.017	10.852
Deviation (%)	−4.2	3.2	−2.7	−1.8	6.7
CV (%)	3.4	4.4	2.0	1.3	0.6

stable in the reconstituted extracts under the tested conditions. In addition, the stability of ribavirin in dried extracts stored at −20 °C was investigated. The variations over time of ribavirin, expressed as deviations of the starting levels (i.e., after immediate analysis), were less than 5% (from −1.8 to 4.7%) in samples kept at −20 °C for 72 h (Table 4). These results indicate that, in contrast to certain data previously reported [8], storage of dried residues ensures the good preservation of ribavirin for at least 3 days.

3.4. Analysis of patient samples

This method has been applied to measure ribavirin concentration in a number of plasma samples from both HCV-infected and HCV–HIV-coinfected patients and has proved to be robust and sensitive enough for routine therapeutic drug monitoring of ribavirin plasmatic levels. Furthermore, the method is being applied in ongoing research studies. In our patient samples, observed ribavirin plasma concentrations ranged from 0.56 to 4.62 μg/ml, in accordance with the concentration values reported by others [28,29]. Fig. 1C shows a representative chromatogram of a plasma sample from a HCV-positive patient receiving ribavirin. The estimated ribavirin concentration in this sample was 3.23 μg/ml.

4. Conclusions

A simple HPLC assay with UV detection for the measurement of ribavirin concentrations in human plasma was established and validated, and was shown to be specific, sensitive, and accurate over a concentration range of 0.05–10 μg/ml. The applicability and the ruggedness of the method have been demonstrated in the analysis of plasma samples of HCV-infected patients. Analysis of the stability of ribavirin under various conditions showed that the drug concentration remains stable in plasma stored at room temperature for 24 h or at −20 °C for up to 1 month as well as in samples undergoing three freeze–thaw cycles and thermal virus inactivation for 60 min at 60 °C. The drug is also stable in processed samples, both in dried extracts kept at −20 °C for 3 days and in reconstituted samples for at least 4 days at 20 °C.

Acknowledgements

We thank Roche for providing pure ribavirin. This work was funded by Azienda Ospedaliera of Padua, by PRIN 2005 (grant no. 2005060941) and MURST EX60% to AL, and by Istituto Superiore di Sanità of Italy (grant no. 40F.57) and by PRIN 2005 (grant no. 2005064229) to GP.

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