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Review

Ribavirin: Analytical determinations since the origin until today

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Abstract

Ribavirin (RV) (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), is a synthetic purine nucleoside analog with a broad spectrum of antiviral activity. To better understand the mechanism of action of RV, as well as its pharmacokinetic characteristics, an assay that can allow specific, sensitive, and accurate measurement of RV in biologic samples is critical. In this way, diverse analytical methods have been established. In this work, we have recompiled these methods with the aim to present the different options for the RV determination.

Keywords: Ribavirin; Review; Pharmaceutical formulations; Biological samples; Antiviral therapy

Contents

1. Introduction

Only a few substances are known to demonstrate a broad spectrum of antiviral activity against DNA and RNA viruses both in vitro and in vivo. Among these agents, ribavirin (RV) (Fig. 1) is a well-known synthetic compound, structurally related to the naturally occurring ribonucleoside guanosine.

The World Health Organization estimated that about 170 million peoples are infected by hepatitis C virus (HCV) worldwide. HCV-associated chronic hepatitis is a major cause of morbidity and mortality. Standard treatment for chronic HCV hepatitis is the association of pegylated α-interferon (PEG-IFN α-2a or PEG-IFN α-2b) and RV.

RV is a purine nucleoside analog that was first synthesized in 1972. It was reported to have broad-spectrum activity against a variety of DNA and RNA viruses. Several mechanisms of RV action have been proposed, including inositol monophosphate dehydrogenase inhibition, mutagenesis, direct inhibition of the RNA-dependent RNA polymerase, and immune-modulating action. Therapeutically, RV inhalation has been a primary treatment of lower respiratory tract diseases (including bronchiolitis and pneumonia) caused by respiratory syncytial virus (RSV) in

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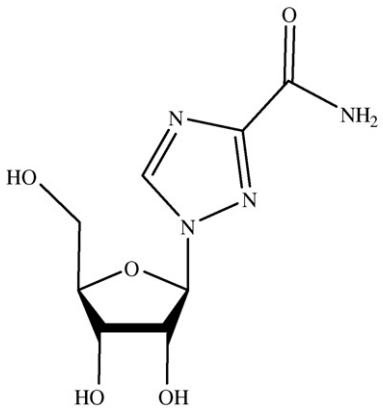

Fig. 1. Chemical structure of ribavirin.

hospitalized infants and young children. Orally administered RV was used with some success for the treatment of various strains of influenza A and B. Although oral therapy of RV alone is not effective for the treatment of hepatitis C virus infection, combination of the drug with either interferon α-2a/2b or peginterferon α-2a/2b was widely accepted for the treatment of chronic hepatitis C with an increased rate of sustained response by two- to three-fold and a decreased rate of relapse following discontinuance of the therapy.

RV undergoes two pathways of metabolism in vivo. The first pathway is a reversible phosphorylation in nucleated cells, and the second is degradation involving deribosylation and/or amide hydrolysis. As the result of deribosylation, RV is biotransformed to RV base (1,2,4-triazole-3-carboxamide). In order to better understand the exposure of both RV and RV base, it is necessary to determine the plasma concentration of these two compounds in biological fluids.

Intracellularly, RV is phosphorylated to ribavirin monophosphate (RMP), ribavirin diphosphate (RDP), and ribavirin triphosphate (RTP). Red blood cells (RBC) have the capacity to phosphorylate RV to RMP, RDP, and RTP, but are devoid of phosphatase activity to convert them back to RV. As a result, high levels of phosphorylated RV accumulate over time, leading to haemolytic anaemia.

The toxicology and pharmacology of RV were assessed in experimental animals, as well as in humans, where their effects on the immune system and blood compartment were investigated.

In this paper, we have reviewed the literature about the RV determination. The cited methods have been classified according to used analytical techniques.

2. Radioimmunoassay

Austin et al. reported a radioimmunoassay for the measurement of RV that is capable of determining plasma concentration as low as 0.01 μM (2.45 ng mL^{-1}) [1]. However, the assay lacks specificity and cross-reacts with many RV metabolites, thus, limiting its usefulness in pharmacokinetic studies. Later, an enzyme immunoassay (EIA), performed directly on fixed infected monolayers of HEp-2 cells in microtiter plates, was compared with the conventional plaque reduction assay (PRA) method for the determination of antiviral activity of RV against respiratory syncytial virus (RSV) [2].

3. Spectrophotometric methods

A survey of the literature revealed that only a single visible [3] and two UV spectrophotometric methods have been reported [4,5]. These methods possess deficiencies, such as a low λ_{max} value or low sensitivity. It is therefore of interest to develop simple and sensitive procedures with higher λ_{max} for the determination of RV in pure and pharmaceutical formulations. Three simple and sensitive visible spectrophotometric methods (A–C) have been described for the assay of RV [6]. They are based on the oxidation of RV with excess sodium metaperiodate and estimating the either products formed.

Also, eight spectrophotometric methods for determination of RV have been developed and validated [7]. These methods were based on the oxidation of the drug by different inorganic oxidants: ceric ammonium sulfate, potassium permanganate, ammonium molybdate, ammonium metavanidate, chromium trioxide, potassium dichromate, potassium iodate, and potassium periodate. The oxidation reactions were performed in perchloric acid medium for ceric ammonium sulfate and in sulfuric acid medium for the other reagents. With ceric ammonium sulfate and potassium permanganate, RV concentration in these samples was determined by measuring the decrease in the absorption intensity of the coloured reagents at 315 and 525 nm, respectively. With the other reagents, RV concentration was determined by measuring the intensity of the developed coloured reaction products at the wavelengths of maximum absorbance: 675, 780, 595, 595, 475, and 475 nm for reactions with ammonium molybdate, ammonium metavanadate, chromium trioxide, potassium dichromate, potassium iodate, and potassium periodate, respectively. Under the optimum conditions, linear relationships were found between the absorbance readings and the concentrations of RV in the range of 4–1400 μg mL^{-1}. The proposed methods were successfully applied to the analysis of RV in pure drug material and capsules.

The determination of RV in eye drops by first derivative spectrophotometry [8],- as well as spectrophotometric method [9] in the range of 5–100 μg mL^{-1} has also been described.

A kinetic method for the determination of RV, manganese-(II) and tiazofurin was developed by Milovanović et al. [10]; in the presence of ammonium carbonate, traces of Mn-(II) catalyse the oxidation of Nile Blue A by hydrogen peroxide, which enables its kinetic determination. By investigating the effect of antiviral and antitumour substances on this reaction, it was established that some of them modify the catalytic activity of Mn-(II): RV increases the catalytic effect of Mn-(II), while tiazofurin acts as an inhibitor.

4. Fluorimetric methods

A simple and sensitive fluorimetric method for determination of antiviral drugs: RV, acyclovir, and amantadine hydrochloride

has been proposed by Darwish et al. [11]. Cerium-(IV) has been used as an oxidizing agent for the determination of some pharmaceutical compounds by monitoring the fluorescence of cerium-(III) induced from their oxidation reaction. These reactions were the basis behind the development of sensitive fluorimetric methods for the determination of these compounds. Previous studies have demonstrated the liability of RV, acyclovir, and amantadine HCl for oxidation by strong oxidizing agents including Ce-(IV). Therefore, their oxidation with Ce-(IV) was attempted to be utilized for development of fluorimetric method for their determination in pharmaceutical formulations.

5. Chemiluminescence methods

No chemiluminescence (CL) methods have been previously reported in the open literature for its measurement. In recent years, CL methods coupled with flow injection (FI) analysis have been applied successfully for many drug measurements owing to their great sensitivity and wide linear working range with simple instrumentation. In this way, a novel FI–CL method for the determination of RV was developed by Lu et al. [12] based on the enhancing effect of the analyte on CL emission of luminol oxidized by sodium persulfate in alkaline solution. Also, this method can be regarded as a basis for the development of HPLC–CL or electrophoresis–CL determination of RV and its metabolite in biological fluids.

6. High-performance liquid chromatography (HPLC)

Many HPLC-based methods (HPLC–UV, LC–MS, etc.) have been reported for the determination of RV in biological matrices [13–26,30–35]. Most of these are reversed phase (RP) HPLC methods with UV detection.

A method for RV quantification by HPLC with UV detection was reported by Paroni et al. [13]. This method, however, has an assay sensitivity of 100 ng mL^{-1}, which is inadequate for the pharmacokinetic evaluation of RV in animals and man following oral administration.

The purpose of the study presented by Homma et al. [14] was to use HPLC for the determination of unchanged and phosphorylated RV levels in whole blood. The level of phosphorylated RV can be estimated by comparing the difference between the total and the unchanged levels, which are measured in samples with and without dephosphorylation, respectively. In addition, concentrations of total (unchanged plus phosphorylated) and unchanged drug in erythrocytes can be estimated from the corresponding plasma and whole blood levels, with knowledge of each subject's hematocrit. Thus, this method is an indirect one based on the conversion of RV phosphates to RV, with quantification of RV. Such a method may be useful for predicting drug toxicity or evaluating potential drug interactions. As an example, this HPLC method was used to explore the in vitro effects of dipyridamole, a nucleoside transport inhibitor, on RV disposition in plasma and erythrocytes.

However, due to the extreme polar nature of the RV molecule, a tedious solid-phase extractions (SPE) using a phenyl boronic acid (PBA) phase had to be carried out to isolate RV and the internal standard (IS) from the matrix [15]. Furthermore, mobile phases with very low organic solvent (<2%), or no organic at all had to be used in RP HPLC in order to obtain adequate retention for RV and separate it from endogenous interference.

HPLC method was previously developed to determine RV levels in order to assess the disposition of RV in erythrocytes [14]. Since phosphorylated metabolites are the main form of intracellular RV, whole blood samples were treated with acid phosphatase prior to column extraction and analysis. However, the dephosphorylation procedure used in previous study [14] was too tedious to use for routine monitoring of cellular RV. Inoue et al. [16] have simplified the dephosphorylation procedure. The modified method was tested for therapeutic drug monitoring of cellular RV levels in two patients during the first 8 weeks of HCV treatment by combination therapy with RV and interferon α-2b.

Lin et al. [17] reported an LC–MS/MS RV assay sensitive to 10 ng mL^{-1} of RV in rat and monkey plasmas, which has been used to evaluate pharmacokinetics of RV in animals.

Recently, an LC–MS/MS method using a silica column, with an LLOQ at 10 ng mL^{-1} for RV analysis in human plasma and serum, was reported by Shou et al. [18].

On the other hand, as a liver-targeting drug, the evaluation of liver concentration of RV following prolonged treatments has become increasingly important. To facilitate the monitoring of RV concentration in liver, a quantitative method has been developed by Yeh et al. [19] to determine the concentration of total RV (RV, RMP, RDP, and RTP) in monkey liver and subsequently in human liver biopsy samples using LC–MS/MS. The

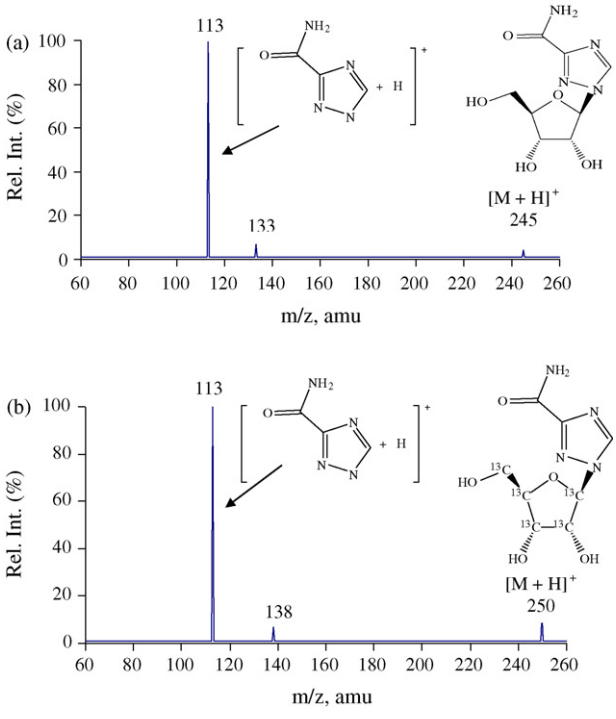

Fig. 2. Product ion scan spectra of (a) ribavirin and (b) [^{13}C]-ribavirin.

product ion scan spectra of RV and [^{13}C]RV are presented in Fig. 2.

6.1. Ribavirin and ribavirin base

Only a few HPLC–UV methods are available for the quantification of both RV and RV base in biological samples [13,20]. However, the reported sample preparation required many steps of manipulation, including dilution with water, filtration with 30,000 Da cut-off, extraction with dichloromethane, filtration through double-bed column, elution, dryness, and reconstitution. The HPLC analysis cycle time was greater than 10 min with a LLOQ of 20 ng mL^{-1} when 1.0 mL of sample was used.

Solid-phase extraction using MCX cartridge (Waters Corp., Milford, MA, U.S.A.) was investigated with 0.1 M HCl or 20 mM potassium phosphate buffer (pH ∼ 6.0) as the equilibration and loading solvents. Unfortunately, very low recovery was obtained for both analytes due to their extremely polar nature. Although a phenyl boronic acid (PBA) cartridge could be used to isolate RV from biological matrix through an exclusive extraction of poly-ol species via interaction with surface-bound borate groups [14–16,21], RV base was expected to be present in the wash waste during the loading step in SPE due to its lack of poly-ol groups in the molecule. Thus, protein precipitation with 10% methanol in acetonitrile was employed. Typical chromatograms of blank serum, blank serum with IS (3-methylcytidine methosulfate), serum supplemented with a medium-range calibrator, and patient serum containing RV are shown in Fig. 3 [21].

Initial LC–MS/MS experiments were carried out by using underivatized silica columns (Betasil and Hypersil silica columns from Thermo-electron,- and Kromasil and Luna silica columns from Phenomenex) with the mobile phase consisting of high organic and low aqueous phase, acetic acid and TFA

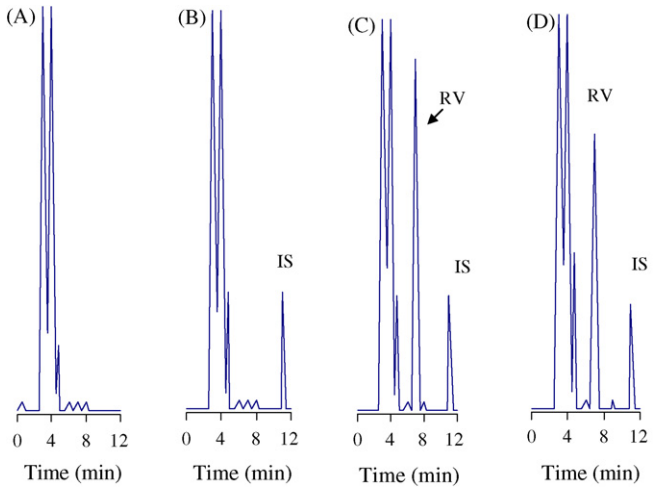

Fig. 3. Typical HPLC chromatograms of extracts of control human serum (A), control human serum spiked with 2.5 μg mL^{-1} of IS (B), control human serum spiked with 2.5 μg mL^{-1} of IS and 3.0 μg mL^{-1} of RV (C), and serum from a patient receiving RV (RV concentration, 2.5 μg mL^{-1}) (D).

[17,18]. Significant matrix effect was observed for the analytes, especially for RV base due to the presence of endogenous ingredients in the extracts after protein precipitation. Chromatographic separation could be performed on a C_{18} column using aqueous or near aqueous (<5% organic solvent) mobile phase in order to retain the extremely polar analytes on the column, but the aqueous mobile phase can be detrimental to many conventional HPLC columns [15,18]. Thanks to the advance of HPLC column technology, several new columns have become available for chromatography of highly polar compounds under aqueous mobile phase condition. Among these columns, Waters Atlantis dC-$_{18}$ column is a di-functionally bonded and silica-based reversed-phase column with super retention of polar compounds. It not only thrives 100% aqueous mobile phase, but also does not exhibit excessive retention of hydrophobic compounds. Thus, a Waters Atlantis dC-$_{18}$ column was used in the current assay with both RV and RV base eluted under 100% aqueous mobile phase. However, the use of aqueous mobile phase has been speculated to yield poor ionization efficiency or poor electrospray sensitivity since water possesses a high surface tension, needs large heat for evaporation, and is a much poorer solvent for electrospray than organic solvents, such as acetonitrile or methanol in LC–MS/MS interface. In the current assay, such phenomenon was overcome by post-column addition of acetonitrile via a "Tee" before the column effluent enters the mass spectrometer, which yielded a 10–15% increase in signal intensity.

A liquid chromatographic method with tandem mass spectrometric detection (LC–MS/MS) for the simultaneous determination of RV and RV base was developed and validated over the concentration range of 10–5000 ng mL^{-1}, respectively, using a 0.025 mL monkey plasma sample [22]. RV, RV base, and the internal standards were extracted from monkey plasma via protein precipitation. After evaporation of the supernatant, the extract was reconstituted with 5% methanol (containing 0.1% formic acid) and injected onto the LC–MS/MS system. Optimum chromatographic separation was achieved on a Waters Atlantis dC-$_{18}$ (150 mm × 2.1 mm, 5 μm) column with mobile phase run in gradient with 100% water containing 0.5% formic acid and 90% acetonitrile (containing 0.5% formic acid). The flow rate was 0.4–0.6 mL min^{-1} with total cycle time of approximately 7.0 min. Post-column addition of acetonitrile (containing 0.1% formic acid) at 0.3 mL min^{-1} was used to increase the ionization efficiency in the MS source. The method was validated for sensitivity, linearity, reproducibility, stability, and recovery. Lack of adverse matrix effect and carry-over was also demonstrated. The intra- and interday precision and accuracy of the quality control (QC) samples were <9.0% relative standard deviation (R.S.D.) and 10.8% bias for RV, and 10.3% R.S.D., and 11.3% bias for RV base. The current specific, accurate, and precise assay is useful in support of the toxicokinetic and pharmacokinetic studies of these compounds.

6.2. Ribavirin and viramidine

A dose-limiting adverse effect associated with the long-term therapeutic use of RV is the potential for causing haemolytic

anaemia. This adverse effect often necessitates reduction of dose and/or discontinuation of RV therapy in a significant proportion of patients. A second-generation analog of RV, therefore, which retains clinical efficacy, but demonstrates a lower potential for causing haemolytic anaemia would be highly desirable.

Viramidine (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine) is an analog and prodrug of RV. Viramidine is converted to RV through adenosine deaminase. The in vitro partition of viramidine and RV between RBC and plasma has indicated that monkey shows similar distribution pattern to human. Similar metabolic profiles in monkey RBC have been observed following single or 10-day dosing of [^{14}C]viramidine or [^{14}C]RV. However, viramidine dosing yielded 1/2 of the radioactivity in monkey RBC than following RV dosing suggesting a potentially better safety profile for viramidine.

To further elucidate the potential safety advantage of viramidine over RV, it is of interest to determine RV levels in RBC from monkeys dosed with RV or viramidine for an extended period (36 weeks).

For simultaneous analyses of RV and viramidine, Lin et al. recently reported an LC–MS/MS method for the simultaneous determinations of RV and viramidine with the aim to determine the absorption, pharmacokinetics, metabolism, and excretion of viramidine in rats and monkeys [23], as well as human serum [24], with an LLOQ of 10 ng mL^{-1}. The same investigation group reports a more sensitive LC–MS/MS method for the simultaneous determination of viramidine and RV in human plasma [25]. The method involved the addition of [^{13}C]viramidine and [^{13}C]RV as internal standards, protein precipitation with acetonitrile, HPLC separation, and quantification by MS/MS system using positive electrospray ionization in the multiple reaction monitoring mode. The precursor → product ion transitions were monitored at 245 → 113, 250 → 113, 244 → 112, and 249 → 112 for RV, [^{13}C]RV, viramidine, and [^{13}C]viramidine, respectively. The calibration curves for viramidine and RV were linear over a concentration range of 1–1000 ng mL^{-1}. For both viramidine and RV, the lower limit of quantification (LLOQ) was 1 ng mL^{-1}. For viramidine, intra- and interday analyses of QC samples at 1, 5, 250, and 1000 ng mL^{-1} indicated good precision (%R.S.D. between 1.0 and 7.0%) and accuracy (%bias between −4.3 and 5.2%). For RV, intra- and interday analyses of QC samples at 1, 5, 250, and 1000 ng mL^{-1} indicated similar precision (%R.S.D. between 0.8 and 8.3%) and accuracy (%bias between −5.8 and 9.4%). Both viramidine and RV were stable in human plasma stored at room temperature for at least 3 h, 4 °C for at least 6 h, and for at least three freeze–thaw cycles. This accurate and highly specific assay provides a useful method for evaluating the pharmacokinetics of viramidine and RV in man following administration of viramidine.

A HPLC–MS/MS method has been developed for the simultaneous determinations of total viramidine (viramidine, viramidine monophosphate, viramidine diphosphate, and viramidine triphosphate) and total RV (RV, RMP, RDP, and RTP) in monkey RBC [26]. The method involves the addition of internal standards and perchloric acid, conversion of viramidine or RV phosphorylated metabolites to viramidine or RV, purification with an aminopropyl (NH_2) solid-phase extraction cartridge, and LC–MS/MS analysis. The MS/MS is selected to monitor *m/z* 245 → 113, 250 → 113, 244 → 112, and 249 → 112 for RV, [^{13}C]RV, viramidine, and [^{13}C]viramidine, respectively, using positive electrospray ionization. The calibration curves are linear over a concentration range of 100–10,000 ng mL^{-1} with a lower limit of quantification of 100 ng mL^{-1} for both compounds. Mean interassay recoveries for RV are 101, 98.9, and 96.0%, with %R.S.D. values between 1.95 and 4.50% for 100, 1000, and 10,000 ng mL^{-1} quality control (QC) samples, respectively. Mean interassay recoveries for viramidine are 96.3, 101, and 102%, with %R.S.D. values between 3.61 and 7.22%, for 100, 1000, and 10,000 ng mL^{-1} QC samples, respectively. Over curve dilution QC at 400 μg mL^{-1} for both viramidine and RV are used to ensure the dilution accuracy (25× dilutions) for monkey samples.

The method has been used to simultaneously determine the total concentrations of RV and viramidine in monkey RBC following 5, 15, and 36 weeks dosing of viramidine or RV (60 mg kg^{-1}). The concentrations of total RV following RV dosing are 1242 μM at week 5, 1257 μM at week 15, and 1146 μM at week 36. The concentrations of total RV following viramidine dosing are 634 μM at week 5, 716 μM at week 15, and 683 μM at week 36. Only small amounts of viramidine are detected in RBC following viramidine dosing, 7.80 μM at week 5, 6.63 μM at week 15, and 10.4 μM at week 36. The results suggest that RV levels in RBC were at steady state at week 5 of RV or viramidine dosing. At steady state, RV levels in RBC are approximately 2× after RV dosing than viramidine dosing. The relatively small percentage of viramidine in RBC suggests that viramidine either poorly penetrated into RBC or was extensively converted to RV following entry into RBC.

7. Capillary electrophoresis

A popular alternative to the use of HPLC is capillary electrophoresis (CE), in which analytes are separated on the basis of their charge-to-size ratio. This different separation mechanism provides a distinctly different selectivity to HPLC, often readily separating components that are difficult to separate by HPLC. When combined with the higher separation efficiency, CE analyses are often quicker than those by HPLC and in some cases can minimize or even eliminate sample pretreatment. In this sense, the electrophoretic separation of RV and 5-methylcytidine (IS) by CE was examined by Breadmore et al. [27]. The aim of this study was to investigate the possibility of using CE for therapeutic drug monitoring of RV in human serum and plasma, particularly with regard to minimizing sample preparation and enhancing analytical separation. A fully validated CE method is presented, employing SPE of RV and UV-absorbance detection. Patient data obtained with the CE assay are compared to those from a corresponding HPLC method.

The analytical characteristics and applications of some methods described in the literature are summarised in Table 1.

Table 1
Analytical methods for RV determination

System	Procedure	Other analytes	Analytical characteristics	Applications	Reference
Bioassay	Inhibition of measles virus cytopathic effect in BS-C-1 cells	Active metabolic products of RV	Based upon maximum dilutions of samples, detected concentrations as low as 0.006 μg mL^{-1} in serum or 0.03 μg mL^{-1} in urine	Human serum and urine	[28]
Radioimmunoassay	To prepare an effective immunogen, RV was monosuccinylated and coupled to ovalbumin using tritium-labeled RV and rabbit antiribavirin serum	Rabbit antibody cross-reacted with the major metabolite of RV, 1,2,4-triazole-3-carboxamide	Quantitative for RV at concentrations of 1 pmol/100 μL in urine or plasma samples	Mock samples	[1]
UV–vis; A: 660 nm; B: 520 nm; C: 540 nm	A: oxidation of RV with excess sodium metaperiodate; B: iodate with metol–sulfanilamide, in presence of Mo (VI) and iodide; C: amount of periodate consumed (celestine blue in presence of telurium (IV))	–	Linear range: A 4–28 mg mL^{-1}; B 2–12 mg mL^{-1}; C 1–5 mg mL^{-1}; Molar absorptivity (L mol^{-1} cm^{-1}): A 0.3×10^4; B 0.7×10^4; C 1.39×10^4; R.S.D: A 0.48%; B 0.57%; C 0.65%	Pharmaceutical formulations	[6]
UV–vis; at 630 nm	Oxidation with Ce (IV) ammonium sulfate (Method A) or potassium persulfate (Method B); products of oxidation in both methods coupled with 3-methylbenzothiazolin 2-one hydrazone, producing a deep blue colour	Acyclovir (ACV)	A: linear ranges of 5–50 and 10–60 μg mL^{-1} with DL of 0.18 and 0.63 μg mL^{-1} for ACV and RV, respectively B: linear ranges of 5–45 and 20–50 μg mL^{-1} with DL of 0.11 and 1.40 μg mL^{-1}, respectively	Pharmaceutical formulations	[29]
Kinetic determination	Measured amount of H_2O_2 solution stored in one compartment of a special vessel, ammonium carbonate, potassium nitrate, and Mn (II), RV or tiazofurin solutions placed in second compartment and Nile Blue A and water in third compartment, at 25 °C	Mn (II), tiazofurin	Linear range from 0.05 to 0.4 μg mL^{-1} for RV and from 0.3 to 2.6 μg mL^{-1} for tiazofurin	Mn (II) in mineral water and RV in pharmaceutical preparations	[10]
Fluorimetric method; monitoring fluorescence of the induced Ce-(III) at λ_{ex} 255 and λ_{em} 355 nm	Based on the oxidation of these drugs by Ce-(IV) in presence of perchloric acid	Acyclovir and amantadine hydrochloride	Linear range of 50–1400 ng mL^{-1}; DL and QL 20–49, and 62–160 ng mL^{-1}, respectively; R.S.D. 1.58%	Pure and pharmaceutical dosage forms	[11]
FI–CL	Enhancing effect of RV on CL emission of luminol	–	Linear range from 0.01 to 1.0 μg mL^{-1}; detection limit 0.004 μg mL^{-1}; R.S.D. 2.8%	Pharmaceutical preparations	[12]
HPLC–UV; peaks detected at 207 nm	Elution using distilled water as the mobile phase at a flow-rate of 1 mL min^{-1} gave the best separation of RV, uridine, carboxamide, and the endogenous interfering peaks of serum and urine;	Urine concentrations of the 1,2,4-triazole-3-arboxamide produced by hydrolysis of the ribosylic moiety of the molecule	Retention times 4, 6.9, and 13 min for the carboxamide, RV and uridine, respectively; other described metabolites showed retention times shorter than 3 min; linear response from 20 to 1000 ng mL^{-1}	In serum at therapeutic levels	[13]
RP–HPLC with variable wavelength UV–vis detector; detection at 207 nm	At ambient temperature on a Microsorb™ C_{18} stainless steel column (5 μm particle size, 25 cm × 4.6 mm) protected by a Guard-Pak™ precolumn with a C_{18} insert; using 0.02 M ammonium phosphate buffer, pH 5.10 containing 1% methanol as the mobile phase; strongly adsorbed substances eluted with a gradient of 0–80% acetonitrile in water; flow-rate 1.0 mL min^{-1}	–	Linear range: 1 ng–10 pg per injection; minimum detectable amount standard RV 1 ng (4.1 pmol) injected; minimum detectable level in serum, lung tissue or tracheal aspirates is 0.1 μg mL^{-1}	Biological fluids and tissues	[30]
HPLC	Extraction with a boronate affinity gel, which uses a 3-methylcytidine IS	–	Sensitive (to 0.4 μM), specific (no interference with 34 commonly prescribed drugs), reproducible (CV from 5.4 to 22.4%), and linear from 0.5 to 50.0 μM	Serum, plasma or cerebrospinal fluid	[31]
HPLC–UV; detection at 207 nm	Microbondapak C_{18} column using a mixture of 0.01 M dibasic potassium phosphate and methanol (95:5)	–	Low value of R.S.D. and recovery in range 99.1–101.5% indicates good precision and non interference	Stability studies; pharmaceutical dosage forms	[32]

RP–HPLC–UV; detection at 235 nm	Using a C_{18} RP column; mobile phase solvent, 10 mM ammonium phosphate buffer (pH 6.5) at a flow rate 0.7 mL min^{-1} for analysis of enzyme-treated samples; for nontreated samples, pH of the mobile phase adjusted to 2.5	Phosphorylated anabolites in erythrocytes	Retention times of RV and IS were 6.5 and 13.1 min, respectively; detection limit of RV was 40 pmol, corresponding to 0.08 mM for plasma or 0.2 mM for whole blood	Whole blood	[14]
HPLC–UV	Using SPE with phenylboronic acid columns	–	Linear from 1 to 64 μM in 100 μL serum; DL 0.1 μM; intra-assay R.S.D. was 3.2% at treatment levels (9.7 μM) and 11.5% at 0.4 μM	Serum	[15]
HPLC	–	Magnesium/procaine/xylitol solution, lidocaine in DMSO solution and 5-aminolevulinic acid gel	–	–	[33]
HPLC–UV; detection at 207 nm	SPE on phenyl boronic acid cartridges; with a C18-bonded silica column and a mobile phase containing 10 mM ammonium phosphate buffer (pH adjusted to 2.5)	–	Linear range from 0.1 to 10 μg mL^{-1}; lower limit of quantification and limit of detection were set at 0.2 and 0.06 μg mL^{-1}, respectively, for extracted samples	In 24 serum samples from patients with chronic hepatitis C treated with RV and interferon α-2	[21]
RP–HPLC–UV; detection at 225 nm	Whole blood diluted with a six-fold volume of ice-cold distilled water subjected to acid phosphatase digestion to convert phosphorylated RV metabolites to free RV, resulting mixture, spiked with an IS; phenyl boronic acid column extraction	–	Linear range from 5.3 to 1.024 μM; validation CV for intra- and interday assays were 2.9–5.8% and 4.3–8.3%, respectively	In two hepatitis C patients receiving interferon α-2b-plus RV combination therapy	[16]
HPLC–UV	Using an Atlantis 3 μm dC_{18} column (150 mm × 4.6 mm), protected by a Security Guard with C_{18} (4 mm × 3 mm), at 35 °C with a column thermostat; with gradient; mobile phase composed of Buffer A (KH_2PO_4 50 mM with *o*-phosphoric acid, final pH 3.23) and Buffer B (acetonitrile)	–	Retention time 4.3 min; linear range from 78.125 to 10,000 ng mL^{-1}; QL and DL 78.125 and 19.5 ng mL^{-1}, respectively; intra- and interday (%R.S.D.) precision ranged from 1.48 to 2.40 and from 1.73 to 5.09, respectively	Plasma concentrations in HCV-positive patients	[34]
LC–MS–MS equipped with a positive electrospray ionization in multiple reaction mode; MS–MS reaction selected to monitor the 245 → 113 and 226 → 152 transitions for RV and IS, respectively	With addition of acyclovir as an IS and protein precipitation with acetonitrile followed by separation by an Intertsil Silica column	–	Linear range of 10–5000 ng mL^{-1}; lower limit of quantification 10 ng mL^{-1}; R.S.D. 8–11%, intra- and interday analysis of QC samples at 30, 1500 and 3500 ng mL^{-1} indicate that the method was precise (R.S.D. < 18%) and accurate (bias < 13%)	Rat and monkey plasma	[17]
LC–MS–MS	Samples (0.1 mL) extracted from matrix by simple protein precipitation; supernatants evaporated to dryness, reconstituted and injected onto system; on a silica column operated with an aqueous–organic mobile phase; Bamethan used as IS; protein precipitation extraction automated on 96-well format with use of robotic liquid handlers	–	Linear range of 10–10,000 ng mL^{-1}; LLOQ was set at 10.0 ng mL^{-1}; at the LLOQ, R.S.D. ($n = 6$) 11.2% and the relative error from the nominal value was +12.8% for the plasma matrix	Human plasma and serum	[18]
LC–MS–MS; selected to monitor *m/z* 245–113 for RV and *m/z* 250–113 for [^{13}C]RV using positive electrospray ionization	By addition of an IS and perchloric acid, conversion of RV phosphorylated metabolites to RV, purification with a SPE cartridge	–	Linear range from 100–10,000 ng mL^{-1}; QL 100 ng mL^{-1}; mean interassay accuracy for QC at 100, 1000, and 10,000 ng mL^{-1} are 101.8, 99.4, and 98.8%, respectively; mean interassay precision (%R.S.D.) for QC at 100, 1000, and 10,000 ng mL^{-1} are 5.0, 5.0, and 2.5, respectively	Human red blood cells	[35]

System	Procedure	Other analytes	Analytical characteristics	Applications	Reference
LC–MS–MS; monitor 245 → 113 and 250 → 113 transitions using positive electrospray ionization for RV and [^{13}C]RV	RV and its phosphorylated metabolites extracted with $HClO_4$; metabolites converted to RV using acid phosphatase and further purified using a NH_2 SPE cartridge; [^{13}C]RV added with the extraction solution as IS	–	Linear range from 1 to 100 μg g^{-1}; LOQ 1.0 μg g^{-1}; mean interassay accuracy for QC at 1, 10, and 100 μg/g are 108, 99.7, and 99.7%, respectively; mean interassay precision (%R.S.D.) for QC at 1, 10, and 100 μg g^{-1} are 5.34, 5.24 and 4.59%, respectively	Monkey liver	[19]
LC–MS–MS; in the multiple-reaction mode with positive electrospray ionization monitoring the transitions from *m/z* 245 to 113 and 259 to 128 for RV and IS	With addition of IS (acyclovir), protein precipitation with acetonitrile, solvent evaporation, reconstitution of residue, and separation on an Intertsil Silica column	Viramidine	For viramidine, LQ 10 ng mL^{-1}, with R.S.D. 9% and bias 1.3%; linear range, 10–5000 ng mL^{-1}; for RV, LQ 10 ng mL^{-1}, with R.S.D. 8.5% and bias 1.8%; linear range, 10–5000 ng mL^{-1}	Rats and Cynomolgus monkeys	[23]
CE–UV	Using reverse polarity in a 100 mM borate electrolyte, pH 9.1, with 5 mM spermine added to reduce the electroosmotic flow; SPE employing phenyl boronic acid cartridges	–	With 500 μL sample and reconstitution of dried extract into 100 mL of 33% 100 mM phosphate buffer, pH 6.4/67% acetonitrile, DL and QL were 0.05 and 0.10 μg mL^{-1}, respectively	Human plasma and serum	[27]

8. Conclusions

Several analytical methods have been developed for the analysis of RV. These are based on either radioimmunoassay or HPLC with UV-absorbance or MS detection. The latter approach offers adequate sensitivity and specificity for sample analysis with only protein precipitation for sample pretreatment, however, the cost and expertise of employing HPLC–MS is a considerable disadvantage for many therapeutic drug monitoring laboratories. While the use of UV detection is simpler and cheaper to operate, it requires the use of SPE to remove interferences and to reach suitable detection levels. This is achieved by using a highly specific phenyl boronic acid extraction column that exclusively extracts polyol species via interaction with surface bound borate groups. Due to the extremely polar nature of RV, analytical separations are performed on a C_{18} column in aqueous or near aqueous conditions (<5% organic solvent), which can be detrimental to many analytical columns, while strongly retained analytes are removed by cleaning overnight with a mobile phase containing a high amount of organic solvent. Other popular alternative to the use of HPLC is capillary electrophoresis.

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