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Measurement of intracellular ribavirin mono-, di- and triphosphate using solid phase extraction and LC–MS/MS quantification

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ABSTRACT

Ribavirin (RBV) is a nucleoside analog used to treat a variety of DNA and RNA viruses. RBV undergoes intracellular phosphorylation to a mono- (MP), di- (DP), and triphosphate (TP). The phosphorylated forms have been associated with the mechanisms of antiviral effect observed in vitro, but the intracellular pharmacology of the drug has not been well characterized in vivo. A highly sensitive LC–MS/MS method was developed and validated for the determination of intracellular RBV MP, DP, and TP in multiple cell matrix types. For this method, the individual MP, DP, and TP fractions were isolated from lysed intracellular matrix using strong anion exchange solid phase extraction, dephosphorylated to parent RBV, desalted and concentrated and quantified using LC–MS/MS. The method utilized a stable labeled internal standard (RBV-$^{13}C_5$) which facilitated accuracy (% deviation within ±15%) and precision (coefficient of variation of ≤15%). The quantifiable linear range for the assay was 0.50 to 200 pmol/sample. The method was applied to the measurement of RBV MP, DP, and TP in human peripheral blood mononuclear cells (PBMC), red blood cells (RBC), and dried blood spot (DBS) samples obtained from patients taking RBV for the treatment of chronic Hepatitis C virus infection.

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1. Introduction

Ribavirin (RBV) is one of the only broad spectrum antiviral drugs available in the world [1]. Though RBV has been a fundamental component of the treatment of chronic Hepatitis C virus (HCV) infection for decades, its mechanism of antiviral activity has not been established in vivo. It is also associated with a major dose-limiting toxicity, hemolytic anemia. Our lack of understanding of the clinical pharmacology of RBV is a critical barrier to the optimal use of this drug in the treatment of HCV and other viruses.

RBV is a nucleoside analog that most closely resembles guanosine and adenosine [2]. RBV undergoes intracellular phosphorylation to a mono- (MP), di- (DP), and triphosphate (TP). It is the phosphorylated forms of the drug that have been associated with the antiviral effects observed in vitro [2–5]. RBV is a substrate for concentrative nucleoside uptake transporter 2 [6] and is widely taken up into many cell types in the body. Red blood cells (RBC) lack dephosphorylation enzymes, thus the TP moiety is thought to accumulate in RBC leading to hemolytic anemia [5].

Characterizing the intracellular pharmacology of RBV and determining concentration–effect relationships for the drug would inform dosing decisions and improve treatment outcomes [7,8]. We have developed a novel method for measuring the individual phosphate moieties of RBV in human peripheral blood mononuclear cells (hPBMC), red blood cells (RBC), RBC lysate derived from dried blood spots (DBS) and other types of cells including hepatocytes. While prior methods with similar solid phase extractions have quantitated parent RBV concentrations in whole blood and total

Abbreviations: RBV, ribavirin; NA, nucleoside analog; MP, monophosphate; DP, diphosphate; TP, triphosphate; IS, internal standard; hPBMC, human peripheral blood mononuclear cells; SPE, solid phase extraction; RBC, red blood cells; DBS, dried blood spots; UP, ultrapure; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantitation; CV, coefficient of variation; A, adenosine; dA, 2′deoxyadenosine; G, guanosine; dG, 2′deoxyguanosine; C, cytidine; dC, 2′deoxycytidine; T, thymidine; U, uridine; QC, quality control; ME, matrix effect; RE, recovery; PE, process efficiency; STD, standard; ULOQ, upper limit of quantification; pmol, picomole; HPLC, high pressure liquid chromatography; HCV, hepatitis C virus.

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phosphorylated RBV in hPBMCs [9,10] as well as RBV-MP and RBV-TP in vitro [10], this paper describes the development and validation of a highly sensitive and unique method for the separation and quantitation of intracellular phosphorylated RBV MP, DP, and TP *in vivo* using samples obtained from patients being treated with RBV for chronic HCV infection. It is the first method to describe measurement of RBV and its phosphorylated metabolites in multiple cell matrices from human subjects.

2. Materials and methods

2.1. Chemicals and materials

The following chemicals were acquired from the stated manufacturers: Ribavirin (RBV, $M_W = 244.2$), Sigma Aldrich, St. Louis, MO; Ribavirin Monophosphate (RBV-MP, $M_W = 324.2$) and Ribavirin triphosphate, (RBV-TP, $M_W = 484.1$), Moravek Biochemicals, Inc., Brea, CA; Ribavirin isotopic internal standard, (RBV $^{13}C_5$, $M_W = 249.2$), Toronto Research Chemicals, North York, ON.

Analytical grade reagents were purchased from Fisher Scientific, Fairlawn, NJ, (acetonitrile, methanol, formic acid, potassium chloride, phosphoric acid, and ammonium acetate) as well as Whatman 903 DBS cards, bags and desiccant for DBS preparation and storage. Sodium acetate and acid phosphatase were purchased from Sigma Aldrich, St. Louis. Ultrapure (UP) water was prepared in house from deionized water with a Barnstead Nanopure System (Thermo Fisher Scientific, Waltham, MA). Consumables included Waters Sep-Pak Accell Plus QMA Cartridge, 3cc (500 mg) (Waters Corporation, Milford, MA) and Varian Bond-Elut LRC Phenylboronic Acid (PBA) Cartridge 100 mg/10 mL (Agilent, Santa Clara, CA); and blood products for lysed cellular matrix (Bonfils, Denver, CO).

2.2. Preparation of hPBMC and RBC cellular matrices

Methods for sample collection, separation of hPBMC and RBC from whole blood, QMA separation of phosphorylated moieties and dephosphorylation have been previously published [12]. Briefly, RBV MP, DP, and TP concentrations were measured from hPBMCs and RBCs using isolation procedures developed specifically for the type of cells to be analyzed. The isolation procedure for hPBMCs included RBC removal with RBC lysis media (Gibco, Invitrogen) which is essential since RBV MP, DP, and TP are found at high levels in RBCs [8,12,13]. Once cell samples were isolated, purified, and counted, the cells were lysed with 500 μL cold 70:30 methanol:ultrapure water (v:v) and stored at −80 °C. For DBS, 30 μL of whole blood was spotted and dried for at least 2 h and up to overnight on Whatman 903 cards [14,15]. A 3 mm punch was lysed in 500 μL 70:30 solution via a 10 min sonication prior to storage at −80 °C [14]. It is this lysed cellular matrix (70:30) that was subjected to QMA separation to obtain the MP, DP and TP moieties using a potassium chloride gradient followed by desalting, concentrating and analysis with LC–MS/MS.

2.3. Preparation of standard, quality control, and internal standard solution

The RBV standard (STD) preparation stocks were prepared with 1 mg/mL (4095 pmol/μL) concentrations in UP water from reference powder. Preparation stocks of parent RBV were prepared at concentrations of 500, 50, and 5 pmol/μL, which were further diluted in UP water to create the final working standard solutions. The working standard concentrations range from 0.5 to 200 pmol/sample. Sample was defined as 20 μL working stock added to the 2 mL 1 M KCL eluate (for triphosphate) resulting from blank lysed cellular matrix carried through the QMA and dephosphorylation process [12]. A RBV isotopic internal standard ($^{13}C_5$) stock was prepared in UP water at a concentration of 40 pmol/μL. This stock was diluted to a working concentration of approximately 10 pmol/sample for use in the assay. Standard and internal standard solutions were stored at 4 °C.

Individual quality control (QC) preparation stocks were prepared from RBV-triphosphate (RBV-TP) reference standard. Quality assurance procedures were performed on the RBV-TP stocks received from the manufacturers to assess both purity and potency, as described previously [16]. A potency determination of 99.9% and a purity determination of 92.6% were multiplied to arrive at a correction factor of 0.925 for the RBV-TP stock used in this validation. Applying this correction factor gave an initial RBV-TP QC preparation stock concentration of 1851 pmol/μL which was stored at −80 °C. QCs were prepared by appropriate dilution with blank lysed cellular matrix (10×10^6 cells/mL) in 25 mL volumetric flasks. Four sets of QCs were prepared from the RBV-TP QC preparation stock for validation: 1.5 pmol/sample (QC Low); 15 pmol/sample (QC Med); 150 pmol/sample (QC High) and 0.5 pmol/sample (QC LLOQ). The QC LLOQ was prepared by dilution of 40 μL of QC Med in 1.2 mL total volume blank lysed cellular matrix freshly prepared each day of analysis. The volume extracted for each QC above was 0.2 mL. This volume was defined as sample. QCs were stored at −80 °C.

2.4. SPE: Desalting and concentration of parent RBV

Once separated by QMA [12], the isolated MP, DP, and TP RBV fractions, in 5 mL 75 mM KCL, 7 mL 90 mM KCL, and 2 mL 1 M KCL, respectively, were dephosphorylated to parent RBV. Working standard solutions (20 μL) were added to blank hPBMC samples obtained from the QMA and internal standard working stock solution (20 μL) was added to all tubes except the blank. 1.5 mL of 250 mM ammonium acetate, pH 8.5, was added to all the tubes followed by vortex mixing. Varian Bond-Elut LRC phenylboronic acid (PBA) SPE (Varian/Agilent) cartridges were used to de-salt and concentrate the samples. First, the PBA SPE cartridges were prepared with three washes: 1 × 1.0 mL 0.25% phosphoric acid in methanol, 2 × 1.0 mL 250 mM ammonium acetate pH 8.5. After sample application, cartridges were washed with 2 × 1.0 mL of 250 mM ammonium acetate pH 8.5, 1 × 3.0 mL H_2O, then 2 × 1.0 mL methanol [17]. The analytes were eluted with one application of 1.0 mL 20% formic acid in methanol. Samples were dried for 25 min under nitrogen at 40 °C in a Zymark TurboVap (Zymark Corp., Hopkinton, MA). Final reconstitution was with 100 μL UP water. The sample was vortex mixed, centrifuged to maximize collection (100 × *g* × 2 min) and transferred to a 150 μL low volume insert (Waters Corporation, Milford, MA) followed by a 10 μL injection onto the LC–MS/MS system.

2.5. LC–MS–MS instrumentation and analytical conditions

Both a TSQ Quantum® and TSQ Vantage® triple quadrupole mass spectrometer were used for clinical sample analysis. Validation runs were completed on the TSQ Quantum® triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA) HPLC system coupled with a Surveyor HPLC pump and autosampler. The assay was moved to the Vantage to achieve greater sensitivity at the lower end concentrations as well as the advantage of having a heated source. A cross validation showing comparable sample results between the instruments is displayed in Fig. 1. The Vantage was coupled with an Accela® pump and a PAL® Autosampler from CTC Analytics and operated in positive ESI mode for this analytical method. A 20 μL PEEK loop was used for sampling and data were acquired with Xcalibur™ 2.0.7 software from ThermoScientific. Chromatography was achieved on a Develosil C_{30} Reversed-Phase–Aqueous, 140 Å, 150 × 2.0 mm, 3 μm particle size column purchased from Phenomenex (Torrance, CA). The mobile

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