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Development and validation of a dried blood spot assay for the quantification of ribavirin using liquid chromatography coupled to mass spectrometry

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ABSTRACT

Efficient, inexpensive and sensitive assays for the measurement of drugs are of interest for pharmacokinetic and pharmacodynamics (PK–PD) analysis. Dried blood spots (DBS) are a unique bioanaltyical matrix with the potential to fulfill this interest for the measurement of numerous analytes. Here we describe the development and validation of a reversed-phase high performance liquid chromatographic (LC), tandem mass spectrometry (MS/MS) assay for the determination of ribavirin (RBV) in DBS. A 3 mm punch from spotted and dried whole blood was extracted in methanol utilizing isotopically labeled internal standard for LC–MS/MS analysis. Validation was performed over a range of 0.05 μg/mL to 10.0 μg/mL and the method was shown to be precise (coefficient of variation ≤15%) and accurate (within ±15% of control). These acceptance criteria were met for hematocrit ranges of 20–54%, for center versus edge punches and for spot volumes from 10 to 60 μL. RBV was stable for up to 140 days at room temperature and −20 °C as well as for three freeze/thaw cycles. Correlation of RBV in DBS versus in plasma yielded $r^2 \geq 0.98$ demonstrating that DBS can be used as an alternative to plasma for PK–PD studies in human subjects.

1. Introduction

Ribavirin (RBV) is a nucleoside analog used for the past 15 years in the treatment of hepatitis C. Although the mechanism of action is still debated, RBV has been essential for increased cure rate of hepatitis C in combination with other drugs. Therapeutic drug monitoring of RBV is important because this drug causes hemolytic anemia leading to dose reduction and even the cessation of treatment [1]. However, existing assays to gain PK–PD information require venous blood draw, separation of plasma and a lengthy extraction process.

Recently, dried blood spots (DBS) have become of interest for the quantification of various drugs for pharmacokinetic (PK) studies [2,3]. Analysis of DBS by LC–MS/MS has been used for over 20 years to quantify a wide variety of analytes for different purposes, including genetic disease screening and therapeutic drug monitoring [4]. DBS is especially attractive for these studies because samples can be easily and less invasively obtained via finger or heel stick, processed cheaply and quickly and stored more efficiently compared to plasma samples. Additionally, the low volume requirement (~25 μL) is useful when dealing with special patient populations such as pediatrics or those suffering from anemia. It also allows for measurement of RBV in samples obtained from patients at sites without laboratory capabilities and there are no special shipping costs or requirements associated with DBS samples [5]. We present here the development and validation of an assay to quantify RBV in DBS using a sensitive LC–MS/MS technique.

2. Methods

2.1. Chemicals and materials

RBV was purchased from Sigma Life Sciences (St. Louis, MO) and RBV isotopic internal standard (RBV-IS) was purchased from Toronto Research Chemicals (TRC Canada). HPLC grade methanol, formic acid and acetonitrile were purchased from Fisher Scientific

Abbreviations: RBV, ribavirin; DBS, dried blood spots; LC–MS/MS, liquid chromatography tandem–mass spectrometry; PK–PD, pharmacokinetics/dynamics; STD, standard; QC, quality control; LLOQ, lower limit of quantitation; ALQ, above limit of quantitation; HCT, hematocrit; RT, room temperature; UPH2O, ultra-pure water.

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(Fairlawn, NJ) as well as Whatman 903 protein saver cards, desiccants and humidity indicators. Human blank whole blood with K3 EDTA was acquired from Biological Specialty Corporation and K2 EDTA whole blood was obtained from volunteers who consented for IRB-approved protocols.

2.2. Preparation of stocks, standard calibrators, quality controls (QCs) and internal standard (IS)

Prep stocks at a concentration of 1 mg/mL were prepared for RBV standard calibrators and QCs separately by dissolving approximately 5 mg of RBV into an equal volume of ultrapure water (UPH_2O). Each solution was used to make the standard and QC working stocks to be diluted for the appropriate DBS concentration. To prepare the calibration standards, 25 μL of working stock was added to 475 μL of whole blood followed by immediate spotting onto Whatman 903 protein saver cards at 30 μL per spot, except for the spot volume experiment in Section 3.5. The pipet tip was not allowed to touch the paper when spotting and cards were set to dry for 2 h and up to overnight at room temperature. Standards (STDs) H-A were made at concentrations of 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5.0 and 10.0 μg/mL. Quality controls were prepared in an identical fashion using their specific 1 mg/mL prep stock at levels of lower limit of quantitation (LLOQ): 0.05 μg/mL, QL (low): 0.15 μg/mL (3x LLOQ), QM (medium): 3.0 μg/mL and QH (high): 8.0 μg/mL (80% of highest standard).

Internal standard solution (RBV-IS) was prepared in two steps. First, a 40 pmol/μL stock solution was diluted to 0.5 pmol/μL. Secondly, 5 mL of this solution was transferred to a new 15 mL conical tube and diluted to a final concentration of 0.25 pmol/μL. All solutions were stored at 4 °C.

2.3. DBS extraction

Once cards were dried, a single 3 mm diameter circle was punched from the edge (except for Section 3.7 of this manuscript) for each STD and QC and placed in 200 μL of methanol followed by the addition of 20 μL RBV-IS. The samples were then subjected to 10 min of sonication. Samples were dried under nitrogen at 40 °C, reconstituted in 100 μL of UPH_2O and placed in auto-sampler vials for injection onto the LC–MS/MS system.

DBS samples were collected from patients consented to participate in IRB approved protocols. Each whole blood sample was collected in an EDTA Vacutainer tube followed by immediate spotting of 30 μL onto Whatman 903 protein saver paper and extracted as described above. Samples from human subjects were used to compliment stability testing in QCs. A total of 28 DBS samples collected from human subjects were paired to plasma results obtained from a previously validated method [6] to explore the correlation between the two methods.

2.4. LC–MS/MS instrumentation

RBV and RBV-IS were quantified using a Thermo Scientific TSQ Vantage® triple quadrupole mass spectrometer coupled with a Thermo Scientific Accela® UHP pump and CTC analytics HTC PAL® autosampler based on previous methods [7–9]. The system utilized an electrospray ionization (ESI) probe (Ion Max HESI II®) operated in positive mode. Selective and highly selective (SRM and hSRM) reaction monitoring [precursor/product] m/z transitions were: RBV (245.056/133.110) and RBV-IS (250.100/113.110). The instrument injection volume was 10 μL and separation was achieved using an isocratic mobile phase (0.1% formic acid in 2% acetonitrile: 98% UPH_2O) and a flow rate of 200 μL/min. A Develosil 3 μ Reverse Phase-Aqueous, C30, 140A, 150 × 2.0-mm column was purchased from Phenomenex for chromatographic separation. Total run time was 4.0 min with an approximate retention time of 3.0 min for RBV. Data were analyzed using Xcalibur™2.0.7 software from Thermo Scientific.

2.5. Validation procedure

Validation was carried out according to the FDA guidelines for typical bioanalytical assays [10]. Important items to show are accuracy and precision of the assay, matrix effect (ME), recovery (RE) and process efficiency (PE) as well as general stability of QCs and clinical samples. DBS also has its own set of unique properties that should be assessed including spot volume, punch location, hematocrit and dilution/punch stacking effects [11]. The acceptance criteria for all of these tests are within ±15% deviation from control/nominal and ≤15% coefficient of variation (CV).

Stability is of particular interest for RBV DBS samples because RBV is a nucleoside analog which is transported, phosphorylated and accumulated within red blood cells [12]. Since this accumulation increases with continued dosing, we found it vital to assess the effect of increased intracellular concentrations on parent RBV levels at various time points of dosing (i.e. day 1 up to week 48). This is especially important since the matrix for DBS consists mainly of red blood cells.

3. Validation results

3.1. Determination of accuracy and precision

3.1.1. Accuracy, precision and performance

Performance of the calibration standards was shown by averaging the r^2 values from each of the three analytical runs. Each calibration sample contained internal standard and peak area ratios versus concentration were fitted with $1/x^2$ weighted linear regression. Acceptance criteria were similar to QCs with the lowest standard (STD H at 0.05 μg/mL) treated the same as the LLOQ (within ±20%, all other levels within ±15%). Any standard outside of this range was dropped from the curve as long as 6/8 (75%) calibration standards were within this range. An r^2 of 0.980 or higher was considered acceptable. A representative standard curve is shown in Fig. 1 and curve performance is summarized in Table 1a.

Accuracy and precision were assessed according to FDA guidelines for general bioanalytical matrices [10]. Six replicates of each of the four prepared QC levels (LLOQ: 0.05, QL: 0.15, QM: 3.0 and QH: 8.0 μg/mL) were extracted and analyzed against a standard calibration curve consisting of eight levels from 0.05 to 10.0 μg/mL as described above. This was repeated in three separate analytical runs to determine both inter- and intra-accuracy (% deviation from nominal) and precision (% coefficient of variation (CV)). The assay was considered valid if both of these parameters were within ±15% at all levels except for the LLOQ which was considered valid within ±20%. Table 1b gives a summary of inter- and intra-assay accuracy and precision. For the LLOQ, accuracy was within ±9.3% and precision was ≤6.3% while all other levels were within ±11.4% deviation from nominal and ≤7.9% CV.

3.1.2. Precision in clinical samples

Intra-assay precision using four human subject samples at both room temperature and −20 °C was also assessed. Each of the four patient samples was run in triplicate per storage condition (room temperature (RT) and −20 °C) with mean RBV concentration ranges of 1.91–7.57 μg/mL. Precision was determined to be within acceptance criteria at 12.9%. This was consistent with QC precision assessment signifying a precise method of measurement for RBV in DBS.