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Short and Robust HPLC-UV Method to Determine Serum Ribavirin Concentration without Evaporation Step

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Abstract

Objective: Measurement of ribavirin (RBV) is important for therapeutic drug monitoring in hepatitis C patient. A simple and fast high performance liquid chromatography (HPLC) method developed and validated to measure ribavirin concentration in serum samples without an evaporation step.

Design and method: About 500 µl serum sample, 50 µl internal standard and 20 mM ammonium acetate buffer (pH=8.5) were mixed for 30 seconds and centrifuged. The supernatant was transferred into preconditioned phenyl boronic acid cartridges for solid phase extraction. All cartridges were washed two times with 1 mL of 20 mM ammonium acetate buffer under vacuum not exceeding 10 psi. Ribavirin and internal standard were eluted with 300 µl of 3% formic acid. An aliquot of 100 µl was injected into HPLC system.

Results: The method was linear in the range of 0.1-8.0 mg/l with 0.05 mg/l as limit of detection. The correlation coefficient of method comparison was 0.975 with p value of 0.116 showing a good reproducibility of results. The mean accuracy was checked at three different concentrations and found to be between 107-110% for all three levels. The extraction efficiency was 65.5% for ribavirin in the range of 0.1-8.0 mg/l and 71.2% for internal standard at 50 mg/l. The intra assay precisions were determined at 0.5, 2.5, and 5.0 mg/l and % CV were found to be 2.2%, 5.0%, 4.5% respectively. The injection reproducibility at three levels was 5.5%, 6.1% and 3.3%. Removal of gravity flow and evaporation step made this method faster and easy for routine analysis of ribavirin samples.

Conclusion: The newly developed HPLC method was faster, accurate and sensitive. It applied for determining serum ribavirin level in hepatitis C patient in our hospital.

Keywords: Ribavirin; HPLC; Analysis

Introduction

Ribavirin (RBV) is a guanosine ribonucleoside analog which has spectrum of antiviral activity against DNA and RNA viruses [1,2]. Chronic hepatitis C virus (HCV) is treated with α-2-interferon along with ribavirin analogs. Combination therapy has greatly improved the rates of biochemical and virological response compared to the patients treated with interferon alone [3-6]. Ribavirin absorbs quickly and maximum plasma concentration achieve within 1.5 hour after oral administration. It slowly releases from kidney and gains steady state concentration after 4 weeks. Ribavirin produces significant side effects like hemolytic anemia and varying biological responses. Therefore, monitoring of ribavirin concentration is important to reduce adverse effects, to drive dose modification and to optimize management of HCV-infected patients receiving combination treatment [7-9].

Analytical methods for the determinations of ribavirin reviewed in 2007 [10]. Ribavirin was analyzed by tandem mass spectrometry in monkey and rat brain [11,12]. Capillary electrophoresis and high performance liquid chromatographic methods with UV detection were used for the determination of ribavirin in human plasma [13,14]. A high performance liquid chromatographic method with solid phase extraction was also reported using phenylboronic acid cartridges for sample clean up followed by evaporation [15]. Extraction of RBV from plasma was performed using a novel method based on ultrafiltration in one step that allows direct injection into the high-performance liquid chromatography without any prior steps of dryness or reconstitution [16]. The ultrafiltration technique is not commonly available in most of the lab and it limits the number of injection to 200-300 per column. The HPLC column gives high pressure after 200 samples with reduced resolution and bad peak shape. After reviewing most of the published methods for the determination of ribavirin in serum sample, we observed that all of these methods either involved the evaporation of purified serum sample after liquid - liquid extraction or after solid phase extraction which consumed time and labor especially when ribavirin extracted with 3% formic acid. After loading serum sample on solid phase cartridges, the washing and elution process involved the gravity flow of solvents which did not work for several samples. The third problem was direct injection of elution solvent into the HPLC column which gave a high solvent front merged with ribavirin peak.

In this study we have developed a faster cleaning method of serum sample by using phenylboronic acid cartridges with vacuum elution of washing and extraction solvent. The final elution solvent (3% formic acid) was directly injected into HPLC system without any evaporation step. The vacuum manifold was used under low vacuum to make extraction process faster without any blockage of cartridges which was observed many times when solvent passed under gravity force from PBA cartridges. A precise and low flow rate was used on 3 µ, 3 mm × 150 mm C_{18} column which completely resolved ribavarin peak with solvent front which was not possible with many published HPLC columns, mobile

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phases and flow rates. There was no single HPLC method available so far which used vacuum manifold for sample cleaning and extraction as well as direction injection of elution solvent into HPLC column. A precise low flow rate was used to completely resolved ribavirin peak with solvent front. We have also evaluated the effect of interferences due lipemic, icteric and hemolysis on assay results of which was not reported in any of published chromatographic methods before. The method is fully validated according to FDA method validation guideline and successfully applied for the pharmacokinetic study and determination of Ribavirin trough level of hepatitis C patient treated with ribavirin along with interferon in National Guard Health Affairs, department of pathology and laboratory medicine, Riyadh, Kingdom of Saudi Arabia. The developed method fully validated by using standard method validation guideline as described below.

Materials and Methods

Reagent and chemicals

Ribavirin (Catalogue # R9644), internal standard (N^6-Methyladenosine 5-monophosphate sodium, Catalogue # M2780), formic acid and sodium dihydrogen phosphate were purchased from Sigma-Aldrich of highest purity. Bond elute phenylboronic acid (PBA, 100 mg, 1 ml) cartridges were obtained from Agilent, HPLC grade methanol and acetonitrile were purchased from Fisher Scientific. HPLC column 150 mm × 3 mm with 5 µm particle size was obtained from chromsystem Germany (Part 38130). Purified water was obtained from Millipore water purification system. Drug free blood samples were provided from our blood bank unit.

Instrumentation

High Performance Liquid Chromatographic system was Waters 2790 separation module which composed of binary pump and an auto-sampler. A tunable dual wavelength 2487 UV detector was used for monitoring both analytes and data was processed by using Waters empower 2 software. Vacuum manifold was obtained from supelcosil for sample preparation. An injection volume of 100 µl was loaded into the HPLC system.

Calibration and quality control preparation

For stock standard solution, accurately weighted 100 mg of ribavarine standard was transferred into 100 ml volumetric flask. The material was dissolved in HPLC grade water by sonication for 5 minutes and filled up to the mark. The stock standard solution (1000 mg/l) was further diluted to prepare working standard solution of 5, 25, 50, 100, 200 and 400 mg/l in water. Six Calibration standards of 0.1, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/l were prepared by spiking 200 µl of each of these working standard solutions in 10 ml of ribavirin drug free serum in six different 15 ml glass tubes. The six levels of spiked serum calibration standards were mixed for 30 minutes, aliquoted into 500 µl and stored at -20°C until used. Stock standard solution (1000 mg/l) for quality control preparation was prepared by separately weighing about 100 mg of ribavirin and dissolved into 100 mL of HPLC grade water by 5 minutes sonication. Working quality control solutions of 25, 125, 250 mg/l were prepared in water by diluting stock solution. Three quality controls in serum samples (0.5, 2.5, 5.0 mg/l) were made by spiking 200 µl of each working quality controls solutions into 10 mL of ribavirin free serum sample separately. The three level of spiked serum quality controls were mixed, aliquoted into 500 µl and stored at -20°C until used.

Internal standard and reagent preparation

For stock internal standard solution, 50 mg of internal standard were accurately weighted and transferred into 100 ml volumetric flask. The content of flask was dissolved in HPLC grade water and made up to the mark. The working internal standard solution was prepared by transferring 10 mL of stock internal standard solution into 100 ml flask and volume was made up to the mark with HPLC grade water to get the final concentration of 50 mg/l. The working standard solution was aliquoted into 5 ml and stored at -40°C until used. Mobile phase A was made with 20mM buffer of KH_2PO_4 adjusted to the pH 3.0 with orthophosphoric acid and filtered with 0.45 µm. Mobile phase B was made by mixing 90% of HPLC grade acetonitril with 10% of HPLC grade water. Precipitation and extraction reagents were 20 mM ammonium acetate (pH 8.5 with ammonia) and 3% formic acid in HPLC grade water respectively.

Extraction procedure

The extraction of sample was performed by transferring 500 µl of ribavirin free serum, six level calibration standards, three levels quality controls and patient samples into separate 1.5 ml eppendorf centrifuged tubes. 50 µl of working internal standard and 500 mL of ammonium acetated were added in each tube, vortexed for 5 min and centrifuged at 10000 RPM for 5 min. The supernatants were loaded into phenylboronic acid cartridges (PBA) which were previously washed with 1 ml methanol followed by 1 ml of 20mM ammonium acetate (pH 8.5) by using vacuum manifold at the vacuum not exceeding 10 mm Hg. The SPS cartridges were drained by vacuum followed by washing with 1 ml of 20 mM ammonium acetate (pH 8.5) three times. After third washing, all the cartridges were dried under vacuum for 5 minutes. The ribavarin and internal standard were eluted from the cartridges with 300 µl of 3% formic acid at a vacuum not exceeding 10 mm Hg. The eluents were transferred into HPLC vials and 100 µl injected into HPLC systems.

HPLC analysis

A 100 µl of blank, calibration, quality controls and patient samples were injected into C_{18} HPLC column (150 mm × 3.0 mm, 3 µm, Chromsystem Germany) where ribavirin and internal standard were separated using mobile phase A (20mM buffer of KH_2PO_4 adjusted to the pH 3.0 with orthophosphoric acid) and mobile phase B (90% of HPLC grade acetonitril with 10% of HPLC grade water). The gradient elution was started with 0.5 ml/min of 97% mobile phase A and 3% of mobile phase B for first 10 minutes and then changed into 50% mobile phase A and B at for next 5 minute. The initial conditions were turned on for 5 min before the next sample injection. The tunable UV detector was operated at 230 nm to monitor ribavirin and changed to 262 nm after 15 min to monitor internal standard. The linear equations for the relationship between the peak areas of ribavirin and their concentration were determined by least-squares method using internal standard quantification mode of calculation. Quality controls and patient samples were quantified by six point calibration standard curve based on peak area toward concentration in serum sample. Chromatogram of blank without internal standard addition was free from all interferences at the typical retention time of ribavirin and internal standard.

Results and Discussion

Method development

Bond elut phenylboronic acid (PBA) cartridges were used because its boronat group had strong affinity to cis-diol containing compounds like ribavarin. The internal standard N^6-Methyadenosine 5-monophosphate was selected to have similar cis-diol group which provided better recovery. The UV detection was performed at 230 nm and 262 nm which were the wavelength of maximum absorbance of ribavarin and internal standard respectively. The saturation and

washing of PBA cartridges were carried out as described in the general protocol of extraction method from Agilent. Both ribavarin and internal standard were eluted with 300 µl of 3% formic acid without any organic solvent which saved cost. The final elution solvent (3% formic acid) was directly injected on five different HPLC columns (supelcosil C_{18} 150 mm × 2.1 m, 3 µm, Hypersil BDS C_{18} 150 mm × 2.1 m × 3 µm, Atlantic T3 150 mm × 4.6 m × 3 µm, Zorbax C_{18} 250 mm × 4.6 m × 5 µm and Chromsystem C_{18} HPLC column 150 mm × 3 mm × 3 µm) with different mobile phase of different composition and flow rate. Ribavarin and internal standard were well resolved from solvent front only on Chromsystem C_{18} HPLC column (150 mm × 3 mm, 3 µm). The low flow rate was selected in order to have well separated peak of ribavarin from solvent front and to overcome the back pressure problem after 500-600 injections. An aliquot of 100 µl was loaded on HPLC column in order to have better signal to noise ratio at low concentration of ribavarin which was very useful if low sample volume (250 µl) was available for some patients. Waters tunable absorbance detector provided a facility to detect both molecules on two different wavelength and different retention time in a same injection.

Specificity and selectivity

Six different pool of serum samples were collected from different sources and analyzed by the assay procedure in duplicate with and without adding internal standard. The chromatograms of these blank samples showed no interfering peak from other component of blood at the retention time of ribavirin and internal standard. These blank samples were analyzed to demonstrate that the developed method was selective and specific for the analysis of ribavirin in serum samples. Chromatograms of a blank sample, serum calibration standard and a patient sample are shown in Figures 1-3 respectively.

Linearity and range

Six different amount of ribavirin was spiked in drug free serum ranging from 0.1-8.0 mg/l. After thorough mixing, these calibration

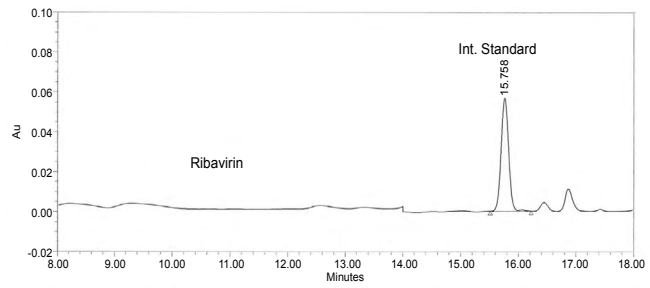

Figure 1: Chromatogram of Blank Plasma Sample containing Internal Standard only.

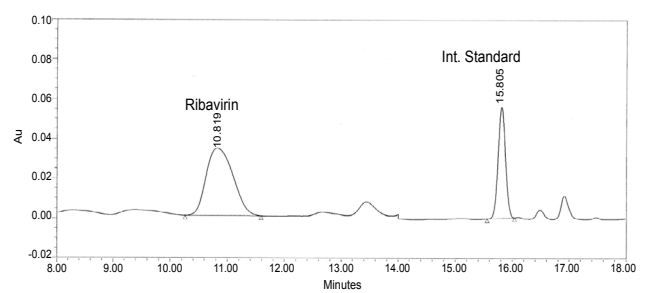

Figure 2: Chromatogram of plasma calibration standard containing ribavirin (4.0 mg/L).

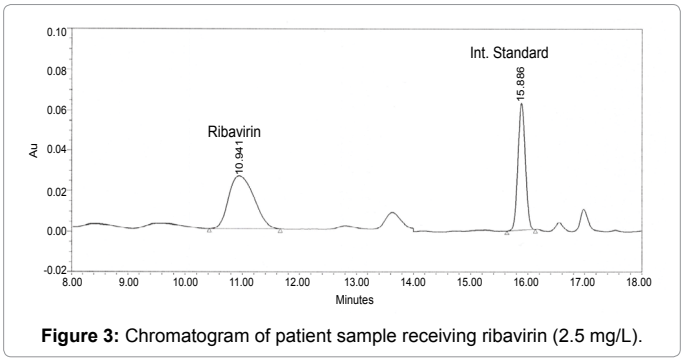

Figure 3: Chromatogram of patient sample receiving ribavirin (2.5 mg/L).

serums samples were extracted and analyzed in duplicate by the assay procedure. A linear calibration curve was obtained by plotting the peak area ratio of ribavirin to the peak area of internal standard against the corresponding six concentrations. Linear regression analysis was performed and results were calculated (Table 1).

Limit of detection and lower limit of quantification (LOD and LLOQ)

A serum sample of 0.05 mg/l and 0.1 mg/l were prepared by spiking ribavirin standard solution into drug free serum samples and analyzed 10 times by the assay procedure. The recovery and precision of the results were calculated and it was found that limit of detection was 0.05 mg/l whereas lower limit of quantification was 0.1 mg/l Table 1 according to FDA method validation guideline.

Accuracy and precision

Known amount of ribavirin at three different concentrations 0.5 mg/l, 2.5 mg/l and 5.0 mg/l levels were spiked in drug free serum samples and analyzed 5 times on two different days by newly developed HPLC method. Mean accuracy of 110.1%, 108.3% and 107.7% were obtained on 0.5 mg/l, 2.5 mg/l and 5.0 mg/l respectively. The intra assay precision determined at 0.5, 2.5, and 5.0 mg/l were found to be 2.2%, 5.0% and 4.5% respectively. The injection reproducibility were found to be 5.5 %, 6.1% and 3.3% at three levels calculated from the results of 5 replicate injections of extracted quality control samples on same day (Table 1).

Comparison and extraction efficiency

In an experiment, about 40 patient sample of varying ribavirin concentration were analyzed on different days by developed HPLC-UV method and results were compared to tandem mass spectrometry method. Statistical analysis was performed by comparing the mean values of both methods and bias. No significant bias and differences in patient results was found as indicated from the statistical analysis of two mean values (Table 1).

Extraction efficiency of the method was checked by preparing six calibration standards in serum sample followed by extraction procedure. Six calibration standards were also prepared in water and analyzed by the assay procedure without extraction. The experiment was performed twice on two different days. The mean recovery of ribavirin and internal standard, calculated from the speak area of ribavirin and internal standard in both serum (with extraction) and water sample (without extraction), were 65.5 and 71.2% respectively (Table 1).

Ruggedness and interference study

The developed method was used for the analysis of ribavirin patient samples in routine laboratory. Therefore the ruggedness was also checked in order to see the effect on retention time from day to day

Linearity	**Range (mg/L)**		**Slope**		**Intercept**	**R^2**
	0.1 - 8.0		0.310		-.006	0.999
Sensitivity	**LOD (mg/L)**	**% Recovery**	**% CV**	**LLOQ (mg/L)**	**% Recovery**	**% CV**
	0.05	122	19.2	0.1	110	5.5
Accuracy (N=10)	**Level 1 (0.5 mg/L)**		**Level 2 (2.5 mg/L)**		**Level 3 (5.0 mg/L)**	
	110.1%		108.3%		107.3%	
Precision (N=10) % CV	**Level 1 (0.5 mg/L)**		**Level 2 (2.5 mg/L)**		**Level 3 (5.0 mg/L)**	
	2.2		5.0		4.5	
Reproducibility (N=10) CV	**Level 1 (0.5 mg/L)**		**Level 2 (2.5 mg/L)**		**Level 3 (5.0 mg/L)**	
	5.5		6.1		3.3	
Comparison	**Slope**	**Intercept**	**Bias**	**p Value**	**R^2**	
	0.953	0.056	-0.03	0.116	0.975	
Extraction Efficiency	**% Recovery of Ribavirin**		**% Recovery of Internal Standard**			
	65.5		71.2			
System Suitability Test Evaluation						
Components	**Retention Time (Min)**	**Theoretical Plates**	**Tailing Factors**	**Resolution**		
Ribavirin	19.614	29160	1.2	33		
Internal Standard	32.708	17294	1.1			

Table 1: Validation data for Linearity, Accuracy, Precision, Comparison, and Extraction, Efficiency and System Suitability.

use. To determine the ruggedness of the chromatographic methodology developed, experimental conditions were purposely altered and chromatographic characteristics were evaluated. The pH of the mobile phase was adjusted to 2.5 and 3.5. The normal pH for the method was 3.0. Similarly the concentration of elution reagent (3% formic acid) and mobile phase composition were slightly changed and effects on retention time and results were monitored. In all these changes the percent variation in retention time was less than 5.0% for both ribavarin and internal standard with no significant affect in the results of quality control and patient samples.

For checking the effect of Billirubin, Hemolysis and Lipemia, a pool of patient sample was spiked with increasing amount of billirubine, heymolysat and lipid 0 µl, 10 µl, 20 µl, 30 µl and 40 µl and analyzed in duplicate. There is no significant difference in results was found as shown in Figure 4.

System suitability

The peaks of ribavarin and internal standards were well separated from each other and from other adjacent peaks whereas theoretical plates and tailing factors for both analytes were calculate by Empower software and summarized in Table 1.

Stability studies

Three patient samples were initial analyzed and then stored at 4°C and -20°C. After 5 day and 15 day, these samples were again analyzed by the assay procedure with freshly prepared calibration standard in order to assess the stability. The calculated recovery showed that the patient samples were stable at both temperature tested up to 15 days (Table 2).

Application

The developed HPLC method was successfully applied for serum ribavirin baseline pharmacokinetic profile and monitoring of trough ribavirin concentration from HCV patient treated with combination therapy after 4, 8, 12 and 24 weeks.

Conclusion

In conclusion, the present analytical method development provides a simple, faster and robust analysis of ribavirin in human serum sample which could be used for the monitoring of ribavirin concentration in patient with HCV infection in order to enhance the effectiveness of interferon therapy with ribavirin, optimized therapy and reduce adverse events particularly ribavirin related anemia. The present method offers advantages of speediness, simplicity, sensitivity and accuracy, and is applicable to the selective determination of ribavirin with shorter and faster extraction using vacuum and without evaporation step. This method has also evaluated for the most common interferences due to lipemic, icteric and hemolysis of serum samples and found to be free from the effects.

Days	**At 4°C**			**At -20°C**		
	Patient 1 (mg/L)	**Patient 2 (mg/L)**	**Patient 3 (mg/L)**	**Patient 1 (mg/L)**	**Patient 2 (mg/L)**	**Patient 3 (mg/L)**
Initial	0.469	1.258	2.622	0.469	1.258	2.622
5 Days	0.479	1.195	2.479	0.497	1.127	2.393
15 Days	0.485	1.200	2.275	0.426	1.273	2.532

Table 2: Stability Studies.

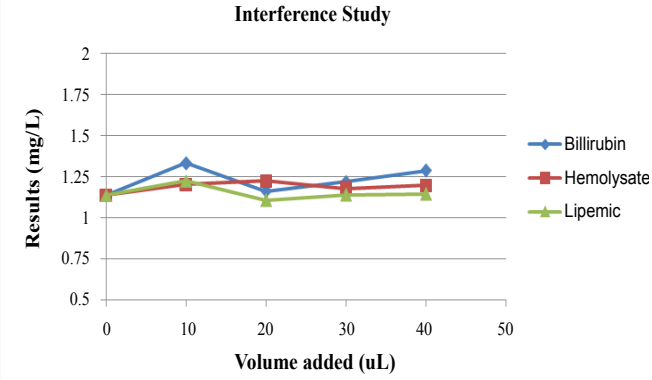

Figure 4: Inference Study.

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