International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

RP-HPLC-UV method validation for Ribavirin used in topical applications

Muhammad Hamza[1,2], Qudsia Kanwal[1], Ahmad Raza[3], Komal Zehra[3], Muhammad Yasir[1*], Dur e Najaf Iqbal[1], Nuzhat Jamil[4], Muhammad Muddassar Afzal[5], Mudasir Majeed[6], Muhammad Zubair Kamran[1]*

[1]*Department of Chemistry, The University of Lahore, Lahore, Pakistan*

[2]*Additive Manufacturing Institute, Shenzhen University, Shenzhen, China*

[3]*Department of Chemistry, Superior University, 17Km Raiwind Road, Kot Araian, Lahore, Pakistan*

[4]*Nanotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan*

[5]*Department of Chemistry, University of Education, Lahore, Faisalabad Campus, Pakistan*

[6]*Department of Applied Chemistry, Government College University, Faisalabad, Pakistan*

Abstract

In this research paper, accurate, cost effective and a straightforward reverse phase- High Performance Liquid Chromatography process was established and verified for the estimation of Ribavirin in topical dosage form. Chromatography separation technique was done on USP L17 (4.6mm×15cm) column possessing particles size of 5µm with liquid mobile phase consisting of sulfuric acid to a pH of 2.5±0.1 having column temperature 65±0.5ºC. Flow rate was kept 1mL per min. Estimation of ribavirin was done at 207nm with the help of UV-detector. Linearity was performed for Ribavirin and calibration curve was obtained which is linear for which is well within the limit. The method is developed & verified for accuracy, precision, linearity, range, specificity, and limit of detection (LOD), limit of quantization (LOQ), ruggedness & robustness. A simple, green, cost & time effective, robust and precise HPLC procedure for estimation of ribavirin was established. This procedure was then verified according to ICH guidelines Q2 (R1) Validation of Analytical methods. Ribavirin was determined accurately and precisely in topical dosage form. Overall this method was developed and performed for the determination of ribavirin amount in any dosage form within 2 minutes in a precise and accurate way.

Keywords: RP-HPLC-UV, Method validation, Isocratic elution, Ribavirin, Dermatitis.

Full length article **Qudsia Kanwal*, e-mail: qudsia.kanwal@chem.uol.edu.pk; *Muhammad Yasir*, e-mail: muhammad.yasir@chem.uol.edu.pk
Doi # https://doi.org/10.62877/4-IJCBS-24-26-20-4

1. Introduction

In 1972, ribavirin was artificially synthesis by Joseph and Roland [1]. They reported it as an active medicine to a number of ribonucleic acid and deoxyribonucleic acid viruses [2]. Ribavirin has another name tribavirin. IUPAC Name of RBV is 1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide. RBV used against hepatitis C, RSV inflammation and some viral hemorrhagic fevers. Sofosbuvir, Simeprevir, Peginterferon alfa-2a or Peginterferon alfa-2b used along RBV for the medication of hepatitis C [3-4]. Ribavirin guanosine analogue produces broad activity against several DNA and RNA viruses. Ribavirin is approved only for the treatment of (RSV) that causes inflammation in children, it has been used for the medication of (LSV) infection [5-7], influenza A and B [8] and other viruses. Ribavirin was studied only for the treatment of HCV [9], in the early of 1990s. [10]. Ribavirin along pegylated interferon alfa [11-14], used for treatment of severe inflammations likes hepatitis B and HIV [13-15-16]. Ribavirin along other medicines may improve its efficiency in the treatment of hepatitis C. Ribavirin is only medicine used for treatment of a numerous hemorrhagic fevers caused by viruses, that includes Crimean-Congo hemorrhagic fever, Hantavirus infection, Lassa fever and Venezuelan hemorrhagic fever [17-19]. The USAMRIID stated that "RBV has no effect in vivo and in vitro activity in the case of Filo-viruses (Ebola

virus [20] and Marburg viruses), Flavi-viruses (Dengue, Omsk hemorrhagic fever, Kyasanur forest disease, Yellow fever,)"[21] for the medication of rabies, ribavirin along amantadine, ketamine and midazolam introduced as an effective vaccine [22].

Investigational results show that RBV have powerful results for the medication of canine distemper viruses and pox-viruses [23-24]. Herpes simplex virus can be treated with the help of ribavirin. Ribavirin single or in conjugation along FLC has potent antifungal activity *in vivo* and *in vitro*. Action mechanism of ribavirin is incredible. Feeling tired, fever, headache, irritable mood, muscle pains and nausea are common side effects. Allergic reactions, liver problems and red blood cell breakdown are serious side effects. Usage of ribavirin during pregnancy time is harmful for children. Effectual contraception for at least 7 months during and after use is advised for both parents both men's and women's. [4] The medication has two "black box" cautions from The US FDA: One up lifts issues that medication through ribavirin prior to or in the time of pregnancy by male or female cause's defects in new born children, the second one is related to the threat of breakdown of RBCs. [25] Ribavirin is not used along Zidovudine as a result risk of anemia increased; [25]synchronous use with Didanosine should be avoided as a result risk of mitochondrial toxicity increased [26]. Literature review express that a lot of publications for the estimation of Ribavirin single or with other Active pharmaceutical ingredients APIs [27-30] with the help of numerous spectrophotometric methods e.g. mass spectrophotometer and thin layer chromatographic (TLC) method [30-38], In the Egypt, they have started a national program against obliteration of HCV main objective was to medicate 300,000 hepatitis C patients with in a year. In this analytical procedure three co-direction medicines for the medication of HCV; DAC, RBV and SF were present in human plasma and simultaneously determined and verified, easy and accurate RP-HPLC process with the help of an I.S. propyl paraben. Liquid to liquid extraction with the help of $CH_3COOC_2H_5$ was carried out for separation of samples. Extraction was carried out by using chromatograph of Scharlau column C18 (250 mm2 × 4.6 mm2, 5µm). Liquid mobile phase consists of acetonitrile and water, gradient elution was achieved at the rate of flow 1mLmin-1. Ultra violet rays analysis was done with the help of photodiode detector, and results were found as 207nm for ribavirin (RBV), 260nm for Sofosbuvir (SOF) and 312nm for Daclatasvir (DAC). This analytical procedure for method validation was carried out related to FDA rules and regulations for bio-analytical verification of procedure. Calibration curves were found to be straight from the limit of 0.5µgmL-1 to 80µgmL-1, 0.1µgmL-1 to 40µgmL-1 and 0.5µgmL-1 to 80µgmL-1) having average recoveries (100.64% to 108.28%, 98.48% to 105.91% and 97.68% to 101.38%) for Ribavirin, Sofosbuvir and Daclatasvir. The intra and inter day accuracy and precision calculations were found to be within the sustainable values. Then stability was disclosed for these examined drugs those drugs were found to be stable in the process of sample formation and sampling, its formulation and injection of samples. This analytical procedure can applied for routine HCV patient's analysis of plasma for their treatment by using this combined therapy that may be added in therapeutic monitoring of drug and follow-up of patients in Egypt and around the world that are fighting against the HCV.

So that, this was really important to establish an accurate, fast, selective and single run time procedure of RP-HPLC, for estimation and analysis the quantity of Ribavirin in pharmaceutical drugs which leads to time saving and cost effective process for laboratory analysis. This research report express that it was an accurate, simple, time saving, selective and cost effective Reversed phase-High Pressure Liquid Chromatography procedure for the determination of Ribavirin quality and quantity through isocratic elution with the help of UV-detector, just in 3 minutes by single run exhibits good percentage of recovery and precision.

2. Materials and methods

2.1. Chemicals

Ribavirin (98% purified) was purchased from Shrooq Pharmaceutical Pvt. Ltd. Sulphuric acid and Acetonitrile (HPLC grade) were purchased from Merck pharmaceutical company. Highly Purified distilled water was used.

2.2. Equipments

Shimadzu HPLC Model LC-20 (LC Lab solution software) fitted with manual sample injector, quaternary pump along degasser, thermo-stated column section, UV-detector and Perkin Elmer HPLC Auto Sampler 600 Series fitted with thermo-stated column section, UV-detector, auto sample injector and quaternary pump degasser were utilized in this procedure.

2.3. Chromatographic conditions

HPLC scanning was done by using Agilent Technologies® USP L17 column (150x4.6mm, 5µm) possessing column section temperature at 65ºC. An isocratic elution was done with mobile phase consisting of Sulphuric acid at pH 2.5 at 1mL/mim flow rate. UV detector was set at λ_{max} 207nm while injecting rate is 20µL for sample and standard solutions in every run. In this HPLC analysis the retention time for Ribavirin was found about 3.47 minutes.

2.4. Standard Solution Preparation

Accurately weight 20mg of ribavirin RS in volumetric flask of 0.1L and add 0.05L diluent which is used as a mobile phase, Shake it for 15 minutes and then fill the volumetric flask with mobile phase up to 0.1L.

2.5. Preparation of Sample Solution

For the preparation of samples, took 15 mg, 20 mg and 25 mg of Ribavirin in volumetric flask of 0.1L and add 60ml of diluent, shake for 15 minutes to prepare homogeneous mixture, and after that fill volumetric flask with liquid mobile phase up to 0.1L. After that filtrate the resulting solution with the help of Whatman filter paper of 0.5µm pore size.The final concentration of ribavirin was obtained 75 %, 100 % and 125 % respectively.

2.6. Chromatographic Conditions

Mobile phase consist of water and sulphuric acid having pH 2.5±0.1 is filtered from 0.5 µm pore size filter paper and degassed with the help of ultrasonic bath for 15 minutes. Chromatographic separation was achieved on USP L17 (4.6mm×15cm) column possessing 5µm particles size, 1.0 mL/min flow rate, 20 µL injection volume, and column

was kept at 65±0.5ºC temperature. UV analysis was carried out at 207 nm.

3. Results and Discussions

3.1. Optimization of chromatographic conditions

For advancement of active and specific liquid chromatographic process for the estimation of the Ribavirin various mixture combinations of selective column and mobile phases were applied for maximum separation. After examining the solubility and molecular structure of medicine, analytical procedure establishment was initiated based on hydrophobic interactions and molecular polarity of ribavirin. On these basis, mobile phase mixture (water and sulphuric acid) having pH 2.5 & C18 Column was selected in start. The procedure establishment was achieved at rate of flow 1.0mLmin^{-1} and the temperature of column was 65°C.At initial stage, a mobile phase is consist of sulphuric acid aqueous solution was used with the help of 20µL volume's injection having concentration of 0.2mgmL^{-1} of ribavirin sample. Current procedure of chromatic technique acceptability and appropriate was found to be achieved according to FDA requirements. Achieving the optimization condition of chromatogram, this procedure was verified to the guidelines of ICH for validation of analytical procedure.

3.2. Method Validation

This analytical procedure was verified according to the guideline of ICH, Q2 (R1) for the verification of analytical parameters and validation procedures are as follow:

3.3. Linearity

During method validation procedure, linearity was estimated by analyzing five different concentrations solution of ribavirin, with concentration 50%, 75%, 100%, 125% & 150% respectively, formed through the stock standard solution and its three duplicates of each concentration solution, were examined based on the guidelines of ICH, Q2 (R1). Correlation coefficient value calculated for Ribavirin was 0.99937. Calibration curve's regression values express relationships between concentrations and peak areas were found satisfactory. Values of linearity for Ribavirin are shown in **Error! Reference source not found.**.

3.4. Accuracy and recovery

Ribavirin accuracy process was achieved through running the samples of 75%, 100% and 125% ribavirin active pharmaceutical drug concentrations, with three different replicated solutions of following concentrations. Values of recovery were achieved through performing the trails of following concentrations three solutions, computing the amount recovered and the amount used in recovery process. Ribavirin values of recovery was found in limit, ranging from 98% to 102% and computing value of standard deviation and relative standard deviation of trails of active pharmaceutical gradient was found within or less then the limit, 2%. The value of ribavirin recovery is shown in **Error! Reference source not found.**.

3.5. Precision/Repeatability

Repeatability of the following procedure was calculated through repeating trails test of six various concentration sample solutions of Ribavirin possessing concentrations respectively i.e. (100µg/mL, 150µg/mL, 200µg/mL, 250µg/mL and 300µg/mL). The results of various concentration repeated trail samples were accurate having less than 2% RSD that predicts following process is repeatable. Precision parameters e.g. SD, RSD and standard error of means were found within the limit's range as shown in **Error! Reference source not found.**. Correspondingly, Intermediate precision was evaluated through analysis of the three various concentration samples, during second day through different instruments by two different analysts and their values of intermediate precision was also found within limit having RSD values less than 2% for all trails results.

3.6. Robustness

Robustness of the following analytical procedure was achieved that explains there is no valuable change in the result whenever alter the conditions of chromatograph, i.e. injection volume (20µL±5µL), flow rate (2.0 mlmin^{-1}±0.5 mlmin^{-1}), detection wavelength (207nm± 2nm) and Colum temperature (65±5 ºC). Trails of ribavirin were carried out through injecting three replicates of test solution in each changed chromatographic condition. The process of robustness expresses stability and the reliability of this analytical procedure. Values of robustness were found within limit, having RSD less than 2% for every single column run that is 0.008%.

3.7. Ruggedness

Ruggedness is a parameter applied for reliability evaluation and its results when exterior factor e.g. analyst, instrument and laboratory are changed. This parameter ruggedness expresses that due to external factor there is not significant variation found in the results, sample solutions of Ribavirin possessing concentrations of 100µg/mL and 300µg/mL, were analyzed at two different instruments (Shimadzu LC 20 and Perkin Elmer Auto sampler HPLC series 600) within two consecutive days. Values of ruggedness were found within limit, having RSD less than 2% for every single column run that is 0.008%.

3.8. Selectivity/Specificity

Intervention from solution, consist of all additives except active pharmaceutical ingredient, and diluent is verified by running their triplicates at the same chromatograph condition at same instrument, along with sample and standard solutions. No peak or interference of diluent and solution found up to the elution time of ribavirin. Chromatograms of diluent and solution are correlated with the results achieved from 100% target sample possessing concentration of 100 µgmL−1. Selectivity/specificity results express that there is no intervention from all additives like inactive materials in the pharmaceutical drug formulation. The mean value of standard error for the trails of ribavirin was 0.34 and value of RSD for the trails of ribavirin was found 0.45% and these results of selectivity/specificity process verified this process.

3.9. LOD and LOQ

Limit of detection and limit of quantification are computed from the slope of linearity curve and standard deviation of the y- intercept. Ribavirin five various concentration solutions are applied to found linearity curve.

Table 1. Summary of linearity data of HPLC method.

Active Material	Conc. (%)	Slope (N=3) Mean±SD	Intercept (N=3) Mean±SD	R^2±SD	R^2± RSD (%)
RBV	50-150	36432.32±229.21	1589165.27±14775.32	0.999±0.003	0.082

Table 2. Summary of accuracy data of HPLC method.

Initial Amount (mg)	Amount Added (mg)	Amount Found (mg)±SD	Average Recovery (%)±SD	RSD (%)	LOD (mg)	LOQ (mg)
20	15	15.16±0.03	101.04±0.26	0.36		
20	20	20.21±0.03	101.09±0.19	0.16	1.57	7.853
20	25	25.27±0.03	101.07±0.22	0.06		

Table 3. Summary of repeatability and intermediate precision data of HPLC method.

	Repeatability		Intermediate Precision Day 1		Intermediate Precision Day 2	
Conc. (N=3) (mg)	Assay (%)	RSD (%)	Assay (%)	RSD (%)	Assay (%)	RSD (%)
15	100.99	0.20	100.99	0.22	101.20	0.23
20	101.03	0.59	101.24	0.16	100.89	0.26
25	100.90	0.20	101.10	0.16	101.12	0.29

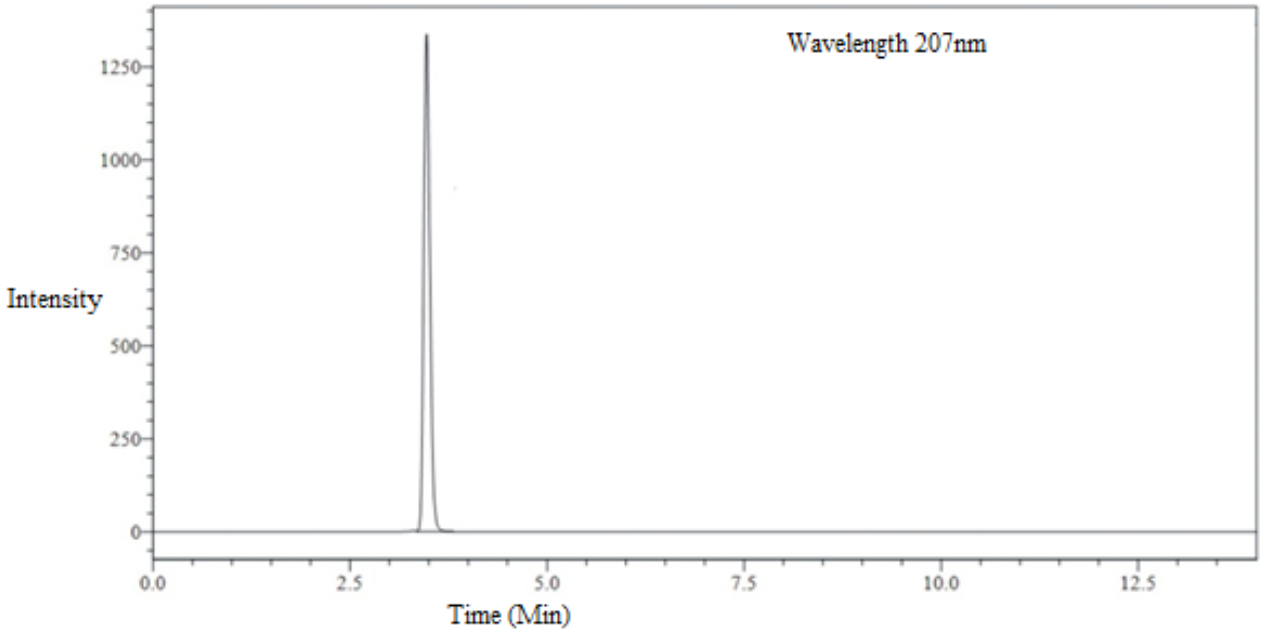

Figure 1: Ribavirin retention time at 207 nm

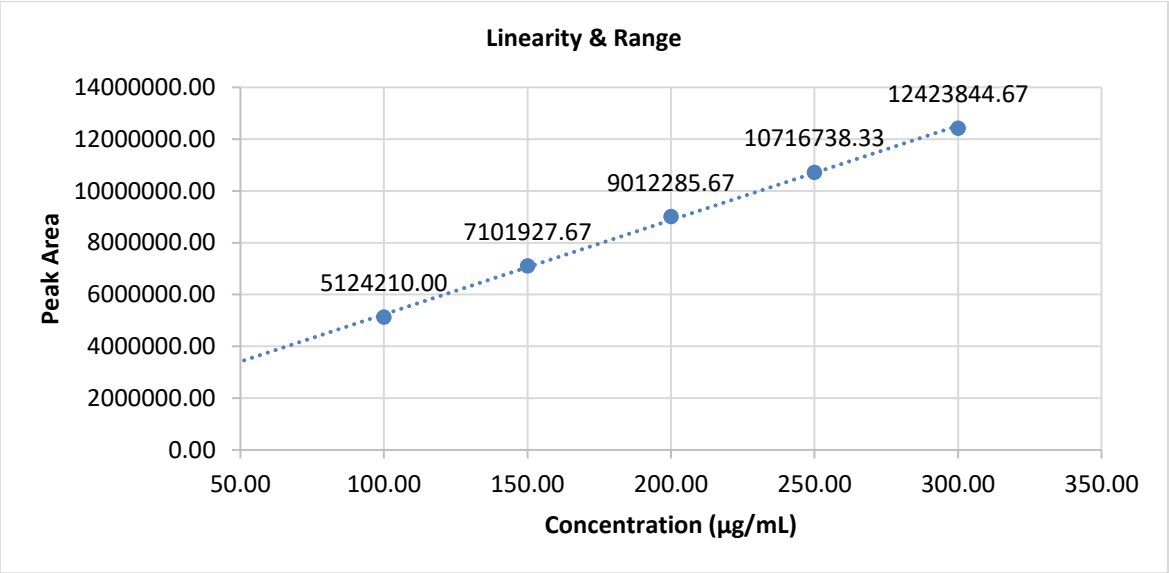

Figure 2: Ribavirin calibration cure at 207nm

Ribavirin various concentration solution ranging from 100% - 300% concentrations are applied. The values of LOD and LOQ for the Ribavirin were found 1.57mg and 7.853mg respectively.

3.10. Solution stability

Solution stability of ribavirin is verified by correlating the freshly prepared sample solution and stored sample solution for 24hrs at room temperature .The results of both freshly prepared and that of stored sample solutions exhibited no considerable change regarding their stability.

3.11. Range

During this analytical method development, the method validation process was analyzed with the help of highest concentrated solution 300% and the lower concentration 50% of sample that is also applied in the determination of accuracy, linearity and precision. Value of range and all parameters expresses that all the parameters of system validation and system suitability were found within limit, according to the guidelines of ICH.

4. Conclusions

The procedure of determination of Ribavirin, in pharmaceutical drug through reverse phase HPLC by isocratic elution, is found to be accurate, delicate, selective and rapid. This process is applicable for routine analysis of drugs in any kind of pharmaceutical (either bulk or finish) and during stability analysis for single run of sample time is less than 5 minutes. This specific process imparts a vital role for development of other pharmaceutical methods for the quantitative analysis of other active pharmaceutical drugs for future application. This process is verified according to the guidelines of ICH and results founds are acceptable, which indicates this process is important addition to very limited previously reported procedure for the determination of Ribavirin by RP-HPLC.

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