SYSTEMATIC DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OFRIBAVIRIN, RITONAVIR AND LOPINAVIR IN SOLID DOSAGE FORM

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Running title: RP-HPLC Method Validation for the Simultaneous Estimation of Ribavirin, Ritonavir andLopinavir

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Abstract: To perform batch release testing and to conduct stability studies of Ribavirin, Ritonavir and Lopinavir in pharmaceutical solid products, a stability-indicating analytical method is required to separate the active pharmaceutical ingredient peak from the peaks of all potential degradation products, process related impurities, potential packaging leachable, excipients, and also separate these compounds from each other. The main objective of the present study was to develop and validate simple, precise, sensitive and accurate RP-HPLC method for the simultaneous estimation ofRibavirin, Ritonavir and Lopinavir in solid dosage form. Successful separation of Ribavirin, Ritonavir and Lopinavir was achieved on CromosilC18 column (5 µm 250 × 4 mm) with isocratic elution ofMethanol:0.1 % Orthophosphoric acid84:16 (v/v) as a mobile phase. The Ultraviolet detection was monitored at a wavelength of 223 nm at flow rate 0.8 mL/min. The validation of proposed method was carried for linearity, precision, accuracy and robustness were determined in accordance with ICH guidelines. The method has good specificity and specified impurities can be effectively separated with good resolution.

The proposed method is found to have linearity in the 40–200 µg/mLfor Ribavirin and Lopinavir then 10–50 µg/mL for Ritonavirwith correlation coefficients of not less than 0.999 respectively.All method validation criteria were within the range of acceptance. Relative standard deviation (%RSD) was observed to be <2% for inter- and intra-day precision.Besides, the recovery rate was observed close to 100% for both the drugs confirming the accuracy of the method. Minor alterations in the chromatographic conditions have revealed robustness and ruggedness of the developed method.The method successfully estimated the Ribavirin, Ritonavir and Lopinavir in formulation tablet. The proposed method can be applied for quality control assay of Ribavirin, Ritonavir and Lopinavir, with the advantages of simplicity, accuracy, robustness, good selectivity, and high sensitivity.

Keywords: Ribavirin, Ritonavir and Lopinavir, Assay, RP-HPLC, Validation.

DOI: 10.53555/ecb/2022.11.12.284

Introduction:

Ribavirin: Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), is a synthetic purine nucleoside analog with a broad spectrum of antiviral activity. Different mechanisms of RBV action have been proposed, such as inositol monophosphate dehydrogenase inhibition, mutagenesis and direct inhibition of the RNA-dependent RNA polymerase. Ribavirin is used for a variety of viral hemorrhagic fevers like Lassa Lassa fever, Crimean-Congo hemorrhagic fever Crimean-Congo hemorrhagic fever, Venezuelan hemorrhagic fever, hantavirus infection chronic hepatitis C respiratory syncytial virus [1-3].

Ritonavir: The chemical name of Ritonavir is 1,3-thiazol-5-ylmethyl N-[(2S, 3S, 5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2- (propan-2-yl)-1,3-thiazol 4yl]methyl}) carbamoyl] amino} butanamido]- 1,6 - diphenylhexan-2-yl] carbamate. Ritonavir is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature noninfectious viral particles. Protease inhibitors are almost always used in combination with at least two other antiHIV drugs [4-7].

Lopinavir: The chemical name of Lopinavir is (2S)-N-[(2S,4S,5S)-5-[2-(2,6dimethylphenoxy) acetamido]-4-hydroxy-1,6- diphenylhexan-2-yl]-3- methyl-2-(2-oxo1,3-diazinanyl) butanamide. Lopinavir inhibits the HIV viral protease enzyme by forming an inhibitor-enzyme complex therapy by preventing the cleavage of the gag-pol polyproteins. Immature, non-infectious viral particles are subsequently produced. Lopinavir is an inhibitor of the HIV1 protease. Ritonavir inhibits the CYP3Amediated metabolism of lopinavir, thereby providing increased plasma levels of lopinavir[7-8].

To perform batch release testing and to conduct stability studies for solid pharmaceutical products, a stability-indicating analytical method is required to separate the active pharmaceutical ingredient (API) peak from the peaks of all potential degradation products, process related impurities, potential packaging leachables, excipients, and also separate these compounds from each other. The finished product release specifications should include a content determination test with acceptance criteria and limits for Ribavirin, Ritonavir and Lopinavir present in the formulation [9-10]. The drug product stability guideline Q1A (R2) issued by the International Conference on Harmonisation (ICH) suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and, hence, supporting the suitability of the proposed analytical procedures. Analytical method validation ensures that various HPLC analytical techniques shall give reliable and repeatable results; it is a crucial step in developing new dosage forms as it provides information about accuracy, linearity, precision, detection, and quantitation limits. According to the ICH guideline, "the objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose." It is now obligatory in the process of drug development to supply the validation data for the responsible authorities. Guidelines for analysis method validation include ICH and USP guidelines [11–14].

In the past, numerous techniques for the simultaneous or single measurement of Ribavirin, Ritonavir and Lopinavir have been developed, which have made use of a variety of equipment, such as the UV spectrophotometer, the high-performance liquid chromatography, the HPLC-MS, and the UPLC [15-17]. In contrast, the current approach, which was developed via RP-HPLC for the determination of Ribavirin, Ritonavir and Lopinavir in dosage forms, was found to be simple, exact, quick, and cost-effective to perform. Although multiple RP-HPLC approaches for measuring Ribavirin, Ritonavir and Lopinavir were discovered in various works of literature, they were found to be complex and time-

consuming. To promote green chemistry, the present research sought to create a new RP-HPLC technique for the measurement of Ribavirin, Ritonavir and Lopinavir in dosage form that was accurate, sensitive, cost-effective, and stability-indicating, while employing the fewest amount of hazardous chemicals possible [18-20].

Material and Methods:

Chemical and reagents:

Dr. Reddy's Laboratories provided a gift sample of Ribavirin, Ritonavir and Lopinavir (99.73%, 99.41% and 100.2% pure) (Hyderabad, India). The HPLC-grade solvents used in this study were obtained from Merck Ltd. in Bangalore, India, including acetonitrile, methanol, Perchloric acid and water. All of the chemicals used were of the highest quality for HPLC.

Instruments and chromatography condition:

Agilent 1100 series HPLC instrument with Quaternary G1311 A pump, COLCOM G1316A thermostat column temperature control, Thermostatic auto sampler G 1329A with sample volume of 0. 1–1500 µL and variable programmable UV detector G 1314 A. The instrument was operated and integrated with Agilent chem. station LC software.

The Ribavirin, Ritonavir and Lopinavir were resolved on a reverse-phase Cromosil C18 column (250 mm× 4 mm, 5 µm). Whereas Mixture of Methanol:0.1 % Orthophosphoric acid 84:16 (v/v) in aisocratic mode used as mobile phase. The selected diluent is mixture of Methanol:0.1 % Orthophosphoric acid 84:16 (v/v). Before the first injection, the column was saturated for 30 min with the initial mobile phase. The temperature was maintained at 25°C. Injection volume was decided to maintain at 20 µL. The PDA was set by optimizing wavelength to give the best response for two peaks at 223 nm to acquire the chromatogram. A software system called Chemstationwas used to collect the chromatographic data for this study. The standard *Ribavirin, Ritonavir and Lopinavir* were identified by comparing the retention time and spectra obtained from the sample and standard solutions [20-22].

Selection of lambda max:

Lambda max is selected using a UV spectrophotometer, and this decision is critical for the sensitivity of the RP-HPLC technique. By using an optimum wavelength, it is possible to detect an exact absorbance for any medication. In the current investigation, pure Ribavirin, Ritonavir and LopinavirAPIconcentrations of 100 ppm were determined by scanning in the range of 200–400 nm using a high-performance liquid chromatography system (Agilent 1100 system).

Preparation of Standard Stock Solution:

Weigh and transfer accurately about 100 mg of Ribavirin, 25 mg of Ritonavir and 100 mg of Lopinavirworking standard to a 100 mL volumetric flask. Add about 10 mL of methanol, sonicate to dissolve and dilute to volume with methanol.

Preparation of Standard Solution:

Pipette out 3.0 mL of Standard Stock Solution transfer it into 25 mL volumetric flask and dilute to volume with mobile phase and mix well. (Theoretical Concentration: 120 ppm, 30 ppm and 120 ppm of Ribavirin, Ritonavir and Lopinavir)

Preparation of Sample solution:

Weigh and crush 20 tablets to fine powder. Accurately weigh and transfer crushed powder equivalent to 1000mg of Ribavirin, 250mg of Ritonavir and 1000mg of Lopinavir, into 500

mL volumetric flask. Add 300 mL of mobile phase, sonicate for 30 minutes with intermittent shaking. Allow to cool at room temperature and dilute with diluent to volume and mix. Filter the solution through 0.45µm Teflon filter discarding first few mL of filtrate. Pipette out 3 mL of filtrate to 50 mL volumetric flask, dilute with mobile phase to volume and mix.

Method of analysis:

The chromatographic conditions were maintained as previously mentioned, and the baseline stabilisation procedure was carried out for 30 minutes total. Following stabilisation, the repeatability of the Blank and the prepared concentration solution of the standard drug was tested in the respective peak regions of the Blank and the prepared concentration solution of the standard drug. For the purpose of quantification, the solution of the sample was injected. We estimated the response factor based on the standard peak ratio and the sample peak ratio. The same technique was done six times to ensure that the created method was adhering to the established standard of repeatability [23-25].

- **Validation of RP-HPLC method:**

Accuracy:

Accuracy is defined as the degree of agreement between the measured value and the genuine value. The three separate Ribavirin, Ritonavir and Lopinavir standard and sample solutions were obtained from concentrations ranging from 80 to 120 mg in the concentration range. These solutions were injected into the test subjects in order to determine the correctness of our devised procedure. A sample solution was generated for three duplicate concentrations, and the results were quantified in the meanwhile. The percentage of recovery was calculated using the methodology shown below. The recovery rate must be in the range of 98–102 percent in order to be considered satisfactory.

Precision:

Precision may be defined as a measurement of real value between different outcomes of the same amount or quantity range. Analyzing six duplicate concentration solutions from 100 ppm on the same day and three separate days allowed researchers to examine intra-day and inter-day fluctuations in the concentrations. Calculation of the percent relative standard deviation (RSD) was accomplished by the use of the following equation: The normal acceptance restriction for percent RSD acceptance is less than 2 percent of the total.

Linearity:

For the purpose of evaluating the linearity of Ribavirin, Ritonavir and Lopinavir solution, several concentration solutions ranging from *40–200 µg/mL* for Ribavirin and Lopinavir then *10–50 µg/mL* for Ritonavir (40, 64, 72, 80, 88, 96, and 120 ppm) were injected into the test tube. Each concentration solution was examined six times in the column under the identical conditions by injecting it into the column six times. The linearity calibration curve was developed based on the area peak obtained from the chromatogram and each concentration of Ribavirin, Ritonavir and Lopinavir solution used in the experiment. It was possible to calculate the coefficient of correlation (R2) using regression analysis calculations since the slope and intercept values were known. It is recommended that the standard limit of regression analysis be greater than 0.999.

System suitability:

The appropriateness of the system was determined in accordance with the United States Pharmacopeia (USP). To determine the metrics such as column efficiency, resolution, peak symmetry factor, percentage coefficient in peak area or height, it was necessary to employ the prepared concentration solution of Ribavirin, Ritonavir and Lopinavir for six duplicate injections. A percentage RSD, theoretical plate value, tailing factor, and system accuracy were all calculated using the observed value as a starting point [26-28].

Specificity and selectivity:

It was necessary to test the specificity and selectivity of the newly devised approach in order to identify the excipients that were interfering with the estimate of the Ribavirin, Ritonavir and Lopinavir. The blank solution, which did not include the Ribavirin, Ritonavir and Lopinavir, was produced and injected. The chromatogram produced from the blank was compared to the chromatograms obtained from the standard and sample, and the difference was examined to determine if excipients interfered with the drug quantification.

Robustness and ruggedness:

The robustness and ruggedness of the created RP-HPLC technique were proved by modest modifications in the chromatographic conditions that were used in the development. In order to determine if any impacts were induced by the changes in parameters such as column temperature, flow rate, and the usage of various percentage ratios of mobile phase, the parameters were varied. The difference in chromatographic conditions was taken into consideration in the current investigation.

The following modifications to the Chromatographic conditions will be evaluated:

- Change in column Temperature (±5°C)
- Change in wavelength (±5 nm)
- Change in Flow rate (± 0.1 ml\min) 10% change [29-30].

Results and Discussion:

Development of RP-HPLC method:

The development of an RP-HPLC technique for the measurement of Ribavirin, Ritonavir and Lopinavir in dosage form was completed. Mixture of Methanol:0.1 % Orthophosphoric acid 84:16 (v/v) in a isocratic mode used as mobile phase. A variety of chromatographic conditions, including flow rate, column temperature, and the components ratio in the mobile phase used, were tested in order to generate a crisp and symmetric peak with an appropriate retention period. In order to get a superior peak, the column Cromosil C18 column (5 µm 250 × 4 mm) was employed. Because of the mobile phase employed in the procedure, the characteristics of the chromatographic settings such as retention duration, theoretical plate number (N), retention factor, and selectivity could be tailored to meet the needs of the researchers. The determination of wavelength was carried out using anAgilent 1100 system equipped with a photodiode array detector in order to ensure adequate sensitivity of Ribavirin, Ritonavir and Lopinavir using the RP-HPLC technique (PDA). In order to discover the wavelength maxima, the standard solution of Ribavirin, Ritonavir and Lopinavir was scanned throughout a range of 200–400 nm (Figure 1, Figure 2,Figure 3) with a better peak being identified at 223 nm.

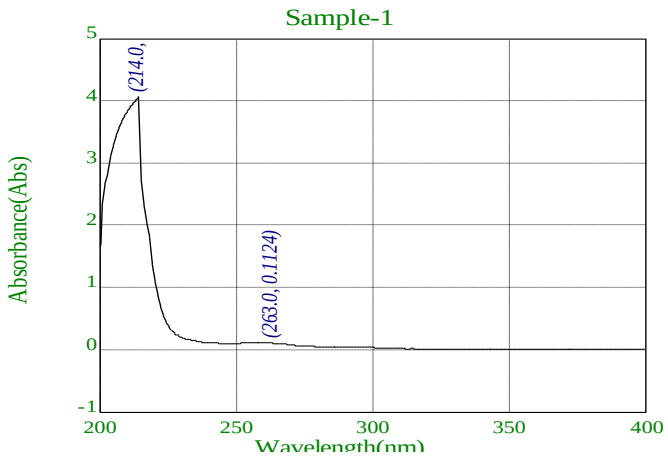

Figure 1: UV spectra of Lopinovir

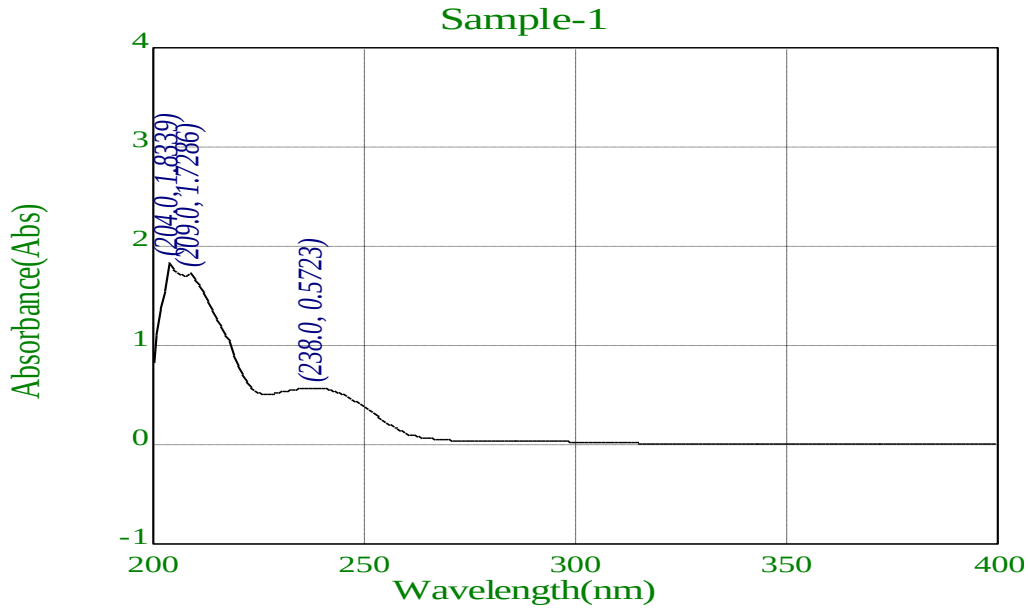

Figure 2:UV spectra of Ritonavir

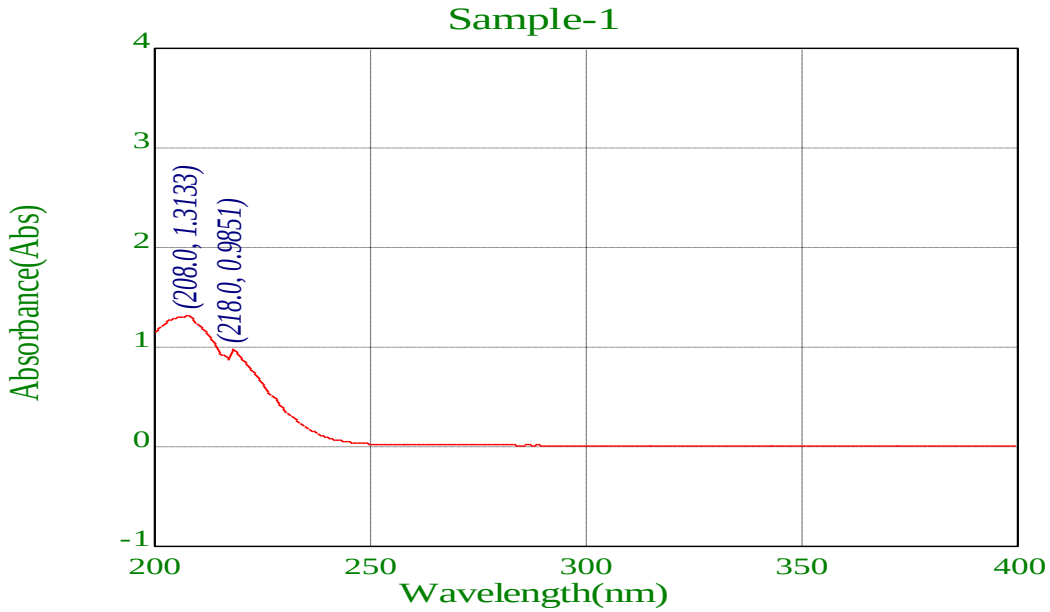

Figure 3:UV spectra of Ribavarin

- **Validation of RP-HPLC method**

Accuracy:

Placebo powder of Ribavirin, Ritonavir and Lopinavirtabletswas spiked with Ribavirin, Ritonavir and Lopinavirdrug Substance at three different levels: 80%, 100% and 120% of the label claim in triplicate (in total nine determinations) and then proceeded with Sample solution as described under. As demonstrated in Table 1, the information received was deemed to be accurate. According to the findings, the percentage recovery of standard and sample Ribavirin, Ritonavir and Lopinavir was near to 100 percent, respectively. It was determined that the acquired result is within the range of typical recovery values (98.0 percent to 102.0 percent).

Table 1: Accuracy studies of developed method

Sample Name	Lopinavir	Ribavarin	Ritonavir
	% Recovery		
Acurracy 80%_Sample 1	97.83	97.06	100.70
Acurracy 80%_Sample 2	97.37	97.61	101.10
Acurracy 80%_Sample 3	98.21	98.24	100.80
Acurracy 100%_Sample 1	100.26	100.03	102.00
Acurracy 100%_Sample 2	99.16	100.40	101.10
Acurracy 100%_Sample 3	99.78	99.01	101.70
Acurracy 120%_Sample 1	102.40	101.75	101.20
Acurracy 120%_Sample 2	101.95	101.92	101.60
Acurracy 120%_Sample 3	101.21	100.21	101.00
Average	**99.80**	**99.58**	**101.24**
Overall RSD	**1.82**	**1.73**	**0.43**

Precision:

In this research, precision was examined in terms of system precision, technique accuracy, and intermediate accuracy. Using six duplicate injections of the same standard from the same vial, the accuracy of the system was measured and quantified in terms of percent relative standard deviation (percent RSD), tailing, plate count, and resolution. The sample was subjected to the above-mentioned method a total of six times. The percent assay for each analyte was represented as a percentage of the standard deviation (percent RSD). Intermediate precision was performed on two different systems, one using a Waters e2695 Alliance system with a 2996 PDA and the other using a 2489 ultraviolet (UV) detector, by different analysts by analyzing six different samples of extract, and the results were expressed as a percent relative standard deviation. The results of this investigation demonstrated a more exact and accurate approach for detecting Ribavirin, Ritonavir and Lopinavir in the dose form than previous methods (Table 2).

Table 2: Method Precision and Intermediate precision Results

Sample Name	Lopinavir	Ribavarin	Ritonavir
Method Precision % Assay			
Sample 1	99.60	100.69	98.59
Sample 2	101.78	100.63	100.97
Sample 3	100.61	102.65	102.01
Sample 4	99.86	100.43	100.08
Sample 5	102.08	101.84	102.60
Sample 6	100.86	102.25	99.86
Average	**100.80**	**101.41**	**100.68**
RSD	**0.99**	**0.94**	**1.47**
Intermediate Precision % Assay			

Sample 1	100.16	100.03	100.26
Sample 2	100.23	102.21	100.19
Sample 3	100.20	101.04	102.22
Sample 4	99.90	100.29	100.00
Sample 5	100.42	102.51	101.40
Sample 6	100.32	101.29	101.82
Average	**100.21**	**101.23**	**100.98**
RSD	**0.18**	**0.98**	**0.94**
Overall Average	**100.50**	**101.32**	**100.83**
Overall RSD	**0.74**	**0.92**	**1.19**

Linearity and range:

The linearity calibration curves were found to have an R2 value of 1.0000. The calibration curve that was drawn in the concentration range of 40–200 μg/mL for Ribavirin and Lopinavir then 10–50 μg/mL for Ritonavir (Table 3). The equation and regression coefficient (R2) are presented in Fig. 4, 5, 6. According to the results, the relative standard deviation was in the range of 1.0 to 1. A higher correlation value was discovered between the observation derived from peak value and the concentration of the drug solution than had previously been seen.

A series of Standard preparations of Ribavirin, Ritonavir and Lopinavir were prepared over a range of 50% to 150% of the working concentration of Ribavirin, Ritonavir and Lopinavir. Since the working concentration is 120 ppmof Ribavirin, 30 ppm of Ritonavir and 120 ppm ofLopinavir, the range proposed is about *40–200 μg/mL* for Ribavirin and Lopinavir then *10–50 μg/mL* for Ritonavir.

Table 3: Linearity of Ribavirin, Ritonavir and Lopinavir

% Concentration	Lopinavir	Ribavarin	Ritonavir
35%	756.38	1819.40	217.50
60%	1429.55	3190.27	430.84
100%	2122.14	4750.12	618.46
130%	2826.57	6161.12	837.15
160%	3498.77	7457.97	1033.75
Slope	**17.2**	**35.62**	**20.38**
Intercept	**62.14**	**401.3**	**15.89**
Regression	**0.999**	**0.999**	**0.999**

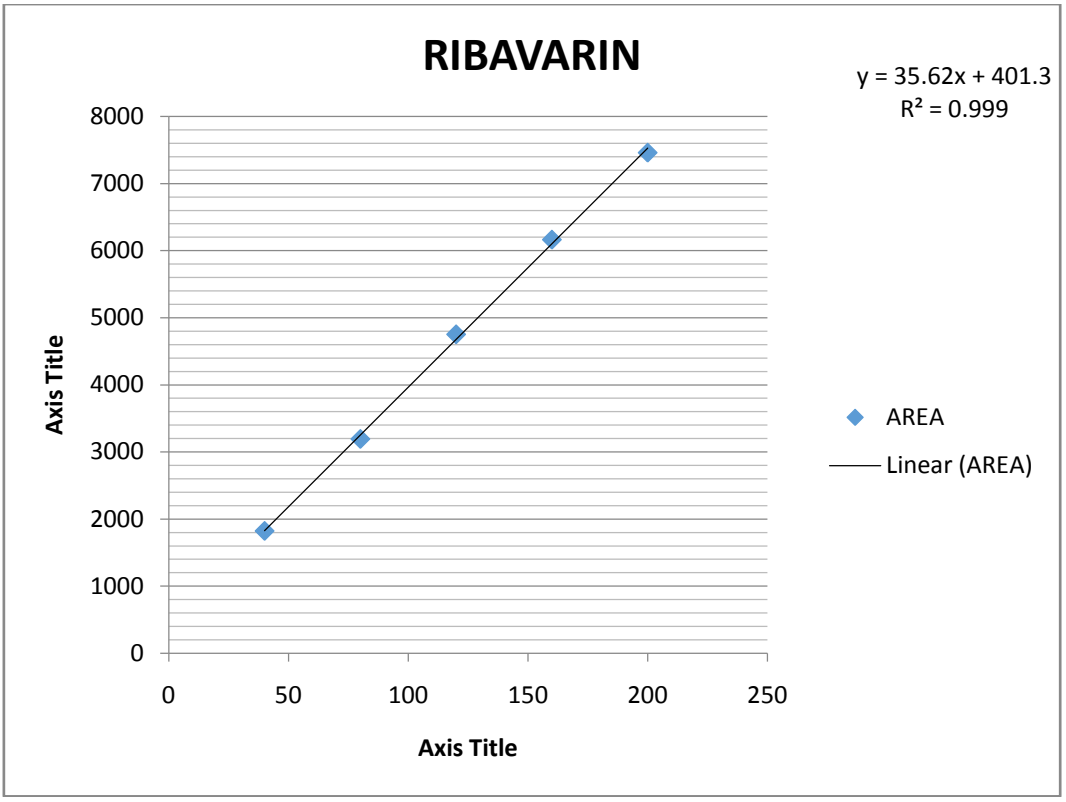

Figure 4:Linearity Graph for Ribavarin

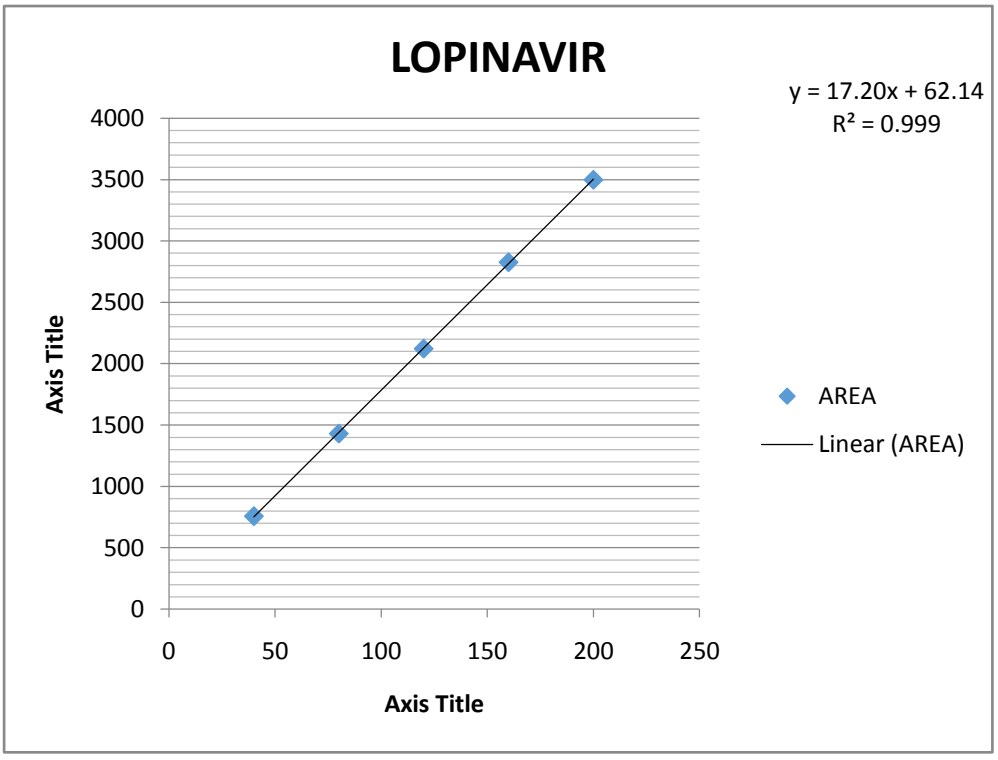

Figure 5: Linearity Graph for Lopinavir

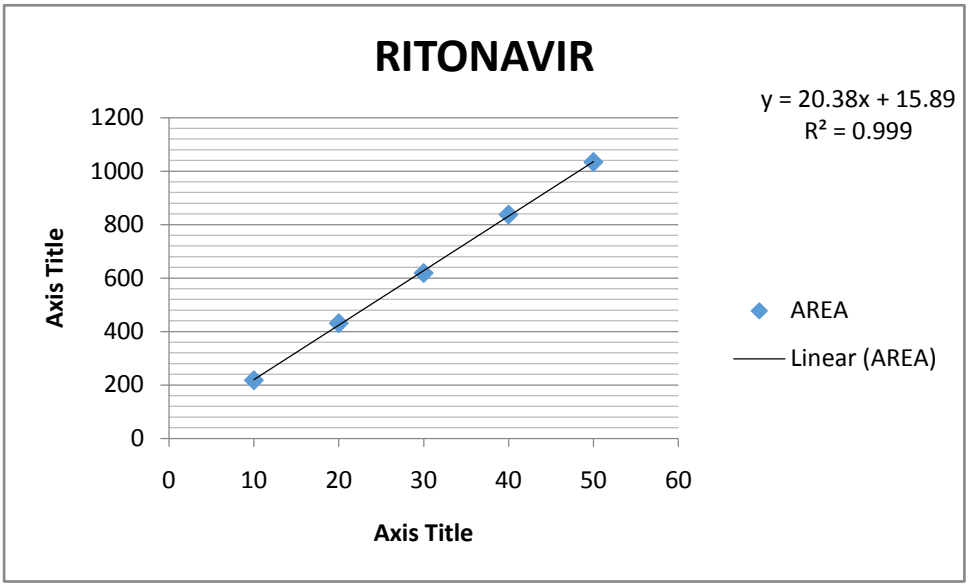

Figure 6: Linearity Graph for Ritonavir

System suitability:
For the system suitability investigations, a standard Ribavirin, Ritonavir and Lopinavir solution with a concentration of 120 ppmof Ribavirin, 30 ppm of Ritonavir and 120 ppm ofLopinavir was used, and the results were evaluated. The time required for separation under chromatographic conditions was determined to be 15 minutes. A variety of frameworks, including the tailing factor, retention duration, theoretical plate number (N), and system accuracy, were determined to be within acceptable limits of 2 percent. The results obtained were within the acceptability requirements specified in the United States Pharmacopeia at the time of testing. Detailed data were displayed in Table 5, which may be seen here.

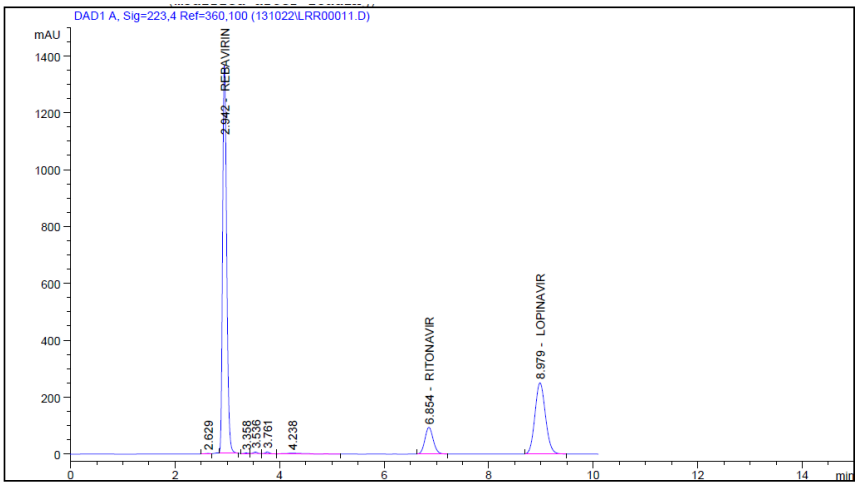

Figure 7: HPLC chromatogram of Developed HPLC Method

Specificity and selectivity:
When comparing the results of the current study to those of past research studies on the same medication, it was discovered that the retention period with readily accessible mobile phase was superior. The approach was quantified accurately and with high resolution. It was not possible to draw any conclusions from the blank sample. A cost-effective mobile phase

consisting of Mixture of Methanol: 0.1 % Orthophosphoric acid 84:16 (v/v) is utilised in this procedure. Figure 3 shows the retention time for standard and sample LCZ concentrations. The retention time for standard and sample Ribavirin, Ritonavir and Lopinavir concentrations was 15 minutes. When compared to other approaches that have been developed, the retention time attained was found to be shorter.

Robustness:

In order to determine the robustness of the currently developed luliconazole RP-HPLC method, minor variations in chromatographic parameters, such as the rate of flow of mobile phase (1.0 ml/min and 1.2 ml/min), different wavelengths (218 and 223), and temperature of the column (20 degrees Celsius and 30 degrees Celsius), were applied. The collected results did not reveal any statistically significant differences in peak area or retention duration. The percentage recovery of Ribavirin, Ritonavir and Lopinavir for the standard solution was almost identical to 99.0 percent, whereas the percentage recovery of Ribavirin, Ritonavir and Lopinavir for the sample solution was nearly identical to 99.2 percent. According to the findings, the percent RSD was less than 2.0 percent under various situations, indicating that the current approach is robust and tough. The values of % RSD as shown in Table 6 indicate better robustness of the method [28-30].

Table 4: Robustness for Luliconazole

Robustness parameter		**% RSD**			**Remark**
		Lopinavir	**Ribavarin**	**Ritonavir**	
Wavelength (nm)	222	0.88	0.48	0.31	Pass
	223	0.36	0.21	0.52	Pass
	224	1.27	0.24	0.84	Pass
Mobile Phase	(83:17)	1.25	1.01	0.91	Pass
	(85:15)	0.36	0.87	0.23	Pass
	(89:21)	1.06	0.43	0.71	Pass
Flow (mL/min)	0.7	0.84	0.72	0.65	Pass
	0.8	0.36	0.65	0.74	Pass
	0.9	0.84	0.32	0.82	Pass

Conclusion:

The RP-HPLC technique was used to produce an accurate, precise, robust, reliable, and repeatable approach for the quantitative measurement of Ribavirin, Ritonavir and Lopinavir in dosage form. The solvent that was utilised as the mobile phase has shown a very high-resolution rate while requiring less retention time. The procedure was carried out in accordance with ICH and FDA rules, and the report received fulfilled all of the required standards. The accuracy, precision, and linearity of the procedure were all evaluated in order to ascertain the quality of the drug content. The RP-HPLC technique presented here enables for the measurement of Ribavirin, Ritonavir and Lopinavir in a repeatable manner. According

to the statistical data, the proposed approach may be effectively used to our regular determination method with success. The specificity report demonstrated that the excipient had no effect on the results. A kinetics investigation using plasma and biological fluids may be included to this research as a further extension. The fact that this innovative approach demonstrated a greater cost-effectiveness ratio when compared to the previously reported studies was a significant finding.

Acknowledgments:
Oriental University, Sanwer Road, Indore, Madhya Pradesh 453555, India, gave financial assistance to the writers.
Authors Funding:
None

Conflicts of interest
No conflicts of interest are declared by the authors.

Authors' contribution:
AkashTambe and Dr. DeshrajChumbhale were involved in the sample selection, the planning and execution of lab research, the interpretation of data, and the writing of the report. Dr. DeshrajChumbhaleefforts include data analysis and chemical identification. The final document was interpreted and approved by each author.

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