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A NEW APPROACH TO DEVELOP A STANDARDIZED METHOD FOR ASSESSMENT OF RIBAVIRIN, RITONAVIR AND LOPINAVIR FROM PHARMACEUTICAL FORMULATION USING HPTLC

Running title: Assessment of Ribavirin, Ritonavir and Lopinavir from Pharmaceutical Formulation Using HPTLC

Akash Tambe[1] and Deshraj Chumbhale*[2]

[1]Research Scholar, Faculty of Pharmacy, Oriental University, Indore, Madhya Pradesh 453555 India
[2]* ResearchSupervisor, Faculty of Pharmacy, Oriental University, Indore, Madhya Pradesh 453555 India
Email ID- atskyworld@gmail.com
***Correspondence:** atskyworld@gmail.com

Abstract: To guarantee quality and appropriate concentrations of active substances for their bio-potency, pharmaceutical formulations should be standardized in terms of the quality of the basic materials utilized, manufacturing techniques, and composition. This study presents the first detailed description of a sensitive, selective, accurate, and reliable HPTLC technique for measuring ribavirin, ritonavir, and Lopinavir concentrations in pharmaceutical preparations. Chromatographic development was performed using a mobile phase of N-Butanol, glacial acetic acid, and water (in that order) at a volumetric concentration of (5:1:2 v/v/v). The plates were precoated with silica gel 60 F254. Densitometric analysis was carried out at a wavelength of 254 nm. About 0.39 ± 0.01, 0.48 ± 0.01 and 0.52 ± 0.01 were determined to be the RF values for Ribavirin, Ritonavir, and Lopinavir, respectively. In compliance with ICH standards, the method's linearity, accuracy, robustness, and other features were verified. This novel, user-friendly, and cost-effective technology is designed to assist enterprises and researchers in the quick and cost-effective routine analysis of ribavirin, ritonavir, and Lopinavir in bulk, preformulation studies, and pharmaceutical formulation.

Keywords: Ribavirin, Ritonavir and Lopinavir, HPTLC, Fingerprint, Formulation

1 Introduction:

High performance thin layer chromatography (HPTLC) is becoming more popular as an analytical method because to its low cost, ability to detect and quantify quantities as small as micrograms, and growing acceptance in the scientific community. The ability to examine numerous samples at once is a distinct benefit of this technology. Much less solvent is required compared to HPLC. As a result, there will be less chance of contamination and less resources spent on analysis. In addition, the chromatogram may be scanned many times using HPTLC, either with the same parameters or with new ones each time. Moreover, a multicomponent formulation may have numerous component tests performed simultaneously [1].

- **Ribavirin:**

The synthetic purine nucleoside analogue ribavirin (1- β- D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is effective against a broad variety of viruses. Mutagenesis, suppression of inositol monophosphate dehydrogenase, and direct inhibition of RNA-dependent RNA polymerase are just a few of the many hypothesized modes of action for RBV. Ribavirin is used to treat Lassa fever, as it is with other viral hemorrhagic fevers. Crimea's hemorrhagic fever Hemorrhagic fevers of Venezuela, Crimea, and the Congo, hepatitis C respiratory syncytial virus, and Hantavirus infection [2-3].

- **Ritonavir:**

1, 3-thiazol-5-ylmethyl 3-Hydroxy-2-(2S, 3S, 5S)-N-[(2S, 3S, 5S) (2S) Ritonavir's molecular formula reads C.I. -3-methyl. Carbamoyl 2-[methyl ([(propan-2-yl)-1, 3-thiazol-4yl] methyl)] alpha-amino butyric acid specifically, diphenylhexan-2-yl carbamate (1, 6). The protease inhibitor ritonavir has been shown to be effective against HIV-1. Human immunodeficiency virus type 1 is the name given to this pathogen (HIV-1). Protease inhibitors may block the activity of the HIV protease. Ritonavir inhibits protease activity by binding to the active site of the enzyme. This blocking prevents the formation of noninfectious immature viral particles, which would otherwise result from the breakdown of viral polyproteins. Protease inhibitors are often used in combination with two or more other anti-HIV drugs [4-7].

- **Lopinavir:**

The scientific name for Lopinavir is (2S) N-[(2S, 4S, 5S) (2S, 4S, 5S) (2S, 4S, 5S). The acetamido ester of 2-(2, 6-dimethylphenoxy) [(4-Hydroxy-1, 6-diphenylhexan-2-yl). The 2-oxo-1, 3-diazinyl-3-methylbutanamide. Lopinavir inhibits the HIV protease enzyme by forming an inhibitor-enzyme complex that prevents the cleavage of gag-pol polyproteins. Thereafter, harmless, immature virus particles are created. Lopinavir is a medication that blocks the enzyme HIV1 protease. By blocking CYP3A-mediated Lopinavir metabolism, ritonavir raises Lopinavir concentrations in the blood [7-8].

Separating the peak of the API from the peaks of all possible degradation products, process-related contaminants, potential packaging leachables, and excipients, as well as separating these compounds from each other, is essential for any stability-indicating analytical approach. Testing and stability studies on solid pharmaceutical batches need this method. A content determination test with acceptance criteria and restrictions for the amount of ribavirin, ritonavir, and Lopinavir contained in the formulation should be included in the completed product release requirements [9-10]. To determine a medicine's inherent stability, stress tests are required under the International Conference on Harmonization (ICH) drug product stability guideline Q1A (R2). This will enable degradation products to be identified, providing evidence for the usefulness of the suggested analytical approaches. The goal of validating an analytical technique, as outlined by the ICH guideline, is to show that the method is appropriate for its proposed application. Validation data are now required to be sent to the relevant authorities throughout the pharmaceutical development process. Analytical methods must be validated in accordance with standards such as ICH and USP [11–14].

High-performance thin-layer chromatography (HPTLC) is an automated kind of chromatography that uses sorbents with smaller particle and pore sizes, as well as a development chamber that uses less mobile phase, for greater efficiency (TLC). A high-tech densitometer called a UV/Visible/Fluorescence scanner performs a qualitative and quantitative analysis of the complete chromatogram. To estimate the concentrations of Ribavirin, Ritonavir, and Lopinavir in pharmaceutical dosage forms, scientists have developed a number of analytical methods, such as high-performance liquid chromatography, ultra-performance liquid chromatography, and liquid chromatography-mass spectrometry (LC-MS). The aforementioned techniques for measuring medicines in biological samples are laborious, time-consuming, and expensive compared to high-performance thin-layer chromatography (HPTLC). The development of a simple, economical, accurate, and speedy method of analysis, such as HPTLC, is crucial for preserving the quality of regularly given drugs like doxycycline hyclate. The goal of this project is to create and verify an HPTLC technique for determining the dosages of Ribavirin, Ritonavir, and Lopinavir in tablet and capsule form[15-17].

2 Materials and methods:

2.1 Chemicals and reagents:

A gift sample of 99.73%, 99.41%, and 100.2% pure ribavirin, ritonavir, and Lopinavir was given by Dr. Reddy's Laboratories (Hyderabad, India). The additional compounds were analytical reagent grade and were bought from Merck India.

2.2 HPTLC instrumentation and experimental conditions:

To apply samples, we used the semi-automatic TLC sampler Linomat V (Camag, Switzerland) and the Wincatsprogramme. The plates were made in a 20 cm by 10 cm glass box with parallel troughs (Camag, Switzerland). To take pictures of the TLC plates, we used a TLC scanner III. The tests were done on silica gel 60F254-coated aluminium plates (E. Merck, Darmstadt, Germany). Before chromatography, the plates were activated at 60 degrees Celsius for five minutes after being pre-washed with methanol. A 20 x 10 cm TLC plate was filled with 10 µl of standard solutions for Ribavirin, Ritonavir, and Lopinavir to create a calibration curve. The mobile phase on each plate was a 5:1:2 (v/v/v) mixture of N-Butanol, glacial acetic acid, and water. It took 15 minutes for the mobile phase to get completely saturated in the chamber at 25 degrees Celsius and 60% relative humidity. Densitometric scanner III in absorbance mode at 256 nm was used to scan the Ribavirin, Ritonavir, and Lopinavir plates in less than 10 minutes. Continuous radiation between 200 and 400 nanometers was radiated from a deuterium lamp. The information was analyzed by means of Wincats to produce a linear regression equation[18, 19].

2.3 Preparation of Standard Stock Solution:

Ribavirin 100 mcg, Lopinavir 100 mcg, and ritonavir 25 mcg should all be weighed out and placed in a volumetric vial with a 100 ml capacity. The mixture has to be diluted to volume with methanol once 10 mL have been added.

2.3.1 Preparation of Standard Solution:

The standard stock solution is pipetted into a 25 mL volumetric flask, and the mobile phase is added to make up the difference in volume. There are 120 ppm of ribavirin, 30 ppm of ritonavir, and 120 ppm of Lopinavir in theory.

2.3.2 Preparation of Sample solution:

Crush 20 pills into a powder and weigh the resulting powder. Measure out 1000 milligramme of Ribavirin, 250 milligramme of Ritonavir, and 1000 milligramme of Lopinavir into a 500 millilitres volumetric flask. The required amount of mobile phase is 300 mL. Sonicate while being shaken for 30 minutes. After it has cooled to room temperature, mix it with a diluent until it reaches the appropriate volume. While filtering the solution over a 0.45 µm Teflon membrane, discard the first millilitres. To dilute the filtrate by volume, transfer 3 mL using a micropipette into a 50 mL volumetric flask and add the mobile phase.

2.4 Validation of HPTLC method [20]:

2.4.1 Linearity:

Analytical findings should be linear with respect to the amount of analyte present in the sample, and this is what we mean by linearity. A five-point calibration curve was created by plotting the peak area against the concentration. The linearity of Ribavirin, Ritonavir, and Lopinavir solutions was evaluated by injecting a series of solutions into the test tube, with concentrations ranging from 40 to 200 µg/mL for Ribavirin and Lopinavir and 10 to 50 µg/mL for Ritonavir.

2.4.2 Range:

The range of an analytical method is defined as the concentration interval between the highest and lowest concentrations of analyte in a sample for which it has been shown that the analytical technique has a suitable degree of precision, accuracy, and linearity.

2.4.3 Precision:

The precision of an analytical technique may be defined as the standard deviation of a series of measurements taken from the same homogeneous sample under the same experimental circumstances. There are three levels of accuracy: intermediate, reproducible, and repeatable. The precision, repeatability, and accuracy of the innovative analytical approach were verified based on ICH requirements. We have calculated the intraday, daily, and weekly accuracy of the procedure. The reliability of the device was established by applying the identical cream solution six times in triplicate. Analyzing six separate, same-day applications of newly prepared standard solutions allowed researchers to calculate the assay's intra-day precision. Analyzing six days' worth of data from applying the same concentration of a standard solution was used to calculate the intermediate

precision. The % CV is used to assess the reproducibility of peak area measurements and sample application.

2.4.4 Specificity:

When an analyte is said to be "specific," it means that it may be evaluated accurately even in the presence of other substances that could be expected to be there. They typically consist of a degradant, matrix, and contaminant. Ribavirin, Ritonavir, and Lopinavir were analyzed, and their prevalence in various forms was used to establish the method's specificity. The existence of Ribavirin, Ritonavir, and Lopinavir in the Cream sample was established by comparing the RF and spectra of the spot to those of the sample. By comparing the spectra at the beginning, middle, and end of the spot/bands, we were able to establish the relative purity of ribavirin, ritonavir, and Lopinavir.

2.4.5 Robustness:

How well an analytical technique holds up under normal circumstances may be inferred from its robustness, which is the degree to which it remains unchanged in the face of tiny but purposeful changes in method parameters. The estimate was carried out by making small adjustments to the chosen parameters (mobile phase composition, mobile phase volume, and duration of mobile phase saturation) within a set range (±10%), without substantially impacting the efficiency of the method or the outcomes. The findings were calculated using the%RSD of the differences in the data for each condition of the variable.

2.4.6 Accuracy:

Accuracy is defined as the extent to which the found value agrees with the value generally accepted as the genuine value or with the value generally accepted as a reference value. This is often referred to as "being honest." Utilizing the standard addition method, three different addition concentrations of Ribavirin, Ritonavir, and Lopinavir were used in the recovery trials (50, 100, and 150 percent).

2.4.7 System suitability:

Resolution and repeatability of analysis were tested to ensure the system met requirements. A freshly prepared reference solution of ribavirin, ritonavir, and Lopinavir at a concentration of 200 ng/spot was scanned and densitogrammed six times under the same chromatographic conditions to assess the system's applicability. Peak areas and retention factors were recorded for each concentration of ribavirin, ritonavir, and Lopinavir. The mean peak area, standard deviation, and coefficient of variation (CV) were then calculated.

3 Result and discussion:

3.1 TLC fingerprint and co-chromatography:

The use of chromatographic fingerprint analysis to determine the authenticity and species of traditional medicines is a sensible and effective approach. It employs chromatographic methods to identify patterns unique to medicinal plants. The fingerprint pattern of components may be used to determine not only whether or not markers of interest are present, but also the ratio of all analytes identified. High performance thin layer chromatography (HPTLC) is a useful tool for evaluating the quality of medicinal plants because of its ease of use, low cost, and minimal technical requirements, despite having some disadvantages such as a smaller developing range and lower plate efficiency compared to HPLC and GC. It has also been put to good use in creating the chromatographic imprint of therapeutic herbs. Growing polarity-separate fractions on two or more thin-layer plates is another way to circumvent the aforementioned limitations. Thus, HPTLC's distinctive ability of producing a picture-like image when paired with a digital scanning profile to produce the herbal chromatographic fingerprint is more appealing to the field of herbal analysis. Ribavirin, Ritonavir, and Lopinavir may be distinguished from one another using the information and parameters that this HPTLC could offer (Figure 1).

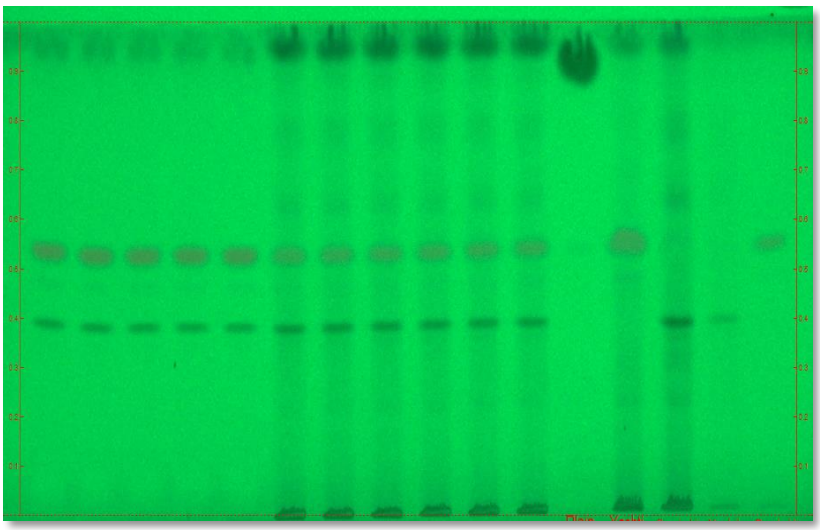

Figure 1: HPTLC profile of Ribavirin, Ritonavir and Lopinavir

3.2 Validation of RP- HPTLC method:

3.2.1 Accuracy:

Sample solutions were prepared by adding the drug material to powdered ribavirin, ritonavir, and Lopinavir tablets at three different concentrations: 80%, 100%, and 120% of the label claim in triplicate (for a total of nine determinations). The data submitted was validated as correct per Table 1. The percentages of recovered Ribavirin, Ritonavir, and Lopinavir from both the standard and sample preparations were 99.27%, 100.29%, and 98.26%, respectively. The data collected was found to be consistent with expected recovery levels.

Table 1: Recovery study of the method for Ribavirin, Ritonavir and Lopinavir

Sr. No	Recovery level (%)	Ribavirin	Ritonavir	Lopinavir
1	80	98.4	99.28	98.22
2	100	100.28	101.36	100.32
3	120	99.13	100.23	98.52
Average		99.27	100.29	99.26

3.2.2 Precision:

Precision at all levels from the system level to the technique level to the intermediate level was investigated. The accuracy of the system was assessed and quantified by using six replicate administrations of the same standard from the same ampoule to measure % relative standard deviation (RSD), tailing, plate count, and resolution. Six iterations of the aforementioned procedure were performed on the sample. Expressing the test percentage for each analyte as a percentage of the standard deviation (% RSD). Six different extract samples were analyzed on two different systems, one using a Waters e2695 Alliance system with a 2996 PDA and the other using a 2489 ultraviolet (UV) detector, to determine the intermediate precision. The data was presented as a percentage of the relative standard deviation. This research demonstrated a more reliable approach than previously used for recognizing Ribavirin, Ritonavir, and Lopinavir in their dose form (Table 2).

Table 2: Method validation parameters for quantitation of Ribavirin, Ritonavir and Lopinavir

Sr. No.	Parameters	Ribavirin	Ritonavir	Lopinavir
1	Specificity	Specific	Specific	Specific
2	System precision.(% RSD)	0.98	1.12	0.89

3	Method Precision.(% RSD)	1.25	1.37	1.16
4	Intermediate precision. (% RSD)	1.57	1.02	1.08

3.2.3 Linearity and range:

It was determined that the R2 values of the linearity calibration curves were both 1.00. The range of 10-50 µg/mL was used for the Ritonavir calibration, whereas the range of 40-200 µg/mL was used for the Ribavirin and Lopinavir calibrations. (Table 3). The regression coefficient and equation are shown in Figure 2. (R2). According to the findings, the relative standard deviation was a constant 1.0. The peak value observation was found to have a stronger than expected relationship to the concentration of the drug in the solution. Ribavirin, ritonavir, and Lopinavir standard formulations were created with active concentrations from 50 to 150 percent. The proposed range is between 40 and 200 µg/mL for Ribavirin and Lopinavir and between 10 and 50 µg/mL for Ritonavir, given that the working concentrations are 120 ppm, 30 ppm, and 120 ppm, respectively.

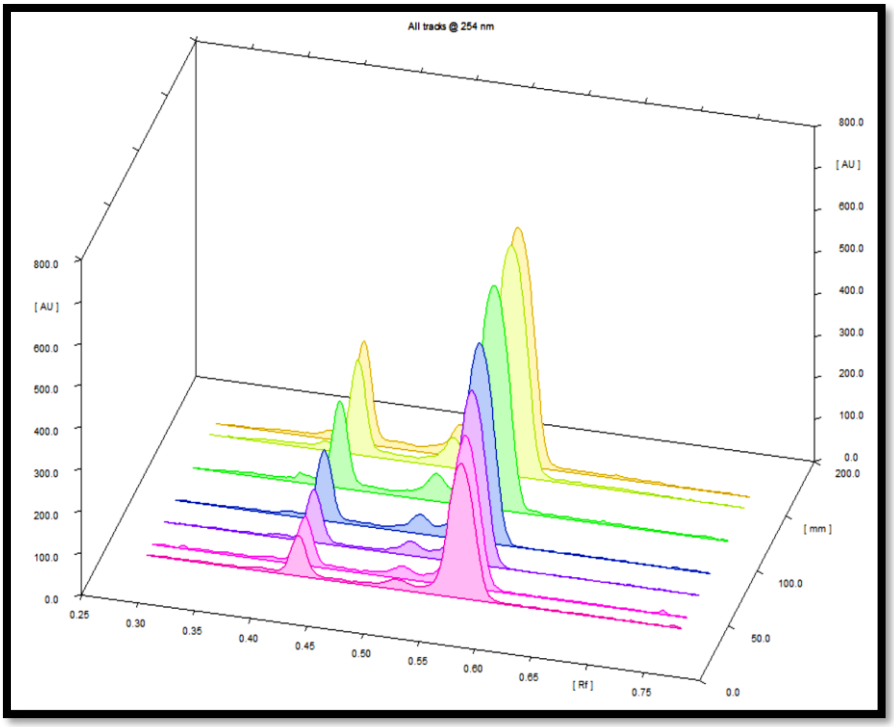

Figure 2: 3-D chromatogram of linearity of Ribavirin, Ritonavir and Lopinavir

Table 3: Summary of linear regression and validation data

Parameters	Ribavirin	Ritonavir	Lopinavir
Linearity range	40–200 µg/mL	40–200 µg/mL	10–50 µg/mL
Linear regression equation	y = 0.19694x + 0.25244	y =0.13162x + 0.16696	y =0.23162x + 0.15696
Slope ± SD	0.19694	0.13162	0.12365
Intercept ± SD	0.25244	0.16696	0.16694
Correlation coefficient ®	0.9994	0.9993	0.9993
Determination coefficient (r2)	0.9984	0.9991	0.9998

3.2.4 Solution Stability:

Sample solution was prepared and was kept at room temperature (20 ± 2°C and 30 ± 2°C) on a shelf protected from direct light. The solution was analyzed after 1 h, 3 h, 6 h, 12 h, 18 h and 24 h. Because of the time needed for sonication and filtration, the fastest possible analysis was carried out within 20 min and hence results of the remaining analysis times were compared with it. The average peak areas values are presented in Table 4. The average peak areas of of Ribavirin, Ritonavir, and Lopinavir solutions does not varied significantly from the reference time after 1 day of sample preparation.

Table 3 System stability study

Sr.no	Time of analysis (hrs)	Peak Area (AU)		
		Ribavirin	Ritonavir	Lopinavir
1	0	10104	23087	31087
2	3	10202	22596	38596
3	6	10090	22869	39869
4	12	10078	22731	30731
5	18	10143	22921	31921
6	24	10105	22843	32843

3.2.5 Specificity and selectivity:

The peak purity tests of Ribavirin, Ritonavir, and Lopinavir acid spots were assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot **[12]**. The results ofspectral comparison for Ribavirin, Ritonavir, and Lopinavir were found to be specific at peak start–peak apex and at peak apex–peak end, respectively. The closeness of peak purity values to 1 indicates that the spots were only attributed to a single compound. Good correlation (r= 0.992) was also obtained between standard and sample spectra of Ribavirin, Ritonavir, and Lopinavir. The UV spectra comparison of the spots of the standards and all extracts were presented in Figure 3.

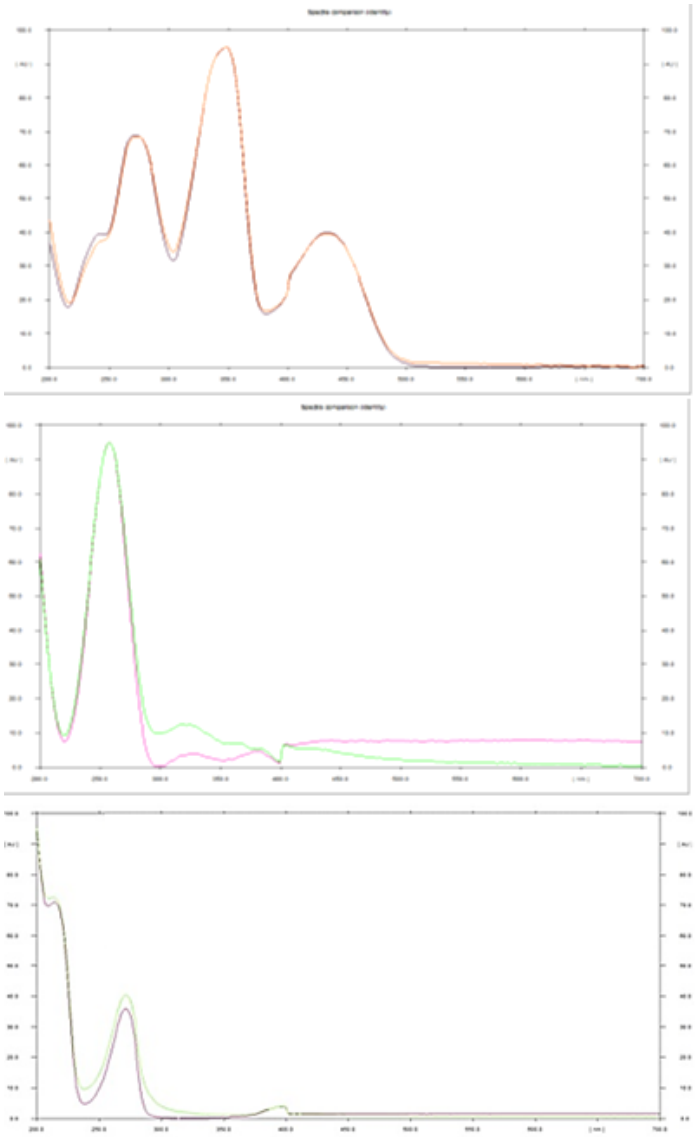

Figure 4: Comparative spectra of Ribavirin, Ritonavir and Lopinavir

3.2.6 Robustness:

The standard deviations of peak areas were calculated for the aforementioned four parameters (variation in composition of the mobile phase, volume of the mobile phase, time from spotting to development and time from development to scanning) and coefficients of variation were found to be less than 2.0%in all cases as shown in Table 5. The low RSD values indicate the robustness of the method [28-30].

Table 4: Robustness study for the developed method

SN	Parameter studied	Ribavirin % RSD	Ritonavir % RSD	Lopinavir % RSD
1	Composition of mobile phase	1.37	0.99	1.29
2	Volume of mobile phase	1.11	1.38	1.23
3	Time from spotting to development (5–60 min)	1.08	0.87	1.02
4	Time from development to scanning	1.30	1.21	1.32

	(5–60 min)			

4 CONCLUSION:

A quantitative approach for assessing ribavirin, ritonavir, and Lopinavir in dosage form that is accurate, precise, robust, reliable, and repeatable has been created using RP- HPTLC technology. The resolution and retention times of the mobile phase solvent are very high and low, respectively. The operation and related report were conducted in compliance with ICH and FDA guidelines. To determine the substance's efficacy, the procedure's exactness, precision, and linearity were tested.Ribavirin, Ritonavir, and Lopinavir may be quantified reliably using the RP- HPTLC technique described in this article. The statistical evidence suggests that the proposed technique can be effectively integrated into our current decision process. According to the specificity analysis, the excipient did not have any role in the results. It is possible to expand upon the existing experiment by include kinetics research using plasma and biological fluids. This innovative approach was shown to be more cost-effective than the previously stated study, which was a noteworthy discovery.

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Conflicts of interest:

No conflicts of interest are declared by the authors.

Authors' contribution:

Akash Tambeand 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. DeshrajChumbhale efforts include data analysis and chemical identification. The final document was interpreted and approved by each author.

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