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"Quantitative Analysis of Ritonavir Tablets by RP-HPLC"

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ABSTRACT
This study presents a comprehensive validation of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative determination of Ritonavir. The chromatographic separation was achieved using a Shimpack Solar C18 column (250 mm × 4.6 mm, 5 µm) in conjunction with a potassium buffer. The isocratic mobile phase consisted of a 60:40 (v/v) mixture of dihydrogen phosphate and acetonitrile, delivered at a constant flow rate of 1.0 ml/min. Detection and quantification of Ritonavir were performed at a wavelength of 246.8 nm. The optimized method yielded a precise retention time (RT) of 4.063 minutes for RITONAVIR, with a limit of detection (LOD) of 1.203 µg /ml and a limit of quantification (LOQ) of 4.011µg/ml.
Keywords:-Shimpack Solar, dihydrogen phosphate, Acetonitrile, conjunction, Ritonavir.

I. INTRODUCTION

The development and validation of analytical methods are essential steps in the research, development, and production of pharmaceuticals. According to current good manufacturing practice ($_C$GMP) regulations and guidelines issued by the Food and Drug Administration (FDA), it is vital to employ robust, reliable analytical methods with high sensitivity and reproducibility.

Typically, the development of such methods builds upon existing literature or prior research, often using similar instrumentation. In modern practices, it is rare to develop a high-performance liquid chromatography (HPLC) method without referencing well-established, literature-based methodologies.

HPLC has become the leading analytical technique in the pharmaceutical industry for quantifying intact drugs and their degradation products. Carefully selecting chromatographic conditions ensures the HPLC method achieves the required specificity. Additionally, ultraviolet (UV) spectroscopy is widely utilized for routine drug analysis due to its simplicity and effectiveness.

As a result, HPLC and UV spectroscopy were chosen as the analytical techniques for the proposed methods. The developed chromatographic methods were validated following the guidelines of the International Conference on Harmonisation (ICH) or the United States Food and Drug Administration (USFDA), addressing all critical parameters.

To ensure the precision of the analytical results and validate the methods, statistical tools are applied. These include confidence interval determination, regression analysis for constructing calibration curves, and optimization of critical response parameters. This comprehensive approach guarantees the accuracy and reliability of the analytical methods.

II. CHROMATOGRAPHY:

Chromatographic techniques have been utilized for centuries to separate substances, such as plant-derived dyes. The discovery of chromatography is attributed to the Russian botanist Tswett, who, in 1903, successfully separated leaf pigments using a polar solid stationary phase. It wasn't until the 1930s that researchers like Kuhn and Leder, along with Reichstein and van Euw, further advanced this technique for the separation of natural products.

In 1941, Martin and Synge were awarded the Nobel Prize for their pioneering work in liquid-liquid chromatography. They introduced the concept of theoretical plates as a metric for assessing chromatographic efficiency. The term "chromatography" itself is derived from the Greek words "chroma" (color) and "graphein" (to write), meaning "color-writing."

TYPES OF HPLC TECHNIQUES:
Based on modes of chromatography:
Normal phase chromatography
Reverse phase chromatography
Based on principle of separation:
Adsorption chromatography
Ion exchange chromatography
Size exclusion chromatography

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Affinity chromatography
Based on elution technique:
Isocratic separation
Gradient separation
Based on the scale of operation:
Analytical HPLC
Preparative HPLC

III. HPLC METHOD DEVELOPMENT:

The development of novel analytical methods is necessitated by the absence of official methods for certain products. Alternatively, existing non-pharmacopoeial products may require the design of new methods to enhance cost-efficiency, reduce analysis time, and improve precision and robustness. When proposing an alternative method to supplant an existing one, a comprehensive comparison of laboratory data is required, including an evaluation of the advantages and limitations of both methods.

To ensure the reliability and accuracy of quantitative analysis, several critical factors must be meticulously considered. These include:

1. Rigorous sample preparation to prevent contamination and ensure homogeneity.
2. Judicious selection of the chromatographic column to achieve optimal separation and resolution.
3. Optimization of the flow rate to ensure efficient separation and minimize analysis time.
4. Proper selection of the detector wavelength to maximize sensitivity and selectivity.
5. Accurate regulation of column temperature to maintain consistent separation conditions.

By carefully controlling these factors, analysts can ensure the accuracy, precision, and reliability of quantitative analytical results.

IV. MATERIAL AND METHODS

INSTRUMENTS USED:

List of Equipment

S.NO	Equipment's	Model	Company
1	Electronic Balance	ER200A	ASCOSET
2	Ultra-Sonicator	SE60US	ENERTECH
3	Heating Mantle	BTI	BIO TECHNICS INDIA
4	Thermal oven	---------	NARANG
5	pH Meter	AD102U	ADWA
6	Filter Paper 0.45 microns	---------	MILLI PORE
7	HPLC	E-2695	WATERS
8	UV Spectrophotometer	-------	SYSTRONICS

CHEMICALS AND REAGENTS

KH_2PO_4HPLC Grade
Methanol HPLC Grade
Acetonitrile
Inertsil, C18
Peroxide
Sodium hydroxide
Phosphoric acid
Hydrochloric acid

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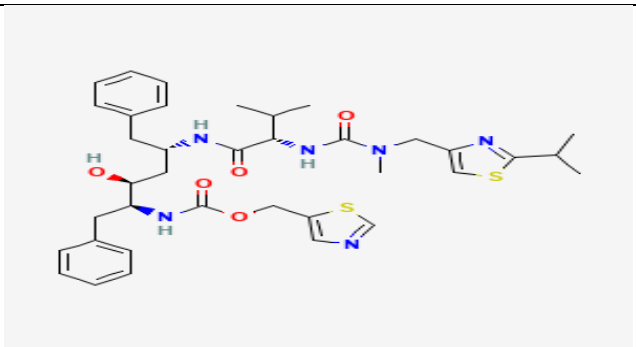
V. DRUG PROFILE:

Name of the drug	Ritonavir
Chemical structure	
Chemical name	1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate
Chemical formula	$C_{37}H_{48}N_6O_5S_2$
Molecular weight	720.94 g/mol
Category	Protease Inhibitor
Dosage form	Oral, tablet
Dose	400 mg
Brand Name	Kaletra

Ritonavir is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose Ritonavir and other protease inhibitors. It is now more commonly used as a booster of other protease inhibitors and is available in both liquid formulations and as capsules.

While Ritonavir is not an active antiviral agent against hepatitis C virus (HCV) infection, it is added in combination therapies indicated for the treatment of HCV infections as a booster. Ritonavir is a potent CYP3A inhibitor that increases peak and trough plasma drug concentrations of other protease inhibitors such as Paritaprevir and overall drug exposure.

VI. EXPERIMENTAL WORK:

PREPARATION OF STANDARD SOLUTION:

Accurately weigh and transfer 100mg of Ritonavir 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water.

Transfer the above solution into 1ml into 10ml volumetric flask dilute to volume with water.

PREPARATION OF SAMPLE STOCK SOLUTION:

Commercially available 20 tablets ware weighed and powdered the powdered equivalent to the 150mg of (100 Ritonavir) of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20min (or) shake 10min and makeup with water.

Transfers above solution 1ml into 10ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45µm filter before injecting into HPLC system

PREPARATION OF MOBILE PHASE:

Transfer 1000ml of HPLC water into 1000ml of beaker and KH2PO4 (1M- 136.09gms) adjust pH 4.6 (OPA)

Transfer the above solution 700ml KH2PO4 of, 300ml of Methanol is used as mobile phase. They are mixed and sonicate for 20min.

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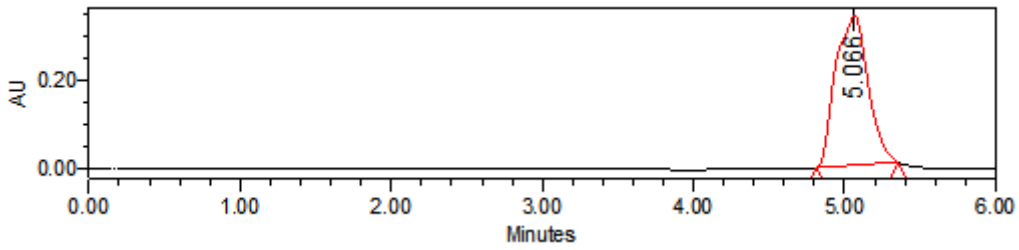
VII. DEVELOPMENT OF HPLC METHOD:

The analytical method used for quantifying Ritonavir underwent thorough validation, adhering to the guidelines established by the International Conference on Harmonization (ICH). This validation process was conducted to confirm that the method met the required standards of specificity, sensitivity, accuracy, precision, and robustness, ensuring its reliability for the accurate determination of Ritonavir.

Chromatographic Conditions:

Sr. No.	Trial	Mobile Phase	Name of the peak	Retention time (min)	Flow rate	Time to run	Tempo in the column
1.	1	OPA: ACN(70:30)	Ritonavir	5.066	0.8 ml/min	6min	25°C
2.	2	OPA: ACN(60:40)	Ritonavir	4.970	0.8ml/min	10min	25°C
3.	3	K_2HPO_4: Methanol (60:40)	Ritonavir	3.857	1.0ml/min	8min	25°C
4.	4.	K_2HPO_4: Methanol (50:50)	Ritonavir	3.713	0.8ml/min	8min	25°C
5.	5.	KH_2PO_4: Methanol (65:35)	Ritonavir	4.169	1.0ml/min	6min	25°C
6.	6.	KH_2PO_4: Methanol (70:30)	Ritonavir	4.063	1.0ml/min	7min	25^0C



Trail-1

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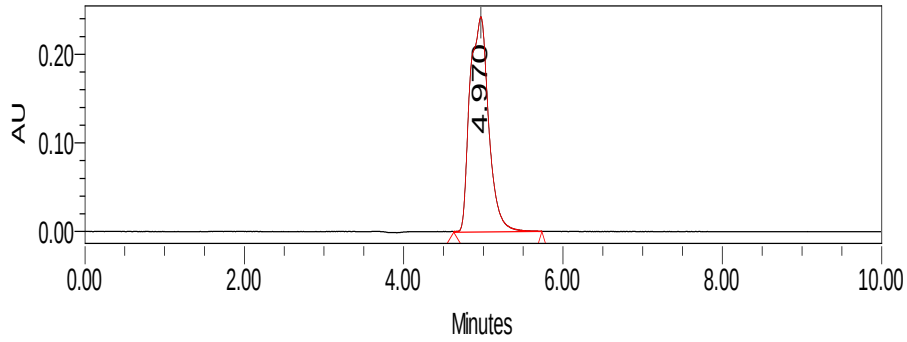

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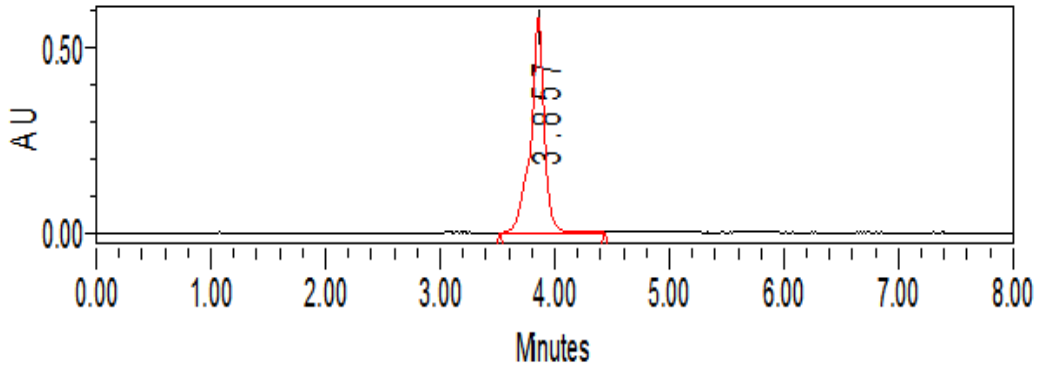

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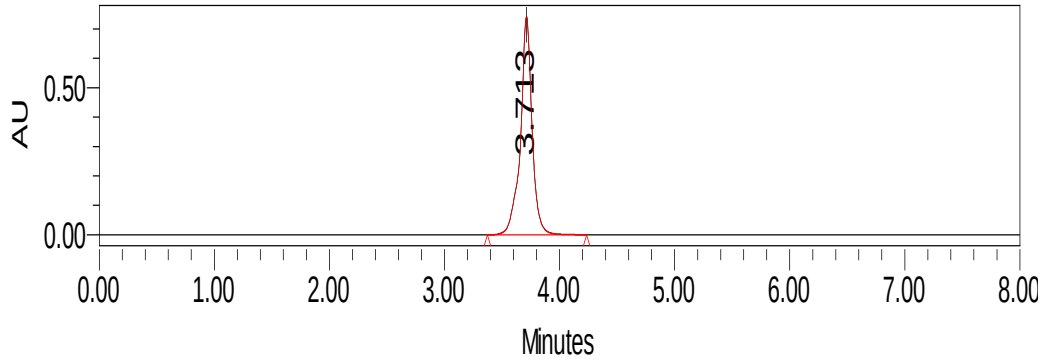

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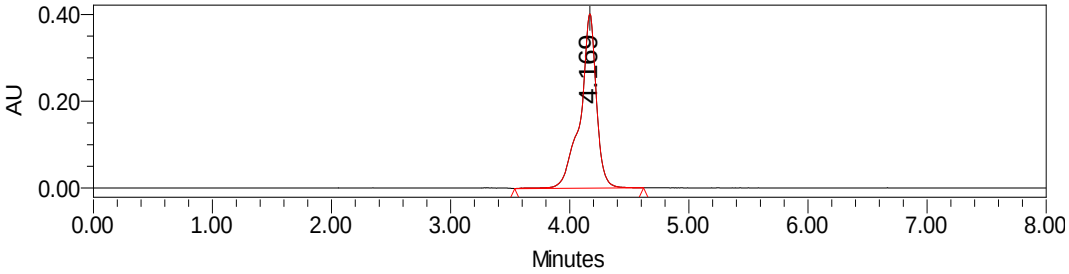

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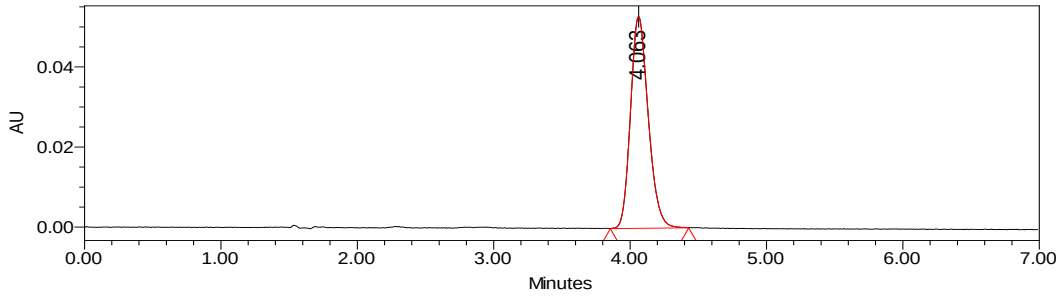

Trail-6

VIII. METHOD VALIDATION:

a) SYSTEM SUITABILITY

The system suitability study produced satisfactory outcomes, as detailed in the table. Six consecutive injections of the standard solution showed consistent retention times, theoretical plate counts, tailing factors, and resolution for both analytes, confirming the reliability and efficiency of the chromatographic system for analysis.

System Suitability

Parameter	Ritonavir	Acceptance Criteria
Retention time	4.4478	+-10
Theoretical plates	4562.8	>2500
Tailing factor	1.272	<2.00
% RSD	0.3	<2.00

b) SPECIFICITY:

The chromatograms show identical retention times for the standard, sample, and commercial Ritonavir product, confirming that the excipients in the formulation do not interfere with the analysis. Additionally, the clear separation between the blank peak and the drug peak highlights the method's excellent selectivity..

Specificities

S. No	Sample Name	Ritonavir area	Rt
1	Standard	1317572	2.658
2	Sample	1308642	2.648
3	Blank	-	-
4	Placebo	-	-

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c) PRECISION:

The results of the variability study, presented in the table, demonstrate the precision of the method. The percentage relative standard deviation (%RSD) of peak areas was calculated for multiple runs, and the results show that the %RSD values are consistently below 2%. This indicates that the method exhibits excellent precision, with minimal variability in the results**.**

Precision

S. No	RT	Area(µV*sec)	%Assay
injection1	3.985	471546	99
injection2	3.974	469631	99
injection3	3.966	472161	99
injection4	3.967	470511	99
injection5	3.954	469680	99
injection6	3.954	472215	99
Mean	3.966667	470,957.33	99
Std. Dev.	0.011928	1,180.42546	0.25
% RSD	0.301%	0.251%	0.25

d) ACCURACY:

The accuracy study results, outlined in the table, confirm the method's exceptional accuracy. Recovery testing was used to assess the measured value against the spiked amount of Ritonavir, yielding a 100% recovery rate. These findings demonstrate the method's precision and reliability for accurately quantifying Ritonavir.

Data of Accuracy

Sr. No	Accuracy level	Injection	Sample area(µV*sec)	RT (min)	Sample weight	µg/ml added	µg/ml found	% Recovery	Mean
1	50%	1	231712	3.944	75	49.500	48.72	98	99
		2	232515	3.934	75	49.500	48.89	99	
		3	232900	3.929	75	49.500	48.97	99	
2	100%	1	479259	3.933	150	99.00	100.77	102	101
		2	471073	3.930	150	99.00	99.05	100	
		3	469867	3.927	150	99.00	98.80	100	
3	150%	1	708284	3.927	225	148.50	148.93	100	100
		2	704696	3.920	225	148.50	148.18	100	
		3	704515	3.922	225	148.50	148.14	100	

e) LINEARITY:

A strong linear relationship was observed between the peak areas and Ritonavir concentrations, covering a range from 50% to 150% of the nominal concentration. The correlation coefficient (r) of 0.998 confirms the method's outstanding linearity within this range..

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Data for Linearity

Sr. No	Conc (µg/ml)	RT(min)	Area(µV*sec)
1.	50	3.907	231712
2.	75	3.902	355533
3.	100	4.029	470509
4.	125	3.909	589226
5.	150	3.905	701718
Correlation coefficient (r^2)			0.9998

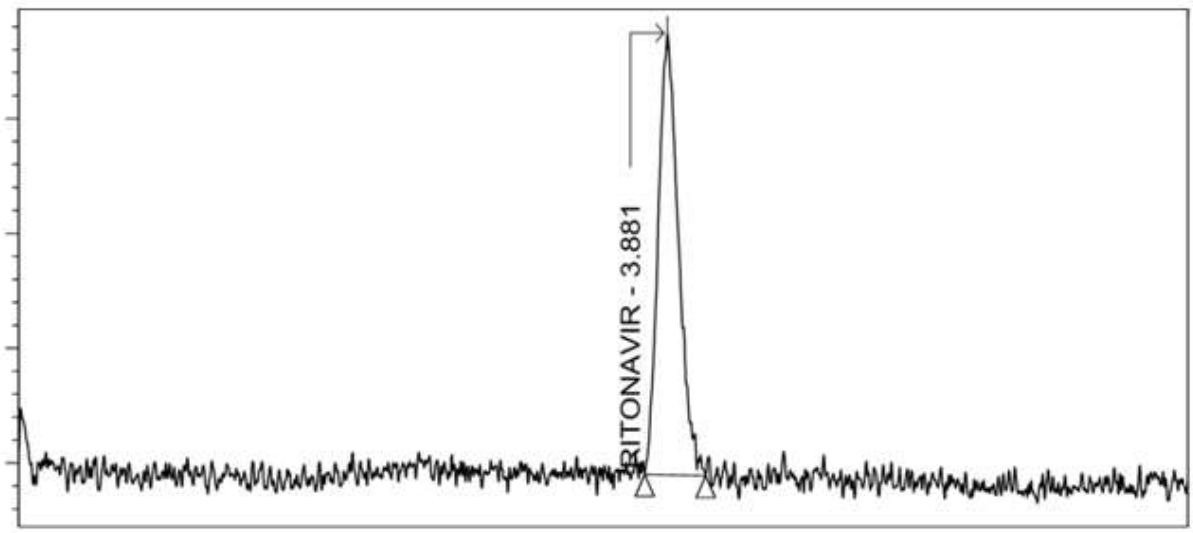
f) LOD (LIMIT OF DETECTION):

The Limit of Detection (LOD) is defined as the minimum concentration of a standard component at which its peak can be distinguished from the background noise.

LOD = 3.3* σ/S
Where;
σ = standard deviation
S = slope
LOD for Ritonavir = 1.203

LOD data for Ritonavir

S. No	Sample name	RT	Area(µV*sec)
1	Ritonavir	3.881	16215



Chromatogram for LOD

g) LOQ (LIMIT OF QUANTIFICATION):

The Limit of Quantification (LOQ) is defined as the minimum concentration of a standard component at which its peak can be accurately detected and quantified with acceptable precision and accuracy.

LOQ = 10*σ/S
Where;

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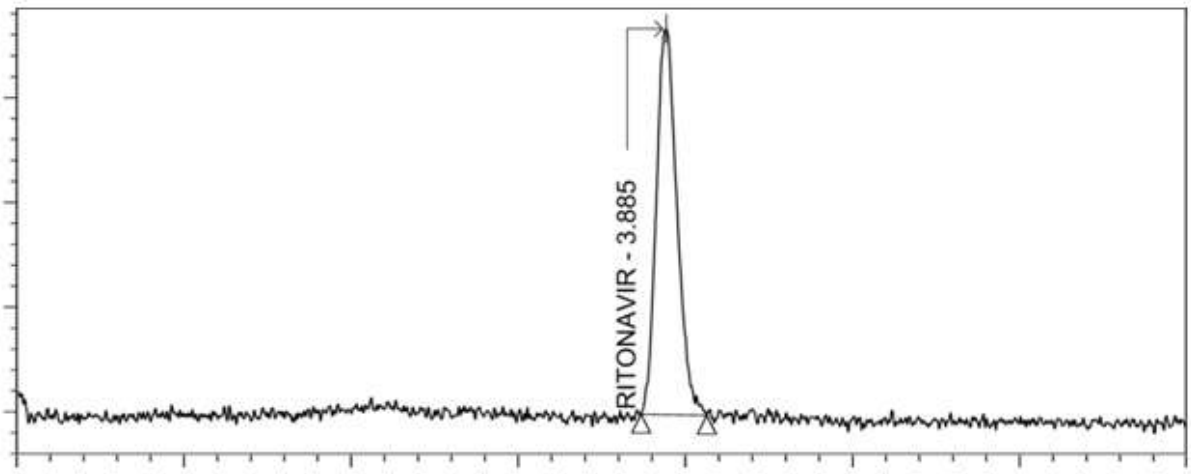
σ = standard deviation
S = slope

LOQ for Ritonavir=4.011

LOQ data for Ritonavir

S.no	Sample name	RT	Area(µV*sec)
1	Ritonavir	3.885	31885



Chromatogram for LOQ

h) ROBUSTNESS:

The robustness of the method was assessed by intentionally varying the flow rate and temperature. As shown in the table, these variations had no significant effect on the analytical results, confirming that the method is robust and capable of withstanding minor changes in experimental conditions..

Data for Robustness

Parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.8ml/min)	3.251	4483	1.22
Increased flow rate(1.2ml/min)	4.786	5419	1.23
Decreased temperature(20^0c)	4.636	4531	1.22
Increased temperature(30^0c)	5.863	5766	1.21
Decreased comp rate(5%)	4.636	4531	1.22
Increased comp rate(5%)	4.786	5419	1.23
Decreased pH(0.2)	3.974	4768	1.20
Increased pH(0.2)	3.985	4794	1.20

DEGRADATION STUDY:

The Ritonavir tablet sample solution was subjected to various stress conditions, including acid, base, oxidant, water, heat, and light, to assess its stability and specificity. The outcomes of this forced degradation study demonstrate that the method is stability-indicating and specific, with Ritonavir remaining stable under the tested conditions, ensuring the accuracy and reliability of the analytical results..

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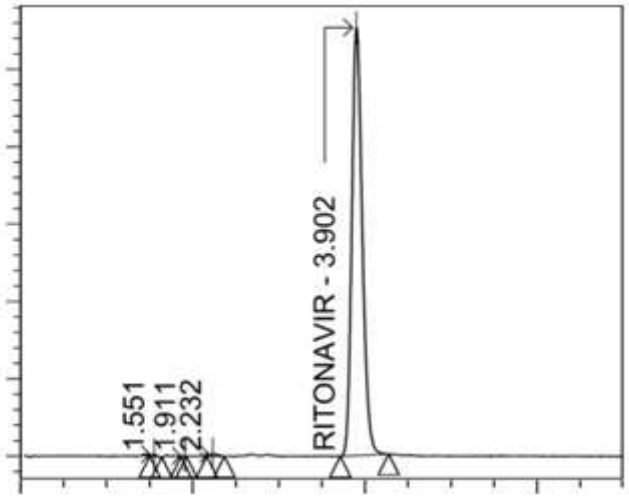
Ritonavir degradation data

Drug Exposed	Ritonavir	
	Response Area(µV*sec)	%Assay
0.1N HCl	422925	88.93
0.1N NaOH	440297	92.58
30% H_2O_2	442820	93.11
105 ^oC	423250	89.00
Sunlight	447370	94.07
Untreated	466567	98.11

RITONAVIR STABILITY DIRECTION:
Acid>Heat >Base >Peroxide >Sunlight

STABILITY INDICATING NATURE AND SPECIFICITY:

A chromatographic study was conducted to assess the stability of Ritonavir under various stress conditions, including acidic (0.1 N HCl), basic (0.1 N NaOH), oxidative (peroxide), photolytic (sunlight), and thermal (105°C) degradation. The chromatograms demonstrated excellent separation of Ritonavir from its degradation products, showcasing the method's effectiveness in evaluating Ritonavir's stability. These findings further validate the method's precision and reliability.



Acid degraded sample chromatogram

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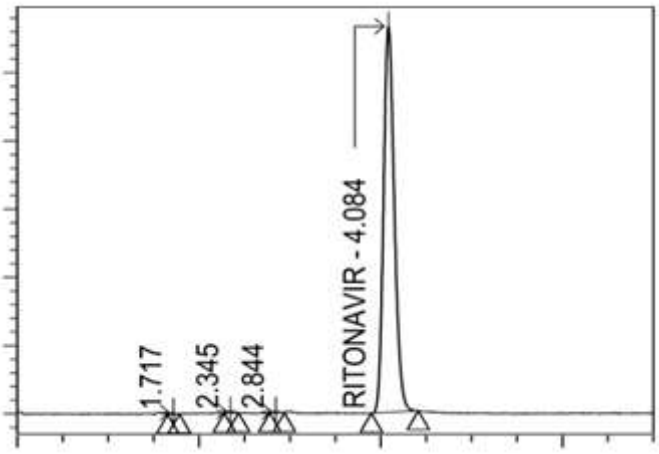
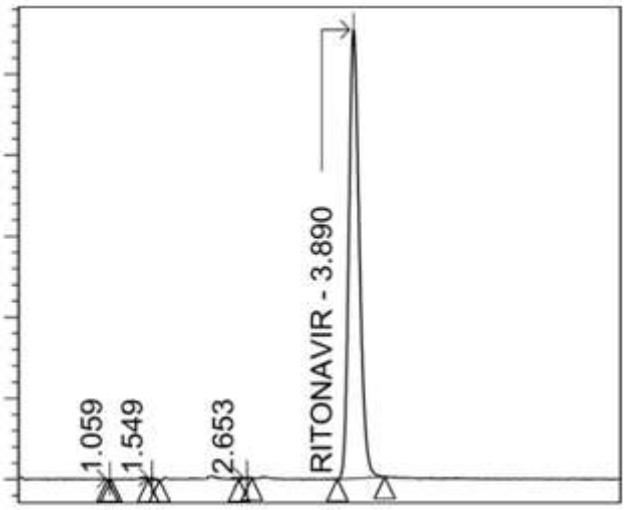
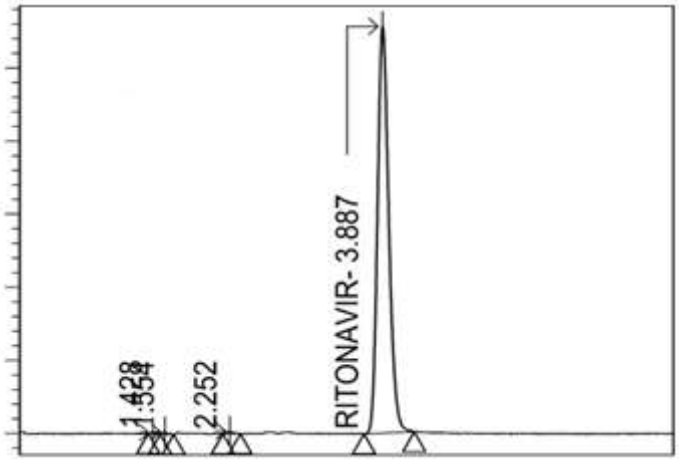


Base degraded sample chromatogram



Oxidant degraded sample chromatogram



Thermal degraded sample chromatogram

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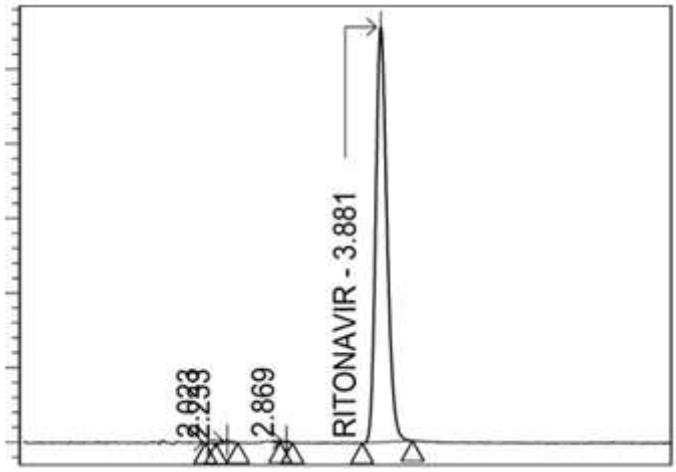
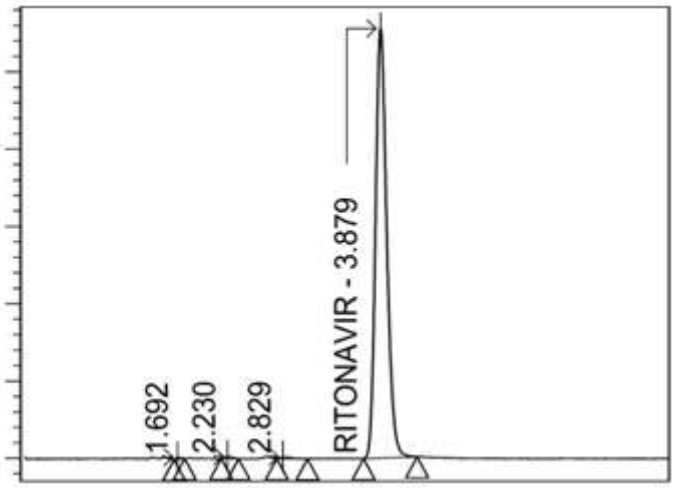


Photo degraded sample chromatogram



Water degraded sample chromatogram

IX. CONCLUSION AND SUMMARY:

CONCLUSION OF RITONAVIR

Ritonavir quantification was accurately performed and validated using a stability-indicating RP-HPLC method that is selective, precise, specific, efficient, robust, cost-effective, and requires minimal run time.

SUMMARY OF RITONAVIR

Summary of validation data forRitonavir

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 4562.8 1.272 4.4478 0.3%	 Not less than 2500 Not more than2 Not more than 2%

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2	Specificity a) Blank interference b) Placebo interference	Specific	Specific
3	Method precision(%RSD)	0.25	Not more than 2.0%
4	Linearity parameter Slope Intercept Correlation coefficient(r^2)	50-150mcg/ml 0.998	 Not less than 0.999
5	Accuracy (Mean % recovery) 50% 100% 150%	 99% 101% 100%	 97.00 – 103.00%
6	Robustness a) Flow rate variation b) Temperature variation	All the system suitability parameters are within the limits.	

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