RESEARCH PAPER

Method Development and Validation for Ritonavir with Comprehensive Impurity and Toxicity Profiling

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Abstract

Introduction / **Objective**: The selectivity, potency, and stability of antiviral compounds, including Ritonavir (RTV), require high-precision analytical testing and impurity profiling for confirmation. As a widely used pharmacokinetic enhancer in COVID-19 therapy, the regulation and clinic validation of impurities and their toxicity are of the utmost importance. The main goal of the present work was to develop and confirm a simple, accurate, and stability, indicating HPLC method for determining the Ritonavir content and its related impurity (Impurity, A Free Base) includes theoretical and experimental toxicity assessments. **Methods**: Chromatographic separation was performed on a Phenomenex C18 column (250 4.6 mm, 5 m). The mobile phase was 20:80 of water and acetonitrile, flow rate 1.0 mL/min. The method validation tested different things using ICH Q2(R1) rules, Accuracy, Precision, Specificity, and Linearity. The toxicity assessment for Impurity A was done by the *insilico* prediction tools (PROTOX 3.0) and checked with an in vitro study of toxicity in PBMC cells. **Results**: The method demonstrated (R = 0.9999) in a range of 1.64 to 29.94 g/mL, with recovery rates from 92% to 99% and a relative standard deviation below 2%. Ritonavir and its impurity- A with distinct retention times is 4.3 min and 2.3 min respectively. In silico studies stated that the drug has a medium acute oral toxicity as low with a LD50 values of more than 2000 mg/kg with mild hepatotoxic and neurotoxic while MTT results showed an IC50 value >500 µg/mL, suggesting low cytotoxicity. **Discussion**: The validated HPLC method is a reliable, accurate, and economical way for the routine analysis of Ritonavir and its contaminants. The use of computational and in vitro toxicity assessments used to identify impurity hazards and comply with quality control standards of antiviral drugs. **Conclusion**: A consistent, precise, and cost-effective HPLC method was successfully developed and validated for Ritonavir and its related impurity. The integration of in silico and in vitro toxicity studies strengthens impurity risk assessment and supports the safe and effective quality control of antiviral pharmaceutical products.

Keywords: Ritonavir; Impurity Profiling; HPLC; Validation; Cytotoxicity; In silico Toxicity; MTT Assay; PROTOX 3.0

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1.Introduction:

For pharmaceutical formulations to remain safe, standard, and clinically effective, reliable and validated analytical techniques must be developed (RTV) drug against viruses to treat COVID 19 (1) and other viral illness. The action of drugs by stopping CYP3A system from breaking down, Ritonavir helps other drug, like Nirmatrelvir, get into the blood better and be more powerful (2,3,4).

The standards established by the International Council for Harmonization ICH, such as Q2(R2) for validation and Q14 for analytical development, impose strict

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requirements for impurity identification and control. Regulations including ICH Q3A/B and USP or EP monographs position impurity profiling as an essential aspect of quality control, particularly for pharmaceuticals like Ritonavir Impurity-A (5,6), which may induce adverse reactions or safety concerns.

HPLC is the best way to measure impurities like very selective and reproducible and can be used for drugs that are sensitive to heat (7,8). Detailed measurement of APIs and their related chemicals, providing confidence that the drug remains uniform from development through to manufacturing and stability testing. Ritonavir is an antiretroviral medication (Fig-1) and one of the top oral antivirals used in combined COVID-19 treatment (9,10). During quality checks, Impurity-A for Ritonavir was added to impurity profile (Fig-2) and stress tested to find possible break down process (11,12).

Over recent years, green and cost-efficient analytical methods have become popular for their ability to cut down on solvent waste and lessen environmental strain from analytical labs. At the same time, computational toxicity tools like PROTOX 3.0 and cell-based toxicity tests such as the MTT assay have offered valuable data on impurities related to biological concerns, all while reducing the need for testing on animals. The goal of the current work is aimed at designing and validating a straightforward, precise, and robust HPLC method for quantifying Ritonavir and its pharmacopeial impurity (Impurity-A Free Base). Additionally, comprehensive in silico and in vitro toxicity profiling was performed to evaluate the potential safety risks associated with the impurity.

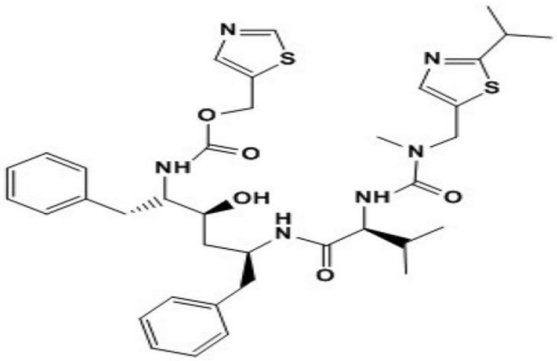


Fig 1: Structure of Ritonavir (13)

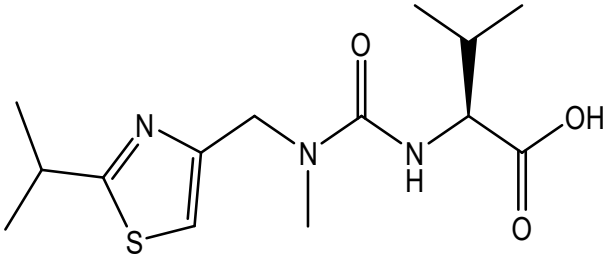


Fig 2: Structure of Ritonavir Impurity A (Free Base) (14)

1.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a flexible, non-destructive analytical method that works well with substances that are sensitive to heat. It is essential in the medical, chemical, biological and pharmaceutical research application industries because it allows extremely selective separations using a variety of stationary phases and detection techniques. (15)

1.2 Method Development

The selection of suitable mobile phase, columns, detectors, and quantization algorithms is a common step in method development. Important considerations include the following: current methods may be expensive, time-consuming, energy-intensive, or challenging to automate; they may lack adequate sensitivity or selectivity for the analytes of interest; they may be erroneous, prone to artifacts or contamination, or exhibit poor precision and reliability. Higher analyte identification, reduced detection limits, increased overall efficiency, precision and accuracy are all possible outcomes of equipment advancements (16,17).

1.3 Validation of Analytical Methods:

Method validation verifies that an analytical process yields precise, repeatable, and accurate results within a predetermined range. It offers recorded proof of dependable performance, and ICH recommendations state that important attributes including accuracy, precision, sensitivity and linearity should be assessed for all kinds of analytical techniques.
(18,19).

2.Materials and Procedures:

2.1 Chemicals and Substances

Ritonavir raw material was received from AA Scientifics, Hyderabad, Telangana, India. Reference standard with stated purity was 99.7% was obtained.

HPLC Grade water were supplied by Sd Fine (India), Mumbai, India (HPLC grade) and Acetonitrile were obtained from Loba Chem; Mumbai, India (HPLC grade). Ultrapure water was obtained from a Milli-Q purification system by Millipore. Further reagents and solvents of analytical quality were used. Marketed formulation, Ritonavir (labeled claim, ritonavir- 100 mg) was purchased from licensed pharmacy.

2.2 Equipment and Chromatographic Conditions:
The HPLC evaluation was executed on a Waters 2690/ 5 Model equipped with PDA detector wavelength was setted from 190 nm to 800 nm, and the pump mode was isocratic. The sample injection was performed with a 20 µL loop. C18 Phenomenex column (250 mm × 4.6 mm i.d, 5 µm particle size) was employed. In order to dilute the mobile phase with HPLC grade water and acetonitrile (20:80 v/v) and the flow-rate fixed to 1 mL/min. The temperature of the apparatus was room temperature. The volume of injection was 10 µL for all standards and samples. Empower SPs software was used to acquire the data and measure the identified peak locations.

2.3 Preparation of Mobile Phase:
After adding 80 mL (80%) of HPLC-grade acetonitrile and 20 mL (20%) of HPLC-grade water, the mixture was vacuum-filtered over a 0.45 µm filter membrane. Ten minutes in a water bath with ultrasonic, the dissolved gasses were eliminated.

2.4 Preparation of Standard Solution:
10mg of Ritonavir were dissolved in order to prepare the standard solution. The accurately weighed material was moved into a dry volumetric flask with a capacity of 10 ml. Little Diluents (Acetonitrile) was added to the reference drug and sonicated to dissolve the drugs. The same solvent was mixed to adjust the final volume. (Final Concentration for Ritonavir was 997.9 ug /mL).

2.5 Preparation of Sample Solution:
We bought commercially accessible 20 tablets of Ritonavir, precisely weighed, and ground into a powder in a mortar. Diluent was added to a 10 mL dry volumetric flask containing 10 mg of Ritonavir. To guarantee total dissolution, the mixture was filtered through a 0.45 µm filter and sonicated for 15 minutes. After that, acetonitrile was used to adjust the volume to the desired level. A 100 µg/mL solution was obtained by pipetting 1 mL of this stock solution into a 10 mL volumetric flask, and diluent was used to make up the remaining volume.

Table 1: Summary results of Ritonavir

RITONAVIR (API)				RITONAVIR (Tablet)			RITONAVIR Impurity (Imp- A Free base)		
S. No	Sample ID/ Injection	RT	Area	Sample ID/ Injection	RT	Area	Sample ID/ Injection	RT	Area
1	Ritonavir API Inj- 01	4.3	507350	Ritonavir Tab Inj- 01	3.3	272889	Impurity A Free base Inj- 01	2.3	42180
2	Ritonavir API Inj-02	4.3	506312	Ritonavir Tab Inj- 02	3.3	272210	Impurity A Free base Inj-02	2.3	41980
3	Ritonavir API Inj- 03	4.3	508126	Ritonavir Tab Inj- 03	3.3	271998	Impurity A Free base Inj- 03	2.3	41993
	Average	**4.3**	**507262**	**Average**	**3.3**	**272366**	**Average**	**2.3**	**42051**
	Std Dev	**0**	**910.1**	**Std Dev**	**0**	**466**	**Std Dev**	**0**	**111.9**
	%RSD	**0.1**	**0.2**	**%RSD**	**0.0**	**0.2**	**%RSD**	**0**	**0.3**

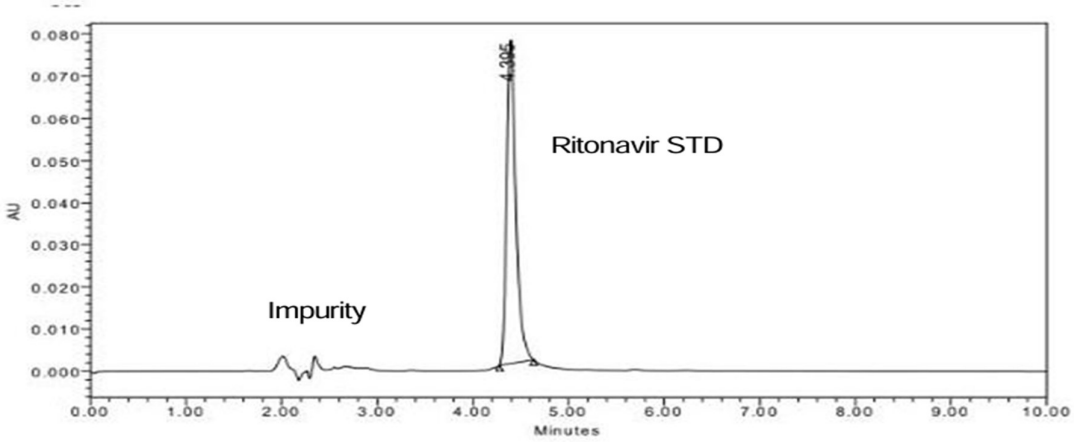


Fig 3: HPLC Chromatogram of Ritonavir API (100ug/ ml)

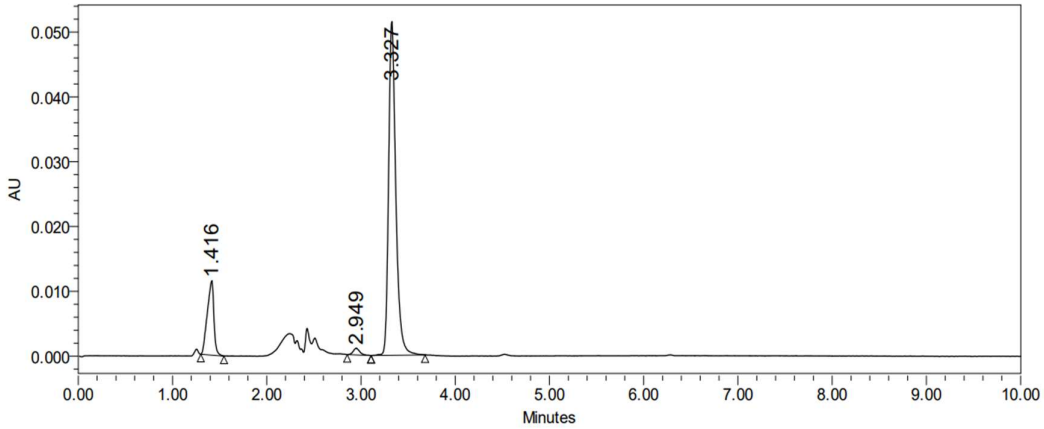


Fig 4: HPLC chromatogram of Ritonavir Tablet(100ug/mL)

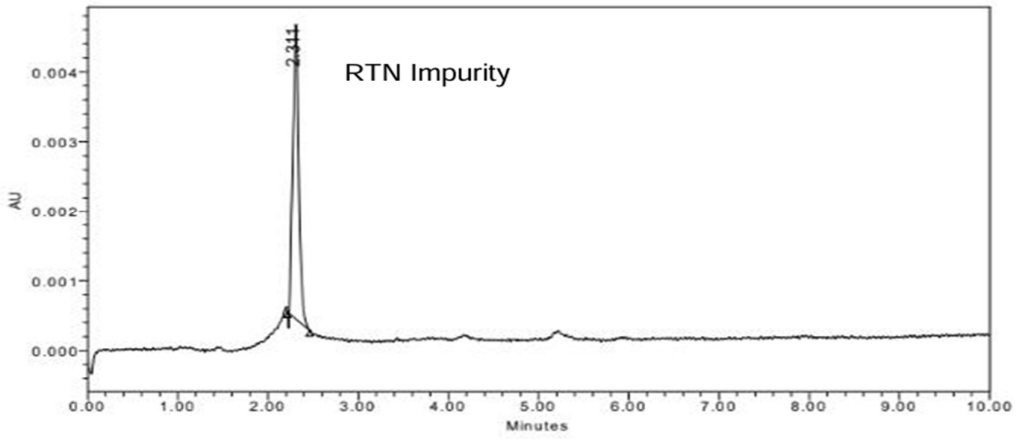


Fig 5: HPLC chromatogram of RTN Imp A (100ug/mL)

2.6 Preparation of Sample Solution (Tablets)

Twenty precisely measured readily accessible Ritonavir tablets were broken down in mortar. Diluent was added to a 10 mL dry volumetric flask containing an amount equal to 10 mg of Ritonavir. To ensure complete dissolution, the solution was filtered using a 0.45 µm filter and subjected to sonication for 15 minutes. Following this, acetonitrile was used to obtain the correct amount. To make a 100 ug/ml solution, 1 mL of the stock was added to a 10 mL volumetric flask and then solvent was added to reach the final volume.

2.6.1 Preparation of Stock Solution-08:
A 0.3 mL portion of the stock solution described earlier was placed into a 10 mL flask. Next, 9.7 mL of acetonitrile was added, and the mixture was adjusted to the 10 mL mark with the same diluent (acetonitrile) to achieve a solution concentration of 29.94 µg/mL.

2.6.2 Preparation of Stock Solution-07:
A 8 mL portion of the stock was placed into a 10 mL volumetric flask, then 2 mL of acetonitrile was added. The flask was filled to the mark with acetonitrile to create a solution with a concentration of 23.95 µg/mL.

2.6.3 Preparation of Stock Solution-06:
A 7 mL of the stock solution was placed into a 10 mL volumetric flask, add 3 mL of acetonitrile, then fill up to the mark with acetonitrile to make it 16.77 g/mL.

2.6.4 Preparation of Stock Solution-05:
Take 9 mL part of the stock and moved to a 10 mL flask. Add 1 mL of acetonitrile and then adjust up to mark. The final strength of 15.09 g/ml.

2.6.5 Preparation of Stock Solution-04:
Take 7 mL of the stock and placed to 10 mL flask. Add 3 mL of acetonitrile and then adjust up to mark. The final strength of 10.56 g/ml.

2.6.6 Preparation of Stock Solution-03:
A 7.5 mL of the stock was placed in a 10 mL flask, and added 2.5 mL of acetonitrile and then adjust up to mark. The final strength of 7.92 g/ml.

2.6.7 Preparation of Stock Solution-02:
Take 5.6 mL of stock solution was placed in a 10 mL flask. Added 4.4 mL of acetonitrile and then adjust up to mark. The final strength of 4.44 g/ml.

2.6.8 Preparation of Stock Solution-01:
Take 3.7 mL of stock solution was placed in a 10 mL flask. Added 6.3 mL of acetonitrile and then adjust up to mark. The final strength of 1.64 g/ml.

2.6.9 Preparation of HQC:
The 10 mL container was up to line with 0.32 mL of stock. Then, 9.68 mL of acetonitrile was added to the flask, and the total volume was adjusted to the calibration line with the solution, yielding a concentration of 31.94 g/mL.

2.6.10 Preparation of MQC:
We placed 5.2 mL of the solution in a 10 mL flask and then added 4.8 mL of acetonitrile. We then used the diluent acetonitrile to fill the flask to the mark, which gave us a strength of 16.61 g/mL.

2.6.11 Preparation of LQC:
An aliquot of 1.1 mL of the mentioned prior stock solution was transferred into a 10 mL volumetric flask, followed by the addition of 8.9 mL of acetonitrile, and the volume was made up to the mark with the diluent (acetonitrile) to obtain a final concentration of 1.83 µg/mL.

3. VALIDATION:
ICH Q2(R1) recommendations for accuracy, precision, linearity, selectivity, linearity and recovery were followed in the validation of the developed technique.

3.1 SELECTIVITY:
An analytical method's selectivity is its capacity to measure the target analyte precisely even in the presence of possible interferences in the sample matrix.
Procedure:
Inject diluent and evaluate the interference at RT of API. Inject each (Blank and LLOQ) calculate the peak area and retention time after entering the chromatographic procedure.

Table-2: Results of Selectivity – Ritonavir

S No	Sample ID	Analyte RT	Analyte Area
1	BLK-01	1.3	510
2	BLK-02	1.3	512
3	BLK-03	1.3	500
4	BLK-04	1.3	502
5	BLK-05	1.3	515
6	BLK-06	1.3	510
7	LLOQ-01	3.31	30742
8	LLOQ-02	3.31	30692
9	LLOQ-03	3.31	30665
	Average		**30699.7**
	Standard Deviation		**39.1**
	%RSD		**0.1**

Table 3: Results of Selectivity _ Interferences for Ritonavir

Sample ID	% Interference of Analyte-Ritonavir

BLK-01	1.66
BLK-02	1.67
BLK-03	1.63
BLK-04	1.64
BLK-05	1.68
BLK-06	1.66

Observation: Selectivity was determined by injecting blank and lowest limit of quantification (LLOQ) samples to ensure that the analyte retention time is free of interference. The percentage interference observed was below 2%, confirming method specificity.

3.2 LINEARITY:

Linearity is the ability of an analytical procedure to yield outcomes from tests that are precisely proportionally to the analyte level in samples within a given range. One must evaluate a direct correlation at each stage of the process. When a typical stock solution is diluted, the active pharmaceutical ingredient becomes clearly visible.

Procedure:

Injections were made into the chromatographic system at each point, and the peak areas obtained were measured. Calibration curves were plotted to evaluate linearity, with concentration along the X axis and the peak region along the Y axis. The r value was calculated.

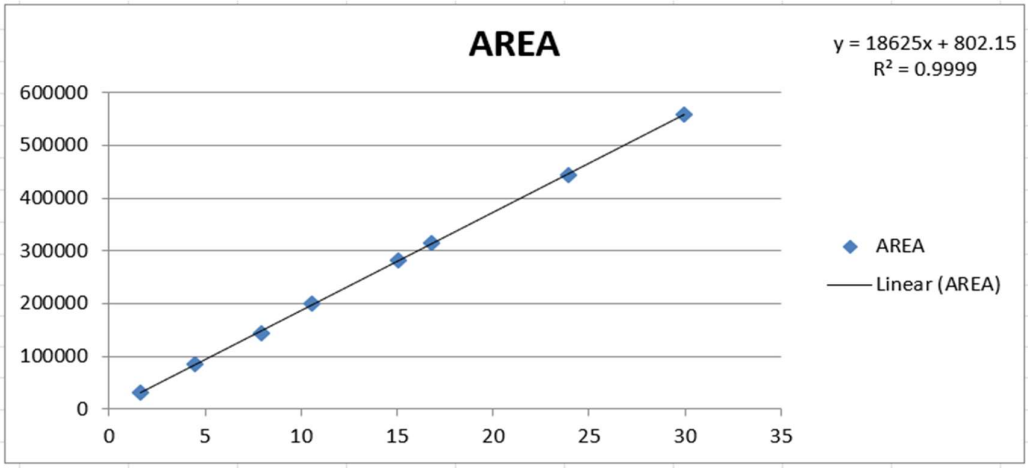


Fig 6: Calibration plots of Ritonavir

Table 4: Results of Linearity – Ritonavir

S.NO	SOLUTION	CONC	AREA
1	SS01	1.64	30743
2	SS02	4.44	84470
3	SS03	7.92	144704
4	SS04	10.56	200410
5	SS05	15.09	283053
6	SS06	16.77	314003
7	SS07	23.95	444506
8	SS08	29.94	559049
	Average		**257617.3**
	Standard Deviation		**180114.6**
	%RSD		**69.9**
	Correlation Coefficient		**0.9999**

Observation: This study shows linear the range from 1.64 to 29.94 ug/ml. Measurement charts were created by plotting the peak area against concentration. The slope measures 18,625 and the intercept through 802.15. The R squared (0.9999) indicates the approach is highly linear.

3.3 ACCURACY:

The degree of agreement between the test findings produced by the methods and the actual value is known as accuracy, and it must be shown over the specified range of the analytical process.

Procedure:

Prepared samples at three different strengths LQC,

MQC, and HQC at measured levels within predetermined limitations, the effectiveness of this approach was assessed and how much of each solution falls within the set limits. Developed method proved good and strong. The percent recoveries of ritonavir were between 92 and 98.

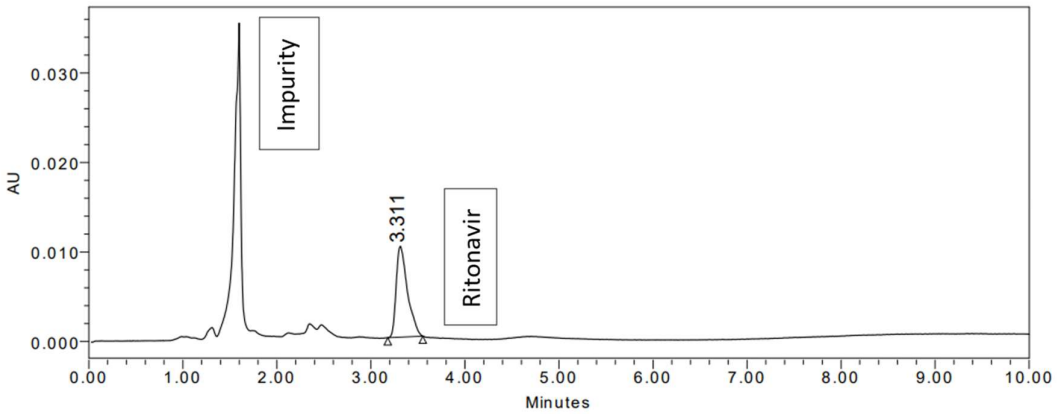


Fig 7: Chromatograms of Accuracy- Ritonavir

Table 5: Accuracy Batch- Ritonavir

S No	Sample	Concentration (ng/ml)	Analyte Area	Calculated Concentration	Accuracy
1	SS01	1.64	30743	1.61	98.1
2	SS02	4.44	84470	4.49	101.2
3	SS03	7.92	144704	7.73	97.6
4	SS04	10.56	200410	10.72	101.5
5	SS05	15.09	283053	15.15	100.4
6	SS06	16.77	314003	16.82	100.3
7	SS07	23.95	444506	23.82	99.5
8	SS08	29.94	559049	29.97	100.1

Table 6: Results of Accuracy: Concentration Vs Analyte for Ritonavir

S No	Sample	Concentration (ng/ml)	Analyte Area	Calculated Concentration	Accuracy
1	LQC-01	1.83	32256	1.69	92.4
2	LQC-02	1.83	31985	1.68	91.6
3	LQC-03	1.83	31853	1.67	91.2
4	LQC-04	1.83	32452	1.70	92.9
5	LQC-05	1.83	32842	1.72	94.1
6	LQC-06	1.83	31689	1.66	90.7
7	MQC-01	16.61	302532	16.20	97.5
8	MQC-02	16.61	306253	16.40	98.7
9	MQC-03	16.61	315623	16.90	101.8
10	MQC-04	16.61	304256	16.29	98.1
11	MQC-05	16.61	314236	16.83	101.3
12	MQC-06	16.61	302658	16.21	97.6
13	HQC-01	31.94	572523	30.69	96.1
14	HQC-02	31.94	581036	31.15	97.5
15	HQC-03	31.94	579842	31.09	97.3
16	HQC-04	31.94	579782	31.08	97.3
17	HQC-05	31.94	581123	31.16	97.5
18	HQC-06	31.94	586352	31.44	98.4

Table 7: Results of Accuracy: Percentage of Recovery.

S No	Sample	Ritonavir % Recovery

1	LQC	92.2
2	MQC	99.1
3	HQC	97.3
Mean		96.2

Observation: Evaluated by analyzing samples at test of three times at HQC, MQC and LQC. It is averaged recovery of 92% to 99% and a %RSD less than 2 for the quality control samples indicates that the test is reproducible.

3.4 PRECISION:

The precision of a method indicates the extent of congruence among multiple measured quantities extracted from repeated samples of the same substance under specified conditions.

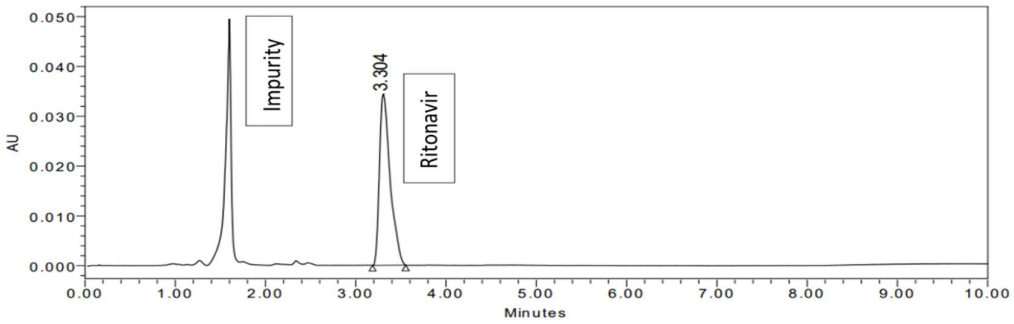


Fig 8: Chromatogram of Precision- Ritonavir

Table 8: Results of Precision- Ritonavir

Injection	Area for Ritonavir
Injection-1	572523
Injection-2	581036
Injection-3	579842
Injection-4	579782
Injection-5	581123
Injection-6	586352
Average	580109.7
Std Dev	4440.5
%RSD	0.77

Observation: Performed 6 repeat injections to check the precise and the results shows that %RSD at 0.77, well below the ICH limit of 2.0. The results support that the proposed method has outstanding repeatability.

4.Impurity Profiling

Unwanted chemical components that could be present in a medicinal ingredient or product are known as impurities. They may rise either from the drug manufacturing process, the materials used to make it, or they can form over time when the drug is stored or when it breaks down. Their presence can influence the drug's efficacy, safety, and overall stability, make their identification and control an essential aspect of pharmaceutical quality assurance (20).

4.1 Selection of Impurities

Impurity A (Free Base), associated with Ritonavir, is included in the USP and European Pharmacopoeia monographs as a known contamination that needs to be tested for precautionary measures. Product safety and efficacy depend on regulatory approval from organizations like the FDA and EMA.A crucial component of quality management is impurity monitoring. The drug's efficacy is changed by the impact of free base and Ritonavir salt on potency and solubility. Furthermore, Impurity-A occurs in stressed conditions such exposure to water or high temperatures, necessitating the development of stability indicating procedures.

5. In-silico Toxicity

Digital systems, which rely on algorithms developed, assist in detecting whether substances could pose harmful risks. These systems eliminate the requirement for animal testing and may decrease the necessity for such practices early in drug formulation. Platforms like PROTOX 3.0 and similar systems can analyze the outcomes of drug byproducts, such as causing liver damage, genetic modifications, or cancer. Using these tools in the initial phases of medicine design can result in safer drugs and lessen the use of animals.

5.1 Benefits of Study:

The use of computational methods provides notable advantages in pharmaceutical studies, including rapid processing of a large number of chemical arrangements. These systems facilitate efficient examination of vast molecular data and decrease resource expenditure.

5.2 PROTOX 3.0 software

In general, in silico studies the median lethal dose (LD_{50}) serves as a key indicator of acute toxicity. It helps to identify the safety or toxicity depends on the value. If LD_{50} is more than 5000 mg/kg represent safe. At the same time LD_{50} from 300 to 5000 mg/kg has toxic. Furthermore, the value has LD_{50} of 50 mg/kg or less, reported as highly toxic or fatal. This sorting method is a useful resource to gauge the initial hazard levels of impurities and active compounds at the outset of safety assessments.

Table 9: Impurity in In-silico Toxicity

Impurity	LD50 (mg/kg)	Predicted Organ Toxicity	Regulatory Concern
Ritonavir Impurity A	>2000	Respiratory Toxicity, Hepatotoxicity, Neurotoxicity &Clinical Toxicity	Ph. Eur./USP

Fig 9: Toxicology Chart for RTN Impurity: Ritonavir Impurity A

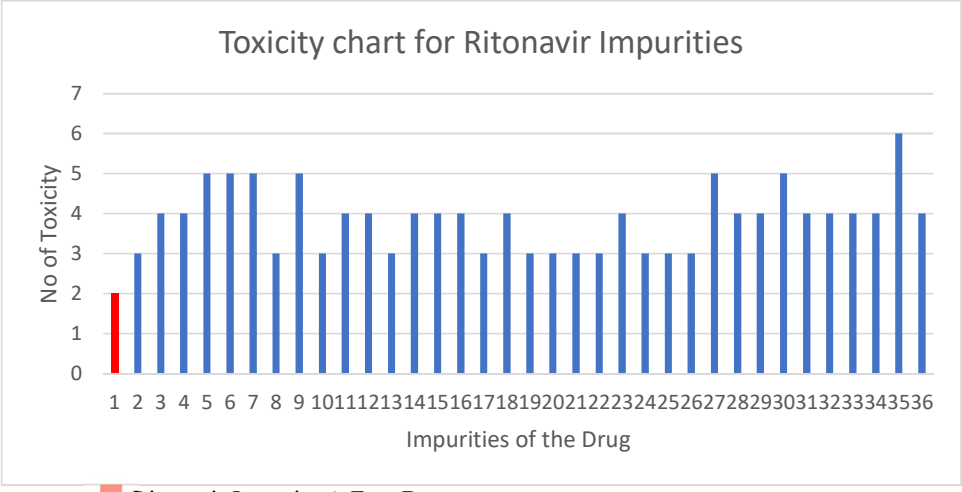
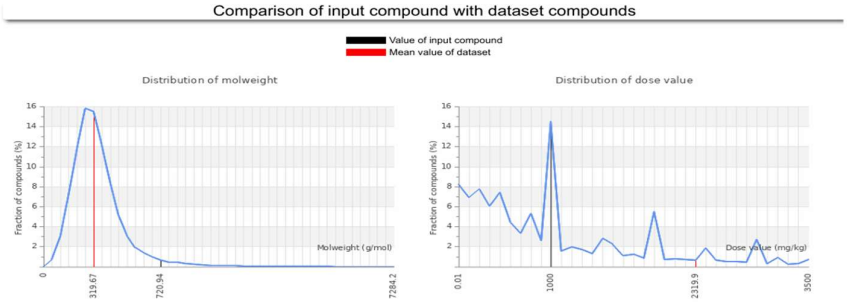


Fig 10: RTN Impurity – Impurity A Free Base



5.3 Cytotoxicity:

Testing for cellular activity is immense and moral work for medicine study hence cytotoxicity valuation is an essential part of medication development. These studies provide valuable information on a drug's therapeutic index and aid in predicting *in-vivo* toxicity.

Cytotoxicity testing procedures evaluate the ability of a substance to induce cellular damage or death and are an essential validation technique in both basic investigation and the development of drugs. (20). To ensure that antiviral medications like Ritonavir and Nirmatrelvir have selective antiviral activity without harming host cells, cytotoxicity testing is particularly important.

5.4 Types of Toxicity Studies:

Toxicity investigations are classified according to the type of toxic effects seen and the time of discovery. Before pharmaceutical substances are used in clinical settings, these assessments are essential for figuring out their safety profile. The following are the primary

categories:

• **Acute Toxicity**: A test to check for fatalities or temporary side effects following a single large dosage of a drug.

• **Sub-acute / Sub-chronic Toxicity:** For potential aggregation or effects on specific organs, continuous exposure over medium-term durations is assessed.

• **Chronic Toxicity:** Extensive investigations to understand long-term exposure harmful effects, particularly related to medical application.

• **Genotoxicity:** The evaluation of a material's ability to damage or change DNA.

• **Reproductive and Developmental Toxicity:** Assess progeny survival, embryonic development, and fertility.

• **Carcinogenicity**: Extensive studies on the chemical's ability to cause cancer.

The foundation for establishing safe exposure limits and ensuring that medications are appropriate for consumption by humans is these research studies.

5.5 Importance in Drug Development

Toxicity assessment is a crucial component of a new medication's initial strategy. Monitoring the cellular and systemic safety of active pharmaceutical compounds and their impurities makes it feasible to comprehend how a chemical interaction with biological mechanisms. By assessing toxicity, the therapeutic dose range is rendered safe and useful for human use.

Cytotoxicity screening supports the choice of appropriate lead compounds by providing important safe concentration ranges for ensuing pharmacological and effectiveness investigations. In order to ensure conformity with global norms like ICH S6(R1) and OECD test standards, this information are also required for regulatory filings. Overall, toxicity studies support mitigates potential health risks and form the scientific basis for risk–benefit evaluation during drug development and approval processes (21).

5.6 In Vitro Cytotoxicity Assays

In vitro cytotoxicity tests are a common way to measure how toxic compounds are to cells grown in a lab. These tests are carried out in sterile lab environments to determine if a compound causes cell damage. When cells are exposed to a test compound, these tests are fast, reliable, and low-cost methods to measure cell vitality and activity. The MTT test ranks among the top tests due to its ease of use, accuracy, and reliability (22).

The MTT test finds how active cells turns to a yellow tetrazolium salt called 3 [4, 5 dimethylthiazol-2-yl] 2 5 diphenyltetrazolium bromide into insoluble purple formazan. The change happens through the work of the cell mitochondria. The strength of the resulting colour, as measured by spectrophotometry, clearly correlates with the number of living cells. This method enables the ability to assess how exposure to medications affects cytotoxicity, proliferation, and metabolic integrity. (22).

This test is the best method for detecting poisons and medicines. Its cytotoxicity research provides valuable information on cellular safety and selectivity for antiviral medications such as ritonavir, supporting compliance with regulations and preclinical efficiency assessment. (23)

5.7 MTT Assay- General View

- In this experiment, metabolically active cells convert the yellow tetrazolium molecule, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), enzymatically into granules of impermeable purple formazan.
- After 24 to 72 hours of exposure to different doses of the test drug, MTT is applied to the wells containing the cultured cells. After the incubation period, spectrophotometry is used to measure the formazan's absorbance at 570 nm.
- Percentage of live cells compared to control samples determines cell survival rates.
- Dimethyl sulfoxide DMSO dissolves the formazan in the test to give clean optical density reading.
- In addition to its solubilizing function, DMSO has a number of applications in cell biology. In addition to having radioactive and cryoprotective properties, it can scavenge free radicals and encourage cell differentiation.
- DMSO has other uses in cell metabolism as well to its dissolution role. It can scavenge free radicals, promote cell differentiation, and offer radioprotective and cryoprotective qualities. Due to its ability to break down a wide range of organic, inorganic, hydrophilic, and hydrophobic compounds, it is a versatile reagent in cytotoxicity analysis. (24).

5.8 Advantages:

The MTT test is a well-known in vitro method for assessing cell viability and cellular toxicity.

Ease and simplicity of Use: This method requires minimal manual work and is easy to utilize.

Quantitative and reproducible: The accurate and reliable count of living cells that this colorimetric test yields can be easily measured using a spectrophotometer.

Flexibility: It can detect small variations in the number of live cells; the method is highly valuable for cytotoxicity and drug screening research.

Versatility: It can be applied to a variety of cell types, including adhering and suspended cultures.

Non-radioactive and Safe: In this approach radioactive elements are not used, hence its safer and more environmentally friendly.

Broad Applications: This method can be used to evaluate the development of cells, cell metabolism, and cytotoxic adverse reactions to possible new medications or pollutants. This approach has become a typical procedure in pharmaceutical safety and cell-based research, providing accurate results for recognizing drugs and efficacy evaluation. (24).

6. MTT Assay

6.1 PRINCIPLE:

The MTT assay relies on metabolically active cells converting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT), a yellow-colored tetrazolium salt, into insoluble purple formazan crystals. The reduction in mitochondria is caused by the mitochondrial succinate dehydrogenase enzyme, which is specific to living cells. The enzymes in the mitochondria will break down the tetrazolium ring when employing this method to increase cell viability, forming purple formazan inside the cell.

After treatment, the resulting crystals are dissolved using a suitable organic solvent, such as dimethyl sulfoxide (DMSO). The intensity of the purple colour, which is measured via spectroscopy at 570 nm, is directly connected to the number of live cells.

This idea allows researchers to measure metabolic rate and survival of cells. This test is widely used to assess the safety of pharmaceutical goods, find new drugs, and gauge the degree of chemical toxicity(25,26).

6.2 MATERIALS REQUIRED:

To evaluate in vitro cytotoxicity, the paragraph that follows supplies and chemicals were used:

- **Cell Line:** Peripheral Blood Mononuclear Cells (PBMC)
- **Culture Medium:** RPMI medium enchanced with 10% Fetal Bovine Serum (FBS)
- **Reagents:**

o MTT reagent (*3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide*) – 5 mg/mL stock solution (Sigma, USA)

o Dimethyl Sulfoxide (DMSO) – for solubilizing formazan crystals

o Phosphate-Buffered Saline (1X PBS) – for washing cells

o Antibiotic solution with 100 µg/mL penicillin and 100 µg/mL streptomycin.

o 96 micro tissue culture plates and washing basin supplied to Tarson (India).

To ensure reproducibility and avoid interference, the processes were conducted underneath septic circumstances using either laboratory or cell culture grade chemicals.

6.3 PROCEDURE

Cell culture

PBMCs (peripheral blood mononuclear cells) cultivated in liquid medium (RPMI) plus 10 % of bovine blood serum (FBS), 100 micrograms per millilitre penicillin, and 100 micrograms per millilitre streptomycin. The fluid was kept at 37° Celsius with 5% carbon dioxides.

6.4 MTT assay

In vitro cytotoxicity testing of the test specimen (Ritonavir (RT), Ritonavir Impurity-A Free base (1 - AFB)) was performed on PBMC cells using the MTT method. The harvested PBMC cells were aggregated in a 15 ml tube. The cells were then plated into a 96-well tissue culture plate at a density of 1×105 cells/ml cells/well (200 µL) in RPMI media with 10% FBS and 1% antibiotic solution for 24–48 hours at 37°C. The wells were cleaned in a serum-free RPMI medium at five distinct concentrations using a sterile PBS sample.

The cells were cultured for 24 hours at 37°C in a humidified 5% CO2 incubator after each sample was reproduced three times. Following incubation, each well received 10 µL of MTT at a concentration of 5 mg/ml. The cells were then incubated for an additional two to four hours, or until purple precipitates were clearly visible under an inverted microscope.

After aspirating the medium and MTT (220 µL) from the wells, 200 µl of 1X PBS was used for washing. Additionally, formazan crystals were dissolved by adding 100 µL of DMSO and shaking the plate for five minutes. A microplate reader (Thermo Fisher Scientific, USA) was used to measure the absorbance at 570 nm for each well. Graph Pad Prism 6.0 software was then used to determine the IC50 value and the percentage of cell viability. (26).

Formula Cell viability % = Test OD/Control OD X 100

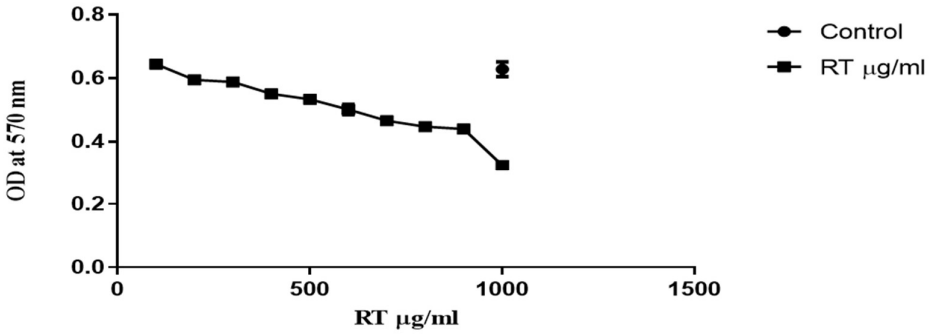


Fig 11: Graph of sample Ritonavir (RT)

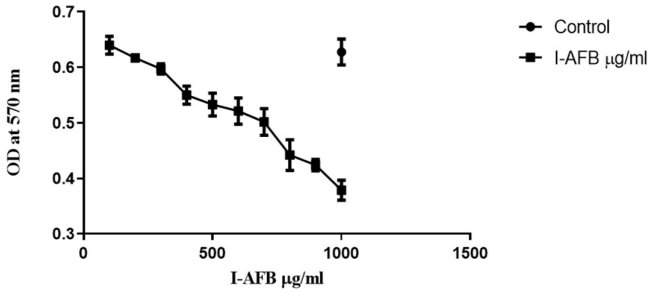


Fig 12: Graph of Ritonavir Impurity A Free base (I- AFB)

4.1: Results of Cell Viability (%)

Table:10: Cell Viability of same sample Ritonavir (RT)

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	1000 µg/ml	54.0496	52.32	49.2331	51.867572
3	900 µg/ml	71.7355	69.76	68.7117	70.069065
4	800 µg/ml	73.719	74.08	66.2577	71.352226
5	700 µg/ml	75.0413	74.88	72.8528	74.258028
6	600 µg/ml	85.2893	77.44	76.9939	79.907707
7	500 µg/ml	88.595	85.44	81.135	85.05667
8	400 µg/ml	90.7438	87.36	85.2761	87.793292
9	300 µg/ml	98.5124	91.84	90.9509	93.767772
10	200 µg/ml	99.3388	95.36	90.0307	94.909839
11	100 µg/ml	109.091	100.64	98.3129	102.68126

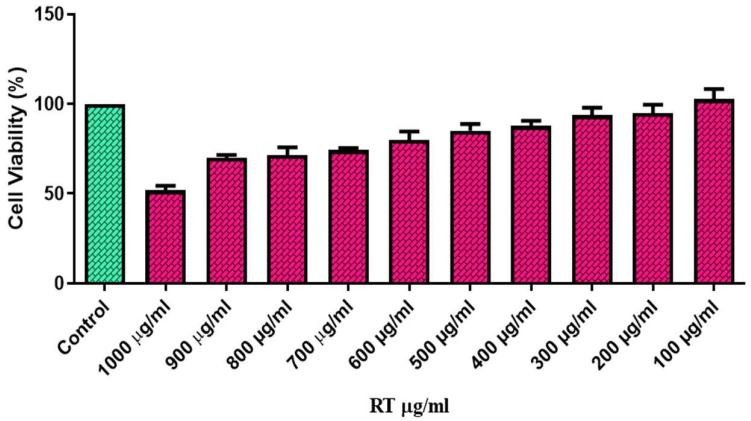


Fig 13: Results of Cell Viability for Ritonavir

Table 11: Cell Viability of sample Ritonavir Impurity A Free base (1-AFB)

S. No.	Tested sample concentration (µg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1	Control	100	100	100	100
2	1000 µg/ml	61.6529	63.84	55.8282	60.440371
3	900 µg/ml	68.0992	68.64	66.1043	67.614489

4	800 µg/ml	70.2479	75.84	65.4908	70.526244
5	700 µg/ml	81.3223	77.44	81.135	79.965761
6	600 µg/ml	81.8182	84.48	82.9755	83.091214
7	500 µg/ml	84.9587	84.64	85.1227	84.907126
8	400 µg/ml	88.595	87.36	87.1166	87.690535
9	300 µg/ml	99.0083	96.96	90.0307	95.33298
10	200 µg/ml	101.157	99.36	94.7853	98.4341
11	100 µg/ml	102.975	102.72	100.46	102.05178

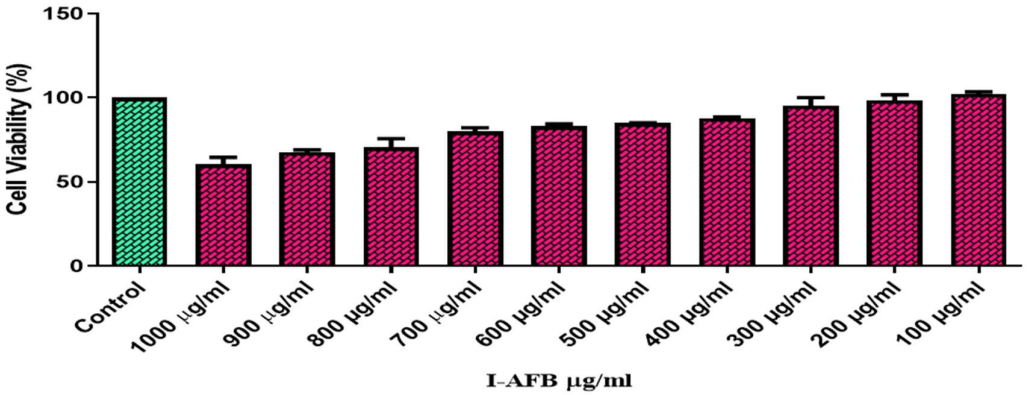


Fig 14: Results of Cell Viability for Ritonavir Impurity – A Free Base

Table 12: OD Values of sample at 570 nm (RT and I -AFB)

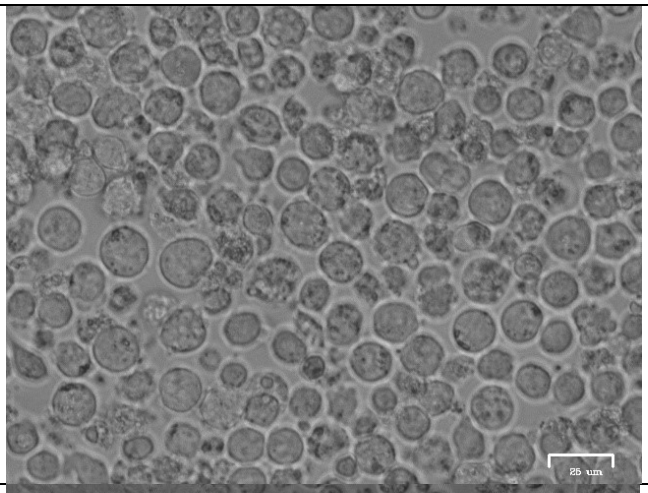
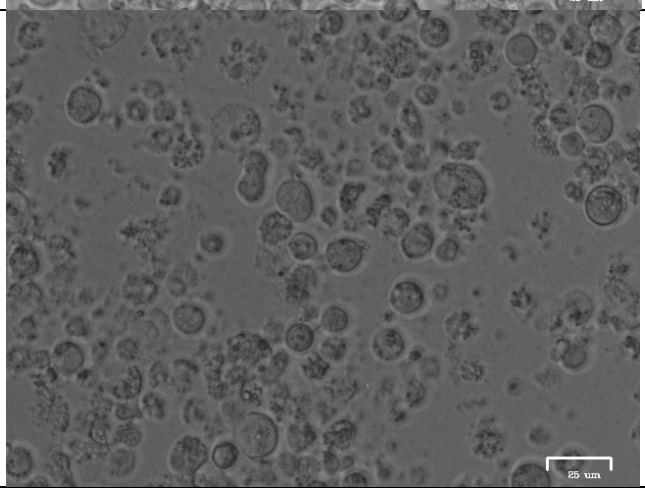
S. No.	Tested sample concentration (µg/ml)	OD value at 570 nm (in triplicates) RT			OD value at 570 nm (in triplicates) I-AFB		
1	Control	0.605	0.625	0.652	0.605	0.625	0.652
2	1000 µg/ml	0.327	0.327	0.321	0.373	0.399	0.364
3	900 µg/ml	0.434	0.436	0.448	0.412	0.429	0.431
4	800 µg/ml	0.446	0.463	0.432	0.425	0.474	0.427
5	700 µg/ml	0.454	0.468	0.475	0.492	0.484	0.529
6	600 µg/ml	0.516	0.484	0.502	0.495	0.528	0.541
7	500 µg/ml	0.536	0.534	0.529	0.514	0.529	0.555
8	400 µg/ml	0.549	0.546	0.556	0.536	0.546	0.568
9	300 µg/ml	0.596	0.574	0.593	0.599	0.606	0.587
10	200 µg/ml	0.601	0.596	0.587	0.612	0.621	0.618
11	100 µg/ml	0.66	0.629	0.641	0.623	0.642	0.655

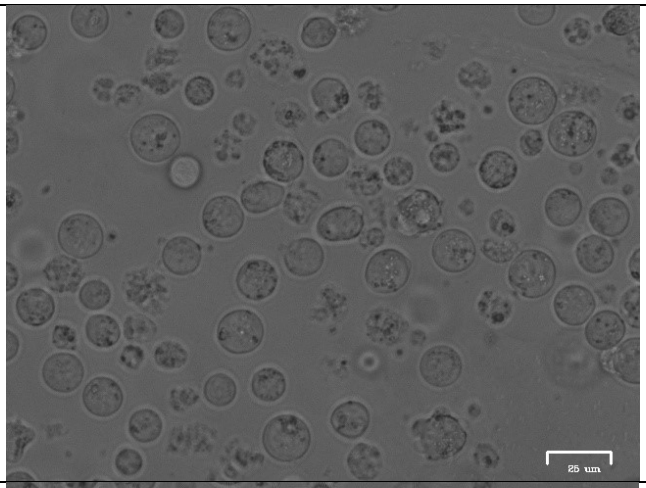
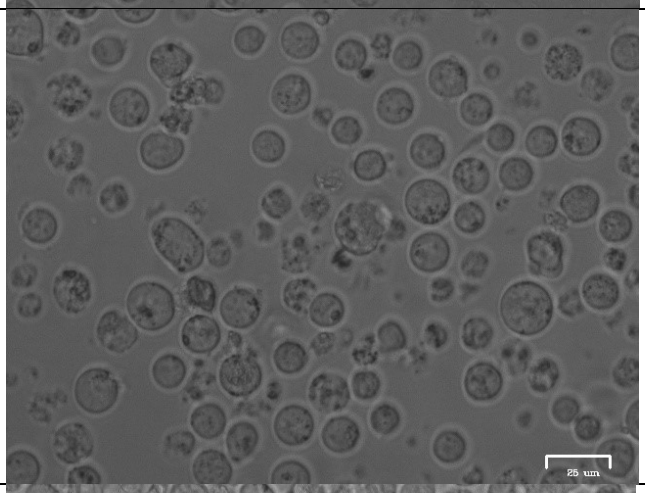
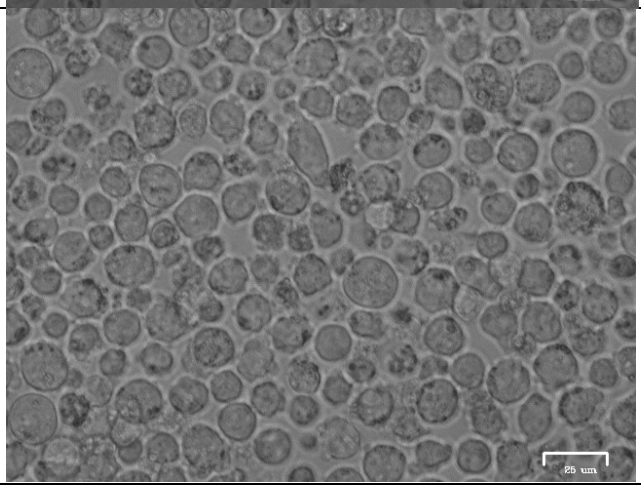
Table 13: IC50 Value of tested sample (RT and I -AFB)

Log(inhibitor) vs Normalized Response	Ritonavir (RT)	Ritonavir Imp A Free Base(I-AFB)
Best-fit values		
LogIC_{50}	2.784	2.732
IC50 value of test sample (µg/ml)	608.4	539.4
95% CI (profile likelihood)		

LogIC50	2.682 to 2.888	2.609 to 2.856
IC50	480.9 to 772.1	406.3 to 717.0
Degrees of Freedom	29	29
R squared	0.71	0.6663
Sum of Squares	6704	10077
Sy.x	15.2	18.64
# of X values	30	30
# Y values Analyzed	30	30

Fig 15: Images of control cells and treated cells- Ritonavir (RT)

CONC.	**IMAGE**
CONTROL	
1000 µg/ml	

800 µg/ml	
500 µg/ml	
300 µg/ml	

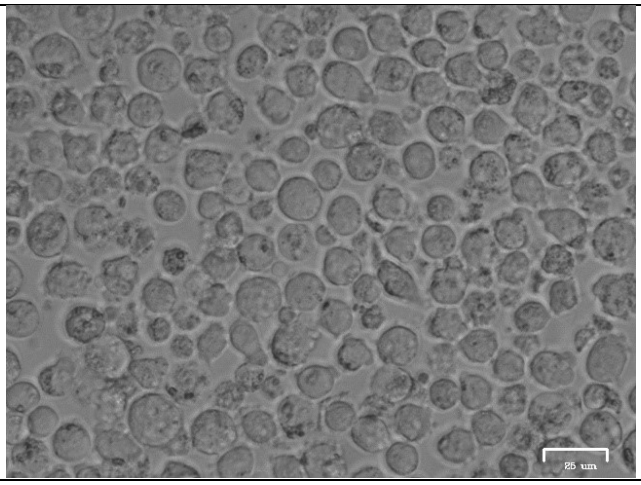
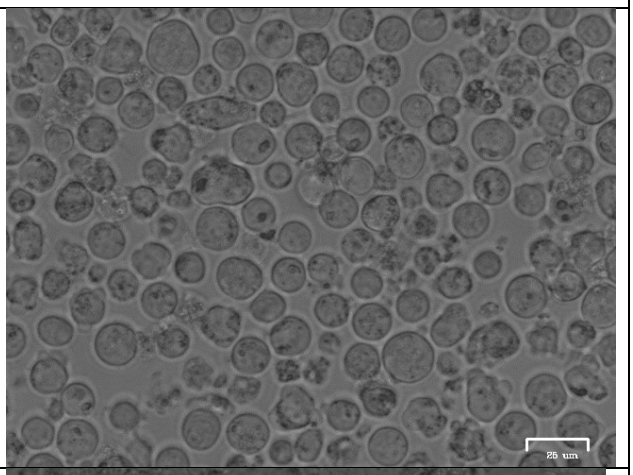
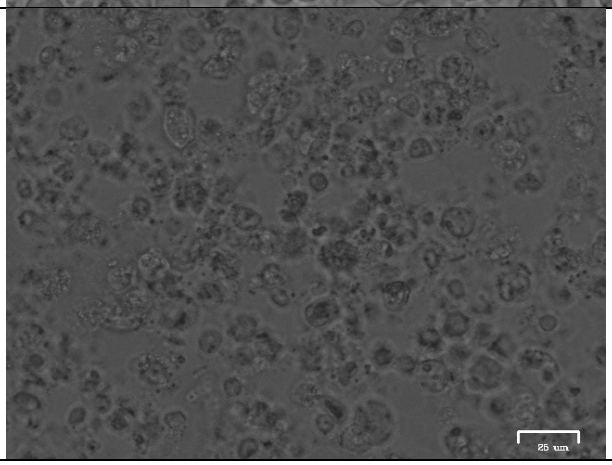
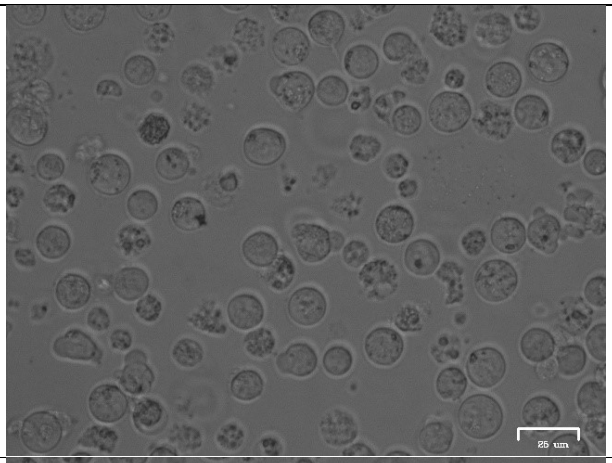
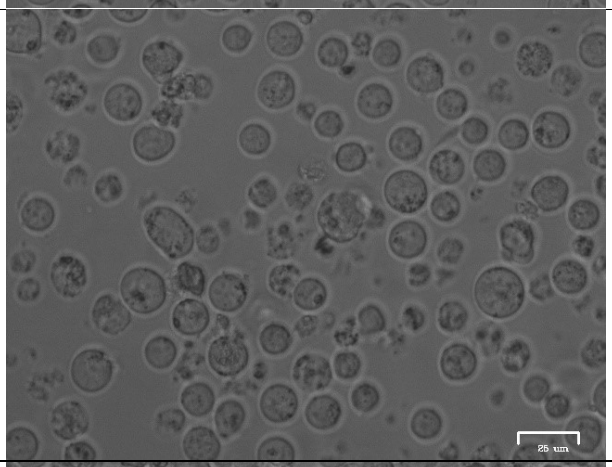
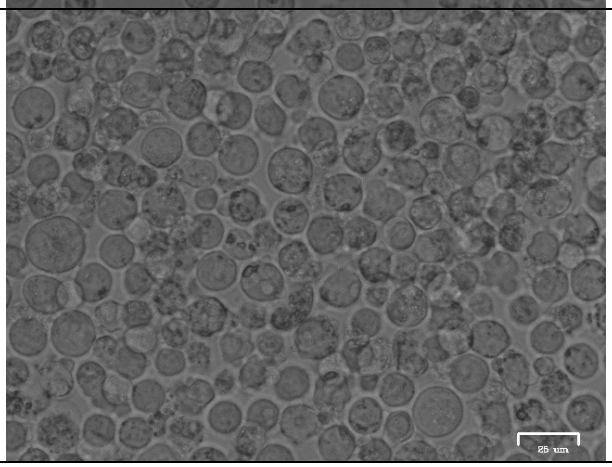
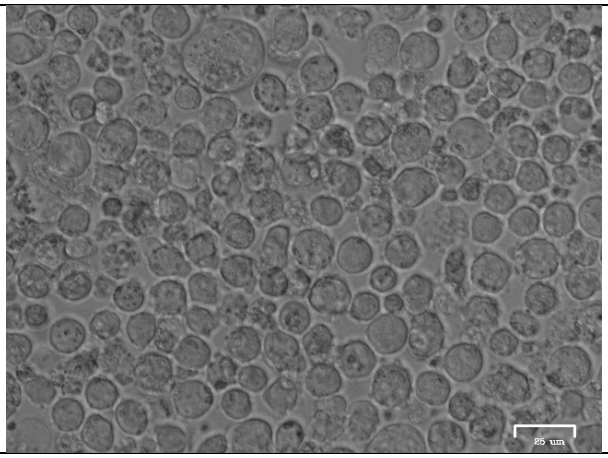
100 µg/ml	

Fig 16: Images of control cells and treated cells- Ritonavir Impurity A Free Base (I- AFB)

CONC.	**IMAGE**
CONTROL	
1000 µg/ml	

800 µg/ml	
500 µg/ml	
300 µg/ml	

100 µg/ml	

7.Results and Discussion

Outstanding selectivity and resolution were demonstrated by the devised HPLC technique as shown in Fig 3-5 and there was no discernible interference at the retention durations that corresponded to the target analytes (Table-1). With a correlation value (R^2) of 0.9999, Ritonavir demonstrated excellent linearity within the strength range of 1.64–29.94 µg/mL, supporting the calibration model's resilience (Tables 2-4 and Fig-6). To check the accuracy of the method, recovery tests were used, and they showed an average recovery rate of 96.2%, which means the testing method was high dependability and repeatability as shown in Fig-7 and in Tables 5-7.

Comprehensive impurity research revealed Ritonavir Impurity-A to be an important related compound (Fig - 8 &Table-8). Mucosal tissues are known to be irritated and hypersensitized by this contaminant, which is a reactive cyclic anhydride. As Impurity-A's free base may have inconsistent stability and absorption properties, managing it throughout drug development is essential. According to ICH Q3A/B, these contaminants need to be closely monitored and controlled in order to meet the standards for medication efficacy and dependability.

An in-silico toxicity study that revealed no appreciable signs of acute oral poisoning (LD50 > 2000 mg per kilogram) concluded that Ritonavir Impurity-A was safe (Table-9 & Fig 9-10). The importance of managing contaminants at every stage of manufacturing and maintenance is shown by analyses that projected mutation impacts and mild liver injury. These studies confirm that contaminant detection methods are essential for antiviral medications to ensure effectiveness and compliance.

8.Conclusion:

Ritonavir and its impurities (Impurity-A Free Base) can now be consistently, promptly and affordably checked. The method demonstrated good specificity, linearity, accuracy, and precision and fully complied with ICH Q2(R1) validation parameters. With distinct peaks and minimal interference, this separation proved to be successful. Periodic evaluations may measure the effectiveness of Ritonavir in both bulk and dosage forms. The chromatographic separation was efficient, with well-resolved peaks and minimal interference. Ritonavir's potency can be evaluated in the bulk and dose forms by routine tests.

Overall, the efficacy of Ritonavir Impurity-A is better supported by the combined in vitro and in silico toxicity testing. The predictive models immediately predicted a minimal risk of toxicity and minimized the possibility of hepatotoxicity or mutation. The contaminant was not extremely damaging to cells at the measured dosages, according to the lab data (Fig 11-15 & Table 10-13). A thorough approach for testing and analysing impurities in antiviral medications is added by this work. Pharmaceutical quality assurance and stability analysis are made easier by the proven HPLC technique's integration with predictive toxicity assessment, which ensures patient safety and regulatory compliance.

9.AUTHORS CONTRIBUTION:

The Authors Confirm their Contribution to the paper as follows: Study Conception and design were contributed by SJ; data collection and contributed by BR; analysis and interpretation of results were contributed by KN, TP and JN: Manuscript drafted by EM, SD. All the authors reviewed the results and approved the final version of the manuscript.

10. CONSENT FOR PUBLICATIONS

Not Applicable

11. CONFLICT OF INTEREST

The Author declares no conflict of interest, financial or otherwise

12. FUNDING

None

13. ACKNOWLEDGEMENTS

Declared none

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15. LIST OF ABBREVIATIONS:

HPLC- High Performance light Chromatography
RTN – Ritonavir
1-AFB- Ritonavir Impurity A Free Base
C18- Octadecyl carbon Chain
%RSD- Relative Standard Deviation
COVID-19- Coronavirus Disease 2019
ICH- International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
SARS-CoV-2- Severe acute respiratory syndrome coronavirus 2
3 CL Protease- 3-chymotrypsin-like protease
GC- Gas Chromatography
PDA- Photodiode Array detector
API- Active Pharmaceutical Ingredient
HQC-High Quality Control
MQC- Medium Quality Control
LQC- Low Quality Control
RT- Retention Time
LLOQ- Lowest Limit of Quantification
LD- Lethal Dose
1-AFB- Ritonavir Impurity A Freebase
OECD- Organization for Economic Co-operation and Development.
MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO- Dimethyl sulfoxide
PBMC- Peripheral Blood Mononuclear Cells
RPMI- Phenol red-free media
OD- Optical Density
IC50- Half-maximal inhibitory concentration