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DEVELOPMENT AND VALIDATION OF AN RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR SULPHATE AND RITONAVIR IN BULK AND TABLET DOSAGE FORMS

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Abstract:

A robust and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for the simultaneous estimation of Atazanavir Sulphate and Ritonavir in bulk and tablet dosage forms. The development process involved a systematic optimization of chromatographic parameters, including the selection of wavelength, mobile phase composition, flow rate, and column. UV-spectrophotometric analysis revealed maximum absorbance for Atazanavir Sulphate and Ritonavir at 249 nm and 239 nm, respectively. However, a detection wavelength of 210 nm was chosen for HPLC due to optimal peak response for both drugs.

Several chromatographic trials were conducted to optimize the conditions. The final optimized method employed a Nucleodur C18 column (150 mm × 4.6 mm × 5 µm), with a mobile phase composed of acetonitrile, methanol, and phosphate buffer (pH 3.0 adjusted with orthophosphoric acid) in the ratio 44:11:45 v/v/v. The flow rate was maintained at 1.5 mL/min and the injection volume was 20 µL. Under these conditions, Atazanavir and Ritonavir were eluted with sharp, symmetrical peaks and good resolution, with retention times of approximately 3.13 and 6.10 minutes, respectively.

Method validation was performed in accordance with ICH guidelines. Specificity studies confirmed the absence of interference from excipients, with no peaks observed in blank or placebo chromatograms. System suitability parameters, including theoretical plates, resolution, tailing factor, and symmetry factor, were all within acceptable limits, confirming the adequacy of the system.

Linearity was demonstrated for Atazanavir Sulphate (34–102 µg/mL) and Ritonavir (10–30 µg/mL), with correlation coefficients (r^2) of 0.999 for both drugs. Precision was established by evaluating method repeatability; %RSD values were 0.12% for Atazanavir and 1.39% for Ritonavir, indicating excellent reproducibility. Accuracy was confirmed through recovery studies at 80%, 100%, and 120% levels, yielding recoveries within the range of 99.91% to 100.40% for Atazanavir Sulphate and 100.12% to 100.31% for Ritonavir.

Robustness was evaluated by altering flow rates slightly (±0.1 mL/min), and the method showed negligible variations with %RSD well below 2%, proving its consistency. Ruggedness, assessed by intraday and intermediate precision with different analysts, yielded %RSD values below 1% for both drugs, confirming the method's analyst-independence and reproducibility.

The method also demonstrated high sensitivity with limits of detection (LOD) and quantitation (LOQ) of 0.002 µg/mL and 0.016 µg/mL for Atazanavir Sulphate, and 0.001 µg/mL and 0.004 µg/mL for Ritonavir, respectively.

In conclusion, the developed RP-HPLC method is simple, accurate, precise, rapid, and cost-effective. Its high sensitivity, robustness, and reproducibility make it highly suitable for routine quality control analysis of Atazanavir Sulphate and Ritonavir in both bulk drug substances and tablet dosage forms.

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INTRODUCTION:

High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique widely used in pharmaceutical, chemical, and biological research for the separation, identification, and quantification of components in a mixture. It operates on the principle of liquid chromatography, where a liquid mobile phase carries the sample through a stationary phase packed in a column. The different components of the sample interact differently with the stationary phase, leading to their separation based on polarity, size, or other chemical properties.

HPLC is preferred for its high resolution, accuracy, and sensitivity. It is especially useful in the analysis of drugs, bioactive compounds, and degradation products. The system includes a solvent reservoir, pump, injector, column, detector, and data processor. Various types of HPLC methods—such as reverse-phase, normal-phase, ion-exchange, and size-exclusion—are selected based on the nature of the sample and the objective of the analysis.

Detection is typically carried out using UV-Vis, fluorescence, or mass spectrometry detectors. HPLC is essential in quality control, pharmacokinetic studies, and stability testing due to its ability to deliver precise and reproducible results. Its role in method development and validation makes it a cornerstone of modern analytical chemistry.

Validation Parameters in HPLC Method Validation

HPLC method validation ensures that an analytical method is reliable, accurate, and suitable for its intended purpose. The key validation parameters as per ICH guidelines (Q2(R1)) include:

1. Specificity: Ability to measure the analyte clearly in the presence of other components like impurities, degradants, or excipients.
2. Linearity: The method's ability to produce results that are directly proportional to the concentration of analyte within a given range.
3. Accuracy (Recovery): Closeness of test results to the true value. Typically assessed by recovery studies at different concentration levels (e.g., 80%, 100%, 120%).
4. Precision: Reproducibility of the method under the same conditions.
   - Repeatability: Intra-day variation (same analyst, equipment, day).
   - Intermediate Precision: Variation between days, analysts, or instruments.
5. Limit of Detection (LOD): The lowest amount of analyte that can be detected but not necessarily quantified.
6. Limit of Quantification (LOQ): The lowest amount of analyte that can be quantitatively determined with acceptable precision and accuracy.
7. Robustness: The method’s capacity to remain unaffected by small, deliberate variations in method parameters (e.g., pH, temperature, flow rate).
8. Ruggedness: Reproducibility of results under varied conditions like different laboratories or analysts.
9. Range: The interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity.

These parameters collectively ensure the reliability and scientific validity of the HPLC method.

MATERIALS AND METHODS:

Equipments Used

S.No	Name	Make
1	Weighing Balance	SHIMADZU
2	pH Meter	ELCHEM
3	Sonicator	SAISONIC
4	HPLC System	SHIMADZU LC-2010 with UV Detector
5	UV-Visible Spectrophotometer	PERKIN ELMER with LAMBDA 25

Chemicals Used

S.No	Name	Grade / Make
1	Phosphate Buffer	HPLC Grade (MERCK)
2	Acetonitrile	HPLC Grade (MERCK)
3	Methanol	HPLC Grade (MERCK)
4	Orthophosphoric Acid	HPLC Grade (RANCHEM)
5	Purified Water	HPLC Grade (Milli-Q)

Active Pharmaceutical Ingredients (Pure Drugs)

S.No	Name	Specification
1	Atazanavir Sulphate & Ritonavir	Reference Standard

Marketed Formulation

S.No	Name	Manufacturer
1	Atazotec Tablets	Sai Mirra Innopharm

Solubility Study

Solubility of the drugs was tested in various solvents. It was observed that both Atazanavir sulphate and Ritonavir showed good solubility in organic solvents like acetonitrile and methanol, but were poorly soluble in water and alkaline medium.

Solubility of Drugs in Different Solvents

Solvent	Solubility
Water	– (Insoluble)
Acetonitrile	+ (Soluble)
Methanol	+ (Soluble)
0.1 N NaOH	– (Insoluble)

Method Development

Determination of λ max of Atazanavir Sulphate (UV-Visible Spectrophotometry)

Sample Preparation:

- Accurately weighed 25 mg of Atazanavir sulphate was transferred into a 50 mL clean, dry volumetric flask.
- Added 50 mL of methanol, sonicated for 5 minutes, and filtered through a 0.45 µm nylon membrane filter.
- From this, 5 mL was pipetted into another 50 mL volumetric flask and diluted to volume with methanol.
- The resulting solution was scanned in the UV-Visible spectrophotometer between 200–400 nm to determine λ

Determination of λ max of Ritonavir (UV-Visible Spectrophotometry)

Sample Preparation:

- Accurately weighed 25 mg of Ritonavir was transferred into a 50 mL clean, dry volumetric flask.
- Added 50 mL of methanol, sonicated for 5 minutes, and filtered through a 0.45 µm nylon membrane filter.
- 5 mL of this solution was pipetted and diluted to 50 mL with methanol.
- The solution was scanned between 200–400 nm using UV-Visible spectroscopy.

Determination of Isosbestic Point of Atazanavir Sulphate and Ritonavir

Sample Preparation:

- Accurately weighed 25 mg each of Atazanavir sulphate and Ritonavir were transferred into a 50 mL clean, dry volumetric flask.
- 50 mL of methanol was added, sonicated for 5 minutes, and filtered through a 0.45 µm nylon membrane filter.
- Equal volumes (5 mL each) of both solutions were mixed and diluted to 50 mL with methanol.
- The combined solution was scanned between 200–400 nm to determine the isosbestic point.
- Isosbestic point observed

Method Development Trials

Several trials were carried out using different mobile phase combinations and flow rates on a Shimadzu LC-2010 HPLC system with UV detector and isocratic pump, controlled via LC Solution software.

- Flow rates tried: 0.8 mL/min to 1.5 mL/min
- Objective: Achieve sharp, well-resolved, and reproducible peaks for both drugs.

OPTIMIZED METHOD

Buffer Preparation:

- Dissolved 6.8 g of potassium dihydrogen orthophosphate in 1000 mL of HPLC grade water.
- Adjusted pH to 3.0 with orthophosphoric acid.
- Filtered through a 0.45 µm nylon membrane filter and degassed by sonication.
- Used as diluent and for mobile phase preparation.

Mobile Phase Preparation:

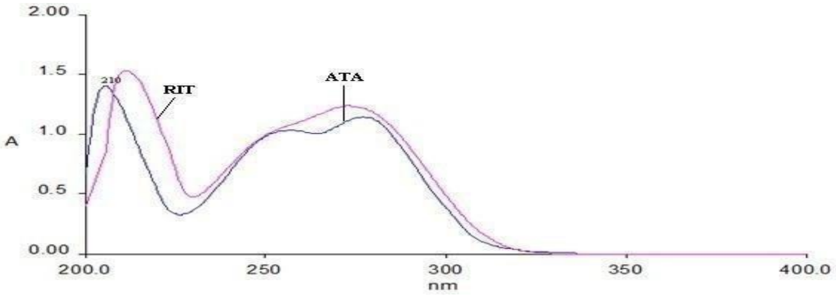
- Mixed Acetonitrile : Methanol : Buffer (pH 3.0) in the ratio 44:11:45 v/v.
- Sonicated for 10 minutes and filtered through a 0.45 µm nylon membrane filter.

Standard Solution Preparation:

- Accurately weighed 68 mg of Atazanavir sulphate and 20 mg of Ritonavir into a 100 mL volumetric flask.
- Dissolved and made up to volume with methanol.
- From this, 5 mL was diluted to 50 mL with the mobile phase.
- Final concentrations: Atazanavir sulphate – 68 µg/mL, Ritonavir – 20 µg/mL

RESULTS AND DISCUSSION:

Selection of wavelength:



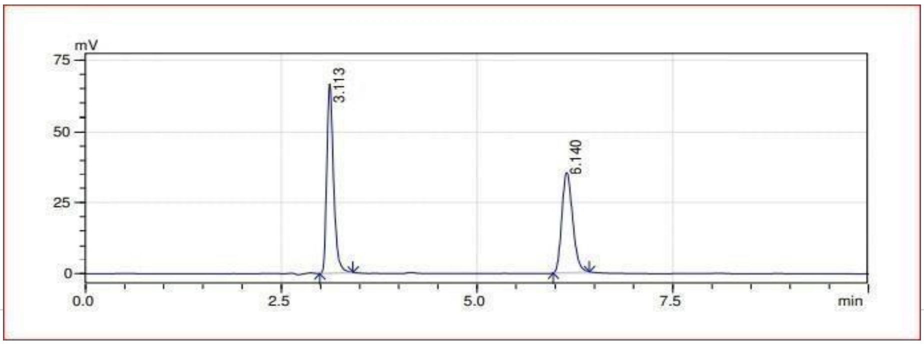
Observation: It was concluded that Atazanavir sulphate shows maximum absorbance at 249nm.

It was concluded that Ritonavir shows maximum absorbance at 239 nm.

Comparative Table of Chromatographic Conditions

Trial	Column	Mobile Phase (ACN:MeOH:Buffer pH 3.0)	Flow Rate (mL/min)	Wavelength (nm)	Injection Volume (µL)	Observation
Trial-1	Nucleodur C8 (100 mm × 1.8 mm × 5 µm)	65 : 5 : 30	0.8	210	20	Peaks eluted, but poor resolution
Trial-2	Nucleodur C8 (100 mm × 1.8 mm × 5 µm)	25 : 5 : 70	0.9	210	20	Theoretical plates > 2000, but column changed for better efficiency
Trial-3	Nucleodur C18 (150 mm × 4.6 mm × 5 µm)	60 : 10 : 30	1.0	210	20	Peak broadening observed in Ritonavir
Trial-4	Nucleodur C18 (150 mm × 4.6 mm × 5 µm)	50 : 10 : 40	1.2	210	20	Atazanavir eluted well, Ritonavir had delayed retention time
Optimized	Nucleodur C18 (150 mm × 4.6 mm × 5 µm)	44 : 11 : 45	1.5	210	20	Both drugs eluted properly with good resolution and peak shape (final method)

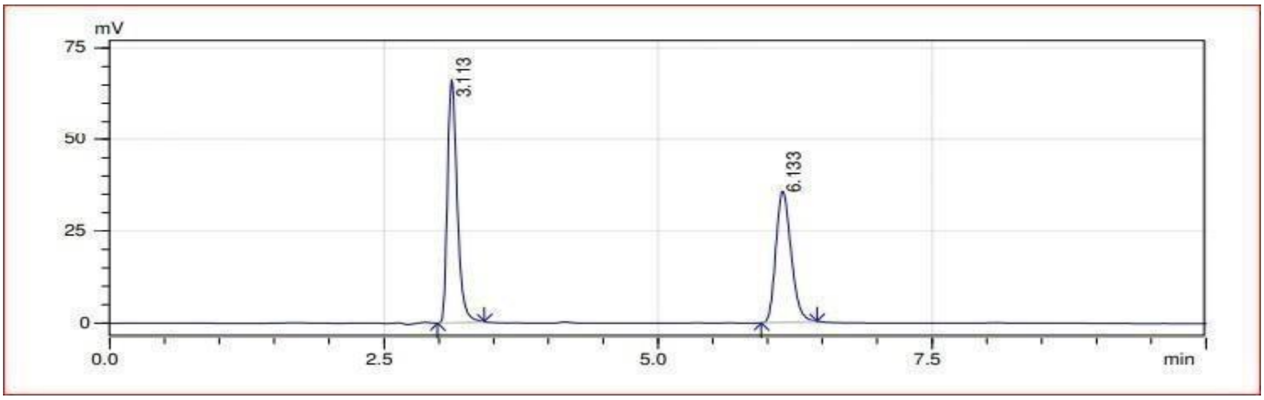
Optimized TRAIL :5



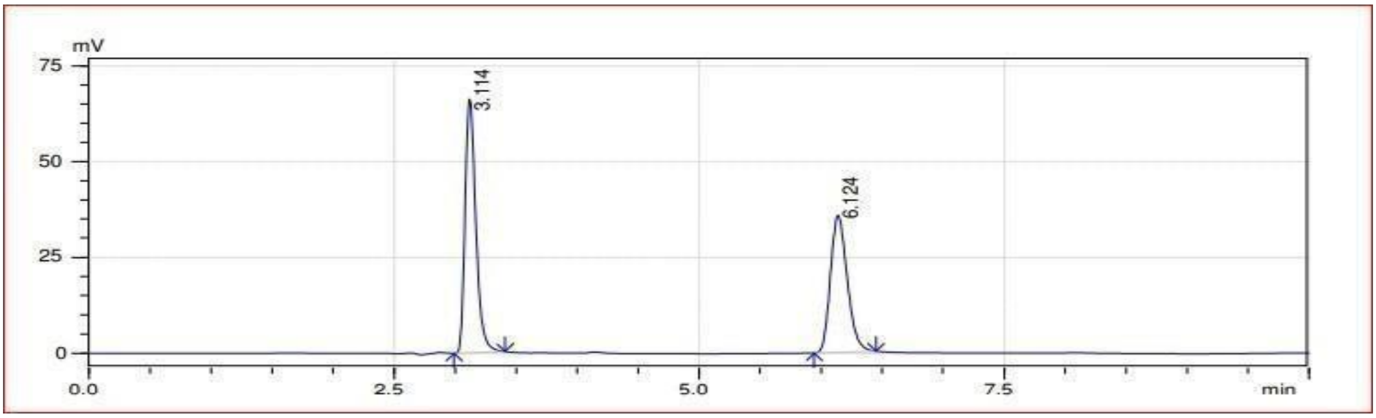
ASSAY:

Assay Data for Atazanavir Sulphate and Ritonavir (Standard and Tablet)

S.No.	Standard Peak Area		Tablet Peak Area	
	Atazanavir	Ritonavir	Atazanavir	Ritonavir
1	1,970,033	568,671	1,972,526	555,272
2	1,974,636	560,152	1,976,472	555,303
3	1,945,756	560,190	1,977,694	553,068
Average	1,963,475	560,181	1,975,564	554,547



Standard chromatogram of Atazanavir sulphate and Ritonavir.



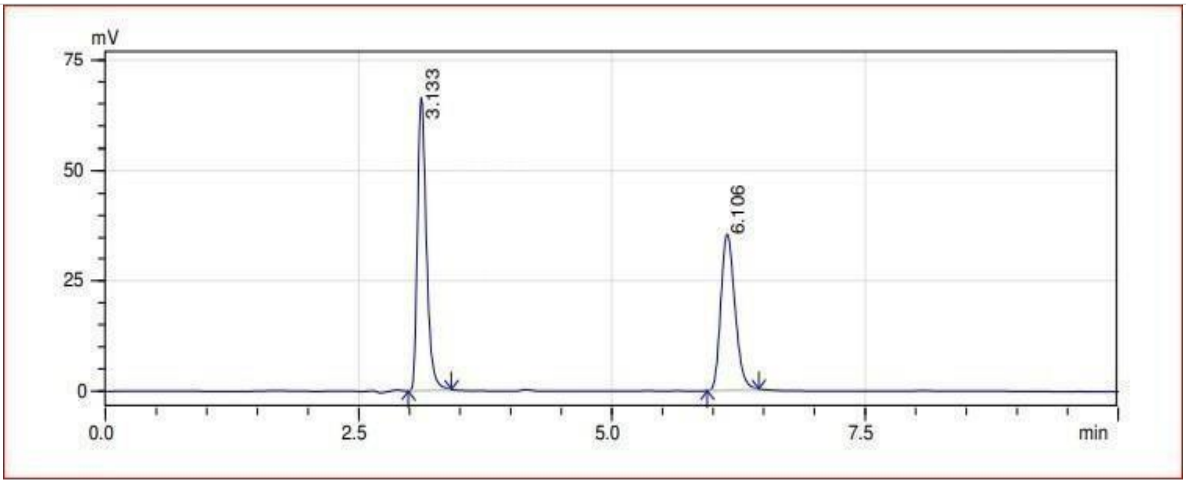
Chromatogram of marketed formulation of Atazanavir sulphate and Ritonavir

Method Validation

Specificity

Name of the Solution	Retention Time (min)
Blank	No peak
Placebo	No peak
Standard	3.06 and 6.18
Sample	3.13 and 6.10

No interference was observed at the retention times of Atazanavir sulphate and Ritonavir, indicating the method is specific for both analytes.



Result:
The method was found to be specific, as it did not show any extra peaks and there was no interference from excipients at the retention times of Atazanavir sulphate and Ritonavir.

System Suitability Parameters

S.No.	Parameter	Atazanavir Sulphate	Ritonavir
1	Theoretical Plates	6336	7379
2	Symmetry Factor	1.05	1.04
3	Resolution	6.65	6.65
4	Tailing Factor	1.15	1.32

Acceptance Criteria:

- Theoretical Plates: ≥ 2000
- Symmetry Factor: ≤ 2
- Tailing Factor: ≤ 2

From the above table, it was concluded that all the system suitability parameters were within acceptable limits, indicating the suitability of the developed HPLC method for the simultaneous estimation of Atazanavir Sulphate and Ritonavir.

LINEARITY

The linearity of the developed RP-HPLC method was evaluated by analyzing five different concentrations of Atazanavir sulphate and Ritonavir. The peak areas were recorded and a calibration curve was plotted for both drugs.

Linearity Data for Atazanavir Sulphate and Ritonavir

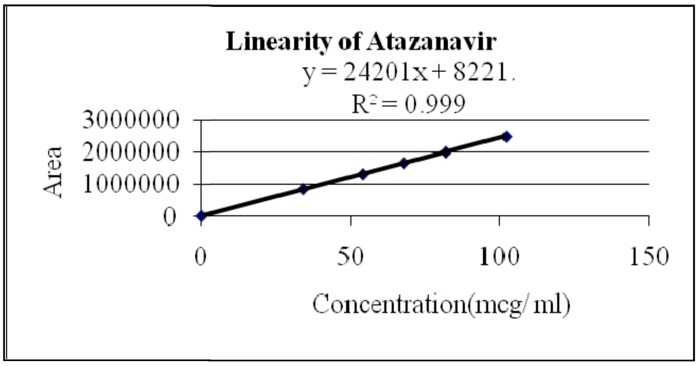
S.No.	Atazanavir Conc. (µg/mL)	Peak Area (Atazanavir)	Ritonavir Conc. (µg/mL)	Peak Area (Ritonavir)
1	34	842,922	10	292,661
2	54	1,310,821	16	455,824
3	68	1,658,694	20	577,054
4	82	1,996,529	24	691,459
5	102	2,468,854	30	861,146

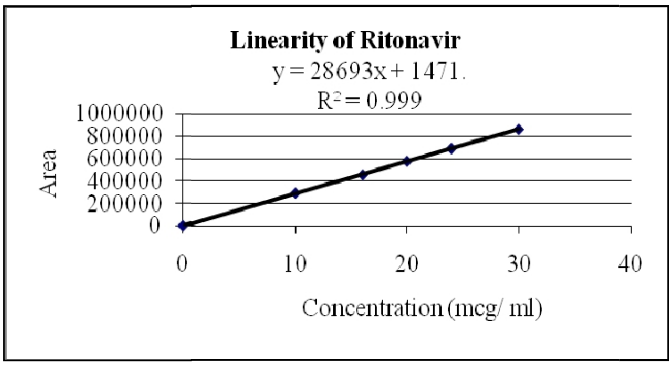
Linearity Parameters

Parameter	Atazanavir Sulphate	Ritonavir
Slope	8221	1471
Y-Intercept	24,201	28,693
Correlation Coefficient (r²)	0.999	0.999

Acceptance Criteria:

- Correlation coefficient (r²) should not be less than 0.999.





The correlation coefficients for both Atazanavir sulphate and Ritonavir were found to be 0.999, indicating that the method is linear over the concentration range tested.

PRECISION

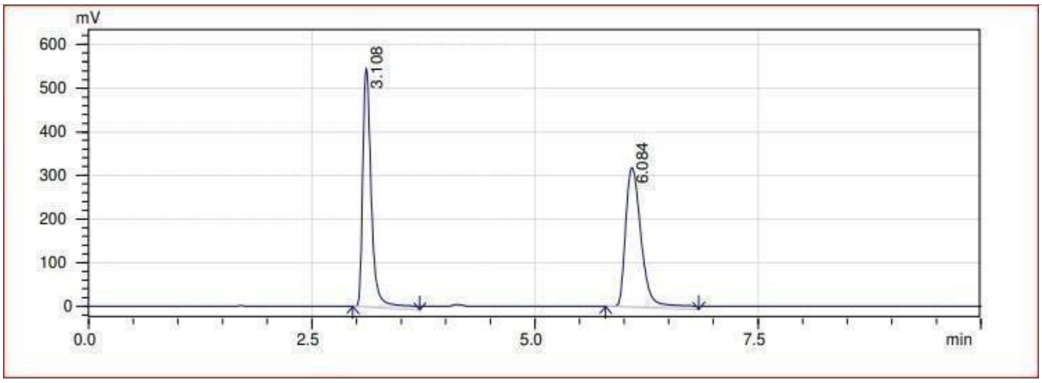
Method precision was evaluated by injecting six replicates of the sample solution and measuring the retention time (Rt) and peak area of Atazanavir sulphate and Ritonavir under the same chromatographic conditions.

Method Precision for Atazanavir Sulphate and Ritonavir

S.No	Atazanavir Sulphate		Ritonavir	
	Rt (min)	Peak Area	Rt (min)	Peak Area
1	3.10	1,975,564	6.03	534,547
2	3.10	1,976,472	6.08	535,303
3	3.11	1,977,694	6.09	533,068
4	3.11	1,975,450	6.09	531,885
5	3.10	1,980,537	6.08	550,278
6	3.13	1,973,346	6.10	545,065
Mean	—	1,976,511	—	538,358
SD	—	2435.14	—	7494.10
%RSD	—	0.12%	—	1.39%

Acceptance Criteria:

- %RSD should be ≤ 2.0% for both drugs.



The %RSD values for both Atazanavir sulphate (0.12%) and Ritonavir (1.39%) are within the acceptable limits, indicating that the method is precise.

Accuracy Results for Atazanavir Sulphate and Ritonavir

Level	Drug	Average % Recovery	% RSD
80%	Atazanavir Sulphate	100.01%	0.17
	Ritonavir	100.31%	0.27
100%	Atazanavir Sulphate	100.40%	0.31
	Ritonavir	100.12%	0.61
120%	Atazanavir Sulphate	99.91%	0.24
	Ritonavir	100.31%	0.29

All values are within acceptable limits (% Recovery: 98–102%, RSD: ≤2%), so the method is accurate.

Intraday Precision and Intermediate Precision – Analyst 1

Injection	Atazanavir Sulphate			Ritonavir		
	Rt (min)	AUC	% Assay	Rt (min)	AUC	% Assay
1	3.116	1987964	99.78	6.105	544358	100.42
2	3.112	1986984	99.14	6.062	546722	100.26
3	3.107	1979854	99.56	6.047	541098	100.00
4	3.127	1985269	99.60	6.027	544037	100.32
5	3.106	1985060	99.41	6.040	546826	100.65
6	3.107	1998324	99.80	6.041	539046	98.94

Parameter	Atazanavir Sulphate	Ritonavir
Mean % Assay	~99.55%	~100.10%
% RSD	0.31	0.60

% RSD values are below 2%, indicating the method is rugged and precise under intraday conditions.

Table: Comparison of Intraday and Intermediate Precision (Ruggedness)

Parameter	Atazanavir Sulphate			Ritonavir		
	Mean % Assay	% RSD	AUC Range	Mean % Assay	% RSD	AUC Range
Intraday Precision (Analyst-1)	99.55%	0.25	1979854–1998324	100.10%	0.60	539046–546826
Intermediate Precision (Analyst-2)	99.62%	0.51	1974107–1997980	100.32%	0.64	542741–549121

Interpretation:

- Both analysts produced consistent results with low %RSD (<2%), proving the ruggedness of the method.
- Atazanavir showed slightly lower variability with Analyst-1 (%RSD = 0.25) compared to Analyst-2 (%RSD = 0.51).
- Ritonavir's results were also reproducible between analysts with %RSDs under 1%.

The results confirm that the developed HPLC method is rugged, reproducible, and analyst-independent, meeting ICH validation criteria.

Robustness – Effect of Flow Rate on Atazanavir Sulphate and Ritonavir

Drug	Flow Rate (mL/min)	Mean Peak Area	%RSD	Observation
Atazanavir	1.2	1,984,363	0.81	Slightly higher variation
	1.3 (Standard)	1,995,076	0.28	Optimized flow with low variation
	1.4	1,992,000	0.12	Very low variation, consistent performance
Ritonavir	1.2	543,056	0.10	Stable results
	1.3 (Standard)	559,432	0.04	Highly robust at standard flow rate
	1.4	550,988	0.07	Consistent with low variation

- Both Atazanavir Sulphate and Ritonavir showed good robustness across ±0.1 mL/min flow rate changes.
- The %RSD values were well below 2%, indicating method reliability and consistency.
- Optimum robustness was seen at 1.3 mL/min for both drugs.

Limit of Detection (LOD) & Limit of Quantitation (LOQ)

LOD and LOQ were calculated using an instrumental signal-to-noise (S/N) ratio method.

Parameter	Atazanavir Sulphate	Ritonavir
LOD (µg/mL)	0.0023	0.0006
LOQ (µg/mL)	0.0161	0.0047
LOD S/N Ratio	3.1	3.1
LOQ S/N Ratio	9.9	10.1

- The method is highly sensitive, capable of detecting and quantifying very low concentrations of both drugs.
- Both LOD and LOQ values are well within acceptable limits, proving the method's suitability for trace analysis.

SUMMARY AND CONCLUSIONS:

In the present study, a novel RP-HPLC method was successfully developed for the simultaneous estimation of Atazanavir Sulphate and Ritonavir in both bulk and pharmaceutical dosage forms. The method was validated according to ICH guidelines for key analytical parameters including accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity, limit of detection (LOD), and limit of quantification (LOQ). The summary of the method development and validation findings is as follows:

A) Method Development:Optimized Trial (Trial 5): The most suitable chromatographic conditions were achieved using a C18 Nucleodur column (150 mm × 4.6 mm, 5 µm). The mobile phase comprised acetonitrile, methanol, and phosphate buffer (pH 3.0 adjusted with orthophosphoric acid) in a ratio of 44:11:45 v/v/v. The flow rate was maintained at 1.5 mL/min, and detection was performed at 210 nm using UV detection.

- The percentage assay results were 99.05% for Atazanavir Sulphate and 99.20% for Ritonavir, indicating the method's high accuracy and efficiency in quantifying the drugs.

B) Method Validation Parameters:

- Linearity: The calibration curves for both drugs showed excellent linearity with correlation coefficients (r^2) of 0.999 for Atazanavir Sulphate and 0.999 for Ritonavir across the studied concentration ranges.
- Precision: The method demonstrated good reproducibility, with % RSD values below 2%, fulfilling the criteria for intra-day and inter-day precision.
- System Suitability: The system suitability parameters confirmed well-resolved peaks, with resolution values greater than 2 for both analytes.
- Accuracy (Recovery Studies): The percentage recovery was in the range of 99.91%–100.40% for Atazanavir Sulphate and 100.12%–100.31% for Ritonavir, indicating excellent method accuracy without interference from excipients.
- Robustness: Deliberate variations in chromatographic conditions (such as flow rate and mobile phase composition) did not significantly affect retention times, proving the method's robustness and reliability under small operational changes.
- Sensitivity (LOD and LOQ):
    - LOD: 0.002 µg/mL for Atazanavir Sulphate and 0.001 µg/mL for Ritonavir
    - LOQ: 0.016 µg/mL for Atazanavir Sulphate and 0.004 µg/mL for Ritonavir

CONCLUSION:

The developed RP-HPLC method is simple, sensitive, accurate, precise, robust, and cost-effective for the simultaneous estimation of Atazanavir Sulphate and Ritonavir in bulk and tablet dosage forms. Due to its short run time, minimal sample requirement, and good resolution between peaks, it is highly suitable for routine quality control and analysis in pharmaceutical industries. The method ensures high reliability, repeatability, and ease of operation, making it practical for commercial and laboratory applications.

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