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ESTIMATION OF ATAZANAVIR AND RITONAVIR BY USING HPLC

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Abstract:
A new, simple, rapid, accurate and precise Reverse Phase High Performance Liquid Chromatographic method has been developed for the validated of Atazanavir & Ritonavir, in Active pharmaceutical Ingredient form as well as in combined tablet dosage form. Chromatography was carried out on Symmetry ODS C18 (4.6mm × 250mm, 5µm) column using a mixture of Methanol: Acetonitrile (35:65v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 273 nm. The retention time of the Atazanavir and Ritonavir, was 2.085, 5.262 ± 0.02min respectively. The method produce linear responses in the concentration range of 30-70mg/ml of Atazanavir and 6-14mg/ml of Ritonavir,. The mean % assay of marketed formulation was found to be 100.04%, and % recovery was observed in the range of 98-102%. Relative standard deviation for the precision study was found <2%.The developed method is simple, precise and rapid, making it suitable for estimation of Atazanavir and Ritonavirin API and combined tablet dosage form. The method is useful in the quality control of bulk and pharmaceutical formulations.

Keywords: *Atazanavir & Ritonavir, RP-HPLC, Validation, ICH Guidelines.*

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INTRODUCTION:

Introduction to HPLC:

In the modern pharmaceutical industry, high-performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development and production. It is ideal for the analysis of many drugs in both dosage forms and biological fluids due to its simplicity, high specificity and good sensitivity.

High Performance Liquid Chromatography (HPLC) is a technique that has arisen from the application to liquid chromatography the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-1970 and was improved with the development of column packing material and the additional convenience of on-line detectors. The various components of HPLC are pumps (solvent delivery system), mixing unit, gradient controller and solvent degasser, injector (manual or automatic), guard column, analytical columns, detectors, recorders and/or integrators. Recent models are equipped with computers and software for data acquisition and processing. The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-5 cm3/min. The mobile phase acts as a carrier for the sample solution. The chemical interactions of the mobile phase and sample with the column determine the degree of migration and separation of components contained in the sample. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.

Types of Chromatography:

1. Normal-phase chromatography

Mechanism: Retention by interaction with the polar surface of the stationary phase with polar parts of the sample molecules.

Stationary phase: SiO2, Al2O3, -NH2, -CN, -Diol, -NO2, etc.

Mobile phase: Heptane, hexane, cyclohexane, CHCl3, CH2Cl2, dioxane, methanol, etc.

Application: Separation of non-ionic, non-polar to medium polar substances. Disadvantage: Lack of reproducibility of retention times as water or protic organic solvents change the hydration state of the silica or alumina chromatographic media.

2. Reversed-phase chromatography

Mechanism: Retention by interaction of the stationary phase's non-polar hydrocarbon chain with non-polar parts of the sample molecules.

Stationary phase: n-octadecyl (RP-18), n-octyl (RP-8), ethyl (RP-2), phenyl, (CH2)n-CN, (CH2)n-diol, etc.

Mobile phase: Methanol, Acetonitrile, water, buffer (sometimes with additives of THF orDioxane), etc.

Application: Separation of non-ionic and ion forming non-polar to medium polar substances (carboxylic acids, hydrocarbons). If ion forming substances (as carboxylic acids) are to be separated, a pH control by buffers is necessary.

3. Reversed-phase ion-pair chromatography

Mechanism: Ionic sample molecules are ionically bound to an ion-pair reagent. The ion- pair reagent contains an unpolar part suitable for interaction with the unpolar hydrocarbon chain of the stationary phase.

Stationary phase: Reversed phase materials (RP-18, RP-8, CN), etc.

Mobile phase: Methanol, Acetonitrile, buffer with added ion-pair reagent in the concentration range of 0.001 to 0.01 M, etc.

Application: Ionic substances often show very poor retention in reversed phase chromatography. To overcome this difficulty an ion-pair reagent is added to the eluent.

4. Ion-exchange chromatography

Mechanism: Retention of reversible ionic bonds on charged groups of the stationary phase

Stationary phase:

	Strong	Weak
Cation exchanger	SO_3^-	COO^-
Anion exchanger	NR_3^+	NHR_2^+

Mobile phase: Aqueous buffer systems.

Application: Separation of substances which can form ions such as inorganic ions, organic acids, organic bases, proteins, nucleic acids.

Advantages of HPLC

1) It provides specific, sensitive and precise method for analysis of the different complicated sample.

2) There is ease of sample preparation and sample introduction.
3) There is speed of analysis.
4) The analysis by HPLC is specific, accurate and precise.
5) It offers advantage over gas chromatography in analysis of many polar, ionic substances, high molecular weight substances, metabolic products and thermo labile as well as nonvolatile substances.

Applications of HPLC

a) Natural Products: HPLC is an ideal method for the estimation of various components in plant extracts which resemble in structure and thus demand a specific and very sensitive method e.g., analysis of digitalis, cinchona, liquorice, and ergot extracts.

b) Stability studies: HPLC is now used for ascertaining the stability of various pharmaceuticals. With HPLC the analysis of the various degradation products can be done and thus stability indicating HPLC systems have been developed.

c) Bioassays and its complementation: Complex molecules as antibiotics and peptide hormones are mainly analyzed by bioassay which suffers from high cost, necessity replicates, poor precision and length of time required. Also bioassay gives an overall estimate of potency and gives no guidance about the composition. Thus HPLC can be used to complement bioassays and give an activity profile. It has been used for analysis of chloramphenicol, penicillins and clotrimoxazole, sulfas and peptides hormones.

d) HPLC has also been used in the cosmetic industry for quality control of various cosmetics.

Instrumentation

The basic components of HPLC are:

1. Pumping System
2. Sample Introduction Device
3. Chromatographic Column
4. Detector
5. Data handling Device

1. Pumping System: The HPLC pump is very important component of the system. It delivers the constant flow of the mobile phase or phases so that the separation of the components of the mixture occur in a reasonable time. Its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps are used in HPLC to propel the liquid mobile phase through the system are as under;

a. Displacement pump: It produces a flow that tends to independent of viscosity and backpressure and also output is pulse free. But it possesses limited capacity (250 ml).

b. Reciprocating pump: It has small internal volume (35 to 400 μl). It has high output pressure (up to 10,000 psi) and constant flow rates. But it produces a pulsed flow.

c. Pneumatic or constant pressure pump: They are pulse free, suffer from limited capacity as well as a dependence of flow rate on solvent viscosity and column back pressure. They are limited to pressure less than 2000 psi.

There are two type of elution process, i.e. isocratic and gradient

Isocratic: In this system, the things are kept constant throughout the run. In the case of pumping of mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is ±1% of the set flow

Gradient: There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of mobile phase is continuously varied during the particular run. The gradient accuracy of ±1% of the step gradient composition is typical.

2. Sample Introducing Device

It is not possible to use direct syringe injection on column like GC, as the inlet pressure in LC is too high. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening attributable to this step is negligible. The injection system itself should have no dead (void) volume. There are three important ways of introducing the sample into injection port.

a. Loop injection: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector.

b. Valve injection: In which, a variable volume is introduced by making use of an injection valve.

c. On column injection: In which, a variable volume is introduced by means of a syringe through a septum.

3. Chromatographic Column

Column is a heart of chromatography. The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μm or less. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing:

The packing used in modern HPLC consists of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

a. Porous, polymeric beds: Porous, polymeric beds based on styrene divinyl benzene co-polymers used for ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

b. Porous layer beds: Consisting of a thin shell (1-3µm) of silica or modified silica on a spherical inert core (e.g. Glass). After the development of totally porous micro particulate packings, these have not been used in HPLC.

c. Totally Porous silica particles (dia. < 10µm): These packing have widely been used for analytical HPLC in recent years. Particles of diameter > 20µm are usually dry packed. While particles of diameter < 20µm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

4. Detector

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. There are several detectors available in the market. However UV Visible detector, photo diode array detector, fluorescence detector, conductometric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector, while the MS detector is outstanding. Detectors are usually of two types:

a. Bulk property detectors: It compares overall changes in a physical property of the mobile phase with and without an eluting solute e.g. refractive index, dielectric constant or density.

b. Solute property detectors: It responds to a physical property of the solute, which is not exhibited by the pure mobile phase e.g. UV absorbance, fluorescence or diffusion current.

5. Data handling Device

Computer-based system that controls all components of HPLC instrument (eluent composition (mixing of different solvents); temperature, injection sequence, etc.) and acquires data from the detector and monitors system performance (continuous monitoring of the mobile phase composition, temperature, back pressure, etc.)

MATERIALS AND METHODS:

Atazanavir & Ritonavir Procured from Sura labs, Water and Methanol for HPLC from LICHROSOLV (MERCK), Acetonitrile for HPLC from Merck, Triethylamine from Merck.

Hplc method development:

Trails :

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Atazanavir and Ritonavir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.5ml of the above Ritonavir and 0.1ml of Atazanavir stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water and Water: Acetonitrile and Methanol: TEA Buffer: ACN with varying proportions. Finally, the mobile phase was optimized to Methanol: Acetonitrile in proportion 35:65 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, Symmetry and Zodiac column. Symmetry ODS C18 (4.6mm × 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions:

Instrument used	:	Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature	:	Ambient
Column	:	Symmetry ODS C18 (4.6mm × 250mm, 5µm)
Mobile phase	:	Methanol: Acetonitrile (35:65v/v)
Flow rate	:	1ml/min
Wavelength	:	273 nm
Injection volume	:	20 µl
Run time	:	10 min

Method validation:

Preparation of mobile phase:

Preparation of Mobile Phase:

Accurately measured 350 ml (35%) of Methanol, 650 ml of Acetonitrile (65%) were mixed and degassed in digital ultra sonicater for 20 minutes and then filtered through 0.45 µm filter under vacuum filtration.

Diluent Preparation:
The Mobile phase was used as the diluent.
RESULTS AND DISCUSSION
Optimized Chromatogram (Standard)
Mobile phase : Methanol: Acetonitrile (35:65v/v)
Column : Symmetry ODS C18 (4.6mm × 250mm, 5µm)
Flow rate : 1 ml/min
Wavelength : 273 nm
Column temp : Ambient
Injection Volume : 20 µl
Run time : 10 minutes

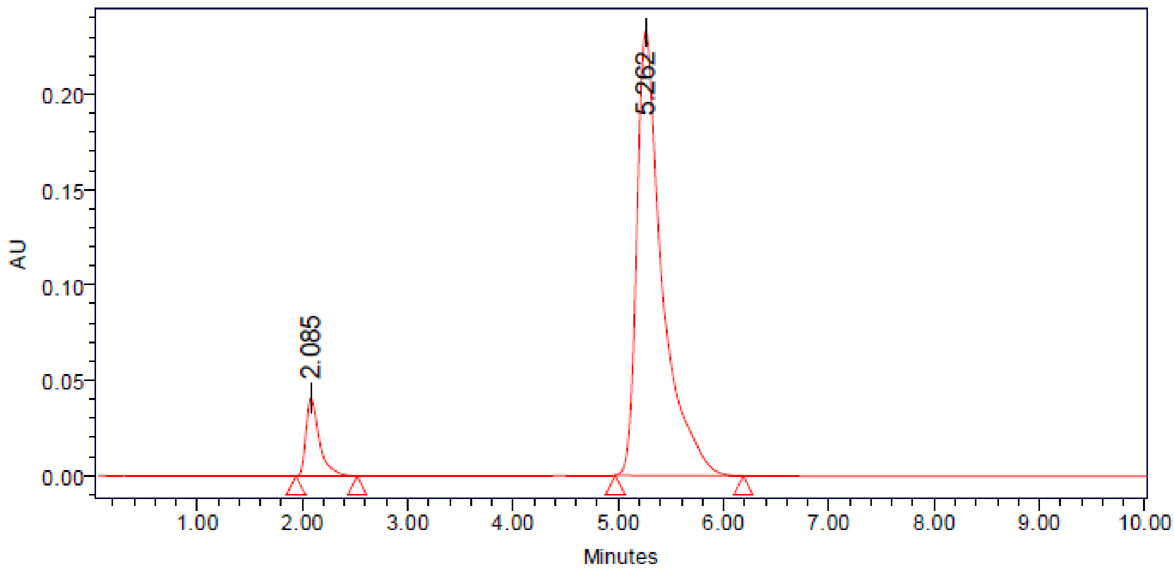

Fig-: Optimized Chromatogram

Table: - Peak Results for Optimized Chromatogram

S. No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Atazanavir	2.085	289632	3527		1.66	6746
2	Ritonavir	5.262	4658750	28537	8.58	1.83	8639

Observation: From the above chromatogram it was observed that the Atazanavir and Ritonavir peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.
Optimized Chromatogram (Sample)

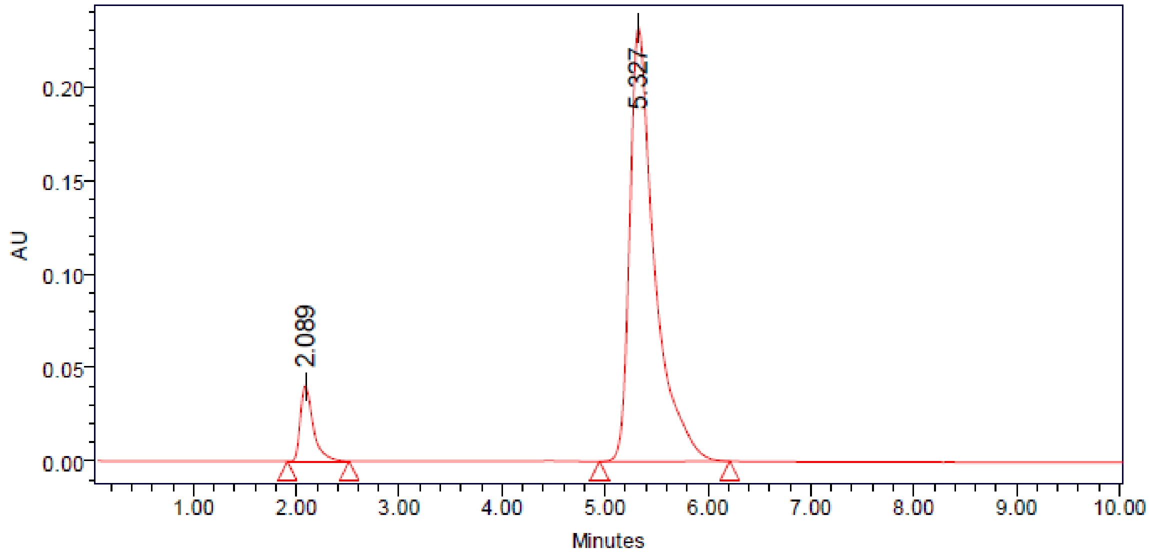

Figure: Optimized Chromatogram (Sample)

Table: Optimized Chromatogram (Sample)

S. No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Atazanavir	2.089	298699	3659		1.69	6858
2	Ritonavir	5.327	4758696	29587	8.65	1.86	8788

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

Table-: Peak Results for Assay Standard

S No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Atazanavir	2.090	289653	3565		1.66	6786	1
2	Ritonavir	5.289	4685783	28653	8.61	1.84	8658	1
3	Atazanavir	2.089	289746	3597		1.67	6797	2
4	Ritonavir	5.338	4658974	286597	8.58	1.81	8623	2
5	Atazanavir	2.089	285688	3588		1.64	6781	3
6	Ritonavir	5.327	4658797	254876	8.62	1.82	8696	3

Table-: Peak Results for Assay sample

S. No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Atazanavir	2.088	296853	3658		1.65	6858	1
2	Ritonavir	5.276	4785657	29864	9.76	1.84	8753	1
3	Atazanavir	2.087	298546	3699		1.68	6873	2
4	Ritonavir	5.268	4788983	29862	9.83	1.83	8786	2
5	Atazanavir	2.085	296855	3673		1.65	6858	3
6	Ritonavir	5.262	4789857	29865	9.77	1.85	8797	3

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Atazanavir and Ritonavir in pharmaceutical dosage form was found to be100.04%.

System Suitability:

Table-: Results of system suitability for Atazanavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Atazanavir	2.090	289855	3527	8658	1.81
2	Atazanavir	2.090	285748	3542	8643	1.84
3	Atazanavir	2.089	289589	3613	8675	1.85
4	Atazanavir	2.089	285465	3585	8693	1.84
5	Atazanavir	2.085	285980	3573	8655	1.82
Mean			287327.4			
Std. Dev			2195.570609			
% RSD			0.7641			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table-: Results of system suitability for Ritonavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Ritonavir	5.289	4658746	28563	8658	1.81	
2	Ritonavir	5.289	4652588	28456	8646	1.82	
3	Ritonavir	5.338	4674834	28951	8631	1.83	
4	Ritonavir	5.327	4685827	28755	8646	1.81	
5	Ritonavir	5.262	4652149	28966	8695	1.82	
Mean			4664828.8				
Std. Dev			14905.06121				
% RSD			0.3195				

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Linearity:

Atazanavir:

Concentration µg/ml	Average Peak Area
30	185659
40	245476
50	309659
60	365847
70	428696

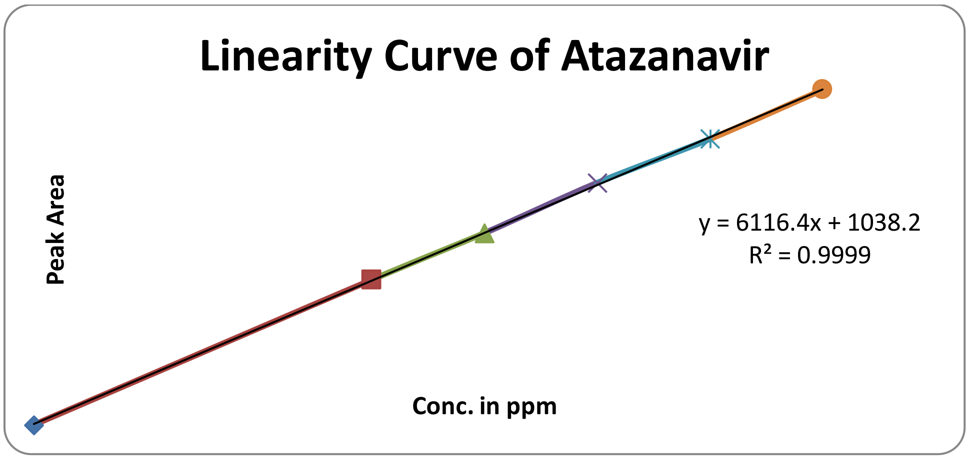

Figure: Linearity for Atazanavir

Ritonavir

Concentration µg/ml	Average Peak Area
6	2658796
8	3556973
10	4458748
12	5265873
14	6169885

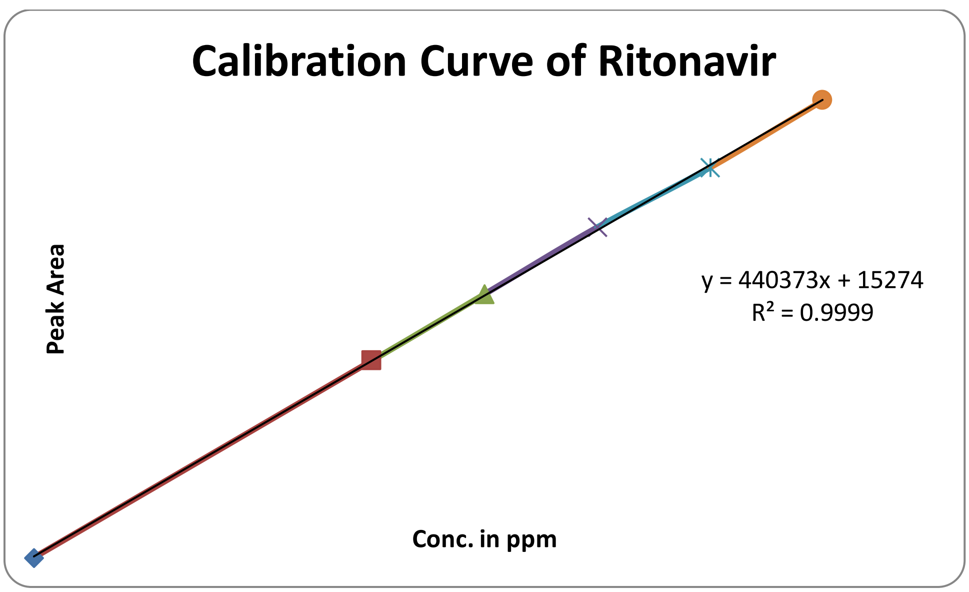

Figure: Calibration Curve for Ritonavir

Repeatability

Table-: Results of Repeatability for Atazanavir:

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Atazanavir	2.086	289659	3568	6788	1.65
2	Atazanavir	2.083	289548	3527	6757	1.66
3	Atazanavir	2.083	285699	3599	6793	1.65
4	Atazanavir	2.081	284575	3546	6748	1.66
5	Atazanavir	2.081	285701	3596	6743	1.65
Mean			287036.4			
Std. Dev			2388.336			
% RSD			0.832067			

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table-: Results of method precession for Ritonavir:

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Ritonavir	5.178	4685983	28568	8660	1.84	8.60
2	Ritonavir	5.199	4698548	28573	8696	1.85	8.60
3	Ritonavir	5.235	4658755	28599	8653	1.81	8.60
4	Ritonavir	5.202	4635982	26986	8679	1.84	8.60
5	Ritonavir	5.206	4658797	26855	8691	1.85	8.60
Mean			4667613				
Std. Dev			24754.48				
% RSD			0.530346				

Acceptance criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:

Day 1:

Table-: Results of Intermediate precision for Atazanavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Atazanavir	2.083	298658	3658	6896	1.66
2	Atazanavir	2.083	298575	3676	6848	1.67
3	Atazanavir	2.089	296588	3699	6823	1.67
4	Atazanavir	2.083	295683	3625	6855	1.66
5	Atazanavir	2.082	296535	3697	6871	1.67
6	Atazanavir	2.080	296527	3643	6894	1.66
Mean			297094.3			
Std. Dev			1226.273			
% RSD			0.412755			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Table-: Results of Intermediate precision for Ritonavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Ritonavir	5.229	4785699	29866	8799	1.83	
2	Ritonavir	5.203	4785643	298623	8758	1.84	
3	Ritonavir	5.133	4715267	293542	8763	1.83	8.66
4	Ritonavir	5.229	4752144	298765	8755	1.84	
5	Ritonavir	5.151	4715688	296536	8793	1.84	
6	Ritonavir	5.112	4785983	295878	8765	1.83	
Mean			4756737				
Std. Dev			34512.01				
% RSD			0.72554				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

Day 2:

Table-: Results of Intermediate precision Day 2 for Atazanavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Atazanavir	2.078	278599	3786	6986	1.67
2	Atazanavir	2.082	275986	3788	6924	1.68
3	Atazanavir	2.080	274563	3794	6931	1.67
4	Atazanavir	2.089	274155	3757	6953	1.68
5	Atazanavir	2.083	274566	3747	6925	1.67
6	Atazanavir	2.089	274583	3790	6980	1.68
Mean			275407.8			
Std. Dev			1684.552			
% RSD			0.611657			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2

Table-: Results of Intermediate precision for Ritonavir

S no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Ritonavir	5.077	4589853	27853	8546	1.82	
2	Ritonavir	5.151	4526542	27462	8594	1.80	
3	Ritonavir	5.112	4523655	27485	8522	1.81	8.62
4	Ritonavir	5.133	4524570	27456	8575	1.80	
5	Ritonavir	5.203	4526544	27659	8537	1.81	
6	Ritonavir	5.133	4526588	27851	8541	1.80	
Mean			4536291				
Std. Dev			26268.18				
% RSD			0.579066				

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

Accuracy:

Table-: The Accuracy Results for Atazanavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	153881	25	24.986	99.95%	100.00%
100%	306723.7	100	49.982	99.963%	
150%	460176.7	150	75.072	100.095%	

Table-: The Accuracy Results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	233876.3	5	4.964	99.26%	99.94%
100%	455389.3	10	9.995	99.94%	
150%	680035	15	15.096	100.633%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Robustness

Atazanavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	289659	2.090	6746	1.65
Less Flow rate of 0.9 mL/min	298657	2.736	6853	1.69
More Flow rate of 1.1 mL/min	275476	1.673	6684	1.62
Less organic phase	265395	2.736	6636	1.64
More organic phase	245870	1.673	6421	1.67

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Ritonavir:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	4658748	5.289	8639	1.82
Less Flow rate of 0.9 mL/min	4875986	6.746	8760	1.81
More Flow rate of 1.1 mL/min	4525322	4.032	8453	1.80
Less organic phase	4425644	6.746	8696	1.83
More organic phase	4258671	4.032	8240	1.84

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION:

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the Quantitative estimation of Atazanavir & Ritonavir in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.

Atazanavir sodium is freely soluble in ethanol, methanol, and water and practically insoluble in Acetonitrile.

Ritonavir is freely soluble in water, soluble in methanol, insoluble in acetone.

Methanol: Acetonitrile (35:65v/v) was chosen as the mobile phase. The solvent system used in this method was Economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectro photometric methods.

This method can be used for the routine determination of Atazanavir & Ritonavir in bulk drug and in Pharmaceutical dosage forms.

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