ISSN: 0975-766X
CODEN: IJPTFI
Research Article

Available Online through
www.ijptonline.com

METHOD DEVELOPMENT AND VALIDATION OF ATAZANAVIR AND RITONAVIR IN A COMBINED DOSAGE FORM BY RP-HPLC METHOD

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Received on 09-08-2011 *Accepted on 25-08-2011*

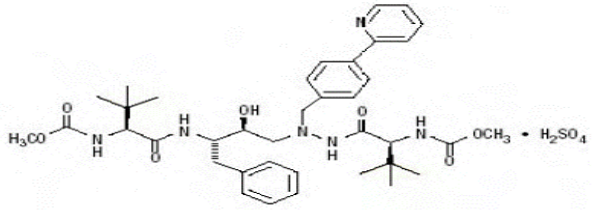
Abstract

A simple, precise, accurate, and rapid HPLC method has been developed, and validated for the determination of Atazanavir and Ritonavir simultaneously in combined tablet dosage form. The mobile phase used was a mixture of phosphate buffer pH 4 and Acetonitrile (43:57% v/v). The detection of Atazanavir and Ritonavir was carried out by UV detector at 240 nm. The retention time of Atazanavir and Ritonavir were found to be 4.2 and 5.2 respectively. Results of the analysis were validated statistically, and by recovery studies. The proposed method can be successfully used to determine the drug contents of marketed formulation.

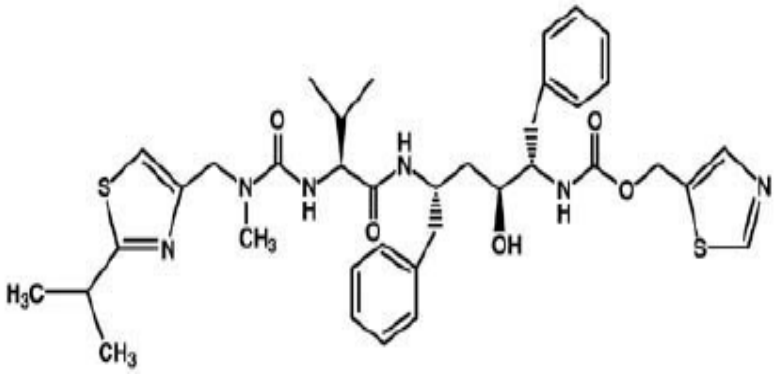
Key Words: Atazanavir and Ritonavir, Reverse Phase High Performance Liquid Chromatography and validation.

Introduction

Atazanavir sulphate is an azapeptide inhibitor of HIV-1 protease. It is a white to pale yellow crystalline powder and slightly soluble in water. Its molecular weight is 802.9 (sulfuric acid salt). The free base molecular weight is 704.9. The chemical name for atazanavir sulfate is (3S,8S,9S,12S)-3,12-Bis(1,1-dimethylethyl)-8hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13pentaazatetradecanedioic acid dimethyl ester, sulfate.[1] Its molecular formula is $C_{38}H_{52}N_6O_7 \bullet H_2SO_4$ and its structure is



Ritonavir is an inhibitor of HIV protease with activity against the Human Immunodeficiency Virus. Ritonavir is a white-to-light-tan powder. It has a bitter metallic taste and freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water. Its molecular weight is 720.95. Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12- tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)][2] and its structure is



Synthivan is a combination of Atazanavir and Ritonavir for the treatment of HIV infection.

HIV infection is a condition cause by the human immunodeficiency virus (HIV). The condition gradually destroys the immune system, which makes it harder for the body to fight infections[3]. Treatment consists of highly active antiretroviral therapy, or HAART.[4] This has been highly beneficial to many HIV-infected individuals since its introduction in 1996, when the protease inhibitor-based HAART initially became available.[5] Atazanavir and Ritonavir are protease inhibitors which selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells, preventing formation of mature virions.[6]

Combination therapy of Atazanavir and Ritonavir is used in treatment of HIV infection, eg. Synthivan

Atazanavir-Ritonavir is supplied as tablets with two brand names**:**

SYNTHIVAN: Atazanavir-300mg, Ritonavir-100mg

ATAZOR R: Atazanavir-300mg, Ritonavir-100mg

Various methods such as high performance liquid chromatography (HPLC)[7], high-performance liquid chromatography-tandem mass spectrometry[8,9] have been reported in the literature for the determination of these compounds in various biological samples and pharmaceutical preparations.

REAGENTS, STANDARDS AND SAMPLES:

Water HPLC Grade, Atazanavir Working Standard, Ritonavir Working Standard, Acetonitrile, Potassium dihydrogen phosphate and Synthivan tablets.

CHROMATOGRAPHIC PARAMETERS:

Equipment	: High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector.
Column	: Symmetry C18 (4.6 x 100mm, 3.5µm, Make: ACE) or equivalent
Flow rate	: 0.9 mL per min
Mobile phase	: PH-4 Phosphate buffer : acetonitrile [43:57]
Wavelength	: 240 nm
Injection volume	: 20µl
Column oven	: Ambient
Run time	: 8 min

Preparation of Phosphate buffer:

Weigh 7.0 grams of Potassium dihydrogen Phosphate into a 1000ml beaker, dissolve and diluted to 1000ml with HPLC water. Adjusted the pH to 4.0 with Orthophosphoric acid.

Preparation of mobile phase:

Mix a mixture of above buffer 430 mL (45%) and 570 mL of Acetonitrile HPLC (55%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile Phase as diluent.

Assay

Preparation of the Atazanavir and Ritonavir Standard and Sample Solution:

Standard Solution Preparation:

Accurately weigh and transfer 30 mg of Atazanavir and 10 mg of Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and

dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer 568.2 mg of Atazanavir and Ritonavir Tablet powder into a 100 ml clean dry volumetric flask and add about 70 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for the Atazanavir and Ritonavir peaks and calculate the % Assay by using the formulae.

System Suitability:

Tailing factor for the peaks due to Atazanavir and Ritonavir in Standard solution should not be more than 1.5

Theoretical plates for the Atazanavir and Ritonavir peaks in Standard solution should not be less than 2000

Precision

Preparation of stock solution:

Accurately weigh and transfer 30 mg of Atazanavir and 10 mg Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution:

Accurately weigh and transfer 30 mg of Atazanavir and Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Accuracy

Preparation of Standard stock solution:

Accurately weigh and transfer 30 mg of Atazanavir and 10 mg Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 15 mg of Atazanavir and 5 mg of Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 30mg of Atazanavir and 10 mg of Ritonavir working standards into a 10 ml clean

dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 45mg of Atazanavir and 15 mg of Ritonavir working standards into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of Atazanavir and Ritonavir of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Linearity

Preparation of stock solution:

Accurately weigh and transfer 30 mg of Atazanavir and 10 mg Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (60 ppm of Atazanavir & 20 ppm of Ritonavir):

0.2 ml of stock solution has taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (90 ppm of Atazanavir & 30 ppm of Ritonavir):

0.3 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (120 ppm of Atazanavir & 40 ppm of Ritonavir):

0.4 ml of stock solution has taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (1500ppm of Atazanavir & 50ppm of Ritonavir):

0.5 ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (180 ppm of Atazanavir & 60 ppm of Ritonavir)

0.6 ml of stock solution has taken in 10 ml of volumetric flask dilute up to the mark with diluent.

Limit of Detection

ATAZANAVIR

Preparation of 120 µg/ml solution:

Accurately weigh and transfer 30 mg of Atazanavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.6% solution At Specification level (0.072 µg/ml solution):

Further pipette 1ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Further pipette 1ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Pipette 0.6 ml of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

RITONAVIR

Preparation of 40 µg/ml solution:

Accurately weigh and transfer 10 mg of Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of 2.4% solution At Specification level (0.96 µg/ml solution):

Further pipette 1ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent

Further pipette 1ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Pipette 2.4 ml of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

LIMIT OF QUANTIFICATION:

ATAZANAVIR

Preparation of 120 µg/ml solution:

Accurately weigh and transfer 30 mg of Atazanavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stocksolution).

Further pipette 0.4 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 2.0% solution At Specification level (0.24 µg/ml solution):

Further pipette 1ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Pipette 2 ml of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

RITONAVIR

Preparation of 40 µg/ml solution:

Accurately weigh and transfer 10 mg of Ritonavir working standard into a 10 ml clean dry volumetric flask add about 7 ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.4 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.8% solution At Specification level (0.32 µg/ml solution):

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Pipette 0.8 ml of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

ROBUSTNESS:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature

Variation was made to evaluate the impact on the method.

a). The flow rate was varied at 0.8 ml/min to 1.0ml/min.

Standard solution 120 ppm of Atazanavir and 40 ppm of Ritonavir was prepared and analysed using the varied flow rates along with method flow rate.

b). The Organic composition in the Mobile phase was varied from 45% to 65%.

Standard solution 120 µg/ml of Atazanavir and 40 µg/ml of Ritonavir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Calculation:

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = Average Peak Area obtained with test preparation

AS = Average Peak Area obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Results and Discussions

System suitability:

Standard solution is injected five times and Flow rate was maintained at 0.9 ml/min. temperature of column kept ambient and the column effluents were monitored at 240 nm, chromatograms were taken and System suitability parameters were computed. The system suitability was calculated as per ICH guidelines (figure no.1,2).

Figure No-1: Atazanavir and Ritonavir sample.

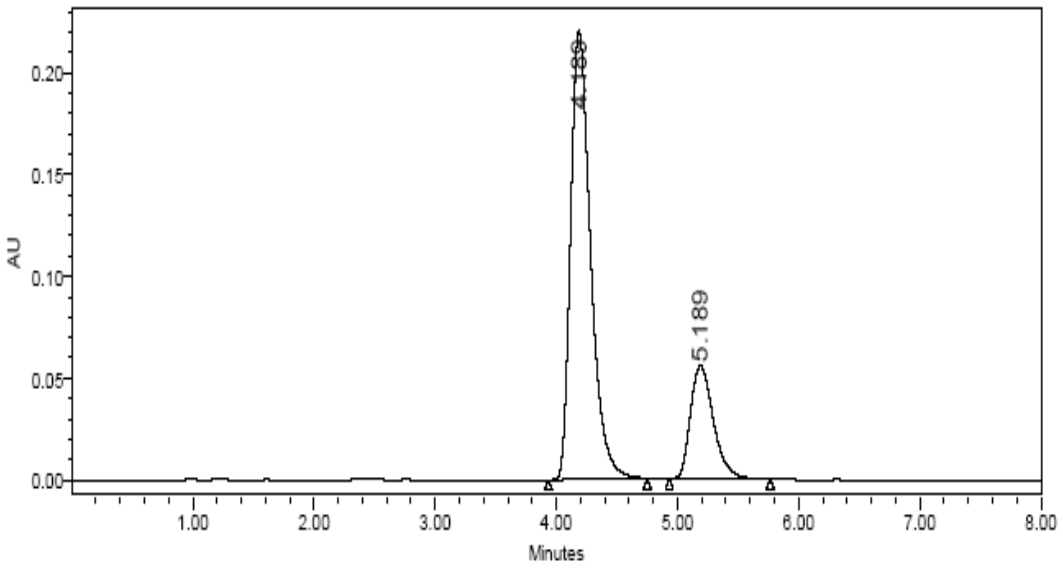
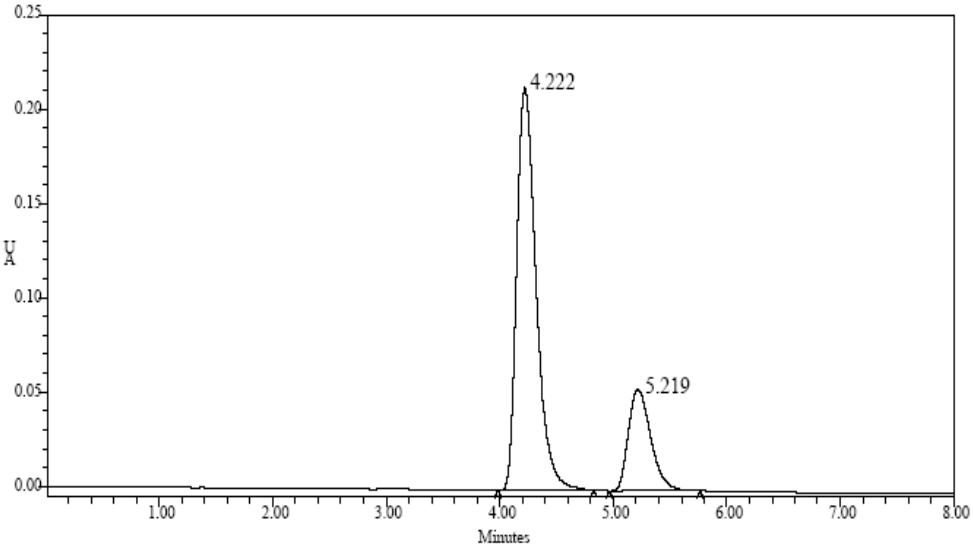


Figure No-2: Atazanavir and Ritonavir standard.



Precision

The sample solution prepared as mentioned in sample preparation was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. (table no.1 & 2).

Table No-1: Summary of atazanavir precision:

Injection	**Area**
Injection-1	2532223
Injection-2	2520121
Injection-3	2508140
Injection-4	2517743
Injection-5	2519377
Average	2519521
Standard Deviation	8579.4
%RSD	0.34

Table No-2: Summary of ritonavir precision:

Injection	**Area**
Injection-1	742716
Injection-2	738788
Injection-3	749890
Injection-4	743884
Injection-5	743329
Average	743721
Standard Deviation	3986.7
%RSD	0.54

INTERMEDIATE PRECISION:

Table No-3: Summary of Atazanavir intermediate precision:

Injection	**Area**
Injection-1	2568708
Injection-2	2578435
Injection-3	2583364
Injection-4	2581777
Injection-5	2593887
Average	2581234
Standard Deviation	9078.3
%RSD	0.35

Table No-4: Summary of ritonavir intermediate precision:

Injection	**Area**
Injection-1	760558
Injection-2	761801
Injection-3	760207
Injection-4	763501
Injection-5	764051
Average	762024
Standard Deviation	1716.7
%RSD	0.23

ACCURACY (RECOVERY STUDIES):

To check the degree of accuracy of the method, recovery studies were performed in triplet by standard addition method at 50%, 100% and 150% concentration levels.

Results of recovery studies are shown in table no.5 and 6.

Table No-5: Summary of Recovery studies of Atazanavir.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1273593	15.0	15.2	101.3%	100.5%
100%	2525197	30.0	30.1	100.4%	
150%	3783688	45.0	45.1	100.3%	

Table No-6: Summary of Recovery studies of Ritonavir.

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	372636	5.08	5.11	100.6%	100.3%
100%	717252	10.0	9.84	98.4%	
150%	1116613	15.2	15.3	100.8%	

LINEARITY:

Linearity was studied by preparing standard solutions at different concentration levels.

Table No-7: Summary of Linearity for Atazanavir

S.No	Linearity Level	Concentration	Area
1	I	60ppm	1296416
2	II	90ppm	1959240
3	III	120ppm	2503083
4	IV	150ppm	3192029
5	V	180ppm	3815691
Correlation Coefficient			0.999

Table No-8: Summary of Linearity for Ritonavir.

S.No	Linearity Level	Concentration	Area
1	I	20ppm	376247
2	II	30ppm	577388
3	III	40ppm	753267
4	IV	50ppm	940468
5	V	60ppm	1109090
Correlation Coefficient			0.999

Figure No-3: Linearity Graph of Atazanavir.

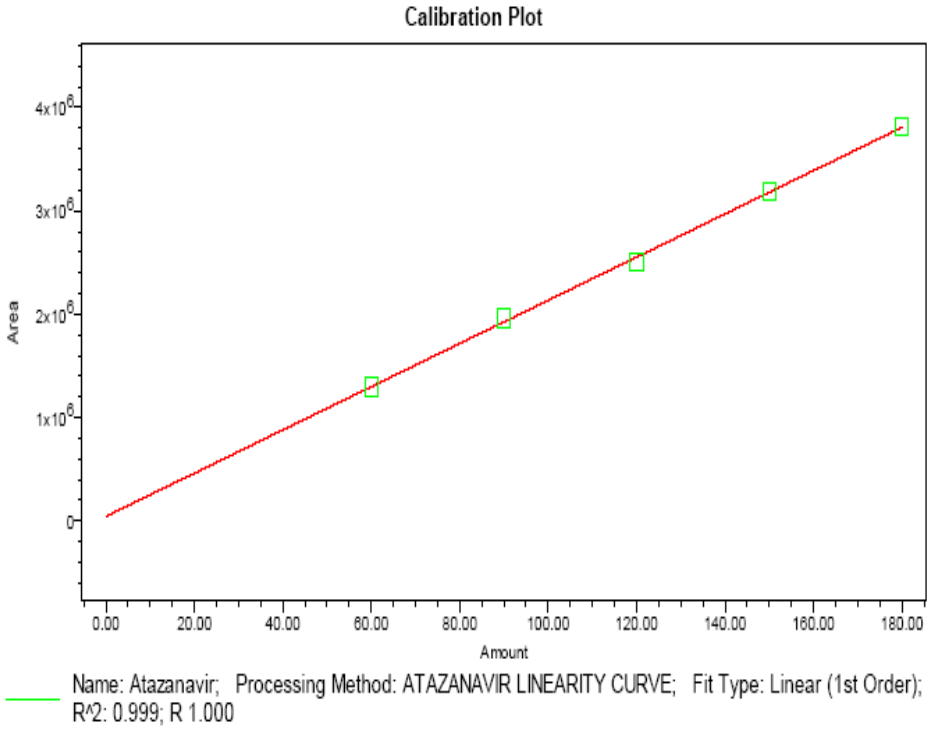
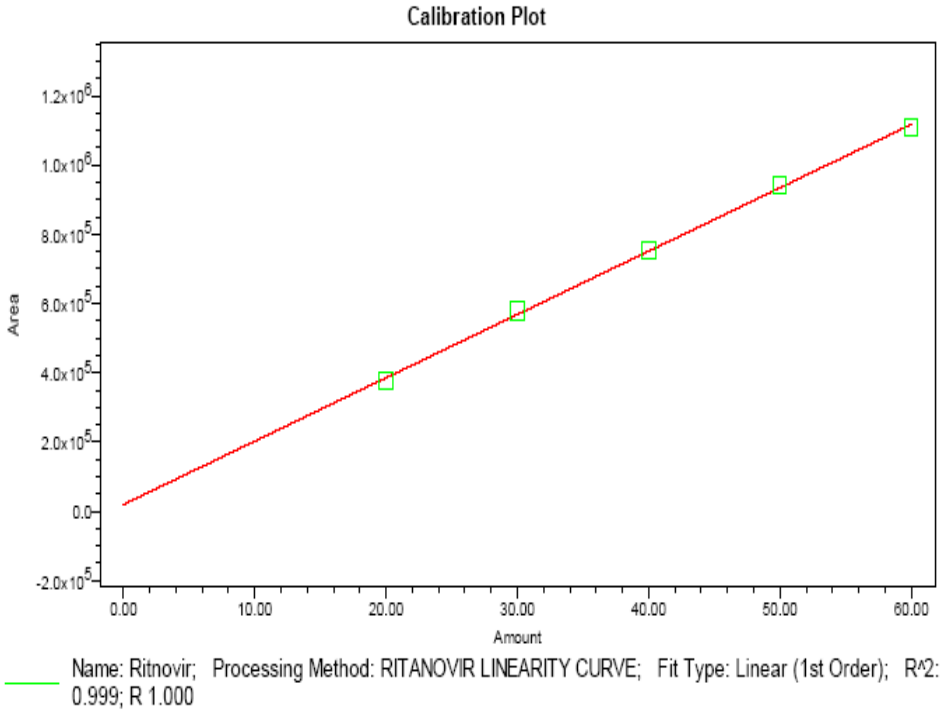


Figure No-4: Linearity Graph of Ritonavir.



LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

ATAZANAVIR:

For Atazanavir the LOD concentration obtained is 0.072 µg/ml (or) 0.6% with respect to working concentration of 0.1mg/ml and the LOQ concentration obtained is 0.24 µg/ml (or) 2.0% with respect of working concentration of 0.1mg/ml (table no.9).

Table No-9: Summary of LOD and LOQ.

Component	Working concentration[mg/ml]	LOD concentration[µg/ml]	Signal to Noise Ratio
Atazanavir [LOD]	0.120	0.072	3.04
Atazanavir [LOQ]	0.120	0.24	10.1

RITONAVIR:

For Ritonavir the LOD concentration obtained is 0.096µg/ml (or) 2.4% with respect to working concentration of 0.025mg/ml and the LOQ concentration obtained is 0.32µg/ml (or) 0.8% with respect to working concentration of 0.025mg/ml (table no.10).

Table No-10: Summary of LOD and LOQ.

Component	Working concentration[µg/ml]	LOD concentration[µg/ml]	Signal to Noise Ratio
Ritonavir [LOD]	0.040	0.096	3.31
Ritonavir [LOQ]	0.040	0.32	9.95

ROBUSTNESS:

The flow rate was varied at 0.8 ml/min to 1ml/min.

The results are summarized:

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The method is robust only in less flow condition.

System suitability results for Atazanavir:

S.No	***Flow Rate (ml/min)***	***System Suitability Results***	
		USP Plate Count	***USP Tailing***
1	*0.8*	*3431.8*	*1.4*
2	*0.9*	*3047.1*	*1.4*
3	*1.0*	*3104.8*	*1.4*

System suitability results for Ritonavir:

S.No	**Flow Rate (ml/min)**	**System Suitability Results**	
		USP Plate Count	**USP Tailing**
1	0.8	3845.1	1.3
2	0.9	3492.3	1.3
3	1.0	3495.9	1.3

The Organic composition in the Mobile phase was varied from 45% to 65%.

The results are summarized

On evaluation of the above results, it can be concluded that the variation in 10% Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is not robust even by change in the Mobile phase ±1

System suitability results for Atazanavir:

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	10% less	3462.1	1.2
2	*Actual	3047.1	1.4
3	10% more	3004.6	1.4

System suitability results for Ritonavir:

S.No	**Change in Organic Composition in the Mobile Phase**	**System Suitability Results**	
		USP Plate Count	**USP Tailing**
1	10% less	3807.6	1.2
2	*Actual	3492.3	1.3
3	10% more	3477.4	1.4

Conclusion:

The proposed method is simple, specific, accurate and precise and hence can be used in routine for estimation of atazanavir and ritonavir in tablet dosage. Statistical analysis of the results has been carried out revealing high accuracy and good precision. The percentage RSD for all parameters was found to be less than two, which indicates the validity of the method and assay results obtained by this method are in fair agreement.

Acknowledgement:

The authors are thankful to Mr. K. Chandra Shekar, Managing Director of Pharma train, Balanagar, Hyderabad. Author thankful to A. Sunil Kumar Reddy, principal, Bharat Institute of Technology(Pharmacy) his support during this work.

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