INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Research Article

METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ATAZANAVIR AND RITONAVIR IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

Anusha Tiyyagura[1]*, Ashwini Gunda[1], Annapurna Renee Chitturi[1], Aravind sai[2], Abbaraju prasana Laxmi[3] and Avinash Kodoori[4]

[1]University College of Technology, Osmania University, Hyderabad, Andhra Pradesh, India.
[2]Swinburne University, Melbourne, Austraila.
[3]Bharat Institute of Technology, Ibrahimpatnam, Hyderabad, Andhra Pradesh, India.
[4]University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India.

ABSTRACT

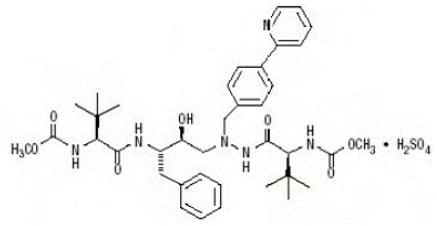
A simple, precise, specific and accurate reverse phase HPLCmethod has been developed for the determination of Atazanavir and Ritonavir in pharmaceutical dosage forms.Thechromatographic separation was achieved on Symmetry C8 (4.6 x 100mm, 5µm, Make: ACE)column using a mixture of Buffer:Acetonitrile (45:55) as the mobile phase at a flow rate 0.9ml/min. The retention time of Atazanavir and Ritonavir was 2.9 min and 4.1min.The analyte was monitored using UV detector at 235 nm.Results of analysis were validated statistically and byrecovery studies. The method was validated according to theICH guidelines with respect to linearity, accuracy, precision and robustness. The proposedmethod can be successfully used to determine the drug contents of marketed formulation.

Keywords: Atazanavir and Ritonavir, RP-HPLC and validation.

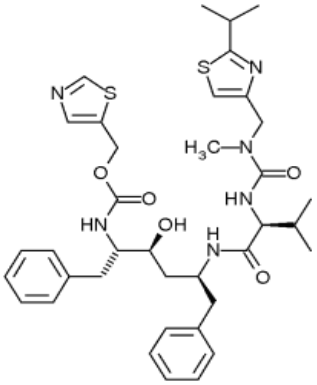
INTRODUCTION

Atazanavirantiretroviral drug (protease inhibitor)is chemicallymethyl *N*-[(1*S*)-1-{[(2*S*,3*S*)-3-hydroxy-4-[(2*S*)-2[(methoxy-carbonyl)amino]-3,3-dimethyl-*N'*-{[4-(pyri din-2-yl) phenyl] methyl}butanehydrazido]-1-phenylbutan-2-yl]carbamoyl}-2,2-dimethyl propyl] carbamate sulfate having a molecular formula of $C_{38}H_{52}N_6O_7.H_2SO_4$with a Molecular weight of 802.9 g/mol. Atazanavir (ATV) is an azapeptide HIV-1 protease inhibitor (PI). The compound selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells, thus preventing formation of mature virions[1-5].

Ritonavir is also an antiretroviral drug belonging to the class of protease inhibitors. Ritonavir chemically is 3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl}) carbamoyl] amino} butanamido]-1,6-diphenylhexan-2-yl] carbamate, having a molecular formula of$C_{37}H_{48}N_6O_5S_2$with a molecular weight 720.946 g/mol. Protease inhibitors, such as Ritonavir prevent viral replication by inhibiting the activity of proteases, e.g.HIV-1 protease, enzymes used by the viruses to cleave nascent proteins for final assembly of new virions[5-10].



Atazanavir sulfate



Ritonavir

An extensive literature review on the methods reported for the simultaneous estimation of Atazanavir and Ritonavir gives out information that there are few separate methods reported for the quantitative estimation of Atazanavir sulfate in bulk, pharmaceutical dosage forms and in plasma by HPLC, likewise a very few methods have been reported for the quantitative estimation of Ritonavir by HPLC but till date no method has been reported for the simultaneous quantitative estimation of Atazanavir and Ritonavir by HPLC[11-17]. There is just one spectrophotometric method reported for the simultaneous estimation of Atazanavir sulfate and Ritonavir in tablets. The present developed method was used determine the Atazanavir and Ritonavir present in the formulation and method validated according to the ICH guidelines[18-19].

Reagents, standards and samples

Water HPLC grade, Atazanavir working Standard, Ritonavir working standard, Potassium Dihydrogen Orthophosphate, Acetonitrile, orthophosphoric acid.

Chromatographic parameters

Equipment: High performance liquid chromatography equipped with Auto Sampler and UV detector.
Column: Symmetry: C-8(4.6x100 mm, 5um, Make: ACE)
Flow rate: 0.9ml per min
Mobile phase: pH-2.5 Phosphate buffer: Acetonitrile [45:55]
Wavelength : 235nm
Injection volume: 20µl
Column oven: Ambient
Run time: 8 min

Preparation of Phosphate buffer

7.0 grams KH_2PO_4 was weighed and transferred into a 1000ml beaker to which HPLC water was added an the pH was adjusted to 2.5 with Ortho phosphoric acid.

Preparation of mobile phase

Mixture of above buffer 450 ml (45%) and 550 ml of Acetonitrile HPLC (55%) was prepared and degassed in ultrasonic water bath for 5 minutes. The solution was later filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

Mobile Phase as diluent.

Assay

Preparation of Atazanavir and Ritonavir Standard & Sample Solution

Standard Solution Preparation

25 mg of Atazanavir and 25mg of Ritonavir working standard were accurately weighed and transferred into a 25 ml clean dry volumetric flask and about 20 ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Sample Solution Preparation

Equivalent weight of 25 mg of Atazanavir and equivalent weight of 25mg of Ritonavir sample were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure

20 µL of the standard, sample solutions were injected into the chromatographic system and the areas for the Atazanavir and Ritonavir peaks were measured and the %Assay was calculated using the formulae.

Calculation

% Assay =

$$\frac{AT}{AS} \times \frac{WS}{WT} \times \frac{DT}{DS} \times \frac{P}{100} \times \frac{Avg\ wt}{LC} \times 100$$

Where;

AT = average area counts of sample preparation.

AS = average area counts of standard preparation.

WT = Weight of sample taken in mg.

WS = Weight of working standard taken in mg.

DT = Dilution factor of sample

DS = Dilution factor of standard

P = Percentage purity of working standard

LC = Label claim of sample in mg/ml.

System Suitability

Tailing factor for the peaks due Atazanavir and Ritonavir should not bemore than 2.0.

Theoretical plates for the Atazanavir and Ritonavir peaks should notbe less than 2000.

Precision

Preparation of stock solution

25 mg of Atazanavir and 25mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted 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.

Acceptance Criteria

The % RSD for the area of five standard injections results should not be more than 2%

Calculation

$$\%RSD = \left(\frac{\text{Standard Deviation}}{\text{Mean}}\right) * 100$$

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.

a) System ID Precision

b) Preparation of stock solution

25 mg of Atazanavir and 25mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure

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Acceptance Criteria

The % RSD for the area of five standard injections results should not be more than 2%.

Calculation

$$\%RSD = \left(\frac{\text{Standard Deviation}}{\text{Mean}}\right) * 100$$

Accuracy

Preparation of Standard stock solution

25 mg of Atazanavir and 25mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it

completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation Sample solutions

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

12.5 mg of Atazanavir and 12.5mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

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

25 mg of Atazanavir and 25mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

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

37.5 mg of Atazanavir and 37.5 mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir & 0.6ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Procedure

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected and the amount found and amount added for Atazanavir & Ritonavir were calculated and also the individual recovery and mean recovery values were calculated.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%

Calculation

$$\%\text{Recovery} = \left(\frac{\text{Amount found}}{\text{Amount added}}\right) * 100$$

Linearity

Preparation of stock solution

25 mg of Atazanavir and 25 mg of Ritonavir working standard were accurately weighed and transferred into a 25ml clean dry volumetric flask about 20ml of diluent is added and sonicated to dissolve it completely and the volume is made up to the mark with the same solvent. (Stock solution).

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

0.6ml & 0.2 ml of stock solution was taken into a 10ml of volumetric flask and diluted up to the mark with diluent.

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

1.2ml & 0.4 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

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

1.8ml & 0.6 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – IV (240ppm of Atazanavir & 80ppm of Ritonavir)

2.4ml & 0.8 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level – V (300ppm of Atazanavir & 100ppm of Ritonavir)
3.0ml & 1.0 ml of stock solution was taken in 10ml of volumetric flask diluted up to the mark with diluent.

Procedure
Each level was injected into the chromatographic system and the peak area was measured.Graphs of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) were plotted and the correlation coefficient was calculated.

Acceptance Criteria
Correlation coefficient should be not less than 0.999.

Limit of Detection (Atazanavir)
Preparation of 180µg/ml solution
25 mg of Atazanavir working standard was accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.04% solution At Specification level (0.07µg/ml solution)
Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and dilute up to the mark with diluent. Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent. 0.4ml of the above solution was again pipetted out into a 10 ml of volumetric flask and diluted up to the mark with diluent.

Limit of Detection (For Ritonavir)
Preparation of 60µg/ml solution
25 mg of Ritonavir working standard was accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 0.6 ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.15% solution At Specification level (0.09µg/ml solution)
Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent**.** Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. 0.15 ml of the above solution was again pipetted out into a 10 ml volumetric flask and diluted up to the mark with diluent.

Limit of Quantification (Atazanavir)
Preparation of 180µg/ml solution
25 mg of Atazanavir working standard was accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 1.8ml of Atazanavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.14% solution At Specification level (0.25µg/ml solution)
Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.1.4ml of the above solution was pipetted out into a 10 ml of volumetric flask and diluted up to the mark with diluent.

L**imit of Quantitation (Ritonavir)**
Preparation of 60µg/ml solution
25 mg of Ritonavir working standard was accurately weighed and transferred into a 25ml clean dry volumetric flask and about 20ml of diluent was added and sonicated to dissolve it completely and volume is made up to the mark with the same solvent. (Stock solution). Further from the above stock solution 0.6 ml of Ritonavir is pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of 0.5% solution At Specification level (0.30µg/ml solution)
Further 1ml of the above stock solution was pipetted out into a 10ml volumetric flask and diluted up to the mark with diluent. 0.5 ml of the above solution was pipetted out into a 10 ml of volumetric flask and diluted 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 1.0 ml/min**
Standard solution 180ppm of Atazanavir & 60ppm 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 60% to 50%**
Standard solution 180 µg/ml of Atazanavir & 60 µg/ml of Ritonavirwas prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Precision data for Atazanavir and Ritonavir

Run	Atazanavir (Area)	Ritonavir (Area)
1	2328864	894680
2	2432878	928763
3	2432878	893719
4	2432571	927703
5	2414270	921964
Average	2408292	913366
Standard Deviation	45119.17	17689.7
%RSD	1.87	1.94

Intermediate Precision data for Atazanavir and Ritonavir

Run	Atazanavir (Area)	Ritonavir (Area)
1	2411522	927107
2	2409643	927276
3	2418907	927597
4	2414020	924706
5	2405748	925078
Average	2411968	926353
Standard Deviation	4915.6	1351.5
%RSD	0.20	0.15

Accuracy results for Atazanavir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1163259	12.5	12.48	99.8%	100.1%
100%	2349046	25.0	25.2	100.8%	
150%	3483810	37.5	37.39	99.7%	

Accuracy results for Ritonavir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	455728	12.5	12.5	100.5%	100.1%
100%	907126	25.0	25.0	100.0%	
150%	1389787	37.5	37.3	99.6%	

Linearity Results

S.No	Linearity Level	Concentration	atazanavir Area	Ritonavir (Area)
1	I	60ppm	829813	336774
2	II	120ppm	1630131	624165
3	III	180ppm	2396048	899783
4	IV	240ppm	3250765	1224557
5	V	300ppm	3933987	1465377
Correlation Coefficient			0.999	0.999

System Suitability Result

S.NO.	Parameters	Atazanavir	Ritonavir
1	Theoretical plates	3870.1	3935.4
2	Tailing factor	1.3	1.0
3	Resolution	4.8	4.8
4	Relative retention time	2.96	4.1

Robustness (flow rate) Atazanavir

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	3157.6	1.2
2	0.9	3870.1	1.3
3	1.0	2698.7	1.2

Robustness (flow rate) Ritonavir

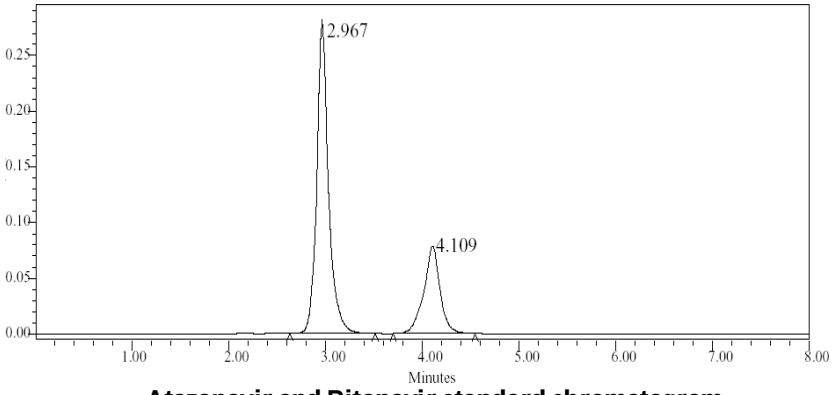
S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	2921.3	1.0
2	0.9	3870.1	1.0
3	1.0	2882.8	1.1

Organic Composition alteration for Atazanavir

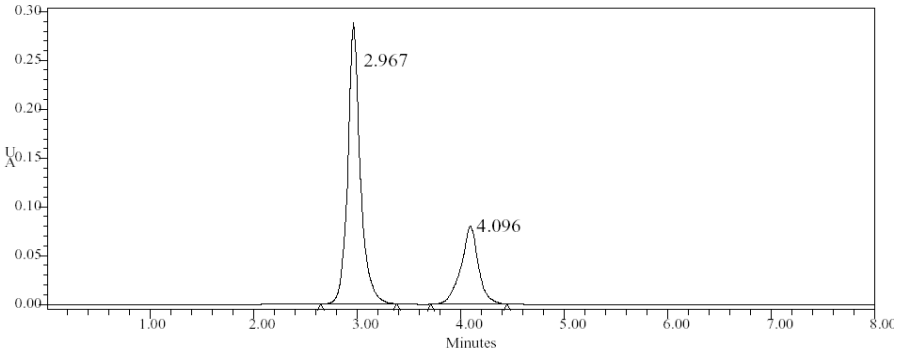
S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	3172.6	1.2
2	*Actual	3870.1	1.3
3	10% more	2612.1	1.2

Composition alteration for Ritonavir

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2951.2	1.0
2	* Actual	3935.4	1.0
3	10% more	2831.6	1.1

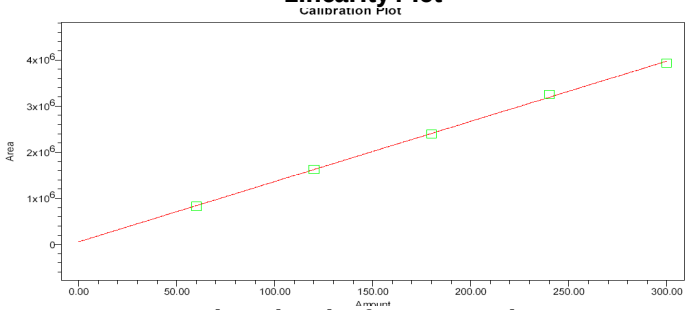


Atazanavir and Ritonavir standard chromatogram

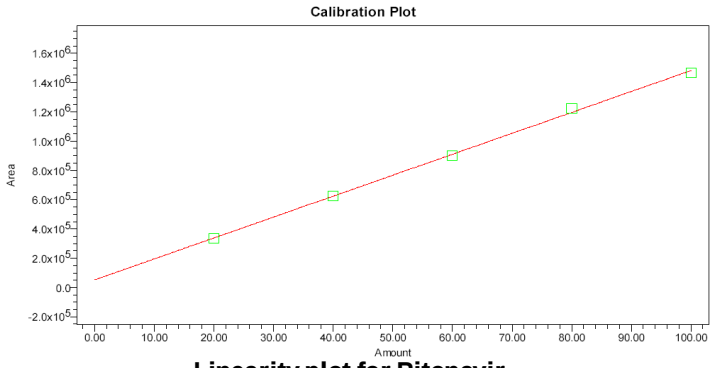


Atazanavir and Ritonavir sample chromatogram

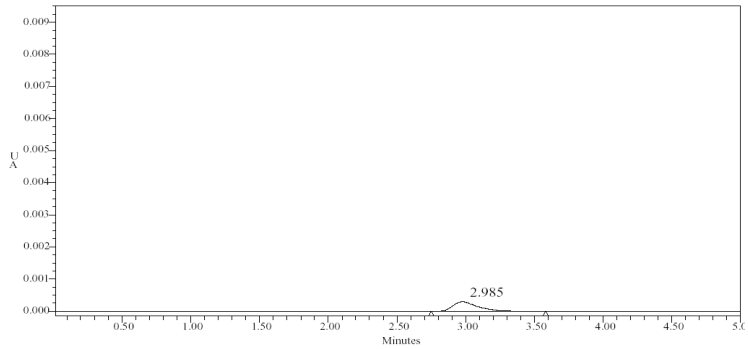
Linearity Plot



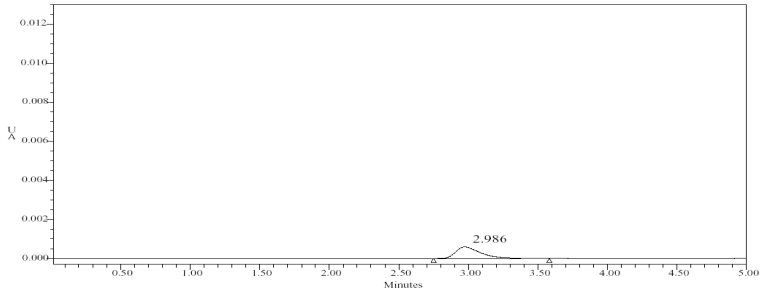
Linearity plot for Atazanavir



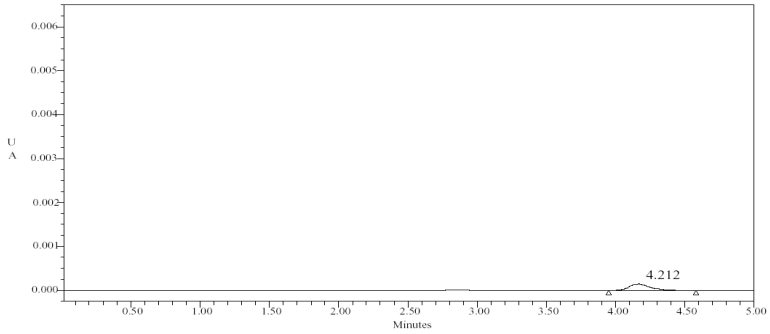
Linearity plot for Ritonavir



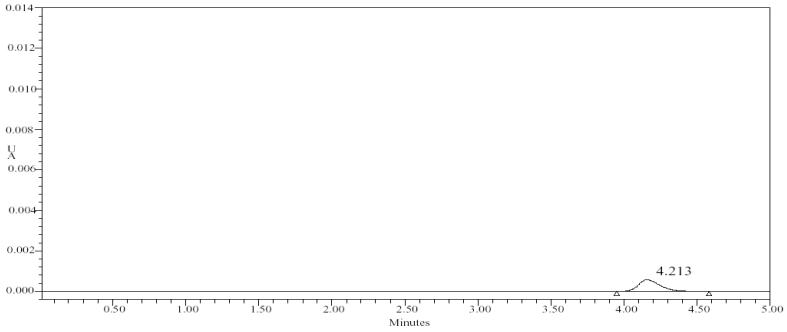
LOD for Atazanavir



LOQ for Atazanavir



LOD for Ritonavir



LOQ for Ritonavir

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 235 nm, chromatograms were taken and System suitabilityparameters were computed. The system suitability was calculated as perICH guidelines.Five replicates of standard samples were injected and the parameters like theoretical plate number (N), tailing factor (T), resolution(R), and relative retention time were estimated. From the results it can be concluded that all the system suitability parameters pass the criteria.

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.

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.The results were found to be within the limits of 98% to 102%

Linearity

Linearity was studied by preparing standard solutions at different concentration
levels.Correlation coefficient was found to be not less than 0.999.

LOD and LOQ

Atazanavir: LOD & LOQ for Atazanavir were found to be 0.07 ug/ml and 0.25ug/ml.

Ritonavir: LOD & LOQ for Ritonavir were found to be 0.09ug/ml and 0.30 ug/ml.

Robustness

The flow rate was varied at 0.8 ml/min to 1.0 ml/min as a part of testing the robustness of the method. The results of the changed flow rate indicate that the method is robust even by change in the flow rate ±10%. Similarly, the organic composition in the mobile phase is altered from 60% to 50%. The resultsindicate that the method is robust even by change in the organic phase ±10%.

CONCLUSION

The results of the validation study indicate that the analytical method developed for the determination of assay is found to be accurate and precise.The percentage RSD for all parameters was found to be less than two,which indicates the validity of the method and assay results obtained bythis method are in fair agreement. The method is both repeatable and rugged.

ACKNOWLEDGEMENT

The authors are thankful to Managing Director of Pharma Train, Kukatpally,and, and Hyderabad for providing necessary facilities and we are also thankful to Mr. Suman for his moral support and guidance during this work.

REFERENCES

1. http://packageinserts.bms.com/pi/pi_reyataz.pdf
2. British Pharmacopoeia, British pharmacopoeia commission, London, UK. 2001; 1: 305.
3. http://www.medicinenet.com/atazanavir/article.htm
4. http://www.nlm.nih.gov/medlineplus/druginfo/meds/a603019.html
5. WWW.drugbank.com
6. http://en.wikipedia.org/wiki/Ritonavir
7. http://www.rxlist.com/norvir-drug.htm
8. http://www.norvir.com/
9. http://www.nlm.nih.gov/medlineplus/druginfo/meds/a696029.html
10. Rao JV. e-Journal of chemistry 2011;8(1):453-456.(www.e-journals.net)
11. Srinivas Rao. Journal of Pharmaceutical and Biomedical Analysis. 2011;55(1):31-47.
12. Arianna Loregia. Journal of Pharmaceutical and Biomedical Analysis. 2011;42(4):500-505.

13. Estelle Cateau Journal of Pharmaceutical and Biomedical Analysis. 2005;39(3-4):791-795.
14. Chiranjeevi. International journal of pharmaceutical sciences and research IJPSR. 2011;2(3).
15. Anindita Behera. Der Pharmacia letter. 2011;3(1):145-151.
16. Nanda RK. Der Pharma Chemica. 2011;3(3):84-88.
17. ICH Q2A. Guidelines on validation of analytical procedure; Definitions and terminology, Federal Register. 1995;60:11260.
18. ICH Q2B. Guidelines on validation of analytical procedure; Methodology, Federal Register. 1996;60:27464.