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

Anusha Tiyyagura¹, Ashwini Gunda¹, Annapurna Rene Chitturi¹, Aravind sai², Abbaraju prasana Laxmi³ and Avinash Kodoori⁴

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

ABSTRACT
A simple, precise, specific and accurate reverse phase HPLC method has been developed for the determination of Atazanavir and Ritonavir in pharmaceutical dosage forms. The chromatographic 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 4.1 min. The analyte was monitored using UV detector at 235 nm. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to linearity, accuracy, precision and robustness. The proposed method can be successfully used to determine the drug contents of marketed formulation.

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

INTRODUCTION
Atazanavir is a retroviral drug (protease inhibitor) which is chemically methyl \( N-(\{(2S,3S)-3-hydroxy-4-(\{(2S)-\{3,3-dimethyl-N'-(\{4-\{(pyridin-2-yl)phenyl\}methyl\}butanehydrazido\}-1-phenylbutan-2-yl)carbamoyl\}-2,2-dimethyl propyl\}carbamate sulfate having a molecular formula of \( C_{38}H_{52}N_{6}O_{7} \cdot H_{2}SO_{4} \) 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.

Ritonavir is also an antiretroviral drug belonging to the class of protease inhibitors. Ritonavir chemically is 3-thiazol-5-yilmethyl \( N-(\{(2S,3S,5S)-3-hydroxy-5-(\{(2S)-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 \( C_{37}H_{48}N_{6}O_{5}S_{2} \) 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.

![Atazanavir sulfate](attachment:image-url)
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. 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.

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 KH₂PO₄ 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 formulæ.

Calculation

\[
\% \text{ Assay} = \frac{\text{AT} \times \text{WS} \times \text{DT} \times \text{P} \times \text{Avg wt}}{\text{AS} \times \text{WT} \times \text{DS} \times 100 \times \text{LC}} \times 100
\]

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 be more than 2.0. Theoretical plates for the Atazanavir and Ritonavir peaks should not be 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

\[
\% \text{RSD} = \left( \frac{\text{Standard Deviation}}{\text{Mean}} \right) \times 100
\]

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

\[
\% \text{RSD} = \left( \frac{\text{Standard Deviation}}{\text{Mean}} \right) \times 100
\]

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 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). 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**

\[
\% \text{Recovery} = \left( \frac{\text{Amount found}}{\text{Amount added}} \right) \times 100
\]

**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 in 10ml of volumetric flask 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 diluted 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 Quantitation (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.

Limit 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 Ritonavir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

<table>
<thead>
<tr>
<th>Run</th>
<th>Atazanavir (Area)</th>
<th>Ritonavir (Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2328864</td>
<td>894680</td>
</tr>
<tr>
<td>2</td>
<td>2432878</td>
<td>928763</td>
</tr>
<tr>
<td>3</td>
<td>2432878</td>
<td>893719</td>
</tr>
<tr>
<td>4</td>
<td>2432571</td>
<td>927703</td>
</tr>
<tr>
<td>5</td>
<td>2414270</td>
<td>921964</td>
</tr>
<tr>
<td>Average</td>
<td>2408292</td>
<td>913366</td>
</tr>
<tr>
<td>%RSD</td>
<td>1.87</td>
<td>1.94</td>
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<table>
<thead>
<tr>
<th>Run</th>
<th>Atazanavir (Area)</th>
<th>Ritonavir (Area)</th>
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</thead>
<tbody>
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<td>1</td>
<td>2411522</td>
<td>927107</td>
</tr>
<tr>
<td>2</td>
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<td>927276</td>
</tr>
<tr>
<td>3</td>
<td>2418907</td>
<td>927597</td>
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<td>4</td>
<td>2414020</td>
<td>924706</td>
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<td>5</td>
<td>2405748</td>
<td>925078</td>
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<tr>
<td>Average</td>
<td>2411968</td>
<td>926353</td>
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<tr>
<td>%RSD</td>
<td>0.20</td>
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</table>

<table>
<thead>
<tr>
<th>%Concentration (at specification Level)</th>
<th>Area</th>
<th>Amount Added (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
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<tr>
<td>50%</td>
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<td>12.5</td>
<td>12.48</td>
<td>99.8%</td>
<td></td>
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<tr>
<td>100%</td>
<td>2349046</td>
<td>25.0</td>
<td>25.2</td>
<td>100.8%</td>
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<tr>
<td>150%</td>
<td>3483810</td>
<td>37.5</td>
<td>37.39</td>
<td>99.7%</td>
<td>100.1%</td>
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</table>
### Accuracy results for Ritonavir

<table>
<thead>
<tr>
<th>%Concentration (at specification Level)</th>
<th>Area</th>
<th>Amount Added (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
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<tbody>
<tr>
<td>50%</td>
<td>455728</td>
<td>12.5</td>
<td>12.5</td>
<td>100.5%</td>
<td>100.1%</td>
</tr>
<tr>
<td>100%</td>
<td>907126</td>
<td>25.0</td>
<td>25.0</td>
<td>100.0%</td>
<td></td>
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<tr>
<td>150%</td>
<td>1389787</td>
<td>37.5</td>
<td>37.3</td>
<td>99.6%</td>
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</table>

### Linearity Results

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<tr>
<th>S.No</th>
<th>Linearity Level</th>
<th>Concentration</th>
<th>Atazanavir Area</th>
<th>Ritonavir (Area)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>60ppm</td>
<td>829813</td>
<td>336774</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>120ppm</td>
<td>1630131</td>
<td>624165</td>
</tr>
<tr>
<td>3</td>
<td>III</td>
<td>180ppm</td>
<td>2396048</td>
<td>899783</td>
</tr>
<tr>
<td>4</td>
<td>IV</td>
<td>240ppm</td>
<td>3250765</td>
<td>1224557</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>300ppm</td>
<td>3933987</td>
<td>1465377</td>
</tr>
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</table>

Correlation Coefficient: 0.999

### System Suitability Results

<table>
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<tr>
<th>S.NO.</th>
<th>Parameters</th>
<th>Atazanavir</th>
<th>Ritonavir</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Theoretical plates</td>
<td>3870.1</td>
<td>3935.4</td>
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<tr>
<td>2</td>
<td>Tailing factor</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>Resolution</td>
<td>4.8</td>
<td>4.8</td>
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<tr>
<td>4</td>
<td>Relative retention time</td>
<td>2.96</td>
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### Robustness (flow rate) Atazanavir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Flow Rate (ml/min)</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8</td>
<td>3157.6</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>3870.1</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>2698.7</td>
<td>1.2</td>
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</tbody>
</table>

### Robustness (flow rate) Ritonavir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Flow Rate (ml/min)</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8</td>
<td>2921.3</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>3870.1</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>2882.8</td>
<td>1.1</td>
</tr>
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### Organic Composition alteration for Atazanavir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Change in Organic Composition in the Mobile Phase</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>10% less</td>
<td>3172.6</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>*Actual</td>
<td>3870.1</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>10% more</td>
<td>2612.1</td>
<td>12</td>
</tr>
</tbody>
</table>

### Composition alteration for Ritonavir

<table>
<thead>
<tr>
<th>S.No</th>
<th>Change in Organic Composition in the Mobile Phase</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% less</td>
<td>2951.2</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>*Actual</td>
<td>3935.4</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>10% more</td>
<td>2831.6</td>
<td>1.1</td>
</tr>
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</table>
Atazanavir and Ritonavir standard chromatogram

Atazanavir and Ritonavir sample chromatogram

Linearity plot for Atazanavir

Linearity plot 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 suitability parameters were computed. The system suitability was calculated as per ICH 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 results indicate 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 by this method are in fair agreement. The method is both repeatable and rugged.

ACKNOWLEDGEMENT
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