# STABILITY INDICATING RP–HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATAZANAVIR AND COBICISTAT IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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A novel approach was used to develop and validate stability indicating RP-HPLC method for the simultaneous determination and degradation of Atazanavir and Cobicistat in bulk and pharmaceutical dosage forms. Separation was achieved in isocratic mode with a dikma $C_{18}$ (4.6 × 250 mm), 5 µm column and mixture consisting of 0.1% OPA buffer, methanol, acetonitrile (40 : 20 : 40) as a mobile phase with a flow rate of 1ml/min and run time as 10min.Uv detection is performed at 239 nm and the sample temperature was maintained ambient. The described method for the simultaneous determination of Atazanavir and Cobicistat shows good precision results which were below 2.0% RSD and linear over a range of 100-500 µg/ml for Atazanavir and 50–250 µg/ml for Cobicistat respectively with the correlation coefficient $r^2 = 0.999$. The developed method was validated according to ICH guidelines for various parameters.

**KEYWORDS** : RP – HPLC method, Atazanavir, Cobicistat, simultaneous determinations, forced degradation.

## INTRODUCTION [1, 2, 11, 12]

Atazanavir is HIV – 1 protease inhibitor uesd for the treatment of HIV – 1infection in combination with other antiretroviral agent. Atazanavir is chemically known as Methyl N - [(1S) - 1 - {[2S, 3S] - 3 - hydroxyl - 4 - [(2S) - 2 [(methoxy carbonyl) amino] - 3, 3 dimethyl - N ' - {[4 - (pyridine - 2 - yl) phenyl] methyl} butane hydrazido] - 1 - phenyl butane - 2 - yl] carbonyl} - 2, 2 dimethyl propyl] carbamate. Atazanavir (protease inhibitor) mimics the transition state of the peptide cleavage reaction catalysed by HIV – 1 protease *i.e.*, it works by blocking the growth of HIV. The basic mechanism involved is , Atazanavir selectively inhibits the virus specific processing of viral Gag and Gag - pol polyproteins in HIV - 1 infected cells by binding to the active site of HI protease, thus preventing the information of mature virions. Atazanavir is not active against HIV - 2. Atazanavir is an azapeptide HIV - 1 protease inhibitor (PI) with the activity against human immunodeficiency virus Type - 1 (HIV - 1). HIV - 1 protease is an enzyme required for the proteolytic cleavage of the viral poly protein

precursor into the individual functional protein in infectious HIV - 1. Atazanavir binds to protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of viral poly protein results in the in the formation of immature viral particles.

Cobicistat is known as Thiazol - 5- yl methyl N - [1 - benzyl - 4 - [[2 - [[(2 - isopropyl thiazol - 4 - yl) methyl methyl carbamoyl] amino] - 4 - morpholinobutanoyl] amino] 5 - phenyl phenyl] carbamate. Cobicistat is a potent inhibitor of Cytochrome P 450 3A (CYP 3A) which act as a pharmaco enhancing or a boosting agent for anti viral drugs used in the treatment of HIV infection. Cobicistat inhibits the liver enzymes that metabolize other medications used to treat the HIV. Cobicistat is a novel pharmacokinetic boosting agent without the activity on HIV. The basic mechanism involved is, inhibition of CYP 3A mediated metabolism by Cobicistat increases the systemic exposure of CYP 3A substrates, Atazanavir, and Darunavir and therefore enables increased anti viral activity at lower dosage. Cobicistat does not have any anti - viral activity on its own.

## LITERATURE

Literature survey reveals that many spectroscopic and chromatographic method were reported for the estimation of these drugs individually in bulk and pharmaceutical dosage forms. However the analytical method lack stability indicating nature further there was no reported analytical method for the estimation of both drugs in pharmaceutical dosage forms in the presence for their degradation products. In the present degradation there was made to develop a simple, precise, rapid, accurate, stability indicating RP – HPLC assay method for simultaneous estimation of Atazanavir and Cobicistat.

## MATERIALS AND CHEMICALS

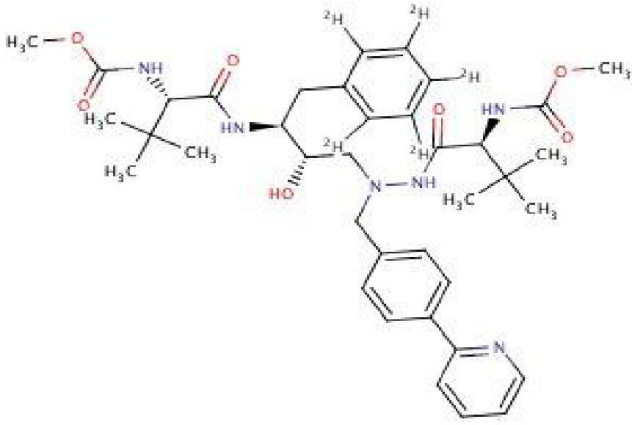
Atazanavir and Cobicistat the active pharmaceutical ingredient (API) was obtained from the HIQ labs, Hyderabad, India. HPLC grade of potassium hydrogen phosphate was obtained from FINER chemical limited, India. HPLC grade of water and methanol was obtained from LICHROSOLV (MERCK), India. Acetonitrile for HPLC was obtained from MOLYCHEM, India. Orthophosphoric acid obtained from MERCK, India.

## PREPARATION OF SOLUTIONS

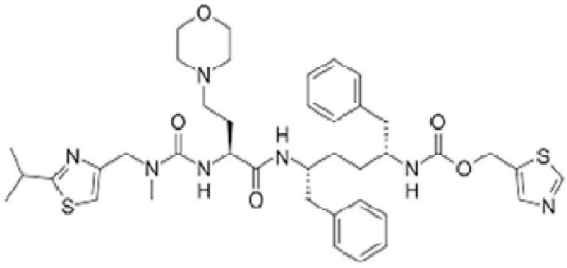
**Preparation of phosphate buffer :** Accurately pipette out 1ml of orthophosphoric acid was taken in 1000ml of volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted pH 3 with sodium hydroxide.

**Preparation of mobile phase :** Accurately measure 400 ml of above phosphate buffer and 200 ml of HPLC grade methanol and 400ml oh HPLC grade acetonitrile, mixed and degassed in an ultrasonic water bath for 10min and then filtered through 0.45µ filter paper under vaccum filtration.

**Diluent preparation :** The mobile phase is used as diluents



**Chemical structure of Atazanavir**



**Structure of cobicistat**

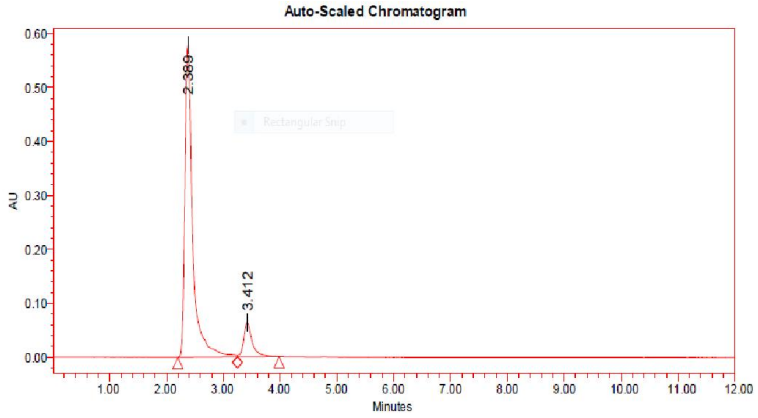
**Preparation of standard solution :** Accurately weigh and transfer 20 mg of Atazanavir and 10 mg of Cobicistat working standard into 10ml clean dry volumetric flask and add about 7ml of diluents and sonicate to dissolve it completely and make volume upto the mark with the same solvent (stock solution). Further pipette out 1.5 ml of the above stock solution into 10 ml volumetric flask and dilute upto the mark with the diluent.

**Preparation of sample solution :** Accurately weigh and transfer 20 mg of Atazanavir and 10mg of Cobicistat working standard into 10ml clean dry volumetric flask and add about 7ml of diluent and sonicate to dissolve it completely and make up the volume to the mark with the same diluent (stock solution). Further pipette out 1.5 ml of the above stock solution into a 10ml volumetric flask and dilute upto the mark with diluents.

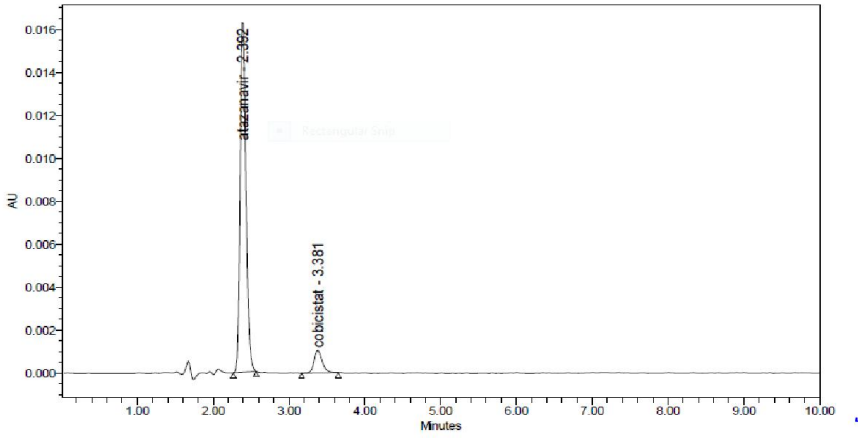
## METHOD DEVELOPMENT

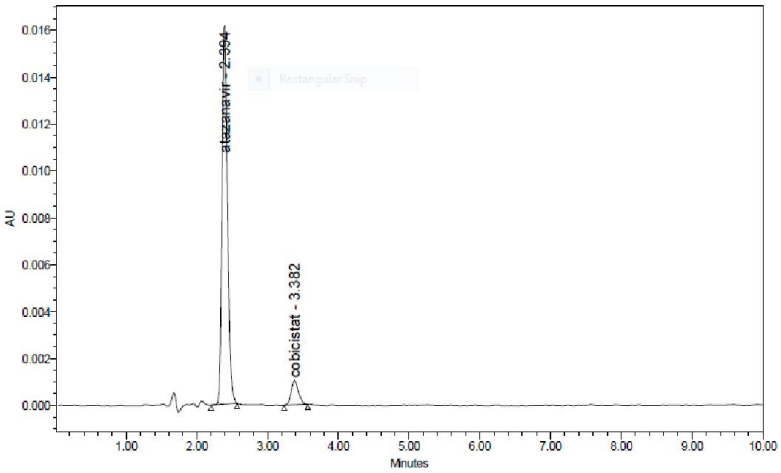
Seperation of Atazanavir and Cobicistat was achived on Dikma column (4.6 × 250 mm, 5 µm) column as stationnary phase using mobile phase consists of OPA buffer pH 3, Aceotonitrile and methanol in the ratio of 40 : 40 : 20 v/v isocratic elution was achieved at a flow rate 1 ml/min with ambient column temperature the injection volume was 20 µl .this chromatogram were recorded at 239 nm using photodiode array detector.

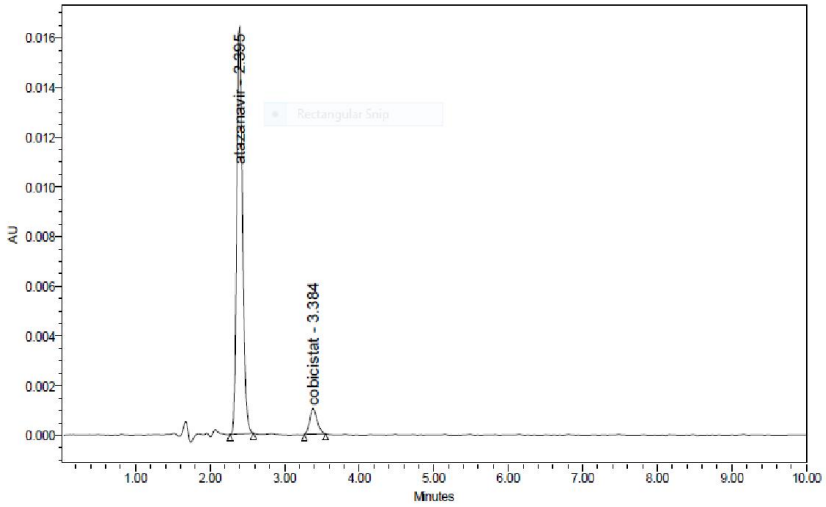
**Chromatogram of optimized conditions:**




**Chromatogram of Precision /repeatability:**

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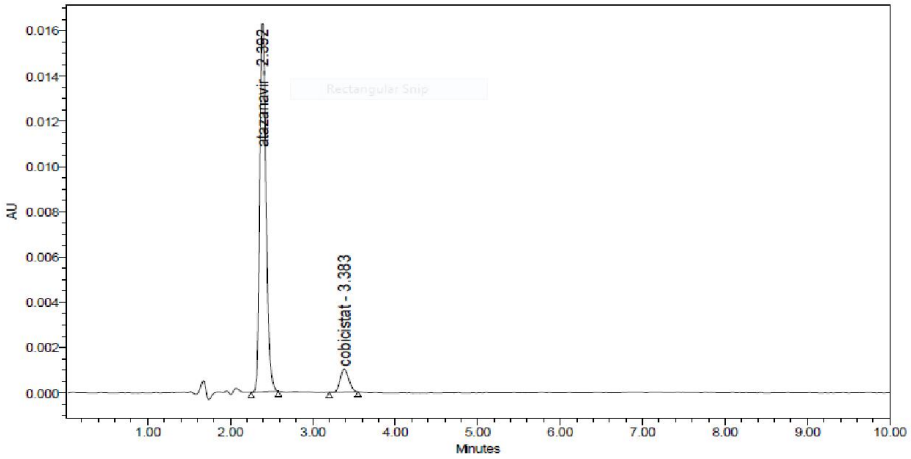
cobicistat - 3.384
AU
Minutes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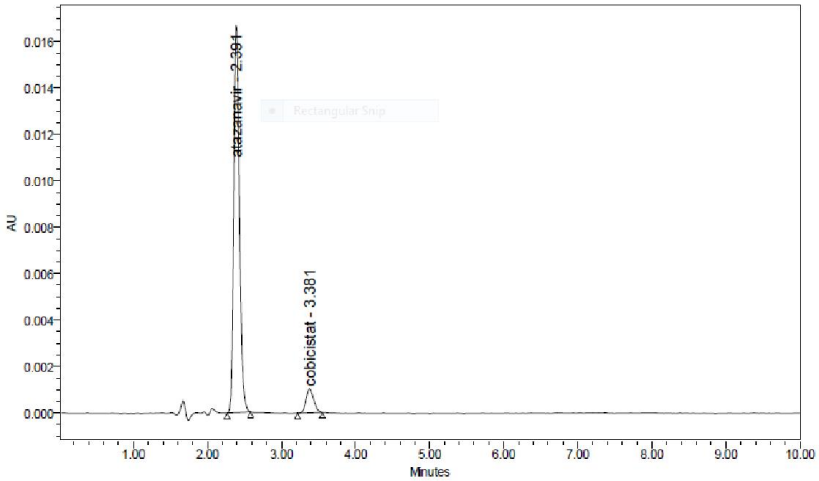
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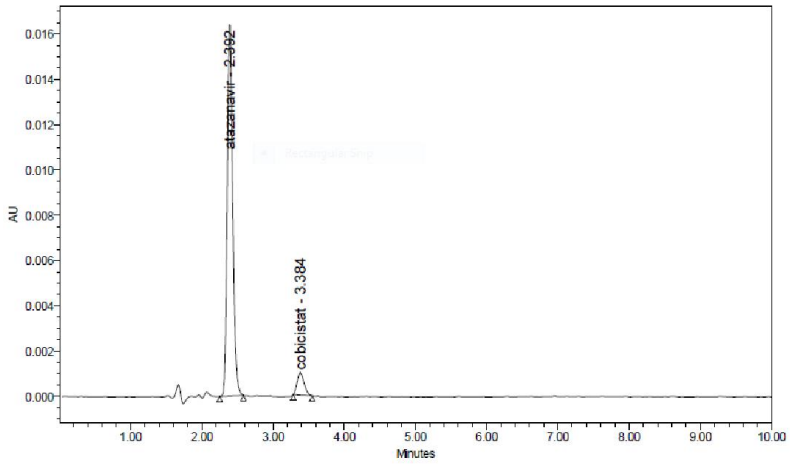
Minutes




# METHOD VALIDATION [3, 4, 5]

## PRECISION :

**Repeatability :** The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits and the results were shown in the table 1.

**Table 1. Results of Precision**

| Injection | Area for Atazanavir | Area for Cobisistat |
|---|---|---|
| Injection-1 | 87799 | 7524 |
| Injection-2 | 86973 | 7519 |
| Injection-3 | 86232 | 7524 |
| Injection-4 | 87604 | 7581 |
| Injection-5 | 85975 | 7558 |
| Injection-6 | 87018 | 7565 |
| **Average** | 86933.8 | 7545.2 |
| **Standard Deviation** | 723.5 | 26.2 |
| **%RSD** | 0.8 | 0.3 |

**Intermediate precision or ruggedness:**

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

**Procedure:** The standard solutions were prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for

the area of six replicate injections was found to be within the specified limits and the obtained results were shown in table 2.

**Table 2: Results of Intermediate Precision**

| Injection | Area of atazanavir | Area of cobicistat |
|---|---|---|
| Injection – 1 | 86017 | 7508 |
| Injection – 2 | 86172 | 7587 |
| Injection – 3 | 86652 | 7576 |
| Injection – 4 | 86680 | 7534 |
| Injection – 5 | 86818 | 7558 |
| Injection – 6 | 86585 | 7517 |
| Average | 86933.8 | 7546.7 |
| Standard deviation | 723.5 | 32.1 |
| %RSD | 0.8 | 0.4 |

**ACCURACY:**

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions in triplet injections. Calculate the Amount found and Amount added for Atzanavir & Cobisistat and calculate the individual recovery and mean recovery values in table 3 and 4.

**Table 3. Results of Accuracy for Atazanavir**

| %concentration (at specification level) | Area | Amount added (mg) | Amount found (mg) | %recovery | Mean recovery |
|---|---|---|---|---|---|
| 50% | 43148.6 | 10 | 10.01 | 100.08 | 100.43 |
| 100% | 86625.0 | 20 | 20.09 | 100.46 | |
| 150% | 30313.3 | 30 | 30.23 | 100.75 | |

**Table 4. Results of Accuracy for cobicistat**

| %concentration (at specification level) | Area | Amount added (mg) | Amount found (mg) | % recovery | Mean recovery |
|---|---|---|---|---|---|
| 50% | 3818.7 | 5 | 5.04 | 100.75 | 100.50 |
| 100% | 7587 | 10 | 10.01 | 100.08 | |
| 150% | 11447 | 15 | 15.01 | 100.67 | |

**Table 5. Results of Linearity for atazanavir**

| S. No. | Linearity level | concentration | Area |
|---|---|---|---|
| 1 | Level – 1 | 100 | 30018 |
| 2 | Level – 2 | 200 | 58216 |
| 3 | Level – 3 | 300 | 86174 |
| 4 | Level – 4 | 400 | 117088 |
| 5 | Level – 5 | 500 | 147293 |
| Correlation coefficient | | | 0.999 |

**Table 6. Results of Linearity for Cobicistat**

| S.no | Linearity level | Concentration | Area |
|---|---|---|---|
| 1 | Level – 1 | 50 | 2613 |
| 2 | Level – 2 | 100 | 4969 |
| 3 | Level – 3 | 150 | 7547 |
| 4 | Level – 4 | 200 | 9909 |
| 5 | Level – 5 | 250 | 12640 |
| Correlation coefficient | | | 0.999 |

**LINEARITY:**

Aliquots of 0.5, 1, 1.5, 2 and 2.5ml of mixed standard working solutions of Atazanavir and Cobicistat was pipette out from the standard stock solution into 10 ml clean dry volumetric flask and make volume up to the mark with the same diluent to get the concentration of 100 – 500 µg/ml of Atazanavir and 50 – 250 µg/ml of Cobicistat. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient and the results were shown in table 5 and 6.

**ROBUSTNESS :**

As a part of robustness that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10 and the results are shown in table 7.

**Table 7. Results of robustness**

| S.NO | Flow rate (1ml/min) | System suitability parameters | |
|---|---|---|---|
| | | USP plate count | USP tailing |
| 1 | 1.35 | 4531.39 | 1.20 |
| 2 | 1.5 | 4529.07 | 1.18 |
| 3 | 1.65 | 4072.7 | 1.15 |

| S.No | Flow rate (1ml/min) | System suitability parameters | | |
|---|---|---|---|---|
| | | USP Plate count | USP Tailing | USP Resolution |
| 1 | 1.35 | 4857.7 | 1.27 | 5.90 |
| 2 | 1.5 | 4633.60 | 1.20 | 5.75 |
| 3 | 1.65 | 5791.3 | 1.35 | 5.97 |

| S.No. | Change in organic composition in mobile phase | System suitability parameters | |
|---|---|---|---|
| | | USP Plate count | USP Tailing |
| 1 | 10% less | 4683 | 1.21 |
| 2 | Actual | 4529.07 | 1.81 |
| 3 | 10% more | 4383 | 1.21 |

| S.No | Change in organic composition in mobile phase | System suitability parameters | | |
|---|---|---|---|---|
| | | USP Plate count | USP Tailing | USP Resolution |
| 1 | 10% less | 5278.62 | 1.20 | 5.97 |
| 2 | Actual | 4633.60 | 1.20 | 5.75 |
| 3 | 10% more | 5201.62 | 1.20 | 5.97 |

**Table 8: Results for degradation:**

| Sample Name | Atazanavir | | | | |
|---|---|---|---|---|---|
| | Area | % Degraded | Purity Angle | Purity Threshold | Peak purity |
| **Standard** | **86056.0** | **100** | | | |
| Acid | 81872 | 95.14 | 4.86 | 90.00 | Passes |
| Base | 81285 | 94.46 | 5.54 | 90.00 | Passes |
| Peroxide | 82049 | 95.34 | 4.66 | 90.00 | Passes |
| Thermal | 82411 | 95.76 | 4.24 | 90.00 | Passes |
| Photo | 82185 | 95.50 | 4.50 | 90.00 | Passes |

| Sample Name | Cobisistat | | | | |
|---|---|---|---|---|---|
| | Area | % Degraded | Purity Angle | Purity Threshold | Peak purity |
| Standard | 7565.7 | 100 | | | |
| Acid | 7239 | 95.68 | 4.32 | 54.60 | Passes |
| Base | 7298 | 96.46 | 3.54 | 90.00 | Passes |
| Peroxide | 7267 | 96.05 | 3.95 | 90.00 | Passes |
| Thermal | 7245 | 95.76 | 4.24 | 90.00 | Passes |
| Photo | 7264 | 96.01 | 3.99 | 42.53 | Passes |

## DETECTION LIMIT

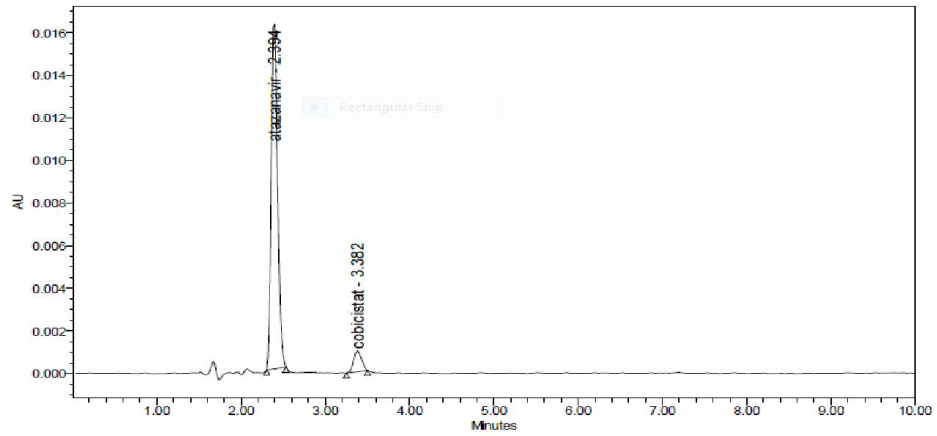
Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3 × SD/S and 10 × SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3).

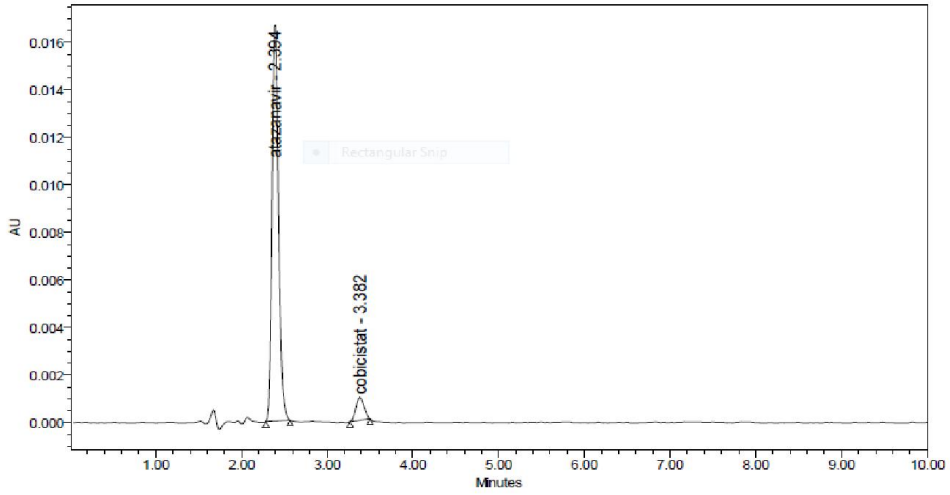
The LOD of Atazanavir Sulphate and Cobicistat was calculated. The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Atazanavir and Cobicistat was calculated.

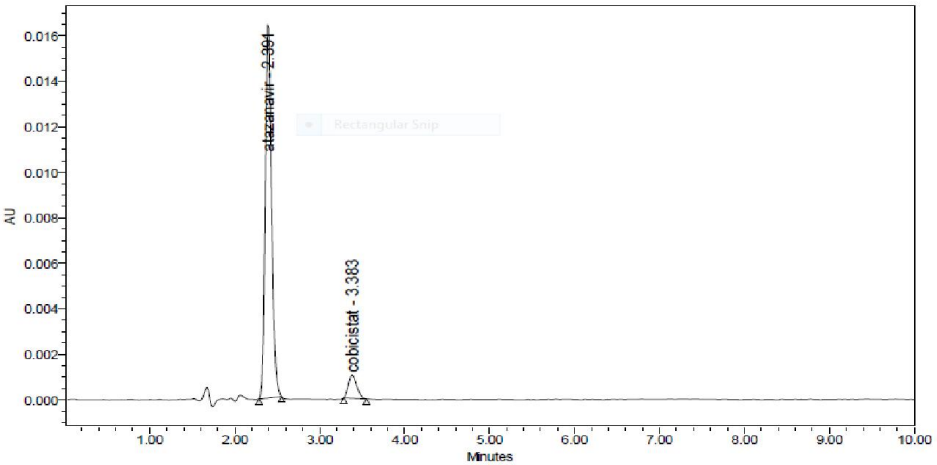
## DEGRADATION STUDIES [8, 9, 10]

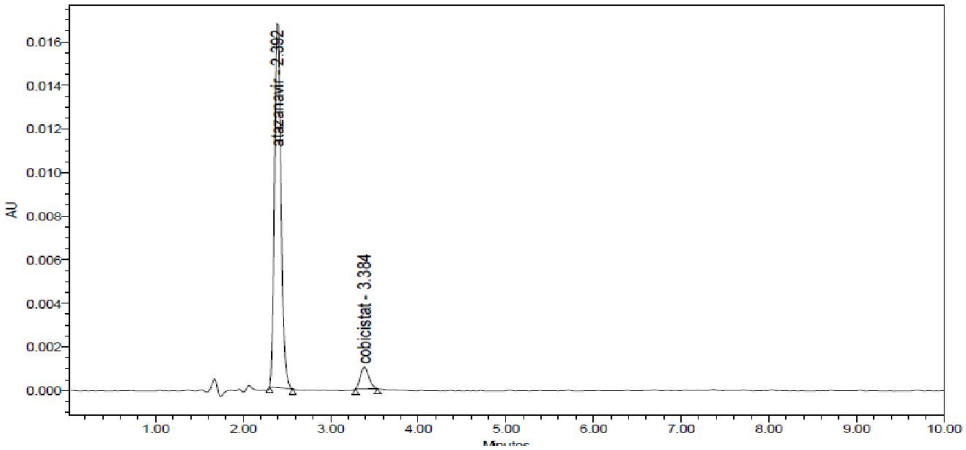
The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Atzanavir and Cobisistat using the proposed method.

**Chromatograms of intermediate precision**

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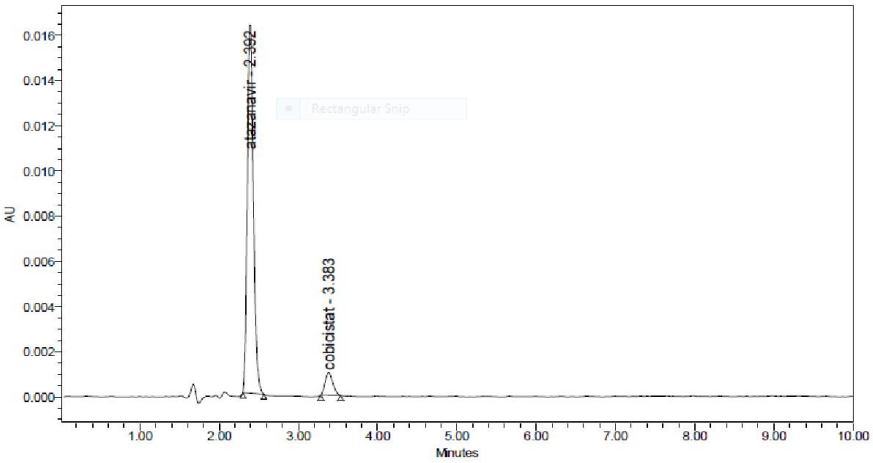
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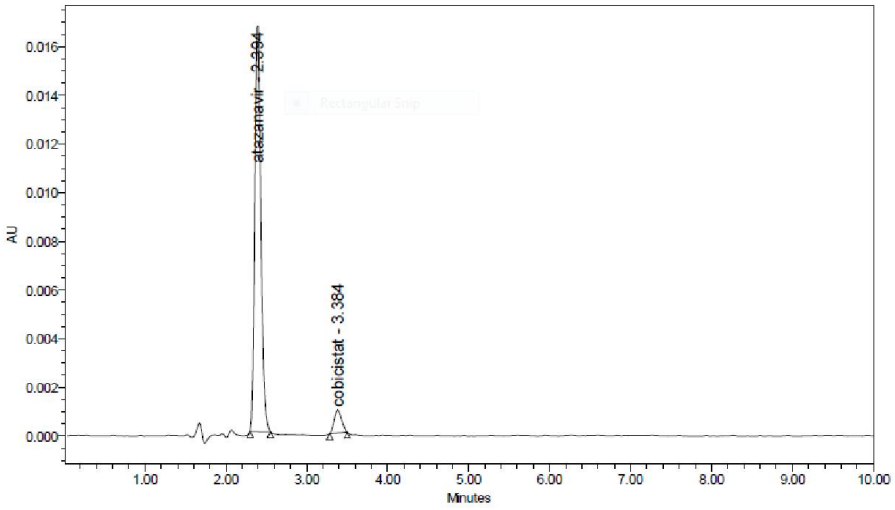
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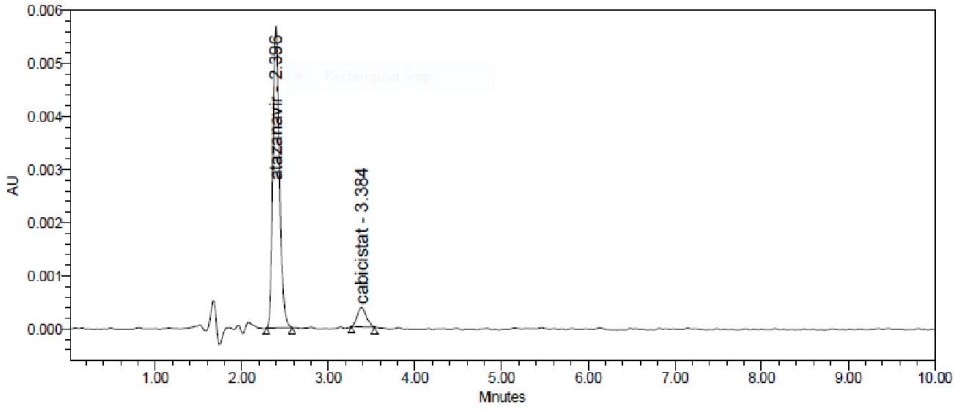
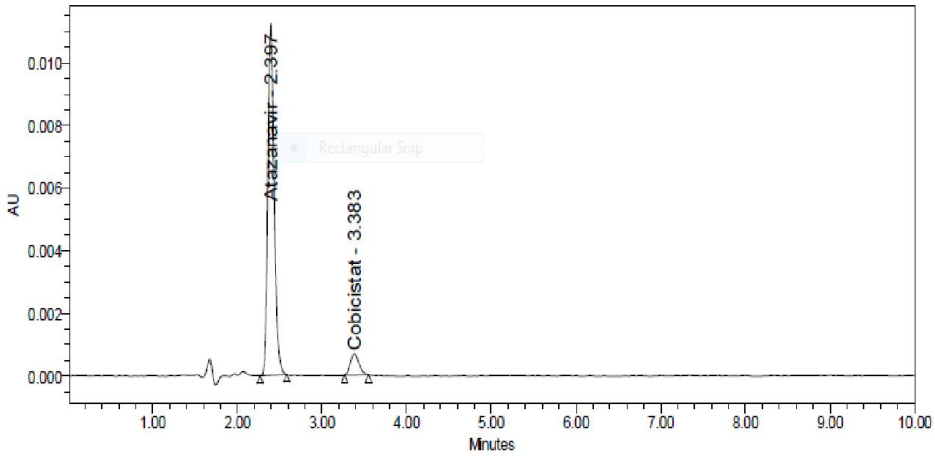
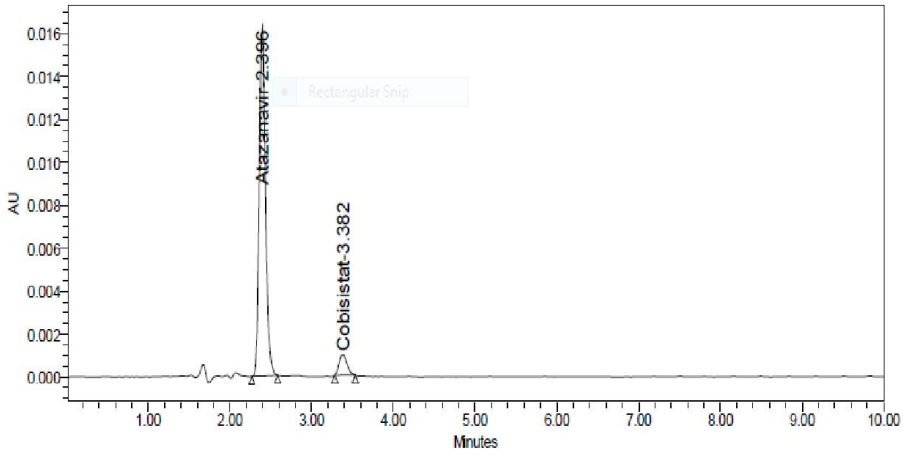
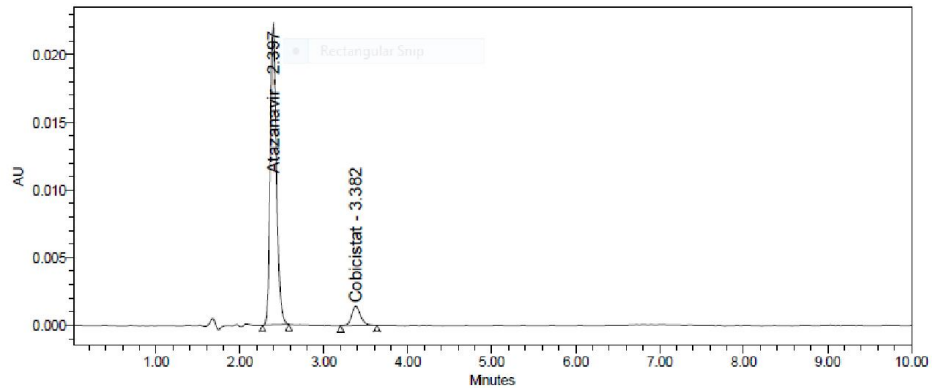
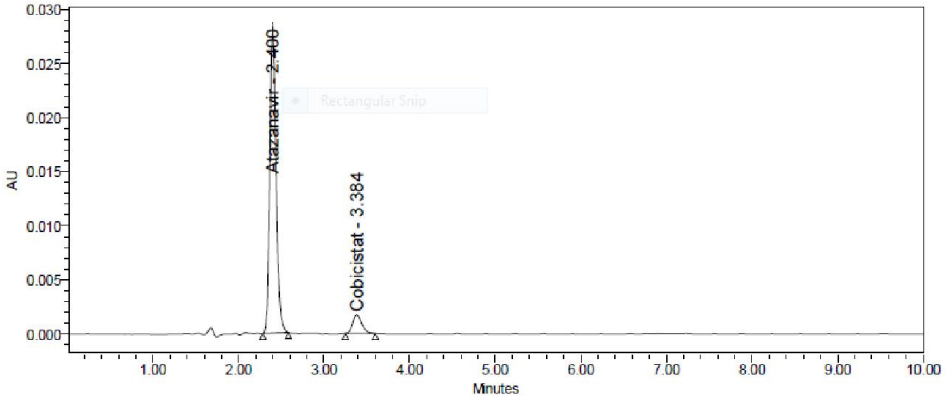
**Chromatograms of linearity**



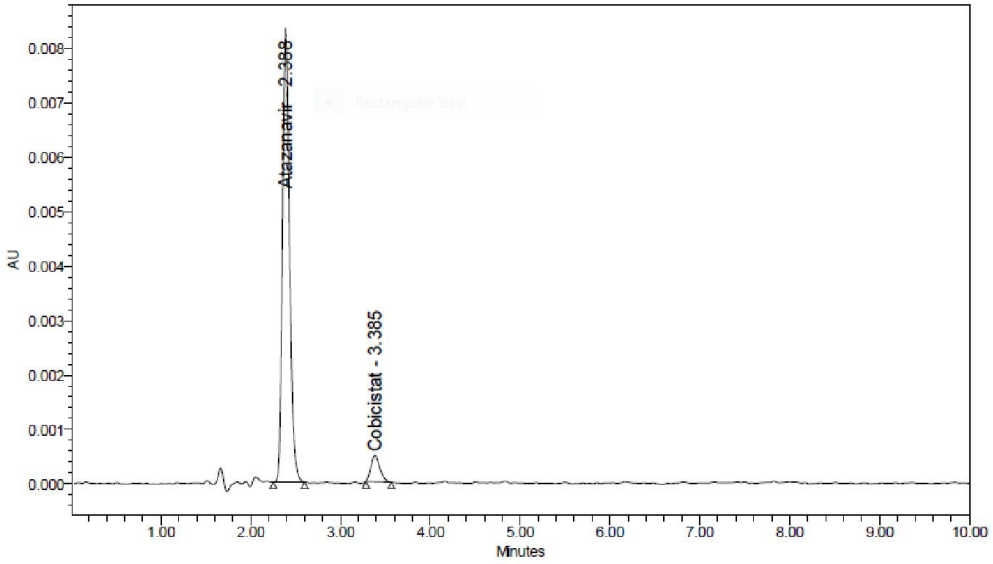



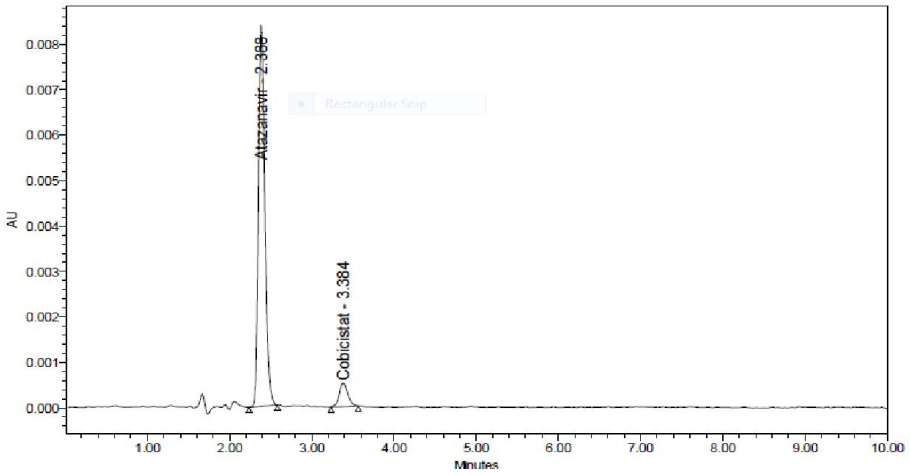



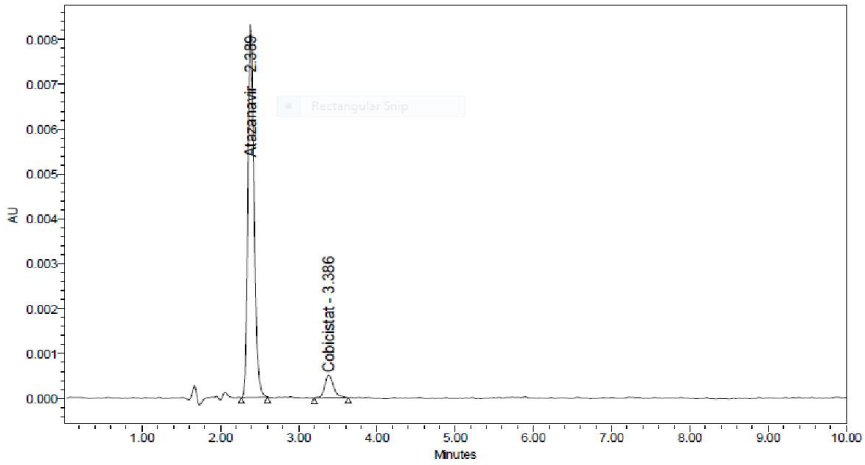



**Chromatogram of accuracy 50%**



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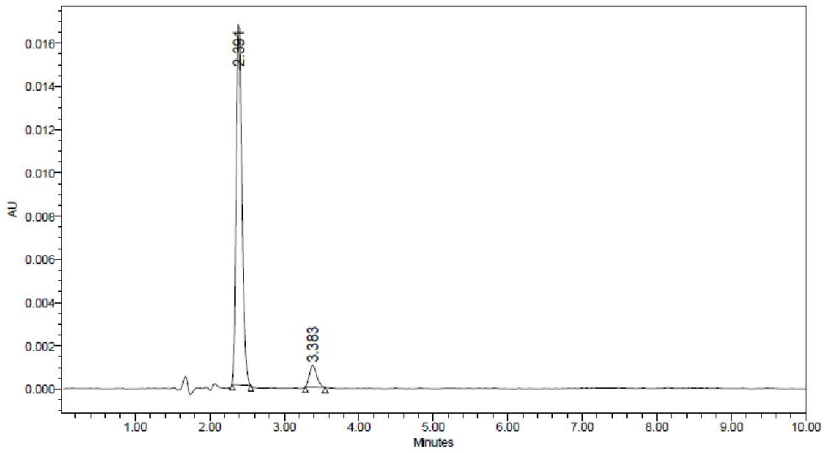
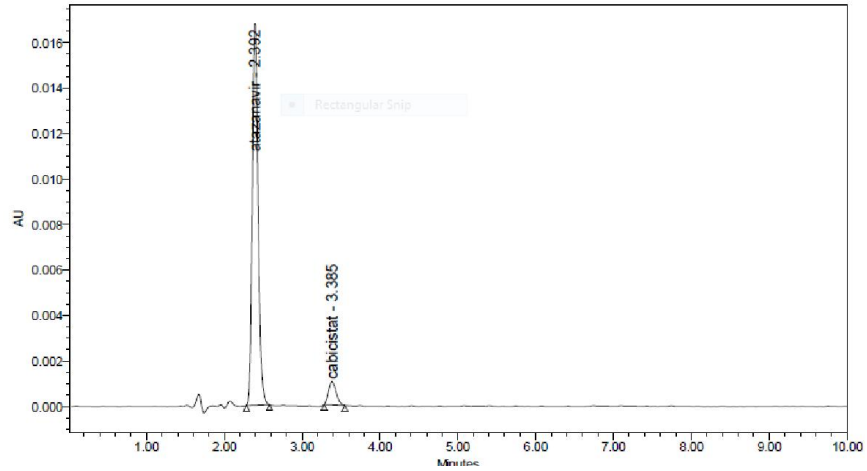
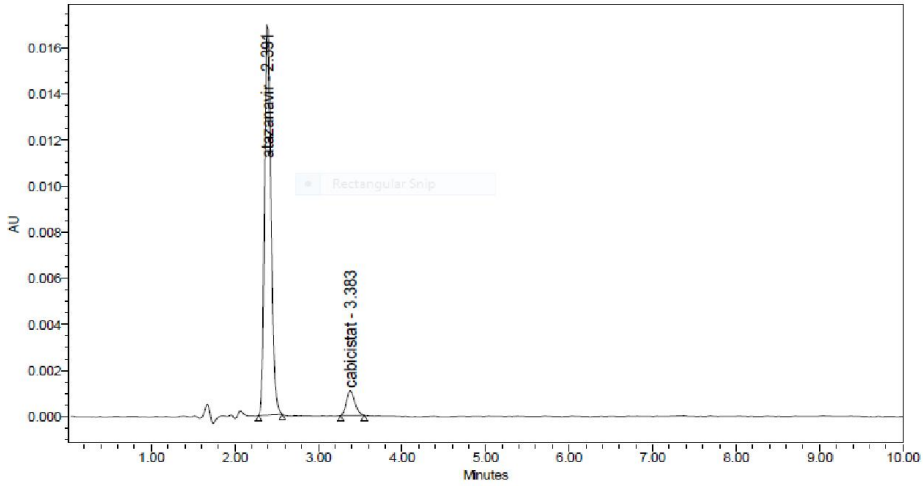
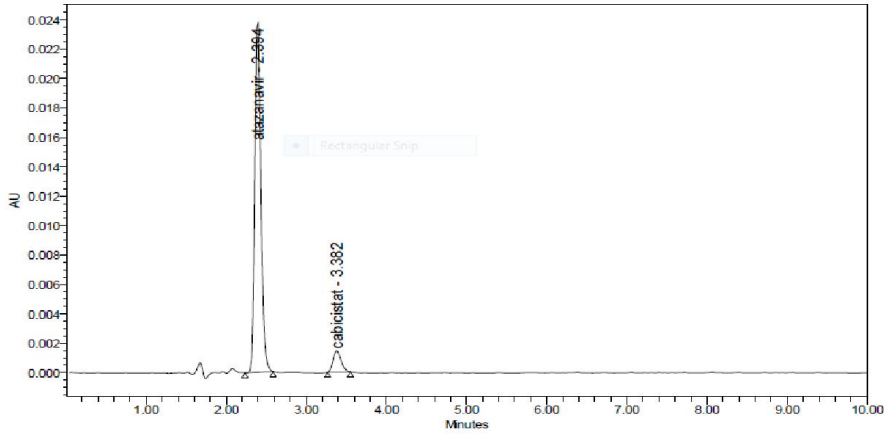
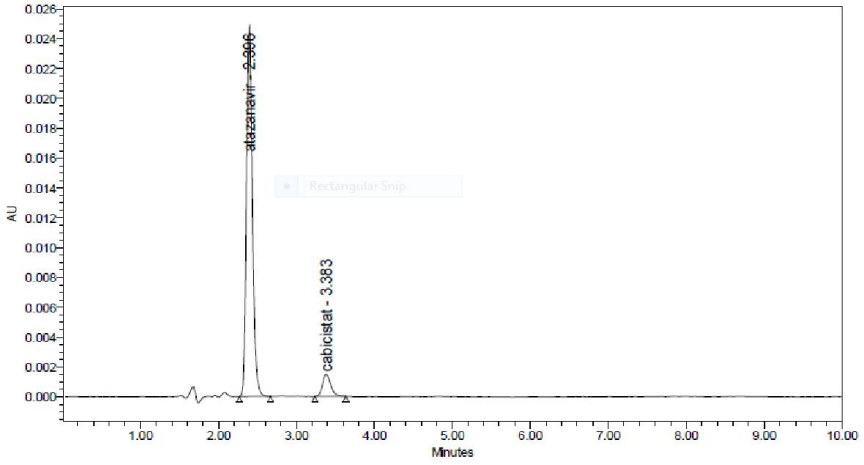


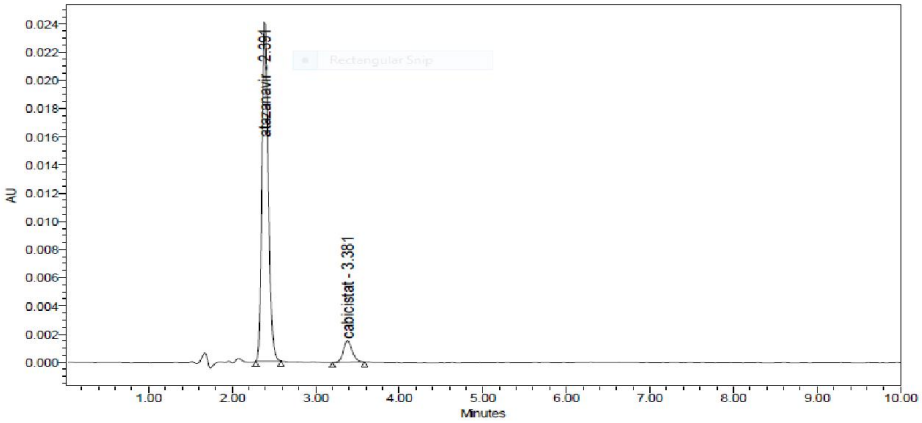


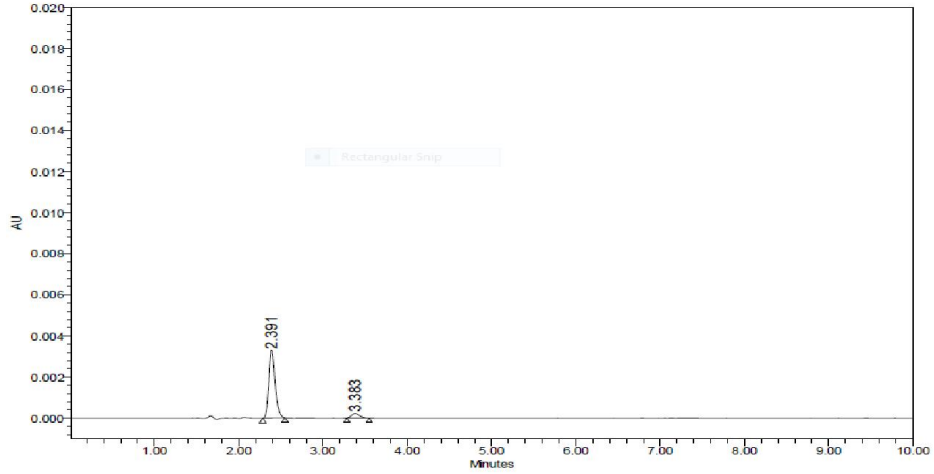

**Chromatogram of accuracy 150%**



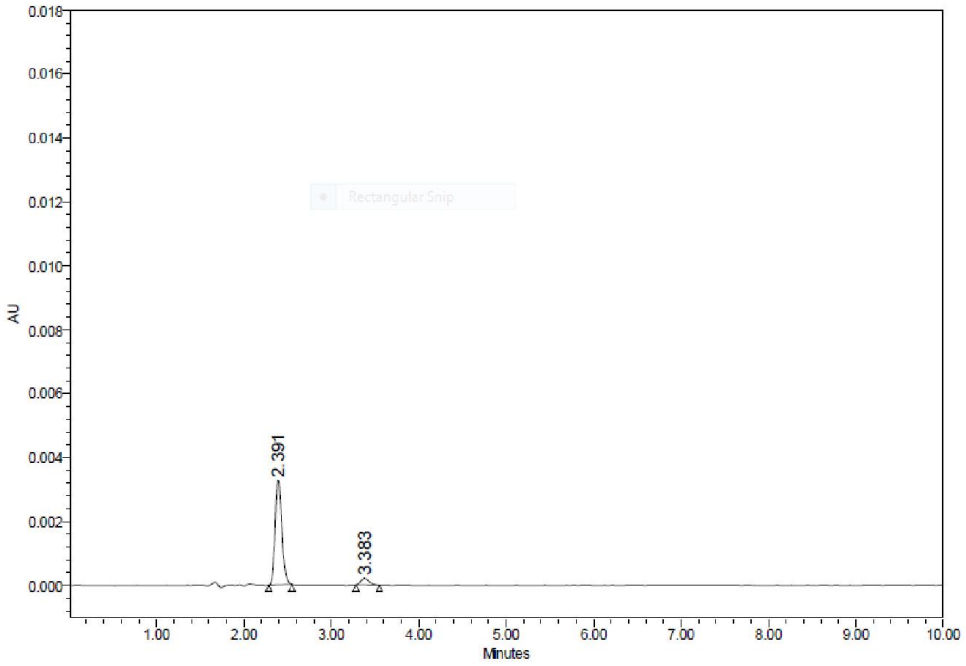


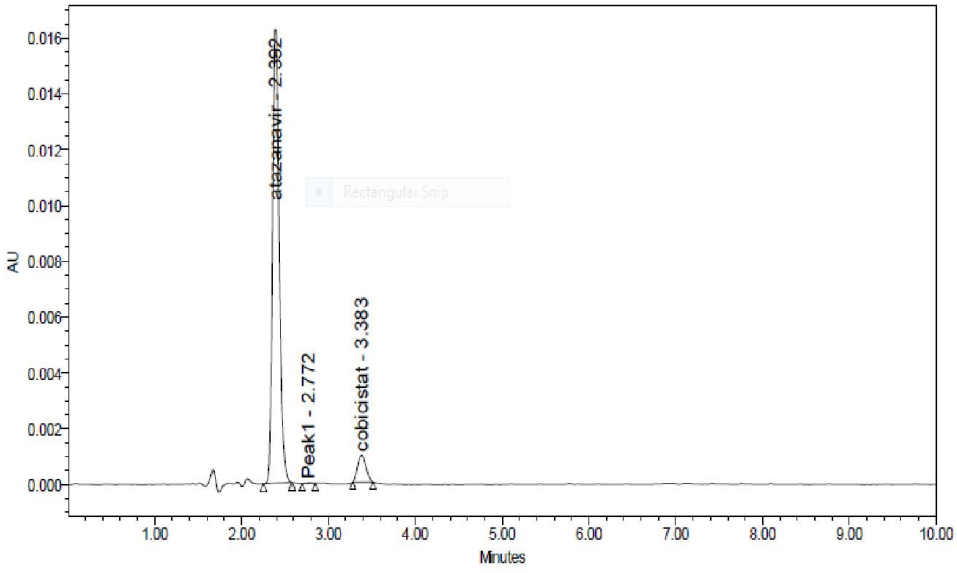

**Chromatogram of limit of detection:**



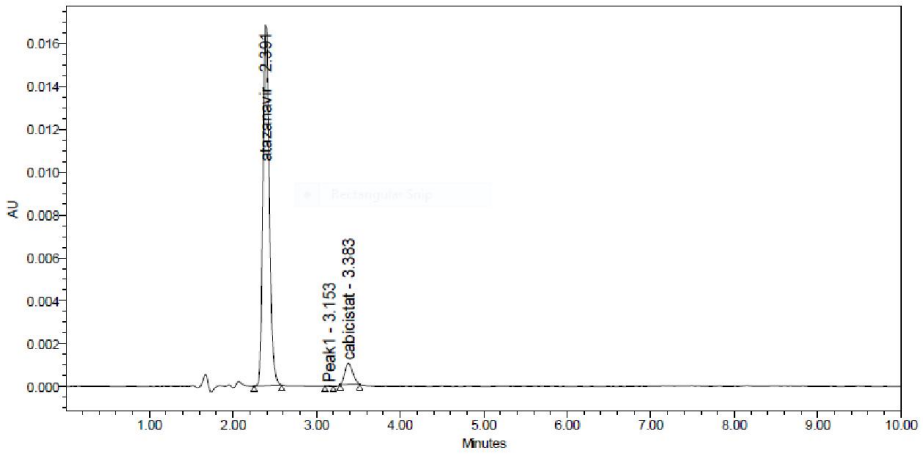
**Chromatogram of limit of quantification**



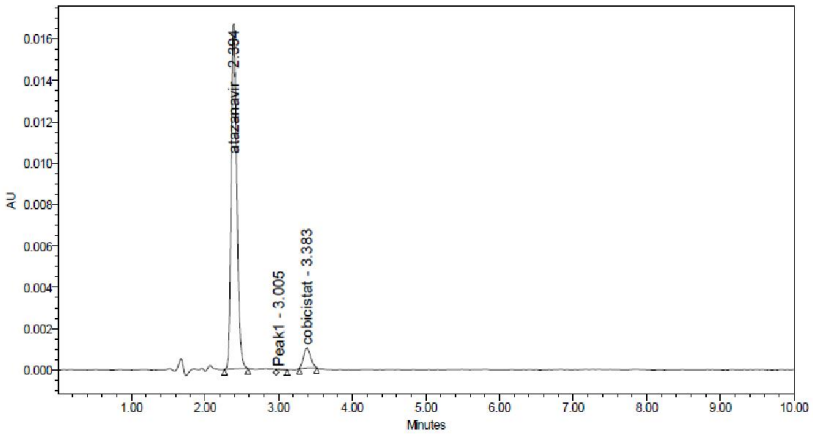

**Chromatogram of acid degradation**



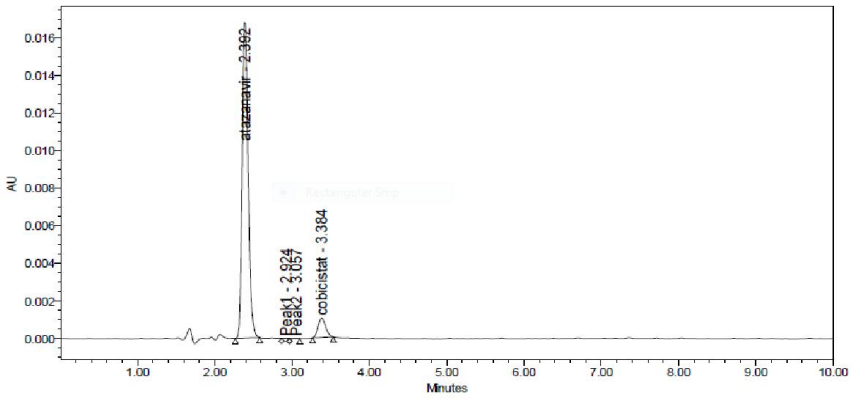
**Chromatogram of base degradation**



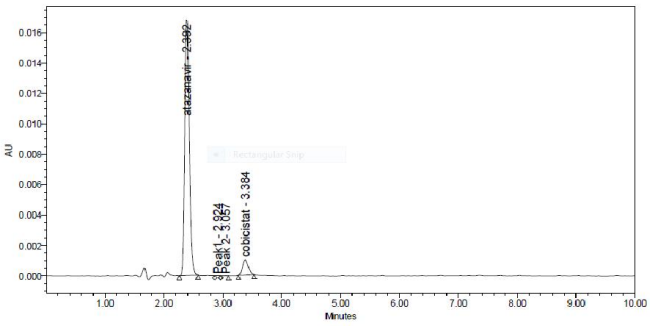

**Chromatogram of oxidative degradation:**




**Chromatogram of thermal degradation:**



**Chromatogram of photo degradation**




**Preparation of stock :**

Accurately weigh and transfer 20 mg of Atzanavir and 10 mg of Cobisistat 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) and the results were shown in table 8.

**Hydrolytic degradation under acidic condition :**

Pipette 1.5 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

**Hydrolytic degradation under alkaline condition :**

Pipette 1.5 ml of above solution into a 10ml volumetric and add 3ml of 0.1 N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

**Thermal induced degradation**

Atzanavir and Cobisistat sample was taken in petridish and kept in Hot air oven at 110°C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

**Oxidative degradation**

Pipette 1.5 ml above stock solution into a 10 ml volumetric flask and 1ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

**Photo degradation :** Pipette 1.5 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24 hrs and the volume was made up to the mark with diluent. Filter the solution with 0.45 microns syringe filters and place in vials.

# Discussion

To optimize RP – HPLC parameters several mobile phase combinations are tried. A satisfactory separation and good peak symmetry for Atazanavir and Cobicistat were obtained with a mobile phase containing a mixture of Acetonitrile : orthophosphoric acid buffer: methanol (40 : 40 : 20) was delivered at a flow rate of 1 ml/min. to get a better reproducibility and repeatability. Quantification was achieved with the UV detection at 239 nm. The retention

time of was found to be 2.401 min and 3.374 min respectively with a resolution of 5.75. linearity was established for Atazanavir in the range of 100 – 500 µg/ml and for Cobicistat in the range of 50 – 250 µg/ml. with the correlation coefficient was found to be $r^2$ = 0.999.and the percentage purity was found to be 100.16% and 100.12% for Atazanavir and Cobicistat respectively which indicates the accuracy of the proposed method.

The RSD values of accuracy for Atazanavir and Cobicistat were found to be below < 2% and the %RSD values of method precision are 0.8 % and 0.3 % for Atazanavir and Cobicistat respectively and %RSD values of System precision are 0.8% and 0.4% for Atazanavir and Cobicistat respectively. LOD values for Atazanavir and Cobicistat were found to be and 3 and 3.02µg/ml respectively. LOQ values for Atazanavir and Cobicistat were found to be 9.98 and 10 µg/ml respectively.

# Conclusion

The present RP – HPLC method for the simultaneous estimation of Atazanavir and Cobicistat in their combined dosage form was established and validated as per ICH guidelines. Linearity was achieved for Atazanavir and Cobicistat in the range of 100-500 µg/ml and 50-250 µg/ml respectively with the correlation coefficient $r^2$ = 0.999. the percentage purity of Atazanavir and Cobicistat was found to be 100.16% and 100.12%. The % RSD was NMT 2% which proved the precision of the developed method. The developed method was simple, precise, rugged, accurate, specific and robust.

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