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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING SIMULTANEOUS ESTIMATION OF ATAZANAVIR AND COBICISTAT BY RP-HPLC METHOD

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Abstract: A simple, accurate, sensitive and Precise RP-HPLC method has been developed and validated for simultaneous estimation Atazanavir and Cobicistat. In RP-HPLC method, good resolution and separation of two drugs were achieved on Hypersil BDS C18 (25cm x 0.46 cm) Column by using 0.05 M Sodium dihydrogen phosphate (pH 5): Acetonitrile(70:30 v/v) as mobile phase with a flow rate of 1 ml/min and 235nm Wavelength. Retention time of Cobicistat and Atazanavir were found to be 3.243 and 7.110 min respectively. The proposed method was accurate and precise. The different analytical parameters such as linearity, accuracy, precision, robustness were determined according to the International Conference on Harmonization (ICH) Q2B guidelines. The method was linear (r = 0.9990) at a concentration range of 10-30 µg/mL and 5-15 µg/mL. Forced degradation study of Atazanavir and Cobicistat was performed by RP-HPLC method which includes Acid, Base, Oxidative, Photo and Thermal degradation. Results of degradation were found within limit. Therefore proposed method can be used for routine analysis of Atazanavir and Cobicistat in tablets.

Keywords: RP-HPLC, Stability indicating method validation, Atazanavir and Cobicistat

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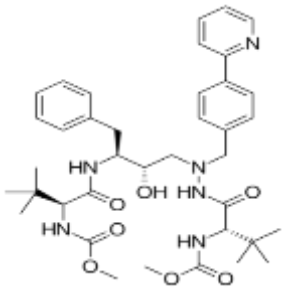
INTRODUCTION

In this present study an attempt was made to develop RP-HPLC method for simultaneous estimation of atazanavir and cobicistat in tablet dosage form.

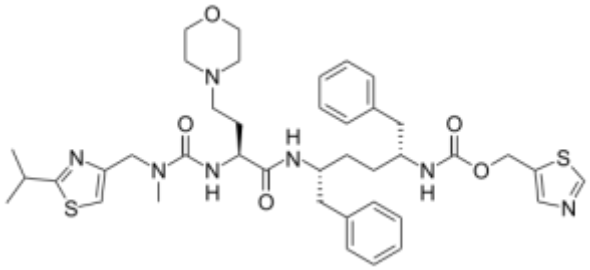
EVOTAZ is a FDA approved combination drug product contains 300 mg of atazanavir and 150 mg of cobicistat. It is indicated in combination with other antiretroviral agents for the treatment of human immunodeficiency virus (HIV-1) infection in adults.[1]

Atazanavir is an antiretroviral drug of the protease inhibitor (PI) class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV). Chemical name is methyl N[(1S)-1-{[(2S,3S)-3-hydroxy-4-[(2S)-2-[(methoxycarbonyl) amino]-3,3-dimethylN'{[-4-(pyridine-2-yl)phenyl]methyl}butanehydrazido]-1-phenylbutan-2-yl] carbamoyl}-2,2-dimethylpropyl] carbamate. Its chemical formula Is $C_{38}H_{52}N_6O_7$ and Molecular Weight is 704.856 g/mol.[2-3]

Cobicistat is a CYP3A inhibitor. It increases the systemic exposure of atazanavir or darunavir in combination with other antiretroviral agents in the treatment of HIV-1 infection. Chemical name is Thiazol-5-ylmethylN-[1-benzyl-4-[[2-[[(2-isopropylthiazol-4-yl)methyl methylcarbamoyl] amino]-4-morpholinobutanoyl] amino]5-phenylpentyl] carbamate. It has a molecular formula of $C_{40}H_{53}N_7O_5S_2$ and a molecular weight of 776.023 g/mol.[4-5]



Atazanavir



Cobicistat

Literature review revealed that very few analytical methods appeared in the literature for the determination of Atazanavir and Cobicistat available in Combined Dosage form and also official assay of Atazanavir and Cobicistat is not described in any pharmacopoeias.[6-15]

In view of the need for a suitable RP-HPLC method for routine analysis of Atazanavir and Cobicistat in formulation, an attempt was made to develop simple, precise and accurate analytical method for the determination of Atazanavir and Cobicistat in tablet formulation.

RESULTS AND DISCUSSIONS

Instrumentation:

Component	Brand / Model / Software
HPLC	Shimadzu LC-20 AT
HPLC Column	C18 (25cm x 0.46 cm) Hypersil BDS
Detector	SPD-20A

MATERIALS AND METHODS

Instrumentation:

Shimadzu LC-10 AT system equipped with UV detector was used. The output signals were monitored and integrated using waters LC Solution software. Analytical balance (AUX-200 Make: Shimadzu). Systronics digital pH meter 361 was used to adjust the pH of the buffer. Degassing of the mobile phase was done by sonication using Dolphin Ultra Sonicator. Filtration was done by using millipore vaccum filter.

Drugs and Chemical:

Pure standards of Atazanivir and Cobicistat standards were kindly gifted from Unique Pharmaceuticals and Acceration Pharmaceuticals Respectively. The HPLC grade methanol, potassium di-hydrogen phosphate, ortho phosphoric acid were purchased from Merck.

Preparation of Solution:

Preparation of Buffer:

Weighed about 6.8gms of Phosphate into a 1000ml beaker and dissolved and diluted to 1000ml with milli-Q water. Adjusted the pH to 5 with 0.1N NaOH and filtered through 0.45µm membrane filter.

Preparation of Mobile Phase:

Acetonitrile and buffer were mixed in the ratio 70:30v/v and sonicated for 10 minutes.

Preparation of Test Solution:

Std Preparation:

Atazanavir standard stock solution: (200 µg/mL)

A 20 mg of Atazanavir was weighed and transferred to a 100 mL volumetric flask volume was made up to the mark with mobile phase.

Cobicistat standard stock solution: (100 µg/mL)

A 10 mg of Cobicistat was weighed and transferred to a 100 mL volumetric flask volume was made up to the mark with mobile phase

Preparation of standard solution of binary mixtures of Atazanavir (20 µg/mL) and Cobicistat(10 µg/mL)

Take 1 mL from the Atazanavir stock solution and 1mL from Cobicistat stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

Test Preparation:

Sample Stock Solution (Cobicistat 100 µg/mL, and Atazanavir 200 µg/mL):

Take Tablet Powder equivalent to 10 mg of Cobicistat, and 20 mg of Atazanavir was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

Working Sample Preparation (Cobicistat 10 µg/mL, and Atazanavir 20 µg/mL):

Take 1 mL from standard stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase

Inject above Solution 20 µl for Assay Analysis.

HPLC METHOD DEVELOPMENT AND OPTIMIZATION[16-20]

Selection of wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study, drug solutions of Atazanavir (20 ppm) and Cobicistat (10 ppm) were prepared in Methanol. These drug solutions were than scanned in UV region of 200-400 nm and overlay spectrums were recorded.

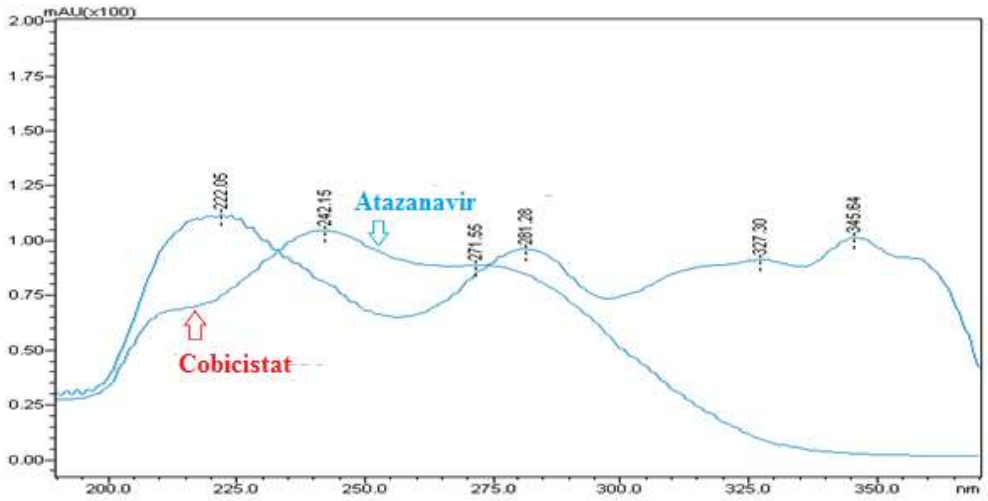


Fig. UV Spectra of Atazanavir (20 ppm) and Cobicistat (10 ppm) in Methanol (Isoabsorptive point 235 nm)

All solutions were scanned between 200 - 400 nm. Wavelength was selected from the overlay spectra of above solutions.

Selection of Mobile Phase

Mobile Phase was selected based on the review of literature. Various mobile phases were tried. Trial contains various mobile phases which consisted of Methanol, Water, Buffers (potassium dihydrogen Phosphate) in different proportions with various pH and different volumes at flow rate 1 mL/min were tried. On the basis of various trials the mixture of Buffer (Potassium phosphate, pH 5.0): Acetonitrile (70:30) was selected.

Parameters	Cobicistat	Atazanavir
Retention Time	3.243	7.110
Theoretical Plates	4282	7241
Asymmetry	1.680	1.370
Resolution	14.523	

Optimization of flow rate

1 mL/min flow rate, proved to be better than the other in terms of resolution, peak shape and shorter retention time.

RP-HPLC optimized chromatographic conditions:

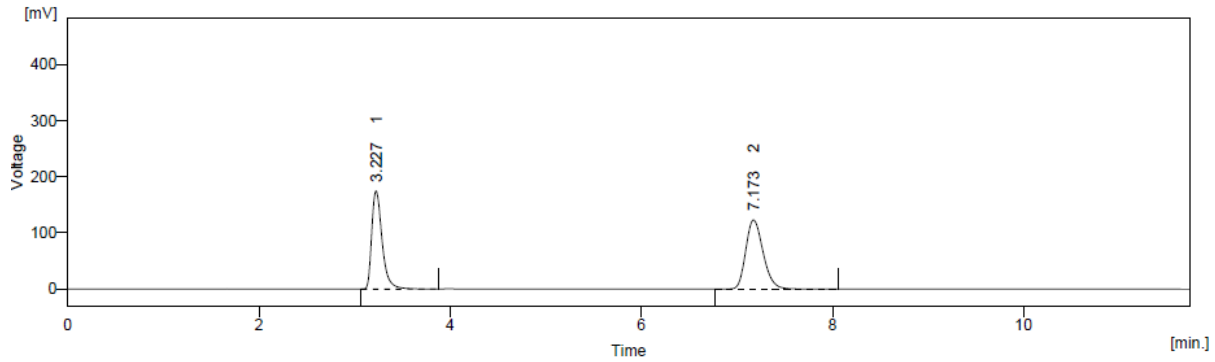
Parameters	Chromatographic Condition
Mode of elution	Isocratic
Mobile Phase	Buffer (Potassium Phosphate, pH 5.0) : Acetonitrile (70:30)
Column	C18 (25cm x 0.46 cm) Hypersil BDS
Flow rate	1 mL/min
Runtime	10 min
Injection volume	20 µL
Detection wavelength	235 nm

VALIDATION OF RP-HPLC METHOD:[21-22]

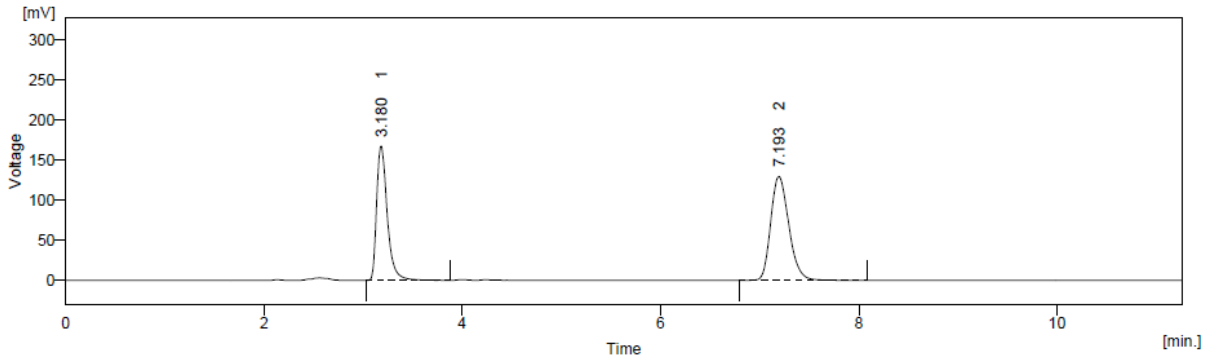
Method validation is the process of proving that an analytical method is acceptable for its intended purpose. It is documented evidence that specific process and procedure repeatedly and continuously produce the pre-determined specifications. For test methods used for pharmaceuticals, the United States Pharmacopoeia, the European Pharmacopoeia, Japanese pharmacopeia, along with other guidelines published by International Conference on Harmonization, and Food and Drug Administration outline the necessary parameters needed for method validation based on the type of material being tested and provide a frame work for the data required in agency submissions.

Quantitative aspects:

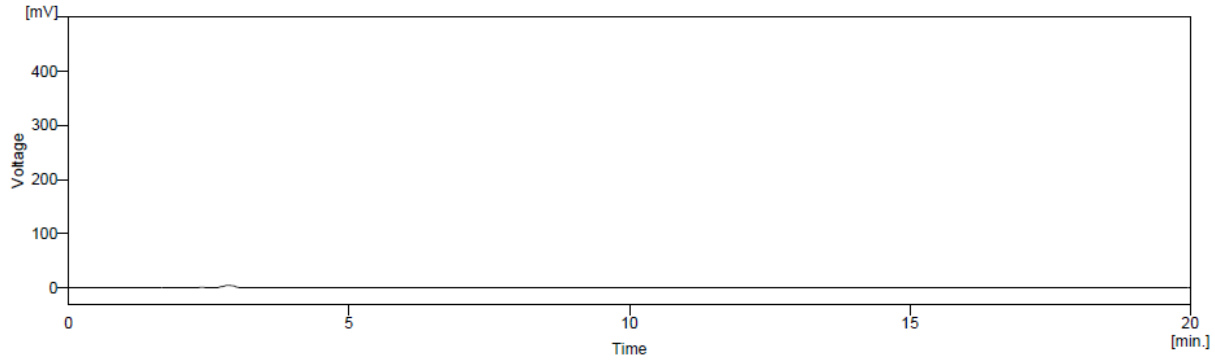
Specificity:



Chromatogram of Cobicistat and Atazanavir STD



Chromatogram of Cobicistat and Atazanavir sample



Chromatogram of Cobicistat and Atazanavir Blank

The Chromatograms of Cobicistat and Atazanavir standards and Cobicistat and Atazanavir sample show no interference with the Chromatogram of Cobicistat and Atazanavir Blank, so the Developed method is Specific.

Linearity:

The linearity for Atazanavir and Cobicistat were assessed by analysis of combined standard solution in range of 10-30 µg/mL and 5-15 µg/mL respectively.

5,7.5,10,12.5,15 mL solutions were pipette out from the Stock solution of Atazanavir (200 µg/mL) and Cobicistat (100 µg/mL) and transfer to 100 mL volumetric flask and make up with mobile phase to obtain 10,15,20,25 and 30 µg/mL, and 5,7.5,10,12.5 and 15 µg/mL for Atazanavir and Cobicistat respectively

Correlation co-efficient for calibration curve Atazanavir and Cobicistat was found to be 0.998 and 0.998 respectively.

The regression line equation for Atazanavir and Cobicistat are as following:

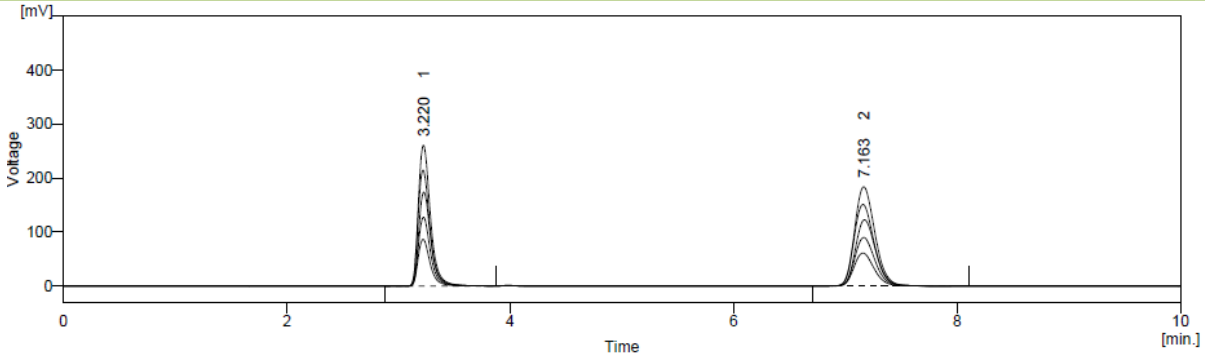
For Atazanavir **y = 78.30x - 35.33** and For Cobicistat: **y = 131.6x - 14.33**

Linearity data for Cobicistat:

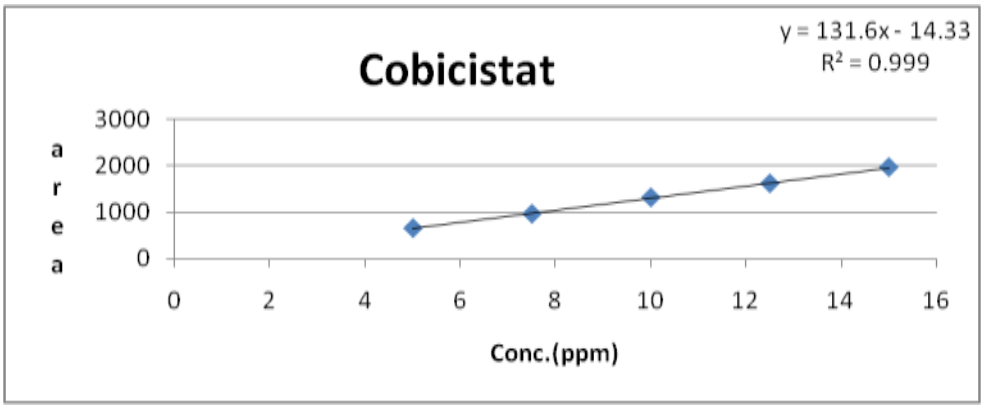
Sr.No	Concentration (µg/mL)	Area
1	5	650.798
2	7.5	960.726
3	10	1313.438
4	12.5	1617.137
5	15	1968.044

Linearity data for Atazanavir:

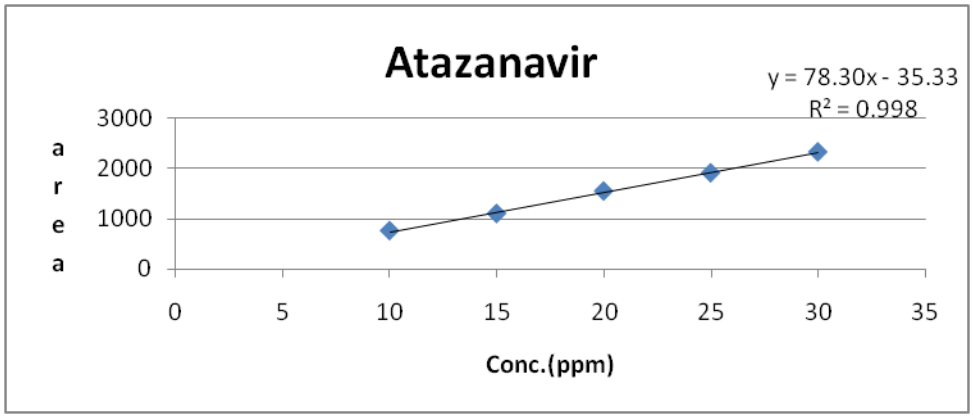
Sr.No	Concentration (µg/mL)	Area
1	10	767.027
2	15	1105.321
3	20	1549.923
4	25	1909.031
5	30	2322.883



Overlay chromatogram of different concentrations of mixtures of Atazanavir and Cobicistat



Calibration Curve of Cobicistat (5-15 µg/mL).



Calibration Curve of Atazanavir (10-30 µg/mL).

Precision

Repeatability

The data for repeatability of peak area measurement for Atazanavir (20 µg/mL) and Cobicistat (10 µg/mL) based on six measurements of same solution of Atazanavir (20 µg/mL) and Cobicistat (10 µg/mL).The % RSD for Atazanavir and Cobicistat was found to be 1.063 and 0.816 respectively.

Repeatability data for Cobicistat:

Cobicistat				
Sr No.	**Conc (µg/mL)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	10	1308.159 1279.002 1313.429 1316.063	1306.810±13.885	1.063

		1313.422		
		1310.784		

Repeatability data for Atazanavir:

Atazanavir				
Sr No.	**Conc (µg/mL)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	20	1543.702	1542.389 ±12.590	0.816
		1546.818		
		1549.959		
		1517.136		
		1549.915		
		1546.805		

Intraday precision

Standard solution containing (10,20,30 µg/mL) of Cobicistat and (5,10,15 µg/mL) of Atazanavir were analyzed three times on the same day and % RSD was calculated.

Intraday precision data for estimation of Cobicistat:

	Cobicistat		
SR. NO.	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	5	641.867 ± 11.567	1.801
2	10	1300.475 ± 14.615	1.124
3	15	1952.366± 15.497	0.794

Intraday precision data for estimation of Atazanavir:

	Atazanavir		
SR. NO.	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	10	757.012 ± 11.609	1.533
2	20	1534.824 ± 14.547	0.948
3	30	2300.794 ± 20.927	0.909

Interday precision

Standard solution containing (10,20,30 µg/mL) of Cobicistat and (5,10,15 µg/mL) of Atazanavir were analyzed three times on the different day and % R.S.D was calculated.

Interday precision data for estimation of Cobicistat:

	Cobicistat		
SR. NO.	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	5	642.713 ± 6.874	1.069
2	10	1298.704± 11.832	0.864
3	15	1946.645± 11.832	0.608

Interday precision data for estimation of Atazanavir:

	Atazanavir		
SR. NO.	**Conc. (µg/mL)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	10	757.396 ± 6.914	0.913
2	20	1532.233± 10.709	0.699
3	30	2295.871 ± 21.479	0.935

Accuracy:

For Cobicistat

5 µg/mL drug solution was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10mL. The area of each solution peak was measured at 235 nm.

For Atazanavir

10 µg/mL drug solution was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10mL. The area of each solution peak was measured at 235 nm.

Recovery data for Cobicistat:

SR. NO.	Conc. Level (%)	Sample amount (µg/mL)	Amount Added (µg/mL)	Amount recovered (µg/mL)	% Recovery	% Mean Recovery ± S.D
1	80 %	5	4	3.958	98.949	100.006 ± 0.994
2		5	4	4.037	100.922	
3		5	4	4.006	100.149	
4	100 %	5	5	4.955	99.098	99.665 ± 0.603
5		5	5	5.015	100.298	
6		5	5	4.980	99.599	
7	120 %	5	6	6.007	100.113	99.687 ± 0.408
8		5	6	5.958	99.300	
9		5	6	5.979	99.649	

Recovery data for Atazanavir:

SR. NO.	Conc. Level (%)	Sample Amount	Amount Added	Amount recovered (µg/mL)	% Recovery	% Mean Recovery ± S.D
1	80 %	10	8	7.934	99.169	100.011 ± 0.732
2		10	8	8.040	100.497	
3		10	8	8.029	100.367	
4	100 %	10	10	9.932	99.318	99.759 ± 0.415
5		10	10	10.014	100.141	
6		10	10	9.982	99.818	
7	120 %	10	12	11.985	99.871	99.750 ± 0.204
8		10	12	11.942	99.515	
9		10	12	11.984	99.863	

Robustness:

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 mL/min) 0.8 mL/min and 1.2 mL/min.
2. pH of Mobile phase was changed (± 0.2) 5.2 and 4.8.
3. Ratio of Mobile phase was changed(±2) Buffer : Acetonitrile (72:28) and Buffer : Acetonitrile (68:32)

Robustness data for Cobicistat:

SR NO.	Area at Flow rate (- 0.2 mL/min)	Area at Flow rate (+ 0.2 mL/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	1338.830	1275.301	1313.947	1230.315	1342.382	1249.476
2	1362.129	1264.321	1348.977	1256.842	1310.592	1280.602
3	1367.337	1291.030	1356.904	1262.155	1352.829	1289.733
% R.S.D	1.119	1.051	1.706	1.365	1.648	1.658

Robustness data for Atazanavir:

SR NO.	Area at Flow rate (- 0.2 mL/min)	Area at Flow rate (+ 0.2 mL/min)	Area at pH (- 0.2)	Area at pH (+ 0.2)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	1601.086	1474.322	1582.457	1476.961	1556.343	1501.967
2	1583.736	1514.251	1567.358	1456.233	1587.000	1488.185
3	1613.390	1523.537	1601.086	1489.528	1596.276	1522.009
% R.S.D	0.931	1.739	1.067	1.140	1.323	1.131

Forced degradation[23]

Forced degradation studies are conducted to evaluate the stability indicating capacity of method of analysis. These stress studies are essential for the product quality.

These studies provide information and discrimination between process related, degradant related and degradants from drug-excipient combination. If the drug product does not show any degradation when it was exposed to stress conditions, further stress study is unnecessary.

Forced Degradation Summary

Acid degradation

The sample is subjected to 0.1 N HCl at 70°C for 3 hours. After time period the content was cooled to room temperature. Further, the volume was adjusted with diluent to get 20 µg/mL for Atazanavir and 10 µg/mL for Cobicistat.

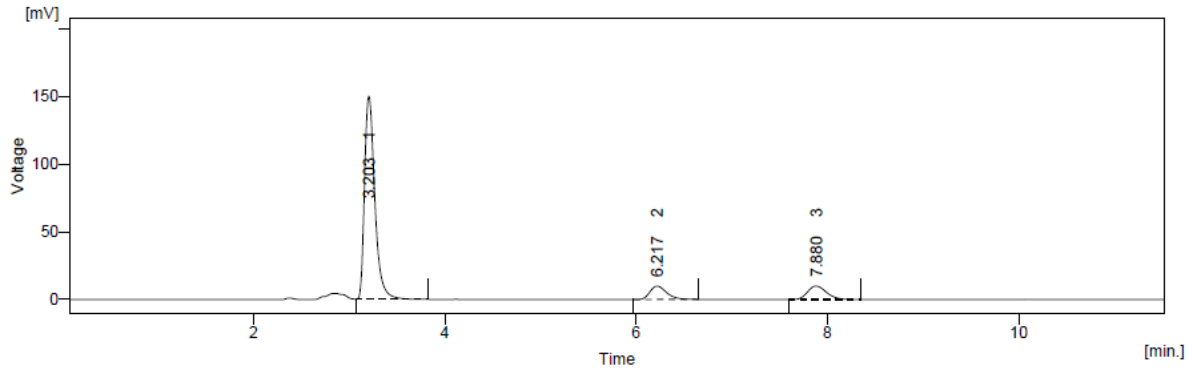


Fig. Cobicistat Acid Degradation Standard

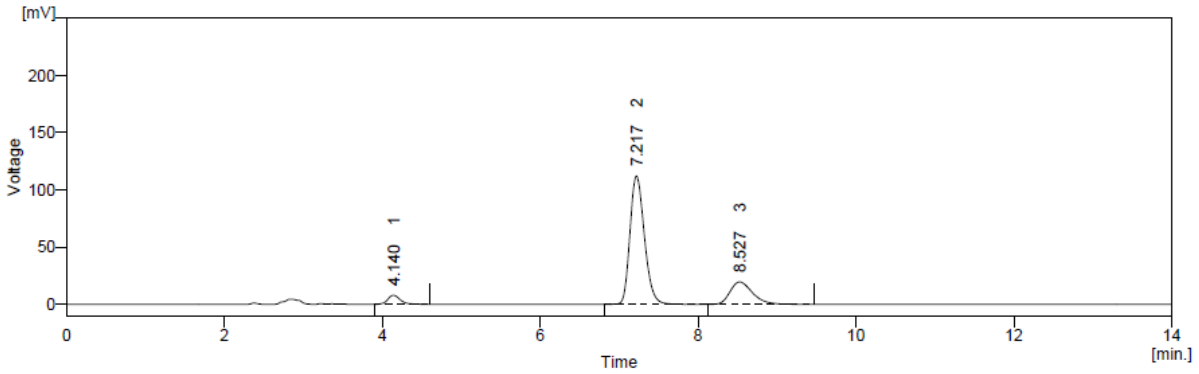


Fig. Atazanavir Acid Degradation Standard

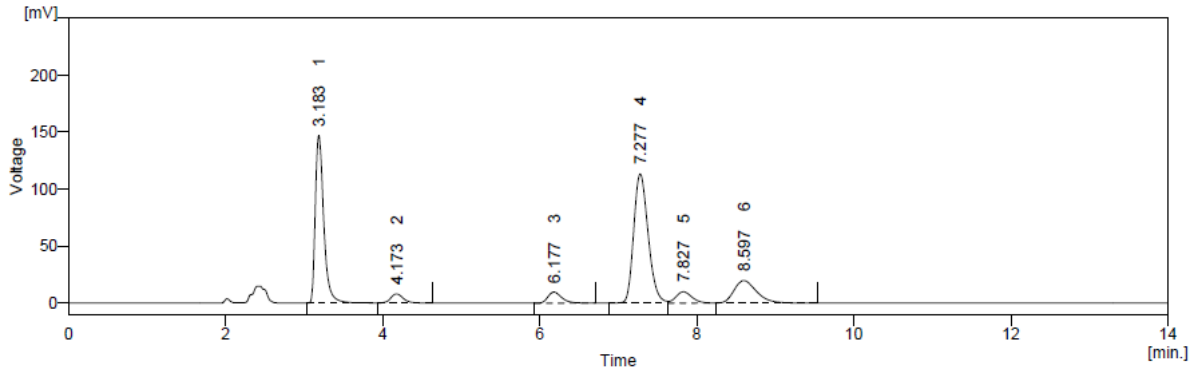


Fig. Atazanavir and Cobicistat Acid Degradation Sample

Base degradation

Basic decomposition studies were performed by exposing the sample to 0.1 N NaOH solutions for 3 hours at 70 ºC. After time period, the content was cooled to room temperature. Then the volume was adjusted with diluent to get 20 µg/mL for Atazanavir and 10 µg/mL for Cobicistat

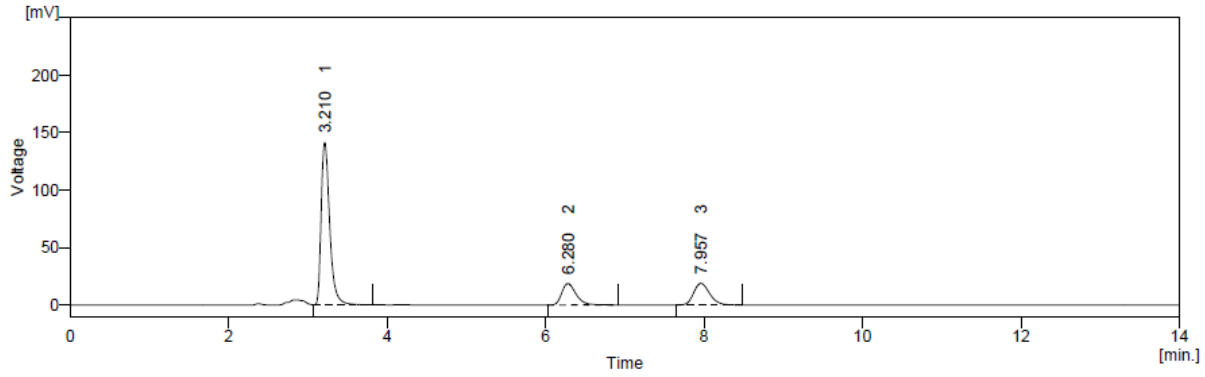


Fig. Cobicistat Base Degradation

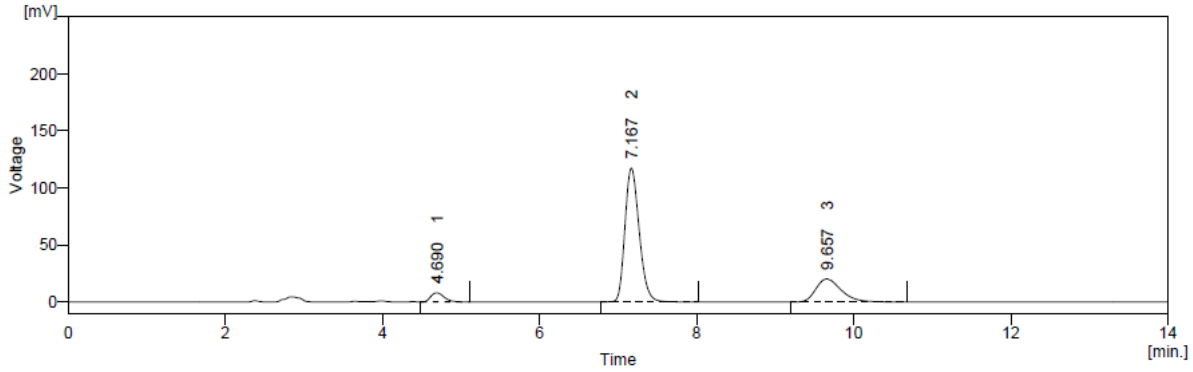


Fig. Atazanavir Base Degradation

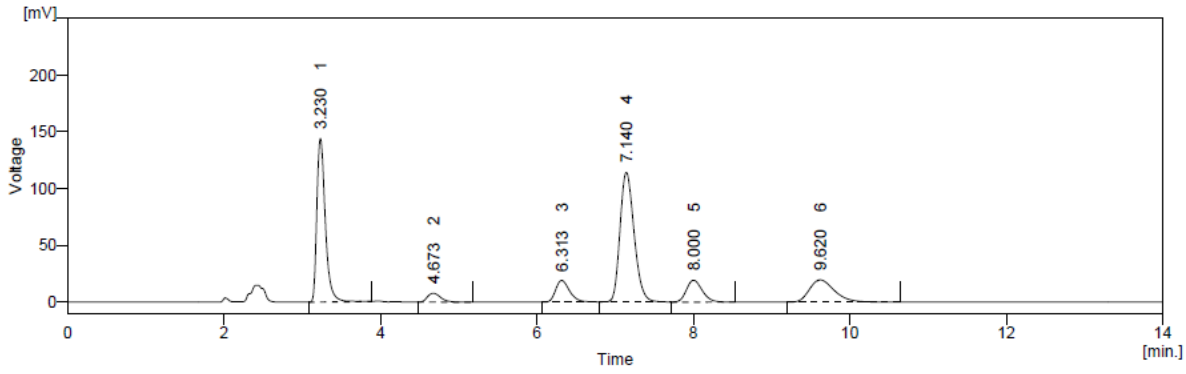


Fig. Atazanavir and Cobicistat Base Degradation Sample

Oxidative degradation

The sample is subjected to 3% Hydrogen peroxide at room temperature for 3 hours. Later the sample content was cooled to room temperature. Then the volume was adjusted with diluent to get 20 µg/mL for Atazanavir and 10 µg/mL for Cobicistat.

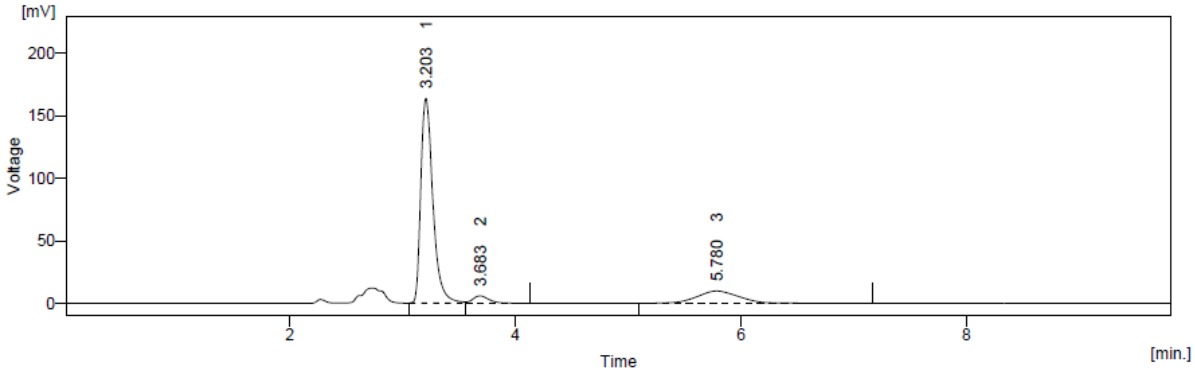


Fig. Cobicistat Oxidation Degradation

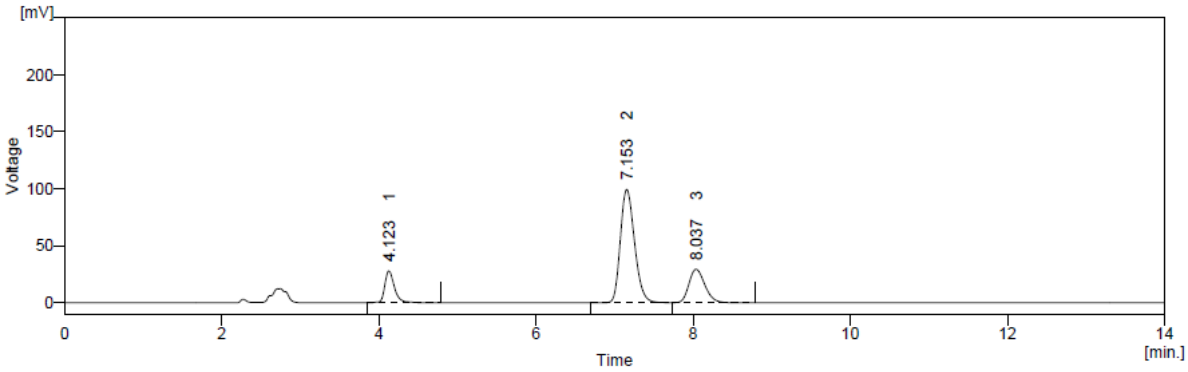


Fig. Atazanavir Oxidation Degradation

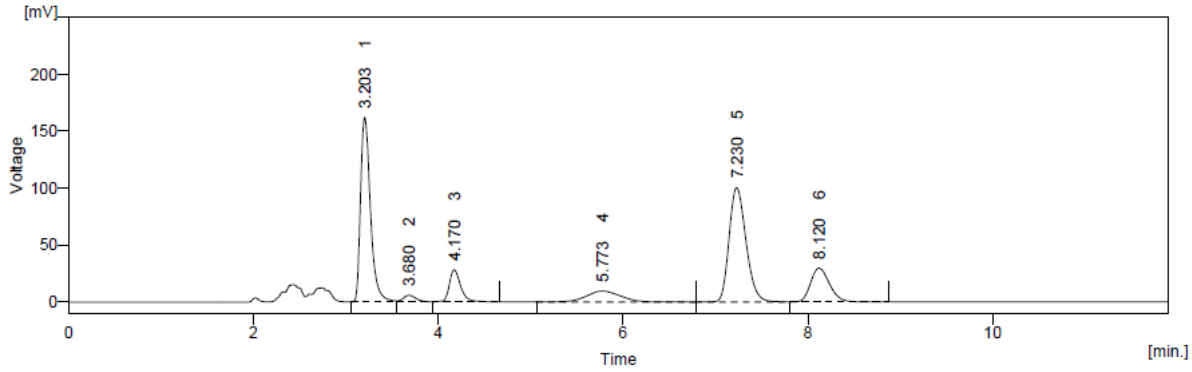


Fig. Atazanavir and Cobicistat Oxidation Degradation sample

Photo degradation

Photo Degradation studies were performed by exposing the sample to sunlight for 4 hours. Later the volume was adjusted with diluent to get 20 µg/mL for Atazanavir and 10 µg/mL for Cobicistat.

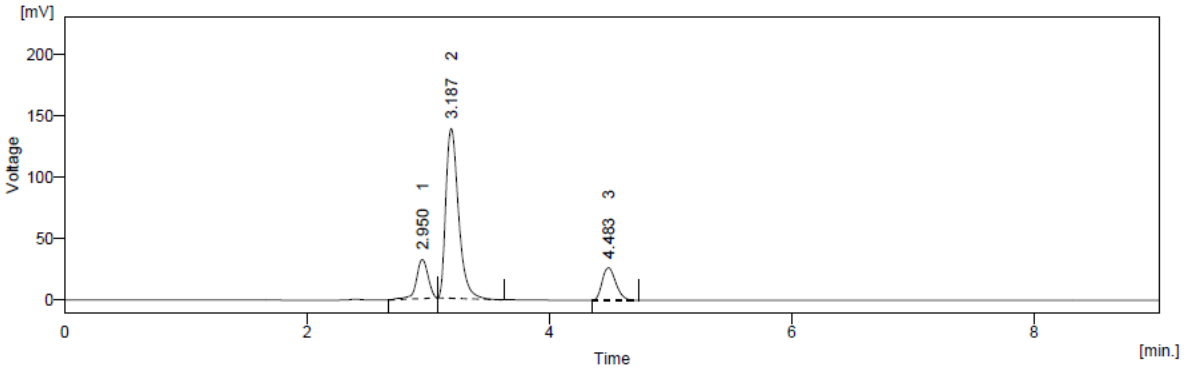


Fig. Cobicistat Photo Degradation

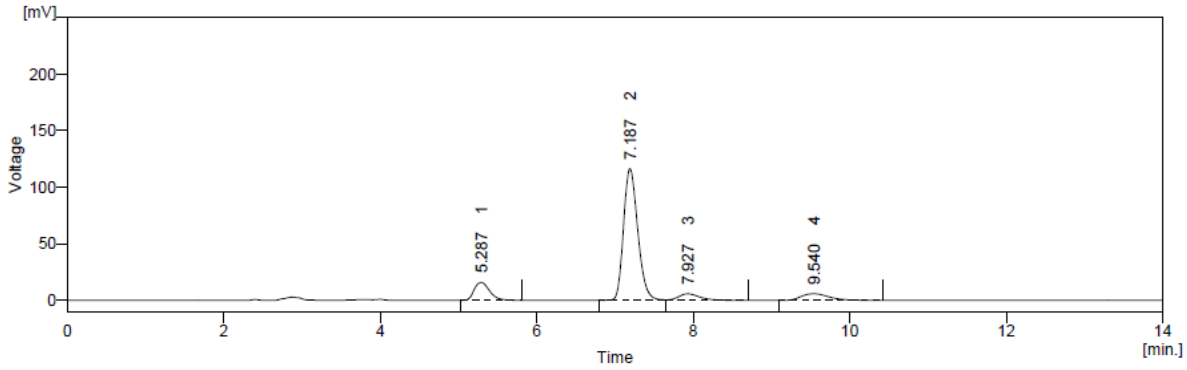


Fig. Atazanavir Photo Degradation

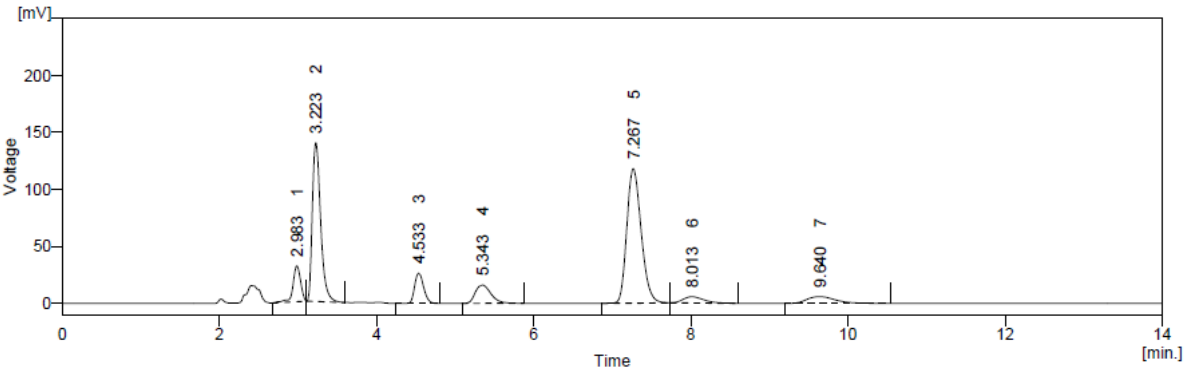


Fig. Atazanavir and Cobicistat Photo Degradation sample

Thermal degradation

Thermal degradation studies were performed by subjecting the sample at 110°C for 3.5 hours. Further, the volume was adjusted with diluent to get 20 µg/mL for Atazanavir and 10 µg/mL for Cobicistat.

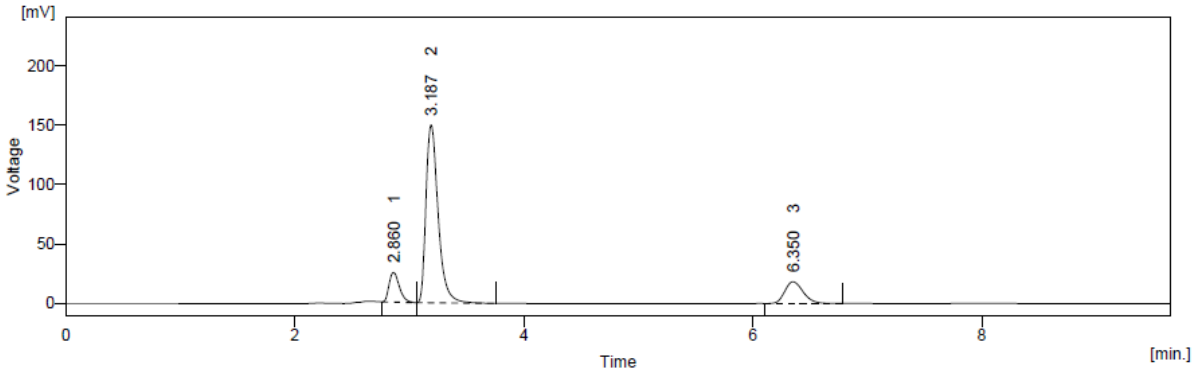


Fig. Cobicistat Thermal Degradation

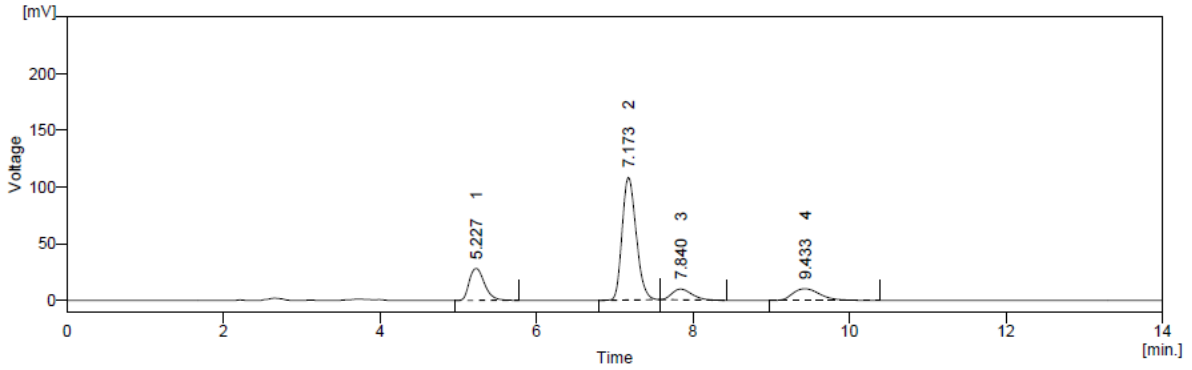


Fig. Atazanavir Thermal Degradation

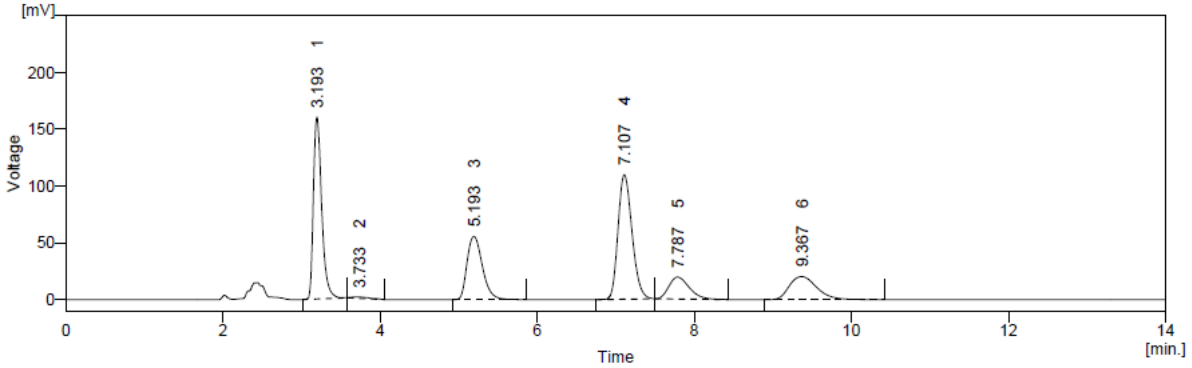


Fig. Atazanavir and Cobicistat Thermal Degradation sample

Calculation for Stability:

Atazanavir and Cobicistat STD for stability

Drugs	Area
Atazanavir	1615.362
Cobicistat	1311.663

Atazanavir % Degradation:

Atazanavir				
Parameter	**Standard**		**Sample**	
	Area	**%Degradation**	**Area**	**%Degradation**
Acid	1328.310	17.770	1348.202	16.539
Base	1301.164	19.451	1339.770	17.061
Oxidation	1253.891	22.377	1278.633	20.845
Photo	1372.283	15.048	1399.346	13.373
Thermal	1350.146	16.418	1356.119	16.049

Cobicistat % Degradation:

Cobicistat				
Parameter	**Standard**		**Sample**	
	Area	**%Degradation**	**Area**	**%Degradation**
Acid	1119.279	14.667	1108.507	15.488
Base	1055.766	19.509	1085.737	17.224
Oxidation	1025.512	21.816	1011.192	22.908
Photo	1007.610	23.181	1026.783	21.719
Thermal	1107.886	15.536	1078.054	17.810

Analysis of marketed formulation by developed method

Sample Stock Solution (Cobicistat 100 µg/mL, and Atazanavir 200 µg/mL):

Take Tablet Powder equivalent to 10 mg of Cobicistat, and 20 mg of Atazanavir was transferred to a 100 mL volumetric flask, Add 60 mL Mobile phase and Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

Working Sample Preparation (Cobicistat 10 µg/mL, and Atazanavir 20 µg/mL):

Take 1 mL from standard stock solution and transferred to 10 mL volumetric flask and made up volume up to the mark with the mobile phase.

Inject above Solution 20 µl for Assay Analysis.

Analysis on marketed formulation:

Tablet	Evotaz	
Label claim	**Cobicistat (150 mg)**	**Atazanavir (300 mg)**
Assay (% of label claim) Mean ± S. D.	99.336±3.837	96.338±3.002

The assay results were comparable to labelled value of each drug in combined dosage form. These results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

SUMMARY AND CONCLUSION

Atazanavir is an antiretroviral drug of the protease inhibitor (PI) class.Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV).

Cobicistat is a licensed drug for use in the treatment of infection with the human immunodeficiency virus.

RP-HPLC method was developed for simultaneous estimation Atazanavir and Cobicistat. In RP-HPLC method, good resolution and separation of two drugs was achieved. 0.05 M Sodium dihydrogen phosphate (pH 5):Acetonitrile(70:30 v/v) was used as mobile phase. Retention time of Cobicistat and Atazanavir were found to be 3.243 and 7.110 min respectively with a flow rate of 1 ml/min. The proposed method was accurate and precise. Therefore proposed method can be used for routine analysis of Atazanavir and Cobicistat in tablets.

Forced degradation study of Atazanavir and Cobicistat was performed by RP-HPLC method which includes Acid, Base, Oxidative, Photo and Thermal degradation. Results of degradation were found within limit.

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