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Stability indicating RP-HPLC Method Development and Validation for Simultaneous Determination of Atazanavir and Cobicistat in Bulk and Pharmaceutical Formulation

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ABSTRACT

A stability indicating reverse phase High performance liquid chromatography (RP-HPLC) method has been developed and subsequently validated for the simultaneous determination of Atazanavir and Cobicistat in bulk and pharmaceutical formulation. Separation was achieved in isocratic mode with a Kinetex C_{18} 100 A (250 mm x 4.6 mm, 5µ) column and mixture consisting of 0.1% OPA(pH 3) and methanol in 80:20 v/v was used as mobile phase with a flow rate of 1 ml/min, column temperature at 25°C and the run time as 10 mins. UV detection was performed at 239 nm and the sample temperature was maintained ambient. The described method for simultaneous determination of Atazanavir and Cobicistat is linear over a range of 8 µg/ml to 120 µg/ml and 5 µg/ml to 60 µg/ml respectively. The method shows good precision results which were below 2.0%RSD. Limit of Detection (LOD) and Limit of Quantification (LOQ) of Atazanavir and Cobicistat was established and found to be 1.49 and 4.97 µg/ml and 1.13 and 3.77 µg/ ml respectively. The developed method was validated according to ICH guidelines for various parameters.The method is simple, rapid, selective and stability indicating method which would be used for regular stability indicating quality control determinations.

Keywords: RP-HPLC; Simultaneous Determination; Atazanavir and cobicistat; stability indicating.

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INTRODUCTION

Atazanavir Sulphate (ATV) [1,2] is a protease inhibitor, 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.

ATV has the chemical name (3*S*, 8*S*, 9*S*, 12*S*)-3, 12-Bis (1, 1-dimethylethyl)-8-hydroxy-4, 11-dioxo-9-(phenyl methyl)-6-[[4-(2-pyridinyl) phenyl] methyl] -2, 5, 6, 10, 13 pentaazatetradecanedioic acid dimethyl ester, sulfate (1:1). ATV is a white to pale-yellow crystalline powder with a molecular formula of $C_8H_{14}N_2O_2$and a molecular weight of Salt form - 802.9 and Free form - 704.9. ATV is slightly soluble in water (4-5 mg/ml, free base equivalent) with the pH of a saturated solution in water being about 1.9 at 24 ± 3°C. Its chemical structure is given in Figure 1.

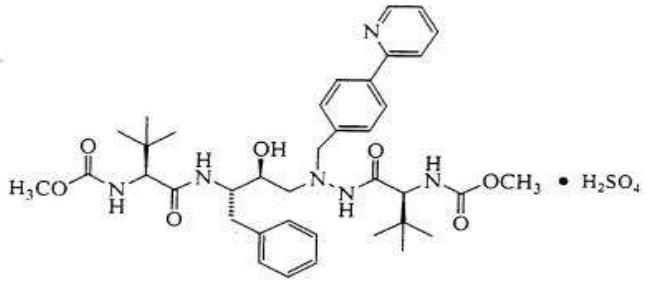

Figure 1: Chemical Structure of Atazanavir Sulphate

Cobicistat (COBI) [1,3] is a potent inhibitor of cytochrome P450 3A (CYP3A) which acts as a pharmaco-enhancing or "boosting" agent for antiviral drugs used in the treatment of HIV infection. Chemically COBI is 1, 3-thiazol-5-ylmethyl [(2R, 5R)-5-{[(2S)-2- [(methyl {[2-(propan-2-yl)-1, 3- thiazol-4-yl] methyl} carbamoyl) amino] -4- (morpholin-4-yl) butanoyl] amino}-1, 6-diphenylhexan-2-yl] carbamate. It is adsorbed onto silicon dioxide and is a white to pale yellow solid powder with a molecular formula of $C_{40}H_{53}N_7O_5S_2$ and a molecular weight of 776.0. COBI solubility is 0.1 mg/ml in water at 20 °C. Its Chemical structure is given in Figure 2.

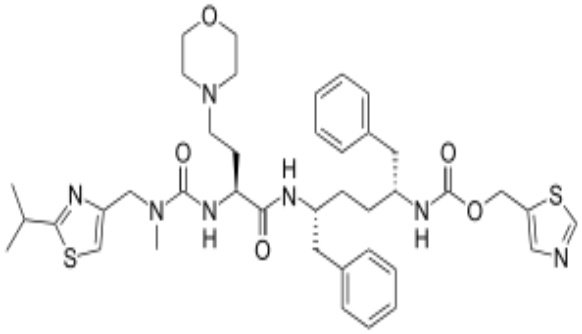

Figure 2: Chemical Structure of Cobicistat

COBI is a mechanism-based inhibitor of cytochrome P450 3A (CYP3A). Inhibition of CYP3A-mediated metabolism by COBI increases the systemic exposure of CYP3A substrates to ATV.

A few spectroscopic and liquid chromatographic procedures [4-7] have been reported for the determination of ATV and COBI individually but there is no method for stability indicating and simultaneous estimation of both the drugs.

Therefore there is need to develop rapid and reliable Stability indicating liquid chromatographic method for simultaneous determination of ATV and COBI in bulk and pharmaceutical dosage forms.

MATERIALS AND METHOD

Instrumentation

The analysis of the drug was carried out on a Shimadzu LC20 –AD, SPD M20A prominanace DAD detector, Rheodyne universal injector 7725 port and Hamilton 50 µl manual injector. Data processing was performed with shimadzu LC Solutions software version 1.25 for LC peak integration.

Chemicals and solvents

Methanol (HPLC Grade- Lobachemie), Orthophosphoricacid (OPA) (HPLC Grade- Fisher scientific) and HPLC Grade water - Merck.

METHOD DEVELOPMENT

Chromatographic Condition

Chromatographic separation was achieved by using Kinetex C_{18} 100 A (250 mm x 4.6 mm, 5µ) column as stationary phase and mixture consisting of 0.1% OPA(pH 3) and methanol in 80:20 v/v was used as mobile phase with a flow rate of 1 ml/min, column temperature at 25°C and the run time as 10 mins. UV detection was performed at 239 nm and the sample temperature was maintained ambient. standard and sample solutions were diluted with diluent filtered through Whattman filter paper (0.45µm) and degassed before use. Typical chromatogram of standard drug and sample were shown in Figure. 3 & 4.

Preparation of Mobile phase

A 80:20 v/v mixture of 0.1% OPA (pH 3) and methanol was prepared by mixing 800mL 0.1% OPA (pH 3) and 200mL of methanol in a 1000 ml volumetric flask. The mixture was filtered through 0.45 µ membrane filter and sonicated before use. The same mixture was used as diluent for preparing working standard solutions of the drugs.

Preparation of stock and working standard solution of ATV and COBI

About 30 mg of ATV and 15 mg of COBI was weighed accurately and transferred into a 100 ml volumetric flask and dissolved with adequate amount of mobile phase. The solution was sonicated for 15 min and then the volume made up with a further quantity of the mobile phase. This solution was suitably diluted with the mobile phase to get a working standard solution of 30 µg/ml of ATV and 15 µg/ml of COBI.

Preparation of Sample Solutions

10 tablets were weighed and crushed to powder and then powder equivalent to 5 tablets sample was weighed and transferred to 250 mL volumetric flask. 200 mL of diluent was added, sonicated to dissolve and diluted to final volume with diluent. The contents are filtered through 0.45µ Nylon syringe filter. Further diluted 5 mL of filtrate to 100 mL with diluent.

Procedure

10µL of standard preparation and sample preparation were injected five times in the Chromatograph. Chromatograms were recorded and the peak responses for ATV and COBI were measured. The System suitability parameters should be met. From the peak responses, the content of ATV and COBI in the sample was calculated. Assay results were shown in table 1.

METHOD VALIDATION

The method was validated for its linearity range, accuracy, precision, sensitivity and specificity. Method validation is carried out as per ICH guidelines [8-9].

System suitability

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Peak Area was evaluated for five replicate injections of the drug. The results shown in Table 2 were within acceptable limits.

Acceptance criteria:

The % RSD for the retention times of principal peak from 5 replicate injections of each Standard solution should be not more than 2.0 %

Linearity

A series of standard solutions (recommended not less than 5) 8 – 120 µg/ml of ATV and 5-60 µg/ml of COBI were prepared. 10µL of each solution was injected in the Chromatograph. Chromatograms were recorded and the peak responses for ATV and COBI were measured. A plot of average peak area versus the concentration in µg/ml or mg/ml is made and from this the correlation coefficient, y-intercept (const. of regression) and slope (coefficient of regression) of the regression line were calculated. The calibration data of ATV and COBI is given in Table 3 and the calibration curve of linearity and Calibration curves are depicted in figure 6 & 7.

Acceptance criteria:

Correlation Coefficient should be not less than 0.999.

Accuracy

To validate, weather the test method can accurately quantify ATV and COBI samples in six times for higher and lower levels, in triplicate by spiking ATV and COBI active material with equivalent amount of placebo were prepared. Samples at levels 50%, 100%, and 150% of the target assay concentration were prepared i.e. 50% of the lowest strength initial concentration to 150% of the highest strength initial concentration level. The Accuracy results were shown in Table 4A & 4B.

Acceptance Criteria:

The mean % Recovery of the ATV and COBI at each level should be not less than 98.0% and not more than 102.0%.

The %RSD of recovery of sample preparations from 50% - 150% should not be more than 2.

Precision

The precision of the test procedure was evaluated for ATV and COBI respectively. The % Assay and Relative Standard Deviation of assay results were calculated. The system precision is checked to ensure that the analytical system is precise. Method precision indicates whether a method is giving consistent results. The result of Precision studies is given in Table 5.

a. System precision: Standard solution prepared as per test method and injected six times.

b. Method precision: Prepared six sample preparations individually as per test method and injected each solution.

Robustness

A study to establish the effect of variation in mobile phase composition, flow rate and wavelength was conducted. Working standard solutions were injected into HPLC system. The system suitability parameters, % assay, theoretical plates and tailing factors were observed.The results were shown in Table 6.

Acceptance criteria:

There should be no significant effect on the result by doing small deliberate changes in the system as well as in method parameters

FORCED DEGRADATION STUDIES

Acid degradation

ATV and COBI working standard solution was refluxed with 3N HCl at 60°C for 1hour and then neutralized by adjusting pH to 7.0 with 5N NaOH. The Solution was further diluted to required concentration with diluent.

Alkali degradation

ATV and COBI working standard solution was refluxed with 2N NaOH at 60°C for 1hour and then neutralized by adjusting pH to 7.0 with 2N HCl. The Solution was further diluted to required concentration with diluent.

Oxidative degradation

ATV and COBI working standard solution was refluxed with 30%H_2O_2 by heating on water bath at 60°C for 1hour.

Photolytic degradation

ATV and COBI working standard solution was exposed to UV (200watt hour/m^2) and Visible (1.2million Lux hours) as per ICH Guidelines.

Thermal Degradation

ATV and COBI working standard solution was exposed to temperature at 105°C for 3days.

Hydrolytic Degradation

ATV and COBI working standard solution was refluxed with water by heating on water bath at 100°C for 1hour.

Humidity Degradation

ATV and COBI working standard solution was exposed to 85% Humidity (Prepared potassium nitrate saturated solution) at 3days. The results for forced degradation were shown in table 7A & 7B and chromatograms were shown in figure 8-13.

RESULTS AND DISCUSSION

Method Development

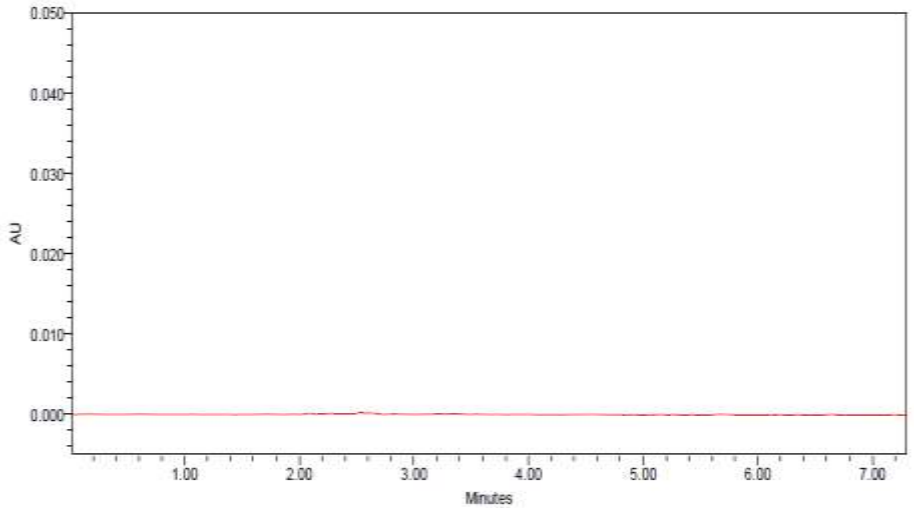

Figure 3: Chromatogram of Blank

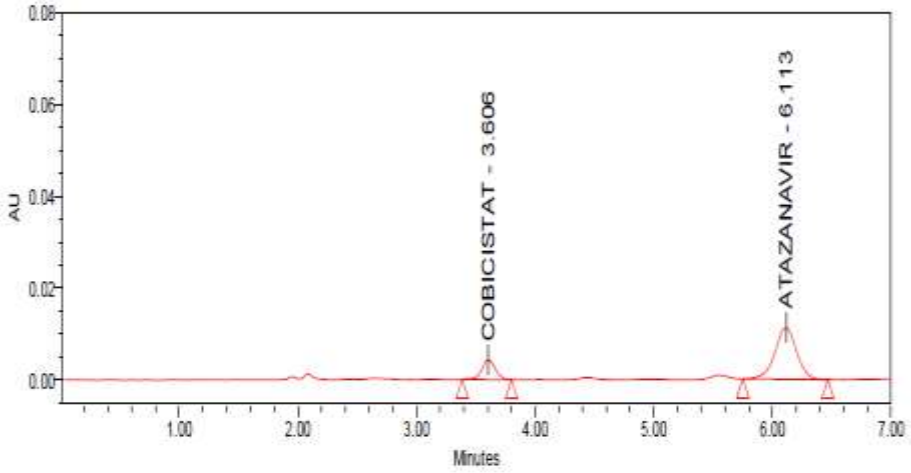

Figure 4: Chromatogram of Standard

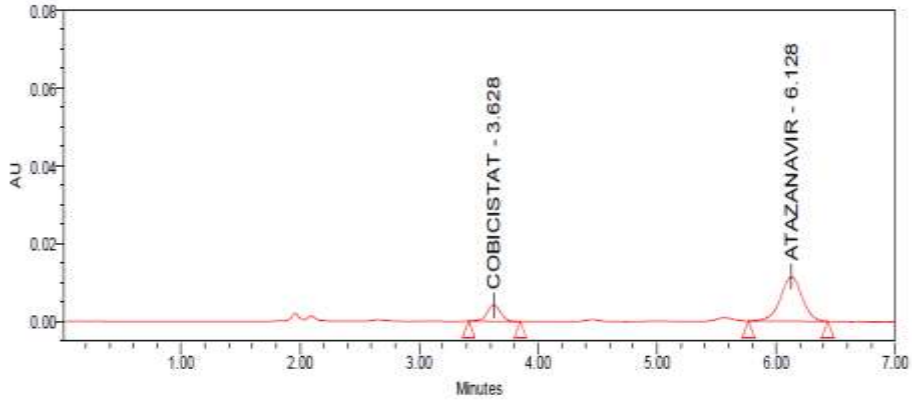

Figure 5: Chromatogram of Formulation

Assay Results:

Table 1: Assay Results of COBI & ATZ

S. No.	Drug	Label Claim (mg)	% Amount Found*	% RSD
1	COMBI	500	99.90	1.594
2	ATZ	1000	100	0.161

* Mean of Three Determinations

Method Validation:

System Suitability:

Table 2: System Suitability Results for COBI & ATZ

S. No.	Drug	Peak Area*	SD	% RSD
1	COMBI	1466117	3140.37	0.214
2	ATZ	6241162	16967.46	0.272

* Mean of Five Determinations

Linearity:

Table 3: Linearity data of ATV and COBI

Concentration of ATV(µg/mL)	Peak Area of ATV*	Concentration of COBI (µg/mL)	Peak Area of COBI*
8	88228	5	22508
16	207654	10	54657
30	380168	19	104652
40	506671	23	134265
48	611524	30	178606
78	986744	37	217891
120	1533953	60	358920
Y-Intercept	-5888		-7740
Slope	12812		6117
R^2	0.999		0.999

* Mean of Three Determinations

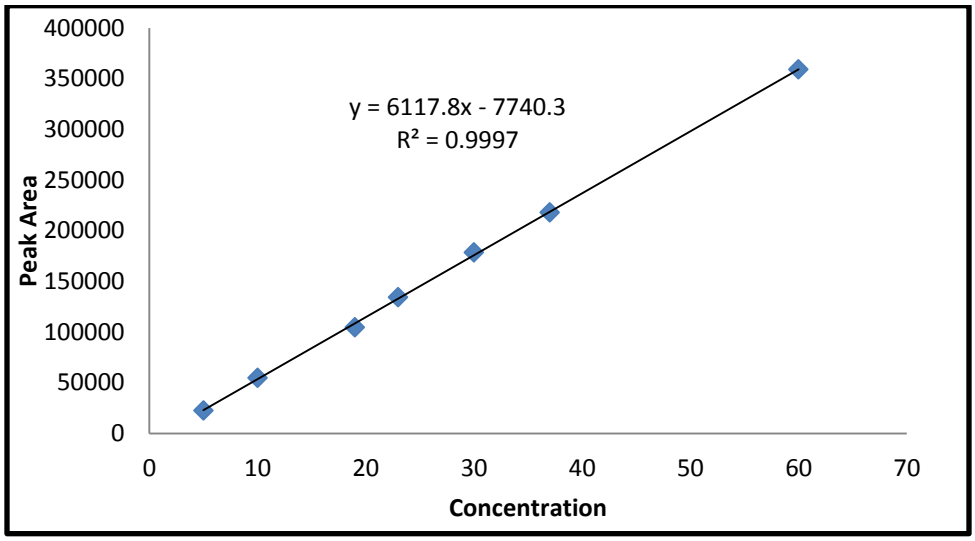

Figure 6: Linearity Plot of COBI

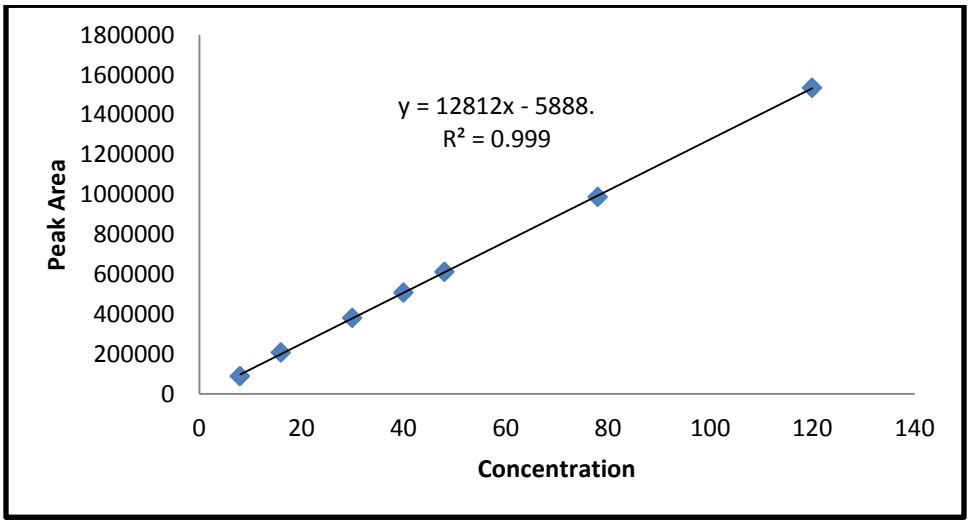

Figure 7: Linearity Plot of ATV

Accuracy:

Table 4A: Accuracy data (Triplicate values at 50, 100 and 150 percent levels) of ATV

Concentration of spiked level	Amount added (µg)	Amount found (µg)	Percent Recovery	Mean (%) Recovery	% RSD
50%	15.4	15.41	100.06	100.28	0.32
	15.07	15.09	100.13		
	15.3	15.4	100.65		
100%	30.5	30.54	100.13	100.16	0.15
	30.14	30.15	100.03		
	30.2	30.3	100.33		
150%	45.4	45.42	100.04	100.04	0.01
	45.16	45.18	100.04		
	45.04	45.07	100.06		

Table 4B: Accuracy data (Triplicate values at 50, 100 and 150 percent levels) of COBI

Concentration of spiked level	Amount added (µg)	Amount found (µg)	Percent Recovery	Mean (%) Recovery	% RSD
50%	7.51	7.53	100.26	99.73	0.53
	7.56	7.50	99.20		
	7.59	7.57	99.73		
100%	15.40	15.11	99.11	99.01	0.95
	15.80	15.49	98.03		
	15.60	15.43	99.91		
150%	22.52	22.56	100.17	99.95	0.20
	22.57	22.52	99.77		
	22.53	22.51	99.91		

Precision:

Table 5A: System Precision data of the proposed method

Injection	Peak Area of ATV	Peak Area of COBI
1	381165	89730
2	380157	89642
3	381153	89529
4	380148	89944
5	380069	89635
6	380159	89699
Mean	380475	89696.5
SD	530.79	139.422
% RSD	0.14	0.16

Table 5B: Method Precision data of the proposed method

Injection	Peak Area of ATV	Peak Area of COBI
1	378561	90327
2	377695	90125
3	378256	90326
4	376523	90152

5	374582	90236
6	378264	90138
Mean	380475	90217
SD	530.79	93.02
% RSD	0.14	0.10

Robustness

Table 6: Robustness data of ATV and COBI

Variations	ATV			COBI		
	% Assay*	Theoretical plates*	Tailing factor*	% Assay*	Theoretical plates*	Tailing factor*
23% of Methanol in the mobile phase	99.4	2975	1.01	99.4	8660	0.98
17% of Methanol in the mobile phase	99.6	2989	1.01	99.6	8678	0.98
Flow rate at 0.7 mL/min	101.1	2998	1.02	101.1	8650	0.97
Flow rate at 0.9 mL/min	99.8	2886	1.01	99.8	8670	0.98
Wavelength at 327	101.2	2978	1.02	101.2	8648	0.97
Wavelength at 331	99.8	2975	1.03	99.8	8652	0.98

* Mean of Six Determinates

Forced Degradation Studies

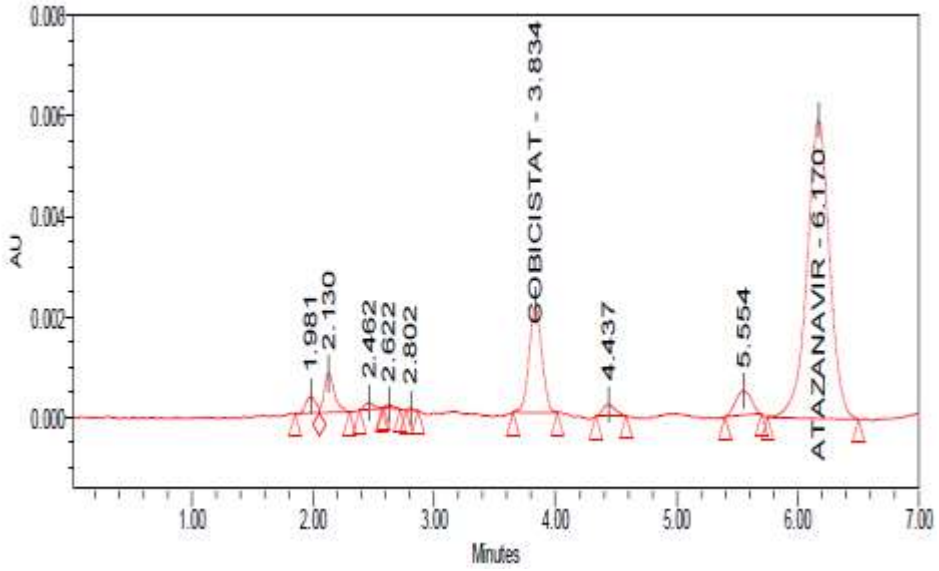

Figure 8: Chromatogram of Acid degradation

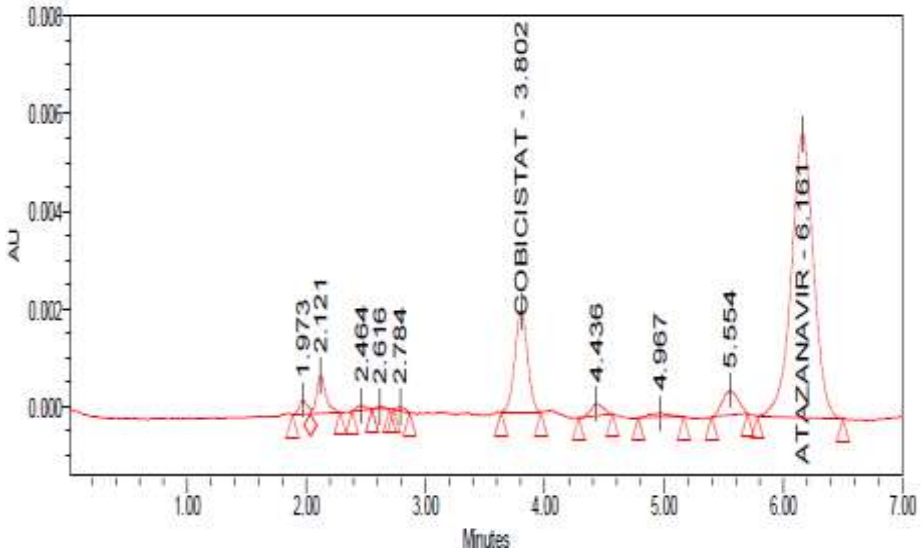

Figure 9: Chromatogram of Alkali degradation

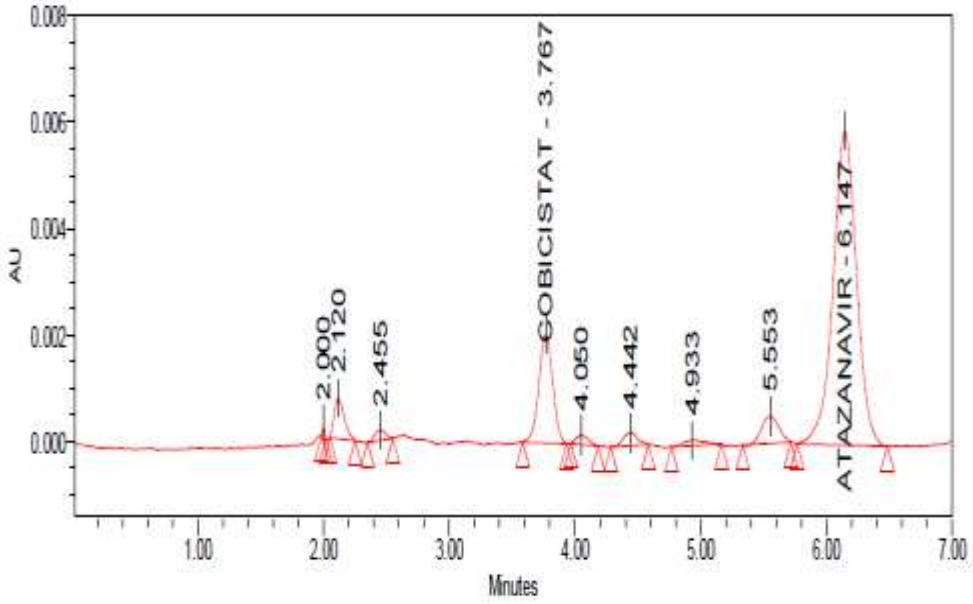

Figure 10: Chromatogram of Oxidation degradation

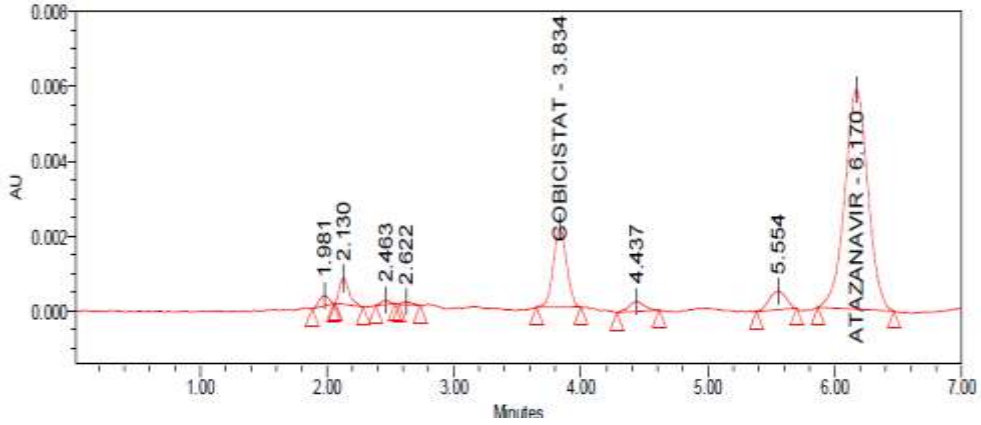

Figure 11: Chromatogram of Photolytic degradation

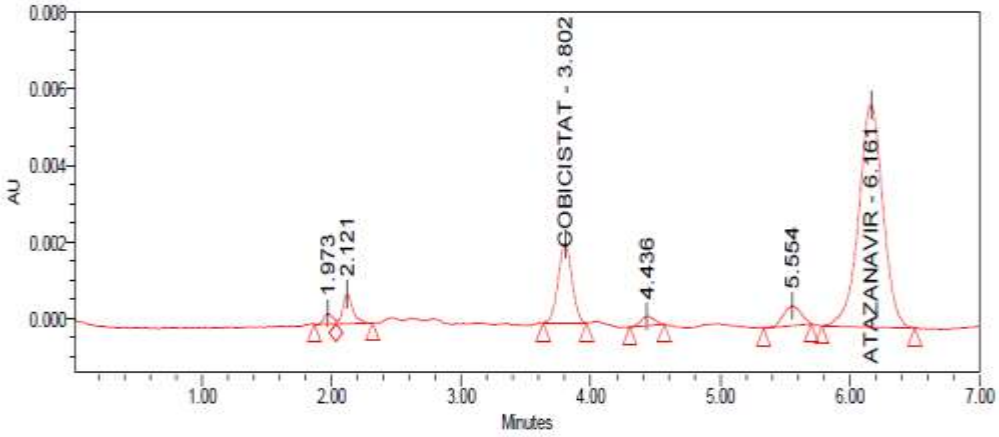

Figure 12: Chromatogram of Thermal degradation

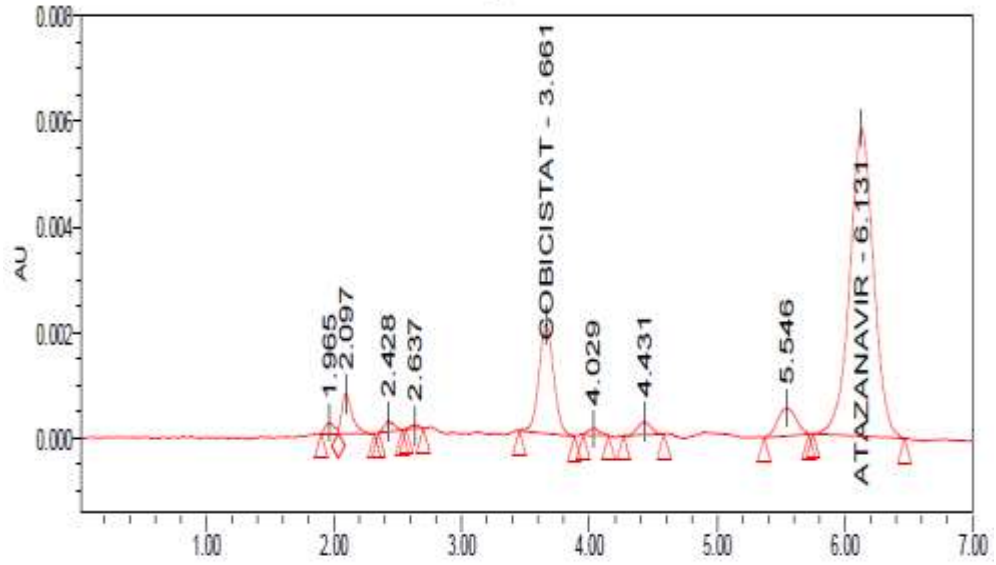

Figure 13: Chromatogram of Humidity degradation

Table 7A: Results of Forced Degradation Studies of ATV

Treatment	% Label Claim	% Degradation	Peak purity		
			Purity Angle	Purity Threshold	Pass / Fail
Control	100	0	0.641	2.133	Pass
Acid	78.7	21.3	1.374	3.209	Pass
Alkali	79.2	20.8	1.253	2.863	Pass
Peroxide	75.2	24.8	1.190	2.941	Pass
Reduction	76.8	23.2	1.988	3.301	Pass
Thermal	77.4	22.6	1.532	3.185	Pass
Photo	73.6	26.4	1.310	3.260	Pass
Humidity	78.4	21.6	1.492	2.883	Pass
Hydrolysis	72.3	27.7	1.208	3.281	Pass
Heat	70.7	29.3	1.270	3.193	Pass

Table 7B: Results of Forced Degradation Studies of COBI

Treatment	% Label Claim	% Degradation	Peak purity		
			Purity Angle	Purity Threshold	Pass / Fail
Control	100.1	-0.1	1.397	4.051	Pass
Acid	75.6	24.4	1.402	4.712	Pass
Alkali	74.3	25.7	1.678	4.079	Pass
Peroxide	76.4	23.6	1.888	4.173	Pass
Reduction	75.1	24.9	1.151	4.218	Pass
Thermal	78.8	21.2	1.642	4.546	Pass
Photo	72.9	27.1	1.025	4.418	Pass
Humidity	73.6	26.4	1.584	4.030	Pass
Hydrolysis	75.5	24.5	1.972	4.604	Pass
Heat	74.7	25.3	1.391	4.744	Pass

The present study was aimed to develop a rapid, stability indicating, precise and accurate HPLC method for the analysis of the ATV and COBI in bulk and in its dosage forms. For this method, 80: 20 v/v mixture of 0.1% OPA (pH 3) and methanol proportions was used as mobile phase as well as diluents and Kinetex 5 µ C18 100 A (250 mm x 4.6 mm) column was found to be suitable to get well defined and resolved peaks free from tailing in the chromatogram. Peaks at 3.6 and 6.1 min was observed in chromatograms of the drug samples extracted from the marketed formulations; there was no interference from the excepients commonly present in the formulations and from mobile phase. It may therefore be inferred that no degradation of ATV and COBI in the pharmaceutical formulations was detected by use of this method. Whereas placebo formulation samples and blank samples, yielded clean chromatograms; with no interference from the excepients and mobile phase and the peaks of the degradation products were well resolved from that of ATV and COBI (RSD> 2%) this is indicative of the specificity of the method. Limit of Detection (LOD) and Limit of Quantification (LOQ) of ATV and COBI was established and found to be 1.49 and 4.97 µg/ml and 1.13 and 3.77 µg/ ml respectively. The described method of ATV and COBI was linear over a range of 8 µg/ml to 120 µg/ml and 5 µg/ml to 60 µg/ml respectively. The regression equation calculated by the least-squares method for ATV and COBI was y = 12812x – 5888; correlation coefficient 0.999 and y = 6117x- 7740; correlation coefficient 0.999 respectively. The method was found to be precise as the RSD <2 %. Accuracy of the method was evaluated by standard addition method and the percent recoveries ranged from99.01 to 100.28% which shows that the method is highly accurate. The deliberate changes in the method have not much affected the peak tailing, theoretical plates and the percent assay. This indicates that the present method is robust. The bench top study suggests that, the working solutions of the drug are stable up to twenty four hours.

CONCLUSION

From the above discussion it can be concluded that the proposed method is precise, accurate and stability indicating. Therefore the proposed method can be used for routine quality control and analysis of the drug during stability studies in bulk samples and in tablet dosage forms.

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