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RP-HPLC METHOD DEVELOPMENT FOR THE ASSAY AND DEGRADATION STUDY OF COBICISTAT AND ATAZANAVIR SULPHATE IN BULK AND COMBINED DOSAGE FORM

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ABSTRACT

Objective: A reversed phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the assay of Atazanavir sulphate (ATV) and Cobicistat (COBI) simultaneously in bulk and pharmaceutical formulations. The method was intentionally developed for the study of stability of the drug samples under different degradation conditions. **Experimental**: Waters HPLC system equipped with auto sampler, Inertsil ODS C_{18} (4.6 x 150mm, 5.0µm) column and photo diode array (PDA) or ultra violet (UV) detector were adopted in method development. Exactly 20 µl of working standard (15 µg/ml of ATV and 30 µg/ml of COBI) is introduced into column at ambient temperature, mobile phase of a mixture of 0.1% orthophosphoric acid buffer of pH 5.5, methanol in the ratio 30:70 v/v was allowed to flow through the column at a flow rate of 1.0 ml/min for a run time of 8.0 minutes, and the components were detected at a wavelength of 242 nm. The chromatographic data was obtained by using Empower -2 software. **Results:** System suitable parameters such as number of theoretical plates, peak area, tailing factor for COBI and ATV were found to be 2529.34, 44562, 1.9 and 3588.27, 217752.7, 1.20 respectively. The retention time and resolution between the two peaks were found to be 1.994 and 3.325min and 4.58 respectively. Assay of COBI and ATV was found to be 100.35 and 100.48 % respectively. The stability of the drug sample under different degradation conditions was determined and found to be within the range of 86.46 to94.72 and 83.97 to 92.82 for COBI and ATV respectively. **Conclusions:** The developed method was found to be simple, rapid and applied for the analysis of drug samples, therefore the proposed method is recommended for the analysis of COBI and ATV in pure and tablet dosage forms in any quality control laboratories.

KEYWORDS: Atazanavir sulphate, Cobicistat, Validation, Stability, Assay.

INTRODUCTION

HPLC Technique

High performance liquid chromatographic (HPLC) technique is generally applied for the separation of drug components in the sample and used for the determination of their content in bulk and pharmaceutical formulations. Since most of the drug molecules are polar in nature because of the presence of relatively high electronegative hetero atoms such as oxygen, nitrogen, sulphur and halogens in their molecular structure or they may be present in the form of salts, hence soluble in polar solvents like water, methanol, acetonotrile etc. The separation of the polar components takes place in reversed phase HPLC technique in which a non polar stationary phase (a non polar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel) and a polar mobile phase (buffer solution and organic solvents like methanol) were used for the separation. This technique is widely used in the study of stability of drug products under different forced degradation conditions and also adopted to identify, separate and quantify the impurities if present or formed during degradation.

Profile of the selected drugs

Cobicistat is a licensed drug used for the treatment of infection with the human immunodeficiency virus (HIV). Cobicistat is a component of the four-drug, fixed-dose combination HIV treatment elvitegravir/cobicistat/emtricitabine/tenofovir marketed as Stribild, and also known as the "Quad Pill". Additionally, in existence are a fixed-dose combination of Cobicistat and protease inhibitor darunavir (darunavir/cobicistat; marketed as Prezcobix, by Janssen Therapeutics), and a fixed-dose combination of cobicistat and protease inhibitor atazanavir (atazanavir/cobicistat;

marketed as Evotaz, by Bristol-Myers Squibb). Both Prezcobix and Evotaz were approved by the FDA in January 2015. Cobicistat is chemically known as thiazol-5-ylmethyl *N*-[1-benzyl-4-[[2-[[(2-isopropylthiazol-4-yl) methyl-methyl-carbamoyl] amino]-4-morpholino-butanoyl] amino]-5-phenyl-pentyl] carbamate. Cobicistat is a white to pale yellow solid with a solubility of 0.1 mg/mL in water at 20°C, the pKa are 1.8, 2.5 and 6.4.The molecular formula and molecular weight of cobicistat are $C_{40}H_{53}N_7O_5S_2$ and 776.023 g/mol respectively.

Atazanavir is chemically known as methyl *N*-[(1*S*)-1-{[(2*S*,3*S*)-3-hydroxy-4-[(2*S*)-2-[(methoxycarbonyl)amino]-3,3-dimethyl-*N'*-{[4-(pyridin-2yl)phenyl]methyl}butanehydrazido]-1-phenylbutan-2-yl]carbamoyl}-2,2 dimethylpropyl]carbamate. Atazanavir sulfate is a white to pale yellow crystalline powder with a solubility of 4 to 5 mg/ml free base equivalents in water at 24°C, the pKa is 4.7. The molecular formula and molecular weight of atazanavir sulphate are $C_{38}H_{52}N_6O_7$ and 802.9416 g/mol respectively. Atazanavir is an antiretroviral drug of the protease inhibitor (PI) class is used to treat infection of human immunodeficiency virus (HIV).[1,2] It is the first PI approved for once-daily dosing, and also appears to be less likely to cause lipodystrophy and elevated cholesterol as side effects. Like other protease inhibitors, it is used only in combination with other HIV medications. The U.S. Food and Drug Administration (FDA) approved atazanavir on June 20, 2003. Evotaz (a fixed-dose combination tablet for oral administration containing 300 mg atazanavir as atazanavir sulfate and 150 mg cobicistat) is a once-daily fixed-dose combination of a protease inhibitor and a pharmacokinetic enhancer for the treatment of HIV-1 infection. Inactive ingredients present in Evotaz are microcrystalline cellulose, croscarmellose sodium, sodium starch glycollate, crospovidone, stearic acid, magnesium stearate, hydroxypropylcellulose, and silicon dioxide in tablet core and hypromellose, titanium dioxide, purified talc, glycerol triacetate, and iron oxide red in film coating. The structures of the cobicistat and atazanavir are represented in Fig.1and Fig.2 respectively.

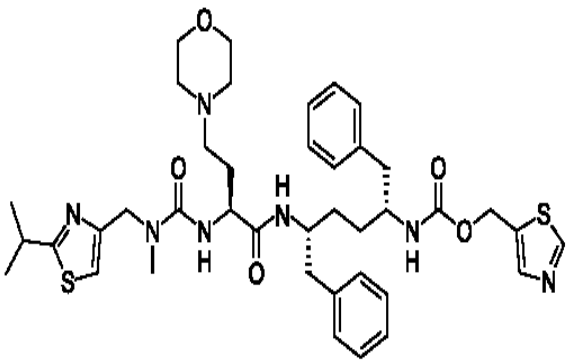

Fig. 1: Molecular structure of Cobicistat (COBI)

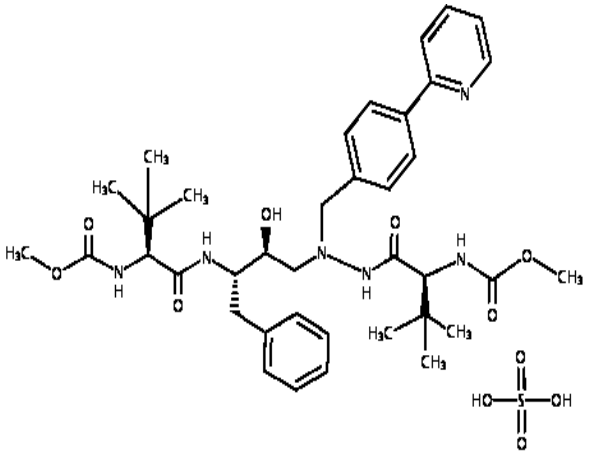

Fig. 2: Molecular structure of Atazanavir sulphate (ATV**)**

Literature review

Literature survey reveals few UV spectrophotometric methods[3-7] and RP-HPLC methods[8-16] for the determination of atazanavir sulphate alone and simultaneously with other retroviral drugs in formulations and biological fluids. In addition, one HPTLC[17] and two LC/MS/MS methods[18-19] were also reported. RP-HPLC methods[20-21] for the analysis of Cobicistat and related impurities in bulk and pharmaceutical dosage forms, Stability indicating HPLC method[22] for simultaneous estimation of emtricitabine, tenofovir disoproxyl fumarate, cobicistat and elvitegravir in pharmaceutical dosage form, A new gradient liquid chromatographic method[23] for simultaneous estimation of Tenofovir, Disoproxil fumarate, Cobicistat, Emtricitabine and Elvitegravin in bulk drug and tablet dosage form.

Objective of the investigation

The aim of the present investigation is to determine potency and to test the stability of drug sample under different degradation conditions. The main objectives of the present work are, to develop and optimize an isocratic HPLC method by choosing different columns, buffer solutions, HPLC grade solvents, mobile phases, composition of mobile phase, detection wavelengths based on the physic-chemical properties of the drug molecules, to validate the developed method in terms of precision, accuracy, sensitivity, linearity, robustness and ruggedness, to determine the assay of cobicistat and atazanavir sulphate simultaneously in pharmaceutical formulations by adopting the optimized method and to study the stability of cobicistat and atazanavir sulphate under different degradation conditions (forced degradation) such as acid, base, peroxide, thermal and photo light, and to find out the degradation of the drug moiety present in the sample.

MATERIAL AND METHODS

Instrumentation

Waters HPLC system equipped with auto sampler and photo diode array and ultra violet detector was used in

the present investigation. The Empower-2 software was used to acquire the chromatographic data.

Standards and reagents

Cobicistat (potency of 99.8) and atazanavir sulphate (potency 99.8) drug products were obtained as a gift samples from Mylan Laboratories and Hetero Drugs Ltd. Hyderabad, Telangana state India respectively. The Evotaz tablets (150 mg of cobicistat and 300mg atazanavir sulphate, Bristol-Myers Squibb Company) were procured from the local pharmacy. HPLC grade reagents such as methanol, acetonitrile, orthophosphoric acid, hydrochloric acid, sodium hydroxide, hydrogen peroxide and water were procured from Merck India.

Preparation of solutions

Preparation of 0.1% OPA buffer: Exactly 1ml of orthophosphoric acid is transferred into a 1000ml volumetric flask, diluted up to the mark with HPLC grade water and the pH of the solution was adjusted to 5.5.

Preparation of mobile phase: 0.1% OPA buffer and methanol were mixed in the ratio 30:70 v/v, sonicated for a few minutes by using ultrasonic water bath. Then the solution was filtered through 4.5 µ filter under vacuum filtration.

Preparation of diluents: Methanol and HPLC grade water were mixed in the ratio 50:50 v/v and used for dilution.

Preparation of standard solution: Exactly 15 mg of Cobicistat and 30 mg of Atazanavir sulphate working standards were accurately weighed, transferred into a 10 ml clean dry volumetric flask, dissolved in diluents and sonicated for a few minutes and filtered through 4.5 µ filter under vacuum filtration (Solution-A)**.** Then precisely 1.0 ml of solution-A is accurately transferred into a 10 ml volumetric flask and diluted up to the mark with diluents. Further exactly 2 ml of the above dilute solution was introduced into a 10 ml volumetric flask and diluted up to the mark with diluents, and final concentration of COBI and ATV was found to be 30 and 60 µg/ml respectively.

Preparation of sample solution: Average weight of ten tablets was determined, made them into a fine powder, and an amount of the powder equivalent to (150 mg of Cobicistat and 300 mg Atazanavir sulphate) the average weight of ten tablets was accurately weighed and transferred into a 100 ml clean dry volumetric flask, dissolved in 70 ml of diluents and sonicated for five minutes and filtered through 4.5 µ filter under vacuum filtration (Solution-B). Then the solution –B was diluted as explained in standard preparation.

Method development

The development of liquid chromatographic method was based on physic-chemical properties such as molecular weight, molecular formula, chemical structure, solubility, PK_a value and UV absorption maxima of selected drugs. The selected drugs were completely soluble in water and methanol; hence a reversed phase liquid chromatographic technique was adopted. The optimum chromatographic conditions were established by different trials by changing one of the chromatographic conditions such as column, mobile phase and its composition, flow rate of the mobile phase, injection volume, run time, column temperature and detection wavelength keeping other constant. Finally in the optimized chromatographic procedure, precisely 20 µl of the standard or sample was injected into Inertsil ODS C_{18} (4.6 x 150mm, 5.0µm) column which was kept at ambient temperature, mobile phase of the mixture of 0.1% OPA buffer of pH 5.5 and methanol in the ratio 30:70 v/v was allowed to flow through the column at a flow rate of 1.0 ml/min and the response of the detector was measured at a wavelength of 242 nm.

METHOD VALIDATION

The objective of validation of an analytical procedure is to demonstrate that the new method is suitable for its intended purpose. Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product similar validation characteristics also apply when assaying for the active or other selected component(s).

System suitability

The system suitable parameters such as tailing factor, plate count, and resolution between two adjacent peaks were determined by injecting working standard solution of COBI and ATV in triplicate into the chromatographic system and chromatograms were obtained under the optimized chromatographic conditions. An investigation of specificity (ability to assess unequivocally the analyte in the presence of other components which may be expected to be present generally impurities, degradants, matrix, etc.) was conducted during the validation of the assay procedure. In assay procedure, representative chromatograms for standard and sample were recorded to demonstrate the specificity. System suitable parameters were evaluated; the parameters of test were compared with standard and found no significant variation in retention time, symmetry and plate count. A typical HPLC chromatogram of standard and sample were presented in Fig. 3 and Fig.4 respectively. The system suitable parameters, retention time, peak area and peak height were presented in Table-1. The purity of the peaks were evaluated by determining purity angle and threshold angle and found to be 0.422 & 0.681 and 0.519 & 0.864 for COBI and ATV respectively. Purity plots for COBI and ATV were presented in Fig.5 and Fig.6 respectively.

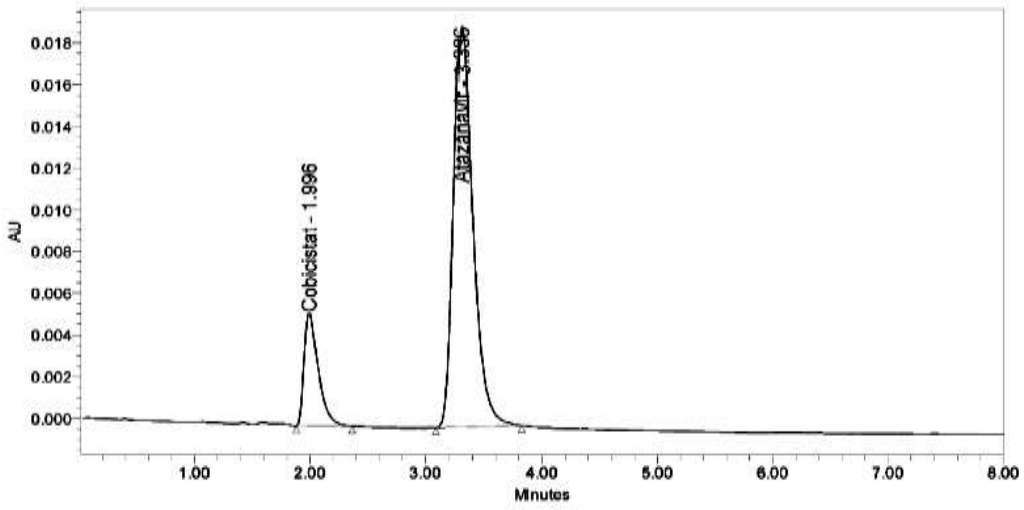

Fig. 3: HPLC chromatogram for working standard solution of COBI and ATV

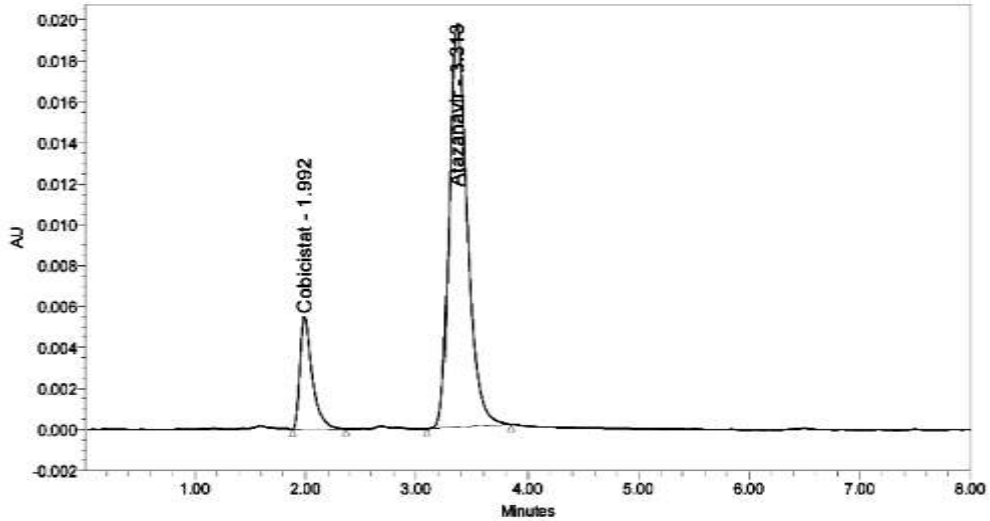

Fig. 4: HPLC chromatogram for sample solution of COBI and ATV

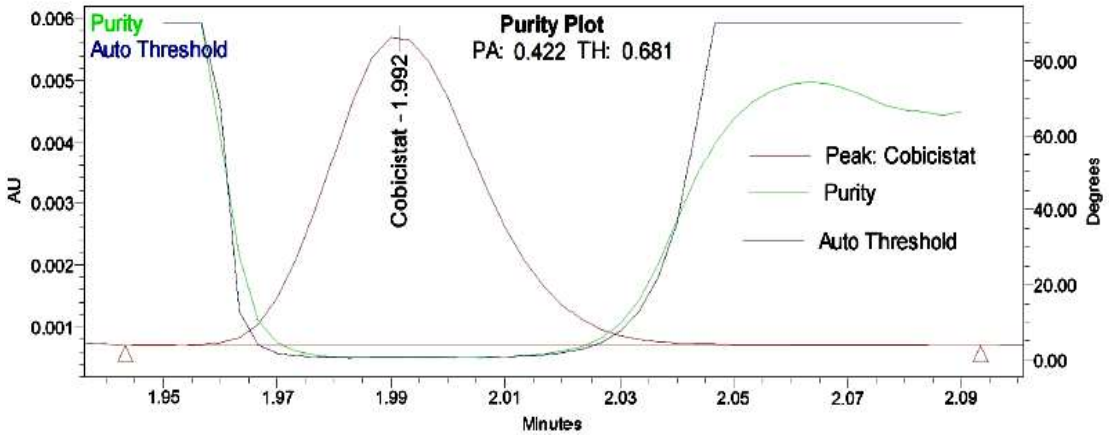

Fig. 5: Purity plot of cobicistat

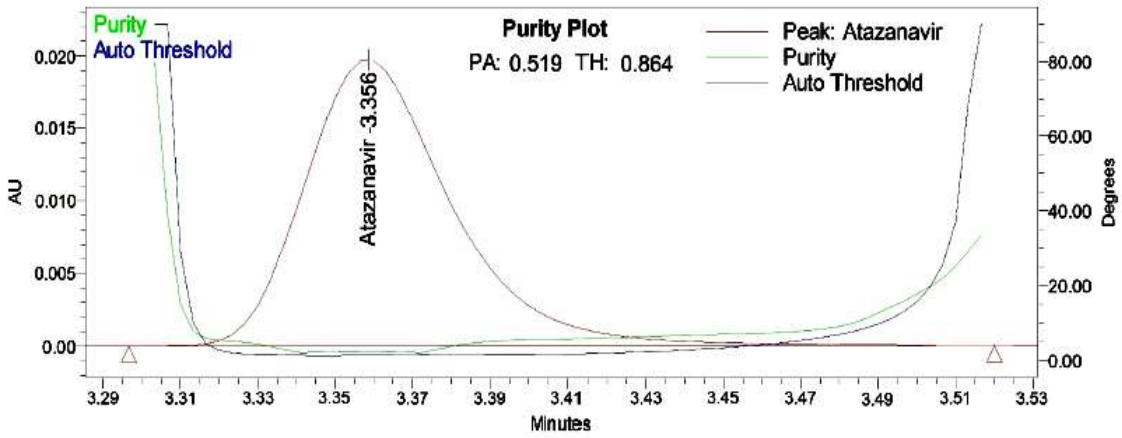

Fig. 6: Purity plot of atazanavir sulphate

Table-1: System suitable parameters, retention time, peak area and peak height

S.No.	Retention time	Peak area	Peak height	USP resolution	USP plate count	USP tailing
	Cibicistat					
1	1.996	44681	5573	4.46	2481.63	1.42
2	1.998	44242	5669	4.68	2182.64	1.43
3	1.988	44763	5644	4.48	2353.64	1.43
Mean	1.994	44562	5628	4.54	2339.3	1.426
	Atazanavir					
S.No.	**Retention time**	**Peak area**	**Peak height**	**USP resolution**	**USP plate count**	**USP tailing**
1	3.336	216381	19212	4.46	3683.48	1.27
2	3.333	218654	19895	4.68	3598.81	1.21
3	3.306	218223	19803	4.48	3482.52	1.12
Mean	3.325	217752	19639	4.54	3588.27	1.12

Sensitivity

Sensitivity of an analytical technique is defined as the instrument response to the low concentration of the analyte. The sensitivity is expressed in terms of limit of detection (LOD) and limit of quantization (LOQ), and were determined from the signal-to-noise ratio. LOD values were determined from the signal-to-noise ratio, in which measured signals from samples with known low concentrations of analyte was compared with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. To find out LOD and LOQ, the stock solution was diluted to low concentrations successively and chromatograms were obtained at each dilution, chromatographic parameters were evaluated, and from the peak area of the sample and blank, LOD and LOQ values were determined and were presented in Table-2. The chromatograms of blank, LOD and LOQ solutions were presented in Fig.7 to Fig.9 respectively.

Preparation of 45µg/ml cobicistat solution: Exactly 15mg of Cobicistat working standard was weighed and transferred into a 10mL clean dry volumetric flask and dissolved in 7mL of diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1.0ml of Cobicistat the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1.5ml Cobicistat the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of 90µg/ml atazanavir solution: Accurately transferred 30mg of Atazanavir working standard into a 10mL clean dry volumetric flask and dissolved in 7mL of diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. Further pipetted 1.0ml of Atazanavir D F the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents**.** Further pipette 1.5ml of Atazanavir D F the above stock solution into a10ml volumetric flask and dilute up to the mark with diluents**.**

Preparation of LOD (1.397 and 0.820 µg/ml of COBI and ATV) solution: Then precisely 2.0 ml of the each stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluents. Further 3.1 ml / 0.9 ml of the above stock solution were pipetted into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation LOQ (4.748 and 2.759 µg/ml of COBI and ATV) solution: Then precisely 5.3ml / 3.1 ml of the

above stock solution was transferred into a 10ml volumetric flask and diluted up to the mark with diluents. Further 4.0 ml / 2.0 of the above stock solution were pipetted into a 10ml volumetric flask and dilute up to the mark with diluents.

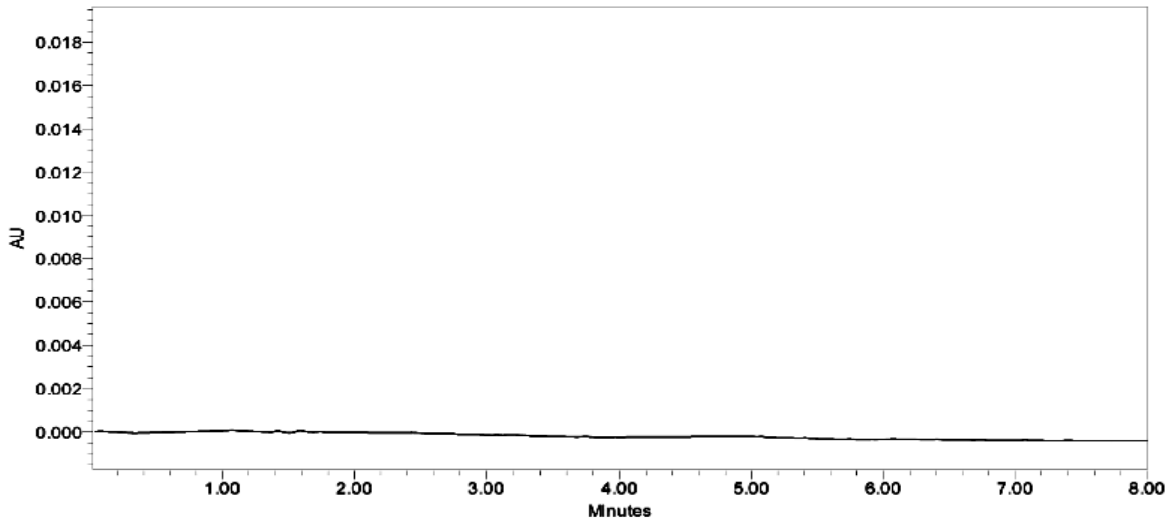

Fig. 7: HPLC chromatogram for blank

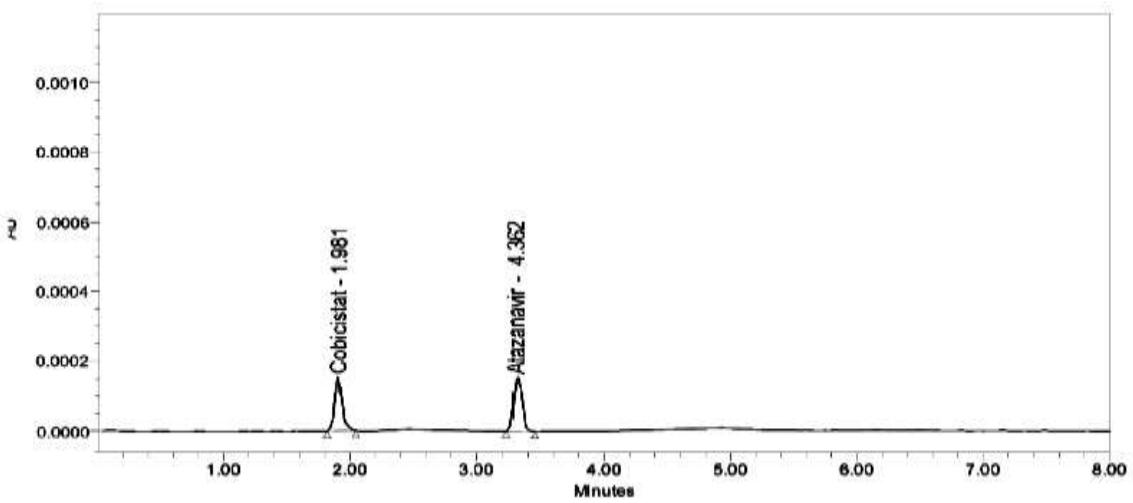

Fig. 8: HPLC chromatogram for LOD concentration level

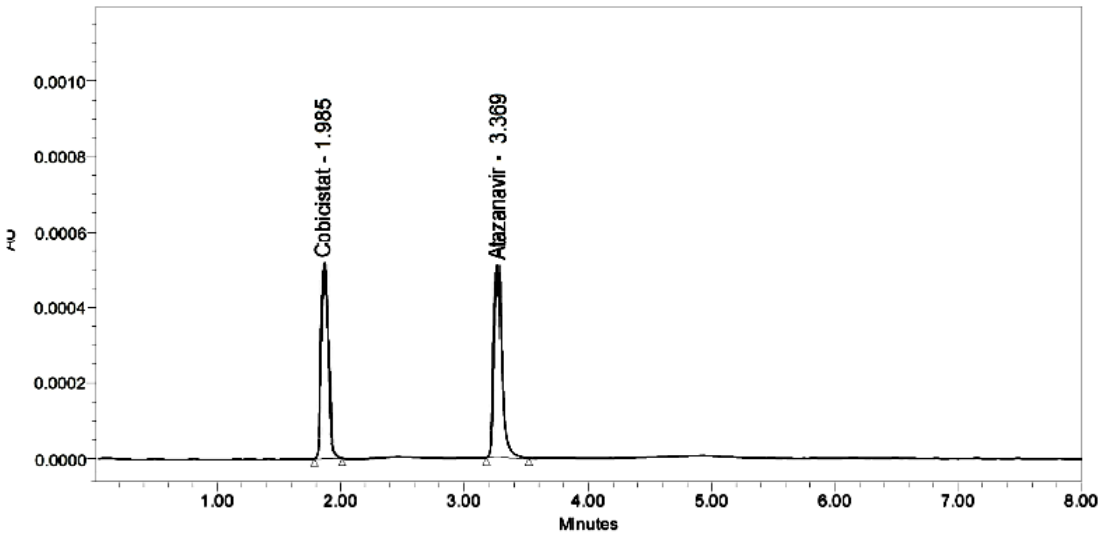

Fig. 9: HPLC chromatogram for LOQ concentration level

Table-2: Results of LOD and LOQ of COBI and ATV

Peak Name	Property	Concentration	Baseline noise (N)	Peak height (S)	S/N ratio	Acceptance criteria
COBI	LOD	1.397 µg/ml	59	173	2.93%	3%
	LOQ	4.748 µg/ml	59	588	9.97%	10%
ATV	LOD	0.820 µg/ml	59	175	2.97%	3%
	LOQ	2.759 µg/ml	59	589	9.98%	10

Linearity studies

Stock solution of cobicistat and atazanavir was prepared by accurately weighing 15 mg of cobicistat and 30 mg of atazanavir working standards and transferring into a 10 ml clean volumetric flask, dissolved in 7 ml of diluents and sonicated for five minutes and made volume up to the mark with the same diluent. Further pipette 1.0 ml of the above solution into a 10 ml volumetric flask and dilute up to the mark with diluents. This solution (stock solution) was used for further dilutions in the study of linearity. Into a series of five 10 ml volumetric flasks, different aliquots of stock solution 0.5 to 2.5 ml (7.5ppm to 37.5ppm and 15ppm to 75ppm of COBI & ATV respectively) were accurately transferred, diluted up to the mark, injected each solution in duplicate into the column, chromatograms were recorded and measured the peak area. Linearity plots (Fig. 10 and Fig. 11) were drawn by taking peak area against concentration of the drugs, and calculated the slope, intercept and correlation coefficient, and were reported in Table-3.

Table-3: Results of linearity studies of COBI and ATV in RP-HPLC method

S.No	Linearity Level	Cibicistat		Atazanavir	
		Concentration µg/ml	Area	Concentration µg/ml	Area
1	I	7.5	14938	15.0	73154
2	II	15.0	30277	30.0	144308
3	III	22.5	43816	45.0	221462
4	IV	30.0	59754	60.0	294616
5	V	37.5	74693	75.0	365770
Correlation coefficient		0.9990		0.9990	
Slope		1986		4896	
Intercept		0.238		380.9	

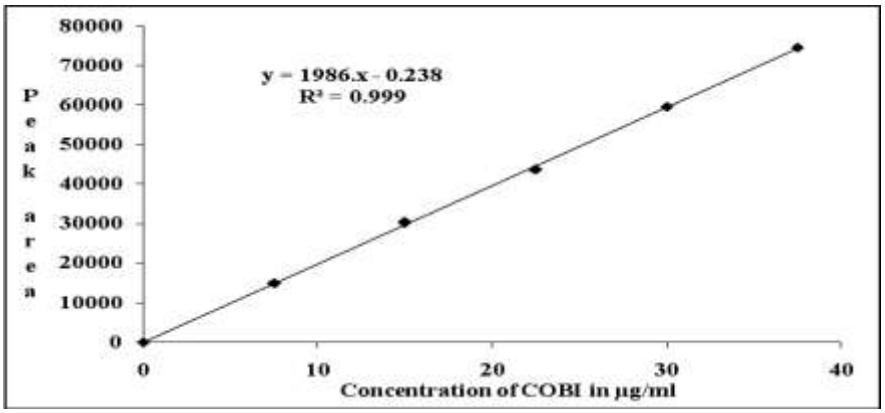

Fig.10: Linearity plot between peak area and concentration of COBI

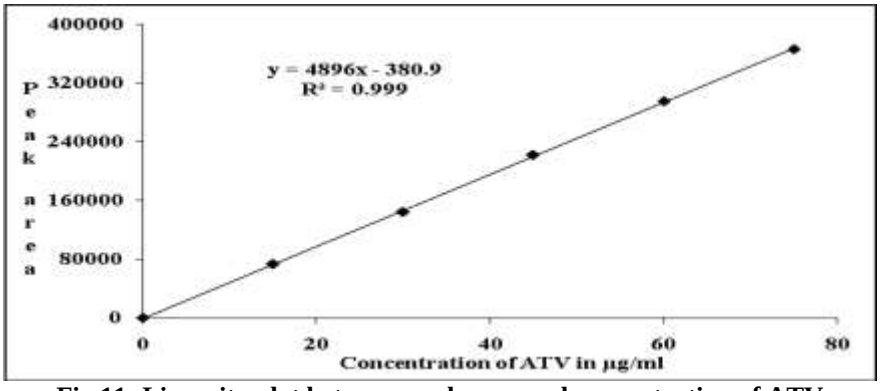

Fig.11: Linearity plot between peak area and concentration of ATV

Precision

System precision

To determine system precision i.e. the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions, working standard solution of COBI and ATV was injected six times into the colomun, chromatograms were obtained as per the prescribed procedure.

Method precision

To determine method precision, working standard solution of COBI and ATV was prepared six times as described in experimental section; chromatograms were obtained as per the prescribed procedure by injecting each solution into the column. The system precision and method precision were presented in Table-4 and Table-5 respectively.

Table-4: System precision of the developed RP-HPLC method

Injection ID	Peak area of COBI	Peak area of ATV
Injection-1	45683	213649
Injection-2	44124	216953
Injection-3	45082	219275
Injection-4	44762	223116
Injection-5	45216	220642
Injection-6	45687	221948
Average	45092.33	219263.8
Standard Deviation	594.088	3487.398
%RSD	1.3175	1.5905

Table-5: Method precision of the developed RP-HPLC method

Injection ID	Peak area of COBI	Peak area of ATV
1	44897	225143
2	45142	219541
3	44976	221487
4	44845	222954
5	45162	221642
6	44658	221748
Average	44946.67	222085.8
Standard Deviation	190.533	1857.196
%RSD	0.4239	0.8362

Accuracy

Preparation of Standard stock solution

Accurately 15 mg of Cobicistat and 30 mg of Atazanavir working standard was weighed and transferred into a 10ml clean dry volumetric flask, added diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. Further pipetted 1.0 ml of the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluents. Further pipetted 1.5 ml of the above diluted solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 50% / 100% and 150% sample solution

Exactly 7.5 / 15 / 22.5 mg of Cobicistat and 15.0 / 30.0 / 45.0 mg of atazanavir working standard was accurately weighed and transferred into three 10 ml clean dry volumetric flask, about 7 ml of diluents was added to each flask and sonicated to dissolve the components completely for five minutes and made volume up to the mark with the same solvent. Then precisely 1.0 ml of the above stock solution was pipetted into three 10ml volumetric flask and dilute up to the mark with diluents, further 1.5 ml of this solution was transferred into another three 10 ml volumetric flask and diluted up to the mark with diluents.

Standard and sample solutions were injected in triplicate into the column, chromatograms were obtained, calculated the percent of recovery from the amount added and amount recovered and calculated mean recovery. The results were presented in Table-6.

Table-6: Accuracy of the developed RP-HPLC method

%Concentration	Peak Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
COBI					
50%	22683	7.5	7.62	101.60	100.48
100%	44352	15.0	14.90	99.33	
150%	67327	22.5	22.62	100.52	
ATV					

50%	109119	15.0	15.0	100.02	100.37
100%	218219	30.0	30.0	100.01	
150%	330804	45.0	45.48	101.08	

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. The flow rate was varied at 0.9 ml/min to 1.1ml/min. Standard solution 45 & 90 ppm of COBI and ATV were prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%. The method is robust only in less flow condition. The organic composition in the mobile phase was varied from 63% to 87%. Standard solution 22.5 µg/ml and 45 µg/ml of COBI and ATV was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded 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. The results of robustness study were presented in Table-7 (a) &(b).

Table-7(a): Results of robustness

S.No.	Change in flow rate	Cobicistat		Atazanavir sulphate	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	10% less	2736.08	1.53	3910.92	1.31
2	*Actual	2481.63	1.42	3683.48	1.27
3	10% more	2540.88	1.53	3456.84	1.29

Table-7(b): Results of robustness

S.No.	Change in Organic Composition in the Mobile Phase	Cobicistat		Atazanavir sulphate	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	10% less	2732.24	1.54	3884.75	1.29
2	*Actual	2481.63	1.42	3683.48	1.27
3	10% more	2865.60	1.66	4002.13	1.36

Ruggedness

To evaluate the ruggedness (intermediate precision) of the method, precision was performed on different days and different laboratories under the optimized chromatographic conditions. The standard solution was injected into the HPLC system for six times and measured the peak area for all six injections. The %RSD for the area of six replicate injections was found to be within the specified limits and presents in Table-8(a) &8(b).

Table-8(a): Results of ruggedness of the developed method

	COBI		ATV	
	Day-1	Day-2	Day-1	Day-2
Injection	Area	Area	Area	Area
Injection-1	45325	44852	216943	219785
Injection-2	44958	44746	215547	218764
Injection-3	44396	44691	211964	219648
Injection-4	44724	44951	221273	219756
Injection-5	45683	45974	217164	217164
Injection-6	44286	44864	213212	221321
Average	44895.33	45013	216017.2	219406.3
Standard Deviation	540.2	479.6791	3295.9	1373.365
%RSD	1.203183	1.065646	1.525752	0.625946

Table-8(b): Results of ruggedness of the developed method

	COBI		ATV	
	Lab-1	Lab-2	Lab-1	Lab-2
Injection	Area	Area	Area	Area
Injection-1	45134	44973	221534	222468

Injection-2	44985	44891	219756	221648
Injection-3	44258	45134	223485	219485
Injection-4	44981	44796	219891	222891
Injection-5	45974	45486	218463	229463
Injection-6	45167	45347	222485	218485
Average	45083.17	45104.5	220935.7	222406.7
Standard Deviation	548.4835	269.5424	1889.686	3860.605
%RSD	1.216604	0.597595	0.85531	1.735832

ASSAY STUDIES

Standard Solution Preparation: Accurately weigh and transfer 15 mg of Cobicistat & 30 mg of Atazanavir working standard into a 10ml clean dry volumetric flask add Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 1.0 ml of Cobicistat & Atazanavir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1.5 ml of Cobicistat & Atazanavir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Sample Solution Preparation: Accurately weigh and transfer equivalent to 15 mg of Cobicistat & 30mg Atazanavir equivalent weight of the sample into a 10ml clean dry volumetric flask add about 7mL of Diluents and sonicated to dissolve it completely and make volume up to the mark with the same solvent. (stock solution). Further pipette 1 ml of Cobicistat & Atazanavir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1.5 ml of Cobicistat & Atazanavir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure: Injected 20 µl of the standard, sample into the chromatographic system and measured the peak area for the Cobicistat & Atazanavir peaks and calculate the percent of assay and the results were given Table-9.

Table-9: Assay of COBI and ATV in Evotaz formulation

Brand name	Peak name	Peak area of Standard	Peak area of sample	Average weight	Labeled claimed	% Assay*
Evotaz	COBI	44562	44808	882.5	150	100.35
	ATV	217752	219232	882.5	300	100.48

Average of three determinations

1.6 STABILITY TESTING

The objective of stability studies is to determine the percent of the sample found to be stable when it was subjected to different chemical and physical degradation conditions such as acid hydrolysis (0.1N HCl), base hydrolysis (0.1N NaOH), oxidation in the presence of hydrogen peroxide (3% H_2O_2), thermal and radiation decomposition for specified period of time. An amount of the fine tablet powder equivalent to 15 mg of cobicistat and 30 mg of atazanavir sulphate was accurately weighed and transferred into 100 ml of round bottom flask and 50 ml of freshly prepared 0.1 N HCl or 0.1N NaOH was added and kept aside, after 24 hours the solution was filtered through 0.45µfilter into a 100 ml standard flask and neutralized with 0.1N NaOH or 0.1 N HCl respectively and made up to the mark. In case of peroxide degradation, the sample powder equivalent to 15 mg of cobicistat and 30 mg of atazanavir sulphate was added to 50 ml of freshly prepared 3%H_2O_2 and allowed for 24 hours, then filtered, and made up to the mark. Then precisely 1.5 ml of the filtrates were transferred into three different volumetric flasks and diluted to 10 ml with mobile phase. In the study of thermal decomposition or UV degradation an amount of tablet powder equivalent to 15 mg of cobicistat and 30 mg of atazanavir sulphate was accurately transferred into a clean and dry watch glass, placed in an oven which was maintained at 80^oC or UV chamber for 24hrs. Then the watch glass was removed and allowed to cool to room temperature. The substance was accurately transferred into 100 ml volumetric flask and dissolved in diluents and made up to the mark, filtered and about 1.5 ml of this filtrate was diluted to 10 ml with mobile phase. Then freshly prepared working standard solution and degradation solution were injected into chromatographic column, chromatograms were recorded as per the test procedure and percent of degradation was calculated from the area of the peaks of the respective chromatograms. Results of purity angle, purity threshold and percent of degradation were presented in Table-10.

Table-10: Results of study of degradation, purity angle and purity threshold

Degradation condition	Purity angle	Purity Threshold	% Degradation	Purity angle	Purity Threshold	% Degradation
	COBI			ATV		
Acid	0.452	0.762	11.37	0.563	0.986	16.03

Base	0.418	0.643	13.54	0.569	0.929	15.48
Peroxide	0.451	0.701	5.28	0.596	0.896	7.18
Thermal	0.452	0.721	6.36	0.563	0.885	8.51
Photo light	0.453	0.711	7.42	0.566	0.895	10.5
Standard	0.422	0.681	NA	0.519	0.9	NA

NA: Not applicable

RESULT AND DISCUSSION

RP- HPLC method was developed for the assay of Atazanavir sulphate (ATV) and Cobicistat (COBI) simultaneously in pure and dosage forms and to the study of stability of the drug sample under different degradation conditions such as acid, base and peroxide hydrolysis, and photo and light decomposition. Waters HPLC system equipped with auto sampler, Inertsil ODS C 18 (4.6 x 150mm, 5.0µm) column and photo diode array (PDA) was adopted in method development and ultra violet detector was used for method validation. The chromatographic data was obtained by using system software known as Empower-2 software. The optimized chromatographic procedure was obtained by different trails.

The system suitable parameters such as number of theoretical plates, peak area, tailing factor were obtained by injecting exactly 20µl of working standard or sample of concentration 15 µg/ml of ATV and 30 µg/ml of COBI into the HPLC column at ambient temperature, mobile phase, the mixture of 0.1% orthophosphoric acid buffer of pH 5.5 and methanol in the ratio 30:70 v/v was allowed to flow through the column at a flow rate of 1.0 ml/min and the components were detected at a wavelength of 242 nm. The number of theoretical plates, peak area, tailing factor were for COBI and ATV were found to be 2529.34, 44562, 1.9 and 3588.27, 217752.7, 1.20 respectively. The retention time and resolution between the two peaks were found to be 1.994 and 3.325min and 4.58 respectively. The purity of the peaks were evaluated by determining purity angle and threshold angle and found to be 0.422 & 0.681 and 0.519 & 0.864 for COBI and ATV respectively. The values of LOD and LOQ for COBI and ATV were found to be 1.397 & 4.748 µg/ml and 0.820 & 2.759 µg/ml respectively. Linearity between peak area and concentration of COBI and ATV was examined and found to be 7.5ppm to 37.5ppm and 15ppm to 75 ppm of COBI & ATV respectively. Slope, intercept and correlation between peak area and concentration of COBI and ATV were found to be 1986, 0.238, 0.9990 and 4896, 380.9, 0.9990 respectively.

System precision was determined for six replicates of the measurements and %RSD was found to be 1.3175 and 1.5905 for COBI and ATV respectively. Method precision was determined for by preparing working standard solution six times and measured peak area and %RSD was determined and found to be 0.4239 and 0.8362 for COBI and ATV respectively. Accuracy of the developed method was determined at three different concentration levels by injecting standard and sample solutions in triplicate into the column, chromatograms were obtained, calculated the percent of recovery from the amount added and amount recovered and then mean percent of recovery was calculated, and found to be 100.48 and 100.37% for COBI and ATV respectively. As part of the robustness, deliberate change in the flow rate (varied to 0.9 ml/min and 1.1ml/min) and mobile phase composition (organic composition in the mobile phase was varied to 63% and 87%) variation was made to evaluate the impact on the method. System suitable parameters were evaluated for each variation and found to be significantly not different. Ruggedness of the method was determined on different days and different laboratories under the optimized chromatographic conditions by injecting standard solution into the HPLC system for six times and measured the peak area, and %RSD was calculated for the six replicate injections was found to be less than 2.0

Assay of COBI and ATV was found to be 100.35 and 100.48 % respectively. The stability of the drug sample under different degradation conditions was determined and found to be within the range of 86.46 to94.72 and 83.97 to 92.82 for COBI and ATV respectively. The developed method was found to be simple, rapid and applied for the analysis of drug samples, therefore the proposed method is recommended for the analysis of COBI and ATV in pure and tablet dosage forms in any quality control laboratories.

CONCLUSIONS

The developed HPLC method was found to be simple, selective and sensitive. The developed method was adopted for the determination of assay of pharmaceutical formulations and extended to study the was the degradation under different forced degradation conditions, hence the reported method was suggested to use in any quality control laboratories for the studies of quality control of cobicistat (COBI) and atazanavir sulphate (ATV) in pure and pharmaceutical formulations.

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