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A Novel Stability Indicating RP-HPLC Method for Simultaneous Estimation of Atazanavir and Cobicistat in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

Reverse phase high performance liquid chromatographic method has been focused with the aim of reducing analysis time and maintaining good efficiency. The present work describes a rapid, simple, precise, selective stability indicating RP-HPLC method for simultaneous estimation of Atazanavir and Cobicistat in tablet dosage forms. A Phenomenix C8 column of 250 × 4.6 mm internal diameter, 5 µm particle size with mobile phase consisting of O.1 M ammonium acetate and methanol of HPLC grade in the ratio of 1:1 was used. The flow rate was maintained at 1.2 ml/min. The effluents were monitored at 268 nm. The detector response was found to be linear over a concentration range of 30-90 mcg for atazanavir and 15-45 mcg for cobicistat. The retention times of atazanavir was found to be 3.576 min and Cobicistat 6.592 min. The correlation coefficients of atazanavir and cobicistat was 0.999 with equation y=43363x and y=59029x. Recovery of the method was 98-102%. According to ICH guidelines, the newly developed method has been validated in terms of accuracy, specificity, linearity, precision and robustness. Inorder to demonstrate the stability indicating power of the developed method forced degradation studies were performed for atazanavir and cobicistat by subjecting them to stress conditions including acidity, alkalinity, oxidation, photolysis and thermal degradation. The described method can be successfully employed for the quality control analysis of Atazanavir and Cobicistat in formulations.

Keywords: Atazanavir; Cobicistat; RP-HPLC method development; Validation; Gradient; Stability indicating

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INTRODUCTION

Atazanavir [1] Sulphate (ATV), chemically is (3S, 8S, 9S, 12S)-3,12-Bis(1,1-dimethyl ethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13-pentaazatetrade anedioic acid dimethyl ester; 1-[4-(pyridine-2-yl)phenyl]-5S,2,5-bis[[N-(methoxycarbonyl)-L-tertleucinyl]amino]-4Shydroxyl-6-phenyl-2azahexane.
It is an oral antiretroviral Protease inhibitors used in the treatment of HIV/AIDS .This drug is official in Indian Pharmacopoeia. The U.S.FDA approved atazanavir on June 20, 2003. Atazanavir 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. It may also not be cross-resistant with other PIs. When boosted with ritonavir it is equivalent in potency to lopinavir for use in salvage therapy in patients with a degree of drug resistance (Figure 1).

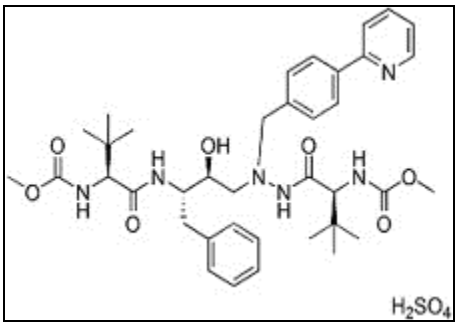

Figure 1: Structure of Atazanavir

Cobicistat [2] is a potent inhibitor of CYP450 3A enzymes, including the important CYP3A4 subtype. It is chemically described 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. Its molecular formula is C40H53N7O5S2. When administered as a fixed dose combination tablet (Elvitegravir 150 mg, Emtricitabine 200 mg, Tenofovir 300 mg, Cobicistat 150 mg) in healthy volunteers, Cobicistat's AUCinf and Cmax each increases 3% with a light meal,and decreases 17% and 24% respectively with a high-fat meal. Cobicistat is a licensed drug for use in the treatment of infection with the human immunodeficiency virus (HIV) (Figure 2).

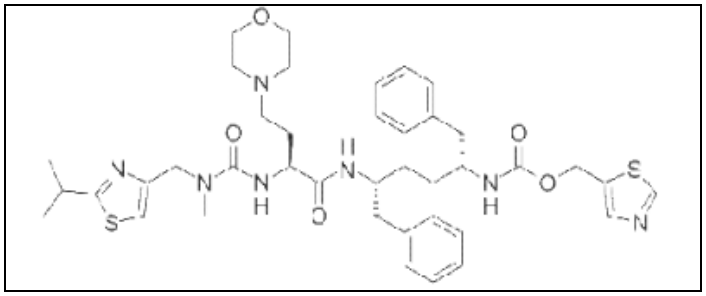

Figure 2: Structure of Cobicistat

MATERIALS AND METHODS

Atazanavir and Cobicistat gift samples were kindly gifted by Rainbow laboratories Hyderabad, India and used as reference standard without further purification. Methanol and sodium dihydrogen phosphate of analytical grade were procured from Qualigens. Hydrochloric acid, ammonium acetate and sodium hydroxide were procured from S.D. Fine Chemicals. Purification of all solvents and solutions were done by using membrane filter and degassed before use by using ultrasonicator. All other solvents used were of HPLC and reagents were of analytical grade.

Instruments and chromatographic conditions

High performance liquid chromatography model [3] of waters equipped with PDA detector is used for integration and processing of chromatograms. A reverse phased HPLC C8 column of dimension 250 × 4.6 mm, 5 µm was used as analytical column. All analysis was done at ambient temperature (30°C). Mobile phase used for the analysis is a mixture of 0.1 M ammonium acetate and methanol in the ratio of 50:50. Flow rate was maintained at 1.2 ml/min. Volume of injection is 10 µl. Before using all the solutions mobile phase was sonicated for 30 min and uv detection was performed at 268 nm.

Preparation of standard stock solutions [4]

Atazanavir standard stock solution:

300 mg of atazanavir was transferred to the 100 ml volumetric flask and the volume was made up to the mark with mobile phase**.**

Cobicistat standard stock solution:
In a 100 ml volumetric flask 150 mg of cobicistat was taken and final volume was made by mobile phase upto the mark. Std stock solutions of atazanavir and cobicistat were diluted appropriately with mobile phase to obtain conc. 60 µg/ml and 30 µg/ml.

Preparation of sample
20 tablets were taken and weight equivalent to 300 mg of ATZ and 150 mg of CBT was taken in a 100 ml volumetric flask and dissolved by sonication in mobile phase and the volume was made upto 100 ml. 2 ml of this solution was diluted to 100 ml with mobile phase to get the desired concentration.

Method development
Various mobile phases containing methanol, water, acetonitrile, and glacial acetic acid in different ratios were tried with different flow rates [5]. The aliquot portions of std stock solutions of atazanavir and cobicistat were diluted appropriately with mobile phase to obtain conc. 60 µg/ml and 30 µg/ml. Diluted concentrations of both the drugs were injected into column and elution pattern and resolution of both drugs were studied. They were scanned in the range of 200 – 400 nm by UV spectroscopy and the optimization was compared. Among the various wavelengths 268 nm was found satisfactory since the detection of drugs with adequate sensitivity was observed (Table 1).

Table 1: Optimised chromatographic conditions

Parameter	Optimized conditions
Instrument	HPLC
Column	Phenomenex (250 × 4.6 mm, 5 µm, c8)
Detector	PDA
Wave length	268 nm
Mobile phase	0.1 M ammonium acetate and methanol (50:50)
Flow rate	1.2 ml/min
Injection volume	10 µl
Temperature	30°C

Assay
20 µl of standard and sample solutions were injected into the chromatographic system and the area of peaks foe atazanavir and cobicistat were measured and % assay was calculated using the formula.

$$\%\ \text{Label claim} = \frac{Wt\ of\ Asp}{Ast} \times \frac{Dsmp}{Dstd} \times \frac{std}{Wt\ of\ Sample} \times \frac{Avg.wt}{Lc} \times P$$

Where, Asp: Area for sample solution; Ast: Area for standard solution; Dst: Dilution factor for standard; Dsp: Dilution factor for sample; Lc: Label claim; A: Average weight; P: Purity of STD

Method validation
As part of method validation as per ICH guidelines, the following parameters are studied [6].

1. System Suitability and System Precision
2. Specificity Studies
    a. Blank Interference
    b. Placebo Interference
    c. Forced degradation studies in different stress conditions to establishing stability indication of the developed method
3. Method Precision
4. Accuracy studies
5. Linearity Studies including LOD/LOQ determination
6. Ruggedness
7. Robustness
8. Analysis of Marketed samples by applying the developed method

System suitability [7]:
System suitability was performed daily during the entire validation period of this method. The results of the system suitability were presented.

Accuracy
The accuracy of the method indicates the closeness between the observed value and the expected value found. Standard addition method was adopted for the accuracy studies. Accuracy of the method was determined by applying the anticipated method for drug mixture containing known amount of atazanavir and cobicistat to 50%, 100% and 150% of the label claim (Tables 2-4) (Figures 3-5).

Table 2: System suitability parameters

S No	Parameters	Atazanavir	Cobicistat
1	Working con	60 µg/ml	30 µg/ml
2	Tailing factor	1.3	1.22
3	Peak area	2005866	5904800.2
4	Retention time	3.564	6.581
5	Theoritical plates	5010	6946
6	Resolution factor	11.4	
7	Y-intercept	0	0
8	Slope	43363	59029
9	Regression coeffecient	0.999	0.999
10	LOD	0.148	0.0388
11	LOQ	0.492	0.1294

Table 3: Recovery result for Atazanavir

% level of sample	Wt of sample	Area at 268 nm	Amount added (µg/ml)	Amount recovery (µg/ml)	% Recovery	Mean recovery
50%	298.2	1002396	30	29.71	90.03	99.07
		1003154	30	29.74	99.13	
		1002740	30	29.72	99.06	
100%	596.4	2006771	60	59.49	99.15	99.05
		2004542	60	59.42	99.03	
		2003343	60	59.39	98.98	
150%	894.6	3006028	90	89.11	99.01	99
		3005179	90	89.08	98.97	
		3006353	90	89.12	99.02	

The accuracy was then deliberated as the percentage of analyte reobtained by the assay.
The % Recovery was then calculated by using formula:

$$\text{mg recovery} = \frac{Asp}{Ast} \times \frac{Dst}{Dsp} \times \frac{P}{100}$$

Table 4: Recovery result for Cobicistat

% level of sample	Wt of sample	Area at 268 nm	Amount added (µg/ml)	Amount recovered (µg/ml)	% recovery	Mean Recovery
50%	298.2	2955228	15	14.97	99.8	99.77
	298.2	2954493	15	14.97	99.8	
	298.2	2952413	15	14.96	99.73	
100%	596.4	5907090	30	29.92	99.73	99.69
	596.4	5905003	30	29.91	99.7	
	596.4	5903601	30	29.9	99.66	
150%	894.6	8858015	45	44.87	99.71	99.69
	894.6	8855018	45	44.85	99.66	
	894.6	8857500	45	44.87	99.71	

$$\% \text{ recovery} = \frac{Mg\ Recovered}{Mg\ Added} \times 100$$

Precision

The precision of each method was ascertained separately from peak area obtained by acual determination of six replicates of fixed amount of drug (50 µg/ml). Relative standard deviations for atazanavir and cobicistat were calculated. Intraday and inter day variation in the peak areas of both the drugs were also calculated (Table 5).

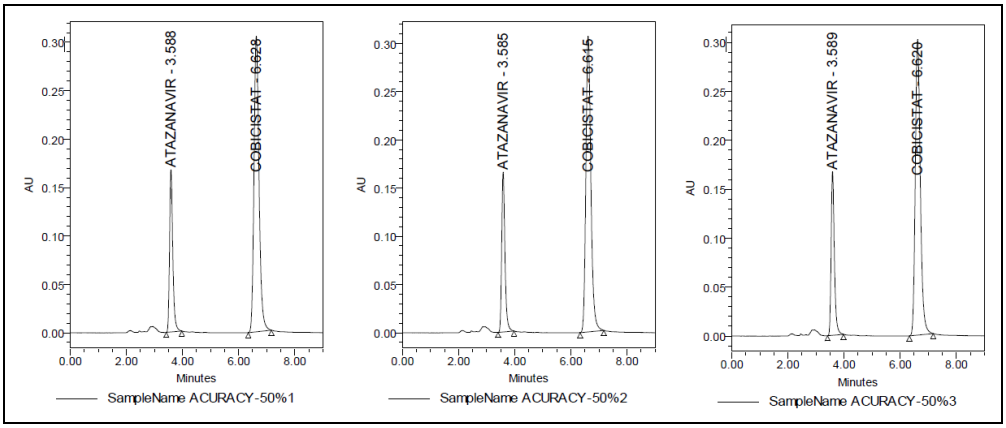

Figure 3: Accuracy chromatograms for 50% solution

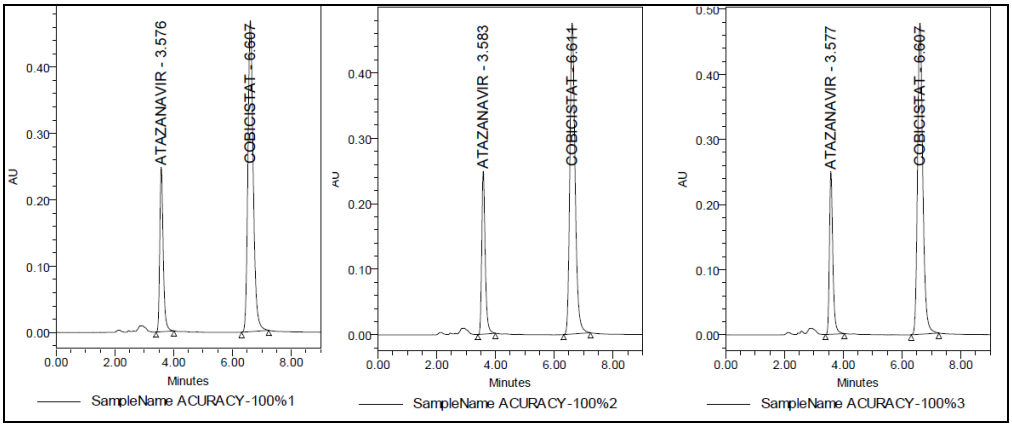

Figure 4: Accuracy chromatograms for 100% solution

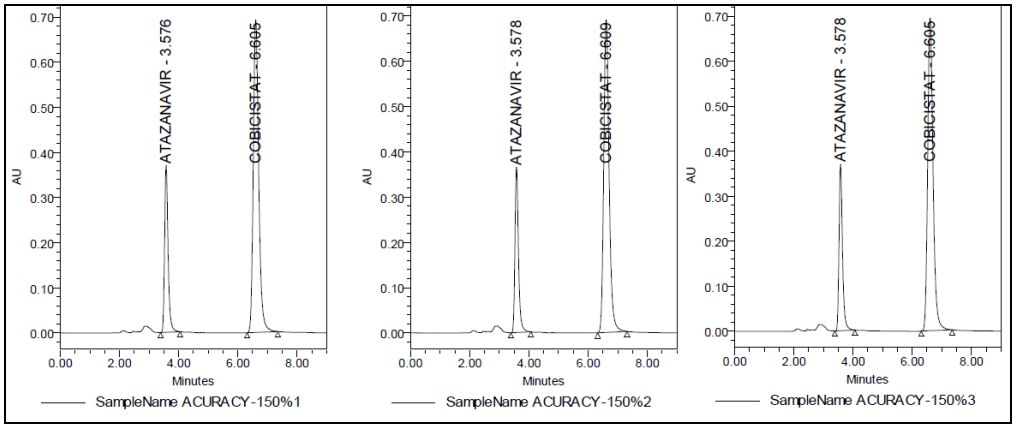

Figure 5: Accuracy chromatograms for 150% solution

Linearity [8]

The linearity of the method is to generate same results which are directly proportional to the concentration of the analyte in samples. Linearity of the method was further evaluated at five concentration levels by diluting the standard solutions to obtain diluted solutions over the ranges 30-90 (µg/ml). The linearity of detector response was

verified by injecting diluted solutions in the concentration range of 30-90 µg/ml for Atazanavir and 15-45 µg/ml for cobicistat. Observations are tabulated in Tables 6 and 7 (Figures 6 and 7).

Table 5: Precision study of Atazanavir and Cobicistat

Injection replicates	Peak area of ATZ sample	Peak area of ATZ (STD)	Peak area of Cobicistat Sample	Peak area of Cobicistat STD
1	2005898	2007343	5905038	5901489
2	2003185	2006255	5903639	5896431
3	2008028	2003539	5905315	5892992
4	2008797	1999397	5904290	5909607
5	2008520	2012748	5907962	5927494
6	2004923	2007392	5902053	5897477
Mean	2006558.5	2005866.2	5904716.17	5904800.2
Standard Deviation	3429.285	6567.082	3139.0905	23807.978
%RSD	0.17	0.3	0.05	0.4

Table 6: Linearity study for Atazanavir

S No	Concentration of drug(µg/ml)	Peak Area
1	30	1002462
2	45	1504073
3	60	2005565
4	75	2507658
5	90	3008048

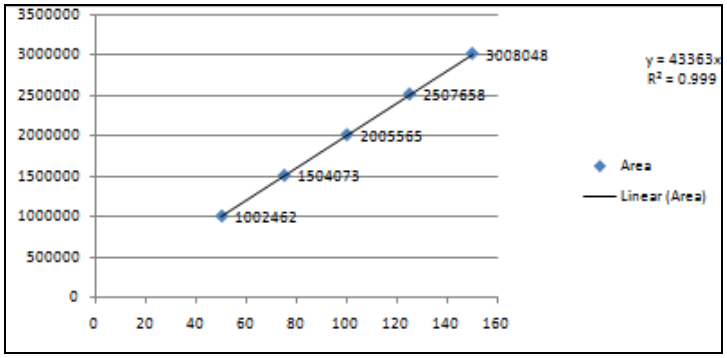

Figure 6: Calibration plot for Atazanavir

Table 7: Linearity study for Cobicistat

S No	Concentration of drug(µg/ml)	Peak area
1	15	2912754
2	22.5	4418718
3	30	5904959
4	37.5	7381425
5	45	8867710

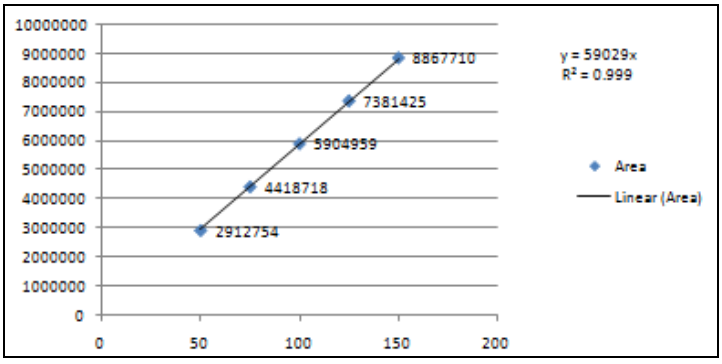

Figure 7: Calibration plot for Cobicistat

Robustness

The robustness of the method was determined to check the reliability of analysis [9]:

The tablet sample of atazanavir and cobicistat was analyzed using proposed method after deliberate variations in method parameters.

The typical variations are:

Variation in flow rate by ±0.1 ml/min

Variation in temperature by ±5°C

The results were tabulated as below in Table 8.

Table 8: Robustness study

Factors	Atazanavir (RT min)	Cobicistat (RT min)
Flow rate (ml/min)		
1.0 ml	4.341	8.075
1.4 ml	3.124	5.8
Temperature		
25ºC	4.331	8.07
35ºC	3.114	5.789

Specificity

Placebo interference study:

Inorder to make out the interference by excipients, Std. solution, marketed formulation the placebo sample was analyzed by the developed method (Figures 8 and 9).

Placebo preparation:

Accurately 146.4 mg of placebo was weighed and transferred into a 100 ml volumetric flask and dissolved by sonication in sufficient mobile phase then volume is made upto 100 ml. 2.0 ml of this solution is diluted to 100.0 ml with mobile phase and mixed. The solution is filtered and the clear filtrate is injected.

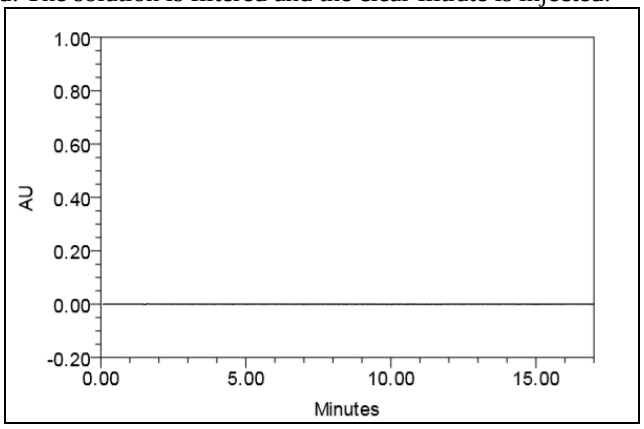

Figure 8: Chromatogram for blank

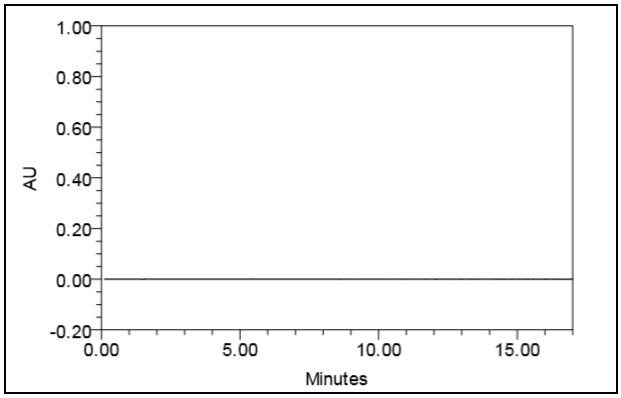

Figure 9: Chromatogram for placebo

Forced degradation studies (Specificity)
Forced degradation of the drug was performed as per the ICH guideline in acidic, alkaline and peroxide media and exposing the drug to heat and sunlight conditions.

Acid degradation:
596.4 mg of sample was transferred into a 100 mL of volumetric flask to this 10 ml of 0.1 N HCl and 50 mL of mobile phase was added and was sonicated for 30 minutes. The sample was neutralized using 10 mL of 0.1 N NaoH. Final volume was made up to 100 ml with mobile phase. 2 ml of the above solution was transferred to 100 ml volumetric flask and diluted upto the mark with mobile phase.

Base degradation:
596.4 mg of sample was transferred into a 100 ml of volumetric flask containing 10 ml of 0.1N NaOH and 50mL of mobile phase and sonicated for 30 minutes. 10 ml of 0.1N HCl was added to neutralize the sample. The volume was made up to the volume with mobile phase. 2 ml of the above solution was transferred to 100 ml volumetric flask and diluted the volume to the mark with mobile phase (Figure 10).

Peroxide degradation:
596.4 mg of sample was transferred into a 100 ml of volumetric flask containing 10 ml of peroxide and 50 mL of mobile phase and sonicated the solution for 30 minutes and made up to the volume with mobile phase. 2 ml of the above solution was transferred to 100 ml volumetric flask and diluted the volume to the mark with mobile phase (Figure 11).

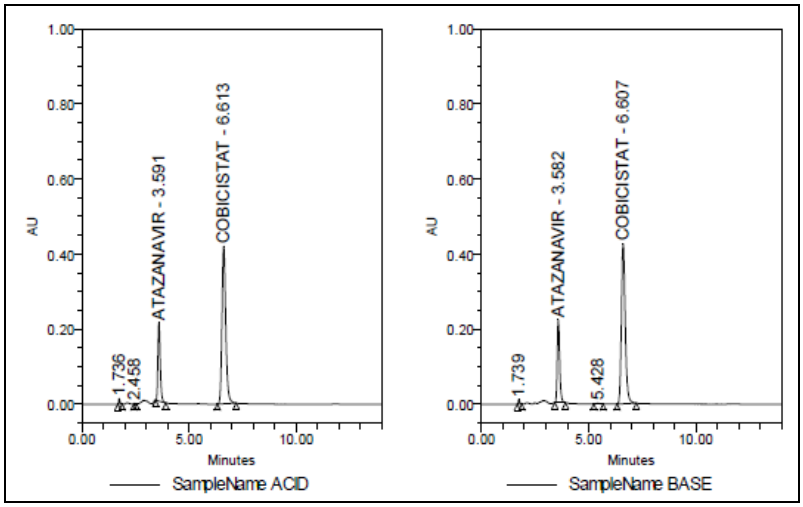

Figure 10: Chromatogram of acid and base stressed degradation of drug product

Heat degradation:
Before weighing the sample, the sample was exposed to 105°C for 30 minutes. 596.4 mg of sample was transferred into a 100 ml of volumetric flask containing 60 ml of mobile phase and sonicated the mixture for 30 minutes. The solution was made up to the mark with the mobile phase. 2 ml of the above solution was transferred to 100 ml volumetric flask and diluted the volume to the mark with mobile phase (Figure 12).

Light degradation [10]:
Before weighing the sample, the sample was exposed to sun light for 24 hours. 596.4 mg of exposed sample was transferred to a 100 ml of volumetric flask. 60 ml of mobile phase was added and sonicated the solution for 30 minutes. The solution in the flask was made up to the mark with mobile phase 2 ml of the above solution was transferred to 100 ml volumetric flask and diluted the volume to the mark with mobile phase.

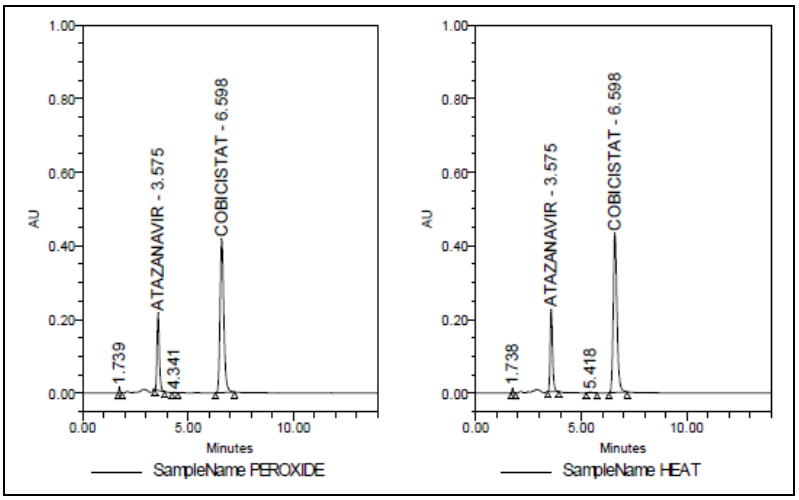

Figure 11: Chromatogram of heat and peroxide stressed degradation of drug product

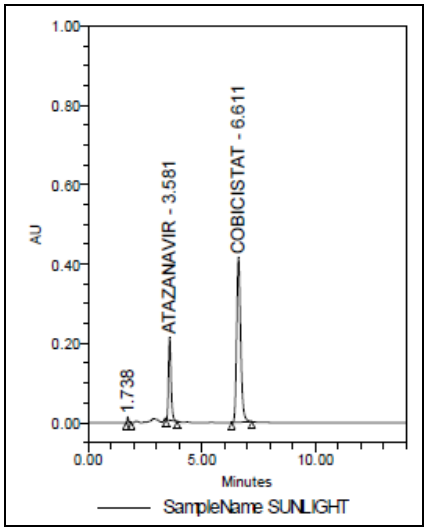

Figure 12: Chromatogram of sunlight stressed degradation of drug product

RESULTS

The retention time of Atazanavir sulphate was 3.564 min and cobicistat 6.581 min. The result demonstrated with the detector response was found to be linear in the concentration range of 30-90 µg/ml for atazanavir and 15-45 µg/ml for cobicistat. The developed method has been validated according to ICH guidelines and shown to be specific, sensitive, precise, linear, accurate, rugged, robust and fast. Atazanavir sulphate and cobicistat were subjected to different accelerated stress conditions and the obtained degradation products, were well resoluted from the pure drug with notably different retention time values.

DISCUSSION

In the present research an effort has been made to develop stability indicating assay method for simultaneous estimation of Atazanavir and Cobicistat by RP- HPLC.

The current experimental study was divided into three parts:

I. Development of RP-HPLC method for simultaneous estimation of Atazanavir and Cobicistat.
II. Validation of the developed method according to ICH guidelines.

III. Forced degradation studies by exposing the sample to different stress conditions.

Development of RP-HPLC method for simultaneous estimation of atazanavir and cobicistat

- The separation was done on PHENOMENEX C8 column (250 mm × 4.6 mm × 5 µm).
- The mobile phase ammonium acetate and methanol (50:50) was found to be more satisfactory because of good resolution of drugs with markedly symmetrical sharp peak.
- A detection wavelength 268 nm was selected.
- This system gave good resolution and optimum retention time with appropriate
- Tailing factor (< 2).
- The total run time of chromatogram was about 10.5 min.
- The observed numbers of theoretical plates were more than 2000 signifying high column efficiency.
- The detector response was found to be linear.
- Assay has been done by optimized HPLC methods.

Validation of the proposed method

Accuracy:

By selecting over the range of 50, 100 and 150% of labeled claim the recovery studies were conducted and results were found within the range 98-102% demonstrating the accuracy of the method**.**

Precision:

Duplicate estimations of Atazanavir and Cobicistat in tablet by projected method have produced quite contemporaneous results proposing the repeatability of the method.

Specificity:

In a view to assertain how accurately and specifically, the analyte of interest can be estimated in presence of other components specificity of the method was determined.

Linearity and range:

Graphs plotted with detector response as a function of labeled claim shown a linear relationship over 50 - 150% of labeled claim. This indicates the capability of method to estimate the drug accurately over a wide range.

Robustness:

The robustness of the method was resoluted to ensure the reliability of analysis. The tablet sample of atazanavir and cobicistat was analyzed using proposed method after purposeful variations in method parameters.

The typical variations are:

Variation in flow rate by ±0.1 ml/min

Variation in temperature by ±5ºC

Stability of analytical solution:

The standard and sample solutions of drugs have shown remarkedly good stability over a period of about 12 hrs.

Forced degradation study

- By exposing the DP to 0.1 N HCl, 0.1 N NaOH and 3% H_2O_2 respectively in dark at room temperature for 8 hrs the acid, alkali and oxidative stress studies were performed.
- Thermal degradation was performed by exposing the DP to 80°C for 8 hrs.
- Photo degradation was attempted by exposing the DP to direct UV radiation for 24 hrs in UV chamber.
- Stressed samples withdrawn at regular intervals and were chromatographed under optimized conditions.
- To resolve the degradants from each other and also from the parent drug, HPLC method was developed.

CONCLUSION

The developed RP–HPLC method was found to be suitable for the analysis of atazanavir sulfate and cobicistat in bulk form and in pharmaceutical dosage forms and was found to be simple, reliable, sensitive, economical and precise.

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