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A NOVEL ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTAENEOUS ESTIMATION OF ATAZANAVIR AND COBICISTAT BY USING RP-HPLC

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ABSTRACT

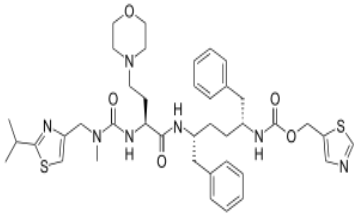
The present study is aimed to develop a simple, accurate, precise method was developed for the simultaneous estimation of the Atazanavir And Cobicistat dosage form by using RP-HPLC. Drugs were run through Xterra150,C_{18} (4.6mm, 5µ) column. Mobile phase containing Potassium dihydrogen Ortho phosphoric acid Methanol taken in the ratio 35:65 pumped through column at a flow rate of 0.8ml/min. Buffer used in this method was Potassium di hydrogen ortho Phosphate ortho phosphoric acid. Temperature was maintained at 25°C. Optimized wavelength for Atazanavir And Cobicistat 260 nm. Retention time of Atazanavir And Cobicistat were found to be 2.536min and 3.266 min. %RSD of the Atazanavir And Cobicistat were and found to be 1.52and 1.58 respectively. Linearity of the method was in the concentration range of 50-150% for Atazanavir and Cobicistat. % Recovery was Obtained as 100.0% for Atazanavir And Cobicistat respectively. The percentage RSD for precision of the method was found to be less than 2%. LOD, LOQ values were obtained from regression equations of Atazanavir And Cobicistat were 2.95ppm, 3.04ppm and 9.87ppm, 10ppm respectively. Regression equation of Cobicistat is y = 26773x + 2906, and of Atazanavir is y = 9958.7x + 959.67. Regression co-efficient of Cobicistat was 0.999.Regression co-efficient of Atazanavir was 0.999.The method was validated according to ICH guidelines.

KEYWORDS: Atazanavir, Cobicistat, Methanol, potassium dihydrogen ortho phosphate.

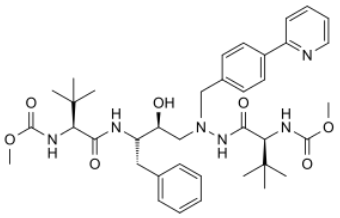
INTRODUCTION

Atazanavir is the anti-HIV agents, HIV protease inhibitors. Atazanavir selectively inhibits the virus –specific processing of viral Gag and Gag-polpolyproteins in HIV-1 injected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions. Atazanavir is not active HIV-2.Chemically it is methy N-[(1S)-1-{[(2S,3S)-3-hydroxy-4-[(2S)-2-[(methoxycarbonyl)amino]-3,3-dimethyl-N'-{[4-(pyridin-2-yl)phenyl} butanehydrazido] -1-phenylbutan-2-yl]carbomoyl}-2,2-dimethylpropyl][1] carbamate. Cobicistat is Thizol-5-ylmethyl-N- [1-benyl-4[[2-[[(2-isopropylthiozol -4-yl)- methyl-methyl-carbomyl] amino]-4-morpholino-butanoyl]amino]-5-phenylphenyl]carbamate.Cobicistat is a licensed drug for use in treatment of infections with human immunodeficiency virus(HIV). Cobicistat is of interest for its ability to inhibit liver enzyme that metabolise other medications used to treat HIV, notably elvitegravir, an HIV integrase Inhibitor. By combining cobicistat with elvitegravir, higher concentrations of the later are achieved in the body with lower dosing, theoretically enhancing elvitegravir's viral suppression while diminishing its adverse side-effects. Literature survey revealed HPLC,[2-6] UV, HPTLC methods for the estimation of Atazanavir and Cobicistat. The present study aims to develop simple, accurate, precise and selective RP-HPLC[10]assay procedure for the analysis of Atazanavir[7] and Cobicistat[7] in bulk drug samples and in combined dosage. The method is optimized and validated as per the International Conference on Harmonization (ICH) guidelines.[8]

Structure of Atazanavir



Structure of cobicistat



MATERIALS AND METHODS

Quantitative HPLC was carried out by using waters 2695 HPLC alliance connected with PDA Detector Waters 996 and Empower Software. Analytical column was Xterra C18 5µm (4.6*150mm). Lab India UV- double beam spectrophotometer **(UV3000)** and UV win 5 software, digital weighing balance **(BSA224SCW),** PH meter **(AB102U)**, ultra Sonicator **(SE60US)**, suction pump**(VE115N)** were used.

Pharmacetical grade Atazanavir and cobicistat (EVOTAZ) were obtained from SL drugs and pharmaceuticals, Hyderabad, India Methanol, Water, Acetonitrile were of HPLC grade and purchased from MERCK. Potassium dihydrogen ortho phosphate was analytical reagent grade supplied by MERCK.

Preparation of phosphate buffer

Weighed 0.50 grams of KH_2PO_4 and 0.301 grams of potassium dihydrogen phosphate was taken into a 1000ml beaker and diluted to 1000ml with HPLC water, adjusted the PH to 7 with ortho phosphoric acid.

Preparation of mobile phase

A mixer of PH 7 phosphate buffer 300 ml (30%), 700 ml of MEOH (70%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45µ filter under vaccum filtration.

Preparation of diluent

Mobile phase is used as diluent.

Selection of column

Column is selected based on solubility, polarity and chemical differences among analytes [column: Xterra C18(4.6* 250mm,5µm(WATERS).

Preparation of Standard solutions

Accurately weighed 10mg of Cobicistat and 10mg of Atazanavir were dissolved in mobile phase and transformed into a 10 ml clean volumetric flask and about 2 ml of DMF is added then it is sonicated to dissolve it completely and made volume up to the mark with diluent.

Preparation of sample solutions

Accurately 10 tablets are weighed and crushed in mortar and pestle and weigh equivalent to 10 mg of Atazanavir and Cobisistat (marked formulation) sample into a 10ml clean dry volumetric flask and about 7ml of diluents is added and sonicated to dissolve it completely and made volume up to the mark with the same solvent.(stock solution) further 3ml of above stock solution was pipetted in to a 10ml volumetric flask and diluted up to the mark with diluent

Method validation

The proposed method was validated as per ICH guidelines.[9]

Specificity (forced decomposition studies)

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank. The chromatograms are shown in the table.

The specificity test was performed for Cobicistat and Atazanavir. It was found that there was no interference of impurities in retention time of analytical peak.

Precision

The precision of the method verified by repeatability and by intermediate precision Repeatability was checked by injecting five individual preparations of Atazanavir and Cobicistat real sample (tablets). The % RSD for the area of five replicate injections was found to be within the specified limits. The % RSD for the area of five standard injections results should not be more than 2 The Method precision study was performed for the % RSD of Cobicistat & Atazanavir was found to be 1.33 & 1.76 (NMT 2).The intermediate precision of the method was also evaluated using different analyst and performing the analysis on different days and by using different make columns of same dimensions.

Linearity

Linearity test solutions for the assay method were prepared from Atazanavir & Cobicistat stock solutions at five concentrations levels from 50 -150% of assay analyte concentrations. Linearity test solutions for the method were prepared by diluting stock solution to the required concentrations.

Accuracy

Accuracy of the assay method was evaluated in triplicate using three concentration levels 50,100 and 150µg/ml on real sample (tablets). Standard addition and recovery experiments were conducted on real sample to determine accuracy the method. The percentage of recoveries for Atazanavir and Cobosistat were calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the system suitability parameters were evaluated. Tailing factor for Atazanavir and Cobicistat was recorded. The flow rate of the mobile phase was 0.8ml/min, to study the effect of floe rate on the retention time, flow was changed by ± 0.2 units from 0.6 to 1.0 ml/min. The effect of the column temperature on retention time was studied at ambient temperatures.

FORCED DEGRADATION STUDIES

1. Acid degradation: Accurately weigh 5 mg standard drug and transfer into 25 ml volumetric flask add 1ml of 1 N Hydrochloric acid and was placed on a water bath maintained at 60º C for 60 min. Then it was cooled to room temperature & make up to the mark with mobile phase. Above 2 ml of above solution was transferred into 5 ml volumetric flask & made up to the volume with mobile phase. With draw 20µl of solution and injected into HPLC system to record the chromatograms.

2. Base Degradation: Transfer 5mg of sample standard stock and add 1ml of 1 N 25ml sodium hydroxide to volumetric flask and was placed on a water bath maintained at 60ºC for 60 min. Then it was cooled to room temperature & make up to the mark with mobile phase. The resultant solution was diluted to obtain test concentration and injected into the system and the chromatograms were recorded to evaluate the degradation of the sample.

3. Oxidation Degradation: Transfer 5mg of sample stock preparation and add 0.5ml of 3% w/v hydrogen peroxide (H202) to 25 ml volumetric flask and maintained at 60º C for 60 min in a water bath. For RP-HPLC study, the resultant solution was diluted to obtain the test concentration with diluents and injected into the system and chromatograms were recorded to evaluate the degradation.

4. Photolytic Degradation: The photochemical stability of the drug was studied by exposing the sample powder and sample placed under the UV light for 254 nm for 8 hours by keeping the

beaker in photo stability chamber. The resultant solution was diluted to obtain the test concentration with diluents and injected into HPLC system and chromatograms were recorded.

5. Heat Treatment: About 5mg of standard drugs were accurately weighed to a 25 ml volumetric flask and was placed on a water bath maintained at 105℃ for 6 hours. Then it was cooled to room temperature. About 20µl of above solution was injected into HPLC system to record the chromatograms.

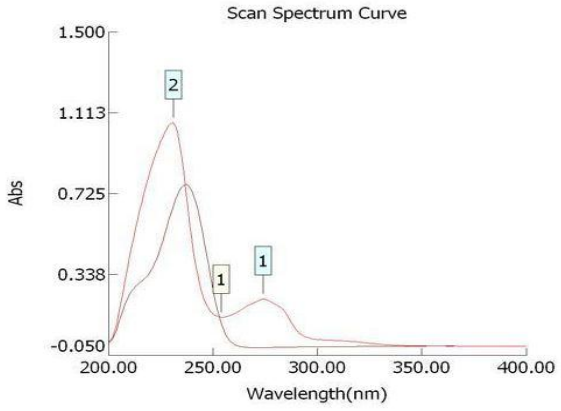
RESULTS AND DISCUSSION

The absorption wavelength for Atazanavir and Cobicistat is determined after several trials. The absorbance spectra of the diluted standard and working solutions of Atazanavir and Cobicistat in methanol are recorded on a UV spectrophotometer. They are scanned in the wavelength 200nm - 400nm range using quartz cuvettes with 10mm path length. The maximum absorbance wavelength was observed at 255nm for two drugs. This is the good agreement with reported wavelengths for these drug combination.

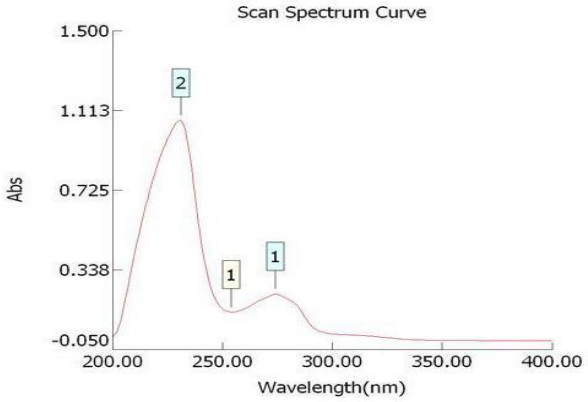
Data of forced degradation studies

Atazanavir and Cobicistat was found to degrade significantly in acid hydrolysis and in base hydrolysis and mild degradation was observed in UV and peroxide stress conditions. The following figure shows that Spectrum of Atazanavir & Cobicistat. Photodiode array detector was employed to check ensure the homogeneity and purity of Atazanavir and Cobicistat peak in all the stressed sample solutions. Assay studies were carried out for stress samples against Atazanavir and Cobicistat qualified working standard. The results are presented in Table no 1. The purity and assay of Atazanavir and Cobicistat was unaffected by the presence of its degradation products and thus confirms the stability indicating power of the developed method.

(a) Tablet sample of Cobicistat



(b) Tablet sample of Atazanavir



(c) Representative chromatograms of standards of Atazanavir and Cobicistat

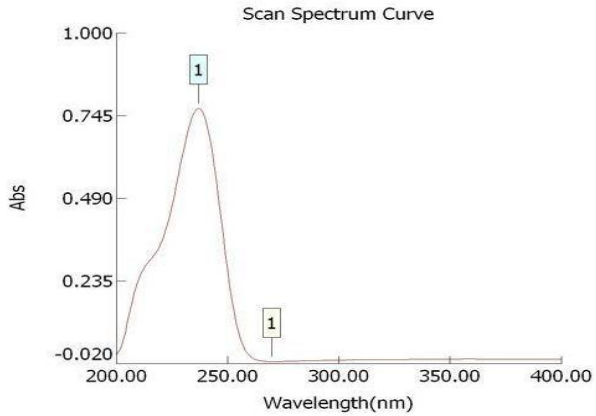


Table 1: Data of forced decomposition studies.

Nature of the Sample	Sample Area	% Assay (Atazanavir)	Sample Area	% Assay (Cobicistat)
Acid	11508045	100.96	1943375	105.32
Base	11574887	101.72	1928103	101.24
Oxidative	11601993	102.03	1992047	100.88
Photolytic	11584887	101.84	2006340	101.01
Water	11237957	107.89	1918103	100.21
Acceptance criteria: There should not be any interference peaks at the analyte Rt.				

Precision

The % RSD during the method precision study of Atazanavir was 2.52 and 1.52% for retention time and peak area respectively. For Cobicistat the retention time was 3.252 and peak area was 1.58 respectively. The % RSD for the area of Atazanavir and Cobicistat were well within 2% conforming good precision of the method. The % RSD values are presented in Table 2.

Table 2: Precision data.

S. NO	ATAZANAVIR		COBICISTAT	
	RT	AREA	RT	AREA
INJECTION 1	2.506	1553631	3.230	2790868
INJECTION 2	2.516	1508002	3.239	2661482
INJECTION 3	2.519	1545624	3.246	2706096
INJECTION 4	2.531	1542374	3.257	2703419
INJECTION 5	2.544	1561368	3.271	2695932
INJECTION 6	2.541	1578456	3.271	2711560
MEAN	2.52	1548242.50	3.252	2711559.50
Std.Dev 23557.44			42750.28	
% RSD 1.52			1.58	

Linearity

Linear calibration plot for above method was obtained over the calibration range 50 to 150 µg/ml. The results show that excellent correlation existed between the peak area and concentration of the analyte.

Linearity of Cobicistat				
S.NO	**Linearity Level**	**Conc. PPM**	**RT**	**Area**
1	50%	10	2.309	1810101
2	75%	20	2.322	2044287
3	100%	30	2.324	2367133
4	125%	40	2.336	2602279
5	150%	50	2.345	2869778
Linearity of Atazanavir				
1	50%	20	4.307	1164173
2	75%	40	4.317	1342535
3	100%	60	4.323	1555931
4	125%	80	4.340	1777973
5	150%	100	4.340	1942319
Acceptance criteria: The correlation coefficient (R^2) should be not less than 0.999.				

Accuracy

Accuracy was determined by analyzing a sample of known concentration (reference standard solutions) and comparing the measured value with the true value and using the method of standard additions. A table 3 summarizes the accuracy results expressed as percent recovery. The method showed good recovery.

Table 3: Results of Accuracy.

% Concentration (at specification level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	1717685	5	5.1	101.2%	100.0%
		5	5.0	101.3%	
		5	5.1	101.4%	
100%	3472797	10	9.93	99.2%	
		10	9.94	99.4%	
		10	9.92	99.3%	
150%	5224472	15	14.6	99.0%	
		15	14.8	99.2%	
		15	14.7	99.1%	

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage in order to perform the robustness study of the proposed method deliberate modifications in flow rate and column temperature were made. The results are shown in Table 4. It can be seen that every employed conditions, the chromatographic parameters are in accordance with established value. A change of ± 0.2 unit of flow rate and column temperature had no impact on chromatographic performance (Table 4). According to the data of robustness test study proposed criteria for system suitability test [tailing factors, theoretical plates number and repeatability (R.S.D)]. It is used to verify that the repeatability of the system are adequate for the analysis intended.

Table 4: Results of Robustness.

Parameter	Atazanavir			Cobicistat		
	RT	Theoretical Plates	Asym metry	RT	Theoretical plates	Asymmetry
1.6 ml/min flow rate	2.0387	4195	1.02	3.607	6330	1.00
1.0 ml/min flow rate	2.385	3811	0.99	3.601	7381	1.02
Column temp at 33ºC	2.387	3867	0.90	3.602	7467	1.01
Column temp at 37ºC	3.181	5271	1.01	3.605	6250	0.99
Buffer: MeoH (30:70v/v)	3.042	5263	1.00	3.895	5263	0.98
Buffer: MeoH (40:60v/v)	2.399	4251	0.98	3.214	6214	0.99

Detection and quantification limits: Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3 and limit of quantification (LOQ)at which S/N is10 were determine experimentally for the proposed methods and results are given in Table 5.

Table 5: Detection and quantification limits.

S. No	Sample	LOD(µg/ml)	LOQ(µg/ml)
1	Atazanavir	3.04	10
2	Cobicistat	2.95	9.87

Assay

The proposed validated method was successfully applied to determine Atazanavir and Cobicistat in tablet dosage form. The result obtained was comparable with corresponding labelled amounts. The result obtained was comparable with corresponding labelled amounts. The result were shown in table 6.

Table 6: Results of Assay.

S. NO	AREA	% Assay	AREA	% Assay
1	2194758	100.70	1494758	102.39
2	2195700	100.74	1456296	99.75
3	2196191	100.76	1457422	99.83
4	2195326	100.72	1456513	99.77
5	2200951	100.98	1454579	99.64
6	2196585	100.78	1439871	98.63
Mean	2196585.17	100.78	1459960.55	100.00
Std Dev	2232.64	0.10	18303.79	1.25
%RSD	1.10	1.10	1.25	1.25

CONCLUSION

The wide linearity range, accuracy, short retention times, and simple mobile phase imply that the proposed method can be successfully employed for routine quantification of Atazanavir and Cobicistat in combined dosage form. The method is economic too as the cost of mobile phase used is less compared to costly solvents that has to be used like acetonitrile for the quantification of Atazanavir and Cobicistat. Also the forced degradation studies imply that this method is stability indicating method development and validated according to ICH guidelines, one can adopt in an industry confidently for routine analysis.

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