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Stability Indicating RP-HPLC Method for Simultaneous Estimation of Atazanavir and Cobicistat in Tablets

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Authors' contributions

The present work was done in collaboration between all the authors MVSSN, BH and PRKV. The three authors were involved for the designing the study. Author MVSSN is involved in conducting the experiments, collection of literature and preparation of the manuscript. Data analyzing and manuscript corrections were done by authors BH and PRKV. All the authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to develop a stability indicating RP-HPLC method for simultaneous quantitative analysis of atazanavir and cobicistat in tablets.
Place and Duration of Study: At Rainbow Pharma Training Lab, Kukatapally, Hyderabad, India and Department of chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, India in between October 2015 and February 2016.
Methodology: Atazanavir and cobicistat was eluted on the Inertsil C8, 150 mm x 4.6 mm, 5 µm analytical column with a mobile phase consisting of 0.1 M ammonium acetate and methanol (50:50 v/v), pumped at 1.2 mL/min flow rate. The column was maintained at 30℃ and 10 µl of the solutions were injected. UV detection was performed at 234 nm. According to ICH guidelines, the

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method was validated.
Results: Under the optimized chromatographic conditions the retention times of atazanavir and cobicistat were 2.559 min and 3.576 min, respectively. Linearity was observed in the concentration range of 45-135 µg/mL for atazanavir and 22.5-67.5 µg/mL for cobicistat. The percent recovery and percent relative standard deviation for both the drugs were in the range of 99.311-100.342% and 0.290-0.401%, respectively. The results of forced degradation studies demonstrated the stability-indicating power of the method.
Conclusion: The proposed method was found to be appropriate for the quality control of atazanavir and cobicistat hydrochloride simultaneously in a bulk drug as well as in a pharmaceutical dosage forms.

Keywords: Antiretroviral drugs; HPLC; forced degradation; analysis; tablets.

1. INTRODUCTION

Atazanavir (Fig. 1), chemically described as methyl N- [(2S)-1- [2- [(2S,3S)-2-hydroxy-3-[[(2S)-2- (methoxycarbonylamino) -3,3-dimethylbutanoyl]amino] -4-phenylbutyl] -2- [(4-pyridin-2-yl phenyl) methyl] hydrazinyl] -3,3-dimethyl-1-oxobutan-2-yl] carbamate, is a azapeptide human immunodeficiency virus type 1 protease inhibitor [1]. As part of antiretroviral therapy, it was approved in 2003 by US Food and Drug Administration for people with human immunodeficiency virus infection [2,3]. The use of atazanavir with other antiretroviral drugs can reduce the viral load and increase CD4 cell counts [4,5].

Cobicistat (Fig. 2), chemically described as 1,3-thiazol-5-ylmethyl N-[(2R,5R)-5-[[(2S)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl) methyl]carbamoyl]amino]-4-morpholin-4-ylbutanoyl] amino]-1,6-diphenylhexan-2-yl]carbamate, is a pharmacokinetic enhancer and Cytochrome P450 3A Inhibitor [6]. By inhibiting cytochrome P450 3A enzyme and intestinal transport proteins, cobicistat increases the bioavailability and absorption of several human immunodeficiency virus medications such as atazanavir, darunavir and tenofovir alafenamide [7].

The combination of atazanavir and cobicistat was approved by US Food and Drug Administration in 2015. This combination is specifically indicated for the treatment of human immunodeficiency virus type 1 infection in adults [8]. Atazanavir acts as human immunodeficiency virus type 1 protease inhibitor and cobicistat increase

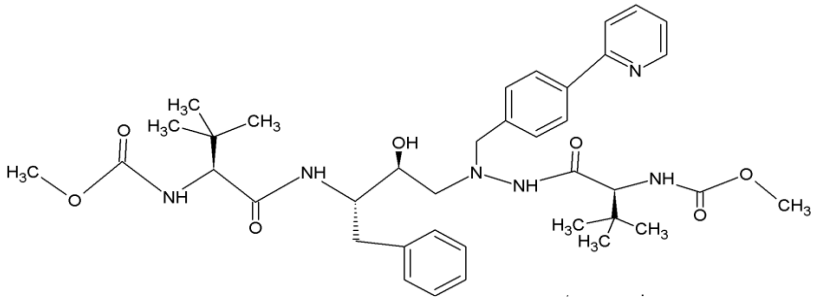

Fig. 1. Structure of atazanavir

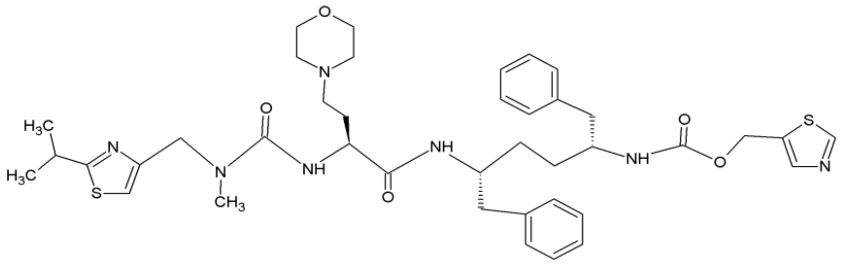

Fig. 2. Structure of cobicistat

the systemic exposure of atazanavir in combination with other antiretroviral agents.

A complete literature survey of analytical methods for the simultaneous estimation of atazanavir and cobicistat is done. Only few methods are reported. A stability indicating RP-HPLC method for the simultaneous determination of atazanavir and cobicistat in bulk and pharmaceutical formulation was presented by Priya and Sankar [9]. They used Kinetex C18 100 A column and 0.1% orthophosphoric acid-methanol (80:20 v/v) as mobile phase with UV detection at 239 nm. Kumar & Getaw [10] determined atazanavir and cobicistat simultaneously in tablets by stability indicating RP-HPLC with UV-detection at 235 nm using a Zorbax X DBC-8 column and a mobile phase consisting of acetonitrile and phosphate buffer (pH-4.2) in the ratio of 70:30 (*v/v*).

A reversed phase HPLC method had been reported by Panigrahy & Reddy [11] for the simultaneous determination of atazanavir and cobicistat in their pharmaceutical dosage forms. The method involves the simultaneous estimation of atazanavir and cobicistat using Agilent ZORBAX eclipse plus C18 column with 0.01 M potassium dihydrogen phosphate (pH 3.5): acetonitrile (30:70, *v/v*) as the mobile phase. Quantitation of atazanavir and cobicistat was achieved with ultraviolet detection at 260 nm. Simultaneous assay of atazanavir and cobicistat in combined dosage form by RP-UPLC has also been reported by Purnima et al. [12]. The separation was achieved on an Endeavorsil C18 column using 0.1% orthophosphoric acid (pH 5.5), methanol and acetonitrile in the ratio 27:18:55 (*v/v*) as mobile phase. Quantitation was achieved by measuring UV absorption at 245 nm.

The objective of the present investigation was to develop a simple, rapid, sensitive and precise stability indicating RP-HPLC method with photodiode array detector for simultaneous quantitative analysis of atazanavir and cobicistat in combined tablets and to validate the method in accordance with ICH guidelines [13].

2. MATERIALS AND METHODS

2.1 Instrumentation

- Waters HPLC system, consisted of a binary HPLC pump model 2695, PDA detector model 2998, vacuum degasser and Waters Empower2 software
- Inertsil C8 (150 mm x 4.6 mm, 5 µ particle size) analytical column

2.2 Standard, Chemicals and Reagents

Atazanavir and cobicistat standards were provided by Lara Drugs Private Limited (Telangana, India). Ammonium acetate (Sd Fine Chemicals Ltd., Mumbai, India) and methanol (Merck Pvt Ltd., Mumbai, India) of HPLC grade were used during the present study. Purified water was obtained from a Milli-Q system. Evotaz tablets, labeled to contain 300 mg of atazanavir and 150 mg of cobicistat (Bristol-Myers Squibb company, Princeton, USA), were purchased from the pharmacy market.

2.3 Chromatographic Conditions

Separation of Atazanavir and cobicistat was achieved on Inertsil C8 (150 mm x 4.6 mm, 5 µ particle size) analytical column as the stationary phase using mobile phase consisted of 0.1 M ammonium acetate and methanol in the ratio of 50:50 *v/v*. The mobile phase was filtered by passing through a membrane filter prior to use. Isocratic elution was achieved at a flow rate of 1.2 mL/min with a column temperature of 30℃. The injection volume was 10 µL. The chromatograms were recorded at 234 nm using photodiode array detector.

2.4 Standard Solutions

The mobile phase was used as the diluent. An accurately weighed amount of about 300 mg of atazanavir and 150 mg of cobicistat were put into a 100 mL volumetric flask, 30 mL of mobile phase was added and the mixture was sonicated to dissolve it. The resulting mixture was made up to volume with the same solvent. Working standard solution was prepared in mobile phase by diluting 3 mL of the above stock solution to 100 mL in a 100 mL volumetric flask to get atazanavir and cobicistat with final concentration of 90 µg/mL and 45 µg/mL, respectively.

2.5 General Assay Procedure

The working standard solutions were prepared by dilution of the stock solution with mobile phase to reach the concentration range 45-135 µg/mL and 22.5-67.50 µg/mL for atazanavir and cobicistat, respectively. Triplicate injections were made for each concentration. The chromatograms were recorded under the earlier

described chromatographic conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration curve.

2.6 Assay of Tablet Dosage Form

For the determination of atazanavir and cobicistat in combined tablet dosage forms, 20 Evotaz tablets were weighed and finely powdered. A suitable portion of powder equivalent to 300 mg of atazanavir and 150 mg of cobicistat was accurately weighed and transferred to a 100 mL volumetric flask. The flask was made up to volume with mobile phase and sonicated for 15 min. The solution was passed through a 0.45 µm membrane filter and diluted appropriately with the same solvent to reach a final concentration of 90 µg/mL (atazanavir) and 45 µg/mL (cobicistat). The sample solution was treated as described under the general assay procedure. Recovered concentrations of atazanavir and cobicistat were calculated from the corresponding calibration graphs.

3. RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic Conditions

The most important phase in the stability indicating RP-HPLC method development is the achievement of adequate resolution of atazanavir, cobicistat and their stress degradants with acceptable peak symmetry in a reasonable analysis time. To accomplish this, several experiments were carried out so as to optimize the stationary phase and mobile phase. For the stationary phase, three analytical columns Kromasil C8 (250 x 4.6 mm, 5 µm particle size), YMC Pack pro C18 (250 × 4.6 mm; 5 µm particle size) and Inertsil C8 (150 x 4.6 mm, 5 µm particle size) were tested. Successful resolution and acceptable peak symmetry of atazanavir and cobicistat was attained by using the Inertsil C8 (150 x 4.6 mm, 5 µm particle size) analytical column. Hence it was used in the present study.

Regarding the mobile phase 0.1 M ammonium acetate and methanol were tested using various proportions and various flow rates. The best resolution of the two drugs within acceptable analysis time was obtained through an isocratic elution using a mobile phase consisting of 0.1 M ammonium acetate and methanol in the ratio 50:50 (*v/v*) at a flow rate of 1.2 mL/min. Quantification was done using photodiode array detection based on peak area measurement. Atazanavir and cobicistat exhibited considerable absorption at 234 nm. Hence the wavelength 234 nm was selected for quantification of the analytes.

The above described chromatographic conditions showed symmetric peaks and sufficient resolution between atazanavir and cobicistat. Fig. 3 shows a typical chromatogram for the separation of two drugs. Atazanavir and cobicistat eluted at retention times 2.559 min and 3.576 min, respectively.

3.2 System Suitability Test

The suitability of the developed method was verified by repeated injections (n=5) of working standard solution (90 µg/mL atazanavir and 45 µg/mL cobicistat). The system suitability parmameters such as USP plate count, USP tailing factor, USP resolution and repeatability of the retention time and peak areas were determined. The results and the acceptable limits are shown in Table 1.

3.3 Linearity and Concentration Ranges

The linearity of the proposed HPLC method was evaluated by analyzing a series of different concentrations (n=5) for each of the two drugs. The linear regression equations were generated by least square treatment of the calibration data. Under the optimized chromatographic conditions, the measured peak areas of atazanavir and cobicistat at 234 nm were found to be proportional to their concentrations. Table 2 presents the linear regression equations,

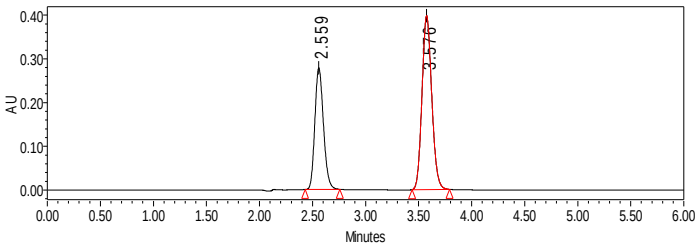

Fig. 3. Chromatogram of atazanavir and cobicistat under optimized conditions

Table 1. System suitability

Parameters	Atazanavir	Cobicistat	Recommended limits
Retention time	2.551 (%RSD - 0.268)	3.574 (%RSD – 0.110)	RSD ≤2
Peak area	1676351 (%RSD – 0.13)	2781823 (%RSD – 0.105)	RSD ≤2
USP resolution	-	6.27	> 1.5
USP plate count	4869	7193	> 2000
USP tailing factor	1.18	1.12	≤ 2

Table 2. Linearity, regression and sensitivity parameters and for the determination of atazanavir and cobicistat

Parameter	Atazanavir	Cobicistat
Linearity range (µg/mL)	45-135	22.5-67.5
Regression equation (y^a= m x^b + c)	y = 18707 x - 7451	y = 61760x - 4472
Slope (m)	18707	61760
% RSD of slope (n=3)	0.186	0.127
Intercept (c)	-7451	-4472
% RSD of intercept (n=3)	0.242	0.217
Regression coefficient (R^2)	0.9999	0.9990
LOD (µg/mL)	0.074	0.056
LOQ (µg/mL)	0.248	0.187

a – peak area of the drug: b - concentration of drug in µg/mL

concentration ranges, regression coefficients, intercept and slope. Regression analysis shows good linearity as indicated from the correlation coefficient values (≥0.9990).

3.4 Sensitivity

Sensitivity of the method was assessed by determining limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated as signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ values for atazanavir and cobicistat were calculated and are presented in Table 1.

3.5 Precision

The precision for the proposed method was studied at a concentration of 90 µg/mL atazanavir and 45 µg/mL cobicistat using six replicate determinations. The percentage relative standard deviation (RSD %) did not exceed 1.0% proving the high repeatability of the developed method (Table 3).

3.6 Accuracy

The accuracy of the proposed method was established by means of the standard addition technique, by adding a known amount of standard drug at three different levels (50%, 100% and 150%) to the preanalyzed sample. Accuracy was expressed as percentage recovery in Table 4. The accuracy of the developed method for the atazanavir and cobicistat ranged from 99.311% to 100.342% and 99.103%-100.060%, respectively indicating acceptable accuracy.

Table 3. Precision of the method

Atazanavir		Cobicistat	
Peak area	**%RSD**	**Peak area**	**%RSD**
1686749	0.290	2781916	0.401
1679478		2759527	
1672187		2785252	
1683510		2789844	
1679810		2773685	
1681125		2786021	

3.7 Robustness

The robustness of the developed method was checked by studying the effect of deliberate changes in the flow rate of mobile phase (±0.1 mL/min) and column temperature (±2℃) on the chromatographic system suitability parameters. According to the results shown in Table 5, these small and deliberate variations did not have any significant effect on the measured system suitability parameters.

Table 4. Accuracy of the method

Spiked level	Concentration of drug (µg/mL)		% recovery	% mean
	Added	Found		
		Atazanavir		
	44.55	43.988	98.739	99.311
50%	44.55	44.122	99.039	
	44.55	44.619	100.156	
	89.10	89.475	100.421	99.785
100%	89.10	88.429	99.247	
	89.10	88.820	99.686	
	133.65	133.754	100.078	100.342
150%	133.65	135.058	101.053	
	133.65	133.510	99.895	
		Cobicistat		
	22.50	22.422	99.654	99.636
50%	22.50	22.357	99.364	
	22.50	22.475	99.889	
	45.00	44.985	99.966	101.060
100%	45.00	44.913	99.808	
	45.00	46.534	103.408	
	67.50	66.154	98.006	99.103
150%	67.50	67.274	99.665	
	67.50	67.256	99.639	

Table 5. Robustness of the method

Parameter	Investigated value	Area	USP plate count	USP tailing	USP resolution
		Atazanavir			
Temperature (℃)	30-2	1915493	5051	1.20	-
	30+2	1490257	4467	1.16	-
Flow rate (mL/min)	1.2-0.1	1916559	5105	1.21	-
	1.2+0.1	1496352	4531	1.17	-
		Cobicistat			
Temperature (℃)	30–2	3182790	7683	1.13	6.90
	30+2	2467019	6900	1.11	6.55
Flow rate (mL/min)	1.2-0.1	3184220	7637	1.14	6.81
	1.2+0.1	2472818	6882	1.12	6.51

3.8 Specificity (Forced Degradation Studies)

The forced degradation study was conducted to make sure that the proposed method was able to separate atazanavir and cobicistat from the possible degradants generated during the acid, base, oxidative, sunlight and thermal degradation. The degradation study was carried out using the tablet powder containing atazanavir and cobicistat at a concentration of 90 µg/mL and 45 µg/mL, respectively. Acidic degradation was performed by sonication of sample with 10 mL of 0.1N HCl for 30 minutes. Alkaline degradation was performed by sonication of sample with 10 mL of 0.1N NaOH for 30 minutes. The acid and alkali degraded samples are neutralized with 0.1 N NaOH and 0.1 N HCl, respectively. Oxidative degradation was performed by sonication of sample with 10 mL of 30% hydrogen peroxide for 30 minutes. Thermal degradation was performed by heating the sample at 105℃ for 30 minutes in oven. The sample was exposed to sunlight for 24 hrs for photolytic degradation. All the forced degraded samples were injecting into the HPLC system. The chromatograms are shown in Figs. 4-8.

Under all degradation conditions, a small percentage of degradation was observed (Table 6). The analysis of the chromatograms of the degraded samples and determination of the

Table 6. Forced degradation studies

Type of degradation	Peak area	% assay	% degradation	Purity angle	Purity threshold
Atazanavir					
Acid	1559282	92.179	7.821	0.324	0.531
Base	1596131	94.358	5.642	0.318	0.524
Oxidative	1574632	93.087	6.913	0.295	0.506
Heat	1549937	91.627	8.373	0.316	0.503
Sunlight	1599045	94.530	5.470	0.300	0.496
Cobicistat					
Acid	2612874	93.645	6.355	0.204	0.274
Base	2601033	93.221	6.779	0.216	0.273
Oxidative	2673049	95.802	4.198	0.229	0.273
Heat	2650873	95.007	4.993	0.220	0.273
Sunlight	2601062	93.222	6.778	0.206	0.273

Table 7. Summary of reported and proposed methods

Drug	Retention time (min)	Total run time (min)	LOD (µg/mL)	LOQ (µg/mL)	RSD (%)	Recovery (%)	Reference
Atazanavir	6.113	8.0	1.49	4.97	0.161	100	Priya &
Cobicistat	3.605		1.13	3.77	1.594	99.90	Sankar [9].
Atazanavir	5.277	20.0	0.5	1.11	1.2	101.8	Kumar &
Cobicistat	6.698		1.30	2.41	1.4	101.6	Getaw [10]
Atazanavir	2.243	10.0	0.6	1.8	0.83	99.92-100.03	Panigrahy &
Cobicistat	6.043		0.2	0.6	0.98	99.91-100.05	Reddy [11]
Atazanavir	0.619	3.0	0.150	0.500	1.069	99.95	Purnima et
Cobicistat	0.443		0.250	0.850	1.153	100.20	al. [12].
Atazanavir	2.559	6	0.074	0.248	0.290	99.311-100.342	Proposed
Cobicistat	3.576		0.056	0.187	0.401	99.103-101.060	method

peak purity angle values demonstrated that the atazanavir peak and cobicistat peak was pure in all situations. The results of forced degradation studies allowed to conclude that the degradants produced as a result of forced degradation did not interfere with the detection atazanavir and cobicistat, and the proposed method can hence be regarded as stability-indicating.

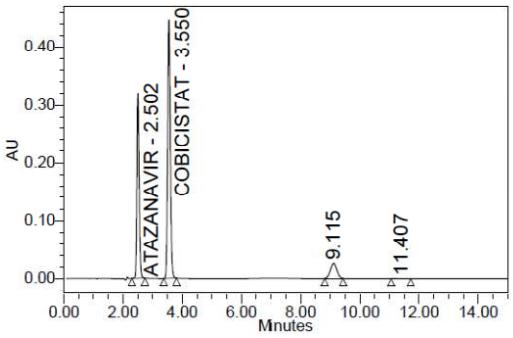

Fig. 4. Chromatogram of acid degraded sample

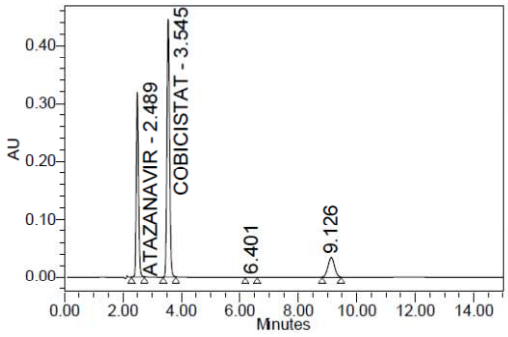

Fig. 5. Chromatogram of base degraded sample

3.9 Selectivity

The chromatograms of mobile phase blank, placebo blank, tablet sample and standard sample were compared to establish the selectivity of method. Placebo blank solution was prepared in the same way of the tablet sample solution by common excipients of the tablet dosage form but without atazanavir and

cobicistat. The chromatograms are presented in Fig. 9-12. The method was selective for the simultaneous assay of atazanavir and cobicistat, since common excipients of the tablet dosage form and components of the mobile phase did not interfere with the peaks of atazanavir and cobicistat.

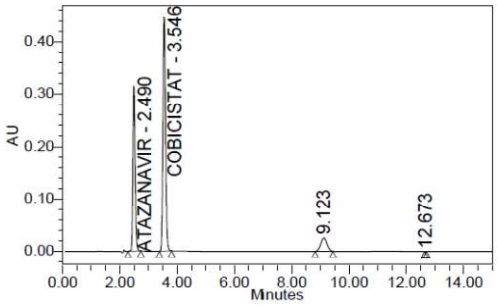

Fig. 6. Chromatogram of H_2O_2 degraded sample

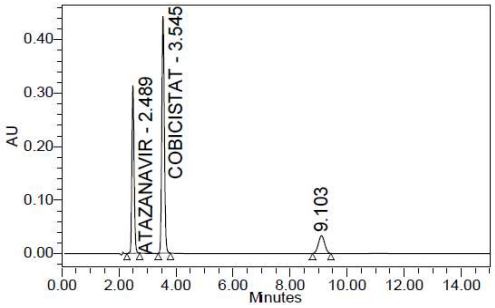

Fig. 7. Chromatogram of heat degraded sample

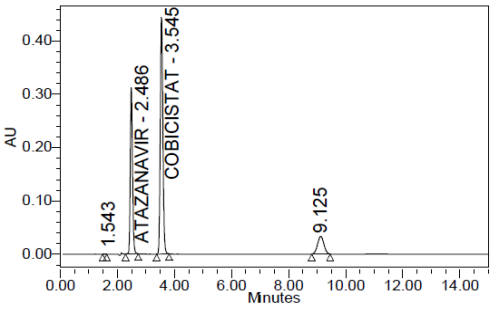

Fig. 8. Chromatogram of photo degraded sample

3.10 Comparison of the Proposed Method with Reported Method

The developed method has the advantages of being more sensitive and precise than the reported RP-HPLC methods [9-11]. The total run time of <8 minutes enables rapid determination of drug combination than the reported methods [9-11]. Unlike the methods of Kumar & Getaw [10] and Panigrahy & Reddy [11], the proposed method does not use acetonitrile in the preparation of mobile phase. Unlike the RP-UPLC method of Purnima et al. [12], the proposed method does not use triple solvent system as mobile phase. The lengthy total run time, use of acetonitrile as organic modifier and use of triple solvent system increases the cost of analysis. Though the RP-UPLC method [12] is sensitive and rapid, requires sophisticated and costly instrumentation which is not available in the most of developing and underdeveloped countries. The details of the proposed and reported methods are summarized in Table 1.

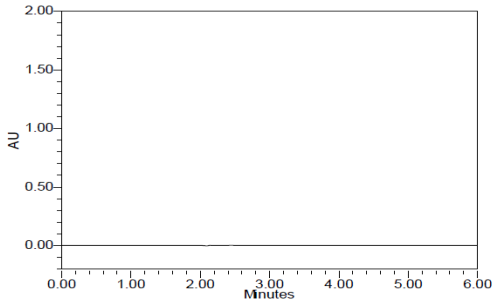

Fig. 9. Chromatogram of mobile phase blank

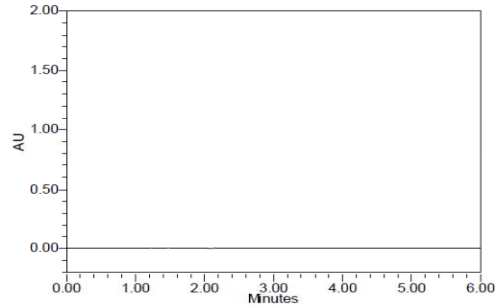

Fig. 10. Chromatogram of placebo blank

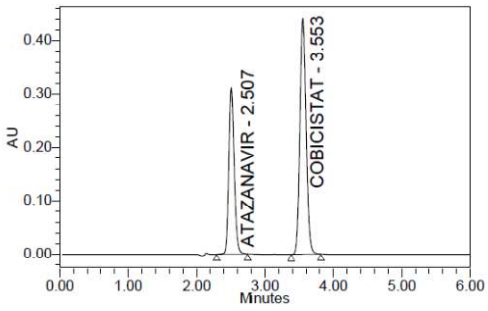

Fig. 11. Chromatogram of table sample

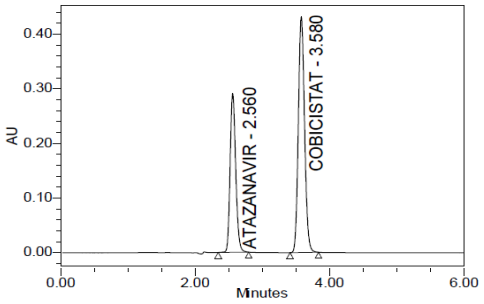

Fig. 12. Chromatogram of standard sample

4. CONCLUSION

A simple, rapid, accurate, precise and sensitive HPLC method with photodiode array detection was developed for the simultaneous determination of atazanavir and cobicistat in bulk and in combined tablets. The method was validated for linearity, limit of detection, limit of quantitation, precision, accuracy, robustness, specificity and selectivity as indicated by the ICH guidelines. The retention time of less than 5 minutes for both the drugs enables rapid determination of drugs. The proposed method is adequate to separate the peaks of atazanavir and cobicistat from the degradants produced during forced degradation studies. Hence, it can be recommended for use in quality control laboratories for the simultaneous assay of atazanavir and cobicistat in the presence of its degradants.

CONCENT

It is not applicable.

ETICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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