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Impurities Profiling and Quantification of Atazanavir Sulphate (ATZ) and its Impurities in their Dosage Forms by Gradient RP-HPLC Method

Nirav Rajendrakumar Soni*, Pragnesh Patani

Department of Quality Assurance, Gujarat Technological University (GTU), Ahmedabad, India

***Corresponding author:** Nirav Rajendrakumar Soni, Department of Quality Assurance, Gujarat Technological University (GTU), Ahmedabad, India, **E-mail**: nirav_sonic@yahoo.com*

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ABSTRACT

Analytical method was developed using HPLC Shimadzu (with power stream) gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on hypersil based deactivated silica C18 (250 mm × 4.6 mm, 5 µm) column using mobile phase composition of 0.05 M Potassium phosphate buffer: Methanol (60 v/v:40 v/v), (15 v/v:85 v/v), (60 v/v:40 v/v), adjusted to pH 4 with 1% orthophosphoric acid. Makeup volume with water. The flow rate of mobile phase was maintained at 1 ml/min with wavelength 225 nm UV detection. The Retention Time (RT) found for Atazanavir sulphate (ATZ), impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with an injection volume of 20 ml and the detection was made at 225 nm. Validation of the method was successfully established by performing various validation parameters such as accuracy, precision, specificity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), ruggedness, robustness, according to ICH guidelines.

Keywords: Atazanavir sulphate; Impurity-A and impurity-5; RP-HPLC; Gradient elution; Force degradation study; Atazor capsules (300 mg); Atavir (300 mg)

INTRODUCTION

Chemically, Atazanavir sulphate(ATZ)1 is a methyl N-[(2S)-1-[2-[(2S,3S)-2-hydroxy-3-[[(2S)-2-(methoxycarbonylamino)-3,3-dimethylbutanoyl]amino]-4-phenylbutyl]-2-[(4-pyridin-2-ylphenyl)methyl]hydrazinyl]-3,3dimethyl-1-oxobutan-2-yl] carbamate. ATZ is an oral antiretroviral drug that specifically belongs to the protease inhibitors class used in the treatment of HIV/AIDS. Literature survey reveals few chromatographic methods for the determination of ATZ in combination with other anti-retroviral drugs in bodyfluids, one assay with quantification of impurity method in active pharmaceutical excipients and one assay in dosage form 10. The present paper aims at reporting precise, accurate, selective, sensitive, robust and rugged validated RP-HPLC method for the estimation of ATZ and its known impurity A and impurities in the marketed dosage form (Figures 1-3) [1].

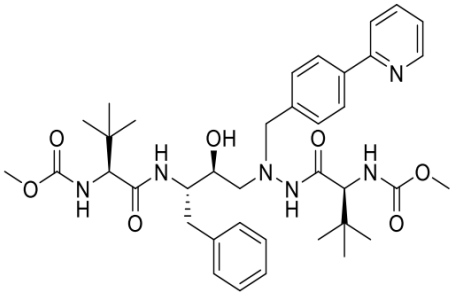

Figure 1: Structure of Atazanavir Sulphate (ATZ)

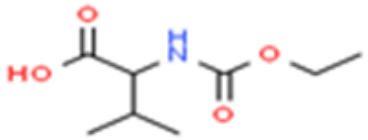

Figure 2: Structure of Impurity A

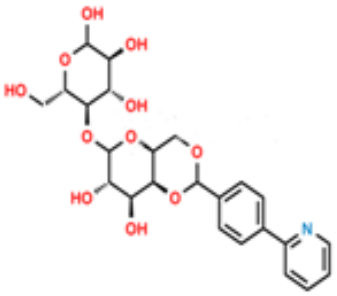

Figure 3: Structure of Impurity 5

In the literature survey, it was found stability method, Chemical synthesis and characterization, Method Development (MD) and Method Validation (MV) for selected anti-HIV class of drugs. Also, simultaneous estimation of analytical method and validation of anti-HIV class. There are a very few methods reported for impurity profiling and quantification of selected anti-HIV drugs by chromatography technique. Quality Control (QC) of pharmaceutical products needs the identification and quantification of the active ingredient and its impurities for safety and efficacy reasons. Impurities and potential degradation products that may exist in medicines can change the chemical, pharmacological and toxicological properties of the product. There is no impurities profiling indicating analytical methods were reported in the literature and since pharmacopeias do not describe a suitable stability-indicating method for the determination of ATZ in pharmaceutical formulations, in the present work an attempt has been made to develop a simple, rapid precise and accurate RP-HPLC method for the determination of ATZ in capsules and its impurities. Potential impurities of ATZ were not separated from the main analytes in the reported methods and it is the most common in their dosage forms. It has produced genotoxicity. Hence, no liquid chromatographic methods (RP-HPLC and UPLC) were reported for the determination of ATZ impurities in their fixed dosage forms. RP-HPLC system enables improved sensitivity, selectivity, rapid analysis, environment friendly due to lower solvent consumption, RP-HPLC equipment was chosen for the determination of ATZ and its impurities in the fixed-dose products. Finally, the present work has selected two impurities namely Impurity A and Impurity 5 respectively [2].

MATERIALS AND METHODS

Reagents and chemical compounds

ATZ standard (purity 99.80%) was gotten from Emcure Pharmaceuticals Limited, Ahmedabad, India. Impurity A and Impurity 5 from Medvin Pharmaceutical Limited, Ahmedabad, HPLC grade of Water, Methanol, Acetonitrile (ACN), Potassium dihydrogen orthophosphate while using AR grade of Sodium Hydroxide (NaOH) and Hydrochloric Acid (HCl). Commercially ATZ capsule called (Label claim 300.0 mg) of ATZ marketed as Atazor-300 mg and Atavir-300 mg were obtained from the medical and retail store [3].

Instrument

It was achieved by using a Shimadzu (with power stream), equipped with PDA 600 UV Detector, UV Spectrophotometer Systronics 119, C18 column particle size (250 mm × 4.6 mm, 5 µm) and for optimum separation time of both impurity A and impurity 5, the flow rate of 1 ml/min was approved. In order to have symmetric peak shapes as per SST (System Suitability Test) and optimum resolution between the both impurity A and impuritiy 5, the C18 column oven temperature was set at 45°C [4].

Chromatographic situations

Gradient elution achieved using mobile phase composition of 0.05 M Potassium phosphate buffer: Methanol (60 v/v: 40 v/v), (15 v/v: 85 v/v), (60 v/v: 40 v/v), adjusted to 4 pH with 1% Orthophosphoric Acid (OPA). Makeup volume with water. The flow rate was maintained at 1 ml/min with 225 nm UV detection. The Retention Time (RT) obtained for Atazanavir sulphate (ATZ), impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with an injection volume of 20 ml and the detection was made at 225 nm [5].

Experiment works

Preparation of stock solution: Weigh and powder 20 capsules. Disperse the content of capsules containing about 20 mg ATZ with 60 ml of the

methanol in the 100 ml volumetric flask. Ultrasonicate for 10 minutes and make up the volume with Methanol. Pass the solution with Whatman filter paper no-1 [6].

Preparation of standard solution: Above the stock solution pipette out 1 ml into 10 ml volumetric flask and make up the final volume with given mobile phase (ATZ-20 µg/ml) [7].

Preparation of mobile phase

Mobile phase A: 0.05 M Potassium dihydrogen phosphate Buffer containing pH-4.0. Take 6.8 gm Potassium dihydrogen orthophosphate (KH_2PO_4) into a 1000 ml beaker. Add 800 ml water and dissolve with water by using magnetic stirrer. Adjust pH 4.0 with 1% orthophosphoric acid. Makeup volume with water [8,9].

Preparation of sample solution

Take 1 ml from stock solution into 10 ml and makeup with the given mobile phase. (ATZ-20 µg/ml) [10].

Validation: The method was authenticated for the following parameters accuracy, precision, linearity, Limit of Detection (LOD), Limit of Quantitation (LOQ), specificity and robustness as per ICH guidelines, 2005 [11].

Linearity: Linearity of ATZ, Impurity A and Impurity 5 described below [12].

Preparation of stock solution of ATZ: Exactly weighed 5 mg of ATZ and diluted in 100 ml volumetric flasks to obtain 50 ppm solution [13].

Preparation of standard solution of ATZ: Taken 1 ml of the above stock solution of ATZ and diluted up to the 10 ml volumetric flask to obtain 5 ppm solution [14].

The linearity and range: The linearity of the method was determined at six different concentration levels. The linearity data obtained for the calibration curve of ATZ (concentration 0.5 µg/ml-7.5 µg/ml) and its Impurity A (concentration 0.5 µg/ml-7.5 µg/ml) and Impurity 5 (concentration 0.2 µg/ml-7.5 µg/ml) were linear over the concentration range of LOQ to 150% respectively. 20 µl of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. Then, a linear regression equation was derived by plotting the graph between the sample dissolved and recovered by the method.

A calibration curve was drawn by taking the concentration on the X-axis and the corresponding peak area on the Y-axis. The slope and Y-intercept of the calibration curve were reported. It is clear that the correlation coefficient (R^2) is equal to unity and comes under the acceptance criteria ($R^2 \geq 0.999$). Therefore, depending upon the calculated values of R^2, the developed method should be considered to have a high degree of linearity. A series of solutions of ATZ, Impurity A and Impurity 5 were prepared over the range of LOQ to 150 % of the specification limit with R^2 should not be less than 0.99 (Figures 4-7) [15].

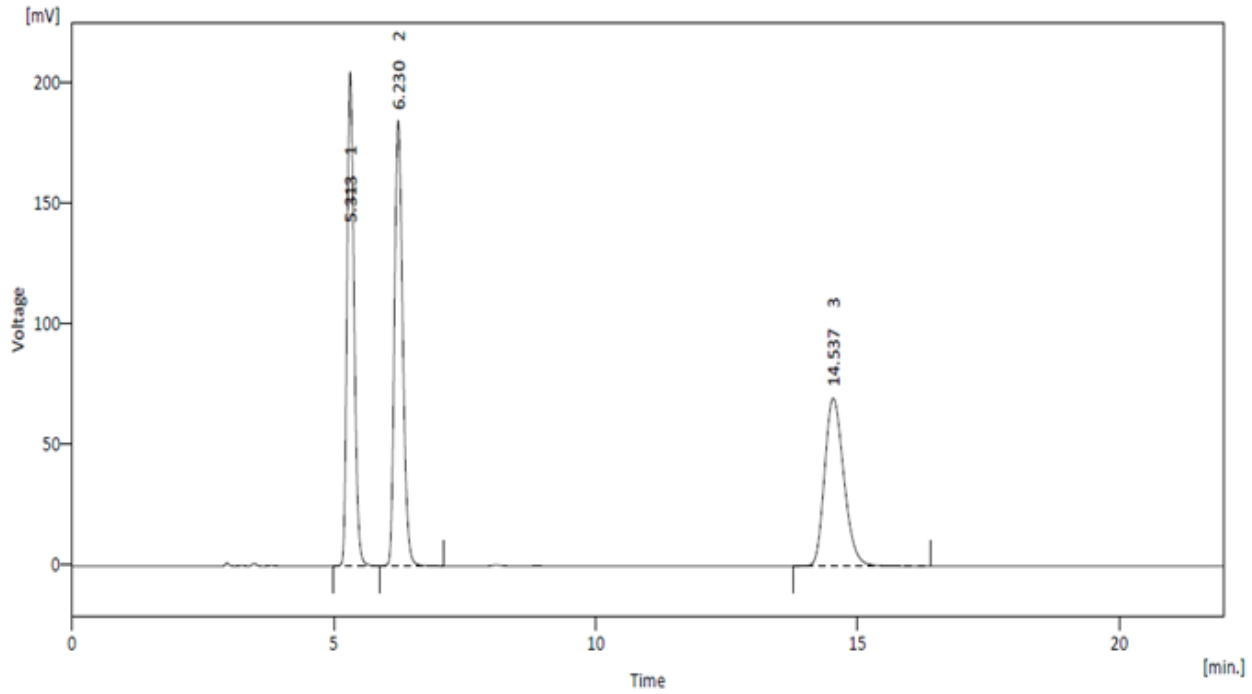

Figure 4: Final developed chromatogram of Atazanavir Sulphate (ATZ)+impurity A+impurity 5 in gradient technique. **Note:—** ATZ+ATZ imp-1+ATZ impurity-2 gradient-4

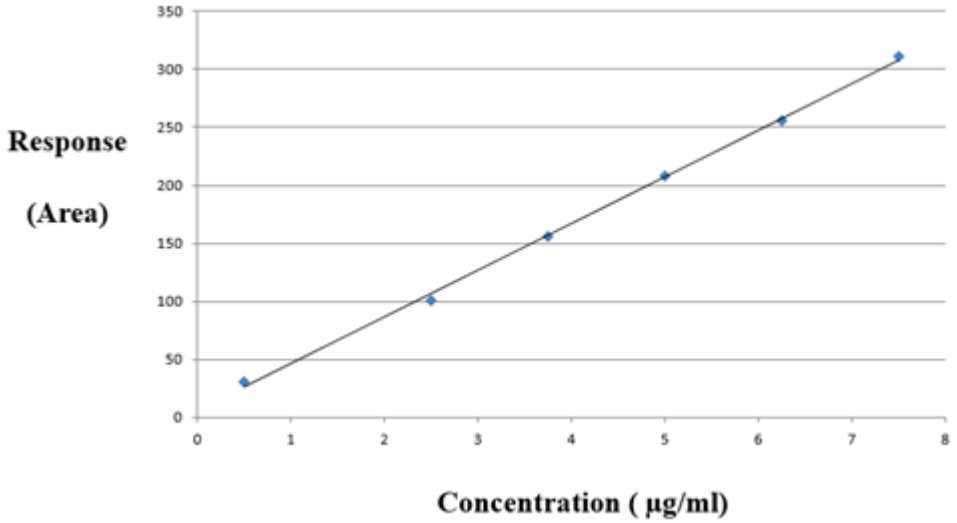

Figure 5: Linearity of Atazanavir sulphate (ATZ). **Note:** y=40.199x+6.3305, R^2=0.9987

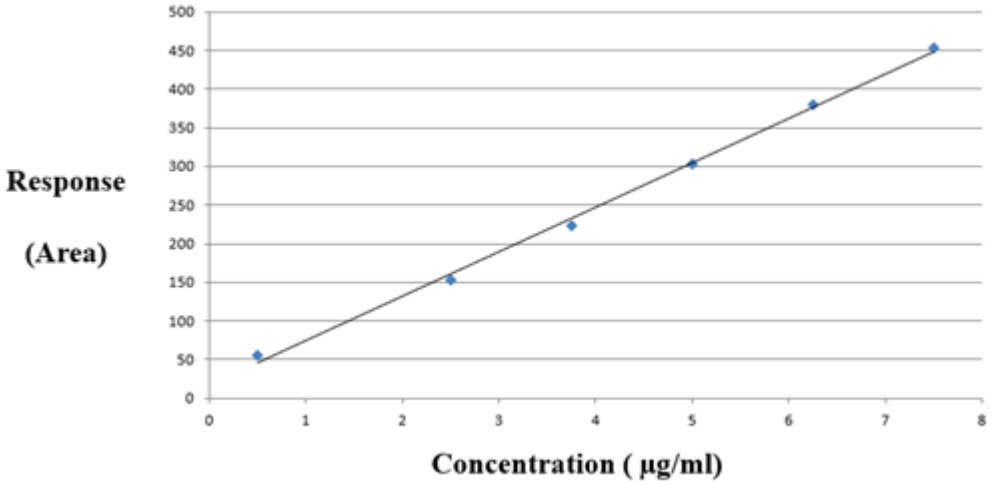

Figure 6: Linearity of impurity A. **Note:** y=57.416x+17.03, R^2=0.9974

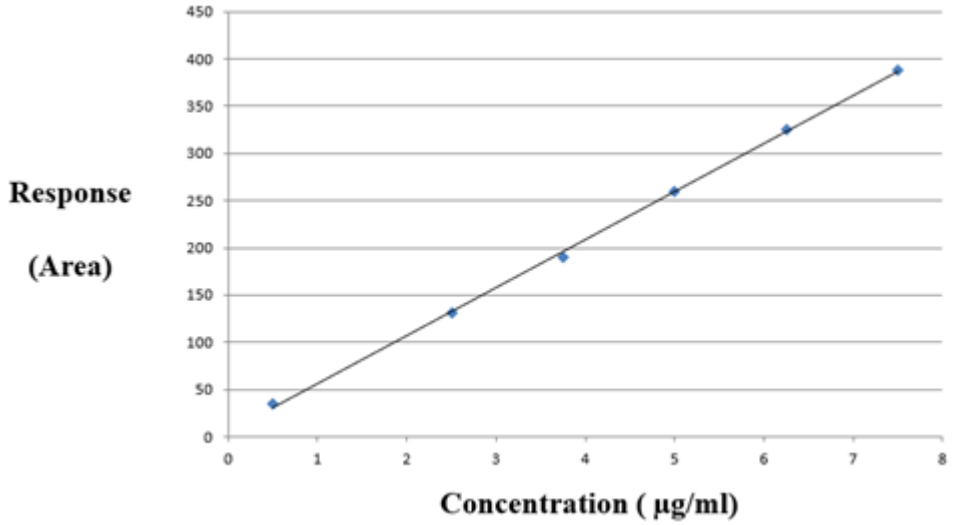

Figure 7: Linearity of impurity 5. **Note:** y=50.877x+5.4661, R^2=0.9998

Intra-day and inter-day precision: The data for intraday precision for ATZ, Impurity A and Impurity 5. The % RSD for intraday precision was found to be 2.561-4.582 for ATZ, 2.787-3.919 for impurity A and 1.423-2.818 for impurity 5 [16].

Accuracy: The accuracy sample of ATZ was spiked with known impurity A and impurity 5 at five different concentration levels LOQ, 80%, 100% and 120 % of the specification limit in triplicate (total 12 determinations) and then progress with sample preparation as described under given the methodology. The acceptance criteria of mean recovery should be in the range of 90.0%-110.0% for LOQ, 80%, 100% and 120% levels [17].

Sensitivity: The Limit of Detection (LOD) and Limit of Quantification (LOQ) were based on the Standard Deviation (SD) of the response and the slope of the constructed calibration curve (n=3), as described in international conference on harmonization guidelines Q2 (R1). The sensitivity of the method was recognized with respect to LOD and LOQ for ATZ and calculated by the slope method as mentioned below [18].

$$LOD=3.3 \times \sigma/S, LOQ=10 \times \sigma/S.$$

Robustness: The robustness of the method was validated in terms of minute deliberate variations in mobile phase composition (± 2%), flow rate (± 0.2 mL/min) and pH (± 0.2). Each solution was inserted or injected in triplicate, peak areas observed and %RSD was calculated [19].

Forced degradation: Forced degradation studies are performed on drug products under different conditions like acidic, alkali, oxidative, thermal and photolytic stress. Each stress condition sample is analyzed in the planned method and peak purity data is recorded to check the similar nature of the drug [20].

Forced degradation study: The major ways of degradation of any drug compounds included hydrolysis, oxidation, heat and photolysis [21].

Hydrolytic degradation: Hydrolytic study under acidic and basic circumstances involves canalization of ionizable functional groups present in the molecule. 0.1 M Hydrochloric Acid and 0.1 M Sodium Hydroxide are employed for generating acidic and basic stress samples, respectively [22].

Oxidative degradation: Many drug compounds undergo Auto-oxidation i.e. Oxidation under usual storage conditions and involving ground state elemental oxygen. Therefore, it is an important degradation pathway of many drugs. Auto-Oxidation is a free radical reaction that requires a free radical initiator to begin the chain reaction. Hydrogen peroxide, metal ions or trace levels of impurities in a drug substance act as initiators for Auto-oxidation. The mechanism of oxidative degradation of drug substances involves an electron transfer mechanism to form reactive anions and cations. Amines, sulphides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulphones and sulphoxide. 3% Hydrogen peroxide is a very common oxidant to produce oxidative degradation products which may arise as minor impurities during long-term stability studies [23].

Thermal degradation: In overall, the rate of a reaction increases with the rise in temperature. Thus, the drugs are susceptible to degradation at higher temperatures (105°C). Many APIs are sensitive to heat or tropical temperatures. For example, vitamins, peptides, etc. Thermal degradation involves different reactions like pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization [24].

Photolytic degradation: The rate of Photo-degradation is contingent upon the intensity of occurrence light and the quantity of light absorbed by the API molecule. The photolytic degradation can happen through oxidative or non-oxidative photolytic reactions. Photolytic degradation is carried out by exposing the drug substance or drug product to a combination of visible and UV light. The non-oxidative photolytic reactions include isomerization, dimerization, cyclization, rearrangements and decarboxylation etc. And while oxidative photolytic reactions occur through either singlet oxygen ($^1O_2$) or triplet oxygen ($^3O_2$) mechanism [25].

Assay of commercial formulation

Preparation of stock solution of ATZ: 50 mg of ATZ and diluted in 100 ml volumetric flasks to obtain 500 ppm solution [26].

Preparation of mix standard solution of ATZ: Take 1 ml of the above prepared stock solution of ATZ and diluted up to the 10 ml volumetric flask to obtain 50 ppm solution [27].

Preparation of sample solution: 50 mg of ATZ and diluted in 100 ml volumetric flasks to obtain 500 ppm solution. Again Take 1 ml of this prepared solution dilute up to the 10 ml volumetric flask to obtain 50 ppm solution [28]. The proposed method was applied for the determination of ATZ impurities estimation in marketed capsules results of its impurities RSD less than 5.0%. The results designate that the method is discriminating for the assay of ATZ without interference from the excipients used in these dosage form [29].

RESULTS AND DISCUSSION

An impurities profiling RP-HPLC was developed to quantification ofATZ at 225 nm. ACN was chosen as an organic modifier in the mobile phase. The satisfactory resolution was achieved with separation was achieved on hypersil BDS C18 (250 × 4.6 mm, 5 µm) column using mobile phase composition of 0.05M potassium phosphate buffer: Methanol (60 v/v: 40 v/v), (15 v/v: 85 v/v), (60 v/v: 40 v/v), pH adjusted to 4 with 1% Orthophosphoric Acid (OPA). Makeup volume with water. The flow rate was maintained at 1 ml/min with 225 nm UV detection. The Retention Time (RT) obtained for Atazanavir Sulphate (ATZ), impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with an injection volume of 20 ml and the detection was made at 225 nm. The present impurities profiling method for the determination of ATZ in pharmaceutical formulations is specific because the drug peak was well separated even in the presence of impurities. Overall, the data demonstrated that the impurities did not interfere with the ATZ peak, indicating the specificity of the above method. The whole separation of the ATZ and both the impurity A and impurity 5 was separated in less than 15 min and the method can be successfully applied to perform long-term and faster identification of known and unknown impurities of any ATZ related to formulation or dosage forms [30].

HPLC method development and optimization

Analytical method was developed using HPLC Shimadzu [with power stream] gradient chromatographic technique. Data were passed through the spinchrom software. Separation was achieved on hypersil BDS C18 (250 × 4.6 mm, 5 µm) column using mobile phase composition of 0.05M potassium phosphate buffer: Methanol (60 v/v: 40 v/v), (15 v/v: 85 v/v), (60 v/v: 40 v/v), pH adjusted to 4 with 1% Orthophosphoric Acid (OPA). Makeup volume with water. Therefore, the flow rate of mobile phase was maintained at 1 ml/min with 225 nm UV detection chosen as the optimized condition for the entire study. The retention time obtained for ATZ, impurity A and impurity 5 was at 5.3 min, 6.23 min and 14.53 min respectively with injection volume 20 µL [31].

Method validation

System Suitability Test (SST): SST was performed to ensure that the whole testing system was suitable for the intended application. The limitations measured were peak area, RT, asymmetrical peak and to counted theoretical plates. In all measurements the peak area varied less than 2.0%, the retention time of ATZ was 5.313 minutes, Impurity A was 6.230 minutes and Impurity 5 was 14.537 minutes. The capacity factor was more than 2, theoretical plates were more than 2000 and the tailing factor was less than 2.0 for the ATZ and its both impurity A and impurity 5. The above proposed method proposals to high sensitivity and ATZ and its impurities can be detected accurately. In all the cases, the ATZ peak was separated from their impurities as per SST criteria (Table 1) [32].

Table 1: Optimized mobile phase of ATZ and its impurities

Parameters	Atazanvir sulphate	Impurity A	Impurity 5	Limit
Retention time	5.313	6.230	14.537	-
Efficiency	6951	7157	7317	>2000
Tailing/Asymmetry	1.343	1.341	1.376	<2.0
Resolution	-	3.336	17.051	>2.0

Linearity: The linearity of the method was determined at six concentration levels. The linearity data obtained for the calibration curve of ATZ (concentration 0.5 µg/ml-7.5 µg/ml) and its Impurity A (concentration 0.5 µg/ml-7.5 µg/ml) and Impurity 5 (concentration 0.2 µg/ml -7.5 µg/ml) were linear over the concentration range of LOQ to 150% respectively 20 µl of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. Then, a linear regression equation was derived by plotting the graph between the sample dissolved and recovered by the method. A calibration curve was drawn by taking the concentration on the X-axis and the corresponding peak area on the Y-axis (Table 2) [33].

Table 2: Linearity of ATZ and its impurity A and impurity 5

		Atazanvir sulphate		Impurity A		Impurity B	
Sr. No	Level	Conc. (µg/ml)	Response (Area)	Conc. (µg/ml)	Response (Area)	Conc. (µg/ml)	Response (Area)
1	LOQ	0.5	31.087	0.5	55.993	0.5	34.86
2	Linearity-1	2.5	101.133	2.5	153.158	2.5	130.737
3	Linearity-2	3.75	156.255	3.75	222.875	3.75	190.671
4	Linearity-3	5	207.854	5	302.874	5	260.029
5	Linearity-4	6.25	255.678	6.25	378.95	6.25	325.376
6	Linearity-5	7.5	311.047	7.5	452.428	7.5	388.485
Slope		41.540		60.369		150.434	
Correlation coefficient		0.9997		0.9974		0.9998	

Precision: The precision of the instruments was plaid by repeatedly injecting (n=6) solutions of ATZ and its impurity (5 µg/ml). The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of ATZ and its impurities at three levels (LOQ, 100% and 150%) against the qualified reference standard. The %RSD of three obtained assay values at 3 different concentration levels was calculated. The inter day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (LOQ, 100% and 150%, n=3). The %RSD of three obtained assay values on three different days was calculated. The % RSD values of intra-day and inter-day was (2.561, 1.217 and 1.288) for ATZ and its both impurity A and impurity 5 reveal that the proposed method is precise (Tables 3 and 4) [34].

Table 3: Repeatability of ATZ and its impurities

ATZ		ATZ IMP 1		ATZ IMP 2	
At 100%	5 µg/ml	at 100%	5 µg/ml	at 100%	5 µg/ml
Std	area	Std	area	Std	area

1	205.206	1	296.875	1	256.403
2	208.988	2	304.97	2	263.618
3	207.37	3	311.882	3	270.28
4	214.41	4	303.515	4	261.543
5	210.381	5	321.496	5	277.167
6	203.358	6	326.784	6	263.966
Avg	208.286	Avg	310.920	Avg	265.496
Std	3.924	Std	11.419	Std	7.256
%RSD	1.884	%RSD	3.673	%RSD	2.733
Limit: %RSD for area NMT 5.0%		Limit: %RSD for area NMT 5.0%		Limit: %RSD for area NMT 5.0%	

Table 4: Intra-day and inter-day precision studies of ATZ and its both impurities

Intraday precision (Ruggedness)										
		Atazanvir sulphate			Impurity A			Impurity 5		
Sr. No	Level	Conc. (µg/ml)	Mean ± SD	%RSD	Conc. (µg/ml)	Mean ± SD	%RSD	Conc. (µg/ml)	Mean ± SD	%RSD
1	LOQ	0.5	31.855 ± 1.364	4.282	0.5	63.004 ± 2.469	3.919	0.2	48.351 ± 1.363	2.818
2	100	5	215.779 ± 5.527	2.561	5	311.464 ± 2.469	3.743	5	262.182 ± 5.889	2.246
3	150	7.5	318.499 ± 10.657	3.346	7.5	456.746 ± 12.732	2.787	7.5	390.022 ± 5.550	1.423

Accuracy: The accuracy Sample of ATZ was spiked with known impurities at five different levels: LOQ, 80%, 100% and 120 % of the specification limit in triplicate (total 12 determinations) and then proceed with sample preparation as described under Methodology. The acceptance criteria of mean recovery should be in the range of 90.0% to 110.0% for LOQ, 80%, 100% and 120% levels. Recovery studies were carried out in triplicate and the percentage recovery and standard deviation of the percentage recovery were calculated. The mean recovery for known both the impurity A and impurity 5 were within the limits. Therefore, the RP-HPLC method for the determination of ATZ impurity A and impurity 5 are accurate (Tables 5 and 6) [35].

Table 5: Recovery results of impurity A

Recovery level	Area of recovery spiked with test	Area of Imp in test	Net area of Std	Area of Std	Amount added (mcg/ml)	Amount recovered (mcg.ml)	%Recovery	Mean ± SD	%RSD
LOQ	114.428	38.965	75.463	150.809	2.500	2.502	100.078	100.098 ± 1.788	1.786
LOQ	115.799	38.965	76.834	150.809	2.500	2.547	101.896		
LOQ	113.103	38.965	74.138	150.809	2.500	2.458	98.32		
80%	158.779	38.965	119.814	150.809	4.000	3.972	99.309	99.585 ± 1.196	1.201
80%	160.691	38.965	121.726	150.809	4.000	4.036	100.894		
80%	157.863	38.965	118.898	150.809	4.000	3.942	98.55		
100%	189.281	38.965	150.316	150.809	5.000	4.984	99.673	99.371 ± 1.175	1.183
100%	190.326	38.965	151.361	150.809	5.000	5.018	100.366		
100%	186.87	38.965	147.905	150.809	5.000	4.904	98.074		
120%	218.674	38.965	179.709	150.809	6.000	5.958	99.303	100.114 ± 0.713	0.712
120%	220.654	38.965	181.689	150.809	6.000	6.024	100.397		
120%	221.098	38.965	182.133	150.809	6.000	6.039	100.642		

Table 6: Recovery results of impurity 5

Recovery level	Area of recovery spiked with test	Area of Imp in test	Net area of std	Area of Std	Amount added (mcg/ml)	Amount recovered (mcg.ml)	%Recovery	Mean	SD	%RSD
LOQ	89.396	25.836	63.56	128.524	2.500	2.473	98.908	99.791	0.898	0.900
LOQ	89.945	25.836	64.109	128.524	2.500	2.494	99.762			
LOQ	90.55	25.836	64.714	128.524	2.500	2.518	100.703			
80%	130.337	25.836	104.501	128.524	4.000	4.065	101.636	100.613	1.065	1.058
80%	128.152	25.836	102.316	128.524	4.000	3.980	99.511			
80%	129.366	25.836	103.53	128.524	4.000	4.028	100.691			
100%	156.591	25.836	130.755	128.524	5.000	5.087	101.736	101.022	0.732	0.725
100%	155.720	25.836	129.884	128.524	5.000	5.053	101.058			
100%	154.710	25.836	128.874	128.524	5.000	5.014	100.272			
120%	182.207	25.836	156.371	128.524	6.000	6.083	101.389	100.564	1.118	1.112
120%	178.971	25.836	153.135	128.524	6.000	5.957	99.291			
120%	181.624	25.836	155.788	128.524	6.000	6.061	101.011			

Robustness: The given procedure refers to its ability to remain unaffected by small and careful variations in method parameters and provides an indication of its reliability for routine analysis. The robustness of the method was evaluated by performing the assay of ATZ and its impurity A and impurity 5 both under different analytical conditions deliberately changing from the original condition. Flow rate of mobile phase, slight changes in mobile phase composition and pH affect the chromatographic response such as retention time and peak area. The % RSD obtained for peak area was 0.64-2.68 indicating that the developed method is robust (Table 7) [36].

Table 7: Robustness study of ATZ, impurity A and impurity 5

Sr. No	Parameter	Condition	ATZ		Impurity A		Impurity 5	
			Mean ± SD	%RSD	Mean ± SD	%RSD	Mean ± SD	%RSD
1	Flow rate+0.2 ml	1.2	201.3923 ± 4.656829	2.312317	291.4833 ± 3.422955	1.174323	251.55467 ± 4.142761	1.6468633
2	Flow rate-0.2 ml	0.8	219.704 ± 4.659113	2.120632	319.11866 ± 2.755327	0.863418	269.3927 ± 3.963373	1.471225
3	Mobile phase+2%	62:38	201.4623 ± 2.17482	1.079517	296.288 ± 7.948018	2.6825312	251.767 ± 3.575102	1.4200044
		17:83						
		62:38						
4	Mobile phase-2%	58:42	221.637 ± 1.698928	0.766536	319.6743 ± 3.453901	1.080444	271.61 ± 1.739094	0.640291
		13:87						
		58:42						
5	pH+0.2 unit	4.2	209.229 ± 3.920163	1.873623	299.0187 ± 4.539861	1.518253	259.4377 ± 1.677521	0.646599
6	pH-0.2 unit	3.8	206.209 ± 5.16774	2.506061	302.065 ± 2.809774	0.930188	259.2493 ± 4.159856	1.604577

Forced degradation studies: The stability-indicating capability of the method was recognized from the separation of ATZ and its impurity A and impurity 5 peak from the degraded samples. The degradation of ATZ was found to be very comparable for both the marketed dosage forms and its standard. Typical chromatograms obtained following the assay of stressed samples. The ATZ has undergone slightly acidic, photolytic and thermal degradations. Summarizes the data of degradation studies. The number of theoretical plates (N) is used to determine the performance and efficiency of the column. It is a measure of band spreading of a peak. The smaller the band spread, the higher is the number of theoretical plates, indicating good column and system performance.

Columns with theoretical plates ranging from 4,000 plates/meter-100,000 plates/meter are ideal for a good separation system. The theoretical plates were found to be more than 2000 and the tailing factor was <2.0 indicating good column proficiency and peak shape. Degradation studies are performed on drug products under different conditions like slightly acidic, basic, oxidative, thermal and photolytic stress. Each stress condition sample is studied in the proposed method and peak purity data is recorded to check the same nature of the drug (Figures 8-15) (Table 8) [37-40].

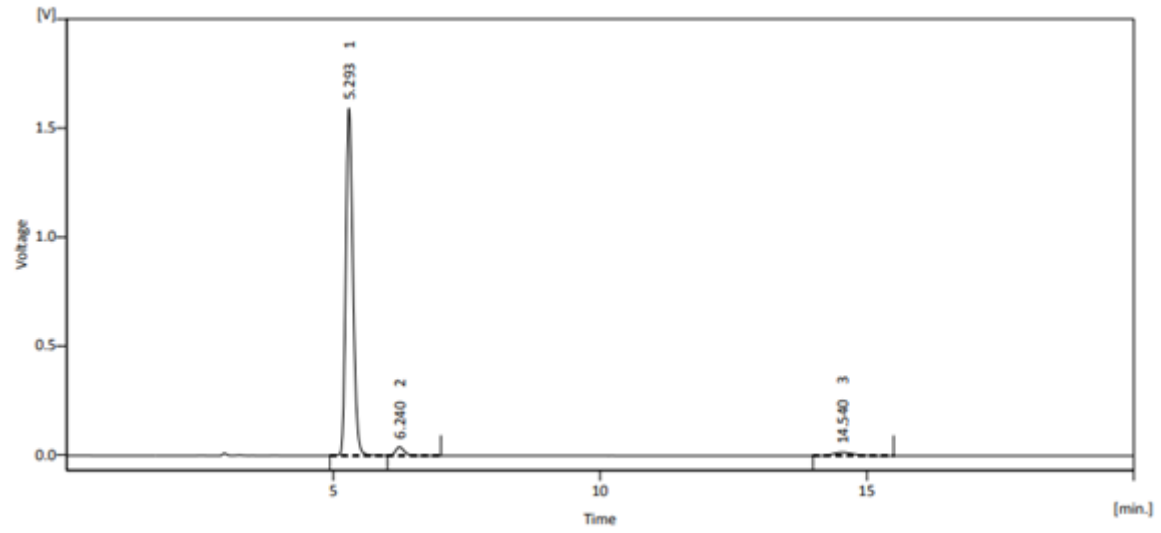

Figure 8: ATZ Standard acid degradation.

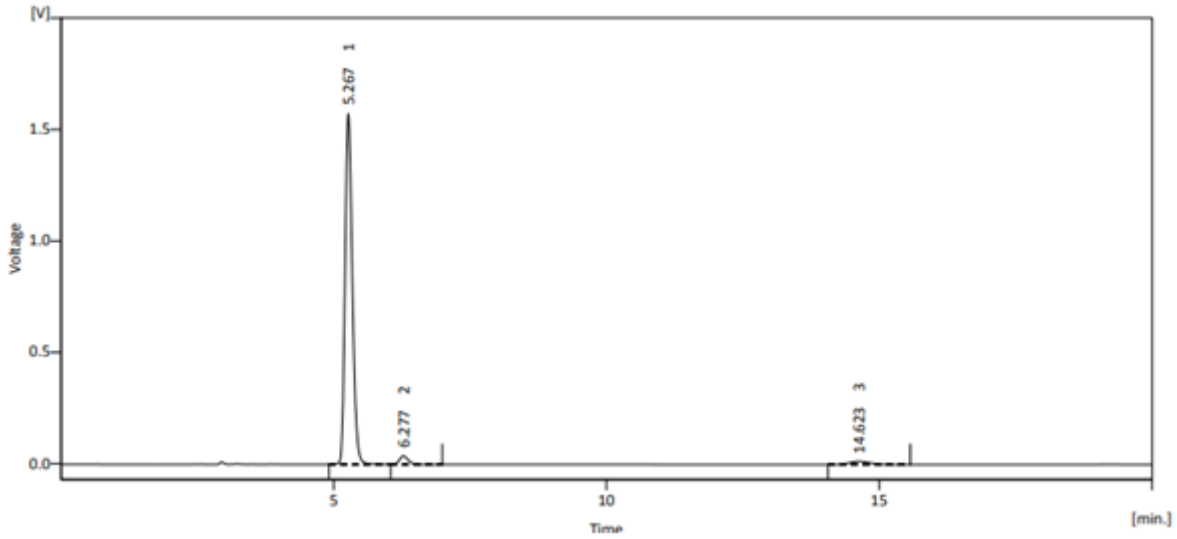

Figure 9: ATZ Sample acid degradation.

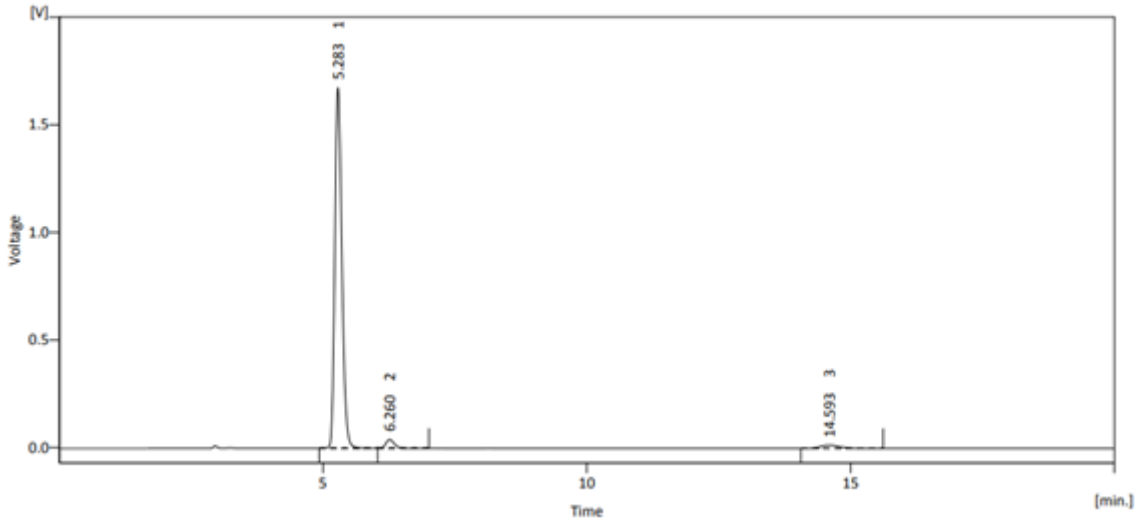

Figure 10: ATZ Standard base degradation.

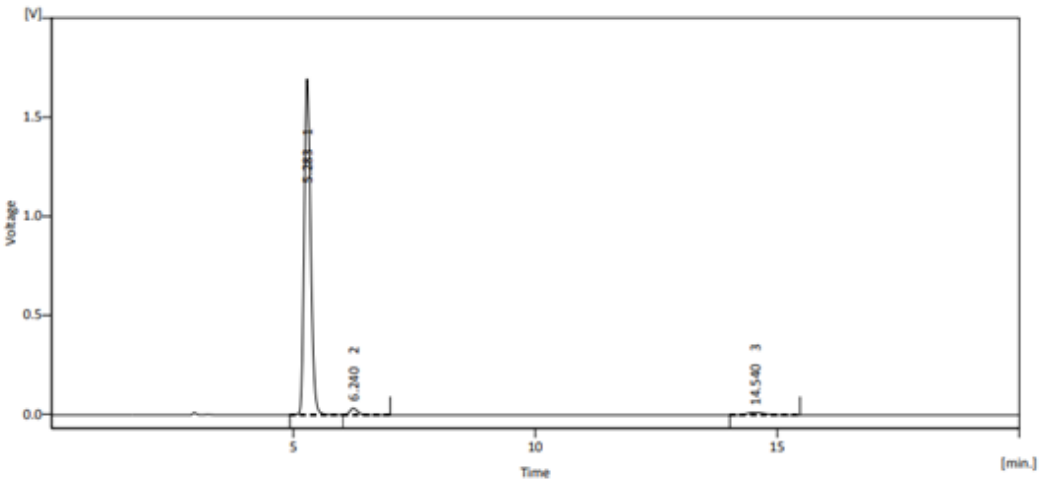

Figure 11: ATZ Sample base degradation.

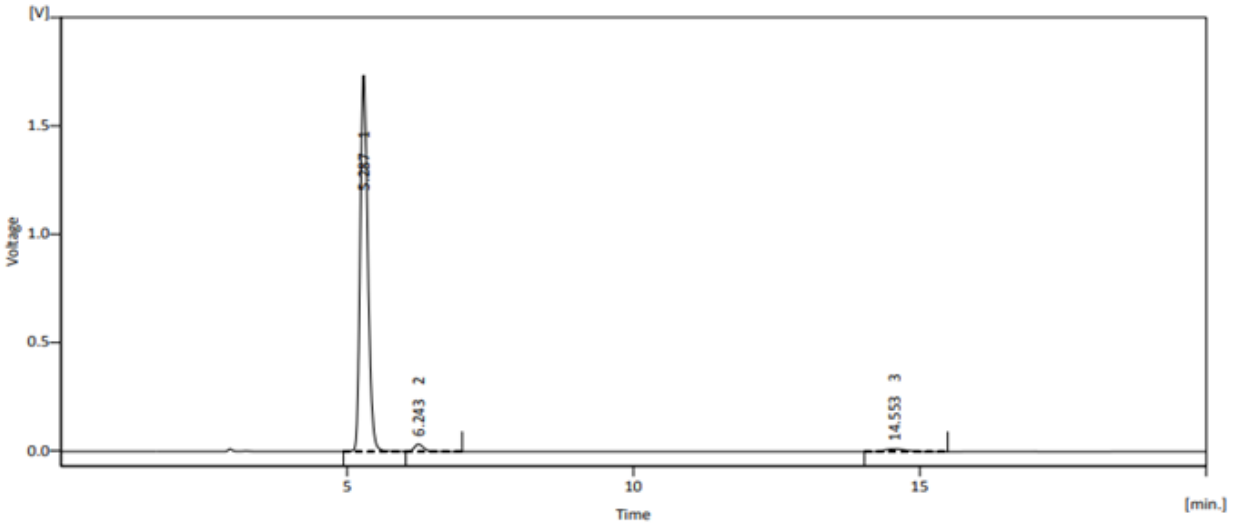

Figure 12: ATZ Standard oxidative degradation.

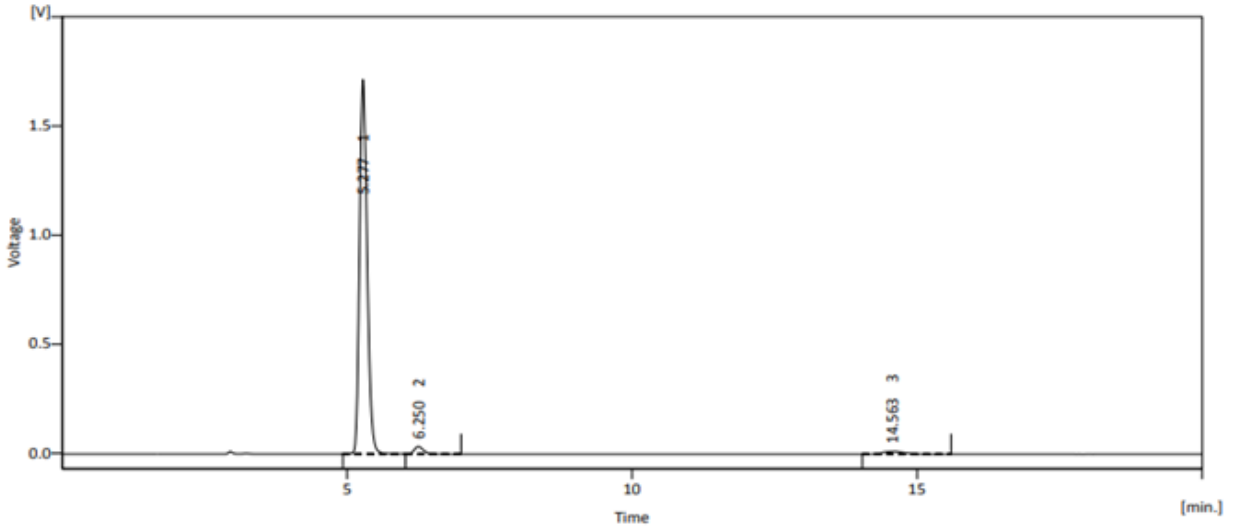

Figure 13: ATZ Sample oxidative degradation.

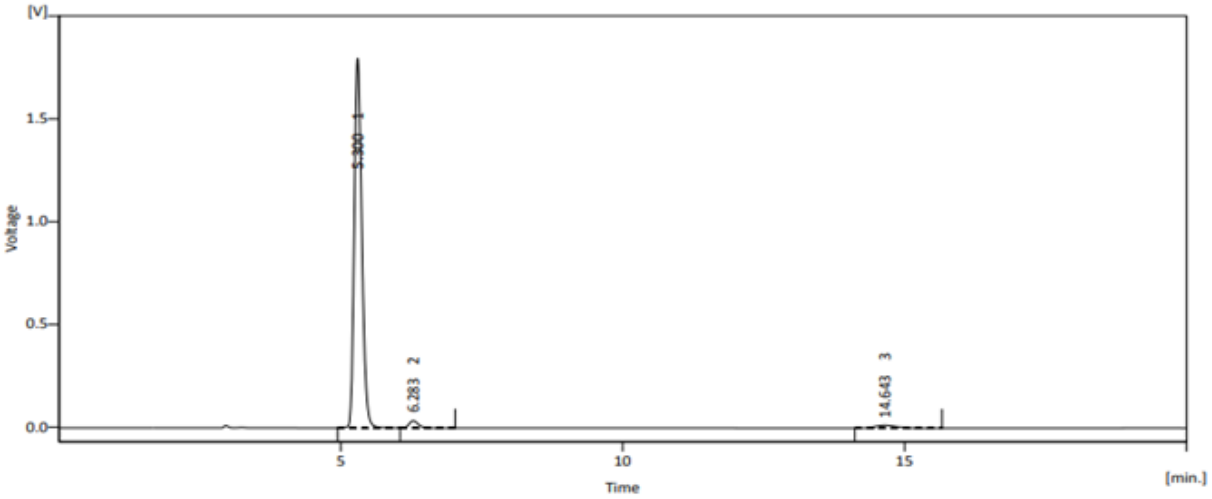

Figure 14: ATZ Standard Photo-degradation.

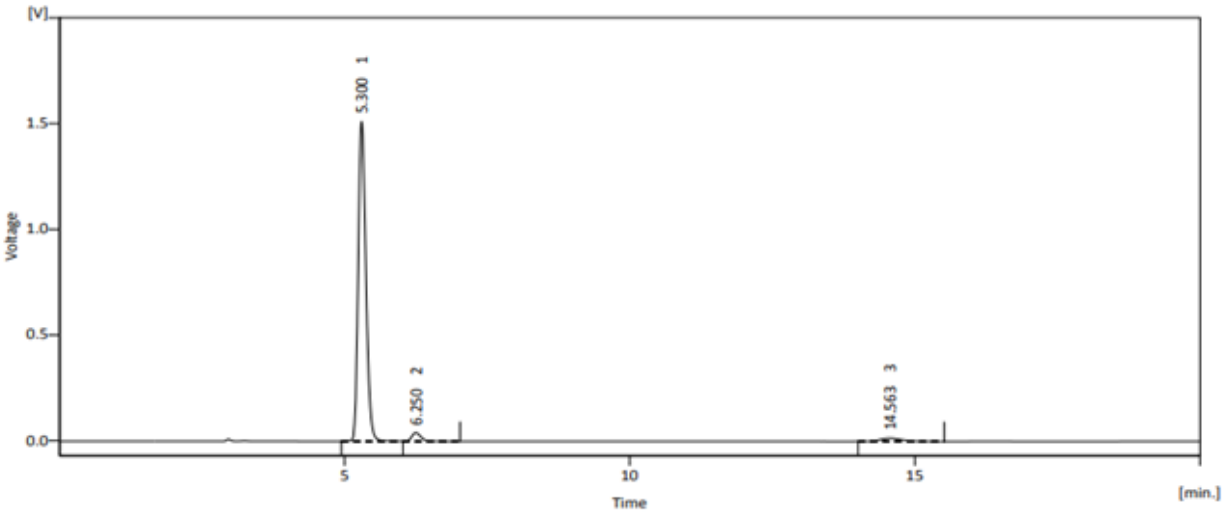

Figure 15: ATZ Sample Photo-degradation.

Table 8: Forced degradation studies of ATZ, impurity A and impurity 5

Sr. No	Stress condition and time	%Standard degradation	%Drug recovered	Std. mean peak area	%Sample degradation	%Drug recovered	Sample. mean peak area
Area of standard-19133.117							
1	Acid hydrolysis (4 hours)	17.99	82.01	14872.066	19.61	76.35	14608.917
2	Alkaline hydrolysis (3 hours)	14.04	85.96	15605.437	13.84	86.16	15795.364
3	Thermal degradation (3 hours)	21.17	78.83	14243.767	21.8	78.2	14123.621
4	Oxidative degradation (48 hours)	11.76	88.24	16190.821	12.85	87.15	15968.178
5	Photolytic degradation (48 hours)	8.45	91.55	16796.639	7.37	92.63	16970.265

Analysis of commercial formulations (Capsules): The proposed method was applied for the determination of ATZ impurities estimation in marketed capsules results of its impurities RSD <5.0%. The results designate that the method is selective for the assay of ATZ without interference from the impurities and other excipients used in these capsules. And the results indicate that the method is selective for the assay of ATZ without intrusion from the impurities used in these capsules. The proposed method was applied for the determination of ATZ in marketed capsules and the result of the assay was found to be in marketed formulation (Atavir 300) in marketed formulation (Atazor-300) 99.73 ± 1.66 and100.57 ± 1.39 respectively (Figure 16) (Tables 9 and 10) [41-43].

Figure 16: Identify the impurities from known and unknown calculations

Table 9: Analysis of commercial formulation

Formulation	Impurity A		Impurity 5		Total impurities	
Brand name	**(Mean ± SD)**	**%RSD**	**(Mean ±SD)**	**%RSD**	**(Mean ±SD)**	**%RSD**
Atavir 300 (Cipla)	0.254 ± 0.003	1.19	0.195 ± 0.005	2.453	0.449 ± 0.007	1.561
ATAZOR-300 (Emcure)	0.256 ± 0.006	2.473	0.199 ± 0.002	1.189	0.455 ± 0.008	1.704

Table 10: Estimation of ATZ

Label claim (mg)	Amount found (mg)	Mean ± SD
Atavir-300 (Cipla)	299.05	99.73 ± 1.66
Atazor-300 (Emcure)	299.12	100.57 ± 1.39

Highlights:

- Method development and validation of ATZ and its impurities
- Identify the known and calculate the unknown impurities
- Force degradation study
- Assay of marketed dosage forms

CONCLUSION

RP-HPLC method for the determination of impurity A and impurity 5 of ATZ in atazanavir sulphate API is robust for a small change in flow and small change in pH of mobile phase and flow plus. The mean recovery for known Impurities is within the limits. Thus, the RP-HPLC method for the determination of impurities of ATZ in atazanavir sulphate API is accurate. The R2 for ATZ, impurity-A and impurity-5 is >0.99. Therefore, the

RP-HPLC method for the determination of impurities of ATZ in atazanavir sulphate API is linear. The % Cumulative RSD is within limits. A simultaneous method for the determination of ATZ and its impurity A and impurity 5 has been developed and validated. Known impurities identify and unknown impurities quantify of marketed dosage form in this method. This method is fast, selective and sensitive for the intended purpose. Selected detection wavelength of 225 nm provides optimum responses and enables the quantification of impurities without placebo interference. Linearity curves drawn for the impurity A and impurity 5 and main drugs were found to have good R^2 values of more than 0.995. Accuracy results were observed in the range of 90%-110% which proved satisfactory recovery. The developed gradient elution of RP-HPLC method is very much useful in the pharmaceutical industry in the modern era with respect to precision, accuracy, sensitivity and efficiency.

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CONFLICT Of INTEREST

This article does not cover any studies with human members or animals performed by any of the authors.

INFORMED CONSENT

This research does not belong to human trial.

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