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A VALIDATED FORCED DEGRADATION METHOD FOR CHARACTERIZATION OF ATAZANAVIR DEGRADANTS BY LC-MS/MS

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Keywords:

Cyclea peltata, HPTLC, Oleanolic acid

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ABSTRACT: The validated stability-indicating analytical method was developed for quantification of Atazanavir using Atazanavir-D9 as an internal standard by UPLC-ESI-MS/MS. The method was validated according to United States Pharmacopeia (USP) guideline with respect to the accuracy, precision, specificity, linearity, solution stability, robustness, sensitivity, and system suitability. Forced degradation study was validated according to International Conference on Harmonisation (ICH). For this, an isocratic condition of mobile phase comprising 10 mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20:40:40%, v/v/v) using the CORTECS C18, 90Å, 2.7 µm, 4.6 mm × 150mm. The total analysis time was 4.0 min, and the flow rate was set to 0.5 ml/min. The mass transitions of Atazanavir and Atazanavir-D9 obtained were m/z 705.3 × 167.9 and 714.9®167.9. The standard curve shows a correlation coefficient (r2) greater than 0.999 with a range of 150.0-450.0 pg/ml using the linear regression model. Forced degradation of the drug product was carried out as per the ICH guidelines with a view to establishing the stability-indicating property of this method and providing useful information about the degradation pathways, degradation products, and how the quality of a drug substance and drug product changes with time under the influence of various stressing conditions.

INTRODUCTION: In HIV-infected patients, virus multiplies within the cells of the body. Viruses are released from the cells and spread throughout the body, and infect other cells. During the production of the viruses, new proteins are made. Protease is the enzyme that forms the new structural proteins and enzymes. ATA and RIT are used to block the activity of protease and results in the formation of defective viruses that are unable to infect the body's cells.

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Due to this, the number of viruses in the body decreases and improves the patient's immunity. From the study of literature, it was found that HPLC methods are available for the estimation of ATA in pharmaceutical formulations and human plasma [1, 2].

Also, HPLC methods are reported for the determination of ATA impurities [3, 4]. HPTLC methods for the content of ATA in tablets are available [7, 9]. HPLC, UPLC, HPLC/HPTLC, HPLC–MS/MS, UPLC–MS/MS methods for the simultaneous determination of ATA and RIT in dosage forms were also reported [10, 19]. The reported methods can be able to quantify the individual drug components of ATA and RIT only. Also, potential impurities of ATA were not identified from the main analyte in the reported methods.

Hence, no liquid chromatographic methods (HPLC and UPLC) were reported for the characterization of ATA impurities in drug substances. In addition to this, characterization degradation products of ATA is not official in any of the pharmacopeia. Since UPLC-MS technique enables improved sensitivity, selectivity, rapid analysis, environment friendly due to reduced solvent consumption.

Fast analytical methods are necessary in the pharmaceutical industry in order to meet narrow timelines for delivering the products as a part of satisfying the customer needs. This task is possible only by the invention of a shorter version of liquid chromatographic methods. Hence, related substances method development was initiated by UPLC to meet these requirements. The main goal of the present study is to develop and validate the novel simple, sensitive, selective, rapid, rugged and reproducible analytical method for the quantitative determination of Atazanavir in drug substances by UPLC-MS/MS using Atazanavir-D9 as an internal standard. The developed method was subjected to forced degradation (FD) studies to check the stability-indicating nature as per International Conference on Harmonization (ICH) conditions. FD studies are able to establish the intrinsic stability of active ingredients in a pharmaceutical dosage formulation and helpful in establishing degradation pathways of finished dosages. These studies can resolve the issues occur during stability exposure by distinguishing degradation products that are related to drug product from those which are produced from excipients present in the formulation. The developed method was validated as per the required parameters of United States Pharmacopeia (USP) and ICH [20, 23].

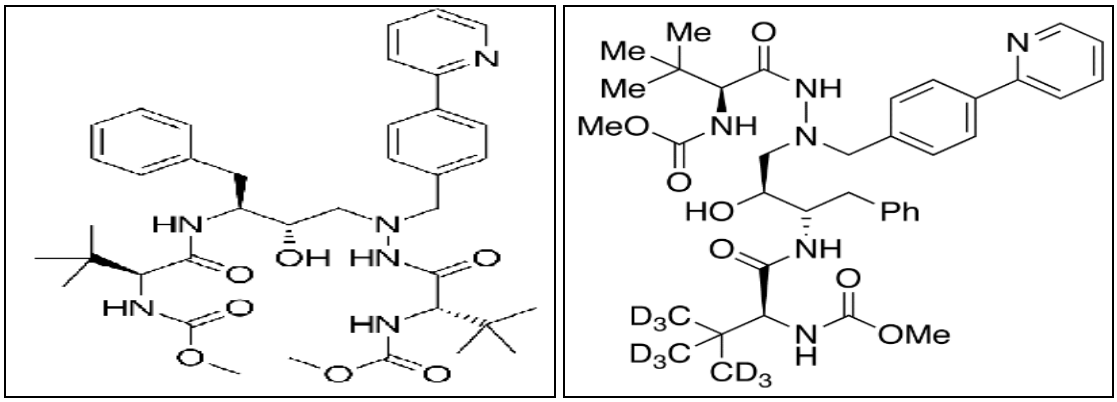

FIG. 1: CHEMICAL STRUCTURES OF A) ATAZANAVIR (ATV) B) ATAZANAVIR-D9 (ATVIS)

MATERIALS AND METHODS:

Materials:

Chemical Resources: Atazanavir (ATV) (ALSA CHIM), Atazanavir-D9 (ATVIS) (ALSACHIM), HPLC grade, Methanol, Acetonitrile (J. T. Baker, Phillipsburg, NJ, USA), Ammonium acetate, Acetic acid (Merck Pvt. Ltd, Worli, Mumbai), Ultrapure water (Milli - Q system, Millipore, Bedford, MA, USA), hydrochloric acid, sodium hydroxide and hydrogen peroxide (3% w/v) used for stress degradation studies are of analytical reagent grade,. The chemicals and solvents were used in this study, analytical and HPLC grade.

Instrument Resources: Chromatographic separation was performed on a Q Sight ® Triple Quad UPLC-ESI-MS/MS system (Perkin Elmer). Combined with Q Sight LX50 UHPLC, data acquisition and processing were accomplished using Simplicity™ 3Q software. Micro balance (ME5 model Sartorius), variable range micro pipette (Eppendorf), Autosampler vials, variable size glass bottles, graduated measuring cylinders, volumetric flasks (Borosil), Ultrasonic bath (Pharmatek Scientifics), Vortexer (Spinix), Refrigerator (LG). Pipette tips 10 µL-1000 µL were employed in the present investigation.

Methods:

Preparation of Standard Stock Solution: Standard stock solution of Atazanavir (1.0 mg/mL) and Atazanavir-D9 (1.0 mg/mL) were prepared by accurately weighing about 10 mg and transferring in to 10 mL volumetric flask and dissolved in Methanol. All stock solutions were stored in refrigerated conditions (2-8 °C) until analysis.

Preparation of 10 mM Ammonium Acetate Buffer (pH: 4.0): Weighed about 0.770g of ammonium acetate, transferred to 1000.0 mL volumetric flask, and makeup to volume (1000.0 mL) with ultra-pure water. Finally, the pH of the solution was adjusted to pH: 4.0 with acetic acid and filtered through a 0.45µ membrane disc filter, and sonicated to degas.

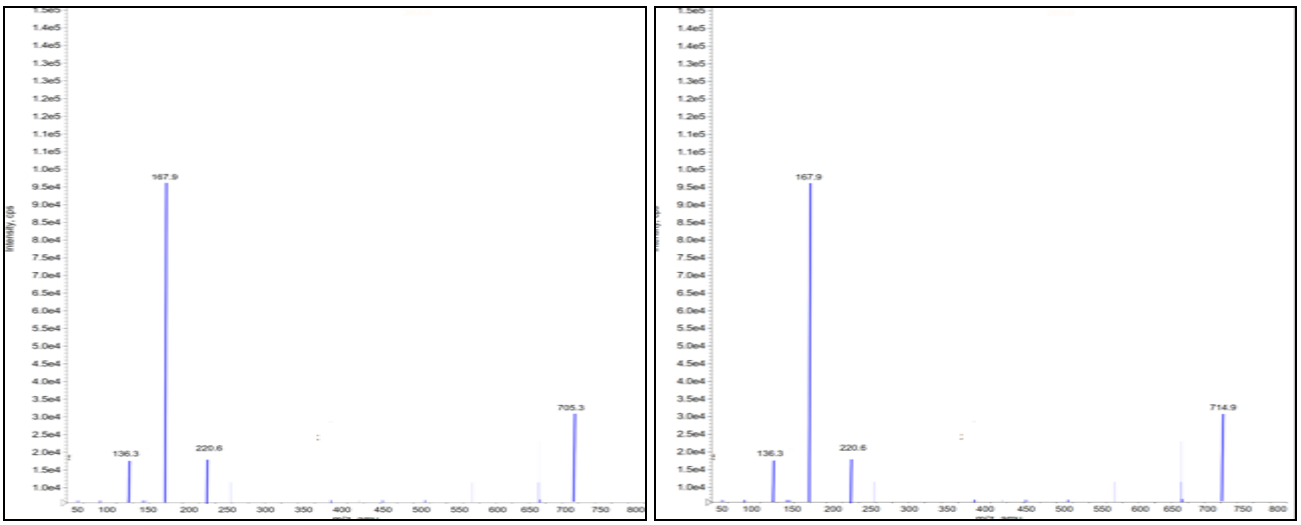

FIG. 2: PARENT ION MASS SPECTRA (Q1) AND (Q3) OF ATAZANAVIR AND ATAZANAVIR-D9

Preparation of Mobile Phase: The mobile phase used was 10 mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20: 40: 40%, v/v/v) and the mobile phase was filtered through 0.45µ membrane filter and sonicated before use.

Preparation of Internal Standard Spiking Solution: The Atazanavir -D9 (internal standard) spiking solution (200.00 pg/mL) was prepared from standard stock solution of Atazanavir-D9 (1000.00 µg/mL) in the mobile phase (10 mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20: 40: 40%, v/v/v)). Internal standard spiking solution (Atazanavir-D9) was stored in refrigerated conditions (2-8 °C) until analysis.

Preparation of Standard Solutions: Standard solutions of different concentrations of Atazanavir (ATV) were prepared from Atazanavir stock solution (1000 µg/mL) in mobile phase 10mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20:40:40%, v/v/v)). To each aqueous standard solution, 100 µL of 200.00 pg/mL of Atazanavir-D9 was added and vortexed for 5 min and injected into the UPLC-ESI-MS/MS for analysis.

Method Development: For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate mass parameters and choice of stationary and mobile phase, Internal standard, extraction solvent. The following studies were conducted for this purpose.

Selection of Internal Standard: For the selection of internal standard, Abacavir, Famciclovir, Entecavir, Valcyclovir, Nevirapine, Acyclovir, Adefovir were tried with optimized mobile phase and column conditions. Finally, Atazanavir-D9 was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. The peak elution times for the Atazanavir and Atazanavir-D9 were found at 1.62 and 0.56 min.

Optimization of Mass Spectroscopic Parameters: The pure drug of Atazanavir (ATV) and Atazanavir-D9 (ATVIS) were prepared in methanol (100.00 pg/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra-high pure nitrogen gas), EP, DP, CE, FP, and CXP were optimized.

The analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 705.3 × 167.9 and 714.9®167.9 for ATV and ATVIS.

The mass spectra of parent and product ions were depicted in **Fig. 2**.

Optimized Chromatographic conditions: Several systematic trials were performed by varying stationary phases and mobile phase ratios to achieve ideal chromatographic conditions.

The chromatographic separation was achieved with 10 mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20: 40: 40%, v/v/v) using the CORTECS C18, 90Å, 2.7 µm, 4.6 mm × 150 mm gave the best peak shape and low baseline noise was observed. The total analysis time was 4.0 min, and flow rate was set to 0.5 ml/min. The temperature was set to 40 °C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

After completion of several systematic trials, a sensitive, precise, and accurate UPLC-MS/MS method was developed for the analysis of Atazanavir (ATV) using Atazanavir-D9 as internal standard. The chromatograms of blank (Mobile phase), LOQ, and ULOQ standards were shown in **Fig. 3, 5**. This was followed by method validation.

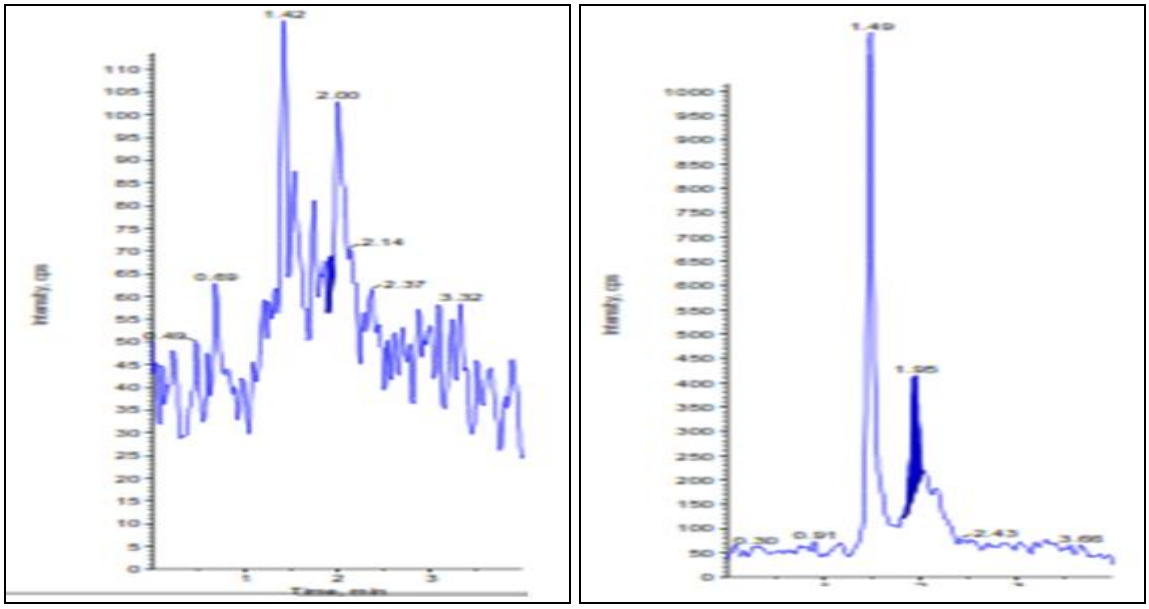

FIG. 3: BLANK CHROMATOGRAM (MOBILE PHASE)

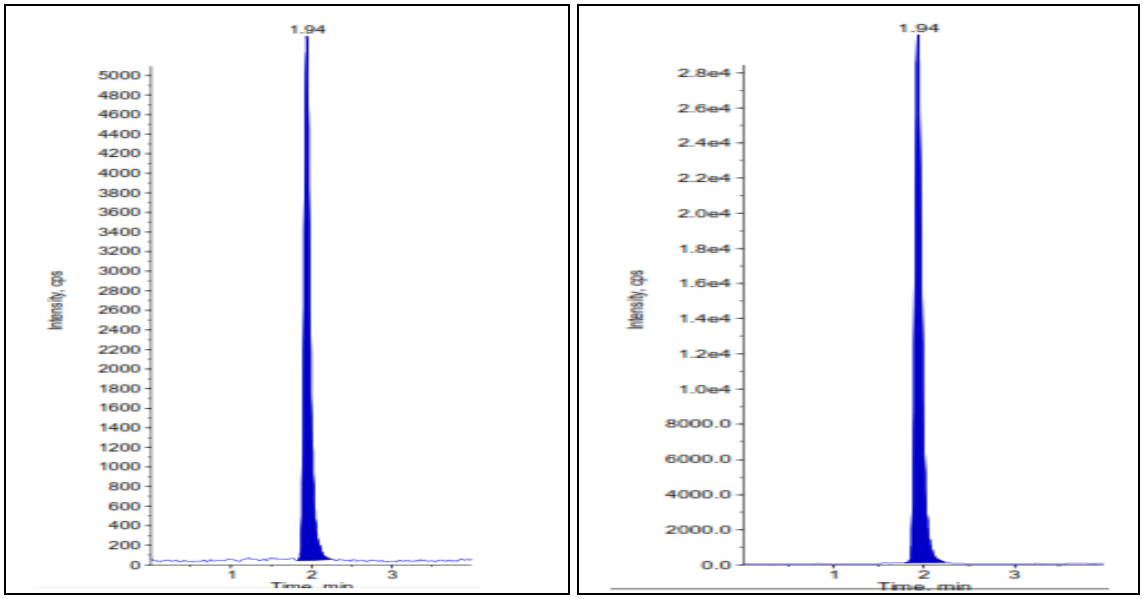

FIG. 4: STANDARD CHROMATOGRAM OF LOQ SAMPLE (50% LINEARITY LEVEL) (ATV AND ATVIS)

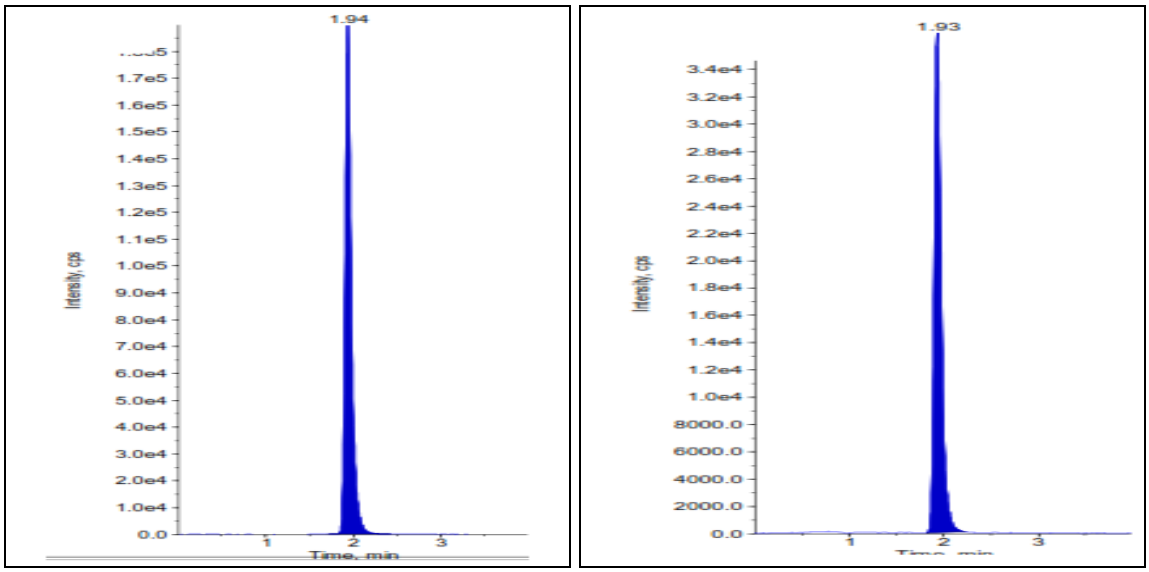

FIG. 5: STANDARD CHROMATOGRAM OF ULOQ SAMPLE (150% LINEARITY LEVEL) (ATV AND ATVIS)

Method Validation: The developed method was validated over a linear concentration range of 150.0-450.0 pg/ml. The validation parameters include selectivity and specificity, LOQ, linearity, precision and Accuracy, Robustness, Recovery, Stability in solution was evaluated under the validation section.

System Suitability: Six replicate injections of aqueous standard 100% level (300.0 pg/mL) along with internal standard (200.0 g/mL) were injected into UPLC-MS/MS, and %RSD was calculated.

Selectivity & Specificity: The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively, the method is called selective. It has been observed that there were no peaks of diluents and placebo at main peaks. In order to test the interference at the retention time of Atazanavir & Atazanavir-D9 & blank samples (Mobile phase) were injected into UPLC-MS/MS.

Hence, the chromatographic system used for the estimation of Atazanavir & Atazanavir-D9 was very selective and specific. Specificity studies indicating that the excipients did not interfere with the analysis. The standard solution showed an asymmetric peak with retention times of 1.94 ± 0.01 min for Atazanavir & Atazanavir-D9.

Linearity and Range: The linearity of calibration curve for Atazanavir was assessed at 50% to 150 % of the target concentration at different levels in the range of 150.0 pg/mL to 450.0 pg/mL in aqueous standards. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained from the least-squares linear regression presented with their correlation coefficient.

TABLE 1: CALIBRATION CURVE DETAILS OF ATAZANAVIR (ATV)

Linearity Level (%)	Nominal Conc.(pg/mL)	Atazanavir (ATV) Mean Peak Area (n=3)	Atazanavir-D9 (ATIS) Mean Peak Area (n=3)	Mean Peak Area Ratio (n=3)
50	150.00	7877	52345	0.150
70	200.00	11027	52142	0.211
90	250.00	14178	51939	0.273
100	300.00	15753	52832	0.298
125	375.00	19691	52431	0.376
150	450.00	23630	52830	0.447
		Correlation coefficient		0.99925
		Y-Intercept		-0.000139
		Slope		0.00099
		Standard Error		0.00443

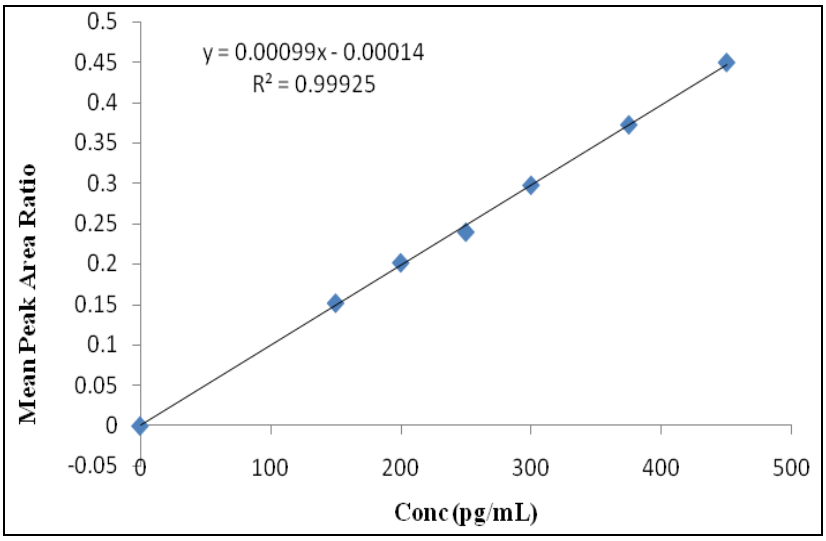

FIG. 6: CALIBRATION CURVE FOR ATAZANAVIR (ATV)

Precision: The intra-day data reflects the precision and accuracy of the method under the same conditions within one day. Intra-day accuracy and precision were obtained by analyzing ten replicates of three different standard samples (200, 300, and 375 pg/mL). Accuracy was determined by the regressed (measured) concentration represented as a percentage of the target (nominal) concentration. The percent relative standard deviation (% RSD) of the regressed (measured) concentrations were used to report precision. The inter-day precision and accuracy were verified by repeating the above procedure on three different days.

TABLE: 2: PRECISION AND ACCURACY OF ATAZANAVIR (ATV) AT THREE DIFFERENT CONCENTRATIONS

Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Mean Concentration measured (n=10;pg/ml; mean ±S.D)	%CV	%Accuracy	Mean Concentration measured (n=30;pg/ml;mean ± S.D)	%CV	%Accuracy
200.00	200.51±0.44	0.22	100.25	200.20±0.26	0.13	101.03
300.00	300.53±0.29	0.10	100.18	300.36±0.29	0.10	100.12
375.00	375.43±0.24	0.06	100.12	376.23±0.57	0.15	100.33

Limit of Detection (LOD) And Limit of Quantification (LOQ): Limit of detection (LOD) and limit of quantification (LOQ) of Atazanavir was determined by the calibration curve method. Solutions of Atazanavir (ATV) were prepared in linearity range and injected in triplicate. The average peak area ratio of three analyses was plotted against concentration. LOD and LOQ were calculated by using the following equations:

$$LOD = 3.3 \times S; \quad LOQ = 10 \times S;$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Robustness: The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, temperature and determine the effect (if any) on the results of the method.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. Robustness was carried by carried out by varying the method parameters like flow rate (± 5%), Column temperature (± 5%), and pH (± 2%). Six replicate injections of aqueous standard 100% level (300.0 pg/mL) along with internal standard was injected into UPLC-MS/MS, and %RSD was calculated.

TABLE 3: ROBUSTNESS OF ATAZANAVIR (ATV)

Validation Sample	%RSD		
	Flow Rate (± 5%)	Column Temp (± 5%)	pH (± 2%)
Atazanavir (300.0 pg/mL)	0.19	0.09	0.25

Stability: The stability of Atazanavir & Atazanavir-D9 in aqueous standards was performed using two replicates of 300.0 pg/mL and 200.00 pg/mL at ambient and refrigerated conditions with different time intervals.

Solution Stability: A Atazanavir at a concentration of 300.0 pg/mL solution and Atazanavir-D9 (IS) solution at 200.0 pg/mL were prepared from fresh stock solutions. A portion of the freshly prepared standard solutions (Atazanavir & Atazanavir-D9) were kept at ambient temperature (25 °C) for 48 h and then analyzed by the proposed method. A second portion of the freshly prepared standard solutions (Atazanavir & Atazanavir-D9) were stored at refrigerated temperature (between 2 °C and 8 °C) for 48 h and then analyzed. The results were compared with those obtained from samples analyzed at the initial moment (0.0 h).

TABLE 4: SOLUTION STABILITY DATA OF AT AZANAVIR & ATAZANAVIR-D9

Stability Sample	Ambient temperature		Refrigerated Temparature	
	%Difference at 0.0 H	%Difference at 48.0 H	%Difference at 0.0 H	%Difference at 48.0 H
Atazanavir (300.00pg/mL)	0.00	0.0375	0.00	0.0539
Atazanavir-D9 (200.00pg/mL)	0.00	0.7392	0.00	0.9021

Filter Validation (Filter Interference: A Atazanavir (ATV) at a concentration of 300.00 pg/mL solution and Atazanavir-D9 (ATVIS) solution at 200.00 pg/mL of were prepared from fresh stock solutions. Some portion of Atazanavir (ATV) and Atazanavir-D9 (ATVIS) standard solutions (300.00 pg/mL and 200.00 pg/mL) was filtered through three different filters, namely 0.45µm PVDF filter, 0.45µm PTFE, and 0.45µm Nylon filter and some portion was centrifuged and injected into the UPLC-MS/MS system.

TABLE 5: FILTER INTERFERENCE RESULTS OF AT AZANAVIR & ATAZANAVIR-D9

Validation Sample	%Difference		
	0.45µm Nylon	0.45µm PVDF	0.45µm PTFE
Atazanavir (300.00pg/mL)	0.11	0.15	-0.04
Atazanavir-D9 (200.00pg/mL)	0.239	0.413	0.562

Forced Degradation Studies:

Preparation of Un-Stressed Sample: Stock solution of Atazanavir (1000 µg/ml) was prepared by dissolving an accurately weighed 25 mg of the sample and dissolved in 25 ml of the diluent in a volumetric flask. Atazanavir (100 ng/ml) solution was prepared with mobile phase and injected into the UPLC-MS/MS System to study the unstressed sample.

Preparation of Degradation Solutions: A total of six degradation samples were prepared for every stress condition. The samples were further diluted with mobile phase followed by filtration through 0.45 µ membrane filter before UPLC-MS/MS analysis.

Preparation of Acid-Induced Degradation Sample: Weighed about 10 mg of Atazanavir was taken in a 10 ml volumetric flask and dissolved 25 ml methanol and diluted with 0.1 N HCl. The solution was set aside for 72 h at ambient temperature. After degradation, the solution was diluted with the mobile phase to get the final concentration of 100 ng/mL and injected into UPLC-MS/MS system.

Preparation of Alkali Induced Degradation Product: Weighed about 10 mg of Atazanavir was taken in a 10 ml volumetric flask and dissolved 10 ml methanol and diluted with 0.1 N NaOH. The solution was set aside for 72 h at ambient temperature. After degradation, the solution was

diluted with the mobile phase to get the final concentration of 100 ng/mL and injected into UPLC-MS/MS system.

Preparation of Hydrogen Peroxide Induced Degradation Product: Weighed about 10 mg of Atazanavir was taken in a 10 ml volumetric flask and dissolved 10 ml methanol and diluted with 3% v/v H_2O_2. The solution was set aside for 72 h at ambient temperature. After degradation, the solution was diluted with the mobile phase to get the final concentration of 100 ng/mL and injected into UPLC-MS/MS system.

Thermal (Dry Heat) Degradation Product: The 10 mg Atazanavir was taken on Petri dishes (10 cm in diameter) and spread as a thin layer of 1 mm, and exposed to 80 ºC for 72 h. After degradation, the degradation sample was dissolved in methanol and diluted with mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

Photolytic Degradation Product: Weighed about 10 mg of Atazanavir was taken in a 10 ml volumetric flask and dissolved 10 ml methanol. The solution was exposed to UV radiation (254 nm) at 1.2 million lux-hours for 72 h (using the photostability chamber Thermolab 400G, New Delhi, India). After degradation, the solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

Neutral Degradation Studies: Weighed about 10 mg of Atazanavir was taken in a 10 ml volumetric flask and dissolved with methanol and diluted to 10 mL with water. The solution was set aside for 72 h at ambient temperature. After degradation, the solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

RESULTS AND DISCUSSION:

Method Development: The main aim of this work was to develop a rapid, selective, and sensitive analytical method for quantitative analysis of Atazanavir in standard samples. In optimizing the chromatographic conditions, the ammonium acetate buffer solution was adopted in the mobile phase of the UPLC in order to suppress the tailing phenomena of chromatographic peaks of Atazanavir and Atazanavir-D9. Besides, the concentration of the ammonium acetate buffer was investigated, and the concentration of 10mM Ammonium Acetate Buffer (pH: 4.0) made the chromatographic peaks sharp and symmetric. The acceptable retention and separation of Atazanavir and Atazanavir-D9 were obtained by using an elution system of 10 mM Ammonium Acetate Buffer (pH: 4.0): Methanol: Acetonitrile, (20: 40: 40%, v/v/v) as the mobile phase.

The UPLC/MS/MS method described here satisfies the requirement of routine analyses since it has a short run time (4.0 min). The MS optimization was performed by direct injection of Atazanavir and Atazanavir-D9 into the mass spectrometer. The mass parameters were optimized to obtain better ionization of Atazanavir and Atazanavir-D9 molecules. The full scan spectrum was dominated by protonated molecules [M+H] m/z 705.3 and 714.9 for Atazanavir and Atazanavir-D9, and the major fragment ions observed in each product spectrum were at m/z 167.9.

Method Validation:

System Suitability: The system suitability parameter can be defined as a test to ensure that the method can generate precise results. In this method %, RSD value obtained was less than 2%.

Selectivity and Specificity: No significant response was observed at retention times of Atazanavir and Atazanavir-D9 in the mobile phase. It can be concluded that the method is specific for the estimation of Atazanavir in the presence of a solvent. The chromatograms of blank samples (Mobile phase) Atazanavir Standards were shown in **Fig. 3**, **5**.

Linearity: Linearity was plotted as a peak area ratio (Atazanavir peak area Atazanavir-D9 peak area) on the y-axis against Atazanavir concentration (pg/ml) on the x-axis. The correlation coefficient for Atazanavir over the concentration range of 150.0 to 450.0pg/mL was 0.9992 **Table 1** and **Fig. 6**. The regression equation for Atazanavir was, y = 0.00099x - 0.00014. Linearity was found to be quite satisfactory and reproducible.

Precision & Accuracy: The precision of the proposed method was evaluated at three different concentration levels, and Accuracy and % RSD for

each concentration value obtained was calculated. Intermediate precision was carried out by analyzing the samples by a different analyst on different days. No statistically significant difference was observed. The % RSD and accuracy was found to be 0.06 to 0.22 and 100.12 to 100.25% for intraday precision. Whereas, for interday precision % RSD and accuracy were found to be 0.10 to 0.15 and 100.10 to 100.33%. The results were summarized in **Table 2**.

LOD and LOQ: The detection and quantification limits were evaluated from the calibration curve plotted in the concentration range of 150.0 – 450.0 pg/mL. LOD and LOQ for this method were found to be 14.70 and 44.50 pg/mL, respectively. These values indicated that the method was sensitive to quantify the drug.

Robustness: The %RSD of Atazanavir was good under most conditions and didn't show any significant change when the critical parameters were modified and the components (Analyte and IS) were well separated under all the changes carried out **Table 3**. Thus the method conditions were robust.

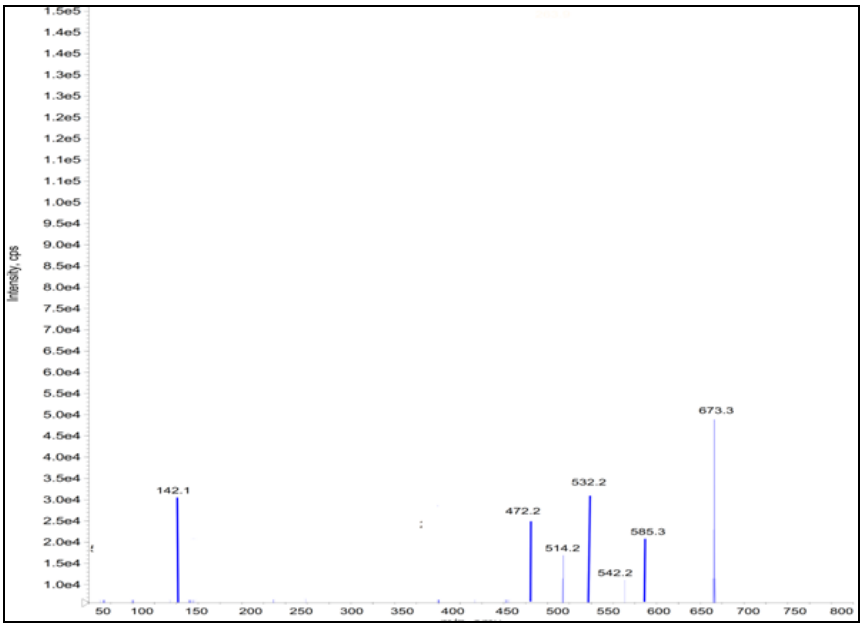

FIG. 7: FRAGMENTATION MASS SPECTRUM OF DEGRADATION PRODUCT FORMED IN ACID HYDROLYSIS OF ATAZANAVIR

Solution Stability: The processing and storage conditions of samples need to maintain the integrity of a drug or at least keep the variation of pre-analysis as minimal as possible. For this reason, stability studies play an important role in analytical method development. In this study, the stability was assessed by considering stock solution stability for Atazanavir and Atazanavir-D9. **Table 4** shows that stable under the studied conditions (Ambient and Refrigerated conditions). Since, in all conditions, the % Difference values were smaller than 2%.

Filter Validation (Filter Interference: The % Difference values for Atazanavir and Atazanavir-D9 of different filter materials was found to be 0.04 to 0.15% and 0.23 to 0.56 **Table 5,** and no significant interference was observed.

Identification of Major Degradation Product Formed under Stress Conditions by UPLC-MS/MS: The fragmentation for the degradants was also carried out for Atazanavir using product ion scan by UPLCMS/MS. In these stress studies, a total of three degradation products (D1–D3) were observed for Atazanavir, among these products three degradation products, D1 (acidic), D2 (Basic), and D3 (Peroxide). Under oxidative degradation, the percentage degradation of the drug was 100%, and complete degradation occurred for acidic and basic hydrolysis after 72 h. Under acidic hydrolysis, the Atazanavir was degraded after 72 h with the formation of the molecular ion. There was no degradation observed under neutral hydrolysis up to 72 h. The degradation products of acid and base hydrolysis were analyzed by UPLC–MS-MS, the total ion chromatograms of both degradates was

showing molecular ions at m/z 673.3 and 627.3, indicating the presence of the degradation product in both cases. The oxidation stress conditions were studied using 3% v/v H_2O_2 up to 72 h at room temperature.

It was found that 3% v/v H_2O_2 was effective in oxidizing the drug even after 72 h. The oxidation degradant product has a molecular ion at m/z 641.3, and its fragmentation ions were shown in **Fig. 8**. No neutral, thermal, and photodegradation were observed for the solution and solid form of the drug after exposure to the water, 80 ºC, UV light up to 72 h, respectively.

It was confirmed that the drug was found to be stable solid as well as in solution forms under neutral, photolytic and thermal stress conditions. The respective degradation of m/z values of all degradate and their fragmentation ions represented in **Table 6** and **Fig. 7** to **9**.

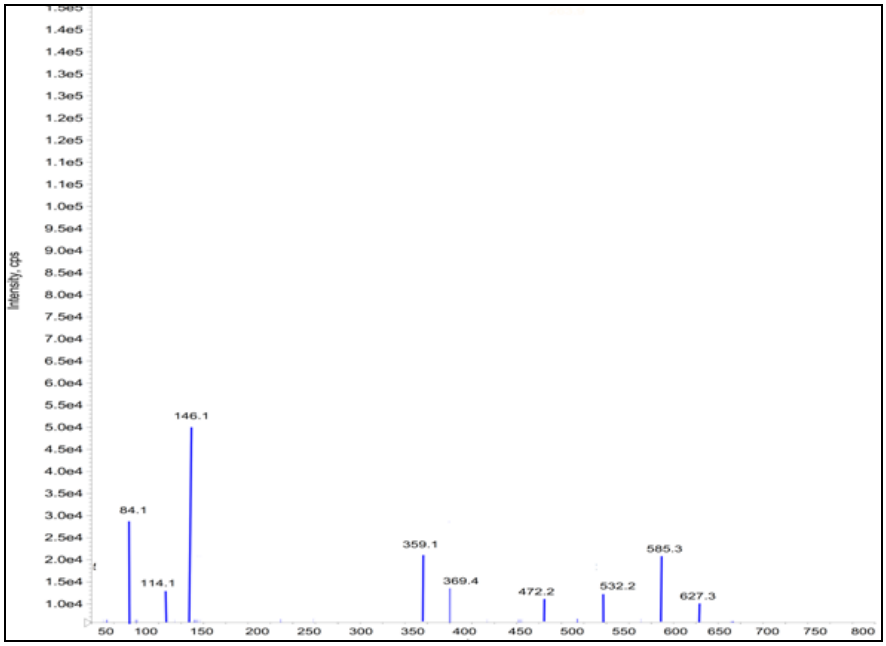

FIG. 8: FRAGMENTATION MASS SPECTRUM OF DEGRADATION PRODUCT FORMED IN BASE HYDROLYSIS OF ATAZANAVIR

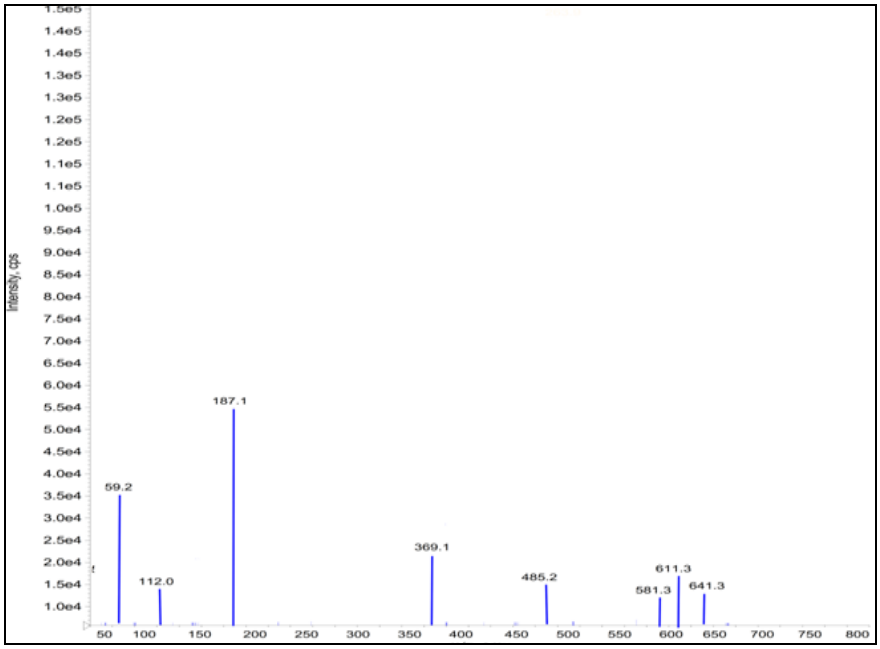

FIG. 9: FRAGMENTATION MASS SPECTRUM OF DEGRADATION PRODUCT FORMED IN OXIDATIVE (PEROXIDE) DEGRADATION OF ATAZANAVIR

TABLE 6: ATAZANAVIR MASS DEGRADATION PROFILE

S. no	Acid Degradation Fragment ions (m/z, amu)	Base Degradation Fragment ions (m/z, amu)	Peroxide Degradation Fragment ions(m/z, amu)
1	142.1	84.1	59.2
2	472.2	114.1	112.0
3	514.2	146.1	187.1
4	514.2	359.1	369.1
5	532.2	369.1	485.2
6	542.2	472.2	581.3
7	585.3	532.2	611.3
8	Degradation product-I (673.3)	Degradation product-II (627.3)	Degradation product-III (641.3)

TABLE 7: DEGRADATION BEHAVIOR OF ATAZANAVIR

Stress condition	Strength of stress	Temperature	Duration	% Degradation	Inference
Acid Hydrolysis	0.1M HCl	Ambient	72 h	100.00	Complete degradation
Basic Hydrolysis	0.1M NaOH	Ambient	72 h	100.00	Complete degradation
Oxidative/Peroxide degradation	3% v/v H_2O_2	Ambient	72 h	100.00	Complete degradation
Thermal	-	80 ºC	72 h	0.00	Degradation not observed
Photolytic Studies (UV-Light)	UV lamp (254 nm) at 1.2 million lux h	Ambient	72 h	0.00	Degradation not observed
Neutral (Water)	Water	Ambient	72 h	0.00	Degradation not observed

CONCLUSION: An UPLC-ESI-MS/MS method for quantification of Atazanavir has been successfully developed and validated. The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, robustness, stability in various matrices. The method is sensitive enough for quantitative detection of the analyte in pharmaceutical preparations.

The degradation behavior of Atazanavir under various stress conditions (hydrolysis, oxidation, photolysis, thermal and neutral) was studied. Since the developed method can be used for checking the quality of Atazanavir in its stability samples, it was inferred that Atazanavir was found to be unstable in hydrolytic (basic and acid) and oxidative conditions and stable in neutral, photolysis, and thermal stress conditions. A total of three unknown degradants were identified and characterized the accurate mass measurements using UPLC–ESI–MS-MS.

In addition, the reported method has a short analysis run time and deuterated internal standard, an advantage over previously reported methods. The proposed method can also be used for routine analysis, quality control, stability studies, and suitable for therapeutic drug monitoring (pharmacokinetic or bioequivalence studies) of pharmaceutical tablets containing Atazanavir. This method may be useful in further investigation and characterization of other process-related impurities and helps in confirming the identity of degradation products formed. The possibility of further research for this developed method is to synthesize and develop reference standards and monitor their presence in the stability samples from the identified degradation products.

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