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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ATAZANAVIR SULFATE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

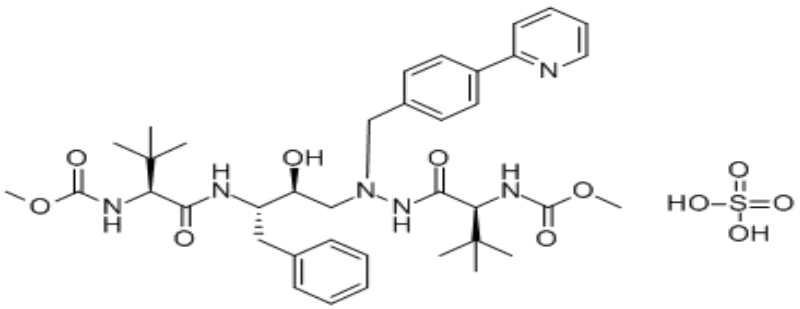
The chromatographic conditions were successfully developed for the separation of Atazanavir sulfate by Agilent C_{18} Column (250mm x 25mm)5µm, flow rate was 1ml/min, mobile phase ratio was Methanol: Acetonitrile: Buffer (45:35:20 v/v), detection wavelength was 249 nm. The Spectroscopic method was done in solvent using mobile phase and the instrument Systronics 1170 with UV win software. The instrument used was Agilent HPLC, Separation module 2695, Uv-Vis detector, Empower-software version 2. The retention time was found to be 3.73 min. The system suitability parameters for Atazanavir sulfate such as theoretical plates and tailing factor were found to be 6754, 1.62. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1))[1]. The linearity study of Atazanavir sulfate using internal standard was found in concentration range of 25 µg-150 µg and correlation coefficient (r^2) was found to be 0.999 respectively, % recovery was found to be 100.5% respectively. % RSD for repeatability and precision was found to be <2. LOD value was 50 ng/mL and LOQ value was found to be 140 ng/mL respectively for Atazanavir sulfate.

KEYWORDS: Atazanavir sulfate, HPLC.

1. INTRODUCTION

Atazanavir[2] (ATV) is an azapeptide HIV-1 protease inhibitor (PI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1. Atazanavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. Atazanavir is pharmacologically related but structurally different from other protease inhibitors and other currently available anti retrovirals.

Atazanavir selectively inhibits the virus-specific processing of viral Gag and Gag-Pol poly proteins in HIV-1 infected cells by binding to the active site of HIV-1 protease[3]. Atazanavir is not active against HIV-2.



Atazanavir

2. MATERIALS AND METHOD

Apparatus
The instrument used for the study was Agilent HPLC, Separation module 2695, Uv-Vis detector with Empower-software version-2.

Reagents and Materials
The solvents used were Methanol, Acetonitrile, Sodium dihydrogen ortho phosphate, Disodium hydrogen ortho phosphate, Tri Ethyl Amine, Ortho phosphoric acid and HPLC Water.

Selection of chromatographic condition
Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drug selected in the present study is polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

Selection of detection wavelength
The sensitivity of method that uses Uv-Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected.

Standard solution of Atazanavir sulfate was scanned in the UV range (200-400nm) and the spectrum was recorded. From the spectrum, 249 nm was selected as the detection wavelength for the present study.

Selection of mobile phase
Initially the mobile phase tried was methanol and water, methanol and Acetonitrile, buffer and water in various proportions. Finally, the mobile phase was optimized to Methanol: Acetonitrile: Sodium dihydrogen ortho phosphate buffer in proportion of 45:35:20 v/v respectively.

Chromatographic condition (Optimized Method)

Column	: Agilent (25×250mm) 5µ
Mobile phase ratio	: Methanol: ACN: Phosphate Buffer (45:35:20 % v/v)
Detection wavelength	: 249 nm
Flow rate	: 1.0 ml/min
Injection volume	: 10µl
Column temperature	: Ambient
Auto sampler temperature	: Ambient
Run time	: 10min
Retention time	: 3.73 min

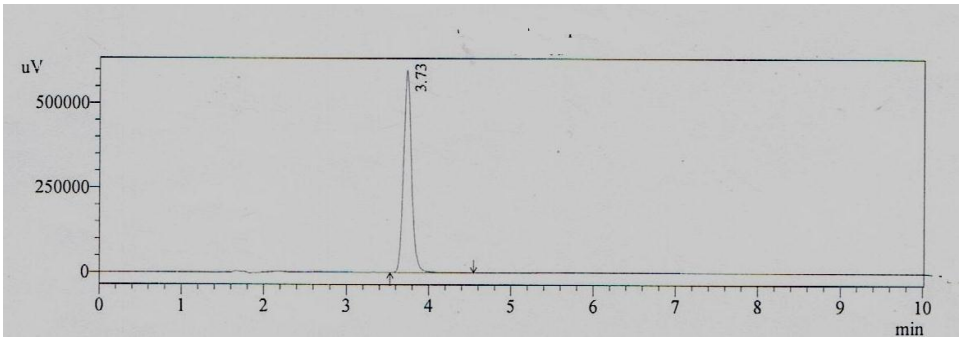


Fig. 1: Chromatogram of Atazanavir sulfate.

Procedure
Preparation of Buffer
About 7.0 g of Sodium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and pH 4.0 was adjusted with Orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase
Mix a mixture of 200 ml of Phosphate buffer (20%), 450 ml of Methanol (45%) and 350 ml of Acetonitrile (35%) degassed in ultrasonic water bath for 5 minutes. Filter through 0.22 µ filter under vacuum filtration. Mobile phase was used as the diluent.

Preparation of Atazanavir sulfate standard preparation
10 mg of Atazanavir sulfate working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and add about 2 ml of diluent and sonicated to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1.0 ml from the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

Sample solution preparation
10 mg of Atazanavir sulfate tablet powder was accurately weighed and transferred into a 10 ml clean dry volumetric flask, add about 2ml of diluent and sonicated to dissolve it completely and making volume up to the mark with the same solvent(Stock solution). Further pipette 10ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

3. RESULTS AND DISCUSSION

Method Validation Parameters

1. Specificity

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.

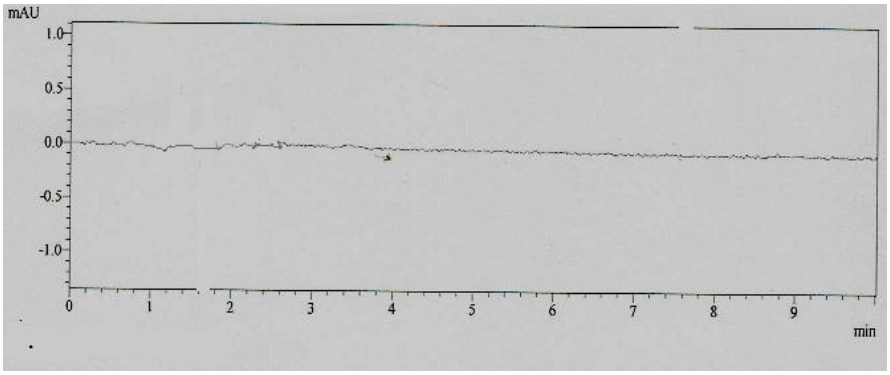


Fig. 2: Chromatogram of Blank.

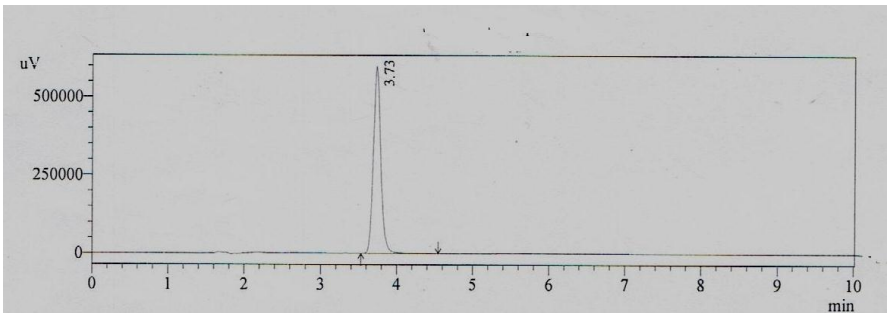


Fig. 3: Chromatogram of Sample.

2. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.Serial dilutions of Atazanavir sulfate (25-150 µg/ml) were injected into the column and detected at a wavelength set at 249 nm. The calibration curve was obtained by plotting the concentration vs. peak area.

Acceptance criteria: Correlation coefficient should be not less than 0.999.

3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 20-200 µg/ml for Atazanavir sulfate respectively

4. Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2 types of recovery studies are there.

a) **Standard addition method:** To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

b) **Percentage method:** For these assay method samples are prepared in three concentrations of 50%, 100%, and 150% respectively.

Acceptance criteria: The mean % recovery of the Atazanavir sulfate at each level should be not less than 95.0% and not more than 105.0%.

Assay procedure

10µL of the blank, standard and sample was injected into the chromatographic system and areas for the Atazanavir sulfate the peak was used for calculating the % assay by using the formulae.

5. Precision

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions.

The % RSD of peak areas of six samples was calculated. The method precision was performed on Atazanavir sulfate formulation.

Acceptance criteria

- The % RSD for the area of six sample injections results should not be more than 2.

Selection of solvent

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400 nm. The overlay spectrum of Atazanavir sulfate was obtained and the isobestic point of Atazanavir sulfate showed absorbance's maxima at 249 nm.

VALIDATION OF THE METHOD

Linearity

Atazanavir sulfate: Serial dilutions of Atazanavir sulfate (25-150µg/ml) were injected into the column and detected at a wavelength set at 249 nm. The calibration curve was obtained by plotting the concentration vs. peak area and the correlation coefficient was found to be 0.999 respectively.

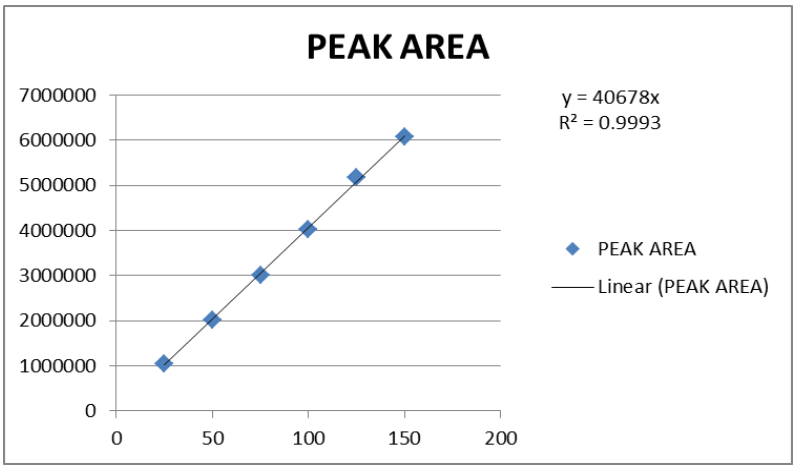


Fig. 4: Calibration graph of Atazanavir sulphate.

Table 1: Calibration data of Atazanavir sulfate.

Level	Conc (ug/ml)	Peak Area
Level-1	25	1053328
Level-2	50	2022943
Level-3	75	3019848
Level-4	100	4024196
Level-5	125	5167667
Level-6	150	6074920

Recovery studies

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. To an equivalent quantity of formulation powder a known quantity of standard Atazanavir sulfate were added at 50%, 100% and 150% level and the contents were re-analyzed by the proposed method.

Table 2: Accuracy results for Atazanavir sulfate.

S.No	Concentration	Measured Concentration (mcg) ± S.D (n=3)
1.	0.1	0.09±0.05
2.	1	0.99±0.04
3.	10	9.99±0.11

Table 3: Assay results for Atazanavir sulfate.

Formulation	Labeled amount (mg)	Mean±S.D (amount recovered) (n=3)	% RSD	Mean ± S.D (% of recovery)	% RSD
Virataz	300	301.5±0.36	0.05	100.5±0.44	0.46

Table 4: Recovery results for Atazanavir sulfate.

Concentration of specific level	Peak Response	Average Peak area	Amount Added	Amount Found	% Recovery	Mean Recovery
50%	5613787	5616407	300	305.1	101.70	99.7%
	5610866					
	5624568					
100%	9928627	9924076	400	397.12	99.28	
	9915165					
	9928437					
150%	15384338	15340585	500	504.05	100.81	
	15288620					
	15348798					

Precision

Table 5: Results of Precision for Atazanavir sulfate.

S. No.	Concentration mcg/ml	Measured Concentration (mcg/ml)± S.D (n=3)	
		Intra day	Inter day
1	0.1	0.099±0.0005	0.099±0.0005
2	1	0.99±0.005	0.99±0.005
3	10	9.99±0.005	9.99±0.005

LOD and LOQ

Table 6: LOD and LOQ for Atazanavir sulfate.

Drug name	LOD	LOQ
Atazanavir sulfate	50 ng/mL	140 ng/mL

4. Stress Degradation

In order to separate Atazanavir sulfate and degradation production products produced under stressed condition, water methanol phases were used and adjusted to obtain a rapid and simple assay method with a reasonable run time, suitable retention time and sharpness of the peak. Satisfactory resolution was obtained using the mobile phase system of Methanol: Acetonitrile: Sodium dihydrogen ortho phosphate buffer (45:35:20) with a flow rate of 1.0 ml/min., the optimum mobile phase. As, Atazanavir sulfate showed maximum absorption at 249 nm, the detector was set at 249 nm. Under the experimental conditions, the chromatogram of Atazanavir sulfate showed a single peak of the around 3.7 min. Chromatograms of sample solution is given.

The International Conference of Harmonization (ICH) guideline entitled "Stability Testing of New Drug Substances and Products" requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substances. The hydrolytic stabilities are required. The following degradation behavior of drug was observed during the above mentioned HPLC studies, followed by the chromatograms of the degradation products at Room Temperature:

a) **Acidic Condition:** On heating the drug solution with the 1N HCl for 4 hours the peak corresponding to the parent peak disappeared, and two new additional signals can be observed on the chromatogram.
b) **Degradation on Alkali:** When the Atazanavir sulfate solution was exposed to basic hydrolysis (1N NaOH) for 4 hours the chromatographic peak corresponding to parent drug peak disappeared and showed additional peaks.
c) **Oxidative Conditions:** When the Nelfinavir solution was exposed to chemical oxidation with H_2O_2 for 4 hours the chromatographic peak corresponding to parent drug diminished completely and new signals can be observed.

Table 7: Degradation details Atazanavir sulfate at Room Temperature.

Condition	Time	% Degradation	R_t values of degradation products
Acid, 1 N HCl	48hr	24	3.1, 3.5, 3.9
Base, 1N NaOH	48hr	64	3.2, 3.8
H_2O_2, 30%	48hr	100	3.5, 3.9, 4.2, 4.5

Table 8: Degradation details Atazanavir sulfate at 60^0C.

Condition	Time	% Degradation	R_t values f degradation products
Acid, 1 N HCl, (60 ^oC)	48hr	100	2.9, 3.9, 4.0
Base, 1N NaOH, (60 ^oC)	48hr	100	3.3, 3.5, 4.1, 4.7
H_2O_2, 30%, (60 ^oC)	48hr	100	3.1, 3.4, 4.2, 4.9, 5.1.

Table 9: Stress induced stability studies of Atazanavir sulfate at Room temperature.

Time in hours	O.1N HCl		O.1N NaOH		O.3% H2O2	
	Amt. found	% of deg	Amt. found	% of deg	Amt. found	% of deg
0	9.97	0.2	9.57	4.1	9.53	4.2
2	9.66	3.7	9.02	9.4	8.76	12.8
4	9.10	8.9	8.62	13.8	8.08	19.3
8	8.53	14.2	7.49	25.3	6.70	32.5
24	7.82	21.6	5.09	48.4	5.55	64.1
48	6.59	24	3.56	64	0	100

Table 10: Stress induced stability studies of Atazanavir at elevated Temperature (60^0C).

Time in hours	O.1N HCl		O.1N NaOH		O.3% H2O2	
	Amt. found	% of deg	Amt. found	% of deg	Amt. found	% of deg
0	9.97	0.2	9.77	3.2	9.52	4.2
2	9.75	3.7	8.74	12.2	9.36	12.8
4	8.62	12.7	7.62	23.4	6.89	29.3
8	6.97	29.3	5.89	41	4.61	52.5
24	3.86	61.3	2.18	78.6	1.58	84.1
48	0	100	0	100	0	100

Initial analyte concentration = 10 µg/ml

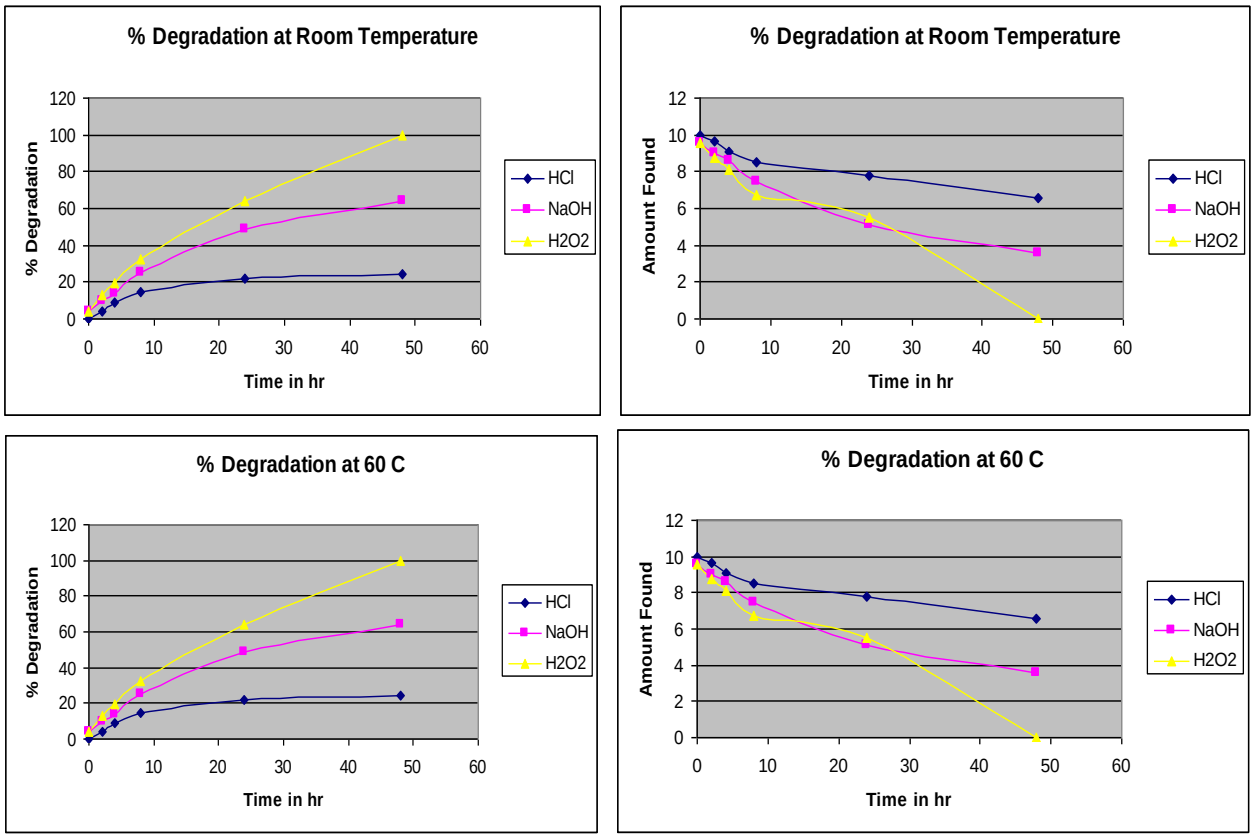


Fig 5: Degradation Pattern of Atazanavir sulfate.

SUMMARY AND CONCLUSION

A new method was established for estimation of Atazanavir sulfate by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Atazanavir sulfate by using Agilent C18 column (25×250mm) 5µ, flow rate was 1ml/min, mobile phase ratio was Methanol: Acetonitrile: Buffer (45:35:20 v/v), detection wavelength was 249 nm. Precision and recovery studies were also found to be with the range. The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for estimation of Atazanavir sulfate in Pharmaceutical dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. Hence the suggested RP-HPLC method can be used for routine analysis of Atazanavir sulfate in API and Pharmaceutical dosage form.

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