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METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ATAZANAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORMS AND ITS STRESS DEGRADATION STUDIES USING UV-VIS SPECTROPHOTOMETRIC METHOD

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ABSTRACT

The present study describes a simple, accurate, precise and cost effective UV-VIS Spectrophotometric method for the estimation of Atazanavir, an anti-HIV drug, in bulk and pharmaceutical dosage form. The solvent used was methanol and the λmax or the absorption maxima of the drug was found to be 250nm. A linear response was observed in the range of 10-50µg/ml with a regression coefficient of 0.999. The method was then validated for different parameters as per the ICH (International Conference for Harmonization) guidelines. This method can be used for the determination of Atazanavir in quality control of formulation without interference of the excipients. Atazanavir sulphate was subjected to stress degradation under different conditions recommended by ICH. The samples so generated were used for degradation studies using the developed method.

KEYWORDS

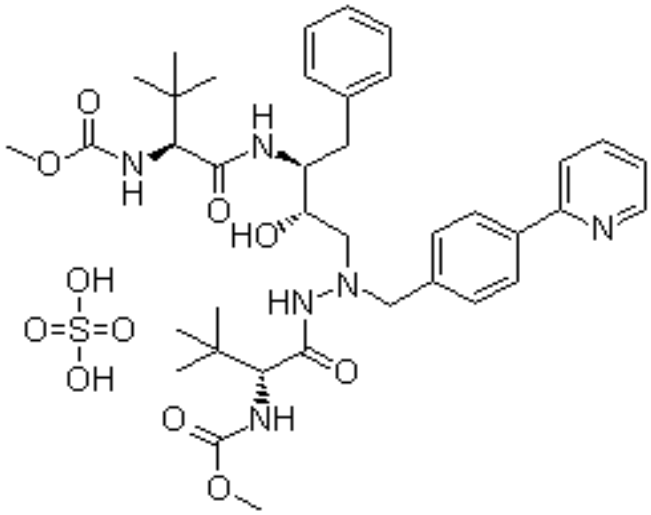
Atazanavir, HIV, λmax, ICH, UV-VIS spectroscopy.

INTRODUCTION

It is a recently introduced azapeptide inhibitor of HIV-1 Protease. It is formulated as 1:1 sulphate salt. The drug was approved by USFDA on June 20, 2003. Literature survey revealed that Atazanavir was quantitatively assayed in biological fluids either individually [2] or in presence of other retroviral drugs using liquid chromatography [3,4]. However, no UV-VIS spectrophotometric method was proposed for the estimation of atazanavir in bulk and pharmaceutical dosage forms. The literature survey also indicates that no stability indicating spectrophotometric method was proposed for atazanavir [5]. The aim of this work is to develop

and validate an analytical method by using UV-VIS spectrophotometry for the estimation of atazanavir in bulk and pharmaceutical dosage forms and also perform stress degradation studies on the drug as per ICH [6,7] Guidelines using the developed method.

Atazanavir sulphate, chemically[1] is (3*S*,8*S*,9*S*,12*S*) - 3,12-Bis (1,1-dimethylethyl) - 8-hydroxy -4,11 – dioxo - 9- (phenylmethyl) -6- [[4- (2-pyridinyl) phenyl] methyl] -2,5,6,10,13 - penta aza tetra decanedioic acid dimethyl ester, sulphate (1:1).



Materials and Methods

The instrument used for the study was an UV-VIS double beam spectrophotometer (Mode

l T60, Analytical Technologies Limited) with 1cm matched pair quartz cells. The solvent used was methanol and was of AR grade, purchased from SD Fine Chemicals Limited, India.

METHOD DEVELOPMENT

Solubility Test: Solubility test for the drug atazanavir was performed by using various solvents. The solvents include Water, Methanol, Ethanol, Acetonitrile, 0.1 N Hydrochloric Acid (HCl), 0.1 N Sodium Hydroxide (NaOH) and Chloroform. However, Methanol was chosen as a solvent for developing the method.

Determination of λ_{max}

Preparation of Stock Solution: Standard stock solution of atazanavir sulphate was prepared by dissolving 10mg of atazanavir sulphate in 10ml of methanol to produce a concentration of 1000µg/ml. 1ml of this stock solution was taken and then diluted up to 10ml by using methanol to produce a concentration of 100µg/ml which is the standard stock solution.

Preparation of Working Standard Solution: From the above stock solution, 2ml was pippetted into a 10ml volumetric flask and the volume was made up to the mark with methanol to prepare a concentration of 20µg/ml. Then the sample was scanned in UV-VIS Spectrophotometer in the range 400-200nm using methanol as a blank and the wavelength corresponding to maximum absorbance (λmax) [8] was found to be 250nm(fig. 1).

Preparation of Calibration Curve: 1ml of the 100µg/ml solution was diluted to 10ml by using methanol to produce 10µg/ml solution. 2ml, 3ml, 4ml and 5ml of 100µg/ml solution were diluted to 10ml using methanol to produce 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml solutions respectively. Then the construction of calibration curve was done by taking the above prepared solutions of different concentration

ranging from 10-50µg/ml. Then, the calibration curve was plotted by taking concentration on x-axis and absorbance on y-axis (in fig.2). The curve showed linearity in the concentration range of 10-50µg/ml. The correlation coefficient (r^2) was found to be 0.999.

Assay of Atazanavir capsules (ATAZOR-300mg):

A quantity of powder equivalent to 50mg of atazanavir was taken in a 50ml volumetric flask and it was dissolved and diluted upto the mark with methanol. The resultant solution was ultrasonicated for 5 minutes. The solution was then filtered using Whatmann filter paper No.40. From the filtrate, appropriate dilutions were made in methanol to obtain the desired concentration (50µg/ml). This solution was then analysed in UV and the result was indicated by % recovery given in table 1.

METHOD VALIDATION

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

The method was validated for different parameters like Linearity, Accuracy, Precision, Specificity, Robustness, Ruggedness, Limit Of Detection (LOD) and Limit Of Quantification (LOQ).

Linearity: Various aliquots were prepared form the stock solution (100µg/ml) ranging from 10-50µg/ml. The samples were scanned in UV-VIS Spectrophotometer using methanol as blank. It was found that the selected drug shows linearity between the 10-50µg/ml (table 3&1).

Accuracy: The accuracy of the method was determined by preparing solutions of different concentrations that is 80%, 100% and 120% in which the amount of marketed formulation(ATAZOR-300mg) was kept constant (10mg) and the amount of pure drug was varied that is 8mg, 10mg and 12mg for 80%, 100% and 120% respectively. The solutions were prepared in triplicates and the accuracy was indicated by % recovery (table 1 & 5).

Precision: Precision of the method was demonstrated by intraday and interday variation studies. In intraday variation study, 9 different solutions of same concentration that is 20µg/ml were prepared and analysed three times in a day i.e. morning, afternoon and evening and the absorbances were noted. The result was indicated by % RSD (table no.1,6,7).

In the interday variation study, solutions of same concentration 20µg/ml were prepared and analysed three times for three consecutive days and the absorbances were noted. The result was indicated by % RSD (table no.8).

Specificity: 10mg of Atazanavir was spiked with 50% (5mg), 100% (10mg), and 150% (15mg) of excipient mix (Magnesium Stearate) and the sample was analysed for % recovery of Atazanavir (table no.1 & 9).

Robustness: Robustness of the method was determined by carrying out the analysis at two different temperatures i.e. at room temperature and at 18ºc. The respective absorbances were noted and the result was indicated by % RSD (tableno.1 & 10).

Ruggedness: Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective absorbances were noted. The result was indicated by % RSD (table no.10).

Limit of Detection (LOD): The limit of detection (LOD) was determined by preparing solutions of different concentrations ranging from 0.1-0.5µg/ml. The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value (table no.1).

Limit of Quantification: The LOQ is the concentration that can be quantitated reliably with a specified level of accuracy and precision.

The LOQ was calculated using the formula involving standard deviation of response and slope of calibration curve (table no.1).

Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the atazanavir using the method developed.

Stress degradation by hydrolysis under acidic condition: To 3 ml of stock solution(1000µg/ml) of Atazanavir, 1 ml of 3 N HCl was added in 10 ml of volumetric flask and the volume was made up to the mark with methanol. Then, the volumetric flask was kept at normal condition for 90 minutes. After 60 min. time interval, 1 ml of solution was pipetted out from this flask, neutralised and diluted with methanol in order to make the volume up to 10 ml and the dilution was carried out to achieve the appropriate concentration (30µg/ml). This solution was taken in cuvette. For the blank, 0.5 ml solution of 3N HCl and 0.5 ml solution of 3N NaOH were diluted with methanol in 10 ml of volumetric flask. After 90 minutes, again 1ml of the solution was pipetted out from the flask and the above procedure was repeated (table no.2 & fig.no.3 &4).

Stress degradation by hydrolysis under alkaline condition: To 3 ml of stock solution of atazanavir 1 ml of 0.1 N NaOH was added in 10 ml of volumetric flask and made up the volume to the mark with methanol. Volumetric flask was kept at normal condition for 90 min. After 60 min time interval, 1 ml of solution was pipetted out from this flask, neutralized and diluted with methanol in order to make the volume up to 10 ml and the dilutions were carried out to achieve the appropriate concentration (20µg/ml). The solution was then taken in cuvette. For the blank, 0.5 ml solution of 0.1N HCl and 0.5 ml solution of 0.1N NaOH diluted with methanol in 10 ml of volumetric flask. After, 90 minutes 1ml of solution was again pippetted out from the flask and the above procedure was repeated (table no.2 & fig.no.5 & 6).

Dry heat induced degradation: Atazanavir sample was taken in a petriplate and exposed to a temperature of 70℃ for 48 hours in an oven. After 48 hours, 10 mg of the sample was diluted with methanol in order to make the volume up to 10 ml. From this solution, dilutions were carried out to achieve the appropriate concentration (20µg/ml) and the solution was taken in cuvette for the UV-VIS Analysis (table no.2 & fig.no.7).

Oxidative degradation: To 1.5 ml of the stock solution of atazanavir (1000µg/ml), 1 ml of 30 % w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with methanol. The volumetric flask was then kept at room temperature for 15 min. For the blank, 1 ml of the 30 % w/v of hydrogen peroxide was kept at normal condition for overnight in 10 ml of volumetric flask. Both solutions were heated on boiling water bath to remove the excess of hydrogen peroxide. Finally, after 15 minutes dilutions were made from the stock solution to achieve the required concentration (30µg/ml). The solution was then taken in a cuvette and analysed (table no.2 & fig.no.8).

Photolytic degradation: Sample of atazanavir was exposed to near ultraviolet lamp in photostablity chamber providing illumination of not less than 1.2 million lux hours. Ten milligrams sample was dissolved methanol and volume made up to 10 ml. From this solution appropriate dilution (30µg/ml) was made using methanol and taken in cuvette for the U.V. analysis (table no.2 & fig.no.9 & 10).

RESULTS AND DISCUSSION

The developed method was found to be precise as the %RSD values for intra-day and inter-day were found to be less than 2%. Good recoveries (99.97% to 101.4%) of the drug were obtained at each added concentration, indicating that the method was accurate. The method was also found to be specific indicated

by the % recoveries ranging from 98.2% to 101.2%. The LOD and LOQ were found to be in sub-microgram level indicating the sensitivity of the method. The method was also found to be robust and rugged as indicated by the %RSD values which are less than 2%. The results of Assay show that the amount of drug was in good agreement with the label claim of the formulation as indicated by % recovery (101.8%). Summary of validation parameters of proposed spectrophotometric method is shown in table 1.The stress degradation studies showed that Atazanavir undergoes degradation in acidic and alkaline conditions whereas it is relatively stable when exposed to dry heat, oxidation and photolytic conditions. Summary of the results of stress degradation studies of Atazanavir are shown in the table 2.

TABLE 1.

SUMMARY OF VALIDATION

PARAMETER	RESULT
Linearity indicated by correlation coefficient	0.999
Precision indicated by %RSD	0.3306%
Accuracy indicated by % recovery	99.97-101.4%
Specificity indicated by % recovery	98.2-101.2%
Limit of Detection	0.2µg/ml
Limit of Quantification	0.665µg/ml
Range	10-50µg/ml
Linear regression equation	y = 0.034x + 0.009
Robustness indicated by %RSD	0.18%
Assay indicated by % recovery	101.8%

TABLE 2.

SUMMARY OF RESULT OF STRESS DEGRADATION STUDIES

Condition	Time	%Degradation
0.1N NaOH(1ml)	60min	43.07%
	90min	87.95%
3N HCl(1ml)	60min	89.75%
	90min	98.79%
30% Hydrogen Peroxide(1ml)	15min	12.65%
Dry Heat 70°	48hr	0.14%
Photolytic	3hr	32.53%
	6hr	41.36%

VALIDATION:

Table 3.

Linearity Table of Atazanavir Sulphate in Working Standard

Concentration (µg/ml)	Absorbance
10	0.36
20	0.721
30	1.02
40	1.395
50	1.659

Table 4.

Optical characteristics

Beer's Law limit (µg/mL)	10-50µg/ml
Molar extinction coefficient (1 mole-1 c.m-1)	360.5
Correlation coefficient	0.999
Regression equation (Y*)	y = 0.034x + 0.009
Slope (a)	0.034
Intercept (b)	0.009

Accuracy:

Table 5.

Accuracy Readings of Atazanavir Sulphate

OBSERVATION / RESULTS						
No. of preparations	Concentration (µg/ml)		% Recovery	Statistical Results		
	Formulation	Pure Drug		Mean	SD	%RSD
S_1 : 80 %	10	8	100.7	100.71	0.685055	0.68
S_2 : 80 %	10	8	101.4			
S_3 : 80 %	10	8	100.03			
S_4 : 100 %	10	10	100.5	100.4	0.953939	0.95
S_5 : 100 %	10	10	99.4			
S_6 : 100 %	10	10	101.3			
S_7 : 120 %	10	12	99.5	99.9566	0.450148	0.45
S_8 : 120 %	10	12	100.4			
S_9 : 120 %	10	12	99.97			

Precision:

Table 6.

Precision Results Showing Repeatability of Atazanavir Sulphate

Concentrations (µg/ml)	Absorbance	Statistical Analysis
20	0.721	Mean =0.723
20	0.723	
20	0.728	

20	0.721	SD = 0.002404 %RSD =0.332
20	0.726	
20	0.722	
20	0.721	
20	0.723	
20	0.724	
20	0.721	

Table no. 7 Intra-assay

Concentrations (µg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Average %RSD
20	0.721	0.731	0.728	
20	0.721	0.731	0.728	
20	0.721	0.731	0.728	
20	0.721	0.731	0.728	
20	0.728	0.721	0.726	
20	0.728	0.721	0.726	
20	0.728	0.721	0.726	
20	0.728	0.724	0.731	
20	0.721	0.728	0.731	
%RSD	**0.50%**	**0.65%**	**0.26%**	**0.47%**

Precision:

Table no. 8 Inter-assay Precision

Concentrations (µg/ml)	%RSD			Average %RSD
	Day 1	Day2	Day3	
20	0.15	0.22	0.22	0.19

Test for Specificity:

Table no. 9
Test for Specificity showing no effect of excipient.

Sample No.	Excipient Conc. (%)	Atazanavir Input (mg)	Atazanavir Recovered (mg)	Atazanavir Recovered (%)	MeanRecovered (%)	S.D.	%R.S.D.
1	100%	10	9.82	98.2%			
2	50%	10	10.05	100.5%	100.2%	1.86854	1.86%

3	150%	10	10.19	101.9%

Ruggedness & Robustness

Table No. 10
Results Showing Ruggedness of Method for Atazanavir Sulphate

Analyst-1				Analyst-2		
Conc. (µg/ml)	**Abs.**	**Statistical Analysis**		**Conc. (µg/ml)**	**Abs.**	**Statistical Analysis**
20	0.728	Mean = 0.727		20	0.726	Mean = 0.7275
20	0.728			20	0.726	
20	0.728	SD = 0.001095		20	0.726	SD =0.001643
20	0.726			20	0.729	
20	0.726			20	0.729	
20	0.726	%RSD =0 .1506		20	0.729	%RSD =0.225
Room Temperature				**Temp. 18°C**		
Conc. (µg/ml)	Abs.	Statistical Analysis		Conc. (µg/ml)	Abs.	Statistical Analysis
20	0.721	Mean = 0.722		20	0.728	Mean = 0.725
20	0.721			20	0.726	
20	0.721	SD =0.001862		20	0.726	SD =0.001517
20	0.724			20	0.724	
20	0.724			20	0.724	
20	0.725	%RSD = 0.257		20	0.725	%RSD = 0.209

Limit of Detection (LOD)

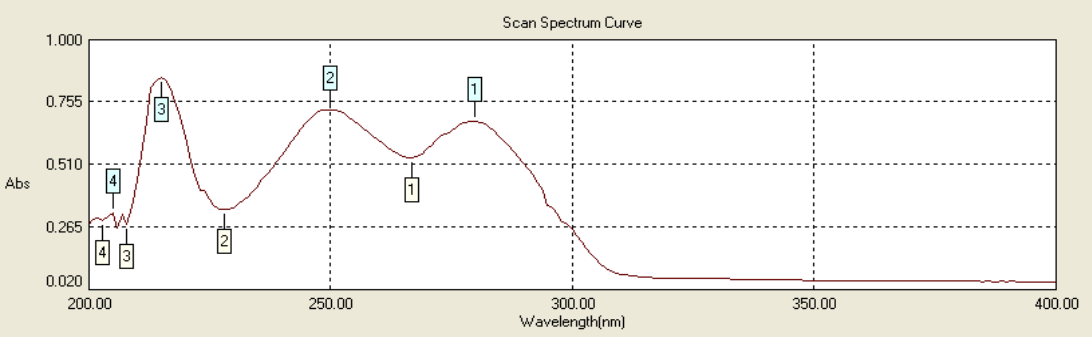
The LOD for Atazanavir Sulphate was found to be 0.2µg/ml.

Limit of Quantification (LOQ)

The LOQ for Atazanavir Sulphate was found to be 0.66µg/ml.

Figures:

Determination of λ_{max}:



λmax of Atazanavir Sulphate showing at 250nm (fig. no. 1)

Peak 2- 250.00- 0.721

Preparation of Calibration Curve:

Calibration Curve of Atazanavir Sulphate

conc.	Abs
0	0
10	0.36
20	0.721
30	1.02
40	1.395
50	1.659

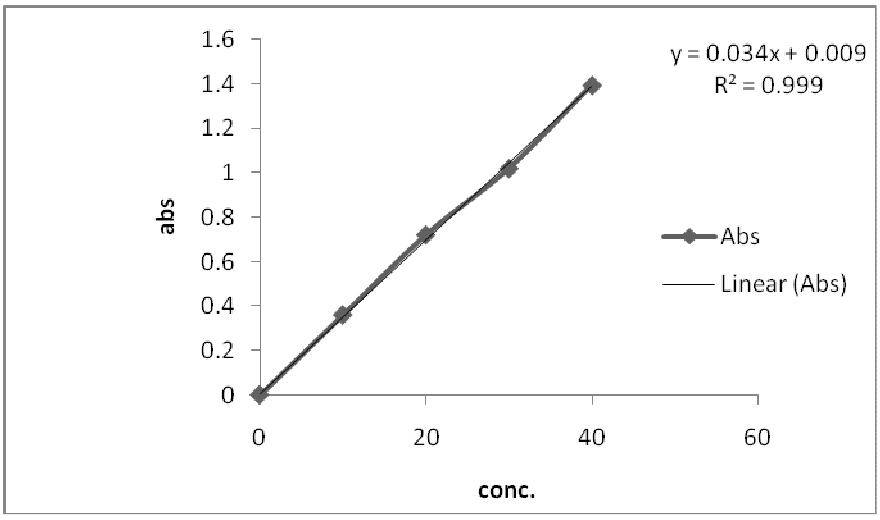
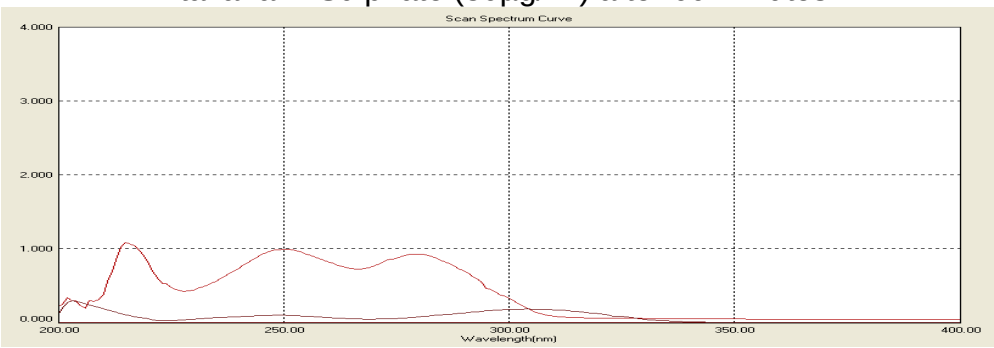


Fig. no. 2 Calibration curve of Atazanavir Sulphate

Degradation Studies:

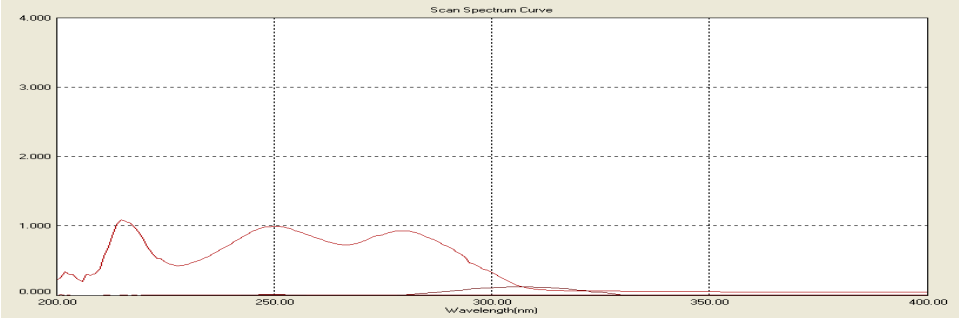
Stress degradation by hydrolysis under acidic condition:

Comparison between standard Atazanavir Sulphate (30µg/ml) & Acid Degraded sample of Atazanavir Sulphate (30µg/ml) after 60 minutes.



Drug got degraded by 89.75% after exposing for 60min. to the acidic condition (fig. no.3)

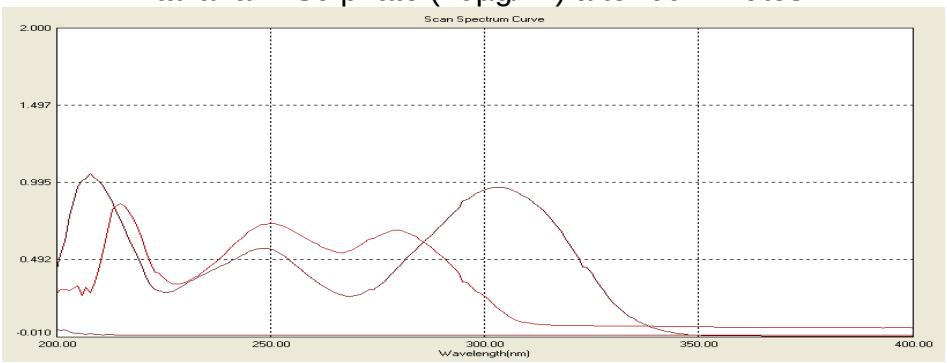
Comparison between standard Atazanavir Sulphate (30µg/ml) & Acid degraded sample of Atazanavir Sulphate (30µg/ml) for 90minutes.



Drug got degraded by 98.79% after exposing for 90min. to the acidic condition (fig. no.4)

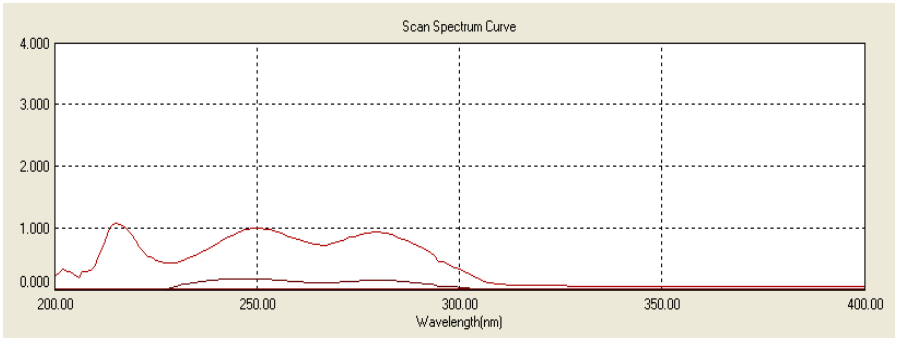
Stress degradation by hydrolysis under alkaline condition condition:

Comparison between standard Atazanavir Sulphate (20µg/ml) & Alkali degraded sample of Atazanavir Sulphate (20µg/ml) after 60 minutes.



Drug got degraded 43.07% after exposing for 60min. to the alkaline condition (fig. no.5)

Comparison between standard Atazanavir Sulphate (30µg/ml) & Alkali degraded sample of Atanavir Suphate after 90minutes



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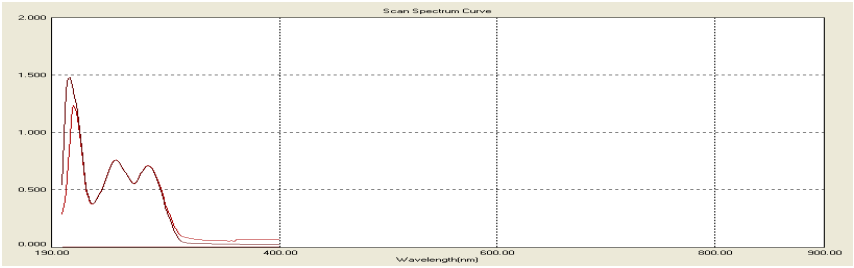
Drug got degraded by 87.95% after exposing for 90min. to the alkaline condition (fig.no.6)

Dry heat induced degradation:

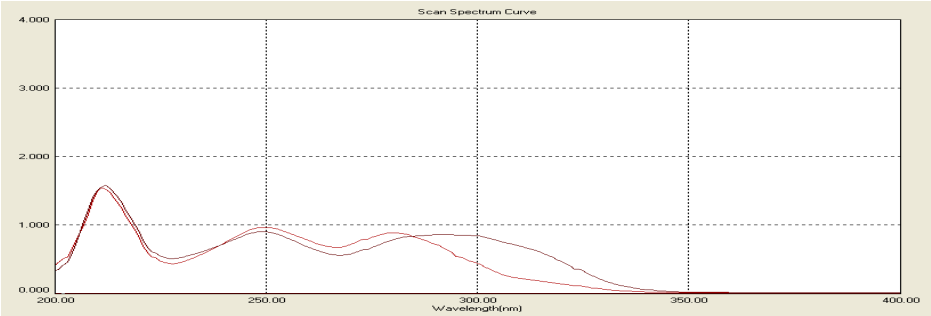
Comparison between standard Atazanavir Sulphate (20µg/ml) & Temperature degraded sample of Atazanavir Sulphate (20µg/ml)



Drug got degraded by 0.14% when exposed to a temp of 70℃ for 48 hours (fig. no.7)

Oxidative degradation:

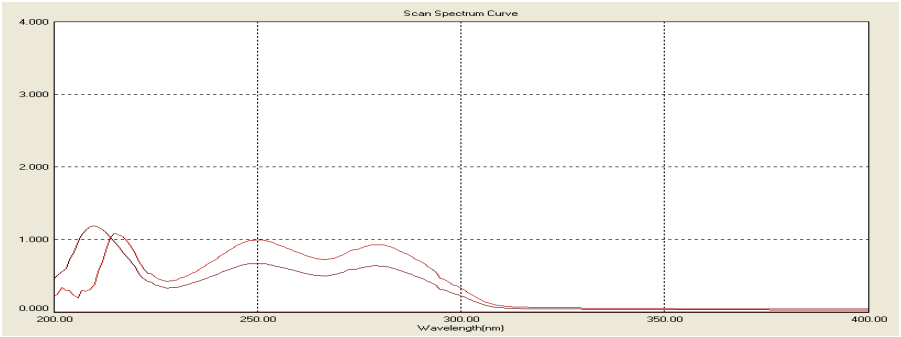
Comparison between standard Atazanavir Sulphate (20µg/ml) & Oxidized sample of Atazanavir Sulphate (20µg/ml).



Drug got degraded by 13.25% when it is treated with 30% (w/v) hydrogen peroxide for 15 minutes (fig. no.8)

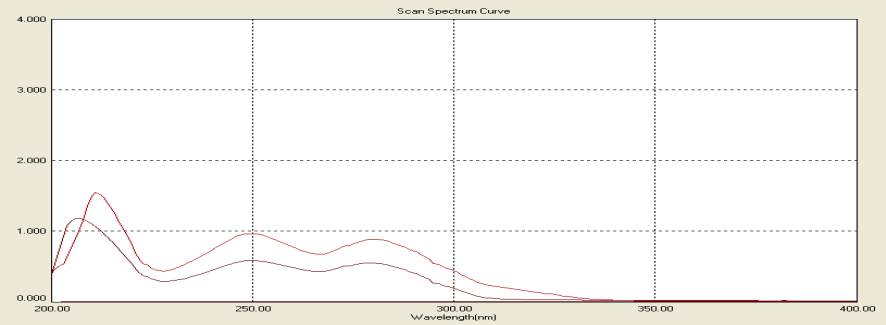
Photolytic degradation:

Comparison between standard Atazanavir Sulphate (30µg/ml) & UV degraded sample of atazanavir sulphate (30µg/ml) after 3 hours



Drug when exposed to UV light for 3hrs, got degraded by 32.53% (fig. no.9)

Comparison between standard Atazanavir Sulphate(30µg/ml) & UV light degraded sample of Atazanavir Sulphate(30µg/ml) after 6 hours



Drug when exposed to UV light for 6hrs, got degraded by 41.3%(fig. no.10)

CONCLUSION

All the above factors lead to the conclusion that the proposed method is accurate, precise, simple, sensitive, robust and cost effective and can be applied successfully for the estimation of atazanavir in bulk and pharmaceutical formulation. The proposed method is also useful for determination of atazanavir stability in sample of pharmaceutical dosage forms.

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