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Development and Validation of New RP-HPLC Method for the Estimation of Atazanavir Sulphate in Bulk and Dosages Form

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ABSTRACT

New stability indicating reverse phase-high performance liquid chromatographic (RP-HPLC) method was developed and validated for the estimation of atazanavir sulphate (ATV) in bulk and dosages forms by using C18 column Phenomenix (250 mm × 4.6 mm, 5 µm), with a mobile phase consisting of a acetonitrile and water (80:20 v/v) at a flow rate of 0.5 mL/min. The detection was carried out at 248 nm and retention time (Rt) of atazanavir sulphate was found to be 3.989 min. The response of detector was linear in the concentration range of 10-50 µg/mL (n=5), with the regression coefficient of determination r2 was found to be 0.999. Atazanavir sulphate was subjected to different stress conditions as per ICH guidelines like acidic alkaline, oxidative, thermaland the results showed that it was more sensitive towards basic degradation.

Keywords: RP-HPLC, Phenomenix, Acidic, Alkaline, Oxidative, Thermal degradation

INTRODUCTION

Atazanavir sulphate is azapeptide inhibitor of human immunodeficiency virus type-1 (HIV-1) protease inhibitors which allows once-daily oral administration [1]. It is a white to pale yellow powder, slightly soluble in water. It is used in the treatment in combined form with other antiretroviral agents. Atazanavir sulphate, chemically designated as 3, 12-bis (1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9-(phenylmethyl)-6-((4-(2-pyridinyl)phenyl)methyl)-,dimethyl ester [2] (Figure 1). Atazanavir formulated as 1:1 sulphate salt is the most recently introduced azapeptide inhibitor of human immunodeficiency virus type-1, which is approved by the United State Food and Drug Administration (USFDA) in June 2003. Combined form of such several drugs shows highly active antiretroviral therapy. The American National Institute of Health and other organizations recommended offering

antiretroviral treatment to all patients with AIDS [3]. Several research papers have been reported in the literature survey it reveals that, atazanavir is quantitatively assayed in biological fluids either individually [4,5] or in combined form, using liquid chromatography [6,7]. However, some UV-VIS spectroscopic methods were proposed for estimation of atazanavir sulphate in bulk and pharmaceutical dosage form [8,9]. In the present paper, developed new RP-HPLC method and stability-indicating study for atazanavir sulphate in bulk as well as in dosage form and validated it by ICH guidelines [10].

MATERIAL AND METHODS

Chemicals required

The solvents methanol, acetonitrile used for work was HPLC grade, hydrochloric acid, sodium hydroxide, hydrogenperoxide and other were of AR grade. Atazanavir sulphate was supplied by Hetro Drug Ltd, Hyderabad, India as gift sample (Figure 1).

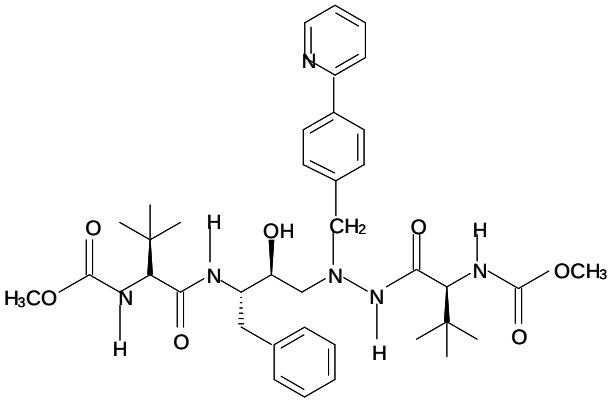


Figure 1: Atazanavir sulphate

Equipment

Agilent technologies 1200 LC system with gradient pump connected to DAD UV detector, electronic balance (sigma 200), hot air oven (universal Hot Air Oven), digital pH meter (Unilab) and syringe Hamilton(Rheodyne-50 µL) were used to carry out this work.

Chromatographic conditions

Chromatographic separation was achieved on Agilent TC C_{18}250 × 4.6 mm, 5 µm columns by using mobile phase composition of acetonitrile: water (80:20 v/v). Flow rate was maintained at 0.5 mL/min with 248 nm UV detection. The retention time obtained for atazanavir sulphate was at 3.989 min with injection volume 20 µL. Dilution was prepared by mixing 800 mL of acetonitrile with 200 mL Milli Q water. All determinations were performed for a run time of 10 min. The optimized chromatographic conditions are shown in Table 1.

Table 1: Optimized chromatographic conditions of atazanavir sulphate.

Parameters	Conditions
Stationary phase (column)	C_{18} (250 mm × 4.6mm, 5 µm)
Mobile phase	Acetonitrile: water (80:20 v/v)
Flow rate	0.5 mL/min.
Run time	10 min.
Volume of injection loop	20 µL
Detection wavelength	248 nm
Retention time	3.999 min.

Method development

Selection and preparation of mobile phase

Various mobile phases containing methanol, water, acetonitrile and acidic buffer were tried with different ratio and at different flow rates. Sharp symmetrical peak with minimum retention time (R_t) was found with the mobile phase composition acetonitrile and water in the ratio 80: 20 v/v which was prepared by mixing 800mL of HPLC grade acetonitrile with 200 mL of Milli Q. water.

Preparation of standard stock solutions

The standard stock solution of 100 µg/mL of the drug atazanavir sulphate were prepared by dissolving 10 mg of pure drug in the methanol (mobile phase) in the 10 mL volumetric flask and the volume was made up to the mark. Resulting solution were further diluted with mobile phase to obtain a final concentration of 100 µg/mL and stored under refrigeration.

Preparation of calibration curve

1-5 mL of standard stock solution were taken in 10 mL volumetric flask and diluted up to the mark with mobile phase in such a way that the final concentration of drug were in the range of 10-50 µg/mL. 20 µL of each solution were injected under the chromatographic condition as described above. Recorded the peak area and calibration curve were constructed by plotting the peak area on the y-axis against respective concentration of drugs on the x-axis. The calibration curve was evaluated by its coefficient of determination (r^2).

Method validation

The developed method was validated for its linearity, accuracy, precision, robustness, sensitivity and specificity [10].

Linearity

100 µg/mL atazanavir sulphate stock solutions was prepared from this stock solution various working standard solution were prepared in the range of 10-50 µg/mL and injected 20 µL in to HPLC. It was found that the atazanavir sulphate had linearity in the range of 10-50 µg/mL. By plotting the graph of peak area verses atazanavir sulphate concentration (replicate analysis n=5 at all concentration level) and the linear relationship was evaluated using the least square method within Microsoft Excel program. The correlation coefficient was found to be 0.999 nm at 248 nm wavelength as shown in the Figure 1.

Accuracy

The accuracy of the method was determined by using one set of different standards addition method at different concentration levels, 50%, 100% and 150%. The solutions were prepared on triplicates and the accuracy was indicated by % recovery was shown in Table 1.

Precision

The precision of the method was calculated from the peak area obtained by actual determination of five replicates of a fixed concentration of the drug 20 µg/mL. Precision was also calculated in terms of inter-day intra-day variation and was calculated in terms of relative standard deviation (RSD).

Robustness

Robustness of the method for atazanavir sulphate was carried out by small change in flow rate and the percentage recovery (RSD) calculated.

Ruggedness

Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective peak area was noted and the result was calculated by % RSD.

Limit of detection and limit of quantification

As per International Conference Harmonization (ICH) guidelines the limit of detection (LOD)and the limit of quantification (LOQ) calculated by using following equation.

LOD=3.3SD/slope

LOQ=10SD/slope

Force degradation studies

Force degradation studies carried out on atazanavir sulphate sample using acid, alkaline, oxidative, and thermal degradation. The sample was exposed to above conditions and the main peak of atazanavir sulphate was studied for the peak purity which effectively separated the degradation products from the pure active atazanavir sulphate (API).

RESULTS AND DISCUSSION

Method development

Chromatographic separation

Several mobile phases of different composition were tried to optimize the separation of atazanavir sulphate by HPLC. A good separation for atazanavir sulphate was found by using mobile phase, acetonitrile and water, Retention time, and the other optimized chromatographic conditions are as shown in Table 1.

Calibration curve

By plotting the calibration curve of average peak area against concentration levels of 10-50 µg/mL of standard atazanavir sulphate, the correlation co-efficient (r^2) was 0.999 which was within the accepted range of ICH guidelines. The slope and intercept for atazanavir sulphate were 0.5052 and 0.4952 respectively as shown in Figure 2.

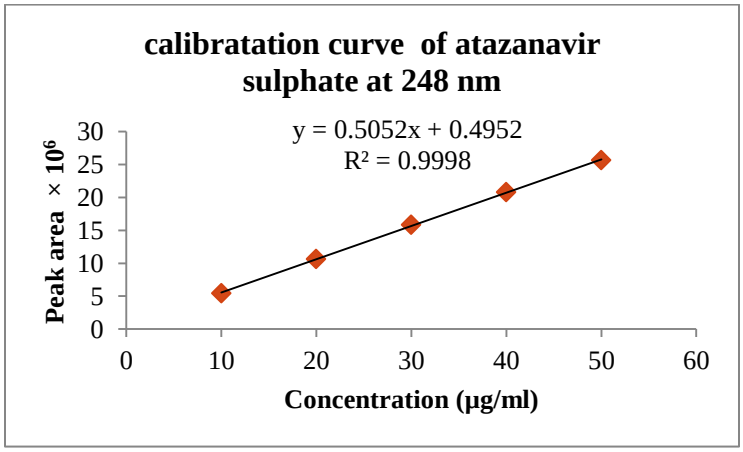
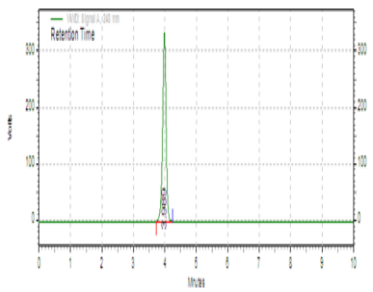


Figure 2: Calibration curve of atazanavir sulphate at 248 nm.

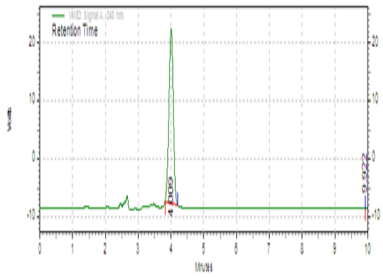
Method validation

Linearity, accuracy and precision

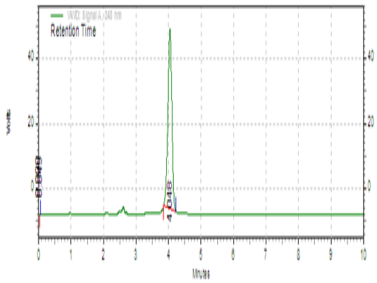
The correlation co-efficient for atazanavir sulphate was 0.999 as shown in Figure 2. The recovery study of drug shows the accuracy of the method, atazanavir sulphate was used at three levels of concentration, 50%, 100% and 150% as shown in Table 2. The precision of the method was demonstrated by inter and intra-day variation studies. In intraday studies five repeated injection of working sample solution were made and response of peaks and % RSD were calculated. In the inter day studies, three repeated injections of working sample were made for different days and the response factor of drug peak and % RSD were calculated as shown in Table 3. From the data, the developed HPLC method was found to be precise.



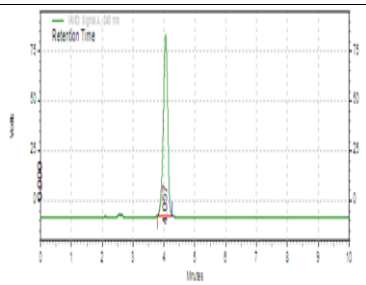
Chromatogram of pure atazanavir sulphate



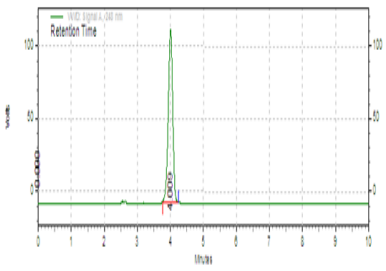
Chromatogram of atazanavir sulphate at concentration 10 µg/ml



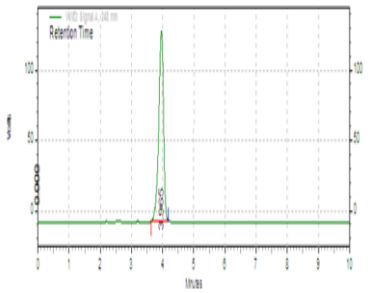
Chromatogram of atazanavir sulphate at concentration 20 µg/ml



Chromatogram of atazanavir sulphate at concentration 30 µg/ml



Chromatogram of atazanavir sulphate at concentration 40 µg/ml



Chromatogram of atazanavir sulphate at concentration 50 µg/ml

Graph 1: Accuracy studies of atazanavir sulphate.

Table 2: Inter-day studies of atazanavir sulphate.

No. of preparations (%)	Concentrations (µg/mL)		Recovery (%)	Statistical results		
	Formulation	Pure drug		Mean	SD	%RSD
S_1: 50	20	10	100.2952	100.71	0.503	0.500
	20	10	101.4752			
	20	10	100.3874			
S_2: 100	20	20	107.6251	105.71	1.856	1.755
	20	20	102.9269			
	20	20	106.5808			
S_3: 150	20	30	103.8782	103.53	0.450	0.450
	20	30	102.8385			
	20	30	103.8997			

Table 3: Intra-day studies of atazanavir sulphate.

Day 1 (10 am)				Day 2 (10 am)			Day 3 (10 am)		
Conc. (µg/mL)	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis
20	10.886656	20.57	Mean=20.55 SD=0.80 % RSD=0.77	10.61156	20.03	Mean=20.06 SD=0.33 %SD=0.33	9.969964	18.76	Mean= 18.99 SD=0.76 % RSD=0.808
20	10.982423	20.76		10.68024	20.16		10.102764	19.02	
20	10.752523	20.31		10.59679	20.00		10.186209	19.19	

Table 4: Results for robustness study at different wavelength

Conc. (µg/mL)	Wavelength 247 nm			Wavelength 249 nm		
	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis
20	10.536488	19.88	Mean=20.26, SD=0.2160, % RSD=1.066	10.807310	20.42	Mean=20.72, SD=0.1771, % RSD=0.8549
20	10.753548	20.31		11.012987	2082	
20	10.652128	20.11		11.105871	21.01	
20	10.721232	20.24		10.886491	20.57	
20	10.974332	20.75		10.980984	20.76	

Table 5: Results for robustness study at different flow rate

Conc. (µg/mL)	Flow rate 0.4 mL/min			Flow rate 0.6mL/min		
	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis	Peak area × 10^6	Calc. amt. (µg/mL)	Statistical analysis
20	10.435428	19.68	Mean=20.22, SD =0.2591, % RSD 1.2814	10.435327	19.68	Mean=20.21 SD=0.2649, %RSD=1.3110
20	10.754047	20.31		10.754048	20.31	
20	10.652229	20.11		10.633328	20.07	
20	10.731132	20.26		10.731233	20.26	
20	10.964212	20.73		10.954021	20.71	

Robustness and ruggedness

Robustness of the method was studied by changing the wavelength 248 ± 0.1 and flow rate of mobile phase 0.5 ± 0.1 mL/min, the result are given in Tables 4 and 5. Ruggedness of the method was studied by carrying out the experiment by different analyst, as shown in Table 6.

Table 6: Results for ruggedness study

Conc. (µg/mL)	Analyst-1			Analyst-2		
	Peak area	Calc. amt. (µg/mL)	Statistical analysis	Peak area	Calc. amt. (µg/mL)	Statistical analysis
20	7820475	18.79	Mean=18.79, SD=0.05 % RSD=0.05	7820875	18.79	Mean=18.79, SD=0.04, % RSD=0.05
20	7830475	18.81		7829875	18.81	
20	7820875	18.79		7820775	18.79	
20	7820875	18.79		7820775	18.79	
20	7820875	18.79		7820675	18.79	

LOD and LOQ

The LOD an LQD value of the developed for atazanavir sulphate was found to be 2.041 and 6.1863 respectively, which were determined by injecting low concentration of the standard solution for six times according to the following formulas:

LOD=3.3 SD/slope

LQD=10 SD/slope

Force degradation studies

Degradation in acidic condition

Acid degradation of atazanavir sulphate was performed by using 0.5 M HCl. Ten micrograms ofatazanavir sulphate was added with 5 mL 0.5 M HCl in clean 10 ml volumetric flask. Then the volumetric flask was kept at room temperature for different time intervals such as 0,min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h. At different time interval, different samples ware diluted with methanol to achieve the concentration of 10 µg/mL. It was then filtered by 0.22 µm filter and then injected in to HPLC. The acid degradation results are given in Table 7.

Degradation in basic conditions

Base degradation of atazanavir sulphatewasperformed by using 0.5 M NaOH. Ten micrograms of atazanavir sulphate was added with 5 ml 0.5 M NaOH in clean 10 mL volumetric flask. Then volumetric flask was kept at room temperature for different time intervals as 0 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h. At different time interval, different samples were diluted with methanol to achieve the concentration of 10 µg/mL. It was then filtered by 0.22 µm filter and then injected in to HPLC. The degradation atazanavir sulphate in basic condition results are given in Table 8.

Oxidative degradation

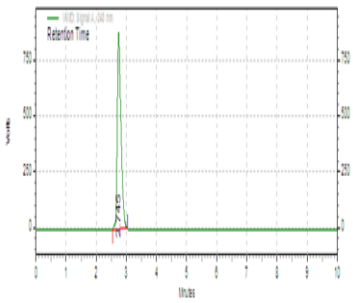
Oxidative degradation of atazanavir sulphate was carried by using3%H_2O_2. Ten micrograms of atazanavir sulphate was added with 5 ml 3% H_2O_2 in clean 10 mL volumetric flask. Then volumetric flask was kept at room temperature for different time intervals as 0, min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h. At different time interval, different samples ware diluted with methanol to achieve the concentration of 10 µg/mL. It was then filtered by 0.22 µm filter and then injected in to HPLC. The degradation results are given in Table 10.

Thermal degradation

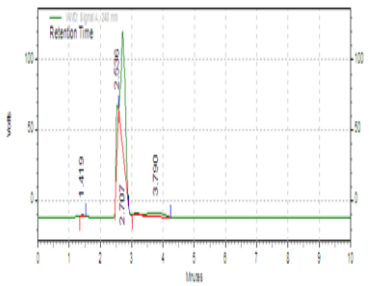
Thermal degradation was carried out byplacing the atazanavir sulphate bulk in a petriplate and exposed to a temperature 80^oC for 0, min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h in an open furnace. After above time interval sample was taken outside and diluted by methanol to achieve the concentration of 10 µg/mL. Filtered by 0.22 µm filter and then injected in to HPLC. The degradation results are given in Table 10.

Table 7: Result showing acidic degradation of atazanavir sulphate

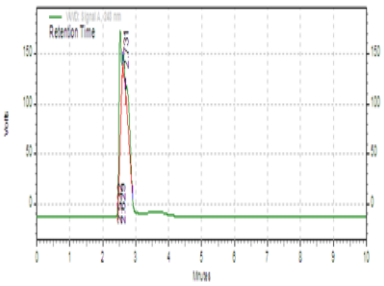
Time in hr.	Peak area	Retention time (Min)	% area	height	% height	% degradation
0	2.7864308	2.74	92.22	2607914	79.5	0
1	2.0823129	2.68	96.07	126674	96.65	25.26
2	1.3112005	2.7	76.21	1386628	72.17	52.94
4	1.2792464	2.74	100	14794246	100	54.09
8	0.3921810	2.76	27.22	326465	26.02	85.92
16	0.1612157	2.73	32.57	383599	16.22	94.21
24	0.0945386	2.62	13.32	314883	13.32	96.60



Pure atazanavir sulphate chromatogram



Acid degradation of atazanavir sulphate (0.5 M HCl, RT, 2 h)

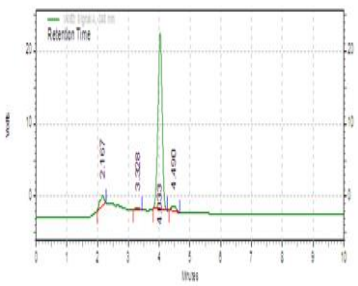


Acid degradation of atazanavir sulphate (0.5 M HCl, RT, 24 h)

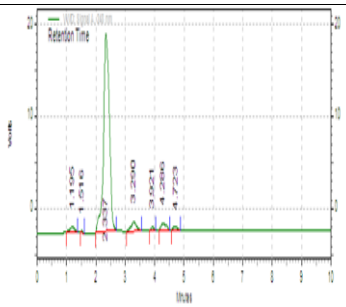
Graph 2: Result showing acidic degradation of atazanavir sulphate

Table 8: Result showing basic degradation of atazanavir sulphate

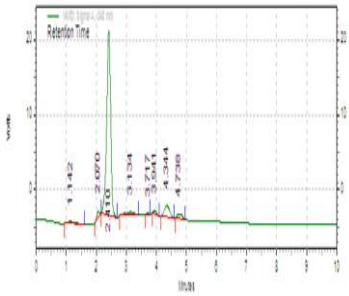
Time in hr.	Peak area	Retention time (Min)	% area	height	% height	% degradation
0	3.859124	4.033	90.79	409164	91.13	0
1	0.87823	3.97	18	81436	19.46	77.24
2	0.41441	3.92	8.5	51924	10.97	89.26
4	0.413232	4.004	2.29	19465	3.4	89.29
8	0.135042	6.74	6.5	41225	9.88	96.50
16	0.106985	3.94	1.99	14643	2.33	97.22
24	0.61130	3.94	1.31	8197	1.69	99.84



Pure atazanavir sulphate chromatogram



Base degradation of atazanavir sulphate (0.5 M NaOH, RT, 2 h.)

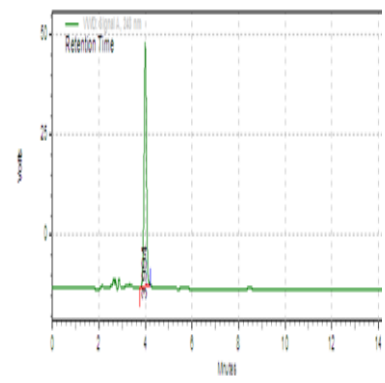


Base degradation of atazanavir sulphate (0.5 M NaOH, RT, 24 h)

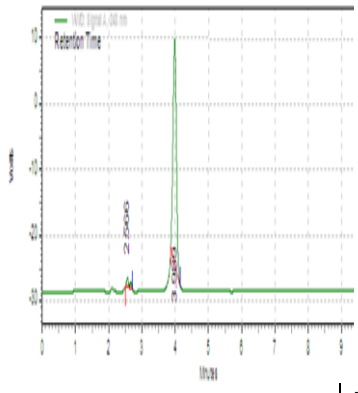
Graph 3: Result showing basic degradation of atazanavir sulphate.

Table 9: Result showing thermal degradation of atazanavir sulphate

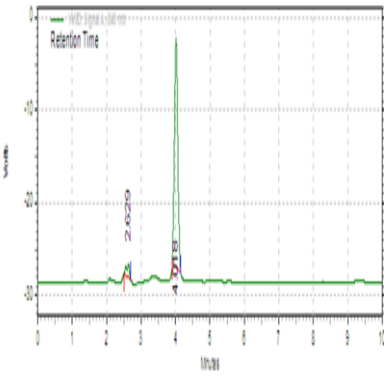
Time in hr.	Peak Area	Retention time (Min)	% Area	Height	% Height	% Degradation
0	7.936384	3.99	67.57	1038697	78.82	0
1	7.177002	3.98	18.00	81436	19.46	9.56
2	4.111263	3.98	96.61	57299	95.91	48.19
4	3.510767	4.01	91.14	466477	89.11	55.75
8	3.492496	4.02	92.78	375514	90.14	55.99
16	3.212981	2.86	93.44	391213	91.24	59.51
24	2.789007	4.01	95.29	415519	94.73	64.85



Pure atazanavir sulphate chromatogram



Thermaldegradation of atazanavir sulphate (80°C in furnace, 2 h.)

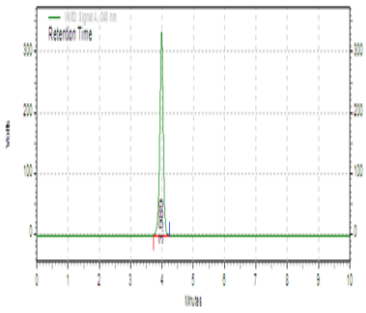


Thermal degradation of atazanavir sulphate (80°C in furnace, 24 h.)

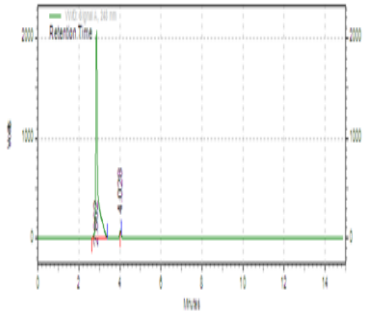
Graph 4: Result showing thermal degradation of atazanavir sulphate.

Table 10: Results showing oxidative degradation of atazanavir sulphate

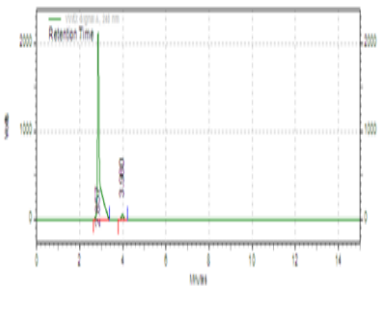
Time in hr.	Peak Area	Retention Time (Min)	% Area	Height	% Height	% Degradation
0	2.5757259	2.85	96.83	34290316	96.93	0
1	2.542115	2.85	96.68	35942994	97.14	40.70
2	2.5124412	2.93	96.44	35801232	97.21	41.47
4	2.4489141	2.85	96.77	33870476	96.7	42.15
8	2.4305977	2.85	96.16	34769175	96.64	43.62
16	2.4303451	2.85	96.83	34775437	97.16	44.04
24	2.3983386	2.86	96.08	34465688	97.34	44.04



Oxidative degradation of pure atazanavir sulphate.



Oxidative degradation of atazanavir sulphate (RT, 3% H_2O_2, 0 Min.)



Oxidative degradation of atazanavir sulphate (RT, 3% H_2O_2, 24 h.)

Graph 5: Result showing oxidative degradation of atazanavir sulphate

DISCUSSION

The linearity range was used within the range of 10-50 µg/mL This RP-HPLC method was successful validated in the optimized condition and validated parameters were found within the parameters limits. The validated parmeters LOD and LQD were found to be 2.041 and 6.1863 respectively. In this present studies atazanavir sulphate was subjected to its stability studies under different conditions as per the ICH guidelines. The acidic hydrolysis study showed that when atazanavir sulphate mixed with acid the retention time get shifted from 3.989 to 2.74 at zero min. The degradation peak found at retention time 2.68 min, 2.70 min, 2.74 min, 2.76 min, 2.73 min and 2.62 min along with pure drug. The peak area showed 96.60% drug gets degraded when it is kept in 0.5 M HCL at RT up to 24 h.

CONCLUSION

Atazanavir sulphate when subjected to alkaline degradation in 0.5 M NaOH at RT up to 24 h the degradation peak found at retention time 3.97 min, 3.92 min, 4.004 min, 4.74 min, 3.94 min, and 3.94 min in the chromatogram. The peak area of chromatogram showed that the drug get degraded 99.84% in basic condition.

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