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An Analytical Method Development and Validation for Simultaneous Estimation of Atazanavir and Ritonavir in Tablet Dosage Forms by Using UPLC.

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ABSTRACT

The present work was undertaken with the aim to develop and validate a rapid and consistent UPLC method in which the peaks will be appear with short period of time as per ICH guidelines. The UPLC separation was achieved on a Symmetry C_{18} (2.1 x 50 mm, 1.7 µm, Make: BEH) or equivalent in an isocratic mode. The mobile phase was composed of phosphate buffer (40%) [pH 2.5] and acetonitrile (60%). The flow rate was monitored at 0.25 ml per min. The wavelength selected for the detection was 249 nm. The run time was 4 min. The retention time found for Atazanavir and Ritonavir were 0.819 and 1.236 min. respectively. The % recovery was found 98.75 – 101.01 % for Atazanavir and 99.05 - 100.39 % for Ritonavir. The linearity was established in the range of 30 to 90 µg/ml for Atazanavir and 10 to 30 µg/ml for Ritonavir. The LOD found for Atazanavir and Ritonavir were 0.026 and 0.048 µg/ml respectively. The LOQ found for Atazanavir and Ritonavir were 0.096 and 0.15 µg/ml respectively. Overall the proposed method was found to be suitable, sensitive, reproducible, specific and accurate for the quantitative determination of the drug in tablet dosage form.

Keywords: Atazanavir, Ritonavir, LOD, LOQ, UPLC.

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INTRODUCTION

Atazanavir Sulphate Methyl is a Antiretroviral drug N- [(1S)-1-{ [(2S,3S) - 3 - hydroxy-4- [(2S)-2-[(methoxycarbonyl) amino] -3, 3-dimethyl-N'-{[4-(pyridin-2-yl)phenyl]methyl} butanehydrazido]-1-phenylbutan-2-yl] carbamoyl}-2, 2 - dimethyl propyl] carbamate sulphate is a azapeptide HIV-1 protease inhibitor. The compound selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells, thus preventing formation of mature virions [1-2].

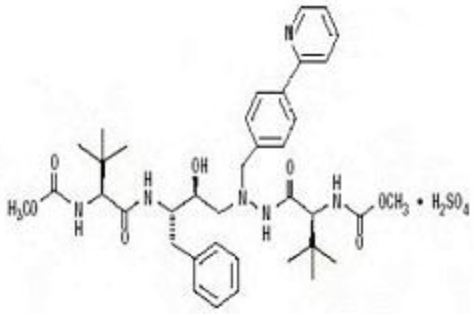

Figure 1: Chemical structure of Atazanavir Sulphate

Ritonavir is a Antiretroviral drug 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2 {[methyl({[2-(propan-2-yl)-1,3-tiazole-4-yl]methyl})carbamoyl]amino} butanamido]-1,6-diphenylhexan-2-yl] carbamate. Ritonavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and, therefore, improper viral assembly results. This subsequently results in noninfectious, immature viral particles [1-2].

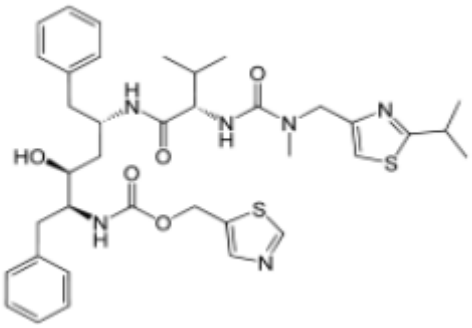

Figure 2: Chemical structure of Ritonavir

Literature survey revealed that very few methods have been reported for the analysis of Atazanavir and Ritonavir combinational dosage forms which include UV spectroscopy [5-7], RP-HPLC [8-13], Densitometric method [14-15], HPTLC [16-17] methods. The present study illustrate development and validation of simple, economical, selective, accurate, precise UPLC method for the determination of Atazanavir and Ritonavir in bulk and pharmaceutical dosage forms as per ICH guidelines [18].

In the present proposed work a successful attempt had been made to develop a method for the simultaneous estimation of Atazanavir and Ritonavir pharmaceutical dosage form and validate it. The goal of this study is to develop rapid, economical UPLC method for the analysis of Atazanavir and Ritonavir in combined dosage form using most commonly employed column (C_{18}) and simple mobile phase preparation. From the economical point of view and for the purpose of routine analysis, it was decided to develop a more economical UPLC method with simple mobile phase preparation for the estimation of Atazanavir and Ritonavir combinational dosage form. The method would help in estimate of drugs in single run which reduces the time of analysis and does not require separate method for each drug. Thus, the paper reports an economical, simple and accurate UPLC method for the above said pharmaceutical dosage forms.

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MATERIALS AND METHODS

Quantitative UPLC was performed on an ultra performance liquid chromatography -Waters Acquity 2996 Alliance, UPLC system connected with PDA Detector and Empower2 Software. The drug analysis data were acquired and processed using Empower2 software running under Windows XP Symmetry C_{18} (2.1 x 50 mm, 1.7 µm, Make: BEH). In addition an analytical balance (AFCOSET Model ER200A), digital pH meter (ADWA Model AD102U), a sonicator (ENERTECH Model SE60US) were used in this study.

Standards and chemicals used

The reference samples of Atazanavir and Ritonavir standards were kindly supplied as gift samples by M/s. Pharma Train Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate and ortho phosphoric acid from Merck Ltd., Mumbai, India, while methanol, acetonitrile (UPLC grade) and triethylamine (UPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used was of UPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India.

Preparation of mobile phase

A mixture of above prepared buffer 400 ml (40%) and 600 ml of UPLC grade Acetonitrile (60%) were mixed and degassed in ultrasonic water bath for 5 minutes. The mobile phase was filtered through 0.45 µm filter under vacuum. The mobile phase was used as diluent.

Preparation of calibration standards

Accurately weighed and transferred 10 mg of Atazanavir and 10 mg of Ritonavir working standard into a 100 ml clean dry volumetric flask and added about 70 ml of diluent. It was sonicated to dissolve the drug completely and made volume up to the mark with the same diluent. From the above prepared stock solution, further dilutions were made to get concentration of 30-90 µg/ml for Atazanavir and 10-30 µg/ml of Ritonavir.

System suitability

System suitability is an integral part of chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solutions at 100% concentration level for Atazanavir and Ritonavir to check the reproducibility of the system. At first the UPLC system was stabilized for 40 min. One blank followed by six replicate analysis of solution containing 100% target concentration of Atazanavir and Ritonavir were injected to check the system suitability. To ascertain the system suitability for the proposed method, a number of parameters such as theoretical plates, peak asymmetry and retention time were taken and results were presented in table no. 1.

Table 1: The system suitability results for Atazanavir and Ritonavir

Parameters	Atazanavir	Ritonavir	Acceptance Criteria
Tailing Factor	1.94	1.67	< 2
Theoretical plates	8682	8953	> 8000
% R.S.D	0.4	1.4	< 2
Resolution	5.12	5.12	> 2

Calibration curves for Atazanavir and Ritonavir

Replicate analysis of solution containing 30-90 µg/ml for Atazanavir and 10-30 µg/ml for Ritonavir sample solutions respectively were injected into UPLC according to the procedure in a sequence and chromatograms were recorded. Calibration curves were constructed by plotting by taking concentrations on X-axis and ratio of peak areas of standards on Y-axis and regression equation were computed for both the drugs and represented in fig. no. 5 and 6. The data are represented in table no.2.

ISSN: 0975-8585

Table 2: Linearity results for Atazanavir and Ritonavir

Atazanavir		Ritonavir	
Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
30	396447	10	110737
45	701780	15	151805
60	920662	20	194649
75	1154631	25	233454
90	1384932	30	268163

Analysis of marketed formulation

Estimation of Atazanavir and Ritonavir (Brand name Viratаz-R) in tablet formulation by UPLC was carried out by using optimized chromatographic conditions. Twenty tablets of formulation (Atazanavir 300 mg and Ritonavir 100 mg) were accurately weighed; the average weight of tablets were calculated and crushed to a fine powder. From the triturate of 20 tablets, an amount equivalent to 786.5 mg of powder was accurately weighed and transferred in a 100 ml clean and dry volumetric flask. Initially about 70 ml of diluent was added to dissolve the powder and further the volume was made upto the mark with the diluent. The solution was sonicated for 15 minutes and filtered through a 0.45 µ filter under vacuum. From the clear solution, pipette out 6 ml and transferred into a 10 ml clean and dry volumetric flask and the volume was made up to the mark. For theoretically, the drug solution contain 60 µg/ml of Atazanavir and 20 µg/ml of Ritonavir. 20 µL of the standard and sample solutions were injected into the chromatographic system and areas for the Atazanavir and Ritonavir peaks were measured. The results are represented in table no.3.

$$\textbf{\% Assay Calculation} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.Wt}{Label\ Claim} \times 100$$

where:
AT = average area counts of sample preparation.
AS = average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = percentage purity of working standard
LC = label claim of Atazanavir/Ritonavir (mg/ml).

$$\textbf{\% Assay for Atazanavir} = \frac{932192}{932544} \times \frac{10}{100} \times \frac{6}{10} \times \frac{100}{26.22} \times \frac{10}{6.0} \times \frac{99.9}{100} \times \frac{786.5}{300} \times 100 = 99.85\ \%$$

$$\textbf{\% Assay for Ritonavir} = \frac{191087}{193855} \times \frac{10}{100} \times \frac{2}{10} \times \frac{100}{26.22} \times \frac{10}{6.0} \times \frac{99.9}{100} \times \frac{786.5}{100} \times 100 = 98.47\ \%$$

Table 3: Quantification of Formulation

Drug	Label Claim (mg/tab)	Percentage Obtained	S.D	%R.S.D
Atazanavir	300	99.85	986.1	0.1
Ritonavir	100	98.47	947.5	0.5

Validation study for Atazanavir and Ritonavir

An integral part of analytical method development is validation. Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. The newly developed UPLC method was validated as per International Conference on Harmonization (ICH) guidelines for parameters like system suitability, accuracy, linearity, precision (repeatability), intermediate precision, limit of detection (LOD), limit of quantification (LOQ) and robustness.

ISSN: 0975-8585

Precision

Precision studies for the samples (Atazanavir and Ritonavir) were carried out by estimating corresponding responses 5 times on the same day and on different day for the 100 % target concentration. The percent relative standard deviation (% RSD) is calculated which is within the acceptable criteria of not more than 2 %. The results are presented in table no. 4 and 5.

Table 4: Precision results for Atazanavir and Ritonavir

Sr. No.	Atazanavir			Ritonavir		
	R_T (min)	Area (µV*sec)	Height (µV)	R_T (min)	Area (µV*sec)	Height (µV)
1	0.833	934062	299932	1.265	198789	57586
2	0.831	931925	296065	1.265	196295	57589
3	0.830	930796	300861	1.267	197691	57904
4	0.832	931360	296009	1.266	196548	57709
5	0.827	935030	303228	1.266	197288	57584
Average		932634.8			197322.1	
Standard Deviation		1822.4			992.8	
%RSD		0.2			0.5	

Table 5: Intermediate precision results for Atazanavir and Ritonavir

Sr. No.	Atazanavir			Ritonavir		
	RT (min)	Area (µV*sec)	Height (µV)	RT (min)	Area (µV*sec)	Height (µV)
1	0.842	934022	299803	1.318	208549	57624
2	0.844	934124	301902	1.318	206667	57650
3	0.843	933493	300867	1.323	202816	56756
4	0.843	934954	302024	1.324	203151	56890
5	0.842	932356	299221	1.326	205775	57239
Average		933789			205391	
Standard Deviation		957.4			2418.3	
%RSD		0.1			1.2	

Linearity

The linearity graphs for the proposed assay methods were obtained over the concentration range of 30-90 µg/ml for Atazanavir and 10-30 µg/ml for Ritonavir. Method of least square analysis is carried out for getting the slope, intercept and correlation coefficient, regression data values and the results were presented in table no. 2. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curves were shown in fig. no. 5 and 6.

Accuracy (Recovery studies)

The amount added and amount found for Atazanavir and Ritonavir and the individual recovery and mean recovery values were calculated. Known amount of Atazanavir and Ritonavir at 50 %, 100 %, 150 % was added to a pre quantified sample solution. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo. The mean percentage recovery of Atazanavir and Ritonavir at each level was not less than 98 % and not more than 102 %. The data are represented in table no. 6.

ISSN: 0975-8585

Table 6: Accuracy results for Atazanavir and Ritonavir

Drug	% Level	Area	Amount present (mg)	Amount added (mg)	% Recovery	Mean Recovery
Atazanavir	50%	463050	5.0	4.94	98.81%	99.52%
	100%	925515	10.0	9.87	98.75%	
	150%	1420126	15.0	15.15	101.01%	
Ritonavir	50%	81880	5.0	5.02	100.39%	99.76%
	100%	162902	10.0	9.99	99.86%	
	150%	242366	15.0	14.86	99.05%	

Robustness

The robustness is evaluated by the analysis of Atazanavir and Ritonavir under different experimental conditions such as making small changes in flow rate (± 0.02 ml/min) and mobile phase composition (± 5%). The results are presented in table no. 7, 8, 9 and 10.

Table 7: Robustness results for Atazanavir (change in flow rate)

Sr. No.	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	UUSP Tailing
1.	0.23	8695	1.98
2.	0.25	8685	1.94
3.	0.27	8956	1.96

Table 8: Robustness results for Ritonavir (change in flow rate)

Sr. No.	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.23	8956	1.76
2	0.25	8958	1.66
3	0.27	9012	1.74

Table 9: Robustness results for Atazanavir (change in mobile phase composition)

Sr. No.	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	8725	1.92
2	Actual	8685	1.94
3	10% more	8869	1.91

Table 10: Robustness results for Ritonavir (change in mobile phase composition)

Sr. No.	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	9016	1.69
2	Actual	8958	1.66
3	10% more	8796	1.79

ISSN: 0975-8585

LOD and LOQ

Limit of detection is the lowest concentration in a sample that can be detected but not necessarily quantified. Under the stated experimental conditions, the limit of quantification is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of detection and limit of quantification were calculated using following formula LOD= 3.3(SD)/S and LOQ= 10(SD)/S, where SD= standard deviation of response (peak area) and S= average of the slope of the calibration curve. The LOD obtained for Atazanavir and Ritonavir was 0.026 and 0.048 µg/ml. The LOQ obtained for Atazanavir and Ritonavir was 0.096 and 0.15 µg/ml.

RESULT AND DISCUSSION

An UPLC method was preferred for simultaneous estimation of Atazanavir and Ritonavir. Preliminary experiments were carried out to achieve the best chromatographic conditions for the simultaneous determination of the drug substances. Several column types and lengths were tried considering other chromatographic parameters. The optimized chromatographic conditions and system suitability parameters for proposed method is represented in table no. 11.

Table No. 11 Optimized chromatographic conditions and system suitability parameters for proposed method

Parameters	Condition
Column	BEH C_{18}(2.1 X 50 mm); 1.7 µm
Detector Wavelength	249 nm
Flow rate	0.25 ml/min
Injection volume	5.0 µl
Column over temperature	Ambient
Sample tray temperature	Ambient
Run time	4 minute
Elution	Isocratic
Gradient programme	NA
Diluent	Acetonitrile and Phosphate buffer pH 2.5 mixed in 60:40 ratio used as diluents.

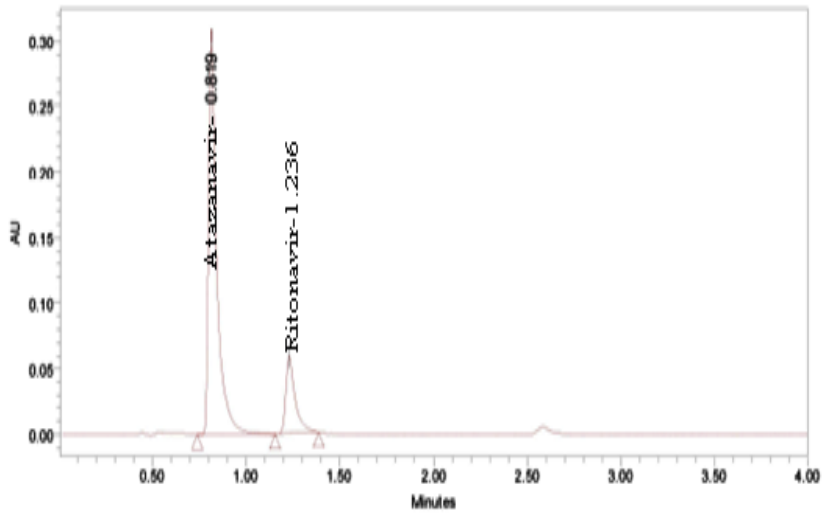

Figure 3: Chromatogram represents for the optimized method

ISSN: 0975-8585

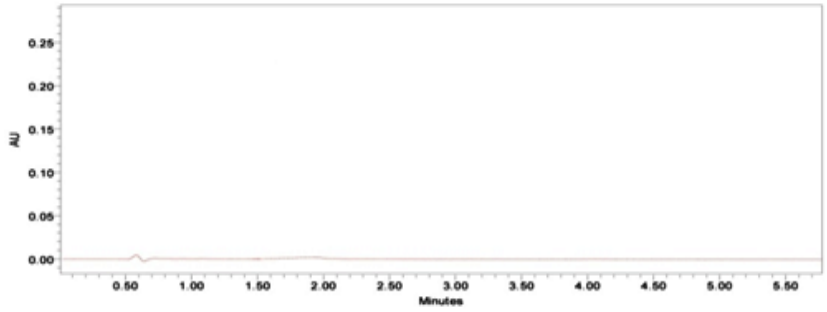

Figure 4: Chromatogram represents for the blank

A C_{18} column with a 2.1 X 50 mm internal diameter and 1.7 µm particle size was chosen. The detection wavelength was selected as 249 nm. The Chromatographic conditions were optimized by changing the mobile phase composition and buffers used in mobile phase. Different experiments were performed to optimize the mobile phase but adequate separation of the drugs could not be achieved. By altering the pH of buffer results a good separation. Different proportions of solvents were tested. Eventually the best separation was obtained by the isocratic elution system using a mixture of Phosphate buffer and Acetonitrile in the ratio of 40:60. The pH of the buffer was adjusted to 2.5 by using OPS. The flow rate was monitored at 0.25 ml/min. A typical chromatogram for simultaneous estimation of the two drugs obtained by using the above mentioned mobile phase was represented in the fig. no. 3 and the results are summarized in table no. 10.

Under these conditions Atazanavir and Ritonavir were eluted at 0.891 and 1.236 minutes respectively with a run time of 4.0 minutes. The Phosphate buffer (pH-2.5): Acetonitrile (40:60) was chosen as the mobile phase. The method shows linearity between the concentration range of 30-90 µg/ml for Atazanavir and 10-30 µg/ml for Ritonavir. The experimental results are represented in table no. 2 and fig. no. 5 and 6.

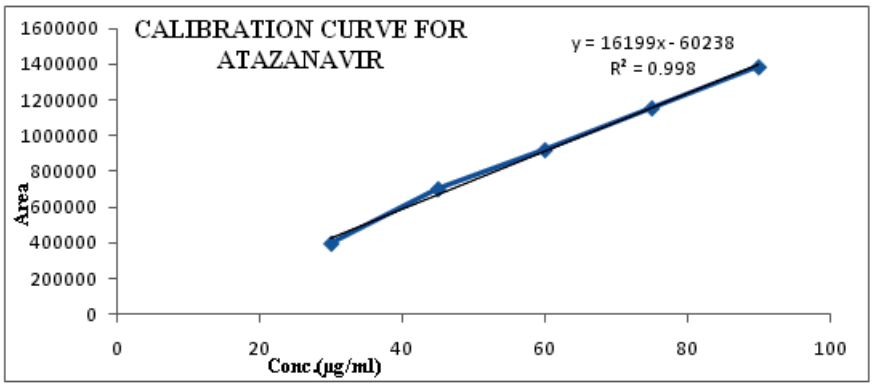

Figure 5 Calibration curve for Atazanavir

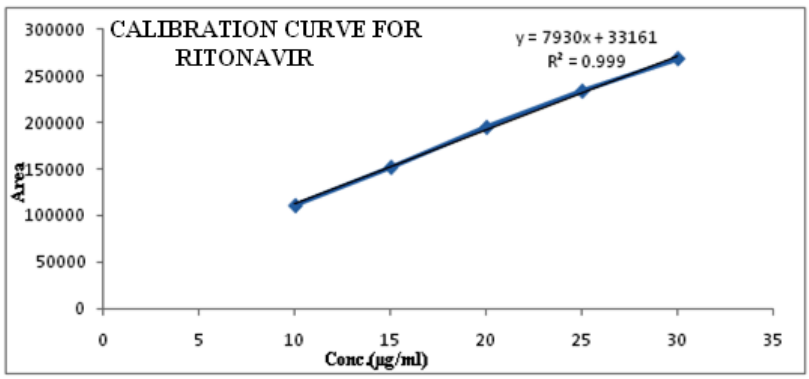

Figure 6: Calibration curve for Ritonavir

The % recovery of Atazanavir and Ritonavir was found to be in the range of 98.75 to 101.01 % and 99.05 to 100.39 % respectively. As there was no interference due to excipients and mobile phase, the method was found to be specific. As both compounds pass the peak purity, the method was found to be specific. The method was robust and rugged as observed from insignificant variation in the results of analysis by changes in flow rate and mobile phase composition. The results are shown in table no. 7, 8, 9 and 10. The LOD and LOQ values were calculated based on the standard deviation of the response and the slope of the calibration curve at levels approximately the LOD and LOQ. The LOD found for Atazanavir and Ritonavir were 0.026 and 0.048 µg/ml respectively. The LOQ found for Atazanavir and Ritonavir were 0.096 and 0.15 µg/ml respectively.

CONCLUSION

The proposed UPLC method was found to be specific, precise, accurate, rapid and economical for simultaneous estimation of Atazanavir and Ritonavir in tablet dosage forms. 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 and this method can be used for routine analysis.

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