Method development and validation for the simultaneous estimation of Atazanavir and Ritonavir in tablet dosage form by RP-HPLC

Nuli Vasavi*, Afroz Patan

Nimra College of Pharmacy, Jupudi, Vijayawada, A.P, India

***Corresponding author: Email: vasavi14nooli@gmail.com, Phone +91-9966136060**

ABSTRACT

The present investigation describes about a simple, economic, selective, accurate, precise reverse phase high performance liquid chromatographic method for the simultaneous estimation of Atazanavir and Ritonavir in pure and pharmaceutical dosage forms of Atazanavir and Ritonavir were well separated using a X-Tera C_{18} (100 x 4.6mm, 3.5µm) and Mobile phase consisting of Buffer(pH-2.5): Acetonitrile (40:60) adjusted to pH- 2.5 at the flow rate 1.2 ml/min and the detection was carried out at 247nm with PDA detector. The Retention time for Atazanavir and Ritonavir were found to be 1.982 & 2.576 respectively. The developed method was validated for recovery, specificity, precision, accuracy, linearity according to ICH guidelines. The method was successfully applied to Metronidazole and Norfloxacin combination pharmaceutical dosage form.

Key Words: RP-HPLC, Atazanavir and Ritonavir Accuracy, Precision.

1. INTRODUCTION

Atazanavir Sulphate Methyl is a Antiretroviral drug *N*- [(1*S*)-1-{ [(2*S*,3*S*) - 3 - hydroxy-4- [(2*S*)-2-[(methoxycarbonyl) amino] - 3, 3 – dimethyl - *N′* - {[4-(pyridin-2-yl)phenyl]methyl} butanehydrazido]-1-phenylbutan-2-yl] carbamoyl}-2, 2 - dimethylpropyl] 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.

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 non-infectious, immature viral particles.

Literature survey revealed that very few methods have been reported for the analysis of Atazanavir and Ritonavir combinational dosage forms which include UV spectroscopy, Reverse Phase High performance Liquid Chromatography, Densitometric method, HPTLC methods. The present study illustrate development and validation of simple, economical, selective, accurate, precise RP-HPLC method for the determination of Atazanavir and Ritonavir in bulk and Pharmaceutical dosage forms as per ICH guidelines.

The goal of this study is to develop rapid, economical HPLC method for the analysis of Atazanavir and Ritonavir in combined dosage form using most commonly employed column (C18) and simple mobile phase preparation. 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. From the economical point of view and for the purpose of routine analysis, it was decided to develop a more economical RP-HPLC 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 RP-HPLC method for the above said pharmaceutical dosage forms.

2. MATERIALS AND METHODS

Quantitative HPLC was performed on a high performance liquid chromatograph -Waters e2695Alliance HPLC system connected with PDA Detector 2487 and Empower2 Software. The drug analysis data were acquired and processed using Empower2 software running under Windows XP X-Tera C_{18} (100 x 4.6mm, 3.5µm) particle size. 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 Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from Merck Ltd., Mumbai, India, while acetonitrile (HPLC

grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used was of HPLC 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 HPLC grade Acetonitrile (60%) were mixed and degassed in ultrasonic water bath for 5 minutes. The mobile phase was filtered through 0.45 µ filter under vacuum.

Preparation of calibration standards: Accurately weighed and transferred 30mg of Atazanavir and 10mg of Ritonavir working standard into a 10ml clean dry volumetric flask and added about 7ml of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution)(3000, 1000 µg/ml). From the above stock solution, 1ml of the solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. (300, 100µg/ml). From this, 4ml of the solution was pipetted into another 10ml volumetric flask and diluted up to the mark with diluent.

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 HPLC 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 2.

Calibration curves for Atazanavir and Ritonavir: Replicate analysis of solution containing 60-180 µg/ml for Atazanavir and 20-60 µg/ml for Ritonavir sample solutions respectively were injected into HPLC 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 drugs and represented in fig:5&6

Analysis of marketed formulation: Accurately weighed and transferred 49.8mg of Atazanavir and Ritonavir tablet powder into a 10ml clean dry volumetric flask and added about 7ml of diluent. It was sonicated to dissolve it completely and made volume up to the mark with the same diluent. (Stock solution).

From the above stock solution, 1ml of the solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent. From this, 4ml of the solution was pipetted into another 10ml volumetric flask and diluted up to the mark with diluent. 20 µL of the standard and sample solutions were injected into the chromatographic system and areas for the Atazanavir and Ritonavir peaks were measured.

Validation study of Metronidazole and Norfloxacin: 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 RP-HPLC 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.

Precision: precision study of sample (Atazanavir and Ritonavir) was carried out by estimating corresponding responses 5 times on the same 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 were presented in Table 3.

Linearity: The linearity graphs for the proposed assay methods were obtained over the concentration range of 30mg of Atazanavir and 10mg of 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 5. The representative chromatograms indicating the sample were shown in fig.2&3. A calibration curve was plotted between concentration and area response and statistical analysis of the calibration curves were shown in fig. 5&6.

Accuracy (Recovery studies): The Amount found and Amount added for Atazanavir & Ritonavir and the individual recovery and mean recovery values were calculated. Known amount of Atazanavir and Ritonavir at 50%, 100%, 150% is 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 is not less than 98% and not more than 102%.

Robustness: The robustness is evaluated by the analysis of Atazanavir and Ritonavir under different experimental conditions such as making small changes in flow rate (±0.2 ml/min), λmax (±5), column temperature (±5), mobile phase composition (±5%), and pH of the buffer solution. The results were presented in Table 4.

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.

3. RESULTS AND DISCUSSION

Reverse phase HPLC method was preferred for the determination 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. C18 column with a 4.6 mm inner diameter and 3.5µm particle size was chosen. The detection wave length was selected as 247nm with 2487 detector. 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 Acetonitrile (40:60) adjusted to Buffer (pH-2.5) at a flow rate of 1.2 ml/min. A typical chromatogram for simultaneous estimation of the two drugs obtained by using a above mentioned mobile phase. Under these conditions Atazanavir and Ritonavir were eluted at 1.982 & 2.567 minutes respectively with a run time of 5 minutes. The representative chromatogram of this simultaneous estimation shown in fig. 3 & 4 and results were summarized in Table 1.

The Buffer (pH-2.5): Acetonitrile (40:60) was chosen as the mobile phase. The run time of the HPLC procedure was 5 minutes at flow rate of 1.2ml/min was optimized which gave sharp peak, minimum tailing factor. The system suitability parameters were shown in Table 1 were in within limit, hence it was concluded that the system was suitable to perform the assay. The method shows linearity between the concentration range 30mg of Atazanavir and 10mg of Ritonavir. The experimental results were shown in table 5 and fig.5&6. The % recovery of Atazanavir and Ritonavir was found to be in the range of 98.96 to 101.84 % and 98.29 to 100.54% 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, column oven temperature, mobile phase composition and wave length separately and analysis being performed by different analysts. The results were shown in Table 4. 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 limit of detection was obtained for Atazanavir and Ritonavir found to be 0.999 and 0.999. The results were shown in Table-6.

Table.1 optimized chromatographic conditions and system suitability parameters for proposed method

Parameter	Chromatographic conditions
Instrument	Waters e2695 Alliance HPLC with Empower2 software
Column	X-Tera C_{18} (100 x 4.6mm, 3.5µm)
Detector	Detector 2487
Mobile phase	Phosphate Buffer (pH2.5): Acetonitrile (40:60)
Flow rate	1.2ml/min
Detection wavelength	247nm
Temperature	Ambient
Injection volume	20µl
Retention time	Atazanavir: 1.982; Ritonavir: 2.576
Theoretical plate count	Atazanavir: 4092.8 ; Ritonavir: 4900.4
Tailing factor	Atazanavir: 1.3; Ritonavir: 1.2
Resolution factor	4.2

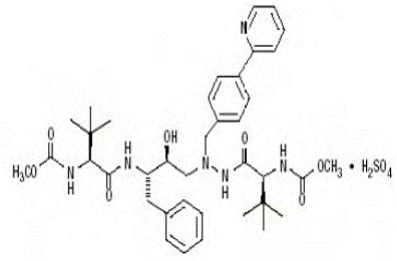

Fig. 1: Structure of Atazanavir Sulphate

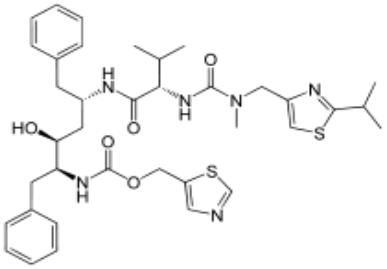

Fig. 2: Structure of Ritonavir

Table.2.System suitability:Flow change observation of atazanavir and ritonavir

	Flow rate (ml/min)	System suitability results	
		Usp plate count	Usp tailing
Atazanavir	1.0	4028.2	1.3
	1.2	4092.8	1.3
	1.4	4010.7	1.3
Ritonavir	1.0	4727.0	1.2
	1.2	4900.4	1.2
	1.4	4712.2	1.2

Table.3.Results of Precision study

Sample	Injection number	Precision	
		RT	Peak area
Atazanavir	1	1.978	1647681
	2	1.976	1647899
	3	1.979	1642958
	4	1.982	1649928
	5	1.974	1649877
	Mean	1.977	1633919
	%RSD(NMT 2.0)		0.10
Ritonavir	1	2.576	595172
	2	2.572	596877
	3	2.573	596609
	4	2.578	597459
	5	2.573	596311
	Mean	2.574	596485.6
	%RSD(NMT 2.0)		0.14

Table 4: Robustness studies Atazanavir and Ritonavir

Sample	Paraameters	Optimized	Used	RT	USP Tailing	Plate count
Atazanavir	Flow rate(±0.2)	1ml/min	1.0	2.541	1.3	4028.2
			1.2	1.982	1.3	4.92.8
			1.4	1.786	1.3	4010.7
	Mobile phase variation	0%	Less	2.432	1.3	4131.8
			Actual	1.982	1.3	4092.8
			More	1.785	1.3	4013.1
Ritonavir	Flow rate(±0.2)	1ml/min	1.0	2.956	1.2	4727.0
			1.2	2.576	1.2	4900.4
			1.4	2.291	1.2	4712.2
	Mobile phase variation	0%	Less	2.952	1.2	4918.2
			Actual	2.576	1.2	4900.4
			More	2.290	1.2	4878.1

Table.5.Linearity data of the Atazanavir and Ritonavir

Observation of Atazanavir		
Linearity Level	**Concentration**	**Area**
I	60ppm	899573
II	90ppm	1254637
III	120ppm	1648501
IV	150ppm	2027034
V	180ppm	2469227
Correlation Coefficient		0.999
Observation Of Ritonavir		
I	20ppm	328807
II	30ppm	457715
III	40ppm	602795
IV	50ppm	744367
V	60ppm	904976
Correlation Coefficient		0.999

Table.6.Limit of Detection and Limit of Quantification

	Atazanavir		**Ritonavir**	
	mcg	Area	mcg	Area
LOD	1.979	580	2.578	687
LOQ	1.978	1962	2.577	2322

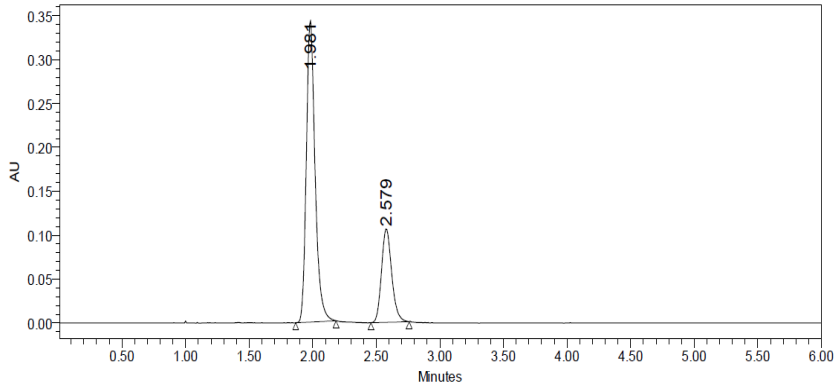

Figure.3.Typical Chromatogram of standard Atazanavir and Ritonavir

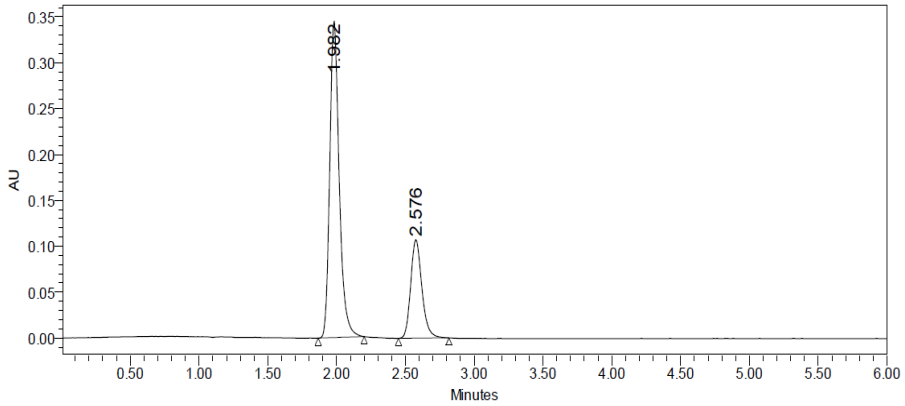

Figure.4.Typical chromatogram of Metronidazole and Norfloxacin tablets in marketed formulation

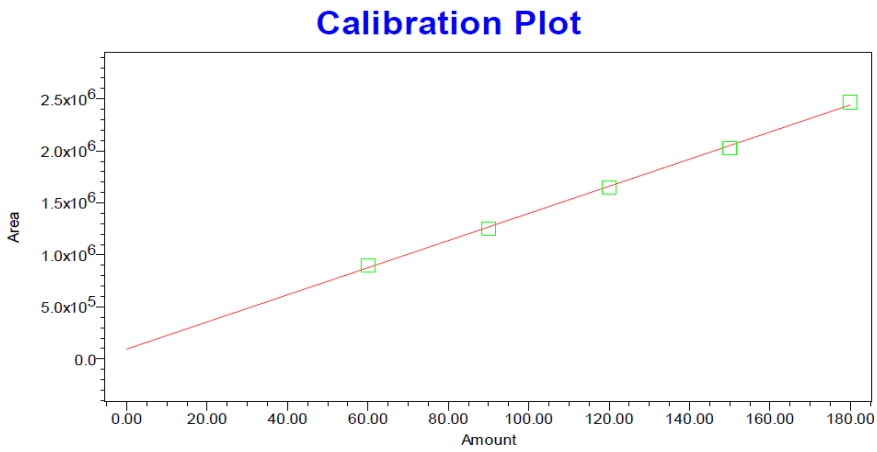

Figure.5.Linearity for Atazanavir

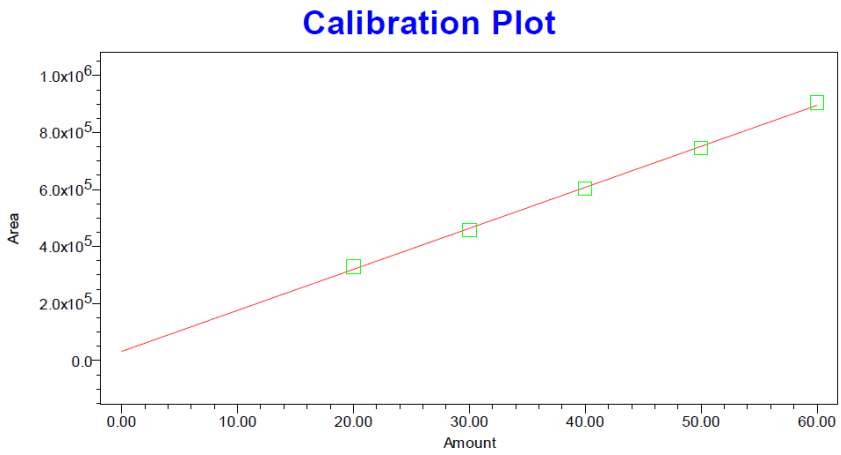

Figure.6.Linearity for Ritonavir

4. CONCLUSION

The proposed RP-HPLC method was found to be specific, precise, accurate, rapid and economical for simultaneous estimation of Atazanavir and Ritonavir in Tablet 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 and this method can be used for routine Analysis.

ACKNOWLEDGEMENT

The authors would like to thank beloved parents and all my well wishers, one and all who have helped me directly and indirectly in completing this project work.

REFERENCES

Adrienne C. Muller, An efficient HPLC method for the quantitative determination of Atazanavir in Human Plasma suitable for Bioequivalence and Pharmacokinetic studies in healthy Human Subjects. Journal of Pharmaceutical and Biomedical Analysis, 53(1), 2010, 113–118.

J. Venkateswara Rao, K. Srinivasu, N. Appala Raju and K. Mukkanti, A Validated RP-HPLC method for the determination of Atazanavir in Pharmaceutical dosage form, E-Journal of Chemistry, 8(1), 2011, 453-456.

M. Padmalatha, K.Vanitha Prakash and Eranna Dopadally, Validated Reversed Phase High Performance Liquid Chromatography method for the estimation of Atazanavir Sulphate in Pharmaceutical formulations. Oriental Journal of Chemistry, 26(1), 2010, 123-127.

R. K. Nanda, A.A. Kulkarni and P.B.Yadav, Simultaneous Spectrophotometric estimation of Atazanavir Sulphate and Ritonavir in tablets, Scholars Research Library Der Pharma Chemica, 3(3), 2011, 84-88.

Ravindra Reddy Y. and A. Swetha Mallesh, Method development and validation of Atazanavir and Ritonavir in a combined dosage form by RP-HPLC

Method, International Journal of Pharmacy & Technology, 3(3), 2011, 3316-3334.

S. Colombo, N. Guignard, C. Marzolini, A. Telenti, J. Biollaz and L.A. Decosterd, Determination of the new HIV-Protease Inhibitor Atazanavir by Liquid Chromatography after Solid-Phase Extraction. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences, 810 (1), 2004, 25-34.

S.G. Khanage, V.K. Deshmukh, P.B. Mohite, V.M. Dhamak and S. Appala Raju, Development of Derivative Spectrophotometric estimation of Atazanavir Sulphate in bulk drug and Pharmaceutical dosage forms. Int.J.Pharm.& Health Sci., 1(3), 2010, 49-154.

Sreenivasa Rao Chitturia, Yallappa Somappa Somannavara, Badrinath Gupta Peruria, Srinivas Nallapatia, and Hemant Kumar Sharmaa, Gradient RP-HPLC method for the determination of Potential Impurities in Atazanavir Sulfate, Journal of Pharmaceutical and Biomedical Analysis, 55(1), 2011, 31-47.

Vishnu P. Choudhari, Nilesh A. Bari, Shailendra P. Kela, Shailesh N. Sharma and Saroj V. Shirse, Spectrophotometric simultaneous determination of Atazanavir and Ritonavir in combined tablet dosage form by Ratio Derivative and Area Under Curve Method, Scholars Research Library Der Pharma Chemica, 4(1),2012, 208-213.