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Development and Validation of Stability Indicating RP-HPLC Method for the Estimation of Atazanavir and Ritonavir in Bulk and its Dosage Form

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ABSTRACT

The main aim of the present study was to develop a simple, accurate, precise, sensitive, selective, reproducible, validated and rapid stability indicating RP-HPLC method for the simultaneous estimation of Atazanavir and Ritonavir in tablet dosage forms .Chromatography was carried out using Unisol C18 (3µm, 4.6 ×150mm) column with a flow rate of 0.8mL/min .The mobile phase consisted Acetonitrile and buffer pH 5.0 in the ratio 50:50. The retention times of Atazanavir and Ritonavir were found to be eluted at 2.1 min and 4.0 min respectively .The LOD value was found to be 0.316µg/ml for Atazanavir and 0.949µg/ml for Ritonavir at signal to noise ratio 3:1. LOQ value was found to be0.433µg/ml for Atazanavir and 3.19µg/ml for Ritonavir at signal to noise ratio 10:1. The accuracy of the method was assessed by recovery study in the dosage form at three concentration levels. The method developed has been statistically validated according to ICH guidelines. The method showed good reproducibility and recovery with % RSD less than 2. The stability-indicating capability of the method was established by forced degradation studies under stress conditions like acid, base, peroxide, UV, thermal, humidity. Hence, the chromatographic method developed for the estimation was said to be rapid, simple, specific, sensitive, precise, accurate, robust, and reliable that can be effectively applied for routine analysis in research institutions, quality control departments in industries.

Keywords: Atazanavir, Ritonavir, RP-HPLC and UV Spectroscopy

1. Introduction

Atazanavir (formerly known as BMS-232632) is an antiretroviral drug of the protease inhibitor (PI) class. Like other antiretrovirals, it is used to treat infection of human immunodeficiency virus (HIV). Atazanavir is distinguished from other PIs in that it can be given once-daily (rather than requiring multiple doses per day) and has lesser effects on the patient's lipid profile (the amounts of cholesterol and other fatty substances in the blood). Like other protease inhibitors, it is used only in combination with other HIV medications. The U.S. Food and Drug Administration (FDA) approved atazanavir on June 20, 2003. Atazanavir (ATV) is an azapeptide HIV-1 protease inhibitor (PI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Atazanavir selectively inhibits the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-1 infected cells by binding to the active site of HIV-1 protease, thus preventing the formation of mature virions. Atazanavir is not active against HIV-2. Atazanavir is chemically methyl 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-dimethylpropyl]carbamate.The chemical structure of Atazanavir is represented in Fig. 1

Ritonavir inhibits the HIV viral proteinase enzyme that normally cleaves the structural and replicative proteins that arise from major HIV genes, such as *gag* and *pol*. *Gag* encodes proteins involved in the core and the nucleocapsid, while *pol* encodes the the HIV reverse transcriptase, ribonuclease H, integrase, and protease. The *pol*-encoded proteins are initially translated in the form of a larger precursoe polypeptide, *gag-pol*, and needs to be cleaved by HIV protease to form other complement proteins. Ritonavir is chemically 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate. The chemical structure of Ritonavir is represented in Fig. 2. Atazanavir and Ritonavir[1-7] combination medication used to treat HIV-1 infection. In the combination atazanavir functions as a protease inhibitor and ritonavir functions to increase levels of atazanavir. The combination was approved for use in India in 2012, and is pending approval in the United States As of 2017.

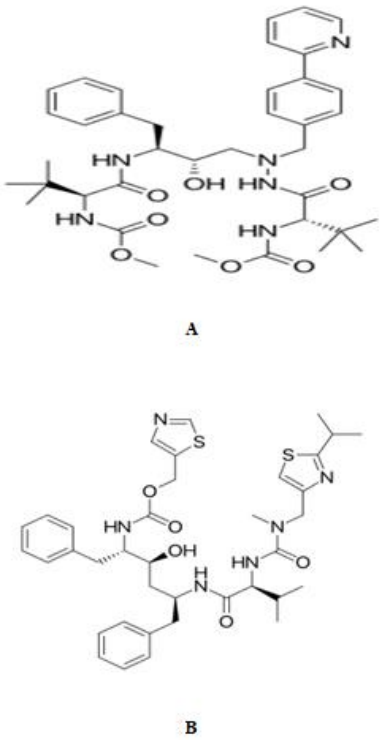

Figure 1. Chemical structures of (A) Atazanavir (B) Ritonavir

Extensive literature review [8-20] was conducted and an attempt was made to develop an unambiguous, valid method for stability indicating RP-HPLC method for the simultaneous estimation of Atazanavir and Ritonavir in tablet dosage forms. The aim of this study is to develop and validate a new simple, accurate and economic stability-indicating RP-HPLC method with less runtime, which would be able to separate and quantify a combination of Atazanavir and Ritonavir in a single run.

2. Materials and methods

Materials and Instruments

Materials

ATAZOR–R (tablets of 300 mg, 100 mg of Atazanavir and Ritonavir respectively) formulation was purchased from the authorized drug store. HPLC grade water, Methanol, Orthophosphoric acid, Triethanol amine.

Instruments

Electronics Balance - Metler Toledo ME204

p^H meter - Eutech instruments pH 700

Ultrasonicator - PCI Analysis

HPLC - Agilent technologies 1200 infinity series, integrated with Openlab software

Vaccum filtration unit - Milli pore (XI 0422050)

Distillation unit - Borosil

Method

Chromatographic Conditions

Mobile phase **:** Acetonitrile and bufferpH 5.0 in the ratio 50:50

Flow rate **:** 0.8mL/min

Column **:** Unisol C18 (3µm, 4.6 ×150mm)

Detector wave length **:** 238nm

Column temperature **:** 25℃

Injection volume **:** 10µL

Run time **:** 10 min

Preparation of Working Standard Solution

20 mg of Atazanavir and 12.5 mg of Ritonavir were accurately weighed and transferred into a 10 mL clean dry volumetric flask. Add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further, 1 ml of the above prepared stock solution is pipetted into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of sample solution

Accurately weigh the samples of 10 tablets. It is crushed in mortor and pestle. Transfer equivalent to 20 mg of Atazanavir and 12.5 mg of Ritonavir sample into a 10 mL clean dry volumetric flask. Add about 7 mL of diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then, it is filtered through 0.44 micron Injection filter. Further, pipette 1 ml of Atazanavir and Ritonavir from the above sample solution into a 10 ml volumetric flask and dilute up to the mark with diluent. The standard solutions were prepared on daily basis from which stock solutions were prepared.

Mobile phase selection

Experiments were conducted with mobile phase consisting of Phosphate Buffer (pH 5.0 adjusted with TEA) and Acetonitrile and trails were conducted taking different combinations of mobile phases to achieve maximum possible theoretical plates, least possible tailing factor and retention time. Based on this data, the best separation was obtained with Acetonitrile and Phosphate buffer (50:50) mobile phase composition.

Method validation

Validation parameters are calculated according to international conference on harmonization (ICH) guidelines –validation of analytical procedures: text and methodology Q2 (R1)

System suitability parameters

The system suitability parameters were determined by preparing standard solution of Atazanavir and Ritonavir and the solution was injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

Acceptance criteria The % RSD for the area and retention times of six standard injections results should not be more than 2%.

Specificity

Standard solution of Atazanavir and Ritonavir was injected into the system and chromatogram was recorded. Diluent (50:50 Acetonitrile: Phosphate Buffer) used as blank and chromatogram was recorded after injection into the system.

Acceptance criteria: Chromatogram of blank should not show any peak at the retention time of analyte peak.

Precision

The precision was determined for Atazanavir and Ritonavir in formulation in terms of intraday precision and interday precision. Sample solution of Atazanavir and Ritonavir was prepared and injected into the system six times in different time intervals within a day (intraday) and at 6 different days (interday). Statistical parameters such as mean, standard deviation and percentage relative standard deviation are calculated.

Acceptance criteria: The % RSD for the area and retention times of six standard injections results should not be more than 2%.

Linearity

Linearity Atazanavir and Ritonavir were found by preparing various dilutions from the working standard solution and recording their responses at the optimized set of chromatographic conditions. The calibration plots were constructed between concentrations versus peak areas and the linearity was found in the range from10 µg/ml to 50 µg/ml for Atazanavir and 6.25 µg/ml to 31.25 µg/ml. The regression equation and correlation coefficient were calculated.

Acceptance criteria: The regression coefficient should NMT 1%

Accuracy

To the pre analyzed sample three different amounts of 50%, 100% and 150 % of working standard was added, at each level 3 replicate samples were prepared and samples were analyzed to determine percentage recovery from the sample. Percentage recovery is calculated for all nine readings from the ratio of amount of drug found .Further statistical parameters such as percentage recovery are calculated.

Acceptance Criteria: The % Recovery for each level should be between 98 to 102

Robustness

Small deliberate changes in method like flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like flow minus (0.6ml/min), Flow plus (1.0ml/min), mobile phase minus, mobile phase plus, was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Acceptance Criteria: The % RSD results should not be more than 2%.

Limit of detection (LOD) and Limit of quantitation (LOQ)

Limit of detection (LOD) is the minimum concentration at which the analyte can be detected without actually being quantitated where as Limit of Quantitation (LOQ) is the minimum concentration at which the analyte response can be taken for quantitation i.e., being able to measure various chromatographic parameters like area of curve, peak height, theoretical plates, %RSD can be measured with reliable accuracy and precision .These are obtained by comparing the signal to noise ratio (S/N) of blank and drug at different concentrations.

3. Results and discussion

System suitability All the system suitability parameters were within the range and satisfactory as per ICH guidelines

Table 1: System Suitability parameters data

Parameters	Atazanavir	Ritonavir
Retention Time (min)	2.1	4.01
Tailing Factor (T)	1.2	1.4
Theoretical Plates (N)	11456	10366
Resolution	--	2.2

Inference: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2. All the system suitable parameters were passed and were within the limits.

Discussion: According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Specificity

Retention times of Atazanavir and Ritonavir were found to be 2.127 mins and 4.010 mins respectively. We did not found any interfering peaks in blank.

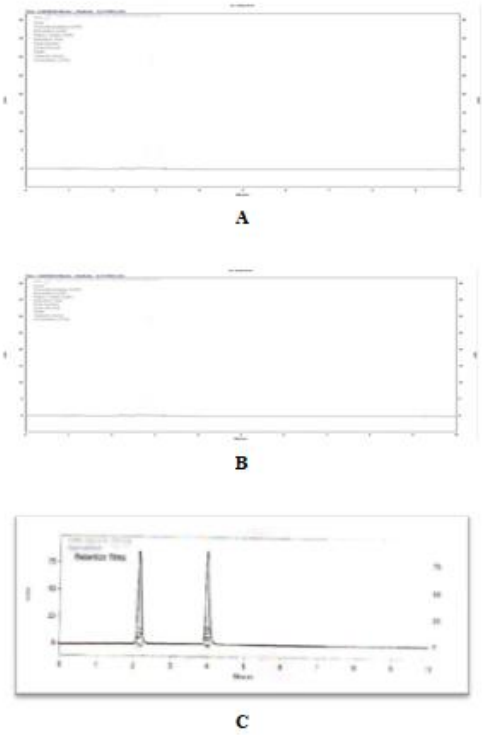

Figure 2. Typical chromatograms of (A) Blank (B) Placebo (C) Standard

Linearity

Five linear concentrations of Atazanavir (12.5-62.50 g/ml) and Acetylcysteine (10-50 µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Atazanavir was y = 3372.x - 9698and of Ritonavirwas y = 1889.x + 18250 Correlation coefficient obtained was 0.998 and 0.999 for the Atazanavir and Ritonavir respectively.

Table 2: Linearity table for Atazanavir and Ritonavir

S. No	Atazanavir			Ritonavir		
	Conc. (µg/ml)	Rt (min)	Peak Area	Conc. (µg/ml)	Rt (min)	Peak Area
1	20	2.5	3870150	20	4.1	4528516
2	40	2.5	8122762	40	4.1	8747174
3	60	2.5	12421486	60	4.1	13568359
4	80	2.5	16411684	80	4.1	17809809
5	100	2.5	20481898	100	4.1	22575114

Precision

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. %RSD of interday precision for retention time and peak area were found to be 0.145 and 1.442 for Atazanavir and 0.195 and 0.098 for Ritonavir respectively. As the limit of Precision was less than "2%" the system precision was passed in this method.

Table 3: Determination of Intraday Precision for Atazanavir and Ritonavir by RP- HPLC

S. No	Peak Area	
	Atazanavir(100µg/ml)	Ritonavir(100µg/ml)
1	20481698	22575114
2	20073583	22347591
3	20415432	22753473
4	19740006	21896076
5	20515025	22391680
6	20272436	22481473
Mean	20249696	22407567
SD	297660	209363
% RSD	0.147	0.934

Accuracy

The percentage recovery was calculated for 50, 100 and 150 % spiked concentrations and they were found to be 99.16%, 99.73% and 101.6% for Atazanavir and 98.01%, 98.75% and 100.3 % for Ritonavir respectively.

Accuracy table of Atazanavir and Ritonavir

Drug name	Conc. (%)	Amount spiked (µg/mL)	Amount recovered (µg/mL)	% recovery	Statistical parameters
Atazanavir	50 100 150	4 24 44	19.8 39.9 61.2	99.1 99.7 101.6	Mean Recovery 99.1-101.6%
Ritonavir	50 100 150	16 36 56	19.6 39.4 60.02	98.01 98.75 100.3	Mean Recovery 98.01-100.3%

Sensitivity

The LOD value was found to be 0.316µg/ml for Atazanavir and 0.949µg/ml for Ritonavir at signal to noise ratio 3:1. LOQ value was found to be 0.433µg/ml for Atazanavir and 3.19µg/ml for Ritonavir at signal to noise ratio 10:1.

Table 5: Sensitivity table for Atazanavir and Ritonavir

Molecule	**LOD**	**LOQ**
Atazanavir	0.316	0.949
Ritonavir	0.433	1.28

Robustness

Robustness conditions like flow minus (0.6 ml/min), flow plus (1.0ml/min), mobile phase minus (75:25), mobile phase plus (85:15), was maintained and samples were injected in duplicate manner. The %RSD for flow rate was found to be 0.47 and 0.36 for Atazanavir and 0.52 and 0.25 for Ritonavir. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Table 6: Robustness data for change in flow rate

Chromatographic Parameters	**Atazanavir**		**Ritonavir**	
	Retention Time	**Peak Area**	**Retention Time**	**Peak Area**
Flow Rate (ml/min)				
0.8	3.13	20457846	5.100	22385435
	3.11	20852365	5.117	22563224
	3.09	20456685	5.113	22632541
Mean	3.10	20653624	5.112	22425325
SD	0.0025	0.0026	0.0031	0.0032
%RSD	0.47	0.36	0.52	0.66
1.2	1.715	20369525	3.623	22654255
	1.718	20865265	3.622	22635582
	1.714	20852333	3.625	22533321
Mean	1.716	20645622	3.622	22568844
SD	0.0032	0.0021	0.0026	0.0028
%RSD	0.36	0.31	0.25	0.38

Ruggedness

The %RSD obtained by different analysts were 0.1 and 0.46 for Atazanavir and 0.58 and 0.60 for Ritonavir respectively. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Table 3: Determination of Intraday Precision for Atazanavir and Ritonavir by RP- HPLC

S. No	**Peak Area**	
	Atazanavir(100µg/ml)	**Ritonavir(100µg/ml)**
1	20481698	22575114
2	20073583	22347591
3	20415432	22753473
4	19740006	21896076
5	20515025	22391680
6	20272436	22481473
Mean	20249696	22407567
SD	297660	209363
% RSD	0.147	0.934

Assay

The tablets (ATAZOR-R) were initially powdered and an amount equivalent to 300mg of Atazanavir, 100mg of Ritonavir was accurately weighed into a 100ml volumetric flask, mixed with 10ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm Millipore membrane filter. Final stock containing 100µg/ml of Atazanavir and Ritonavir respectively was prepared by subsequent dilution with the same mobile phase. 20µl of sample solution was injected into chromatographic system and the peak responses

were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard. The results were shown in Table 9

$$\%Assay = \frac{At}{As} \times \frac{Ws}{100} \times \frac{5}{20} \times \frac{200}{Wt} \times \frac{200}{5} \times \frac{AW}{LC} \times P \times MF$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of Atazanavir / Ritonavir in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

4. Conclusion

A simple, rapid, reliable, robust and optimized reversed phase high performance liquid chromatographic method for the simultaneous estimation of Atazanavir and Ritonavir in formulation was successfully developed and validated as per International Conference on Harmonization guidelines. There are no interfering peaks underperformed degradation conditions. Therefore, a sensitive, accurate and stability indicating method was developed with high degree of practical utility. It can be concluded that the developed reverse phase HPLC method is accurate, precise, linear, rugged and robust. Accordingly, the method can be used for the routine analysis of Atazanavir and Ritonavir in tablets.

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6. Conflicts of Interest

The authors declare that they have no conflict of interest.

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