Method Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Atazanavir and Ritonavir in Bulk and Its Pharmaceutical Formulations

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ABSTRACT

A new rapid, precise and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the estimation of Atazanavir and Ritonavir simultaneously in combined dosage form. The two components Atazanavir and Ritonavir were well resolved on an isocratic method, C18 column, utilizing a mobile phase composition of acetonitrile: 0.02M ammonium acetate buffer (40:60), v/v, pH 4.0) at a flow rate of 1.0 mL/min with UV detection at 205 nm. The retention time of Atazanavir and Ritonavir were 2.8 min and 5.7 min respectively. The developed method was validated for specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness as per ICH guidelines. Linearity for Atazanavir and Ritonavir were found in the range of 18.0-42.0 µg/ml and 5.0-14.0 µg/ml, respectively. The percentage recoveries for Atazanavir and Ritonavir ranged from 98.9-101.0 % and 98.2-100.1 %, respectively. The proposed method could be used for routine analysis of Atazanavir and Ritonavir in their combined dosage forms.

Keywords: Liquid Chromatography; Atazanavir, Ritonavir, Combined dosage forms; Simultaneous estimation, Validation

INTRODUCTION

Atazanavir antiretroviral drug (protease inhibitor)is chemically methyl *N*-[(1*S*)-1-{[(2*S*,3*S*)-3-ydroxy-4-[(2*S*)-2[(methoxy carbonyl)amino]-3,3-dimethyl-*N'*-{[4-(pyridin-2-yl) phenyl] methyl}butanehydrazido]-1-phenylbutan-2-yl]carbamoyl}-2,2-dimethyl propyl] carbamate sulfate having a molecular formula of $C_{38}H_{52}N_6O_7.H_2SO_4$with a Molecular weight of 802.9 g/mol. Atazanavir (ATV) is an azapeptide HIV-1 protease inhibitor (PI). The compound selectively inhibits the virus-specific processing of viral Gag and Gag-Pol poly proteins in HIV-1 infected cells, thus re venting formation of mature virions1-5. Ritonavir is also an antiretroviral drug belonging to the class of protease inhibitors. Ritonavir chemically is 3-thiazol-5-ylmethyl *N*-[(2*S*,3*S*,5*S*)-3-hydroxy-5-[(2*S*)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl}) carbamoyl] amino} butanamido]-1,6-diphenylhexan-2-yl] carbamate, having a molecular formula of$C_{37}H_{48}N_6O_5S_2$with a molecular weight 720.946 g/mol. Protease inhibitors, such as Ritonavir prevent viral replication by inhibiting the activity of proteases, e.g.HIV-1 protease, enzymes used by the viruses to cleave nascent proteins for final assembly of new virions5-10.
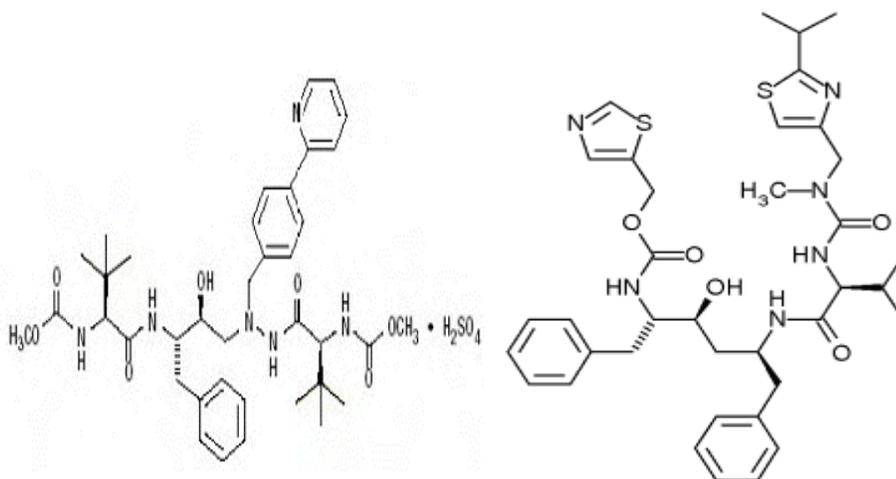
A. Structure of Atazanavir   B. Structure of Ritonavir

An extensive literature review on the methods reported for the simultaneous estimation of Atazanavir and Ritonavir gives out information that there are few separate methods reported for the quantitative estimation of Atazanavir sulfate in bulk, pharmaceutical dosage forms and in plasma by HPLC, likewise a very few methods have been reported for the quantitative estimation of Ritonavir by HPLC but till date no method has been reported for the simultaneous quantitative estimation of Atazanavir and Ritonavir by HPLC[11-17]. There is just one spectrophotometric method reported for the simultaneous estimation of Atazanavir sulfate and Ritonavir in tablets. The

present developed method was used determine the Atazanavir and Ritonavir present in the formulation and method validated according to the ICH guidelines[18-19].

MATERIALS AND METHODS

Materials

HPLC grade Ammonium Acetate, acetonitrile and water were procured from Merck India. All dilutions were performed in standard class-A, volumetric glassware. For the estimation of commercial formulation, Synthivan tablets having AtazanavirRitanovir manufactured by Cipla ltd were procured from the local market.

Instrumentation

Agilent 1120 compact LC chromatographic system, with DAD detector and a fixed injector equipped with 20µL loop was used for the chromatographic separation. The chromatogram was recorded at and peaks quantified by means of Ez Chrome software. Chromatographic separation was carried out on a C18 column [Zodiac sil, 250mm x4.5mm 5µ]. Sartorius electronic balance was used for weighing the samples. Ultra-sonic bath sonicator was used for degassing and mixing of the mobile phase.

Chromatographic conditions

Chromatographic separation of Atazanavir and Ritonavir was carried on a C18 column. The mobile phase was composed of acetonitrile and ammonium acetate buffer (pH 4.0) in the ratio of 40:60 v/v. It was filtered through a 0.45 µ membrane filter and degassed for 15 minutes. The flow rate of the mobile phase was maintained at 1 ml/min. Detection was carried out at 205 nm at ambient temperature.

Method development

Preparation of Standard Stock Solutions

Standard stock solutions were prepared by dissolving 60 mg of Atazanavir and 25 mg Ritonavir working standard in two separate each 100 mL volumetric flasks using 15mL of mobile phase and made up to the mark with mobile phase to obtain a final concentration of 600µg/mL and 250 µg/mL of each Atazanavir and Ritonavir . From the above stock solutions, 5 and 4 ml aliquots each were pipette in to a 100mLvolumetric flask and dissolved in 25mL of the mobile phase and made up to the mark with the solvent to obtain a final concentration of 30µg/mL and 10 µg/mL for Atazanavir and Ritonavir respectively.

Preparation of Sample solutions

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 300

mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, added 70 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 μm membrane Filter. Transferred 2.0 mL of the above solution into a 100 mL volumetric flask and diluted to volume with diluent to obtain a concentration of 30 and 10μg/mL of Atazanavir and Ritonavir respectively.

Method validation

The developed HPLC method for the simultaneous determination of Atazanavir and Ritonavir was validated as per the ICHguidelines[13, 14].

As part of method validation as per ICH guidelines, the following parameters are studied.

1. System Suitability and System Precision
2. Specificity Studies
    A. Blank Interference
    B. Placebo Interference
    C. Forced degradation studies in different stress conditions to establishing stability indication of the developed method.
3. Method Precision
4. Accuracy studies
5. Linearity Studies including LOD/LOQ determination
6. Ruggedness
7. Robustness
8. Analysis of Marketed samples by applying the developed method.

Each parameter was explained separately in different sections under results and discussions.

RESULTS AND DISCUSSION

System suitability and System Precision

System suitability for chromatographic separation was checked on each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), Resolution, Tailing factor. The HPLC system was equilibrated using the initial mobile phase composition, followed by 5 injections of the standard solution of 100% concentration containing 30 μg/mL Atazanavir and 10 μg/ml Ritonavir. These 5 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the Table 1.

Table 1: System suitability parameters for Atazanavir and Ritonavir by proposed method

Name of the Compound	Retention Time	Tailing factor	Theoretical plate	USP Resolution
Atazanavir	2.833	1.79	3103	-
Ritanovir	5.753	1.30	7471	12.392

Specificity

Blank interference

A study to establish the interference of blank was conducted. Diluent was injected into the chromatograph in the defined above chromatographic conditions and the blank chromatograms were recorded. Chromatogram of Blank solution (Figure 2) showed no peaks at the retention time of Atazanavir and Ritonavir   peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Atazanavir and Ritonavir in Atazanavir and Ritonavir tablets. Similarly typical representative chromatogram of standard is also shown (Figure 3)
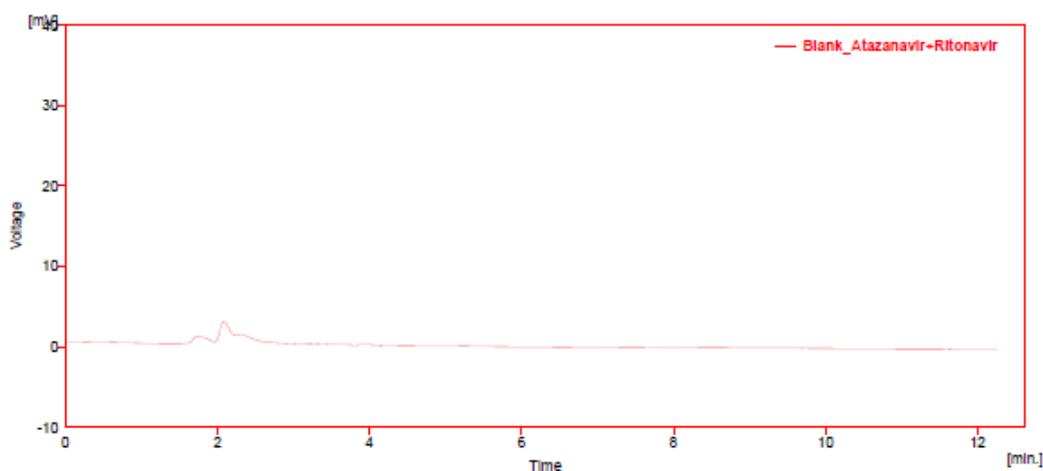
Figure 2: A typical HPLC Chromatogram showing the no interference of diluent for Atazanavir and Ritonavir
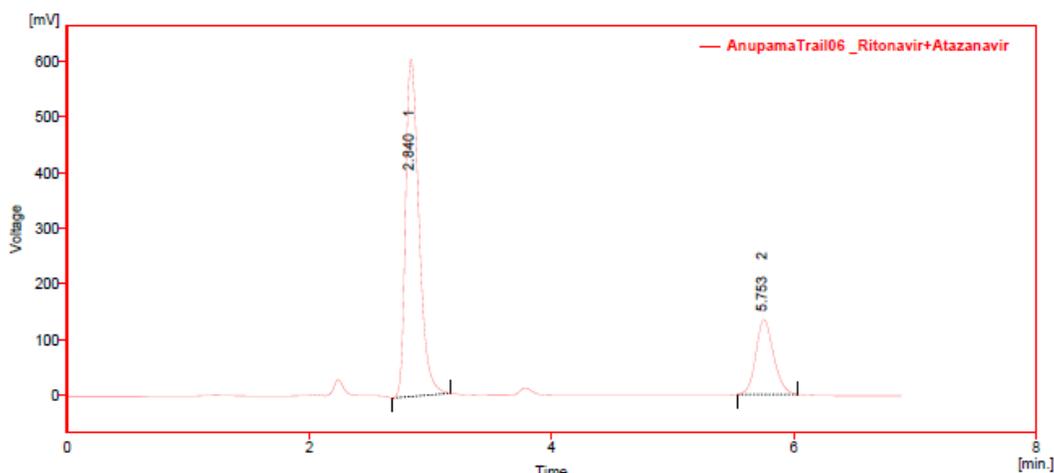
Figure 3: A typical HPLC Chromatogram showing the peak of Atazanavir and Ritonavir

Forced Degradation:

Control Sample:

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 300.mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Figure 4A)

Acid Degradation Sample:

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 300.mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N acid, refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Figure 4B)

Base Degradation Sample:

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 300.mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N NaOH, refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Figure 4C)

Peroxide Degradation Sample : +

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 300.mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of Hydrogen Peroxide, refluxed for 30min at 60°C, then cooled to room temperature, and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Figure 4D)

Thermal Degradation Sample:

Powder collected from 20 tablets are exposed to heat at 105°C for about 5days. Accurately weigh and transfer equivalent to 300.mg of Atazanavir and 100mg of Ritanovir into a 100 mL volumetric flask, add 70 mL of diluent, and sonicate for 30 minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 2.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Figure 4E)

Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by proposed method.
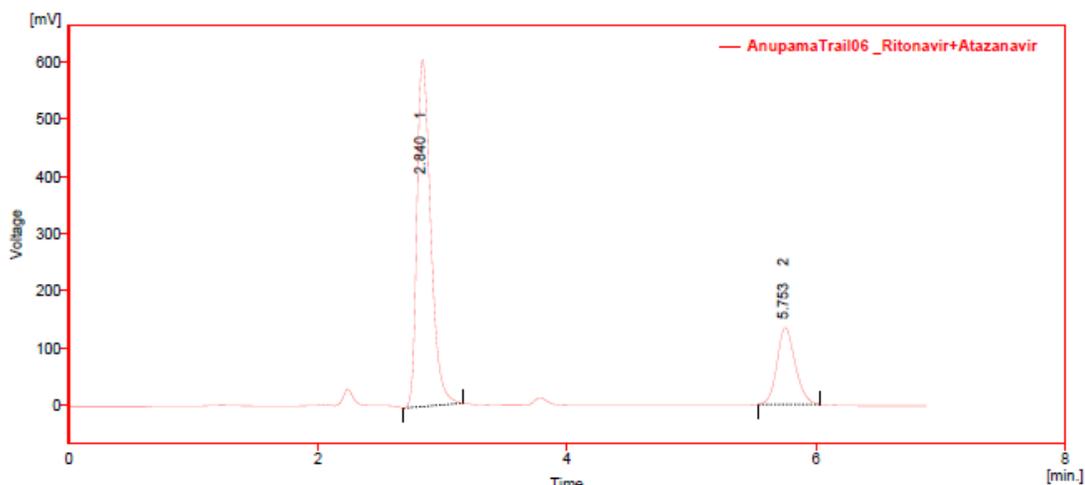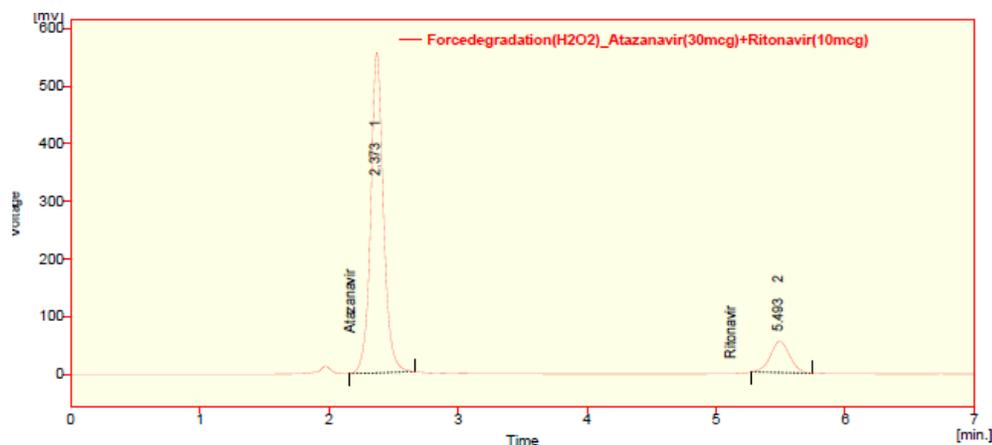

Figure 4A: A typical HPLC Chromatogram showing the Control Sample profile of Atazanavir and Ritonavir by proposed method.
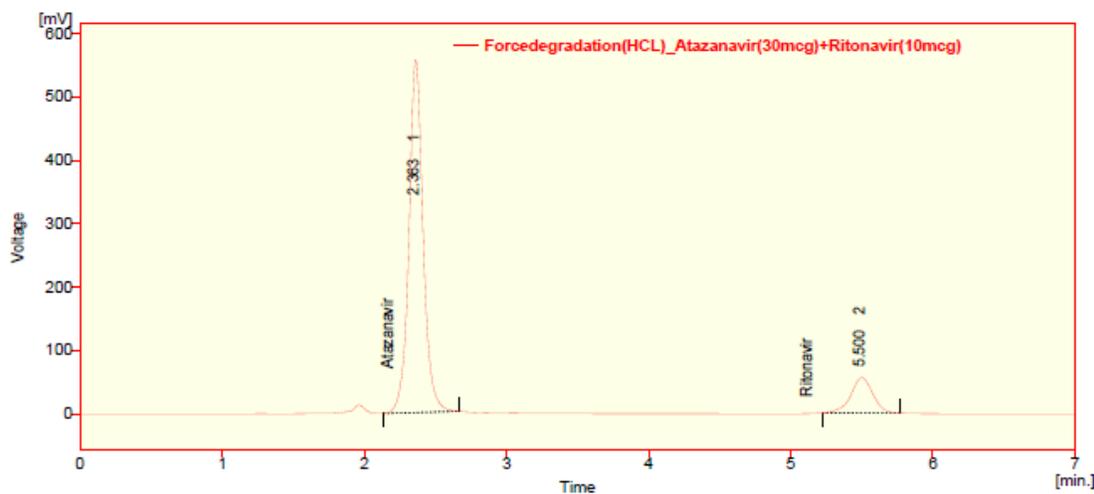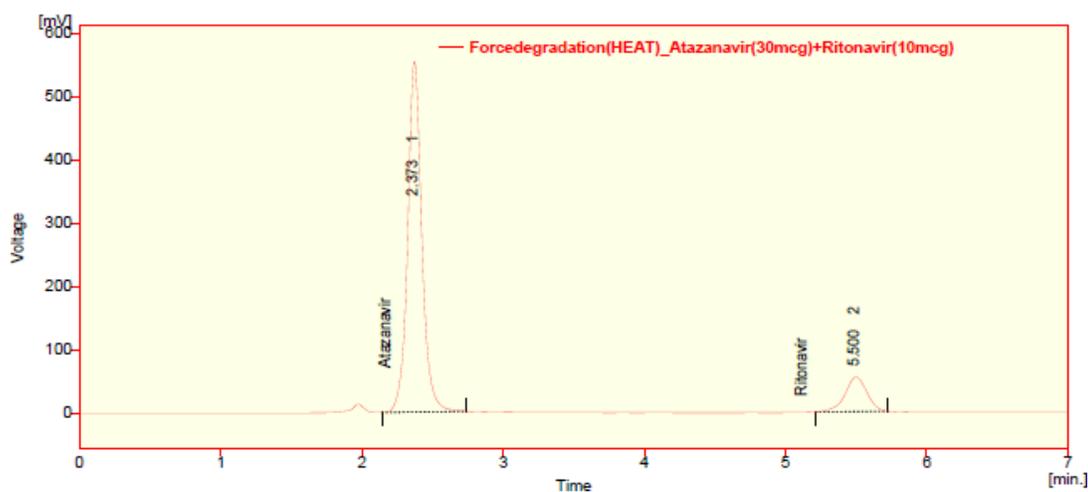

Figure 4B: A typical HPLC Chromatogram showing the profile of Atazanavir and Ritonavir in Acidic hydrolysis by proposed method.
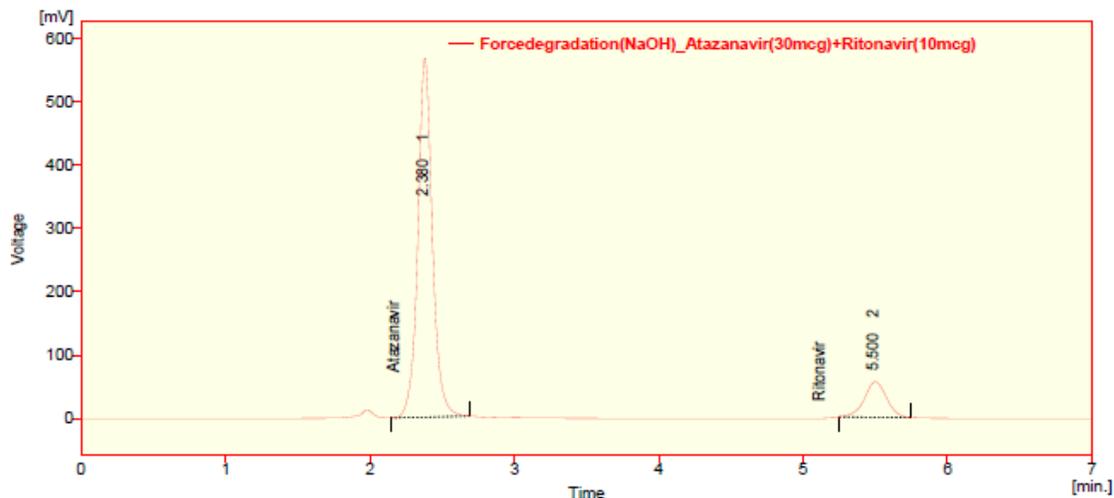
Figure 4C: A typical HPLC Chromatogram showing the profile of Atazanavir and Ritonavir in Base hydrolysis by proposed method.


Figure 4D: A typical HPLC Chromatogram showing the profile of Atazanavir and Ritonavir in Peroxide hydrolysis by proposed method.

Figure 4E: A typical HPLC Chromatogram showing the profile of Atazanavir and Ritonavir in Thermal hydrolysis by proposed method.

Linearity and range

The standard curve was obtained in the concentration range of 18.0-42.0 μg/ml for Atazanavir and 5.0-14.0μg/mL for Ritanovir. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient [r2] of standard curve were calculated and given in Figure-5A(For Atazanavir) and Figure-5B(For Ritanovir) to demonstrate the linearity of the proposed method. The result of regression analysis was given in the Table 2.

From the data obtained which given in Table-2 (For Atazanavir and Ritonavir) the method was found to be linear within the proposed range.

Table 2: Linearity studies for Atazanavir and Ritanovir by proposed method

	Linearity Study for Atazanavir		Linearity Study for Ritonavir	
% Level	Conc. µg/mL	Area	Conc. µg/mL	Area
60	18.00	2451.56	5.04	349.568
80	24.00	3102.59	7.56	468.631
100	30.00	3785.73	10.08	591.079
120	36.00	4438.88	11.34	642.098
140	42.00	5212.03	14.11	757.414
Slope	114.3		45.179	
Intercept	369.5		126.85	
% Y-Intercept	323.3		280.8	
Residual Sum of Squares	39.6		7.31	
CC(r)	0.9995		0.9992	
RSQ(r2)	0.9990		0.9984	
LLD	1.14		0.53	
LLQ	3.46		1.62	
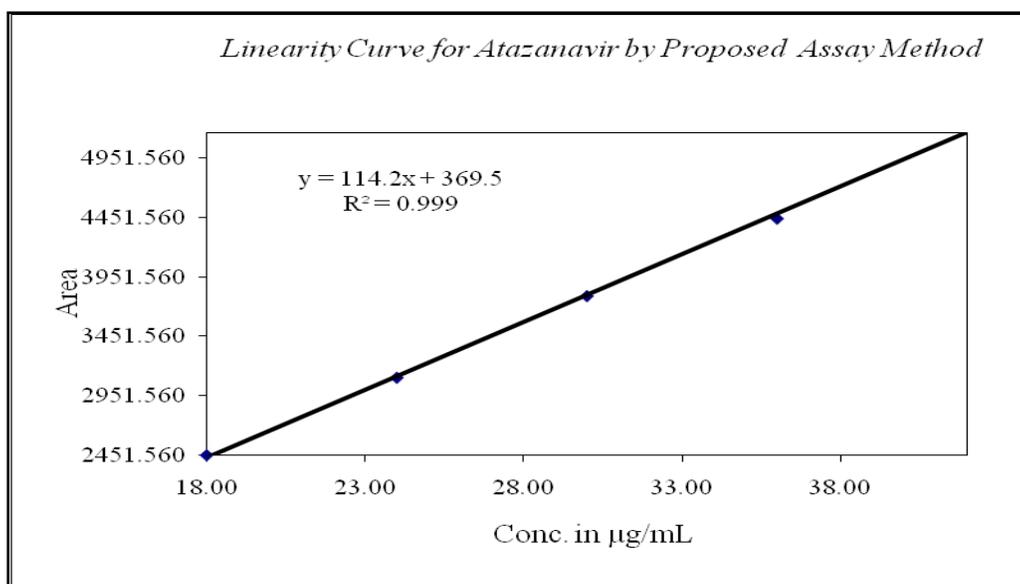
Figure 5A: Calibration curve for Atazanavir

Linearity Curve for Ritanovir by Proposed Assay Method

$y = 45.17x + 126.8$
$R^2 = 0.998$
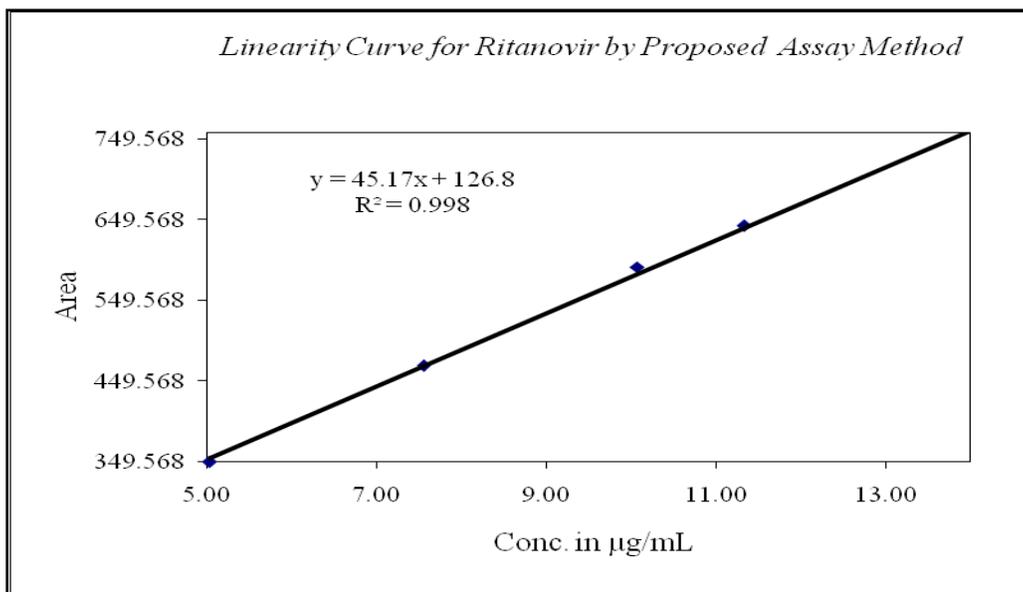
Figure 5B: Calibration curve for Ritanovir

Accuracy

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method. In the current study recovery at three spike levels 50%, 100% and 150% were carried out. The % recovery at each spike level was calculated and was given in Table 3.

Table 3A: Recovery studies for Atazanavir by proposed method

% Level	Recovery Range	% RSD at each level	Over all %RSD
50	98.9-99.5	0.3	0.7
100	99.5-99.9	0.4	
150	98.9-101.0	1.1	

Table 3B: Recovery studies for Ritanovir by proposed method

% Level	Recovery Range	% RSD at each level	Over all %RSD
50	98.2-99.9	0.7	0.6
100	98.4-100.1	0.9	
150	99.4-99.8	0.3	

Precision

The precision of an analytical method is the closeness of replicate results obtained from analysis of the same homogeneous sample. Precision was considered at different levels, i.e. method, system, Inter day and intraday. Precision of the developed method was assessed by measuring the response on the same day (intraday precision) and next two consecutive days (inter day precision). The precision of the method was assessed by six replicate injections of 100% test concentration. Intra and inter-day precision of the method was assessed by determination of standard deviation and % RSD for the analyte response. The result was given in Table 4.

Table 4: Method Precision (Inter and Intraday) studies for Atazanavir and Ritanovir by proposed method

Summary showing Method Precision by Proposed Method			
For Atazanavir		*For Ritanovir*	
Method Precision (Inter &Intra Day)		Method Precision (Inter &Intra Day)	
100.9	99.3	99.9	99.5
99.6	100.4	98.4	100.3
100.5	101.2	98.2	100.0
98.9	98.7	99.3	100.2
100.7	98.6	100.2	99.2
101.1	100.0	101.7	101.7
Overall Avg.	100.0		99.9
Overage Std Dev.	0.95		1.09
Over all %RSD	1.00		1.10

LOD and LOQ

LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S(Where, σ is the standard deviation of the responses and S is the slope of the calibration curves). In the present method σ is the mean of standard deviation of y intercepts of the three calibration curves and S is the mean of slopes of the calibration curves. The result was given in Table5.

Robustness

The robustness of the method was determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as percent organic content, pH of the mobile phase, buffer concentration, temperature, injection volume and flow rate. A deviation of ± 2nm in the detection wavelength, ± 0.1 mL/min in the flow rate, ± 5%change in the organic phase were tried individually. The result was given in the Table 5.

Table 5: Robustness studies for Atazanavir and Ritonavir by proposed method

Parameter		% RSD	
		Atazanavir	**Ritanovir**
Wavelength ±2	203 nm	0.46	0.56
	207 nm	0.32	0.32
Flow Rate mL /min	0.8 mL/min	0.65	0.72
	1.2mL.min	0.87	0.82

OVER ALL SUMMARY OF THE METHOD

Column chemistry, solvent selectivity, solvent strength (volume fraction of organic solvent(s) in the mobile phase), detection wavelength and flow rate were varied to determine the chromatographic conditions for giving the best separation. Several mobile phase compositions were tried to resolve the peaks of Atazanavir and Ritonavir. The optimum results were attained with acetonitrile and ammonium acetate buffer (pH 4.0) in the ratio of 40:60 (v/v) because it could

resolve the peaks of Atazanavir with retention time at 2.8 min and Ritanovir retention time at 5.7 min. The two peaks were symmetric and sufficiently resolved. System suitability was carried out by injecting 5 replicate injections of 100% concentration of Atazanavir and Ritonavir. The resolution was found to be greater than 2 and the other parameters are presented in Table 1.

Specificity of the chromatographic method was tested by injecting mobile phase as blank and sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Atazanavir and Ritonavir at 2.8min and 5.7 min respectively without any interference. Thus the developed method was specific for analyzing the commercial formulations for Atazanavir and Ritonavir. An optimized chromatogram with the retention times of Atazanavir and Ritonavir was shown in the Figure 2.

The peak areas corresponding to the concentration range of Atazanavir 18.0-42.0 µg/mL and Ritznovir 5.0-14.0 µg/ml prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for Atazanavir and Ritonavir, respectively, with mean correlation coefficients (n=3) of 0.999 and higher, the representative calibration curve is shown in Figure3. The regression analysis was given in Table 2.

Accuracy of the proposed method was assessed by standard addition method at 50%,100% and 150% levels of recovery to the pre analyzed sample in triplicate. The recovery of the added standard to the sample was calculated and it was found to be 98.9-101.0 %w/w for Atazanavir and 98.2-100.1%w/w for Ritanovir respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in table 3.

LOD and LOQ were calculated from the average slope and standard deviation of y intercepts of the calibration curve. Limit of detection for Atazanavir and Ritonavir were 1.14 µg/mL and 0.53µg/mL respectively where as limit of quantitation of Atazanavir and Ritonavir were 3.46 µg/mL and 1.62 µg/mL respectively indicating high sensitivity of the method. LOD and LOQ value was given in table 2. The method is precise with a %RSD of less than 2 for both Atazanavir and Ritonavir respectively. The results of intraday and inter day precision was given in table 4. Robustness was carried out by change in the flow rate (±1mL/min), mobile phase variation (±5%) and variation in wavelength (± 2 nm).Solution of 100% concentration is prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The result was given in table 5. The proposed method was applied for the assay of commercial formulation containing Atazanavir and Ritonavir. Each sample was analyzed in triplicate. The mean recovery

values were 100.14 and 100.45 for Atazanavir and Ritonavir. The result of estimation was given in table 6.

Table 6: Assay of Marketed samples for Atazanavir and Ritonavir by proposed method

Drug	Amount Claimed in mg per Tablet	Atazanavir	Ritanovir
Atazanavir	300	300.12	100.14
Ritanovir	100	100.45	100.45

CONCLUSION

The proposed RP-HPLC method for simultaneous assay Atazanavir and Ritonavir in combined dosage forms was validated, and found to be applicable for routine quantitative analysis of Atazanavir and Ritonavir. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of Atazanavir and Ritonavir with no interference from other formulation excipients. Therefore, this method can be employed for the routine analysis for simultaneous estimation Atazanavir and Ritonavir in quality control of formulations and also in the dissolution studies.

ACKNOWLEDGEMENTS

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