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STABILITY-INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF ATAZANAVIR AND RITONAVIR IN PHARMACEUTICAL FORMULATION

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ABSTRACT: A simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC method was developed and validated for the simultaneous determination of Atazanavir (AT) and Ritonavir (RT) in commercial tablets. The method has shown adequate separation for AT, RT from their degradation products. Separation was achieved on a Hypersil BDS-C_{18}, 5 µm, 125 mm × 4.6 mm i.d. column using a mobile phase consisting of buffer (pH3.4) – acetonitrile (50:50, v/v) at a flow rate of 1.5 mL/min and UV detection at 250 nm. The drugs were subjected to oxidation, acid, base hydrolysis, photolysis and heat to apply stress conditions. The linearity of the proposed method was investigated in the range of 7.8–225 µg/mL (r^2 = 0.9993) for AT and 2.7-75 µg/mL (r^2 = 0.9995) for RT. The limit of detection was 2.4 µg/mL for AT and 0.9 µg/mL for RT. The limit of quantitation was 7.8 µg/mL for AT and 2.7 µg/mL for RT. Degradation products produced as a result of stress studies did not interfere with the detection of AT and RT and the assay can thus be considered stability-indicating.

INTRODUCTION: Atazanavir (AT)is an azapeptide, chemically described as methyl N-[(2S)-1-[[(2S,3S)-3-hydroxy-4-[[[(2S)-2- (methoxycarbo nylamino)-3,3-dimethyl butanoyl]amino]- [(4-pyridin-2-ylphenyl)methyl] amino]-1-phenylbutan-2 -yl]amino]-3,3-dimethyl-1-oxo butan-2-yl]carbamate (**Fig. 1**). Its empirical formula is $C_{38}H_{52}N_6O_7$ and its molecular weight is 704.8.Ritonavir(RT), is chemically described as 1,3-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 (**Fig. 2**).

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Its molecular formula is $C_{37}H_{48}N_6O_5S_2$ and its molecular weight is 720.95. Both AT and RT belongs to protease inhibitor class of drugs used to treat HIV infections and AIDS. Both the drugs bind to the protease active site and inhibit the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles.

The drug combination had shown a more beneficial pharmacodynamic and/or pharmacokinetic profile compared to the currently licensed PIs [1]. RT is a potent in vitro and in vivo inhibitor of the HIV virus. It blocks the HIV protease, thereby reducing the viral load in the infected individual. AT is metabolized by CYP3A4 in the liver and is 86% bound to human serum proteins, whereas 98-99% of RT binds to proteins. Monotherapy with RT has been shown to be 90% effective.

However, monotherapy with a single protease inhibitor may result in both viral resistance and possible cross-resistance to the other protease inhibitors. Therefore, combination therapy, which may include the protease inhibitors, is the standard of care. Recently, the Food and Drug Administration (FDA) has approved fixed dose combination of ritonavir and atazanavir sulfate tablets (100 mg/300 mg) for use in combination with other antiretrovirals for the treatment of HIV-1 infection.

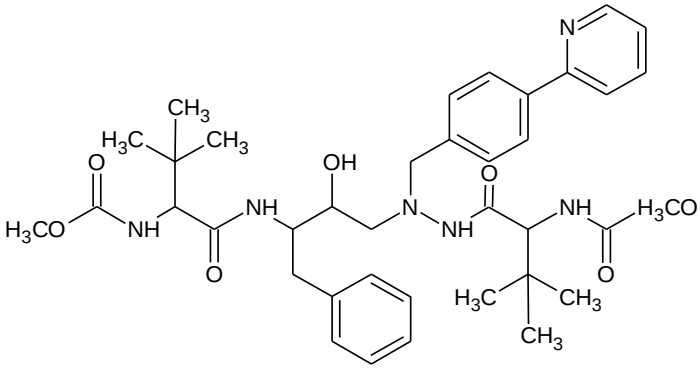


FIGURE 1: CHEMICAL STRUCTURE OF ATAZANAVIR

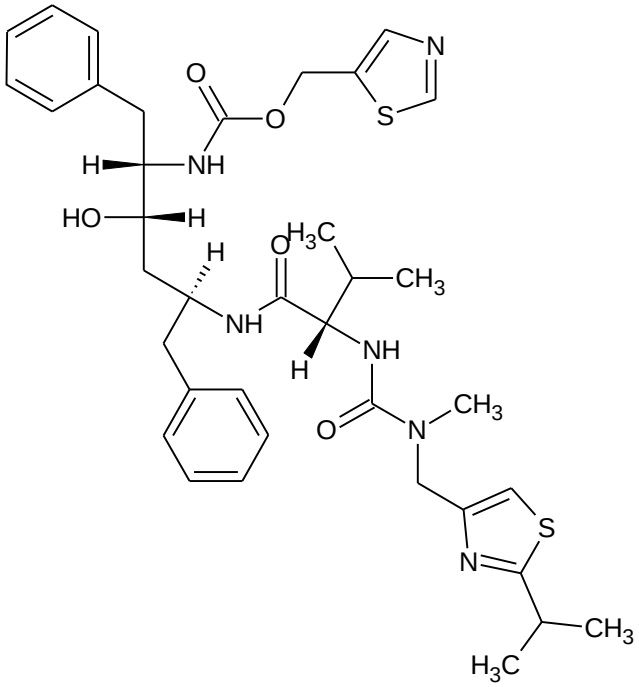


FIGURE 2: CHEMICAL STRUCTURE OF RITONAVIR

Objective of the study: As far as literature is concerned, there are many reported methods for the determination of AT and RT in combined pharmaceutical dosage form by using RP-HPLC [2-4] and in plasma samples using LC-MS/MS [5, 6] methods. All the HPLC methods lack stability indicating nature.

Only one spectrophotometric [7] method was reported. And one stability indicating method by HPTLC [8] has been reported for the simultaneous determination of these compounds.

However, none of the reported analytical methods describe a stability indicating method by HPLC for the simultaneous determination of AT and RT in a combined dosage form. To our knowledge, this is the first report of a stability indicating method for the simultaneous determination of both AT and RT in solid oral dosage forms by HPLC. The present manuscript describes a simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC method for the simultaneous determination of AT and RT in the same tablet dosage form.

MATERIALS AND METHODS:

Chemicals and Reagents: AT (99.6%) and RT (99.7%) were kindly supplied by Mylan laboratories limited, Hyderabad, India and were used without further purification. Ritovaz300 (AT)/100(RT) mg tablets were purchased from local market. Acetonitrile, potassium dihydrogenphosphate, ortho phosphoric acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were obtained from Merck (India).

All reagents used, were at least of analytical grade except acetonitrile which was HPLC grade. HPLC grade water was obtained following distillation in glass and passage through a Milli-Q® system (Millipore, Milford, MA, USA) and was used to prepare all solutions.

HPLC Instrumentation and Conditions: HPLC used for the study was Waters e2695 separation module equipped with waters 2998 photodiode array (PDA) detector and EMPOWER version 2.0 software. The chromatographic separation was achieved on a Hypersil BDS C_{18}, 5µm, 125mm x 4.6mm i.d. column using a mobile phase consisting of buffer (pH3.4) – acetonitrile (50:50, v/v) at a flow rate of 1.5 mL/min.

The eluent was monitored using U.V detection at a wavelength of 250 nm. The column was maintained at a temperature of 30^{o}C and an injection volume of 20µl was used. The mobile phase was filtered through 0.45 µm membrane filter prior to use.

Procedure:

Preparation of Stock and Standard Solutions: Stock solutions of AT (equivalent to 0.3 mg/mL) and RT (0.1 mg/mL) were prepared with mobile phase. Aliquots of standard stock solutions of AT and RT were diluted to yield final concentrations of 37.5, 75.0, 112.5, 150, 187.5, 225 for AT and 12.5, 25.0, 37.5, 50.0, 62.5, 75.0 for RT.

Preparation of Sample Solution for Assay: Twenty Ritovaztablets were weighed, crushed and mixed in a mortar and pestle. A portion of the powder equivalent to one tablet was accurately weighed into each of nine 200 mL A-grade volumetric flasks and 50 mL of mobile phase was added to each flask. The volumetric flasks were sonicated for 20 min to effect complete dissolution of AT and RT and the solutions were then made upto volume with mobile phase. Aliquots of the solutions were filtered through 0.45µm membrane filter and 5 mL of the filtered solution was transferred to a 50 mL A-grade volumetric flask and made upto the volume with mobile phase, to yield concentrations of each of the two drugs in the range of linearity previously described.

Forced degradation studies of API and Tablets: In order to establish whether the analytical method and the assay were stability-indicating, Ritovaz tablets and pure active pharmaceutical ingredient (API) of both AT and RT was stressed under various conditions to conduct forced degradation studies [9]. As these drugs are freely soluble in acetonitrile, it was used as a solvent and diluent in all the forced degradation studies. Forced degradation studies of the API drug samples and tablets of the same were carried out using the following conditions: acid hydrolysis (0.1M hydrochloric acid), base hydrolysis (0.1M sodium hydroxide), heat (105^{o}C for 48 h), photolytic (UV radiation for 48 h) and oxidation (6% hydrogen peroxide).

1. **Oxidation studies:** Solutions for use in oxidation studies were prepared in acetonitrile and 6% H_2O_2 and the resultant solutions were analyzed immediately after preparation.

2. **Acid degradation studies:** Solutions for acid degradation studies were prepared in acetonitrile and 0.1 M hydrochloric acid and the resultant solutions were analyzed 5 minutes after preparation.

3. **Alkali degradation studies:** Solutions for alkali degradation studies were prepared in acetonitrile and 0.1 M sodium hydroxide and the resultant solutions were analyzed 5 minutes after preparation.

4. **Neutral degradation studies:** Solutions for neutral degradation studies were prepared in acetonitrile and the resultant solutions were heated on a water bath at 90^{o}C for 5 minutes prior to analysis.

5. **Temperature stress studies:** Ritovaz tablets and API were exposed to dry heat of 100^{o}C in a convection oven for 8 hours. The tablets and API powders were removed from the oven and 20 tablets were crushed and mixed and an aliquot of powder equivalent to the weight of 1 tablet and API powder were then prepared for analysis as previously described.

6. **Photo stability studies:** Ritovaz tablets and API and solutions of each drug were prepared and exposed to light to determine the effects of irradiation and the stability of the two drugs in solution and in solid state. Approximately 50 mg of each API was spread on a glass dish in a layer that was less than 2 mm in thickness. A solution of each API (1mg/mL) was prepared in acetonitrile. Tablets were prepared in the same way. All samples for photo stability testing were placed in a light cabinet and exposed to light for 40 h resulting in an overall illumination of ≥200W h/m^2 at 25^{o}C with UV radiation at 320-400 nm. Control samples which were protected from light with aluminum foil were also placed in light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

RESULTS AND DISCUSSION:

HPLC method development and optimization: Hypersil BDS C_{18}, 5µm, 125mm x 4.6mm i.d. column maintained at 30^{o}C was used for the separation and the method was validated for the determination of AT and RT in Ritovaz.

The composition, pH and the flow rate of the mobile phase were changed to optimize the separation conditions using stressed samples of the two compounds of interest.

A mobile phase consisting of buffer (pH3.4) – acetonitrile (50:50, v/v) set at a flow rate of 1.5 mL/min was selected for use for further studies after several preliminary investigatory chromatographic runs.

Under the described experimental conditions, all the peaks were well defined and free from tailing. The effects of small deliberate changes in the mobile phase composition, pH and flow rate were evaluated as a part of testing for method robustness. The optimized conditions are tabulated in **table 1**.

TABLE 1: CHROMATOGRAPHIC CONDITIONS

Variable	Condition
Column	Hypersil BDS, 125*4.6, 5µm
Phase	BDS
Mobile phase & diluents	Buffer (pH3.4) – acetonitrile (50:50, v/v)
Flow rate	1.5 mL/min
Temperature	30^0C
Injection volume	20 µL
Wave length	250 nm

Validation of the method: The analytical method was validated with respect to parameters such as linearity, limit of quantification (LOQ) limit of detection (LOD), precision, accuracy, selectivity, recovery, robustness and ruggedness[10].

Linearity: Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 7.8-225 µg/mL and 2.7-75µg/mL for AT (n=6) and RT (n=6) respectively.

Peak areas of AT or RT were plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves.

Correlation coefficients were found to be more than 0.999 for both the drugs. Typically, the regression equations were: y = 9329.3x - 1607 (R^2 = 0.9993) for AT and y = 37273x - 14767 (R^2 = 0.9995) for RT respectively. The linearity curves are depicted in **Figure 3 (A-B).**

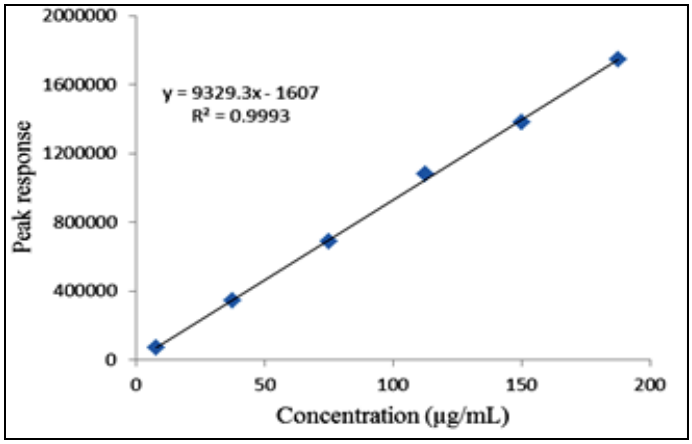


FIGURE 1A: LINEARITY PLOT OF ATAZANAVIR (AT)

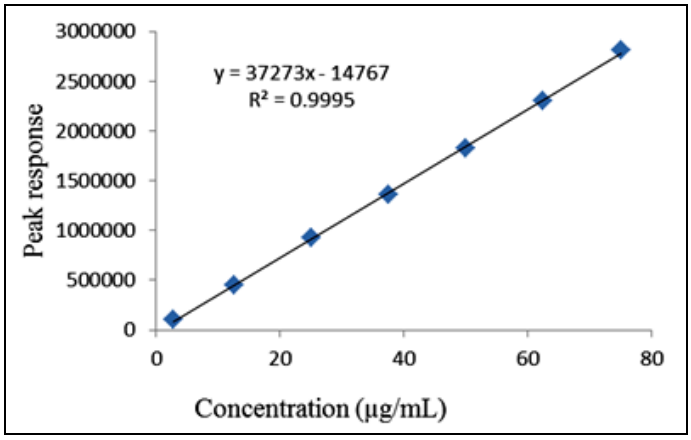


FIGURE 1B: LINEARITY PLOT OF RITONAVIR (RT)

LOQ and LOD: The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the base line noise following triplicate injections. The % RSD for these studies was less than 0.55%. The LOQ that produced the requisite precision and accuracy was found to be 7.8µg/mL and 2.7µg/mL for AT and RT, respectively. The LOD was determined based on signal to noise ratios and was determined using an analytical response of three times the background noise. The LOD for AT and RT was found to be 2.4µg/mL and 0.9µg/mL respectively.

Precision: The intra and inter-day variability or precision data are summarized in Table 2 and were assessed by using standard solutions prepared to produce solutions of three different concentrations of each drug. AT and RT were used in the same solution for the purpose of these studies. Repeatability or intra-day precision was investigated by injecting nine replicate samples of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the same three samples over three consecutive days.

TABLE 2: INTRA AND INTER- DAY PRECISION (n=9)

Name of the compound	Actual concentration	Measured concentration (µg/mL), RSD (%)	
		Intra-day	Inter-day
Atazanavir (µg/mL)	75	75.45, 0.15	75.37, 0.17
	150	150.12, 0.33	150.00, 0.26
	225	225.93, 0.21	225.31, 0.14
Ritonavir (µg/mL)	25	25.15, 0.26	25.10, 0.10
	50	50.24, 0.34	50.19, 0.60
	75	75.33, 0.27	75.26, 0.15

Data expressed as mean for "measured concentration" values.

Accuracy: Accuracy data for the assay following the determination of each of the compounds of interest are summarized in **Table 3**. Accuracy was performed by using recovery studies. A known amount of each standard powder was added to samples of tablet powders, which was then mixed, extracted and subsequently diluted to yield a starting concentration of 150µg/mL for AT and 50µg/mL for RT. These samples were prepared in the same manner as the sample preparation for assay and were analyzed. In each case, the percent relative error in accuracy was calculated and found to be less than 0.30 % with a corresponding percent recovery value greater than 99.7%.

TABLE 3: ACCURACY DATA (n=5)

S. No.	Accuracy									
	AT	RT	AT	RT	AT	RT	AT	RT	AT	RT
	50%		75%		100%		125%		150%	
1	99.6	99.7	99.8	99.8	100.1	100.2	100.0	99.8	100.0	99.9
2	99.9	99.5							100.0	99.9
3	99.8	99.8	99.8	99.8	99.9	100.1	100.1	99.9	100.1	100.0
4	99.2	99.8							100.0	100.0
5	100.1	99.6	99.9	99.7	99.7	100.1	100.0	99.8	100.0	99.9
6	99.9	100.0							99.9	99.9
Average	99.7	99.7	99.8	99.8	99.9	100.1	100.0	99.8	100.0	99.9
%RSD	0.30	0.16	0.09	0.08	0.18	0.08	0.05	0.07	0.05	0.05

Specificity: The results of stress testing studies in addition to that of monitoring standard solutions of each drug indicated a high degree of specificity of this method for both AT and RT. The degradation product(s) of each of the parent compounds was found to be similar for both the Ritovaz and API powders assessed.

Ruggedness and robustness test: As recommended in the ICH guidelines and the Dutch pharmacist's guidelines, a robustness assessment was performed during the development of the analytical procedure. The ruggedness of the method is assessed by comparison of the intra and inter-day assay results for AT and RT that has been performed by two analysts. The % RSD values for intra and inter-day assays of AT and RT in the Ritovaz performed in the same laboratory by two analysts didn't exceed 0.60 % indicating the ruggedness of the method. In addition, the robustness of the method was investigated under a variety of conditions including changes of pH of the eluent, flow rate, buffer composition and detection wavelength. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and the data is summarized in **Table 4.**

Stability studies: AT and RT were found to be relatively stable following oxidation and basic hydrolysis. Considerable degradation was observed for AT in neutral (10%) and acid(17%) hydrolysis. Ritonavir undergone considerable degradation in all the stressed conditions i.e. acid (16%), base (12%), water (26%) and oxidation (20%). Both the drugs have shown degradation upon exposure to dry heat (19% for AT, 25% for RT) and photolytic conditions (20% for AT, 25% for RT). These are summarized in **table 5**. The chromatograms of all the stressed samples were checked for the appearance of any extra peaks and these are depicted in **fig. 4(A-H)**. Peak purity of these samples was verified using a photodiode array (PDA) detector.

The purity of the principle and other chromatographic peaks was found to be satisfactory. This study confirmed the specificity and the stability indicating power of the HPLC method.

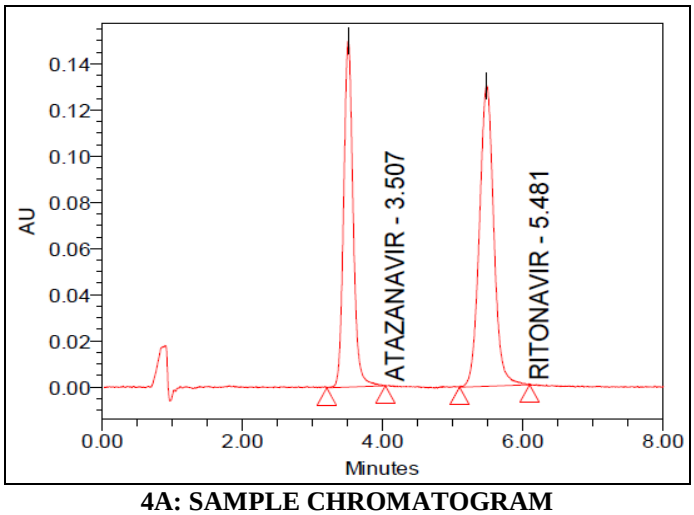
TABLE 4: ROBUSTNESS TESTING OF THE METHOD

Parameter	Modification	Atazanavir (% recovery)	Ritonavir (% recovery)
pH	3.3	101.0	99.5
	3.4	100.6	100.0
	3.5	98.4	99.9
Buffer composition (M)	40	98.8	100.0
	50	101.7	100.5
	60	101.0	99.6
Flow rate (mL/min)	1.4	100.3	101.3
	1.5	100.1	100.5
	1.6	100.8	100.2
Wave length (nm)	248	100.4	100.1
	250	99.8	98.9
	252	100.4	100.2

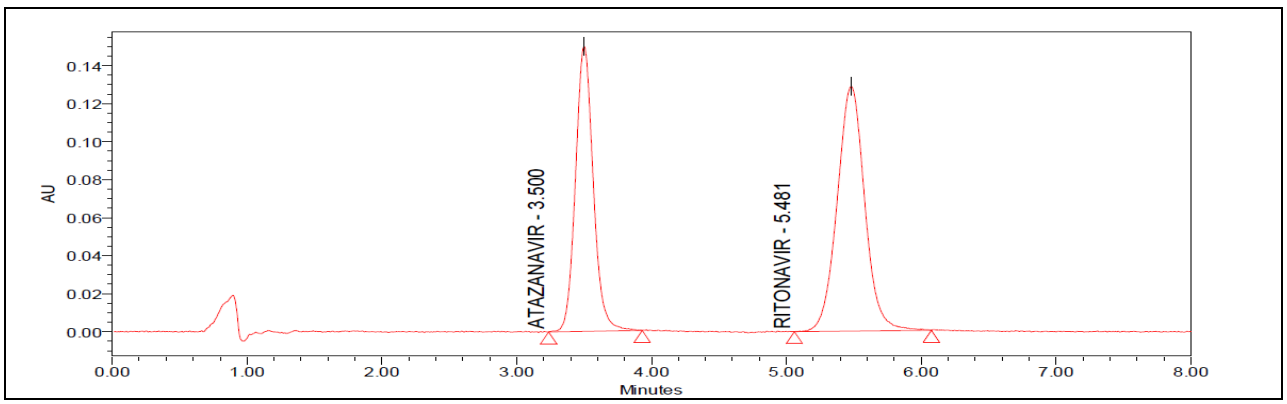
TABLE 5: DEGRADATION STUDY

Type of degradation	Degradant	% Degradation	
		Atazanavir	Ritonavir
Acid hydrolysis	0.1 M Hydrochloric acid	17	16
Base hydrolysis	0.1 M Sodium hydroxide	-	12
Oxidation	6 % Hydrogen peroxide	-	20
Aqueous	Water	10	26
UV visible	Chamber as per ICH	20	25
Thermal	105^0C for 48 Hours	19	25

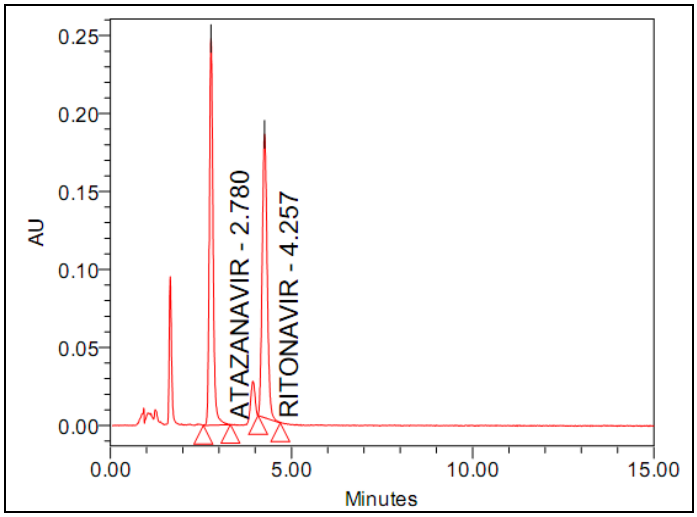
Assay: The validated method was applied to the determination of AT and RT in commercially available Ritovaz tablets. Figure 4illustrates typical HPLC chromatograms obtained following the assay of Ritovaz tablets (A) and from a standard solution (B). The result of the assays (n=9) undertaken yielded 99.0% (%RSD=1.44%) and 99.0% (%RSD=1.14%) of the label claim for AT and RT respectively. The mean retention times of AT and RT were 3.503 and 5.474 min with associated %RSD values of 0.03% and 0.03% respectively. The results of the assay indicate that the method is selective for the analysis of both AT and RT without interference from the excipients used to formulate and produce these tablets.



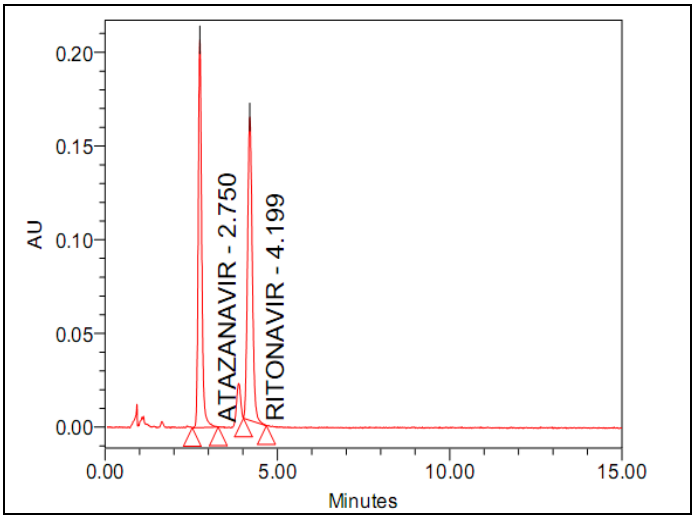
4A: SAMPLE CHROMATOGRAM



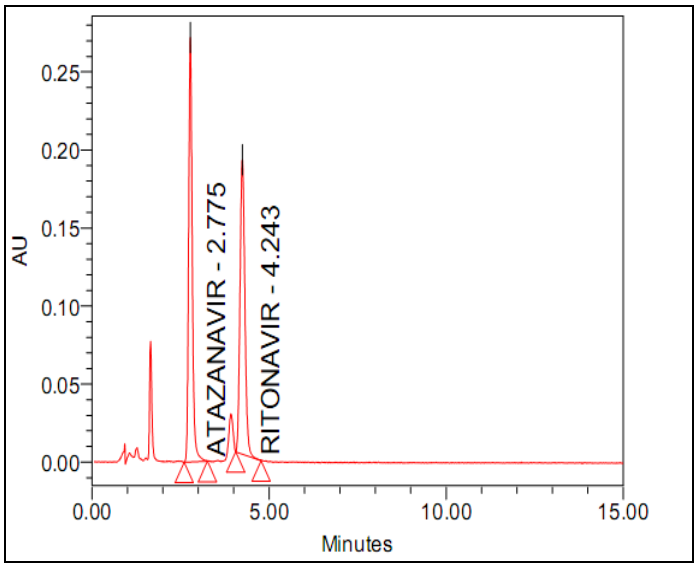
4B: STANDARD CHROMATOGRAM



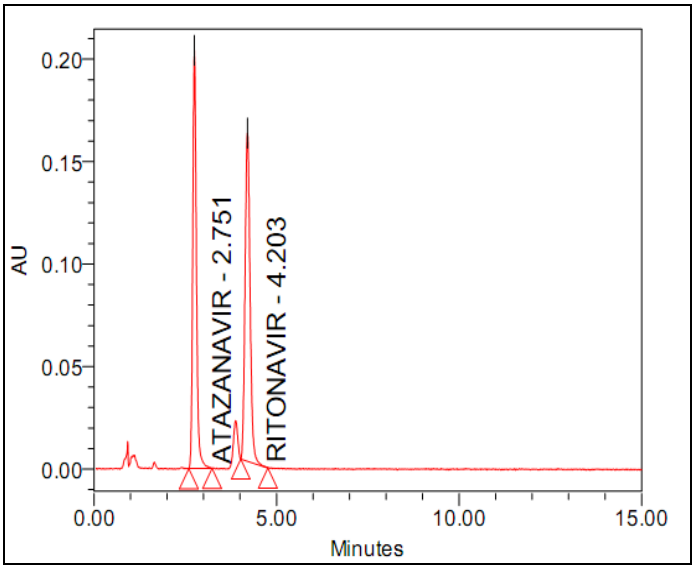
4(C): CHROMATOGRAM ON ACIDIC DEGRADATION



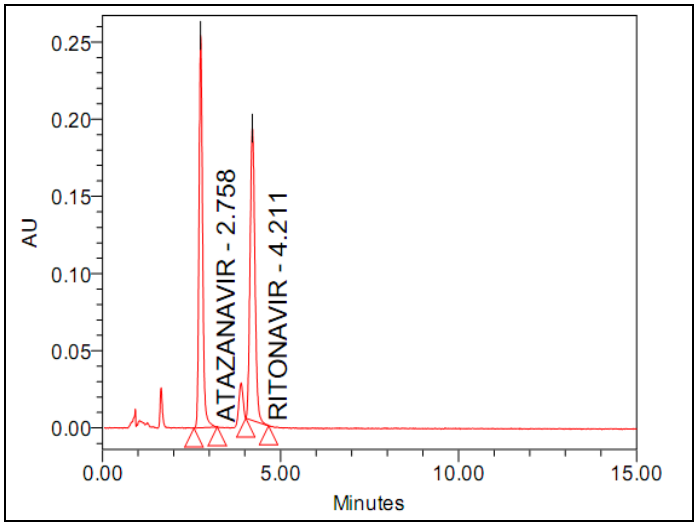
4F: CHROMATOGRAM ON NEUTRAL HYDROLYSIS



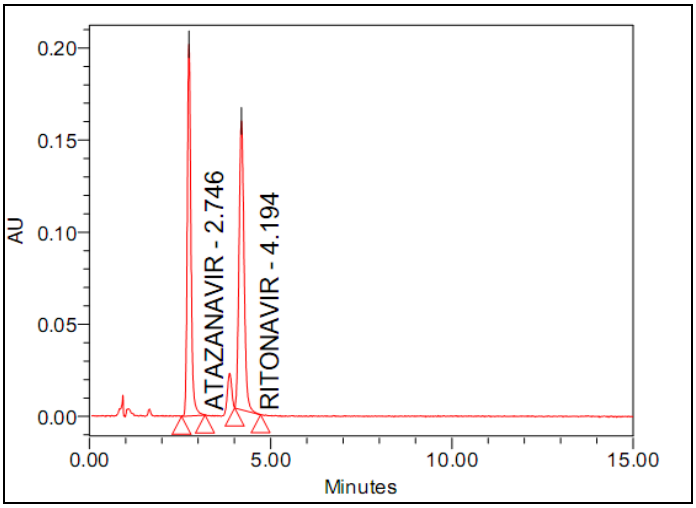
4D: CHROMATOGRAM ON BASIC DEGRADATION



4G: CHROMATOGRAM ON PHOTOLYTIC DEGRADATION



4E: CHROMATOGRAM ON OXIDATIVE DEGRADATION



4H: CHROMATOGRAM ON THERMAL DEGRADATION

CONCLUSIONS: A simple, rapid, accurate and precise stability indicating HPLC analytical method has been developed and validated for the routine analysis of AT and RT in API and tablet dosage forms. The results of the stress testing undertaken, according to the international conference on harmonization (ICH) guidelines reveal that the method is selective and stability indicating. The proposed method has the ability to separate these drugs from their degradation products; excipients found in tablet dosage forms and can be applied to the analysis of samples obtained during accelerated stability studies.

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