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Simultaneous Estimation of Atazanavir and Ritonavir in Combined Tablet Dosage Form

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Abstract

Ritonavir is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Although it was initially developed as an independent antiviral agent, 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). Scan the standard solution and test solution on UV/Visible spectrophotometer, over the spectral range 200 to 400 nm. Use diluent as blank. The UV spectrum of the test solution should exhibit maxima at the same wavelength (±2 nm) as that of a standard solution ATV and RTV show reasonably good response at 279 and 240 nm, respectively in methanol as solvent. For mobile phase mixed 85 mL of Buffer and 15 mL of HPLC grade Methanol. Filtered through 0.45 µ Millipore nylon filter and degassed. Calibration curves were plotted over concentration range of 10 – 30 µg/mL for ATV and of 5 – 15 µg/mL for RTV. Mixed standard stock solutions which contains ATV (200 µg/mL) and RTV (100 µg/mL) was diluted as mentioned. Accuracy was determined in terms of % recovery. Recovery experiments were carried out in triplicate by spiking standard drug to previously analyzed samples of the tablets. Method precision of the instrument was established by repeatedly injecting six standard solutions of ATV (20 µg/mL) and RTV (10 µg/mL) under same conditions on the same day. Intermediate precision was evaluated in terms of intra-day and inter-day precision by analysing 3 different solutions 3 times on the same day and on different days over entire concentration range for both drugs. The solution stability for both drugs in the assay method were carried out by leaving test and standard solutions in tightly capped volumetric flasks at room temperature for 24 hrs. Sample preparation was injected in triplicate and chromatograms were recorded then responses for the analyte peaks were measured and all data recorded were found complies with results.

Keywords: Ritonavir, Atazanavir, Accuracy, Recovery, Method development, validation.

1. INTRODUCTION:

These new "HPLC" instruments could develop up to 6,000psi (400 bar) of pressure, and included improved detectors and columns. HPLC really began to take hold in the mid to late 1970‟s. With continued advances in performance, the name was changed to High Performance Liquid Chromatography (HPLC). High Performance Liquid Chromatography (HPLC) is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as "parts per trillion" (ppt), are easily obtained. HPLC can be applied to just about any sample, such as pharmaceuticals, food, neutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.[1-3]

High Performance Liquid Chromatography is the most widely used analytical separation technique. HPLC has been rapidly developed with the introduction of new pumping methods, more reliable columns and a variety of detectors. Most of the drugs in multicomponent dosage forms can be analyzed by HPLC methods because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.[4]

1.1 Reverse Phase Chromatography:

Reversed phase mode is the most popular mode for analytical and preparative separation of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent.[5] An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity.

1.2 Partition Chromatography:

It is the most widely used liquid chromatographic procedure to separate most kinds of organic molecules. Here the components present in the analyte mixture distribute themselves between the mobile phase and stationary phase as

the mobile phase moves through the column.[6] The stationary phase actually consists of a thin liquid film either adsorbed or chemically bonded to the surface of finely divided solid particles.

- **DRUG PROFILE:**

2.1 Name of drug: Ritonavir[7]

Structure:

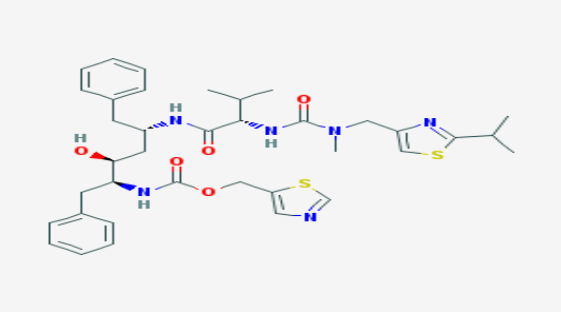

Figure 1: Structure of Ritonavir

Weight: Average: 720.944, Monoisotopic: 720.312760056

Chemical Formula: $C_{37}H_{48}N_6O_5S_2$

Description:

Ritonavir is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose ritonavir and other protease inhibitors. It is now more commonly used as a booster of other protease inhibitors and is available in both liquid formulation and as capsules.

Table 1: Physiciochemical properties of Ritonavir

Property	Value
water solubility	Practically insoluble
logP	3.9
Water Solubility	0.00126 mg/mL
logP	4.24
logP	5.22
logS	-5.8
pKa (Strongest Acidic)	13.68
pKa (Strongest Basic)	2.84
Number of Rings	4
Bioavailability	0

2.2 Atazanavir[8]

Name of Atazanavir

Structure:

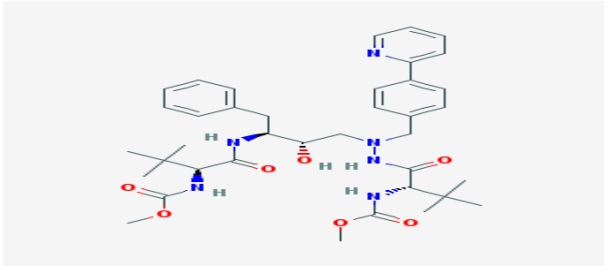

Figure 2: structure of Atazanavir

Weight: Average:704.8555, Monoisotopic: 704.389748048

Chemical Formula:$C_{38}H_{52}N_6O_7$

Description: 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.

Table 2: Physiciochemical properties of Atazanavir

Property	Value
Water Solubility	0.00327 mg/mL
logP	4.08
logP	4.54
logS	-5.3
pKa (Strongest Acidic)	11.92
pKa (Strongest Basic)	4.42
Bioavailability	0

3. MATERIALS AND METHODS

3.1 Apparatus and Instruments:

- A Shimadzu (Kyoto, Japan) HPLC system (LC-20AT) equipped with UV
- detector, Rheodyne manual injector with 20 µL loop volume.
- Balance (Model AX200)
- Toshcon Ultra Sonicator, (Fast clean ultrasonic cleaner)
- pH analyser (Chemiline CL 180 based pH meter)

3.2 Reagents and Materials:

- Ritonavir (Aurbindo Pharmaceutical LTD., Hyderabad)
- Atazanavir (Lauras Lab, Hyderabad)
- HPLC grade Water (Finar Chemicals Pvt. Ltd, vadodara, India)
- HPLC grade Acetonitrile, HPLC grade Methanol (RANKEM Fine Chemicals

Limited, New Delhi, India).

- Ortho Phosphoric Acid, Potassium Dihydrogen Phosphate, Sodium Hydroxide

  (RANKEM Fine Chemicals Limited, New Delhi, India)
- 0.45 µ Millipore nylon filter (Gelman Laboratory, Mumbai, India)
- Whatman filter paper no. 41

Melting Point Determination:

Melting point of the APIs were determined by using melting point apparatus. The observed melting points of APIs were compared with the reported melting point.[9]

3.4 Infrared Spectroscopy:

IR spectrum of Ritonavir and Atazanavir were taken by KBr pellet method on FTIR and characteristic peaks were compared with IR spectrum of Reference standard given in Indian Pharmacopoeia.[10]

3.5 Solubility Determination

The solubility of Ritonavir and Atazanavir were checked in various solvents like distil ATV water, methanol, and Dimethyl Sulphoxide etc.[11,12]

3.6 Selection of wavelength

Scan the standard solution and test solution on UV/Visible spectrophotometer, over the spectral range 200 to 400 nm. Use diluent as blank. The UV spectrum of the test solution should exhibit maxima at the same wavelength (±2 nm) as that of a standard solution ATV and RTV show reasonably good response at 279 and 240 nm, respectively in methanol as solvent.[13-15]

3.7 Marketed Formulation:

Table 3: Market formulation detail of combined dosage form[16]

Brand Name	Contents	Manufacturer	Formulation
Azavir-R	Atazanavir 300mg and Ritonavir 100mg	Mylan Pharmaceuticals Pvt. Ltd	Tablet

3.8 preparation of standard stock solution ATV and RTV

Accurately weighed 20 mg of ATV standard drug powder. It was transferred into 100 mL volumetric flask and diluted up to the mark with HPLC grade Methanol to give a stock solution having strength of 0.2 mg/mL or 200 ppm of DAP. Accurately weighed 10 mg of RTV standard drug powder. It was transferred into 100 mL volumetric flask and diluted up to the mark with HPLC grade Methanol to give a stock solution having strength of 0.1 mg/mL or 100 ppm of RTV.[17]

3.9 Preparation of Combined Standard Stock Solution of ATV and RTV:

1mL of ATV standard stock solution and 1 mL of RTV standard stock solution were pipetted out into 10 mL volumetric flask and diluted up to 10 mL with mobile phase to produce final concentration of 20 µg/mL of ATV and 10 µg/mL of RTV.[17-19]

3.10 Preparation of Mobile Phase:

Mixed 85 mL of Buffer and 15 mL of HPLC grade Methanol. Filtered through 0.45 µ Millipore nylon filter and degassed.

3.11 Preparation of Test Solution:

Separately weighed 20 tablets and average weight of individual tablets were found out and weight equivalent to ATV (10 mg) and RTV (5 mg) was taken into 100 mL volumetric flask and dissolved in 60 mL of HPLC grade Methanol with sonication for 20 minutes. The solution was filtered through 0.45 µ Millipore nylon filter and the residues were washed thoroughly with HPLC grade Methanol. The filtrate and washings were combined into 100 mL volumetric flask and diluted up to the mark with HPLC grade Methanol to get final concentration of 100 µg/mL of ATV and 50 µg/mL of RTV. From this solution 1 mL of was pipetted out into 10 mL volumetric flask and diluted up to 10 mL with mobile phase to produce final concentration of 10 µg/mL of ATV and 5 µg/mL of RTV.

4. Method Validation:

As per the ICH guidelines Q2R1, the method validation parameters studied were solution stability, linearity, accuracy, precision, limit of detection, limit of quantitation, robustness and system suitability test.[20-26]

4.1 Linearity and Range:

Calibration curves were plotted over concentration range of 10 – 30 µg/mL for ATV and of 5 – 15 µg/mL for RTV. Mixed standard stock solutions which contains ATV (200 µg/mL) and RTV (100 µg/mL) was diluted as mentioned. Volume was made up to the mark with mobile phase and mixed.

Each standard preparation was injected in triplicate and the peak area obtained at retention time 4.080 and 5.343 minutes at a flow rate of 1 mL/min were measured at 279 nm for ATV and RTV. Calibration curves were constructed by plotting peak area Vs. concentration.

4.2 Accuracy (% Recovery):

Accuracy was determined in terms of % recovery. Recovery experiments were carried out in triplicate by spiking standard drug to previously analyzed samples of the tablets (10 µg/mL and 5 µg/mL for ATV and RTV) with three levels of standards (80, 100 and 120 % for both). Amounts of ATV and RTV were determined using corresponding regression equations of calibration curves.

4.3 Precision:

4.3.1 Method Precision (Repeatability):

Method precision of the instrument was established by repeatedly injecting six standard solutions of ATV (20 µg/mL) and RTV (10 µg/mL) under same conditions on the same day. Results were reported in term of % RSD which should not be more than 2 %.

4.3.2 Intermediate Precision (Reproducibility):

Intermediate precision was evaluated in terms of intra-day and inter-day precision by analysing 3 different solutions 3 times on the same day and on different days over entire concentration range for both drugs. Results were reported in terms of % RSD.

4.4 Robustness:

Method robustness was performed by applying small changes in flow rate, ratio of mobile phase, and pH. Results were expressed in terms of % RSD.

1) Change in flow rate by ± 0.2 mL/min [Flow rate 0.8 mL/min. and 1.2 mL/min.]

2) Minor changes in the mobile phase composition by ± 2 % absolute [Composition

(a). Buffer: Methanol (87:13), Composition (b). Buffer: Methanol (83:17)]

3) Change in Buffer pH ± 0.2 Unit (pH 4.7 and pH 4.3).

4.5 Solution Stability:

The solution stability for both drugs in the assay method were carried out by leaving test and standard solutions in tightly capped volumetric flasks at room temperature for 24 hrs. The same sample solutions were assayed for interval of 6 hrs up to 24 hrs throughout the study period. Results obtained were compared with those of freshly prepared solutions.

4.6 Estimation of RTV and ATV in Formulation

Sample preparation was injected in triplicate and chromatograms were recorded. Responses for the analyte peaks were measured. The concentration of the drug in sample solution was determined using regression equation of calibration curve.

5. RESULT AND DISCUSSION:

5.1 Melting point determination:

Table 4: Melting point determination

Name of Drug	Reported Melting Point	Observed Melting Point
ATV	207-209^{0}C	206-208^{0}C
RTV	120-122^{0}C	118-121 ^{0}C

5.2 Infrared Spectroscopy:

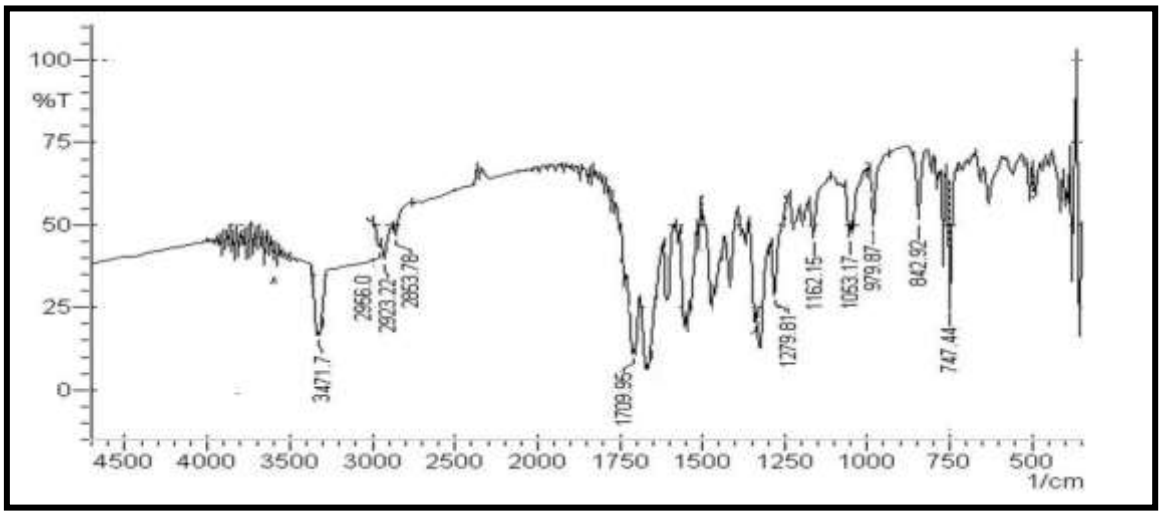

Fig 3: IR Spectra of ATV

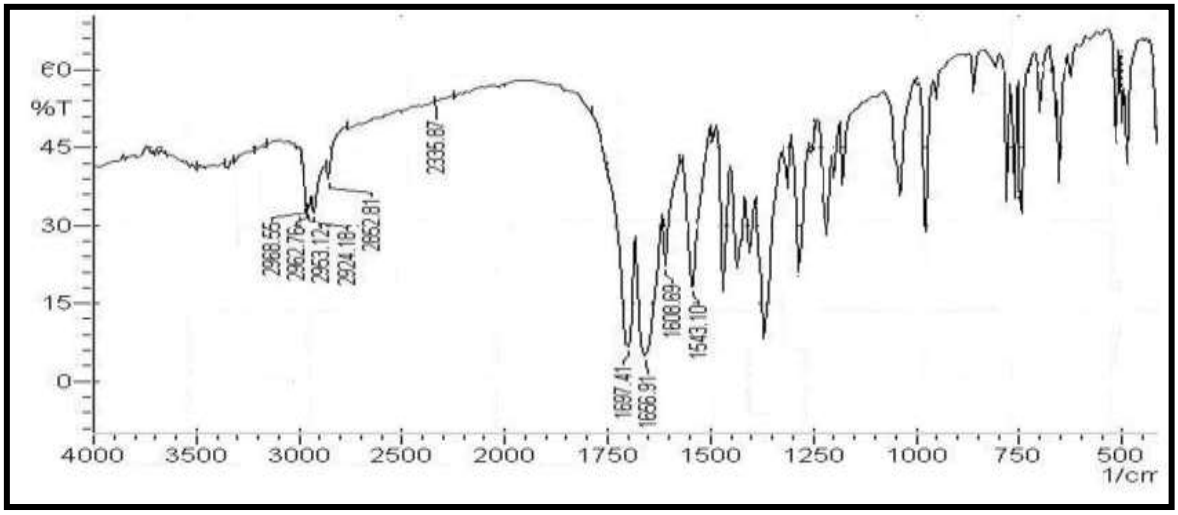

Fig 4: IR Spectra of RTV

5.3 Solubility Studies:

Table 5: Solubility determination of APIs

Sr No.	Drug	Reported	Observed
1	ATV	**Water :** soluble in water **Methanol :** soluble in Methanol **Dimethyl Sulphoxide :** Soluble in Dimethyl Sulphoxide	Complies with Reported solubility
2	RTV	**Water :** soluble in water **Methanol :** freely soluble in Methanol **Dimethyl Sulphoxide :** Soluble in Dimethyl Sulphoxide	Complies with Reported solubility

6. Method Development and Optimization:

6.1 Selection of Wavelength for Determination:

The standard solutions of ATV (20 µg/mL) and RTV (10 µg/mL) were scanned in the range of 200 – 400 nm. The responses of standard solution measured with UV detector showed a good result at 279 nm for the RP-HPLC method.

6.2 Selection of Mobile Phase:

As per the value of Ka and solubility of both the compounds, various compositions of mobile phase were tried. The chromatographic conditions were optimized with a view to develop a method, which can separate both drugs from with good resolution. Both the compounds were separated with mobile phase in different proportions of Methanol and Phosphate Buffer. Good peak shape with clear baseline and resolution was obtained within short retention time of 10 minutes with mixture of Phosphate Buffer : Methanol (85 : 15 v/v) as mobile phase, at a flow rate of 1 mL/min, was satisfactory to obtain well-resolved peaks with better reproducibility and repeatability for ATV and RTV. All other method parameters were developed to obtained good peak shape. A representative chromatogram is shown which satisfies all the system suitability criteria and better resolution of peaks from solvent peak with clear base line separation.

Table 6: Optimization of mobile phase for ATV and RTV

Mobile phase	Ratio	pH	Remark
Water : Methanol	50 : 50	-	Small single peak was obtained.
Water : Methanol	70 : 30	-	Single peak was obtained.
Water : Methanol	90 : 10	-	Single peak was obtained.
Water : ACN	50 : 50	-	Separation was not good.
Water : ACN	60 : 40	-	Separation was not good.
Phosphate Buffer :	50 : 50	4.5	Separation occurred but first peak
Methanol			was after blank and having tailing.
Phosphate Buffer :	70 : 30	4.5	Moderate good separation and
Methanol			resolution but peak tailing.
Phosphate Buffer :	85 : 15	4.5	Good separation, good resolution and
Methanol			good peaks shape with achievement of all system suitability criteria

6.3 Optimized Chromatographic Condition

Column: Hypersil BDS C18 (250 mm × 4.6 mm) 5µ, Thermoscientic

Mobile Phase: Phosphate Buffer : Methanol (85 : 15 v/v) pH 4.5

Detection wavelength: 279 nm

Flow rate: 1 mL/min

Injection volume: 20 µl

Column oven temperature: 25ºC

6.4 Method Validation:

The developed method as described above was validated for various parameters like system suitability, robustness, linearity, precision, accuracy, LOQ and LOD.

6.4.1 Linearity and Range:

The correlation coefficient value should not be less than 0.995 over the working range. Linearity of the method was evaluated

at five concentration levels by diluting the standard stock solution to give solutions of ATV and RTV in the concentration range from 10 – 30 µg/mL and 5 – 15 µg/mL. Results show good correlation between peak area and concentration of analytes. The calibration curves were prepared by plotting the area under the response from the detector versus the concentration of standard drugs

6.4.2 Accuracy (% Recovery):

Acceptance criteria:

Recovery for individual and mean value at each level should be 95.0 % to 105.0 % and with % RSD not more than 2.0 %.Recovery and % RSD were calculated at each level and recorded.

Table 7: Results of recovery study of ATV and RTV (n = 3)

Drug	Amount of sample (µg/mL)	Amount of standard added (µg/mL)	Amount of found (µg/mL)	Amount of recovery (µg/mL)	% Recovery± SD (n = 3)	% RSD
ATV	10	8	17.937	8.025	100.31 ± 1.19	1.19
		10	20.001	10.09	100.89 ± 0.95	0.94
		12	22.068	12.16	101.30 ± 0.66	0.65
RTV	5	4	8.981	4.015	100.38 ± 0.97	0.97
		5	10.012	5.047	100.94 ± 1.2	1.19
		6	10.989	6.023	100.39 ± 0.59	0.59

6.4.3 Precision:

6.4.3.1 Method Precision (Repeatability):

Acceptance Criteria:

The % RSD of assay of six sample preparations should not be more than 2.0 %. Mean, % assay, SD and % RSD of result obtained were recorded.

6.4.3.2 Intermediate Precision (Reproducibility):

Acceptance Criteria: % RSD of intermediate precision should not more than 2.0 %.

Table 8: Results of intra-day and inter-day precision of ATV and RTV (n = 3)

Concentration (µg/mL)		Intra-day precision		Inter-day precision	
		Measured mean area ± %RSD		Measured mean area ± %RSD	
ATV	**RTV**	**ATV**	**RTV**	**ATV**	**RTV**
10	5	507.460 ± 0.48	101.769 ± 0.17	509.291 ± 0.86	101.345 ± 1.89
20	10	1020.596 ± 0.43	203.844 ± 0.71	990.636 ± 1.23	199.057 ± 1.06
30	15	1510.421 ± 0.31	301.321 ± 1.27	1516.091 ± 0.47	304.791 ± 0.30

6.4.4 Estimation of ATV and RTV in Formulation:

The proposed RP-HPLC method was successfully applied for determination of ATV and RTV from Tablet formulation. The percentage of ATV and RTV were satisfactory, which were comparable with the corresponding claim amount.

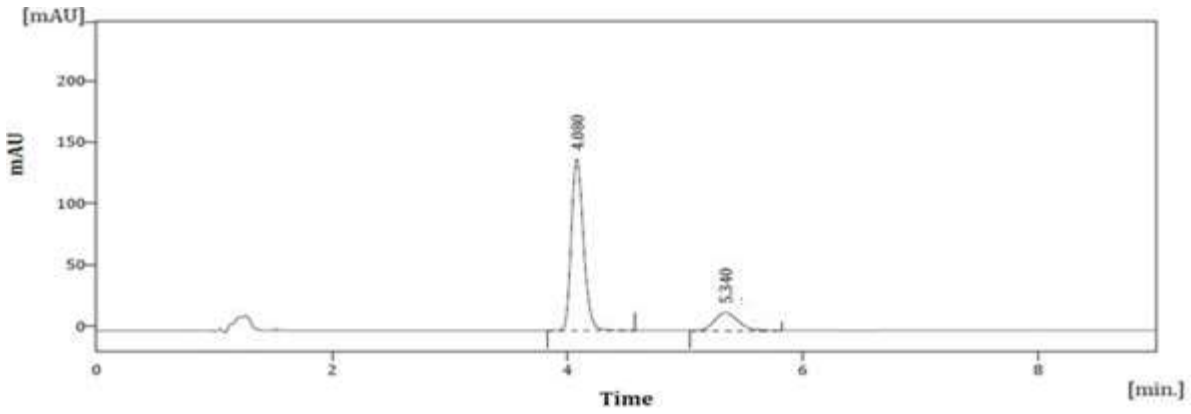

Figure 5: Estimation of ATV and RTV in Formulation

7. CONCLUSION:

The proposed method is simple, accurate, precise and has ability to separate drug from degradation products and excipients found in dosage forms. The HPLC method developed meets the system suitability criteria and resolution of the parent drugs from its degraded products. Detection and quantification limits achieved, describe the sensitivity of developed method. High recovery and acceptable % RSD values confirms that the established RP-HPLC method is accurate and precise. The complete separation of the drug and its degradation product was accomplished within 10 minutes. The method has been successfully applied to perform accelerate stability study of ATV and RTV. Hence, the method can be used for routine quality control analysis and pharmacokinetic study of ATV and RTV.

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