https://doi.org/ 10.48047/AFJBS.6.5.2024. 8492-8503

African Journal of Biological Sciences

Development and Validation of Ultra Performance Liquid Chromatographic Assay for Simultaneous Determination of Atazanavir and Ritonavir in Pharmaceutical Formulations

Induri Madhusudhana Reddy[1*], Vudumula Koti Reddy[2], Sai Rajesh Kollapudi[3], Kishore More[4] and Guntupalli Rajesh[5]

[*1]Department of Pharmaceutical Chemistry, SVS College of Pharmaceutical Education and Research, Tembhurni, Tal Madha, Dist. Solapur, 413211, Maharashtra, India
[2]Department of Pharmaceutical Chemistry, [3]Department of Pharmaceutical Analysis, A.S.N. Pharmacy College, Tenali, Andhra Pradesh, India
[4]Department of Pharmaceutical Analysis, Vikas College of Pharmaceutical Sciences, Rayanigudem - 508 376, Suryapet, Telangana, India
[5]Department of Pharmaceutical Analysis, Malineni Perumallu Pharmacy College, Pulladigunta, Vatticherukur, Prathipadu Road, Guntur, Andhra Pradesh 522017, India

***Corresponding Author**:
Induri Madhusudhana Reddy,
Department of Pharmaceutical Chemistry,
SVS College of Pharmaceutical Education and Research,
Tembhurni, Tal Madha, Dist. Solapur, 413211, Maharashtra, India
imsreddychem@gmail.com

Article History
Volume 6, Issue 5, 2024
Received: 15 May 2024
Accepted: 02 Jun 2024
doi: *10.48047/AFJBS.6.5.2024. 8492-8503*

ABSTRACT

An accurate, rapid, and simple UPLC method was developed and validated for simultaneous determination of atazanavir and ritonavir in formulations. The separation was established on a column BEH Shield C_{18} (50 × 2.1 mm; 1.7 µm) connected at 30 °C. The detector (PDA) was set at 249 nm. The mobile phase consisted of 5% acetonitrile in methanol and 10mM ammonium formate (pH = 4.0; 0.1% formic acid) in a gradient mode. The flow rate was set at 0.3 mL/min. The retention times of atazanavir and ritonavir were 0.321 and 1.779 min, respectively. The proposed method demonstrated linearity in the ranges of 6-36 µg/mL for atazanavir and 2-12 µg/mL ritonavir. The coefficients of determination (R^2) were greater than 0.999, with percentage recoveries greater than 98-102% for each drug. The proposed method was highly precise, as indicated by the low percentage of relative standard deviation values of less than 2% for each drug. The method had the requisite accuracy, precision, and robustness for simultaneous determination of atazanavir and ritonavir. The proposed method could be successfully employed in routine quality control for the simultaneous analysis of atazanavir and ritonavir n pharmaceutical formulations.

Keywords: Atazanavir, Ritonavir, UPLC, ICH Guidelines, Quality Control

INTRODUCTION

Atazanavir is administered with low-dose ritonavir (atazanavir/r), because of its low dose, high potency and less toxic hence, used in the treatment of HIV infection in adults. Atazanavir/r is currently used with a C-C chemokine receptor type-5 (CCR5) inhibitor or an integrase inhibitor in order to give NRTI-sparing treatment. [1-3] Atazanavir chemically is 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. It is soluble in methanol, ethanol and slightly soluble in water. [4-5] Ritonavir chemically is 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. It is freely soluble in methanol and ethanol. [6-9] Various methods have been reported for simultaneous estimation of atazanavir and ritonavir in pharmaceutical formulations and biological matrices, which includes the use of LC-MS/MS [10, 11], LC-ESI-MS/MS [12], RP-HPLC [13-15] and HPTLC [16,17]. Present study involves development of UPLC method using simple mobile phase containing acetonitrile, methanol and buffer for quantitative estimation of atazanavir and ritonavir in dosage forms which is sensitive and requires shorter analysis time. [18]

MATERIALS AND METHODS

Materials:

The working standard atazanavir (as sulfate) (ATV) and ritonavir (RTV) were obtained from Hetero Drugs Ltd., India. A commercial Synthivan tablets (Cipla, Mumbai) containing ATV (300 mg) and RTV (100 mg) were purchased from local pharmacies and used within their shelf-life period. The HPLC grade methanol, acetonitrile and water were procured from Rankem, New Delhi, India. All other chemicals used were of analytical grade purchased from Rankem, New Delhi, India.

Methods:

Preparation of standard solutions

Preparation of standard stock solution: An accurately weighed ATV (150 mg) and RTV (50 mg) were transferred into separate 100 mL volumetric flasks containing 30 mL of diluent (methanol; HPLC grade), sonicated for 15 min to dissolve the analytes and the remaining volume was made up to the mark with diluent. Aliquots of these standard solutions were pipetted out into a 100 mL volumetric flask and the remaining volume was made up to the mark with diluent to get final concentration of ATV (150 µg/mL) and RTV (50 µg/mL).

Preparation of 10 mM ammonium formate: An accurately weighed 0.63 g of ammonium formate was transferred into a 1000 mL volumetric flask containing 400 mL water (HPLC grade). The solution was sonicated for 5 min, add water to 950 mL, adjust the pH 4.0 with 0.1% formic acid and then the remaining volume was made up to the mark with water.

The buffer and standard solutions were filtered through 0.2 µm nylon membrane filter, before use.

Instrumentation

The Waters AcquityTM UPLC M-Class system consisting of a H05UPB062M binary gradient pump, Water 2996 PDA detector, an inbuilt autosampler, and column connected to a multi-instrument data acquisition and processing system with Empower 2.1 version. A Bandline Sonerex sonicator was used for enhancing the dissolution of analytes in diluents. A Digisum DI 707 digital pH meter was used for pH adjustment. A Mettler Toledo Excellence XS analytical balance (Model: XS64) was used for weighing all the materials.

Method Validation

System Suitability: The parameters like resolution (R_s), tailing factor, no. of theoretical plates (N) and, %RSD values of retention time and peak area were analyzed for standard solution containing ATV (18 µg/mL) and RTV (6 µg/mL).

Specificity: The evaluation of the specificity of the method was determined against excipients and each analyte. The interference of the excipients and each analyte were derived by injecting excipients combined with individual analytes, and the retention time of each was taken into consideration. Further, the specificity of the method towards the analyte was established by means of the interference of excipients at the retention time of the analyte peak.

Linearity: From the mixed working standard solution containing ATV (150 µg/mL) and RTV (50 µg/mL), aliquots of the solution (0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 mL) were pipetted out into a series of 10 mL volumetric flasks and then the remaining volume was made up to the mark with mobile phase to get final concentrations of 6.0-36 µg/mL and 2.0-12 µg/mL, respectively. Aliquots (10 µL) of every solution were injected into UPLC system in triplicate. The calibration curves were plotted over the concentration versus peak area and therefore, the regression equations were calculated.

Limit of Detection and Limit of Quantification: The limit of detection (LOD) and limit of quantification (LOQ) values were determined by using calibration curve method according to ICH Q2 (R1) [18] recommendations. The LOD (k=3.3) and LOQ (k=10) values were calculated using the following formula:

$A=k\sigma/S$

A is LOD or LOQ; σ is the standard deviation of the response;

S is the slope of the calibration curve

Accuracy: It was performed by adding known quantities of each standard drug related to three concentration levels - 50, 100 and 150 % - of the labeled claim to the ATV (300 mg) and RTV (100 mg). It was calculated as percentage analyte recovered by the developed method.

Precision: Repeatability of injection was studied by injecting ten standard solutions of ATV (18 µg/mL) and RTV (6.0 µg/mL) on the same day and calculate the peak area %RSD values. For each intra- and inter-assay precision, sample solutions of ATV (18, 24 and 30 µg/mL) and RTV (6.0, 8.0 and 10 µg/mL) were injected into UPLC system in triplicate. Reproducibility was performed by different analysts using same instrument as well as same laboratory.

Robustness: It was analyzed by performing the experiments, during which altered the optimized parameters like buffer pH (varied by ± 0.1) and flow rate (varied by ± 0.02 mL). The retention time, tailing factor and no. of theoretical plates were recorded.

Estimation of Atazanavir and Ritonavir in Tablets:

Twenty tablets were weighed and crushed to get fine powder in a mortar. An accurately weighed powder equivalent to one tablet was transferred into a 100 mL volumetric flask containing 30 mL of diluent (methanol; HPLC grade), sonicated for 20 min to dissolve the contents and the remaining volume was made up to the mark with diluent. The resulting solution was filter through 0.2 µm nylon membrane filter. Appropriate aliquot of the filtered solution was pipetted out into a 100 mL volumetric flask and the remaining volume was made up to the mark with mobile phase to get final concentrations of ATV (24 µg/mL) and RTV (8.0 µg/mL). The solutions were injected into UPLC system in triplicate and analyzed under optimized chromatographic conditions.

RESULTS AND DISCUSSION

Method Development:

It is required to contemplate the sequent steps involved for the development of UPLC method. Specifically, the issue with reference to the choice of mobile phase, selection of column and choice of detector has to be emphasized. The optimized chromatographic conditions (Table 1) were carefully chosen based on retention time, sensitivity, baseline drift and peak shape. The retention time for ATV and RTV at a flow rate of 0.3 mL/min was 0.321 min and 1.779 min, respectively. The analyte peaks were well resolved and free from tailing (Fig. 1). Hence, the optimized method was accurate for the simultaneous estimation of ATV and RTV; subsequently no interfering peaks appeared close to the retention time of the compound of interest.

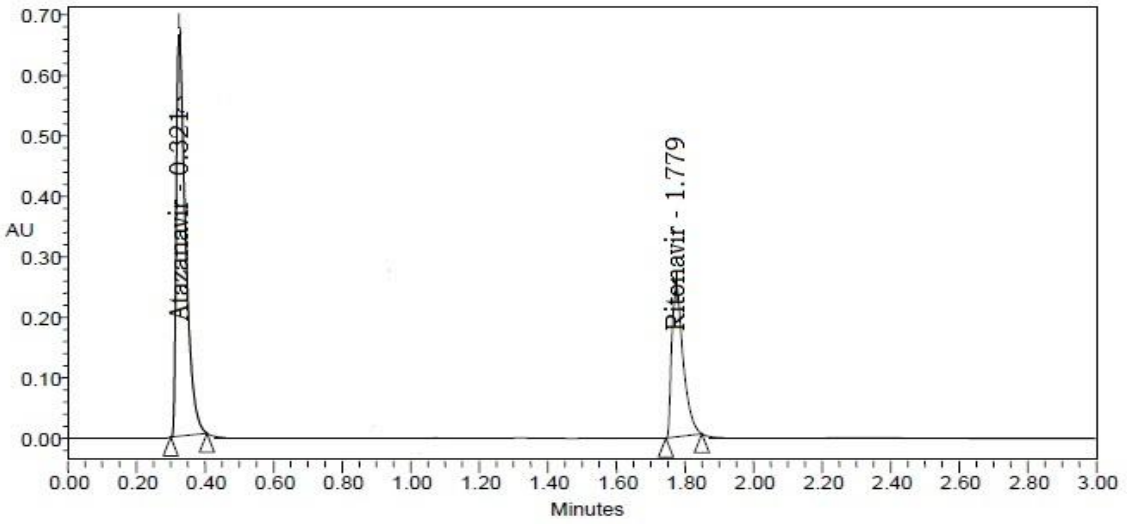

Figure 1: A Standard Chromatogram of Atazanavir (t_R: 0.321) and Ritonavir (t_R: 1.779)

Table 1: Optimized Chromatographic Conditions by UPLC Method

Parameters	**Conditions**
Column	Acquity UPLC BEH Shield RP18 (50 x 2.1 mm, 1.7 µm)
Mobile Phase A	10 mM Ammonium formate (pH = 4.0; 0.1 % formic acid)
Mobile phase B	5 % Acetonitrile in Methanol
Gradient Elution	**As per Table 2**
Flow rate	0.3 mL/min
Wavelength	249 nm
Injection volume	10 µL
Column oven Temp.	30±2^{o}C
Run time	3 Min

Table 2: Gradient Programming of Proposed Method

Time (min)	**Flow Rate (mL/min)**	**% Mobile Phase A**	**% Mobile Phase B**
Initial- 0.8	0.3	65	35
0.8-1.2	0.3	50	50
1.2-3.0	0.3	65	35

Method Validation

System Suitability: The tailing factor for ATV and RTV were 1.26 and 1.32, respectively, thus reflecting good peak symmetry. The resolution value indicated that a good separation of both analytes from each other. The no. of theoretical plates for ATV and RTV were 7823 and 6923, respectively, so indicating good column efficiency (Table 3).

Table 3: Results of System Suitability Parameters

Parameter*	**ATV**	**RTV**	**Limit**
Peak area (%RSD)	0.824	1.034	NMT: 2 %
Retention time (%RSD)	0.902	0.465	NMT: 1 %
Tailing factor	1.26	1.32	NMT: 2
No. of theoretical plates	7823	6923	NLT: 2000
Resolution	6.27		NLT: 2

** Replicates of six determinations; RSD: Relative Standard Deviation; NMT: Not More Then; NLT: Not Less Then*

Specificity: From the specificity studies, it was confirmed that no interference was observed from placebo or individual analytes to the peak of other and also the peak purity results were within the acceptance criteria. Therefore, it was proved that the developed method was extremely specific with respect to the placebo and each analyte to the other.

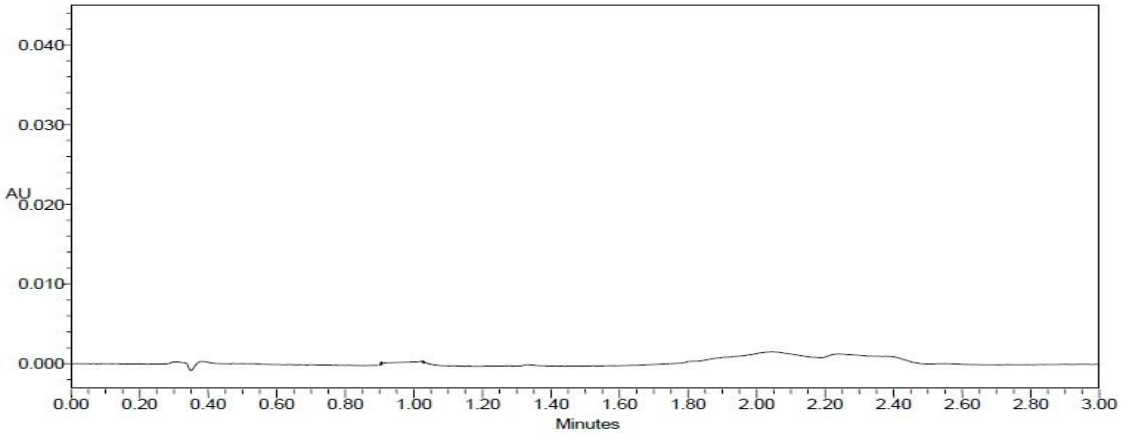

Figure 2: A Typical Chromatogram of Blank

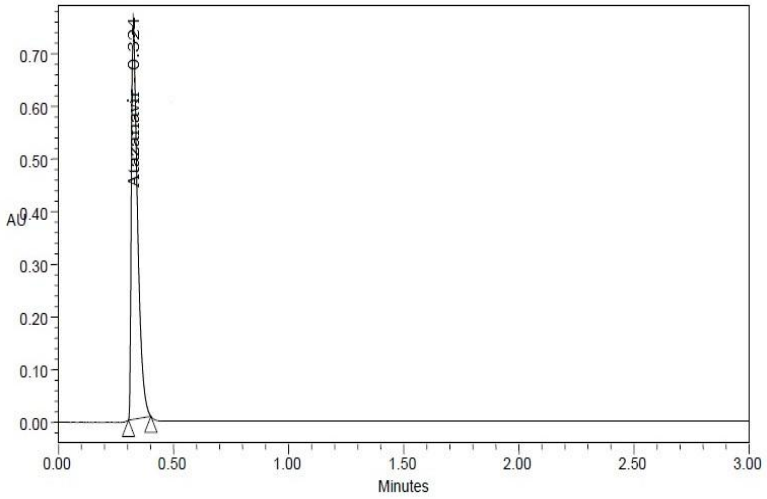

Figure 3: A Typical Chromatogram of Atazanavir

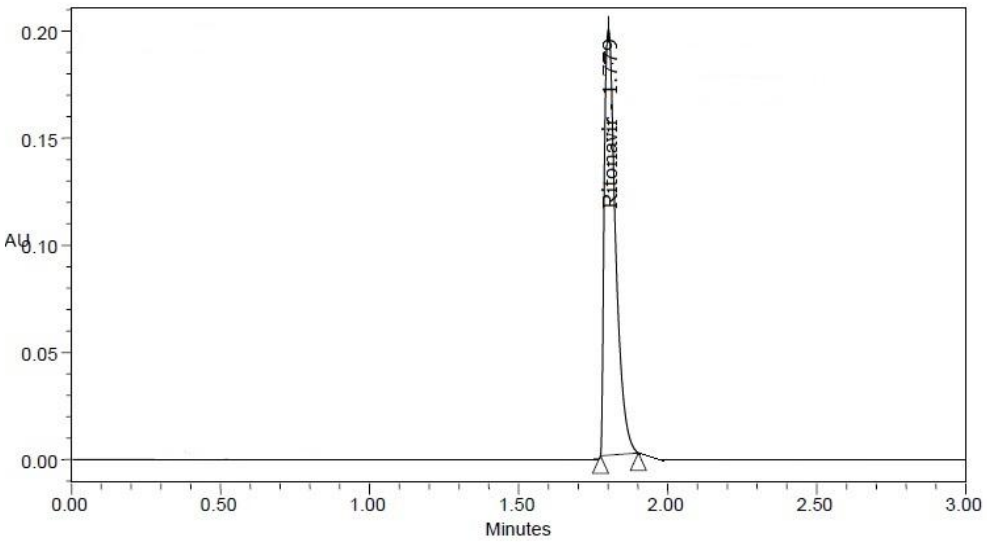

Figure 4: A Typical Chromatogram of Ritonavir

Linearity: The linearity curve was constructed by plotting curve between concentration versus peak area, showed linear in the concentration range of 6.0-36 µg/mL for ATV, and 2.0-12 µg/mL for RTV (Fig. 5, 6). The regression coefficients of ATV (R^2 = 0.9993) and RTV (R^2 = 0.9995) signify that a decent linear relationship exhibited between peak area versus concentration over a wide range. (Table 4)

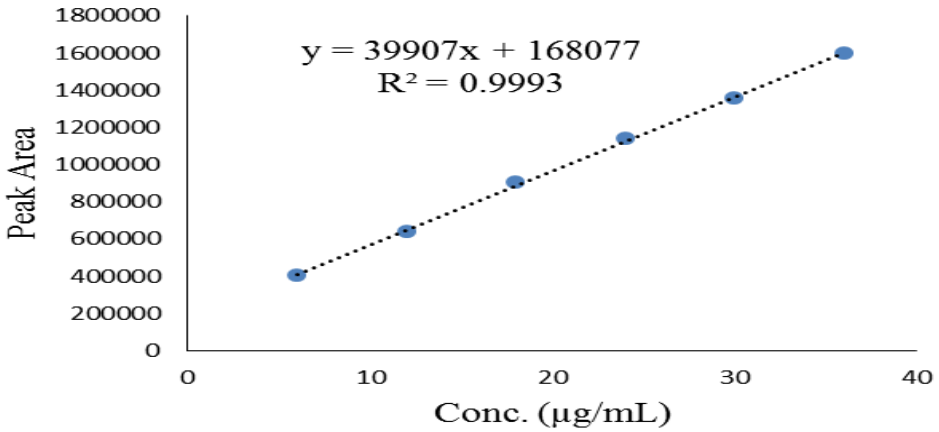

Figure 5: Linearity Curve of Atazanavir

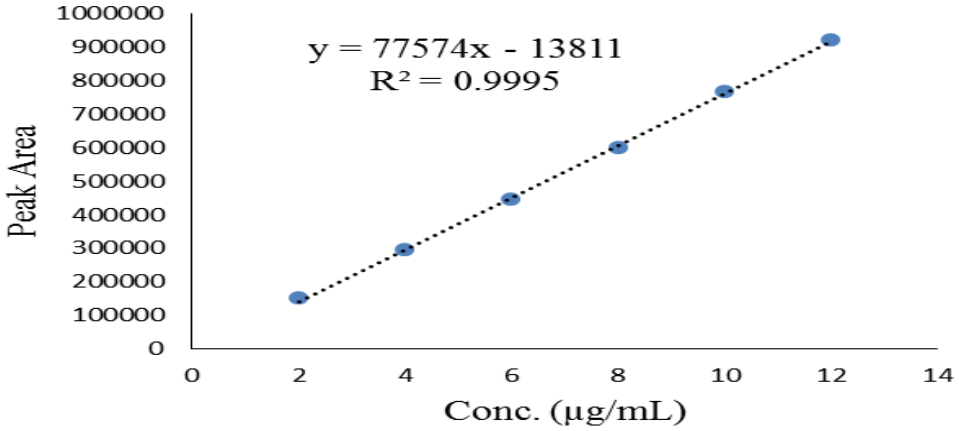

Figure 6: Linearity Curve of Ritonavir

Table 4: Linearity Data of Optimized Method

Anal-yte	Conc. (µg/mL)	Peak Area (Mean ± SD)*	RSD (%)	Linear regression equation
ATV	6.0	400234± 6833	1.707	y= 39907x + 168077 R^2= 0.9993
	12	639234± 8168	1.278	
	18	902345± 10967	1.215	
	24	1139765± 8774	0.77	
	30	1356893± 11834	0.872	
	36	1598235± 7942	0.497	
RTV	2.0	149823± 2134	1.424	y= 77574x - 13811 R^2= 0.9995
	4.0	294338± 5001	1.699	
	6.0	443582± 3536	0.797	
	8.0	600234± 6926	1.154	
	10	765322± 7794	1.018	
	12	921937± 8152	0.884	

**Replicate of three determinations; SD: Standard Deviation; RSD: Relative Standard Deviation*

LOD and LOQ: The LOD values of ATV and RTV were 0.10µg/mL and 0.03µg/mL, respectively, whereas LOQ values were 0.31µg/mL and 0.09µg/mL, respectively. The obtained results indicate that the developed method was more sensitive.

Accuracy: From the recovery data has been confirmed that the percentage recovery was within the range (98 to 102 %) and additionally %RSD values were below 2 %. Hence, the recovery results indicate that the developed method was more accurate (Table 5)

Table 5: Results of Recovery Study by Standard Addition Method

Anal-yte	Amount of standard drug spiked		Amount of sample taken (mg)	% Recovery (Mean ± SD)*	RSD (%)	SEM
	% Spiked	Quantity (mg)				
ATV	50	150	300	100.42± 1.025	1.021	0.5921
	100	300	300	99.66± 0.633	0.635	0.3655
	150	450	300	100.38± 0.592	0.590	0.3419
RTV	50	50	100	100.45± 0.710	0.707	0.4101
	100	100	100	99.46± 0.841	0.846	0.4856
	150	150	100	99.79± 1.457	1.460	0.8412

**Replicate of three determinations; SD: Standard Deviation; RSD: Relative Standard Deviation; SEM: Standard Error of Mean*

Precision: Injection repeatability values (%RSD) of ATV and RTV were found to be 1.019 and 0.496, respectively. The intra- and inter-assay precision results were expressed as %RSD values and were shown in Table 6. The low %RSD values proved that the method was precise. The reproducibility results were observed that there was no significant difference

between %RSD values obtained (table 7), which indicates that the developed method was reproducible.

Table 6: Intra- and Inter- Assay Precision Data of the Proposed UPLC Method

Analyte	Analyte Conc. (µg/mL)	Intra-assay precision*	Inter-assay precision*
ATV	18	0.904	0.708
	24	1.123	1.007
	30	0.974	0.615
RTV	6.0	1.804	0.641
	8.0	1.297	1.096
	10	1.483	1.034

**%RSD Values*

Table 7: Reproducibility Data of the Proposed UPLC Method

Analyte	Analyte Conc. (µg/mL)	Reproducibility*	
		Analyst one	Analyst two
ATV	18	1.048	0.869
	24	0.485	0.834
	30	0.382	0.691
RTV	6.0	0.201	0.959
	8.0	1.557	0.965
	10	1.032	1.407

**%RSD Values*

Robustness: There were no significant changes in the retention time, tailing factor and no. of theoretical plates of ATV and RTV when the flow rate and buffer pH were changed, which indicates that the proposed method was robust (Table 8 & Fig. 7-12).

Table 8: Results for Robustness Study of the Proposed UPLC Method

Parameter	Used	Anal-yte	Retention time	Tailing Factor	No. of Theoretical Plates
			Mean± SD*	Mean± SD*	Mean± SD*
Flow rate (mL/min)	0.28	**ATV**	0.323±0.002	1.23±0.015	7654±77
	0.3		0.323±0.002	1.27±0.015	7881±52
	0.32		0.327±0.002	1.35±0.025	7355±96
	0.28	**RTV**	1.750±0.010	1.43±0.017	6648±78
	0.3		1.784±0.016	1.34±0.020	6974±43
	0.32		1.764±0.006	1.23±0.006	7163±140
pH (Buffer)	3.9	**ATV**	0.325±0.002	1.19±0.020	7623±75
	4.0		0.329±0.004	1.29±0.006	7864±66
	4.1		0.357±0.004	1.42±0.010	7361±67
	3.9	**RTV**	1.805±0.017	1.43±0.006	6438±85
	4.0		1.778±0.003	1.33±0.017	6931±59
	4.1		1.839±0.018	1.39±0.015	6385±58

**Replicates of three determinations; SD: Standard Deviation*

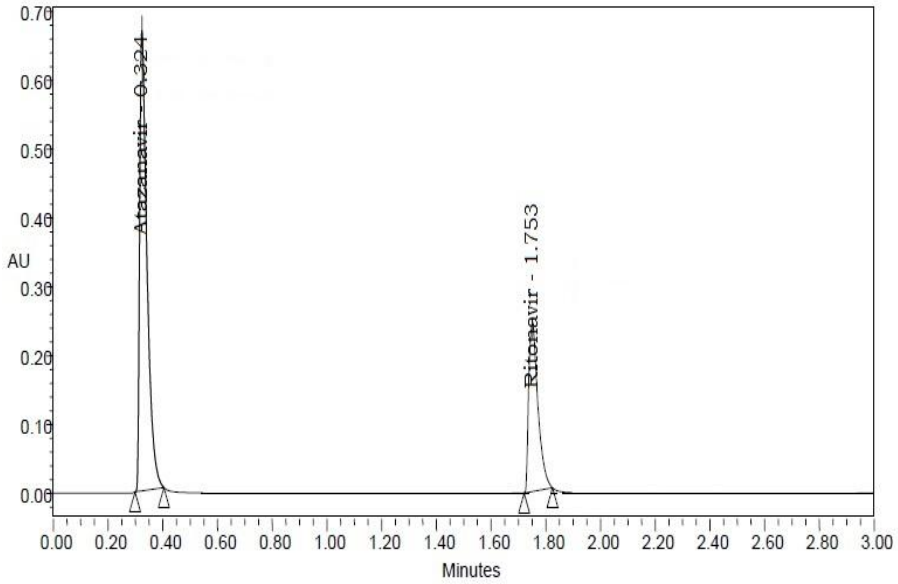

Figure 7: A Chromatogram of Robustness Study at 0.28 mL/min (Flow Rate)

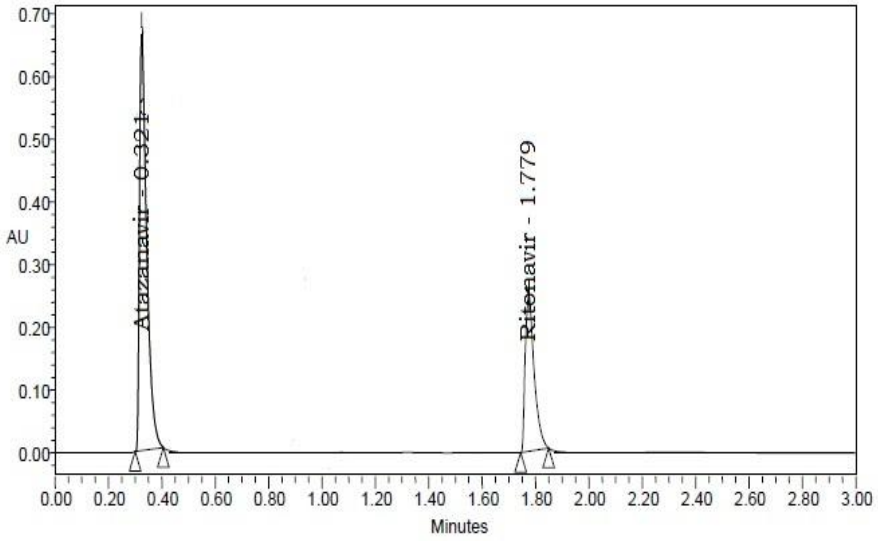

Figure 8: A Chromatogram of Robustness Study at 0.3 mL/min (Flow Rate)

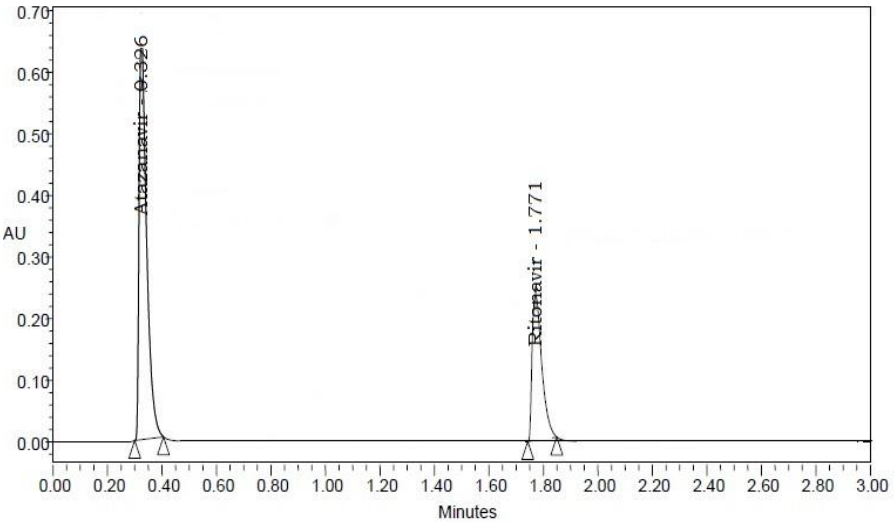

Figure 9: A Chromatogram of Robustness Study at 0.32 mL/min (Flow Rate)

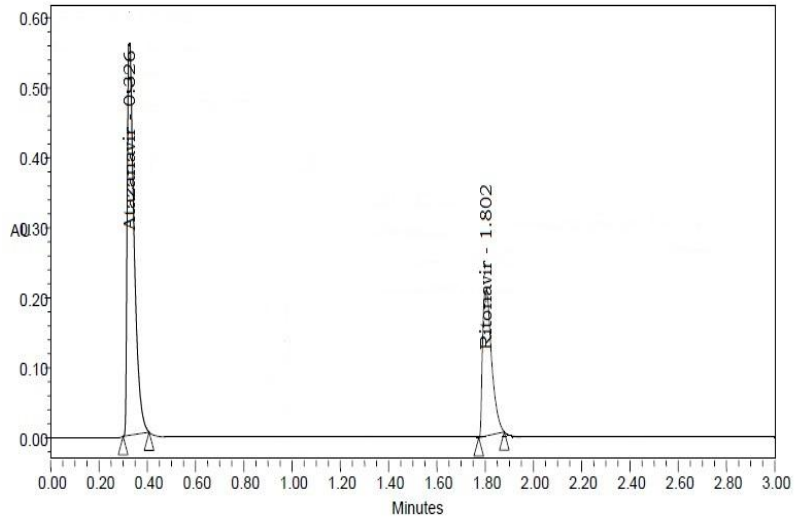

Figure 10: A Chromatogram of Robustness Study at 3.9 (Buffer pH)

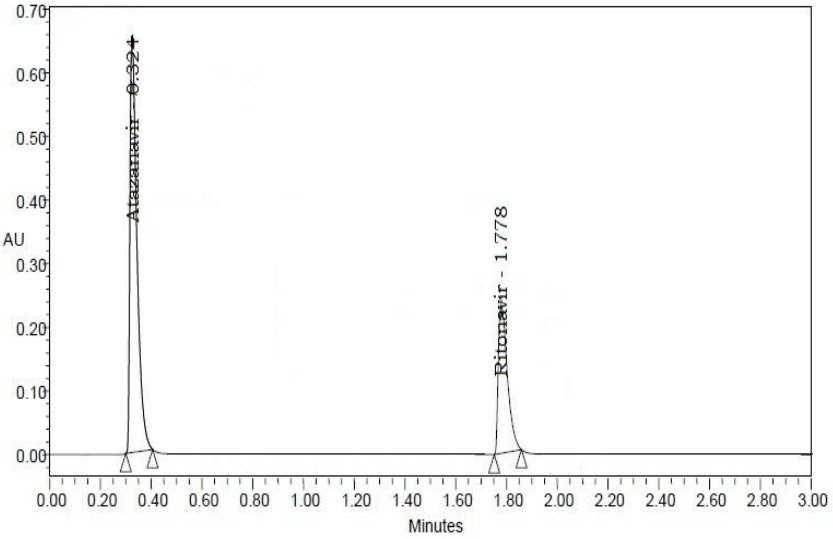

Figure 11: A Chromatogram of Robustness Study at 4.0 (Buffer pH)

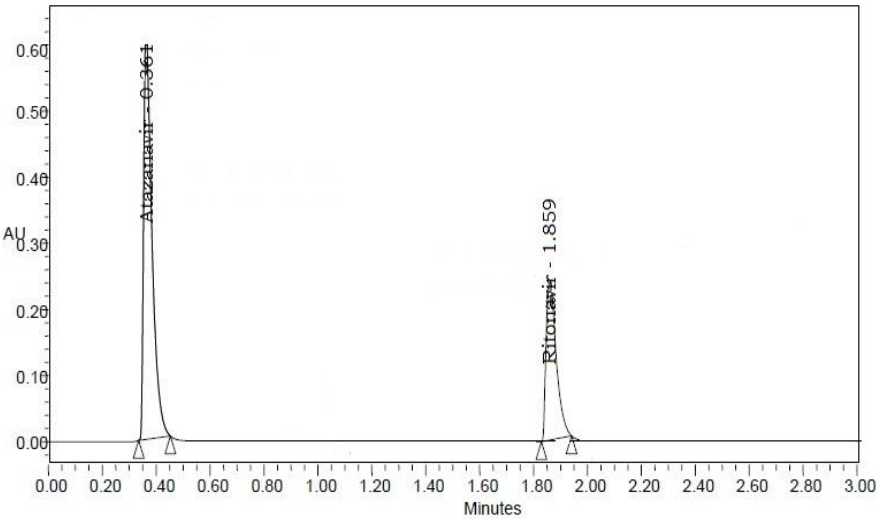

Figure 12: A Chromatogram of Robustness Study at 4.1 (Buffer pH)

Estimation of Atazanavir and Ritonavir in Tablets

The assay results (Table 9) were showed that the developed method was selective for the simultaneous estimation of ATV and RTV without interference of the excipients, which were present in the tablets (Fig. 13).

Table 9: Assay Results of Atazanavir and Ritonavir in Tablets

Brand Name	Analyte	Label claim (mg)	% analyte estimated (Mean ±SD)*	RSD (%)	SEM
Synthivan	ATV	300	100.62±0.702	0.697	0.405
	RTV	100	99.45± 1.079	1.085	0.623

**Replicate of three determinations; SD: Standard Deviation;*
RSD: Relative Standard Deviation; SEM = standard error of mean

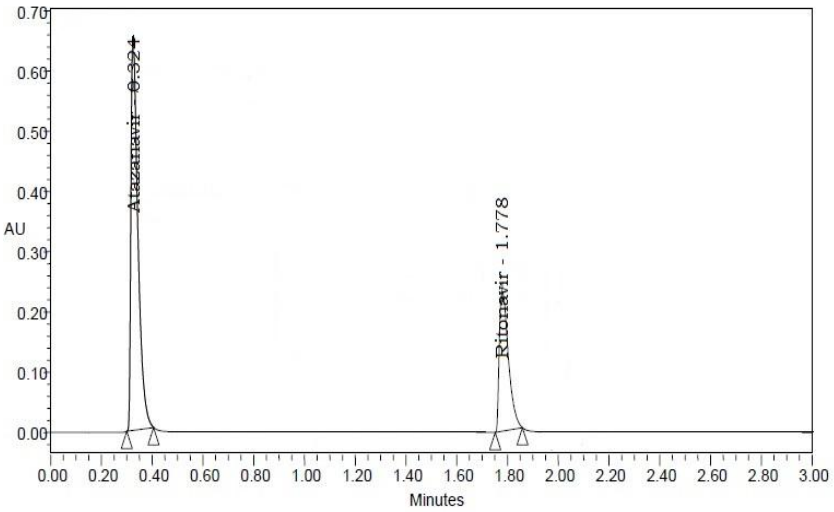

Figure 13: A Sample Chromatogram of ATV and RTV

REFERENCES

1. Dickinson L., Boffito M., Back D., *et al.*, Population pharmacokinetics of ritonavir-boosted atazanavir in HIV-infected patients and healthy volunteers. Antimicrob Chemother 2009; 63(6): 1233-43.
2. King J.R., Kakuda T.N., Paul S., Tse M.M., Acosta E.P., Becker S.L. Pharmacokinetics of saquinavir with atazanavir or low-dose ritonavir administered once daily (ASPIRE I) or twice daily (ASPIRE II) in seronegative volunteers. J Clin Pharmacol 2007; 47(2): 201-8.
3. Chad J.A., Kristin M.D., Robert L.M., Christine K. Atazanavir/ritonavir-based combination antiretroviral therapy for treatment of HIV-1 infection in adults. Future Virol 2011; 6(2): 157-77.
4. Atazanavir. MedlinePlus. National Institutes of Health. October 15, 2012. https://medlineplus.gov/druginfo/meds/a603019.html (Retrieved July 3, 2023).
5. Croom K.F., Dhillon S., Keam S.J. Atazanavir: a review of its use in the management of HIV-1 infection. Drugs 2009; 69(8): 1107-40.
6. Flexner C. HIV-protease inhibitors. New Eng J Med 1998: 338 (18); 1281-92.
7. Molla A., et al., Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat Med 1996; 2 (7): 760-6.
8. Hsu A., Granneman G.R., Bertz R. J. Ritonavir: clinical pharmacokinetics and interactions with other anti-HIV agents. Clinical Pharmacokinetics 1998; 35 (4): 275-91.

9. Cameron D.W. et al., Randomised placebo-controlled trial of ritonavir in advanced HIV-1 disease. The Lancet 1998; 351(9102): 543-9.
10. Laxminarayana B., Nageswara Rao P., Ajitha M., Durga Srinivas L., Rajnarayana K. Simultaneous determination of ritonavir and atazanavir in human plasma by LC-MS/MS and its pharmacokinetic application. Am J Pharm Tech Res 2012; 2(4): 558-71.
11. Xu X.S., Rose A., Demers R., Eley T., Ryan J., Stouffer B., Cojocaru L., Arnold M. Quantitative determination of free/bound atazanavir via high-throughput equilibrium dialysis and LC-MS/MS, and the application in ex-vivo samples. Bio anal 2014; 6(23): 3169-82.
12. Manish Y., Vikas T., Vivek U., Gaurang S., Girin A.B., Sailendra G., Pranav S.S. Comparison of extraction procedures for assessment of matrix effect for selective and reliable determination of atazanavir in human plasma by LC–ESI-MS/MS. J Chromatogr B 2012; 885–886: 138-49.
13. Alagar Raja M., Bhavana, Rao K.N.V., David B., Selva Kumar D. Simultaneous estimation of method development and validation of atazanavir and ritonavir by RP-HPLC method. Asian J Pharm Anal Med Chem 2015; 3(3): 89-99.
14. Sukhadev Pawar D., Manjusha D., Sanjay S., Jyoti M.S. Development and validation of RP-HPLC method for the simultaneous estimation of atazanavir sulphate and ritonavir in bulk and formulations. Int J Pharm Pharm Sci 2013; 5(suppl 3): 905-9.
15. Anusha T., Ashwini G., Annapurna Renee Ch., Aravind Sai, Prasana Laxmi A., Avinash K. Method development and validation for the simultaneous estimation of atazanavir and ritonavir in pharmaceutical dosage form by RP-HPLC. Int J Pharm Chem Biol Sci 2012; 3(1): 44-54.
16. Manoj G., Anil B., Bhanubhai S., Ishwarsinh R., Urvish D., Arpit P., Ketan V. Simultaneous determination of ritonavir and atazanavir in combined tablet dosage form by HPTLC. Asian J Biomed Pharm Sci 2012; 2(15): 15-19.
17. Vasudha B.P., Padmanabh B.D., Ashish G., Sujith S.N. High performance thin layer chromatographic determination of atazanavir and ritonavir in combined tablet dosage form. Der Chem Sinica 2013; 4(5): 21-5.
18. International Conference on Hormonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedure: Text and Methodology {ICH- Q2 (R1)}; November 2015: 1-13.