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RP-HPLC Method Development and Validation for the simultaneous estimation of Atazanavir sulphate and Ritonavir in bulk and tablet dosage form

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Abstract: A simple, robust, precise and accurate reverse phase liquid chromatography method was developed for simultaneous estimation of Atazanavir sulphate and Ritonavir in combined tablet dosage form by RP-HPLC method. Chromatography was carried out on a Nucleodur C_{18} column (150 mm x 4.6 mm x 5µm) using Acetonitrile: Methanol: Phosphate buffer, pH was adjusted to 3.0 with orthophosphoric acid in the ratio of 44:11:45 (V/V) as a mobile phase at a flow rate of 1.5 mL min^{-1} and eluents were monitored at 210 nm. The calibration curves were linear over the range of 34 – 102 µg mL^{-1} for Atazanavir sulphate and 10 – 30 µg mL^{-1} for Ritonavir. The average retention time of Atazanavir sulphate and Ritonavir was found to be 3.133 min and 6.133 min respectively. The method was reproducible with good resolution of Atazanavir sulphate and Ritonavir. The results of the analysis have been validated statistically.

Keywords: Atazanavir Sulphate; Ritonavir; RP-HPLC: Validation; Acetonitrile; Methanol

1. Introduction

Atazanavir sulphate (ATA) is chemically **(Figure 1)** (3S,8S,9S,12S)-3,12-Bis (1,1-dimethylethyl)-8hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)-phenyl]-methyl]-5,6,10,13-penta-aza-tetradecanedioic acid dimethyl ester, sulphate**.** It has antiretroviral activity. Atazanavir sulphate is a protease inhibitor. 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.Ritonavir (RIT) **(Figure 2)** 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]ethyl}) carbamoyl] amino} butanamido]- 1,6-diphenylhexan- 2-yl] carbamate. It has antiretroviral activity. Ritonavir inhibits the HIV viral proteinase enzyme which prevents cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles. Literature survey reveals many analytical methods are available for the determination of Atazanavir sulphate such by UV Spectrophotometry,[1, 2] HPLC,[1, 3-10] HPTLC methods in pharmaceutical preparations.[11] Few analytical methods for the determination of Ritonavir and Atazanavir sulphate such as spectrophotometer, HPLC in pharmaceutical formulation have been reported.

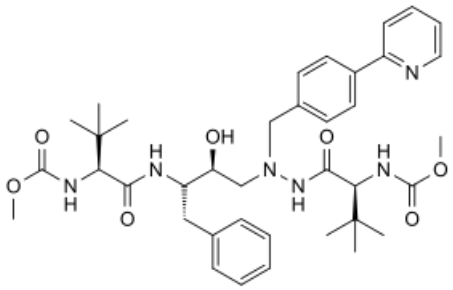

Figure 1. Structure of Atazanavir (ATA)

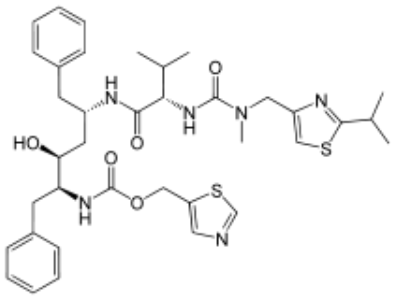

Figure 2. Structure of Ritonavir (RIT)

This paper presents a simple, rapid, reproducible and economical method for the simultaneous estimation of both the drugs in pharmaceutical dosage form using reverse phase HPLC. The proposed method was validated according to ICH guidelines.[12, 13] The method can be successfully employed for the assay of

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Atazanavir sulphate and Ritonavir in bulk and combined dosage form.

2. Results and Discussion

2.1.Method Development

2.1.1.Detection of wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of wavelength. Drug solutions containing 50 µg mL^{-1} of ATA and 50 µg mL^{-1} of RIT was prepared separately in 100 mL volumetric flask and made up the volume with methanol. The above solutions were scanned from 200 nm to 400 nm and their spectra were overlaid. Both the drugs showed significant absorbance at a wavelength of 210 nm so it was selected for further studies. **(Figure 3).**

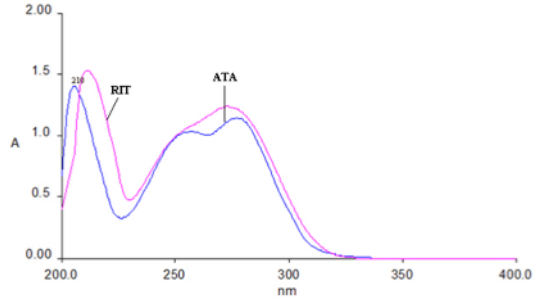

Figure 3. Overlaid spectra of ATA and RIT

2.1.2. Preparation of standard stock solution for ATA & RIT

Standard stock solutions of both the pure drugs containing 6.8 mg & 20 mg of the ATA and RIT respectively was prepared in a 100 mL clean dry volumetric flask using methanol as the solvent. Sufficient amount of methanol was added to dissolve; the contents were sonicated and made up to the volume with methanol. About 2.5, 4, 5, 6 and 7.5 mL of the above solutions were transferred to a separate series of 50 ml of volumetric flasks and made up to volume with mobile phase [(ACT: MeOH: $PB^{3.0}$)$_{44:11:45}$] to obtain a concentration of 34-102 µg mL^{-1} & 10-30 µg mL^{-1} for ATA & RIT respectively (TH_1). The diluted standard solutions with varying concentration were injected in triplicate into the HPLC system separately. Chromatogram peaks of both drugs were recorded at 210 nm using UV detector. The calibration curves were plotted using peak area versus concentration for both the drugs as shown in **Figure 4 & 5.**

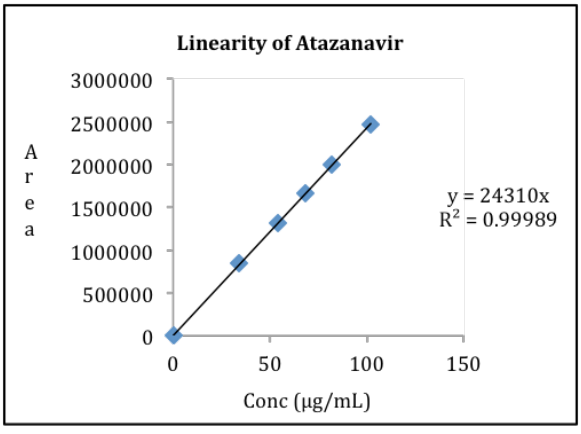

Figure 4. Calibration curve of ATA

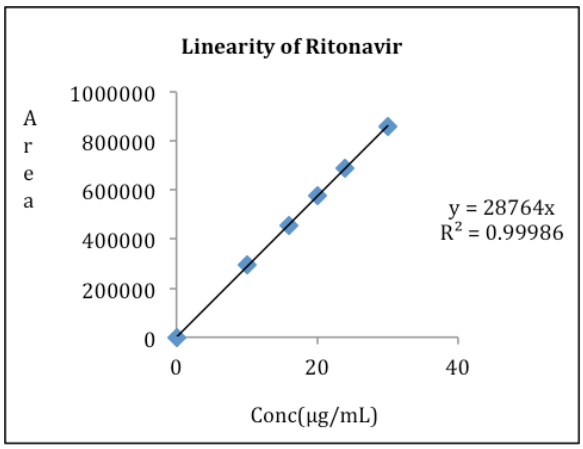

Figure 5. Calibration curve of RIT

2.1.3. Preparation of sample solution containing mixture of ATA & RIT

Twenty tablets (Virataz-r & Atazanavir) were weighed and crushed. Drug powder equivalent to 218 mg of ATA was transferred to 100 mL volumetric flask and dissolved in 70 mL of methanol. It was then Sonicated for 15 min and filtered through 0.45 µm nylon filter. 5 mL of the filtrate was taken into a 50 mL volumetric flask and made up to volume with the mobile phase [(ACT: MeOH: $PB^{3.0}$)$_{44:11:45}$] to get a test solution(TH_2).

2.1.4. Chromatographic method for the determination of ATA and RIT

With the optimized Chromatographic condition a steady baseline was recorded with mobile phase [(ACT: MeOH: $PB^{3.0}$)$_{44:11:45}$] that was followed by the analysis of sample solution. 20 µL of sample solution was injected and the Chromatogram was recorded in triplicate. The retention time of the ATA and RIT were found to be 3.133 and 6.133 min respectively **Figure 6 & 7**. Content of ATA and RIT in tablet was calculated by comparing the mean peak area of the sample with that of the standard. Concentrations of both drugs were calculated as shown in **Table 1.**

Table 1. Analysis of marketed tablet formulation

	Formulations			
	Virataz-r		Atazanavir	
	ATA	RIT	ATA	RIT
LC (mg)	300	100	300	100
AUC	1971418	560338	1971420	560340
RT(min)	3.10	6.07	3.10	6.07
% Est.	99.27 ±0.18	99.26 ±0.13	99.27 ±0.18	99.26 ±0.13

[a]Mean (n=3); [b]Mean±SD (n=3); LC-Label claim; RT-Retention time; %Est.-Percentage estimated

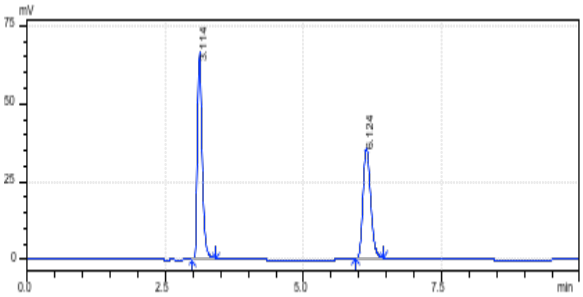

Figure 6. Standard chromatogram of ATA& RIT(TH_1)

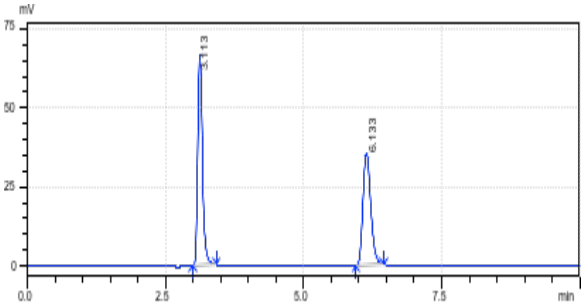

Figure 7. Chromatogram of marketed formulation of ATA & RIT(TH_2)

2.2. Method Validation

2.2.1. Linearity and Range

The linearity of the method was determined at six concentration levels ranging from 34 to 102 µg mL^{-1} for ATA and 10-30 µg mL^{-1} for RIT. The calibration curve was constructed by plotting response factor against concentration of drugs.

2.2.2. Accuracy

To ascertain the accuracy of the proposed method recovery studies were carried out by the standard addition method a known amount of each drug was added to the pre analyzed tablet at three levels 80%, 100% and 120% of the label claim. Recovery studies were carried out in triplicate and the percentage recovery and standard deviation of the percentage recovery was calculated (**Table 2).**

Table 2. Accuracy Study

Acuracy for ATA/ RIT		Amount Spiked (%)		
		80	100	120
Amt added (mg)	ATA	240.59	299.88	361.70
	RIT	79.51	100.04	120.49
Amt received (mg)	ATA	240.31	301.13	360.71
	RIT	79.51	100.29	119.95
[a]% Est.	ATA	100.01 ±0.17	100.40 ±0.31	99.91 ±0.24
	RIT	100.31 ±0.27	100.12 ± 0.61	100.31 ±0.29
% RSD	ATA	0.17	0.31	0.24
	RIT	0.27	0.61	0.29

[a]Mean±SD (n=3)

2.2.3. Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and it is normally expressed as the relative standard deviation for a statistically number of samples. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility. Three repeated injections of standard and sample solutions were made and the response factor of drug peaks and % RSD were calculated (**Table 3 & 4)**.

2.2.4. Limit of Detection and Limit of Quantification:

LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the proposedd RP-HPLC method. LOD and LOQ for both the drugs were calculated by using the values of slopes and intercepts of the calibration curve (**Table 5).**

Table 3. Method of precision

S.No.	ATA		RIT	
	RT	[a]AUC	RT	[a]AUC
1	3.10	1975564	6.03	534547
2	3.10	1976472	6.08	535303
3	3.11	1977694	6.09	533068
Mean		1976577		534306
SD		1068.85		1136.823
% RSD		0.05		0.21

[a]Mean (n=3); RT-Retention time; AUC-Area Under Curve

Table 4. Intermediate precision

Interday Precision data	Inj	ATA		
		RT	[a]AUC	% Est.
Analyst 1	1	3.116	1987964	99.78
	2	3.112	1986984	99.14
	3	3.107	1979854	99.56
	Mean		1984934	99.49
	% RSD		0.22	0.33
Analyst 2	1	3.015	1993291	100.05
	2	3.012	1988365	99.21
	3	3.112	1986281	99.88
	Mean		1989312	100
	% RSD		0.18	0.45
	Inj	**RIT**		
		RT	**[a]AUC**	**% Est.**
Analyst 1	1	6.105	544358	100.42
	2	6.062	546722	100.26
	3	6.047	541098	100.00
	Mean		544059	100
	% RSD		0.52	0.21
Analyst 2	1	6.125	542741	100.12
	2	6.121	543104	99.60
	3	6.101	549121	101.49
	Mean		544989	100
	% RSD		0.66	0.97

[a]Mean±SD (n=3)

Table 5. Limit of Detection and Quantitation

Contents	ATA	RIT
LOD (S/N)	3.1	3.1
LOQ (S/N)	9.9	10.1

2.2.5. Robustness

Robustness of the proposed method was ascertained by deliberately changing the chromatographic conditions such as change in flow rate of the mobile phase (± 0.1 mL min^{-1}), change in composition of the mobile phase. Effect of change in chromatographic parameters on resolution and tailing factor of peak was studied. The condition with variations was shown in table **(Table 6).**

2.2.6. System suitability studies:

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions. The values obtained demonstrated the suitability of the system for the analysis of these drug combinations. System suitability parameters may fall within ± 2 % standard deviation range during routine performance of the method **(Table 7, Figure 6).**

Table 6. Robustness study

Flow rate (mL^{-1}) Peak area				
S. No.	**ATA**		**RIT**	
	1.2 mL	**1.4 mL**	**1.2 mL**	**1.4 mL**
1	1995819	1982858	542654	549370
2	1972906	1983273	543459	542393
3	1984263	1982066	543256	546182
Mean	1984329	1982732	543123	545982
SD	11457	613	419	3493
% RSD	0.58	0.03	0.08	0.64

Mobile phase Peak area				
S. No.	**ATA**		**RIT**	
	50:10:40	**45:11:44**	**50:10:40**	**45:11:44**
1	1982858	1979925	549370	543884
2	1983273	1983419	542393	549614
3	1983466	1983672	545982	546849
Mean	1983199	1982339	545915	546782
SD	311	2094	3489	2866
% RSD	0.02	0.11	0.64	0.52

Table 7. System Suitability Parameters

S. No.	Parameters	ATA	RIT
1	Theoretical plates	6336	7379
2	Symmetric factor	1.05	1.04
3	Tailing factor	1.15	

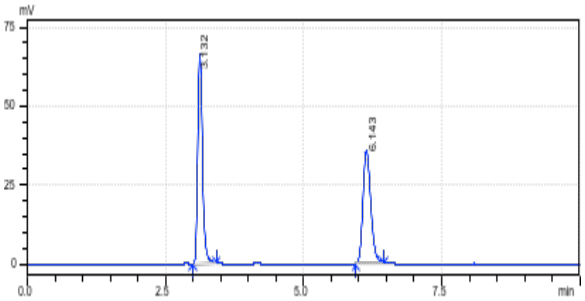

Figure 8. Chromatogram of System Suitability of Atazanavir sulphate and Ritonavir

3. Experimental

Materials & Methods: Standard samples of ATA and RIT were procured from Aurobindo Pharma Pvt. Ltd, Hyderabad, India. The marketed formulation virataz-r& Atazanavir contaning300 mg of ATA and 100 mg RIT fixed dose tablet were procured from local market. HPLC grade water, Acetonitrile and methanol were purchased from Merck, India. Weighing of samples was performed on secura 224-1S Analytical balance Sartorius, USA. Degassing of mobile phase was performed using Branson 1510MTH ultrasonic cleaner, Balkowitsch Enterprises Inc., USA. The Ph of the mobile phase was checked and adjusted using pH-meter (Elchem model). All chromatographic analyses were performed on HP Agilent 1100 (Agilent Technologies, USA) machine with Nucleodur C_{18} column XBD column (AgilentTehnologies, USA). Details of software used for chromatographic analysis was Empower 2, CDS (waters, USA) .Excel 2007 (Microsoft Office 2007, Microsoft) was used for plotting calibration curves.

3.1. Method Development

3.1.1. Chromatographic conditions

Column: Nucleodur C_{18} column(150x4.6 mm: 5µm)
Mobile phase: $[(ACT: MeOH: PB^{3.0})_{44:11:45}]$
Diluents: HPLC grade methanol
Flow rate: 1.5 mL min^{-1}
Detector wavelength: 210 nm
Injection mode: Auto injector (vial)
Injection volume: 20 µL

3.1.2. Preparation of buffer ($PB^{3.0}$)

Phosphate buffer was prepared by dissolving 6.8 gm of potassium dihydrogen orthophosphate in 50 mL of HPLC water pH 3.0 was adjusted with ortho phosphoric acid and volume made up to 1000 mL with HPLC water.

3.1.3. Preparation of Mobile Phase [$(ACT: MeOH: PB^{3.0})_{44:11:45}$]

The mobile phase consisting of Acetonitrile: Methanol: Phosphate buffer in the ratio of (44:11:45) was found to resolve ATA and RIT. The mobile phase was filtered through 0.45 µm membrane filter ultra sonicated for 15 min and degassed. The flow rate was set to 1.5 mL min^{-1} and UV detection was carried out at 210 nm.

3.2. Method Validation

3.2.1. Specificity

It is evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference of any peak at the retention time of ATA and RIT respectively.

3.2.2. Linearity and Range

ATA and RIT standard stock solution was transferred to volumetric flask of 50 mL capacity. The volume was adjusted with methanol to obtain concentration of 34 to 102 µg mL^{-1} and 10 to 30 µg mL^{-1} of ATA &RIT respectively. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

3.2.3. Method of precision

The repeatability was evaluated by assaying three times of sample solution prepared as per the test solution. The intraday and intraday precision study of ATA and RIT was carried out at different concentrations of ATA (34, 54,102 µg mL^{-1}) and RIT (10, 16, 30 µg mL^{-1}).

3.2.4. Accuracy

Accuracy was assessed by standard addition method. The standard drug was spiked to the pre-quantified placebo preparation at three different concentration levels 80, 100 and 120 % taking into consideration the percentage purity of added bulk drug samples. Each concentration was analyzed three times and average recoveries were calculated.

3.2.5. Intermediate precision (Ruggedness)

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e., different analysts, laboratories, instruments, reagents, assay temperatures,

variations in mobile phase and different days etc. (i.e. from laboratory to laboratory, from analyst to analyst). Acceptance criteria for ruggedness the % RSD for the three standard injections should not be more than 2%.

3.2.6. Robustness

As part of robustness, deliberate change in the flow rate and mobile phase composition was made to evaluate the impact on the method. The mixed standard solution is injected in three replicates and % RSD was calculated.

3.2.7. LOD and LOQ

LOD is the smallest concentration of the analyte that gives measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified signal to noise ratio.

Abbreviations

ACN-Acetonitrile; MeOH-Methanol; PB-Phosphate Buffer; ATA- Atazanavir sulphate; RIT-Ritonavir.

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