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Development and Validation of a Simple, Sensitive and Selective RP-HPLC Method for the Quantitative Determination of Ritonavir in Bulk and Pharmaceutical Formulation

Jyothsna Modugula*[1], N.Kumar D.B[2], Marry Prasanthi Maram[3],
Venkata Seshagiri Podili[4], krishnaveni kamepalli[5].
[1]*Department of pharmaceutical Analysis, SIMS College of Pharmacy, Mangaldas nagar-522001, Guntur, Andhra Pradesh, India.*
[2]*Junior Manager, Syngene International Limited, Bommasandra-Jigani link road, Bangalore-560099.*
[3]*Masters of public health, La Trobe University, City campus, 360 Collins Street, Melbourne, Victoria-3000, Australia.*
[4]*Department of Pharmacology, St. Xavier Institute of Pharmacy, Phirangipuram, Guntur, Andhra Pradesh, India.*
[5]*Department of Pharmaceutical Chemistry, St. Xavier Institute of Pharmacy, Phirangipuram, Guntur, Andhra Pradesh, India.*
Email id: jyothsna.pharma10@gmail.com

Abstract: A simple, economic, selective and precise RP-HPLC method has been developed and validated for Ritonavir in bulk and Pharmaceutical formulation. The isocratic LC analysis was performed on Inertsil BDS C18 column (250 mm x 4.6 mm, 5 µ) using mobile phase composed of Methanol and Acetate buffer pH 4.0 (55:45 v/v) at a flow rate of 1.0 ml/min. Quantitation was performed using UV detector at 261 nm. The retention time was found to be 4.521min. The analytical method was validated according to ICH guidelines. The linearity was observed in the range of 20-60 µg/ml with correlation coefficient (r^2) is 0.999 for Ritonavir. The % recovery was found to be 100.10 - 100.71 %. The relative standard deviation values for repeatability and intermediate precision studies were less than 2%. The proposed method was precise, rapid, accurate, and cost-effective and can be used for the routine estimation for Ritonavir in bulk and pharmaceutical formulation.
Index Terms: Ritonavir, ICH guidelines, pharmaceutical formulation, RP-HPLC

1. INTRODUCTION

Ritonavir belongs to the class of organic compounds known as n-carbamoyl-alpha aminoacids and derivatives. These are compounds containing an alpha amino acid (or a derivative thereof) which bears and carbamoyl group at its terminal nitrogen atom [1].

Ritonavir structure- activity studies states that it is a protease inhibitor with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Protease inhibitors block the part of HIV called protease [2].

HIV-1 protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV-1[1]. Ritonavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. Protease inhibitors are almost always used in combination with at least two other anti-HIV drugs. [3]

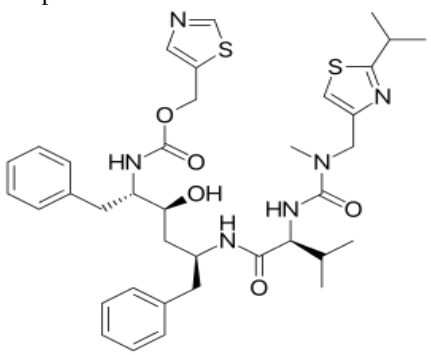

Fig 1. Structure of Ritonavir

2. MATERIALS AND METHODS

2.1. Instruments

- HPLC –Waters Model NO.2690/5 series Compact System Consisting of Inertsil-C18 BDS column.
- Electronic balance (SARTORIOUS)
- Sonicator(FAST CLEAN)

2.2. Chemicals

- Methanol HPLC Grade.
- Sodium acetate, ammonium acetate and Glacial acetic acid were Analytical reagent grade.

2.3. Raw Material

Ritonavir and Lopinavir Working Standards obtained from local market.

2.4. Preparation of Solutions

2.4.1. Mobile Phase

Degassed Methanol and Acetate buffer pH 4.4 in the ratio of 55:45 V/V.

2.4.2. Preparation of Acetate buffer solution pH 4.4

Dissolve 136 g of sodium acetate and 77 g of ammonium acetate in water and dilute to 1000.0 mL with the same solvent; add 250.0 mL of glacial acetic acid and mix.

2.4.3. Preparation of standard solution

The solution was prepared by dissolving 25.0 mg of accurately weighed Ritonavir in 100.0 mL of volumetric flask and add mobile phase then sonicate for 20min. From the above solutions take 10.0 mL of each solution into a 50.0 mL volumetric flask and then make up with mobile phase. The stock solutions equivalent to 50µg was prepared and filtered through 0.45µ membrane.

2.4.4. Preparation of sample drug solution for pharmaceutical formulations

Twenty tablets were weighed accurately and a quantity of tablet powder equivalent to 25 mg was weighed and dissolved in the 30 mL mobile phase and sonicate for 10 min. The content was diluted to 100 mL with mobile phase. This solution was filtered through a 0.45 µm Nylon syringe filter and 10.0 mL of the filtrate was diluted into a 50.0 mL volumetric flask to give a test solution containing 50 µg/mL of Ritonavir.

2.5. Detection of Wavelength maximum

The sensitivity of method depends on UV detection and the proper selection of wavelength is that gives maximum absorbance and good response for the given drug. In setting up the conditions for development of the assay method, the choice of the detection wavelength was based on the scanned absorption spectrum. The UV-spectrum of Ritonavir was scanned in the wavelength range of 200-400 nm against blank. From the spectrum 261nm wavelength was selected for the analysis**.**

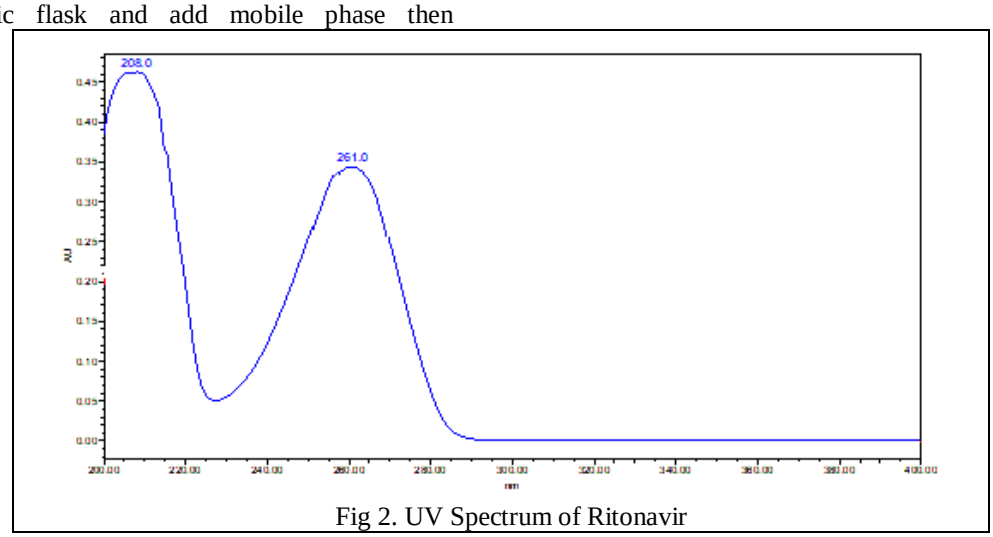

Fig 2. UV Spectrum of Ritonavir

2.6. Optimized Chromatographic conditions

Parameters	**Method**
Stationary phase (column)	Inertsil -BDS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : Acetate Buffer pH 4 (55:45)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	8 min
Column temperature (°C)	Ambient

Volume of injection loop (µl)	20
Detection wavelength (nm)	261nm
Drug RT (min)	4.521min

3. RESULTS AND DISCUSSION

3.1. Method Development

Trials were done to develop a RP-HPLC method which was able to separate and quantify the Ritonavir in short time with an adequate sensitivity and selectivity. So as to achieve the good peak symmetry, various parameters like choice of mobile phase, its composition, flow rate and detection wavelength were considered during optimization of method. During trials with different columns, it was observed that Inertsil-BDS C_{18} (250 x 4.6 mm, 5 µ) gave good results (good symmetric and sharp peak) [4,5]. Hence the same column is used in the analysis. Different mobile phases in different ratios, different flow rate and different pH were tried. Finally methanol and Acetate buffer PH 4.0 in the ratio of (55:45v/v) with a flow rate of 1 ml/min was selected as mobile phase and these chromatographic conditions provided less analysis time, good peak response and good peak symmetric[6,7]. The sensitivity of the method was good at a wavelength of 261 nm. Therefore the same wavelength was selected as analytical wavelength. The chromatogram of Ritonavir after optimization is given in fig. 3.

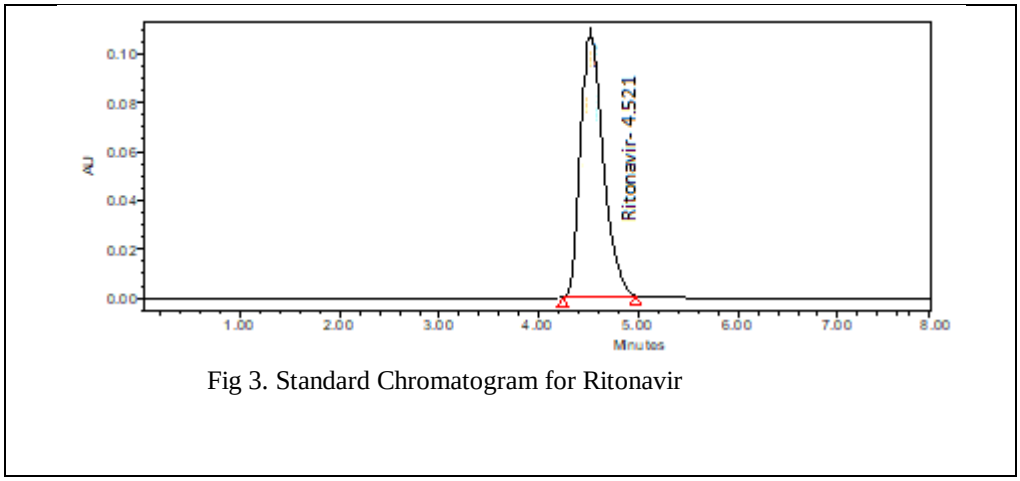

Fig 3. Standard Chromatogram for Ritonavir

3.2. Method validation

The validity of method was tested by determining the validation parameters according to the Instructions given by ICH guidelines for method validation. [8,9]

3.2.1. System suitability:

A Standard solution was prepared by using Ritonavir working standard as per test method and was injected Five times into the HPLC system

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD retention times and peak areas for five replicate injections of Ritonavir.

Table 1. System Suitability parameters for Ritonavir

Parameters	**Ritonavir**	**Recommended limits**
Retention time	4.521	RSD≤ 2
Peak area	1027021	RSD≤ 2
% RSD	0.26	< 2
USP plate count	7541.210	> 3000
USP tailing factor	1.176	≤ 2

3.2.2. Specificity:
Specificity was carried out to determine the interference of any other peaks like impurity peaks or reagent peaks etc. at the retention time of analytical peak. Solutions of standard and samples were prepared as per the test method and are injected into chromatographic systems. The chromatogram of Standard and Sample shows identical retention time and blank injection was performed and there is no interference of blank reagents at the retention time of sample as shown in fig 4-6.

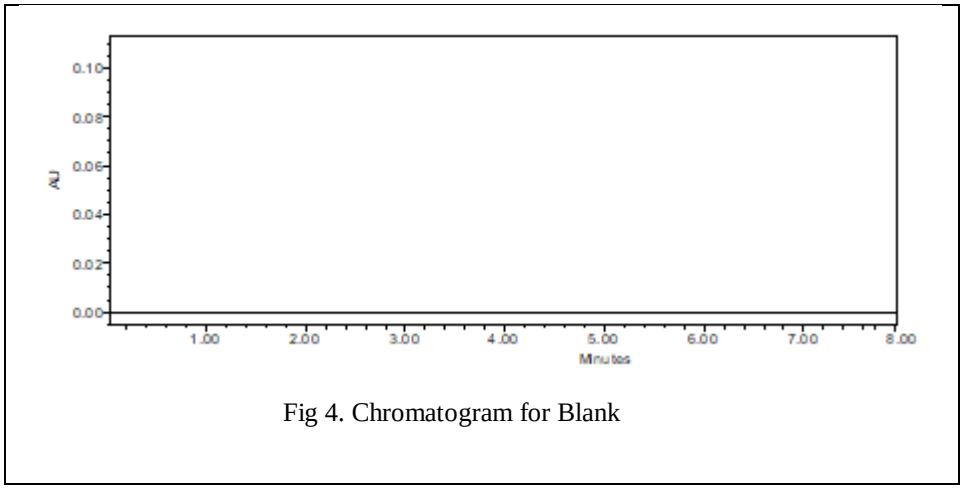

Fig 4. Chromatogram for Blank

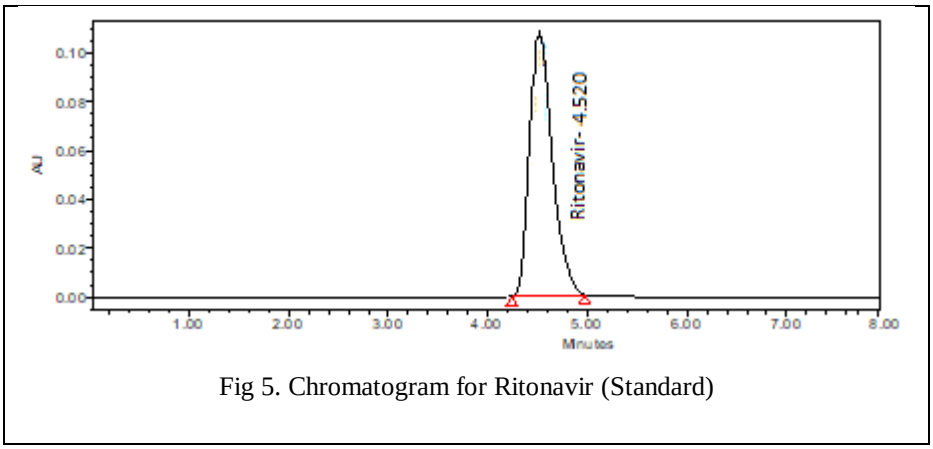

Fig 5. Chromatogram for Ritonavir (Standard)

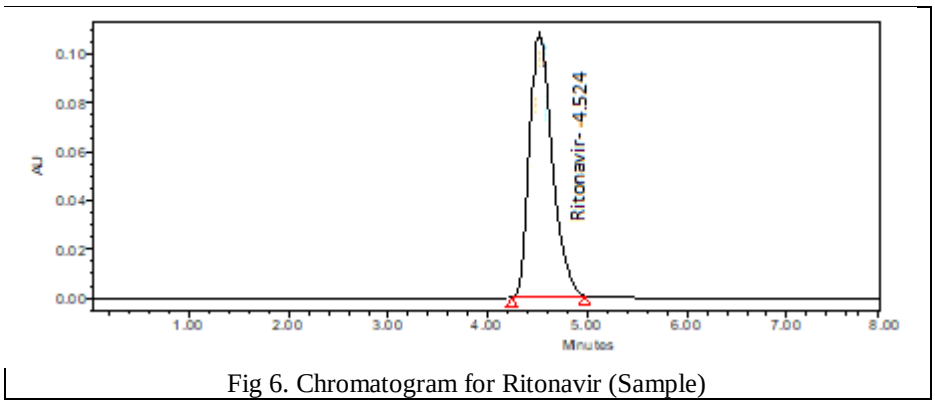

Fig 6. Chromatogram for Ritonavir (Sample)

3.2.3. Precision
Precision is the degree of agreement among individual results. The entire procedure should be applied repeatedly to separate, identical samples drawn from the same homogeneous batch of material. [10]

a. System precision: Standard solution was prepared as per the test method and the

same homogenous sample was injected for five times. Measure the peak area and % assay for each injection. %RSD for peak area and % assay was found to be 0.225 and 0.45 respectively. The chromatograms were shown in fig 7-11.

b. Method precision: Prepared five sample preparations individually as per test method and injected each solution. Measure the peak area and % assay for each injection. %RSD for peak area and % assay was found to be 0.18 and 0.51 respectively Test results are showing that the test method is precise. Refer tables 2 and 3 for system precision and for method precision.

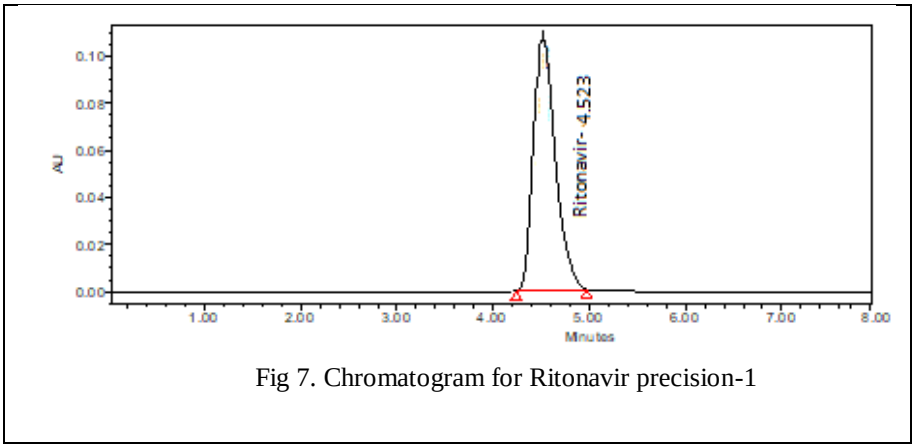

Fig 7. Chromatogram for Ritonavir precision-1

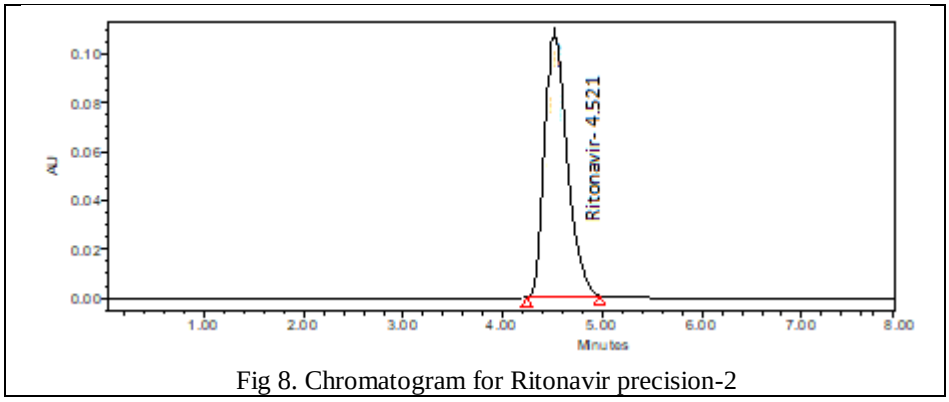

Fig 8. Chromatogram for Ritonavir precision-2

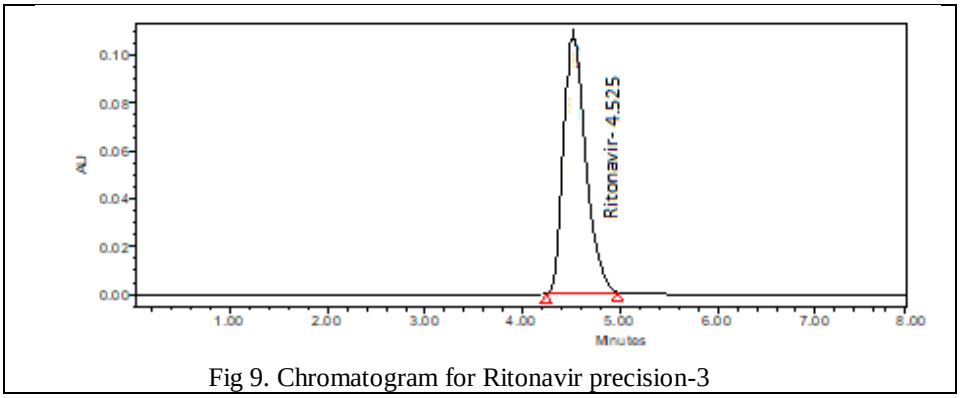

Fig 9. Chromatogram for Ritonavir precision-3

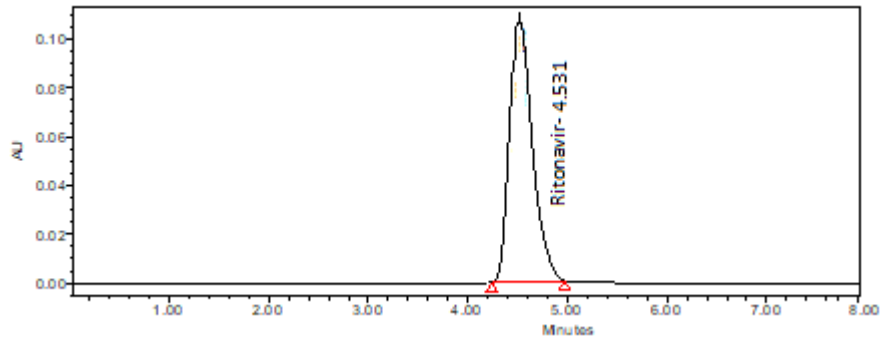

Fig 10. Chromatogram for Ritonavir precision-4

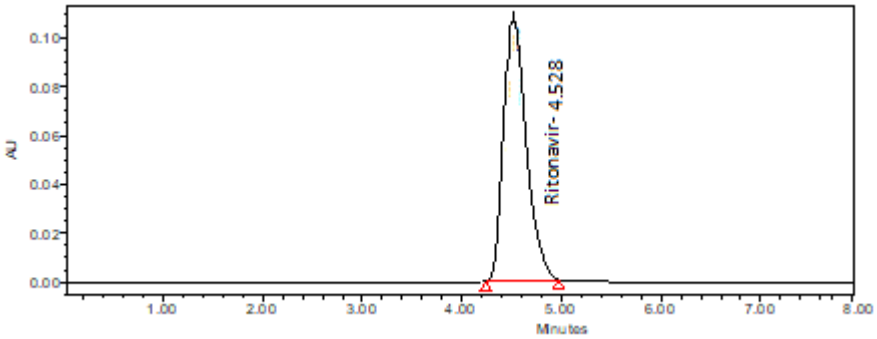

Fig 11. Chromatogram for Ritonavir precision-5

Table 2. Results for System precision

S.No	**Peak area**	**% Assay**
1	1027021	99.52
2	1022421	100.12
3	1027542	100.52
4	1025120	99.65
5	1028124	99.47
Mean	1026046	99.86
SD	2318.665	0.451365
%RSD	0.225	0.45

Table 3. Results for Method precision

S.No	Peak area	% Assay
1	1026721	99.82
2	1025427	100.78
3	1028514	99.52
4	1026592	99.91
5	1023522	100.47
Mean	1026155	100.1
SD	1839.761	0.512396
%RSD	0.18	0.51

3.2.3.1. Intermediate precision (analyst to analyst variability)

Intermediate precision is also known as ruggedness. This was performed by two analysts as per test method. The standard solution was injected for five times and the individual assays were calculated for both Ritonavir and Lopinavir. It should be not less than 98% and not more than 102% and %RSD of assays should be NMT2.0% by both analysts. Individual % assays and %RSD of assay are calculated and tabulated in table: 4

Table 4. Results for Intermediate precision

S.No	Peak area	% Assay
1	1031721	100.82
2	1029447	100.18
3	1028554	99.67
4	1025214	99.95
5	1028522	100.17
Mean	1028692	100.16
SD	2339.3	0.424347
%RSD	0.23	0.42

3.2.4. Accuracy (Recovery)

Accuracy of the method was performed by using standard addition method. This method was conducted by prepared triplicate solutions as per the test method with equivalent amount of Ritonavir and spike the solution to get the concentration equivalent to 50%, 100%, and 150% of the labelled amount as per the test method. The average % recovery was calculated and tabulated in Table-5 and the chromatograms were shown in figure 12-14.

Table 5. Results for Accuracy

S No.	Concentration (%)	Original level (µg/ml)	Amount added (µg/ml)	% Recovery	Mean % Recovery	%RSD
1	50	25	25.24	100.96	100.21	0.65
2	50	25	24.98	99.92		
3	50	25	24.94	99.76		
4	100	50	50.58	101.16	100.71	0.65
5	100	50	50.51	101.02		
6	100	50	49.98	99.96		
7	150	75	75.22	100.29	100.10	0.26
8	150	75	74.86	99.81		
9	150	75	75.16	100.21		

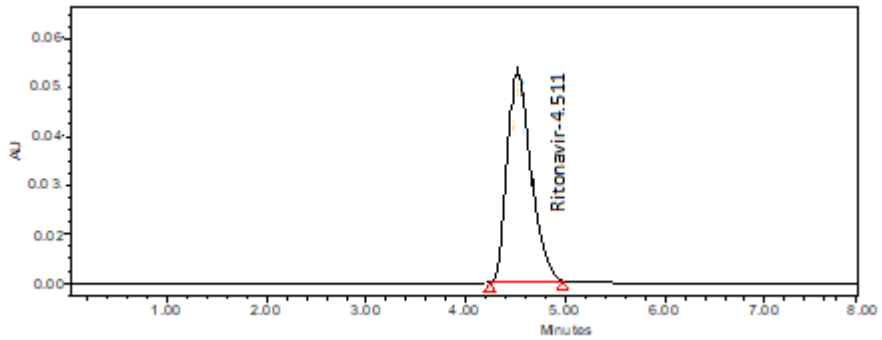

Fig 12. Chromatogram for accuracy-50%

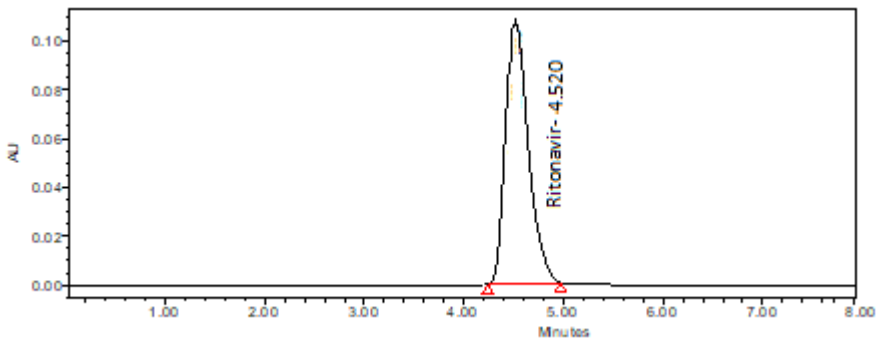

Fig 13. Chromatogram for accuracy-100%

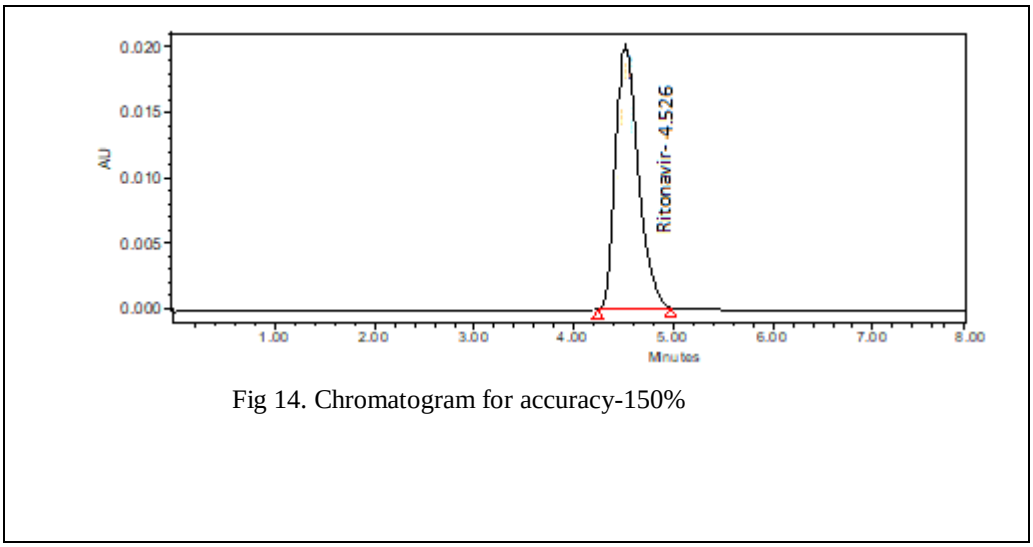

Fig 14. Chromatogram for accuracy-150%

3.2.5. Linearity

Linearity was performed by preparing a series of concentrations (20ppm to 60ppm) by using Ritonavir working standard. Each concentration was injected into chromatographic system and measures the peak area response of solutions. The graph was plotted by taking concentration of the drug on x-axis and peak area on y-axis. The correlation coefficient was found to be 0.999 which shows an excellent correlation existed between absorbance and concentration of the drug within the concentration range tested. The linearity plot was shown in the fig 15-20 and the results were tabulated in Table-6

Table 6. Results for Linearity

Concentration (µg)	**Average Area**	**Statistical Analysis**	
20	413140		
30	604283		
40	815547	Slope y-Intercept Correlation Coefficient	20278 1519 0.999
50	1011252		
60	1220522		

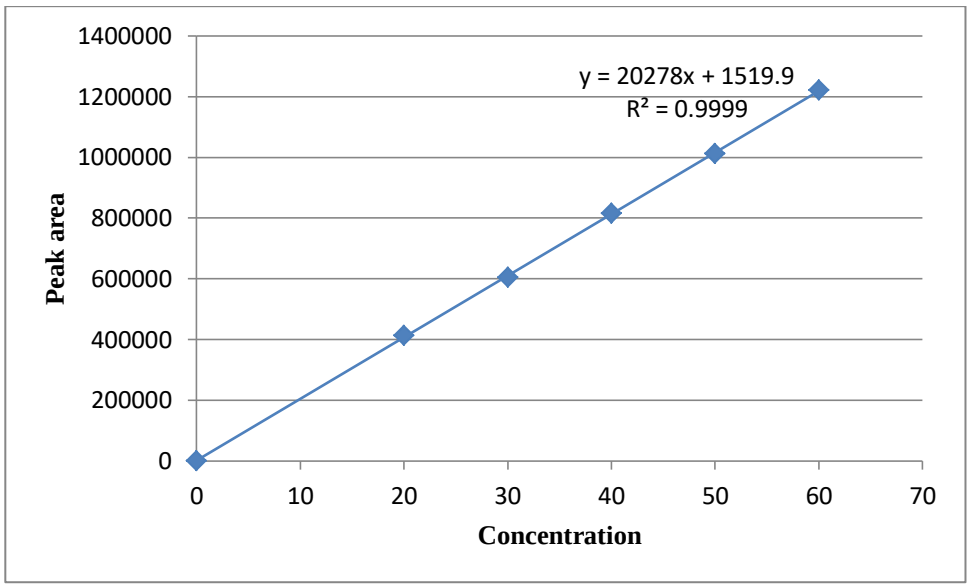

Fig 15. Linearity plot for Ritonavir

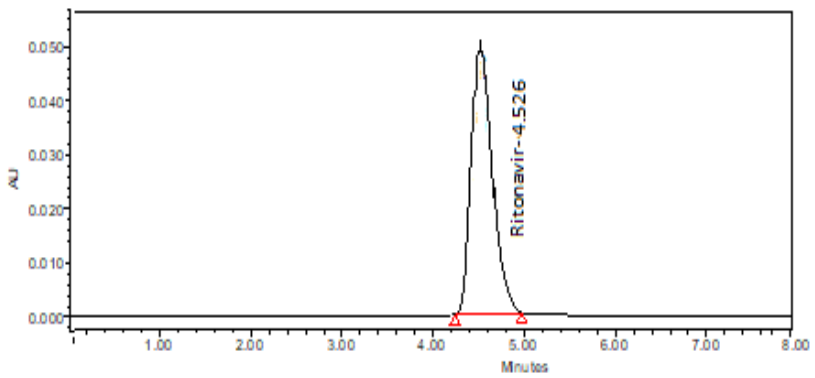

Fig 16. Linearity chromatogram for 20 µg

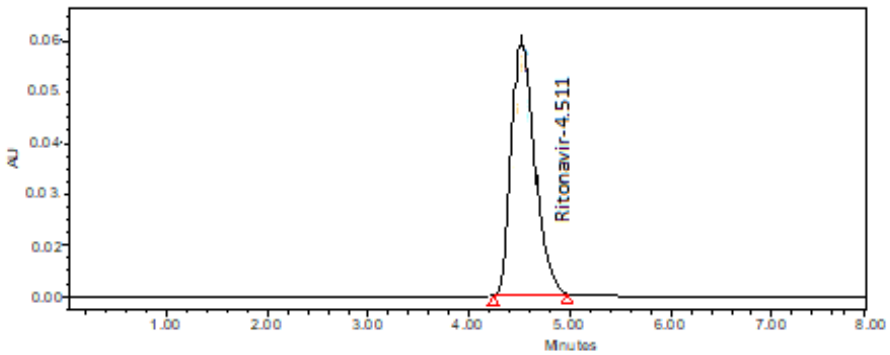

Fig 17: Linearity chromatogram for 30 µg

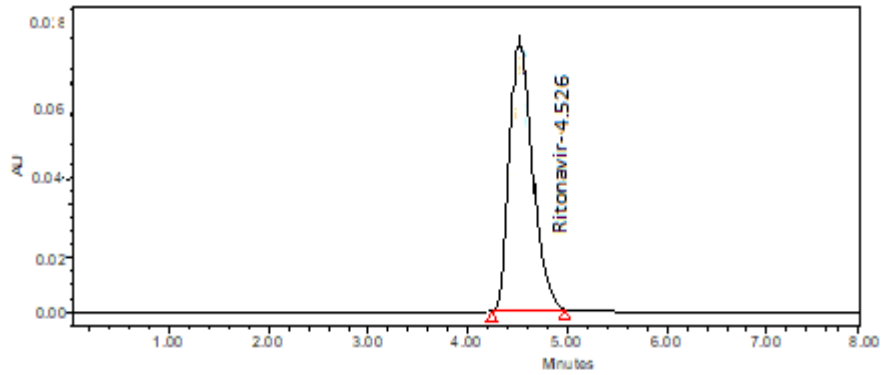

Fig 18: Linearity chromatogram for 40 µg

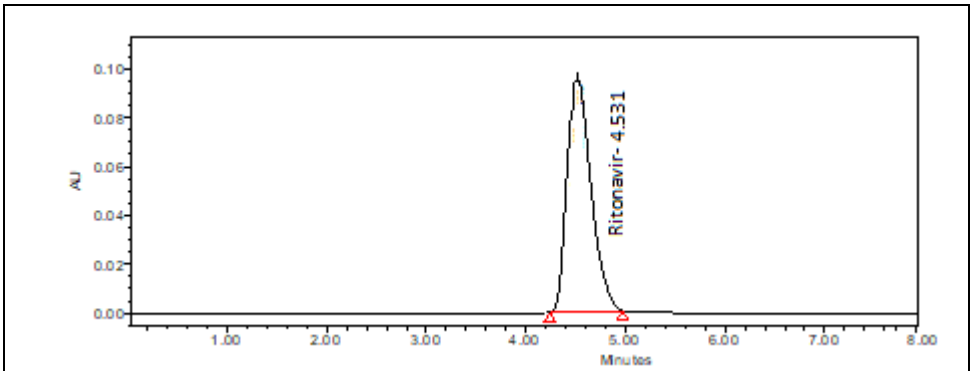

Fig 19: Linearity chromatogram for 50 µg

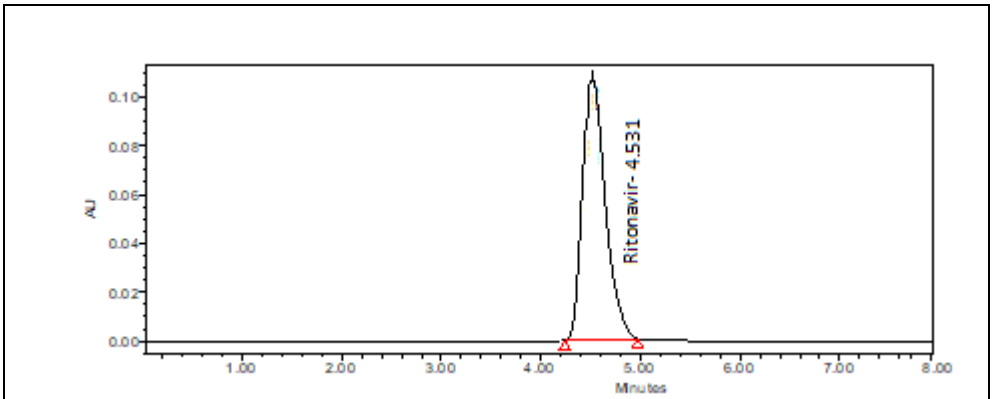

Fig 20: Linearity chromatogram for 60 µg

3.2.6. Robustness:

3.2.6.1. Effect of variation of flow rate

As a part of robustness this study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into the HPLC system by varying the flow rates at 0.8ml/min and 1.2ml/min. The system suitability parameters were evaluated and found to be within the limits for 0.8ml/min and 1.2ml/min flow and the mean retention time and mean area was calculated and tabulated in Table-7.

3.2.6.2. Effect of variation of temperature

A study was conducted to determine the effect of variation in temperature on the developed method. The sample solution was prepared and injected at 25º±2C temperature and the retention times were comparable and the results were tabulated in Table-7.

Table 7. Results for Robustness

Parameter	**Adjusted to**	**System suitability parameters**		
		Mean area	**Mean RT**	**%RSD**
Ritonavir				
Flow rate(ml/min)	1.0-0.2	1029447	4.514	0.81
	1.0+0.2	1023512	4.421	0.35
Temperature (ºC)	25+2.0	1022544	4.152	0.48
	25-2.0	1023654	4.418	0.56

4. SUMMARY AND CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Valsartan and Hydrochlorothiazide in bulk and Pharmaceutical dosage forms.

The advantage of the developed method lies in the simplicity of the solution preparation and the method was accurate (100.10 - 100.71 %.), precise (%RSD=0.45), linear (20 to 60µg/ml) and the correlation coefficient was found to be 0.999. The method is also robust with the deliberate changes of flow rate and temperature will not give any significant changes. For the development of this method less number of reagents was used and the retention time is about 4.521 min so the time taken for the analysis also less. From the above results it was clearly shows that all the validation parameters were within the limits.

It can be concluded that the proposed method can be used for the routine analysis for the RP-HPLC method development and validation for the estimation of Valsartan and Hydrochlorothiazide in bulk and Pharmaceutical dosage forms

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