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DEVELOPMENT OF RRRS DIASTEREOMER IN 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]BUTANOYL]AMINO]-1,6-DIPHENYLHEXAN-2-YL]CARBAMATE BY HPLC

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Abstract:

Ritonavir is an antiretroviral protease inhibitor that is widely used in combination with other protease inhibitors in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). Ritonavir can cause transient and usually asymptomatic elevations in serum aminotransferase levels and, rarely, can lead to clinically apparent acute liver injury. In HBV or HCV coinfected patients, highly active antiretroviral therapy with ritonavir may result of an exacerbation of the underlying chronic hepatitis B or C. Here, a reverse phase high performance liquid chromatographic method has been developed for the validation of RRRS Diastereomer in Ritonavir API form.[1] Chromatography was carried out on a Gemini C18, 150 x 4.6 mm, 5.0 µm column using a mixture of methanol: water (50:50 v/v) as the mobile phase at a flow rate of 0.8 mL/min, the detection was carried out at 240 nm and the retention time of the RRRS Diastereomer was found to be 18.3 and Ritonavir was found to be 16.1. The method produce linear responses in the concentration range of 25-50 µg/mL of Ritonavir. The method precision for the determination of assay was below 4.48 % RSD. The LOD and LOQ values obtained were 0.006µg/mL and 0.018µg/mL respectively.[2] There were no significant changes observed upon changing chromatographic conditions indicating the method to be rugged, robust, precise and linear. Therefore this validated method can be useful in the quality control of bulk of Ritonavir.[3]

Keywords: RRRS Diastereomer, Ritonavir, HPLC, LOD, LOQ and Retention time.

Introduction: The RRRS Diastereomer is isomeric compound. Hence, Diastereomers differ in most, if not all, physical and chemical properties; in fact diastereomers tend to be as different from each other as many constitutional isomers. The basic reason for this difference is that enantiomers are "isometric"; that is, for each distance between two given atoms (whether bonded or not) in one isomer there is corresponding identical distance in the other. No such "isometry" exists in diastereomers or in constitutional isomers by HPLC.

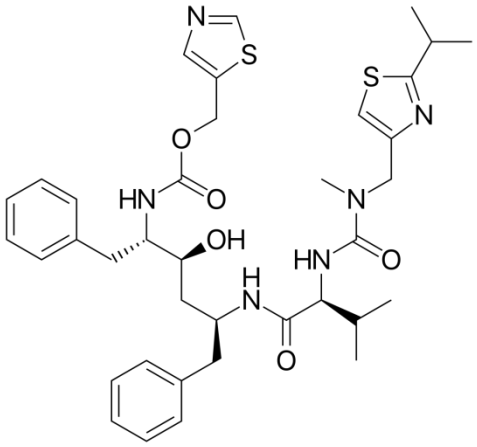


Fig.1: Ritonavir.

Material and Methods:

RELATED COMPOUNDS BY HPLC:

Method of Analysis:

Chemicals:

Orthophosphoric acid (85%) : Merck HPLC grade or equivalent

Potassium hydroxide : Merck or equivalent

Acetonitrile : HPLC grade or equivalent

Methanol : HPLC grade or equivalent

Water : Milli 'Q'.

Chromatographic Conditions:

Column : Gemini C18, 150 x 4.6 mm, 5.0 µm

Flow rate : 0.8 mL/minute

Detector Wavelength : 240 nm

Injection volume : 10 µL

Column temperature : 35^{o}C

Run time : 35 minutes

Mode : Gradient

Diluent : Prepare a mixture of Methanol and Water in the ratio of 50:50 (v/v)

Preparation of Buffer:

Dissolve 1.0 mL of Orthophosphoric acid in 1000 mL of water and mix.

Adjust the pH of this solution 3.00 $\pm$ 0.05 with dilute potassium hydroxide solution.

Filter and degas through the 0.45 µm filter paper.

Preparation of Solvent-A: Use buffer.

Preparation of Solvent-B:

Prepare a mixture of Acetonitrile and Methanol in the ratio of 50:50 (v/v).

Filter and degas through the 0.45 µm filter paper.

Table-1: Gradient Programme:

Time (Minutes)	Solvent-A (%)	Solvent-B (%)
0.01	40	60
20.0	30	70
25.0	30	70
28.0	5	95
30.0	40	60
35.0	40	60

Preparation of Standard stock solution:

Weigh accurately about 5.0 mg of RRRS Diastereomer standard into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix. Dilute 3.2 mL of this solution to 20 mL with diluent.

Preparation of Standard solution: Dilute 2.0 mL of standard stock solution to 20 mL with diluent.

Preparation of Test solution: Weigh accurately about 20.0 mg of test sample into 10 mL volumetric flask, dissolve and dilute to the volume with diluent.

Procedure: Equilibrate / Condition the column for at least 35 minutes. Inject diluent as blank solution into the system and record the chromatogram. Program the data processor to inhibit the peaks due to blank solution and

perform blank correction if necessary. Inject the Standard solution into the system for six replicates and record the chromatograms. Check for the system suitability acceptance criteria, if met the requirements and proceed further.

System suitability acceptance criteria:

The % RSD for the area of RRRS Diastereomer peak obtained from six replicate injections of standard solution should be not more than 10.0. The tailing factor for RRRS Diastereomer obtained from standard solution should be not more than 2.0. The number of theoretical plates for RRRS Diastereomer obtained from standard solution should be not less than 3000. Inject test solution into the system and record the chromatogram. Calculate the % of RRRS Diastereomer in test solution by using the following formula and report the result.

Table-2: Sample Information

S. No.	Name of the compound	~ RT	RRT
01	Ritonavir	16.1	1.00
02	RRRS Diastereomer	18.3	1.13

Validation Parameters & Procedure:

The following parameters should be considered for the validation.

System suitability/System precision:

Preparation of Standard stock solution: Weigh accurately about 5.0 mg of RRRS Diastereomer standard into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Standard solution: Dilute 2.0 mL of standard stock solution to 20 mL with diluent.

Table-3: System suitability / System Precision Results:

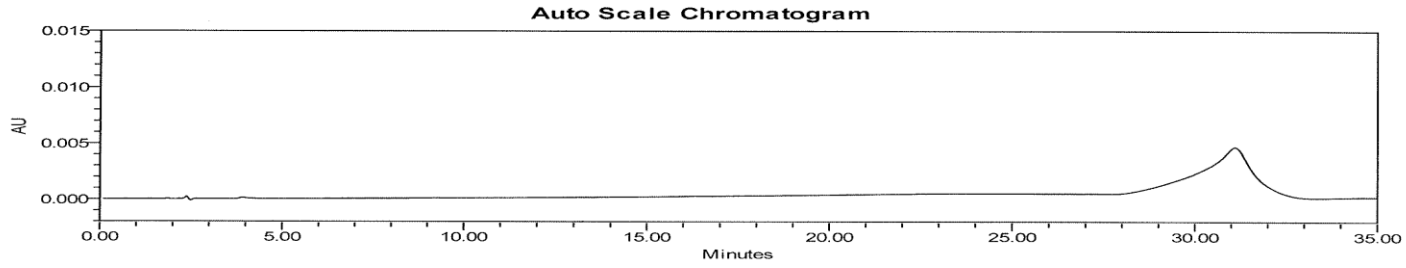
S.No	RRRS Diastereomer		
	Peak Area	Theoretical Plates	Tailing Factor
1	15176	27507	1.02
2	13842	29597	0.99
3	13551	31782	0.94
4	13593	30240	1.13
5	13237	28351	1.06

6	13969	26090	0.94
Mean	**13895**	**28928**	**1.01**
% RSD	**4.87**	**-**	**-**
Online	14117	28596	0.94
Online Average	**13926**	**28881**	**1.00**
Cumulative % RSD	**4.48**	**-**	**-**

Procedure: Inject diluent as blank solution and record the chromatogram. Inject Standard solution into the system for six replicates and record the chromatograms.

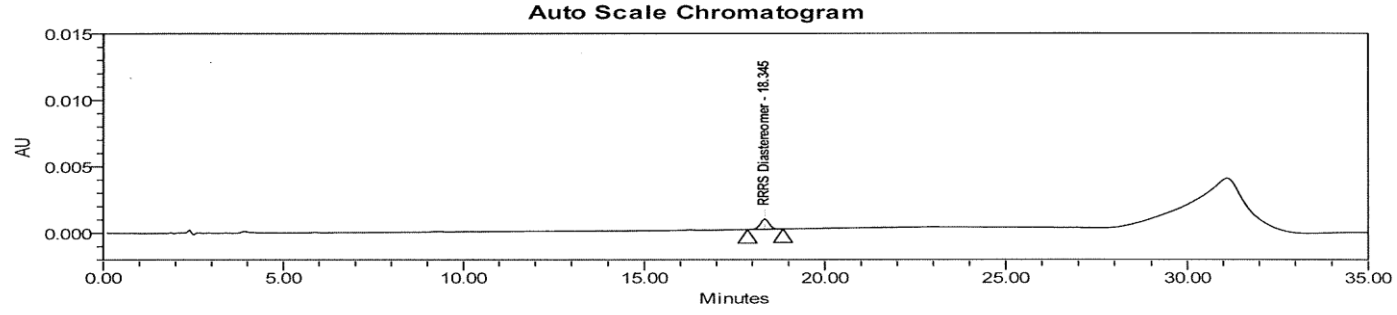
Acceptance Criteria: The % RSD for the area of RRRS Diastereomer peak obtained from six replicate injections of standard solution should be not more than 10.0. The tailing factor for RRRS Diastereomer obtained from standard solution should be not more than 2.0. The number of theoretical plates for RRRS Diastereomer obtained from standard solution should be not less than 3000.

Observation: System suitability complies.



	RT	Area	% Height	% Area	Int Type	Name
1	18.79				Missing	RRRS Diastereomer
Sum						

Fig-2: Blank Preparation.



	RT	Area	% Height	% Area	Int Type	Name	USP Plate Count	USP Tailing
1	18.35	12734	100.00	100.00	BB	RRRS Diastereomer	26584	0.97
Sum		12734						

Fig-3: Standard Preparation.

Specificity:

Individual solution of Ritonavir Identity standard solution (Which contains Specified impurities and Ritonavir), RRRS Diastereomer and blend solution (Ritonavir test sample spiked with RRRS Diastereomer) are injected and analyzed as per the method.

Table-4: Specificity

Name of the Compound	Retention Time (RT)		Relative Retention time (RRT)		
	From Individual solution	From Blend solution	From Individual solution	From Blend solution	Variation
RRRS Diastereomer	18.35	18.27	1.14	1.13	0.01

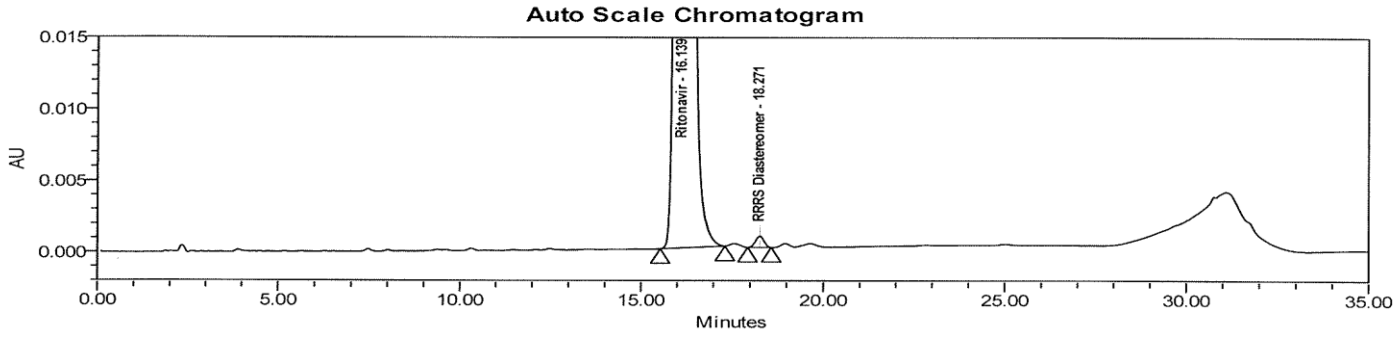
Observation:

RRRS Diastereomer resolved from all possible known impurities. The elution order and Relative retention time (RRT) of RRRS Diastereomer obtained from individual solution and the blend solution were comparable.

Preparation of stock solution:

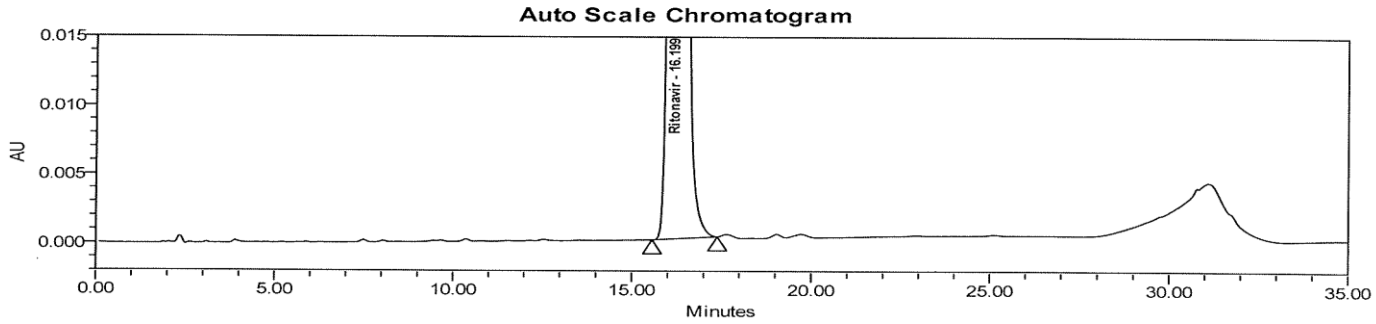
Weigh accurately about 5.0 mg of RRRS Diastereomer standard into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix. Dilute 3.2 mL of this solution to 20 mL with diluent.

Preparation of Blend solution: Weigh about 20.0 mg of the test sample into 10 mL volumetric flask dissolve and dilute to the volume with diluent and mix.



	RT	Area	% Height	% Area	Int Type	Name	USP Plate Count	USP Resolution	RT Ratio
1	16.14	16906415	99.92	99.92	BB	Ritonavir	21498		
2	18.27	12766	0.08	0.08	BB	RRRS Diastereomer	27918	4.79	1.13
Sum		16919181							

Fig-4: Blend Preparation.



	RT	Area	% Height	% Area	Int Type	Name
1	16.20	17673394	100.00	100.00	BB	Ritonavir
2	18.73				Missing	RRRS Diastereomer
Sum		17673394				

Fig-5: Test Preparation.

Procedure:

Inject diluent as a blank and conclude the interference due to blank at the retention time of carryover impurities. Inject individual solution of RRRS Diastereomer impurity solution and blend solution into the system and record the chromatograms. Establish retention time (RT) for RRRS Diastereomer obtained from individual solution and blend solution.[7]

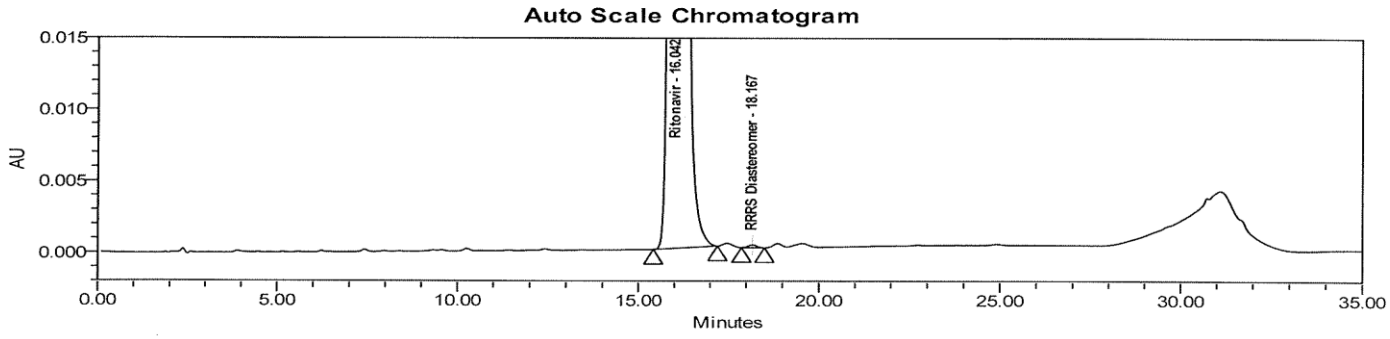
Acceptance Criteria: There should be no interference due to blank and possible known impurities at the retention time of corresponding carryover impurities. Impurity should resolve from each other and possible known impurities. The elution order and retention time (RT) of RRRS Diastereomer obtained from individual solution and the blend solution should be comparable. (± 10% variation for retention time).

Test Solution Stability: Perform the analysis of test sample spiked with RRRS Diastereomer at limit level and carryout solution stability up to 24 hrs.

Procedure: Prepare and inject spiked test sample solution (Blend solution) containing RRRS Diastereomer at specification level and Ritonavir test conc. level record the chromatogram. Inject each interval stability sample solution and record the chromatograms. Compare the % variation of RRRS Diastereomer obtained from initial sample and each time interval of solution stability sample and calculate together. Report the solution stability in hours.[8]

Note: After finding the time interval of solution at which is not stable no need to evaluate solution stability for the samples of remaining time intervals. The time intervals can be reduced and altered based on the intended application.

Acceptance criteria: The variation content (ppm) of carryover impurity obtained from solution stability study and initial result (fresh sample) should be meet ± 30.0% of the specification limit.



	RT	Area	% Height	% Area	Int Type	Name
1	16.04	17684088	99.98	99.98	BB	Ritonavir
2	18.17	3054	0.02	0.02	BB	RRRS Diastereomer
Sum		17687142				

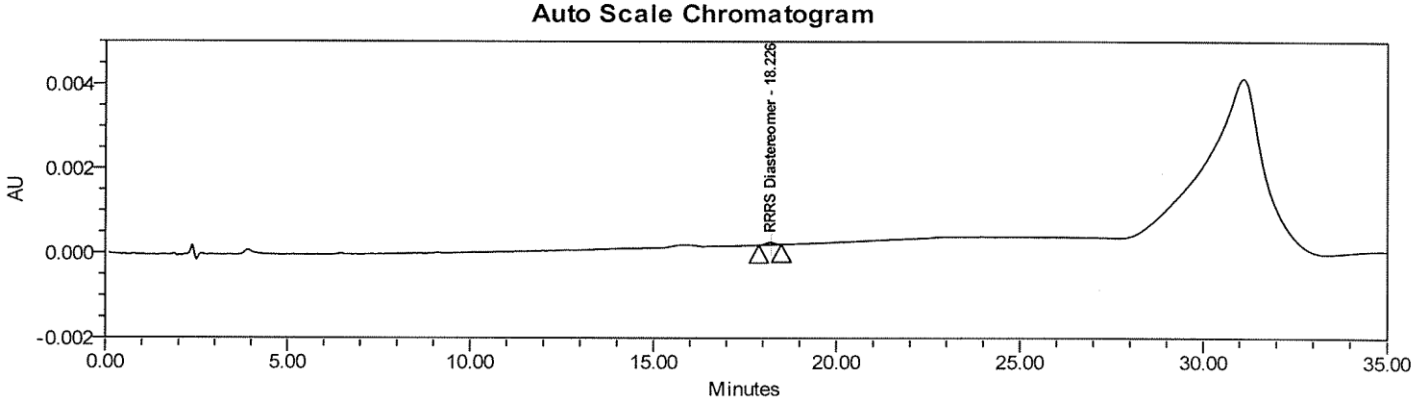
Fig-6: Linearity Preparation.

Detection Limit (DL): This is the measurement of lowest concentration of analyte that can be detected but not to be quantified.

Preparation of DL solution: Prepare DL solution containing carryover impurities with respect to test concentration by diluting each individual standard stock solution, so as to get S/N ratio is about 3:1 to 5:1

Procedure: Inject diluent as blank solution and record the chromatogram. Inject DL solution and record the chromatogram. Calculate the S/N ratio using the software.

Acceptance criteria: The S/N ratio should be about 3:1 to 5:1



	RT	Area	% Height	% Area	Int Type	Name	S_N_Ratio
1	18.23	922	100.00	100.00	BB	RRRS Diastereomer	4.0
Sum		922					

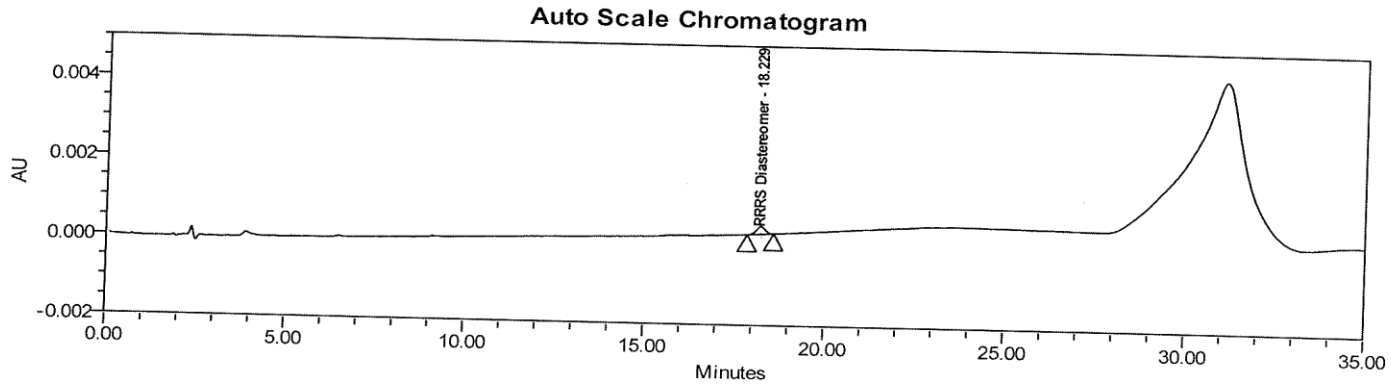
Fig-7: Detection Limit Preparation.

Quantitation Limit (QL): This is the measurement of lowest concentration of analyte that can be quantified with acceptable precision.

Preparation of QL solution: Based on the S/N ratio obtained from DL solution, derive QL concentration of RRRS Diastereomer so as to get S/N ratio is about 10:1

Procedure: Inject QL solution and record the chromatogram. Calculate the S/N ratio by using the software.

Acceptance Criteria: The S/N ratio should be about 10:1.



	RT	Area	% Height	% Area	Int Type	Name	S_N_Ratio
1	18.23	3205	100.00	100.00	BB	RRRS Diastereomer	13.0
Sum		3205					

Fig-8: Quantification Limit Preparation.

Precision at QL: Inject QL solution for six replicates (six times) and record chromatograms. Calculate % RSD for the peak area of RRRS Diastereomer obtained from six replicate injections of QL level.[9]

Acceptance criteria: The % RSD for the peak area of RRRS Diastereomer obtained from six replicate injections of QL level should be not more than 15.0

Accuracy at QL:

Preparation of Test solution: Weigh accurately about 20.0 mg of test sample into 10 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Accuracy at QL solution: Prepare the solution having 100% sample and spike the carryover impurities at QL level.

Note: Should prepare in duplicates.

Procedure: Prepare and inject test solution in single, Accuracy at QL level in duplicate and record the chromatograms. Calculate the % recovery of impurity.

Acceptance criteria: The % recovery should be between 70 and 130 at QL level.

Linearity: Perform linearity with different concentrations of RRRS Diastereomer by analyzing a minimum six concentrations i.e. QL, 50%, 75%, 100%, 125% and 150% w.r.to limit level.

Preparation of Linearity stock solution: Weigh accurately about each 5.0 mg of RRRS Diastereomer standard into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Linearity level -1 solution (QL): Prepare and inject QL solution in dupilcate. (or) Consider first two injections of Precision at QL.

Preparation of Linearity level -2 solution: Take 1.0 mL of linearity stock solution into 20 mL volumetric flask, dilute to the volume with diluent and mix.

Preparation of Linearity level -3 solution: Take 1.5 mL of linearity stock solution into 20 mL volumetric flask, dilute to the volume with diluent and mix.

Preparation of Linearity level -4 solution: Take 2.0 mL of linearity stock solution into 20 mL volumetric flask, dilute to the volume with diluent and mix.

Preparation of Linearity level -5 solution: Take 2.5 mL of linearity stock solution into 20 mL volumetric flask, dilute to the volume with diluent and mix.

Preparation of Linearity level -6 solution: Take 3.0 mL of linearity stock solution into 20 mL volumetric flask, dilute to the volume with diluent and mix.

Procedure: Inject QL level to level-6 solution in duplicate and record the chromatograms. Plot linearity graph between average peak areas of duplicate injections against concentration of RRRS Diastereomer determine the correlation coefficient value for carryover impurities.

Acceptance Criteria: Correlation coefficient value should be not less than 0.99.

Accuracy at QL level:

Accuracy at QL was proved by checking % recovery of RRRS Diastereomer.

Test sample spiked with RRRS Diastereomer at QL level and results were given below table.

Table-5: % Recovery at QL level:

Accuracy at QL Level	% Recovery of RRRS Diastereomer
Prep'n	93.9

Observation:

The % recovery of RRRS Diastereomer at QL level was within the limit.

Acceptance criteria:

The % recovery should be between 70 and 130 at QL level.

Accuracy at 100% level:

Accuracy at 100% level of the method is proved by checking the % recovery of RRRS Diastereomer.

Test sample spiked with RRRS Diastereomer at 100% level and results were given below table.

Table-6: % Recovery at 100% level:

Accuracy at 100% Level	% Recovery of RRRS Diastereomer
Prep'n	99.0

Observation:

The % recovery of RRRS Diastereomer was within the limit.

Acceptance criteria:

The % recovery should be between 80 and 120 for 100% level.

Accuracy:

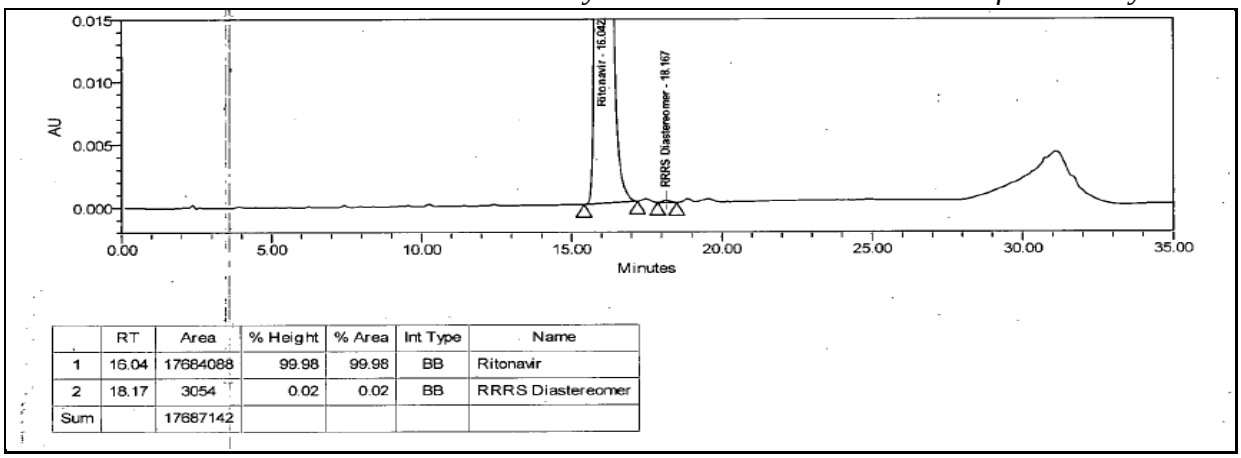
Preparation of Accuracy stock solution: Weigh accurately about each 5.0 mg of RRRS Diastereomer standard into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Test solution: Weigh accurately about 20.0 mg of test sample into 50 mL volumetric flask, dissolve and dilute to the volume with diluent and mix.

Preparation of Accuracy at 50% level solution: Weigh accurately about 25.0 mg of test sample into 50 mL volumetric flask, add 1.0 mL of accuracy stock solution, dissolve and dilute to the volume with diluent and mix.[10] *(This solution contains 32.5ppm RRRS Diastereomer with respect to test solution concentration which covers the 50% of specification level)*[15, 16]

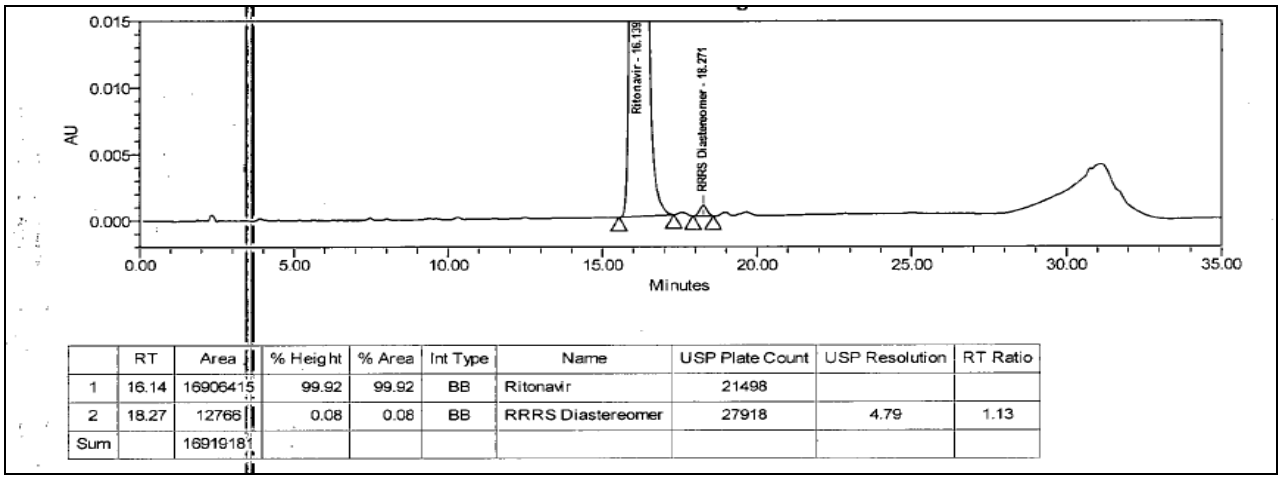
Preparation of Accuracy at 100% level solution: Weigh accurately about 20.0 mg of test sample into 20 mL volumetric flask, add 2.00 mL of accuracy stock solution, dissolve and dilute to the volume with diluent.

(This solution contains 65ppm of RRRS Diastereomer with respect to test solution concentration which covers the 100% of specification level)



	RT	Area	% Height	% Area	Int Type	Name
1	16.04	17684088	99.98	99.98	BB	Ritonavir
2	18.17	3054	0.02	0.02	BB	RRRS Diastereomer
Sum		17687142				

Fig-9: Accuracy at QL level chromatogram.



	RT	Area	% Height	% Area	Int Type	Name	USP Plate Count	USP Resolution	RT Ratio
1	16.14	16906415	99.92	99.92	BB	Ritonavir	21498		
2	18.27	12766	0.08	0.08	BB	RRRS Diastereomer	27918	4.79	1.13
Sum		16919181							

Fig-10: 100% spike recovery solution.

Note: *Should prepare Accuracy at 50% and 100% levels in triplicate preparations and Accuracy at 100% level in six preparations. Use first three preparations of Accuracy at 100% level for accuracy study and total six preparations for method precision study.*[11, 12, 13, 14]

Procedure: Inject test solution in single, each accuracy at 50% and 100% levels in triplicate preparations and Accuracy at 100% level in six preparations. Calculate the % recovery of carryover impurity.

Acceptance criteria: The % recovery should be between 80 and 120 for 50% & 100% level.

Table-7: Summary of % recoveries

Accuracy level	Preparations	% Recovery of RRRS Diastereomer
50%	1	102.1
	2	108.6
	3	106.9
100%	1	101.0
	2	98.8
	3	99.9

Calculation for % Recovery:

$$\textbf{RRRS Diastereomer (\%)} = \frac{AU}{AS} \times \frac{CS}{CU} \times P \times 10000$$

$$\textbf{\% Recovery} = \frac{\text{Content in spiked sample (\%)} - \text{Content in test sample (\%)}}{\text{Concentration added (\%)}} \times 100$$

Where,

AU = Peak area of RRRS Diastereomer in spiked test solution

AS = Average area of RRRS Diastereomer in standard solution

CU = Concentration of spiked test solution (mg/mL)

CS = Concentration of RRRS Diastereomer in standard solution (mg/mL)

P = Purity / Potency of RRRS Diastereomer standard (%)

Method Precision: Perform the analysis by spiking the test sample with carryover impurities at specification level in six different preparations and determine the method precision.

Note: *The data from the Accuracy at 100% level six preparations can be used for method precision.*

Procedure: Prepare and inject spiked test sample in six different preparations into the system and record the chromatograms. (Consider the Accuracy at 100% level in six preparations) Calculate the ppm of RRRS Diastereomer in each preparation as per the method.

Calculate the % RSD for the results obtained from the method precision study.

Acceptance criteria: The % RSD for the results obtained from method precision study should be not more than 10.0

Intermediate Precision: Carry out the precision study on a different day, with different instrument, different analyst and different column using with fresh preparations.

Procedure: Establish the system suitability as per the method. Prepare and inject standard solution in six replicates and record the chromatograms. Prepare and inject spiked test sample solution in six preparations (which is analyzed under method precision study) and record the chromatograms. Calculate the ppm of RRRS Diastereomer in each preparation as per the method. Calculate the % RSD for the results obtained from the

intermediate precision study. Calculate the cumulative % RSD for the results obtained from method precision study and intermediate precision study.[17]

Acceptance criteria: System suitability should comply as per the method. The % RSD for the results obtained from intermediate precision study should be not more than 10.0. The cumulative % RSD for the results obtained from method precision study and intermediate precision study should be not more than 15.0.

Conclusion: Conclude from the above studies whether the method is valid and suitable for the content of RRRS Diastereomer in Ritonavir by HPLC.

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