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ENANTIOMERIC SEPARATION AND QUANTITATIVE DETERMINATION OF RITONAVIR ENANTIOMER IN RITONAVIR DRUG SUBSTANCE BY CHIRAL LIQUID CHROMATOGRAPHY

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ABSTRACT

A stability indicating analytical method was developed for the determination of ritonavir enantiomer in ritonavir drug substance by chiral liquid chromatography. The chromatographic separation was achieved on chiralpak IC which is an immobilized (cellulose tris-(3,5 dichlorophenylcarbamate) phase coated on silica matrix. The separation achieved employing a mobile phase consists of n-hexane, ethanol and diethylamine as mobile phase. the flow rate was 1.0 ml/ minute and ultra violet detector at 240nm. The average retention time for Rotonavir and Enantiomer found to be 15.0 min and 18.0 min the proposed method was validated for selectivity, precision, linearity and accuracy.

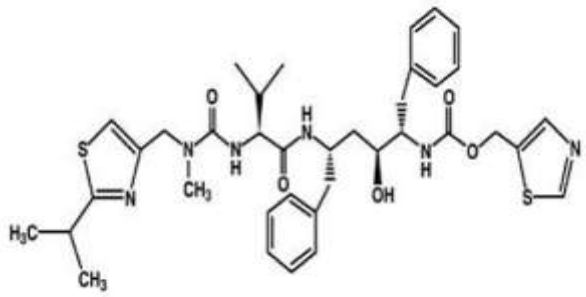
KEYWORDS: Ritonavir, Isocratic, HPLC, Chiral pak IC, n-Hexane, Ethanol and Diethylamine and validation.

Ritonavir is an antiretroviral medication used along with other medications to treat HIV/AIDS This combination treatment is known as highly active antiretroviral therapy (HAART). Often a low dose is used with other protease inhibitors. It may also be used in combination with other medications for hepatitis C. It is taken by mouth.

Ritonavir is of the protease inhibitor class. It is often used to inhibit the enzyme that metabolizes other protease inhibitors. This inhibition leads to higher concentrations of these latter medication.

Ritonavir first came into use in 1996. It is on the World Health Organization's List of Essential Medicines, the most important medications needed in a basic health system.

Ritonavir is chemically designated as 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)]. Its molecular formula is $C_{37}H_{48}N_6O_5S_2$ and its molecular weight is 720.95. Ritonavir is a white-to-light-tan powder. Ritonavir has a bitter metallic taste. It is freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water. Ritonavir is a peptidomimetic inhibitor of the HIV-1 protease. Inhibition of HIV protease renders the enzyme incapable of processing the gag-pol polyprotein precursor which leads to production of non-infectious immature HIV particles.



Structure of Ritonavir

EXPERIMENTAL

Chemicals

Active pharmaceutical ingredient standards, Related impurities and samples were supplied by Mylan laboratories limited, Hyderabad, India. The HPLC grade n-Hexane purchased from Rankem chemicals limited and HPLC grade Ethanol and Diethylamine were purchased from Merck Chemicals limited

Chromatographic conditions and equipment

LC was carried out on a Waters Alliance with 2487 as detector module. The output signal was monitored and processed using Empower Software. The chromatographic column used was an Chiral PAK IC

(250 mm, 4.6 mm, and 5 µm particle size). A mixture of n-Hexane, Ethanol and Diethylamine in the ratio of 85:15:0.1 v/v/v was prepared and used as mobile phase. The flow rate of mobile phase was 1.0 mL/min. The column temperature was maintained at 35ºC and the detection was monitored at a wavelength of 240 nm. The injection volume was 20.0 µL. The diluent was ethanol.

Preparation of solutions

Sample preparation

100.0 mg of the test sample was taken in a 100 mL volumetric flask, dissolved in and diluted to the volume with diluents.

RESULTS AND DISCUSSIONS

In the development of a chiral HPLC method, it is usually desirable to use a chiral stationary phase (CSP) for direct separation of enantiomers, because of the simplicity of operation. Various types of CSP are available; among these cellulose and amylose-based CSP have been proved to be quite versatile. The order of elution of enantiomers is of crucial importance in monitoring enantiomeric purity. The minor enantiomer should be eluted before the major isomer, to avoid possible interference caused by tailing of the major enantiomer. This is especially true when the separation is relatively poor.

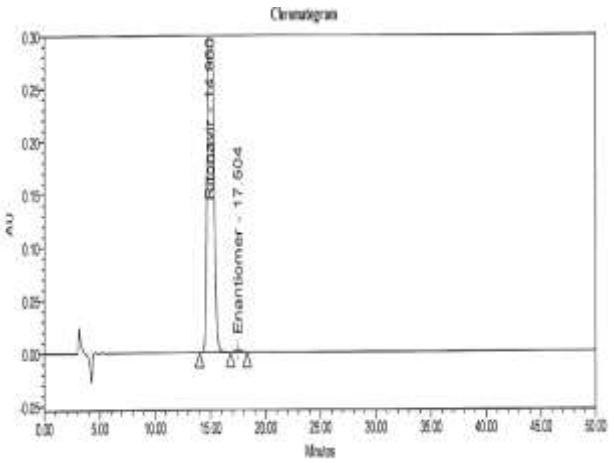


Figure: 1 Chromatogram of Ritonavir and Enantiomer

METHOD VALIDATION

Linearity

A linear calibration plot of the method was obtained over the tested calibration ranges, i.e. LOQ to 120% for enantiomer impurity. The linearity calibration curve is presented in Figure 2. The correlation coefficient obtained was greater than 0.999, indicating a linear response of the enantiomer.

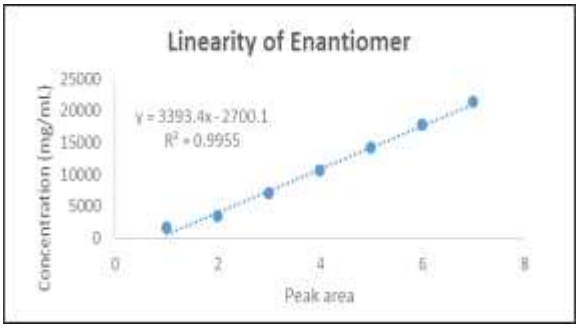


Figure 2. Linearity graph for Enantiomer

Table-1: Linearity for Enantiomer

Level	Concentration (mg/mL)	Peak area
LOQ	2.123915E-04	1571.5
20%	3.034164E -04	3489.0
40%	6.068328E-04	7062.0
60%	9.102492E-04	10670.0
80%	1.213666E-03	14189.0
100%	1.517082E-03	17817.5
120%	1.820498E-03	21315.0

Method precision (repeatability)

The precision of the method was determined by analyzing a sample of Ritonavir solution spiked with Ritonavir enantiomer at 100% of the specification limit (six replicate sample preparations). Data obtained is summarized in Table. 2.

Table. 2: Summary of Percent areas for method precision

Sample No	Enantiomer (%w/w)
1	0.14
2	0.13
3	0.14
4	0.14
5	0.14
6	0.14
Mean	0.14
%RSD	3.0

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The results obtained for Ritonavir and its isomers are listed in table. 3 and 4.

The limit of detection of enantiomer impurity was 0.000076 mg/mL and the limit of quantification of enantiomer impurity was 0.000212 with respect to Ritonavir working concentration.

Table. 3: Limit of detection (LOD) for Ritonavir Enantiomer

Component	Concentration (mg/mL)	Signal to noise ratio	LOD (%)
Enantiomer	0.000076	3.6:1	0.0080

Table. 4: Limit of Quantitation (LOQ) for Ritonavir Enantiomer.

Component	Concentration (mg/mL)	Signal to noise ratio	LOQ (%)
Enantiomer	0.000212	10.4:1	0.02

Accuracy (recovery study)

The accuracy of the method was determined through the recovery test of the samples using known Amount of enentiomer. All the solutions were prepared in triplicate and analyzed. The percentage recovery of enantiomer impurity ranged 78.7 to 100.3 The percentage recovery of the enantiomer is listed in Table 5. The Recovery values were within the acceptance limit.

Table.5: Summary of % recoveries for Enantiomer

Level	Theoretical conc. (mg.mL)	Measures Conc. (mg.mL)	%Recovery	%RSD
LOQ	0.000212	0.000167	78.7	**4.5**
	0.000212	0.000180	84.9	
	0.000212	0.000181	85.4	
50%	0.000759	0.000650	85.8	**3.5**
	0.000759	0.000624	82.3	
	0.000759	0.000669	88.2	
100%	0.001517	0.001430	94.3	**3.1**
	0.001517	0.001350	89.0	
	0.001517	0.001420	93.6	
120%	0.001820	0.001826	100.3	**0.8**
	0.001820	0.001801	98.9	
	0.001820	0.001825	100.2	

Specificity: Forced degradation studies

Degradation was not observed in Ritonavir samples under stress conditions like acid hydrolysis, alkaline hydrolysis, and thermal hydrolysis. However mild degradation was observed when the drug substance exposed to photolysis and hydrogen peroxide. Therefore, it may be concluded that Ritonavir is susceptible to degrade in oxidative and photolytic conditions. Photodiode array detection was used as an evidence of the specificity of the method and to evaluate the homogeneity of the drug peak. The forced degradation result reveals that the degradation products were well resolved from Ritonavir and its enantiomer, confirming the stability-indicating power of the method.

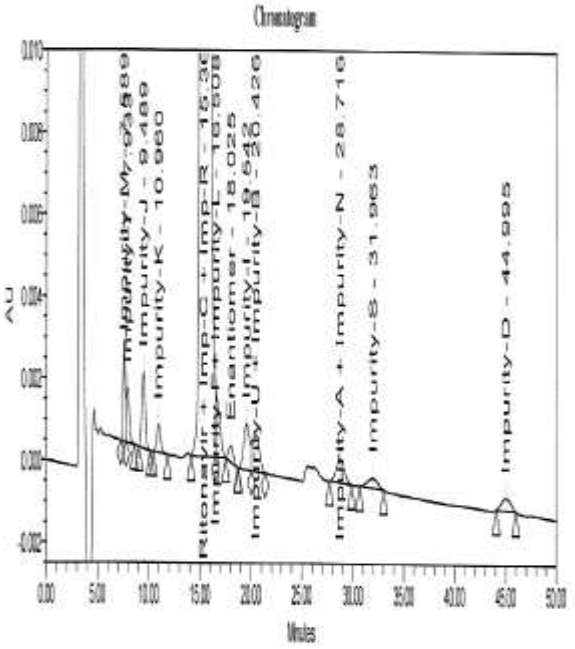


Figure: Typical chromatogram for specificity

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature and mobile phase composition), all analytes were adequately resolved and

elution orders remained unchanged. Resolution between enantiomer impurity and Ritonavir was greater than 2.1 The resolution between the enantiomer impurity and Ritonavir under various conditions are listed in Table 6.

Parameter	Parameter condition	Resolution between Ritonavir and Ritonavir enantiomer
Actual	Flow rate 1.0 ml/min, column oven temperature 35°C and mobile phase composition n-Hexane :ethanol:Diethylamine (85:15:0.1v/v/v)	2.1
Flow rate	0.9 mL (Low flow)	2.8
	1.1 mL (High flow)	2.7
column oven temperature	33°C (Low)	2.7
	37°C (High)	2.5
Mobile phase composition	n-Hexane :ethanol:Diethylamine (84:16:0.1v/v/v) (Low)	2.8
	n-Hexane :ethanol:Diethylamine (815:165:20v/v/v) (High)	2.9

Ruggedness

The ruggedness of a method was defined as degree of reproducibility of results obtained by analysis of the same sample under variety of normal test conditions such as different laboratories, different analysts, different instruments, different days and different lots of reagents. The standard addition and recovery experiments of enantiomer was carried out in Ritonavir bulk samples at the same concentration levels tested in Laboratory A were again carried out at laboratory B using a different instrument by a different analyst. The data obtained from Laboratory B was well in agreement with the results obtained in Laboratory A, thus proving the method ruggedness.

Table. 7: Summary of Ruggedness

Sample	Enantiomer (%w/w)	
	Analyst(1)/Instrument(1)/ Day(1)/Column(1)/Reagent(1)	Analyst(1)/Instrument(1)/ Day(1)/Column(1)/Reagent(1)
Sample-1	0.14	0.15
Sample-2	0.13	0.15
Sample-3	0.14	0.15
Sample-4	0.14	0.15
Sample-5	0.14	0.15
Sample-6	0.14	0.15
%RSD	**3.0**	**0.0**

Table No. 8: Validation parameters of evaluated method

S. No	Parameter	Limit	Value Obtained
1.	**Accuracy(%Recovery)**	80-120%	78.7 to 100.3 for Enantiomer
2.	**Linearity concentrations Range(µ g/mL)** **Regression coefficient (R2 value)**	NLT 0.990	0.995 for Enantiomer
3.	**Precision (% RSD)** **Method precision (Repeatability) (%RSD, n = 6)**	NMT 2%	%RSD is 3.0
4.	**Robustness** **Flow Variation, Column temperature and mobile phase composition**	Resolution between Ritonavir and enantiomer should be >1.5	The resolution between Ritonavir and enantiomer was observed in the range of 2.1 to 2.9.
5.	**Ruggedness (Intermediate Precision) (%RSD analyst to analyst variation)**	NMT 2%	0.0 to 3.0

*RSD = Relative standard deviation.

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the estimation of enantiomeric purity of Ritonavir in drug substance was found to be simple, precise, accurate and high resolution and it can be effectively applied for routine analysis in research institutions, quality control department and approved testing laboratories.

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