Development and Validation of RP-LC Method for Ritonavir in Pharmaceutical Formulations

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ABSTRACT

A new, simple, rapid, selective, precise and accurate isocratic reverse phase high-performance liquid Chromatography assay method has been developed for estimation of Ritonavir in tablet formulations. The separation was achieved by using column X-Terra RP18 (4.6x100 mm), 3.5µ (Make: Waters), in mobile phase consisted of Acetonitrile and pH 6.8 Phosphate buffer (0.01M) in the ratio of (50:50, v/v). The flow rate was 1.0 mL.min-1 and column oven temperature ambient temperature, the injection volume was 10 µL. The separated Ritonavir was detected using UV detector at the wavelength of 239 nm. The retention time of Ritonavir was noted to be 4.35 min respectively, indicative of rather shorter analysis time. The method was validated as per ICH guidelines. The proposed method was found to be accurate, reproducible, and consistent.

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INTRODUCTION

Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Viruses are smallest microorganisms which not only take nutrition from host cell but also direct its metabolic machinery to synthesize new virus particles. Anti-viral drugs are active against these viruses and can target virus-specific steps like cell penetration, uncoating, reverse transcription, virus assembly or maturation.

Antiretroviral Drugs [1] are active against the human immunodeficiency virus (HIV). The first Antiretroviral drug Zidovudine was developed in 1987. Over past 20 years, more than 20 drugs belonging to 3 classes have been developed. Mechanisms of drugs act like Nucleoside Reverse Transcriptase inhibitors (NRTI), Non-Nucleoside reverse transcriptase inhibitors (NNRTIS) and Protease inhibitors.
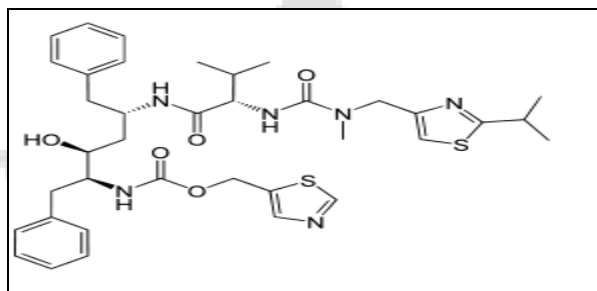

Fig.1 The structure of Ritonavir

IUPAC names**:** 1,3-thiazol-5-ylmethylN-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-[methyl({[2-(propan-2-yl)-1,3-thiazol-4-l]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl] carbamate. Ritonavir is a white or almost white powder, Ritonavir has a bitter metallic taste. Freely Soluble in Methanol and Methylene chloride. Very slightly soluble in Acetonitrile; practically insoluble in water. Ritonavir was originally developed as an inhibitor of HIV protease. It is one of the most complex inhibitors. It is now rarely used for its own antiviral activity but remains widely used as a booster of other protease inhibitors. More specifically, Ritonavir is used to inhibit a particular liver enzyme that normally metabolizes protease inhibitors, cytochrome P450-3A4 (CYP3A4). The drug's molecular structure inhibits CYP3A4, so a low dose can be used to enhance other protease inhibitors. This discovery, which has drastically reduced the adverse effects and improved the efficacy of PI's and HAART, was first communicated in an article published in the AIDS Journal in 1997 by the University of Liverpool. This effect does come with a price: it also affects the

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116

efficacy of numerous other medications, making it difficult to know how to administer them concurrently. In addition, it can cause a large number of side-effects on its own.

Literature review revealed that several methods were developed for quantitative estimation of Antiretroviral drugs such as voltammetric [2], capillary electrophoresis [3], spectrofluorometer [4], spectrophotometer [5], and liquid chromatography-(LC) [6-18]. Moreover, voltammetric, capillary electrophoresis, spectrophotometry, spectrofluorometry involves tedious procedure and too many steps which do not satisfy the determination of the samples. Hence, in the present study new sensitive, economical, stability indicating RP-HPLC method was developed and validated in accordance with ICH guidelines.

MATERIALS AND METHODS

Experimental

Chemicals and Reagents

Analytical-grade Potassium dihydrogen phosphate, Sodium Hydroxide pellets, Acetonitrile and Water HPLC-grade, were from Merck Chemicals. Mumbai, India. Millex syringe filters (0.45 μm) were from Millex-HN, Millipore Mumbai, India.

Instrumentation

Waters 2489 U.V-Visible detector/2695 Separation Module, equipped with Empower 2 software, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model), Analytical Balance (Mettler Toledo Model) were used in the present assay.

Buffer preparation

1.3g of Potassium dihydrogen phosphate was dissolved in 1000ml of milli Q water and pH adjusted to 6.8 ± 0.05 With NaOH solution. The solution was filtered through 0.45μ filter paper and degassed.

Mobile phase preparation

Buffer and Acetonitrile were mixed in the ratio of 50:50v/v respectively, filtered and degassed.

Citation: D. Ramachandran et al. Ijppr.Human, 2016; Vol. 7 (3): 115-125.

117

Diluent preparation

Water and Acetonitrile were mixed in the ratio of 1:1v/v respectively.

Standard preparation:

Weighed accurately and transferred about 100 mg of Ritonavir working standard into 200 ml volumetric flask. About 170 ml of diluent was added and sonicated to dissolve. Volume was made up to with diluent and mixed well. 10 ml of above solution was taken in 50 ml volumetric flask, diluted up to mark with diluent (100 ppm). Solution was mixed well and filtered through 0.45μm filter.

Sample preparation:

Weighed 10 tablets of the Ritonavir and crushed. Average weight of the Ritonavir tablets was transferred into 200 ml volumetric flask. Added 170 ml of diluent and sonicated for 20 minutes with intermittent shaking in cold water, made up to the volume with diluent and mixed well. 10 ml of above solution was taken in 50 ml volumetric flask, diluted up to mark with diluent (100 ppm). The solution was filtered through 0.45 μm PVDF membrane filter.

Chromatographic conditions

Chromatographic analysis was performed on X-Terra RP18 (4.6x100mm), 3.5μ (Make: Waters) column. The mobile phase consisted of Acetonitrile and pH 6.8 Phosphate buffer (0.01M) in the ratio of (50:50, v/v). The flow rate was 1.0 ml/min, column oven temperature ambient temperature, the injection volume was 10 μl, and detection was performed at 239 nm using a photodiode array detector (PDA).

RESULTS AND DISCUSSION

Method development

Spectroscopic analysis of compound Ritonavir showed maximum UV absorbance (λmax) at 239 nm. To develop a suitable and robust LC method for the determination of Ritonavir, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Inertsil-ODS-3V, 150x4.6mm, 5μ with the following different mobile phase compositions like that 50:50 water, Acetonitrile mixture , 50:50 0.01M Phosphate buffer (pH 3.0), Acetonitrile mixture, 50:50 0.01M Phosphate buffer (pH

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118

3.0), Acetonitrile mixture. It was observed that when Ritonavir was injected, Peak Tailing, not satisfactory.

For next trial, the mobile phase composition was changed slightly. The mobile phase composition was 50:50 0.01M Phosphate buffer (pH 6.8), Acetonitrile mixture. Respectively as eluent at flow rate 1.0 ml/min. UV detection was performed at 239 nm. The retention time of Ritonavir was 4.35 minutes and the peak shape was good.

The chromatogram of Ritonavir standard using the proposed method is shown in **(Fig: 2)** system suitability results of the method are presented in **Table-1.**
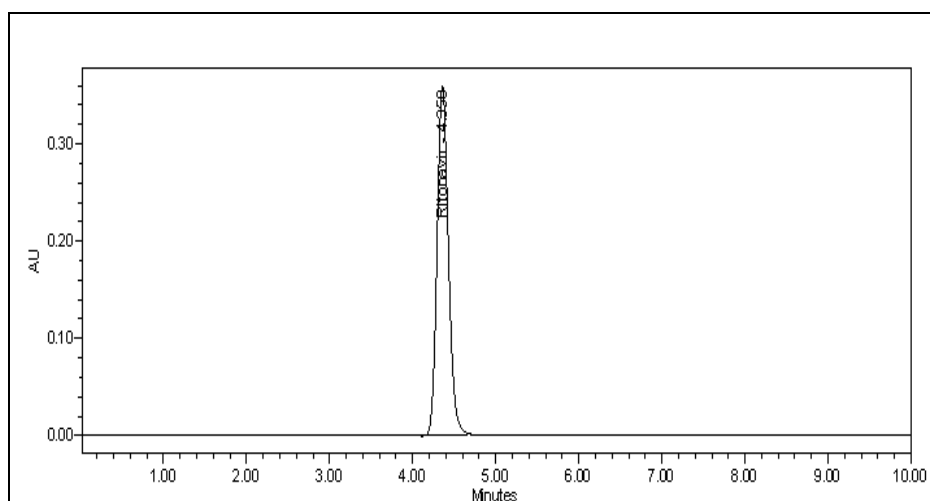

Fig. 2: Chromatogram showing the peak of Ritonavir

METHOD VALIDATION

The developed RP-LC method extensively validated for assay of Ritonavir using the following parameters.

Specificity

Preparation of blank solution:

Water, Acetonitrile were mixed in the ratio of 50:50 and degassed.

Preparation of Placebo solution:

Placebo solution was prepared in duplicate by weighing the equivalent amount of excipients present in the finished drug product and analyzed as per proposed method. Interference due to placebo was evaluated for each of the placebo preparations.

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119

Blank and Placebo interference

A study to establish the interference of blank and placebo were conducted. Diluent and placebo were injected into the chromatograph in the above defined chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of blank solution **(Fig: 3)** showed no peak at the retention time of Ritonavir peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Ritonavir in Ritonavir tablets. Similarly, chromatogram of placebo solution **(Fig: 4)** showed no peaks at the retention time of Ritonavir peak. This indicates that the placebo used in sample preparation do not interfere in estimation of Ritonavir in Ritonavir tablets.
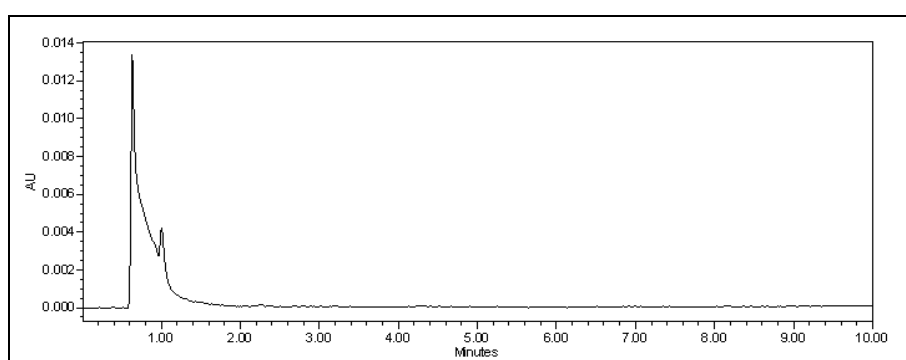

Fig: 3 Chromatogram showing the no interference of diluent for Ritonavir

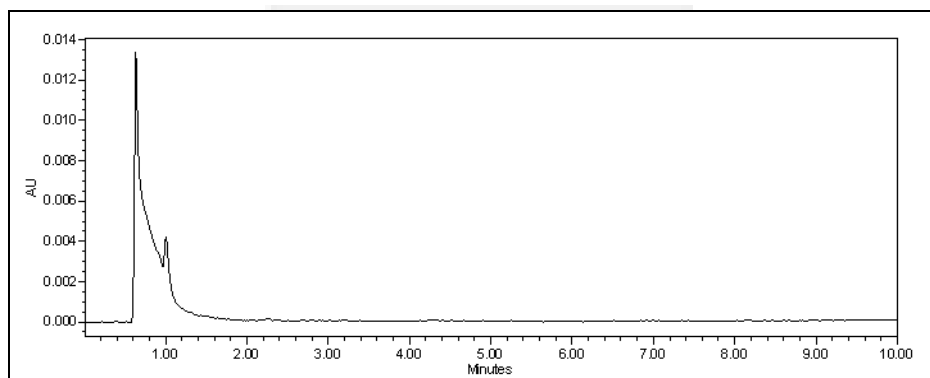**Fig: 4 Chromatogram showing the no interference of placebo for Ritonavir**

Table 1: System suitability parameters for Ritonavir by proposed method

Name of the Compound	Retention Time	Theoretical plates	Tailing factor
Ritonavir	4.35	13123	1.2

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120

System precision:

The standard solution was prepared as per the test method, injected into the HPLC system for six times and evaluated the % RSD for the area responses. The chromatogram was shown in **Figure: 5** and data were shown in **Table: 2**
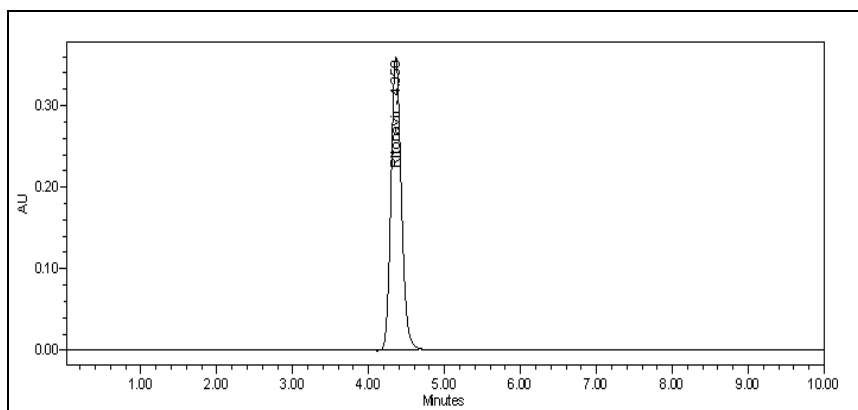

Fig: 5 System precision standard chromatogram

Table: 2 System precision data for Ritonavir

No. of injections	Peak area response
1	3388949
2	3387682
3	3424050
4	3394325
5	3402068
6	3398582
Average	**3399276**
% RSD	**0.4**

Method precision:

The precision of test method was evaluated by doing assay for six samples of Ritonavir tablet as per test method. The content in mg and % label claim for Ritonavir for each of the test

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121

preparation was calculated. The average content of the six preparations and % RSD for the six observations were calculated. The chromatogram was shown in **Figure: 6** and data were shown in **Table: 3**
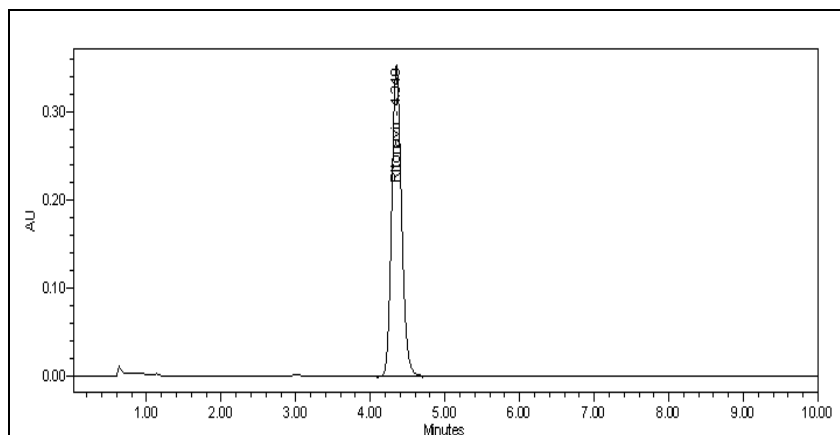

Fig: 6 Method precision sample chromatogram

Table: 3 Method precision data for Ritonavir

Sample Number	% Assay
1	98.2
2	98.1
3	98.5
4	99.4
5	97.7
6	97.9
Mean	**98.3**
% RSD	**0.5**

Linearity of detector response

The standard curve was obtained in the concentration range of 200.0-800.0μg/ml for Ritonavir. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient [r2] of standard curve were calculated and given in **Fig. 7**

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122

to demonstrate the linearity of the proposed method. From the data obtained which given in **Table: 4** the method was found to be linear within the proposed range.

Table: 4 Linearity studies for Ritonavir by proposed method

Level no.	Linearity concentration	Drug concentration (in ppm)	Response for Ritonavir
1	40%	200	1355584
2	60%	300	2053915
3	80%	400	2711167
4	100%	500	3388949
5	120%	600	4063500
6	140%	700	4659774
7	160%	800	5422334
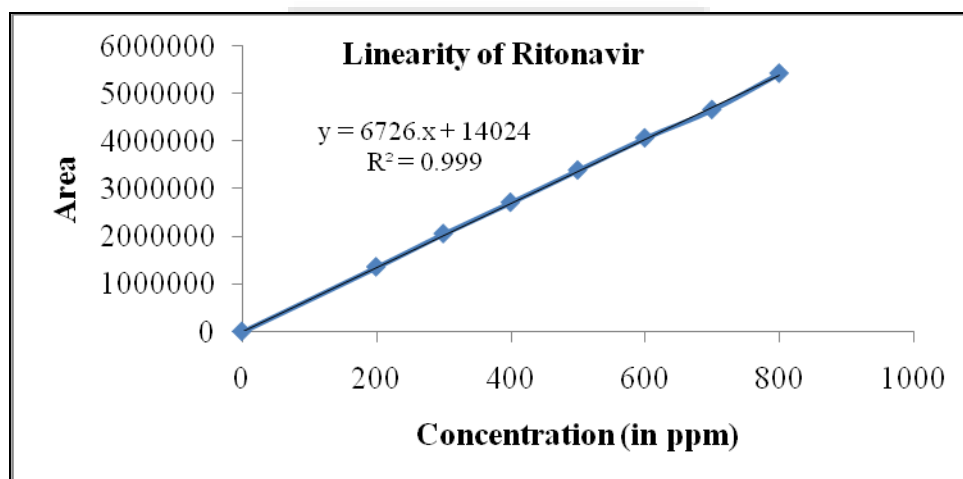
Fig. 7 Calibration curve for Ritonavir

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123

Accuracy

The accuracy of the method was determined on three concentration levels by recovery experiments. The recovery studies were carried out in triplicate preparations on composite blend collected from 20 tablets of Ritonavir, analyzed as per the proposed method. The percentage recoveries with found in the range of 99.5 to 100.2 for Ritonavir. The data obtained which given in **Table: 5** the method was found to be accurate.

Table: 5 Recovery studies for Ritonavir by proposed method

S. No.	% Spike level	Amount added (mg)	Amount recovered (mg)	% Recovery	% Mean recovery	%RSD
1.	50	50.12	50.20	100.2	100.1	0.2
2.		50.06	50.15	100.2		
3.		50.13	50.02	99.8		
1.	100	100.08	100.15	100.1	99.9	0.2
2.		100.02	99.80	99.8		
3.		100.07	99.96	99.9		
1.	150	150.03	149.94	99.9	99.7	0.2
2.		149.98	149.20	99.5		
3.		150.08	149.52	99.6		

CONCLUSION

An RP-HPLC method for estimation of Ritonavir was developed and validated as per ICH guidelines like Accuracy, Precision, Linearity, Specificity and System suitability. The results obtained were within the acceptance criteria.

The proposed method was applied for the determination of Ritonavir in marketed formulation. Hence, the proposed method was found to be satisfactory and could be used for the routine analysis of Ritonavir in tablet dosage form.

Citation: D. Ramachandran et al. Ijppr.Human, 2016; Vol. 7 (3): 115-125.

124

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125