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RP-HPLC method development and validation of ritonavir in bulk and pharmaceutical dosage forms

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ABSTRACT

A Simple, accurate and rapid RP-HPLC method has been developed for the estimation of ritonavir (RIT) in bulk and pharmaceutical dosage forms using a hypersil BDS C 18, 100 x 4.6 mm i.d, 5 µm particle size in isocratic mode; with mobile phase comprising of buffer (0.02M potassium dihydrogen phosphate) and acetonitrile in the ratio 70:30 (v/v).The flow rate was 1 ml/min and detection was carried out by photodiode array detector at 237 nm. The retention time for RIT was found to be 2.55 min. the proposed method has permitted the quantification of RIT over linearity in the range of 25 – 150 µg/ml and its percentage recovery was found to be 99.299 – 100.575 %. The % RSD of intraday and inter day precision were found 0.37% and 0.4% according to International Conference on Harmonization (ICH) Q2B guidelines.

Keywords: Ritonavir, RP-HPLC, Validation and method development.

INTRODUCTION

RIT is a HIV – protease inhibitors[1]. Chemically, RIT is 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]methyl}) carbomoyl]amino} butanamido]-1,6- iphenylhexan- 2yl] carbamate with empirical formula of $C_{37}H_{48}N_6O_5S_2$ and molecular weight 720.9 [2]. The chemical structure of RIT was shown in Fig.1. RIT is absorbed following oral administration and peak plasma concentration occurs in about 2 to 4 hours. Absorption is enhanced when ritonavir is taken with food, and is dose related. Protein binding is reported to be about 98% and penetration into the CNS is minimal. RIT is extensively metabolized in the liver principally by cytochrome P450 isoenzymes CYP3A and CYP2D6. The major metabolite has antiviral activity, but concentration in plasma is low. RIT is mainly excreted in the faeces, with a half-life of 3 to 5 hours. The analytical method for RIT is published in USP [3]. Several methods are reported for the individual and simultaneous estimation of RIT by UV-Visible spectrophotometry. Chiranjeevi et al, (2011)[4] described a development and validation of spectrophotometric method for quantitative estimation of ritonavir in bulk and pharmaceutical dosage forms, Vaishali P. Nageswar et al, (2010)[5] developed a simultaneous estimaton of ritonavir and lopinavir by absorption ratio (Q-analysis) UV

spectrophotometric method in combined tablet dosage form. Some few HPLC method Chiranjeevi et al, (2011)[6] Development and validation of RP-HPLC method for quantitative estimation of ritonavir in bulk and pharmaceutical dosage forms and Sudha et al, (2011)[7] was described a development and validation of RP-HPLC and HPTLC methods for estimation of Ritonavir in bulk and in pharmaceutical formulation with more HPLC analysis time and less sensitivity. From the above literature survey, our interest is to develop a method with shorter analysis time, more sensitivity and reliability for the estimation of RIT both in bulk and pharmaceutical dosage form and validated as per ICH guidelines[8-12].

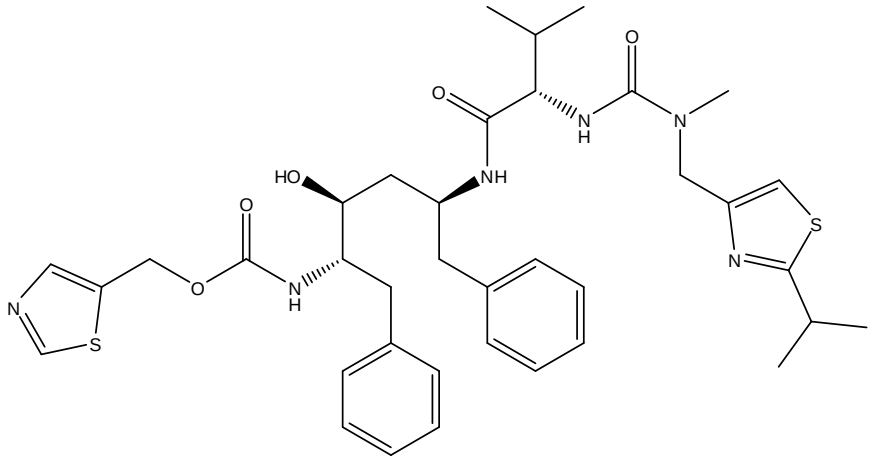

Fig.1: Chemical Structure of Ritonavir

EXPERIMENTAL

Reagents and materials

Pure standard of RIT (99.89%) was obtained as gift sample from Ranboxy Pharma Ltd, New delhi. HPLC grade acetonitrile (Rankem, avantor chemicals, gujarat), HPLC grade water, potassium dihydrogen phosphate, Ortho phosphoric acid, acetic anhydride, acetone (Merck specialities pvt ltd, Mumbai). The tablet was purchased from local pharmacy (EMPETUS tablets Emcure (ARV) Pharma Ltd). High precision weighing balance (wensar instruments, hyderabad), micro pipette (in labs, 10-100 µl) were employed in the study. All the glassware employed in the work cleaned with hot water followed acetic anhydride then acetone and dried in hot air oven whenever required. Working environment was maintained in between 18-22^{o}C.

HPLC apparatus and chromatographic conditions

The analysis was performed on Waters 2695 HPLC system with Waters2996 Photodiode Array detector. Data acquisition was performed by using Empower 2 software. Hypersil BDS, C18 column (100 x 4.6mm, 5µ) was used as stationary phase. Injections were performed by the manual injector with 10µl loop. Different mobile phases were tested in order of their polarity to find out the best conditions for the separation of ritonavir. The selected mobile phase Acetonitrile and Potassium Dihydrogen Phosphate buffer of 0.1% (pH was adjusted to 3.5) in the ratio of 30:70%v/v gave acceptable retention time (RT). The flow rate was maintained at 1.0 mL min^{-1}, with a run time of 7 min. the mobile phase was filtered by using 0.45µ filter and it was degassed by sonication prior to use. All determinations were made at ambient temperature. The eluent was detected at 237 nm.

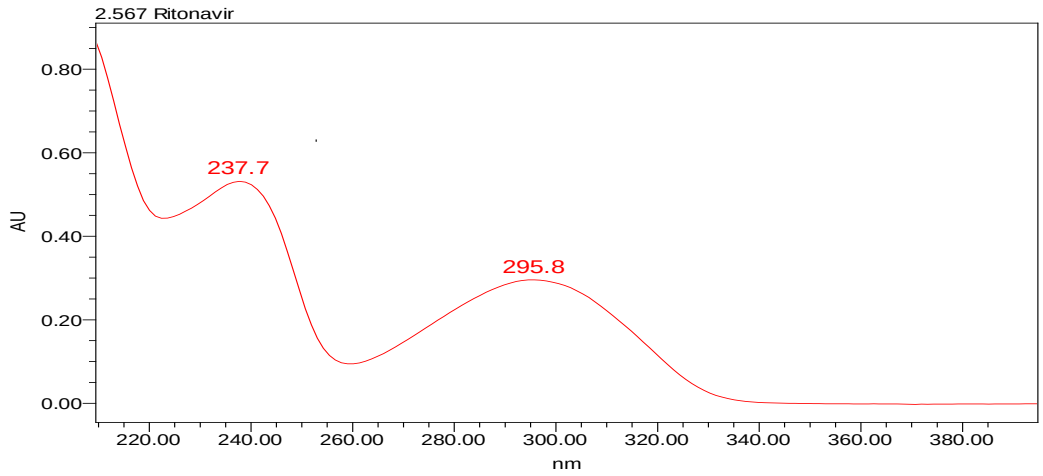

Fig.2: Overlaid spectrum of RIT

PROCEDURE RECOMMENDED

Preparation of mobile

Potassium Dihydrogen Phosphate Buffer (0.02M KH_2PO_4) pH was adjusted to 3.5 and acetonitrile taken in the ratio 70:30 (v/v) were employed as a mobile phase.

Preparation of stock solution

A stock solution was prepared by accurately weighed and transferred 10mg of RIT working standards into a 10ml clean dry volumetric flask, add 7ml of diluent (Water and Methanol 50:50), sonicated for 30 minutes and make up to the final volume with diluents. From the above stock solution, 1ml was pipette out into a10ml volumetric flask and then make up to the final volume with diluent.

Construction of linearity

The concentrations of analyte were prepared from the stock solution by taking suitable volume (0.25 – 1.5 ml) and diluted up to 10 ml to get the desired concentrations for linearity in the range of 25 – 150µg/ml. the prepared solutions were filtered through 0.45µm membrane filter and each of the dilutions was injected three times into the column. The calibration curve for RIT was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis). It was found to be linear in the concentration range 25-150µg/ml with good correlation in between concentration and mean peak area.

Assay of rit

20 tablets were weighed and the contents were removed to obtain the average weight powder. A sample of the powder claimed to contain 100mg of active ingredient, was mixed with 70ml of diluent. The mixture was allowed to stand with intermittent sonication to ensure complete solubility of drug. Further the volume made up with diluent and the resulting solution was passed through 0.45µm membrane filtered. From the filtered stock solution of 1mg/ml an aliquot of this solution (0.2ml) was transferred to a volumetric flask and made up to a sufficient volume with diluent to get desired concentration of 20µg/ml. the prepared dilution was injected three times into the column to obtain chromatogram. From that peak area, the drug content in the tablet was quantified.

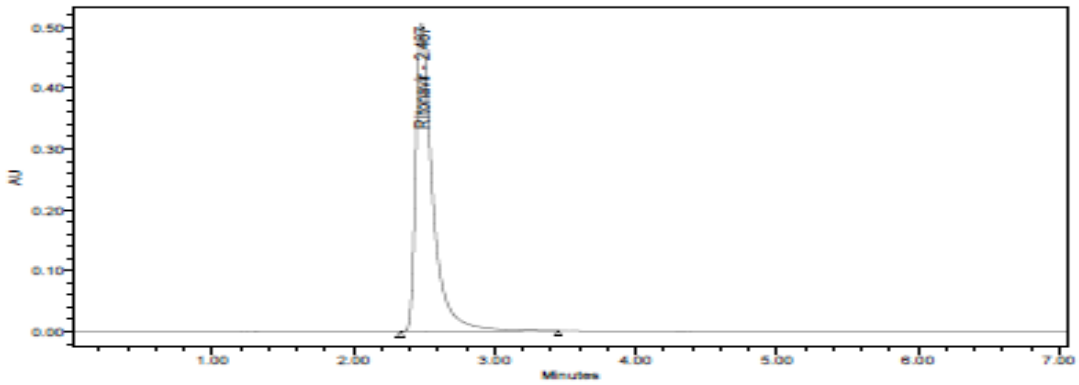

Fig.3; A typical chromatogram of RIT

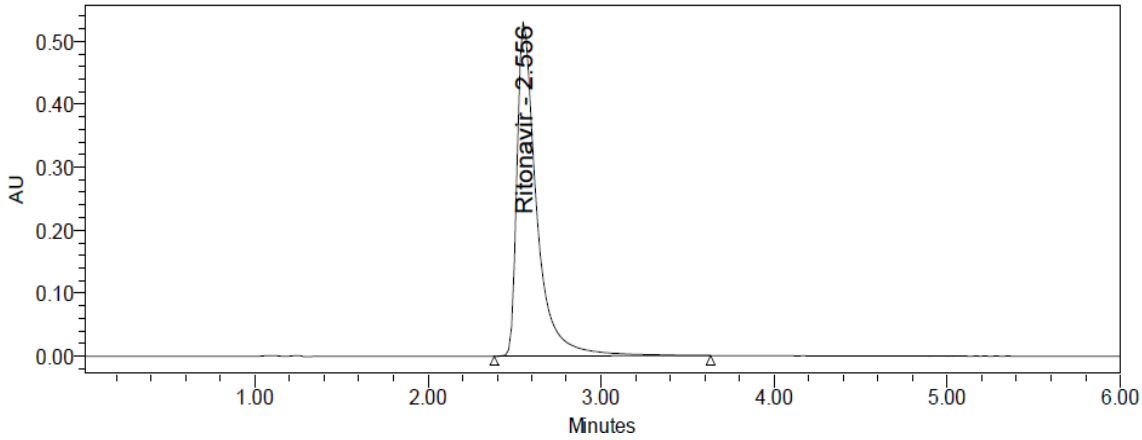

Fig.4: Chromatogram showing the assay of Ritonavir marketed dosage form.

METHOD VALIDATION

As per the International Conference on Harmonization (ICH) guidelines, the method validation parameters such as Specificity, Linearity, Precision, and Accuracy, Limit of Detection / Quantification and Robustness were optimized.

RESULTS

The present RP-HPLC method for the quantification of RIT in bulk and pharmaceutical dosage forms, revealed as simple, rapid, accurate and precise method with significant shorter retention time of 2.487.min. The linearity for the detection of RIT was 25-150µg/ml with (R^2 = 0.999; y = 39440x + 17210) the coefficients of variation based on mean peak area for three replicate injections were not found. Results were shown in table-1 and statistical data of calibration curves were shown in table-2. The intraday and inter day variations of the method were determined using five replicate injections analyzed on the same day and next day over a period of 24 hours. The result revealed the precision with %RSD of 0.37 and 0.4, respectively for intraday and inter day. Results were shown in table-3. To ensure the reliability and accuracy of the method, the recovery studies were carried out by adding a known quantity of drug with pre-analyzed sample and contents were reanalyzed by the proposed method. Accuracy was evaluated by injecting the solution about three times, at three different concentrations equivalent to 50%, 100% and 150% of the active ingredients, by adding a known amount of RIT standard to a sample of known concentration and calculating the recovery of RIT with RSD (%) and recovery for each concentration. The mean % recoveries were in between 99.298 – 100.57% and were given in table-4. The assay for the marketed tablets (EMPETUS tablets) was established with present chromatographic condition developed and it was found to be more accurate and reliable. The average

drug content was found to be 100.01 of the labeled claim and no interfering peaks were found in chromatogram, indicating that the estimation of drug free from inference of excipients. The results were shown in table – 5. To know reproducibility of the method system suitability test was employed to establish the parameters such as tailing factors, theoretical plates, limit of detection and limit of quantification and values were shown in table-6. Ruggedness of the method (intermediate precision) was estimated by preparing three Dilutions of the RIT as per the proposed method and each dilution injected into column. The results were shown in table -7. Robustness of the proposed method was estimated by changing mobile phase composition from buffer: acetonitrile (70:30) v/v to buffer: acetonitrile 65:35 (v/v), changing the flow rate from 1ml to 1.2 ml/min, changing the temperature ($\pm$ 5^{o}c) and system suitability parameters were found to be within acceptable limits[17]. Results were shown in table-8 and indicating that the test method was robust for all variable conditions. Hence the method was sufficiently robust for normally expected variations in chromatographic conditions. The ruggedness and robustness for the method was performed as per ICH guidelines. Limit of detection (LOD) and quantification (LOQ), the limits of detection and quantification were calculated by the method based on the standard deviation (σ) and the slope (S) of the calibration plot, using the formulae LOD = 3.3 σ/s LOQ=10 σ/s. The specificity test of the proposed method demonstrated that the excipients from tablets do not interfere in the drug peak. Furthermore, well shaped peaks indicate the specificity of the method. The typical chromatograms of RIT standard and tablet dosage form were shown in Fig.3 and 4.

Table 1; Concentration Vs Mean Peak Area

Concentration (µg/ml)	**Mean peak area**
25	1088339
50	1927768
75	2965182
100	3945579
125	4931638
150	5967783
Regression Equation	y = 39440x – 17210
Correlation coefficient(r2)	0.999

Table 2; Statistical Data of Calibration Curves of RIT

Parameters	**RIT**
Linearity	25 – 150 µg/ml
Regression Equation	39440x – 17210
Standard deviation of Slope	154.5
Average of intercept	17210.3
Standard deviation of intercept	6341.86
Correlation coefficient (r^2)	0.999

Table 3; Precision of method

Parameters (n=6)	**Intraday**	**Interday**
Mean Peak Area	4156195	4241271
Standard deviation	15377.91	15251.8
%RSD	0.37	0.4

Table 4: Recovery Study of Method

Standard of Drug (µg)	% of drug Added	Amount Present (µg)	Mean amount found (n=3)	Mean % recovery
100	50	50	50.47±0.03	100.09
100	100	100	99.45 ± 0.06	99.45
100	150	150	151.40 ± 0.12	100.93

Average mean % recovery = 100.45, Standard deviation = 0.77, %RSD = 0.767

Table 5: Estimation of amount of RIT

Brand name of	Tablet	Label claim (mg)	Amount of drug estimated (mg)	Mean amount	%RSD
EMPETUS		100	100.01	100.01± 0.44	0.45

Table 6: System suitability Parameters

Parameters	RIT
Retention factor	2.591
Theoretical plates	2896
Tailing factor	1.73

Table 7: Ruggedness of method

S.No	Labeled Claim (mg)	Amount estimated (mg)	Mean ± S.D	%RSD
Analyst - 1	100	100.45	100.45 ± 0.77	0.767
Analyst - 2	100	100.01	100.01 ± 0.45	0.45

Table 8: Robustness of method

Parameter	Variation	Theoretical Plates	Tailing factor	%RSD
Standard	-	4842	1.36	0.37
Flow rate	0.8 ml	4904	1.78	0.6
	1.2 ml	4748	1.77	0.7
Mobile phase	65:35	4753	1.7	0.2
	75:25	4739	1.7	0.3
Temperature	-5^{o}C	4767	1.74	1.0
	+5^{o}C	4876	1.77	0.9

DISCUSSION

The development of HPLC methods for the determination of drugs has received considerable attention in recent years because of their importance in the quality control of drugs and drug products. The goal of this study was to develop and validate a RP-HPLC method for the estimation of RIT in bulk and pharmaceutical commercial preparations. The main objective of method development was to determine the drug content present in the formulation and its % purity. The chromatographic conditions like mobile phase composition, flow rate was optimized and the method was developed, validated successfully. The selected mobile phase system gave a single sharp peak without interfering peaks. Initial development of the method various mobile phases were tried to get sharp peak, finally Potassium Dihydrogen Phosphate buffer (pH was adjusted to 3.5): acetonitrile in the ratio of 70:30 (v/v) was selected which gave a single sharp peak with retention of 2.487 min and tailing factor 1.70. Commercial marketed formulation of RIT was analyzed for its contents and % of content was calculated. The proposed method was found to be simple, rapid, accurate and the method was applicable to routine laboratory analysis. Greater reproducibility was obtained for calibration plots and it was determined by calculating the slope, intercept and %RSD for each standard plot.

CONCLUSION

All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was simple, accurate and sensitive RP-HPLC method for the estimation of RIT in bulk and pharmaceutical dosage forms. The ritonavir tablets analyzed by the validated method showed adequate quality and drug contents in concordance with the labeled amount.

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