

## DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR RITONAVIR BY HPLC

R. ARUN<sup>1</sup>, A. ANTON SMITH<sup>2\*</sup><sup>1</sup>Karpagam University, Pollachi Main Road, Eachanari Post, Coimbatore-641021, Tamil Nadu, <sup>2</sup>Department of Pharmacy, Annamalai University, Annamalai Nagar-608 002, Tamil Nadu, India

Received: 13 Mar 2012, Revised and Accepted: 18 Apr 2012

## ABSTRACT

This paper describes the development and validation of a HPLC method (254nm) for the quantitation of ritonavir in pure form and pharmaceutical formulations. The method shown here is linear ( $r^2 > 0.999$ ), precise (R.S.D.  $< 0.66\%$ ), accurate (recovery of 100.29 % for ritonavir), specific and robust. Three batches of ritonavir tablets were assayed by the validated method. The ritonavir contents in the tablets varied from 99.21 to 100.35 %.

**Keywords:** Ritonavir, Antimalrials, HPLC-UV.

## INTRODUCTION

Ritonavir is an antiretroviral drug, chemically known as 10-Hydroxy-2-methyl-5- (1-methylethyl) -1- [2-(1-methylethyl) -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\*)]. The Chemical structure of Ritonavir is given in (Figure 1) from the protease inhibitor class used to treat HIV infection and AIDS <sup>1</sup>.

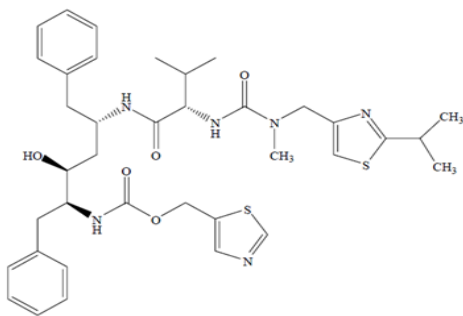


Fig. 1: Chemical Structure of Ritonavir

Ritonavir is frequently prescribed with Highly Active Anti-Retroviral Therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. This inhibition leads to higher plasma concentrations of these latter drugs, allowing the clinician to lower their dose and frequency and improving their clinical efficacy.

It is official in Indian Pharmacopoeia and United States Pharmacopoeia <sup>2, 3</sup>. From the literature survey, it was found that Ritonavir can be estimated by analytical methods such as Reversed Phase High Performance Liquid Chromatographic (RP-HPLC) method, LC-MS and HPTLC method <sup>4,5,6,7,8,9,10,11,12,13,14</sup>. And the simultaneous determination of Ritonavir with other antiretrovirals in human plasma by RP-HPLC <sup>15, 16, 17</sup>.

Hence, the aim of this study was to develop and validate a simple HPLC method, using UV detection to quantify ritonavir in pure form and pharmaceutical formulations. Due to the low molar absorptivity of ritonavir, the UV region was found to be at 254nm. The validated method was applied to the analysis of tablets containing ritonavir (100 mg).

## MATERIALS AND METHODS

## Reagents and materials

Ritonavir reference standards were purchased from Glenmark Laboratories, Mumbai. Tablets were purchased from local pharmacies, manufactured by Cipla Laboratories (Mumbai, India) Ritomune<sup>®</sup>. Ultra-pure water was obtained from a Millipore system

(Bedford, MA, USA). Acetonitrile, Methanol (HPLC grade) was obtained from E-Merck (India) Ltd - Mumbai - India. All other chemicals used in the analysis were AR grade.

## Instrumental and analytical conditions

The HPLC analyses were carried out on Thermo scientific (Spectra serious P4000) system with UV detector, Hypersil (ODS) C8 (250 × 4.6mm, 5 $\mu$ m) column from Thermo, USA. UV detection was performed at 254nm. UV spectra from 190 to 400 nm were online recorded for peak identification. The injection volume of sample was 20  $\mu$ l. An isocratic mobile phase containing acetonitrile, methanol and 0.01M potassium dihydrogen orthophosphate buffer (30:20:50), at the pH 3.0 was carried out with the flow rate of 1ml/min. The separation of ritonavir was evaluated in different proportions of these solvents and, for each condition, retention factor (k) and resolution (R) was calculated.

## Preparation of standard solution

Approximately 100mg of ritonavir reference standard was accurately weighed and transferred to a 100 ml volumetric flask, 10 ml of acetonitrile was added to ensure the complete solubilisation and the volume was adjusted with the mobile phase. Further dilutions were made to get the final concentration of 0.1 mg/ml of ritonavir. The solution was filtered through 0.45 $\mu$  membrane filter.

## Preparation of internal standard solution

Approximately 100mg of lopinavir reference internal standard was accurately weighed and transferred to a 100 ml volumetric flask, 10 ml of acetonitrile was added to ensure the complete solubilisation and the volume was adjusted with the mobile phase. Further dilutions were made to get the final concentration of 0.1 mg/ml of lopinavir. The solution was filtered through 0.45 $\mu$  membrane filter.

## Analysis of fixed dose tablets

Three different batches of Ritomune<sup>®</sup> were analyzed using the validated method. Ritonavir standard was added to the samples, with the aim of increasing the peak area of ritonavir in the chromatograms and thereby improving the detection of this compound. For the analysis, six replicates of each batch were assayed. The tablets were weighed and finely powdered. An accurately weighed portion of the powder, equivalent to about 50mg of ritonavir was transferred to a 100 ml volumetric flask followed by the addition of 25 ml of acetonitrile. The solution was sonicated for 3 minutes and diluted with mobile phase to volume. Further dilutions were made to get the final concentration equivalent to 100  $\mu$ g/ml of ritonavir.

## Validation

## Linearity

Standard solutions containing 0.1mg/ml of ritonavir was prepared, in triplicate. Aliquots of these solutions were diluted in mobile phase to five different concentrations, corresponding to 80, 90, 100, 110,

120 µg/ml of ritonavir. Calibration curves for concentration versus peak area were plotted for ritonavir obtained data were subjected to regression analysis using the least squares method with a weighting factor of  $1/x$ .

### Precision

The intra-day precision was evaluated by analyzing six sample solutions ( $n = 6$ ), at the final concentration of analyses (100 µg/ml) of ritonavir. Similarly the inter-day precision was evaluated in three consecutive days ( $n=18$ ). The ritonavir concentrations were determined and the relative standard deviations (RSD) were calculated.

### Accuracy

Ritonavir reference standards were accurately weighed and added to a mixture of the tablets excipients, at three different concentration levels (110, 120 and 130 µg/ml of ritonavir). At each level, samples were prepared in triplicate and the recovery percentage was determined.

### Specificity

Spectral purities of ritonavir chromatographic peaks were evaluated using the UV spectra recorded by a UV detector. In addition, a solution containing a mixture of the tablets excipients was prepared using the sample preparation procedure and injected on to the chromatograph, to evaluate possible interfering peaks.

### Robustness

Six sample solutions were prepared and analyzed under the established Conditions and by variation of the following analytical parameters: flow rate of the mobile phase (0.8, 1.1 and 1.3 ml/min), acetonitrile, methanol and buffer as mobile phase (25:25:50, 20:20:60, 30:30:40, 30:20:50), mobile phase pH (2.8, 3.2, 3.4) and column temperature (23, 25 and 27°C). The ritonavir contents was determined for each condition and the obtained data was submitted

for statistical analysis (ANOVA test)

### Detection and quantitation limits

Limit of detection LOD (signal-to- noise ratio of 3) and limit of quantification LOQ (signal-to- noise ratio of 10) were measured based on the signal-to-noise ratio. Determination of the signal-to-noise is performed by comparing measured signals samples with known low concentration of analyte with those of blank samples establishing the minimum concentration at which the analyte can be reliably detected and quantified.

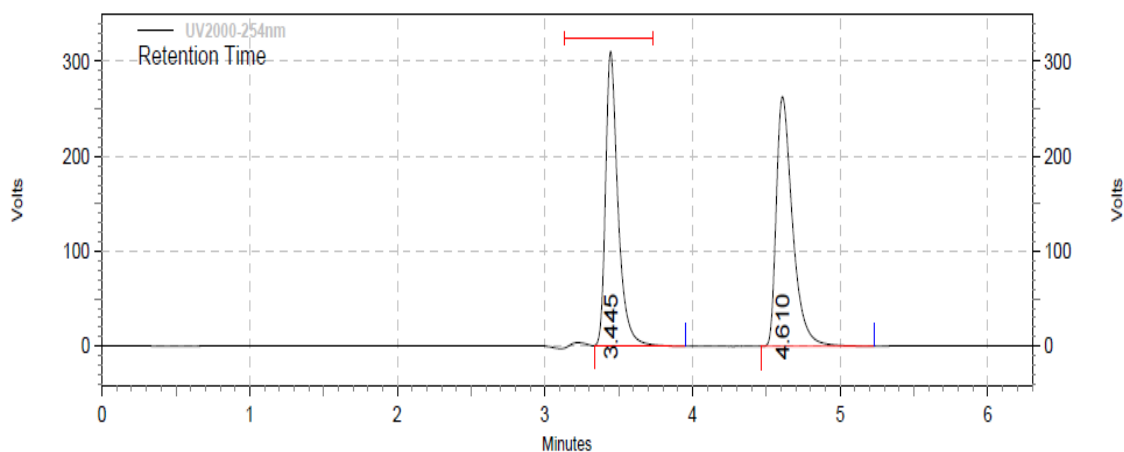
### RESULTS AND DISCUSSION

The chromatographic parameters were initially evaluated using a Thermo Hypersil (ODS) C8 (250 × 4.6mm, 5µm) column and a mobile phase composed of acetonitrile, methanol and 0.01M potassium dihydrogen orthophosphate buffer (30:20:50). Using this column, different proportions of mobile phase solvents were evaluated, to obtain a good peak (Table 1).

**Table 1: Chromatographic Parameters for ritonavir at different mobile phase composition using Thermo Hypersil (ODS) C8 (250 x 4.6mm, 5µm) column**

Mobile phase composition Acetonitrile: Methanol: Buffer	Ritonavir Retention factor (k)
25:25:45	1.66
20:20:60	2.34
30:30:40	2.09
30:20:50	5.18

Under these conditions the retention factor obtained for ritonavir was 3.44 and a short run time (5 min), and so, this condition was adopted in subsequent analysis (Figure 2).



**Fig. 2: Typical sample Chromatogram of ritonavir and internal standard. Retention time of ritonavir 3.44 & lopinavir 4.61minutes**

After the evaluation of the ritonavir UV spectrum in the range of 200-400 nm, the wavelength of 254 nm was selected for detection, due to the adequate molar absorptivity of ritonavir in this region and the higher selectivity of this wavelength regarding possible interfering compounds or solvents in the sample.

### Validation

#### Linearity

A linear correlation was found between the peak areas and the concentrations of ritonavir in the assayed range. The regression analysis data were presented in Table 2.

**Table 2: Regression parameters data for ritonavir**

Regression parameters	Ritonavir
Regression coefficient ( $r^2$ )	0.999
Slope $\pm$ standard error	0.010 $\pm$ 0.02
Intercept $\pm$ standard error(%)	0.06 $\pm$ 0.1
Relative standard error (%)	0.18
Concentration range(µg/ml )	80-120
Number of points	5

The regression coefficients ( $r^2$ ) obtained was higher than 0.999 for the ritonavir (Figure 3), which attest the linearity of the method.

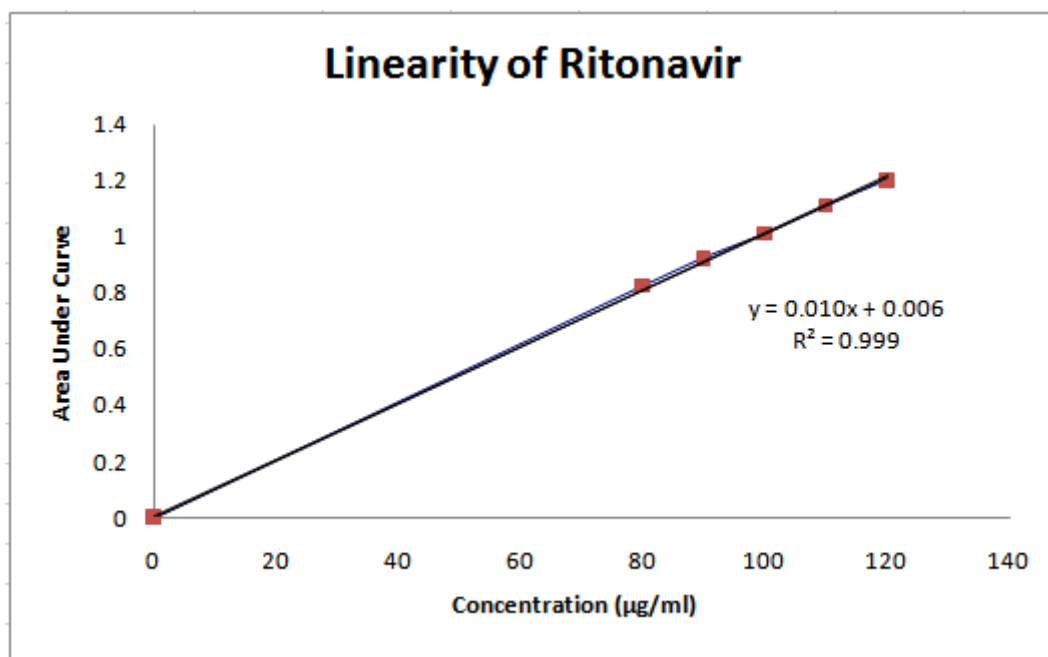


Fig. 3: Linearity profile of ritonavir

#### Precision

Mean contents of ritonavir in the intra-day precision analysis ( $n = 6$ ) was 100 µg/ml (RSD = 0.66%). For the intra-day precision ( $n=18$ ) the mean contents obtained for ritonavir was 50.3 µg/ml (RSD = 0.67%). RSD values lower than 2.0% assure the precision of the method.

#### Accuracy

It was investigated by means of addition of ritonavir reference standards to a mixture of the tablets excipients. ritonavir mean recovery ( $n = 9$ ) was 100.29% (RSD. = 0.210 %) demonstrating the accuracy of the method.

#### Specificity

Peak purities higher than 99.0% were obtained for ritonavir in the chromatograms of sample solutions, demonstrating that other compounds did not co-elute with the main peaks. The chromatogram obtained with the mixture of the tablets excipients showed no interfering peaks in the same retention time of ritonavir.

#### Robustness

Statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method and those obtained in the experiments in which variations of some parameters were introduced. Thus, the method showed to be robust for changes in mobile phase flow rate from (0.8, 1.1 and 1.3 ml/min), acetonitrile, methanol and buffer proportion from (25:25:50, 20:20:60, 30:30:40, 30:20:50), mobile phase pH (2.8, 3.2, 3.4) and column temperature (23, 25 and 27°C).

#### Detection and quantitation limits

According to the determined signal-to-noise ratio, ritonavir presented limits of detection of 14µg/ml and limits of quantitation of 0.57 µg/ml, where the compounds proportion found in the sample solutions injected onto the chromatograph. However, the objective of the method is the quantitation of ritonavir, so that the values obtained for ritonavir should be considered as the limit of method sensitivity.

#### Analysis of fixed dose combination tablets

Samples of fixed dose combination tablets Ritomune® containing 100 mg of ritonavir was analyzed using the validated method. The results obtained were presented in Table 3.

Table 3: Contents of ritonavir in the fixed dose tablets (n=6)

Sample tablet	Batch	Content (%)+S.D.Ritonavir
Ritomune®	A	99.21±0.23
	B	99.80±0.46
	C	100.35±0.42

S.D=Standard Deviation

All the analyzed batches presented ritonavir were very close to the labeled amount. The ritonavir content in the tablets samples varied from 99.21 to 100.35%.

The development of simple and reliable method is essential to assure the identification and quantitative determination of antimalarial drugs, since the problem of counterfeit or substandard antimalarials is well established all over the world. The quality control of the antimalarial pharmaceutical preparations marketed nowadays may help to assure the treatment efficacy and avoid the development of resistance to antimalarial drugs.

#### CONCLUSION

This study was the first report of development and validation of ritonavir in pure form and pharmaceutical formulations in the presence of internal standard lopinavir. The developed method showed to be a simple and suitable technique to quantify the antimalarial and might be employed for quality control analysis, as well as in further studies in other matrices, such as plasma. The ritonavir tablets analyzed by the validated method showed adequate quality and drug contents in concordance with the labeled amount.

#### REFERENCES

- Raffanti S, Haas DW, Goodman and Gilman's: The Pharmacological Basis of 422 Therapeutics, McGraw-Hill (2001) 1371–1372
- Indian Pharmacopoeia, Vol. III, 2007; 1058.
- United States Pharmacopoeia 30, National Formulary 25, 2007; 3143
- Richard M, Hoetelmans W, Marjolijn Van Essenberg, Monique Profijt, Pieter L. Meenhorst, Jan W. Mulder, *et al.* High-Performance Liquid Chromatographic determination of Ritonavir in human plasma, cerebrospinal fluid and saliva. Journal of Chromatography B: Biomedical Sciences and Applications 1998; 705(1):119-126.

5. Rebiere Herve, Mazel Bernard, Civade Corinne, Bonnet Pierre-Antoine. Determination of 19 antiretroviral agents in pharmaceuticals or suspected products with two methods using High-Performance Liquid Chromatography. *Journal of chromatography B* 2007; 850:376-383.
6. Yekkala RS, Ashenafi D, Marien I, Xin H, Haghedooren E, Hoogmartens J *et al.* Evaluation of an International Pharmacopoeia method for the analysis of Ritonavir by Liquid Chromatography. *Journal of pharmaceutical and biomedical analysis* 2008; 48(3):1050-4.
7. Veronica Albert, Pilar Modamio, Cecilia FL and Eduardo LM. Determination of Saquinavir and Ritonavir in human plasma by RP-HPLC and the analytical error function. *Journal of Pharmaceutical and Biomedical Analysis* 2004; 36(4):835-840.
8. Usami Yoshiko, Tsuyoshi OK, Naka Masahiko, Sagisaka Masafumi, Kaneda Tsuguhiro. A simple HPLC method for simultaneous determination of Lopinavir, Ritonavir and Efavirenz. *Journal-Chemical and pharmaceutical bulletin* 2003; 51:715-718.
9. Dias CL, Rossi RC, Donato EM, Bergold AM and Froehlich PE. LC Determination of Ritonavir, a HIV Protease Inhibitor, in Soft Gelatin Capsules. *J Chromatographia* 2005; 62:589-593.
10. Proust V, Toth K, Hulin A, Taburet AM, Gimenez F, Singlas E. Simultaneous High-Performance Liquid Chromatographic determination of the antiretroviral agents' Amprenavir, Nelfinavir, Ritonavir Saquinavir, Delavirdine and Efavirenz in human plasma. *Journal of chromatography B* 2000; 742:453-458.
11. Temghare GA, Shetye SS, Joshi SS. Rapid and Sensitive Method for Quantitative Determination of Lopinavir and Ritonavir in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry. *E-Journal of Chemistry* 2009; 6(1):223-230.
12. Sulebhavikar AV, Pawar UD, Mangoankar KV, Prabhunavelkar ND. HPTLC Method for Simultaneous Determination of Lopinavir and Ritonavir in Capsule Dosage Form. *E-Journal of Chemistry* 2008; 5(4):706-712.
13. Chiranjeevi K and Channabasavaraj KP. Development and validation of rp-hplc method for quantitative estimation of ritonavir in bulk and pharmaceutical dosage forms. *International Journal of Pharmaceutical Sciences and Research*, 2011; 2(3): 596-600.
14. Agn`es Rouzes, Karine Berthoin, Fabien Xuereb, Sarah Djabarouti, Isabelle Pellegrin, Jean Luc Pellegric. Simultaneous determination of the antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by high-performance liquid chromatography-mass spectrometry. *Journal of Chromatography B* 2004; 813: 209-216.
15. Chutima matayatsuk Phechkrajang, Ei Ei Thin, Lawan Sratthaphut, Duangjai Nacapricha, Prapin Wilairat, Chemometrics-assisted UV spectrophotometric method for determination of Lopinavir and ritonavir in syrup, *International Journal of Pharmacy and Pharmaceutical Sciences* 2012; 4(1), 492-496.
16. Kakadiya PR, Pratapa Reddy B, Singh V, Ganguly S, Chandrashekar TG, Singh DK. Low level determinations of methyl methanesulfonate and ethyl methanesulfonate impurities in Lopinavir and Ritonavir Active pharmaceutical ingredients by LC/MS/MS using electrospray ionization. *Journal of Pharmaceutical and Biomedical Analysis* 2011; 55 : 379-384.
17. John Raya, Edna Panga, Dianne Carey. Simultaneous determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma by high-performance liquid chromatography. *Journal of Chromatography B* 2002; 775: 225-230.