DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR PROTEASE INHIBITOR - RITONAVIR

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ABSTRACT
Ritonavir is chemically designated 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 etraazatridecan-13-oic acid, 5-thiazolylmethyl ester. The present study was to validated reverse phase high performance chromatography (RP-HPLC) method for the estimation of ritonavir. The RP-HPLC separation was achieved on Waters HPLC system, pump (Model Waters 515 HPLC) operating at 1ml/min, a syringe loading sample injector of 20µl capacity (Model 7725i), a C18 reverse phase column of 250×4.6mm dimension and 5µ particle size and a dual wavelength UV-Visible detector (Model 2487) by using acetonitrile:water:glacial acetic acid (60:40:0.1%V/V) as mobile phase at an wavelength 240nm. The concentration range 0.01- 10 µg/ml with accuracy, precision and linearity.

KEY WORDS: Ritonavir, RP-HPLC, Protease Inhibitors.

1.INTRODUCTION
Retrovirus is the etiologic causative agent of acquired immunodeficiency syndrome (AIDS). Protease is an enzyme which is essential for the viral growth. These enzyme actions can be inhibited by the protease inhibitors, mainly in this class Indinavir, Ritonavir, Amprenavir, Nelfinavir, Atazanvivir, Saquinavir drugs are used in the treatment of HAART (Highly Active Anti-Retro viral Therapy) (Rang HP, 2007).

The mRNA of virus is transcribed from the provirus, which is translated into two biochemically inert polyproteins. A virus-specific protease than converts the polyproteins into various structural and functional proteins by cleavage at the appropriate positions, HIV-specific protease inhibitors bind to the site where cleavage occurs, and their use in combination with reverse transcriptase inhibitors (Flexner, 1998).

Ritonavir is chemically designated 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 etraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)]. Its molecular formula is C_{37}H_{48}N_{6}O_{5}S_{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 (Abbott Laboratories, 2010). Ritonavir has the following structural formula:

![Ritonavir Structural Formula]

The aim of the present study is to develop a valid, reliable and convenient HPLC-based method For the determination of ritonavir.

2.EXPERIMENTAL
Chemicals and reagents: Ritonavir, Zidovudine powder was kindly gifted by Aurabindo Pharmaceuticals, HPLC grade Acetonitrile, Glacial acetic acid were purchased from Finar chemicals (Ahmedabad) and the water for HPLC was prepared by distillation method (double distillation), which is filtration through a nylon 0.45µm membrane filter (Axiva semi bio tech, Delhi). All solvents were of HPLC grade.

Chromatographic equipment and conditions: The Waters HPLC system is used in the study consisted of a pump (Model Waters 515 HPLC pump) operating at 1ml/min, a syringe loading sample injector of 20µl capacity (Model 7725i), a C18 reverse phase column of 250x4.6mm dimension and 5µ particle size and a dual wavelength UV-Visible detector (Model 2487). The data analysis was done by Wufeng-chrom workstation (Shanghai Wufeng scientific instruments Co.Ltd., Shanghai).

The mobile phase was consisted of Acetonitrile: Water: Glacial acetic acid (60: 40:0.1 % v/v) was pumped at a flow of 1ml/min. The mobile phase was filtered through 0.4 µm membrane filter and degassed before use. The elution was monitored at 240nm and injection volume was 20µl and the total run time of method was set at 15 min.
Standard preparation: The initial stock solution of ritonavir was prepared by dissolving 25mg in 25ml volumetric flask and diluted to the mark with acetonitrile to obtain the standard stock solution of ritonavir (1mg/ml). Standard solutions were obtained by serial dilution of the stock solution (10 µg/ml) and working solutions with concentrations of 0.1, 0.3, 0.5, 1, 3, 5, 10, 30 and 50 µg/ml for the preparation of calibration and quality control (QC) of samples. Ritonavir calibration standard samples were prepared with concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/ml. The each sample of 100 µl of internal standard was added, and 20µl of this solution was injected into HPLC analysis. Analysis of ritonavir with different concentrations i.e. low (0.01 µg/ml), medium (0.5 µg/ml), high (10 µg/ml), which were freshly prepared to evaluate accuracy and precision of HPLC method.

HPLC method validation
Specificity and selectivity: In order to evaluate levels of endogenous compounds with potential interference with the analytical method, analysis of different concentrations was performed by comparing chromatograms of blank mobile phase, sample with Ritonavir and Zidovudine.

Limit of detection and limit of Quantification: The limit of detection (LOD) was defined by the lowest detectable concentration yielding a Signal-to-noise ratio of three, indicating a significant difference between spiked samples and blank samples of three individuals as determined by the Student’s –test. For the concentration to be accepted as the lower limit of quantification (LOQ), the measure of accuracy (percent deviation from the nominal concentration) and precision (relative standard deviation) have to be less than 20%. All samples were assayed in triplicate.

Accuracy, precision, linearity: Intra-day, inter-day accuracy and precision of the methods were determined by the measuring three replicate samples at three different concentrations of Ritonavir, which were mentioned above. These things were validated as per the ICH guidelines (IFPMA 1994, 1996).

3. RESULTS
Method development: Ritonavir was scanned in the UV-Visible spectrophotometer (Shimadzu, UV-1800) at the range of 200-800. The maximum absorbance was found to be at 240nm, so, this wave length has been used for the detection in HPLC. The mobile phase used for the assay has achieved the separation of Ritonavir and Zidovudine (internal standard (IS)) without interference from the other components.

Method validation
Specificity and selectivity: Fig. 1 and Fig. 2 represents chromatograms of Ritonavir and Zidovudine (IS). No interference with endogenous peaks with Ritonavir or Zidovudine at their retention times (Ritonavir $t_R=8.6$ min, Zidovudine $t_R=2.6$ min) in samples was observed.

Limit of detection and limit of Quantification: The LOQ of Ritonavir was found to be 0.05 µg/ml. The LOD of Ritonavir was found to be 0.055µg/ml.

Accuracy, precision, linearity: The accuracy was carried out by the standard addition method. The precision %RSD (Relative standard deviation) values of intraday and interday were found to be 0.771 and 0.618 of Ritonavir. Linearity was obtained between peak area ratio Vs concentration (µg/ml) of Ritonavir in the range of 0.01-10µg/ml respectively. In table2 mentioned the correlation co-efficient of regression ($r^2$) and intercept as well as slope.

4. CONCLUSION
Many methods were available to determine the concentration of Ritonavir, but we present here a validated, reliable and convenient assay for the simultaneous determination of peak area and concentration levels of ritonavir. The developed procedure was most suitable, the developed method is simple, accurate, precise, specific, and could separate the drug.

5. ACKNOWLEDGEMENTS
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![Figure 1: Ritonavir Peak in mobile phase](image1)

![Figure 2: Zidovudine(IS) Peak in mobile phase](image2)
Table 1: The validation of Parameters of Ritonavir

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ritonavir (RP-HPLC)</th>
</tr>
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<tbody>
<tr>
<td>Accura</td>
<td>99.1%</td>
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<tr>
<td>Precision (%RSD)</td>
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<tr>
<td>Interday</td>
<td>0.771</td>
</tr>
<tr>
<td>Intraday</td>
<td>0.618</td>
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%RSD (Relative standard deviation)

Table 2: Regression analysis of the calibration (linearity) curve for Ritonavir

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ritonavir (RP-HPLC)</th>
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<tr>
<td>Concentration(µg/ml)</td>
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<td>Slope</td>
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<tr>
<td>Correlation coefficient</td>
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References


