DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR RITONAVIR BY HPLC

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ABSTRACT

This paper describes the development and validation of a HPLC method (254 nm) for the quantitation of ritonavir in pure form and pharmaceutical formulations. The method shown here is linear (r² > 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) -12 - tetraazatridecan-13-oic acid, 5-thiazolymethyl ester, [[5S -(5R*, 8R*, thiazolyl] -3, 6-dioxo-8, 11-bis (phenylmethyl) -2, 4, 7, 12 - methoxy]-2-methyl-5-(1-methylethyl)-4-thiazolyl]-3, 6-dioxo-8, 11-bis (phenylethyl)-2, 4, 7, 12-tetraazatridecan-13-oic acid, 5-thiazolymethyl ester, [[5S -(5R*, 8R*, thiazolyl] -3, 6-dioxo-8, 11-bis (phenylmethyl) -2, 4, 7, 12 - method, LC-MS and HPTLC method 4,5,6,7,8,9,10,11,12,13,14. And the 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 254 nm. 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®. 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 series P4000) system with UV detector, Hypersil (ODS) C8 (250 x 4.6mm, 5µm) column from Thermo, USA. UV detection was performed at 254 nm. UV spectra from 190 to 400 nm were online recorded for peak identification. The injection volume of sample was 20 µl. 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 1 ml/min. The separation of ritonavir was evaluated in different proportions of these solvents and, for each condition, retention factor ($) and resolution (R) was calculated.

Preparation of standard solution

Approximately 100 mg 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 of ritonavir. The solution was filtered through 0.45 µm membrane filter.

Preparation of internal standard solution

Approximately 100 mg 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 ritonavir. The solution was filtered through 0.45 µm membrane filter.

Analysis of fixed dose tablets

Three different batches of Ritomune® 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 50 mg 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 µg/ml of ritonavir.

Validation

Linearity

Standard solutions containing 0.1 mg/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 (25:25:45, 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 x 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

<table>
<thead>
<tr>
<th>Mobile phase composition</th>
<th>Acetonitrile: Methanol: Buffer</th>
<th>Ritonavir Retention factor (k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25:25:45</td>
<td></td>
<td>1.66</td>
</tr>
<tr>
<td>20:20:60</td>
<td></td>
<td>2.34</td>
</tr>
<tr>
<td>30:30:40</td>
<td></td>
<td>2.09</td>
</tr>
<tr>
<td>30:20:50</td>
<td></td>
<td>5.18</td>
</tr>
</tbody>
</table>

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 4.61 minutes

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

<table>
<thead>
<tr>
<th>Regression parameters</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression coefficient (r²)</td>
<td>0.999</td>
</tr>
<tr>
<td>Slope ± standard error</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td>Intercept ± standard error (%)</td>
<td>0.06±0.1</td>
</tr>
<tr>
<td>Relative standard error (%)</td>
<td>0.18</td>
</tr>
<tr>
<td>Concentration range(µg/ml)</td>
<td>80-120</td>
</tr>
<tr>
<td>Number of points</td>
<td>5</td>
</tr>
</tbody>
</table>

The regression coefficients (r²) obtained was higher than 0.999 for the ritonavir (Figure 3), which attest the linearity of the method.
Precision

Mean contents of ritonavir in the intra-day precision analysis \((n = 6)\) was 100 µg/ml \((\text{RSD} = 0.66\%)\). For the intra-day precision \((n = 18)\) the mean contents obtained for ritonavir was 50.3 µg/ml \((\text{RSD} = 0.67\%)\). 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% \((\text{RSD} = 0.210\%)\) 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 \(27^\circ C)\).

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 Ritonune® 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 \((n=6)\)

<table>
<thead>
<tr>
<th>Sample tablet</th>
<th>Batch</th>
<th>Content (µg/ml) + S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritonune® A</td>
<td>B</td>
<td>99.21 ± 0.23</td>
</tr>
<tr>
<td>Ritonune® B</td>
<td>C</td>
<td>99.80 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>100.35 ± 0.42</td>
</tr>
</tbody>
</table>

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


Fig. 3: Linearity profile of ritonavir

![Linearity profile of ritonavir](image-url)


