Stability indicating RP-HPLC method for simultaneous estimation of Lopinavir and Ritonavir

Sanjay G. Walode* and Monali R. Bhalerao

Department of Pharmaceutical Chemistry, Sinhgad Institute of Pharmaceutical Sciences, Lonavala, Pune, Maharashtra, India

ABSTRACT

Present work describes a precise, accurate and reproducible RP-HPLC method for simultaneous estimation of Lopinavir and Ritonavir. The drugs were resolved using a mobile phase of buffer (diammonium hydrogen phosphate pH 3.5 : acetonitrile (50:50 v/v) on an (250mm x 4.6 mm, 5µm) Water Spherisorb ODS column in isocratic mode. Recovery values of 99.87-101.27 %, percentage relative standard deviation of <2 and correlation coefficient of 0.995–0.995 shows that the developed methods were accurate and precise. For stability study, the drug was exposed to the stress conditions such as acid, base, oxidation, neutral and sunlight. As per ICH guidelines the results of the analysis were validated in terms of specificity, limit of detection, limit of quantification, linearity, precision and accuracy and were found to be satisfactory. These methods can be employed for the routine analysis of capsule containing lopinavir and ritonavir.

Keywords: Reverse phase liquid chromatography, Lopinavir, Ritonavir, Method validation

INTRODUCTION

Lopinavir and ritonavir is a human immunodeficiency virus (HIV) protease inhibitor and chemically designated as (2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethylphenoxy)Acetamido]-4-hydroxy-1,6-diphenylhexane-2-yl]-3-methyl-2-(2-oxo-1,3-diazine-1-yl)butanamide and (55,8S,10S,11S)-10-Hydroxy-2-methyl-5-(1-methyl ethyl)-1-[2-(1-methyl ethyl)-4-thiazoly]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-etaazatridecan-13-oic acid 5-thiazolyl methyl ester respectively. The chemical structure of lopinavir and ritonavir were given in (fig.1). This class of drugs inhibits the HIV protease preventing cleavage of the gag-pol polyprotein, reducing the probability of viral particles reaching a mature infectious state. Administered alone, lopinavir has insufficient bioavailability. However, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4 [2,3] and therefore lopinavir is co-administered with sub-therapeutic doses of ritonavir by oral route of administration. Several HPLC methods have been described in the literature for the determination of lopinavir. These are determination of lopinavir alone and simultaneously with other antiretrovirals in human plasma by RP-HPLC.
The aim of the work was to introduce a simple, accurate and reproducible isocratic stability indicating RP-HPLC method for simultaneous determination of LOP and RIT. The proposed methods were optimized and validated as per ICH guidelines [24].

MATERIALS AND METHODS

LOP and RIT were obtained as gift samples from Cipla Pvt. Ltd., Kurkumbh Disodium hydrogen phosphate, HPLC grade Water and Acetonitrile were procured from Merck Ltd, Mumbai, India. Orthophosphoric acid was purchased from Research Lab., Fine Industries, and Mumbai. The commercial formulation of LPO and RIT (Lopumin capsule) procured from local market.

Instrumentation: The HPLC system, Spectrum system 011-4736-5, Shimadzu Corporation with manual Rheodyne injector facility operates at 20 µL capacity per injection was used. The column used was (250mm x 4.6 mm, 5µm) Water Spherisorb ODS column and the detector consisted of UV/VIS UV-1601 Shimadzu Corporation operated at 220 nm.

Chromatographic Conditions: The mobile phase containing buffer (diammonium hydrogen phosphate pH 3.5) : acetonitrile (50:50 v/v) was found to resolve LOP and RIT. The mobile phase was filtered through 0.45 micron nylon filter and then sonicated for 10 min. The flow rate was set to 10 µl/min. Both the drugs shows good absorbance at 220 nm, this wavelength was selected for further analysis. All determinations were performed at constant temperature (40°C). A typical chromatogram shown in Fig.2

Preparation of standard stock solution:
Weigh accurately about 140 mg of LOP and 35 mg of RIT into a 200 ml of volumetric flask and sufficient amount of methanol sonicate to dissolve equilibrate to room temperature and dilute up to the volume mark with methanol.
Preparation of working standard solution:
From the standard stock solution, 5 ml sample was pipette out and diluted to 10 ml with mobile phase.

Analysis of marketed formulation:
Take 20 capsules remove sample powder and weighed. Find out the average. Accurately weighed capsule powder equivalent to 133.3 mg of LOP and 33.3 mg of RIT and transferred into 250 ml volumetric flask. Add sufficient amount of methanol, sonicate for 20 min. cool and dilute to volume with methanol filtered through 0.45 µm syringe filter. Further dilute 5 ml of filtrate to 50 ml with mobile phase so as to get final concentration of LOP (0.0664 mg/ml) and RIT (0.01664 mg/ml)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Area of Standard</th>
<th>Weight of standard (mg)</th>
<th>Area of sample</th>
<th>Weight of sample (mg)</th>
<th>% Purity</th>
<th>% Label claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir</td>
<td>1164956</td>
<td>35</td>
<td>1155363</td>
<td>133.3</td>
<td>99.17</td>
<td>101.59</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>1042115</td>
<td>35</td>
<td>1055230</td>
<td>33.3</td>
<td>101.25</td>
<td>101.69</td>
</tr>
</tbody>
</table>

VALIDATION:
Limit of detection (LOD) and limit of quantification (LOQ): The signal-to-noise ratio (S/N) method was adopted for the determination of limit of detection and limit of quantification. The limit of detection was estimated as three times the S/N ratio and the limit of quantification was estimated as ten times the S/N ratio.

Specificity:
Specificity is the ability of a method to discriminate between the analyte of interest and other components that may present in the sample. The specificity of the method was evaluated to ensure separation of LOP and RIT and was demonstrated by assaying samples of LOP and RIT capsule.

Linearity:
Different standard solutions were prepared by diluting standard stock solution with mobile phase in concentration 28,42,56,70,77,91,105 ppm for LOP and 7,10.5,14,17.5,19.25,22.75,26.25 ppm for RIT, injected to HPLC system and chromatograms were taken under standard chromatographic conditions.

Precision:
Precision of analytical methods were expressed in relative standard deviation (RSD) of a series of measurements. The intra-day and inter-day precisions of the proposed methods were determined by estimating the corresponding responses (i.e. three concentrations / three replicates each) of the sample solution on the same day and on three different days respectively.

Recovery:
To check the accuracy of the proposed method, recovery studies were carried out by applying standard addition method. A known amount of standard LOP and RIT corresponding to 50, 100 and 150% of the label claim was added to preanalysed sample of capsule. The recovery studies were carried out in triplicate at each level.

FORCED DEGRADATION:
Acid and base induced degradation product:
To 10 ml of standard stock solution, 10 ml of 0.1 N HCl and 10 ml of 0.1N NaOH were added separately. These mixtures were reflux separately for 1 hr at 50ºC. The forced degradation study in acidic and basic media was performed in the dark in order to leave out the possible degradative effect of light. 0.4 ml of each resultant solution was diluted to 10 ml with the mobile phase and resultant solution injected into the system.
Hydrogen peroxide induced degradation product:
To 10 ml of standard stock solution, 10 ml of hydrogen peroxide (30 % v/v H₂O₂) was added. This solution was heated in boiling water bath for 10 min to remove completely the excess of hydrogen peroxide and reflux for 30 min at 50°C. 0.4 ml of resultant solution was diluted to 10 ml with the mobile phase and injected into the system.

Neutral hydrolysis:
Ten milliliters of standard stock solution was mixed with 10 ml water and reflux for 60 min at 60°C. 0.4 ml this solution was diluted to 10 ml with the mobile and resultant solution injected into the system.

Photolytic induced degradation product:
Ten milliliters of standard stock solution was exposed to direct sunlight for 4 hr on a wooden plank and kept on terrace. 0.2 ml of resultant exposed solution was transferred to 10 ml volumetric flask, diluted with the mobile phase and solution was injected into the system.
RESULTS AND DISCUSSION

The parameters were focused for improvisation of retention time, separation of degradation products and column life. The Water Spherisorb ODS column provided good peak shapes and no peak splitting was observed. The retention time for LOP and RIT was found to be 3.446 and 5.115 respectively. LOP and RIT showed good linear responses in concentrations level ranging from 28-105 μg/ml (0.995) for LOP and 7-26.25 μg/ml (0.995) for RIT [Fig.8 (Table 2)].

Table 2: Result Linear Regression data of Lopinavir and Ritonavir

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Parameter</th>
<th>Lopinavir</th>
<th>Ritonavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Range</td>
<td>28-105 μg/ml</td>
<td>7.26-25 μg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Regression coefficient</td>
<td>0.995</td>
<td>0.995</td>
</tr>
<tr>
<td>3</td>
<td>% Y-intercept</td>
<td>-0.24</td>
<td>-0.55</td>
</tr>
<tr>
<td>4</td>
<td>% RSD of peak area response of level-1</td>
<td>0.17</td>
<td>0.56</td>
</tr>
<tr>
<td>5</td>
<td>% RSD of peak area response of level-7</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>% RSD of retention time for level-1</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>% RSD of retention time for level-7</td>
<td>0.01</td>
<td>0.01</td>
</tr>
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</table>

Parameter

RP-HPLC SAL GF Limit of detection 6.58 ng/spot and 11.52 ng/spot Limit of quantitation 34.11 ng/spot and 26.22 ng/spot Retention time 3.446 and 5.115 min
Correlation coefficient (r²)
0.995 And 0.995 Calibration range 0.5-5 µg/ml 25-250 µg/ml Regression equation y = 39809x - 1E+06 and y = 15923x - 1E+06

The measurement at three different concentration levels showed low value of % R.S.D. (<2) and low value of S. E. (<2) for intra-day and inter-day variation, which suggested an excellent precision of the method (Table 3).

<table>
<thead>
<tr>
<th>Name</th>
<th>Theoretical plates</th>
<th>Tailing factor</th>
<th>Similarity factor</th>
<th>% RSD</th>
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<tr>
<td>Lopinavir</td>
<td>4197</td>
<td>1.12</td>
<td>1.01</td>
<td>0.39</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>4899</td>
<td>1.1</td>
<td>1</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Intra-day
101.18 ± 1.66 99.46 ± 1.13 101.95 ± 1.17 100.53 ± 1.52 100.37 ± 1.53 102 ± 0.70

Inter-day
99.14 ± 1.17 101.33 ± 1.63 100.60 ± 1.75 99.84 ± 0.96 101.18 ± 0.88 101.82 ± 0.38

Forced degradation of drug was carried out as per the ICH guidelines (ICH Q2B) by subjecting LOP and RIT to various stress conditions. The percent area decreased at the level of 4.22-29.80 % and it indicates that LOP and RIT undergoes degradation in acidic, basic, oxidative, neutral and photolytic conditions. Summary of force degradation data are given in Table 4.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Degradation condition</th>
<th>Retention time</th>
<th>% Degradation</th>
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<tbody>
<tr>
<td>LOP</td>
<td>RIT</td>
<td>LOP</td>
<td>RIT</td>
</tr>
<tr>
<td>1</td>
<td>Acid</td>
<td>3.441</td>
<td>5.115</td>
</tr>
<tr>
<td>2</td>
<td>Base</td>
<td>3.45</td>
<td>5.112</td>
</tr>
<tr>
<td>3</td>
<td>Oxidation</td>
<td>3.446</td>
<td>5.117</td>
</tr>
<tr>
<td>4</td>
<td>Reduction</td>
<td>3.441</td>
<td>5.113</td>
</tr>
<tr>
<td>5</td>
<td>Photolysis</td>
<td>3.43</td>
<td>5.112</td>
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</table>

REFERENCES
[1]. www.rxlist.com
[7]. International Conference on Harmonization (ICH), Validation of Analytical Procedures: Methodology Q2B, 1996.