Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Rivaroxaban and Clopidogrel Bisulphate in Pharmaceutical Dosage Form

By N. I. Majan, N. I. Patel & Aejaz Ahmed

Al-Illana College of Pharmacy

Abstract- A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Rivaroxaban and Clopidogrel, using a C18 (25cm x 0.46 cm) Hypersil BDS column and a mobile phase composed of buffer (pH 4.5): methanol (70:30). The detection was carried out at wavelength 214 nm. The retention times of Rivaroxaban and Clopidogrel were found to be 3.300 min and 4.740 min, respectively. Linearity was established for Rivaroxaban and Clopidogrel in the range of 2-6μg/ml and 7.5-22.5μg/ml, respectively. The percentage recoveries of Rivaroxaban and Clopidogrel were found to be 100.09% and 99.79%, respectively. Both the drugs were subjected to acid, alkali, oxidation, thermal and photolytic UV degradation. The degradation study shows that both drugs are susceptible in all parameter. Clopidogrel is more susceptible for photo and thermal degradation.

Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

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Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

I. INTRODUCTION

Rivaroxaban ((S)-5-chloro-N-[[2-oxo-3-[4-(3-oxo-morpholin-4-yl) phenyl] oxazolidin-5-yl] methyl] thiophene-2-carboxamide) (fig. 1) is an anticoagulant and the first orally active direct factor Xa inhibitor [1]. Clopidogrel Bisulphate methyl (2S)-2-(2-chlorophenyl)-2-{4H, 5H, 6H, 7H-thieno [3, 2-c] pyridin-5-yl} acetate (fig. 2) is an antplatelet agent structurally and pharmacologically similar to ticlopidine. It is used to inhibit blood clots in a variety of conditions such as peripheral vascular disease[2].

![Figure 1: Structure of Rivaroxaban](image1)

![Figure 2: Structure of Clopidogrel Bisulphate](image2)

Fixed dose combination (FDC) for the probing drugs is not available commercially, yet this FDC has not been listed in any of the common pharmacopoeia.
HPLC\textsuperscript{[21-22]} In combination of this drug only RP-HPLC method were available in mobile phase and also in plasma and urinary\textsuperscript{[23-24-25]}. However no stability indicating RP-HPLC method was set up for this combination.

We are involved newly to conduct investigation relating to stability indicating RP-HPLC method development of Rivaroxaban and Clopidogrel for fill this information gap. It was tried to develop and validate RP-HPLC method with stability indicating properties for this combination (Rivaroxaban and Clopidogrel Bisulphate). We expect the inclusion of this knowledge in the current literature will be benefit for the pharmaceutical industries for support the quality of their products holding these active ingredients and also the execution agencies in broad to evaluate the quality of the marketed preparations.

II. EXPERIMENTAL

Chemical and reagent: Pure Clopidogrel (CLP) and Rivaroxaban (RIV) were obtained as a gift sample from Remus Remedies. As Sample Clopidogrel 75mg and Rivaroxaban 20mg Synthetic Mixture is used. HPLC grade Methanol, Potassium dihydrogen, Ammonium Acetate, HPLC Grade High purity deionized water were obtained from Merck specialties Pvt Ltd., Mumbai.

Instrumentation and materials: The liquid chromatographic system was of Thermo separation Product TSP UV 2000, which consisted a gradient pump, variable wavelength, programmable UV/Vis detector, a manual injection facility with 20 μl fixed loop. The chromatographic analysis was performed using spinchrom software on a C18 Hysepil BDS column (25cm x 0.46 cm with 5μm particle size). In addition, an electronic balance (CP-124S Sartorius, Germany), a pH meter (Electroquip's Digital pH meter) were used in this study.

Chromatographic conditions: The elution of CLP and RIV was obtained by running HPLC in isocratic mode using Phosphate Buffer (pH 4.5): Methanol (70:30). Flow rate was maintained at 1.0 ml/min with run time of 6 min. The retention time for RIV was obtained 3.300 min and CLP was obtained 4.740 min. Detection was performed at 214 nm. Mobile phase was previously filtered through Whatman filter paper no 41.

\textbf{a) Preparation of standard solutions}

\textbf{CLP standard stock solution (150 μg/ml):} A 15 mg of CLP was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase.

\textbf{RIV standard stock solution (40 μg/ml):} A 40 mg of RIV was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase, taken 10 ml from this solution and transferred to 100 ml volumetric flask and volume was made up with methanol.

\textbf{Preparation of standard solution of binary mixtures of CLP (15 μg/ml) and RIV (4 μg/ml):} Take 1 ml from the CLP stock solution and 1 ml from RIV stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

\textbf{b) Preparation of formulation solution}

\textbf{Sample Stock Solution (CLP 150 μg/ml, RIV 40 μg/ml):} Take Tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

\textbf{Working Sample Preparation (CLP 15 μg/ml, and RIV 4 μg/ml):} Take 1 ml from standard stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase.

\textbf{c) Forced degradation study}\textsuperscript{[26]}

\textbf{Acid degradation:} Acid decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. 2 ml of 0.1 N Hydrochloride solutions was added and mixed well and put for 5 hrs. at room temperature. Then the volume was adjusted with diluent to get 15μg/ml for CLP and 4μg/ml for RIV.

\textbf{Base degradation:} Basic decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. 2 ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at room temperature. Then the volume was adjusted with diluent to get 15μg/ml for CLP and 4μg/ml for RIV.

\textbf{Oxidative degradation:} Oxidative decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. 2 ml of 3% H\textsubscript{2}O\textsubscript{2} solutions was added and mixed well and put for 6 hrs at room temperature. Then the volume was adjusted with diluent to get 15μg/ml for CLP and 4μg/ml for RIV.

\textbf{Photo degradation:} Photo degradation studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was kept in UV Chamber for 12 hrs. Then the volume was adjusted with diluent to get 15μg/ml for CLP and 4μg/ml for RIV.

\textbf{Thermal degradation:} Thermal degradation studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 80°C for 5 hrs. Then the volume was adjusted with diluent to get 15μg/ml for CLP and 4μg/ml for RIV.

\textbf{Method validation:} After method development, the method was validated in compliance with ICH guidelines. The method was validated for Accuracy, Precision, Reproducibility, Specificity, Limit of Detection, Limit of Quantitation, Linearity and Range, Ruggedness and Robustness.
III. Result and Discussion

a) Optimization of mobile phase

Trial contains various mobile phase which are considered of Methanol, Water and buffer. Methanol in different proportions and different volumes at different flow rate were tried. On the basis of various trials the mixture of Buffer (pH 4.5): Methanol (70:30), at 1.0 mL/min flow rate, proved to be better than the other mixture in terms of peak shape, theoretical plate and asymmetry.

![HPLC Chromatogram of RIV and CLP Buffer (pH 4.5): Methanol (70:30)](image)

b) System suitability parameter

System suitability is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use. Observed values for system suitability is show in Table 1.

![Figure 3: HPLC Chromatogram of RIV and CLP Buffer (pH 4.5): Methanol (70:30)](image)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RIV</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time</td>
<td>3.300</td>
<td>4.740</td>
</tr>
<tr>
<td>Theoretical Plates</td>
<td>9427</td>
<td>13320</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>1.316</td>
<td>1.160</td>
</tr>
<tr>
<td>Resolution</td>
<td>9.593</td>
<td></td>
</tr>
</tbody>
</table>

![Table 1: Result of system suitability parameters](image)

c) Validation of RP – HPLC method

i. Specificity

The Chromatograms of Clopidogrel and Rivaroxaban standards and Clopidogrel and Rivaroxaban sample show no interference with the Chromatogram of Clopidogrel and Rivaroxaban Blank, so the Developed method is Specific.

![Figure 4: Chromatogram of RIV and CLP standard](image)  
![Figure 5: Chromatogram of RIV and CLP sample](image)
d) **Linearity**

The linearity for RIV and CLP were assessed by analysis of combined standard solution in range of 2-6 μg/ml and 7.5-22.5 μg/ml respectively. Correlation coefficient for calibration curve RIV and CLP was found to be 0.998 and 0.999 respectively. The regression line equation for RIV and CLP are as following:

For RIV: \( y = 55.905x + 0.2138 \)

For CLP: \( y = 16.145x - 0.4796 \)

![Figure 6: Calibration Curve of RIV](image)

![Figure 7: Calibration Curve of CLP](image)

**e) Precision**

The precision of the method was demonstrated by repeatability study, inter-day precision and intra-day precision. In the repeatability study, six replicates of the same concentration of working standard solutions were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 2. In inter-day precision and intra-day precision, three replicates of three different concentration of working standard solution were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 3.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Drug</th>
<th>Amount Taken (μg mL(^{-1}))</th>
<th>Mean Area Found (n=3)</th>
<th>S.D</th>
<th>%R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday precision</td>
<td>RIV</td>
<td>2</td>
<td>113.066</td>
<td>0.556</td>
<td>0.451</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>225.837</td>
<td>1.696</td>
<td>0.751</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>335.157</td>
<td>1.21</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>CLP</td>
<td>7.5</td>
<td>122.28</td>
<td>0.754</td>
<td>0.616</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>243.516</td>
<td>1.319</td>
<td>0.541</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.5</td>
<td>362.858</td>
<td>1.696</td>
<td>0.467</td>
</tr>
<tr>
<td>Interday precision</td>
<td>RIV</td>
<td>2</td>
<td>112.632</td>
<td>0.972</td>
<td>0.863</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>224.325</td>
<td>1.237</td>
<td>0.552</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>333.162</td>
<td>3.512</td>
<td>1.054</td>
</tr>
<tr>
<td></td>
<td>CLP</td>
<td>7.5</td>
<td>122.769</td>
<td>0.388</td>
<td>0.316</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>241.954</td>
<td>1.786</td>
<td>0.738</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.5</td>
<td>360.46</td>
<td>4.525</td>
<td>1.255</td>
</tr>
</tbody>
</table>

**Table 2: Results of repeatability study**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mean Area (n=6)</th>
<th>S.D</th>
<th>%R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIV</td>
<td>224.773</td>
<td>1.058</td>
<td>0.471</td>
</tr>
<tr>
<td>CLP</td>
<td>242.442</td>
<td>1.451</td>
<td>0.599</td>
</tr>
</tbody>
</table>

* n - Number of estimations

**Table 3: Results of intra-day and inter-day precision**

**Accuracy**
Accuracy of the method was confirmed by recovery study from formulation at three levels of standard addition. 2 µg/ml drug solution for RIV and 7.5 µg/ml drug solution for CLP was taken in three different flasks labeled A, B, and C. Spiked 80%, 100%, and 120% of standard solution in it and diluted up to 10 ml. The area of each solution peak was measured at 214 nm. The amount of RIV and CLP was calculated at each level and % recoveries were computed. The results are shown in Table 4. Percentage recovery for Rivaroxaban was 0.718%-1.357%, while for Clopidogrel it was found to be in range of 0.649%-1.110%.

Table 4: Results of recovery study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. Level(%)</th>
<th>Sample amount added(µg/ml) (n=3)</th>
<th>Standard amount added(µg/ml) (n=3)</th>
<th>Mean of amount recovered (µg/ml) (n=3)</th>
<th>% Mean recovery</th>
<th>S.D</th>
<th>% R.S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIV</td>
<td>80</td>
<td>2</td>
<td>1.6</td>
<td>1.601</td>
<td>100.081</td>
<td>1.358</td>
<td>1.357</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>1.992</td>
<td>99.594</td>
<td>0.716</td>
<td>0.718</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>2</td>
<td>2.4</td>
<td>2.415</td>
<td>100.623</td>
<td>0.734</td>
<td>0.729</td>
</tr>
<tr>
<td>CLP</td>
<td>80</td>
<td>7.5</td>
<td>6</td>
<td>6.001</td>
<td>100.028</td>
<td>0.649</td>
<td>0.649</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.5</td>
<td>7.5</td>
<td>7.474</td>
<td>99.656</td>
<td>0.948</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>7.5</td>
<td>9</td>
<td>8.975</td>
<td>99.728</td>
<td>1.107</td>
<td>1.11</td>
</tr>
</tbody>
</table>

n-Number of estimations

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Where, SD = Standard deviation of intercepts

Table 5: Results of LOD and LOQ

<table>
<thead>
<tr>
<th></th>
<th>LOD</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>3.3x(SD/Slope) = 3.3 x(3.02/55.905) = 0.195 µg/ml</td>
<td>3.3x(SD/Slope) = 3.3 x(3.849/16.145) = 0.787 µg/ml</td>
</tr>
<tr>
<td>CLP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>3.3x(SD/Slope) = 3.3 x(3.02/55.905) = 0.195 µg/ml</td>
<td>3.3x(SD/Slope) = 3.3 x(3.849/16.145) = 0.787 µg/ml</td>
</tr>
</tbody>
</table>

f) Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation. (a) Flow rate of mobile phase was changed (±0.2 ml/min) 0.8 ml/min and 1.2 ml/min. (b) Ratio of Mobile phase was changed (±2) Buffer: Methanol (72:28) and Buffer: Methanol (68:32). (c) pH of Buffer was changed (±0.2), pH 4.3 and pH 4.7. The effect of changes was found to be within the acceptance criteria as shown in Table 6. The % RSD should be less than 2%.

Table 6: Results of robustness study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variation</th>
<th>RIV</th>
<th>CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean area (n=3)</td>
<td>S.D</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>72:28</td>
<td>229.51</td>
<td>1.036</td>
</tr>
<tr>
<td></td>
<td>68:32</td>
<td>217.964</td>
<td>0.579</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 ml/min</td>
<td>233.192</td>
<td>3.135</td>
</tr>
<tr>
<td></td>
<td>1.2ml/min</td>
<td>213.584</td>
<td>0.697</td>
</tr>
<tr>
<td>pH</td>
<td>4.3</td>
<td>233.898</td>
<td>3.193</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>222.218</td>
<td>1.783</td>
</tr>
</tbody>
</table>
g) Assay

Triturate 20 tablets, take tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100 ml volumetric flask. Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. Take 1 ml from this stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase. Inject above Solution 20 μl for assay analysis. The solution was filtered through Whatman filter paper no. 42. The results are given in Table 7.

Table 7: Results of assay study

<table>
<thead>
<tr>
<th>Tablet</th>
<th>Label claim</th>
<th>CLP(75mg)</th>
<th>RIV(4mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay (% of label claim*) Mean ± S. D.</td>
<td>98.895±0.267</td>
<td>98.651±0.363</td>
<td></td>
</tr>
</tbody>
</table>

h) Stability indicating method

![Figure 8: RIV and CLP Sample for stability](image)

Table 8: Calculation of CLP and RIV standard for stability

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rivaroxaban</td>
<td>242.859</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>237.109</td>
</tr>
</tbody>
</table>
i) Acid degradation

**Figure 10:** Acid degradation blank

**Figure 11:** RIV acid degradation standard

**Figure 12:** CLP acid degradation standard

**Figure 13:** RIV and CLP acid degradation sample
j) Base degradation

Figure 14: Base degradation blank

Figure 15: RIV base degradation

Figure 16: CLP base degradation

Figure 17: RIV and CLP base degradation sample
k) Oxidation Degradation

Figure 18: Oxidation degradation blank

Figure 19: RIV oxidation degradation

Figure 20: CLP oxidation degradation

Figure 21: RIV and CLP oxidation degradation sample
j) *Photo degradation*

Figure 22: Photo degradation blank

Figure 23: RIV photo degradation

Figure 24: CLP photo degradation

Figure 25: RIV and CLP photo degradation sample
m) Thermal degradation

Figure 26: Thermal degradation blank

Figure 27: RIV thermal degradation

Figure 28: CLP thermal degradation

Figure 29: RIV and CLP thermal degradation sample
The Combined dosage form of Rivaroxaban and clopidogrel are not available commercially. But individually rivaroxaban is used as an anticoagulant and clopidogrel is used as an antiplatelet agent. Various methods are reported for the analysis of individual drug and in combination with other drugs but no stability indicating HPLC method reported for these two drugs in combined dosage form. Therefore, a novel RP-HPLC method has been developed for the simultaneous estimation of Rivaroxaban and Clopidogrel combination. The optimized chromatogram was run for appropriate minutes with mobile phase Phosphate buffer (Ph 4.5): Methanol (70:30). Data related to peak like area, height, retention time, resolution etc. were recorded using software. Thermo scientific, C18(25cm×0.46cm) Hypersil BDS, Mobile Phase Phosphate buffer, pH 4.5: Methanol (70:30) with Flow Rate 1.0 ml/min and Runtime 6 min Injection volume of 20.0 μl. The detection was carried out at wavelength 214 nm. It was found to be simple, precise and accurate. In this stability indicating RP-HPLC methods were developed by degradation of sample and compared with standard. The % RSD was also less than 2 % showing high degree of precision of the proposed method. The proposed method can be used for routine analysis of Rivaroxaban and Clopidogrel in combined dosage form. It can be also used in the quality control in bulk manufacturing.

Acknowledgement

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