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

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ABSTRACT
A simple, precise, rapid, specific and accurate stability indicating reverse phase high performance liquid chromatography method was developed for simultaneous estimation of Rosuvastatin (RST) and Clopidogrel (CPG) in pharmaceutical dosage form. Chromatographic separation was performed on Zorbax-SB (C_{18}) (150mmx4.6mm, 5μ) column, with mobile phase comprising of mixture of buffer (pH 3, adjusted with ortho phosphoric acid), and methanol in the ratio of 60:40 v/v, at the flow rate 1.2 ml/min. The detection was carried out at 254 nm. The retention times of RST and CPG were found to be 1.6 and 2.5 mins respectively with a run time of 6 mins, theoretical levels for RST and CPG were found to be 5050 and 5503 respectively, with a resolution of 5.88. As per ICH guidelines the method was validated for linearity, accuracy, precision, limit of detection and limit of quantitation, robustness and ruggedness. Linearity of RST was found in the range of 10-30 µg/mL and that for CPG was found to be 75-225 µg/mL. The correlation coefficient for RST and CPG were found to be 0.999 and 0.999 respectively. The LOD values for RST and CPG were found to be 2.61 and 2.88 µg/mL respectively. The LOQ values for RST and CPG were found to be 8.70 and 9.62 µg/mL respectively. The results of the degradation studies indicated the suitability of the method to study stability of RST and CPG under various forced degradation conditions viz. acid, base, dry heat, photolytic degradation. The developed method can be applied to the analysis of stability samples of combination dosage form of RST and CPG. This demonstrates that the developed method is simple, precise, rapid, selective, accurate and reproducible for simultaneous estimation of RST and CPG in pharmaceutical dosage form.

Keywords: Clopidogrel (CPG), forced degradation studies, method development, rosuvastatin (RST), RP-HPLC, validation

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INTRODUCTION
Rosuvastatin calcium is an antilipemic agent and chemically it is 7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonylamo)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyheptenoic acid (Mol Wt-481.53), that competitively inhibits hydroxymethylglutaryl-coenzyme A (HMG-CoA) Reductase. HMG-CoA Reductase catalyzes the conversion of HMG-CoA to mevalonic acid, the rate-limiting step in cholesterol biosynthesis and it is used to reduce plasma cholesterol levels and prevent cardiovascular disease [1]. Clopidogrel Sulphate is an antiplatelet agent and is chemically (2S)-2-{(2-chlorophenyl)-2-{4H,5H,6H,7H-thieno[3,2-c]pyridin-5-yl}acetate (Mol Wt-321.82 ).It is used to inhibit blood clots in a variety of conditions such as peripheral vascular disease, coronary artery disease, and cerebrovascular disease and it is an irreversible inhibitor of the P2Y12 adenosine diphosphate
receptor found on the membranes of platelet cells [2].

**Figure 1: Structure of Rosuvastatin**

![Structure of Rosuvastatin]

**Figure 2: Structure of Clopidogrel**

Literature survey revealed that few analytical techniques are available for estimation of Rosuvastatin alone as well as in combine dosage form such as UV, HPLC [3, 4]. Similarly few analytical methods are available for estimation of Clopidogrel alone and its combination with drugs such as UV and HPLC [5, 6]. The combination therapy is used for Coronary Artery Disease, which is the most common cause of death in the world and a major cause of hospital admissions in 2012. There is no Stability Indicating HPLC method reported in the literature for the determination of Rosuvastatin and Clopidogrel by RP-HPLC technique [7 to 15]. Hence here is an attempt to develop a more precise and accurate Stability Indicating HPLC method for the simultaneous determination of Rosuvastatin and Clopidogrel in pharmaceutical solid dosage forms. Various validation aspects of the analysis accuracy, precision, recovery, the limits of detection and quantification etc have been measured as per ICH guidelines [16 to 20].

**MATERIALS AND METHODS**

**Equipment**

Chromatographic separation was performed on HPLC system - Water's 2690 series, PDA Detector 2695 series, equipped with a solvent delivery pump, sample injector and column thermostats. Empower software was applied for data collecting and processing.

**Chemicals and Reagents**

Methanol, Water of HPLC grade was purchased from Merck Chemicals (Mumbai, India). Buffer used was pH-3 (pH adjusted with analytical reagent (AR) grade o-phosphoric acid from LOBA Chemie Pvt). Reference standards Clopidogrel and Rosuvastatin were obtained from Rainbow labs. Atorfit-CV, Capsules with Atorvastatin (10 mg) and Clopidogrel (75 mg), manufactured by Ajanta Pharma Limited, were procured from local market.

**Preparation of Standard Solutions**

Accurately weighed and transferred 10 mg of Rosuvastatin and 75 mg of Clopidogrel working standard into a 50 ml clean dry volumetric flask and added about 30 mL of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution) (200,1500 µg/mL). From this, 1 ml of the solution was pipetted into another 10 ml volumetric flask and diluted up to the mark with diluent (20, 150 µg/mL).

**Preparation of Sample Solution**

Accurately weighed and transferred tablet powder equivalent to 10 mg of RST and 75 mg of CPG into a 50 mL clean dry volumetric flask and added about 30 mL of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution) (200, 1500 µg/mL) From this, 1 mL of the solution was pipette into another 10 mL volumetric flask and diluted up to the mark with diluent.

**Preparation of Buffer**

Take 1000 mL of HPLC grade water. The pH was adjusted to 3.0 with orthophosphoric acid.

**Optimized chromatographic conditions**

- **Diluent**: Methanol
- **Mobile phase**: Buffer pH 3 (Water):
  - Methanol: (60:40)
  - Flow rate: 1.2 mL/min
- **Column**: Zorbax-SB (C18) (150mm x 4.6 mm, 5 μ)
- **Detector wavelength**: 254 nm
- **Injection volume**: 20 μL
- **Run time**: 6 min
- **Mode of Pump**: Isocratic
Figure 3: Chromatogram of RST (20µg/mL) and CPG (150µg/mL) standard

Figure 4: Chromatogram of RST (20µg/mL) and CPG (150µg/mL) sample

Table 2: System Suitability Studies

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rosuvastatin</th>
<th>Clopidogrel</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>5050</td>
<td>5503</td>
<td>Not less than 2000</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.55</td>
<td>1.39</td>
<td>Not more than 2</td>
</tr>
<tr>
<td>Resolution</td>
<td>-</td>
<td>5.88</td>
<td>Not less than 2</td>
</tr>
</tbody>
</table>

METHOD VALIDATION

Linearity

Solutions were prepared containing 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml, 30µg/ml, concentrations of Rosuvastatin and 75µg/ml, 112.5µg/ml, 150µg/ml, 187.5µg/ml, 225µg/ml, concentrations of Clopidogrel which corresponding to 50, 75, 100, 125 and 150% respectively of the test solution concentration. Each solution was injected, linearity was evaluated by linear-regression analysis.

Table 1: Result of Linearity

<table>
<thead>
<tr>
<th>Linearity conc (%)</th>
<th>Volume of Stock taken (mL)</th>
<th>Diluted to (mL)</th>
<th>Final Conc (µg/mL)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RST</td>
<td>CPG</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>10</td>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>75</td>
<td>0.75</td>
<td>10</td>
<td>15</td>
<td>112.5</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>10</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>125</td>
<td>1.25</td>
<td>10</td>
<td>25</td>
<td>187.5</td>
</tr>
<tr>
<td>150</td>
<td>1.5</td>
<td>10</td>
<td>30</td>
<td>225</td>
</tr>
</tbody>
</table>
Figure 5: Calibration curve for Rosuvastatin

Figure 6: Calibration curve for Clopidogrel

Accuracy
Accuracy was determined by the recovery studies at three different concentrations (corresponding to 50, 100 and 150% of the test solution concentration) by addition of known amounts of standard to pre-analysed sample preparation. For each concentration, three sets were prepared and injected.

Table 3: Recovery studies for Rosuvastatin and Clopidogrel

<table>
<thead>
<tr>
<th>Drug</th>
<th>Spiked level%</th>
<th>Amount taken (µg/ml)</th>
<th>Amount found (µg/ml)</th>
<th>Percent recovery</th>
<th>Mean recovery Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>RST</td>
<td>50</td>
<td>9.889</td>
<td>9.909</td>
<td>100.05</td>
<td>99.90</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>19.820</td>
<td>19.794</td>
<td>99.90</td>
<td>97.59</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>30.448</td>
<td>29.702</td>
<td>97.59</td>
<td></td>
</tr>
<tr>
<td>CPG</td>
<td>50</td>
<td>74.620</td>
<td>74.774</td>
<td>100.21</td>
<td>100.06</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>149.550</td>
<td>149.689</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>46.87</td>
<td>229.743</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n- Number of replicate injections

Precision
Intraday variations were determined by using six replicate injections of one concentration and analyzed on the same day and different days. Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.
**Table 4: Results of Intraday Precision**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample Weight</th>
<th>Sample Area-1</th>
<th>Sample Area-2</th>
<th>% Assay</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>483.00</td>
<td>6032493</td>
<td>8538527</td>
<td>98.99</td>
<td>99.78</td>
</tr>
<tr>
<td>2</td>
<td>483.00</td>
<td>6039657</td>
<td>8533252</td>
<td>99.10</td>
<td>99.72</td>
</tr>
<tr>
<td>3</td>
<td>483.00</td>
<td>6039793</td>
<td>8535900</td>
<td>99.11</td>
<td>99.75</td>
</tr>
<tr>
<td>4</td>
<td>483.00</td>
<td>6032497</td>
<td>8531566</td>
<td>98.99</td>
<td>99.70</td>
</tr>
<tr>
<td>5</td>
<td>483.00</td>
<td>6031979</td>
<td>8536214</td>
<td>98.98</td>
<td>99.75</td>
</tr>
<tr>
<td>6</td>
<td>483.00</td>
<td>6038540</td>
<td>8536253</td>
<td>99.08</td>
<td>99.76</td>
</tr>
</tbody>
</table>

Avg Assay: 6035826.5 8535285.3 99.04 99.74

STD 3867.040 2476.001 0.06 0.03

%RSD 0.064 0.029 0.06 0.03

**Robustness**

The robustness was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions. The factors chosen for this study were the flow rate (±0.1 ml/min) and temperature (± 5 °C).

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

LOD and LOQ was calculated from linear curve using formulae:

\[
\text{LOD} = 3.3 \times \sigma / \text{slope}, \quad \text{LOQ} = 10 \times \sigma / \text{slope}
\]

(Where \(\sigma\) = the standard deviation of the response

And, \(S\) = Slope of calibration curve).

The LOD was 2.61 and 2.88 µg mL\(^{-1}\) for RST and CPG respectively and, the respective LOQ were 8.70 and 9.62 µg mL\(^{-1}\).

**Specificity**

Specificity was checked for the interference of impurities in the analysis of blank solution and injecting sample solution under optimized chromatographic conditions to demonstrate separation of both Rosuvastatin and Clopidogrel from impurities.

**Table 5: Results of Robustness study**

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Parameter changing</th>
<th>Area</th>
<th>USP Tailing</th>
<th>USP Plate count</th>
<th>USP</th>
<th>Rt</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Temp1</td>
<td>5775411</td>
<td>1.53</td>
<td>5104</td>
<td>1.660</td>
<td>---</td>
<td>RST</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Temp2</td>
<td>5780683</td>
<td>1.53</td>
<td>5074</td>
<td>1.658</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Flow1</td>
<td>6949049</td>
<td>1.49</td>
<td>5407</td>
<td>2.000</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Flow2</td>
<td>5789017</td>
<td>1.49</td>
<td>4992</td>
<td>1.663</td>
<td>---</td>
<td>2.609 8.696</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Temp1</td>
<td>8151496</td>
<td>1.41</td>
<td>5934</td>
<td>---</td>
<td>2.536</td>
<td>CPG</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Temp2</td>
<td>8156778</td>
<td>1.43</td>
<td>5791</td>
<td>---</td>
<td>2.533</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Flow1</td>
<td>9712712</td>
<td>1.45</td>
<td>6294</td>
<td>---</td>
<td>3.050</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Flow2</td>
<td>8136622</td>
<td>1.37</td>
<td>5937</td>
<td>---</td>
<td>2.539</td>
<td>2.884 9.615</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7: Chromatogram of Blank**

**Figure 8: Chromatogram of placebo**
FORCED DEGRADATION OF RST AND CPG
To determine whether the analytical method and assay were stability-indicating RST and CPG bulk powder were stressed under different forced degradation conditions in forced degradation studies. RST (10 mg) and CPG (75 mg) were weighed and dissolved with 50 mL of mobile phase. These stock solutions were used for forced degradation studies.

Acidic and Alkaline Degradation
HCl (1N, 10 mL) and NaOH (1N, 10 mL) were separately added to 10 mL stock solutions of RST and CPG. These mixtures were separately heated under reflux for 4 h at 70°C in the dark (to exclude the possible degradative effect of light). The solutions (10 mL) were transferred separately to volumetric flasks, neutralized by addition of 10 mL 1N NaOH and 1N HCl, and suitable volume was diluted with mobile phase.

Figure 9A: Chromatogram of Acid Degradation

Figure 9B: Chromatogram of Base Degradation

Oxidative Degradation
Hydrogen peroxide (H2O2; 3% v/v, 10 mL) was added to 10 mL stock solutions of RST and CPG. These solutions were separately heated under reflux for 3 h at 70°C in dark. The solutions were then transferred separately to volumetric flasks, neutralized by addition of 1N NaOH and diluted to suitable volume with mobile phase.

Figure 9C: Chromatogram of Oxidative Degradation

Dry heat Degradation
For dry heat degradation, solid drugs were kept in petriplates in an oven at 100°C for 5h. Thereafter, 10 mg and 75 mg of each RST and CPG were weighed and transferred into 10 mL volumetric flask and suitably diluted with mobile phase.
Photochemical Degradation

10 mL stock solutions of RST and CPG were separately subjected to natural sunlight for 5 h to study the effect of photolytic degradation. Suitable volumes from above stock solutions were diluted with mobile phase.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sample Area</th>
<th>% Assay RST</th>
<th>% Assay CPG</th>
<th>% Deg RST</th>
<th>% Deg CPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>4622474</td>
<td>75.85</td>
<td>82.48</td>
<td>-23.15</td>
<td>-17.22</td>
</tr>
<tr>
<td>Base</td>
<td>4853049</td>
<td>79.63</td>
<td>83.63</td>
<td>-19.37</td>
<td>-16.07</td>
</tr>
<tr>
<td>Peroxide</td>
<td>4957377</td>
<td>81.34</td>
<td>84.28</td>
<td>-17.66</td>
<td>-15.42</td>
</tr>
<tr>
<td>Light</td>
<td>5102035</td>
<td>83.72</td>
<td>87.01</td>
<td>-15.28</td>
<td>-12.69</td>
</tr>
<tr>
<td>Heat</td>
<td>5032159</td>
<td>82.57</td>
<td>85.36</td>
<td>-16.43</td>
<td>-14.34</td>
</tr>
<tr>
<td>Avg Assay:</td>
<td>4094515.7</td>
<td>6029504</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td>2012857.1</td>
<td>2956801</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>49.2</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSIONS

Several mobile phase compositions were tried to resolve the peak of RST and CPG. The mobile phase containing buffer: Water: Methanol in proportion of 60:40 v/v was found ideal to resolve the peak of RST and CPG. Retention time of RST and CPG were 1.6 and 2.5 min respectively (Figure 3 & 4). Result of assay is shown in Table-3. The proposed method was found to be linear in concentration range 10-30μg/ml for RST and 75-225 μg/ml for CPG. The data was shown in (Table 1) and (Figure 5 & 6). System suitability parameters were evaluated and results shown in (Table 2), which were within acceptance criteria. The mean percentage recovery for RST and CPG were found to be 99.18% and 99.18% respectively, which are well within the limit and hence the method was found to be accurate (Table 3). LOD and LOQ values were 2.61μg/mL and 8.70μg/mL for RST and 2.88μg/mL and 9.62 μg/mL for CPG (Table 5). Results of intraday precision were shown in (Table 4). The robustness of the method was investigated by varying experimental conditions such as changes in flow rate and column temperature. The result obtained implies method is robust for routine qualitative analysis (Table 5).

DEGRADATION STUDIES

The degradation studies indicated that both RST and CPG degraded in acidic and basic hydrolysis, oxidation, photolysis and dry

Figure 9D: Chromatogram of Dry Heat Degradation

Figure 9E: Chromatogram of Photolytic Degradation
heat degradation under experimental conditions with maximum degradation under acidic hydrolytic condition. The percent degradation of RST and CPG at photolytic, dry heat, oxidative, basic and acid stress conditions, were found to be 13,14,15,16,17% and 15,16,18,19,23% degradation respectively. Both RST and CPG were highly susceptible towards acidic hydrolysis, resulting in more than 15% degradation, however no additional degradation peak was observed. Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peaks of the drugs under non degradation condition. Summary of degradation studies of the drugs were shown in (Table 6).

CONCLUSION
The proposed RP-HPLC method was validated as per International Conference on Harmonization (ICH) guidelines, and found to be applicable for routine quality control analysis for the simultaneous estimation of RST and CPG using isocratic mode of elution. The results of linearity, precision, accuracy and specificity, proved to be within the limits. The proposed method is highly sensitive, reproducible, reliable, rapid and specific. The results of the degradation studies indicated the suitability of the method to study stability of RST and CPG under various forced degradation conditions viz. acid, base, dry heat, photolytic degradation. The developed method can be applied to the analysis of stability samples of combination dosage form of RST and CPG.

REFERENCES