DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF ROSUVASTATIN CALCIUM AND ASPIRIN IN COMBINED DOSAGE FORM

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ABSTRACT
An isocratic stability indicating RP-HPLC method was developed for the quantitative determination of rosuvastatin calcium and aspirin in combined dosage form. A Kromasil C8, 5 µm column with mobile phase containing, acetonitrile: methanol: water (25:30:45, v/v/v), (pH 3) was used. The flow rate was 1.0 mL/min and effluents were monitored at 243 nm. The retention times of aspirin and rosuvastatin calcium were 2.6 min and 5.7 min, respectively. The linearity for rosuvastatin calcium and aspirin were in the range of 0.2 - 10 µg/ ml and 1.5 - 75 µg/ ml, respectively. The proposed method was validated with respect to linearity, accuracy, precision, specificity and robustness. The method was successfully applied to the estimation of aspirin and rosuvastatin calcium in combined dosage form.

Keywords: Aspirin; Rosuvastatin Calcium; Reversed phase liquid chromatography; Validation

INTRODUCTION
Rosuvastatin calcium (ROS) is chemically (E)-(3R,5S)-7-{4-(4-fluorophenyl)-6-isopropyl 2{methyl (methylsulphonyl amino)} pyrimidin-5-yl} -3,5-dihydroxyhepten-6-oic acid calcium. It is a competitive inhibitor of HMG-CoA reductase (statins class). Used in hyperlipidemia, dyslipidemia and in primary prevention of cardiovascular disease. It is official in IP.[1]

Aspirin (ASP) is chemically 2-acetoxybenzoic acid. Aspirin, It is non-selective cyclo-oxygenase inhibitor used as an antipyretic, analgesic, anti-inflammatory. It is official in IP, BP and USP.
These drugs are now a day’s available in combination therapy. The rationale behind use of this drug combination is for the treatment of dyslipidemia associated with arthersclerotic arterial disease with risk of Myocardial infarction, stroke or peripheral vascular disease.

Literature survey revealed various spectrophotometric and chromatographic methods have been reported for estimation of ROS and ASP individually or in combination with other drug. Different methods for estimation of rosuvastatin calcium in single and in combination with other drugs are as mentioned. For UV spectrophotometric\(^\text{[2-4]}\), HPLC\(^\text{[5-9]}\) and HPTLC\(^\text{[10-11]}\). Different methods for estimation of aspirin in single and in combination with other drugs are as mentioned. For UV spectrophotometric\(^\text{[12]}\), HPLC\(^\text{[13-16]}\) and HPTLC\(^\text{[17]}\). To the best of our knowledge, there is no stability indicating HPLC method reported for analysis of rosuvastatin calcium and aspirin in pharmaceutical formulations. Hence the aim of present study is to establish accurate and sensitive method and, after validation in accordance with International Conference on Harmonization (ICH) guidelines, to use the method for analysis of the drug content of both in capsule dosage form.

**EXPERIMENTAL**

**Apparatus**
The liquid chromatographic system consist of 2690, Waters equipped with a 2487, UV detector, quaternary gradient pump and automated injector. The analytes were monitored at 243 nm. Chromatographic analysis was performed on Kromasil C₈ column having 125 mm× 4.6 mm i.d. and 5 μm particle size. All the drugs and chemicals were weighed on Shimadzu electronic balance.

Reagents and Materials

Analytically pure Rosuvastatin Calcium and Aspirin were obtained as gift samples from Torrent Research Center, Ahmadabad, India and Mercury Laboratories Limited, Vadodara, India, respectively. HPLC grade acetonitrile, methanol and water were obtained from E. Merck Ltd., Mumbai, India. Capsule formulation (UNISTAR*, Unichem Laboratories Ltd) containing labeled amount of 10 mg of Rosuvastatin Calcium and 75 mg of Aspirin was used for the study.

Chromatographic Conditions

The Kromasil C₈ column equilibrated with mobile phase acetonitrile: methanol: water (25:30:45, v/v/v) was used. The flow rate was maintained at 1 mL/min, eluent were monitored with UV detector at 243 nm, and the injection volume was 20 μL. Total run time was kept 10 min.

Preparation of standard stock solutions

Standard ROS (10 mg) and ASP (75 mg) were accurately weighed and transferred to separate 25 ml volumetric flasks and dissolved in few ml of diluent(methanol: acetonitrile, 50:50 v/v). Volumes were made up to the mark with diluent to yield a solution containing 400 μg/ml of ROS and 3000 μg/ml ASP.

Degradation/ Stability studies

This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing.

The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.
Acidic degradation:
Procedure:
To the different 25 ml volumetric flasks, a 1 ml stock solution of each ROS and ASP was taken and in third volumetric flask stock solution of formulation (1 ml) solution was taken.
Each flask was subjected to 5 ml of 0.01 N HCl for 1 hr at 60-70 °C in water bath. After exposure to the degradation condition each solution of standards and formulation was cooled and neutralized using drop wise addition of 5 ml of 0.01 N NaOH. Add 15 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking. Make the volume up to mark with diluent and mix. Filter the solution. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 8 µg/ml ROS and 60 µg/ml of ASP. Each solution was analyzed by the proposed method and Chromatogram recorded.

Alkali degradation:
Procedure:
To the different 25 ml volumetric flasks, a 1 ml stock solution of each ROS and ASP was taken and in third volumetric flask stock solution of formulation (1 ml) solution was taken. Each flask was subjected to 5 ml of 0.01 N NaOH for 1 hr at 60-70 °C in water bath. After exposure to the degradation condition each solution of standards and formulation was cooled and neutralized using drop wise addition of 5 ml of 0.01 N HCl. Add 15 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking. Make the volume up to mark with diluent and mix. Filter the solution. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 8 µg/ml ROS and 60 µg/ml of ASP. Each solution was analyzed by the proposed method and Chromatogram recorded.

Peroxide degradation:
Procedure:
To the different 25 ml volumetric flasks, a 1 ml stock solution of each ROS and ASP was taken and in third volumetric flask stock solution of formulation (1 ml) solution was taken.
Each flask was subjected to 5 ml of 3% \( \text{H}_2\text{O}_2 \) for 1 hr at 60-70 °C in water bath. After exposure to the degradation condition each solution of standards and formulation was cooled. Add 15 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking. Make the volume up to mark with diluent and mix. Filter the solution. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 8 µg/ml ROS and 60 µg/ml of ASP. Each solution was analyzed by the proposed method and Chromatogram recorded.

**Thermal degradation:**

**Procedure:**

Analytically pure samples of ROS, ASP and formulation were exposed in oven at 105°C for 1 h. The solids were allowed to cool and 25 mg each of ROS and ASP were weighed, transferred to two separate volumetric flasks (25 ml) and dissolved in few ml of diluent. In similar way formulation was also treated. Add 20 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking. Make the volume up to the mark with diluent and mix. Filter the solution. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 8 µg/ml ROS and 60 µg/ml of ASP. Each solution was analyzed by the proposed method and Chromatogram recorded.

**Photo degradation:**

**Procedure:**

Analytically pure samples of ROS, ASP and formulation were irradiated with a UV lamp (254 nm) (Camag, Muttenz, Switzerland). 25 mg each of ROS and ASP were weighed, transferred to two separate volumetric flasks (25 ml) and dissolved in few ml of diluent. In similar way formulation was also treated. Add 20 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking. Make the volume up to the mark with diluent and mix. Filter the solution. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 8 µg/ml ROS and 60 µg/ml of ASP. Each solution was analyzed by the proposed method and Chromatogram recorded.
FORCED DEGRADATION STUDY

Figure 2
HPLC chromatogram of ROS (100μg/ml) and ASP (100μg/ml)
Figure 3
Chromatograms of acid degraded ASP (a), ROS (b) and capsule sample solution (c)
Figure 4
Chromatograms of alkali degraded ASP (a), ROS (b) and capsule sample solution (c)
Figure 5
Chromatograms of peroxide degraded ASP (a), ROS (b) and capsule sample solution (c)
Figure 6
Chromatograms of thermal degraded ASP (a), ROS (b) and capsule sample solution (c)
Figure 7
Chromatograms of photo degraded ASP (a), ROS (b) and capsule sample solution (c)

**TABLE 1: % DEGRADATION UNDER STRESS CONDITION**

<table>
<thead>
<tr>
<th>Stress condition/ Duration</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROS (8 µg/ml)</td>
</tr>
<tr>
<td>Acidic/0.01 N HCl/60-70°C/1 hr</td>
<td>17.5</td>
</tr>
<tr>
<td>Basic/0.01 N NaOH/60-70°C/1 hr</td>
<td>1.1</td>
</tr>
<tr>
<td>Oxidative/3%/60-70°C/1 hr</td>
<td>7.9</td>
</tr>
<tr>
<td>Dry heat/105°C/1 hr</td>
<td>0.8</td>
</tr>
<tr>
<td>Photolytic/UV 254 nm/12 hr</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Validation of the Developed Stability Indicating Rp-HPLC Method**[^18]

**Linearity and Range**

Linearity of the method was evaluated by constructing calibration curves at six concentration levels over a range of 0.2–10 µg/ml and 1.5-75 µg /ml of ROS and ASP respectively. The calibration curves were developed by plotting peak area versus concentration (n = 6).

**Accuracy**

Accuracy is the closeness of the test results obtained by the method to the true value.
The accuracy of the method was determined by calculating recoveries of ROS and ASP by method of standard additions. Known amount of ROS (0, 2, 4, 6 µg mL\(^{-1}\)) and ASP (0, 15, 30, 45 µg mL\(^{-1}\)) were added to a pre quantified sample solution (containing ROS and ASP in 4:30 µg/ ml proportion respectively) and analysed by proposed method. The amount of ROS and ASP were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

Precision

Repeatability

Standard solutions of ROS and ASP (8 and 60 µg/ml respectively) were prepared, analyzed by proposed method and chromatograms were recorded. Area of the same concentration solution was measured six times and % RSD was calculated.

Intra and inter day precision

The intra-day and inter-day precision studies were carried out by estimating the corresponding responses 6 times on the same day and on 6 different days for three different concentrations of ROS (2, 6, 10 µg mL\(^{-1}\)) and ASP (15, 45, 75 µg mL\(^{-1}\)), and the results are reported in terms of relative standard deviation.

LOD AND LOQ

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines.

\[
LOD = 3.3 \times \sigma /S; \quad LOQ = 10 \times \sigma /S; \quad \text{Where } \sigma \text{ is the standard deviation of } y \text{-intercepts of regression lines and } S \text{ is the slope of the calibration curve.}
\]

Solution Stability Study

Stability of sample solutions were studied at 25 ± 2°C for 24 h.

Robustness

Robustness of the method was studied by deliberately changing the experimental conditions like flow rate and percentage of organic phase and the effects on the results
were examined. Robustness of the method was determined in triplicate at a concentration level of 8µg/ml and 60 µg/ml of ROS and ASP respectively. The mean and % RSD of peak areas were calculated.

**System Suitability**

A system suitability test was an integral part of the method development to verify that the system is adequate for the analysis of ROS and ASP to be performed. System suitability test of the chromatography system was performed before each validation run. Five replicate injections of a system suitability standard and one injection of a check standard were made. Area, retention time (RT), tailing factor, asymmetry factor, and theoretical plates for the five suitability injections were determined.

**TABLE 2: REGRESSION ANALYSIS OF CALIBRATION CURVE**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ROS</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear Range(µg/ml)</td>
<td>0.2 – 10</td>
<td>1.5 – 75</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Slope</td>
<td>52144</td>
<td>15372.4</td>
</tr>
<tr>
<td>Intercept</td>
<td>4557.4</td>
<td>14027</td>
</tr>
<tr>
<td>Standard deviation of slope</td>
<td>401.79</td>
<td>74.5104</td>
</tr>
<tr>
<td>Standard deviation of intercept</td>
<td>1278.35</td>
<td>1427.4</td>
</tr>
</tbody>
</table>

**TABLE 3: SUMMARY OF VALIDATION PARAMETERS**

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>ROS</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (µg/ml)</td>
<td>0.2 – 10</td>
<td>1.5 – 75</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>99.32 – 100.71</td>
<td>99.95 – 100.04</td>
</tr>
<tr>
<td>Precision ( % RSD )</td>
<td>0.3048</td>
<td>0.8269</td>
</tr>
<tr>
<td>Repeatability ( n = 6)</td>
<td>0.62 – 1.05</td>
<td>0.55 – 0.96</td>
</tr>
<tr>
<td>Intraday (n = 6)</td>
<td>0.75 – 1.11</td>
<td>0.57 – 1.02</td>
</tr>
</tbody>
</table>

RSD is relative standard deviation and 'n' is number of determinations.
TABLE 4: SYSTEM SUITABILITY TEST PARAMETER

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>System Parameters</th>
<th>Suitability</th>
<th>Proposed Method</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ROS</td>
<td>ASP</td>
</tr>
<tr>
<td>1</td>
<td>Retention times (R₁) (min)</td>
<td>5.7</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>No. of Theoretical plates (N)</td>
<td>5754</td>
<td>4165</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>3</td>
<td>Resolution (Rₛ)</td>
<td>10.73</td>
<td></td>
<td>&gt;2</td>
</tr>
<tr>
<td>4</td>
<td>Tailing factor (Aₛ)</td>
<td>1.1</td>
<td>1.0</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

Analysis of Marketed Formulation
The contents of twenty capsules were accurately weighed and powdered in a mortar. An amount equivalent to one capsule (Containing 10 mg of ROS and 75 mg of ASP) was transferred in to 10 ml of volumetric flask containing few ml of mobile phase and sonicated for 10 min. The flask was shaken and the solution was filtered through Whatman filter paper and filter paper was wash with methanol twice and volume was then made up to the mark with mobile phase. The sample solution thus prepared was appropriately diluted with mobile phase to get the solution containing ROS and ASP in 6:45 µg/ml proportion respectively. The solution was analyzed by proposed method and peak areas were measured. The quantification was carried out by keeping these values to the straight line equation of calibration curve.

TABLE 5: ASSAY RESULTS OF MARKETED DOSAGE FORM

<table>
<thead>
<tr>
<th>Dosage forms</th>
<th>Actual concentration in M g</th>
<th>Amount obtained in mg</th>
<th>% Assay ± % RSD (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ROS</td>
<td>ASP</td>
<td>ROS</td>
</tr>
<tr>
<td>Capsule</td>
<td>6</td>
<td>45</td>
<td>5.95</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION
Optimization of mobile phase
The objective of the method development was to resolve chromatographic peaks for active drug ingredients with less asymmetric factor.
The mobile phase acetonitrile: methanol: water (25:30:45, v/v/v), (pH 3) was found to be satisfactory which gave two symmetric and well-resolved peaks for ROS and ASP. The
retention time for ROS and ASP were 5.7 min and 2.6 min, respectively (Figure:-2). The resolution between ASP and ROS was found to be 10.73, which indicates good separation of both the compounds. The asymmetric factors for ROS and ASP were 1.1 and 1.0, respectively. The mobile phase flow rate was maintained at 1 mL/min. Overlaid UV spectra of both the drugs showed that ROS and ASP absorbed appreciably at 243 nm, so detection was carried out at 243 nm.

Forced degradation study also has been done according to ICH guideline.

Following degradation studies were done:

**Acid hydrolysis (figure:-3)**
ROS showed extensive degradation in acidic hydrolytic conditions. ROS was found to be unstable in acidic conditions, while ASP was found to be stable.

During the initial forced degradation experiments, it was observed that high degree of degradation of ROS occurred on heating with 0.1 N HCL solution at 60 - 70°C for 1 hr. Therefore, in later experiments, the acid hydrolysis of drug product in solution state was conducted by heating with 0.01 N HCL solution at 60 - 70°C for 1 hr.

**Base hydrolysis (figure:-4)**
ASP showed extensive degradation in basic hydrolytic conditions. ASP was found to be unstable in acidic conditions, while ROS was found to be stable.

During the initial forced degradation experiments, it was observed that complete degradation of ASP occurred on heating with 0.1 N NaOH solution at 60 - 70°C for 1 hr. Therefore, in later experiments, the acid hydrolysis of drug product in solution state was conducted by heating with 0.01 N NaOH solution at 60 - 70°C for 1 hr.

**Peroxide degradation (figure:-5)**
Both drugs exhibited very low degree of degradation in oxidative conditions on heating with 3 % v/v hydrogen peroxide solution in water at 60 - 70°C for 1 hr

**Thermal degradation (figure:-6)**
Both drugs were found to be relatively stable when the drug substances and drug product were kept for thermal degradation in solid state at 105°C for 1 hr under dry heat.

**Photo degradation study (figure:-7)**
Both drugs were found to be relatively stable to photolytic stress when the drug substances and drug product in solid state, was irradiated for 12 hr at 254 nm in a cabinet with a UV lamp.

Results and observations of these studies are shown in (Table: - 1).

**Method validation**

The calibration curve for ROS was found to be linear in the range of 0.2 - 10 µg/mL with a correlation coefficient of 0.999. The calibration curve for ASP was found to be linear in the range of 1.5 - 75 µg/mL with a correlation coefficient of 0.999. The regression analysis of calibration curves are reported in (Table:-2). Instrument precision was determined by performing injection repeatability test and the RSD values for ROS and ASP were found to be 0.3048 % and 0.8269 %, respectively. The intra-day and inter-day precision studies were carried out and the results are reported in (Table:-3). The low RSD values indicate that the method is precise.

The accuracy of the method was determined by calculating recoveries of ROS and ASP by method of standard addition. The recoveries found to be 99.32%–100.71% and 99.95%-100.04% for ROS and ASP respectively (Table:-3). The high values indicate that the method is accurate.

The detection limits for ROS and ASP were found to be 0.04 µg/mL and 0.08 µg/mL, respectively, while quantitation limits were found to be 0.15 µg/mL and 0.27 µg/mL, respectively. The above data shows that a nano gram quantity of both the drugs can be accurately and precisely determined. Robustness study was performed by deliberately changing the experimental conditions like flow rate from 1 ml/min to 0.9 ml/min and 1.1 ml/min. The composition of mobile phase was changed varying the proportion of methanol by 5 %. In both the conditions the recovery of both the drugs was determined and the RSD was found to be less than 2%.

System suitability test was carried out and the results are summarized in (Table:-4). Stability of standard and sample solution of ROS and ASP were evaluated at room temperature for 24 hr.
Analysis of marketed formulations
The proposed method was successfully applied to the determination of ROS and ASP in their combined dosage form. The % recovery was found to be 99.32 ± 0.92 and 99.97 ± 0.89 respectively, for ROS and ASP (Table:-5) which were comparable with the corresponding labeled amounts.

CONCLUSION
Proposed study describes stability indicating RP-HPLC method for the estimation of ROS and ASP combination in mixture. The method was validated and found to be simple, sensitive, accurate and precise. The method was successfully used for determination of drugs in their pharmaceutical formulation.

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