ABSTRACT

Objective: The present work was focused on the development and validation of reversed-phase high-performance liquid chromatography (RP-HPLC) method which is simple, rapid, accurate, sensitive, economical, and stability-indicating for the quantification of rosuvastatin (RSV) calcium and clopidogrel bisulfate (CLO) in bulk and tablet formulation.

Methods: The separation was attained on RP Princeton (C18) column with dimensions (250 mm × 4.6 mm, 5 μ) employing buffer which is a mixture of water (pH 3.0, adjusted with orthophosphoric acid), and methanol in the ratio (20:80) v/v as mobile phase, at flow rate 1.0 ml/min and detection was carried out at wavelength 240 nm. The retention time under the optimized condition of RSV calcium and CLO was found to be 2.844 min and 4.388 min, respectively.

Results: The linearity of the method was demonstrated in the concentration range of 6–16 μg/ml and 45–120 μg/ml for RSV calcium and CLO with a correlation coefficient (r²) of 0.9999 and 0.9996, respectively. The percentage relative standard deviation was <2% and percentage recovery was found to be 100.12–101.37% and 99.72–101.09% for RSV calcium and CLO, respectively. Assay of marketed tablet formulation was found to be 98.99% and 99.92%, respectively.

Conclusion: The developed RP-HPLC method was found to be simple, specific, sensitive, rapid, linear, accurate, precise, and economical and could be used for regular quality control of RSV calcium and CLO in bulk and tablet formulations.

Keywords: Rosuvastatin calcium, Clopidogrel bisulfate, Reversed-phase high-performance liquid chromatography, Validation, International conference on harmonization guidelines.

INTRODUCTION

Rosuvastatin (RSV) calcium is chemically (3R,5R)-7-[4-(4-Fluorophenyl)-2-[methyl(methylsulfonyl)amino]-6-propan-2-ylpyrimidin-5-yl]-3,5-dihydroxyhept-6enoic acid (Fig. 1) [1]. It is an HMG-CoA reductase inhibitor used in the treatment of hypertension and abnormal lipid [2]. Clopidogrel bisulfate (CLO) is chemically Methyl 2-(2-Chlorophenyl)-2-(6,7-dihydrothieno[3,2-C]pyridine-5(4H)-yl)acetate sulfone (Fig. 2) [3]. It is an anti-platelet agent as an ADP receptor blocker mainly to treat patients with the acute coronary syndrome, myocardial infarction, peripheral vascular disease, and some stroke (ischemic type) patients [4]. An extensive literature survey revealed that several high-performance liquid chromatography (HPLC) methods were reported for the estimation of RSV calcium and CLO in bulk and tablet formulation [5-27]. The International Conference on Harmonization (ICH) guideline entitled “Stability testing of new drug substances and products” requires that stress testing be administered to elucidate the inherent stability characteristics of the active substance [28]. An ideal stability-indicating technique is one that resolves the drug, and its degradation products efficiently. Consequently, the implementation of an analytical methodology to work out RSV and CLO, in the presence of its degradation products is sort of a challenge for pharmaceutical analysts. Therefore, it was thought necessary to study the stability of RSV and CLO under acidic, alkaline, hydrolytic, oxidative, light, and thermal conditions. The reported methods have the drawbacks of long runtime and less economical with a high proportion of organic phase. Hence, an attempt was made to develop RP-HPLC method which is simple, rapid, accurate, precise, specific, economical, sensitive, and stability-indicating for the estimation of RSV calcium and CLO in bulk and tablet formulations.

METHODS

Chemicals and reagents
Pharmaceutical grade RSV calcium and CLO were procured as a gift sample from Cadila Pharmaceuticals Ltd., Ahmedabad (India), Rosmi CV, a tablet formulation, obtained commercially.

Methanol, orthophosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide 30% of analytical grade were used throughout the work.

Instrumentation
Shimadzu HPLC system and PDA detector with Lab Solution software were used.

Chromatographic conditions
Chromatographic separation was achieved on a reversed-phase (RP) Princeton (C18) column with dimensions (250 mm × 4.6 mm, 5 μ) at ambient temperature using a mobile phase consisting of a mixture of buffer (pH 3.0, adjusted with orthophosphoric acid), and methanol in the ratio of (20:80) v/v at a flow rate of 1.0 ml/min. Detection was carried out at 240 nm. The pH of the mobile phase was set at 3.0, and a column temperature of 30°C. The injection volume was 10 μl. The optimized chromatographic conditions are shown in Table 1.
Preparation of standard solution of RSV and CLO

For RSV, an accurately weighed 1.0 mg of RSV was transferred to a 10.0 ml volumetric flask and dissolved in 5.0 ml of methanol. The volume was completed to 10.0 ml with methanol. One milliliter of the resulting solution was pipetted in 10.0 ml volumetric flask and the volume was made up to 10.0 ml with methanol to furnish a solution of concentration 10 μg/ml of RSV and 75 μg/ml of RSV and CLO, respectively.

Method Validation

The developed method was validated following ICH guidelines (ICH Q2R1) for accuracy, precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness [29].

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a standard true value or an accepted reference value, and therefore, the value found. It was computed at three different levels, that is, 80, 100, and 120% of the label claim. Standard addition and recovery experiments were conducted to determine the accuracy of RSV and CLO for the quantification of drugs in the samples.

Precision

The system precision was evaluated by measuring the area of six qualified working standards for RSV and CLO and calculating the percentage of relative standard deviation (RSD). The assay method precision was evaluated by conducting six independent assays of test samples of RSV and CLO against qualified working standards and calculating the percentage of RSD.

Linearity

Linearity test solutions of RSV and CLO were prepared at concentration levels of 6–16 μg/ml and 45–120 μg/ml, respectively. Linearity test solutions were prepared by diluting the stock solution to the required concentrations. Linearity was established by the least-squares linear regression analysis of the calibration data. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. The linear curve of RSV calcium and CLO was shown in Figs. 3 and 4, respectively.

Specificity

The specificity of the developed method was established by comparing the chromatograph of the standard and sample. It was found that there was no interference due to excipients and impurities at the retention time of the drug.

LOD and LOQ

The LOD is the lowest analyte concentration that can be detected. LOQ is the lowest analyte concentration that can be quantified with acceptable accuracy and precision. The LOD and LOQ were calculated from the standard deviation of the response and the slope of the calibration plot. LOD and LOQ were established, under ICH definitions, by use of the equations LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the standard deviation of the regression line and S is the slope of the calibration plot.

Table 1: Optimized chromatographic condition

| Parameters               | Chromatographic condition |
|--------------------------|----------------------------|
| Mobile phase             | Water (pH adjusted to 3.0 with orthophosphoric acid): Methanol (20:80) v/v    |
| Flow rate                | 1.0 ml/min                 |
| Column                   | Princeton C18 (250 mm × 4.6 mm, 5 μ) |
| Detector wavelength      | 240 mm                     |
| Column temperature       | 30°C                       |
| Injection volume         | 10 μl                      |
| Runtime                  | 20 min                     |
| Diluent                  | Methanol                   |
| Retention time           | About 2.844 min for rosuvastatin calcium peak and 4.388 min for clopidogrel bisulfate peak |

Fig. 1: Chemical structure of rosvastatin calcium

Fig. 2: Chemical structure of clopidogrel bisulfate
Robustness
To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between RSV and CLO was evaluated. To study the effect of wavelength on the estimation, the wavelength was altered by ±2 nm, that is, 238 and 242 nm from the actual wavelength, 240 nm. To study the effect of flow rate on estimation, the flow rate was altered by ±0.1 ml/min, that is, 0.9 and 1.1 ml/min from the actual flow rate, 1.0 ml/min.

Stability of the solution
The stability of both the standard and sample solution was checked up to 24 h at room temperature.

Stability-indicating assay
The drug was subjected to acidic (0.1 N HCl), alkaline (0.1 N NaOH), oxidative (0.3% H\textsubscript{2}O\textsubscript{2}), photo (light), thermal (sand bath at 50°C), and hydrolytic (water) condition and the percentage degradation was calculated.

RESULTS AND DISCUSSION
HPLC method development and optimization
Initially, pure drugs solution was chromatographed using a mobile phase consisting of a mixture of buffer (pH 3.0, adjusted with orthophosphoric acid), and methanol in the ratio of (20:80) v/v at a flow rate of 1.0 ml/min gives well-resolved peaks of drugs as well. Detection was carried out at 240 nm. The retention time under the optimized condition of RSV calcium and CLO was found to be 2.844 min and 4.388 min, respectively. The total run time of the chromatogram was about 20 min. A typical chromatogram of a mixture of standard and sample of RSV calcium and CLO was shown in Figs. 5 and 6, respectively.

Validation of the method
System suitability
The suitability of the system was demonstrated by assessing various parameters. It was established by injecting six replicate injections of the standard solution. Theoretical plates were found to be 2816 and 3811, tailing factor of 1.60 and 1.54, and %RSD of peak area was 0.9 and

y = 47831x + 3050.8
R\textsuperscript{2} = 0.9999

Fig. 3: Linear curve of rosuvastatin calcium

Fig. 4: Linear curve of clopidogrel bisulfate

Fig. 5: Chromatogram of mixture of standard RSV and CLO. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.844 min and 4.388 min, respectively

y = 7103.4x + 6386
R\textsuperscript{2} = 0.9996

Fig. 6: Chromatogram of mixture of sample RSV and CLO. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.849 min and 4.387 min, respectively
0.8 for RSV and CLO, respectively (Table 2). All the system suitability parameters were well within limits, indicating that the system was well suitable for performing the analysis.

**Linearity**

Linearity was established by the least-squares linear regression analysis of the calibration data. Calibration plots were linear over the concentration range of 6–16 µg/ml for RSV and 45–120 µg/ml for CLO. Peak areas were plotted against the respective concentrations and linear regression analysis performed on the resulting curves. The linear curve of RSV calcium and CLO was shown in Figs. 3 and 4, respectively. The linear regression equation obtained was $Y=47831x+3050.8$ for RSV and $Y=7103.4x+6366$ for CLO with correlation coefficient 0.9999 and 0.9996, respectively. The results of linearity are shown in Table 3.

**Accuracy**

Accuracy was computed by recoveries studies. The mean percentage recoveries values for three levels were found to be between 100.12–101.37% and 99.72–101.09% for RSV and CLO, respectively. The percentage recoveries values within limits, indicating the method developed was accurate. The results of recovery are shown in Table 4.

**Precision**

The results of intraday precision and interday precision were 0.9 and 0.6 for RSV. The results of intraday precision and interday precision were 0.8 and 0.4 for CLO. The percentage RSD of system, method, and intermediate precision study was well within limits (<2%), indicate that the method was precise.

**LOD and LOQ**

The LOD was found to be 0.65 µg/ml for RSV and 6.90 µg/ml for CLO. The LOQ was found to be 1.98 µg/ml for RSV and 20.91 µg/ml for CLO. The values of LOD and LOQ indicate that the method was greatly sensitive (Table 5).

**Robustness**

Robustness of the method was designed by changing the optimized condition adequately. To evaluate the robustness of the developed method, the chromatographic conditions were deliberately altered and the resolution between RSV and CLO was evaluated. On the assessment

| Parameter          | RSV          | CLO          |
|--------------------|--------------|--------------|
| Theoretical plate  | 2816         | 3811         |
| Retention time (Rt)| 2.844        | 4.388        |
| Tailing factor     | 1.60         | 1.54         |
| % RSD              | 0.9          | 0.8          |

Table 2: System suitability results

| Parameter          | RSV          | CLO          |
|--------------------|--------------|--------------|
| Concentration range (µg/ml) | 6–16        | 45–120       |
| Slope (m)          | 47831        | 7103.4       |
| Tailing factor     | 3050.8       | 6366         |
| Coefficient correlation ($r^2$) | 0.9999      | 0.9996       |

Table 3: Linearity results

| Drug | Level (%) | Amount taken (µg/ml) | Amount found* (µg/ml) | % Recovery* |
|------|-----------|----------------------|-----------------------|-------------|
| RSV  | 80        | 8                    | 8.11                  | 101.37      |
|      | 100       | 10                   | 10.01                 | 100.12      |
|      | 120       | 12                   | 12.05                 | 100.41      |
| CLO  | 80        | 60                   | 59.83                 | 99.72       |
|      | 100       | 75                   | 75.81                 | 101.09      |
|      | 120       | 90                   | 90.75                 | 100.83      |

Table 4: Recovery results

*Average of three determinations. RSV: Rosuvastatin, CLO: Clopidogrel bisulfate

Fig. 7: Chromatogram of RSV and CLO degraded with acid hydrolysis. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.998 min and 4.288 min, respectively, and degradation products of RSV with Rt 2.712 and 2.868 min and degradation products of CLO with Rt 3.522 min

Fig. 8: Chromatogram of RSV and CLO degraded with alkali hydrolysis. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.840 min and 4.366 min, respectively, and degradation products of RSV with Rt 2.322 min and degradation products of CLO with Rt 2.116 and 3.724 min
of the result, it can be deduced that the variation in the changing wavelength, flow rate does not affect the method significantly. %RSD <2% specifies that the developed method was robust. The results of robustness are shown in Table 6.

### Analysis of RSV calcium and CLO from marketed tablets

The percentage assays of tablet formulation were found to be 98.99 and 99.92% for RSV and CLO, respectively. The stability of the drug solution was observed for 24 h. In degradation studies, the drug was exposed to various stress conditions. From the chromatograms of stressed samples, it was found that no interference from degradants was observed at the retention time of RSV calcium and CLO. Optimum degradation was observed in the presence of acid and alkali. Substantial degradation was observed in the presence of water, light, and peroxide. No degradation was observed in the presence of heat for RSV and CLO. The results of the percentage of degradation are presented in Table 7 and Figs. 7-12. Hence, the method was found to be specific.

### Table 5: Summary of validation parameter

| Parameter                              | RSV          | CLO          |
|----------------------------------------|--------------|--------------|
| Calibration range (µg/ml)              | 6–16         | 45–120       |
| Optimized wavelength (nm)              | 240          | 240          |
| Retention time                         | 2.844        | 4.388        |
| Regression equation (Y)                | Y = 4783.1x+3050.8 | Y = 7103.4x+6366 |
| Slope                                  | 47831        | 71034        |
| Intercept                              | 3050.8       | 6366         |
| Coefficient correlation (r²)           | 0.9999       | 0.9996       |
| Precision (%RSD)                       | Intraday: 0.6 | 0.8          | 98.99     |
|                                       | Interday: 0.9 | 0.4          | 99.92     |
| LOD (µg/ml)                            | 0.65         | 6.90         |
| LOQ (µg/ml)                            | 1.98         | 20.91        |

*Average of five determinations, LOD: Limit of detection, LOQ: Limit of quantification. RSV: Rosuvastatin, CLO: Clopidogrel bisulfate, RSD: Relative standard deviation

### Table 6: Robustness results

| Condition                          | RSV       | RSD (%) | CLO       | RSD (%) |
|------------------------------------|-----------|---------|-----------|---------|
| Change in wavelength 238 nm        | 97.28     | 0.5698  | 100.16    | 0.0900  |
| (240±2 nm) 242 nm                  | 98.21     | 0.5733  | 100.08    | 0.1417  |
| Change in flow rate 0.9 ml/min     | 99.55     | 0.6381  | 99.37     | 0.2068  |
| (1.0±0.1 ml/min) 1.1 ml/min        | 99.24     | 0.5078  | 99.56     | 0.2099  |

*Average of three determinations, %RSD: Percentage relative standard deviation. RSV: Rosuvastatin, CLO: Clopidogrel bisulfate, RSD: Relative standard deviation

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**Fig. 9**: Chromatogram of RSV and CLO degraded with neutral hydrolysis. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.846 min and 4.342 min, respectively, and degradation products of RSV with Rt 2.114 and 2.316 min and no degradation products of CLO

**Fig. 10**: Chromatogram of RSV and CLO degraded with oxidative hydrolysis. Rosuvastatin calcium (RSV) and clopidogrel bisulfate (CLO) with Rt of 2.892 min and 4.380 min, respectively, and degradation products of RSV with Rt 0.722 and 2.234 min and no degradation products of CLO
CONCLUSION

The method enables simple, rapid, accurate, precise, specific, economical, and sensitive analysis of RSV calcium and CLO in bulk and tablet dosage form. This method was validated as per ICH guidelines. The method can, therefore, be used for routine quality control analysis of RSV calcium and CLO in bulk and tablet dosage form.

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AUTHORS’ CONTRIBUTIONS

AP and RK designed the study. AP performed the experiment, and analyzed the data, and reviewed it. RK supervised the experiment, reviewed the data, and supported for writing the manuscript.

CONFLICTS OF INTEREST STATEMENT

Authors declare that they have no conflicts of interest that exist in this investigation.

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