DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF ASPIRIN, ROSUVASTATIN, CLOPIDOGREL IN BULK AND PHARMACEUTICAL DOSAGE FORM

POOJA PISAL, GANESH NIGADE, AMOL KALE, SMITA PAWAR*

Department of Pharmaceutical Chemistry, PDEA's Seth Govind Raghunath Sable College of Pharmacy, Saswad, Pune 412301, Maharashtra, India

Email: sjpawar4477@gmail.com

Received: 06 Jul 2018 Revised and Accepted: 01 Sep 2018

INTRODUCTION
Platelet aggregation and thrombus formation play a critical role in the initiation and development of key complications of acute coronary syndromes (ACSs). 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 (CVD), antiplatelet therapy and antithrombotic therapy have been demonstrated to modify clinical outcome favorably, and recent trials of revascularization in ACSs have demonstrated a reduction in the frequency of major cardiac events [1-3]. Yet, all effective antithrombotic agents also increase the risk of bleeding. Especially bleeding that results from vascular accessories associated with surgery, including coronary artery bypass grafting (CABG) [4-6]. Aspirin is chemically acetylsalicylic acid. Aspirin is a non-steroidal anti-inflammatory drug that exhibits anti-inflammatory, analgesic and antipyretic activities. Rosuvastatin calcium, chemically it is 7-[4- (4-fluorophenyl)-2-[methyl(methylsulfonyl)amino]-6-propan-2- yl]pyrimidin-5-yl]3,5-dihydroxyhept-6-en-1-oxid. It is used as anti-platelemic Clopidogrel bisulphate, chemically it is [S-(a)(2-ylypyrimidin-5-yl)]-3,5-dihydroxyhept-6-en-1-oxid. It is used as anti-platelemic and antithrombotic agents.

The combination of aspirin, rosuvastatin, and clopidogrel is available in the market as a capsule and tablet dosage form and is choice of drug for treatment of heart attack prevention. Rosumac gold manufactured by Macleod pharmaceuticals was used for RP-HPLC study. The strengths of the drug in the capsule chosen are aspirin 75 mg, rosuvastatin 10 mg, and clopidogrel 75 mg. The literature survey revealed that no method has been reported for estimation of aspirin, rosuvastatin, and clopidogrel using RP-HPLC. UV spectrophotometric methods have been reported for estimation of aspirin, rosuvastatin and clopidogrel in the pure form [7]. Some methods have been reported for stability indicating assay method for simultaneous estimation of aspirin and rosuvastatin [8, 9] and aspirin and clopidogrel in combined dosage form [10]. The clopidogrel bisulphate was analysed using bioanalytical RP-HPLC method [11]. Also, simultaneous estimation of clopidogrel and atorvastatin was carried out using bioanalytical UFLC method [12]. However, no RP-HPLC method is available for simultaneous determination of aspirin, rosuvastatin and clopidogrel and its pharmaceutical dosage form. Similarly, no studies have been carried out on Stability Indicating Assay Methods for simultaneous determination of aspirin, rosuvastatin and clopidogrel in bulk drugs and pharmaceutical dosage form.

In the present study, stability indicating an RP-HPLC method for the combination was tested by degrading the drugs together under various stress condition like acid, base and neutral hydrolysis, oxidation, thermal and photolytic stress which is recommended by ICH.

MATERIALS AND METHODS
Aspirin, rosuvastatin and clopidogrel pure compounds were available by Research-lab fine chem, Industries, Mumbai, Lapin Ltd., Pune, Cadila Healthcare Ltd, Goa. Rosumac gold capsule manufactured by Macleod Pharmaceuticals, Mumbai, containing the labeled amount of 75 mg aspirin, 10 mg rosuvastatin, and 75 mg Clopidogrel was purchased from local markets. All the chemicals
used were of analytical grade. HPLC grade water and solvents were used to prepare all the solutions.

**HPLC instrumentation and chromatographic conditions**

The chromatographic system used to perform analytical method development and validation of this assay method comprised of (SHIMADZU, HPLC) with LC-20 AD pump, with software LC software and UV-Visible detector SPD-20A. Chromatographic analysis was performed on a BISCOF C18 (250 mm 4.6 mm ID, 5 μm particle size) column. The mobile phase consisted of water at pH 2.51 with 0.1% (v/v) orthophosphoric acid (v/v): acetonitrile (50:50). The flow rate of the mobile phase was adjusted to 1.0 ml/min, and the injection volume was 20 μl. Detection was performed at 237 nm. Isocratic analytical method was used.

**Preparation of stock solution**

Standard stock solution (1000 µg/ml) of aspirin, rosuvastatin, and clopidogrel was prepared in optimized mobile phase. Accurately weighted 100 mg of each drug is transferred to 100 ml volumetric flask and dissolved in the selected mobile phase. It was then sonicated for 20 min. The solution was diluted up to volume with the same mobile phase. In order to obtain the test solution, stock solutions were further diluted to make 1 µg/ml.

In the present work, an analytical method based on LC using UV detection was developed and validated for the determination of aspirin, rosuvastatin, and clopidogrel in a capsule formulation. The analytical conditions were selected, keeping in mind the different chemical nature of aspirin, rosuvastatin, and clopidogrel. The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions.

The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between aspirin, rosuvastatin and clopidogrel peak. After evaluating all these factors, C18 (250 mm, 4.6 mm ID, 5 μm particle size) column was found to be giving satisfactory results.

**Forced degradation studies**

**Table 1: Forced degradation condition**

| Degradation condition | Condition                                      |
|-----------------------|------------------------------------------------|
| Acidic                | 1 h refluxed with 0.1 N HCl for 80 °C           |
| Basic                 | 1 h refluxed with 0.1 N NaOH for 80 °C          |
| Neutral               | 1 h kept at room temperature.                   |
| Oxidation             | 1 h refluxed with 6 % v/v H₂O₂                  |
| Thermal               | 24 h kept in a dry oven at 105 °C               |
| Photolytic            | UV light-254 nm for 5 h.                        |

**Fig. 1:** Chromatogram of aspirin, rosuvastatin, and clopidogrel

**Fig. 2:** Degradation of the sample by acidic conditions (A1-First degradants of aspirin, R1-First degradant of rosuvastatin, R2-Second degradant of rosuvastatin, C1-First degradant of clopidogrel)

**Fig. 3:** Degradation of sample by alkaline condition (A1-First degradant of aspirin, R1-First degradant of rosuvastatin, C1-First degradant of clopidogrel)
RESULTS AND DISCUSSION

The retention time of all three drugs, aspirin, rosuvastatin, and clopidogrel decreased as the pH of the mobile phase increased. This may be due to the ionized state of the drugs. The satisfactory retention time was achieved for the selected drug at 2.51. Various combinations of solvents were tried to get satisfactory retention time and proper resolution of peaks. The C18 column was used which is hydrophobic in nature. The column was selected as it resulted in proper resolution of all three drugs without tailing.

At 50:50 % ratio of acetonitrile: water at pH 2.51 adjusted to 0.1 % (v/v) OPA a symmetrical peak eluted at around 3.9 min, 7.5 min and 16.9 min with good capacity and it was selected for further studies.

1 ml/min flow rate gave symmetrical peak with the acceptable capacity factor. For the present study, 1 ml/min was selected on the basis of less retention time, good peak shape, the acceptable back pressure of aspirin, rosuvastatin calcium and clopidogrel. At a flow rate of 0.8 ml/min peak broadening was observed, and peak shape was irregular.
Method development

A reverse phase HPLC method with stability indicating assay methods was developed keeping in mind the system suitability parameter that is a resolution factor between peaks, tailing factor, the number of theoretical plates, run time. The developed optimized method resulted in elution of aspirin at 4.3 min, rosuvastatin at 7.6 min and clopidogrel at 16.6 min. In figure 1 represents chromatogram of all three drugs. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatography system. Retention time, the number of theoretical plates, peak resolution and peak tailing factor were evaluated for seven replicate injections of the standard working concentration. The results given in table 2 were within the acceptable limits.

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meets the requirements for the intended analytical application [13-14]. The developed HPLC method was validated according to ICH guideline for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, method precision, robustness, limit of detection and limit of quantitation [15].

Precision

System precision

Five replication injection of 1 µg/ml standard solutions showed percent RSD less than 2, which indicates the acceptable reproducibility and thereby the precision of the system [14, 16]. System precision results are tabulated in table 3.

Method precision

Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration. Seven injections of the sample from the same homogeneous mixture at working concentration showed %RSD less than 2 indicate that the developed method was precise by the test of repeatability [16, 17] and hence can be understood that the method gives consistently reproducible results in table 4.

Linearity

Linearity of aspirin, rosuvastatin, and clopidogrel was performed using a standard solution in the range of 1-7 µg/ml of each drug. The plots of peak area Vs respective concentration of aspirin, rosuvastatin, and clopidogrel were found to be linear in the range of 1-7 µg/ml. The correlation coefficients were greater than 0.99 for each drug, which meets the method validation, acceptance criteria [16, 17] and hence the method is said to be linear (fig. 8-10).

Table 2: Results of system suitability studies

| Parameters                      | Aspirin | Rosuvastatin | Clopidogrel |
|---------------------------------|---------|--------------|-------------|
| Retention time (min)            | 4.3     | 7.6          | 16.6        |
| Tailing factor                  | 1.1     | 1.0          | 0.909       |
| Capacity factor                 | 1.052   | 2.354        | 1.3         |
| Theoretical plate               | 10313   | 12884        | 16149       |
| Resolution factor               | 4.1     | 5.10         | 2.10        |

Table 3: Results of system precision

| Concentration | Aspirin | Rosuvastatin | Clopidogrel |
|---------------|---------|--------------|-------------|
| 1 µg/ml       | 208.800 | 42.651       | 187.546     |
| 1 µg/ml       | 207.741 | 43.757       | 189.822     |
| 1 µg/ml       | 205.800 | 42.651       | 187.546     |
| 1 µg/ml       | 210.872 | 42.789       | 189.097     |
| Mean          | 207.682 | 43.066       | 187.848     |
| SD            | 2       | 0.51         | 1.76        |
| % RSD         | 0.99    | 1.20         | 0.94        |

# n: 5 i.e. number of injections, #SD: standard deviation, # % RSD: percentage relative standard deviation

Table 4: Result of method precision

| Compounds       | Intra-day       | Inter-day       |
|-----------------|-----------------|-----------------|
|                 | Concentration (µg/ml) n=7 | % RSD | Concentration (µg/ml) n=7 | % RSD |
| Aspirin         | 1-7             | 0.08            | 1-7             | 1.74  |
| Rosuvastatin    | 1-7             | 0.19            | 1-7             | 0.17  |
| Clopidogrel     | 1-7             | 0.11            | 1-7             | 0.09  |

# % RSD: percentage relative standard deviation, # n: 7 i.e. number of injections

Table 5: Data for linearity studies

| Compounds       | Concentration range (µg/ml) | Regression equation | R² |
|-----------------|----------------------------|---------------------|----|
| Aspirin         | 1-7                        | y=258.7x+86.52      | 0.999|
| Rosuvastatin    | 1-7                        | y=50.583x+9.5243    | 0.9989|
| Clopidogrel     | 1-7                        | y=165.46x+37.867    | 0.9988|

# n: 7 i.e. number of injections, # R: Correlation coefficient.
Accuracy and recovery

Recovery studies were performed at three different levels 80, 100 and 120 %. The sample was spiked with known amounts of aspirin, rosuvastatin and clopidogrel. The % RSD of accuracy study was found to be within specific limits, and thus we can conclude that the developed method is suitable for assay and there is no interference of excipients of the pharmaceutical dosage form. The recovery data from the study were reported in table 6-8.

Robustness

Deliberate variations were made in the method and % RSD was calculated plus and minus flow rate and mobile phase ratios were the variations that were analyzed. Small but deliberate variations were made in the method parameters, and it was found that % RSD values for all the variations were in acceptable limits. This indicated that the method is robust and it can be used within small variations of flow rate and mobile phase, without having a measurable effect on the result.

Table 6: Accuracy study of aspirin

| Spiked concentration (µg/ml) | Measured concentration (µg/ml) mean±SD | % RSD | % Recovery |
|-----------------------------|----------------------------------------|-------|------------|
| 80                          | 79.9±0.89                              | 1.19  | 99.81      |
| 100                         | 101±1.09                               | 1.07  | 100.09     |
| 120                         | 119±1.79                               | 1.74  | 99.87      |

# %RSD: percentage relative standard deviation
Table 7: Accuracy study of rosuvastatin

| Spiked concentration (µg/ml) | Measured concentration (µg/ml) mean±SD | % RSD | % Recovery |
|-----------------------------|----------------------------------------|-------|------------|
| 80                          | 78.9±1.15                              | 1.44  | 99.77      |
| 100                         | 102±0.89                               | 0.88  | 100.15     |
| 120                         | 119±0.20                               | 0.17  | 100.32     |

# % RSD: percentage relative standard deviation, #SD: standard deviation

Table 8: Accuracy study of clopidogrel

| Spiked concentration (µg/ml) | Measured concentration (µg/ml) mean±SD | % RSD | % Recovery |
|-----------------------------|----------------------------------------|-------|------------|
| 80                          | 79.2±0.46                              | 0.58  | 100.04     |
| 100                         | 99.7±0.25                              | 0.30  | 99.47      |
| 120                         | 120±0.21                               | 0.17  | 100.06     |

# % RSD: percentage relative standard deviation, #SD: standard deviation

Table 9: Result of Robustness of standard drugs

| Parameters                      | Aspirin | Rosuvastatin | Clopidogrel |
|---------------------------------|---------|--------------|-------------|
| Flow rate                       | 3.98    | 6.54         | 16.17       |
| 1.2                             | 3.69    | 6.40         | 16.24       |
| mean±SD                         | 3.83±0.20 | 6.47±0.09 | 16.2±0.04   |
| Mobile phase ratio (Organic: Aqueous) | 4.01    | 6.60         | 16.89       |
| 5:5:45                          | 3.90    | 6.48         | 16.75       |
| 53:47                           | 3.95±0.77 | 6.54±0.08 | 16.82±0.09  |

# % RSD: percentage relative standard deviation, #SD: standard deviation

Sensitivity

The sensitivity of measurement of aspirin, rosuvastatin and clopidogrel by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and limit of detection (LOD). LOD and LOQ were calculated by the use of the equations LOD = 3.3σ/S and LOQ = 10σ/S where σ is the standard deviation of intercepts of calibration plots and S is the average of the slopes of the corresponding calibration plot (table 10).

Table 10: LOD and LOQ for aspirin, rosuvastatin and clopidogrel

| Compounds         | LOD (µg/ml) | LOQ (µg/ml) |
|-------------------|-------------|-------------|
| Aspirin           | 0.005       | 0.01        |
| Rosuvastatin      | 0.01        | 0.05        |
| Clopidogrel       | 0.009       | 0.002       |

# LOD: limit of detection, # LOQ: limit of quantitation.

Forced degradation studies

Table 11: Forced degradation study

| Types of degradation | Peak area (mV) | % of degradation |
|----------------------|----------------|-----------------|
| Aspirin              |                |                 |
| Acidic               | 36.469         | 14.54           |
| Base                 | 520.681        | 26.97           |
| Neutral              | 34.024         | 10.2            |
| Oxidative            | 545.874        | 27.01           |
| Thermal              | 244.697        | 3.87            |
| Rosuvastatin         |                |                 |
| Acidic               | 37.714         | 23.35           |
| Base                 | 171.653        | 12.24           |
| Neutral              | 39.115         | 19.27           |
| Oxidative            | 3.834          | 4.35            |
| Photo                | 2.479          | 2.28            |
| Clopidogrel          |                |                 |
| Acidic               | 6.990          | 4.58            |
| Base                 | 19.507         | 9.78            |
| Neutral              | 2.794          | 17.21           |
| Oxidative            | 3.199          | 2.20            |
| Photo                | 8.102          | 8.70            |
| Thermal              | 3.298          | 8.71            |
| Acidic               | 6.990          | 4.58            |
The results obtained from the above set of observations prove that the method is useful in the qualitative and quantitative analysis of the standard drug with pharmaceutical dosage form. Moreover, various analytical methods for estimation of aspirin, rosuvastatin and clopidogrel alone were reported and in combination with other drugs. However, there is no HPLC and stability indicating assay method is reported for analysis and for simultaneous estimation of aspirin, rosuvastatin and clopidogrel combination and the novel method developed in this report is the first of its kind. The developed method is based on the use of a very economical solvent, had short chromatographic time and hence can be performed with ease.

CONCLUSION

The developed RP-HPLC method is simple, economical, specific, accurate and precise for the simultaneous estimation of aspirin, rosuvastatin, and clopidogrel in the combined capsule dosage form. The developed method offers good resolution between aspirin, rosuvastatin and clopidogrel. It was successfully validated in terms of system suitability, linearity, range, precision, accuracy, specificity, LOD, LOQ and robustness as per ICH guidelines. So, the described method is suitable for routine analysis and quality control of pharmaceutical preparations comprising these drugs either as an individual or in combination. Developed stability indicating analytical method can be used to check the stability of the compounds and was found suitable to determine % degradation of drugs in pharmaceutical dosage form.

ACKNOWLEDGEMENT

The authors acknowledge, PDEA's Seth Govind Raghunath Sable College of Pharmacy, Saswad, Maharashtra, India, for providing the necessary facilities to carry out the research.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declare none

REFERENCES

1. Trialists Collaboration A. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high-risk patients. Br Med J 2002;324:71-6.
2. Boersma E, Harrington RA, Moliterno DJ, White H, Theroux P, Van de Wert F, and Simoons ML. Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. Lancet 2002;359:189-98.
3. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Clopidogrel in unstable angina to prevent recurrent events trial investigators. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. New England J Med 2001;345:494-502.
4. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK. Clopidogrel in unstable angina to prevent recurrent events trial (CURE) investigators. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet 2001;358:527-33.
5. Pursuit Trial Investigators. Inhibition of platelet glycoprotein Ib/IIa with epifibatide in patients with acute coronary syndromes. Platelet glycoprotein Ib/IIa in unstable angina. Receptor suppression using integrin therapy. New England J Med 1998;339:436-43.
6. PRISM Study Investigators. A comparison of aspirin plus tiotidine with aspirin plus heparin for unstable angina. New England J Med 1998;338:1498-505.
7. Singh RD, Yadav H, Hinge M, Patel A. Development and validation of analytical methods for simultaneous estimation of rosuvastatin, clopidogrel and aspirin in the pharmaceutical dosage form. J Pharm Sci Bioscientific Res 2016;1:65-70.
8. Badawy A, Mostafa N, Lamie N, El-Aal A. Stability-indicating methods for the determination of rosuvastatin in its oxidative degradation products. Int J Pharm Res 2012;4:31-36.
9. Panchal A, Sanghvi G, Vachhani A, Seth N, Vaishnavi D. Simultaneous determination of aspirin and rosuvastatin calcium in capsules by using an RP-HPLC couple with photodiode array detection. Int Lett Chem Phys Astron 2014;3:218-20.
10. Gandhi B, Mohapatra K. High performance liquid chromatographic determination of aspirin and clopidogrel in tablets. Indian J Pharm Sci 2007;69:123-5.
11. Nagavi J, Gurupadasuya B. Simultaneous estimation of clopidogrel and hep in human plasma using the bio-analytical RP-HPLC fast liquid chromatographic method. Int J Curr Pharm Res 2015;7:30-5.
12. Jain HK, Deore DD. Bioanalytical method development and validation for estimation of clopidogrel bisulphate in human plasma by RP-HPLC. Int J Appl Pharm 2016;4:18-21.
13. Sharma BK. Instrumental method of chemical analysis. 2nd ed. Goal publication House 1991;23:7-10.
14. Dong M. Regulatory aspects of HPLC analysis: HPLC system and method validation, modern HPLC for practicing scientists. John Wiley and Sons. New Jersey; 2006. p. 1.
15. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. Validation of Analytical Procedures: Text and Methodology ICH Q2 (R1); 2005.
16. FDA, Guidance R. Validation of chromatographic methods center for drug evaluation and research (CDER). Food Drug Administration; 1994. p. 2.
17. FDA, ORA validation and verification guidance for human drug analytical methods. Food Drug Administration; 2003. p. 1.