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Original Article

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

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ABSTRACT

Objective: To develop a novel, accurate, precise and linear reverse phase high-performance liquid chromatography (RP-HPLC) and Stability Indicating Assay Method (SIAMs) for simultaneous, qualitative and quantitative estimation of aspirin, rosuvastatin and clopidogrel in bulk and pharmaceutical dosage form as per International Conference on Harmonization (ICH) guidelines.

Method: In the present work, good chromatographic separation was achieved by isocratic method using a BISCOF HPLC C_{18} column (250 mm ×4.6, 5 µm) and a mobile phase consisting of water at pH 2.51 with 0.1 % (v/v) orthophosphoric acid (OPA): acetonitrile in the ratio 50:50, at a flow rate of 1 ml/min. The effluents obtained were monitored at 237 nm with the UV-visible detector.

Results: The retention time of aspirin, rosuvastatin, and clopidogrel was found to be 4.3 min, 7.6 min and 16.6 min respectively. For linearity seven-point calibration curves were obtained in a concentration range from 1-7 µg/ml for aspirin, rosuvastatin and clopidogrel with correlation coefficient 0.999, 0.9989, 0.9988 respectively. The high recovery values (99%-101%) indicate a satisfactory accuracy. The low percent relative standard deviation (% RSD) values in the precision study reveal that the method is precise. 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.

Conclusion: The developed RP-HPLC method is simple, economic, specific, accurate and precise for the simultaneous estimation of aspirin, rosuvastatin, and clopidogrel in the combined capsule dosage form. The 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.

Keywords: RP-HPLC, Stability indicating method, Aspirin, Rosuvastatin, Clopidogrel, ICH guideline

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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-ylpyrimidin-5-yl]-3,5-dihydroxyhept-6-enoate. It is used as antilipidemic. Clopidogrel bisulfate, chemically it is [S-(a)(2-chlorophenyl)-6,7-dihydrothieno (3,2-C) pyridine-5 (4H) acetic acid methyl ester sulfate]. It is an antithrombotic agents.

The combination of aspirin, rosuvastatin, 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 conditions like acid hydrolysis, base hydrolysis, and oxidation, thermal and photolytic stress as per ICH guideline.

MATERIALS AND METHODS

Aspirin, rosuvastatin and clopidogrel pure compounds were supplied by Research-lab fine chem. Industries, Mumbai, Lupin 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 was 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 C_{18} (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 the obtained 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, C_{18} 250 mm, 4.6 mm ID, 5 µm particle size) column was found to be giving satisfactory results.

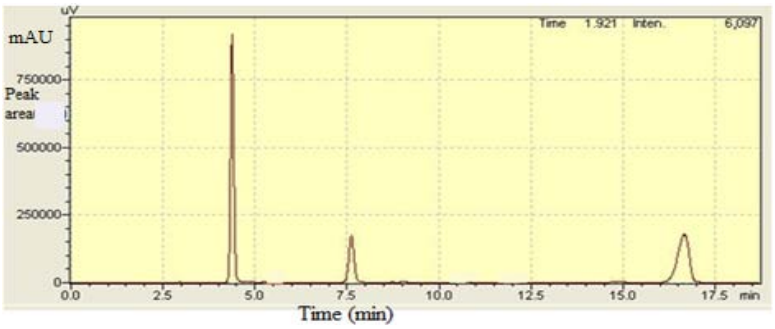

Fig. 1: Chromatogram of aspirin, rosuvastatin, and clopidogrel

Forced degradation studies

Table 1: Forced degradation condition

Degradation 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 H_2O_2.
Thermal	24 h kept in a dry oven at 105 °C.
Photolytic	UV light-254 nm for 5 h.

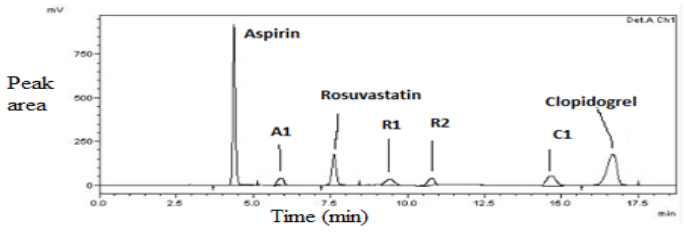

Fig. 2: Degradation of the sample by acidic conditions (A1-First degradants of aspirin, R1-First degradant of rosuvastatin, R2-Second degradant of rosuvastatin, C1-First degradant of clopidogrel)

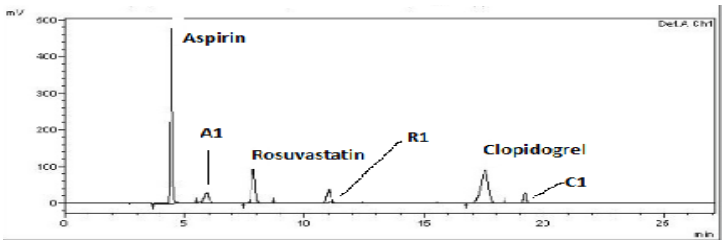

Fig. 3: Degradation of sample by alkaline condition (A1-First degradant of aspirin, R1-First degradant of rosuvastatin, C1-First degradant of clopidogrel)

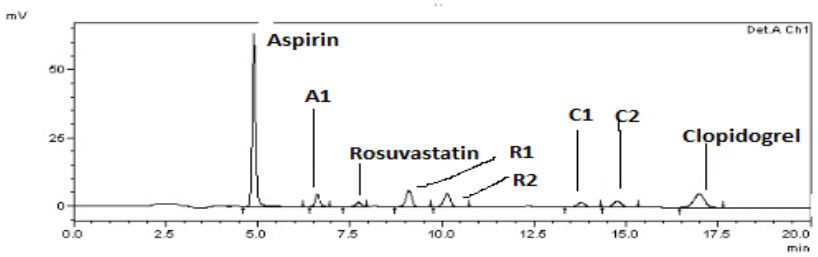

Fig. 4: Degradation of the sample by neutral condition (A1-First degradants of aspirin, R1-First degradant of rosuvastatin, R2-Second degradant of rosuvastatin, C1-First degradant of clopidogrel, C2-second degradant of clopidogrel)

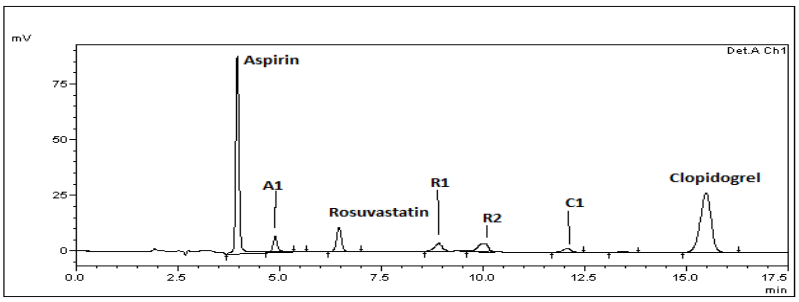

Fig. 5: Degradation of the sample by Oxidative condition (A1-First degradants of aspirin, R1-first degradant of rosuvastatin, R2-Second degradant of rosuvastatin, C1-first degradant of clopidogrel)

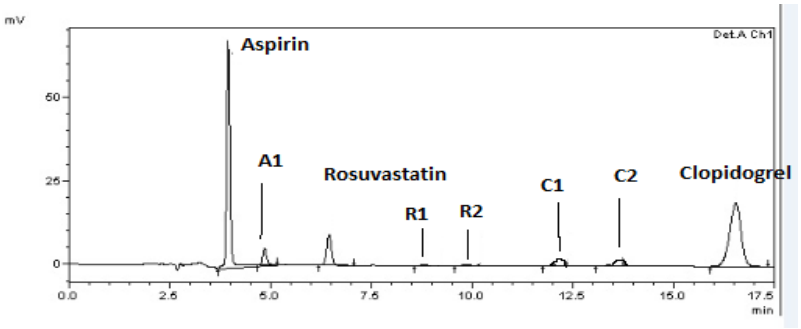

Fig. 6: Degradation of the sample by thermal condition (A1-first degradants of aspirin, R1-first degradant of rosuvastatin, R2-Second degradant of rosuvastatin, C1-First degradant of clopidogrel, C2-Second degradant of clopidogrel)

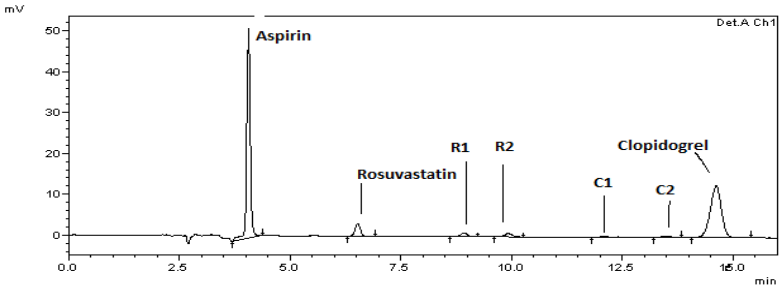

Fig. 7: Degradation of the sample by photolysis condition (R1-first degradant of rosuvastatin, R2-second degradant of rosuvastatin, C1-first degradant of clopidogrel, C2-second degradant of clopidogrel)

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 C_{18} 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 fig. 1 represent 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.

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

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 3: Results of system precision

Concentration	Peak area (mV)		
	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
1 µg/ml	206.197	43.483	185.229
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	R^2
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.

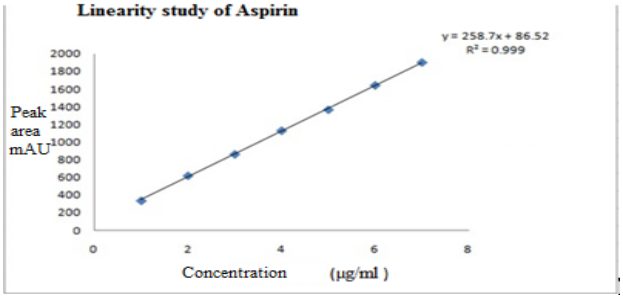

Fig. 8: Linearity plot of aspirin

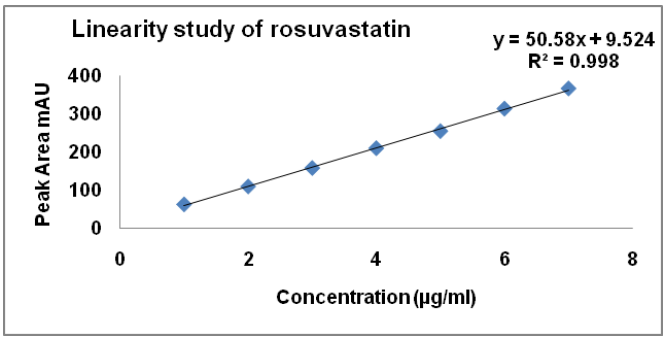

Fig. 9: Linearity plot of rosuvastatin

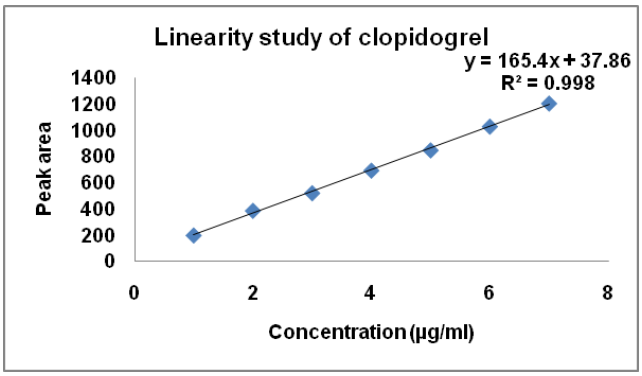

Fig. 10: Linearity plot of clopidogrel

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	Retention time (min)		
Flow rate	**Aspirin**	**Rosuvastatin**	**Clopidogrel**
0.8	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)	Retention time (min)		
55:45	4.01	6.60	16.89
53:47	3.90	6.48	16.75
mean±SD	3.955±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). LOQ and LOD 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		% 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		8.07
Rosuvastatin			
	R1	R2	
Acidic	37.714	3.787	23.35
Base	171.653	-	12.24
Neutral	39.115	61.825	19.27
Oxidative	3.834	4.228	4.35
Photo	2.479	3.137	2.88
Clopidogrel			
	C1	C2	
Acidic	6.990		4.58
Base	19.507		9.78
Neutral	2.794	3.772	17.21
Oxidative	3.199		2.20
Photo	8.102	1.274	8.70
Thermal	3.298	5.124	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.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declare none

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