Research Article

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

*D. Sai Malakondaiah, V. RanjeetKumar, M. NaveenKumar, A. Sai Ram, T. Rama Mohan Reddy, A. Ajitha, V. Uma Maheshwara Rao

Department of Pharmaceutical Analysis and Quality Assurance, CMR College of Pharmacy, Kandlakoya (v), Medchal road, Hyderabad – 501 401, A.P, India.

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 $Zorbax\text{-}SB(\ C_{18})(150mmx4.6mm,\ 5\mu)$ 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

Received 16 Sept 2014 Received in revised form 30 Sept 2014 Accepted 02 Oct 2014

***Address for correspondence:**
D. Sai Malakondaiah,
Department of Pharmaceutical Analysis and Quality Assurance, CMR College of Pharmacy, Kandlakoya (v), Medchal road, Hyderabad – 501 401, A.P, India.
E-mail: sai333dama@gmail.com

INTRODUCTION

Rosuvastatin calcium is an antilipemic agent and chemically it is 7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonamido)-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].

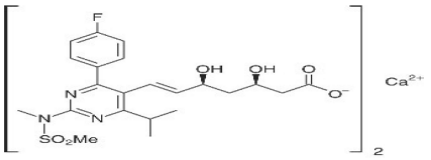

Figure 1: Structure of Rosuvastatin

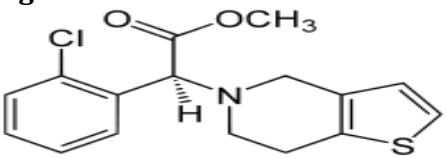

Figure 2: Structure of Clopidogrel

Literature survey revealed that few analytical techniques are available for estimation of Rosuvastastin 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 Rosuvastastin 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 Rosuvastastin and 75 mg of Clopidogrel working standard into a 50 ml clean dry volumetric flask and added about 30mL 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, 1ml of the solution was pipetted into another 10ml 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 50mL clean dry volumetric flask and added about 30mL of diluent. It was sonicated to dissolve completely and made volume up to the mark with the same diluent. (Stock solution) (200, 1500µg/mL) From this, 1 mL of the solution was pipette into another 10ml volumetric flask and diluted up to the mark with diluent.

Preparation of Buffer

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

Optimized chromatographic conditions

Diluent**:** Methanol
Mobile phase: Buffer p^H - 3(Water): Methanol: (60:40)
Flow rate: 1.2mL/min
Column: *Zorbax-SB (C18) (150mmx4.6mm, 5µ)*
Detector wavelength: 254nm
Injection volume : 20 µL
Run time : 6 min
Mode of Pump : Isocratic

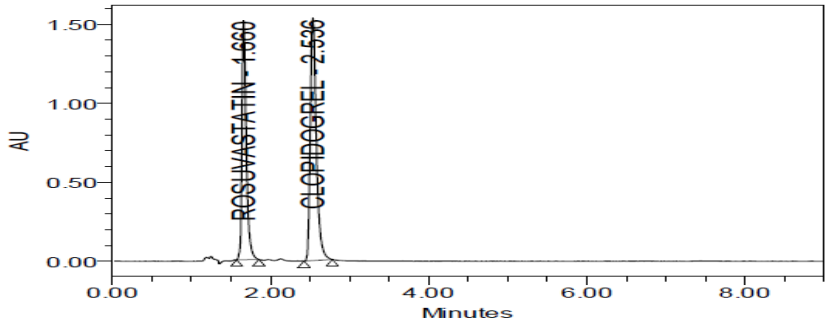

Figure 3: Chromatogram of RST (20µg/mL) and CPG (150µg/mL) standard

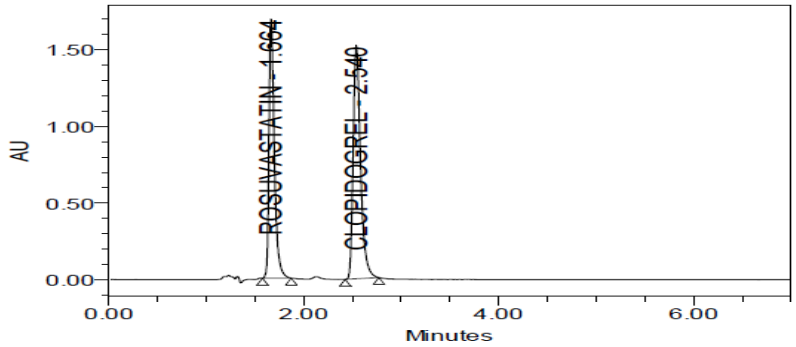

Figure 4: Chromatogram of RST (20µg/mL) and CPG (150µg/mL) sample

Table 2: System Suitability Studies

Parameters	Rosuvastatin	Clopidogrel	Acceptance criteria
Theoretical plates	5050	5503	Not less than 2000
Tailing factor	1.55	1.39	Not more than 2
Resolution	-	5.88	Not less than 2

METHOD VALIDATION

Linearity

Solutions were prepared containing10µg/ml, 15 µg/ml, 20µg/ml, 25µg/ml, 30µg/ml, concentrations of Rosuvastastin 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

Linearity conc (%)	Volume of Stock taken (mL)	Diluted to (mL)	Final Conc (µg/mL)		Peak Area	
			RST	CPG	RST	CPG
50	0.5	10	10	75	3012443	4262310
75	0.75	10	15	112.5	4525437	6395832
100	1	10	20	150	6031777	8535360
125	1.25	10	25	187.5	7540647	10650622
150	1.5	10	30	225	9050365	12721156

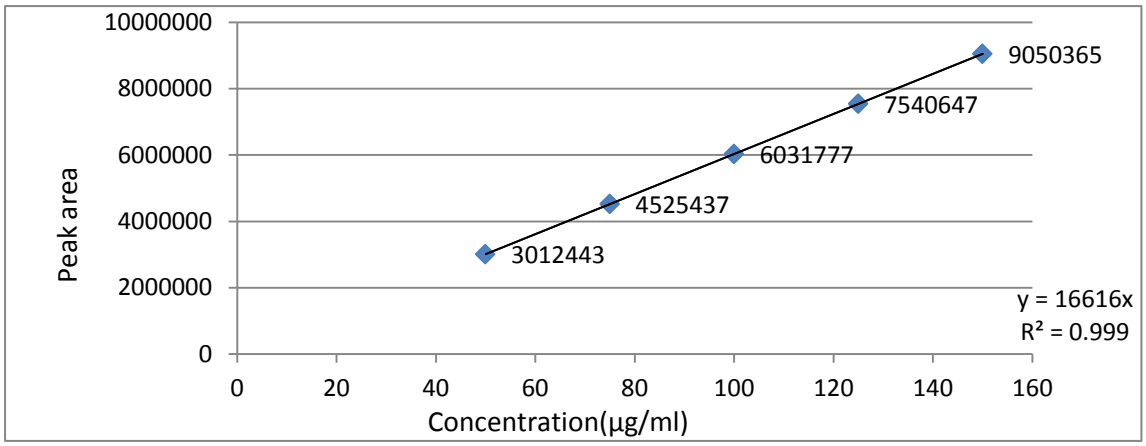

Figure 5: Calibration curve for Rosuvastatin

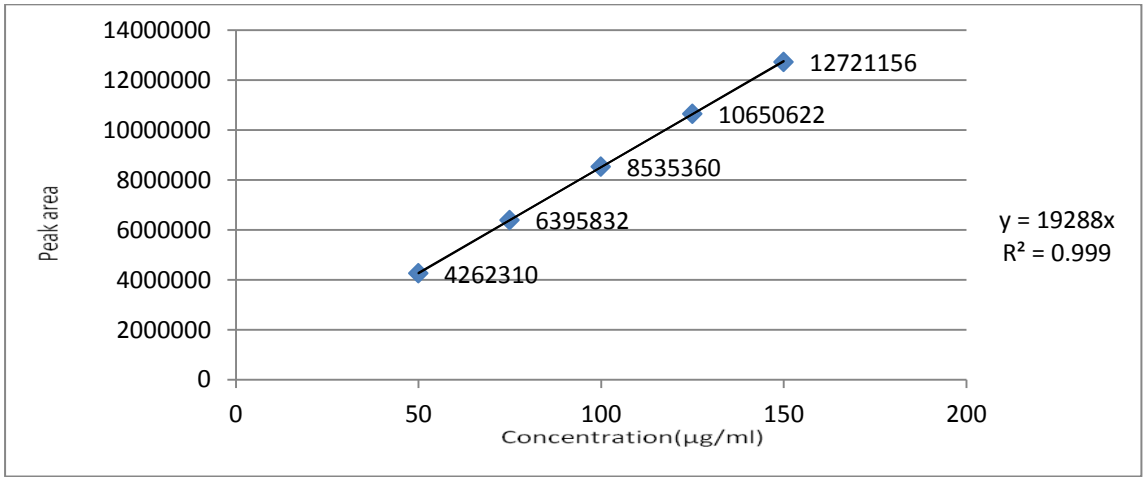

Figure 6: Calibration curve for Clopidogrel

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

Drug	Spiked level%	Amount taken (µg/ml)	Amount found (µg/ml)	Percent recovery n=3	Mean recovery Percent
RST	50	9.889	9.909	100.05	
	100	19.820	19.794	99.90	99.18
	150	30.448	29.702	97.59	
CPG	50	74.620	74.774	100.21	
	100	149.550	149.689	100.06	99.18
	150	46.87	229.743	97.28	

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

S.No	Sample Weight	Sample Area-1	Sample Area-2	% Assay	% Assay
1	483.00	6032493	8538527	98.99	99.78
2	483.00	6039657	8533252	99.10	99.72
3	483.00	6039793	8535900	99.11	99.75
4	483.00	6032497	8531566	98.99	99.70
5	483.00	6031979	8536214	98.98	99.75
6	483.00	6038540	8536253	99.08	99.76
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 (±0.1ml/min) and temperature (±5 ^{0}C).

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

LOD and LOQ was calculated from linear curve using formulae

LOD= 3.3 * σ / slope, LOQ= 10 * σ / slope

(Where σ = 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 Rosuvastastin and Clopidogrel from impurities.

Table 5: Results of Robustness study

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

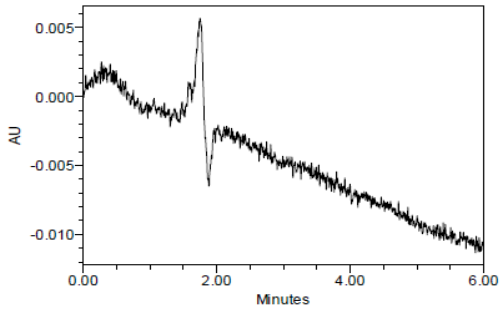

Figure 7: Chromatogram of Blank

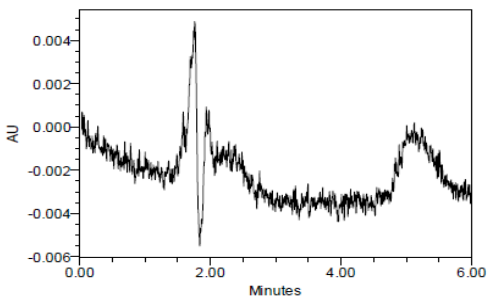

Figure 8: Chromatogram of placebo

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.

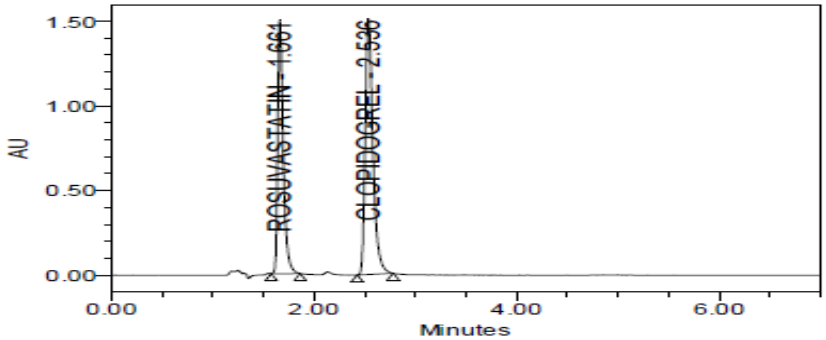

Figure 9A: Chromatogram of Acid Degradation

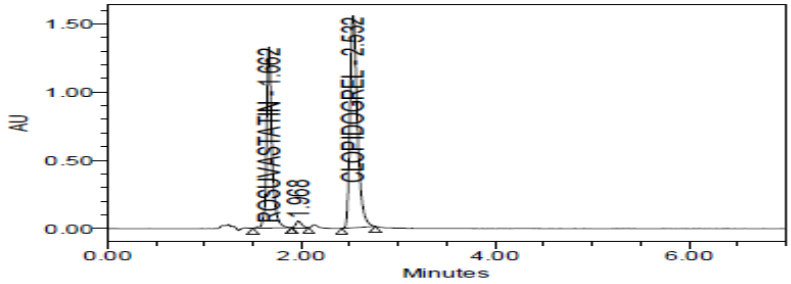

Figure 9B: Chromatogram of Base Degradation

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.

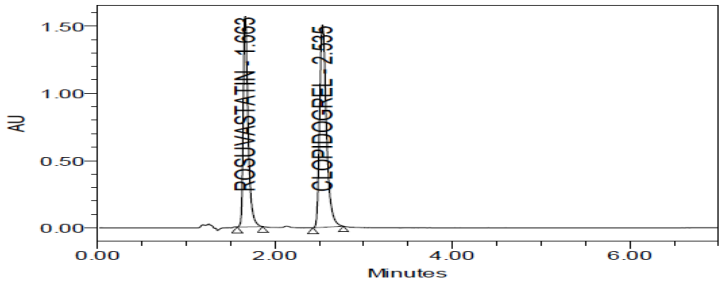

Figure 9C: Chromatogram of Oxidative Degradation

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.

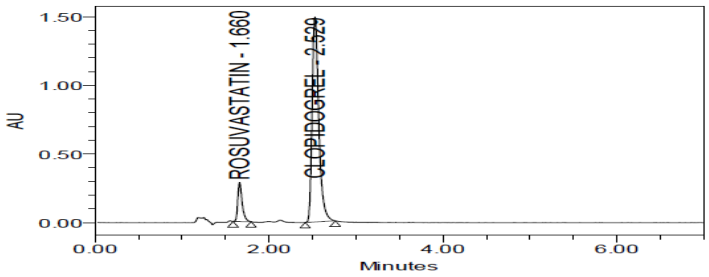

Figure 9D: Chromatogram of Dry Heat Degradation

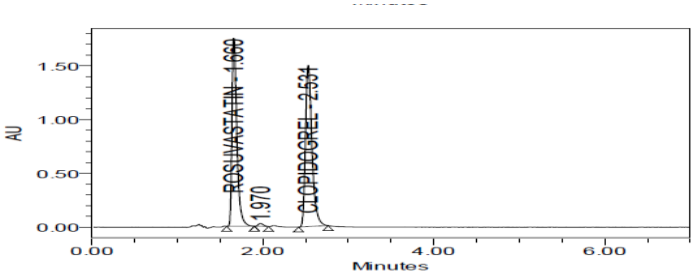

Figure 9E: Chromatogram of Photolytic Degradation

Photochemical Degradation

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

Table 6: Forced Degradation Studies

Parameters	Sample Area		% Assay		% Deg	
	RST	CPG	RST	CPG	RST	CPG
Acid	4622474	7057791	75.85	82.48	-23.15	-17.22
Base	4853049	7156580	79.63	83.63	-19.37	-16.07
Peroxide	4957377	7211910	81.34	84.28	-17.66	-15.42
Light	5102035	7445975	83.72	87.01	-15.28	-12.69
Heat	5032159	7304765	82.57	85.36	-16.43	-14.34
Avg Assay:	4094515.7	6029504				
STD	2012857.1	2956801				
%RSD	49.2	49				

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 range10-30µg/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

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

1. Drug profile of Rosuvastatin [Online] Available at http://www.medicinenet.com/ Rosuvastatin/article.htm.
2. Drug profile of Clopidogrel [Online] Available at http://www.medicinenet.com/Clopidogrel /article.htm.
3. Ashwini J. Purkar, AR. Balap, SB. Jadhav and PD. Chaudhari, Development and Validation of UV Spectrophotometric Method for Simultaneous Determination of Rosuvastatin Calcium and Aspirin in its Pure and Pharmaceutical Dosage Forms, International Journal Of Pharmaceutical And Chemical Sciences, ISSN: 2277-5005, Vol. 1 (3) Jul-Sep 2012, 10008-1012.
4. Sandhya Donthula , Meriga Kiran Kumar 1 , G. Shiva Teja, A New Validated RP-HPLC Method For Determination Of Rosuvastatin Calcium In Bulk And Pharmaceutical Dosage Form , Der Pharmacia Lettre, 2011, 3(3): 350-356.
5. Suhas Grav, Venkatamahesh, Development And Validation Of Derivative UV Spectrometric Methods For Quantitative Estimation Of Clopidogrel In Bulk And In Pharmaceutical Dosage Forms, International Journal of ChemTech Research CODEN(USA): IJCRGG ISSN : 0974-4290,Vol.4, No.2, pp 497-501, April-June 2012,47-501.
6. Nalini K. Sahoo, Madhusmita Sahu, Podilapu S. Rao, Validation Of Assay For Bulk Clopidogrel And For Some Tablet Forms By Reverse-Phase High-Performance Liquid Chromatography , J. Taibah Univ. Sci. (2014), 1 to 6.
7. Ch. Sai Krishna Reddy, B.V.D.Tulasi, A.Rohini,- Simultaneous Estimation Of Rosuvastatin Calcium And Clopidogrel Bisulphate By Uv Spectroscopy, International Journal Of Chemtech Research : Vol.5, No.1, Pp 127-130, 2013.
8. Purohit Zalak, Rohit Minal, Development And Validation Of Uv Spectrophotometric Methods For Simultaneous Estimation Of Clopidogrel Bisulfate And Rosuvastatin Calcium In Bulk And Formulation, ISSN 2320-5407, International Journal of Advanced Research (2014), Volume 2, Issue 2, 729-734.
9. Avani Sethi- Simultaneous Estimation of Rosuvastatin and Clopidogrel by Rp-HPLC-IRJP 2012, 3(11).
10. Sk Md Mobeen, G Sarvanan -Development And Validation Of Rp-hplc Method For The Estimation Of Rosuvastatin Calcium And Clopidogrel Bisulphate In Pharmaceutical Dosage Form - Inventi Rapid: Pharm Analysis & QA -2012.
11. Vidya Sagar, G.; Angala Parameswari - Development And Validation Of RP-HPLC Method For Simultaneous Determination Of Rosuvastatin Calcium And Clopidogrel In Capsule Form- Journal Of Pharmacy Research . 2012 Vol-5.
12. Nima Pandya, R.C. Mashru- Simultaneous Estimation Of Rosuvastatin Calcium And Clopidogrel bisulfate In Combined Pharmaceutical Formulation- IJDFR Volume 3 Issue 5, Sep.-Oct. 2012.
13. H. O. Kaila, M. A. Ambasana, R. S. Thakkar, H. T. Saravaia, and A. K. Shah-A new improved RP-HPLC method for the assay of rosuvastatin tablets-Indian J Pharm Sci. 2010 Sep-Oct; 72(5): 592–598.

14. Indian Pharmacopoeia 2007, Vol 2, Government of India, Ministry of health and welfare published by Indian Pharmacopoeia Commission, Pg: 576, 578.
15. United States of Pharmacopoeia, United States Pharmacopoeia Convention, Rockville, 2009, Pg: 2231.
16. H.H. Willard, L.L. Merritt, J.A. Dean, F.A. Settle, Instrumental Methods of Analysis,7th edition, CBS Publishers and Distributers, New Delhi, Pg: 518-521, 580-610.
17. R. Snyder, J. Kirkland, L. Glajch, Practical HPLC method development, 1997, II Ed, A Wiley International publication, Pg: 235,266-268,351-353.653-600.686-695.
18. D.A. Skoog, J. Holler, T.A. Nieman, Principle of Instrumental Analysis, 5th Edition, Pg: 778-787.
19. Sharma B.K., Instrumental Methods of Chemical Analysis, GOEL Publication House, Meerut, Pg. 133-161, 68-80, 114-165, 286-320.
20. ICH guidelines, validation of analytical procedure: methodology Q2B; I.C.H. Hormonized Tripartite Guidelines, 1996.