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PRECISE METHOD DEVELOPMENT AND VALIDATION OF SELECTED FIXED DOSE COMBINATIONS OF ANTILIPIDIMIC DRUG IN PHARMACEUTICAL FORMULATION BY RP-HPLC

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ABSTRACT

In this research work, an improved RP-HPLC method was developed for the simultaneous estimation of Rosuvastatin, Aspirin and Clopidogrelin pharmaceutical fixed dosage form. There are various mobile phase and stationary phase tried among these the proposed reverse phase liquid chromatographic method was performed phenomenex® Gemini C18 column (150 x 4.6mm i.d., 5µm) and Acetonitrile: Methanol: 0.1% of Triethylamine (45:05:50, v/v), pH of the aqueous phase adjusted at to 3.0 with 10% orthophosphoric acid at a 1.0ml/min of flow rate. The peak measurement was performed by UV-detector at λmax of 235nm based on the peak area with linear calibration curves established at concentration of 2-10µg/ml for aspirin, clopidogrel and 1-5µg/ml for rosuvastatin (where R2> 0.999 for all three drugs). The overall runtime was achieved within 10 minutes. The improved method was validated according to International Conference on Harmonisation (ICH) guidelines to confirm specificity, linearity, accuracy and precision. The proposed validated method was successfully applied for the analysis of API and fixed dosage form.

KEYWORDS

API, Fixed dosage combination, HPLC method, Aspirin, Rosuvastatin and Clopidogrel.

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INTRODUCTION

Rosuvastatin (RST) belongs to the statin class of drugs used to treat hypercholesterolemia both in patients with established cardiovascular disease as well as those who are at a high risk of developing atherosclerosis. These drugs inhibit the rate limiting key enzyme known as 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase involved in cholesterol biosynthesis. Until the approval of RS in 2003, AT was the most

efficacious drug in the statins class but, recent studies reported RS as a potent inhibitor of HMG-CoA reductase having a higher LDL-lowering effect as compared with other statins (Sreejanarthanan, 2016[1], Satoskar RS 2013[2]).

Aspirin (ASP), chemically known as acetyl salicylic acid, it is used as an anti-plateleting agent; aspirin is adhering and aggregating platelets secrete TXA-2, which leads to further platelet recruitment and activation. TXA-2 formation is catalyzed by the enzyme Cyclo-Oxygenase. This anti-aggregatory effect is considered as the major mechanism for the protection against thrombotic events [Sathiyasundar R *et al*, 2014][3]. Additional proposed protective effects of aspirin include anti-inflammatory properties and anti-thrombin actions.

Clopidogrel bisulfate (CLP), chemicallyas (+)-S-Methyl-2-(2-chlorophenyl)-2-(6, 7-dihydrothieno [3, 2-c] pyridin-5- (4H)-yl) acetate; sulfuric acid. It's a pro-drug activated in microenzyme (i.e. CYP 450 and CYP2C19), CLP is irreversible inhibiting of P2Y12 receptor on platelet cell membranes, and preventing adenosine diphosphate (ADP) from activating platelets and eventual cross-linking by the protein fibrin. It is used in the treatment of coronary artery disease and cerebrovascular disease. CLP and ASP in this combination has more potential for the treatment of unstable coronary syndromes [Sathiyasundar R *et al*, 2014[4], Gianluca *et al*, 2010[5]].

RST Calcium (Figure No.1) is official with Indian Pharmacopoeia (IP) [2010], which describes HPLC methods for determination of RST. Detailed survey of literature reveals several methods for the estimation of RST in pharmaceutical dosage forms using HPLC [Kaila *et al*, 2010][6], RST has been estimated with ezetimibe using HPLC and high performance thin layer chromatography [Varghese and Ravi, 2011][7] and Stability indicating [Mehta *et al*, 2008][8] method for quantification of RST are also reported. Quantification of RS in biological fluids, such as high performance chromatography [Pasha *et al*, 2006[9], Kumar *et al*, 2006[10]], RST in combination with other drugs [Vittal *et al*, 2006][11] and [Shah *et al*, 2011[12], Nasir *et al*, 2011[13]] with UV detection. Application of microbore HPLC in combination with tandem MS for the quantification of RST in human plasma [Oudhoff *et al*, 2006][14]. Ion pair liquid- liquid extraction using liquid chromatography with electrospray ionization tandem mass spectrometry [Lan *et al*, 2007] and Quantification of the N-desmethyl metabolite of rosuvastatin in human plasma by automated SPE by HPLC with tandem MS detection [Hull *et al*, 2004][15].

ASP is an official in Pharmacopoeias (IP, BP, USP). Several methods have been reported for the determination of ASP in pharmaceutical formulation by HPLC individually and simultaneous estimation with other combinations [Arayna *et al*, 2011[16], Deepak *et al*, 2012[17] and Dipali *et al*, 2013[18]], in biological fluids by LC–MS/MS [Satheesh *et al*, 2010][19]. A stability indicating assay method of ASP by HPLC [Nageswara Rao *et al*, 2012][20] has been reported.

CLP is not official in any of the pharmacopoeias. Several methods has been reported for the determination of CLP in the pharmaceutical formulation by HPLC individually and with other combinations [Hemant and Pravin, 2013, Sahoo *et al*, 2014[21]]. Stability Indicating HPTLC method [Sinha *et al*, 2009][22], LC-MS/MS method for the simultaneous determination of CLP and its metabolites [Marta *et al*, 2012][23] and UFLC method used for the estimation of clopidogrel and pantoprazole in human plasma samples [Nagavi *et al*, 2014] are also reported.

Recently, a couple of method have been reported for the simultaneous estimation of Rosuvastatin, Telmisartan, Ezetimibe and Atorvastatin by RP-HPLC [Sree Janardhanan *et al*, 2012][1] RST, ASP and CLP simultaneous estimation by UPLC method [Kaila *et al*, 2010[6]; Mahmoud *et al*, 2013[24]]. There is no HPLC method was reported for the simultaneous estimation of RST, ASP and CLP in formulation and plasma sample. The aim of the presentstudy is to improve a RP-HPLC method for the simultaneous estimation of RST, ASP and CLP, in the pharmaceutical formulation sample. The

developed method was validated as per the ICH guideline.

MATERIAL AND MATERIALS

HPLC instrumentation and conditions

Chromatographic separations were carried out on a Phenomenex® C18 analytical column (150mm × 4.6mm i.d., 5μm) connected with the mobile phase consisted of Acetonitrile: Methanol: 0.1% Triethylamine, pH of the mobile phase were adjusted to 3.0 with 10% orthophosphoric acid. In order to increase the sensitivity for the less concentrated compound and to decrease the background from mobile phase a wavelength of 235 nmwas selected for detection. An injection volume of the sample was 20μl. The HPLC system was used in an air-conditioned laboratory atmosphere (25 ± 2°C).

Stock and working standard solutions

Standard stock solutions of ASP, RST and CLP were prepared using mobile phase as a diluting solvent, the standard solutions containing a mixture of ASP (10.0µg mL^{-1}), RST (5.0µg mL^{-1}) and CLP (10.0µg mL^{-1}) were prepared in the mobile phase. Calibration curves of ASP, RST and CLP peak area ratio versus drug concentrations were established in the range of 2.0 -10.0µg/mL for ASP, CLP and 1.0 -5.0µg/mL for RST.

RESULTS AND DISCUSSION

Initial studies were carried out based on literature by trial and error method to identify the basic requirements of liquid chromatographic method developments such as (i) type ofstationary phase (C18, C8 and C6), (ii) range of pH,(iii) flow rate (iv) type of mobile phase additives (Diethylamine, Tryethylamine, THF, etc.), based on the studies we find out best chromatographic separation. Toobtain a reasonable analytical retention time, good quality of separation (resolution, capacity factor), there is need to optimize the chromatographic separation.

Selection of stationary phase

There are different types of stationary phase available for the reverse phase HPLC and we tried varies analytical column like, phenyl, C18, C8 and C6. Well resolved peak separation and excesses of asymmetric factor, less peak resolution were observed on C8 and C6 columns. Moreover a phenyl column is not suitable for these samples. Among these C18 provide good peak separation and satisfactory retention time, resolution and capacity factor.

Selection of mobile phase

Initially acetonitrile was selected as the organic phase and HPLC water was selected as an aqueous phase, then various ranges of pH (pH was adjusted with 10% orthophosphoric acid) were tried. In the above combination of mobile phase were tested indifferent proportion (50:50, 40:60, 60:40, 70:30) and at 50: 50 (MeCN: water (pH 2.5 to 3.5) ratio only we observed valuable retention time but poor resolution, capacity factor and poor peak separation, then introduced methanol to overcome this problem. Then methanol introduced in mobile phase in different ratios from 5.0 to 20 % v/v.

Selection of Additives

From the selected above mobile phase we added 0.05 to 1.0% diethylamine and there areno significant changes in resolution, and the peak overlapping. Then we tried acetic acid (0.1- 0.5%) in aqoues phase small variation in resolution, soatlast we tried with 0.05 - 1.0% of triethylamine, it produced significant improvements in resolution and good peak shape. The finalized Chromatographic Condition for Rosuvastatin, Aspirin and Clopidogrel was given in Table No.1.

METHOD VALIDATION

Linearity was established at five levels over the concentration ranges of 2.0-10µg mL^{-1} for ASP, CLP and 1.0-5.0µg mL^{-1} for RST in the standard solution. Typically, the mean (n = 6) regression equations were: y =423720x -22042 for ASP, y = 414820 x +63042 for CLP and y = 886934x - 67489 for an RST with R^2 values more than 0.999 for all the analytes. Since the correlation coefficients are good indicators of linearity performance of an analytical procedure a one way ANOVA was performed (Table No.2). For all the drugs, the calculated F_{calc} values less than the F_{Crit} at 5%

significance level, indicating that there was no significant difference between replicate obtained for each concentration level. The LOD and LOQ were estimated 3.92, 11.02ng mL^{-1} for ASP, 1.23, 3.99ng mL^{-1} for CLP and 7.05, 20.21ng mL^{-1} for RST was found. There is no placebo interference with drug main peak.

That the optimized assay method is selective and specific to the formulation placebo used in this study. Accuracy, assessed by spike recovery, in which the percentage recovery of the analytes at each levels (n = 3) and mean % recovery (n = 9) were determined. The recoveries of ASP, CLP, and RST at each level were found well within the acceptable criteria of bias ± 2.0%. The mean % recovery (n = 9) for each analyte was also tested for significance by using the Student t - test. Since the t_{Calc} is less than the theoretical t value (t_{Crit} = 2.306), at 5.0% significance level, the null hypothesis (the recovery is unity or 100%) were accepted. The intra and inter-day precision was confirmed since, the % CV were well within the target criteria, the optimized method was robust with small deviated in mobile phase composition, pH, flow rate and there is no variation on retention and resolution of the selected analytes.

Application of the method

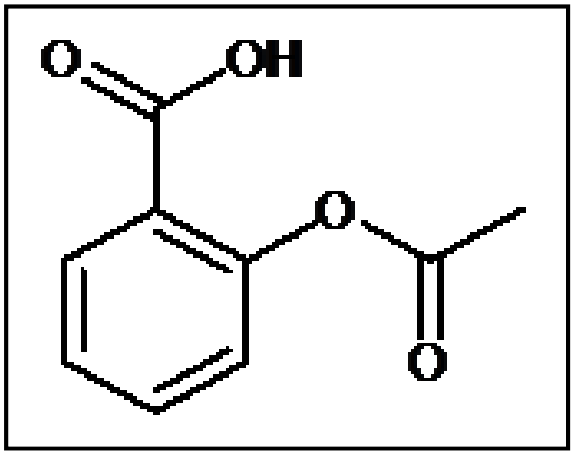
The optimized and validated formulation assay method was applied to the quantitative analysis of real samples (Rosuva Gold 20 Capsule) containing RST-20, ASP-75mg with CLP-75mg, (Rozagold 10 Capsule) Containing RST-10mg with ASP-75mg with CLP-75mg, were assayed by the proposed HPLC method. The mean % recoveries achieved when analyzing, Rosuva Gold 20 Capsule were, 98.2% for RST and 99.1% for ASP, 100.2% for CLP and Rozagold 10 Capsule were, 98.6% for RST and 98.9% for ASP, 99.8% for CLP, with the values within parenthesis being the % CV of the six replicates. The % CV of the assay results were <2, indicating the precision of the analytical methodology. This optimized method can be applied for both quantitative and qualitative analysis of bulk and pharmaceutical formulation in individual and combination of Rosuvastatin, Aspirin and Clopidogrel fixed dose combination.

Table No.1: Optimized chromatographic condition for rosuvastatin, aspirin and clopidogrel

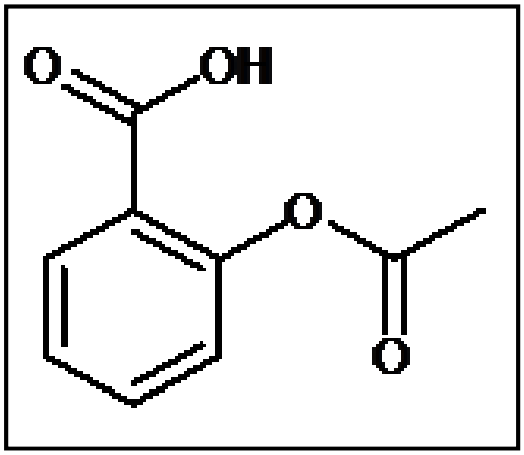
S.No	Chromatographic Parameters	Optimized Criteria
1	Mobile Phase	Acetonitrile: Methanol: 0.1% of Triethylamine (45:05:50, v/v)
2	Stationary Phase	Phenomenex® C18 analytical column (150mm × 4.6 mm i.d., 5μm)
3	Temperature	Ambient
4	Diluent	Mobile phase as a diluent
5	Detection wavelength	235nm
6	Flow rate	1.0ml/min
7	Injection volume	20µl

Table No.2: Validation parameter of rosuvastatin, aspirin and clopidogrel

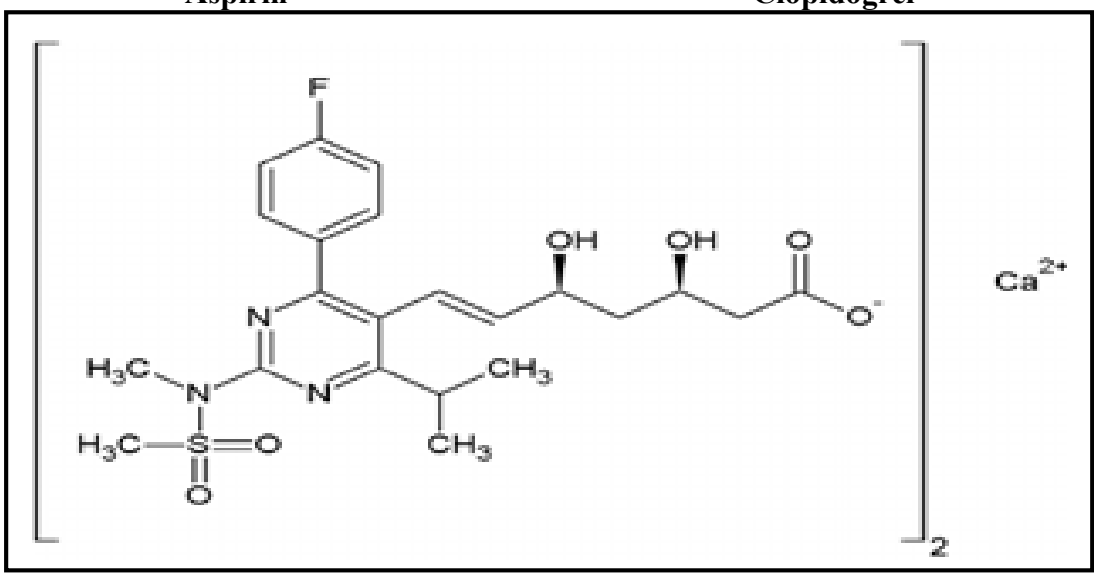
S.No	Parameters	ASP	RST	CLP
1	Linearity range (µg/ml)	2-10µg/ml	1-5µg/ml	1-5µg/ml
2	Slope	423720x	886934x	423720x
3	Correlation coefficient R2	0.9994	0.9997	0.9996
4	Rt	3.10 min	4.5 min	5.97 min
5	Tailing factor	0.3	0.8	0.7
6	LOD	3.92ng/ml	1.23ng/ml	7.05ng/ml
7	LOQ	11.02ng/ml	3.99ng/ml	20.21ng/ml
8	Theoretical plates (USP)	5234	6002	4585
9	Accuracy (n=6)	99.8% ± 0.2	98.9% ± 0.4	100 % ± 0.3
10	Precession (n=6)	100.5 ± 0.3	99.8± 0.2	99.5± 0.2



Aspirin



Clopidogrel



Rosuvastatin Calcium

Figure No.1: Chemical structure of Rosuvastatin, Aspirin and Clopidogrel

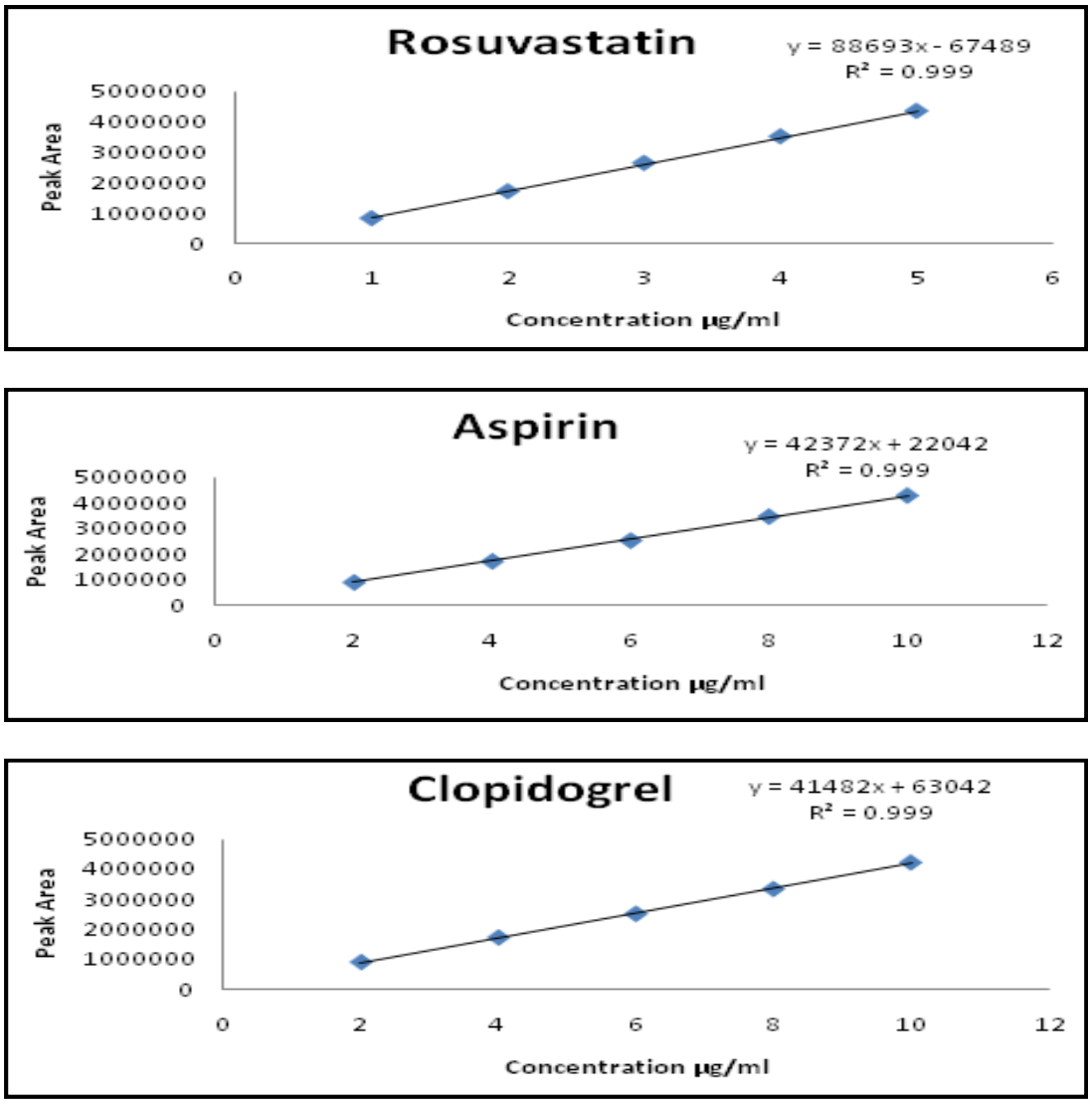


Figure No.2: Linearity graph of rosuvastatin, aspirin and clopidogrel

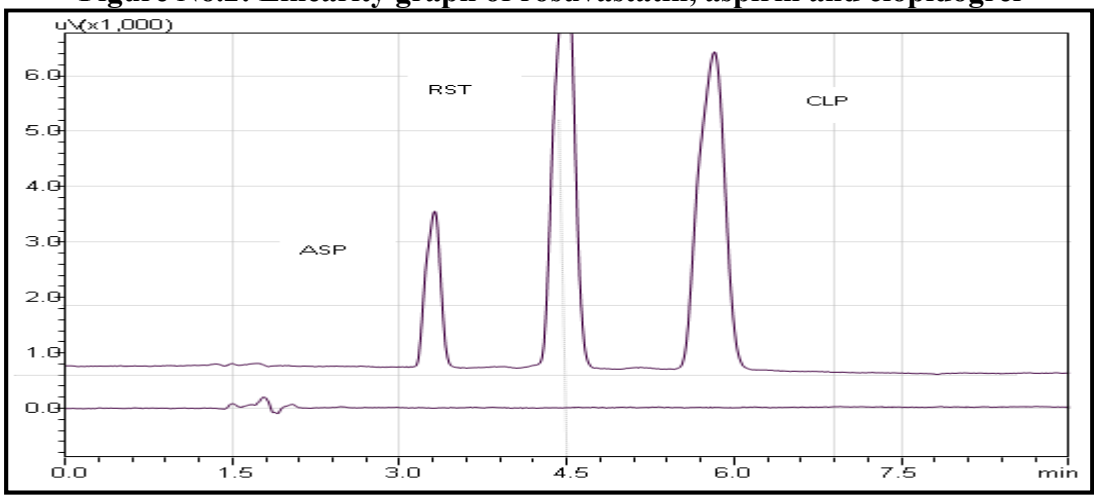


Figure No.3: Finalized chromatogram of aspirin, rosuvastatin and clopidogrel

SUMMARY AND CONCLUSION

This optimized method can utilize for the simultaneous estimation of the following analytes RST, ASP and CLP in bulk and pharmaceutical formulations (tablets). The overall runtime was achieved within 10 minutes and the peak elution were 3.10, 4.5, 5.97 min of RST, ASP, CLP respectively. The improved method was validated according to International Conference on Harmonisation (ICH) guidelines to confirm specificity, linearity, accuracy and precision. The proposed validated method was successfully applied for the analysis of API and fixed dosage form, commercially available on the Indian market.

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CONFLICT OF INTEREST

All authors' declared no conflict of interests

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