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RP-HPLC METHOD DEVELOPMENT AND METHOD VALIDATION OF SIMVASTATINE CLOPIDOGRIL ASPIRINEIN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

In this study, to develop a RP-HPLC method for the simultaneous estimation of Simvastatin, Aspirin and Clopidogrel in pharmaceutical combined dosage form. There are many trial and error method performed among these phenomenex ® Gemini C18 column (150 x 4.6mm i.d., 5µm) used as a stationary phase and Acetonitrile: Methanol: 0.05% of Triethylamine (55/15/30 v/v/v), used as a mobile phase and the pH of the aqueous phase adjusted at to 3.5 with 10% ortho phosphoric acid at a 1.0ml/min of flow rate. The peak measurement was performed by UV-detector at λmax of 240nm based on the peak area with linear calibration curves established at concentration of 2-10µg/ml for aspirin, clopidogrel and Simvastatin (where R_2> 0.999 for all three drugs) respectively. The overall runtime was achieved within 10 minutes. The 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 bulk and combined marketed dosage forms.

KEYWORDS

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

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INTRODUCTON

Simvastatin (SMT), is belongs to the statin derivatives used to hypercholesterolemia both in patients with established cardiovascular disease as well as those who are at a high risk of developing atherosclerosis. Simvastatin is made from the fungus Aspergillus terreus (Cechinel-Filho V)[1]. The main uses of simvastatin are to treat dyslipidemia and to prevent atherosclerosis-related complications such as stroke and heart attacks in those who are at high risk. It is recommended to be used as an addition to a low-cholesterol diet (Stephen R B *et*

al, 2010)[2]. Simvastatin is contraindicated with pregnancy, breastfeeding, and liver disease. All statins act by inhibiting 3-hydroxy-3-methylglutaryl (HMG) coenzyme A reductase. HMG-CoA reductase, the rate-limiting enzyme of the HMG-CoA reductase pathway, the metabolic pathway responsible for the endogenous production of cholesterol (Nilesh P *et al*, 2008[3], McTaggart V *et al*, 2003, Nagraju P *et al*, 2010[4]), which demonstrates that both Simvastatin are the leading drugs in the statins class (Anantha K D *et al*, 2009[5], Satoskar R S 2013[6]).

Aspirin (ASP), It's 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 [R. Sathiyasundar *et al*, 2014][7]. Additional proposed protective effects of ASP include anti-inflammatory properties and anti-thrombin actions.

Clopidogrel bisulfate (CLP), chemically as (+)-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, It's 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 [R.Sathiyasundar *et al*, 2014[8], Gianluca *et al*, 2010[9]].

SMT Calcium (Figure No.1) is official with Indian Pharmacopoeia (IP), BP and USP, which describes HPLC methods for determination of SMT. Detailed survey of literature reveals several methods for the estimation of SMT in pharmaceutical dosage forms using HPLC [Dewani *et al*, 2015][10], SMT has been estimated with ezetimibe using HPLC and high performance thin layer chromatography [Varghese and Ravi, 2010][11] and Stability indicating [Mehta *et al*, 2008][11] method for quantification of SMT are also reported. Quantification of RS in biological fluids, such as high performance chromatography [Pasha *et al*, 2006[13], Kumar *et al*, 2006[14]], SMT in combination with other drugs [Vittal *et al*, 2006][15] and [Shah *et al*, 2011[16], Nasir *et al*, 2011[17]] with UV detection. Application of microbore HPLC in combination with tandem MS for the quantification of SMT in human plasma [Oudhoff *et al*, 2006][18]. 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 Simvastatin in human plasma by automated SPE by HPLC with tandem MS detection [Hull *et al*, 2004][19].

ASP is an official in Pharmacopoeias (IP, BP, USP). There are many methods available for the determination of ASP in pharmaceutical formulation by HPLC individually and simultaneous estimation with other combinations [Arayna *et al*, 2011[20], Deepak *et al*, 2012[21] and Dipali *et al*, 2013[22]], in biological fluids by LC–MS/MS [Satheesh *et al*, 2010][23]. A stability indicating assay method of ASP by HPLC [Nageswara Rao *et al*, 2012][24] 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]. Stability Indicating HPTLC method [Sinha *et al*, 2009][25], LC–MS/MS method for the simultaneous determination of CLP and its metabolites [Marta *et al*, 2012][26] and UFLC method used for the estimation of clopidogrel and pantoprazole in human plasma samples [Nagavi *et al*, 2014][27] are also reported.

Recently, a couple of method have been reported for the simultaneous estimation of Simvastatin, Telmisartan, Ezetimibe and Atorvastatin by RP-HPLC [Sree Janardhanan *et al*, 2012][28], SMT, ASP and CLP simultaneous estimation by UPLC method [Kaila *et al*, 2011[29]; Mahmoud *et al,* 2013[30]]. There is no HPLC method was reported for the simultaneous estimation of SMT, ASP and CLP in formulation and plasma sample. The aim of the present study is to improve a RP-HPLC method for the simultaneous estimation of SMT, ASP and CLP, in the pharmaceutical formulation sample. The

developed method was validated as per the ICH guideline.

MATERIAL AND METHODS

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: 0.05% Triethylamine, pH of the mobile phase were adjusted to 3.5 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 240nm was 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 SMT, ASP and CLP were prepared using mobile phase as a diluting solvent, the standard solutions containing a mixture of ASP (10.0µg mL^{-1}), SMT (10.0µg mL^{-1}) and CLP (10.0µg mL^{-1}) were prepared in the mobile phase. Calibration curves of ASP, SMT and CLP peak area ratio versus drug concentrations were established in the range of 2.0 -10.0µg/mL for ASP, CLP, and 2.0 -10.0µg/mL for SMT.

RESULTS AND DISCUSSION

This research work carried out based on literature by trial and error method to identify the basic requirements of RP- liquid chromatographic method to initiated developments such as, type of stationary phase (C18, C8 and C6), range of pH, flow rate and type of mobile phase additives (Diethylamine, Tryethylamine, THF, etc.), based on the studies we find out best chromatographic separation. To obtain a reasonable analytical retention time, good quality of chromatographic separation (resolution, capacity factor).

We tried varies stationary phase (reverse phase) like, C18, C8 and C6, phenyl analytical column. 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. For mobile phase Selection, 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 in different proportion (50:50, 60:40, 70:30) and at 50: 50 (MeCN: water (pH 2.5 to 4.0) 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 35% v/v. For addition of mobile phase additives we tried with 0.01 to 1.0% diethylamine and there are no significant changes in resolution, and the peak overlapping. Then we tried with acetic acid (0.01- 0.5%) in aqoues phase small variation in resolution, so at last we tried with 0.05 - 1.0% of triethylamine, it produced significant improvements in resolution and good peak shape. The finalized Chromatographic Condition for Simvastatin, Aspirin and Clopidogrel was given in Table No.1.

METHOD VALIDATION

The proposed liquid chromatographic method was validated by following ICH guidelines. Validation parameters like selectivity, specificity, linearity, limit of detection and quantification, accuracy, precision, stability and robustness were addressed.

Specificity

The specificity of the method was evaluated by assessing the chromatograms of most commonly used excipients (starch, lactose monohydrate, methyl cellulose, titanium dioxide and magnesiumstearate) with that of the standard drugs. There was no excipient peaks co-eluted with the analytes, indicating that the optimized assay method is selective and specific in relation to the excipients used in this study. All placebo chromatograms showed no interference peaks Figure No.2.

Linearity

The linearity of the method was established at five levels over the concentration linear calibration curves established at concentration of 2-10µg/ml for Aspirin, Clopidogrel and Simvastatin respectively and nominal range of analyte approximately from

20 to 200%. Peak areas (y) of SMT, ASP and CLP were plotted versus their respective concentrations (χ) and linear regression analysis performed on the resultant calibration curves (n=6). The slope and intercept of the calibration curve were (mean *n* = 6): y =423720x +22042 for ASP, y = 424820 x +183042 for CLP and y = 886934x +32511 for an SMT 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. 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.

Limits of Detection (LOD) and Limits of Quantitation (LOQ)

In accordance with ICH recommendations, the approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits.LOD and LOQ values were estimated as [(standard deviation of repeatability)/ (slope of the regression equation)] by multiplying with 3.3 and 10 respectively. Using the above equations, the LOD and LOQ were estimated at 28ng/ml and 82ng/ml for SMT, 15ng/ml and 59ng/mL for ASP, 18ng/ml and 54ng/ml for CLP respectively.

Accuracy

The accuracy of the method was determined by analyzing Quality Control (QC) standards prepared at three levels of 80, 100and 120% of the expected assay value or label claim of the analytes in the commercial formulation. QC samples were prepared as three replicates at each concentration level by spiking the standard drugs with the placebo excipients, which were left overnight to allow matrix-analyte interactions to occur. The %recovery of the analytes at each level (n = 3) and mean % recovery (n = 9) were determined and %accuracy was expressed as [(calculated amount/predicted amount) × 100]. Accuracy, assessed by spike recovery, in which the % recovery of both enantiomers it is at each level (n = 3) and mean % recovery (n = 9) were found to be 98.87, 99.21 and 99.19SMT, ASP, and CLP respectively.

Precision

The precision was established by injecting three different concentrations of each analyte (2.0, 6.0, 10.0µg/mL for SMT, ASP and CLP each in six replicates, for intraday precision (repeatability) and on three consecutive days for the intermediate precision (reproducibility). Precision was expressed by the %RSD of the analyte peak area. Results for all studied compounds met the proposed requirement %RSD ≤ 2%. The intra and inter-day precision (n = 6.0) was confirmed since, the % CV were well within the target criterion of ≤ 2.0 and ≤ 2.0 respectively.

Robustness

The robustness of the proposed method was assessed to provide an indication of its reliability during normal usage with respect to small, but deliberate variations in experimental parameters such as variations in MeCN concentration (55 % ± 0.5), the flow rate (1.0 ± 0.05) and the pH (3.5 ± 0.05 Unit) did not alter the assay values of both enantiomers more than 1.0 % and therefore it would be concluded that the method conditions are robust.

Application of the method

The optimized method was validated and applied for formulation assay to the quantitative analysis of real samples (Simlip FC) containing SMT-20mg and (Antiban-ASP, Asogrel-A)Containing ASP-75mg with CLP-75mg, were assayed by the proposed HPLC method. The mean % recoveries achieved when analyzing all brand tablets and capsules were, 98.87, 99.21 and 99.19 SMT, ASP and CLP respectively, 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 Simvastatin, Aspirin and Clopidogrel fixed dose combination.

SUMMARY

This optimized method can utilize for the simultaneous estimation of the following analytes SMT, ASP and CLP in bulk and pharmaceutical formulations (tablets). The overall runtime was achieved within 10 minutes and the peak elution

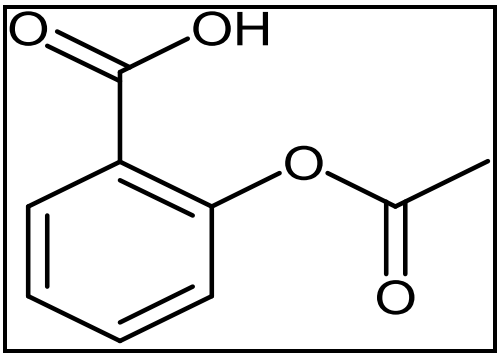
were 3.74, 4.85, 6.03 min of ASP, SMT, CLP respectively. The improved method was validated according to International Conference on Harmonisation (ICH) guidelines to confirm specificity, linearity, accuracy and precision. The proposed validated method was successfully applied for the analysis of bulk and combined solid oral dosage form; commercially it's available on the Indian market.

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

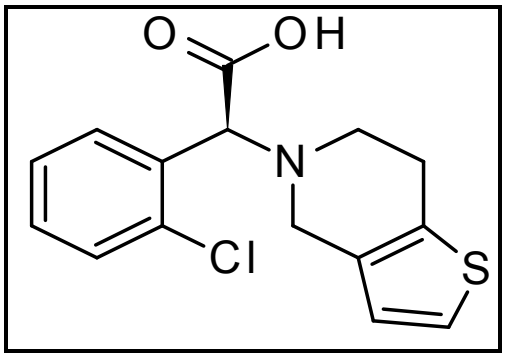
S.No	Chromatographic Parameters	Optimized Criteria
1	Mobile Phase	Acetonitrile: Methanol: 0.05% of Triethylamine (55/15/30 v/v/v), used as a mobile phase and the pH of the aqueous phase adjusted at to 3.5 with 10% ortho phosphoric acid.
2	Stationary Phase	Phenomenex® C18 analytical column (150mm × 4.6mm i.d., 5μm)
3	Temperature	Ambient
4	Diluent	Mobile phase as a diluent
5	Detection wavelength	240nm
6	Flow rate	1.0ml/min
7	Injection volume	20µl

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

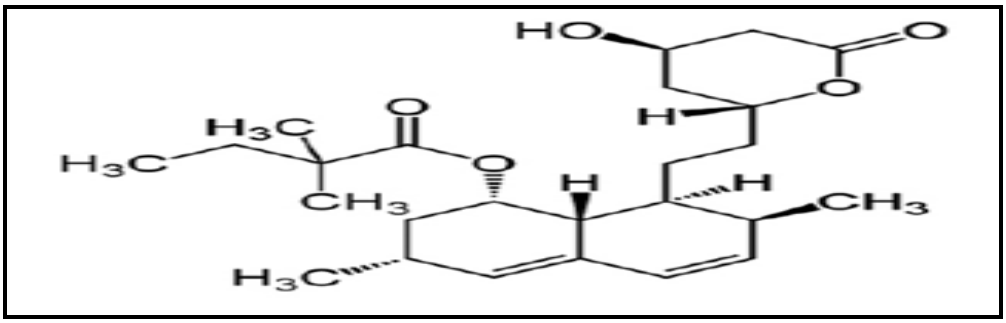
S.No	Parameters	ASP	SMT	CLP
1	Linearity range (µg/ml)	2-10µg/ml	2-10µg/ml	2-10µg/ml
2	Slope	423720x	886934x	424820x
	Intercept	22042	32511	183042
3	Correlation coefficient R2	0.9994	0.9997	0.9995
4	Rt	3.74 min	4.85 min	6.03 min
5	Tailing factor	0.3	0.8	0.7
6	LOD	15ng/ml	28ng/ml	18ng/ml
7	LOQ	59ng/ml	82ng/ml	54ng/ml
8	Theoretical plates (USP)	6146	7465	5456
9	Accuracy (n=6)	99.21% ± 0.2	98.87% ± 0.4	99.19 % ± 0.3
10	Precession (n=6)	99.02 ± 0.3	98.56± 0.2	98.87± 0.2



Aspirin

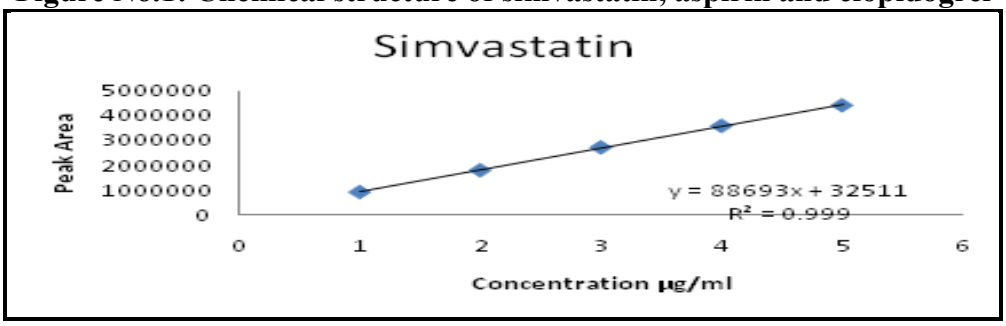


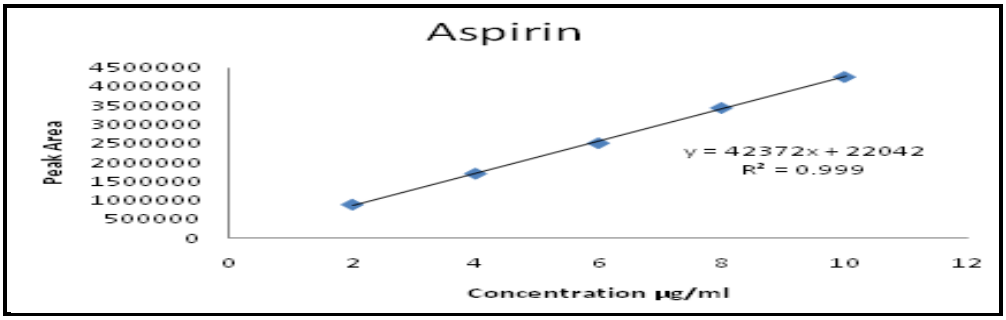
Clopidogrel



Simvastatin Calcium

Figure No.1: Chemical structure of simvastatin, aspirin and clopidogrel





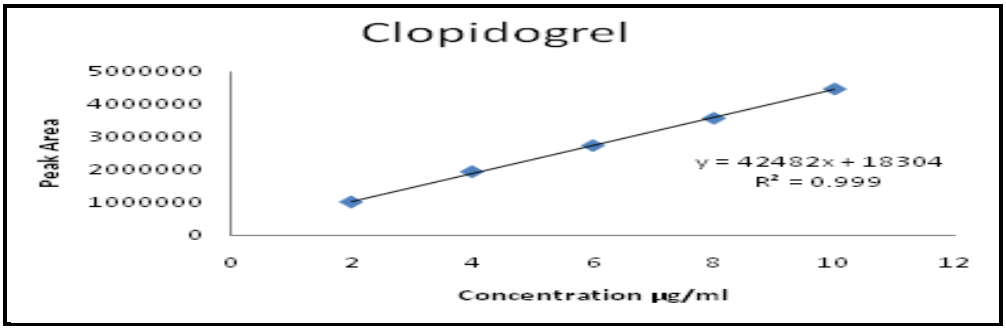


Figure No.2: Linearity graph of simvastatin, aspirin and clopidogrel

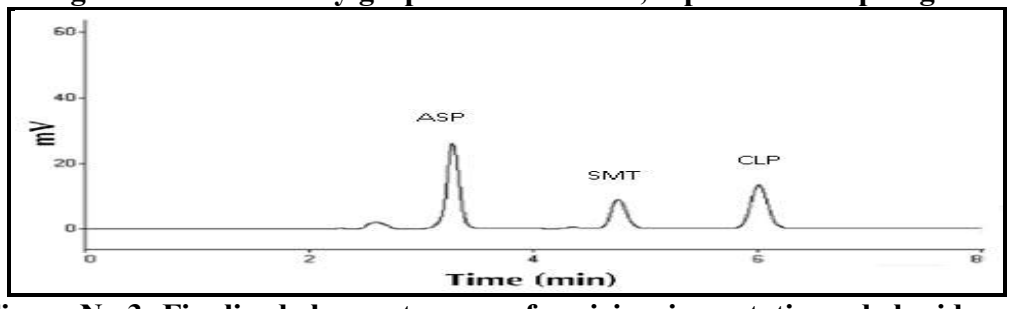


Figure No.3: Finalized chromatogram of aspirin, simvastatin and clopidogrel

CONCLUSION

A rapid, simple, robust and efficient isocratic reversed-phase high-performance liquid chromatography method was developed, optimized with trial/error method and validated for the simultaneous determination of the SMT, ASP and CLP, in pharmaceutical formulations.

The analytical results obtained lead to the conclusion that the developed method performs well with regard to precision, accuracy, rapidity, sensitivity and robustness, with single mobile phase allows to detect SMT, ASP and CLP. Therefore, it could be successfully employed for the analysis of these antiretroviral drugs in formulations samples.

This optimized method has to be utilized for the simultaneous quantitative analysis of SMT, ASP and CLP in pharmaceutical formulation. The method can be applied for the marketed (commercial) formulation the % recoveries values achieved were within the parenthesis being the % CV of the six replicates and the % CV of the assay results were < 2.0, indicating the precision of the analytical methodology.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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