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Original Article

AN IMPROVED RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ASPIRIN, ATORVASTATIN, AND CLOPIDOGREL IN PHARMACEUTICAL FORMULATION USING EXPERIMENTAL DESIGN METHODOLOGY

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ABSTRACT

Objective: In this study an improved RP-HPLC method was developed for the simultaneous estimation of Aspirin, Atrovastatin, and Clopidogrel in pharmaceutical dosage form. For improving the separation, an experimental design approach was employed.

Methods: Factors–independent variables (Organic modifier, pH of the mobile phase and flow rate) were extracted from the preliminary study and as dependent three responses variables viz. Capacity factor of tR_1, resolution between Atorvastatin and internal standard, retention time of tR_4 were selected. To improve method development and optimization, Derringer's desirability function was applied to simultaneously optimize the chosen three responses.

Results: The procedure allowed deduction of optimal conditions and the predicted optimum was Acetonitrile: Methanol: 0.1% of Triethylamine (52:05:43, v/v/v), pH of the aqueous phase adjusted at to 3.0 with 10 % *ortho* phosphoric acid, and the separation was achieved within 8 minutes. The method showed good agreement between the experimental data and predictive value throughout the studied parameter space.

Conclusion: The optimized assay condition 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 commercially available dosage form.

Keywords: Central composite design, Multiple response optimizations, HPLC method, Aspirin, Atrovastatin, and Clopidogrel.

INTRODUCTION

Aspirin (ASP) is chemically known as 2-(acetyloxy)-benzoic acid, small doses of aspirin inhibit the synthesis of TXA2 by platelets but higher doses also inhibit prostacyclin formation in the vessel walls as well [1]. Atrovastatin (ATV) is chemically known as [R-(R, R)]-2-(4-fluorophenyl)-5,5,-dihydroxy-5-(1-methylethyl)–3-phenyl4 [(phenylamino) carbonyl] -lH-pyrrole-1-heptanoic acid a potent inhibitor of the enzyme hydroxymethylglutaryl co-enzyme A-reductase (HMG-COA reductase, the rate limiting enzyme in cholesterol synthesis in the liver [2]. Clopidogrel bisulfate (CLP), is chemically (+)-(S)-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c] pyridine- 5 (4H) acetic acid methyl ester sulphate is a potent oral antiplatelet agent often used in the treatment of coronary artery disease, peripheral vascular disease and cerebro vascular disease.

The major risk factors for a fatal cardiovascular disease are high blood cholesterol, high blood pressure, smoking, diabetes, poor diet and overweight. The common cardiovascular diseases are aneurysm, angina, atherosclerosis, cerebrovascular accident (stroke), cerebrovascular disease, congestive heart failure, coronary artery disease, myocardial infarction (heart attack) and peripheral vascular diseases Usage of fixed dose combinations in cardiovascular diseases have many advantages such as reduction in cost, adverse effects and dose, ease of use by patients, improved patient compliance and medication adherence.

The review of literature revealed that various analytical methods involving RP-HPLC, Spectrophotometry have been reported for ASP [3], ATV [4] and CLP [5] in single form and in combination with other drugs. The simultaneous determination of CLP and ASP in combined oral dosage forms was also reported [6]. However, recently a simultaneous estimation of all the three drugs in a single combination dosage form with the aid of chemometric assisted UV spectroscopic and liquid chromatographic method, has been described by [7]. The reported analytical method mostly focused on UV- spectrophotometry (H-Point addition) method. It has a limited focus on HPLC method development and does not provide details on separation attributes like capacity factor, resolution, and asymmetric factor.

It is noticed that none of the above methods applied a systematic optimization procedure for the simultaneous HPLC estimation of ASP, ATV, and CLP, but employed a time-consuming trial-and error approach resulting only in an apparent optimum and information concerning the sensitivity of the factors on the analytes separation and interaction between factors is not available. In this work chemometric procedure is applied to realize the above objective.However, HPLC method intended to be applied for the pharmaceutical or industrial environment, there is a need to optimize multiple responses (analysis time and resolution) simultaneously [8, 9]. To achieve global optimization of multiple responses the Derringer's desirability function (Multi-Criteria decision making) has been applied [10]. Hence there was a need to develop an improved HPLC method, for simultaneous estimation of ASP, ATV, and CLP in pharmaceutical formulations, with aid of chemometric protocol.

The aim of this work is to (i) develop an improved HPLC method for the simultaneous analysis of ASP, ATV, and CLP in pharmaceutical dosage form and (ii) provide information on the sensitivity of chromatographic factors and their interaction effects on the separation characteristics.

Experimental

Apparatus

In this study was performed with a Shimadzu (Japan) chromatograph equipped with an LC-20 AD and LC-20 AD vp solvent-delivery module, an SPD-20A PDA detector, and a Rheodyne model 7125 injector valve fitted with a 20 µL sample loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. The mobile phase was

degassed using Branson sonicator (Branson Ultrasonics Corporation, USA). Absorbance spectra were recorded using an UV-Visible spectrophotometer (Model UV-1601PC, Japan) employing quartz cell of 1.00 cm of path length. The chromatographic analyses were done on an phenomenex analytical column Gemini C18 (150 mm × 4.6 mm I. D and 5 µm particle size).

MATERIALS AND METHODS

Working standards of aspirin, atrovastatin, and clopidogrel were gifts from RanbaxyLaboratery Ltd., New Delhi, India. Warfarin (Internal Standard) was gifts from Sasun Lab. Ltd., Pondy. The marketed formulation was purchased from whole saler (Ecosprin® Gold 20). Acetonitrile (MeCN) and Methanol (MeOH) of HPLC grade and Triethylamine (TEA) and other reagents of analytical-reagent grade were from SD Fine Chemicals (Mumbai, India). The HPLC-grade water was prepared by using Milli-Q Academic, Millipore, Bangalore, India.

Software

Experimental design, data analysis and desirability function calculations were performed by using Design-Expert 8.0.0 (Stat-Ease Inc., Minneapolis) and individual desirability function was performed by JMP-Software 9.0.0 (SAS) trail version.

Standard solutions

Standard stock solutions of ASP, ATV and CLP (1mg/ml) were prepared in the mobile phase. Working standard solutions were freshly obtained by diluting the standard stock solutions with mobile phase during the analysis time. Calibration curves reporting peak area ratios of ASP, ATV and CLP to that of the IS versus drug concentrations were established in the range of 2-10 µg/mL for ASP, CLP, and 1-5 µg/mL for ATV, in presence of warfarin (5µg/mL) as internal standard. Standard solution prepared for the optimization procedure constituted 4 µg/mL of ASP, CLP, and ATV for 2 µg/mL, respectively.

Sample preparation

Twenty capsules were weighed and mixed thoroughly, an amount of capsule powdered equivalent to 40mg for ASP, CLP, and 10mg of ATV were accurately weighed and transferred in a 100 ml Volumetric flask: suitable quantity of IS was added followed by 75 ml of mobile phase. The mixture was subjected to sonication for 15 min then complete extraction of drugs and the solution was made up to the mark with the mobile phase to obtain a concentration of ASP, CLP, 4 µg/mL and ATV for 2 µg/mL, respectively. The solution was centrifuged at 2500 rpm for 10 min; the clear supernatant was collected and filtered through a 0.45 µm membrane filter (pall tech, India) and 20 µl of this solution was injected for HPLC analysis.

Validation study

In accordance with the ICH guidelines on the validation of analytical methods Q2A and Q2B, the following validation characteristics were examined: specificity, linearity, Accuracy, precision, Limit of Detection (LOD) and Limit of Quantification (LOQ). For specificity study, placebo containing starch, lactose monohydrate, aerosil, hydroxy propyl methylcellulose, and titanium dioxide, magnesium stearate was used. There is no interference from the extracted blank, extraction solvent, and excipients used for the drug preparations on the retention times of the 3 compounds of interest. Linearity was established at five levels over the concentration ranges of 2-10 µg/mL for Aspirin, Clopidogril, and 1-5 µg/mL for Atrovastatine (approximately from 20 to 200% of nominal range of analyte) [11] with regression coefficient values more than 0.999, which showed reproducibility. LOD and LOQ is 4.06 ng/ml, 12.32 ng/ml of ASP, 1.37 ng/ml, 4.17 ng/ml of CLP, and 7.85 ng/ml, 23.78 ng/ml of ATV was founded respectively.

Chromatographic procedure

Chromatographic separations were carried out on a Phenomenex® C18 analytical column (150 mm × 4.6 mm i. d., 5 μm) connected with a Phenomenex® C18 guard cadridge (4 mm × 3 mm i. d., 5 μm). The mobile phase consisted of Acetonitrile: Methanol: 0.1% Triethylamine, pH of mobile phase adjusted to 3.0 with 10% ortho phosphoric acid. In order to increase the sensitivity for the less concentrated compound and to decrease the background from mobile phase a wavelength of 220 nm was selected for detection. An injection volume of the sample was 20 μl. The HPLC system was used in an air-conditioned laboratory atmosphere (20 ± 2°C).

RESULTS AND DISCUSSION

Optimization design and analysis

The central composite design was applied to optimize the separation and to assist the development of better understanding of the interaction of several chromatographic factors on separation quality [12]. In this work, the important chromatographic factors were selected and optimized by a central composite design experiment. The selection of factors for optimization was based on preliminary experiments and prior knowledge from literature, as well as certain instrumental limitations. From preliminary experiments, the key factors selected for optimization processes were MeCN concentration (A), pH of the mobile phase (B) and flow rate (C). table 1 shows the levels of each factors studied for finding out the optimum values and responses. As can be seen in this table, the ranges of each factors used were: MeCN concentration (45-55%), pH (3.0-4.0) and flow rate (1.0-1.4 ml/min). As response variables, the capacity factor (k) of (tR_1) and retention time of CLP (tR_4), then resolution of ATV-IS ($Rs_{2,3}$) was chosen. All experiments were performed in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Replicates (n=6) of the central points were performed to estimate the experimental error. For an experimental design with three factors, the model including linear, quadratic, and cross terms can be expressed as

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

Where Y is the response to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represent factors A, B and C respectively. To obtain a simple and yet a realistic model, the insignificant terms (P>0.05) are eliminated from the model through 'backward elimination' process. The statistical parameters obtained from the ANOVA for the reduced models are given in table 2.

Since R^2 always decreases when a regress or variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regress or variables into account, is usually selected. In the present study, the adjusted R^2 were well within the acceptable limits of $R^2 \geq 0.80$ which revealed that the experimental data shows a good fit with the second-order polynomial equations [13]. For all the reduced models, P value of <0.05 are obtained, implying these models are significant. The adequate precision value is a measure of the "signal (response) to noise (deviation) ratio". A ratio greater than 4 is desirable [14]. In this study, the ratio was found to be in the range of 10.061 to 44.35, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (CV) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10%. The CV for all the models was found to less than 10%. As can be seen in table 2, the interaction term with the largest absolute coefficients among the fitted models is AB (+0.43) of tR_4 model. The positive interaction between A and B is statistically significant (P=0.0001) for tR_4. The study reveals that changing the fraction of MeCN from low to high results in a rapid decline in tR_4 both at the low and high level of flow rate. Especially this interaction is synergistic, as it led to a decrease in run time. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for optimization of the chromatographic separation [15]. In order to gain a better understanding of the results, the predicted models are presented in Fig. 1 as the perturbation plot. For an optimization design, this graph shows how the response changes as each factor moves from a chosen reference point, with all other factors held constant at the reference value. A steep slope or curvature in a factor indicates that the response is sensitive to that factor. Hence, the plot shows that factor A mostly affected the analysis time (tR_4) followed by factor B and then C.

Table 1: Central composite rotatable design arrangement and responses[a]

Factor levels				Responses	
MeCN	**Flow**	**pH**	**k_1**	**tR_4**	**$Rs_{2,3}$**
50.00	1.20	3.50	1.01	14.90	3.68
45.00	1.40	4.00	0.85	22.61	7.06
58.41	1.20	3.50	0.82	08.51	0.57
41.59	1.20	3.50	1.40	28.64	10.31
50.00	1.20	3.50	1.01	14.81	3.45
50.00	1.54	3.50	0.59	11.67	3.56
50.00	1.20	3.50	1.01	14.89	3.64
50.00	1.20	2.66	1.02	06.37	3.43
55.00	1.00	4.00	1.23	13.89	2.83
50.00	1.20	4.34	1.43	20.33	4.62
50.00	1.20	3.50	1.01	15.58	3.30
50.00	1.20	3.50	1.01	15.76	3.69
50.00	0.86	3.50	1.80	20.64	4.05
45.00	1.40	3.00	0.95	12.28	6.51
45.00	1.00	3.00	1.68	16.87	6.57
50.00	1.20	3.50	1.01	15.76	3.69
45.00	1.00	4.00	1.55	31.41	8.29
55.00	1.40	4.00	0.64	10.08	2.21
55.00	1.00	3.00	2.09	13.64	1.68
55.00	1.40	3.00	0.62	07.47	1.59

[a] Randomized

Table 2: Reduced response models[a] and statistical parameters obtained from ANOVA (After backward elimination)

Response	Regression model	Model p-value	%CV	R-Square	Adj.R-Square
K	+0.90 +8.225E-003A- B-0.12 C+0.087BC	<0.0001	4.95	0.9936	0.9798
tR4	+15.29-5.27A 2.82B+3.75C+0.43AB-2.75AC +1.15A^2	<0.0001	7.26	0.9832	0.9681
$Rs_{2,3}$	+3.58-2.67A-0.21B+0.44C	<0.0001	6.76	0.9925	0.9857

[a] Only significant coefficients with P < 0.05 are included. Factors are in coded levels.

Table 3: Criteria for the optimization of the individual responses

Response	Lower limit	Upper limit	Criteria	
			Goal	Importance
k_1	0.5	2.0	Range	1
tR_4	7.5	13.0	Minimize	3
$Rs_{2,3}$	1.5	2.5	Maximize	3

Table 4: The comparison of experimental and predictive values of different objective functions under optimal conditions.

Optimum Conditions	MeCN (%)	pH	Flow (ml/min)	k_1	tR_4	$Rs_{2,3}$
Desirability value (D) = 0.942	52.06	3.0	1.4			
				0.74	7.40	2.50
Predictive				0.72	7.23	2.60
Experimental						
Error (%)				2.70	2.35	3.84

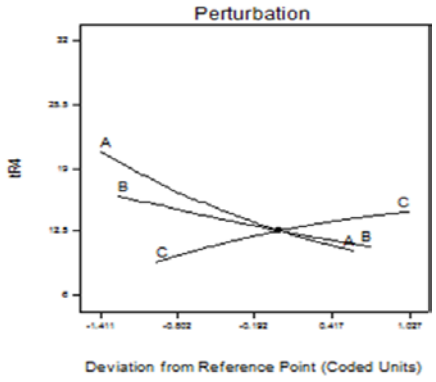

Fig. 1: Perturbation plot showing the effect of each of the independent variables on tR_4 while keeping other variables at their respective midpoint levels

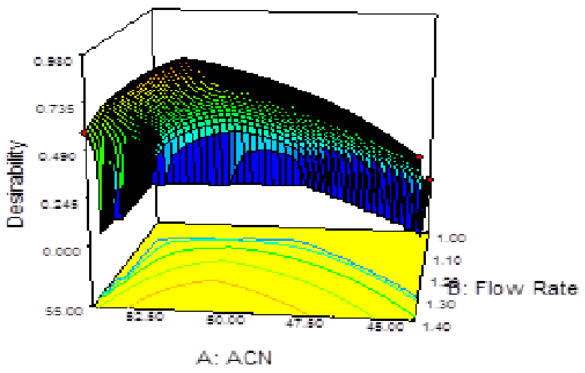

Fig. 2: Graphical representation of the overall desirability function D. MECN concentration, (A)is plotted against flow rate (B) with factor C held constant at pH 3.0

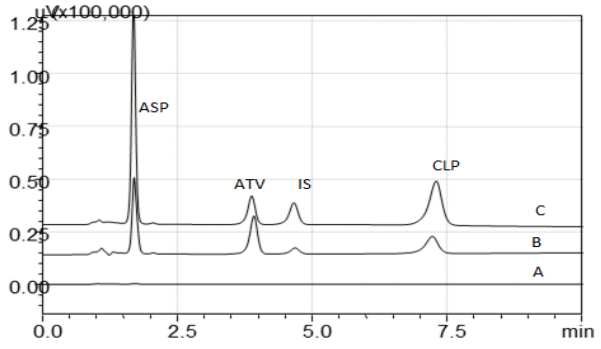

Fig. 3: Overlay chromatogram corresponding to (A) a placebo solution; (B) working standard of ASP (4µg/ml), ATV (2 µg/ml), IS (5 µg/ml) and CLP (4µg/ml); (C) a real sample of Ecosprin® Gold20 Capsules containing ASP (4µg/ml), ATV (2 µg/ml), IS (5 µg/ml) and CLP (4µg/ml)

Multi-criteria decision making

In the present study, to optimize three responses with different targets, Derringer's desirability function, was used. The Derringer's desirability function, D, is defined as the geometric mean, weighted, or otherwise, of the individual desirability functions. The expression that defines the Derringer's desirability function is:

$$D = \left[d_1^{p1} \times d_2^{p2} \times d_3^{p3} \times \ldots\ldots \times d_n^{pn} \right]^{1/n} \qquad (2)$$

Where *p*i is the weight of the response, *n* the number of responses and *di* is the individual desirability function of each response obtained from the transformation of the individual response of each experiment. The scale of the individual desirability function ranges between $d_i = 0$, for a completely undesired response, to $d_i = 1$ for a fully desired response. Weights can range from 0.1 to 10. Weights lower than 1 give less emphasis to the goal, whereas weights greater than 1 give more emphasis to the goal in both cases [16], *d*i varies in a non linear way while approaching the desired value. But with a weight of 1, d_i varies in a linear way. In the present report we chose weights equal to 1 for all the six responses.

A value of D different to zero implies that all responses are in a desirable range simultaneously and consequently, for a value of D close to 1, the combination of the different criteria is globally optimal, so as the response values are near target values. The criteria for the optimization of each individual response are shown in table 3, and it is proposed for selecting an optimum experimental condition for analyzing routine quality control samples.

As can be seen under criteria (Table 3), were responses $Rs_{2,3}$ maximized, capacity factor in the range and tR_4 minimized, were in order to shorten the analysis time. Importance can range from 1 (the least important) to 5 (the most important), which gives emphasis to a target value. For instance, high importance value of 3 was assigned to tR_4 response as short analysis time is usually preferred for routine analysis. Following the conditions and restrictions above, the optimization procedure was carried out. The response surface obtained for the global desirability function is presented in Fig. 2. The coordinates producing the maximum desirability value (*D*= 0.942) were MeCN concentration of 52.06%, pH of the mobile phase 3.0 and flow rate of 1.4 ml/min.

The predicted response values corresponding to the latter value of *D* were: $k_1 = 0.74$, $tR_4 = 7.4$ min $Rs_{2,3} = 2.5$. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram is shown in Fig. 3. The agreement between experimental and predicted responses for the predicted optimum is shown in table 4. The errors for retention factor, retention time and resolution were 2.70, 2.35, and 3.84 %., respectively which were found to be in good agreement [17], with a difference of 1–6%.

Assay method validation

The optimised assay method is specific in relation to the placebo used in this study because there was no excipients peak co-eluted with the analytes and IS (Fig. 4). An excellent linearity was established at five levels in the range of 2-10 µg/mL for Aspirin, Clopidogril, and 1-5 µg/mL for Atrovastatin with R^2 of more than 0.999. The LOD and LOQ were estimated as 4.06 ng/ml, 12.32 ng/ml of ASP, 1.37 ng/ml, 4.17 ng/ml of CLP, and 7.85 ng/ml, 23.78 ng/ml of ATV. Accuracy (*n*=9), assessed by spike recovery, were found to be 99.86 for ASP, ATV and CLP was within acceptable ranges of 100±2%. The intra and inter-assay precision (*n*=6) was confirmed since, the %C. V. Were well within the target criterion of ≤ 2. [18]. Robustness study reveals that small changes did not alter the retention times, retention factor and resolutions more than 3% and therefore it would be concluded that the method conditions are robust.

Application of the method

The proposed RP-HPLC method was applied to the simultaneous estimation of real samples (Ecosprin® Gold20 Capsules) containing ASP, ATV and CLP. Representative chromatograms are presented in Fig. 3. The results achieved when analyzing Ecosprin® Gold20 Capsules was 75 mg of ASP, CLP and 20 mg of ATV, with the values within parenthesis being the % CV of the six replicates. Good agreement was found between the assay results and the label claim of the product. The % CV of Capsules was < 2, indicating the precision of the analytical methodology.

CONCLUSION

Statistically based experimental designs proved to be a valuable approach in optimizing selectivity-controlling parameters for the determination of ASP, ATV, and CLP in pharmaceutical dosage form. The significant factors were optimized by applying central composite design and surface response methodology. The objective responses, resolution and the analysis time, were then simultaneously optimized by applying Derringer's desirability function, a multi-criteria decision making tool. The improved method showed higher sensitivity and shorter analysis time than the existing method making it viable to be implemented for routine quality control analysis in a pharmaceutical laboratory. The method validation studies supported the selection of the assay conditions was specific, accurate, linear, precise, and robust.

CONFLICT OF INTERESTS

Declared None

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