Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 3, 2012

Research Article

DEVELOPING AND OPTIMIZING A VALIDATED RP-HPLC METHOD FOR THE ANALYSIS OF AMLODIPINE AND EZETIMIBE WITH ATORVASTATIN IN PHARMACEUTICAL DOSAGE FORMS APPLYING RESPONSE SURFACE METHODOLOGY

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Received: 03 Apr 2012, Revised and Accepted: 06 Jun 2012

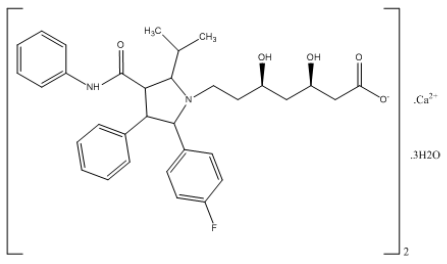
ABSTRACT

This paper deals with multiple response simultaneous optimization using the Derringer's desirability function for the development of a reversed-phase HPLC methods for the simultaneous determination of Amlodipine and Ezetimibe with atorvastatin in commercial pharmaceutical preparations. The ranges of the independent variables used for the optimization were MeCN: 35-40%, buffer conc.: 10-20 mM and flow rate: 0.8-1.2 ml/min. The influence of these independent variables on the output responses: capacity factor of the first peak (k_1), resolutions ($Rs_{2,3}$), and Retention time (tR_4) were evaluated. Using this strategy, mathematical model were defined and response surface were derived for the separation. The coefficients of determination R^2 were more than 0.8871 for all the models. The three responses were simultaneously optimized by using Derringer's desirability functions. Optimum conditions chosen for assay were MeCN, MeOH, 20 mM K_2HPO_4 (pH 5.0) solution (36.74 : 20 : 43.26 v/v/v) and flow rate 1.20 ml/min. Total chromatographic analysis time per sample was approximately 7.79 min with AMD,NFD(IS),EZT and ATV eluting with retention times of 3.58,5.54,6.71 and 7.79. The LODs were 0.17, 0.18, 0.03 and 0.44 ng /mL and the LOQs were 0.52, 0.57, 0.083 and 1.34 ng/mL for AMD, NFD, EZT and ATV respectively. The optimized assay condition was validated as per the ICH guidelines and applied for the quantitative analysis of Avas-AM, and Aztor-EZ tablet.

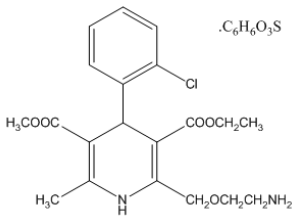
Keywords: Central composite design, Derringer's desirability function, Response surface methodology, HPLC, Amlodipine, Nifedipine, Atorvastatin, Ezetimibe.

INTRODUCTION

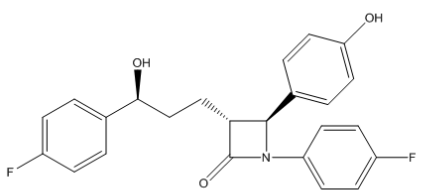
Cardiovascular diseases are the first cause of mortality worldwide, causing around the 30% of the global deaths, a number of which will significantly increase in the following years according to the world Health Organization (WHO). In fact 80% of the deaths take place in low and middle income countries due to the troubles to access medicines and also due to their unhealthier diet [1,2]. Actually, in appropriate diet and other bad habits like alcohol and tobacco consumption are some of the factors closely related to the risk of suffering from a cardiovascular illness. Other important risk factors are hypertension, dyslipidemia and diabetes. The suffering from some of these pathologies simultaneously is known as metabolic syndrome. Since various factors are involved in metabolic syndrome a combined cardiovascular therapy is necessary. This therapy usually involves different antihypertensive: Amlodipine (AMD), Nifedipine (NFD) (Fig. 1) and lipid lowering drugs: Atorvastatin (ATV), Ezetimibe (EZT) (Fig. 1). Combination drug products of AMD and ATV, ATV and EZT are hence widely marketed and used in the treatment of concomitant hypertension and dyslipidemia. Therefore the simultaneous determination of these analytes becomes interesting and important.



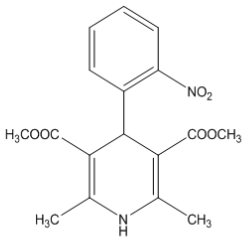
Atorvastatin calcium



Amlodipine besylate



Ezetimibe



Nifedipine(IS)

Fig. 1: The chemical structures of analytes and internal standard (IS).

Amlodipine besylate (AMD), chemically Methylethyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate is a calcium antagonist that inhibits the trans membrane influx of calcium ions into vascular smooth muscles and cardiac muscles and has been used in the treatment of hypertension and angina pectoris[3]. Atorvastatin calcium (ATV), chemically 7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbomyl)-5-propan-2-yl-pyrrol-1-yl]-3, 5-dihydroxy-heptanoic acid, calcium salt (2:1) trihydrate is a potent inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis and has been demonstrated to be effective in reducing both cholesterol and triglyceride[4]. Ezetimibe (EZT), chemically 1-(4-fluoro-phenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone is the first member of novel class of lipid-lowering drug that inhibits intestinal uptake of dietary and biliary cholesterol and related phytosterols.EZT does not appear to compromise the absorption of fat soluble vitamins, triglycerides and bile acids[5-7]. AMD besylate is official with British Pharmacopoeia[8] and European Pharmacopoeia[9] which describes HPLC methods for determination of AMD besylate, but do not address simultaneous determination with ATV calcium. A detailed survey of the literature for AMD reveals several methods based on different techniques, viz. HPLC[10],HPTLC[11], supercritical fluid chromatography[12],and UV spectrometry[13] for its determination in pharmaceutical dosage forms. Similarly, a literature survey for ATV calcium reveals methods based on HPLC [14], CE[15], and UV spectrometry[16] for its determination in pharmaceuticals. Owing to the presence of interferences of time consuming analysis, these analytical methods cannot be applied for the analysis of samples containing mixtures of AMD and ATV. However , an intensive literature search revealed to the best of our knowledge that only six methods[17-22] are available for the determination of these analytes in pharmaceutical mixtures. Among that only one method developed by T.Sivakumar et al involve optimization using Multi criteria decision making approach but the analysis time is 9 minutes and the other five methods does not applied a systematic optimization procedure for the separation and quantitation of these drugs, 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. Till date there are only few analytical methods reported for the estimation of EZT. Shaik jafar sadik basha et al[23] have reported HPLC determination of EZT and its metabolites which is a time consuming one .Some bio analytical methods were based either on HPLC coupled to UV detector[24,25]/radio detector[26]. To the best of our knowledge, currently there is no HPLC method employing optimization technique has been reported for the simultaneous estimation of EZT, ATV calcium and AMD besylate. Therefore the simultaneous determination of these analytes becomes motivating and significant.

Developing and optimizing an isocratic HPLC[27, 28] methods is a complex procedure that requires simultaneous determination of several factors, viz., the type and composition of the organic phase, column temperature, flow rate, pH, type of the stationary phase, etc. For decades HPLC separations were based on a trial and error methodology, but employing 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. To achieve this objective, any one of the chemometric methods which includes the overlapping resolution maps, [29] factorial design [30] and response surface methodology[31-35] can be applied. The best experimental design approach for the purpose of modeling and optimization are the response surface design [31]. However, the HPLC method intended to be applied for the pharmaceutical or industrial environment, the analysis time is usually optimized simultaneously without losing resolution[36]. When one needs to optimize more than one response at a time the use of multi-criteria decision making (MCDM), a chemometric technique is the best choice. However, this method optimizes only one response by targeting all other responses to appropriate constraints. When there is a mix of linear and non-linear responses, or when all response models are of linear or non-linear, Pareto-optimality, utility function or Derringer's desirability function can be used. The Pareto-optimal method and the Derringer's approach have their own advantages and that the decision on which method to use depends on the problem and the availability of chromatographic expertise.

There are many ways in which the individual desirabilities can be combined. If the combined criterion is a simple arithmetic average, it is called as utility function and if it is a geometric mean it is referred as Derringer's desirability function. The idea of combining desirabilities as geometric mean was first presented by Harrington[36] but it was put into a more general form by Derringer[37]. The advantage of the Derringer's desirability function is that if one of the criteria has an unacceptable value, then the overall product will also be unacceptable, while for the utility functions, this is not the case. Further, Derringer's method offers the user flexibility in the definition of desirability functions. Derringer's desirability function was introduced in chromatography by Deming, [36] implementing resolution and analysis time as objective functions to improve separation quality. Among the various above options, the Derringer's desirability function was applied to explore the user flexibility of this technique in selecting optimum chromatographic conditions for the determination of drugs in a variety of sample matrices. We have recently employed the same MCDM approach (Derringer's desirability function) for the development and optimization of a HPLC method for the simultaneous estimation of pantoprazole and domperidone[33], amlodipine and atorvastatin[34] in quality control and plasma samples.

In the present work, a HPLC method was developed, optimized and validated for the simultaneous determination of Amlodipine, ezetimibe and atorvastatin in commercial pharmaceutical preparations using chemometric procedure. The significance of the studied factors was evaluated with the aid of factorial design whilst the optimum chromatographic conditions were estimated by a central composite design using both a graphical and a mathematical (Derringer's desirability function) global optimization approach. Finally, the proposed method was tested for linearity, specificity, inter and intra-day precision, accuracy, and robustness. Two commercially available pharmaceutical products were analyzed in order to check the validity of the proposed method.

MATERIALS AND METHODS

Apparatus

Chromatographic measurements were made on a Shimadzu (Tokyo, Japan) model which consisted of a LC10AD and LC10 ADvp solvent delivery module, SPD 10A UV-Visible detector, a Rheodyne injector (model 7125, USA) valve fitted with a 20µl loop, and UV detector (SPD-10A). 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.

Softwares

Experimental design, data analysis and desirability function calculations were performed by using Design-Expert® trial version 7.0.0. (Stat-Ease Inc., Minneapolis). The rest of the calculations for the analysis were performed by use of Micro soft Excel 2007 software (Microsoft, USA).

Chemicals and reagents

Working standards of Amlodipine, Ezetimibe, Atorvastatin and Nifedipine (IS) were donated by M/S. Sunglow Pharma, Puducherry, India. Acetonitrile (MeCN) and methanol (MeOH) were of HPLC grade and dipotassium hydrogen phosphate and orthophosphoric acid was of analytical-reagent grade supplied by M/S SD Fine Chemicals, Mumbai, India. The HPLC grade water was prepared by using Milli-Q Academic, Millipore, Bangalore, India. The pharmaceuticals Avas-AM tablets (ATV-10 mg with AMD-5 mg), and Aztor-EZ tablets (ATV-10 mg with EZT-10 mg) were purchased from Micro Labs Limited (Pondicherry, India) and Sun Pharmaceutical industries, (Jammu &Kashmir, India) respectively.

Standard solutions

Stock standard solutions of ATV, EZT and AMD (1mg/ml) were prepared in mobile phase. The prepared stock solution was stored at 4° C protected from light. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the analysis day. Calibration curves reporting peak area ratios of ATV, EZT and AMD to that of the NFD(IS) versus drug concentrations were established in the range of 0.5-5.0µg/ml for ATV & EZT, and 0.25-2.5µg/ml for AMD in presence of Nifedipine (2.5µg/ml) as internal standard. Standard solution prepared for the optimization procedure constituted ATV, EZT, AMD and IS at 10.0, 10.0, 10.0, and 6µg/ml, respectively.

Sample preparation

Twenty tablets were weighed and finely powdered. An amount of pharmaceutical products powder equivalent to 10 mg of ATV with 5 mg of AMD and 10 mg of ATV with 10mg of EZT, were accurately weighed and transferred in a 50ml volumetric flask ; suitable quantity of IS was added followed by 25 ml of mobile phase. This mixture was subjected to sonication for 10 min for complete extraction of drugs and the solution was made up to the mark with mobile phase to obtain a concentration of ATV, EZT , AMD and IS as 5.0, 5.0, 2.5 and 2.5µg/ml, respectively. The solution was centrifuged at 4000 rpm for 10 min; the clear supernatant was collected and filtered through a 0.2µm membrane filter (Gelman Science, India) and 20 µl of this solution was injected for HPLC analysis.

Chromatographic procedure

Chromatographic separations were carried out on a Phenomenex® C18 analytical column (150mm×4.6mm i.d., 5µm) connected with a Phenomenex® C18 guard cadridge (4mm×3mm i.d., 5µm). The mobile phase consisted of MeOH-MeCN-dipotassium hydrogen phosphate buffer (pH 5.0), adjusted with 10% phosphoric acid. Wavelength of 231 nm was selected for detection. An injection volume of the sample was 20µl. The HPLC system was used in an air conditioned laboratory atmosphere (20 ± 2°C).

Validation

Validation studies were conducted using the optimized assay conditions based on the principles of validation described in the ICH guidelines "Text on Validation of Analytical Procedures" [38]and "Q2B, Validation of Analytical Procedures: Methodology" [39]. Key analytical parameters, including, specificity, accuracy, precision, linearity, detection limit and quantitation limit were evaluated. For specificity study, placebo containing starch, lactose monohydrate, aerosil, hydroxypropyl methylcellulose, titanium dioxide and magnesium stearate was used. The calibration curves were tested using one-way ANOVA at 5% significance level.[34] Calibration curves were constructed in a low region of 0.05-1.0% of the target analyte concentration for the limit of detection and quantification [40]. Also, robustness of the proposed method was assessed with respect to small alterations in the MeCN concentration (36.74 ± 0.5%), the pH value (5.0 ± 0.2) and the buffer concentration (20 ± 2.0 mm).

RESULTS AND DISCUSSION

Optimization design and analysis

Before starting an optimization procedure, it is important to investigate the curvature term using Factorial design with center points. ANOVA generated for 2^k Factorial design shows that curvature is significant for all the responses (k_1, $Rs_{(2,3)}$ and tR_4) since p-value is less than 0.05. This implies that a quadratic model should be considered to model the separation process[41]. In order to obtain second order predictive model, central composite design (CCD) is employed, which is a design type under RSM. CCD is chosen due to its flexibility and can be applied to optimize an HPLC separation by gaining better understanding of factor's main and interaction effects[42]. The selection of key factors examined for optimization was based on preliminary experiments and prior knowledge from literature The factors selected for optimization process were MeCN concentration (A), buffer molarity (B) and flow rate (C). The capacity factor for the first eluted peak (k_1), the resolution of the critical separated peak, IS and EZT, ($Rs_{2,3}$), the retention time of the last peak, ATV, (tR_4), were selected as responses. In the preliminary study, resolution between peak (Rs2,3) were found to be close to 1.5, hence these two peaks were considered as critical peaks and included as one of the response for the global optimization. Nifedipine (IS) was used as an internal standard since it presented acceptable resolution and retention time with all the analytes.

All experiments were conducted 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. (Table 1), summarizes the conducted experiments and responses. The quadratic mathematical model for three independent factors is given in Eq. (1):

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

Where Y is the response to be modeled, β is the regression coefficient and X_1, X_2 and X_3 represents factors A, B and C, respectively. Statistical parameters obtained from ANOVA for the reduced models are given in (Table 2).The insignificant terms (P > 0.05) were eliminated from the model through backward elimination process to obtain a simple and realistic model. Since R^2 always decreases when a regressor variable is eliminated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected[43].

Table 1: Experimental responses and central composite rotatable design arrangements[a]

Design points	Factor levels			Responses		
	A **(%,v/v)**	***B*** **(mM)**	***C*** **(ml/min)**	K_1	$Rs_{2,3}$	Tr_4
1	35.00	10.00	0.80	1.60	7.18	13.41
2	40.00	10.00	0.80	0.97	2.91	8.30
3	35.00	20.00	0.80	1.78	5.86	13.42
4	40.00	20.00	0.80	1.25	2.85	8.21
5	35.00	10.00	1.20	1.54	6.45	8.90
6	40.00	10.00	1.20	0.94	2.64	5.52
7	35.00	20.00	1.20	1.79	6.78	8.90
8	40.00	20.00	1.20	0.88	2.66	5.46
9	33.30	15.00	1.00	2.42	8.87	14.76
10	41.70	15.00	1.00	0.99	1.98	6.54
11	37.50	6.59	1.00	1.50	3.52	8.33
12	37.50	23.41	1.00	1.33	4.10	8.14
13	37.50	15.00	0.66	1.47	5.70	13.18
14	37.50	15.00	1.34	1.27	5.02	6.51
15	37.50	15.00	1.00	1.34	5.11	8.73
16	37.50	15.00	1.00	1.34	4.94	8.73
17	37.50	15.00	1.00	1.34	5.11	8.73
18	37.50	15.00	1.00	1.34	4.94	8.73
19	37.50	15.00	1.00	1.34	5.11	8.73
20	37.50	15.00	1.00	1.34	4.94	8.73

[a]**Randomized**

Table 2: Response models and statistical parameters obtained from ANOVA for CCD

Responses	Regression model	Adjusted R^2	Model *P* value.	%C.V	Adequate precision
K_1	+28.86-1.32 A+0.012 A^2	0.8626	<0.0001	9.31	24.97
$Rs_{2,3}$	+30.24 - 0.78A + 0.58B - 0.012B^2	0.9624	<0.0001	7.00	43.63
tR_4	+212.62 - 8.37A+ 0.35B -55.49C + 0.87AC + 0.0813A^2 - 0.012175B^2+6.62122C^2	0.9804	<0.0001	4.01	37.303

In the present study, the adjusted R^2 were well within the acceptable limits of $R^2 \geq 0.80$[44] which revealed that the experimental data shows a good fit with the second-order polynomial equations. For all the reduced models, *P* value of < 0.05 is 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[45]. In this study, the ratio was found to be in the range of 24.97–43.63, which indicates an adequate signal and therefore the model is significant for the separation process. The coefficient of variation (C.V.) 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% [45]. The C.V. 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 *AC* (+ 0.87) of tR_4 model. The positive interaction between *A* and *C* is statistically significant (< 0.0001) for tR_4. The study reveals that changing the fraction of MeCN from low to high results in a rapid decline in the retention time of ATV both at the low and high level of buffer molarity. Further at low level of factor *A*, an increase in the buffer molarity results in a marginal decrease in the retention time. This may be due to reduced silanol effects as a result of higher buffer molarity used. Therefore, when the MeCN concentration is set at its lowest level, the buffer concentration has to be at its highest level to shorten the run time. Especially this interaction is synergistic, as it led to a decrease in run time.

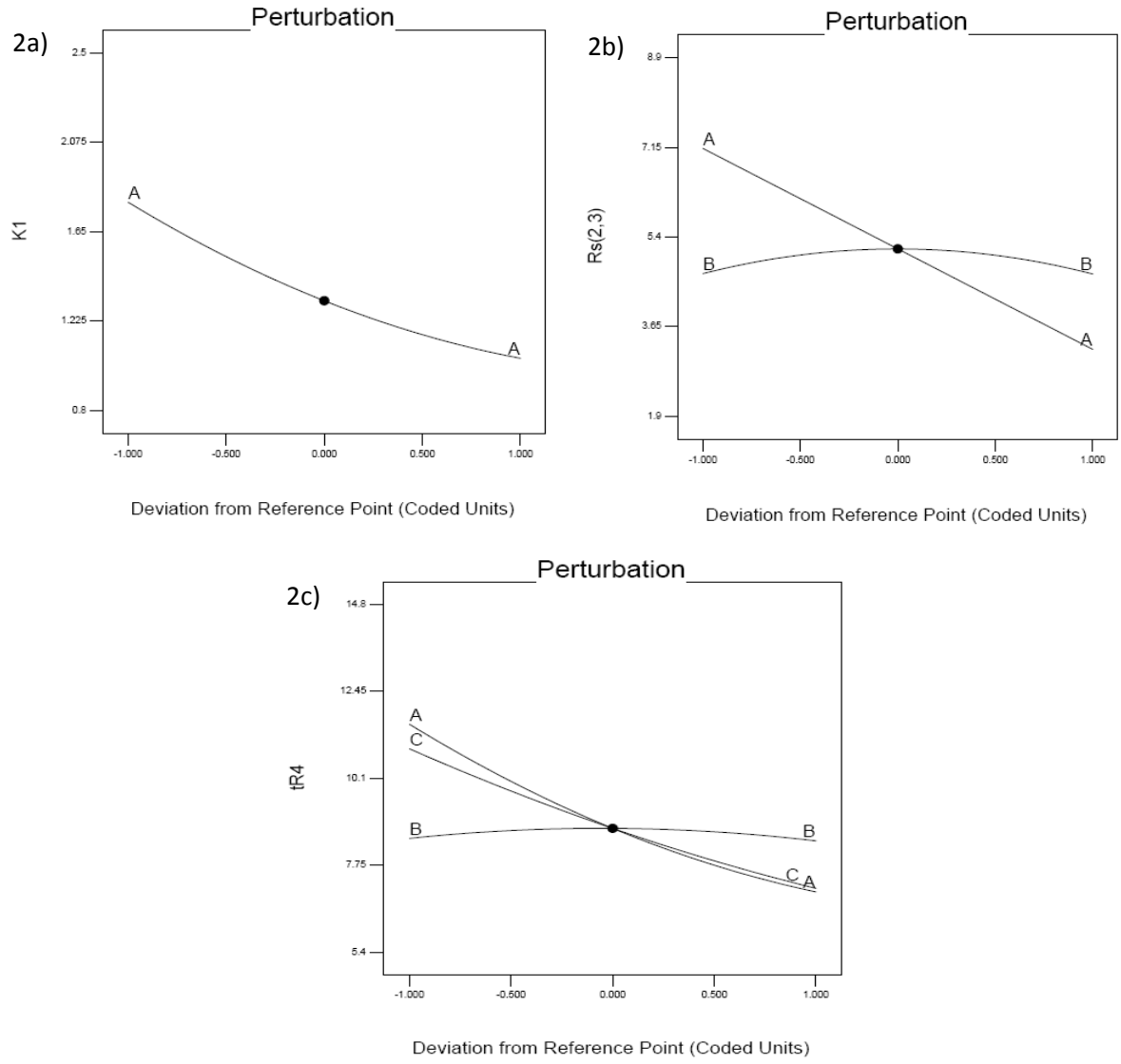
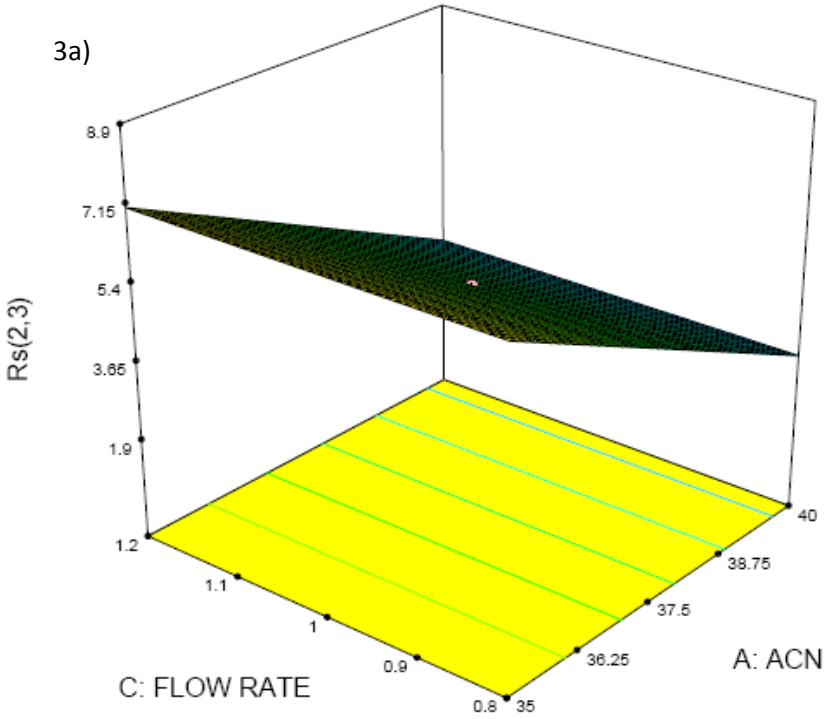
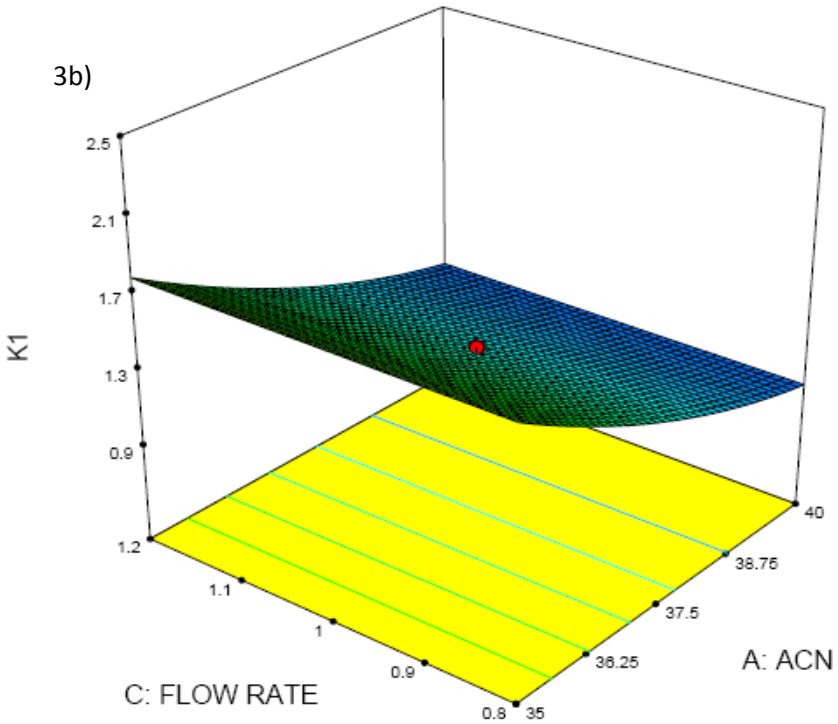


Fig. 2: **Perturbation plots showing the effect of each of the independent variables on a) *k*1, b) $Rs_{2,3}$, and c) tR_5. Where *A* is the concentration of acetonitrile, *B* the buffer molarity and *C* the mobile phase flow rate.**

In (Fig.2) perturbation plots are presented for predicted models in order to gain a better understanding of the investigated procedure. This type of plots show the effect of an independent factor on a specific response, with all other factors held constant at a reference point [31]. A steepest slope or curvature indicates sensitiveness of the response to a specific factor. (Fig. 2c) shows that MeCN (factor *A*) had the most important effect on Retention time tR_4 followed by factor *C* and then *B*. The factors (MeCN concentration and buffer molarity) had significant effect on $Rs_{2,3}$ and only one factor A had significant effect on k_1. In (Fig. 2 a) and (b), k_1 and $Rs_{2,3}$ values increased as the levels of MeCN concentration (factors *A*) decreased and $Rs_{2,3}$ values increased at the level of buffer molarity (factors *B*) is at mid point.

Response surfaces plots for k_1, $Rs_{2,3}$ and, tR_5 are illustrated in Fig.3 (% acetonitrile concentration is plotted against the flow rate with buffer concentration held at constant at the center value). Analysis of the perturbation plots and response plots of optimization models revealed that factor *A* and *C* had the significant effect on separation of the analytes, whereas the factor *B*, i.e. the buffer molarity, is of little significance.





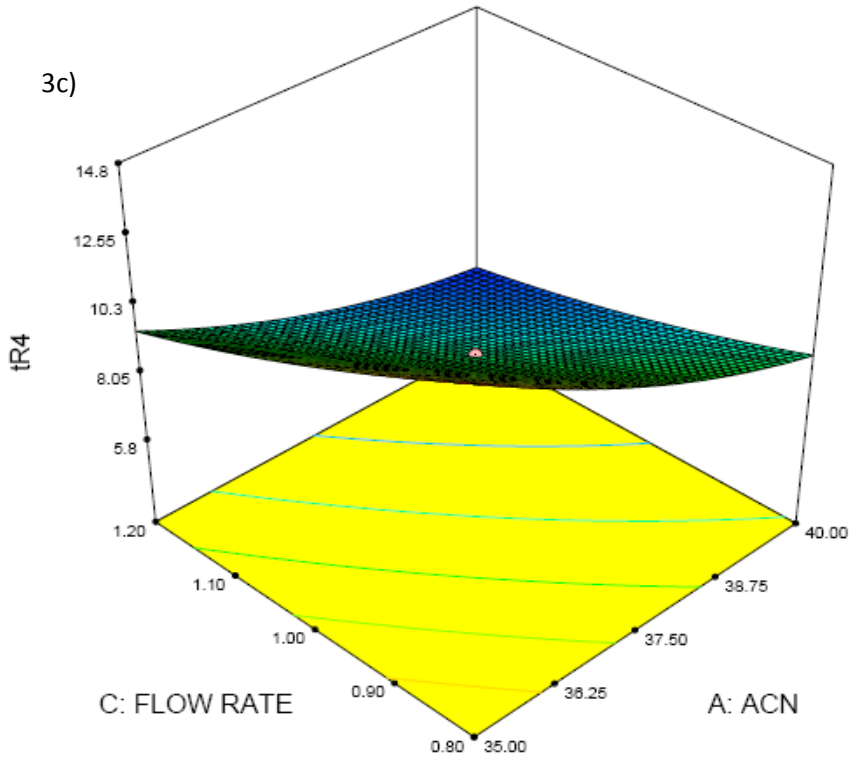


Fig. 3: Response surfaces related to percentage acetonitrile concentration (*A*) and Flow rate (*C*): (a) capacity factor of the first peak (*k*1), (b) resolution of the critical pair ($Rs_{2,3}$), and (*C*) retention time of the last peak (tR_4)

Global Optimization

In the present study, the identified criteria for the optimization were: resolution between the critical peaks, capacity factor, and elution time. Derringer's desirability function was used to optimize three responses with different targets[37]. 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:

$$D = [d_1^{p^1} \times d_2^{p^2} \times d_3^{p^3} \times \ldots \times d_n^{pn}\ ]^{\frac{1}{n}} \qquad (2)$$

Where *pi* is the weight of the response, *n* the number of responses and *di* is the individual desirability function of each response. Desirability function (*D*) can take values from 0 to 1. Weights can range from 0.1 to 10. Weights lower than 1 give less importance to the goal, whereas weights greater than 1 give more importance to the goal. In the present study, *pi* values were set at 1 for all the three responses. A value of *D* close to 1, indicates that the combination of the different criteria is matched in a global optimum [31].The criteria for the optimization of each individual response are shown in (Table 3). Criteria I have been proposed for selecting an optimum experimental condition for analyzing routine quality control samples. As can be seen under criteria I, the responses tR_4 was minimized, in order to shorten the analysis time. On the other hand, $Rs_{2,3}$ was minimized to allow baseline separation of EZT and IS. In order to separate the first eluting peak (AMD) from the solvent front, *k*1 was targeted at 1.5. Importance can range from 1 to 5, which gives emphasis to a target value. 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. 4). From the figure it can be concluded that there was a set of coordinates producing high desirability value (D = 0.716) were MeCN concentration of 36.74%, buffer molarity of 20 mM and flow rate of 1.20 ml/min. The predicted response values corresponding to the latter value of *D* were: k_1 = 1.44, $Rs_{2,3}$ = 5.26, and tR_4 = 7.39 min. The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram is shown in (Fig. 5).

In order to investigate the predictability of the proposed model, the agreement between experimental and predicted responses for the predicted optimums I are shown in (Table 4). The Percentage of prediction error was calculated by Eq. (3). The average errors for K_1, $Rs_{2,3}$, and tR_4 were 2.08, 8.36 and 5.41 % respectively, indicating good correlation between the experimental and the predicted responses[46].

Predicted Error = Experimental – Predicted / Predicted x 100 (3)

Table 3: Criteria for the optimization of individual responses

Responses	Lower limit	Upper limit	Criteria I	
			Goal	Importance
K_1	0.88	1.79	Target = 1.5	4
$R_{2,3}$	1.98	8.87	Minimize	4
tR_4	5.46	14.76	Minimize	3

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

Optimum conditions	MeCN(%)	Buffer (Mm)	Flow(ml/min)	K_1	$Rs_{2,3}$	tR_4
I	Desirability Value (D) = 0.716					
	36.74	20.00	1.20			
	Experimental value			1.47	4.82	7.79
	Predicted value			1.44	5.26	7.39
	Average % error			2.08	8.36	5.41

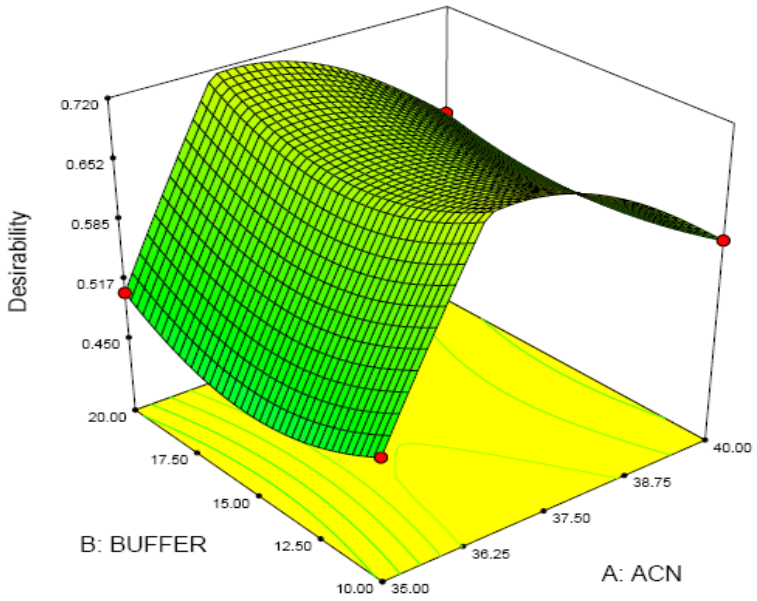


Fig. 4:**Graphical representation of the overall desirability function *D.* (*D* = 0.716) were MeCN Conc. (*A*) of 36.74%, Buffer Molarity(*B*) of 20 mM, and flow rate (*C*) of 1.20 ml/min.**

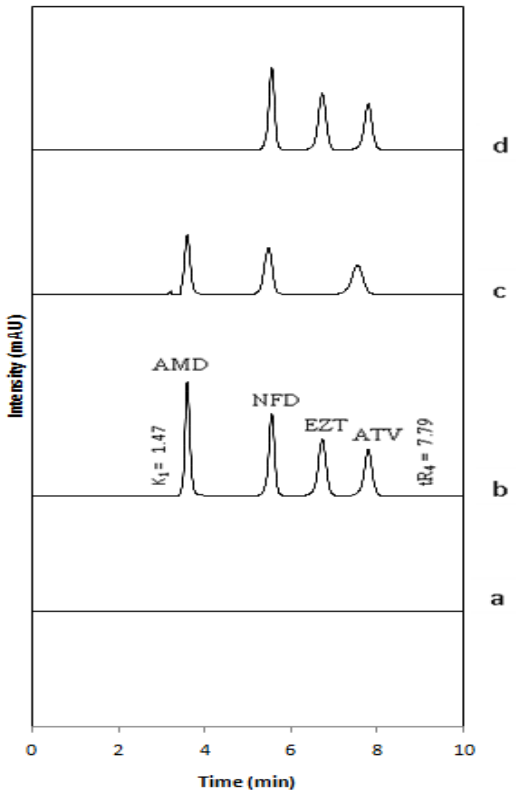


Fig. 5: **Chromatograms corresponding to (a) a placebo solution; (b) a synthetic mixture of AMD (10.10 µg/ml), IS(7.01 µg/ml), EZT(9.98 µg/ml) and ATV (9.97 µg/ml); (c) a real sample of Avas-AM tablets containing AMD (2.52 µg/ml), IS (2.5 µg/ml) and ATV (4.96 µg/ml); (d) a real sample of Aztor-EZ tablets containing ATV (4.97 µg/ml), IS (2.49 µg/ml) and EZT(4.98 µg/ml); under optimum assay conditions I for formulation.**

Assay method validation

The last step of the present study was to check method's validation for specificity, linearity, accuracy, intra/inter-day precision, and robustness. The optimized HPLC method was specific in relation to the placebo used in this study. All placebo chromatograms showed no interference peaks (Fig.5a). An excellent linearity was established at five levels in the range of of 0.5-5.0µg/ml for ATV & EZT, and 0.25-2.5µg/ml for AMD in presence of Nifedipine (2.5µg/ml) as internal standard with R^2 of more than 0.999 for all the analytes. The slope and intercept of the calibration curve were 0.2011 and + 0.0056 for ATV, 0.2346 and - 0.005 for EZT, and 0.4505 and + 0.0215 for AMD respectively. Since the correlation coefficient are not good indicators of linearity performance of an analytical procedure[47] a one way ANOVA was performed. For all the analytes, the calculated F- Value (F*calc*) was found to be less than the theoretical F-Value (F*crit*) at 5% significance level, indicating that there was no significance difference between replicate determinations for each concentration level. The LODs were 0.17, 0.18, 0.03 and 0.44 ng /mL and the LOQs were 0.52, 0.57, 0.083 and 1.34 ng/mL for AMD, NFD, EZT and ATV respectively. Accuracy (n = 9), assessed by spike recovery, were found to be 99.42, 99.65, 99.74 and 99.62% for AMD, NFD, EZT and ATV respectively, which were within acceptable ranges of 100 ± 2%.[46] The intra and inter-assay precision (n = 6) was confirmed since, the %C.V. were well within the target criterion of≤ 2 and ≤ 3, respectively [48]. Robustness study reveals that small changes did not alter the retention times, retention factor, and resolutions and therefore it would be concluded that the method conditions are robust.

Application of the method

As a final step, two commercial tablet products Avas-AM tablets (ATV-10 mg with AMD-5 mg), and Aztor-EZ tablets (ATV-10 mg with EZT-10 mg) were assayed by the proposed HPLC method. Representative chromatograms are presented in (Fig. 5). The results achieved when analyzing Aztor-EZ tablets were, 9.98 (0.21) mg of ATV and 10.04 (0.5) mg of EZT; and Avas-AM tablets were, 9.99 (0.47) mg of ATV and 4.99 (0.26) mg of AMD with the values within parenthesis being the %C.V. of the six replicates. Good agreement was found between the assay results and the label claim of the product. The %C.V. for the tablets were < 2, indicating the precision of the analytical methodology.

CONCLUSIONS

An efficient isocratic reversed-phase high-performance liquid chromatography method was developed, optimized and validated for the simultaneous estimation of the analytes AMD, NFD (IS), EZT and ATV in pharmaceutical formulations (tablets). The developed HPLC method could be of immense relevance and value since in India atorvastatin is chiefly prescribed in combination with ezetimibe and amlodipine. This method reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic factors and their interaction effects on the attributes of separation. Time of analysis, resolution, and quality of the peaks were simultaneously optimized by applying useful tools of chemometrics: central composite design and Derringer's desirability function. The validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust. Therefore, this HPLC method can be used as a routine quality control analysis in a pharmaceutical environment. The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determination of drugs in pharmaceutical formulation.

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