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ORIGINAL ARTICLE

Chemometric technique for the optimization of chromatographic system: Simultaneous HPLC determination of Rosuvastatin, Telmisartan, Ezetimibe and Atorvastatin used in combined cardiovascular therapy

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Abstract Developed and optimized a validated isocratic reverse phase HPLC separation of Rosuvastatin, Telmisartan, Ezetimibe and Atorvastatin in pharmaceutical preparation using response surface methodology. The separation was carried out by using phenomenex C_{18} column (15 cm × 4.6 mm id, 5 μm particle size) and UV detection at 239 nm. The ranges of the independent variables used for the optimization were MeCN: 33–38%, buffer conc.: 10–20 mM and flow rate: 1–2 ml/min. The influence of these independent variables on the output responses: capacity factor of the first peak (k_1), resolutions of the 2nd and 3rd peak ($Rs_{2,3}$), and capacity factor of the fifth peak (k_5) were evaluated. Using this strategy, a mathematical model was defined and a response surface was derived for the separation. The three responses were simultaneously optimized by using Derringer's desirability functions. Optimum conditions chosen for the assay were MeCN, MeOH, 20 mM K_2HPO_4 (pH 3.0 ± 0.2) solution (34.27:20:45.73 v/v/v) and flow rate 2 ml/min. Total chromatographic analysis time per sample was approximately 10 min. The optimized assay condition was validated as per the ICH guidelines and applied for the quantitative analysis of Rosavel EZ, Avas-EZ and Lipisar 20 tablet. The developed method was simple, accurate and precise. Hence, it can be employed for the routine analysis in quality control laboratories.

1. Introduction

High performance liquid chromatography method development and optimization is a well-known procedure exceptionally for the simultaneous determination of pharmaceutical dosage forms. Since HPLC utilizes a wide selection of several chromatographic factors, viz., the type and composition of the organic phase, column temperature, flow rate, buffer molarity, pH, type of the stationary phase, etc., optimization of the experimental conditions is a complicated process. To

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achieve this objective, any one of the chemometric methods which includes the overlapping resolution maps (Lews et al., 1996), factorial design (Valliappan et al., 2002) and response surface methodology (Myers and Montgomery, 1995; Sivakumar et al., 2007, 2007a,b, 2008) can be applied. In general, the chemometrics can be used to accomplish a variety of goals in chromatography laboratory: (i) speeding methods development, (ii) make better use of chromatographic data and (iii) explain the chromatographic process (Matthijs et al., 2004).This kind of knowledge provides important clues in the attainment of optimum experimental conditions in the development of chromatography methods (Morgan, 1991).The best experimental design approach for the purpose of modeling and optimization is the response surface design (Myers and Montgomery, 1995). However, for the HPLC method intended to be applied for the pharmaceutical or industrial environment, the analysis time is usually optimized without losing resolution (Deming, 1991).

When one needs to optimize more than one response at a time the use of multicriteria decision making (MCDM), a chemometric technique is the best choice. The different approaches of MCDM include the path of steepest ascent, constrained optimization procedure, Pareto-optimality, utility function, Derringer's desirability function. The path of steepest ascent can be employed only when all the response models are linear. Constrained optimization procedure can be used when all response models are non-linear, or when there is a mix of linear and non-linear responses. 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 linear or non-linear, Pareto-optimality, utility function or Derringer's desirability function can be used. Pareto-optimality method can basically identify the Pareto optimal region by graphical means, but requires some additional criterion or the advice of an expert to select one particular Pareto optimum point (Hadjmohammadi and Safa, 2004). 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 desirability 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 (1965) but it was put into a more general form by Derringer (Derringer and Suich, 1980). 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 (1991), implementing resolution and analysis time as objective functions to improve separation quality. Safa and Hadjmohammadi (2005) employed Derringer's desirability function for the simultaneous optimization of resolution and analysis time in micellar liquid chromatographic separation of a group of nine phenyl thiohydantoin amino acids. Recently, Hayashi and Matsuda (1994) proposed a chemometric tool based on the Function of Mutual Information (FUMI) theory to improve prediction of the uncertainty in HPLC. Kotani et al. (2003) employed FUMI theory for the prediction of measurement R.S.D. and detection limits in HPLC-electrochemical detection of catechins without repetitive measurement of chromatograms, saving considerable amounts of chemicals and experimental time. Among the various above options, the Derringer's desirability functions were successfully employed.

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 (Sivakumar et al., 2007b), amlodipine and atorvastatin (Sivakumar et al., 2007) in quality control and plasma samples.

Atorvastatin (AT) (Fig. 1), (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid and rosuvastatin (RS) (Fig. 1), (3R,5R,6E)-7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonamido)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid belong 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 rosuvastatin in 2003, atorvastatin was the most efficacious drug in the statins class (Jones et al., 1998) but recent studies reported rosuvastatin as a potent inhibitor of HMG-CoA reductase having a higher LDL-lowering effect as compared with other statins (Jones et al., 2003; McTaggart, 2003), which demonstrates that both rosuvastatin and atorvastatin are the leading drugs in the statins class.

Ezetimibe (EZ) (Fig. 1), (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)azetidine-2-one is a selective cholesterol absorbtion inhibitor, which potently inhibits the absorption of biliary and dietary cholesterol (Van Heek et al., 1997) from the small intestine without affecting the absorption of fat-soluble vitamins, triglyceride or bile acids. Clinical studies have shown that co-administration of ezetimibe with statins could provide significant reductions in both the low-density lipoproteins (LDL) and the total cholesterol with slight increase in the high-density lipoproteins (HDL) (Ballantyne et al., 2003; Davidson et al., 2002; Kerzner et al., 2003; Melani et al., 2003). Also co-administration of ezetimibe with statins could significantly reduce the risk of coronary heart disease (CHD) events in patients with hypercholesterolemia.

Telmisartan (TL) (Fig. 1), 2-(4-{[4-methyl-6-(1-methyl-1H-1,3-benzodiazol-2-yl)-2-propyl-1H-1,3-benzodiazol-1-yl]methyl} phenyl)benzoic acid is a new highly selective, non-peptide angiotensin II type 1 (AT1)-receptor antagonist angiotensin that lowers blood pressure through blockade of the renin–angiotensin–aldosterone system (RAAS) (Neutel and Smith,1998) and widely used in treatment of hypertension. It can selectively block the angiotensin type I (AT1) receptor, which is responsible for vasoconstriction and for salt and water retention. The therapy with this drug offers a good quality of life for hypertensive patients due to the absence of side effects and its once daily administration. Telmisartan has become one of the most important advances in the treatment of hypertension.

Cardiovascular therapy usually involves different combination of antihypertensive and lipid lowering drugs. Therefore the simultaneous determination of these analytes becomes

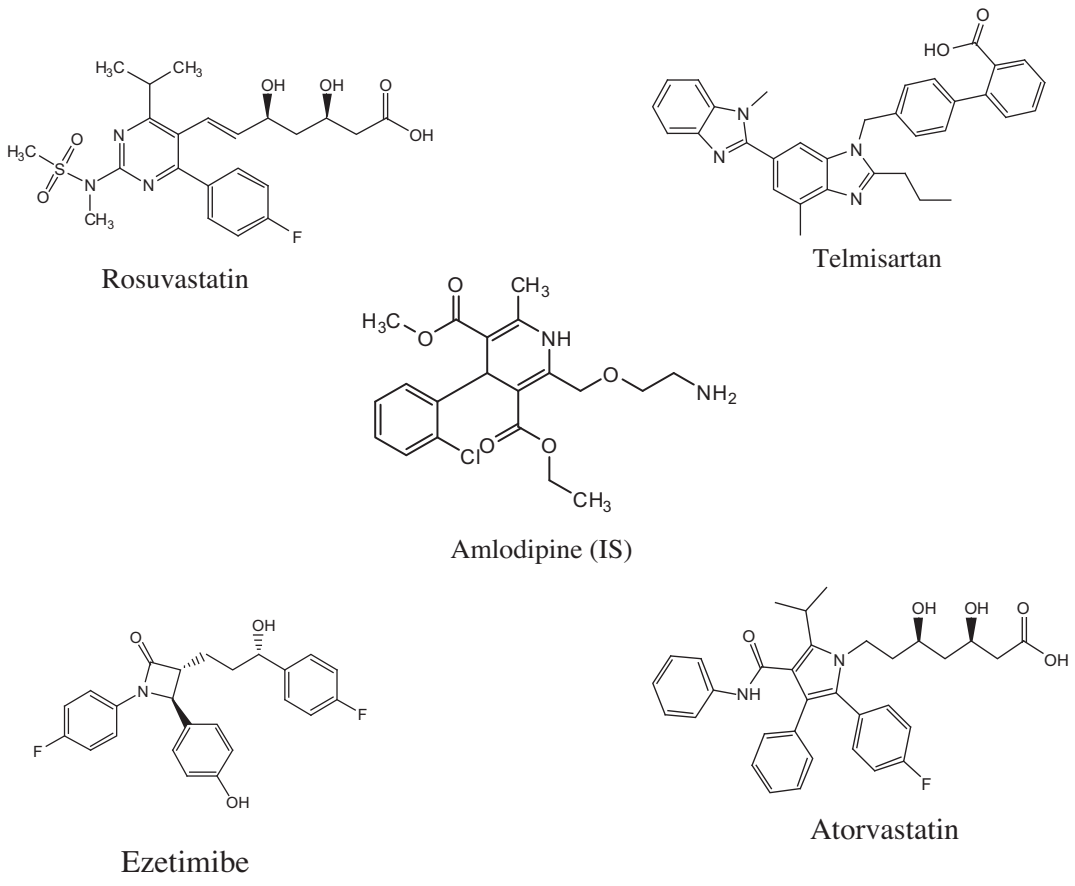

Figure 1 The chemical structures of analytes and internal standard (IS).

motivating and significant. In the present work, a HPLC method was developed, optimized and validated for the routine quality control analysis of RS, TL, EZ and AT from commercial preparations.

The present manuscript describes (i) the development, optimization and validation of an isocratic reversed-phase HPLC method for the routine quality control analysis of RS, TL, EZ and AT in a pharmaceutical laboratory and (ii) provide information on sensitivity of the chromatographic factors and their interaction effect on the separation characteristics. In the first step, the factorial design was employed to identify the significance of the curvature term for all the (k_1, $Rs_{2,3}$ and k_5) chromatographic responses. Subsequently, the chromatographic factors that had the significant effect were optimized using a central composite design and response surface analysis. Derringer's desirability function was successfully employed to explore the user flexibility of this technique in selecting optimum chromatographic conditions for the determination of drugs in a variety of sample matrices.

2. Material and methods

2.1. 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 20 µl 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.

2.2. 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).

2.3. Chemicals and reagents

Working standards of rosuvastatin, ezetimibe, telmisartan, atorvastatin and amlodipine (IS) were donated by M/S. Pharma analytical Lab., Puducherry, India. Acetonitrile (MeCN) and methanol (MeOH) were of HPLC grade and dipotassium hydrogen phosphate and phosphoric acid were 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 Rosavel EZ, (RS-10 mg with EZ-10 mg), Avas-EZ (AT-10 mg with EZ-10 mg) and Lipisar 20 tablet (TL-20 mg with AT-10 mg) were purchased from Sun pharmaceuticals (J&K,

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

Design points	Factor levels			Responses		
	A (%*v*/*v*)	*B* (mM)	*C* (mL min^{-1})	k_1	$Rs_{2,3}$	k_5
1	33	10	1	1.319	11.194	12.084
2	33	10	1	1.381	9.921	11.735
3	38	10	1	0.427	8.549	5.086
4	38	10	1	0.856	7.413	5.835
5	33	20	1	1.684	11.15	11.956
6	33	20	1	1.587	10.013	11.577
7	38	20	1	0.856	8.6	5.695
8	38	20	1	0.895	7.571	5.677
9	33	10	2	0.978	10.178	10.126
10	33	10	2	1.086	9.23	10.161
11	38	10	2	0.434	7.094	4.619
12	38	10	2	0.492	6.432	4.483
13	33	20	2	1.684	10.182	11.913
14	33	20	2	1.331	9.342	11.611
15	38	20	2	0.865	7.231	4.913
16	38	20	2	0.693	6.616	4.981
17	31.3	15	1.5	1.896	11.292	14.84
18	31.3	15	1.5	1.886	11.039	15.4
19	39.7	15	1.5	0.76	6.755	4.487
20	39.7	15	1.5	0.745	6.793	4.626
21	35.5	6.59	1.5	0.849	8.57	7.363
22	35.5	6.59	1.5	0.92	8.655	8.195
23	35.5	23.41	1.5	1.08	8.934	7.805
24	35.5	23.41	1.5	0.805	8.578	6.717
25	35.5	15	0.66	1.187	9.61	7.845
26	35.5	15	0.66	1.139	9.334	7.899
27	35.5	15	2.34	1.166	8.021	7.762
28	35.5	15	2.34	0.928	7.922	6.974
29	35.5	15	1.5	1.191	9.133	7.74
30	35.5	15	1.5	0.938	8.895	7.085
31	35.5	15	1.5	0.929	8.921	7.814
32	35.5	15	1.5	1.193	9.128	6.965
33	35.5	15	1.5	1.191	9.133	7.74
34	35.5	15	1.5	0.938	8.895	7.085
35	35.5	15	1.5	0.929	8.921	7.814
36	35.5	15	1.5	1.193	9.128	6.965
37	35.5	15	1.5	1.191	9.133	7.74
38	35.5	15	1.5	0.938	8.895	7.085
39	35.5	15	1.5	0.929	8.921	7.814
40	35.5	15	1.5	1.193	9.128	6.965

[a] Randomized.

Table 2 Response models[a] and statistical parameters obtained from ANOVA for CCD.

Responses regression model	Model *P*-val.	%CV	Ad. prec.	Adjusted R^2
$K_1 = +1.06–0.34 * A + 0.10 * B - 0.067 * C + 0.075 * A^2 - 0.070 * B^2$	<0.0001	13.45	23.317	0.8576
$Rs_{(2,3)} = +8.86–1.34 * \mathrm{A} - 0.48 * C$	<0.0001	4.14	44.697	0.9125
$K_5 = +7.46 - 3.13 * A + 0.090 * B - 0.31 * C + 0.24 * B * C + 0.83 * A^2$	<0.0001	5.34	63.504	0.9759

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

India) Micro Labs Limited (Baddi, India) and INTAS pharmaceuticals Ltd. (Ahamedabad, India) respectively.

2.4. Standard solutions

Stock standard solutions of RS, TL, EZ and AT (1 mg/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 RS, TL, EZ, and AT to that of the IS versus drug concentrations were established in the range of 0.5–5 μg/ml for RS, EZ, AT and 1–10 μg/ml for TL in the presence of amlodipine (2.5 μg/ml) as

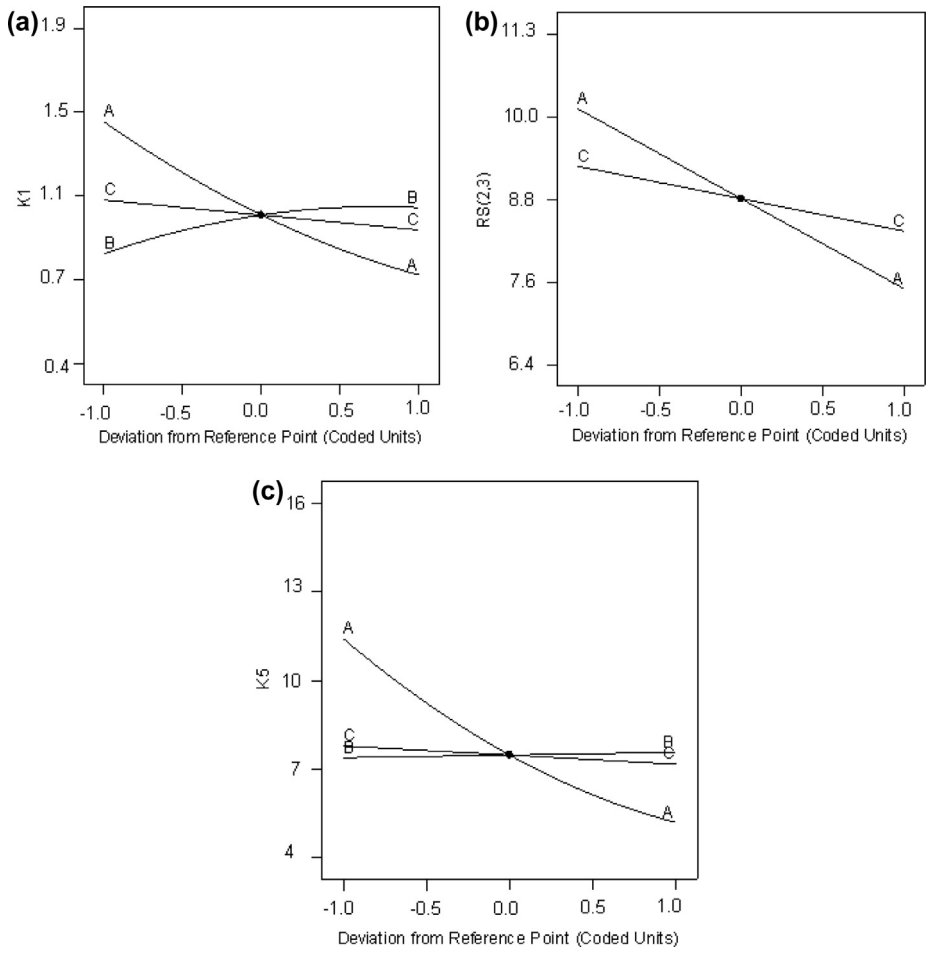

Figure 2 Perturbation plots showing the effect of each of the independent variables on k_1, $Rs_{2,3}$ and k_5. Where A is the concentration of acetonitrile, B the buffer molarity and C the mobile phase flow rate.

internal standard. The standard solution prepared for the optimization procedure constituted RS, TL, EZ, AT and IS at 10.0, 10.0, 10.0, 10.0 and 5 μg/ml, respectively.

2.5. Sample preparation

Twenty tablets were weighed and finely powdered. An amount of pharmaceutical products powder equivalent to 10 mg of RS with 10 mg of EZ, 10 mg of AT with 10 mg of EZ, and 10 mg of AT with 20 mg of TL were accurately weighed and transferred into a 50 ml 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 a mobile phase to obtain a concentration of RS, TL, EZ, AT and IS as 2.5, 5.0, 2.5, 2.5 and 2.5 μg/ml, respectively. The solution was centrifuged at 4000 rpm for 10 min; the clear supernatant was collected and filtered through a 0.2 μm membrane filter (Gelman Science, India) and 20 μl of this solution was injected for HPLC analysis.

2.6. 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 MeOH–MeCN–dipotassium hydrogen phosphate buffer (pH 3.0), adjusted with 10% phosphoric acid. Wavelength of 239 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).

2.7. 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"(International Conference on Harmonization, Q2A, 1995) and "Q2B, Validation of Analytical Procedures: Methodology" (International Conference on Harmonization, Q2B, 1997). 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 curve was tested using one-way ANOVA at 5% significance level (Thanikachalam Sivakumar et al., 2007). Calibration curves were constructed in a low region of

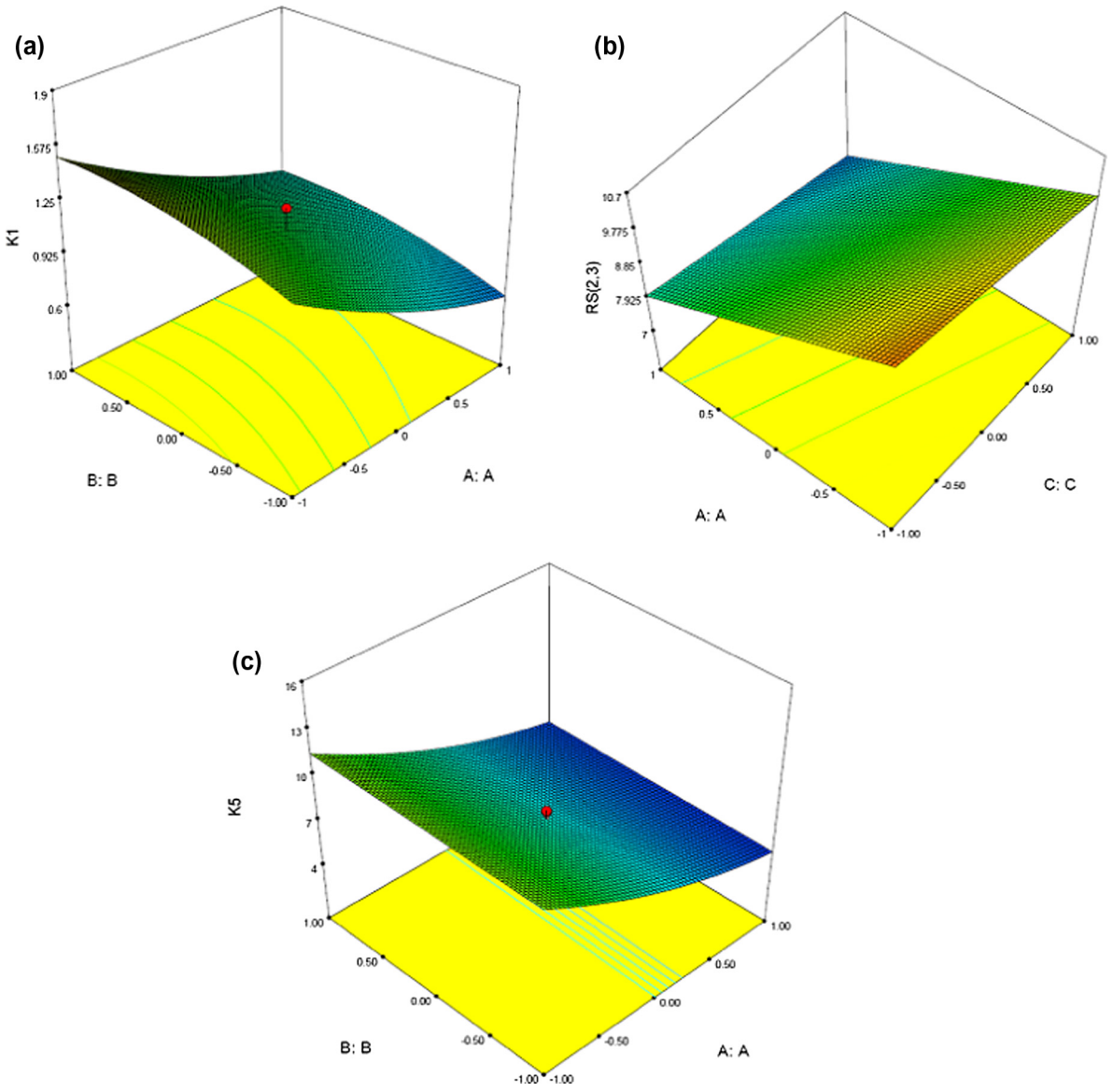

Figure 3 In figure (a) and (c), response surfaces related to percentage acetonitrile concentration (*A*) and Buffer molarity(*B*): Flow rate was kept constant and in figure (b), response surfaces related to percentage acetonitrile concentration (*A*) and Flow rate (*C*): Buffer molarity was kept constant (a) capacity factor of the first peak (k_1), (b) resolution of the critical pair ($Rs_{2,3}$), (c) capacity factor of the last peak (k_5).

Table 3 Criteria for the optimization of individual responses.

Response	Lower limit	Upper limit	Weight	Criteria I		Criteria II	
				Goal	RI	Goal	RI
k_1	0.427	1.896	1	Target = 1.2	3	Target = 1.4	5
$Rs_{2,3}$	6.436	11.292	1	Range	2	Target = 8	3
k_5	4.483	15.4	1	Target = 10	1	Target = 10.9	3

RI-relative importance.

0.05–1.0% of the target analyte concentration for the limit of detection and quantification (Crowther, 2001). Also, robustness of the proposed method was assessed with respect to small alterations in the MeCN concentration (34.27 ± 0.5%), the pH value (3.0 ± 0.2) and the buffer concentration (20 ± 2.0 mM).

3. Results and discussion

3.1. Data analysis and optimization design

The experimental design approach to HPLC method development relies on two stages of experimentation: screening and

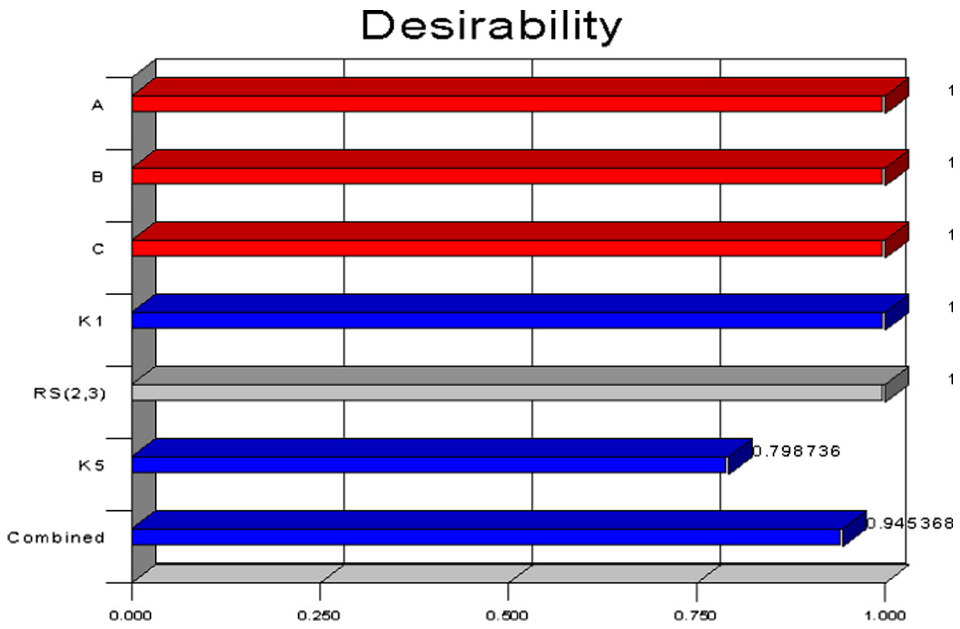

Figure 4 Bar graph showing individual desirability values of various objective responses and their association as a geometric mean (D) corresponding to formulation samples.

Table 4 Comparison of experimental and predictive values of different objective functions under optimal conditions.

Optimum conditions	MeCN (%v/v)	Buffer (mM)	Flow (mL min^{-1})	k_1	$Rs_{2,3}$	k_5
I	34.27	20.00	2.00			
		Experimental		1.34	3.18	9.82
		Predictive		1.22	9.04	9.22
II	33.26	19.34	2.00			
		Experimental		1.46	8.68	10.54
		Predictive		1.40	9.57	10.90
Average error				7.0	7.9	4.9

optimization. The purpose of the screening is to identify the factors that had significant effect on the responses and to investigate the curvature term using Factorial design with center points. Factorial design consisting of eight factorial runs along with six other experiments at the center of the design points was carried out to estimate the experimental error. The design experiments were produced in the random order. Before starting an optimization procedure, ANOVA was generated for 2^k factorial design which shows that curvature is significant for the variable (k_5) since p-value is less than 0.05. This implies that a quadratic model should be considered to model the separation process (Ting et al., 2009). 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. (Wang et al., 2006a,b) The selection of key factors examined for optimization was based on preliminary experiments and prior knowledge from the 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, RS and TL ($Rs_{2,3}$) and the capacity factor of the last peak, AT (k_5), were selected as responses. All experiments were conducted in a 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):

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

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. 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 elimi-

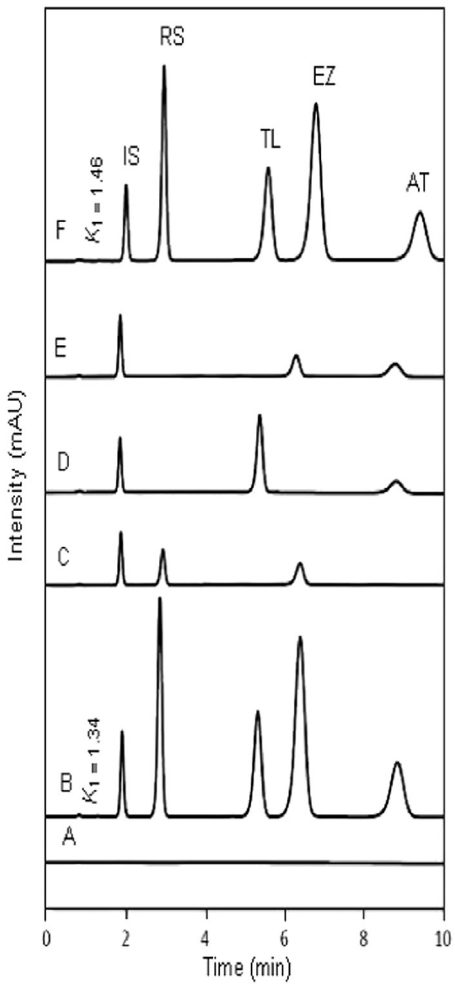

Figure 5 Chromatograms corresponding to (A) a placebo solution; (B) a synthetic mixture of IS (4.8 μg/ml), RS (10.2 μg/ml), TL (10.01 μg/ml), EZ (9.98 μg/ml) and AT (9.97 μg/ml); (C) a real sample of Rosavel-EZ containing IS (2.6 μg/ml), RS (2.5 μg/ml) and EZ (2.52 μg/ml); (D) a real sample of Lipisar 20 tablets containing IS (2.49 μg/ml), TL (4.97 μg/ml) and AT (2.50 μg/ml); (E) a real sample of Avas-EZ containing, IS (2.50 μg/ml), EZ (2.48 μg/ml) and AT (2.5 μg/ml) under optimum assay conditions I for formulation and (F) a synthetic mixture of IS (4.9 μg/ml), RS (9.94 μg/ml), TL (9.91 μg/ml), EZ (10.1 μg/ml) and AT (9.92 μg/ml) under optimum assay conditions II for plasma.

nated from a regression model, in statistical modeling the adjusted R^2 which takes the number of regressor variables into account, is usually selected (Parajo et al., 1992). In the present study, the adjusted R^2 were well within the acceptable limits of $R^2 \geqslant 0.80$ (Lundstedt et al., 1998) which revealed that the experimental data show 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 (Beg et al., 2003). In this study, the ratio was found to be in the range of 23.31–63.50, 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% (Beg et al., 2003). The C.V. for all the models was found to be less than 10%, except for k_1 (13.45%). Hence, the diagnostic plots, (a) normal probability plot of residuals (Choisnard et al., 2003) and (b) plot of residuals versus predicted values (Lui and Peng, 2004) were analyzed for response k_1. Since, the assumptions of normality and constant variance of the residuals were found to be satisfied; the fitted model for k_1 was accepted.

The interaction term with the largest absolute coefficients among the fitted models is BC ($+0.24$) of k_5 model can be seen in Table 2. The positive interaction between B and C is statistically significant (<0.0001) for k_5. The study reveals that changing the fraction of MeCN from low to high results in a rapid decline in the k_5 of AT both at the low and high levels of buffer molarity. Further at low level of factor A, an increase in the buffer molarity results in a marginal decrease in k_5. 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 k_5. Especially this interaction is synergistic, as it led to a decrease in k_5.

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 shows the effect of an independent factor on a specific response, with all other factors held constant at a reference point (Sivakumar et al., 2007b). A steepest slope or curvature indicates sensitiveness of the response to a specific factor. Fig. 2 (c) shows that MeCN (factor A) had the most important effect on capacity factor k_5 followed by factor C and then B. In Fig. 2a k_1 values increased as the levels of buffer concentration (factors B) increased while in Fig. 2a and b k_1 and $Rs_{2,3}$ values decreased as the levels of flow rate (factors C) increased.

Response surfaces plots for k_1, $Rs_{2,3}$ and k_5 are illustrated in Fig. 3 (% acetonitrile concentration is plotted against the flow rate with buffer concentration held at constant at the center value for $Rs_{2,3}$ plot, for k_1 and k_5, % acetonitrile concentration is plotted against the buffer concentration with flow rate held at constant). Analysis of the perturbation plots and response plots of optimization models revealed that factors A and C had the significant effect on separation of the analytes, whereas factor B, i.e. the buffer molarity, is of little significance.

3.2. Derringer's desirability function

In the present study, the identified criteria for the optimization were: resolution between the critical peaks, capacity factors k_1 and k_5. Derringer's desirability function was used to optimize three responses with different targets (Derringer and Suich, 1980). 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:

$$D = \left[d_1^{p^1} \times d_2^{p^2} \times d_3^{p^3} \times \cdots \times d_n^{p^n}\right]^{\frac{1}{n}} \quad (2)$$

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 (Sivakumar et al., 2007b). 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 response k_5 was targeted at 10, in order to shorten the analysis time. On the other hand, $Rs_{2,3}$ was kept in a range to allow baseline separation of RS and TL. In order to separate the first eluting peak (IS) from the solvent front, k_1 was targeted at 1.2. Importance can range from 1 to 3, 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 a set of coordinates producing high desirability value ($D = 0.945$) were MeCN concentration of 33.27%, buffer molarity of 20 mM and flow rate of 2.00 ml/min. The predicted response values corresponding to the above optimum condition are given in Table 4.

To substantiate the flexibility of the optimization strategy and to search for an optimum experimental condition for analyzing plasma samples, criteria II was established by varying the response goals and their importance values (Table 3). For instance, high value of k_1 has to be selected for the separation of IS from the initial disturbances of plasma components. Therefore, k_1 was targeted at 1.4 and high importance value of 5 was assigned. Following the response goals above, the optimization procedure was carried out for which optimal conditions II with the maximum desirability value ($D = 0.837$) were MeCN concentration of 33.26%, buffer molarity of 19.34 mM and flow rate of 2.00 ml/min. In order to investigate the predictability of the proposed model, the agreement between experimental and predicted responses for both the predicted optimums I and II are shown in Table 4. The Percentage of prediction error was calculated by Eq. (3). The prediction efficiency of the model confirmed by performing the experiment under the optimal conditions I and II is also presented Fig. 5B and F, respectively. This approach offers flexibility to the chromatographer to slide the k_1 values depending upon the environment of the analyte under consideration.

$$\text{Predicted Error} = \text{Experimental} - \text{Predicted}/\text{Predicted} \times 100 \quad (3)$$

3.3. Assay method validation

The present study was to check method's validation for specificity, linearity, accuracy, intra/inter-day precision, and robustness. The method specificity was assessed by comparing the chromatograms of standard drugs with those of placebo solutions obtained from the most commonly used excipients in pharmaceutical formulation, which included lactose monohydrate, aerosil, starch, hydroxy methylcellulose, magnesium stearate and titanium dioxide, There were no excipient peaks co- eluted with analyte and IS, 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 (Fig. 5). An excellent linearity was established at five levels in the range of 0.5–1.0 μg/ml for IS, RS, EZ and AT and 1.0–10 μg/mL for TL with R^2 of more than 0.997 for all the analytes. The slope and intercept of the calibration curve were 0.375 and 0.0003 for RS, 0.614 and 0.043 for TL, 0.382 and 0.002 for EZ and 0.335 and 0.0023 for AT, respectively. Since the correlation coefficients are not good indicators of linearity performance of an analytical procedure (Danzer and Currie, 1998) a one way ANOVA was performed. For all the analytes, the calculated F-value ($Fcalc$) was found to be less than the theoretical F-value ($Fcrit$) at 5% significance level, indicating that there was no significance difference between replicate determinations for each concentration level. The LOD and LOQ were estimated as 0.88 and 2.66 ng/ml for RS, 0.50 and 1.51 ng/mL for TL, 1.00 and 3.02 ng/ml for EZ, and 0.75 and 2.27 ng/mL for AT, respectively. Accuracy ($n = 9$), assessed by spike recovery, were found to be 99.72%, 99.85%, 99.78% and 99.66% for RS, TL, EZ and AT, respectively, which were within acceptable ranges of 100 ± 2% (Kleinschmidt, 2005). The intra and inter-assay precision ($n = 6$) was confirmed since, the % C.V. were well within the target criterion of $\leqslant 2$ and $\leqslant 3$, respectively (Kleinschmidt, 2005). Robustness study reveals that small changes did not alter the retention times, retention factor and resolutions more than 4% and therefore it would be concluded that the method conditions are robust.

3.4. Application of the method

The proposed RP-HPLC method was applied to the quantitative analysis of real samples, three commercial tablet products Rosavel EZ tablet (RS-10 mg with EZ-10 mg), Avas-EZ tablet (AT-10 mg with EZ-10 mg) and Lipisar 20 tablet (TL-20 mg with AT-10 mg) were assayed by the proposed HPLC method. Representative chromatograms are presented in Fig. 5. The results achieved when analyzing Rosavel EZ tablets were, 9.98 (0.97) mg of RS and 10.01 (1.11) mg of EZ; Avas- EZ tablets were, 10.02 (1.38) mg of AT and 10.02 (1.05) mg of EZ and Lipisar 20 tablet were, 20.06 (0.44) mg of TL and 10.08 (1.38) mg of AT 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 tablets were < 2, indicating the precision of the analytical methodology. The mean recoveries for each analyte were also tested for significance to establish whether the recovery means are different from the label claim of the tablets by employing Student's t-test.

4. Conclusions

Statistically based experimental designs proved to be a valuable approach in optimizing selectivity-controlling parameters for the simultaneous estimation of the analytes RS, TL, EZ, and AT in pharmaceutical formulations (tablets). The developed HPLC method could be of immense relevance and value since cardiovascular therapy usually involves different combination of antihypertensive and lipid lowering drugs. Therefore the simultaneous determination of these analytes becomes significant. This method reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic factors and their interaction ef-

fects 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 and plasma samples.

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