Research Article

Chemometrics Assisted RP- HPLC Method for the Simultaneous Determination of Levocetirizine, Ambroxol and Montelukast in Pharmaceutical Formulation

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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 Ambroxol(AMB) and Montelukast(MLS) with Levocetirizine (LCT) in commercial pharmaceutical preparations with Probenecid(PRO) as Internal standard. The ranges of the independent variables used for the optimization were MeCN: 30-40%, 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 coefficient of determination R^2 was more than 0.8972 for all the models. The three responses were simultaneously optimized by using Derringer's desirability functions. Optimum conditions chosen for assay were MeCN, MeOH, 11.39 mM K_2HPO_4 (pH 7.0) solution (40:30:30 v/v/v) and flow rate 0.81 ml/min. Total chromatographic analysis time per sample was approximately 4.56 min with PRO(IS), AMB, LCT and MLS eluting with retention times of 2.44,3.44,4.11 and 4.56 minutes respectively. The LODs were 0.20, 0.16, 0.23 and 0.34 ng /mL and the LOQs were 0.56, 0.47, 0.58 and 1.12 ng/mL for PRO (IS), AMB, LCT and MLS respectively. The optimized assay condition was validated as per the ICH guidelines and applied for the quantitative analysis of Airitis plus capsules, and Montair-LC tablets.

Keywords: Central composite design, Derringer's desirability function, HPLC.

INTRODUCTION

The incidence of allergic diseases such as allergic rhinitis and asthma is increasing to epidemic proportions (allergic rhinitis: 10-50%; and asthma: 5-15%), both in the developed and the developing world, with a reduced quality of life of the patients, lower productivity and increasing medical costs. The increasing evidence on the links between allergic rhinitis and asthma comes from epidemiological, immunological and clinical studies[1]. Epidemiologically, up to 40% of patients with allergic rhinitis also have asthma, and up to 80% of patients with asthma experience nasal symptoms. Furthermore, patients with allergic rhinitis are at three times the risk of developing asthma compared with those without allergic rhinitis. In children who develop rhinitis within the first year of life the chances of developing asthma are twice as great as in those who develop rhinitis later in life. Again, rhinitis frequently precedes asthma, and treating allergic rhinitis has beneficial effects on asthma, suggesting that upper airway disease is a risk factor for asthma[2, 3]. This therapy usually involves antihistamines: Levocetirizine di hydrochloride(LCT), anti leukotrienes: Montelukast sodium(MLS) and bronchosecretolytic and expectorants: Ambroxol hydrochloride(AMB). Combination drug products of LCT and MLS, LCT and AMB are hence widely marketed and used in the treatment of Upper resopiratory tract diseases. Therefore the simultaneous determination of these analytes becomes motivating and significant.

Levocetirizine di hydrochloride(Fig.1a), chemically 2[2-[4-[(R)-(4-Chlorophenyl)

phenyl methyl]-1-piperazin-1-yl] ethoxy]acetic acid dihydrochloride, is a third generation non-sedative antihistamine and used in the form of Levocetirizine dihydrochloride for the treatment of allergic rhinitis and chronic idiopathic urticaria. It is an active R-enantiomer of cetirizine, orally active, potent, selective and long acting H1-histamine receptor antagonist with no anticholinergic activity [4, 5].

Montelukast sodium(Fig 1 b), chemically 2-[1-[[(1R)-1-[3-[2-(7-chloroquinolin-2-yl)ethenyl] phenyl]-3-[2-(2-hydroxypropan-2yl)phenyl]propyl]sulfanyl]methyl]cyclopropyl]aceticacid sodium salt is a selective and orally active leukotriene receptor antagonist that inhibits the cysteinyl leukotriene (CysLT$_1$) receptor in the lungs and bronchial tubes. It is used in the form of Montelukast sodium for the treatment of asthma and to relieve symptoms of seasonal allergies [6-9].

Ambroxol hydrochloride (Fig 1c), chemically trans-4-[(2-amino-3, 5-dibromophenyl-methyl) amino] cyclohexanol hydrochloride is a semi synthetic derivative of vasicine obtained from Indian shrub Adhatoda vasica.It is a metabolic product of bromhexine. It is used as bronchosecretolytic and expectorant drug in the treatment of upper respiratory tract diseases[10].

It has been demonstrated by recent studies that the treatment of asthma and allergic rhinitis with concomitant administration of an anti leukotriene (Montelukast sodium) and an antihistamine (Levocetirizine), shows significantly better symptom relief compared with the modest improvement of rhinitis symptomatically with each of the treatments alone. Levocetirizine and Ambroxol combination have been widely used clinically for their anti-allergic and expectorant properties.

Literature review reveals that some analytical methods have been reported for Levocetirizine dihydrochloride [11-14] and Montelukast sodium[15-17] individually as stability indicating and in biological fluids or in combination with other drugs in pharmaceutical dosage forms. Recently HPLC and HPTLC methods has been reported for simultaneous estimation of Levocetirizine dihydrochloride and Montelukast sodium in pharmaceutical dosage forms which are either tedious or expensive methods [18-20]

An HPLC [21] and an UV [22] method has been reported for the simultaneous determination of Levocetirizine and Ambroxol combination in tablet dosage form. Further detailed literature survey reveals analytical methods like UV, HPLC, and LC-MS [23-26] have been reported for the determination of Levocetirizine and Ambroxol individually and with other combinations.

To the best of our knowledge, currently there is no HPLC method employing optimization techniques have been reported for the simultaneous estimation of LCT dihydrochloride, AMB hydrochloride and MLS sodium. Therefore the simultaneous determination of these analytes becomes encouraging and important.

Developing and optimizing an isocratic HPLC[27, 28] method 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 Ambroxol(AMB), Montelukast(MLS) and Levocetirizine (LCT) 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.

EXPERIMENTAL
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 Ambroxol(AMB), Montelukast(MLS), Levocetirizine (LCT) and Probenecid (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 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 Montair-LC tablets (LCT-5 mg with MLS-10 mg), and Airitis plus capsules (LCT-5 mg with AMB-75 mg) were purchased from local pharmacy, chidambaram, India.

Standard solutions

Stock standard solutions of LCT, AMB and MLS (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 LCT, AMB and MLS to that of the PRO (IS) versus drug concentrations were established in the range of 0.5-5.0µg/ml for MLS, 0.25-2.5µg/ml for LCT and 3.75-37.5 µg/ml for AMB in presence of Probenecid (2.5µg/ml) as internal standard. Standard solution prepared for the optimization procedure constituted LCT, AMB, MLS and IS at 10.0, 10.0, 10.0, and 6µg/ml, respectively.

Sample preparation

Twenty tablets were weighed and finely powdered and Twenty capsules were taken and the contents were weighed. An amount of pharmaceutical products powder equivalent to 5 mg of LCT with 10 mg of MLS and 5mg of LCT with 75mg of AMB, were accurately weighed and transferred in to 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 LCT, MLS , AMB and IS as 2.5, 5.0, 37.5 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 7.0), adjusted with 10% phosphoric acid. Wavelength of 216 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 (40 ± 0.5%), the pH value (7.0 ± 0.2) and the buffer concentration (11.39 ± 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, AMB and LCT, ($Rs_{2,3}$), the retention time of the last peak, MLS, (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. Probenecid (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):

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

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].

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 16.37–52.54, 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% except for $Rs_{(2, 3)}$ (48.71). Hence, the diagnostic plots, (a) normal probability plot of residuals [46] and (b) plot of residuals versus predicted values [47] were analyzed for response $Rs_{(2, 3)}$. Since, the assumptions of normality and constant variance of the residuals were found to be satisfied, the fitted model for the $Rs_{(2, 3)}$ was accepted.

As can be seen in (Table 2), the interaction term with the largest absolute coefficients among the fitted models is AC (+ 0.528) 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 MLS 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.

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. 2a) shows that flow rate (factor C) had the most important effect on capacity factor K_1 followed by factor A and then B. (Fig. 2 b) shows that the factors A and B (MeCN concentration and buffer molarity) had significant effect on $Rs_{2,3}$ and(Fig. 2 b) shows that factors A and C (MeCN concentration and flow rate) had significant effect on tR_4. 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_4 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.

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:

$$D = [d_1^{p_1} \times d_2^{p_2} \times d_3^{p_3} \times \ldots \times d_n^{p_n}]^{\frac{1}{n}} \qquad (2)$$

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 targeted at 2 to allow baseline separation of AMB and LCT. In order to separate the first eluting peak

(PRO) from the solvent front, $k1$ 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 bar diagram 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 = 0.938) were MeCN concentration of 40%, buffer molarity of 11.39 mM and flow rate of 0.81 ml/min. The predicted response values corresponding to the latter value of D were: k_1 = 1.5, $Rs_{2,3}$ = 2.0, and tR_4 = 4.611 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).

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 PRO from the initial disturbances of plasma components. Therefore, k_1 was targeted at 2.0 and high importance value of 5 was assigned. Following the response goals above, the optimization procedure was carried out for which optimal condition II with the maximum desirability value of $D = 0.815$ was obtained. 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 average errors for K_1, $Rs_{2,3,}$ and tR_4 were 2.47, 2.00 and 2.02 % respectively, indicating good correlation between the experimental and the predicted responses[48].

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

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 0.5-5.0µg/ml for MLS, 0.25-2.5µg/ml for LCT and 3.75-37.5 µg/ml for AMB in presence of Probenecid (2.5µg/ml) as internal standard with R^2 of more than 0.998 for all the analytes. The slope and intercept of the calibration curve were 0.4021 and + 0.0086 for LCT, 0.4386 and - 0.008 for AMB, and 0.4507 and + 0.0515 for MLS respectively. Since the correlation coefficient are not good indicators of linearity performance of an analytical procedure[49] 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 0.20, 0.16, 0.23 and 0.34 ng /mL and the LOQs were 0.56, 0.47, 0.58 and 1.12 ng/mL for PRO (IS), AMB, LCT and MLS respectively. Accuracy ($n = 9$), assessed by spike recovery, were found to be 99.46, 99.25, 99.54 and 99.68% for PRO (IS), AMB, LCT and MLS respectively, which were within acceptable ranges of 100 ± 2%.[48] 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[50]. 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, the pharmaceuticals Montair-LC tablets (LCT-5 mg with MLS-10 mg), and Airitis plus capsules (LCT-5 mg with AMB-75 mg) were assayed by the proposed HPLC method. Representative chromatograms are presented in (Fig. 5). The results achieved when analyzing Montair-LC tablets were, 4.98 (0.24) mg of LCT and 10.06 (0.65) mg of MLS; and Airitis plus capsules were, 4.99 (0.44) mg of LCT and 74.96 (0.63) mg of AMB 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 and capsules 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 PRO (IS), AMB, LCT and MLS in pharmaceutical formulations (tablets and capsules). The developed HPLC method could be of immense relevance and value since in India Levocetirizine is chiefly prescribed in

combination with Montelukast and Ambroxol. 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 formulations and plasma samples.

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	30.00	10.00	0.80	2.178	1.806	10.258
2	40.00	10.00	0.80	1.482	2.972	4.712
3	30.00	20.00	0.80	2.465	5.236	11.737
4	40.00	20.00	0.80	1.637	1.513	4.933
5	30.00	10.00	1.20	1.1	1.76	6.674
6	40.00	10.00	1.20	0.653	2.568	3.12
7	30.00	20.00	1.20	1.333	4.694	7.845
8	40.00	20.00	1.20	0.756	1.261	3.277
9	26.59	15.00	1.00	2.242	8.802	14.645
10	43.41	15.00	1.00	0.978	2.341	3.327
11	35.00	6.59	1.00	1.102	2.313	5.321
12	35.00	23.41	1.00	1.336	0.00	5.505
13	35.00	15.00	0.66	2.509	0.00	8.62
14	35.00	15.00	1.34	0.73	0.00	4.234
15	35.00	15.00	1.00	1.266	0.043	5.507
16	35.00	15.00	1.00	1.303	0.00	5.566
17	35.00	15.00	1.00	1.324	0.00	5.718
18	35.00	15.00	1.00	1.266	0.043	5.507
19	35.00	15.00	1.00	1.303	0.00	5.566
20	35.00	15.00	1.00	1.324	0.00	5.718

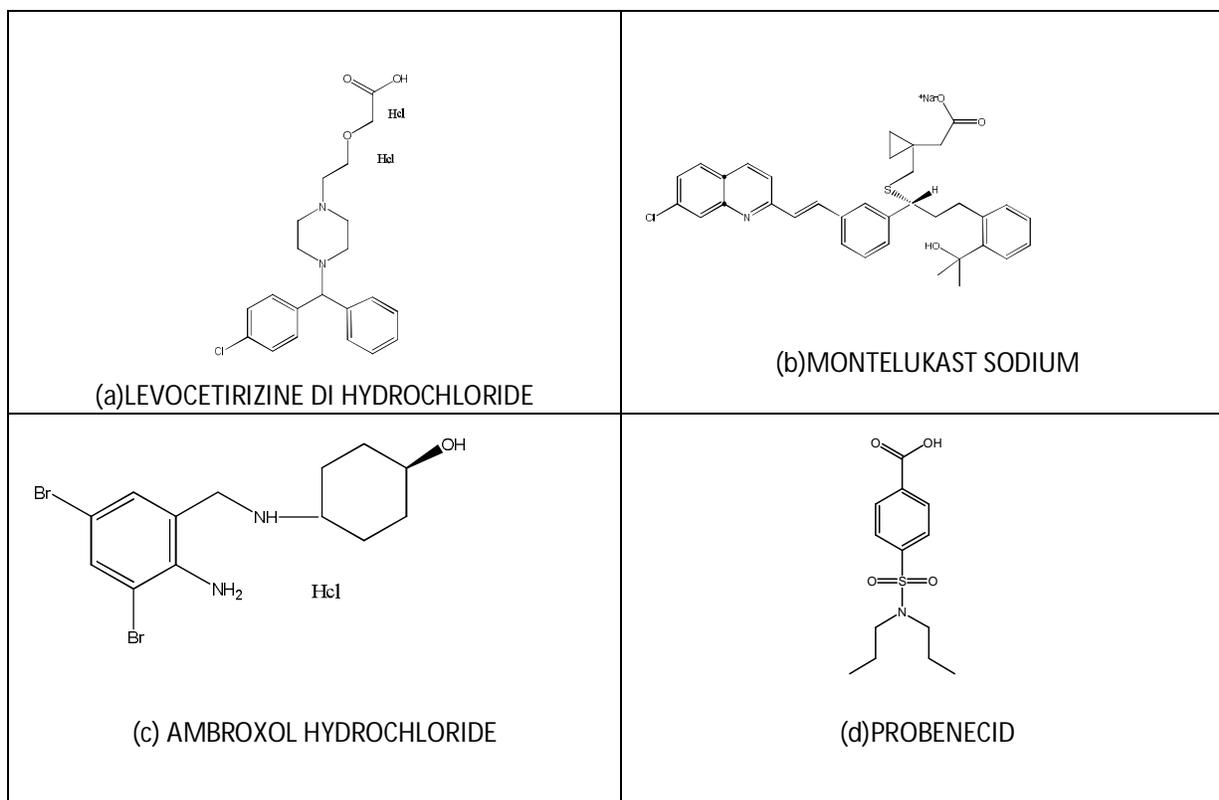**Table 2: Response models and statistical parameters obtained from ANOVA for CCD**

Responses	Regression model	Adjusted R^2	Model P value.	%C.V	Adequate precision
K_1	$+15.40-0.42A+0.061B-9.94C+0.063AC+4.05A^2-1.48B^2+2.62C^2$	0.9893	<0.0001	3.97	52.545
$Rs_{2,3}$	$+88.16-5.24A+1.03B-0.046AB+0.082A^2+0.078B^2$	0.8605	<0.0001	48.71	16.376
tR_4	$+104.87-4.19A-25.124C+0.528AC+0.044A^2$	0.9557	<0.0001	9.68	34.178

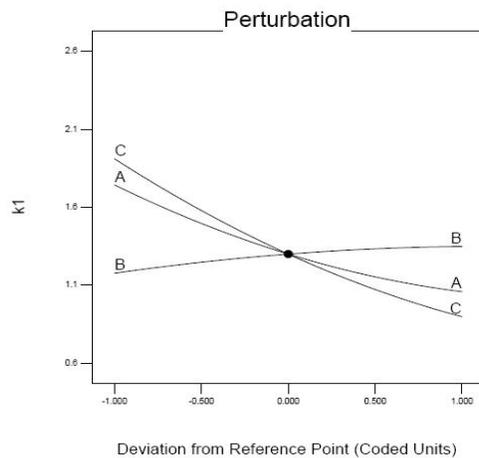**Table 3: Criteria for the optimization of individual responses**

Responses	Lower limit	Upper limit	Criteria I		Criteria II	
			Goal	Importance	Goal	Importance
K_1	0.653	2.509	Target = 1.5	3	Target = 2	5
$Rs_{2,3}$	0.00	8.802	Target = 2	3	Target = 2	3
tR_4	3.12	14.645	Minimize	5	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_5
I (For Formulations)	Desirability Value (D) = 0.939					
	40.00	11.39	0.81			
	Experimental value			1.44	2.05	4.56
	Predicted value			1.50	2.00	4.61
II (For Plasma)	Desirability Value (D) = 0.815					
	32.70	20.00	0.85			
	Experimental value			2.02	2.02	8.66
	Predicted value			2.00	1.99	8.40
	Average error			2.47	2.00	2.02

(a)LEVOCETIRIZINE DI HYDROCHLORIDE

(b)MONTELUKAST SODIUM

(c) AMBROXOL HYDROCHLORIDE

(d)PROBENECID

Fig.1: The chemical structures of analytes and internal standard (IS)
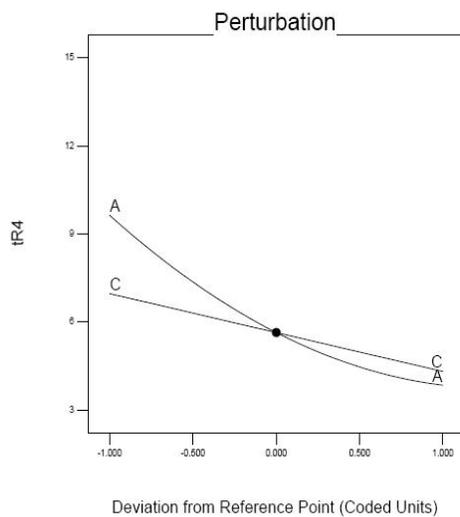
2a)

2b)
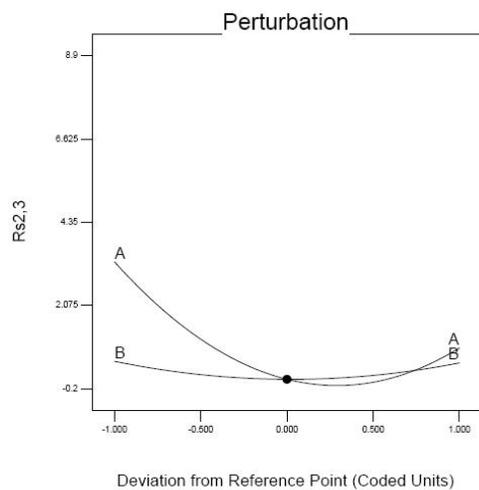
2c)

Fig. 2 : **Perturbation plots showing the effect of each of the independent variables on a) $k1$, 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**
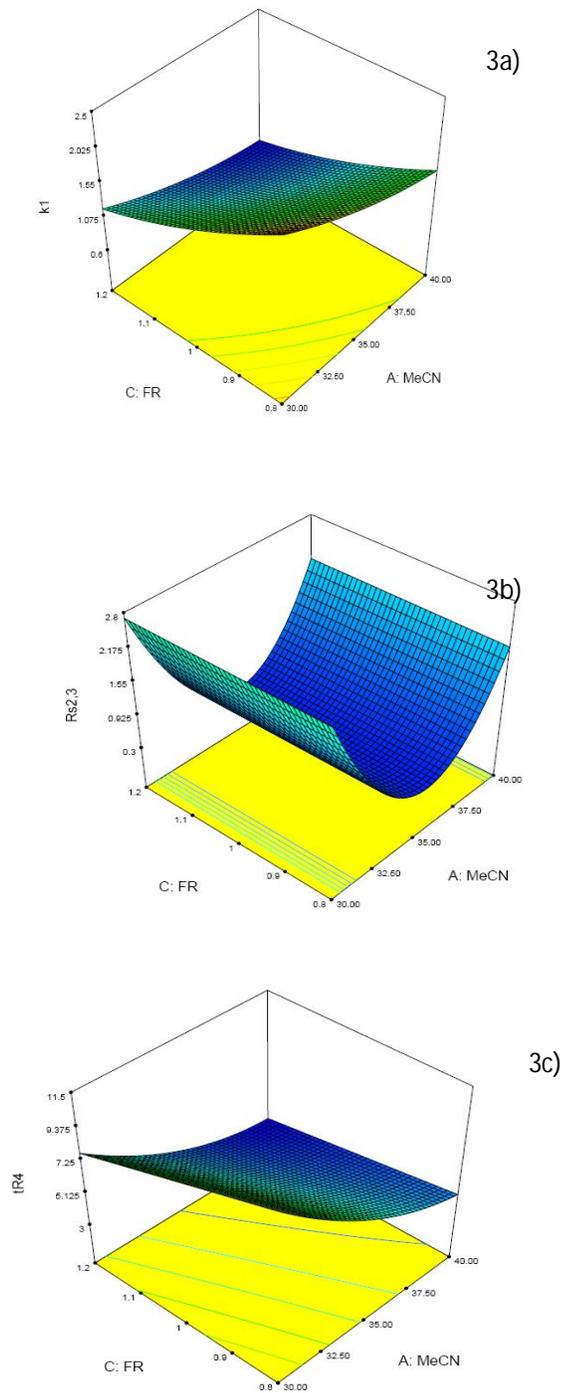
Fig. 3: Response surfaces related to percentage acetonitrile concentration (*A*) and Flow rate (*C*): (a) capacity factor of the first peak (*k*1), (b) resolution of the critical pair (*Rs*$_{2,3}$), and (*C*) retention time of the last peak (*tR*$_4$)
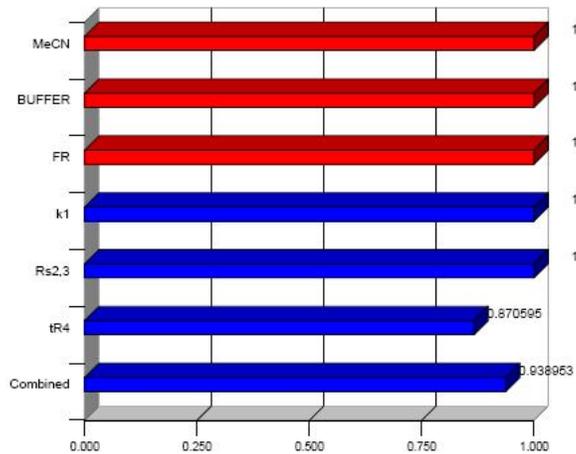
Fig. 4: Graphical representation of the overall desirability function *D*. (*D* = 0.938) were MeCN Conc. (*A*) of 40%, Buffer Molarity(*B*) of 11.39 mM, and flow rate (*C*) of 0.81 ml/min and individual desirabilities of the three responses and factors
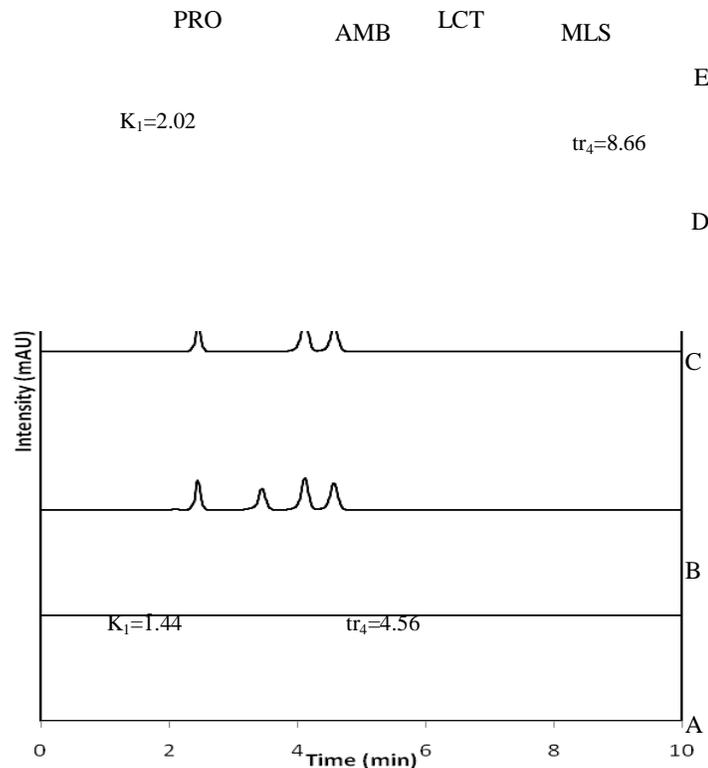

Fig. 5: Chromatograms corresponding to (A) a placebo solution; (B) a synthetic mixture of PRO-IS (7.5 µg/ml), AMB(10.01 µg/ml), LCT(9.98 µg/ml) and MLS (9.96 µg/ml); (C) a real sample of Montair-LC tablets containing PRO-IS (2.5 µg/ml) , LCT (2.48 µg/ml), and MLS (4.96 µg/ml); (D) a real sample of Airitis plus capsules containing PRO-IS (2.49 µg/ml) , LCT (2.48 µg/ml), and AMB(37.5 µg/ml); under optimum assay conditions I for formulation. (E) a synthetic mixture of PRO-IS (7.5 µg/ml), AMB(9.98 µg/ml), LCT(9.96 µg/ml) and MLS (9.99 µg/ml) under optimum assay conditions II for plasma

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