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HPLC method for the simultaneous determination of Levocetirizine, Ambroxol and Montelukast in human Plasma employing response Surface Methodology

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Abstract

Multiple response simultaneous optimizations employing the Derringer's desirability function for the development of reversed-phase HPLC methods for the simultaneous determination of Ambroxol(AMB) and Montelukast(MLS) with Levocetirizine (LCT) and Probenecid(PRO) as Internal standard in human plasma samples is described. 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, 20.00 mM K_2HPO_4 (pH 7.0 ± 0.5) solution (32.7:30: 37.3 v/v/v) and flow rate 0.85 ml/min. The eluate was monitored using an UV detector set at 230 nm. Peak area ratio of the analyte and internal standard was used for the quantification of plasma samples. Total chromatographic analysis time per sample was approximately 8.665 min. The validation of the proposed analytical method was conducted in accordance to the recommendations of the guidelines "Bioanalytical method validation" [FDA-CDER, 2001]. The method was found to be simple, sensitive and hence it could be applied in bioavailability studies.

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Key words:
Central composite design, Derringer's desirability function, Response surface methodology, HPLC, Ambroxol, Montelukast, and Levocetirizine.

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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 respiratory 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]aceti cacid 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].

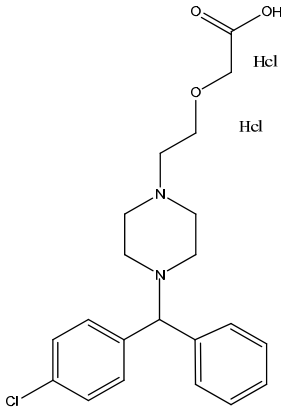
It has been demonstrated by recent studies that the treatment of asthma and allergic rhinitis with concomitant administration of an anti leukotriene (Montelukast sodium) and an antihistamine (Levocetirizine), shows significantly better symptom relief compared with the modest improvement of rhinitis symptomatically 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]

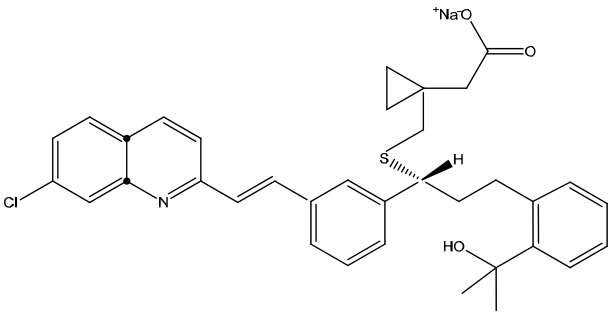
An HPLC [21] and an UV [22] method has been reported for the simultaneous determination of Levocetirizine and Ambroxol combination in tablet dosage form. Further detailed literature survey reveals analytical methods like UV, HPLC, and LC-

MS [23-26] have been reported for the determination of Levocetirizine and Ambroxol individually and with other combinations.

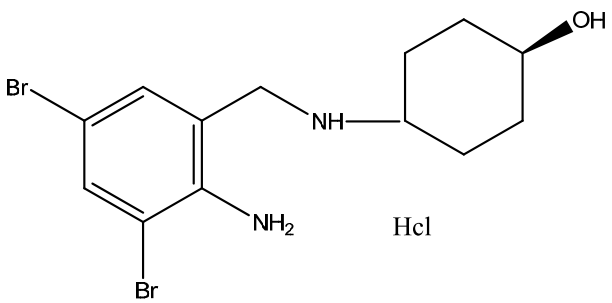
To the best of our knowledge, currently there is no HPLC method employing optimization techniques have been reported for the simultaneous estimation of LCT dihydrochloride, AMB hydrochloride and MLS sodium in human plasma. Therefore the simultaneous determination of these analytes becomes encouraging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



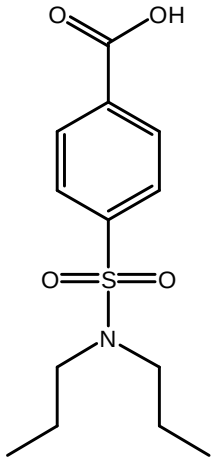
(a)LEVOCETIRIZINE DI HYDROCHLORIDE



(b)MONTELUKAST SODIUM



©AMBROXOL HYDROCHLORIDE



(d)PROBENECID

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

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 human plasma 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 validation of the proposed analytical method was conducted in accordance to the recommendations of the guidelines "Bioanalytical method validation" [FDA-CDER, 2001].

MATERIAL 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 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 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 drug free human plasma was purchased from blood bank of Rotary Central TTK VHS (Chennai, India).

Stock and working 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.

Extraction procedure for plasma sample

The 1ml blank plasma in a glass-stoppered 15 ml centrifuge tube were spiked with the working solutions of AMB, MLS, LCT and IS to achieve a concentration of 250 ng ml each. The samples were then alkalinized by addition of 100 µl of 3M KOH, vortex-mixed for 30 seconds and a certain volume of extraction solvent was added. The mixture was gently shaked for 5 min and centrifuging on a laboratory centrifuge (Remi®, R&C, Remi Equipment, Mumbai, India) at 3500 rpm (1878 × g) for 5 min. The supernatant organic layer was transferred to eppendorf tubes and the contents were evaporated to dryness under vacuum at 60°C using an Eppendorf concentrator. The residue was reconstituted in 100 µl of mobile phase and vortex mixed for 30 seconds. [38] Aliquots of 20 µl were injected into the chromatographic system. The same procedure was carried out for blank plasma samples to check the cleanness of the extracts. To assess the efficiency of the extraction procedure, the spiked plasma sample was extracted according to the above procedure, but the addition of IS after extraction. The percentage recovery was estimated by comparing the peak areas of each analyte spiked sample with that from the blank plasma sample to which the drug was added previous the evaporation step.

$$\% \text{ Recovery} = \frac{\text{E (spike)/IS}}{\text{E(non spike)/IS}} \times 100$$

Where, E (spike) is the area of the each analyte in spiked plasma sample; E (non spike) is the area of each analyte obtained by addition of the drug previous to the evaporation step.

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 230 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

The plasma assay method was validated in accordance to the recommendations of the guidelines "Bioanalytical method validation" [FDA-CDER, 2001].

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[39]. 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[40]. 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):

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

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 [41].

Table 1: Central composite rotatable design arrangement and responses[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

[a] Randomized

Table 2: Reduced response models[a] and statistical parameters obtained from ANOVA for CCD

Responses	Regression modle	Adjusted R^2	Model P value.	%C.V	Adequate precision
K_1	+1.30-0.34A+0.086B-0.51C +0.063AC+01A^2-0.037B^2+0.10C^2	0.9893	<0.0001	3.97	52.545
$Rs_{2,3}$	+0.059-1.18A-0.021B-1.14AB+2.03A^2+0.47 B^2	0.8605	<0.0001	48.71	16.376
tR_4	+5.64- 2.89A-1.33C+0.53AC+1.10 A^2	0.9557	<0.0001	9.68	34.178

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

Table 3: Criteria for the optimization of the individual responses

Responses	Lower limit	Upper limit	Criteria I	
			Goal	Importance
K_1	0.653	2.509	Target= 2	5
$Rs_{2,3}$	0.00	8.802	Target = 2	3
tR_4	3.12	14.645	Minimize	4

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
For Human Plasma	Desirability Value (D) = 0.815					
	32.7	20.00	0.85			
	Experimental value			1.99	2.02	8.241
	Predicted value			2.00	1.99	8.405
	Average error			0.5	1.40	1.961

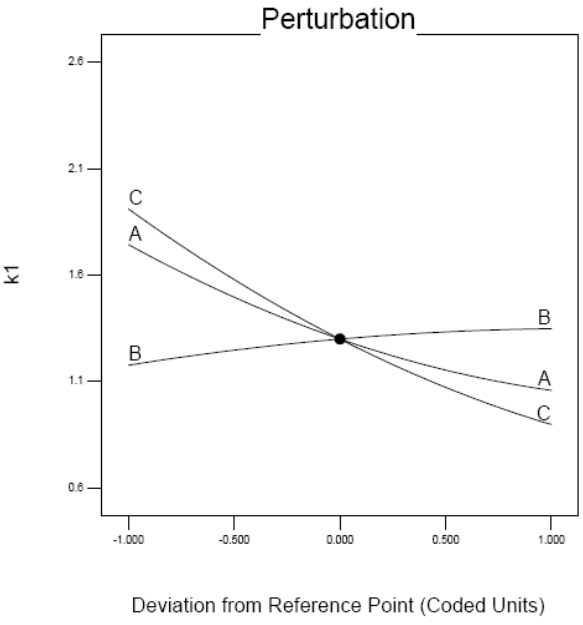
In the present study, the adjusted R^2 were well within the acceptable limits of $R^2 \geq 0.8605$[42] which revealed that the experimental data shows a good fit with the second-order polynomial equations. For all the reduced models, P value of < 0.05 is obtained, implying these models are significant. The adequate precision value is a measure of the signal (response) to noise (deviation) ratio". A ratio greater than 4 is desirable[43]. 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% [43]. 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[44] and (b) plot of residuals versus predicted values[45] 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 (+ 0.53) 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.



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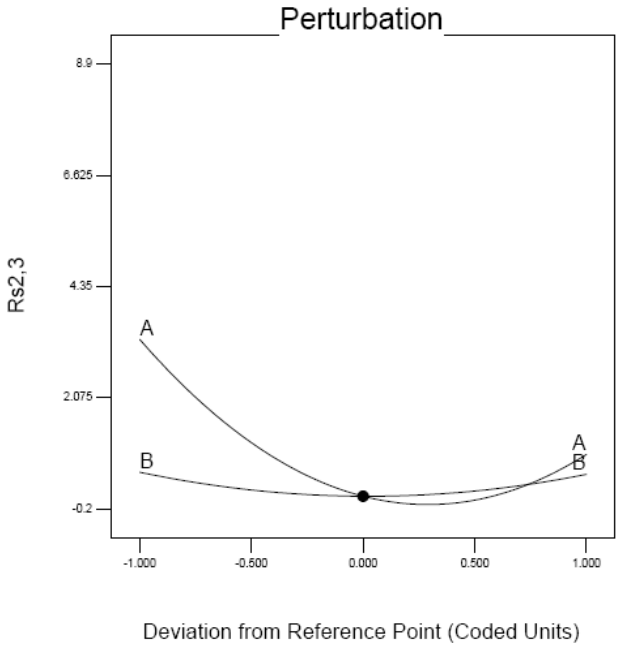

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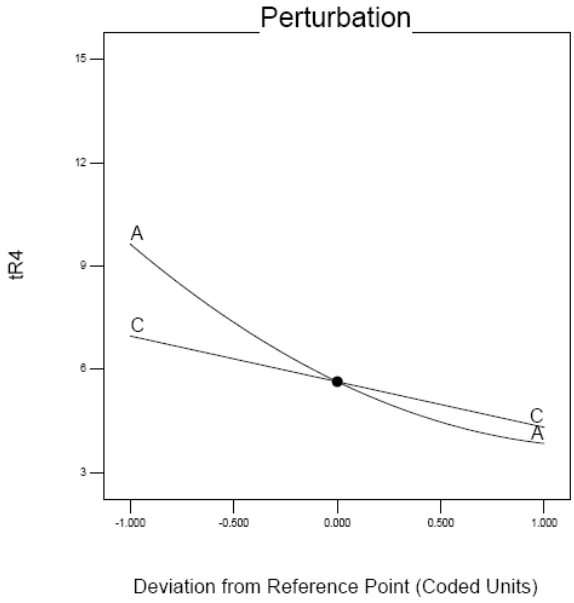
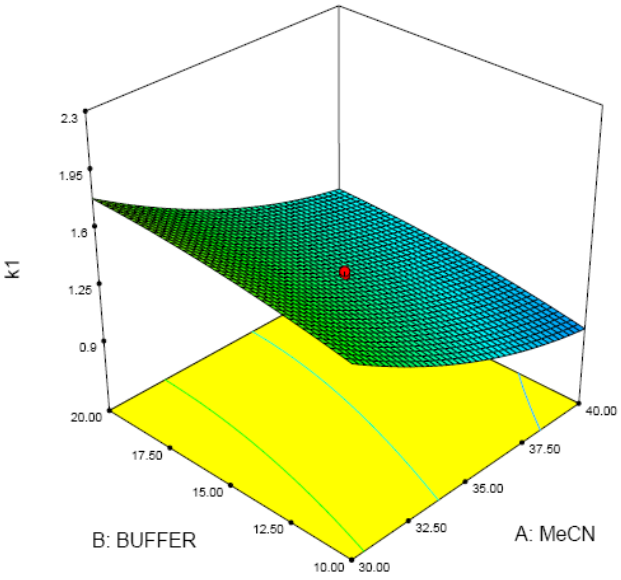


2 (c)

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.



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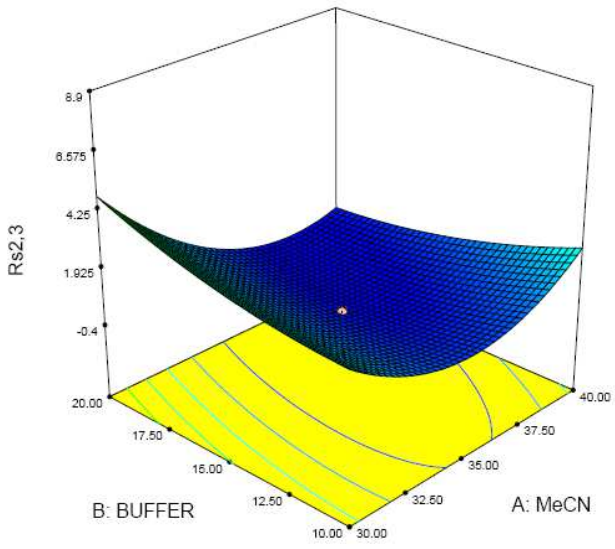
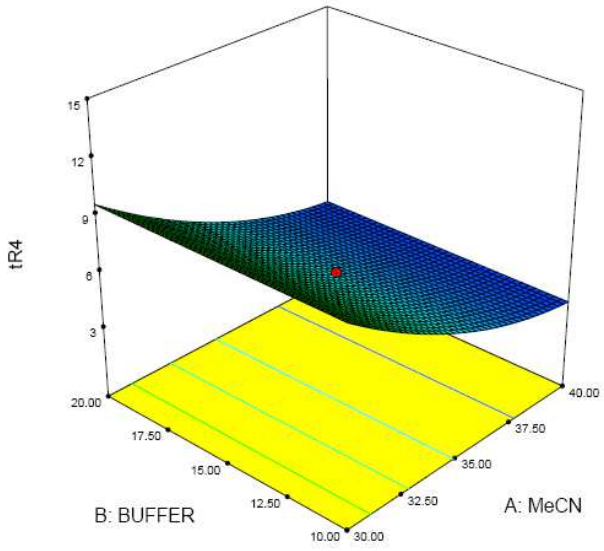


3 (b)



3 (c)

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 four 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:

$$D = [d_1^{p_1} \times d_2^{p_2} \times d_3^{p_3} \times \ldots \times d_n^{p_n}]^{\frac{1}{n}} \quad (3)$$

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 four responses. A value of *D* close to 1, indicates that the combination of the different criteria is matched in a global optimum [24].The criteria for the optimization of each individual response are shown in (Table 3). Optimum condition for analyzing the plasma samples, Criteria were established by varying the response goals and their importance values. For instance, larger value of k_1 has to be selected for the separation of Probenecid from the initial disturbance of plasma components. There, k_1 was targeted at 2.0 and high importance value of 5 was assigned. Following the conditions and restrictions above, the optimization was carried out. The function is maximized at an overall desirability of about D = 0.815, is presented in (Fig. 4) which provides an optimum condition for the analysis of plasma samples. The predicted response values corresponding to the latter value of *D* were: k_1 = 2.00, $Rs_{2,3}$ = 1.99, and tR_4 = 8.405 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).The observed difference between the predicted and experimental responses are found to be in good agreement, within a difference of 2.0% is shown in (Table 4). This approach offers flexibility to the chromatographer to slide k_1 values depending upon of the analyte under consideration.

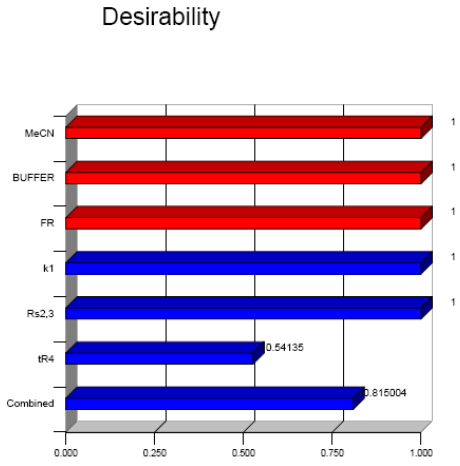


Fig. 4: Graphical representation of the overall desirability function *D*. (*D* = 0.815) were MeCN Conc. (*A*) of 32.7%, Buffer Molarity(*B*) of 20 mM, and flow rate (*C*) of 0.85 ml/min and individual desirabilities of the three responses and factors.

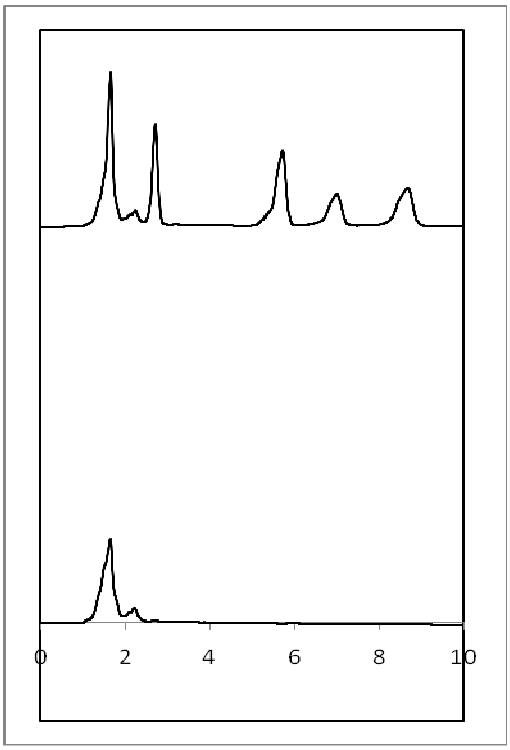


Fig. 5: Chromatograms of Ambroxol (AMB), Levocetirizine (LCT), Montelukast (MLS), and Probenecid (IS) obtained under optimal separation and extraction conditions. (A) extract of human blank plasma (B) spiked plasma sample with 250 ng/ml each of the analyte.

Validation of plasma assay method

Linearity was established at six levels in the range of 10, 25, 50, 250, 500, 1000 ng mL^{-1} for AMB, LCT and MLS. Typically, the mean (n = 6) regression equations were: y = 0.006 x – 0.030 for AMB with R^2 more than 0.998, y = 0.002 x – 0.010 for LCT with R^2 more than 0.996, and y = 0.004 x + 0.016 for MLS with R^2 more than 0.999 for the analytes. The LOQ values for AMB, LCT and MLS were 6.45 ng ml, 8.7ng ml, and 9.45 ng ml respectively. In the optimized chromatographic and extraction conditions, specificity was indicated by the absence of any endogenous interference from plasma matrix at retention times of AMB, LCT, MLS and IS peaks (Fig. 5). Accuracy and precision was determined by replicate analysis (n = 6) of 3 concentration levels of

each analyte (25, 250 and 1000 ng ml). The accuracy and precision were well within the acceptance criterion of ±15%. Stability of AMB, LCT, MLS in the spiked plasma samples was examined by replicate analysis (n = 6) at three concentration levels: 25, 250 and 1000 ng ml. The stability of analytes and the IS stock solution in MeCN (250 ng ml each) was also checked over a 12 h period, at 3 h sampling interval. The percentage responses for the aged solutions were calculated using freshly prepared solutions. The results shows that sample and standard solutions of analytes and IS were stable for 12 h, as during this time the result does not decrease below the minimum percentage (95%).

CONCLUSION

In this study, an isocratic RP-HPLC-UV method for the simultaneous determination of AMB, LCT and MLS in human plasma samples was developed and optimized. Time of analysis and resolution were simultaneously optimized by applying chemometrics tools: CCD and Derringer's desirability function. The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determination of drugs in plasma samples. Total chromatographic analysis time per sample was approximately 8.665 min. The validation study supported the selection of the assay conditions by confirming that the assay was specific, accurate, linear, precise, and robust. The method was found to be simple, sensitive and can be applied successfully in routine analysis for the estimation of AMB, LCT and MLS in biological samples.

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