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Simultaneous Optimization of the Resolution and Analysis time in RP-HPLC Method for Abacavir and Lamivudine using Derringer's desirability function

T.Sudha[1]*, P.Shanmugasundram[2]

[1]*Research Scholar, Vel's University, Department of Pharmaceutical Analysis, The Erode College of Pharmacy, Erode, India.
[2]Department of pharmaceutical Analysis, School of pharmaceutical Sciences, Vel's University, Chennai,India.

***Corres.author: jvchrsty@yahoo.co.in**
Mobile: 9362857380

Abstract: A High performance liquid chromatographic method has been developed and optimized for antiretroviral drugs (Abacavir and Lamivudine). Multiple response simultaneous optimization using the Derringer's desirability function was employed for the development of RP-HPLC. The possibilities of the simultaneous drug analysis allow a decrease of time during the assay and save reagents and solvents. The ranges of independent variables used for the optimization were MeOH (65-75%v/v), pH (6.0 -7.0), flow rate (0.8 -1.2 ml/min). The influence of these variables on the output responses such as capacity factors of the first peak (k_1), resolutions ($Rs_{1,2}$) and retention time (tR_2)were evaluated. The experimental responses were fitted into a second order poly nominal and the three responses were simultaneously optimized to predict the optimum conditions for the effective separation of the studied components. Optimum conditions chosen for assay were MeoH: phosphate buffer (74.3:25.7%v/v) (pH 6.85, buffer strength 0.05M) and flow rate of 1.2 ml/min.The eluate was monitored using an UV detector set at 260 nm. Total chromatographic analysis time was approximately 5.0 min. The optimized assay condition was validated as per International Conference on Harmonization guidelines to confirm specificity, linearity, accuracy, limit of detection, limit of quantification and precision.
Key words: Central composite design, Derringer's desirability function, Abacavir, Lamivudine, RP-HPLC.

Introduction

Abacavir[1] (Aba)(fig 1a) is a Nucleoside Reverse Transcriptase Inhibitors (NRTI) with activity against human immunodeficiency Virus type-I (HIV-I). It is in combination with Lamivudine[2](Lam) (fig1b). It is also a Nucleoside Reverse Transcriptase Inhibitor (NRTI)with activity against human deficiency virus type-I and Hepatitis B. The drugs individually, as well as in multicomponent dosage form are available in the market. A number of methods have been published for the estimation of above said analytes. There are spectrophotometric estimation of Abacavirsulphate[3], spectrophotometric estimation of lamivudine[4], Lamivudine in human plasma by RP-HPLC[5],titrimetric and spectrophotometric estimation of lamivudine[6]. Simultaneous analysis of abacavir and lamivudine in human plasma by LC-MS/MS[7]was reported. Determination of abacavir, lamivudineand Zidovudine in pharmaceutical tablets human serum and in drug dissolution studies by HPLC [8] were also reported in the literature. To the best of our knowledge a chemometric approach for development and validation of HPLC method for simultaneous estimation of Aba and Lam has not been reported.

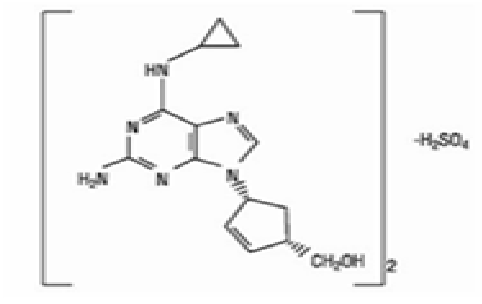

Figure 1a: Structure for Abacavir

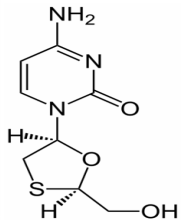

Figure 1b: structure for Lamivudine

Optimization strategies are followed when attempting to optimize, for instance a formulation, product, process or an analytical method, e.g. a chromatographic method to separate components in a given matrix[9].In an optimization, one tries to find the optimal settings or conditions for a number of factors. Factors are parameters that can be set and reset at given levels e.g. temperature, pH, reagents concentration, reaction time etc and that affect the responses or the outcome of a method or procedure. The factors and their level rangesfrom the experimental domain within which one tries to find the global optimum, i.e. the overall best conditions. Factors also might interact, for instance, a two –factor interaction occurs when the influence of one factor on the response is different at different levels of the second factor. In case only one factor needs to be optimized, a simple univariate is performed. However, usually two or more factors are studied. This can be done using multivariate optimization strategies[10,11]. The multivariate statistical methods mostly used in chromatography and indeed in chemistry in general can be conveniently classified according to how one decides experiments are to be executed. All methods require the user to supply minimum and maximum values for each factor that defines the experimental domain to be investigated during the optimization procedure. In optimization procedure, Response Surface Methodology (RSM) is most commonly used. RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing process. It also has important applications in the design, development and formulation of new product, as well as in the improvement of existing product design[12,13] Response Surface Methodology also quantifies the relationship between the controllable input parameters and the obtained response surfaces. The designprocedure of Response Surface Methodology is as follows: 1. Designing of a series of experiments for adequate and reliable measurements of the response of interest. 2. Developing a mathematical model to the second order response surface with the best fittings.3.Finding the optimal set of experimental parameter that produce a minimum or maximum value of response. 4. Representing the direct and interactive effects of process parameter through two and three dimensional plots[14].

HPLC utilizes a wide selection of chromatographic factors like, the type and concentration of organic modifier, pH, buffer molarity, temperature and flow rate etc. Optimization of the experimental conditions is a complicated process[15,16]. Therefore, a systematic approach such as experimental design to optimize chromatographic separation is more essential. However in the HPLC method intended to be applied for the pharmaceutical or industrial environment, the analysis time is essentially optimized without losing resolution [17]. When one needs to optimize more than one response at a time, the Multiple Criteria Decision Making (MCDM), a chemometric technique, is employed. Some of these criteria critically evaluate the chromatographic response function, chromatographic optimization function, the informing power, the separation number and the product resolution [18]. The different approaches of MCDM include the path of steepest ascent, constrained optimization procedure, Pareto-optimality, utility function, Derringer's desirability function. The desirability function approach is one of the most frequently used multi response optimization techniques in practice. There are many ways in which the individual desirability can be combined. The total desirability is defined as geometry mean of the individual desirability. The advantage of the desirability function is that one of the criteria has an unacceptable value. Then the overall product will also be unacceptable, while for utility functions, this is not the case. Further, Derringer's method offers the user flexibility in the definition of desirability function.Derringer's desirability function was introduced in chromatography by Derringer implementing resolution and analysis time as objective functions as they improved separation quality. The Derringer's desirability function was applied to explore the user flexibility of this technique in selecting optimum chromatographic conditions for the determination of drugsin a variety of sample matrices. This analysis included calculating case statistics to identify outlines and examining diagnostic plots such as 1. normal probability plots 2. Residual plots. Maximization and minimization of the second order poly nominal

was fitted usually performed by desirability function method and mapping of the fitted response was achieved using computersoftware such as Design Expert [19,20]

Experimental

Chemicals and Reagents

HPLC grade of methanol was procured from Merck, Mumbai, India. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate and phosphoric acid were obtained from SD fine chemicals, Mumbai, India. The HPLC grade water was prepared by using Milli-Q Acedamic, Millipore, Bangalore, India.Pure standard of lamivudine was donated by M/S pharma Train, Hyderabad, India. Abacavir pure standard was gifted by Hetero labs, India. Tablet formulation Abamune-L (300 mg of Lam and 600 mg of Aba) was purchased from local pharmacy

Chromatographic conditions

HPLC was performed with Shimadzu prominence equipment comprising LC20AD solvent delivery modules, SPD 20A UV-visible detector, a Rheodyne model 7125 injection valve fitted with a 20µl loop, and SPD-20A detector. Compounds were separated on a 250 mm X 4.6 mm i.d., 5µm particle, phenomenex, Gemini C_{18} column and a personal computer. The equipment was situated in an air conditioned laboratory (20±2˚C). The chromatographic software Autochro 3000 (Shimadzu) was used for data acquisition and treatment of chromatographic data.The wave length was selected by scanning working standard solution of both investigated compounds over 200 to 400nm. All measurements were made with 20µl injection volume and UV detection at 260nm because both components showed reasonable good response at this wavelength.

Preparation of Standard solution

25mg of Aba and 25mg of Lam were separately weighed accurately and transferred into a 25ml volumetric flask and dissolved with methanol, then diluted to make up with the same, (1mg/ml). From this 2.5ml of the solution was transferred into 50ml standard flask and made up to the volume with mobilephase (50µg/ml).

Preparation of sample solution

Marketed formulation Abamune – L contains (300mg of Lam and 600mg of Aba). Twenty tablets were weighed accurately; the average mass per tablet was determined and finely powdered. The powder equivalent to 25 mg of each was accurately weighed and a minimum quantity of methanol was added to dissolve the substance. The total volume was brought upto25ml with more methanol (1000µg/ml) in a volumetric flask. The solutions were sonicated for 10 min. and then filtered through Whatmann filter paper no: 41. Insoluble excipients were separated out. The filtrate was collected after rejecting the first portion of the filtrate. 2.5ml of the clear solution was further diluted and madeupto 50ml with mobile phase to obtain 50µg/ml. Further dilution was made by diluting 3ml to 10ml with mobile phase to obtain 6µg/ml. 20µl of each solution was injected and the chromatogram was recorded. The analysis was repeated for six times. The content of the drug was calculated from the peak area recorded.

Result and Discussions

Optimization of Design and Analysis

In order to understand the selectivity of the chromatographic factors such as the separation of analytes, simultaneous optimization of resolution and retention time, chemometric protocol of response surface design and Derringer's desirability function were successfully employed. The central composite design can be applied to optimize the separation and to assist the development of better understanding of the interaction of several chromatographic factors on separation quality. In this work, the important chromatographic factors were selected and optimized by a central composite design experiment. Factors selected and optimized were based on preliminary experiments and prior knowledge from the literature. The factors selected for optimization process were MeoH concentration (A), buffer pH (B) and flow rate (C). The ranges of factors used were MeoH concentration (65– 75), buffer pH (6.0 – 7.0) and flow rate (0.8 – 1.2 ml/min).The levels of each factor studied for finding out the optimum values and responses were shown in table-.1. The capacity factor for the first eluted peak LAM, (k_1), the resolution of the lam and aba peak ($Rs_{1,2}$)and the retention time of last peak (Rt_2)were

selected as responses. 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. For an experimental design with three factors, the model including linear, quadratic and cross terms can be expressed as -

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

Where Y is the response to be modeled, β is the regression coefficients and X_1, X_2, 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[21]. The adjusted R^2values were well within the acceptable limits of $R^2 \geq 0.80$[22], which revealed that the experimental data shows a good fit with 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[23]. The ratio was found to be in the range of 7.07 – 13.467 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%.The C.V. for all models was found to be less than 10%, except for *Rs* (11.55%). Hence, the diagnostic plots, (1). Normal probability plot of residuals [24] and (2) Plot of residuals versus predicted values[25] were analysed for response *Rs.* The normal probability plot (fig 2a) indicates whether the residuals follow a normal distribution, in which case the points will follow a straight line. In fig 2a the points on this plot lies fairly close to the straight line, so the model seems appropriate. The plot of residuals versus predicted values fig 2b is a measure of how many standard deviationsthe actual value deviates from the value predicted. From this plot, it is possible to conclude that they were randomly distributed around zero and there is no evidence of outliers (no point lies away from the mean more than three times standard deviation). Since the assumptions of normality and constant variance of the residuals were found to be satisfied, the fitted model for the *Rs* was accepted. In table -2 the interaction with the largest absolute coefficients among the fitted model is AB(+0.023) of K_1model. The positive interaction between A and B is statistically significant (< 0.0001) for K_1. The study reveals that changing the fraction of MeoH from low to high results in a rapid decline in the retention time of LAM both at the low and high level of buffer pH. Further at low level of factor *A*, an increase in the buffer pH results in a marginal decrease in the retention time. This may be due to reduced silanol effects as a result of higher buffer pH used. Therefore, when the MeoH concentration is set at its lowest level, the buffer pH has to be at its highest level to shorten the run time. The existence of such interactions emphasizes the necessity to carry out active multifactor experiments for the optimization of chromatographic separation. In order to gain a better understanding of the results, the predicted models are presented in the form of perturbation plot (3a,3b,3c) and 3D response surface plots (fig 4a,4b and 4c). Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. Perturbation plot provides silhouette, views of the response surface plots where it shows how the response changes as each factor moves from a chosen reference point, with all factors held constant at the reference value. A steepest slope or curvature indicates the sensitiveness of the response to a specific factor. Figure 3c shows that MeoH concentration (factor A) had most important effect on retention time (tR_2) following by factor C. The rest of the factors had significant effect on K_1 and $Rs_{1,2}$. In figure 3a showsK_1 values increased as the level of flow rate increased and K_1values decreased as the level of MeoH concentration increased. The value of resolution ($Rs_{1,2}$) increased with increasing levels of A and B. Analysis of the perturbation plots and response plots of optimization models revealed that factor A,B and C had the significant effect on the separation of the analytes. Derringer's desirability function was employed for global optimization of three responses and to select different optimal conditions for the analysis of formulation.in the present study. The identified criteria for the optimization were: resolution between the peaks, capacity factor and elution time. 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:

$$D = [d_1^{p2} x\ d_2^{p2} x\ d_3^{p2} x \ldots\ldots\ldots x\ d_n^{pn}]^{1/n}$$

Where pi is the weight of the response, n the number of responses and di is the individual desirability function of each response. Desirabilityfunction (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. The criteria for the optimization of each individual response are shown in (Table 3). In criteria, the

responses tR_2 was minimized in order to shorten the analysis time and $Rs_{1,2}$ was maximized to separate the LAM and Aba. In order to separate the first eluting peak (LAM) from the solvent front k_1 was maximized. 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 5. From the figure it can be concluded that there was set of coordinates producing high desirability value (D= 0.728) were MeoH concentration of 74.3%, buffer pH of 6.8and flow rate of 1.2ml/min. The optimized assay conditions were MeoH: phosphate buffer (74.3:25.7%v/v) (pH 6.85, buffer strength 0.05M) as mobile phase at a flow rate of 1.2 ml/min. and UV detection at 260nm. The predicted response values corresponding to the later value of D were K1=1.02, Rs1,2 = 3.74 and tR2 = 3.5min.The prediction efficiency of the model was confirmed by performing the experiment under the optimal condition and the corresponding chromatogram was shown in figure6. The observed difference between the predicted and experimental responses are found to be in good agreement, within a difference of2.0% is shown in table –4.

Table-1: Central composite arrangement and responses

Run	Factor levels			Responses		
	Methanol concentration (A%v/v)	**Buffer pH (B)**	**Flow rate (C ml/min)**	**k_1**	**$Rs_{1,2}$**	**tR_2**
1	70.00	5.66	1.00	1.00	3.3	3.55
2	78.41	6.50	1.00	0.94	5.41	4.50
3	70.00	7.34	1.00	0.95	1.13	5.46
4	65.00	7.00	0.80	1.00	6.00	6.26
5	75.00	7.00	1.20	1.07	4.57	3.66
6	75.00	6.00	1.20	1.00	4.00	3.51
7	65.00	6.00	1.20	1.00	5.88	4.30
8	70.00	6.50	0.66	1.09	5.91	7.01
9	61.59	6.50	1.00	0.96	6.60	8.38
10	75.00	6.00	0.80	1.05	5.05	5.46
11	70.00	6.50	1.34	1.00	4.93	3.66
12	65.00	6.00	0.80	1.11	4.36	5.31
13	65.00	7.00	1.20	1.00	6.56	4.56
14	75.00	7.00	0.80	1.05	0.51	5.41
15	70.00	6.50	1.00	1.05	5.70	4.65
16	70.00	6.50	1.00	1.05	5.70	4.65
17	70.00	6.50	1.00	1.05	5.70	4.65
18	70.00	6.50	1.00	1.05	5.70	4.65
19	70.00	6.50	1.00	1.05	5.70	4.65
20	70.00	6.50	1.00	1.05	5.70	4.65

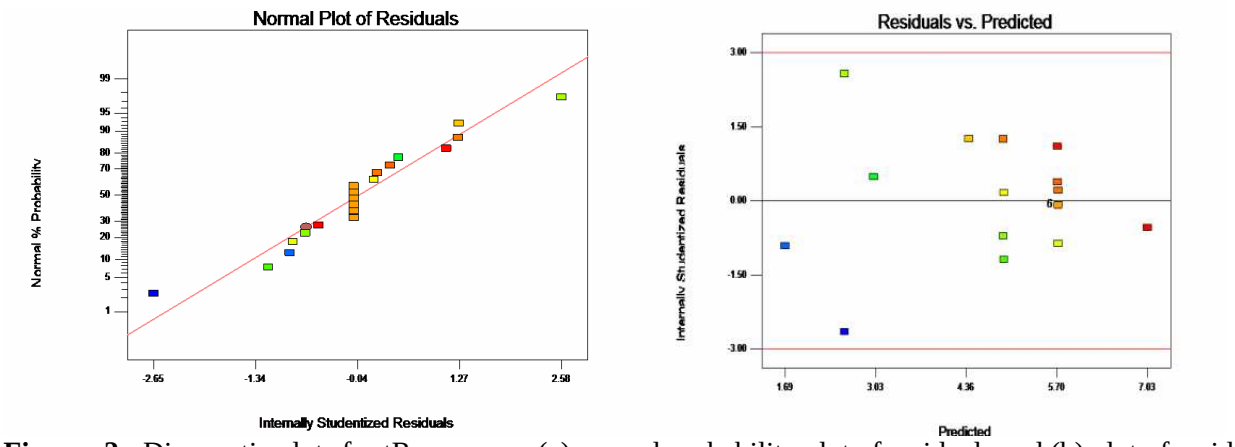

Figure -2: Diagnostic plots for tR_2 response (a) normal probability plot of residuals and (b) plot of residuals versus predicted values

Table -2: Reduced 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	+1.06+1.93A-9.08B-0.021C+0.023AB+0.020BC-0.026A^2-0.017B^2	0.9646	<0.0001	2.92	7.07
$Rs_{1,2}$	+5.72-0.78A-0.39AB-1.19B^2	0.9565	<0.0001	10.33	11.26
tR_2	+4.59-0.65A-0.88C+0.15A^2	0.9606	<0.0001	11.52	13.46

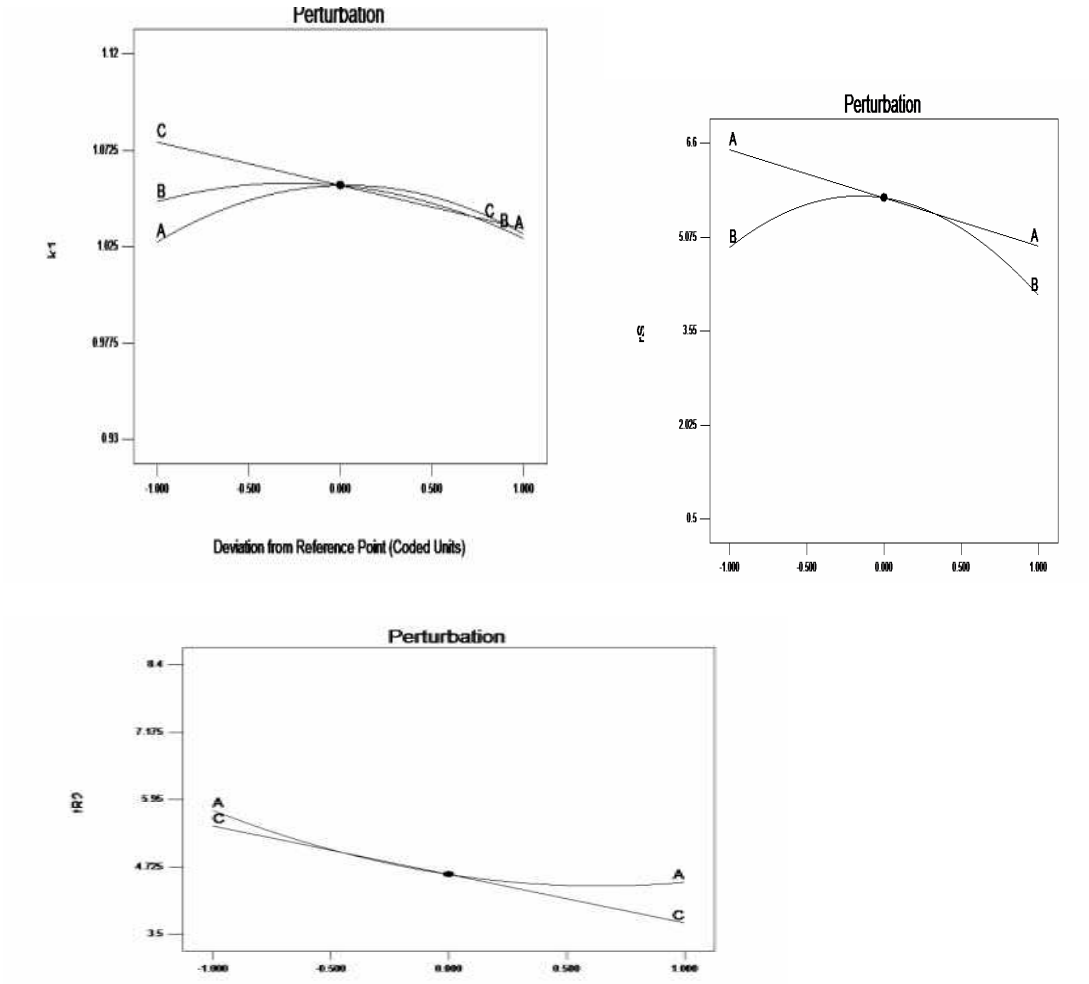

Figure-3: Perturbation plots for three responses (a) K_1, (b) $Rs_{1,2}$ and (c) tR_2

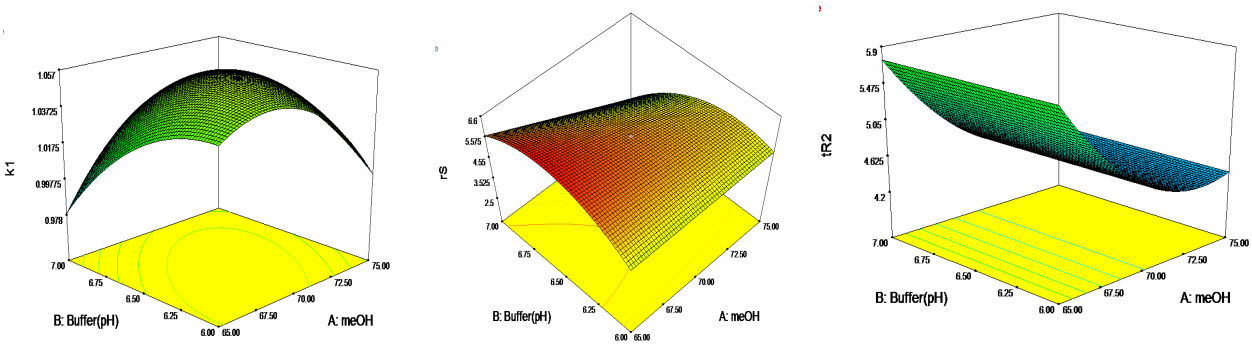

Figure-4: Response Surface plots for the responses (a) K_1, (b) $Rs_{1,2}$ and (c) tR_2

Table- 3: Criteria for the Optimization of the Individual Responses

Response	Lower limit	Upper limit	Criteria/Goal
k_1	0.94	1.11	Maximize
$Rs_{1,2}$	2.0	4.0	Maximize
tR_2	3.51	8.38	Minimize

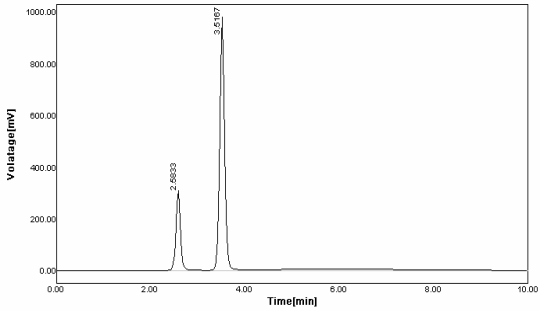

Figure-5: Optimized chromatogram for Lamivudine and abacavir

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

Optimum conditions	Methanol (%v/v)	Buffer (pH)	Flow rate (ml/min)	K_1	$Rs_{1,2}$	tR_2
Predictive	74.3	6.85	1.2	1.02	3.74	3.55
Experimental	74.3	6.85	1.2	1.0	3.72	3.51
Average error				1.96	0.53	1.12
Desirability Value (D)=0.728						

Method Validation

The proposed method was validated as per ICH guidelines[26,27]

Linearity

The linearity of analytical method is ability to elicit test results that are directly proportional to the analyte concentration in samples within a given range.Working stock solutions were prepared by diluting the stock solution with mobile phase to obtain concentration from 2-12µg/ml of both drugs (Aba & LAM).The solutions were injected and the chromatograms were recorded at 260 nm. It was found that the above concentration range was linear with the concentration range of 2-12 µg/ml. The reports were shown in table- 5.

Table -5: Method validation Parameters

Parameters	Lamivudine	Abacavir
Range(μgmL^{-1})	2-12	2-12
Y=mx + c	Y= 5874.19 X 1972.36	Y = 9809.32 X + 630.49
Correlation coefficient	0.9993	0.9997
Slope (m)	5874.19	9809.32
Intercept (c)	1972.36	630.49
LOD (μgmL^{-1})	0.0589	0.0012
LOQ(μgmL^{-1})	0.0204	0.0115
Accuracy (%)	99.13	100.41
Precision (%RSD)	0.57	1.05
Analyst I (%RSD)	1.367	1.335
Analyst II (%RSD)	1.262	1.286

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. Precision is usually investigated at three levels: repeatability, intermediate precision, and reproducibility. Precision study was done with the Lam and aba standards. 6 µg/ml solutions of both Lam and Aba were prepared from the stock solution and injected five times and the areas of five injections were recorded in HPLC. The % RSD was found to be 0.57 and 1.05 for Lam and aba respectively. The % RSD for the area of five replicate injections was found to be within the specified limit. It shows that the drug is having good precision.

Accuracy

Accuracy was confirmed by recovery studies. To the pre ansalysed formulation a known quantity of (Aba&Lam) raw material solution were added at different concentration levels of 80%, 100% and 120%. The amount of drug recovered was calculated. The percentage recovery of Lam and Aba were found in the range from 98.14 to 100.14 % and 99.88 to101.23% respectively. The %RSD value for Lam and Aba were found to be 0.8690and 0.7162% respectively. The %RSD value was found to be less than 2%. . The low percentage RSD value indicated that there was no interference due to the excipients used in formulation. Hence the accuracy of the method was confirmed.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. The percentage RSD value for analyst I was found to be 1.367 and1.262% for lam and aba respectively. The percentage RSD valuefor analyst II was found to be 1.335 and 1.286 % for lam and aba respectively.

Conclusion

The analytes Abacavir and Lamivudine have been simultaneously analysed in pharmaceutical formulations by using HPLC. Time of analysis, resolution and quality of the peaks were simultaneously optimized by applying useful tools of chemometrics: response surface design and Derringer's desirability function. The results of the study demonstrate the benefit of applying this approach in selecting optimum conditions for the determinations of drugs in pharmaceutical formulations. This method reduces overall assay development time and provides essential information regarding the sensitivity of various chromatographic variables on separation attributes.The validation study supported the selection of the assay conditions by confirming that the assay was accurate, linear, precise and robust.

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