J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(2): 625-636, 2023
Doi: 10.33483/jfpau.1181816

ORIGINAL ARTICLE / ÖZGÜN MAKALE

UTILITY OF QUALITY BY DESIGN APPROACH IN RP-HPLC METHOD DEVELOPMENT FOR QUANTIFICATION OF LAMIVUDINE AND EFFAVIRENZ IN COMBINATION FORMULATION

RP-HPLC KULLANILARAK TASARIM YAKLAŞIMI YOLUYLA KOMBİNE FORMÜLASYONDA BULUNAN LAMİVUDİN VE EFFAVİRENZ'İN TAYİNİNE YÖNELİK YÖNTEM GELİŞTİRİLMESİ

Bhagavan Rajesh Babu KOPPİSETTY[1]*, Rajendra Prasad YEJELLA[1], Krishna Manjari Pawar AMGOTH[1], Srinivasa Rao YARRAGUNTLA[2], Vasudha DADI[2], Hemant Kumar TATAPUDI[3]

[1]Andhra University, College of Pharmaceutical Sciences, Department of Pharmaceutical Analysis, Visakhapatnam, Andhra Pradesh, India

[2]Vignan Institute of Pharmaceutical Technology, Department of Pharmaceutical Analysis, Visakhapatnam, Andhra Pradesh, India

[3]NSHM Knowledge Campus, Department of Pharmaceutical Technology, Kolkata, West Bengal, India

ABSTRACT

Objective: *For the measurement of lamivudine(LAM) and effavirenz (EVZ) in combination formulation, an uncomplicated and reliable liquid chromatographic approach has been proposed.*
Material and Method: *The design of experiments (DoE) was used to set up the experimental conditions for the multivariate optimization of the RP-HPLC method. The crucial method parameters were determined by a risk assessment. The mathematical models were created using three independent variables: percentage of acetonitrile, percentage of methanol, and buffer pH. The*

* **Corresponding Author / Sorumlu Yazar:** Bhagavan Rajesh Babu Koppisetty
e-mail / e-posta: koppisettybrbabu@gmail.com, **Phone / Tel.:** +919490597959

Submitted / Gönderilme : 29.09.2022
Accepted / Kabul : 15.04.2023
Published / Yayınlanma : 20.05.2023

impacts of these independent elements were thoroughly investigated using the central composite design (CCD), which was utilized to analyze the response surface methodology.
Result and Discussion: *The LAM and EVZ retention time and resolution were both concurrently optimized using the desirability function. Acetonitrile, methanol, and phosphate buffer (pH 7.0) in the proportions of 40:20:40 v/v each were used in the optimized and anticipated data from the contour diagram, with detection occurring at a wavelength of 215 nm. Baseline separation of both pharmaceuticals with high resolution and a run time of under 6 minutes was accomplished under these ideal conditions. The validated test parameters followed ICH recommendations. As a consequence, the findings demonstrated that the Quality by Design methodology could be successfully used to optimize the RP-HPLC technique for the concurrent quantification of LAM and EVZ.*
Keywords: *Central composite design, effavirenz, lamivudine, quality by design, RP-HPLC*

ÖZ

Amaç: *Kombinasyon formülasyonunda lamivudin (LAM) ve effavirenzin (EVZ) ölçümü için karmaşık olmayan ve güvenilir bir sıvı kromatografik yaklaşım önerilmiştir*.
Gereç ve Yöntem: *Deney tasarımı (DoE), RP-HPLC yönteminin çok değişkenli optimizasyonu için deneysel koşulları ayarlamak üzere kullanıldı. RP-HPLC yönteminin çok değişkenli optimizasyonu, deneysel koşullar, deney tasarımı (DoE) kullanılarak gerçekleştirildi. Kritik yöntem parametreleri bir risk değerlendirmesi ile belirlendi. Matematiksel modeller üç bağımsız değişken kullanılarak oluşturulmuştur: asetonitril yüzdesi, metanol yüzdesi ve tampon pH'ı. Bu bağımsız öğelerin etkileri, yanıt yüzeyi metodolojisini analiz etmek için kullanılan merkezi bileşik tasarım (CCD) kullanılarak ayrıntılı bir şekilde incelenmiştir.*
Sonuç ve Tartışma: *LAM ve EVZ tutma süresi ve çözünürlüğü, arzu edilebilirlik işlevi kullanılarak aynı anda optimize edildi. Her biri 40:20:40 v/v oranlarındaki asetonitril, metanol ve fosfat tamponu (pH 7.0), 215 nm'lik bir dalga boyunda meydana gelen algılama ile kontur diyagramından optimize edilmiş ve beklenen verilerde kullanılmıştır. Her iki farmasötik maddenin yüksek çözünürlükte ve 6 dakikanın altında çalışma süresiyle temel ayrımı, bu ideal koşullar altında gerçekleştirildi. Doğrulanmış test parametreleri, ICH önerilerini izledi. Sonuç olarak, bulgular Tasarıma Göre Kalite metodolojisinin LAM ve EVZ'nin eş zamanlı ölçümü için RP-HPLC tekniğini optimize etmek üzere başarılı bir şekilde kullanılabileceğini göstermiştir.*
Anahtar Kelimeler: *Effavirenz, lamivudin, merkezi kompozit tasarım, RP-HPLC, tasarıma göre kalite*

INTRODUCTION

Lamivudine (LAM) is an inhibitor of the reverse transcriptase enzyme that has been shown to be effective against the "HIV-1, HIV-2, and hepatitis B viruses". LAM, chemically "4-amino-1-[(2*R*, 5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one". Lamivudine triphosphate is the active 5'-triphosphate metabolite of lamivudine, a synthetic nucleoside analogue that is phosphorylated intracellularly (L-TP). Viruses' DNA chains are broken when enzymes like HIV reverse transcriptase and HBV polymerase incorporate this nucleoside analogue. Chemically EVZ, "(4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1*H*-3,1-benzoxazin-2-one", acts as a competitive inhibitor of DNA polymerase and is an inhibitor of reverse transcriptase that does not rely on nucleosides. Figure 1 illustrates the structure of both drugs. In the management of hepatitis B and HIV illnesses, the combination of two or more antiviral medicines represents a significant achievement. Patients are more compliant with multidrug combination treatment due to the lower medication load each day. The combination of lamivudine and effavirenz available with tenofovir disoproxil fumarate, zidovudine ,stavudine, didanosine etc. In these FDC the dosage of lamivudine and effavirenz available as 150/300 mg and 300/600 mg respectively.

Several HPLC techniques have been published in the literature for the determination of lamivudine alone [1-4], effavirenz alone [5-7], lamivudine and effavirenz with other drugs, in pharmaceutical products and biological samples [8-11]. There is no published QbD-based RP-HPLC technique for the concurrent measurement of lamivudine and effavirenz. The use of "Quality by Design" (QbD) or "Design of Experiments" (DoE) is advocated to provide robustness throughout the statistical

quality control monitoring of analytical technique validation and the research of variables that have a negative influence on the quality of pharmaceutical analysis. The conventional way of developing a method consists of trial and error and adjusting one parameter at a time. Due to considerations such as the restricted access of chromatographic supplies such the column, solvents, and chemicals , as well as the crucial physicochemical features of the analyte, this method frequently has difficulty setting robust chromatographic conditions. Recently, FDA has approved multiple NDAs using the QbD approach to analytical methods like HPLC and UV spectrophotometery. These NDAs have regulatory flexibility for movement within the stated method operational design region (MODR). Quality by Design (QbD) has been an integral part of developing new pharmaceuticals since its deployment by the FDA, influencing its robustness. A current treatment of the HPLC technique's robustness using QbD necessitates the evaluation of all elements that have the greatest effect on the method's outcomes. It is impracticable, challenging, and more expensive to experimentally verify several factors at once. The reversed-phase technique [12-15] is the method of preference in analysis due to its ease of use, adaptability, and diversity of applications, which include handling compounds with a variety of polarities and molecular masses.

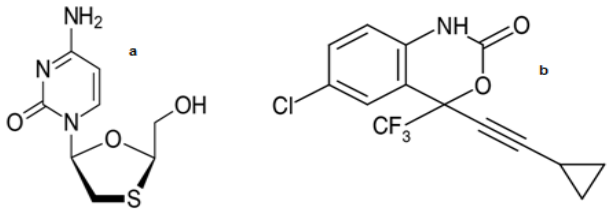

Figure 1. Structure of Lamivudine (a) and Effavirenz (b)

MATERIAL AND METHOD

Chemicals and Reagents

Both of these drug standards brought from the pharmaceutical industry. Merck India Ltd., Mumbai, supplied LC-grade methanol, water, and LC-grade acetonitrile. SD Fine chemicals, Mumbai supplied potassium dihydrogen phosphate and orthophosporic acid. Odivir kit (Lamivudine 300 mg, Effavirenz 600 mg, Cipla Limited) was obtained from a local pharmacy.

HPLC Instrumental Condition

The technique was created using a Waters Acquity (HPLC) system (Milford, MA, USA) with a PDA detector (model #2996) and Empower-2 software. The two analytes were separated by using methanol : acetonitrile: phosphate buffer pH 7 (20:40:40, v/v) with an isocratic flow rate of 1 ml/min, at room temperature. Separation and analysis were executed on an Xterra C18 column (250 mm x 4.6 mm ; 5 µm) in a temperature-controlled laboratory (25°C was maintained for all chromatographic runs). At 215 nm, the PDA detector was utilized to monitor the two drugs. The solvents were degassed in an ultrasonic bath after being filtered through a 0.22 µm membrane filter. As a diluent, mobile phase was utilized.

Software

Utilizing Design-Expert version 12, calculations for the desirability function, experimental design (CCD), and data analysis were made.

Preparation of Buffer Solutions

Potassium dihydrogen orthophosphate weighing 1.38 grams was added to a 1000 ml volumetric flask, dissolved with HPLC water and diluted to 1000 ml. Ortho-phosphoric acid was used to bring the pH down to 7.

Preparation of Standard Solutions

A sample containing 25 mg of each drug is weighed before being moved to a volumetric flask measuring 25 ml. After adding 15 ml of acetonitrile, the solution is sonicated for 15 minutes. To create a stock solution containing 1000µg/ml, the volume is brought up to the required level using acetonitrile. Working standard solutions of 100 µg/ml are created by withdrawing 2.5 ml from the standard stock solutions, transferring it to 25 ml volumetric flasks, and topping off the volume with diluent.

Preparation of Pharmaceutical Samples

"Odivir-kits (Tablets)" each tablet with labelled claim content "300 mg lamivudine" and "600 mg efavirenz" were weighed, the mean weight was recorded, and the pulverised LAM and EVZ were carefully weighed and placed into a volumetric flask of capacity 50 ml which is previously cleaned and dried. The flask's contents were mixed in diluent, subjected to a 30-minute sonication, diluted to the desired volume, and designated as a sample stock solution on the label. PVDF 0.45 µm filters were used to filter the sample stock solution. Filtered sample stock solution in the quantity of one milliliter was placed in a ten milliliter volumetric flask and adjusted to the necessary strength.

Software Aided Method Optimization

According to the literature, several design methodologies were offered to test the robustness of the procedure. They were utilized when screening techniques needed to be optimized for separation and while assessing robustness and optimizing formulations, products, or methods. The significant chromatographic variables in the present research were chosen based on preliminary studies and existing study of literature. Because of its effectiveness in terms of the number of runs needed, the CCD screening design may be a useful option for examining the robustness of a limited number of variables (three or less). Several variables, including the fraction of acetonitrile and methanol in the mobile phase and the buffer's pH, were addressed during method development. As a result, CCD was used to assess the impact of three distinct parameters in chromatography on three identified critical response characteristics. 20 test runs were incorporated into the design and aided in factor screening by assessing each component's major influence to get study results. A 3 x 2 factorial design indicates the presence of two levels and three factors. These were a "low (-1)" and a "high (+1)" level, factors on the other were (X_1) fraction of methanol in mobile phase (20 % and 40 %), (X_2) proportion of acetonitrile in mobile phase (20 % and 40 %), and (X_3) pH of buffer (5 and 7). Table 1 displays the retention times for LAM (Y_1), EFZ (Y_2), and resolution (Y_3), which were employed as the experimental design's responses. The data obtained were incorporated into Design-Expert version 12. In order to explore, examine response behavior around optimal values of the variables, and get the greatest system performance, response surface quadratic approach was utilized. To assess the model's significance, analysis of variance (ANOVA) was used. Conditions were chosen from this optimized method, and their performance in terms of accuracy, precision (< 2% RSD), and robustness as a targeted response, was verified. Twenty experiments were conducted using the conditions and outcomes reported in Table 2.

Method Validation

"International Conference on Harmonization (ICH) (2005) Q2R(1)"[16] criteria were used to validate the optimized chromatographic technique for "system suitability, linearity, limit of detection, limit of quantitation, precision, accuracy, specificity, and robustness".

System Suitability Test

LAM and EFZ concentrations of 15µg/ml and 30µg/ml, respectively, in six replicates of standard solutions were injected prior to the sample analysis to test the system appropriateness characteristics of Resolution, tailing factor, and theoretical plate count. The % RSD should always be less than 2.0%. In each chromatogram, the acceptance requirements for system suitability standards were determined.

Table 1. Experimental plan of CCD showing factors with levels

Factor	Code	Range levels	
		Low (-1)	High (+1)
% Methanol composition in mobile phase	X_1	20	40
% Acetonitrile composition in mobile phase	X_2	20	40
pH of Buffer	X_3	5	7
Responses			
Retention time LAM	Y_1	-	-
Retention time EVZ	Y_2	-	-
Resolution factor	Y_3	-	-

Table 2. Coded values for factor level and observed responses in CCD for 20 analytical trials

Exp. (Run)	Type	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	Center	30	30	6	3.184	5.384	4.48
2	Axial	30	30	4.31	2.121	4.212	4.21
3	Axial	46.81	30	6	3.981	5.123	4.09
4	Axial	30	46.81	6	1.567	3.982	3.91
5	Axial	30	30	7.68	2.01	3.674	3.1
6	Center	30	30	6	3.184	5.384	4.48
7	Factorial	20	20	5	4.512	5.687	2.15
8	Factorial	20	20	7	2.412	3.998	3.45
9	Center	30	30	6	3.184	5.384	4.48
10	Factorial	20	40	7	1.689	3.921	4.555
11	Factorial	40	40	7	3.119	4.12	2.04
12	Center	30	30	6	3.184	5.384	4.48
13	Factorial	20	40	5	2.512	5.287	4.32
14	Factorial	40	40	5	3.376	5.981	3.98
15	Axial	30	13.18	6	4.982	4.772	0.54
16	Axial	13.18	30	6	3.763	6.021	6.98
17	Factorial	40	20	5	4.265	5.391	2.13
18	Center	30	30	6	3.184	5.384	4.48
19	Center	30	30	6	3.184	5.384	4.48
20	Factorial	40	20	7	2.982	6.102	5.85

Linearity

The suggested approach's linearity was established at five levels over the 5-25µg/ml for LAM and 10-50µg/ml for EFZ ranges. Each linearity solution was administered in triplicate using the appropriate sample concentrations. By utilizing linear regression analysis to plot the peak area versus the concentration, the calibration curve was created.

Accuracy and Precision

The accuracy was tested by spiking a predetermined quantity of standard to the tablet solution in triplicate at levels of 50, 100, and 150%, and then performing the optimized technique on the samples. Then, percentage recoveries for both medications were determined. For acceptance, the target concentrations' mean recovery was set at 100 ± 2%. By examining the intermediate precision and repeatability, the precision of the optimized technique was ascertained. Intermediate precision is expressed through changes in laboratories, such as various days, analysts, equipment, etc. The phrase

"inter-assay precision" also applies to intermediate precision. Repeatability describes the accuracy over a brief period of time while using the same operating variables. To evaluate the method's precision, six homogeneous LAM and EFZ samples were analyzed.

Limit of Detection(LOD) and Limit of Quantitation(LOQ)

Using the standard deviation approach, LOD and LOQ of LAM and EVZ were assessed. Based on the response's standard deviation (σ) and calibration curve's slope (S), LOD was determined at 3.3 σ /S and LOQ at 10 σ /S.

Robustness

The robustness of the technique is the capacity to withstand modest and intentional changes in the method parameters. By infusing the system suitability solution with slight purposeful modifications to the chromatography's parameters (mobile phase ratio, flow rate, and analytical wavelength), the robustness of the optimized technique was examined. It was calculated using the percentage of the RSD.

RESULT AND DISCUSSION

Preliminary Studies and Factor Selection

An initial investigation was conducted to find a simple, robust, and inexpensive RP-HPLC technique for estimating LAM and EFZ in mixtures. Using data from preliminary studies and other published works, the key chromatographic parameters were chosen. Studies aimed at selecting the factor levels for use in screening and optimization studies highlighted the requirement to improve mobile phase conditions in order to separate LAM and EFZ in a small space of time. The simultaneous estimate of both drugs was shown to be more suited for the mobile phase composition of phosphate buffer pH, volume of methanol and acetonitrile, which led to a significant impact in retention time. As a result, it is regarded as one of the essential criteria for method development.

QbD Assisted Method Development

The current work on the optimization of the analytical approach used CCD design. It is an effective and extensive DoE centered on meticulous exploration of three essential elements of the RP-HPLC technique (percentage of methanol, acetonitrile, and pH of buffer).

To assess method robustness, a “multivariate approach DoE with CCD” was used in the RP-HPLC technique to examine the concurrent modifications of the parameters on selected responses, includes LAM retention time(Y_1), EVZ retention time(Y_2), and resolution(Y_3). Based on an analysis of this data and the influence of three factors on responses, it was possible to develop mathematical models to determine the link between the factors and the examined responses. After analyzing the data, we found that the response surface quadratic model provided the greatest match for CCD. ANOVA was used in Design Expert to further validate the model. The retention time adjusted R-squared values for LAM(Y_1) and EVZ(Y_2) were in reasonable agreement with the predicted values, with a deviation of less than 0.2. The current model may not be the best predictor of the response, as indicated by the negative predicted R-squared for resolution(Y_3). Signal-to-noise ratio is measured with adequate precision. The obtained responses for the Y_1, Y_2, and Y_3 were 9.1254, 5.811, and 7.1743, respectively, which suggests an adequate precision; a ratio of larger than 4 is preferred. Navigation of the design space is done using the quadratic model. The model is considered significant since the responses' model F-values for the retention times of LAM (Y_1), EVZ (Y_2), and resolution (Y_3) were 6.48, 4.13, and 3.53 respectively. There is only a 0.36 percent probability for Y_1 and 0.19 percent chance for Y_2, and a 3.10 percent probability of resolution (Y_3) thanan F-value, suggesting that this might be caused by noise. Consequently, the p value of the significant responses was less than 0.05, indicating that the terms of the model are significant. The high adjusted R-square value and low standard deviation show that the fitted models and experimental data are well correlated. The results are presented in Table 3. Predictions regarding the responses for specific levels of each factor may be made using the equations in terms of coded factors. This equation can be used to figure out how important each factor is by comparing the factor coefficients. Here are the final equations for Y_1, Y_2, and Y_3:

LAM (Y_1) = +3.18 + 0.2185X_1 - 0.6750X_2 – 0.3405X_3 + 0.2464X_1X_2 + 0.1729X_1X_3 + 0.2879X_2X_3 + 0.2527X_1^2 - 0.0414X_2^2 – 0.3860X_3^2;

EVZ: (Y_2) = +5.37 + 0.0872X_1 - 0.2341X_2 – 0.3742X_3 - 0.1144X_1X_2 + 0.2381X_1X_3 - 0.2811X_2X_3 + 0.1669X_1^2 - 0.2556X_2^2 - 0.4090X_3^2;

Resolution: (Y_3) = +4.49 - 0.3907X_1 + 0.5113X_2 + 0.1060X_3 -0.6544X_1X_2 + 0.0306X_1X_3 - 0.8406X_2X_3 + 0.3298X_1^2 - 0.8405X_2^2 -0.3349X_3^2.

Table 3. ANOVA regression analysis for models and responses

Response	Mean	SD[a]	%CV[b]	Press value	R^{2c}	Adjusted R^2	Predicted R^2	Adequate Precision	SS[d]	d[e]	MS[f]	F[g]	P
$R_T$of LAM(Y_1)	3.12	0.4768	15.28	17.28	0.8537	0.7221	0.6121	9.1254	13.27	9	1.47	6.48	0.0036
R_T of EVZ(Y_2)	5.03	0.5853	11.64	27.63	0.6947	0.4199	0.4629	5.811	7.79	9	0.8661	4.13	0.0082
Resolution (Y_3)	3.91	0.9515	24.34	72.92	0.7607	0.5454	-0.6273	7.1743	28.78	9	3.20	3.53	0.0031

R_T: Retention time, a: Standard deviation, b: Coefficient of variations, c: Coefficient of Regression, d: Sum of squares, e: Degrees of freedom, f: Mean sum of squares, g: Fischer's ratio

It is evident from the coefficient values in the aforementioned equations and their corresponding that factors like methanol volume (X_1) has positive impact on retention time of LAM and EVZ, Y_1 and Y_2. Acetonitrile volume (X_2), and pH of buffer (X_3), had a negative impact on retention time of LAM and EVZ, Y_1 and Y_2. The pH of buffer (X_3) had a positive impact on resolution (Y_3), whereas methanol volume (X_1) has a negative effect and acetonitrile volume (X_2) had positive effects. The interactions between X_1 and X_2 had a positive impact on Y_1 and a negative impact on Y_2 and Y_3, whereas the interactions between X_2 and X_3 had a positive impact on Y1 and a negative impact on Y_2 and Y_3 and a positive impact on Y_1, Y_2, and Y3. All chromatographic responses were positively impacted by the squares of factor $X1^2$ whereas X_2^2, and X_3^2, had negative impact on all responses of the model.

In order to understand how the factors and their interactions affected the response, response surface and contour plots were examined. The contour plots had curvature, indicating a nonlinear relationship between the variables and the responses. Figures 2 and 3 illustrate contour plots in 2D (A) and 3D (B) demonstrating the impact of methanol volume (X_1) and acetonitrile volume (X_2) on retention time of LAM (Y_1) and EVZ (Y_2). A curved rising trend was noticed for the methanol volume (X_1) that demonstrated higher retention time of LAM (Y_1), as well as EVZ (Y_2) and there was a curved downward trend was observed for acetonitrile volume (X_2), which showed lower retention time of LAM (Y_1), as well as EVZ (Y_2). Therefore, lower levels of X_1 and high levels of X_2 were suggested to achieve retention time of LAM (Y_1) and EVZ (Y_2). Figure 4 depicts the curvature impacts of the methanol volume (X_1) and acetonitrile volume (X_2) on resolution using 3D and 2D contour plots. Both X_1 and X_2 showed a rising curvature trend, indicating greater resolution at higher levels. To achieve resolution, the optimal values of X_1 and X_2 were chosen.

Consistent with the goals and boundaries of each response, a composite desirability was used to find the ideal combination of conditions. The entire experimental region was investigated for the compositions, whereby constraints established were satisfied to the highest extent possible, i.e., unity, as demonstrated in Figure 5 and Figure 6. The accomplishment of planned goals within the established limits was demonstrated by the desirability function "R," which is equal to unity. After determining the optimal RP-HPLC chromatographic conditions, we decided as volume of methanol (X_1) , volume of acetonitrile (X_2) and buffer pH 7.0 in ratio (20 : 40 : 40 % v/v) and retention time as a result in which LAM (Y_1) 1.729±0.0162, retention time of EVZ (Y_2) 3.85±0.013, and resolution (Y_1) 4.52±0.021 min, respectively, as shown in Figure 7.

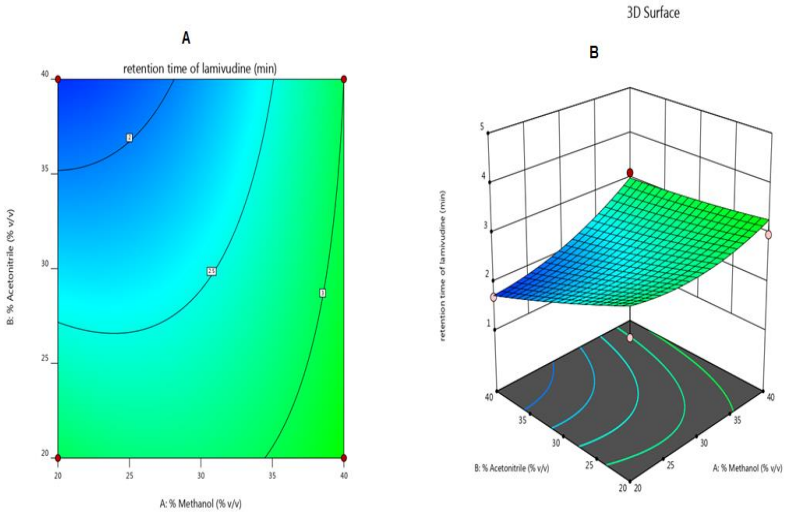

Figure 2. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on retention time of LAM (Y_1)

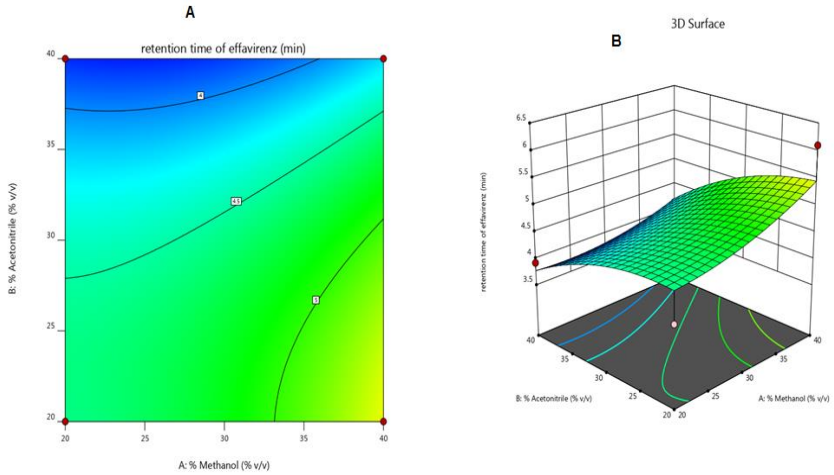

Figure 3. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on retention time of EVZ (Y_2)

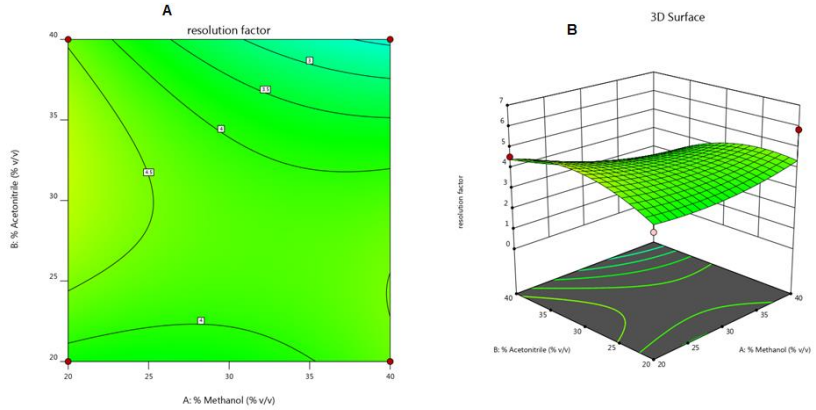

Figure 4. 2D (A) and 3D (B) contour plots showing the effect of % methanol (X_1) and % acetonitrile (X_2) on resolution (Y_3)

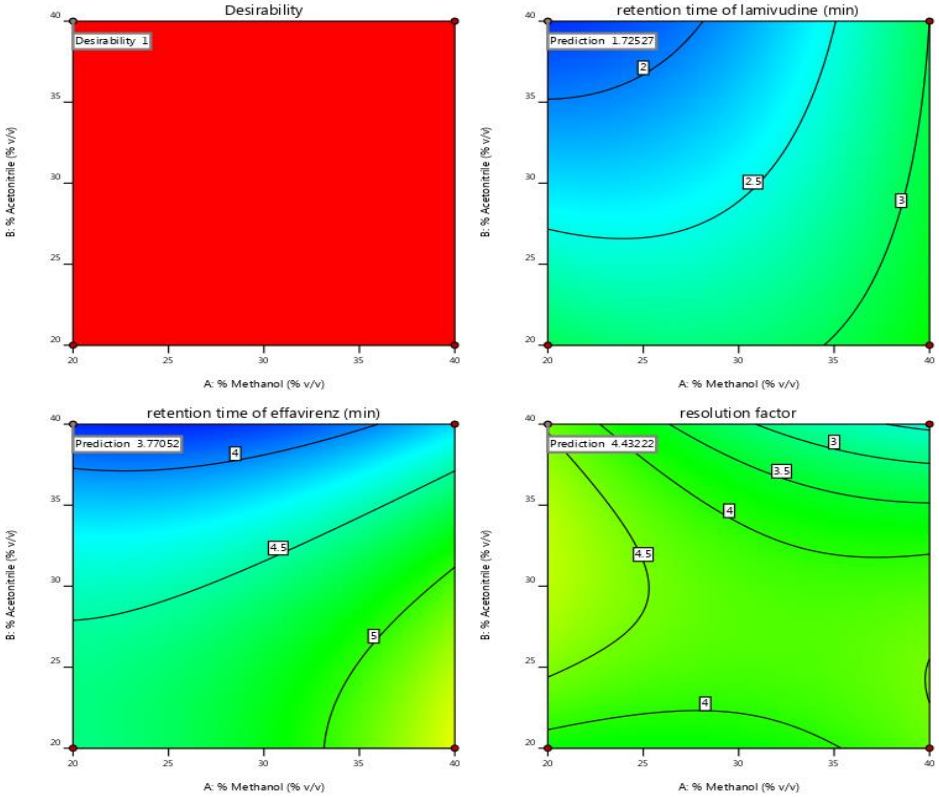

Figure 5. 2D desirability plot

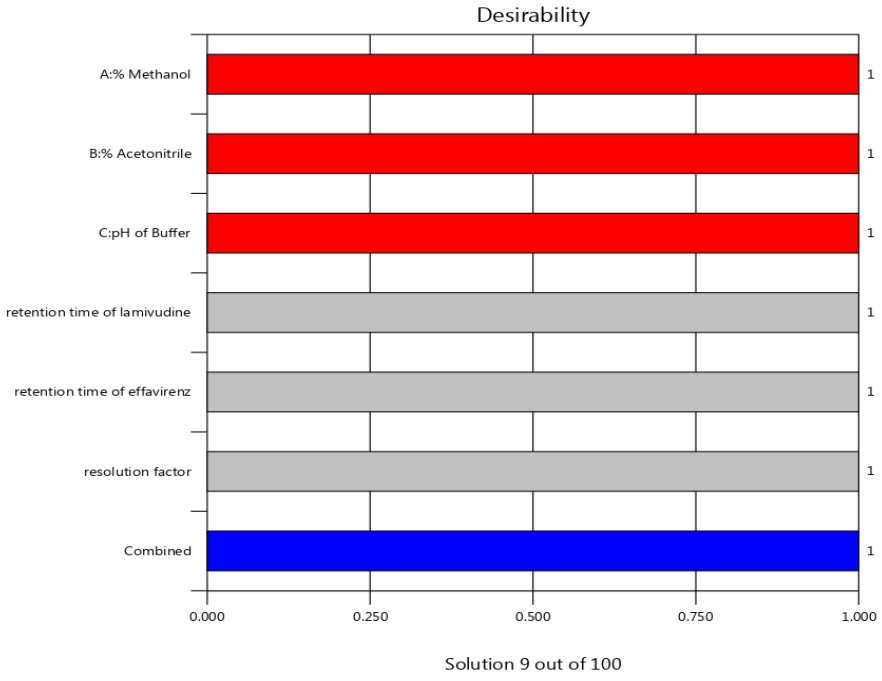

Figure 6. Desirability plot

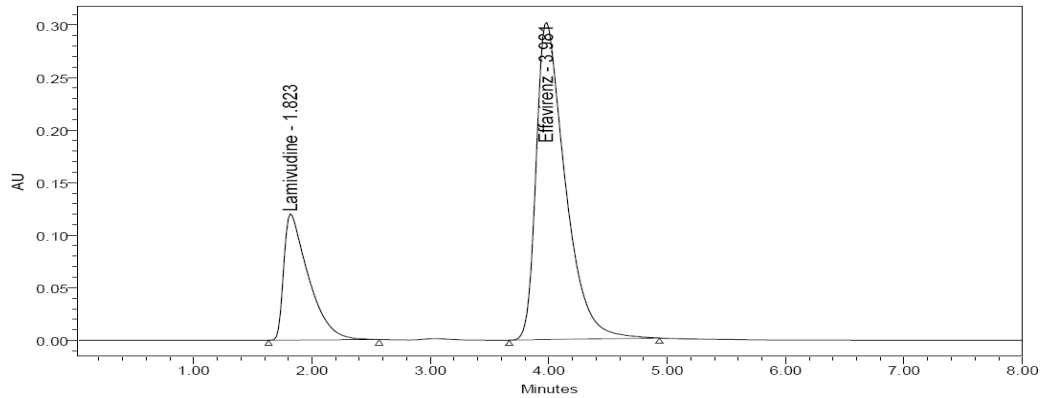

Figure 7. Optimized RP-HPLC chromatogram for LAM and EVZ at 215 nm

Method Validation

System suitability tests, according to the ICH, are an essential aspect of liquid chromatographic procedures. The performance of columns, as evaluated by theoretical plates count for both medicines, was greater than 2000, the resolution was 4.52, and tailing was less than 2. For six replicate injections, the percent relative standard deviation was 0.87 at the indicated concentration of 15 µg/ml for LAM and 1.09 at the indicated concentration of 30 µg/ml for EVZ, respectively. Because the % RSD < 2%, it demonstrated high injection repeatability. The proposed method's linearity was verified by plotting the linearity curve for LAM and EVZ over a range of concentrations, from 5 to 25µg/ml and 10 to 50µg/ml, respectively, with correlation coefficients of (r^2=0.999) for both medicines, as shown in Table 4.

Table 4. Validation results for LAM and EVZ

PARAMETERS		LAM	EVZ
System Suitability Parameters			
No. of theoretical Plates	Mean±SD*	2716.6 ± 88.98	2546.8 ± 73.88
	% RSD	1.65	1.81
Resolution	Mean±SD*	-	4.52 ± 0.021
	% RSD		0.24
Tailing factor	Mean±SD*	1.05 ± 0.003	1.01 ± 0.0019
	% RSD	0.21	0.18
Linearity			
Range (µg/ml)		5-25	10-50
Slope		48973	15368
Intercept		-48000	43802
Correlationcoefficient		0.9991	0.9992
Accuracy			
% Recovery		99.3-101.43	99.02-99.89
% RSD**		1.87	1.73
Precision			
Repeatability		0.87	1.09
Intermediate precision		0.91	1.23
LOD(µg/ml)		0.12	0.18
LOQ((µg/ml)		0.36	0.54

*Mean of six determinations, **Set of three determinations

The peak area and concentration have a strong correlation, as shown by the obtained correlation coefficient (r2=0.999). Samples at 50%, 100%, and 150% of the nominal concentrations for both drugs

were created for the recovery investigation and recovery rates for LAM and EVZ were 99.3-101.43% and 99.02-99.89%, respectively. Table 4 displays data demonstrating a low level of error (% RSD 1.87 and 1.73 for LAM and EVZ, respectively) with the established approach. The outcomes of the intermediate precision and repeatability tests are presented in Table 4. For both medicines, the procedure was repeatable and accurate, since the precision values were below 2%. Results showed that the LOD and LOQ for LAM were 0.12 and 0.36 µg/ml, while those for EVZ were 0.18 and 0.54 µg/ml. The robustness of the RP-HPLC technique to slight variations in the optimum experimental modifications revealed its insensitivity to such modest alterations. The mobile phase composition and buffer pH had a substantial influence on LAM and EVZ retention time and resolution.

For the first time, the current study involves the methodical QbD-based study and development of a rapid, accurate, and cost effective RP-HPLC technique for concurrent quantification of LAM and EVZ. The experimental design covers the exploration of important components, such as methanol volume, acetonitrile volume, and buffer pH. The modeling software aided in a superior comprehension of the elements impacting optimization of the procedure and separation of LAM and EFZ. CCD was utilized in order to improve the resolution in a reasonable period of time(6 min) in response to LAM and EFZ. In the optimized model, the 20:40:40 v/v ratio of methanol, acetonitrile, and pH 7.0 phosphate buffer confirms the appropriateness for estimating LAM and EFZ. The validation study indicated that the method was selective, specific, accurate, linear, precise, and robust, which contributed in the determination of the optimal conditions. As a result, using the response surface methodology gives a better understanding for method development and robustness testing. This approach proposed meets the design space principle and is in line with regulatoryflexibility and suitable for regulatory submission.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. L.Rathaiah, Chairman of Lavu educational society for providing necessary facilities to carry out the above research work.

AUTHOR CONTRIBUTIONS

Concept: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Design: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Control: R.P.Y., K.M.P.A.,S.R.Y.; Sources: R.P.Y., H.K.T.; Materials: S.R.Y., V.D.; Data Collection and/or Processing: S.R.Y., V.D.; Analysis and/or Interpretation: B.R.B.K., H.K.T.; Literature Review: B.R.B.K., V.D., H.K.T.; Manuscript Writing: B.R.B.K.; Critical Review: B.R.B.K., R.P.Y., K.M.P.A., S.R.Y., V.D., H.K.T.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

REFERENCES

1. Bahrami, G., Mirzaeei, S., Kiani, A., Mohammadi, B. (2005). High-performance liquid chromatographic determination of lamivudine in human serum using liquid-liquid extraction; application to pharmacokinetic studies. Journal of Chromatography B, 823(2), 213-217. [CrossRef]
2. Checa, A., Oliver, R., Hernandez-Cassou, S., Saurina, J. (2008). Reversed-phase liquid chromatographic method with spectrophotometric detection for the determination of antiretroviral drugs. Analytica Chimica Acta, 616, 85-94. [CrossRef]
3. Kano, E.K., Reis Serra E.E., Koono, M., Andrade, S.S. (2005). Determination of lamivudine in human plasma by HPLC and its use in bioequivalence studies. International Journal of Pharmaceutics, 297, 73-79. [CrossRef]

4. Nalini, C.N., Nithya, A., Afreen, A., Banu, A.A. (2018). Analytical methods for determination of lamivudine and their applicability in biological studies. Research Journal of Pharmacy and Technology, 11(11), 5166-5172. [CrossRef]
5. Mogatle, S., Kanfer , I. (2009). Rapid method for the quantitative determination of efavirenz in human plasma. Journal of Pharmaceutical and Biomedical Analysis, 49, 1308-1312. [CrossRef]
6. Matthews, C.Z., Woolf, E.J., Mazenko, R.S., Haddix-Wiener, H. (2000). Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection. Journal of Pharmaceutical and Biomedical Analysis, 28, 925-934. [CrossRef]
7. Nacenta, M.S., Pua, Y.L., Cortes, L.F.L., Mallolas, J. (2001). Determination of efavirenz in human plasma by high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B., 763, 53-59. [CrossRef]
8. More, S., Tandulwadkar, S., Nikam, A., Rathore, A. (2013). Separation and determination of lamivudine, tenofovir disoproxil fumarate and efavirenz in tablet dosage form by thin-layer chromatographicdensitometric method. Journal of Planar Chromatography-Modern TLC, 26(1), 78-85. [CrossRef]
9. Bhavsar, D.S., Patel, B.N., Patel, C.N. (2012). RP-HPLC method for simultaneous estimation of tenofovir disoproxil fumarate, lamivudine, and efavirenz in combined tablet dosage form. Pharmacutical Methods, 3(2), 73-78. [CrossRef]
10. Anandakumar, K., Abirami, G., Murugan, S., Ashok, B. (2013). RP-HPLC method for simultaneous estimation of lamivudine, tenofovir disoproxil fumarate and efavirenz in tablet formulation. Journal of Analytical Chemistry, 68, 815-821. [CrossRef]
11. Godela, R., Kammari, V., Gummadi, S., Beda, D. (2021). Concurrent estimation of lamivudine, tenofovir disoproxil fumarate, and efavirenz in blended mixture and triple combination tablet formulation by a new stability indicating RP-HPLC method. Future Journal of Pharmaceutical Science, 7, 94. [CrossRef]
12. Kumar, T.H., Prajna C.K.K., Rao, K.V., Rao, Y.S. (2019). RP-HPLC method for estimation of tramadol hydrochloride and paracetamol ın pharmaceutical formulation. GSC Biological and Pharmaceutical Sciences, 8(1), 89-97. [CrossRef]
13. Vidyadhara, S., Rao, Y.S., Ramu, A., Sasidhar, R.L. (2013). Method development and validation for the simultaneous estimation of cinitapride and pantoprazole in solid dosage forms by RP-HPLC. Oriental Journal of Chemistry, 29(3), 1213. [CrossRef]
14. Nagamani, P., Manjunath, S.Y., Kumar, T.H. (2020). Development and validation of RP-HPLC method for estimation of amlodipine besylate and celecoxib in pharmaceutical formulation. Journal of Drug Delivery and Therapeutics, 10(6), 31-36. [CrossRef]
15. Lakshmi Teja, G., Srinivasa Rao, Y., Vara Prasada Rao, K., Hemant Kumar, T. (2015). RP-HPLC method for estimation of atomoxetine hydrochloride in bulk and pharmaceutical dosage form. Research Journal of Pharmaceutical Biological and Chemical Sciences 6(2), 1208-1214.
16. ICH. (R1), (2005). Validation of analytical procedures: text and methodology. Vol. Q2.