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Optimization of RP-HPLC Method for Simultaneous Estimation of Lamivudine and Raltegravir in Binary Mixture by Using Design of Experiment

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ABSTRACT
A simple, sensitive, cost effective and robust RP-HPLC method for the simultaneous estimation of the Lamivudine (LAM) and Raltegravir (RAL) in laboratory prepared binary mixture was developed, optimized and validated. Separation was achieved on phenomenex C18 column (150 X 4.6 mm id, 5µ particle size) and mobile phase was composed of 75% methanol: 15% Acetonitril: 10 % (0.05mM) phosphate buffer (at pH 3.0), with flow rate 1.2 ml/min at 254nm. Developed method was optimized by using Box Behnken Design (BBD) in response surface methodology (RSM). The independent variables such as the concentration of methanol, pH in mobile phase and flow rate were selected for the optimization and Retention time (Rt) were used as responses for both drugs. Derringer's desirability function was used to concurrently optimize the selected responses. The LOD and LOQ were found to be 1.04 and 3.18 µg/ mL for LAM and 0.36 and 1.08µg/mL of RAL. The percentage recoveries were found to be less than 2% for LAM and RAL. Retention time of LAM and RAL was 3.13±0.07 and 7.27±0.01 minutes respectively.
Conclusion: The developed and optimized method was fully validated. The validated method further can be potentially used for estimation of these drugs in combined dosage form.
Keywords: response surface methodology, box behnken design, RP-HPLC, lamivudine, raltegravir

INTRODUCTION

Lamivudine (LAM) is chemically (2R, cis)-4-amino-1-(2-hydroxymethyl-1, 3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. It is an HIV-1 nucleoside analogue reverse transcriptase inhibitor [1, 2]. Similarly, Raltegravir (RAL) is chemically N-[(4-Fluorophenyl) methyl]-1,6-dihydro-5-hydroxy-1-methyl-2[1-methyl-1-[[(5-methyl-1,3,4-oxadiazol-2-yl) carbonyl] amino] ethyl]-6-oxo-4 pyrimidine carboxamide mono potassium salt. It is a human immunodeficiency virus (HIV) integrase strand transfer inhibitor [1, 2]. The chemical structure of LAM and RAL were shown in **Figure 1**.

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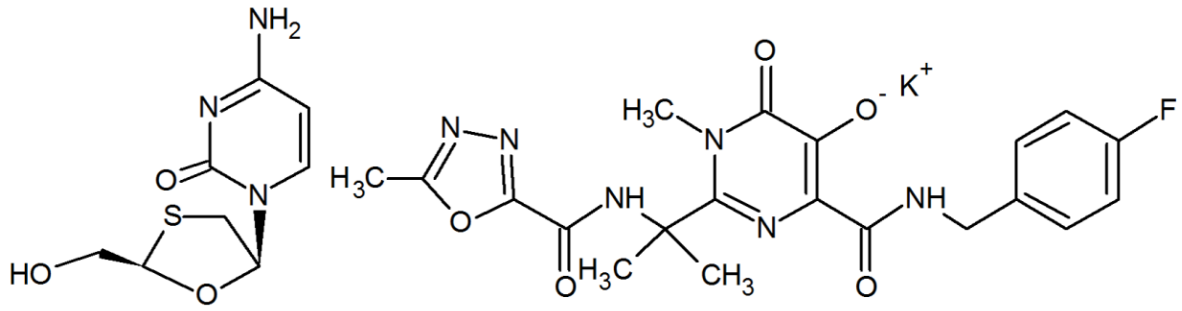

Figure 1. The chemical structure of a) Lamivudine and b) Raltegravir

Recently, RAL (300mg) and LAM (150mg) a combined formulation was approved by FDA for the treatment of HIV-1 infection. The action of RAL (300mg) and LAM (150mg) in combination are showing equivalent action to that of individual doses of RAL (400 mg) and LAM (150 mg) taken simultaneously. In the combined formulation, content of RAL was less than that of single formulation of RAL with having similar action. Therefore, intake of RAL can be reduced by using combined formulation [1, 2]. Presently; it is not commercially available in market. So the study was performed in the laboratory prepared binary mixture of LAM and RAL [1].

Literature survey reveals that various analytical methods for estimation of LAM have been reported such as UV [3-10], HPLC [2, 3, 11-17], HPTLC [3, 18-19] and LC-MS [20-21] in either individually or combined dosage form and biological sample. Similarly, for estimation of RAL, few analytical methods such as UV [22-25], HPLC [2, 26-31, 35], UPLC [32], LC-MS [33-34]-and HPTLC [35] have been reported in either alone or combined dosage form and biological sample. To best of our knowledge one HPLC method for simultaneous estimation of LAM and RAL in bulk active pharmaceutical ingredient (API) dosage form has been recently published [2]. This reported method has not showing a systematic optimization procedure for the separation and quantitation of LAM and RAL. Although, these methods employed a time consuming trial and error approach for giving potential information concerning the sensitivity of the factors on the analytes separation. But it did not provide the information concerning interaction between factors. [2]

Correspondingly, this manuscript described the optimization of an isocratic RP-HPLC method for the routine quality control analysis of LAM and RAL in laboratory prepared binary mixture. In spite of that Development and optimization of isocratic RP-HPLC method is a tedious process that involves instantaneous determination of several factors [37-40]. Therefore, Design of Experiment (DOE) which includes Box Behnken Design (BBD) [41] in Response Surface methodology (RSM) was used to optimize the developed method [38, 44]. It is recognized to provide risk-based understanding of the analytical as well as major factors affecting the performance of analytical method [42, 43]. Furthermore, it provided thorough

understanding of the possible risk and associated with interaction among the method variables, respectively [45, 46].

Therefore, the aim of present study was to develop, optimize and validate sensitive, robust and cost-effective RP-HPLC method using DOE approach for estimation of LAM and RAL in laboratory prepared binary mixtures. In addition, concentration of methanol, pH in mobile phase and flow rate were chosen as factors and their effect was seen at a response i.e. retention time of both the analytes that can be provide as an assay method for combination drug product of LAM and RAL.

EXPERIMENTAL

Materials

Pure drugs LAM (99.95 %) and RAL (99.95%) were kindly supplied by Richer Pharmaceuticals (Prasanthinagar, Hyderabad, India) and Emcure Pharmaceuticals (Pune, India) respectively. Methanol and Acetonitril (HPLC grade) from Qualigen, Potassium dihydrogen phosphate and Dihydrogen phosphate (AR grade) were purchased from E-Merck Ltd. (Mumbai, India). Ultra-purified HPLC grade water was obtained from the Milli - Q® system (Synergy Pak®- ICW-3000, Billerica) water purification unit. Mobile phase was filtered using 0.45µ nylon filters made by Millipore water, sonicated and degassed by using Ultra Sonicator bath.

Instrumentation and Chromatographic Conditions

A Shimadzu HPLC system consist of LC-10AT-vp Solvent delivery system (pump), SPD – 10Avp –UV visible detector, Rheodyne injector with 20µL loop volume, Spinchrom CFR software was used for data collections and processing. The mobile phase was composed of 75% methanol: 15% Acetonitril : 10 % (0.05mM) phosphate buffer (at pH 3.0), in the various ratios with a flow rate of 1.2 ml/min. Separation was achieved using Phenomenex Luna C18 column (150mm X 4.6 mm in diameter) with an average particle size of 5µ and the column was kept at an ambient temperature. The column effluent was monitored at 254 nm by UV detection.

Softwares

Experimental design, data analysis and desirability function calculations were performed by using Design Expert® trail version 10.0.0. (Stat-Ease Inc., Minneapolis, USA).

Preparation of 0.05mM phosphate buffer solution

Potassium dihydrogen phosphate (2.95 gm) and Dihydrogen phosphate (0.545 mg) were weighed and made up to 500ml with water (pH 3).

Preparation of stock solution

10 mg of LAM and RAL were weighed accurately and dissolved separately with methanol in 10 mL volumetric flask. The solution was diluted with mobile phase to obtain a concentration of 1000 µg/mL. The aliquot portions of stock solution were further diluted with mobile phase to obtain standard solutions over a concentration range of 10-100µg/mL of LAM and 5-30 µg/mL of RAL. The solution was filtered through 0.45µ nylon filters before analysis.

Preparation of laboratory prepared sample solution

The binary mixture of LAM and RAL was prepared in the ratio of 1:2 respectively. Accurately weighed LAM (150mg) and RAL (300 mg) was transferred to a 100 mL volumetric flask and methanol (70 mL) was added. Then suitable amount of common excipients i.e. croscarmellose sodium, hypromellose (2910), lactose monohydrate, magnesium stearate, microcrystalline cellulose, and silicon dioxide, which are used in the tablet formulation, were added in this mixture [1]. The content was sonicated for 15 min and flask was allowed to stand at room temperature for 5 min. Thereafter, the mixture was diluted up to the mark with methanol to obtain the sample stock solution (1500 and 3000 µg/mL) of LAM and RAL, respectively. The solution was filtered through 0.45µm membrane filter. Sample stock solution (2 mL) was transferred to a 10 mL volumetric flask, and diluted to the mark with mobile phase to obtain working sample solution (300 and 600 µg/mL) for LAM and RAL, respectively. Further 0.5 mL sample stock solution was transferred to a 10 mL volumetric flask, and diluted to the mark with mobile phase to obtain working sample solution (15 and 30 µg/mL) for LAM and RAL, respectively.

Experimental design

The optimization of HPLC method was performed by using Design Expert® 10.0.0 software (Stat-Ease Inc., Minneapolis, USA). In DOE, response surface methodology along with three factor three levels Box– Behnken design (BBD) was chosen. Here, percentage of methanol (A), pH (B) and flow rate (C) in the variation levels of 55-75 % v/v, pH 2.5-3.5and 0.8-1.6 ml/min were selected as independent variables and retention time was selected as response for LAM and RAL, respectively. Response surface analyses were done to identify the effect of different independent variables on the observed responses. It was carried out to measure the response (retention time of LAM and RAL) in each run of total 17 run, which were conducted in randomized order.

Method Validation

Linearity and range

The linearity was evaluated by measuring concentrations range 10-100µg/mL of LAM and 5-30 µg/mL of RAL standard solutions. The calibration curve was constructed by plotting concentration of standard solutions against mean peak areas and the regression equation was computed.

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

LOD and LOQ were calculated from the standard deviation of the response and slope of the calibration curve of each drug using the formulae, limit of detection (3.3×σ/S) and limit of quantitation (10×σ/S), where, σ is standard deviation of response and S is the slope of calibration curve.

Precision and Accuracy

Precision of the developed method was evaluated by performing repeatability on same day and intermediate precision studies on different days in three replicates. Repeatability and intermediate precision was carried out for three different concentrations (20, 60 and 100 µg/mL for LAM and 10, 20 and 30 µg/mL for RAL). %RSD of the all assays were obtained and calculated.

The accuracy of the method was determined in triplicate at three concentration levels of 80%, 100% and 120% by spiking the prequantified samples with a known amount of LAM and RAL standard. Recovery studied was calculating in term of % RSD for aforementioned drugs. The good recoveries of standard addition method suggested good accuracy of the proposed methods.

Selectivity and specificity

To check the selectivity of the proposed method, mixture of LAM and RAL was performed in laboratory prepared sample solutions of the binary mixture. The comparison of its area with the area of the standard solution was done along with the percentage recovery of both the analytes. The specificity of the method was established by comparing the chromatograms of LAM and RAL from standard and laboratory prepared sample solutions of the binary mixture.

Robustness

The robustness was studied by analyzing the same samples of LAM and RAL by deliberate variation in the method parameters. The change in the responses of LAM and RAL were observed. Robustness of the method was studied by changing the percentage of methanol in mobile phase by ± 5 %, pH by ±0.5 and flow rate by± 0.4 mL/min.

Determination of LAM and RAL in laboratory prepared sample solution

The responses of sample solutions were measured at 254 nm for quantitation of LAM and RAL by the proposed method. The amount of LAM and RAL present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for LAM and RAL, respectively.

Table 1. Values of independent variables and responses of LAM and RAL by DOE Software

Run	Factors			Retention time			
	A: methanol in mobile phase (%v/v)	B: pH	C: Flow rate (mL/min)	LAM		RAL	
				Actual	Predicted	Actual	Predicted
1	-1	-1	0	2.97	2.98	6.99	7.08
2	0	0	0	3.15	3.15	7.43	7.43
3	0	-1	-1	2.93	2.92	6.92	6.86
4	-1	0	-1	2.97	2.97	6.97	6.94
5	-1	1	0	3.01	2.93	7.13	7.10
6	0	0	0	3.15	3.15	7.43	7.43
7	0	-1	1	3.13	3.06	7.27	7.21
8	-0	1	-1	3.11	3.18	7.01	7.07
9	-1	0	1	2.67	2.73	7.28	7.25
10	1	1	0	3.16	3.15	7.41	7.32
11	0	0	0	3.15	3.15	7.44	7.43
12	0	0	0	3.16	3.15	7.43	7.43
13	1	0	1	3.03	3.02	7.28	7.31
14	0	1	1	2.67	2.68	7.12	7.18
15	1	-1	0	3.14	3.22	7.11	7.14
16	0	0	0	3.15	3.15	7.43	7.43
17	1	0	1	3.21	3.14	7.13	7.16

Table 2. Predicted response models and statistical parameters obtained from ANOVA for BBD

Response (Rt)	Type of model	Polynomial equation model for y	Adjusted R^2	PRESS Value	Model p value	%CV	Adequate precision
LAM	Quadratic	3.15+0.12*A-0.027*B-0.090*C-0.0003*AB +0.030*AC-0.16*BC-0.036*A^2-0.046*B^2-0.15*C^2	0.8306	0.55	0.0034	2.20	10.394
RAL	Quadratic	7.43+0.070*A+0.047*B+0.12*C+0.040*AB- 0.040*AC -0.060*BC-0.093*A^2-0.18*B^2-0.17*C^2	0.8423	0.62	0.0027	1.03	10.096

RESULTS AND DISCUSSIONS

Method optimization

The chromatographic conditions were optimized in order to develop an RP-HPLC method for the simultaneous measurement of laboratory prepared binary mixtures of LAM and RAL.

Preliminary study for selection of mobile phase

The suitability of mobile phase combination, flow rate, and pH was decided on the basis of linearity, sensitivity, system suitability, selectivity, lesser time required for analysis (low

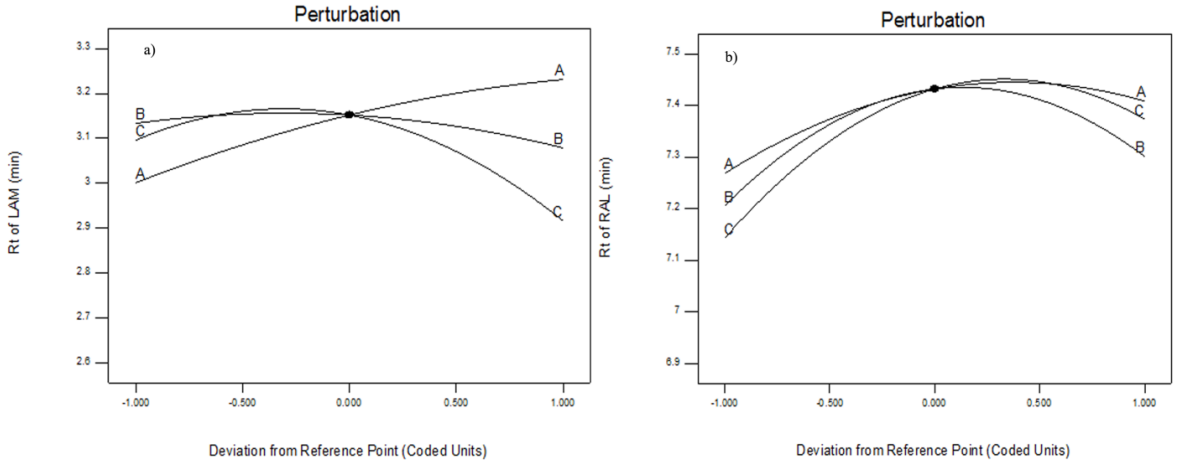

Figure 2. The Perturbation plots of a) Lamivudine and b) Raltegravir

retention time), peak parameters. Mobile phase for this method was selected on the basis of analysis of own experience, literature report of similar studies and traditional trial and error methods. Though, various combination of HPLC grade organic solvent of different polarities such as methanol, chloroform and Acetonitril with buffers was tried in different ratio to resolve the peak of LAM and RAL. Finally, after several tried combinations as suggested by BBD, mobile phase composed of 75% methanol: 15% Acetonitril : 10 % (0.05mM) phosphate buffer (at pH 3.0) showed efficient chromatographic separation of LAM and RAL (10µg/mL) with retention time of 3.13±0.07 minutes and 7.27±0.01 minutes, respectively as shown in **Figure 5**. In the RP-HPLC method development use of methanol than other organic solvents is a commercial approach for routine analysis of analytes alone or in combination.

Optimization of HPLC method by DOE

BBD approach is often used for optimization of isocratic HPLC conditions in chemometric methods. For optimization, main factors were selected on the basis of initial experiment and from literature. The three factors; percentage of methanol in mobile phase (A), pH in mobile phase (B) and flow rate (C) and responses (retention time) of LAM and RAL were selected, respectively. Response surface methodology (RSM) was carried out to identify the effect of different independent variables on the observed responses.

Table 1 described total 17 experimental runs obtained by using BBD with their observed responses and predicted responses. During model selection, the best-fitted models for the Retention time of LAM and RAL were Quadratic model, based on lowest PRESS value, adjusted R^2 value closer to 1 and p values less than 0.05. The quadratic model for three independent factors was validated with analysis of variance (ANOVA) using software and the results were shown in **Table 2**.

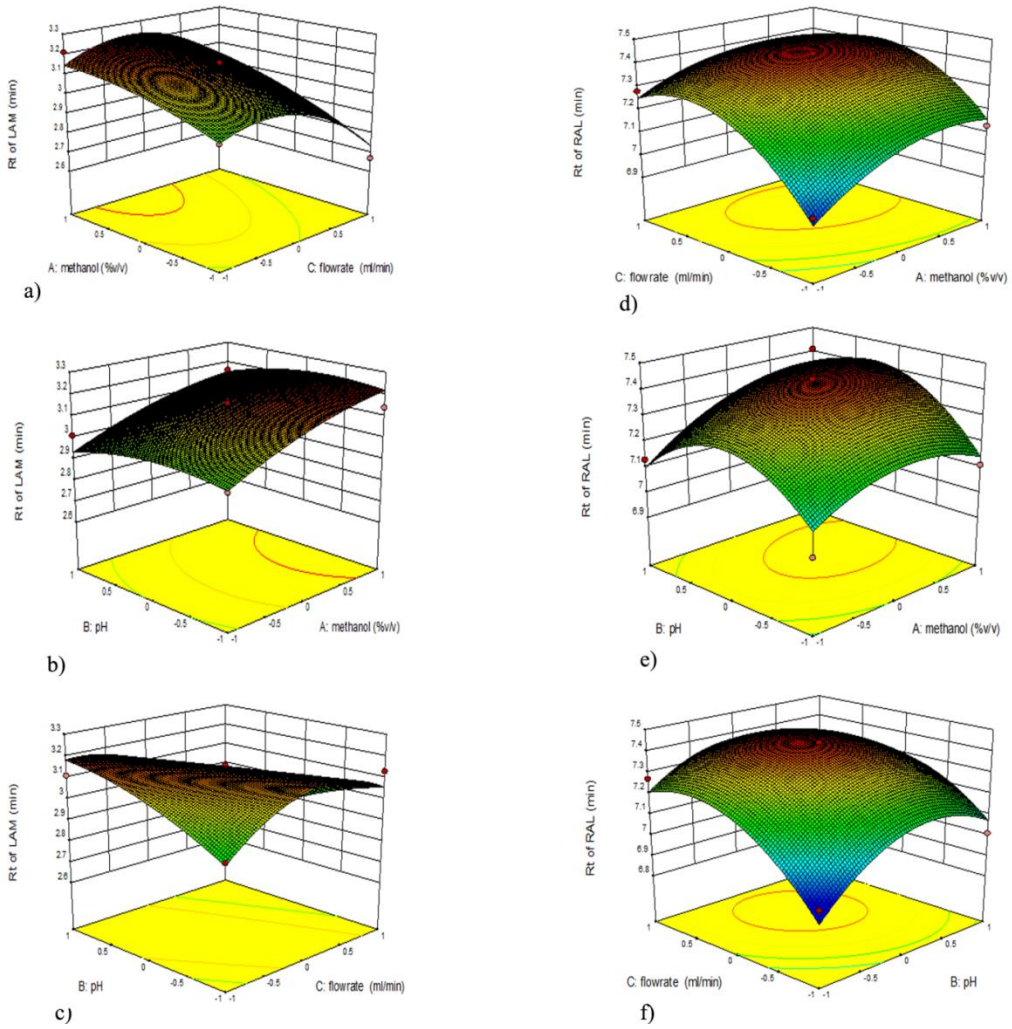

Figure 3. Three dimensional response surface plots for effects of factor A (% methanol) , effects of factor B (pH) and effects of factor C (flow rate, mL/min) on Retention time of Lamivudine and Retention time of Raltegravir

An adequate precision a measure of the signal to noise ratio, greater than 4 is desirable and obtained ratio for LAM and RAL were 10.394 and 10.096, respectively. It was indicated an adequate signal. A coefficient of variation (% CV) was less than 10% which measures the reproducibility of the model. The adjusted R^2 values were within the acceptable limit, which were found to be 0.8306 and 0.8423 for LAM and RAL respectively. It was showed a good relationship between the experimental data and (quadratic models) polynomial equations. The polynomial equation in terms of the actual components and factors was shown in **Table 2.** A positive value represents an effect that favors optimization and negative value shows an inverse relationship between the factor and response [44, 47]. **Table 2** illustrated that A, C, BC and C^2 were significant (<0.0001) model term for Rt of LAM. Similarly for Rt of RAL; A, C, A^2, B2 and C^2 were significant (<0.0001) model term.

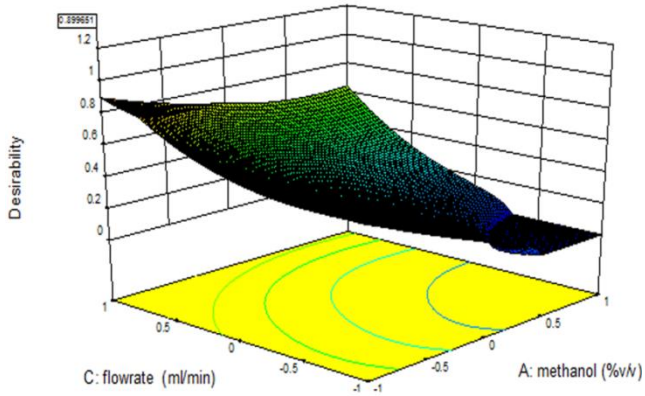

Figure 4. Maximum derringer's desirability function

Table 3. Comparison of experimental and predicted values under optimum condition

Optimum condition		**Response (Rt)**	**Predicted value**	**Experimental value**	**%Residual value**
Factor	Condition				
Methanol (%)	75%				
pH	3.0	LAM	3.22	3.14	2.48
Flow rate	1.2mL/min	RAL	7.14	7.11	0.42

Figure 2 and **Figure 3** presented the Perturbation plots and 3-D response surface plots. It was constructed to evaluate the effect of factors on response of both drugs and also used for the predicted model to better understand the investigated procedure. This type of plots represented the effect of an independent factor on a specific response with all other factors assumed constant at a reference point [38]. A steepest slope or curvature represents the sensitiveness of the response to specific factor.

Figure 2(a) and **2(b)** demonstrated that the concentration of methanol in mobile phase (factor A) and flow rate (factor C) had the most significant effect on the Rt of LAM and RAL as compared with other factors i.e. pH of mobile phase (factor B). It was shown a relationship with retention time of LAM and RAL respectively. Hence, pH was not significantly affect the retention time of LAM and RAL, respectively.

Figure 3 (a) and **(d)** shown that when increasing the concentration of methanol, retention time of both drugs were increased. By increasing the flow rate, retention time of LAM was gradually decreased while retention time of RAL shown a relationship with flow rate. Thus, plots were revealed that at the intermediate levels of flow rate the retention time was found to be optimized. Furthermore, **Figure 3 (b) (c) (e)** and **(f)** indicated that a relationship between pH of the mobile phase and retention time of both drugs. It was found that the increase in pH of mobile phase did not significantly affect the retention time.

Table 4. Analytical parameters of proposed HPLC method for simultaneous estimation of LAM and RAL

Parameters	LAM	RAL
Wavelength (nm)	254	254
Linearity range (µg/mL)	10-100	5-30
Regression coefficient(R^2)	0.998	0.992
Regression equation (Y)	3298.4x+7485.3	41263.2x+16565.5
Slope ±S.D[a].	3298.4±51.97	41263.2±113.24
%RSD[b] of slope	1.57	0.27
Intercept ±S.D[a].	7485.3±1041.70	16565.5±4526.3
Rt	3.13±0.07	2.27±0.01
LOD[c] (µg/mL)	1.04	0.36
LOQ[d](µg/mL)	3.18	1.08

The difference between the predicted and the observed results was found within ±2.50 % as shown in **Table 3**. The percent residual value was calculated by using the given formula (1):

$$Percent\ residual = \frac{Predicted\ results - Observed\ results}{Predicted\ results} x100 \quad (1)$$

In the present study, Derringer's desirability function (D) was used to optimize the one response with same target. The desirability of the optimized factor was shown in **Figure 4.** The desirability values generally in the range of 0-1. If the value is near to zero means the solution of the method is not strong whereas the value toward 1 means the solution or method is very strong [44]. The obtained desirability value was found to be; D=0.899 which indicated that the method is effective. Thus, these coordinates were used to select an optimum experimental condition to analyze LAM and RAL in combination.

Method Validation

Linearity and Ranges

The standard calibration curve was linear over the concentration range 10-100µg/ml for LAM and 5-30µg/ml for RAL. The regression coefficients were found to be 0.998 for LAM and 0.992 for RAL. The regression equations of the area and % Relative standard deviation of slope values in six replicates of both drugs were shown in **Table 4**.

Limit of detection & Limit of quantification

The LOD and LOQ of LAM were found to be 1.04 and 3.18 µg/ mL, respectively, while for RAL were 0.36 and 1.08µg/mL, respectively. **Table 4** indicated that the method was very sensitive to quantify both the drugs.

Table 5. Intraday and Interday precision of the method for binary mixtures of LAM and RAL

Drug	Amount (µg/mL)	Intraday precision (n=3)				Interday precision (n=3)			
		% Recovery	S.D[a].	%RSD[b]	S.E[c].	% Recovery	S.D[a].	%RSD[b]	S.E[c].
LAM	20	101.25	0.31	0.30	0.13	96.35	0.28	0.29	0.11
	60	100.97	0.14	0.14	0.06	100.91	0.38	0.38	0.16
	100	98.51	0.18	0.19	0.07	98.51	0.95	0.96	0.39
RAL	10	98.83	0.85	0.86	0.34	99.46	0.25	0.25	0.10
	20	97.61	1.22	1.25	0.50	97.93	1.43	1.45	0.58
	30	97.41	1.83	1.88	0.74	102.98	0.36	0.35	0.15

Table 6. Recovery study of the method by using the standard addition method for LAM and RAL

Drug	Initial amount (µg/mL)	%Recovery level	Amount added (µg/mL)	% Recovery	S.D[a].	%R.S.D[b]
		80	16	101.25	0.63	0.62
LAM	20	100	20	96.83	1.04	1.07
		120	24	101.60	1.10	1.09
		80	16	102.50	0.63	0.61
RAL	20	100	20	96.50	0.50	0.51
		120	24	101.4	0.86	0.85

Precision and Accuracy

The experiment was repeated three times in one day (intra-day precision) with different time interval. The average % RSD values and Standard error values of LAM and RAL were found within range of 0.14-1.88% and 0.06-0.74 respectively. Similarly, the experiment was repeated on three different days (inter-day precision).The average % RSD values and standard error of LAM and RAL were found in range of 0.25-1.45% and 0.10-0.58, respectively. The method showed good precision for both drugs and data were summarized in **Table 5**.

The accuracy study has been performed by the standard addition method at three concentration level 80%, 100% and 120% by spiking with standard. The percentage recovery were found in the range of 96.5-102.5% and percentage relative standard deviation (%RSD) values were found to be less than 2% in all cases. Satisfactory results were obtained and shown in **Table 6.**

Specificity and Selectivity

Selectivity of the method was examined by preparing several laboratory-prepared binary mixtures of above cited drugs at various concentrations within the linearity ranges as mentioned in **Table 4**. The percentage relative standard deviation (RSD %) of LAM and RAL were found to be less than 2%. Percentage relative standard error (%S.E.) was found within the range of 0.08-0.42% and 0.13-0.26% for LAM and RAL, respectively. The results were shown in **Table 7** and satisfactory results were obtained. The proposed Liquid chromatography was successfully applied for determination of LAM and RAL in laboratory

Table 7. Determination of LAM and RAL in laboratory-prepared binary mixtures by the proposed HPLC method

Mixture	Nominal amount (µg/mL)		Found (µg/mL) (Mean ±S.D[a].)		%R.S.D[b]		E[r] (%)[c]	
	LAM	RAL	LAM	RAL	LAM	RAL	LAM	RAL
1	10	20	9.96±0.04	20.19±0.12	0.37	0.58	0.15	0.24
2	20	10	19.81±0.21	9.86±0.04	1.03	0.37	0.42	0.15
3	20	20	20.08±0.04	20.17±0.13	0.18	0.64	0.08	0.26
4	10	5	9.86±0.03	5.01±0.02	0.30	0.31	0.12	0.13
5	30	30	30.21±0.11	29.85±0.16	0.35	0.52	0.14	0.21

Table 8. Analysis results for laboratory prepared sample solution of LAM and RAL

LAM			RAL		
Labelled amount (mg)	Amount found (mg)	% Mean ± SD[a]	Labelled amount (mg)	Amount found (mg)	%Mean ± SD[a]
150	150.03±0.59	100.02±0.40	300	299.89±1.96	99.96±0.65

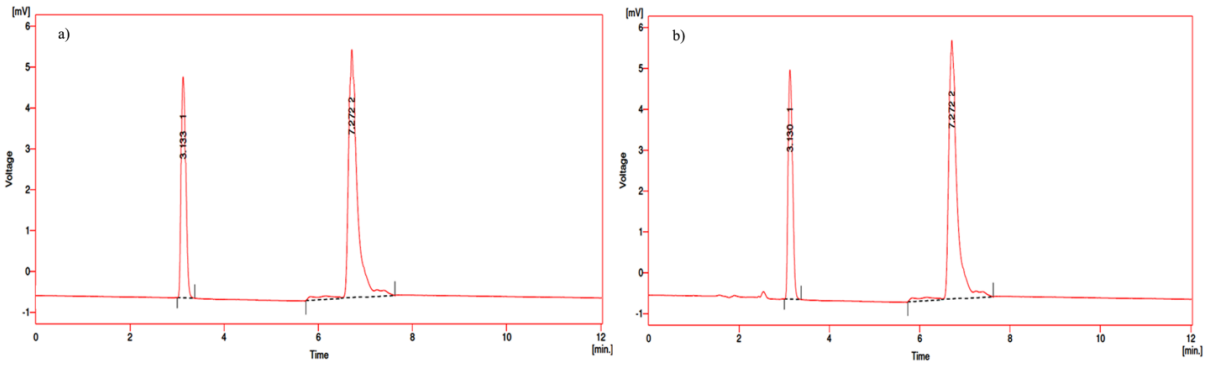

Figure 5. HPLC chromatogram of a) LAM (10µg/mL) (1) and RAL (10µg/mL) (2) in standard binary mitures b) LAM (10µg/mL) (1) and RAL(10µg/mL) (2) in laboratory prepared sample solutions

prepared sample solution. The percentage recoveries were found to be 100.02±0.40 and 99.69±0.65 for LAM and RAL respectively. The obtained results for both drugs were comparable with the corresponding claim percentage. Results were shown in **Table 8**.

Specificity was studied for the examination of the presence of interfering components in the working solution of LAM and RAL. The results were indicated that the retention time of LAM and RAL was 3.13±0.07 and 7.27±0.01 minutes respectively, shown in **Figure 5**. There were no variation in the retention time of the both the compounds as compared with the standard drug solution. They were free from interference from formulation excipients and solvent from each other. The results showed that the proposed HPLC method was selected and specific for determination LAM and RAL simultaneously.

Table 9. Results of robustness study for LAM and RAL

Variable	Optimized value	Range	LAM		RAL	
			%Mean±S.D.[a]	Rt± S.D.[a]	%Mean±S.D.[a]	Rt± S.D.[a]
Methanol (%)		70	100.23±0.15	3.14±0.02	100.09±0.11	7.26±0.02
	75	75	98.43±0.56	3.13±0.03	98.80±0.08	7.27±0.02
		80	100.03±0.12	3.13±0.01	100.03±0.12	7.28±0.01
Mobile phase pH		2.5	100.5±0.36	3.13±0.02	100.19±0.83	7.27±0.02
	3	3	98.80±0.05	3.14±0.01	98.87±0.10	7.26±0.02
		3.5	100.02±0.13	3.14±0.02	100.09±0.22	7.28±0.02
Flow rate (mL/min)		0.8	99.96±0.59	3.14±0.02	99.53±0.78	7.27±0.02
	1.2	1.2	98.77±0.01	3.13±0.01	99.11±0.57	7.28±0.02
		1.6	100.35±0.44	3.13±0.01	100.02±0.13	7.27±0.03

Robustness

Robustness study was performed by slight variations in the optimized conditions such as concentration of methanol in mobile phase mobile phase by ±5%, pH of mobile phase by ± 0.5 and flow rate of the mobile phase by ±0.4mL. The results were not significantly affected by the slight variations and results were shown in **Table 9.** Thus, the proposed method was found to be robust.

CONCLUSION

A simple, rapid and sensitive RP-HPLC was effectively developed for the simultaneous estimation of LAM and RAL using UV- visible detection in binary mixture. The proposed RP-HPLC method was concurrently optimized by using Box Behnken design in response surface methodology and Derringer's desirability function. It gave more information in less time by reducing the number of experiments. The various validation characteristics were applied and determined, to assure the sensitivity of the method. This study also confirmed that, the chromatographic techniques provide a complete profile of separation process. Therefore, this optimized and validated RP-HPLC-UV method can be potentially used for estimation of these drugs in bulk form either alone and in combination as a routine quality control analysis.

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