RESEARCH ARTICLE

An efficient RP-HPLC-PDA Method for Estimating Dolutegravir and Lamivudine in Combined Pharmaceutical Formulations using a Box-Behnken Design Approach

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ABSTRACT

This study used a quality-by-design (QbD) method to build a novel, high-performance reverse-phase fluid chromatography (RP-HPLC) system with diode array detection (DAD) for the simultaneous measurement of dolutegravir (DLG) and lamivudine (LMV) tablets. In accordance with the International Council for Harmonization's (ICH) requirements, chromatographic conditions were adjusted utilizing the Box-Behnken design (BBD), and the resultant technique was verified for linearity, system appropriateness, precision, accuracy, sensitivity, robustness, and stability of the solution. At retention durations of 2,271 and 3,431 minutes, LMV and DLG peaks were separated using a C-18 column with 150 x 4.6 mm and 5 µm particles. The mobile phase was 0.1% orthophosphoric acid (OPA): acetonitrile (ACN) (50:50, v/v) at a flow rate of 0.8 mL/min and a pH of 3 at 25°C. Peaks were seen at 254 nm and the volume of sample injection was 20L. The percent strength of the commercially available tablet is 98.89 and 98.76 for LMV and DLG, respectively, using a standard calibration curve. The developed RP-HPLC technique's stability is shown by the suggested RP-HPLC method's capacity to identify LMV and DLG in the existence of their degradation products. As per ICH requirements, the findings of the validation parameters for linearity, system appropriateness, accuracy, precision, robustness, and sensitivity were all within acceptable limits. It is simple to use, accurate, efficient, and reasonably priced to employ the innovative RP-HPLC technology with BBD application for QbD.

Keywords: Lamivudine, International Council for Harmonization, Dolutegravir, Analytical quality-by-design, Box-Behnken design.

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INTRODUCTION

In order to manage human immunodeficiency virus 1 (HIV1) infections, healthcare professionals often prescribe a combination of antiretroviral medications, including dolutegravir (DLG). This drug is known for its efficacy in suppressing the virus and improving the life quality of HIV patients. These are substances that prevent the HIV-1 integrase from integrating its genome into the host cell by stopping the strand transfer process. They are commonly referred to as integrase strand transfer inhibitors (INSTIs). This medication has little toxicity and great tolerance because its impact has no similarity in human host cells.[1,2] Lamivudine (LMV) is a drug that is utilized to manage and treat HIV infections and hepatitis B. It belongs to the category of synthetic nucleoside analogs, which means it mimics the structure of nucleosides that are present in DNA and RNA (Figure 1). Once it enters the cells, it undergoes a process called intracellular phosphorylation that leads to the formation of its active metabolite, known as lamivudine triphosphate or L-TP. This compound plays a crucial role in inhibiting the replication of the virus and preventing it from spreading throughout the body. HIV reverse transcriptase and HBV polymerase incorporate this nucleoside analog into viral DNA, breaking the DNA chain.[1,2] The therapeutic uses of antiretroviral drugs have advanced, with the outcome of different drug combinations having fewer side effects.

However, cutting-edge research in multidrug therapy has found that the two-drug combination of LMV and DHG effectively controls HIV disease.[3,4] A fixed-dose combination containing 300 mg of LMV and 50 mg of DHG has therefore been

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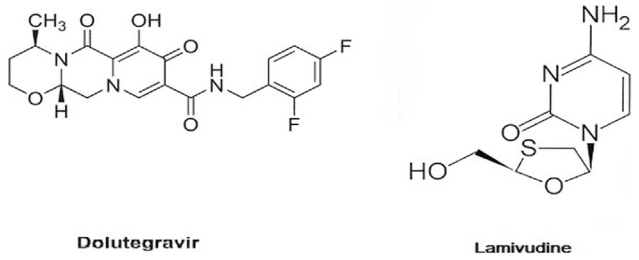

Figure 1: Structures of dolutegravir and lamivudine

suggested by the Committee on Human Medicinal Products (CHMP) to efficiently treat HIV-1 in adult and adolescent patients without known or suspected protection against integrase inhibitors.[5-9] In order for a medicine to be examined either alone or in combination with other pharmaceuticals in the pharmaceutical sector, an effective analytical technique is necessary. Numerous analytical techniques, including the UV and reverse phase high performance liquid chromatography (RP-HPLC) methods, have been reported to estimate lamivudine and dolutegravir separately, according to a thorough literature search.[11,12] Several RP-HPLC methods are available to estimate concomitant LMV, DHG, and tenofovir disproxil fumarate or butcaver sulfate or abacavir in triple combinations.[9,15-18] Dolutegravir and Lamivudine fixed dose film-coated tablets to treat HIV-1 were authorized in April 2019, according to the FDA's official release. The researchers also estimated DLG through a UV spectrophotometer with the Quality by design (QbD) method.[19] Through RP-HPLC, the amount of DLG and LMV was evaluated in a study with the box behnken design (BBD) method in nanoliposomes.[20] Some limitations of these research methods may be impediments to analysis, such as high cost, column congestion due to the use of high resistance buffers, and solvents such as tetrabutyl, and less accurate results are obtained due to high flow rates.[21-24] There are currently a number of benefits to using QbD while developing and validating analytical procedures. The most important advantages are time savings, accuracy of results, and cost efficiency. Consequently, many academics have used QbD to conduct qualitative and quantitative analyses.[19] In order to build a quick, precise, timely, and cost-effective technique for this medication combination, the RP-HPLC-PDA method was established in this work with the use of BBD. Creating this new technique has benefited greatly from the current literature. The capacity to differentiate the column's stationary phase and the short retention duration with excellent resolution are both taken into account in this investigation. Positive outcomes were discovered when certain BBD optimization requirements were met. Additionally, the RPHPLC-PDA technique was created and verified in accordance with International Council for Harmonisation (ICH) standards. This strategy is innovative, simple, accurate, and succinct. This approach is unique and cost-effective since both medicines have a shorter retention period. To our knowledge, there have been no reports of the simultaneous measurement of LMV and DLG using the RPHPLC-PDA technique with BBD in tablet form. The traditional analysis will gain from this method.

MATERIALS AND METHODS

LMV and DLG purity standards (99% purity) were bought from Hetero Drugs Ltd., a Chemical company based in Hyderabad, India. DLG 50 mg and LMV 300 mg (manufactured by Ecure Pharmaceuticals) was procured from a medical shop and branded Twinaqt. The solvents like methanol, acetonitrile, OPA, and Milli-Q water HPLC-grade were utilized.

Instrumentation

The HPLC 2695 system is a highly efficient and versatile system that is often utilized alongside an integrated PDA detector and Empower 2 software, both of which are developed by Waters Union. This combination of technology provides users with a powerful tool for accurate and reliable analytical testing. Column specifications are DIKAM Spursil EP C18 (150 x 4.6 mm, 5 µm). Meltronics generator (BVK company) was used to degas the solvent and improve the solute's solubility. The Adwa – AD 1020 pH meter is also used in the analysis.

BBD Application to Optimize Chromatography Condition

Analytical target profile (ATP) in immediate assessment, the highest level of by making the separation parameters optimal, the chromatogram will become better. The peaks should be nicely resolved and have little drag under ideal circumstances. Additionally, ATP must adhere to the quantitative technique's quality requirements.[22]

Risk Assessment Research

Risk assessment studies are conducted to keep track of how different variables affect the target method quality profile (TMQP). Before examining the risk valuation, evaluating the relationship of the TMPQ parameters to each other is necessary, empowered by critical analytical attributes (CAA). Identifying the primary reason for worry and the origin of issues like faults, deviations, mistakes, or failures might be simple throughout the risk assessment study process. Lists of each risk factor are yet another potential output of risk assessment research. After that, the risk variables are categorized as low, medium, and high. Seven parameters were screened for in the current study. Three of the seven variables were taken into account for the research. The reaction of each medication, including retention duration and tailing coefficient, was taken into account to optimize the chromatographic settings. In this experiment, flow rate, buffer pH strength, and column length were taken into consideration.[21,22]

Optimization

To improve the chromatographic conditions, BBD was applied. Software utilized by Design Expert, Stat-Ease Inc., Minneapolis, USA, software version 13.0.3.0. For the purposes of optimizing retention duration and drug coefficient, responses for each drug were taken into account. The flow rate, the buffer's pH level, and the column length were all taken into account in this experiment. The provided collection of variables was the subject of seventeen experiments.

Standard Solutions Preparation

In a clean 100 mL volumetric flask, properly transfer and weigh 300 mg of lamivudine and 50 mg of standard dolutegravir.

Pour roughly 7 mL of diluent and start sonication for a full dissolution. Then, using the same solvent, increase the volume to the required level (Stock Solution). In 3.0 mL of the abovementioned solution was piped into a 10 mL volumetric container, and then diluted to the required concentration. Buffer-OPA has been created in the lab by mixing a little quantity of milli Q water with 1-mL of OPA from 85% water in 1000 mL of a volumetric container. After thorough mixing, milli Q water was added to the mixture to increase the volume to 1000 mL.

Calibration Curve

The calibration curves created at λ_{max} (254 nm) for both medications were optimized. Each medication was made in six dilutions. For DLG, a range of 5 to 25 µg per mL has been determined, and for LMV, a range of 30 to 150 µg per mL. Before creating the calibration curve, the LoQ and LoD for both medications were decided upon. Table 1 displays the specifics (Figure 2). For both medications, three quality control samples were created: high-level quality controls (HQC), medium-level quality controls (MQC), low-level quality controls (LQC). LQC (30 µg/mL), HQC" (150 µg/mL), and MQC (90 µg/mL) were the concentrations for LMV, while MQC (15 µg/mL), LQC (5 µg/mL), and HQC (25 µg/mL) were the values for DLG.

Preparation of Sample Solutions

A tottal of 20 drugs were weighed as well as crushed precisely. Each tablet's typical weight was calculated. Weigh one powdered tablet's worth of material, add 7 mL of diluent, 300 mg of lamivudine, and 50 mg of dolutegravir to a 100 mL volumetric flask. Sonicate the mixture until it is fully dissolved, then add solvent (stock solution) to fill the remaining volume to the mark. Add the diluent up to the mark after adding 3 mL of the abovementioned "stock solution" into a volumetric container with a 10 mL capacity. The solution's ultimate concentrations were 15 µg/mL DLG and 90 µg/mL LMV.

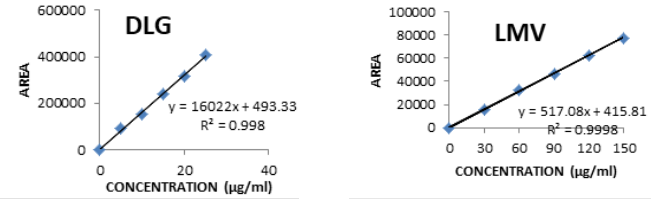

Figure 2: Linearity of LMV and DLG

Table 1: Calibration curve details

	LMV	*DLG*
Linearity Range (µg/mL)	30-150	5-25
Calibration curve equation	y=517.08x + 415.81	Y=16022x + 493.33
Determination coefficient (R^2)	0.9998	0.998
Correlation coefficient (R)	0.9993	0.9993
LoQ (µg/mL)	3.56	1.38
LoD (µg/mL)	1.75	0.5

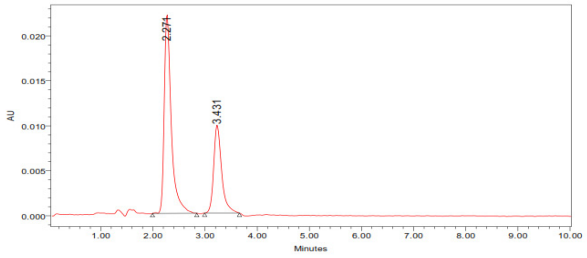

Figure 3: Optimized chromatogram of LMV and DLG

Method Development

A C 18-column DIKAM Spursil EP C18 (5 µm, 150 x 4.6 mm) reversed stationary phase was employed for the chromatographic separation. The composition of mobile phase was 50:50 ratio of acetonitrile (ACN) and 0.1% v/v OPA buffer, pH 3, used at a flow rate of 0.8 mL/min. The analytes were determined at a wavelength range of 254 nm by employing a PDA detector. The analysis process was carried out for a duration of 10 minutes at a temperature of 25°C, with the utilization of isocratic separation. The retention times for LMV and DLG were recorded as 2.271 and 3.431 minutes, respectively. Figure 3 shows the optimized chromatograms of LMV and DLG.

System suitability

Six replica injections of newly made standard solutions were employed to evaluate the system's suitability. The percent RSD value (2%) fell within allowable limits. The experiment took into account the theoretical plate count, resolution, and tailing factor. As a result, the percent RSD values for each parameter for both medicines were determined.

Linearity

Both the LMV and the DLG medicines were produced using standard solutions. The linearity of medications was assessed over the specified content ranges (30–150 µg/mL and 5–25 µg/mL for LMV and DLG, respectively). He carried out each experiment three times. The linearity was shown using the multivariate least-squares approach.

Accuracy

Intraday and intraday analyses served as evidence of the accuracy of the method. A content of 100% (90 and 15 µg/mL for LMV and DLG) was used in precision studies. Drug analysis was conducted on 6 consecutive days at the given concentrations to check the inter-day precision.

Precision

To assess precision, the summation method of measuring drug recovery was used. To predetermine concentrations, concentrations of 0, 50, 100, and 150% of standard drugs were added (90 µg/mL for LMV and 15 µg/mL for DLG). The results obtained and Found them to be acceptable.

Ruggedness

This approach is reliable. This was verified by looking at data collected by several analysts using various analytical tools on many days.

Robustness

It was tested by intentionally varying 3 parameters of the optimal chromatographic conditions, including the composition of mobile phase (+5%), detection of wavelength (+2 nm) and flow rate (+0.1 mL/min).

LoQ and LoD

Empower2 software used the signal to noise ratio technique to calculate LoQ and LoD.

Specificity

A specificity evaluation for this technique was conducted to determine if drug retention time interference from contaminants will affect analyte separation, identification, and quantification. Under the predetermined chromatographic conditions, a blank solution was injected onto the column during the experiments before injecting a standard drug solution.

RESULTS

Optimization: Following risk assessment research, three parameters were taken into account while using BBD. Factors are buffer pH, flow rate, and column length. Time of retention and tailing factor were observed as dependent variables for both drugs.

LMV Tailing Factor

The built-in value of drug tailing coefficient showed it to be significant. The final equations are the coded coefficients A (+0.0120), B (+0.0345), C (-0.0234), AB (+0.0898), AC (-0.0012), BC (-0.0625), A2 (+ 0.2132), B2 (+0.0475), and C2 (+0.0938) values in the final formula. The built-in model graph in Figure 3 shows predicted and actual color point values, and Figures 4 and 5 details the 3D surface design points.

Retention Time of Dolutegravir

A quadratic model was utilized for the ANOVA. This trivial model summarizes the quadratic model and the missing goodness test. A built-in value for retention time of drug showed not significance. The built-in model graph in Figure 6 shows the predicted and actual color point values, and Figure 7 shows the 3D surface design point (Table 2).

System suitability

Further 6 injections of newly made standard solutions were made. Both medicines ' theoretical plate, resolution, and tailing characteristics were evaluated as three responses. The test findings' %RSD (<2.0%) was within allowable limits.

Standard solutions were prepared and demonstrated linearity over the content range of 30 to 150 µg/mL for LMV and 5 to 25 µg/mL for DLG. A total of 6 dilutions of each drug were prepared and responses were measured in triplicate. The linearity of calibration curves was examined using multivariate least-squares analysis. Additionally, a curve between concentration and peak area was recorded. The outcomes are revealed in Table 1 and Figure 2. Using the provided formulae, LoD and LoQ were determined.

LoQ = 10xavg.std./slope

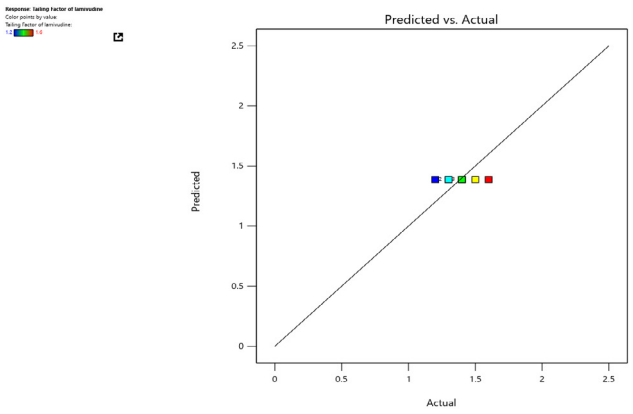

Figure 4: Green represents the optimal value for the lamivudine tailing factor

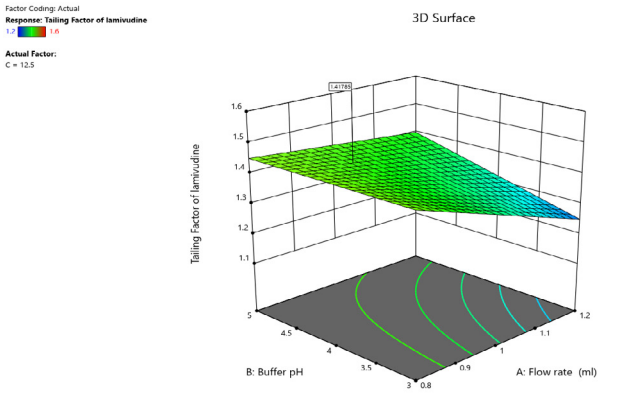

Figure 5: BBD trial tailing factor and LMV's built-in 3D surface design points

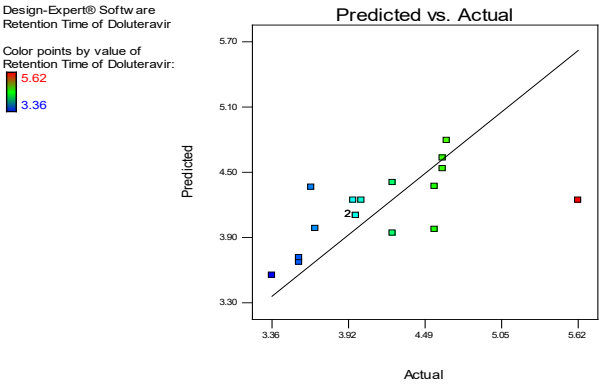

Figure 6: Dolutegravir retention time measured in colour points; optimal value is green

LoD = 3.3 x avg.std/slope

Slope = Calibration curve slope

Avg. std = average standard deviation responses.

With the help of equation, the LMV LoQ and LoD were assessed to be 3.56 and 1.75 µg/mL, correspondingly, and the DLG LoQ and LoD were set to 1.38 and 0.5 µg/mL, respectively. According to ICH rules, all findings were obtained within acceptance requirements. The suitability system are summarised in Table 3.

Table 2: ANOVA for response surface linear model

ANOVA table [Partial sum of squares - Type III]						
Source	*Sum of squares*	*df*	*Mean square*	*F-value*	*p-value*	*Prob > F*
Model	1.85	3	0.62	2.46	0.1088	non significance
A-Flow Rate	3.620E-003	1	3.620E-003	0.014	0.9061	
B-BufferpH Strength	1.33	1	1.33	5.30	0.0385	
C-Column Length	0.40	1	0.40	1.58	0.2038	
Residual	3.26	13	0.25			
Lack of Fit	1.48	10	0.15	0.25	0.9583	non significance
Pure Error	1.77	3	0.59			
Cor Total	5.10	16				

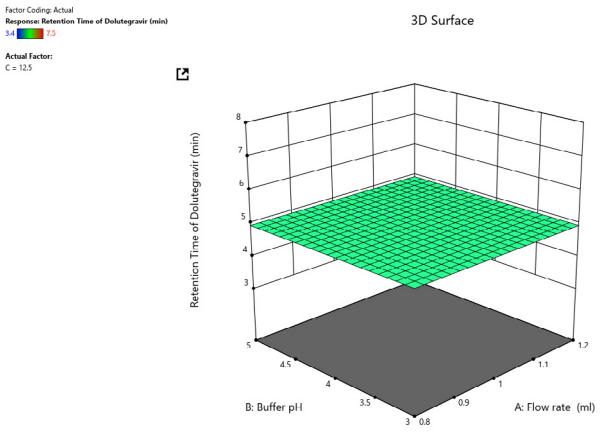

Figure 7: BBD trial retention period and DLG's built-in 3D surface design points

Precision and Accuracy

They were determined utilizing repeated experiments (n = 6). Intraday and intraday analyses finally determined the accuracy of the established technique. Outcome analysis in % RSD was calculated and found to be acceptable (less than 2.0%). The accuracy of this technique was measured. He examined each content level in six replicates—0, 50, 100, and 150% and determined the percentage recovery for each. The percentage RSD values that were obtained were satisfactory. The outcomes are shown in Tables 4 and 5.

Robustness

This procedure's reliability was looked at. Three things were taken into account. We purposefully changed the ideal chromatographic conditions a little and tracked the outcomes. The wavelength for detecting eluted materials was modified by +2%, the mobile phase flow rate was altered by +10%, and the composition of mobile phase was altered by +5% under optimal circumstances. Experimental results show that small changes in the given environments do not cause large variations in the response. Retention time, tailing factor, number of plates, and peak resolution. Three replicate injections were used for each modified condition. Experimental results confirmed the robustness of this method is depicted in Table 6.

DISCUSSION

One of the primary goals of this work was to develop his RP-HPLC-PDA technique utilizing the BBD methodology to accurately assess LMV and DLG in bulk and pharmaceutical dose forms. His LMV and DLG were thus evaluated using several chromatographic parameters in both bulk and prescription dose forms. The experiment's outcomes were thus within accepted limits. Results integrated into ANOVA were driven by the optimization of chromatographic conditions using BBD. Dependent variable retention time and a number of plates have been optimized. The significance of the model is not shown by the outcomes of the *p-value* as well as the f-value analyses. Additionally, the dependent variable model

Table 3: Suitability test for system

		DLG			*LMV*		
S.No.	*Sample*	*Resolution*	*Tailing factor*	*Plate count*	*resolution*	*Tailing factor*	*Plate count*
1	STD-1	3.7	1.37	5410	NA	1.30	5610
2	STD-2	3.8	1.40	5398	NA	1.31	5615
3	STD-3	3.75	1.38	5412	NA	1.32	5610
4	STD-4	3.8	1.39	5400	NA	1.30	5625
5	STD-5	3.8	1.38	5433	NA	1.33	5620
6	STD-6	3.75	1.39	5397	NA	1.31	5620
	Average	3.76	1.38	5408		1.31	5616
	SD	0.05	0.01	46.11		0.01	47.08
	%RSD	1.48	0.60	1.45	0.86	0.87	

Table 4: Precision analysis

Precision									
	LMV					*DLG*			
QC Samples	*Inter day*		*Intra day*		*Qc samples*	*Inter day*		*Intra day*	
	SD	*%RSD*	*SD*	*%RSD*		*SD*	*%RSD*	*SD*	*%RSD*
LQC (30 µg/mL)	220.65	0.24	596.25	0.32	LQC (5 µg/mL)	542.11	0.36	546.88	0.63
MQC (90 µg/mL)	259.99	0.05	682.39	0.09	MQC (15 µg/mL)	230.43	0.09	110.25	0.03
HQC (150 µg/mL)	540.32	0.01	5621.32	0.73	HQC (30 µg/mL)	620.65	0.18	1168.98	0.22

Table 5: Developed method accuracy

Accuracy						
	DLG			*LMV*		
%Excess Drug Added	*Avg %Recovered*	*SD*	*%RSD*	*Avg %Recovered*	*SD*	*%RSD*
0	100.32	0.96	0.95	101.23	0.98	0.97
50	101.45	0.68	0.67	100.52	0.71	0.70
100	99.95	0.52	0.52	99.98	1.14	1.12
150	100.42	0.32	0.31	100.75	1.02	1.01

Table 6: Developed method robustness

	LMV			*DLG*		
	Mean Area	*SD*	*%RSD*	*Mean Area*	*SD*	*%RSD*
Flow rate at 0.6 mL/min	2,08,165	199.65	0.08	54,212	99.82	0.13
Flow rate at 1.0 mL/min	2,08,260	228.32	0.12	54,190	101.65	0.12
0.1%OPA (38%) + ACN (62%)	2,08,190	202.16	0.07	54,386	100.89	0.11”
0.1%OPA (42%) + ACN (58%)	2,08,263	284.13	0.21	54,128	110.45	0.19
Wavelength 250 nm	2,08,188	222.88	0.13	54,862	109.87	0.14
Wavelength 260 nm	2,08,285	286.75	0.25	54,769	112.83	0.18

plots for the expected and actual responses as well as the 3D responses, demonstrate a respectable level of agreement between the predicted and adjusted R2. For DLG and LMV, the “calibration curves were discovered to be linear across the concentration ranges of 5 to 25 g/mL and 30 to 150 g/mL, respectively. At a volume of 20 L, elution was discovered at 254 nm. The drug retention durations for LMV and DLG, respectively, were 2.271 and 3.341 minutes under optimal chromatographic conditions. The produced chromatogram’s theoretical tailing factor and LMV values were 1.70 and 3031, respectively. The DLG was 1.39 and 4598. The peaks in the shown columns are well-defined, sharp, and symmetrical. The received resolution a 3.6 rating. Statistics were utilized to verify the suggested method’s linearity. The created method’s accuracy and precision were confirmed by the percent RSD values, which in terms of precision and accuracy were less than 2.0%. Several research articles are accessible for the RP-HPLC simultaneous measurement of both medications. However, there are no research papers using his BBD for simultaneous quantitation of his LMV and DLG in tablet forms. The present study, optimization of chromatographic conditions, had been carried out using his QbD tool. This makes the technique novel. Pre-measured by RP-HPLC, the retention times of DLG and LMV were 6.36, 2.16, 3.4, and 5.0 minutes, respectively. Under the specified chromatographic conditions, the retention periods of both medications were found to be 2.271 minutes for LMV and 3.341 minutes for DLG in this study. Short run periods enable the observation of both peaks. This technique can evaluate more samples in less time, making it helpful for regular analysis. For instance, if the run duration is 5 minutes, it’s possible to see both peaks, necessitating further sample analysis. A time-saving approach ultimately translates into cost advantages. The development of this method is novel because of its short retention time and the advantages of using the BBD.

CONCLUSION

The devised chromatographical technique is simple to use, selective and precise. Additionally, its stability is shown, enabling the simultaneous estimate of LMV and DLG in bulk and medicinal dose form. Using the BBD in chromatograph optimization for medication determination is a revolutionary strategy that may save time and cost while improving analysis quality by emphasizing process quality. According to the QbD pooled analysis of variance (ANOVA), the findings did not exhibit a significant statistical difference. Furthermore, the process of sampling was found to be relatively straightforward, and the subsequent analysis was conducted in a time-efficient manner. Elution uses an isocratic process. We are unaware of any attempts to evaluate this pharmacological combination using QbD methods in bulk as well as tablet

forms. Nevertheless, the amounts of all active compounds were measured, and sharp peaks were produced. It was successful in determining the concentrations of LMV and DLG in bulk as well as dose forms using the devised chromatographic technique. It is possible to examine LMV and DLG estimates in plasma and other bodily fluids by extending this. It may also be used to calculate the stability samples for quality control.

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