

Development and Validation of Stability Indicating Method for Simultaneous Estimation of Ilaprazole and Levosulpiride in Bulk and Capsules Using Design of Experiment Approach

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DOI: 10.31080/ASPS.2020.04.475

Received: December 26, 2019

Published: January 08, 2020

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Abstract

A stability indicating High Performance Thin Layer Chromatography (HPTLC) was developed for the simultaneous estimation of Levosulpiride and Ilaprazole in bulk and capsule dosage form. The chromatographic conditions were optimized using Design of Experiment (DoE); where in the critical factors for separation of both drugs were identified using Taguchi design. Further, optimization was done using Central Composite design and it was then subjected to forced degradation study. The separation was achieved using mobile phase Toluene: Ethyl acetate: Methanol: Tri ethyl amine (TEA) (4.52: 2.5: 2.02: 0.2% v/v/v/v) with R_f values of 0.46 and 0.71 for Levosulpiride and Ilaprazole respectively. Linearity was observed in the concentration range of 3750-22500 ng/band for Levosulpiride and 500-3000 ng/band for Ilaprazole. Developed method was validated according to ICH guidelines. The developed method can be used for separation and simultaneous estimation of aforesaid drugs in capsule dosage form as well as in presence of degradation products.

Keywords: Central Composite Design; Levosulpiride; Ilaprazole; HPTLC; Stability Indicating

Introduction

Ilaprazole is a Proton Pump Inhibitor that suppresses gastric acid secretion by specific Inhibition of The H⁺/K⁺-ATPase in the Gastric Parietal cell thus reducing gastric acidity [1,2]. Levosulpiride is a substituted benzamide derivative and a selective dopamine D₂ antagonist with antipsychotic and antidepressant activity. The Prokinetic effect of Levosulpiride is mediated through the blockade of enteric (neuronal and muscular) inhibitory Dopamine D₂ receptors. Levosulpiride acts as a moderate agonist at the 5-HT₄ receptor. The serotonergic (5-HT₄) component of Levosulpiride may enhance its therapeutic efficacy in gastrointestinal disorders. Together, they exhibit beneficial effects in Gastro Esophageal Reflux Disease (GERD) [3,4].

Ilaprazole is a benzimidazole derivative having IUPAC name; 2- [(4-methoxy-3-methylpyridin-2-yl) methylsulfinyl]-6-pyrrol-1-yl-1H-benzimidazole whereas Levosulpiride is chemically (s)-(-)-5-Aminosulfonyl-N-[(1-ethyl-2-pyrrolidinyl) methyl]-2-methoxybenzamide. The structures of both drugs are shown in Figure 1.

Few analytical methods such as HPLC, LC/MS, and UV spectrophotometry are available for estimation of LEVO and ILA individually [5-11]. So far only one HPLC method has been reported for their simultaneous estimation in their combined dosage form [12]. Also, the reported method did not use any methodical approach like DoE and did not reveal any information related to stability of the components in the capsule. Further, the reported method uti-

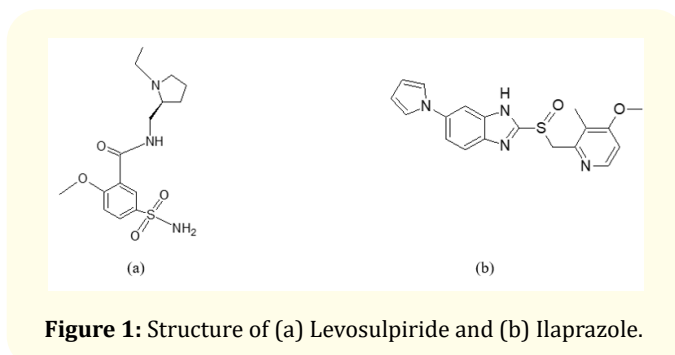


Figure 1: Structure of (a) Levosulpiride and (b) Ilaprazole.

lizes high amount of methanol in mobile phase for separation of LEVO and ILA. So, there is a need to develop HPTLC method (cost effective and rapid) using DoE approach along with study of various factors during forced degradation of LEVO and ILA. The developed method was then validated as per ICH guidelines.

Materials and Methods

Materials and software

Working standards of Levosulpiride and Ilaprazole were supplied as gift samples from Aeon Formulations Pvt. Ltd., Puducherry, India. All solvents Toluene, Ethyl acetate, Methanol, TEA (HPLC grade) were purchased from Finar chemicals Ltd., Ahmedabad, India. Capsules containing 75/10 mg of LEVO/ILA were purchased from local market. Experimental design (Taguchi/ CCD), desirability functions and data analysis calculations were performed using Design-Expert® version 7.0.0.

Preparation of standard stock solutions

LEVO and ILA (10 mg) were weighed accurately and transferred separately in 10 mL volumetric flasks, dissolved and diluted to the mark with methanol to obtain standard solution having concentrations of LEVO and ILA (1000 µg/mL). From above solution 7.5 mL of LEVO and 1 mL of ILA were transferred into 10 mL volumetric flask and volume was made up to mark with methanol to get combined working standard solution of LEVO (750 µg/mL) and ILA (100 µg/mL). The prepared solutions were used for spotting on TLC plates.

Preparation of test solution

Twenty capsules of Iladac L containing 75 mg of LEVO and 10 mg of ILA were accurately weighed and average weight was calculated. All the capsules were crushed to fine powder and quantity equivalent to 75 mg of LEVO and 10 mg of ILA were weighed and transferred to 100 mL volumetric flask. To the same flask, 50 mL methanol was added, and sonicated for 10 min and volume was made up to the mark with methanol. Flask contents were filtered using whatman filter paper no. 41 and used further for spotting on TLC plates.

Chromatographic conditions

The plates were prewashed by methanol and activated at 60°C for 2 min prior to chromatography. Spotting on plates were done by Camag Hamilton syringe (100 µL) on pre-coated silica gel aluminium plate 60F254, (10 × 10 cm; E. Merck, Darmstadt, Germany) using a Linomat V Camag (Muttentz, Switzerland) sample applicator. Before the application of sample it was filtered to 0.22 µm Nylon filter. All solutions were applied at a constant application rate of 0.1 µL/s. The slit dimension was kept at 5 × 0.45 mm and 10 mm/s scanning speed was employed. The mobile phase composed of Toluene: Ethyl acetate: Methanol: TEA (4.5: 2.5: 2: 0.2 v/v/v/v) was used for separation of LIVO and ILA. Linear ascending development was carried out in 10 × 10 cm twin - trough glass chamber saturated with the mobile phase to a distance of 80 mm. The chamber was saturated with mobile phase for 20 min at room temperature (25 ± 2°C) and at relative humidity of 55 ± 5%. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometer scanning performed on Camag TLC scanner III in the absorbance mode was at 255 nm. The deuterium lamp emitting a continuous UV spectrum in the range of 200- 300 nm was used for scanning of components.

Optimization of chromatographic conditions using Design of Experiment (DoE)

There are many factors that affect the separation of components in chromatography. The conventional method involves trial and error method for development of chromatographic method. The limitation of the conventional approach is that it does not give any idea about interaction of different factors as it is OFAT (one factor at a time) analysis. DoE helps in understanding the effect of interaction of various factors on the separation of components.

Hence, to screen important factors; amongst all possible factors screening design (Taguchi) was initially used [13]. The important factors selected as change in mobile phase ratio [amount of methanol and TEA was kept constant (mL)], development distance (mm), saturation time (min), time from spot to chromatogram (min), time from chromatogram to scan (min), band size (mm) and wavelength (nm). Taguchi design was performed using Design Expert 7.0.0 that gave an 8-run trial. Critical factors were selected on the basis of % contribution of factors as shown in Table 1.

The critical factors were now considered as independent variables and retardation factor of both drugs were considered as dependent variable to perform second order design: central composite design using same software. All experiments were performed in triplicate and retardation factors values were reported for all trials. The mobile phase ratio (X_1), development distance (X_2) and saturation time (X_3) were selected as factors. The higher and lower values of factors were selected as mentioned in Table 2. The trials were conducted as mentioned in Table 3. Retardation factor (R_f) and area of the drug were taken as responses (Y). The non-linear computer generated quadratic model is given as

$$Y = b_0 - b_1 X_1 + b_2 X_2 - b_3 X_3 - b_4 X_1 X_2 - b_5 X_1 X_3 + b_6 X_2 X_3 - b_7 X_1^2 + b_8 X_2^2 + b_9 X_3^2$$

Where, b_0, b_1, \dots, b_9 etc are coefficients. X_1, X_2, X_3 are factors.

Validation parameters

The developed method was validated as per ICH guidelines [14] for parameters mentioned below: linearity, precision, accuracy, specificity, limit of detection (LOD), limit of quantitation (LOQ), and robustness.

Linearity and range

The linear response of LEVO and ILA were determined by analyzing six independent levels of the calibration curve in the range of 3750 – 22500 ng/band for LEVO (5, 10, 15, 20, 25, 30 µL of 750 µg/mL) and 500 -3000 ng/band for ILA (5, 10, 15, 20, 25, 30 µL of 100 µg/mL). Linearity study was performed in triplicate. The calibration curves of peak area Vs concentration were plotted for both drugs individually and regression analysis was performed for each of them. The linear equation, correlation coefficient value, intercept was calculated using MS Excel sheet for each drug.

Precision (Reproducibility)

Method precision

Method precision was performed by preparing the test solution (as mentioned above) for six times and 20 µL of each test solution was applied on same TLC plate having 15000 ng/band of LEVO and 2000 ng/band of ILA. Plate was developed and analyzed using the avowed chromatographic conditions. The areas of six replicate bands were measured and %RSD was calculated.

Intraday and interday precision

It was performed by using test solution (750 µg/mL of LEVO and 100 µg/mL ILA) and applying 15, 20, 25 µL by Linomat V for on same TLC plate on same day for intra day precision and different days for interday precision. The areas of three replicate spots were measured and %RSD was calculated.

Accuracy (% Recovery)

The recovery experiments were carried out in triplicate by spiking previously analyzed samples of injection (LEVO 7500 ng/band and ILA 1000 ng/band) with three different concentration of standards at 80%, 100% and 120% of LEVO (6000, 7500, 9000 ng/band) and ILA (800, 1000, 1200 ng/band). The % recovery was then calculated.

Specificity

The specificity of the method was ascertained by analyzing standard drug and test solutions. The band for LEVO and ILA in individual samples were confirmed by comparing the peak purity spectra of standard and sample, at three different levels i.e. peak start (S), peak apex (A), and peak end (E) to check the interference of powdered material or other components present in dosage form.

LOD and LOQ

The LOD and LOQ were estimated from the set of 3 calibration curves used to determine Method linearity. LOD and LOQ were measured by using following mathematical expressions.

$$\text{LOD} = 3.3 \times (\sigma/S)$$

$$\text{LOQ} = 10 \times (\sigma/S)$$

Where, σ = the standard deviation of Y- intercept of 3 calibration curves S = the mean slope of the three calibration curves.

Robustness

The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Typical parameters evaluated during method robustness are change in mobile phase composition, change in Development distance, and change in chamber saturation time.

Preparation of solutions for forced degradation study

Forced degradation studies are performed to degrade the drug/s purposely so as to establish a degradation pattern for the same. In addition, it also provides us idea about the possible degradation pathways for API [15,16]. Usually, the degradation conditions are chosen so as to get 10-30% degradation of drug/s based on trial and error method. Forced degradation study of LEVO and ILA (mixture) was carried out in solution state under acidic, alkaline hydrolysis, oxidative, wet heat degradation for API. Moreover, forced degradation of individual API of LEVO and ILA were carried out to identify the degradation products formed from API only. Solutions were prepared by diluting 7.5 mL of standard stock solution of LEVO (1000 µg/mL) and 1 mL of ILA (1000 µg/mL) in 10

mL volumetric flasks individually. To it, 2 mL of 0.5 N hydrochloric acid, 0.5 N sodium hydroxide, 3% v/v hydrogen peroxide were added separately for acid, base and oxidative degradation. The solution was diluted up to mark with methanol and refluxed at 60°C for 20 min. The resulting solution (20 µL) was applied to HPTLC plate and the chromatogram was developed under above mentioned conditions. For wet heat degradation study, the prepared solution was directly refluxed and analysed using same conditions. The degraded samples were spotted on TLC plates and developed under same conditions for analysis. The developed chromatograms were studied in reference with pure standard drug/s chromatograms to prove any interference from the degraded products.

Result and Discussion

HPTLC method development

Preliminary screening

The optimum wavelength for detection was set at 255 nm which was obtained by scan standard drug solution in the range of 200-400 nm against methanol as a blank in UV-Visible spectrophotometer. Initially, neat solvents like toluene, ethyl acetate, and methanol were tried. Afterwards, mixture of these solvents in different proportions were tried and in order to improve peak shape and symmetry of LEVO; triethyl amine was added to mobile phase prior to development of plate. The mixture of Toluene: Ethyl acetate: Methanol: TEA (4.5: 2.5: 2: 0.2 v/v/v/v) was proven to be better than the other in terms of resolution and peak shape.

Optimization of chromatographic conditions using DoE

The optimization of chromatographic conditions DoE was used and devised in three stages. DoE helps in understanding the effect of interaction of various factors on the separation of components. During first stage, screenings of critical factors were done using first order design such as Taguchi design. The effect of listed factors (Table 1) on retardation factor of both drugs were studied by performing 8 different trials and reported as % contribution.

Factors	% Contribution	
	Rf of LEVO	Rf of ILA
Change in mobile phase ratio (mL)	19.84	31.37
Development distance (mm)	48.99	31.37
Saturation time (min)	19.84	17.65
time from spot to chromatogram (min)	0.40	7.84
time from chromatogram to scan (min)	10.12	1.96
band size (mm)	0.40	1.96
wavelength (nm)	0.40	7.84

Table 1: Effects of factors on Responses.

Looking at the data, it was concluded that three factors; change in mobile phase ratio (mL), development distance (mm) and saturation time (min) showed higher % contribution and found to be critical. Before moving to second stage, the values for critical parameters were set as shown in table 2.

Factors	Variables	Levels			Target
		Low (-)	Nominal (0)	High (+)	
A	Amount of Toluene and Methanol in mobile phase composition (mL)	Toluene - 4.5 Methanol-2	Toluene-5 Methanol-2.5	Toluene-5.5 Methanol-3	Maximum resolution between the bands
B	Development distance (mm)	75	80	85	
C	Saturation time (min)	10	15	20	

Table 2: Variables selected in Central Composite Design.

In second stage, second order design CCD was selected. CCD is the most popular response surface design. It combines a two-level fractional factorial, center and axial points. Box-Behnken design (BBD) could have been selected also as with three factors it would have given only 14 trials. The reason for selection of CCD was less impact of erroneous points on mathematical model as more no of trials are performed. As chromatographic separations are strongly affected by slight variations in the experimental conditions, more reliable mathematical models can be developed using CCD instead of BBD [17].

Run	X ₁ Mobile phase ratio (mL)	X ₂ Development distance (mm)	X ₃ Saturation time (min)	Y ₁ Rf of LEVO	Y ₂ Rf of ILA
1	5:2.5:2.5:0.2	80	23.4	0.5	0.8
2	5:2.5:2.5:0.2	80	15	0.52	0.73
3	4.5:2.5:2:0.2	85	20	0.47	0.68
4	5.8:2.5:3.3:0.2	80	15	0.54	0.87
5	4.1:2.5:1.6:0.2	80	15	0.57	0.72
6	4.5:2.5:2:0.2	75	10	0.6	0.77
7	5.5:2.5:3:0.2	75	20	0.59	0.8
8	5:2.5:2.5:0.2	80	15	0.56	0.73
9	5:2.5:2.5:0.2	80	6.6	0.65	0.94
10	4.5:2.5:2:0.2	85	10	0.6	0.74
11	5.5:2.5:3:0.2	75	10	0.52	0.84
12	4.5:2.5:2:0.2	75	20	0.53	0.73
13	5:2.5:2.5:0.2	80	15	0.56	0.74
14	5.5:2.5:3:0.2	85	20	0.59	0.8
15	5.5:2.5:3:0.2	85	10	0.59	0.82
16	5:2.5:2.5:0.2	80	15	0.59	0.73
17	5:2.5:2.5:0.2	71.6	15	0.49	0.75
18	5:2.5:2.5:0.2	88.4	15	0.54	0.77
19	5:2.5:2.5:0.2	80	15	0.51	0.73
20	5:2.5:2.5:0.2	80	15	0.52	0.74

Table 3: Trials and results obtained using CCD design.

The values of response Y₁ (R_f of LEVO) and Y₂ (R_f of ILA) ranges from 0.47-0.65 and 0.68-0.94 respectively. Here, Amount of Ethyl acetate and TEA kept constant because they were crucial for separation of both drugs. The selection of model for analyzing the response was done after comparing several statistical parameters including SD, R-squared values and predicted residual sum of square (PRESS). The model having low SD, higher R-square value and lower PRESS value were selected. On this basis, quadratic model was best fit for analyzing both the responses. Table 4 shows multiple regression analysis. The predicted R-Square of 0.0208 and 0.1224 are in reasonable agreement with the adjusted R-Square of 0.4601 and 0.7777 for Y₁ and Y₂ respectively.

Source	S.D		R-Squared		Adjusted R-Squared		Predicted R-Squared		PRESS	
	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂
Linear	0.04	0.04	0.291	0.53	0.158	0.44	-0.2	0.23	0.04	0.05
2FI	0.03	0.05	0.630	0.53	0.460	0.32	0.02	0.13	0.03	0.06
Quadratic	0.03	0.02	0.743	0.88	0.512	0.77	-0.1	0.12	0.04	0.06
Cubic	0.03	0.03	0.861	0.92	0.562	0.75	-2.0	-15.3	0.12	1.16

Table 4: Regression analysis for responses Y₁ and Y₂.

The higher value of correlation coefficients signifies an excellent correlation between the independent variables. All the above considerations indicate an excellent adequacy of the regression model.

For estimation of significance of the model, the analysis of variance (ANOVA) was applied. The ANOVA for Y1 and Y2 was summarized in Table 5.

Using 5% significance level, a model is considered significant if the p-value (significance probability value) is less than 0.05. As

shown in table 5, the Model F-values of 1.22 and 61 retardation factor (R_f) of LEVO and ILA, respectively, implies the model is significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. Therefore, saturation time(X_3) and X_1X_3 are significant model terms for LEVO and mobile phase ratio (X_1), saturation time (X_3) and X_3^2 are significant model terms for ILA.

Source	Sum of Squares		Df		Mean Square		F Value		p-value Prob > F	
	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂	Y ₁	Y ₂
X ₁	1.149E-004	0.026	1	1	1.149E-004	0.026	1.22	61.0	0.7535	0.0002
X ₂	6.476E-004	3.231E-004	1	1	6.476E-004	3.231E-004			0.4601	0.5458
X ₃	0.011	0.011	1	1	0.011	0.011			0.0085	0.0040
X ₁ X ₂	2.112E-003	4.500E-004	1	1	2.112E-003	4.500E-004			0.1924	0.4776
X ₁ X ₃	9.112E-003	2.000E-004	1	1	9.112E-003	2.000E-004			0.0135	0.6334
X ₂ X ₃	2.112E-003	0.000	1	1	2.112E-003	0.000			0.1924	1.0000
(X ₁) ²		2.527E-003		1	2.368E-004	2.527E-003				0.1110
(X ₂) ²		1.065E-005		1	1.289E-003	1.065E-005				0.9119
(X ₃) ²		0.023		1	5.158E-003	0.023				0.0004
Residual	0.015	8.267E-003	13	7	3.964E-004	8.267E-004				

Table 5: ANOVA for responses Y₁ and Y₂

The mathematical relationship in the form of a polynomial equation generated by Design-Expert® 7.0 software for the measured responses, R_f of LEVO (Y₁) and R_f of ILA (Y₂), are shown below equations respectively.

$$Y_1 = +0.55 + 2.902E-003 X_1 + 6.889E-003 X_2 - 0.028 X_3 + 0.016 X_1 X_2 + 0.034 X_1 X_3 - 0.016 X_2 X_3$$

$$Y_2 = +0.73 + 0.043 X_1 - 4.866E-003 X_2 - 0.029 X_3 + 7.500E-003 X_1 X_2 + 5.000 E-003 X_1 X_3 + 0.000 X_2 X_3 + 0.013 (X_1)^2 + 8.610E-004 (X_2)^2 + 0.043(X_3)^2$$

The above equations represent the quantitative effect of independent variables (X_1 , X_2 , and X_3) and their interactions on the responses (Y₁ and Y₂). A positive sign represents a synergistic effect, while a negative sign indicates an antagonistic effect. The theoretical values of Y₁ and Y₂ were obtained by substituting the values of X_1 - X_3 into the above equation.

Design space and optimal region selection

The relationship between the dependent and independent variables was further elucidated using perturbation and response surface plots. A perturbation graph was plotted to find those factors that affect the response most significantly. A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that particular factor. In case of response R_f of LEVO, Saturation time (X_3) shows a steeper slope as compared to Mobile phase ratio and Development distance which exhibit slight slope. Whereas in case of R_f of ILA, development distance shows a steep slope, Mobile phase ratio (X_1) and Saturation time (X_3) exhibit curvature. Figure 2 represents perturbation plot for responses Y₁ and Y₂.

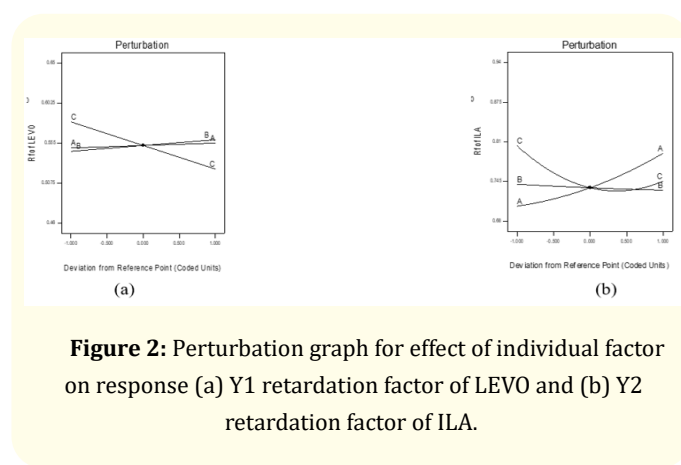


Figure 2: Perturbation graph for effect of individual factor on response (a) Y1 retardation factor of LEVO and (b) Y2 retardation factor of ILA.

Three-dimensional (3D) and contour response surface plots for the measured responses were formed, based on the model polynomial functions to assess the change of the response surface. Also the relationship between the dependent and independent variables can be further understood by these plots. Figure 3 (a) and (b) represents the effect of factors X_1 , X_2 , and X_3 on the response Y₁ and Y₂.

As seen in Figure 3 a as the factor mobile phase ratio (X_1) increase, the response R_f of LEVO (Y₁) is also increase and as the factor Development distance (X_2) increase, the response R_f of LEVO (Y₁) is decrease. There is no effect of factor Saturation time (X_3) on the response R_f of LEVO (Y₁) and in Figure 6.10 b as the factors Mobile phase ratio (X_1), Development distance (X_2)² and Saturation time (X_3) increases, there is an increase on the response R_f of ILA (Y₂).

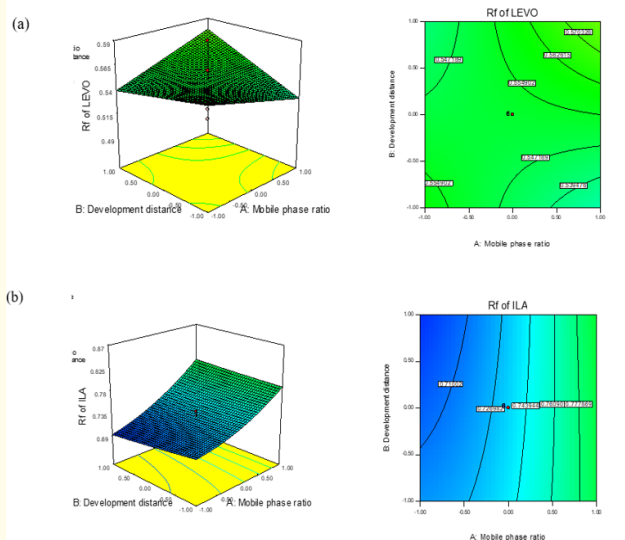


Figure 3: Contour and 3D Response surface plot showing the effect of mobile phase, development distance and saturation time, on retardation factor of (a) LEVO and (b) ILA.

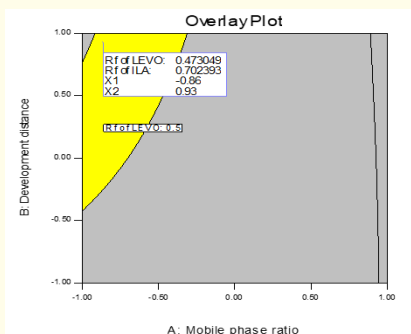


Figure 4: Overlay Plot of Experimental Design.

Overlay Plot (Figure 4) of was obtained by overlay of all three responses and created a yellow color region, selecting of any point from this region will satisfy the optimum value of responses. The yellow area in overlaid plot indicate all the constraints are satisfied in this region.

Validation of chosen model

In the third stage, the chosen model is validated. Hence, after studying the effect of the independent variables on the responses, the levels of these variables that give the optimum response were determined. Optimization was performed to find out the level of independent variables (X_1 , X_2 , and X_3) that would yield a minimum Rf value of LEVO with maximum value of ILA. Using a Design-Expert® 7.0 software optimization process, predicted values of dependent and independent factors (3 sets) were chosen randomly from yellow region. For confirmation, a fresh mixture in triplicate was prepared at the optimum levels of the independent variables, and the resultant mixture were evaluated for the responses. The experimental values of LEVO and ILA are given in the Table 6 respectively, which were in close agreement with the predicted values. The % error was less than 10% indicating the good predictability of the chosen model.

HPTLC method validation
Linearity

Linear responses were observed in the concentration range of 3750-22500 ng/band for LEVO and 500 - 3000 ng/band for ILA. Regression analysis was performed and regression equations were found to be $y = 0.211x + 4082$ and $y = 2.333x + 4943$ for LEVO and ILA respectively Correlation co-efficient for calibration curve of LEVO and ILA were found to be 0.998 and 0.996 respectively. Overlain chromatogram of sample and standard LEVO and ILA are shown in figure 5.

Amount of solvents (mL)		Development distance (mm)	Saturation time (min)	Retardation factor (R_f) of LEVO		% PE	Retardation factor (R_f) of ILA		% PE
Toluene	Methanol			Experimental value	Predicted value		Experimental value	Predicted value	
4.57	2.07	84.65	19.85	0.49	0.47	4.08	0.72	0.70	2.7
4.52	2.02	80.35	19.85	0.46	0.48	-4.34	0.70	0.71	-1.42
4.59	2.09	83.3	19.85	0.48	0.47	2.08	0.73	0.70	4.10

Table 6: Validation of developed model.

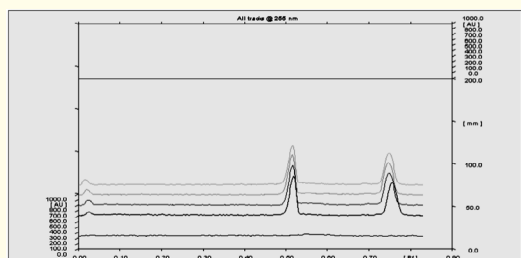


Figure 5: Overlaid chromatogram of sample and combined standard of LEVO (15000 ng/band) and ILA (2000 ng/band) at 255 nm.

Precision

For method precision, Peak areas were recorded for 15000 ng/band of LEVO and 2000 ng/band of ILA six times and %RSD was calculated. It was found to be 0.513 and 0.527 for LEVO and ILA respectively. Mean % RSD for intra-day precision of LEVO and ILA was found to be 0.924 and 0.903 respectively whereas %RSD for inter day precision of LEVO and ILA was found to be 1.07 and 1.5 respectively. In each study, %RSD was low indicating the developed method is precise and can be used reliably.

Accuracy

It was performed by calculating total amount of both drugs recovered from the spiked mixtures. The average % recovery of LEVO and ILA was found to be 99.52 and 100.11 respectively. These results indicated that developed method is accurate.

Specificity

The peak purity of LEVO and ILA were assessed by comparing its HPTLC chromatogram with standard at peak start, apex, and peak end positions of the band. Peak purity spectra of test LEVO and ILA are shown in Figure 6(a) and (b) respectively.

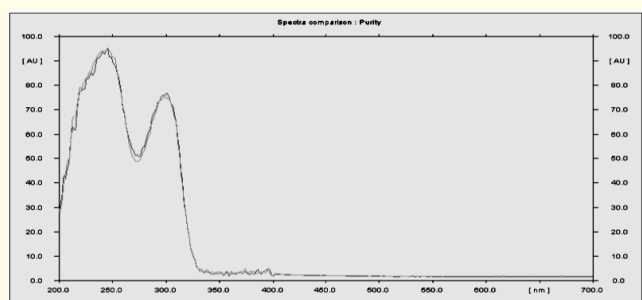


Figure 6: (a) Peak purity spectra of LEVO in standard and sample at peak start, peak apex and peak end.

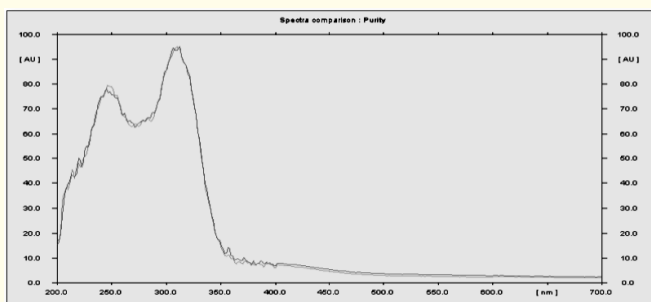


Figure 6: (b) Peak purity spectra of ILA in standard and sample at start, peak apex and peak end.

The values of correlation coefficient r (s,m) and r (m,e) were found to be 0.9993 and 0.9838 for LEVO whereas they were 0.9987 and 0.9922 for ILA. The values of r near to 1 indicate that no interference from test solution is observed and the method is specific.

LOD and LOQ

The LOD for LEVO and ILA were found to be 742.90 ng/band and 14.2517 ng/band respectively. The LOQ for LEVO and ILA were found to be 2251.22 ng/band and 43.18 ng/band respectively.

Robustness

Robustness study was performed by changing the mobile phase composition, development distance and saturation time in triplicate. The peak areas were recorded for different conditions and % RSD was calculated for each condition. The % RSD were found to

be 0.49, 0.46 and 0.76 respectively. Lower values of %RSD showed that method would give reproducible results with deliberate change in chromatographic conditions.

Analysis of capsule by proposed method

The assay results obtained by using the proposed method for the analysis of a marketed formulation containing LEVO (75 mg) and ILA (10 mg) were in good agreement with the labeled amounts of LEVO and ILA. The % drug obtained was $99.80 \pm 0.83\%$ of LEVO and $101.73 \pm 0.37\%$ of ILA. No interference of the excipients with the peaks of interest appeared; hence the proposed method is applicable for the routine estimation of LEVO and ILA in capsule.

Forced degradation study

The rate of degradation in acid was found to be faster as compared to the alkali in both LEVO and ILA. In all degradation study the solutions were refluxed for 20 min at 60 °C. In acidic degradation using 0.5 N HCl about 3.53% degradation was observed in LEVO and 65.14% degradation was observed in ILA. The rate of degradation in alkali was found to be slower as compared to the acid in both LEVO and ILA. Using 0.5 N NaOH about 9.36% degradation was observed in LEVO and 15.51% degradation was observed in ILA corresponding but no visible peaks were obtained. The reason may be the formation of non-absorbing compounds. The LEVO and ILA were found to be labile to oxidative degradation. With use of 3% H₂O₂, about 10.26% degradation was observed in LEVO and 31.93% degradation was observed in ILA. The LEVO and ILA were found to be slightly labile to wet heat degradation. After applying exposure to 60°C for 20 min, about 7.94% degradation was observed in LEVO and 84.97% degradation was observed in ILA (Figure 7). The data for forced degradation can be seen in Table 7.

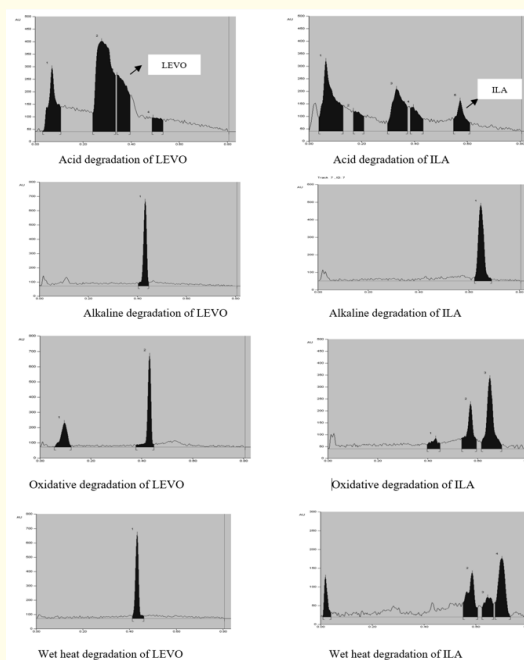


Figure 7: Chromatograms of degradation of LEVO and ILA.

Sr. No.	Stress type	Peak area of non degraded standards		Peak Area*		% Degradation	
		LEVO (11250 ng/band)	ILA (1500 ng/band)	LEVO (11250 ng/band)	ILA (1500 ng/band)	LEVO	ILA
1	Acid hydrolysis	6462.5	8445.1	6234.3	2944.2	3.53	65.14
2	Alkali hydrolysis			5857.9	7135.8	9.36	15.51
3	Oxidation			5799.9	5749.3	10.26	31.93
4	Wet heat degradation			5949.5	1269.8	7.94	84.97

Table 7: Summary of forced degradation study of LEVO and ILA.

Conclusion

Simple and rapid HPTLC method was developed and validated for simultaneous estimation of LEVO and ILA in its bulk and capsule dosage form. The developed method utilizes commonly available solvents toluene, ethyl acetate, methanol and triethyl amine. The chromatographic conditions were further optimized using Design of experiment concept that is time saving, cost effective and scientific. Using screening design (Taguchi) critical factors affecting the separation of both drugs were identified in the initial stage. The effect of critical factors of the retardation/resolution of drugs was studied using central composite design (CCD). The developed method was successfully applied for estimation of content of both drugs from capsule and the drugs were subjected to acidic, basic and oxidative degradation. The method was successfully applied in estimating the drugs in presence of their degradation products.

Acknowledgements

The authors are very grateful to the principal of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar and Aeon Formulations Pvt. Ltd., Pondicherry for providing us gift samples of standard compounds for method development.

Author Contribution

Dr. Archita Patel and Ms. Margi Shah have devised the idea behind research together and statistical analysis and DOE was planned by Archita Patel. Ms. Margi Shah conducted the experiments in the laboratory. Mr. Nisith Teraiya have helped in carrying out the forced degradation study. The manuscript first draft was prepared by Archita Patel.

Compliance with Ethical Standards

Funding No funding/grants have been received from any agency for conducting the research work.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethical Approval

The article does not contain any studies with human participants or animals performed by any authors.

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