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Application of Plackett-Burman and central composite designs for screening and optimization of factor influencing the chromatographic conditions of HPTLC method for quantification of efonidipine hydrochloride

Suraj R. Chaudhari* and Atul A. Shirkhedkar

Abstract

We report here an analytical method for expeditious estimation of efonidipine hydrochloride in tablet formulation with statistical screening and optimization designs using NP-HPTLC. TLC silica gel 60 F₂₅₄ aluminum plates and ethyl acetate to dichloromethane to triethylamine (3:2:0.5 v/v) were chosen for chromatographic separation of efonidipine hydrochloride. The R_f value for efonidipine hydrochloride turned out to be 0.35 ± 0.25 and quantitative evaluation was done at 251 nm. Plackett-Burman and face-centered central composite design (CCD) were used to obtain the most peak area and well-resolved compact band with an adequate retention factor of efonidipine hydrochloride. Plackett-Burman design at two-level with six independent variables has been employed for screening of prominent factors that affect the responses. The prominent factors have been selected and are optimized through face-centered CCD. The results obtained from face-centered CCD showed that most peak area can be obtained with development distance 8.50 cm and chamber saturation 17 min. Furthermore, the current NP-HPTLC investigation has been validated according to the ICH guidelines for accuracy, precision, sensitivity, robustness, ruggedness, and specificity. The detection and quantification limit was found that 10.41 ng and 31.57 ng, suggesting that the analysis could be accurately and precisely detected the analyte up to nanogram quantity. The current NP-HPTLC investigation is rugged, accurate, and highly sensitive and could be used for routine analysis of efonidipine hydrochloride.

Keywords: Calcium channel blocker, Efonidipine hydrochloride, HPTLC, Plackett-Burman design, Response surface methodology

Introduction

Efonidipine hydrochloride (EFH) is a dihydropyridine class of the calcium channel blocker chemically 2-[benzyl(phenyl)amino]ethyl 5-(5,5-dimethyl-2-oxo-1,3,2λ⁵-dioxaphosphinan -2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydro

pyridine-3-carboxylate hydrochloride. It is an antihypertensive and antianginal drug with dihydropyridine ring in its chemical structure Fig. 1. EFH effectively blocks Ca²⁺ channels of types L and T and has a better clinical profile (Tanaka and Shigenobu 2002). EFH has an excellent pharmacological profile, because it has a phosphate moiety in the 5th position of the dihydropyridine ring which makes it different from other dihydropyridine agents (Masuda et al. 1995; Masumiya et al. 1997; Masumiya et al. 2000).

* Correspondence: chaudharisuraj72@gmail.com

Central Instrumental Facility (CIF), Department of Pharmaceutical Chemistry, R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Maharashtra 425405, India



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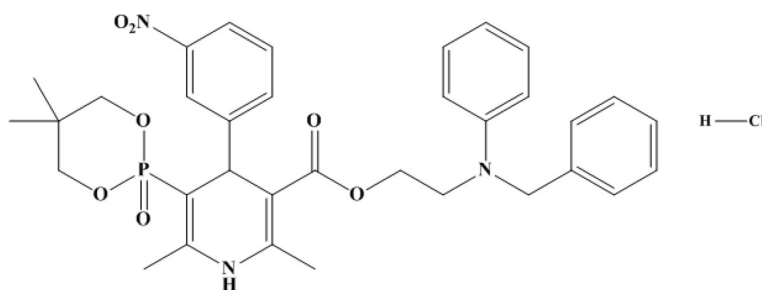


Fig. 1 Chemical structure of efonidipine hydrochloride

EFH blocks Ca^{2+} channels of types L and T as a result of that vasodilation and decreased automaticity of the heart and causes negative chronotropic effect and lower the heart rate (Tanaka and Shigenobu 2002).

From the other instrumental techniques of pharmaceutical analysis, high-performance thin-layer chromatography (HPTLC) is the most significant technique because the advances in the stationary phase and the commencement of densitometric detection are increasingly being used nowadays in the area of drug analysis. HPTLC is a more efficient tool over liquid chromatography because the separation of several samples in parallel on the same plate allowing high-throughput and rapid low-cost analysis and also a small amount of organic solvents are needed and which somewhat reduces the disposal of hazardous organic solvents (Shewiyo et al. 2012). While developing the method for pharmaceuticals using HPTLC, the analyst has to optimize some controllable variables including activation of the plate, chamber saturation, volume and composition of the mobile phase, detection wavelength, type of stationary phase, and type of detector. The “trial and error” strategy has been used for long years to optimize these controllable variables; this approach comprises of changing one variable at a time whereas other variables have to maintain steady to get efficient separation of pharmaceuticals. Moreover, this traditional approach can consume a lot of time and money to optimize the robust conditions for the analysis of pharmaceuticals.

Therefore, the experimental designs are extensively used from the past few years for optimization of analytical methods because it requires less time, efforts, and resources than the traditional approach. It is a stunning tool and enables a simultaneous evaluation of several variables at multiple levels in a limited number of experiments. The two types of experimental design are used for the optimization of method, i.e., screening and optimization design (response surface methodology). The screening design is basically comprised of full factorial design, factorial design, and Plackett-Burman design (PBD) which are used to identify the most crucial independent variable influencing response. Response surface methodology (RSM) consisting of central composite design (CCD), Box-Behnken design, and

Doehlert design is employed for the optimization of the most crucial independent variable and to identify the optimum level of each factor (Müller et al. 2020; M. Cavazuti 2013; Tauler et al. 2009).

PBD is a type of two-level fraction factorial screening design. The most important aspect of this design is combining different combinations of the independent variables with a variable level. Through PBD, maximum $N-1$ independent variables can be examined, where N is a multiple of 4. PBD is a very efficient screening design for the evaluation of independent variables with very few experimental runs which result in the preservation of chemicals, times, and the most important saving of human resources (Ahuja et al. 2004).

A review of the literature revealed that few analytical methods for quantification of EFH have been reported. The HPLC method for determination of EFH (Kumar et al. 2017), LC-MS/MS, and chiral LC-MS/MS methods have been reported for the determination of EFH in human plasma (Liu et al. 2015; Liu et al. 2016).

To date, no HPTLC method has been studied to quantify of EFH in tablet formulation and due to the lack of reports in the literature for the investigation of the impact of independent variables of the HPTLC method on the responses using PBD and CCD. Therefore, the scientific novelty of the proposed work is to explore PBD and RSM experimental design approach for screening and optimization of independent variables and validation of a method for quantification of EFH in tablet formulation using normal phase (NP)-HPTLC method.

Materials and methods

Instrumentation

A CAMAG Linomat 5 applicator with sample syringe of 100 μL (Hamilton, Bonaduz, Switzerland) for sample application under a gentle stream of nitrogen for immediate evaporation and evaluation of thin-layer chromatography (TLC) plates were done using CAMAG TLC 3 scanner, and data analysis was performed using winCATS version 1.3.0-Planar Chromatography Manager software (CAMAG, Muttensz, Switzerland). The HPTLC system facilitates with deuterium, and halogen-tungsten lamp and measurement

were performed using absorbance/reflectance mode. The slit dimensions of TLC 3 scanner were set at 6.00×0.45 mm, data resolution was $100 \mu\text{m}/\text{step}$, and scanning speed was kept at $20 \text{ mm}/\text{s}$. A twin-trough chamber was used for the development of TLC plates. Shimadzu AUX-120 analytical balance was used for weighing operations.

Materials and chemicals

HPLC grade dichloromethane, ethyl acetate, triethylamine, and all other solvents were purchased from Merck Life Science, Mumbai, India, and Avantor Performance Materials, Thane, India. TLC silica gel 60F₂₅₄ aluminum sheets (20×20 cm) were purchased from Merck, Darmstadt, Germany. EFH of pharmaceutical grade was acquired as a gift sample from Zuventus Healthcare Ltd. Mumbai, India. Efnocar-40 was labeled to contain 40 mg EFH per tablet, a marketed product of Zuventus Healthcare Ltd. Mumbai, India.

Preparation of standard solution

A stock solution of EFH ($1 \text{ mg}/\text{mL}$) was prepared by transferring the accurately weighed 10 mg of EFH in 10 mL of methanol, and the solution was stored at 5°C up to the time of further dilution and analysis. The stability of the standard solution was accomplished under optimized conditions.

Chromatographic procedure

All measurements were executed on TLC silica gel 60 F₂₅₄ aluminum plates. Before use, each plate was cut into 10×10 cm and prewashed with methanol. The standard and sample solution of EFH (μL) were applied to the TLC plate, 15 mm from the bottom and 15 mm from the side edges, in the form of bands (6 mm bandwidth). Five milliliters of the mobile phase consisted of ethyl acetate to dichloromethane to triethylamine (3.0:2.0:0.5 v/v) was poured into the twin-trough chamber. The chamber was saturated with an optimized mobile phase for 17 min at room temperature ($28 \pm 2^\circ\text{C}$). The linear ascending plate development was achieved until a migration distance of 8.5 cm from the origin. Subsequently to development, the plates were dried in air for almost a minute. The quantification of analytes was done at 251 nm using absorbance/reflectance mode, and the rest of the data analysis was carried out using winCATS software.

Statistical tool

Minitab® 17.1.0, USA software was used for developing PBD for the screening of independent variables and Design Expert (Version 7.0.0), Stat-Ease, Inc. Minneapolis, MN, 55413, for designing a CCD and RSM for optimization of independent variables.

Experimental

Experimental design and statistical analysis for the optimization of chromatographic conditions

For estimation of EFH with high specificity and selectivity in pure form and pharmaceutical preparation, the developed TLC method was optimized to obtain maximum peak area and that would give a dense and well-resolved compact band with an adequate retention factor (R_f) for EFH which was desired. Therefore, the present method was subjected to PBD for a screening of independent variables and optimization of the significant independent variables using RSM.

Plackett-Burman design screening of independent variables

The PBD is an efficient screening design to identify the crucial variables among a large number of variables that ultimately affect the results. PBD was utilized to determine the significant independent variable that affects the peak area and R_f . The independent variables (factors) selected for PBD were wavelength, saturation time, the volume of dichloromethane (DCM), volume of triethylamine, development distance, and activation of the plate and were investigated to identify the significant factor for the peak area and R_f chosen as responses with a confidence level of all intervals at 95%. The level of independent variables employed in PBD is tabulated in Table 1. In the proposed work, a total of 12 experimental runs was obtained using Minitab® 17.1.0 to analyze six factors and their effect on responses. One and all factors were studied at two levels, i.e., for low level – 1 and high level + 1. Experimental runs of PBD are depicted in Table 2. The PBD based on a first-order polynomial model for mathematical modeling which was used is shown in equation (1):

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the responses (peak area and retention factor), β_0 is the model intercept, β_i is a linear coefficient, and X_i is the level of independent variables (Naveena et al. 2005; Vanaja and Shobha Rani 2007). The independent

Table 1 Level of independent variables selected in Plackett-Burman design for screening the independent variables

Sr. No.	Independent variables	Unit	Experimental value	
			Low (– 1)	High (+ 1)
1	Wavelength	nm	248	254
2	Saturation time	min	15	25
3	Volume of dichloromethane	mL	1.5	2.5
4	Volume of triethylamine	mL	0.3	0.7
5	Development distance	cm	7.5	8.5
6	Activation of plate	min	8.0	12

Table 2 Plackett-Burman design experimental runs for screening the significant independent variables affecting on the responses

Run order	Wavelength	Saturation time	Volume of DCM	Volume of triethylamine	Development distance	Activation of plate	Peak area	Retention factor
1	1	1	-1	1	-1	-1	4985.45	0.77
2	-1	-1	-1	-1	-1	-1	6736.95	0.58
3	1	-1	1	1	-1	1	5383.96	0.52
4	-1	1	1	-1	1	-1	5565.23	0.5
5	1	-1	1	-1	-1	-1	5954.12	0.46
6	-1	-1	-1	1	1	1	9045.56	0.43
7	-1	1	1	1	-1	1	4005.6	0.49
8	1	-1	-1	-1	1	1	8195.95	0.48
9	1	1	1	-1	1	1	7902.53	0.47
10	-1	-1	1	1	1	-1	9265.45	0.55
11	-1	1	-1	-1	-1	1	4601.98	0.45
12	1	1	-1	1	1	-1	8315.76	0.46

variables with probability value (p value < 0.05), i.e., more than 95 % confidence level of all intervals, are thought-about to have a significant effect on peak area and R_f and by using CCD and RSM, the significant independent variables are optimized. The above experiment was performed using the concentration of 1000 ng/band.

Optimization of HPTLC conditions using face-centered CCD and RSM

As a consequence obtained after performing PBD for a screening of independent variables, the two most influential significant independent variables were selected for further optimization and to point out the ideal value of each independent variable that would give maximum peak area of EFH. Hence, in the proposed work, an RSM using a 2-factor, 3-level face-centered CCD was applied to optimize and analyze the variables of NP-HPTLC chromatographic separation condition. The two major significant independent variables selected from PBD affecting the response namely development distance (X_1) and saturation time (X_2) were selected and investigated at three different coded levels as 7.5, 8.0, and 8.5 for X_1 and 15, 20, and 25 for X_2 . Peak area (Y) was picked from PBD as a dependent response variable to be optimized, and the goal was to set to obtain maximum peak area. Thirteen experimental runs were obtained from the face-centered CCD matrix generated from the Design Expert® Version 7.0.0 (State-Ease, Inc., Minneapolis, MN) consisting 8 non-center points and 5 center points as shown in Table 3. The impact of each independent variable on responses and model competency was reviewed using analysis of variance (ANOVA) respectively with Fisher's statistical analysis and p value. Besides, the area of the peak was examined by a second-order polynomial equation and the data fitted by a multiple regression procedure (Dinç-Zor et al. 2020;

Priyadharshini and Bakthavatsalam 2016; Imam et al. 2014; Varshosaz et al. 2010). The statistical relationship between the peak area (Y) to the selected independent variables X_1 and X_2 is shown by the following quadratic polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (2)$$

where Y is the measure response (peak area), X_1 and X_2 are significant independent variables, β_1 and β_2 are the linear regression coefficients, β_{12} is the interactive regression coefficient, and β_0 is a constant term. Further, to get maximum peak area, RSM was taken into consideration to analyze the effect of a significant independent variable on response. At the last, the maximum peak area was traded-off through a graphical optimization process.

Calibration curve of EFH

Calibration solutions were made in the concentration range of 30–180 $\mu\text{g/mL}$ for EFH through appropriate dilutions of a stock standard solution. From the resulting solution, a fixed volume of 10 μL solution was applied on NP-TLC plates in the form of bands to get final concentrations of EFH. The plates were developed using an optimized mobile phase. To check the linearity in the above-stated concentration range, samples were applied six times per concentration and peak area values were recorded. Eventually, the calibration curve was developed by plotting the analyte concentrations against the peak area.

Validation of method

The present work was validated for accuracy, precision, sensitivity (limit of detection and limit of quantification), ruggedness, robustness, and selectivity considered as the requisite parameters to make sure of the validation of

Table 3 Face-centered CCD matrix with two independent variables studied accompanied by results marked

Runs	Development distance (mm) (X_1)	Saturation time (min) (X_2)	Peak area
1	8.0	25	4526.56
2	8.5	20	6512.32
3	7.5	25	5264.65
4	8.0	20	6865.48
5	8.0	20	6795.53
6	8.0	20	6656.69
7	8.0	20	6845.12
8	7.5	15	5156.98
9	7.5	20	5951.18
10	8.5	25	4156.69
11	8.5	15	6965.74
12	8.0	20	6685.48
13	8.0	15	5985.56

the analytical method. NP-HPTLC method efficiency has been assessed using International Conference of Harmonisation (2005).

Accuracy

The accuracy of the NP-HPTLC method was investigated by calculating the percentage (%) recovery of EFH using the standard addition method. Percentage recovery investigation was evaluated by over spotting the known amounts of EFH (480, 600, and 720 ng/band) to the pre-analyzed sample solution, and the results were re-examined by the NP-HPTLC method. The amount of EFH was determined from assessing the peak areas and by keeping these values to the calibration curve equation.

Precision

Precision was studied with respect to intra-day and inter-day precisions using three different concentrations of the calibration curve of EFH and was appraised in terms of % RSD. Intra-day precision was estimated by assessing the sample solutions of EFH (900, 1200, and 1500 ng/band) at different time intervals on the same day. Inter-day precision was estimated by assessing the sample solution of EFH (900, 1200, and 1500 ng/band) for three consecutive days according to ICH guidelines. The peak area values found have been used to estimate the values of mean, standard deviations (SD), and % RSD. Repeatability was studied at a concentration of (900 ng/band) of EFH using six times.

Sensitivity

The sensitivity of the NP-HPTLC method was calculated in terms of LOD and LOQ. The sigma method was employed for LOD and LOQ. The sigma method is based upon the slope of the calibration plot and SD of

the responses. The LOD and LOQ were calculated as 3 and 10 times the noise level. The LOD and LOQ for the NP-HPTLC method were computed from the formula $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$, where, σ = standard deviation of the linearity plot and S = mean slope of the linearity plot. A series of sample solutions of EFH (300–800 ng/band) were spotted on the NP-TLC plates and analyzed to appraise LOD and LOQ.

Robustness and ruggedness

The slight changes in the detection wavelength (nm), volume of mobile phase (mL), and activation of plate (min) were made, and the impacts on the responses were studied. Robustness experiment was evaluated using the (1500 ng/band) concentration of EFH, and the mean, SD, and % RSD values for peak area were measured. Ruggedness procedure was executed by two different analysts within similar experimental and environmental conditions. Ruggedness experiment was assessed using the concentration of 1500 ng/band.

Applicability of HPTLC method for analysis of pharmaceutical preparation

To determination of EFH in pharmaceutical dosage form with high sensitivity and selectivity, ten Efnocar-40 tablets were weighed accurately and finely powdered in a mortar. A tablet-powdered weight equivalent to 40 mg of EFH was transferred into clean and dry 100 mL of volumetric flask. The powder of EFH was dissolved in 50 mL of methanol using manual shaking for 2 min and subjected for sonication for about 20 min. The solution was filtered through 0.45 μ m filter paper (Millifilter, Milford, MA, USA) and the volume was made up to the mark with the same solvent to obtain the concentration of 400 μ g/mL. From the resulting solution, appropriate

volume was taken and diluted with methanol; the final concentration of EFH was 60 µg/mL. From this, 10 µL sample solution was spotted on NP-TLC plates using Linomat 5 applicator to get the concentration of 600 ng/band and analyzed as per the "Chromatographic procedure" section. The area of the peak was estimated to calculate the % amount found.

Results and discussion

Stability of standard solution

To verify the stability of the solution, the stock standard solution was analyzed for about a week when it was stored at room temperature ($28 \pm 2^\circ\text{C}$) and refrigerated at 4°C for 1 week. The results were analyzed using checking the peak areas during this phase, and it was noted that there was no significant difference. The solution was wherefore stable.

Optimization of mobile phase composition

The selection and optimization mobile phase is a crucial step in TLC method development because it ultimately affects the quality of separation (Shewiyo et al. 2012). The selection of the effective mobile phase system for EFH analysis was taken into consideration and was studied on and based on "trial and error" basis. At the beginning, a single solvent such as *n*-hexane and ethyl acetate was selected; it was pointed out that EFH stayed as it is on the plate without any movement on taking *n*-hexane and ethyl acetate. Hence, the mixture of mobile phase system and ratio were assessed like *n*-hexane to ethyl acetate, *n*-hexane to methanol, ethyl acetate to methanol, and ethyl acetate to dichloromethane. In the first two systems, EFH remained as it is without any movement, while it is co-eluted within the third system. The movement of EFH was observed in the mixture of ethyl acetate and dichloromethane with acceptable R_f value, but tailing was observed. Therefore, to decrease the tailing effect, the few drops of triethylamine were added on the basis of pKa of EFH (strongest basic- 2.33). Here, the triethylamine reduced the tailing and gets better the sharpness of the spot. At last, the good results were obtained using a solvent system consisting of ethyl acetate to dichloromethane to triethylamine in ratio (3:2:0.5 v/v) which gave an excellent compact band of EFH. Hence, this solvent system was considered for screening and optimization.

Screening of significant independent variables affecting the peak area and retention factor by Plackett-Burman design

The effect of the above six factors taken into consideration in the proposed work on peak area and R_f was statistically studied using PBD. The result of the study revealed that the impact of saturation time and development distance have a positive influence (p value < 0.05) on peak area,

and the effect of wavelength, saturation time, volume of DCM, volume of triethylamine, development distance, and activation of plate has negative influence (p value > 0.05) on the R_f . Consequently, the factors having more than 95% confidence level were taken into account, and hence, the saturation time and development distance were selected for the further optimization stage and their influence on peak area was observed. The statistical results of screening for significant factors are shown in Table 4. The first-order polynomial model equation for peak area (Y_1) and retention factor (Y_2) can be written as:

$$Y_1 = -21,463 + 42.1(\text{wavelength}) - 153.4(\text{saturation time}) \\ - 634 \text{ volume of DCM} + 852(\text{volume of triethylamine}) \\ + 277.0(\text{development distance}) - 70(\text{activation of plate}) \quad (3)$$

$$Y_2 = 0.07 + 0.00444(\text{wavelength}) + 0.00200(\text{saturation time}) \\ - 0.0300(\text{volume of DCM}) + 0.117(\text{volume of triethylamine}) \\ - 0.00633(\text{development distance}) - 0.0200(\text{activation of plate}) \quad (4)$$

The coefficient of determination (R^2) value of 87.77% and 47.57%, respectively, for peak area and retention factor indicated that up to 87.77% and 47.57% variability in peak area and retention factor could be estimated. Experimental data was statistically analyzed by F test for ANOVA and the results are depicted in Table 5. The model F value of 5.98 for peak area signifies that the model was significant (relationship between the response and independent variables); it means that independent variables in the proposed model improve the fit. While for retention factor, the F value is 0.76 indicating that there is no relationship between the response and independent variables. The significance of each independent variable was checked using the probability value, and hence, p value < 0.05 implies a significance of each factor. Figure 2 illustrates Pareto charts of the standardized effects for responses. According to results obtained from ANOVA, the development distance and saturation time have been identified as important independent variables that only influence the peak area and were taken into consideration for further studies.

Optimization of thin-layer chromatographic conditions by face-centered CCD and RSM

The significant independent variables were identified from the PBD, i.e., development distance (X_1) and saturation time (X_2) selected for further optimization of chromatographic condition. Likewise, the low, middle, and high levels of corresponding independent variables for the face-centered CCD (-1 , 0 , and $+1$) were estimated

Table 4 Plackett-Burman design for statistical analysis and effect of independent variables on peak area and retention factor

Factors	Effect	Coefficient	Standard error coefficient	T value	p value
Peak area					
Constant	–	6663	273	24.41	0.000
Wavelength	253	126	273	0.46	0.663
Saturation time	– 1534	– 767	273	– 2.81	0.038
Volume of DCM	– 634	– 317	273	– 1.16	0.298
Volume of triethylamine	341	170	273	0.62	0.560
Development distance	2770	1385	273	5.08	0.004
Activation of plate	– 281	– 141	273	– 0.52	0.628
Retention factor					
Constant	–	0.5133	0.0284	18.10	0.000
Wavelength	0.0267	0.0133	0.0284	0.47	0.658
Saturation time	0.0200	0.0100	0.0284	0.35	0.739
Volume of DCM	– 0.0300	– 0.0150	0.0284	– 0.53	0.620
Volume of triethylamine	0.0467	0.0233	0.0284	0.82	0.448
Development distance	– 0.0633	– 0.0317	0.0284	– 1.12	0.315
Activation of plate	– 0.0800	– 0.0400	0.0284	– 1.41	0.218

Table 5 Analysis of variance for peak area and retention factor using Plackett-Burman design

Source	DF	Adj. SS	Adj. MS	F value	p value
Peak area					
Model	6	3,207,985	5,345,164	5.98	0.034
Linear	6	3,207,985	5,345,164	5.98	0.034
Wavelength	1	191,774	191,774	0.21	0.663
Saturation time	1	7,061,677	7,061,677	7.90	0.038
Volume of DCM	1	1,206,350	1,206,350	1.35	0.298
Volume of triethylamine	1	348,509	348,509	0.39	0.560
Development distance	1	23,025,404	23,025,404	25.76	0.004
Activation of plate	1	237,271	237,271	0.27	0.628
Error	5	4,469,113	893,823		
Total	11	36,540,098			
Retention factor					
Model	6	0.043800	0.007300	0.76	0.633
Linear	6	0.043800	0.007300	0.76	0.633
Wavelength	1	0.002133	0.002133	0.22	0.658
Saturation time	1	0.001200	0.001200	0.12	0.739
Volume of DCM	1	0.002700	0.002700	0.28	0.620
Volume of triethylamine	1	0.006533	0.006533	0.68	0.448
Development distance	1	0.012033	0.012033	1.25	0.315
Activation of plate	1	0.019200	0.019200	1.99	0.218
Error	5	0.048267	0.009653		
Total	11	0.092067			

DF Degrees of freedom, Adj. SS Adjusted sum of square, Adj. MS Adjusted mean square

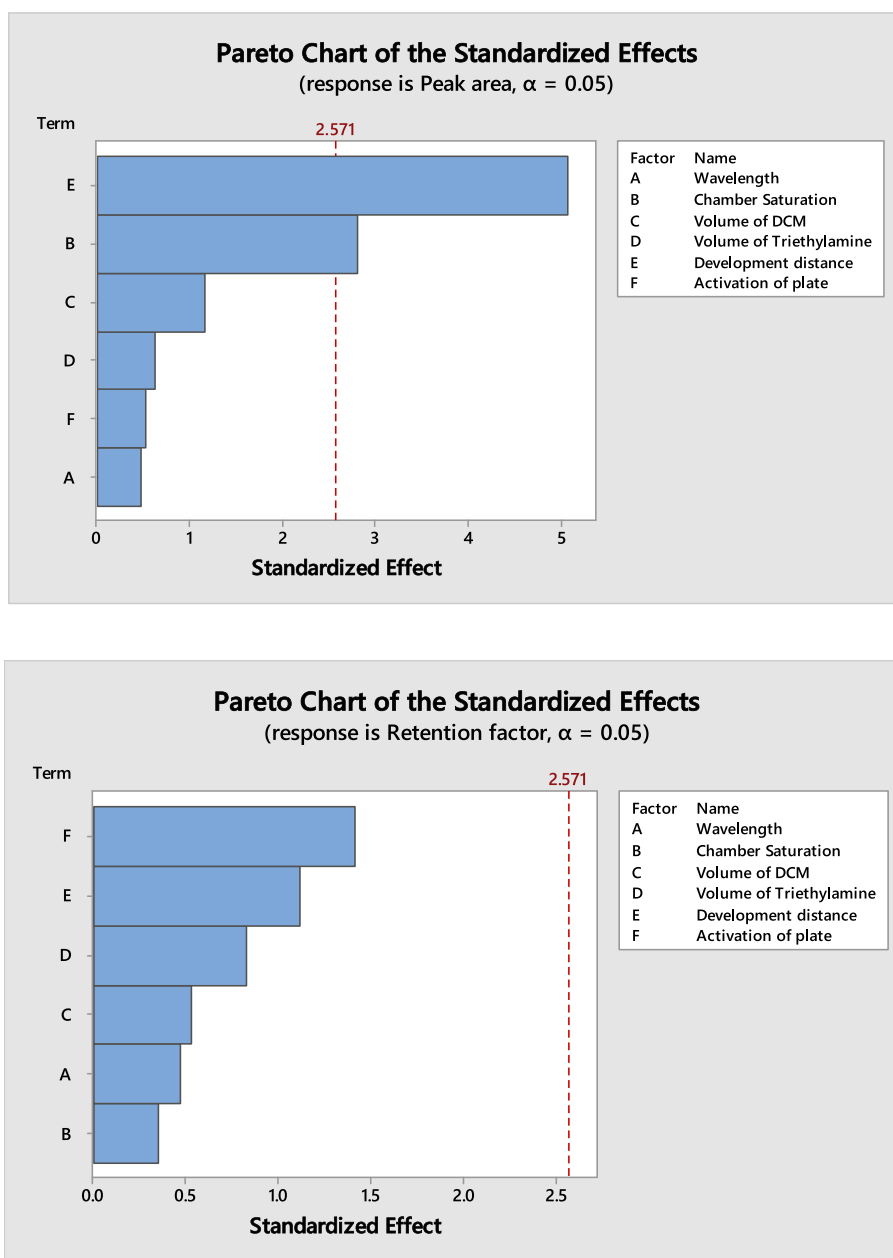


Fig. 2 Pareto chart of the standardized effects for peak area and retention factor

as per the results obtained through screening of independent variables affecting responses, and on the basis of results obtained, peak area was selected as a dependent variable. Further, the statistical model was validated using ANOVA, and analysis was done with Fisher’s statistical analysis, p value, and coefficient of determination (R^2) which measures the goodness of fit of the regression model (Priyadharshini and Bakthavatsalam 2016). By exploring multiple regression analysis, the second order polynomial equation was generated to describe the relationship between the peak area (Y) to the

selected independent variables X_1 and X_2 being represented in the following expression:

$$Y = +6677.54 + 210.32X_1 - 693.40X_2 - 729.18X_1X_2 - 215.49X_1^2 - 1191.18X_2^2 \tag{5}$$

where Y is the peak area, X_1 development distance (cm), and X_2 saturation time (min). By considering the ANOVA, results are shown in Table 6. The F value of

Table 6 Face-centered CCD ANOVA results for peak area

Source	Sum of square	Df	Mean square	F value	p value > F
Model	1.064E+007	5	2.129E+006	41.19	0.0001
X ₁	2.654E+005	1	2.654E+005	5.14	0.0578
X ₂	2.885E+006	1	2.885E+006	55.83	0.0001
X ₁ X ₂	2.127E+006	1	2.127E+006	41.16	0.0004
X ₁ ²	1.283E+005	1	1.283E+005	2.48	0.1592
X ₂ ²	3.919E+006	1	3.919E+006	74.84	0.0001
Residual	3.617E+005	7	51674.84		
Lack of fit	3.263E+005	3	1.088E+005	12.29	0.0174
Pure error	35393.43	4	8848.36		
Total	1.100E+007	12			

Df Degrees of freedom

the model was 41.19, lack of fit *F* value 12.29, and the value of *p* value (Prob > *F*) 0.0001, implying that the established model was significant. The *R*² was 0.9671 which is suitable for peak area and indicating excellent agreement between the predicted values. The “Pred R-Squared” of 0.7875 is in reasonable agreement with the “Adj R-Squared” of 0.9437.

The mutual interaction of each independent variable and the effect resulting from their interaction are calculated using a graphical representation of the regression model presented in Fig. 3. Frequently, the 3D response surface plots were established by plotting the dependent variable versus two of the independent variable, while the remaining variables were kept constant at an optimal level (Dinç-Zor et al. 2020). Figure 3 depicted the relationship between peak area, and two independent variables can be observed in this figure, which demonstrates that the maximum peak area could be acquired while the development distance was high (+ 1) and saturation time was low (− 1). Finally, the optimum peak area was chosen from the design technique by a graphical optimization trade-off process achieved from the overlay plot (Fig. 4). The optimized value of independent variables that will give maximum peak area was the development distance (8.5 cm) and saturation time (17 min). Figure 5 depicts the chromatogram of EFH developed in the optimized chromatographic conditions was optimized from face-centered CCD and RSM. The optimized chromatographic conditions gave excellent separation of EFH with *R_f* of 0.35 ± 0.25 with maximum peak area.

Linearity studies

The proposed method shows an excellent relationship between the EFH concentration and peak areas in the range of 300–1800 ng/band for EFH. The calibration curve was very linear well and indicated a high correlation coefficient (*r*² = 0.999) with a low intercept value (506.5) and the relative standard deviation (% RSD) for all results less than 2.

3D overlay of HPTLC densitogram of calibration curve of EFH 300–1800 ng/band.

Validation of method

The optimized chromatographic conditions were subsequently explored to validation for different parameters accuracy, precision, sensitivity (limit of detection and limit of quantification), robustness, ruggedness, and selectivity.

Accuracy

The accuracy of the method was investigated using the known amount EFH which is spiked into the pre-analyzed sample solution. The % recovery was found in the range 98.57–101.40% and 0.34–0.75% for EFH. The % recoveries and % RSD values obtained were in the range and meet the acceptance conditions for the experiment; therefore, the proposed method is correct.

Precision

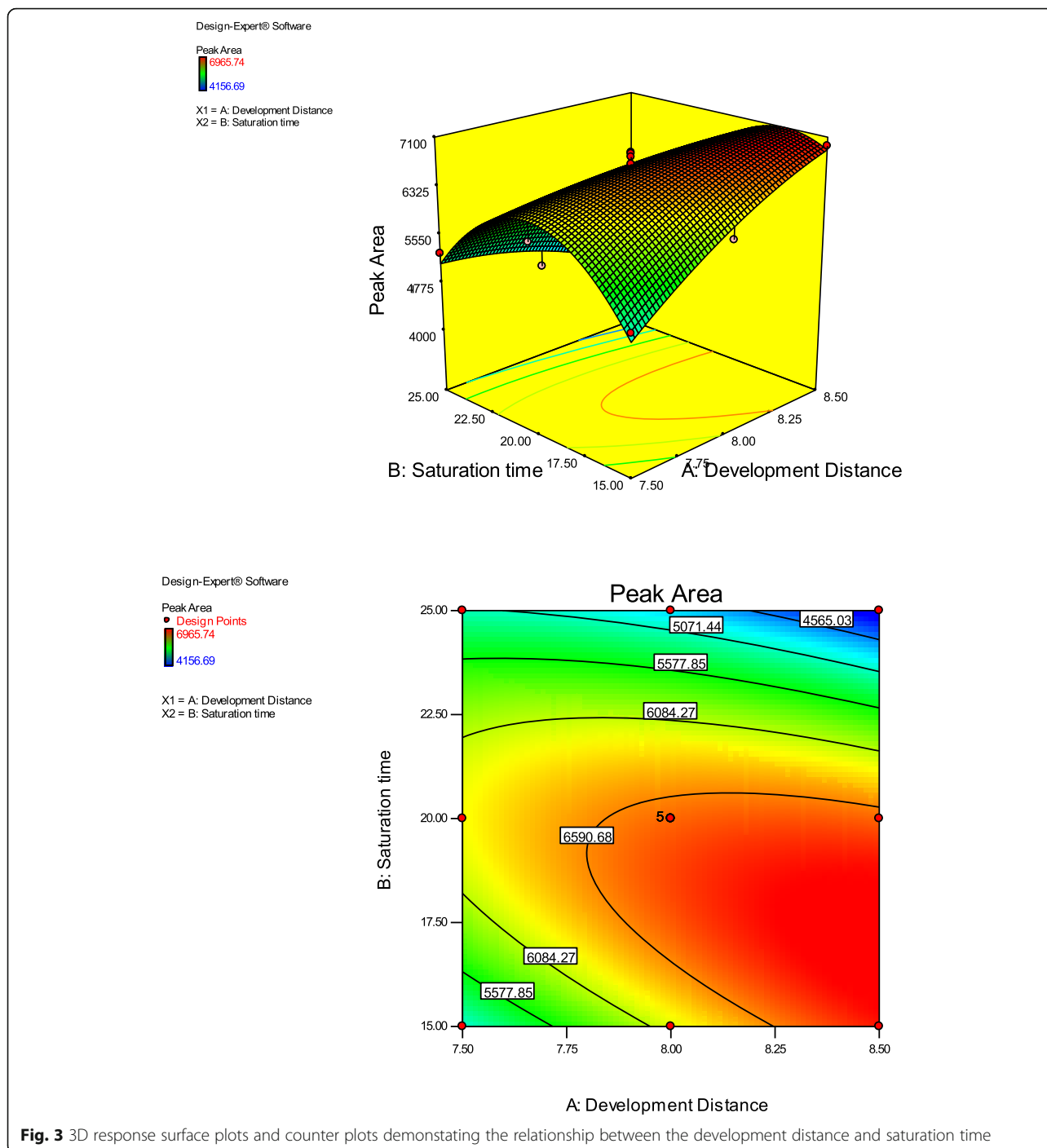
The precision of the method has been examined by intra-day, inter-day precision, and repeatability displayed in % RSD of the areas of peak. Intra-day and inter-day precision were achieved by executing three replicates of three distinct sample solutions of EFH (900, 1200, and 1500 ng/band) and repeatability using the EFH concentration of (900 ng/band). The established method shows excellent precision and the % RSD for intra-day and inter-day precision was between 0.41–1.54% and 1.14–1.66% and for repeatability 0.65%, indicating that the proposed method is precise.

Sensitivity

The LOD and LOQ were calculated using the sigma method. Within optimized conditions, the LOD and LOQ were found to be 10.41 ng and 31.57 ng. This shows that the proposed method could be accurately and precisely determined the nanogram quantity of analyte that exhibits the sensitivity of the method.

Robustness and ruggedness

The factors selected for this experiment were the detection wavelength (nm), volume of mobile phase (mL), and activation of plate (min). The impact of each factor on responses was assessed. The results of the robustness experiment showed that the % RSD values of less than 2% were obtained when the minor but deliberate changes in the parameters were initiated. It may therefore be would suggest that this proposed method is robust. The proposed method proved to be rugged when the experiment was carried out using a concentration of 1500 ng/band by two different analysts under similar experimental and environmental conditions. The results of the experiment show that the % RSD value was less than 2%.



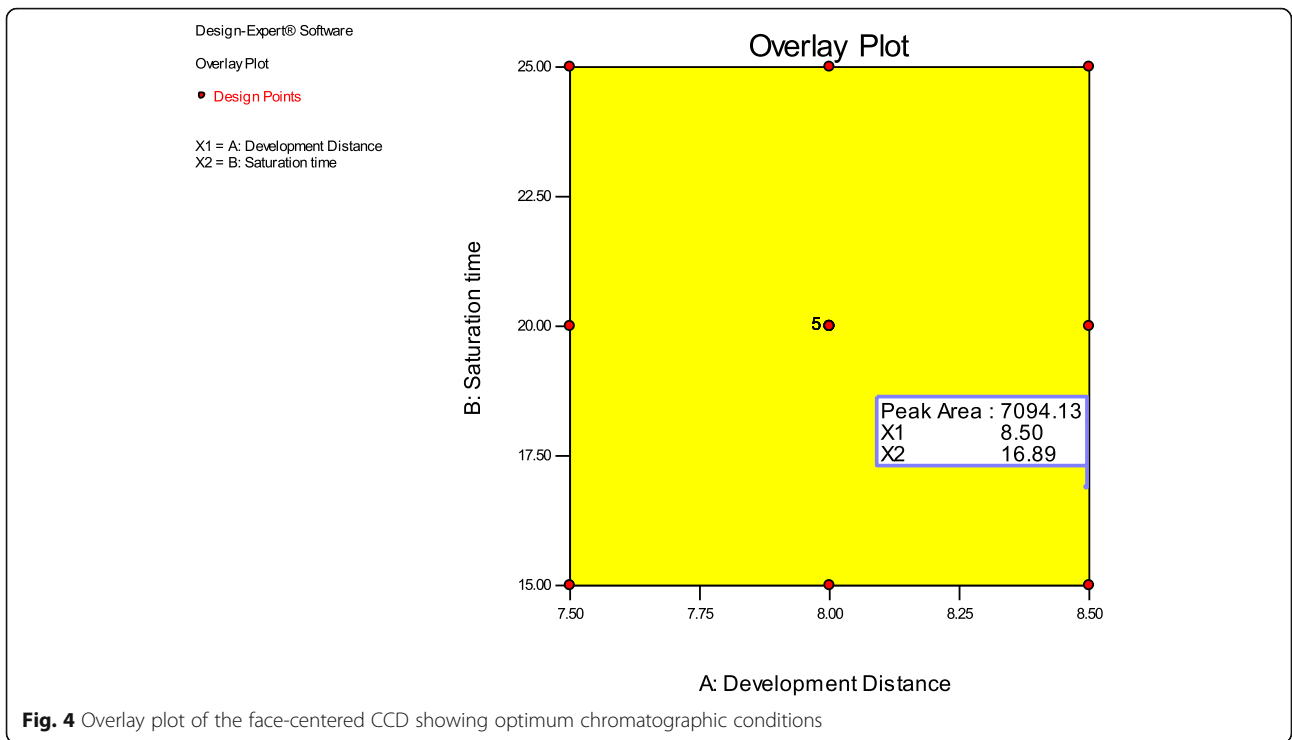
Specificity

Specificity is the ability of the proposed analytical method to evaluate the analyte present in the sample formulation. The peak-purity spectrum of EFH was estimated by correlating the spectra of EFH standard and EFH extracted from the Efnocar-40 tablets shows good resolution and a dense band at R_f of 0.35 ± 0.25 was at the same R_f standard. The peak purity of EFH in Efnocar-40 tablets was studied by

comparing the spectra at the peak-start (S), peak-apex (A), and at the end of peak. The correlation within these spectra means that the purity of the EFH (correlation $r(S, M) = 0.9998$, $r(M, E) = 0.9997$).

Analysis of pharmaceutical preparation

The proposed method was subjected to commercial Efnocar-40 tablets. The marketed preparation of EFH



was analyzed using the established method; the amount of EFH present in Efnocar-40 tablets was $100.03 \pm 1.63\%$. A well-resolved and compact band at R_f of 0.35 ± 0.25 was observed in the densitogram of EFH during analysis, and there was no excipient

interference present in the marketed preparation indicating that the proposed method can be employed in routine quality control laboratories for analysis of pharmaceutical dosage form of EFH. The validation parameters are summarized in Table 7.

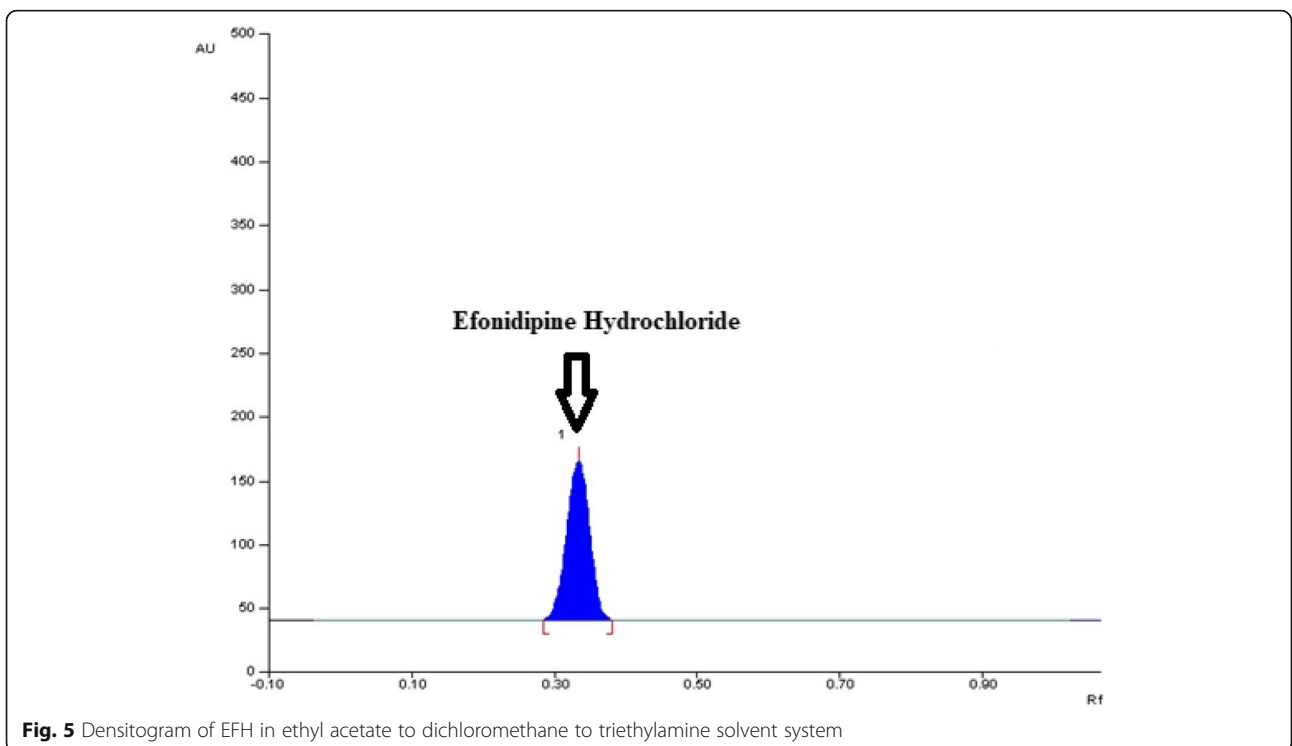


Table 7 Summary of linear regression data and validation parameters of HPTLC method

Parameters	HPTLC
Linearity	
Range (ng/band)	300–1800
Slope	4.812
Intercept	506.5
Correlation coefficient	0.999
Accuracy	
% recovery (% RSD)	98.57–101.40%, 0.34–0.75
Precision	
Intra-day precision ($n = 3$) (% RSD)	0.41–1.54
Inter-day precision ($n = 3$) (% RSD)	1.14–1.66
Repeatability ($n = 6$) (% RSD)	0.65
Sensitivity	
Limit of detection (ng)	10.41
Limit of quantification (ng)	31.57
Robustness	
	Robust
Ruggedness	
Analyst-I (mean \pm SD)	101.63 \pm 0.45
Analyst-II (mean \pm SD)	101.69 \pm 0.70
Analysis of pharmaceutical preparation (mean \pm SD)	100.03 \pm 1.63

n Number of determinations

Conclusion

The proposed work describes the implementation of Plackett-Burman design and the central composite design for screening and optimization of independent variables of the HPTLC method and to achieve excellent EFH separation and detection in pure form and in commercial pharmaceutical preparation. The main objective of this work is to evaluate significant independent variables that affect the EFH peak area and retention factor and to get the maximum peak area and appropriate retention factor. Among six independent variables, development distance and saturation time as the significant independent variables affecting the EFH peak area.

Further, central composite design and response surface methodology were subjected for the optimization of development distance and saturation time. The results of experimental designs showed that a small change in development distance and saturation time had a direct effect on peak area of EFH. Thus, it is necessary to pay special attention to the precise supervision of these two independent variables during the optimization of chromatographic conditions. Moreover, validation of accuracy, precision, sensitivity, robustness, ruggedness, and specificity was demonstrated to be suitable for routine analysis of EFH in the pharmaceutical matrix. The LOD and LOQ values were 10.41 ng and 31.57 ng experimentally detected and quantified for EFH making the method sensitive due to the nanogram quantity of drug

accurately and precisely determined. Hence, it has been accomplished that the established method is simple, sensitive, and specific for quantification EFH in bulk and pharmaceutical formulation.

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Authors' contributions

Suraj Chaudhari executed the statistical experimental design and method development and validation work. Atul Shirkhedkar organized a preliminary draft of the article. The authors read and approved the final manuscript.

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Authors declare that they have no competing interests.

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