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DESIGN OF EXPERIMENT UTILIZATION TO DEVELOP AND VALIDATE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUE FOR ESTIMATION OF SERTACONAZOLE NITRATE

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ABSTRACT: A novel method of estimation and validation of Sertaconazole nitrate (SER) by Reverse Phase-High Performance Liquid Chromatography coupled with Ultra-violet detection was developed which had high potential in determining drug concentration with more precision and high accuracy. The process of elution was conducted using Phenomenex C_{18} (250 mm × 4.6 mm i.d., 5.0 µm) using 0.01M monobasic sodium phosphate and acetonitrile in a ratio of 28:72% v/v as mobile phase at 4.5 pH and a flow rate of 1.0 mL/min. Detection was carried out using a UV detector at 260 nm. This precise method was linear between a range of 10 to 500 µg/ml with R^2 close to one (0.999). The limit of detection (LOD) and limit of quantification (LOQ) of SER was found to be 0.1 µg/ml and 0.15 µg/ml respectively. The method was validated for accuracy, precision, linearity, LOD, LOQ, and robustness. Validation studies demonstrated that this HPLC method is simple, specific, rapid, reliable and reproducible. The 3-level 2-factor face-centred central composite design was employed using Design Expert Software ver. 7.0.0 to examine the effect of independent chromatographic factors like pH of the mobile phase and the ratio between 0.01M monobasic sodium phosphate and acetonitrile on the dependent factors like peak area, theoretical plates and tailing factor. The ANOVA studies proved that the model employed for this study was significant. This method can be used as a more convenient and efficient option for the analysis of SER to establish the quality of the drug substance during routine analysis with consistent and reproducible results.

INTRODUCTION: Candidiasis is one of the widely occurring opportunistic infections majorly caused by *Candida albicans*, which normally inhabits moist areas of the human body like oropharynx, gastrointestinal tract and vagina.

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The most commonly seen symptoms of candidiasis are vaginal thrush, oral thrush, armpit infection, infected fingernails, *etc*. This infection generally affects top layers of the skin, namely stratum corneum, where these stubborn fungal organisms adhere by the expression of adhesions. This contact with host cells triggers hyphae growth, where it reaches the bloodstream due to invasins and can prove to be fatal. Epidemiologically, *Candida albicans* accounts for 8-10% of total fungal infections in the USA, while in India, around 12% of the mycotic infections are superficial candidiasis, which clearly indicates that this

pathogen is the predominant cause of these distressing and persistent infections representing a serious public health challenge with increasing medical and economic importance [1]. Several classes of drugs play a significant role to effectively target these stubborn mycotic species and maintain healthy conditions of the skin, of which imidazole derivatives are one of the most popularly, used antifungal agents. SER belongs to this class of drugs, which inhibits the 14α-demethylase enzyme, a cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol which is a critical component of the fungal cell membrane. Inhibition of the synthesis of ergosterol may result in increased cellular permeability causing leakage of cellular contents thereby preventing fungal cells from multiplying and impairing hyphae growth [2].

However, due to several limitations and shortcomings related to SER and its current dosage forms, development of advanced drug delivery system which can overcome these problems is the need of the hour. Hence, efficient methods which can quantify drug concentrations are required to be established. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) is the most sensitive, selective and simplified way of estimating SER with minimum time and expense, and improved industrial applicability. Siras and Mandlik (2017) developed a rapid and sensitive HPLC method for SER determination in bulk and tablet dosage form [3] while Muzaffar and Singh (2016) developed the same in microemulsion [4].

Hence, in the present article, an attempt has been made to establish a convenient, reproducible and selective method for estimating Sertaconazole nitrate in the bulk drug as well as in the novel drug delivery systems in HPLC by applying 3-level 2-factor face-centered central composite design and exploiting possibilities of different independent process variables on various dependent responses.

MATERIALS AND METHODS:

Materials: Sertaconazole nitrate was a gift sample from Cipla Pvt., Ltd., (Mumbai), which were found to be >99% pure. HPLC grade acetonitrile was purchased from SD Fine-Chem Limited (Mumbai, India). Sodium phosphate was obtained from Sigma-Aldrich Pvt., Ltd., Mumbai. Other chemicals such as pH adjusting agents like glacial acetic acid and triethanolamine (TEA) were of HPLC grade. HPLC grade water was used for the preparation of aqueous mobile phase in all experiments.

Methodology:

Equipment: Sophisticated equipment, HPLC LC-2010 HT (Shimadzu, Japan) equipped with a Serial dual plunger and autosampler was used for chromatographic separation. Phenomenex C_{18} (250 mm × 4.6 mm i.d., 5.0 µm) was employed for the analysis. The detection was carried out with the UV-Visible SPD M20A detector. LC solutions software was used for the interpretation of the results.

Experimental Design for Developed Method of HPLC: Optimization techniques help in designing the experiments by using an appropriate model. It is beneficial to evaluate and identify the most imperative parameters with a minimum number of runs. During the optimization steps, the area of the peak, theoretical plates and tailing factor were the responses which were screened to minimize the analysis time and maximize the peak resolution and optimal peak asymmetry of the developed method. Furthermore, 3-level 2-factor face-centered central composite design was selected to determine the best experimental conditions for formulating drug loaded solid lipid nanoparticles. Thirteen experiments were conducted using the factors and corresponding levels as described in **Table 1**.

Levels of 0.01M sodium phosphate: acetonitrile ratio were selected as 20:80 %v/v, 25:75% v/v, 30:70% v/v. Similarly, the levels of the pH of the mobile phase were selected as 2.0, 4.5 and 7.0. Peak area (Y1), theoretical plates (Y2), and tailing factor (Y3) were the responses for these studies [5].

TABLE 1: INDEPENDENT VARIABLES, DEPENDENT VARIABLES, AND LEVELS OF FACE-CENTERED 3^2 CENTRAL COMPOSITE DESIGN

Factors		Levels		
Independent	**Symbol**	**-1**	**0**	**+1**
0.01M sodium phosphate: acetonitrile ratio	A	20:80	25:75	30:70
pH of the mobile phase	B	2.0	4.5	7.0
	Dependent			
Peak area		Y1		
Theoretical plates		Y2		
Tailing factor		Y3		

Preparation of Calibration Curve (CC): A stock standard solution (1mg/mL) of SER was prepared in HPLC grade methanol. Working standard solutions (100 µg/mL) of the selected internal standard (IS), Ketoconazole were prepared by making appropriate serial dilutions of the solution in the mobile phase. CC was prepared by serial dilution of SER stock solution in the range of 10, 20, 40, 50, 100, 200, 300, 400, 500 µg/ml. 500 µl of 10 µg/mL Ketoconazole standard solution (IS) was added to the specified dilutions of the drug solutions in the Eppendorf tubes. Further, these solutions were centrifuged for 20 min at 10,000 rpm, and the supernatant was extracted and injected into the chromatographic system for analysis [6]. All these solutions were stable for seven days when stored at room temperature (20-25 ºC) and were used within one week. The bulk spiked CC samples were stored at −20 ºC and brought to room temperature before use.

Validation of the Developed Method: Validation of the developed method was carried out as per ICH guidelines [Q2 (R1)] which include various parameters like selectivity, specificity, system suitability, linearity, accuracy, precision, ruggedness, limits of detection and quantification. This included the examination of the injections of six consecutive replicate of the standard sample solutions. Selectivity is a tool to determine the ability of the analytical method to differentiate and quantify the analyte in the presence of some components expected in the sample. Sensitivity was determined by analyzing control in replicates (n = 6) spiked with the analyte at the lowest level of the calibration standard, that is 0.01 µg/ml [7].

System Suitability Tests: The system suitability parameters were determined by injecting six times the standard solution containing SER at a concentration of 200 µg/mL and Ketoconazole (IS) at a concentration of 10 µg/ml. The retention time (R_t), Area (A), height (H), tailing factor (T) and theoretical plate number (N) were the various parameters which were tested on the sample containing the combination solution of 200 µg/mL of SER and 10 µg/mL of IS [8].

Linearity: Calibration curves were constructed with standard solutions, containing the two compounds simultaneously, ranging from 10-500µg/mL. Linearity was determined through the calculation of a regression line by the method of least squares, representing the peak areas a function of the standard concentration [9].

Precision and Accuracy: Precision was determined by repeatability (intraday estimation of drug) and intermediate precision (accuracy) for three consecutive days. The intra-day assay precision and accuracy were estimated by analyzing six replicates containing SER at three different concentrations, *i.e*., 10, 100 and 500µg/mL. The inter-day assay precision was determined by analyzing the three concentrations on six different runs. The criteria for acceptability of the data included accuracy within ± 2% deviation (DEV) from the nominal values and precision within 2% relative standard deviation (RSD). For intra-day, accuracy and precision at each concentration were assayed on the same day. The inter-day accuracy and precision were evaluated for three subsequent days [10].

Ruggedness: From the stock solution, sample solutions of SER (10 µg/mL, 100 µg/mL and 500 µg/mL) were equipped and analyzed by two different analysts employing analogous operational and environmental surroundings. The peak area was calculated for identical concentration solutions six times [11, 12].

Limit of Detection and Limit of Quantification: Five standard solutions were prepared by serial dilution of SER stock solution (1000 μg/ml) in the range of 50, 100, 110, 120, 130, 140 and 150 µg/ml in order to determine the limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated according to LOD = 3.3 σ/S and LOQ = 10 σ /S, where σ is the standard deviation of the response and S is the slope of the calibration curve [13].

RESULTS AND DISCUSSION:

Optimization: Response Surface Based on 3^2 Central Composite Design: A 3^2 central composite design (CCD) was utilized to obtain the surface response graphs to determine the optimum conditions and to examine the interactions between factors employed for the design. This design allowed the response surface to be modeled by performing the number of experiments equal to

2k+2k+1, where k is the number of variables (k=3), which makes a total of 13 experiments to be executed as per CCD design [14].

TABLE 2: EXPERIMENTAL CONDITIONS OF ESTIMATION OF SER BY HPLC ACCORDING TO THE CENTRAL COMPOSITE DESIGN AND OBSERVED RESPONSE VALUES

Exp no.	Run	A	B	Y1	Y2	Y3
1	7	-1	-1	28775	1079.28	5.124
2	3	1	-1	58871	2589.01	2.184
3	4	-1	1	32558	1521.23	3.104
4	12	1	1	87752	3278.12	1.481
5	10	-1	0	30255	1299.7	4.201
6	11	1	0	78998	2841.2	2.014
7	9	0	-1	81587	8058.89	1.521
8	8	0	1	92982	8126.73	1.218
9	1	0	0	108852	8587.9	0.184
10	2	0	0	107452	8452.7	0.195
11	5	0	0	107588	8512.7	0.225
12	6	0	0	108794	8415.9	0.218
13	13	0	0	108487	8542.1	0.207

A: 0.01M sodium phosphate: acetonitrile ratio; -1 = 20:80% v/v, 0= 25:75% v/v, +1= 30:70% v/v B: pH of the mobile phase;-1= 2.0, 0= 4.5, +1= 7.0. Y1: Peak area; Y2: theoretical plates; Y3: tailing factor.

Three-dimensional response surface plots are given in **Fig. 1** and are highly imperative to study the effects of the factors and their interaction on the selected responses. The result of the response of peak area showed that the intermediate values of the factors were found to be the best as the peak area was found to be maximum, which was desirable when the values of acetonitrile: sodium phosphate ratio was 75:25% v/v. This is represented in **Fig. 1a**.

Similarly, theoretical plates were found to be the maximum at the middle values of the selected factors. It is understood that the number of theoretical plates indicates the better resolution of the peaks. The maximum value of the theoretical plates was found when the acetonitrile: sodium phosphate ratio was 75:25% v/v. This relation has been indicated in **Fig. 1b**.

The tailing factor is an important criterion in the selection of the best peaks of HPLC. The desirable tailing factor should be as low as possible so that the symmetry of the peak is maintained and the effect of the other factors can be neglected. It was observed that the tailing factor was improved at an intermediate level of the selected pH of the mobile and selected ratios of acetonitrile: sodium phosphate, as shown in **Fig. 1c**. The tailing factor was also found to be least in these conditions [15].

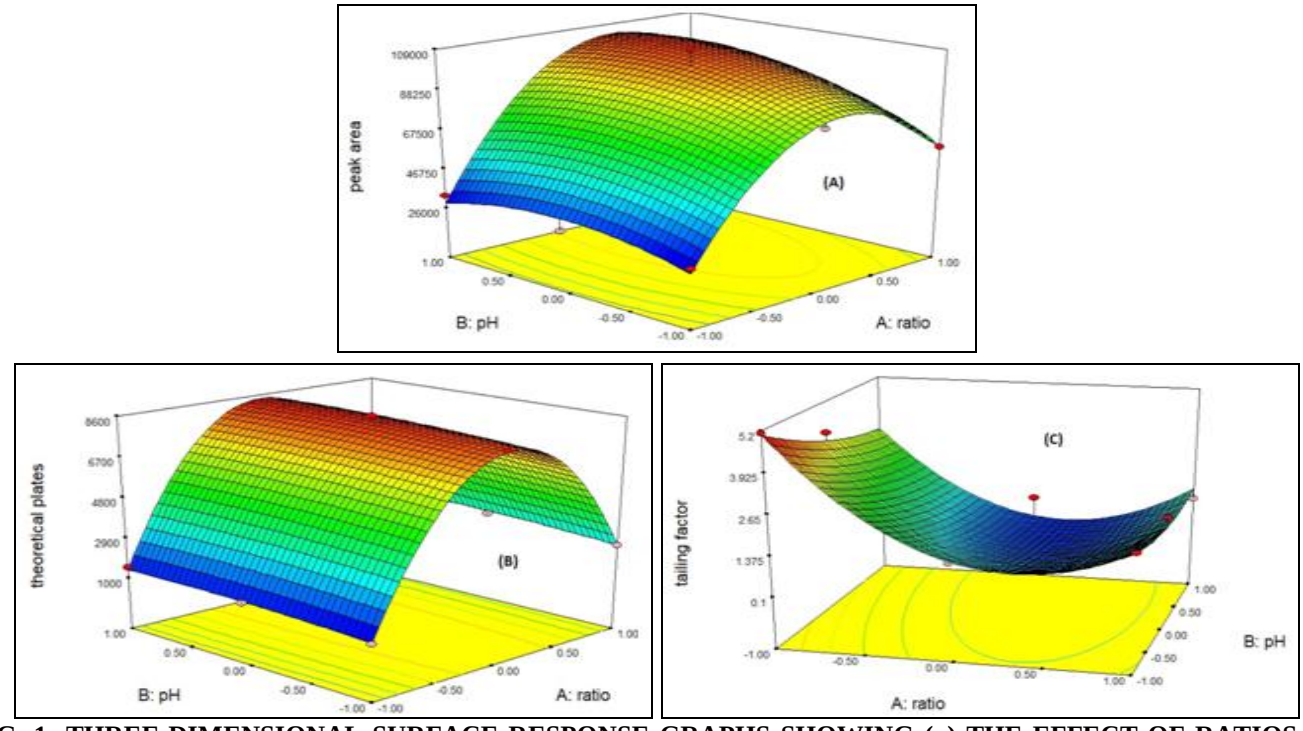

FIG. 1: THREE-DIMENSIONAL SURFACE RESPONSE GRAPHS SHOWING (a) THE EFFECT OF RATIOS OF 0.01M SODIUM PHOSPHATE: ACETONITRILE CONTENT IN MOBILE PHASE AND ITS pH ON THE PEAK AREA (b) THE EFFECT OF RATIOS OF 0.01M SODIUM PHOSPHATE: ACETONITRILE CONTENT IN MOBILE PHASE AND ITS pH ON THE THEORETICAL PLATES (c) THE EFFECT OF RATIOS OF 0.01M SODIUM PHOSPHATE: ACETONITRILE CONTENT IN MOBILE PHASE AND ITS pH ON THE TAILING FACTOR

The model was authenticated by analysis of variance (ANOVA) employing Design Expert software version 8.0.0. The ANOVA tests demonstrated that the models materialized to be adequate, with a significant lack of fit (P<0.0001) and with a satisfactory coefficient of correlation (r). It was found that the final optimized mobile phase comprised of acetonitrile: sodium phosphate ratio was 75:25% v/v [16].

The quadratic equations of all the three responses are given as follows:

$Y1= 100435 + 22338.83A + 734317 B + 6274.5 AB - 41605.5A^2 - 8947.5B^2$

$Y2= 8347.21 + 801.35A + 199.82 B + 61.79 AB – 6156.42 A^2 – 134.06 B^2$

$Y3= 0.771 – 1.125A – 0.504 B + 0.33 AB + 2.04 A^2 + 0.31 B^2$

The equations describe the relation of dependent variables on the independent variables mathematically. Equation (1) indicates that there is a positive correlation of the sodium phosphate: acetonitrile ratio and pH of the mobile phase with the response peak area, while the interaction terms of the independent variables are negatively correlated. This means that as the ratio between the solvents and their pH will increase, the peak area will also increase and *vice versa*. However, very low values of the independent variables will also lead to low peak areas.

Similarly, equation (2) shows a positive relation of the sodium phosphate: acetonitrile ratio and pH of the mobile phase with the response theoretical plates, while the interaction terms of the independent variables are negatively correlated. This means that as the ratio between the solvents and their pH will increase, the theoretical plates will also increase and *vice versa*.

However, very low values of the independent variables will also lead to low peak areas. Equation (3) shows a negative relation of the independent variables with the dependent variable- tailing factor, while all the interaction terms are positively correlated. This means that as the ratio between the solvents and their pH will increase, the peak area will decrease and *vice versa*. Hence, optimum values of all the dependent variables are desired to be chosen.

Over-lay Plots: Overlay plots can predict the values of the dependent variables from the values of the independent variables. It was found that the best values of independent variables acetonitrile: sodium phosphate ratio was found to be 72:28% v/v and pH of the mobile phase was found to be 4.5.

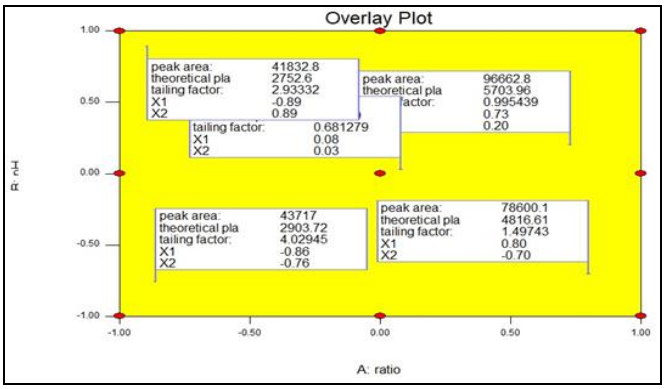

FIG. 2: OVERLAY PLOT FOR THE DETERMINATION OF THE BEST VALUES OF INDEPENDENT VARIABLES

System Suitability Tests: System suitability tests were performed for the proposed method of estimating SER and to check its applicability and commerciality. Also, many parameters like resolution (Rs), retention time (Rt), Area (A), height (H), tailing factor (T) and theoretical plate number (N), capacity factor (K') and asymmetry were checked to ensure column efficiency, selection of the chromatographic conditions and its repeatability **Table 3**.

TABLE 3: SYSTEM SUITABILITY PARAMETERS

Parameter	Compound	
	SER	Ketoconazole
*Resolution (Rs)	1.887	
Retention Time (Rt)	5.136	7.023
Area (A)	79050	56550
Height (H)	7579	6027
Tailing Factor (T)	0.187	0.123
Theoretical Plate number (N)	8965.339	9998.23

*Resolution between SER and IS.

The Relative standard deviation (RSD) of peak areas of six consecutive injections was found to be less than 2%, which indicated good injection repeatability and adequate precision. The tailing factor (T) for the SER was found to be close to 1, reflecting good peak symmetry. Resolution between SER and Ketoconazole was found to be 4.81, which showed good separation of peaks. Higher values of theoretical plate number (N) demonstrated good column efficiency.

Fig. 3 depicts the HPLC chromatogram which was obtained when 300 µg/ml of SER and 100 µg/ml of IS (Ketoconazole) was spiked in the rat plasma and estimated for its concentration by the developed method of HPLC [17].

Linearity: The regression equation and determination coefficients were estimated by assessing the linearity of the developed method over a drug concentration range of 10 - 500 µg/ml. The coefficient was found to be 0.998, and the regression equation which was generated was found to be y = 265.4x + 477.2. The linearity of the developed method to estimate SER has been clearly shown in **Fig. 4**.

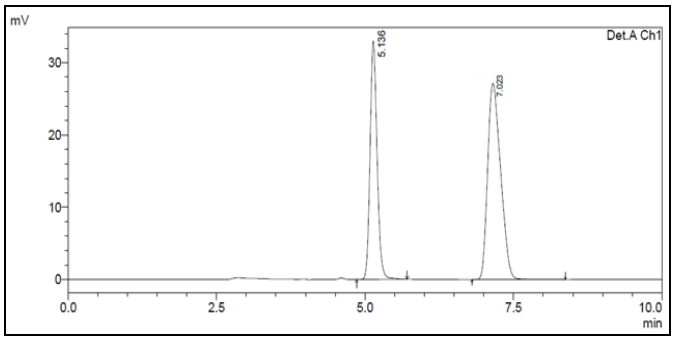

FIG. 3: HPLC CHROMATOGRAM OF RAT PLASMA SPIKED WITH SER PURE DRUG AND IS

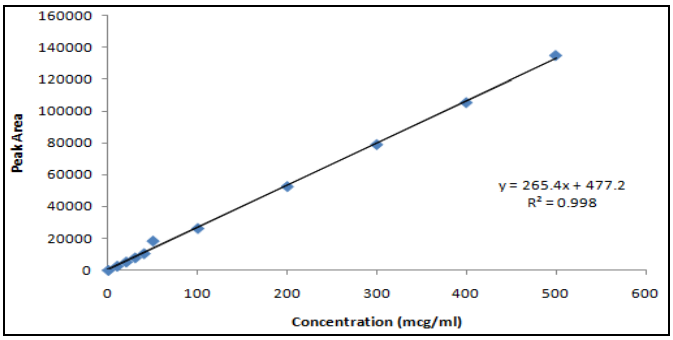

FIG. 4: LINEAR STANDARD CURVE DETERMINATION OF SER (CONCENTRATION RANGE 10-500 µg/ml)

Precision and Accuracy: Samples containing the drug in concentrations of 10, 100 and 500µg/ml were estimated intra-day and inter-day to confirm the precision and accuracy of the developed method. The results of the conducted study are given in **Table 4**. All the data obtained fulfill the acceptance criteria. Intra-day and inter-day precision (% R.S.D.) of the methods were lower than 2% and were within the acceptable limits to be in concurrence with the guidelines for the United States Pharmacopeial norms method validation. Accuracy was with the deviation between the nominal concentration and calculated concentration for SER well below the limit of ± 2%. The results obtained for the determination of precision and accuracy were reproducible and robust [18].

Ruggedness: To determine that the method development for estimation of Sertaconazole is competent, robust and efficient, ruggedness is assessed by carrying out a same analysis under the same conditions by two different analysts. As seen in **Table 5**, drug estimations were not greatly affected due to change in the analysts as the values of % RSD were within the allowed limits.

Limit of Detection (LOD) and Quantitation (LOQ): The LOD and LOQ for SER were found to be 0.1 µg/ml and 0.15 µg/ml respectively. This indicates that the amount of drug which can be detected by HPLC can be as low as 100 µg/ml and the amount of drug which can be quantified by the same can be as low as 150 µg/ml.

TABLE 4: INTRA- AND INTER-DAY PRECISION AND ACCURACY DETERMINATION OF SER CONCENTRATION IN SPIKED RAT PLASMA SAMPLES

Spiked concentration* (µg/ml)	Mean measured concentration (µg/ml ± SD)		Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
10	10.02 ± 0.128	9.733 ± 0.28	1.62	2.98	0.78	1.19
100	99.125 ± 1.83	101.833 ± 1.60	1.59	1.85	0.98	1.39
500	514.257 ± 2.89	522.789 ± 2.547	1.73	1.59	0.53	1.65

*(n = 6 at each concentration for intra-day and n= 6 for interday precision).

TABLE 5: VALUES OF RUGGEDNESS STUDIES FOR THE DEVELOPED METHOD

*Concentration (µg/ml)	The amount determined by analyst 1 (% ± SD)	RSD (%)	The amount determined by analyst 2 (% ± SD)	RSD (%)
10	9.02 ± 0.11	1.58	10.18 ± 0.25	1.08
100	102.389 ± 1.78	1.37	99.88 ± 1.96	1.22
500	503.483 ± 6.483	1.88	509.805 ± 12.89	1.45

*(n=6)

CONCLUSION: A simple, sensitive and robust method for the determination of SER, an antimycotic agent, by HPLC was developed and validated. Ketoconazole was employed as an IS, and no interfering peaks were observed at the elution times of SER and are. System suitability parameters like linearity, precision, accuracy, resolution, theoretical plates, retention times, *etc* of the proposed method were checked and were found to be appropriate. Linearity was demonstrated over the concentration range of 10 to 500 µg/ml. LOD and LOQ were found to be 0.1 µg/ml and 0.15 µg/ml respectively.

The method was accurate, reproducible, specific, and provided excellent separation and enable the quantification of SER. Face centered 3-level 2-factor central composite design was applied to study and understand the effect of various independent factors like pH of the mobile phase and ratio of sodium phosphate: acetonitrile on the dependent factors like peak area, theoretical plates and tailing factor. These effects were found to be noteworthy, and the p-values $\leq$ 0.001, which proved that the model employed here was highly significant. The best values of acetonitrile: 0.01M sodium phosphate was found to be 72:28% v/v and that of pH of the mobile phase was found to be 4.5. These values were determined by the values of the dependent variables.

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CONFLICT OF INTEREST: None

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