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Advances in Bioresearch

ORIGINAL ARTICLE

Application of Quality by Design (QBD) based Approach in Development and Validation Of RP-HPLC Method for Estimation of Silymarin from Bulk and Different Pharmaceutical Dosage Form

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ABSTRACT
A simple, sensitive, precise, accurate and robust reverse phase high performance liquid chromatographic method has been developed for estimation of Silymarin (SM) in various pharmaceutical formulations. Chromatographic separation was carried out using Phenomenex C18 column (250 X 4.6 mm, 5µm particle size) by using Photodiode array detector (model 2998) at 287nm. Isocratic elution was carried out in mobile phase compose ACN and water (80:20) the flow rate was kept 1 mL/min. The retention time for SM was found to be 2.49 min. The method has been validated according to International Conference of Harmonization (ICH) Q2 (R1) guidelines with respect to specificity, system suitability, accuracy, precision, and robustness. The described method was linear over a range of 05-25 µg/mL slope was y = 22714x - 2717.4 and R^2 was found to be 0.9991. The mean percent recoveries of marketed and in house cream formulation were found 100.3%, 100.4% 100.11% and 99.02%, 99.3%, 99.8% respectively. The One-way ANNOVA test was used to check the intermediate precision data obtained under different experimental setups. Robustness of method were check by using two independent factors; mobile phase composition, flow rate was used to design Response surface central composite design (CCD) was used to study in depth the effects of these independent factors. The optimization data analysis was carried out by multiple linear regression analysis using Design Expert® ver.7.0.0 software. This method can be adopted in routine analysis of Silymarin in tablet and inhouse cream dosage form and it involves relatively low-cost solvent and no complex extraction technique.
***Key words**: Central Composite Design, HPLC, Silymarin, ANNOVA, Design Expert*

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INTRODUCTION

Silymarin is a plant-derived flavonoid which is extracted from the fruits and seeds of milk thistle (*Silybum marianum* L. Gaertn.), which belongs to the family Asteraceae [1]. The extract of milk thistle has been used as a general medicinal herb used to treat the disorders of the spleen, liver, and gallbladder since as early as the 4th century BC, and was first reported by Theophrastus [2]. In modern times, it has been primarily used in liver disorders including hepatitis, alcoholic liver diseases, and cirrhosis [3-5]. Extensive chemoprevention studies have been performed in several *in-vitro* and *in-vivo* animal models to test the efficacy of silymarin and establish its mechanism of action against skin carcinogenesis [6, 7]. Silymarin is available in both topical and oral formulations and has been used in multiple conditions in dermatology, such as melanoma and nonmelanoma skin cancers [8], melasma, rosacea, psoriasis, atopic dermatitis, acne, wound healing, cosmeceuticals, as well as in anti-aging therapy. The photoprotective mechanisms of silymarin and silybin on the skin are mainly due to their ability to reduce and suppress the harmful effects of solar UV radiation, such as UV-induced oxidative stress, inflammation, immune responses, and DNA damage, as well as the induction of apoptosis [9]. Pharmacological studies have revealed that silymarin is nontoxic even at higher than physiological doses, which suggests its safe use

for the treatment of various diseases [3]. No significant interaction of silymarin has been reported. The safety and efficacy of this herbal drug in liver disease.

Silymarin is a complex mixture of four flavonolignan isomers, namely, silybin, isosilybin, silydianin, and silychristin with an empirical formula C25H22O10 [Figure 1] [10]. Among the isomers, silybin is the major and most active component and represents approximately 60–70%, followed by silychristin (20%), silydianin (10%), and isosilybin (5%). Silymarin also contains taxifolin, which has significant free radical scavenging properties [11].

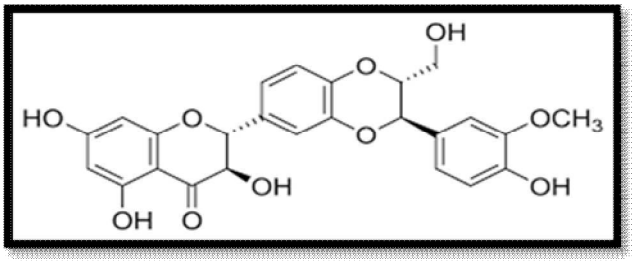

Figure 1. Structure of Silymarin

INSTRUMENTS AND EQUIPMENT

The HPLC analysis was carried out using dual pumps Waters Alliance 2695 separation module and Detector is a Photodiode array (model 2998) with wavelength range of 190-800 nm having rheodyne sample injection port with a 10 µl loop. The separation was made on a Phenomenex C18 with column specification (250 × 4.6 mm, 5 µm) and the analysis of data was carried out by using Waters Empower 3 Software. The other equipment used in analysis consists of digital weighing balance (AUX 220, Shimadzu Corporation)), electric hot air oven (MSI-66, Meta Lab Scientific industries, India), Ultrasonic bath (PCI Analytics Pvt. Ltd, Mumbai, India).

MATERIAL AND METHODS

Drug sample Silymarin was obtained as a gift sample from Yucca Pvt Ltd., Mumbai, India., certified to contain 95 %, w/w and used without further purification for analysis. The experiment called for HPLC-quality methanol and acetonitrile. Other chemical was procured from S D Fine-Chem Ltd. in Mumbai. Double distillation assembly used for preparation of double-distilled water. Silymarin tablets were 70 mg (Silybon 70 mg) purchase from medical store and in housed cream formulation was prepared.

EXPERIMENTAL

Chromatographic condition

During analysis chromatographic conditions used consist of Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) and mobile phase used as acetonitrile and water 80:20% v/v maintain at a flow rate of 1.0 mL/min. The eluents may be seen at 287 nm. Every analysis was performed at room temperature.

Preparation of Standard Stock Solution: -

10 mg of Silymarin was accurately weighed and transferred to a 10ml volumetric flask and volume was made upto10 mL with methanol (Stock solutionA-1000µg/ml). Form Stock solution A 1mL was taken into a 10mL volumetric flask and volume was made up to 10ml with Mobile phase (Stock solution B-100 µg/mL).

Procedure for Calibration curve:

Standard solution of Silymarin in concentration range of 5µg/mL to 25 µg/mL obtained by transferring (0.5, 1.0, 1.5, 2.0, 2.5 mL) of Silymarin stock solution (B-100 µg/mL) to the series of 10ml volumetric flask.

Procedure for analysis of tablet formulation:

Twenty tablets were weighed accurately and powdered. A quantity of tablet powder equivalent to 10mg of SM was accurately weighted and transferred into a10ml of volumetric flask, 7mL of methanol was added. The content was ultrasonicated for 15min. The volume was then diluted to the mark and mixed well. A small portion was with-drawn and filtered through a 0.45µm filter to ensure the absence of particulate matter.

Procedure for Analysis of Cream Formulation:

Weight the amount of cream equivalent 10mg of SM adds in 10ml of methanol. The content was ultrasonicated for 15min. The volume was then diluted to the mark and mixed well. A small portion was

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with-drawn and filtered through a 0.45µm filter to ensure the absence of particulate matter. Prepared further dilution.

Validation of HPLC method

ICH Q2 (R1) guideline is commonly used for analytical method validation [7]. The standard SM stock solution was added in the tablet formulation stock solution at to achieve 80%, 100%, and 120% QC range along the calibration curve to evaluate the precision and accuracy. Every study was done in triplicate over the course of three days. Low percent RSD values were viewed as an indication of precision, and the percentage of recovery that reached the true added values was viewed as a sign of accuracy. The analytical data was evaluated for variance of analysis, and the intermediate precision was calculated by using F (theoretical) and F (observed). Following equation used for determination of detection and quantitation limit: DL = (3.3σ / S) and QL = (10σ / S), σ where is the standard deviation (SD) of response (y-axis) and s is calibration curve slope. The percentage organic concentration, flow rate, and wavelength of the system were slightly but purposefully changed under the ideal chromatographic circumstances mentioned previously, and the variations in the parameters were recorded. This was done to test the method's robustness [9].

Statistical Analysis

Statistical analysis was conducted using one way ANOVA. An F value indicated statistical significance. Unless indicated otherwise, all data are expressed as mean ± standard deviation and rounded to one decimal place, except.

RESULTS AND DISCUSSION

Determination of λmax and selection of wavelengths

From the stock B standard solution, 2.0 ml of SM was transferred into 10ml volumetric flask and the volume was adjusted to the mark to obtain strength 20µg/ ml. The solution was scanned in the UV range 200- 400 nm. SM showed maximum absorbance at 287nm.shown in fig.no -2.

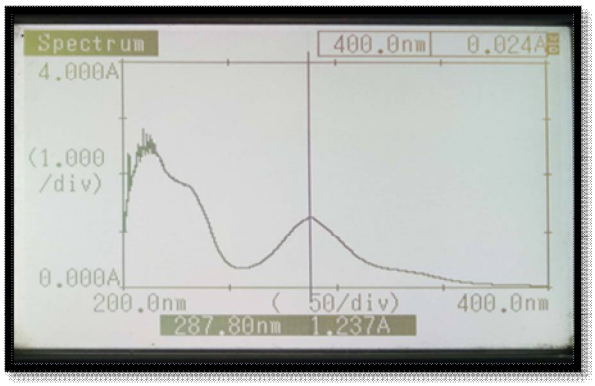

Figure 2. UV spectra of Silymarin

Chromatographic conditions for analysis:

The retention of SM was adequate at min with acceptable system suitability when stationary phase Phenomenex C18 column used with mobile phase of acetonitrile: water (80:20, v/v) at flow rate of 1.0 mL/min. At the ideal wavelength of 287 nm, all eluents were detected. Figure 3 displays the Silymarin chromatogram with optimized chromatographic conditions.

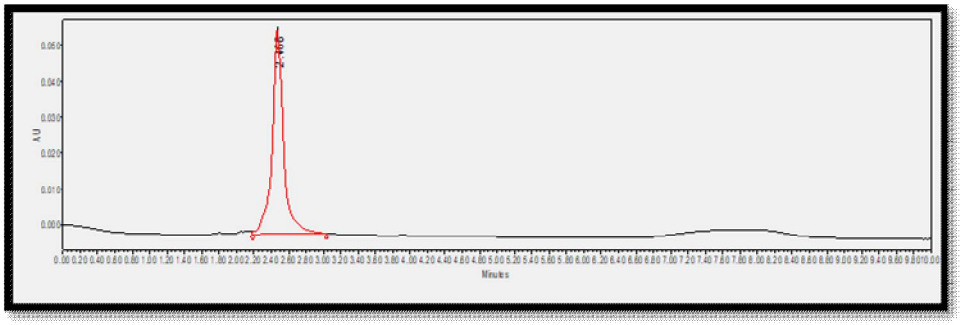

Figure 3. Chromatogram of Standard SM

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Specificity

The HPLC chromatograms recorded for the blank solution and placebo showed no peaks at the retention time of SM. Shown in fig.no.64

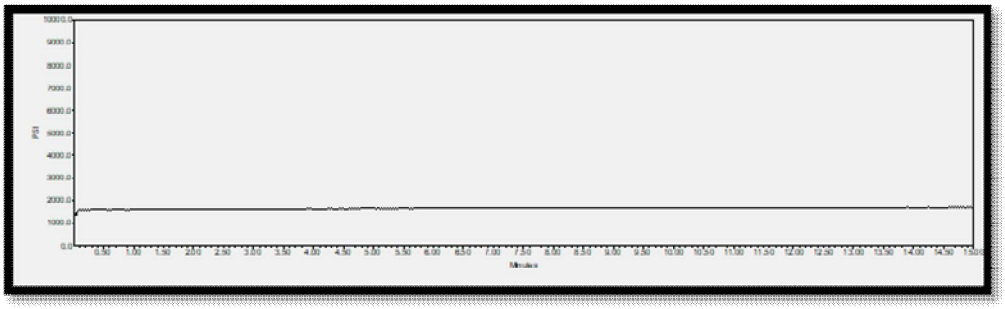

Figure 4. Chromatogram of blank

Linearity

After calibration experiment it was observed that SM was linear in the range of 5- 25µg/mL. The plot of peak area vs. concentration was subjected to least square regression. The equation was y = 22714x - 2717.4, where X is concentration (µg/mL) and Y is the peak area. The regression coefficient was found to be 0.9991. Linearity data shown in table no .1 and calibration curve shown in fig no.5.

Table 1. Linearity data of Silymarin

Sr. No	Conc.	Area
1	5	115313
2	10	221402
3	15	336984
4	20	444808
5	25	571462

Average of three determinations*

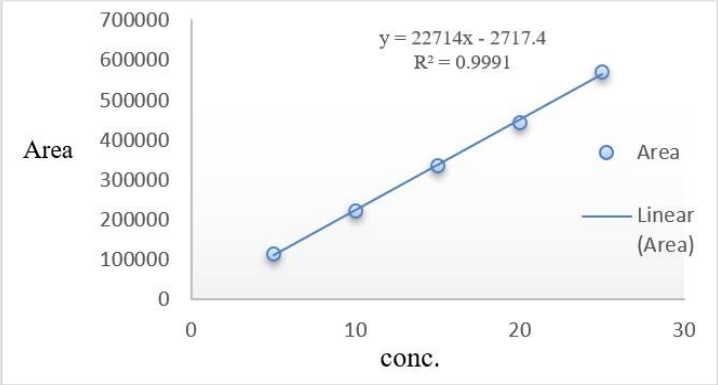

Figure 5. Calibration curve of silymarin

Accuracy and precision

The accuracy was evaluated by standard addition method at three different concentrations of drug (80%, 100%, and 120%) according to label claim. % Recovery of drug added drug was taken as a measure of accuracy. The result obtained for accuracy and precision experiment for tablet and in house cream formulation as shown in Table no.2 and 3 respectively. From the data obtained for accuracy and precision studies, it has been found that mean value of amount of drug found very close to amount of drug added. To determine intra-day and inter-day variability, one way ANOVA was conducted and the F-value for each level was determined by taking ratio of between mean square to within mean square.

Detection Limit

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope, the detection limit for SM was found to be 0.90233µg/mL.

Quantitation Limit

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope, the quantitation limit for SM was found to be 2.734µg/mL

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Table 2. Accuracy and Precision data of Tablet Formulation

Amount Added	Amount Found (µg/mL)			Within mean square	Between mean square	F value
	Day 1	Day 2	Day 3			
80% (18 µg/mL)	18.1	18.1	18.2	0.002017	0.03735	0.05399
	17.9	17.9	17.89			
	18.2	18.2	18.1			
Mean	18.066	18.066	18.0633			
Recovery (%)	100.33	100.33	100.35			
SD	0.1247	0.1247	0.129			
%RSD	0.69	0.69	0.71			
100% (20 µg/mL)	19.99	20.3	20.1	0.005017	0.016617	0.3019
	19.89	20.1	19.97			
	20.2	20.1	20.3			
Mean	20.0266	20.166	20.031			
Recovery (%)	100.1	100.8	100.15			
SD	0.129	0.0942	0.0531			
%RSD	0.645	0.467	0.265			
120% (22 µg/mL)	22.1	21.97	22.05	0.00095	0.000255	0.372549
	22.02	21.98	22			
	21.9	22.01	22			
Mean	22.06	21.986	22.016			
Recovery (%)	100	99.63	100.7			
SD	0.08219	0.01669	0.02357			
%RSD	0.373	0.077	0.107			

Table 3. Accuracy and Precision data of Cream formulation

Amount Added	Amount Found (µg/mL)			Within mean square	Between mean square	F value
	Day 1	Day 2	Day 3			
80% (18 µg/mL)	17.8	17.87	17.9	0.005633	0.002317	0.411
	17.86	17.89	17.8			
	17.7	17.8	17.79			
Mean	17.786	17.85	17.83			
Recovery (%)	98.87	99.16	99.05			
SD	0.06599	0.03858	0.0496			
%RSD	0.371	0.2156	0.27			
100% (20 µg/mL)	19.87	19.95	19.79	0.007217	0.0080	1.11
	19.9	19.92	19.86			
	19.78	20	20.01			
Mean	19.78	19.95	19.88			
Recovery (%)	98.9	99.75	99.4			
SD	0.061847	0.0329	0.0917			
%RSD	0.312	0.16	0.461			
120% (22 µg/mL)	21.79	21.88	21.79	0.011833	0.000817	0.069014
	21.94	21.9	21.78			
	21.79	21.91	22			
Mean	21.84	21.89	21.856			
Recovery (%)	99.27	99.5	100.92			
SD	0.323	0.0569	0.1014			
%RSD	0.323	0.0569	0.463			

Assay

The chromatograms of the drug samples did not show a change in the retention time. There were no interferences from excipients, which are commonly present in the solution. in Marketed formulation. (Tablet of Silymarin) and In house cream formulation the drug content was found to be 99.45% with a % RSD of 1.84 and 97.8 with a %RSD 0.489 respectively as shown in Table no. 4 and 5.The % RSD value indicated the suitability of the method for the routine analysis of SM in marketed formulation and in house cream formulation.

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Table 4. Assay of Marketed tablet formulation

Amount of drug in vial (mg)	Amount of drug found (mg)	Amount found in %	Average (%)	±SD	%RSD
10mg	9.82	98.2	98.77	0.498	0.504
10mg	9.71	99.1			
10 mg	10.1	99.02			

Table 5. Assay of In house Cream formulation

Amount of drug in vial (mg)	**Amount of drug found (mg)**	**Amount found in %**	**Average (%)**	**±SD**	**%RSD**
10mg	9.82	98.2	97.8	0.4	0.4089
10mg	9.74	97.4			
10mg	9.78	97.8			

System suitability testing

System suitability tests are an integral part of method development and are used to expose adequate performance of the chromatographic system. Retention time, Number of Theoretical plates (N). The results given in table no .6 were in acceptable limits.

Table 6. System Suitability Data

Sr.No	Parameter	Result
1	Retention time (min)	2.468
2	Theoretical plate	2571.5
4	USP Tailing	1.1
4.	K-Prime	1.5
5.	Height	56899

Robustness

Robustness of method was check by change in mobile phase composition and Flow rate on the effect of Retention time Tailing and no of theoretical plate was used to design Response surface central composite design (CCD) was used to study in depth the effects of these independent factors. The optimization data analysis was carried out by multiple linear regression analysis using Design Expert® ver.7.0.0 software. The statistical data R^2, F value and polynomial equation was indicate that there is no significate effect shown in table no7, 8, 9. and Figures 6, 7, 8, 9, 10, 11. It indicates that our method was robust.

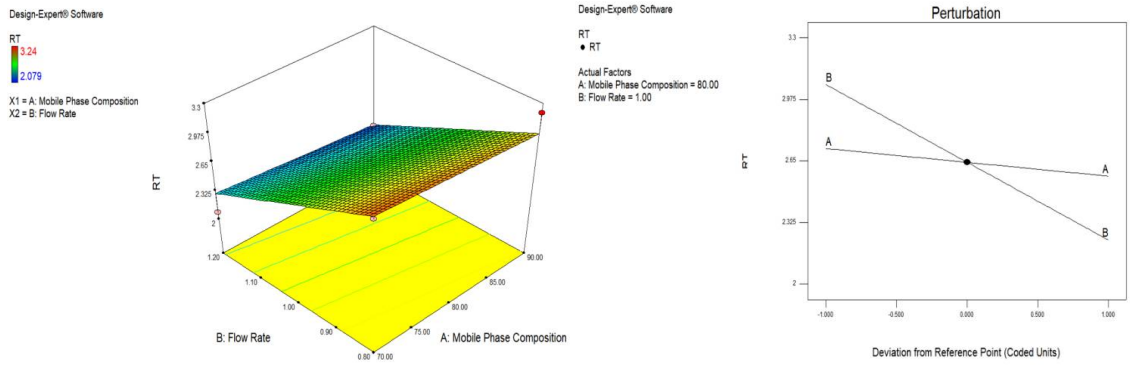

Figure 6. 3-D Plot and Perturbation Plot of Retention time.

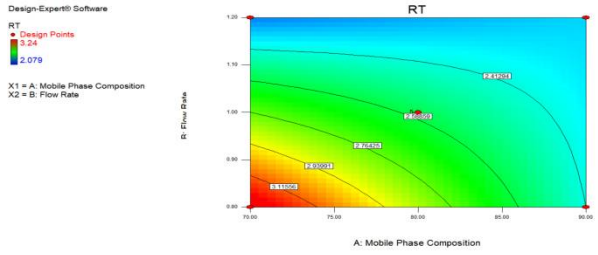

Figure 7. Counter Plot of Retention time

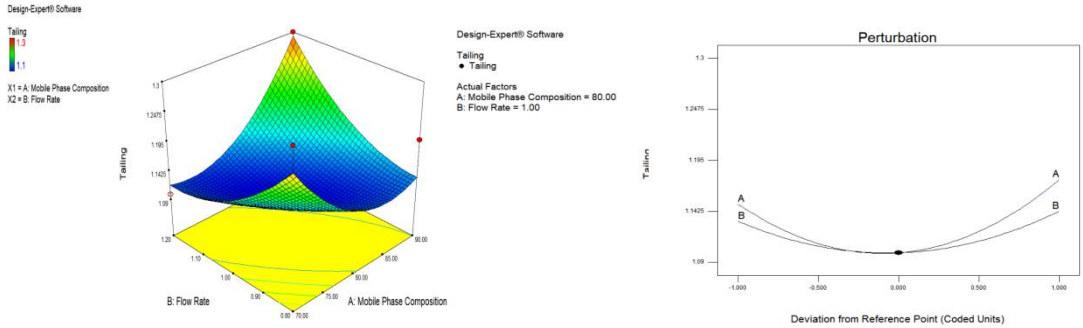

Figure 8. 3-D plot and perturbation plot of USP Resolution

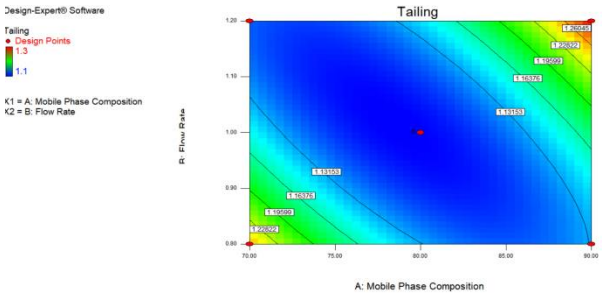

Figure 9. Counter Plot of USP Resolution

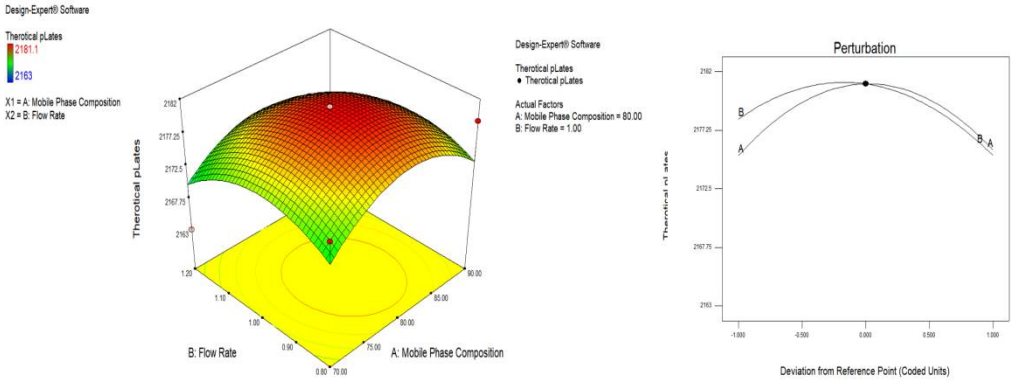

Figure 10. 3-D plot and perturbation plot of Theoretical Plates

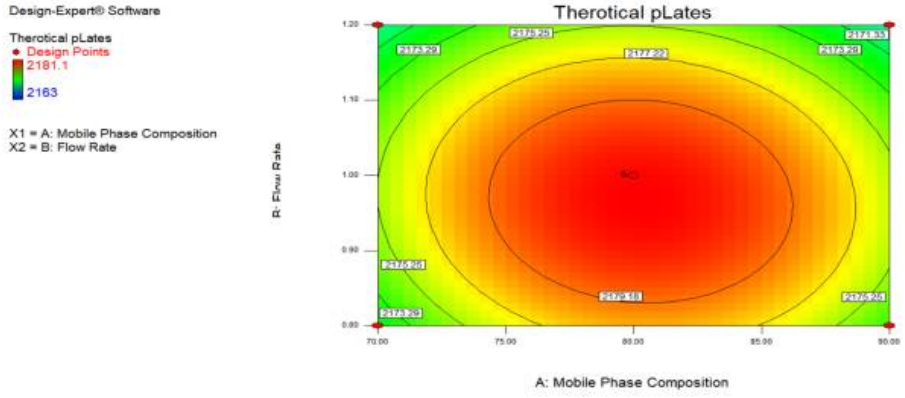

Figure 11. Counter Plot of Theoretical Plates

Table 7. ANOVA for Retention Time

Source	Sum of Squares	df	Mean square	F value	p-value Prob>F
Model	1.20	3	0.40	10.27	0.0029
A-Organic phase conc.	0.31	1	0.31	8.06	0.0194
B- Flow rate	0.65	1	0.65	16.77	0.0027
AB	0.23	1	0.23	6.0	0.0368
Residual	0.35	9	0.039	-	-
Lack of fit	0.35	5	0.069	62.58	0.0007
Pure Error	0.03	04			
Cor Total	1.55	12			
R-Squared	0.7740				

Table 8. ANOVA for USP Tailing

Source	Sum of Squares	df	Mean square	F value	p-value Prob>F
Model	0.057	5	0.011	5.92	0.0187
A-Organic phase conc.	1.250	1	1.250	0.65	0.4478
B- Flow rate	2.145	1	2.145	0.11	0.7489
AB	0.023	1	0.023	11.64	0.0113
A^2	0.027	1	0.027	14.05	0.0072
B^2	9.783	1	9.78	5.06	0.0593
Residual	0.014	7	9.783E-003		
Lack of fit	0.014	3	4.512E-003		
Pure Error	0.000	4	0.00		
Cor Total	0.071	12			
R-Squared	0.08087				

Table 9. ANOVA for Theoretical Plate

Source	Sum of Squares	df	Mean square	F value	p-value Prob>F
Model	326.48	5	65.30	2.10	0.1803
A-Organic phase conc.	0.39	1	0.39	0.012	0.9144
B- Flow rate	17.58	1	17.58	0.57	0.4767
AB	1.0	1	1	0.032	0.8628
A^2	216.02	1	216.02	6.95	0.0336
B^2	129.98	1	129.98	4.18	0.0802
Residual	217.67	7	31.10		
Lack of fit	217.66	3	72.55	36277.14	0.0001
Pure Error	8.000E-003	4	2.000E-003		
Cor Total	544.15	12			
R-Squared	0.600				

CONCLUSION

A simple and efficient RP-HPLC method was successfully developed and validated for the quantitative determination of SM in Pharmaceutical formulation and In house cream formulation. The developed method offers several advantages including simplicity of sample preparation procedures, good recovery, negligible matrix effect, and acceptable sensitivity comparable to the previously reported HPLC method which employs more complex sample pretreatment procedure. Validation experiments proved that the LC conditions used were able to prove the robustness of method. Also, the method had desired accuracy, precision and linearity. The Chromatograms obtained conclude that SM Drug was well resolved from. Analysis of formulation proved a practical range of the method. Thus, QbD approach was successfully applied to achieve optimum chromatographic conditions for SM and an accurate, precise and specific LC method was developed for the drug in solution.

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Consent for publication
Not applicable

Competing interest
The author declares that they do not have any competing interests.

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