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RESEARCH ARTICLE

AQbD Oriented New LC-ESI/MS Method for Quantification of Sirolimus in Drug and Spiked Plasma Sample

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Abstract: ***Background:*** The ICH guidelines Q8 (Analytical Quality by Design (AQbD) technique) give the procedure for optimization of analytical parameter involved method development and validation of Sirolimus (SL) in spiked Plasma and in dosage form by using advanced analytical technique.

Objective: This research paper is based on Quality by Design (QbD) finalized conditions for a method for the determination of concentration of Sirolimus by using liquid chromatography–tandem mass spectrometry (LC-ESI/MS).

Method: Sirolimus is a potent immunosuppresant drug. Critical Process Attributes (CPA) is considered to be an influential parameter in separation, identification and quantification processes by UHPLC-ESI-MS/MS which are pH modifier,organic content, buffer strength, flow rate, Ionization chamber temperature, sheath gas, spray voltage and auxiliary gas that alters critical Analytical Attributes (CAA), like peak area and retention time (Rt). These factors were evaluated first in a factorial design (TAGUCHI) and then extensively in a Central composite design (CCD) to zero-in on the mobile phase for the quantification of Sirolimus(SL) standard drug and along with its internal standard (SL IS) in spiked plasma samples and in the formulation. Pareto chart from initial factorial design (Taguchi) model suggested for which of the CPA factors be given the importance that is to be exhaustively analyzed in the CCD and response surface analysis. An UHPLC instrument with the octadecylsilica column (C18) of dimensions (5 µm, 2.1 × 50 mm) was used and selectivity of the column was modified using methanol: water (65:35) methanol as an organic modifier as the mobile phase at a flow rate of 0.6 mL min-1. While spray voltage and Ionization chamber temperature for the method is maintained at the level predicted by the response analysis. Detection was performed using Triple quadrupole MS/MS by multiple-reaction monitoring via a positive electrospray ionization source. The ICH guidelines had elaborated about the parameters to be studied in method validation, i.e., selectivity, linearity, accuracy, precision repeatability system-suitability tests, method robustness,ruggedness, sensitivity and stability were accomplished.

Results: The results are very clearly indicated that linearity with r value = 0.9980 in the concentration range of 10–500.0 ng mL-1, an intra- and inter-assay precision of 2.4 and 4.1%, respectively, and recovery studies were found to be between 100.8 and 105.6%. The lower limit of quantification was 0.086 ng/mL in 50 µL of human plasma sample.

Conclusion: The present method gives a robust QbD-compliant quantitative UHPLC –ESI-MS/MS method for Sirolimus drug containing plasma samples (spiked).

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1. INTRODUCTION

Analytical Quality by Design (AQbD) [1-5] based method development for immune suppressive agent. Sirolimus (SLS) is an immunosuppressive agent. Sirolimus is a macrocyclic lactone produced by Streptomyces hygroscopicus. The chemical name of SLS (also known as Rapamycin) is (3S, 6R, 7E, 9R, 10R, 12R, 14S, 15E, 17E, 19E, 21S, 23S, 26R, 27R, 34aS) 9,10,12,13,14,21,22,23,24, 25,26,27,32,33,34, 34a-hexadecahydro-9,27-dihydroxy-3 - [(1R) - 2 [(1S, 3R, 4R) – 4 – hydroxyl – 3 - methoxycyclohexyl]-1-methylethyl]-10,21 – dimethoxy 6, 8, 12, 14, 20, 26 – hexamethyl - 23, 27-epoxy-3H – pyrido [2,1-c][1,4] oxa azacyclohentriacontine1,5,11,28,29 (4H,6H,31H) -pentone. Its molecular formula is $C_{51}H_{79}NO_{13}$ and its molecular weight is 914. 17 g/mol. SLS is a white to offwhite powder and freely soluble in benzyl alcohol,methanol, chloroform, acetone,acetonitrile and insoluble in water. SLS available as

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1573-4110/18 $58.00+.00

oral solution as well as tablet formulation.SLS available 1 mg and 2 mg tablet formulation [6, 7]. The molecular structure is shown in Fig. (**1**). It acts by selectively blocking the transcriptional activation of cytokines by inhibiting cytokine production. It is bioactive only when bound to immunophilins.

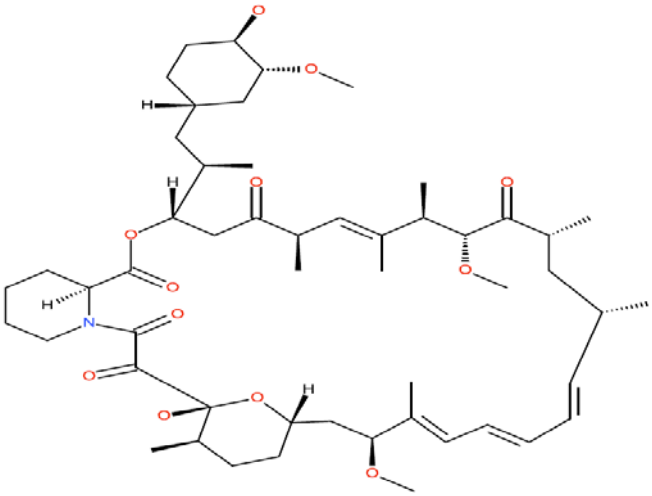

Fig. (1). Structure of Sirolimus (SLS).

As per scientific literature, analysis of SLS has been reported as individual ingredients and in combination with other compounds in the plasma sample by using LC-MS and other Liquid chromatographic technique [8-14]. But nobody reported QbD technique for SLS.

The developed method discusses about the process of optimizing both the LC and ESI/MS parameter simultaneously in a TAGUCHI orthogonal array and Central composite design methods. Combined evaluation reduces the experimental run to a greater extent. The developed method does find to be compliant with the ICH laid acceptable limits for the Bioanalytical method development. The Pareto chart from initial factorial design (Taguchi) model suggested for which of the CPA factors be given the weightage that is to be exhaustively analysed in the CCD and response surface analysis. An UHPLC SIL 20 AC HT with 20 AD instrument with the octadecylsilica column (C_{18}) of dimensions (5 µm, 2.1 × 50 mm) was used and selectivity of the column was modified using methanol:Water (55:45) having the mobile phase at a flow rate of 0.6 mL min^{-1}. While spray voltage for the method is maintained at the level predicted by the response analysis. Detection was performed using Triple quadrupole MS/MS by multiple-reaction monitoring via a positive electrospray ionization source. The ICH –Q2B [15-18] had elaborated about the parameters to be studied in method validation, i.e., selectivity, linearity, accuracy, precision repeatability system-suitability tests, method robustness/ruggedness, sensitivity and stability were accomplished. The results are clearly showing that linearity with r value = 0.9934 in the concentration range of 10–500.0 ng mL^{-1}, an intra- and inter-assay precision having % RSD less than the 5.0 % and recovery studies were found to be in the acceptable range of 98 and 101.2 %. The lower limit of quantification was 0.989.34 ng mL^{-1} in 50 µL of human plasma sample. The objective of this study was to validate a method for quantification of Sirolimus in plasma (spiked samples) and stability.

2. MATERIALS AND METHODS

The HPLC grade water and methanol were purchased from Merck (India) and used as such. Extrapure water was produced in the Questlife sciences, Chennai, with the help of the MilliPure water purification system (EMD Millipore, Billerica, MA, USA). Standard substances SLS and Deuterated Sirolimus (SLS IS), were kindly gifted by Quest Life Sciences, Chennai. Analytical grade ammonium acteate and formic acid were used. All other chemicals used were of AR grade.

2.1. Instrumentation and its Conditions

LC-ESI/MS positive electrospray ionization of SLS produced the abundant protonated molecule (MH+) atm/z 932.189 m/z, under positive ionization conditions and subsequently fragmented in ESI /MS mode to the product ion spectra, which has mass by charge ratio as 864.2 Fig. (**2**). Quantitative analysis was carried out by MRM at m/z 932.189 →864.2 for SLS and its IS.

The several stationary and mobile phases were tested, whereby a combination of a C18 column and a mobile phase Methanol and water in (55:45 ratio) gives the the optimum separation and sensitivity toward SLS. The MS parameters and collision energies were set as derived from the FFD optimised method.

2.2. LC-MS/MS Condition

LC-ESI/MS analysis was performed using a TSQ Quantum triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). The injection volume was 10 µL. The SLS samples were introduced into the mass spectrometer via the ESI source and analyzed in the positive ion mode. The pre-ionized SLS (M+) was detected in the multiple ion reaction monitoring (MRM) mode using the following transitions: *m/z* 932.2–864.25 for SLS.

The QbD optimized LC-ESI/MS conditions were as follows: spray voltage: 3000 kV; sheath gas: nitrogen, 50 (arbitrary units, bar); auxiliary gas pressure, 10 bar; ion transfer capillary temperature: 200 °C**;** collision gas: Ar; and capillary offset voltage: 35 V**.** HPLC was carried out using a Shimadzu SIL 20 AC HT auto sampler with LC 20 AD HPLC pump both of them was controlled by CBM 20 A module. Software used to acquire data was LCQUAN 2.5.6 software which is a 21 CFR Part 11-compliant solution for method development in LC ESI/MS. For the separation of SLS from its matrix a QbD optimized isocratic mobile phase was used. This comprises methanol and water (55 parts) – (45 parts) at a flowrate of 0.6 mL/min. During the experimental run the column and Autosampler tray were preserved in 37 and 4 °C, respectively.

2.3. Sample Preparation

The 100 µL of human plasma samples were spiked with 50 µL each of SLS and SLS IS (100 ng mL^{-1}). To this 1.8 mL of extraction mixture [containing acetonitrile (80 %) (a protein precipitant) and 20 %)] was added and then subjected

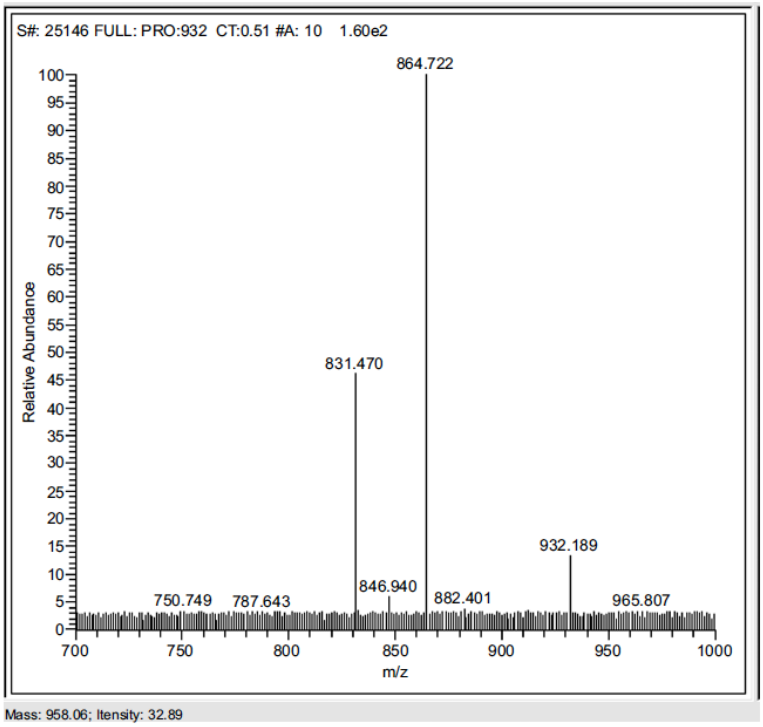

Fig. (2). Mass Spectrum of SLS.

to vortex mixing followed by centrifugation at 13000 g. After this 10 µL was injected directly into the triple quadrupole LC-ESI/MS system. Precipitation of plasma proteins was initiated by adding 200 µL acetonitrile, 50 µL of both SLS and SLS IS solutions were added to 100 µL aliquots of human plasma containing 5 µL of calibration standards. This mixture was vigorously vortexed for 10 min and centrifuged at 12,000 g for 15 min, after which an aliquot (200 µL) of the supernatant was evaporated to dryness in the stream of nitrogen gas. An aliquot equivalent to 5 µL of this solution was then directly injected into the LC-ESI/MS system.

2.4. Preparation of Stock Solution

Primary stock solutions containing 1 µg mL^{-1} concentration of SLS and SLS IS were dissolved in methanol and serial dilutions of this solution were made in mobile phase to achieve working standard solutions at concentrations of 10.0,20.0, 50.0, 150.0,250.0,350.0,450.0 and 500.0 ng mL^{-1} of both SLS and SLS IS. Solutions to outline the lower limit of quantification (LLOQ) and low quality control (LQC), medium QC (MQC), high QC (HQC) and dilution QC (DQC) samples were prepared in bulk by diluting the primary stock solution. The IS working solution (1 µg mL^{-1}) was prepared by diluting an aliquot of stock solution (1 mg mL^{-1}) of SLS IS with methanol. All stock solutions were stored in glass bottles at –20 °C in the dark.

2.6. Selection of Optimization Parameters of LC-ESI/MS Technique by Using DOE

Experimental design for evaluating the critical process attributes (CPA) were organic content concentration, spray voltage, sheath gas and auxillary gas pressure, buffer strength (Ammonium acetate), pH modifier (formic acid) concentration and flow rate. These factors were expected to influence critical analytical attributes (CAA), i.e. area and retention time (Rt) significantly [19]. Fractional factorial design (TAGUCHI) and central composite design (CCD) were considered for finding the critically influencing levels of each factors, this two step experimental runs will greatly reduce the total experiments atleast by 30 for a Six factor process. Factorial design with TAGUCHI was designed to determine the main effect without the effects of the interaction. Experiments were run as per the design and pareto chart was derived based on the effects shown by the factors on the responses Fig. (**3**) and Fig. (**4**). The selected parameter by FFD is optimized by CCD process [20].

The six factors that were found to influence responses and the ones that need to be analyzed extensively in CCD for their influence on CAA‘s were spray voltage, set at low level to 3500 and high level at 4500 v. Similarly, for sheath gas pressure between 10 and 50 bar, auxillary gas pressure between 10 and 20 bar, organic solvents Methanol 55% and 75 %, ionisation probe temperature 200-300 and flow rate at 0.2 and 0.7 ml min^{-1}. With the help of DESIGN EXPERT ® trial version 10.0 a statistics program for design of experiment, factors and responses was chosen and fed into this program. A total of 6 run based on the generated TAGUCHI orthogonal array design Table **1**, and their levels, Table **2**, points were carried out. A factorial model (main effect with the elimination of terms having values greater than 0.1) was chosen after aliases were eliminated from the model. Most of the influence comes mainly from spray voltage, Methanol

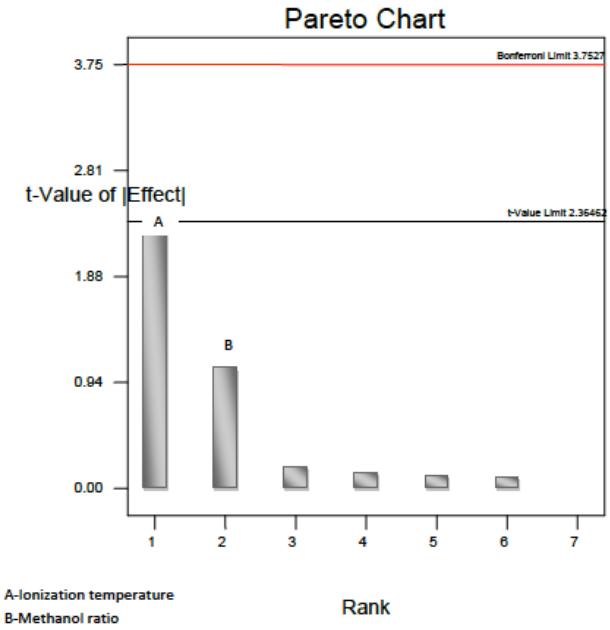

Fig. (3). A Pareto Chart Showing Effects of CPA on First Response Rt (Retention Time).

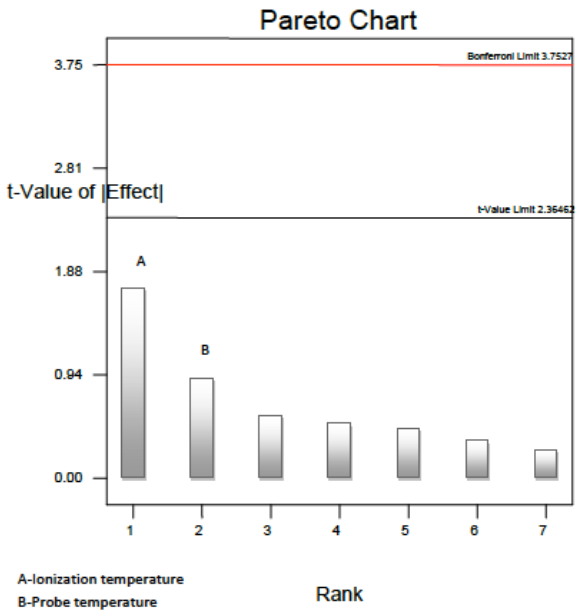

Fig. (4). A Pareto Chart Showing effects of Influential CPA on Second Response Area.

Table 1. Factorial design for optimizing LC-ESI/MS conditions for determining SLS in plasma samples in response variable with Peak Area and Retention time

% Methanol	Spray Voltage	Probe Temperature	Flow Rate	Column Content	Sheath Gas	Auxillary Gas	Retention Time in Min	Response 2
-1	-1	1	1	1	-1	1	2.82	422846
-1	1	-1	1	-1	1	-1	2.78	490436
-1	-1	-1	-1	1	1	1	1.84	423651
-1	1	1	-1	-1	-1	-1	2.5	532382
1	-1	1	-1	1	1	-1	2.6	425635
1	-1	-1	-1	-1	-1	-1	2.95	510786
1	1	-1	1	-1	1	1	2.15	362520
1	1	1	1	1	-1	1	2.24	485621

Table 2. Factorial Design method variable with levels.

Method Variable	Low (-1)	High (1)
1.Methanol %	55	75
2.Spray Voltage	3500	4500
3. Flow Rate	0.2	0.6
4.Probe temperature	200	300
5.Sheath Gas	10	50
6. Auxiliary Gas	10	20

content and flowrate, whereas sheath gas and auxillary gas pressure were having influence only at > 0.1% on the response. The Pareto chart was derived and the significance level was set at 0.05. The most influencing factors found were spray voltage with more than 98 % and Probe temperature by 0.5 % for Rt, while all other factors were found to influence only by a meagre 0.05% for the first reponse of (R1) retention time. To finalize the factor that has highest influence on area, the generated model shows only one factor greatly influences and that is Methanol with 99% contribution, while the flow rate has a very small percentage of influence on area, therefore, fixed the parameters at 0.1 % and 0.3 - 0.6 ml min^{-1} . From these two models, it was decided to take up the methanol content,spray voltage and Probe temperature to be optimized further for their contribution on how they influence the (R1) retention time and area(R2), respectively. This is shown in Table **2**. The content range of methanol used as an organic modifier was set between 55 and 75%. When the content of Methanol was less than 55%, the analysis time was too long, which did not meet the goal (approximately more than 5 min) of the present research to develop a rapid analytical method. The content of Methanol cannot be more than 75%, wherein the system was not stable and selectivity gets reduced. The range of flow rate was 0.3–0.7 mL min^{-1} because the elution time gets prolonged at a flow rate of less than 0.3 mL/min. In the Pareto chart, the first and second most significant factors influencing the Rt were content of organic layer and (flow rate have little contribution of less than 0.025 %) spray voltage which have more than 99 % of contribution towards the area of the analyte, while less than 0.02 % on Rt.

2.7. Central Composite Design (CCD)

The selected factors (content of organic layer modifier, spray voltage and probe temperature) that need further exploration in a CCD, a response surface design for improving Rt and area.The generated design is shown in Table **3**. The experimental point ranges of the selected factors for the response surface method were the same as for fractional factorial design but the center level has been introduced so as to get the median value of extremes Table **4**, A total of 11 experiments for CCD were carried out and the responses were noted down. Analysis of variance (ANOVA) test was performed to select function model and to evaluate significance of factors over responses. The significance level was set at $p < 0.05$. The selected function model for Rt and area was reduced 2F1 and reduced quadratic model for Rt and area, respectively. For Rt, the most influencing factors were interaction terms of methanol and spray voltage. Terms were reduced 2F1 factors of methanol and spray voltage, while none of the other factors show influence which was evident from

Table 3. Shows the experimental factors and levels used in central composite design.

Factor	Name	Level(-1)	Level(0)	Level(+1)
A	Spray Ionization voltage	3500	4000	4500
B	Probe temperature	200	250	300
C	Mobile phase(methanol ratio %v/v)	55	65	75

Table 4. Summary of Design Matrix as per the Central Composite Rotatable Design for Sirolimus.

Run	Blocks type	Factor 1	Factor 2	Factor 3	Response 1	Response 2
		A-Ionization Spray voltage	B-Probe temperature^{o} C	C-Mobile phase ratio(methanol ratio)	Retention time in min	Peak Response area
1	Axial	4000	320.71	65	2.7	2732562
2	Fact	3500	200	55	2.6	2934589
3	Fact	4500	300	55	2.5	2254365
4	Axial	4000	250	79.14	2.6	2454657
5	Fact	3500	300	75	2.4	3025452
6	Axial	4707.10	250	65	1.96	2654258
7	Centre	4000	250	65	2.4	2256325
8	Fact	4500	200	75	2.1	2653789
9	Axial	4000	250	50.85	2.6	2569852
10	Axial	4000	179.28	65	2.4	2565392
11	Axial	3292.83	250	65	2.1	2389147

their p value < 0.05, so only methanol influenced the area other than the most influencing term spray voltage, all the others did not find a place in the model. Once the model has been generated, optimization criteria were set for the area to be maximized and minimization of the retention time. In the response surface 3D graph and from the perturbation plot, the area and the retention time opposed to each other because they were inversely proportional to the content of organic modifier and spray voltage. The factors with the most significant influence on sensitivity were content of organic modifier and spray voltage, while probe temperature has less effect on area where as interaction terms of the main factors could find a place in the model for Rt values. This is mainly due to nature of mobile phase changes with polarity altering the sensitivity.

2.8. Method Validation

Selectivity of the method was ensured by checking that no interference peaks were observed at the Rt of both SLS and SLS IS with blank plasma samples. Linearity of the method was validated by injecting nine calibration samples ranging from Lower Limit of Quality Control sample (LL QC), LQC, MQC, HQC and one concentration in between each level and the standards injected were as follows 10.0, 50.0, 100.0, 150.0, 250.0, 350.0, 450.0 and 500.0 ng ml^{-1}. Peak area was plotted against concentration and regression analysis with weightage of x^{-2} was given in finding the regression values.

The accuracy and precision were determined by injecting the three QC samples LQC, MQC and HQC repeatedly for six times and the results were analyzed for their % RSD. For accuracy, MQC level sample was spiked at 50 ,75 and 100 % of MQC and analyed by the proposed method.

The sensitivity of the method was established by injecting the LLOQ for six times and the their % RSD was verified to be in the acceptable limits (20 % for LLOQ and 15 % for ULOQ). The stability of the analytes (SLS and SLS IS) in spiked plasma in different storage conditions was examined by analyzing low and high QC samples (n=3 at each concentration) and compared their deviation from the nominal concentration. Bench-Top stability was done till the value fall below the acceptable limits. Freeze-thaw stability was determined after three freeze-thaw cycles of the QC samples by storing at -70 ° C for 24h and thawing to room temperature before analysis was performed and it was done for three times. Short-term stability was determined after exposure of the QC samples at 4 °C for 24 h for three days whereas long term stability was done upto14 days. Autosampler stability too was found out. All the samples would be stored in the freezer at -20 °C and at intervals specified in the respective stability studies, their concentration was verified with the developed QbD optimized method. All the results were based on their percentage difference from their nominal values [21]. (US Food and Drug Administration, 2001). Fig. (**2**) shows representative LC-ESI/MS MRM chromatograms obtained from the analysis of plasma spiked with SLS (1 ng/mL).

3. RESULTS

MS parameters that have considerable influence on peak area were column temperature and spray voltage which were taken into the CCD for studying elaborately. The effect of selected parameters is clearly shown in Perturbation plots Fig. (**5**) and Fig. (**6**). The other MS parameters auxillary gas and sheath gas pressure were unable to influence both the responses much evident from the perturbation plots of Rt and area, therefore, fixed at 10 and 50 bar respectively. The LC parameters flow rate does influence the Rt but not statistically significant enough to be considered for CCD. Similar to it, the flowrate was not considered for the next level of investigation as is the earlier case of sheath and auxillary gas pressure. Flow rate was fixed as follows. Flowrate 0.5 ml min^{-1} lesser rate would increase the time of investigation beyond 4 min. The another important LC parameter is Organic modifier in mobile phase. As per FFD results, the concentration of methanol influence the peak area as well as Retention time of drug. Initially, it was found that below 55%v/v mL/ min flow rate of mobile phase the peaks became broad and above 75%v/v proper separation was not observed. The concentration of methanol should be used as a LC parameter and minimum volume of organic solvent is safe for environment. Therefore, the optimization of methanol ratio was important LC parameter for the optimization process. When the factors got reduced from 7 to 3 CCD was generated and 11 experiments were conducted. The model generated for Rt reduced polynomial equation model suggesting the interaction terms between spray voltage, methanol and probe temperature. Whereas peak area and the sec-

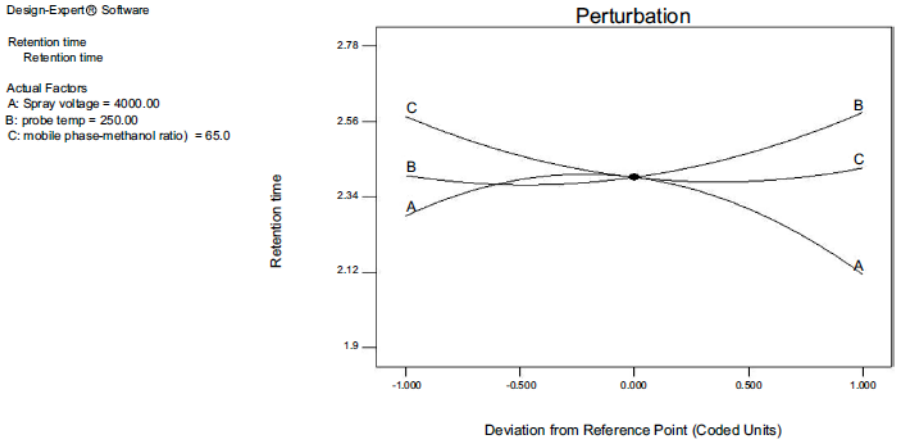

Fig. (5). Perturbation Plot Showing the effects of selected factors on Rt.

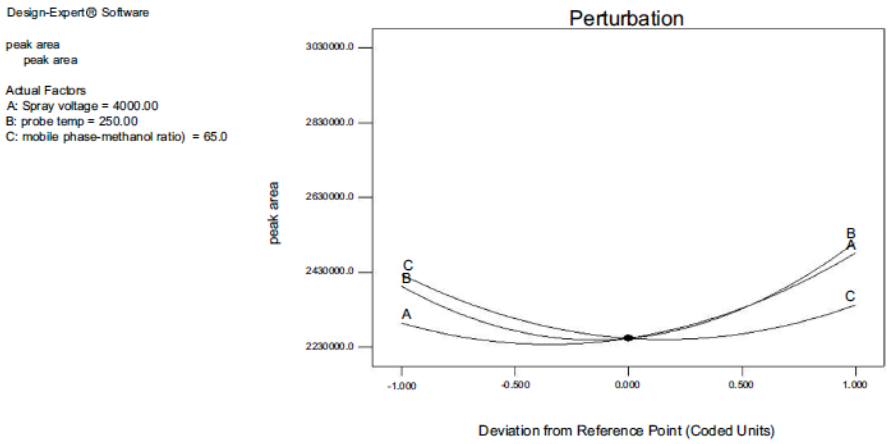

Fig. (6). Perturbation Graph Showing the effects of Selected factors on Area.

ond response of the model has interaction term of spray voltage and Probe temperature. AMBIGUITY IN THE SENTENCE, AUTHOR'S ASSISTANCE REQUIRED

The design matrix generated the rotatable Central Composite and the Design is shown in Table **4**. The system was fully optimized using the 11 experiments. This design is composed of a three level factorial design with eleven experimental run. In this study, the levels of each factor were selected based on prior scouting experiments. Many more experiments would have been required if this method was optimized with the standard univariate approach. The ionization spray voltages and probe temperature play key roles in ionization of compounds because if sufficient ionization spray voltage is not supplied the compound will not be completely ionized and becomes unionized in bulk. The higher rate of ionization spray voltage i.e. more than 4500 v, affects the charged particle size i.e. it produces less than 1 nm size particle which undergoes ion evaporation, further reducing the charged particle. So we planned to optimize the mass ionization spray voltage. The spray voltage usually ranges between 3000 to 5000. For sirolimus we used the spray voltage is between 3500 to 4500. The optimized level of ionization voltage is 3500. The second important mass parameter is probe temperature. The probe temperature affected the daughter ion formation, it produced the effect on substance peak area.

The probe temperature is used to regulate the movement of ions and maintain the ionized state of molecule in ionization chamber. The movement of ions in ionization champer is increased by keeping the high value of probe temperature. The decrease in probe temperature reduces the movement of ions. According to probe temperature and ionization voltage the peak response area will be affected. The more the probe temperature the more will be the peak area and due to this the more will be the daughter ion movement. Therefore, the optimization process is applied for both probe temperature and ionization voltage.The high probe temperature affects the movement of charged particle and causes interation between charged particle to form clustering of charged particle. So to avoid the clustering the probe temperature should be optimized a uniform flow of particle. The optimized level of ionization voltage (3500 v) and Probe temperature 200 ° C produces a completly charged particle in less probe temperature.

The developed method we used is mobile phase which is methanol:water (65%:35%) only.We did not use any environmental toxic solvent and even the volume of methanol was also reduced from 65 % to 55% by using optimization process. The 55 % ratio of methanol gives good separation and collects separated ions in narrow and sharp peak in chromatogram. Table **4** shows the ranges of each factor: Ionization voltage is 3500 v to 4500 v; Mobile phase ratio is 55.00-75.00 %v/v and probe temperature is 200-300º C. The Optimized method parameters auxillary gas and sheath gas pressure were unable to influence both the responses were clearly evident from the perturbation plots of Rt and area, therefore fixed at 10 and 50 bar, respectively. Here, the main goal is to develop the method with minimum run time as well as good peak retention and environment safe mobile phase flow. This facilitates the accurate quantification of drug within a short period with using environment safe mobile phases. Hence, the retention time of eluting compound peak (RT), and peak response area were taken as response. The Derringers Desirability value of optimized method is 0.86 which is an ideal value. The effect of CCD parameters is clearly shown in 3D dimensional picture Fig. (**7**). The overall desirability value was found to be with in the limit. The diagram Fig. (**8**) shows the effect of Desirability for Optimized method (0.86).

The predicted Rt was 2.42 and obtained values were 2.44. In the case of area predicted value the observed values were found to be very close. Standard calibration curves were linear over an SLS concentration range of 10-500 $ngmL^{-1}$ in human plasma (spiked). Linear regression of these curves resulted in a linear fit of $y = 0.04159x + 0.000474$ ($r = 0.9934$) with a weightage of x^{-2}. Assay sensitivity was determined by analyzing LLOQ samples (n = 6) in three separate validation batches. The values are shown in Table **7**. The precision values were found to be in the range < 3.7 % (RSD) and accuracy studies were between 97-102 % as shown in Table **8**. At three QC levels, the calculated concentrations at each level are shown in Table **8**. The same table shows a summary of intra- and inter-assay precision and accuracy data for QC samples in plasma (spiked) containing SLS. Results show that these results suggest that the acceptable accuracy and precision of the described method. The LLOQ was set at 0.078 ng mL^{-1} for SLS using 100 µL of

Table 5. Regression Equation and Statistical Parameters obtained from ANOVA.

Regression Equation	Adjusted R2	Model p Value	% CV	Adequate Precision
Retentiontime = (-.81214+6.16072E-003A-0.017585B-0.14029C-7.91429E-007B^2+3.88571E-005B^2+1.02143E-003C^2	0.8876	0.0399	5.15	8.50
Peak area = +3.26364E+007 -4248.10361A-58647.51564B-4.59859E+005C-6.53197AB +27.24875AC +713.40529BC+0.53717A^2+79.17227B^2+1295.69429C^2	0.9997	0.0188	1.5	12.50

Table 6. Comparison of experimental and predictive values of experimental runs under optimum conditions.

Optimum Conditions	Selected Optimized Run					
	A: Ionisation spray voltage	B:Probe temperature	C: Mobile phase ratio	Peak area	Retention time in min	Desirability
1	3500	200	55	2975519	2.42	0.850
	Experimental Value					
	Predictive Value			2933519.2	2.46	
	Predicted Error (%)			0.0112	0.0081	

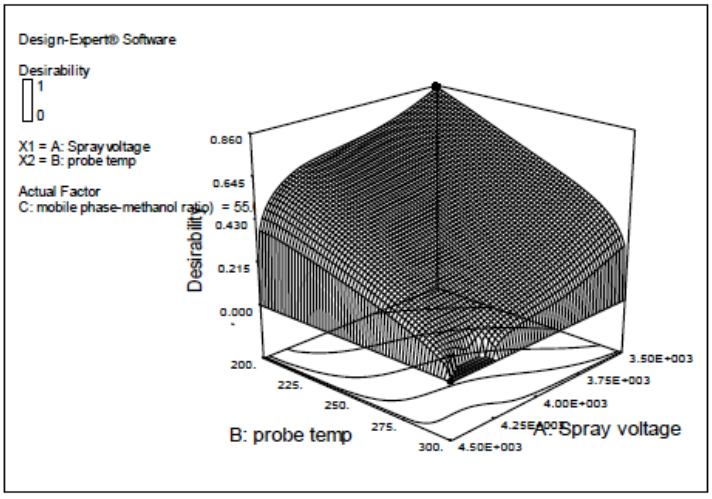

Fig. (7). Response Surface Plot Showing the Desirability for the selected factors.

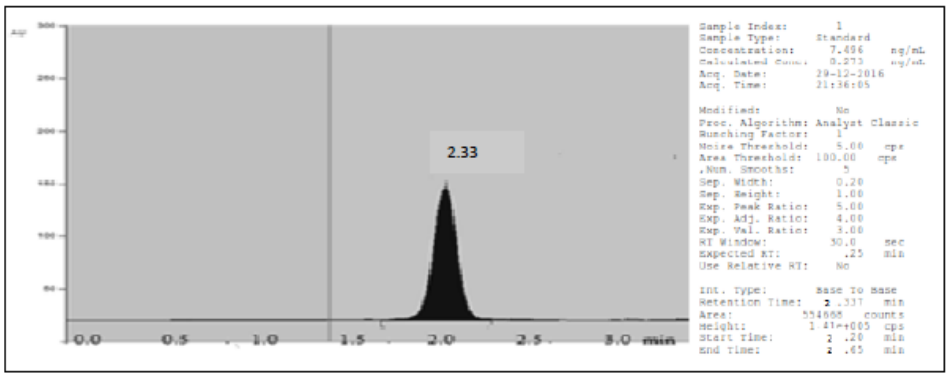

Fig. (8). Chromatogram of SLS.

Table 7. Parameters of linear regression equations for Sirolimus.

Parameters	Sirolimus*
Calibration range (ng mL^{-1})	10-500 ng/ml
Correlation coefficient (r)	0.9943
Slope	4159.78
Intercept	50260.9
LOD (µg mL^{-1})	2.035ng/ml
LOQ (µg mL^{-1})	989.34ng/ml

LOD = Limit of Detection, LOQ = Limit of Quantification
* average of six determinations

Table 8. Accuracy study of Sirolimus by using the proposed method.

% Level	Amount Added (ng mL^{-1})	Amount found* in ug mL^{-1} (Mean ± S.D)	%Recovery* (Mean ± S.D)
50	50	49.23 ± 0.04	98.5 ± 0.42
75	75	74.25 ± 0.02	99.6 ± 0.21
100	100	101.97 ± 0.05	101.2 ± 0.54

*Average of five determinations

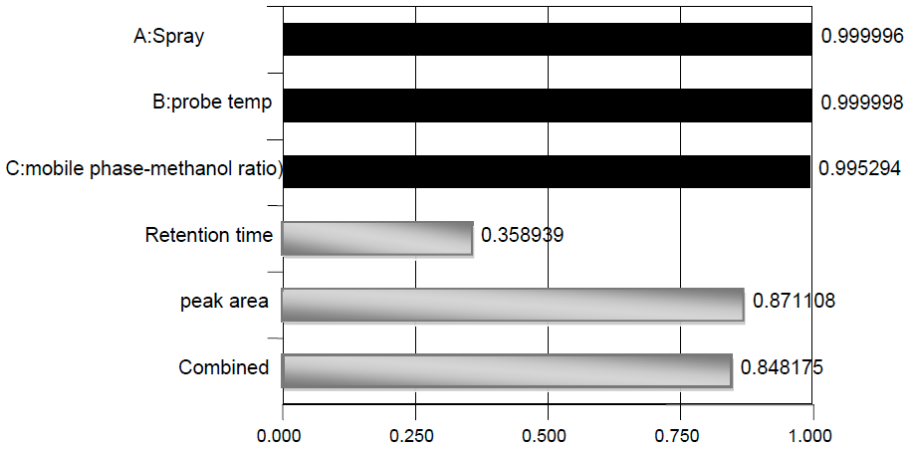

Fig. (9). Overall Desirability bargraph of Optimized method for SLS.

spiked plasma (a representative chromatogram is shown in Fig. (**9**), noting that the signal-to-noise ratio for SLS is >10 times the baseline at 0.078 ng mL^{-1}. The average extraction recoveries determined at LQC, MQC and HQC concentrations were between 88 - 95% for SLS. The developed assay was applied to a laboratory prepared spiked samples and softgel capsules containing SLS. The mean concentration and recoveries for them were found to be in good agrrement with the nominal values and that was with precision % < 5. The overall process efficiency of SLS quantified in spiked plasma was consistent at three concentration levels, varying from 98.33% to 102.6%, while that of the IS was 92.7%. In addition, this demonstrates that the precipitation of plasma via the addition of acetonitrile can be successfully used to extract SLS from spiked plasma. The stability results of SLS during sample handling three freeze–thaw cycles, short-term temperature storage 3 days, long-term stability 15 days and the stability of the processed samples in the auto sampler for 30 h were evaluated and shown in Table **9**. These sample preparation and storage steps had little effect on the quantification of LQC and HQC samples. Extracted QCs and calibration standards were allowed to stand at 4 °C for 24 h prior to injection without affecting quantification.

CONCLUSION

A simple, QbD optimized, robust,sensitive Tandem mass spectrometry method was developed and validated for the determination of SLS concentration in spiked plasma utilizing a protein precipitation and low pressure gradient elution monitored over MRM mode. This assay procedure provides a linear dynamic range from 10 to 500 ng mL^{-1},

Table 9. Stability of Sirolimus in spiked Plasma.

S.no	Storage Condition	Concentration in ng/mL	Accuracy in %
1	Freeze thaw stability (After 3 cycles	50	98.20
		100	99.3
2	Bench top stability (After 15 hrs)	50	101.2
		100	99.2
3	Bench top extraction stability	50	98.3
		100	102.2
4	Long term stability (After 5 days)	50	100.2
		100	100.5
5	Injector stability (After 2 days)	50	98.04
		100	100.6

Table 10. Constraints details of Sirolimus by using the proposed method.

Constraints						
		Lower	Upper	Lower	Upper	
Name	Goal	Limit	Limit	Weight	Weight	Importance
A:Spray voltage	Minimize	3500	4500	1	1	5
B:probe temp	Minimize	200	300	1	1	5
C:mobile phase-methanol ratio)	Minimize	55	75	1	1	5
Retention time	Minimize	1.9	2.78	1	1	3
peak area	Maximize	2.25437E+006	3.02545E+006	1	1	5

Solutions							
Number	Spray Voltage	Probe Temp	Mobile Phase-Methanol Ratio)	Retention Time	Peak Area	Desirability	
1	3500.0	200.0	55.000	2.467	2933519.214	0.850	Selected

leading to an LLOQ of 0.078 ng mL^{-1} using 100 µL of blank plasma. Moreover, the previously reported method addresses only separation of sirolimus with traditional approach. While our proposed method is able to quantify Sirolimus in plasma (spiked) within a short run time with less mobile phase and low ionization spray voltage. The proposed method is rugged nature and this study proves that the method may be applied for various researches and other preclinical studies.

AUTHOR CONTRIBUTIONS

M.Sumithra conceived and designed the experiments and analyzed the data Also, wrote the first draft of the manuscript.

RAVICHANDIRAN.V and SHANMUGASUNDARAM .P contributed in the writing of the manuscript.

Agree with manuscript results and conclusions: Jointly developed the structure and arguments for the paper:. Made critical revisions and approved final version:

All authors reviewed and approved of the final manuscript

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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