IJPSR (2015), Vol. 6, Issue 10 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 April, 2015; received in revised form, 08 June, 2015; accepted, 26 July, 2015; published 01 October, 2015

QUALITY BY DESIGN APPROACH TO OPTIMIZATION OF TACROLIMUS LOADED PLGA NANOPARTICLES

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Keywords:

Quality by Design (QbD), PLGA, nanoparticles, Critical quality attributes (CQAs), *I*-Optimal design, Design space (DS).

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ABSTRACT: The aim of the present study was to implement QbD approach to identify, evaluate and control critical process and formulation parameters and their impact on critical quality attributes (CQAs) and to establish design space with multifactorial combination which would provide high degree of assurance to achieve quality target product profile (QTPP). A risk assessment was performed with various process and formulation parameters to determine their impact on particle size and encapsulation efficiency (CQAs) of nanoparticles and further high risk parameters were optimized using *I*-Optimal design. Design space was generated by setting limits to CQAs as per acceptance criteria of Tacrolimus loaded PLGA (TC PLGA) nanoparticles. The optimized formulation from design space was further characterized by XRD, DSC, SEM, *in-vitro* dissolution study. The nanoparticles obtained were free flowing, spherical, smooth and uniform. *In-vitro* dissolution study showed ability of nanoparticles to sustain drug release for period of 4 weeks. In conclusion, it can be demonstrated that, QbD approach was successfully implemented for understanding of process and formulation parameters and further develop robust design space for TC PLGA nanoparticles to achieve sustained drug release from complex PLGA delivery system.

INTRODUCTION: A polymeric parenteral nanoparticle (NP) has potential and is effective drug delivery due to their ability to control drug release for extended period of time [1, 2]. It can effectively deliver the drug to the specific sites as well as with higher therapeutic benefit while minimizing side effects related to high drug dose and extended drug release of certain therapeutic medicaments [3, 4].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(10).4342-53

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(10).4342-53

Moreover, due to their size, it can penetrate specific tissues such as endothelium of cancer and inflamed tissue, target cells or in the blood brain barrier (BBB); and can provide sustained release with improved pharmacokinetic and pharmacodynamic profiles of loaded drugs [5, 6]. Nanoparticles (NP) in size range from 10 to 1000 nm can be prepared using biodegradable and/or synthetic polymers. Based on different method of preparation drug can be absorbed onto the nanoparticle surface and/or can be entrapped inside the polymer to form particle matrix [5]. The certain potent drugs which show higher variability in bioavailability can be loaded in nanoparticles with potential application of improvement in pharmacokinetic and pharmacodynamic of drugs with reduction in side effects [2, 3, 7].

Poly-lactic-co-glycolic acid (PLGA) is one of most successfully used biodegradable polymer with minimal systemic toxicity with its use as carrier for drug delivery in the form of microparticles, nanoparticles, implant and medical devices. PLGA is the only FDA and EMA approved polymer for parenteral administration, which can provide the sustained release of an encapsulated or matrix embedded drug [1, 8, 9].

PLGA based nanoparticles can protect drugs from enzymatic degradation and enhance their stability. Due to its inert nature, it can be used for specific delivery of drugs, proteins, peptides or nucleic acids to their target tissue. Fabrication of PLGA nanoparticles and their characteristics are depending upon different critical parameters such as, method of preparation, process and formulation parameters involved in manufacturing of nanoparticles which can be governed by controlling all these critical parameters [10, 11].

Tacrolimus, a macrolide lactone immunosuppressant from the fungus *Streptomyces tsukubaensis* approved by USFDA for the prophylaxis treatment of multiple organ (heart, pancreas, bone marrow, small bowel, and lung) transplants and for the treatment of T cell-mediated autoimmune diseases [12] . The tacrolimus has very low solubility and high permeability (cLogP = 5.78) and is therefore a biopharmaceutical classification system (BCS) class II drug; its molecular weight is 822.05, elimination half-life of 8.7-11.3 hrs. It is a substrate for CYP3A5 and MDR1 genes polymorphisms of the genes which is one of the major reasons for the large inter-individual variations in the pharmacokinetic characteristics of tacrolimus [13, 14].

Also, it has incomplete absorption from the GIT and has first-pass effect as a result drug shows poor oral bioavailability which varies from 4% to 93% [13, 15]. Higher inter-individual variability of tacrolimus induces toxicity such as nephrotoxicity, hypertension, diabetogenic effects and neurotoxicity (tremor, seizure and encephalopathy) and became causes for discontinuation of the tacrolimus therapy [14, 16, 17]. Prograf® injection (Astellas, Chuo, TKY, Japan) is approved product by USFDA.

A current scenario in product development inclined towards the quality building into product and is motivated through Quality by design (QbD) holistic and system-based approach. QbD approach is based on concepts from ICH Q8, Q9, and Q10 guidelines, guiding industry movement towards building quality into pharmaceutical through manufacturing, bridging the gap between development and manufacturing using sound knowledge to demonstrate and assure the product's safety, quality and efficacy throughout lifecycle [17, 18, 19, 20].

QbD based study includes detailed risk assessment of process and formulation development with understanding their correlation to product quality, safety and efficacy and further optimization critical parameters through well established design of experimental tools to obtain design space with multifactorial combination.

In present study, the purpose is to develop Tacrolimus sustained release PLGA based formulation using system-based QbD approach to understand the process and formulation parameters influence and their risk on critical quality attributes (CQAs) and to fetch design space (DS) with desired quality target product profile (QTPP).

MATERIAL AND METHODS:

Materials:

Tacrolimus, PLGA (lactide: glycolide ratio 50:50, molecular weight = 50,000–75,000) and PluronicF-68 (poloxamer 188) was supplied as gift sample by Emcure Pharmaceuticals, Pune; Evonik (Mumbai); and VerGo Pharma Ltd (Verna, Goa) respectively. Trehalose dihydrate was purchased from Sigma-Aldrich (Mumbai). HPLC grade water was obtained from a Milli-Q water purification system (Millipore Co., Milford, MA, USA) and was used throughout the study. Methanol, acetone and acetonitrile were of HPLC grade.

TC-PLGA nanoparticle using nanoprecipitation method:

TC-PLGA and empty PLGA nanoparticles were prepared using the nanoprecipitation method [1, 2, 3].

Briefly, PLGA and Tacrolimus were dissolved in acetone and further added to the aqueous solution containing surfactant, poloxamer 188 under continuous stirring at room temperature for 3-4 hrs. The traces of acetone from nanoparticles dispersion was evaporated under vacuum. The nanoparticles were centrifuged at 20000 rpm for 30 mins at ambient temperature and washed with milipore water 2-3 times to remove free drug and residual traces of acetone.

Separated nanoparticles were dissolved in milipore water and trehalose dihydrate as a cryoprotectant and subjected to freezing at −20°C for 12 h in glass vessels, and further sublimation lasted at −54°C for 36 h at a vacuum pressure of 0.001 mbar (Christ freeze dryer, Christ Alpha 1-2 LD). Lyophilized samples were collected and kept in a tightly closed container and stored in dessicator at 2-8°C. The % yield of empty and Tacrolimus-loaded nanoparticles were calculated and reported.

Initial Risk assessment for TC-PLGA nanoparticles formulation:

An overall risk assessment of the TC-PLGA nanoparticle formulation was performed. Based on prior scientific knowledge, identification of formulation and process parameters was carried out for manufacturing method impacting the entrapment efficiency and particle size of TC-PLGA loaded nanoparticles as CQAs as these parameters were are likely affect QTPP (Quality Target Product Profile) of Tacrolimus nanoparticles. The high risk components were studied using design of experimentation (DoE) to reduce their risk to low level by controlling these parameters in specific accepted range.

TABLE 1: RISK ASSESSMENT TO IDENTIFY VARIABLES AFFECTING DRUG PRODUCT QUALITY.

Drug Product CQAs	Initial risk assessment of the Process and Formulation variables					
	PLGA Conc.	Stabilizer Conc.	Stirring Speed	Type of Organic solvent	Organic: Aqueous Phase ratio	Rate of Addition of Organic phase
Entrapment efficiency	High	High	Medium	Low	Low	Low
Drug Release	High	Low	Medium	Low	Medium	Low
Particle Size	High	High	High	Medium	Medium	Medium

TC-PLGA nanoparticles optimization study: Optimal Design:

Based on the initial risk assessment and the preliminary feasibility study, response surface method, *I*-Optimal design with coordinate exchange points for two factors; PLGA: poloxamer ratio (X1) and stirring speed (X2) as critical attributes. 'Design Expert version 9' (State Ease, USA) software was used to generate design matrix and for purpose of statistical analysis. *I*-Optimal design generated 16 experimental runs with two levels and multiple coordinate points within two levels. The purpose of *I*-optimal design was to optimize the factor settings, requiring greater precision in the estimated model. Polynomial equations were generated for each response entrapment efficiency (Y1) and particle size (Y2) of TC PLGA nanoparticles by optimizing formulation and process parameter.

Design space was constructed by setting the criteria for the CQAs using contour plots. Validation of the applied model was carried out by performing the 3 checkpoints nanoparticles formulations at different levels of factors within design space and tested to evaluate correlation between actual results with predicted results. Linear regression line was drawn between actual and predicted values and percentage error was calculated.

TABLE 2: FORMULATION AND OPTIMIZATION OF TC PLGA NANOPARTICLES USING *I*-OPTIMAL RSM DESIGN

Exp	X_1: PLGA: poloxamer ratio	X_2: Stirring Speed (rpm)	Y_1 Particle Size (nm)	Y_2* Entrapment Efficiency (%)	Polydispersity Index (PDI)
1	0.80	500.00	148.40	71.17±1.20	0.053
2	1.08	500.00	193.30	75.15±1.15	0.209
3	1.08	500.00	190.20	74.84±2.10	0.218

4	0.94	500.00	169.50	79.93±1.10	0.114
5	1.20	650.00	442.40	63.15±1.26	0.994
6	1.20	650.00	471.90	61.56±0.83	0.619
7	0.86	650.00	154.60	73.82±0.95	0.112
8	0.97	800.00	173.70	72.50±1.36	0.103
9	0.97	800.00	192.90	71.63±2.15	0.160
10	0.97	800.00	194.40	69.85±1.40	0.173
11	1.13	825.00	203.50	64.62±1.50	0.221
12	0.82	825.00	185.20	57.19±1.36	0.161
13	1.20	1000.00	322.80	52.13±0.86	0.264
14	0.80	1000.00	237.70	55.37±1.26	0.552
15	1.01	1000.00	252.00	58.74±2.15	0.275
16	1.01	1000.00	261.40	60.49±1.20	0.609

For all batches rate of addition of TC and PLGA solution to aqueous phase containing poloxamer 188 was 5 ml/min; PLGA: TC ratio kept constant to 4:1; Volume of batch varied to maintain PLGA: poloxamer ratio and PLGA: drug ratio; Temperature maintained 25^0C±2^0C (Cryostat bath).*All values are expressed as mean ± SD (N=3)

Entrapment efficiency:

Entrapment efficiency was calculated by measuring the entrapped drug to that of unentrapped Tacrolimus. Nanoparticles dispersion was centrifuged at 20000 rpm for 30 mins and supernatant liquid was collected. 2 ml of supernatant liquid was transferred to 10 ml volumetric flask, 0.5 ml of sulphuric acid was added and acetonitrile used to make up the volume. The solution filtered through 0.22 µm PES syringe filter measured the absorbance using UV spectrophotometer at 291 nm. The % drug entrapment efficiency was calculated by following formula.

% Drug Entrapment Efficiency =

Total drug (mg) – Free drug (mg)/ Total weighed drug (mg) X 100.

Particle size:

The average particle size analysis and size distribution (PDI) of the prepared nanoparticles were measured using a Nanosizer 90ZS (Malvern Instruments, Southborough, MA). The intensity of scattered light was detected at 90^0 to an incident beam. 2 ml of TC PLGA nanoparticles was placed in polystyrene cuvette and then particle size measurements performed at 25^0C. The particle size analysis of a sample consisted of three measurements, and the results are expressed as mean size ± SD.

Morphological characterization of TC-PLGA nanoparticles: To examine the sphericity and surface characteristics, the morphology study of TC PLGA nanoparticles was observed using scanning electron microscopy. The nanoparticles were coated with gold (<20 nm thick) using sputter (JFC-1100, JEOL, University of Pune) for 5 min at 20 mA. Observation was performed at an accelerating voltage of 5 kV and a working distance of 10 mm under an argon atmosphere in a high-vacuum evaporator. The magnification for the SEM images was 20,000X.

Thermal analysis and XRD analysis of TC-PLGA nanoparticles:

Thermal analysis was performed to evaluate the interaction between Tacrolimus and PLGA and the changes in the physical status of Tacrolimus, differential scanning calorimetric (DSC) equipped with a thermal analysis data system (DSC 2920, TA Instruments, Alzenau, Germany). The instrument was calibrated using indium as the standard. The endothermic melting temperature for tacrolimus, PLGA, physical mixture of tacrolimus/PLGA, and TC-PLGA nanoparticles were determined with a DSC. 5 milligrams of samples were scanned from 20 °C to 160 °C at a rate of 10 °C/min.

X-ray diffractometry (XRD) was performed to identify the crystalline or amorphous nature of Tacrolimus before and after the manufacturing process, Powder X-ray diffraction patterns (XRD) was taken with a PAN analytical Xpert pro X-ray diffractometer by using a Ni-filtered Cu Kα radiation over the 2θ range of 10-90°. Samples were finely ground in glass substrate and the experimental parameters were set as: voltage, 40 kV; current, 20 mA; angular speed, 4°/min. The

XRD patterns of Tacrolimus, PLGA, poloxamer 188 and physical mixture of TC/PLGA and TC-PLGA nanoparticles were recorded.

***In-vitro* drug release study of TC-PLGA nanoparticles:**

In-vitro drug release study from TC-PLGA nanoparticles was measured in PBS medium (pH 7.4) up to 30 days. TC-PLGA nanoparticles were individually dispersed in 10 ml of the releasing medium (PBS) and were placed into a cellulose membrane dialysis tube (MW cutoff = 12,000–14,000). The dialysis tube was placed in volumetric flask containing 90 ml of PBS medium (pH 7.4) on reciprocating shaking bath at 37±5°C and100 rpm. Samples (5 ml at each time point) were withdrawn at 1, 3, 6, 12, 24, 48 hrs and 7, 15, 30 Days, replaced with the fresh medium to maintain sink conditions. The obtained samples were centrifuged at 20000 rpm for 30 min; 2 ml supernatant obtained was filtered through 0.22 µm PES and sample prepared by sulphuric acid method and analyzed by UV-spectrophotometer. The release study was performed in triplicate.

Accelerated stability studies:

Three checkpoint TC-PLGA nanoparticle formulations from design space were subjected to a stability testing as per ICH guidelines at a temperature of 40 ± 2^0C and RH 25 ± 5% for period of 3 M. Optimized freeze dried TC-PLGA nanoparticles were filled in glass container and were further analyzed for particles size, and entrapment efficiency.

Statistical evaluation:

All obtained data were analyzed by the Student's t-test (a = 0.05) and calculated values were expressed as their mean ± SD for statistical significance.

RESULT AND DISCUSSION:

TC-PLGA nanoparticles were successfully prepared using nanoprecipitation method as it is best method for encapsulation of hydrophobic drugs into nanoparticles. Preparation of nanoparticles involved assessment of many critical parameters which include formulation and process parameters. Optimization of these parameters could lead to development of robust nanoparticle formulation. An overall risk assessment of the process parameter and formulation components involved in TC-PLGA nanoparticles manufacturing was performed to determine which process parameter and formulation components have a high risk of impacting the drug product CQAs. The results of the initial process and formulation risk assessment are presented in **Table1**. Further, for development of the robust formulation of TC-PLGA nanoparticles, the design of experimentation was conducted by taking the high risk parameters as critical process and material attributes and by selecting suitable statistical model, the DoE study was performed to see the effect on the CQAs and to achieve desirable QTPP.

Statistical model to conduct DoE is selected based on different criteria's such as the nature of the problem and/or study, type of study (screening/optimization), nature and number of critical parameters and their levels, type of effect to know (main effects or two-way interactions), available resources i.e. feasibility of time and cost involvement. By considering the all above criterias, '*I*-Optimal RSM design' was selected for conducting the experimental study. '*I*-Optimal RSM design' is Response Surface Methodology design which can be used for optimization purpose for process as well as formulation parameters.

It has better ability to identify multiple factor interactions along with main effect on the selected responses as compared to full factorial and fractional factorial designs. '*I*-Optimal RSM design' also have ability to provide the curvature effect of selected high risk parameters due to inclusion of center points in the design, this design is resistant to alias effects observed in case of factorial design (Taguchi, Placket Burman, Resolution IV designs). The parameters selected for DoE in preparation of nanoparticles were PLGA: poloxamer ratio and stirring speed based on risk assessment as these were critical to product CQAs; particle size and entrapment efficiency. From preliminary screening study, experimental levels of critical parameters were established as 0.80 to 1.20 of PLGA: Poloxamer ratio and stirring speed, 500 to 1000 rpm. By taking these parameters at three levels, 16 experimental runs were obtained as shown in **Table 2.**

For selected statistical design, it is important that any experimental design has sufficient power to ensure that the conclusions drawn are meaningful. Power can be estimated by calculating the signal (minimum significant effect to detect) to noise (standard deviation) ratio from fraction design space (FDS) graph. We evaluated *I*-Optimal RSM design for suitability to give fitted surface as precisely as possible by using FDS tool. 100% FDS shown in **Fig.1** indicates that design will provide a fitted response surface that is precise throughout the region of interest at 95% TI.

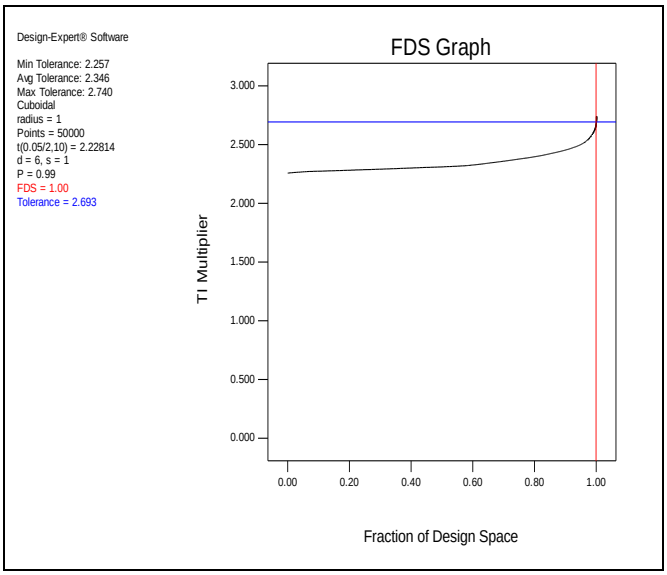
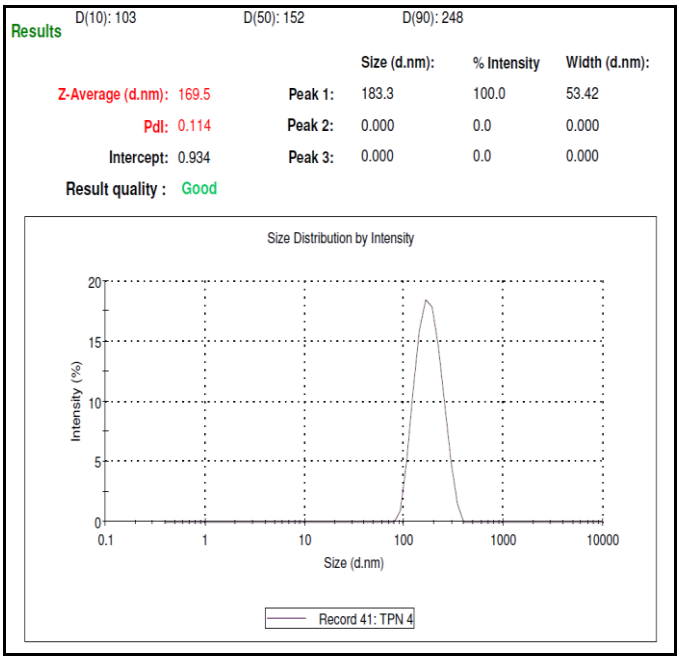


FIG.1: FDS PLOT FOR CENTRAL COMPOSITE DESIGN TO OPTIMIZE TC PLGA NANOPARTICLES.

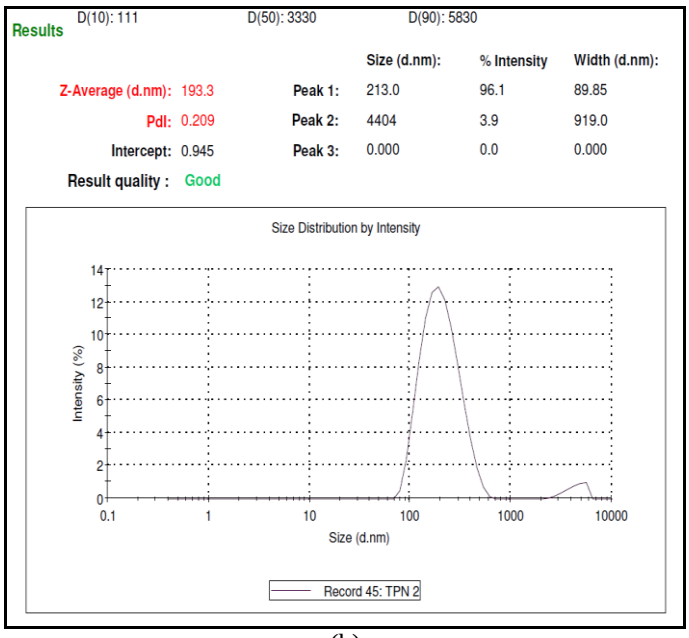
Response surface study:

Particle size of TC-PLGA nanoparticles analyzed using Malvern zeta seizer was shown in **Fig.2 (a) & (b)**. Particle size was significantly affected by PLGA/Poloxamer ratio and stirring speed which can be depicted from 'Prob> F' (< 0.05) value from ANOVA **Table 3**. 3D response surface plots were then generated, using the statistical model obtained from multiple regression analysis, in order to investigate more in depth the effects of changing the independent variables on the considered responses i.e. particle size shown in **Fig.3 (a).** The independent variables have opposite effect on particle size means increase in PLGA: Poloxamer ratio leads to increase in particle size due to resultant increase in organic phase viscosity whereas increase in stirring speed leads to decrease in particle size. As particle size get affected polydispersity index also get changed by these independent variables which significantly affecting entrapment and drug release from TC-PLGA nanoparticles. Entrapment efficiency was significantly affected by PLGA/Poloxamer ratio and stirring speed (Prob> F is less than 0.05); entrapment was increased with decrease in PLGA/Poloxamer ratio as shown in **Fig.3 (b),** this might be due to higher ratio of PLGA/Poloxamer leads to more availability of polymer with lesser quantity of stabilizer resulted into poor entrapment whereas entrapment was improved with decrease in ratio upto 0.94 due to increased poloxamer proportion. At higher concentration of poloxamer 188, uniform layer around the Tacrolimus leads to higher entrapment upto 79.93 % as compared to 52.13 at higher ratio i.e. 1.20 and 74.84 at medium ratio of 1.08 as shown in **Table 3**.

Stirring speed showed the same pattern as that of particle size, entrapment decreased with increasing stirring speed from low level to high level. This negative effect of stirring speed on EE was attributed due to attrition of nanoparticles with other particles, blades of stirrer and wall of container upto certain extent and due to reduction of particle size. This is one of the critical issues which need to focus prominently at scale up level and optimization of this will lead to robust and controlled formulation with respect to entrapment efficiency and drug release.

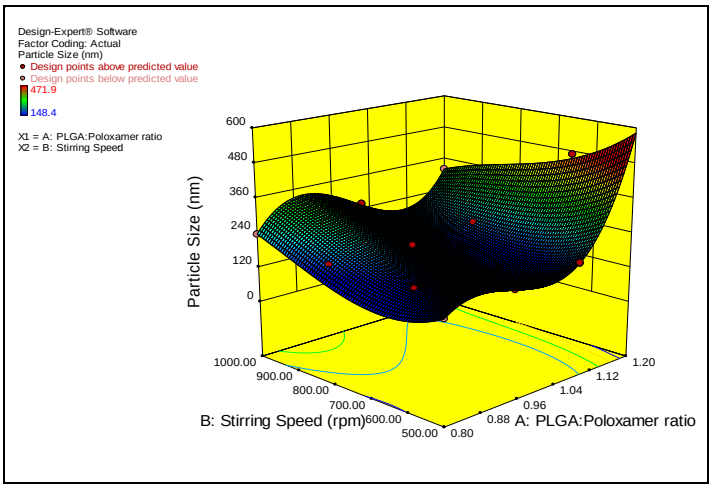


(a)

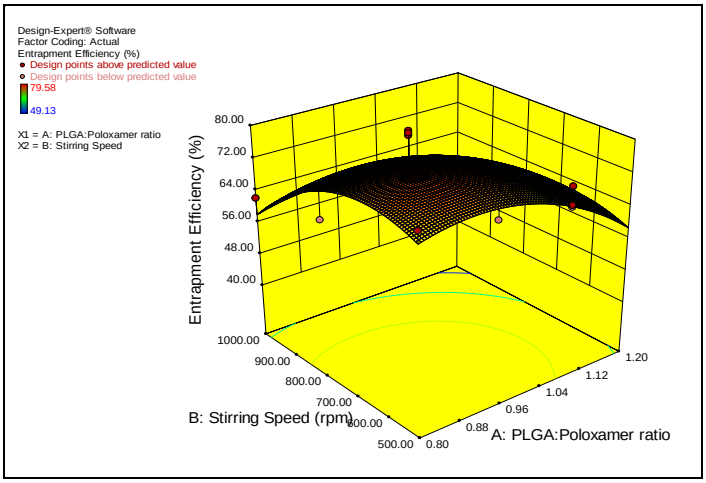


(b)

FIG.2: PARTICLE SIZE OF TC PLGA NANOPARTICLES MEASURED USING MALVERN ZETA SIZE ANALYZER A) TPN 4; B) TPN 2.



(a)



(b)

FIG.3: 3D SURFACE PLOT SHOWING EFFECT OF PLGA/POLOXAMER RATIO AND STIRRING SPEED ON a) PARTICLE SIZE, b) ENTRAPMENT EFFICIENCY OF TC PLGA NANOPARTICLES.

TABLE 3: ANNOVA FOR CQAs OF TC PLGA NANOPARTICLES.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
ANOVA for Particle Size - Cubic model					
Model	1.397E+005	9	15518.47	123.98	< 0.0001
X_1	1998.90	1	1998.90	15.97	0.0071
X_2	1541.19	1	1541.19	12.31	0.0127
X_1X_2	13738.57	1	13738.57	109.76	< 0.0001
X_1^2	17879.96	1	17879.96	142.85	< 0.0001
X_2^2	4570.77	1	4570.77	36.52	0.0009
$X_1^2X_2$	8042.79	1	8042.79	64.26	0.0002
$X_1X_2^2$	303.72	1	303.72	2.43	0.1703
X_1^3	10249.39	1	10249.39	81.88	0.0001
X_2^3	282.27	1	282.27	2.26	0.1839
Residual	751.02	6	125.17		
Lack of Fit	0.45	1	0.45	2.971E-003	0.9586
Pure Error	750.57	5	150.11		
Cor Total	1.404E+005	15			
ANOVA for Entrapment Efficiency - Cubic model					
Model	1031.18	5	206.24	7.09	0.0045
X_1	294.36	1	294.36	10.12	0.0098
X_2	398.24	1	398.24	13.69	0.0041
X_1X_2	0.34	1	0.34	0.012	0.9155
X_1^2	148.85	1	148.85	5.12	0.0472
X_2^2	222.79	1	222.79	7.66	0.0199
Residual	290.87	10	29.09		
Lack of Fit	287.35	5	57.47	81.82	< 0.0001
Pure Error	3.51	5	0.70		
Cor Total	1322.04	15			

In-vitro drug release study:

In vitro drug release from TC-PLGA nanoparticles was mainly controlled by the PLGA grade [4], its proportion to drug, poloxamer quantity in the ratio and stirring speed. *In-vitro* study revealed that release rate decreases significantly with increasing the poloxamer ratio up to certain limits [1]. Initial burst release occurred when PLGA: poloxamer ratio used either higher or lower shown in **Fig.4**.

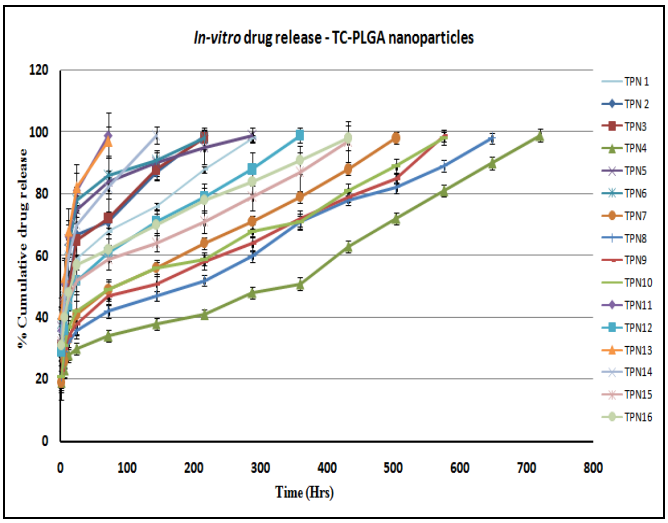


FIG.4: *IN-VITRO* DRUG RELEASE STUDY OF TC PLGA NANOPARTICLES

At higher ratio; more PLGA and low poloxamer availability might have resulted into porous nanoparticles due to which nanoparticles were unable to retard drug release whereas at lower ratio; low PLGA and high poloxamer availability resulted into reduction of particle size and higher PDI could lead to higher drug release due to increase in surface area of nanoparticles.

When optimum ratio of PLGA: Poloxamer were taken, it could fabricate nanoparticles with desired particle range and entrapment with sustained *in-vitro* drug release. This might be due to forming proper coating or uniform layer of Poloxamer-PLGA at interface of nanoparticles which resulted into increase in coat thickness surrounding the drug particles thereby increasing the distance traveled by the drug throughout coat. Poloxamer and PLGA (50:50) were able to retard the drug release from the nanoparticles for period of 4 weeks. Poloxamer being a block polymer (PEO-PPO-PEO) can be considered good stabilizer for designing sustained release PLGA nanoparticles of low or highly soluble drug with optimum proportion to polymer.

Stirring speed affected drug release from TC-PLGA nanoparticles, high initial burst release (33.85% to 47.79%) within 12 hours was observed at higher stirring speed.

This might be due to the drug migration was high at higher stirring speed and more amount of drug remain on nanoparticles surface and at lower stirring speed, drug migration was less due to minimum shear rate on nanodroplets [22]. Also at higher stirring speed, decrease in nanoparticles size leads to lager surface area and faster drug release as compared to optimum stirring speed.

X-ray diffraction pattern for TC-PLGA nanoparticles:

X-ray diffraction pattern for Tacrolimus, PLGA, poloxamer 188 and TC PLGA nanoparticles are shown in Figure No 5. The presence of numerous distinct peaks in the X-ray diffraction spectrum indicates that Tacrolimus is present as a crystalline material with characteristic diffraction peaks appearing at a diffraction angle of 12^0, 14^0, 19^0, 21^O and 24^0. PLGA exhibit amorphous nature which shows absence of sharp peaks and poloxamer 188 exhibiting crystalline nature with two sharp peaks. TC-PLGA nanoparticles formulation shows peaks at same degree as that of pure Tacrolimus but with less intensity which might be due to the drug entrapped in NP was in an amorphous or disordered crystalline phase of molecular dispersion. This indicates that there was no any interaction of drug and polymer in TC-PLGA nanoparticles.

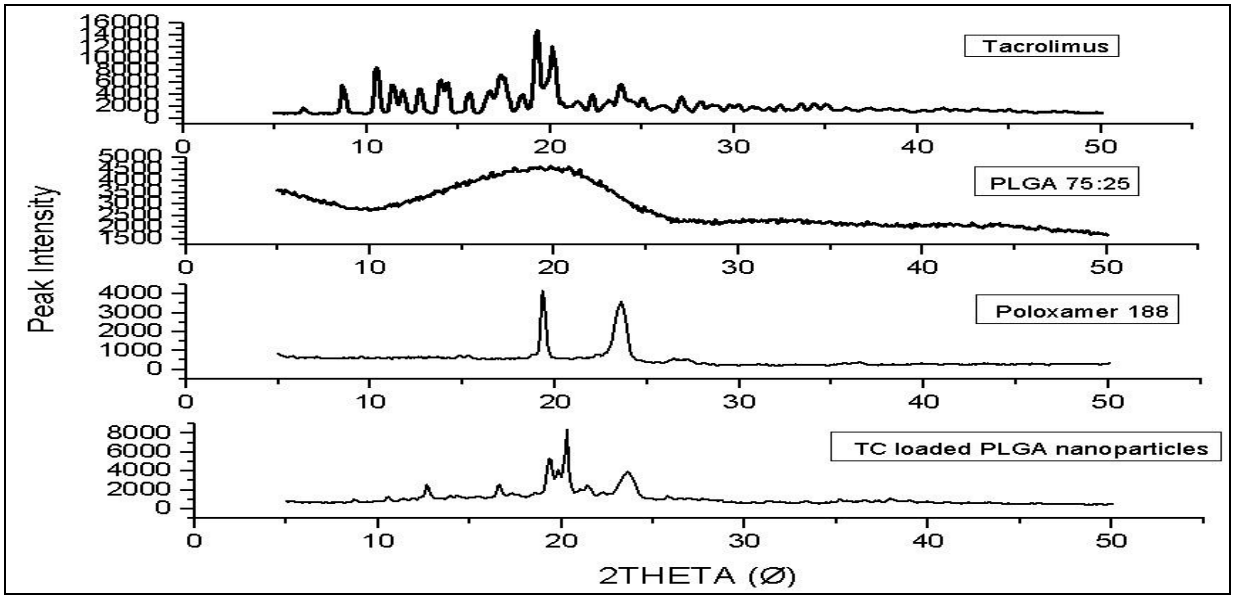


FIG.5: X-RAY DIFFRACTION PATTERN FOR TC, PLGA, POLOXAMER 188 AND TC PLGA NANOPARTICLES.

Differential Scanning Calorimetry of TC PLGA nanoparticles:

DSC studies were performed in order to characterize drug status inside the NP as well as the melting points of each component. The thermal characteristic of TC PLGA nanoparticles (d) was compared to Tacrolimus (a), PLGA (b) and physical mixture of PLGA and TC (c) as shown in **Fig.6.** The melting point of Tacrolimus is 127–129^0C, whereas DSC thermogram of tacrolimus showed sharp endothermic peak at 128^0C and was also visible in the pattern obtained from the physical mixture of Tacrolimus which indicates the crystalline nature of it. The glass transition temperature (Tg) of PLGA was found to be 34-36^0C and no melting endothermic peak was observed, as PLGA appears amorphous in nature. Physical mixture of TC and PLGA showed peak at melting point temperature but with less intensity might be due to physical masking of peak as higher drug: polymer ratio (1:4), whereas TC-PLGA nanoparticles exhibit peak with change in intensity with broadening of peak but no significant shifting of the position of peak. This indicates that the drug is only physically entrapped in the polymer matrix and there is no interaction between drug and polymers.

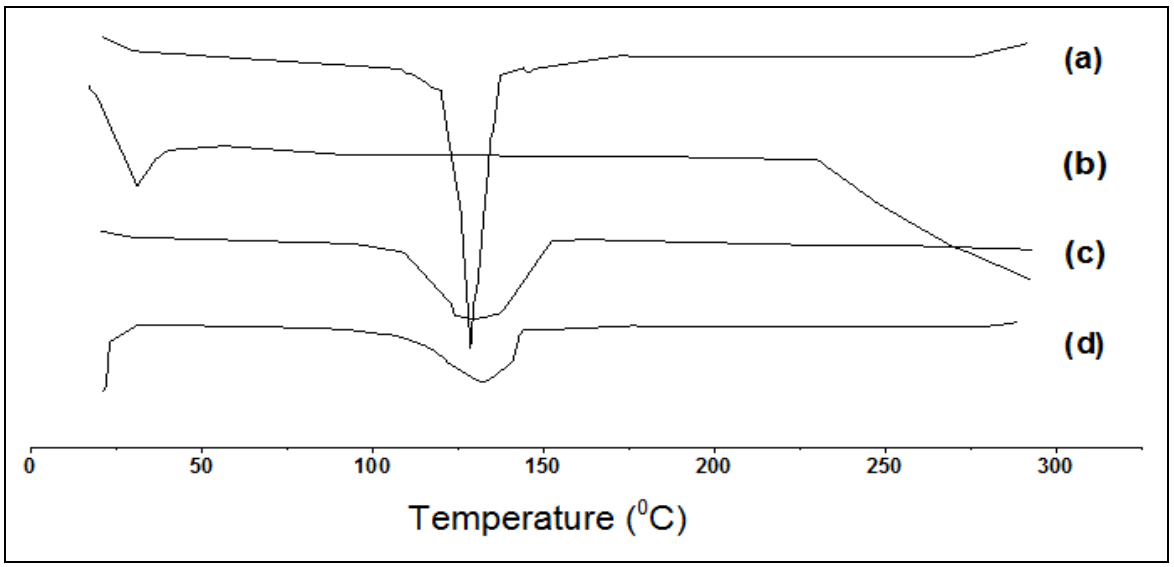


FIG.7: DSC THERMOGRAM FOR TACROLIMUS, PLGA, PHYSICAL MIXTURE OF TC AND PLGA AND TC PLGA NANOPARTICLES.

Scanning electron microscopy of TC PLGA nanoparticles:

In the SEM observation shown in **Fig.7**, the prepared TC PLGA nanoparticles were spherical in shape with a smooth surface and homogeneous without aggregation. SEM image shows that the drug particles entrapped within the carrier matrix, and very less drug particles are surface bound.

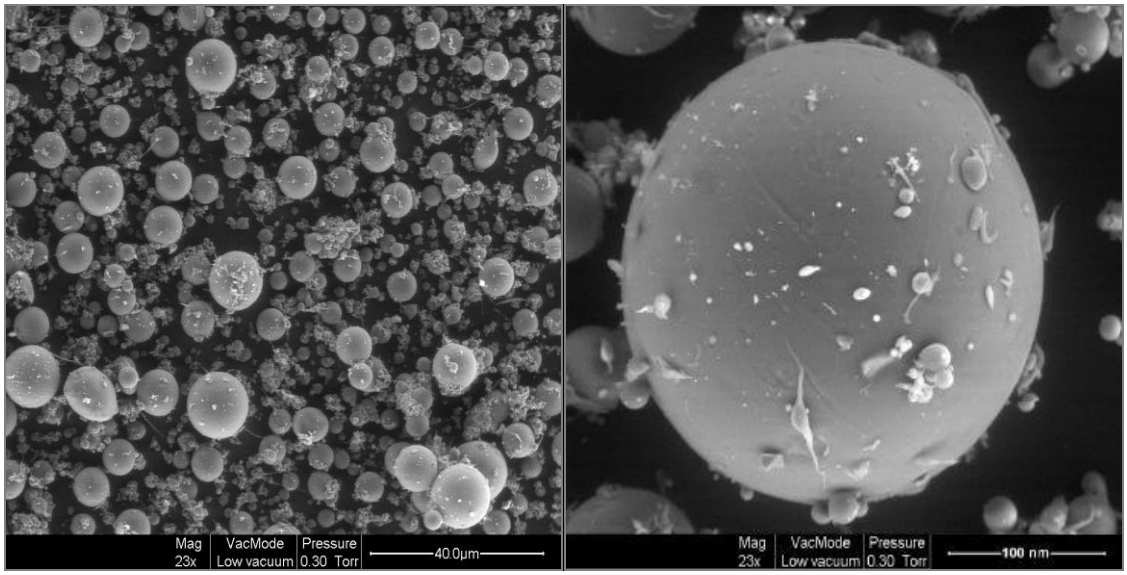


FIG.6: SCANNING ELECTRON MICROSCOPY (SEM) OF TC-PLGA NANOPARTICLES.

Optimization of design space and validation of model:

The design space (operating within these multivariate factor ranges provides the assurance of quality) for TC PLGA nanoparticles was established by setting the acceptance criteria for particle size (150-350 nm) and entrapment efficiency (55-78.14 %). Design space (overlay plot) by overlapping contour plot of 2 responses were constructed shown in **Fig. 8** characterizes acceptable ranges of PLGA: poloxamer ratio and stirring speed which provides assurance that CQAs will be within acceptable criteria. Robustness can be assured of TC-PLGA nanoparticles formulation if operated in the range of design space. Validation of the applied model was carried out by performing the 3 formulations with combinations of factors within obtained design space and predicted and observed results were compared for particle size and entrapment efficiency and obtained results were compared with predicted results as shown in **Table 4**.

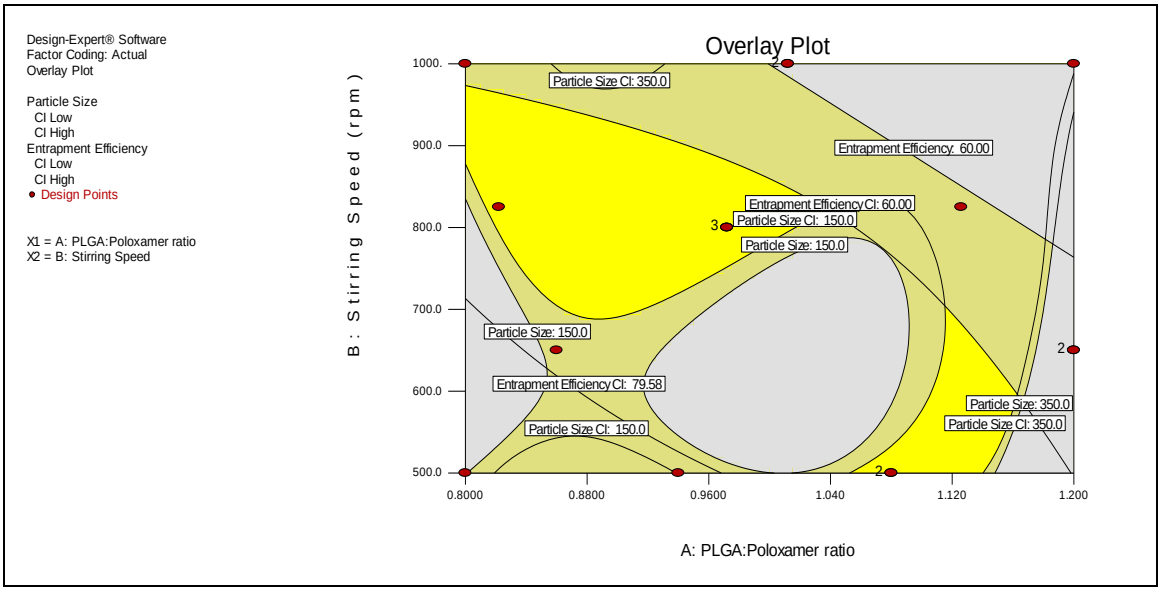


FIG.8: FUNCTIONAL DESIGN SPACE FOR TC-PLGA NANOPARTICLES FRAMED WITH TOLERANCE INTERVALS.

TABLE 4: VALIDATION OF DESIGN SPACE FROM *I*-OPTIMAL RSM DESIGN.

Formulation Code	Composition (mg/tab)		Response	Predicted Value	Experimental Value	Standard Error
	X_1	X_2				
TPN 17	1.12	600	Y_1 -Particle Size	208.3	216.9	2.465
			Y_2 -Entrapment Efficiency	66.82	64.59	1.255
TPN 18	0.8220	825	Y_1 -Particle Size	184.9	198.73	3.127
			Y_2 -Entrapment Efficiency	70.14	68.67	1.019
TPN 19	0.9720	800	Y_1 -Particle Size	187.2	199.64	2.965
			Y_2 -Entrapment Efficiency	66.13	68.65	1.334

Stability Results:

TC PLGA nanoparticles kept for stability were evaluated for particle size, and assay shown in **Table 5**. There was no change observed in particle size of nanoparticles, whereas PDI obtained was within 0.322 to 0.426 for three batches indicated good polydispersity after stability. Assay was calculated based on the entrapment efficiency of individual batch and calculated with respect to 5 mg Tacrolimus equivalent nanoparticles. Assay of stability batch was slightly decreased after 3 M of stability study but it was within accepted specification.

TABLE 5: ACCELERATED STABILITY RESULTS FOR TC PLGA NANOPARTICLES.

Batch No	Assay * (% w/w)		Particle Size (nm)		PDI	
	Initial	3 M	Initial	3 M	Initial	3 M
TPN 17	99.87±2.5	97.95±1.59	208.3	200.34	0.315	0.426
TPN 18	102.52±1.7	99.43±1.65	184.9	193.22	0.346	0.322
TPN 19	101.75±1.71	96.57±1.94	187.2	204.64	0.281	0.362

* Assay was calculated based on entrapment efficiency of TC PLGA nanoparticles and nanoparticles for assay were weighed equivalent to Tacrolimus 5 mg.

CONCLUSION: In present investigation QbD approach was implemented to develop TC PLGA nanoparticles prepared using nanoprecipitation technique for understanding and optimizing effect of process and formulation variables with desired particle size, entrapment efficiency and sustain drug release for period of 4 weeks. Based on initial risk assessment, PLGA: Poloxamer 188 ratio and stirring speed were found as the two major critical parameters for meeting goals set in QTPP which were taken for DoE and *I*-Optimal design was selected to see the effect on CQAs; particle size and entrapment efficiency. Morphological studies of the optimized batch showed that the TC PLGA

nanoparticles were smooth, spherical with a uniform surface. The release kinetics from TC PLGA nanoparticles exhibited a biphasic pattern with an initial burst release followed by a slower diffusion sustained drug release for a period of 4 weeks. The application of RSM design demonstrates a useful tool for optimization of TC PLGA nanoparticles.

The results of multiple regression analysis led to a statistical model that described adequately the influence of the selected variables at different levels on the chosen response and thereby, minimize the number of experimental trials and reduce the formulation development cost. Thus, it can be concluded that based on understanding of variables and process PLGA nanoparticle drug delivery system can be implement to any active substance to provide sustain drug delivery after parenteral administration.

ACKNOWLEDGMENT: Author would like to acknowledge and thank to Emcure Ltd., Pune, India; Evonik (Mumbai); and VerGo Pharma Ltd (Verna, Goa) for providing Tacrolimus, PLGA and Poloxamer.

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How to cite this article:
Shirsat AE and Chitlange SS: Quality by Design Approach to Optimization of Tacrolimus Loaded PLGA Nanoparticles. Int J Pharm Sci Res 2015; 6(10): 4342-53.doi: 10.13040/IJPSR.0975-8232.6(10).4342-53.