5. Formulation Development of Cubosomes of Tacrolimus

5.1. Introduction

Cubosomal drug delivery system is selected for the present study with an objective of achieving therapeutic plasma levels of Tacrolimus via transdermal route, given to the capability of cubosomes of deeper penetration in to the skin for achieving sufficient plasma concentration.(1, 2) Out of the numerous methods available for preparation, bottom up (ethanol injection) method was used for the preparation of cubosomes of Tacrolimus. A systematic Quality-by-design (QbD) approach employing statistical design of experiments was utilized to exhaustively evaluate the impact of material attributes and process parameters on critical formulation attributes.(3)

5.2. Materials and Methods

5.2.1. Materials

Both Tacrolimus and Glyceryl Monooleate were obtained as a gift sample from Concord Biotech Ltd., India and Mohini Organics, Mumbai, India, respectively. Polyvinyl alcohol (PVA)-6000, poloxamer 407, and poloxamer 188 were purchased from Acros Organic, USA and BASF supplier of India respectively. Double distilled water was prepared in lab, filtered with the help of membrane filter having a pore size of 0.2µ (stored in air tight container) and consumed within a maximum of 7 days.

5.2.2. Screening of formulation parameters for cubosomes of Tacrolimus

5.2.2.1. Solubility:

Solubility of TAC was studied in various solvents on the basis of the saturation solubility. For performing the study, incremental amount of TAC was added to 1 mL of solvent till it became hazy. Then, to separate undissolved drug, drug dispersion was centrifuged at 3000 rpm. Supernatant from the above solution was collected and analyzed using HPLC method as described in chapter 3 section 3.4.1. after suitable dilution as required.(4)

5.2.2.2. Selection of lipid:

Based on the solubility of Tacrolimus in lipids [Glyceryl Monooleate (GMO), Glyceryl Monosterate (GMS)], screening of the lipid was carried out for the preparation of nanocarrier i.e. cubosome. Solubility in these lipids was performed on hot plate magnetic stirrer above the melting point of the selected lipids same as described in section 5.2.2.1.

5.2.2.3. Selection of stabilizer

For the selection of stabilizer various characteristics were taken into account like vesicle size, stability, entrapment efficiency and stability of the prepared formulation. Different stabilizers like Polyvinyl alcohol (PVA), Poloxamer 188, and Poloxamer 407 were used to prepare a formulation and were studied for the vesicle size, stability (for 10 days) and entrapment efficiency. For Tacrolimus, concentration of GMO and stabilizers were kept constant i.e. 2.0 % and 1.0 % w/v respectively.

5.2.2.4. Concentration of stabilizer

Concentration range of the stabilizer was chosen based on the entrapment efficiency, and vesicle size of the prepared formulation. Here, for cubosomes of Tacrolimus, amount of added GMO was set at constant conc. of 2 % w/v. Then, various batches were prepared having different concentrations of PVA.

5.2.2.5. Concentration of lipid

Concentration range of lipid was selected based on the entrapment of the drug and vesicle size of the cubosomes. Different batches were prepared with varying amount of GMO wherein the amount of added stabilizer was set at constant conc. of 0.5 % w/v.

5.2.2.6. Selection of stirring speed

An effect of stirring speed was studied on a formulation wherein the stirring speed ranged from 500 to 1500 rpm while keeping the concentration of GMO and PVA constant.

5.2.2.7. Selection of stirring time

The effect of stirring time was studied on the quality of the formulation wherein stirring time was kept in a range of 15-20 minutes while keeping the concentration of GMO and PVA constant.

5.2.2.8. Temperature

Temperature range of 50-80 °C was selected for the study while all other parameters were kept constant.

5.2.2.9. Volume of organic phase

Volume of an organic phase in a range of 1-3 mL was selected while all other parameters were kept constant.

5.2.2.10. Rate of addition of organic phase

A rate of addition of organic phase was selected in a range of 0.5-1.5 mL/min while all other parameters were kept constant.

5.2.3. Preparation and optimization of Tacrolimus loaded cubosomes

Bottom up approach was utilized for preparation of TAC Cubosomes as shown in fig 5.1. For the preparation of cubosomes of Tacrolimus, two solutions were prepared: A) organic phase and B) aqueous phase. For the preparation of organic phase (A), X mg of **Glyceryl monoolein (GMO)** was solubilized in 2 ml of ethanol in a 10 ml glass beaker and then, 10 mg of Tacrolimus was added to it. 10 mL of Y % w/v **polyvinyl alcohol (PVA)** as stabilizer in double distilled water was used as aqueous phase (B). Both solutions were kept at 50 °C under continuous stirring for 5-10 min. Then, organic phase A was added to aqueous phase B in a drop-wise manner with continuous stirring at 500 rpm. The rate of addition of organic phase A was maintained at 1 mL/min. Stirring was continued for 15 min at room temperature. After that, the mixture was introduced into a rotary evaporator at 50 °C and rotated at speed of 100 rpm under vacuum until most of the ethanol was removed and the volume of the prepared batch was reduced to 2.5 mL. The resulting Cubosomal dispersion was exposed to centrifugation with predefined process parameters i.e. for a period of 10 minutes at 5000 rpm and the temperature was set as 25°C for facilitating the sedimentation of free drug. Care was taken while separating the supernatant of cubosomal

dispersion so as to not disturb free drug pellet which is deposited at the bottom of the centrifugation tube. Finally, the resulting separated cubosomal dispersion was stored for utilization in future tests in glass vials at room temperature.(2, 5)

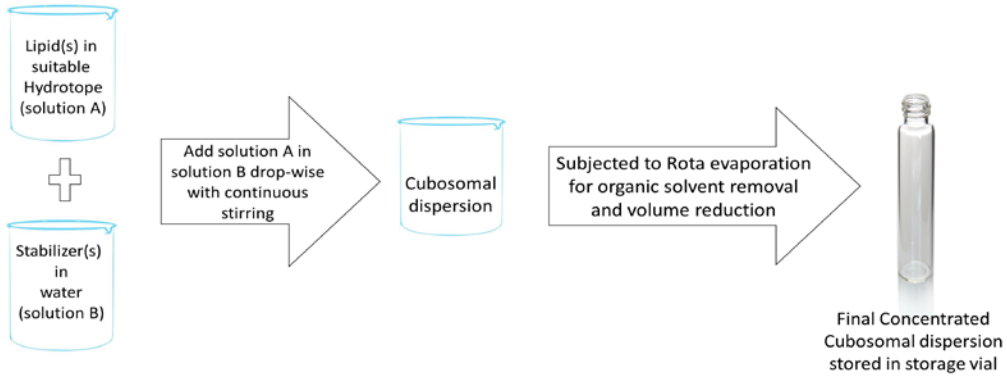


Figure 5.1: Bottom up approach for preparation of Tacrolimus loaded cubosomes

5.2.4. Quality target product profile and critical quality attributes of formulation

Firstly, QTPP for Tacrolimus loaded cubosomes was established on the basis of scientific, therapeutic, industrial and regulatory factors. Then, three response variables, vesicle size (VS), PDI (poly-dispersity index) and % entrapment efficiency were selected as CQA and this selection was done based on literature review, and experiment trials.(3)

5.2.5. Identification of independent variables and qualitative risk assessment using Ishikawa diagram

All possible variables which were linked with the development of TAC loaded cubosomes were demonstrated with the help of Ishikawa diagram. These factors were considered as ‘low, intermediate and high risk’ on the basis of their predicted effect on CQAs as described in Table 5.1.

Table 5.1: Quality risk assessment criteria for various attributes

Low Risk	Factors with wide range of acceptability. No investigation required.
Moderate Risk	Acceptable risk. Small change does not significantly affect the quality of product
High Risk	Unacceptable risk. Acceptable range of attributes needed to be studied.

5.2.6. Screening of various attributes (CQA) using 2-level design

There are various moderate and high risk attributes which can affect the quality of product. These parameters were screened for most significant attributes using randomized 2-level fractional factorial design developed by Design Expert 7.0.

5.2.6.1. Vesicle size (VS) and size distribution

Cubosomal dispersion was taken and diluted 10X using pre-filtered double distilled water. Further, it was taken in a disposable sizing cuvette. The cubosomal dispersion was evaluated using dynamic light scattering (DLS) with Nano-ZS Zetasizer, Malvern Instruments Ltd., UK for VS and poly-dispersity index (PDI).(5, 6)

5.2.6.2. Percent Entrapment efficiency

Acetonitrile was employed for the solubalization and dilution of Cubosomal samples of Tacrolimus and analyzed using developed HPLC analytical method described in chapter 3.(6) % Entrapment efficiency was calculated using following formula 5.1(6):

$$\%EE = \frac{Amount\ of\ entrapped\ drug}{Total\ drug\ added}\ X\ 100$$

Equation 5.1

5.2.7. Formulation optimization of Tacrolimus loaded cubosomes using 3^2 Factorial Design

For explicitly studying the relation between the vital factors and CQA of Tacrolimus loaded cubosomes, 3^2 Factorial Design was employed. This facilitated the use of reduced number of experimental batches while mixture components and other numeric factors could be handled simultaneously.(7) A randomized design matrix was generated, experimental data was statistically evaluated for achieving an optimized solution and the design space was created with help of Design Expert® 7.0.0. Software was employed for selection of a suitable model for the study. ANOVA was performed which was followed by F-test for the identification of significant model terms. CQA was calculated with the help of mathematical equations wherein, the equation was simplified by the removal of insignificant terms. The correlation between critical factors and CQAs was studied with the help of contour and 3-D response surface plots. For the verification of the model, three check point batches with optimized composition were produced.(3)

5.2.8. Preparation of checkpoint batches as per the overlay plot

Data obtained from the prepared batches was added as suggested by the software, this data was analyzed at 95 % confidence and prediction level using Design Expert 7.0 for the optimized area. Three randomized points were selected from this optimized area and checkpoint batches were prepared according to it. The composition of these checkpoint batches are given in table 5.2.

Table 5.2: Composition of checkpoint batches

Sr. No.	Concentration GMO (% w/v)	Concentration of PVA (%w/v)	Predicted % EE	Predicted vesicle size (nm)	Predicted PDI
1	2.90	1.01	93.8	163.5	0.183
2	3.19	1.32	99.89	175.5	0.195
3	2.30	1.29	92.29	150.9	0.157

5.2.9. In-vitro characterization of optimized Tacrolimus cubosomes

5.2.9.1. Shape and surface morphology

Transmission electron microscopy (TEM) was employed for the evaluation of shape and surface morphology of the TAC loaded cubosomes. For performing the test, the dispersion was smeared on a carbon-coated grid, and any extra solution was cleaned and carbon-coated grid was dried at room temperature for a period of 5 hrs. TEM (CM 200, Philips, Netherlands) was employed with the following process parameters i.e. the operating voltage was set in a range of 20-200 kV to visualize cubosomes at suitable enlargement with an accelerating voltage of 20 kV.(5, 6)

5.2.9.2. Zeta potential

Nano-ZS zetasizer by Malvern Instruments Ltd., UK, was employed for analysis of zeta potential of TAC loaded cubosomes. For this, the dispersion of Tacrolimus loaded cubosomes was taken and was diluted up to 10 times and the dilution was performed using pre-filtered distilled water. Then, the dispersion was taken in disposable folded capillary cells and was evaluated for zeta potential. A Smoluchowski equation was used by zetasizer for the calculation of zeta potential centered on an amount of doppler shift occurring due to electrophoretic mobility of colloidal particles in response to electric field.(5, 6)

5.2.9.3. Vesicle size and size distribution

The dispersions of Tacrolimus loaded cubosome were diluted upto 10X using pre-filtered distilled water. Further the dispersions was taken into disposable sizing cuvette and the VS and PDI was analyzed with a help of Nano-ZS zetasizer which calculates VS and PDI based on dynamic light scattering (DLS). For calculation of mean diameter of cubosomes, an instrument examines angular scattering of a laser beam during its passage through the dispersed cubosomal sample and use the Mie theory of light scattering.

5.2.9.4. % Entrapment efficiency

A % EE of the prepared formulation of cubosomes of TAC was found out as described in section 5.2.6.2.(5, 6)

5.2.9.5. Total drug content

The formulation which was prepared was analyzed for total drug content. For performing this, 1 mL of the cubosomal dispersion eq. to 4 mg of TAC was withdrawn and was dissolved in 10 mL ACN. A prepared samples of TAC were then analyzed using developed HPLC method as described in chapter 3 section 3.4.1.(5)

5.2.9.6. Small Angle X-rays Scattering

Bruker Nanostar Xeuss 2.0 model was employed for conducting SAXS experiments furnished with a rotating anode and three-pinhole collimation. The device employs Cu-Kα radiation having a λ_{max} of 1.54 Å and a sample to detect length of approx. 105 cm. Anode was set at 45 kV and 100 mA current. The samples were transferred in a 2 mm quartz capillary (from Charles-Supper, USA) having 10μm wall thickness. For keeping reference, scattering from glassy carbon film was employed. The temperature of sample holder was maintained by Peltier unit. The obtained data was taken on a HISTAR gas filled multi-wire detector. Further, the 2D data was circularly averaged for the conversion of data to 1D. The scanning of samples was performed for a period enough to obtain atleast two million counts. Further, these were normalized with the transmission coefficient of the sample and the acquisition time. The scattering emerging from silver behenate was employed for the calibration of Detector.(5, 6)

5.2.9.7. Headspace Gas Chromatography (HS-GC) testing for residual solvent

A. Standard preparation

In 10 ml volumetric flask, 0.13 mL of ethanol equivalent to 0.1 g of ethanol was taken and final volume was made up using DMF (dimethyl formamide) which gave final concentration of 10000 ppm. In other volumetric flask with 10 ml capacity, 1 ml of above obtained solution was taken and final volume was made up using deionized water to achieve final conc. of 1000 ppm.(8)

B. Sample preparation

A volume of formulation (0.105) equivalent to 0.1 g was shifted in volumetric flask with 10 ml capacity and final volume was made up with DMF. From the above solution 1 ml was shifted in volumetric flask (10 ml) and volume was made up by using deionized water. Sample was injected into column (capillary column: CR-624, Dimensions: 30m, 0.53mm, 3.00 µm) at 80 °C using nitrogen as carrier gas. Others parameters like carrier gas flow rate, H2 gas flow rate, air flow rate, injection volume, injector temperature, and detector temperature were set to 40 mL/min, 30 mL/min, 300 mL/min, 0.2 µl, 260 °C, and 260 °C respectively. Total run time was set at 20 min.(8)

5.2.9.8. In-vitro drug release

(A) Activation of dialysis membrane

Dialysis membrane-130 (la 393), having a molecular weight cut off of 12000-14000 DA and an estimated capacity of 2.41 ml/cm, procured from Himedia Laboratories Pvt. Ltd, was used for the study. For the activation of dialysis bag, 10 cm long dialysis membrane was cut and kept under running tap water overnight to remove glycerol followed by treatment with sodium sulphide solution (0.3% W/V) at temp. of 80 °C for period of 1 min for removal of sulphur compound(s). Further, washing was given with hot water maintained at a temp. of 60 °C. Washing was followed by a step of acidification by employing 0.2% v/v sulphuric acid and then it was subsequently washed with hot water for the removal of the acid. Then, the dialysis membrane was dipped overnight in the diffusion medium before *in-vitro* release.(9)

(B) In-vitro drug release

A dialysis membrane having molecular weight cut off in range of 12-14K Dalton in the Franz diffusion cell was employed for conducting *in-vitro* drug release study. In case of Franz diffusion cell, a donor compartment had a volume capacity of 7 mL. To perform in-vitro drug release, as a diffusion medium, 1 % ethanolic phosphate buffer pH 7.4 was

prepared.(10) For performing the study, plain drug suspension in water, and cubosomes of TAC both eq. to 2 mg were situated in the donor compartment. Further, from the receptor compartment, samples (1.0 mL) were removed at steady time intervals (0.5, 1, 2, 3, 4, 5, 6, 8, 12 and 24 hour) and an identical volume (1.0 ml) was replaced by a fresh diffusion medium. Withdrawn samples were then quantified with the help of method developed for HPLC as described in chapter 3. For the above mentioned experiments, three readings were taken and further the average of these three values was considered.(6)

5.3. RESULTS AND DISCUSSION

5.3.1. Screening of formulation parameters for cubosomes of Tacrolimus

5.3.1.1. Solubility

As shown in fig. 5.2, Tacrolimus has maximum solubility in Acetonitrile, i.e. 625.4 mg /mL while minimal solubility in Dimethyl Formamide i.e. 29.78 mg/mL. Moreover, it has a solubility of 40.32 mg/mL and 32.81 mg/mL in GMO and Glyceryl monostearate (GMS) respectively.

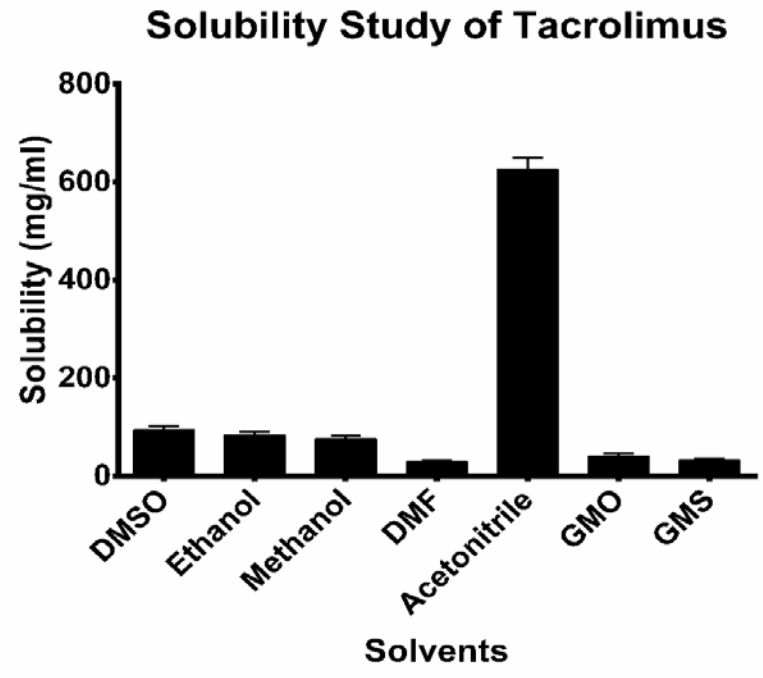


Figure 5.2: Solubility study of Tacrolimus

5.3.1.2. Selection of lipid

On the basis of the solubility of Tacrolimus in lipids [Glyceryl Monooleate (GMO), Glyceryl Monosterate (GMS)], screening of the lipid was carried out for the preparation of nanocarrier i.e. cubosome. It was observed that Tacrolimus has maximum solubility in GMO i.e. 48.04 mg/ml. So, GMO is selected for further process.

5.3.1.3. Selection of stabilizer

Table 5.3: Screening of stabilizers for Tacrolimus

Batch No.	Name of stabilizer	Conc. of stabilizer (% w/v)	Conc. GMO (%w/v)	% EE	Vesicle size (nm)	Visual observation
1	PVA	1.0	2	76.38 ± 3.80	155.36 ± 7.11	Stable
2	Poloxamer 407	1.0	2	61.74 ± 3.54	169.43 ± 10.40	Stable
3	Poloxamer 188	1.0	2	58.28 ± 2.29	486.04 ± 18.83	Unstable

As shown in table 5.3, we can conclude that all formulations are stable except batch 3 which contains poloxamer 188 and no significant difference was observed on vesicle size of the prepared formulation. However, we can see noticeable difference in obtained % entrapment efficiency of a formulation. In case of PVA, maximum % entrapment efficiency i.e. 76.38 % was obtained, thus it was selected for further development.

5.3.1.4. Selection of concentration of stabilizer

For the selection of concentration range of stabilizer different batches were prepared wherein the concentration range of stabilizer was kept between 0.25-1% and the amount of GMO was set at constant concentration of 2% w/v. From a data collected as shown in the table 5.4, concentration range of stabilizer selected for the optimization was 0.5-1.5 %w/v.

Table 5.4: Selection of concentration of stabilizer for cubosomes of Tacrolimus

Batch No.	Conc. of GMO (% w/v)	Conc. of PVA (%w/v)	% EE	Vesicle size (nm)
1	2.0	0.25	21.09 ± 3.23	543.67± 50.11
2	2.0	0.5	67.16 ± 3.26	209.42± 16.27
3	2.0	1.0	81.39 ± 2.29	150.53± 13.36
4	2.0	1.5	86.28 ± 4.36	207.19± 16.82

5.3.1.5. Selection of concentration of lipid

For the selection of concentration range of the lipid, different batches were prepared wherein the concentration of GMO was kept in a range of 1-5% w/v as shown in table 5.5. The amount of PVA for all the batches was set at constant concentration of 0.5% w/v as shown in table 5.5 According to data showed in table 5.5, concentration range selected for the optimization of cubosomes of Tacrolimus was 1-4 %w/v.

Table 5.5: Selection of concentration of GMO for cubosomes of Tacrolimus

Batch No.	Conc. of PVA (% w/v)	Conc. of GMO (%w/v)	% EE	Vesicle size
1	0.5	1.0	36.18 ± 7.47	146.2 ± 14.62
2	0.5	2.0	51.84 ± 6.16	181.91 ± 12.13
3	0.5	3.0	59.7 ± 2.81	213.39 ± 29.0
4	0.5	4.0	66.16 ± 12.25	266.43 ± 28.61
5	0.5	5.0	76.11 ± 5.23	395.61 ± 30.03

5.3.1.6. Selection of stirring speed

Various batches of cubosomes of TAC were prepared as shown in table 5.6 keeping the concentration of GMO and stabilizer constant i.e. 2.0 %w/v and 1.0 %w/v respectively at varying stirring speed. From the obtained data, it was established that stirring speed does not have any substantial impact on

vesicle size and % entrapment efficiency of TAC in cubosomes. Thus lowest rpm was selected to prepared cubosomes of TAC i.e. 500.

Table 5.6: Selection of stirring speed for preparation of cubosomes of Tacrolimus

Batch No.	Stirring Speed (RPM)	% EE	Vesicle size (nm)
1	500	80.16 ± 3.79	161.5 ± 6.94
2	1000	82 ± 4.9	145.8 ± 14.91
3	1500	78.9 ± 4.56	150.3 ± 14.72

5.3.1.7. Selection of stirring time

Various batches of cubosomes of TAC were prepared as shown in table 5.7 keeping the concentration of GMO, stabilizer constant i.e. 2.0 %w/v, 1.0 %w/v respectively. The stirring speed was set at 500 rpm. Different stirring time was taken for all batches as shown in the table 5.7. From the collected data, it was established that stirring time does not have any substantial impact on vesicle size and % entrapment efficiency of Tacrolimus in cubosomes. Thus lowest stirring time was selected to prepare cubosomal dispersion of TAC i.e. 15 min.

Table 5.7: Selection of stirring time for preparation of cubosomes of Tacrolimus

Batch No.	Stirring Time (min)	% EE	Vesicle size (nm)
1	15	78.96 ± 4.25	150.7 ± 15.87
2	17.5	80.28 ± 5.30	156.7 ± 18.13
3	20	79.09 ± 4.67	141 ± 16.55

5.3.1.8. Selection of temperature

Various batches of cubosomes of Tacrolimus were prepared wherein the concentration of GMO and stabilizer was kept constant i.e., 2.0 %w/v, 1.0 %w/v respectively. Stirring speed and stirring time was kept at 500 rpm and 15 min respectively. For all the batches temperature was varied as mentioned

in the table 5.8. During the preparation of cubosomal dispersion, temperature of organic phase and aqueous phase was maintained as shown in table 5.8. A conclusion was drawn from the collected data that, temperature does not have any significant impact on vesicle size and % entrapment efficiency of Tacrolimus in cubosomes. Thus, lowest temperature i.e. 50 °C was maintained for preparing cubosomal dispersion of TAC.

Table 5.8: Selection of temperature for preparation of cubosomes of Tacrolimus

Batch No.	Temperature °C	% EE	Vesicle size (nm)
1	50	81.44 ± 3.9	142.8 ± 13.43
2	60	81.27 ± 4.09	153 ± 18.31
3	70	79.98 ± 2.91	162.7 ± 15.36

5.3.1.9. Selection of volume of organic phase

Various batches of cubosomes of Tacrolimus were prepared wherein the concentration of GMO and stabilizer was kept constant, i.e. 2.0 %w/v, 1.0 %w/v respectively. Stirring speed and stirring time were set at 500 rpm and 15 min respectively. Further the temperature was kept at 50 °C. Different volumes of organic phase was taken for all batches as shown in table 5.9. From the data collected, it was concluded that volume of organic phase does not have any significant impact on vesicle size and % entrapment efficiency of Tacrolimus in the cubosomes. However, in case of batch no. 1, creaming of prepared formulation was occurred. Thus, 2 mL volume of organic phase was kept constant for preparing further batches of cubosomal dispersion.

Table 5.9: Selection of volume of organic phase for preparation of cubosomes of Tacrolimus

Batch No.	Volume of organic phase (mL)	% EE	Vesicle size (nm)
1	1	75.48 ± 5.78	140 ± 17.14
2	2	80.95 ± 4.78	145.6 ± 19.24
3	3	83.68 ± 5.48	136.9 ± 14.46

5.3.1.10. Selection of rate of addition of organic phase

Various batches of cubosomes of Tacrolimus were prepared wherein the concentration of GMO and stabilizer was kept constant, i.e. 2.0 %w/v, 1.0 %w/v respectively. Stirring speed and stirring times were set at 1000 rpm and 17 min respectively. Further the temperature was kept at 60 °C and 2 ml of the organic phase was added. For all batches rate of addition of organic phase was different as shown in table 5.10. From the data collected, it was concluded that rate of addition of organic phase does not has any significant impact on vesicle size and % entrapment efficiency of Tacrolimus in cubosomes. However, creaming of cubosomal dispersion took placed over a period of 1 week in case of batch no. 1. Thus, rate of addition of organic phase to aqueous phase was kept at 1.0 mL/min for preparing further batches of cubosomal dispersion.

Table 5.10: Selection of rate of addition of organic phase for preparation of cubosomes of Tacrolimus

Batch No.	Rate of addition of organic phase (mL/min)	% EE	Vesicle size (nm)
1	0.5	74.88 ± 5.84	139.8 ± 20.60
2	1.0	80.47 ± 5.39	154.4 ± 16.77
3	1.5	84.73 ± 5.72	151.8 ± 16.99

5.3.2. Preparation and optimization of Tacrolimus loaded cubosomes

5.3.2.1. Establishing QTPP and CQA

Various OTPP elements and CQA with their target and justification are described in table 5.11. Among the various QTPP listed, vesicle size, PDI and % entrapment efficiency need to be controlled according to their limits as these were identified as critical quality attributes (CQAs) in governing the product quality to attain pre-defined QTPP. Therefore, these three characteristics were selected as CQA.

Table 5.11: QTPP and CQA elements with justification for cubosomes of Tacrolimus

QTPP elements		Target	Justification
Route of administration		Transdermal	To circumvent drug metabolism and gastro-intestinal related side effects
Dosage form		Cubosomes	Better skin permeability, high drug loading, controlled release of drug
Formulation quality attributes	Vesicle size#	Minimize	To ensure enhanced permeability through skin and uniform drug release
	Polydispersity index (PDI)#	Minimize (<0.3)	To ensure the formation of monodisperse formulation
	Zeta potential	> ± 30 mV	To ensure that the dispersion is stable
	Shape, Surface characteristic	Cubical, smooth	To ensure that cubosomes are formed
	% entrapment efficiency#	Maximize	To reduce the cost and drug wastage
	In-vitro drug release	For 24 hrs	To ensure controlled release of drug for the maintainance of plasma drug concentration
Ex-vivo permeability		Better transdermal flux	To ensure that the PK and PD parameters of

		the prepared formulation are similar or superior to marketed formulation
Stability	Not less than 3 month	To ensure that the prepared formulation is stable till expiry date of formulation
Safety	Non-toxic and non-irritant to skin	To ensure that the prepared formulation are safe
Pharmacokinetic	Similar or better than oral suspension and marketed product	To fulfill the requirement of bioequivalence
Pharmacodynamic		For the demonstration of therapeutic efficacy

Critical Quality Attributes (CQAs)

5.3.3. Identification of independent variables and qualitative risk assessment using Ishikawa diagram

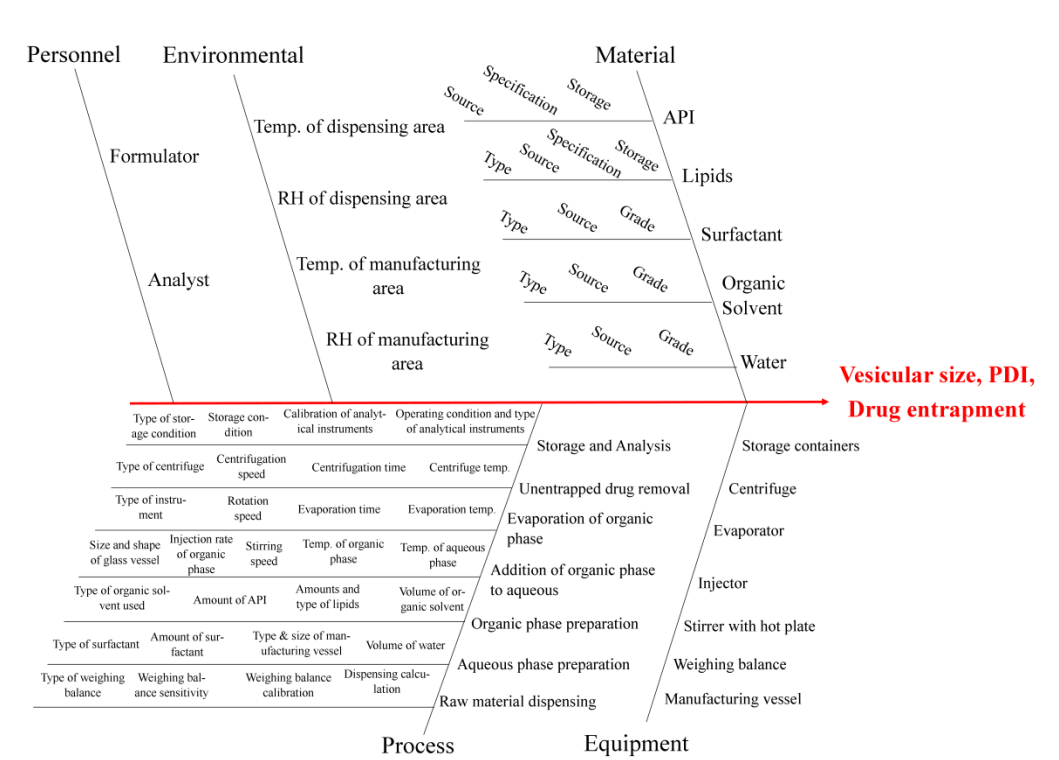


Figure 5.3: Ishikawa diagram showing probable variables that may influence the CQA

During the various brainstorming sessions, all likely variables related with development of Tacrolimus loaded cubosomes by bottom up approach were recognized and were categorized into these categories i.e. Material, Equipment, Process, Environment and Personnel.(3) An ishikawa diagram was constructed for illustrating the cause and effect relation between identified variables and CQAs (Fig. 5.3). Figure 5.3 shows all variables that affect product quality in terms of vesicular size, PDI, % entrapment efficiency. Among these variables, most significant variables that affect the product quality was separated using 2-level fractional factorial design.

Table 5.12: Qualitative risk assessment of independent variables

Factors	Process step	Impact on CQA	Constant levels
Source and specifications of API	Raw material selection and storage	Low risk	Authentic and reliable source with COA
Storage condition		Low risk	At room temp.
Type of lipid		Moderate risk	Glyceryl Monooleate
Source and specifications of lipid		Low risk	Authentic and reliable source with COA
Storage condition of lipids		Low risk	Store at recommended condition
Type of surfactant		Moderate risk	Polyvinyl alcohol low molecular weight (PVA-6000)
Source and specifications of surfactant		Low risk	Authentic and reliable source with COA
Storage conditions of surfactant		Low risk	At recommended condition
Type of organic solvent		Moderate risk	Ethanol
Source and specifications of organic solvent		Low risk	Authentic and reliable source with COA
Grade of water		Low risk	Double distilled prepared in house
Weighing balance	Dispensing	Low risk	1 mg

sensitivity			
Weighing balance calibration		Low risk	Calibration
Temperature and RH of dispensing area		Low risk	At room temp., Ambient RH
Dispensing calculations		Low risk	Calculated using Microsoft excel
Type, size and material of construction (MOC)	Manufacturing and storage vessel	Low risk	20 mL beaker, 250 mL round bottom flask of class A borosilicate glass
Temp. and relative humidity	Manufacturing area	Low risk	At room temperature, ambient RH
Volume of aqueous phase	Aqueous phase preparation	Low risk	20 mL
Amount of surfactant		High risk	To be optimized
Volume of organic phase	Organic phase preparation	High risk	To be optimized
Amount of API		Moderate risk	10 mg
Amount of lipids		High risk	To be optimized
Injection rate of organic phase	Addition of organic phase in aqueous phase	High risk	To be optimized
Stirring speed and time		High risk	To be optimized
Temperature of aq. and organic phase		High risk	To be optimized
Type of evaporator	Evaporation of	Low risk	Rota evaporator

Evaporation time	organic phase	Low risk	30 min
Evaporation temperature		Low risk	60 °C
Evaporation vacuum		Low risk	600 mmHg
Stirring speed during evaporation		Low risk	100 rpm
Type of centrifuge	Unentrapped drug removal	Low risk	Cooling centrifuge (Remi)
Type and MOC of centrifuge tube		Low risk	15 mL conical centrifuge tube with screw cap
Centrifugation speed		Low risk	3000 rpm
Centrifugation time		Low risk	15 min
Centrifugation temperature		Low risk	25 °C
Type and material of storage vessel	Storage and Analysis	Low risk	10 mL storage vial (Tarson)
Storage condition		Low risk	At room temperature
Analytical instrument		Low risk	Agilent gradient HPLC-infinity 1220
Calibration of analytical instruments		Low risk	Calibrated
Methods used for analysis		Low risk	Developed and calibrated
Formulator	Personnel	Low risk	Common for all experiments and analysis
Analyst		Low risk	

Factors with high risk were carried forward for quantitative risk assessment.

5.3.4. Screening of various attributes (CQA) using 2-level design

2-level fractional factorial screening design was used for the statistical assessment of factors with high risk. The low (-1) and high (+1) levels of all independent variables were decided dependent on a preliminary trials and are mentioned in Table 5.12. Randomized design matrix of 30 experimental batches was generated using Design Expert 7.0 statistical software and presented in Table 5.13 & 5.14. The data was statistically processed by Design expert to generate reduced equation for all the CQA(s) (vesicle size, PDI, % entrapment) wherein the value of $P < 0.05$ was taken as a level of significance.

Table 5.13: Various parameters affect the product quality along with their levels for screening by fractional factorial design

Sr. No.	Independent variable	Unit	Levels	
			-1	+1
A	Concentration of lipid	% w/v	1	4
B	Concentration of surfactant	% w/v	0.5	1.5
C	Stirring speed	Rpm	500	1500
D	Stirring time	Min	15	20
E	Rate of addition of organic phase	mL/min	0.5	1.5
F	Volume of organic phase	mL	1	3
G	Temperature of both phases	°C	50	70

Table 5.14: 2-Level fractional factorial batches suggested by Design Expert 7.0

Batch no	Run order	Run Order							CQA		
		A	B	C	D	E	F	G	Vesicle Size (nm)	% Entrapment efficiency	PDI
A1	26	-1	-1	-1	+1	-1	-1	+1	215.9	42.68	0.325
A2	13	-1	+1	-1	-1	+1	-1	-1	160.5	58.49	0.204
A3	14	-1	+1	+1	+1	-1	-1	+1	149.3	56.19	0.195
A4	23	-1	-1	+1	-1	-1	-1	+1	222.7	50.84	0.296
A5	10	+1	+1	+1	+1	+1	+1	+1	245.7	97.28	0.315
A6	28	-1	-1	-1	-1	+1	+1	-1	201.6	41.98	0.32
A7	18	+1	+1	-1	-1	-1	+1	-1	230	96.41	0.331
A8	8	-1	+1	-1	+1	+1	+1	-1	151.6	55.81	0.212
A9	30	+1	-1	+1	-1	+1	-1	-1	325.4	70.18	0.427
A10	12	+1	+1	-1	+1	-1	+1	+1	235.4	101.15	0.297
A11	1	-1	-1	-1	+1	-1	+1	-1	190.8	39.47	0.295
A12	6	-1	-1	-1	+1	+1	+1	+1	201.3	45.51	0.319
A13	7	-1	-1	+1	+1	+1	-1	-1	199.4	40.91	0.289
A14	21	+1	-1	-1	+1	+1	-1	-1	301.7	75.84	0.466
A15	22	+1	+1	-1	+1	-1	-1	-1	234.4	96.84	0.286
A16	15	-1	+1	+1	-1	-1	-1	-1	160.8	55.1	0.216
A17	2	+1	+1	-1	-1	+1	+1	+1	244.7	97.08	0.291
A18	19	+1	+1	+1	-1	-1	+1	+1	231.5	96.47	0.286
A19	11	-1	+1	+1	-1	+1	+1	-1	155.7	54.14	0.211
A20	25	+1	+1	+1	+1	+1	-1	-1	241.2	95.81	0.279
A21	27	+1	-1	-1	-1	+1	-1	+1	312.8	71.8	0.445
A22	20	+1	-1	-1	-1	-1	+1	+1	296.4	69.48	0.401
A23	29	+1	-1	+1	+1	+1	+1	-1	329	73.94	0.412
A24	9	-1	-1	+1	-1	-1	+1	-1	149.2	54.18	0.208

A25	5	+1	-1	+1	+1	-1	-1	-1	317.6	70.04	0.394
A26	3	-1	+1	+1	-1	+1	-1	+1	154.7	59.41	0.203
A27	17	+1	+1	-1	-1	-1	-1	+1	233.9	102.4	0.294
A28	16	-1	-1	+1	+1	-1	+1	+1	207.1	38.49	0.316
A29	4	+1	-1	+1	+1	+1	-1	+1	312.8	68.94	0.406
A30	24	-1	+1	-1	-1	-1	+1	+1	149.8	56.48	0.217

It is clearly evidenced from the Pareto and normal charts (Fig. 5.4) that factors having major effect on vesicle size and PDI of a prepared cubosomes were conc. of lipids and surfactant. Likewise, the conc. of lipid has major effect on % entrapment efficiency. So, inference was drawn from these observations that concentration of lipid and surfactant will be considered as CMA in the concluding optimization step.

An impact of stirring speed, injection rate, stirring time, organic solvent volume, and temperature of both phases were found insignificant on both CQA. Hence, constant level of these factors (stirring speed, 500 rpm, injection rate, 1.0 mL/min; stirring time 15 min, temperature of both phases 50 °C and organic solvent volume, 2 mL) were selected during the optimization.

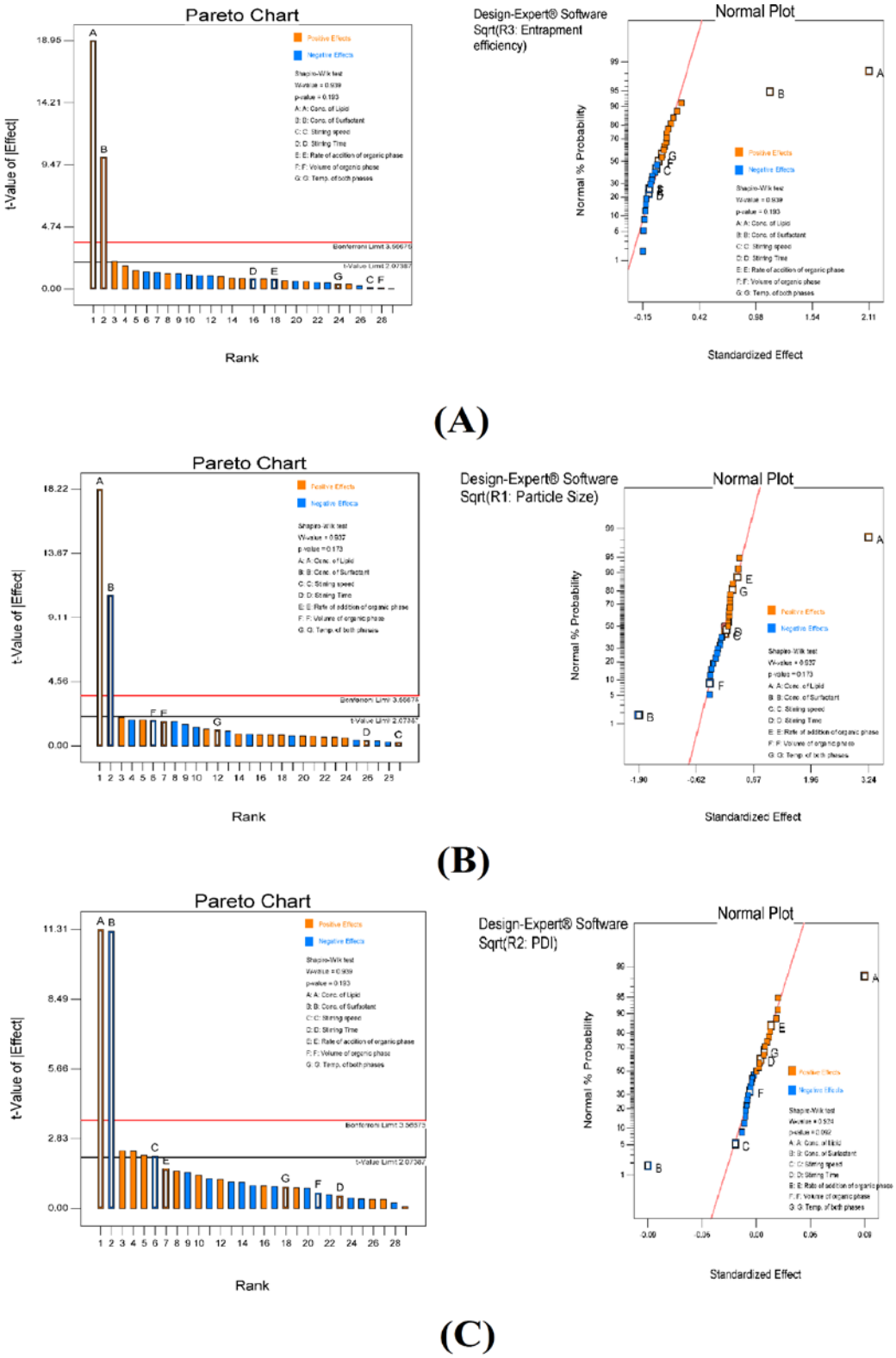


Figure 5.4: Pareto and normal plots for (A): % Entrapment efficiency, (B) Vesicle size, (C) PDI

5.3.5. Formulation optimization using 3^2 Factorial Design

On the basis of the results of screening design, two independent variables were identified and their relationships with CQA were exhaustively investigated using 3^2 factorial design. The low (-1), intermediate (0) and high (+1) levels of independent variables are listed in Table 5.15.

Table 5.15: Independent variables along with their level for optimization by 3^2 factorial design

Independent variables		Unit	Levels		
			-1	0	+1
A	Concentration of PVA	% w/v	0.5	1.0	1.5
B	Concentration of GMO	% w/v	1	2.5	4

A randomized matrix of thirteen batches was generated by Design-Expert 7.0 and presented in Table 5.16. These batches were formulated according to their run order and were assessed for CQA using the methods described earlier. Table 5.16 also represents the resulting CQA of these batches.

Table 5.16: Randomized design matrix for 3^2 factorial design

Batch no	Run order	Independent variables		CQA (Dependent variables)		
		A: Conc. of PVA (%w/v)	B: Conc. of GMO (%w/v)	% entrapment efficiency	Vesicle size (nm)	PDI
10	1	1.0	2.5	93.4	151.3	0.136
7	2	0.5	4.0	65.1	294.1	0.388
8	3	1.0	4.0	81.34	191.0	0.302
3	4	1.5	1.0	53.21	156.0	0.221
2	5	1.0	1.0	52.8	129.1	0.206
12	6	1.0	2.5	91.48	140.9	0.164
5	7	1.0	2.5	89.74	146.7	0.137
4	8	0.5	2.5	74.09	254.8	0.328
6	9	1.5	2.5	93.44	173.8	0.189

13	10	1.0	2.5	90.73	165.0	0.171
11	11	1.0	2.5	92.66	160.8	0.201
9	12	1.5	4.0	98.13	229.4	0.286
1	13	0.5	1.0	41.73	195.4	0.309

Based on the data obtained from experiments for **% entrapment efficiency**, quadratic model was suggested by the software for both mix order and process order. ANOVA and coded coefficients of full quadratic model for % entrapment efficiency are mentioned in Table 5.17 & 5.18, respectively. The categorization of terms as significant or insignificant was done on the basis of the p-value i.e. model term having p-value less than 0.05 (α-level) is significant while others are insignificant.(3) Hierarchy based removal of insignificant model terms was done to simplify model and obtain reduced equation.

Table 5.17: Analysis of variance of full quadratic model for % entrapment efficiency

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob > F**	
Model	4292.77	5	858.55	145.44	< 0.0001	Significant
A: Conc. of PVA	679.68	1	679.68	115.14	< 0.0001	Significant
B: Conc. of GMO	1562.67	1	1562.67	264.72	< 0.0001	Significant
AB	116.10	1	116.10	19.67	0.0030	Significant
A^2	76.82	1	76.82	13.01	0.0087	Significant
B^2	1332.97	1	1332.97	225.81	< 0.0001	Significant
Residual	41.32	7	5.90			
Lack of fit	32.73	3	10.91	5.08	0.0752	Not Significant
Pure error	8.59	4	2.15			
Cor total	4334.09	12				

ANOVA table for % entrapment efficiency showed significant interaction, quadratic and linear mixture effects among selected dependent variables (CQA). Significant quadratic terms indicated that the relationship between these dependent variables and % entrapment efficiency follow a curved line. An insignificant lack-of fit showed competence of a model in justifying difference in the observations.

Table 5.18: Coded coefficient of full as well as reduced quadratic model for % entrapment efficiency

Term	Full model			Reduced model		
	Coefficient Estimate	Standard Error	VIF	Coefficient Estimate	Standard error	VIF
Intercept	90.87	1.01	-	90.87	1.01	-
A: Conc. of PVA	10.64	0.99	1.00	10.64	0.99	1.00
B: Conc. of GMO	16.14	0.99	1.00	16.14	0.99	1.00
AB	5.39	1.21	1.00	5.39	1.21	1.00
A^2	-5.27	1.46	1.17	-5.27	1.46	1.17
B^2	-21.97	1.46	1.17	-21.97	1.46	1.17

Coefficients table for % entrapment efficiency showed VIF values near to 1 for 2-way interaction terms while it was <10 for quadratic terms indicating that the predictors are not correlated and regression coefficients are well estimated. Regression equations for full and reduced models in un-coded units are presented as Eq. 5.2 and 5.3, respectively. The (+) and (-) symbol preceding every coefficient indicates direct or inverse connection of that model term with the % entrapment efficiency of prepared batches

Full model:

$$\% \text{ Entrapment efficiency} = 90.87 + 10.64A + 16.14B + 5.39\,AB - 5.27A^2 - 21.97\,B^2$$

Equation 5.2

Reduced model:

$$\% \text{ Entrapment efficiency} = 90.87 + 10.64A + 16.14B + 5.39\ AB - 5.27A^2 - 21.97\ B^2$$

Equation 5.3

Reduced model in uncoded unit:

$$\% \text{ Entrapment efficiency} = -21.48 + 45.52\ (\text{conc. of PVA}) + 52.40\ (\text{conc. of GMO}) + 7.18\ (\text{conc. of PVA})(\text{conc. of GMO}) - 21.10\ (\text{conc. of PVA})^2 - 9.76\ (\text{conc. of GMO})^2$$

Equation 5.4

Here, we observed that concentration of PVA and GMO have a positive effect on the % entrapment efficiency of cubosomes which means that as the concentration of PVA and GMO increases it also increases the % entrapment efficiency of cubosomes of TAC. Moreover, equations of full and reduced model for the % entrapment efficiency are the same because all the terms evaluated are significant ($p < 0.05$) in statistical terms as showed in table 5.17.

Based on the experimental data of **vesicle size**, quadratic model was suggested by software for mix order and linear model for process order. ANOVA was performed by the software for suggested models for vesicle size and is mentioned in table 5.19. The categorization of terms as significant or insignificant was done on the basis of the p-value i.e. model term having p-value less than 0.05 (α-level) is significant while others are insignificant. Hierarchy based removal of insignificant model terms was done to simplify a model and obtain reduced equation.

Table 5.19: Analysis of variance of full quadratic model for vesicle size

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	27620.64	5	5524.13	44.32	< 0.0001	Significant
A: Conc. PVA	5710.34	1	5710.34	45.81	< 0.0003	Significant
B: Conc. GMO	9126.00	1	9126.00	73.21	< 0.0001	Significant
AB	160.02	1	160.02	1.28	0.2945	Not

						Significant
A^2	9964.01	1	9964.01	79.94	< 0.0001	Significant
B^2	93.35	1	93.35	0.75	0.4155	Not Significant
Residual	872.56	7	124.65			
Lack of fit	478.75	3	159.58	1.62	0.3184	Not Significant
Pure error	393.81	4	98.45			
Cor total	28493.20	12				

ANOVA table for vesicle size showed significant interaction, quadratic and linear mixture effects among selected CQAs. Significant quadratic terms indicated that the relationship between these CQAs and vesicle size follow a curved line. An insignificant lack-of fit showed adequacy of a model in justifying difference in the observations.

Table 5.20: Coded coefficient of full as well as reduced quadratic model for vesicle size

Term	Full model			Reduced model		
	Coefficient Estimate	Standard Error	VIF	Coefficient Estimate	Standard error	VIF
Intercept	153.31	4.64	-	153.31	4.64	-
A: Conc. of PVA	-30.85	4.56	1.00	-30.85	4.56	1.00
B: Conc. of GMO	39.00	4.56	1.0	39.00	4.56	1.00
AB	-6.33	5.58	1.00	**Not significant**		
A^2	60.06	6.72	1.17	60.06	6.72	1.17
B^2	5.81	6.72	1.17	**Not significant**		

Coefficients table for vesicle size of Tacrolimus loaded cubosomes showed VIF values near to 1 for 2-way interaction terms while it was <10 for

quadratic terms indicating that the predictors are not correlated and regression coefficients are well estimated.(11) Regression equations for full and reduced models in uncoded units are presented as Eq. 5.5 & 5.6 respectively. The (+) and (-) symbol preceding every coefficient symbolizes direct or inverse connection of that model term with vesicle size of prepared cubosomal batches.

Full model:

$$\text{Vesicle Size} = 153.31 - 30.85\,A + 39.00\,B - 6.33\,AB + 60.06\,A^2 + 5.81\,B^2$$

Equation 5.5

Reduced model:

$$\text{Vesicle Size} = 90.87 + 10.64A + 16.14B - 5.27A^2$$

Equation 5.6

Reduced model in uncoded unit:

$$\text{Vesicle Size} = 385.33 - 521.13\,(\text{conc. of PVA}) + 21.51\,(\text{conc. of GMO}) + 240.26\,(\text{conc. of PVA})^2$$

Equation 5.7

From the equation 5.7, it can be concluded that concentration of PVA has negative effect on vesicle size of cubosomes means as the concentration of PVA increases vesicle size of cubosomes decreases. Opposite phenomena was observed with the concentration of GMO. Moreover interaction effect between the concentration of PVA and GMO was not observed on vesicle size. In reduced model equation 5.6, interaction effect AB, and BB were removed as its p value was greater than 0.05.

Based on the experimental data of **polydispersity index (PDI)**, software suggested quadratic model for mix order and linear model for process order. Analysis of variance (ANOVA) was performed by the software for suggested models for PDI and presented in table 5.21. The categorization of terms as significant or insignificant was done on the basis of the p-value i.e. model term having p-value less than 0.05 (α-level) is significant while others are insignificant. Hierarchy based removal of insignificant model terms was done to simplify model and obtain the reduced equation.

Table 5.21: Analysis of variance of full quadratic model for PDI

Source	Sum of squares	Df	Mean square	F value	p-value Prob > F	
Model	0.072	5	0.014	18.50	< 0.0007	Significant
A: Conc. of PVA	0.018	1	0.018	23.18	< 0.0019	Significant
B: Conc. of GMO	9.6×10^{-3}	1	9.6×10^{-3}	12.34	< 0.0098	Significant
AB	4.9×10^{-5}	1	4.9×10^{-5}	0.063	0.8091	Not Significant
A^2	0.015	1	0.015	18.76	< 0.0034	Significant
B^2	0.013	1	0.013	16.51	0.0048	Significant
Residual	5.45×10^{-3}	7	7.78×10^{-4}			
Lack of fit	2.54×10^{-3}	3	8.47×10^{-4}	1.17	0.4265	Not Significant
Pure error	2.9×10^{-3}	4	7.27×10^{-4}			
Cor total	0.077	12				

ANOVA table for PDI showed significant interaction, quadratic and linear mixture effects among selected CQAs. Significant quadratic terms indicated that the relationship between these CQAs and PDI follow a curved line. An insignificant lack-of fit showed the adequacy of the model in justifying the difference in the observations.

Table 5.22: Coded coefficient of full as well as reduced quadratic model for PDI

Term	Full model			Reduced model		
	Coefficient Estimate	Standard Error	VIF	Coefficient Estimate	Standard error	VIF
Intercept	0.17	0.012	-	0.17	0.012	-
A: Conc. of PVA	-0.055	0.011	1.00	-0.055	0.011	1.00

B: Conc. of GMO	0.040	0.011	1.0	0.040	0.11	1.00
AB	-3.5×10^{-3}	0.014	1.00	**Not significant**		
A^2	0.073	0.017	1.17	0.068	0.017	1.17
B^2	0.068	0.017	1.17	0.068	0.017	1.17

Coefficients table for PDI of Tacrolimus loaded cubosomes showed VIF values near to 1 for 2-way interaction terms while it was <10 for quadratic terms indicating that the predictors are not correlated and regression coefficients are well estimated.(11) Regression equations for full and reduced models in un-coded units are presented as Eq. 5.8 & 5.9, respectively. The (+) and (-) symbol preceding every coefficient indicates direct or inverse relationship of that model term with PDI.

Full model:

$$PDI = +0.17 - 0.055\ A + 0.040\ B - 3.5X10^{-3}\ AB + 0.073\ A^2 + 0.068\ B^2$$

Equation 5.8

Reduced model:

$$PDI = +0.68 - 0.68\ A - 0.12\ B + 0.29\ A^2 + 0.03\ B^2$$

Equation 5.9

Reduced model in uncoded unit:

$$PDI = +0.68 - 0.68\ (\text{conc. of PVA}) - 0.12\ (\text{conc. of GMO}) + 0.29\ (\text{conc. of PVA})^2 + 0.03\ (\text{conc. of GMO})^2$$

Equation 5.10

From the eq. 5.10, it can be concluded that PDI of a cubosomal dispersion of is inversely related to the concentration of PVA and GMO, which means that a concentration of PVA and GMO decreases PDI improves. Moreover, eq. 5.10, also indicates absence of interaction effects of concentration of GMO and PVA on PDI because its p value is greater than 0.05 as shown in table 5.21.

Model summary for all independent variables are presented in Table 5.23. A low SD value and high R^2 value symbolized an improved prediction of observations by the model. Predicted R^2 value was reported to be in good

agreement with adjusted R^2 value for further supporting the prediction potential of the model.

Table 5.23: Summary of full quadratic model for all independent variables

Responses	Full model				
	SD	Mean	R-sq	R-sq (adjusted)	R-sq (predicted)
% Entrapment efficiency	2.43	78.30	0.9905	0.9837	0.9353
Vesicle size	11.16	183.72	0.9694	0.9475	0.8093
Polydispersity index (PDI)	0.028	0.23	0.9297	0.8794	0.6852

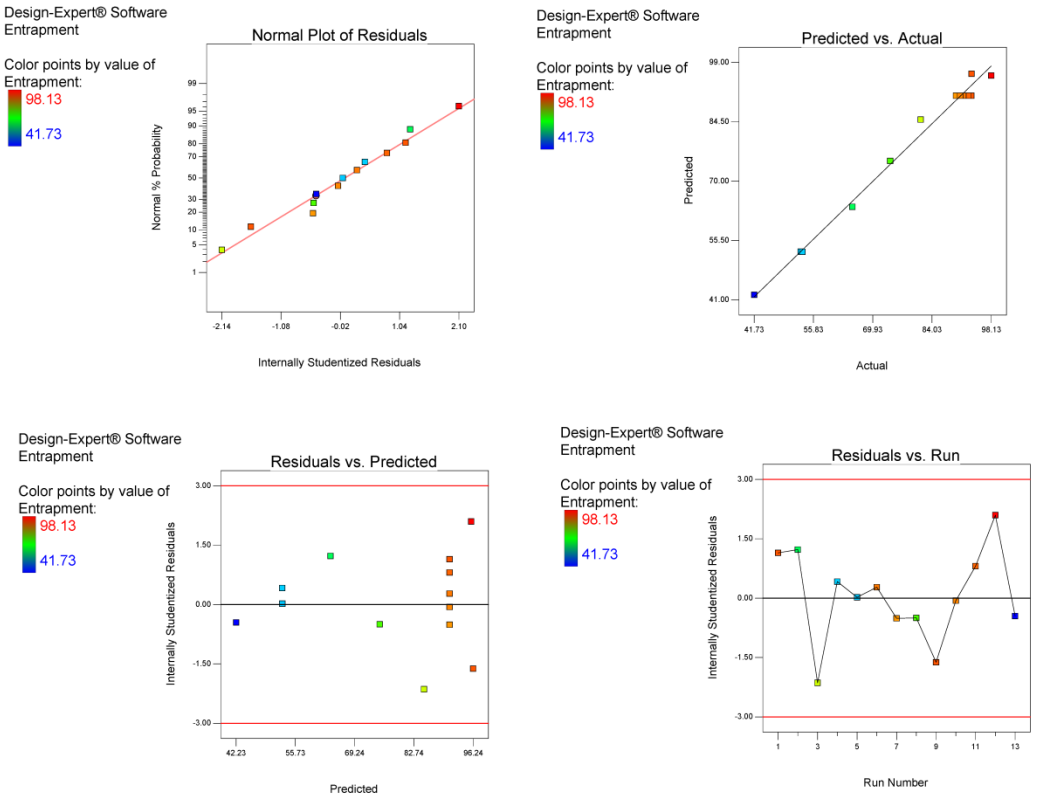


Figure 5.5: Residuals plots for % entrapment efficiency

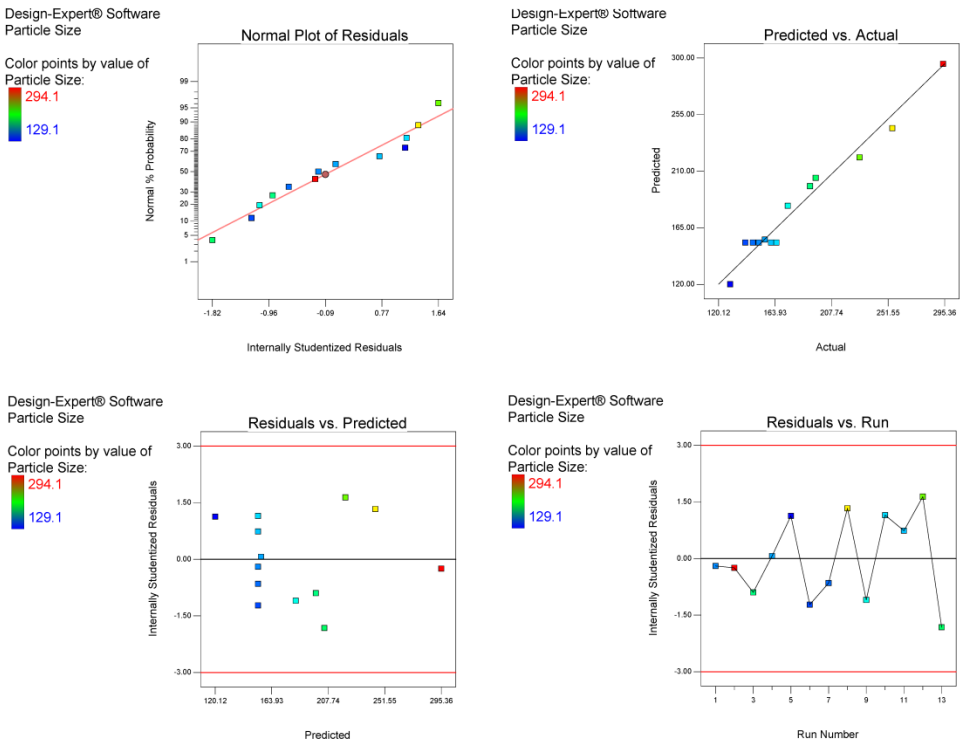


Figure 5.6: Residuals plots for vesicle size

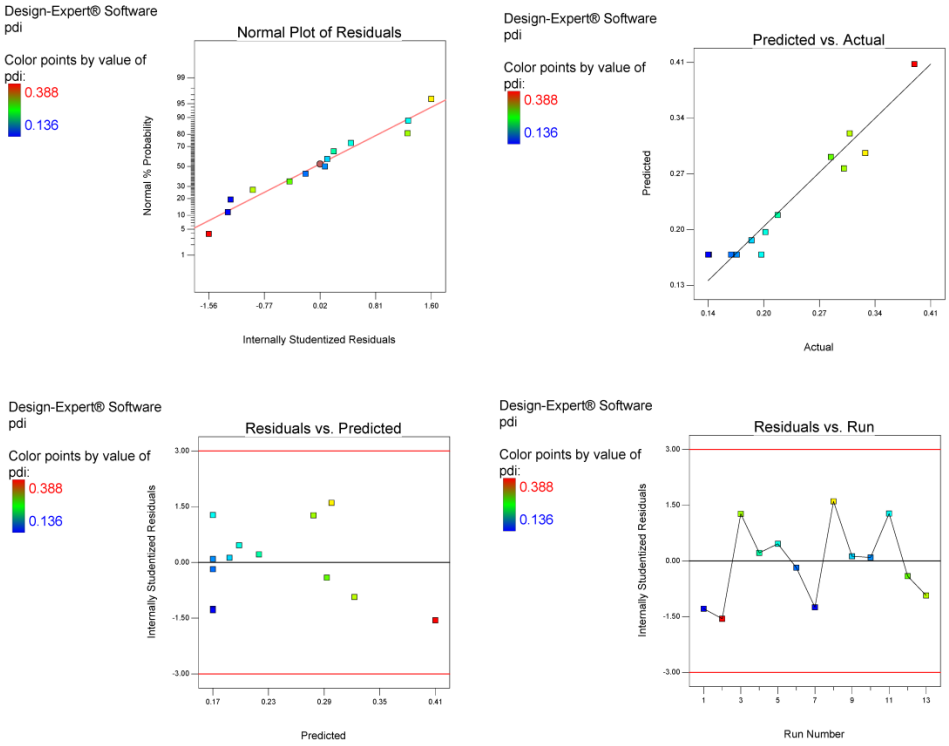


Figure 5.7: Residuals plots for Polydispersity index (PDI)

Four different residual plots viz., normal plot of residual, residual versus ascending predicted response values, residual versus experimental run order and predicted versus actual were generated for all three CQAs and presented in Fig. 5.5, 5.6 & 5.7. It was observed that the data was normally distributed in normal plot as it could be seen that the residuals followed a straight line. Further, the prediction of constant variance was validated as random scattering with the absence of any megaphone pattern in residual versus predicted plot was seen. Likewise, the absence of lurking variables was validation by the random scattering without any pattern in residual versus run plot. The selected model gave convenient assumption of the values given that the data points were consistently split by 45-degree line.(12)

Contour and response surface plots of % entrapment efficiency, vesicular size and PDI are presented in Fig. 5.8, 5.9, & 5.10 respectively. These graphs were used to depict how the CQA is related to respective independent factor while keeping other independent variable at constant levels.

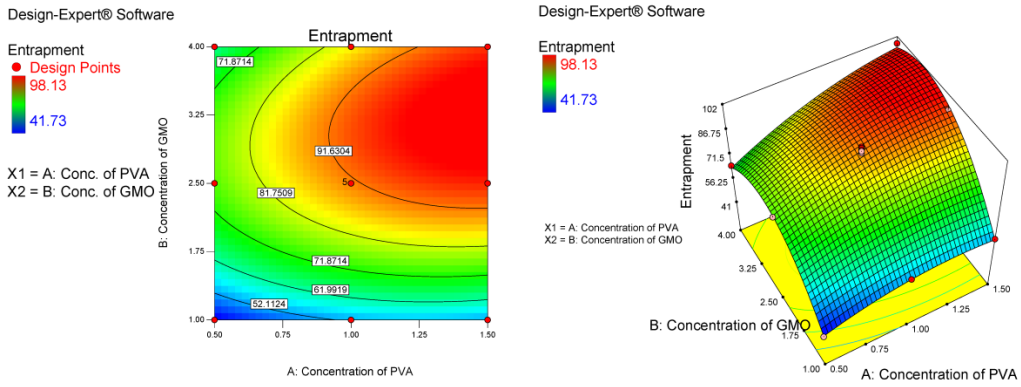


Figure 5.8: Contour and surface response plot of % entrapment efficiency

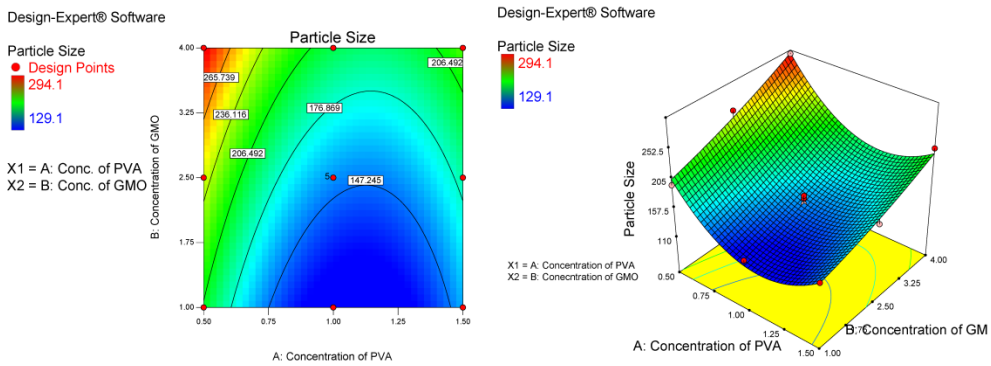


Figure 5.9: Contour and surface response plot of vesicle size

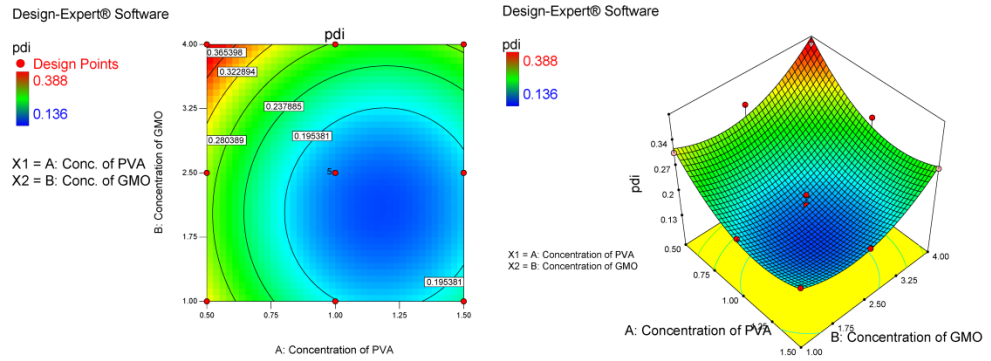


Figure 5.10: Contour and surface response plot of polydispersity index (PDI)

Numerical optimization was performed by the software for defined optimization criteria as presented in Table 5.24. The programming of software was done in a manner so that it generated the optimization solution with least vesicle size, PDI and maximum % entrapment efficiency wherein all independent variables were kept in the investigational range.

Table 5.24: Criteria for optimization of Tacrolimus loaded cubosomes

Constraints name	Goal	Lower limit	Upper limit	Weight	Importance
Conc. of PVA	In range	0.5	1.5	1	+++
Conc. of GMO	In range	1	4	1	+++
%	Maximum	80	100	1	+++

Entrapment					
Vesicle size	Minimum	129.1	250	1	+++
PDI	Minimum	0.136	0.3	1	+++

Overlay contour plots for all three CQAs (% entrapment, vesicle size and PDI) were generated (Fig. 5.11) for obtaining the design space (yellow area in graph) by employing the defined criteria shown in above mentioned table 5.24.

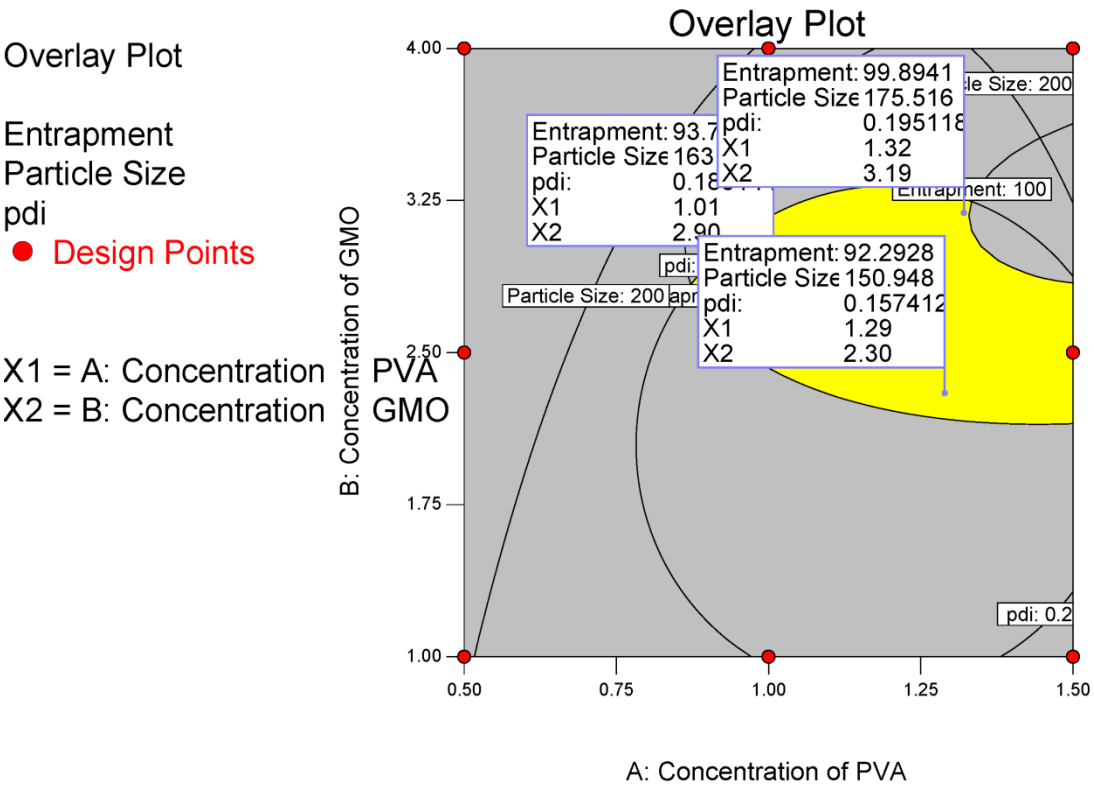


Figure 5.11: Overlay plot for the optimization of Tacrolimus loaded cubosomes using full quadratic model

Further, the optimized desirability plot is presented in Fig. 5.12. The optimized desirability plot showed a composite desirability of 0.809945 for the solution provided by the software.

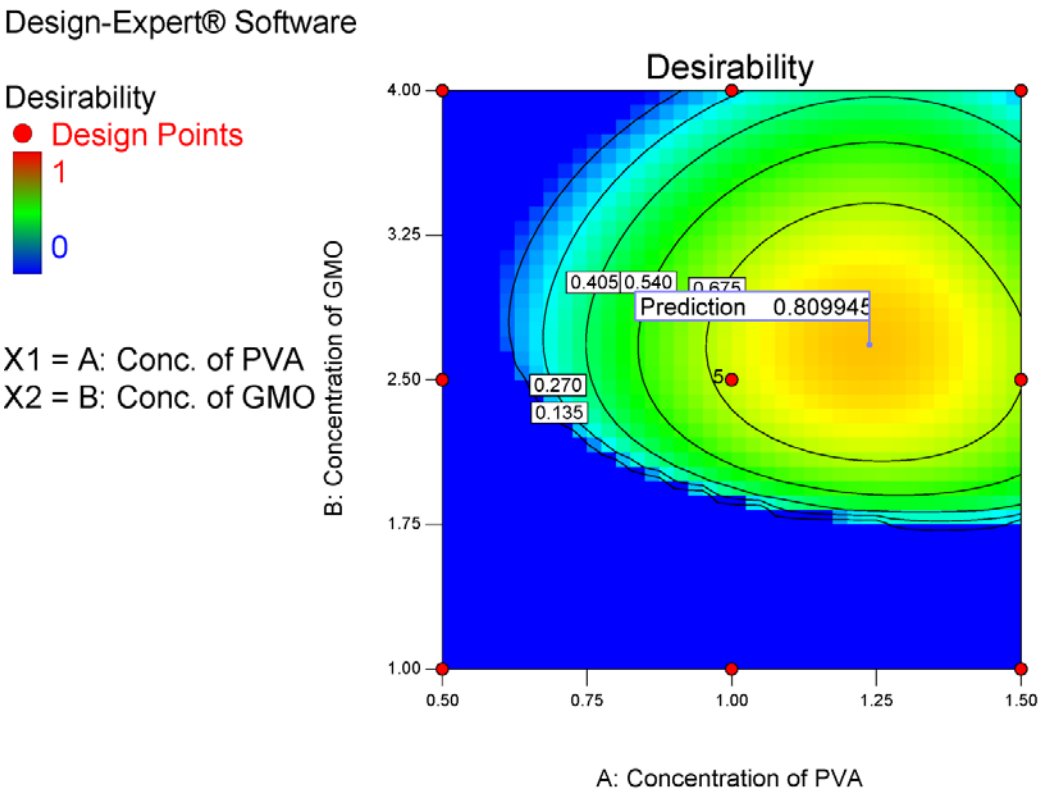


Figure 5.12: Desirability contour plot for optimization of Tacrolimus loaded cubosomes using full quadratic model

5.3.6. Results of Checkpoint batch

Upper limit and lower limit of the independent variables and CQAs at 95 % confidence and prediction level are given in table 5.25 & 5.26. Three checkpoint batches were prepared according to these levels and results of these checkpoint batches are represented in table 5.27. The legitimacy of the model was established as it was found that the average values of both CQA were observed to be within low and high levels of 95% confidence interval.

Table 5.25: Levels of independent variables as per the point prediction analysis.

Factor	Name	Level	Low level	High level
A	Conc. of PVA	1.24	0.50	1.50
B	Conc. of GMO	2.68	1.00	4.00

Table 5.26: Levels of responses at 95 % confidence and prediction intervals

Response	SE Mean	95 % CI* low	95 % CI* high	SE Pred	95 % PI* low	95 % PI* high
% Entrapment	1.03	94.23	99.12	2.64	90.43	102.92
Vesicle size	4.75	145.42	167.90	12.13	127.96	185.35
PDI	0.012	0.14	0.19	0.030	0.093	0.24

*CI = Confidence interval; PI = prediction interval

Table 5.27: Results of checkpoint batches obtained using optimized overly plot of Tacrolimus loaded cubosomes

Sr. No.	Conc. GMO (% w/v)	Conc. of PVA (%w/v)	Predicted value			Results obtained		
			% Entrapment	Vesicle size (nm)	PDI	% Entrapment	Vesicle size (nm)	PDI
1	2.90	1.01	93.8	163.5	0.183	90.84	156.9	0.175
2	3.19	1.32	99.89	175.5	0.195	96.42	170.1	0.215
3	2.30	1.29	92.29	150.9	0.157	91.08	145.6	0.148
Avg						92.78	157.5	0.179

5.3.7. In-vitro characterization of optimized Tacrolimus loaded cubosomes

5.3.7.1. Shape and surface morphology

TEM was utilized for determining the shape and surface morphology of an optimized formulation and the image is presented in figure 5.13. The image showed cubical shape of the prepared Tacrolimus loaded cubosomes with smooth surface.(6) The size of the cubosomes seen in the image was found in-line with the results of vesicle size data obtained from Malvern zetasizer.

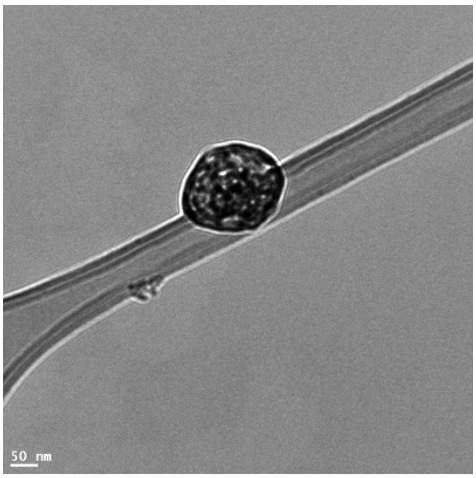


Figure 5.13: TEM images of Tacrolimus loaded cubosomes

5.3.7.2. Zeta potential

Zeta potential most commonly indicates the stability of a prepared colloidal formulation. Various compositions used in the preparation of colloidal dispersion contribute in the formation of zeta potential on the vesicles. Thus, zeta potential indicates a degree of repulsion between the charged particles of colloidal dispersion. High zeta potential means that vesicles of colloidal dispersions are highly charged and they do not prepare agglomerate due to the high repulsive force between them and vice versa. The optimum zeta potential required for the stability of colloidal dispersion is ± 30 mV according to various literatures.(13, 14) Zeta potential of the prepared Tacrolimus cubosomal formulation was measured using Malvern Zetasizer and was found to be -7.06 due to the absence of the any charged materials in the formulation. The obtained zeta potential is way below than the required for the stability of cubosomal dispersion. The negative was obtained due the presence of the free oleic acid in lipid. However, prepared cubosomal formulation of Tacrolimus does not form any aggregate during the entire storage period due to the stealthing effect provided by the surfactant (PVA) used in the preparation. A stealthing effect is the phenomena by which non-ionic surfactant stabilizes the nanoparticulate systems so that they can't form aggregate.(2)

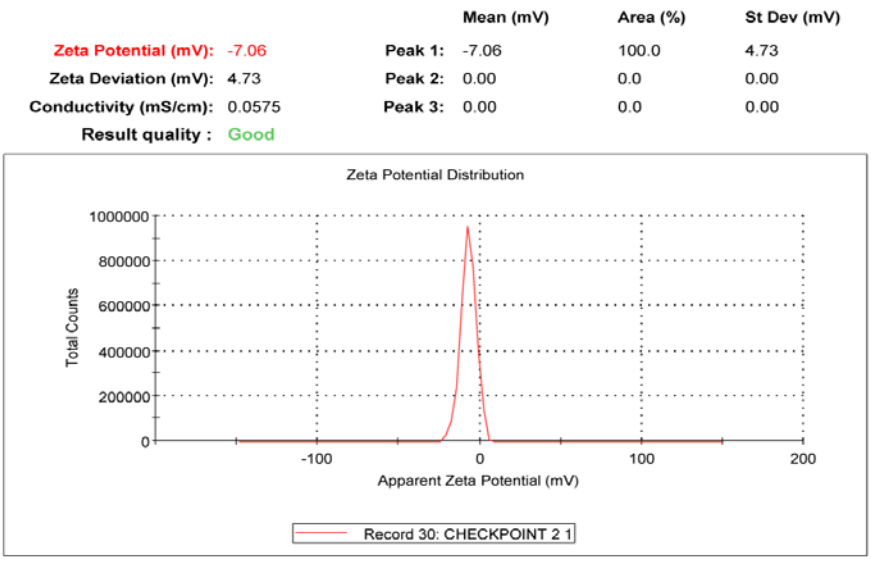


Figure 5.14: Zeta potential of the optimized Tacrolimus loaded cubosomes

5.3.7.3. Vesicle size and size distribution

Mean vesicle size and PDI of the cubosomal Tacrolimus dispersions were measured using the Malvern Zetasizer and was reported to be 173.8 nm and 0.189 respectively. An obtained vesicle size of cubosomes is sufficient for the transdermal permeation of Tacrolimus. Additionally, low PDI value of the optimized batch suggests the formation of the mono-dispersed dispersion.(15) According to the literature, if nanocarriers have vesicle size below 300 nm, it can efficiently reach to the deeper layer of a skin i.e. transdermal delivery from where systematic absorption of drug take places.(16) Here, cubosomes having vesicle size less than 300 nm was successfully prepared. Due to the smaller size they efficiently reached to the dermis layer of a skin and drug was absorbed systematically from here to obtained desired therapeutic concentration of drug in blood.

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	173.8	Peak 1:	179.1	100.0	60.70
PdI:	0.189	Peak 2:	0.000	0.0	0.000
Intercept:	0.908	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

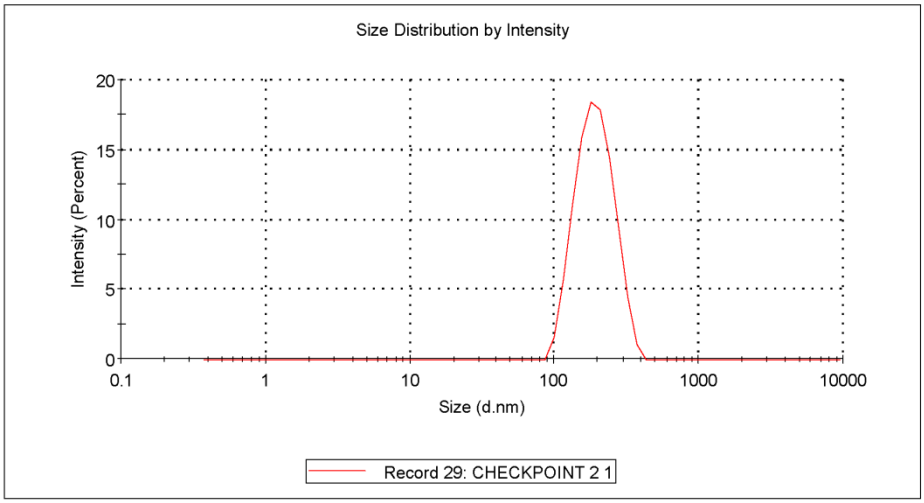


Figure 5.15: Vesicle size of the optimized Tacrolimus loaded cubsomes

5.3.7.4. % Entrapment efficiency

Mean % Entrapment efficiency of the optimized formulation was observed to be 92.78 %. The lipophilic property of the entrapped drug is responsible for the high % entrapment efficiency of the optimized formulation (Tacrolimus - log p value: 3.3). Moreover, cubosomes have a distinct advantage of providing high entrapment efficiency of encapsulated drug according to literature survey.(15)

5.3.7.5. Total drug content

Total drug content of prepared cubosomal dispersion of Tacrolimus was found to be 98.57 % which means that 1 mL of cubosomal dispersion contain 3.66 mg of TAC.

5.3.7.6. Small Angle X-rays Scattering

SAXS was employed for the investigation of liquid-crystalline structure of prepared cubosomes and the results are shown in figure 5.16. It showed one well-defined scattering patterns and one diffuse diffraction pattern at Q value of 0.3, and 3.0 – 5.0 A^{-1} region with relative positions on curve respectively.

The peak at Q value of 0.3 A^{-1} indicates characteristic scattering peaks due to cubic phase. The key feature of this X-Ray scattering diagram was diffuse scattering pattern of low intensity in the region of 3.0 – 5.0 A^{-1} indicating presence of water channels inside Cubosomes which is a unique feature among all nanocarriers.(6)

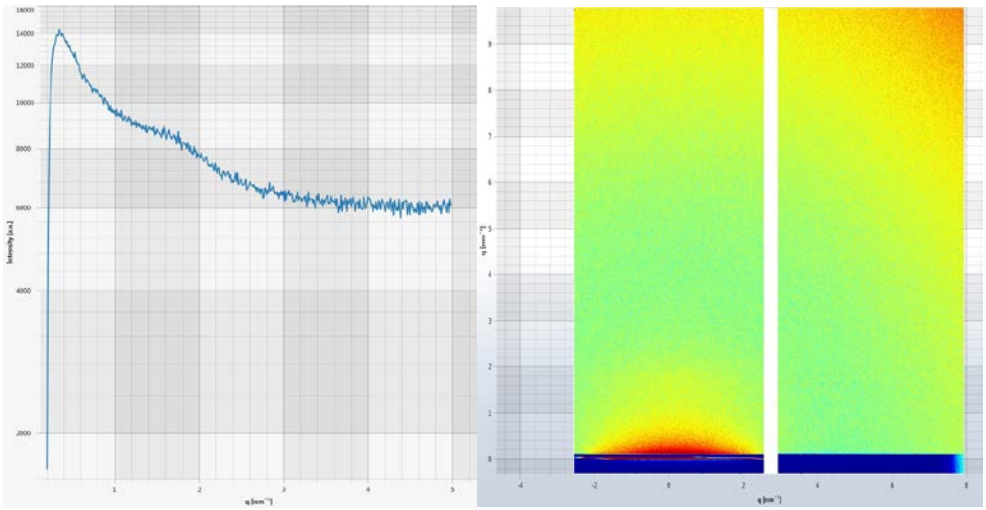
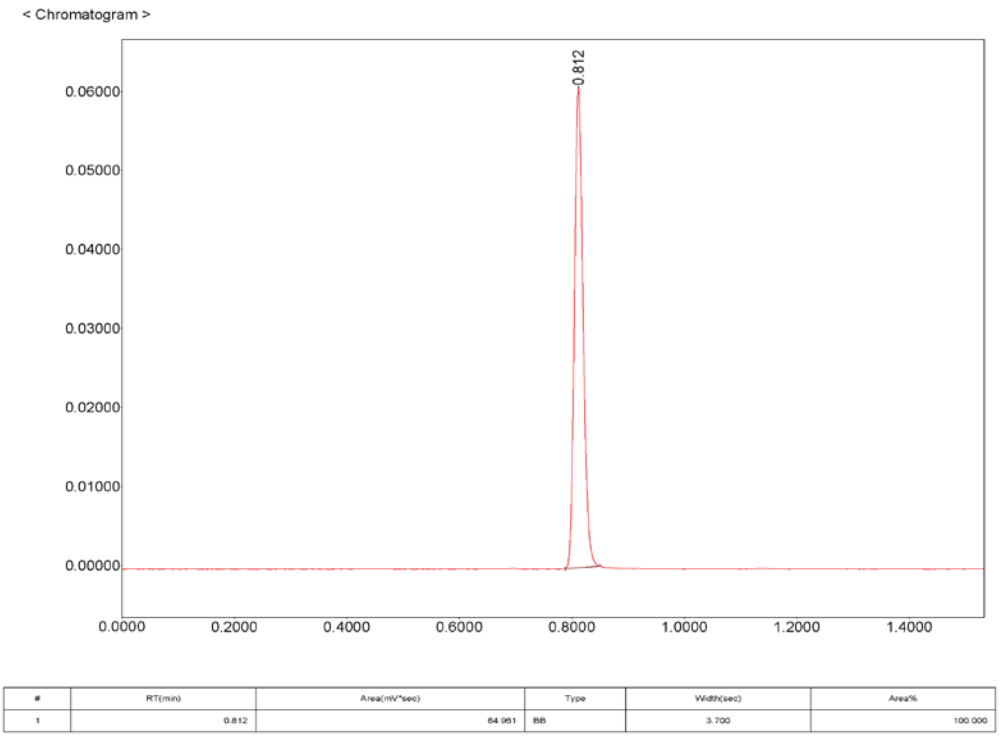


Figure 5.16: SAXS analysis of cubosomes of TAC

5.3.7.7. Headspace Gas Chromatography (HS-GC) testing for residual solvent

For the preparation of cubosomal dispersion of Tacrolimus, ethanol was utilised. For therapeutic purpose, the content of this organic should be in limit as defined by various regulatory bodies. In case of ethanol, the limited quantity is 1500 ppm according to the ICH guidelines Q3C (R6) for residual solvents.(17) Thus residual content of ethanol was calculated using HS-GC and was found to be 64.96 ppm which is significantly less than the permitted level ppm according to the ICH guidelines Q3C (R6) for residual solvents.(18)



#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	0.812	64.961	BB	3.700	100.000

Figure 5.17: Residual estimation of ethanol in cubosomes of TAC

5.3.7.8. In-vitro drug release

In vitro drug release from optimized cubosomes of TAC was evaluated in comparison with plain drug suspension. A cumulative percent release of drug at various time intervals are concise in Table 6.28 and in Fig. 6.18. Release data of cubosomes of TAC showed >70 % TAC release in 24 hrs while only 23.08 % drug was released from plain suspension in 24 hrs due to insufficient solubility of drug in phosphate buffer pH 7.4 indicating control release behavior of prepared cubosomes. Sink condition was maintained by using 1 % ethanolic phosphate buffer pH 7.4 as diffusion medium in receptor compartment. Various mathematical models were applied to a data of drug release from cubosomes and listed in table 6.29. The R^2 values for first order model was found higher suggesting a diffusion controlled system where release rate was dependent on remaining drug concentration within the cubosomes.(19) TAC is practically insoluble in water. Thus, when its suspension was prepared in phosphate buffer pH 7.4 and filled in diffusion bag, it was not able to solubilize in phosphate buffer pH 7.4. To permeate the

drug molecule across the dialysis bag it must be present in solubilized form. On other hand, cubosomes have advantage of improving surface area which is in contact with phosphate buffer pH 7.4. Thus, more amount of drug dissolved in a phosphate buffer pH 7.4 and diffuses to the receptor compartment. Due to this reason more amount of TAC was able to permeate the diffusion membrane and higher in-vitro release of TAC obtained in case of cubosomes of TAC.(20, 21)

Table 5.28: *In-vitro* drug release profile of TAC from drug suspension and prepared cubosomes of TAC

Time (h)	Cumulative percent drug release	
	TAC drug suspension	Cubosomes of TAC
0.5	2.85 ± 0.15	5.51 ± 0.11
1	3.85 ± 0.26	9.81 ± 0.11
1.5	5.09 ± 0.25	11.83 ± 0.18
2	6.04 ± 0.15	12.65 ± 0.15
3	7.73 ± 0.21	16.44 ± 0.53
4	8.93 ± 0.25	17.45 ± 0.35
5	10.28 ± 0.22	21.62 ± 0.42
6	11.57 ± 0.30	27.10 ± 0.76
7	13.44 ± 0.31	31.34 ± 0.84
8	15.16 ± 0.40	35.89 ± 1.30
9	16.70 ± 0.24	43.52 ± 1.16
10	17.27 ± 0.25	48.97 ± 0.74
11	18.44 ± 0.45	52.32 ± 2.24
12	19.53 ± 0.91	57.51 ± 2.21
24	23.08 ± 1.26	76.09 ± 3.59
48	23.40 ± 0.57	79.06 ± 3.97

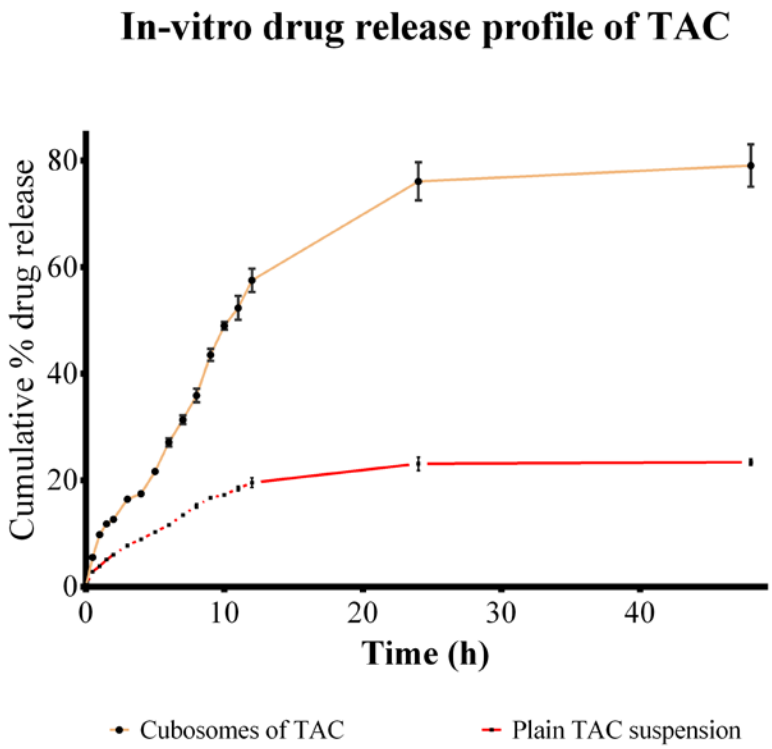


Figure 5.18: In-vitro drug release profile of TAC

Table 5.29: Various statistical model for release kinetic with their R^2 value

Statistical model	Cubosomes of FBX	
	R^2	n
Zero order	0.8762	-
First order	0.9769	-
Higuchi	0.9024	-
Korsmeyer Peppas	0.9656	0.715
Hixon Crowell	0.9750	-

5.4. References

1. Karami Z, Hamidi M. Cubosomes: remarkable drug delivery potential. Drug discovery today. 2016;21(5):789-801.

2. Patel B, Thakkar HP. Cubosomes: Novel Nanocarriers for Drug Delivery. Nanocarriers: Drug Delivery System: Springer; 2021. p. 227-54.

3. Sangshetti JN, Deshpande M, Zaheer Z, Shinde DB, Arote R. Quality by design approach: Regulatory need. Arabian Journal of Chemistry. 2017;10:S3412-S25.

4. Kumar L, Verma R. Determination of saturated solubility of propranololusing UV visible spectrophotometer. Der Pharmacia Lettre. 2016;8(17):196-201.

5. Parinaz S, Renata I, Ben J. Impact Of Preparation Method And Variables On The Internal Structure, Morphology, And Presence Of Liposomes In Phytantriol-pluronic (r) F127 Cubosomes. Colloids and Surfaces B: Biointerfaces. 2016:845-53.

6. Avachat AM, Parpani SS. Formulation and development of bicontinuous nanostructured liquid crystalline particles of efavirenz. Colloids and surfaces B: biointerfaces. 2015;126:87-97.

7. Chakraborty P, Dey S, Parcha V, Bhattacharya SS, Ghosh A. Design expert supported mathematical optimization and predictability study of buccoadhesive pharmaceutical wafers of loratadine. BioMed research international. 2013;2013.

8. Witschi C, Doelker E. Residual solvents in pharmaceutical products: acceptable limits, influences on physicochemical properties, analytical methods and documented values. European Journal of Pharmaceutics and Biopharmaceutics. 1997;43(3):215-42.

9. Innes A, Farrell A, Burden R, Morgan A, Powell R. Complement activation by cellulosic dialysis membranes. Journal of clinical pathology. 1994;47(2):155-8.

10. Sheshala R, Anuar NK, Samah NHA, Wong TW. In vitro drug dissolution/permeation testing of nanocarriers for skin application: A comprehensive review. AAPS PharmSciTech. 2019;20(5):1-28.

11. 10.7 - Detecting Multicollinearity Using Variance Inflation Factors [Available from: https://online.stat.psu.edu/stat462/node/180/.

12. Diagnostics Plots [Available from: https://www.statease.com/docs/v11/contents/analysis/diagnostics/diagnostics-plots/.

13. Samimi S, Maghsoudnia N, Eftekhari RB, Dorkoosh F. Lipid-based nanoparticles for drug delivery systems. Characterization and biology of nanomaterials for drug delivery. 2019:47-76.

14. Freitas C, Müller RH. Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN™) dispersions. International journal of pharmaceutics. 1998;168(2):221-9.

15. Salah S, Mahmoud AA, Kamel AO. Etodolac transdermal cubosomes for the treatment of rheumatoid arthritis: ex vivo permeation and in vivo pharmacokinetic studies. Drug delivery. 2017;24(1):846-56.

16. Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, et al. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics. 2018;10(2):57.

17. Agency EM, editor ICH guideline Q3C (R5) on impurities: guideline for Residual Solvents. Int Conf Harmon Tech Requir Regist Pharm Hum Use; 2015.

18. Guideline IHT. Impurities: Guideline for residual solvents Q3C (R5). Current Step. 2005;4:1-25.

19. Wu IY, Bala S, Škalko-Basnet N, Di Cagno MP. Interpreting non-linear drug diffusion data: Utilizing Korsmeyer-Peppas model to study drug release from liposomes. European Journal of Pharmaceutical Sciences. 2019;138:105026.

20. Ahirrao M, Shrotriya S. In vitro and in vivo evaluation of cubosomal in situ nasal gel containing resveratrol for brain targeting. Drug development and industrial pharmacy. 2017;43(10):1686-93.

21. Peng X, Zhou Y, Han K, Qin L, Dian L, Li G, et al. Characterization of cubosomes as a targeted and sustained transdermal delivery system for capsaicin. Drug design, development and therapy. 2015;9:4209.