6. Formulation Development of Cubosomes of Febuxostat

6.1. Introduction

Cubosomal technology is selected for the present study with an objective of achieving therapeutic plasma levels of FBX 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 FBX. A systematic Quality-by-design (QbD) approach employing statistical design of experiments was utilized to exhaustively evaluate influence of material traits and method parameters on critical formulation attributes.(3)

6.2. Materials and Methods

6.2.1. Materials

FBX and Glyceryl Monooleate were obtained as a gift sample from Ami drugs and specialty chemicals Pvt. 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. Preparation of double distilled water was performed in lab, filtered with 0.2µ membrane filter (store air tight container) and utilized within a maximum of 7 days.

6.2.2. Screening of formulation parameters for cubosomes of Febuxostat

6.2.2.1.Solubility(4):

FBX was solubilized in different solvents for performing solubility study of FBX and this study was based on the saturation solubility. For performing the study, FBX was added in incremental amount to 1 mL of the 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 UV spectrophotometer as described in chapter 3 section 3.5.2. after suitable dilution.

6.2.2.2.Selection of lipid:

Screening of lipids was carried out for preparation of nanocarrier i.e. cubosome. This was performed by checking the solubility of FBX in various lipids like Glyceryl Monooleate (GMO), and Glyceryl Monosterate (GMS). Solubility in these lipids were performed on hot plate magnetic stirrer above the melting point of selected lipids as described in section 6.2.2.1.

6.2.2.3.Selection of stabilizer

For the selection of stabilizer various characteristics were taken into account like vesicle size, % entrapment efficiency and stability of the prepared formulation. Different stabilizers like Polyvinyl alcohol (PVA), Poloxamer 188, and Poloxamer 407 were used to prepare formulation and further formulations were tested for the stability (for 10 days), vesicle size and % entrapment efficiency. For FBX, concentration of GMO and stabilizer were kept constant i.e. 5 % and 1.0 % w/v respectively.

6.2.2.4.Concentration of stabilizer

Concentration range of the stabilizer was chosen based on the vesicle size, % entrapment efficiency, and zeta potential of the prepared formulation. Here, for cubosomes of FBX, concentration of GMO was set at 5% w/v. Then, various batches were prepared having varying concentrations of PVA.

6.2.2.5.Concentration of lipid

Optimization of the conc. on range of lipid was based on % entrapment efficiency 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 concentration of 0.5 % w/v.

6.2.2.6.Selection of stirring speed

The effect of stirring speed was studied on the formulation wherein the stirring speed was ranged from 500 to 1500 rpm while the concentrations of GMO and PVA were kept constant.

6.2.2.7.Selection of stirring time

A effect of stirring time was studied on quality of formulation wherein stirring time was taken in the range of 5-30 minutes while the concentrations of GMO and PVA were kept constant.

6.2.2.8.Temperature

While keeping all other parameters constant, a temperature range of 50-80 was selected for solution of GMO and FBX in ethanol and solution of stabilizer during study.

6.2.2.9.Volume of organic phase

While all other parameters were kept constant a volume of organic phase to be added was varied in a range of 2-4 mL.

6.2.2.10. Rate of addition of organic phase

A rate of addition of organic phase in a range of 0.5-1.5 mL/min was selected for investigation keeping all the others variables constant.

6.2.3. Preparation and optimization of Febuxostat loaded cubosomes(1, 5)

Bottom up approach was utilized for preparation of Cubosomes of FBX which is shown in *fig. 6.1*. For the preparation of cubosomes of FBX 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 taken in glass beaker with 10 ml capacity and dissolved in 4 ml of ethanol, then 40 mg of FBX was added to it. For aqueous phase (B), Y mg of PVA was dissolved in 20 ml of water. Initially, both solutions were kept at a temperature of 50 °C and were continuously stirred for 5-10 min. Afterward, organic phase was added to aqueous phase in a drop-wise manner with continuous stirring and the addition of organic phase was maintained at rate of 1 mL/min and 500 rpm on magnetic stirrer. The resulting medium was continuously stirred for 15 min at room temperature using a magnetic stirrer. This medium was then introduced to the rotary evaporator at a temperature of 50 °C and 100 rpm under vacuum for

removing ethanol from the dispersion and the volume of the prepared batch was reduced to 10 mL. The resulting Cubosomal dispersion was exposed to centrifugation with 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 centrifuge tube. Finally, the resulting separated cubosomal dispersion was stored for utilization in future tests in glass vials at room temperature.

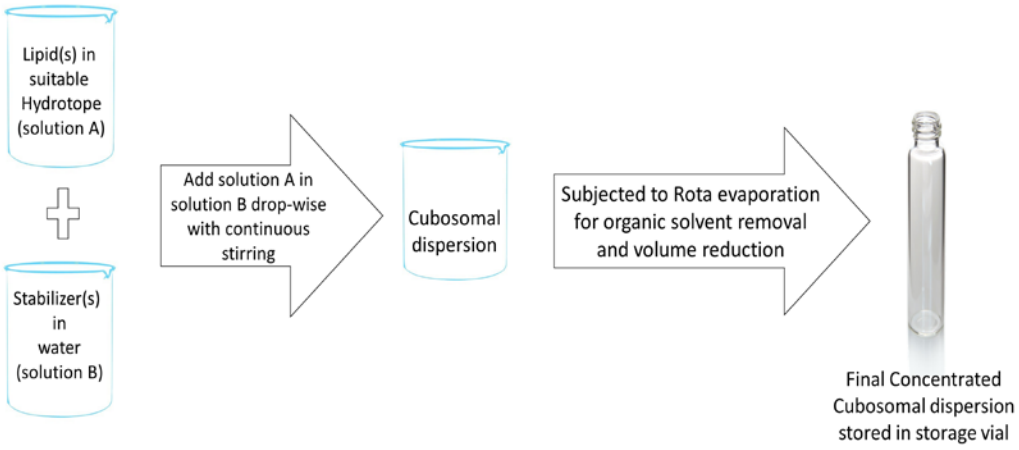


Figure 6.1: Bottom up approach for preparation of FBX loaded cubosomes

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

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

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

All possible variables which were linked with the development of FBX loaded cubosomes were demonstrated with the help of Ishikawa diagram. These factors were categorized as 'low, intermediate and high risk' on the basis of their predicted effect on CQA as described in Table 6.1.

Table 6.1: 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.

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

The quality of the product is affected by various moderate and high risk attributes. These parameters were screened for most significant attributes using randomized 2-level fractional factorial design developed by Design Expert 7.0.

6.2.6.1. Vesicle size and size distribution(5, 6)

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

6.2.6.2. % Entrapment efficiency(5, 6)

Free FBX was isolated from entrapped FBX for the estimation of % entrapment efficiency in cubosomes by centrifuging it at 6000 rpm for a period of 15 min at temp. of 25°C using Remi Centrifuge. Then, supernatant of the centrifuge tube which contains cubosomal dispersion of FBX was separated carefully without disturbing the hard pallet of free drug, which was formed at the

bottom of the centrifuge tube. Cubosomal dispersion of FBX was broken down using ACN:Methanol (9:1) for quantitative analysis of FBX. The absorbance of prepared sample was then calculated with UV visible spectrophotometer at wavelength of 315 nm as described in chapter 3. The % entrapment efficiency was calculated with the help of following formula:

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

Equation-6.1

6.2.7. Formulation and optimization of Febuxostat loaded cubosomes using 3^2 Factorial Design

For explicitly studying the relation between the vital factors and CQA of FBX 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 the 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 crucial factors and CQA was studied employing contour and 3-D response surface plots. For the verification of the model, three checkpoint batches were prepared.(3)

6.2.8. Preparation of checkpoint batches as per the overlay plot

After deriving and adding the data of the prepared batches based on the 3^2 factorial Design, the data was analyzed at 95 % confidence and prediction level by employing Design Expert 7.0 for optimized area. Checkpoint batches were prepared according to the three randomized points which were selected from the

above mentioned optimized area. The composition of these checkpoint batches are given in table 6.2.

Table 6.2: Composition of checkpoint batches

Sr. No.	Concentration GMO (% w/v)	Concentration of PVA (%w/v)	Predicted entrapment	Predicted vesicle size (nm)	Predicted PDI
1	7.93	0.96	92.03	174.9	0.218
2	8.34	1.29	97.51	188.51	0.206
3	6.63	1.37	83.74	147.74	0.181

6.2.9. In vitro characterization of optimized Febuxostat cubosomes

6.2.9.1.Shape and surface morphology(5, 6)

A shape and surface morphology of the FBX loaded cubosomes was evaluated with the help of Transmission electron microscopy. For performing the test, the dispersion was smeared on carbon-coated grid, and any extra material was removed and carbon-coated grid was dried at room temp. for a period of 5 hrs. Transmission electron microscope (CM 200, Philips, Netherlands) was employed the following process parameters i.e. the operating voltage was set in a range of 20-200 kV to visualize cubosomes at suitable magnification with an accelerating voltage of 20 kV.

6.2.9.2.Zeta potential(5, 6)

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

6.2.9.3.Vesicle size and size distribution(5, 6)

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

6.2.9.4.% Entrapment efficiency(5, 6)

The % entrapment efficiency of prepared formulation of cubosomes of FBX was found out as described in chapter 6 section 6.2.6.2.

6.2.9.5.Total drug content(5)

For estimation of total drug content of prepared formulation, 1 mL of the cubosomal dispersion which was eq. to 4 mg of FBX was precisely withdrawn and was dissolved in 10 mL of ACN. A prepared sample of FBX were then analyzed with UV visible spectrophotometer at wavelength of λ_{max} 315 nm as described in chapter 3 section 3.5.2.

$$\%\boldsymbol{Total\ drug\ content} = \frac{\boldsymbol{Amount\ of\ total\ drug\ found\ (entrapped + unentrapped)}}{\boldsymbol{Total\ drug\ added}} \boldsymbol{X100}$$

Equation-6.2

6.2.9.6.Small Angle X-rays Scattering(5, 6)

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 10 μm 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 at least 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.

6.2.9.7.Headspace Gas Chromatography (HS-GC) testing for residual solvent(8)

A. Standard preparation

In 10 ml volumetric flask, 0.13 mL of ethanol equivalent to 0.1 g 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 concentration of 1000 ppm.

B. Sample preparation

A volume of formulation (0.105 mL) equivalent to 0.1 g was taken in volumetric flask having 10 ml capacity and final volume was made up using DMF. From above solution 1 ml was shifted in volumetric flask with 10 ml capacity 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)

6.2.9.8.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 the glycerol followed by treatment with sodium sulphide solution (0.3% W/V) at 80 °C for a period of 1 minute to facilitate the removal of sulphur compounds. Further, it was rinsed using hot water maintained at a temp. of 60 °C for period of 2 minutes. Next, it was acidified using 0.2% v/v sulphuric acid solution and was subsequently washed with hot water for the elimination of acid. Then, the dialysis membrane was dipped overnight in the diffusion medium before *in-vitro* release study.(9)

(B) In-vitro drug release(6)

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, the donor compartment has a volume capacity of 20 mL. To perform an 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 (1 mL), and cubosomes of FBX (1 mL) both equivalent to 3.4 mg were placed 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 the identical volume (1.0 ml) was replaced by a fresh diffusion medium. Samples were analyzed with the help of method developed for UV visible spectrophotometer as described in chapter 3. For the above mentioned experiments, three readings were taken and further the average of these three values was considered.

6.3. RESULTS AND DISCUSSION

6.3.1. Screening of formulation parameters for the cubosomes of FBX

6.3.1.1.Solubility

As shown in fig. 6.2. that FBX has maximum solubility in Dimethyl Formamide i.e. 56.96 ± 5.14 mg/mL while minimal solubility in Acetonitrile 7.34 ± 0.57 mg /mL. Moreover, it has a solubility of 48.04 ± 3.91 mg/mL and 32.81 ±2.50 mg/mL in GMO and Glyceryl monostearate (GMS) respectively.

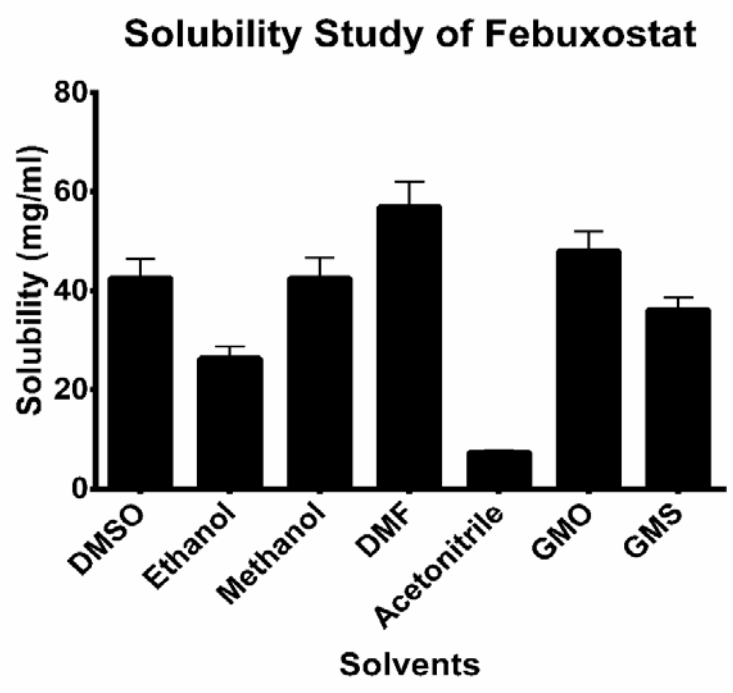


Figure 6.2: Solubility study of FBX

6.3.1.2.Selection of lipid

On the basis of the solubility of FBX in the lipids [Glyceryl Monooleate (GMO), Glyceryl Monosterate (GMS)], screening of the lipid was carried out for the preparation of nanocarrier i.e. cubosome. It was reported that FBX has the maximum solubility in GMO i.e. 48.04 ± 3.91 mg/mL. Therefore, GMO is selected for further process.

6.3.1.3.Selection of stabilizer

Table 6.3: Screening of stabilizers for FBX

Batch No.	Name of stabilizer	Conc. of stabilizer (% w/v)	Conc. GMO (%w/v)	% EE	Vesicle size (nm)	Stability
1	PVA	1.0	2	35.52±3.61	141.58±15.68	Stable
2	Poloxamer 407	1.0	2	29.76 ± 2.86	164.26 ± 23.54	Stable
3	Poloxamer 188	1.0	2	26.4 ± 3.28	635.71 ± 62.81	Unstable

From the data reported in table 6.3, it can be concluded that all formulations are stable except batch 3 which contains poloxamer 188. Further, there was no significant change reported in the vesicle size of the prepared formulations. However, we can see noticeable difference in the entrapment efficiency of the prepared formulation. In case of PVA, maximum entrapment efficiency found out was 35.52 %, thus it is selected for further development.

6.3.1.4.Selection of concentration of stabilizer

For the selection of concentration range of stabilizer, batches were prepared wherein the concentration of PVA was kept in a range of 25-1.5% w/v and the concentration of GMO was kept at a constant of 5% w/v as shown in table 6.4. From the data collected, concentration ranging from 0.5 to 1.5% w/v of stabilizer was selected for optimization of cubosomes of FBX due to the better entrapment and vesicle size of prepared batches. As the concentration of PVA increases it improves the stability of prepared cubosomal dispersion, hence improved the vesicle size and % entrapment.

Table 6.4: Selection of concentration of stabilizer for cubosomes of FBX

Batch No.	Concentration of GMO (% w/v)	Concentration of PVA (%w/v)	% EE	Vesicle size (nm)
1	5.0	0.25	12.09 ± 2.06	804.36 ± 69.27
2	5.0	0.5	37.16 ± 3.68	304.04 ± 41.38
3	5.0	1.0	61.39 ± 2.68	154.9 ± 17.68
4	5.0	1.5	66.28 ± 4.92	180.24 ± 23.68

6.3.1.5.Selection of concentration of lipid

For the selection of concentration range of the lipid, batches were prepared wherein the concentration of GMO was kept in a concentration ranging from 1-

10% w/v and the concentration of PVA was kept at a constant of 0.5% w/v as shown in table 6.5. From the data collected, a concentration range of 5.0 to 10% w/v of GMO was selected for the optimization of cubosomes of FBX.

Table 6.5: Selection of concentration of GMO for cubosomes of FBX

Batch No.	Conc. of PVA (% w/v)	Conc. of GMO (%w/v)	% EE	Vesicle size (nm)
1	0.5	1.0	9.16 ± 1.84	170.77 ± 16.9
2	0.5	3.0	21.07 ± 3.57	206.05 ± 20.74
3	0.5	5.0	34.86 ± 1.68	296.49 ± 15.26
4	0.5	7.5	56.21 ± 3.74	426.49 ± 42.98
5	0.5	10.0	64.08 ± 4.06	539.4 ± 35.97

6.3.1.6.Selection of stirring speed

Various batches of cubosomes of FBX were prepared as shown in table 6.6 wherein the concentration of GMO and stabilizer was kept constant i.e. 5.0 %w/v and 1.0 %w/v respectively at varying stirring speed. From the obtained data, it was concluded that stirring speed does not have any significant impact on the vesicle size and % entrapment of a drug in cubosomes of FBX. Thus, lowest stirring (500 rpm) was selected for the preparation of cubosomal dispersion.

Table 6.6: Selection of stirring speed for preparation of cubosomes of FBX

Batch No.	Stirring Speed (RPM)	% EE	Vesicle size (nm)
1	500	59.16 ± 3.18	161.6 ± 12.3
2	1000	62.08 ± 2.94	159.2 ± 21.84
3	1500	60.29 ± 4.32	150.1 ± 15.68

6.3.1.7.Selection of stirring time

Various batches of cubosomes of FBX were prepared as shown in table 6.7 wherein the concentration of GMO and stabilizer were kept constant i.e. 5.0% w/v and 1.0% w/v. Further, the stirring speed was set at 500 rpm. Different

stirring time were taken for all these prepared batches as shown in table 6.7. From the data collected, it was concluded that stirring time does not have significant impact on the vesicle size and % entrapment of a drug in cubosomes of FBX. Thus lowest stirring time was selected to prepare cubosomal dispersion which is 15 min.

Table 6.7: Selection of stirring time for preparation of cubosomes of FBX

Batch No.	Stirring Time (min)	% EE	Vesicle size (nm)
1	15	60.99 ± 4.20	150.7 ± 21.15
2	17.5	61.12 ± 3.66	156.4 ± 17.28
3	20	59.28 ± 2.57	161.5 ± 15.35

6.3.1.8.Selection of temperature

Various batches of cubosomes of FBX were prepared as shown in table 6.8 wherein the concentration of GMO and stabilizer were kept constant i.e. 5.0% w/v and 1.0% w/v. Further, stirring speed and stirring time were kept at 500 rpm and 15 min respectively. Various temperature were selected to prepare various batches as shown in table 6.8. During the preparation of cubosomal dispersion, temperature of organic phase and aqueous phase was maintained as shown in table 6.8. From the collected data, it was concluded that the temperature does not have any significant impact on the vesicle size and % entrapment efficiency of a drug in cubosomes of FBX. Thus, for preparing cubosomal dispersion, lowest temperature of organic and aqueous phase was maintained i.e. 50 °C.

Table 6.8: Selection of temperature for preparation of cubosomes of FBX

Batch No.	Temperature °C	% EE	Vesicle size (nm)
1	50	62.09 ± 3.84	156.8 ± 15.64
2	60	59.88 ± 3.58	160.8 ± 13.37
3	70	63.15 ± 2.91	165.7 ± 18.54

6.3.1.9.Selection of volume of organic phase

Various batches of cubosomes of FBX were prepared as shown in table 6.9 wherein the concentration of GMO and stabilizer were kept constant i.e. 5.0% w/v and 1.0% w/v. Further, stirring speed and stirring time were set at 500 rpm and 15 min respectively. Additionally, temperature was set at 50°C. Different volumes of organic phase were taken for the prepared batches as shown in table 6.9. From the data collected, an inference was drawn that volume of organic phase does not have any significant impact on the vesicle size and entrapment of a drug in cubosomes of FBX. However, in case of batch no. 1, creaming of prepared formulation occurred. Thus, 4 mL volume of organic phase was kept constant for preparing further batches of cubosomal dispersion.

Table 6.9: Selection of volume of organic phase for preparation of cubosomes of FBX

Batch No.	Volume of organic phase (mL)	% EE	Vesicle size (nm)
1	2	60.2 ± 4.09	149.5 ± 14.82
2	4	63.29 ± 3.55	153.8 ± 16.22
3	6	61.44 ± 2.3	162.2 ± 18.34

6.3.1.10. Selection of rate of addition of organic phase

Various batches of cubosomes of FBX were prepared as shown in table 6.10 wherein the concentration of GMO and stabilizer were kept constant i.e. 5.0% w/v and 1.0% w/v respectively. Further, stirring speed and stirring time were set at 500 rpm and 15 min respectively. Additionally, temperature was set at 50°C. 4 ml of the organic phase was taken. Different rate of addition of organic phase was taken for the prepared batches as shown in the table 6.10. From the data collected, it was concluded that the rate of addition of organic phase does not have any significant impact on the vesicle size and entrapment of a drug in cubosomes of FBX. 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 the aqueous phase was kept at 1.0 mL/min for preparing further batches of cubosomal dispersion.

Table 6.10: Selection of rate of addition of organic phase for preparation of cubosomes of FBX

Batch No.	Rate of addition of organic phase (mL/min)	% EE	Vesicle size (nm)
1	0.5	64.52 ± 2.68	161.7 ± 14.92
2	1.0	59.63 ± 3.21	155.8 ± 19.16
3	1.5	60.34 ± 2.66	149.2 ± 13.08

6.3.2. Preparation and optimization of FBX loaded cubosomes

6.3.2.1.Establishing QTPP and CQA

Various OTPP elements and CQA with their target and justification are mentioned in table 6.11. 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 6.11: QTPP and CQA elements with justification for cubosomes of FBX

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	Minimize	To ensure formation

	index (PDI)#	(<0.3)	monodisperse formulation
	Zeta potential	> ± 30 mV	For the enhancement of stability of the dispersion
	Shape, Surface characteristic	Cubical, smooth	To ensure formation of cubosomes
	% entrapment efficiency#	Maximize	For the reduction of cost, drug wastage to be reduced
	In-vitro drug release	For 24 hrs	To ensure controlled release of drug to maintain plasma drug concentration
Ex-vivo permeability		Better transdermal flux	For achieving equivalent or better pharmacokinetic (PK)/ pharmacodynamics (PD) when compared to the marketed formulation.
Stability		Not less than 3 month	For ensuring prepared formulation's stability during its complete shelf life.
Safety		Non-toxic and non-irritant to skin	For ensuring prepared formulation's stability
Pharmacokinetic		Similar or better than oral suspension and marketed product	To fulfill bioequivalence requirement
Pharmacodynamic			For the illustration of therapeutic efficacy

Critical Quality Attributes (CQAs)

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

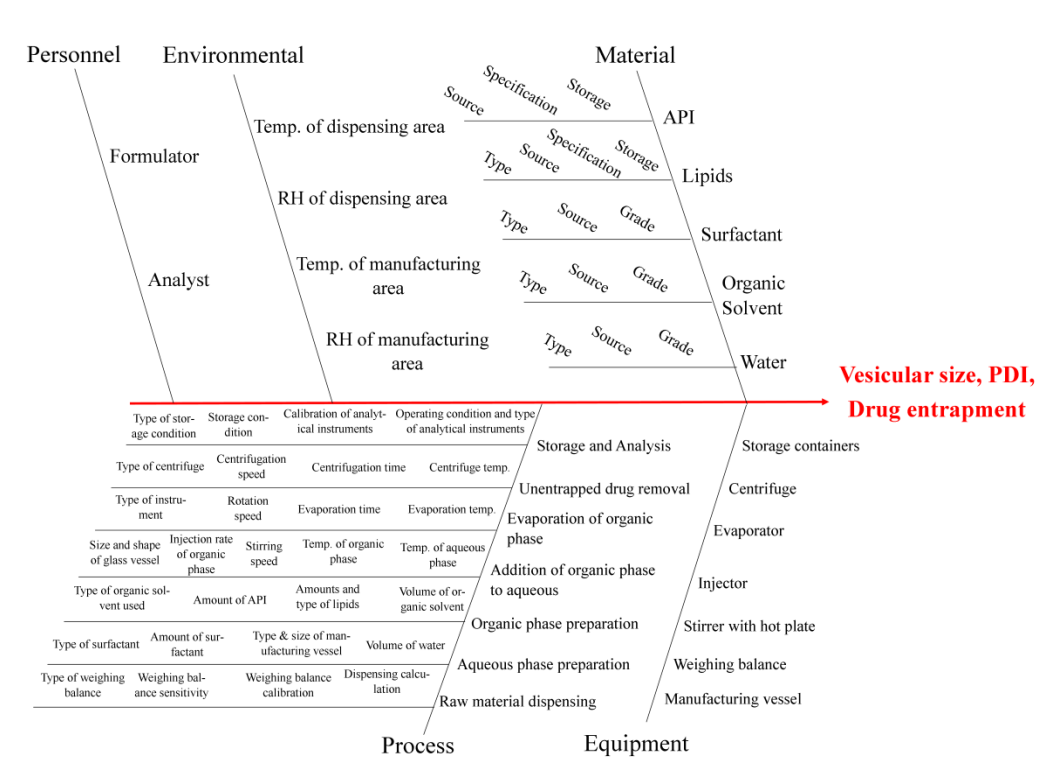


Figure 6.3: Ishikawa diagram showing probable variables that may influence the CQA

During the various brainstorming sessions, all likely variables related with development of FBX loaded cubosomes by bottom up approach were recognized and were categorized into these categories i.e. Material, Process, Equipment, Personnel and Environment.(3) An ishikawa diagram was constructed for the illustrating the cause and effect relation between identified variables and CQA (Fig. 6.3). Figure 6.3 shows all the variables that affect the product quality in terms of vesicular size, PDI, % entrapment efficiency. Among these variables, most significant variables that affect the product quality were separated using 2-level fractional factorial design.

Table 6.12: 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 sensitivity	Dispensing	Low risk	1 mg
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	50 °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.

6.3.4. 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 the independent variables were decided dependent on the preliminary trials and are mentioned in Table 6.12. Randomized design matrix of 30 experimental batches was generated using Design Expert 7.0 statistical software and presented in Table 6.13 & 6.14. The data was statistically processed by Design expert to generate reduced equation for all the CQA(s) (vesicle size, PDI, % entrapment efficiency) wherein the value of $P < 0.05$ was taken as a level of significance.

Table 6.13: 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	5	10
B	Concentration of surfactant	% w/v	0.5	1.5
C	Stirring speed	Rpm	500	1500
D	Stirring time	Min	5	30
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 6.14: 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
B1	22	-1	-1	-1	+1	-1	-1	+1	306.8	42.67	0.394

B2	23	-1	+1	-1	-1	+1	-1	-1	181.3	59.38	0.168
B3	14	-1	+1	+1	+1	-1	-1	+1	176.5	61.08	0.184
B4	2	-1	-1	+1	-1	-1	-1	+1	296.4	40.29	0.401
B5	4	+1	+1	+1	+1	+1	+1	+1	335.6	92.84	0.274
B6	11	-1	-1	-1	-1	+1	+1	-1	315.5	43.91	0.378
B7	19	+1	+1	-1	-1	-1	+1	-1	307.1	93.04	0.284
B8	24	-1	+1	-1	+1	+1	+1	-1	186.2	56.8	0.173
B9	1	+1	-1	+1	-1	+1	-1	-1	452.6	73.81	0.501
B10	21	+1	+1	-1	+1	-1	+1	+1	294	90.82	0.295
B11	13	-1	-1	-1	+1	-1	+1	-1	312.8	39.4	0.411
B12	25	-1	-1	-1	+1	+1	+1	+1	297.1	43.81	0.386
B13	18	-1	-1	+1	+1	+1	-1	-1	316.3	42.01	0.39
B14	27	+1	-1	-1	+1	+1	-1	-1	439.1	69.44	0.523
B15	8	+1	+1	-1	+1	-1	-1	-1	289	94.26	0.325
B16	6	-1	+1	+1	-1	-1	-1	-1	176.5	58.93	0.201
B17	29	+1	+1	-1	-1	+1	+1	+1	311.6	93.09	0.306
B18	30	+1	+1	+1	-1	-1	+1	+1	324	90.91	0.295
B19	7	-1	+1	+1	-1	+1	+1	-1	175.5	60.44	0.179
B20	20	+1	+1	+1	+1	+1	-1	-1	312.5	93.07	0.312
B21	5	+1	-1	-1	-1	+1	-1	+1	444.6	69.48	0.483
B22	26	+1	-1	-1	-1	-1	+1	+1	437.3	72.19	0.502
B23	12	+1	-1	+1	+1	+1	+1	-1	423.5	69.38	0.516
B24	17	-1	-1	+1	-1	-1	+1	-1	289.1	37.06	0.384
B25	28	+1	-1	+1	+1	-1	-1	-1	409.1	70.18	0.499
B26	16	-1	+1	+1	-1	+1	-1	+1	169	57.16	0.169
B27	10	+1	+1	-1	-1	-1	-1	+1	333.1	89.07	0.296
B28	3	-1	-1	+1	+1	-1	+1	+1	279.8	41.6	0.406
B29	9	+1	-1	+1	+1	+1	-1	+1	428.3	65.28	0.432
B30	15	-1	+1	-1	-1	-1	+1	+1	193.2	62.33	0.196

It is clearly visible from the Pareto and normal charts (Fig. 6.4) that factors having major effect on vesicle size and PDI of the prepared cubosomes were concentration of lipids and surfactant. Likewise, the concentration of lipid has major effect on the % entrapment efficiency. On the basis of the observation made, inference was drawn that concentration of lipid and surfactant will be considered as CMA in the concluding optimization step.

The impact of injection rate, stirring speed, stirring time, organic solvent volume, and temperature of both phases were found insignificant on both CQA. Hence, median level of these factors (injection rate, 1.0 mL/min; stirring speed, 500 rpm, stirring time 15 min, temperature of both phases 50 °C and organic solvent volume, 4 mL) were selected during the optimization.

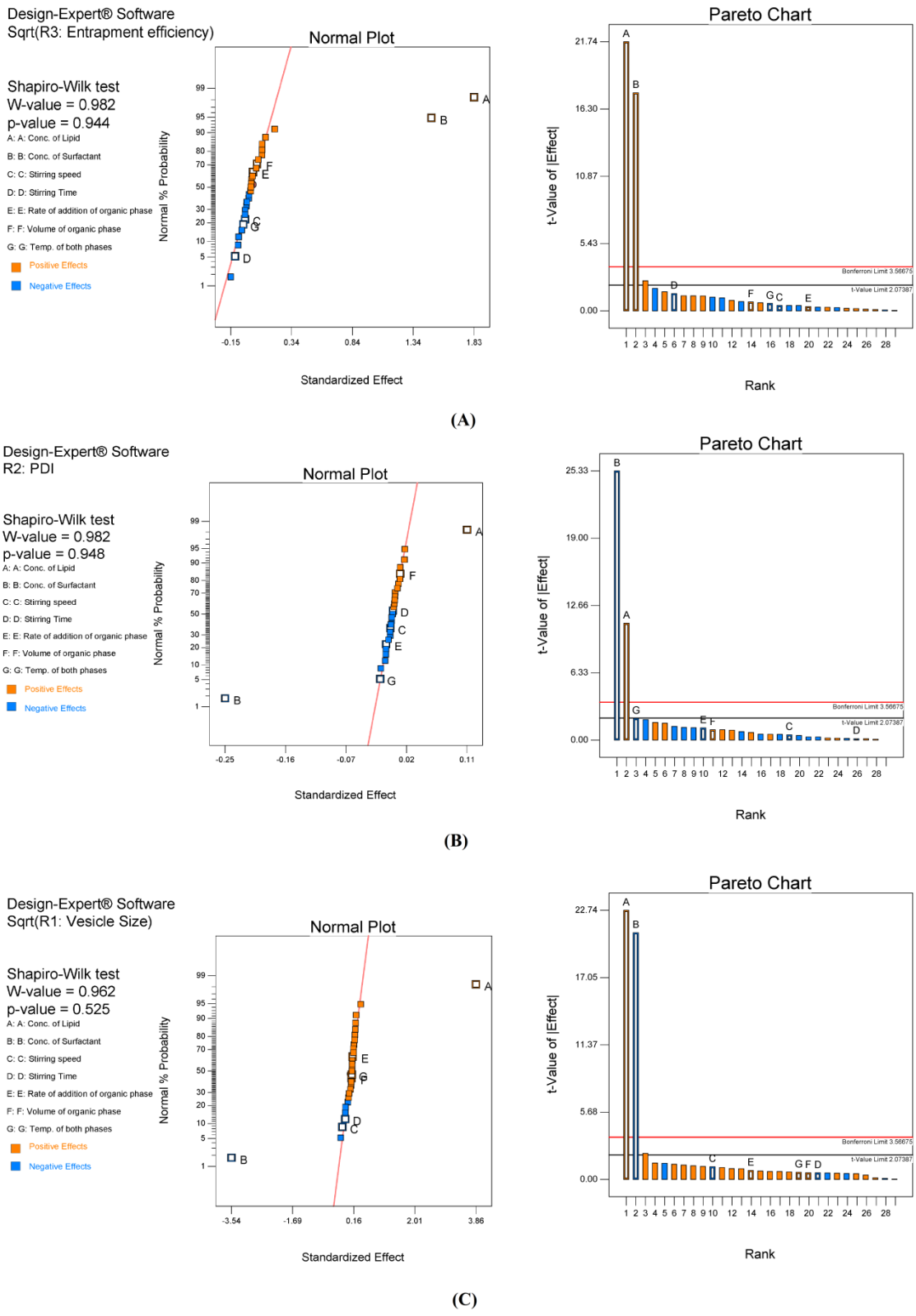


Figure 6.4: Pareto and normal plots for (A): % Entrapment efficiency, (B) PDI and (C) Vesicle size

6.3.5. Formulation optimization using 3^2 Factorial Design

Based on 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 6.15.

Table 6.15: 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	5	7.5	10

A randomized matrix of thirteen batches was generated by Design-Expert 7.0 and presented in Table 6.16. These batches were formulated according to their run order and were assessed for CQA using the methods described earlier. Table 6.16 also represents the resulting CQA of these batches.

Table 6.16: 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	1.00	7.50	89.81	165.4	0.186
7	2	0.50	10.00	75.93	429.4	0.480
12	3	1.00	7.50	89.37	158.6	0.203
8	4	1.00	10.00	89.47	289.4	0.274
5	5	1.00	7.50	90.83	153.1	0.191
3	6	1.50	5.00	55.77	174.6	0.177
11	7	1.00	7.50	91.61	145.2	0.215
13	8	1.00	7.50	93.1	141.8	0.194
4	9	0.50	7.50	68.65	322.8	0.383

1	10	0.50	5.00	35.68	280.7	0.344
6	11	1.50	7.50	88.39	199.2	0.244
9	12	1.50	10.00	96.31	328.8	0.281
2	13	1.00	5.00	53.08	135.9	0.208

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 6.17 & 6.18, 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 the model and obtain reduced equation.

Table 6.17: Analysis of variance of full quadratic model for % entrapment efficiency

Source	**Sum of squares**	**Df**	**Mean square**	**F value**	**p-value Prob > F**	
Model	4369.23	5	873.85	125.39	< 0.0001	Significant
A: Conc. of PVA	604.21	1	604.21	86.70	< 0.0001	Significant
B: Conc. of GMO	2288.53	1	2288.53	328.38	< 0.0001	Significant
AB	0.021	1	0.021	$3.017 \text{ X } 10^{-3}$	0.9577	Not Significant
A^2	224.22	1	224.22	32.17	0.0008	Significant
B^2	729.78	1	729.78	104.72	< 0.0001	Significant
Residual	48.78	7	6.97			
Lack of fit	39.92	3	13.31	6.00	0.0581	Not Significant
Pure error	8.87	4	2.22			
Cor total	4418.01	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 the adequacy of the model in justifying the difference in the observations.

Table 6.18: 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	89.97	1.10	-	89.97	1.10	-
A: Conc. of PVA	10.04	1.08	1.00	10.04	1.08	1.00
B: Conc. of GMO	19.53	1.08	1.00	19.53	1.08	1.00
AB	0.072	1.32	1.00	**Not Significant**		
A^2	-9.01	1.59	1.17	-9.01	1.59	1.17
B^2	-16.26	1.59	1.17	-16.26	1.59	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.(11) Regression equations for full and reduced models in coded units are shown as Eq. 6.3, and 6.4 while in un-coded form shown in Eq. 6.5. The (+) and (-) symbol preceeding every coefficient indicates a direct or inverse connection of that model term with the % entrapment efficiency of prepared batches.

Full model:

$$\% \text{ Entrapment efficiency} = 89.97 + 10.04A + 19.53B + 0.072AB - 9.01A^2 - 16.26B^2$$

Equation 6.3

Reduced model:

$$\% \text{ Entrapment efficiency} = 89.97 + 10.04A + 19.53B - 9.01A^2 - 16.26B^2$$

Equation 6.4

Reduced model in uncoded unit:

$$\% \text{ Entrapment efficiency} = -170.59 + 91.72 (\text{conc. of PVA}) + 46.77 (\text{conc. of GMO}) - 36.04 (\text{conc. of PVA})^2 - 2.60 (\text{conc. of GMO})^2$$

Equation 6.5

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 FBX. 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 6.17.

Based on the experimental data of **vesicle size**, quadratic model was suggested by the software for mix order and linear model for process order. Analysis of variance (ANOVA) was performed by the software for suggested models for vesicle size is mentioned in table 6.19. 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 the model and obtain reduced equation.

Table 6.19: Analysis of variance of full quadratic model for vesicle size

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	$1.062 \times e^5$	5	21242.93	206.68	< 0.0001	Significant
A: Conc. PVA	18183.01	1	18183.01	176.91	< 0.0001	Significant

B: Conc. GMO	34716.83	1	34716.83	337.78	< 0.0001	Significant
AB	7.56	1	7.56	0.074	0.7940	Not Significant
A^2	27483.03	1	27483.03	267.40	< 0.0001	Significant
B^2	7297.82	1	7297.82	71.00	< 0.0001	Not Significant
Residual	719.46	7	102.78			
Lack of fit	348.21	3	116.07	1.25	0.4027	Not Significant
Pure error	371.25	4	92.81			
Cor total	1.069 x e^5	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 the adequacy of the model in justifying the difference in the observations.

Table 6.20: 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	155.23	4.21	-	155.23	4.21	-
A: Conc. of PVA	-55.05	4.14	1.00	-55.05	5.14	1.00
B: Conc. of GMO	76.07	4.14	1.0	76.07	4.14	1.00
AB	1.37	5.07	1.00	**Not significant**		
A^2	99.75	6.10	1.17	99.75	6.10	1.17
B^2	51.40	6.10	1.17	51.40	6.10	1.17

Coefficients table for vesicle size of FBX 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 coded units are presented as Eq. 6.6 and 6.7 while in un-coded form shown in Eq. 6.8, respectively. The (+) and (-) symbol preceding every coefficient symbolizes a direct or inverse connection of that model term with vesicle size of prepared cubosomal batches.

Full model:

$$\text{Vesicle Size} = 155.23 - 55.05\ A + 76.07\ B - 1.37\ AB + 99.75\ A^2 + 51.40\ B^2$$

Equation 6.6

Reduced model:

$$\text{Vesicle Size} = 155.23 - 55.05\ A + 76.07\ B + 99.75\ A^2 + 51.40\ B^2$$

Equation 6.7

Reduced model in uncoded unit:

$$\text{Vesicle Size} = 907.02 - 916.38\ (\text{conc. of PVA}) - 94.04\ (\text{conc. of GMO}) + 399.01\ (\text{conc. of PVA})^2 + 8.22\ (\text{conc. of GMO})^2$$

Equation 6.8

From the equation 6.8, it can be concluded that concentration of PVA has a negative effect on the 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 6.6, interaction AB was removed because it has a p value greater than 0.05 as shown in table 6.19.

Based on the experimental data of **polydispersity index (PDI)**, quadratic model was suggested by the software for mix order and linear model for process order. Analysis of variance (ANOVA) was performed by the software for suggested models for PDI is presented in table 6.21. 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 the model and obtain reduced equation.

Table 6.21: Analysis of variance of full quadratic model for PDI

Source	Sum of squares	Df	Mean square	F value	p-value Prob > F	
Model	0.098	5	0.020	45.03	< 0.0001	Significant
A: Conc. of PVA	0.043	1	0.043	97.75	< 0.0001	Significant
B: Conc. of GMO	0.016	1	0.016	35.89	< 0.0005	Significant
AB	2.56×10^{-4}	1	2.56×10^{-4}	0.59	0.4680	Not Significant
A^2	0.027	1	0.027	61.28	< 0.0001	Significant
B^2	1.82×10^{-3}	1	1.82×10^{-3}	4.20	0.0795	Not Significant
Residual	3.04×10^{-3}	7	4.35×10^{-4}			
Lack of fit	2.52×10^{-3}	3	8.4×10^{-4}	6.43	0.0520	Not Significant
Pure error	5.22×10^{-4}	4	1.91×10^{-4}			
Cor total	0.10	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 competence of the model in justifying the difference in the observations.

Table 6.22: 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	0.20	8.66×10^{-3}	-	0.20	8.66×10^{-3}	-
A: Conc. of PVA	-0.084	8.51×10^{-3}	1.00	-0.084	8.51×10^{-3}	1.00
B: Conc. of GMO	0.051	8.51×10^{-3}	1.0	0.051	8.51×10^{-3}	1.00
AB	-8×10^{-3}	0.010	1.00	**Not significant**		
A^2	0.098	0.013	1.17	0.098	0.013	1.17
B^2	0.026	0.055	1.17	**Not Significant**		

Coefficients table for PDI of FBX 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 coded units are presented as Eq. 6.9 and 6.10 while in un-coded form shown in Eq. 6.11, respectively. The (+) and (-) symbol preceding every coefficient indicates a direct or inverse relationship of that model term with PDI.

Full model:

$$PDI = + 0.20 - 0.084\ A + 0.051\ B - 8.0X10^{-3}\ AB + 0.098\ A^2 + 0.026\ B^2$$

Equation 6.9

Reduced model:

$$PDI = + 0.20 - 0.084\ A + 0.051\ B + 0.098\ A^2$$

Equation 6.10

Reduced model in uncoded unit:

$$PDI = +0.80 - 0.91\ (\text{conc. of PVA}) - 0.035\ (\text{conc. of GMO}) + 0.39\ (\text{conc. of PVA})^2$$

Equation 6.11

From the eq. 6.11, 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. 6.11, also indicates absence of interaction effects of AB and BB as its p value is greater than 0.05 as shown in table 6.21.

Model summary for all independent variables are presented in Table 6.23. A low SD value and high R^2 value symbolized a 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 6.23: Summary of full quadratic model for all independent variables

Responses	Full model				
	SD	**Mean**	**R-sq**	**R-sq (adjusted)**	**R-sq (predicted)**
% Entrapment efficiency	2.64	78.31	0.9890	0.9811	0.9314
Vesicle size	10.14	224.99	0.9933	0.9885	0.9684
Polydispersity index (PDI)	0.021	0.26	0.9698	0.9483	0.7683

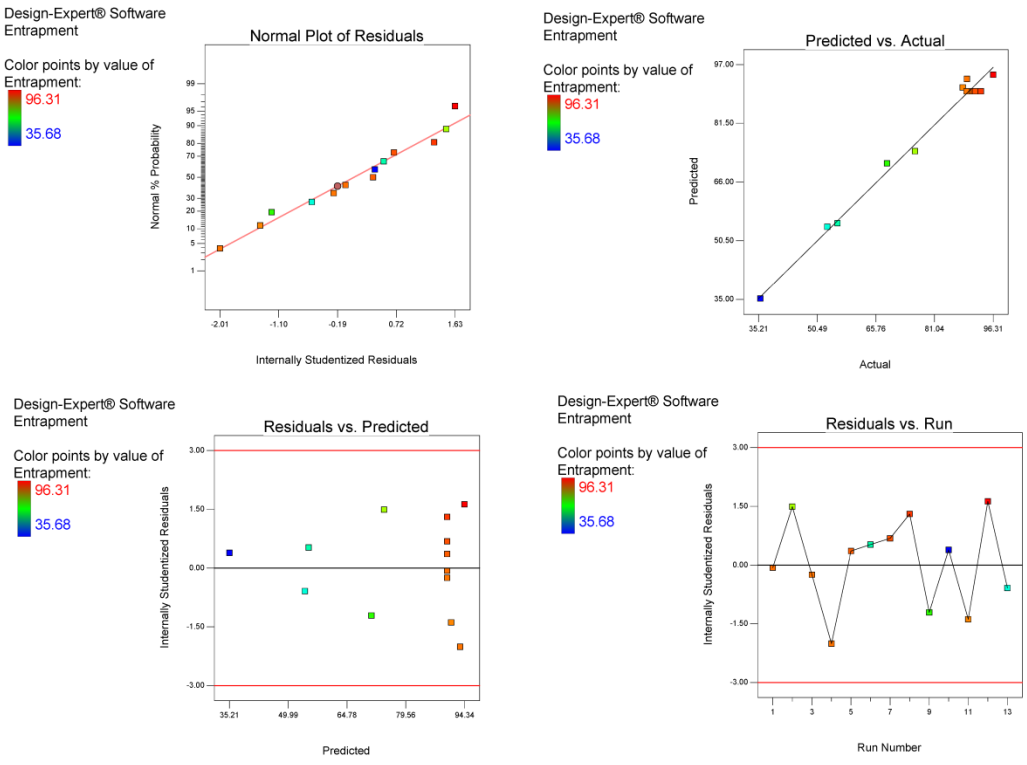


Figure 6.5: Residuals plots for % entrapment efficiency

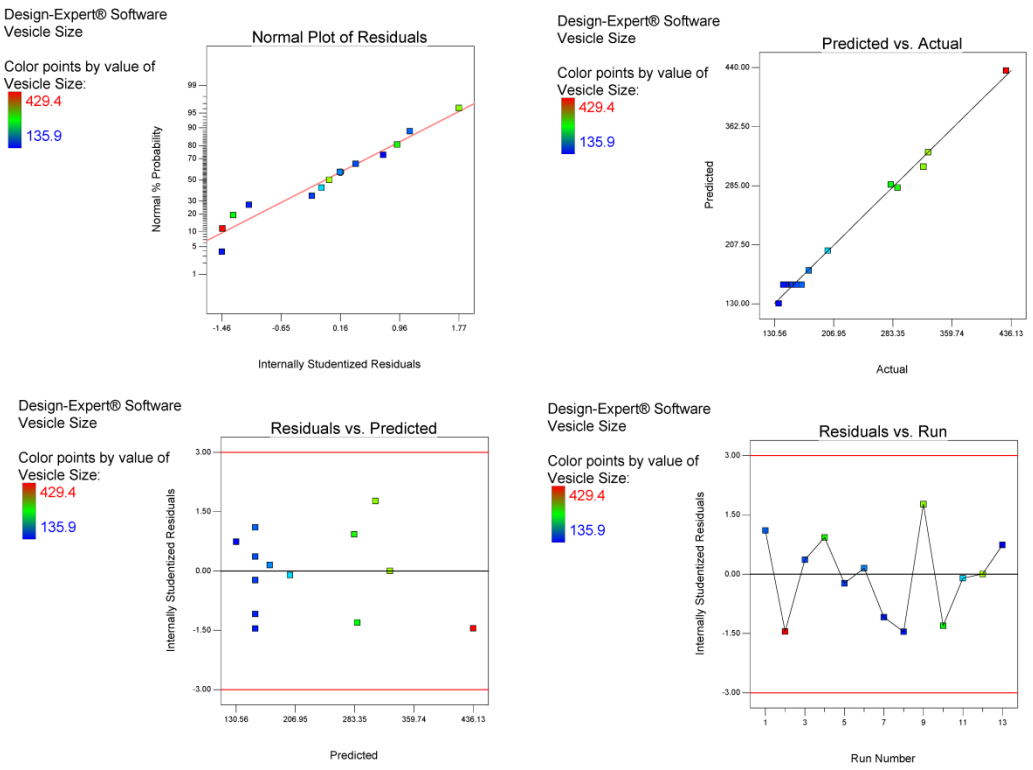


Figure 6.6: Residuals plots for vesicle size

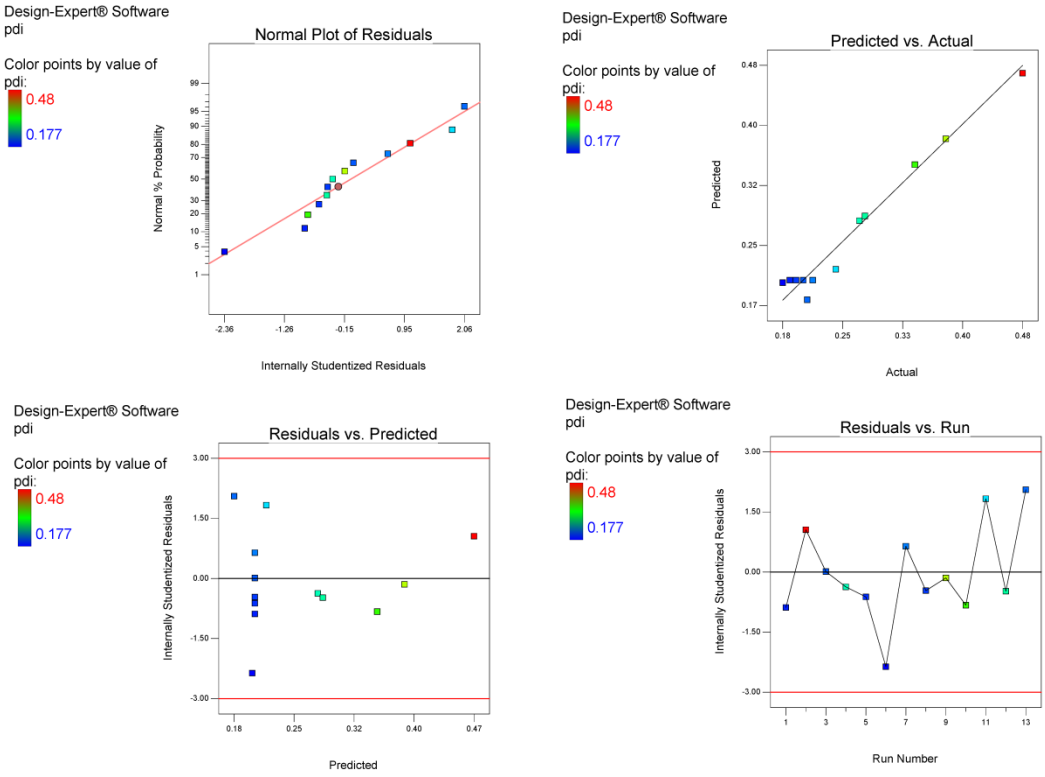


Figure 6.7: Residuals plots for Polydispersity index (PDI)

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. 6.5, 6.6 and 6.7. It was observed that the data was normally distributed n 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. 6.8, 6.9 and 6.10 respectively. These graphs were used to depict how the CQA is related to respective independent factor while keeping other independent variable at constant levels.

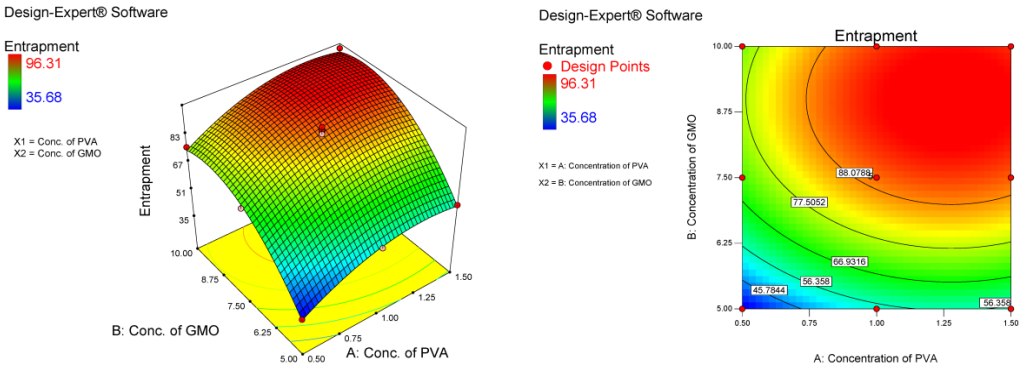


Figure 6.8: Contour and surface response plot of % entrapment efficiency

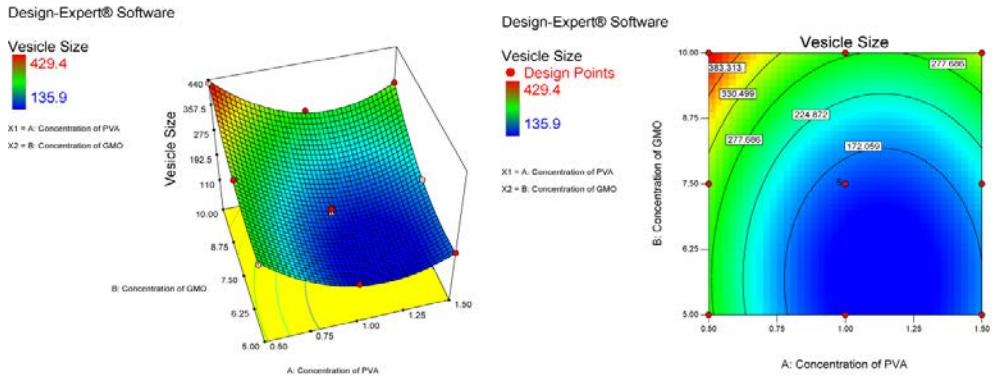


Figure 6.9: Contour and surface response plot of vesicle size

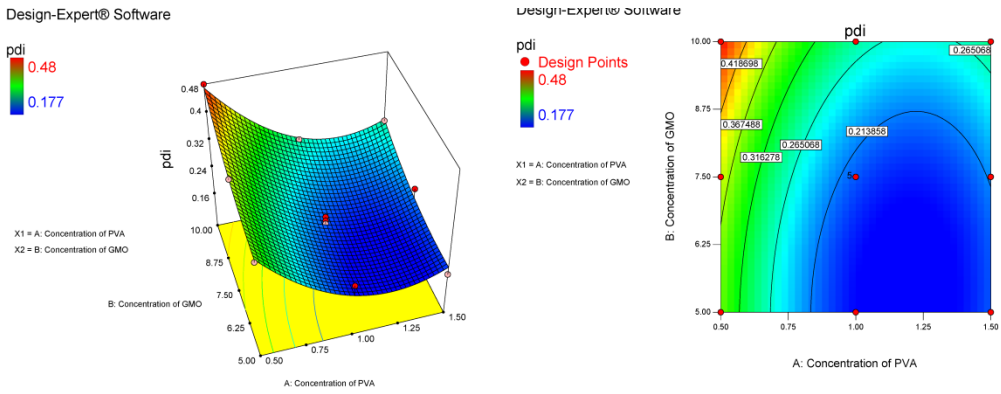


Figure 6.10: Contour and surface response plot of polydispersity index (PDI)

Numerical optimization was performed by the software for defined optimization criteria as presented in Table 6.24. 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 6.24: Criteria for optimization of FBX 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	5	10	1	+++
% Entrapment	Maximum	80	100	1	+++
Vesicle size	Minimum	135.9	200	1	+++
PDI	Minimum	0.177	0.3	1	+++

Overlay contour plots for all three CQAs (% entrapment, vesicle size and PDI) were generated (Fig. 6.11) for obtaining the design space (yellow area in graph) by employing the defined criteria shown in the above table.

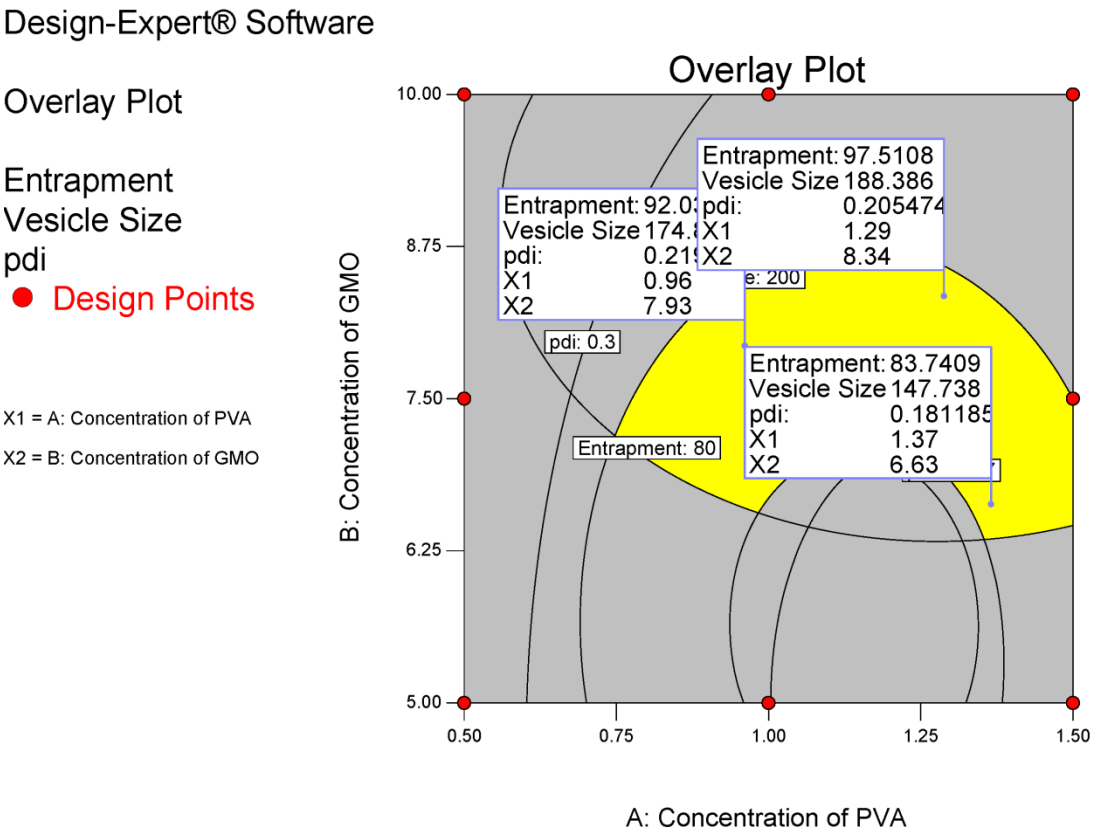


Figure 6.11: Overlay plot for the optimization of FBX loaded cubosomes using full quadratic model

Further, the optimized desirability plot is presented in Fig. 6.12. The optimized desirability plot showed a composite desirability of 0.832 for the solution provided by the software.

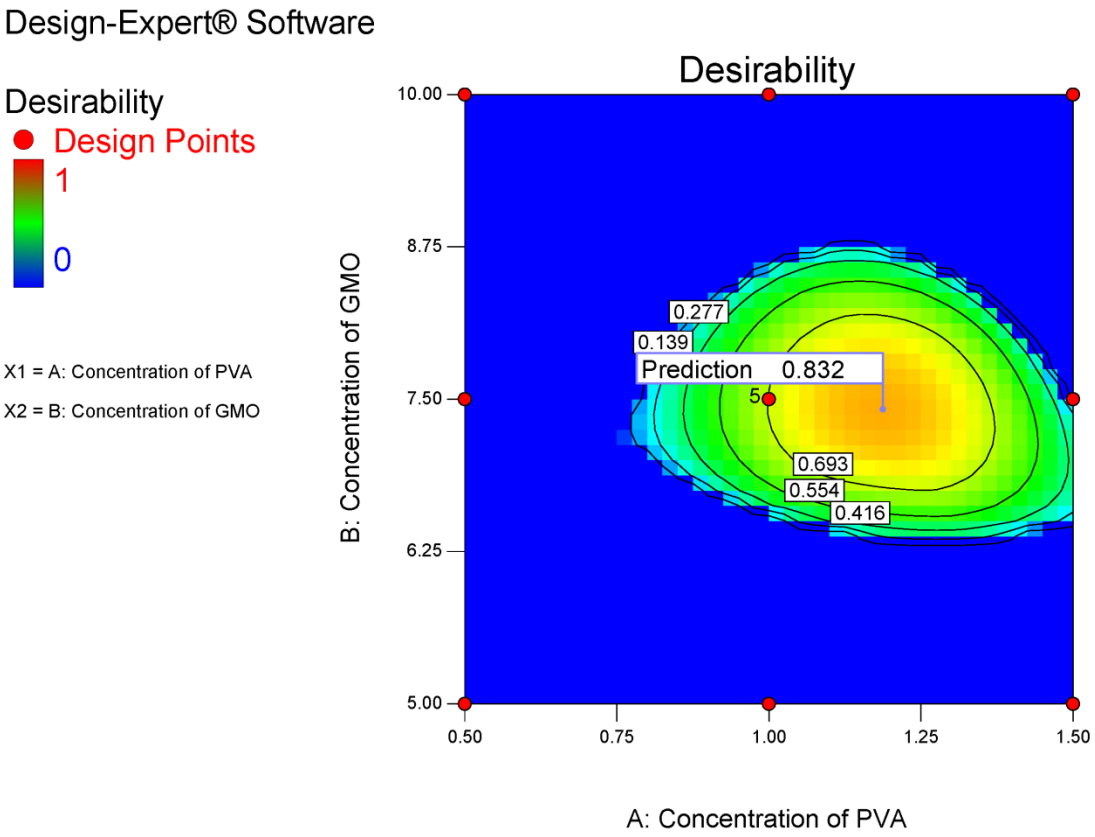


Figure 6.12: Desirability contour plot for optimization of FBX loaded cubosomes using full quadratic model

6.3.6. 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 6.25 & 6.26. Three checkpoint batches were prepared according to these levels and results of these checkpoint batches are represented in table 6.27. 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 6.25: Levels of independent variables as per the point prediction analysis

Factor	Name	Level	Low level	High level
A	Conc. of PVA	1.19	0.50	1.50
B	Conc. of GMO	7.42	5.0	10.00

Table 6.26: 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 efficiency	1.10	89.18	94.39	2.86	85.02	98.55
Vesicle size (nm)	4.23	136.09	156.08	10.98	120.11	172.06
PDI	0.019	0.16	0.20	0.023	0.13	0.24

*CI = Confidence interval; PI = prediction interval

Table 6.27: Results of checkpoint batches obtained using optimized overly plot of FBX 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	7.93	0.96	92.03	174.8	0.219	89.21	163.9	0.222
2	8.34	1.29	97.51	188.39	0.205	92.37	166.4	0.198
3	6.63	1.37	83.74	147.74	0.181	86.18	151.6	0.159
Avg						89.25	160.6	0.193

6.3.7. In-vitro characterization of optimized FBX loaded cubosomes

6.3.7.1.Shape and surface morphology

Transmission Electron Microscopy (TEM) of the optimized formulation was performed for determining the shape and surface morphology and the image is presented in figure 6.13. The image shows cubical shape of the prepared FBX loaded cubosomes with smooth surface.(6) The size of the cubosomes seen in the image was found in-line of the results of vesicle size data obtained by Malvern Zetasizer.

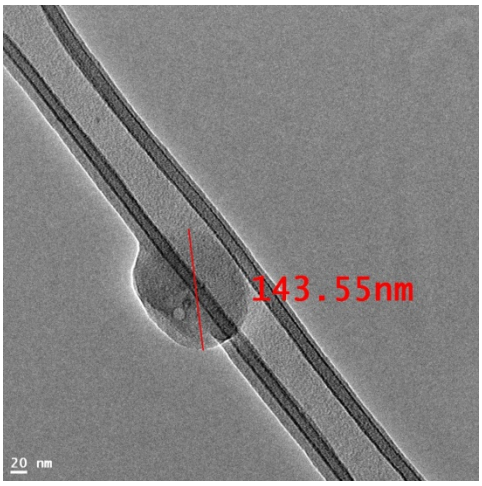


Figure 6.13: TEM images of FBX loaded cubosomes

6.3.7.2.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 prepared the agglomeration 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 FBX cubosomal formulation was measured using Malvern Zetasizer and was reported to be -17.2 due to the absence of any charge materials in the formulation as shown in fig 6.14. The obtained zeta potential is way below than the required for the stability of cubosomal dispersion. The negative zeta potential was obtained due the presence of the free oleic acid in the lipid. However, prepared cubosomal formulation of FBX do not form any aggregate during the entire storage period due to the stealthing effect provided by the surfactant (PVA) used in the formulation.(1)

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-17.2	Peak 1:	-13.1	97.8	12.9
Zeta Deviation (mV):	14.4	Peak 2:	33.7	2.2	4.77
Conductivity (mS/cm):	0.0461	Peak 3:	0.00	0.0	0.00
Result quality :	See result quality report				

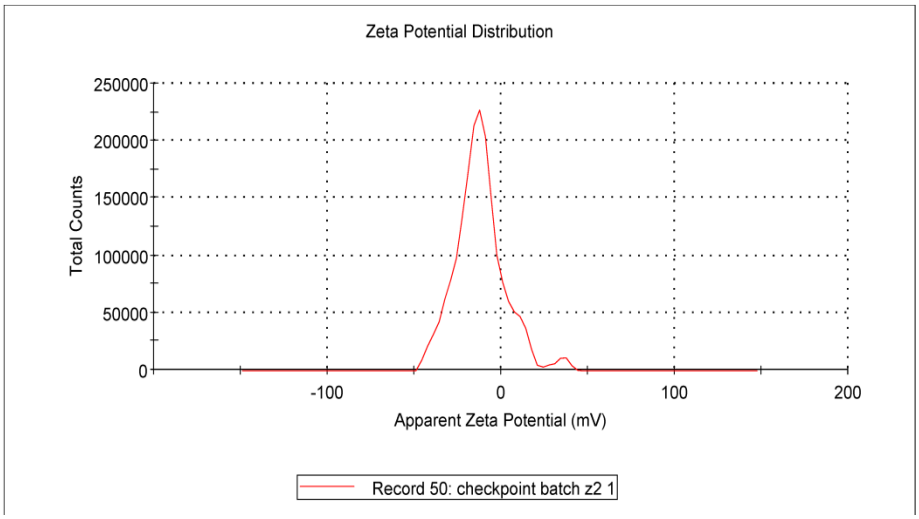


Figure 6.14: Zeta potential of the optimized FBX loaded cubosomes

6.3.7.3.Vesicle size and size distribution

Mean vesicle size and size distribution (PDI value) of the cubosomal FBX dispersions were measured using the Malvern Zetasizer and found to be 157.5 nm and 0.165 respectively as shown in fig 6.15. Observed vesicle size of the cubosomes is sufficient for the transdermal permeation of the FBX. Moreover, low PDI value of the optimized batch suggests the formation of monodispersed 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 of less than 300 nm were successfully formulated. Due to the smaller size they efficiently reach the dermis layer of the skin and drug can be absorbed systematically from here to obtain desired therapeutic concentration of drug in blood.

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 157.5	Peak 1:	175.4	98.8	69.62
PdI: 0.165	Peak 2:	4761	1.2	740.4
Intercept: 0.951	Peak 3:	0.000	0.0	0.000
Result quality : Good				

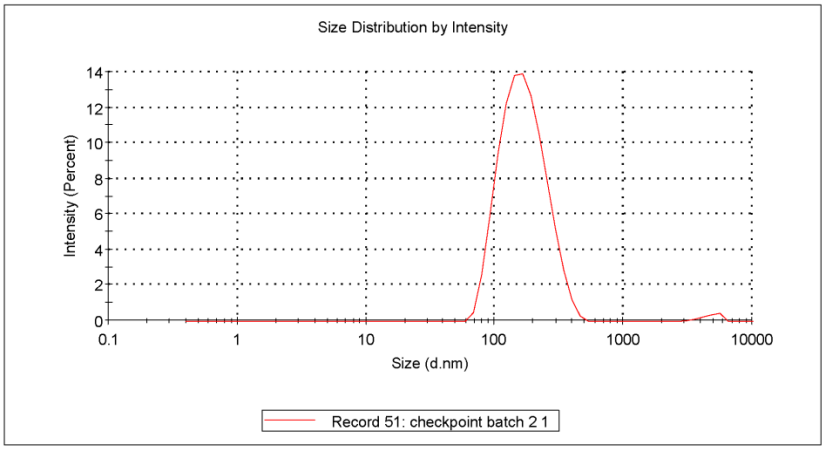


Figure 6.15: Vesicle size of the optimized FBX loaded cubsomes

6.3.7.4.Entrapment efficiency

Mean % Entrapment efficiency of the optimized formulation was observed to be 85.2 %. The lipophilic property of the entrapped drug is responsible for the high % entrapment efficiency of the optimized formulation (FBX- log p value- 3.3). Additionally, cubosomes have a distinct advantage of providing high entrapment efficiency of encapsulated drug according to the literature survey.(1, 2)

6.3.7.5.Total drug content

Total drug content of the prepared cubosomal dispersion of FBX was found to be 97.28 % which means that 1 ml of cubosomal dispersion contains 3.32 mg FBX.

6.3.7.6.Small Angle X-rays Scattering

SAXS was used for the investigation of the liquid-crystalline structure of the prepared cubosomes and the results are shown in fig 6.16. It showed a sequence of two well-defined scattering patterns and one diffuse diffraction pattern at Q value of 0.12, 0.9 and 0.35-0.75 A-1 region with relative positions on curve respectively. The peak at Q value of 0.12 A^{-1} indicates characteristic scattering peaks due to cubic phase whereas peak at 0.9 A^{-1} revealed scattering

pattern due to whole cubosome liquid crystalline structure. The key characterestic of this X-Ray scattering diagram was diffuse scattering pattern of low intensity in the region of 0.35-0.75 A^{-1} indicating presence of water channels inside Cubosomes which is a unique feature among all nanocarriers.(6)

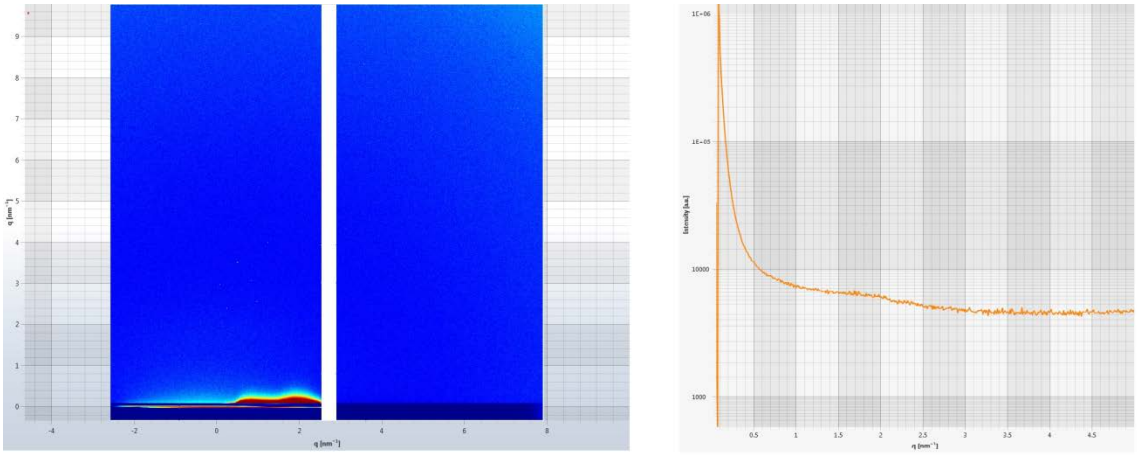
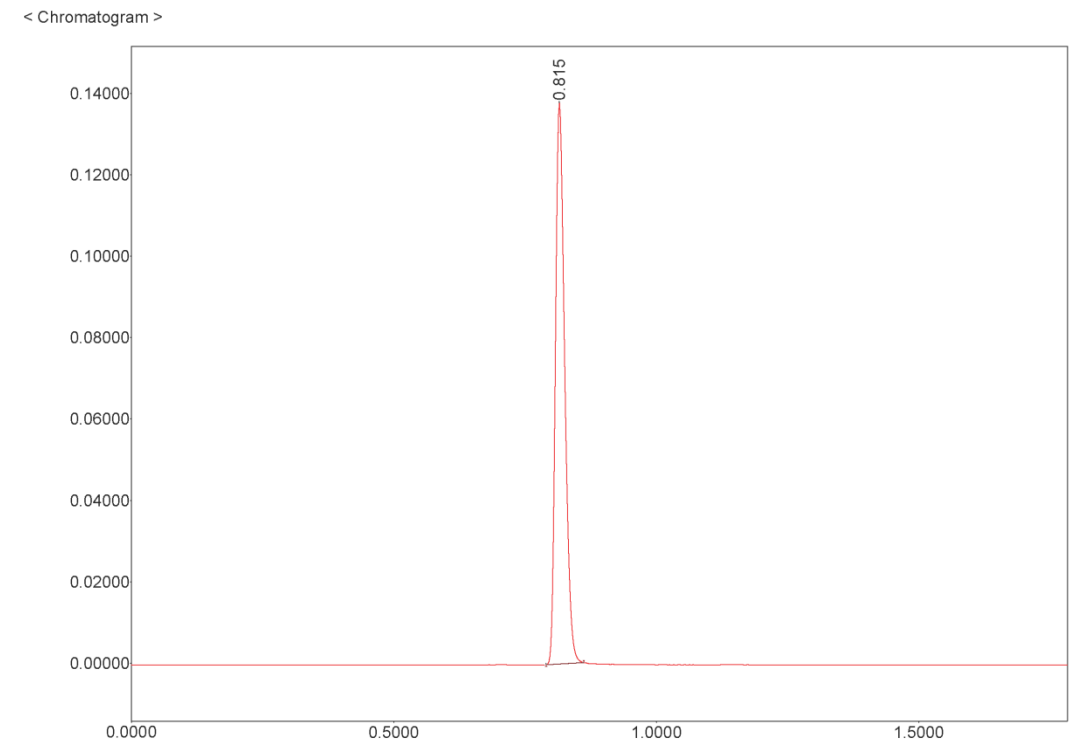


Figure 6.16: Scaterring pattern of optimized cubosomes of FBX

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

The cubosomal dispersion of FBX was prepared using organic solvent i.e. ethanol. For therapeutic purpose, the content of this organic solvent 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 found to be 167.95 ppm as shown in fig 6.17 which is significantly less than the permitted limit according to the ICH guidelines Q3C (R6) for residual solvents.(17)



#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	0.815	167.951	BB	4.300	100.000

Figure 6.17: Residual estimation of ethanol in final optimized batch of cubosomes of FBX

6.3.7.8.In-vitro drug release

In vitro drug release from optimized cubosomes of FBX was evaluated in comparison with the pain drug suspension. The 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 FBX showed >60 % FBX release in 24 hours while only 39.97 % drug was released from plain suspension in 24 hours due to insufficient solubility of drug in phosphate buffer pH 7.4 indicating the 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 the data of drug release from the cubosomes and listed in table 6.29. The R^2 values for Korsmeyer Peppas was found higher suggesting a diffusion controlled system where release rate was

dependent on remaining drug concentration within the cubosomes. Moreover, n value of 0.607 for Korsmeyer Peppas model suggests non-fickian diffusion of drug from cubosomes.(18) FBX 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 solubilize form. Thus due to insolubility of FBX in water, only limited amount of drug was able to permeate through the dialysis bag. 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 donor compartment. Due to this reason more amount of FBX is able to permeate the diffusion membrane and higher in-vitro release of FBX obtained in case of cubosomes of FBX.(19, 20)

Table 6.28: *In-vitro* drug release profile of FBX from drug suspension and prepared cubosomes of FBX

Time (h)	Cumulative percent drug release	
	FBX drug suspension	Cubosomes of FBX
0.5	2.88 ± 0.08	7.43 ± 0.17
1	5.19 ± 0.09	9.50 ± 0.36
1.5	6.30 ± 0.1	11.58 ± 0.43
2	8.33 ± 0.1	13.56 ± 0.37
3	10.24 ± 0.12	15.44 ± 0.49
4	12.44 ± 0.12	18.03 ± 0.99
5	13.30 ± 0.14	22.46 ± 0.99
6	15.17 ± 0.2	26.49 ± 1.27
7	17.10 ± 0.29	31.89 ± 1.53
8	20.04 ± 0.32	34.39 ± 1.11
9	21.98 ± 0.36	37.05 ± 0.88
10	23.42 ± 0.40	39.97 ± 0.80

11	24.25 ± 0.4	42.56 ± 1.21
12	26 ± 0.52	44.49 ± 1.72
24	32.07 ± 0.87	61.08 ± 2.52
36	35.45 ± 1.08	75.73 ± 3.31
48	38.69 ± 2.85	77.68 ± 3.56

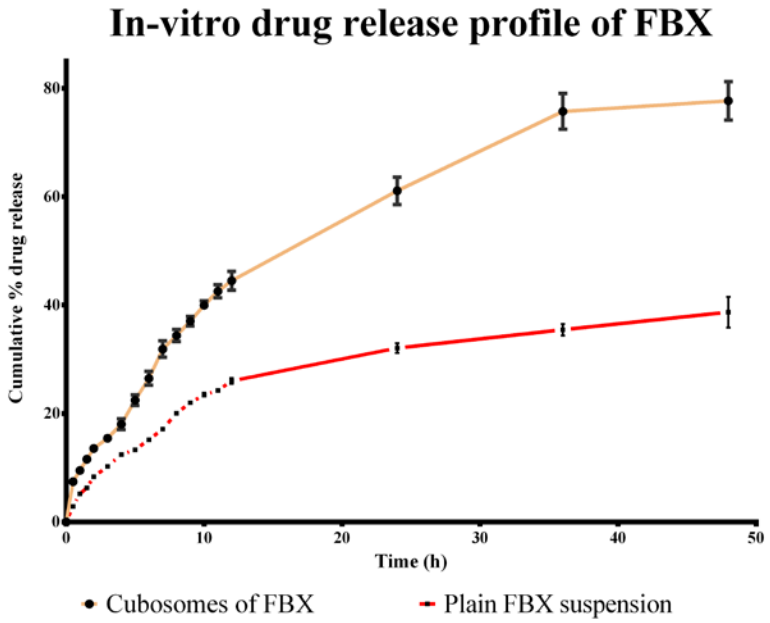


Figure 6.18: In-vitro drug release profile of FBX

Table 6.29: Various statistical model for release kinetic with their R^2 value

Statistical model	Cubosomes of FBX	
	R^2	n
Zero order	0.7267	-
First order	0.9498	-
Higuchi	0.9568	-
Korsmeyer Peppas	0.9813	0.607
Hixon Crowell	0.9114	-

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