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FORMULATION AND EVALUATION OF LIPOSOMES OF FENOFIBRATE PREPARED BY THIN FILM HYDRATION TECHNIQUE

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Keywords:

Liposomes, Rotavapor, Fenofibrate, Central composite designs, Contour plots, Surface plots, Design of experiment

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ABSTRACT: The objectives of the present study were to use design of experiments (DOE) for formulation and optimization liposomal formulations and study the impact of process variables on quality attributes of the complex liposomal formulation system. Several factors may have contributed to the slow pace of commercialization of liposome drug products during the last decade: 1) the difficulties associated with identifying the formulation and process design critical quality attributes of these complex systems and 2) Higher manufacturing cost due to low preparation reproducibility and low entrapment of therapeutic active agents. Thus, a central composite design (CCD) was used for the optimization of the liposomal formulation. In the present study, the hydration volume, hydration time, sonication time and drug: lecithin ratio were chosen as input variables for the liposomal preparation and whereas; like particle size, peak shape, polydispersity Index, drug loading, entrapment efficiency and redispersion behavior were investigated as quality attributes. Additionally, contour plots and response surface plots were also utilized to understand the fundamental relationships between input variables and quality attributes. *In-vitro* dissolution utilizing United States Pharmacopeia (USP) apparatus II showed enhanced dissolution for the entire drug: lecithin ratios in the comparison to the equivalent amount of fenofibrate. The input variables showed significant effect on the quality attributes of the liposomal formulation as studied by the contour plots and surface response plots. This study will provide further understanding of the impact of process variables on the quality of the liposomal formulation.

INTRODUCTION: Current advances in synthetic, analytical and purification chemistry along with the progress of specialized tools such as high throughput screening, combinatorial chemistry and proteomics have led to a sharp influx of discovery compounds entering in to development. Most of these compounds are highly lipophilic, since the *in-vitro* screening techniques place considerable emphasis on interaction of the compounds with defined molecular targets.

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In recent years it has been estimated that up to 40% of the new drugs discovered by the pharmaceutical industry are poorly soluble or lipophilic compounds causing difficulties in formulation steps.

Poor aqueous solubility is one of the major hurdles the development of new compounds into oral dosage forms, since absorption is limited by dissolution for these compounds [1]. Significant issues associated with poorly water-soluble compounds can be listed as poor bioavailability, fed / fasted variation in bioavailability, lack of dose-response proportionality, suboptimal dosing, use of harsh excipients (use of co-solvents), use of extreme acidic and basic conditions to enhance solubilization, uncontrollable precipitation after dosing and non-compliance by the patient.

Modifications on the chemical structure to enhance solubility may drastically change pharmacological activity by modifying the affinity of drug for its receptor. Development of water-soluble derivatives is costly; however, this approach has demonstrated efficacy and safety of new chemical species.

Fenofibrate is a highly lipophilic drug clinically used to lower lipid levels [25, 26]. However, its therapeutic efficacy has been compromised for years due to the virtual insolubility in water and physiological fluids [2]. Problems with solubility commanded to the growth of novel fenofibrate formulations aimed to increase the total bioavailability through numerous diverse methods [3]. Primarily, micronized formulations increased solubility by decreasing particle size and growing surface area [4]. Tablets having combination of the standard particle size reduction process with a micro coating linkage of hydrophilic polyvinyl pyrrolidone resulted in enhanced dissolution rates and better bioavailability [5]. Consequently, insoluble drug delivery microparticle (IDD-P) tablets containing phospholipid agents that alter surface characteristics to inhibit aggregation were established, as a result preserved the extended drug surface area of micro particles which has accelerated the dissolution and resulted in good absorption [6]. Nanoparticle formulations causes' reduction in particle size, resulting in improved ratio of surface area to volume and enhanced bioavailability [7, 8, 9]. To follow optimal drug action, functional molecules a carrier might be used for transportation to the site of action and released to perform their task, thus the carrier should be non-hazardous, biodegradable, and of appropriate shape and size to accommodate wide diversity of substances.

Liposomes, discovered in 1965 by Bangham and his colleagues are spherical colloidal particles containing an aqueous core surrounded by phospholipid bilayer which replicates cell membrane [10]. Liposomes were a better model to investigate membrane structure and functionality as it is mainly composed of phospholipids [11]. Liposome's structural resemblance to the cellular membrane has allured scientists to explore the potency of using liposomes as drug carriers to transport therapeutics with peculiar properties to targeted parts of body since 1940's [12, 13, 14, 15].

Phospholipids are intended to be relatively non-toxic as they are natural ingredients and they are degradation by different enzymes present in the body. Liposomes are microscopic lamellar structures formed on the admixture of soya lecithin with subsequent hydration in aqueous media. Liposomes have been widely evaluated for controlled and targeted drug delivery for treatment cancer, viral infections and other microbial diseases. Liposomes are found to be suitable for localization of topically applied drugs at or near the site of application, due to fact that they may act as slow releasing vehicles.

Liposomes as drug-carriers represent versatile technology with more advantages compared to other delivery systems including, but not limited to the followings: biocompatible, biodegradable, marked decrement in drug toxicity, marked increment in therapeutic efficacy, retention and permeability is improved. Due to its similarity with cell membrane structure liposomes can incorporate both lipophilic and hydrophilic drug into phospholipid bilayer and aqueous core. Other advantage is it can simultaneously encapsulate other active ingredients into its complex formulation [16, 17, 18, 19].

Despite the enormous amount of effort spent during the past 44 years (greater than 114,000 scientific publications) and the well-formed consensus within the scientific community about the potential of liposomes as drug carriers, currently relatively few products (12 therapeutic products) are available on the market and all of these were approved between 1995 and 2004. Several factors may have contributed to the slow pace of commercialization of liposome drug products during the last decade: 1) the difficulties associated with identifying the formulation and process design critical quality attributes of these complex systems; 2) the high manufacturing cost due to low preparation reproducibility and low entrapment of therapeutic active agents (in particular for hydrophilic drugs); and 3) the high regulatory burden associated with product safety of these complex parenteral product.

A systemic methodology is essential for effective optimization of liposomal formulation. Optimi-zation approaches can in overall be categorized as following: Univariate method, sequential methods

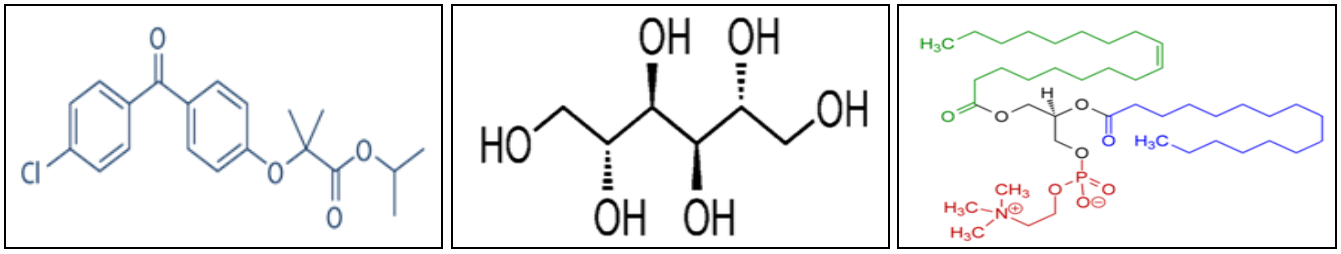
and simultaneous methods. The Univariate method, the easiest one, is usually applied to conditions when the outcome of few number of factor deprived of mutual interactions is studied. This approach has broadly been used for optimizing liposomal formulation. Conversely, the proportions of the various components in the liposomes are dependent to make the vectors which are effective and of small size.

Hence, we have used central composite design (CCD) for the optimization of the liposomal formulation. Central composite design (CCD) is also known as response surface methodology (RSM) which is a prompt procedure used to empirically develop a practical connection amongst the experimental outcome and the set of input variables / designs. It determines the optimal point of experimental factors essential to get desired response. A factor is well-defined as an input variable and its value can be fixed throughout an experiment. The output (response) variable is a measured quantity and its value is altered by points (level) selected for the factors.

CCD lessens the number of experimental runs which are required to create a mathematical trend in the experimental design region. The range should not be too vast, leading to non-realistic experiments and not too narrow, distant from the optimum region [20, 21, 22, 23, 24]. In the current study, a central composite design, 4 factors, face centered was employed to develop the liposomal suspension and identify the critical parameters.

MATERIALS AND METHODS:

Materials: Fenofibrate was obtained from Sigma-Aldrich (St. Louis, MO). Lecithin was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Mannitol was purchased from Roquette America Inc. (Keokuk, IA. Chloroform, methanol, acetonitrile and water were purchased from pharmco-aaper, and were of analytical grade. Sodium lauryl sulfate (SLS) was obtained from spectrum pharmaceuticals (New Brunswick, NJ). All materials were used as received.Fig.1 represents the chemical structure of model drug (Fenofibrate API) and excipients.



FENOFIBRATE **MANNITOL** **LECITHIN**

FIG. 1: CHEMICAL STRUCTURE OF API AND EXCIPIENT

Methods: Preparation of Fenofibrate Liposomes: Fenofibrate liposomes were prepared by thin film hydration technique using Buchi rotavapor R 200 [27, 28]. Liposomal formulations were prepared by dissolving variable amounts of lecithin and drug (2: 0.1, 3.5: 0.1 and 6: 0.1) in approximately 50 ml of chloroform [12, 13, 14]. The solution was then taken in 500 ml round bottom flask. Chloroform was evaporated under vacuum 100 mmhg using rotary evaporator (R10 Rotavapor, Buchi) at 63 ºC to form a thin film. Evaporation was continued for approximately 15 min until dry residue is formed. The organic solvent is eliminated slowly by this method to yield a thin lipid film on the interior surface of the flask. To ensure complete evaporation of the organic solvent, the films were vacuum dried for overnight. The film was than hydrated with different amount (90 ml, 105 ml, 120 ml) of phosphate buffer (pH 7.4) solution containing 1g of mannitol and rotated for different hydration time (15 min, 30 min, 45 min) at 45 ºC. The liposomal suspension was kept overnight to get complete lipid hydration at 4 ºC.

Size Reduction by Sonication: For this method a sonic dismembrator by Fisher scientific (model F50) having a probe sonicator was used. The power delivered to the solution can be selected as percentage amplitude which was kept at 40%. The liposomal suspension was sonicated for different time (14 min, 21 min and, 28 min) by probe sonicator at 40% amplitude, using depth 19 mm measured from the base of vessel at room temperature.

Determination of Particle Size and Poly-dispersity Index: Particle size measurements were carried at 25 ºC by photon correlation spectroscopy on Malvern Zetasizer Nano Z® instrument (Malvern, Model ZEN3600) armed with a 4 mW He-Ne laser (633 nm). Samples were put in transparent disposable cuvette and the dispersant viscosity and refractive index was set to 0.8872 cP and 1.330 at 25 ºC. Particle size was analyzed by the Dispersion Technology Software provided by Malvern Instruments. All samples were kept in refrigerator at 4 °C prior to characterization.

Determination of Zeta Potential: A dynamic light scattering instrument (Malvern Instruments Inc., Malvern, UK) was used to measure zeta potential of the liposomal formulation. The sample was analyzed at 25 °C, using a dispersant refractive index of 1.33. In the current study, the zeta potential of the prepared liposomal formulation was measured in disposable folded capillary cells (Model #DTS1061). Samples should be bubble free for accurate measurement of zeta potential. Measurement was done with Malvern nano series zeta sizer instrument at 25 ºC.

Determination of Drug Loading: Drug loading was determined using HPLC. 1 mL of liposomes formulation was dissolved in 10 mL methanol: water mixture (ratio 7: 3). The solution was than subjected to brief sonication. The resulting solution is than filtered using 0.22 µm filters. The filtrate was than analyzed through HPLC. Different formulations showed different drug loading based on the drug and lecithin ratio [29].

HPLC Analysis: The quantitative analysis of fenofibrate was performed by reverse-phase gradient high-performance liquid chromatography. Analysis was performed using an Agilent 1100 System (Agilent Technologies, Palo Alto, CA), equipped with an XTerra™ RP18 (5 µm 4.6 * 250 mm column (Waters technologies, Ireland). The column temperature was kept constant at 25 ºC and UV detection was carried out at 287 nm for fenofibrate respectively. The mobile phase consisted of a mixture of acetonitrile (ACN) and water solvent (70:30% v/v). The injection volume was 10 µL. The flow rate was 1.5 mL/min, and the total run time was set for 9 min. The retention time was found to be 6.5 min. This reverse-phase gradient method provided baseline resolution and excellent peak characteristics for fenofibrate. Quantification of drug concentrations was performed by analyzing the peak area using Agilent Chemstation® analytical software 28.

Determination of Entrapment Efficiency: The liposomal formulation was centrifuged at 4000 rpm for 18 min at 4 °C temperature by using remi cooling centrifuge to separate the free drug. A supernatant contains the liposomes in suspending stage and free drug on the wall of centrifuge tube. The supernatant was again centrifuged at 12000 rpm for 38 min at 4 °C temperature. As a result, a transparent solution of supernatant and liposome pellet was attained. The pellet consisting of liposomes was redispersed in distilled water prior to other studies.

The liposomes devoid of unentrapped free drug were mixed with 10 ml of mixture of methanol: water ratio (7:3 v/v) followed by 5 min of sonication. As a result of sonication, the liposomes were disrupted to discharge the drug. The discharged drug was determined for the drug entrapment. The amount of fenofibrate was estimated by using HPLC system. Percentage entrapment efficiency was determined as

$$\text{Percentage Entrapment Efficiency} = W_c / W_t \times 100$$

Where amount of drug content (entrapped) in the liposomes is denoted as W_c and total amount of drug in the dispersion is denoted as W_t.

Redispersibility: The liposomal formulation was centrifuged at 4000 rpm for 18 min at 4 °C temperature by using remi cooling centrifuge (Beckman coulter, Allegra™ X-22R) to separate the free drug. A supernatant contains the liposomes in suspending stage and free drug on the wall of centrifuge tube. The supernatant was again centrifuged at 12000 rpm for 38 min at 4 °C temperature. As a result, a transparent solution of supernatant and liposome pellet was attained.

The pellet was than diluted to 10 ml with deionized water. The solution was then taken in 14 ml Eppendorf tubes and vortexed on a vortex mixture at speed 3 for 5 min. After vortexing the solution was then taken in transparent cuvettes and particle size was determined after 10 min.

Design of Experiments (DOE): To evaluate the effect of liposomal formulation process parameters on various product attributes, a DOE was generated wherein drug: lecithin ratio, hydration volume, hydration time and sonication time were selected as independent variables. Their units and range are depicted in **Table 1**. Particle size (nm), peak shape, polydispersity index, drug loading, entrapment efficiency and redispersion behavior were the product attributes measured. A central composite design consisting of 17 experiments was created using the minitab 17 software as shown in **Table 2**.

TABLE 1: INPUT VARIABLE ALONG WITH THEIR UNITS AND RANGES

Independent variable	Levels		
	Low	Medium	High
Drug: lecithin ratio	0.1:2	0.1:3.5	0.1:6
Hydration time (min)	15	30	45
Hydration volume (ml)	90	105	120
Sonication time (min)	14	21	28

TABLE 2: CENTRAL COMPOSITE DESIGN WITH INDEPENDENT INPUT VARIABLES

Compo -sition	Hydration volume (ml)	Hydration time (min)	Drug: lecithin Ratio	Sonication time (min)
1	105	30	0.1:3.5	21
2	120	45	0.1:2	14
3	90	45	0.1:2	28
4	90	45	0.1:6	28
5	105	15	0.1:3.5	21
6	90	30	0.1:3.5	21
7	105	30	0.1:6	21
8	90	15	0.1:6	14
9	120	15	0.1:6	28
10	120	45	0.1:6	14
11	105	30	0.1:2	21
12	105	30	0.1:3.5	28
13	120	15	0.1:2	28
14	105	45	0.1:6	21
15	105	30	0.1:3.5	14
16	120	30	0.1:3.5	21
17	90	15	0.1:2	14

Statistical Analysis:

Contour Plots: To investigate the effect of different input variables hydration time, hydration volume, drug: lecithin ratio and on quality attributes, the contour plots were developed using minitab 17 (CA, USA). The contour plots were prepared using the experimental results from the testing dataset.

Response Surface Plots: To investigate the effect of different input variables on quality attributes, the response surface plots were developed using minitab 17 (CA, USA). The response surface plots were prepared using the experimental results from the testing dataset.

Characterization of Liposomes:

Differential Scanning Calorimetry (DSC): Differential scanning calorimeter was used for conducting thermal analysis. Nitrogen is used as purging gas at a flow rate of 50 ml/min, which provides outstanding sensitivity in the DSC cell. For the calibration of the instrument intended for temperature and cell constant, Indium with 99.99% purity is used. Sapphire and empty cells were heated for baseline and heat capacity calibration sapphire respectively. Powder samples of weight equal to 5 - 10 mg were encapsulated hermetically in the standard aluminum pans with pin hole. Constant pressure was maintained throughout the analysis when pin hole is made on the lid of each pan. Samples were subjected to a DSC heating program from 30 ºC to 220 ºC at heating rate of 5 ºC/ min.

Fourier Transform Infrared Spectroscopy (FTIR): Nicolet iS5 Fourier transform infrared spectrophotometer with iD5 ATR diamond accessory is used spectral analysis. A small amount of sample was mixed with inert potassium bromide (KBr) acting as background. 500 mg of KBr and 5mg of the dried liposomes are triturated thoroughly in mortar with a pestle and then compressed into a semi-transparent film. The film was scanned in the spectrophotometer through a region of 400 to 4000 cm^{-1} with an average scan of 64 and 2 cm^{-1} resolution. The resulting spectrum was further investigated for the depths relating to the functional groups present in the sample molecules.

***In-vitro* Dissolution Studies:** *In-vitro* release studies were carried in USP Type- II (Paddle) dissolution apparatus was used to perform in-vitro dissolutions studies for the liposomes. The release behavior of the liposomal formulation and pure drug was studied in distill water (containing 0.3 gm of sodium lauryl sulfate). The media was conditioned at 37 ºC with rotation speed of 75 rpm to confirm sufficient wetting. A sample of 10 ml from the liposomal formulation was taken in dissolution medium and is sustained at a temperature of 37 ± 0.5 ºC with rotation speed of 75 rpm.

Small aliquots (5ml) of sample with help of stainless steel cannula were taken from each dissolution vessel at regular time intervals of 5, 10, 15, 30, 45, 60, 90 and 120 min. Consequent to each sample removal, an equal amount of fresh media was replaced to retain the total volume of dissolution media constant. Dissolution samples were filtered using 0.22 µm syringe filters and subject for analysis using HPLC. Because of sustaining the constant volume of the media, it allows for the ease of calculations. The liposomal formulations were tested in triplicate for their release profile and the average was considered as the absolute value (n=3).

RESULTS AND DISCUSSION:

Effect of Process Parameters on Quality Attributes Studied by Contour Plots and Surface Response Plot: The particle size and particle size distribution of liposomes containing FBT were determined using determined by Malvern zeta sizer (DLS, Malvern instruments Inc., Malvern, UK). The polydispersity index (PDI) which measures the width of the size distributions was used to monitor the quality of the data. The particle size of the liposomal formulation was found to be between ranges of 99.5 nm to 290 nm **Table 3**.

The composition 4 showed lowest particle size that is 99.5 nm because of highest sonication time and composition 17 showed particle size 290 nm which can be related to lowest sonication time. Hydration time also affects particle size which is explained in detail in further section. The polydispersity indices obtained from readings are in range of 0.136 to 0.675 which indicate that the liposomes were monodispersed to polydispersed. The PDI values greater than 0.3 when hydration volume is 90 and higher sonication time.

TABLE 3: RESULTS FOR ALL QUALITY ATTRIBUTES FOR THE LIPOSOMAL FORMULATIONS

Composition	Particle size (nm)	PDI	Peak shape	% Drug Loading	% Entrapment efficiency	Redispersion (nm)
1	186	0.411	2	106.95	77.19	140
2	168	0.675	3	75.96	54.31	142
3	135	0.38	3	75.74	63.17	105
4	99.5	0.158	1	88.46	2.91	105
5	199	0.219	1	93.62	57.78	246
6	208	0.136	1	112.55	43.82	166
7	184	0.313	2	104.36	18.79	173
8	173	0.351	1	96.21	18.80	125
9	191	0.191	1	168.98	18.92	184
10	184	0.228	3	97.04	26.00	133
11	234	0.501	2	90.48	56.20	111
12	169	0.502	3	93.62	75.72	157
13	121	0.399	2	91.80	49.70	105
14	134	0.380	2	72.56	49.47	106
15	187	0.389	3	109.13	77.40	142
16	164	0.341	2	109.44	75.74	137
17	290	0.426	3	91.86	57.32	154

Effect of Process Parameters on Particle Size: Particle size is very critical parameter that governs the formulation quality. It has also potential to govern the formulation's solubility, dissolution rate and eventually the pharmacokinetic profile of the formulation. Hence, it is important to determine the effect of different process variables on the particle size.

Here, in our current research we have studied the impact of four input parameters hydration time, hydration volume, sonication time and drug: lecithin ratio on the particle size distribution.

The data were analyzed using contour response plots and surface response plot depicted in the **Fig. 2** and **3**. Particle size ranges from 100 nm to 150 nm for all hydration volumes and when hydration time is 32 to 45 min. It can be observed that particle size is between less than 100 nm to 200 nm throughout the range of drug: lecithin ratio when hydration time is above 40 min.

Thus, with increase in hydration time and drug: lecithin ratio there is decrease in particle size. Particle size is less than 200 nm throughout the range of sonication time and drug: lecithin ratio

greater than 0.1: 3. At sonication time higher than 28 min and low hydration volume 90 ml the particle size is greater than 100 nm. Particle size is less than 200 nm for all sonication time and hydration volume is higher than 105 ml. At sonication time 28 min the particle size is less than 100 nm. This may be accredited to differences in lipid tail length, molecular shape and membrane fluidity. The higher the hydration time and sonication time, the lower is the particle size.

As the hydration time increases, the ease of hydration of vesicles increases and so the ease of downsizing of the particles / liposomal vesicles increases resulting in the lower particle size. And then, higher the sonication time, higher is the time the vesicles being exposed to sonic energy and thus helps in disrupting the large multilamellar vesicles (LMV) to small unilamellar vesicles (SUV). This results in the lower particle size to the liposomal vesicles.

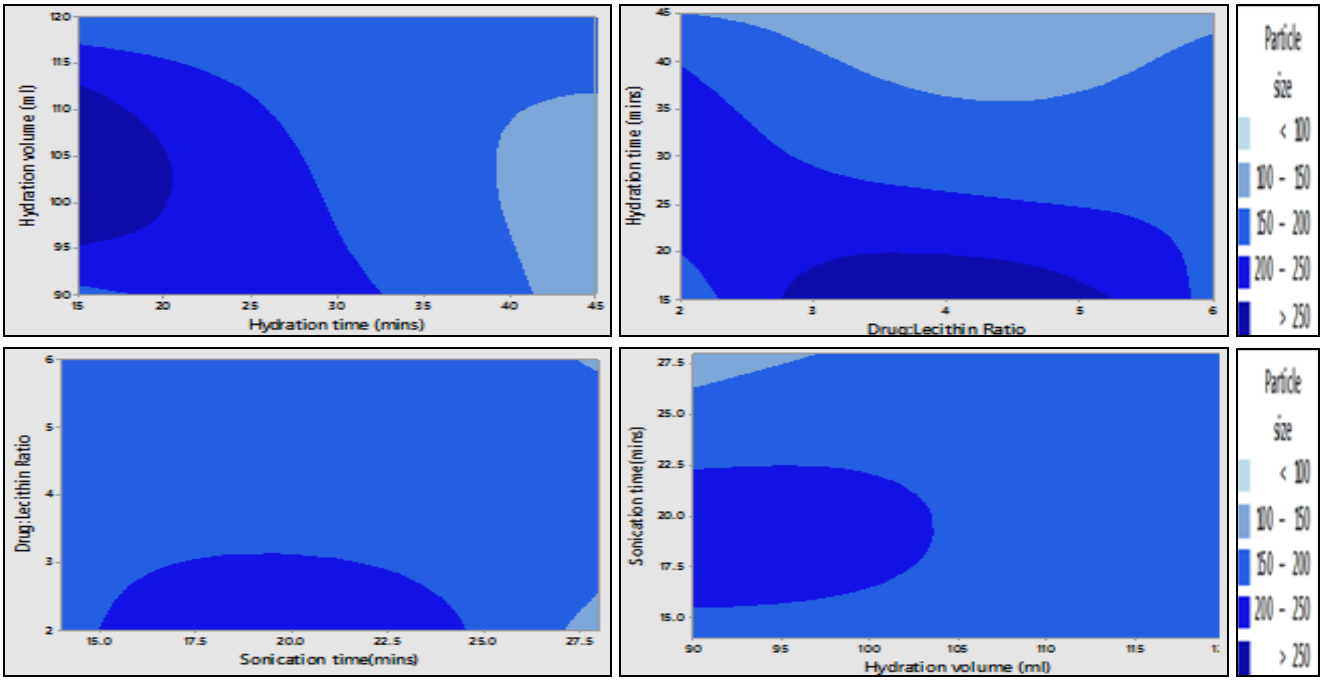


FIG. 2: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTES PARTICLE SIZE

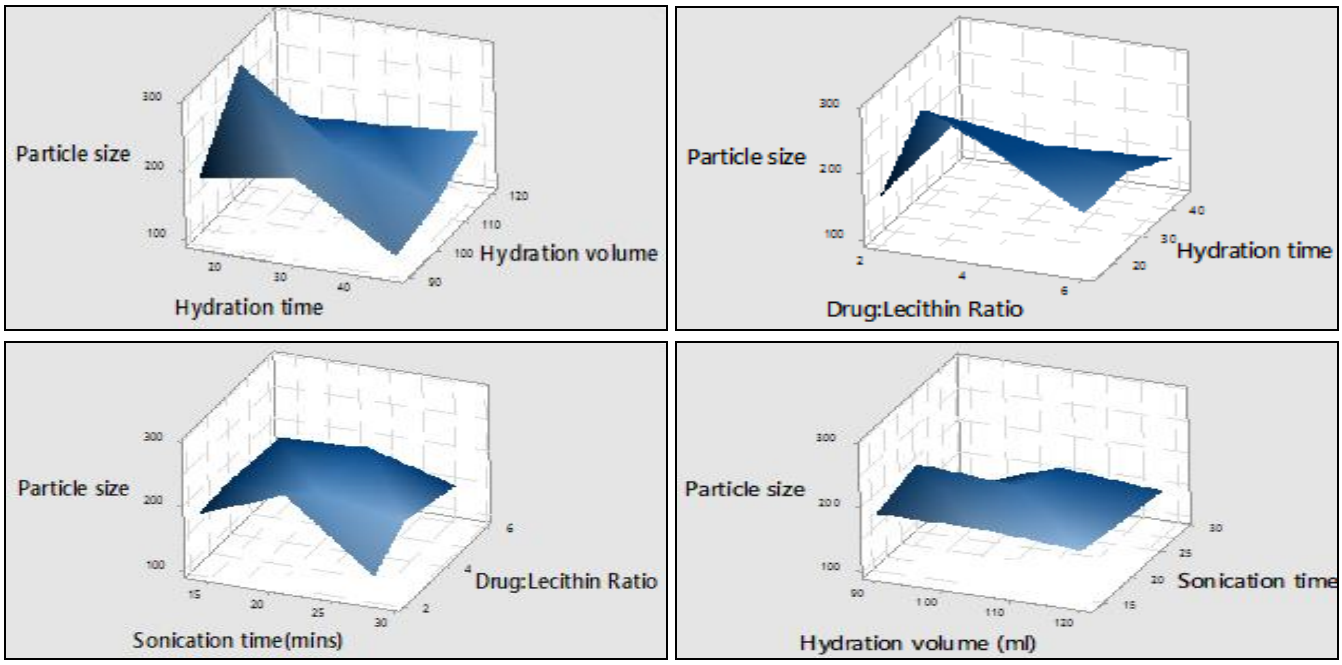


FIG. 3: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE PARTICLE SIZE

Effect on Process Parameter on Peak Shape: Peak shape can be monomodal, bimodal or multimodal depending on the various parameters affecting it. It suggests the homogeneity of the particle size distributions. Peak shape is mono-modal for all hydration time when hydration volume is 90 - 100 ml and for all hydration volume when hydration time is 15 - 25 min as observed from **Fig. 4** and **5**. Peak shape is monomodal when hydration time is 15 - 25 min drug: lecithin ratio greater than 2. Peak shape is monomodal for every drug: lecithin ratio when the sonication time is greater than 20 min. Peak shape is monomodal with increase in sonication times and hydration volume. Thus, as the sonication time increases and hydration volume decreases, the downsizing process of the vesicles is more effective, and thus helps in giving a formulation with uniform particle size and so the monomodal peaks.

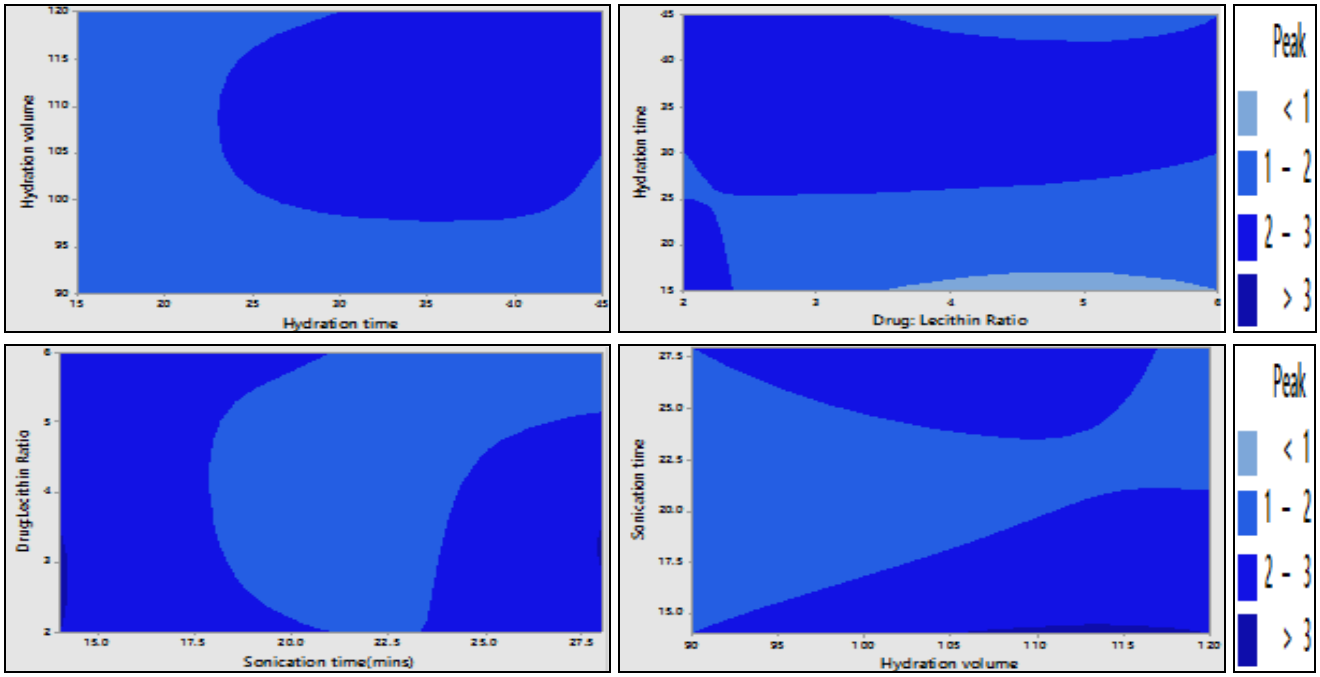


FIG. 4: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE PEAK SHAPE

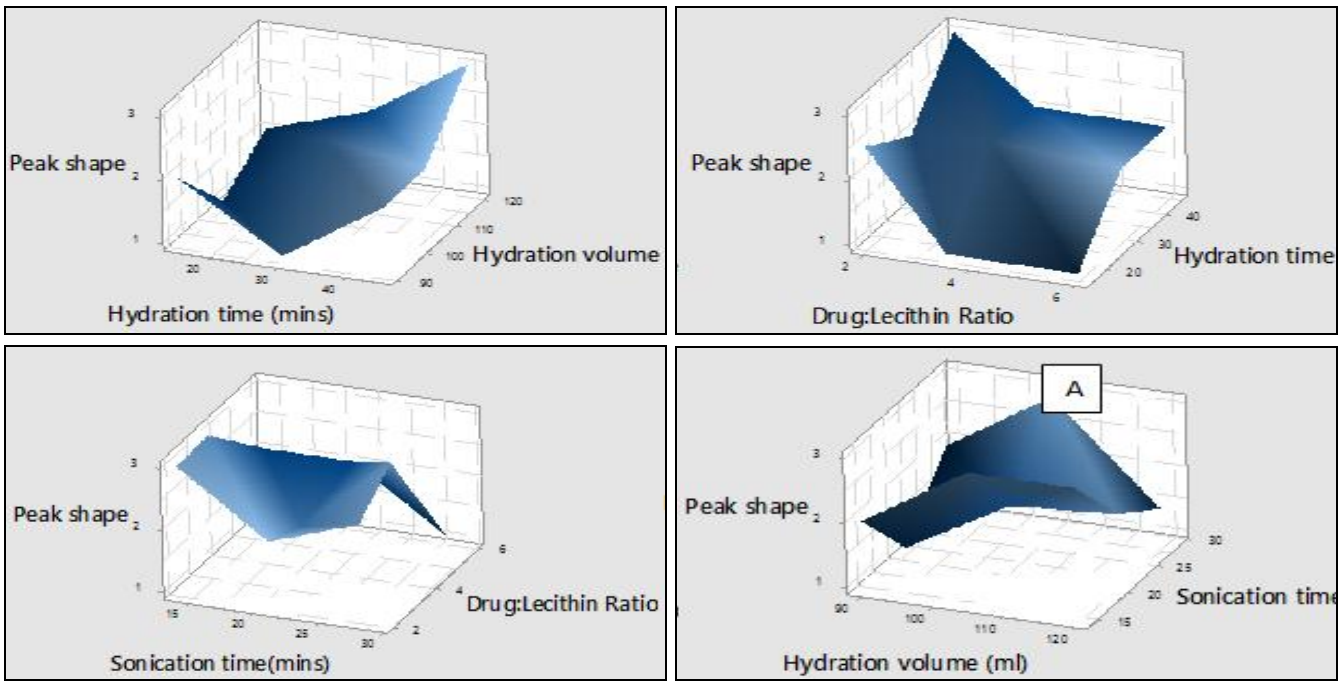


FIG. 5: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE PEAK SHAPE

Effect on Process Parameter Polydispersity Index: The dispersity is a measure of the heterogeneity of sizes of molecules or particles in a mixture. The polydispersity index (PDI) which measures the width of the size distributions was used to monitor the quality of the data. Studies were carried out to determine PDI as described above in the methods. In **Fig. 6** and **7**, the lower PDI value region (PDI less than 0.3) is towards the lower hydration time and lower hydration volume. But this region of lower PDI values extended over the broad range of hydration time and hydration volume axes which indicates that their effect on PDI is not much significant. The **Fig. 6** showed lower PDI values at higher drug: lecithin ratios at all hydration times and low PDI values lies towards the higher drug: lecithin ratios at all sonication times.

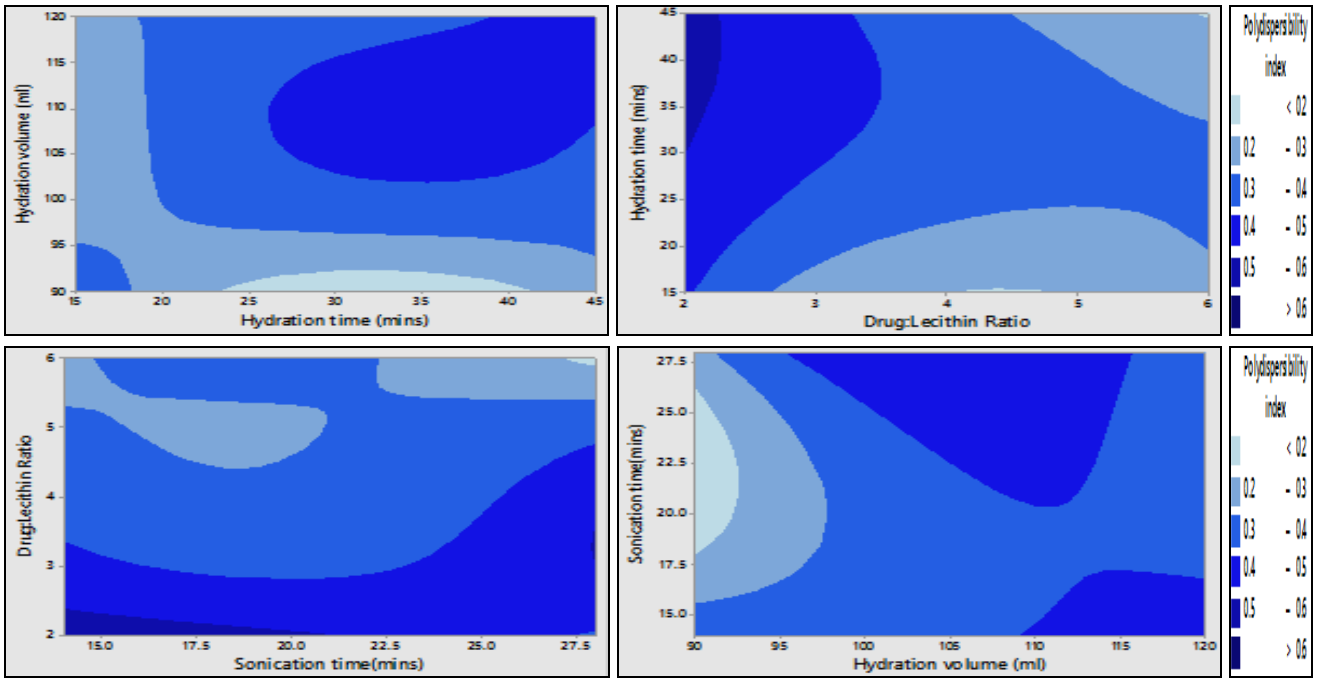


FIG. 6: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE TWO MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE POLYDISPERSITY INDEX

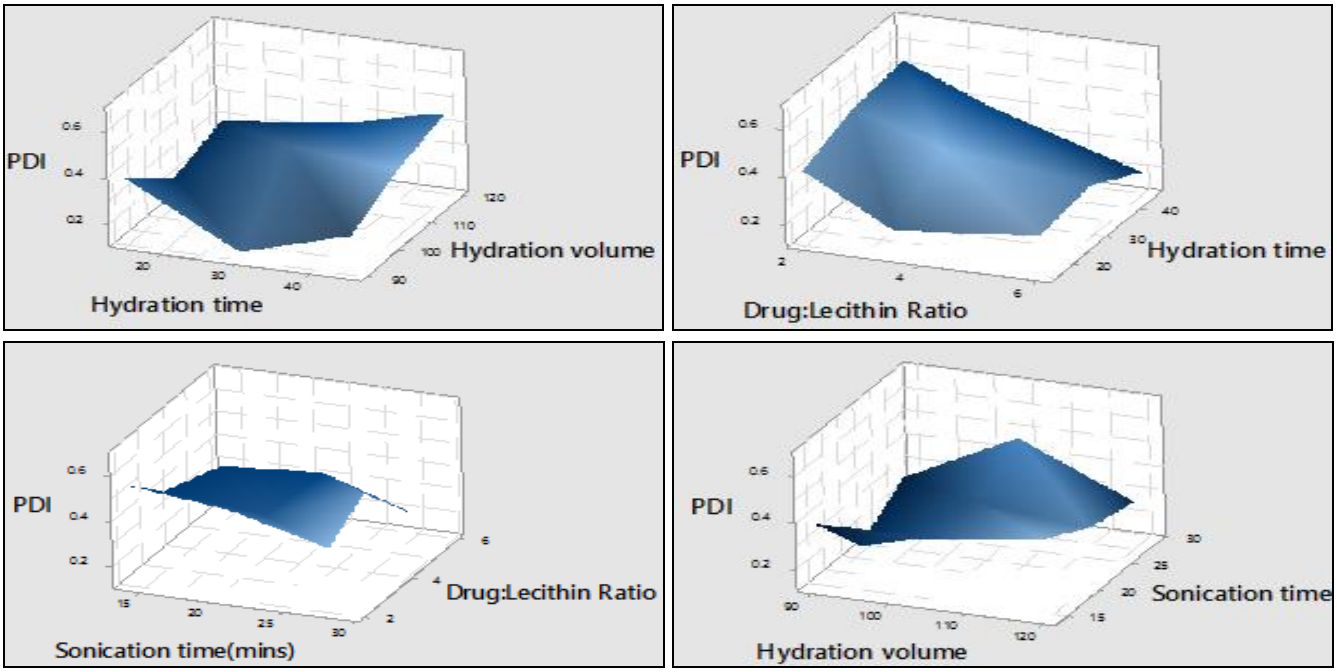


FIG. 7: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE POLYDISPERSITY INDEX

It is observed that the lower PDI value region (PDI less than 0.3) is spread over broad range of sonication time and hydration volume (spread over large area of graph along the axes), indicating less significance of their effect on PDI. Data suggested the PDI is greater than 0.4 throughout the range of sonication time and drug: lecithin ration greater than 0.1: 3.5. The PDI is greater than 0.3 when sonication time is more than 15 min and hydration volume is between 90 - 95 ml. Thus, as the sonication time increases and hydration volume decreases, the downsizing process of the vesicles is more effective, and thus helps in giving a formulation with uniform particle size and so the monomodal peaks.

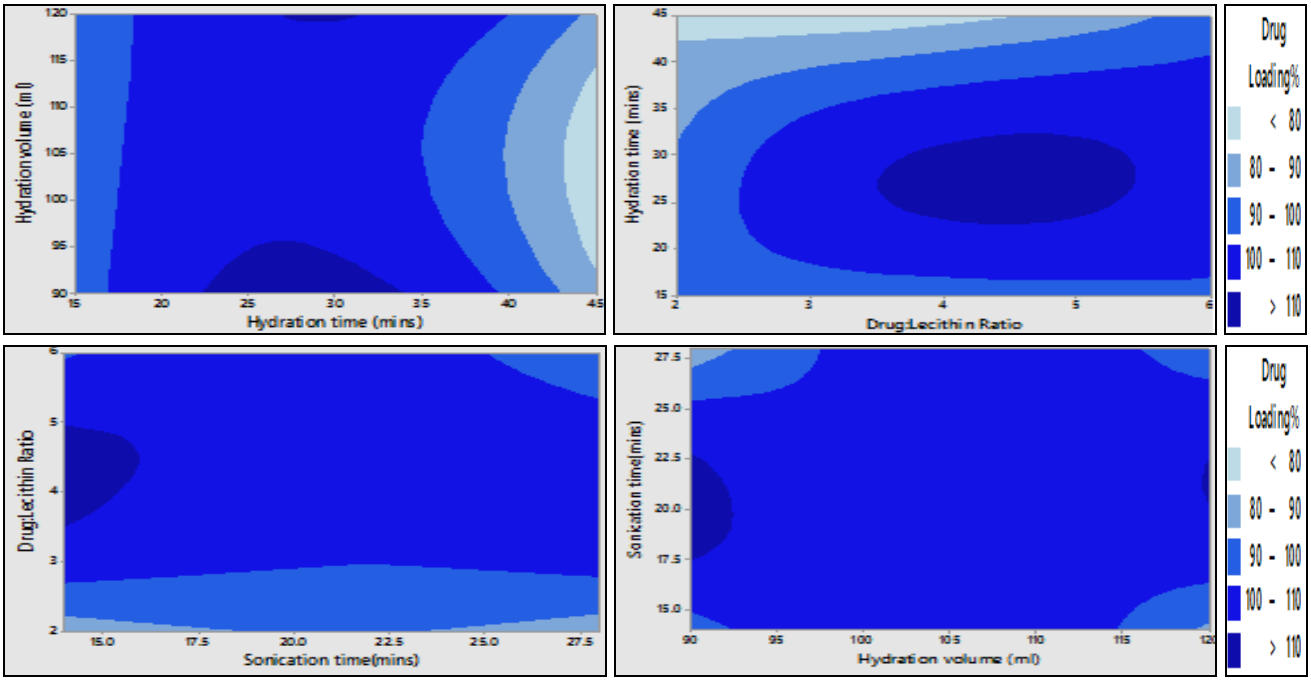


FIG. 8: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE % DRUG LOADING

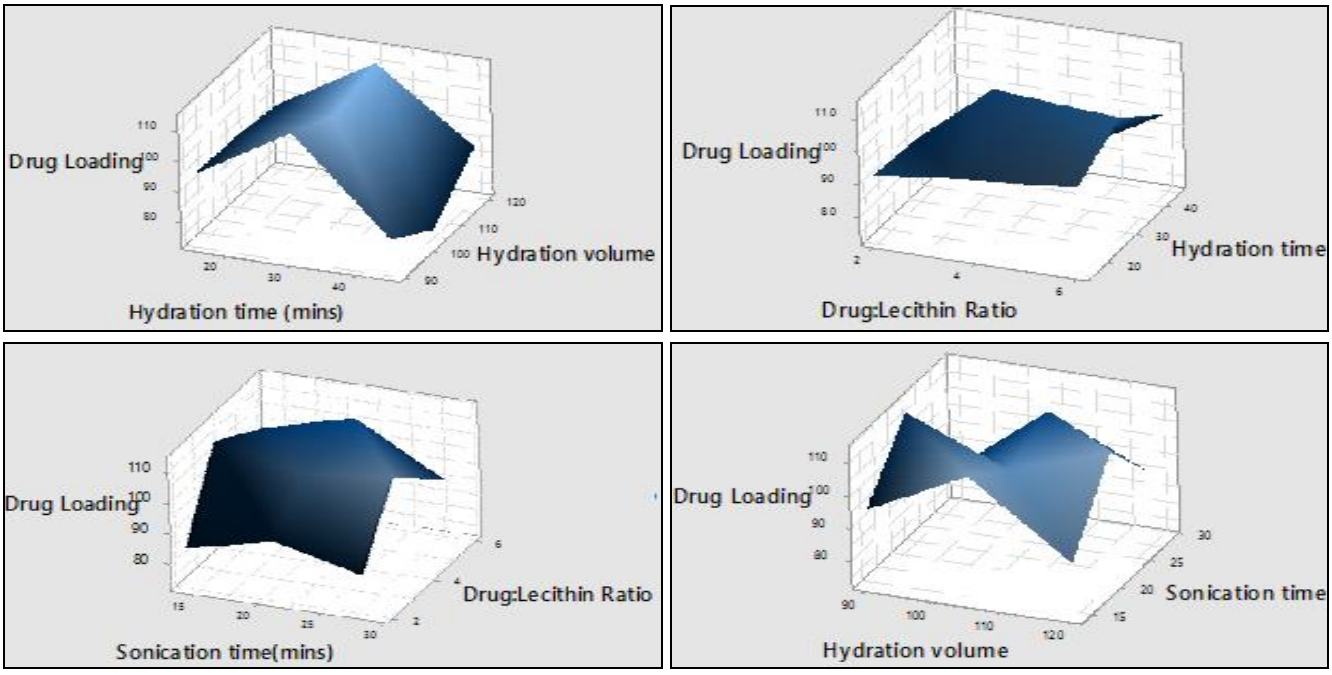


FIG. 9: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE % DRUG LOADING

Effect of the Process Parameters on the % Drug Loading: Liposome drug loading is described as the percentage of drug entrapped in comparison to the lipid utilized (drug to lipid ratio). Drug loading is a challenge for hydrophilic drugs compared to hydrophobic drugs. By increment in drug concentration the drug loading can be enhanced for hydrophilic drug. As observed from **Fig. 8** and **9**, drug loading is between 90 to 110% when hydration time is 17 to 42 min and all hydration volume and drug loading is 90 to 110% with every Drug: lecithin Ratio and increasing hydration time till 30 min. As drug: lecithin ratio decreases (0.1:2) and hydration volume increases it shows less drug loading. Data suggested the % drug loading ranges from 90 - 110% with drug: lecithin ratio greater than 2 and sonication time and the % drug loading is 90 - 110% with sonication time ranging from 15 to 25 min and hydration volume is 95 to 115 ml.

Thus, drug loading depends on drug: lecithin ratio which has a positive effect. As lecithin concentration increases the drug loading increases as drug has more lipid bilayer to disperse into. The sonication time has negative effect on drug loading. This may be attributed to the higher energy applied to the system which makes its unstable.

Effect of the Process Parameters on Entrapment Efficiency: Encapsulation efficiency is defined as the percentage of drug entrapped into the liposomes in comparison to the total amount of drug. For hydrophobic (water - insoluble) drugs the encapsulation is in hydrophobic regions / phospholipid bilayer of the liposomes. For efficient drug entrapment, three concepts can be applied, 1) the lipid concentration is kept constant and visceral aqueous volume is increased, 2) the particle size of liposomes is maintained same while intensifying the vesicle population, 3) the phospholipid bilayers expedite the diffusion of drugs. Data suggested the entrapment efficiency greater than 70 when hydration volume is 110 - 120 ml and hydration time is 25 - 35 min and the entrapment efficiency greater than 70 when hydration time is 25 - 35 min and drug: lecithin ratio is 3 to 4. The entrapment efficiency is greater than 60 for all sonication time and when Drug: lecithin ratio is between 2 and 5 as observed from the **Fig. 10** and **11**. The entrapment efficiency is greater than 60 for all sonication time and when hydration volume is more than 95 ml.

Thus, drug: lecithin ratio and hydration volume are the important parameters affecting entrapment efficiency. The percentage entrapment efficiency increases with increase in Drug: lecithin ratio up to 0.1:3.5 which can be attributed to their dependency on lipid concentration but as the lipid concentration is further increased there is decrease in the percentage entrapment efficiency which may be due to sustained release of drug for longer period.

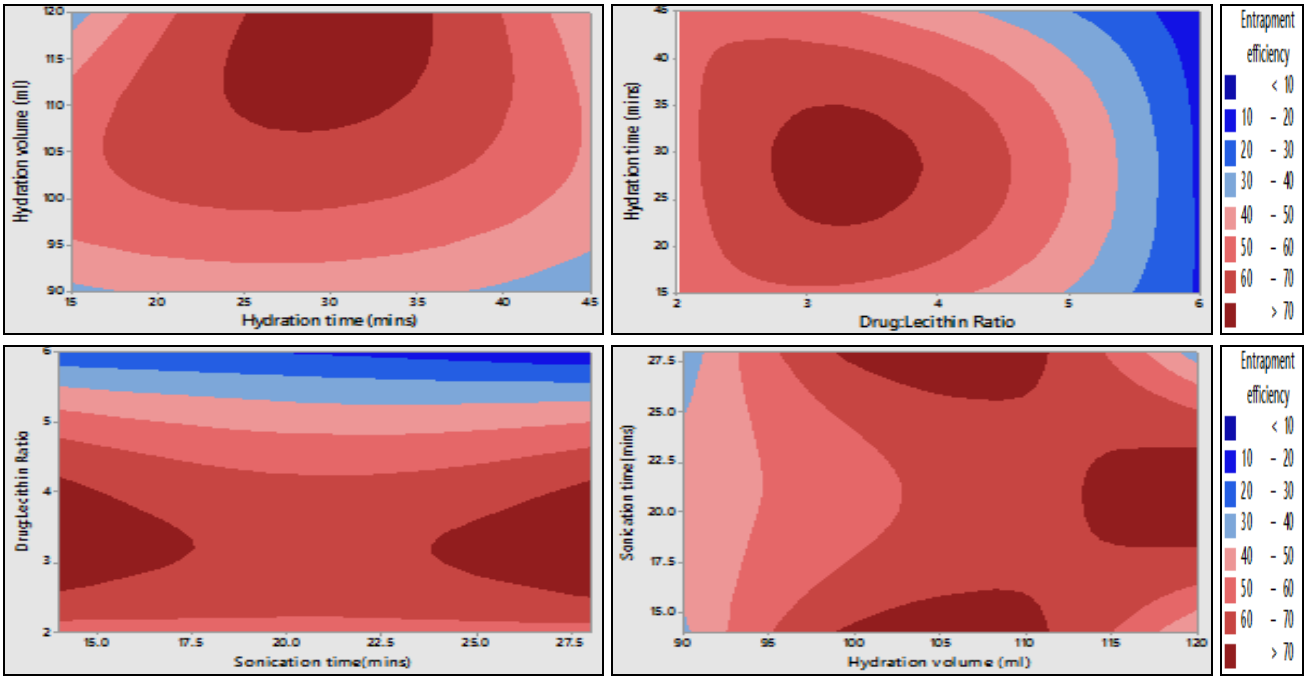


FIG. 10: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE ENTRAPMENT EFFICIENCY

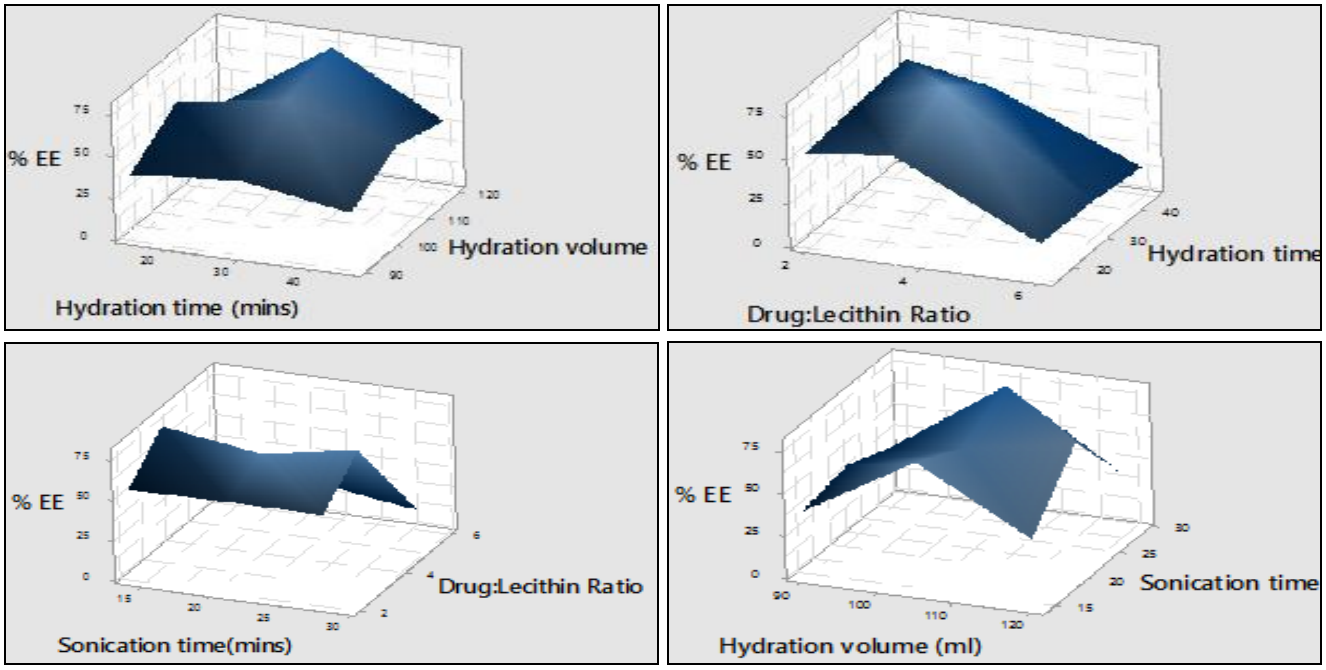


FIG. 11: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE ENTRAPMENT EFFICIENCY

Effect of Process Parameters on Redispersion: Redispersibilty is carried out to check the change in particle size after aggregation. Data suggested from **Fig. 12** and **13** particle size ranges from less than 120nm to 160 nm for all hydration volumes and when hydration time is 32 to 45 min. From figure 12 and 13 it can be observed that particle size is between less than 120 nm to 160 nm for all Drug: lecithin ratios when hydration time is above 25 min. Thus, increase in hydration time and Drug: lecithin there is decrease in particle size. Particle size is less than 180 nm all sonication time and drug: lecithin ratio. At sonication time higher than 28 min the particle size is greater than 120 nm. Particle size is less than180 nm for all sonication time and hydration volume. At sonication time 28 min the particle size is less than 120 nm.

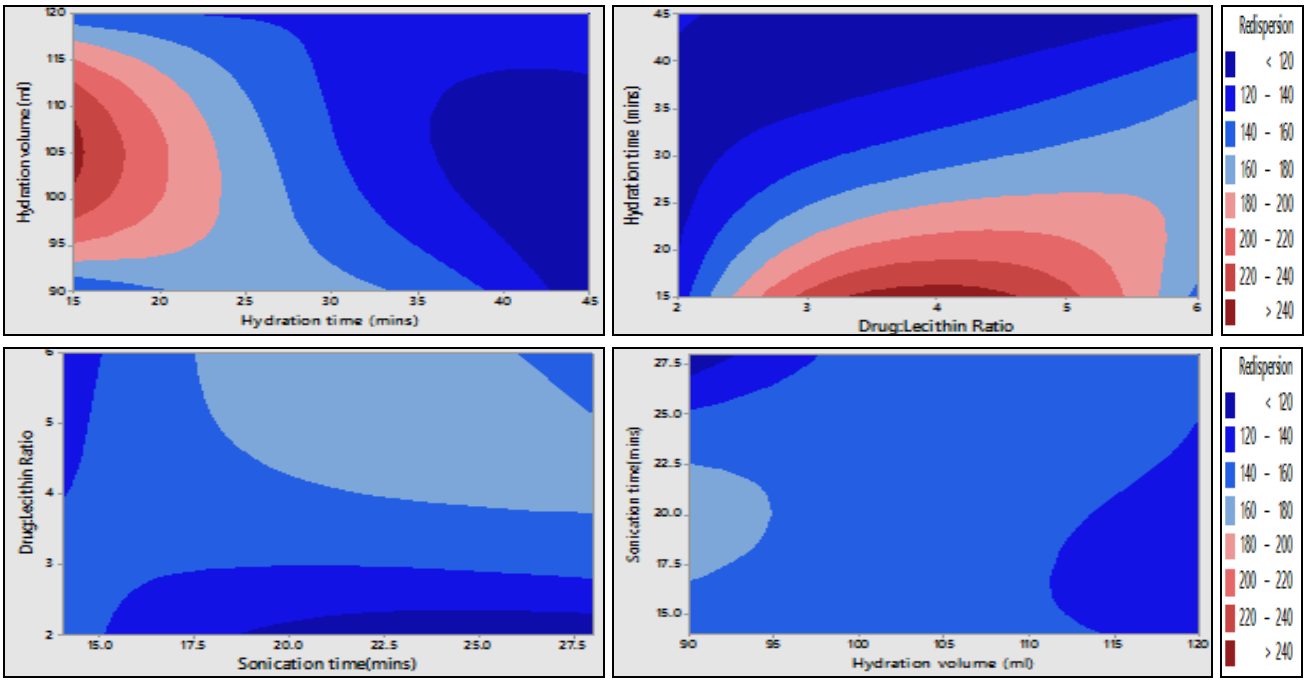


FIG. 12: EXPERIMENTAL RESPONSE CONTOUR PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE REDISPERSION

The higher the hydration time and sonication time, the lower is the particle size. As the hydration time increases, the ease of hydration of vesicles increases and so the ease of downsizing of the particles/liposomal vesicles increases resulting in the lower particle size. And then, higher the sonication time, higher is the time the vesicles being exposed to sonic energy and thus helps in disrupting the large multilamellar vesicles (LMV) to small unilamellar vesicles (SUV). This results in the lower particle size to the liposomal vesicles. The zeta potential data can be related to the ease of redispersion.

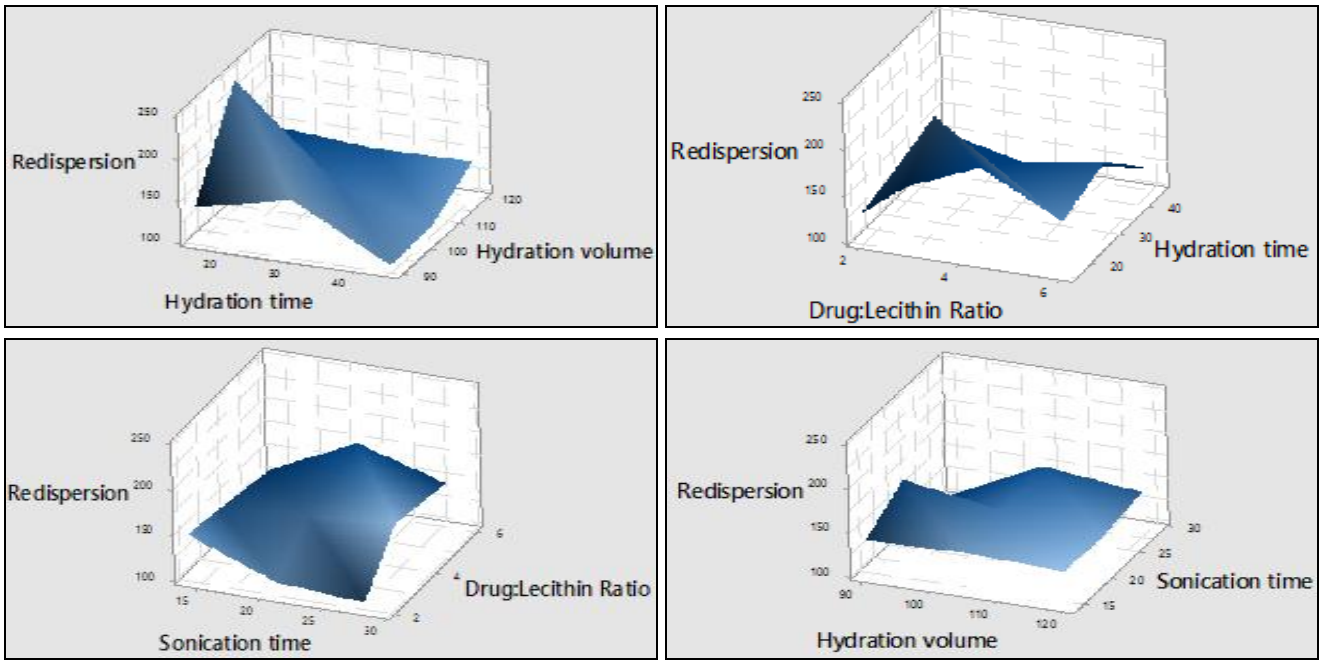


FIG. 13: EXPERIMENTAL RESPONSE SURFACE PLOTS DEPICTING THE INFLUENCE OF THE MAIN PROCESS PARAMETERS ON QUALITY ATTRIBUTE REDISPERSION

Zeta Potential: Zeta potential was for the liposomes containing FBT were determined using the method described previously. All the formulations exhibited a negative charge indicating the association of lecithin molecules at the liposomal surface regarding the absence of positively charged molecules. The zeta potential was found to be in the ranges between -20.1 mv and -28.3 mv. Thus, the liposomal formulations were found to be stable [30].

Differential Scanning Calorimetry (DSC): Differential scanning calorimetry study was carried out all drug loaded liposomal formulations helps in understanding the polymorphic changes underwent by the drug (FBT) in the presence of other excipients used in the formulation.

DSC thermogram of pure FBT showed a sharp endothermic peak (melting point) at 81.90 ºC. The DSC thermogram of soya lecithin showed endotherm at that of mannitol exhibited the endothermic peak at 166.21 ºC. The thermograms of Lecithin showed the broader and indistinct endothermic peak at 168.21 ºC.

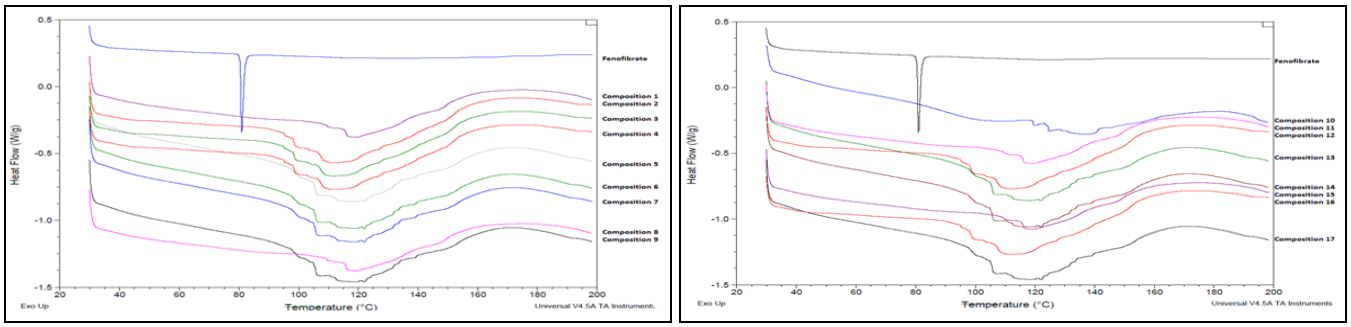


FIG. 14: DSC ANALYSIS OF DRUG LIPOSOMAL FORMULATION

The thermogram of FBT loaded liposomes interestingly showed the disappearance of the melting endothermic peak of FBT and mannitol. The melting peak of lecithin was found to be shifted from higher melting point to lower melting point, signifying that all lipid components interact with each other to great extent while forming the lipid bilayer. The nonappearance of the melting endothermic peak suggested significant interaction of FBT with lipid component leading to enhanced entrapment of the drug **Fig. 14** [32]. The data suggests that fenofibrate is molecularly dispersed in the liposomes and significant physical interaction between FBT and lipid components.

Fourier Transform Infrared Spectroscopy (FTIR): The interaction of the fenofibrate and lecithin was examined through FTIR studies to ensure the chemical integrity of the drug. FENO has four functional groups that can acts as a proton acceptor, two hydroxyl groups (-O-H) groups and two oxygen atoms of carbonyl (C=0) but it lacks proton donor. FTIR spectrum of pure fenofibrate showed characteristics bands at 2985 cm^{-1} corresponding to C-H aliphatic stretch, 3000 cm^{-1} shows C-H aromatic stretch. 1466 cm^{-1} and 1539 cm^{-1} C=C stretch, 1729.06 cm^{-1} due to ester group and 1651.49 cm^{-1} due to C=O group. The chemical structure of mannitol contains -OH groups. The IR spectrum of mannitol (figure) shows transmission at 3287.79 cm^{-1} represents the symmetric and asymmetric hydroxyl stretching vibration due to many intermolecular H-bond and aromatic bond at 2948.15 cm^{-1} due to C-H stretching. The IR spectrum of lecithin **Fig. 15** shows transmission at 2924.56 cm^{-1} due to CH_3 stretching 825.14 cm^{-1} due to $N+-(CH_3)_3$ stretching and 1739.54 cm^{-1} to C=O. There were no major shifts in the characteristics peaks of the FBT in the liposomal drug formulation indicating the manufacturing method or the excipients had no significant effect on the chemical stability of FBT. However, small shifts and broadening of the characteristics peaks might be due to weak interaction forces between the drug and the lecithin.

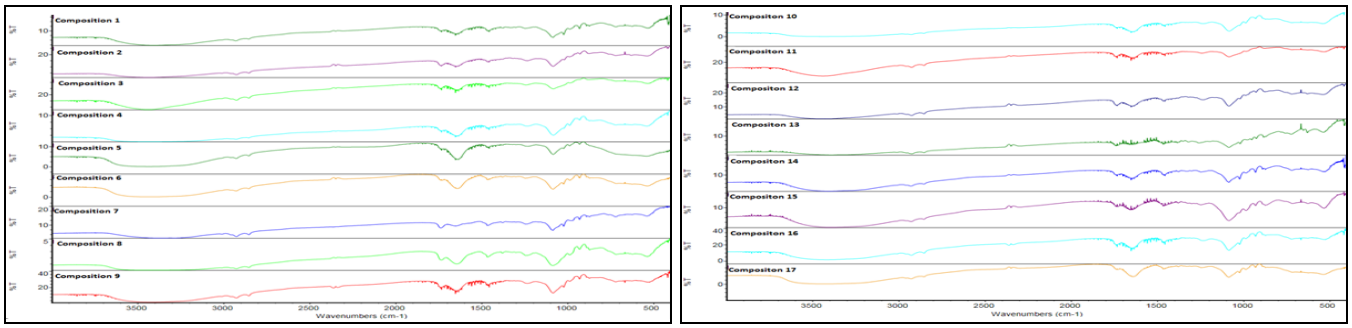


FIG. 15: FTIR ANALYSIS OF DRUG LIPOSOMAL FORMULATION

***In-vitro* Dissolution Studies:** One of the principal factors affecting oral absorption is the dissolution rate. According to Noyes-Whitney equation, increase in the surface area of particles by size-reduction enhances the dissolution rate [31]. Dissolution studies were performed in deionized containing SLS to evaluate the effect of changing process parameters on the dissolution rate of FBT liposomal formulation. Dissolution studies performed for pure fenofibrate in water having SLS showed dissolution profiles having 21.90%, 13.98% and 7.00% drug release. All the formulations with the equivalent amount of drug showed improved dissolution in comparison to pure drug release. This significant enhancement in the dissolution rate is addition to decrease in the particle size.

All the Compositions having drug: lecithin ratio 0.1: 3.5 has shown percentage drug release ranging from 22.61 % to 44.69 % in the **Fig. 16A** and **16B**. composition 14 has shown 44.69% drug release and composition 6 has shown 22.61% drug release. Thus, it can be interfered that percentage drug release of liposomal formulation is increased in comparison to the pure FBT which was 13.98%. It can be inferred from composition 14 which has shown the best dissolution that the formulation which has highest hydration time showed more dissolution. The particle size in range of 120 - 190 nm showed better dissolution. While from composition 6 which showed slowest dissolution, it can be inferred that the lower hydration time affects the dissolution. The particle size is the other factor affecting the dissolution.

The dissolution increases with decrease in particle size. The compositions having drug: lecithin ratio 0.1: 6 has shown percentage drug release ranging from 44.054% to 74.608% as shown in the **Fig. 16C**. Composition 4 has shown 74.60% drug release after 120 min while Composition 9 showed 44.05% drug release after 120 min. Thus, it can be interfered that percentage drug release of liposomal formulation is increased in comparison to the pure FBT which was 21.90%. It can be inferred that the hydration time affects the dissolution. At higher hydration time the dissolution is better than compared to lower hydration time. This is understood from the results for composition 4 which has highest hydration time and composition 9 which has lowest hydration time. The particle size greater than 150 nm showed better dissolution than compared to less than 150 nm. The particle size is the other factor affecting the dissolution. The dissolution increases with decrease in particle size. The compositions having drug: lecithin ratio 0.1: 2 has shown percentage drug release ranging from 21.39% to 45.22% as shown in the figure. composition 3 has shown 45.22% drug release while composition 17 has shown 21.39% drug release. Thus, it can be interfered that percentage drug release of liposomal formulation is increased in comparison to the pure FBT which was 7.00%. It can be inferred that the hydration time affects the dissolution. At higher hydration time the dissolution is better than compared to lower hydration time. This is understood from the results for composition 3 which has highest hydration time and composition 17 which has lowest hydration time. The particle size less than 200 nm showed better dissolution than compared to greater than 200 nm. The particle size is the other factor affecting the dissolution. The dissolution increases with decrease in particle size.

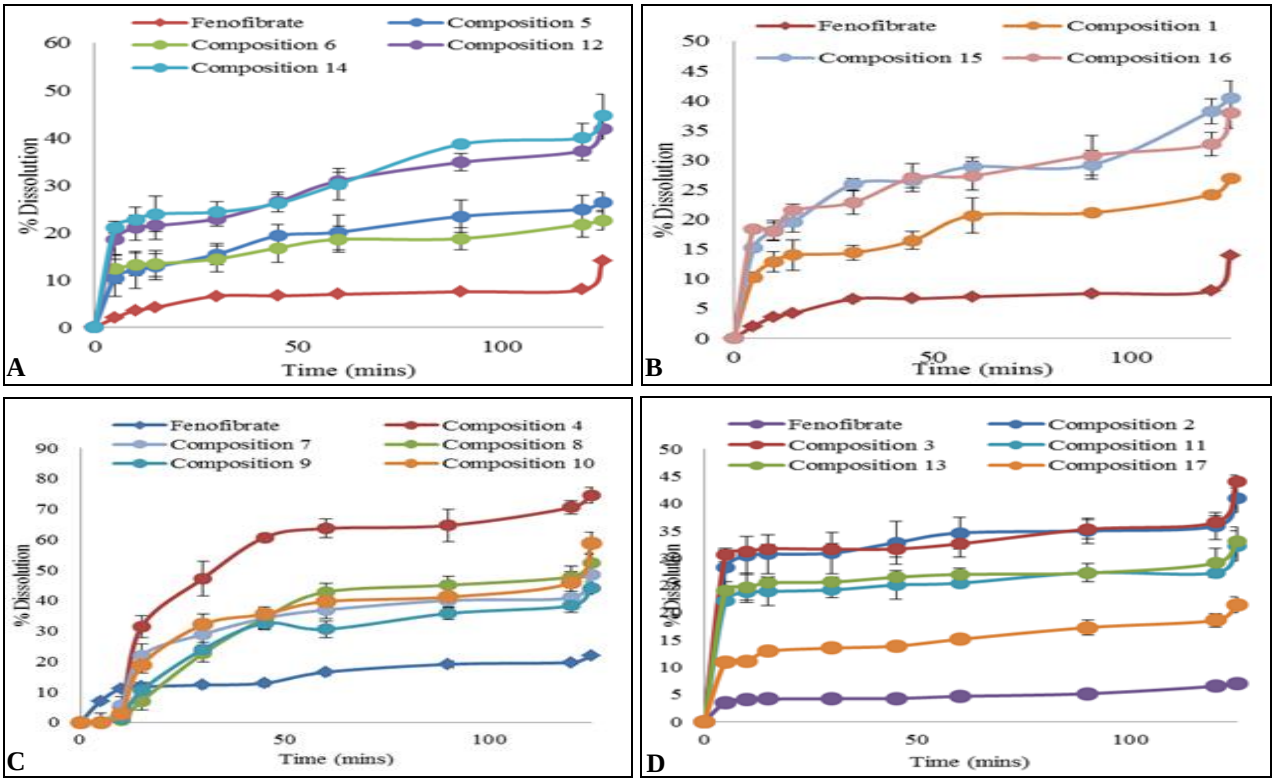


FIG. 16: DISSOLUTION PROFILES OF LIPOSOMAL FORMULATIONS HAVING DIFFERENT DRUG: LECITHIN RATIO IN DEIONIZED WATER (0.3 gm SLS) RPM 75 A) RATIO 0.1: 3.5 B) RATIO 0.1: 3.5 C) RATIO 0.1: 6 D) RATIO 0.1: 2

CONCLUSION: The current study demonstrated the usefulness of the application of design of experiments to gain a comprehensive under-standing of formulation and processing parameters affecting liposome formulations prepared via thin film hydration technique. Drug to lecithin ratio, hydration volume, hydration time and sonication time were identified as critical parameters affecting particle size, polydispersity Index, peak shape, drug loading, drug entrapment and redispersion. Using the generated experimental screen, a design space for liposome preparation was

established, within which preparation variability is minimized and product quality can be optimized. The particle size of the liposomal formulation was found to be between ranges of 99.5 nm to 290 nm. The decrease in particle size is related to higher hydration time (45 min) and sonication time (28 min) as observed from the contour plots and surface plots. Peak shape was found to be mono-modal, bi-modal or multi modal for the liposomal formulation based on the effect of the quality attributes. The increase in drug: lecithin ratio and sonication time showed positive effect on peak shape as studied from surface plots and contour plots. The higher hydration volume and hydration time showed negative effect.

The polydispersity indices obtained from readings are in range of 0.136 to 0.675 which indicate that the liposomes were monodispersed to poly-dispersed. Higher the drug: lecithin ratio, lower is the PDI value. This might be due to the varying amount of lecithin. The drug loading for the liposomal formulation was between 72.56 to 112.55%. Thus, drug loading depends on drug: lecithin ratio which has a positive effect. As lecithin concentration increases the drug loading increases as drug has more lipid bilayer to disperse into.

The sonication time has negative effect on drug loading. This may be attributed to the higher energy applied to the system which makes its unstable. The entrapment efficiency was found to be 2.4% to 77.41% for the liposomal formulations. Entrapment efficiency is greater than 70 with drug: lecithin ratios 0.1: 3.5 and higher hydration volumes. Redispersibilty data shows there was no major difference in the particle size.

DSC thermogram of FBT, showed sharp endothermic peak however, the FBT loaded liposomes showed disappearance of melting peak for FBT, indicating the molecularly dispersion of FBT in liposomes and significant physical interaction between FBT and lipid components. The FTIR spectrum showed distinctive peaks for FBT which were also observed in the liposomal formulation hence, the drug had no strong interaction with lecithin chemically. Dissolution studies of all formulations showed enhanced dissolution compared to the pure FBT. The formulations having higher hydration time and less particle size showed enhanced drug release in comparison to the drug release of equivalent amount of fenofibrate. The drug to lecithin ratio and hydration volume has significant impact on drug entrapment capacity of the formulation and *in-vitro* dissolution rate. Lastly, the methods and principles used in the current study can be applied to liposomes containing other molecules and can provide time and cost savings to industrial formulation scientists, which will result in a more robust liposome preparation process.

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CONFLICT OF INTEREST: The authors declare that they have no competing interests.

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