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Design and development of optimized nanosuspension of Fenofibrate; An Invitro approach

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***Background*:** The aim of the study is to prepare nanosuspension in order to improve the dissolution rate and bioavailability of lipophilic fenofibrate.

***Method*:** Anti-solvent precipitation followed by sonication technique was adapted, and poloxamer188 was selected as surfactant. This method was selected by using various trails consisting of Solvent to antisolvent volume ratio (1:10, 1:15, 1:20),Amount of stabilizer (30 mg, 40 mg, 50 mg), Stirring speed (800RPM, 1000RPM, 1200RPM) and Sonication time (5 min, 10 min, 30 min, 60 min) etc. Later factorial design was adopted to

***Results*:**From the statistical optimization, it was evident thatthe model predicted that the formulation with particle size 121 nm, Polydispersibility index 0.437 and drug release at 4 hr is 79.5% for fenofibrate nanosuspensions. The dissolution rate of fenofibrate nanosuspension was increased obviously. Therefore, nanosuspensions may be a suitable delivery system to improve the bioavailability of those drugs with poor water solubility.

1. Introduction

Hyperlipidemia is also known as high cholesterol or also known as elevated concentration of all lipids or any in blood. When body has higher levels than the required amount of cholesterol it forms plaque in arteries. It forms thick, hard plaque and can clog arteries. It prevents oxygen and blood flow and can cause heart attack and stroke. Elevated plasma cholesterol levels and LDL leads to atherosclerosis in humans. As per 2020 census, WHO report, 60% of cardiovascular cases occurred in India. Total cholesterol lower than 200 mg/dl is considered as safe. [1,2] TC greater than 240 mg/dl is treated as greater risk. Triglycerides, another type of fat is carried in the blood as VLDL. TG less than 150 mg/dl is considered as safe. TG 200-499 mg/dl is a risk factor. LDL, a bad cholesterol produced by the liver carries cholesterol, other lipids from liver to various sites of the body muscles, tissues, organs and heart. LDL less than 100 mg/dl is safe and considered as high in the range of 160-189 mg/dL. HDL is a good cholesterol and below 40mg/dl is risk for cardiovascular diseases. [3,4]

S.no	Classification of antihyperlipidemic drugs	Examples	Mechanism of action
1.	HMG Co-A reductase inhibitors	Atrovastatin, Fluvastatin, Lovastatin, Pravastatin, Rosuvastatin, simvastatin	These drugs are similar to HMG Co-A reductase, inhibit the biosynthesis of cholesterol in the liver which decrease plasma levels of Tc, LDL and Apo B.
2.	Bile acid sequestrants	Cholesteramine	These are positively charged

		Colestipol.	resins which bind to negatively charged bile acids in intestine to form insoluble complex that are not absorbed and excreted in feces. They increase HDL and lipoprotein levels.
3.	Fibric acid derivatives (Fibrates)	Fenofibrate, Gemfibrozil	lipolysis, hepatic fatty acid uptake, removes LDL particles by increasing LDL catabolism, HDL formation and stimulation of reverse cholesterol transport.
4.	Niacin derivatives	Niacin, Nicotinic acid	Inhibits the synthesis of hormone sensitive lipase, so it decreases the triglycerides lipid lysis which is the producer of free circulating fatty acids which help in formation of triacylglycerol, so it inhibits the secretion of VLDL and it lowers the LDL levels.
5	Cholesterol absorption inhibitors	Ezetimibe	Inhibits the small intestinal absorption of phytosterols and cholesterols.

Table no 1. Showing Classification of antihyperlipidemic drugs with their mechanism of action[5,6,7,8,9,10,11,12,13]

The bioavailability of poorly water-soluble hydrophobic drugs [Biopharmaceutics Classification System (BCS) Class II] is limited by their solubility and dissolution rate, posing great challenges in their formulation development.[14] One approach to address this problem is by producing nanometer or micrometer size particles leading to an increase in surface area to improve the dissolution rate of hydrophobic drugs.[15] This is usually done using two broadly classified methods: (i) top-down processes and (ii) bottom-up processes. Numerous top-down and bottom-up methods have been developed to produce micro or nano drug particles. Liquid antisolvent precipitation (LASP), a bottom-up approach, is a relatively rapid, less-energy intensive and costeffective method. [16,17]

Nanosuspension is defined as a sub-micron colloidal system it's contains the poorly soluble drug, waver in a suitable dispersion medium stabilised by the surfactants. [18].Nanosuspension usually consists of colloidal carriers like polymeric resins which are inert. They help in the enhancement of drug solubility and thus bioavailability. Nanosuspension also imparts stability within the formulation. [19,20]

II Preformulationstudies: The organoleptic characteristics like color, odor and texture were observed by sensory organs. The melting point was determined using capillary fusion method where a small amount of drug was filled in a capillary sealed from one side and kept inverted i.e. sealed end downwards into the melting point apparatus. The temperature at which drug started liquefy was recorded and compared with literature value.

- **Determination of Wavelength of Fenofibrate:**1000 µg/mL of stock solutions of Fenofibrate was prepared by dissolving 50 mg of drug in 50 mL of methanol and diluting 1ml of above solution to 10 ml with methanol. The 10 µg/ mL of drug concentrations were then prepared and scanned using UV spectrophotometer to determine the λmax of drug. Observed λmax of Fenofibrate was at 290 nm.
- **Solubility of Fenofibrate pure drug:** The solubility study of Fenofibrate was carried out in various solvents. Accurately weighed 20 mg of drug was added to screw capped vials containing 10 mL of solvent. The vials were kept in a water bath shaker at 37±0.5 ℃ and shaken for 24h. The mixtures were then filtered through milliporefiltermembrane of pore size 0.45 µm, diluted and drug was analyzed using UV spectrometer. The result of solubility study is shown in the Table.
- **Determination of Calibration curve of Fenofibrate:**

  10 mg of fenofibrate weighed accurately and transferred in to 100 mL volumetric flask. Sufficient quantity of ethanol was added to the flask to dissolve the drug and the solution was sonicated for 15 min and then diluted up to 100 ml with same solvent, so as to obtain concentration of 100 µg/mL. This stock solution was further diluted for

calibration curve.A series of fenofibrate solution ranging from 5 to 30 µg/ mL were prepared from standard solution. Different aliquots (0.5, 1.0, 1.5 2.0, 2.5 and 3.0 mL) of a standard Fenofibrate (1000 µg/mL) solution were transferred into a series of 100 ml calibrated flasks and all were made up to the mark with ethanol and absorbance was measured at 290 nm against blank.

- **DSC**:Thermal analysis was performed using differential scanning calorimetry (DSC Q1000, TA instrument). Approximately 2 mg of sample was weighed in a crimped aluminum pan and measured under nitrogen purge with a heating rate of 10 °C/min over the temperature range of 0-300 °C. It was observed that Fenofibrate showed a sharp endothermic peak at 98^0c.
- **XRD**: Area under peak is high for FBT pure drug, so the peaks are sharp indicating crystalline geometry.

IIIMaterials and Methods

Materials

Fenofibrate has a very low aqueous solubility but a high permeability. Fenofibrate was purchased from Jai Radhe Sales (Ahmadabad, India). The solvent acetone (ACS reagent, 99.5%) was purchased from Sigma Aldrich (St. Louis, MO). The stabilizers like Polyvinyl Alcohol, PVP K-30, Sodium Lauryl Sulphate, Poloxamer 188 and Poloxamer 407 were a donation from Nisso America Inc. (New York, NY) and Dow Chemicals (Midland, MI), respectively.

Methods

Selection of solvent and anti-solvent

The solubility of selected drug was studied in different solvents and their combinations. Selection of good and poor solvent was done based upon the solubility of the drug in respective solvents. About 10 mg of drug was added to 10 ml of solvent in specific gravity bottles. This amount was sufficient to obtain a saturated solution. These specific

gravity bottles were shaken at 100 RPM for 24 h at 25ºC by keeping in a cryostatic constant temperature reciprocating shaker bath. The bottles were then opened and solutions were filtered with the help of whatmanfilter paper (0.22 µm). The absorbance of the solution was measured at the respective λ_{max} of the drugs. This method was repeated for three times.

Method of Preparation by Anti-solvent precipitation followed by sonication technique

The drug powder was dissolved in 10ml of suitable solvent to form a clear solution. 0.5 g of Fenofibrate was dissolved in 10ml of DMF. This solution was then transferred in to a beaker containing aqueous surfactant solution drop wise with continuous stirring under a magnetic stirrer until the entire solvent has been evaporated. In case of Fenofibrate the drug being insoluble in water precipitates out as fine particles. The stirring was continued for further 15 minutes and the precipitated drug suspension was then sonicated using a Probe sonicator. The drug suspension was kept in an ice bath and sonicated with 80% amplitude at 2s pulse rate for 15min. The procedure was repeated using different sonication time for optimization of the technique. Nanosuspensions were prepared using different types of stabilizers at various concentrations using the above procedure.

Selection of stabilizer:

Different stabilizers like Polyvinyl Alcohol, PVP K-30, Sodium Lauryl Sulphate, Poloxamer 188 and Poloxamer 407 were screened by preparing nanosuspensions and measuring their saturation solubility, mean particle size, polydispersity index (PDI) and zeta potential for selection of the best one which can be utilized for further research work.

Optimization of preliminary parameters

Preliminary parameters were optimized by varying one parameter at a time while keeping others constant, so that effect of varied parameters could be evaluated. Each batch was

repeated thrice (n=3) for the confirmation of repeatability. The parameters were optimized to achieve minimum particle size and maximum saturation solubility. Optimized parameters were

- Solvent to antisolvent volume ratio (1:10, 1:15, 1:20),
- Amount of stabilizer (30 mg, 40 mg, 50 mg),
- Stirring speed (800RPM, 1000RPM, 1200RPM),
- Sonication time (5 min, 10 min, 30 min, 60 min) etc.

Factorial design for optimization of key parameters

A Full factorial Design for two factors at three levels each was selected for the formulation and optimization of the nanosuspensions using variables. The concentrations of **diffusing drug and the amount of stabilizer (Polaxomer 188) used was selected as the two factors** and were accordingly varied, and the factor levels were suitably coded. The suggested formulations given by the DesignExpert software (Version 12.0.7.1, Stat-EaseInc., Minneapolis, MN) weresystematically prepared and characterized for particles size, polydispersity index and drug release. These parameters, the particle size (nm), the polydispersity index and the percentage of drug released after 4 hours were taken as the response variables.

In this design, two factors were evaluated, each at three levels, and experimental trials were performed for all possible combinations. All other formulation variables and processing variables were kept invariant throughout the study. The effect of the two independent variables diffusing drug concentration (X1) and stabilizer concentration (X2) on the response (Y) was observed. The regression equation for the response was calculated using the

following equation

$$Y = b0 + b1X1 + b2X2 \qquad \text{Eq...(1)}$$

$$Y = b0 + b1X1 + b2X2 + b12X1X2 \quad \text{Eq...(2)}$$

$$Y = b0 + b1X1 + b2X2 + b11X12 + b22X22 + b12X1X2 \quad \text{Eq...(3)}$$

Where, Y is the dependent variable, while b0 is the intercept, bi (b1 and b2), bij (b12) represents the regression coefficient for the second order polynomial equation and Xi represents the levels of independent formulation variables. Mathematical modeling was carried out by using equation (3) to obtain a second order polynomial equation. The values of dependent variable obtained at various levels of two independent variables (X1 and X2) were subjected to multiple regressions to yield a second order polynomial equation. The main effects of X1 and X2 represent the average result of changing one variable at a time from its low to high value. The interaction (X1X2) shows how the particle size and saturation solubility changed when two variables were simultaneously changed.

Characterization of fenofibrate nanosuspension:

- **Size measurement and zeta potential analysis:**

The particle size and the polydispersity Index (PI) of the particles was measured by dynamic laser light scattering after suitable dilution. (90 Plus Brookhaven Instruments, USA). The measurement was done at 25ºC at a scattering angle of 90º. The zeta potential of the preparations was also measured using the clear disposable Zeta cell by electrophoretic mobility method (Zetasizer Ver. 6.11 Malvern).

- **Drug entrapment efficiency (DEE):**The freshly prepared nanosuspension was centrifuged at 20,000 rpm for 20 min at 5ºC temperature using cool ultracentrifuge (Remi Cool Centrifuge, Makers Remi, Vasai, India). The amount of unincorporated drug was measured by taking the absorbance of the appropriately diluted 25 ml of supernatant solution at 290 nm for Fenofibrate using UV spectrophotometer against

blank/control nanosuspension. DEE was calculated by subtracting the amount of free drug in the supernatant from the initial amount of drug taken. The experiment was performed in triplicate for each batch and the average was calculated (Mandal et al 2010). The entrapment efficiency (EE %) could be achieved by the following equation:

$$\mathbf{EE\ \% = [(W_{initial\ drug} \_ W_{free\ drug}) / W_{initial\ drug}] * 100}$$

- **Scanning electron microscopy (SEM):** The particle size and morphology of the dried nanoparticles was observed using scanning electron microscopy (SEM, JSM-6360, JEOL Inc., Japan). Small drop of the nanosuspension was air dried followed by oven drying and were fixed on an SEM stub using double-sided adhesive tape and coated with Au at 20 mA for 6 min through a sputter-coater (Ion sputter JFC 1100, Japan). A scanning electron microscope with a secondary electron detector was used at an accelerating voltage of 30 kV.

- **Transmission electron microscopy:** The particle size and morphology were confirmed by observation with Transmission Electron Microscope (JEM-2100, 200 kV, Jeol, Japan). The sample after suitable dilutions were fixed with copper grid and studied without staining at 200kV.

- **In vitro drug release kinetic experiments:** In vitro drug release of the nanosuspensions was determined by the dialysis membrane diffusion technique in Ethanol–water (50:50) system. One milliliter of the nanosuspensions was placed in the dialysis membrane (Mw cutoff 12000-14000 Hi-media), fixed on donor part of Kesery-Chein apparatus of surface area 1.76 cm2 and receptor volume of 20 mL. The entire system was kept at 37°C with continuous magnetic stirring. Samples (1 ml) were withdrawn from the receptor compartment at predetermined time intervals and

replaced by fresh medium. The amount of drug dissolved was determined by UV spectrophotometer at 290 nm for Fenofibrate.

4. RESULTS AND DISCUSSION

- **Preformulation Studies:**

Drug	Parameter	Reported	Observed
Fenofibrate	Appearance	Whitecrystallinesolid	Lightyellowish-white
	Odor	None	None
	Meltingpoint	79-82℃	81℃

Table 2: Preformulation Studies of Fenofibrate

Determination of Wavelength of Fenofibrate:

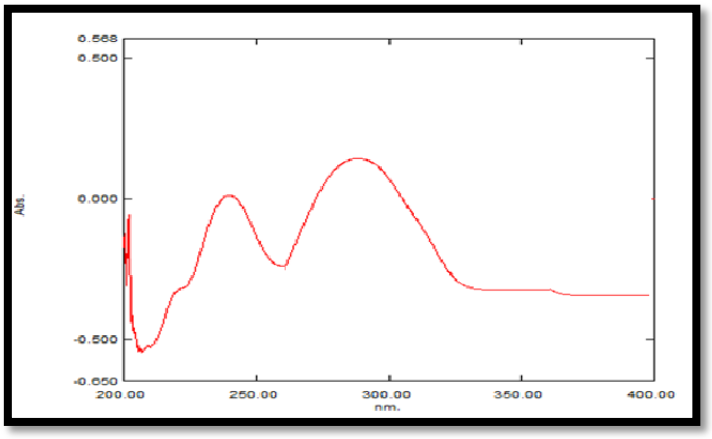
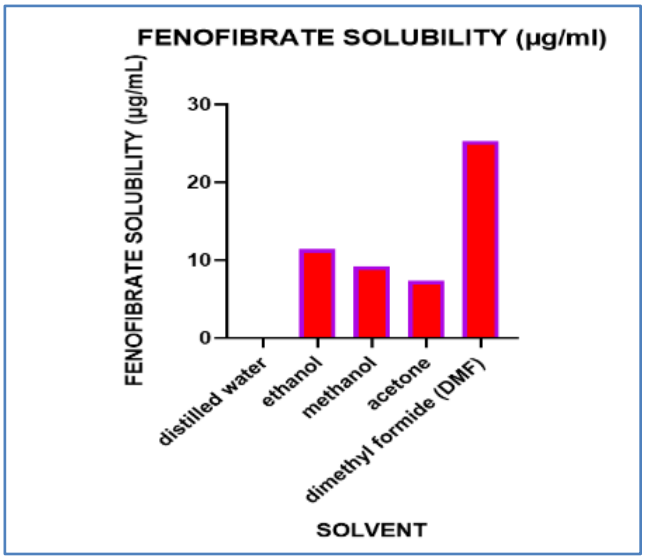


Fig 1 Wavelength of Fenofibrate at 290 nm

- **Solubility of Fenofibrate pure drug:**

Solvent	Fenofibrate solubility (µg/ml)
Distilled water	**Not soluble**
Ethanol	**11.38±0.6**



Methanol	**9.2±0.5**
Acetone	**7.3±0.14**
Dimethyl formide	**25.3±0.5**

Table no 3, Fig no 2Fenofibrate drug solubility in various solvents

❖ **Determination of Calibration curve of Fenofibrate**

Parameters	FBT-result
λ_{max} (nm)	290
Linearity range (µg/ml)	0-30
Regression equation	0.03X+0.0016
Intercept	0.0016
Slope	0.03
Correlation coefficient (R^2)	0.9998

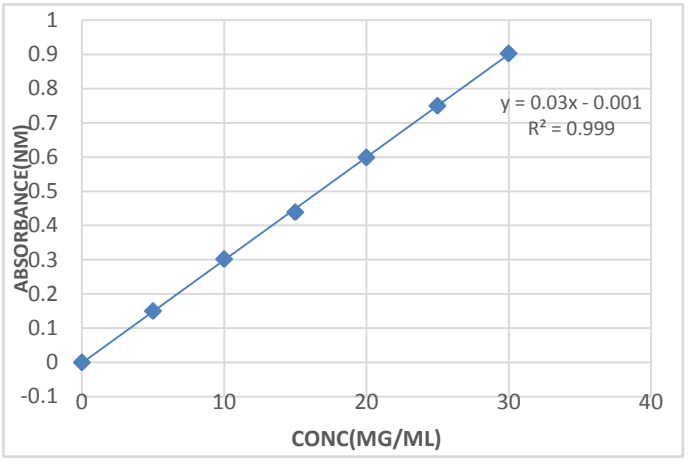


Table no 4, Fig no 3 Fenofibrate drug calibration curve

❖ **DSC**

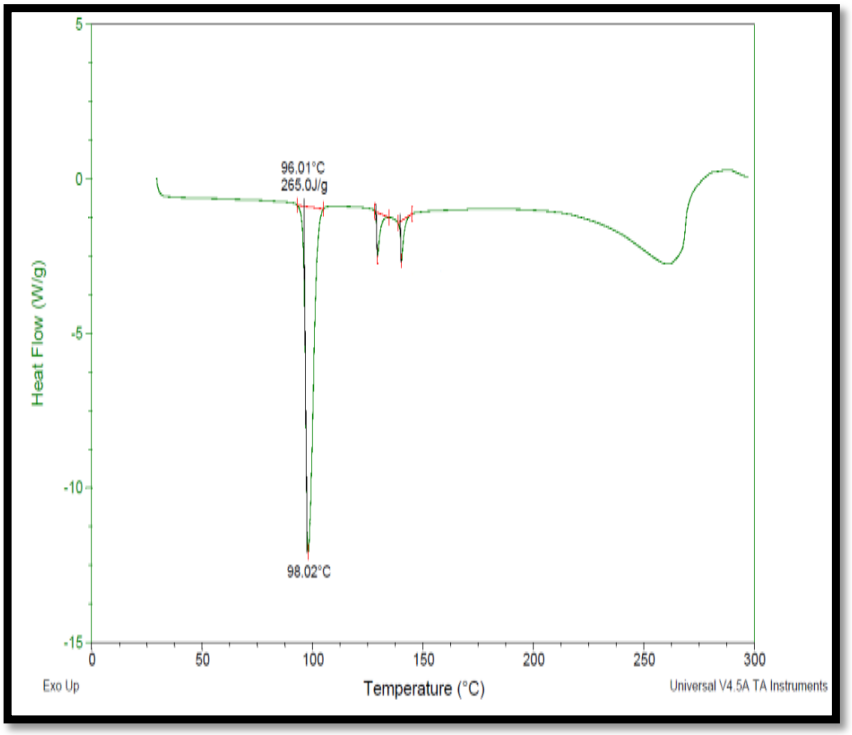


Fig no 4 DSC of Fenofibrate

❖ **XRD**

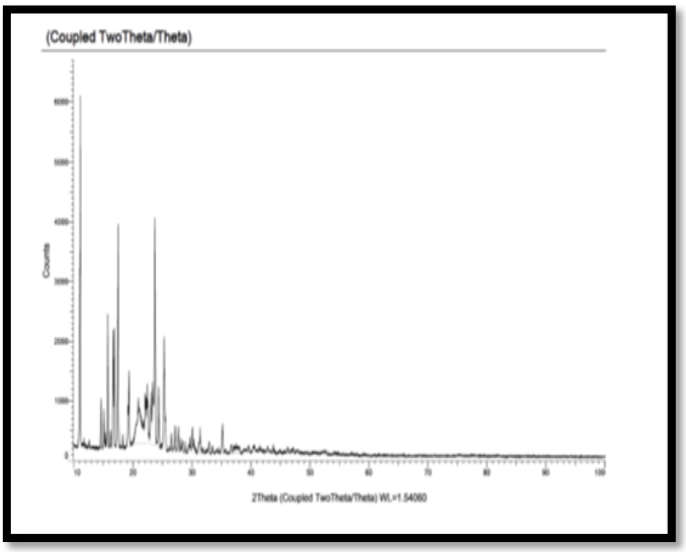


Fig no 4 XRD of Fenofibrate

❖ **Selection of solvent and antisolvent**

Selection of solvent and antisolventforFenofibratewas performed on the basis of solubility as well as particle size in various solvents and their combinations. Results indicated that Fenofibratehad showed (30mg/ml) in Dimethyl formamide (DMF) and practically insoluble in water, so they were selected as solvent and antisolvent respectively as shown in Table 1.

Drug	**Solvents**	**Solubility (mg/ml) (Mean±S. D.) ***
Fenofibrate	**DMF**	**30±0.01**
	DMSO	15±0.05

	Ethanol	1±0.02
	Water	Insoluble

***Indicates average of three readings**

Table 5: Solubility of Fenofibrate in suitable solvents

Fenofibrategave particle sizes of 325.7 nm, 504.4 nm and 645 nm,respectively in DMF, DMSO and Ethanol as shown in Fig 1, 2 and 3. Hence DMF was selected as solvent as it produced nanoparticles of smaller size as shown in Fig 4.

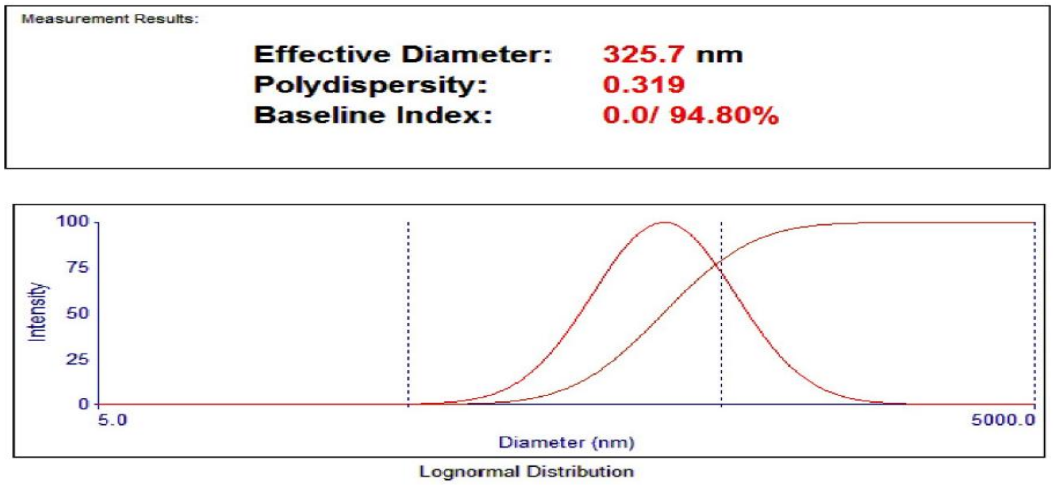


Fig 5: Particle size using the Solvent **DMF**

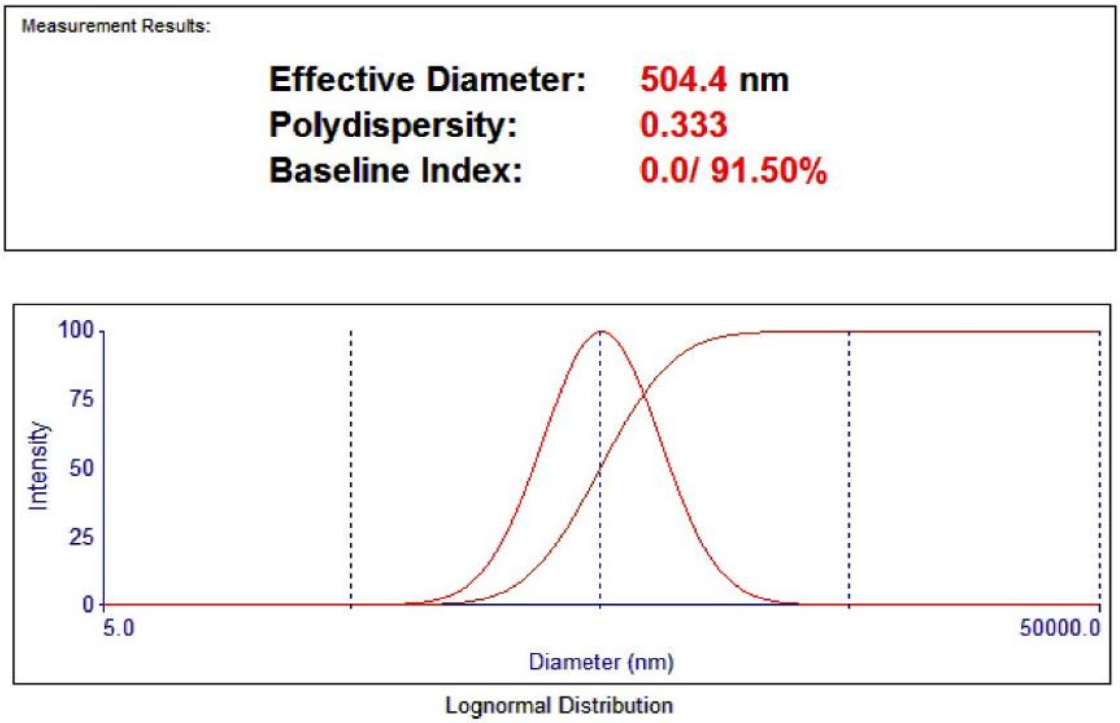


Fig 6: Particle size using the Solvent DMSO

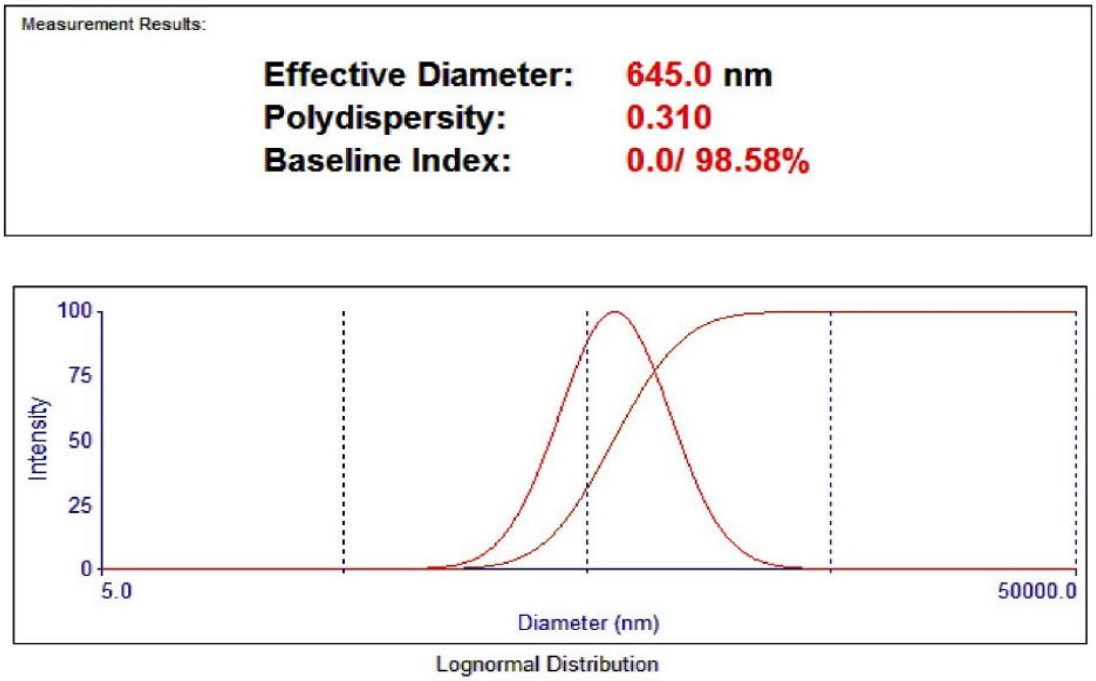


Fig 7: Particle size using the Solvent Ethanol

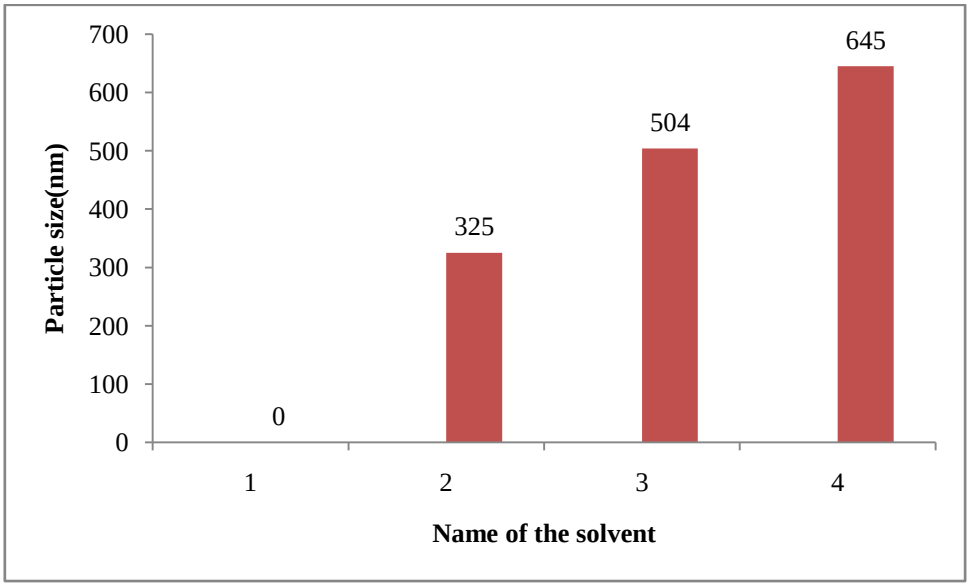


Fig 8: Impact of solvent on Particle size

Effect of Solvent-Antisolvent Ratio: From the preliminary studies the effect on particle sizes of different solvent:antisolvent ratios (1:20, 1:15, 1:10) was observed which produced particles of 276.9 nm, 424.9 nm, 684 nm, respectively, as shown in Fig 5, 6 and 7. As such formulation with S:AS ratio at1:20 showed smaller particle size (Fig 8) and it was selected for the preparation of optimized nanosupensions.

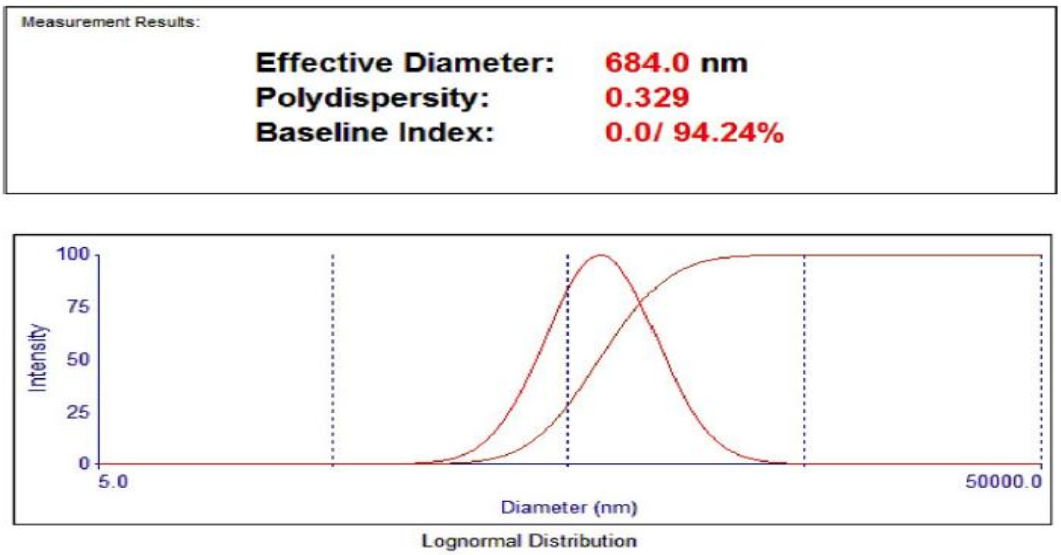


Fig 9: Particle size Analysis of Formulation with S: AS of 1:10

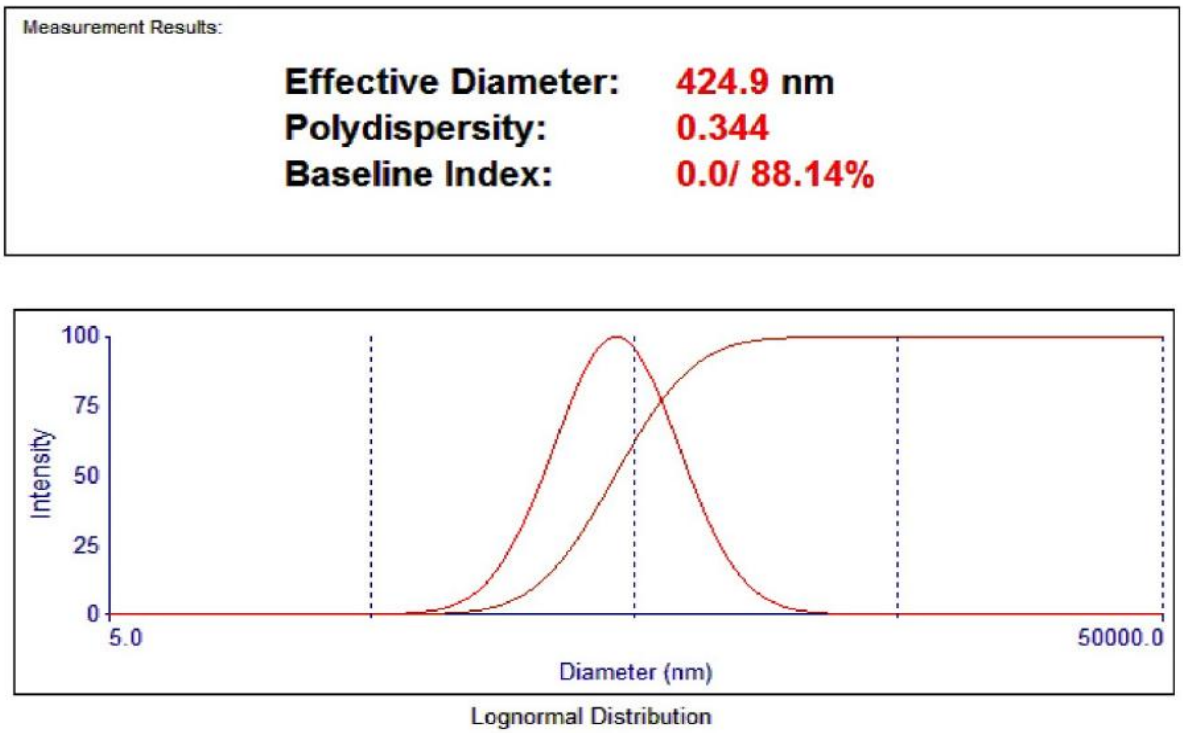


Fig 10: Particle size Analysis of Formulation with S: AS of 1:15

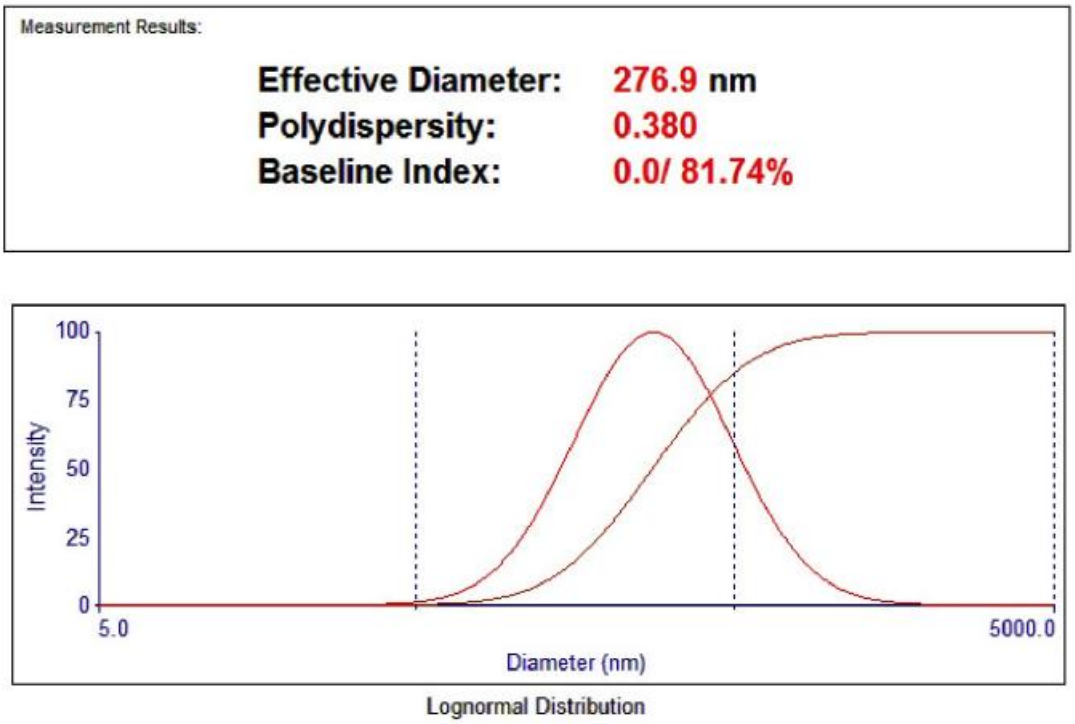


Fig 11: Particle size Analysis of Formulation with S: AS of 1:20

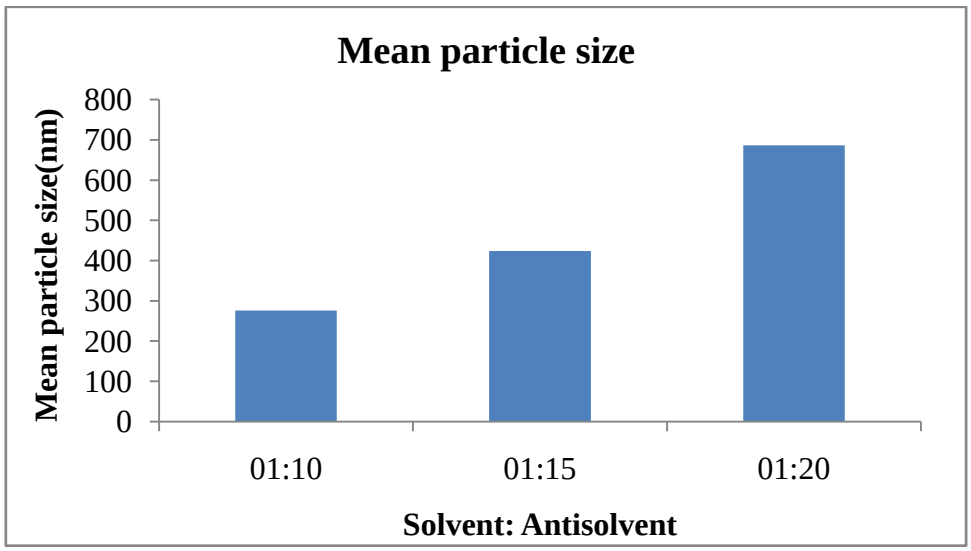


Fig 12: Impact of solvent: antisolvent on Particle size

The selection of proper S: AS ratio is important for the formulation as it affects the extent of supersaturation.

Effect of Stirring time:

The effect of stirring time (5, 10, 30 and 60 min) on the particle sizewas studied, which showed particle size of 293.6, 287.1, 259.7 and 288.3 nm, respectively. The results are shown in Fig 9, 10, 11 and 12, No sign of aggregation due to stirring have been observed, and the particle size doesn't show dependence on stirring time as shown in Fig 13.

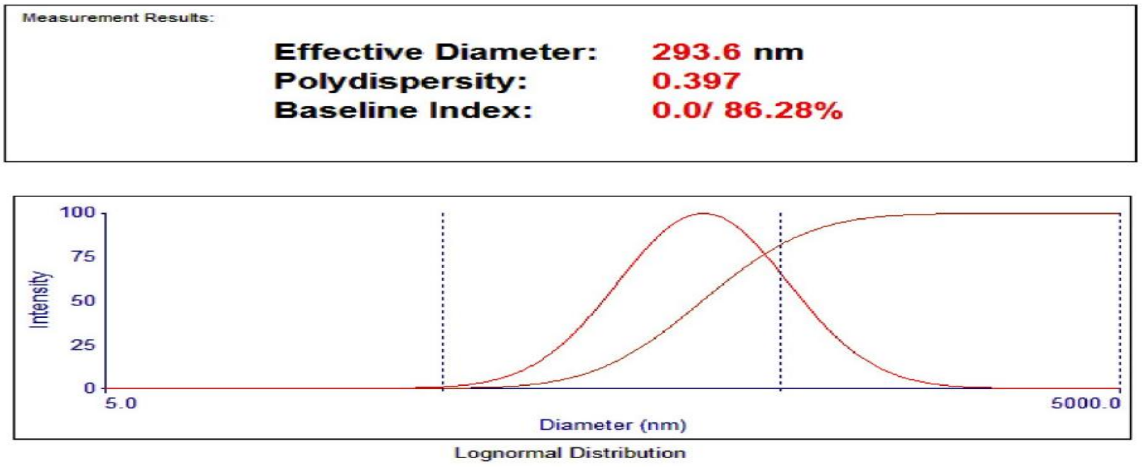


Fig 13: Particle size analysis of formulation with stirring time 5 min

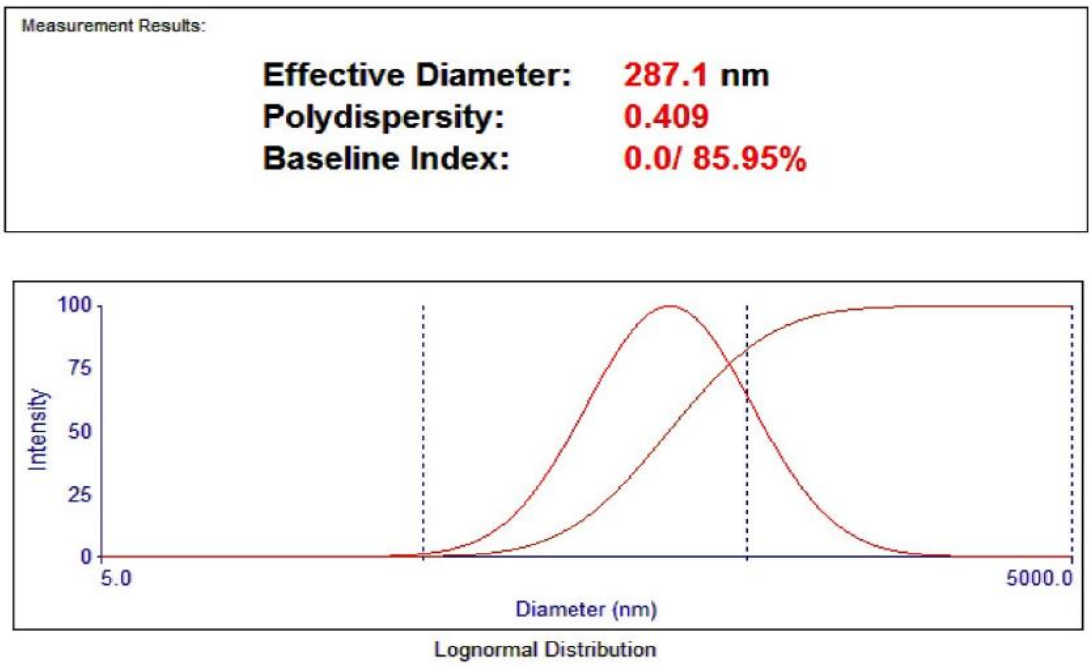


Fig 14: Particle size analysis of formulation with stirring time 10 min

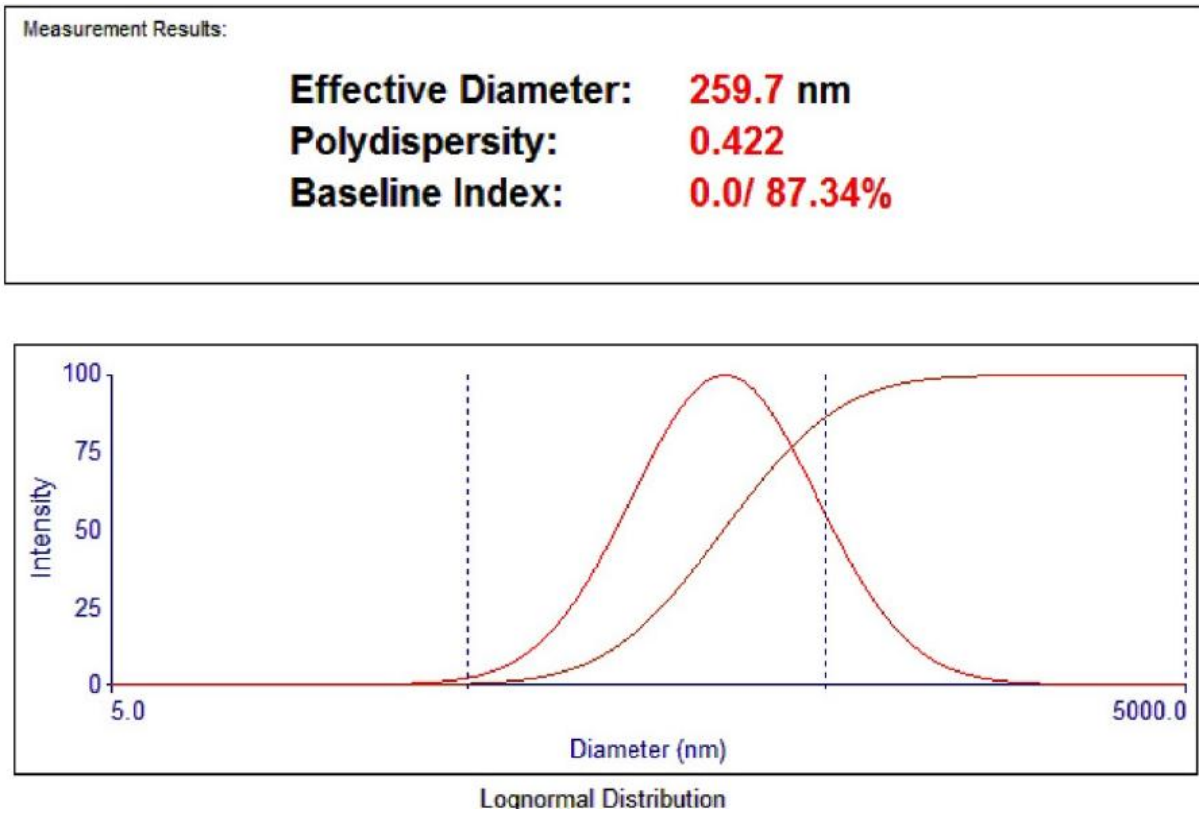


Fig 15: Particle size analysis of formulation with stirring time 30 min

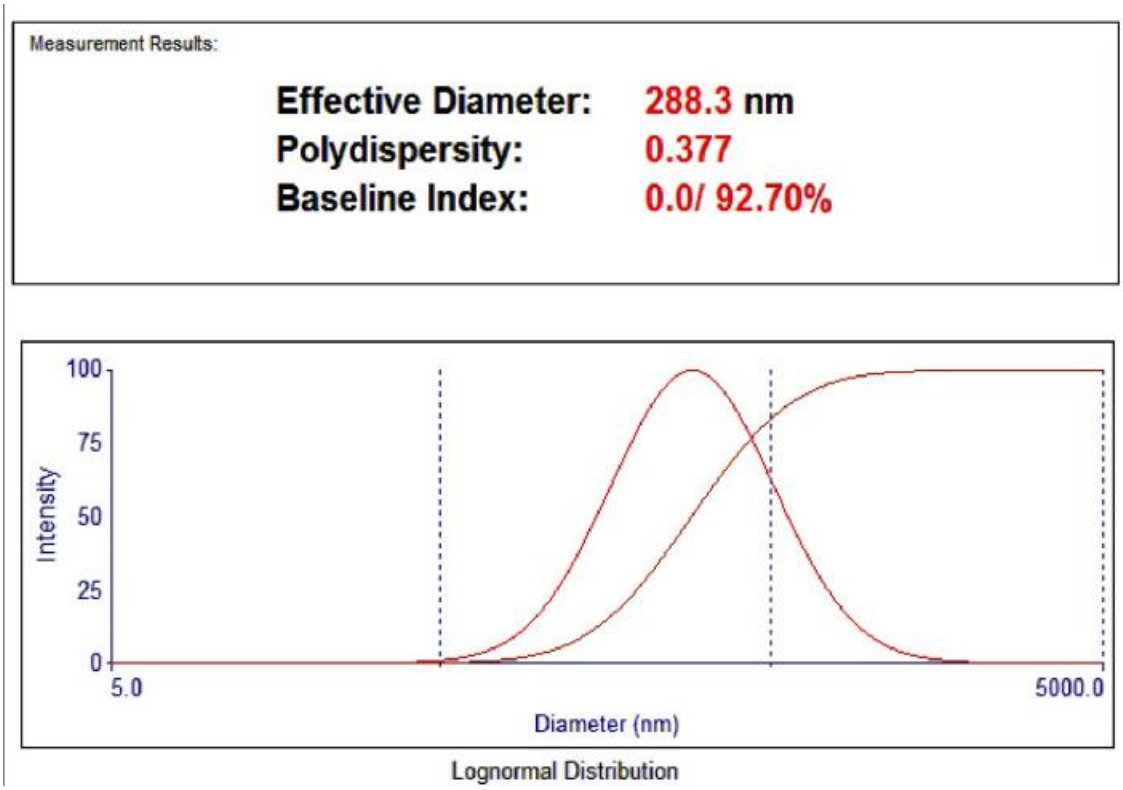


Fig 16: Particle size analysis of formulation with stirring time 60 min

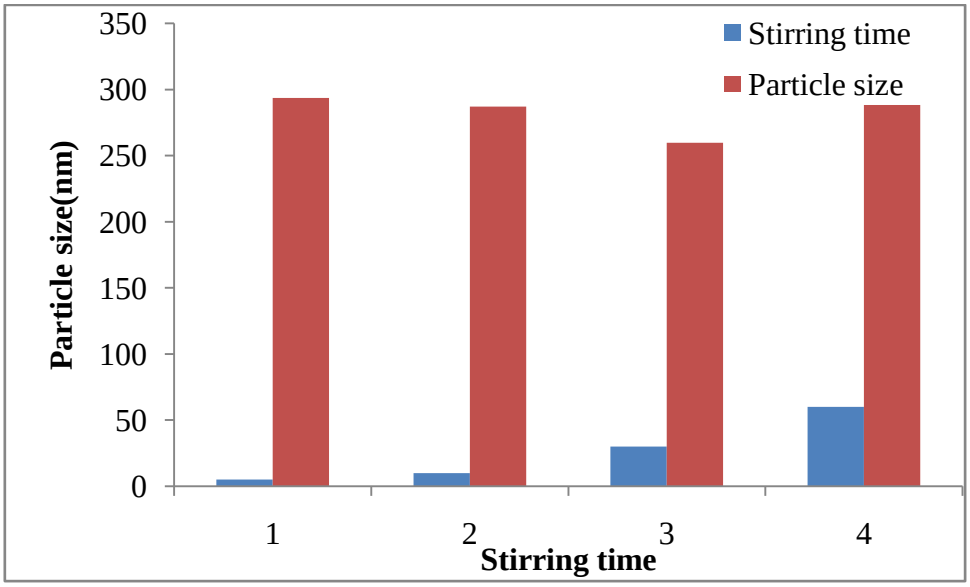


Fig 17: Effect of stirring time on particle size

Effect and amount of Stabilizer:

In the present study suitability of stabilizer alone for stabilization of the nanosuspension has been investigated. Different stabilizers like polyvinyl alcohol, PVP K-30, sodium lauryl sulfate, poloxamer 188 and poloxamer 407 were screened for preparing nanosuspensions. Among all the stabilizers nanosuspension prepared with Poloxamer 188 gave stable nanosuspension.

Fig 18: a: Nanosuspension prepared by using Polaxomer 188 as Stabilizer, b: Pure drug

In order to produce stable NS similar hydrophobicity should result in better surface coverage.Once the suitable stabilizer was fixed, the quantity of stabilizer was fixed, from the preliminary studies, 0.5 % concentration was found to beoptimum for Polaxomer 188. The Polaxomer-based formulations at concentration of 0.5 % gave smaller and more uniform nanoparticles as observed by particle size analysis and SEM.

Impact of diffusing drug concentration:

The effect of diffusing drug concentration on the particle size was studied. The nanosuspensions were made with different diffusing drug concentrations 20, 40, 60, 80, 100 mg/ml. The particle size varies with the change in drug concentration (Fig 15, 16, 17, 18 and 19) giving particle size of 566 nm, 430nm, 218nm, 284nm and 479 nm, respectively. Preparations with 60 mg/ml diffusing drug concentration were selected as the optimum for

Poloxamer-based formulations as shown in Fig 20. However, at very high concentration the particle size increases as very high supersaturation may result in increase in the particle growth by promoting condensation/coagulation due to higher diffusion controlled growth and agglomeration.

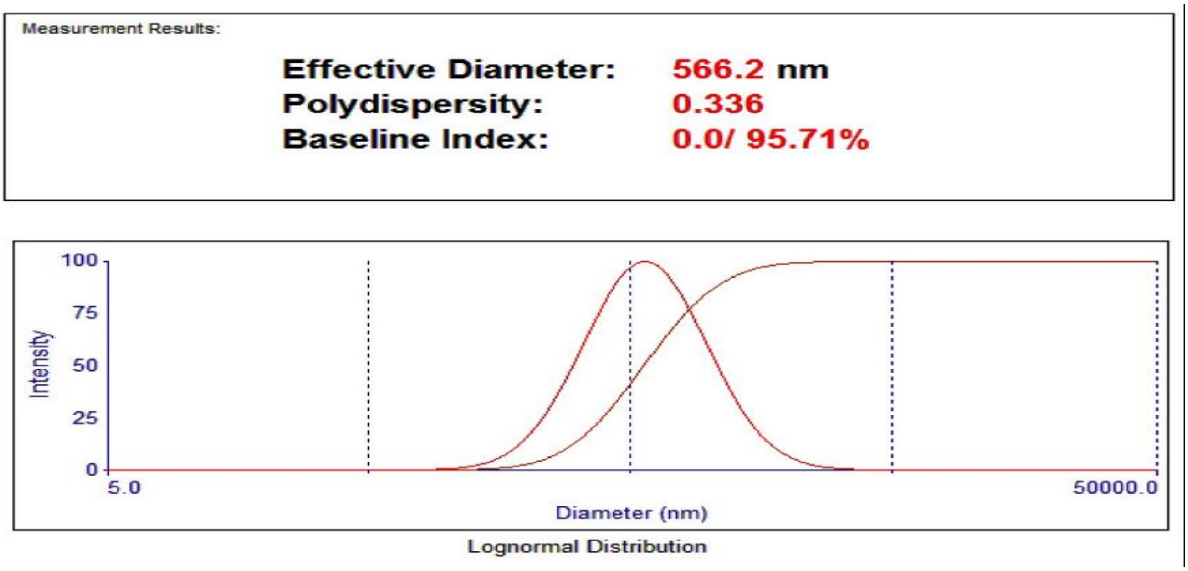


Fig 19: Particle size analysis of formulation based on Polaxamer 188 with diffusing drug concentration of 20 mg/ml

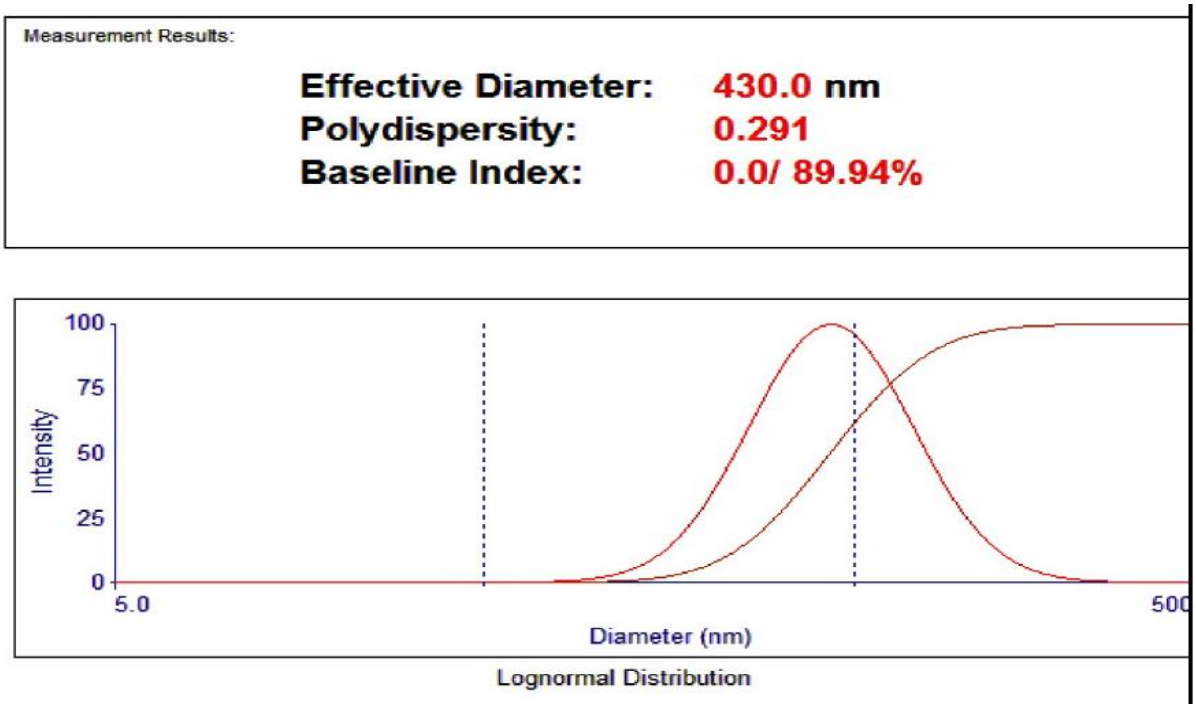


Fig 16: Particle size analysis of formulation based on Polaxamer 188 with diffusing drug

concentration of 40 mg/ml

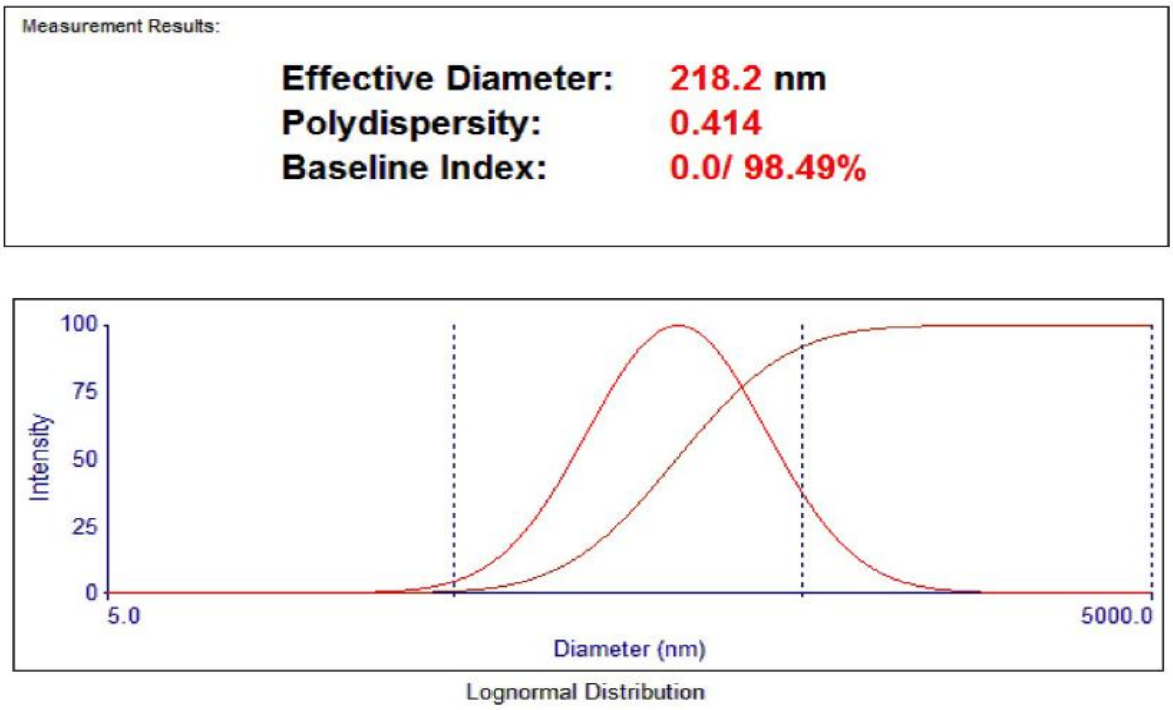


Fig 20: Particle size analysis of formulation based on Polaxamer 188 with diffusing drug concentration of 60 mg/ml

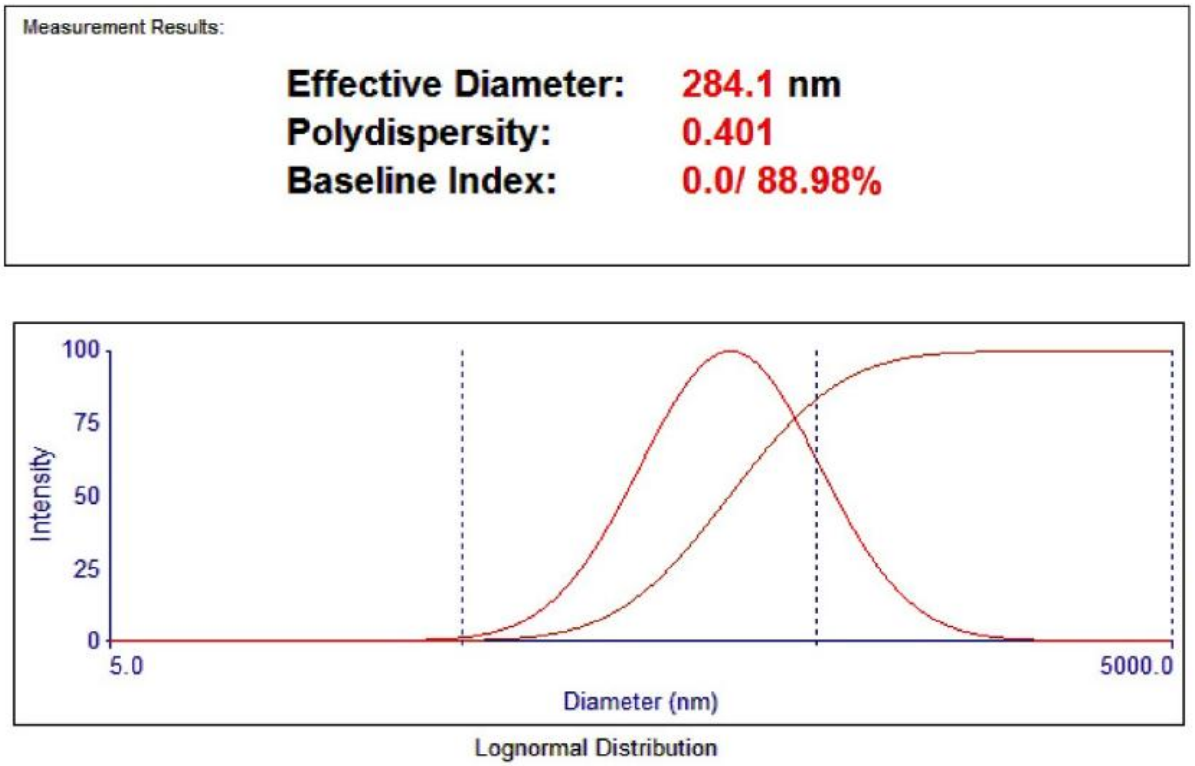


Fig 21: Particle size analysis of formulation based on Polaxamer 188 with diffusing drug

concentration of 80 mg/ml

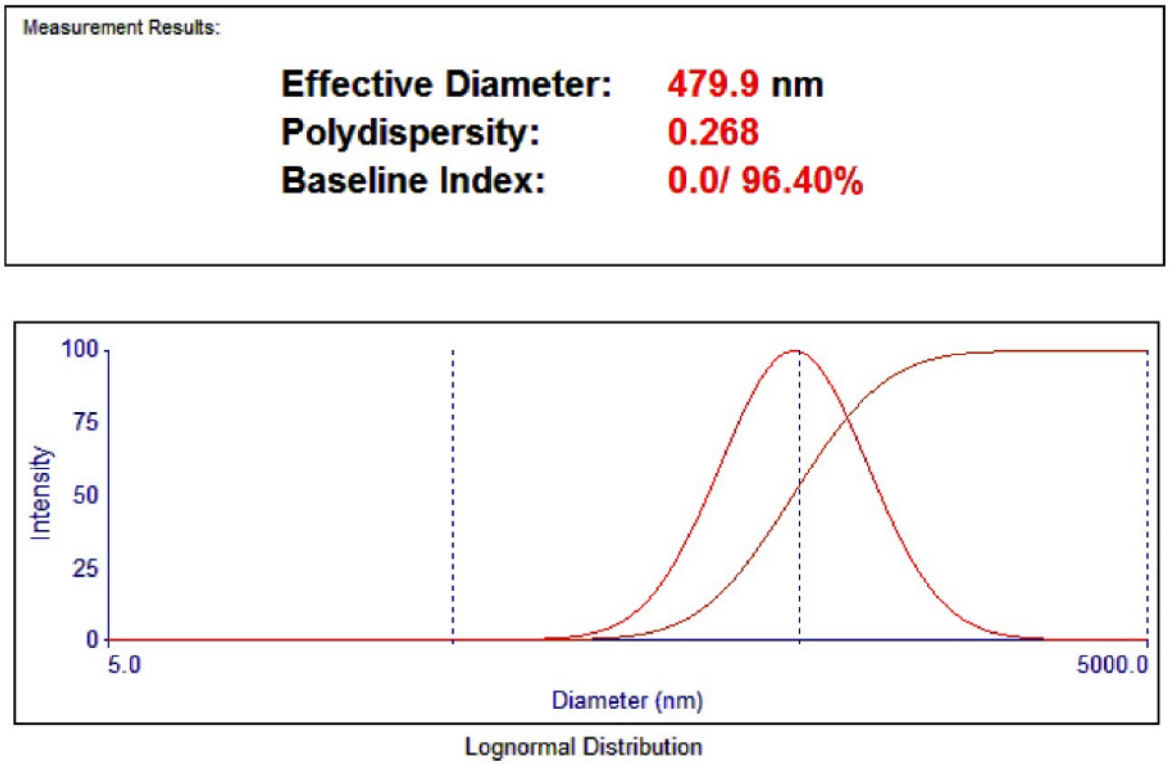


Fig 22: Particle size analysis of formulation based on Polaxamer 188 with diffusing drug concentration of 100 mg/ml

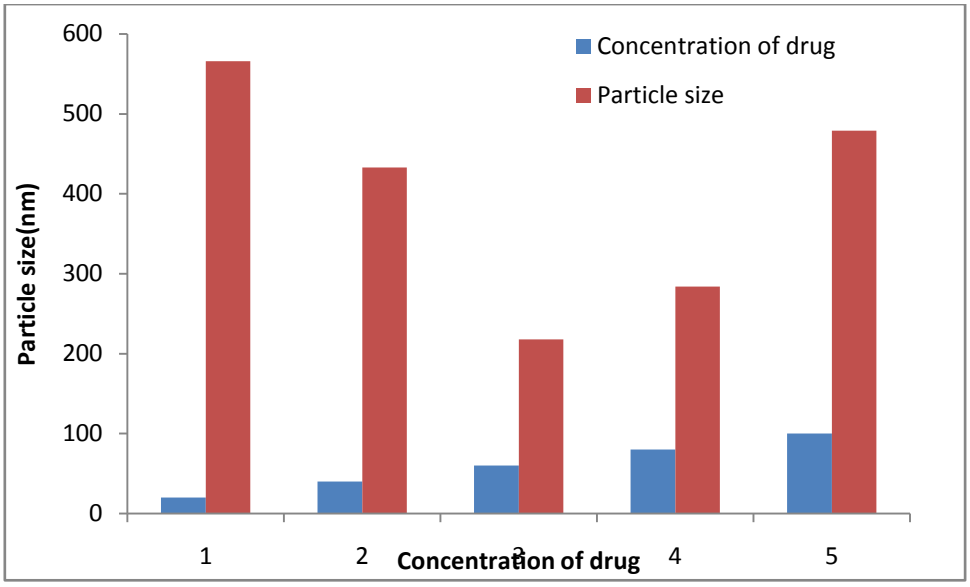


Fig 23: Relation between particle size and concentration of drug

Effect of stirring speed:

Stirring speed is important processing parameter for preparation of nanosuspension. For the optimization of stirring speed 800 RPM, 1000 RPM and 1200 RPM were selected. From the above studies it was found that stabilizer Polaxomer 188 with 0.5% w/v concentration was selected, diffused drug concentration was 60mg/ml. Nanosuspensions were prepared according to the procedure given in experimental section. Prepared nanosuspensions were evaluated with mean particle size and saturation solubility to select the stirring speed for further formulation work, as shown in Fig 21, 22, 23. 1000 RPM stirring speed was selected which was showing minimum mean particle size and maximum saturation solubility.

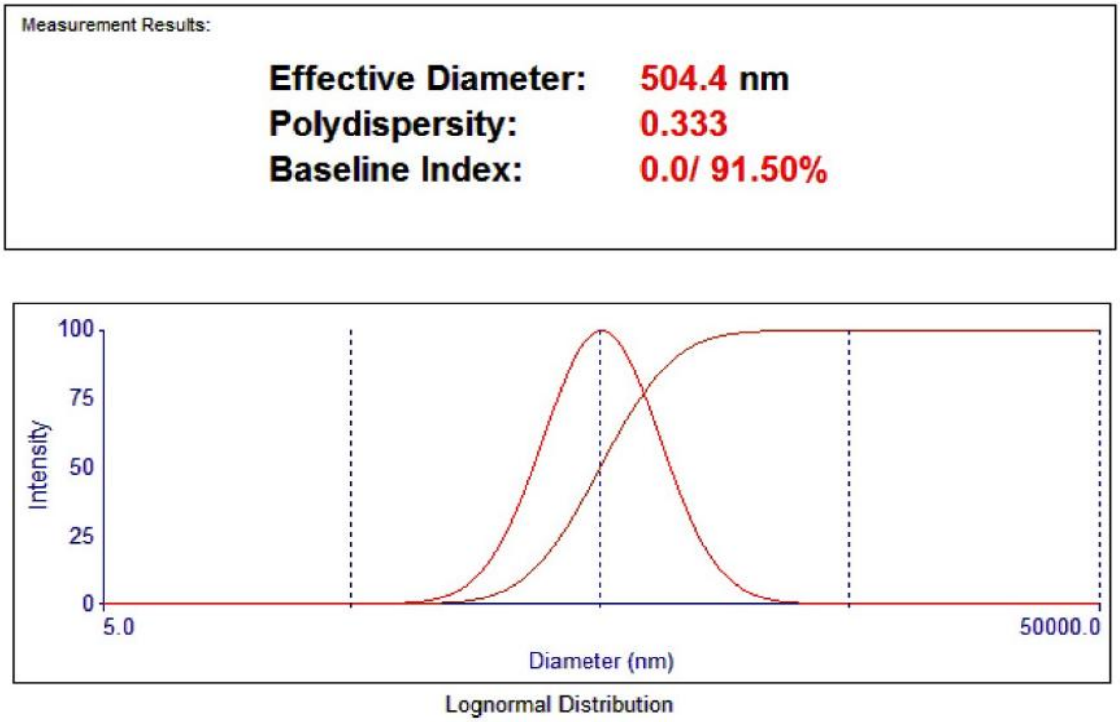


Fig 24: Particle size with RPM of 800.

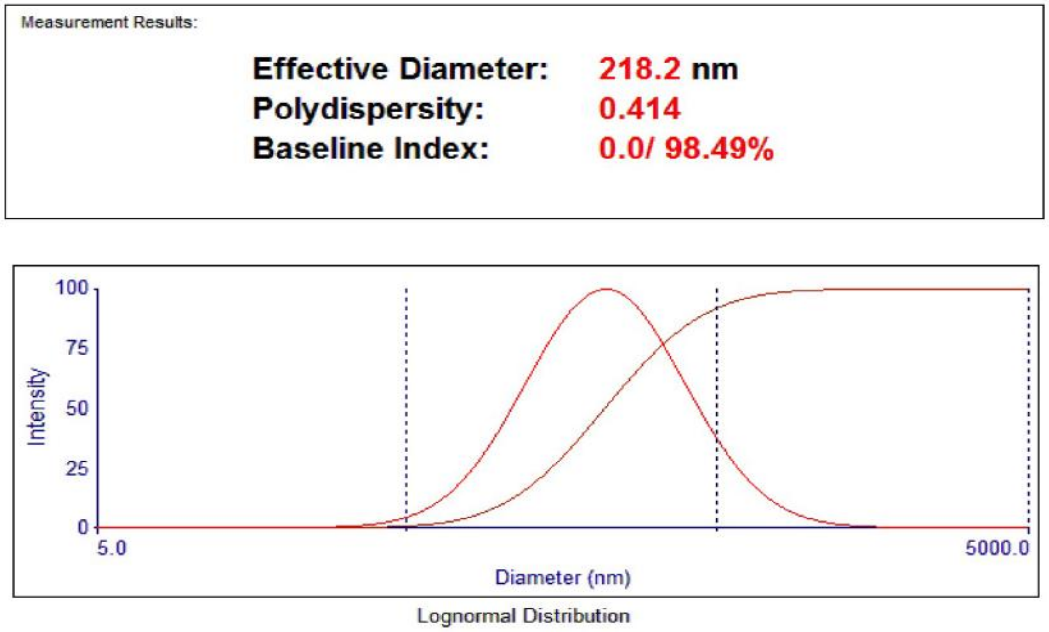


Fig 25: Particle size with RPM of 1000.

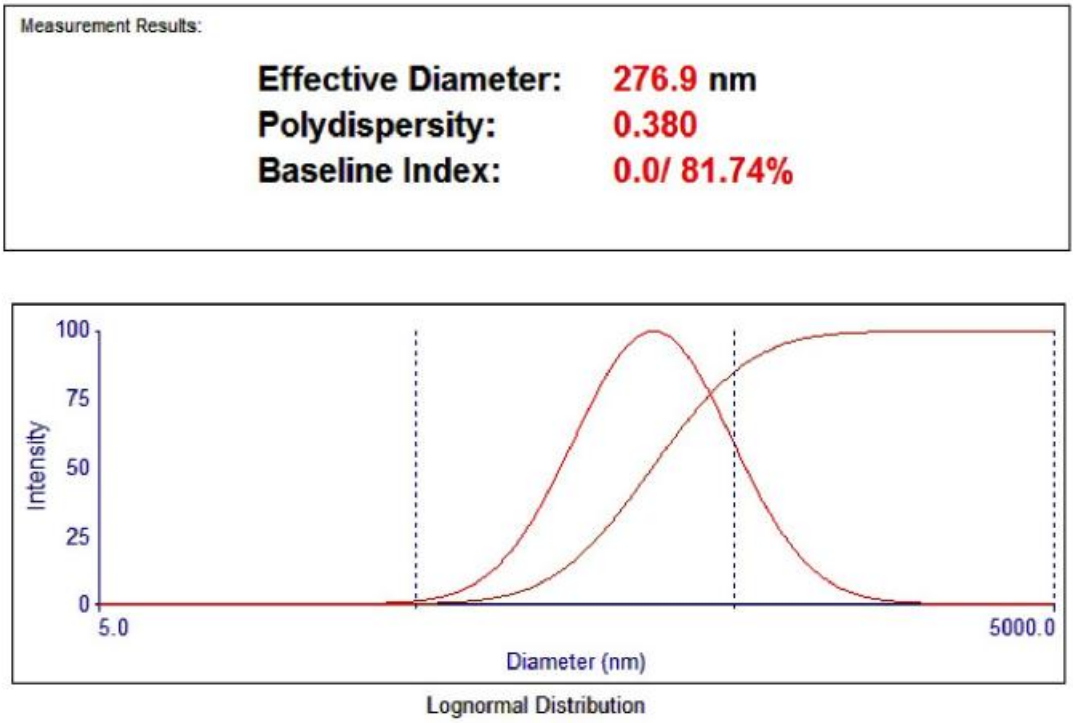


Fig 26: Particle size with RPM of 1200.

Effect of sonication

Once the precipitation of drug particle had occurred in suspension, to convert into the uniform nanosized particles probe sonicator was used. 10 min, 20 min and 30 minperiods were screened for sonication time. Nanosuspension was prepared according to the procedure given before. Prepared nanosuspensions were evaluated with different evaluation parameters like mean particle size and saturation solubility to select theoptimized time period of sonication for further formulation work. 30 min sonication time was selected which was showing minimum mean particle size and maximum saturation solubility.

Full Factorial Design

Factorial Design for two factors at three levels with -1, 0 and +1 equivalent to a 3^2 factorial design was chosen as the experimental design. This is an effective second-order experimental design associated with a minimum number of experiments to estimate the influence of individual variables (main effects) and their second-order effects. Further, this design has an added advantage of determining the quadratic response surface, not estimable using a factorial design at two levels.

Toinvestigate the factors systematically, a full factorial design was employed. The suggested formulations given by the Design-Expert software (Version 12.0.7.1, StatEaseInc., Minneapolis, MN) were systematically prepared and characterized for particles size, polydispersityindex and drug release.

Table 6 Experimental range and the levels of the independent variables in a 3^2 full factorial design

Run No.	Variable level in coded form	
	X_1	X_2
1	-1	+1
2	+1	-1
3	-1	-1
4	+1	+1
5	0	0
6	0	-1

7	**+1**	**0**
8	**-1**	**0**
9	**0**	**+1**

Table 7 Actual values

Coded values	**X_1(Diffusing drug conc in mg)**	**X_2 (Amount of stabilizer %w/v)**
-1	**40**	**0.2**
0	**60**	**0.5**
+1	**80**	**0.8**

Data analysis, optimization and cross-validation ofmodel:

Dataanalysis:

Three responses i.e. Y_1 (Particle size), Y_2 (Polydispersibility Index) and Y_3 (% drug release at end of 4 hr) were selected for statistical optimization and fitted to linear, interactive and quadratic models. The summary of statistics of Fenofibratewaspresented in Table 2 and the comparative R^2, adjusted R^2, predicted R^2, PRESS, s.d., F-values and p-values were determined using the Design Expert. A suitable polynomial model for describing the data was selected based on correlation (R^2) and PRESS values. Response Y_1, respone Y_2 and response Y_3 followed linear model.Hence these models were selected for further optimization. These models showed higher R^2 and F-values and lower PRESS andp-values.

Table 8: Summary of model statistics for responses Y_1, Y_2 and Y_3 for Fenofibrate

Model	R^2	AdjustedR^2	Predicted R^2	PRESS	s.d.	F-value	p-value	Remarks
FenofibrateNanosuspension								
Response Y_1 (Particle size in nm)								
Linear	0.9670	0.9576	0.9311	13.70	0.9686	102.51	< 0.0001	**Suggested**
Interactive	0.9783	0.9674	0.9613	7.70	0.8482	3.13	0.1274	
Quadratic	0.9904	0.9783	0.9318	13.57	0.6922	2.50	0.1971	
Response Y_2 (Polydispersibility Index)								
Linear	0.9812	0.9758	0.9600	8.07	0.7367	182.29	< 0.0001	**Suggested**
Interactive	0.9820	0.9729	0.9312	13.87	0.7788	0.2638	0.6259	
Quadratic	0.9915	0.9786	0.8954	21.10	0.6986	1.79	0.2781	
Response Y_3 (% Drug released at 1 hr)								
Linear	0.9896	0.9866	0.9790	3.57	0.50	333.49	< 0.0001	**Suggested**
Interactive	0.9911	0.9866	0.9810	3.24	0.50	0.98	0.358	
Quadratic	0.9971	0.9935	0.9765	4.00	0.35	4.2	0.1036	

The results of the second order response surface model fitting in the form of ANOVA for fenofibratenanosuspension is given in Table 3. These parameters were used to construct the independent variables on the responses.

Interpretations from data analysis:3 responses Y1, Y2 and Y3 for Fenofibrate of particle size, PDI and %drug release were selected.

- ✓ Responses fitted to linear, quadratic and interactive model
- ✓ The 3 responses followed linear model
- ✓ Showed higher R2, f values: lower PRESS and p values
- ✓ The smaller the PRESS value- better model's predictive ability
- ✓ If f value is more , the responses are said to be doing better for optimization
- ✓ If p value <o.o5, then it defines that the results are statistically significant.
- ✓ R2 value is said to be coefficient of determination, which should be in the range from 0 to 1 (i.e., >0.9- high correlation; <0.4- low correlation)

So, the responses indicating that there is a good correlation between the independent and dependent variables statistically significant.

Table 9: ANOVA for the responses of Fenofibratenanosuspension

Source	SS	df	MS	F-value	p-value
Response Y_1(Linear Model)					
Model	192.33	2	96.17	102.51	< 0.0001*
X_1-Diffusing drug conc	88.17	1	88.17	93.98	< 0.0001*

in mg					
X_2-Amount of stabilizer in%	104.17	1	104.17	111.04	< 0.0001*
Residual	6.57	7	0.9381		
Total	198.90	9			
Response Y_2(Linear Model)					
Model	197.88	2	98.94	182.29	< 0.0001*
X_1- Diffusing drug conc in mg	92.04	1	92.04	169.58	< 0.0001*
X_2- Amount of stabilizer in %	105.84	1	105.84	195.00	< 0.0001*
Residual	3.80	7	0.5428		
Total	201.68	9			
Response Y_3(Linear Model)					
Model	168.33	2	84.17	333.4	< 0.0001*
X1- Diffusing drug conc in mg	160.17	1	160.17	634.6	< 0.0001*
X2- Amount of stabilizer in %	8.17	1	8.17	32.4	0.0007*
Residual	1.77	7	0.28		
Total	170	9			

* Significant ($p<0.05$), SS: Sum of squares; MS: Mean sum of squares

The F-value for the responses, Particle size(Y_1) Polydispersibility Index (Y_2) &(% Drug released at end of 4hr(Y_3) werefound to be 102.51, 182.29 &333.4 which indicated that the models were significant. The values of Prob>F (less than 0.05) for all the responses indicated the significance of the models.

The goodness of fit of the model was checked by the coefficient of determination (R^2). The R^2 values of Particle size (Y_1),Polydispersibility Index (Y_2) &% Drug released at end of 4hr (Y_3) responses offenofibratenanosuspension was found to be0.9670, 0.9812& 0.9896 indicated a good correlation between the independent and dependent variables. The model was found to be significant with respect to adjusted coefficientof determination (Adj R^2 > 0.9000) values. In all the cases 'Predicted R^2' values were in reasonable agreement with the Adj R^2values.

The effect on particle size (Y_1) was observed to be significant by ANOVA and the polynomial equation was found as follows:

$$Y= 108.50-48.05X_1+5.65X_2+13.05X_1^2+19.78X_2^2-0.60X_1X_2$$

The negative sign for coefficient of X_1 indicates that particle sizedecreases with increase in the drug concentrations. 3D plots (Fig.24, 25) shows thatthe particle sizes are towards upper level at low drug concentration and decreases with increase in concentration. Particle size was decreased because increasing drug concentration results in supersaturation, which causes rapid precipitation on diffusion (Suzanne et al 2011). Therefore, the drug particles were reduced to nano-size ranges, which were efficiently shielded by stabilizer to prevent agglomeration. A smaller concentration of stabilizer induces agglomeration or aggregation and particle size was towards higher level and too much stabilizer promotes Oswald's ripening (a phenomenon in which small crystals, more soluble than large ones, dissolve and re-precipitate onto larger particles).Moreover high concentration of stabilizer could also result in enhanced viscosity of the solution which would hinder the transmission of ultrasonic vibration and the diffusion between

the solvent and anti-solvent during precipitation(Xia et al 2010). Optimum stabilizer concentration was found between 0.35 to 0.55%.

The effect on % Drug Release (Y_2) was observed to be significant byANOVA and the polynomial equation was found as follows:

$$Y= 14.60+0.90X_1+0.094X_2-0.75X^2_1-0.42X_2-0.42X^2_2+0.83X_1X_2$$

Slow dissolution can be partly attributed to hydrophobicity as evidenced by poor wetting of the drug surface. This causes the particles to aggregaterather than disperse. Dissolution rate in the nanosuspension was improved because of increased surface area. The positive sign for coefficient of X1 indicates that as thedrug concentration increases, the % Drug Release (Y2) also increases. 3D figures as shown in (Fig. 26, 27) show nearly linear ascending pattern for the values of drug release with decreasing particle size. At higher drug concentration with optimum stabilizer concentration drug release was towards higher level.

Similarly, the effect Polydispersity index (Y_3) was observed to besignificant by ANOVA and the polynomial equation was found as follows:

$$Y=0.89+6.652X_1-0.027X_2-0.062X_1{}^2-0.22X^2_2-0.033X_1X_2$$

The uniformity of size indicated by PI value was found to be moredependent of stabilizer concentration. The negative sign for coefficient of X_2indicated that the PI decreases with increase in the stabilizer concentration (Fig 28, 29). Particles were less homogenous at very high drug concentration of stabilizer.

Particle size:

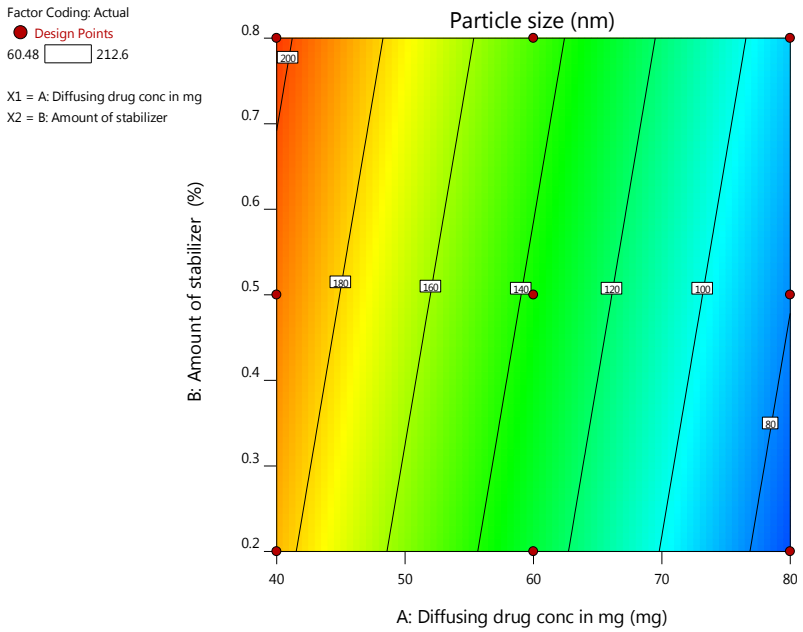


Fig 27: Contour plot showing the influence of diffusing drug concentration (X_1) and amount of stabilizer (X_2) on Particle size (nm)

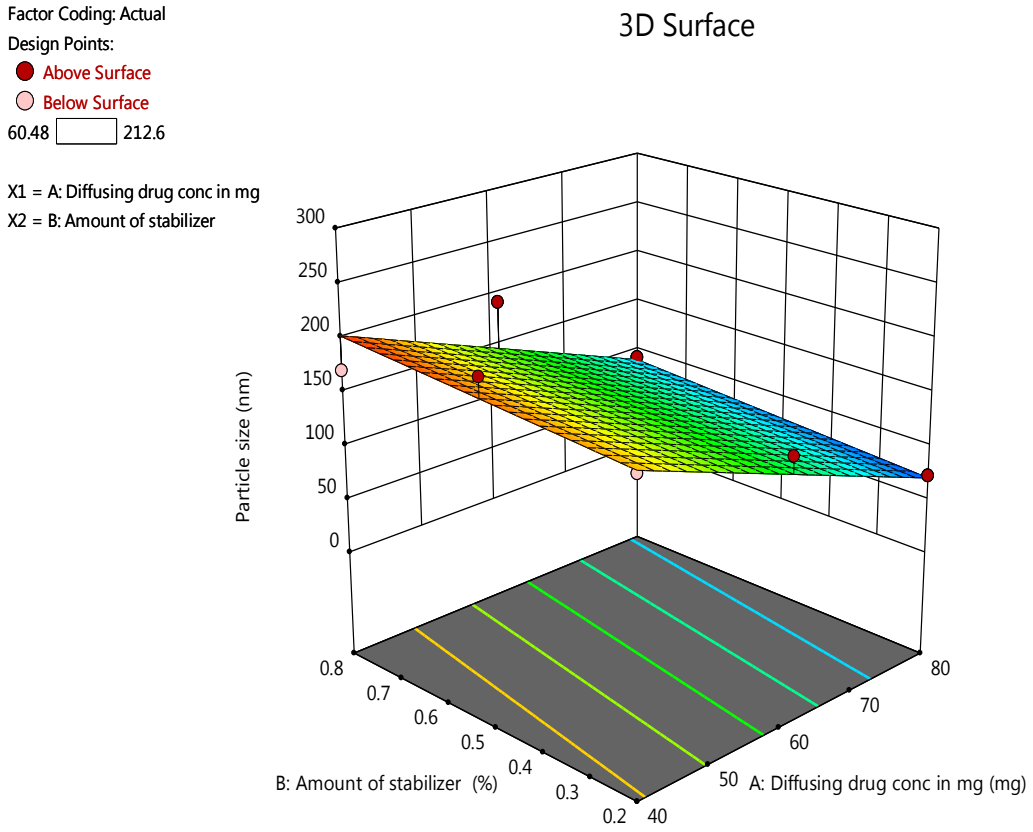


Fig 28: Response surface plot showing the influence of diffusing drug concentration (X_1) and amount of stabilizer (X_2) on Particle size (nm)

Polydispersibility Index:

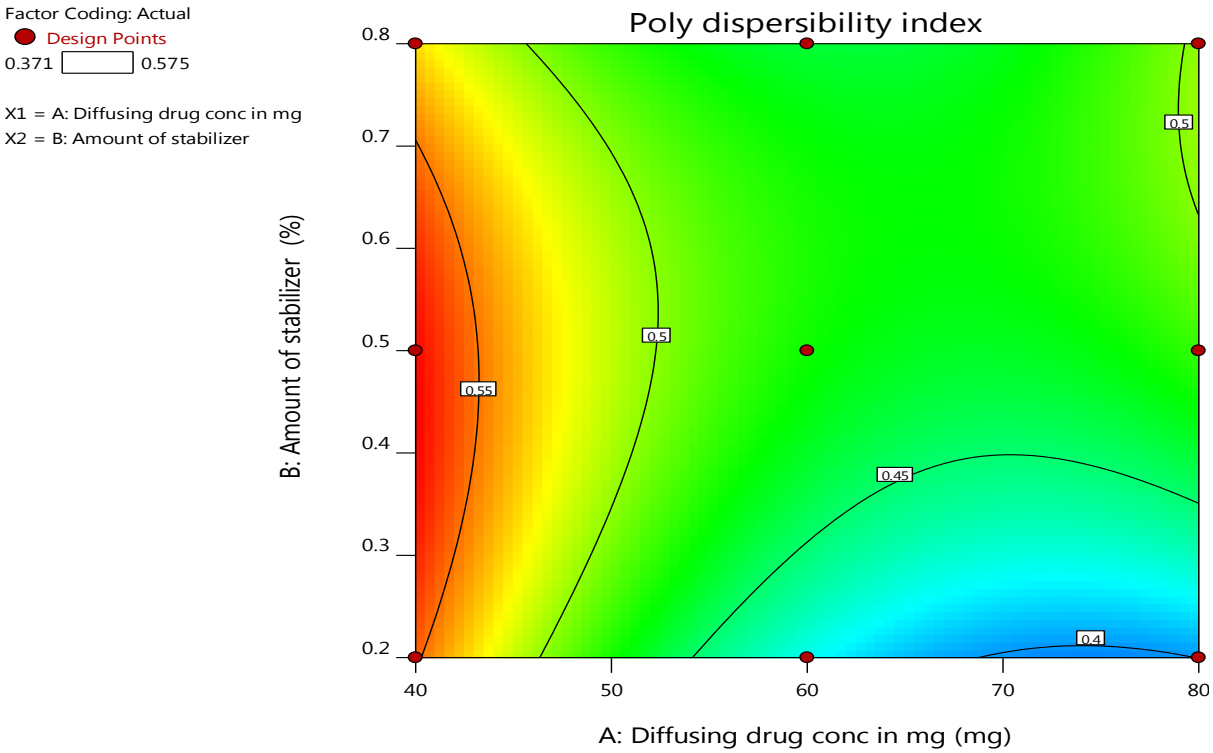


Fig 29: Contour plot showing the influence of diffusing drug concentration (X_1) and amount of stabilizer (X_2) on PDI

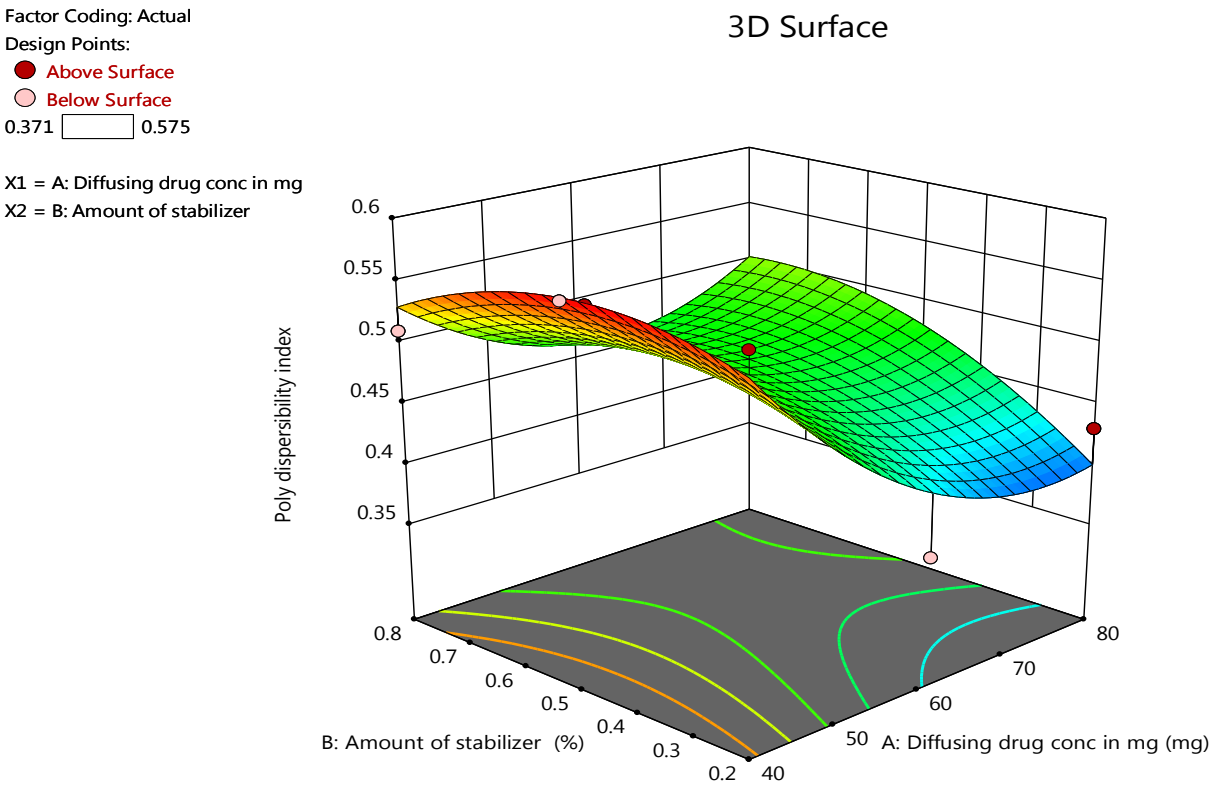


Fig 30: Response surface plot showing the influence of diffusing drug concentration (X_1) and amount of stabilizer (X_2) on PDI

Cumulative % drug release at 4 hr

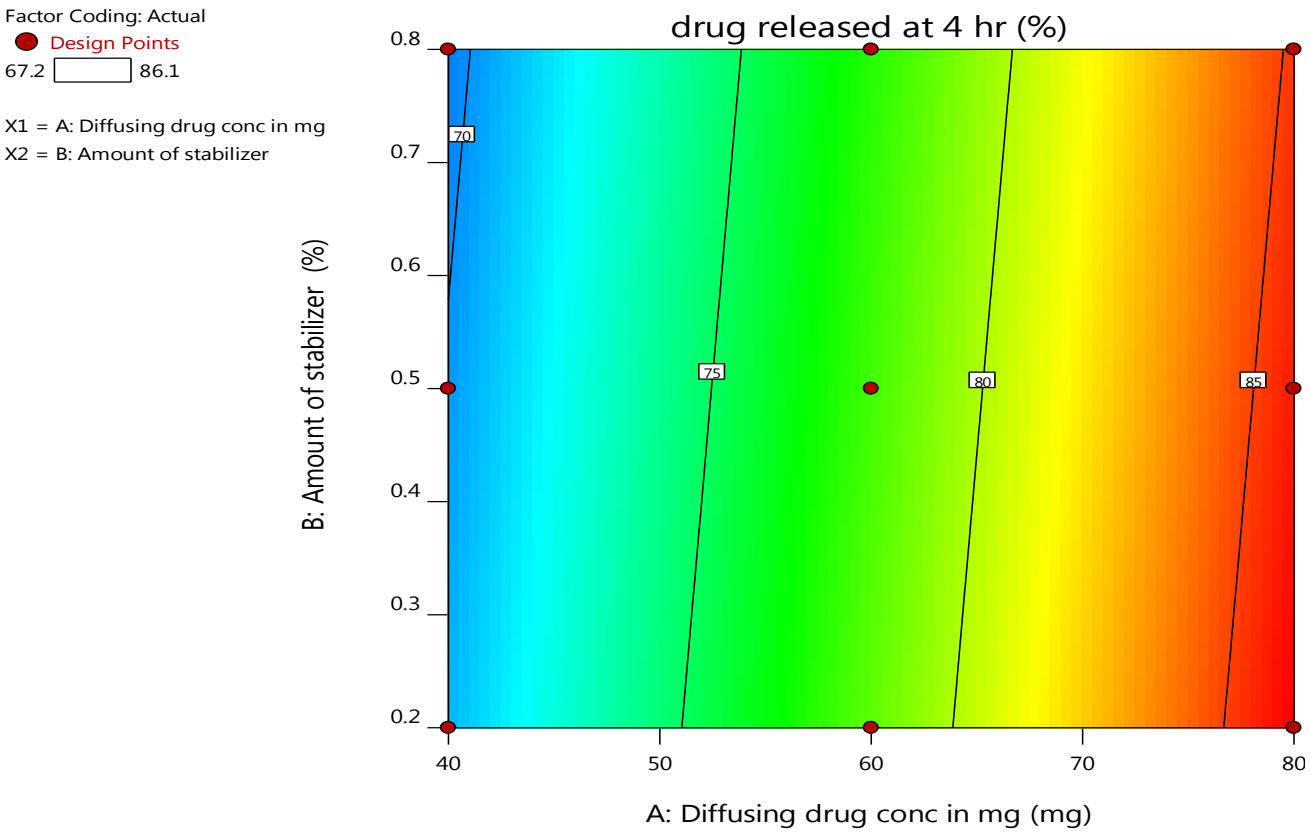


Fig 31: Contour plot showing the influence of diffusing drug concentration (X1) and amount of stabilizer (X2) on Cumulative % drug release at 4 hr

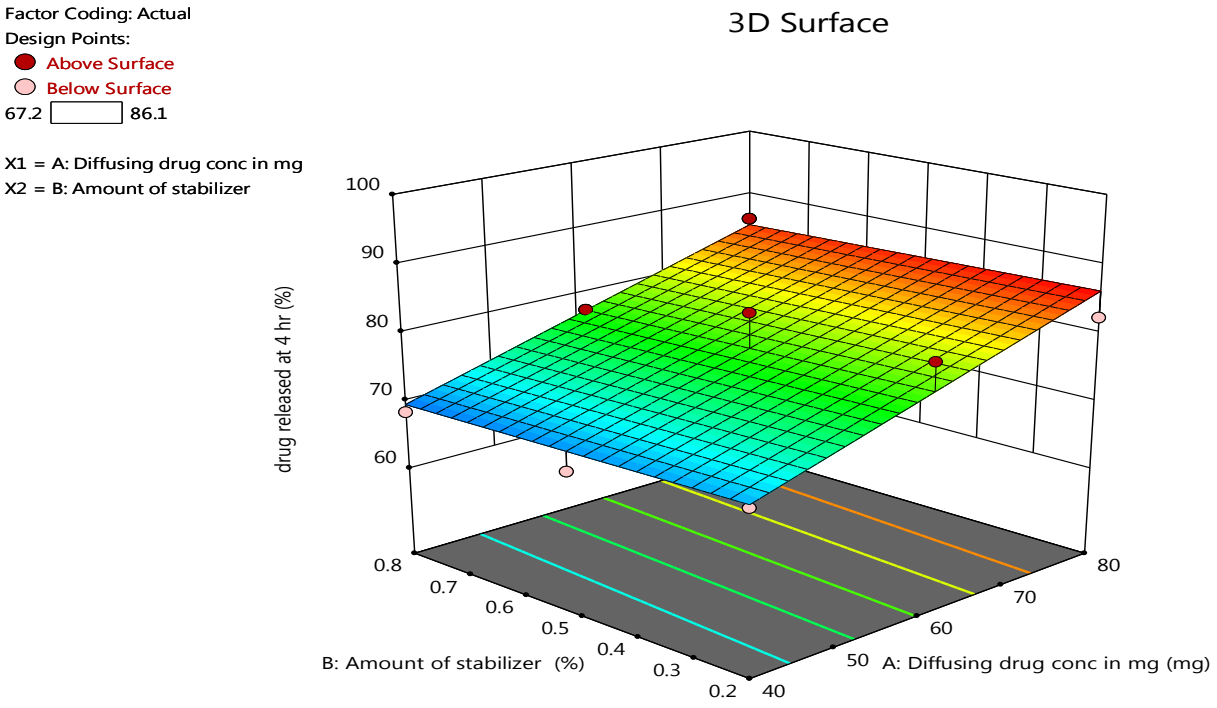


Fig 32: Response surface plot showing the influence of diffusing drug concentration (X_1) and amount of stabilizer (X_2) on Cumulative % drug release at 4 hr

Optimization:

The higher desirability value indicates the more suitability of the formulation and the optimized formula can directly be obtained from the desirability function response surface plots and (or) overlay plots. The desirability function (as shown in Figs. 30) was found to be higher (near to 1) for the optimized formula indicating the suitability of the formulations. The optimal values of independent test variables were obtained from the overlay plots (Fig. 31) and are presented in Table 4. The optimized formulation of Fenofibrate contained 63.1 mg of drug and 0.3% of stabilizer.

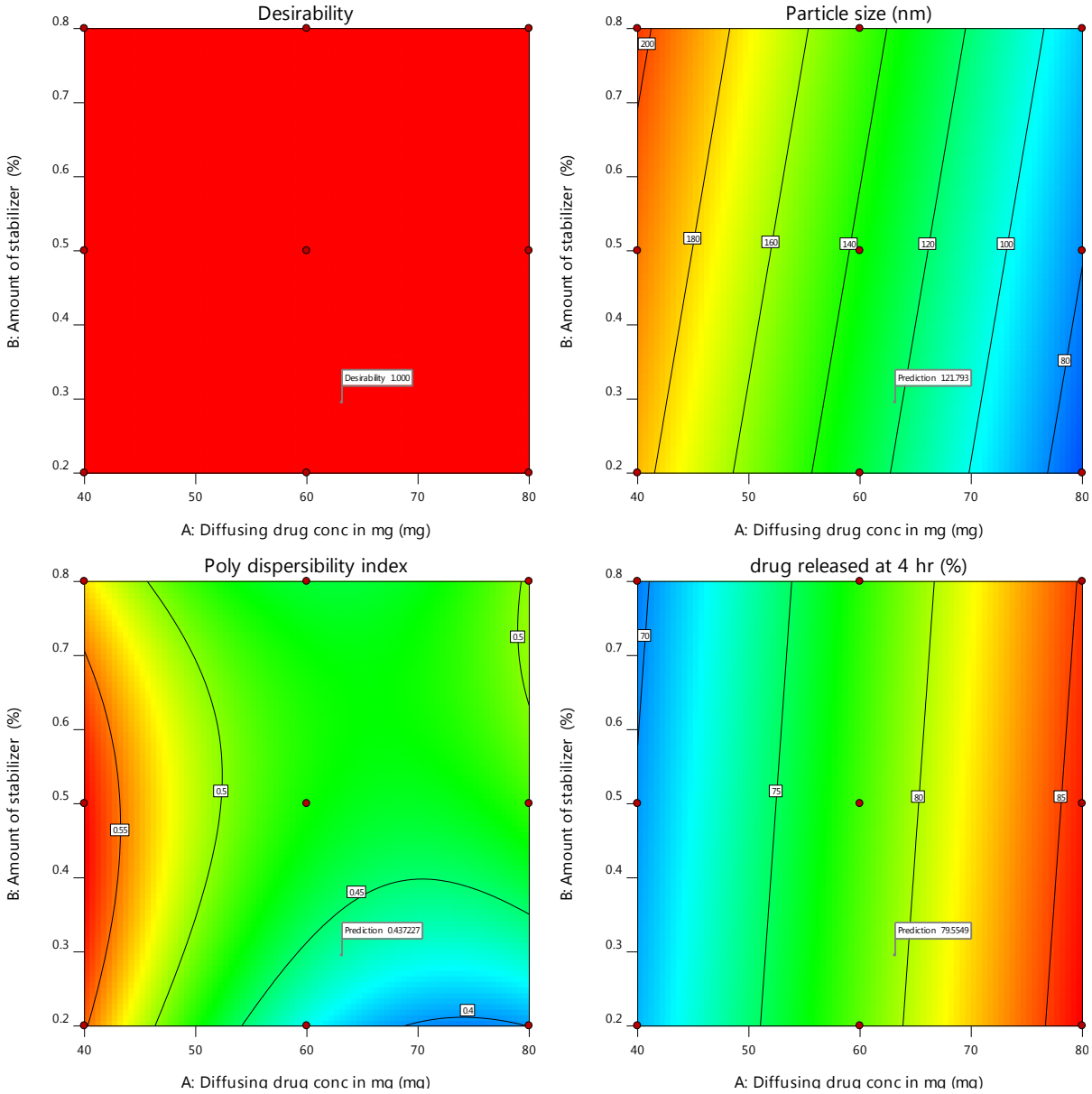


Fig 33: Desirability plots for nanosuspensions of Fenofibrate

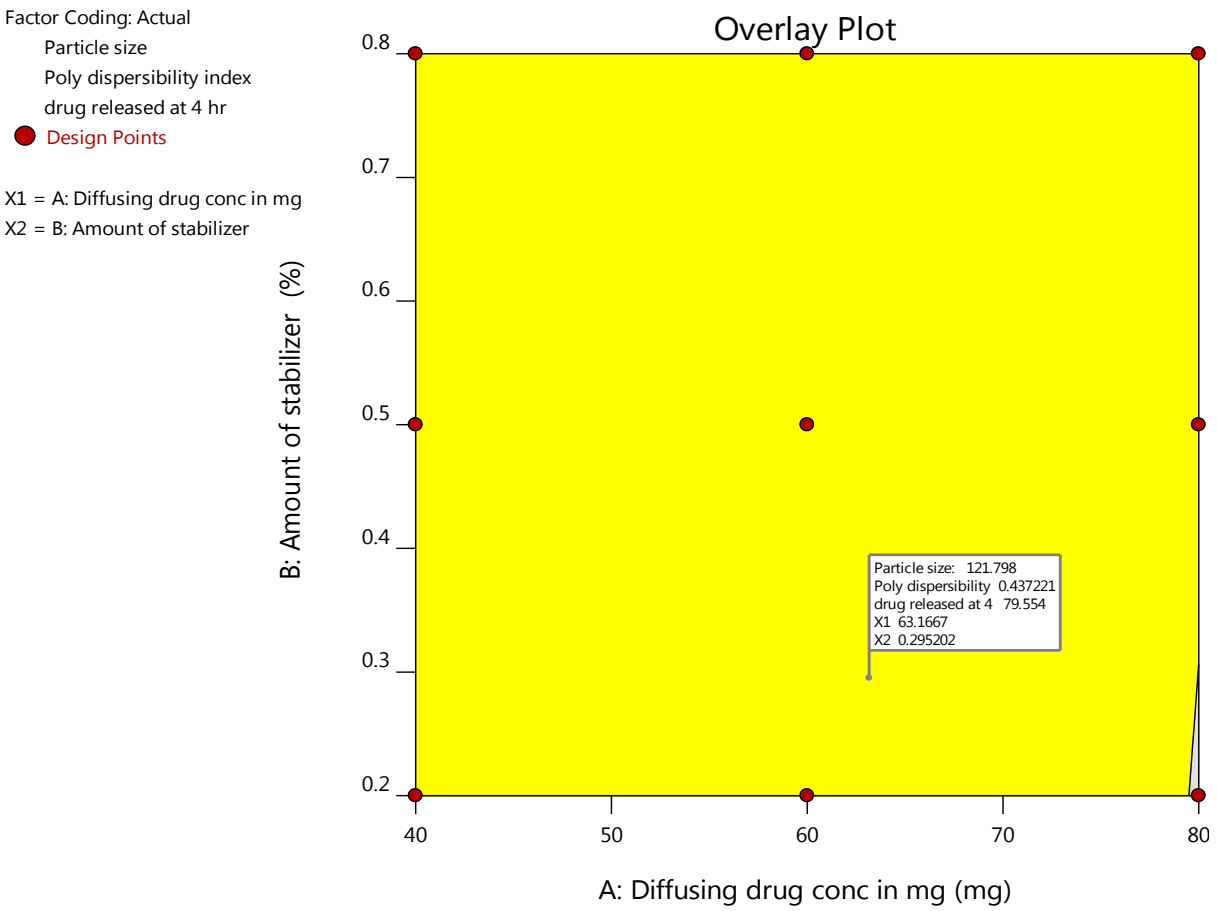


Fig 34: Overlay plot for nanosuspensions of Fenofibrate

Cross-validation ofmodel:

The model predicted that the formulation with particle size 121 nm, Polydispersibility index 0.437 and drug release at 4 hr is 79.5% forfenofibratenanosuspensions.

Hence, formulation wasprepared with the above optimized concentrations. The prepared optimized nanosuspensions fulfilled all the evaluation tests described and the results are shown in Table.

S. No	Name of the Ingredient	Quantity
1	Fenofibrate	63.1 mg
2	Polaxomer 188	0.3%w/v

Table 10 optimized formulation

IV. Characterization of fenofibrate nanosuspension:

❖ Size measurement, PDI and zeta potential analysis:

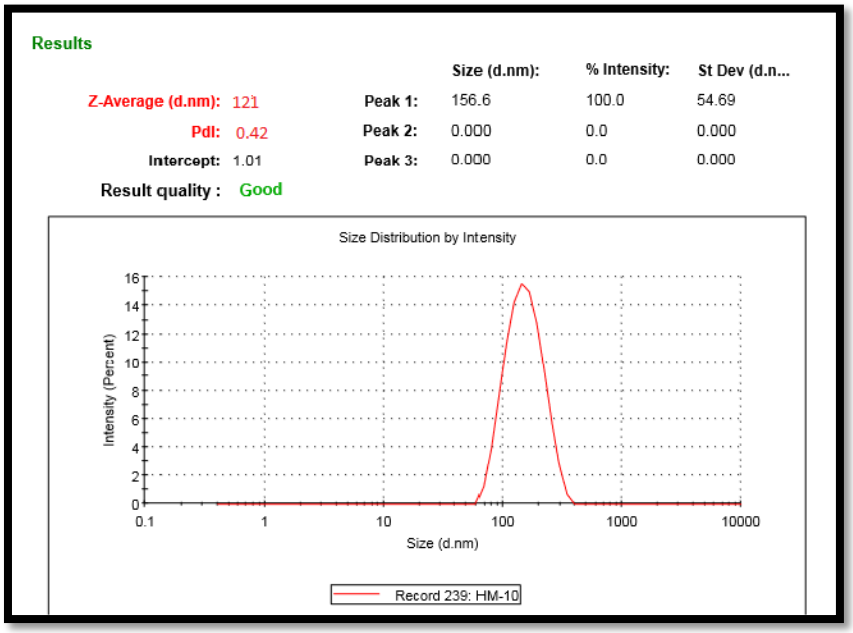
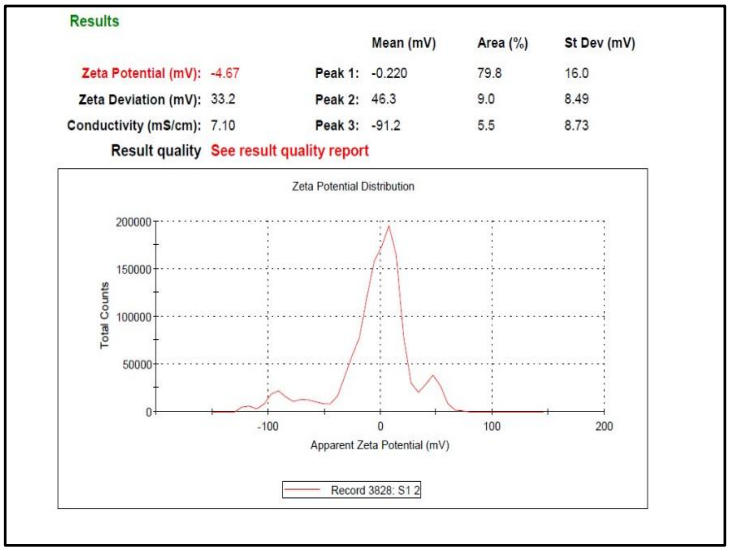


Fig 35 Size measurement and zeta potential analysis



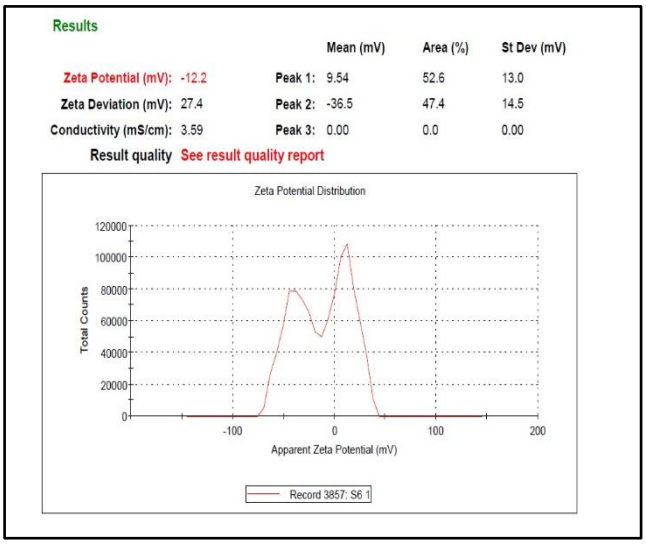


Fig 36, 37 Zeta potential analysis of pure drug fenofibrate, zeta potential analysis of fenofibrate nanosuspension

ZETA POTENTIAL	PURE DRUG	FBT NSP
	-4.67 mV(approx. range -1 to -5mV)	-12.2 mV (approx. range potential of -10mV)

Table 11 zeta potential of optimized fenofibrate nanosuspension

- **Drug entrapment efficiency (DEE):** The experiment was performed in triplicate for each batch and the average was calculated (Mandal et al 2010). The entrapment efficiency (EE %) could be achieved by the following equation:

EE % = [(Winitial drug _Wfree drug) /Winitial drug] *100

Entrapment efficiency was found to be 90.01+/-0.51 %

- **Scanning electron microscopy (SEM):**

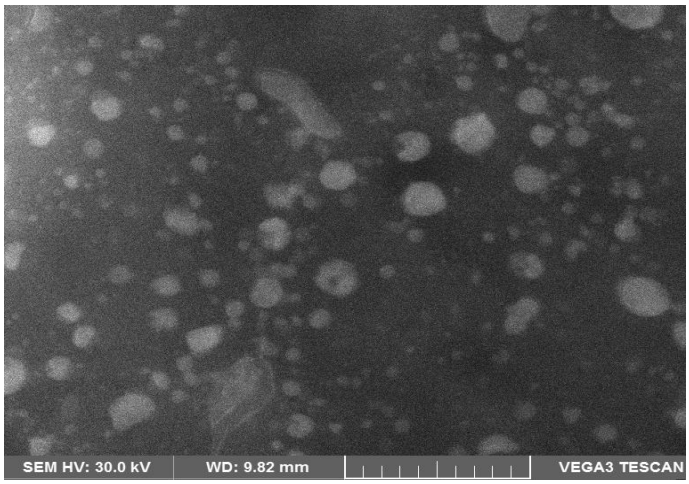


Fig 38 Scanning electron microscopy (SEM) of fenofibrate nanosuspension

- **Transmission electron microscopy:**

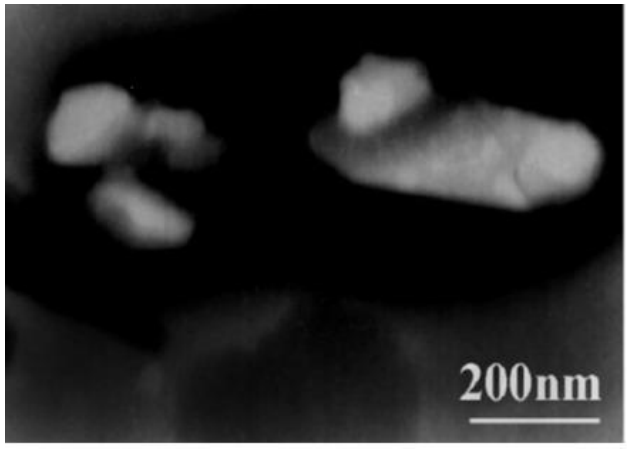


Fig 39 Transmission electron microscopy of fenofibrate nanosuspension

- **In vitro drug release kinetic experiments**

Parameter	Test condition
Qty. of media	900 ml, Sodium phosphate buffer pH 7.4
Apparatus & Type	USP Type 2 (Paddle)
Agitation Speed	50 RPM
Temperature	37°±0.5°C
Time Points	0,0.5,1,1.5,2,2.5,3,3.5,4,4.5,5,5.5,6,6.5,7,7.5 hours
Volume Withdrawn	10 mL

Table 12 Dissolution Conditions

Time (hr)	Marketed formulation	In House Formulation
0	0	0
0.5	62.5	55.3
1	99.9	59.2
1.5	-	63.5
2	-	67.4
2.5	-	69.5
3	-	72.2
3.5	-	76.3
4	-	79.5

4.5	**-**	**83.7**
5	**-**	**89.2**
5.5	**-**	**91.7**
6	**-**	**94.5**
6.5	**-**	**97.3**
7	**-**	**99.2**
7.5	**-**	**99.9**

Table 13 comparison of percentage drug release between FBT nanosuspension and marketed formulation

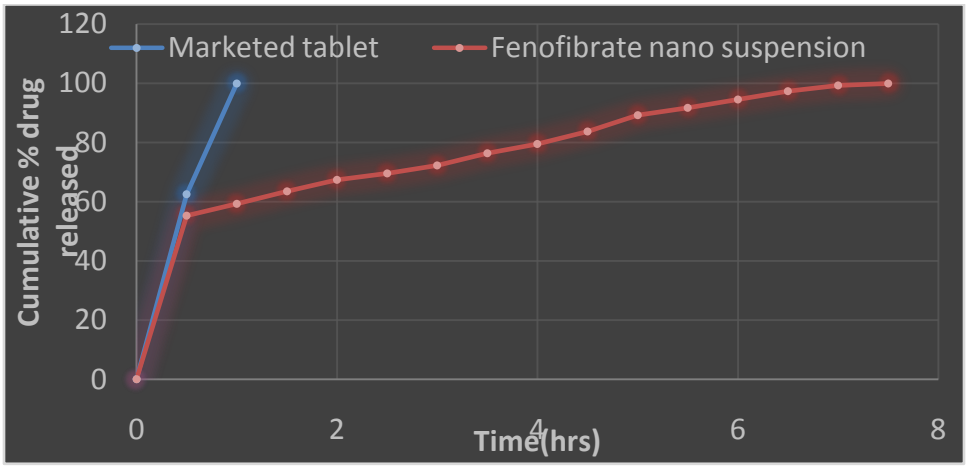
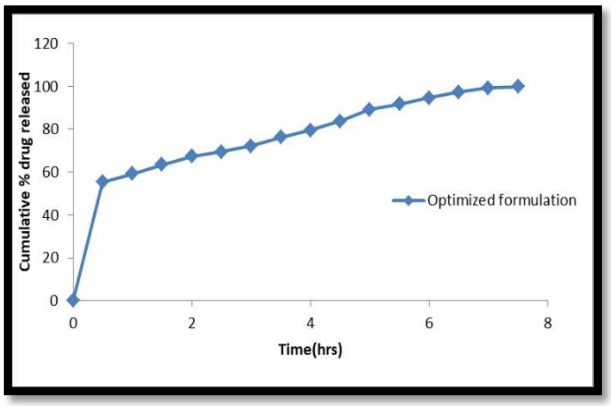


Fig 40 cumulative percentage drug release of FBT nanosuspension

The drug release from the Fenofibrate nanosuspension was compared with the marketed formulation, it was observed that at end of 8hr, 99.9 of drug release**.**



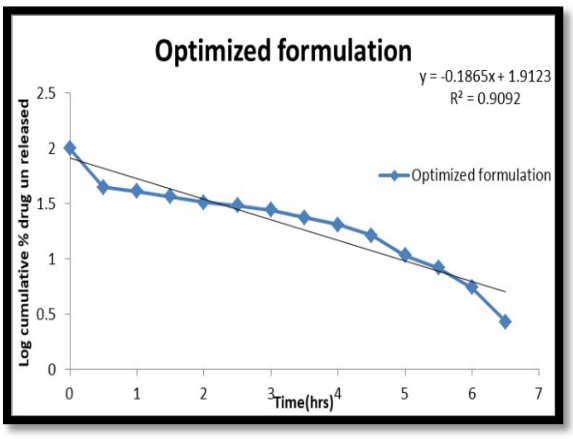


Fig 41 zero order plot of optimized FBT nanosuspension

Fig 42 first order plot for optimized FBT NSP

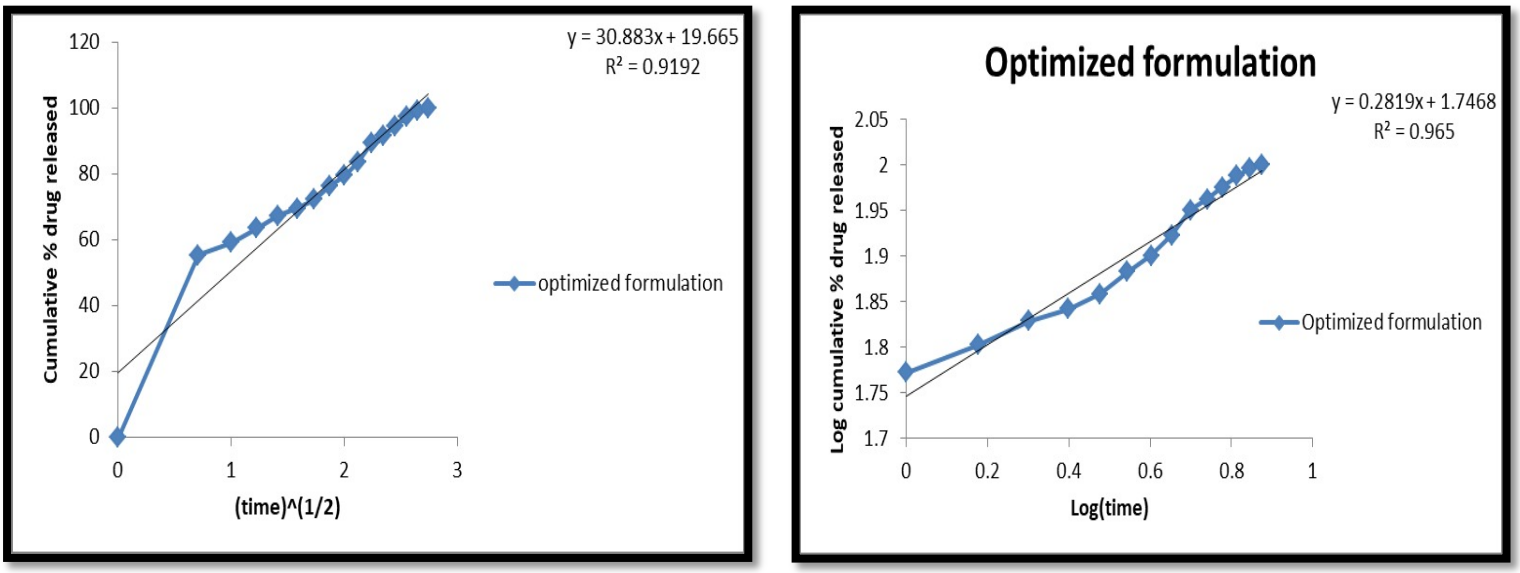


Fig 43,44 Higuchi first order plot for optimized FBT Nanosuspension,Peppas plot of optimized FBT Nanosuspension

Conclusion: Anti-solvent precipitation followed by sonication technique can be successfully employed to produce a stable fenofibrate nanosuspension. This method showed significant improvement in aqueous solubility as well as dissolution characteristics which may improve its oral bioavailability.

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