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A CENTRAL COMPOSITE DESIGN APPROACH FOR QUANTIFYING LIPID NANOPARTICLES USING UV-TRANSMITTANCE

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ABSTRACT

The present work reported an economic approach for SLN development using a Bath-Sonicator. In our method Transmittance (T%) measures the effects of Dextran, Tween 80, Sonication time and Lipid Concentration over formation of Particles below 300 nm. In our brute-force methodology, the modified Solvent-evaporation method has been used for nanoparticle formation. The variables have been optimised using Central Composite Design, to find best SLN formulation with highest T%. The Particle Size analyser has been used for conforming the effect of variables over T% and for supporting our determinations. The Quadratic model from response surfaces for \T%-300 nm was found significant. The best SLN preparation is made with 0.5-1% Tween 80, 12 mg Dextran, 8 mg LC and 18 min Sonication time. It has maximum T%-300 nm (83.75%) respectively, with Zeta potential as 23 mV. This proposed method can be used for formulating SLNs, along with the determination of Particle size range. The method is economic, by involving a UV Spectrophotometer based approach. Results reveal that there is no insignificant difference between Particle sizes as determined using Spectrophotometer and Malvern Zetasizer.

KEYWORDS: Solid Lipid Nanoparticles (SLN); Central Composite Design (CCD); Transmittance (T%); Lipid Concentration (LC); Sonication Time (ST).

1. INTRODUCTION

Solid lipid nanoparticles are the carriers with average diameter between 50-1000 nm, and capable of carrying lipophilic and hydrophilic drugs. They are said as suspensions of an aqueous continuous phase composed of fully or partially crystalline lipid nanoparticles.[1] Schoenitz et al have defined SLN as an emulsifier stabilized lipid matrix with particle diameter within a range of 100-500 nm.[2] They are better than other colloidal carriers[1,3,4], can also be scaled-up.[5] Lipids in SLN when compared to polymers are easy to process, degrade to weaker acids and have no effect on peptide stability.[3,6,7] Moreover, SLN can be modified for their controlled release.[8] The hydrophobic core of lipids incorporates lipophilic proteins, while hydrophilic ones undergo partition towards aqueous phase during encapsulation.[9] Hence, SLN provides a tool for peptide delivery by overpowering different stability issues and shields several benefits of lipid as emulsion and polymeric nanoparticle systems.[10] The current study aimed to study effect of independent variables like Sonication Time (ST), Lipid concentration (LC), Tween 80 & Dextran on Particle size. According to our knowledge the modified method used for SLN Formulation is unique, due to Bath-Sonicator. Gulseren et al have hypothesized that ultrasonication induces mechanical, chemical and thermal changes in proteins like Bovine Serum Albumin.[11,12]

The optical properties of nanoparticles makes UV/Vis Spectroscopy an important tool for identifying and characterising them. Spectral Transmission can be used as method of obtaining Particle size distribution in a deflocculated medium. Bailey developed method for determining Particle size from Transmission in visible and near Infra-red parts of spectra.[13] Similarly Eerdenbrugh et al have investigated effect of micron and nano-sized particles over UV absorption spectra. It was found that Tyndall scattering diminishes with bigger particles at lower wavelength.[14] Hence in our study Transmittance (T%) has been selected for determining Particle size variation. The T% at a particular wavelength represents the minimum percentage of particles present pertaining to that wavelength as particle size, which corresponds to Mie Theory. The validity of results have been proven by measuring Particle Size distribution of developed Nano formulations.

Optimisation using statistical software reduces number of experiments and improves quality. Statistical designs identifies mathematical models for making decision on optimum effect of variables on selected response.[15] Response surface methodology is popular in many phases of drug delivery[16], as it helps in predicting response from a designed model. CCD is flexible

and sensitive for estimation of quadratic model.[15] In our study Lysozyme was selected as model protein due to its commercial availability and proper characterization. The activity of Lysozyme relies on amino acids Glutamate and Aspartate. In our work proteins and peptides will be encapsulated in natural bio-lipids, with further analysis.

2. MATERIAL AND METHODS

2.1. Materials

Lysozyme was purchased from Himedia Laboratories (USA). Stearic Acid was purchased from CDH New Delhi, while Tween 80 was procured from SD Fine Chemicals (Maharashtra, India). Dextran was a gift from IGL, India. All other reagents were of analytical grade and were used as received.

2.2. Preperation of Stearic Acid – Lysozyme SLNs

SLN were prepared using modified solvent emulsification-evaporation sonication method used somewhere else also.[17] The Stearic acid as lipid was dissolved in Chloroform. The aqueous phase contained Lysozyme, with Dextran and Tween 80 solution as per optimised process. When both phases were isothermal the dispersed phase was added to aqueous continuous phase, kept in a Sonicator (150 W, Time is adjusted as per Optimisation Process; Zexter (GG Technologies), New Delhi-India). The Sonicator was operated at regular intervals at 20^{o}C and again restarted. The SLN formulations were prepared using different concentrations of Stearic acid, Tween 80 and Dextan with constant amount of Lysozyme, with varying Sonication cycles. However, the range for different co-functional agents was fixed in Response surface diagram after several preliminary experiments.

2.3. Experimental Design

The independent variables selected for Optimisation were (X1) Tween 80 (concentration in percentage), (X2) Dextran (concentration in mg), (X3) Sonication cycles (minutes) and (X4) LC (mg). The independent variables were simplified for optimisation after a series of experiments. The experimental methodology used was CCD with help of Design Expert ver 7.0 (Stat-Ease, Minneapolis, MN, USA) with $\alpha = 2$ and 6 centre points was selected. The dependent variable selected was T% at 300 nm. Table I represents the levels of independent variables used in the experiment. The final design consist of 30 runs Table II.

Table. I: Central Composite Design (CCD) - Independent Variables with Levels.

	Factor Level in Design.				
	-2	**-1**	**0**	**+1**	**+2**
(X1) Tween 80 (%)	0	0.5	01	1.5	02
(X2) Dextran (mg)	9	10	11	12	13
(X3) Sonication Cycles (min)	0	6	12	18	24
(X4) Lipid (mg)	1	8	15	22	29

Table. II: Central Composite experimental design. Response of Transmittance at 300 nm.

Run	A:Tween 80	B:Dextran	C:Sonication Time	D:Lipid Concentration	Transmittance @300nm
	Factor 1	Factor 2	Factor 3	Factor 4	Response 3
	Percentage	mg	min	mg	300 nm
1	0.5	12	6	8	96
2	1	11	12	1	77.18
3	1	11	12	29	62.96
4	2	11	12	15	84.32
5	1	11	12	15	77.21
6	0.5	10	18	8	79.28
7	1.5	10	6	22	83.53
8	0.5	10	18	22	67.13
9	1	11	24	15	58.32
10	1.5	12	18	8	77.16
11	0.5	12	18	8	83.75
12	1	9	12	15	81.61
13	1	13	12	15	91.08
14	1.5	10	18	22	67.81
15	1	11	12	15	78.21
16	1	11	0	15	96.44
17	1.5	12	18	22	67.63
18	1	11	12	15	75.69
19	1	11	12	15	75.34
20	1.5	12	6	22	88.53
21	0.5	10	6	8	86.35
22	0.5	12	6	22	93.04
23	0.5	12	18	22	73.33
24	0.5	10	6	22	81.66
25	1.5	10	6	8	87.33
26	1	11	12	15	75.48
27	1	11	12	15	76.32
28	1.5	10	18	8	79.07
29	0	11	12	15	89.04
30	1.5	12	6	8	93.25

2.4. Transmittance Measurement

The prepared SLN formulations were diluted, 100 times by double distilled water. The number of Particles within range of 300 nm were measured using Transmittance %. Transmission was determined using a UV Spectrophotometer (UV-1800 Series, Shimadzu Corporation, Japan). The method of using Transmittance is based on principal of Tyndall effect.[18]

2.5. Statistical Analysis

Our aim was to analyse and enumerate effect of significant factors on Particle Size using T% with a mathematical model. The major factors were taken as independent variables in CCD. All the factors were studied through second order polynomial Equation-I:-

$$\text{Response} = a^0 + a^1(X1) + a^2(X2) + a^3(X3) + a^4(X4) + a^{11}(X1)^2 + a^{22}(X2)^2 + a^{33}(X3)^2 + a^{44}(X4)^2 + a^{12}(X1)(X2) + a^{13}(X1)(X3) + a^{14}(X1)(X4) + a^{23}(X2)(X3) + a^{24}(X2)(X4) + a^{34}(X3)(X4)$$

Equation. 1.

In Equation-1 a^0 = Intercept, a = 0, 1, 2, 3, 4 while (a^1, a^2, a^3, a^4) are linear coefficients, (a^{11}, a^{22}, a^{33}, a^{44}) are quadratic coefficients and (a^{12}, a^{13}, a^{14}, a^{23}, a^{24}, a^{34}) are interaction effects.[15] Within the equation (+) sign of coefficient represents an increase of response due to variable, while reverse effect is with (-) coefficient.[16,20] The effect of variables is analysed using Contour plot. Statistical analysis was applied using ANOVA to prove significance of our results.

2.6. Particle size and Zeta potential determination

The particle size distribution of SLN dispersions was observed using Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). This was required for knowing the Zeta Potential and uniformity in Particle size distribution. The instrument was operated at 25°C using a clear disposable zeta cell. The Zeta Potential and Particle size distribution analysis was measured for some formulations, having extremity in variable concentrations. These results were compared with measured Transmittance % and tabulated in Table III.

Table. III: Comparison between T% - 300 nm representing Particle Size, Cumulative Particle size distribution and Zeta Potential.

Run Number	Tween 80	Dextran	Sonication Time (ST)	Lipid Concentration (LC)	From UV-Visible Spectrophotometer	From ZetaSizer	
					Transmittance (T%) -300 nm	Cumulative Percentage of Particles below 300 nm	Zeta Potential
2	1	11	12	1	77.18	93.90	-9.7
5	1	11	12	15	77.21	100.00	-10.5
9	1	11	24	15	58.32	74.90	-12.4
10	1.5	12	18	8	77.16	0.00	-12.6
11	0.5	12	18	8	83.74	94.23	-23.5
15	1	11	12	15	78.21	100	-12
23	0.5	12	18	22	73.33	100	-10.8

3. RESULTS AND DISCUSSION

3.1. Design Statistics and its feasibility

The significance of our design can be proved by Variance Inflation Model which is very much close to 1, representative of non-correlation with predicted value. The least standard error was found to be for quadratic coefficients proving their goodness. The Polynomial equation for determining T % at 300 nm is as follows.

$$T\% - 300\,nm\,(R1) = 76.37 - 1.07(X1) + 2.48(X2) - 7.95(X3) - 3.67(X4) - 1.43(X1)(X2) - 0.46(X1)(X3) + 0.057(X1)(X4) - 1.46(X2)(X3) + 0.27(X2)(X4) - 1.70(X3)(X4) + 2.81(X1)^2 + 2.73(X2)^2 + 0.49(X3)^2 - 1.34(X4)^2$$

Equation. II.

The F Value of the Quadratic plot as in Table 2 was 56.09 ($p < 0.0001$) indicating significant relation between Response R1 with set of X variables. The high R^2 value 98.13% represents the response variability around mean.[15] The model represented a lower Coefficient of Variation of 2.28 giving better dispersibility around mean.[16,19] The model at R1 shows stationary point, since most of the quadratic regression terms at T%-300 nm are positive.[15] The significance of ANOVA here, shows the importance of effects related to critical factors.[21] The high F value in ANOVA Table of (Table-IV) signifies a significant effect.

Table. IV: ANOVA Table for CCD (R2 T%-300 nm).

Source	Sum of Squares	df	Mean Squares	f Value	p-Value
Model	2617.55	14	186.97	56.09	< 0.0001
X1 -Tween 80	27.42	1	27.42	8.23	0.0117
X2 - Dextran	147.16	1	147.16	44.15	< 0.0001
X3 - Sonication Time	1516.54	1	1516.54	454.99	< 0.0001
X4 - Lipid Concentration	322.40	1	322.40	96.73	< 0.0001
X1X2	32.69	1	32.69	9.81	0.0069
X1X3	3.41	1	3.41	1.02	0.3281
X1X4	0.051	1	0.051	0.015	0.9032
X2X3	34.15	1	34.15	10.25	0.0060
X2X4	1.14	1	1.14	0.34	0.5669
X3X4	46.19	1	46.19	13.86	0.0020
$X1^2$	217.26	1	217.26	65.18	< 0.0001
$X2^2$	204.64	1	204.64	61.40	< 0.0001
$X3^2$	6.59	1	6.59	1.98	0.1802
$X4^2$	49.00	1	49.00	14.70	0.0016
Residual	50.00	15	3.33		
Lack of Fit	43.59	10	4.36	3.40	0.0946
Pure Error	6.41	5	1.28		
Cor Total	2667.54	29			

3.2. Influence of Variables at Transmittance 300 nm (T%-300nm)

According to quadratic Equation-II, the linear regression coefficients a^2 is positive while a^1, a^3 and a^4 are negative. The model included in-significant terms a^{13}, a^{14} and c^{24} have been removed from study. Removal of these terms were effecting actual and predicted R very marginally, but they were kept to maintain order of model. All the cross term reaction coefficients were negative except a^{14} and a^{24} which showed support of LC for Tween 80 and Dextran after interaction. The positive quadratic coefficients a^{11}, a^{22} and a^{33} have a curved path above x axis except a^{44}. The "Predicted R-Squared value" of 94.83 and "Adjusted R-Squared value" of 97.89 are within 20 % of each other, with an adequate precision of 40.31. In our quadratic model Variance Inflation Model is very much close to 1 clarifying a no-correlation with predicted value. The least standard error was found to be for quadratic coefficients proving their goodness.

The response plots for estimating effect of T%-300 nm are interpreted using 2 conserved variables at ideal positions. In our study variables effecting smaller and bigger particles, in nanoformulations are similar. The effects of variables are reported at 300 nm to determine effective ones responsible for size reduction studied at higher wavelengths. It is clearly indicated through Fig I-A that Dextran and Tween 80 have equal impact over T%, but still

higher T% is achieved at 0.5% of Tween-80 concentration and at 12 mg Dextran. The effect of ST and Dextran on T%-300 nm, studied in Fig I-B represents that maximum T% is achieved at Dextran concentration of 12 mg and at lower sonication time of 6 min. A very well sharp lessening in T% is with changing ST from 6 – 18 minutes. LC along with ST effects T%-300nm, signified through Fig I-C. It's seen that interaction between ST and LC behaved differently when studied at T%-300 nm. The maximum T%-300 nm in Fig I-C is observed at 8 mg LC and 6 min of ST stating more quantity of SLNs is formed with particle size around or below 300 nm. The effect is somehow in contrast to Fig I-A, where T%- 200 nm is maximum at 8 mg LC and 18 min ST. Vitorino et al and Peres et al in their work have reported that Greater LC results in a higher particle size distribution due to inefficient ST and increase in viscosity[20,21], which supports our study. Vitorino et al, in his study used double factorial design and emphasised role of Sonication in reducing Particle size.[22] In our study an excess of sonication has been performed to assess the effect with increasing LC at constant emulsifier and stabiliser concentrations. The increase in ST with LC proves synergistic in increasing Particle size represented by lowering of T%. The synergism may be due to inefficient sonication at higher viscosity resulting in particulate aggregation. However, as reported by other authors[22,15,24] that there is concentration dependent growth of particle size which is true as per our study.

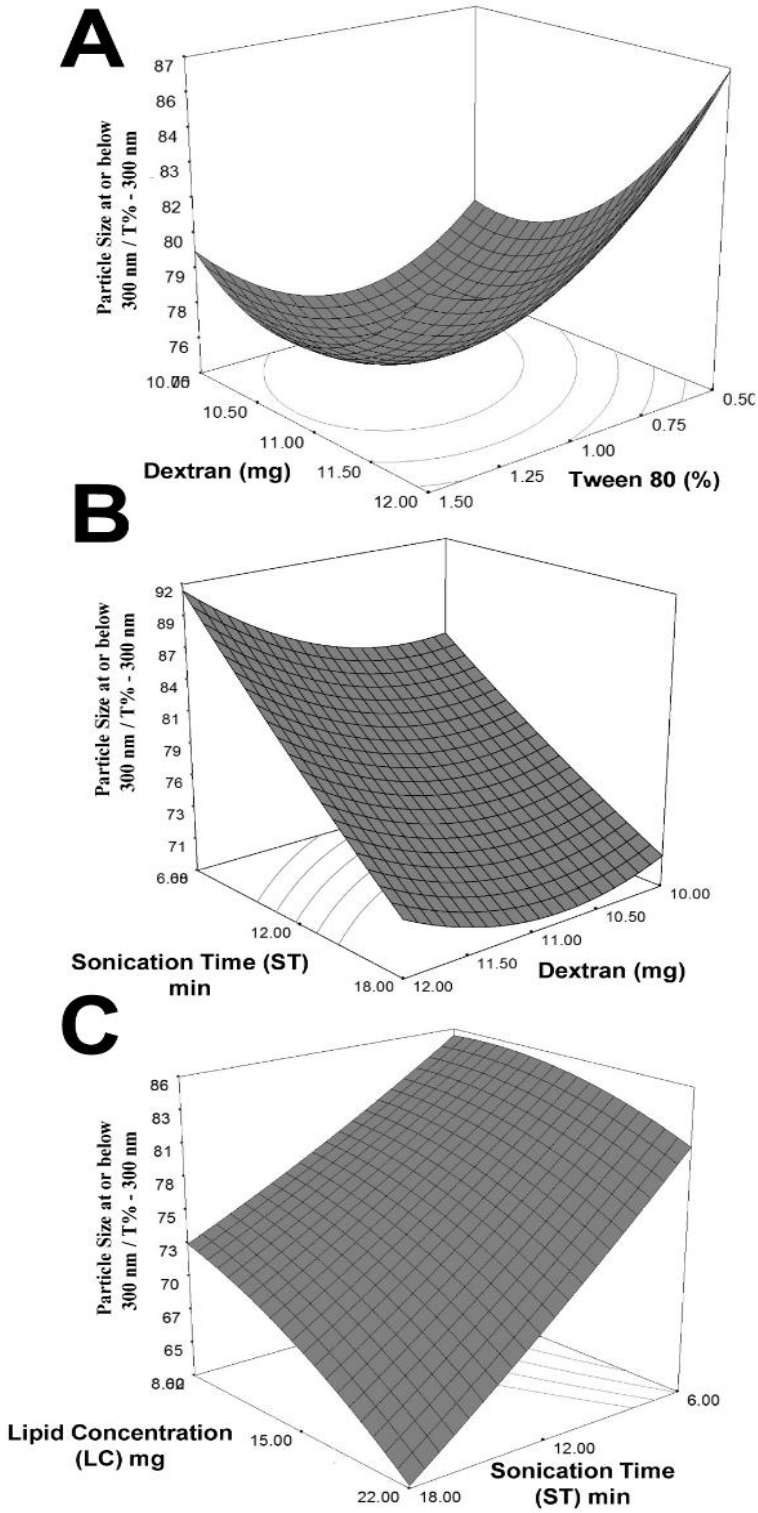

Fig. I - Response Plots showing impact of 2 variables over Transmittance % at 300 nm

(A) Impact of Tween 80 and Dextran

(B) Effect of Dextran and ST

(C) Influence of ST & LC

3.4. Comparison of T% with Particle size Analysis

The T%-300 nm data of SLN formulations has been statistically compared with their Particle Size distribution measurements (Malvern Instruments Ltd, Worcestershire, UK). ANOVA, statistically is used to prove significance of our model.

3.5. Comparison of variables over Transmittance (T%)

In our study we used T%-300 nm of SLNs formulations for estimating the effect of variables over Particle size. The factors under investigation are the crucial ones for obtaining protein loaded SLNs economically. However, for comparison the effect from response surfaces has been represented graphically in Figure II. The graphs have been drawn using maxima and minima of selected variables.

3.5.1. Effect of Lipid concentration

Lipid forms bulk of our nanoparticles and caused formation of particles with different sizes. It effects particle size mostly independently and interacts with other variables by effecting encapsulation efficiency, pore size and release. The graph in Fig II-A signifies effects of changing LC over T% along with maxima and minima of variables under study. It is clear that T% increases with reducing LC at all levels of maxima and minima. Thus, highest T% - 300 nm is achieved at lowest LC of 8 mg ie smallest particle size SLNs are obtained. Schubert and Muller-Goymann[24] reported that higher LC results in concentration-dependent increase in particle size which is consistent with our study. In addition, LC higher than experimental leads to formation of microparticles.[15] In our study LC of 22 mg has shown to increase Particle size in several responses. Hence, highest T%- 300 nm is achieved at 8 mg LC, with 12 mg Dextran and 0.5 % Tween 80.

In our study Stearic acid was selected, as its Melting point is higher than body temperature, which will promote sustained release of drug. In addition the long chain fatty acid shows a slower transformation rate from stable α form to β form. Such lipids have lesser imperfections giving a stable structure.[2]

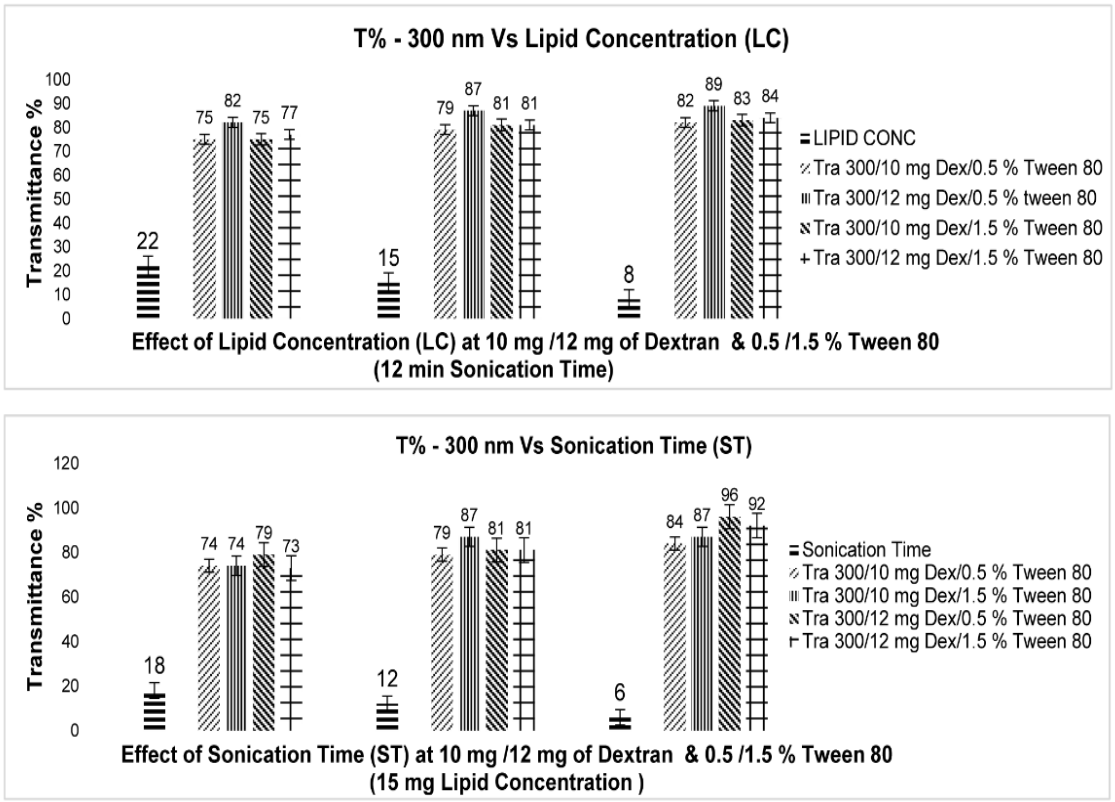

Fig. II - Graphical representation between Transmittance % 300 nm with different maxima and minima of variables under study. (A) - T% vs Lipid Concentration (LC) (B) - T% vs Sonication Time (ST) Data were expressed as mean values (±SD, n = 3).

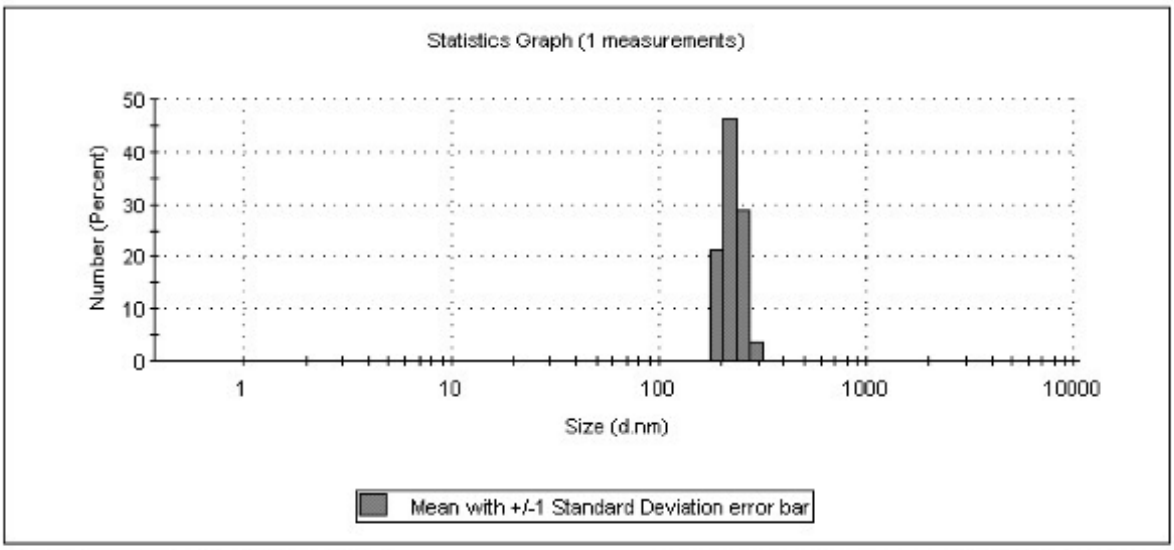

Fig. III – Histogram of our selected Formulation representing Particle Size Variation

3.5.2. Effect of Sonication Time

Sonication is used as a method in our study to induce size reduction. The effect of Sonication over T% is studied using Tween 80, Dextran and LC. Here, Sonication effects formation of smaller particles as seen in several comparative graph in Fig II-B. It's evident from all graphs that T% - 300 nm increases with increasing Sonication time. Hence, smaller particles are formed by higher ST, but bigger particles are made at lower ST resulting in formation of aggregates. It is also established from our study that formation of Particles of different sizes depends on LC along with ST. The volume distribution of nanoparticles among bigger and smaller is also reliant on sonication.[22] An increase in ST removes solvent and causes formation of lipid layer over the water solubilised drug. But, still the fair chance of aggregate formation depends on α-form of lipids, which is reduced by higher sonication time.[26]

4. CONCLUSIONS

The usage of suitable formulation strategies enhances its acceptability by Regulatory authorities. In our study using CCD as response surface methodology proved useful in optimising the formulation. Hence, our results are in contrast to many supporting lesser usage of surfactants like Tween 80. Development of such formulations may definitely promote prolonged release action of proteins at desired site.

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