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Optimizing the Concentration of Ampiphiles in the Development of Colloidal Drug Carriers for Paclitaxel.

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ABSTRACT

In the development of solid lipid nanoparticle (SLN) role of ampiphiles is crucial as they control important characteristic of formulation. The potential characteristics of these carrier controlled by amiphiles are particle size, encapsulation efficiency, stability and controlled release.Paclitaxel(PTX) is anticancer drug used widely in various carcinomas. The present study was undertaken to establish the optimum concentration of ampiphiles in the development of SLN loaded with PTX by microemulsification technique. In this study 3^2 Full Factorial Design was employed for optimizing the concentration surfactant and co-surfactant for the nanoparticles. Soy lecithin was used as surfactant and Sodium taurocholate co- surfactant with shell composition of Stearic acid (SA) and Di-palmitoyle phosphotidylcholine (DpPc).The optimization of ampiphile concentration was done by studying dependent variables as particle size less than 200 nm, % encapsulation efficiency(% EE) of more than 75 percent. Total nine batches of drug loaded SLN were prepared with varying concentration of surfactant and co-surfactant. Results showed that SLN prepared with concentration of 20% of soy lecithin and 0.8% of sodium taurocholate produced the SLN with optimum characteristic as compared with other formulations. They showed particle size of 192 nm, and encapsulation efficiency of 81%.The *In-Vitro* release performance was found to be 69% drug released up to 48 hrs and better *In-Vitro* cytotoxicity in MCF7 breast cancer cell line.

Keywords: Paclitaxel, Colloidal carriers, Optimization, 3^2 Full Factorial Design.

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INTRODUCTION

Nanotechnology is one of the most promising approaches to deal with cancer, which is a life threatening ailment all over the world [1, 2].

Regulatory agencies have given due constrain due to which less number of nanotech based products have entered the market [3]. In the spectra of nanotechnology various colloidal carriers are studied .The much focus is given to solid lipid nanoparticles and most important in developing formulation to anticancer drugs [4].

Solid lipid nanoparticles (SLN) are a novel nano particulate system which is attracting major attention as an alternative colloidal carrier system to polymeric nanoparticles, liposome's and nanoemulsion.The increasing interest gained by SLN as a colloidal drug carrier is due to their properties like possible targeting by suitable chemical modification, good protection of encapsulated drug, high encapsulation load, no biotoxicity of carrier and ease of production and scale up at low cost [5].

Paclitaxel (PTX) is one of the several cytoskeletal drugs that target tubulin. Cells treated with paclitaxel results in defects such as mitotic spindle assembly, chromosome segregation, and cell division. Extensive efforts have been put to prepare nanotech based formulation for paclitaxel till date [6, 7].

In developing the SLN the issue of their utility is governed by various characteristics of SLN including particle size, entrapment efficiency and their release profile. These characteristics are primly controlled by the amount of emulsifier added during their scale up [8-10]. Factorial design is a systematic approach in optimizing the results in a large number of study populations. The results generated by this systematic approach are widely used in the field of formulation development and in biological studies [11- 14].

Stearic acid (SA) and Di-palmitoyle phosphotidylcholine (DpPc) are biodegradable and biocompatible solid lipid and phospholipid which are approved by FDA and useful in the fabrication of nanotech based products [15, 16].

In present research work we have undertaken the development of PTX loaded SLN using biodegrable lipid shell made up of SA and DpPC.The concentration of ampiphiles is crucial in the formation of SLNs. So, to optimize the characteristics of SLN present research work emphasises on the concentration of surfactant and co-surfactant added with respect to particle size and drug encapsulation profile of prepared SLN.

MATERIALS

Paclitaxel was supplied generously by Yunan Hande Biotech Co.Ltd.China as gift sample, Stearic acid was purchased from S.D. fine Chem. Mumbai, DpPC was supplied by Genzyme Switzerland as gift sample, Soy lecithin and Sodium Taurocholate were supplied by Across Organics New Jersy USA., MCF7 Breast cancer cell line were purchased from NCI Pune.All reagents were used as supplied by suppliers without further purification.

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METHODS

Compatibility Studies

FTIR Study

The IR spectroscopy was done by using FTIR (Spectrum One, Perkin Elmar, USA) to confirm the identity of the pure drug and to detect the interaction of the drug with carriers. The IR studies were carried out by the pressed pellet technique using a KBr press. Dried KBr, drug, lipids, were compressed to form pellets. The prepared pellet was placed in the sample holder and kept in the instrument to record the IR peaks.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) analysis was conducted to ascertain the compatibility of drug with the polymer using Mettler Toledo DSC 822 (STIC, Cochin, India).The DSC thermogram of pure PTX,physical mixture wih lipid and that of one formulation was recorded.

Determination λ_{max} of Paclitaxel.

***Stock solution*:** Paclitaxel 5 mg in 50 ml Methanol: Phosphate buffer saline pH 7.4 (9:1) ratio solution.

Scanning: From the stock solution, 10 µg / ml solution of Paclitaxel was prepared in Methanol: Phosphate buffer saline pH 7.4 (9:1) ratio and scanned between 200-400 nm.

3^2 Full Factorial Experimental Design.

To optimize the concentration of independent variables, ((X1) soy lecithin and (X2) sodium taurocholate) factorial design was developed. In this design the concentration of X1 and X2 was altered with respect to each other at 3 different levels. The three levels are designated as +1(high), 0(middle) and -1(low).

The dependent variable are particle size less than 200 nm and entrapment efficiency of more than 75%.The details of design is given in table no I.

Table No. I

Sr.No.	Independent Variable		Code level		
			High	Medium	Low
1	X1	Soy Lecithin (%w/w)	25	20	15
2	X2	Sodium Taurocholate (%w/w)	1.2	0.8	0.4

Independent Variables with Code Levels

Preparation of paclitaxel loaded solid lipid nanoparticles by micro emulsification technique: [17]

Solid lipid nanoparticles of paclitaxel were prepared by micro emulsification technique using soy lecithin as surfactant, sodium glycholate as co surfactant. The concentration of shell was mixture of SA and DpPC in the ratio of 1:1 and the ratio of drug to shell was 1:20. Total 9 batches of SLN were prepared by varying the concentration of soy lecithin(X1) and sodium tairocholate(X2) (Independent Variables) using 3^2 full factorial design.(Table No.II) SA and DpPC were melted at a temperature of 80^0c, to the melted lipids PTX was added with 5 min. stirring followed by sonication. To this mixture soy lecithin was added and stirred for 2 minutes. Aqueous phase containing co surfactant sodium glycolate was heated at 80^o.c and added to melted lipid phase with mechanical stirring at 80^0.c for 10-15 minutes, formed o/w microemulsion.

The micro emulsion was carefully added drop wise using a 5 cc glass syringe fitted with 21 gauge needle, into ice cold water in a beaker with continuous stirring. The mixture was stirred at 3000rpm and SLN dispersion was stirred for 3 hrs after complete addition of microemulsion.The SLN dispersion was subjected to ultrasonication for 10 minutes.

Formed SLNs were collected and lyophilized. Lyophilized formulations were stored in previously sterilized (autoclave) glass containers and all the preparations steps were carried out at room temperature (23-25^0C).The prepared formulations were stored in freezer.

Table No.II

Batch Code	Soy Lecithin (X1) %w/w (Level)	Sodium Taurocholate (X2) %w/w (Level)
F1	25 (+1)	1.2 (+1)
F2	25 (+1)	0.4 (-1)
F3	25 (+1)	0.8 (0)
F4	20 (0)	1.2 (+1)
F5	20 (0)	0.4 (-1)
F6	20 (0)	0.8 (0)
F7	15 (-1)	1.2 (+1)
F8	15 (-1)	0.4 (-1)
F9	15 (-1)	0.8 (0)

The 3^2 Full Factorial Design Layout of Paclitaxel Loaded SLN

Particle Size [18]

The size distributions along the volume mean diameter of the nanoparticles were measured by Dynamic Light Scattering Particle Size Analyzer (Nanotrac Particle Size Analyzer,USA). The range of the analyzer is 0.8 nm to 6.54 µm.

Determination of Encapsulation Efficiency [19]

Paclitaxel loaded SLNs were estimated by centrifugation method. The prepared SLNs were placed in centrifugation tube and centrifuge (Lastocraft) run at 15000 rpm for 30 min.

The supernatant (1ml) was withdrawn and diluted with methanol. The unentrapped paclitaxel was determined by UV spectrophotometer at 227 nm. The samples from the supernatant were diluted suitably before going for absorbance measurement. The free paclitaxel in the supernatant gives the total amount of unentrapped drug. Encapsulation efficiency is expressed as the percent of drug trapped.

$$\% EE = \frac{\text{Total amount of drug} - \text{Free dissolved drug}}{\text{Total amount of drug}} X\ 100$$

Shape and Surface Morphology [20]

Shape and surface morphology of nanoparticles was done by Scanning Electron Microscopy (JSM-T330A, JEOL). SEM is the most commonly used method for characterizing drug delivery systems because of its simplicity in sample handling and ease of operation. SEM has been used to determine particle size, surface topography, texture and to examine the morphology of fractured surface.

Zeta Potential

The surface charge of the NPs in water was determined by Zeta meter (3+ zeta potential,USA) analyzer at room temperature. The suspension of NPs was diluted by ultrapure water. The zeta potential was measured.

***In vitro* drug release studies** [21]

The release of paclitaxel from the nanoparticles was measured in triplicate in PBS (PH 7.4). SLNs equivalent to 10 mg of paclitaxel were placed in dialysis bag soaked overnight with dissolution fluid. Dialysis bag containing formulation was suspended in 200 ml of PBS (PH 7.4) solution in a capped beaker and placed in an orbital shaker, which was maintained at 37^0 centigrade and shaken horizontally at 120 min^{-1}. The temperature of buffer is maintained at 37±1 ^{0}c., release medium was withdrawn at time intervals of 0.5,1, 2, 4, 8, 16, 24, and 48 hrs and replaced by the same volume of PBS. The sample was diluted appropriately with methanol and estimated by UV-Visible Spectrophotometer at 227 nm.
Results of *In-Vitro* drug release studies obtained from absorbance data were tabulated and shown graphically as cumulative percentage drug released vs. Time.

Release Kinetic Study

The data obtained by performing In-Vitro drug release study was subjected to model fitting in different kinetic equation. This kinetc modlling helps us to define the mechanism of drug release form SLN.

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***In-Vitro* cytotoxicity study** [22]

In vitro cytotoxicity of prepared SLNs was done by using Breast cancer cell line (MCF-7 cell line) and compared with pure drug .The concentration of pure drug and drug loaded in the SLN of 2.5µg/ml was taken.To perform in-vitro cytotoxicity study trypan blue dye test was used. Cell concentration was diluted to 1x 10^6 cells/ml using phosphate buffered saline (pH 7.4). Trypan blue dye (0.4%) was prepared in PBS pH 7.4 and 0.1ml of MCF7 breast cancer cell line was mixed. To this one formulation from each group of SLN prepared, was added. All the samples were diluted with phosphate buffered saline (pH 7.4) to make concentration of PTX as 2.5 mcg/ml, were added and incubated for 24 hours in incubator at 37^0C. After this, the mixture was added with 0.1ml of trypan blue dye and incubated for 5 minutes at 37^0C. Blue coloured dead cells and unstained viable cells were counted by using a Neubauer chamber using low power objective.Percentage of dead cells were calculated and recorded. The percentage viability and % dead cells were calculated by the following formula,

$$\% \text{ viability} = \frac{\text{Viable cell}}{(\text{Viable cell} + \text{Dead cell})} \times 100$$

$$\% \text{ Dead Cells} = 100 - \%\text{Cell Viability}$$

RESULTS

Compatibility Studies

FTIR

Overlain I.R spectra of pure paclitaxel(1) , and its physical mixture with lipids(2,3,4) and IR spectra of formulation (5) were also recorded, which are shown in (Figure No.I).For pure paclitaxel the prime peaks were observed at 1731.70, 1703.84, 1645.08, 1368.44 and 1251.56

All these characteristic peaks of paclitaxel were present in spectra's of physical mixture (2, 3 and 4) and in formulation (5) with lipid and were shifted marginally with respective composite peaks. Thus, indicating compatibility between drug and lipids. It shows that there was no significant change in the chemical integrity of the drug.

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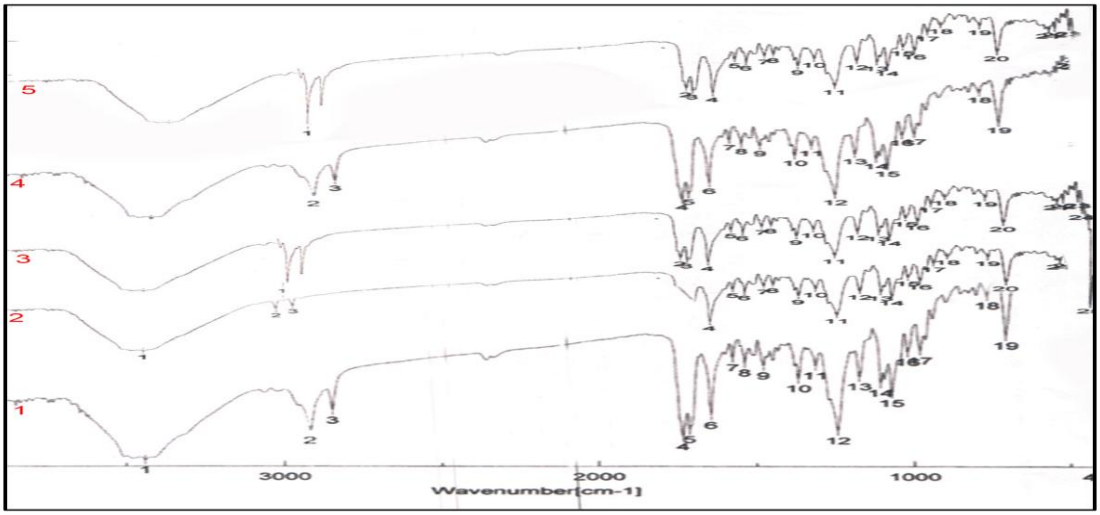
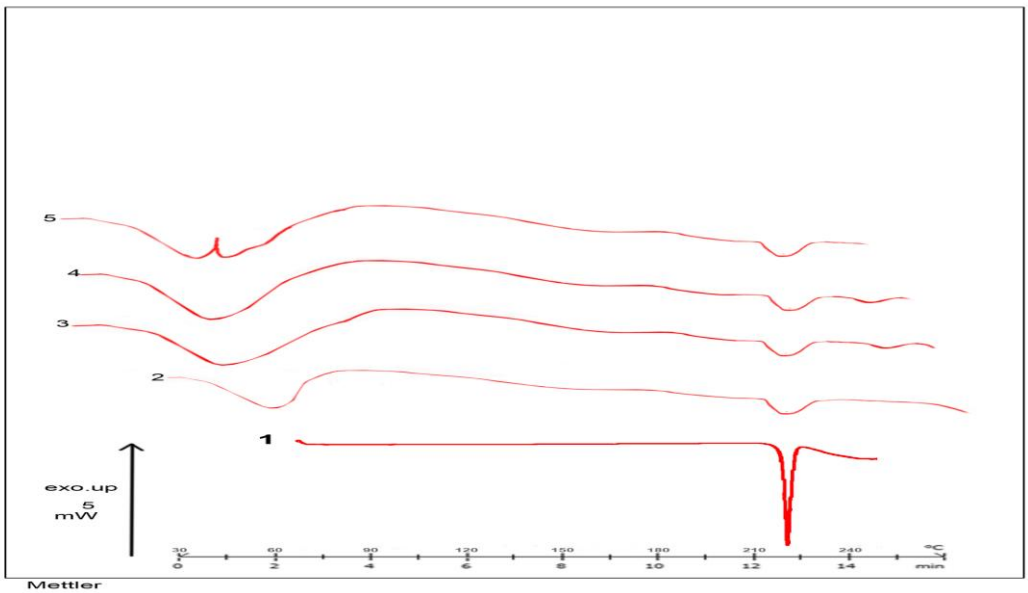


Overlain FTIR Spectra of 1-.Paclitaxel, 2- PTX+SA, 3- PTX+ DpPC, 4-PTX+SA+DpPC, 5-SLN Formulation F6

DSC

Overlain DSC curve (Fig.no.II) showed the endothermic peaks for drug paclitaxel at 217^0C and was found to be sharp. The endothermic peak corresponding to drug was broadened in the physical mixture of drug with lipid and in formulationthis may be attributed to less availability of drug in comparison to lipids.Futher the endothermic peaks of lipids were also observed. According to these data, there was no interaction between drug paclitaxel and ingredients used in sln preparation



Overlain DSC Thermogram of 1-.Paclitaxel, 2- PTX+SA, 3- PTX+ DpPC, 4-PTX+SA+DpPC, 5-SLN Formulation F6

Determination of λ_{max} for Paclitaxel

The Absorption spectrum of pure drug was scanned between 200-400 nm with 10 µg/ml concentration prepared in Methanol: Phosphate buffer saline pH 7.4 (9:1) ratio solution. The maximum absorbance was observed at 227 nm.(Fig.No.III)

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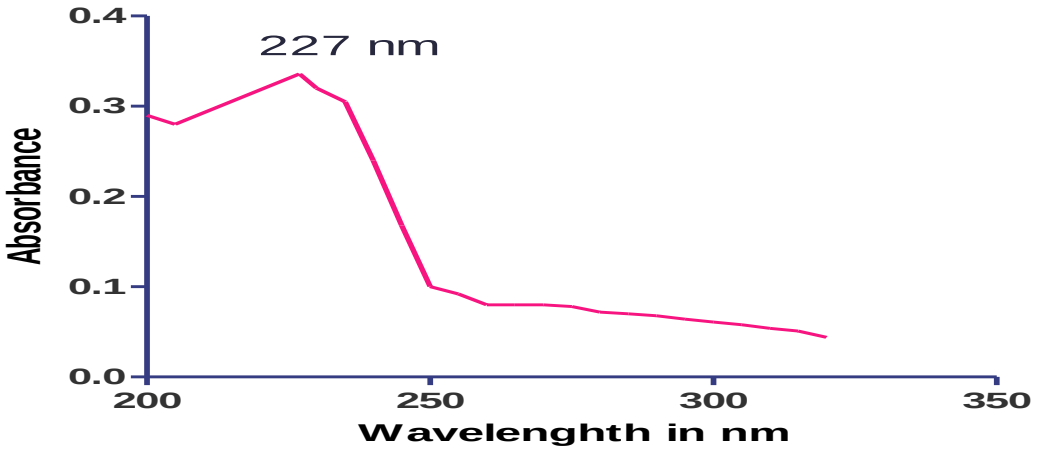

Absorbance Maxima for Paclitaxel in Phosphate buffer and Methanol.

Particle Size Determination

The Particle size distribution for prepared SLN is tabulated in table no. IV.The concentration of surfactant and co surfactant significantly affected the particle size distribution pattern. The particle size for high level of surfactant with its respective concentration with cosurafctant (Formulation F1, F2 and F3) was in the range of 103 nm-120 nm.

Formulation F7, F8 and F9 which were prepared with low level of surfactant and its combination with cosurfactant was found to have particle size range of 292 nm-313 nm.

The particle size analysis showed that formulation prepared with middle level of surfactant with different cosurfactant concentration have size range of 170 nm to 192 nm.

Table No.IV

Formulation Code	Particle Size in nm	% Entrapment Efficiency	Zeta Potential meV	%Drug Released after 48 hours	%Cell Death
F1	103	45	-28	86.78	80
F2	109	52	-26	84.84	78
F3	120	58	-26	81.32	76
F4	170	68	-27	75.96	72
F5	180	70	-29	72.25	71
F6	192	81	-28	70.18	71
F7	292	85	-29	69.50	60
F8	305	87	-28	66.70	57
F9	313	88	-26	61.76	53

Each value represent Average of three findings.

Particle Size, % Entrapment Efficiency, Zeta potential, % Drug Release after 48 hours, and *In-Vitro* cytotoxicity of F1-F9 SLN Formulations.

Encapsulation Efficiency

Encapsulation efficiency is an important characteristic of prepared formulation as it defines the quantity of formulation to be taken for given dose. As anticancer drugs are usually quite expensive and the EE is also crucial for clinical applications since more NPs would have to be used for a given dose if the EE is low. Table no. IV shows % EE of prepared SLN formulations. Due to high concentration of surfactant we obtained small SLN which shown lowest EE in the range of 45% to 58%.The low concentration of surfactant resulted higher entrapment efficiency in the range of 85% to 88%.The drug EE for middle level of surfactant was found to be in the range of 68% to 81%.

Statistical Analysis for Particle Size and Encapsulation Efficiency

The emulsifier is to stay in the oil - water interface to lower the surface tension of the lipid monolayer, it is nothing but to reduce the surface energy of interface, and thus facilitate the nanoparticle formation. Understandably less surface energy would be needed to form NPs of given size range. The emulsifier amount used in the micro-emulsification process can be used to quantitatively control the nanoparticle size. The amount of surfactant added shown significant difference in the size distribution. As the concentration of surfactant was raised there is high accumulation of these molecules at interface and more stabilizing the interface which results in smaller particle size. While in low concentration of surfactant is in insufficient to reduce the interfacial tension and ultimately results in particles with larger size.

The role of cosurfactant is to have maximum surface contact of surfactant and the interface. The results showed that for all 3 levels of surfactant, as the concentration of cosurfactant was increased there is significant reduction of particle size. This explains the effect of cosufactant as, to increase the potential of surfactant to reduce surface energy of interface.

The concentration of cosurfactant also potentiates the effect surfactant on EE percentage. The high level of cosurfactant in all formulations resulted in decreased EE as compared with middle level and low level. This low EE is attributed to the larger surface area to volume ratio for small size NPs and thus causing more drug loss into the aqueous phase in the NPs preparation process

The obtained results were analyzed by Design Expert Software and generated the results with contour plots and surface plots which are shown in fig. no.VIII to XI.

The resulting equation for analyzing the effect of ampiphiles on particle size and encapsulation efficiency were found to be

$$\text{Particle size} = +190.778 - 96.333*(X1) - .833*(X2) + 1.75*(X1)*(X2) + 26.333*(X1)^2 - 15.167*(X2)^2$$

$$\%E.E. = +70.44 - 17.5*X1 - 1.83*X2.$$

The results of statistical analysis are tabulated in table no.III.

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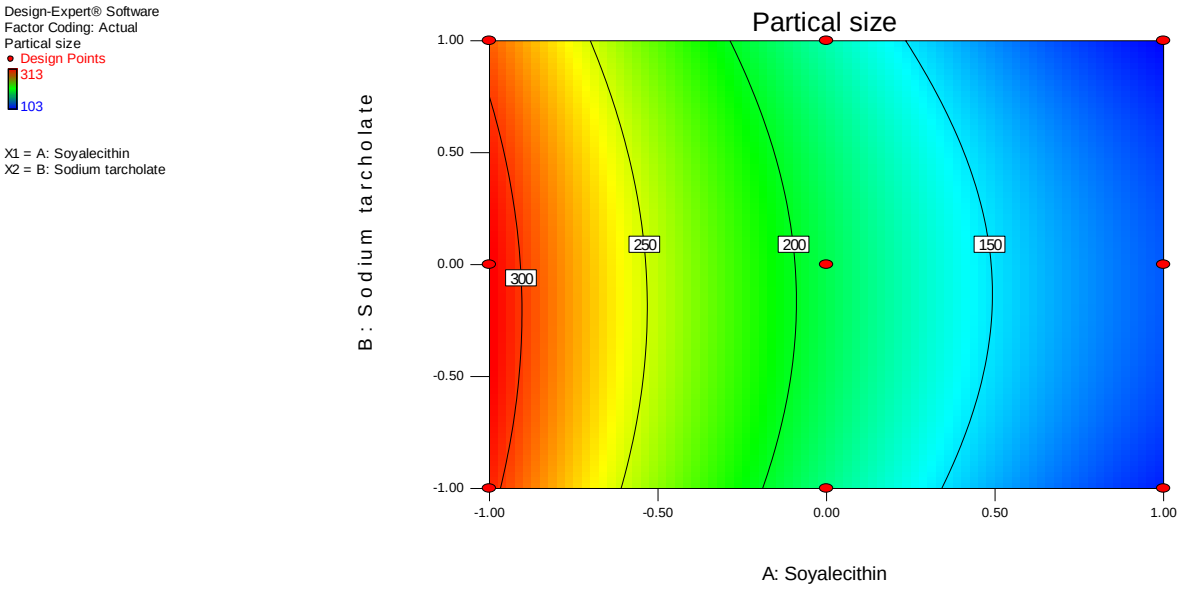
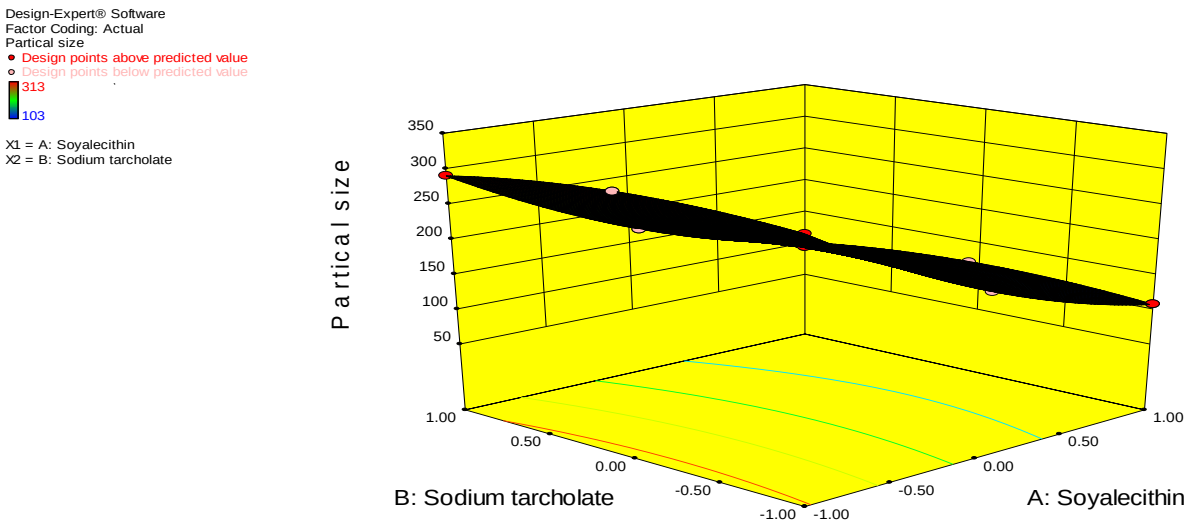
The resulting polynomial equations revealed that the amount of surfactant X1 is having more pronounced effect on particle size distribution and % EE as compared to co-surfactant X2.The combined effect of X1 and X2 was found to be high as compared to individual effect of X1 and X2. The polynomial equation indicated that as there is increase in the concentration of ampiphiles there is reduction in particle size but along with this it will also reduce the % EE of the formulation. So to get proper balance between particle size and high % EE their concentration needs to be well controlled. Thus it showed that potential control on particle size and % EE is well defined by the both ampiphiles but majorly contributed by surfactant. The formulation F6 was recorded as optimized formulations as per the design drawn; it shows particle size less than 200nm and % EE more than 75%.

Figure No. VIII



Contour Plot for Effect of Soy lecithin and Sodium Taurocholate on Particle Size

Figure No. IX



3-D Plot for Effect of Soy lecithin and Sodium Taurocholate on Particle Size

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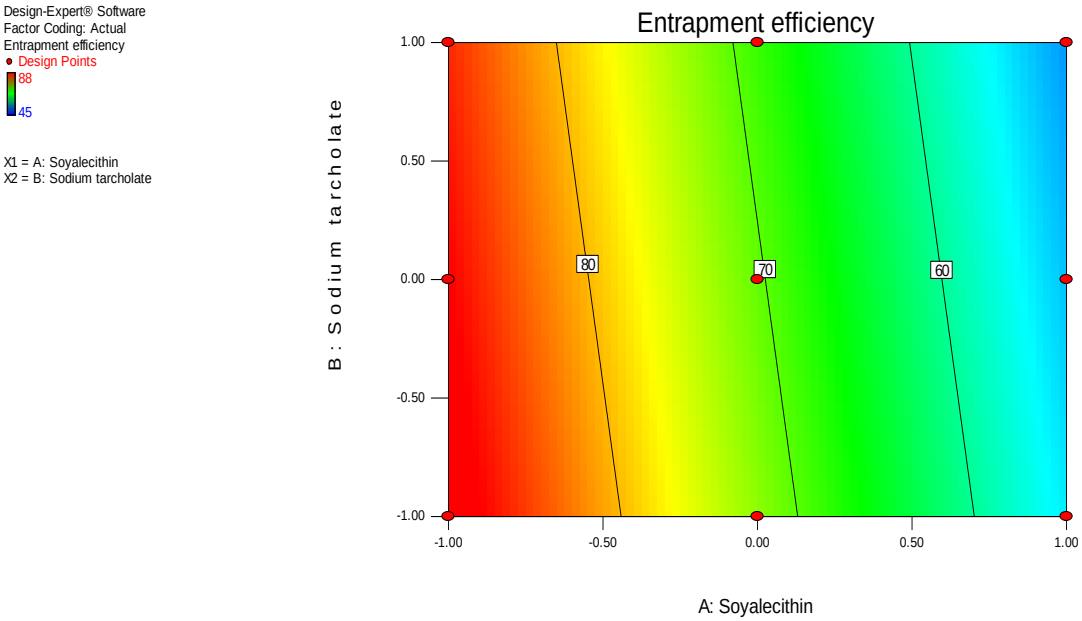
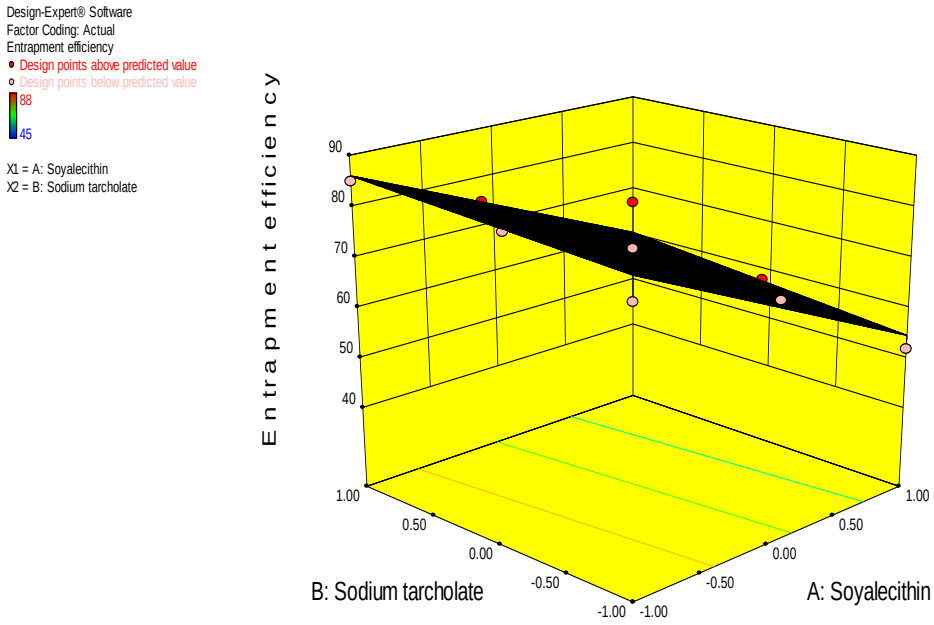
Figure No. X



Contour Plot for Effect of Soy lecithin and Sodium Taurocholate on % Entrapment Efficiency

Figure No. XI



3-D Plot for Effect of Soy lecithin and Sodium Taurocholate on % Entrapment Efficiency

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Table No.III

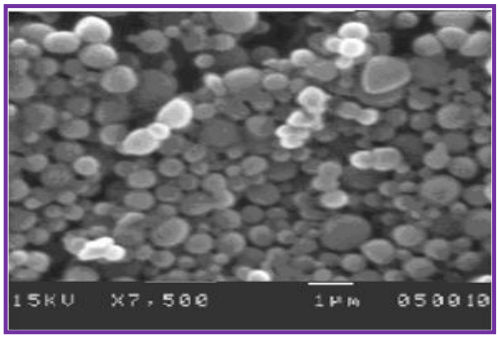
	Regression statics for	
	Particle Size	% EE
Model	Quadratic model	Quadratic
Standard	1.08	5.72
Predicted R	0.9995	0.8171
R Squared	0.9999	0.9043
Adjusted R	0.9998	0.8724
Adiquate Precision	238.214	11.701

Results of Regression Statistics for 3^2 Factorial Design

Size and Shape

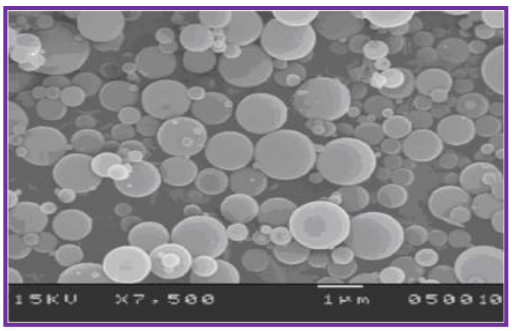
Scanning Electron Microscopy shown that SLN prepared with high and middle level of surfactant were having smooth surface area with spherical structure. While SLN prepared with low level of surfactant have irregular structure which indicated insufficient stabilization of interface during manufacturing. The SEM of F1, F6 and F9 formulations are shown in fig no.IV, V and VI respectively

Figure No.IV



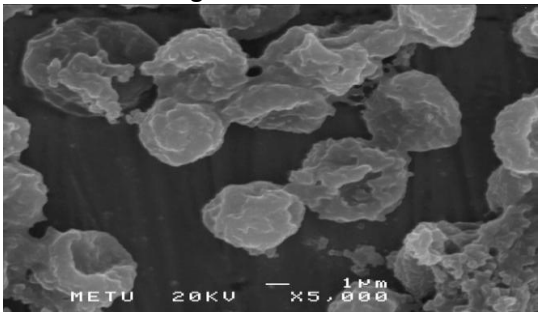
SEM Study of formulation F1

Figure No.V



SEM Study of formulation F6

Figure No.VI



SEM Study of formulation F9

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Zeta Potential

Surface charge is an important indication for the stability of a colloidal system in medium as well as for their interaction with the biological cells in vivo. The zeta potential values of the SLN shown, which demonstrated that the more ampiphiles used in the preparation process the larger absolute value of the negative charge would be resulted. The negative charge of the SLN could be due to both of the polymer and the emulsifier. The values of zeta potential are tabulated in table no. IV.

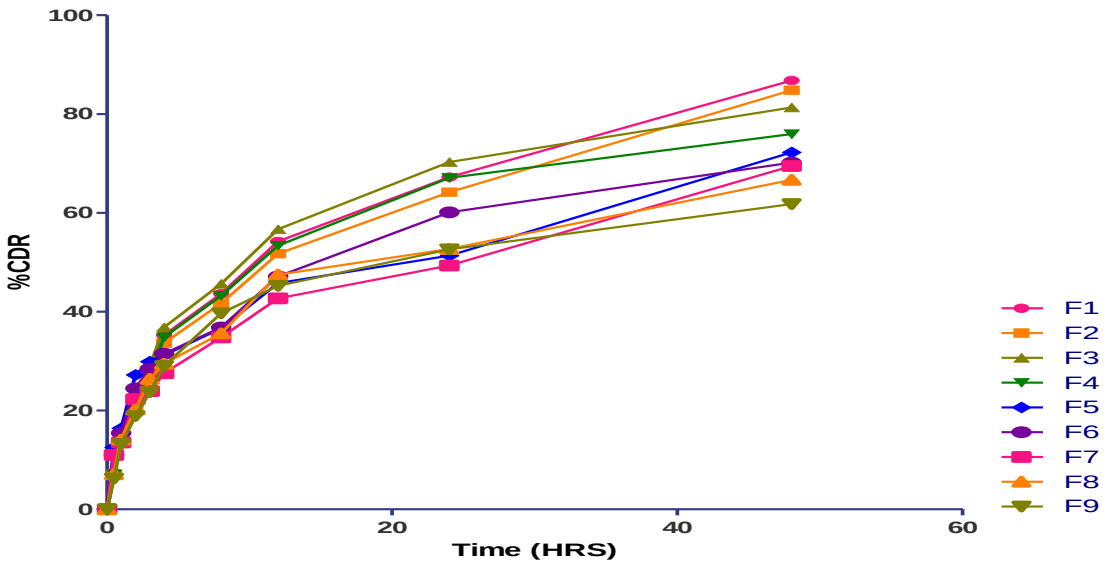
***In-Vitro* drug release Study**

In vitro drug release studies of all PTX loaded SLN were performed using dialysis membrane method. The dissolution medium used was PBS pH 7.4.

The release data obtained is for all formulation is depicted in fig.no.VII

The release of PTX from all formulations showed an initial rapid release phase, releasing approximately 24.38 to 40.06 % of paclitaxel during the first 4 hrs, and release rate was reduce thereafter, indicating that the release of paclitaxel reached a slow release status. It was observed that the drug release from the formulations increases as the particle size of the formulation decreases and all the formulations showed a biphasic release with initial burst effect. The initial burst effect is seen because of drug present at the surface of SLN which is released rapidly during initial stages of study.Formualtions F1-F3 shown rapid drug release as compared with other formulations. Percent drug released after 48 hours is shown in table no. IV.

Figure No.VII



Cumulative % Drug Released Vs Time Plot for Formulations F1-F9

Release kinetic Study

The results obtained by *In-Vitro* release study were subjected to kinetic modeling so as to check the mechanism of drug release. For this release data was fitted in four different

kinetic equations namely Zero Order, First Order, Higuchi's Classical Diffusion Model and Peppa's Model. The results of regression analysis are showed in table no. V.

The all formulations were having high R^2 value for Higuchi's Classical Diffusion model. This indicates that drug release from SLN is dependent on diffusion of drug from the polymeric matrix.

Table No.V

Kinetic Model	FORMULATION CODE								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
	R^2	R^2	R^2	R^2	R^2	R^2	R^2	R^2	R^2
Zero Order	0.8190	0.8190	0.7573	0.7798	0.7924	0.7714	0.8296	0.7243	0.6928
First Order	0.9749	0.9651	0.9188	0.9317	0.9233	0.8973	0.9386	0.8270	0.7938
Higuchi Model	0.9753	0.9753	0.9482	0.9590	0.9520	0.9554	0.9753	0.9284	0.9122
Peppa's Model	0.3515	0.4865	0.6103	0.5406	0.4865	0.5310	0.5406	0.5881	0.6113

Kinetic Model fitting study of F1-F9 SLN formulations.

In-vitro cytotoxicity

In-vitro cytotoxicity study was performed on Breast Cancer Cell Line (MCF-7 cell line) using Trypan Blue dye test. The test was carried out to assess the cellular cytotoxicity of paclitaxel from SLNs.The results are represented in table no.IV. Formulation F1-F3 showed %cell death of 76%-80%, F4 to F6 formulation showed 71%-.72% and F7 to F9 showed 53% to 60%.

The results were found to be in close agreement as that observed in *In-Vitro* release study where F1-F3 shown high release,F4-F6 shown intermediate release while F7-F9 were shown more retarded release. This indicated that more PTX from of formulations F1 to F3 is released during incubation period which was in accordance with the *In-Vitro* dissolution study.

CONCLUSION

Among various physicochemical and pharmaceutical properties of the drug loaded SLN of biodegradable polymers, the particle size and entrapment efficiency are two the most important characters that determines their *In-Vitro* and *In-Vivo* performance, and thus therapeutic effects of the formulated drug. It is thus crucial to find a way to control the particle size and surface coating in the microemulsification process. Our study showed that the amount of ampiphiles used has significant impact on the particle sizes. Too little amount of surfactant and co-surfactant would not be enough to cover the entire surface of the nanoparticles to stabilize the oil droplets in the O/W emulsion, thus leading to nanoparticles of large size. Too much amount of ampiphiles, however, would facilitate formation of smaller nanoparticles, which would result in lower drug encapsulation efficiency.

In this research we have optimized the amount of ampiphiles, which seems to be 20%w/w of soy lecithin and 0.8% w/w sodium taurocholate. We demonstrated that at this concentration of ampiphile for the formulation of anticancer drug paclitaxel in SLN showed

ideal particle size, drug encapsulation efficiency, *In-Vitro* drug release and better performance *In-Vitro* cellular uptake and cytotoxicity. It should be pointed out, however, that these advantages should be further confirmed by *In-Vivo* evaluation such as the pharmacokinetics, the half-life in the blood system, the biodistribution of the formulated drug and the xenograft tumor model.

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