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Research Article

GENISTEIN-LOADED NANOSTRUCTURED LIPID CARRIERS FOR INTRAVENOUS ADMINISTRATION: A QUALITY BY DESIGN BASED APPROACH

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ABSTRACT

Genistein (Gen) is a naturally occurring soy isoflavonoid, ing anticancer, antiproliferation & antioxidant-like properties which make it suitable as an anticancer medicine. The disadvantage of poor solubility and less oral bioavailability restrict its use as potential anticancer agent. In the present study, Gen was entrapped into solid-liquid lipid mixture with the aid of surfactant by using modified solvent evaporation technique. The present work was carried out with the aim to screen the component as well as process variables to ease the formulation process which ends up with the finished product having characteristics of best nanoformulation in all respects. Optimized levels by employing numerical optimization technique for each factor viz. surfactant concentration (X1), Lipid concentration (X2) & amount of organic solvent (X3) were 0.3 %, 0.78 % & 8.51 ml respectively. The resultant formulation exhibited a particle size of 122.22 nm, and entrapment efficiency of 92.8 %, & zeta potential of -21.25 mV with unimodal size distribution. Findings of the haemocompatibility studies suggested that optimized formulation was pretty safe for intravenous administration. In a nutshell, GenNLC seems to be a superior alternative carrier system for the formulation industry to obtain the higher entrapment with excellent stability of the formulation.

Keywords: Nanostructure lipid carriers, quality by design, optimization, Heamocompatibility.

INTRODUCTION

Genistein (Gen) (4', 5, 7-trihydroxyisoflavone) is a naturally occurring soy isoflavonoid, ing anticancer, antiproliferation, antioxidant-like properties which makes it suitable to be used as anticancer medicine for the treatment of various cancers including genital and ovarian cancer. Genistein is well known in the prevention of Polycystic ovarian disease (PCOD)[1] which is indicated by its epidemiological survey that the people having more intake of soy flavonoids are less prone to prostate and other genital cancers. It acts by the down-regulation of genes related to cell proliferation and cell cycle, persuades apoptosis, inhibits activation of NF- κB and reduces Akt protein level which corresponds to down-regulation of androgen-mediated carcinogenesis[2-4]. In spite of its strong antineoplastic activities, genistein did not step forward from 'bench to besides' due to its flaws for human use. It is sensitive to heat, light, and oxidation and also suffers from less oral bioavailability due to poor water solubility. These flaws possess some technical problems to use it as anticancer medicine. No anticancer marketed formulation of genistein is known till date probably due to its physicochemical limitations [5]. Several studies have concluded that therapeutic activity of these nutraceuticals can be enhanced by incorporation of these molecules into lipid systems which can prevent their in vivo degradation and provide with the better bioavailability. Overall, it would be of great therapeutic importance if we develop a lipid-based delivery system of genistein that will protect its degradation and enhance its bioavailability.

The lipids employed to prepare lipid nanoparticles are usually physiological lipids (biocompatible and biodegradable) so, that drugs can be delivered at the required site of action with the controlled release with low acute and chronic toxicity [6, 7]. The innovative concept of Nanostructured lipid carriers (NLCs) provided insights into the reduction of drawbacks of the historic SLN formulation as they are composed of solid lipid as well as liquid lipid mixture in a particular ratio. Due to the binary lipid (spatially different lipids) concept used in NLC, they are amorphous in nature and prevent the drug leakage during storage. During storage, the lipid with crystalline nature undergoes transitions from one form to another which leads to expulsion of the drug from the lipid cage. If these lipid transitions occur after the administration of the drug inside the body, it will interfere with the release mechanism of the specifically engineered formulation. NLC is the latest generation colloidal nanoparticles with improved stability and drug loading efficiency. The average diameter of NLCs ranges from 50- 500 nm. Despite of liquid lipid incorporation, NLCs are solid at room temperature as the less amount of liquid lipid is used in comparison to solid lipids[8-11].

Formulation of nano products is somewhat typical as large no. of the process and product variables influence the product characteristics and considering each and every variable during processing are time consuming and require a huge amount of investment. To circumvent the existing problems, the concept of Quality by Design (QbD) is getting more popular in the field of medicines which utilizes the principles of statistics along with DMAIC strategies. A software based concept of Design of Experiments (DoE) (Design Expert®) curtails the number of experimental trials to a larger extent. In short, QbD is a methodical approach which utilizes the principles of statistics and DoE and the promises to produce the high quality of the product which satisfies the customer needs in all respect. The various steps of DoE include: (a) define objective (b) identify critical quality attributes (c) screening of most influencing factors (d) experimental design (e) analysis of responses (f) optimization

process (g) validation of methodology[6, 9, 12-15]. Despite various designs available, we had selected Placket Burman (PB) factorial based design for the screening of large no. of factors which were further optimized for their optimum levels by Response surface based Box Behnken design (BBD) matrix followed by desirability approach based numerical optimization. The facts and figures available in the literature till date does not qualify for the inclusion of Qbd based optimization technique for the formulation of genistein loaded NLCs.

In this perspective, the present study was aimed at utilizing the potential advantages of NLC systems along with the concepts and principles of QbD which will be able to accommodate the higher amount of drug with lesser production cost. It will be able to deliver the drug in a controlled manner for a longer period with the improved bioavailability of the phytoconstituent.

MATERIALS AND METHODS

Genistein (Gen) was purchased from Swapanroon Drugs Pvt. Ltd. Pune (India). Tristearin and D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS) were acquired from Sigma Aldrich, India. Glycerol tristearate was purchased from TCI chemicals, India. TPGS was received as a kind gift from Antares Pharma, USA. Captex 355, Captex 300P, Capmul & Capmul MCM were provided as a gift sample by Abitac, USA. Miglyol was received as a kind gift from Chika Pvt. Ltd., Mumbai. Compritol 888ATo was received as a generous gift from Gattefosse international, Mumbai. Dynasan 114, 118 & Imwitor 900K were provided as a gift sample from Cremer oleo GmbH, Germany. Poloxamer 188, pluronic 407 & Solutol HS15 were generously donated by BASF, Mumbai. All the other chemicals used for the experiment were purchased from Fischer Scientific, Mumbai. Ultrapure-Milli-Q water was used throughout the experiment. All the solvents used for the study were of HPLC grade.

Solubility in Liquid lipids

The solubility of the drug was analyzed in various liquid lipids. Briefly, an excess amount of drug was added to each vial containing 1.0 ml of liquid lipid, the vial was sealed properly and was kept for sonication in a bath sonicator for 10 minutes to facilitate the proper mixing of the components. Then the mixtures were shaken for 48 h in a water bath shaker (Remi, Mumbai, India) maintained at room temperature. The final mixtures were centrifuged at 5000 rpm for 15 minutes, and aliquots of supernatants were analyzed for drug content by using UV-VIS spectrophotometer at λ max of 261.5 nm after dilution with methanol [12, 13, 16].

Solubility in Solid lipids

To test the solubility of the drug in solid lipid qualitatively, 1 gm of lipid was taken in the test tube and heated on water bath above 5- 10℃ of their melting point. The drug was added to the melted lipid gradually under constant stirring and examined visually [13, 17].

Quantitative solubility determination

The quantitative solvency of the medication was resolved in the chosen strong lipids. A known amount of the drug was taken in the test tube, and the measured amount of lipid was added step by step to the test tube with constant warmth (temperature above the melting point of lipid) and blending. The amount of lipid required to shape a reasonable straightforward arrangement of the drug was determined [10, 13].

Physical compatibility of Solid lipid with Liquid lipids

Lipid blend of selected solid lipids and liquid lipid was taken in different glass vials which were melted to congeal at room temperature. The glass vials were visualized for separation of layers in congealed mass, and then the Solid liquid lipid mixture (Smix) was smeared over a glass slide and examined microscopically for the examination of amorphous mass [13].

Selection of solid lipid to liquid lipid ratio

Melting point determination technique was followed to determine the ratio of Smix. Briefly, selected solid lipid and liquid lipid with the ratio from 95:05 to 05:95 were melted above their melting point of solid lipid, and a congealed mass of the Smix was prepared which was further evaluated for melting point determination by capillary method. The selected ratios of the Smix were visualized microscopically by an optical microscope (Dewinter® empowered with Capture Pro® software), and the most suitable ratio was determined. The melting point of the finally selected Smix ratio was further confirmed by differential scanning calorimeter (DSC6000, Pyrix 6, Serial Number: 002082704; Software Version: 11.0.0.0449) at a scanning rate of 10°C/ min over the temperature range of 10-400°C [18-20].

Selection of Surfactant

For the preparation of NLCs, surfactants were chosen by their capacity to emulsify Solid liquid lipid blend. 100 mg of the Solid liquid lipid blend was added to 3 mL of Dichloromethane (DCM) which was further added to amalgamate with 10 mL of 5 % surfactant solution under constant stirring. The organic phase was expelled at 40° C. Percentage transmittance of the resultant mixture was analyzed utilizing UV spectrophotometer at λ max of 638.2 nm after dilution with milli-Q water [12, 13, 21-24].

Method of preparation

Solvent evaporation and emulsification method with slight modifications were explored for the formulation of Gen loaded NLCs. The components and variables needed for the formulation were selected on the basis of literature review, and we further put in the statistical designs for their optimization. The technique consists of the following steps: 0.78 % of Smix (60:40 solid lipid: liquid lipid {SL: LL}) was dissolved in 8.5 ml Dichloromethane (DCM) along with 10 mg of Gen (for 25 ml formulation). The 0.3 % of TPGS solution was prepared in milli Q water. Aqueous and organic phases were kept at 60˚C & 900 RPM for 4 minutes to augment the uniform mixing of components after which the aqueous phase was stirred by using high shear IKA T25 digital Ultra turrax homogenizer and the organic phase was added drop wise. The operational parameters for homogenizer were 13000 RPM for 15 minutes after that the formulation was sonicated using Ningbo Haishu Sklon probe Ultrasonicator for 4 minutes on-off cycle at 40 % amplitude. Finally, the formed colloidal NLC suspension was kept untouched at room temperature for 24 h to check any signs of instability afterward which was characterized for particle size, polydispersity index, zeta potential, surface morphology and entrapment efficiency using suitable techniques [12, 13, 25, 26].

Optimization of process and product Parameters for NLCs

Risk Assessment Studies

QbD strategies can be applied for the optimization of different product and process parameters for the higher quality NLCs. The various elements of the QbD are:
Quality target product profile (QTPP) (Table 8) was prepared which identified critical quality attributes (CQAs) of the drug products. CQAs were of 2 types: Critical material attributes

(CMAs) & critical process parameters (CPPs) (Table 9 & 10).CMAs and CPPs were classified[18, 25, 26]. Further failure mode effect analysis (FMEA) was employed to define the ranks to the selected CTQs on the basis of their relative significance (Table 11).

Screening of Factors by Placket Burman Design

To select the factors which were highly significant for the formulation of NLCs, a factorial based placket burmen design was exploited using Minitab ver. 17. Pareto charts were prepared as a result of placket burmen analysis which defined the significant factors for each response based on 80/20 rule or the principle of factor sparsity. This beauty of PB design lies in its property of creating lesser no. of experimental runs even with large no. of factors [12, 14].
The factors were studied at two levels i.e. low (-1) and high level (+1) respectively. All factors and responses along with their lower and higher levels are described in Table 12.

Optimization by Box Behnken methodology

Response surface methodology based Box Behnken design (BBD) was used for the construction of polynomial orders which is a collaboration of various statistical techniques and is useful for the mathematical problems where confounding of the factors exists. To checks the effect of independent factors on dependent factors we had performed Box Behnken analysis[20, 27, 28]

A 3 factor, 3 level (3^3) BBD with 16 no. of experimental runs was constructed when 3 independent variables were studied at different levels (+1 higher level; -1 lower level) and center points during analysis. The effect of 3 factors i.e. lipid concentration (X1), Surfactant concentration (X2) & amount of organic solvent (X3) was calculated for 3 dependable variables i.e. particles size (Y1), polydispersity index (Y2) & entrapment efficiency (Y3). The independent and dependent factors were chosen as per the requirements of the NLC systems. The design was executed in a randomized order to avoid the biasness between factors by using Design Expert® software (7.0, Stat Ease Inc., Minneapolis). The analysis was performed and the relationship between factors and response was generated in the form of polynomial equations for each response. From the equations, a vast no. of information was collected and represented in the form of 3-D surface plots. Principles of Analysis of Variance (ANOVA) were utilized for the data analysis and values of coefficient of variation, lack of fit, regression coefficient were determined.

Further, optimization was done by employing the desirability approach based numerical optimization. The concept of design space was utilized well by keeping the responses under constraints and percentage biases between experimental and practical values of the optimum formulation was calculated which is elaborated in table no. 18 & 19; (Figure 8).

CHARACTERIZATION TESTS

HPLC method development

High-performance liquid chromatography with reverse phase was used for quantification of Gen in the prepared NLC formulation. HPLC system was consisted of waters 1525 binary HPLC pump (Waters, USA), rheodyne 7725i manual injector (Waters, USA), C18 reverse-phase (4.6 x75 mm; 3.5 µm) Symmetry® C18 column and waters 2998 photodiode array detector (Waters, USA)[14, 29, 30]. Acetonitrile: 2mM phosphoric acid buffer in Milli-Q water (50:50) was used as mobile phase and was run at a rate of 1.0 ml/min. The column temperature was maintained at 30±1˚C during the whole process and the peak was detected at 262 nm. HPLC peak area and retention time were integrated by using the Breeze2 software and were utilized for the calculation of drug content at all times. Standard calibration curves in required media were plotted from 500-3000 ng mL^{-1} of Gen [30, 31].

TEM (Transmission Electron Microscopy)

The surface morphology of the formulation was investigated using transmission electron microscopy (TEM, FEI TECNAI $G^2$20 TWIN MODEL 943205022121). Samples were prepared by placing a drop of nanoparticle suspension which was diluted previously with water, onto a copper grid and kept fortnight for air drying. The air-dried samples were then directly examined under the TEM.

Particle size and Polydispersity index (PDI)

The mean Particle size and Particle size distribution were determined by Particle size analyzer (Delsa Nano C Beckman Culter).

Zeta Potential

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. Its measurement brings detailed insight into the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions, and suspensions. It was determined by using Particle size analyzer (Delsa Nano C Beckman Cutler).

Encapsulation Efficiency (EE), Total drug content (TDC) & Loading Efficiency (LE)

Entrapment efficiency, drug loading and total drug content are the main parameters to determine the amount of drug that goes inside the nanoparticles. The entrapped drug will show the property of controlled release. We had determined all the three parameters with the help of indirect method and the amount of drug was analyzed quantitatively by using HPLC (WATERS; Breeze 2 software). Briefly, the 1.5 ml of formulation was centrifuged (Eltek Cooling Centrifuge) at 14000 RPM for 15 minutes at temperature 10°C using Nanosep (Pall Corporation, 100 K Omega). The clear liquid was collected from the lower chamber of the tube and was further diluted 375 times with HPLC grade methanol. The samples were filtered through syringe filters (Axiva, PES 0.45 micron) and the peak area was measured against the standard. The encapsulation efficiency was expressed as a percentage of the amount of drug encapsulated in the nanoparticles to the total drug content.

$$\text{EE (\%)} = \frac{Total\ drug\ content - free\ drug}{Total\ drug\ content} X100$$

$$\text{LE (\%)} = \frac{Total\ drug\ content - free\ drug}{Total\ amount\ of\ lipid\ added} X100$$

Total drug content (TDC) measures the amount of total drug (entrapped + free drug) present in the formulation. For TDC determination, 1 ml of formulation was taken and fully dissolved in the solvent which was further diluted with methanol to analyze the peak area with the help of HPLC against the standard. The total amount of drug in 'mg' was calculated by using the calibration curve.

Cumulative Percentage Drug Release (%CDR)

Calculation of %CDR is a determinant of in vitro release behavior of the drug inside the formulation. Dialysis bag diffusion technique was employed to study the release behavior as well as release kinetics of the formulation. Briefly, a predetermined amount of formulation was added to activated dialysis membrane (8-12 kDa) (Himedia labs, India) molecular weight cut off which

was tied from both the ends. The bag was incubated in 50 ml of release medium (PBS 7.4) maintained at 37.5 ±0.5 °C at 150 rpm. At predetermined times intervals, the media was replaced with fresh buffer and the samples were filtered and analyzed by HPLC. Cumulative percentage drug release was calculated. Data was fitted to various kinetic models (zero order, first order, Higuchi kinetics & Korsmeyer Peppas model) to get the release kinetics. Sink conditions were maintained throughout the release period.

Haemocompatibility studies

Plasma Clotting

Plasma clotting studies were performed with platelet poor citrated plasma which was collected from the citrated whole blood after centrifugation at 1344 x g for 15 minutes and stored at 2-8˚C. The activated partial thromboplastin time (APTT) and Prothrombin time (PT) were determined by the appearance of a clot which indicates the end of the reaction. Gen NLC formulation equivalent to 10 µg/ml and 100 µg/ml was added to plasma samples and were stirred for 1 h at 37˚C. For the PT determination, UNIPLASTIN (Tulip) was added to the plasma samples whereas, for APTT, LIQUICELIN-E (Tulip) along with Calcium chloride was added to the plasma containing samples. 0.9% (v/v) saline with plasma was kept as normal control [32]. For APTT determination, 100 µl of LIQUICELIN-E was added to 100 µl of citrated plasma containing the sample and was incubated for 3-5 minutes which was followed by the addition of equal amount of calcium chloride solution and incubated the whole for 15 minutes. Time required for the clot appearance was calculated by using stop watch. Similar procedure was followed for the determination of Prothrombin time with PT reagent kit. PT can also be reported as percentage activity. This percentage is intuitive property and is based on the observation that normal pooled plasma possess 100 % PT activity and is taken as 100 and further on dilution, the clotting time as well as % activity goes on decreasing and a calibration curve was plotted between percentage activity vs. clotting times. The % PT activity of the samples was evaluated from the same calibration curve [33]. All the experiments were performed at 37˚C and in triplicate batches. (n=3)

Evaluation of Hemolysis

Formulations intended for intravenous administration should be tested for hemolytic potential of the same as the excipients present or drug may cause damage to red blood cells. To asses this, we had used the method mentioned by Vijay Kumar et al.[30]. Briefly, fresh human blood was purchased and processed to extract plasma as stated earlier. Study consisted of four groups viz. test, placebo, positive and negative control. RBCs were taken out from the whole blood and washed with equal amount of saline by centrifugation at 1344xg for three times. To re-suspend the pellet, normal saline was used and the pellet was diluted 10 times. 10 and 100 µg/ml of each group (Test, Placebo, Positive & Negative control) were taken and mixed with the erythrocyte suspension up to 1 ml. Positive control (100% lysed erythrocytes) and spontaneous negative control were prepared by diluting an equal volume of erythrocyte suspension with 1 % Triton X100 and normal saline respectively. The samples were incubated for 15 minutes and then the aliquots were withdrawn from each sample at predetermined time intervals (0.5, 1,2,4,8 hr). The aliquots withdrawn were centrifuged and supernatants were kept at room temperature for 30 minutes for oxidation of hemoglobin into oxyhemoglobin. The absorbance was measured spectrophotometrically by microplate reader (Biorad, Germany) at 540 nm. The percentage hemolysis was calculated by using the following formula

$$\% \text{ hemolysis} = \frac{Abs\ sample - Abs\ spontaneous\ control}{Abs\ Positive\ control}\ X\ 100$$

Where Abs_{sample} is the absorbance of the samples (nanoparticles formulations with and without drug), $Abs_{spontaneous\ control}$ is the absorbance of the spontaneous control (0.9% saline solution), and $Abs_{positive\ control}$ is the absorbance of the 100 % control (1% Triton X 100). All the samples were analyzed in triplicate batches (n=3).

Evaluation of erythrocyte membrane integrity

Formulation components may affect the integrity of erythrocyte membrane which can be accessed on the basis of Lactate Dehydrogenase enzyme (LDH) which is released when the erythrocyte membrane got ruptured. The activity of LDH can be accessed photometrically by using LDH commercial kit (Tulip). The same procedure was followed as described in hemolysis assay instead samples were incubated for 1 hr. The study consisted of same four groups viz. test, placebo, positive and negative control at 10 & 100 µg/ml. After 1 hr, sample was taken from each tube and centrifuged at 1344xg for 10 minutes. The LDH released in the supernatant was detected spectrophotometrically by microplate reader (Biorad, Germany) at 500nm. The concentration o LDH released was calculated from the following equation[29, 32]:

$$LDH(UL^{-1}) = \frac{Abs_{sample} - Abs_{control}}{Abs_{standard}} X150$$

Where Abs_{sample} is the absorbance of supernatant of erythrocyte suspension containing samples, $Abs_{control}$ is the absorbance of the RBCs suspension without nanoparticles added to the substrate reaction. $Abs_{standard}$ is the absorbance of the supernatant of RBC suspension with LDH standard (150 UL^{-1}, according to the manufacturer specifications). All the samples were analyzed in triplicate batches (n=3).

Platelet aggregation tests

Platelet aggregation tests were performed to identify the changes in the platelets after treatment with the formulation. Citrated whole blood was incubated with test formulation (10 & 100 µg/ml of the drug), PBS (as a spontaneous control for platelet aggregation), pure drug suspension and placebo formulation. Method stated by Bender et al.[32] was followed to execute the work. The samples were incubated for 30 min with gentle agitation (400 RPM). The peripheral blood smears were stained with Leishman's stain (Span Diagnostic, India) for 5-6 minutes after incubation followed by rinsing with water which was then allowed to dry and the dried smears were analyzed by an optical microscope in immersion objective. The images were captured using the digital system (Dewinter Trinocular Microscopic Unit, Dewinter Technologies). Platelet aggregation was also evaluated by counting the no. of platelets before and after the addition of formulation to the citrated whole blood by hematological counter (Multisizer 4, Backmann coulter, USA).

Statistical analysis

All the results were expressed as mean ± standard deviation. Results were statistically analyzed by utilizing the principles of analysis of variance (ANOVA) followed by Bonferroni post-test using GraphPad Prism® Ver. 5. results with $p<0.05$ (95 % CI) were considered as statistically significant.

RESULTS AND DISCUSSION

Selection of solid lipid and liquid lipids

Selection of liquid lipids (LL) and solid lipids (SL) was made on the basis of saturation solubility of the drug with the same. The lipids showing maximum solubility were selected for the formulation [12, 13]. Sufficient solubility of the drug was observed in Capmul MCM & Capmul MCMC8, however Capmul MCM

showed the highest solubility of 30.21 mg/ml (Table 1 & 2) [34]. Among the solid lipids, Tristerin was found be best fitted for the preparation of NLCs because of its maximum solubility (23.14 mg/g) and higher compatibility with the drug (Table 3, 4 & 5). Congealing of the Smix was observed with the help of optical microscopy as shown in Figure 1. Failing to congeal may be attributed to lowering of combined melting temperature for the lipid mix.[24]. Black particles in the microscopic picture assured the amorphous nature of the molecules as the amorphous substances possess isotrophicity which means they do not transmit light with cross-polarizing filters because they have a single refractive index, so they appear black [35]. The black particles in the pictures confirmed the presence of amorphous mixtures of both the lipids which showed that SL & LL were completely mixed when melted. Hence, from the above studies, Tristearin & Capmul MCM were selected as lipid phase for the NLC preparation [30].

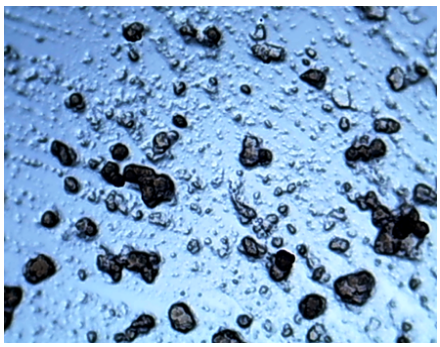

Figure 1: Optical microscopic picture of solid lipid liquid lipid binary mixture (SLB) (Tristearin + Capmul MCM)

Table 1: Comparative solubility of Genistein in different oils

Oil	Solubility
Miglyol	-
Captex 355	+
Capmul MCM EP	+++
Captex 300P	+
Capmul MCM C8	+++

Table 2: Quantitative solubility of phytoconstituent in Liquid lipids

Oil	Solubility (mg/mL)
Miglyol	0.87±0.13
Captex 355	4.10±0.32
Capmul MCM EP	30.21±0.34
Captex 300P	3.42±0.16
Capmul MCM C8	8.54±0.35

Data expressed as mean±S.D; n=3

Table 3: Solubility of drug in Solid lipids

Solid Lipid	Solubility
Imwitor 900K	+++
Compritol 888ATO	++
Tristearin	+++
Dynasan 118	+
Dynasan 114	++
Glyceryl monostearate	++
Glyceryl monooleate	+

Table 4: Quantitative Solubility of drug in selected solid lipids (SL)

Solid Lipid	Solubility(mg/g)
Imwitor 900K	11.3±0.24
Tristearin	23.14±0.46

Table 5: Compatibility study of selected SL with LL

Solid liquid lipid binary mixture	Congealing
Imwitor 90K+Capmul MCM	-
Tristearin + Capmul MCM	+

Selection of solid lipid to liquid lipid ratio

NLCs were preferred over the SLNs because of their higher entrapment efficiency and more stability of the formulation. The liquid lipids present in the formulation were able to carry more drug as compare to solid lipids alone[10, 11]. Increasing the LL content in the formulation could enhance the EE of the formulation but simultaneously, their melting point range should be studied as increasing LL content will decrease the melting points of the solid lipid binary mixtures (SLBs) and the consistency of the formulation will be compromised. Various combinations of Smix were made varying in the ratio from 90-10% SL and 10-90 % LL (Table 6) which were sorted for proper congealing and were further evaluated for melting points be capillary method as given in Figure 2. The SLB ratios with melting points between 50°C - 60°C was selected for the formulation as higher liquid lipid favors higher drug solubilization but consistency of the formulation at room temperature will be compromised as at room temperature, nanoparticles will not be able to keep pace with solid or semi-solid form [13]. In this study, SL: LL from 10:90- 30:70 were considered as below 30% of SL content, the mixtures were not able to congeal at room temperature. Further the congealing of the combinations with higher SL content was confirmed by optical microscopy, Fig.3. Only the 3 combinations of SL:LL i.e 65:35, 60:40 & 70:30 were chosen out of which SL: LL 60:40 was found to be best for formulation of PTXNLC not just on the basis of its optical microscopic image but also because of its ability to entrap sufficient drug and sufficiently high melting point to keep pace with the consistency of the formulation at room temperature. The melting point of the selected mixture was further confirmed by DSC given in figure 4 [34].

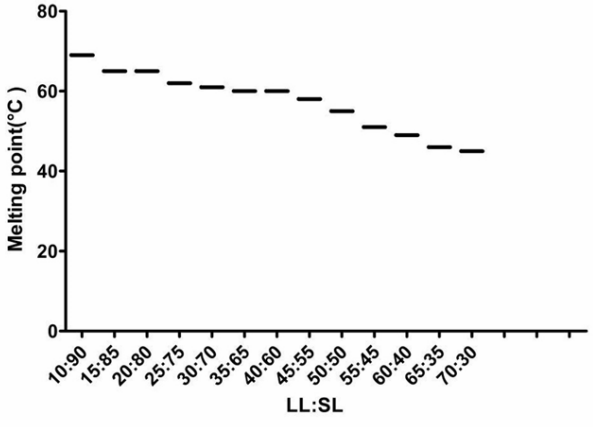

Figure 2: Melting point ranges of Solid lipid liquid lipid binary mixtures (LL:SLs)

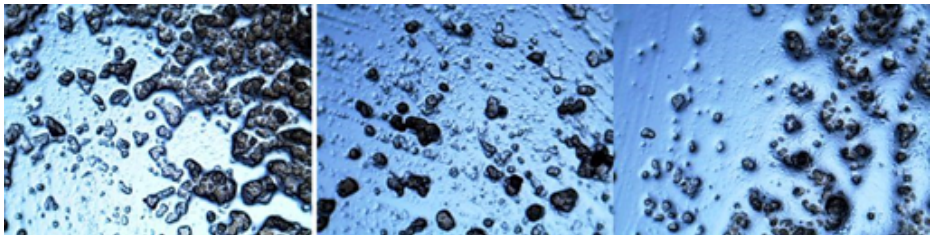

Figure 3: Optical microscopic pictures of selected SLBs Optical microscopic pictures of selected SLBs

Table 6: Selection of solid lipid to liquid lipid ratio (SL:LL)

Sr. No	Ratio (SL:LL)	Congealing	Melting point (°C) (By capillary method)	Microscopy
SL: Tristearin, LL: Capmul MCMC8				
1.	10:90	-	-	-
2.	15:85	-	-	-
3.	20:80	-	-	-
4.	25:75	-	-	-
5.	30:70	+	45.3	-
6.	35:65	+	46.4	-
7.	40:60	+	49.1	-
8.	45:55	+	51.0	--
9.	50:50	+	55.5	--
10.	55:45	+	58.2	
11.	60:40	+	60.1	Good
12.	65:35	+	60.6	Good
13.	70:30	+	61.4	Good
14.	75:25	+	61.9	--
15.	80:20	+	64.2	-
16.	85:15	+	64.6	-
17.	90:10	+	68.5	-

-indicates separation; + indicates formation of congealed mixtures
-- indicates improper mixing of SL with LL

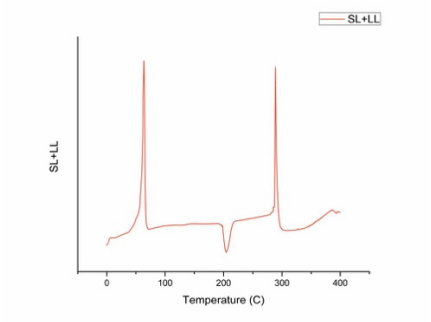

Figure 4: DSC curve of selected solid lipid liquid lipid mix in ratio of 60:40

Table 7: Percentage transmittance values of different surfactants

Surfactant	Transmittance (%)
Solutol HS15	84±2.14
Tween 80	91.6±4.02
Poloxamer 188	92.5±3.25
TPGS Vitamin E	96.3±2.14
Pluronic 407	89.56±1.15
Brij 78	84.5±2.81

Selection of surfactant

Selection of surfactants is a crucial step for the formulation as surfactants play an important role for the solubilization of the drug, particle size optimization and entrapment efficiency of the formulation. Selection was made on the basis of emulsification ability of the drug which was calculated by the percentage transmittance value. This observation is based on the concept that smaller particles exhibit higher percentage transmittance and hence higher the emulsification ability of the surfactant[12, 13, 21-23]. From the data, it was concluded that Vitamin E TPGS fabricated the emulsion with higher percentage transmittance as compared to others (Table 7). The other surfactants tested for their emulsification ability were also successful in producing sufficiently good emulsion but TPGS was preferred over the others because of its benefits like anticancer activity which will provide a synergistic effect, nontoxicity, safety, biodegradability and antioxidant properties [30, 36-40].

The Placket Burman design (PBD)

The PBD helps in initial screening of large number of factors based on the type of material and formulation characteristics of the components. It resulted in 20 no. of runs with 12 factors at their maximum and minimum levels with 3 responses. Details of variables and responses as well as summary of PBD are mentioned in Table no. 12 & 13. Each response was analyzed with the help of ANOVA and Pareto charts were constructed for each response separately which shows the most significant factors for that particular response. Based on the analysis of ANOVA, it was concluded that particle size was mostly influenced by the brand of lipid, a brand of surfactant and lipid concentration. Similarly, entrapment efficiency was mostly influenced by brand of lipid, a brand of surfactant and surfactant concentration whereas for Polydispersity index, the significantly affecting factors were the brand of lipid, a brand of surfactant and organic solvent concentration (fig.6) ($p<0.05$). As different brand of the same lipid and different brand of the same surfactant are

influencing all the 3 parameters, we had simplified our problem by manually segregating the factors after characterizing all the responses for all combinations of surfactant and lipid brand. After the experiments, it was concluded that Sigma Aldrich based tristearin as lipid and Sigma Aldrich based TPGS vitamin E as a surfactant were best suited for the formulation of NLCs and the remaining three parameters i.e., lipid concentration (affecting particle size), surfactant concentration (affecting entrapment efficiency) & organic solvent concentration (affecting polydispersity index) were chosen as variables for further response surface methodology.

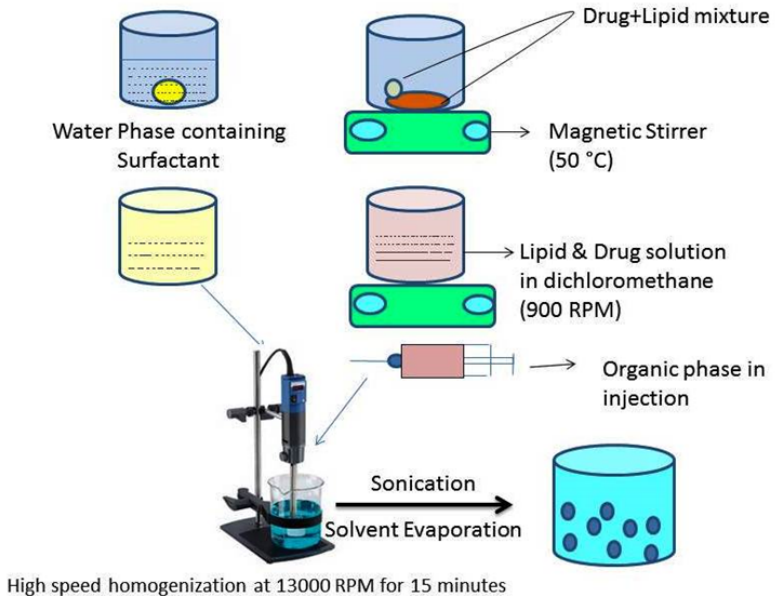

Figure 5: Method of preparation of GenNLC

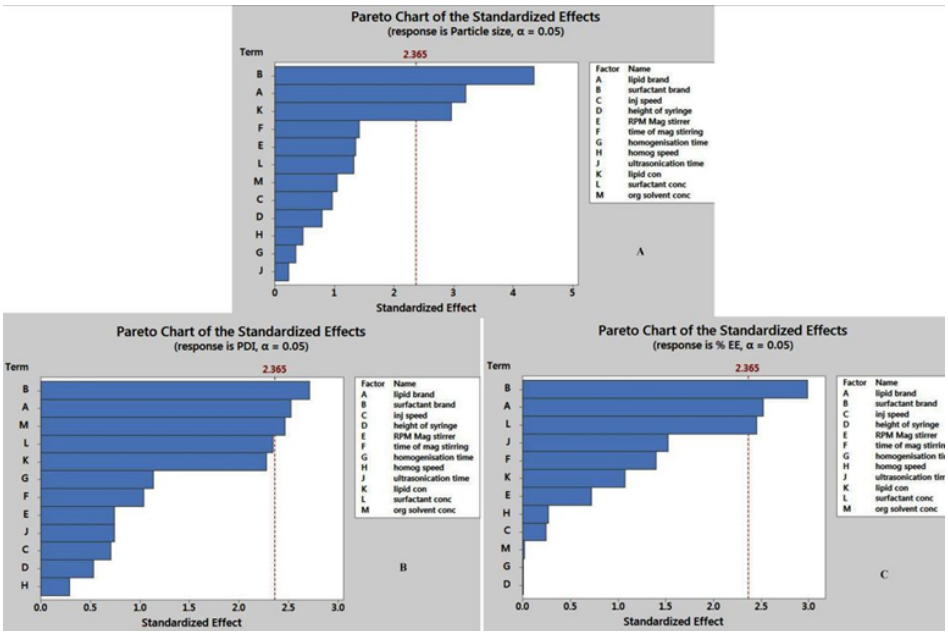

Figure 6: Pareto chart showing the influence of variables (A) Influence of process variables on Particle Size (B) Influence of process variables on PDI (C) influence of process variables on Entrapment Efficiency

Table 8: Quality Target Product Profile (QTPP)

QTPP Elements	Target	Justification
Dosage form	Nanostructured lipid carriers (NLCs)	Lipid based systems that help in enhancing the bioavailability of the poorly water soluble drug and nano systems helps in targeting the drug to the particular area in case of cancer.
Dosage design	Delayed release	Decreases dosage frequency as well as toxicity caused by drugs.
Administration route	Intravenous	Required to target the drug to the cancerous area.
Finished product	Lyophilized powder	It will be stable an easy for packaging.
Stability	Minimum 08 months	To maintain the therapeutic potential of the drug.

Table 9: Control Impact Matrix

CONTROL	IMPACT		
Critical quality attributes (CQAs)	**High**	**Medium**	**Low**
In our Control	• Type of raw materials (oils, solid lipids, surfactants etc. • Concentration of lipids, surfactants • Type of water/organic solvent used • Amount of Water phase/ organic phase • Speed of magnetic stirrer/homogenizer/sonicator • Time of homogenization • Method of preparation • Injection speed • Needle size used • Temperature of the system	Efficiency of formulator	----------
Out of Control	• Purity of raw materials • Partition coefficient of the drug • Solubility profile of drug • Efficiency of measurement system • Environmental conditions (Room temperature, humidity, pressure etc.)	Efficiency of Analyst, Chemist.	Contamination

Table 10: Critical Process parameters and material attributes

Sr. No	CPPs	CMAs
1.	Method of preparation used	Type of Liquid lipid, solid lipids & surfactants
2.	Speed of magnetic stirrer/homogenizer/sonicator	Concentration of SL, LL & surfactant used
3.	Time of homogenization/ sonication	Type of water/ organic solvent used
4.	Injection Speed	Ration of Aqueous phase/ organic phase
5.	Temperature of the system	Needle size
6.	Efficiency of measurement system	

Table 11: Risk Estimation Matrix

CTQ (CMAS+ CPPs)	Particle Size	Poly dispersity Index	Entrapment Efficiency
Type of lipids used	High	Low	High
Amount of lipids	High	Medium	High
Type of surfactant	High	Low	Low
Surfactant conc.	High	High	Medium
Solvent type	High	Low	Medium
Humidity	Low	Low	Low
Solvent ratio	High	Low	High
Temperature of the system	High	Low	High
Speed of homogenizer	High	Medium	Medium
Homogenization time	High	Low	High
Sonication time	High	Medium	Low
Stirring speed	Medium	Low	Medium
Type of Analyst	Low	Low	Low
Stirring time	High	Low	Low
Injection Speed	High	Medium	Low
Needle Size	Medium	Low	Low
Method of preparation	High	Low	Medium
Room Temperature	Low	Low	Low

Table 12: Details of factors used for Placket Burman design

Codes	Independent Variables	Low level (-1)	High Level (+1)	Unit	Type of factor
A.	Brand of the same lipid	TCI Chemicals	Sigma aldrich	-	Category
B.	Brand of the same surfactant	Antares	Sigma aldrich	-	Category
C.	Injection Speed	5	10	ml/min	Numeric
D.	Height of the syringe	2	5	cm.	Numeric
E.	Speed of magnetic stirrer	900	1200	Rpm	Numeric
F.	Time of magnetic stirring	4	8	Min.	Numeric
G.	Homogenization time	10	15	Min.	Numeric
H.	Homogenization Speed	12500	15000	Rpm	Numeric
I.	Ultrasonication Time	4	8	Min.	Numeric
J.	Lipid Concentration	0.75	1.0	% w/v	Numeric
K.	Surfactant Concentration	0.1	0.3	% w/v	Numeric
L.	Amount of Organic solvent	5	10	ml	Numeric
Dependent Variables					
Particle Size (nm)					
Poly dispersity index					
Entrapment efficiency (%)					

Table 13: Summary of Placket- Burman design

Plackett-Burman Design												
Factors: 12 Replicates: 1												
Base runs: 20 Total runs: 20												
Base blocks: 1 Total blocks: 1												
Design Table (randomized)												
Run	Blk	A	B	C	D	E	F	G	H	J	K	L	M
1	1	-	+	+	-	-	-	-	+	-	+	-	+
2	1	+	+	-	-	-	-	+	-	+	-	+	+
3	1	-	+	+	+	+	-	-	+	+	-	+	+
4	1	+	+	+	+	-	-	+	+	-	+	+	-
5	1	-	-	-	-	-	-	-	-	-	-	-	-
6	1	+	-	+	+	-	-	-	-	+	-	+	-
7	1	-	-	-	+	-	+	-	+	+	+	+	-
8	1	-	+	+	-	+	+	-	-	-	-	+	-
9	1	+	-	-	+	+	-	+	+	-	-	-	-
10	1	+	-	+	-	+	+	+	+	-	-	+	+
11	1	-	-	-	-	+	-	+	-	+	+	+	+
12	1	+	+	+	-	-	+	+	-	+	+	-	-
13	1	-	+	-	+	-	+	+	+	+	-	-	+
14	1	+	-	-	-	-	+	-	+	-	+	+	+
15	1	-	+	-	+	+	+	+	-	-	+	+	-
16	1	+	+	-	+	+	-	-	-	-	+	-	+
17	1	+	+	-	-	+	+	-	+	+	-	-	-
18	1	-	-	+	-	+	-	+	+	+	+	-	-
19	1	-	-	+	+	-	+	+	-	-	-	-	+
20	1	+	-	+	+	+	+	-	-	+	+	-	+

The Box-Behnken design (BBD): the response surface methodology

We had utilized the principles of 3 factors, 3 level (3^3) response surface based BBD methodology to identify the effect of independent variables {i.e. surfactant concentration (X1), lipid concentration (X2) and amount of organic solvent (X3)} on dependent variables {i.e. particle size (Y1), entrapment efficiency (Y2) & ploy dispersity index (Y3)} (Table 14). The design matrix composed of total 16 runs (detail is given in table 16) and analysis of the results was made by using the ANOVA principles ANOVA using Design expert® software at 95 % confidence interval. As a result, quadratic equations were generated and their corresponding positive and negative signs showed the positive or negative effect of that particular variable on the respective response. Best fitting of the regression model was determined from their F values. To differentiate the interactive effects of 2 independent variables on dependent variable, we had plotted 3D surface contour plots which are shown in Figure 7. Results of the quadratic models analysis were depicted in the form of lack of fit value as well as p-value [14, 15, 28]

Table 14: Summary of 3 factor 3 level Box Behnken design

Study Type Response Surface				**Runs** 16			
Initial Design Box-Behnken				**Blocks** No Blocks			
Design Model Quadratic							
Factor	**Name**	**Units**	**Type**	**Low (-1)**	**Medium (0)**	**High (+1)**	
X1	surfactant conc	%	Numeric	0.100	0.200	0.30	
X2	lipid conc	%	Numeric	0.75	0.875	1.00	
X3	organic solvent	ml	Numeric	5.00	7.5	10.00	
Response	**Name**	**Units**	**Obs**	**Analysis**	**Minimum**	**Maximum**	**Constraint**
Y1	PS	nm	16	Polynomial	119.300	383.800	Minimize
Y2	EE	%	16	Polynomial	68.400	95.400	Maximize
Y3	PDi		16	Polynomial	0.109	0.284	Minimize

Table 15: Statistical ANOVA based results of quadratic model

Response	**Quadratic model**						
	F- Value	**P- Value***	**R-Square**	**R-Sq (adj)**	**CV%**	**Lack of fit**	**Remark**
P.Size(nm)	184.04	<0.0001	0.9964	0.9553	2.93	0.1998	Significant
EE (%)	34.00	<0.0002	0.9808	0.9519	1.81	0.3712	Significant
PDI	26.69	<0.0004	0.9756	0.9391	7.85	0.1340	Significant
R-Sq (adj)= R Square adjusted; CV= Coefficient of variation, *p-value<0.05 is considered as statistically significant.							

Table 16: Box Behnken experimental design representing experimental runs with different combinations of input factors

Run	Surf conc % (X1)	Lipid conc % (X2)	Amt of org solvent (ml) (X3)	Particle size (nm) (Y1)	EE% (Y2)	PDI (Y3)
1	0.10	0.75	7.50	329.3	68.4	0.23
2	0.10	0.88	10.00	383.3	78.8	0.198
3	0.10	0.88	5.00	342.3	80.4	0.284
4	0.20	0.75	5.00	254.5	84.4	0.163
5	0.20	1.00	10.00	274.9	87.7	0.123
6	0.30	0.88	5.00	182.4	95.4	0.162
7	0.30	0.88	10.00	134.8	93.4	0.131
8	0.20	1.00	5.00	290.6	90.3	0.142
9	0.20	0.88	7.50	273.3	91.3	0.142
10	0.30	1.00	7.50	132.3	92.3	0.139
11	0.10	1.00	7.50	346.4	81.02	0.241
12	0.30	0.75	7.50	119.3	94.8	0.109
13	0.20	0.75	10.00	243.8	81.7	0.113
14	0.20	0.88	7.50	268.3	88.4	0.134
15	0.20	0.88	7.50	261.4	90.2	0.134
16	0.20	0.88	7.50	263.3	88.5	0.123

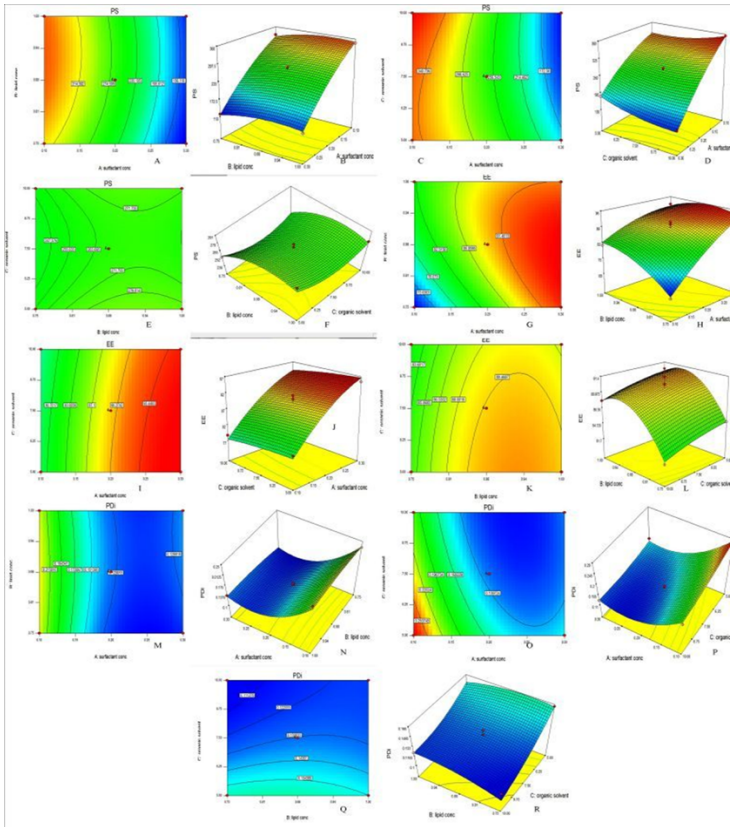

Figure 7: Graphical representation of effect of independent variables (Surfactant concentration (X1), lipid concentration (X2) & amount of organic solvent (X3)) on dependent variables (particles size (Y1), entrapment efficiency (Y2) & polydispersity index (Y3), A-F represents the 3D plots & contour plots for particle size, while G-L represents the 3D and contour plots related to entrapment efficiency & M-R represents the 3D and contour plots of polydispersity index.

Influence of variables on particle size: Particle size of GENNLC varied from 119.3 to 383.9 nm during all the experimental runs. The F value of 184.04 allowed sufficient fitting of data with non-significant lack of fit (0.1998). P value of <0.0001 at 95% confidence interval indicated the best fitting of the used models for that particular response. The model was found to be highly precise and reliable as indicated by the low value of coefficient of variation (2.93). The values of predicted R squared (0.9553) and adjusted R squared (0.9910) were in reasonable agreement so this model can be used to navigate the design space. The results of the statistical analysis were represented in table 15. Based on the observations (Table 17 Figure 7(A-F)), it was analyzed that the studied response was negatively affected by surfactant concentration (X1) & amount of organic solvent (X3) while lipid concentration has positive effect on the same, i.e., if we go on decreasing the surfactant concentration and amount of solvent, particle size of the nanoformulation will increase while it will increase with increase in lipid content and the surfactant concentration seems to be the most influencing parameter for this particular response as indicated by its high coefficient value. Concentration of surfactant has remarkable effect o the particle size of the formulation as it is responsible for the emulsification of the nanoparticles which leads to churning of bigger lipid particles into the smaller one. On the other hand if we go on increasing the lipid concentration without sufficiently increasing the surfactant concentration as well as amount of organic solvent, it will results in formation of bigger particles due to the formation of aggregates. Increased viscosity of the formulation can reduce the shear strength of the stirrer. In the same manner, amount of organic solvent possess inverse relationship with particle size which can be due to a decrease in viscosity of the lipid contents with a higher amount of organic solvent, thereby imparting high shear stress which would break the emulsion droplets without any coalescence [14, 15, 28].

Table 17: Quadratic equation generated by Box Behnken Design

*Y	Particle size (Y1)	Entrapment efficiency(Y2)	Polydispersity index (Y3)
X_0	+266.57	+89.60	+0.13
*A	-104.06	+8.41	-0.052
*B	+12.16	+2.75	+03.750E-003
*C	-4.13	-1.11	-0.023
*A*B	-1.02	-3.78	+4.75E-003
*A*C	-22.15	-0.1	+0.014
*B*C	-1.25	+0.025	+7.75E-003
*A2	-20.00	-2.25	+0.053
*B2	-14.75	-3.22	-6.00E-003
*C2	+14.13	-0.35	+8.00E-003
*Y= response; X0 = intercept; A-C= Factors			

Influence of variables on entrapment efficiency (EE): The entrapment efficiency of GENNLCs varied from 68.4 %- 95.4 % for different formulation variable combinations. The parametric values generated by ANOVA defined the suitability of the model for this response. The modal F value of 34.00 ($p<0.0002$) indicted the chosen model was a right choice for relating EE with the various factors and the non-significant lack of fit (0.3712) indicated the suitability of the model in fitting of data. A good correlation was seen between the chosen factors and responses which were indicated by the R squared value (0.9808). The reasonable agreement was observed between R squared and adjusted R squared value as indicated in table 15."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 22.082 indicates an adequate signal (Table 15). It was observed from the second order polynomial equation that both surfactant and lipid concentration (X1 & X2) affects the EE positively while amount of organic solvent has its negative effect on the same. Increasing lipid concentration will create thicker layer to restrict the further diffusion of the drug out of the lipid cover and also more the lipid content available, more will be the solubility of the drug [14, 28]. Similarly, higher the surfactant concentration, higher will be the emulsification which will help in emulsification of the particles and the drug will be distributed uniformly. Lesser surfactant concentration and higher lipid concentration will cause increase in viscosity of the formulation which will result in higher viscous resistance against a shear force which will hinder the formation of nanodroplets and also the lesser amount of drug will get solubilized into viscous lipid matrix which ultimately results into decrease entrapment efficiency [16]. On the other way, higher amount of organic solvent will lead to leaching of the drug from the lipid core which ultimately will decrease the EE of the formulation [6].

Influence of process variables on polydispersity index (PDI): The value of PDI varies from 0.109-0.284 for various combinations of process parameter at their minimum and maximum levels. Te second order polynomial equation was generated by using principles of ANOVA with the help of Design Expert® software and is shown in table 17.The modal F value was found to be 26.69 which was good enough at $p<0.0004$, 95 % confidence interval. It showed that the model was highly significant and reliable. The suitability of the model was indicated by its non-significant lack of fit value. All the values are listed in Table 15. Good correlation between independent and dependent variables was indicated by its higher R squared value (0.9756). Values of "Prob > F" less than 0.0500 indicate model terms were significant. In this case, A, C, A^2 were significant model terms (Table 15. The influence of various parameters on PDI was evaluated by relating Figure 7 (M-R) & Table 17. It was noted that surfactant concentration (X1) & amount of organic solvent (X3) affects the response negatively while lipid concentration (X2) has its positive effect on the PDI. Enhanced lipid concentration will enhance the value of PDI due to its direct impact on the thickness of formulation contents. The Higher viscosity of the lipid matrix will suppress their segregation or will promote the aggregation of the nanoparticles by suppression of their negative charge which would result in the irregular distribution of the particles and hence higher will be the PDI[6, 14, 28]. Nevertheless, significant decrease in PDI was observed with increase in surfactant concentration (X1) and amount of organic solvent (X3) which can be attributed to the marked reduction in interfacial tension between aqueous and organic phase which will provide homogeneity to the particles and result in decreased PDI[14, 18, 26, 28, 41].

Optimization of PTXNLCs: A desirability based approach i.e. numerical optimization technique was utilized for the optimization of the desired Gen NLC formulation. By the use of desirability function, we can convert all the different units into the one function and can easily optimize the results. Here, we had fixed constraints for each response and the values are listed in tabl3 18 & 19 & figure 8. From the optimized formulation, percentage biasness was calculated between experimental values and predicted values as shown in table 19. A good agreement between the predicted and experimental values confirmed the reliability of the response surface design for optimization of the GenNLC formulation.

Table 18: Desirability approach based numerical optimization of various factors

Independent variables	Predicted levels
Surf conc (X1)	0.3 %w/v
Lipid conc (X2)	0.78 %w/v
Amt of org solvent (X3)	8.51 ml

Table 18: Results of numerical optimization approach

Responses	Predicted value	Experimental value	% bias[a]
Particle Size	119.3 nm	122.20 nm	-2.43 %
Entrapment Efficiency	94.252 %	92.8 %	1.54 %
Polydispersity Index	0.120	0.116	3.33 %
Overall desirability	0.964		
Drug Loading	4.3 ± 0.21 %		
Total Drug Content (TDC)	4.5 mg in 25 ml formulation		

All results were expressed as mean ±SD, n=3. [a]Bias is calculated as {(predicted value- experimental value) / predicted value} x 100.

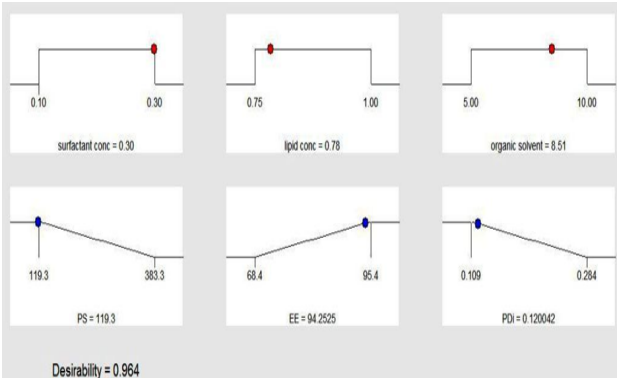

Figure 8: Predicted levels of various responses based on numerical optimization of desirability approach

HPLC analytical method development

Methanol was chosen as a solvent for the construction of calibration curve of genistein. The calibration curve was found to be linear from 500- 3000 ng/ml of Gen with correlation coefficient value (R^2) of 0.951. The method followed in our study was already reported method[31, 42], so we had mentioned only the R^2 value here.

Transmission electron microscopy (TEM)

The final GenNLC formulation was studied under transmission electron microscopy to confirm the particle size of the same and nanoparticles were found to be in the range of 90-125 nm size throughout the image area as shown in figure 9. The particle size observed under particle size analyzer as well as under TEM was compliant to each other.

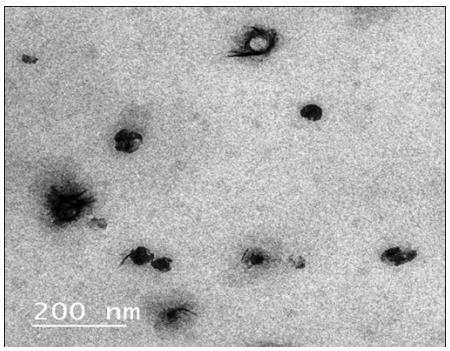

Figure 9: Transmission electron microscopic image of optimized GenNLC formulation

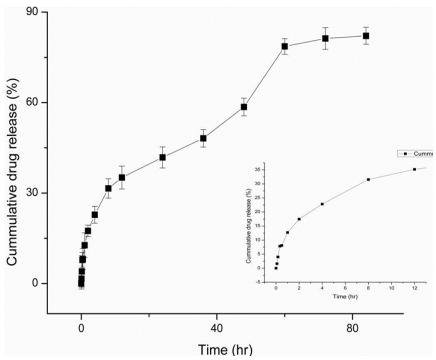

Figure 10: In vitro drug release profile of optimized GenNLC in phosphate buffer saline pH 7.4. Vertical bars represent S.D, n=3.

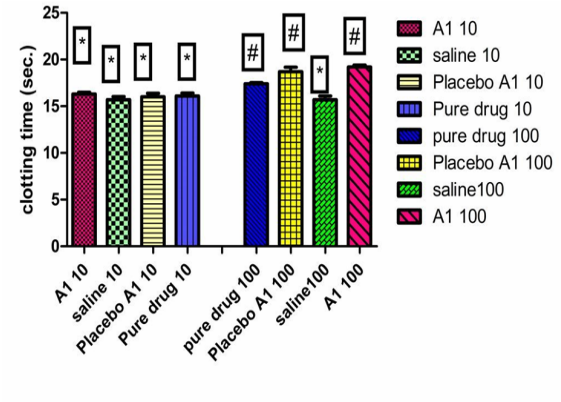

Figure 11: Prothrombin time of GenNLC, placebo NLC, pure drug and saline at various concentrations. Vertical bars represent S.D, n=3. * Data is non significantly different at p<0.05 from saline (control); # data is significantly different at p<0.05 from saline

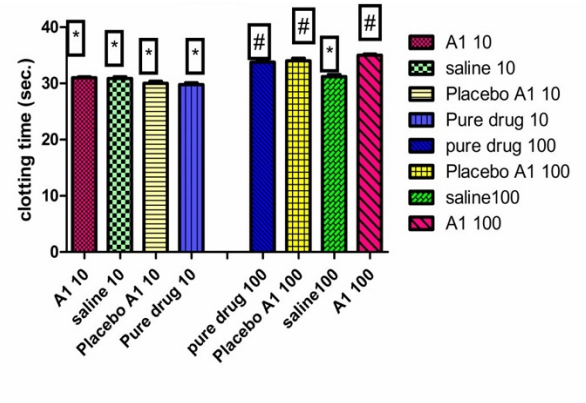

Figure 12: Activated partial thrombin time of GenNLC, placebo NLC, pure drug and saline at various concentrations. Vertical bars represent S.D, n=3. * Data is non significantly different at p<0.05 from saline (control); # data is significantly different at p<0.05 from saline

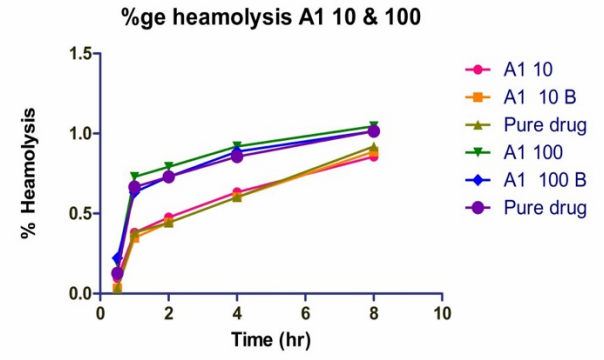

Figure 13: Heamolysis profile of GenNLC, placebo NLC and pure drug at different concentrations

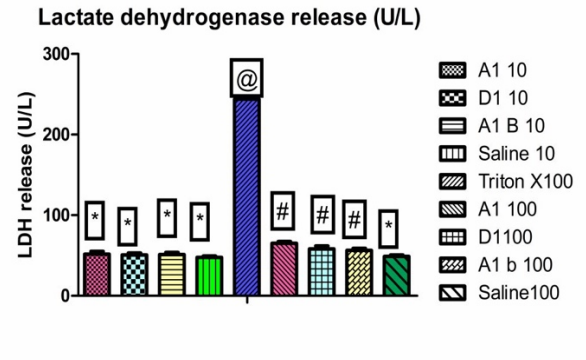

Figure 14: LDH activity of GenNLC, placebo NLC, pure drug, Triton X100 (positive control) and saline at various concentrations. Vertical bars represent S.D, n=3. *Data is non significantly different at p<0.05 from saline (negative control); # data is significantly different at p<0.05 from saline. @ Positive control.

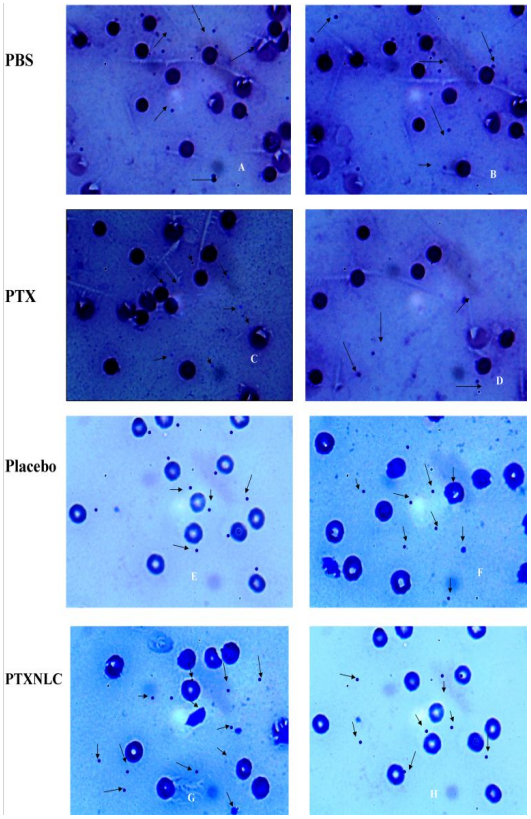

Figure 15: Optical microscopy images of whole blood samples after treatment with PBS (A,B), Gen (C,D), Placebo (E,F) & GenNLC (G,H) at 10 & 100 mcg/ml concentrations. Pictures were taken at 100x with oil immersion lens after staining the samples with Leishman's stain.

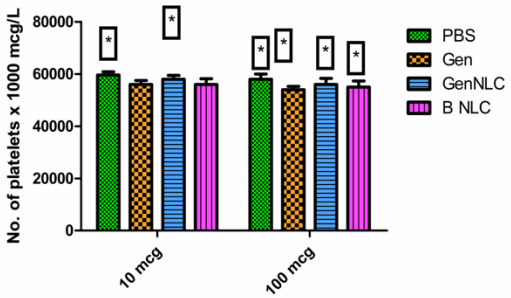

Figure 16: No. of platelets after addition of PBS, Gen, GenNLC & Placebo NLCs at 10 % 100 mcg/ml. Vertical bars represent S.D, n=3. *Data is non significantly different at p<0.05 from PBS (negative control).

Table 20: Correlation coefficients & release exponent values for various release kinetics models during in vitro release kinetics from GenNLC

Release Kinetics Models	Correlation coefficient (R^2)	Release exponent (n)
Zero Order	0.8170	-
First Order	0.9146	-
Higuchi model	0.9840	-
Korsemeyer- Peppas model	0.9821	0.457

Particle size (PS), Polydispersity index (PDI), Entrapment efficiency (EE %) & Zeta potential (ZP) determination

The optimized formulation was characterized for PS, PDI and ZP with the help of Particle size analyzer (Delsa Nano C) which works on the principles of Brownian motion, and light scattering whereas the value & charge of zeta potential are determined by the chemical nature of the polymer, oil & most importantly on the nature of surfactant used. The average particle size of the GenNLC formulation was found to be 122.20± 2.34 nm and PDI of the same was about 0.116. The excellent particle size and uniform particle size distribution is essential for any nanoformulation to work [27, 43].Zeta potential of the formulation was determined to study the stability behavior of the formulation in vitro and in vivo which was found to be -21.25 mV. It confirmed the stability of the colloidal system which is high enough to keep the particles aside and prevent the aggregates formation[14,28,32,36,38]. Also, the negative charge of the nanoparticles will delay their protein binding and thereby results in longer circulation half-life of the nanoparticles. Entrapment efficiency was calculated by the indirect method and quantified by the help of HPLC. EE of the optimized formulation was found to be 92.8 % which proved the ability of the selected SLB mix as well as colloidal system in carrying the good amount of drug

inside to release the appropriate amount of drug for an extended period.

Cumulative percentage drug release (%CDR)

In vitro release study was performed to assess the capability of the developed NLC formulation in controlling the release in vivo. The in vitro release profile of the optimized NLC formulation was shown in figure 10. The formulation did not show any burst release of the drug which is due to the absence of free drug in the formulation [30]. The formulation was showing sustained release of the drug up to 4 days and the maximum percentage release shown by the formulation was 82 % till the end of 4th day. The lipidic system present in the formulation is responsible for the sustained release of the drug as the drug belongs to class II according to the biopharmaceutical classification system (BCS) and it possess great affinity for the lipidic system and thereby the mobility of the drug out of the matrix system was averted. The release mechanism was also studied by substituting the release profile data to various kinetics models and their correlation coefficients and release exponent values are mentioned in Table 20. From the R^2 values of all the models, it was decided that our developed formulation was best fitted to Higuchi model of release kinetics and its fickian diffusion based release mechanism was explained by release exponent value of Korsmeyer Peppas model which was found to be 0.457 ($n<0.5$ for fickian diffusion controlled mechanism)[28].

Evaluation of Haemocompatibility

Plasma clotting: To test the compatibility of the formulation with the blood, Optimized NLC formulation, blank formulation, saline and pure drug were added to citrated plasma samples at 10 & 100 µg/ml. The different concentrations were chosen according to the minimum and maximum amount of sample required for i.v bolus administration [32, 44]. The pathway of blood coagulation includes intrinsic and extrinsic pathways which quote for APTT (activated partial thromboplastin time) and PT (Prothrombin time) respectively [33] . Although APTT and PT were reported as clotting time (sec.) but now a day's PT can also be reported as percentage activity, PT ratio (patient to normal clotting activity) and INR (international normalized ratio). We had used clotting time (sec.) to represent APTT as well as PT and also represented PT as percentage activity. In PT analysis, NLC (16.2±0.65s), GenNLC (16.3±0.34s) & Gen (16.1±0.54s) all at 10 µg/ml were not statistically different in clotting times as compared to saline (15.7± 0.56s) at the same concentration. However, at 100 µg/ml these formulations were significantly different from saline (15.9±0.67) at same concentration (NLC 18.7±0.83, GenNLC 19.2± 0.37, Gen (17.4±0.23) at ($p<0.05$) (Fig. 11). These effects of clotting were explained in a better way with the help of percentage prothrombin activity. At 10 µg/ml, GenNLC, NLC blank & Gen possess high prothrombin activity viz. 85 %, 86 % & 88 % respectively while at 100 µg/ml, lower values of prothrombin activities were observed. (GenNLC-69 %, NLC blank- 73 % & Gen -71 %). At the end, we concluded that at a lower concentration, drug loaded formulation as well as pure drug suspension does not much influence the extrinsic pathway but at higher concentrations, the % prothrombin levels were greatly influenced. For the evaluation of intrinsic pathway, APTT clotting time was evaluated for Gen, GenNLC, NLC blank & saline at concentrations of 10 & 100 µg/ml. It showed that Gen (29.8±0.54s), GenNLC (31.56±0.35s) & NLC blank (30.01±0.65s) in plasma does not influence the clotting time significantly in comparison to saline (30.8±0.56s) at 10 µg/ml. However a significant increase in clotting time was observed for Gen (33.8±0.23s), GenNLC (35.04±0.37 s) & NLC blank (34.03±0.82) at 100 µg/ml in comparison to saline (31.3±0.67) at same concentration; $p<0.05$ (Fig. 12). The normal clotting time varies from 30-45 s [45]. So, increase in clotting time at higher concentration of formulation will not much influence the clotting pathways as the values were under the range. These results demonstrate the ability of the NLC system for intravenous administration even at higher concentrations of the formulation.

Evaluation of haemolysis: The assessment of heamolysis is a critical factor during the preparation of nanoformulations intended for i.v use as to aid the formulation; we use some excipients which sometimes may prove fatal for the therapeutic use. To carry out the work, we tested the optimized formulation at 10 & 100µg/ml and the compared the same with the pure drug suspension, placebo formulation at the same concentrations. According to Brazilian standards, the limit for spontaneous haemolysis is not more than 1 %. Any of the compound or formulation, intended for i.v use should not cause more than 1 % of haemolysis of erythrocytes [46]. Our Optimized formulation, pure drug and blank formulation caused less than 1 % of heamolysis at the end of 8th hr when incubated at lower concentrations (10 µg/ml) but at 100µg/ml, the limits of heamolysis was exceeded to certain extent during the last hour of the study which may be due to interaction of the components like capmul MCM C8 oil and TPGS with the blood cells [29] (Fig 13). At higher concentrations, the surfactant molecules can penetrate the cell wall and can dissolve the lipids present there. Solubilization of the membrane lipids cause destruction of erythrocytes [32]. This hypothesis would be sufficient to explain the reason behind exceeding heamolysis limit by the nanoformulations.

Evaluation of membrane integrity of erythrocytes: Membrane integrity of the erythrocytes was estimated by the LDH enzyme assay. To execute the test, erythrocyte suspension. All results are presented in figure 14. Insignificant increase in LDH enzyme was observed for GenNLC(51.561±3.45), NLC blank(51.0±2.78) & Gen(50.625±2.45) as compared to saline (47.5±1.89) at lower concentrations while at higher concentrations, the significant difference was observed for GenNLC(65.0±2.43), NLC blank(56.25±2.67) & Gen(58.125±3.76) in comparison to saline (48.9±1.78) at same concentration ($p<0.05$). These results specified that erythrocytes were able to maintain their integrity at lower concentrations. The erythrocytes at lower concentrations of nanosuspensions neither got ruptured (release of haemoglobin content) nor they released LDH to the medium. However, at higher concentrations of nano-formulation, significant levels of membrane damage were observed which could be due to two reasons. Firstly, at higher concentrations of nano-formulations, the erythrocytes were exposed to higher concentrations of the drug (entrapped + unentrapped), and due to the anticancer effect of the drug, it might have caused damage to erythrocyte membrane which leads to release of a higher amount of LDH. Secondly, the formulations contain surfactant molecule which when exposed to higher concentrations, can cause damage to the cells or tissues [32, 46].

Platelet aggregation test: The potential of nanoparticle formulation for intravenous administration was also studied by calculating the platelet aggregation on addition of formulation to the citrated whole blood. The samples containing formulation and whole blood were visualized under optical light microscope and the no. of platelets was also counted. As platelet aggregation is associated with high risk of causing myocardial infarction, transient ischemia, thromboembolism, and stroke, therefore assessment of platelet aggregation seems to be an essential part of the study. We had performed this testing on GenNLC formulation, pure drug suspension and phosphate buffer saline (PBS) at 10 & 100 mcg/ml which were were treated with citrated whole blood, and no. of platelets were counted after 30 minutes of incubation. At lower concentrations, the insignificant difference in platelet count was observed for all the samples as compared to PBS while at 100 mcg/ml, Gen suspension showed a significant decrease in no. of platelets in comparison to PBS and all other groups (Figure 16). As the significant decrease in no. of

platelets was observed for Gen alone at 100 mcg/ml not for GenNLC at same concentration, this observation can be explained on the basis of potential of phytoconstituent's anticancer effect. In addition to the platelet count, platelet aggregation was also observed by optical light microscopy, and platelets were indicated by white arrows in the photographs in figure 15. Supportively, no platelet aggregation was observed of any of the samples which substantiate the nontoxicity of GenNLC formulation for intravenous use [30].

CONCLUSION

The present study provides a deep insight into the alluring features of NLCs for intravenous delivery of a bioflavonoid. Quality by design principles were used to successfully develop the Gen loaded nanostructured lipid carrier formulation which was characterized by state of art facilities. The final formulation was superlative in terms of particle size, entrapment efficiency, stability and sustained release behavior. Further, the potential of GenNLCs for intravenous administration was authenticated by employing various techniques. The prepared formulation was compatible with the blood cells throughout the study and was said to be suitable for intravenous use. In this purview, the NLCs open a new area in the formulation field for the intravenous delivery of highly lipophilic phytoconstituents by extrapolating the fascinating findings of NLCs in enhancing the stability of the formulations.

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AUTHOR CONTRIBUTIONS

Author Ms. Pooja Mittal was the lead author who had actually performed all the experiments. Prof. Brahmeshwar Mishra had guided the lead author throughout the whole experiments. Authors Mr. Gunjan V Bonde, Mr. Gufran Ajmal & Mr. Harsh Vardhan had helped in overall editing and preparation of manuscript. Author Mr. Ramit Kapoor had arranged all the chemicals needed to carry out the experiments. Author Ms. Ashu Mittal had helped in statistical analysis of the results.

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