Bioavailability Enhancement of Vitamin E TPGS Coated Liposomes of Nintedanib Esylate Formulation Developed Using Quality by Design Approach

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Research Article

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Abstract

Purpose

Nintedanib esylate (NE) is a kinase inhibitor designated for the cure of non-small cell lung cancer suffers from first-pass metabolism which resulted in low oral bioavailability (~4.7%). The intent of this exploration was to increase the oral bioavailability of NE by means of TPGS coated liposomes.

Methods

The NE-loaded TPGS coated liposomes were formulated by high-speed homogenization by optimizing process parameters like phospholipids: cholesterol molar ratio, drug loading and sonication time through the design of experiments. The drug's behaviour was studied using a variety of techniques, including physicochemical characterization, *in-vitro* and *in-vivo* studies.

Results

The NE-liposomes had a particle size of 125±6.68 nm, entrapment efficiency of 88.64±4.12% and zeta potential of +46±2.75 mV. X-ray diffraction analysis revealed that NE had been converted to an amorphous state, while transmission electron microscope images showed spherical shape and smooth coating of TPGS on surface of liposomes. The formulation showed Higuchi kinetics with sustained drug release of 88.72 ± 3.40% in 24 hours. Cellular uptake of C-6 labelled liposomes was observed in A-549 cells and cytotoxicity testing revealed that NE-liposomes were more effective than marketed formulation Ofev®. Formulation remained in simulated fluids and for three months in stability chamber and. Liposomal oral bioavailability was ~6.23 times greater in sprague dawley male rats compared to marketed formulation Ofev®, according to *in-vivo* pharmacokinetic data.

Conclusion

NE-Liposomal formulations are better for oral administration compared to the marketed capsules because of the prolonged drug release and increased oral bioavailability as a result, the developed formulation can become a successful strategy in cancer chemotherapy.

Introduction

Drug delivery via oral administration is one of the extremely used practice because of its non-invasiveness, low cost, patient compliance and lack of discomfort. Nevertheless, most of chemotherapeutic drugs, on the other hand typically have limited bioavailability due to their first-pass effect, lipophilicity, low permeability, and p-glycoprotein (P-gp) efflux. In addition, they feature a narrow therapeutic window and a high-risk of adverse effects [1]. Thus, an effective formulation that maximises bioavailability is required to facilitate the development of oral chemotherapy [2].

Nintedanib esylate (NE) is an antagonist of tyrosine kinase receptors that inhibits three receptors: the platelet-derived growth factor receptor (PDGFR), the fibroblast growth factor receptor (FGFR), and the vascular endothelial growth factor receptor (VEGFR). The drug is used to treat idiopathic pulmonary fibrosis (IPF) and several types of cancers, most notably non-small cell lung cancer (NSCLC)[3]. It is accessible in the market as Ofev®100 mg and 150 mg capsules taken twice daily through oral route [4]. The drug is available in crystalline form and its solubility in water is pH-dependent, with a greater solubility at acidic pH (pH<3) and a poor solubility at neutral pH and classified under BCS class II [5]. The properties such as lipophilicity, flexibility, saturation, solubility, polarity and size also predicted through SwissADME software [6]. Since NE undergoes first-pass metabolism in the liver via esterase's and is a P-gp substrate, it has a ~4.7**%** oral bioavailability with a half-life of 10-15 hours which lead to a decrease in patient compliance due to the need for repeated prescriptions. Drug delivery via oral route is the most appropriate, safe, and cost-effective [7] [8-10] however while reaching the target site via the circulatory system, the drug undergoes first-pass metabolism [11-13]. The drug has a positive food effect as it increases the

maximum concentration (Cmax) and area under the curve (AUC) [14, 11] and the critical properties of an anticancer molecule to be considered for oral delivery are stability, solubility, permeability, and metabolism. It is possible to use alternate processing and specific formulation techniques if these properties prevent the development of a drug formulation [15, 16]. Through the oral route, As a result of nanotechnology, the limitations of conventional capsules overcame by presenting the medications as polymeric nanoparticles [17], nanocrystals [18, 5], peptide nanoparticles [19], nano-micelles [20], liposomes [21, 22] etc. To circumvent the issues discussed previously, liposomal formulations are being considered as potential substitutes for increasing NE's oral bioavailability [12] [23].

Liposomes have many benefits, including the ability to administer drugs through multiple routes, permit active and passive targeting and provide longer circulation times. However, it has fewer drawbacks like stability concerns, handling issues, transportation, and the inability to obtain a uniform dosage [24]. Liposomes are becoming increasingly common in the pharmaceutical manufacturing owed to their ease of fabrication and scaling-up. Liposome forming processes from the top down and bottom up are both versatile and well-established [25, 26].

Vitamin E TPGS is an amphiphilic PEGylated vitamin E that significantly enhances vitamin E's pharmaceutical properties and is thus widely used in the food and pharmaceutical industries. It is quite stable in the absence of hydrolysis under normal conditions. Since TPGS has a hydrophilic–lipophilic balance (HLB) value stuck among 15 and 19, it is extremely water soluble and thus appropriate for use as an efficient surfactant capable of emulsifying hydrophobic molecules [27]. Simultaneous inclusion of TPGS in the liposomal formulation has shown increase in the solubility, prevent multidrug resistance facilitated by P-glycoprotein, and increase the anticancer drugs oral bioavailability [28].

The main objective of this research exploration is to create physiochemically stable liposomes with improved dissolution efficiency to prolong the duration of beneficial pharmacologic effects. The liposomal preparation's physical and chemical properties were examined along with the pharmacokinetic profiling of NE after oral administration to rats as till date no effort was made to increase the oral bioavailability of this drug. Hence, liposomes could be a promising drug delivery mechanism for drugs that are poorly bioavailable.

Materials And Methods

Materials

NE and cyclobenzaprine internal standard (IS) are obtained as a gratis sample from M/s. Aurobindo pharma limited, Hyderabad, India as a gratis sample. Soya lecithin, cholesterol and stearyl amine coumarin-6 (C-6), MTT reagent, TPGS was purchased from Sigma Aldrich. (Bengaluru, India), A-549 cell line purchased from NCCS, India.

Development and validation of *in-vitro* analytical methods

Based on information available in the literature [29], an in-house HPLC method was developed. HPLC with shimadzu Prominence-i LC-2030C 3D photo diode array detector or UV-visible detector (Shimadzu or equivalent) with Empower3 as Data handling system. Analytical column of Inertsil ODS 3V, 150 X 4.6 mm, 5 µm kept at 40°C. Mobile phases used were A: mixed acidified water (altered water pH to 3.00 with orthophosphoric acid and passed with 0.45 µm filter) and B: Acetonitrile in the ratio of 75:25(v/v) in the isocratic method. The optimal mobile phase composition was determined using peak symmetry, retention time, and standard solutions 1 to 20 µg/mL for calibration curves were made in acidified water diluted with phosphate buffer and 5 µl sample injected at flow rate of 1.0 mL/min, evaluated at 240 nm for 9 min run time and validation parameters were determined as per ICH guidelines.

Bioanalytical study

Numerous analytical techniques were reported in the literature [30, 31] for the bioanalytical study. Drug concentration in plasma was determined using a liquid chromatograph with shimadzu Prominence-i LC-2030C 3D photo diode array detector

or UV-visible detector (Shimadzu or equivalent) with Empower3 as data handling system. Analytical column of Inertsil ODS 3V, 150 X 4.6 mm, 5 µm maintained at 40°C was used by varying the mobile phases compositions mentioned in *in-vitro* method. The optimal mobile phase composition was set using the peak symmetry, and retention time for the drug and internal standard (cyclobenzaprine). The calibration curve has been set up within 20 - 3000 ng/mL range and IS (known concentration) were made utilizing 50 mM sodium phosphate buffer, pH 7.4 as diluting agent. Sample was prepared by taking fresh plasma (100 µl), NE (50 µl) and IS (50µl) followed by vortex for 1 min. 500 µl of acetonitrile was included and vortexed for 5 minutes to extract the drug from plasma. Centrifuge samples for 10 minutes at 12000 rpm while maintaining a temperature of 20° C. Supernatant collected and evaporated at 45° C by using a vacuum drier. After the solvent was completely evaporated, samples were reconstituted in 100 µl acetonitrile and centrifuged for 5 minutes at 10000 rpm. 50 µl of the supernatant was injected into the HPLC for analysis and validation parameters were determined as per ICH guidelines.

Preparation of liposomes

A thin-film hydration approach was used to formulate liposomes. Soya lecithin (19 mg), cholesterol (19 mg), stearyl amine (2 mg), TPGS (5 mg) and NE (5 mg, 10% w/w) were liquefied in a 20 ml blend of chloroform (10% v/v) and kept under vacuum in a rotary evaporator (BUCHI, Flawil, Switzerland) for 30 minutes at 100 rpm to get rid of the solvent, and then proceeded in a vacuum oven for 4 hours to get rid of the organic solvent completely, and then hydration with HEPES buffer pH 7.4 was performed by repeated gentle heating at 100 rpm to get a concentration of 5 mg/mL. After that, the blend was sonicated in a cold-water bath for an optimum period to obtain smaller liposomes [32-34]. The developed preparation, were then characterized for encapsulation efficiency and *in-vitro* drug release profile.

Optimization of variables for development of design space

Critical quality attributes (CQA) are inextricably interconnected to both the critical material attributes (CMA) and the critical process parameters (CPP). Relevant CQAs were recognised and prioritised based on personal knowledge and expertise. Experimentation was conducted to determine the extent to which CMA and CPP variation affected the CQA. Quality risk management (QRM) is a scientific method with justification for assessing the risk associated with a material attribute and process parameter that enables the identification of CMA and CPP which influence product CQAs (38). The investigation was conducted to bring quality into the product by means of design of experiments (DoE) software to further upgrade the catalogue and assess the significance of individual variables and interactions with the aim to achieve a deeper insight into the product development.[35, 36].

The phospholipid: cholesterol molar ratio, probe sonication time, and percent w/w loading of drug in liposomes were to be optimized. Particle size (PS) or vesicle size (VS), polydispersity index (PDI), zeta potential (ZP), and entrapment efficiency (EE) were among the characterization parameters. Formulation understanding experiments, i.e., DoE, were conducted considering the findings of the initial risk matrix analysis (IRMA). The impact of each independent CMA (phospholipids: cholesterol molar ratio, DL) and CPP (sonication time) on dependent CQAs (e.g. size and EE) were investigated in order to establish design space (DS) in overlay plot through timely assessment of CQAs that were modelled with the goal of producing a quality product. Response surface study using Box-Behnken design (BBD) was applied (Table 1) for optimization technique for creation of DS to accomplish anticipated CQA [36] by operating Design-Expert® software (Version 12, Stat-Ease Inc., Minneapolis, MN) [37]. Depending on IRMA, DoE was executed for formulation variables with greater risk priorities [38]. Thus simultaneous optimization of phospholipid: cholesterol mole ratios (viz. 2:1, 1:1, and 1:2), drug loading (5%, 10% and 15% w/w of drug/total ingredients (50 mg)) and sonication time (15 sec, 30 sec and 45 sec) was done keeping stearyl amine, TPGS, sonication parameters (3 cycles at amplitude 80 with a 1 minute gap) [39] and volume of final dispersion constant .

Characterization of optimized formulation

Size, size distribution and ZP

Optimized formulation was evaluated in terms of their vesicle size and distribution using an average of five measurements, whereas the ZP was determined using an average of twenty measurements based on particles mobility in an electric field. Additionally, the PDI ranging from 0 to 1 (with 0 representing monodispersed particles), was calculated.

Entrapment Efficiency

The drug content was determined using the direct method using equation (1) [40], which involved centrifugation at 40000 g for 1 hour at 40ºC to form the pellet. The pellet was re-dispersed in aqueous medium, and the yield of liposomes was measured after a single washing to extract free medication. To disrupt liposomes, 1 ml of Triton X 100 (0.5%) was applied, followed by an equivalent amount of methanol to dissolve the compound. The amount of NE was then calculated by means of a revalidated HPLC method, and EE was calculated [32].

$$\text{Encapsulation efficiency} = \frac{\text{Quantity of drug present in pellet}}{\text{Quantity of drug taken initially for formulation of liposomes}} \text{ X } 100 \quad \ldots (1)$$

Freeze drying study

Stability of the colloidal particles is one of the serious issues that are hampering their progress. Improving shelf life of the colloidal particles by freeze drying is gaining popularity these days. The various sugar molecules are used to serve this purpose, which act as lyoprotectants. Freeze drying was done using an optimized cycle containing 2.5% w/v and 5% w/v of mannitol and trehalose respectively. All formulations were evaluated after freeze dying for cake appearance, size, as well as entrapment efficiency after re-dispersion [41, 42].

Shape and morphology of Liposomes

To determine if any solid-state transition in the liposomes has taken place during lyophilization or not X-Ray Diffraction (PXRD) (Miniflex, Rigaku Corporation, Japan) analysis were carried out. Samples of NE, lyoprotectant and lyophilized NE liposomes were prepared and PXRD was taken at 40kV, 40mA, a step size of 0.01 degree at room temperature from 0º to 50º range. Determination of the morphology and shape of the prepared formulation were carried out through transmission electron microscopy (TEM) (FEI 187 Tecnai G2F20, Netherlands).

In-vitro release profile study

The dialysis membrane system was utilized to release the medication from the liposomes *in-vitro* by activating it below running water for 6 hours to eliminate glycerol [43]. Sulphur was eliminated by treating the membrane with 0.3% sodium sulfide for 1 minute, then acidifying it with 0.2% sulfuric acid (the membrane was cleansed using running water after acidification to eliminate excess acid). Phosphate buffer with 0.1% Tween 80 (pH 7.4) was utilized as a release medium [44, 45]. Tween 80 was used to make the released compound more soluble. It aids in the preservation of the sink condition in the release medium. liposomes (1 mL) were placed into dialysis bags (sigma) with a molecular cutoff of 12000 and placed gently in 15 ml of release medium at 37^{o}C and 100 rpm in a shaking water bath. At every time interval, the sample volume (500 µL) was withdrawn (passed through PVDF filter 0.45 µm) and swapped with fresh dissolution medium (500 µL). Samples were then analyzed for drug release by HPLC method developed previously by injecting 10 µl sample collected at each time point. The DDSolver software was used to measure the percent cumulative drug release [46].

Stability studies in simulated fluids

Simulated gastric fluid (SGF pH 1.2) and simulated intestinal fluid (SIF pH 6.8) were used to evaluate steadiness of liposomes and to monitor formulation stability under enzymatic conditions [47]. 9 ml of each media applied to 1 ml of reconstituted volume (5 mg/ml) of freeze-dried formulation and incubated for 2 hours in SGF and 6 hours in SIF [48, 49]. The particle characteristics of liposomes were then assessed for a variety of parameters.

Stability studies in temperature and humidity chambers

Liposomal formulation (n=3) was exposed to storage at 25°C, 60% RH and 2-8°C for 3 months [50] for the assessment of stability and verified for the re-dispersibility, vesicle size and size distribution.

Cytotoxicity study using A-549 cell line

A-549 cells were trypsinized and altered to 50,000 cells/ml in entire medium from confluent cultures. The cell suspension was poured in 96 well tissue culture plates (0.2 ml/well or 1×10^4 cells/well) and incubated overnight (37°C for 48 hours in humidified 5% CO_2) for cell attachment. Subsequent attachment, the medium was exchanged with complete medium (0.2 ml) containing the NE-suspension prepared from Ofev® capsules and NE-liposomes at equivalent free drug concentrations 0.1, 1, 10, 100 µg/ml respectively. The cells were incubated (37°C for 48 hours in humidified 5% CO_2) and cell viability was evaluated by MTT assay. MTT assay was carried out by washing the cells with HBSS (Hank's balanced salt solution) and incubated with 0.2 ml fresh DMEM (Dulbecco's Modified Eagle's Medium) containing 0.5 mg/ml MTT. After 3 hours of incubation, the MTT-containing medium was withdrawn. MTT formazan was dissolved in 0.2 mL dimethyl sulfoxide, and the optical density at 550 nm was checked using a Bio-Tek ELISA plate reader for quantitative analysis [51].

Cellular uptake studies

Fluorescent liposomes were formulated by including coumarin-6 in the organic phase instead of NE in the previously optimized formulation. A-549 cells were sowed overnight in 6-well culture slides (BD Falcon). The cells were incubated with coumarin-6 NE liposomes for 12 hours before being washed with HBSS (5X) to get rid of extracellular particles. The cells were examined using a confocal microscope (Olympus FV1000) at 400X magnification for qualitative analysis [33].

Pharmacokinetic study

Pharmacokinetic testing on male sprague-dawley rats (150–250 g) distributed at random were (n=6, 2 groups) fasted for 12 hours preceding the experiment was done to determine oral bioavailability using equation (2). The SD rats were given liposomes and Ofev® suspension dosage of 10.3 mg/kg via oral gavage [52]. Under mild ether anesthesia, blood samples (0.5 mL) were obtained via the retro-orbital plexus of the eye into heparinized micro-centrifuge tubes at 0.5, 1, 2, 4, 8, 12, 24, 36 h post dosing. To avert changes in the central compartment volume, 0.5 mL of normal saline was given after each sampling. The plasma was isolated from the blood samples by centrifuging them at 10000 g for 5 minutes at 15°C. After adding 25 µl (known concentration) of internal standard to 125 µl of plasma, the mixture was vortexed for 60 sec. After that, 500 µL of acetonitrile was used to precipitate proteins, and the mixture was vortexed for 5 minutes before being centrifuged at 5000 g for 10 minutes. The supernatant was then extracted and dried out in a vacuum oven at 40°C. Following that, dried up samples were re-dissolved and vortexed in 100 µl acetonitrile. Separated supernatants were evaluated using a validated bioanalytical method for plasma concentration. The pharmacokinetic parameters (AUC, $AUC_{0\text{-}t}$, C_{max}, t_{max}) were determined using the pk solver [53].

$$\text{Relative bioavailability} = \frac{\text{AUC (liposomes)}}{\text{AUC (Suspension)}} \text{ X } 100 \qquad \ldots (2)$$

Results

Development and validation of *in-vitro* analytical method

The drug's *in-vitro* analytical technique was noticed to be linear across the range of 1 to 20 µg/mL (r^2 = 0.999 ± 0.005) having an LOD of 0.08 µg/mL and LOQ of 0.25 µg/mL. The samples were injected and recovery (%) in the release media was determined. Values of accuracy, repeatability (intraday precision) and intermediate precision of analytical method remained within an R.S.D. ≤2%) at (n=3) and at concentrations of 3, 8, and 15 µg/mL, in all the cases which suggest that method was highly repeatable within the range of analysis. The extraction effectiveness (%) in case of spiked samples were 103.78 ±

1.82, 99.25 ± 1.94, 98.67 ± 1.45 respectively for the above concentrations. The technique was noticed to be robust, rugged, and well suited for further analysis of the prepared formulation.

Bioanalytical study

The developed bio-analytical method was validated, and the calibration curve showed linearity (r^2 = 0.9978 ± 0.0009) over 20 - 3000 ng/mL range. Quality control samples were injected and recovery (%) in the release media was determined. Values of accuracy, precision of analytical method were within an R.S.D. ≤15%) at concentrations of 20, 500, and 1500 ng/mL (n=5), in all the cases which suggest that method was highly repeatable within the range of analysis. The extraction effectiveness (%) in case of spiked samples were 104.64 ± 2.76, 98.54 ± 2.83, 102.65 ± 2.12 respectively at above concentrations. LLOQ was observed to be 20 ng/mL. So, the procedure was well suited for further analysis of the pharmacokinetic samples.

Preparation, optimization and characterization of liposomes for development of design space

Thin film hydration procedure was used to produce drug loaded liposomes. Various process parameters like molar ratio of phospholipids: cholesterol, DL and sonication time were further optimized.

To optimize varied response variables, a response surface study using BBD with three factors and three levels was chosen. The factors examined were decoded to facilitate the ANOVA analysis. Counter plots were taken help to illustrate the relationship between factors and responses. The ANOVA for the response surface quadratic for particle size was significant (P<0.05), indicating that the chosen equation particle size=+124.23-38.75A+17.50B+90.00C+46.35A2+61.35C2 is fit, and the contour plot for particle size shows an interaction amongst the critical factors and response variables, respectively. The two-dimensional graph for particle size illustrates the effect of PC ratio and DL (%) on particle size. At higher concentrations of phosphatidyl choline, particle size decreases, DL has little effect on particle size, and ST has a significant effect on particle size.

The ANOVA for response surface quadratic for %EE was observed as significant (P<0.05), signifying that the chosen equation percent EE =+86.33+7.12A-4.63B-11.50C+2.75AB-4.50AC+0.0BC-11.79A2-12.79B2-16.04C2 is fit. and the contour plot for percent EE, indicate an interaction among critical factors and response variables. The two-dimensional plot for percent EE illustrates the influence of PC ratio and DL% on percent EE. When the PC is decreased, the EE decreases, and when the DL is increased, the percent EE decreases as the preparation unable to hold the drug.

The experimental results for size and EE were within the permissible limits as shown in the overlay plot. The composition choosen from the overlay plot were prepared and validated at a PC ratio of 70:30, 10% DL, and 30 sec ST. Thus, a composition is mentioned that contains the desired amount of CMA and CPP. The optimised formulation is 121±3.68μ in size and has an EE of 87.54±4.35%, PDI of 0.296±0.041 and ZP of +45±2.61. The PS and EE were acceptable and matchup to the target product profile in terms of quality (QTPP) as shown in table 2 and Fig. 1.

Changes in the ratio of phospholipids to cholesterol cause major changes in the particle size, size distribution, and encapsulation ability of liposomes. With a lower cholesterol molar ratio, particle size and PDI decreased (table 2). Since a constant amount of stearyl amine was used in each formulation, ZP showed no substantial difference in any of the cases. EE was also impacted by the change in ratio of phospholipids: cholesterol. This might be due to the reason that cholesterol prevents the chain tilting of phospholipid and provides stability to lipid bilayer. With regards to vesicle size, PDI, and EE, a molar ratio of 1:1 to 3:1 was found to be optimal. Surprisingly, increasing cholesterol levels caused a decrease in entrapment performance and a rise in vesicle size. Higher levels of cholesterol can compete with the medicament for packing space within the bilayer, prohibiting the medicament to get inside lipid bilayer; and above certain concentration of cholesterol, normal bilayer structure of vesicular membranes is disrupted, resulting in the drug being excluded [54].

DL was optimized by adjusting the dosage of the drug in relation to the amount of lipids consumed (50 mg). Results in Table 2 and Fig. 1 show that as the initial DL increased, the particle size and EE changed significantly. Entrapment performance was found to be higher at 10% theoretical loading and formulation was found to be stable. For 5% loading EE was slightly less and for 15% loading there was a drug loss during the processing of formulation. As the theoretical loading increases, the EE slightly increased and then decreased. This is because drug has a destabilizing effect on the liposomal membrane. This is due to a greater interaction of the drug with the liposomal membrane, which allows the liposomes to expand in size. As a result, 10% DL was chosen for further research based upon the size, PDI, stability, and EE of liposomes [55].

From results shown Table 2 and Fig. 1, increasing the time of probe sonication caused a substantial decline in the vesicle size of liposomes. Longer probe sonication produced a lot of energy and generated a lot of heat, which caused liposome destabilization and breakage. As a result, sonication for 30 sec with three cycles at amplitude 80 at 1-minute intervals was preferred.

Freeze drying study

Following an optimized freeze-dried method, various prepared liposomal formulations were freeze-dried (Virtis, SP Scientific, USA freeze dryer). The temperature of the condenser was -60°C, and the pressure in each stage was 200 Torr. 5 ml of each formulation was poured into 15 ml glass vials covered with porous foil and freeze dried with trehalose and mannitol as lyoprotectants at 2.5 and 5% w/v, respectively. Following lyophilization, the appearance and size of cake of each formulation were evaluated [41]. The cake obtained for mannitol was intact and fluffy, while the cakes for trehalose were collapsed, which could be clearly seen in the figure. Nature of the cake showed that the mannitol was effective in preventing liposome aggregation. 2.5% and 5 % trehalose showed fluffy cake, 5% mannitol was more intact than 2.5% with a reconstitution time of 20 sec as shown in Fig. 2. The proportion of sizes (Sf/Si), i.e., size following reconstitution of freeze-dried preparation and size prior to freeze-drying, was near 1.05 for 5% mannitol.

Shape and morphology of Liposomes

XRD patterns of NE, Mannitol and freeze-dried NE-liposomes were shown in Fig. 3. NE had unique peaks at 2θ angles of 14.64, 18.79, 19.31, 20.11, 21.20, 22.45 and 26.71 degrees which confirm it as a crystalline drug as crystals are arranged perfectly and have a high degree of periodicity resulting in high intensity peaks. Mannitol's diffraction pattern revealed sharp peaks at 2θ angles, indicating its crystalline nature. The characteristic NE and mannitol peaks were not detected in lyophilized NE-liposomes, indicating that NE is encapsulated within liposomes. Additionally, the peak intensity of NE-liposomes was significantly lower showing hallow pattern, and the peak width was significantly larger than that of NE, indicating that it had converted to an amorphous state.

TEM analysis carried out for liposomes demonstrated the spherical shape of liposomes and revealed distinct layers with a smooth TPGS coating as shown in Fig. 4. Both the zeta sizer and the TEM (FEI, Tecnai G2 F20, Cryo FE-TEM, USA, 200 kV) revealed a strong correlation in particle size.

In-vitro release profile study

The dialysis membrane containing liposomes release the drug slowly through the pores of the membrane. The various times points at which sampling was done are 15 min, 30 min, 1, 2, 4, 6, 8, 12, 18, 24 and 30 h. The samples were evaluated, and the cumulative percent drug discharge was drawn against time as shown in Fig. 5. The enhanced rate of drug release initially was due to surface coating of drug on liposomes supported by the hydrophilic and solubilizing properties of TPGS [56]. The formulation demonstrated prolonged release performance, with 29% of the medication released in 1 hour and 88% released in 24 hours. The cumulative drug release was fitted using the Higuchi kinetics (R^2=0.948), Hixon crowel (R^2=0.948) zero-order (R^2=0.948), first-order (R^2=0.948) release models. It is observed that the profile shows biphasic drug release pattern with Higuchi kinetics.

Stability studies

Stability studies in simulated fluids

Drug loaded liposomes, after incubation in various simulated GIT fluids (pH 1.2, pH 6.8) showed, no major changes ($p<0.05$) in vesicle size, PDI, ZP, or EE as shown in Table 3. The robustness of the preparation is due to the TPGS [57].

Stability studies in temperature and humidity chambers

The freeze dried formulation using mannitol was kept in the stability chambers appeared to be stable up to three months under 25°C/60% RH and 2-8°C. the samples at different time point had not shared any significant changes in particle size and PDI when the samples were analyzed after 3 months. The entrapment efficiency remained constant, which proved that the formulation is quite robust for storage condition of the refrigerator. The sample is devoid of cake breakdown or contraction, retained its appearance with a reconstitution time of approximately 20 sec as shown in Table 4 which confirms the stability of the formulation.

Cytotoxicity study using A-549 cell line

Cytotoxicity study was carried out in log concentration range 0.1-100 µg/ml in A-549 cancer cell line for NE-liposomes and NE-suspension prepared from Ofev® capsules. Cytotoxicity of each formulation increased proportionally on increasing the concentration of drug. The % cell viability at log concentrations 0.1 to 100 µg/ml for NE-liposomes treated cell was 71%, 54%, 43% and 2% for NE-suspension treated cell was 80%, 73%, 50% and 5% respectively. IC50 amount of NE-suspension was found 30.75 µg/ml while IC50 value of NE-liposomes was noticed as 14.72 µg/ml. Significant decrease in IC50 value of NE-liposomes proved their higher cytotoxicity in contrast to NE-suspension ($p<0.05\%$) as shown in Fig. 6. The possible reason behind more cytotoxicity of NE-liposomes could be the more penetration due to lipidic nature of liposomes and faster attachment of cationic particles with anionic cell membranes.

Cellular uptake studies

Liposomes were taken up by cells within 12 hours of incubation. The green fluorescence of coumarin-6 loaded into liposomes indicates that it is being taken up by the A-549 cancer cells. Fig. 7 demonstrated the efficient internalization and cellular uptake concentrated within and outer surface of cells due to the positive charge on liposomes (because of stearyl amine) and negative charge on the cell membrane.

Pharmacokinetic study

Plasma drug concentration-time profile of liposomes and drug suspension (Ofev® capsules) obtained from an *in-vivo* sample in rats was plotted as shown in Fig. 8 and the pharmacokinetic parameters calculated from experimental data obtained following oral administration are summarised in Table 5. One compartmental model was used to modify the results. When the AUC_{0-t} of liposomal preparation was matched to the drug suspension, it was discovered that the liposome preparation enhanced the bioavailability of NE by ~6.23 times.

Discussion

A pharmaceutically challenging molecule, NE has poor biopharmaceutical properties that necessitate the use of delivery strategies to improve its clinical efficacy. Molecular drugs with poor biopharmaceutical properties can now be delivered orally using nanotechnology in the form of nano carriers carrying drug. An effective method for producing liposomes coated with TPGS was devised using design of experiments. The variables involved in the preparation of liposomes have been extensively optimised. It was discovered that the amount of phospholipids to cholesterol percentage had a substantial impact on the formulation, so the design of experiments was chosen. However, when the cholesterol rises above 50%, the liposomes' quality attributes suffer, possibly due to the membrane vesicles being disrupted at higher concentrations. As a

result, the ideal PC/cholesterol ratio, was selected as 70% and 30% from the results suggested by the software and can be explained by the cholesterol molecules assembling inside PC molecules to provide firmness in the resulting vesicular assemblies. Additionally, the rise in cholesterol's amount will lead to integration into the vesicles and the subsequent leakage of medicament could be responsible for the sharp decline in encapsulation efficiency [58]. Liposomes prepared with a higher drug loading are more likely to become membrane destabilised and the findings corroborate previous research in the literature. As a result, additional studies were conducted using 5, 10, 15% w/w drug loading and liposomes with 10% w/w drug loading was chosen and it was found that these liposomes possessed desirable qualities i.e., 125±6.68 nm, with entrapment efficiency of 88.64±4.12% at 10% theoretical loading as shown in Table 2.

To explain why the particle diameter and EE decreased as the sonication time increased, it might be ascribed to mechanical collisions and heat generated in the microenvironment, respectively. As a result, the vesicles break down, releasing the drug that was previously trapped inside. Therefore, Table 2 shows that sonication time for 30 sec at amplitude 80 with three cycles at a one-minute interval was found to be the most effective.

Stearyl amine was added to the liposome surface in order to enhance the liposome's ability to bind strongly to the cell membrane due to the charge interactions. TPGS-coated liposomes will accumulate in tumour tissue and increase tumour inhibition without affecting the structure of other organs [59]. Because of this, TPGS-coated liposomes can be used as a cancer chemotherapy strategy for model drugs that are poorly water soluble and the surface coating is visualized in TEM image of TPGS coated liposomes (Fig. 4). Spherical shape and particle diameter were not affected by TPGS treatment, as demonstrated by dynamic light scattering analysis. The developed formulations were subsequently freeze dried with a view to improve the shelf life. Mannitol 5% w/v was found to be best lyoprotectant capable of preserving particle characteristics followed by freeze drying and will increase its storage stability, to attain the highest possible quality attributes (Table 2). NE within liposomes was converted into amorphous form after freeze drying as confirmed by XRD. Without the need for additional energy input, such as sonication or vortexes, gentle shaking of the lyophilized formulation resulted in spontaneous reconstitution (<20 sec). Reconstitution of a lyophilized formulation preserved all of the previous quality attributes of the formulation, like particle diameter, encapsulation efficiency, and the stability of liposomes.

When liposomes were coated with TPGS, drug release profiles (Fig. 5) presented a significant delay in release and followed higuchi kinetics, which is indicative of drug release via diffusion from matrix systems. These findings also corroborate the fact that when coated with TPGS, the liposomes lose their vesicular nature and become particulate. Due to the presence of positive charge outside because of stearyl amine, it can repel the hydrogen ions of simulated gastrointestinal fluids [60] and TPGS coatings also Prevents the phospholipids from being exposed to the harsh conditions of the gastrointestinal tract, which is an indicative of the robustness of formulation stability [61]. The formulation was found intact on subjecting to stability study for three months as per ICH guidelines for refrigerated products. Fig. 7 revealed that prepared formulations were efficiently internalised into A549 cells as TPGS imparts superior binding and cationic charge provided by the stearyl amine interacts with negatively charged cell membrane. The MTT assay showed that the formulation was more cytotoxic than that of the commercially available formulation.

The in vivo pharmacokinetics clearly reflect the ability of liposomes to improve the relative oral bioavailability of drug by 6.23-fold when compared with marketed formulation which is available in capsule form. Increased oral bioavailability may be a result of these lipid-based nanocarriers' improved permeation throughout the GIT. Additionally, these systems are susceptible to chylomicron uptake, allowing for bypass of the drug's p-gp efflux, which is one of the main reasons for NE’s low bioavailability. Additionally, these alternative routes of absorption allow drugs to bypass the hepatic first-pass metabolism, resulting in an increase in oral bioavailability when these nanoformulations are used. Therefore, the outcome of this investigation would certainly lead to a new paradigm in healthcare, allowing the use of liposomes not only to improve the bioavailability but also add therapeutic value in treating cancers.

Conclusion

The current research explored the possibility of using a liposomal approach to upsurge the oral bioavailability of the drug NE, which belongs to BCS class 2. We prepared liposomes with a particle size range of 125 nm and analyzed using validated analytical methods. Liposomes coated with TPGS demonstrated a superior encapsulation efficiency of up to 84% for NE. The transmission electron microscopy analysis revealed that the TPGS-incrusted liposomes are well constructed at the nanoscale. *In-vitro* drug release pattern demonstrated the liposomes coated with TPGS to have a more controlled release property. Due to an increase in saturation solubility, freeze dried liposomes demonstrated an enhanced *in-vitro* release profile. Stability findings in simulated media demonstrated that the preparation is stable in the GI tract for oral administration, while accelerated stability studies established the liposomes' stability. Additionally, the TPGS-coated liposomes enhanced liposomal stability, thereby protecting the NE from the reticuloendothelial system. The pharmacokinetic analysis revealed that drug-loaded liposomes have a bioavailability increase of ~6.23 times that of the free drug. As a result, the current study established that liposomes can be used to boost the bioavailability of BCS class 2 medications. However, the therapeutic effect and side effects of liposomes will need to be investigated further in the future and compared to those of the free drug.

Abbreviations

ACN: Acetonitrile; AUC: Area under the curve; BCS: Biopharmaceutical Classification System; C-6: coumarin-6; DMSO: Dimethyl sulfoxide; EE: entrapment efficiency; HPLC: High Pressure Liquid Chromatography; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDI: Poly dispersity index; PVDF: Polyvinylidene Flouride; PDI: Polydispersity Index; PBS: Phosphate buffer saline; RH: Relative Humidity; RPM: Rotations per Minute; Sprague Dawley; S.D: Standard Deviation; SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid; TEM: Transmission Electron Microscopy; XRPD: X- Ray powder Diffraction;

Declarations

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Tables

Table 1 BBD to explore CMA, CPP and CQA

Factors: CMA+CPP			Levels		
			-1	0	+1
A	phospholipids: cholesterol molar ratio		25	50	75
B	DL (%)		5	10	15
C	sonication time (sec)		15	30	45
	Responses	Goal	Ranges Acceptable		
Y1	Size (nm)	Range	120-130		
Y2	EE (%)	Range	70-87		

Table 2 Optimization results to study CMA and CPP

	Variables			Responses	
Run	A: phospholipids: cholesterol molar ratio	B: DL	C: sonication time	Size	EE
	%	%	sec	nm	%
1	75	10	15	301±4.35	90±1.67
2	75	5	30	120±3.26	81±2.58
3	50	15	45	126±2.15	43±1.75
4	50	10	30	128±1.34	82±1.42
5	25	10	15	380±2.89	58±2.87
6	50	10	30	122±3.21	78±2.87
7	75	10	45	95±3.48	82±2.63
8	25	5	30	180±2.92	64±3.61
9	50	10	30	117±3.42	85±2.45
10	25	15	30	251±5.85	45±3.15
11	50	15	15	273±6.15	67±2.86
12	50	5	15	247±5.85	72±3.75
13	25	10	45	148±4.38	45±2.62
14	75	15	30	135±3.65	65±2.73
15	50	5	45	99±2.75	59±3.66

Values articulated as mean standard deviation (n=3)

Table 3 Stability studies in simulated fluids

Media	Size (nm)		PDI		ZP (mV)	
	Initial	Final	Initial	Final	Initial	Final
SGF pH 1.2	125±6.68	122.14±5.6	0.286±0.036	0.37±0.03	+46±2.75	43.42±3.72
SIF pH 6.8	125±6.68	117±13.79	0.286±0.036	0.35±0.05	+46±2.75	41.53±3.43

Values articulated as mean standard deviation (n=3)

Table 4 Stability studies in temperature and humidity chambers

Condition	Size (nm)		PDI		ZP (mV)		% EE	
	Initial	3 Months	Initial	3 Months	Initial	3 Months	Initial	3 Months
4°C	125±6.68	119±3.81	0.286±0.036	0.289±0.02	+46±2.75	46±2.93	88.64±4.12	85.82±3.8
25°C	125±6.68	124±4.53	0.286±0.036	0.293±0.01	+46±2.75	47.5±2.81	88.64±4.12	86±4.12

Values articulated as mean standard deviation (n=3)

Table 5 Parameters relating to the pharmacokinetics study after oral administration

Dosage form	AUC (ng ml^{-1} h^{-1})	$T_{1/2}$ (h)	Cmax (ng/ml)
Drug suspension (Ofev® capsules)	1945.88± 87.26	2.24 ± 0.57	501± 42.48
Drug loaded liposomes	12138.75± 188.3	5.94 ± 0.97	1214± 72.74

Values articulated as mean of standard deviation (n=6)

Figures

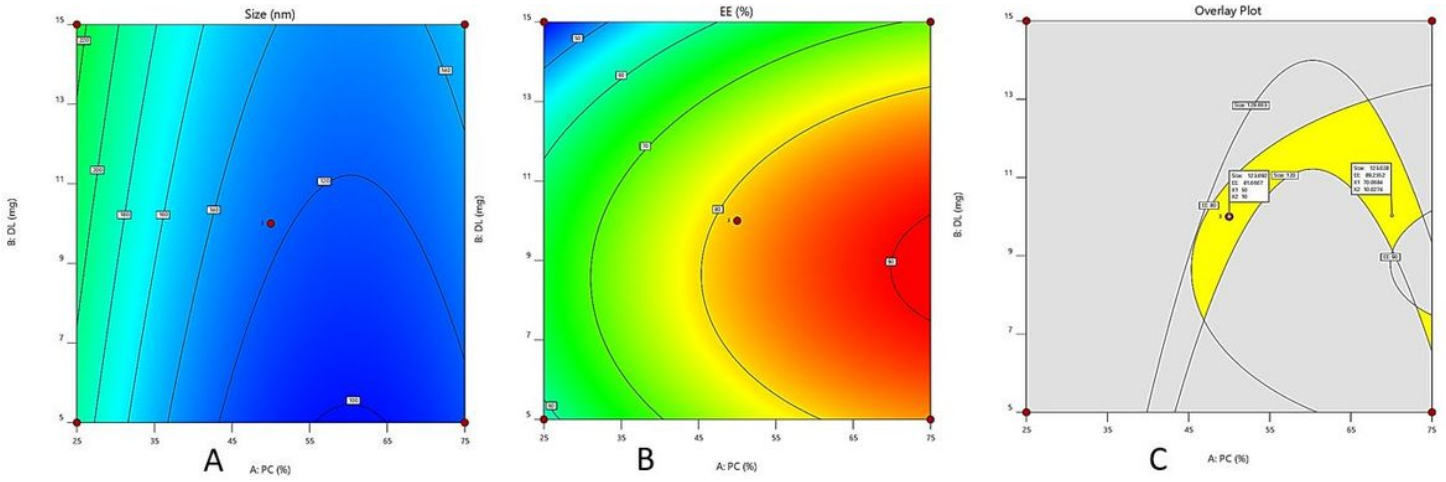

Figure 1

A. Counter plot showing effect of PC ratio and DL on vesicle size. B. Counter plot showing effect of PC ratio and DL on EE. C. Overlay plot showing optimized formulation in yellow region.

Figure 2

Optimized freeze-dried drug loaded liposomes containing different sugars as lyoprotectants. Images from left to right indicates 2.5% w/v trehalose showing fluffy cake, 5% w/v trehalose fluffy cake, 2.5% w/v mannitol showing intact porous cake and 5% w/v mannitol showing intact stable cake.

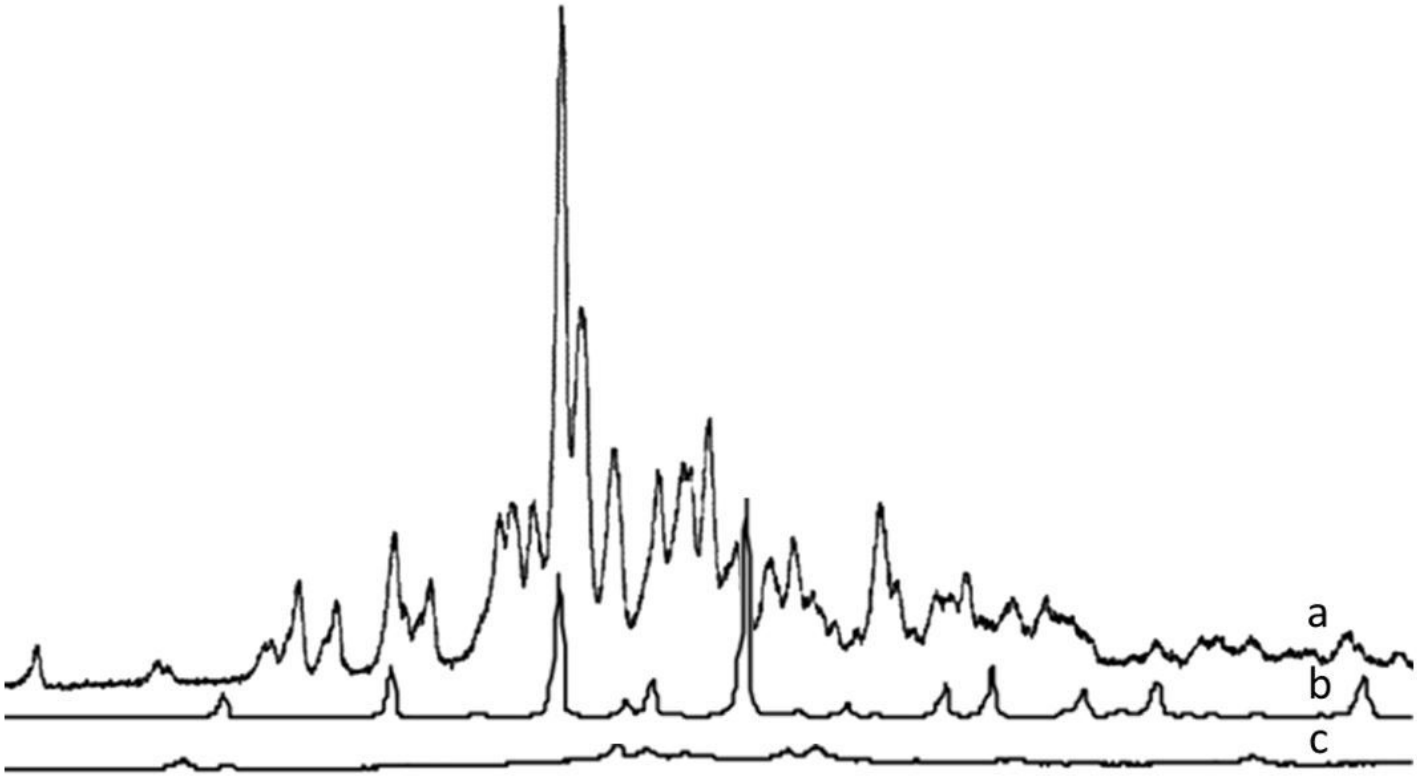

Figure 3

PXRD showing a. nintedanib esylate with sharp peaks b. mannitol with sharp peaks c. freeze dried liposomes without any sharp peaks.

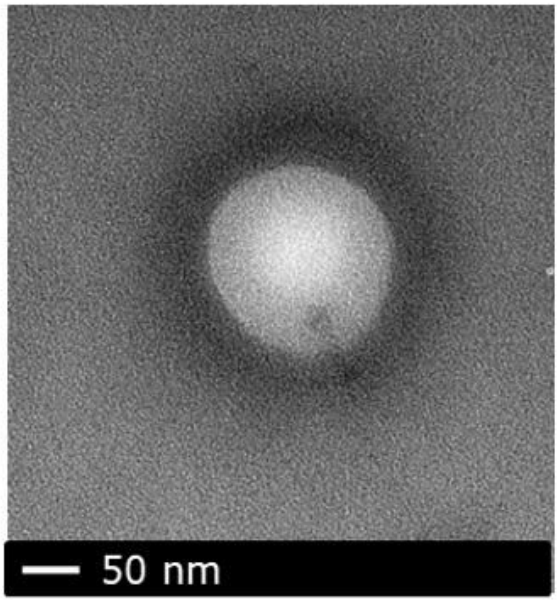

Figure 4

TEM image of liposome with spherical shape and smooth surface with TPGS coating.

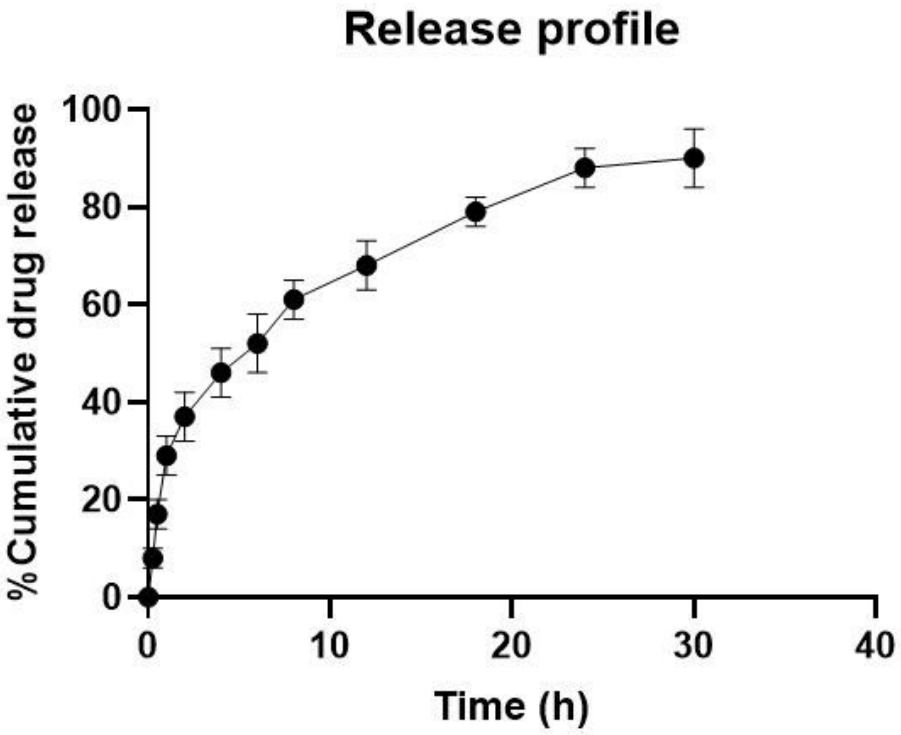

Figure 5

Release profile of drug from liposomes in buffer of pH 7.4. Values articulated as mean standard deviation (n=3).

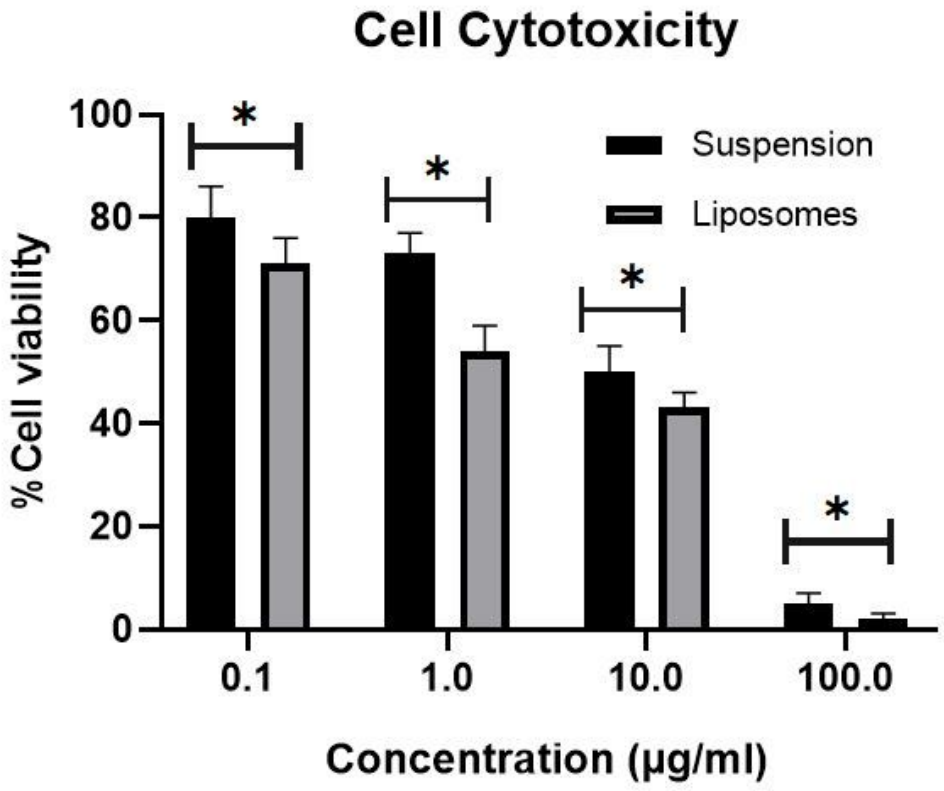

Figure 6

Cytotoxicity of liposomal preparation in comparison with commercial drug suspension at various concentrations (*significant difference at p<0.05 percent).

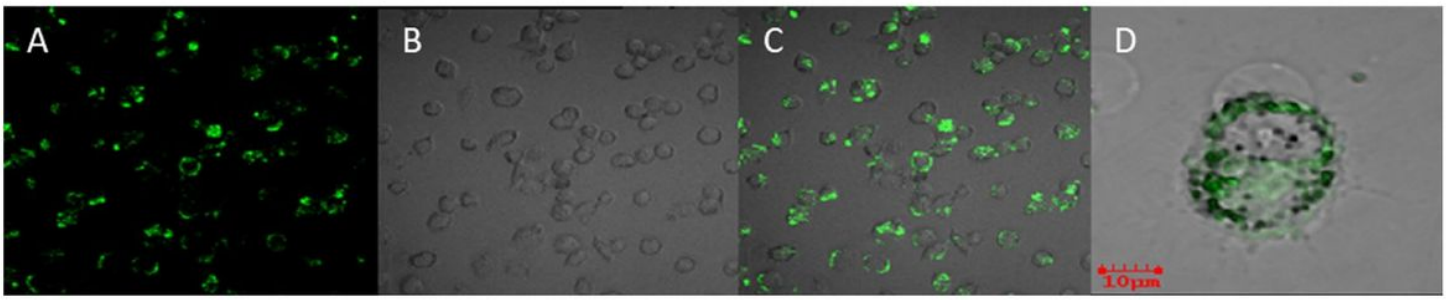

Figure 7

Uptake of liposomes A. image taken using excitation wavelength of dye, showing green fluorescence across A-549 cancer cell lines. B. Differential interference contrast image of A-549 cells. C. Superimposed image of dye and DIC image of figure A and B. D. 3D view of C.

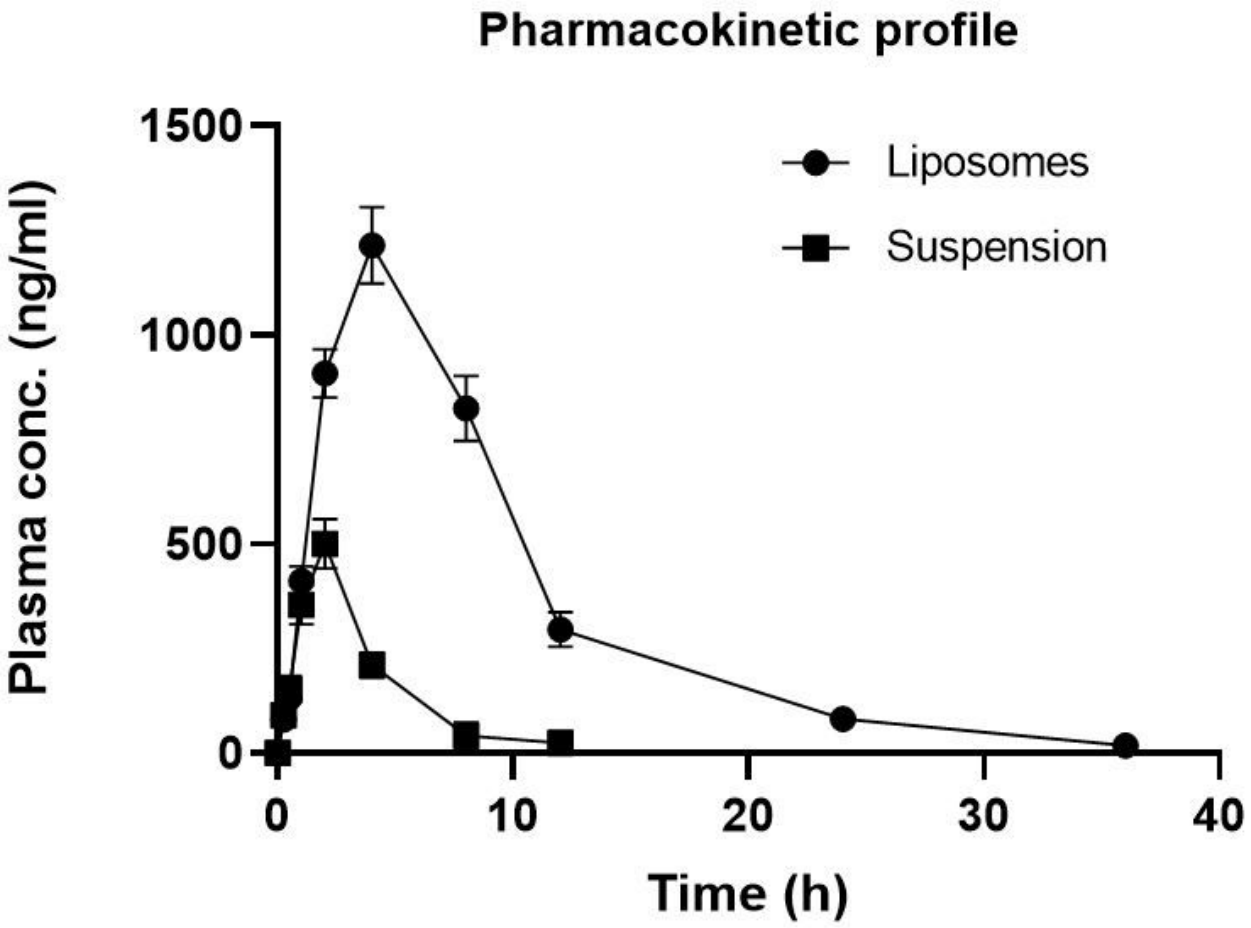

Figure 8

Plasma concentration profiles of marketed formulation (Ofev® capsules) and liposomes following oral administration in rats (10.3 mg/kg). Values articulated as mean standard deviation (n=3).