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DEVELOPMENT, EVALUATION AND TARGETING OF DOXORUBICIN LOADED SOLID LIPID NANOPARTICLES TO THE LYMPHATIC SYSTEM

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ABSTRACT
The distribution of therapeutic drugs to the sick organ has proven to be a difficult issue for formulation scientists. A good drug delivery approach to the sick organ is intended to lessen side effects and improve the therapeutic index. This study aims to assess the ability of doxorubicin-loaded solid lipid nanoparticle (SLN) formulation to target the lymphatic system through the use of response surface methodology in experiment design. Doxorubicin, Compritol 888 ATO (X1), and Pluronic F68 (X2) were used as independent elements in the construction of the Box-Behnken DOE, whereas particle size (Y1) and entrapment efficiency (Y2) were used as dependent factors. Hot homogenization and ultrasonication were used to create the SLN formulation. The produced SLN was analysed using FTIR and SEM. The solid lipid nanoparticle loaded with doxorubicin was found to have an optimised particle size of 190 nm and an entrapment effectiveness of

66.58%, respectively. These values are adequate for the particle to reach the lymphatic system. No interaction between doxorubicin and Compritol 888 ATO was detected by FTIR. The results demonstrated that the targeting efficiency of both the standard oral suspension and the doxorubicin-loaded SLN at the mesenteric lymph node increased significantly ($P<0.05$), as did the pharmacokinetic parameters, such as area under the whole blood concentration-time curve, Cmax, and Tmax.
KEYWORDS: Solid Lipid Nanoparticle, Lymphatic delivery, Doxorubicin

INTRODUCTION:

Globally, cancer is one of the main causes of morbidity and death. According to estimates, 13.1 million people would lose their lives to cancer by 2030.[1] Anthracyclines have long been the cornerstone of cancer treatment, particularly doxorubicin. The use of conventional doxorubicin in clinical practice has been limited due to side effects, mainly cardiotoxicity, despite its broad-spectrum antineoplastic efficacy.This was particularly true for patients who needed to have their doses increased due to advanced disease.[3] The first liposomal encapsulated anticancer medication to obtain clinical approval was doxorubicin hydrochloride (HCl) liposomal injection. It exhibits efficacy against various cancers such as solid tumours, lymphomas, and transplantable leukaemias. [4] The most common tumours that doxorubicin is used to treat include those of the bladder, breast, stomach, lung, ovaries, thyroid, multiple myeloma, soft tissue sarcoma, and Hodgkin's lymphoma. Adriamycin, cyclophosphamide (AC), Taxotere, AC, Adriamycin, bleomycin, vinblastine, dacarbazine, bleomycin, etoposide, AC, vincristine, procarbazine, and prednisone, as well as cyclophosphamide, Adriamycin, vincristine, prednisone, and 5-fluorouracil, AC, are some of the frequently used regimens containing doxorubicin. [5] Doxil is primarily used to treat AIDS-related Kaposi's sarcoma and ovarian cancer that has progressed or returned following platinum-based chemotherapy.[6]In recent decades solid lipid nanoparticles (SLN) has gained much importance in the field of medicine. Solid lipid nanoparticles combines the merits of colloidal drug carriers like liposomes, polymeric nanoparticles and emulsions but at the same time avoid or minimize their drawbacks. Many naturally occurring or synthetically prepared biocompatible, biodegradable polymers are used for the formulation of SLN. [7] The goal of this research is to use solid lipid nanoparticles (SLN) for enhanced treatment for lymphatic cancer. Anticancer medicines often present a number of promising challenges, including normal tissue toxicity, poor selectivity, poor stability, and a high rate of drug-resistant tumour cells [8, 9, 10]. By designing the anticancer drugs like SLN, which evade a number of absorption processes as the reticulo-endothelial system (RES), opsonization, and several efflux transporters, these issues can be resolved. [11] The intestinal lipase enzyme breaks down fatty substances into monoglycerides and triglycerides, which then reassemble inside enterocytes in the presence of bile to produce colloidal lipoproteins. These lipoproteins enter lymph vessels through lymph capillaries and are transported into the circulation by the inferior vein. [12, 13, 14]

Design of experiment (DOE), a statistical method of testing a large number of formulations and process factors in a minimum number of experiment runs, can be used to determine the link between variables affecting the formulation and response (output) of that formulation. The main effect, their interaction, the quadratic effects, and the response surface's form are frequently estimated using response surface models. [15] There is no embedded factorial or fractional factorial design in the Box-Behnken design, which is an independent quadratic design. [16] In this design, the treatment combinations are positioned in the centre and at the midpoints of the process space's edges. There are three layers of each factor needed for these rotational designs. When three elements are being explored, the Box-Behnken design requires a minimum of one experimental run, which gives it an advantage over the central composite design. [17, 18, 19]

MATERIALS AND METHOD

Materials: Doxorubicin was obtained from Cipla Pharmaceutical Pvt Ltd, Mumbai, Compritol888 ATO was obtained from Gattefosse Mumbai, India and Pluronic F68 was obtained from Research lab, Mumbai, India. Methanol and Acetonitrile were obtained from CDH Chemicals, New Delhi, India. Nylon 66 membrane filter was purchased from Himedia, New Delhi, India.

Design of experiments: In order to maximise the solid lipid nanoparticle formation utilising the software design expert, Box-Behnken DOE was built [20]. The treatment combinations in this design are located in the centre and at the midpoints of the process space's edges. Three levels of each factor are needed for these rotational or nearly rotatable devices. The model's quadratic equation is defined as:

$$Y= \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

Where Y is the measured response obtained from each factor level combinations; β0 is the intercept and β1 to β33 are the regression coefficient computed from the response Y; X1, X2, X3 are independent factors. The contour plot for particle size and response surface plot for particle size and entrapment efficiency are found out [21, 22, 23].

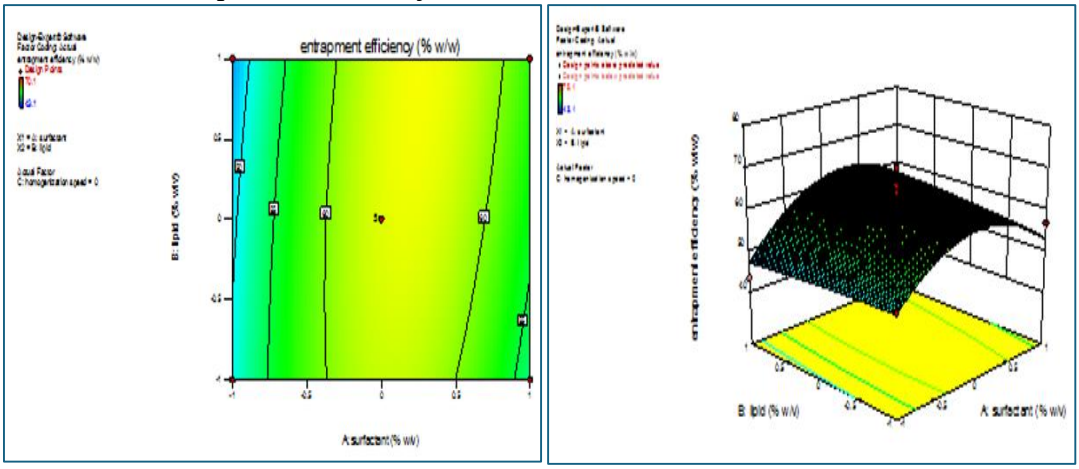

Fig. 1: Counter plot and response surface plot of entrapment efficiency

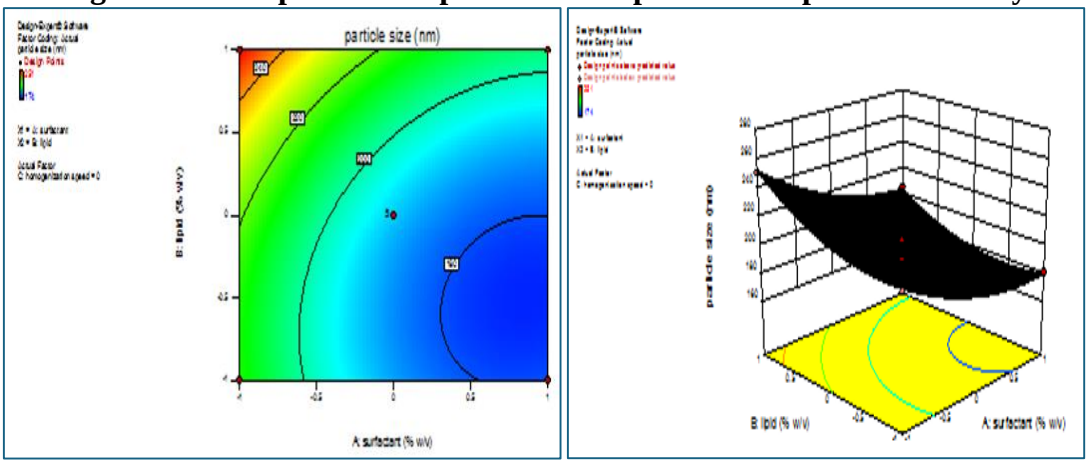

Fig. 2: Contour plot and response surface plot of particle size distribution

Preparation of solid lipid nanoparticles: Doxorubicin-loaded solid lipid nanoparticles were created via ultrasonography and heat homogenization. The appropriate amount of Compritol 888 ATO was precisely weighed, and it was melted at 75oC. Additionally, 100 mg of doxorubicin mesylate was precisely weighed and distributed throughout the lipid melt. After dissolving the necessary quantity of Pluronic F 68 in distilled water, the mixture was heated to 75ºC. After obtaining a clear homogenous phase, a hot aqueous phase was added to the

lipid melt and homogenised for two minutes at 10,000 rpm using a high-speed homogenizer. Throughout this homogenization time, the temperature was kept at 75ºC. The resulting hot primary emulsion was then ultrasonified for three minutes at 40% amplitude and 30/10 second pulse ON/OFF using a probe sonicator. To avoid recrystallization and precipitation during sonication, the temperature was adjusted to 4-5 degrees Celsius above the lipid's melting point (74 degrees Celsius). The resultant nanoemulsion was diluted to 100 millilitres by cooling it in an ice bath. After that, a stirred ultra filtration machine was used to filter the diluted dispersion. The filtering medium is a nylon 66 membrane with a pore size of 0.4µ; to aid in filtration, a positive nitrogen pressure of 3 Kg/cm2 was applied. After collecting the filtrate, it was once again filtered through a polycarbonate membrane filter with a pore size of 0.05µ. The residue on the membrane was then cleaned three times with distilled water and removed from the filter unit. The nanoparticulate suspension was subsequently put into glass vials and prefrozen to -80ºC. The samples that had been prefrozen were subsequently freeze dried for 24 hours at -80ºC using Subzero Lab Instruments, Chennai. Following collection, the freeze-dried nanoparticles were kept refrigerated. [23, 24, 25]

Morphological characterization: Scanning electron microscopy was used to observe the size and form of the particles. The freeze-dried nanoparticles were placed on a platinum ribbon that was held up by a disc. A platinum sputter module was used to coat the nanoparticles with platinum for five minutes at a current of 20 mA in a higher vacuum evaporator. After that, the particles were examined at various magnifications, and pictures were captured. [26]

Entrapment efficiency and drug loading: 50 mg of the freeze-dried nanoparticles were filtered through a 0.22µm membrane filter after being vortexed for an hour with 5 mL of distilled water.Next, using fake nanoparticles that had also been created as reagent blanks and treated similarly to the drug-loaded nanoparticles, the drug content in the filtrate was measured using an ultraviolet (UV) spectrophotometer set at 258 nm [26, 27, 28]. As a gauge of encapsulation effectiveness, the percent encapsulation was computed as the ratio of the drug content in the freeze-dried powder to the initial dosage administered.

Entrapment efficiency = practical drug loading / theoretical drug loading

In-vitro release study: Using the dialysis bag approach, the drug release from Doxorubicin loaded SLN was carried out in phosphate-buffer (PB) solution (Ph 6.8). A dialysis membrane featuring a 100kDa molecular weight cutoff was employed. Prior to use, the membrane was sealed in PB solution for 12 hours. A dialysis bag containing the dispersion was closed on both ends. The dialysis bag was put in a beaker with 100 mL of phosphate buffer (PB, pH 6.8) as the dissolving media at 37±2ºC, and it was magnetically agitated at 100 rpm. Samples were taken out at prearranged intervals, and the sink state was preserved by adding new, previously heated PB solution at the same temperature [30, 31]. Doxorubicin content in the samples was measured using a UV spectrophotometer (1700, Shimadzu, Japan) set to λmax 235 nm. The drug content was measured at λmax 235 nm and the drug release research of SLN was also carried out in 0.1N HCl.

Particle size and zeta potential: Zetasizer was used to examine the produced nanoparticles' zeta potential (ZP) and particle size distribution. Generally speaking, a ZP absolute value of more than 60 mV produces outstanding stability, but values of 30, 20, and less than 5 mV often produce good to acceptable short-term stability. However, because the large molecular weight polymer utilised in the nanoparticles performs steric stabilisation, the particles exhibit good stability in suspension but low zeta potential. [32, 33, 34]

Determination of doxorubicin in blood plasma: HPLC analysis was used to assess the doxorubicin content in lymph fluid and blood plasma. Doxorubicin stock solutions were made at a concentration of 1 mg/mL using HPLC-grade methanol and acetonitrile in a 40:60 ratio, then kept cold at 4ºC. In a 100µL drug-free pooled blood plasma and 20µL lymph fluid, calibration standards of rat blood plasma and lymph fluid were generated at concentrations of

10, 20, 30, 50, and 100µg/mL. The peak area versus doxorubicin concentration was plotted to create the calibration curves. One hour after the doxorubicin standard solution and designed SLN were administered, 100µL of lymph fluid was removed and dissolved in 200µL of HPLC-grade methanol to extract the drug and in 200µL of HPLC-grade acetonitrile to precipitate the sample's proteins. To settle down the precipitate, centrifuge the sample at 5000 rpm, and then collect the supernatant. After that, the mixture was run through a syringe filter with a pore size of 0.2µ, and HPLC analysis was performed. The above process was followed for HPLC analysis14, and lymph fluid was extracted at intervals of 1, 2, 4, and 6 hours. Rat blood samples were obtained at intervals of 0.5, 1.5, 3, 6, 9, and 24 hours in order to separate 200µL of plasma, which was used to calculate the oral bioavailability of doxorubicin. After centrifugation, supernatant was collected and filtered using 0.2µ syringe filter and was used for HPLC analysis. [35, 36]

Pharmacokinetic analysis and evaluation of lymphatic targeting efficiency: One compartmental model was used to estimate the pharmacokinetic parameters related to the oral administration of a medication at a dose of 50 mg/kg. The graph was used to determine the pharmacokinetic parameters, such as Cmax and Tmax, and the trapezoidal rule was used to compute the area under the whole blood concentration (AUC), extrapolating the time value to infinity. Additionally, the rate of drug transport from the standard solution and SLN formulation to the lymph fluid was compared in order to determine the targeting efficiency of doxorubicin to the lymphatic system. [37]

Accelerated stability studies: Using optimised solid lipid nanoparticles, the accelerated stability research was conducted in compliance with ICH (International Conference on Harmonisation) Q1A criteria. Doxorubicin-loaded sealed vials of freshly made solid lipid nanoparticles were kept in a stability chamber with a constant temperature of 25°C±2°C and a relative humidity of 50% RH±5% RH. Particle size and drug content of the nanoparticles that underwent stability testing were examined during a three-month period.[38, 39]

RESULTS AND DISCUSSION

Preparation of doxorubicin solid lipid nanoparticles: Pluronic F68, Compritol 888 ATO, and homogenization speed optimisation. The produced particles' particle size was assessed and the lipid content was adjusted between 1 and 5 percent of the aqueous phase. The formulation containing 1% Compritol 888 ATO was discarded due to phase separation that happened a few hours after formulation. The formulation with the 5% lipid content looked creamier and was thicker. When compared to the formulation's particle size at 3% w/v lipid, the particle size at 2% w/v lipid did not show statistical significance. In order to optimise other parameters, the lipid concentration (%w/v) of 2 was dropped after comparing the particle sizes of the formulations. Instead, 3% w/v of lipid was fixed arbitrarily. Since it controls the size and drug entrapment, optimising surfactant concentration is crucial. Particle size was monitored and the surfactant concentration was adjusted between 0.5 and 4% of aqueous solution during preoptimization studies. The formulation with 4% surfactant concentration produced a high and stable foam, and when the temperature was raised to 80ºC, pluronic F68 surfactant precipitated as a thin film. When the formulation was using 2% w/v surfactant, the particle size at 3% w/v surfactant was statistically significant ($P<0.05$). In order to optimise other parameters, the surfactant concentration (% w/v) of 3 was rejected after comparing the formulation's particle size. Instead, 2% w/v surfactant was fixed arbitrarily. 10000 rpm was chosen as the ideal homogenization speed. The emulsion began to cream at 8000 rpm, and the Pluronic F 68 surfactant separated from the system due to heat created in the system at 14000 rpm. The ideal duration for sonication was three minutes at 40% amplitude; any longer than this, and the temperature rose to 100ºC. The preoptimization study's variables and levels were applied to the Box-Behnken design. Particle size (Y1) and entrapment efficiency (Y2) are two examples of responses that were incorporated to the

design and produced response surface graphs and contour plots. The software provided methods for batch optimisation and identified the best batch based on statistical analyses.

Morphological characterization: The prepared doxorubicin-loaded SLN were seen under a scanning electron microscope (SEM) to have a smooth surface, spherical shape, and a size of around 200 nm. This finding demonstrated that the heat homogenization process was a successful means of preparing doxorubicin-loaded SLN with an optimum shape and size of about 200nm.

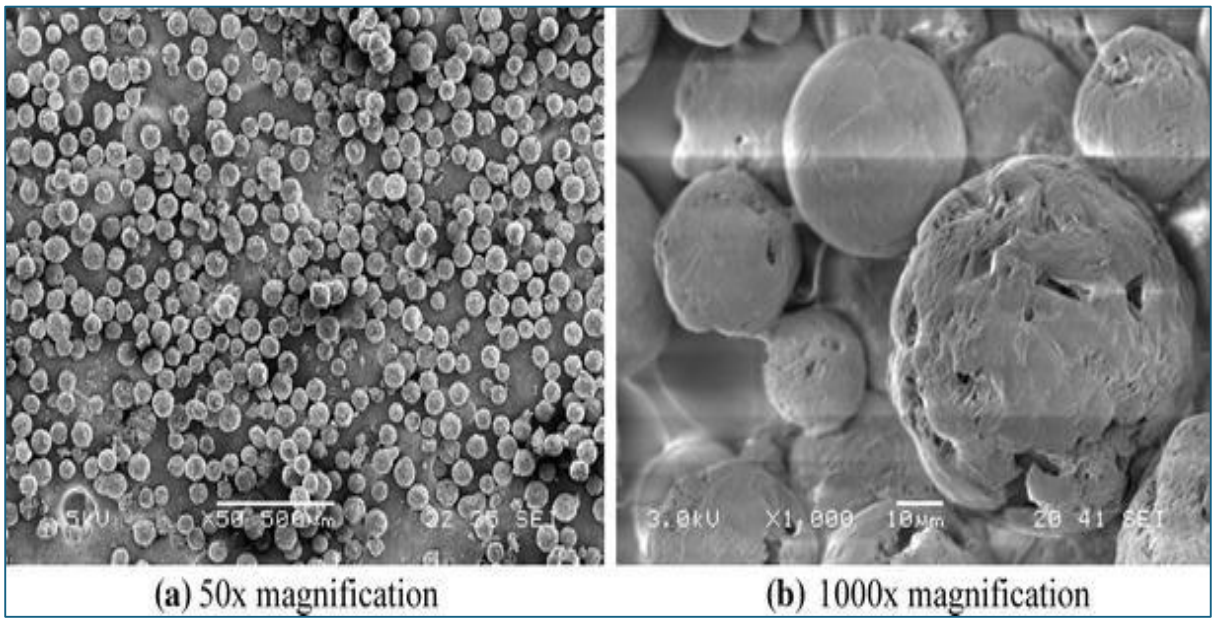

Fig. 3: SEM image of SLN

Zeta potential of doxorubicin loaded solid lipid nanoparticle: The zeta potential of the formulation was negative, which could be attributed to the negative nature of the polymer glyceryl behenate. The formulation's average zeta potential was determined to be -28.11±2.56 mV.

Invitro release studies: Results from an in-vitro release study indicate that the medicine's sustained release from the optimised formulation was achieved within 24 hours, with over 98% of the drug released. The results of the release kinetics investigations indicate a complex order drug release. The release exponent value, n, of the Peppas model (0.953), suggests that the drug release mechanism is supercase II transport, whereas the correlation coefficient (r^2 value=0.911) implies that the release mechanism is diffusion.

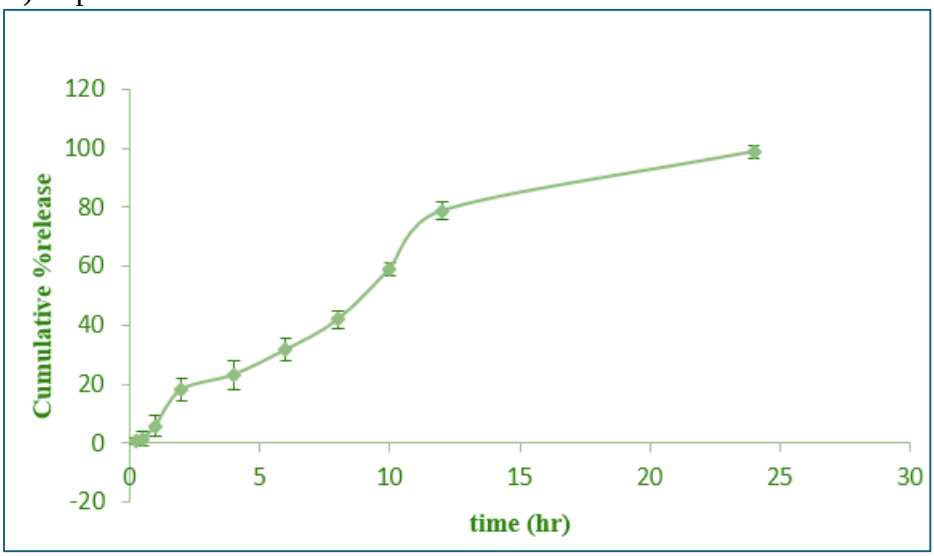

Fig. 4. Invitro drug release of SLN of DOX

Table 1:Drug concentration obtained for SLN and solution

Time	Drug Concentration in Lymph-SLN mcg/ml	Drug Concentration in Lymph-Solution mcg/ml	Average Drug Concentration from SLN in Blood Plasma	Average Drug Concentration from Solution in Blood Plasma
0.5	-	-	4.11	3.72
1	86.11	21.06	-	-
1.5	-	-	4.43	3.89
2	139.23	30.45	-	-
3	-	-	5.11	4.43
4	156.11	34.76	-	-
6	163.11	35.11	5.97	3.89
9	198.65	-	6.09	1.32

SLN, solid lipid nanoparticles, 5mg of Dox

The average drug concentration determined by HPLC analysis for plasma samples taken at intervals of 0.5, 1.5, 3, 6, 9, and 24 hours is displayed in table 1. the mean drug concentration measured in the plasma of rats after formulated SLN and standard drug solution were administered. The outcome showed that doxorubicin from SLN has better bioavailability when compared to traditional dose forms. Additionally, the frequency of drug dose is decreased by the continuous drug release from SLN. The medication concentration determined by HPLC analysis for lymph sample collections made between the times of 1, 2, 4, and 6 hours. the speed at which doxorubicin is transported between the normal drug solution and the prepared SLN. By preventing the unchecked growth of lymphocytes, the notable increase in doxorubicin transfer from the SLN to the lymphatic system will, in turn, result in an efficient treatment of lymphoma within the body. The outcome showed that the anticancer medication doxorubicin formulated as SLN offers a notable benefit over traditional dose form in the treatment of acute lymphoblastic leukemia/lymphoma.

Pharmacokinetic analysis and evaluation of lymphatic targeting efficiency: The outcome demonstrated that doxorubicin's bioavailability from SLN is 1.9 times higher than that of its conventional solution, pointing to SLN's increased AUC. The student's t test was used to statistically analyse the data (AUC0–24), and the results indicated a significant difference ($P<0.001$) between the two formulations. It was discovered that the percentage dose of doxorubicin transferred by the lymphatic system from SLN and standard solution was 0.06% and 0.28%, respectively. This suggests that, in comparison to the usual solution, the doxorubicin-loaded SLN demonstrated a 4.6-fold increase in doxorubicin transfer to the lymphatic system. A substantial difference ($P<0.001$) was found between the two formulations after the data was statistically analysed using the student's t test.

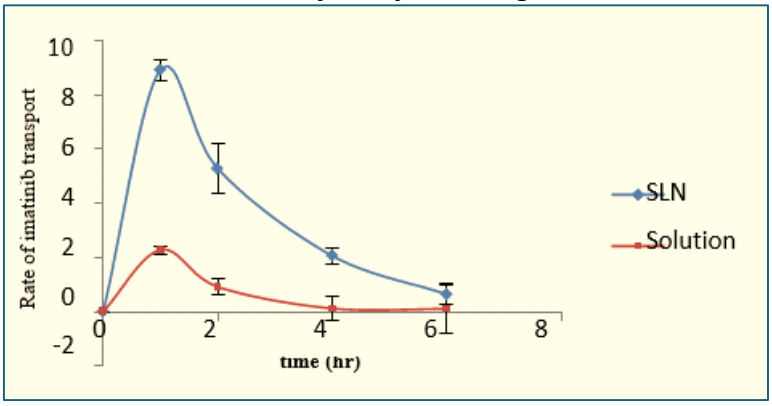

Fig.5:The rate at which oral administration of compounded SLN and standard drug solution causes doxorubicin to be transported through intestinal lymph fluid

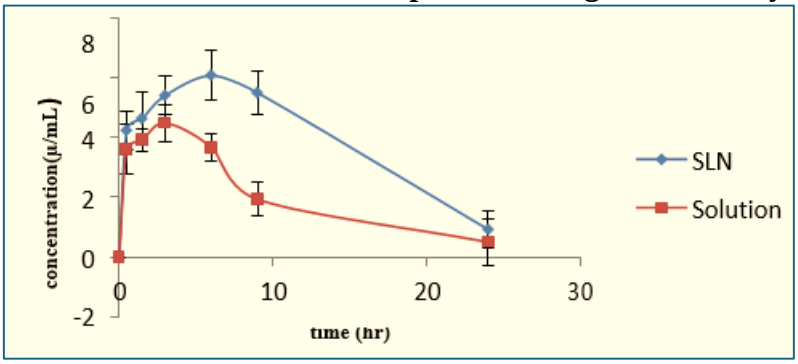

Fig.6:Blood plasma was used to measure the average drug concentration following oral administration of designed SLN and standard drug solution.

Accelerated stability studies: The lyophilized SLN's particle size dramatically increased from 192.7 to 199.5 nm after three months of storage. The particles can still be targeted to the lymphatic system, though, because their size is smaller than 250 nm, which is the maximum size at which a particle can be effectively targeted to the lymphatic system. Even after three months of storage, the drug content of the nanoparticles did not significantly decrease.

CONCLUSION

The findings demonstrated that SLN increased doxorubicin's lymphatic transport by 4.6 times and its bioavailability by 1.9 fold when compared to doxorubicin solution. Because of their physical and chemical properties, such as particle size and surface coating with biodegradable polymers, the majority of NP typically accumulates at the target lymph location. The medicine can be targeted to the lymphatic system because, as was previously indicated, SLN is easily absorbed by the lymphatic system during systemic circulation. We may infer from this work that doxorubicin-loaded SLN are viable options for doxorubicin lymphatic delivery systems.

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