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Research Article

Formulation and Evaluation of PEGylated Lipid Coated Niosomes of 5-Fluorouracil for Parenteral Drug Delivery

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ABSTRACT

Niosomes have been extensively investigated for drug delivery, drug targeting, controlled release and enhancing solubility. The aim of present investigation was to develop and characterize the 5-flurouracil niosomal drug delivery system that will-protect from body immune system (stealth niosomes), prolong drug release. The niosomes were prepared by ether injection method using span 60 as a nonionic surfactant, niosomal dispersion was bath sonicated for different period of time to reduce particle size at or above the phase transition temperature of surfactant. The preformed niosomes were incubated with PEGylated lipid (5mol %) for 30 minuites. The un-entrapped drug was separated by centrifugation. It was observed that with increase in total molar concentration of surfactant and cholesterol, % entrapment efficiency increased. To formulate formulations total concentration of lipid 300µ moles was selected. It was observed that increase in sonication time reduces the particle size of the niosomes. After 15 minutes of sonication average particle size was found to be 244nm. PEGylated lipid coated niosomes were found larger than the conventional ones. In 6-hrs in vitro drug release study PEGylated lipid coated niosomes showed controlled release of 5- FU. Stability study indicates that the PEGylated niosomes showed more drug retention stability than conventional niosomes.

INTRODUCTION

The quest never ends. From the very beginning of the human race; the quest is going on for newer and better alternatives, and in case of drugs it will continue; continue till we find a drug with maximum efficacy and no side effects. Many drugs, particularly chemotherapeutic agents, have narrow therapeutic window, and their clinical use is limited and compromised by dose limiting toxic effect. Thus, the therapeutic effectiveness of the existing drugs is improved by formulating them in an advantageous way. In the past few decades, considerable attention has been focused on the development of new drug delivery system (NDDS).

Niosomes are non-phospholipids vesicular alternatives to liposomes. They are non-ionic surfactant vesicles or surfactant membrane vesicles.

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Handjani-Vila et.al. (1979) were first to report vesicular system on hydration of cholesterol and single alkyl chain non-ionic surfactants. Niosomes are formed by self -assembly of non-ionic amphiphiles with aqueous media resulting in closed bilayer structures. This assembly requires some input of energy in the form of heat or physical agitation –thus it is rarely spontaneous. The result is an assembly wherein hydrophobic parts are shielded from aqueous solvent and hydrophilic head groups enjoy maximum contact with the same[1]. Higher chemical stability of surfactants than phospholipids are used in preparation of Liposomes.

Niosomes behave in-vivo like liposomes, prolonging the circulation of entrapped drug and altering its organ distribution and metabolic stability[2]. Encapsulation of various anti neoplastic agents in these carrier vesicles has been shown to decrease drug induced toxic side effects, while maintaining, or in some instances, increasing the anti-tumor efficacy[3], such vesicular drug carrier systems alter the plasma

clearance kinetics, tissue distribution, metabolism and cellular interaction of the drug[2,4]. They can be expected to target the drug to its desired site of action and/or to control its release[5]. The delivery of drugs to tumors remains a critical problem in the treatment of cancer. Achieving therapeutic drug levels within a tumor is impeded by transport barriers due to the complex physiology and morphology of the tumor[6]. Furthermore, chemo-and radio therapeutic agents are typically toxic to healthy cells in addition to tumor cells, and undesirable side effects are common in anticancer therapy[7]. In an attempt to overcome these problems, different types of drug delivery systems have been developed that use macromolecules, vesicles, or particles as carriers for therapeutics. Treatment schedule and therapy period for anticancer treatment is also very long making the treatment a costly affair Short elimination half life (9 min) and skin toxicity remains a critical problem with a anticancer agent like 5-flurouracil. Niosomal drug delivery system was chosen[8]. Intravenously administration of Vesicles (niosomes) targets the tumors but vesicles are taken up by the cells of mononuclear phagocytes system in dozens of minutes. Even faster they can loose drug cargo. It was also proposed that rapid clearance of vesicles is due to uptake by the cells of immune system and disintegration upon interaction with plasma lipoproteins.

The objectives of present investigation were to develop and characterize the 5-flurouracil niosomal drug delivery system that will-protect from body immune system (stealth niosomes) and also prolong drug release.

MATERIALS AND METHODS

5- Fluorouracil was grerously donated by Biochem Laboratories, Pvt. Ltd. Mumbai. [N-(carbonyl-methoxypolyethylene lycol-2000)-1, 2-distearoyl-sn-glycero-3-phosphoethanolamine, Sodium Salt], (MPEG 2000–DSPE, Sodium Salt) produced from 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine gift sample from Lipoid Gmbh, Germeny, Span 60 (Sorbitan mono-octadecanoate), cholesterol from Loba Chemie, Mumbai. All other chemicals used were of analytical grade obtained from Loba Chemicals Pvt. Ltd. Mumbai.

Preparation of Niosomes

Niosomes were prepared by Ether Injection Method (EIM). Surfactant (span 60) cholesterol (300 µ mol) (dissolved in 20 ml of diethyl ether were injected slowly (0.25 ml/min.) into 5 ml of aqueous solution (PBS 7.4) by using syringe infusion pump containing the drug 10mg/ml. The aqueous phase was stirred on hot plate magnetic stirrer (Remi India). The temperature of aqueous phase was maintained at 650 throughout the experiment to evaporate the diethyl ether. The niosomal dispersion was bath sonicated for different period of time to reduce particle size at or above the phase transition temperature of surfactant. The preformed niosomal dispersion was incubated with PEGylated lipid (5mol %) for 30 minutes[9]. The un-entrapped drug was separated by centrifugation (Eltek) at 10, 000 rpm for 30 minutes. The pellets so obtained were re-dispersed with aqueous phase i.e. PBS 7.4 (5 ml) at 60-65 0 C for 30 minutes. All batches are shown in Table 1.

Characterization of Niosomes

Entrapment efficiency (EE)

It was determined indirectly i.e. by calculating the amount of un-entrapped drug. This was found out by centrifugation of the niosomal dispersion at 10000 r.p.m for 30 minutes and evaluating the supernatant spectrophoto-metrically at 266 nm with suitable dilution and filtration.

$$\% \text{ EE} = \frac{\text{Drug}_{\text{initial}} - \text{Drug}_{\text{supernatant}}}{\text{Drug}_{\text{initial}}} \times 100$$

Effect of sonication time and PEGylated lipids were studied which affect on entrapment efficiency.

Particle Size determination

It was determined with the help of particle size analyzer (Malvern, Mastersizer 2000, U.K) by diluting a drop of the dispersion with phosphate buffer pH 7.4. Effect of sonication time and coating PEGylated lipids were studied which affect particle size.

In vitro drug release

Drug release testing is a fundamental part of drug product development and manufacturing and is also employed as a quality control tool to monitor batch-to-batch consistency of the drug release from niosomes. 5-FU release from niosomes was determined using the dialysis-bag (HiMedia Laboratories Pvt. Ltd. Mumbai) method The membrane is permeable to the solvent and

to the low molecular weight drug, such as 5-Fluorouracil but impermeable to the niosomes.

Four milliliters of suspension was placed in a dialysis bag and suspended in 400 ml of PBS, which was constantly stirred at room temperature on magnetic stirrer. Aliquots (4ml) were withdrawn at hourly intervals and replenished simultaneously with equal volume of fresh PBS. The 5-FU concentration in the samples was analyzed spectrophotometrically at 266nm.
In a control experiment, it was found that in the absence of 5-Flurouracil, no absorbance at 266 nm was measured in solution, which indicated that the niosomes do no interfere with the measurement of 5-flurouracil concentration in the release medium.

Stability study
The one uncoated and PEGylated coated batch was selected for stability study which were dispersed in PBS 7.4. Stability studies with respect to percent drug entrapment at different temperature at conditions i.e. 40 ^{0}C and 37 ^{0}C. the percent drug retained after every 2, 4, 6 weeks at both temperatures was determined.

RESULTS AND DISCUSSION

Optimization of total molar concentration of lipids
Literature review suggests that equimolar ratio of surfactants and cholesterol yields stable niosomes[1,10]. Different ratios of surfactant and cholesterol were used starting from 75:75 respectively to 250:250. Hence optimization was carried out starting from lowest ratios. i.e. 25:25 Consider batches N1 to N8. It was observed that with increase in total molar concentration of surfactant and cholesterol, % entrapment efficiency increased.

When total molar concentration was 50 µ moles (batch N1), % Entrapment efficiency was 4.04 ± 4.84 which increased to with total molar concentration of 300 µ moles (Batch N6) (Fig.1). To formulate further formulations total concentration of lipid 300µ moles was selected.

Encapsulation efficiency
It was observed that increase in bath sonication time from 0 to 15 min. decreases the encapsulation efficiency from 21.50 ± 4.394 % to 15.12 ±1.234% in case of without coating of lipid. This is due to the decrease in particle size of the niosomes there was no any effect of coating of PEGylated lipid on encapsulation efficiency of niosomes. (Table 2)

Particle size
It was determined with the help of particle size analyzer (Malvern, Mastersizer 2000 UK) by diluting a drop of the dispersion with phosphate buffer pH 7.4. The sonication required strict control of experimental conditions, such as the time of sonication, temperature, shape of the vessel used, volume of solution, sonication power, and positioning of the vessel holding the lipid dispersion in the bath in order to obtain reproducible vesicles of the required diameter. It was observed that increase in sonication time reduces the particle size of the niosomes. After 15 minutes of sonication average particle size was found to be 244nm.

When PEGylated liposomes are in the suspension, the PEG chains adopt a random coil conformation at the lipid-water interface. This might be a case with niosomes. The average particle sizes are shown in Fig. 2. There is a clear trend in the present study that the PEGylated niosomes are larger than the conventional ones. This is considered to be due to the additional steric size that is introduced by the PEG chains, which pass through the membrane and then relax around niosomes.

***In vitro* drug release profile**

Drug release from uncoated and PEG2000-DSPE coated niosomes
It was observed that decrease in particle size increases the drug release further increasing the surface area of niosomes which lead to increased drug release, because the increased total surface area exposed to the release medium increased the amount of 5-fu diffusion from niosomes. In 6-hrs in vitro drug release study the uncoated batches showed 37.16, 39.12, and 45.36 % from batches S1, S2, S3 respectively. The PEGylated lipid coated batches showed 23.38, 26.36, and 30.26 % from the batches S4, S5, S6 (Fig. 3). PEGylated lipid coated niosomes showed controlled release of 5- FU.

The drug release data have been analyzed with mathematical models, such as the Higuchi model and the Korsmeyer-Peppas (K-P) equation. Nowadays, one of the most often used mathematical equations to describe the release rate of drugs from drug delivery systems is the Higuchi model. The basic equation of the Higuchi model is

Mt/ A = [D (2c0- cs) c st] 0.5 for c 0> c s------ (1)

Table 1: Formulation design of niosomes

Niosomal batch code	Span 60:Cholesterol (µ molar ratio)	Bath sonication time (Minutes)	Post coating of MPEG 2000–DSPE, Sodium Salt (5 mol %)
S1	150:150	0	-
S2	150:150	3	-
S3	150:150	5	-
S4	150:150	0	+
S5	150:150	3	+
S6	150:150	5	+

- : Indicates non presence of coating of MPEG 2000–DSPE; + : Indicates presence of coating of MPEG 2000–DSPE

Table 2: Effect of bath sonication time and coating of PEGylated lipid on encapsulation efficiency

Niosomal batch code	Span 60:Cholesterol (µ molar ratio)	Bath sonication time (Minutes)	Post coating of MPEG 2000–DSPE, Sodium Salt (5 mol %)	% Encapsulation efficiency (Mean ± S.D)
S1	150:150	0	-	21.50 ± 4.394
S2	150:150	3	-	20 ± 2.133
S3	150:150	15	-	15.12 ±1.234
S4	150:150	0	+	21.00 ± 3.12
S5	150:150	3	+	20.10±2.133
S6	150:150	15	+	14.99±4.112

where A is the surface area of the release device exposed to the release medium, D is the drug diffusivity in the polymer carrier, c 0is the initial drug concentration in the carrier and cs the solubility of the drug in the carrier. The Higuchi law was originally aimed at and applied to dissolution and growth from solid particles. Peppas pointed out that Equation 2 assumed a pseudo-steady state, which could not often be applied to "real" controlled release systems. However, the Higuchi law was extended to consider different geometries and matrix characteristics as well as the case of diffusion controlled drug release from colloid systems, such as small oil droplets in oil in water emulsions and liposomes. The following equation organizes the cases of the drug release from a dosage form, and the cumulative drug amount, M, released at time t is given by

$$\frac{M_t}{M_0} = kt^n \quad \text{Up to} \quad \frac{M_t}{M_0} \leq 0.7 \qquad \ldots\ldots (2)$$

Where M_0 is the initial amount of drug in the drug delivery device (in this case, niosomes) at time t = 0, k is the release constant and n is the release exponent, indicative of the drug release mechanism. If n = 1, the release is zero order. If n is between 0.5 and 1, the release follows an anomalous transport (Non-Fickian) mechanism, which is controlled by factors other than diffusion, including the drug encapsulation matrix and liposome membrane structure. If n = 0.5, the drug release is controlled by Fickian diffusion and the drug release can be modeled using Higuchi's law. All the factors influencing the relationships between Mt and t. A, D, c0 and c, are represented by the release rate constant k. In the measurement of 5-FU release using the dialysis bag method, the drug molecule entrapped in the niosomes goes through 4 steps before it is measured in the release medium, shown in Fig. 4.

(1) 5-FU molecule diffuses to the inside surface of the niosomes
(2) 5-FU passes through the lipid bilayer and/ or PEG layer, a barrier for the release;
(3) 5-FU diffuses from the outside surface of the niosome to the inside surface of the dialysis bag;
(4) The dialysis membrane may also act as a barrier for 5-FU to pass through.
The most important rate limited step in the drug release mechanism is step (2), i.e. 5-FU release from niosome by membrane transport.

Table 3: Coefficient of correlation of all batches

Batch code	Equation	R^2
S1	y = 15.13505x + 0.878581	0.98
S2	Y = 16.41718x + 0.978655	0.98
S3	y = 18.67087 x + 2.601131	0.98
S4	y = 10.75341x - 2.82633	0.93
S5	y = 12.08316x - 2.88842	0.93
S6	y = 13.12284x - 1.97399	0.98

Table 4: Parameters in the K-P equation (2) for 5-FU release kinetics from PEGylated Niosomes containing PEG2000-DSPE

Batch code	n	Release mechanism	K $(hr)^{-n}$	R^2
S4	1.1	-	1.38	0.92
S5	1.1	-	1.63	0.93
S6	0.73	Non-Fickian diffusion	2.16	0.98

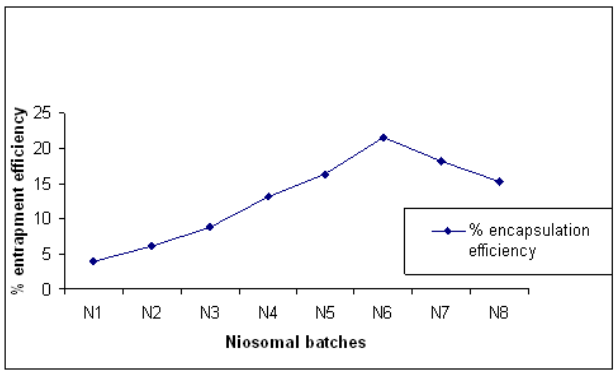


Figure 1: Effect of total molar concentration of surfactant: cholesterol on entrapment efficiency

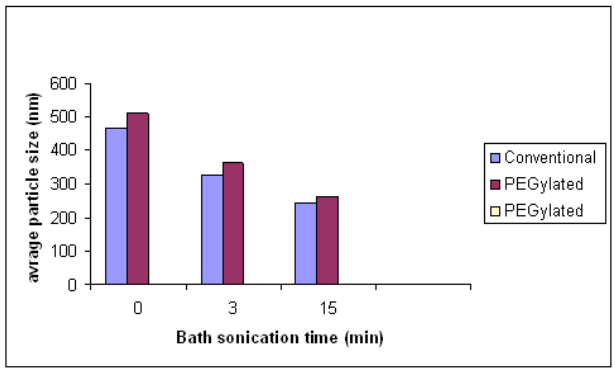
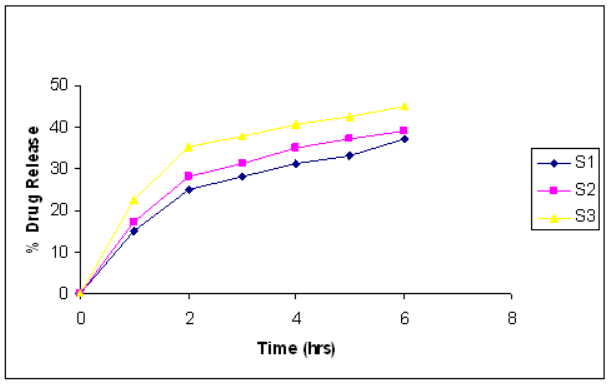


Figure 2: Effect of PEGylated lipid coating on particle size



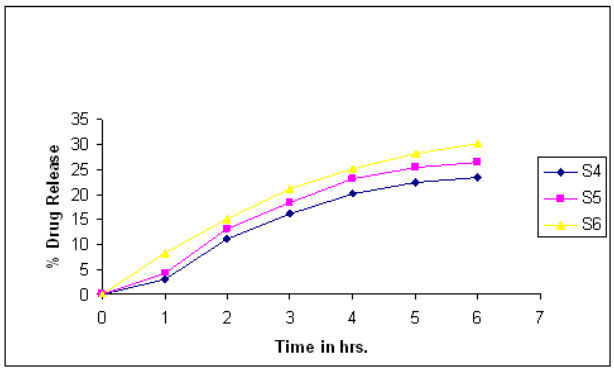
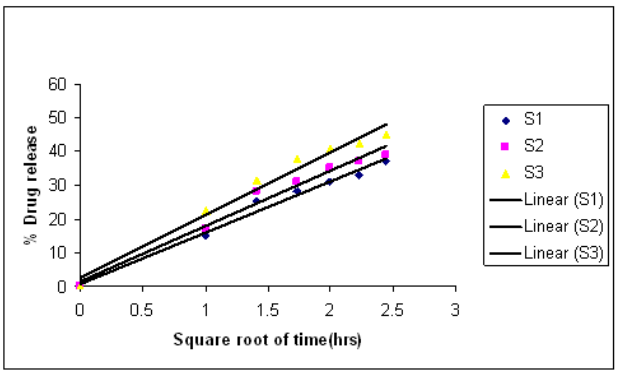


Figure 3: Drug release profile from uncoated and PEG2000-DSPE coated niosomes



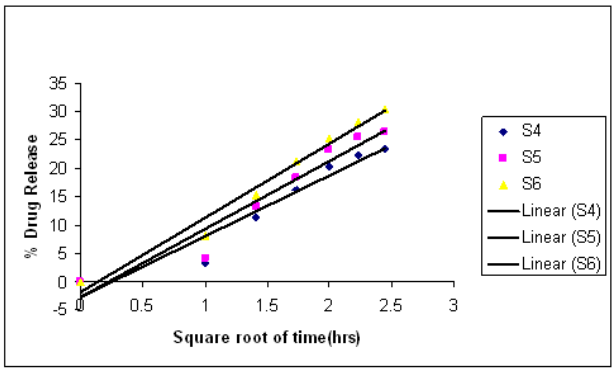


Figure 5: Application of Higuchi model to all batches

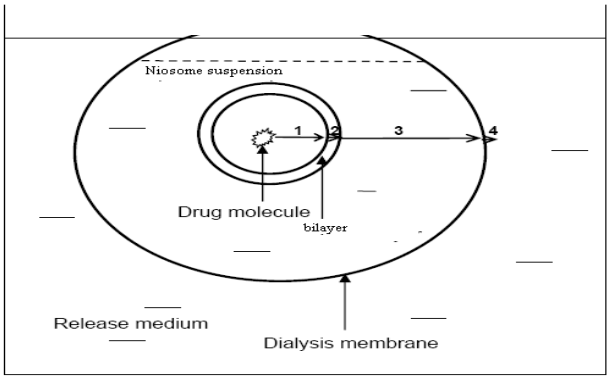


Figure 4: Steps for the release of drug molecule from niosomes to the release medium.

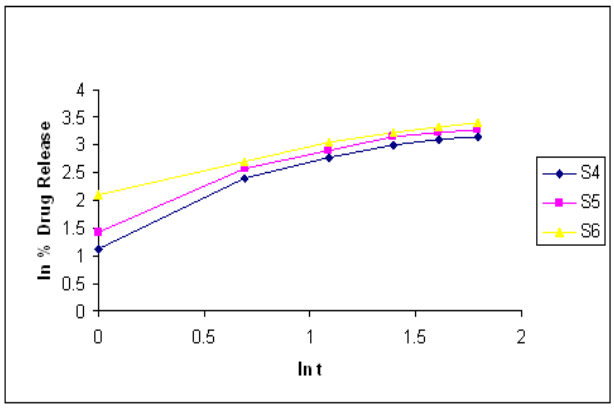


Figure 6: K-P equation fits for 5-FU release (ln % Drug release versus ln t) from niosomes containing 5mol% PEG 2000-DSPE.

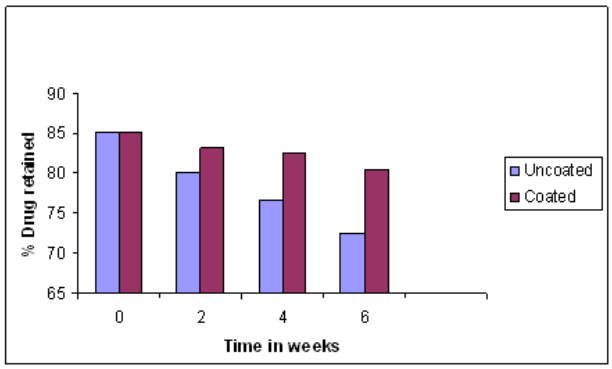


Figure 7: Stability study of coated and uncoated niosomes at 40 ^{0}C

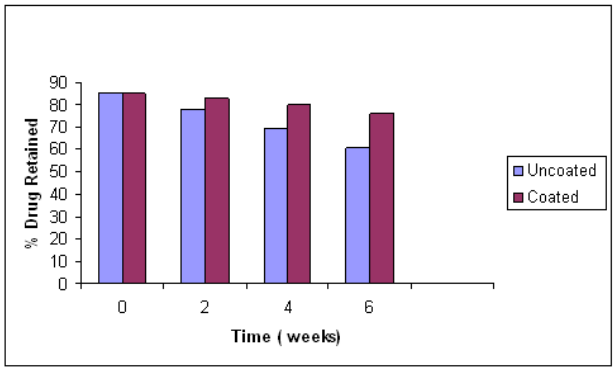


Figure 8: Stability study of coated and uncoated niosomes at 37 ^{0}C

Application of Higuchi model

The graph of the %Drug released vs. square root of the time plotted is as shown in Fig. 5. All batches showed regression greater than 0.93. From Table 3 we can conclude that 5-FU released from the niosomes by diffusion mechanism.

Influence of PEG incorporation on the release mechanism

The 5-FU release percentage (P% = (Mt/ M∞) x 100%) from niosomes containing PEG-lipid showed that good correlation was obtained between experimental data and the K-P equation (Eq 2) for the batch S6 , as shown in (Fig 6), with R2 values greater than 0.90. The release rate constant, k, and the release exponent, n, were obtained from the intercept and slope, respectively, of the plot of ln (Mt/ M∞) (i.e. ln P %) versus ln t in Fig. 6. The values of k and n are tabulated in (Table 4).

The batch S6 follows the non-fickian diffusion controlled release. It was observed that k increases along with decrease in particle size. Increasing the surface area of niosomes led to increased k values because the increased total surface area exposed to the release medium increased the amount of 5-FU diffusion from niosomes. An equivalent trend was observed for the release of a fluorescent hydrophilic probe from lecithin liposomes prepared by extrusion[10].

Stability study

The temperature of storage of these niosomal dispersions must be controlled as a change in temperature f the system often leads to a change in the fundamental nature of the system or an increase in the release of the encapsulated drug[11].

Since batch S3 and S6 were prepared carefully with identical processing parameters and stability studies were conducted as given earlier. These two batches were selected for stability study because submicron size suitable for parenteral drug delivery.

Study at 40 ^{0}C

Fig. 7 indicates that PEG coating enhances the drug retention stability of niosomes. After 6

weeks uncoated niosomes showed 72.45% of drug retention and PEGylated niosomes showed 80.45% drug retention

Stability at 35-37 ^{0}C

Fig. 8 indicates that PEG coating enhances the drug retention stability of niosomes. After 6 weeks uncoated niosomes showed 60.67% of drug retention and PEGylated niosomes showed 75.96 % drug retention

CONCLUSION

Both conventional niosomes containing span 60 and cholesterol, and PEGylated niosomes containing PEG2000 DSPE, as drug delivery carriers, were investigated upon preparation, characterized for encapsulation efficiency, particle size , in vitro drug release mechanism and stability studies. Niosomes prepared by using ether injection method showed low entrapment efficiency due to hydrophilic nature of the drug molecule. The entrapment efficiency further reduced due to the sonication of the vesicles due to decrease in particle size or increase in surface area. After 15 minutes sonication submicron sized vesicles formed will be useful for the parenteral drug delivery system. As little as 5 mol% PEG-lipid incorporation significantly reduced the release kinetics. In addition, the drug release mechanism changed from diffusion controlled to interfacial controlled when the molar ratio of PEG into niosomes was 5 mol%, in which the 5-FU release followed a t0.7 law. The interfacial structure introduced by increasing PEG coverage also increased the activation energy for 5-FU transport across the lipid bilayer. PEGylated niosomes showed controlled release, physical stability, chemical stability which leads to the biological stability because all are interrelated. PEGylated niosomes will enhance the elimination half life of the 5-Fu, targeting to the tumors and will overcome the obstacles in cancer therapeutics.

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