ORIGINAL ARTICLE

Combination Therapy of Lamivudine and Zidovudine using Sterically Stabilized Liposomes: Development and Characterization

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Abstract

Aim: Lamivudine and zidovudine are nucleoside reverse-transcriptase inhibitors with activity against human immunodeficiency virus type 1 (HIV-1). However, only temporary and limited benefits are observed in HIV-infected patients treated with a single drug or in combination. The limited ability of these agents to decrease viral burden, the rapid development of resistance and toxic side effects have limited their long-term efficacy. Hence, in the present work, an attempt is being made to provide for stable drug delivery system with an improved therapeutic index for both the drugs in the form of stealth liposomes. **Methodology:** Various liposome batches were prepared by thin film hydration technique and were characterized for preformulation and post formulation parameters such as compatibility, particle size, zeta potential, percentage entrapment, surface morphology, *in vitro* drug release profile, and stability using specified methods. **Results:** Fourier transform infrared study indicated that there is no significant chemical interaction between the components. Transmission electron microscopy photograph confirmed that vesicles were homogeneous and spherical in shape. Percentage drug entrapment of stealth liposomes was found to be within the range of 56.21-71.92%. *In vitro* dissolution was carried out for 24 h and the percentage drug release for all the formulations was in the range of 79.29% and 94.23%. Stability studies showed that the liposomes were stable in −20°C and refrigerated 4°C for 1 month without significant changes in drug entrapment. **Conclusion:** The present study has given us a fair understanding that liposomes with a low amount of cholesterol and long alkyl chain length phosphatidylcholines are a better candidate for stealth liposomes encapsulated with a combination of antiretroviral drugs.

Key words: Cholesterol, lamivudine, phospholipids, stealth liposomes, zidovudine

INTRODUCTION

Since 2000, around 38.1 million people became infected with human immunodeficiency virus (HIV) and 25.3 million people died of AIDS-related illnesses. According to the data published by UNAIDS in 2014, nearly 36.9 million people are living with HIV out of which 15.8 million people are accessing antiretroviral therapy.[1] Despite over 30 years of clinical research, HIV remains incurable and until now a preventive vaccine is not available. While recent advances may eventually lead to vaccine therapy for HIV, current therapies depend on effective antiretroviral drug delivery. The development of combination therapy of antiviral drugs has reduced HIV morbidity and mortality but not significantly enough to provide hope for the complete eradication of the disease from the globe. A number of recent clinical trials provide hope that additional preventive measures may significantly reduce the rate of new HIV infections.[2]

Sustained treatment of HIV infection requires using a combination of antiretroviral agents acting on different stages of viral replication. However, antiretroviral therapy

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has several notable disadvantages such as multiple drug-drug interactions, additive toxicity from the combination therapy leading to poor adherence, and the potential to develop multidrug resistance. Several antiretroviral drugs have poor aqueous solubility and permeability properties. These drugs are also substrates for efflux transporter systems (i.e., P-glycoprotein), which exist in cells of the gastrointestinal tract, lymphocytes, and brain capillaries. This leads to poor gastrointestinal bioavailability and reduced drug concentrations in anatomical sites where HIV replicates. The development of combination therapy that provides a sustained release of antiretroviral drugs at these sites may improve long-term treatment success rates and retard the development of drug-resistant viruses.[3]

Among all carriers utilized for target-oriented drug delivery, vesicular drug delivery system in the form of liposomes is most extensively investigated. Liposomes are gaining popularity because of their inert biological nature, freedom from antigenic, pyrogenic or allergic reactions and similarity of primary components of liposomes with the natural membrane.[4] Liposome is a spherical vesicle with a membrane composed of a phospholipids and cholesterol bilayer used to deliver drugs. The main disadvantage of the liposome is following intravenous administration they are rapidly cleared from the circulation by the reticuloendothelial system (RES). To overcome this sterically stabilized liposomes called stealth liposomes have been developed. To make the drug-loaded liposomes, escape from RES-mediated clearance, which routinely removes foreign material from the blood, the surface of the liposome is coated with a protective compound, polyethylene glycol (PEG).[5]

Lamivudine (3TC) and zidovudine (AZT) are nucleoside reverse-transcriptase inhibitors commonly used in the treatment of AIDS in HIV-infected patients. However, only temporary and limited benefits are observed in HIV-infected patients treated with conventional therapy of a single drug or in combination. The limited ability of these agents to reduce viral burden, the rapid development of resistance and toxic side effects have limited their long-term efficacy. One major problem associated with the administration of these drugs to patients is their poor ability to penetrate and target infected cells. Rapid drug clearance and systemic toxicity like bone marrow depression are also some of the major drawbacks, which limit the use of these drugs for antiretroviral therapy.[6]

Therefore, the purpose of the present study was to formulate combination therapy of lamivudine and zidovudine into PEGylated liposomes to improve their entrapment, increase their residence time in systemic circulation, and reduce the RES uptake.

MATERIALS AND METHODS

Lamivudine was obtained as a gift sample from CIPLA India Ltd., Zidovudine was obtained as a gift sample from Strides Arco Labs Bangalore, soy phosphatidyl choline (SPC), dipalmitoyl phosphatidyl choline (DPPC), distearoyl phosphatidyl choline (DSPC), PE 18:0/18:0-PEG 2000 (PE-PEG) were kindly donated by lipoid, Germany; cholesterol was purchased from Sigma-Aldrich; and all other solvents and chemicals used were of analytical grade and purchased from commercial sources.

Preparation of stealth liposomes

Lamivudine and zidovudine-loaded stealth liposomes were prepared by thin film hydration technique [Table 1]. Constant amount of drug and phospholipids with variable concentration of cholesterol was dissolved in 10 ml solvent system of chloroform and methanol mixture (3:1, v/v) in a 250 ml round bottom flask. The organic solvent system was removed using a rotary evaporator (HS 3001 NS) under reduced pressure to obtain a thin film on the wall of the flask. During the process, the conditions such as speed (150 rpm) and temperature (55°±2°C) were maintained constant. The flask was removed and left overnight in a desiccator under reduced pressure to remove the solvent residuals completely. Then, the lipid film was hydrated using phosphate buffer saline pH 7.4 at 60±2°C. The resultant suspension was vortexed for about 2 min and allowed to stand for 2-3 h to allow complete swelling of the lipid film. Then, the suspension was sonicated using probe sonicator for about 5 min and extruded through a polycarbonate membrane of 0.2 µm aperture size.[7-9]

Characterization of drug-loaded stealth liposomes

Fourier transform infrared (FTIR) study

All the excipients such as SPC, DPPC, DSPC, PE -PEG, cholesterol, pure drugs (3TC and AZT) individually, physical mixture of drugs, and excipients were mixed separately with infrared (IR) grade KBr in the ratio of 1:100 and corresponding pellets were prepared by applying 15000 lb of pressure in a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4000-400/cm in Magna-IR 750 Series II (Nicolet, USA) FTIR instrument.[10]

Transmission electron microscopy (TEM)

A drop of the sample was placed onto a carbon-coated grid and allowed to dry to a thin film. Before drying of this film on the grid, it was negatively stained with 1% phosphotungstic acid. For this, a drop of staining solution was pipetted onto the film, and the excess drained off with filter paper. The grid was allowed to air dry thoroughly and then examined using a TEM with an accelerating voltage of 80 kV.[11]

Vesicle size and size distribution (polydispersity index [PDI])

Size analysis was done on Malvern instrument v 2.0 (Nano ZS). The average vesicle size and size distribution are important parameters because they influence the

physicochemical properties and biological fate of the liposomes after administration. The mean diameter and size distribution were determined using a particle size analyzer (Zetamaster, Malvern Instruments Ltd., Sparing Lane South, Worchester Shine, England). The size distribution of liposome was expressed as the PDI. Samples analysis was done by diluting 1 ml of liposome dispersion up to 10 ml with double distilled water filtered with 0.1 μm filter; further samples were placed in cuvette and analyzed.[12]

Zeta potential

Zeta potential of the formulation was determined using Zetasizer (Nano ZS, Malvern). 1 ml of liposome suspension was diluted up to 100 ml using deionized water, and the sample was placed in clear zeta cells, and results were recorded. Before putting the fresh sample, cuvettes were washed with the deionized water and rinsed using the sample to be measured before each experiment.[13,14]

Percentage drug entrapment

The percentage drug entrapped was determined by centrifugation. Liposomal suspension of 10 ml was placed in the centrifugal tube and it was balanced on the other side with an equivalent weight. The centrifugation was carried out at 4500 rpm for 10 min. The supernatant was removed, and the concentration in the supernatant was determined spectrophotometrically at 271 nm and 265 nm, respectively, for both the drugs. The percentage of drug entrapment in liposomes was calculated using the equation.

Percentage drug entrapment = (Total drug − Drug in supernatant)/total drug × 100

Percent drug entrapment was confirmed by lysing the liposomes with n-propanol after centrifugation and measuring absorbance at 271 nm and 265 nm.[15-17]

Drug release kinetics

Dialysis bags (dialysis membrane 110, Hi Media, India) were immersed in distilled water for 1 h to remove preservatives followed by rinsing in phosphate-buffered saline (PBS) solution. The drug encapsulated liposomes were placed in PBS and loaded in the dialysis bag. The bag was sealed at both ends and immersed in 4 ml of PBS with 10% methanol. The release of the drug was evaluated at a pH of 7.4 which was maintained using phosphate buffer.[18]

100 mg of a lyophilized product containing known amount of drugs was suspended in phosphate buffer saline, which was placed in a dialysis bag. It was immersed in 50 ml of PBS (pH 7.4), maintained at 37±1°C and stirred with the help of a magnetic stirrer. Aliquots were withdrawn at specific time intervals from the receptor compartment, and the sample was replaced with fresh PBS (pH 7.4) to maintain sink condition. The absorbance of the samples was measured at 271 nm and 265 nm after suitable dilution, if necessary, using appropriate blank.[19-21]

Stability studies

The ability of liposomes to retain the drug was assessed by storing the liposomal suspension at different temperatures such as freezer temperature (−20°C), refrigerator temperature (4±1°C), room temperature (25±2°C), and 37±2°C for 1 month. Samples were withdrawn periodically, and drug content analysis was carried out as mentioned in the determination of percentage drug encapsulation.[22]

RESULTS AND DISCUSSION

Optimization of process parameters

As a preliminary study, the formulation technique was optimized by studying various process parameters such as rotational speed of a rotary evaporator, vacuum pressure, the medium of hydration, time of hydration. It was found that thickness and uniformity of the lipid film vary depending on the rotational speed of the evaporating flask. The optimum speed was found to be 150 rpm. The film obtained after rotary evaporation was kept overnight under vacuum to dry and remove the solvent residuals if any and to avoid the formation of an emulsion, which may form due to the presence of residual solvents in lipid film during hydration. 2 min vortexing was found to be appropriate to obtain the liposomal suspension free from aggregates. The process of vortexing did not influence the percentage drug encapsulation which was confirmed by calculating the entrapment before and after doing vortexing. Further, it was observed that liposomes prepared using phosphate buffer pH 7.4 as the hydration medium showed better entrapment efficiency compared to those prepared with water as the hydration medium.

Compatibility study (FTIR analysis)

Physical mixtures of the different phospholipids and pure drugs showed identical spectrum with respect to the spectrum of the pure drugs, indicating that there is no chemical interaction between the drug molecules and phospholipids used [Figures 1-3].

Percentage yield

Percentage yield of the formulations was carried out and was found to be within the range of 92.86-97.15%. Liposomes consisting of a high concentration of cholesterol showed maximum percentage yields, whereas the liposomes prepared with SPC showed high percentage yields when compared with those prepared from DPPC and DSPC.

Shape and surface morphology

Images obtained under an optical microscope confirmed the formation of the phospholipid vesicles on hydration of

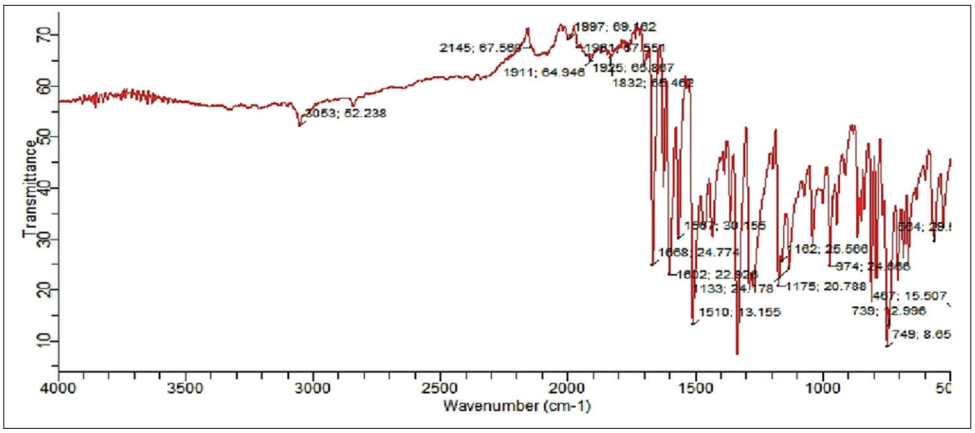

Figure 1: Fourier transform infrared of zidovudine

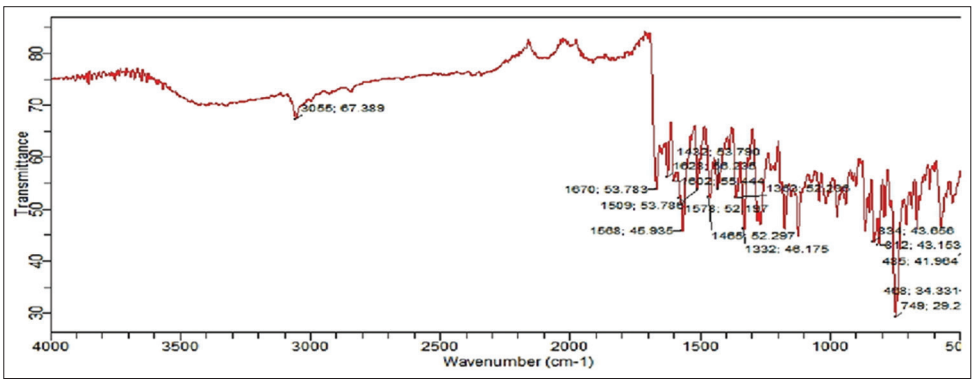

Figure 2: Fourier transform infrared of lamivudine

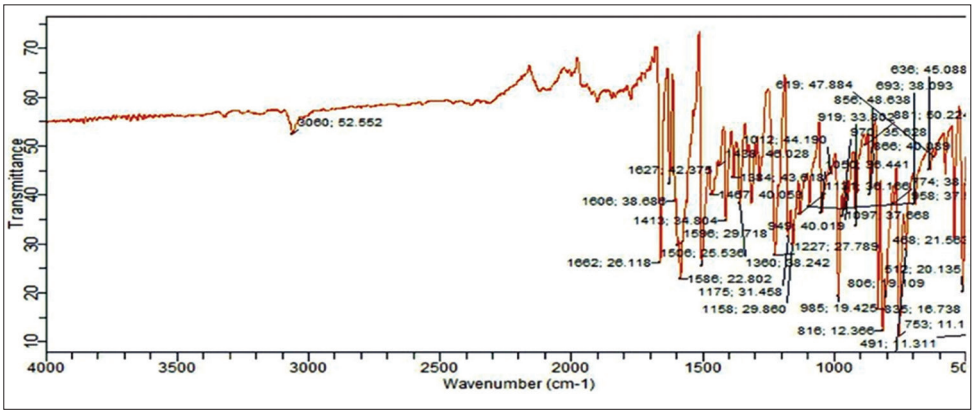

Figure 3: Fourier transform infrared of physical mixtures

thin lipid film formed by the flash rotary evaporator. It was found that the formed vesicles were spherical in shape. The morphology of the PEGylated liposomes was observed by TEM. The image from negative-staining showed that the PEGylated liposomes were of discrete and round structure ranging in size from 150 to 450 nm [Figure 4].

Particle size

The mean particle size of the stealth liposomes was done by Malvern systems particle size analyzer. The mean particle size of the vesicles is significantly increased with an increase in cholesterol concentration and size range was found to be between 224.6 and 397.1 nm. Furthermore, liposomes prepared with SPC showed larger particle sizes when compared with those prepared from DPPC and DSPC [Table 2 and Figure 5].

Percentage drug entrapment

Percentage drug entrapment of the liposomal formulations was found to be within the range of 56.21-71.92%. Moderate

Table 1: Composition of stealth liposomes

Formulation	Drug (3TC+AZT) (mg)	Cholesterol (mg)	SPC (mg)	DPPC (mg)	DSPC (mg)	PE-PEG (mg)	Phospholipids: Cholesterol: Drug: PE-PEG (ratio)
F1	50+50	200	400	-	-	20	4:2:1:0.2
F2	50+50	150	400	-	-	20	4:1.5:1:0.2
F3	50+50	100	400	-	-	20	4:1:1:0.2
F4	50+50	200	-	400	-	20	4:2:1:0.2
F5	50+50	150	-	400	-	20	4:1.5:1:0.2
F6	50+50	100	-	400	-	20	4:1:1:0.2
F7	50+50	200	-	-	400	20	4:2:1:0.2
F8	50+50	150	-	-	400	20	4:1.5:1:0.2
F9	50+50	100	-	-	400	20	4:1:1:0.2

F1-F9: Factorial formulations, 3TC: Lamivudine, AZT: Zidovudine, SPC: Soy phosphatidyl choline, DPPC: Dipalmitoyl phosphatidyl choline, DSPC: Distearoyl phosphatidyl choline, PE-PEG: PE 18:0/18:0-PEG 2000

Table 2: Percentage yield, particle size, zeta potential, and percentage drug entrapment

Formulation	Percentage yield	Particle size (nm)	Zeta potential (mV)	Percentage drug entrapment
F1	97.15	397.12	−24.7	64.57
F2	96.54	363.23	−24.9	68.47
F3	95.17	332.71	−25.4	71.92
F4	96.01	321.21	−32.3	60.17
F5	94.98	302.57	−32.5	63.28
F6	93.28	280.24	−32.9	67.58
F7	95.45	271.26	−38.1	56.21
F8	93.94	252.82	−38.2	61.68
F9	92.86	224.64	−38.5	64.71

F1-F9: Factorial formulations, nm: Nanometers, mV: Millivolts

entrapment of drugs in the liposomes could be ascribed to their hydrophilic nature. Due to their hydrophilic nature, they probably not get intercalated preferentially in the lipid layer of the cell membrane. Liposomes prepared with SPC showed high entrapment efficiency when compared with those prepared from DPPC and DSPC. It was also observed that drug entrapment efficiency increased with increase in the concentration of phospholipids [Table 2 and Figure 6].

Zeta potential

The experimental data shown in Table 2 reflect that zeta potential values are influenced by the lipid composition. Zeta potential values of about −24.7-−38.3 mV were observed for liposomes, which are probably related to the steric effect of PE 18:0/18:0-PEG 2000 [Table 2].

In vitro dissolution study

The *in vitro* release studies of drug-loaded stealth liposomes were carried out in phosphate buffer saline (PBS) as a

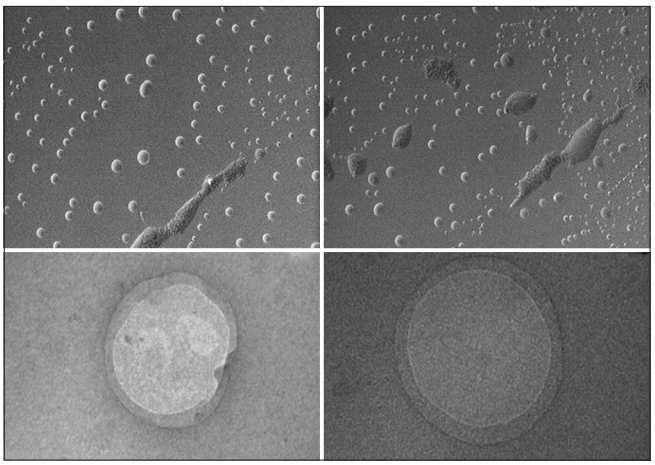

Figure 4: Percentage yield and percentage drug entrapment

dissolution medium for a period of 24-h, respectively. The release showed a biphasic release with an initial burst effect of releasing more than 10% of the drug within the first 1 h followed by a sustained release pattern until 24 h. The percentage cumulative drug release of formulations were found to be 89.87%, 91.54%, 94.23%, 82.43%, 86.87%,

90.03%, 79.29%, 83.12%, and 86.65% at the end of 24th h [Table 3 and Figure 7].

The drug release behavior of liposomes prepared incorporating a high concentration of cholesterol showed improved drug retention capacity and, therefore, percentage drug release was decreased as follows: 89.87%, 82.43%, and 79.29% for the F1, F4, and F7, respectively. Similarly, liposomes prepared with SPC retained drug remarkably lower, when compared with liposomes prepared with DPPC and DSPC. The percentage drug release pattern was found to be in the following order DSPC < DPPC < SPC which may be because increase in alkyl chain length of lipids increases the phase transition temperature, due to stronger vanderwaals interaction between the lipid chains. Thus, higher energy is required to disrupt the ordered packing thereby resulting in slower or sustained drug release.

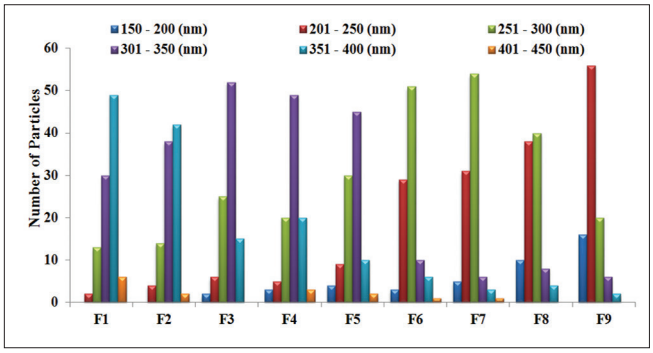

Figure 5: Frequency size distribution of stealth liposomes

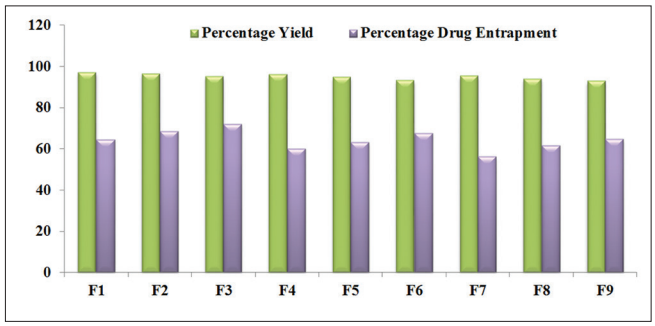

Figure 6: Percentage yield and percentage drug entrapment

Stability studies

The selected batch of stealth liposome was evaluated for physical and chemical stability by storing the liposomal formulation at 4 different temperatures as previously described for a period of 1-month and evaluated for any changes in the percentage drug entrapment. There are no significant changes in the percentage drug entrapment for the formulations stored at −20°C and 4°C. However, it was found that there was a considerable reduction in percentage drug entrapment of the liposomes stored at room temperature as well as the elevated temperature at 37±2°C [Figure 8].

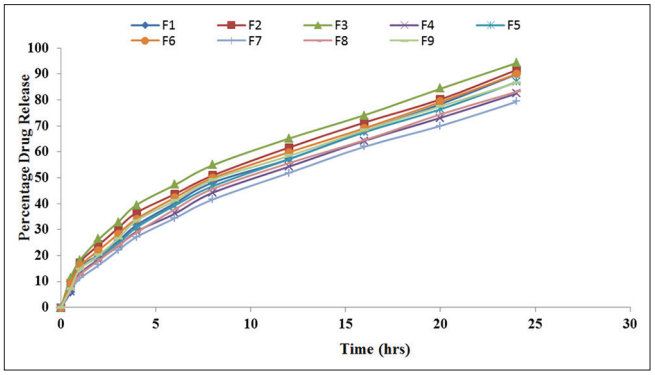

Figure 7: *In vitro* dissolution profile of stealth liposomes (F1-F9)

Table 3: *In vitro* release profile of formulations F1-F9

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
0.5	6.01	9.32	11.34	6.24	8.45	9.21	6.55	6.78	7.13
1	15.23	17.43	18.21	13.02	15.23	16.32	11.13	12.53	14.76
2	20.32	24.12	26.35	18.61	19.65	22.12	16.35	18.12	20.13
3	25.51	30.54	32.82	23.5	24.51	28.23	21.93	23.76	26.51
4	31.81	36.62	39.61	29.3	30.9	34.23	27.22	28.93	33.56
6	40.14	43.71	47.24	36.16	39.3	42.32	34.41	37.82	41.12
8	48.15	50.87	54.85	44.23	46.77	49.87	41.63	45.71	49.21
12	57.14	61.64	65.09	54.26	57.13	59.79	51.81	55.61	58.37
16	68.12	71.34	74.15	64.25	67.57	69.12	61.97	64.51	68.32
20	78.37	80.23	84.34	73.11	76.41	79.23	69.99	74.44	77.55
24	89.87	91.54	94.23	82.43	86.87	90.03	79.29	83.12	86.65

F1-F9: Factorial formulations

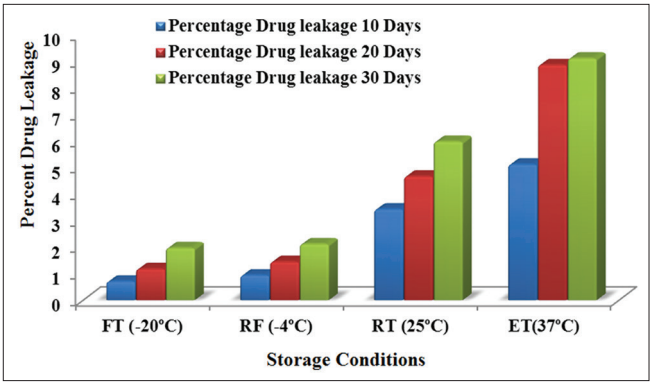

Figure 8: Percent drug leakage from stealth liposomes (F9) at different storage conditions

CONCLUSION

In the present study, a combination of two hydrophilic drugs, lamivudine and zidovudine, was successfully encapsulated into stealth liposomes composed of different phospholipids with stealth properties. Drug-loaded stealth liposomes were shown to be influenced by the concentration of cholesterol and alkyl chain length of lipids used. The presence of an optimum amount of cholesterol was found to enhance encapsulation efficiency and increasing the amount of cholesterol was found to decrease the encapsulation as well as drug release. So, the present study has given us knowledge that liposomes with a low amount of cholesterol are better candidates for liposomes loaded with the combination of lamivudine and zidovudine. This study confirmed that the retention of drugs can be enhanced by employing long alkyl chain length phosphatidylcholines. Future research can be directed toward *in vivo* tissue distribution studies. Finally, this vesicular drug delivery technology can be further explored for the drugs which show less half-life and narrow therapeutic indices.

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