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Development and evaluation of co-encapsulated stavudine and lamivudine niosomes for the controlled delivery

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ABSTRACT

Stavudine and lamivudine are nucleoside reverse transcriptase inhibitors used for the treatment of HIV. We describe here the co-encapsulation of stavudine and lamivudine in niosomal MLVs composed of tween80, span60 and variable amounts of cholesterol. The main aim of this study is to reduce the dose, dosing frequency and overcome the resistance of existing single drug regimen therapy. The influence of drug-lipid ratio was studied and amount of the drug could be encapsulated was optimized. The effect of cholesterol and other process related parameters were studied to obtain the niosomal vesicles with desired quality. All the prepared formulations were characterized for their physico chemical properties such as appearance, vesicle size, vesicle size distribution, percent drug entrapment, viscosity, zeta potential, stability profile and in-vitro drug release. Stability of niosomes in terms of their drug leakage and drug retention behavior was studied as per ICH guidelines for three months by storing the niosomes at refrigerated temperature (4 ± 2 °C) and room temperature (25 ± 2 °C). Niosomes stored under refrigerated condition showed greater stability and results were found to be within the specification in both storage conditions. The maximum percentage drug entrapment (92.64%) was achieved with the formulation containing the drug –lipid ratio of 150:40% w/w. In vitro release data showed that release profile followed zero order kinetics and drug release mechanism was of diffusion. Stavudine and lamivudine niosomes with good stability and appreciable controlled drug release with good retention of the drug even after 24h were prepared successfully.

Key words: Niosome, MLVs, SEM, Zeta potential, Stability.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus is a condition in humans in which the immune system begins to fail, leading to life threatening opportunistic infections [1]. It is established that effective antiretroviral therapy requires long term treatment using higher dosage regimen to reduce and to maintain the viral suppression. However the therapy using conventional formulations such as tablets, capsules and suspension do not eliminate the viral reservoirs in an anatomical and intracellular site. To reduce the frequency of administration and to improve the patient compliance, a controlled release and site specific formulation is desirable. In treating AIDS the multiple drug regimen therapy is required to overcome the resistant mutants. Stavudine and lamivudine combination therapy was proved as effective against retro virus. The main limitations on the therapeutic effectiveness of stavudine and lamivudine are their dose-dependent hematological toxicity, very short biological half lives (stavudine 0.8h; Lamivudine 5h) and lamivudine has high first pass metabolism [2]. This necessitates frequent administration of large doses (200 mg every 4h), since it is crucial to maintain the systemic drugs concentration within the therapeutic level throughout the treatment course. In order to overcome these disadvantages niosomes were selected as carrier and effective drug delivery system to deliver stavudine and lamivudine by which bio-distribution of drugs can be altered to provide a greater degree of targeting of drugs to selected tissues and in a controlled manner. Niosomes are non-ionic surfactant vesicles having bilayered structures, which can entrap both hydrophilic and lipophilic drugs. Niosomes act as a controlled release formulation, which establishes and maintains the drug concentration at the target site for longer period of time. Thus the objective of the study was to co-

encapsulate the anti-retro viral drugs into non-ionic surfactant vesicles (niosomes) of suitable size range with controlled release characteristics.

MATERIALS AND METHODS

Stavudine and lamivudine were gifted by Aurobindo Pharma Ltd (India). The non-ionic surfactants tween 80 (poly sorbate 80), span 60 (sorbitan mono stearate) and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All organic solvents and the other chemicals were of analytical grade.

Optimization of process parameters

Before formulation development, various factors that influence the product such as vacuum, speed of rotation, hydration medium and hydration time were studied in order to prepare drug encapsulated niosomes with desired qualities.

Preparation of stavudine and lamivudine niosomes

Multilamellar niosome vesicles (MLVs) were prepared by thin-film hydration method. An accurately weighed quantity of surfactant and cholesterol mixture was dissolved in 10 ml of chloroform in a 100ml round- bottom flask. The organic solvent was then removed at 55°C, under reduced pressure in a rotary evaporator (Cyberlab corporation MODEL: 2011). The flask was kept under vacuum-attached desicator overnight for removal of chloroform completely. The dried lipid film was hydrated with 10 ml of phosphate buffered saline (PBS) (PH 7.4) containing stavudine and lamivudine by a gentle rotation in water bath at 50 °C for 10 min. The resulting multi lamellar non-ionic surfactant vesicle dispersions were then left to mature for 2-3h. Formulations were sonicated three times at 50 Hz in a bath-sonicator (Model 3.5 L 100H) for 15 min with 5-min interval between successive times [3] Various formulations of niosomes co-encapsulated with satvudine and lamivudine had shown in table 1.

Table1: Formulae of various formulations of stavudine and lamivudine co-encapsulated niosomes

Formulation Code	Drug (mg)		Tween80 (mg)	Span60 (mg)	Cholesterol (mg)
	Stavudine	Lamivudine			
F1	5	5	100	-	-
F2	5	5	100	-	20
F3	5	5	100	-	40
F4	5	5	125	-	40
F5	5	5	150	-	40
F6	5	5	150	-	50
F7	5	5	175	-	40
F8	5	5	-	100	-
F9	5	5	-	100	20
F10	5	5	-	100	40
F11	5	5	-	125	40
F12	5	5	-	150	40
F13	5	5	-	150	50
F14	5	5	-	175	40

Determination of % drug encapsulation efficiency

% drug entrapment efficiency of co-encapsulated niosomes of stavudine and lamivudine were studied by centrifuging the vesicle suspensions at 10000g at 4 °C for 2 cycles of 15 min with 10 min interval. After decanting the supernatant the pellet was washed with PBS (PH 7.4). The amounts of stavudine and lamivudine in the supernatant and also in the pellet were analyzed spectroscopically at 272 and 266 nm respectively, after disrupting the niosomal pellet using ethanol [4]. The % drug entrapment was calculated by the following formula,

% drug entrapment = amount of drug in pellet / total amount of drug *100

Particle size measurement

The size, shape and lamellae of vesicles in non-sonicated formulations were observed by optical microscopy using a calibrated eye-piece micro meter. The scanning electron microscope analysis was carried out to study the shape and surface morphology of vesicles of desired size after sonication [5].

Fourier transform, infrared (FTIR) study

All the excipients such as tween80, span60 and cholesterol individually, physical mixture of excipients, pure drugs stavudine and lamivudine individually, physical mixture of excipients and drugs 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^{-1} in (Bruker 10066117) FTIR instrument [5].

Determination of viscosity
Viscosity of formulations was determined using Brook field Viscometer (Brook field Engineering labs.INC. MIDDLE BORO, MA 02346 U.S.A) with spindle number of 63, speed 30 at room temperature [6].

Osmotic shock
The effect of osmotic shock on niosomal formulations was investigated by monitoring the change in vesicle diameter after incubation of niosome suspensions in media of different tonicity such as 2% NaCl (hypertonic), 0.9% NaCl (isotonic), and 0.5% NaCl (hypotonic). Suspensions were incubated in the media for 3h and the change in vesicle size was measured by optical microscopy with calibrated eyepiece micrometer [7].

Measurement of zeta potential
The Zeta Master apparatus (Malvern Instruments, Malvern, UK) was used to calculate zeta potential by determining the electrophoretic mobility. It was obtained by performing an electrophoresis experiment on the sample and measuring the velocity of particles. There exists an electrical double layer at the interface between solid particle surface and the surrounding liquid medium. The inner electrical layer is tightly bound to the surface whereas the outer layer is loosely attached due to electro static forces and Brownian motion developed. The niosomal particle moves in the liquid medium along with its associated charges as a unit. The ionic potential at the surface between this unit and the surrounding medium is called as Zeta potential and it is measured in milli volts [5], [8].

Stability analysis
The stability analysis for the best stvudine and lamivudine co-encapsulated niosomes were carried out as per ICH guidelines for 3 months. The niosomes were stored in an ambient temperature (refrigerated temperature; 4 ± 2 °C) and accelerated condition (room temperature; 25 ± 2 °C) Perioadically samples were withdrawn and analyzed for the drug content following the same method described in % drug encapsulation efficiency [9], [10], [11].

In vitro drug release
Modified USP XXI dissolution rate model was used for the determination of drug release from niosomal preparation. This model consists of a beaker (250ml) and a plastic tube of diameter 17.5mm opened from both the ends. Sigma membrane (Sigma 12000 MW cutoff) was tied at one end of the tube & the other end left free. This assembly was dipped into the beaker containing 250ml of the dissolution medium. The temperature was maintained at 37 °C. 10ml of niosomal suspension was added into the tube and a paddle type stirrer was placed in the center of the beaker. The speed of the stirrer was maintained at 100 rpm. Dissolution sample of 5ml was withdrawn periodically every one hour up to 24h and analyzed spectrophotometrically at 272nm and 266nm respectively for stavudine and lamivudine. With the help of the standard curve prepared earlier, drug concentration was measured. Results were the mean values of three runs [5], [8].

Release Kinetics
In order to find out the order and mechanism of stavudine and lamivudine release from niosomal formulations the *in vitro* drug release data was subjected to the following mathematical models such as zero-order kinetic model, first-order kinetic model, Higuchi's kinetic model and the Korsmeyer- Peppas model.

RESULTS AND DISCUSSION

Optimization of process related variables
The process-related variables such as sonication time, hydration medium, hydration time, speed of rotation of flask evaporator were investigated in vesicle formation with different concentration of tween80 and cholesterol with a fixed amount (10mg) of stavudine and lamivudine. The studies again carried out with span60 and cholesterol same as that with tween80.

Effect of sonication time
Spherical niosome vesicles were not observed after continuous sonication of more than 5 min, suggesting that exposure of vesicles to ultrasound for more than 5 min may damage the vesicles.

Rotational speed of evaporator flask
The thickness and uniformity of the film depended upon the rotational speed of the flask. A speed of 100rpm was found to give uniform thin film resulting in spherical vesicles on hydration. It was found that lower and higher rpm than the optimum speed produced thick film that was found to form aggregates of vesicles upon hydration.

Hydration medium

Phosphate buffered saline pH 7.4 was found to produce greater drug entrapment and stable suspension when compared to water as the hydration medium.

Percentage drug encapsulation efficiency

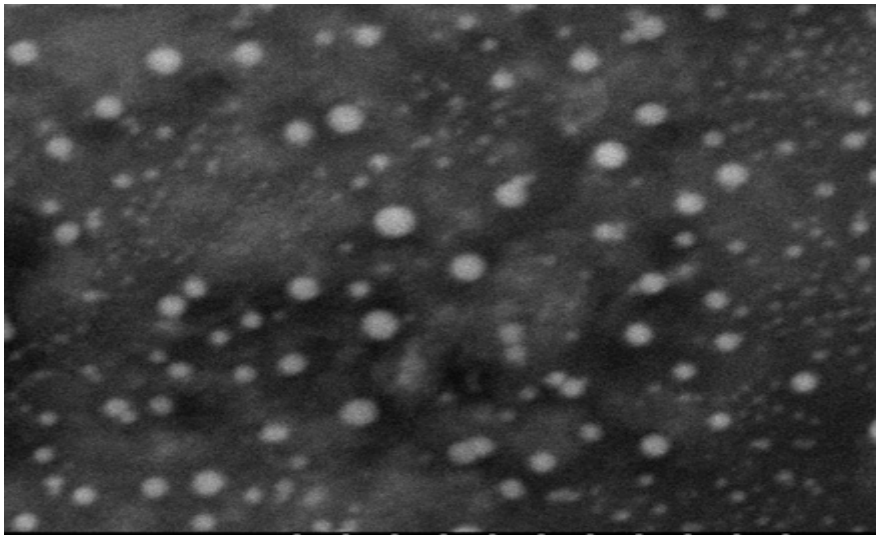
Role of cholesterol content in drug entrapment

Cholesterol was found to play a major role in % drug entrapment of the drugs in niosomal membranes. In the study stavudine and lamivudine niosomal formulations prepared without cholesterol was found to have less drug entrapment (F1 and F8 when compared to F2 and F9 respectively with span 60 and tween 80). High % of drugs entrapment was found in tween80 and span60 formulation at a cholesterol/surfactant ratio of 150:40 %w/w (table 2). Inclusion of cholesterol increases the viscosity of the formulation indicating more rigidity of the bilayer membrane [12]. Moreover, drug partitioning will occur more easily in highly ordered systems of surfactant and cholesterol. The ability of the lamellar surfactant phase to accommodate drug, depends upon the structure of the surfactant phase.

Table 2: Data showing % encapsulation efficiency (%EE), % release and viscosity of various niosomes

Formulation Code	%EE		% drug release		Viscocity (in poise)
	Stavudine	Lamivudine	Stavudine	Lamivudine	
F1	34	33	95	93	0.83
F2	40	39	83	85	0.85
F3	48	45	76	83	0.86
F4	81	83	63	62	0.89
F5	92	92	51	50	0.9
F6	61	59	62	58	0.87
F7	70	69	67	68	0.88
F8	22	21	94	91	1.2
F9	31	30	86	87	1.6
F10	39	41	75	76	1.7
F11	67	70	65	64	1.9
F12	89	80	57	57	2.2
F13	67	65	75	73	1.8
F14	53	50	67	69	1.7

Fig 1: SEM photograph of F5 formulation



Role of surfactant in drug entrapment

The effect of different concentrations of tween80 on drug entrapment is depicted in table 2. An increase in amount of surfactant beyond the ratio of cholesterol/surfactant of 100:20 %w/w (F2 and F9 respectively with tween 80 and span 60) resulted in spherical vesicles along with aggregates. This showed that surfactant beyond certain concentration with low amount of cholesterol does not form stable vesicles with good entrapment. Thus it was decided to increase the amount of surfactant with increasing amount of cholesterol (F3-F7 and F9-F14 respectively with tween 80 and span 60). It was found that 150:40 %w/w of surfactant cholesterol ratio produced greater drug entrapment in both the surfactants. As tween80 is having lower HLB value, the formulations prepared with tween 80 were found to have greater drug entrapment. The best formulation (F5) was found to have greater drug entrapment

of 92% for both stavudine and lamivudine. The higher entrapment may be due to the solid nature, hydrophobicity and high phase transition temperature of the surfactant [13], [14], [15].

Vesicle size and shape

Vesicles after sonication were found to remain non-aggregated for 3 months when compared to non-sonicated vesicles. SEM photograph revealed that niosomes were spherical and surface morphology was uniform (Fig 1). Zeta sizing showed the size distribution pattern as shown in table 3 and fig 2.

Fig 2: Graph for vesicle size distribution

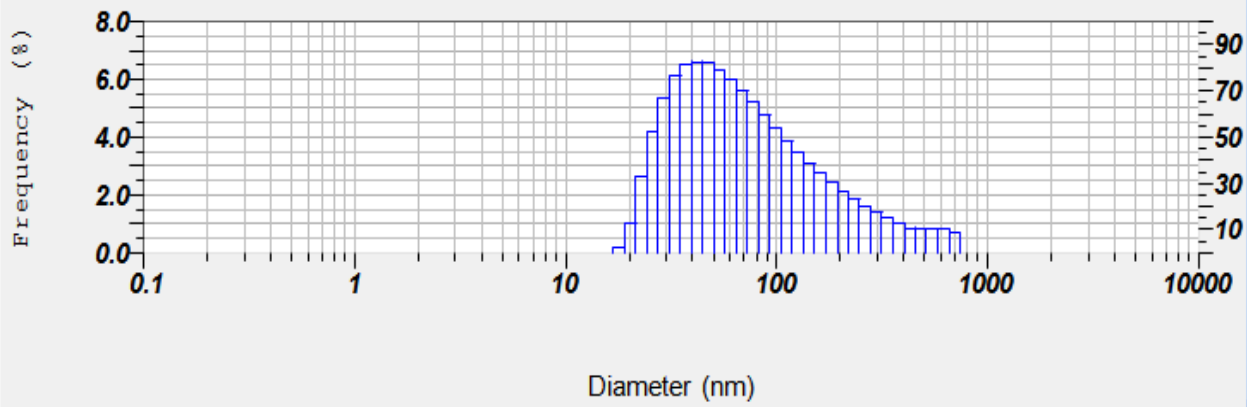


Table 3: Data for vesicle size distribution

Peak No.	S. P. Area Ratio	Mean	S. D.	Mode
1	0.98	97.3 nm	95.3 nm	42.1 nm
2	0.02	652.2 nm	40.8 nm	648.0 nm
3	---	--- nm	--- nm	--- nm
Total	1.00	105.8 nm	116.7 nm	42.1 nm

Fourier transform, infrared (FTIR) study

Drug excipient compatibility was studied before preparing the formulation by using FTIR-spectrophotometer, which is one of the most important analysis described about the stability of formulation, presence of drug and drug release. Minor shifts were observed when figure5 compared with spectrum of pure drugs (figure 5 and 6) and excipients (figure3 and figure4) like, C-C bending (945.33 to 949.35), C-O stretching of ester (1091.22 to 1086.71), C-H stretching (2955.75 to 2920.08), carbonylic C=O stretch of ester (1711.37 to 1711.87), N-H stretch (3425.01 to 3446.31). These shifts observed may be due to the formation of hydrogen bonds, Vander walls attractive forces or dipole moment which are weak forces seen in the polar functional groups of drug and excipients. The frequency of absorption due to the carbonyl group depends mainly on the force constant which in turn depends upon inductive effect, field effect, and stearic effects. The shifts seen due to the above mentioned reasons may however support the formation of favorable vesicle shape, structure with good stability and sustained drug release.

Fig 3: FTIR spectrum of pure tween80

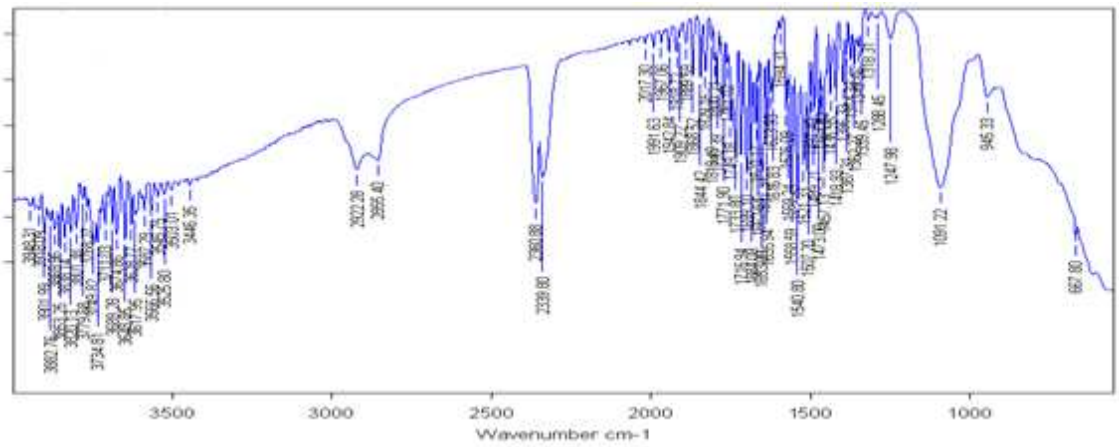


Fig 4: FTIR spectrum of pure cholesterol

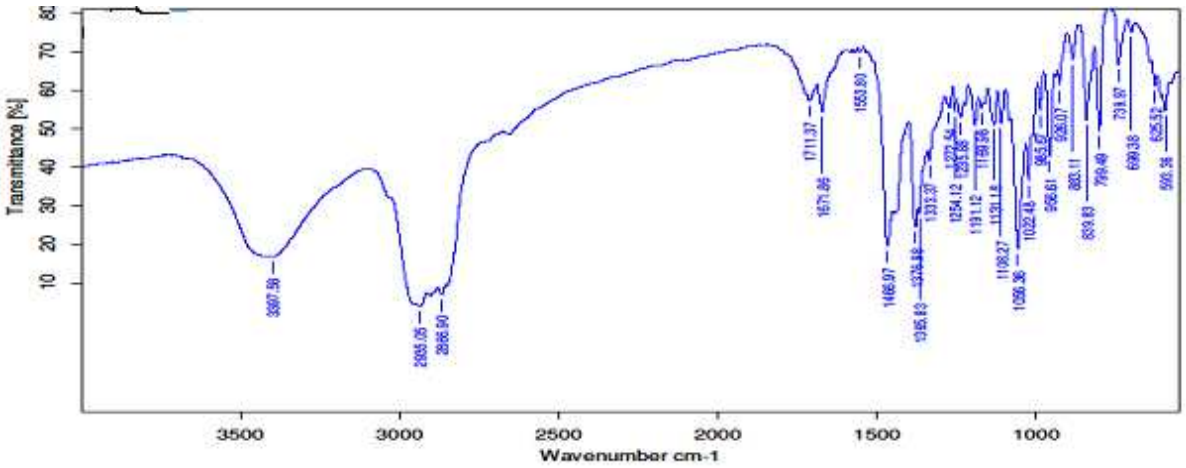


Fig 5: FTIR spectrum of pure stavudine

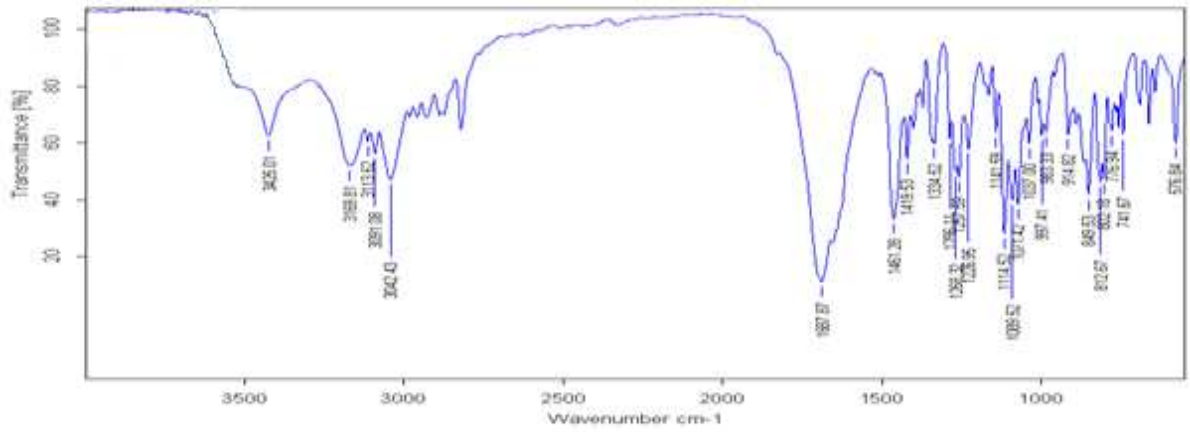


Fig 6: FTIR spectrum of pure lamivudine

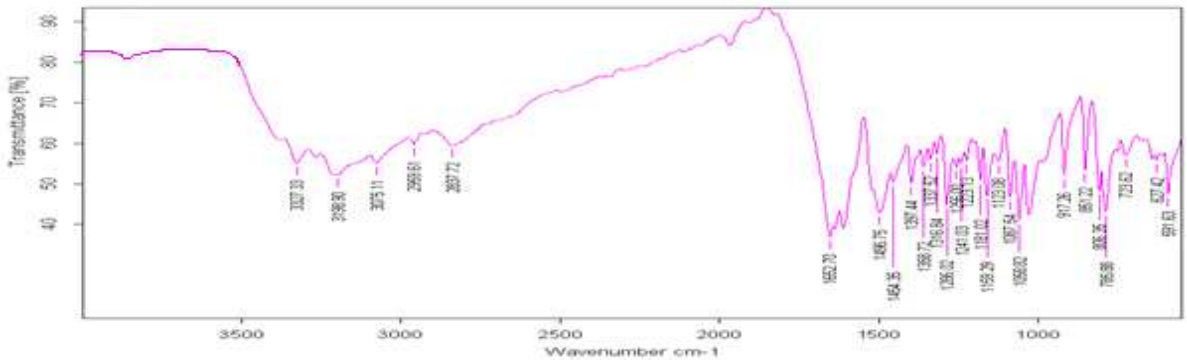


Fig 7: FTIR spectrum of physical mixture of f5 formulation

Stability profile

The stability study for the best niosomes was carried out as per ICH guidelines for 3 months. There is no evident for aggregation, fusion or disruption of the vesicles during the study period of 3 months and it was found that the prepared formulations were able to retain their multilamellar nature and shape uniformity to an appreciable extent. Leakage of drug from the prepared niosomes was analyzed in terms of percentage drug content. At refrigerated condition the niosomal formulation F5 showed 98.74 ± 0.41 % and 98.5 ± 0.62 % for stavudine and lamivudine respectively (4 ± 2 °C). At room temperature (25 ± 2 °C) formulation F5 showed 95.5 ± 0.82 % for both stavudine and lamivudine. Thus it was found that storage under refrigerated condition showed greater stability. But in both the storage conditions drug content was found to be within the specification of 95-105% through out the study period of 3 months (Fig 8 and fig 9).

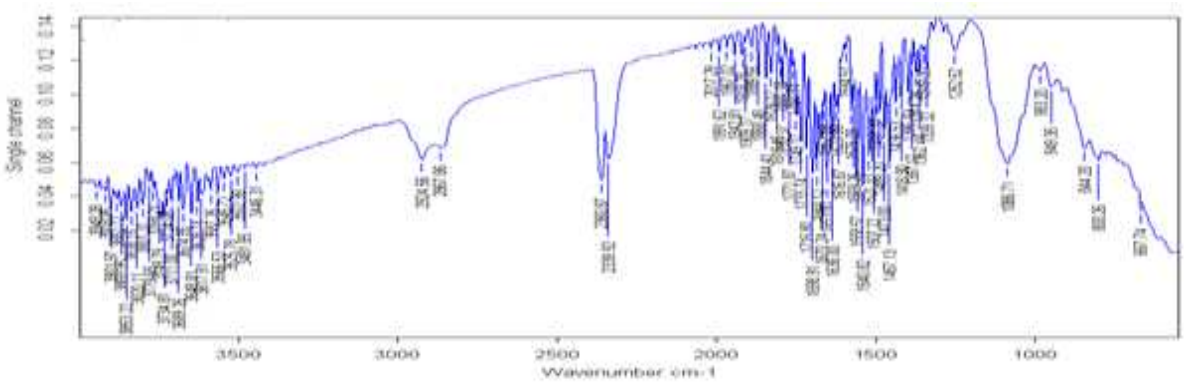


Fig 8: Stability profile of f5 formulation for stavudine at different storage temperatures

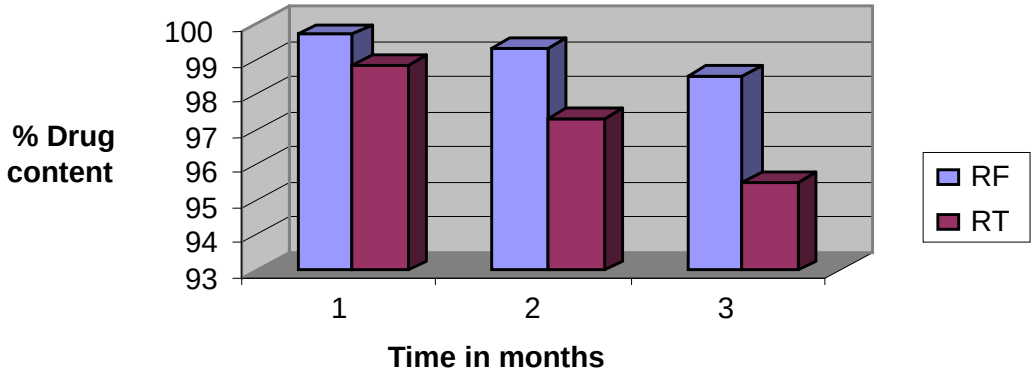
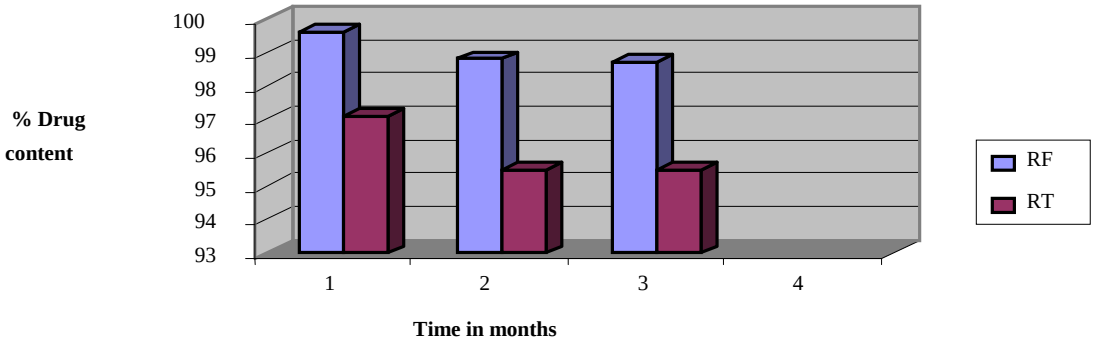


Fig 9: Stability profile of f5 formulation for Lamivudine at different storage temperatures



Osmotic shock

Formulations were treated with hypotonic (0.5% NaCl), normal saline (0.9% NaCl), and hypertonic (2% NaCl) solutions. In hypetonic solutions, all formulations shrank uniformly. Formulations incubated with normal saline showed a slight increase in size when compared to other media (Table 4). This demonstrates that the niosomes could be diluted with normal saline for parenteral use [16].

Table 4: Results of osmotic shock

Formulation code	Appearance of vesicles in different media			
	PBS 7.4	Normal saline	Hypotonic solution	Hypertonic solution
F5	Normal size	No Change	Increased size	Shrank
F12	Normal size	No Change	Increased size	Shrank

Viscosity

It was found that formulations prepared with Span60 and cholesterol were more viscous, while tween80 formulations were less viscous at room temperature (Table 2)

Zeta potential

Zeta potential of F5 formulation was found to be -24.3 ± 2.83 with good result quality. This showed that F5 was found to be appreciably stable.

***In- vitro* release**

The initial drug release in 1st h from all the formulation was 9-14%. Fast drug release in first one hour may be due to the release of bound drug from the lipophilic region of niosomes, which will help to achieve the optimal loading dose [17], [18]. Formulation F5 was found to produce 50.49 ± 0.29 and 51.27 ± 0.33 % for lamivudine and stavudine respectively for 24h which contains optimum amount of surfactant tween 80 and cholesterol (cholesterol/surfactant ratio of 150:40 %w/w). The percentage release was found to increase i.e 84.75 ± 0.75 and 83.26 ± 0.36 % for lamivudine and stavudine respectively with the low concentration of cholesterol (cholesterol/surfactant 100:20%w/w), and without cholesterol it was 93.22 ± 0.22 % and 94.81 ± 0.1 % for lamivudine and stavudine respectively. Increasing cholesterol concentration markedly reduces the efflux of drug. Inclusion of cholesterol fills the pores in vesicular bilayers and abolishes the gel-liquid phase transition of niosomal system resulting in less leaky niosomal formulations. This confirms that cholesterol in the formulation acts as a membrane stabilizing agent that helps to sustain drug release. Release of stavudine and lamivudine from tween80: cholesterol (150:40) was slow compared to other ratios. Initial rapid release up to 1 h followed by extended release greater than 24 h was observed in tween80 formulations. Same sort of release profile with slight changes was obtained for the niosomes prepared with span 60 as the surfactant. Differences might be due to vesicle size, lamellarity and membrane fluidity as a function of chain length of surfactant and cholesterol (Fig 10-13).

Fig 10: *In vitro* release pattern of niosomes for stavudine with tween80

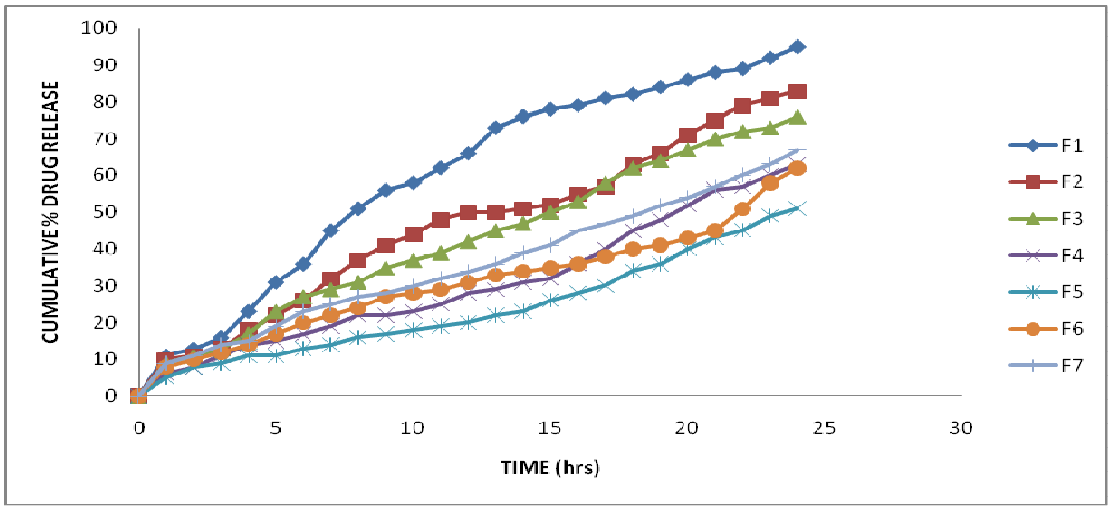


Fig 11: *In vitro* release pattern of niosomes for lamivudine with tween80

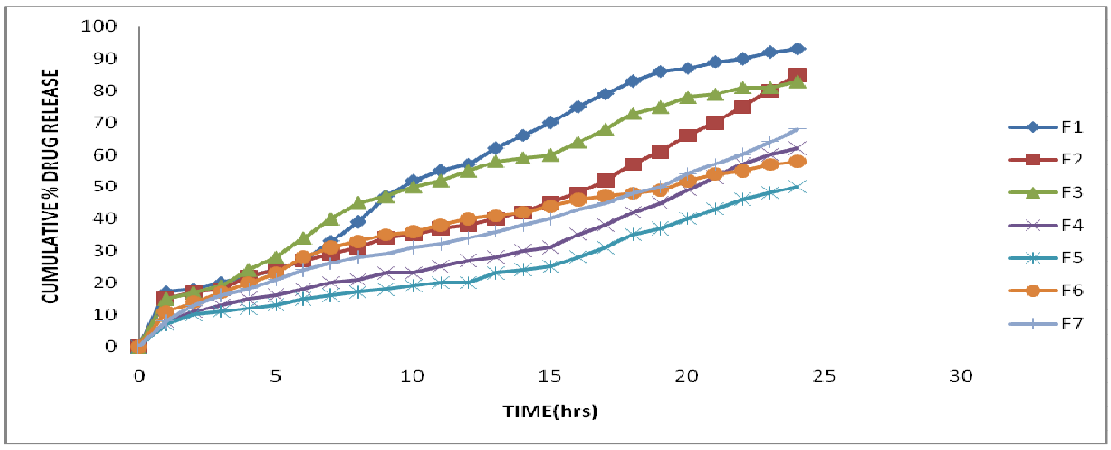


Fig 12: *In-vitro* release pattern of niosomes for stavudine with span60

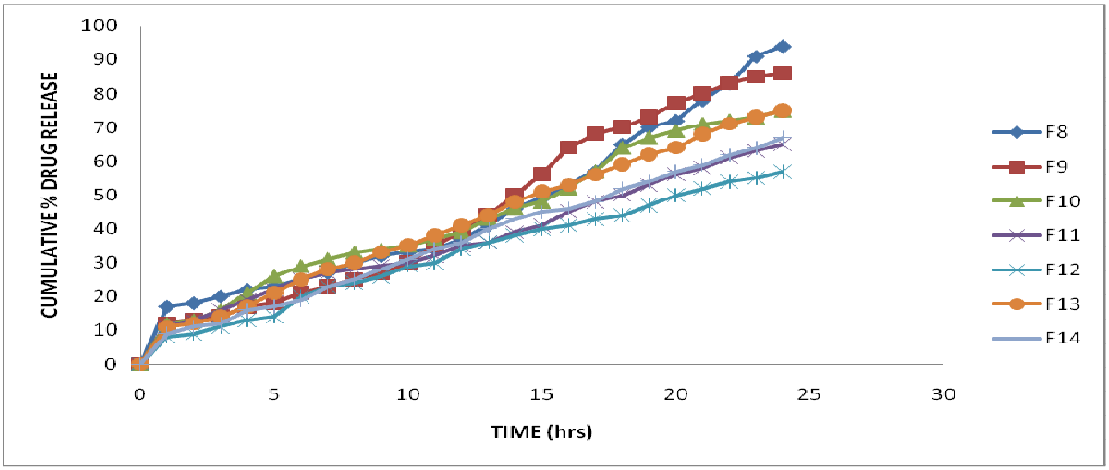
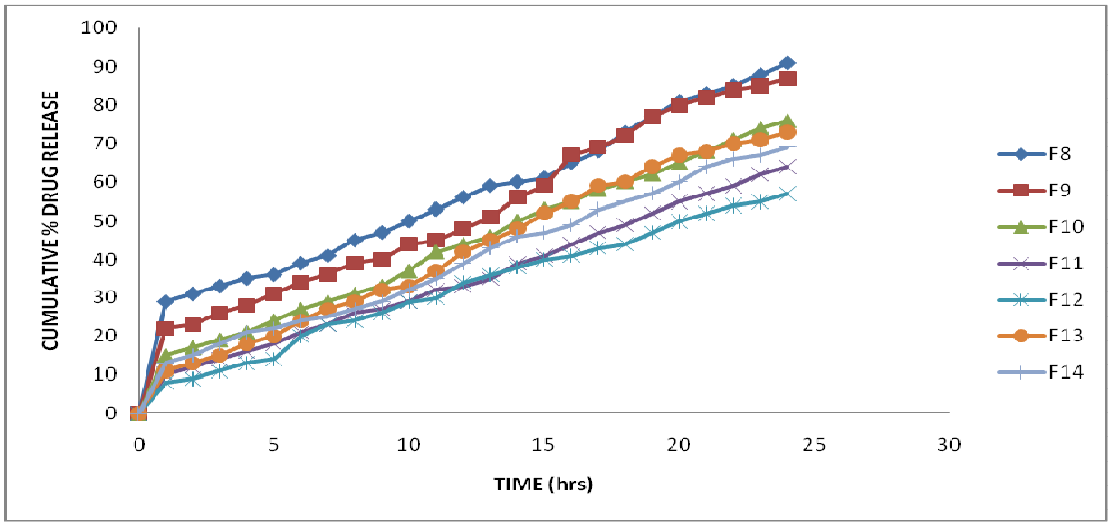


Fig 13: in vitro release pattern of niosomes for lamivudine with span60



Release kinetics

In vitro release data was subjected to various kinetic models to understand rate, order and mechanism of drug release. Regression equation for respective models were followed and found that drugs release followed zero order kinetics (R^2; 0.992-0.998). The data was found to fit better with Higuchi's kinetics model (R^2; 0.991-0.998). The Peppas model showed that 'n' value of all formulations was less than 0.5. Thus the reselts revealed that drug release followed zero order kinetics and mechanism of drug release was of Fickian type of diffusion.

Table 5: Mathematical model showing order and mechanism of drug release

Formulation code	Zero order plot regression (R^2)		First order plot regression (R^2)		Higuchi's plot regression(R^2)		Peppas plot 'n' value	
	Lamivudine	Stavudine	Lamivudine	Stavudine	Lamivudine	Stavudine	Lamivudine	Stavudine
F1	0.998	0.992	0.919	0.889	0.993	0.994	0.485	0.491
F2	0.995	0.993	0.981	0.874	0.994	0.991	0.408	0.48
F3	0.994	0.992	0.965	0.949	0.998	0.998	0.422	0.5
F4	0.999	0.995	0.877	0.897	0.998	0.996	0.462	0.46
F5	0.996	0.995	0.946	0.955	0.992	0.994	0.397	0.484
F6	0.999	0.995	0.857	0.937	0.994	0.994	0.492	0.465
F7	0.996	0.996	0.921	0.932	0.998	0.994	0.409	0.36
F8	0.992	0.991	0.897	0.94	0.994	0.992	0.32	0.5
F9	0.997	0.995	0.98	0.973	0.991	0.991	0.4	0.4
F10	0.997	0.993	0.964	0.935	0.996	0.992	0.44	0.43
F11	0.997	0.991	0.963	0.946	0.993	0.992	0.5	0.47
F12	0.994	0.991	0.909	0.922	0.998	0.993	0.49	0.19
F13	0.993	0.998	0.943	0.937	0.993	0.997	0.5	0.5
F14	0.995	0.998	0.963	0.938	0.994	0.996	0.41	0.48

CONCLUSION

In the present study, stavudine and lamivudine were successfully incorporated into niosomes with desired qualities. Prepared niosomes were shown to be influenced by the type of surfactant, surfactant – cholesterol ratio and bilayer cholesterol content. Presence of optimum amount of cholesterol (40mg) was found to enhance encapsulation efficiency and increasing amount of cholesterol was found to decrease the encapsulation efficiency and to increase ddrug permeability. Thus, the present study has given us knowledge that niosomes with optimum (low) amount of cholesterol are better candidates for niosomes of stavudine and lamivudine.

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