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DESIGN AND *IN VITRO* CHARACTERIZATION OF STAVUDINE LOADED NON IONIC SURFACTANT VESICLES

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ABSTRACT

The present investigation was aimed to prepare, characterize and optimize stavudine loaded nonionic surfactant vesicles (niosomes) for improvement in therapeutic index and efficacy of stavudine. Stavudine loaded niosomes were prepared by ether injection method, thin film hydration method, sonication method and reverse phase evaporation method using cholesterol and different surfactants. The formulations were then characterized with respect to vesicle size, entrapment efficiency, in *vitro* drug release profile and stability under specific conditions of temperature and humidity.Vesicles formed were descrete and spherical. Vesicles formed with tweens were larger in size compared to vesicles of spans. The entrapment efficiency of the formulations was found in between 60.12%-80.56%.Higher entrapment efficiency was observed with the formulations prepared from spans. The formulation T2 showed the highest entrapment efficiency with 80.54%. The cumulative percent drug release was in the range of 59.70% to 81.21% in 24 hrs. Formulation T3 shown highest cumulative percent drug release of 81.21%. Stability studies indicated 4-8ºC was the most suitable condition for storage of Stavudine loaded niosomes.The results of the study revealed that stavudine loaded niosomes are capable of releasing the drug for extended periods of time.

Keywords: Stavudine, niosomes, thin film hydration method, sonication *in vitro*, stability study.

INTRODUCTION

Human immunodeficiency virus infection / acquired immunodeficiency syndrome (HIV/AIDS) is a disease of the human immune system caused by infection with human immunodeficiency virus (HIV). During the initial infection, a person may experience a brief period of influenza-like illness. This is typically followed by a prolonged period without symptoms. As the illness progresses, it interferes more and more with the immune system, making the person much more likely to get infections, including opportunistic infections [1]. Stavudine is a synthetic thymidine analogue reverse transcriptase inhibitor which is active *in vitro* against HIV-1 and HIV-2. Stavudine is well absorbed and reaches peak plasma concentrations within 1 hour. Savudine has high bioavailability with short half life of 2.3 hours [2]. Long term therapy of AIDS with the drugs of short half life like stavudine leads to increase in non compliance and dose related side effects. The dosage form which is capable of releasing the drug gradually can be helpful to overcome the problem [3]. Targeted

drug delivery is a method of delivering medication to a patient in a manner that increases the concentration of the medication in some parts of the body relative to others. Targeted drug delivery seeks to concentrate the medication in the tissues of interest while reducing the relative concentration of the medication in the remaining tissues. This improves efficacy of the drug and reduce side effects. Drug targeting is the delivery of drugs to receptors or organs or any other specific part of the body to which one wishes to deliver the drugs exclusively. The drug's therapeutic index, relies in the access and specific introduction of the drug with its candidate receptor, whilst minimizing its introduction with non –target tissue [4,5].

Niosomes are nonionic surfactant vesicles which can be used as carriers for amphiphillic and lipophilic drugs. Niosomes are microscopic lamellar structures which are formed using non-ionic surfactants and cholesterol. Niosomes has been used to encapsulate colchicines, estradiol, tretinoin, dithranol**,** enoxacin and for application such as anticancer, anti-tubercular, anti-leishmanial, anti-inflammatory, hormonal drugs and oral vaccine. The ability of nonionic surfactant to form bilayer vesicles instead of micelles is dependent on the hydrophilic-lipophilic balance values (HLB) of the surfactant, the chemical structure of the components and the critical packing parameter [6,7].

The present work was aimed at developing an alternative vesicular drug delivery system for stavudine in the form of niosomes which will have advantages of controlled drug release and site specificity, increased drug stability and high drug pay load.

MATERIAL AND METHODS

Chemicals

Stavudine received as a gift sample from HETERO Labs Ltd, Hyderabad, INDIA. Cholesterol was procured from S.D Fine Chemicals Pvt Ltd, Mumbai, INDIA. All other chemicals used in the study were of analytical grade.

Preparation of non-ionic surfactant vesicles:niosomes

Different methods were employed to prepare stavudine loaded niosomes. Drug concentration was kept constant where as surfactants and cholesterol were used in different ratios like 100:100:100 and 120:80:100 respectively. Surfactants like Span 40, span 60 and tween 80 s were used. Cholesterol was used as a stabilizer of the bilayer membrane and to prevent leakage.

Modified ether injection method:

cholesterol and surfactant at ratios of (100:100 and 80:120) dissolved in 10 ml of chloroform and mixture was injected slowly at the rate of 0.25 ml/ min through 14 gauge needle in to 15 ml of hydrating medium (phosphate buffer pH 7.4) containing stavudine (100 mg). The solution was then stirred on magnetic stirrer at 60^0C. As the lipid solution was injected slowly into aqueous phase, the vaporization of solvent thus resulting in spontaneous formation of nonionic surfactant vesicles [8].

Sonication method:

In this method vesicles were produced by sonication of the solution. The drug solution in buffer was added to surfactant and cholesterol mixture in a 10 ml vial. The prepared mixture was sonicated at 60^0Cfor 3 min using bath sonicator which resulted in small and uniform sized niosomes [9].

Thin film hydration method:

In this method cholesterol and surfactant were dissolved in chloroform. The resulted solution was kept in round bottom flask. The solvent was evaporated at room temperature using rotary vacuum evaporator. The thin film of cholesterol and surfactant was formed on the inner wall of round bottom flask. The phosphate buffer solution containing stavudine was added to the flask as hydrating medium. The flask was shaken for 15 minutes at 70^0C [10].

Modified reverse phase evaporation (REV) method:

Surfactant and cholesterol mixtures were dissolved in chloroform. The aqueous phase, consisting of Stavudine in phosphate buffer solution pH 7.4 was then added to the organic phase. The mixture was sonicated for 7 minutes until a stable white emulsion was formed. The organic solvent was slowly evaporated at 60◦C by a rotary vacuum evaporator. The semisolid gel like mass formed was then diluted with PBS then left for 30 minutes. The resulting non ionic surfactant vesicle dispersion was then left to cool. The milky appearance of the resulting dispersions was an initial indication of the formation of niosomes. This was confirmed by optical microscopy for each batch of niosomes prepared [11].

Table 1: Composition of niosome formulations prepared by different methods (mg)

Formulation code	Methods	Span 40	Span 60	Tween 80	Cholesterol	stavudine
E1	Modified ether injection method	100			100	100
E2			100		100	100
E3				100	100	100
E4		120			80	100
T1	Thin film hydration method	100			100	100
T2			100		100	100
T3		120			80	100
S1	Sonification method	100			100	100
S2			100		100	100
S3				100	100	100
S4		120			80	100
R1	Modified reverse phase evaporation method	100			100	100
R2			100		100	100
R3				100	100	100
R4		120			80	100

Physicochemical characterization of Stavudine loaded niosomes

Size and shape analysis:

Vesicle size of reconstituted noisome formulation was determined by optical microscopy. Eye piece was calibrated using stage micrometer at 45 X magnification. Size of each division of eye piece micrometer was determined using the formula:

Size of each division = [Number of divisions of stage micrometer/ Number of divisions of eye piece micrometer] x 100

The average sizes of 100 vesicles were counted after reconstituting niosome formulation with phosphate buffer solution. The dispersion was observed under optical microscope at 45 x magnification [12].

Entrapment efficiency:

The percentage of incorporated stavudine (entrapment efficiency) was determined using UV-visible spectrophotometer. After centrifugation of the aqueous suspension at 18000 rpm for 40 min at 5^{o}C, amount of the free drug was detected in the supernatant and the amount of the incorporated drug was determined as the result of the initial drug minus the free drug [13].

The percentage drug entrapment was calculated as per Equation,

Entrapment Efficiency= [Total drug- Free drug / Total drug)] x 100

***In Vitro Release*:**

Niosomes equivalent to 10 milligrams of stavudine taken into a tube with both ends open. One end of the tube was closed with dialysis membrane. Now the tube containing drug loaded niosomes was kept in a beaker containing 200 ml of PBS pH 7.4. The tube was arranged in such a way that, it just touches the surface of buffer solution. The whole set up was placed on a magnetic stirrer and rotated at 50 rpm. The temperature of buffer was maintained at $37^0 \pm 1^0$c. 5 ml aliquot of release medium were withdrawn at time intervals of 1, 2, 4, 8, 16, 24 hrs and replaced by the same volume of PBS. These samples were filtered through 0.45 µm membrane filter. The Filtrate was diluted suitably and estimated by UV- spectrophotometer at 266 nm [14].

Stability Studies

The purpose of the stability testing was to determine the stability of the niosomes over time under a variety of conditions, namely, temperature and humidity. The formulations T1 and R3were divided into three sets and were stored at 4 ± 2 ^{o}C in a refrigerator, ambient temperature and humidity, and 37 ± 2 ºC/65 ± 5 %RH in a humidity control oven (Lab Care, Mumbai). Throughout the study, proniosomal formulations were stored in aluminium foil-sealed glass vials. After three months, the drug entrapment efficiency and *in vitro* release of the formulations were determined by the methods discussed previously [15].

RESULTS

Physicochemical evaluation of Stavudine loaded niosomes

Stavudine loaded niosomes were prepared by methods like modified ether injection method (E1, E2, E3 and E4), thin film hydration method (T1, T2 andT3), sonication method (S1, S2, S3 and S4) and modified reverse phase evaporation method (R1, R2, R3 and R4). Formulations were optimized on the basis of vesicle size, entrapment efficiency and in vitro release profile. It was observed that vesicle size decreases with increase in surfactant ratio and decrease in cholesterol ratio in all methods. Overall larger vesicle size was observed with the formulations prepared with ether injection method and smaller with the reverse phase evaporation method. The results of entrapment efficiency revealed that formulations with spans shown higher entrapment efficiency than formulations with tweens (Table2). Formulations prepared with span 60 shown higher entrapment efficiency than other formulations. Formulation T2 has the maximum entrapment efficiency of 72.80% whereas E3 with minimum of 60.12%. The entrapment efficiency decreases with increase in surfactant ratio and decrease in cholesterol ratio in all methods (Table2).

Table 2: Characterization of Stavudine loaded niosomes

Formulation code	Surfactant: cholesterol ratio	Vesicle size(µm)	Entrapment efficiency (%)	Drug release (%)
E1(span 40)	100:100	5.24	49.77	68.73
E2(span 60)	100:100	5.28	52.00	73.20
E3(tween 80)	100:100	6.17	31.55	60.12
E4(span 40)	120:80	5.13	46.22	64.96

T1(span 40)	100:100	3.35	49.60	73.55
T2(span 60)	100:100	3.55	51.90	80.54
T3(span 40)	120:80	3.27	48.90	70.16
S1(span 40)	100:100	2.45	64.50	68.57
S2(span 60)	100:100	2.08	64.90	79.05
S3(tween 80)	100:100	5.86	56.20	62.95
S4(span 40)	120:80	2.35	54.80	65.46
R1(span 40)	100:100	2.71	69.60	70.12
R2(span 60)	100:100	2.72	72.80	79.70
R3(tween 80)	100:100	5.75	67.00	64.12
R4(span 40)	120:80	2.12	68.80	68.43

***In vitro* release study**

The drug release across the dialysis membrane was slow from the drug loaded niosomal vesicles. Formulations E1, E2, E3 and E4 prepared by modified ether injection method showed release of 73.20, 62.73, 76.26 and 74.40% respectively in 24 hrs. Formulations T1, T2 andT3 prepared by thin film hydration method showed release of 78.55, 77.54 and 81.21% respectively in 24 hrs (Table 2). Formulations S1, S2, S3 and S4 prepared by sonification method showed release of 63.57, 63.05, 68.95 and 79.53% respectively in 24 hrs. Formulations R1, R2, R3 and R4 prepared by modified reverse phase evaporation method showed release of 64.34, 59.70, 78.12and 68.43% respectively in 24 hrs. The formulation prepared with tween 80 shown higher release of drug among the formulations of respective methods. In all methods of preparation the release pattern follows tween 80>span 40>span 60. In vitro release profile is shown in fig 2.

Stability studies

Stability study results revealed that formulations are relatively more stable at 4 ± 8^{o}C than the other conditions. Entrapment efficiency and *in vitro* release data of formulations T1 and R3 after stability studies period are shown in Table Nos.3&4.

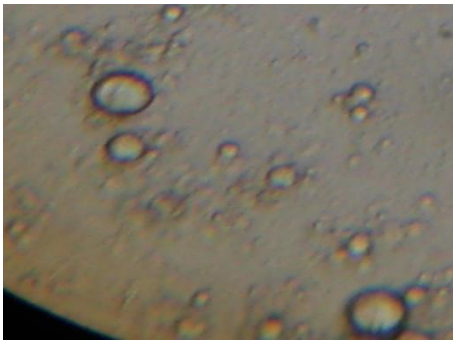
Table 3: Entrapment efficiency of optimized formulations of Stavudine niosomes after three months stability study

Entrapment efficiency (%)				
Formulation code	**Before stability studies**	**After stability studies**		
		At refrigeration temperature (4-8°C)	**At room temperature (25±2°C)**	**At oven temperature (45±2°C)**
T1	73.55	72.43	71.16	70.64
R3	64.12	63.85	62.36	61.75

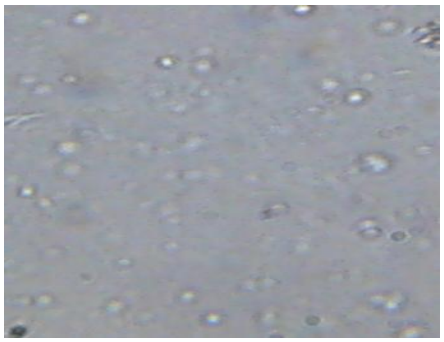
Table 4: *In vitro* release data of optimized formulations of Stavudine niosomes after three month stability study.

In-vitro release data (%)				
Formulation code	Before stability studies	After stability studies		
		At refrigeration temperature (4-8°C)	At room temperature (25±2°C)	At oven temperature (45±2°C)
T1	78.55	78.86	80.26	81.76
R3	78.12	79.05	81.02	81.94

Fig 1: Optical photomicrographs of niosome formulations (S3 and R2)

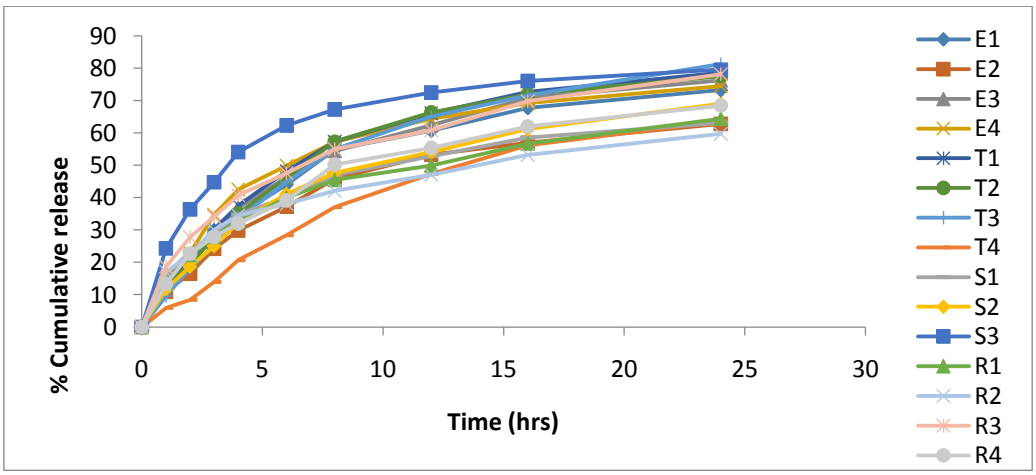


A) Optical photomicrograph S3



B) Optical photomicrograph R2

Fig 2: *In vitro* drug release profile of niosomal formulations prepared by diffrent methods



DISCUSSION

Vesicle size analysis of niosomes revealed that vesicles formed with Spans were smaller in size than vesicles formed with Tweens; this is due to greater hydrophobicity of Spans than Tweens. Further it is observed that niosomes with lesser amount of cholesterol were smaller in size than those niosomes of same surfactant having higher concentration of cholesterol (E1>E4, T1>T3,

S1>S4 and R1>R4). It is because Cholesterol can increase the chain order of liquidated bilayer and decrease the chain order of the gel state bilayer. Entrapment efficiency of vesicles mainly depends on the type of surfactants, amount of surfactant forming the bilayers and intrinsic properties of surfactants like HLB value, chemical structure, liphophilicity, phase transition temperature and alkyl chain length and cholesterol content. It was found that surfactants which have low HLB value, higher liphophilicity, higher phase transition temperature and longer alkyl chain length shown higher entrapment (Span 60). Entrapment efficiency of niosomes prepared with tweens was less than niosomes prepared with spans. It was also observed that variation in cholesterol content leads to variation in entrapment efficiency. Niosomes with low concentration of cholesterol showed decreased entrapment efficiency than those niosomes having same surfactant but higher amount of cholesterol (E1>E4, T1>T3, S1>S4 and R1>R4). It is because Cholesterol can increase the chain order of liquidated bilayer and decrease the chain order of the gel state bilayer. From *in vitro* release data it can be concluded that niosomes prepared with tweens showed higher release profile when compared to the niosomes prepared with spans of same concentration. This is due to fact that tweens are more hydrophilic having higher HLB value, shorter alkyl chain length and low phase transition temperature than spans. Further it is observed that niosomes prepared with same surfactant having surfactant:cholesterol ratio 120:80 shows higher release of drug than that of prepared with surfactant:cholesterol ratio 100:100. It is due to the fact that at higher concentration of cholesterol the rigidity of bilayer membrane increases and permeability decreases. The in-vitro release pattern of niosomes showed bi-phasic release with an initial burst effect over the first hour. Thereafter, drug release followed a steady pattern. Stability study results revealed that there was an overall increase in the drug release. These results may be attributed to phase transition of surfactant and lipid causing vesicles leakage to some extent during storage. It was found that no significant variations were observed in the entrapment efficiency (%) and *in vitro* release values when niosome formulations were stored at refrigeration temperature.

CONCLUSION

Stavudine loaded niosomes were prepared with aim to improve the availability of the drug at the site of action, sustain the drug release and to improve the dose related side effects. From the results it was concluded that stavudine loaded niosomes gave promising results with respect to vesicle size, entrapment efficiency, *in vitro* release studies and stability studies. Potential application of the prepared noisome are reducing dosing frequency, increased bioavailability, sustained release of drug, drug delivery to the target cell, minimal side effects and increased patient compliance.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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