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Original Article

STAVUDINE LOADED NONIONIC SURFACTANT VESICLES: OPTIMISATION OF FORMULATION, EFFECT OF PROCESS VARIABLES AND CHARACTERISATION

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ABSTRACT

Objective: The present work deals with the preparation, characterization and optimization of stavudine loaded nonionic surfactant vesicles (niosomes) for improvement in therapeutic index and efficacy of stavudine.
Methods: Stavudine loaded niosomes were prepared by employing different methods using cholesterol and surfactants. The formulations were characterized for vesicle size, entrapment efficiency, in *vitro* drug release profile and stability under specific conditions of temperature and humidity. Further formulations were analyzed for effect of process variables like type and concentration of surfactant, concentration of cholesterol and method of preparation on vesicle size, drug entrapment efficiency and in vitro drug release profile.
Results: Vesicle size analysis revealed that vesicles were descrete and spherical. Vesicles formed with Spans are smaller in size than vesicles formed with Tweens. The entrapment efficiency of the formulations was found in between 29.33%- 68.50%. The formulation RF1 showed the highest entrapment efficiency with 68.5%. The cumulative percent drug release was observed to be in-between 67.45% to 83.46% in 24 hrs. Highest cumulative percent drug release was observed for formulation TF2 with 83.46%. Stability study indicated 4-8ºC is the most suitable condition for storage of Stavudine loaded niosomes.
Conclusion: The results of the study revealed that stavudine loaded niosomes are capable of releasing the drug for extended period of time

Keywords: HIV/AIDS, niosomes, thin film hydration, in vitro release, and 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) [1]. HIV is a retrovirus that primarily infects components of the human immune system such as CD4+ T cells, macrophages and dendritic cells [2].Principal of therapy is long-term suppression of HIV replication. Stavudine is a synthetic thymidine analogue reverse transcriptase inhibitor that is devoid of 3'-OH group 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 very high bioavailability with short half life of 2.3 hours [3]. 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 capable of releasing the drug gradually can be helpful to overcome the problem [4].

Stavudine first converted to its triphosphate derivative by kinase enzyme of host cell. Then the derivative competes with viral nucleoside triphosphate for access to viral reverse transcriptase (RT) enzyme and hinders the production of c-DNA from RNA. Hence the drug blocks the HIV replication and infection of the new cells [5].

Targeted drug delivery system improves efficacy of the drug and reduce side effects [6]. Therefore, targeting of drug selectively to the diseased cells is expected to increase the therapeutic efficiency as well as decrease the side effects resulting from the interaction of drug with normal cells [7]. Current pharmaceutical scenario is aimed at development of drug delivery systems with maximum therapeutic benefits for safe and effective management of diseases. The concepts are based on controlled drug delivery [8].The controlled rate & mode of drug delivery to pharmacological receptor and specific binding with target cells as well as bioenvironmental protection of the drug to the site of action are specific features of targeting [9]. The vesicular systems are preferred over other formulations because of their specific characteristics such as lack of toxicity, biodegradation, capacity of encapsulating both hydrophilic and lipophilic molecules, capacity of prolonging the existence of the drug in the systemic circulation by encapsulation in vesicular structures, capacity of targeting the organs and tissues, capacity of reducing the drug toxicity and increasing its bioavailability [10,11].

Niosomes are nonionic surfactant vesicles in aqueous media resulting in closed bilayer structures that can be used as carriers of amphiphillic and lipophilic drugs. Niosomes are microscopic lamellar structures formed on admixture of non-ionic surfactant of the alkyl or dialkyl polyglycerol ether class and cholesterol and the enclosed interior usually contains a buffer solution at appropriate pH. In niosomes, the vesicles forming amphiphile is a non-ionic surfactant stabilized by addition of cholesterol and small amount of anionic surfactant such as dicetyl phosphate [12].The surfactant molecules tend to orient themselves in such a way that the hydrophilic ends of the non-ionic surfactant point outwards, while the hydrophobic ends face each other to form the bilayer [13]. The surfactant bilayer has its hydrophilic ends exposed on the outside and inside of the vesicle, while the hydrophobic chains face each other within the bilayer [14]. Liposomes are expensive; their ingredients like phospholipids are chemically unstable because of their predisposition to oxidative degradation. So niosomes are now widely studied as an alternative to liposome to overcome the drawbacks related to liposomes [15].The present study was aimed to prepare stavudine loaded nonionic surfactant vesicles.

MATERIALS AND METHODS

Stavudine was supplied as gift sample by HETERO Labs Ltd, Hyderabad, INDIA. Cholesterol was purchased from S.D Fine Chemicals Pvt Ltd, Mumbai, INDIA. All other chemicals were of analytical grade.

Preparation of non-ionic surfactant vesicles (niosomes)

Niosomes were prepared by employing different methods. Surfactants and cholesterol were taken in different ratios whereas drug concentration was kept constant i.e. 100:100:100 and 120:80:100 respectively. Span 20, span 80 and tween 80 surfactants were used. Cholesterol was used as a stabilizer of the bilayer membrane and to prevent leakage. The following methods were used for prepation of niosomes:

Modified ether injection method

In this method cholesterol and surfactant at different ratios (100:100 and 80:120) were dissolved in 10 ml of chloroform and was injected slowly at the rate of 0.25 ml/ min through 14 gauge needle in 15 ml of hydrating medium (phosphate buffer pH 7.4) containing stavudine (100 mg). The solution was stirred on magnetic stirrer by maintaining the temperature at 60 0C. As the lipid solution was injected slowly into aqueous phase, the vaporization of solvent takes place, resulting in spontaneous vesiculation and formation of nonionic surfactant vesicles. Different batches of nonionic surfactant vesicles were prepared in order to optimize the formulaton [16].

Sonification method

It is the prominent method where vesicles have been produced by sonification of the solution. The drug solution in buffer was added to surfactant and cholesterol mixture in a 10 ml vial. The prepared mixture is sonicated at 600Cfor 3 min using bath sonicator which yields small and uniform sized niosomes [17].

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 vaccum evaporator. The thin layer of cholesterol and surfactant was formed on the inner wall of round bottom flask. The aqueous phase phosphate buffer solution containing stavudine was added to the flask as hydrating medium. The flask was shaken for 15 minutes at 700C. This leads to the formation of milky white niosomal suspension [18].

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 and was 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 ratary vaccum evaporator. The semisolid gel like mass formed was then diluted with PBS then left for 30 minutes. All vesicle preparations were carried out at about 60◦C.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 [19].

Evaluation of Stavudine loaded niosomes

Size and shape analysis

Vesicle size of reconstituted niosomes 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 [20].

Entrapment efficiency

To determine percentage drug entrapment of Stavudine in niosome vesicles, 5 ml of niosomal dispersion was centrifuged at 15,000 rpm for 1 hour at 4° C in a centrifuge tube. The pellet settled at the bottom was collected and the supernant liquid were collected separately. The supernant liquid containing free unentrapped drug was measured using UV-visible spectroscopy method. The back calculation was made to determine the entrapped drug remained in the pellet [21]. The percentage drug entrapment was calculated as per Equation,

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

In Vitro Release

The *in vitro* release rate was determined by dialysis tubing method using glass tube of diameter 1.5cm with an effective length of 8cm that was previously covered with cellophane membrane. Measured amount of niosomes were placed in the cylinder. The cylinder was placed in 200 ml of phosphate buffer solution, pH 7.4, which acted as receptor compartment. The cylinder was arranged in such a way that, it just touches the surface of buffer solution. The temperature of receptor medium was maintained at 37±1°C and agitated at 50rpm speed using magnetic stirrer. Aliquots of 5ml sample were withdrawn at 1,2,3,4,6,8,12,16 and 24 hours. At each sampling time, the volume of receptor compartment was maintained with an equal volume of phosphate buffer solution, pH 7.4. The drug in withdrawn samples was estimated by UV Spectrophotometer at 266 nm [22].

Stability Studies

Stability studies are done to determine the stability of niosomes by storing them at different conditions like temperature and humudity. Throughout the study period, optimized niosomal formulations SF1 and RF2 were stored in aluminium foil-sealed glass vials. Formulations were stored at 4-8^{o}C in a refrigerator, at 25± 2 ^{o}C and at 45±2°C in humidity control oven (Lab Care, Mumbai). After one month, the drug entrapment efficiency and *in vitro* release of the formulations were determined by the methods discussed previously [23].

RESULTS

Physicochemical evaluation of Stavudine loaded niosomes

Stavudine loaded niosomes were prepared by various methods like modified ether injection method (EF1, EF2, EF3 and EF4), thin film hydration method (TF1, TF2 andTF3), sonification method (SF1, SF2, SF3 and SF4) and modified reverse phase evaporation method (RF1, RF2, RF3 and RF6). Formulations were optimized on the basis of vesicle size, entrapment efficiency and in vitro release profile. Vesicle size of the niosomes was determined by optical microscopy. Average size of niosomes prepared with tween 80 was largest compared to other formulations (Table2). It was also 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. Formulations prepared with span 20 shown higher entrapment efficiency than other formulations in all methods. Formulation RF1 has the maximum entrapment efficiency i.e.68.5% whereas EF4 has minimum i.e.29.33% (Table2).The entrapment efficiency decreases with increase in surfactant ratio and decrease in cholesterol ratio in all methods (Table2). Overall, higher entrapment efficiency was observed with the formulations prepared with reverse phase evaporation method than with the ether injection method.

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

Formulation code	Methods	Span 20	Span 80	Tween 80	Cholesterol	Stavudine
EF1	Modified	100			100	100
EF2	ether		100		100	100
EF3	injection			100	100	100
EF4	method			120	80	100
TF1	Thin film	100			100	100
TF2	hydration		100		100	100
TF3	method	120			80	100
SF1		100			100	100
SF2	Sonification		100		100	100
SF3	method			100	100	100
SF4		120			80	100
RF1	Modified	100			100	100
RF2	reverse		100		100	100
RF3	phase			100	100	100
RF4	evaporation method	120			80	100

Table 2: Evaluation of Stavudine loaded niosomes

Formulation code	Surfactant: cholesterol ratio	Vesicle size(µm)	Entrapment efficiency (%)	Drug release (%)
EF1(span 20)	100:100	5.8	45.77	75.43
EF2(span 80)	100:100	3.71	36.44	68.41
EF3(tween 80)	100:100	6.17	31.55	76.26
EF4(tween 80)	120:80	6.11	29.33	78.43
TF1(span 20)	100:100	4.31	46.30	79.50

TF2(span 80)	100:100	1.8	44.9	83.46
TF3(span 20)	120:80	3.74	45.5	82.29
SF1(span 20)	100:100	3.12	63.9	67.45
SF2(span 80)	100:100	1.45	63.3	68.76
SF3(tween 80)	100:100	3.79	56.2	68.95
SF4(span 20)	120:80	2.54	53.4	80.01
RF1(span 20)	100:100	2.79	68.5	76.05
RF2(span 80)	100:100	1.58	67.1	77.71
RF3(tween 80)	100:100	2.81	67.0	78.12
RF4(span 20)	120:80	2.69	67.8	78.42

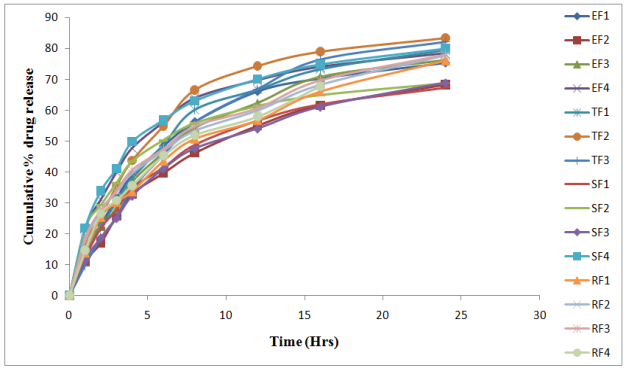

Fig 2: *In vitro* drug release profile of niosomal formulations prepared by diffrent method.

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

Entrapment efficiency (%)				
		After stability studies		
Formulation code	**Before stability studies**	**At refrigeration temperature (4-8°C)**	**At room temperature (25±2°C)**	**At (45±2°C) temperature**
SF1	63.9	63.5	63.1	62.4
RF2	67.1	66.5	66.1	65.5

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

In-vitro release data (%)				
		After stability studies		
Formulation code	**Before stability studies**	**At refrigeration temperature (4-8°C)**	**At room temperature (25±2°C)**	**At (45±2°C) temperature**
SF1	67.45	67.55	67.65	67.61
RF2	77.71	77.81	77.92	77.88

In vitro release study

The *in vitro* release rate was determined using glass tube of diameter 1.5cm with an effective length of 8cm that was previously covered with cellophane membrane in a beaker containing 200 ml of phosphate buffer solution (pH 7.4). The drug release across the cellophane membrane was slow from the drug loaded niosomal vesicles. Formulations EF1, EF2, EF3 and EF4 prepared by modified ether injection method showed release of 75.43, 68.41, 76.26 and 78.43% respectively in 24 hrs. Formulations TF1, TF2 andTF3 prepared by thin film hydration method showed release of 79.50, 83.46 and 82.29% respectively in 24 hrs (table 2). Formulations SF1, SF2, SF3 and SF4 prepared by sonification method showed release of 67.45, 68.76, 68.95 and 80.01% respectively in 24 hrs. Formulations RF1, RF2, RF3 and RF4 prepared by modified reverse phase evaporation method showed release of 76.05, 77.71, 78.12and 78.42% respectively in 24 hrs. The formulation prepared by tween 80 has shown higher release of drug among the formulations of respective method. In all methods of preparation the release pattern follows tween 80>span 80>span 20. In vitro release profile is shown in fig 2.

Stability studies

The stability study results revealed that formulations are relatively more stable at 4 ± 8oC than the other conditions. Entrapment efficiency of the formulation SF1 was 63.5, 63.1 and 62.4% at 4-8°C, 25±2°C and 45±2°C storage condition respectively after one month of stability study compared to the initial entrapment efficiency (63.9%). Similarly for formulation RF2 entrapment efficiency was 66.5, 66.1 and 65.5% at 4-8°C, 25±2°C and 45±2°C storage condition respectively after one month of stability study compared to the initial entrapment efficiency (67.1%). At storage condition 4-8°C, 25±2°C and 45±2°C the in vitro release of formulation SF1 was 67.55, 67.65 and 67.61% respectively & RF2 was 77.81, 77.92 and 77.88% respectively.

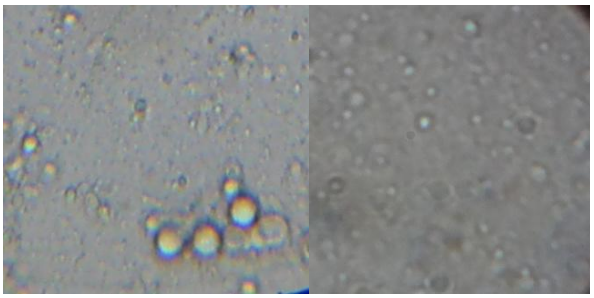

Fig1: Optical photomicrograph of niosome formulations (SF4 and RF4)

DISCUSSION

Results of vesicle size of niosomes indicated that vesicle formed with Spans is smaller in size than vesicles formed with Tweens; this is due to greater hydrophobicity of Spans than Tweens. The results also reveal that the niosomes prepared using span 20 is larger in size than niosomes prepared using span 80. A mean size of niosome increases proportionally with increase in HLB value of surfactant like span 80 (HLB-4.3) and span 20 (HLB-8.6) because surface free energy decreases with increase in hydrophilicity of surfactant. Further it is observed that niosomes with lesser amount of cholesterol in it is smaller in size than those niosomes of same surfactant having higher concentration of cholesterol in it (EF3>EF4, TF1>TF3, SF1>SF4 and RF1>RF4). It is because Cholesterol can increase the chain order of liquidated bilayer and decrease the chain order of the gel state bilayer. It was also observed that overall vesicle size of noisome prepared by reverse phase evaporation method is smallest and that of prepared by ether injection method is largest.

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 having low HLB value, higher liphophilicity, higher phase transition temperature and longer alkyl chain length shows higher entrapment. Thus depending upon these properties niosomes prepared with Span20 shown higher entrapment efficiency than span 80. Entrapment efficiency in the case of niosomes prepared with tweens was less than niosomes prepared with spans. It was also observed the variation in entrapment efficiency of drug due to variation in cholesterol content. Niosomes with lesser content of cholesterol showed decreased entrapment efficiency than those niosomes having same surfactant but higher amount of cholesterol (EF3>EF4, TF1>TF3, SF1>SF4 and RF1>RF4). It is because Cholesterol can increase the chain order of liquidated bilayer and decrease the chain order of the gel state bilayer. It was also noted that overall entrapment efficiency of niosome prepared by reverse phase evaporation method is higher and that of prepared by ether injection method is lower.

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. The burst release in the first hour can be attributed to the drug present on vesicular surface as well as to the unentrapped drug in the niosomal suspension.

The stability studies were carried out for the optimized formulations SF1 and RF2 at 4-8oC in a refrigerator, room temperature 25 ± 2 ºC and 45 ± 2 ºC in humidity control oven (Lab Care, Mumbai, India) as per ICH guidelines for a period of one month. 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. From the stability data it can be concluded that 4-8ºC is the most suitable condition for storage of Stavudine loaded niosomes.

CONCLUSION

Stavudine loaded niosomes were prepared 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. Hence, niosome formulations of Stavudine showed promising results under *in vitro* conditions and thus there exist a scope for evaluation of the developed niosomel formulations for pharmacokinetics studies using appropriate test models.

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