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Research Article

Formulation and optimization of novel vesicular drug delivery system of acyclovir using 3^2 factorial design

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ABSTRACT

Acyclovir, the first agent to be licensed for the treatment of herpes simplex virus infections, is the most widely used drug for infections such as cutaneous herpes, genital herpes, chicken pox, varicella zoster infections with an oral bioavailability of only 10 to 20 % (limiting absorption in GIT to duodenum and jejunum), half-life about 3 hrs, soluble only at acidic pH (pKa 2.27). Liposomal drug delivery systems of acyclovir have been designed and optimized using 3^2 full factorial designs. PEGylated Liposomes were prepared by thin film hydration method. From the preliminary trials, the constraints for independent variables X_1 (amount of Acyclovir), X_2 (amount of Phospholipids) and X_3 (amount of cholesterol) have been fixed. The derived polynomial equations for Particle size, % drug entrapment and zeta potential were verified by check point formulation. The prepared formulations were further evaluated for drug content, in vitro drug releases pattern, and stability. The application of factorial design gave a statistically systematic approach for the formulation and optimization of PEGylated Liposome desired particle size and high entrapment efficiency. Concentration of drug, Lipid and cholesterol were found to influence the particle size and entrapment efficiency of acyclovir loaded PEGylated Liposome. The release was found to follow first order release kinetics with Higuchi mechanism for optimized batches. These preliminary results indicate that acyclovir loaded PEGylated Liposome could be effective in sustaining drug release for a prolonged period.

Keywords: Acyclovir; 3^2 Factorial Design; PEGylated Liposomes; sustained release

INTRODUCTION

Acyclovir, the first agent to be licensed for the treatment of herpes simplex virus infections, is the most widely used drug for infections such as cutaneous herpes, genital herpes, chicken pox, varicella zoster infections. Acyclovir is currently marketed as capsules (200 mg), tablets (200, 400 and 800 mg) and topical ointment (T Tran et al., 2004). Oral acyclovir is mostly used as 200 mg tablets, five times a day. In addition, long term administration of acyclovir (6 month or longer) is required in immune compromised to a patient with relapsing herpes simplex infection (Emmert DH et al., 2000). The presently available conventional therapy is associated with a number of drawbacks such as highly variable absorption and low bioavailability (10–20%) after oral administration (A. G. Wagstaff et al., 1994). Furthermore, with an increase in a dose, there is a decrease in bioavailability. Moreover, because the mean plasma half-life of the drug is 2.5 h, five times a day, administration is required. The main problem with the therapeutic effectiveness of acyclovir is its absorption, which is highly variable and dose dependent thus reducing the bioavailability to 10–20% (E. G. Jalon De et al., 2003). Acyclovir is soluble in acidic pH and is predominantly absorbed from upper gastro intestinal tract (GIT) to the duodenum to jejunum regions (S. Rossi et al., 2003). In order to make oral therapy of acyclovir more patients compliant there is a need of using different approaches like matrix tablets, nanoparticles (M. Ruhnese et al., 1985) and polymeric films (I. Fuertes et al., 2006).

Constant efforts have been perceived in order to design such an ideal drug delivery system, which improves therapeutic index of drugs and also improves patient compliance. One such area which has attracted increasing attention of pharmaceutical scientist and has shown very promising results is “Targeted Drug Delivery System” (Chein Y W et al., 1992). Amongst all carriers utilized for target oriented drug delivery, vesicular drug delivery system in form of liposomes is most extensively investigated. Though liposome has been proven to be a versatile carriers for a wide variety of drug, there are two major inherent drawbacks of conventional liposomes is rapid uptake of the drug carrier by phagocytic cells of RES and Poor or shorter circulation time (Poste G et al., 2003; Senior JH 1987; Papahadjopoulos D et al., 1987).

The inherent drawback of conventional liposome can be overcome by using a novel approach of surface

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modification by using sterically stabilized liposomes or "Stealth Liposome". The most promising results of liposome modification were achieved for avoiding RES detection and prolonging retention in circulation by covalent attachment of hydrophilic polymer groups to the liposome surface to inhibit interaction with blood proteins and other plasma components and thus liposome uptake by the macrophage system is inhibited. And there is an increase in their circulation time. Longer circulation of liposome in blood resulted in higher uptake of drug in an implanted tumor (Poznansky M et al., 1984; Gabizon A et al., 1988; Gabizon, A et al., 1989). In short, short, their purpose is two-fold: (1) to increase the bioavailability of drugs or supplements by bypassing the digestive tract, and (2) to minimize any potential toxicity or side effects of these agents by remaining in the circulation for a long time and releasing their payloads slowly. As a bonus, they're passively targeted to tumors and to inflamed tissues, where they're preferentially absorbed because of the increased permeability of the capillaries that nourish these tissues.

In the present investigation, Phospholipone 80 was used as lipid. Liposomes were prepared by thin film hydration method. From the preliminary trials, the constraints for independent variables X_1 (amount of Acyclovir), X_2 (amount of Phospholipone 80) and X_3 (amount of Cholesterol) has been fixed. The derived polynomial equations for Particle size, % drug entrapment and zeta potential were verified by check point formulation. The prepared formulations were further evaluated for shape, in vitro drug release pattern and short-term stability. The application of factorial design gave a statistically systematic approach for the formulation and optimization of PEGylated liposome's with desired particle size and high entrapment efficiency.

MATERIAL AND METHOD

Materials

Acyclovir was a gift sample from Glenmark Pharmaceuticals Ltd., Mumbai (India). Phospholipone-80 was obtained as a gift from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol and Polyethylene Glycol (PEG) were purchased from HiMedia (Mumbai, India). Chloroform & Methanol were purchased from Finar Chemicals Ltd. (Ahmedabad, India). All other solvents and chemicals used were of analytical grade and purchased from commercial sources.

Method

Method of Preparation of PEGylated Liposome by using thin film hydration method

The weighed quantity of phospholipids and cholesterol was dissolved in mixture of anhydrous chloroform & methanol (3:1 v/v) in a sterile round bottom flask, and subjected to evaporation at 45°C for 2 h using rotary evaporator (Evator, Medica Instruments). The thin film formed was kept in vacuum drier for 24 h to ensure complete removal of chloroform from the film. The film was allowed to hydrate using PBS (pH 7.4) containing 150 mg Acyclovir and 15% w/v mannitol as a cryoprotectant by hand shaking for 10 minutes and further kept for 1 h at room temperature. The formed liposomes were subjected to sonication for 15 minutes for size reduction. The non-entrapped drug was removed by centrifugation (Remi- R- 8C), at 2000 rpm for 1 h at 4°C temperature; this step is called as liposome purification (Seth AK et al., 2002; Gonzalez-Mira E et al., 2011). Final liposomal dispersion was filled in sterile glass vials covered with special stoppers for lyophilization. The liposomal dispersions were preserved by addition of sodium azide 0.05 % w/v related to totaling aqueous phase. (Arunothayanun P et al., 1999)

Experimental Design

Experimental design and response surface methodology is an important tool to identify various factors influencing the responses by carrying out limited number of trial. In this work, a 2^3 factorial design (3factors -2 Levels) was significant to identify formulation variables affecting size, entrapment Efficiency and the zeta potential of Acyclovir containing PEGylated Liposome. (Poste G et al., 2003; Senior JH 1987; Papahadjopoulos D et al., 1987; Poznansky M et al., 1984; Gabizon A et al., 1988; Gabizon, A et al., 1989). Three independent factors, the concentration of Acyclovir (X_1), the concentration of Phospholipone 80 (X_2), and the concentration of cholesterol (X_3), were set at two different levels. High and low levels of each factor were coded as 1 and - 1, respectively. The range of a factor must be chosen in order to adequately measure its effects on the response variables. This design was selected as it provides sufficient degrees of freedom to resolve the main effects as well as the factor interactions. Stepwise regression analysis was used to find out the control factors that significantly affect response variables. The response is measured for each trial and then interactive equation is fitted by carrying out multiple regression analysis and F-statistic to identify statistically significant terms.

$$Y(size)=\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Second order polynomial equation… 1

Where Y is dependent variable, β_0 arithmetic mean response of eight batches, and β_1 estimated coefficient for factor X_1. The main effects (X1, X2 and X3) represent an average result of changing one factor at a time from its low to high value. The interaction term (X_1X_2) shows how the response changes when two factors are simultaneously changed.

Independent factors: Concentration of Acyclovir (X_1), concentration of Phospholipone 80 (X_2) and concentration of cholesterol (X_3)

Dependent variable**:** Y_1(Size); Y_2(% entrapment efficiency) and Y_3 (Zeta Potential).

Factor combination as per the experimental design for Acyclovir is shown in Table 1.

IN VITRO CHARACTERIZATION

Size

The size analysis and polydispersity index (PDI) of Acyclovir loaded PEGylated liposome was performed PCS (Photon correlation Spectroscopy) using Malvern Zeta Sizer Nano ZS (Malvern Instrument, UK) (Arunothayanun P et al., 1999). Diluted liposome suspension was added to the sample cuvette and then cuvette is placed in zeta sizer. Sample is stabilized for two minutes and reading was measured. The average particle size was measured after performing the experiment in triplicate.

Percentage Entrapment Efficiency

The Liposome dispersions were subjected to high speed centrifugation at 15000 rpm for 45 min at 4^0c using Beckman Coulter, Allegra 64R Centrifuge USA. The supernatant containing the free drug was isolated, and after suitable dilution with methanol, analyzed for drug content using HPTLC Method. The encapsulation capacity is the amount of drug that got entrapped out of a total amount of drug added during liposome preparation with respect to the total concentration of lipids used in liposome preparation.(Benoit JP et al., 2002). The encapsulation capacity values were calculated by using the following equation:

$$Encapsulation\ Capacity = \frac{Amount\ of\ Encapsulated\ Drug}{Total\ Amount\ of\ Drug\ Added} \times 100$$

Zeta Potential

The surface charge on liposome dispersion were measured using Malvern Zeta Sizer Nano ZS (Malvern Instrument, UK). The liposomes were dispersed in DDW and the Zeta Potential was measured. The magnitude of zeta potential gives an indication of potential stability of a colloidal system hence particles in suspension have large negative or positive zeta potential tends to repel each other there by inhibiting flocculation or aggregation. (Molpeceres J et al., 2000)

SURFACE MORPHOLOGY

TEM (Transmission Electron Microscopy)

TEM analysis was performed for optimized Liposome Dispersion (SL-2), using Philips CM 200 Transmission Electron Microscopy. Initially, Copper Grid coated with Carbon Film was immersed into SL-2 Dispersion. After being stained by 2% Phosphotongustic acid (PTA) solution. It was dried at room temperature for about 45 mins. And then morphology investigation was performed. (Carstensen JT, 1995)

SEM (Scanning Electron Microscopy)

Shape and Surface morphology of lyophilized SL-2 was performed using Scanning Electron Microscope, VEGA MV2300/40 (TES 5130MM. TESCAN). The liposomes were fixed on a double sized sticky tape that was previously mounted on aluminum stubs and the coated gold in argon atmosphere. (Arunothayanun P et al., 1999; Benoit JP et al., 2000)

FTIR

FTIR (FourierTransform Infrared) spectroscopy is a failure analysis technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. FTIR spectra of pure drug (Acyclovir), cholesterol and prepared PEGylated Liposomes were recorded using Jasco FTIR-4100 instrument. About 2–3 mg of sample was mixed with dry potassium bromide and the sample was scanned through the wave number range of 4000–400 cm^{-1}.

DSC

DSC experiments were carried out to evaluate thermal properties and to characterize the physical state of drugs in pure form and in liposomal formulations. (Fannin TE et al., 1981; Subramanian N et al., 2004).Differential scanning calorimetric (DSC) studies were carried out using differential scanning calorimeter (Model number TA - 60, Shimadzu, Japan) for the samples, such as pure drug Acyclovir, Phospholipone 80, cholesterol, acyclovir loaded vesicles. For this purpose 2 mg of samples of each was sealed thematically in standard aluminum pans. Thermograms were obtained at a scanning rate of 5°C/min. Each sample was scanned between 25°C and 225°C using nitrogen as the purge gas. For calibrating enthalpy, indium was sealed in aluminum pan with sealed empty pan as a reference. Lactose is used as a cryoprotectant. (Padamwar MN et al., 2006)

In Vitro Drug Release

The in vitro release of Acyclovir from liposomes dispersion (SL-2) was determined by dialysis method. After reconstituting the freeze dried liposomes in 10ml PBS (pH 7.4), an aliquot of each liposomal dispersion was placed in a dialysis tube (Himedia Laboratories Pvt. Ltd., Mumbai) with molecular weight cutoff 14000 Da. Then, dialysis tube was immersed in a beaker containing 200 ml of release medium, i.e. PBS (pH 7.4) and stirred with a magnetic stirrer at 150 rpm to maintain sink condition. The samples (5ml) were taken at predetermined time intervals from release medium and replaced by same volume of a fresh medium. Concentration of Acyclovir was determined after filtering the samples through 0.22µm syringe filter and was assayed by HPTLC Method (Dhobale Shankar M. et al., 2013). Dissolution test details for dissolution are summarized in Table-2.(Anthony Armstrong N et al., 1996; Stensrud G et al., 2000)

RESULT AND DISCUSSION

In the present experiment acyclovir loaded, PEGylated liposomes were prepared by using thin film hydration method. All eight batches of acyclovir loaded PE-

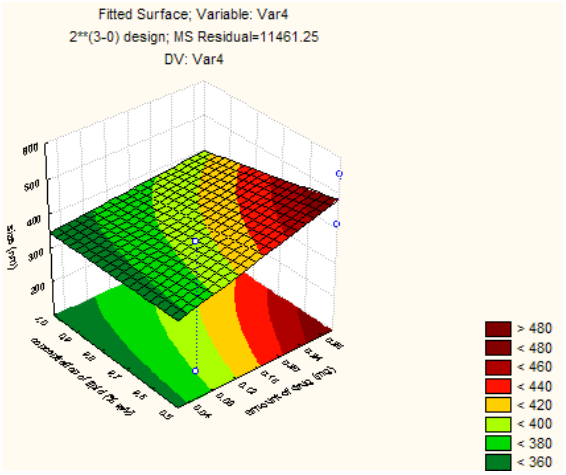

Figure 1: Response surface plot showing effect of factorial variables on Size

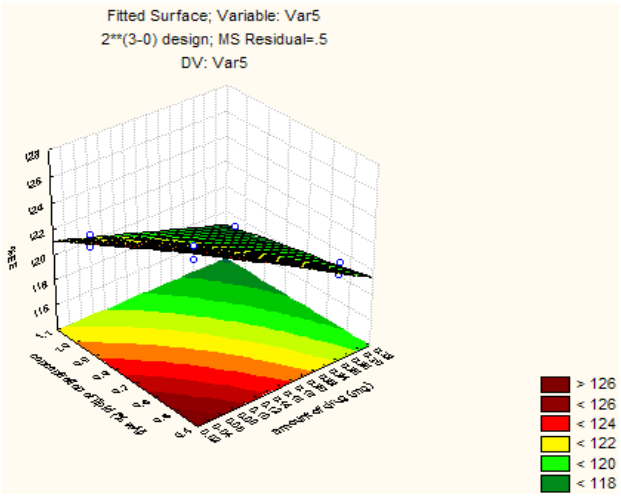

Figure 2: Response surface plot showing effect of factorial variables on % drug entrapment

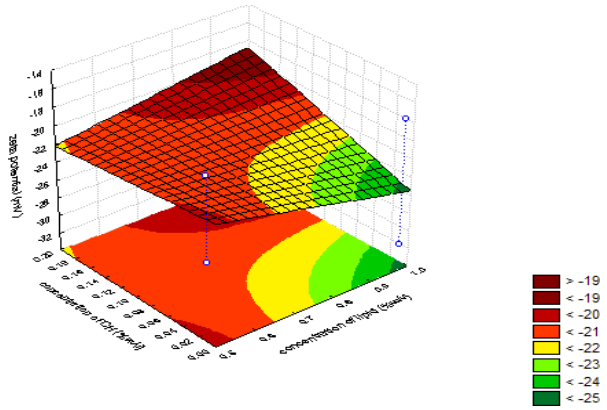

Figure 3: Response surface plot showing effect of factorial variables on zeta potential

Gylated liposomes were homogeneous and opaque white in color. A 23 full Factorial Design was applied in the Liposomal formulation process. All batches of liposomes were evaluated for size, % entrapment efficiency and Zeta Potentialshown in table 3. Out of all batches SL-2 was used for further study.

Size-Based on PDI Value of 0.189, Batch (SL-2) revealed a narrow and unimodal size distribution. (fig.1)

Y_1 (size) =399.00+ $39.75X_1$-$25.25X_2$+$164.50X_3$-$18.50X_1X_2$-$8.75X_1X_3$+ $38.75X_2X_3$…..2

Y_1 the average particle diameter of different batches of Stealth Liposomes ranged from 143±4.8 to 280±4.1 nm as summarized in table 3. The influence of formulation variables on the particle size of the PEGylated Liposome is presented in Eq. (2) and found to be statistically significant (p value 0.088, r^2=0.9156). The particle size analysis revealed positive relationship with all the three formulation variables namely, an amount of drug (X_1), lipid conc. (X_2) and Cholesterol conc. (X_3). However, as evidenced by a strong positive coefficient for X_1 and X_3 in eq. 2, the amount of drugs and the stabilizer

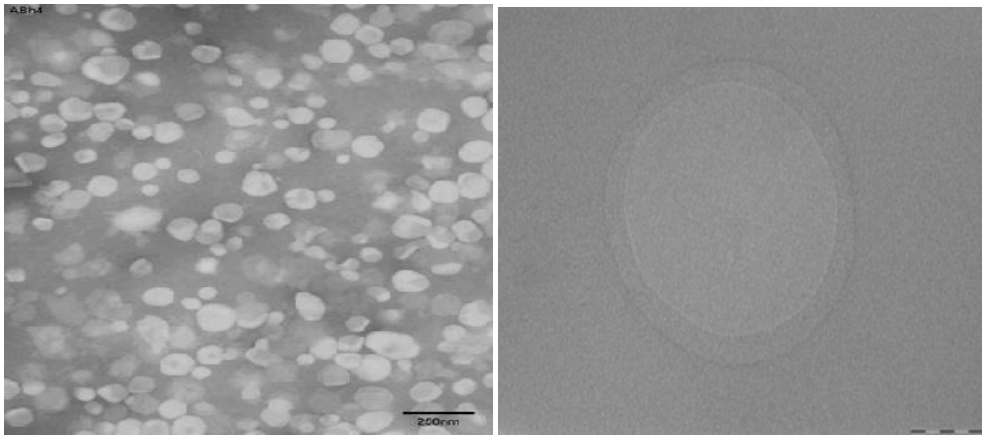

Figure 4: TEM image of PEGylated cholesterol stabilized liposomes

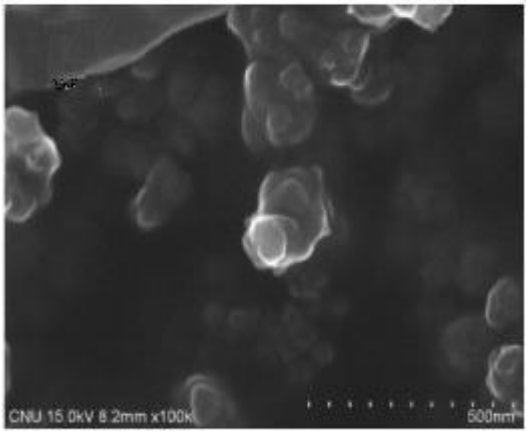

Figure 5: SEM of PEGylated Liposome (SL-2)

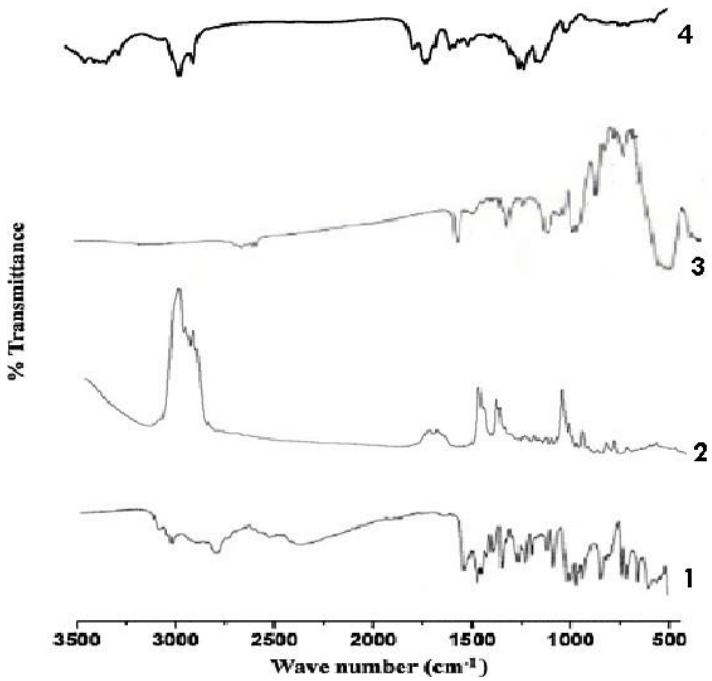

Figure 6: FTIR spectra of [1] Free Acyclovir, [2] free Cholesterol, [3] Free Phospholipid and [4] PEGylated Liposome

concentration are the major factors influencing size of liposomes.

A concentration dependent increase in particle size with the stabilizer could be the result of deposition of the pluronic F127 onto a lipid vesicle. On the other hand, and increasing lipid concentration also lead to a concentration dependent increase in the particle size. This was in agreement on the report of MullarGoy-

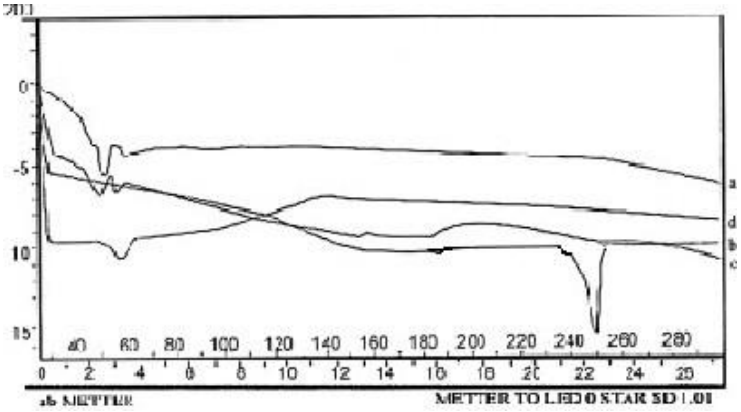

Figure 7: DSC analysis of (a) PEGylated liposomes, (b) Free Acyclovir (c) cholesterol and (d) Phospholipone 80

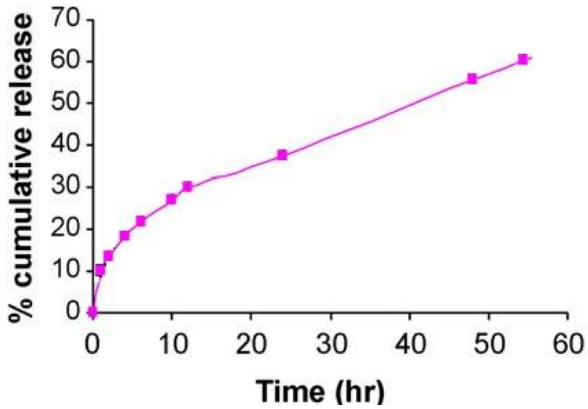

Figure 8: In vitro Release of Acyclovir from PEGylated Liposome

Table 1: Factor combination as per the experimental design for Acyclovir

Batch	Coded Value*		
	X_1	X_2	X_3
SL-1	1	1	1
SL-2	1	1	-1
SL-3	1	-1	1
SL-4	1	-1	-1
SL-5	-1	1	1
SL-6	-1	-1	1
SL-7	-1	-1	-1
SL-8	-1	1	-1

mann besides, the size distribution analysis of Liposome dispersion revealed a narrow and unimodal distribution with PDI value of 0.1

Response 2: Percentage Entrapment Efficiency-The percent entrapment efficiency was calculated mathematically by using following equation:

Y_2 (entrapment efficiency) $=121.00-2.500X_1-1.50X_2+0.25X_3+0.50X_1X_2-0.25X_1X_3+0.25X_2X_3$.....3

These parameters are dependent upon the process of preparation, physicochemical properties of drug, and formulation variables. The drug entrapment efficiency varied from 81.7% to 99.87% and showed a good correlation coefficient (0.9981). (Fig.2) Results of the equation indicate that the effect of X_1 is more significant than X_2.

Response 3: Zeta Potential-The Zeta Potential (mV) was calculated mathematically by using following equation:

Y_3 (Zeta Potential) $= -21.59-11.41X_1-6.41X_2-3.45X_3-8.84X_1X_2+0.086X_1X_3-1.90X_2X_3$.....4

The experimental data shown in Table VII reflects that zeta potential values are influenced by lipid composition. Zeta potential values of about -14.30 to -23.8 were observed, which is probably related to steric effect of the Phospholipid. (Fig. 3)

TEM of SL-2 showed spherical morphology with smooth surface (Fig. 4). However, the particles appeared smaller in diameter when compared to PCS measurement. This was obvious as PCS gave a hydrodynamic diameter which is generally higher than a ge-

Table 2: Dissolution test details for dissolution of PEGylated Liposome

Sr.No.	Specification	Standard values
1	Apparatus	Dialysis Tube
2	Speed	150 rpm
3	Volume of media	200 ml
4	Dissolution Media used.	PBS (pH 7.4)
5	Stirrer	Magnetic Stirrer
6	Aliquot taken at each time interval	5 ml.
7	Temperature	37+ 0.5°C.
8	Time	Up to 60 hrs.

Table 3: Results of batches of acyclovir loaded PEGylated liposomes

Batch	Code			Size (nm) (Y_1)	Entrapment Efficiency (%) (Y_2)	Zeta Potential (Y_3)
	X_1	X_2	X_3			
SL-1	(+1)	(+1)	(+1)	280	93.7	-16.5
SL-2	(+1)	(+1)	(-1)	217	89.13	-18.1
SL-3	(+1)	(-1)	(+1)	187	87.14	-18.4
SL-4	(+1)	(-1)	(-1)	190	91.12	-14.3
SL-5	(-1)	(+1)	(+1)	124	99.87	-23.8
SL-6	(-1)	(-1)	(+1)	160	86.12	-21.9
SL-7	(-1)	(-1)	(-1)	135	84.11	-20.1
SL-8	(-1)	(+1)	(-1)	143	81.7	-17.3

*All values are expressed as Mean ±SD, n = 3

Table 4: Variables in 3^2 factorial Design

Independent Variable	Level	
	Low	High
X_1: Amount of Acyclovir (mg)	10	150
X_2: Amount of Phospholipone80 (mg)	30	60
X_3: Amount of Cholesterol (mg)	06	12
Response Variable		
Y_1= Size Y_2= % Entrapment Efficiency Y_3= Zeta Potential		

nuine diameter while during TEM analysis, most of the water was removed, and analysis was performed on the dried sample. This may explain smaller diameters in case of TEM. SEM reveals that the liposomes were found to be well dispersed and spherical (Fig.5). The particle size was significantly smaller andmore uniform. FTIR spectra of free drug, cholesterol, phospholipid and prepared PEGylated Liposomes showed insignificant changes (Fig.6). For pure drug major bands were observed at 3586.98 cm–1 (OH stretching), 3381.85 cm–1 (NH stretching), 2231.24 cm–1 (C-N stretching) and 1591.95 cm–1 (C=Ogroup). In case of free acyclovir and PEGylated Liposomes, there were no significant changes observed in the major bands. Thus, no interaction between the drug and other ingredients was evidenced from FTIR, and the chemical structure of the drug has not changed before and after the precipitation process.

DSC studies were indicated that acyclovir melting peak was depleted in the calorimetric curve of loaded liposomes, evidencing the presence of amorphous drug in the liposome samples. As the drug melting endotherms was not seeing there is possibility that the drug might be molecularly dispersed within lipid matrix (fig.7).In vitro drug release from acyclovir-loaded, PEGylated liposomes is shown in Fig. 8. It was found that the formulation produced an initial burst release in which acyclovir release was more than 12.2±1.5% for PEGylated liposomes within the initial sampling time (60 min). The burst release was observed, which might be due to the release of surface adsorbed drug. After the initial burst release, a constant drug release was found and maximum of 65.6±3.2% drug was released in 58 h of a time period with PEGylated. A Higuchi plot for the acyclovir-loaded PEGylated liposomes (cumulative amount released vs. square root of time) was linear.

SUMMARY AND CONCLUSION

In the present study, an attempt has been made for surface modified novel vesicular drug delivery system for Acyclovir, which will ensure reduced dosing frequency, improvement in oral bioavailability, increased efficiency and therapeutic index. Increased stability via encapsulation, selective passive targeting to lymphocyte and macrophages. The identity of Acyclovir was

confirmed by physical characteristics, spectrophotometric analysis such as UV spectrophotometric, HPTLC, FTIR, Thermal behaviour like melting point and DSC.A computer aided optimization process using 3^2 factorial design was employed to investigate the effect of three independent variables, i.e., an amount of acyclovir, an amount of Lipid and Amount of cholesterol. Size, % entrapment efficiency and zeta potential were taken as response variables. Total eight batches of liposomes were formulated as per 3^2 factorial designs. Each batch was evaluated for physical properties. Prepared Acyclovir loaded liposomes were evaluated for size, shape, % entrapment efficiency, zeta potential, % drug release and stability. After comparing the effect of concentration of Acyclovir, Phospholipid and cholesterol on an evaluation parameters of liposomes optimized formulation (SL-2) was selected because of better drug entrapment efficiency, size. The Optimized formulation (SL-2) was evaluated for SEM, DSC, FTIR, and XRD, and *in-vitro study.*

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