Design and Development of Vancomycin Liposomes

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ABSTRACT

Vancomycin hydrochloride is water soluble and poorly absorbable glycopeptide antibiotic act by inhibition of the synthesis of peptidoglycan a major component of bacteria cell wall. It is highly effective against the *Staphylococcus aureus* and other *Staphylococcus* species microorganisms. Structurally vancomycin hydrochloride has six peptide bonds with a molecular weight of approximately 1500 Da. Liposomes, the colloidal vesicular structures due to their biphasic environment can act as carriers for both lipophilic & hydrophilic drugs. The encapsulation of antimicrobials in liposomes potentially offers enhanced pharmacokinetics and pharmacodynamics and decreased toxicity. This delivery system has the advantages of targeted, long circulation, low toxicity, sustained-release, no immunogenicity and protecting the encapsulated drugs from the destructive action of the external media. The present research work is planned to develop liposomal formulation of Vancomycin hydrochloride and to study the possibility of permeability enhancement. Liposomes are prepared by using various permeation enhancers like propylene glycol, poly ethylene glycol 400, poly ethylene glycol 600, Tween 80 and Span 60. The prepared liposomes are characterized by optical microscopy, scanning electron microscopy, particle size determination, encapsulation efficiency, FTIR spectroscopy studies and *in vitro* diffusion studies using dialysis membrane. Among six different liposomes F2 formulation (containing propylene glycol) has showed promising results with respect to drug entrapment and percentage drug release.

Key words: Liposomes, Phospholipids Permeability Enhancement, Poly ethylene glycol, Propylene glycol, Surfactants, Vancomycin Hydrochloride.

INTRODUCTION

Vancomycin hydrochloride (vancomycin) is a glycopeptide antibiotic act by inhibition of the synthesis of peptidoglycan a major component of bacteria cell wall. It shows a high antibacterial activity against *Staphylococcus aureus* and other *Staphylococcus* species.[1-3] Vancomycin hydrochloride is considered for the treatment of septicimia, lower respiratory tract, skin, and bone infections caused by gram positive bacteria. Vancomycin hydrochloride is reported to be effective at a minimum inhibitory concentration of 2 µg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA).[4-9] Structurally Vancomycin hydrochloride has six peptide bonds with a molecular weight of approximately 1500 Da.[10] It is water soluble and poorly absorbed from the gastrointestinal (GI) tract.[10] There are very few investigations reported for improving the GI absorption of Vancomycin hydrochloride by using strategies such as multiple emulsions[11] liposomes[12] and using sodium glycocholate as absorption promoter.[13]

Liposomes, the colloidal vesicular structures due to their biphasic environment can act as carriers for both lipophilic & hydrophilic drugs. High hydrophilic drug (log p<-0.3) are located exclusively in the aqueous domains, whereas highly lipophilic drugs (log P>5) are entrapped within the lipid bilayers of the liposomes.[14] The encapsulation of antimicrobials in liposomes potentially offers enhanced pharmacokinetics and pharmacodynamics and decreased toxicity.[15] This delivery system has the advantages of targeted, long circulation, low toxicity, sustained-release, no immunogenicity and protecting the encapsulated drugs from the destructive action of the external media.[16-21] Hence in the present research work it is planned to develop liposomal formulation

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of Vancomycin hydrochloride and to study the effect of different permeability enhancers. Liposomes are prepared by solvent injection method using various excipients like soya lecithin, cholesterol and co-solvents like ethanol, chloroform, propylene glycol (PG), poly ethylene glycol 400 (PEG-400), poly ethylene glycol 600 (PEG-600) and surfactants like Tween 80 and Span 60 etc. The prepared liposomes are characterized by optical microscopy, scanning electron microscopy, particle size determination, encapsulation efficiency, FTIR spectroscopy studies and *in vitro* diffusion studies using dialysis membrane.

MATERIALS AND METHODS

Materials

Vancomycin hydrochloride was a gift sample from M/s. TherDose Pharma Private Limited, Hyderabad. Soya lecithin and dialysis membrane-50 (Molecular Weight. cut off 12000 to 14000) was obtained from M/s. Hi-Media Laboratories, Mumbai. Cholesterol was obtained from M/s. Finar Chemicals Limited, Ahmedabad. Chloroform, ethanol, propylene glycol, polyethylene glyco l400 and polyethylene glyco l600 were obtained from M/s. Sisco Research Laboratories Pvt. Ltd. Andheri (E), Mumbai. Tween 80 and Span 60 were obtained from M/s. Loba Chemie Pvt.Ltd. Potassium dihydrogen phosphate was obtained from M/s. Merck Specialities Pvt. Ltd., Mumbai. Sodium hydroxide was obtained from M/s. Qualigens Fine Chemicals, Mumbai. All other materials used in this study were of analytical grade.

Preparation of vancomycin liposomes

Liposomes were prepared by ethanol injection method using different formulations as shown in Table 1. In this method, initially weighed quantities of lecithin (50 mg) and cholesterol (20 mg) were dissolved in 10 ml volume of chloroform: ethanol (1:1) solvent mixture in a 50 ml beaker. To this lipid phase accurately weighed quantity of vancomycin (10 mg) was added and dissolved. In case of formulations F2 to F6, the co solvents and surfactants were added to lipid phase. In another beaker 10 ml of phosphate buffer pH 7.4 was taken and kept for stirring at 200 rpm on thermostatically controlled magnetic stirrer (Remi Magnetic Stirrer, Model: LBMS-5886) at a temperature of 45ºC. To this aqueous phase, drug containing lipid phase was added by injection at one jet. The mixture was continued stirring for 1 hour to allow the solvent evaporation and to obtain uniform vesicular dispersion. Finally the liposome dispersion was stored in airtight container at 2-8°C.

Characterization of Liposomes

Optical microscopy

The prepared vancomycin liposomes were viewed under phase contrast optical microscope (Olympus DSX 100) for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing a drop of liposome dispersion on a glass slide and cover slip was placed over it and this slide was viewed under optical microscope at 100X magnification. Photographs were taken to prepared slides using digital camera.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to characterize the surface morphology of the prepared vesicles. One drop of liposomal dispersion was mounted on a clear-glass stub, air-dried, coated with Polaron E 5100 sputter coater (Polaron, Watford, United Kingdom), and visualized under a scanning electron microscope (Leo-435 VP; Leo, Cambridge, United Kingdom

Table 1: Preparation of vancomycin liposomes formulation

INGREDIENTS	F1	F2	F3	F4	F5	F6
Lecithin	50 mg	50 mg	50 mg	50 mg	50 mg	50 mg
Cholesterol	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Chloroform	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
Ethanol	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
Vancomycin hydrochloride	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
Phosphate Buffer pH 7.4	10 mL	10 mL	10 mL	10 mL	10 mL	10 mL
PG	-	10 mg	-	-	-	-
PEG-400	-	-	10 mg	-	-	-
PEG-6OO	-	-	-	10 mg	-	-
Tween-80	-	-	-	-	10 mg	-
Span-6O	-	-	-	-	-	10 mg

Particle size determination

The mean particle size was obtained by particle size analyzer (Malvern). The instrument measures the particle size based on the laser diffraction theory. The apparatus consists of a He-Ne laser beam of 632.8 nm focused with a minimum power of 5 mW using a Fourier lens to a point at the center of multielement detector and a sample holding unit (Su cell). The sample was stirred using a stirrer before determining the vesicle size. The vesicle dispersion was diluted about 100 times in the deionized water. Diluted liposomal suspension was added to sample dispersion unit containing stirrer and stirred at high speed in order to reduce interparticles aggregation and laser beam was focused

Drug entrapment efficiency

The drug entrapment efficiency was calculated using the total drug content of liposome dispersion and unentrapped drug content of the dispersion. The total dug content of the dispersion is determined estimating total drug entrapped and unentrapped. 5 ml of liposome dispersion was taken in a volumetric flask. The dispersion was subjected to sonication in bath sonicator (M/s. Remi) for 30 minutes. Then the mixture was filtered and estimated after suitable dilution at 280 nm wavelengths by using UV Visible Spectrophotometer (Shimadzu, UV1800). For the free unenetrapped drug, 5 ml of the liposome dispersion subjected to centrifugation at 18000 rpm using Remi centrifuge for 40 min at 5^0C. The supernatant clear solution was collected separately and the free drug present in the supernatant was estimated after suitable dilution at 280 nm wavelength by using UV Visible Spectrophotometer. The entrapment efficiency of all the formulation was calculated by using following formula.

$$Entrapment\ Efficiency = \left\{1 - \frac{Unentrapped\ drug\ content}{Total\ drug\ content}\right\} x\ 100$$

Fourier Transform Infrared Spectroscopy (FTIR)

To investigate any possible interaction between the drug and the excipients utilized under investigation FTIR spectrophotometry was used. The IR Spectra of pure drug (vancomycin) and the combination of drug with excipients were carried out by using FTIR spectrophotometer on Spectrum II Perkin Elmer with KBr background. Sample preparation includes grinding a small quantity of the sample with a purified salt usually potassium bromide finely to remove scattering effects from large crystals. The powder mixture was crushed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer can pass. The pressed sample was carefully removed from the die and was placed in the FTIR sample holder. The IR spectrum was recorded from 4000 cm^{-1} to 400 cm^{-1}.

In vitro diffusion studies

In vitro diffusion studies were carried by using Franz diffusion cell appratus. The capacity of the receptor compartment was 20 ml and the area of the donor compartment exposed to receptor compartment was 1.41cm^2. Dialysis membrane-50 with molecular weight cut off 12000 to 14000 Da from Hi-Media Laboratories Pvt. Ltd having flat width of 24.26 mm and diameter of 14.3 mm with approximate capacity of 1.61 mL/cm was used for the study. The membrane was soaked overnight in phosphate buffer pH 7.4. 10 ml of prepared liposomal dispersion which contains 10 mg of drug was taken and placed in the donor cell. Dialysis membrane was placed in between donor cell and receptor cell. 20 ml of phosphate buffer (pH 7.4) was taken in receptor cell to touch the bottom surface of dialysis membrane. The temperature of the receptor phase was maintained at 37 $\pm$ 0.5^0C and the receptor compartment was stirred with magnetic stirrer to maintain homogeneous condition. The aliquots of 3 ml were withdrawn at different time intervals. Fresh medium was used to replace with equal volume of the sample withdrawn. The samples were analyzed at 280 nm in a UV-Visible spectrophotometer and amount of drug released at different time intervals was calculated.

Microbiological assay

The microbiological assay of vancomycin was carried out by cup plate method. The nutrient agar medium was prepared, sterilized and inoculated with *Staphylococcus aerius* micro-organism at a temperature between (40 to 50)^{0}C and immediately pored the inoculated medium into petri plates to give a depth of (4 to 5) mm uniformly and kept aside for solidification. Small cavities of 10 mm diameter were made on solidified agar petri plates by using sterilized cylinder shaped borer. 500 µl of the prepared standard solutions and sample solutions (i.e equivalent to 5 µg/ml and 10 µg/ml drug concentration) were added into each cavity. These petri plates are left for 1 to 4 hours at room temperature as a pre-incubation diffusion to minimize the effects of variation in time between different solutions. Prepared petri plates were incubated for 24 hours at 32-35^{0}C and measured the diameter of circular inhibited zones.

RESULTS AND DISCUSSION

Optical microscopy

The microscopy photographs of prepared liposomes formulations F2 and F3 (as shown in Figure 1) which were viewed under phase contrast optical microscope indicated the lamellar structure of liposome. The shape of the vesicles is spherical and the vesicles are discrete in distribution.

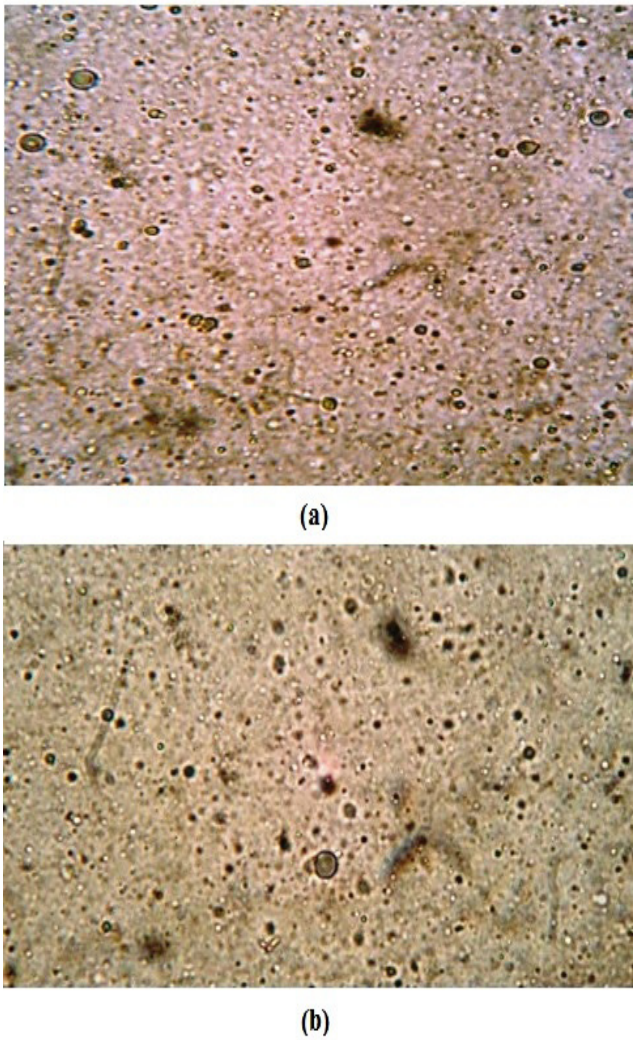


Figure 1: Optical Microscopic image of formulations (a) F2 (b) F3

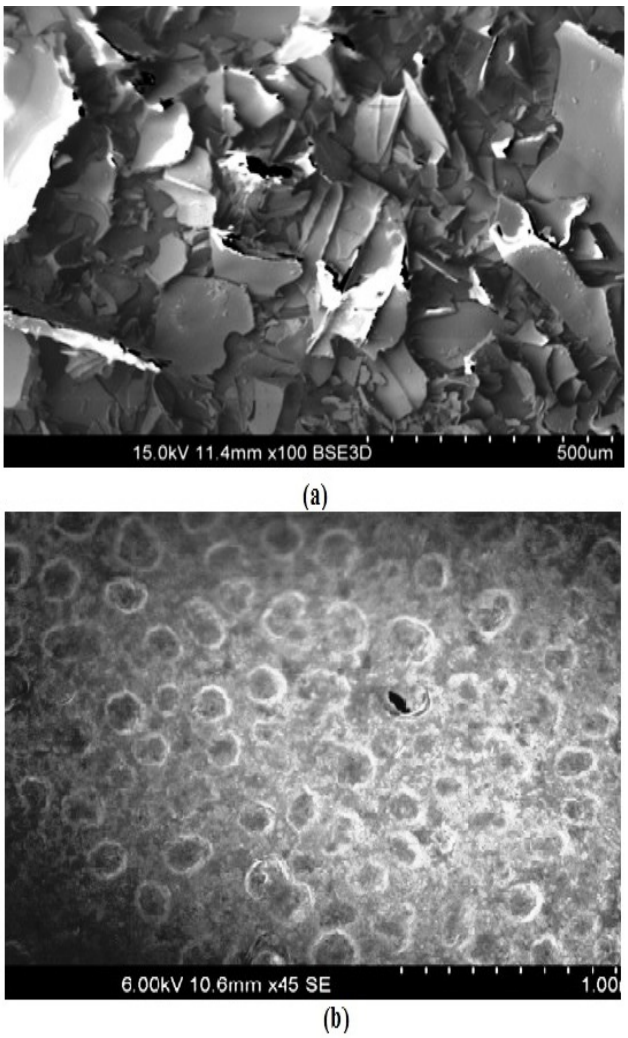


Figure 2: SEM Image of (a) vancomycin pure drug (b) F2 formulation

Scanning Electron Microscopy

The Surface morphology of prepared liposome formulation of F-2 and vancomycin pure drug was studied by using Scanning Electron Microscopy and the images are shown in Figure 2. The images indicated the pure drug is irregular in shape. The liposome formulation F-2 showed spherical structures with smooth regular surface.

Particle size determination

The particle size distribution analysis was performed by using particle size analyzer (Malvern) and the results (Figure 3) showed that the average particle size of the liposome vesicle for formulation F2 was found to be 78.3 nm with poly disparity index of 0.184. These results indicated that vesicle size is in nano particulate range and the size distribution is uniform.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra for vancomycin and vancomycin liposomes are shown in Figure 4. vancomycin FTIR spectra showed that the characteristics peaks of functional group COOH at 3387.38 cm^{-1}, R-CH_2-CH_3 at 2935.51 cm^{-1}, R-NH-R at 2842.19 cm^{-1}, R-CO-NH_2 at 1632.81 cm^{-1}, R-O-R at 1093.52 cm^{-1}, R-NH_2 at 687.81cm^{-1}. The FTIR spectra of prepared liposomes also showed all the major characteristic peaks at 3447.29, 2924.48, 2853.28, 1634.82, 1079.81, 668.28 cm^{-1} with minor shift. These results indicated that there is no interaction between drug and excepients used in the formulation.

Drug Content and drug entrapment efficiency

The percentage drug content and entrapment efficiency values are shown in Table 2. The total drug content liposome formulations F1 to F6 were found to be in range 94.21% to 98.62%. The entrapment efficiency (shown in Table 2) of formulations F1 to F6 was found to be in range 69.66% to 78.66%. Among these formulations; F2 i.e. liposomes prepared by using propylene glycol showed highest entrapment efficiency of 78.66%.

Size Distribution Report by Intensity

v2.1

Malvern

Sample Details

Sample Name:	Van
SOP Name:	NeP.sop
General Notes:	

File Name:	vk.dts	**Dispersant Name:**	Water
Record Number:	1871	**Dispersant RI:**	1.330
Material RI:	1.60	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.00	**Measurement Date and Time:**	Wednesday,09:04:2014

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	344.5	**Measurement Position (mm):**	0.85
Cell Description:	Disposable sizing cuvette	**Attenuator:**	4

Results

			Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	78.3	**Peak 1:**	81.8	100.0	106.7
PdI:	0.184	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.950	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

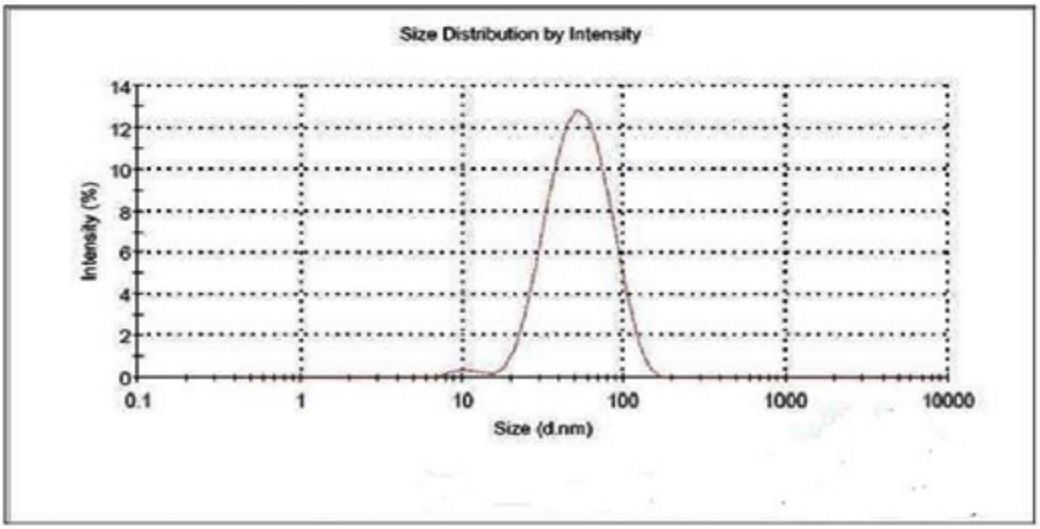


Figure 3: Particle size distribution of formulation F2

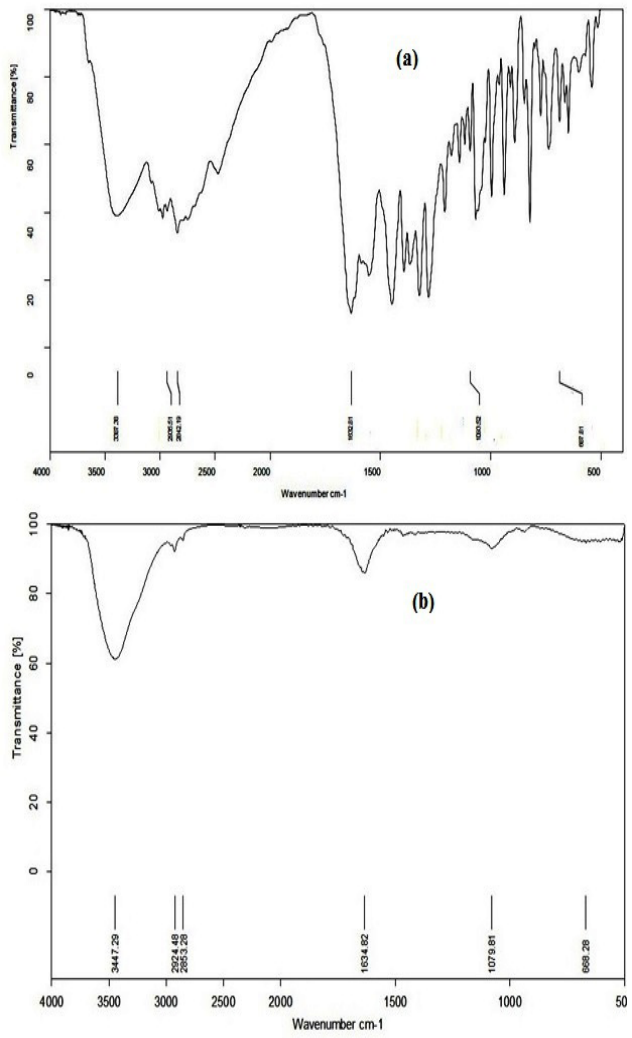


Figure 4: FTIR spectra of (a) vancomycin (b) formulation F2

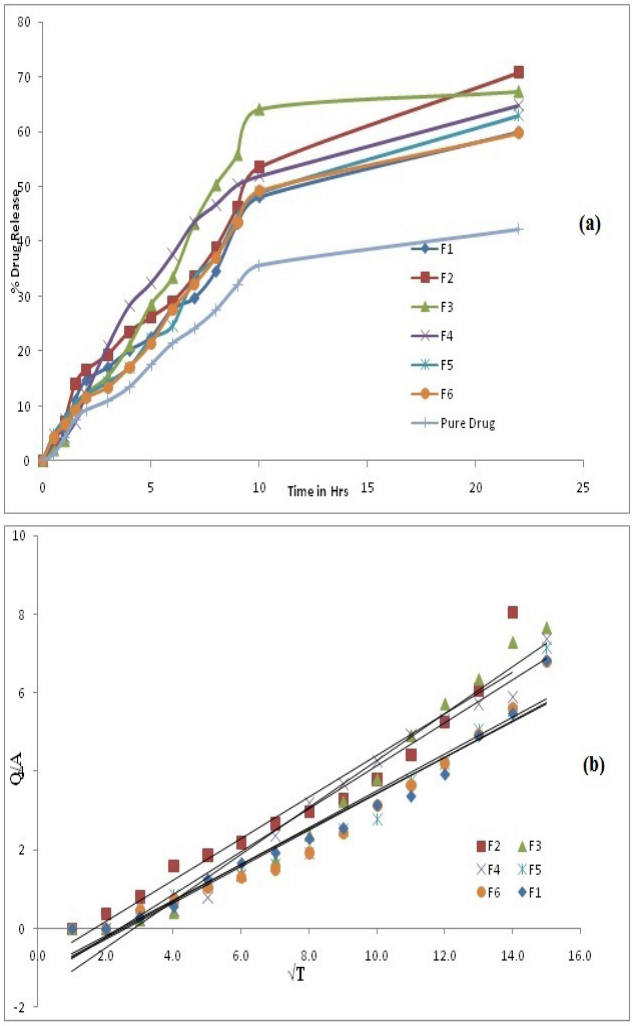


Figure 5: *In vitro* diffusion studies and diffusion constant of Prepared Liposomes

Table 2: Drug content, drug entrapment efficiency and Percentage drug of liposomal formulations

Formulation	Drug Entrapped	Drug Content	Percentage drug release at 22 hrs
F1	72.10	96.12	60.00
F2	78.66	98.62	70.68
F3	77.33	97.12	67.33
F4	75.66	96.65	64.77
F5	74.10	96.32	62.88
F6	69.66	94.21	59.66
Pure drug	-	-	42.30

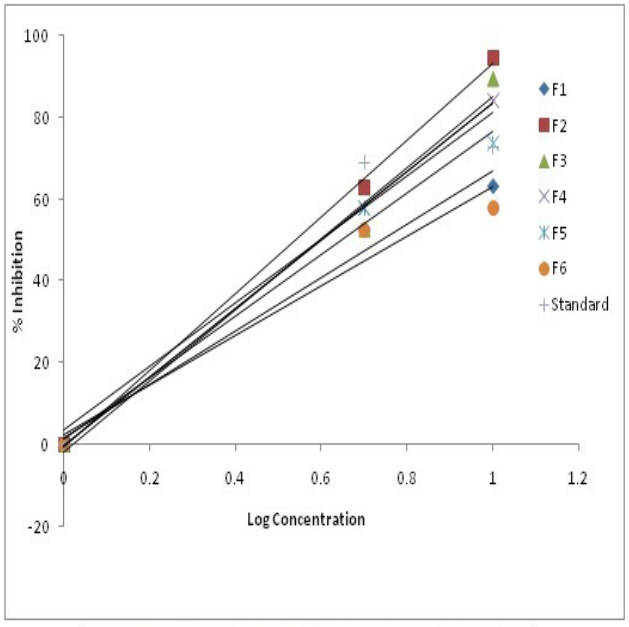
All the values are in %

Whereas formulation F6 i.e. liposomes prepared by span 60 showed lowest percentage entrapment efficiency of 69.66%.

In vitro diffusion studies of prepared liposomes

The results of *in vitro* drug diffusion studies are shown in Table 2. The drug diffusion through semi permeable membrane for the pure vancomycin was found to be 42.30 percent. Percentage drug release of formulations F1 to F6 were found to be in range (59.66 to 70.68)% at 22 hours. Among them the formulation F-2 showed highest percentage drug release of 70.68%. Graphs were plotted (as shown in Figure 5) by calculating diffusion rate constant with Q/A Vs square root of time, whereas Q is the percentage drug released and A is the area of Franz diffusion cell. These results sug-

Table 3: Percentage Zone of Inhibition for prepared formulations			
Formulation	**Concentration (µg/ml)**	**Log. Concentration**	**% Inhibition**
F1	5	0.70	52.63
	10	1.00	63.15
F2	5	0.70	63.15
	10	1.00	94.7
F3	5	0.70	52.63
	10	1.00	89.47
F4	5	0.70	57.89
	10	1.00	84.21
F5	5	0.70	57.89
	10	1.00	73.68
F6	5	0.70	52.63
	10	1.00	57.89
STD.	5	0.70	69.23
	10	1.00	73.07
	15	1.18	88.46
	20	1.30	100



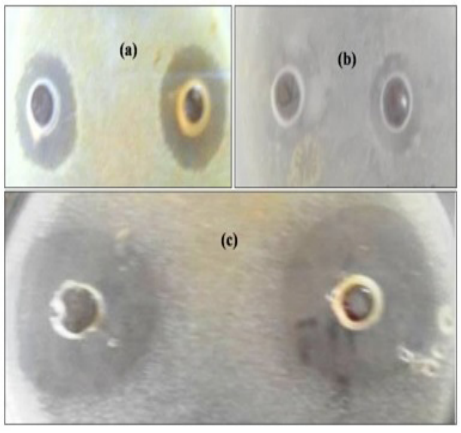


Figure 6: Microbiological assay studies and Zone of Inhibition for (a) Standard (b) Control (c) Formulation F2

gested that all types of co solvents such as PG, PEG-400, PEG-600, Tween-80, and Span-60 have enhanced the rate of percentage drug release than the pure drug. Among them the liposomal formulation prepared with PG (F2) has shown highest percentage of drug release. These results indicate the penetration ability of the drug through membranes.

Microbiological assay

The results in Table 3 showed that the percentage inhibition for standard solution was increased on increasing the drug concentration. The inhibition was found to be linear in the concentration range of 5 to 20 µg/ml. The percentage inhibition of formulations F1 to F6 at 5 µg/ml drug concentrations was found to be in range (52.63 to 63.15)% and at 10 µg/ml concentration the percentage inhibition was found to be in range (57.89 to 94.7)%. Among them the formulation F2 showed highest percentage with 94.7% zone of inhibition after 24 hours at 10 µg/ml concentration when compared with other formulations and control. Graphs from all formulations (as shown in figure 6) showed linearity with straight lines on plot between log concentration and % inhibition and showed that on increasing drug concentration the percentage of inhibition also increased. The results also showed that there was a steady release of the drug and is capable of inhibiting microorganism *staphylococcus aureus* for 24 hrs. This indicated that the prepared vancomycin liposomes are efficient and also can inhibit the growth of microorganism *staphylococcus aureus* due to high drug diffusion.

CONCLUSION

Vancomycin hydrochloride has good water solubility but fails to absorb due to poor permeability. In the

present work attempts were made to entrap the drug in liposome formulation using lecithin and cholesterol. Further the liposomes were prepared with incorporation of permeability enhancers propylene glycol, PEG-400, PEG-600, Tween-80 and Span-60. The prepared liposomes are uniform discrete with spherical vesicular structure. They showed good entrapment efficiency and *in vitro* drug diffusion. Among different formulations of vancomycin liposomes F2 formulation (containing propylene glycol) has shown maximum entrapment efficieny and *in vitro* drug release when compared to other formulations and pure drug. The microbiological study also suggested that the formulation F2 is best among the six formulations. The results clearly indicated the usefulness of liposome formulation containing propylene glycol as permeability enhancer for the improvement of vancomycin release through membranes.

CONFLICT OF INTEREST

There is no conflict of interest.

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