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Research Article

Preparation and characterization of amphotericin B mannosylated liposomes for effective management of visceral leishmaniasis

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Abstract

Visceral leishmaniasis (VL) is a chronic debilitating disease prevalent in tropical and subtropical regions, caused by protozoan parasites of the genus *Leishmania*. Annually, it is approximated the occurrence of 0.2 to 0.4 million novel cases of the disease worldwide. The cast film method was used to prepared cationic and mannosylated liposomes. The surface of the Amphotericin B (Amp B)-bearing cationic multilamellar liposomes was covalently coupled with p-aminophenyl- α -D-mannoside using glutaraldehyde as a coupling agent, which was proved by agglutination of the vesicles with concanavalin A. The prepared liposomes were characterized for shape, size, % drug entrapment, vesicle count, zeta potential and *in vitro* drug release. Vesicle sizes of cationic and mannosylated liposomes were establish to be 2.71 ± 0.12 and $1.62 \pm 0.08 \mu\text{m}$, respectively. Zeta potential of cationic liposomes was higher ($28.38 \pm 0.3 \text{ mV}$), as compared to mannosylated liposomes ($15.7 \pm 0.8 \text{ mV}$). % drug release from cationic and mannose-coupled liposomes was established to be 45.7% and 41.9%, respectively, after 24 hrs. In the present work, cationic and mannosylated liposomes of Amp B were prepared, optimized and characterized for effectual organization of VL.

Keywords: Mannosylated liposomes, Amphotericin B, Leishmaniasis, % drug release.

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INTRODUCTION

Leishmaniasis is caused by intracellular parasites of the *Leishmania* species and transmitted through the bite of sandflies. Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), post-kala-azar dermal leishmaniasis (PKDL), and mucocutaneous leishmaniasis (MCL) are the four main types of the disease. The most common form is CL, which causes cutaneous lesions, while the most severe form is VL also known as Kala-azar, which causes a fatal systemic disease is the most severe form of the disease, having a mortality rate of almost 100% in untreated cases. It is characterized by uneven bouts of fever, considerable weight loss, swelling of the spleen, liver and anemia. Available drugs for the treatment of leishmaniasis are toxic, need multiple injections and have limited efficacy. Additionally, drug resistance emerges in a few cases¹⁻³. Amp B though a widely used drug for the treatment of Leishmaniasis, suffers from major drawbacks such as chills, fever, nausea, hemolytic toxicity, nephrotoxicity and drug resistance and rapidly⁴. Amp B is a potent polyene antibiotic available as a micellar solution (Fungizone®), liposome (AmBisome®), which was the first liposomal drug delivery system, Amp B lipid complex (Abelcet®) and Amp B colloidal dispersion (Amphocil®), for effective treatment of VL⁵. Targeting of drugs offers enormous advantages, but is equally challenging, such as physiological barriers, biochemical challenges to recognize and validate the molecular targets

and the pharmaceutical challenges to devise appropriate techniques of conjugating targeting ligands to the nanosystems. The challenge in drug targeting is not only the targeting of drug to a precise site, but also keeping it for the preferred duration to elicit the desired pharmacological action⁶. For Leishmaniasis, the macrophages are the macrophages of reticuloendothelial source, so a liposomal delivery system has to be intended in such a way that they are simply recognized by macrophages since of active targeting. Liposomal drug delivery systems have entertained substantial attention due to their precise attractions, including effective encapsulation with an extensive variety of hydrophobicity levels and pKa values, extending and targeting release of remedial agents and minimizing clinical drug dose and reducing toxic effects. Liposomal drug delivery systems are not only enabled to deliver higher drug concentrations at the target cells or organs, but also lower drug concentrations in the kidneys and lungs, which reduce the toxicity of Amp B⁷. It was anticipated that ligand-mediated active targeting to the macrophages would considerably enhance the rate and extent of drug accumulation to the macrophages. It may also lesser the required doses of liposomal Amp B in diseases such as hepatosplenic fungal infections and Leishmaniasis associated with granulocytopenia. Mannose/fucose receptors, expressed abundantly in the liver, spleen, and alveolar macrophages, have been most widely utilized for bioactive targeting to the macrophages⁸. The receptor assists

the endocytosis of glycoproteins, which are finished with fucose, mannose, and glucosamine. The mannose-grafted liposomal form was judged for more efficient active targeting to the macrophages and would significantly increase the rate and extent of macrophage accumulation of drug⁹. Mannose-bearing liposomes have proved additional proficient in the transportation of drugs, contrasted to ordinary liposomes. Toxicity studies also demonstrate no apparent drug toxicity in mannose-bearing liposomal forms. This may reduce the need doses of liposomal Amp B in diseases such as hepatosplenic fungal infections, leishmaniasis and pulmonary aspergillosis associated with granulocytopenia¹⁰. For the present study, mannosylated liposomes were selected for active targeting of drug. This method is hypothesized to give improved permeation and active targeting to the reticular endothelial system (RES), which, in turn, would give a direct assault of the drug to the site where the pathogen resides, rendering the other organs free and secure from the toxic manifestations of the drug. In the current work, cationic and mannosylated liposomes of Amp B were prepared, optimized and characterized for effectual management of visceral leishmaniasis.

MATERIALS AND METHODS

Amp B was kindly provided as a gift sample from Life Innovation Care, (H.P.). Soya phosphatidylcholine (PC; 98%), cholesterol (CH; 95%), chloroform, methanol, dimethyl sulfoxide (DMSO), stearylamine (SA), p-aminophenyl- α -D-mannoside, and concanavalin A were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All additional chemicals were of analytical grade and used as procured.

Determination of λ_{\max} of Amp B

Accurately weighed 10 mg of drug was dissolved in 0.5 ml DMSO and volume of the solution was adjusted to 10ml with methanol in 10 ml of volumetric flask. The resulted solution 1000 μ g/ml and from this solution 1 ml pipette out and transfer into 10 ml volumetric flask and volume make up with methanol solution prepare suitable dilution to make it to a concentration range of 1-10 μ g/ml. The resulting solution was scanned in the range 200-600 nm using Shimadzu (1601) UV/Visible spectrophotometer.

FTIR spectroscopy

Infrared spectrum of any compound gives information about the groups present in that particular compound. IR spectrum of Amp B was taken out using KBr pellets. Various peaks in IR spectra were interpreted for different groups and were matched with reference IR spectra¹¹.

Solubility studies

Solubility is defined in quantitative terms as the concentration of solute in a saturated solution at a certain temperature and in qualitative terms it may be defined as the spontaneous interaction of two or more substances to form a v/v homogeneous molecular dispersion¹². Solubility of Amp B was determined in different solvents. Amp B (10 mg) was suspended in 10 ml of different solvents in tightly closed test tubes. These tubes were shaken for about 72 hrs using Wrist action Shaker (Yorco, New Delhi) and solubility was determined.

Preparation of multilamellar liposomes

Multilamellar vesicles (MLVs) containing Amp B were prepared by the method described by Lopez-Berestein et al., 1983¹³. Dissimilar formulations were made by taking lipids viz. soya phosphatidylcholine (PC), cholesterol (CH) in dissimilar molar ratios. These were dissolved in minimum quantity of chloroform: methanol (2:1) mixture in a RB flask

and then methanolic solution of drug (80 μ g/ml) with minimum amount of DMSO was added. A thin film of the lipids was casted on the inner surface of the round bottom flask by evaporating the solvent under reduced pressure using rotary flash evaporator (Stereoglass Rotavap, Italy). The flask was rotated continuously until the film was dried. Final traces of solvents were taken away under vacuum, when stored overnight. The dried lipid film was hydrated with 10 ml of PBS (pH 7.4) followed by continuous vortexing of the flask for about an hour to obtain multi lamellar liposomes. Liposomal suspension was allowed to stand for further 3 to 4 hrs in dark at RT to allow complete swelling of the vesicles. The suspension was then centrifuged at 2000 rpm using sephadex G-75 minicolumn to take away the free drug and stored in dark at low temperature. The PC: CH ratio was optimized which was then utilized for preparation of cationic liposomes. Cationic liposomes containing Stearylamine were also prepared in the same way by dissolving Soya PC, Cholesterol and Stearylamine in minimum quantity of chloroform: methanol (2:1) mixture and further procedure was followed in a similar way as that mentioned for the multilamellar liposomes. Cationic multilamellar vesicles were prepared and the optimized formulation of cationic MLV was utilized further to produce mannose coupled liposomal formulation. Here the molecule of mannose is liganded to the surface of cationic liposomes through chemical linkage there by resulting in mannose coupled / mannosylated liposomes.

Preparation of mannosylated multilamellar liposomes

The surfaces of the Amp B bearing multi lamellar liposomes were covalently coupled with p-aminophenyl- α -D-mannoside. Covalent coupling of p-aminophenyl- α -D-mannoside to the surfaces of liposome was carried out according to the method of Torchilin *et al.* 1978¹⁴. Cationic liposome (1ml) suspension (30 mg lipid/ml 0.025M sodium phosphate buffer pH 7.2 containing 0.15M NaCl) was mixed with 20 mg of p-aminophenyl- α -D-mannoside. Glutaraldehyde was added slowly to the liposome suspension upto 15 mM final concentrations and the mixture was incubated for 5 min at 20°C. The NH₂ group of cationic liposomes was coupled with NH₂ group of p-aminophenyl- α -D-mannoside using glutaraldehyde as coupling agent¹⁵. Uncoupled sugar derivatives and glutaraldehyde were removed by dialysis against the same buffer.

Optimization of various parameters

Different formulation and the process variables viz. PC: CH ratio, stearylamine to PC: CH ratio and sonication time were optimized to get small multilamellar liposomes with maximum drug entrapment efficiency and maximum vesicle count. The PC: CH ratio was optimized by preparing liposomes by above- mentioned method using various ratio of PC: CH (Table 1) and they were characterized for vesicle size and drug entrapment efficiency. Likewise, the concentration of Stearylamine and sonication time was optimized by varying the PC: CH: SA ratio from 7:3:0.5 to 7:3:2.5 (Table 2) and sonication time from 30 to 120 second (Table 3) to get small vesicles with high drug entrapment efficiency. The prepared liposomes were characterized for vesicle size using laser diffraction based particle size analyzer (Cilas, 1064 L, France) and drug entrapment efficiency. On the basis of optimization studies, formulation LIP3C* was selected for conjugation of its surface with p-aminophenyl- α -D-mannoside according to the procedure already mentioned. The mannose coupled liposomes (LIP 3 C-M*) were characterized for average size, shape and percent drug entrapment (Table 4).

Table 1: Optimization of PC: cholesterol ratio

S. No.	Formulation Code	PC:CH Ratio	Average size (µm)	% Entrapment	No. of vesicles per mm ³ X1000
1	LIP 1	9:1	2.22±0.11	57.67±2.8	27
2	LIP 2	8:2	2.56±0.12	60.35±3.0	31
3	LIP 3*	7:3	2.69±0.13	62.15±3.1	39
4	LIP 4	6:4	2.72±0.12	61.67±3.0	25
5	LIP 5	5:5	2.74±0.13	59.87±2.9	15

*Optimum parameter, value expressed are mean ± SD where n= 3

Table 2: Optimization of SA ratio

S. No.	Formulation Code	PC:CH:SA	Average size (µm)	% Entrapment	No. of vesicles per mm ³ X1000
1	LIP-3A	7:3:0.5	2.42±0.12	56.05±2.8	34
2	LIP-3B	7:3:1.0	2.56±0.13	57.20±2.6	32
3	LIP-3C*	7:3:1.5	2.71±0.12	65.32±3.3	35
4	LIP-4D	7:3:2.0	2.85±0.14	61.33±3.0	27
5	LIP-5E	7:3:2.5	2.93±0.15	51.56±2.5	21

*Optimum parameter, value expressed are mean ± SD where n= 3

Table 3: Optimization of sonication time in cationic liposomes with regard to percent drug entrapment

S. No.	Formulation Code	Sonication Time (seconds)	Average size (µm)	% Entrapment	No. of vesicles per mm ³ X1000
1	LIP 3C	30	2.69±0.13	43.31±2.1	22
2	LIP 3C	60	1.71±0.08	41.40±1.9	27
3	LIP 3C*	90	0.94±0.04	40.15±2.2	30
4	LIP 3C	120	0.35±0.01	30.15±1.6	31

*Optimum parameter, value expressed are mean ± SD where n= 3

Table 4: Characterization of mannositated liposomes

S. No.	Formulation Code	Average size (µm)	% Entrapment	No. of vesicles per mm ³ X1000
1	LIP 3 C-M*	1.62±0.08	39.12±1.9	25

Characterization

Vesicle shape

Coupled liposomes were visualized by Philips Morgani 268 Transmission Electron Microscope. A drop of the dissimilar formulations was placed on dissimilar carbon coated copper grids to leave a thin film on the grids. Then, the film was negatively stained with 1% phosphotungstic acid (PTA) by placing a drop of the staining solution on to the film and the excess of the solution was drained off with a filter paper. The grid was allowed to dry thoroughly and formulations were viewed under a transmission electron microscope and photographs were taken at suitable magnification.

Vesicles Size and size distribution

The size and size distribution of vesicles was established by laser diffraction particle size analyzer (Cilas, 1064 L, France). The liposomal suspension was dispersed in distilled water and then it was put into the sample chamber of particle size analyzer and measurement of vesicular size was carried out.

Entrapment efficiency

Entrapment efficiency of Amp B in uncoupled and coupled liposomes was determined using the method described by Fry *et al.*, 1978¹⁶. According to the method, about 1gm sephadex G-75 was allowed to swell in 10 ml of 0.9% NaCl

solution in distilled water in a glass screw capped bottle for 5 hours at room temperature. The hydrated gel was filled to the top in the barrel of 1ml disposable syringe plugged with whatman filter pad. The barrel was then placed in the centrifuge tubes. The tubes were centrifuged at 2000 rpm for 3 minutes to remove excess saline solution. Eluted saline was removed from the centrifuge tubes and exactly 0.2 ml of liposome suspension (undiluted) was applied drop wise on the top of the gel bed in the center. Columns were again centrifuged at 2000 rpm for 3 minutes to expel and remove void volume containing liposome in to the centrifuge tubes. Elute was removed and 0.25 ml saline was applied to each column, and centrifuged as previously. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton-X100 solution followed by filtration and subsequent determination of the drug content using spectrophotometric method.

Vesicles count

To characterize liposomal formulation for vesicular count, the liposomal formulation was diluted (5times) with PBS (pH 7.4) and liposomes /mm³ were counted by optical microscopy. The liposomes in 80 small squares were counted and calculated using the following formula¹⁷.

Total numbers of liposomes/mm³: Total number of liposomes counted x Dilution x 4000/ Total number of squares counted

Zeta potential

The zeta potential of liposomal formulations was measured in 0.1 M of KCl buffer at 25°C, using the Zetasizer 3000 HS (DTS ver. 4.10; Malvern Instruments, Malvern, UK).

In vitro ligand agglutination assay

Mannosylated liposomes were assessed for *in vitro* ligand-specific activity by mannose-binding concanavalin A (Con A), as reported by Copland et al. 2003¹⁸, with slight modification. A 100-μL sample of the original liposomal dispersion (both cationic and mannose coated) was diluted 10 times with PBS (pH 7.4), and 1 ml of varying concentrations of Con A (100-700 μg/ml) in PBS containing 1 mM of MnCl₂ and 1 mM of CaCl₂ (pH 7.4) at 25°C. A time-dependent increase in turbidity for 4 hours was monitored spectrophotometrically (Shimadzu 1601 UV spectrophotometer, Japan) at 550 nm.

In vitro drug release

The in-vitro drug release profile of Amp B was studied for cationic and coupled liposomal formulations. The liposomal formulation was centrifuged to remove unentrapped drug from liposomes. Then, 1 ml of pure liposomal suspension was packed into dialysis tube, which in turn was placed in a beaker containing 20 ml of PBS (7.4 pH). The solution containing the dialysis tube was stirred on a magnetic stirrer while keeping the temperature constant at 37±1°C throughout the study. Samples were withdrawn at different time intervals with subsequent analysis of samples for drug content (at 405.9 nm) using Shimadzu 1601 UV spectrophotometer (Japan).

Stability studies

Stability is of immense importance in the development of pharmaceutical formulations and plays an important role in the final design of a safe and efficacious drug delivery system.

The stability study on prepared formulation was performed by storing the formulation at low temperature that is 4°C and room temperature 27±2°C for 45 days and formulations were assessed periodically for the change in vesicle size, number of vesicles and residual drug content.

RESULTS AND DISCUSSION

The drug was found to be yellow colored, odorless crystalline powder, which slowly got discolored on exposure to light. The absorption maximum of the drug Amp B in dimethyl sulfoxide and methanol were measured through Shimadzu (1601) UV/Visible spectrophotometer and was found to be 364.1 nm, 382 nm and 405.9 nm Figure 1. Solubility profile of the drug in different solvents at room temperature indicates that the drug was practically insoluble in water, ether, acetone, chloroform and slightly soluble in methanol, DMF and soluble in dimethyl sulfoxide (DMSO) and propylene glycol. Infra red spectrum of Amp B confirms the presence of different groups. The various peaks obtained in the IR spectrum matched with the IR spectrum given in the official pharmacopoeia Figure 2. The various formulation variables (PC: CH and SA: lipid ratio) as well as process variables (sonication time) were optimized to get small multi lamellar liposomes with high entrapment efficiency. The PC: Cholesterol ratio was optimized by varying the ratio of PC: CH from 9:1 to 5:5 and the formulations were characterized for vesicle size and entrapment efficiency. It is found that vesicle size increased with increase in the cholesterol content. The size is increased from 2.22 ± 0.11 to 2.74 ± 0.13 μm. while the drug entrapment efficiency was increased from 57.67 ± 2.8% to

62.15 ± 3.1% as on increasing the PC: CH ratio from 9:1 to 7:3, then it was decreased to 59.87 ± 2.9% on further increasing the ratio from PC:CH 7:3 to 5:5, similar effect was observed on vesicle count. The cholesterol is an important component for changing the fluidity of the bilayers of the liposomes. The cholesterol molecules are aligned between the molecules of PC and rigidized the bilayers of the liposomes. At the optimum ratio of PC:CH (7:3), PC bilayers are saturated completely with cholesterol molecules hence no further significant increase in vesicle size was observed vice versa the entrapment of drug was observed. More over the higher number of vesicle is also responsible for higher drug entrapment of the drug in vesicles. Similarly, the stearylamine amount was optimized to prepare cationic liposomes and 7:3:1.5 ratio of PC: CH: SA was found to be optimum with maximum entrapment efficiency of 65.32 ± 3.2%. The incorporation of stearylamine in the bilayers of liposome enhances the vesicles size of liposomes, which could be due to similar change in bilayers and repulsion in the bilayers of liposomes result in increase in size and drug entrapment efficiency. The formulations were sonicated for 30, 60, 90 and 120 seconds. The vesicle size reduced from 2.69 ± 0.13 μm to 0.35 ± 0.01 μm as on increasing the sonication time from 30 sec to 120 sec. It was observed that the percentage entrapment efficiency reduces with increase in the sonication time, which could be due to decrease in size. Formulation sonicated for 120 seconds lost its integrity and the percentage entrapment efficiency was decreased to very low values (30.15% for LIP-3C). Hence, 90 seconds was taken as optimum sonication time for uncoupled as well as coupled formulations. The liposomes LIP-3C* prepared using optimum and cationic charge lipid (SA) i.e 7:3:1.5 was utilized for coupling its surfaces with mannose molecules. Covalent coupling of p- aminophenyl-α-D-mannoside to stearylamine-liposome was carried out according to the method reported by Torchilin *et al.* 1978¹⁹. The presence of mannose residues on the surface of liposomes was detected by agglutination of the vesicles with concanavalin A. In the case of mannopyranoside-linked liposomes, the percent of total NH₂ that gets modified with the mannopyranoside linkage was determined by titrating amino groups of cationic liposomes with trinitrobenzene sulphonic acid in the presence of 0.1% triton X-100. The morphological studies exhibited that the uncoupled and coupled liposomes are spherical in shape as it can be clearly seen by transmission electron microscopy. The percentage drug release from cationic formulations viz. LIP3-A, LIP3-B, LIP3-C*, LIP3-D was 5.4%, 5.1%, 4.9%, 4.1% at an interval of 2 hrs and 50.6%, 49.3%, 45.7%, 41.5% at an interval of 24 hrs, respectively. The mannose coupled liposomes show 3.9% and 41.9% drug release in 2 hrs and 24 hrs, respectively. The reduction in drug release for the coupled liposomes as compared to the uncoupled is due to the enhancement of membrane integrity and the layer of the mannose on the liposomal surface, which have impact on the drug diffusion kinetics across the liposomal membrane. The lower values of percentage drug release in the case of mannose-coupled liposome than uncoupled liposomes may be due to greater membrane integrity of the liposomes when they are coupled with mannose. Zeta-potential measurements of cationic liposomes reveal that these liposomes exhibit positive zeta potential (28.38 ± 0.3 mV), and mannosylated liposomes showed lower zeta potential (15.7 ± 0.8 mV). Cationic liposomes were positively charged because of the incorporation of amine functionalities via SA at the surface. Moreover, coating with mannose shields the positive charge, and hence, zeta potential may have been lowered. The average vesicular size was found to increase on storage, which was least in case of the formulations stored at 4°C than those, stored at 27±2°C. This can be related to fusion of

vesicles, which increases with increase in temperature, indicating fusogenicity to be a temperature dependent process. Hence, ideal storage condition was found to be at 4°C. The number of vesicles in formulations stored at 27±2°C was reduced to a larger extent when compared to those stored at 4°C. This can be attributed to the vesicle disruption or fusion of vesicles (mentioned earlier) at high temperature as revealed by the microscopic observations. Initial drug content was assumed to be 100%. Study revealed significant loss of drug (15-17%) in case of formulations stored at 27±2°C as compared with very little loss (2-5%) of drug for formulations stored at 4°C. As we have already discussed that at higher temperature vesicles disruption and fusion takes place rapidly which could be possible reason for loss of

drug from formulation stored at 27±2°C? More over drug leaching is higher at increased temperature and this again could have resulted in loss of drug Figure 3-6.

CONCLUSIONS

The lipid and carbohydrate composition of carbohydrate-coated liposomes have been shown to be important for their targeted delivery to leishmania parasite infected cells. The carbohydrate-coated liposomes appear to be promising carriers that can expand the specificity of their delivery to the target.

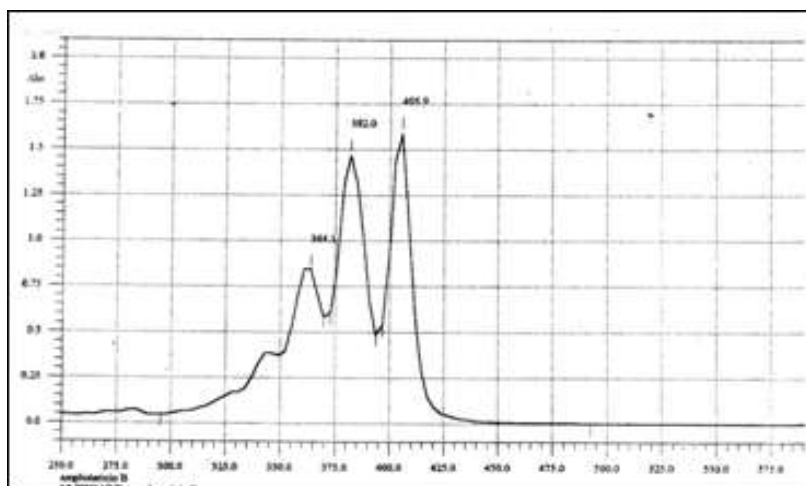


Figure 1 UV visible scan of amphotericin B

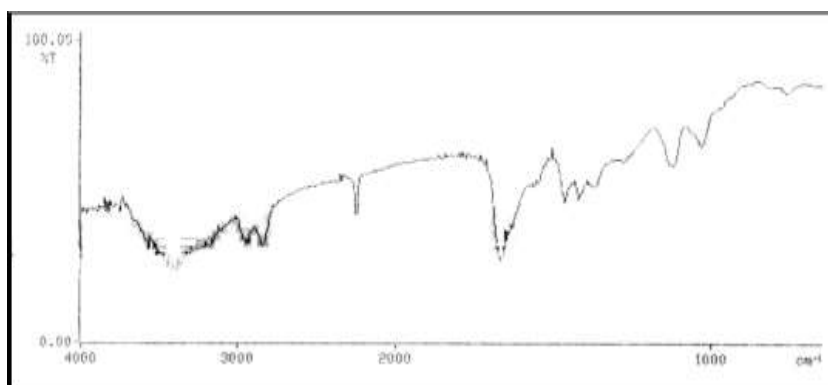


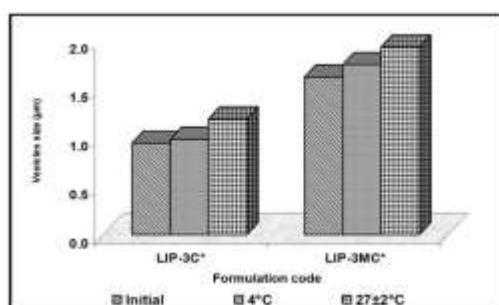
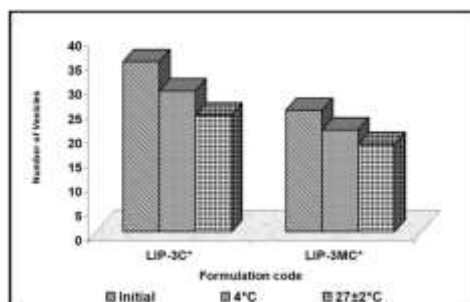
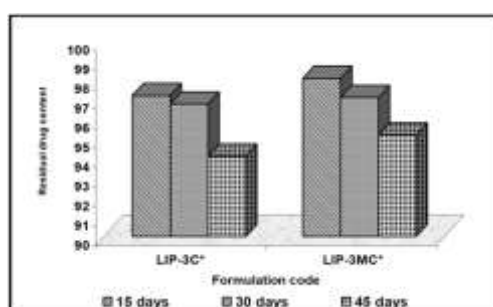
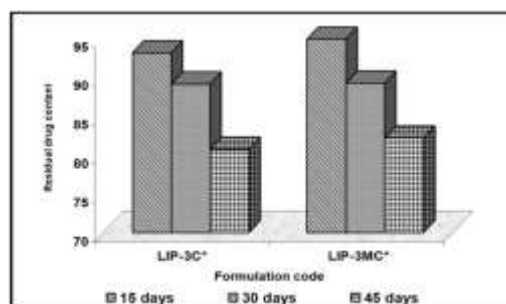
Figure 2 IR spectrum of the sample drug (amphotericin B)

Table 5: *In vitro* drug release from cationic liposomal formulation

S. No.	Time (Hrs)	Cumulative Percent Drug Released			
		LIP 3A	LIP 3B	LIP 3C*	LIP 3D
1	1	4.6	4.5	4.1	3.7
2	2	5.4	5.1	4.9	4.1
3	3	9.1	8.9	8.1	6.7
4	4	13.9	13.5	12.4	11.3
5	5	19.3	18.8	16.8	13.2
6	6	23.2	22.8	20.2	18.1
7	24	50.6	49.3	45.7	41.5

Table 6: *In vitro* drug release from mannosylated liposomal formulation

S. No.	Time (Hrs)	Cumulative % drug release LIP3C-M*
1	1	3.2
2	2	3.9
3	3	7.6
4	4	11.4
5	5	15.8
6	6	19.6
7	24	41.9

**Figure 3 Effect of storage on the size of vesicles****Figure 4 Effect of storage on the number of vesicles per mm³ x 1000****Figure 5 Effect of storage on the residual drug content in various liposomal formulations at 4°C****Figure 6 Effect of storage on the residual drug content in various liposomal formulations at 27±2°C****REFERENCES**

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