



FORMULATION AND INVITRO EVALUATION OF LIPOSOMAL DRUG DELIVERY SYSTEM OF NYSTATIN

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ABSTRACT

Nystatin is widely used for the treatment of fungal disease. The intention of the present study was to formulate Nystatin liposomes for a sustained drug delivery system. It has the advantages of dose reduction, less dosing frequency, minimize the side effect, prolong the action of drug and thus achieve better patient compliance. The liposomes were prepared by physical dispersion and ether injection method. Soya lecithin and cholesterol were used for encapsulating the drug, it facilitates to release the medicaments in sustained manner. Chloroform, ether and methanol were used as a solvent. Phosphate buffer pH 6.8 was used as a hydration medium for loading the drug. The final liposome was evaluated in various quality parameters of drug entrapment efficiency, morphological analysis, particle size analysis, in-vitro drug release studies and stability studies. In the two methods of Nystatin liposome formulation the physical dispersion method showed prolonged action when compared to ether injection method. In the parameters of drug entrapment and stability physical dispersion method shows better results.

Keywords: Physical dispersion, ether injection, soya lecithin, cholesterol, morphological analysis, Nystatin.

1. INTRODUCTION

1.1 Liposomes

Liposomes are microscopic vesicles containing aqueous volume enclosed by lipid bilayer membrane. A.D. Bangham and R.W. Thorne first described about liposomes in 1964 when observed under electron microscope while analysing phospholipids dispersion in aqueous environment. They observed spontaneous arrangement of phospholipids into “bag-like” circular structures. Gerald Weissman, one of the colleagues of Bangham suggested the structures as liposomes. This discovery helped as a multipurpose tool in several fields like biology, biochemistry and medicine. Liposomes gained popularity in vesicular research due to their attributes of biocompatibility and similar structural features of biological cells. Several reports showed the applicability of

liposomes for the safe and effective administration of therapeutic molecules of different classes like antitubercular, anticancer, antifungal, antiviral, antimicrobial, antisense, lung therapeutics, skin care, vaccines genes etc. Liposomes have proven their commercial importance from the first product 'Doxil', a PEGylated doxorubicin liposomal formulation to the latest 'Marqibo', vincristine sulfate liposomal formulation.

1.2 STRUCTURAL COMPONENTS PRESENT IN LIPOSOMES

Liposome vesicles are composed of phospholipids as an important structural component of the bilayered membrane and cholesterol is the other component mostly stabilizes the membrane.

a. Phospholipids

Phospholipids are amphipathic molecules present in membrane. They contain hydrophilic head and hydrophobic tail. The hydrophilic head has phosphorus molecule as phosphoric acid group, and two hydrophobic tails have long hydrocarbon chain groups. Phosphoglycerides, phosphoinositides and phosphosphingosides are three classes of phospholipids.

b. Phosphoglycerides

Phosphoglycerides are the mostly used phospholipids which contain three OH groups in glycerol moiety and among them two OH groups are linked to two fatty acids and phosphoric acid linked with one OH group. Phosphoglycerides are differed with their attached 'polar head alcohol group' esterified with phosphoric acid. All phosphoglycerides will have two nonpolar "tails" of fatty acid (C16 or C18) and among them one is saturated and other is unsaturated which always attaches to middle or β -hydroxyl group.

c. Lecithins (= phosphatidyl cholines): Lecithin is synonym for phosphatidylcholine which is a phospholipid containing phosphate obtained either from yolk of egg or from soya beans. Lecithin contains unsaturated non polar fatty acids, glycerol and phosphoric acid attached to nitrogen base choline.

d. Cephalins: Cephalins have similar basic structure to that of lecithins. The choline present in lecithin is replaced with ethanolamine or serine and examples are phosphatidyl ethanolamine and phosphatidyl serine. Cephalin exists in α and β forms based on position of two attached fatty acids. The primary amino group present in ethanolamine is weak base compared to quaternary ammonium group of choline. Hence, cephalins are more acidic and less soluble in alcohol than lecithins.

e. Plasmalogens: Plasmalogens contains only 10% of phospholipids and are structurally same like other two phosphoglycerides with change of one fatty acid replaced with unsaturated ether. The nitrogen base attached to phosphoric acid of plasmalogens can be choline, ethanolamine or serine and hence, names are phosphatidal choline, phosphatidal ethanolamine and phosphatidal serine.

f. Phosphoinositides Phosphoinositides are phospholipids which have cyclic hexahydroxy alcohol called inositol attached to phosphoric acid. The phosphoinositides on hydrolysis gives glycerol, fatty acids, inositol and phosphoric acid with 1 or 2 or 3 moles. Because of this monophosphoinositide diphosphoinositide and triphosphoinositide are found. Phosphoinositides are glycolipids which contains carbohydrate residue.

g. Phosphosphingosides

Sphingomyelins are structurally different from that of other phospholipids by lacking glycerol moiety and presence of nitrogenous sphingosine or dihydrosphingosine along with choline. These are electrically charged molecules with polar head phosphocholine.

h. Cholesterol

Cholesterol is lipid containing steroidal ring with attached hydroxyl group. The OH group present in cholesterol is united with phosphate head group of the phospholipids on biological cell membrane to keep them firm and fluid. Cholesterol has a molecular formula, $C_{27}H_{45}OH$. It is a white crystalline solid and is optically active.

1.3 ADVANTAGES OF LIPOSOMES

1. Reported methods showed liposomes are non-toxic biocompatible and completely biodegradable.
2. Liposomes increases therapeutic index and efficacy of drugs.
3. Drug molecules will be stable inside liposomes.
4. Drug toxicity can be decreased when formulated into liposomes.
5. Liposome reduces exposure to sensitive tissues with toxic drugs.
6. Binds to specific site to achieve targeted drug delivery.
7. Liposomes are suitable in delivering aqueous as well as lipid soluble molecules.

1.4 DISADVANTAGES OF LIPOSOMES

1. Phospholipids undergo hydrolysis and oxidation.
2. Leakage of loaded drug molecules.
3. Short shelf life and stability.

2. METHODOLOGY**2.1 Preformulation studies**

Preformulation testing is an investigation of physical and chemical properties of a drug substance alone and when combined with excipients. It is the first step in the rational development of dosage form. The objective of preformulation testing is to generate information useful to the formulation in developing stable and stable and bioavailable dosage forms. The use of preformulation parameters maximize the chances in formulating an acceptable, safe, efficacious and stable product.

2.1.1. Solubility

Solubility of Nystatinin water, methanol, phosphate buffer pH 6.8 was determined at room temperature with the help of magnetic stirrer.

Approximate solubility of drug was indicated from the following limits:

- a. **Very soluble:** 1 part of the substance is soluble less than 1 part of the solvent.
- b. **Freely soluble:** 1 part of the substance is soluble in 1 to 10 parts of the solvent.

Soluble: 1 part of the substance is soluble in 10 to 30 parts of the solvent.

d. **Sparingly soluble:** 1 part of the substance is soluble in 30 to 100 parts of the solvent.

e. **Slightly soluble:** 1 part of the substance is soluble in 100 to 1,000 parts of the solvent.

f. **Very slightly soluble:** 1 part of the substance is soluble in 1000 to 10,000 parts of the solvent.

g. **Practically insoluble or insoluble:** More than 10,000 parts of the solvent is required to dissolve 1 part of substance.

2.1.2 Melting Point

Melting point determination was done by using melting point apparatus. Small amount of pure drug of Nystatin was taken in a capillary tube and it was kept in the melting point apparatus and the melting point was noted.

2.1.3 Drug – excipients interaction studies:

FT-IR spectra were taken for the dried samples using FT-IR 8400S (Shimadzu, Japan) to determine the possible interactions between the drug and polymers. The plain drug, individual lecithin and cholesterol, combination of drug with cholesterol and lecithin in three different ratio (1:1, 1:2 and 1:3) were taken and mixed with KBr. The samples were compressed to form a pellet using a hydraulic press. The prepared pellets were transformed into disk. The disk was applied to the centre of the sample holding device and scanned from 4,500 to 400 cm^{-1} using FT-IR spectrophotometer⁷⁶.

2.1.4 Formulation of liposomes loaded with Nystatin:

The formulation of liposomes loaded with Nystatin was prepared by two different techniques namely, physical dispersion method and ether injection method. In both the techniques ratio of cholesterol was kept as same and the lecithin concentration was increased as 1:1, 1:2 and 1:3.

a. Physical dispersion method:

Liposomes were prepared by physical dispersion method using different ratio of soya lecithin and cholesterol was kept as constant. In this method the soya lecithin and cholesterol were dissolved in chloroform. Then it was spread over flat bottom conical flask and allowed to evaporate at room temperature for overnight without disturbing the solution for a formation of lipid film. The drug was dissolved in phosphate buffer pH 6.8. It act as an aqueous medium. Then the aqueous medium was added to the lipid film for hydration. For this the flask was inclined to one side and aqueous medium was introduced down the side of flask and flask was slowly returned to upright orientation. Then the conical flask was kept on water bath and the temperature was maintained at $37 \pm 2^\circ\text{C}$ for 2 hours for the completion of hydration. The conical flask was gently shaken until the lipid layer was removed from wall of conical flask and formation a liposomes suspension. Then the formed liposomes suspension was stored at 4°C for one day for the maturation of liposomes. The prepared liposome suspension was centrifuged at 15,000 rpm for 20 mins. Then the precipitate was collected and diluted with distilled water for further studies. Different batches of liposomes were prepared as per the general method described above and composition for the preparation of liposomes.

b. Ether injection method:

Liposomes were prepared by ether injection method using different ratio of soya lecithin and cholesterol was kept as constant. In this method the cholesterol and soya lecithin were dissolved in ether and methanol. The

drug was dissolved in phosphate buffer pH 6.8. It act as an aqueous medium. The aqueous medium was heated to 60°C. The method involves injecting drop by drop of ether-lipid solutions into the above warmed aqueous medium. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes. Then the product was collected and it was stored at 4°C for maturation of liposome. Then prepared liposomal suspension was centrifuged at 15,000 rpm for 20 mins. The precipitate was diluted with distilled water for evaluation studies. Different batches of liposomes were prepared as per the general method described above and composition for the preparation of liposomes is given.

Table No. 2.1 Formulation of Nystatin liposomes

S. No.	Ingredients	Physical dispersion method			Ether injection method		
		F 1	F 2	F 3	F 4	F 5	F 6
1.	Cholesterol	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg
2.	Lecithin	100 mg	200 mg	300 mg	100 mg	200 mg	300 mg
3.	Nystatin	10 gm	10 gm	10 gm	10 gm	10 gm	10 gm
4.	Ether	-	-	-	7 ml	7 ml	7 ml
5.	Methanol	-	-	-	3 ml	3 ml	3 ml
6	Chloroform	5 ml	5 ml	5 ml	-	-	-
7.	Phosphate buffer pH 6.8	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml

2.2 EVALUATION OF LIPOSOMES:

2.2.1. Determination of percentage drug entrapment efficiency:

Drug entrapment efficiency was calculated by using centrifugation method. 10ml of liposome suspension was taken and centrifuged at 15,000 rpm for 20 mins. The supernatant liquid was collected and suitably diluted. Then the absorbance was taken at 290 nm with the help of UV double beam spectrophotometer using pH 6.8 as a blank.

2.2.2. Morphology analysis:

The prepared Nystatin liposomes for all the formulations were viewed under 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 40X magnification. Photographs were taken to prepared slides using digital camera.

2.2.3. *In vitro* drug release study:

The *in vitro* release for all the formulated Nystatin liposomes were carried out for 8 hours in phosphate buffer pH 6.8. The studies were carried in USP dissolution apparatus II (Paddle) at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and 50 rpm speed. 900 ml of phosphate buffer pH 6.8 was used as a dissolution medium. Equivalent to 100 mg of Nystatin liposome was taken in a dissolution jar contains dissolution medium and the paddle was rotated at 50 rpm. 1 ml of samples were withdrawn at every 30 min. upto 480 minutes and make upto 10 ml with pH 6.8 and analyzed for Nystatin content at 290 nm with pH 6.8 as blank using double beam UV double beam spectrophotometer.

2.2.4. Particle size determination:

The particle size determination is done by using Malven particle size analyzer. Groups of particles are dispersed in a liquid medium and measured as they are circulated between the flow cell, which is placed in the measurement unit, and a dispersion bath in the sampler. The dispersion bath incorporates a stirrer and an ultrasonic sonicator. A pump delivers the dispersed suspension to the flow cell. The pump is specially designed to ensure both liquid medium and the particles are circulated. It can be controlled from a PC. Organic solvents can be used as dispersion media.

2.2.5. Stability studies:

The behavior of the liposome to retain the drug was studied by storing the liposome at two different temperature conditions, i.e., 4°C (refrigerator RF), $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for a period of 1 month. The liposomal preparations were kept in sealed vials. At 30th day the samples were analyzed for the drug content following the same method described in % drug encapsulation efficiency and *in vitro* drug release. And also the liposomes were studied for their morphology.

3. RESULTS AND DISCUSSION

The research study was aimed to formulate Nystatin liposomes to sustain the action of drug for over the period of 8 hours. The liposomes were prepared by physical dispersion method and ether injection method. Soya lecithin and cholesterol were used for encapsulating the drug and also to release the drug in sustained manner. Chloroform, ether and methanol were used as a solvent. Phosphate buffer pH 6.8 was used as a hydration medium for loading the drug.

Preformulation studies such like solubility analysis, melting point and FT– IR studies were carry out before the formulations. After formulation, the liposomes were evaluated for various parameters like percentage drug entrapment efficiency, microscopic analysis, particle size analysis, *in vitro* drug release studies and stability study.

3.1 Preformulation studies

a. Solubility

Nystatin is practically insoluble in water and in alcohol is insoluble in chloroform, in ether, and in benzene; and is slightly to sparingly soluble in methyl alcohol, n-butyl alcohol, and in n-propyl alcohol.

b. Melting point

The melting point was confirmed the Nystatin present in raw material of drug. It was found to be 160 °C within the specification range. So it confirmed Nystatin present in raw material of drug.

c. Organoleptic evaluation: The drugs were examined for its organoleptic properties like colour and odour and it was observed that Tazarotene was light yellow crystalline powder and hydroquinone white crystalline powder.

Table 3.1: Organoleptic property of Nystatin

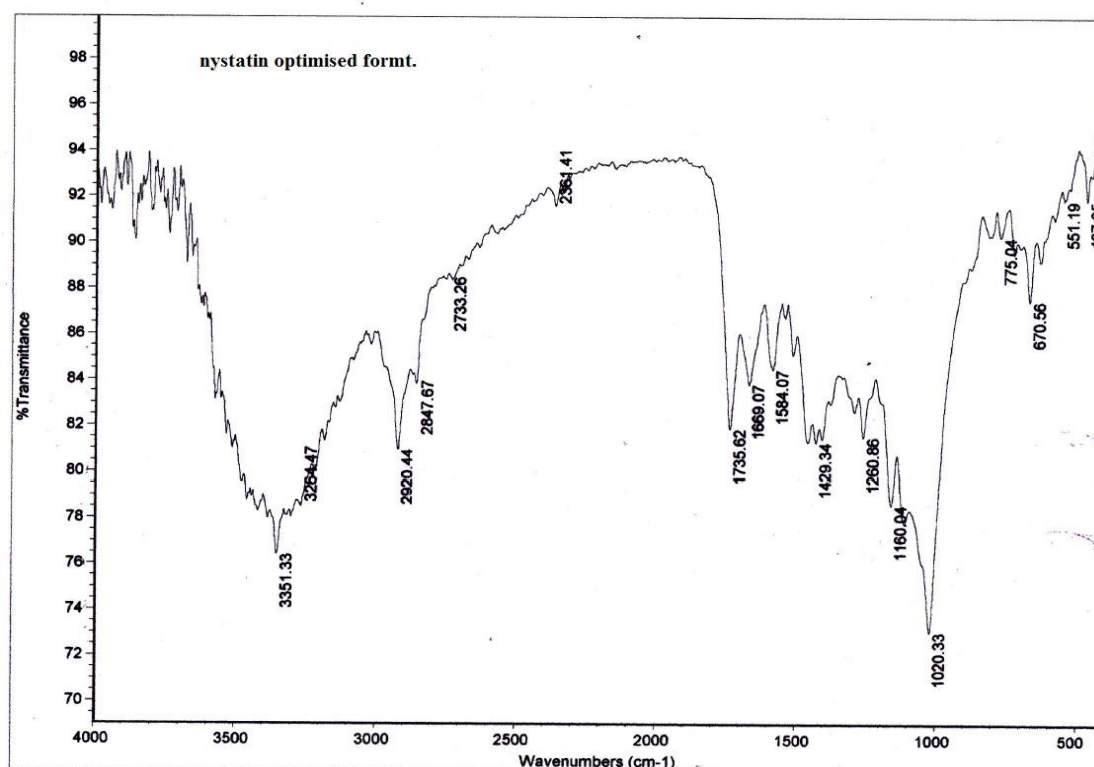
Parameter	Nystatin
Colour	yellow to light tan hygroscopic powder
Odor	Odorless
Taste	Cereals, bitter, foul taste

D) DRUG – EXCIPIENTS INTERACTION STUDIES:

The FT–IR studies of pure Nystatin and Nystatin + cholesterol + soya lecithin were conduct to study the interaction between the drug and excipients.

This indicated that there was no chemical interaction between Nystatin and the other excipients used in the formulations. The spectral datas are presented and spectral peaks were presented graphically.

Figure 3.2: FT – IR Spectrum of Nystatin + cholesterol + soya lecithin



3.2 EVALUATON OF NYSTATIN LIPOSOMES

3.2 .1 Percentage drug entrapment efficiency

The percentage drug entrapment efficiency of liposomes were prepared by physical dispersion method and ether injection method. The formulations was formulated by varying the cholesterol – soya lecithin ratio. It was

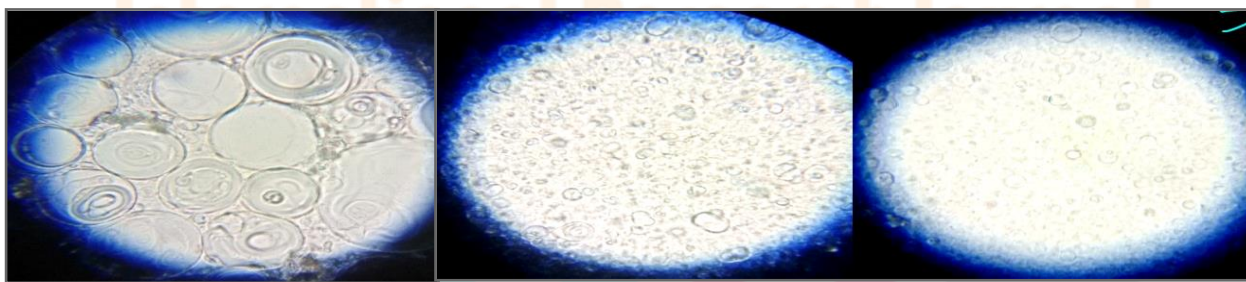
found to be that percentage drug entrapment efficiency of formulations F1, F2 and F3 were 85.20 %, 77.50 % and 72.60 % respectively and formulations F4, F5 and F6 were 29.81%, 40.35% and 41.27% respectively. The results may adjudge physical dispersion method have better drug entrapment efficiency than ether injection method.

Table 3.2: Determination of entrapment efficiency of prepared formulations

Formulation	Percentage drug entrapment efficiency
F1	85.20 %,
F2	77.50 %
F3	72.60 %
F4	29.81%
F5	40.35%
F6	41.27%

8.2.2 Morphology analysis

The morphology characters of liposomes were analyzed by optical microscopy and the images were taken using digital camera. The formulation F 1, F 2, F 3, F4, F 5 and F 6 microscopic images were showed in Figure no.8.3 to 8.8. Prepared liposomes F1 to F6 shows well identified morphology characters.

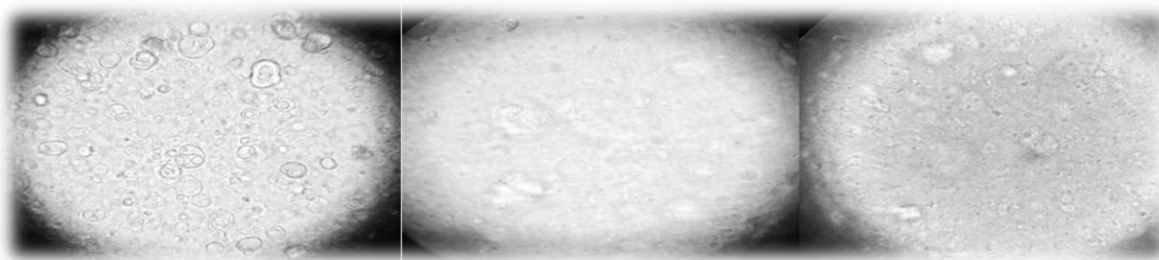


Formulation-F1

Formulation-F2

Formulation-F3

Research Through Innovation

**Formulation-F4****Formulation-F5****Formulation-F6**

3.2.3 Particle size analysis

The particle size analysis was carried out by particle size analyzer for all the prepared liposome formulations. The particle size for all the formulated liposomes were found to be in the range of 22.83 μm to 1.73 μm . The particle size data showed that when the concentration of soya lecithin was increased the particle size was decreased for all the formulations of Nystatin liposomes in prepared by both methods. The particle size of Nystatin liposomes of F3 was found to be lower when compared with other formulations this may be due to higher concentration of soya lecithin.

Table No. 3.3 Particle size of all the formulations of Nystatin liposomes

S. No.	Formulations	Particle size range
1.	F 1	19.58 μm
2.	F 2	17.62 μm
3.	F 3	1.73 μm
4.	F 4	22.83 μm
5.	F 5	2.34 μm
6.	F 6	1.81 μm

3.2.4 *In vitro* drug release studies

In vitro release studies were performed to evaluate the release of drug from the prepared Nystatin liposomes. The result of the *in vitro* release studies of all formulations.

Table No. 3.4 Cumulative percentage drug released of Nystatin from liposomes

S. No	Time (Mins)	F - 1	F - 2	F - 3	F - 4	F - 5	F - 6
1.	30	9.83 \pm 0.94	8.92 \pm 0.52	10.18 \pm 0.63	8.81 \pm 0.48	7.36 \pm 0.58	5.83 \pm 0.33
2.	60	16.76 \pm 0.59	14.19 \pm 0.61	13.53 \pm 0.80	16.82 \pm 0.67	15.84 \pm 0.37	8.17 \pm 0.94
3.	120	30.70 \pm 2.54	22.22 \pm 1.47	19.38 \pm 2.42	33.21 \pm 1.88	27.73 \pm 0.71	17.23 \pm 0.57

4.	180	44.49±1.85	37.58±3.54	31.93±2.44	48.31±2.30	39.37±0.43	27.79±0.42
5.	240	58.43±1.72	49.65±3.87	39.32±2.61	61.44±2.32	48.79±2.58	34.25±1.51
6.	300	73.00±3.11	60.45±3.00	46.73±2.38	77.56±1.47	59.42±1.36	44.03±2.03
7.	360	86.04±2.81	72.84±3.56	58.58±2.37	90.06±1.57	67.72±1.36	54.52±1.88
8.	420	94.83±2.11	82.70±2.63	70.47±2.51	99.89±1.58	80.19±1.61	66.89±1.55
9.	480	101.03±2.47	96.92±2.72	85.19±2.51	-	87.02±1.73	81.72±1.03

All the values expressed as mean± standard deviation, n = 3

The Nystatin liposomes were prepared by physical dispersion method and ether injection method using different ratio of cholesterol and soya lecithin. The cumulative percentage drug release was compared with different formulations.

The cumulative percentage drug release of formulations F 1, F 2 and F 3 were found to be 101.03±2.47, 96.92±2.72 and 85.19±2.51 respectively in 8 hours. The formulation F 1 show faster release than formulations F 2 and F 3 due to the lower concentration of soya lecithin. The cumulative percentage drug release of formulations F 4 was found to be 99.89±1.58 at the end of 7 hours. And the cumulative percentage drug release of formulations F 5 and F 6 were found to be 87.02±1.73 and 81.72±1.03 respectively in 8 hours. The formulation F 4 show faster release than formulations F 5 and F 6. While the concentration of soya lecithin was increase it decrease the release of drug.

The prepared liposomes F 1 to F 6 showed sustained release of drug. When increased ratio of soya lecithin also sustain the release of drug was increased in both method of preparations.

3.2.5 STABILITY STUDIES

All the formulations of Nystatin liposomes were relatively stable at 4°C storage condition. The drug leakage percent amounts of original entrapped in liposomes were very small and the amount retained in vesicle had no significant difference after one month as compared to the amount immediately after preparation. But at the storage condition of 25°C±2°C, all the formulations of Nystatin liposomes were unstable. In addition, the result of drug entrapment studies showed higher leakage at higher temperature. This may be due the higher fluidity of lipid bilayer at higher temperature, resulting into higher drug leakage.

Table No. 3.5 Stability study of percentage drug entrapment of Nystatin liposomes compared with percentage drug entrapment of immediately after preparation.

S. No.	Formulations code	Immediately after preparation (%)	After one month	
			At 4°C	At 25°C±2°C
1.	F 1	85.20 %,	79.14%	75.51%
2.	F 2	77.50 %	74.53%	68.79%
3.	F 3	72.60 %	69.82%	63.47%
4.	F 4	29.81%	24.73%	22.31%
5.	F 5	40.35%	35.47%	32.43%
6.	F 6	41.27%	37.25%	31.62%

After one month, Nystatin liposomes formulations F 1 to F 6 were showed difference in *in vitro* drug release profile. Dissolution rate was decreased in all Nystatin liposomes formulations at both storage conditions like 4°C and 25°C±2°C. The results of *in vitro* drug release of all the formulations at both storage conditions were compared with before and after stability studies.

Table No. 3.6 *In vitro* drug release data of all the Nystatin liposome formulations after stability study, compared with before stability

S. No.	Formulation code	Immediately after preparation	After stability study	
			At 4°C	At 25°C±2°C
1.	F 1	101.03±2.47	93.31	74.63
2.	F 2	96.92±2.72	91.53	69.78
3.	F 3	85.19±2.51	79.41	68.24
4.	F 4	99.89±1.58	92.84	88.83
5.	F 5	87.02±1.73	79.31	64.42
6.	F 6	81.72±1.03	74.28	66.29

At storage condition 4°C showed better stability than another condition. This may due to their elevated temperature reduce the stability. But in both storage condition higher proportion of soya lecithin contains formulations like F 3 and F 6 showed better stability than other their formulations.

CONCLUSION

This study concluded that Nystatin was successfully prepared as a liposomal drug delivery system by using two different techniques such as physical dispersion method and ether injection method. In this liposomes preparations, cholesterol ratio was constant and soya lecithin concentrations were gradually increased (like 1:1, 1:2 and 1:3). The liposomes prepared by physical dispersion method showed better percentage drug entrapment when compared with ether injection method. The morphological characters of prepared liposomes were determined with the help of optical microscope. The particle size was analyzed by Malven particle size analyzer. The results of the particle size showed, when the concentration of soya lecithin was increased the size of the particle was reduced. The *in vitro* release showed that as the concentration of soya lecithin was increased the release rate of drug was retarded. Among the two methods ether injection method showed prolonged action when compared to physical dispersion method. The stability studies for all the formulations were performed by keeping the formulations at two different temperatures 4°C±2°C and 25°C±2°C for a period of 30 days. After the stability period the formulations were tested for morphological analysis, percentage drug entrapment and *in vitro* drug release and compared with before stability study. There was no change in morphological characters at 4°C±2°C, but there was a slight reduced in particles size at 25°C±2°C. The percentage drug entrapment was reduced in all the formulations at both the conditions. The *in vitro* drug release was reduced for all the formulations. Liposomes prepared by physical dispersion method showed better stability compared with ether injection method.

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