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RESEARCH ARTICLE

Preparation and Evaluation of Imiquimod Loaded Liposomal Dispersion: Part-I

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ABSTRACT:

The aim of present investigation was to encapsulate Imiquimod into liposomes and optimization of process and formulation variables, characterization of liposome with respect to shape, lamellarity, drug entrapment efficiency to opt maximum efficacy and to reduce side effects. Preliminary work like development of suitable analytical method, preparation of Calibration curve, Melting point, Partition coefficient, Permeability coefficient determinations, Physicochemical compatibility of Imiquimod and excipients like phospholipid and cholesterol were carried out and results were reported and used for the further work. Liposomes were prepared by thin film hydration technique. Various process parameters like Vacuum of 25 inch Hg, 100 rpm, 2 hour time of hydration, 50 ºC temperature, Drug : Soya Lecithin : Cholesterol ratio 1:12:2, aqueous phase: organic phase ratio1:3 gave thin, smooth, uniform and translucent film with higher percent drug encapsulation efficiency.

KEYWORDS: Thin film hydration technique, liposome, Soya Lecithin, Cholesterol.

INTRODUCTION:

Imiquimod gel is used to treat certain types of actinic keratoses (flat, scaly growths on the skin caused by too much sun exposure) on the face or scalp. Imiquimod gel is also used to treat superficial basal cell carcinoma (a type of skin cancer) on the trunk, neck, arms, hands, legs, or feet and warts on the skin of the genital and anal areas. Imiquimod is in a class of medications called immune response modifiers. It treats genital and anal warts by increasing the activity of the body's immune system.

To achieve desirable therapeutic effect, topical delivery of Imiquimod needs to reside at the sites of infection for prolonged period. Hence there is a need to develop effective drug delivery system that should prolong the contact of drug with affected part. Liposomes have been widely used as drug carrier in topical treatment of various diseases. They are capable to incorporate a variety of hydrophilic and hydrophobic drugs, to enhance the accumulation of drug at the administration site and to reduce side effects. Liposomes can provide sustained and/or controlled release of entrapped drug. Liposomal system allows for a high accumulation of drug in the skin, with relatively low permeation flux as compared to the conventional dosage form.

MATERIALS AND METHODS:

MATERIALS:

Imiquimod was received as a gift sample from Glenmark Pharmaceuticals, Mumbai. Hydrogenated Soya Phosphatidyl Cholines (HSPC), also known as a Hydrogenated Soya Lecithin (neutral charge), cholesterol (neutral charge) were received as a gift sample from Gattefosse, Germany. Tocopheryl acetate was received as a gift sample from Cadila Healthcare, India.

METHOD:

Multilamellar vesicles of Imiquimod were prepared by thin film hydration technique as described by Bangham et al. using rotary flash evaporator. Following steps were used for the preparation of liposomes:

1. Drug **:** HSPC **:** Cholesterol in the ratio 1:10:1 (weight ratio) and tocopheryl acetate (equivalent to 1% w/w of HSPC taken) were dissolved in 14 ml dichloromethane **:** methanol (1:1).

2. The solution was taken in a 250 ml round bottom flask and the flask was rotated in rotary flash evaporator at 80 rpm for 15 minutes in thermostatically controlled water bath at 40°C under vacuum 25 inch Hg.

3. The organic solvent was slowly removed by this process such that a very thin film of dry lipids was formed on the inner surface of the flask. The dry lipid film was hydrated with 5 ml of distilled water and the flask was once again rotated at 80 rpm and at a temperature above the transition temperature of lipids for 2 hr.

4. Liposomal dispersion obtained after hydration was sonicated for 30 minutes by bath sonicator to produce small and more uniform sized population of liposomes.

5. The liposomal dispersion obtained was stored in refrigerator till further use.

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OPTIMIZATION OF PROCESS PARAMETERS:

1. OPTIMIZATION OF VACUUM:

The vacuum used for drying of film was raised from 20 to 30 inch of Hg and the effect on encapsulation efficiency, type of lipid film formed and shape of liposomes was optimized.

2. OPTIMIZATION OF RPM:

The rpm used for drying of film was varied from 80 to 120 and the effect on encapsulation efficiency, type of lipid film formed and shape of liposomes was optimized.

3. OPTIMIZATION OF HYDRATION TIME:

The hydration time was increased from 60 minutes to 120 minutes and the effect on encapsulation efficiency, type of lipid film formed and shape of liposomes was optimized.

4. OPTIMIZATION OF TEMPERATURE:

Temperature used for drying of film was varied from 35 ºC to 60 ºC. 35 ºC was consider as room temperature and the effect on encapsulation efficiency, type of lipid film formed and shape of liposomes was optimized.

5. SONICATION OF LIPOSOMES FOR PARTICLE SIZE REDUCTION:

Liposomal dispersion obtained after hydration was subjected to ultrasonic downsizing on an icebath for 30 minutes in bath sonicator for particle size reduction.

OPTIMIZATION OF FORMULATION PARAMETERS:

1. OPTIMIZATION OF DRUG: PHOSPHOLIPID RATIO:

The ratio of phospholipids was increased proportionally keeping drug concentration same-10mg and the effect on encapsulation efficiency was checked.

2. OPTIMIZATION OF PHOSPHOLIPID: CHOLESTEROL RATIO:

The ratio of cholesterol was increased keeping drug and phospholipid ratio constant and the effect on encapsulation efficiency was optimized.

3. OPTIMIZATION OF RATIO OF AQUEOUS PHASE: ORGANIC PHASE:

Ratio of aqueous phase: organic phase was optimized keeping Drug:HSPC:Cholesterol ratio optimized in previous step.

CHARACTERIZATION OF LIPOSOMES:

Combination of various characterization methods are used to characterize liposomes. Liposomes containing Imiquimod were characterized for following attributes.

1. PARTICLE SIZE

The particle size of optimized batch was obtained by particle size analyzer (Malvern laser light scattering Masterizer model 4700). 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 [R-5] 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 dispersions were 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.

2. ZETA POTENTIAL:

Zeta potential of liposomes was determined using Zetasizer HSA 3000.

3. % ENTRAPMENT EFFICIENCY:

1.0 ml of Imiquimod loaded liposomal suspension was centrifuged at 5000 rpm for 15 minutes to separate the different phases. Supernatant was than analyzed for drug content by UV-VIS spectroscopy at 226 nm. It expresses amount of free drug, which remains unentrapped in formulation. The entrapment efficacy was calculated by following equation:

$$\% \text{ Entrapment efficiency} = \frac{Wa - Ws}{Wa} \times 100$$

Where,

Wa = Amount of drug added into formulation,

Ws = Amount of drug (free) present in supernatant after centrifugation

4. IN VITRO DRUG RELEASE STUDY OF LIPOSOMAL DISPERSION:

For in-vitro release study, the optimized batch of Imiquimod loaded liposomal dispersion (1 ml) was added to a dialysis bag (12,000 Da molecular weight, Sigma), which was previously soaked in medium overnight. The dialysis bag was tied from both the sides and added into 500 ml conical flask containing 375 ml of a phosphate buffer solution (pH 7.4) with 0.8% v/v Tween 20 as a medium. The flask was kept at 37^0C in shaker incubator. At predetermined time intervals, 5 ml aliquots was taken and replaced with the same amount of fresh medium. The amount of Imiquimod released from the liposomal dispersion was measured by UV spectrophotometer. Dispersion of plain Imiquimod was also analyzed by similar manner as discussed above for comparison purpose.

RESULTS & DISCUSSION:

OPTIMIZATION OF PROCESS PARAMETER:

1. OPTIMIZATION OF VACUUM:

Table 1: Optimization of Vacuum

Vacuum Inch Hg.	Batch	Drug: HSPC: Cholesterol Molar Ratio	% Entrapment Efficiency ± S.D.	Observation and Inference
20	B1	1:10:1	51.32 ± 2.1	Aggregation of liposomes, thick lipid film, lower entrapment efficiency
25	**B2**	**1:10:1**	**60.25 ± 3.2**	**Thin, uniform, translucent film formed with higher entrapment efficiency**
30	B3	1:10:1	45.20 ± 2.6	Non- uniform film and lower entrapment efficiency.

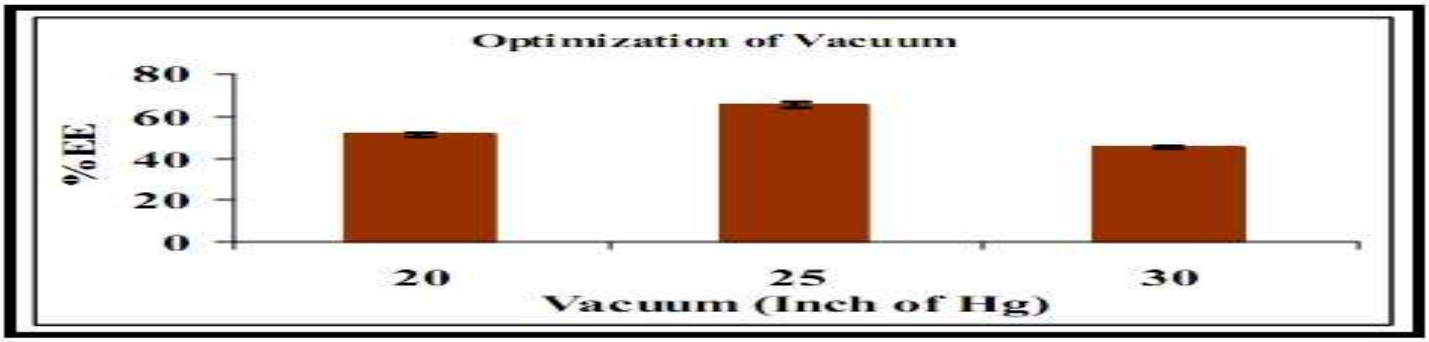

Figure 1: Optimization of Vacuum

The vacuum used for drying of film was raised from 20 to 30 inch Hg. Vacuum of 20 in. of Hg was found to be insufficient for complete removal of the solvents and resulted in aggregation of the liposomes on hydration. Also the film formed was thick. The vacuum of 30 in. of Hg resulted in rapid evaporation of solvents, leading to entrapment of air bubbles on lipid film surface. Also the film formed was non- uniform. The vacuum of 25 inch Hg gave thin, smooth, uniform and translucent film with higher percent drug retention.

2. Optimization of RPM:

Table 2: Optimization of RPM

RPM	Batch	Drug: HSPC: Cholesterol Molar Ratio	% Entrapment Efficiency±S.D.	Observation and Inference
80	B4	1:10:1	45.66 ± 2.8	Thick lipid Film
100	**B5**	**1:10:1**	**61.55 ± 1.6**	**Thin, uniform translucent**
120	B6	1:10:1	50.69 ± 1.9	Thin but non-uniform film,

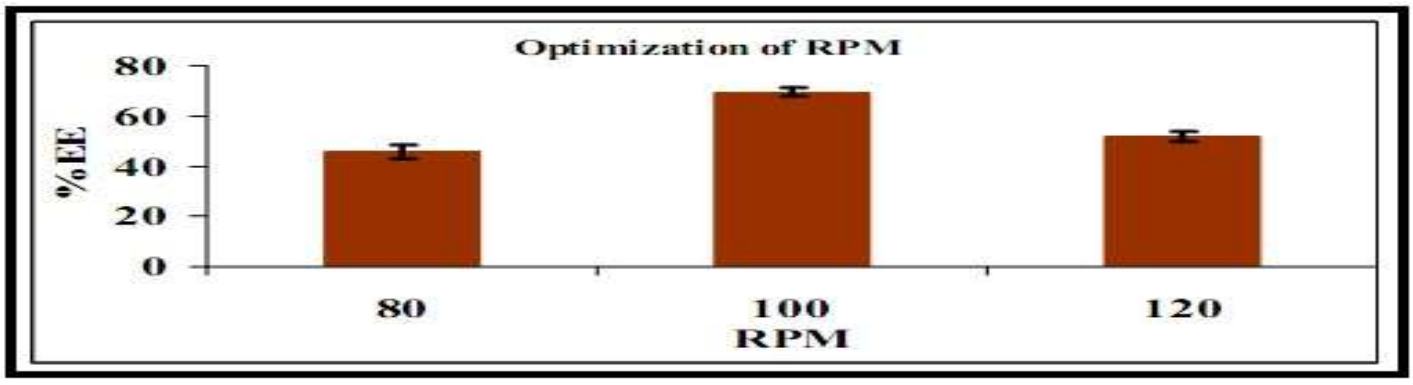

Figure 2: Optimization of RPM

The rpm used for drying of film was varied from 80 to 120. On rotating at 80 rpm thick lipid film was formed with lower percent drug retention. The reason may be the slow rate of evaporation of organic solvent at 80 rpm. On rotating at 120 rpm thin but non uniform lipid film was formed with lower percent drug retention which may also

due to leaking of liposomes. On rotating at 100 rpm gave thin, uniform and translucent film with higher percent drug retention. The reason may be 100 rpm gives desired rate of evaporation for removal of organic solvent and formation of thin, uniform and translucent film.

3. OPTIMIZATION OF HYDRATION TIME:

The hydration time was increased from 90 minutes to 150 minutes. Proper hydration time is necessary for maturation of liposomes but excess may lead to leaking of vesicles. 90 minutes hydration time was found insufficient and formed liposomes were of irregular shaped with low percent entrapment efficiency. 150 minutes hydration time caused leaking of liposomes and formed liposomes were of irregular shaped. 120 minutes hydration time gave complete removal of film with formation of milky dispersion and the liposomes formed were of spherical shaped.

Table 3: Optimization of Hydration time

Hydration Time minute	Batch	Drug: PC: Cholesterol Molar Ratio	% Entrapment Efficiency±S.D.	Observation and Inference
90	B8	1:10:1	36.85±1.6	Not sufficient time
120	**B9**	**1:10:1**	**61.90±2.2**	**Complete removal of thin film and formation milky emulsion**
150	B10	1:10:1	65.32±2.6	Leaking of liposomes

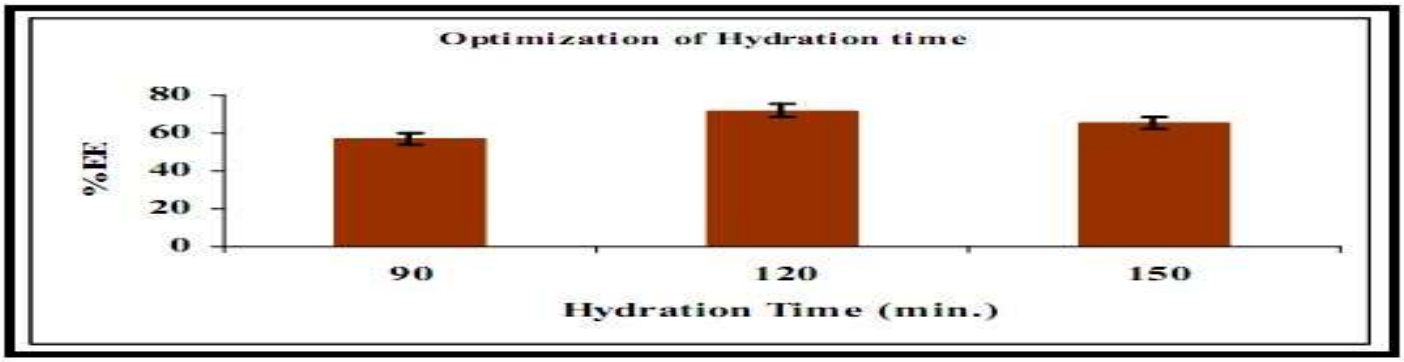

Figure 3: Optimization of Hydration time

4. OPTIMIZATION OF TEMPERATURE

Table 4: Optimization of temperature

Temperature (ºC)	Batch	Drug:PC:Cholesterol Molar Ratio	% Entrapment Efficiency±S.D.	Observation and Inference
35	B7	1:10:1	56.33±1.8	Irregular shaped vesicles with poor entrapment efficiency.
50	**B8**	**1:10:1**	**61.90±1.2**	**Good vesicle formation with high entrapment efficiency.**
60	B9	1:10:1	47.69±1.5	Irregular shaped vesicles with poor entrapment efficiency.

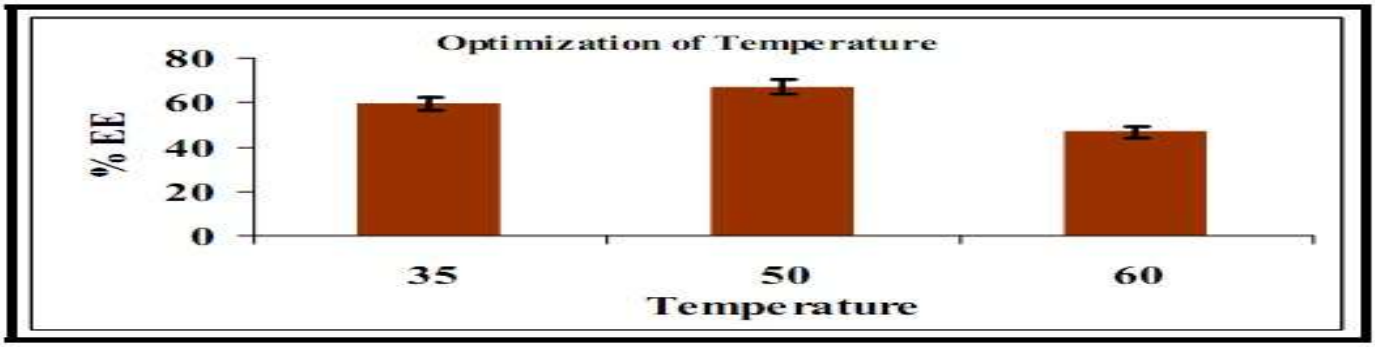

Figure 4: Optimization of Temperature

The most important parameter that must be considered in liposome preparation is the gel- liquid crystalline temperature (Tm) of the membrane lipid. The main effect of temperature is during hydration of liposomes. Temperature used for hydration of film was varied from 35ºC to 60ºC. 35ºC was considered as room temperature. At room temperature poor percent drug retention was obtained. Also the vesicles formed were of irregular shaped.

On raising temperature to 50 ºC good drug retention was found and vesicles formed were of spherical shaped. Also the dispersion formed of liposomes was homogenous in nature. On raising temperature to 60 ºC same poor drug retention and irregular shaped vesicles were formed. It may be due to degradation of lipids. At low temperatures in the gel phase, the acyle chains are in a conformationally well-ordered state, essentially in the all-trans configuration. At higher temperatures, above the chain melting temperature, this chain order is lost, owing to an increase in gauche conformer content. Keeping hydration temperature above phase temperature for long time may lead loss in chain order of phospholipids.

OPTIMIZATION OF FORMULATION PARAMETERS:

1. OPTIMIZATION OF DRUG: PHOSPHOLIPID RATIO:

Table 5: Optimization of Drug:Phospholipid ratio

Drug: PC Molar Ratio	Batch	% Entrapment Efficiency±S.D.
1:2	B11	15.62±1.8
1:4	B12	26.90±1.2
1:6	B13	39.12±2.9
1:8	B14	45.17±2.8
1:10	B15	58.62±1.8
1:12	**B16**	**62.08±2.1**
1:14	B17	62.19±2.6

The ratio of phospholipid was increased proportionally keeping drug concentration same-10 mg and the effect on encapsulation efficiency was checked. The ratio 1:12 was found to be optimized giving highest encapsulation efficiency. On increasing amount of HSPC can improve the number of lamellae and thereby layers of interstitial water are also increased to entrap more drugs or it may also increase number of liposomes per ml. On further increasing ratio does not changed encapsulation efficiency much.

2. OPTIMIZATION OF PHOSPHOLIPID: CHOLESTEROL RATIO:

Table 6: Optimizations of Phospholipid: Cholesterol ratio

Drug:PC:Cholesterol ratio	Batch	% Entrapment Efficiency±S.D.
1:12:1	B18	58.63±1.3
1:12:2	**B19**	**60.23±1.5**
1:12:3	B20	55.39±1.8

Cholesterol is also an essential constituent of the biomembrane and one of its primary functions is to modulate the physiochemical properties of the lipid bilayers. Cholesterol is considered as a fluidity buffer of the lipid membrane due to its properties of either disordering the gel state or ordering the liquid state of the lipid in the fully hydrated state.

The ratio of cholesterol was increased keeping drug and phospholipid ratio constant. The ratio1:12:2 gave highest encapsulation efficiency due to stabilizing effect of cholesterol. On increasing proportion of cholesterol reduces the drug entrapment. The ratio1:12:3 not only decreased entrapment efficiency but simultaneously showed cholesterol free cholesterol crystals in microscopy. On increasing cholesterol concentration it decreases the fluidity or micro viscosity of the bilayers by filling empty spaces among the phospholipid molecules anchoring them more strongly into the membrane thereby making membrane more rigid and ultimately decrease encapsulation efficiency.

3. OPTIMIZATION OF RATIO OF AQUEOUS PHASE: ORGANIC PHASE

Table 7: Optimization of ratio of Aqueous phase: Organic phase

Ratio	Batch	% Entrapment Efficiency±S.D.
1:2	B21	58.89±2.1
1:3	**B22**	**62.71±2.6**
1:4	B23	51.98±2.9

Ratio of aqueous phase: organic phase was optimized by keeping Drug:HSPC:Cholesterol ratio 1:12:2. It was found that on increasing ratio entrapment efficiency increased. The ratio1:3 gave optimum encapsulation efficiency with good vesicle formation. On further increasing organic phase decreases encapsulation efficiency.

CHARACTERIZATION OF LIPOSOMES:

1. PARTICLE SIZE:

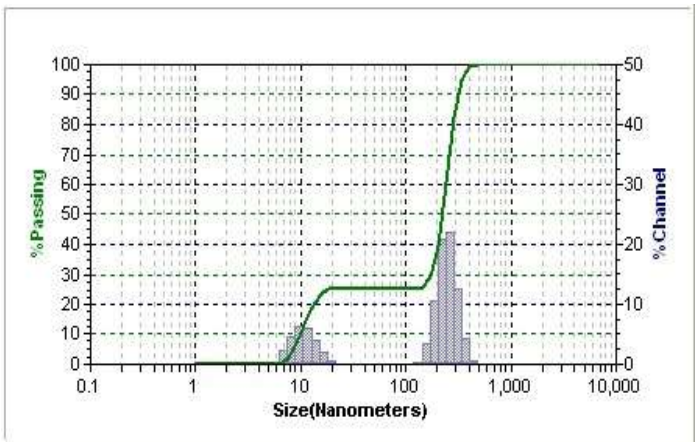

Figure 5: Particle size of optimized batch

Figure 5 shows particle size measurement of optimized liposomal batch. The average particle size was found to be 12.52 µm.

2. Zeta Potential

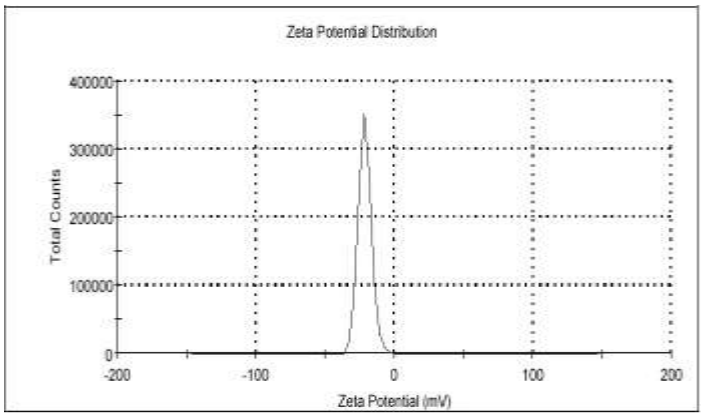

Figure 6: Zeta Potential of Sonicated Liposomes

The zeta potential is an indication of the stability of the colloidal systems and indicates charge present on the colloidal systems. Here, zeta potential was found to be -32.18 mV which indicates high negative surface charge on liposomes indicate higher stability because of the anticipated surface repulsion between similar charged particles hence inhibiting aggregation of the colloidal liposomal particles.

3. % ENTRAPMENT EFFICIENCY:

The percentage entrapment efficiency of optimized liposomal batch was found to be 62.71±1.5 %.

4. IN VITRO DRUG RELEASE STUDY OF LIPOSOMAL DISPERSION:

Results obtained after in-vitro drug release study of liposomal dispersion using dialysis bag was shown below. It showed sustained release of drug from liposomal dispersion as it took 13 hours to reach 99.17 % level whereas plain drug dispersion offered 99.6 % drug release in 8 hr.

Table 9: In-vitro % drug release of liposomal & plain drug dispersion (n=3)

Time (hr.)	Liposomal Dispersion ± S.D.	Plain drug dispersion ±
0.5	5.89±1.341	9.9±1.026
1	13.06±2.074	19.3±0.975
2	24.83±2.138	39.1±1.268
3	31.10±1.143	58.6±1.395
4	39.18±0.931	76.2±2.135
5	46.03±1.816	84.9±2.086
6	52.78±2.762	90.6±1.856
7	61.26±1.591	95.3±1.158
8	72.83±2.860	99.6±1.543
9	82.17±1.013	-
10	88.06±1.172	-
11	94.15±1.820	-
12	97.92±2.403	-
13	99.17±1.362	-

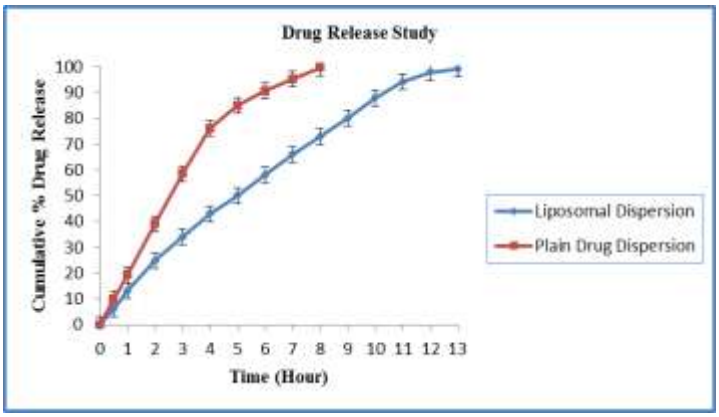

Figure 7: Comparison of % drug release study of liposomal & plain drug dispersion

CONCLUSION:

Liposomes were prepared by thin film hydration technique. Various process parameters like Vacuum of 25 inch Hg, 100 rpm, 2 hour time of hydration, 50 ºC temperature, Drug : Soya Lecithin : Cholesterol ratio 1:12:2, aqueous phase: organic phase ratio1:3 gave thin, smooth, uniform and translucent film with higher percent drug encapsulation efficiency. By microscopic examination it was observed that prepared liposome were multilamellar vesicles with spherical shape. The particle size of optimized liposomal batch was found to be 12.52 µm. Percentage drug entrapment in optimized batch was observed to be 62.71±1.5 %. The value of zeta potential of optimized batch was found to be -32.18 mV indicative of higher stability.

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