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(Research Article)

Formulation and Evaluation of 5-Fluorouracil Loaded Liposomes

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ABSTRACT

The liposome was prepared using 5-fluorouracil as a model drug with the intention for intravenous application. Different batches were prepared by the film hydration method followed by sonication. Different ratios of soya phospholipids, cholesterol, mannitol and sorbitol were used to prepare liposome to determine their effect on particle size, entrapment efficiency, in-vitro drug release (using Franz diffusion cell) and stability. The Liposome particle size varied between 6.24±0.09 to 25±0.99 µm, entrapment efficiency varied from 58.6 to 10.2%. Stability studies showed that the best stability was shown at 2-8°C for 1 month and least stability was shown at 45° C for the same time period. Batch-L was selected best on the basis of its good entrapment efficiency, possessing a release profile over 25 hours and having considerable particle size.

Key Words: Liposome, Evaluation, Film hydration, Sonication, Soya lecithin, 5-Fluorouracil, Mannitol, Sorbitol

INTRODUCTION

The modern scientific and technological advancements in the pharmaceutical field had created bank of interest in development of various novel dosage form, in the recent years to provide a sustained/targeted/prolonged drug delivery. To pursue an optimal drug action, the active moiety could be transported by a suitable carrier system which deliver the drug at the site of action and then trigger it to execute its task and for this the carrier itself must be bio-degradable, provide passive targeting, increase the efficiency and therapeutic index, provide stability via encapsulation, reduce the toxicity of the encapsulated agent and at the same time should improve the pharmacokinetic profile of the drug.

Liposome is microscopic lamellar structures composed of phospholipids, cholesterol, drug solutions/suspensions and surfactants. These are prepared by mechanical dispersion .Several formulations have been developed and evaluated for controlled/targeted drug delivery for cancers, viral infections and other microbial diseases. These were also found to be suitable for intravenous administration as it delivers the biologically active molecule at the site of action directly without getting interrupted anywhere in the path to the target tissue.

The 5-flurouracil is an anti-metabolite belongs to pyrimidine analogue category of anticancer drugs. It interferes with DNA synthesis by blocking the thymidylate synthetase conversion of deoxyuridylic acid to thymidylic acid. It is readily but incompletely absorbed after oral dosing and is highly variable. It has got the short biological half life of 10-20 minutes which makes them uncomfortable to be dispensed as solid dosage form. Thus an effective delivery system is required which carries the drug directly to the site of action for which liposome is a unique technique, especially for solid tumors or neoplasm tumors having low vascularity. Fluorouracil injection is indicated in the palliative management of some types of cancer, including colon, oesophageal, gastric, rectum, breast, biliary tract, stomach, head and neck, cervical, pancreas, renal cell and carcinoid are the side effects of 5-fluorouracil. Encapsulation of 5-fluorouracil in liposome may increase the biological half life, providing prolonged drug delivery and minimize the commonly occurring side effects.

This work investigated on development and evaluation of liposome using 5-Fluorouracil as model drug. Liposome was prepared by using film hydration technique followed by sonication. Various ratios of soya lecithin, cholesterol, sorbitol and mannitol were used to determine their effect on vesicle size, entrapment efficiency, *in-vitro* drug release and stability studies.

MATERIALS AND METHOD

Materials

5-Flurouracil was received as a gift sample from (S.D. Fine Chem. Ltd., Mumbai), phospholipid (Himedia Laboratories Pvt. Ltd., Mumbai), cholesterol (S.D.Fine Chem. Ltd., Mumbai), Chloroform (MorvelLaboratories Pvt. Limited, Mehsana), Mannitol (Embralds Pharmacen New Delhi) and sorbitol (Shivam industries, Mumbai) were used in the study. All other chemicals and solvents were of analytical grade.

Preperation Methodology

Multi-lamellar vesicles of 5-fluorouracil were prepared by film hydration method using rotatry evaporator followed by sonication, which is generally based on the principle of passive loading with the aid of mechanical energy via sonication[1,2] The Soya lecithin (neutral charge), cholesterol (neutral charge), mannitol and sorbitol were the basic component to prepare liposome. The present study basically incorporates drug solution: Soya lecithin: cholesterol, mannitol and sorbitol in different ratios[2,3]. Twelve different batches of liposome were prepared in order to select an optimum formulation. Phospholipids and cholesterol was dissolved in chloroform under vacuum in rotary evaporator to prepare thin film of phospholipid. Aqueous drug solution contained mannitol/sorbitol was added to hydrate the films in order to prepare liposome[4]. Then mixture was subjected to sonication for size reduction of liposome. After 5 cycles of 20 minute sonication centrifuge the mixture at 5000 rpm for 15 minutes at 4°C temperature to separate liposome. Supernatant liquid containing the liposomal suspension (liposome in suspended state) and the free drug was present at the wall of centrifugation tube[4,5]. The supernatant was collected and again centrifuged at 15000 rpm at 4°C temperature for 30 minutes. A clear solution of supernatant and pellets of liposome were obtained. Pellets were re-suspended in distilled water and finally dried at the 40°C temperature and 250 mmHg under vacuum[6].

EVALUATION

The liposome are basically formulated and processed with the main aim to characterize and ensure their predictable *in-vitro* and *in-vivo* performances. The liposomes being formulated by different methods may differ in their physio-chemical features. These differences may have a pronounced effect on the in-vitro and *in-vivo* performances (disposition). There are several examples which proved the ideal requirements for proper selection of liposome structure to optimize therapeutic effect. The basic characterizing parameters for their evaluation can be broadly classified into three categories named, physical, chemical and biological parameters. Physical parameters govern several parameters such as size, shape, surface features, lamellarity, and phase behavior and drug release profile. Chemical characterization gives us the sign of purity and potency of the liposomal constituents. Biological characterization parameters demonstrate the safety and suitability of the formulations for the *in-vivo* use or the therapeutic application. Although physical and chemical parameters are very useful in comparing the meaning full data of different liposomal batches prepared. In contrast to physical and chemical parameters, the biological parameters reflect the ensurity of their suitable use in human[7,8]. The combination of all the above parameters is highly useful to formulate a meaningful liposomal preparation. The prepared liposomes were evaluated for size and size distribution, encapsulation efficiency, *in-vitro* drug release and stability studies[9,10].

Table 1: Composition of prepared batches

Batch codes	Phospholipid (ml)	Cholesterol (ml)	Chloroform (ml)	Drug (g)	Water (ml)	Mannitol (g)	Sorbitol (g)
A	1.5	1.5	5	12	1	2	-
B	1	2	5	12	1	2	-
C	2	1	5	12	1	2	-
D	1.5	1.5	5	12	1	3	-
E	1	2	5	12	1	3	-
F	2	1	5	12	1	3	-
G	1.5	1.5	5	12	1	-	2
H	1	2	5	12	1	-	2
I	2	1	5	12	1	-	2
J	1.5	1.5	5	12	1	-	3
K	1	1	5	12	1	-	3
L	2	2	5	12	1	-	3

A. Preparation of Standard Curve of 5-Fluorouracil in Phosphate Buffer (pH- 7.4)

The stock solution of drug was prepared by dissolving accurately weighed 5-fluorouracil (100 mg) in 100 ml volumetric flask and further dilutions (5, 10, 15, 20, 25, 30, 35 µg/ml) were prepared using buffer (pH 7.4). Any dilution was used to measure suitable wavelength (λ_{max}). Rest of the dilutions was scanned for absorbance, using UV-VIS spectrophotometer (UV 1700S Pharm Spec, Shimadzu, Japan). Plot of concentration versus absorbance was plotted.

B. Infra-Red (IR) Spectroscopy

An infrared spectrum is an important record which gives information about the types of bonds and group present in compound. For IR spectroscopy the drug and KBr where placed at 105°C in oven to remove moisture. Then KBr and drug (100:1) were grounded into powder. Take few mg of mixture in die and compress at 10 tons for a minute to prepared thin pellet. Prepare pellet was placed at IR window to analyses by IR spectrophotometer (Shimadzu FTIR 8400S, software IR spectrum).

C. Microscopy, Particle Size and Particle Size Distribution

The particle sizes of conventional and stealth liposomes were viewed and photographed using phase contrast microscope. All the prepared batches of liposomes were viewed under microscope was determined by placing a drop of liposomal dispersion on glass slide and average size was determined.

D. Drug Entrapment Efficiency

The liposomal suspension was ultra-centrifuged at 5000 rpm for 15 minutes at a temperature of 4°C using the remi-cooling centrifuge in order to separate the drug. The supernatant liquid contained the liposomes in the suspended stage and the free drug at the wall of centrifugation tube. The supernatant liquid was again collected and re-centrifuged at 15000 rpm at 4°C temperature for a span of 30 minutes. A clear solution of supernant and pellets of liposomes was obtained. The pellet containing only liposomes was re-suspended in buffer (pH 7.4) until further processing. The liposomes free from unentrapped drug were soaked in 10 ml of methanol and then sonicated for 10 min. The vesicles were broken to release the drug, which was then estimated for the drug content[11,12]. The absorbance of the drug at suitable wavelength. The percent % entrapment of drug was calculated by the following formula:

% Drug Entrapped (PDE) = Amount of drug in sediment × 100 / Total amount of drug

E. Transmission Electron Microscopy

TEM was used to elucidate the surface morphology and also to analyze the size of vesicles. This method is also used to characterize the liposomal formulation in order to ensure its stability. The prepared liposomes suspension were processed by using copper grids to adsorb liposome particles from the suspension, then stained in 2.5 % uranyl acetate for 30 seconds and dried . The specimens were observed under JEM-1010 Transmission Electron Microscope (JEOL, Japan) operated at 200 kV.

F. Differential Scanning Calorimetry (DSC) Study

Differential scanning Calorimetry (DSC) experiments were performed with differential scanning calorimeter (model TA-60, Shimadzu, Japan) of Sample of pure 5-flurouracils. The liposomal dispersions were weighed in an aluminum cuvette and sealed with an aluminum lid. The cuvette was placed in the DSC and heated from 200-2000°C at a heating rate of 100°C/min in nitrogen atmosphere. The scan was recorded and plotted showing heat flow (w/g) on the Y-axis and temperature (0°C) on the X-axis[13].

G. *In-vitro* Drug Release Study from Liposomes

The Franz-diffusion cell was employed drug release studies from liposomal system with an aim to predict the *in-vivo* behavior of the formulated liposomes. The dimension of Franz-diffusion cell was 25 mm diameter and 4.90 cm^2 area for diffusion. Regenerated cellulose acetate membrane (thickness of 60–65 µm and 0.45 µm pore size) was sandwiched between the lower cell reservoir and the glass cell top containing the sample and secured in place with a pinch clamp. The receiving compartment was fully filled with phosphate buffer of pH 7.4 . The above system was maintained at 37±0.5°C by magnetic heater, resulting in a membrane-surface temperature of 32°C[14]. A Teflon TM coated magnetic bar continuously stirred the receiving medium to avoid diffusion layer effects. A sample (2 ml) was placed evenly on the surface of the membrane in the donor compartment. From receptor compartment the samples (2 ml) were withdrawn from the at 1, 2, 4, 6, 8, 10, 12 and 24 hours, replaced with 2 ml of fresh buffer. Samples were assayed spectrophotometrically for in-vitro drug release studies. All experiments were carried out in triplicate and average values were presented.

H. Stability of Liposomes

Physical stability study was performed with the main aim to examine the leak out of the drug from liposomes during storage. Liposomal suspensions of 5-Fluorouracil of selected batch (code L) were sealed in 20-ml glass vials and stored at refrigeration temperature (2-8°C), room temperature (25±2°C) and 45°C for a period of 1 month. The remaining amount of drug present in the vesicles was determined after separation from the unentrapped drug in the same procedure as mentioned earlier in the heading drug entrapment efficiency[15].

RESULTS AND DISCUSSION

A.Standard Calibration Curve Data of 5-Fluorouracil

The obtained values are shown in table 2.and the obtained calibration is shown in figure 1.

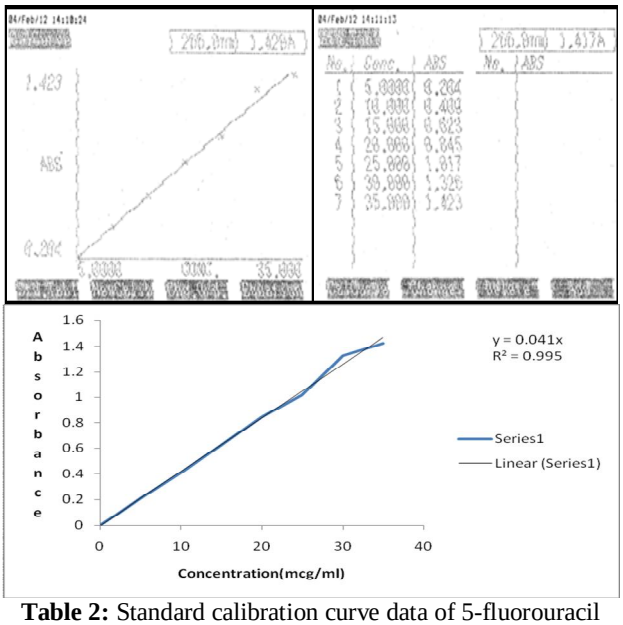

Table 2: Standard calibration curve data of 5-fluorouracil and standard curve of 5-fluorouracil in phosphate buffer pH 7.4

S. No.	Concentration (µg/ml) (X)	Absorbance (Y)
1	0	0.00
2	5	0.204
3	10	0.409
4	15	0.623
5	20	0.845
6	25	1.017
7	30	1.326
8	35	1.423

B. FTIR Spectroscopy

FTIR tracings of 5-fluorouracil, soya phospholipids, cholesterol ,sorbitol, mannitol and of the optimized batch of prepared liposomes are shown below:

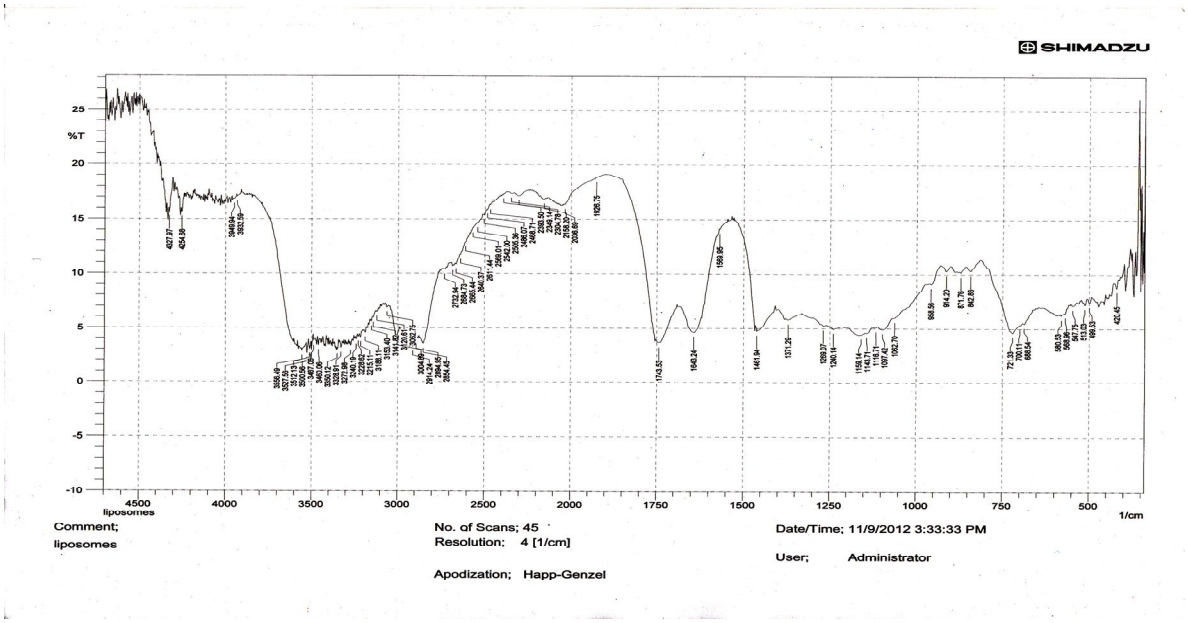

Figure 2: FTIR Spectra of optimized batch Liposome

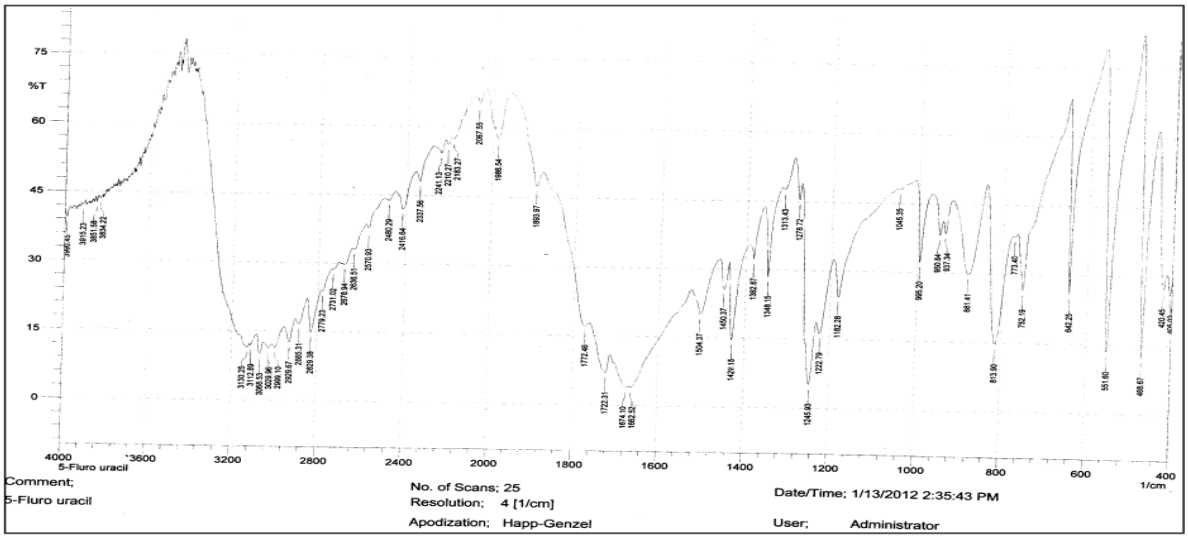

Figure 3: FTIR Spectra of 5-fluorouracil

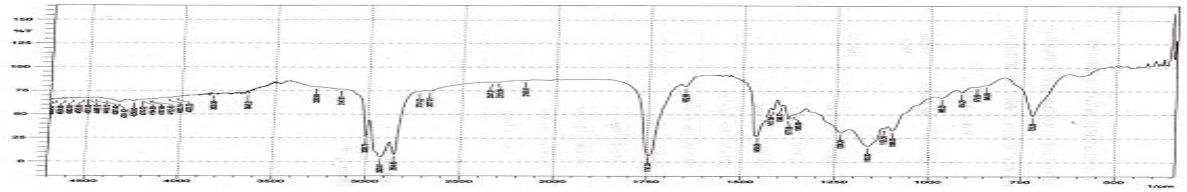

Figure 3: FTIR Spectra of Soya phospholipid

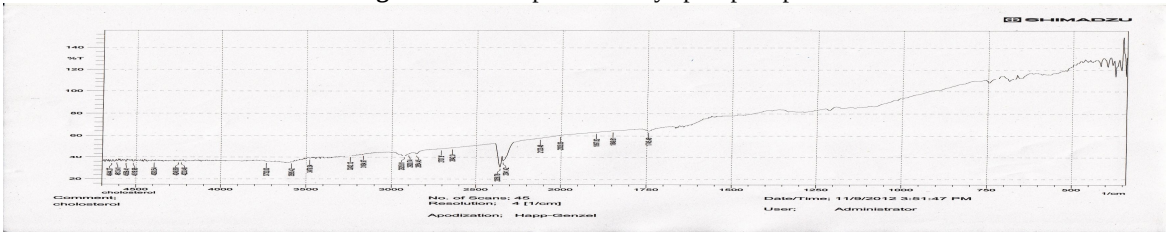

Figure 4: FTIR Spectra of Cholesterol

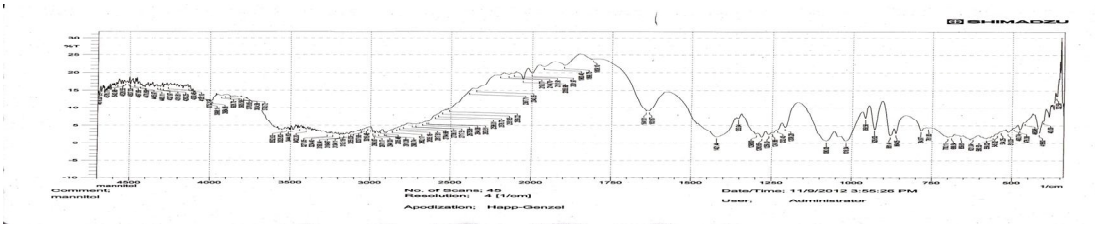

Figure 5: FTIR Spectra of Cholesterol

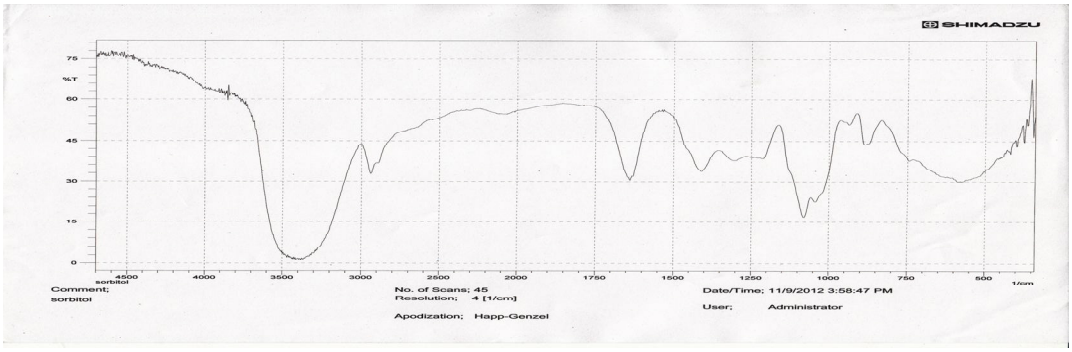

Figure 6: FTIR Spectra of Sorbitol

C. Results of Phase Contrast Microscopy

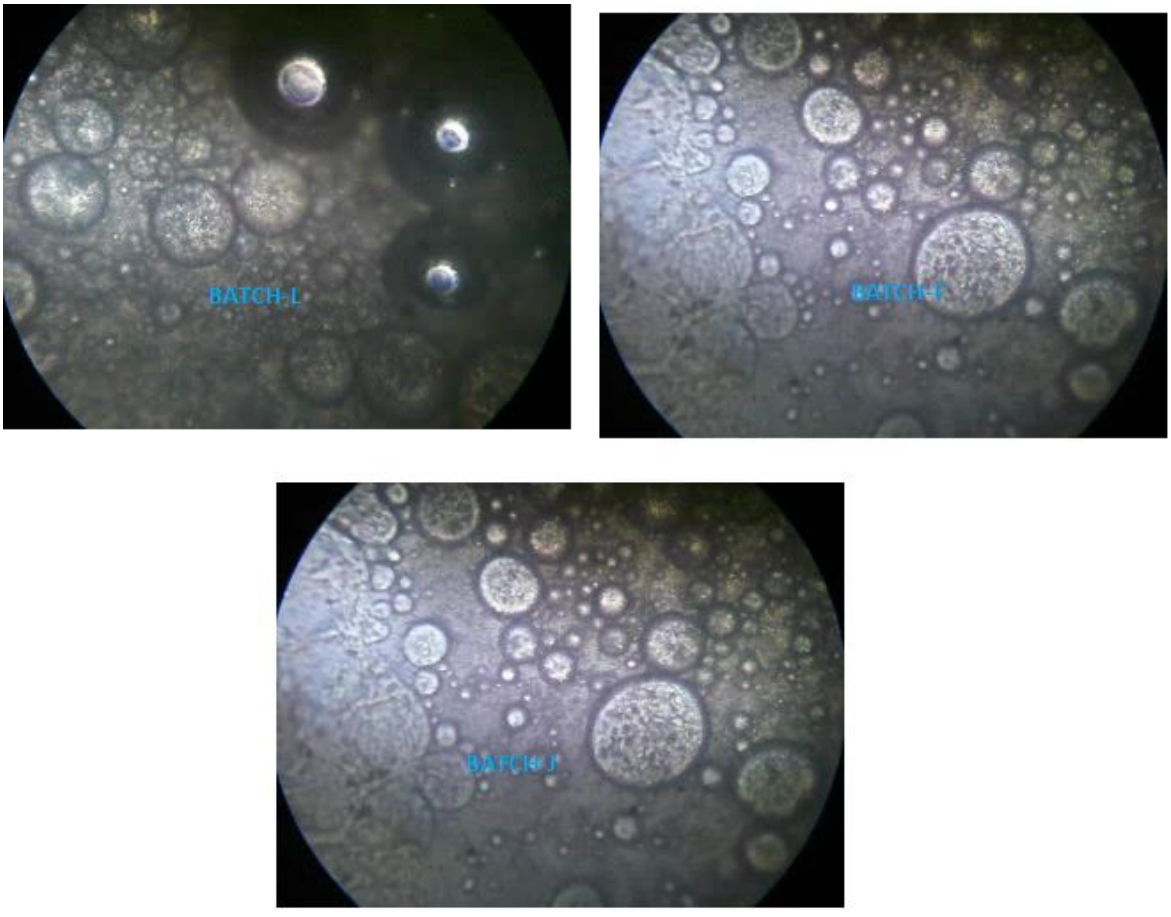

Figure 7: Phase contrast microscopy of best batch

D. Particle Size Distribution and Drug Entrapment

The particle size distribution and their drug entrapment values are given in table 3a and b.

Table-3a: Particle size distribution and their drug entrapment values (Batch A-F)

Batch	A	B	C	D	E	F
%DRUG ENTRAPPED	19.1	10.2	23.6	42.7	38.7	52.4
MEAN PARTICLE SIZE (µm± SD)	17±0.65	25±0.99	16±0.97	11±0.98	14±0.78	7.25±0.08

Table-3b: Particle size distribution and their drug entrapment values (Batch G-L)

Batch	G	H	I	J	K	L
%DRUG ENTRAPPED	16.2	14.2	28.3	49.3	38.7	58.6
MEAN PARTICLE SIZE (µm± SD)	18±1.12	21±0.09	15±0.85	9±0.45	13±0.76	6.24±0.09

E. Transmission Electron Microscopy

The obvious morphology of liposomes produced by thin film method can be investigated by transmission electron microscopy with negative staining technique.

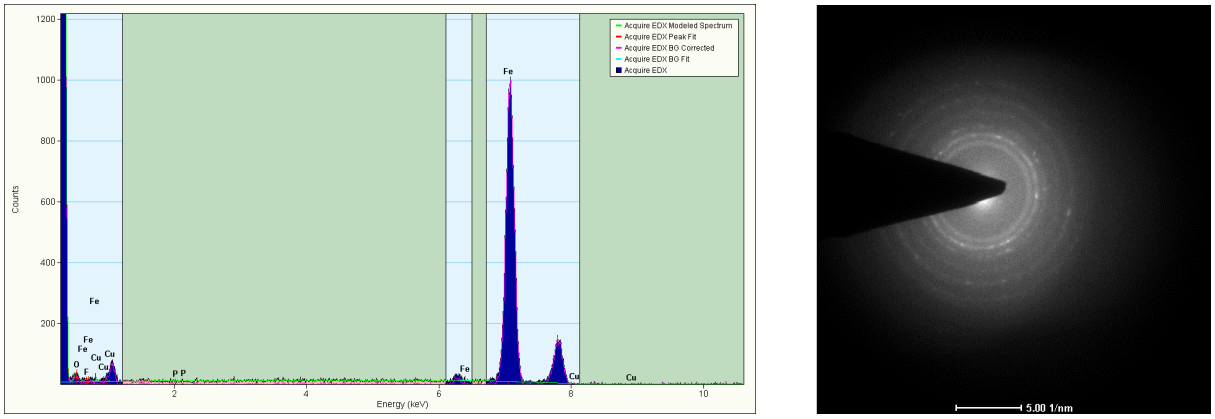

Figure 8: Transmission electron microscopy pictures of the prepared liposomes

F. Differential Scanning Calorimetry (DSC)

The DSC thermogram of 5-fluorouracil showed in figure 7 which showed a long and sharp characteristic **endothermic peak** at 288.049°C due to its phase transition system and it to proved the presence of authentic molecule of 5-fluorouracil.

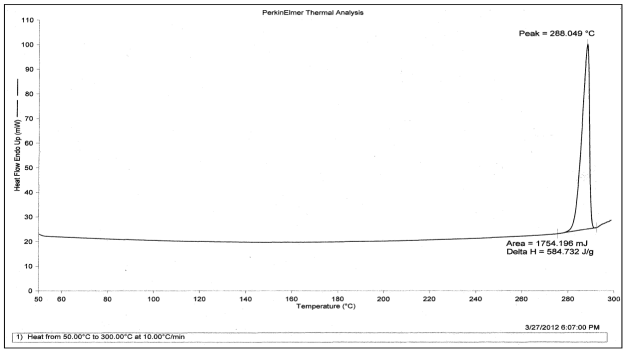

Figure 9: DSC thermo gram of 5-fluorouracil

G. In-vitro Drug Diffusion / Release Study from Liposomes

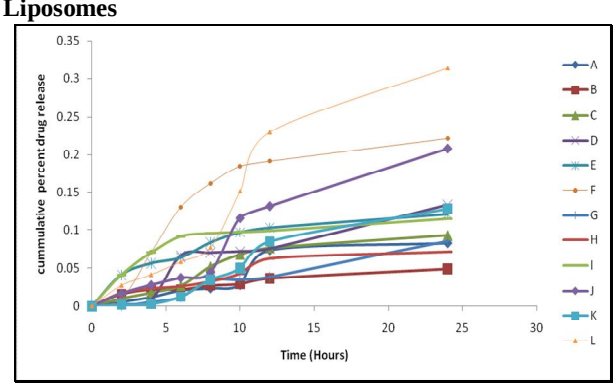

Figure 10: Relese profile of the best batch (batch –L)

G. Stability of Liposomes (Batch-L)

Amount of drug remain				
Temperature	**Weeks**			
	1	**2**	**3**	**4**
2-8°C	98 %	98%	98%	97%
25°C	97%	91%	90%	86%
45°C	87%	64%	46%	30%

H. Release Mechanism of Best Selected Batch – L

Model Fitting	R^2	K	Parameters for
Zero order	0.8503	0.0037	Korsmeyer-Peppas Equation
1st order	0.8503	0	n = 1.132
Higuchi Matrix	0.9082	0.3063	k = 0.0757
Peppas	0.9315	0.0757	Best fit model = Peppas Korsmeyer
Hix.Crow.	0.8503	0	Mechanism of release: Supercase II Transport

CONCLUSION

Among the various method employed for the preparation of liposomes in litreature, the film hydration technique followed by sonication lead to the formation of liposome particle of homogeneous size distribution, which had good entrapment efficiency and stability.

The % entrapment efficiency was varied between 58.2 to 10.2 and size varied between 6.24±0.09 to 25±0.99 µm. The drug release profile indicated that on increasing the phospholipids concentration the drug release was higher, which may be due to the hydrophilic nature of phospholipid and on increasing cholesterol concentration there was decrease in drug release, which may be due to the hydrophobic nature of cholesterol (batch-L).

Drug release was found to be more on increase sorbitol/mannitol concentration from 2-3 mg. The Stability data showed on increasing the temperature the stability of liposomes was found to be decreased .Among sorbitol and mannitol the liposome prepared with sorbitol showed more drug release as compare to mannitol at same concentration (2-3mg).

Finally it was concluded that concentration of phospholipids, cholesterol, sorbitol and mannitol affects drug release and stability.

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