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FORMULATION AND CHARACTERIZATION OF AMPHOTERICIN B LIPOSOMES PREPARED BY THIN FILM HYDRATION METHOD

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ABSTRACT

Amphotericin B is a polyene antifungal drug used intravenously for systemic fungal infections. Simple solution of amphotericin B is having many side effects while liposomal amphotericin B preparations exhibit fewer side-effects having similar efficacy. Various preparations of liposomal amphotericin B have recently been introduced and all of these are more expensive than plain amphotericin B. Fungisome and Abelcet are liposomal complex formulation of amphotericin B and being the latest and cheapest addition to the lipid formulations of amphotericin B. AmBisome is a liposomal formulation of amphotericin B for injection which is having less side effects as compared to all other formulations of Amphotericin B. Liposomal formulation of amphotericin B for injection, prepared by thin film hydration technique was selected in the present study. Different formulations variables (solvents ratio and pH of complex formation) and process variable (numbers of homogenization cycles) were carried out to control the impurities levels and particle size of liposomes. Formulation prepared at pH 3.0 with 1:2 solvent ratio (Methanol: Chloroform) was given least impurities. Formulation prepared at 1400 bar pressure with 15 homogenization cycles was shown desire particle size. The optimized formulation was exhibited more than 90% release of drug for a period of 7 days. The stability study (40±2°C/ 75±5% RH) of the Amphotericin B liposomes was evaluated for 3 months and it was found to be stable.

Keywords: Amphotericin B, Liposomes, Thin film hydration technique, Homogenization.

INTRODUCTION

Amphotericin B is a polyene antifungal drug, often used intravenously for systemic fungal infections. It was originally extracted from Streptomyces nodosus, a filamentous bacterium. Two amphotericins, amphotericin A and amphotericin B are known, but only B is used clinically, because it is significantly more active in vivo. Amphotericin A is almost identical to amphotericin B (having a double C=C bond between the 27th and 28th carbons), but has little antifungal activity. Currently, the drug is available as plain amphotericin B, as a cholesteryl sulfate complex (ABCD), as a lipid complex (ABLC), and as a liposomal formulation (LAmB). The latter formulations have been developed to improve tolerability for the patient, but may show considerably different pharmacokinetic characteristics compared to plain amphotericin B [1].

As with other polyene antifungal, amphotericin B binds with ergosterol, a component of fungal cell membranes, forming a transmembrane channel that leads to monovalent ion (K^+, Na^+, H^+ and Cl^-) leakage, which is the primary effect leading to fungal cell death. Recently, however, researchers found evidence that pore formation is not necessarily linked to cell death, the actual mechanism of action may be more complex and multifaceted. Mammalian and fungal membranes both contain sterols, a primary membrane target for amphotericin B. Because mammalian and fungal membranes are similar in structure and composition, this is one mechanism by which amphotericin B causes cellular toxicity. Amphotericin B molecules can form pores in the host membrane as well as the fungal membrane. This impairment in membrane barrier function can have lethal effects. Bacteria are not affected as their cell membrane does not contain sterols [2, 3, 4].

From studies, it appears that liposomal amphotericin B preparations exhibit fewer side-effects, while having similar efficacy. Various preparations have recently been introduced. All of these are more expensive than plain amphotericin B. AmBisome is a liposomal formulation of amphotericin B for injection, developed by NeXstar Pharmaceuticals (acquired by Gilead Sciences in 1999). It was marketed by Gilead in Europe and licensed to Astellas Pharma (formerly Fujisawa Pharmaceuticals) for marketing in the USA, and Sumitomo Pharmaceuticals in Japan. Fungisome is a liposomal complex of amphotericin B, and being the latest and cheapest addition to the lipid formulations of amphotericin B, it has many advantages. It is marketed by Life care Innovations of India. Other formulations include Amphotec (Intermune) and Abelcet (Sigma-Tau Pharmaceuticals). Abelcet is not a liposomal preparation but rather a lipid complex preparation. Ampholip is a lipid complex formulation of amphotericin B marketed by Bharat Serums & Vaccines Ltd, Mumbai, India [5].

MATERIALS AND METHODS

Materials: Amphotericin B was obtained from symbiotic – Gujarat; Distearoyl phosphatidyl glycerol (DSPG), Hydrogenated Soy Phosphatidyl Choline (HSPC) and Cholesterol were obtained from Avanti Polar Lipids - USA. The laboratory grade chemicals used for the work are Sucrose, Disodium succinate hexahydrate, Alpha Tocopherol, Chloroform, Methanol, Sodium hydroxide, Hydrochloric acid, Triton X-100, Acetonitrile are purchased from Merck Chemicals Pvt., Ltd. Mumbai.

Compatibility studies: IR spectroscopy can be used to investigate and predict any physicochemical interactions between different components in a formulation and therefore it can be applied to the selection of suitable chemically compatible excipients. The aim of the present study was to test, whether there is any interaction between the carriers and drug; The following IR spectroscopy was recorded.

Preparation of Amphotericin B liposomes: The Amphotericin B liposomes with DSPG, HSPC and Cholesterol were prepared by dried thin film hydration technique using rotary evaporator. An accurately weighed quantity of DSPG was dissolved in equi volume mixture of methanol and chloroform by acidifying with HCl at 60°C. Amphotericin B was dispersed in equi volume mixture of methanol and chloroform. HSPC, cholesterol, are dissolved separately in equi volume mixture of methanol and chloroform at 60°C. Complex was formed between lipids and Amphotericin B by mixing lipid solution to drug suspension at 60°C by adjusting the pH between 3-4 with HCl & NaOH. Alpha tocopherol was added to the drug-lipid complex and rotated in a rotavapor by applying vacuum of about 25mmHg at 40°C and 75 RPM, until it formed a thin film. Required quantities of sucrose and disodium succinate were dissolved in W.F.I, added to the above thin film in Round Bottom flask for hydration and rotated until it forms a yellow colour suspension. The above suspension was homogenized by high shear homogenizer at 10000 RPM for 10 min followed by high pressure homogenizer for 20 cycles to reduce particle size of liposomes. The nano-sized Amphotericin B liposomes was filtered through 0.2µ PES/PVDF filter and loaded in to lyophillizer [6,7].
Different formulations variables (solvents ratio and pH of complex formation) and process variable (numbers of homogenization cycles at 1400 bar pressure) cycles were carried out to control the impurities levels and particle size of liposomes.

Characterization of Liposomes

Amphotericin B Assay
Assay Standard preparation: Weigh and transfer accurately 50 mg of Amphotericin B working standard in to 100 mL volumetric flask, add 70 mL of diluent and sonicate to dissolve and make up to volume with diluent. Further dilute the resultant solution from 5 mL to 100 mL with diluent (Concentration: 0.025mg/mL).

Sample preparation: Reconstitute the vial with 12.5 ml of WFI and mix the reconstituted solution. Then transfer accurately whole reconstituted solution in to a clean and dry 200 mL volumetric flask, added 150 ml of diluent, mixed well, sonicated for 5 minutes and make up to the volume with diluent. Further dilute the resultant solution from 5 mL to 50 mL with diluent. (Concentration: 0.025mg/mL)
Separately inject standard and sample preparation into the liquid chromatography record the chromatograms

Related substances:
Reconstitute the vial with 12.5 ml of WFI and mix the reconstituted solution. Then transfer accurately whole reconstituted solution in to a clean and dry 200 ml volumetric flask, added 150 ml of diluent, mixed well, sonicated for 5 minutes and make up to the volume with diluent. Further dilute the resultant solution from 5 mL to 50 mL with diluent. Impurity

B should not be more than 5% and total impurities should not be more than 15%.

Particle size analysis and Zeta potential: The mean diameter and surface charge of liposomes was determined by laser diffractometer (Mastersizer X, Malvern Instrument, UK). Liposomes were diluted from 1 to 10 fold prior to determination of particle size and Zeta potential [8,9].

Transition Electron Microscopy (TEM): The Morphology and surface appearance Liposomes were examined by using TEM (Using Hitachi-S-3700N). Transition electron microscopy was carried out to study the morphological characteristics of Amphotericin B Liposomes. The samples for the TEM analysis were prepared by placing the Liposomal solution on one side of adhesive stub. Then the dried liposomes were coated with gold (100A°) before microscopy. Finally the morphology of the Liposomes was observed with the transition electron microscopy [8].

In vitro Release studies: The in vitro release of drug from the liposomal formulation was carried out by using dialysis membrane employing in two sides open ended cylinder. One side of dialysis membrane was closed and 12 ml of liposomal suspension containing known amount of drug was placed in a dialysis membrane previously soaked overnight. After closed the second side of dialysis membrane, it was placed in 200 ml of PBS (pH 7.4) maintained at 37°C and stirred with the help of a magnetic stirrer. Aliquots (4ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh PBS (pH 7.4) to maintain constant volume. 1 ml of Acetonitrile was added to each aliquot to precipitate the lipids and dissolve the entrapped Amphotericin B and then the samples were analyzed by UV spectrophotometry at a λ max of 383 nm [10, 11, and 12].

Stability Studies: The stability of a pharmaceutical delivery system may be defined as the capability of a particular formulation, in a specific container. The short-term stability was conducted to monitor physical and chemical stabilities of the liquid form of Amphotericin B liposomal formulations at 40°C and room temperature for up to three months. The stability parameter, such as Assay was determined as function of the storage time.

RESULTS AND DISCUSSIONS

Compatibility studies: The compatibility between the drug, lipids and other excipients was evaluated using FTIR peak matching method. There was no appearance or disappearance of peaks in the drug-lipid mixture, which confirmed the absence of any chemical interaction between the drug, lipid and other chemicals.

Optimization of process and formulation variables for Amphotericin B liposomes: Different formulations variables (solvents ratio and pH of complex formation) and process variable (numbers of homogenization cycles at 1400 bar pressure) cycles were carried out to control the impurities levels and particle size of liposomes. Results of formulation variables i.e. effect of pH during complex formation and effect of solvents ratios on impurities were given in table 1 and 2 respectively. Effect of homogenization cycles on particle size were shown in table 3.

The effect of pH during complex formation was played a significant role on impurities profile. Drug-Lipid complex was not formed at pH 3.5 while it formed at pH 3.0 and 2.25. The Related substance of F2 formulation was found to be least i.e. 3.2% in table 1.There was also a significant effect of solvents ratio on impurities of amphotericin B liposomes as shown in table 2. As the ratio of solvents increased, impurities level was decreased significantly. The Related substance of F2 C formulation was found to be 3.82% (Figure 2). Formulation F2 C was selected for further process variables.

High pressure homogenization was carried out with F2 C formulation at 1400 bar pressure up to 20 cycles to check out the effect of particle size. As the numbers of homogenization cycles were increased, particle size of liposomes was decreased significantly. No significant reduction in particle size was observed after 15 homogenization cycles. The particle size distribution was analyzed for optimized formulation F_2 C3 of Amphotericin B Liposomes by wet method. The particle size and Zeta Potential for F_2 C3 was obtained 73nm (Figure 3) & -17.8mV (Figure 4) respectively.

The Assay was determined for the optimized formulation F2 C3. The assay for F_2 C3 formulation was found to be 95.78% (Figure 2).

In vitro Release studies: The in vitro dissolution profile for F_2 C3 formulations (Figure 5) was carried out by membrane diffusion method. The dissolution was carried out for a period of 7 days in saline phosphate buffer pH 7.4. Initial burst release (1day) of liposomes was shown 20% and more than 90% of drug was release within 7 days.

Transition Electron Microscopy (TEM): The Morphology and surface appearance of Liposomes were examined by using TEM (Figure 6). The TEM image for F_2 C3 formulation showed that the liposomes have spherical shape.

Stability Studies: The stability of the Amphotericin B liposomes was evaluated for optimized formulation of F_2 C3 after storage at accelerated condition at 40±2°C/ 75±5% RH for 3 months. The Description, assay & impurities of the samples were determined as a function of the storage time. The Liposomes stored at 40°C were found to be stable for duration of 3 months. The results were showed in Table 4.

CONCLUSIONS

In the present study, attempts were made to prepare Amphotericin B Liposomes for controlled release by thin film hydration technique. Different formulations variables (solvents ratio and pH of complex formation) and process variable (numbers of homogenization cycles at 1400 bar pressure) were carried out to control the impurities levels and particle size of liposomes. The prepared liposomes were evaluated for assay, Impurities, particle size, zeta potential and TEM. There was a significant effect of pH, solvents ratio and homogenization on impurities level and particle size respectively. The optimized formulation was exhibited more than 90% release of drug for a period of 7 days. From the experimental results it was evidenced that the controlled release of Amphotericin B liposomes was successfully formulated with less side effects.

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Table 1: Effect of pH on impurities

Formulation code	Volume of 2.5M HCl	pH	Impurities
F_1	0.5 ml	3.5	3.0%
F_2	1.0 ml	3.0	3.2%
F_3	1.5 ml	2.25	7.2%

Table 2: Effect of solvent ratio on impurities

Formulation code	Ratio of solvent (Methanol: Chloroform)	Impurities
F_2 A	1:0.5	18.2%
F_2 B	1:1	8.6%
F_2 C	1:2	3.8%

Table 3: Effect homogenization cycles on particle size

Formulation	Number of homogenizer cycles (At 1400 bar)	Particle size
F_2 C1	5	200 nm
F_2 C2	10	120 nm
F_2 C3	15	73 nm
F_2 C4	20	70 nm

Table 4: Stability data at 40±2°C/75±5% RH

Test	Initial	1 month	2 months	3 months
Assay	95.78 %	95.0 %	94.50 %	94.25 %
Impurity	3.82%	3.92%	3.95%	3.98%
Particle size	73nm	75nm	81nm	84nm

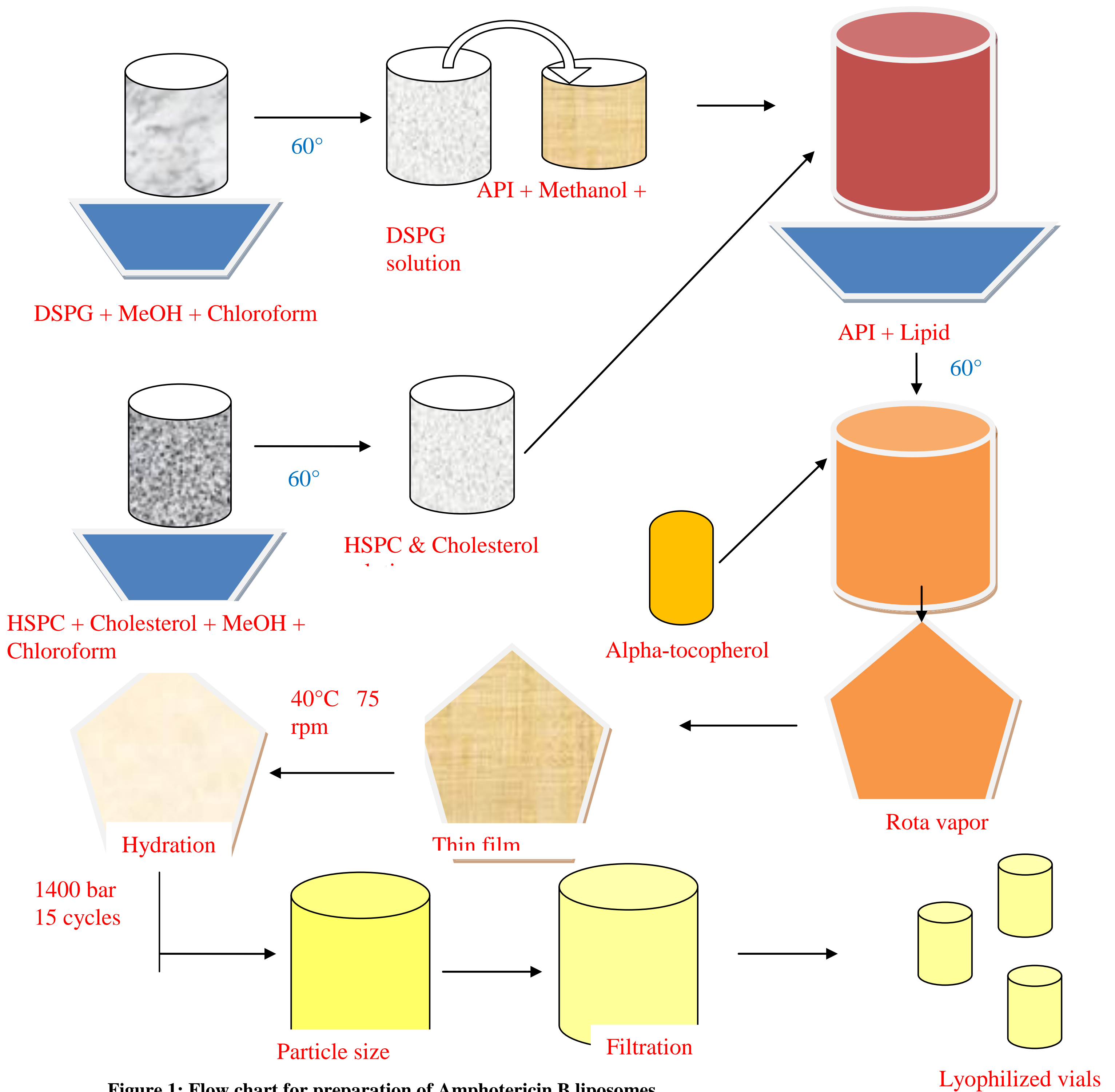

Figure 1: Flow chart for preparation of Amphotericin B liposomes

Celon Innovation Center

Data Discription : Assay&Rs

Sample ID:Sample

Vial No: 2

Injection Volume: 0

Data File: C:\Enterprise\Projects\Amphotericine B\Result\Jan 2013\28012013_Asy.rslt\28012013002-Rep1.dat

Method: C:\Enterprise\Projects\Amphotericine B\Result\Jan 2013\28012013_Asy.rslt\Amphotericine_meth.met

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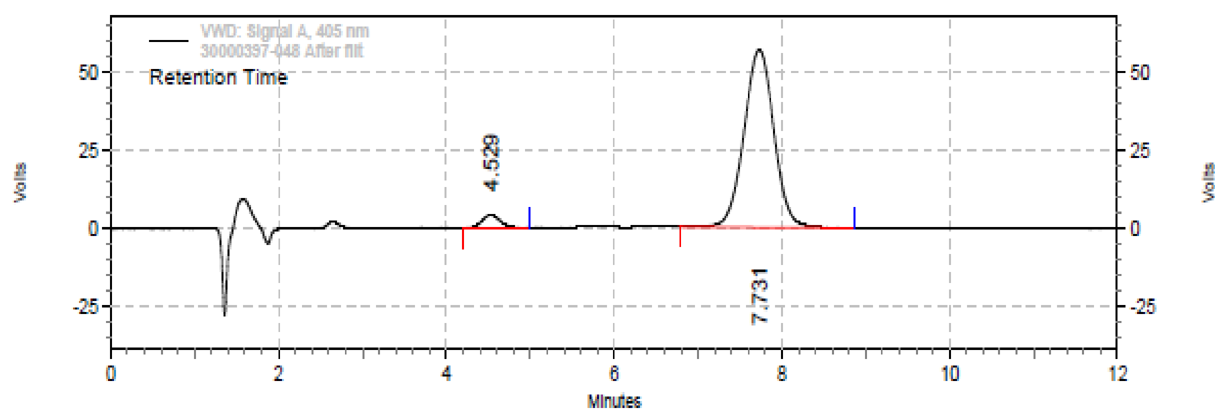

VWD: Signal A,
405 nm Results

Peak Number	Name	Retention Time	Area	Area %
1		4.529	946398	3.82
2		7.731	23728547	95.78
Totals			24774818	100.00

Figure 2: Assay and Related substances for F_2 C3 Formulation

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	73.01	Peak 1:	78.28	95.8	29.51
PdI:	0.194	Peak 2:	4128	4.2	1035
Intercept:	0.967	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

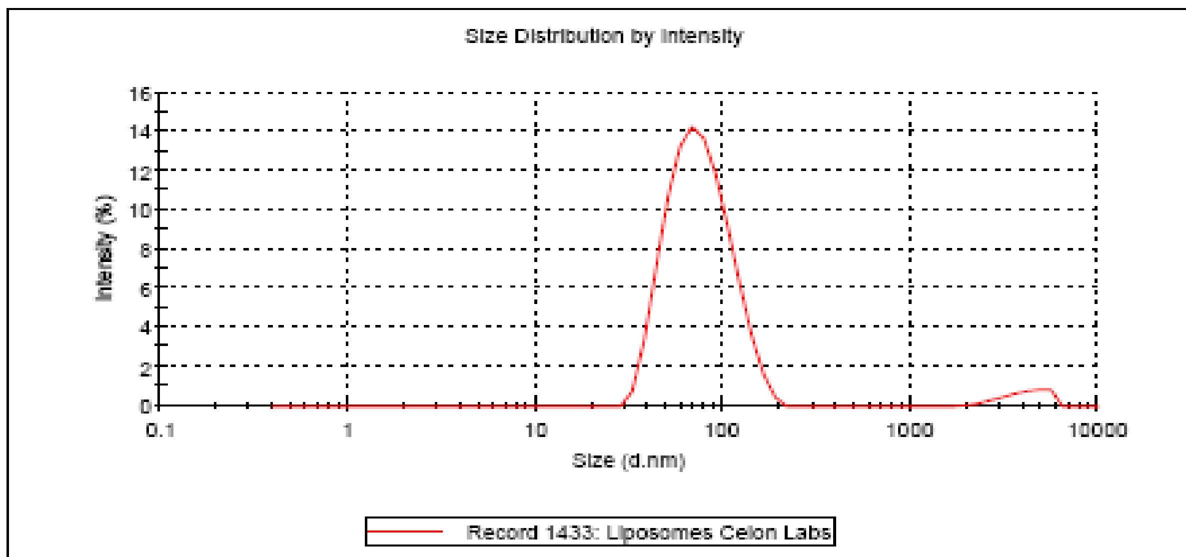

Figure 3: Particle size for F_2 C3 Formulation

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-17.8	Peak 1:	-17.8	100.0	7.48
Zeta Deviation (mV):	7.48	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm):	0.540	Peak 3:	0.00	0.0	0.00
Result quality :	Good				

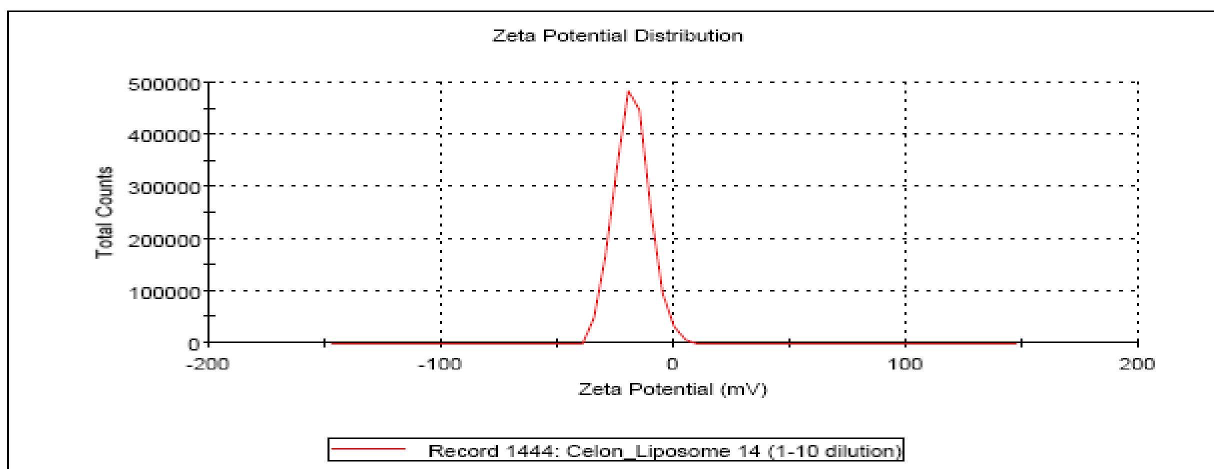

Figure 4: Zeta Potential for F_2 C3

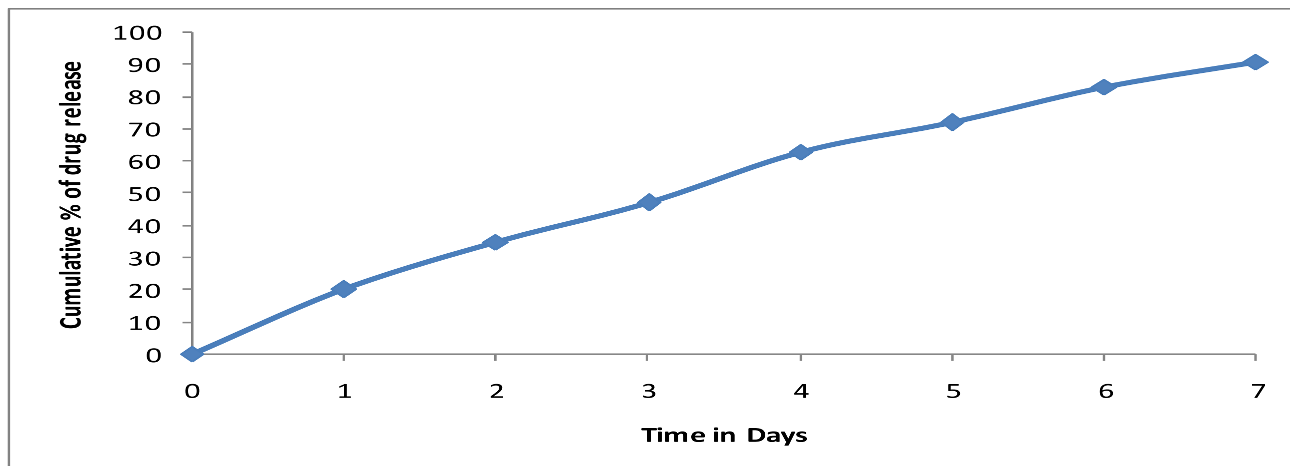

Figure 5: In-vitro release profile for F_2 C3 formulation

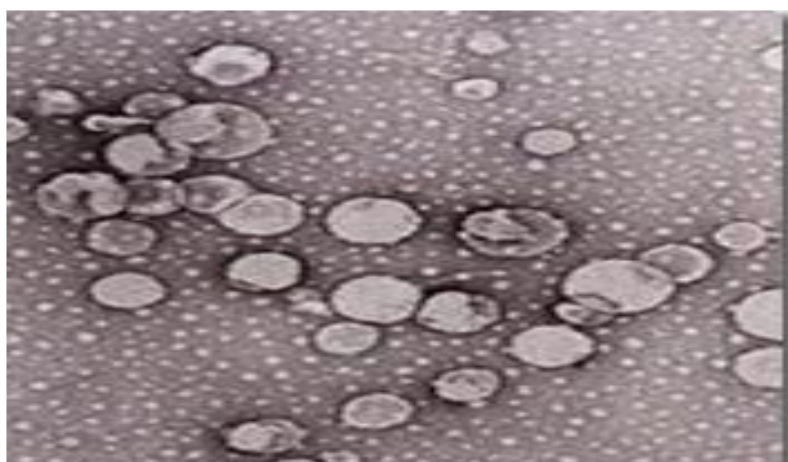

Figure 6: TEM image for F_2 C3 formulation

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