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Formulation and *In-vitro* Characterisation of Fulvestrant loaded liposomes for breast cancer therapy

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A– research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: The intrinsic limits of conventional cancer therapies prompted the development and application of various nanotechnology strategies for more effective and safer cancer treatment, one of which is liposomes.
Objectives: Considerable technological success has been achieved in this field, but the main obstacles to nano-medicine becoming a new paradigm in cancer therapy stem from the complexities and heterogeneity of tumour biology, an incomplete understanding of nano–bio interactions and the challenges regarding chemistry, manufacturing and controls required for clinical translation and commercialization.
Methods: In this study, three different formulations of Fulvestrant liposomes which differ in drug/polymer ratios (A=1:1; B=1:2 and batch C=2:1) were prepared using film hydration technique and thereafter characterized in-vitro by determining the particle sizes, the zeta potentials, drug loading poly dispersible index, drug release profile and the cellular uptake by MCF-7 cell line.
Results: The Fulvestrant liposomes produced were spherical and are of various sizes with average particle size of 191 (d.nm) for formulation A, 760.7 for formulation B and 205.5 (d.nm) formulation C. Formulation B also has the highest drug loading and the poly dispersible index (PDI) values, 30.19 % and 0.750, respectively.The zeta potential values ranged from -14.9mV to -3.40mV for all formulations which suggest relatively stable dispersed liposomal nanoparticle in liquid state. The cumulative drug released for formulation B was 100% and well prolonged for over 1,200 h. Both formulations A and C however did not achieve 100% released of entrapped drug even though the released were equally prolonged and even though the released kinetics for all the three formulations essentially follow the same Korsmeyer mathematical model, as adjudged by the co-efficient of determination values R2. Formulation B was well absorbed in MCF-7 breast cancer cell line and effect time base cytotoxic activity.
Formulation B with Lecithin: Cholesterol ratio of 1:2 showed the highest drug loading, the highest percentage cumulative released of the drug (in a sustained manner for a prolonged period of time) and adequately internalized in the MCF-7 breast cancer cell line in vitro.
Conclusion: Therefore formulation B is more suitable for breast cancer treatment due to its excellent physicochemical characteristics, its good permeation, internalization and the time based killing of the breast cancer cells.
Keywords: Liposome, Nanoparticles, Fulvestrant, Breast cancer**.**

INTRODUCTION

One in every five cancer patients suffers from breast cancer worldwide. Also there are more than 2 million new cases reported annually, (Malik *et al*., 2012). Breast cancer is the second leading cause of cancer death for women in the United States. Approximately 40,290 women and 440 men are estimated to die from the disease annually, (Ellis *et al.,* 2000). Breast cancer is estrogen-dependent, reducing estrogen secretion by oophorectomy, hypophysectomy, or adrenalectomy may lead to regression of breast cancer, (Bhatial *et al.,* 2004).

Clinical treatment of breast cancer depends on the extent of the disease. Early detection is the key to successful management of breast cancer in all age groups. The treatment most time involves combination of chemotherapy, surgery and radiotherapy (Ellis *et al.,* 2000).

The numbers of chemotherapeutic agents available for the treatment of breast cancer are growing. Fulvestrant is an estrogen receptor antagonist with no agonist effect. It competitively blocks the estrogen receptors (ER) thereby preventing the gradual progression of breast tumor. However in contrast to other cytotoxic drugs which are administered in cyclic regimens constant exposure of cancer cells to Fulvestrant is very critical to its cytotoxic activity, (Ning *et a.,l* 2005). Just like any other normal formulation cytotoxic drugs, conventional Fulvestrant is active against hormonal responsive breast cancers cell as well as normal healthy living cells. This is mainly responsible for the clinical adverse drug reaction experienced by patients on these medications. Acceptability and tolerability are great concerns among health care professionals whenever patients are to be placed on conventional chemotherapies. This has greatly limited the chemotherapeutic management of breast cancer. Also drug permeation into cancer cells from the conventional formulations is very poor due to low distribution and fast elimination of the drug from the system, (Sinha *et al*., 2006).

Liposomes are lipid base nano-formulations, spherical shaped, colloidal in nature and composing of lipid bilayer generally in which an outer lipid bilayer surrounds a central aqueous space, (Rivera, 2003). Liposomes are effective drug carrier, and can be fabricated to achieve a controlled and target delivery of incorporated drugs thereby reducing the systemic toxicity and adverse reaction to the administered drugs, (Kohno *et al.,* 1998; Huwyler *et al.,* 2008). Multiple cytotoxic agents can be incorporated into the same liposome; hydrophilic drugs are entrapped in the aqueous compartment while hydrophobic drugs are incorporated within the lipid bilayer. Liposome has been recognized as an effective nano drug delivery system and it may be used extensively in formulating most cytotoxic agents with great success. Entrapped drug molecules are protected from enzymatic and metabolic degradation; thereby increasing the biological half life of the incorporated drugs. The increased uses of liposome as nano drug delivery system is often said to be due to its ability to increase drug concentration within tumor cells there by effecting selective death of the malignant cells. Also because liposomes are formulated using natural biocompatible and biodegradable material that shares some similarity with biological membranes and body tissues, they are generally safe, and can be fabricated to increase their selective target delivery of drug molecules to only tumor cells, (Hussain, *et al.,* 2006).). In order to ameliorate the adverse drug reactions such as nausea, vomiting and hair lost associated with the use of Fulvestrant, a liposomal formulation of Fulvestrant is desirable.

MATERIALS AND METHODS

Fulvestrant was obtained from Sigma-Aldrich Co., St Louis, MO, USA, Lecithin and Cholesterol from HiMedia Laboratories, Mumbai, Indian; all other reagents are analytical grades.

Methods

Pre-formulation study

To gain an understanding of possible interactions among various excipients to be used in and active drug to be used in this study, samples of the pure drug (Fulvestrant), cholesterol and soya lecithin, were mixed separately with infrared (IR) grades KBr in the ratio of 100:1 and corresponding pellets were prepared by applying 5.5 metric tons of pressure in a hydraulic press. The pellets were thereafter scanned in an inert atmosphere over a wave number range of 4000–400 cm–1 in Magna IR 750 series II (Nicolet,USA) FTIR instrument.

The procedure was further repeated for a mixture of Fulvestrant, cholesterol, and soya lecithin.

Liposome formulation and preparation

Three different batches of liposomes were prepared by lipid film hydration method, (Mukherjee *et al.,*

2007). The weighed amount of Fulvestrant, soya lecithin and cholesterol were taken as per the experimental combination (Table I). Since all are hydrophobic, they were all dissolved in 30 mL of chloroform and the mixture thereafter transferred into a 250 mL round bottom flask placed in a rotary vacuum evaporator with an aspirator A3S (Tokyo Rikakikai, Co. Ltd., Tokyo, Japan). The rotary vacuum evaporator was fitted with a cold water circulating bath (Space-N Service, Kolkata, India) and operated at 150 rpm while the water bath was maintained at 32 °C until all the solvent was completely evaporated. The flask was thereafter transferred into a desiccator over night for complete removal of residual chloroform. The lipid film formed in the round bottom flask was however hydrated after 24 hours by adding 100 mL of freshly prepared phosphate buffer solution (PBS) into the flask while continuously rotating the flask in the vacuum evaporator maintained at 60 °C and 100rpm until the lipid film was completely in the aqueous phase. The dispersions were thereafter sonicated using a bath-sonicator (30 ± 2 KHz) (Instrumentation India, Kolkata, India) at 60 °C for 1 h. After sonication, the preparations were kept at room temperature for 1 h for vesicle formation and thereafter they were kept at 4 °C in an inert atmosphere for 24 h. The preparations were thereafter centrifuged at 5000 rpm at 4 °C for 5 min. The liposomes formed were separated from the PBS by filtration and each batch was lyophilized with lyophilizer (Instrumentation India ltd, Kolkata, India) after primary drying conducted at – 40 °C for 8 h under vacuum.

Table 1: Batch formulation of Fulvestrant liposome

Code	QTY of Fulvestrant (mg)	QTY of Lecithin (mg)	QTY of Cholesterol (mg)	Ratio
A	1	1	1	1:1
B	1	2	4	1:2
C	1	2.5	1.25	2:1

Morphology

Specimens for cryo-Transmission electron microscope (cryo-TEM) were prepared using carbon coated copper grids (400 mesh, Agar Grids). The grids were hydrophilized through treatment by glow-discharge. The blotting procedure and the quenching of specimens were performed using an improved version of the controlled environment vitrification system (Bellare *et al.,* 2000).The liposomal dispersions were vitrified on different carbon grids; these were subsequently transferred to the cryo-TEM for investigation. The vitrification was accomplished by blotting a very thin film on a carbon-coated grid. The grid was then plunged into liquid nitrogen very rapidly. This prevented the solution from crystallizing and, amorphous ice, which is a prerequisite for this technique, is formed. This vitrification procedure assures that the individual particles are visualized as similar as possible to their natural appearance in the liposomal suspension.

pH measurement

The pH values of the reconstituted lyophilized liposomes were measured using a pH meter (Sigma, Toledo, USA) at 25 ± 0.5 °C.

Size distribution and zeta potential

Liposomes size distribution and zeta potential measurements were performed with the help of Zetasizer nano ZS with DTS software (Malvern Instruments Ltd., UK). NIBS® (non-invasive backscatter optics) technology was employed in all determinations. Briefly the lyophilized fulvestrant formulations were reconstituted in microcentrifuge tubes with aliquot quantities of phosphate buffer solution, pH 7.4, the microcentrifuge tubes were thereafter introduced into the instrument to read the results for each formulation, (Mukherjee *et al.,* 2008).

Drug loading study

A weighed amount (5 mg) of liposome was lysed with methanol, centrifuged and the absorbance of supernatant was measured at 520 nm using an ultraviolet-visible (UV/VIS) spectrometer (Beckman Instruments, Fullerton, CA, USA). The same procedure was used for the batch without the drug. The absorbance due to drug was the difference between the readings obtained from the preparation with drug and without drug (blank) to avoid any minor error due to the excipients. The percentage drug loaded, was calculated using equation (1)

$$\% \text{ drug Loaded} = \frac{\text{amount of Fulvestrant in liposome}}{\text{amount of liposome}} \times 100$$

***In-vitro* drug release study**

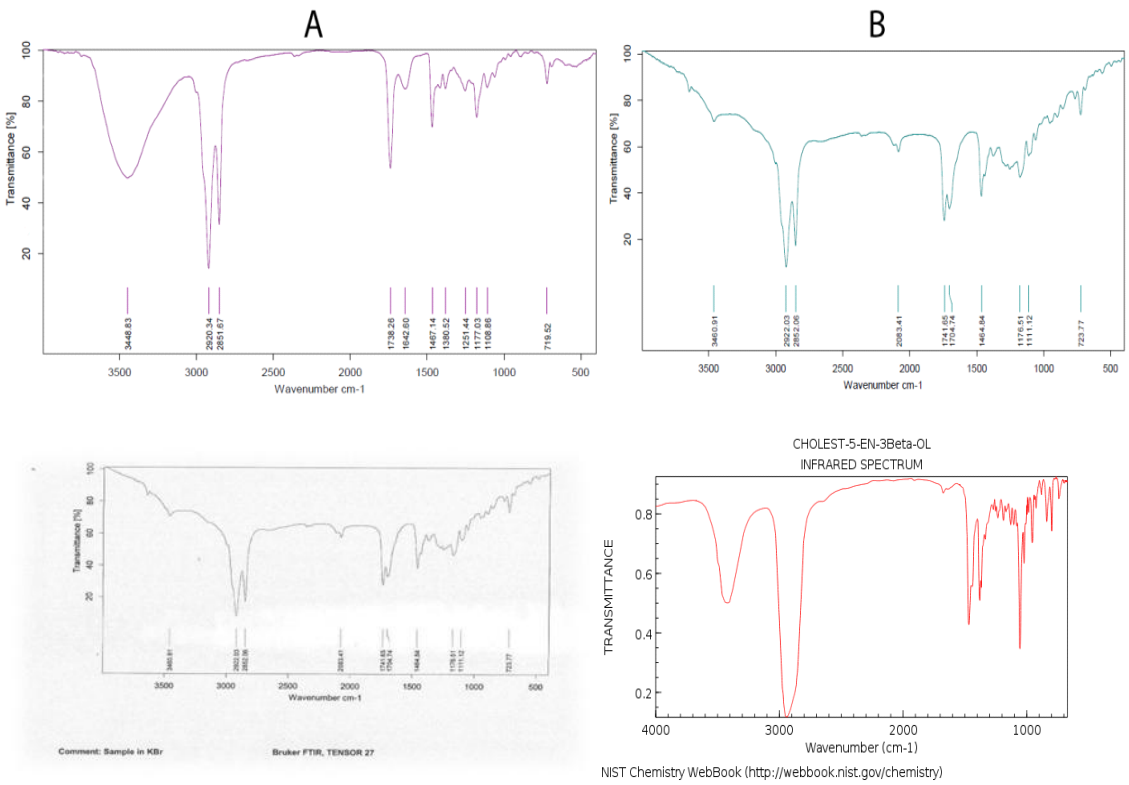
In a 250 mL conical flask, 50 mL of phosphate-buffered saline (PBS) was measured. A weighed amount of lyophilized liposomes (5 mg) was reconstituted in 1 mL PBS and was taken into a dialysis bag (Himedia dialysis membrane-60, Mumbai, India). The two ends of the dialysis sac were tightly bound with cotton thread. The bag was hung inside the conical flask with the help of a glass rod so that the portion of the dialysis bag containing the formulation could dip into the buffer solution. The flask was kept on a magnetic stirrer. Stirring was maintained at 300 rpm with the help of a magnetic bead at room temperature. Sampling was done by withdrawing 1 mL from the medium and 1 mL blank was added. The samples were analyzed spectrophotometrically at 520 nm. The concentration of the released drug from the liposomes was calculated using the standard curve obtained using the Beer-Lamber relationship.

Cellular uptake study

Florescence microscopy was used to visualize the uptake of the liposomes within the cancer cells. For fluorescence imaging of cellular uptake, MCF-7 cells (at 104 cells/mL) were cultivated for 24 hours on cover slips in six well culture plates (3 mL/well). Fulvestrant liposome suspension, 50 µl/mL, was then added to the cell culture medium. The cells were washed three times after incubation for 0, 12, 24 hours and then fixed using 4% paraformaldehyde aqueous solution. After fixing for 15 minutes, they were rinsed with phosphate buffered saline (pH 7.4) solution. After that the cover slips were taken out carefully and placed on the slide and air-dried. Finally, they were observed using a florescence microscopy, (ABV-202i Kyowa Japan.).

Results and Discussion

The hydrophobic nature of Fulvestrant allows it to become incorporated into the bilayer region of the vesicles. However, this rather limits the amount of drug that can be delivered, so we have aimed to formulate it as liposomes. Evaluation of drug, excipients interaction is an important study, which gives insight to the stability of the formulation, and drug release from the formulation (Mukherjee *et al.*, 2005a; Lia *et al.*,2008). FTIR is among the various methods used in studying possible, drug-excipient interactions. It provides a distinct idea regarding actual interaction between various functional groups present in drug and excipients, (Cunha-Filho *et al.*, 2007).

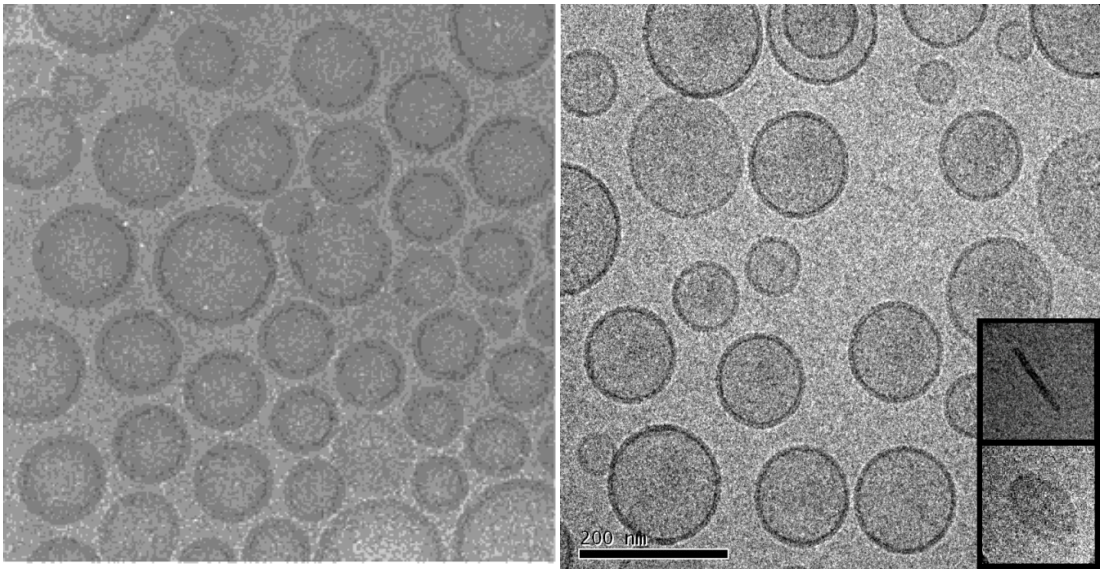


(C) (D)

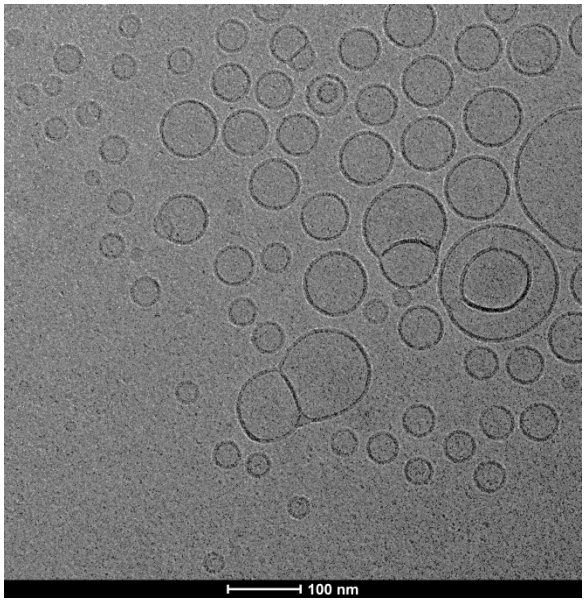
Figure I: FTIR of Fulvestrant (A); mixture of drug + excipients (B); Lecithin(C) and Cholestrol (D)

In the present study, the FTIR spectrum of pure drug (Fulvestrant), pure Cholestrol, pure lecithin and mixture of the pure drug plus the excipients were as shown in Figures 1 (A to D). There were no significant physicochemical interactions as there were no major shifting nor loss of the peaks in the mixture of the drug and the excipients, since all the major peaks of the pure drug were still present in the FTIR of the physical mixture of the drug and the excipients. However, the minor shifting observed may be due to minor physical interactions such as formation of weak hydrogen bonds due to Van der Waal's forces of attraction or due to dipole- dipole interactions between the - OH groups of cholesterol and S=O group of the drug wave number range (3300 The morphology of the preformed Fulvestrant liposome are presented in Figure 2

to 3450 cm^{-1}). Also there were similar interactions or weak hydrogen bonding between the $–NH_2$ of the soya lecithin and –OH groups of the drug which may account for the formation of a spherical structure of the nanoparticles. There were also some interactions between 400 to 1000 cm^{-1} wave length which is the stretching region for alkyl of soya lecithin and aromatic rings of the pure drug, (Gustafsson *et al.,* 1999; Williams *et al.,* 2002). These various interactions were reported to be necessary and vital for the production of stable spherical liposomes. The shape, size and drug release from the liposome all depend on these interactions (Gokhale *et al.,* 1996; Cabens *et al.,* 1998).



(a) (b)



(c)

Figure 2: Cryogenic transmission electron microscopy (Cryo-TEM) Fulvestrant liposomes (batch A, B and C)

Cryogenic transmission electron microscopy (Cryo-TEM) is one of the most advance techniques that is used to characterize the nanovesicles most especially liposome and other temperature sensitive lipid nanoparticles. Cryo-TEM has the unsurpassed ability to visualize individual lipid based nanoparticles and is thus used to assess morphology, size and size distribution of these particles. The distinct bilayer structure of prepared liposome was well defined and it's shown in figure 2 (a-c). All the three batches of

the prepared liposome were smooth, spherical shapes of various sizes, all within nanoscale ranges. Also there was a clear distribution of the Fulvestrant liposome vehicles. This was further confirmed by particle size analysis, which showed that size distribution varied from 59.59 nm to 760.7 nm range for formulation A and C, while Formulation B showed a denser particle distribution with 91.8 %. The size distribution analysis from DLS and cryo-TEM are reported in table 2. Furthermore the particles were found to be in submicron size with a relatively narrow range of distribution as supported by the PDI values.

Table 2: Some formulation parameters for three batches of Fulvestrant liposomes

Code	Z-Ave (d.nm)	PDI	Zeta Potential (mV)	Conductivity (mS/cm)	Loading Capacity (%) ± SD (n = 3)
A	191	0.438	-14.8	2.49	28.17 ±0.014
B	760.7	0.75	-3.40	0.771	30.19 ±0.083
C	205.5	0.533	-14.9	2.50	28.19 ±0.008

Both DLS and cryo-TEM are methods to analyze liposome size and structure, but differences in the analysis with DLS typically measuring mean sizes were larger than those obtained through cryo-TEM. This is a consequence of the enhanced scattering from the minimal number of aggregated liposomes that are present even at the high dilutions used in DLS experiments, (Holzer, *et al.,* 2009; Crawford, *et al.,* 2011). The drug loading capacities vary with varying phospholipids concentrations. Increase in the ratio of cholesterol significantly improves the loading capacity of the prepared liposomes by 2.02 %, while decrease in the ratio of cholesterol shows no significant changes in the loading capacity of the prepared liposome, ($p \leq 0.005$), (Kousik, *et al.,* 2010). This increase may be due to the ability of the cholesterol matrix to accommodate a large amount of the drug molecules in the polymeric network until it reached its saturation point. However, the saturation point (that is the maximum amount of drug molecules which can be accommodated in the polymer matrix of a definite quantity) has not been determined in the present study. The zeta potential of the different batches varied from -14.9 mV to -3.4 mV. The zeta potential values can be used to quantify the nature of the electrostatic potential at the surface of a liposomes. An absolute zeta potential value of between -30 mV and +30 mV are generally acceptable for stable colloidal system and it suggests that the liposomes will remain in a suspended state for a longer period and are not susceptible to quick agglomeration in the liquid state. The three batches of prepared liposome will be relatively stable in liquid form since their zeta potential values falls within the acceptable range, therefore the prepared nanoparticles could be stored in both liquid form or lyophilized state which will be reconstituted before administration (Meiber, *et al.,* 2009; Basu, *et al.,* 2012). Fulvestrant released from the liposome was in a sustained manner over a period of 50 days from all the three experimental formulations. It was observed that formulation B with the highest values of Z-Ave, PDI, zeta potential and drug loading capacity shows slowest release of the drug from the liposome core. This may be due to longer diffusion pathway the drug needs to travel to get released from the liposome polymers (Zhang *et al.,* 2004). This ultimately leads to a sustained diffusion of the drug from the liposome. This may be very useful in the formulation of a sustained release liposomal Fulvestrant.

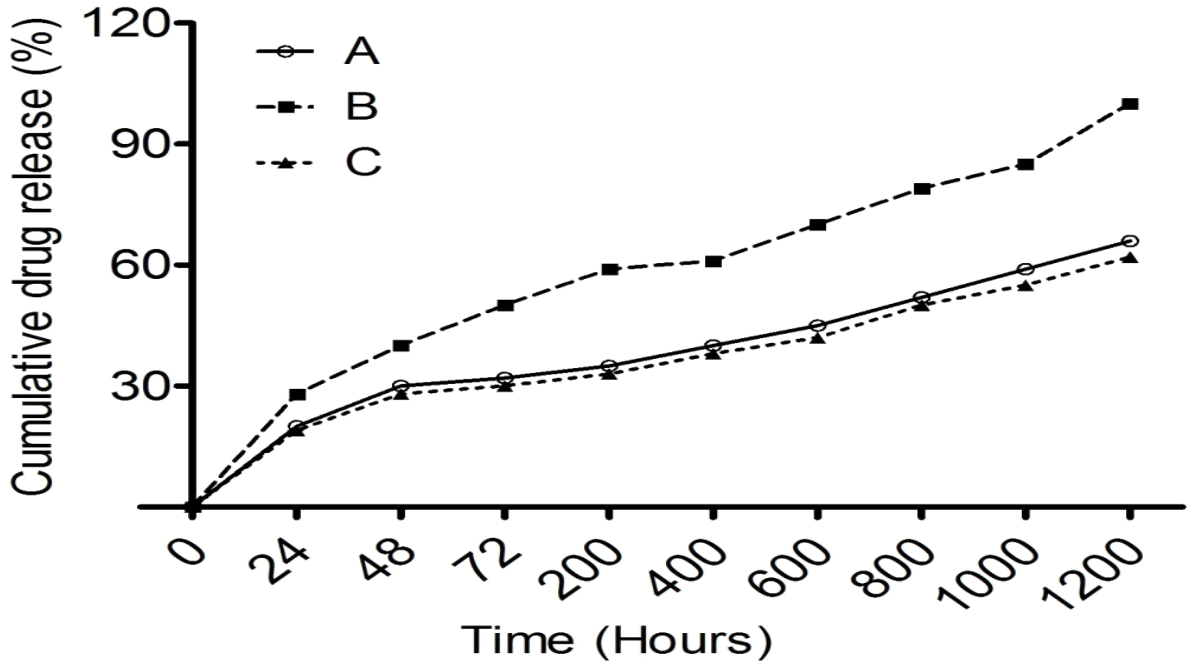


Figure 3: Percentage cumulative drug released.

Fulvestrant released from the liposome was in a sustained manner which was over a period of 50 days from all the experimental formulations. While formulation B achieved 100 ± 0.001 % within 50 days, the maximum drug released for formulation A and C are 66 ± 0.003 % and 62 ± 0.007 % . Presence of rigid cholesterol nucleus along with the acyl chain of phospholipids is known to reduce the free movement of drug which ultimately causes the membrane of the liposome to condense and decrease its fluidity, thereby acting as barrier to the entrapped drug and finally might retard the drug released for such a prolonged period, (Damel, *et al.,* 1976). Also incorporation of extra amount of hydrophobic polymer, cholesterol, as in the case of formulation B might have caused more tortuous polymeric networks to deliver the drug for prolonged period of time maximally, among the experimental formulations. The method provided a narrow-ranged densely-dispersed nanoparticles having capability of release of drug more than 50 days. Thus, by regulating different process parameters this will ensure consistent production of liposomes with a low polydispersity index and improved loading and prolong released of the drug.

Table 3: Correlation co-efficiencies for the three batches of Fulvestrant Liposomes (n= 6)

Code	Zero Order	First order	Higuchi	Korsmeyer
A	R^2 = 0.7984	R^2 = 0.945	R^2 = 0.9398	R^2 = 0.9846
B	R^2 = 0.7940	R^2 = 0.9310	R^2 = 0.940	R^2 = 0.9880
C	R^2 = 0.8013	R^2 = 0.9442	R^2 = 0.945	R^2 = 0.9845

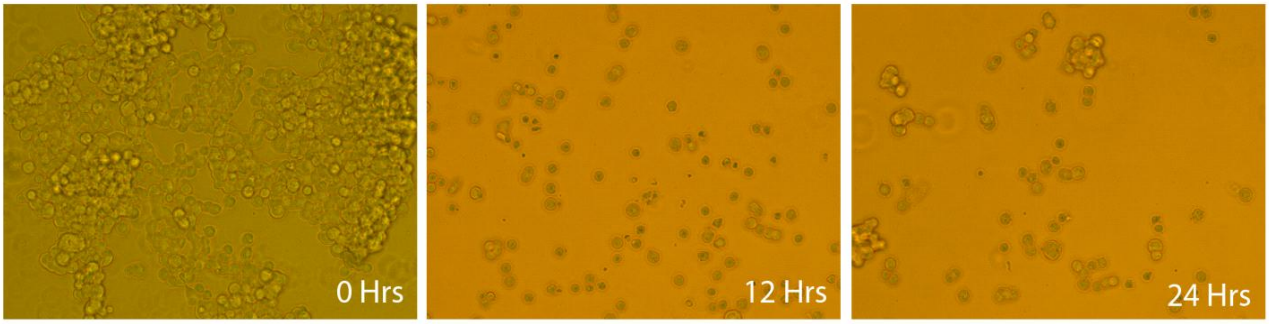


Figure 4; Florescence microscopy of MCF-7 Vs Time (A= 0 h), (B= 12 h) and (C= 24 h)

To evaluate the drug release kinetics patterns for the three batches of the produced liposomes, the drug release data were assessed using zero order, first order, Higuchi, and Korsmeyer equations. The calculated Co-efficient of determination R^2 values was as presented in table 3. The co-efficient of determination, R^2, is useful because it gives the proportion of the variance (fluctuation) of one variable that is predictable from the other variable, (Li *et al.*, 2011). It is a measurement that allows us to determine how certain one can be in making predictions from a certain model/graph. The drug release is said to follow the release models or equation with the highest R^2 values, (Korsmeyer *et al.*, 1983; Hixson and Crowell 1931). In this study all liposome formulations evaluated shows that the drug released from liposome matrix follows Korsmeyer kinetics. More linearity (as assessed by R^2 values) also implies anomalous diffusion controlled drug released by more than one process, (Pattnaik, *et al.*, 2012).

To investigate the cellular uptake of Fulvestrant liposome by MCF-7 cells, a short-term in vitro particle endocytosis test was carried out using (FITC dyed), Fulvestrant liposome. Figure 4 shows that liposomes penetrated the cell membrane and were well internalized. The cytotoxic effect of Fulvestrant markedly decreased the cell number and viability Figure 4 (a-c). The effective kill of the MCF-7 cells may include the enhanced intracellular drug accumulation by liposomal uptake, (Li, *et al.*, 2008; Zhang, *et al.*, 2007)

Conclusion

The outcome of the present investigation proposes a novel formulation of Fulvestrant liposomes prepared by lipid film hydration technique. The liposomal particles in a nano-size range with a desired drug polymer ratio were produced. The size, drug loading and the drug release kinetics can be optimally controlled. Further, Fulvestrant liposomes were absorbed well in breast cancer cells in vitro, suggesting their suitability in breast cancer treatment. Preferential uptake of Fulvestrant nanoparticles by MCF-7 cells is preferable as adverse drug reactions due to non specific distribution of the convention drug will be reduced or totally eliminated, thereby improve patient tolerability and quality of life.

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