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Preparation, Characterization and *In Vitro* Release Study of Liposomes Loaded with Artemether

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ABSTRACT

The purpose of the study was to prepare and evaluate liposomes containing Artemether, a lipophilic drug having short half life of 2-3hrs after oral administration. Thin film hydration method was used for the preparation of artemether-encapsulated conventional and PEGylated liposomal suspensions using various drug: lipid ratio and their characteristics, such as particle size, zeta potential, encapsulation efficiency, and *in vitro* drug release were investigated. The drug encapsulation efficiency of PEGylated liposomes was high when compared to conventional liposomes. The average particle size of both conventional and PEGylated liposomes was obtained in nanometers with PDI ranging from 0-0.356. Zeta potential of conventional liposomes was found to be more negative when compared to PEGylated liposomes. *In-vitro* drug diffusion studies was carried for period of 16 hrs where PEGylated liposomal formulation showed more sustained release compared to conventional liposomes. The conventional and PEGylated liposomal formulations followed zero order and Higuchi kinetics respectively. The artemether containing liposomes were successfully formulated and evaluated.

Keywords: Artemether, thin film hydration, liposomes, conventional, PEGylated.

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INTRODUCTION

Liposomes are microscopic spheres with an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayer configuration. A liposome can be used as a carrier for both hydrophobic and hydrophilic drug by dissolving or encapsulating in a lipid bilayer respectively. The incorporation of drugs into liposomes have several advantages as they protect their contents from interaction with plasma components, while favorably altering the pharmacokinetics and bio-distribution of free compound since liposomes do not readily penetrate biological membrane[1]. The lipophilic drug is usually bound to the lipid bi-layer or 'dissolved' in the lipid phase. A lipophilic drug is more likely to remain encapsulated during storage due to its partition coefficient. Since the lipophilic drug is associated with the lipid bi-layers it will not leach out as readily to the 'external' water phase. Generally the encapsulation efficiency is higher for lipophilic drugs than hydrophilic drugs[2]. Moreover, Polyethylene glycol (PEG) modification on the liposomal surface is known to be effective in preventing their uptake by the reticuloendothelial system (RES)[3]. Parasitic diseases are of immense global significance as around 30% of world's population experiences parasitic infections. Amongst various parasitic infections, malaria is the most life threatening disease and accounts for 1 million to 2 million deaths round the globe every year. In humans, malaria is caused by four distinct blood–borne Apicomplexan parasite species: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Amongst these, the most severe malaria is caused by *P. falciparum* which is responsible for almost all malaria related deaths[4]. Artemether, a derivative of artemisinin has been widely used drug for severe malaria. Artemether is also a rapidly acting antimarial agent which is enlisted in WHO List of essential medicines for the treatment of severe multi-resistant malaria. It is active against *P.vivax* as well as chloroquin sensitive and chloroquin resistant strains of *P.falciparum*[5]. Artemether being very effective drug, is not administered alone. Always it is being included in the combination therapy i.e ACT (Eg. artemether + lumifantrin). The rationale behind this idea is Artemether provides rapid symptomatic relief by reducing the number of parasites present before it, but has very short half life of 2-3 hrs and the combined drug has prolonged half life (4.5 days) which can eliminate the residual parasites[6]. If artemether is administered alone it causes recrudescence. Moreover, Artemether as monotherapy is available only in the form of IM injection whose half life is extended to 6-8 hrs but it is very painful due to oily nature of formulation and erratic absorption on IM administration. Therfore encapsulation of artemether in liposome may increase the half –life providing prolonged drug delivery. The objective of the present work is to

formulate effective and sustained release conventional and PEGylated artemether liposomal formulation and study the *in vitro* parameters and stability studies of prepared liposomes.

MATERIALS AND METHOD

Artemether was obtained as gift sample from Sequent Research Limited, Mangalore, India. 18:0 MPEG 2000-DSPE and DSPC was obtained as gift samples from Lipoid, Frigenstrasse, Ludwigshafen. Cholesterol and chloroform were purchased from Loba Chemie Pvt. Ltd, Mumbai. Sodium dihydrogen phosphate, Sodium hydroxide pellets and methanol were purchased from Hi Media Laboratory Pvt. Ltd., Mumbai, India.

Preparation of Artemether liposomes using thin film hydration method as follows

The molar ratios of lipids as shown in table 1 were accurately weighed and taken in RBF and dissolved in a mixture of chloroform: methanol (2:1) by rotating in rotary flash evaporator. The drug was dissolved in the above mixture and was again rotated in rotary flash evaporator for 20 min. Round bottom flask was then attached to a rotary evaporator by means of a clip ,and vaccum was applied through a vacuum pump and rotated at 80 rpm with the round bottom flask being immersed in a water bath with a thermostat set at a temperature above the phase transition temperature of the phospholipid to obtain a thin dry lipid film. Hydration of the dry lipid film was accomplished by adding 10ml of PBS pH 7.4 and the temperature of the hydrating medium was maintained above the gel liquid crystal transition temperature (Tm) of the phospholipid , before adding to the dry lipid . Hydration was carried out for period of 3 hrs at 80 rpm to get homogenous milky white suspension of MLVs. Sonication- Suspension was subjected to ultra-probe sonication for 10 minutes at an amplitude of 60% with a pulse of 5 cycles. The same procedure was used to prepare the liposomes using mixture of phospholipids (F5 as shown in table 1) but the transition temperature of the phospholipid with a higher phase transition temperature was selected as the main Tm, obtained from literature[7,8,9].

Table 1: Drug: lipid ratio taken in the formulation

Formulation code	Lipid ratio used		
	Cholesterol	DSPC	MPEG-2000 DSPE
F1	1	2	-
F2	1	3	-
F3	1	4	-
F4	1	3	0.2
F5	1	4	0.2

***In Vitro* Characterization of Liposomes**

Optical photomicroscopy[10]

MLVs suspension (100 µl) was placed on a clean glass slide, a cover slip was placed on it by taking care that air bubbles do not form. Focused under 40X magnification of MOTIC digital photographic microscope to view the MLVs. The optical photomicrographs were taken.

Particle size and size distribution[11]

Average particle size (in nanometers) and size distribution (polydispersibility index) was measured using a Malvern nano zeta sizer instrument.

Zeta potential[8,12]

Measurement of zeta potential of the liposomal formulation (SUVs) was done by using a Malvern nano zeta sizer instrument.

Entrapment efficiency (EE)[12]

Entrapment efficiency of liposomes was determined by centrifugation method. 1 ml of liposomal suspension was subjected to centrifugation on a laboratory centrifuge (Remi) at 4000 rpm for a period of 10 min. The clear supernatant and pellet was obtained. Supernatant were removed carefully to separate non-entrapped artemether and to the pellet in the centrifugation tube 5ml of methanol was added and sonicated for 10 minutes to break the vesicles and diluted suitably which was then analyzed for drug concentration.

***In vitro* drug release study**[2,11]

After separation of non-entrapped artemether, liposome dispersion was placed on one side of the cellophane membrane in a vertical Franz diffusion cell. Other side of the membrane was in contact with the dissolution medium. Entire dissolution assembly was placed on a magnetic stirrer at temperature of 37 °C. Dissolution medium was 100 ml of PBS pH 7.4 containing 0.1 % SLS. Aliquots (5 ml) of dissolution medium was withdrawn at different time intervals- 15 min, 30 min, 45 min, 60 min, 2 h, 4 h, 8 h, 10 h and 12 h. Whenever sample was withdrawn equal volume of fresh dissolution medium was added to the cell to maintain a constant volume. Drug concentrations in the dissolution medium were determined by UV spectrophotometric method.

Kinetic Studies[13,14]

To analyze the *in vitro* release data various kinetic models were used to describe the release kinetics. Zero order kinetics: Drug dissolution from pharmaceutical dosage forms that release the drug slowly, assuming that area does not change and no equilibrium conditions and are represented by the equation:

$Qt = Q0 + K0t$

Qt is the amount of drug dissolved in time t,

Q0 is the initial amount of drug in the solution,

K is the zero order release constant.

First order kinetics: The application of this model to drug dissolution studies used to describe absorption and/ or elimination of drugs. To study the first order release rate kinetics the release rate data were fitted to the following equation:

$Log\ Qt = log\ Q0 + K1t/2.303$

Qt is the amount of drug released in time t,

Q0 is the initial amount of drug in the solution,

K1 is the first order release constant.

Higuchi model

Higuchi developed several theoretical models to study the release of water soluble and low soluble drugs incorporated in semi-solid and/ or solid matrixes. Mathematical expressions were obtained for drug particles dispersed in a uniform matrix behaving as the diffusion media, the equation is:

$Qt = KH.\ t1/2$

Qt is the amount of drug released in time t,

KH is higuchi dissolution constant. Higuchi describes drug release as a diffusion process based in the Fick's law,

square root time dependent.

Korsmeyer and Peppas model:

This model is generally used to analyse the release of pharmaceutical polymeric dosage forms, when the release mechanism is not well known or when more than one type of release phenomena could be involved.

$Mt/M = K.\ tn$

Mt / M is the fraction of drug release

K is the release constant

t is the release time

n is the diffusion exponent for the drug release that is

dependent on the shape of the matrix dosage form.

Stability studies

Due to insufficient time, stability study was carried out for the sonicated liposomal suspension of UVs at two different conditions i.e. refrigeration temperature (5° ± 3°C) and room temperature (25±2˚C/40% RH ± 5% RH) for 4 weeks. Drug entrapment efficiency and drug release studies were carried out after 4 weeks. Sampling was done, suitable dilutions were made with PBS pH 7.4

and UV absorbance was determined. The entrapment efficiency was calculated from the regression equation[15].

RESULTS AND DISCUSSION

Total five liposomal formulations loaded with artemether were prepared by thin film hydration technique with varying lipid and cholesterol ratio (table 1) as per the procedure given in the methodology. On physical evaluation, all the five formulation appeared as milky white suspension. The prepared liposomal formulations were characterized for various physiochemical parameters such as optical photomicroscopy, average particle size and size distribution, zeta –potential, % entrapment efficiency, *in vitro* drug release and kinetic study. The liposomal dispersion was suitably diluted on glass slide and viewed under microscope with magnification of 40x and photomicrographs were taken. F1 formulation did not show any vesicles with lipid bilayer and this may be because of the drug to lipid ratio chosen i.e DSPC: Cholesterol in the ratio 2:1, whereas the photomicrographs (Figure 1) of all the other formulations F2 to F5 gave clear picture of formation of spherical vesicle with lipid bilayer. Based on this result F2, F3, F4, F5 were selected for further study.

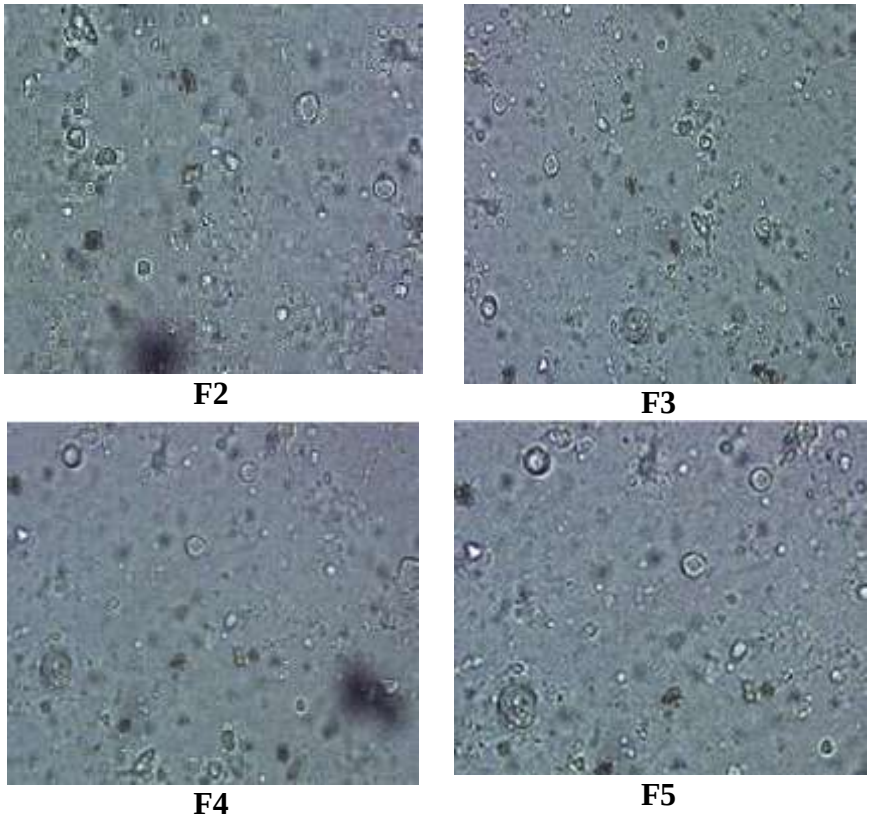
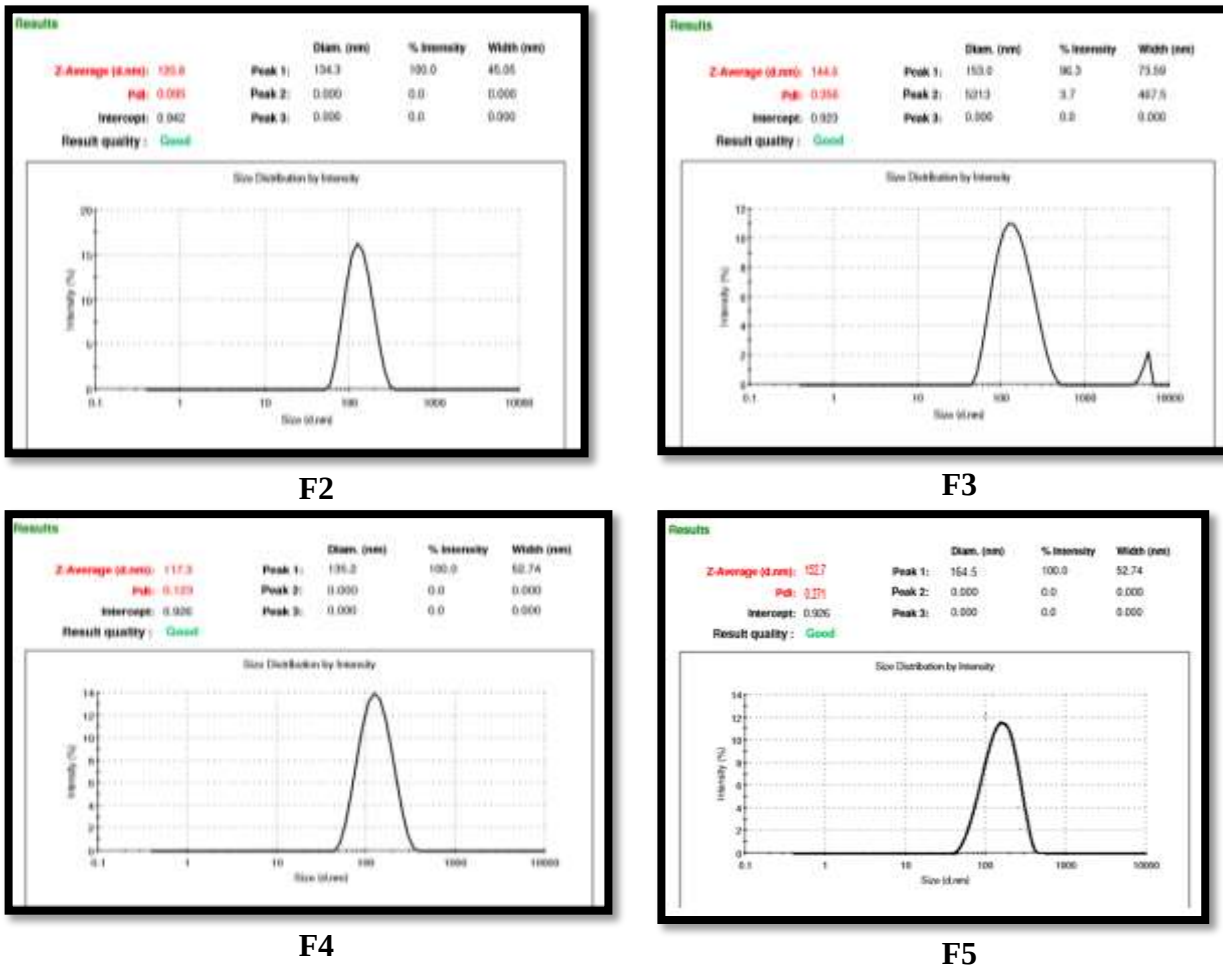


Figure 1: Optical photomicrographs of F2, F3, F4 and F5 formulation respectively under 40 x magnification

The average vesicle size and size distribution are important parameters because they influence the physicochemical properties and biological fate of the liposomes after administration. The size distribution of artemether liposomes was measured by Dynamic Light Scattering phenomenon using Nano Zeta Sizer. The particle size of F2, F3, F4,F5 is shown in Figure 2. In conventional liposomes (F2 and F3) as the ratio between lipids increased size also increased and same was in case of PEGylated liposomes (F4 and F5) as shown in table 2.



F2 **F3**

F4 **F5**

Figure 2: Average particle size of F2, F3, F4, F5

Table 2: Physical characteristics of prepared liposomes

Formulation code	Physical characteristics				
	%E.E	Practical size(nm)	Zeta potential(mV)	PDI	% release
F2	47.05	120.8	-27.3	0.095	90.18
F3	53.69	144.6	-25.4	0.356	93.18
F4	62.2	117.3	-18.4	0.123	84.24
F5	68.92	152.7	-20.5	0.271	80.99

Zeta potential of conventional liposomes F2 and F3 was negative (table 2) due to the presence of terminal carboxylic groups in the lipids. The value of the zeta potential for PEGylated liposomes (stealth) F4 and F5 (table.3) was less negative, due to the presence of the PEG chains on the

liposome surface which reduces the mobility of the liposomes (and hence the zeta potential). Liposomes prepared using DSPC: MPEG - 2000 DSPE:CH (4:0.2:1) i.e F5, gave the best entrapment efficiency. Increase in size of the liposomes also increased the entrapment efficiency. PEGylated liposomes (F4 and F5) showed higher entrapment efficiency compared to that of the conventional liposomes (F2 and F3) as shown in table 2. The *in vitro* release profile of liposomes is shown in Figure 3. Initial burst release was observed for all the formulations. PEGylated formulations showed a higher burst release compared to the conventional formulations. This burst release was observed due to the presence of drug on the surface in the adsorbed form and the prolonged release in the latter stage can be attributed to the slow diffusion of the drug through lipid bilayer. In vitro study was carried out for a period of 16 hrs. Comparing the results (table 3) of both conventional and PEGylated liposomal formulations, it was found that PEGylated liposomes showed a more sustained action owing to the presence of PEG coating on the surface, which release the drug slowly over a prolonged period of time. F5 formulation showed more prolonged release and in 16 hrs it gave 80.99% release of drug.

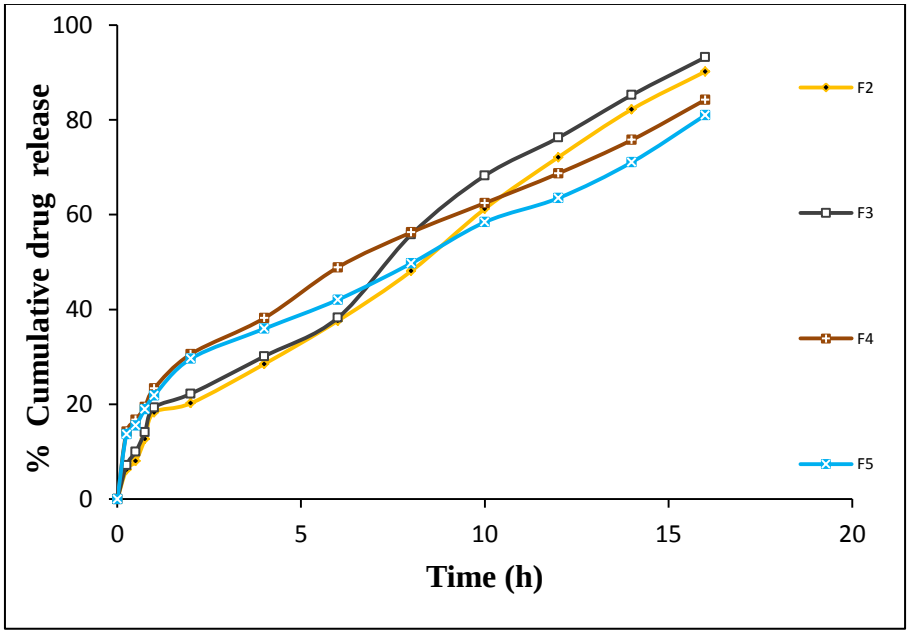


Figure 3: Comparison of release profile of various formulations

Table 3: *In vitro* drug release profile of different liposomal formulation

Time (Hr)	%CDR*			
	F2	F3	F4	F5
0	0	0	0	0
0.25	6.23 ± 0.23	7.13 ± 0.11	14.23 ± 0.27	13.71 ± 0.09
0.5	8.02 ± 0.34	9.98 ± 0.12	16.71 ± 0.26	15.55± 10.42
0.75	12.7 ± 0.23	14.13 ± 0.04	19.42 ± 0.16	18.97 ± 0.85
1	18.29 ±0.11	19.29 ± 0.07	23.35 ± 0.33	21.85 ± 0.49

2	20.23 ±0.45	22.23 ± 0.35	30.6 ± 0.18	29.63 ± 0.81
4	28.51 ± 0.67	30.13 ± 0.27	38.19 ± 0.73	35.95 ± 0.82
6	37.61 ± 0.83	38.24 ± 0.18	48.89 ± 0.18	42.06 ± 0.49
8	48.12 ±0.24	55.82 ± 0.37	56.27 ± 0.18	49.75 ± 0.16
10	61.23 ± 0.37	68.23 ± 0.18	62.45 ± 0.13	58.42 ± 0.37
12	72.11 ±0.76	76.23 ± 0.35	68.7 ± 0.24	63.49 ± 0.61
14	82.22 ± 0.22	85.22 ± 0.76	75.76 ± 0.02	71.07 ± 0.70
16	90.18 ±0.67	93.18 ± 0.19	84.24 ± 0.13	80.99 ± 0.15

* Average of three trials (n=3)

In order to study the exact mechanism of drug release from liposomes loaded with artemether, drug release data were fit into various mathematical models, zero order, first order, higuchi matrix and peppas and regression co-efficient were depicted in table 4. These values were compared with each other for model fitting equation. Based on the highest regression values (r), the best fit model for F2 and F3 was zero order and for F4 and F5 it was higuchi matrix. All the formulations were then fitted into korsmeyer-peppas model and n values are reported in table 5. For all the formulations, the 'n' value was in the range of 0.45-0.89 indicating non fickian diffusion.

Table 4: Kinetics release study of various liposomal formulations

Formulation code	Zero Order	First Order	Higuchi Matrix	Peppas Plot	
				R^2 values	n values
F2	0.9901	0.9337	0.9618	0.9810	0.6274
F3	0.9858	0.9339	0.9675	0.9815	0.6039
F4	0.9525	0.9774	0.9947	0.9912	0.589
F5	0.9579	0.9666	0.9862	0.9850	0.516

Table 5: Stability data of various liposomal formulations

Formulation code	% Drug Entrapment					
	Initial		After 2 week		After 4 week	
	5±3˚C	25±2˚C/40% RH ± 5%RH	5±3˚C	25±2˚C/40% RH ± 5% RH	5±3˚C	25±2˚C/40% RH ± 5% RH
F2	47.05	47.05	46.89	45.27	45.26	44.27
F3	53.69	53.69	53.18	52.66	52.35	51.26
F4	62.2	62.2	61.79	60.15	60.51	59.27
F5	68.92	68.92	68.05	67.72	67.65	66.39

Stability study of the vesicles is the major determinant for the stability of the formulations. Due to insufficient time, study was carried to evaluate drug entrapment at accelerated condition (25 ± 2 ˚C/40% RH ± 5% RH) and refrigeration temperature (5 ± 3˚C) for a period of 1 month as per ICH QA1R2 guidelines. Stability studies was not be carried out at higher temperature (>room temperature) because phospholipid was used as the component for liposomes and get deteriorated at higher temperature. The stability data of liposomes at 5±3 °C and 25±2°C/40%RH ± 5% RH is given in Table 5. According to the data obtained, formulations stored at refrigeration temperature

showed higher drug entrapment when compared to the formulations stored at room temperature. PEGylated liposomes (F4 and F5) were found to be more stable than the conventional liposomes (F2 and F3) and showed much lesser extent of drug leakage compared to the conventional liposomes.

CONCLUSION

Artemether being effective drug in the treatment of malaria, due to its short half life it is not administered alone. To prolong the half life and to be used alone as monotherapy, Artemether loaded conventional and PEGylated liposomes were successfully prepared and evaluated. The drug: lipid ratios selected plays important role in formation of vesicles. Comparing the *in vitro* studies it can be seen that PEGylated formulations gave more prolonged release when compared to conventional formulations in 16 hrs. Thus we conclude that the administration of Artemether in form liposomal drug delivery will be the better therapy in treatment of malaria.

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