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Investigation of Nano Lipid Vesicles of Lornoxicam for Targeted Drug Delivery

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Authors' contributions

This work was carried out in collaboration among all authors. Author PP designed the study. Author AD wrote the protocol and the first draft of the manuscript. Author JKP managed the literature searches and performed the formulation and evaluation. Authors PP and AD corrected the manuscript, provided critical review and editing of the final document. Author SH plotted graph and tables. Authors CSS and RNC checked the manuscript for perfection. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the present study was to formulate nano lipid vesicles of lornoxicam targeting to the specific site (inflamed area), and investigating it's *in vivo* anti-inflammatory activity in animals (rats).
Methods: Liposomes of lornoxicam were prepared by thin film hydration method. Lornoxicam was loaded in stealth liposomes, conventional liposomes and coated conventional liposomes. Stealth liposomes were prepared by incorporating PEGylated lipids MPEGDSPE. Conventional liposomes

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were formulated using phospholipids Lipoid SPC-3 and cholesterol. Conventional liposomes were later coated with the hydrophilic biocompatible polymer chitosan which produced cationic liposomes. All the formulations were optimized to get the best entrapment efficiency.

Results: The average size of the unsonicated liposomes was found to be 844.4 nm, whereas the average particle size of sonicated liposomes was found to be 195.5 nm. Coating of lipid vesicles was confirmed by zeta potential values using a nano zeta sizer instrument which showed that the chitosan coated liposomes exhibited a positive zeta potential compared to the uncoated liposomes which had a negative zeta potential values. The PDI was found to be 0.4, indicated good dispersion of uniformly sized lipid vesicles. All coated conventional, uncoated conventional and PEGylated liposomal formulations followed Higuchi model drug release profile. Stability study showed higher drug content at refrigeration temperature when compared to the formulations stored at room temperature, after a period of 4 weeks. Chitosan coated liposomes were found to be more stable as the coating with chitosan prevents the oxidation of phospholipids. *In vivo* study was carried out in rats for their anti rheumatoid which showed that there was a significant reduction in edema volume in the rat group administered with the liposomal formulation.

Conclusion: PEGylated liposomes were found to be more effective and stable than the uncoated conventional liposomes.

Keywords: Lornoxicam; stealth liposome; conventional liposome; anti rheumatoid efficacy.

1. INTRODUCTION

Liposomes have been comprehensively used as carriers for many molecules in cosmetic and pharmaceutical industries. Many industries like food and farming industries have investigated the use of liposomes as delivery systems that can entrap unstable compounds like antimicrobials, antioxidants, flavors and bioactive elements as well as shield their functionality [1]. Liposomes have the ability to trap both hydrophobic and hydrophilic compounds which in turn can avoid the stability issues of the entrapped combinations, and release the entrapped drug [2]. They are biocompatible, biodegradable, low toxic, and have the aptitude to trap both hydrophilic and lipophilic drugs, can be used as site-specific drug delivery to tumor tissues. These exceptional qualities of liposomes have made interesting and commercially successful as drug delivery systems [3]. They have been investigated as carriers for many active agents such as antineoplastic, antimicrobial drugs, chelating agents, steroids, vaccines and genetic materials [4]. On the basis of the ability of liposomes to interact with cells and/or blood components, at least two types of liposomes currently can be designed like non-interactive sterically stabilized (long-circulating) liposomes (LCL) and highly interactive cationic liposomes (HCL) [5]. Liposomes cause aggregation in the blood by their mutual reaction (Vander Waals interaction or hydrophobic interaction) with various blood plasma (proteins and are captured by the reticuloendothelial system (RES). For example, kupfer cells in the liver or fixed macrophages in the spleen take up the liposomes before they can reach their intended target. Capture by the RES has rendered selected delivery of the liposomes to target tissues or cells very difficult. In addition to capture by the RES, the liposomes are subjected to electrostatic, hydrophobic and Vander Waals interactions with plasma proteins [6]. These interactions result in destabilization of the liposomes leading to rapid clearance of the vesicles from circulation, often before reaching their target. Several approaches taken in an effort to increase the circulation time of liposomes and thus ensure delivery of the liposome contents to the target tissue include the following masking the liposomes from the reticuloendothelial system recognition [7]. The main feature of long circulating liposomes is ability to extravasate at body sites where the permeability of the vascular wall is increased. Currently, best way to fabricate long-circulating liposomes is to attach hydrophilic polyethylene glycol (PEG) polymer covalently to the outer surface. Such PEG-coated liposomes are known as stealth or sterically stabilized liposomes, the first mentioned term referring to their mononuclear phagocytic system (MPS) escaping capability, and the second mentioned term refers to the steric stabilization mechanism held responsible for the induction of long circulation times. Magic gun approach-particulate drug carriers PEGylated liposomes avoid detection and shattering by phagocytes by virtue of their cloaks of hydrated PEG (polyethylene glycol) molecules. They increases the bioavailability of drugs or supplements by passing the digestive

tract and then to minimize any potential toxicity or side effects of these molecules by remaining in the circulation for a prolong time and releasing their content slowly [8].

Targeted deliveries are of two types: passive targeting, which is easy to accomplish but limited in scope, and active targeting, which is difficult to accomplish but filled with potential for many applications. Passive targeting generates accidentally from a physical property of certain tissues, and the active targeting depends on deliberate chemical modifications of the PEG molecules. The other main advantage of PEGylated liposomes is their ability to target the drugs or supplements to the tissues or organs specifically that need them most. This increases the delivery efficiency for the drugs, and also decreases the chances of toxicity to other organs. Passive targeting does not depend on any particular property of the liposome (whether PEGylated or not), but, it depends on a particular physical property of two kinds of tissues, like developing tumors and inflamed tissues. The smaller blood vessels (capillaries) that develops these tissues show increased permeability (leakiness) compared with the capillaries found in normal tissues. This phenomenon is called the enhanced permeation and retention (EPR) effect. EPR-effect is a phenomenon of enhanced extravasations of macromolecules (i.e., molecular weight higher than the renal excretion threshold) from tumor blood vessels and there by retention in tumor tissues, which is not observed in normal vasculature. [9] Mostly the effect of EPR can be observed in all human cancers with exception of hypo vascular tumors such as prostate cancer or pancreatic cancer. Cationic liposomes are structures that are made of positively charged lipids and are increasingly being researched for use in gene therapy due to their favorable interactions with negatively charged DNA and cell membranes. Cationic liposomes are also known as cationic lipoplexes. [10] Lornoxicam (chlortenoxicam), a non-steroidal anti-inflammatory drug (NSAID) of the oxicam class with analgesic, anti-inflammatory and antipyretic properties. The kidney is the second most frequent target of serious adverse effects of NSAIDs. The renal adverse effects of NSAIDs related to the inhibition of COX are reduction in renal blood flow (RBF) and glomerular filtration rate (GFR), sodium-water retention, and hyperkalemia. Lornoxicam is currently available as parenteral and oral formulations [11]. It is different from traditional oxicams by a relatively short elimination half life (3 to 5 h), which may be beneficial from a tolerability stand point. It has been reported that lornoxicam is as effective as the opioid analgesics morphine, pethidine (meperidine) and tramadol in relieving postoperative pain following gynaecological or orthopaedic surgery, and as effective as other NSAIDs after oral surgery. It was also reported that lornoxicam is as effective as other NSAIDs in relieving symptoms of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute sciatica and low back pain. In a chronic model of arthritis, lornoxicam, significantly reduced the PGE(2) level in paw exudate compared to other NSAIDs and did not change PGE(2) level in the brain hypothalamus, indicated that its mechanism of action by which it exhibits the analgesic or anti-inflammatory effect is mainly related to the local area rather than the central effect [11].

Therefore the current study was designed to formulate liposomes of lornoxicam to target specific site (inflamed area), and also investigating it's *in vivo* anti-inflammatory activity in animals (rats).

2. MATERIALS AND METHODS

2.1 Materials

Lornoxicam was provided by Micro labs., Bangalore, India. 18:0 MPEG 2000-DSPE and Soya lecithin (Lipoid S PC-3) were provided by Lipoid, GmBH, Frigenstrasse, Ludwigshafen. Cholesterol and Chitosan were provided by Loba Chemie Pvt. Ltd., Mumbai, India. Triton X-100 was provided by National chemicals Ltd, Vadodara, India. All other chemicals/reagents used were of analytical grade.

2.2 Methods

Liposomes were prepared by passive loading technique - thin film hydration method as per the method described by Bangham *et al*., 1965. [12] The molar ratios of lipids (phospholipids- MPEG DSPE-0.2 for stealth liposomes / Lipoid S PC-3- 10, 9, 8, 7 for conventional liposomes and cholesterol- 2, 3, 4, 5) were accurately weighed and dissolved in minimum quantity (about 2 ml) of a mixture of chloroform: methanol (2:1) (analytical grade), along with the required dose of lornoxicam (134.4735 µm) in a 250 ml round bottom flask having a ground glass neck to obtain a clear solution. Round bottom flask was then attached to a rotary evaporator by means of a elastic rubber band, evacuated with vacuum for

few minutes through a vacuum pump and rotated at 60 rpm with the round bottom flask being immersed in a water bath with a thermostat set at a temperature above the phase transition temperature (Tm) of the phospholipids to obtain a thin dry lipid film. When a mixture of 2 phospholipids was used, the transition temperature of the phospholipids with a higher phase transition temperature was selected as the main Tm.

Hydration of the dry lipid film was accomplished by adding PBS buffer pH 7.4 and the temperature of the hydrating medium was maintained above the gel liquid crystal transition temperature (Tm) of the phospholipids with the highest Tm, before adding to the dry lipid. After the addition of hydrating medium, the lipid suspension was maintained at a temperature above the Tm of the phospholipids used during the hydration period with the vacuum pump switched off. In the present work high transition lipids like MPEG-2000 DSPE and Lipoid S PC-3 were used, the lipid suspension was transferred to a 250 ml round bottom flask and placing the flask on a rotary evaporation system for a hydration period of one hour (hydration time) without vacuum, at a temperature higher than the Tm of the phospholipids used (which was maintained using thermostat water bath) which produced a homogenous milky yellowish white suspension of MLVs free of visible particles [13].

Hydration time was optimized such that there was enough swelling of the lipid film in order to obtain MLVs with more interlamellar distance. Once a stable MLV suspension was produced, it was subjected to ultra-probe sonication by transferring the colloidal suspension on to a glass vial. The probe tip of the ultra sonicator was just dipped into the suspension (care should be taken such that the probe tip does not touch the bottom of the glass vial during sonication). Sonication was done in 2 cycles, first the liposomal suspension was sonicated at 80% amplitude with a pulse of 0.5 cycles per second for a period for 3 min, followed by 3 min rest (excess heat may be generated during probe sonication, which may damage the lipids). After 3 min, second cycle was processed for 3 min at 80% amplitude with 0.5 s pulse for another 3 min [10]. Different molar ratios of lipids were used to formulate the liposomes (Tables 1 and 2). All molar ratios were optimized to get best entrapment efficiency (EE).

Drug to lipid ratio were experimented as (D / L) - 0.2, 0.25, 0.3, 0.35, 0.4, 0.5. Drug to lipid ratio was optimized as 0.2. D / L= 0.2 means 1 / L = 0.2 or 1 / 0.2 =L, Hence L= 5 i.e. Drug: lipid ratio is 1: 5. Hydration volume was experimented as: 2 ml, 2.5 ml, 3 ml, 3.5 ml, 4 ml, 5 ml, 6 ml, 8 ml and 10 ml. Hydration volume was optimized to 10 ml, based on the amount of phospholipid taken.

Table 1. Lipid ratio for conventional liposomes

Formulation code	Lipid ratio used	
	Lipiod S PC-3	Cholesterol
F1	10	2
F2	9	3
F3	8	4
F4	7	5

Table 2. Lipid ratio for stealth liposomes

Formulation code	Lipid ratio used		
	Lipiod S PC-3	MPEG-DSPE	Cholesterol
F5	10	0.2	2
F6	9	0.2	3

2.3 Optimized Ratios

2.3.1 Conventional liposomes

F1: Lipoid S PC-3: CH-10: 2, F2: Lipoid S PC-3: CH-9: 3, F3 – Lipoid S PC-3: CH – 8: 4, F4 – Lipoid S PC-3: CH – 7: 5

2.3.2 Stealth liposomes

F5: Lipoid S PC-3: MPEG-DSPE: CH-10: 0.2: 2, F6: Lipoid S PC-3: MPEG-DSPE: CH-9: 0.2: 2

2.3.3 Coating of the liposomes with cationic hydrophilic polymer chitosan

Coating of MLVs was done by mixing an aliquot of the liposomal suspension with the chitosan solution in 0.5% v/v of glacial acetic acid. Chitosan solution (containing 0.1% w/v, 0.3% w/v, 0.5% w/v and 0.7% w/v) was added drop wise into the respective liposome suspension placed on the magnetic stirrer under controlled stirring rate of 50 rpm at room temperature. After the coating of liposomes, incubation at 10ºC in the refrigerator for 1 h in a 50 ml beaker. This was sonicated at 80% amplitude, 0.5 s pulse for 3 min with a rest period of 3 min, followed by sonication for further 3 min [14].

2.3.4 Optimized ratios conventional liposomes (chitosan coated)

F 1- Lipoid S PC-3: CH-10: 2, F 2- Lipoid S PC-3: CH- 9: 3

2.4 Evaluation

2.4.1 Differential scanning calorimetry (DSC)

DSC of the phospholipid samples was performed in order to determine the exact transition temperature (Tm in °C) of the mixtures of phospholipids [15].

2.4.2 Optical photomicroscopy

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 45 X magnification of MOTIC digital photographic microscope to view the MLVs. The sizes (µ) of the MLVs were also measured using the microscopic scale [13].

2.4.3 Average particle size and size distribution

Average particle size (nm) and size distribution (as the polydispersibility index) of the liposomal suspension (SUVs) was measured using a Malvern nano zeta sizer instrument [15].

2.4.4 Zeta potential

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

2.4.5 Entrapment efficiency (EE)

After sonication, 1 ml of the vesicle suspension was taken in a 1 ml micro centrifuge tube, centrifuged at 80,000 rpm for 1 h at 4°C in a cold centrifuge to get a white pellet. The supernatant was separated to determine the unentrapped amount of drug. To the pellet, 500 µl of 0.1 N NaOH, was added and vortexed thoroughly for 3 min to get a white suspension. To this 5 ml of Triton X-100, was added to get a clear solution, this was further vortexed for 2 min to ensure that the vesicles are lysed completely to release the drug. The supernatant was analyzed for percent drug entrapped using a Shimadzu UV spectrophotometer (Shimadzu-1700, Kyoto, Japan), at λ_{max} of 373.20 nm [13,15].

The entrapment study (EE) was calculated using the formula:

EE (%) = [(Drug in Pellet (Entrapped Drug) (mg/ml) / Total Drug Added (mg/ml)] x100

2.4.6 *In vitro* release studies

The liposomal suspension (1 ml) of SUVs was placed on one side of the sigma dialysis membrane in a vertical franz diffusion cell. Other side of the membrane was in contact with the dissolution medium (200 ml of PBS of pH 7.4). Entire dissolution assembly was placed on a magnetic stirrer at temperature of 37°C. Aliquots (5 ml) of dissolution medium was withdrawn at different time intervals- 5 min, 15 min, 30 min, 45 min, 60 min, 2 h, 4 h and 8 h. Whenever sample was withdrawn equal volume of fresh dissolution medium was added to the beaker to maintain a constant volume. Drug concentrations in the dissolution medium were determined by UV spectrophotometric method. All the experiments were carried out in triplicates and the results were expressed as Mean ± Standard Deviation. [15]

2.4.7 *In vivo* studies

2.4.7.1 Anti rheumatoid efficacy studies

Male Wistar-Lewis (5-6 weeks old rats weighing 200±10 g) were used. Animal groups were selected with the same initial body weight. Animals were kept under environmental conditions (22±0.5°C with relative humidity 40-60%), alternate light-dark cycles, food and water. The animals were allowed to acclimatize for 1 week before the experiment. They were housed in cages in which the floor was covered with saw dust to minimize the possibility of painful contact with hard surface. Adjuvant arthritis was induced as per the method described by Pearson and Wood by injecting 0.6 ml (1 mg/ml) of Complete Freund's Adjuvant (CFA, heat killed and dried *Mycobacterium tuberculosis* (1 mg/ml) in 0.85 ml mineral oil, and 0.15 ml mannide mono-oleate) to the sub plantar region of the left hind paw. The parameter of interest of adjuvant-induced arthritis is the swelling of the left paw, which is typically established in 19 days after induction. Rats were divided into 5 groups as normal standard which received saline by oral route; CFA-control group received only 0.6 ml of CFA, CFA (control), CFA + test 1, CFA+ test 2 and CFA + test 3 with 6 animals in each group. CFA + test 1 group of rats received CFA to the left hind paw + conventional uncoated LXM liposomal formulation (Lipoid S

PC-3: CH-10: 2 at a dose of 1.6 mg, which is equivalent to 2.1 ml of the formulation after considering the drug entrapment data of LXM in liposomes), CFA + test 2 group of rats received chitosan coated conventional liposomes (Lipoid S PC-3: CH-10: 2 at a dose of 1.6 mg, which is equivalent to 1.5 ml of the formulation after considering the drug entrapment data of LXM in liposomes), CFA + test 3 group of rats received PEGylated LXM liposomal formulation (Lipoid S PC-3: MPEG-DSPE: CH-9: 0.2: 3 at a dose of 1.6 mg, which is equivalent to 1.8 ml of the formulation after considering the drug entrapment data of LXM in liposomes) and standard group received LXM solution, i.e., 0.4 ml of LXM injection (4 mg/ml) equivalent to 1.6 mg LXM) was administered as a single intravenous injection through the tail vein on the day 0. The onset day of arthritis was determined as the day on which left hind paw swelling or its redness was detectable. For determining the arthritic reaction, a marking was made in the tebio-tarsal joint of the right and left hind paws and the paw volume (in ml) of each paw was determined on the 0^{th}, 5^{th}, 10^{th}, 21^{st}, 26^{th}, and 29^{th} day after induction of adjuvant arthritis, using a water displacement plethysmograph. The severity of the induced adjuvant disease was determined by measurement of the non injected right paw (secondary lesion) with a plethysmograph and by measuring the body weight every three days after arthritis induction [16,17,18,19, 20,21,22].

2.4.7.2 Arthritis assessments

The rats were assessed daily for signs of arthritis between days 7 and 25 post-CFA. During treatment paw volume was measured every other day with a plethysmograph.

2.4.8 Stability studies

Stability study was carried out for the sonicated liposomal suspension of SUVs at two different temperatures i.e. refrigeration temperature (4±2 ˚C) and room temperature (27±2ºC) for 4 weeks. Sampling was done, suitable dilutions were made with PBS 7.4 and UV absorbance was determined. The entrapment efficiency was calculated from the regression equation Y= 0.0267x obtained from the standard plot of LXM in PBS pH 7.4 at 373.20 nm [23].

2.5 Statistical Analysis

The data were presented as Mean ± S.E.M. One way analysis of variance (ANOVA) followed by post Dunnet multiple comparison tests to compare the efficacy of the formulations by using graph pad prism version 4.03.354 software.

3. RESULTS AND DISCUSSION

Liposomes of lornoxicam were prepared by thin film hydration method using a rotary flash evaporator under nitrogen atmosphere. Lornoxicam was loaded in stealth liposomes, conventional liposomes and coated conventional liposomes. Stealth liposomes were prepared by incorporating PEGylated lipids like MPEG-DSPE and conventional liposomes using phospholipids like Lipoid S PC-3 (Hydrogenated soybean phosphatidylcholine) and cholesterol. Conventional liposomes were then coated with chitosan which produced cationic liposomes. Coating was confirmed by the measurement of zeta potential of the formulation by using a nano zeta sizer instrument which showed that the chitosan coated liposomes exhibited a positive zeta potential compared to the uncoated liposomes which had a negative zeta potential values. All the formulations were optimized to get the best entrapment efficiency.

3.1 Differential Scanning Calorimetry (DSC)

The DSC results of phase transition temperature (Tm) of lipoid S PC-3 and the mixture of lipoid S PC-3 with MPEG-2000 DSPE, was found to be 79.30 ˚C and 58.10ºC respectively Fig. 1 (a) and (b).

3.2 Optical Microscopy

Optical microscopy confirms the desired shape of conventional and stealth liposomes Fig. 2 (a) and (b).

3.3 Particle Size Analysis

Particle size analysis of the sonicated liposomes and size distribution was performed using a Malvern zeta sizer instrument. The size analysis of particles indicated that the size of liposomes without sonication was found to be greater compared to that of with sonication. The average particle size of unsonicated liposomes (F6-Lipoid S PC-3: MPEG-DSPE: CH; 9: 0.2: 3) was found to be approximately 844.4 nm where as the average particle size of sonicated liposomes was found to be 195.5 nm. PDI value before sonication was found to be 1.00 and after sonication reduced to 0.488.

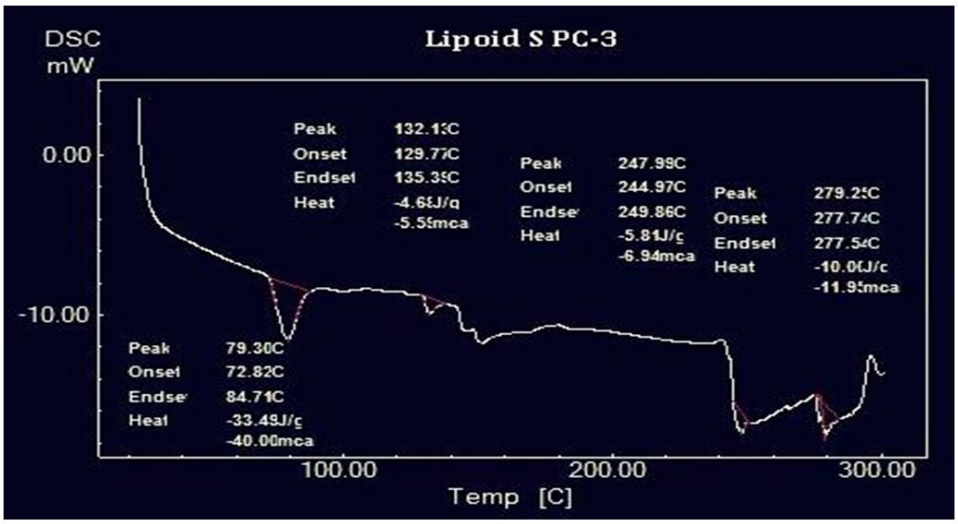

Fig. 1a. DSC graph for Lipoid S PC-3

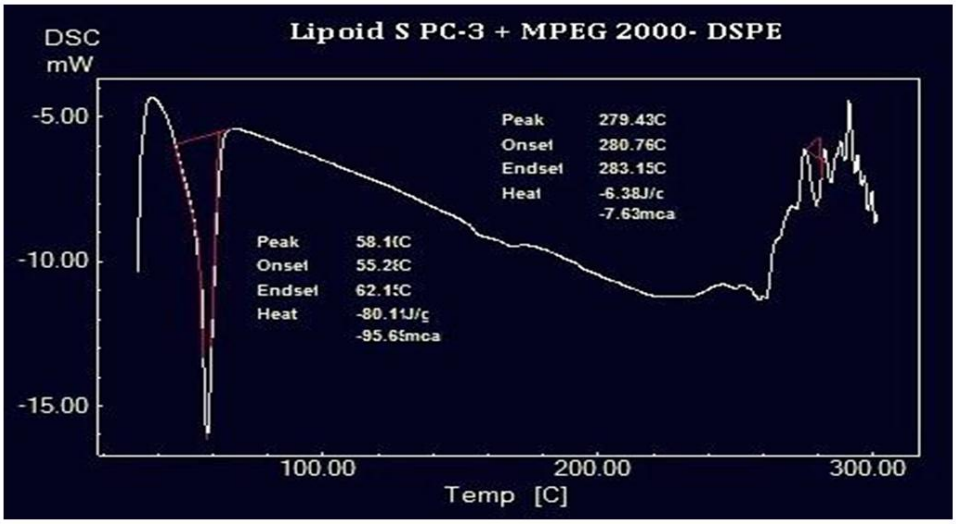

Fig. 1b. DSC graph for Lipoid S PC-3: MPEG 2000- DSPE

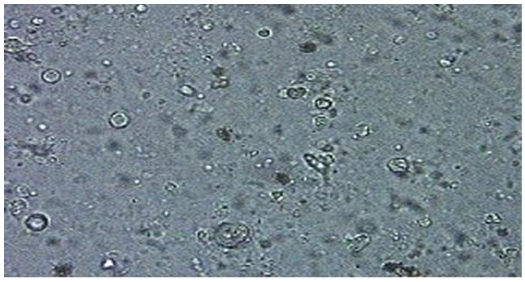

Fig. 2a. Optical photomicrograph of Conventional liposome

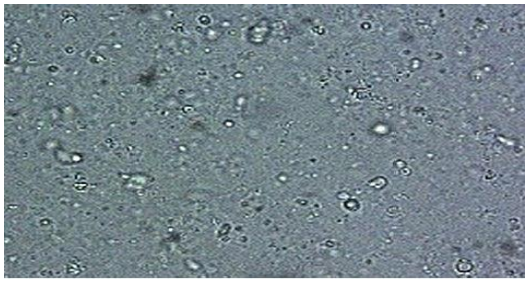

Fig. 2b. Optical photomicrograph of Stealth liposome

3.4 Zeta Potential Analysis

Zeta potential of the sonicated liposomes was performed by malvern nano zeta sizer instrument and formulation F4 was found to be positive (+ 25.0 mv), indicated the successful coating of the formulation with chitosan. The zeta potential of liposome without chitosan coat, found to be negative charge (-1.94 mv,) which, after the incubation with chitosan at various concentrations (0.1% w/v, 0.3% w/v, 0.5% w/v and 0.7% w/v), was turned out to be positive values (Table 3). As the concentration of coating solution increased from 0.1% to 0.7%, the zeta potential also increased and at 0.5% and 0.7% the zeta potential remained more or less same indicated the complete coating of the vesicles. This result is also in line with that earlier reported in the literatures. The presence of chitosan coating on the surface of liposomes confirmed with change of surface charge. Therefore, the main interaction between liposomes and chitosan was found to be electrostatic attraction.

3.5 Entrapment Efficiency

A drug: lipid ratio (1:5) gave the best entrapment efficiency. Increase in size of the liposomes also increased the entrapment efficiency due to the increment of cholesterol concentration. PEGylated liposomes (F6) showed higher entrapment efficiency than that of PEGylated liposome (F5). PEGylated liposomes showed higher entrapment efficiency because of high binding affinities of PEG compared to that of the conventional liposomes (F1 and F2) (Fig. 3).

Table. 3 Chitosan coated lipid vesicles

S. no	Concentration of chitosan (% w/v)	Zeta potential
1	0.0	-1.94
2	0.1	+5.96
3	0.3	+16.1
4	0.5	+25.0
5	0.7	+25.12

3.6 *In vitro* Release Studies

In vitro drug release studies were carried out using a franz diffusion cell in 200 ml PBS pH 7.4 as the dissolution medium at 37 $^{\circ}$C and at 50 rpm. Initial burst release was observed for all the formulations, attributed to their surface hydrophilicity. Higher burst release was observed with PEGylated and chitosan coated formulations, which was found to be far more significant compared to the other formulations. However PEGylated formulations showed a higher burst release compared to that of the chitosan coated formulations. This burst release was observed due to the presence of drug on the surface in the adsorbed form. All the formulations released the drug for a period of 8 h. Formulation F5 released 74.1% of the drug within 8 h, while formulation F6 released 71.4% of the drug within 8 h. Formulation F1 coated (conventional coated) released 33.8% of the drug in 8 h, where as formulation F2 coated (conventional coated) released 31.9% of the drug in 8 h. These results indicated sustained release of LXM, because of stabilization of lipid bilayers by cholesterol. Hence, a depot effect was achieved especially with the coated liposomal formulations (Fig. 4).

3.7 Comparative Release Kinetic of Different Prepared Liposomes

In order to determine the release mechanism of the drug from liposomes, the *in vitro* release data were fitted into Zero order, First order, and Higuchi model. The release data were also kinetically analysed using the Korsmeyer-Peppas model. The data were processed for regression analysis using MS-EXCEL statistical function. The results of kinetics analysis of *in vitro* drug release data for all formulations are given in Table 4. Formulations F1, F2, F3, F4, F5, F6 and F1 coated and F2 coated follows Higuchi model release kinetics. By using Korsmeyer and Peppas model, if n= 0.45 it is Case 1 or fickian diffusion, 0.45< n> 0.89 is for anomalous behaviour or non-fickian transport, n= 0.89 for Case 11 transport, and n> 0.89 for Super Case 11 transport. Fickian release usually occurs by molecular diffusion of the drug due to a chemical potent gradient**.** A case 11 relaxation release is the drug transport mechanism associated with stresses and state transition in hydrophilic polymers (PEGylated and chitosan coating), which swell in water or biological fluids. This term is also includes polymer disentanglement and erosion. In the present investigation, the release from the hydrophilic polymers followed the combination of diffusion and erosion as the 'n' values ranged from 0.594 to 0.738 for LXM as per Korsmeyer and Peppas model, which in turn justified suitability of polymers for the preparation of liposomes (Table 4).

3.8 *In vivo* Studies

3.8.1 Antirheumatoid efficacy

The arthritic lesions i.e., swelling of the left hind paw, lesions on the fore paw appeared from the 19th day in CFA-control group of rats and standard group of rats, where as the lesions were observed from the 21st day, in various test groups (test 1, test 2 and test 3) after arthritis induction of rats treated with the liposomal formulations.

The total edema volume in CFA control group of rats gradually increased as the days passed and reached a peak on 21st day. The gradual decrease in edema volume was observed, in case of CFA treated groups; test 1, test 2, test 3 and standard groups of rats. Rats treated with test 3 formulation showed a significantly lesser peak value on the 21st day compared to that of test 1, test 2 and standard groups, where as the rat group treated with the test 2 formulation showed a peak lesser than the standard. Compared to CFA control group of rats, CFA + test 3 group showed a highly significant % decrease ($P<0.01$) in edema volume on 26th day and 29th day. CFA + test 1 and CFA + test 2 group showed a significant ($P<0.05$) % decrease in edema volume on the 26th day and the 29th day compared to CFA control group of rats. Formulation test 3 (PEGylated liposomal formulation) showed the highest anti rheumatoid efficacy than the test 2 (chitosan coated liposomal formulation), test 2 showed better efficacy than the test 1(uncoated liposomal formulation) and the standard free LXM showed the least efficacy.

Order of efficacy decreases as mentioned below:

Test 3 > Test 2 > Test 1 > Standard

Hence in treating rheumatoid arthritis with intra-articular delivery of LXM (which can lead to joint inflammation with pain) and the oral delivery of LXM (uniform distribution in all the tissues in the body leads to unwanted adverse effects), an alternative delivery of LXM using stealth liposome of LXM can be administered by intravenous route, where the drug selectively reaches to the target site, thereby reduces the damage of other organs. Thus, the delivery system may offer advantages like reduced dose, decreased dosing frequency, thereby improving patient compliance. (Tables 5, 6 and 7), (Fig. 5 a, b, c, d, e and f)

3.9 Stability Studies

The stability of the liposomes is significant from formulation to storage till delivery, as they are thermodynamically unstable systems, tend to fuse, grow into bigger vesicles resulting in breakage of the system on storage which poses a problem of drug leakage. Further, unsaturated phospholipids undergo oxidation easily. Hence, in the present work only saturated phospholipids like Lipoid S PC-3, and MPEG-DSPE were used to formulate the liposomes, to avoid oxidation as a result antioxidant like α-tocopherol was used. A high Tm value of saturated phospholipids further adds to a good physical stability. The stability data of liposomes at 4±2 °C and 27±2 °C is given in Table 8. According to the data, formulations stored at refrigeration temperature showed higher drug content compared to the room temperature, after a period of 4 weeks. Stealth liposomes (F5 and F6) were found to be better stable than the conventional liposomes (F1, F2, F3 and F4) and showed much lesser extent of drug leakage.

Table 4. Release kinetic profile of different liposomal formulation

Formulation code	Zero order	First order	Higuchi model	Korsmeyer-peppas model	
	R^2	R^2	R^2	R^2	n
F1	0.7892	0.8705	0.9332	0.908	0.670
F2	0.7517	0.8334	0.9193	0.911	0.676
F3	0.7385	0.8094	0.9190	0.924	0.664
F4	0.7538	0.8535	0.9257	0.908	0.703
F5	0.8267	0.9575	0.9819	0.934	0.738
F6	0.8709	0.9594	0.9845	0.935	0.730
F1 coated	0.7419	0.7928	0.9228	0.901	0.616
F2 coated	0.7874	0.8335	0.9380	0.920	0.594

Table 5. Volume of water displaced in the plethysmograph

Animal groups	Weight (gms)	Edema volume (ml)					
		0 day	6th day	12th day	21st day	26th day	29th day
CFA-control	210±10	4.576±0.015	4.978±0.012	4.978± 0.017	5.180±0.015	5.356±0.014	5.389±0.011
Standard	210±10	4.219±0.013	4.747±0.011	4.809± 0.012	4.551±0.011	4.492±0.014	4.481±0.012
CFA +test 1 (Uncoated)	210±10	4.300±0.014	4.746±0.015	4.839± 0.014	4.978±0.013	4.824±0.011	4.514±0.013
CFA+ test 2 (Coated)	210±10	4.253±0.011	4.686±0.016	4.810± 0.009	4.743±0.010	4.484±0.011	4.345±0.013
CFA+ test 3 (PEGylated)	210±10	4.357±0.014	4.817±0.011	4.782± 0.010	4.648±0.008	4.520±0.013	4.420±0.009

Tabular value represent Mean ± SEM changes from 0 time readings n=6/group

Fig. 3. Comparison of Entrapment efficiency of various formulations

Table 6. Edema volume of different groups of animal on various days

Animal groups	Edema volume (ml)		
	21st day	26th day	29th day
CFA- control	0.604	0.780	0.813
Standard	0.332	0.273	0.262
CFA+ test 1 (Uncoated)	0.678	0.524	0.214
CFA+ test 2 (Coated)	0.490	0.595	0.092
CFA+test 3(PEGylated)	0.291	0.163	0.063

Edema volume = Final edema- Initial edema volume

Table 7. Percentage decrease in edema volume in various animal groups

Animal groups	%decrease in edema volume		
	21st day	26th day	29th day
Standard	45.03	65.0*	67.77*
CFA+ test 1 (Uncoated)	12.25	32.82*	73.67**
CFA+ test 2 (Coated)	18.87	23.71	88.68**
CFA+test 3 (PEGylated)	51.80*	79.10**	92.25**

**P<0.05- significant, **P< 0.01- Highly significant*

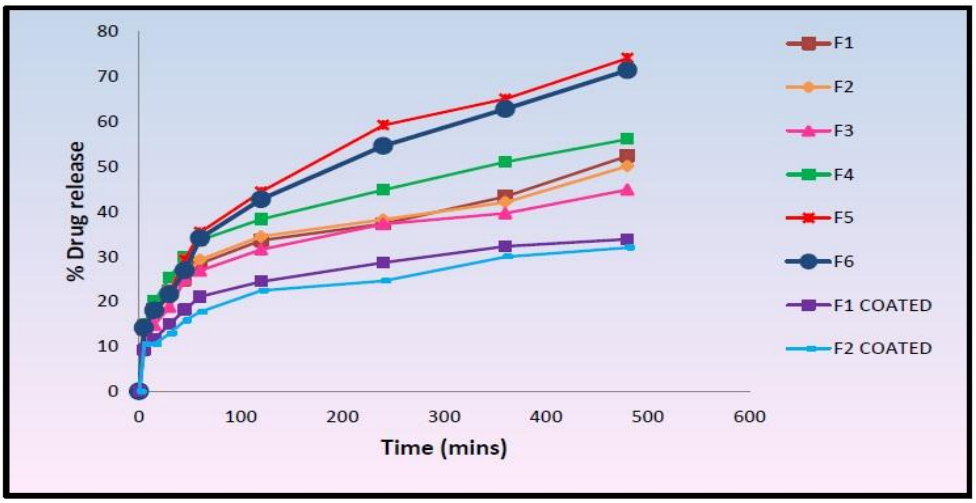

Fig. 4. Comparison of release profile of various formulations

Fig. 5a. Injection of CFA to the sub plantor region of the left hind paw

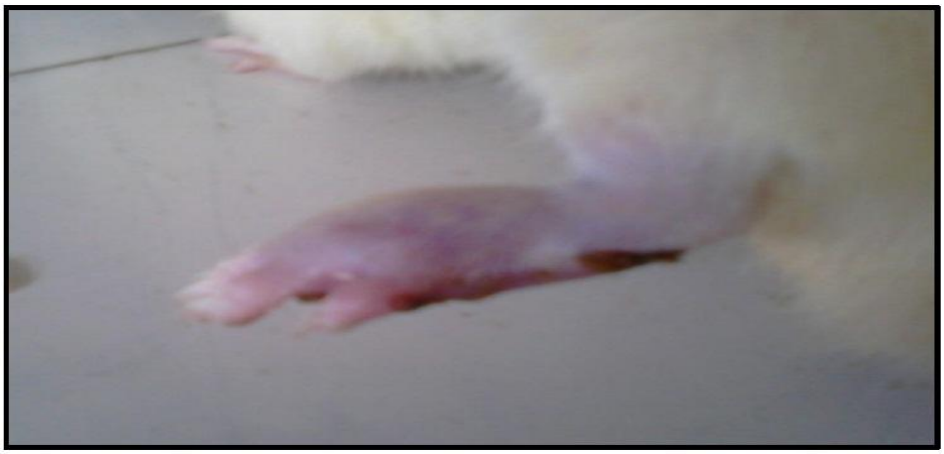

Fig. 5b. CFA - Control group shows severe inflammation on 29th day

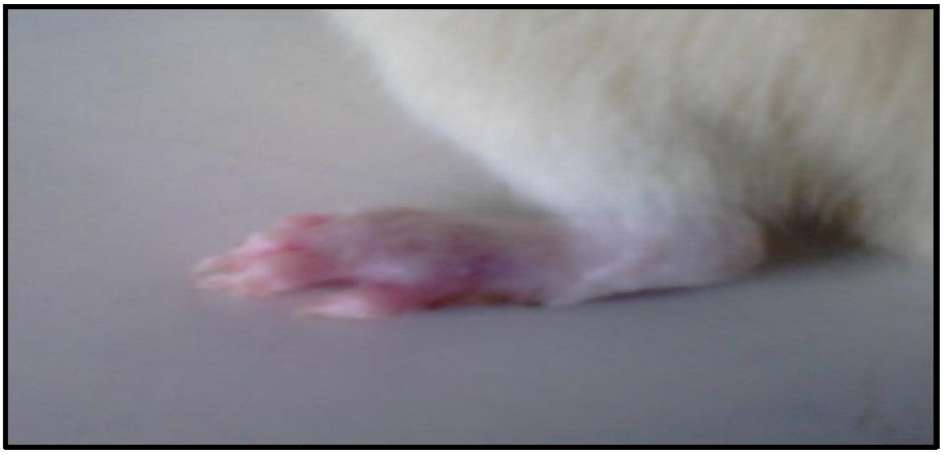

Fig. 5c. CFA + Standard group rat shows a moderate inflammation on 29th day

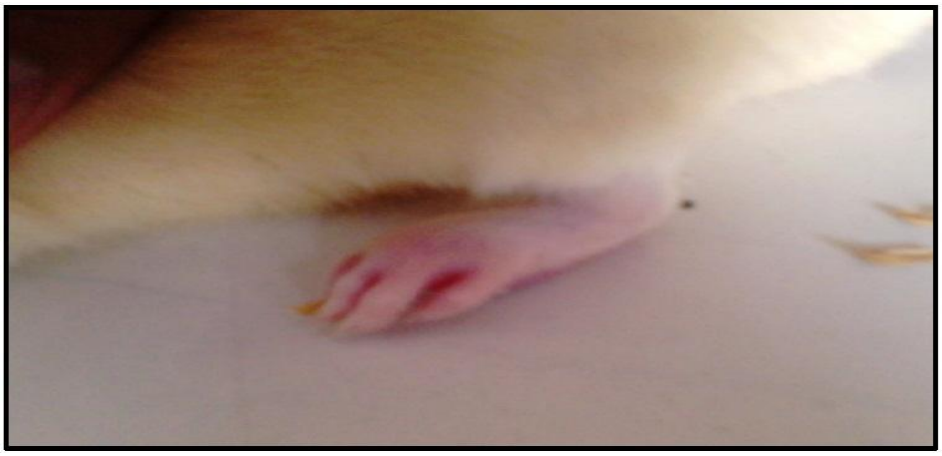

Fig. 5d. CFA + test 1 group rat shows a moderate recovery on 29th day

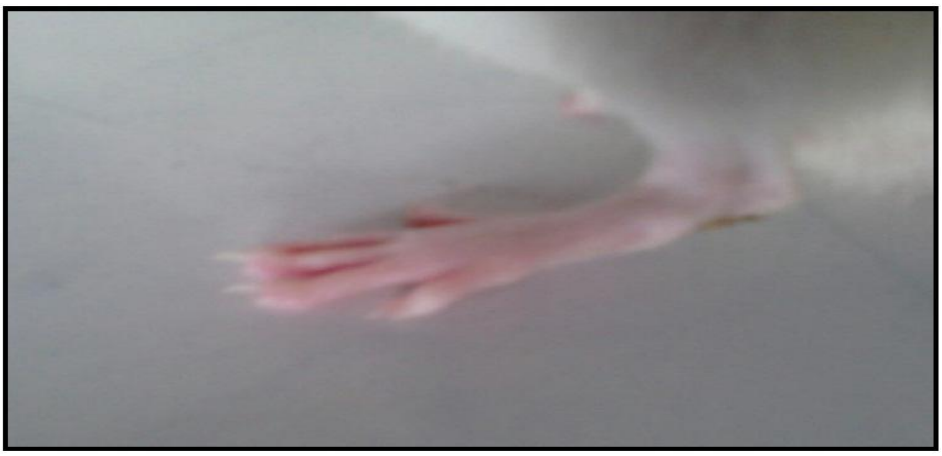

Fig. 5e. CFA + test 2 group rat shows good recovery on 29th day

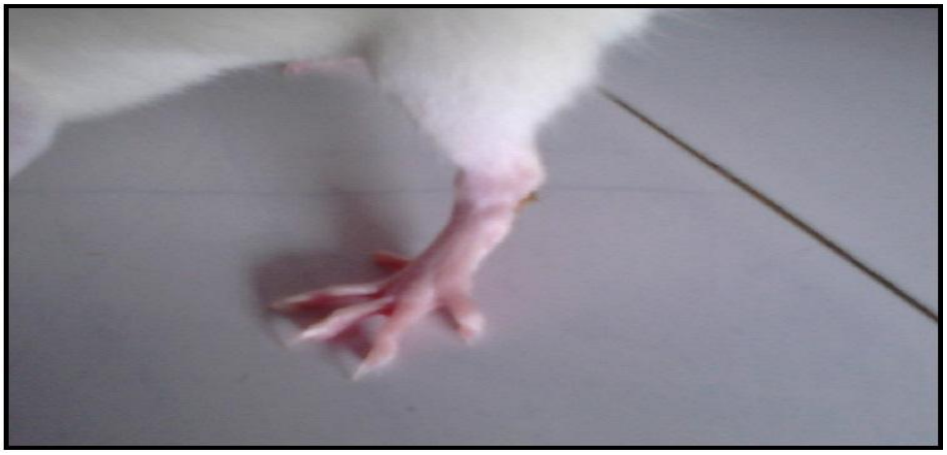

Fig. 5f. CFA + test 3 group rat shows complete recovery on 29th day

Table 8. Stability study of various liposomal formulations

Formula-tion code	Entrapment study									
	Intial		After 1 week		After 2 week		After 3 week		After 4 week	
	4±2°C	27±2°C	4±2°C	27±2°C	4±2°C	27±2°C	4±2°C	27±2°C	4±2°C	27±2°C
F1	13.02	13.02	12.92	12.56	11.82	11.34	11.12	10.45	10.40	10.01
F2	22.84	22.84	22.71	22.43	22.33	22.11	21.89	21.48	21.29	21.04
F3	37.07	37.07	37.03	36.85	36.54	36.19	35.96	35.32	35.11	34.89
F4	40.82	40.82	40.75	40.51	40.39	40.12	39.54	39.04	38.65	38.29
F5	45.22	45.22	45.16	45.03	44.75	44.39	44.10	43.51	43.06	42.46
F6	45.97	45.97	45.72	45.54	45.45	45.26	45.15	44.74	44.61	44.37
F1 coated	54.41	54.41	54.31	54.19	54.10	53.78	53.75	53.54	53.50	50.69
F2 coated	52.30	52.30	52.18	52.03	51.83	51.59	51.49	51.26	51.15	50.69

However, chitosan coated liposomes (F1 coated and F2 coated) were found to be highly stable compared to the stealth and conventional liposomes. Chitosan coated liposomes also showed a significantly lesser drug leakage, apart from better physical stability due to the steric repulsion created by the surface positive charge on the liposomes leading to steric stabilization of colloidal suspension.

4. CONCLUSION

For the treatment of rheumatoid arthritis, the orthopedicians, instead of going for intra articular delivery of lornoxicam which can lead to joint inflammation with pain and in case of oral delivery lornoxicam gets uniformly distributed to all the tissue in the body leading to unwanted adverse effects, the orthopedicians in there clinical practice can switch into an alternative delivery using lornoxicam using stealth liposomes of lornoxicam which can be administered by intravenous route where the drug selectively reaches the target site with reduced toxicity to other organs, moreover it also reduces the dose of lornoxicam.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study is not against the public interest, and has been performed in accordance with the Instutional Animal Ethics Committee (IAEC) after scrutinizing the proposal has granted permission to carry out animal study for the project titled Investigation of nanolipid vesicles of lornoxicam for targeted drug delivery.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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