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Research Article

Influence of egg lecithin composition on physicochemical characteristics of pramipexole liposomes

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ABSTRACT

The objective of this investigation was to study the effect of varied concentration and ratio of egg lecithin, cholesterol and drug (Pramipexole) on physico-chemical characteristics of liposomes prepared by thin film hydration method. Pramipexole-loaded liposomes of egg lecithin were prepared and characterized for vesicle size, TEM, zeta potential, entrapment efficiency (%EE), phospholipid content, in vitro drug release and drug leakage. Liposomes obtained were spherical in shape, 151 to 231 nm in size, and negatively charged (−18 to −32 mV). The %EE of pramipexole in the liposomes ranged from 18 to 61%. The effects of variations in cholesterol, egg lecithin and pramipexole contents on the liposomal characteristics were determined. It was found that the liposomes prepared with unimolar ratio of cholesterol, egg lecithin and pramipexole exhibited better characteristics than other liposomal formulation.

Keywords: Liposomes; Pramipexole; Egg Lecithin; Cholesterol.

INTRODUCTION

Liposomes are synthetic lipid spheres composed by fatty acid on polymers with a bilayered membrane structure surrounding an aqueous core that can encapsulate small molecules. Liposomes have the advantages of being both nontoxic and biodegradable because they are made up of naturally occurring substances. The exclusive ability of liposomes to entrap drugs both in an aqueous and a lipid phase make them attractive for hydrophilic and hydrophobic drugs. The percentage entrapment of hydrophilic drugs by liposomes depends on the composition of bilayer and preparation method of the liposomes. Moreover, such encapsulation has been shown to reduce drug toxicity while retaining or improving the therapeutic efficacy (Spuch 2011). Liposomes have achieved significant attention as drug delivery carriers because they protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the BBB (Elbayoumi 2010).

Due to the presence of multiple endogenous transporters, BBB permits a selective entry of nutrients and minerals across it and limits the entry of foreign substances like drugs as well as neuropharmaceutical agents. This reduces the efficiency of CNS treatment. The conventional drug delivery systems which release the drug into general circulation fail to deliver drugs effectively to brain and are, therefore, not very useful in treating certain diseases that affect CNS including Alzheimer's disease, dementia, Parkinson's disease, mood disorders, AIDS, and viral and bacterial meningitis (Chen 2010).

Phospholipids derived from plant or animal sources and synthetic phospholipids are used for formulating liposomal formulations. Natural phospholipids obtained by solvent extraction and chromatographic procedures differ in phosphatidylcholine content and fatty acid composition. Natural phospholipids are used in oral, dermal and parenteral pharmaceutical applications including liposomes. The phospholipid and fatty acid composition of the lecithin depends and varies with raw material. Depending on the source, lecithins differ in composition, including fatty acid chain length and degree of unsaturation as well as in polar head in choline group. The different physico-chemical characteristics of lecithins depends on the source of phospholipids and hence may influence the formulation properties. Phospholipids can be determinant for the targeting properties for liposomal formulations intended for site specific delivery of drugs (Van 2014). Heterogenicity in composition and high degree of instability due to presence of unsaturation in the fatty acyl chains raised the arguments on the use of natural phospholipids. Moreover, use of phospholipids isolated from animal sources like egg lecithin in the pharmaceutical formulations may sometimes be complicated by the occurrence of animal diseases and are possibly contaminated by trace amounts of proteins, nucleic acids, and other lipids.

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Clinical efficacy exhibited by pramipexole for successful treatment in early Parkinson's disease for several years in the absence of levodopa and as adjunctive therapy with levodopa in late disease is a potential standard for treatment of Parkinson's disease (Olanow 2006, Horstink et al., 2006). The occurrence of dopaminergic motor complications was found to be higher in the initial levodopa treated group, thus a long-term benefit on the development of these complications with initial pramipexole treatment are suggested. Pramipexole, dopamine agonist has been found to delay onset of dyskinesias and wearing off and is also used for the treatment of Restless Leg Syndrome (Eduardo et al., 2012).

Thus present study was designed with objective to investigate the effect of variation in concentration of egg lecithin, cholesterol and pramipexole on the liposomal characteristics and determine the optimum formulation ratio. The prepared liposomes were characterized for vesicle size, TEM, zeta potential, entrapment efficiency (%EE), phospholipid content, in-vitro drug release and drug leakage.

MATERIALS AND METHODS

Chemicals: Pramipexole Hydrochloride was a gift sample obtained from Torrent Research Center, India. Egg lecithin was procured from TCI chemicals (USA), Cholesterol was bought from Sigma Aldrich, India. All other reagents and chemicals were of AR grade.

Preparation of liposomes

Cholesterol, Egg lecithin and pramipexole were taken in different ratios as depicted in Table 1 for the preparation of liposomes by thin film hydration technique. Multilamellar vesicles were prepared by thin film hydration method using rotary vacuum evaporator (Samad 2007). Lipid phase mixture containing accurately weighed quantities of pramipexole (1.05 mg – 0.5 M/2.11 mg – 1 M/4.22 mg – 2 M/6.33 mg – 3 M), cholesterol (0.967 mg – 0.25 M/1.935 mg – 0.5 M/3.87 mg – 1 M) and phospholipids i.e egg lecithin (1.61 mg – 0.25 M/3.22 mg – 0.5 M/6.44 mg – 1 M) were prepared and transferred to the round bottom flask and to it 10 ml chloroform was added. The removal solvent mixture from lipid phase was done by evaporation using rotary vacuum evaporator at 50 ± 2 ℃ for 30 min under reduced pressure of 25mm Hg at 100 rpm to obtain thin lipid film on the wall of the flask. Consequently flask was kept under the vacuum over night to ensure absolute removal of the residual solvent. The dry film thus obtained was hydrated using 30 ml phosphate buffer saline pH 7.4 at 25 ℃. Liposomal dispersions so obtained were vortexed for about 2 min, sonicated for 15 min at room temperature and kept uninterrupted at room temperature for nearly 4 h for complete swelling of film and to obtain absolute vesicular suspension. The liposomes thus formulated were stored in suitable container in refrigerator.

Evaluation of liposomes

Morphology

Digital optical microscope was used to study the morphology of prepared liposomes. All the liposomes of different batches were observed under microscope to determine the shape. Liposomal dispersion in a drop was set up on a glass slide and examined under the digital microscope (Motic Microscope version 3.2).TEM analysis was done to confirm the results.

Determination of vesicular size

Determination of vesicle size of the prepared liposomal batches was done by photon correlation spectrophotometer, which is based on analysis of the fluctuations in light scattering owing to the brownian motion of the particles using a Zeta sizer ZS 90 (Malvern Instrument Ltd., UK). The liposomal suspension was diluted with double distilled water (1:100) and light scattering was monitored at a 90 angle. All measurements were done thrice.

Transmission Electron Microscopy (TEM)

All batches of the liposomes were characterized for their morphology through Transmission Electron Microscopy (TEM) (Jeol Model JM 2100) at SAIF, Kochi. Vaguely turbid solution containing a small amount of liposomes suspended in water/ethanol was obtained. The solution was subjected to sonication to disperse the vesicles and a drop of the solution was casted on carbon-coated grids of 200 mesh and the morphology of liposomes was studied under High Contrast Transmission Electron Microscopy (TEM).

Determination of Zeta Potential

Prepared liposomes were characterized for zeta potential by filling NE in Folded Capillary Cell using Zetasizer ZS 90, (Malvern Instrument Ltd., UK) using Electrophoretic Light Scattering (ELS) technique. Droplet size of prepared liposomes were studied by photon correlation spectrophotometer, which determines the fluctuations in light scattering due to the brownian motion of the vesicles using a Zeta sizer ZS 90 (Malvern Instrument Ltd., UK). The liposomal suspension was diluted with double distilled water (1:100) and light scattering was monitored at a 90 angle. All measurements were done thrice.

Phospholipid content

The phospholipid content determinations of the liposomes were done by UV-Visible Spectrophotometer (Model UV-1700, Shimadzu, Japan) using 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) as standard as per earlier reported colorimetric method employing principle of complexation of soy lecithins and ferrothiocyanate (Stewart 1980). 2 ml liposomal suspension after centrifugation and separation were subjected to drying in vacuum oven overnight and the obtained dry residue was then dissolved in 2 ml chloro-

form and 1 ml thiocyanate reagent. The samples were then vortexed for 1 minute and centrifuged at 2000 rpm for 5 minutes. The lower chloroform layer was separated and absorbance was measured at 486 nm. The amount of DPPC was calculated from the regression equation obtained from the calibration curve (10-100 µg/ml) of DPPC using chloroform as blank.

Drug Entrapment Efficiency

The liposomal suspensions (1 ml) were subjected to centrifugation at 2.8 x 10^5 g (Compufuge, Remi, India) for 20 min at 4°C and then washed with buffer to obtain Pramipexole containing liposomes without unentrapped Pramipexole. The supernatant was removed and fresh pH 7.4 phosphate buffer was added. The same procedure was repeated thrice to ensure complete removal of unentrapped drug. The entrapped concentration of Pramipexole was obtained by dissolving Pramipexole containing liposomes in absolute alcohol and analyzed by validated HPLC method.

In-Vitro drug release studies

In vitro drug release studies were conducted using modified Franz diffusion cell (Receptor compartment-17 ml). Dialysis membrane was sandwiched between the lower reservoir cell and the glass top cell (Diameter- 1.6 cm, area- 2.0096 cm^2) containing the liposomal sample and held in place with a pinch clamp. The liposomal suspensions (1 ml) were centrifuged at 2.8 x 10^5 g for 20 min at 4°C and then washed with buffer to obtain liposomes free from unentrapped drug. Liposomes thus obtained were then re-dispersed in fresh 1 ml phosphate buffer pH 7.4. It was then placed in donor compartment and the receptor compartment was filled with phosphate buffer pH 7.4 (17 ml). The diffusion cell was maintained at 37+0.5°C with stirring at 500 rpm throughout the experiment. Fluid from receptor compartment (100 µl) was withdrawn at predetermined time intervals for the period of 5 h and analyzed by validated HPLC method. Each time the sample was withdrawn, the receptor compartments were replaced with 100 µl fresh phosphate buffer.

Drug in-vitro release kinetics was computed (PCP Disso version 3.1 and DD Solver- Excel based programs) by using zero order kinetic (Equation 1), first order kinetic (Equation 2), Higuchi square root of time (Equation 3), Hixson-Crowel model (Equation 4) and Korsemeyer-Peppas model (Equation 5).

$$Q_t = K_0 . t \dots\dots\dots\dots\dots 1$$

$$Q_t = InQ_0 - K_1 . t \dots\dots\dots\dots\dots 2$$

$$Q_t = K_H . \sqrt{t} \dots\dots\dots\dots\dots 3$$

$$\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = K_{HC} . t \dots\dots\dots 4$$

$$\frac{M_t}{M_\infty} = K . t^n \dots\dots\dots\dots .5$$

Where, Q_t Amount of drug released in time t

Q_0 Initial amount of drug in the system

K_0 Constant for zero order drug release

K_1 Constant for first order drug release

K_H Constant for Higuchi square root time dependant drug release

K_{HC} Constant for Hixson-Crowell drug release model

M_t/M^∞ Fraction of drug released at time t over the total amount of released drug

Mt Fraction of drug released at time t

M∞ Total amount of drug in the system

k Constant of apparent release

n Diffusion exponent

Drug Leakage studies

Drug leakage studies were conducted at three different temperatures (4°C, 25°C and 37°C). Liposomal suspension (30 ml) of different batches were made free from unentrapped drug, washed subsequently and reconstituted in fresh phosphate buffer pH7.4 (30 ml). Liposomal suspensions free from unentrapped drug (10 ml) from different batches were transferred to three different amber colored vials. The liposomes were then stored at three different temperatures (4°C, 25°C and 37°C) in the incubator and were evaluated daily for drug content for 10 days. liposomal suspension (1ml) were withdrawn everyday (previously shaken and briefly sonicated (1 min) for dispersal) and centrifuged. The supernatant was discarded and separated liposomes were dissolved in methanol (10 ml) and diluted suitably with mobile phase and analyzed by injecting in HPLC column for the estimation of pramipexole concentration.

Data Analysis

Data were expressed as the mean standard deviation (±SD).The group means were compared and evaluated by the unpaired *t*-test. The statistical significance of differences among more than two groups was determined by one-way ANOVA. A value of $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Morphology

Liposomes were observed as dispersed spheres with few aggregations. The TEM images of liposomes prepared with Egg lecithin (LNE 1-8) are depicted in Figure 1.Formulations containing unimolar ratio of drug, cholesterol and egg lecithin were more uniform in size with lower degree of variations (LNE-3) whereas liposomal structures with other formulations showed lesser uniformity. As depicted Figure 1 the liposomes from almost all the formulations were separate and spherical individual entities with decreased aggregation

Table 1: Formulations of liposomes prepared using egg lecithin

Sr. No.	Formulation Code	Composition (molar ratio)		
		Pramipexole	Egg lecithin	Cholesterol
1	LNE-1	1	1	0.25
2	LNE-2	1	1	0.5
3	LNE-3	1	1	1
4	LNE-4	0.5	1	1
5	LNE-5	2	1	1
6	LNE-6	3	1	1
7	LNE-7	1	0.5	1
8	LNE-8	1	0.25	1

Table 2: The physical chemical characteristics of liposomal formulations

Sr. No.	Vesicular size (diameter nm)±SD	Zeta Potential (mV)	Entrapment Efficiency (%)	Phospholipid content (%)
LNE1	173.41	-32.21 ± 4.46	37.61 ± 2.63	48.03 ± 5.99
LNE2	186.12	-27.99 ± 5.74	43.71 ± 1.37	48.29 ± 2.76
LNE3	209.99	-23.38 ± 2.25	57.65 ± 2.71	49.71 ± 1.83
LNE4	168.54	-25.61 ± 4.68	61.31 ± 3.32	47.36 ± 2.47
LNE5	217.76	-24.02 ± 3.71	26.70 ± 0.19	47.67 ± 2.31
LNE6	231.48	-21.10 ± 6.53	18.08 ± 0.78	48.03 ± 6.65
LNE7	162.53	-20.45 ± 6.23	42.91 ± 4.85	41.16 ± 1.82
LNE8	151.29	-18.78 ± 5.49	37.34 ± 3.35	36.69 ± 7.86

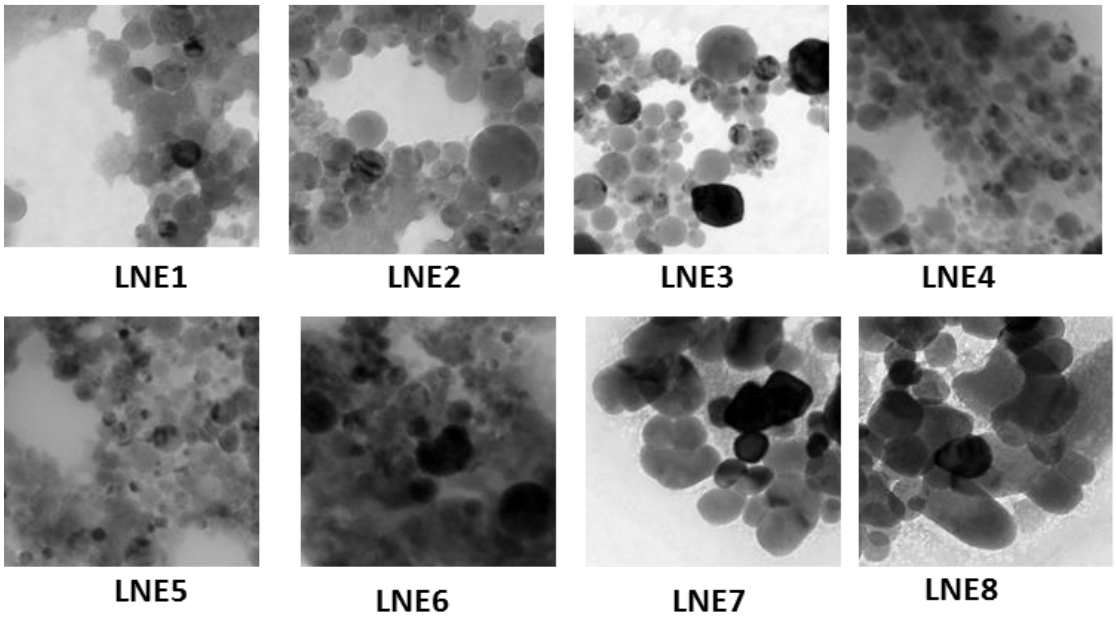


Figure 1: TEM images of the liposomal vesicles prepared with pramipexole, egg lecithin and cholesterol at three different levels

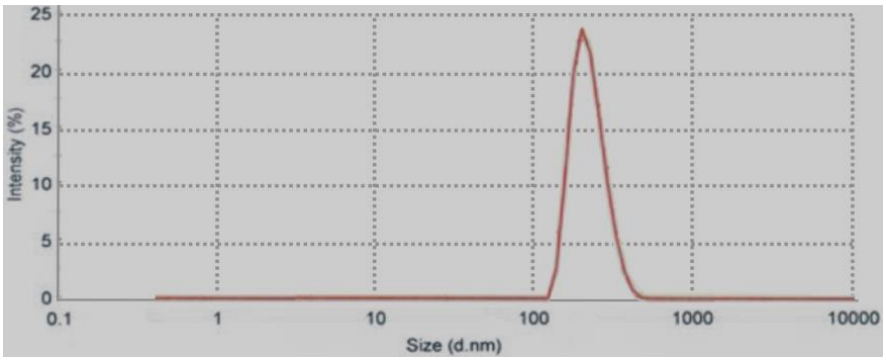


Figure 2: Size distribution for pramipexole loaded liposomal formulations prepared with cholesterol and egg lecithin (LNE3)

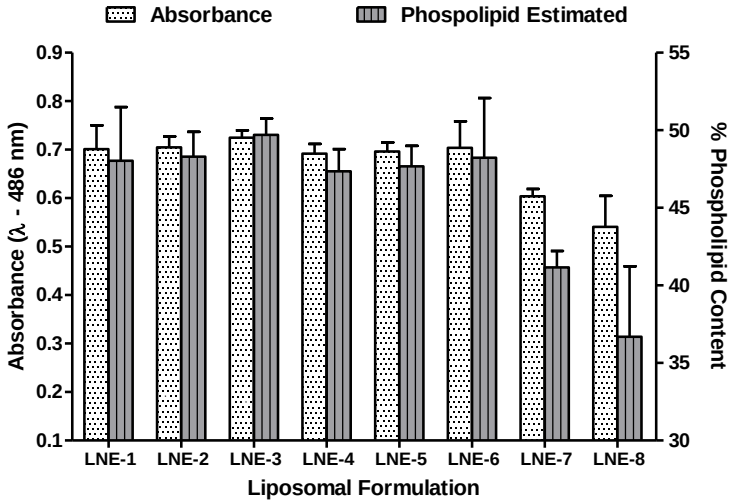
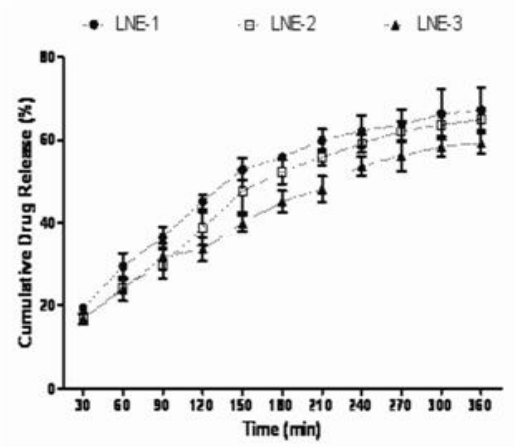


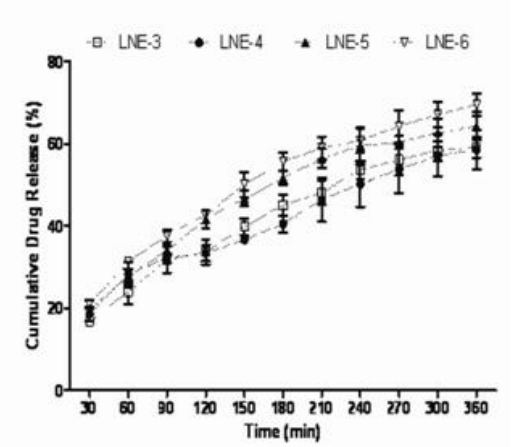
Figure 3: Absorbance and phospholipid content (%) of liposomal formulations prepared with egg lecithin

Table 3: In-vitro pramipexole release kinetics data for liposomal formulations prepared with egg lecithin

Formulation	Zero Order		First Order		Higuchi		Peppas		Hixon-Crowell	
	R	K_0	R	K_1	R	K_H	R	K	R	K_{HC}
LNE-1	0.8078	0.2539	0.9343	-0.004	0.9854	4.0106	0.9836	3.4261	0.9035	-0.0011
LNE-2	0.8869	0.2389	0.969	-0.0037	0.9868	3.7414	0.9875	2.1965	0.9494	-0.0011
LNE-3	0.8743	0.215	0.9581	-0.0031	0.9901	3.3715	0.9895	2.6776	0.9377	-0.0009
LNE-4	0.8199	0.2083	0.9325	-0.003	0.9847	3.2763	0.983	4.0873	0.9047	-0.0009
LNE-5	0.8333	0.2391	0.9445	-0.0037	0.9901	3.7691	0.9904	3.4398	0.9181	-0.001
LNE-6	0.8299	0.2542	0.9574	-0.0041	0.9926	4.0095	0.9932	3.8998	0.9286	-0.0011
LNE-7	0.7719	0.2703	0.9652	-0.0046	0.9907	4.2816	0.9829	7.1054	0.9272	-0.0013
LNE-8	0.7081	0.2855	0.9499	-0.0051	0.9779	4.5347	0.9773	8.791	0.9057	-0.0014

Korsemeyer-Peppas	
n	kKP
0.132 ± 0.15	456.48 ± 623.72
0.262 ± 0.09	30.27 ± 19.23
0.391 ± 0.15	12.92 ± 15.40
0.488 ± 0.14	1.95 ± 1.20
0.232 ± 0.12	48.98 ± 54.64
0.258 ± 0.19	56.65 ± 61.34
0.540 ± 0.04	2.78 ± 0.93
0.541 ± 0.29	6.99 ± 9.04





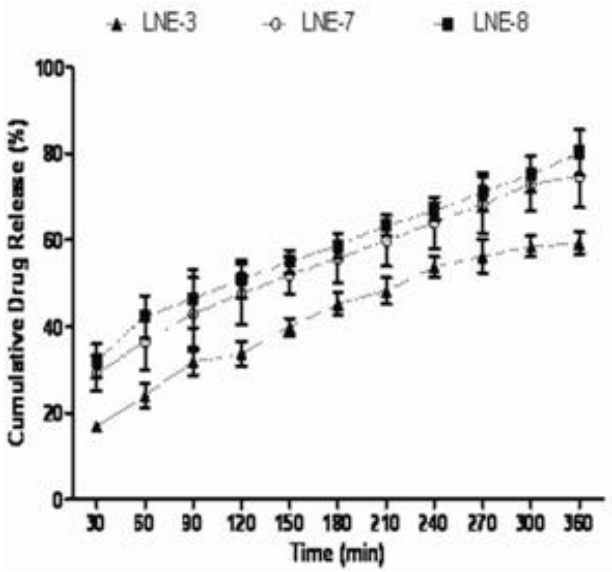


Figure 4: Cumulative in-vitro pramipexole release from liposomal formulations LNE-1 LNE-8

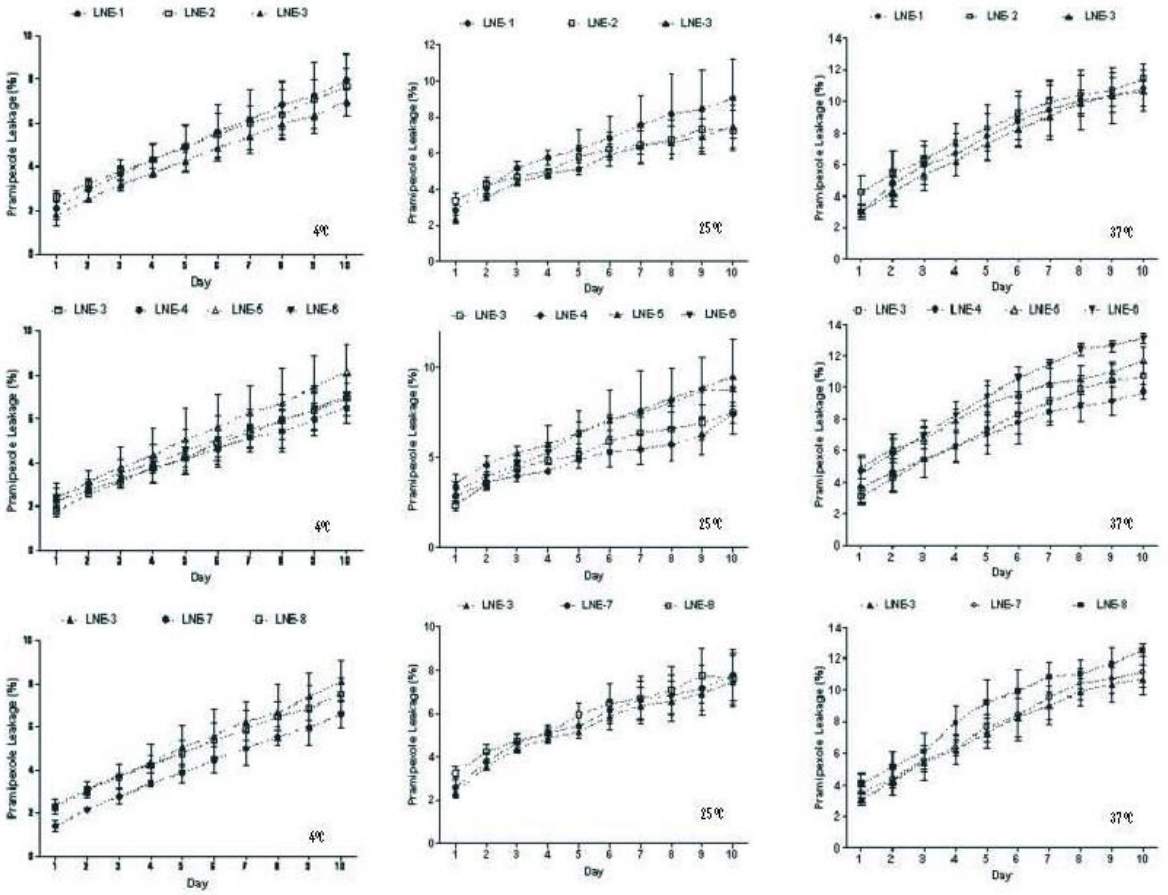


Figure 5: Pramipexole leakage (% ± SD) from the liposomal formulation prepared with egg lecithin stored at 4 °C,25°C and 37°C

Vesicular size

The size-range of all the formulations (LNE-1 to LNE-8) was found to be 158 to 225 nm. The mean vesicle diameter of LNE-3 (unimolar ratio of cholesterol, drug and egg lecithin) formulation was found to be 209.99 nm. The diameters of liposomes containing pramipexole, cholesterol and egg lecithin were analyzed to characterize the effect of variable cholesterol, pramipexole and egg lecithin content. As shown in Table 2, significant increase in the size were observed with altered cholesterol content (LNE-1 (173.41 nm), LNE-2 (186.12 nm) and LNE-3 (209.99 nm)). 2. The results on the sizes of the liposomes increased with the incorporation of cholesterol are consistent with the literature (Liu 2000).

However, vesicular diameter was increased linearly in a consistent manner (LNE-4 (168.54 nm), LNE-3 (209.99 nm), LNE-5 (217.76 nm) and LNE-6 (231.48 nm)) with increasing the peak width, reflecting decrease in the homogeneity of liposomal populations with increasing pramipexole contents. Further, decreased vesicle diameter was observed with decreasing egg lecithin. This might be due to the decreased total phospholipids content into the lipid bilayer. From the obtained data, it could be suggested that vesicle size increases with increasing cholesterol, egg lecithin and pramipexole molar ratio. Whereas more uniform size distribution could be obtained in unimolar ratio.

Transmission Electron Microscopy

TEM images of the different liposomal formulations demonstrated spherical vesicles (Figure1). Although the effect of cholesterol on size was evident, it can be anticipated that the increasing cholesterol content improved the dispersity of the liposomal suspension as evident from the microphotographs ad well as computed deviation from mean vesicular diameter. Increased number of formed vesicles was observed in formulations prepared with increasing quantities of pramipexole. Decreased transmittance as well as increased turbidity was in accordance with liposomal suspensions of increased drug quantity (LNE-5 and LNE-6). Aggregated vesicles with decreased sphericity and irregularity in size were associated with LNE-7 and LNE-8 prepared with decreasing egg lecithin content. Liposome size as well as number was increased with increasing pramipexole content, whereas size irregularity and vesicular aggregation was observed in formulations with lower egg lecithin content as compared to the formulation containing unimolar composition of drug, cholesterol and egg lecithin (LNE-3).

Size Distribution and Zeta potential

The size-range of all the formulations (LNE-1 to LNE-8) was found to be 158 to 225 nm. The mean vesicle diameter of LNE-3 (unimolar ratio of cholesterol, drug and egg lecithin) formulation was found to be 209.99 nm. Log-Size distribution curves confirmed the normal size distribution of the vesicles. However it was found that uniform size distribution could be obtained in unimolar ratio of pramipexole, Egg lecithin and cholesterol (LNE3) (figure 2.).

As depicted in Table-2, The zeta potential of the pramipexole loaded liposomes (LNE-3) containing unimolar composition of drug, cholesterol and egg lecithin was -24.38 ± 2.25 mV. Liposomes prepared varying (increased) molar ratio of cholesterol demonstrated decreased negativity of the electrostatic charge. The incorporation of cholesterol seems to reduce the repulsive forces. The zeta potential of liposomes prepared with increasing content of drug decreased the negativity of the surface charge.

The zeta potential of liposomes decreased for the formulation (LNE-8 (egg lecithin 11% w/w) (-18.78 ± 5.49); LNE-7 (egg lecithin 20% w/w) (-20.45 ± 6.23) and LNE-3 (egg lecithin 33% w/w) (-23.38 ± 2.25 mV)) prepared with increasing content egg lecithin. Since lecithin (phosphatidylcholine) is negatively charged at pH 7 and it is believed that the electrostatic repulsive forces are the major contributing factor to the force field between lipid vesicular surfaces (Chain 1934). Addition of increased quantity of egg lecithin increased the negativity of the surface charge and hence possible aggregating behavior of liposomes. Since pramipexole is a charged moiety as pH 7.4, the inclusion of charged molecules can be anticipated to decrease the negativity due to interaction between charged moiety and polar head groups (Duangjit et al., 2011). The incorporation of the charge inducing molecules within the vesicles usually occurs spontaneously during the thin film hydration process.

Phospholipid Content Estimation

Phospholipid content of the liposomal formulation prepared with egg lecithin (LNE-1 to LNE-8) was determined and found to be between 50% and 72%. As shown in Figure 3,with increased cholesterol (LNE-1 (48.03 ± 5.99 %), LNE-2 (48.29 ± 2.76 %) and LNE-3 (49.71 ± 1.83 %)) very minor increase in the phospholipid content was determined owing to increasing integrity of phospholipid bilayer. Increasing molar composition, however, did not alter the phospholipid content of the vesicles with maximal phospholipid content (49.71 ± 1.83 %) was estimated in the liposomes containing unimolar ratio of drug, egg lecithin and cholesterol, LNE-3. Liposomes from LNE-4 (47.36 ± 2.47 %), LNE-5 (47.67 ± 2.31 %) and LNE-6 (48.03 ± 6.65 %) demonstrated almost analogous phospholipid content. However, phospholipid incorporated in the vesicles was increased with increasing egg lecithin added during film formation and was found to be 36.69 ± 7.86 % (LNE-8), 41.16 ± 1.82 % (LNE-7) and 49.71 ± 1.83 % (LNE-3) for the liposomes containing 11 % w/w, 20 % w/w and 33 % w/w egg lecithin.

Entrapment Efficiency

Pramipexole entrapment efficiency (%) of liposomal suspension is depicted in Table -2.

Maximum pramipexole loading of 57.65 ± 2.71 % was achieved using unimolar composition of drug, egg lecithin and cholesterol. Decreased entrapment was associated with decreased cholesterol as evident from the entrapment efficiency (%) of 37.61 ± 2.63 from LNE-1 (11 % w/w cholesterol), 43.71 ± 1.37 from LNE-2 (20 % w/w cholesterol) and 57.65 ± 2.71 from LNE-3 from (33 % w/w cholesterol). The drug bearing capacity of liposomes was found to be invariably dependent on drug-egg lecithin-cholesterol ratio employed in the liposomal composition. A considerable enhancement in the pramipexole entrapment was observed with increase in the amount of pramipexole up to unimolar ratio (0.5

M- LNE-4 (61.31 ± 3.32 %); 1 M- LNE-3 (57.65 ± 2.71 %). The increased amount of drug loading of pramipexole with increasing its added concentration during the formulation could be due to the saturation of the media with pramipexole that forces the drug to be encapsulated into liposomes (El-Samaligy 2006). While keeping the amount of cholesterol and egg lecithin, further increase in amount of the drug, however, was found to decreased the entrapped drug (26.70 ± 0.19 % and 18.08 ± 0.78 % entrapment for liposomes prepared with 50 % w/w (LNE-5) and 60 % w/w (LNE-6) respectively). Amount of egg lecithin added during film formation also influenced the drug entrapment with significant decrease in the amount of pramipexole encapsulated with decreasing quantities of egg lecithin. This may be due to the fact that increased number of liposomes and hence higher volume of liposomal core can be prepared from a higher phospholipid concentration, which resulted in the corresponding increase in the entrapment efficiency (Hwang et al., 2012). Drug entrapment of 37.34 ± 3.35 %, 42.91 ± 4.85 % and 57.65 ± 2.71 % was estimated for the vesicles prepared with 11 % w/w (LNE-8), 20 % w/w (LNE-7) and 33 % w/w (LNE-3) respectively. Albeit the pramipexole encapsulation was found to increase with cholesterol addition, increase in the drug above unimolar composition decreased the entrapment. The results revealed that the incorporation of cholesterol enhanced the percent entrapment of pramipexole, owing to its cementing effect on the membrane packing which prevent drug leakage from the bilayer membranes leading to enhanced drug retention in liposomes (Aggarwal 2001).

In-vitro release

A depicted in figure maximum pramipexole release was 80.07± 5.24 and 74.54 ± 7.03% respectively at the end of 6 h (Table 3). Results of an in vitro study of the release of pramipexole from egg lecithin and cholesterol liposomal formulations are shown in Figure 3.The release profiles of pramipexole from liposomes of varying cholesterol contents demonstrated biphasic release with almost 25 % of the entrapped drug was released from LNE-1 (29.55 ± 3.17 %), LNE-2 (24.25 ± 0.93 %) and LNE-3 (23.96 ± 2.86 %) in the first hour. However, during the following 5 h a slow and steady release was observed. This data suggest that increasing cholesterol decrease the drug release from the liposomes. It has been reported that, a highly ordered lipid particles in presence of cholesterol prevents drug loss and its permeation across the liposomal membrane (Wissing 2004). As shown in the Figure 3. liposomal formulations of containing 11 % w/w cholesterol displayed the higher extent of release pramipexole as compared to liposomes prepared with 20 % w/w and 33 % w/w. The percent pramipexole retained after 6 h was 33, 36 and 40 for LNE-1, LNE-2 and LNE-3 respectively. The decrease in the pramipexole release for the formulation with varying cholesterol composition was statistically significant ($P < 0.048$).

However, non-significant difference in the pramipexole release was observed between the liposomal formulations prepared using increasing amount of drug (11 – 50 % w/w). Moreover, no significant difference was observed between LNE-3 and LNE-6 ($P < 0.05$) suggesting the higher concentration of drug estimated in the medium from the vesicles with higher amount of drug ($P = 0.441$). It is therefore implied that the release might be control by the concentration drug which was confirmed by the release kinetics.

A significant effect of egg lecithin content in the percent pramipexole released was noted from LNE-3, LNE-7 and LNE-8 formulations prepared with 33% w/w, 20% w/w and 11 % w/w egg lecithin. Decreased pramipexole release was observed with increasing egg lecithin in the liposomal formulations and was inversely correlated with pramipexole release. In case of unimolar composition of drug, cholesterol and egg lecithin (LNE-3) 60 % of the total entrapped drug was released at the end of 6 h.

Flux of drug release decreased with time for almost all the formulation suggesting higher initial flux is due to higher initial liposomal pramipexole concentration which decrease with time demonstrating decreased flux and hence release. This is well supported by the higher initial flux from the liposomal formulations with higher entrapped drug.

However, flux values were significantly increased for the liposomes prepared with decreasing quantity of egg lecithin and despite of lower drug entrapment from LNE-7 and LNE-8. It is possible that the lipid wall containing cholesterol may be unsaturated in terms of the quantity of egg lecithin required for integration of liposomal wall. These results are in agreement with the findings of Valenta & Janisch (Cladera et al., 2003).

The initial fast rate of release is commonly ascribed to drug detachment from liposomal surface while the later slow release results from sustained drug release from the inner core. Further to determine the factor governing the release rate from the liposome, the release data were analyzed by Zero-, first-order, Higuchi, Peppas, Hixon-crowell and Korsemeyer-Peppas equations to assess the kinetics and mechanism of pramipexole release from the liposomes. Higuchi's model was found to be the best fitting model in almost all the cases suggesting that drug release from the liposomes was driven mainly by a diffusion-controlled mechanism. Exponent (n) values for Korsemeyer-Peppas kinetic model were less than 0.5 indicating the diffusion of the drug is governed by and proportional to drug concentration and entrapped drug suggesting Fickian diffusion The higher initial flux in all the formulation is therefore Fickian diffusion of the drug associated with initial high drug content in the core which decreases subsequently as demonstrated by gradual decrease in the flux. Overall inclusion of cholesterol reduced the release rate and increased the amount of

entrapped drug. The drug entrapment, vesicular morphology and size distribution, drug release suggests the optimal performance for LNE-3.

Stability Evaluation for Drug Leakage

As depicted in Figure 4. drug leakage for liposomal formulations prepared with egg lecithin was significantly affected by storage temperature as lower was estimated at 4 ℃ while higher pramipexole leakage was estimated at 37 ℃ on storage at respective temperature for 10 days. Increasing the cholesterol content decreased the pramipexole leaked over the period of time (LNE-3 (33% w/w cholesterol) (6.97 ± 0.63%; 7.48 ± 1.17% and 10.66 ± 0.95%) as compared to LNE-1 (11% w/w cholesterol) (7.95 ± 1.21%, 9.03 ± 2.21% and 11.84 ± 1.49%) and LNE-2 (20% w/w cholesterol) (7.68 ± 0.80%, 7.94 ± 1.10% and 11.44 ± 0.57%) on day 10 for the formulations stored at 4 ℃, 25 ℃ and 37 ℃ respectively). Since bilayer–drug interaction has been demonstrated to influence the membrane permeability (Glavas-Dodov et al., 2005, Kirby 1984, Chen et al.,2001) liposomal formulations prepared with increasing quantities of pramipexole demonstrated higher drug leakage at all the storage temperatures (LNE-4 (20% w/w pramipexole) (6.52 ± 0.69%; 7.41 ± 0.47% and 9.72 ± 0.48%); LNE-3 (33% w/w pramipexole) (6.97 ± 0.63%; 7.48 ± 1.17% and 10.66 ± 0.95%); LNE-5 (50% w/w pramipexole) (7.06 ± 0.92%; 9.48 ± 2.09% and 11.72 ± 0.81%); LNE-6 (60% w/w pramipexole) (8.18 ± 1.27%; 8.76 ± 0.69% and 13.09 ± 0.35%) on day 10 for the formulations stored at 4 ℃, 25 ℃ and 37 ℃ respectively). The pramipexole leakage was decreased with increasing content of egg lecithin following storage at 4℃, 25℃ and 37℃ for 10 days (LNE-3 (33% w/w egg lecithin) (6.97 ± 0.63%; 7.48 ± 1.17% and 10.66 ± 0.95%); LNE-7 (20% w/w egg lecithin) (6.58 ± 0.64%; 7.79 ± 1.19% and 11.16 ± 0.96%); LNE-8 (11% w/w egg lecithin) (7.50 ± 0.77%; 7.91 ± 1.18% and 12.52 ± 0.43%)) .

Effect of cholesterol, drug and egg lecithin content on drug leakage was positively correlated with the drug entrapment as well as drug release data and was predominantly governed by the amount of drug entrapped in the liposomal core as well as membrane properties affected by varying cholesterol and egg lecithin. The observed decreased leakage could be a result of decreased permeability of the liposomal bilayer membrane prepared with increasing quantities of cholesterol. Cholesterol produces an optimum hydrophobicity that decreases membrane fluidity and formation of the transient hydrophilic holes responsible for drug release through liposomal membranes. It has been reported that the phospholipid bilayer packing geometrical structures have been changed after cholesterol incorporation (Cocera et al., 2003) and increased fluidity at higher storage temperature may enhance the drug leakage as observed. On the other hand, additional report also suggests further increase in cholesterol a certain concentration may increase the release by disrupting the regular linear structure of the vesicular membrane (Duangjit 2011). Previous studies have shown that cholesterol increases the disorder of gel-state phospholipid bilayers by forcing apart the rigid hydrocarbon chains and increase vesicle permeability (Ladbrooke 1968, Senior Judith 1982). This can be due to the fact that the presence of cholesterol in liposomal preparations reduces the leakage or permeability of encapsulating material by decreasing membrane fluidity (Betageri 1993)**.** However, opposite reports are also available in the literature which suggests that in addition to composition, the method preparation of liposome may be an additional determinant for the liposomal characteristics (New, Roger 1990).

CONCLUSION

Liposomes containing varied concentration of Egg lecithin, Cholesterol and Pramipexole were prepared by thin film hydration method. It has been found that amount of egg lecithin, cholesterol and pramipexole incorporated during the liposomal formulation affects the physicochemical characteristics of liposomal formulation. It was also found that vesicle size increased with increase in egg lecithin, cholesterol and pramipexole molar ratio while increased cholesterol exhibited decreased negativity in zeta potential values. Decrease in cholesterol, drug and egg lecithin decreases the percentage entrapment efficiency of the Liposomes. Higuchi's model was found to be the best fitting model in almost all the cases suggesting that drug release from the liposomes was driven mainly by a diffusion-controlled mechanism. However the best suitable characteristics were demonstrated by liposomes of unimolar composition of Egg lecithin, cholesterol and drug.

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