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Research Article

Formulation, In-vitro and Ex-vivo Characterization Study of Frusemide Liposomal Drug Delivery System

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ABSTRACT

Frusemide falls under Biological Classification System (BCS) IV. The aim of the study is to enhance permeation of the drug. In this study frusemide is entrapped by using aqueous solution (phosphate buffer pH 7.4) by the Phospholipid (PL) complex containing ethanol and Tween 80. Formulated liposome was carried out for optimization of the drug loading efficacy by using 12000 to 15000 Molecular Weight cut off of Dialysis Membrane. Central composite design was used to optimize formulation to increase drug loading. Contour plot of drug loading was drawnfrom the drug loading calculation as a primary response factor. Ex-vivo permeation study was carried out of the optimized formulation where inner part of the intestine is exposed towards the donor compartment of Franz diffusion cell. Tween 80 decreases the size of liposomal formulation forming small unilamelar vesicles that forms the most stable nanoliposome suspension. Three of the responded factors was noted in the experiment to degrade the stable product i.e. temperature in storage of extraction and formulation product and size of particles formed. The permeation study showed that there is 1.5 fold increase in flux of frusemide if it is used in liposomal dosage form.

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Introduction

Liquids are easier to swallow and act more quickly than solid dosage forms, Liposome add one more advantage by removing solubility and permeation as a rate limiting factors for bioavailability in the case of BCS class IV drug such as frusemide. It is incompletely absorbed after oral administration to healthy subjects and also in patients with various diseases (Lee and Chiou, 1998). It is a BCS class IV drug that is of low solubility (6 mg/L in urine) and low permeability (log P o/w 1.4) (Chatap *et al*., 2014). Natural lecithin can be obtained from different sources such as egg yolk, soyabean which is the safe and easily available main component of the liposome formulation. Lecithin is safe and edible for several purpose. Smaller liposome (0.1-1 μm) is even presence in the liver and spleen while liposome larger than 3 μm is deposited in the lungs (Liu *et al*., 1991). Lecithin

extraction is complex process including its storage. Ethanol injection is one of the techniques frequently used to produce liposome which favors both simplicity and safety. Formulation of liposome is a dispersed system. Suspensions are heterogeneous system consisting of 2 phases (internal and external). Preparing suspension dosage form is a process in which one substance i.e. dispersed Phase is distributed throughout external phase. Each phase can exist in solid, liquid, or gaseous state. A Pharmaceutical liposomal suspension is a coarse dispersion in which internal phase (active ingredient) in a phospholipid is dispersed uniformly throughout the external phase (phosphate buffer). The internal phase consisting of liposomal particles having a range of size (5 µm to below) which is maintained uniformly throughout the suspending vehicle with aid of single or combination of suspending particles.

Due to the advancement of phospholipid engulfing drug and targeting the specific cell to tissue, formulation of liposomal suspension could catch the eye of researcher toward the tiny technology.

Natural extracts are usually degraded because of oxidation and other chemical reactions before they are delivered to the target site. Freeze-drying has been a standard practice employed to the production of many liposomal products.

The amphiphilic nature of the pharmacosome reduces the interfacial tension. The interactions of surfactants with simpler bio membranes such as phosphatidic acid and phosphatidyl choline liposome were first studied (Deo *et al*., 2007) and then cholesterol and protein were added to make the liposome more realistic. Tween 80 is likely to improve systemic exposure of P-gp substrates after oral administration of certain drug (Zhang *et al*., 2003). During formulation to achieve good physical, chemical and biological stability, we have to concentrate on number of methods according to type of drug delivery, properties of incorporated material etc (Yadav *et al*., 2011). Stability on long term storage is a factor to be taken into account when liposome is to be used as a pharmaceutical dosage form (Crommelin *et al*., 1986).

Liposomes are known to have considerable potential as drug carriers such as liposomal suspension, freeze dried and cream-based systems which are followed in utilization among many other liposomal formulations.

Liposomes are versatile in that the entire membrane of the liposome can be composed of either natural or man-made phospholipids used as carriers for numerous molecules in cosmetic and pharmaceutical industries. For reasons of product stability, the utility of an aqueous suspension of liposome as a marketable product was questionable till last decade but at present drastic economical competition could hold of such nano sized advance drug delivery system. Pharmaceutical science has extensively studied the use of liposome encapsulation to grow delivery systems that can entrap unstable compounds (for E.g. antimicrobials, antioxidants, flavors and bioactive elements) and shield their functionality. The properties of the liposome can be changed entirely depending on the phospholipids used. The basic components of liposome are phospholipids which are stabilized by surfactant, with other stabilizers sometimes added to the mixture depending on the specific use of the liposome. The limitations and benefits of liposome drug carriers lie critically on the interaction of liposome with cells and their target to be achieved after administration.

The primary aim of the research is to formulate and evaluate the liposomal suspension of frusemide. The general objective is to extraction of lecithin from egg yolk, formulate the liposomal suspension, determine the drug loading optimize formulation through central composite (CCD) design and compare the permeability (liposomal vs. aqueous solution).

Material and Methodology

Frusemide was being provided as gift sample from mark formulations Pvt. Ltd. Kathmandu Nepal. Eggs were bought from Prabhat Poultry hatchery, Dhulikhel from which the lecithin was extracted. Tween-80 along with other chemicals was of analytical grade. FTIR was used to detect the API in Nepal Remedies Pvt. Ltd. while zeta sizer was used to determine the zeta potential and size of the particle in Donggukk University, South Korea.

Extraction Procedure

Lecithin is a Phospholipid (PL). Extraction of lecithin is a phytochemical process from an egg yolk. However, heating had a negligible effect on PL extraction (Palacios and Wang, 2005).

Eggs (5) about to 63 gram per piece, was broken and the egg yolk was separated from albumin protein part in a beaker. 75 ml of acetone was added to deoil the yolk part and the mixture is centrifuge at 4 ºC or below up to 4000 rpm (Remi, India) for 5 mins. After cooling centrifugation further acetone is applied to wash the light yellow from the deoiled egg. The crude phospholipid is now extracted using chloroform and ethanol. The ratio of chloroform and ethanol is maintained by 1:4 ratio i.e. 40 ml: 160ml. The minor residue can be further extracted with small amount of chloroform-ethanol solvent. Both the primary and minor residue was left in dark for 3 to 4 hours. Separating funnel was used to separate the extracted lecithin.

Purification was done by adding petroleum ether which solubilizes the lecithin complex. Acetone was added to precipitate the complex. Finally, solvent bath was carried out in the rotary evaporator to evaporated (Buchi Switzerland) to evaporate at 60 ºC

Preparation of aqueous drug solution

100 mg of drug was added to 85 ml of Phosphate buffer pH 7.4 and left to sonication for 10 mins.

Preparation of organic phase

Tween 80 and Lecithin dissolved in 15 ml of absolute ethanol (99.5%).

Method of formulation

Organic phase was injected into aqueous phase using syringe (gauge 22). Then it was sonicated in a bath for 15 minutes. Then the resultant was centrifuged in a cooling centrifuge (Remi, India) at 10000 rpm (0°C). Prepared liposome were stored at (-20) freeze and oxygen-free environment.

Solubility Study

Frusemide with very less solubility index was the major problem for R and D as well as formulation. The aqueous solubility of frusemide at room temperature has been reported to be 0.01825 mg/ ml (Shin and Kim, 2003). The combined release and permeation behavior of frusemide loaded into thermally carbonized mesoporous silicon (TCPSi) micro particles was studied in order to evaluate the potential of TCPSi-loading to improve permeation of frusemide, a BCS class IV compound (Heikkilä *et al*., 2007). The author has stated flux of TCPSi-loaded frusemide across Caco-2 monolayers was over five folds compared to pre-dissolved frusemide. The improved permeation could be confirmed also from dose-corrected (% dose-permeated) results. At pH 6.8 and pH 7.4, where corresponding doses could be used in control solutions, more than fourfold permeability values were obtained with TCPSi-loaded frusemide. Effects on transepithelial electrical resistance (TEER) and mannitol permeability were monitored and suggest that monolayer integrity was not compromised by the drug-loaded TCPSi micro particles. Its aqueous solubility increases as function of the pH of the medium from 0.18 mg/ml at pH 2.3 to 13.36 mg/ml at pH 10. From the curve plotted of Phosphate buffer pH 5.8 the solubility was found to be 1.0878 mg/ml and pH 7.4 was found to be 1.9012 mg /ml. After the feasibility studied saline Phosphate buffer pH 7.4 selected. To prepare saline phosphate buffer pH 7.4, 2.38 gm disodium hydrogen phosphate was dissolved in 800 ml of distilled water in volumetric flask. 0.19 gram of potassium dihydrogen phosphate and 8 gm of sodium chloride was added. Finally diluted to 1000 ml and the pH were adjusted.

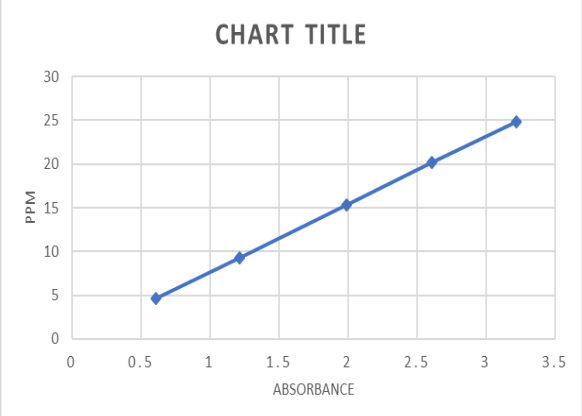

Fig. 1: Calibration curve of Phosphate buffer pH 7.4 (From the absorbance and concentration calibration curve was plotted with intercept, $y = 1.025x - 0.5151$ and $R^2 = 0.9982$)

Azzam, Eisenberg, and Maysinger showed that a conventional polymer micelle of PEG-b-PCL is unstable in serum-containing culture media with

or without cells, but stable in phosphate-buffered saline (Azzam *et al*., 2007).

Drug Loading Capacity

The product stored was thawed. Sample is measured to pour into the dialysis membrane (DM) and tied with non-reacting strings. The DM is partially permeable, having molecular weight cut off between 12,000 and 15,000 with pore size 2.4 nm. Thus, the DM containing liposome acts as donor compartment. And the vessel with phosphate buffer pH 7.4 (250 ml) in which the donor sac was dipped into receiver compartment (Fig. 2)

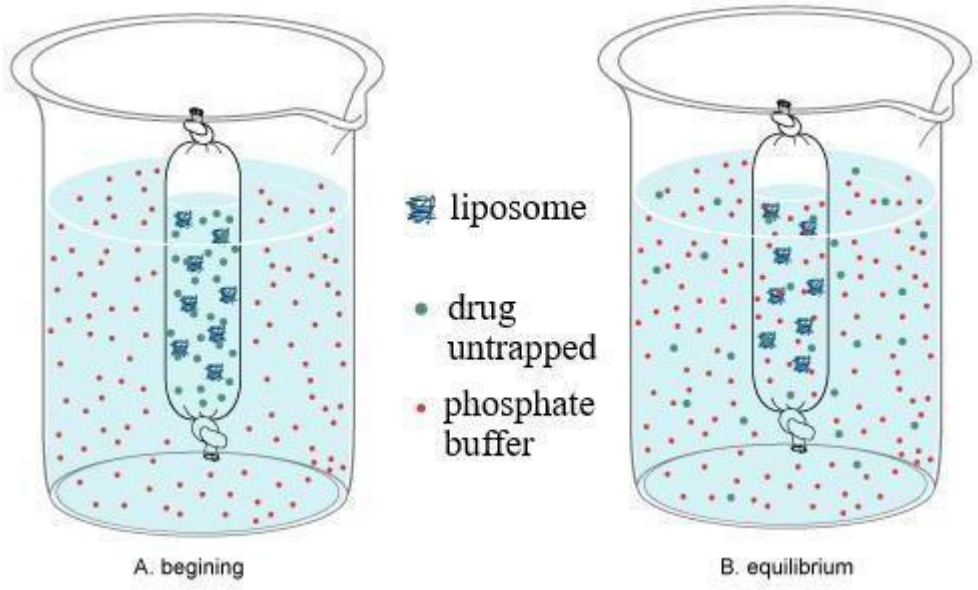

Fig. 2: Drug Loading Capacity

Formulated liposome was carried out for optimization of the drug loading efficacy. Each formulation was comforted to DM. 10 ml of the sedimented particle was frozen in an oxygen free environment and the suspension is poured in the DM. Prepared DM was dipped in the beaker with specific amount of phosphate buffer pH 7.4(250 ml) stirred with magnetic stirrer at 150 rpm. Finally after 24 hour drug loading efficiency was calculated taking the absorbance from receiver compartment with suitable dilution factor.

$W_{lipo} = W_{drug}\text{-}W_{res}$

Drug Loading Efficiency (%) $= W_{lip}/W_{drug} * 100$

Where,

$W_{lipo}$is drug trapped inside liposome

W_{drug} is drug amount used in formulation

W_{res} is residual drug amount left in receiver compartment

Ex-vivo Permeation Study

The lower part of small intestine of goat was washed with phosphate buffer pH 7.4 to act as permeation membrane. Seven stations Franz diffusion cell (Electrolab, India) was used for performing ex-vivo permeation studies. The receiver compartment has the capacity of 5 ml while the donor compartment has also capacity of 5ml. inner part of the intestine is exposed towards the donor compartment. Prepared skin was mounted between donor and receiver compartments of diffusion cell. Then the formulation was positioned over the inside of goat intestine donor compartment. The receiver compartment of the diffusion cell was filled with phosphate buffer (pH 7.4). The media or the receptor fluid was stirred magnetically at a speed of 150 rpm and 2 ml of sample was taken each hour and replaced the same with receptor fluid for 5 hours continuously. The sample was analyzed for drug content at 274 nm UV absorbance.

Particle Size Determination

Optical microscope

Liposome formation in formulation was confirmed observing particles via trinocular microscope (10X) to confirm the shape. (Fig. 5). In expectation of producing the SUV feasible research is carried with varying the tween 80 and lecithin complex. Dilution of the formulation was done with phosphate buffer pH 7.4 and analysis was done through surface shape study.

The Presence of liposome was confirmed using optical microscope (10X) before sending for "zeta sizer analysis". Optimized formulation was chosen on the basis of drug loading and liposome formation.

Zeta sizer

While the optimized formulation was prepared and sent to South Korea which was analyzed by zeta sizer ZS 90 for analysis of specified particle size of liposome and zeta potential? (Figure 6) The loperamide HCl encapsulated liposome had a mean particle size of 103±3 nm and a polydispersity index (PDI) of 0.228±0.075. The low polydispersity (PDI) and small mean particle size is indicator of the size of the majority of the particles in the dispersions. This procedure resulted in high loperamide HCl encapsulation efficiency of >99% (Hua *et al*., 2017).

FTIR (Fourier Transform infrared spectroscopy)

Formulated (optimized liposome was carried out for FTIR study to identify the compound with specified functional group. Advance tool of Agilent technology was used in the range of 5000 to 400 wave number (cm-1). Sample standard and liposome was poured in the booth and the spectrum was recorded. (Fig. 3and 4)

Result and Discussion

FTIR spectroscopy was performed to compare the spectrum of pure drug and liposome complex. The spectra showed the no variation in peak shift. This confirmed the API and found no interaction between drug and phospholipid complex.

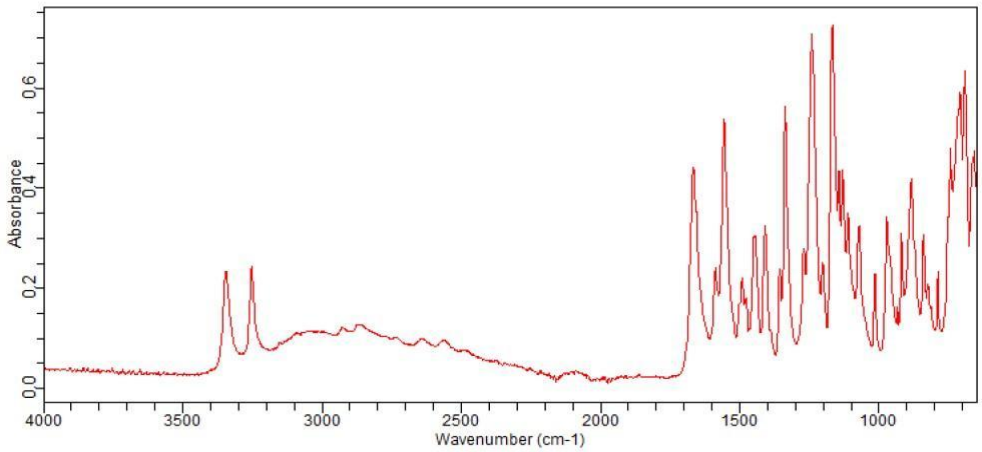

Fig. 3: FTIR of Pure Frusemide

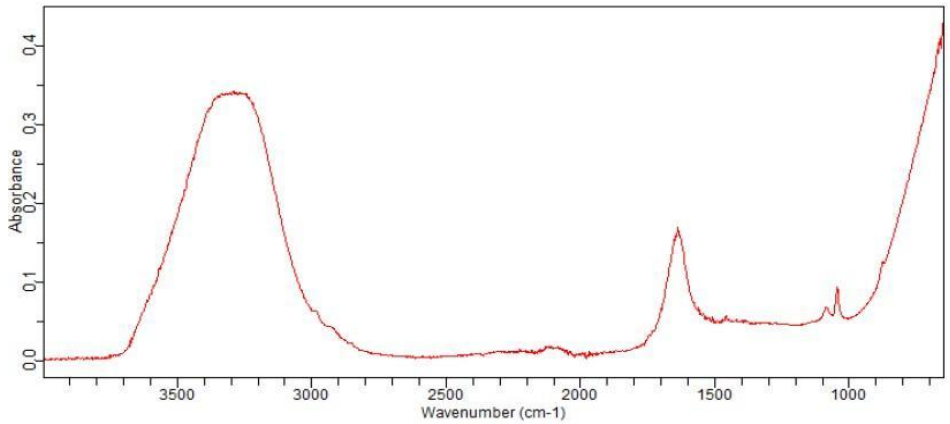

Fig. 4: FTIR of Liposomal Frusemide

The nature of the liposome was found to be sphere overlapped one over another confirmed by the optical microscope. The image of liposome formed is shown in Fig. 5, Microscopic view of liposome has demonstrated that the character of the particle seen through micrometer had spherical. The sample prepared using die i.e. methylene blue was further diluted with 10 times and seen under the optical microscope (10X).

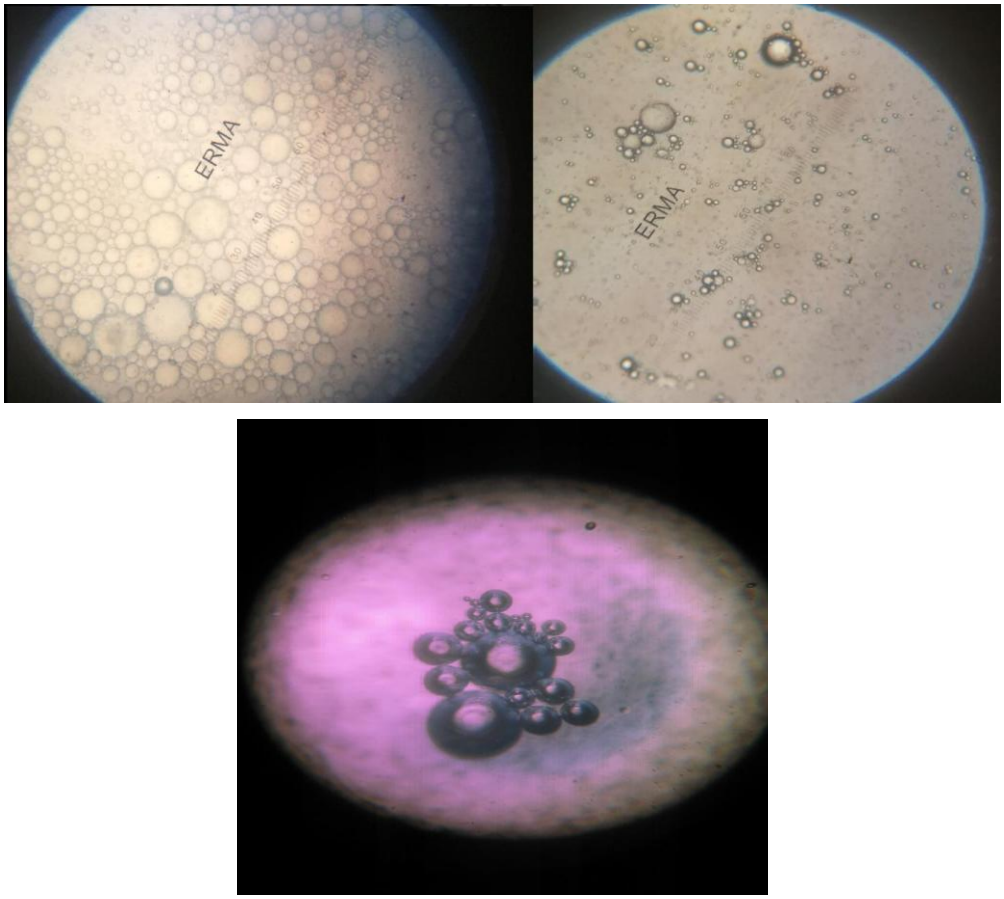

Fig. 5: Microscopic View of Liposome

Records View | Intensity PSD (M) | Zeta Potential (M) | Molecular weight (M) | Autotitration (M) | Aggregation Point (M

Record	Type	Sample Name	Measurement Date and Time	T	Z-Ave	PdI	Aggregation Index	ZP	Mob
				°C	d.nm			mV	µmcm/Vs
79	Size	ashi1 1	Friday, October 05, 2018 1:06:20 AM	25.0	233.4	0.354			
80	Size	ashi1 2	Friday, October 05, 2018 1:09:25 AM	25.0	222.9	0.314			
81	Size	ashi1 3	Friday, October 05, 2018 1:12:30 AM	25.0	256.3	0.453			
82	Zeta	ashi1 1	Friday, October 05, 2018 1:13:35 AM	25.0				-26.5	-2.074
83	Zeta	ashi1 2	Friday, October 05, 2018 1:16:40 AM	25.0				-26.2	-2.054
84	Zeta	ashi1 3	Friday, October 05, 2018 1:17:27 AM	25.0				-25.1	-1.965

Fig. 6: Reported Data from Zeta Sizer

So the zeta potential of the sample was measured and found between -25.1 to -26.5 (Figure 6), while the diameter size of the sample was found to be 237.5333 ± 17.0793nm reported in data. The zeta potential and particle size indicated that the optimized formulation is well dispersed and uniformly distributed.

"So, the evaluation shows two factors responsible for the size of formulated liposome i.e. size of" "needle" used and amount of surfactant used. The use of Tween 80 is to decrease the size of liposomal formulation forming SUV that forms the most stable nanoliposome suspension.

F1 was found to have high drug loading efficiency with the lecithin 4 gm and 2.47 ml Tween-80.

Table1: Formulation Design

Formulation	PL(gm)	Tween-80 (ml)
F1	4	2.47
F2	4	1.2
F3	1	0.3
F4	4	0
F5	0	1.2
F6	8.24	1.2
F7	7	0.3
F8	7	2.1
F9	1	2.1

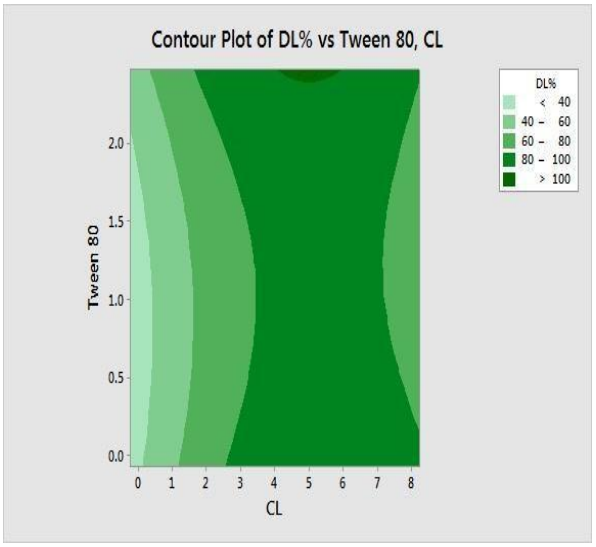

Fig. 7: Contour Plot

Formulation design was prepared by Central composite design (CCD) USING Minitab-15 software. Lecithin (PL) and tween 80 varied to generate the optimized formulation through contour plot (Fig. 7) response of drug loading capacity.

Table 2: Drug loading efficiency (%)

Formulation	*Drug entrapped efficiency*
F1	93.9554 ± 0.1102
F2	83.6450 ± 0.9903
F3	74.6134 ± 0.4099
F4	82.5273 ± 0.2963
F5	2.6217 ± 0.1506
F6	78.6227 ± 0.4570
F7	85.4841 ± 0.3868
F8	82.2981 ± 0.0889
F9	81.4604 ± 0.0347

Contour plot (Figure 7) of drug loading was performed from the drug loading calculation as a primary response factor. From data more than 4 gm lecithin and 2.47 ml tween-80 was obtained. So formulation with increasing lecithin up to 6 gm was performed and was decreased by drug loading efficacy. Afterward while increasing the tween-80 2.5 mg showed decreasing of drug loading capacity. Optimized formulation was formulated with 5 gm of lecithin and 2.47 gm of tween-80 which was found with drug loading efficacy of 94.723 ± 0.3194.

Permeation and Flux

In the study of penetration of Frusemide molecules through inter-compartment barriers in biological systems, the correlation of permeation will be expressed through flux. Permeation study was carried out from the optimized formulation. The cumulative % that is permeated at 5 hours was found to be 5.5569 % and 8.76693% of drug in phosphate buffer pH 7.4 and LSF complex respectively. While the flux of drug in buffer and liposome was found to be 0.021745 mghr-1cm-2 and 0.033988 mghr-1cm-2 respectively. There was 1.56 fold increases in flux of frusemide if it is formulated in liposomal dosage form.

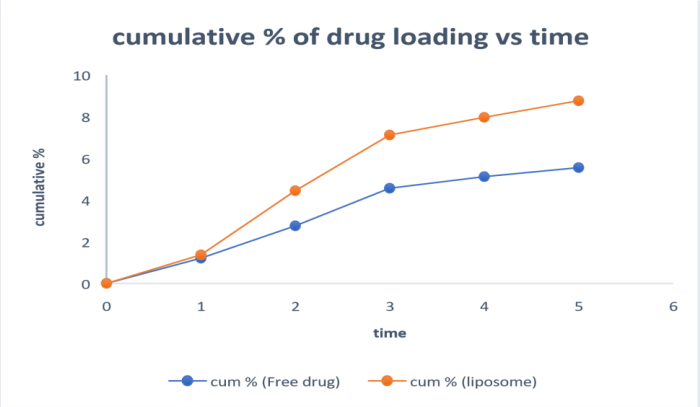

Fig. 8: Cumulative % Permeation of drug loading vs. time

Stability

The major limitation of the research was caught in the stability of the product. Here three of the responded factors was noted in the experiment to degrade the product i.e. temperature in storage of extraction and formulation product and size of particles formed. It was found that lecithin should be stored in freeze condition to prevent the oxidation degradation stopping the movement of particle after extraction. And same after the formulation but the formulation was stored in 0 to 4^{o}C. Freezing the formulation could break the liposomal particles and leaving at room temperature could catch the self-oxidation to degrade the particle. Stability of the particle may vary from the several properties component used and method and formulation. Stability test (Table 3) showed the yield of drug loading with that is stable in respect to time.

Table 3: Stability Test

Time (hour)	Drug content (%)	
	0-4°C	Room temperature
0 hr.	94.723	94.723
12 hrs.	94.716	91.226
24 hrs.	94. 715	89.057
48 hrs.	94.639	64.214

Conclusion

Stability of Lecithin is important for product stability using liposomal dosage form of the drugs whose bioavailability is influenced by permeation can be enhanced and dose can be reduced to achieve the desired therapeutic effect.

Conflict of Interest

Authors declared that there is no conflict of interest regarding this study.

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