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Formulation and *In Vitro* evaluation of liposomal drug delivery system of pregabalin

T Malyadri *, Anjaneyulu Devarakonda, Bollineni Divya, Perla Pravallika SK Mosin, Thatiparthi Vanisri and Medarametla Kishore Babu

QIS Group of Institutions, QIS College of Pharmacy, Prakasam (Dt.), Ongole-A.P.

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Abstract

In this study that Pregabalin was successfully prepared as a liposomal drug delivery system by using two different techniques such as physical dispersion method and ether injection method. In this liposome's preparations, cholesterol ratio was constant and soya lecithin concentrations were gradually increased (like 1:1, 1:2 and 1:3). The liposomes prepared by physical dispersion method showed better percentage drug entrapment when compared with ether injection method. The morphological characters of prepared liposomes were determined with the help of optical microscope. The particle size was analyzed by Malven particle size analyzer. The results of the particle size showed, when the concentration of soya lecithin was increased the size of the particle was reduced. The in vitro release showed that as the concentration of soya lecithin was increased the release rate of drug was retarded. Among the two-methods ether injection method showed prolonged action when compared to physical dispersion method. The stability studies for all the formulations were performed by keeping the formulations at two different temperatures 4ºC±2ºC and 25ºC±2ºC for a period of 30 days. After the stability period the formulations were tested for morphological analysis, percentage drug entrapment and in vitro drug release and compared with before stability study. There was no change in morphological characters at 4ºC±2ºC, but there was a slight reduced in particles size at 25ºC±2ºC. The percentage drug entrapment was reduced in all the formulations at both the conditions. The in vitro drug release was reduced for all the formulations. Liposomes prepared by physical dispersion method showed better stability compared with ether injection method.

Keywords: liposomes; Pregabalin; Drug delivery; *In vitro; In vivo*

1. Introduction

1.1. Novel Drug Delivery System

Novel Drug Delivery system (NDDS) refers to the approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as needed to safely achieve its desired therapeutic effects. NDDS is a system for delivery of drug other than conventional drug delivery system. NDDS is a combination of advance technique and dosage form which are far better than conventional dosage form. The aim of NDDS is to provide a therapeutic amount of drug to the appropriate site in the body to accomplish promptly and then maintain the desired drug concentration. NDDS combining polymer science, pharmaceutics and molecular biology[1].

1.2. LIPOSOMES-An Introduction

Liposomes are colloidal, vesicular structure composed of one or more bilayers surrounding an equal number of aqueous compartment. Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural nontoxic phospholipids. Due to their size and hydrophobic and hydrophilic character (besides biocompatibility),

* Corresponding author: T Malyadri

liposomes are promising systems for drug delivery. The sphere like shell encapsulated a liquid interior which contain substances such as peptides, protein, hormones, enzymes, antibiotics, anti-fungal and anti-cancer agents[2].

1.3. Application of Liposomes

Liposomes for Brain Targeting
Liposome in Eye Disorders
Liposome for Respiratory Drug Delivery System
Liposomes in parasitic diseases and infections
Macrophage activation and vaccination
Liposomes in anticancer therapy
Liposomes in bioengineering
Liposomes in agro-food industry

1.4. Mechanism of Liposome Formation[3]

In aqueous medium, the lipid molecules in self-assembled structures are oriented in such a way that the polar portion of the molecule remains in contact with the polar environment and at the same time shields the non-polar part. Among the amphiphiles used in the drug delivery, viz. soap, detergents, polar lipids, the latter (polar lipids) are often employed to form concentric bilayer structures. However, in aqueous mixtures these molecules are able to form various phases, some of them are stable and others remain in the metastable state. At high concentrations of these polar lipids, liquid-crystalline phases are formed that upon dilution with an excess water can be dispersed into relatively stable colloidal particles.

2. Classification of liposomes[4]

2.1. Liposome classification based on structural features Multilamellar Large Vesicles (MLV)

- Unilamellar vesicles (UV)
- Oligolamellar vesicles (OLV)
- Multivesicular vesicles (MVV)

2.2. Passive loading techniques

In these passive loading technique, the drug is encapsulated by incorporating an aqueous phase of a water-soluble (hydrophilic) drug or an organic phase of a lipid- soluble drug initially or at predetermined stage during the preparation of the liposomes. The huge drug encapsulation efficiency can be achieved with the help of these passive loading technique which is more suitable for lipid-soluble drugs with a high resemblance to the lipid membrane.

Different methods discuss under this class start with a lipid solution in organic solvent and end up with lipid dispersion in water. The a choice of component are typically combined by co-dissolving the lipids in an organic solvent and the organic solvent is then separated by film deposition under vacuum. when residual solvent is removed, the solid lipid mixture is hydrated with the help of aqueous buffer. The lipids spontaneously swell and hydrate to form liposome. Liposomal encapsulation technology (LET) is the latest delivery method used by medical researcher to transmit drugs that act as healing promoters to the definite body organs. LET is state of art method of preparing sub-microscopic bubbles called liposome[5].

2.2.1. Mechanical Dispersion Method

In these method variety components are mainly combined by co-dissolving the lipids in an organic solvent and after that the organic solvent is then separated by film deposition under vaccum. When all the solvent is evaporated, the solid lipid mixture is hydrated using aqueous phase. The lipids spontaneously swell and hydrate to form liposomes[6].

The following are types of mechanical dispersion methods

2.2.2. Lipid film hydration method

The lipid-film hydration procedure is the most common and simple method for preparation of MLV by dissolving the phospholipids in the organic solvents: dichloromethane, chloroform, ethanol and chloroform-methanol mixture (2:1 v/v; 9:1 v/v; 3:1 v/v). A thin and homogeneous lipid film is formed when solvent is evaporated under vacuum at the temperature: 45-60 ºC. Nitrogen gas is involved in order to completely remove the residual solvent. A solution of distilled

water, phosphate buffer, phosphate saline buffer at pH 7.4 and normal saline buffer are used in hydration step. The time for the hydration process varied from 1 h to 2 h at the temperature 60- 70 ºC. In order to obtain full lipid hydration, the liposomal suspension is left overnight at 4 ºC. The lipid-film hydration method can be used for all different kinds of lipid mixtures[7].

2.2.3. Micro-emulsification method

An equipment called as microfluidizer is used to prepare small vesicle from concentrated lipid suspension. The lipids can be introduced into the fluidizer as suspension of large MLVs. This equipment pumps the suspension at very high pressure through the 5 mm screen. Then it is forced long micro channel, which direct two streams of fluid collide together at right angle and very high velocity. The fluid collected can be recycled through the pump and interaction chamber until vesicles of spherical dimension are obtain[8].

2.3. Sonication

Sonication is perhaps the most extensively used method for the preparation of SUV. Here, MLVs are sonicated either with a bath type sonicator or a probe sonicator under a passive atmosphere. The main disadvantages of this method are very low internal volume/encapsulation efficacy, possible degradation of phospholipids and compounds to be encapsulated, elimination of large molecules, metal pollution from probe tip, and presence of MLV along with SUV. There are two sonication techniques[9].

2.3.1. Probe sonication

The tip of a sonicator is directly engrossed into the liposome dispersion. The energy input into lipid dispersion is very high in this method. The coupling of energy at the tip results in local hotness; therefore, the vessel must be engrossed into a water/ice bath. Throughout the sonication up to 1 h, more than 5% of the lipids can be de-esterified. Also, with the probe sonicator, titanium will slough off and pollute the solution[10].

2.3.2. Bath sonication

The liposome dispersion in a cylinder is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method, in contrast to sonication by dispersal directly using the tip. The material being sonicated can be protected in a sterile vessel, dissimilar the probe units, or under an inert atmosphere[11]

2.3.3. French pressure cell

French pressure cell involves the extrusion of MLV through a small orifice. An important feature of the French press vesicle method is that the proteins do not seem to be significantly pretentious during the procedure as they are in sonication. An interesting comment is that French press vesicle appears to recall entrapped solutes significantly longer than SUVs do, produced by sonication or detergent removal. The method involves gentle handling of unstable materials. The method has several advantages over sonication method. The resulting liposomes are rather larger than sonicated SUVs. The drawbacks of the method are that the high temperature is difficult to attain, and the working volumes are comparatively small (about 50 mL as the maximum)[12].

2.3.4. Membrane extrusion

In this method, MLVs is reduced by passing them through a membrane filter of defined bore size. There are two types of membrane filter. The tortuous bath type and the nucleation track type. The former is used for sterile filtration. In this random bath arises between the cross fiber in the matrix. Liposomes that are larger than the channel diameter get struck when one tries to pass them though such membrane. The nucleation track is composed of thin continuous sheet of polycarbonate. They will offer less resistance to passage of liposomes as these consist of straight sided pore holes off exact diameter bored from one side to another. This method can be used to process both LUVs and MLVs[13].

2.3.5. Dried reconstituted vesicles

In DRV method freeze drying of a dispersion of empty SUVs are to be done and then dispersion of it with the aqueous fluid containing the material to be entrapped. This leads to a hydration of solid lipids in finely reduced sized form. Though, the step of freeze-drying is introduced to freeze and lyophilize a performed SUVs dispersion rather than to dry the lipids from an organic solution. This leads to an ordered membrane structure as compared to random matrix structure, which on addition of water can rehydrate, fuse and reseal to form vesicles with a high encapsulation efficiency. The water soluble hydrophilic materials to be entrapped are added to the dispersion which are empty SUVs and they are dried

together, so the material for inclusion is present in the dried precursor lipid before the final step of addition of aqueous medium[14].

2.3.6. Freeze-thawed liposome

SUVs are rapidly frozen and thawed slowly. The short-lived sonication disperses aggregated materials to LUV. The creation of UV is as a result of the fusion of SUV throughout the processes of freezing and thawing. This type of synthesis is strongly inhibited by increasing the phospholipid concentration and by increasing the ionic strength of the medium. The encapsulation efficacies from 20% to 30% were obtained[15].

2.3.7. Solvent Dispersion Method

- Ether injection (solvent vaporization)
- Ethanol injection
- Double emulsion method
- Reverse phase evaporation method
- Stable plural lamellar vesicles

2.3.8. Detergent Removal Method[16]:

In this method the phospholipids are brought into close contact with the aqueous phase via detergents, which associate with phospholipids molecules. The structures formed as a result of this association are known as micelles. They are composed of several hundreds of component molecules. The concentration of detergent in water at which micelles start to form is called CMC. Below CMC the detergent molecule exist in free solution. As the detergent molecule is dissolved in water at concentration higher than the CMC, micelle form in large amounts. As the concentration of detergent added is increased more amount of detergent is incorporated into the bilayer, until a point is reached where conversion from lamellar form to spherical micellar form take place. As detergent concentration is further increased, the micelles are reduced in size.

- Dialysis
- Column Chromatography
- Dilution

ACTIVE LOADING[17]

The exploitation of liposomes as drug delivery system is encouraged with the advancement of well-organized encapsulation procedures. The membrane from the lipid bilayer is in general impermeable to ions and larger hydrophilic molecules. Ions transport can be synchronized by the ionophores though permeation of neutral and weakly hydrophobic molecule can be inhibited by concentration gradients.

A few weak acid or bases yet, can be transported throughout the membrane because of various transmembrane gradient, such as electric, ionic (pH) or specific salt (chemical potential) gradient. Some method exist for improved incorporation of drugs, including remote (active) loading method which load drug molecules into preformed liposome using pH gradient and potential difference across liposomal membrane.

A concentration variation in proton concentration across the membrane of liposomes can drive the loading of amphipathic molecule.

Active loading methods have the following benefit over passive encapsulation Technique

- It will lead to high encapsulation efficiency and capacity.
- Using these method leakage of the encapsulated compounds can be reduced.
- "Bed side" loading of drugs therefore limiting loss of retention of drugs by diffusion, or chemical degradation while storage.
- These process is flexible for constitutive lipid, as drug is loaded after the formation of carrier unit.
- It also reduce the safety hazard by avoiding biologically active compounds in the preparation step during dispersion.
- The transmembrane pH gradient may be occurred by various method. Based upon the nature of drug to be encapsulated[18].

3. Methodology

3.1. Preformulation studies[18]

Preformulation testing is an investigation of physical and chemical properties of a drug substance alone and when combined with excipients. It is the first step in the rational development of dosage form. The objective of Preformulation testing is to generate information useful to the formulation in developing stable and stable and bioavailable dosage forms. The use of Preformulation parameters maximize the chances in formulating an acceptable, safe, efficacious and stable product.

- Solubility
- Melting Point

3.1.1. Drug – excipients interaction studies[19]

FT-IR spectra were taken for the dried samples using FT-IR 8400S (Shimadzu, Japan) to determine the possible interactions between the drug and polymers. The plain drug, individual lecithin and cholesterol, combination of drug with cholesterol and lecithin in three different ratio (1:1, 1:2 and 1:3) were taken and mixed with KBr. The samples were compressed to form a pellet using a hydraulic press. The prepared pellets were transformed into disk. The disk was applied to the center of the sample holding device and scanned from 4,500 to 400 cm-1 using FT-IR spectrophotometer[76].

3.1.2. Formulation of liposomes loaded with Pregabalin hydrochloride

The formulation of liposomes loaded with Pregabalin was prepared by two different techniques namely, physical dispersion method and ether injection method. In both the techniques ratio of cholesterol was kept as same and the lecithin concentration was increased as 1:1, 1:2 and 1:3.

3.1.3. Physical dispersion method[20]

Liposomes were prepared by physical dispersion method using different ratio of soya lecithin and cholesterol was kept as constant. In this method the soya lecithin and cholesterol were dissolved in chloroform. Then it was spread over flat bottom conical flask and allowed to evaporate at room temperature for overnight without disturbing the solution for a formation of lipid film. The drug was dissolved in phosphate buffer pH 6.8. It act as an aqueous medium. Then the aqueous medium was added to the lipid film for hydration. For this the flask was inclined to one side and aqueous medium was introduced down the side of flask and flask was slowly returned to upright orientation. Then the conical flask was kept on water bath and the temperature was maintained at 37± 2ºC for 2 hours for the completion of hydration. The conical flask was gently shaken until the lipid layer was removed from wall of conical flask and formation a liposomes suspension. Then the formed liposomes suspension was stored at 4ºC for one day for the maturation of liposomes. The prepared liposome suspension was centrifuged at 15,000 rpm for 20 mins. Then the precipitate was collected and diluted with distilled water for further studies[35]. Different batches of liposomes were prepared as per the general method described above and composition for the preparation of liposomes is given in Table 5.

3.1.4. Ether injection method[21]

Liposomes were prepared by ether injection method using different ratio of soya lecithin and cholesterol was kept as constant. In this method the cholesterol and soya lecithin were dissolved in ether and methanol. The drug was dissolved in phosphate buffer pH 6.8. It act as an aqueous medium. The aqueous medium was heated to 60°C. The method involves injecting drop by drop of ether-lipid solutions into the above warmed aqueous medium. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes. Then the product was collected and it was stored at 4°C for maturation of liposome. Then prepared liposomal suspension was centrifuged at 15,000 rpm for 20 mins. The precipitate was diluted with distilled water for evaluation studies. Different batches of liposomes were prepared as per the general method described above and composition for the preparation of liposomes is given in Table No. 1.

Table 1 Formulation of Pregabalin liposomes

S. No.	Ingredients	Physical dispersion method			Ether injection method		
		F 1	F 2	F 3	F 4	F 5	F 6
1.	Cholesterol	100 mg	100 mg	100 mg	100 mg	100 mg	100 mg
2.	Lecithin	100 mg	200 mg	300 mg	100 mg	200 mg	300 mg
3.	Pregabalin	10 gm	10 gm	10 gm	10 gm	10 gm	10 gm
4.	Ether	-	-	-	7 ml	7 ml	7 ml
5.	Methanol	-	-	-	3 ml	3 ml	3 ml
6	Chloroform	5 ml	5 ml	5 ml	-	-	-
7.	Phosphate buffer pH 6.8	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml

3.1.5. Evaluation of liposomes22

Determination of percentage drug entrapment efficiency

Drug entrapment efficiency was calculated by using centrifugation method. 10 ml of liposome suspension was taken and centrifuged at 15,000 rpm for 20 mins. The supernatant liquid was collected and suitably diluted. Then the absorbance was taken at 233 nm with the help of UV double beam spectrophotometer using pH 6.8 as a blank.

Morphology analysis

The prepared Pregabalin liposomes for all the formulations were viewed under for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing a drop of liposome dispersion on a glass slide and cover slip was placed over it and this slide was viewed under optical microscope at 40X magnification. Photographs were taken to prepared slides using digital camera[33].

In vitro drug release study:

Apparatus: USP TYPE II (Paddle)

RPM : 50

Temperature: 37 ºC ± 0.5 ºC

Time : 30 min. interval Up to 8 hrs

The *in vitro* release for all the formulated Pregabalin liposomes were carried out for 8 hours in phosphate buffer p H 6.8. The studies were carried in USP dissolution apparatus II (Paddle) at 37ºC ± 0.5ºC and 50 rpm speed. 900 ml of phosphate buffer p H 6.8 was used as a dissolution medium. Equivalent to 100 mg of Pregabalin liposome was taken in a dissolution jar contains dissolution medium and the paddle was rotated at 50 rpm. 1 ml of samples were withdrawn at every 30 min. upto 480 mins and make upto 10 ml with pH 6.8 and analyzed for Pregabalin content at 233 nm with pH 6.8 as blank using double beam UV double beam spectrophotometer[79].

Particle size determination[23]

The particle size determination is done by using Malven particle size analyzer. Groups of particles are dispersed in a liquid medium and measured as they are circulated between the flow cell, which is placed in the measurement unit, and a dispersion bath in the sampler. The dispersion bath incorporates a stirrer and an ultrasonic sonicator. A pump delivers the dispersed suspension to the flow cell. The pump is specially designed to ensure both liquid medium and the particles are circulated. It can be controlled from a PC. Organic solvents can be used as dispersion media.

Stability studies[24, 25]:

The behavior of the liposome to retain the drug was studied by storing the liposome at two different temperature conditions, i.e., 4ºC (refrigerator RF), 25°C±2ºC for a period of 1 month. The liposomal preparations were kept in sealed

vials. At 30th day the samples were analyzed for the drug content following the same method described in % drug encapsulation efficiency and in vitro drug release. And also the liposomes were studied for their morphology.

4. Results and discussion

The research study was aimed to formulate Pregabalin liposomes to sustain the action of drug for over the period of 8 hours. The liposomes were prepared by physical dispersion method and ether injection method. Soya lecithin and cholesterol were used for encapsulating the drug and also to release the drug in sustained manner. Chloroform, ether and methanol were used as a solvent. Phosphate buffer pH 6.8 was used as a hydration medium for loading the drug. Preformulation studies such like solubility analysis, melting point and FT – IR studies were carryout before the formulations. After formulation, the liposomes were evaluated for various parameters like percentage drug entrapment efficiency, microscopic analysis, particle size analysis, *in vitro* drug release studies and stability study.

4.1. Preformulation studies

4.1.1. Solubility

The drug should be dissolve in solvents and also dissolution medium so the solubility analysis for the drug was important. The solubility of raw drug was determined by dissolving in distilled water, methanol and phosphate buffer pH 6.8. The drug was found to be freely soluble in water, soluble in methanol and phosphate buffer p H 6.8.

Melting point

The melting point was confirmed the Pregabalin present in raw material of drug. It was found to be 224ºC within the specification range. So it confirmed Pregabalin present in raw material of drug.

4.1.2. Drug – excipients interaction studies:

The FT – IR studies of pure Pregabalin, cholesterol, soya lecithin and Pregabalin+ cholesterol + soya lecithin were conduct to study the interaction between the drug and excipients.

IR spectral analysis showed that the fundamental peaks and patterns of the spectra were similar both in pure drug and combination containing drug and highest proportion of excipients. This indicated that there was no chemical interaction between Pregabalin and the other excipients used in the formulations. The spectral data are presented in **Table No. 2** and spectral peaks were presented graphically in **Figure No. 1 – 2.**

4.1.3. FT – IR spectrum of pure pregabalin

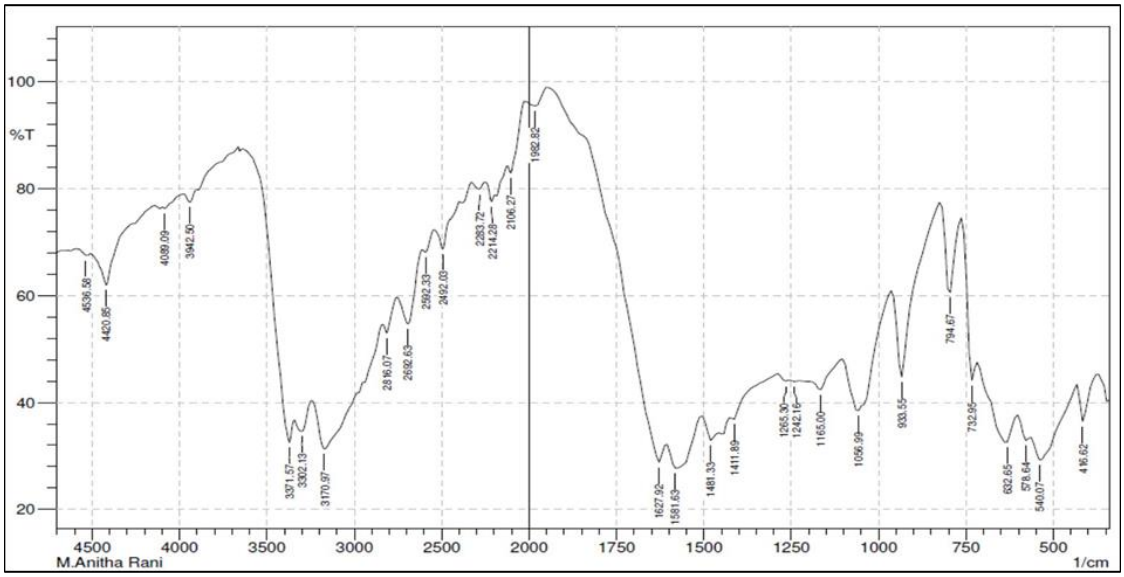

Figure 1 FT – IR Spectrum of pure Pregabalin

Table 2 FT – IR Spectrum of pure Pregabalin

Wave length (cm^{-1})	Functional group
3372	N-H stretching
1582	Amino N-H bending
1466	CH_3 bending alkanes
1057	C-N Stretching
957	Alkene C-H bending

FT – IR SPECTRUM OF COMBINATION OF Pregabalin, CHOLESTEROL AND SOYA LECITHIN

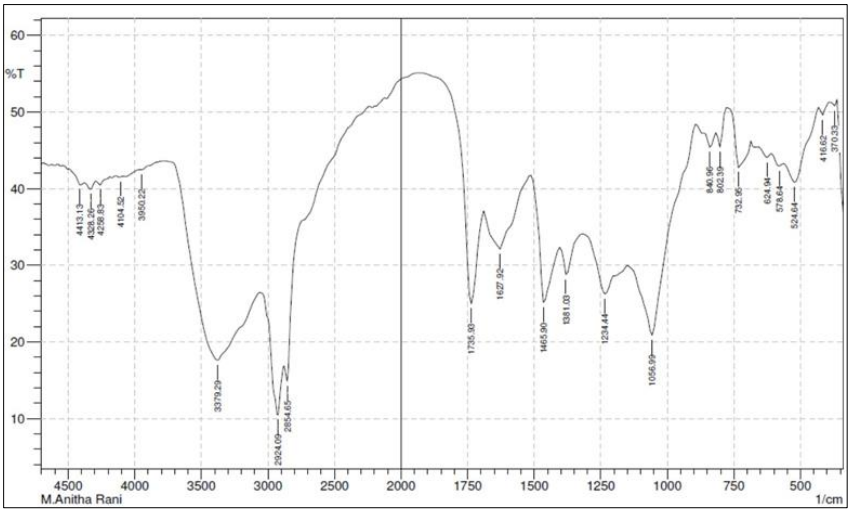

Figure 2 FT – IR SPECTRUM OF COMBINATION OF Pregabalin, CHOLESTEROL AND SOYA LECITHIN

Table 3 FT – IR Spectrum of combination of Pregabalin + cholesterol + soya lecithin

Wave length (cm^{-1})	Functional group
3372	N-H stretching
1582	Amino N-H bending
1466	CH_3 bending alkanes
1057	C-N Stretching
957	Alkene C-H bending

Table 4 FT – IR Spectrum of pure Pregabalin, cholesterol, soya lecithin and combination of Pregabalin + cholesterol + soya lecithin

Functional group	N-H stretching (cm^{-1})	Amino N-H bending (cm^{-1})	CH_3 bending alkanes (cm^{-1})	C-N Stretching (cm^{-1})	Alkene C-H bending (cm^{-1})
Drug	3372	1582	1466	1057	957
Cholesterol	3421	-	1466	1057	955
Soya lecithin	3379	1620	1464	1104	864
Combination of drug + cholesterol + soya lecithin	3372	1582	1466	1057	957

4.1.4. Evaluaton of metormin HCL liposomes

Percentage drug entrapment efficiency

The percentage drug entrapment efficiency of liposomes were prepared by physical dispersion method and ether injection method. The formulations was formulated by varying the cholesterol – soya lecithin ratio. It was found to be that percentage drug entrapment efficiency of formulations F 1, F 2 and F 3 were 86.60 %, 79.90 % and 73.10 % respectively and formulations F 4, F 5 and F 6 were 30.47%, 39.58% and 39.69% respectively. The results may adjudge physical dispersion method have better drug entrapment efficiency than ether injection method.

Morphology analysis

The morphology characters of liposomes were analyzed by optical microscopy (Olympus Opto System, India) and the images were taken using digital camera. The formulation F 1, F 2, F 3, F4, F 5 and F 6 microscopic images were showed in **Figure No. 3.-8** Prepared liposomes F 1 to F 6 shows well identified morphology characters.

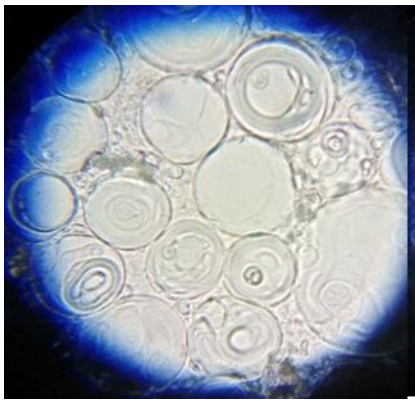

Figure 3 Microscopic image (45 X) of F 1 formulation

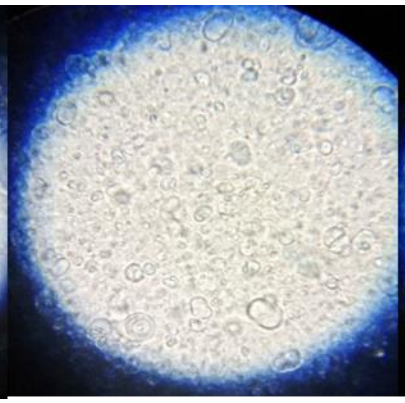

Figure 4 Microscopic image (45 X) of F 2 formulation

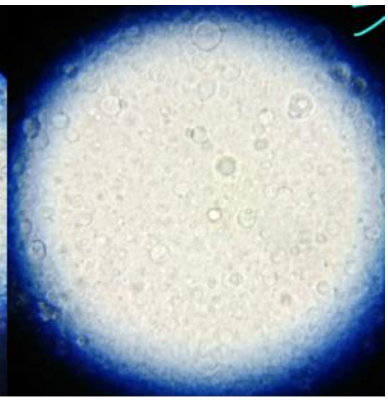

Figure 5 Microscopic image (45x) of F 3 formulation

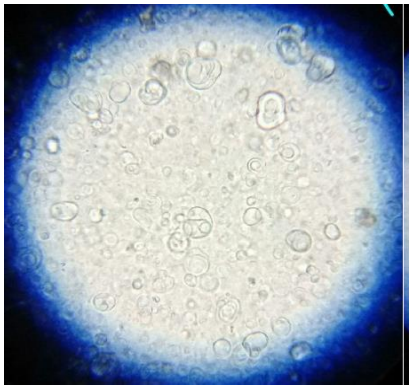

Figure 6 Microscopic image (45x) of F 4 formulation

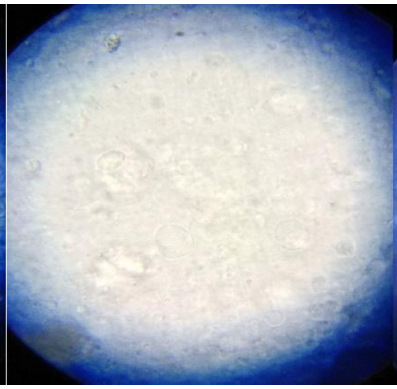

Figure 7 Microscopic image (45x) of F 5 formulation

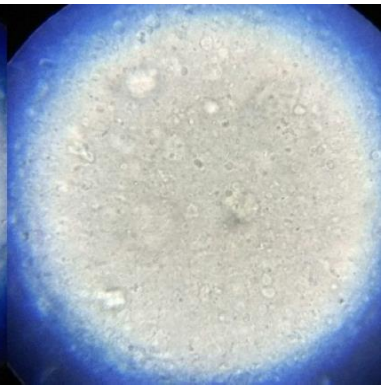

Figure 8 Microscopic image (45x) of F 6 formulation

Particle size analysis

The particle size analysis was carried out by particle size analyzer for all the prepared liposome formulations. The particle size for all the formulated liposomes were found be in the range of 30.617 µm to 0.031µm and graphically showed**.** The particle size data showed that when the concentration of soya lecithin was increased the particle size was decreased for all the formulations of Pregabalin liposomes in prepared by both methods. The particle size of Pregabalin liposomes of F 3 and F 6 were found to be lower when compared with other formulations this may be due to higher concentration of soya lecithin.

Table 5 Particle size of all the formulations of Pregabalin liposomes

S. No.	Formulations	Particle size range
1.	F 1	30.617 – 1.563 µm
2.	F 2	19.023 – 1.563 µm
3.	F 3	0.071 -0.031µm
4.	F 4	24.133 – 1.563 µm
5.	F 5	0.081 – 0.031 µm
6.	F 6	0.071 -0.031µm

In vitro drug release studies

In vitro release studies were performed to evaluate the release of drug from the prepared Pregabalin liposomes. The result of the *in vitro* release studies of all formulation were presented in Table No. 6.

Table 6 Cumulative percentage drug released of Pregabalin from liposomes

S. No	Time (Mins)	F – 1	F - 2	F - 3	F – 4	F - 5	F – 6
1.	30	9.31±0.94	8.92±0.52	8.16±0.63	8.64±0.48	7.53±0.58	2.74±0.33
2.	60	15.76±0.59	12.19±0.61	11.24±0.80	16.63±0.67	13.73±0.37	5.34±0.94
3.	90	24.47±1.13	18.60±1.72	15.84±1.26	24.16±1.28	19.19±0.94	9.79±1.27
4.	120	32.70±2.54	25.22±1.47	20.78±2.42	31.48±1.88	26.48±0.71	15.75±0.57
5.	150	40.48±2.20	30.44±3.18	26.11±2.36	39.54±2.12	33.31±0.48	21.56±0.95
6.	180	45.49±1.85	36.58±3.54	30.60±2.44	47.31±2.30	38.18±0.43	26.58±0.42
7.	210	50.25±1.90	41.20±3.80	35.19±2.47	55.12±2.44	42.81±1.27	30.09±0.97
8.	240	57.43±1.72	47.65±3.87	38.49±2.61	62.44±2.32	47.44±2.58	33.31±1.51
9.	270	65.91±1.45	53.68±3.55	43.26±2.61	69.45±2.12	52.66±1.57	37.28±1.57
10.	300	74.00±3.11	58.45±3.00	46.98±2.38	76.56±1.47	57.29±1.36	42.40±2.03
11.	330	81.77±2.78	63.71±3.21	50.45±2.37	83.81±1.64	61.95±1.91	46.19±2.00
12.	360	88.04±2.81	69.84±3.56	55.85±2.37	91.06±1.57	66.51±1.36	51.41±1.88
13.	390	92.43±2.07	75.66±3.03	62.30±2.37	97.61±1.86	73.10±0.10	57.40±1.75
14.	420	95.83±2.11	80.70±2.63	69.23±2.51	100.58±1.58	79.13±1.61	64.06±1.55
15.	450	99.76±2.02	86.52±3.09	75.36±2.51	-	82.08±1.66	71.34±1.40
16.	480	103.03±2.47	91.92±2.72	82.12±2.51	-	85.06±1.73	79.05±1.03

All the values expressed as mean± standard deviation, n = 3

The Pregabalin liposomes were prepared by physical dispersion method and ether injection method using different ratio of cholesterol and soya lecithin. The cumulative percentage drug release was compared with different formulations. The cumulative percentage drug release of formulations F 1, F 2 and F 3 were found to be 103.03±2.47, 91.92±2.72 and 82.12±2.51 respectively in 8 hours. The formulation F 1 show faster release than formulations F 2 and F 3 due to the lower concentration of soya lecithin. The cumulative percentage drug release of formulations F 4 was found to be 100.58 ± 1.58 at the end of 7 hours. And the cumulative percentage drug release of formulations F 5 and F 6 were found to 85.06±1.73 and 81.39±1.12 respectively in 8 hours. The formulation F 4 show faster release than formulations F 5 and F 6. While the concentration of soya lecithin was increase it decrease the release of drug. The prepared liposomes F 1 to F 6 showed sustained release of drug. When increased ratio of soya lecithin also sustain the

release of drug was increased in both method of preparations. The Figure No. 9 and 10 shows the formulation F 1, F 2 and F 3 and F 4, F 5 and F 6 respectively in 8 hours.

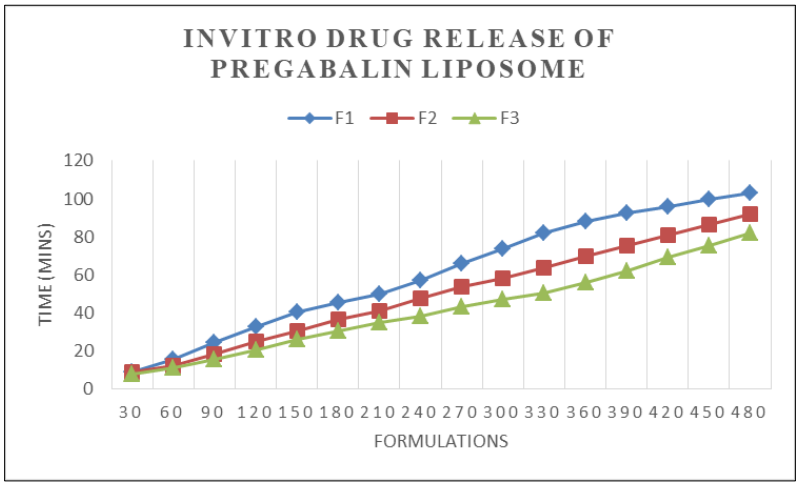

Figure 9 Comparative cumulative percentage drug release of Pregabalin liposome formulations of F 1, F 2 and F 3

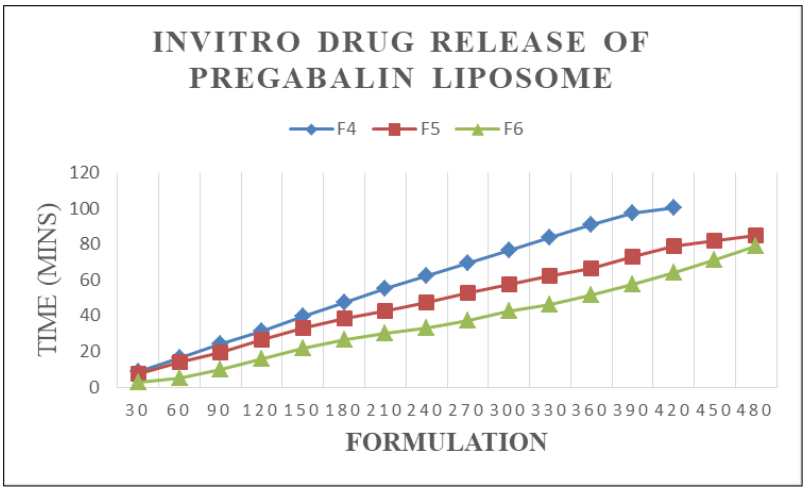

Figure 10 Comparative cumulative percentage drug release of Pregabalin liposome formulations of F4, F5 and F6

4.1.5. Stability studies

Table 7 Stability study of percentage drug entrapment of liposomes Pregabalin liposomes compared with percentage drug entrapment of immediately after preparation

S. No.	Formulations code	Immediately after preparation (%)	After one month	
			At 4°C	At 25°C±2ºC
1.	F 1	86.60	85.92%	76.87%
2.	F 2	79.90 %	77.99%	70.98%
3.	F 3	73.10 %	72.08%	66.89%
4.	F 4	30.47%	29.35%	24.89%
5.	F 5	39.58%	38.44%	35.39%
6.	F 6	39.69%	38.36%	36.69%

All the formulations of Pregabalin liposomes were relatively stable at 4ºC storage condition. The drug leakage percent amounts of original entrapped in liposomes were very small and the amount retained in vesicle had no significant difference after one month as compared to the amount immediately after preparation. But at the storage condition of 25°C±2°C, all the formulations of Pregabalin liposomes were unstable. In addition, the result of drug entrapment studies showed higher leakage at higher temperature. This may be due the higher fluidity of lipid bilayer at higher temperature, resulting into higher drug leakage.

The morphological characters of Pregabalin liposomes for F 1 – F 4 didn't show any characteristic changes after it was stored at 4ºC and 25ºC±2ºC for a period of one month. F 5 and F 6 formulations were showed slightly reduced in the size after it was stored at 25ºC±2ºC for a period of one month but there was no changes for the same formulation when it was stored at 4ºC. Microscopic images of all the formulations (F 1 – F 6) of Pregabalin liposomes were compared with before and after stability studies. After one month, Pregabalin liposomes formulations F 1 to F 6 were showed difference in *in vitro* drug release profile. Dissolution rate was decreased in all Pregabalin liposomes formulations at both storage conditions like 4°C and 25ºC±2ºC. The results of *in vitro* drug release of all the formulations at both storage conditions were compared with before and after stability studies and the results were shown in Table No. 8 and Figure No. 11.

Table 8 *In vitro* drug release data of all the Pregabalin liposome formulations after stability study, compared with before stability

S. No.	Formulation code	Immediately after preparation	After stability study	
			At 4°C	At 25ºC±2ºC
1.	F 1	103.03±2.47	91.81	73.38
2.	F 2	91.92±2.72	86.77	68.26
3.	F 3	82.12±2.51	77.91	64.37
4.	F 4	100.58±1.12	91.74	87.41
5.	F 5	85.06±1.73	78.81	61.81
6.	F 6	79.05±1.03	73.98	63.32

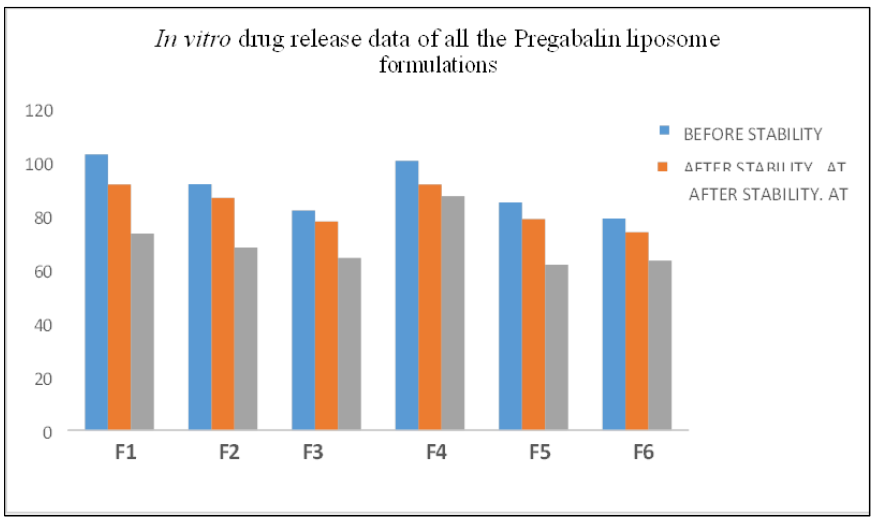

Figure 11 *In vitro* drug release data of all the Pregabalin liposome formulations after stability study, compared with before stability

At storage condition 4°C showed better stability than another condition. This may due to their elevated temperature reduce the stability. But in both storage condition higher proportion of soya lecithin contains formulations like F 3 and F 6 showed better stability than other their formulations.

5. Conclusion

This study concluded that Pregabalin was successfully prepared as a liposomal drug delivery system by using two different techniques such as physical dispersion method and ether injection method. In this liposomes preparations, cholesterol ratio was constant and soya lecithin concentrations were gradually increased (like 1:1, 1:2 and 1:3). The liposomes prepared by physical dispersion method showed better percentage drug entrapment when compared with ether injection method. The morphological characters of prepared liposomes were determined with the help of optical microscope. The particle size was analyzed by Malven particle size analyzer. The results of the particle size showed, when the concentration of soya lecithin was increased the size of the particle was reduced. The *in vitro* release showed that as the concentration of soya lecithin was increased the release rate of drug was retarded. Among the two methods ether injection method showed prolonged action when compared to physical dispersion method. The stability studies for all the formulations were performed by keeping the formulations at two different temperatures 4ºC±2ºC and 25ºC±2ºC for a period of 30 days. After the stability period the formulations were tested for morphological analysis, percentage drug entrapment and *in vitro* drug release and compared with before stability study. There was no change in morphological characters at 4ºC±2ºC, but there was a slight reduced in particles size at 25ºC±2ºC. The percentage drug entrapment was reduced in all the formulations at both the conditions. The *in vitro* drug release was reduced for all the formulations. Liposomes prepared by physical dispersion method showed better stability compared with ether injection method.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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