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Preparation and *in vitro* evaluation of Tacrolimus loaded liposomal vesicles by two methods: A comparative study

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ABSTRACT: Psoriasis is an autoimmune disorder characterized by hyper proliferation of the epidermal layer at the typical body sites, including nails, scalps, cleft and knees. Liposomes are the best carrier for effective delivery of bioactives in the deep skin areas. In the present study, a comparative study of liposomes was carried out using thin film hydration and curvature tuned methods. Liposomes were prepared using cholesterol, soy lecithin and Span 80. The liposomes were evaluated for physical characterization, morphology, surface charge and release properties. Liposomes prepared by the thin film hydration method (formulation TACTL2) had better properties compared to liposomes prepared by the curvature tuned method (formulation TACCT2) in terms of entrapment efficiency (78.5±1.8% *vs* 53.4±5.6%), particle size (411.8 nm *vs* 630 nm), surface morphology(round and dispersed particles *vs* aggregates and lumps), and release profile (70.1% *vs* 32.87% in 12 h). Based on the results it is concluded that the thin film hydration method is better than a curvature tuned method for the preparation of liposomes.

KEYWORDS: Curvature tuned method; film hydration method; liposomes; particle size; psoriasis.

1. INTRODUCTION

Psoriasis is an autoimmune disease caused by the hyper proliferation of the epidermal layer of typical body sites such as nails, scalps, cleft and knees [1-2]. This is the major inflammatory skin disorder recognized by the presence of red or white patches or scales on the skin, which are very painful [3]. The interaction between genetic, and environmental factors are involved in the pathogenesis of psoriasis. However, it is difficult to explore that how the environmental factors break the body balance and affect disease onset and its development [4]. This disease affects the mental and physical quality of the life of the person. The disease is mostly associated with disorders like diabetes mellitus, hypertension, and gastrointestinal diseases, including inflammatory bowel disease, hepatic disease, infection, and mood disorders [5].

For the treatment of skin conditions various nanocarriers have been developed due to their local applications. During the past few years significant attention has been focused on the development of nanocarrier based drug delivery systems [6-10]. The development of unique and targeted therapies of existing drugs is more successful and less risky. These encapsulated systems require lower dose than the existing delivery systems [11]. Liposomal vesicles have shown promising results in topical delivery of bioactives for the effective management of various skin disorders.

Liposomes are the bilayer phospholipids molecules prepared using various fatty acids along with cholesterol and various solvents in the aqueous media. Their size range is varied from 50 nm to several micrometers. Liposomes can entrap both hydrophilic and lipophilic drugs, which make them extraordinary delivery system to deliver the drugs across the skin or dermal layer of the skin [12]. These are effective carrier to deliver hydrophilic and lipophilic drugs into the skin. Liposomes are prepared by several methods, namely, film hydration, ethanol injection, extrusion, bubble, french press, curvature tuned, and reverse phase evaporation. An organic solvent or detergent is used to dissolve phospholipids. Alternatively, high shear homogenization can be carried out to hydrate the phospholipids and generate liposomes. In addition to phospholipids, certain surfactants of the polyoxyethylene monoalkyl ether in combination with cholesterol also used to synthesize stable vesicles [13-17].Stability issues, irreproducibility, sterilization issues, low drug entrapment efficiency, difficult to control particle size, scale up, and rapid clearance of

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vesicles due to non-specific uptake by the cells of reticuloendothelial system are the major limitations of liposome-based drug delivery vesicles [18].

Thin film hydration method is the simplest ways to prepare liposomes. In this method, a superimposed lipid film bilayer forms due to the evaporation of organic solvent or co-solvent in a rotary film evaporator. The lipid film is hydrated with an aqueous solution to form liposomes. Major parameters that affect the vesicle property are angle of evaporation, mass per batch, flask rotation speed and the process of hydration [19].However, thin film hydration method has limited applications for commercial scale production of liposomes due to the limited scale up capacity, and the presence of traces of organic solvent in final product [20].The pH jumping method, known as a curvature tuned method, is based on the phenomenon of spontaneous vesiculation and curvature theory of lipid bilayers to formulate liposomes. This method is relatively fast and solvent-free method for the preparation of stable unilamellar liposomes. However, this method also has certain limitations such as irreproducibility due to uncontrollable manufacturing conditions, low encapsulation efficiency, and potential damage of encapsulated material due to the high energy input [21]. This method is based on the rapid change in pH, which breaks down multilamellar vesicles into unilamellar vesicles.

The aim of the present study is to compare and is to explore the thin film method and curvature tuned method for the preparation of liposomes for the topical delivery of Tacrolimus. To achieve this objective, Tacrolimus loaded liposomes were prepared using both the selected methods. The effect of formulation methods was examined in terms of particle size, polydispersity index, zeta potential, entrapment efficiency, morphological characterization, and *in vitro* drug release profile of Tacrolimus from the prepared liposomes.

2. RESULTS AND DISCUSSION

2.1. Particle size, polydispersity index and zeta potential

The prepared liposomes were characterized in terms of particle size, polydispersity index (PDI) and zeta potential (Table 1). For the transport of drugs to the deeper skin layer, the vesicle size should be in the range of 810 nm to 120 nm. However, the penetration in deep layers of tissues increases with the decrease in vesicle size [22]. It has been reported that the temperature, duration of heating, and pH are major driving forces affecting particle size and their morphological properties [18].In the present study, the processing temperature for thin layer film method and curvature tuned method were 55°C and 60°C, respectively. The pH was maintained at 7.4, and jumped from 7.4 to 11.0 in thin layer film method, and curvature tuned method, respectively. The results indicated that the vesicle size ranged from 411 to 890 nm. The vesicle size was 411.8 nm and 630 nm, respectively, for the liposomes prepared by thin layer film method (formulation TACTL2) and curvature tuned method (formulation TACCT2). The results suggested that the thin layer film method is better that the curvature tuned method in terms of vesicle size.

Table 1. Results of different physical characterization parameters of liposomal preparations.

Formulation code	Weight ratio (SL: CH: SP80)	Particle size (nm)	Polydispersity index (PDI)	Entrapment efficiency (%)
TACTL1	7:3:1	634.8±9.8	0.402	69.1±0.7
TACTL2	8:2:1	411.8±11.3	0.327	78.5±1.8
TACTL3	9:1:1	675.1±2.6	0.385	59.4±0.8
TACCT1	7:3:1	745.2±0.7	0.402	49.5±0.9
TACCT2	8:2:1	630.0±0.8	0.322	53.4±5.6
TACCT3	9:1:1	890.04±12.3	0.510	35.4±0.3

TACTL: liposomes prepared by thin film hydration, and TACCT: liposomes prepared by curvature tuned method

PDI values for all the formulations were in the range of 0.327 to 0.510 (Table 1). The results of PDI of vesicles prepared by the thin layer film method (formulation TACTL2) and vesicles prepared by the curvature tuned method (formulation TACCT2) are shown in Figure 1. Stability of the dispersing particles is influenced by their surface charge. Zeta potential (ζ) is used as the index of surface charge of particles. If zeta potential is high, the particles are stable due to high electrostatic repulsion between particles. On the contrary, a low zeta potential value increases the probability of forming particle aggregates. The zeta potential value of vesicles prepared by the thin layer film method (formulation TACTL2) and vesicles prepared by the curvature tuned method (formulation TACCT2) was found to be -56.76 mV and - 9.83 mV,

respectively (Figure 2). The difference in the parameters such as temperature and pH could be the possible reason for the difference in properties of the liposomes such as particle size, polydispersity index and zeta potential in both the methods. The results suggested that the vesicles prepared by the thin layer film method were more stable than the vesicles prepared by the curvature tuned method. These two formulations were selected for further comparative study.

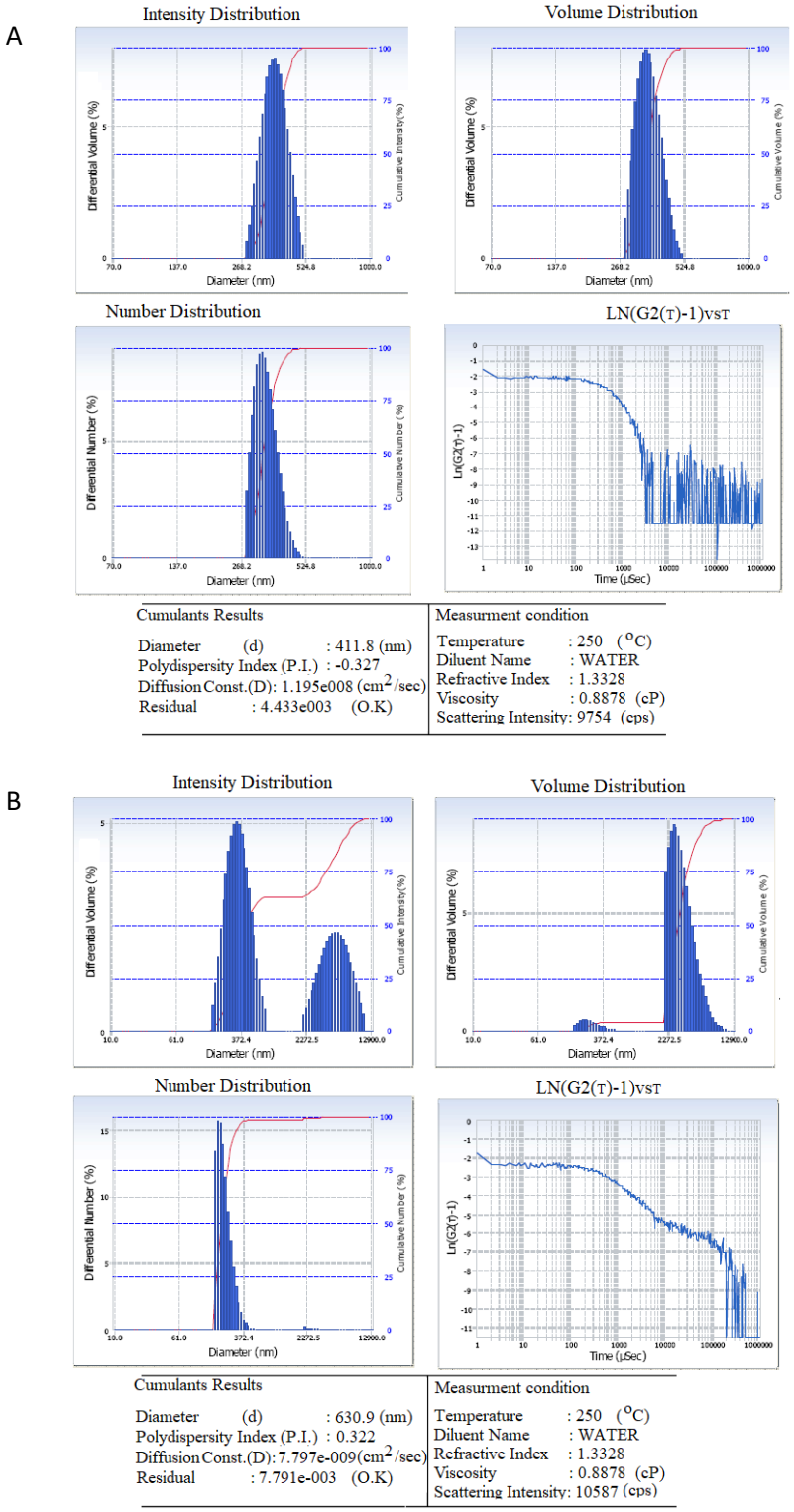

Figure 1. (A) Results of particle size distribution study of the liposomes prepared by the thin film hydration method (formulation TACTL2). (B)Results of particle size distribution study of the liposomes prepared by the curvature tuned method (formulation TACCT2).

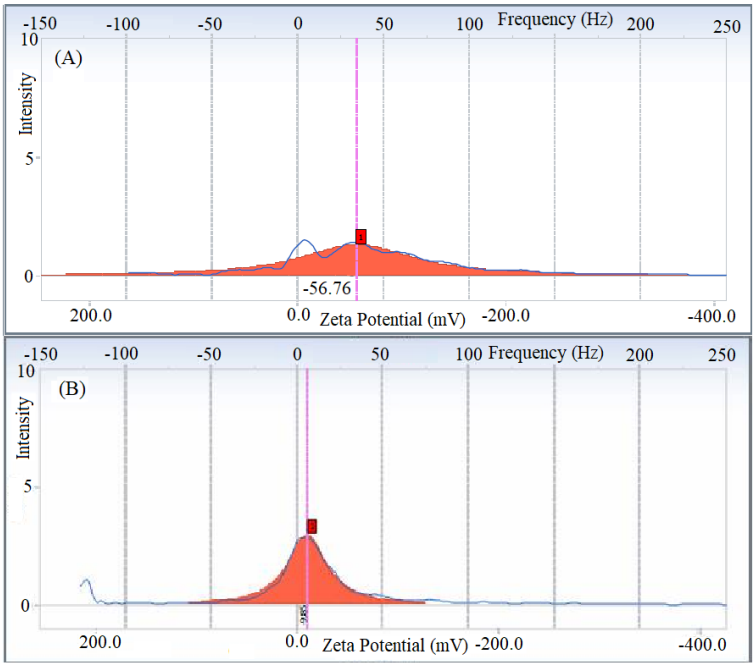

Figure 2. Results of zeta potential study of the liposomes prepared by (A) the thin film hydration method (formulation TACTL2), and (B) the curvature tuned method (formulation TACCT2).

2.2. Entrapment efficiency

The results of entrapment efficiency are shown in Table 1. The cholesterol concentrations significantly affected the entrapment efficiency depending on the method of preparation. Ola *et al*., reported increased entrapment efficiency with an increased concentration of cholesterol due to the enhanced rigidity of layer resulting in a higher stability, reduced permeability of the membrane and greater drug retention [23]. Fang *et al*., observed that the increased cholesterol concentration beyond a certain concentration disrupted bi-layered structure resulting in loss of drug entrapment [24].Entrapment efficiency also depends on other independent variables (hydration time, temperature, preparation method *etc*.) and dependent variables (particle size, morphology, particle aggregation *etc*.). The entrapment efficiency of vesicles prepared by the thin layer film method (formulation TACTL2) and vesicles prepared by the curvature tuned method (formulation TACCT2) was 78.5±1.8% and 53.4±5.6%, respectively, despite of same concentration or ratio of formulation. Tacrolimus is a highly lipophilic compound which leads to the higher entrapment efficiency upon hydration in thin layer film method. In case of the vesicles prepared by the curvature tuned method, this behavior may be explained by the leakage of vesicle membrane due to the particle aggregation, which may facilitate the drug diffusion and thus decrease drug entrapment. This suggested that the thin layer film method is better than the curvature tuned method for the preparation of liposomes.

2.3. Morphology

TEM images were recorded to confirm the formation of liposomes. TEM images confirmed that the liposomes prepared by the thin layer film method (formulation TACTL2) were round and dispersed particles (Figure 3). Whereas the TEM image of liposomes prepared by the curvature tuned method (formulation TACCT2) confirmed formation of aggregates and lumps. These results are also supported from the lower zeta potential values which indicating are latively lower stability of vesicles as compared to those prepared by thin layer film method. Further, the liposomes were prepared at a higher processing temperature (60°C) and pH jump in the curvature tuned method. The higher temperature may increase packing energy of the bilayer and thus promote aggregation of vesicles. The higher temperature and change in pH can also change in the shape of lipid and led to the aggregation [18]. Thus, the results suggested that the thin film hydration method is better than a curvature tuned method for the preparation of liposomal vesicles.

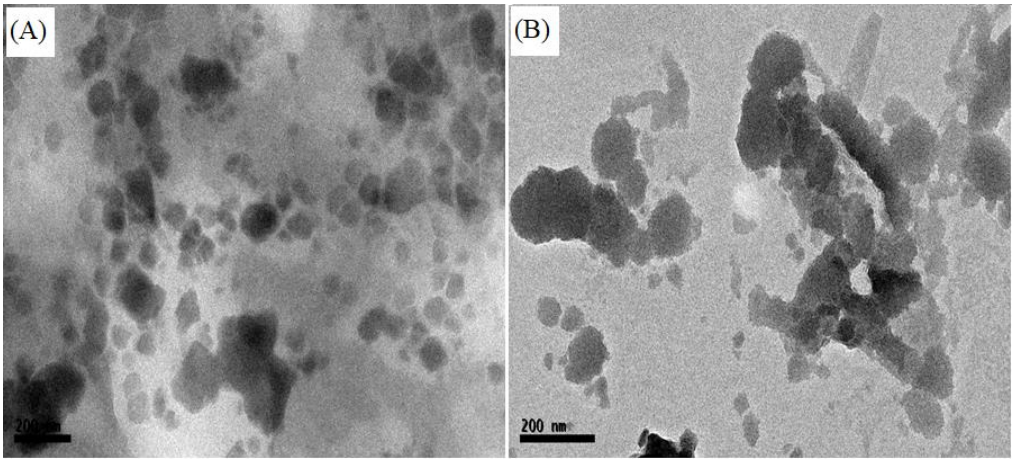

Figure 3. TEM Images of liposomes prepared by thin film hydration method (formulation TACTL2) (A), and curvature tuned method (formulation TACCT2) (B).

2.4. *In vitro* release studies and release kinetics

The benefits of topical liposomal gels are due to the maintenance of desired drug concentration for prolonged times at the site of administration [25]. In the present study, a comparative *in vitro* release profile of Tacrolimus from the liposomal dispersions prepared by both the methods. More sustaining release profile of Tacrolimus was observed from the liposomes prepared by the curvature tuned method. The release of Tacrolimus from vesicles prepared by the thin layer film method (formulation TACTL2) and vesicles prepared by the curvature tuned method (formulation TACCT2) was 70.1% and 32.87%, respectively in 12 h (Figure 4). The difference in release profile could be due to the change in size and morphology of liposomal vesicles prepared by both the methods. In case of the liposomes prepared by curvature tuned method, the formation of particle aggregate increases particle size and thus decreased the effective surface area. This caused a decrease in release rate from the liposomes.

To understand the kinetics governing the drug release profile, the *in vitro* release data was fitted into zero-order and first-order equations. The *in vitro* release data was fitted to the Higuchi's equation to confirm the order of release, which gives steady state drug release. To find out the drug release mechanism, and to verify the fact that whether the process of diffusion is Fickian or non-Fickian, the *in vitro* dissolution data was plotted according to Korsmeyer–Peppas'equation. The linearity of the plots was confirmed by the calculation of correlation coefficient (r^2). From the kinetic data of *in vitro* release profile, it was evident that the drug release kinetics was found to follow Higuchi's. The calculated slope values of Korsmeyer–Peppas' equitation were greater than 0.5 and less than 1, which confirmed the release through diffusion with swelling mechanism (Table 2).

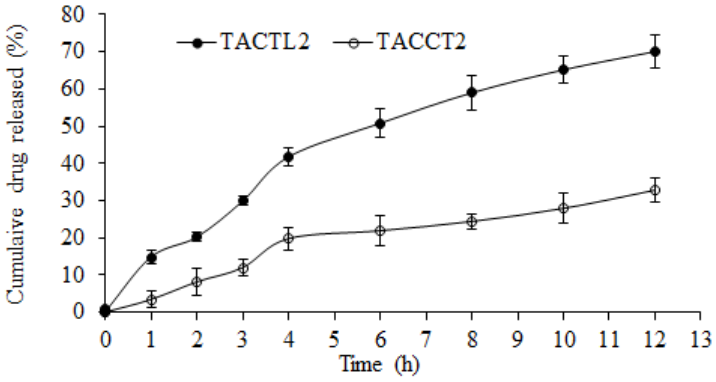

Figure 4. *In vitro* release profiles of Tacrolimus from the liposomes prepared by thin film hydration method (formulation TACTL2) (A), and curvature tuned method (formulation TACCT2) (B) in phosphate buffer, pH 5.5 at 37 ± 0.5°C.

Table 2. Results of kinetic equation parameters for drug release from liposomes prepared by the thin film hydration method (formulation TACTL2), and the curvature tuned method (formulation TACCT2).

Formulation code	Zero order	First order	Higuchi's model	Peppas' model	
	r^2	r^2	r^2	r^2	n
TACTL2	0.9331	0.9915	0.9808	0.9795	0.664
TACCT2	0.9335	0.9892	0.9555	0.9492	0.870

3. CONCLUSIONS

Liposomes are lipid bilayer membrane vesicles which have potential applications in skin targeting of drugs. Various methods have been reported for the preparation of liposomes. Each method has its own merits and demerits. In the present study, we carried out a comparative study of liposomes prepared by thin film hydration and curvature tuned methods. At laboratory scale, the thin film hydration method produced promising results in terms of vesicle size, entrapment efficiency and improved release profile. Thus, the study recommends this method for the preparation of liposomes for the dermal delivery of bioactives.

4. MATERIAL AND METHODS

4.1. Materials

Tacrolimus was obtained from M/s United Biotec Ltd., Baddi, India. Soya lecithin and cholestrol were purchased from Himedia, 23, Vadhani Industrial Estate, L.B.S. Marg, Mumbai - 400 086, India. Span 80 was purchased from Avra Laboratories Pvt. Ltd. Hydrabad, India. Sodium hydroxide, hydrochloric acid, disodium hydrogen phosphate (anhydrous, reagent grade) (Na_2HPO_4), sodium dihydrogen phosphate (anhydrous, extra pure) (NaH_2PO_4), and glycerol (99.5%, reagent grade) were purchased from Sigma-Aldrich Mumbai, India. Milli-Q water (18.2 MΩ cm) was obtained using a Simplicity 185 Millipore-Water System to prepare buffer and liposomes.

4.2. Methods

4.2.1. Thin film hydration method

Soya lecithin (SL), cholesterol (CH), and/or Span 80 (SP80) along with Tacrolimus (TC) were accurately weighed and taken in a nitrogen flushed round-bottom flask containing 500 mL of solvent (chloroform: methanol, 2:1 v/v). The amount of drug (0.1% w/w) and a mass ratio of TC:SL (1:1.5) was kept constant while different SL:CH:SP80 (7:3:1, 8:2:1, 9:1:1, 7:3:2, a total mass of 100 mg) ratios were used to synthesize liposomes. The flask was rotated at 100 rpm. The organic solvent was evaporated using a rotary film evaporator under reduced pressure at 55°C to form a thin film of lipid. The flask was kept under vacuum in a desiccator to ensure complete removal of residual solvents. The obtained thin lipid film was hydrated using phosphate buffer saline (pH 7.4) in 10% v/v ethanolic solution at 55±2°C. The resulting dispersion was kept in bath sonicator and vortexed for 10 min and 5 min, respectively, and kept undisturbed for 2-3 h for complete hydration.

4.2.2. Curvature tuned method

A lipid mixture (as used in thin film hydration method) was hydrated in 10 mL of media (Milli-Q water). The temperature was maintained at 60°C. The mixture was vortexed in a 10 mL falcon tube (with glass beads) for 1-3 min and 6 mL of the buffer solution (pH 7.4) (3% v/v glycerol) was added. Glycerol interacts with the polar head group of lipids and produce antioxidative property. This allows long-term storage of the liposomal formulations [26]. The mixture was stirred for 15 min at constant temperature. The pH was subsequently increased to a maximum (pH 11) using NaOH and adjusted to pH 7.4 using HCl for a fixed time (pH jumping time, Δjt). The resulting mixture was stirred to achieve equilibration (Δeq). Finally, the stirring and heating was stopped, and the mixture was cooled to room temperature. The resultant mixture was centrifuged at 1500 rpm for 15 min to collect liposomes and stored at 4 °C.

4.3. Characterization of liposomes

4.3.1. Determination of particle size, polydispersity index andzeta potential

The mean particle size and poly dispersity index (PDI) were recorded for the liposomes prepared by both methods. Particle size and size distribution of Tacrolimus liposomes were determined by dynamic light

scattering technique. Before analysis, the samples were diluted with millipore water at 1:10 dilution. The zeta potential values were determined using Delsa Nano 4C model of Beckman Coulter, Inc., USA.

4.3.2. Determination of entrapment efficiency

Liposomal formulations were centrifuged at 15,000 rpm for 15 min to obtain pellet. To disrupt the liposomes, the samples were treated with 0.01% Triton X-100 [27]. Methanol (0.5 mL) was added to solubilize the loaded drug. The samples were centrifuged at 10,000 rpm for 7 min. The concentration of the encapsulated drug was determined using HPLC method with Phenomenex C18 column (75 x 3 mm I.D., 2.6 µm). the analysis was performed using mixture of water with 0.1% Triflouroacetic (TFA) acid and acetonitrile with 0.1% v/v TFA with a flow rate of 1mL/min over 7min and detection at 205 nm. The instrument was shimadzu prominence LC 20 AD with lab solution software used to estimate the results. (Instrument details). The entrapment efficiency was calculated using below formula [Eq. 1]:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug taken}} \text{X } 100 \qquad \text{[Eq. 1]}$$

4.3.3. Morphological characterization

Morphological characterization of the prepared liposomes was carried out using transmission electron microscopy (TEM) (JEM2100, JEOL, Japan). The liposomal dispersion was placed on a Formvar® carbon coated copper grid and allowed to dry. The samples were stained with uranyl acetate and dried. The images of dried samples were recorded using Digital Micrograph® software (Gatan, Inc., USA).

4.3.4. In vitro release studies

In vitro release study of Tacrolimus from liposomes was carried out using Franz diffusion cell [28]. Briefly, 1 g of formulation were taken into the donor compartment. The donor compartment was placed in 15.0 mL of release media (phosphate buffer, pH 5.5) and stirred at 100 rpm. The temperature was maintained at 32±0.5°C [25]. Dialyzing medium (1 mL) was withdrawn at predetermined time intervals (0, 1, 2, 3, 4, 6, 8, 10 and 12 h) followed by immediate replacement with fresh media to maintain sink condition and the drug was analyzed by HPLC.

To estimate the precise drug release kinetics from the prepared liposomes the *in vitro* drug release data was analyzed for release kinetics models *viz*. zero order (cumulative amount of drug released against time) and first order (log cumulative percentage of drug remaining against time) models. The drug release mechanism was confirmed using Higuchi's (cumulative percentage drug release against the square root of time) and Korsemeyer-Peppas' (log cumulative percentage of drug release against time) equations [29].

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