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Formulation and Evaluation of Hydrogels Containing Liposomes Entrapped with Tacrolimus

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ABSTRACT

The main aim of the present study was to formulate and evaluate prolonged-release Tacrolimus liposomal gel for the transdermal delivery. Tacrolimus is a potent immunosuppressant used in the treatment of atopic dermatitis or psoriasis. The results indicated that the concentration of cholesterol in the formulations affected the particle size and entrapment efficiency. When the concentration of cholesterol increased particle size was also increased but decrease in entrapment efficiency. The sonication method has no significant effect on the encapsulation efficiency of the liposome. The surface morphology of liposome was spherical. The viscosity of liposomal gel decreases with the increasing rate of shear hence showed with the non-Newtonian flow. The release profile of free Tacrolimus exhibited 100% diffusion in 6 hours, whereas significantly less ($p<0.05$, t-test) amount of drug was released from liposomal gel. Liposomal gel prolonged the drug release for 12 hours. The drug release kinetic showed first-order release kinetics and found to follow the super case II release mechanism. Stability results exhibited no significant change in physical appearance, drug content, and percentage drug release when stored at 4ºC±2ºC. Therefore, Tacrolimus liposomal gel sustained the drug release for a longer duration, hence decreases the number of application of drugs and also improves patient compliance.

INTRODUCTION

Targeting of the drug to particular organs and tissues has become one of the critical challenges of the new century. The search for new drug delivery systems and new modes of action represent one of the frontier areas, includes a multidisciplinary scientific approach to provide major advances in improving therapeutic index and bioavailability at site-specific delivery[1]. The new drug delivery system should ideally fulfill two requirements: Firstly, it should deliver the drug at a rate directed by the needs of our body, until the treatment is completed, secondly; it should channel the drug to the site of action[2]. These new drug delivery systems can reduce solubility problems, protect the drug from the external environment such as photodegradation, pH changes and also reduce dose dumping by controlling the release profile. Such, controlled targeting at the site of action not only reduces the time of exposure at non-targeting tissues and toxicity but also improved patient compliance and convenience[1].

Novel drug delivery systems are new strategies of drug delivery, based on interdisciplinary approaches that combine polymer science, pharmaceutics, bio-conjugate chemistry, technology, and molecular biology. Some of the new drugs require new delivery systems because the traditional systems are inefficient and ineffective, due to this reason new technology and devices are now available in the market. These systems can include physical as well as biochemical mechanisms. Physical mechanisms include osmosis, diffusion, erosion, dissolution, and electron transport, whereas biochemical mechanisms include monoclonal antibodies, dosage frequency, controlling the site of release and maintaining constant drug levels[3].

Introduction to Liposomes:

Liposomes are microscopic vesicles with diameter between 20 nm to 20 µm, which consist of one or more concentric phospholipids bilayers surrounding an aqueous membrane. When phospholipids are dispersed in an aqueous medium, liposomes form spontaneously as a result of the interaction of water and the phospholipids molecules which are amphiphilic, i.e. they possess hydrophilic and hydrophobic regions. This allows a wide range of materials to be incorporated since hydrophilic drugs are entrapped in the aqueous regions and hydrophobic materials are located in the hydrocarbon region. The liposomal system allows for the high accumulation of the drug in the skin, with relatively low permeation flux as compared to the

conventional dosage system. This will no doubt continue to use of the old drug with better and established therapeutic index with minimum side effect.[4,5]

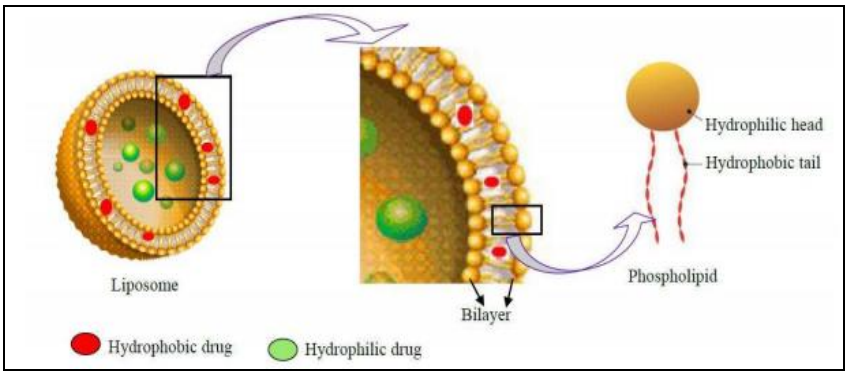

Figure No. 1: Structure of liposome and phospholipids

Introduction to the drug: TACROLIMUS[7-12]

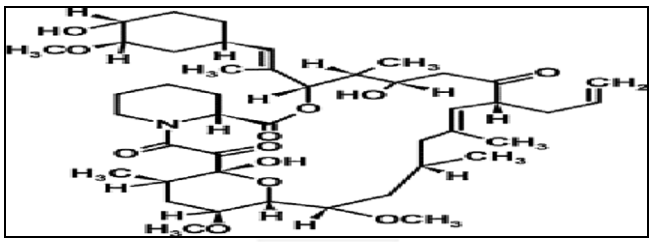

Figure No. 2: Structure of Tacrolimus

Therapeutic category: Immunosuppressant Drug

Macrolide antibiotic.

Calcineurin Inhibitors.

Description: Tacrolimus occurs as white to off white powder.

Solubility: Soluble in methanol, ethanol, acetonitrile, ethyl acetate, diethyl ether, chloroform, dichloromethane, sparingly soluble in hexane, petroleum ether and insoluble in water.

Mechanism of action:

Mechanism of action of tacrolimus includes suppression of the immune system by inhibiting the production of Interleukin-2. Our body has a type of immune response called acquired immune response which creates a memory for the body after an infection from a certain

pathogen so that when the body suffers from the same infection again, it will be able to fight against the infection even more vigorously.

T-cells which are a type of lymphocytes or White Blood Cells (WBC) are quite necessary for the body's acquired immune response and interleukin-2 promotes the development and proliferation of T-cells. Tacrolimus by inhibiting interleukin-2; which is a protein and a cytokine signaling molecule of the immune system, also inhibits the body's immune response. Thus, it helps the body in retaining newly transplanted organs. So, the mechanism of action of tacrolimus is focused on the inhibition of interleukin-2.

MATERIALS AND METHODS

MATERIALS

Table No. 1: List of chemicals used

Sr. No.	Ingredients	Suppliers
1.	Tacrolimus	Yarrow chem products, Mumbai, India.
2.	Phosphatidylcholine	Yarrow chem products, Mumbai, India.
3.	Cholesterol	Thomas Baker chemicals private limited, Mumbai.
4.	Chloroform	S.D. Fine Chem. Ltd, Mumbai, India.
5.	Methanol	S.D. Fine Chem. Ltd, Mumbai, India.
6.	Carbapol 934	S.D. Fine Chem. Ltd, Mumbai, India.
7.	Potassium dihydrogen phosphate	S.D. Fine Chem. Ltd, Mumbai, India.
8.	Sodium hydroxide	S.D. Fine Chem. Ltd, Mumbai, India.
9.	Acetonitrile	Avant or performance materials India Limited.
10.	Triethanolamine	S.D. Fine Chem. Ltd, Mumbai, India.

METHODS

Table No. 2: Excipients used in the preparation of liposomal hydrogel

Sr. No.	Excipient	Function
1	Cholesterol	To prevent leakage of drug formulation
2	Soya lecithin	Penetration enhancer
3	Carbopol	Gel base
4	Methylparaben	Preservatives
5	Propylparaben	Preservatives
6	Chloroform	Solvent
7	Methanol	Solvent

Formulation Design:

Table No. 3: Composition of Tacrolimus liposome by thin-film hydration technique

Sr. No.	Ingredients	Formulation Code					
		F1	F2	F3	F4	F5	F6
1	Tacrolimus (mg)	10	10	10	10	10	10
2	Phosphatidylcholine (mg)	100	100	100	100	100	100
3	Cholesterol (mg)	10	20	30	40	50	60

Table No. 4: Composition of Tacrolimus liposome by sonication technique

Sr. No.	Ingredients	Formulation Code					
		F7	F8	F9	F10	F11	F12
1	Tacrolimus (mg)	10	10	10	10	10	10
2	Phosphatidylcholine (mg)	100	100	100	100	100	100
3	Cholesterol (mg)	10	20	30	40	50	60
4	Sonication Time (mins)	20	20	20	20	20	20

Preparation of Tacrolimus Liposome by Thin film hydration method:

Briefly, drug, Phosphatidylcholine, and cholesterol were dissolved in 10 ml solvent system of chloroform and methanol mixture (2:1, v/v) in a 250 ml round bottom flask. The organic solvent system was removed by using rotary evaporator under reduced pressure to obtain a thin film on the wall of the flask. During the process, the conditions such as speed (150 rpm) and temperature 45º ± 2 ºC. The flask was removed and left overnight in a desiccator under reduced pressure to remove the solvent residuals completely. Then the lipid film was hydrated using phosphate buffer saline pH 7.4 at 60±2 ºC. The resultant suspension was vortexed for about 2 minute and a milky white suspension is formed finally. The suspension is allowed to stand for 2 hours to complete the swelling process. Then the suspension was sonicated using water bath sonicator for about 2 minute[13].

Preparation of Tacrolimus Liposome by Sonication Method:

Lipid mixtures with the same compositions as those used for the thin film hydration techniques were used to form liposomes by sonication. The chloroform-solvated lipid mixtures were dried and placed under vacuum at -45 °C for 3 hours. The dried lipid mixtures

were then hydrated in pH 7.4 buffer solution and sonicated for 20 mins using a bath sonicator. Fresh liposome solutions were prepared before the beginning of each experiment[14].

Preparation of Tacrolimus Liposomal Hydrogel:

The liposomal hydrogel was prepared using 1% Carbopol 934 as a gel base. Briefly, the accurately weighed quantity of Carbopol 934 powder was dispersed into distilled water under constant stirring with a glass rod, and allowed to hydrate for24 hours at room temperature for swelling. Then liposomal hydrogel formulations were prepared by incorporation of liposome's containing Tacrolimus and were mixed into the Carbopol gel with a mechanical stirrer (50 rpm, 5 min). Methylparaben and propylparaben were used as preservatives. The dispersion was neutralized using triethanolamine (0.5%w/w).[15,16]

RESULTS AND DISCUSSION

Tacrolimus loaded liposomes were prepared by two methods i.e. thin-film hydration method and sonication method. A total of 12 batches of liposomes were prepared. In thin-film hydration method ratio of cholesterol and soya lecithin was varied whereas in sonication method concentration as well as sonication time was changed and effect of these parameters on morphology, particle size, zeta potential and entrapment efficacy of liposome was assessed. Then liposome was dispersed in Carbopol 934 gel to form Tacrolimus liposomal hydrogel and subjected to various evaluations.

Pre-formulation studies:

Analytical method determination of Tacrolimus:

Determination of λ_{max}:

UV method was used to determine the λ_{max} of pure drug Tacrolimus. The λmax of the Tacrolimus was found to be 273 nm in concentrated sulphuric acid: acetonitrile solution in 1:25 ratio and same wavelength was used for further studies. The UV spectrum of Tacrolimus is shown in figure 03.

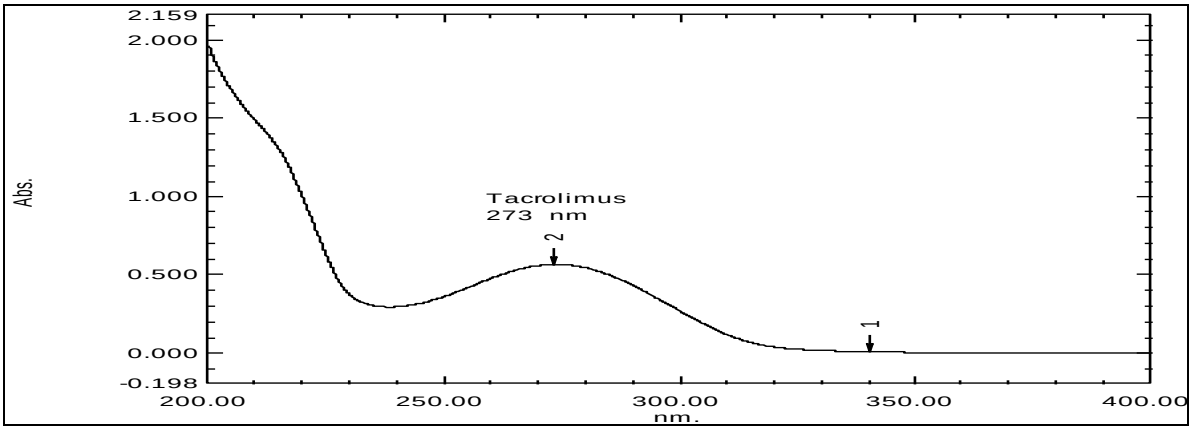

Figure No. 3: UV spectrum of Tacrolimus

Determination of calibration curve

The calibration curve of Tacrolimus was determined by UV spectrophotometer at 273 nm using concentrated sulphuric acid: acetonitrile solution (1:25 ratio) as blank. Tacrolimus pure drug obeyed Beer-Lambert's law in the range of 6-11µg/ml. The calibration curve was obtained by plotting absorbance against drug concentrations (figure 03. and 04). The data were analyzed in MS-Excel-2010. The correlation coefficient (r^2) was found to be 0.997 in concentrated sulphuric acid: acetonitrile solution (1:25 ratio), which indicate linearity.

Table No. 5: Spectrophotometric data of Tacrolimus

Sr. No.	Concentration (µg/ml)	Absorbance at 273 nm			Average	Standard deviation(±SD)
		Trial 1	Trial 2	Trial 3		
1	0	0.000	0.000	0.000	0.000	0.00000
2	6	0.267	0.251	0.266	0.261	0.007318
3	7	0.318	0.306	0.329	0.317	0.009393
4	8	0.364	0.368	0.366	0.366	0.001633
5	9	0.405	0.408	0.401	0.404	0.002867
6	10	0.472	0.467	0.461	0.466	0.004497
7	11	0.513	0.518	0.531	0.520	0.007587

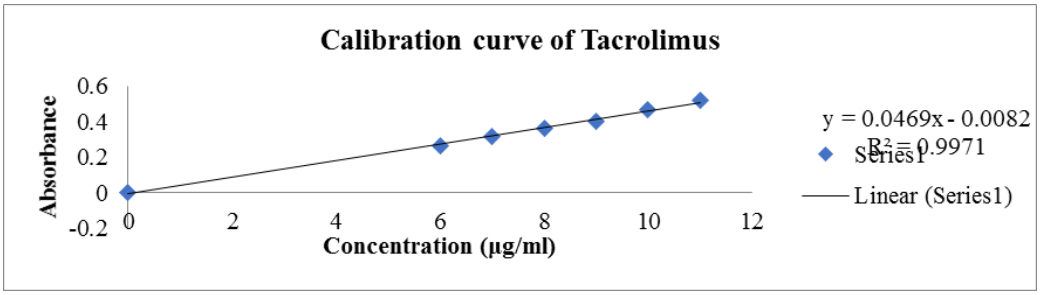

Figure No. 4: Calibration curve of Tacrolimus

Table No. 6: Optical characteristic and statistical data of the proposed method

Parameters	Values
	Concentrated sulphuric acid: acetonitrile solution (1:25 ratio)
λmax (nm)	273
Beer's law limit (µg /ml)	6-11
Slope (b)	0.046
Intercept (a)	0.008
Regression equation; (y= a+bx)	y = 0.046x - 0.008
Correlation coefficient (r^2)	0.997

Solubility profile of Tacrolimus:

The solubility of Tacrolimus was determined by the shake-flask method. One important goal of the preformulation effort is to determine drug solubility and stability in different solvents. The solubility profile of Tacrolimus in different solvents is shown in table 07. Tacrolimus was found to be soluble in concentrated sulphuric acid: acetonitrile solution (1:25 ratio), ethyl acetate, acetonitrile and methanol and practically insoluble in distilled water. The obtained results are in agreement with other researchers, where Tacrolimus is insoluble in water and soluble in ethanol, acetonitrile and ethyl acetate have been reported.[9]

Table No. 7: Solubility profile of Tacrolimus in different solvents

Solvent	Solubility ±SD (mg/ml)	Description term
Distilled water	0.982±0.032	Practically insoluble
Conc. H_2SO_4: acetonitrile (1:25)	47.43±0.261	Soluble
Ethyl acetate	38.93±0.197	Soluble
Acetonitrile	59.11±0.224	Soluble
Ethanol	46.27±0.218	Soluble

Melting point:

In the present work, the melting point was determined by the capillary tube method. The melting point value is one of the physical data routinely used for characterizing a substance in the solid-state, especially in the case of pharmaceuticals, for which the melting point range is often also taken as an estimate of the purity. The melting point observed in our work was found to be 126.0±0.5°C, which is identical to the melting point reported by the Drug Bank database. Other investigators reported 125-130°C as a melting point of tacrolimus[6]. In this temperature range, the solid structure is transformed into a stable liquid without altering the chemical entity of the substance. Hence, the obtained result confirms the purity of the drug.

Determination of partition coefficient

The partition coefficient of the Tacrolimus is found to be Log p=3.46 Sufficient aqueous and lipid solubility, a log P (octanol/ water) between 1 and 3.5 is required for permeate to transverse stratum corneum and an underlying aqueous layer, which means expected improved absorption of Tacrolimus.

Compatibility studies using FTIR

The compatibility of the drug with other excipients was determined using FTIR studies. Infra-red spectrum of drug and polymers were recorded over the KBr disc method and obtained spectra were shown in figure 05-07. The FTIR spectrum of the raw tacrolimus powder shows absorption bands of O-H stretching at 3445.85 cm^{-1}, C=O(ester) stretch at 1741.09 cm^{-1}, C=C stretching vibration at 1451.49 cm^{-1} and the enteric C-O pair of stretches at 1090.22 cm^{-1}. The obtained spectrum showed that there was no major shifting in the frequencies which confirmed that the drug was compatible with all excipients used in the formulation. This further confirms the purity of Tacrolimus. Results of all the IR-spectra are tabulated in table 08.

Table No. 8: Results of the FTIR spectrum of Tacrolimus

Functional group	Observed peaks cm^{-1}		
	Tacrolimus (Pure)	Tacrolimus liposome	Tacrolimus liposomal gel
O-H stretching	3445.85	3444.92	3448.43
C=O(ester) stretch	1741.09	1735.50	1718.97
C=C stretching	1451.49	1457.23	1452.11
etheric C-O (stretch)	1090.22	1088.16	1038.73

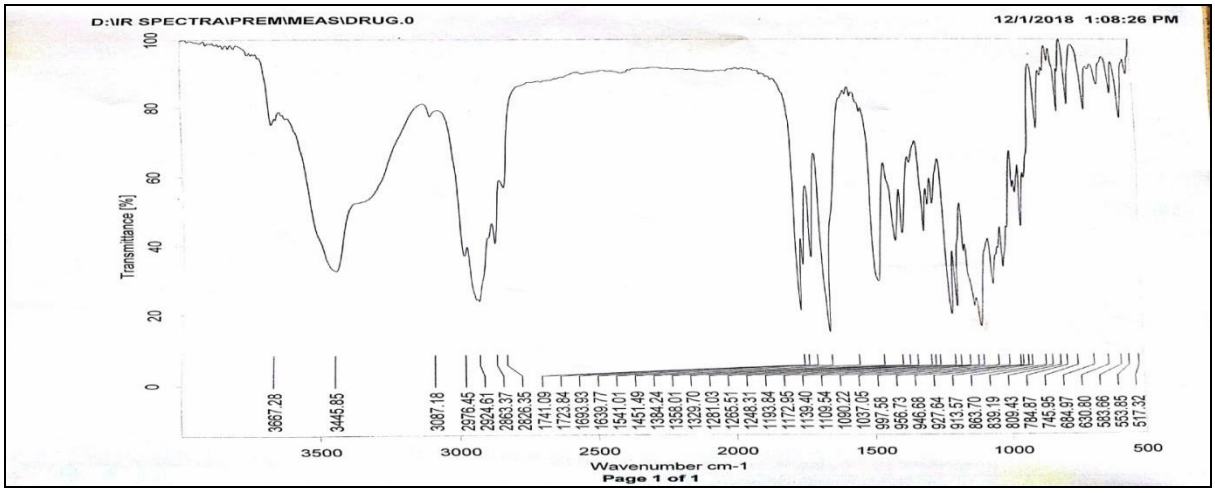

Figure No. 5: Infrared spectrum of Tacrolimus

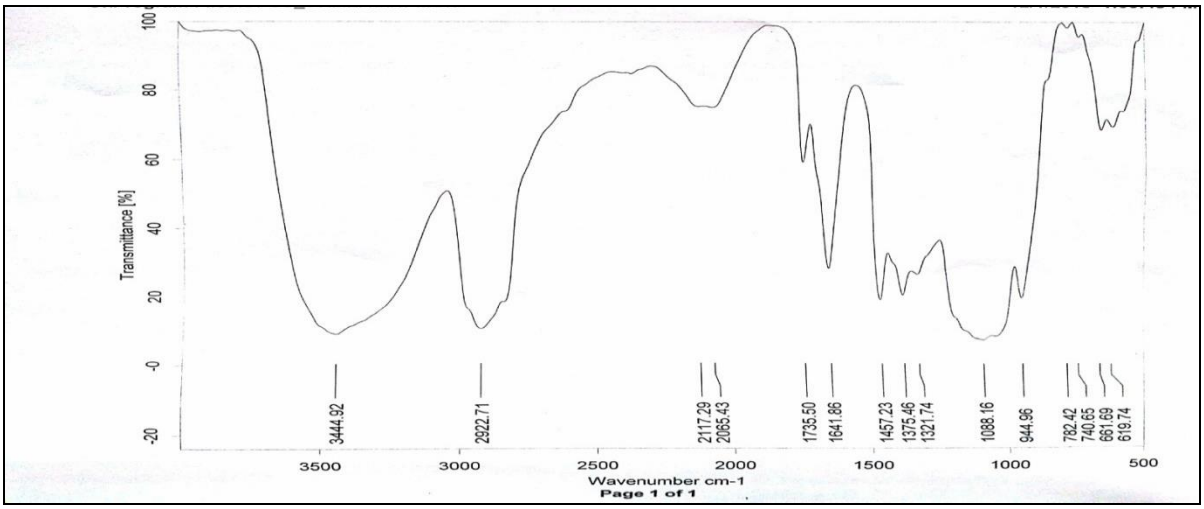

Figure No. 6: Infrared spectrum of polymer

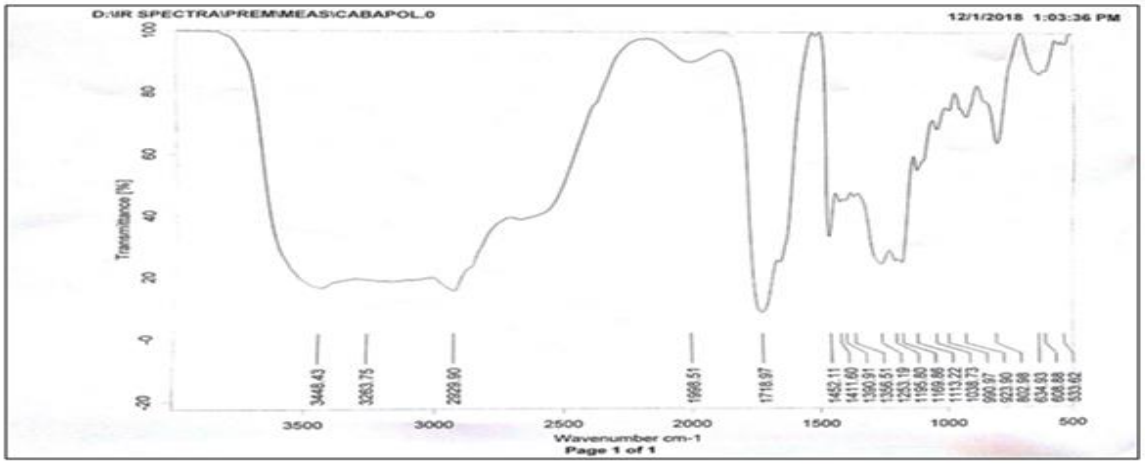

Figure No. 7: Infrared spectrum of Tacrolimus liposomal gel

Characterization of Liposome:

Entrapment Efficiency of Tacrolimus liposome:

The entrapment efficiency of Tacrolimus liposome was carried out using ultracentrifugation method. As shown in Table 09, it was found that the prepared liposome exhibited a good percentage of entrapment (EE %), with values ranging from 53.22±0.36 % to 92.47±0.83 %. This might be due to the lipophilic nature of Tacrolimus molecule; it is incorporated into the liposomal bilayer between the lipophilic chains of the phospholipid's molecules. It was observed that entrapment efficiency decreased with increase in the concentration of cholesterol. Formulation F6 containing lecithin: cholesterol in 1:0.6 ratios showed decreased entrapment efficiency. Cholesterol molecules are placed between the adjacent phospholipids molecules in the liposomal bilayer and hence occupy some space and compete with *Tacrolimus* for incorporation into the bilayer. Additionally, cholesterol makes the bilayer more rigid, which makes the incorporation of the *drug* molecules harder. In this study, 1:0.6 molar ratio of cholesterol to the phospholipids caused a dramatic decrease in encapsulation efficiency, which is speculated to be due to defect in the regular linear structure of the liposomal bilayer. Disruption of the regular linear structure of liposomal bilayer causes a prominent decrease in encapsulation efficiency for both lipophilic and hydrophilic molecules[17]. Formulation F1 showed higher entrapment might be due to a lower concentration of cholesterol. A potential benefit of cholesterol in liposomes is that although the encapsulation efficiency could be decreased, the escape or release of drug molecules from the liposomal membrane could also be decreased due to an increase in membrane rigidity[17]. The mean encapsulation efficiency of the liposomal formulation prepared using a sonication method was essentially similar to the thin-film hydration method. Thus, the sonication methods have no significant impact on the encapsulation efficiency of prepared liposome.[18]

Particle size, zeta potential and polydispersity index:

Tacrolimus loaded liposomes were evaluated for particle size, zeta potential, and polydispersity index. The vesicle size ranged from (125.8 ±1.541 nm) for F7 to (373.6 ±1.526 nm) for F6. The vesicle sizes of liposome were found to be less than 400 nm, and as such are effective for transdermal applications. Particle sizes of liposomes prepared with the thin-film hydration method are larger than sonication method.

Particle size analysis showed that liposome size increased with increase in the concentration of cholesterol. Cholesterol is the most common membrane additives found in liposomes. The addition of cholesterol (a rigid steroid molecule) to the surfactant was required to form stable nonionic surfactant-based vesicles. The results indicated that liposomal size increased linearly with increasing cholesterol concentration in agreement with previous studies.[19]

To explain the increased particle size of liposomes with increasing cholesterol content, it is important to understand the mechanism by which cholesterol is incorporated in the bilayer membrane. Being amphipathic, cholesterol can insert itself into the bilayer membrane with its hydrophilic head oriented towards the aqueous surface and aliphatic chain line up parallel to the hydrocarbon chains in the center of the bilayer. It is known that cholesterol increases the chain order of the liquid-state bilayer and strengthen the non-polar tail of the nonionic surfactant. At low cholesterol concentration, it is feasible to expect that cholesterol would have resulted in close packing of surfactant monomers with increasing curvature and reducing size. However, increasing cholesterol content, with its known lipophilic nature (log P of 7.02), and consequently reducing nonionic surfactant content, would have resulted in increased hydrophobicity of the bilayer membrane and may had imparted disturbance in the vesicular membrane, thus, increasing vesicle radius in a way to establish a more thermodynamic stable form.[19-20]

Particle sizes of liposomes decreased with increase in sonication time i.e. particle size of formulation F1 decreased from 146.9±1.443nm to 125.8±1.541nm after sonication. Sonication is one of the most popular methods used for producing liposomes of known size. The principal effect of sonication is cavitation, which is responsible for many physical effects of ultrasound on lipid membranes. It is well known that ultrasound mechanical waves generate cavitation bubbles in liquids. Bubbles whose size is near the resonant size for the applied frequency begins to oscillate nonlinearly and eventually collapse. As a result of such collapse, a violent implosion occurs that produces extremely high temperatures, high pressures, and shock waves. In work conducted using liposomes, it has been postulated that such ultrasonic high energy randomly and uniformly shatters large liposomes into smaller discoid sections called bilayers phospholipids fragments. These fragments fold up into thermodynamically stable liposomes. Conversely, tiny unstable vesicles, formed during sonication, may fuse to form slightly larger, stable vesicles.[21]

The polydispersity index of the prepared formulation vesicles had values ranging from 0.119to 0.228, showing a homogenous population of the vesicles. It was detected that on increasing the concentration of surfactant, the index of polydispersity decreased.

Surface charge of Tacrolimus liposome was determined using zeta potential analysis. The zeta potential values of all liposomal formulations were found to be negative and in the range of -16.2 mV to -25.18 mV owing to the net charge of the lipid content in the Nano-formulations. In general, charged liposomes were more stable against aggregation and fusion than uncharged liposomes. The magnitude of zeta potential in all prepared formulations is sufficiently high to prevent coagulation and provide stability for the vesicles[21].

Table No. 9: Characterization studies of Tacrolimus liposomes

Formulation	Entrapment Efficiency (%)	Particle size (nm)	PDI	Zeta potential (mV)
F1	92.47±0.83	164.6 ±1.443	0.119	-21.28
F2	86.88± 0.74	178.36 ±0.885	0.178	-25.18
F3	81.08± 0.81	198.61 ±1.506	0.221	-16.20
F4	72.70± 0.73	246.83 ±0.978	0.225	-19.25
F5	64.38± 0.56	312.03 ±0.863	0.228	-24.18
F6	56.24±0.86	373.6 ±1.526	0.223	-20.93
F7	90.11±0.27	125.8 ±1.541	0.226	-18.88
F8	83.82± 0.54	160.75±0.985	0.198	-17.92
F9	79.11± 0.75	173.47±0.899	0.209	-20.33
F10	71.09± 0.63	214.44 ±1.174	0.226	-23.51
F11	62.91± 0.46	293.51 ±1.332	0.219	-16.95
F12	53.22±0.33	347.83 ±1.118	0.224	-19.92

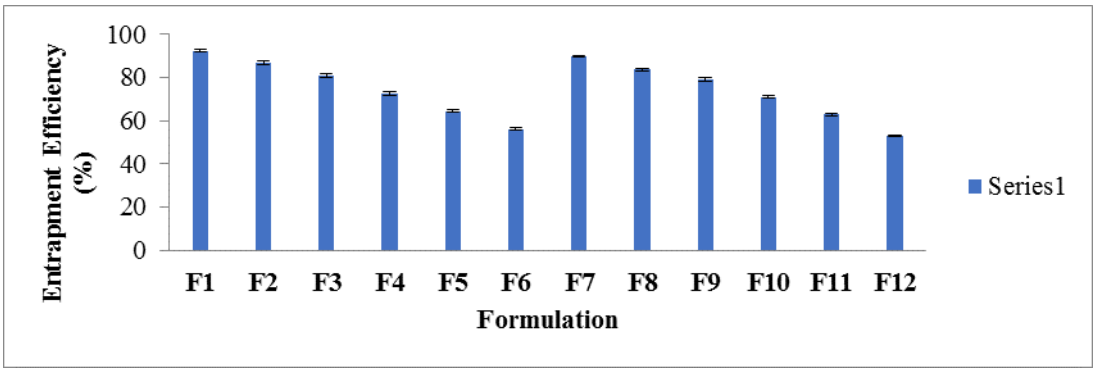

Figure No. 8: Comparative entrapment efficiency profile of liposome formulations

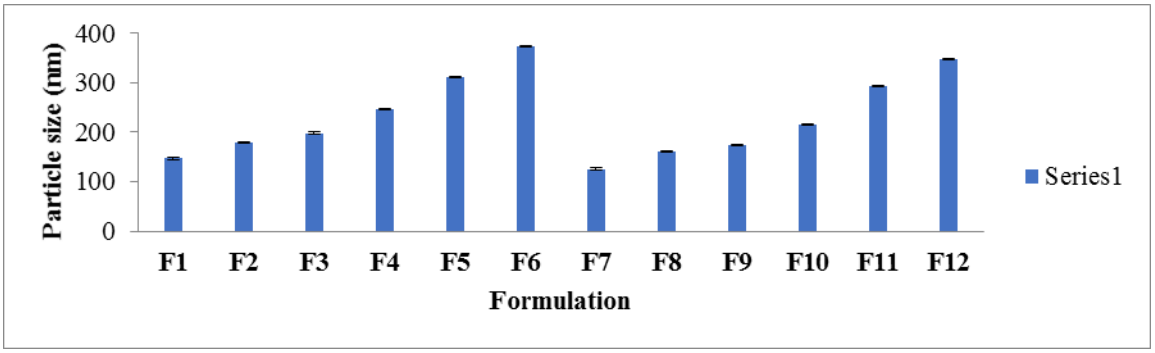

Figure No. 9: Comparative particle size of different liposome formulations

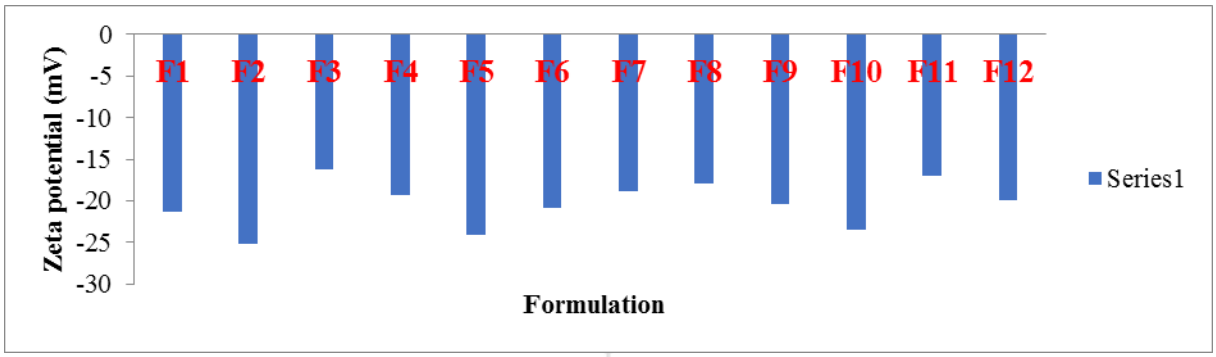

Figure No. 10: Comparative zeta potential of different liposome formulations

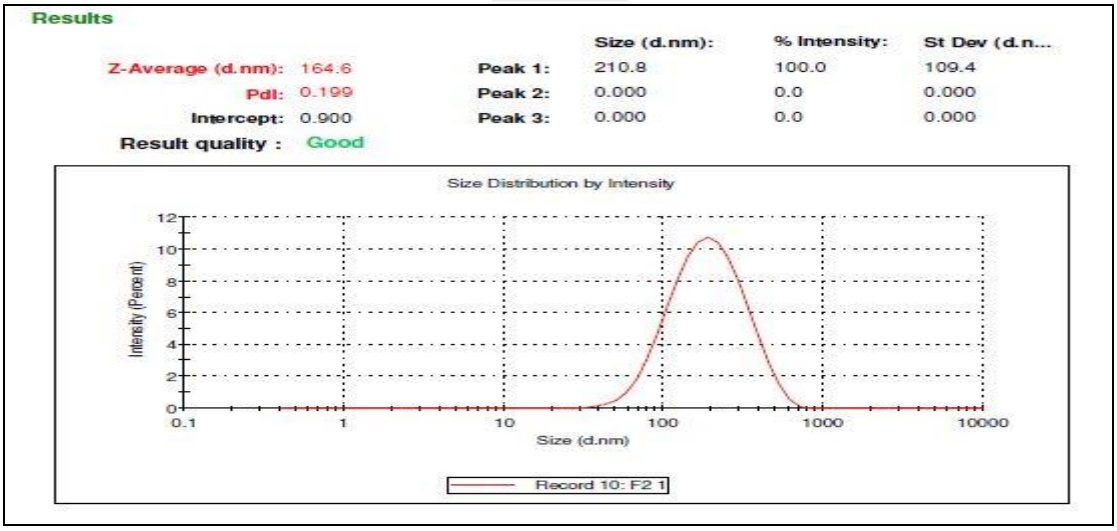

Figure No. 11: Typical particle size distribution of Tacrolimus liposome

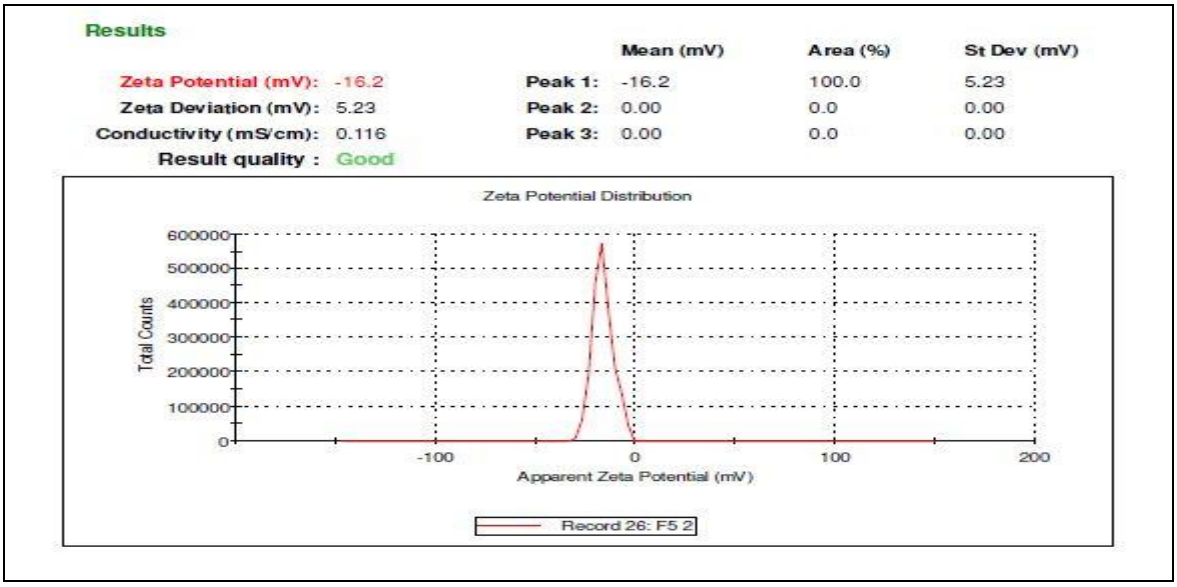

Figure No. 12: Zeta potential analysis of Tacrolimus liposome

Surface morphology of liposome:

The morphology of the liposome vesicles was assessed by optical microscope and scanning electron microscopy for justifying the vesicular characteristics. Tacrolimus-loaded liposome were spherical vesicles with uniform size distribution In SEM image analysis, the surface morphology of all liposome samples showed porous structures which can lead to fast reconstitution.

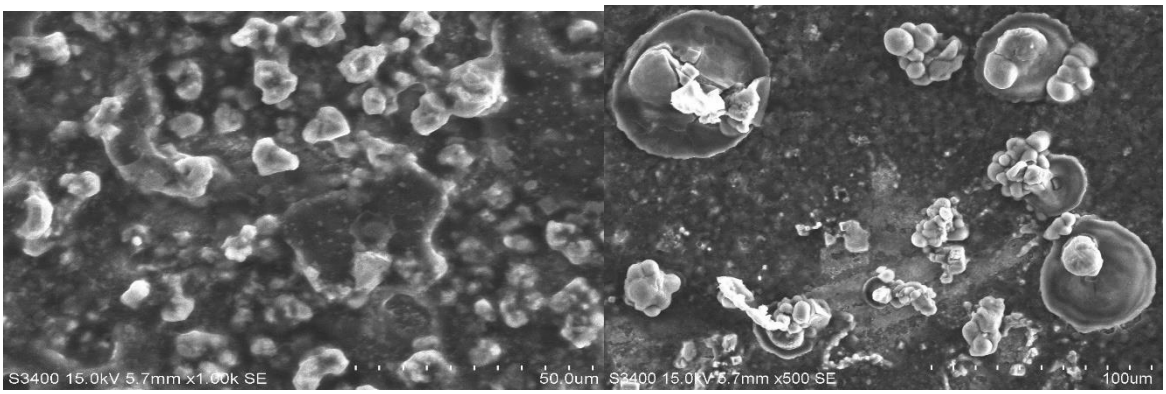

Figure No. 13: Scanning electron microscopy of Tacrolimus liposome

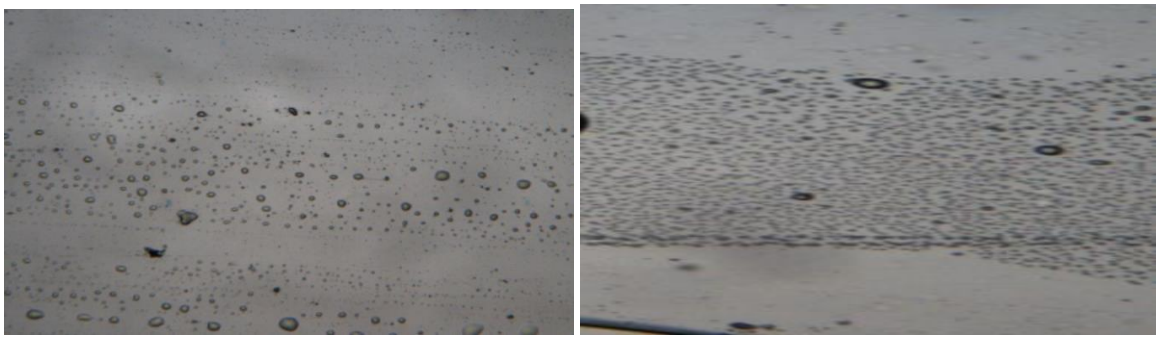

Figure No. 14: Optical microscopy of an optimized batch of Tacrolimus liposome

Evaluation of Tacrolimus liposomal gel:

Colour and Homogeneity:

The Tacrolimus liposomal gel formulation has a smooth texture and appeared to be light yellow. A visual test for homogeneity of the drug product may be useful, at least for an exhibit batch, to ensure no separation of phases, no syneresis, and no foreign matter. As all batches possess the same concentration of carbopol (1%), all batches of gel formulations were homogenous with no sign of grittiness. Results of homogeneity of Tacrolimus liposomal gel is depicted in table 15.

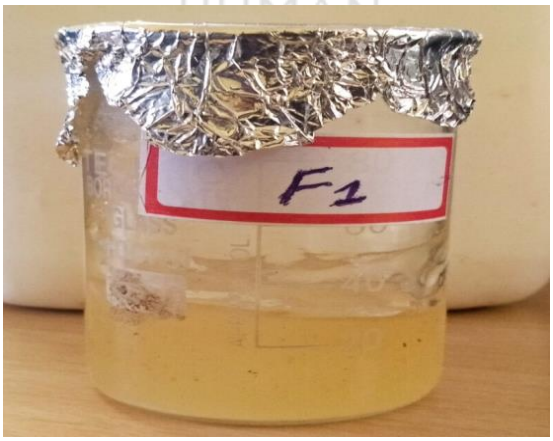

Figure No. 15: Tacrolimus liposomal gel

pH determination:

The pH of gel formulation was determined by using a calibrated pH meter. The readings were taken for an average of three samples. The pH of all developed formulations was in the range

of 5.3±0.15 to 6.2±0.21, which fall in the normal pH range of the skin. The pH of the skin is 5.4.[120]Results of pH of Tacrolimus liposomal gel is described in table 10.

Spreadability Test:

Spreadability data indicated that the liposomal gel formulations are easily spreadable. The therapeutic efficacy of gels depends on its spread. Spreadability of Carbopol based liposomal gel was found in the range of 9.41-13.88 g.cm/s. The gel spreading helps in the uniform application of the gel to the skin, so the prepared gels must have a good spreadability and satisfy the ideal quality in topical application. Furthermore, this is considered important factors in patient compliance with treatment. Results of spreadability of are described in table 10.

Drug content analysis:

The drug content estimation was determined using UV spectrophotometer. Drug content of all the formulations was found between 94.35±0.79to 98.52±0.86% w/w, which represents uniformity in drug content. Results of drug content estimation are depicted in table 10.

Table No. 10: Evaluation of Tacrolimus liposomal gel

Formulation	Colour	Homogeneity	pH	Spreadability g.cm/sec	Drug content (%w/w)
F1	Light yellow	Smooth	5.35±0.64	10.10±0.63	98.27±0.63
F2	Light yellow	Smooth	5.43±0.55	10.39±0.74	98.52±0.86
F3	Light yellow	Smooth	5.85±0.39	9.41±0.86	96.80±0.57
F4	Light yellow	Smooth	5.68±0.62	11.52±0.53	98.13±0.55
F5	Light yellow	Smooth	6.08±0.36	9.23±0.64	94.35±0.79
F6	Light yellow	Smooth	6.20±0.21	10.61±0.91	97.47±0.83
F7	Light yellow	Smooth	5.38±0.76	11.82±0.68	98.08±0.38
F8	Light yellow	Smooth	6.15±0.83	13.88±0.69	95.49±0.24
F9	Light yellow	Smooth	5.37±0.68	12.44±0.53	96.77±0.92
F10	Light yellow	Smooth	5.75±0.66	13.12±0.51	97.49±0.56
F11	Light yellow	Smooth	5.30±0.15	10.11±0.56	98.21±0.53
F12	Light yellow	Smooth	6.11±0.84	11.57±0.88	97.33±0.76

Viscosity and Rheological studies:

Generally, the viscosity of gel formulations reflects consistency. The consistency of the substance is one of the most important features to transdermal gel formulations due to being

applied to the thin layers of the skin so that the gel viscosity plays an important role in controlling of drug permeation. In this study, Brookfield viscometer (spindle no. 5) was used to determine the viscosity of Tacrolimus liposomal gel. An in-gel system, consistency depends on the ratio of solid fraction, which produces the structure to a liquid fraction. The viscosity of all batches was almost similar, as all batches contain the same concentration of carbopol (1%w/w). The viscosity of gel formulation was found in the range of 28800±20-30500±15cps at 5 rpm. The obtained viscosities are tabulated in table 11. The rheological studies were studied by plotting a graph of shear rate Vs viscosity (figure 16).

The viscosity of liposomal gel decreases with an increasing rate of shear showed with the non-Newtonian flow (shear thinning); this behavior is preferred due to its low flow resistance when applied at high shear conditions. This pseudo-plasticity results from a colloidal network structure that aligns itself in the direction of shear, thereby decreasing the viscosity as the shear rate increases. Hence, it confirms the characteristic of high spreadability due to the decrease in viscosity when applying certain force, and at the same time has the property of remaining at the application site without drainage[22].

Table No. 11: Viscosity of Tacrolimus liposomal gel

Shear Rate (RPM)	Viscosity in cps											
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
5	30200	30215	30350	30380	30440	30500	28800	28950	29010	29155	29580	29945
10	22415	22880	22975	23170	23785	23860	21675	21710	21755	21830	23850	22390
15	16860	16930	17330	17670	17950	17305	16680	16720	16780	16815	17185	17410
20	10540	11585	11600	11715	11790	11825	10210	10330	10570	10795	10980	11345
25	8315	8650	8715	8800	8855	8910	8280	8475	8500	8590	8670	8695

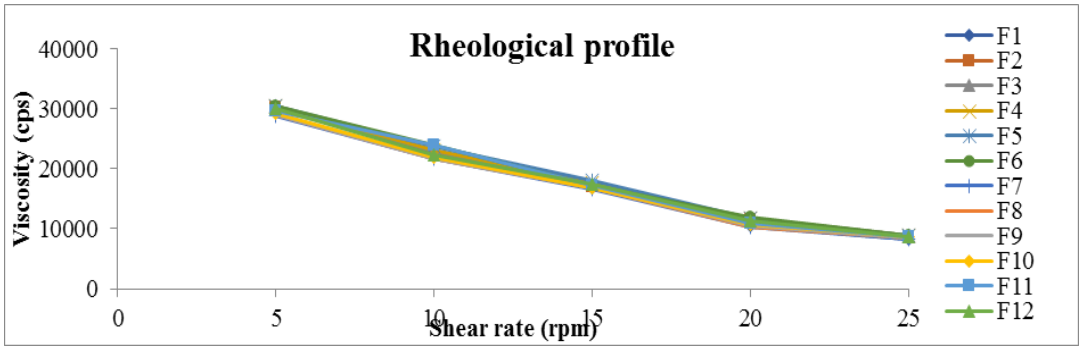

Figure No. 16: Rheological profile of Tacrolimus liposomal gel

***In-vitro* drug release studies:**

The *in-vivo* release rate of the drug from liposomal formulations depends on the design of the liposomal formulation, the route of delivery into the body including potential interactions between the liposomal formulation and any delivery technology that is used, and finally the interaction of the liposomes with the fluid and tissue once in the body. With respect to the formulation, the key factors affecting the release rate include the specific composition and relative proportion of the components in the vesicles (e.g., surfactants can fluidize the membrane increasing the rate of release, whereas cholesterol can have the opposite effect), the vesicle size distribution, the lamellarity of the liposomes (i.e., faster release from unilamellar liposomes), the nature of the drug (e.g., molecular weight, lipophilicity, and charge), the physical state of the drug (e.g., precipitated or in solution), and the release mechanism[23].

In-vitro drug release studies were carried out using Franz diffusion cell using a dialysis membrane. Liposomal gel equivalent to 10 mg of Tacrolimus was taken in donor compartment and 25 ml of distilled water containing 0.005% hydroxypropyl cellulose (adjust pH to 4.5 with phosphoric acid)was taken in the receiver compartment. It has been recommended that the drug concentration in the sink phase in release experiments should be kept below 10% of saturation. If the drug is poorly soluble in water like Tacrolimus, non-aqueous solvents or solubilizing agents may be added to the sink. Hence 0.005% hydroxypropyl cellulose was selected as solubilizing agents[108]. *In-vitro* drug release study was carried out for 12 hours. The cumulative amount of drug release was calculated for each formulation. The release profiles of Tacrolimus from different batches of liposomal gel and plain gel are illustrated in (figure 17-18 and table 12-13). Diffusion of free Tacrolimus in the plain gel through the dialysis membrane was used as control. The release profile of free

Tacrolimus shows 100% diffusion in 6 hours, whereas significantly less ($p<0.05$, t-test) amount of drug was released from liposomal gel. Entrapment of Tacrolimus in liposomes expectedly reduced both the drug release rate and the cumulative amount released. Among liposomal gel formulations (F1-F6) prepared by thin-film hydration technique, formulation F1exhibited faster drug release compared to other formulation. This might be a lower concentration of cholesterol in this batch. Hence, formulation F6 exhibited relatively slower drug release, as it contains higher lipid: cholesterol ratio (1:0.6). The initial fast rate of release is commonly ascribed to drug detachment from liposomal surface (free drug) while the later slow release results from sustained drug release from the inner lamellae.

Liposomal gel formulations (F1-F6) prepared by thin-film hydration technique exhibited faster drug release compared to liposomal gel prepared by sonication method (F7-F12) in the same lipid: cholesterol composition. Liposomal vesicles obtained from thin-film hydration technique and sonication techniques were multi laminar vesicles (MLVs) and small unilaminar vesicles (SUVs). It was observed that SUVs exhibited slower drug release relative to MLVs of the same lipid composition. In general, the release profiles of liposomal dispersions were biphasic, showing a relatively large burst effect over the first 2 hours, followed by a slower release phase. The burst effect varies with the liposome type and lipid composition. Inclusion of cholesterol reduced the initial release rate, the effect being dependent on the lecithin: cholesterol molar ratio[123]. Hence, Tacrolimus liposomal gel prolonged the drug release for 12 hours.

Table No. 12: *In- vitro* release data of Tacrolimus liposomal gel (Thin film hydration method)

Time in Hour	% Cumulative Drug Release						
	Plain gel	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
0	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000
1	33.79±0.42	18.64±0.51	16.07±0.43	13.65±0.33	13.06±0.56	12.46±0.54	11.55±0.32
2	58.11±0.53	31.32±0.72	28.15±0.62	25.08±0.47	23.47±0.73	20.72±0.40	21.14±0.64
4	86.45±0.36	44.49±0.60	42.19±0.40	40.33±0.76	36.65±0.61	31.26±0.35	28.16±0.36
6	100.0±0.77	58.22±0.65	55.86±0.36	51.83±0.79	44.55±0.92	42.45±0.72	37.77±0.77
8	100.0±0.82	66.45±0.37	63.09±0.33	60.11±0.82	54.72±0.48	50.08±0.19	45.36±0.86
10	100.0±0.84	75.58±0.39	71.93±0.71	69.05±0.45	62.78±0.36	59.83±0.65	54.08±0.90
12	100.0±0.91	83.38±0.36	80.11±0.82	76.43±0.54	71.66±0.54	67.39±0.38	63.73±0.72

Table No. 13: *In- vitro* release data of Tacrolimus liposomal gel (sonication method)

Time in Hour	% Cumulative Drug Release					
	F7	F8	F9	F10	F11	F12
0	0.00±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
1	16.64±0.35	15.65±0.92	14.65±0.42	12.41±0.49	11.53±0.56	10.09±0.42
2	28.16±0.27	25.42±0.54	23.16±0.28	21.58±0.35	18.68±0.60	10.22±0.38
4	41.08±0.42	40.04±0.49	38.88±0.33	33.06±0.47	28.33±0.14	26.54±0.91
6	53.12±0.83	49.83±0.38	49.75±0.46	42.71±0.66	37.18±0.23	34.36±0.82
8	61.37±0.48	58.11±0.81	56.63±0.39	53.44±0.92	45.27±0.44	42.12±0.37
10	72.58±0.39	68.85±0.38	66.48±0.33	60.83±0.38	53.11±0.82	51.93±0.72
12	80.17±0.41	76.16±0.39	73.03±0.48	68.35±0.87	62.06±0.37	60.82±0.33

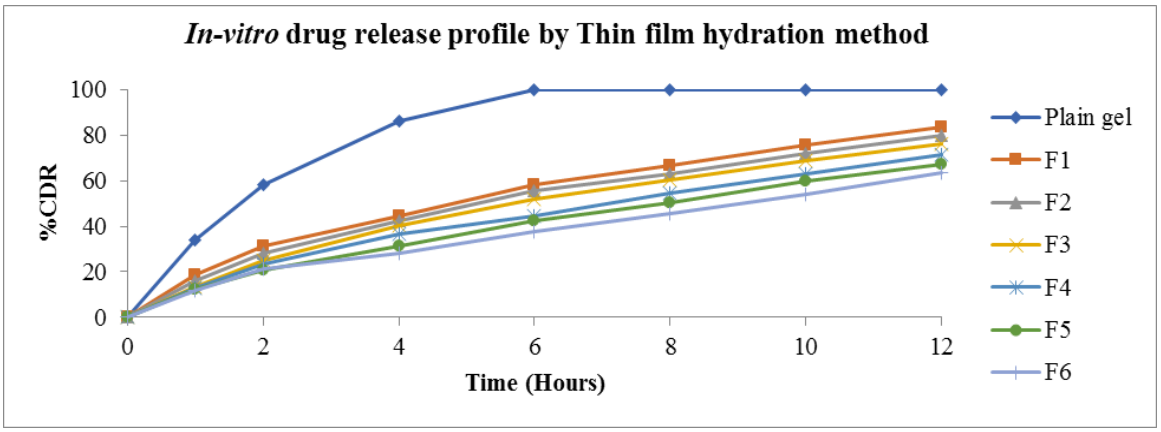

Figure No. 17: Comparative drug release profile of gel formulations (F1-F6 and plain gel)

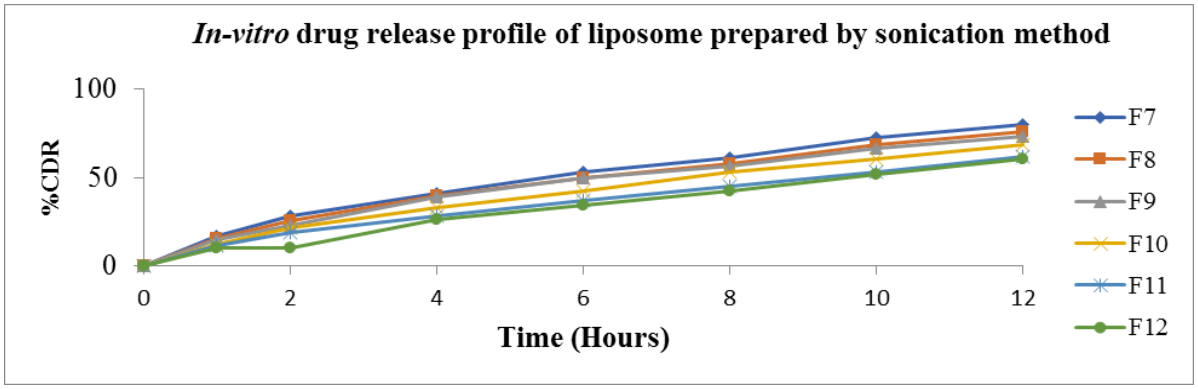

Figure No. 18: Comparative drug release profile of gel formulations (F7-F12)

Drug Release Kinetics:

To predict and correlate the *in-vitro* drug release from various Tacrolimus liposomal gel, it is necessary to fit into a suitable mathematical model. Investigation for the drug release from the gel was done by different kinetic equations (Zero order, First order, and Higuchi's equation). The release mechanism was understood by fitting the data to KorsemeyerPeppas model and shows the different release kinetics pattern and release mechanism of the gel from the different formulations as well as the best-fit release pattern for the gel formulations (table 14). When respective correlation coefficients were compared, it was found to follow the first-order model dominantly with a correlation coefficient close to 1 (0.991-0.998)[124], compared to zero-order (0.407-0.979) and Higuchi model (0.891-0.989). Further, to understand the drug release mechanism, the data were fitted into Korsmeyerpeppas exponential model $M_t / M_a = Kt^n$. Where M_t / M_a is the fraction of drug released after time 't' and 'k' is kinetic constant and 'n' release exponent which characterizes the drug transport mechanism. The values for 'n' were in the range of 1.118-1.667, which indicate that the *in-vitro* drug release exhibited with super case-II transport mechanism. It is well known that when the chain relaxation process is very slow compared with diffusion, the case II transport occurs. Super case II mechanism mostly refers to the degradation of the polymeric chain.

Table No. 14: Release exponent values and rate constant values for different formulations

Formulation Codes	Kinetic Models					
	Zero order	First order	Higuchi	Korsmeyer Peppas		Kinetic Model
	R^2	R^2	R^2	R^2	n	
Plain gel	0.407	0.992	0.891	0.499	1.667	First-order
F1	0.866	0.994	0.989	0.633	1.161	First-order
F2	0.887	0.998	0.987	0.690	1.197	First-order
F3	0.908	0.995	0.981	0.702	1.167	First-order
F4	0.920	0.995	0.979	0.684	1.158	First-order
F5	0.940	0.995	0.971	0.698	1.118	First-order
F6	0.939	0.993	0.967	0.654	1.129	First-order
F7	0.899	0.993	0.986	0.664	1.166	First-order
F8	0.904	0.991	0.985	0.725	1.162	First-order
F9	0.905	0.995	0.983	0.702	1.169	First-order
F10	0.933	0.997	0.974	0.708	1.167	First-order
F11	0.945	0.996	0.968	0.716	1.133	First-order
F12	0.979	0.994	0.921	0.775	1.195	First-order

Stability Studies

The physical appearance, drug content and *in-vitro* drug release of liposomal formulations stored at 4ºC ±2ºC and 25ºC±2ºC/60% for 1, 2, and 3 months were evaluated. Formulation F6 and F12 were selected based upon *in-vitro* release profile. The results of stability studies are given in table 15 and 16.

Results from the stability studies showed that there was no significant ($p>0.05$) change in the appearance, drug content and *in-vitro* release behaviors of the liposomal gel when stored at 4ºC ±2ºC temperature. At room temperature, there was slightly but insignificantly increase in drug release for liposomal gel formulation. This might be due to leakage of drug from the liposome. The result suggested that liposomal product can be stored in refrigeration conditions to minimize stability problems of liposomes.

Table No. 15: Results of stability studies of liposomal gel formulation F6

Months	4°C±2ºC			25°C/60% RH		
	Appearance	Drug content (%)	% CDR	Appearance	Drug content (%)	% CDR
Initial	Light yellow	97.47±0.83	63.73±0.720	Light yellow	97.47±0.83	63.73±0.720
1	No change	97.45±0.42	63.78±0.432	No change	97.44±0.69	63.90±0.861
2	No change	97.41±0.58	63.84±0.861	No change	97.39±0.51	64.86±0.367
3	No change	97.37±0.35	63.88±0.531	No change	97.36±0.73	65.06±0.538
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
Status	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	significant

Table No. 16: Results of stability studies of liposomal gel formulation F12

Months	4°C±2ºC			25°C/60% RH		
	Appearance	Drug content (%)	% CDR	Appearance	Drug content (%)	% CDR
Initial	Light yellow	97.33±0.76	60.82±0.330	Light yellow	97.33±0.76	60.82±0.330
1	No change	97.31±0.44	60.86±0.377	No change	97.30±0.82	61.42±0.275
2	No change	97.29±0.38	60.94±0.428	No change	97.27±0.57	61.98±0.443
3	No change	97.26±0.36	60.98±0.552	No change	97.24±0.61	62.76±0.528
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
Status	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	significant

CONCLUSION

Transdermal drug delivery (TDD) offers many benefits, but it has proven difficult to discover drugs small and lipophilic enough to permeate effectively through the skin barrier. Transdermally delivered drugs often have better patient compliance than more painful/invasive alternative routes that require needle injection. Transdermal routes also offer the benefit of bypassing the first-pass metabolism in the liver that orally delivered drugs undergo. Hence in the present research work, an attempt has been made to develop Tacrolimus liposomal gel for transdermal drug delivery. First Tacrolimus liposomes were prepared by thin-film hydration method and sonication method. Then liposomal dispersion

was dispersed in 1% Carbopol gel base to obtain the liposomal gel. The study has demonstrated various aspects and from the results obtained, the following conclusions are drawn.

- The preformulation parameters i.e. melting point, partition coefficient and solubility of the drug were evaluated. The results found to be satisfactory and all the values obtained comply within pharmacopoeial standards.

- Compatibility between drug and excipients were determined by IR studies and the result showed that there was no possible interaction between pure drug Tacrolimus and excipients used in the preparation of liposomal gel.

- Absorbance maximum of pure drug Tacrolimus was determined using UV-spectroscopy and drug showed maximum absorbance at 273 nm.

- The entrapment efficiency of Tacrolimus liposome depended on the concentration of lipid: cholesterol i.e. when the concentration of cholesterol increased entrapment efficiency was decreased and vice versa.

- Particle sizes of liposomal vesicles were less than 400 nm, which is the required size for transdermal formulation.

- Scanning electron microscopy revealed a nearly spherical shape of the liposome.

- Surface charge of liposome was negatively charged; prevent the vesicles aggregation and accumulation. Hence increase the stability of liposome.

- Tacrolimus liposomal gels were evaluated for homogeneity, pH, spreadability, and viscosity. The values obtained were found to be satisfactory and complies with the standard range.

- The rheological study showed that all gel formulations followed the non-Newtonian flow (shear thinning) as the shear rate increase viscosity of all formulation decreases which are required character for any transdermal gel.

- *In-vitro* drug release studies were conducted for 12 hours using Franz diffusion cell. The results revealed that Tacrolimus liposomal gel prolonged the drug release for a prolonged period and was significantly prolonged than plain gel.

➢ The release data was fitted to various mathematical models such as zero order, first order, Higuchi and Korsmeyer-peppas, to evaluate the kinetics of drug release. The drug release showed first-order release kinetics and followed Super case II release mechanism.

➢ Short term stability studies were conducted for optimized formulation F6 and F12 for the period 3 month. There was no significant change in physical appearance, drug content and release profile of prepared formulation stored at refrigerator but drug release was slightly increased when stored at room temperature. Hence, it is suggested to store liposomal at refrigerator temperature.

From the above experimental data, it can be concluded that Tacrolimus liposomal gel formulations were successfully prepared and can be applied transdermally. Thus, Tacrolimus liposomal gel formulation can be used in the future for the treatment of autoimmune disease with improved bioavailability.

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