ENHANCEMENT OF DISSOLUTION CHARACTERISTICS AND ORAL BIOAVAILABILITY OF TACROLIMUS FROM PRONIOSOMAL DRUG DELIVERY SYSTEM

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ABSTRACT

The current research was designed to improve the oral delivery of tacrolimus by loading into maltodextrin based proniosome powders. Proniosome powders proved to be the potential carriers for efficient oral delivery of lipophilic or amphiphilic drugs. These ‘proniosomes’ minimize problems of niosome physical stability such as aggregation, fusion and leaking, and provide additional convenience in transportation, distribution, storage, and dosing. The proniosome powders were fabricated by various ratios of span 60 and cholesterol and evaluated for micromeritic properties and the results indicates adequate micrometric properties. The optimized formulation showed smaller vesicle size, high surface charge and entrapment efficiency. The optimized proniosome formulation was evaluated by optical and scanning electron microscopy, FT-IR, differential scanning calorimetry, and powder X-ray diffraction to understand the morphological, solid state properties of the drug reveal the absence of chemical interaction, drug transformation from crystalline to amorphous and molecular state. Multimedia dissolution profiles [at pH 1.2, 4.5 and 6.8] were carried out to demonstrate improved dissolution characteristics compared to pure drug/Marketed formulation.

KEY WORDS
Proniosomes, Maltodextrin, Span 60, Tacrolimus, Dissolution

INTRODUCTION:

A number of vesicular drug delivery systems have emerged encompassing various routes of administration to achieve controlled and targeted drug delivery such as liposome, niosome, transfersomes, ethosomes and proniosome were developed. Provesicular concept has evolved to resolve the stability issue pertaining to the conventional vesicular systems i.e. liposomes and niosomes. In recent years, vesicles have become the vehicle of choice in drug delivery. Lipid vesicles were found to be of value in immunology, membrane biology, diagnostic techniques and most recently, genetic engineering. Vesicles can play a major role in modeling biological membranes, and in the transport and targeting of active agents. Encapsulation of a drug in vesicular structures can be predicted to prolong the existence of the drug in systemic circulation and perhaps, reduces the toxicity if selective uptake can be achieved1.

Proniosomes are vesicular systems, in which the vesicles are made up of non-ionic based surfactants, cholesterol and other additives which may be hydrated immediately before use to yield aqueous niosome dispersions. In the current work, proniosomal formulation for tacrolimus was developed using simple slurry method.
MATERIALS AND METHODS:

Materials:
Tacrolimus was gifted from Mylan Laboratories Limited Hyderabad, India, Maltodextrin was gifted by Sigma Aldrich Chemicals, Hyderabad, Cholesterol and Span 60 was purchased from SD Fine Chemicals, Mumbai. Methanol and Chloroform were purchased from Merck Specialties Pvt. Ltd, Mumbai.

Solubility studies of Tacrolimus
An excess amount of tacrolimus was added to each vial containing 1 mL of the vehicle. After sealing, the mixture was vortexed using a vortex mixer at a maximum speed for 10 min in order to facilitate proper mixing of tacrolimus with the vehicle. Mixtures were then shaken in an orbital shaker maintained at room temperature until equilibrium (24 h). Mixtures were centrifuged at 15,000 rpm for 10 min, and the resulting supernatant was filtered through the Whatman filter paper. The filtrate was quantified by the developed HPLC method using methanol and water (60:40 % v/v) as mobile phase and 294nm wavelength was used.

FORMULATION OF PRONIOSOMAL POWDER: 

Pronosome powders were prepared by using slurry method. The composition of different proniosomal formulations is represented in Table 1. Emulsifier/lipid mixture comprising of span 60, cholesterol and drug as per formulation ratios were accurately weighed and dissolved in 20mL of solvent mixture containing chloroform and methanol (2:1). The above solvent solution was transferred into a 250mL round bottom flask and required amount of maltodextrin was added to form slurry. The flask was attached to a rotary flash evaporator (Hei-VAP advantage/561-0300, Heidolph, Germany) and the organic solvent was evaporated under reduced pressure at a temperature of 45±2°C. After ensuring the complete removal of solvent, the resultant powders were further dried overnight in a vacuum oven at room temperature so as to obtain dry, free-flowing product. The obtained proniosome powders were stored in a tightly closed container at 2-8°C for further characterization.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>TPN1</th>
<th>TPN2</th>
<th>TPN3</th>
<th>TPN4</th>
<th>TPN5</th>
<th>TPN6</th>
<th>TPN7</th>
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<tbody>
<tr>
<td>Drug (mg)</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Maltodextrin (mg)</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Span 60(mg)</td>
<td>175</td>
<td>150</td>
<td>125</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Cholesterol(mg)</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>100</td>
<td>125</td>
<td>150</td>
<td>175</td>
</tr>
<tr>
<td>(2:1) Chloroform and Methanol (mL)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Characterization of proniosome powders

Morphological evaluation
The morphology of the proniosomes was evaluated by optical microscopy. The proniosome powder was placed on a cavity glass slide and few mL of water was added drop wise along the side of the cover slip. The formation of vesicles was monitored through an optical microscope (METZER optical instrument) and photographs were taken.

Micromeritic properties
The flow properties of powder are plays an important role in handling and processing operations. The flow properties were studied through measuring the Angle of repose, Carr’s compressibility index and Hausner’s ratio. The conventional fixed funnel method was used for angle of repose determination. The bulk and tapped density of the proniosome powders evaluated for calculating the Carr’s compressibility index and Hausner’s ratio.

Re-dispersible properties
The number of vesicles formed after hydration is one of the important parameter to evaluate the proniosome powder. The proniosome powder was hydration with pH 6.8 phosphate buffer and the formed niosomes were counted by optical microscope using a haemocytometer. The niosomes in 80 small squares were counted and calculated by using the following formula.

Total number of niosomes per cubic millimetre= Total number of niosomes counted x Dilution factor x 4000/ Total number of squares counted.
Determination of vesicle size, Zeta Potential
The proniosomal powders were hydrated with phosphate buffer (pH 6.8) and subjected to bath sonication for 3 min and the resultant dispersion was used for the determination of size, zeta potential. The mean size, size distribution and zeta potential (ZP) of proniosomes was determined photon correlation spectroscopy using Zetasizer NanoZS90. Each sample was diluted to a suitable concentration with phosphate buffer pH 6.8. Size analysis was performed at 25°C with an angle of detection of 90ºC. Size, polydispersity index of proniosomes and their mean zeta potential values (±SD) were obtained from the instrument.

Percentage Drug Entrapment
The percentage drug entrapped (PDE) was determined by Ultra-centrifugation. The liposomal formulations were subjected for ultracentrifugation (ultra-Centrifuge – Remi laboratories, Mumbai, India) at 5000 rpm for 15 min in an ultracentrifuge in order to separate the entrapped drug from the free drug. Then the clear supernatant was separated and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer/ HPLC. This indicates amount of free drug. The liposomal pellet was redispersed in Methanol and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer 294 nm/ HPLC at 213 nm. This indicates amount of drug entrapped. The entrapment capacity of liposomes was calculated as follows

\[
PDE = \left(\frac{T - C}{T}\right) \times 100
\]

Where T is the total amount of drug that is detected both in the supernatant and sediment, and C is the amount of drug detected only in the supernatant [1-3]. The PDE of Tacrolimus proniosomes was calculated after determining the amount of entrapped drug by dialysis\textsuperscript{18}. The dialysis was performed by adding the proniosomal dispersion to a dialysis tube (donor compartment) and then dipping the tube into a beaker containing 200 mL of PBS pH 7.4 with 0.05% SLS (receptor compartment) on a magnetic stirrer, rotated at a speed of 100 rpm for 3 hours. After 3 hours, the solution in the receptor compartment was estimated for unentrapped drug at 294 nm by using a UV spectrophotometer at 213 nm HPLC.

Percent Entrapment = Total Drug - Diffused Drug/Total Drug\times 100

In-vitro drug release study
In vitro dissolution study of proniosomal powders and pure drug/Marked capsule was performed using USP type II (paddle) apparatus in multimedia dissolution media of pH 1.2, pH 4.5 and pH6.8 to maintain sink conditions [4,5]. The volume of dissolution medium used was 900 mL and maintained at a temperature of 37 ± 1°C with 50 rpm of paddle speed set throughout the experiment. 5 mL of aliquot of was collected at predetermined time intervals at 30, 60, 90, 120min and replaced with fresh dissolution medium to maintain constant volume\textsuperscript{40}. The samples were filtered by passing through 0.45 µm PVDF membrane filter (Millipore) and analyzed by HPLC at 213 nm /Samples were analysed for Tacrolimus using UV-Visible spectrophotometer at 294 nm. Cumulative % of drug released was calculated and plotted against time (t).

HPLC analysis of Tacrolimus
Assay of tacrolimus samples were analysed by using HPLC method and HPLC method adapted from USP of tacrolimus [20]. The HPLC system consisted of a vacuum degasser, photodiode array detector. LC mode and 4.6-mm X 15-cm; 3-µm packing L1 column used for the chromatographic separation. The mobile phase was Solution A: 6 mM phosphoric acid, Solution B: Acetonitrile and tert-butyl methyl ether (81:19), Solution C: Solution A and Solution B (4:1), Solution D: Solution A and Solution B (1:4), pumped at a constant flow rate of 1.5 mL/min. the eluent was monitored using Photodiode array detector at a wavelength of 220 nm. The column was maintained at 60°C, auto sampler at 4° and injection volume of 20µl was injected. The mobile phase was filtered through 0.45µm PTFE filter to use. Under these conditions, the retention time (t\textsubscript{R}) of Tacrolimus was approximately 5.3 min. The peak purity was checked with the photodiode array detector.

Solid State Characterization Studies [6-8]:

Differential scanning calorimetry (DSC)
The DSC analysis was used to evaluate the molecular state of the encapsulated drug in optimized proniosomal formulation(TPN4), Pure drug, maltodextrin. Each sample was placed in an aluminum pan and then crimped with an aluminum cover. The DSC curves of the samples were obtained by a differential scanning calorimeter (DSC-6300, SIIO, Japan). The heating and cooling rates were 10°C/min and 250°C/min, respectively. All measurements were...
performed over 0–400 °C under a nitrogen purge at 50 mL/min.

**Powder X-ray diffractometry (PXRD)**

Every crystalline substance gives a pattern; the same substance always gives the same pattern; and in a mixture of substances each produces its pattern independently of the others."

crystalline properties of the optimized proniosome formulation, pure drug and maltodextrin were analysed by using X-ray diffractometer (Stereoscans S120, Cambridge, UK). The measuring conditions were as follows: CuKa radiation, nickel filtered; graphite monochromator; 45 kV voltage; and 40 mA current with X'celerator detector. All samples were run at 1° (2θ) min 1 from 3° to 45° (2θ).

**Scanning electron microscopy (SEM)**

The surface morphology and the internal textures of the pure drug, maltodextrin and proniosome powder formulation were investigated by scanning electron microscope. Samples were fixed on a brass stub using double sided adhesive tape and were made electrically conductive by coating with a thin layer of gold and SEM images were recorded at 15 kev accelerating voltage.

**Fourier transform infrared (FT-IR) spectroscopy**

Infrared spectra of pure drug, maltodextrin, non-ionic surfactant and optimized proniosome powder formulation were obtained using FT-IR spectrophotometer (Bruker, Alpha-T, Lab India) by the conventional KBr pellet method.

**In-vivo or Ex-vivo permeation studies:**

**Permeation data analysis**

The cumulative amount of drug permeated (Q) was plotted against time. The steady state flux (Jss) was calculated from the slope of linear portion of the cumulative amount permeated per unit area vs. time plot. The permeability coefficient (Kp) of the drug through intestine was calculated by dividing steady state flux with initial concentration of tacrolimus in donor compartment [9]. The enhancement ratio (ER) was calculated by using the following equation: \( ER = \frac{\text{Jss of proniosome formulation}}{\text{Jss of control}} \).

**In situ intestinal absorption study [10-12]**

The in situ intestine absorption of tacrolimus from proniosome formulations was assessed by adapting single-pass perfusion method. Male wistar rats weighing between 180-200 gm used in the study were obtained from Mahaveera Enterprises (146-CPCSEA no: 199; Hyderabad, India). The animals were housed in separate cages in a clean room and maintained under controlled condition of temperature and the rats had free access to food and water. The study was conducted with the prior approval of Institutional Animal Ethical Committee, St. Peter’s Institute of pharmaceutical sciences. Euthanasia and disposal of carcass was in accordance of the guidelines. Before perfusion experiments, the rats fasted overnight with free access to water was anesthetized by thiopental sodium injection (60 mg/kg body weight) administered via intraperitoneal route and placed on a thermostatic surface to maintain body temperature. Under anesthesia, an incision was made through a midline to expose the abdominal content. The lower part of the small intestine segment used for perfusion was exposed and semi-circular incisions were made on both ends and cannulated with PE tubing followed by ligation with silk suture. After cannulation the surgical area was covered with cotton soaked in physiological saline (37°C) throughout the experimentation. To remove the adhered mucosal contents, the intestine segment was flushed with phosphate buffered saline (PBS) (pH 7.4 at 37°C) and stabilized by perfusing the blank PBS for 15 min. The perfusates prepared by dispersing proniosome powders (TN4P and control formulation (processed without phospholipids and span 80) equivalent to 5 mg of tacrolimus containing phenol red (7.5 μg/mL) in PBS were passed at a steady flow rate of 0.2 mL/min (NE-1600, New Era Syringe Pumps, USA) and the perfusate was collected for every 15 min. At the end of the perfusion (90 min) the circumference and length of the perfused intestine was measured and the samples were stored at -20°C until further analysis by HPLC. Each experiment was performed in triplicate. Prior to analysis, the perfusate samples were allowed to thaw, deproteinized with methanol, centrifuged and the drug content in the supernatant was quantified for DTX by HPLC.

**Stability studies**

The optimized proniosomal formulation was filled in glass vials and charged into stability for 180days at controlled room temperature and in refrigerator (2-8°C). Samples were withdrawn at predefined time intervals (Initial, 30, 60, 90 and 120days) and hydrated with phosphate buffered saline pH (6.8) and observed under optical microscope for any sign of drug morphology. Further the samples were analysed for...
percentage potency of tacrolimus present optimised formulation and compared to the initial evaluated results to establish the stability of proniosomal formulation.

Statistical analysis
The data obtained was subjected to Student ‘t’ test and one way analysis of variance (ANOVA) and the significance of difference between formulations was calculated by student–Newman–Keuls (compare all pairs) [13] with Instat Graphpad prism software (version 4.00; GraphPad Software, San Diego California). The level of statistical significance was chosen as less than P < 0.05.

RESULTS AND DISCUSSIONS
Preparation and physico-chemical evaluation of proniosome powders
Maltodextrin had porous structure and high surface area and easy adjustment of amount of carrier required to support the surfactant and several researchers reported that Maltodextrin based proniosomes proved to be the potential carriers for efficient oral delivery poorly soluble drugs. Tacrolimus loaded maltodextrin based proniosomes with span 60 as nonionic surfactants prepared by a slurry reported in the literature. Colloidal carrier drug delivery approach of proniosome approach has resolved many stability issues pertaining to aqueous niosome dispersions. The advantages, niosome dispersions suffer from stability problems like aggregation, hydrolysis, drug leakage and production scale up. In this perspective for improve the stability of the vesicular systems several strategies have been used. To improve the stability and entrapment efficiency of vesicular formulations cholesterol is the first choice of additive used as a structural lipid. The concentration of nonionic surfactant and cholesterol will influence the morphology and stability of the vesicular systems like niosomes drug delivery system. The various ratios of cholesterol and span 60 were evaluated for the optimum composition of span 60 to cholesterol ratio by keeping the total lipid constant. The phase transition temperatures of various spans are, span 20 is 16°C, span 40°C is 42°C, span 60 is 53 °C and span 80 is 12 °C, the span 60 having high phase transition temperature and span 80 having the low phase transition temperature. Highest phase transition temperature of span having high entrapment could be observed as reported in the literature. Span 60 was selected as choice of nonionic surfactant due to the high phase transition temperature, it intern led’s to high entrapment efficiency, to facilitate stable vesicle formation and to improve the oral delivery of tacrolimus from proniosomes.

Morphological evaluation of prepared proniosome powders by optical microscope
The niosomes formed from proniosome powder after hydration and gentle shaking with water as shown in Figure 1. multilamellar vesicles are formed after gentle agitation with and all vesicles are in spherical shape. The morphology of the proniosomes was evaluated by optical microscopy

Fig. 1. Optical microphotographs showing (A) proniosome powder, (B) formation of vesicles on maltodextrin after hydration with phosphate buffer (pH 6.8), (C) Niosome dispersion from proniosome powder (TPN4) upon gentle agitation.

Micromeritic properties of proniosome powders
The micromeritics of the proliposome powders is vital in handling and processing operations because the dose uniformity and ease of filling into container is dictated by the powder flow properties. In general, three types of flow measurements can be used to evaluate the nature of powder flow i.e. angle of repose; Carr’s index and Hausner’s ratio and the results were depicted in Table 2. The smaller the value of angle of repose, lesser the internal friction or cohesion between the particles and greater the flow characteristics and vice-versa. It is apparent from the results that small angle of repose (<20°) assure good flow properties for proliposome powder formulations. In addition to angle of repose,
Carr’s index and Hausner’s ratio were also less than 17 and 1.25 respectively ensuring acceptable flow for proniosomes powder formulations and results are reported in Table 2.

Table 2: Micromeritic properties of various proniosome powder formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Angle of repose (°)</th>
<th>Compressibility</th>
<th>Hausner’s ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPN1</td>
<td>16.8±0.21</td>
<td>10.8±0.08</td>
<td>1.09±0.15</td>
</tr>
<tr>
<td>TPN2</td>
<td>19.2±0.23</td>
<td>11.4±0.06</td>
<td>1.18±0.19</td>
</tr>
<tr>
<td>TPN3</td>
<td>19.8±0.32</td>
<td>15.5±0.25</td>
<td>1.22±0.13</td>
</tr>
<tr>
<td>TPN4</td>
<td>18.1±0.35</td>
<td>16.2±0.37</td>
<td>1.17±0.21</td>
</tr>
<tr>
<td>TPN5</td>
<td>19.3±0.27</td>
<td>15.7±0.28</td>
<td>1.23±0.12</td>
</tr>
<tr>
<td>TPN6</td>
<td>19.6±0.14</td>
<td>16.8±0.26</td>
<td>1.20±0.14</td>
</tr>
<tr>
<td>TPN7</td>
<td>19.2±0.26</td>
<td>16.4±0.41</td>
<td>1.17±0.09</td>
</tr>
</tbody>
</table>

Number of vesicles per cubic millimetre

The hydrated niosomes were counted by optical microscope using a haemocytometer. The niosomes in 80 small squares were counted, calculated and tabulated table 3. Total number of niosomes per cubic millimetre

Determination of vesicle size, Zeta potential and Entrapment efficiency and No. of vesicles per mm³:

Several researchers are reported that the vesicle size and size distribution of proniosomes is most important parameter, which needs to monitor during proniosomes preparation for its best performance. Proniosomal sample was placed under digital microscope (Metzer, India) and hydrated with milli Q water. Then formation of vesicle was observed within the niosomal dispersion. Results of average vesicle size and distribution were calculated for count and distribution. The maximum benefit from the proniosome formulations can be speculated when abundant numbers of vesicles are formed after hydration in the gastrointestinal tract. Among all the formulations, the proniosome formulation containing span 60 and cholesterol at a ratio of 1:1 (TPN4) has exhibited good number of vesicles which is min well correlation with the size and entrapment efficiency results.

Vesicle size and size distribution is an important parameter for the vesicular systems [34]. The mean size of the vesicles was in the range of 200–480 nm. Small value of polydispersity index (PI) (<0.1) indicates a homogenous population, while a PI (>0.3) indicates a higher heterogeneity. The zeta potential and entrapment efficiency of the formulations was between -26.5 and -36.5 mV and 60– 92%, respectively. Determination of entrapment efficiency is an important parameter in case of proniosome as it majorly effects the drug release. Entrapment efficiency is expressed as the fraction of drug incorporated into proniosome relative to total amount of drug used. The size and surface charge of the vesicles seems to be dependent on the cholesterol concentration. We could notice small sized vesicles with high zeta potential when equimolar ratio of formulation containing span 60 and cholesterol (TPN4) and also.

Table 3: Physico-chemical characterization of various proniosome formulations

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulation</th>
<th>size (nm)</th>
<th>PI</th>
<th>Zeta potential (mV)</th>
<th>Entrapment efficiency ± SD (%)</th>
<th>No. of vesicles per mm³ x 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TPN1</td>
<td>434±23</td>
<td>0.356</td>
<td>33.1 ± 4.8</td>
<td>94.9±0.244</td>
<td>3.02</td>
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<tr>
<td>2</td>
<td>TPN2</td>
<td>343±12</td>
<td>0.281</td>
<td>27.7 ± 5.2</td>
<td>85,12±1.48</td>
<td>3.81</td>
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<tr>
<td>3</td>
<td>TPN3</td>
<td>265±07</td>
<td>0.278</td>
<td>35.3 ± 3.1</td>
<td>91.02±0.63</td>
<td>3.21</td>
</tr>
<tr>
<td>4</td>
<td>TPN4</td>
<td>206±12</td>
<td>0.178</td>
<td>41.4 ± 2.3</td>
<td>96.5±0.205</td>
<td>4.14</td>
</tr>
<tr>
<td>5</td>
<td>TPN5</td>
<td>334±23</td>
<td>0.294</td>
<td>34.1 ± 3.5</td>
<td>92.7±0.249</td>
<td>3.23</td>
</tr>
<tr>
<td>6</td>
<td>TPN6</td>
<td>412±16</td>
<td>0.312</td>
<td>28.0 ± 4.2</td>
<td>94.1±0.509</td>
<td>3.91</td>
</tr>
<tr>
<td>7</td>
<td>TPN7</td>
<td>480±21</td>
<td>0.357</td>
<td>26.5 ± 2.6</td>
<td>88.1±2.19</td>
<td>4.21</td>
</tr>
</tbody>
</table>
**In vitro studies:**

The dissolution profiles of proniosomes formulation were shown in figure 2B. The amount of tacrolimus released from proniosomes was ranging between 50 to 85% in 90 min and was higher compared to control (30%) (Table 4) at all multimedia dissolution. The dissolution efficiency of insoluble drug tacrolimus has been significantly improved when encapsulated in proniosomes (Table 5). This might be due to the enhanced solubility of tacrolimus by span 60 molecules. However, we could not notice any remarkable change in the dissolution behavior with different proliposome formulations. Interestingly, the proniosome formulation (TPN4) promoted higher dissolution of tacrolimus compared to other formulations. The improved dissolution of the drug from the proniosome powder formulations might be due to the altered physical state of the drug entrapped within the niosome bilayer and enhanced effective surface area available for dissolution medium. From the above results TPN4 formulation has selected as optimized one which is used for further study. The dissolution profiles and cumulative dissolution of tacrolimus from proniosome powder formulations (TPN1-TPN7) and control (pure drug) in pH 1.2, pH 4.8 and pH 6.2 are depicted in Fig. 2 and 3, respectively.

Table 4: Dissolution studies of tacrolimus proniosome powder formulations in pH 1.2.

<table>
<thead>
<tr>
<th>Time</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>Pure drug</th>
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<tr>
<td>0</td>
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<td>0.0</td>
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<tr>
<td>30</td>
<td>21.0</td>
<td>18.2</td>
<td>18.4</td>
<td>35.6</td>
<td>23.5</td>
<td>24.0</td>
<td>20.0</td>
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<td>60</td>
<td>41.0</td>
<td>36.0</td>
<td>35.5</td>
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<td>42.6</td>
<td>30.2</td>
<td>25</td>
</tr>
<tr>
<td>90</td>
<td>66.0</td>
<td>55.4</td>
<td>55.5</td>
<td>80.5</td>
<td>69.8</td>
<td>74.1</td>
<td>60.5</td>
<td>30.5</td>
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</table>

Fig. 2. Dissolution profiles of tacrolimus from proniosome formulations in pH 1.2.

Table 5: Dissolution studies of tacrolimus proniosome powder formulations in pH 4.5

<table>
<thead>
<tr>
<th>Time</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
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<td>30</td>
<td>20.4</td>
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<td>36.2</td>
<td>31.5</td>
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<tr>
<td>90</td>
<td>75.3</td>
<td>53.1</td>
<td>56.8</td>
<td>85.8</td>
<td>75.0</td>
<td>68.0</td>
<td>65.5</td>
<td>42.8</td>
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</tbody>
</table>
Fig. 3. Dissolution profiles of tacrolimus from proniosome formulations in pH 4.

Table 6: Dissolution parameters of tacrolimus from proniosome powder formulations in pH 6.8.

<table>
<thead>
<tr>
<th>Time</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>Pure drug</th>
</tr>
</thead>
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<tr>
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<td>0.0</td>
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<td>0.0</td>
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<td>19.8</td>
<td>36.8</td>
<td>21.4</td>
<td>20.9</td>
<td>19.0</td>
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<td>33.5</td>
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<td>30.5</td>
<td>24.8</td>
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<tr>
<td>90</td>
<td>68.3</td>
<td>51.1</td>
<td>54.8</td>
<td>83.8</td>
<td>68.0</td>
<td>65.0</td>
<td>64.5</td>
<td>31.7</td>
</tr>
</tbody>
</table>

Fig. 4. Dissolution profiles of tacrolimus from proniosome formulations in pH 6.8.

Table 7: Cumulative % Drug Release from various Proniosome formulations of Tacrolimus after 90 min (TPN1-TPN7 & Pure Drug)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulation</th>
<th>Cumulative % drug release in pH 1.2</th>
<th>Cumulative % drug release in pH 4.5</th>
<th>Cumulative % drug release in pH 6.8</th>
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<tr>
<td>1</td>
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<td>54.8</td>
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<tr>
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<tr>
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**Solid state characterization**

Scanning electron microscopy, differential scanning calorimetry, X-ray powder diffraction and fourier transform infrared spectroscopy used evaluation of the molecular interactions between drug and carrier. The thermotropic behavior and the physical state of the drug in proliposome powders were evaluated by performing DSC analysis. The DSC thermograms of tacrolimus, neusilin US2 and proliposome formulation (PL-DX4) were recorded (Fig. 5). The neusilin US2 used as a carrier exhibited a difused peak at 226.94°C corresponding to its melting point. The absence of conspicuous peak in proliposome formulation over the melting range of API unravels the transformation of the physical state of the drug (crystalline to amorphous) which was further confirmed by PXRD analysis.

The PXRD patterns of tacrolimus, maltodextrin and proniosomal powder were represented in figure 6. The pure drug showed numerous characteristic high intensity diffraction peaks at 2θ of 8.8, 11.08, 13.97, 17.73 and 19.34 demonstrating the crystalline nature of the drug. The absence or reduced intensities of characteristic tacrolimus peaks in proliposome formulation suggest the change in physical state i.e. amorphization of drug.

Figure 7 illustrates the FT-IR spectra of tacrolimus, neusilin US2 and proliposome formulation (TPN4). The pure drug tacrolimus exhibit characteristic peaks bands at 1713, 1248 and 709 cm–1, as well as the benzene ring bands at 1450, 1500 and 1600 cm–1. The peaks at 1713, 1248, 709, 1450, 1500 and 1600 cm–1were disappeared in proliposome formulation and the intensity of peaks at 1713, 1248 and 709 cm–1 was reduced. However no additional peaks in proliposome formulation indicate the absence of chemical interaction between the drug and formulation ingredients.

![Figure 5a: DSC thermogram of Tacrolimus](image-url)
Figure 5b: DSC of Maltodextrin

Figure 5c: DSC of Optimized Formulation F4

PXRD:

Figure 6a: PXRD of Tacrolimus,  Figure 6b: PXRD of Optimized formulation F4
Surface morphology
The surface morphology of the pure drug, Maltodextrin and proniosome powders were examined by SEM and the images are represented in figure 8. The absence of typical crystalline structures of tacrolimus in proniosome formulation indicates the transformation of drug to amorphous or molecular state. Further, the porous structure of maltodextrin as evident in figure was illegible in proniosome powders because of the deposition of surfactant and lipid on the surface of maltodextrin.

Ex-vivo intestine permeation study
The rate and extent of absorption is not only dependent on the dissolution rate rather the permeation across the gastrointestinal tract is also a limiting factor for absorption. Therefore, to have an insight on the ability of proniosome formulations for improved absorption, ex-vivo permeation study was carried out using the rat intestinal segment. Since majority of the drugs get absorbed in vivo from the small intestine it is more relevant to use the rat tissue [38]. Henceforth, we have used ileum portion to assess the potential of proniosome powder formulations for improving the permeation of tacrolimus across the intestine barrier. The cumulative amount permeated (CAP) across the intestine was calculated and represented in table 3.
The cumulative amount of tacrolimus permeated from control was 1578 ± 114 lg and was significantly increased to 1908 ± 109, 2392 ± 215 with marketed (P < 0.05) and TPN4 formulation (P < 0.01), respectively (Table 5). The flux was also increased significantly with marketed and proniosome formulation compared to control (P < 0.01). Interestingly the permeation was significantly higher for proniosome formulation compared to marketed tablet formulation (P < 0.01). The enhancement ratio well above 1 indicates improved permeation and in our findings, we could notice an ER greater than 1 for proniosome and marketed formulations compared to control. Based on the improvement in the permeation the formulations can be ranked in the following descending order PN4 > Marketed > Control. The remarkable improvement in the permeation can be owed to the combination of several mechanisms: (i) presence of nonionic surfactant could obviate the barrier function due to the fluidization of the intercellular lipid bilayer; (ii) better membrane contact and permeation enhancement property of the non-ionic surfactants might have led to altered permeability characteristics of the membrane which otherwise result in improved partitioning of the drug into the bilayer; (iii) direct transfer of vesicles across the epithelial membrane. To summarize the promising results reveal the potential of proniosome carriers for improved absorption of tacrolimus across the biological membrane.

In situ perfusion study
The in-situ perfusion study facilitates to ascertain the potential of proniosomes for improved absorption of tacrolimus across GI tract. The effective permeability coefficient (Peff), absorption rate constant (Ka) and enhancement ratio were calculated and represented in table 6. The obtained Peff values for control, TPN5, TPN7 and TPN4 were 3.93±0.23, 5.60±0.31, 6.84±0.30 and 7.72±0.38 cm/sec (×10-4) respectively. The absorption rate constant (Ka) indicative of rate of absorption was also significantly higher for proniosomes compared to control. The enhancement ratio above 1 indicates an enhanced permeation and in our case with all the proniosome formulations we could observe an ER greater than 1 which suggests the potential of proniosomes for improved oral delivery.

Pharmacokinetic study
The objective of the present study is to check the feasibility of tacrolimus loaded proniosomes to improve the oral bioavailability. It is speculated that the positive charge containing vesicles favour the drug absorption due to increased vesicle interaction with the cell surface because of electrostatic attraction. The mean serum concentration vs. time profiles of TACROLIMUS following peroral administration of different proniosomes in comparison to control are shown in figure 7. The pertinent pharmacokinetic parameters were derived and presented in table 7. The obtained results reveal a higher Cmax for proliposomes compared to control (p<0.001). However, the time to reach the peak concentration (Tmax) remained constant from all the formulations. The higher biological half-life and mean residence time of tacrolimus from proniosomes with respect to control is obviously due to the slower elimination rate of tacrolimus from these formulations (Table 7). The AUC values which indicate the extent of absorption was 11.789±1.08, 10.36±0.91and 19.76±1.03 µg h mL-1 following oral administration of TPN5, TPN7 and TPN4 respectively and were significantly higher compared to control (7.077±0.932 µg h mL-1) (p<0.001). The relative bioavailability (F) of tacrolimus following oral administration of proniosomes was also significantly higher compared to control (p<0.001). Overall, it is apparent from the results that the rate and extent of absorption of tacrolimus has been markedly improved from proniosomes compared to control. Based on the AUC and F values, the formulations can be ranked in the following descending order TPN4 > TPN7 > TPN5 > Control. The improved bioavailability of tacrolimus from proniosomes could be due to the contribution of several mechanisms either alone or in combination which include i) by virtue of the surfactant property of the phospholipids can reduce the interfacial barrier and provide intimate contact with epithelial cell membrane thus favouring the partitioning of tacrolimus into the hydrophobic domain of the cell membrane ii) fusion of the niosomes with the epithelial cells by endocytosis also might be responsible for the augment in absorption across GI membrane iii) direct transfer of niosomes formed at the vicinity of the GI tract may lead to an
improved bioavailability due to avoidance of first pass metabolism.

**Stability study**

The physical appearance % retention of tacrolimus was monitored for the optimized proniosome powder formulation (TPN4) upon storage at refrigerated and ambient room temperature for a period of 180 days. At definite time intervals, the proniosome powder was hydrated to form niosomes and we could observe the formation of vesicles without any signs of drug crystallization. The entrapment efficiency was also monitored and the results indicate that there was no appreciable change in the % retention of tacrolimus when stored at refrigerated temperature (Fig. 9). In contrast, the formulation was destabilized at room temperature resulting in drug leakage with less entrapment efficiency with time. The stability studies suggest that the proniosome formulation was comparatively more stable when stored at refrigerated conditions compared to room temperature.

**CONCLUSION:**

In the present work, Proniosomal powder containing tacrolimus was prepared and evaluated. The optimized formulation typically contains span 60, cholesterol, maltodextrin and API. Formulated proniosomes gave satisfactory results for various evaluation parameters like particle size, Zetapotential, Powdered XRD, DSC, Scanning electron Microscopy, FTIR entrapment efficiency, *In-vitro* Dissolution and *In-vivo/Ex-In-vivo*. From this study, we concluded that solubility and bioavailability of tacrolimus can be improved by incorporating it into proniosomal system.

**REFERENCES:**


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