Nano Particle Based Topical Dosage Form of Tacrolimus for Psoriasis

M.Phil Thesis

by

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Nano Particle Based Topical Dosage Form of Tacrolimus for Psoriasis

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DECLARATION

I hereby declare that the thesis titled **"Nano Particle Based Topical Dosage Form of Tacrolimus for Psoriasis"** submitted at Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad for the award of degree of Master of Philosophy in Pharmacy (Pharmaceutics) is the result of research -work carried out by me under the supervision of **Dr. Naveed Ahmed** during the period 2016-2018. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship. I am aware of the terms copyright and plagiarism. I shall be responsible for any copyright violation found in this work.

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Dedicated to my beloved parents,

respected teachers, my family and friends

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AV	Aloe vera
BCS	Bio pharmaceutics classification system
Cht	Chitosan
COX-2	Cyclo-oxygenase 2
CYP2	Cytochrome P 2
DLS	Dynamic light scattering
DL	Drug loaded
DNA	Deoxyribonucleic acid
EE	Encapsulation efficiency
FTIR	Fourier transform infrared spectroscopy
FDA	Food and drug administration
g	Grams
GRS	Generally recognized as safe
GADD	Growth arrest and DNA detriment
H	Hours
HPLC	High performance liquid chromatography
LOX	Lipoxygenase
LC	Loading capacity
M	Molarity
m^2	Meter square
mg	Milli-gram
ml	Milli-liter
MV	Mili volts
µm	Micrometer

N	Normality
nm	Nanometers
NF-KB	Nuclear factor kappa beta
PDI	Polydispersity index
PVA	Poly vinyl alcohol
PG	Propylene glycol
PEG	Polyethylene glycol
RPM	Revolutions per minute
ROS	Reactive oxygen species
SC	Stratum corneum
SEM	Scanning electron microscope
TAC	Tacrolimus
TEA	Triethanolamine
TGF-B	Transforming growth factor beta
TNF	Tumor necrosis factor
TP	Thymidine phosphorylase
UV	Ultra violet
ZP	Zeta potential

ABSTRACT

Tacrolimus nano particle loaded Chitosan/aloe Vera films were formed to be applied as a potential treatment for psoriasis topically. Formulation was based on the intrinsic properties of the used materials. The method adopted for nano particles preparation was nanoprecipitation or solvent evaporation process. The formulation was optimized in terms of the particle size and stability at various levels of polyvinyl alcohol concentration, phase volume ratios, stirring speed and solvent injection rate. Optimized Tacrolimus loaded nanoparticles prepared were of particle size 236+2 nm with polydispersity index values of 0.11. Particles observed through scanning electron microscopy (SEM) showed that particles were of spherical shape with particle size 150-300 nm. The entrapment efficiency was 77% showing the successful entrapment of Tacrolimus into nanoparticles and percentage drug release within 72 hours from nano particles was more than 80% at pH 5.4. Fourier transforms infrared spectroscopy (FTIR) analysis shows that no electrostatic interaction occurred in the nano system. X-rays diffraction (XRD) studies show the successful loading of drug in to polymer. The nanoparticles optimized were loaded into final formulation (film) based on chitosan and Aloe Vera. The prepared film was studied for swelling, erosion and water transmission test. All the tests show that by increasing aloe vera concentration in the film increases the water up take capacity. Water vapor transmission rate (WVTR) also shows that water diffusion and permeability increase as AV concentration increases.

Keywords: Eudragit E100 nano particles, Encapsulation efficiency, Particle size, Alove Vera and Film.

Chapter 1

Introduction

1. INTRODUCTION

1.1. Background

In treatment of mild to moderate Psoriasis topical route for drug delivery is considered very helpful when used in conjugation to systemic therapy. However, some major concerns are there in compliance and efficacy of topical route. Studies indicate that around 70% of patients were not satisfied with the treatment (Finlay and Ortonne, 2004). Undesirable skin interactions and lack of effective drug delivery are the main reasons for patient dissatisfaction and non-compliance (Fouere *et al.*, 2005). Nevertheless, new approaches in drug formulation have significantly increased the hope of improvement in topical delivery of drugs making it more beneficial and increasing patient compliance (Bos and Spuls, 2008). The present research work endeavors to development of novel composite system based on nanoparticles relating to topical management of Psoriasis.

Historically, to counter the issues related to safety and efficacy, new drug discovery and development was in practice, but it takes very long to develop a new drug entity and incurs huge cost. Later it was realized that drug absorption and distribution significantly effects safety and efficacy.

It has been observed that the drug deviates from the desired site of action and give a generalized effect. Sir Paul Ehlrich coined the term "magic-bullets" for the targeted drug delivery which reach the desired site and imparts action thereby increasing safety and efficacy (Morganti *et al.*, 2001). This concept gave rise to an alternate approach of drug delivery in which drug is delivered at specific receptors while other tissues and organs remain unaffected. Carefully designed carriers can cargo drug to bring favorable variations in interacting microenvironment and its delivery. Transportation of the drug to the diseased location can be improved by modification in properties of biological membranes which act as barriers as well as altering the physicochemical properties of drug itself. Furthermore, site specific drug bioavailability and drug-receptor interaction can be enhanced by modulating the composition of carrier systems. These factors result in improved compliance and patient safety by improving pharmacodynamic response. Various routes of administration have been explored for the delivery of these novel carriers however, topical route is still considered most

efficient and practical in treatment of dermatologic ailments like psoriasis, eczema and atopic dermatitis (Goebel *et al.*, 2011).

In contrast to creams and ointments, the conventional formulations, the composition and special exterior and interior design of these novel formulations makes thems unique (Hadgraft, 1996). The choice of system is affected by various pharmaceutical and disease factors. Alteration in methods of manufacturing and composition effects the nature of such nanostructures especially their size, and surface properties. The novel carrier systems film will be useful in addressing issues associated and will improve patient compliance.

1.2. Psoriasis

According to National Psoriasis Foundation, around 7.5 million Americans are suffering from psoriasis and it is one of the most prevailing autoimmune diseases in the United States. Globally this number has reached 125 million. Both the direct and indirect costs of psoriasis management is $11 billion annually with work loss accounting for greater than 40% of this number (Kimball *et al.*, 2011).

Psoriasis is thought to be immune mediated inflammatory skin disease. It is characterized by the appearance of pink colored plaques and white flakey skin. It is classified into three grades mild, moderate, and severe. Patients having less than 3 percent of their body covered by plaques are classified as mild, having between 3 and 10 percent are moderate, and greater than 10 percent covered comes under severe (Shah *et al.*, 2002).

Psoriasis was initially considered to be a disease caused by a malfunctioning of keratinocytes, as when normal control division of these epidermal cells is lost, leading to epidermal hyperplasia and psoriatic plaques formation. But, more recently, immunosuppressant molecular research and animal studies have provided the facts that the immune system is one of the responsible and contributing factor for the disease. Moreover, psoriasis has a genetic component that is not fully elaborated yet completely. The disease etiology is a multifunctional nature resulting from the interaction between genotype and environmental factors (Richter and Brisson, 2004). Potential triggers for the disease include injury, microbial infection, stress, alcohol drinking, smoking and drug abuse. After initial stimulation, there occurs a cascade of

events, including the activation of dermal dendritic cells and proliferation of auto-reactive T cells occurs leading to the production of T cell, dendritic cell, macrophage, and keratinocyte production of cytokines (including IL-1,2, 6 and 8, IFN-γ, and TNF-α). TNF-α, one of the vital cytokines responsible for the disease, is able to stimulate and propagate extensive immunological activity (Prüfer and Jirikowski, 1996). It induces keratinocytes to produce chemokines and other cytokines. These mediators allow csirculating immune system cells to enter the skin due to an increase in expression of dermal endothelial adhesion molecules. This series of events leads to a cycle of cytokine production, over-proliferation of keratinocytes, influx of additional immune cells, and the overall inflammation associated followed by psoriasis lesion formation (Wan *et al.*, 2017).

Psoriasis appears mainly as increased keratinocytes proliferation in the basal layer of the epidermis. In non-psoriatic skin, turnover occurs over 30 days, but in psoriasis, the rate of keratinocyte proliferation occurs approximately 7 10-fold faster. This increase in basal layer cell proliferation is not matched by an increased rate in cell removal from the top layers of skin, and so, plaque buildup occurs (Voinova *et al.*, 1999).

There are numerous treatment options currently on the market for psoriasis, but no known cures. These treatment options and their possible side effects are summarized in table 1.1 in order from treatments for severe psoriasis to more mild cases (Yuan and Verma, 2006).

Topical treatment is considered first line in mild to moderate psoriasis and in case of severe psoriasis it is often considered as initial treatment. In fact, approximately 80% of patients are treated topically because the disease is located in the skin and therefore local, non-systemic approaches for treatment are ideal (Myers *et al.*, 2006).

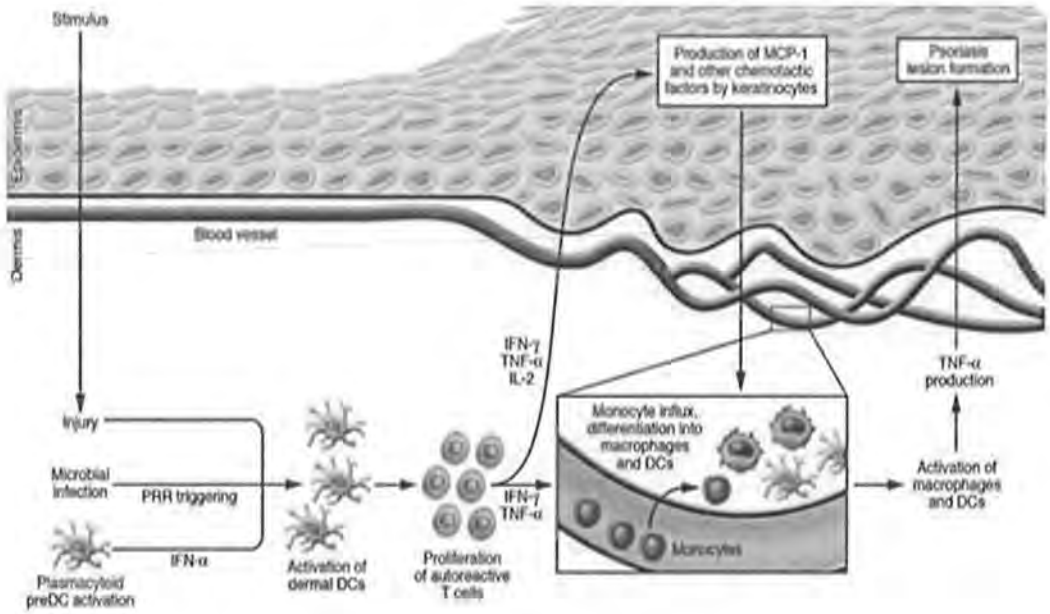

Figure 1.1: Pathophysiology of psoriasis

1.3. Topical drug delivery challenges in psoriasis

Stratum Corneum (SC), the outermost skin layer is active and not inert to the penetration of xenobiotics. Although it permits absorption of most materials to some extent but it does not allow free movement of any molecule across the membrane. It has been demonstrated that main route of absorption across SC is intracellular lipids (Morganti *et al.,* 2001). Hydration of SC imparts significant effect in absorption rate of a given solute. Level of hydration is a function of concentration gradient of water between surface of skin and dermis (Hadgraft 1996).

An increase in cholesterol levels and decrease in ceramides levels have been observed in rigidized psoriatic skin (Wetz *et al.*, 1989). In this case standardized moisturizing factors (SMFs) like water are almost absent. This absence of SMFs and numerous other factors targeting psoriatic skin via topical route is challenging.

Numerous topical formulations are being used in Psoriasis management but none of them is considered an ideal treatment. There are various factors to this including,

conventional vehicle intrinsic adverse effects, changes in physicochemical properties drug and carrier and level of drug absorption through skin (Menter *et al.*, 2009). Hence systemic carriers-based approach can be used to achieve desired physicochemical effects and mitigate the intensity and frequency of adverse effects of drugs (prufer and Jirikwoski, 1996). Conventional formulations like creams, gels, lotions, ointments etc. are when used for topical delivery of anti-psoriatic agents, drug related problems arise resulting in low compliance. Therefore, it must be appropriately designed such that the issues associated with thickened and dehydrated psoriatic skin may be addressed (Piacquadio and Kligman, 1998).

1.3.1. Challenges for topical drug delivery

- First pass effect
- Reservoir capacity of skin
- Toxicities of drug and irrational potential
- Due to disease state and age variability in per cutaneous absorption
- Heterogenicity of skin in metabolism and turnover
- Lack of understanding to reduce percutaneous absorption through modern technology

1.4. Topical Management of Psoriasis and Novel Drug Delivery System

For treatment of psoriasis novel drug delivery systems offer exclusive beneficial features. Keeping in view these benefits there are ongoing efforts to utilize novel drug delivery systems to improve the management of psoriasis by applying topical drug formulation.

1.4.1. Role of novel drug delivery systems

- Biodegradable and biocompatible system
- Utilization of versatile nanocarrier system
- Protection of drug molecules from various problems
- Targeting of receptors by passive method
- Loading of hydrophobic and hydrophilic drugs
- Alteration in physicochemical properties

1.5. Skin

Human skin is a largest extensive and accessible organ of human body that plays a key role in 1) protection of the body from exogenous materials, 2) protection of the body from dehydration, 3) assisting in thermal regulation of body, 4) excretion of water, salt, and other substances to maintain homeostasis, and 5) serving as a crucial sensory organ for tactile and thermal stimuli. These diversified functions are possible due to the complex anatomical structure of the skin (Tobin, 2006).

1.5.1. Skin anatomy

The skin is composed of three main layers: the epidermis, dermis, and underlying hypodermis. The epidermis is the topmost layer that comes in direct contact with the outside environment. It is a stratified, avascular epithelial layer. Beneath the epidermis is the dermis having thickness of 3-5 mm. it contains a matrix of collagenous fibers, elastic connective tissues, proteoglycans, glycosaminoglycans, and structural glycoproteins. It contains the main cellular components including fibroblasts, endothelial, and mast cells and is embedded with blood vessels, lymphatic vessels, nerve endings, sweat glands, and pilosebaceous units. When there occurs any inflammation, macrophages, lymphocytes, and leukocytes may permeate this dermal matrix (Kilfoyle, 2011).

The lowest layer of skin is the hypodermis which serves to attach the skin to the underlying structures. The hypodermis is made up of a network of adipocytes and serves as a site of, metabolism, thermal insulation, energy storage and shock absorption source (Schaefer *et al.*, 1979).

Since the capillaries in the dermis are situated close to the dermal-epidermal junction, the dermis is not measured to contain a significant barrier for drug delivery into the skin. Therefore, further structural discussion will focus on the epidermis where numerous studies (utilizing tape-stripping, lipid extraction, sandpaper, etc.) have shown the major barrier restraining the absorption of chemicals into the skin exists. The epidermis is mainly composed of stratified, squamous epithelium that is divided into the stratum corneum and the viable epidermis. The stratum corneum, which makes up the major skin barrier, has been described as having a brick and mortar structure where the bricks are flattened, dead, anucleated cells, called corneocytes and

the mortar consists of multiple lipid bilayers. The corneocytes contain a core of keratins that accumulate during epidermal differentiation, measure between 20 and 40 μm, and are constantly shed and replenished during the normal cycle of skin turnover (Menon, 2002).

The intercellular lipid bilayers, having the composition of ceramides, cholesterol, fatty acids, and cholesterol esters that establish themselves into multiple broad bands 23-25 folds, are synthesized in the viable epidermis and are then exocytosed into the intercellular space. The viable epidermis consists of melanocytes, 10-20 layers of keratinizing epithelial cells, Langerhans cells, and Merkel cells. The viable epidermis can be further broken down into the: Stratum basale – location of cellular replication, consisting of cuboidal cells in a singular layer connected to each other by desmosomes and to the basement membrane by hemidesmosomes; stratum spinosum - consisting of polygonal shaped cells that contain an increased amount of keratin filaments and desmosomes; stratum granulosum - consisting of two to five layers of flattened, rhombic cells with their axes parallel to the surface of the skin that transitions the epidermis between living cells and a dead horny layer; and stratum lucidum - thin layer between the stratum granulosum and the stratum corneum that is not typically identifiable. The primary task of the viable epidermis is the production of the stratum corneum (Junginger *et al.*, 1994).

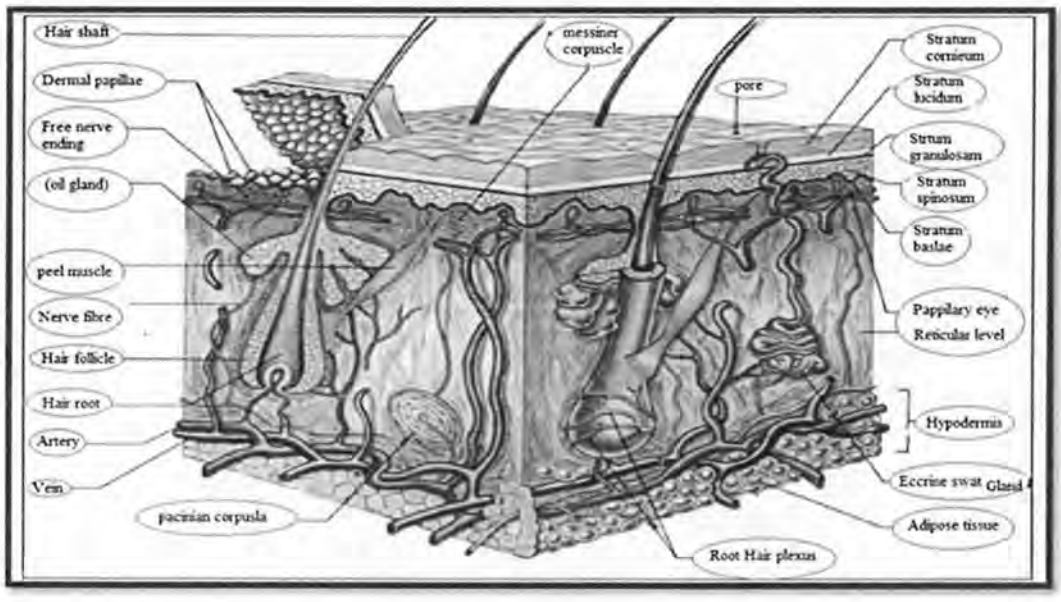

Figure 1.2: Human skin Anatomy (Williams)

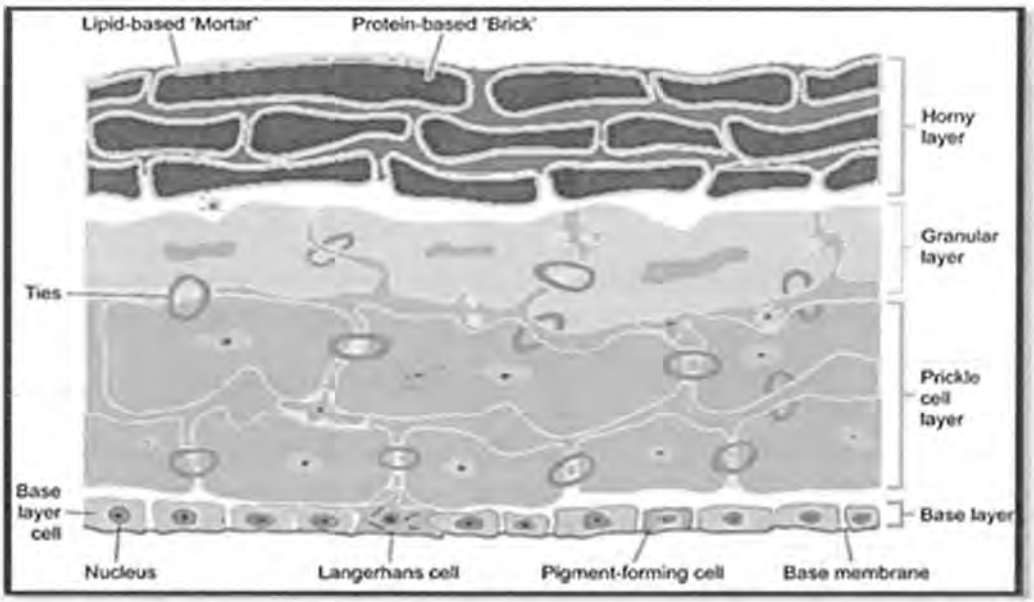

Figure 1.3: Structure of epidermis (Namazi, 2005; Chinchole *et al.*, 2016)

1.5.2. Common skin barrier problems in psoriasis

- Thickness of inflamed skin lesions along with scales
- Sensitivity of skin
- Hairy tethered skin
- Imbalance in skin lipids
- Differentiation of corneocytes and excessive growth
- Natural factor of moisturization and dry deficient skin

1.6. Topical drug delivery

For drug delivery human skin provides a viable target implementing various clinical benefits. It includes escaping from hepatic metabolism, reduction in plasma level fluctuation, improved patient compliance, termination of action with ease (Singh, 2006). In spite of these advantages, its utility as a gateway for drug delivery is limited by the barrier function of the stratum corneum. Skin delivery is subdivided into two main types, topical drug delivery and transdermal drug delivery. The main distinction between these two groups is that drugs delivered trans dermally are intended for systemic use while drugs delivered topically are intended for local delivery into the skin. Topical therapy of skin disorders allows high drug levels at the site of disease

and targets pathological sites of the skin with slight systemic absorption. Drug localization of this sort is vital in the treatment of dermatological conditions such as skin cancer, psoriasis, eczema, and all microbial infections (Wertz, 1996).

A common hurdle in both transdermal and topical drug delivery is bypassing the stratum corneum. To this end, numerous methods, including various active and passive methods, have been researched and developed. Active methods include the use of iontophoresis, sonophoresis, electroporation, and microneedles (Henry *et al.*, 1999). Penetration can be enhanced by applying passive methods that comprise of various form of drug delivery systems. Good delivery systems along with penetration enhancers should be pharmacologically inert, chemically stable, well-suited with the formulation, nonirritating, well-designed, and have revocable effects on the skin (Smith, 1995).

Delivery across the stratum corneum occurs, as seen in figure 1.2, via two main pathways: the shunt routes (across the hair follicle or sweat ducts) and the trans epidermal pathway (across the intact stratum corneum) (Barry, 2001). Shunt ways include sweat glands and pilosebaceous glands. Although they exist in dermis but they are reachable to external environment. Theoretically, stratum corneum barrier can be bypassed. Invaginating epidermis into dermis provides large surface area which in response enhances possible permeation. The trans epidermal route can be further subdivided into intercellular (through the intercellular lipids) and intracellular (through the corneocytes) pathways (Meidan *et al.*, 2005).

Historically, the assumption has been that topically applied substances predominantly use the stratum corneum pathway to permeate through skin because the stratum corneum accounts for greater than 99% of the entire skin surface (Barry, 2002) However, in the late 1960s, researchers showed that under appropriate conditions, each route may be dominant and that in most cases, both routes play a role in drug delivery Penetration typically occurs through a combination of these pathways with highly hydrophilic, charged, and/or large compounds preferentially utilizing the shunt routes and highly lipophilic compounds preferring the intercellular pathway (Mckenzie, 1962).

Due to the lipid rich environment, the stratum corneum is selectively permeable to lipophilic compounds, allowing diffusion into the lower skin strata (Mitragotri, 2003).

In some cases, lipophilic compounds may remain in the lipid environment of the intercellular space acting as a drug depot that slowly permeates to the viable epidermis. Typical penetration of compounds into skin involves partitioning into more hydrophilic epidermis and stratum corneum along with diffusion and metabolism in stratum corneum and epidermis (Ponec, 2002).

1.7. Drugs Used as Psoriasis Therapies

The following drugs are mainly used in management of psoriasis.

Table 1.1. Treatment options for psoriasis and their potential side effects.

Treatment Options	Potential Side Effects
Systemic treatment with anticancer and immune system suppressants	Systemic toxicity
Biological agents (TNF-α blockers) to reduce systemic inflammation	Auto-immune disease
Phototherapy / Photochemotherapy	Carcinogenicity
Topical (vitamin D3 analogs, corticosteroids, retinoids, etc.)	Skin irritation and skin thinning

1.8. Model drug tacrolimus

Fungus Streptomyces tsukubaensis is main source of tacrolimus which is an immunosuppressant macrolide. It is very beneficial for management of Psoriasis by constraining inflammation and pro inflammatory cytokines (Honbo *et al.*, 1987). For topical management of intertriginous and facial psoriasis it was indorsed by American academy of Dermatology. Nevertheless, its high molecular weight (822.05 D) and BCS class 2 properties avoids its penetration through skin which in turn reduces its efficacy in topical treatment of psoriasis (Abruzzo *et al.*, 2017). Protopic® is the only existing marketed formulation of tacrolimus (TAC) for topical use. TAC formulation was based on oil matrix especially propylene carbonate (PC) which made it problematic to remove from surface of skin. Its greasy and oily nature also causes unpleasant sensation to patient. Basic aim was improvement of permeation with prime focus on percutaneous permeation. Nanotechnology based various approaches had been established for topical delivery of TAC (Wan *et al.*, 2017).

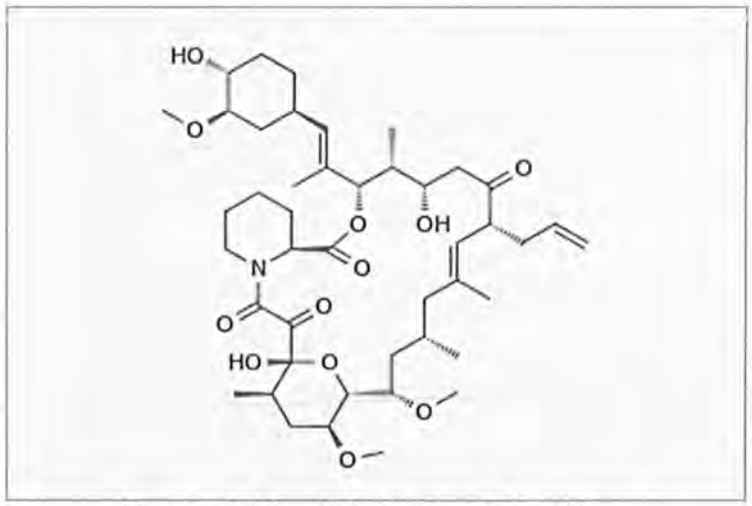

Figure 1.4: Chemical structure of tacrolimus

1.9. Nanoprecipitation

For encapsulation of drug molecules especially for hydrophobic drug, one of the firstly established technique is nanoprecipitation which is also known as interfacial deposition or solvent evaporation method (Fessi *et al.*, 1989). In the form of nanospheres and Nano capsules this technique is widely used for encapsulation purposes. This is reproduceable and easy procedure that has been extensively utilized in nanoparticles formulation. It has several benefits over other encapsulation methods which includes better reproducibility, scale up easiness, circumvention of huge quantity of toxic solvents, simplicity, less energy input and narrow size distribution.

1.9.1. Mechanism of particle formation by nanoprecipitation

Nanoparticles having narrow size distribution can be formulated by nanoprecipitation technique which is comparatively an easy and reproducible method (Fessi *et al.*, 1989) It mostly involves two phases mainly aqueous and organic phase that are miscible. Aggregation, growth and nucleation three phases that play an important role in particle formation through this technique. This phenomenon is derived by the supersaturation, it is basically ratio of polymer solubility to polymer concentration. Nucleation rate is controlled by the supersaturation hence it plays a vital role. Therefore, poor mixing always yields particles of larger size which is not desired (Budhian *et al.*, 2007; Lince *et al.*, 2011). Liquid having low surface tension that includes organic solvents is forcefully pulled by the liquid having higher surface tension (Aqueous phase). Interfacial turbulence is originated due to these alterations

that exist between the two liquid phases. It also produces thermal inequalities in between the system (Mora-Huertas *et al.*, 2010).

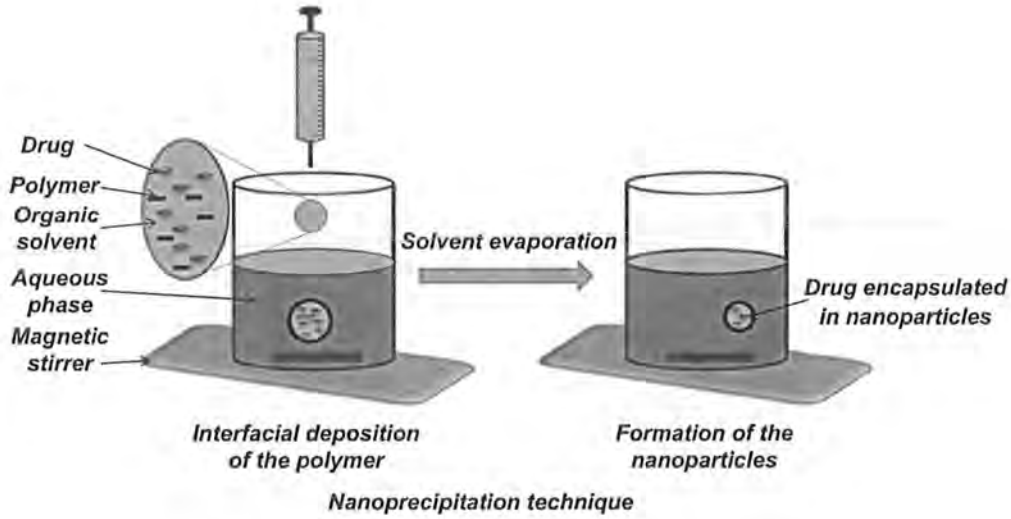

Figure 1.5: Nano precipitation technique

1.10. Naturally occurring treatments: Aloe Vera

Plant research represents a continuous efforts and trials to discover new compound against disease causing agents. Natural and semi synthetic products are main source of marketed antibiotics, among them 20% are mainly subjected to biological and pharmacological tests (Mothana and Lindequist, 2005), among 400 existing species most biologically active *Aloe barbadensis* which is living under satisfactory condition nearly from 50 years (Joshi, 1998; West and Zhu, 2003; Yagi *et al.*, 2003). it contains many polysaccharides and monosaccharides i.e. vitamins B1, B2, B6 and C and enzymes like amylases, lipases and phosphatases and several organic and inorganic ingredients (Vogler and Ernst, 1999; Grindlay and Reynolds, 1986). Acetylated mannose is chief useful component of Aloe vera (Djeraba and Quere, 2000; Lee *et al.*, 2001).

Gel of aloe vera is available in the form of powdered concentrate. Dermal ischemia frostbite and intra-arterial drug abuse can be inhibited by using this gel matrix. It is evident from the animal studies that mechanism of action is through inhibition of thromboxane A2 (Atherton, 1998).

Number of animal studies and in vitro studies explain anti-inflammatory properties are due to activity of bradykinase (Sahu *et al.*, 2013). Bradykinin causes pain and it is

broken down by the bradykinase which is derived by the aloe plant. C-glucosyl chromone is a novel anti-inflammatory compound and it is also extracted from aloe vera. This plant decreased prostaglandin E2 construction as well as cyclo-oxygenase pathway (Vázquez *et al.*, 1996). Freshly formulated gel of aloe vera lessen acute inflammation but it is ineffective in chronic inflammation. Sterols of aloe vera gel are found to eliminate inflammation caused by the croton oil in mice. Among them lupeol is most active anti-inflammatory sterol, decreasing inflammation depending on dose. The data propose that explicit plant sterols may also subsidize to the anti-inflammatory activity of gel (Madan *et al.*, 2008).

For nutraceuticals, skin care and cosmetics aloe vera extracts are extensively utilized (Cragg and Newman, 2001). Anti-oxidant protein also known as metallothionine is produced in the skin when aloe vera is administered. It in turns suppress superoxide dismutase and glutathione peroxidase as well as hunts hydroxyl radicals (Sato *et al.*, 1990). In some studies, burning sensation of skin and contact dermatitis was also reported after aloe vera topical application.

Table 1.5. Chemical composition and properties of *Aloe vera*

Constituents	Properties and activity
Amino acids	Basic building blocks of proteins in the body and muscle tissues
Anthraquinones	Analgesic, antibacterial
Enzymes	Antifungal and antiviral activity but toxic at high concentrations
Hormone	Wound healing and anti-inflammatory
Minerals	Essential for good health
Salicylic acids	Analgesic
Saponins	Cleansing and antiseptic
Steriods	Anti-inflammatory agents, lupeol has Antiseptic and analgesic properties
Sugar	Anti-viral, immune modulating activity of acemannan
Vitamins	Antioxidant (A, C, E), neutralizes free radicals

1.11. Literature Review

Azuma *et al.*, (2016) assessed the effect of Tacrolimus/chitin nanofibril (CNF) by application of skin swabs on an experimental AD and psoriatic model. CNF also showed the anti-inflammatory effects by suppressing the activation of cyclooxygenase-2, nuclear factor-kappa B and inducible nitric oxide synthase. It may be concluded that TAC CNF is a potential functional biomaterial for suppression of psoriasis and AD.

Katas *et al.*, (2012) Studied the chitosan loaded hydrocortisone (HC) nanoparticles (CS NPs) as a percutaneous drug delivery system synthesized by ionic gelation method. This formulation was considered as excellent candidate for sustain delivery of HC despite of its low permeation from CS NPs in aqueous cream. These experimental findings proved that HC-loaded CS NPs could be used as a promising system for delivery of drugs especially for anti-inflammatory drugs. They are found to improve efficacy and also decrease toxic effects.

Lam Ms *et al.*, (2012) Prepared microemulsion cream formulation of tacrolimus ointment against in hapten-induced murine model of skin dermatitis was developed which enhanced the penetration through skin. The enhanced penetration was possible by of reducing dose. The developed cream formulation in this experiment was found to be significantly deposited at the targeted site with substantial reduction in cytokine expression.

Alam *et al.*, (2012) Developed the clobetasol propionate (CP) topical o/w Nano emulsion through aqueous phase titration method. It was found clearly that the CP loaded Nano emulsion significantly increased their anti-inflammatory activity and nucleoside triphosphate diphosphohydrolases activity in lymphocytes. Absence of irritation in *In vivo* irritation studies in spite of high amount of surfactant proved the safety of developed Nano emulsion for human use.

Pople and Singh (2011) Studied the optimization and development of tacrolimus loaded modified Nano lipid carrier (T-MNLC) by using high pressure homogenization in order to enhance the drug solubility in carrier lipid matrix by using lipophilic solubilizer for topical delivery. T-MNLC displayed sufficient stability attributed to reduce total lipid concentration in carrier. Thus, the study highlighted the increased

encapsulation efficiency of colloidal lipid carriers prepared by novel T-MNLC using lipophilic solubilizers with benefit of improved skin localization performance, stability and internalization in psoriatic skin.

Keck *et al.*, (2010) Developed physically and chemically stable prednicarbate Nano emulsion with positive charge as a Nano carrier system for the treatment of psoriasis. In context of dermal delivery, use of positively charged carriers are beneficial, owing to the positive charge which enhances an increased adsorption through negatively charged skin, increasing retention time and bioavailability at desired site.

Songkro *et al.*, (2011) Formulated micro/Nano emulsion as vehicles for delivery of natural substance plaunoi extract (*Croton stellatopilosus*) containing plaunotol as a chemical constituent. Latter has shown to possess antimicrobial activity for treatment of dermatitis and psoriasis. A 2% w/w plaunoi-loaded emulsion showed improved antibacterial activity for *Staphylococcus aureus (S. aureus), Staphylococcus epidermidis (S. epidermidis)* when compared with the blank formulations. Th skin irritation was not noticed in all plaunoi loaded Nano emulsion (NE-P) treated rabbits. Both plaunoi loaded micro emulsions (ME1-P) and ME2-P exhibited minor erythema. These experimental results suggested a potential use of Nano emulsion for topical delivery of plaunoi extract.

Muller *et al.*, (2010) Demonstrated the cyclosporine A(CyA) amorphous nanoparticles suspension by using wet bead milling method and studied the physical/chemical long-term stability. The micro/nanosized amorphous cyclosporin was studied using tape stripping in the pig ear skin test to improve skin penetration which explained the superiority in penetration. Improved dermal CyA delivery was feasible due to formulations of amorphous CyA nanoparticles.

Batheja *et al.*, (2010) Investigated topical delivery of lipophilic molecules as carriers for tyrosine derived nanospheres containing diclofenac sodium as model drug. Tyrosine derived nanospheres as either gel formulation or aqueous dispersion are biocompatible for topical delivery systems since they did not induce any morphological changes or short-term cytotoxicity in stratum corneum. Hence, gel formulation of tyrosine-derived nanospheres offer an adjustable and promising platform for benign and effective topical delivery of lipophilic therapeutics for treatment of dermatological conditions as required.

Kang *et al.*, (2010) Developed the topical preparations of taxifolin glycoside (TXG). The TXG-loaded Pep1-elastic liposomes formulation was synthesized by conjugating Pep-1 peptides to drug containing EL via the thiol maleimide reaction and examined for their efficacy and skin permeation. Moreover, the multiple immunological parameters including IL-4, IgE, and IFN-γ in NC/Nga mice were formulation normalized with serum levels approaching those of healthy mice. It may be concluded that TXG-loaded Pep1-EL preparations may offer a potential therapeutic means for the treatment of psoriasis, and should be further investigated.

1.12. Aim and Objectives

The aim of the present work was;

To develop polymeric Nano particulate carrier system loaded with Tacrolimus for Management of Psoriasis.

Objectives

- Preparation and optimization of nanoparticles
- To study the physicochemical behavior of nanoparticles
- Evaluation of the prepared nanoparticles for drug loading, encapsulation efficiency, drug release studies.
- Preparation and evaluation of final dosage form of optimized nanoparticles (film).

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Tacrolimus (LC Lab, USA), Chitosan, Eudragit 100 (Eu E100), Monobasic potassium phosphate, poly vinyl alcohol (Sigma Aldrich, Germany) Glacial acetic acid, Sodium Hydroxide (NaOH), Sodium Chloride (NaCl), Hydro Chloric acid (HCL), Glycerol (Merck, Germany), Ethanol (BDH-Laboratory suppliers England), Distilled water and aloe vera.

2.2. Instruments and Equipment

Analytical balance (Ohaus corporation, PA-216C, USA-pH Meter), Centrifuge Machine (Hermle-GmbH-Z-326K, Germany), Magnetic stirrer hot plate (PCSIR-MSHP-1A111, Islamabad, Pakistan), Vortex Mixer (Bante instruments), Pharmaceutical Freezer (Panasonic MDF-137, Japan), Pharmaceutical refrigerator, (PHS, 25GW, Chicago, USA), Lypholizer (Alpha 1 -2LD plus, Chirst, Germany), HPLC (Aglient 8543), Zeta sizer (Nano ZS, Malvern instruments, Malvern UK), Screw gauge (Panasonic, MRP-161H, Japan), Ultra-sonicator (Elm sonic - Gmbh, E60H), Stability Chamber, Germany), Spectrum-100 FTIR-Spectrophotometer (L160000A, Perkin Elmer, USA), Magnetic stirrers, Pipettes, glass vials, Eppendorf tubes and Volumetric flasks.

2.3. Methods

2.3.1. Solutions preparation

2.3.1.1 Organic phase preparation for blank formulation

For preparation of organic phase, 200 ml Eudragit E100 was dissolved in sufficient quantity of ethanol to make final volume (up to10 ml) by continuous stirring on magnetic stirrer.

2.3.1.2 Poly vinyl alcohol solution (0.5%) preparation

For preparation of 50 ml of poly vinyl alcohol solution 250 mg of PVA was added in a beaker with continuous stirring at 40°C for 4h and sufficient quantity of distilled water was added to make the volume. Continuous stirring was provided with help of

magnetic stirrer to completely dissolve the PVA. Volume was made up with help of distilled water up to 50 ml.

2.3.1.3 Organic phase solution for drug loaded emulsion

To prepare drug loaded emulsion 200 ml of Eudragit E100 polymer was transferred into ethanol in a beaker with continuous stirring and 10ml of organic phase was prepared. After that 10 mg of Tacrolimus was added with continuous stirring until complete dissolution. Final volume was made up to 10 ml.

2.3.1.4 Acetic acid solution (1 %) preparation

1% acetic acid solution was prepared by addition of glacial acetic acid 1 ml into distilled water. Continuous stirring was provided until dissolution and final volume was made up with distilled water up to 100 ml.

2.3.1.5 Preparation of 2% sodium hydroxide solution (NaOH)

Accurate weighed NaOH (2g) was added into distilled water having sufficient quantity. Distilled water was used to make final volume up to 100 ml.

2.3.1.6 Chitosan solution (2%) preparation

Accurately weighed Chitosan (2g) was dispersed in 99 ml of distilled water and continuous stirring was provided. Final volume was made up with help of 1% acetic acid solution up to 100 ml.

2.3.2. Preparation of phosphate buffer saline

Phosphate buffer was used as a medium to check the drug release from nano particles and also for the film that was impregnated with nano particles. To achieve desired purpose 1 liter of distilled water was used to dissolve 0.028 moles of $NaHCO_3$, 0.102 moles of NaCl, 0.002 moles of Na_2SO_4 and 0.002 moles of Na_2HPO_4. Entire set was equilibrated at 32°C with continuous stirring. pH of system was adjusted at 5.4 using 1N NaOH or 1N HCL solution as required.

2.4. Preparation of Calibration Curve of Tacrolimus by HPLC

Different solutions of tacrolimus working standard at concentration of 0.04 mg/ml in different medias i.e. Acetonitrile, Methanol, Ethanol and water were scanned over the

UV range of 190 to 400 nm to determine the lambda max 214 nm. Five different dilutions of Tacrolimus working standard at concentration i.e.10, 20, 30, 40 and 50 were selected in ethanol. All the five solutions of different concentration of all the medias were studied. The reading of the blank was subtracted from over all reading in order to get correct value and the calibration curve were drawn.

2.5. HPLC Method of Analysis for Tacrolimus

The mobile phase consisted of acetonitrile and water at 3:1 and the diluent used was the same mobile phase.

Diluent: Mobile phase

Chromatographic System:

Table 2.1: Chromatographic System

Column	C18, 4.6mm ×250 mm (5µ)
Lambda max	214 nm
Injection volume	20 µL
Flow rate	1.5 ml/min
Temperature	50 °C
Retention time	5 min approx.
Run time	10 min
Concentration	0.04 mg/ml

2.6. Optimization of nano particles

For optimization of nano particles, hit and trial method was utilized. The parameters like injection speed, stirring rate and ratio of organic to aqueous phase were studied under surfactant with variable concentrations. Two responses particle size and stability were measured for each trial.

2.6.1. Optimization of Critical Process Parameters

2.6.1.1 Effect of surfactant concentration

By taking different concentration (mg/ml) of PVA i.e. 0.25, 0.5, 0.75, 1 and 1.5, five samples were prepared and all others parameters were kept constant and their effects were studied.

Table 2.2: Effect of surfactant concentration

Formulation code	Surfactant conc.	Tacrolimus mg	Volume ratios Aq/OC	Injection rate ml/min	Stirring rate RPM
F1	**0.25**	20	1:4	4.30	650
F2	**0.50**	20	1:4	4.30	650
F3	**0.75**	20	1:4	4.30	650
F4	**1**	20	1:4	4.30	650
F5	**1.5**	20	1:4	4.30	650

2.6.1.2 Volume of aqueous phase to organic phase

Volume of organic phase was kept constant (10 ml) and volume of aqueous phase was varied in ratio of (3,4,5,6 and 7 ml) for preparation of nano particles and their response was studied.

Table 2.3: Volume of aqueous phase to Organic phase

Formulation code	Volume ratios OC/AQ	Tacrolimus mg	Surfactant conc	Injection rate	Stirring rate
F6	**1:3**	20	0.5	4.30	650
F7	**1:4**	20	0.5	4.30	650
F8	**1:5**	20	0.5	4.30	650
F9	**1:6**	20	0.5	4.30	650
F10	**1:7**	20	0.5	4.30	650

2.6.1.3 Effect of injection rate

Organic phase (1 ml) was injected into aqueous phase at the described speed of 2.30, 3, 3.30, 4 and 4.30 minutes injection speed.

Table 2.4: Effect of injection rate (ml/min)

Formulation code	Injection rate ml/min	Tacrolimus mg	SURFACTANT con	Volume ratios OC/AQ	Stirring rate RPM
F11	**2**	20	0.5	1:3	650
F12	**2.30**	20	0.5	1:4	650
F13	**3**	20	0.5	1:5	650
F14	**3.30**	20	0.5	1:6	650
F15	**4.30**	20	0.5	1:7	650

2.6.1.4 Effect of stirring speed (rpm)

Five samples were prepared at different rpm. The speeds selected were 600, 650, 700, 750, and 800 rpm and all other variables were taken constant.

Table 2.5: Effect of stirring speed

Formulation code	Stirring rate RPM	Tacrolimus mg	SURFACTANT con	Volume ratios OC/AQ	Injection rate ml/min
F16	**600**	20	0.5	1:3	4.30
F17	**650**	20	0.5	1:4	4.30
F18	**700**	20	0.5	1:5	4.30
F19	**750**	20	0.5	1:6	4.30
F20	**800**	20	0.5	1:7	4.30

2.7. Preparation of nano particles

2.7.1. Preparation of blank nano particles

Nanoprecipitation method or solvent evaporation method was used for preparation of nano particles with slight modification. Hot plate was used for this purpose and 40 ml of 0.5 % PVA solution was placed at 650 rpm at 30°C with continuous stirring. Organic phase (10 ml) was taken into a syringe and injected slowly in PVA solution by maintaining the speed at 1 ml in 4.30 minutes. Organic phase was evaporated in open air until its smell completely disappeared from the beaker. Centrifugation was performed at 13000 rpm for 30 minutes to attain nano particles pellets.

2.7.2. Preparation of tacrolimus loaded nano particles

To prepare tacrolimus NPs, organic phase contains tacrolimus already dissolved in it, the rest of process was kept constant over here as for preparation of blank NPs.

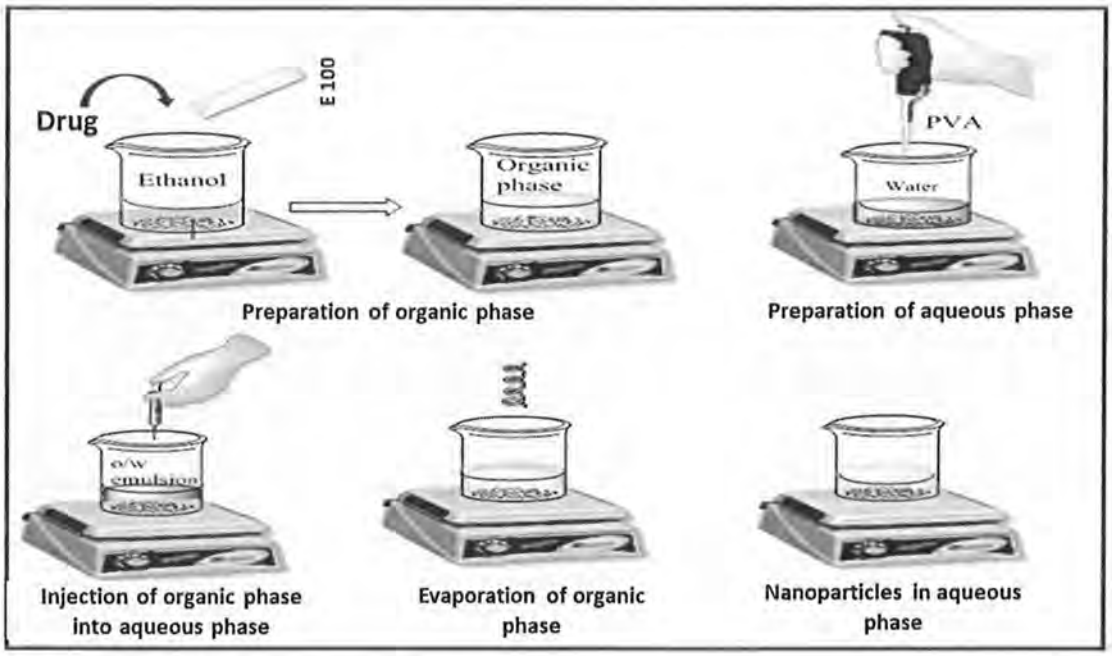

Figure 2.1. Preparation of blank and drug loaded NPs by nano precipitation method

2.8. Extraction of *Aloe Vera* Gel

Alove vera fresh leaves were collected early at morning time. Then they were washed with distilled water to remove dirt and air particles from surface of these leaves. Scalpel shape knife was used to cut the skin carefully for separation of clear gel. Distilled water was used for extensive washing which in turn removed the exudates from the surface. After that gel was broken down into filets. After homogenizing the filets in a blender, they were centrifuged at 13000 rpm and filtration was performed. Thereafter extracted gel was stored at 4°C.

2.9. Characterization

2.9.1. Characterization of nano particles

For determination of novel formulation potential for desired application, characterization of nano particles/nanocarriers was performed.

2.9.2. Particle size

Particle size analysis was performed to check the formation of particles in different ranges especially in submicron size. For optimization of prepared formulation particle size measurement was performed. Mean particle size of blank nano particle along with drug loaded nano particles was performed with help of dynamic light scattering process. Nano emulsion was diluted ten folds with distilled water and then analyzed for particle size distribution. The experiment was done in triplicate.

2.9.3. Zeta potential of prepared nano particles

Zeta potential of nano particles was measured by DLS. Dilutions were made by the same procedure as for particle size.

2.9.4. Polydispersity index (PDI)

DLS technique was used for determination of PDI. Samples for PDI were organized by same process as for the purpose of particle size.

2.9.5. Drug loading and encapsulation efficiency

Indirect method was used to find drug loading and encapsulation in nanoparticles. This method assessed drug content in the supernatant layer which contains free drug. For this method, formulation was centrifuged at 13000 rpm for 1 h. Supernatant was filtered via 0.2 µm syringe filter. After that it was analyzed for presence of free drug with help of HPLC at 214 nm. Results were calculated by computing with that of standard results of calibration curve formed by HPLC.

2.9.6. Percentage yield

For percentage yield calculation, tacrolimus nano particles were fabricated in a beaker and centrifuges at 13000 rpm. After that an empty falcon tube was weighed. Pellets collected after centrifugation were lyophilized. After this procedure percentage yield was calculated by the given formula.

Nano particles yield (%) = Weight of nano particles obtained/Weight of drug+ Polymer taken initially ×100

2.9.7. Morphology

Shape and surface characteristics of the prepared nano particles were observed by scanning electron microscope (SEM). Lyophilized nano particles were used in order to determine surface morphology. The dilution of the prepared nano particles was done in the same way as already discussed in case of DLS.

2.9.8. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis gives information about the interaction between formulation and different constituents. For FTIR analysis, Tacrolimus, Eudragit E100, lyophilized blank nano particles and lyophilized drug loaded nano particles were examined and their spectra were studied.

2.9.9. X-ray diffraction (XRD)

To study the Tacrolimus physical state with in Nano formulation, X-ray Diffraction (XRD) was applied at (25°C, 20 kV and 5 mA) and absolute intensity was recorded against 2 Theta in the range of 10 -90°.

2.10. Preparation of Film

2.10.1. Preparation of blank film

A standardized solution in Aq. Acetic acid (1%) was prepared by dissolving chitosan (2%) w/v. Aloe vera gel that was already extracted was added into already prepared chitosan solution in different ratios of 0:1, 1:1, 1:2, 2:1 and 1:0 v/v. Glycerol (plasticizer) was added in blended mixture (water/glycerol 2.5% v/v). This blended system was kept at hot plate for stirring at 650 rpm for 1 h at 25°C. After homogenization, this solution was transferred in petri dishes and dried for 48 h at 50°C in oven. The neutralization of the film membranes was performed by drenching them in 4% NaOH and ethanol 1:1 for 10 minutes followed by washing with ethanol and distilled water.

2.10.2. Preparation of tacrolimus nano particles loaded film

According to above stated procedure chitosan mixture and aloe vera gel were homogenized. In final formulation slurry of optimized nano particles that was equivalent 0.1% w/v was added in each of these mixtures. By redispersion of the pellets in 5 ml of distilled water this slurry was formed containing nano particles. Mixture were kept on stirring for 30 minutes and were casted into petri dishes. Membrane neutralization was done according to early stated method.

2.11. Method for In Vitro Drug Release

2.11.1. *In vitro* drug release from NPs

Already lyophilized 10 mg powder was added to 50 ml of PBS solution that was maintained at 32°C on shaking water bath for estimation of drug release at pH 5.4. Experiment was performed by withdrawing samples (2 ml) after definite interval of time. By using previously formed standard curve of tacrolimus formed in PBS, samples were analyzed for release studies by analyzing through HPLC at 214 nm. For determination of drug release mechanism, various kinetic models were applied.

2.11.2. *In vitro* release from TAC NPs

To study tacrolimus release in final formulated films, 10 mg drug equivalent nano particles loaded in chitosan film were placed in 50 ml PBS with an aloe vera content of 33% maintained at 32°C in shaking bath assembly. By withdrawing samples (2 ml)

after quantified intervals of time 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 h, analysis was performed via HPLC at 214 nm to determine drug release.

2.12. Kinetics of Drug Release

Various mathematical drug release models were applied to study release mechanism and mass transport from developed systems. Application of these models give drug release data for tacrolimus nano particles and films.

2.12.1. Zero order kinetic model (Dash *et al.*, 2010)

Zero order kinetic model gives information about controlled and constant drug release from given formulation or dosage form. A graph that is plotted between CDR and time is used to determine fitness of drug release. Zero order release rate constant can be obtained from slope of such graph. It can be determined by the following equation.

$$W = k1t$$

K1= Release constant of zero order

T= time in hours

W= CDR (Cumulative drug release)

2.12.2. First order kinetic model (Ritger and Peppas, 1987):

This model elaborates that drug concentration present in defined system impacts drug release pattern. A graph drawn between logs of time and CDR elucidates kinetics of first order.

$$\text{Ln (100-W)} = \text{ln 100 - k2t}$$

k2= Release constant for first order

2.12.3. Hixon crowel's model (Costa and Lobo, 2001):

This model suggests that drug release is limited by dissolution rate. It also explains that release of drug is not limited by diffusion that might occur from the polymeric matrix system. Following equation represents this system.

$$(100)1/31001/3\text{-W} = \text{-k3t}$$

k3= Hixon release constant

2.12.4. Higuchi model (Singhvi and Singh, 2011):

Higuchi model represents drug release from matrix reservoir system through non-crodible manner. This model assumes that faultless conditions of sink are maintained in medium of release. A plot between square root time and CDR is utilized to demonstrate this model.

$$W = k4t$$

2.12.5. Korsmeyers papas model (Dash *et al.*, 2010):

Korsmeyer peppas model describes release of drug versus elapsed time with an exponential function. It is elucidated by equation given below.

$$Mt/M\infty = k5tn$$

N= drug release diffusion exponent (Value describes drug release mechanism)

K5= Constant incorporating geometrical and structural behavior of device measuring drug release.

2.13. Film Characterization

2.13.1. Swelling index

Swelling index of film was measured by gravimetric method. Dried films of 1cm*1cm were equally cut and immersed in beaker having 20 ml PBS (pH 5.4) maintained at 37 °C. After fixed time intervals of 1, 2, 3, 4, 5 and 24 h immersed swollen films were removed carefully. By gently tapping the surface of the film with filter paper, excess of water was wisely removed from the surface and weight of swollen films was recorded at the time intervals specified earlier. Percent swelling was determined by using the formula given below. Bar chat was also plotted for blank and tacrolimus/nanoparticles loaded film at various ratios.

$$Swelling(\%) = (W2 - W1) * 100 / W1$$

W1= weight of the initial dry film

W2 = weight of swollen film

2.13.2. Erosion

Solubility of materials in body fluid can be determined by calculating erosion. Percentage erosion of dried film after 24 h of immersion in phosphate buffer saline can be estimated by this method. Film samples are subjected to 24 h swelling and dried in oven for 24 h at 50°C. After that dried films were weighed. Erosion can be calculated by the formula given below.

Erosion (%) =(W1-W3) *100/W1

2.13.3. Water vapor transmission rate (WVTR)

A USP modified method was used to determine extent of water vapor transmission via film. To attain desired purpose films are sealed onto mouths of gelling tubes that are filled with 5 g of $CaCl_2$. These films are without any defects or pinholes. They were kept in oven to obtain constant weight at 50°C. Weight of each vial can be determined by using analytical balance. After that desiccator containing sodium chloride was used to place the vials in it. By calculating the weight of vials vapor penetration can be determined from 1 to 5 days. A plot of time and weight gain was attained on Microsoft excel. This slope represents vapors diffusing per unit time from the film.

Water vapor transmission rate was expressed as per square meter, gram unit and per day ($mg/m^2/d$). It is calculated from straight333 line slope divided by film area exposed (cm^2).

WVTR= slope (mg/day) / Film area (cm2)

By multiplying film thickness with WVTR, water vapor permeation can be calculated. Experiments were performed in triplicate and average value was reported as standard.

Chapter 3

Results

3. RESULTS

3.1. Calibration curve of tacrolimus in ethanol

Standard calibration curve of tacrolimus in ethanol was made by plotting graph between different concentration of dilutions against respective AUC (Table 3.1) by utilizing MS excel software as represented in Figure 3.1.

Table 3.1: Concentration versus AUC of Tacrolimus in Ethanol

s.no	Concentration	AUC
1	80% (10µg/ml)	2210316.80
2	90% (20µg/ml)	2486601.00
3	100% (30 µg/ml)	2762896.00
4	110% (40 µg/ml)	3039058.00
5	120% (50 µg/ml)	3315468.00

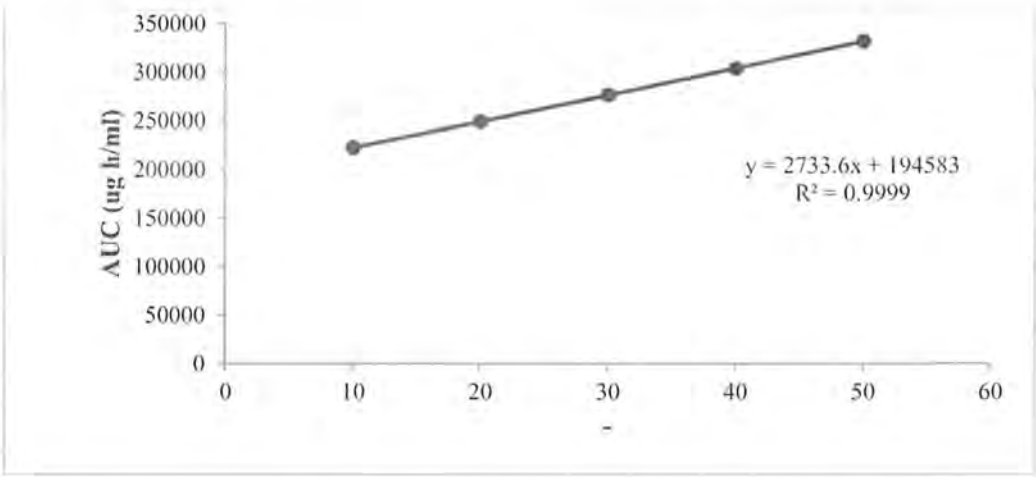

Figure 3.1: Calibration Curve of Tacrolimus by HPLC (Mean:111619)

3.2. Optimization of Process parameters for NPs

Different process parameters and their effect i.e. injection rate, surfactant concentration, volume of aqueous to organic phase and stirring speed were studied in response to particle size and physical stability. Results are compiled in the following tables.

3.2.1. Effect of PVA concentration on particle size

Five samples of PVA of varying concentrations (0.25, 0.5, 0.75, 1 and 1.5 %) were prepared. Figure 3.2 represents the results. Particle size and physical stability was prominently affected by low and high concentration of PVA. Low concentration of PVA produces particles of smaller size but issue of sedimentation persists.

Table:3.2 Effect of PVA concentration on particle size

Formulation	Tac.mg	PVA.con	OC/AQ ratio	Injection rate ml/min	S speed RPM	Stability	Particle size nm
F1	20	**0.25**	1:4	4.30	650	Unstable	134
F2	20	**0.50**	1:4	4.30	650	Stable	166
F3	20	**0.75**	1:4	4.30	650	Stable	246
F4	20	**1**	1:4	4.30	650	Stable	321
F5	20	**1.5**	1:4	4.30	650	Unstable	654

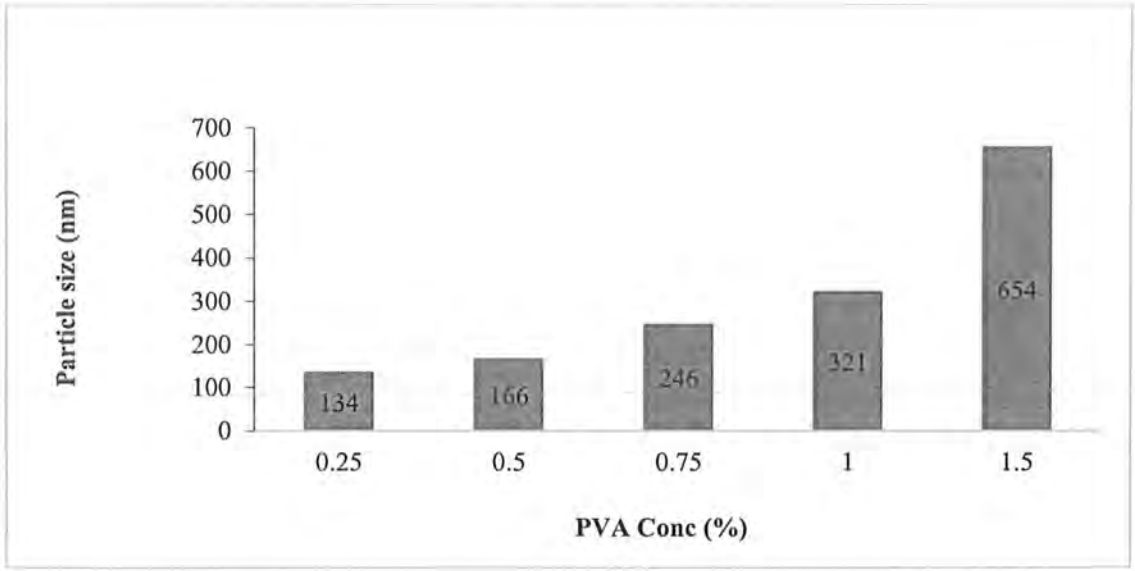

Figure 3.2: Effect of PVA concentration on particle size

3.2.2. Effect of volume of aqueous phase to organic phase on particle size and stability

For preparation of nano particles, volume of organic phase was kept constant and by changing the ratios of aqueous phase (3, 4, 5, 6 and 7 ml). Results are displayed in given figure 3.3. It can be claimed that particle size enhanced by increasing the concentration of aq. phase.

Table 3.3: Effect aqueous to organic phase on particle size and stability

Formulation	Tac.mg	PVA.con	Oc/Aq ratio	Injection rate ml/min	S speed RPM	Stability	Particle size nm
F6	20	0.5	**1:3**	4.30	650	Stable	121
F7	20	0.5	**1:4**	4.30	650	Stable	167
F8	20	0.5	**1:5**	4.30	650	Stable	187
F9	20	0.5	**1:6**	4.30	650	Stable	560
F10	20	0.5	**1:7**	4.30	650	unstable	680

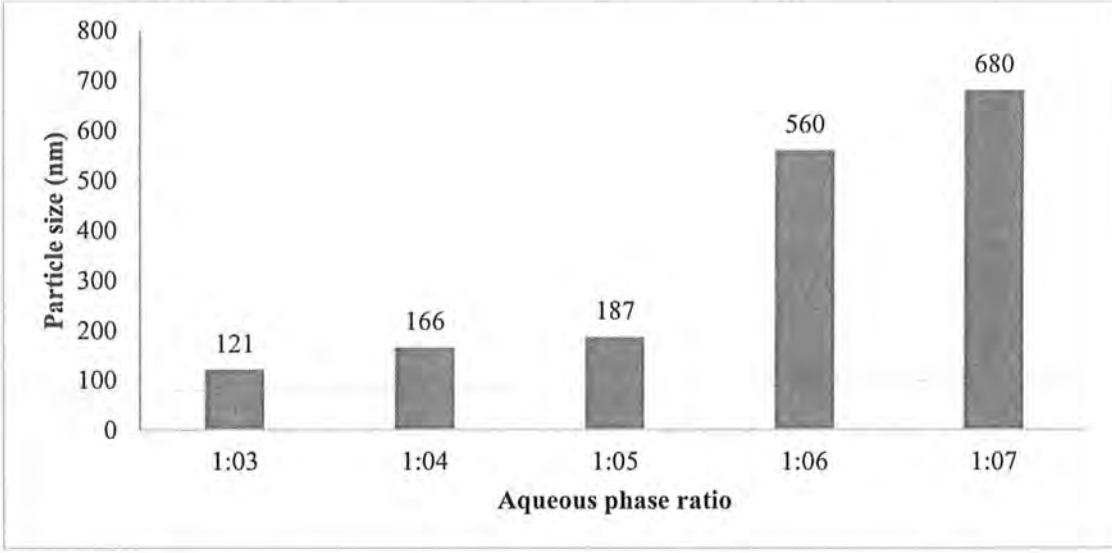

Figure 3.3: Effect aqueous to organic phase on particle size and stability

3.2.3. Effect of injection rate on particle size and stability

1 ml of organic phase was injected into aqueous phase by maintaining injection speed of 2.30, 3, 3.30, 4 and 4.30 ml/min. Results are displayed in figure 3.4. It is evident from study that fast injection speed gives larger particles while slow injection rate gives smaller particles.

Table 3.4: Effect of injection rate on particle size and stability

Formulation	Tac.mg	PVA.con	OC/AQ ratio	Injection rate ml/min	S speed RPM	Stability	Particle size nm
F11	20	0.5	0.5	**2**	650	unstable	651
F12	0	0.5	0.5	**2.30**	650	Stable	411
F13	20	0.5	0.5	**3**	650	Stable	203
F14	20	0.5	0.5	**3.30**	650	Stable	192
F15	20	0.5	0.5	**4.30**	650	Stable	166

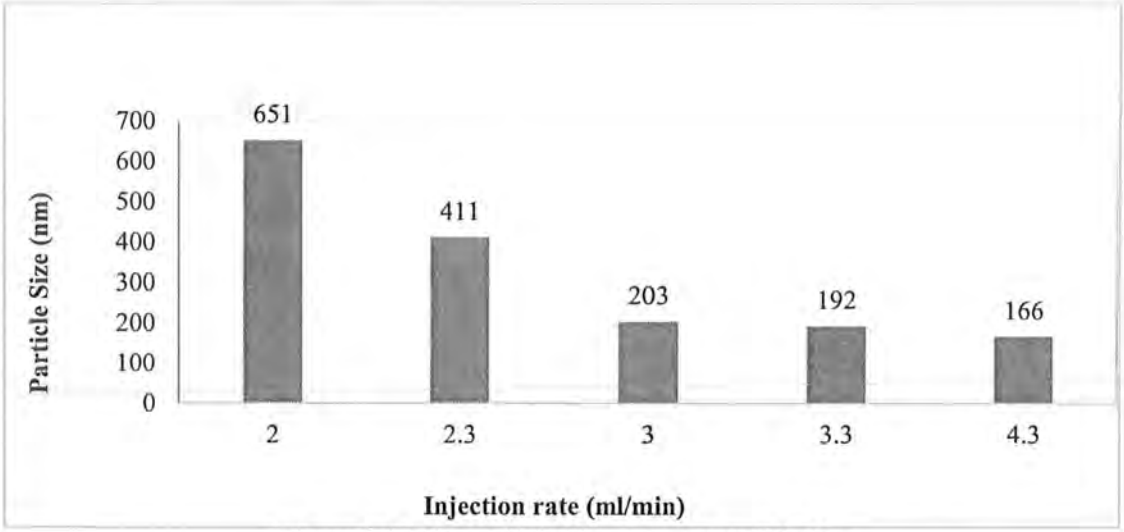

Figure 3.4: Effect of injection rate on particle size

3.2.4. Effect of stirring speed on particle size

Samples were prepared by varying the stirring speed. For this purpose, five different stirring speeds (600, 650, 700, 750 and 800 rpm) were selected. Results are displayed in figure 3.9. It is evident that no prominent effect was observed by varying the stirring speed but particle size increased a little bit at 800 rpm.

Table 3.5: Effect of stirring speed on particle size

Formulation	Tac.mg	PVA.con	OC/AQ ratio	Injection rate ml/min	S speed RPM	Stability	Particle size nm
F16	20	0.5	0.5	4.30	**600**	unstable	112
F17	20	0.5	0.5	4.30	**650**	stable	167
F18	20	0.5	0.5	4.30	**700**	stable	173
F19	20	0.5	0.5	4.30	**750**	Stable	178
F20	20	0.5	0.5	4.30	**800**	Stable	191

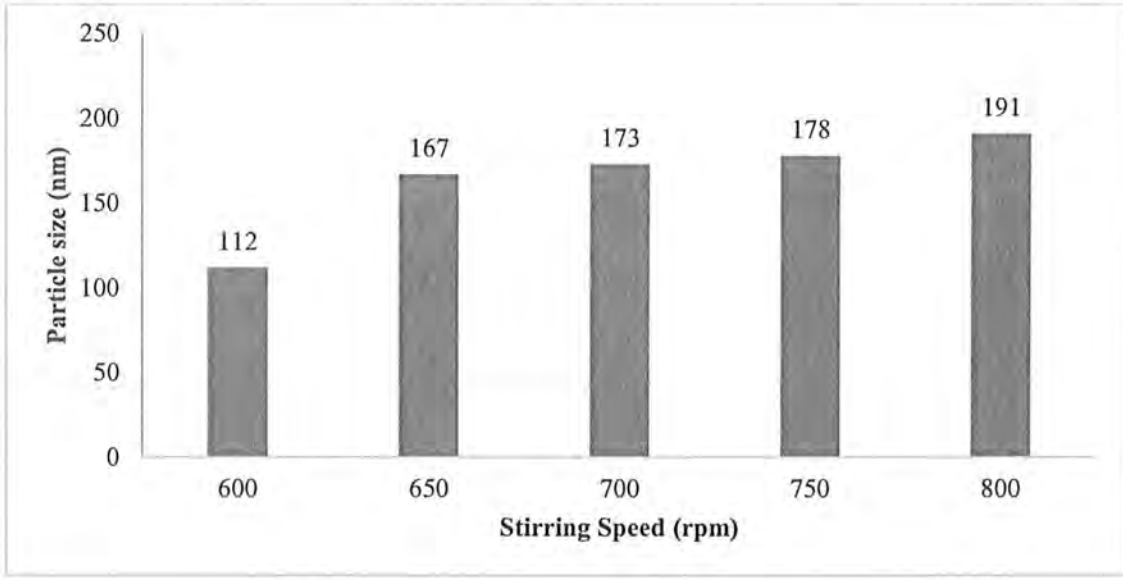

Figure 3.5: Effect of stirring speed on particle size and Stability

3.3. Characterization of Nano particles

3.3.1. Size of blank and drug loaded nano particles

DLS technique was used for measurement of particle size (Nano ZS, Malvern instruments, Malvern UK). It was evident from previous studies that dilution did not alter the particle size and as well as distribution (Jain *et al.*, 2016). Nano particles Emulsion was diluted with distilled water to determine the particle size. Dilution was performed 10 times with milli-Q water to measure light scattering intensity within range of instrument sensitivity. The average size of the blank and drug loaded Nano particle was 167.8 nm and 237.9 nm respectively as shown in figures 3.6 and 3.7.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 167.8	Peak 1:	192.3	100.0	73.28
PdI: 0.112	Peak 2:	0.000	0.0	0.000
Intercept: 0.949	Peak 3:	0.000	0.0	0.000
Result quality : Good				

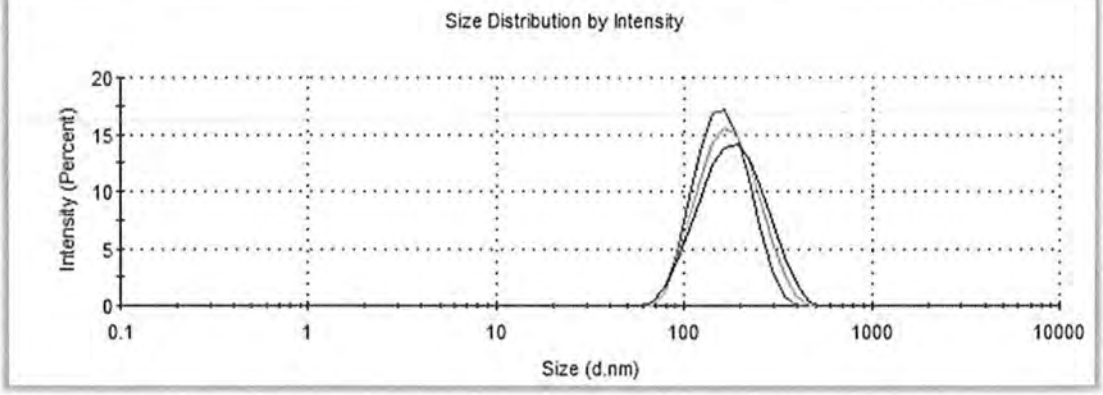

Figure 3.6: Size of Blank Nano particles

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 237.9	Peak 1:	266.7	100.0	93.17
PdI: 0.101	Peak 2:	0.000	0.0	0.000
Intercept: 0.968	Peak 3:	0.000	0.0	0.000
Result quality : Good				

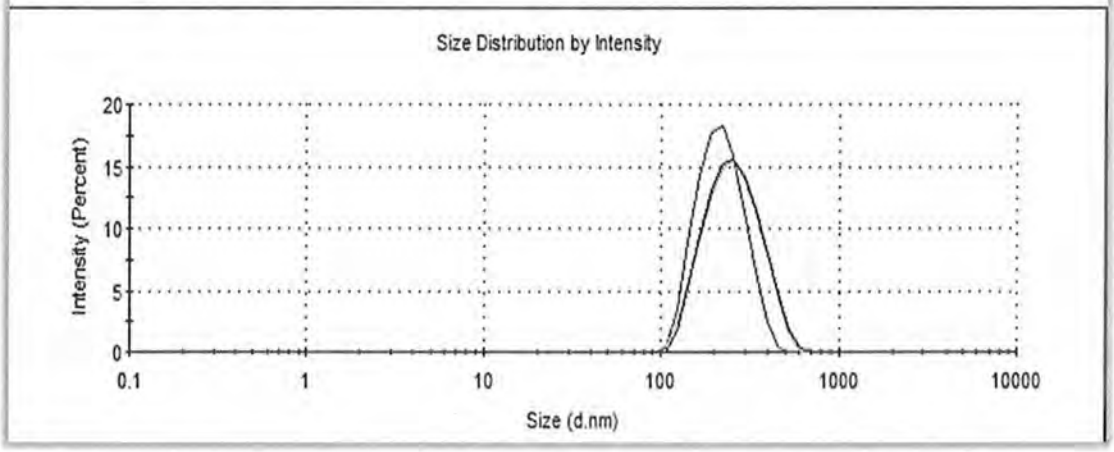

Figure 3.7: Size of Drug loaded Nano particles

3.3.2. Zeta Potential of blank and Tacrolimus Nano particles (mV)

It is an important parameter to investigate the stability of formulation. It was measured by DLS and was found to be 21 mV for blank and 31 mV for drug loaded nano particles.

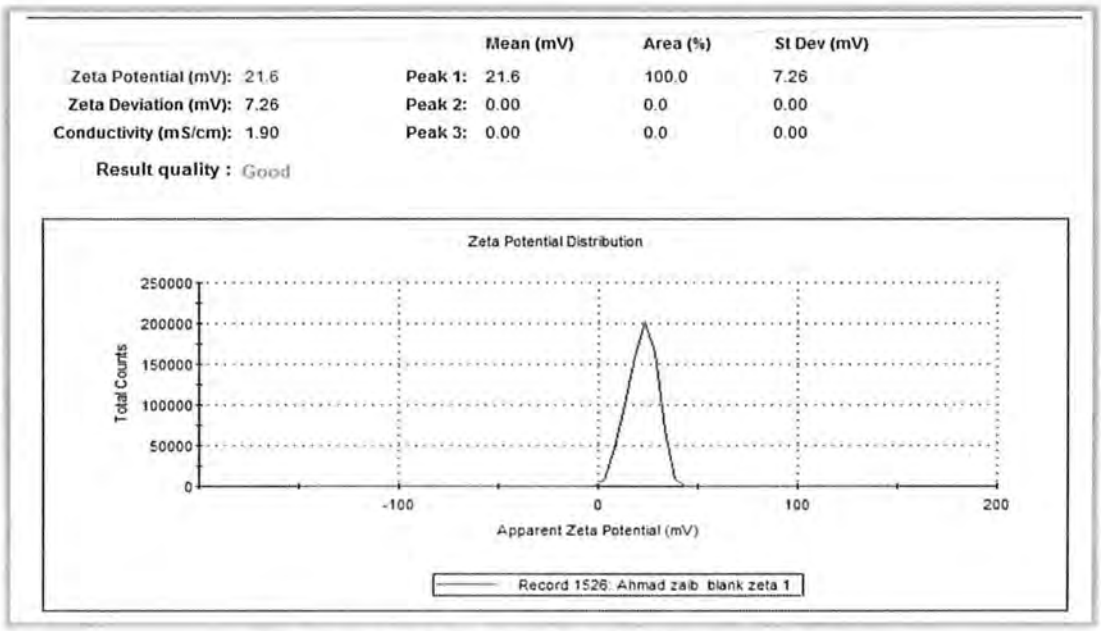

Figure 3.8: Zeta Potential of Blank Nano particles

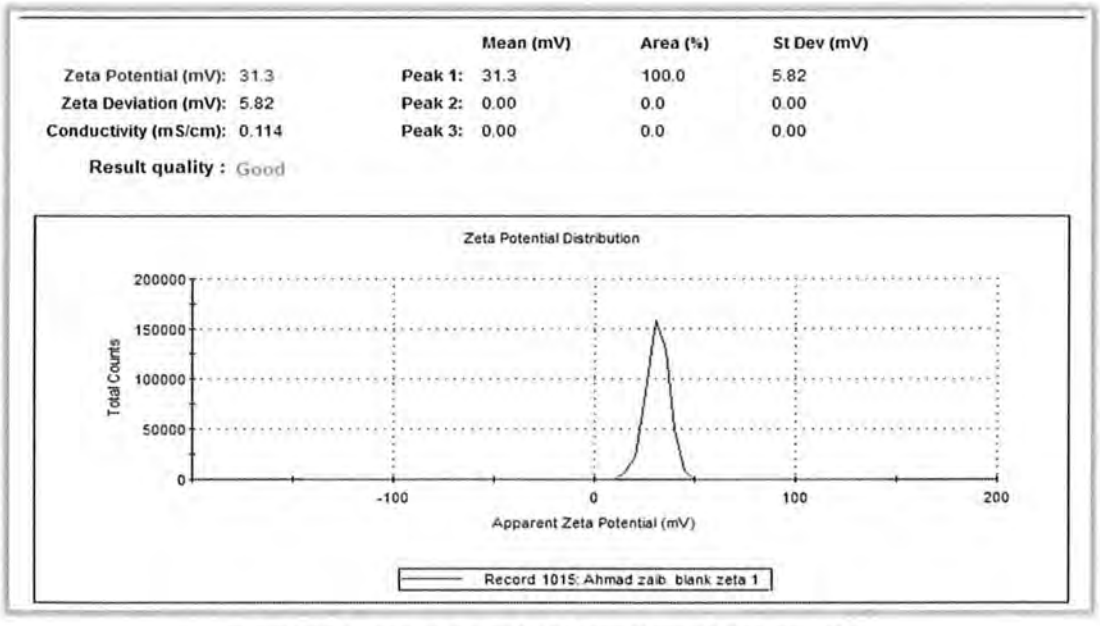

Figure 3.9: Zeta Potential of Tacrolimus Nano particles

3.3.3. Polydispersity index

Experiments were performed in triplicate. PDI was found to be 0.11 and thus indicated uniformity in size and homogeneous particle distribution.

3.3.4. Encapsulation efficiency and drug loading

Experiment was performed in triplicate to determine the encapsulation efficiency of tacrolimus loaded nano particles. It was analyzed through HPLC and found to be 77.5 %.

EE (%) =W1-W2/W1

20-15.4/20=77.5 %

W1 = Total Tacrolimus added
W2 = Free Tacrolimus in supernatant

Table 3.7: Indirect method to estimate the EE and LC

Indirect method				
AUC	**Percent entrapment efficiency**	**Mean of entrapment efficiency**	**Loading capacity**	**Mean loading capacity**
331360	77.32	77.5± 3.6	57.22396	64.61349 + 6.5
331590	77.00		69.68209	
341267	78.5		66.93443	

3.3.5. Percentage yield

Given formula was used to calculate the percentage yield of tacrolimus loaded Eu E100 nano particles and it was 75%.

*Nanoparticles yield % =166/20+200*100*

=75.4%

3.3.6. SEM images of drug Loaded Nano particles

For determination of morphology of drug loaded nano particles scanning electron microscopy was done. SEM images are shown in the following figure 3.10, at various intensifications. It is obviously shown in both figures that NPs have a round shape.

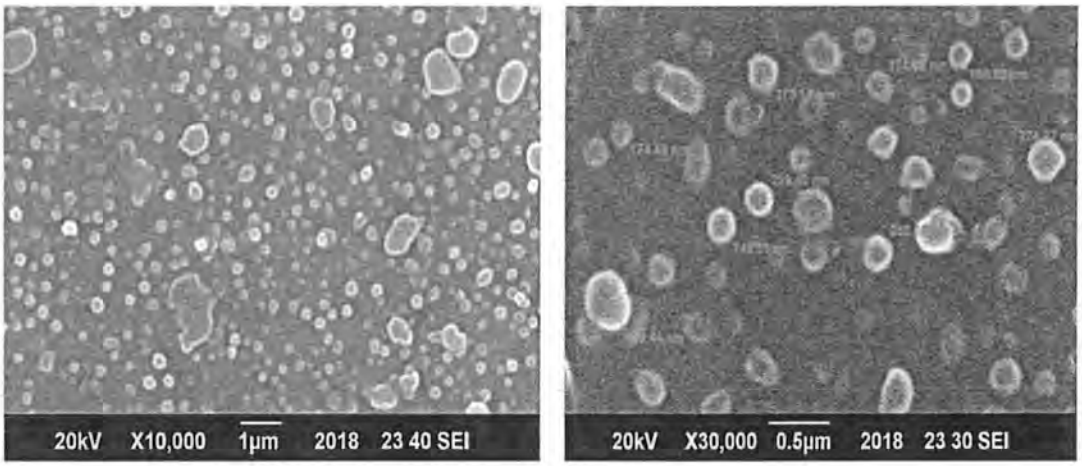

Figure 3.10: SEM images of optimized tacrolimus loaded nanoparticles

3.3.7. Fourier transforms infrared spectroscopy

FTIR examination was conducted in order to find to the physical compatibility among the ingredients exploited in Tacrolimus loaded nano particles. FTIR spectra indicated the lack of any electrostatic interaction in Tacrolimus loaded nano particles and characteristics peaks of pure dug Tacrolimus i.e. phenol, carbonyl and aldehyde groups are maintained in all formulations. In the same way appearance of any new group was totally absent as clearly perceived in figure 3.11.

Table 3.8: FTIR spectrum peaks interpretation:

Groups	ethanol	Eudragit E 100	PVA	Tacrolimus	Formulation
Phenols	.....	3450-3610	3450-3610	3450-3610	3450-3610
Alcohols OH	3200-3500	3200-3500	3200-3500	3200-3500	3200-3500
Carbonyl C=O	.....	3100-3200	.....	3100-3200	3100-3200
Aromatic C=O	.....	1700-1750	.....	1700-1750	1700-1750
Alkene =CH	.....	600-870	3200-3500	600-870	600-870
Ester C=O	.....	1100-1300	.....	1100-1300	1100-1300

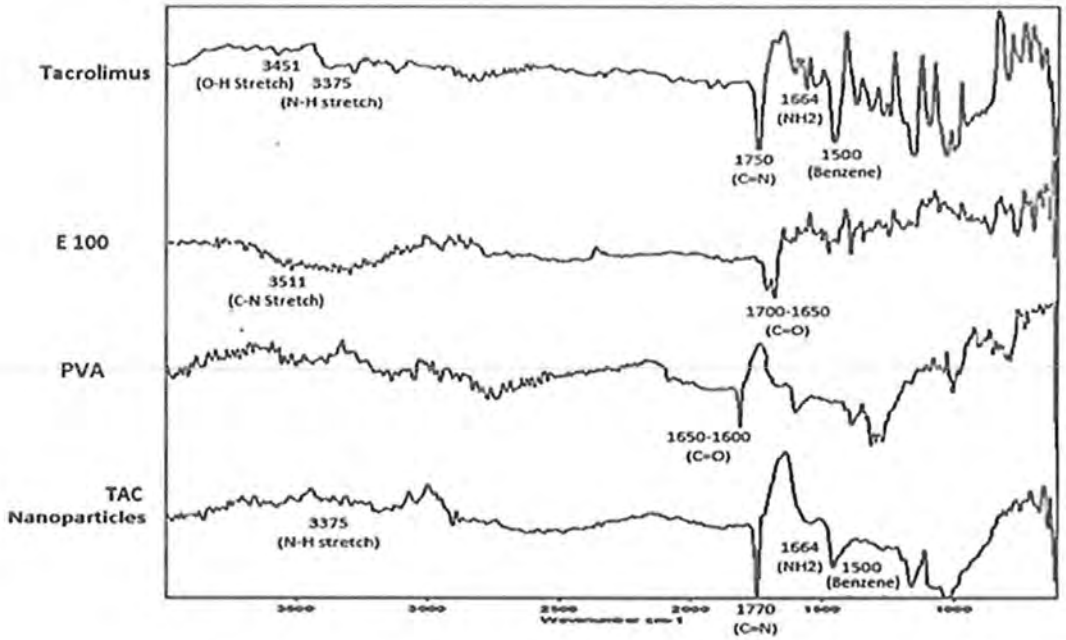

Figure 3.11: FTIR spectrum of components of nanoparticles

3.3.8. X-ray diffraction (XRD)

XRD analysis in figure 3.12 explained that the drug was in crystalline hydrated form. All peaks seemed in range from 10-90 were absent in XRD analysis of Tacrolimus loaded nano particles and intensity of the peaks were relatively low, showing that all drug was successfully merged into polymeric carrier.

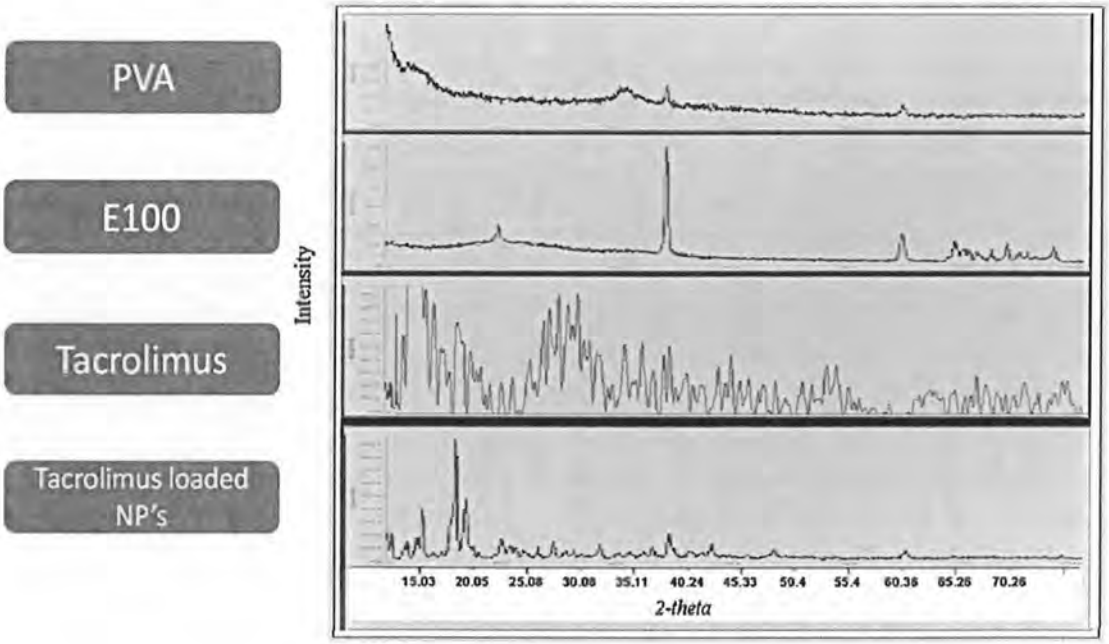

Figure 3.12: XRD analysis

3.4. Preparation of Blank and Drug NP Loaded Membrane

According to section 2.10.1 and 2.10.2 blank and drug loaded NPs loaded film was prepared by changing the concentration of Aloe vera and chitosan. Table 3.9 given below describes visual film forming properties. Chitosan imparted film forming properties to the composite film. Fifty percent of chitosan was found to form good film and thus it was selected for additional studies.

Table: 3.9 Characteristics of composite films:

	Aloe Vera: Chitosan ratio	% Aloe Vera content	Glycerol Conc	Physical observation
Cht	0:1	0	3%	Good film
Cht/AV1	1:1	50	3%	Good film
Cht/AV2	2:1	66	3%	Film not Formed
Cht/AV3	1:2	33	3%	Good film

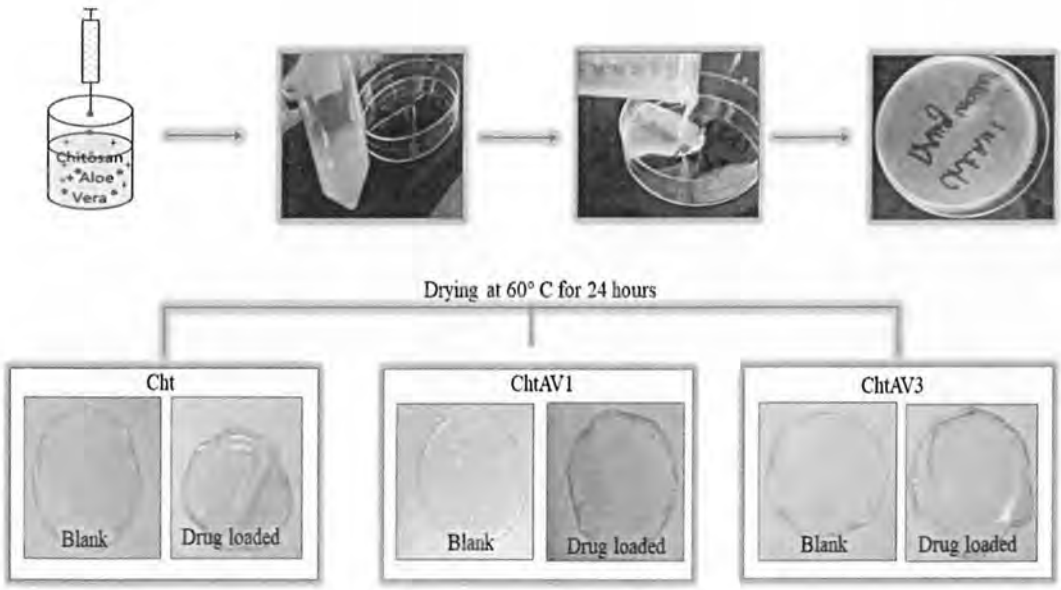

Figure 3.13: Preparation of Tacrolimus nano particles loaded Cht/AV film

3.5. Release study of Tacrolimus in phosphate buffer saline at pH 5.4 from nano particles and Film

To study the drug release from Tacrolimus nano particles and final dosage form at pH 5.4 at 32˚C skin temperature, the dialysis bag method was used for evaluation of release of drug from NPs and Film, as discussed in chapter 2. The graph visibly showed that approximately 70% of all entrapped drug was released in 60 hours and nearly 65% of the entrapped drug was released from the Film in 60 hours as clearly shown from figure 3.14. The film encapsulation provided more controlled release to the Tacrolimus.

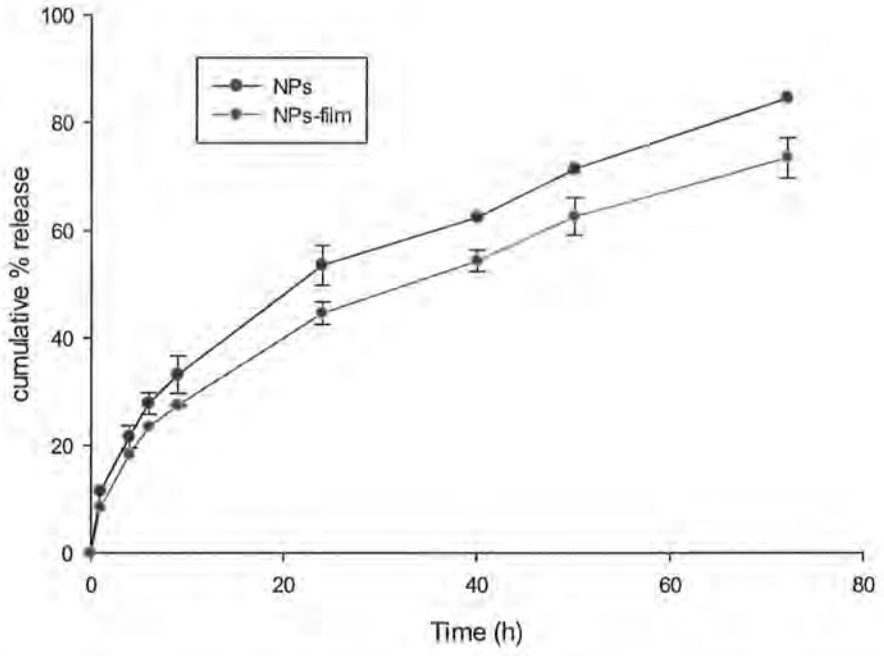

Figure 3.14: Release study of Tacrolimus in phosphate buffer saline at pH 5.4 From nano particles and Film

3.6. Release Kinetics

The mechanism by which drug release occurred from the Tacrolimus loaded nano particles was find out by applying several mathematical models including Higuchi, first order, zero order, Hixon-Crowel and krosmeyer Peppas. Rate constant ($R^{2)}$ value concluded that the release from the both nano particles and Film followed Higuchi model. The following tables 3.10 and 3.11 shows the result of most suitable model in term of R^2 value.

Table 3.10: Finest release model for nanoparticles:

Zero order	First order	Higuchi	Korsmeyer-peppas	Hixon Crowell
R^2	R^2	R^2	R^2	R^2
0.639	0.9104	0.9958	0.543	0.710

Table 3.11: Finest release model for Film

First order	Zero order	Higuchi	Korsmeyers Peppas	Hixon Crowell
R^2	R^2	R^2	R^2	R^2
0.933	0.677	0.9744	0.743	0.541

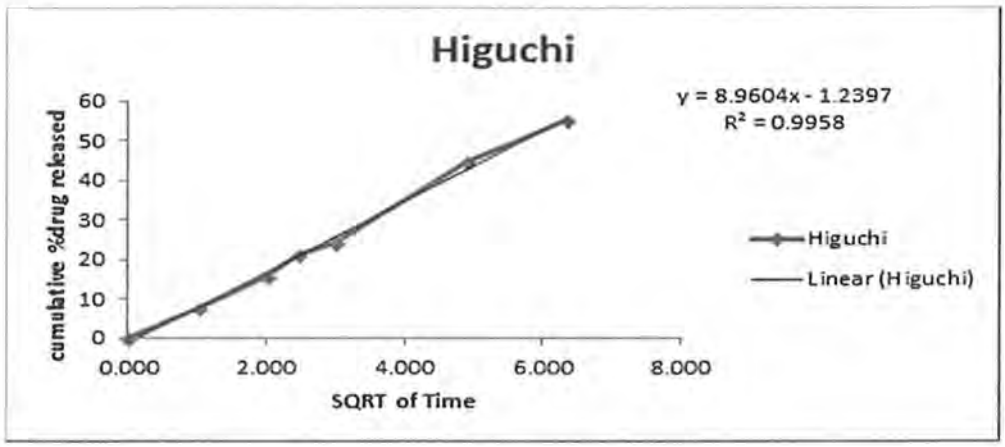

Figure 3.15: Best release model for drug release Nano particles

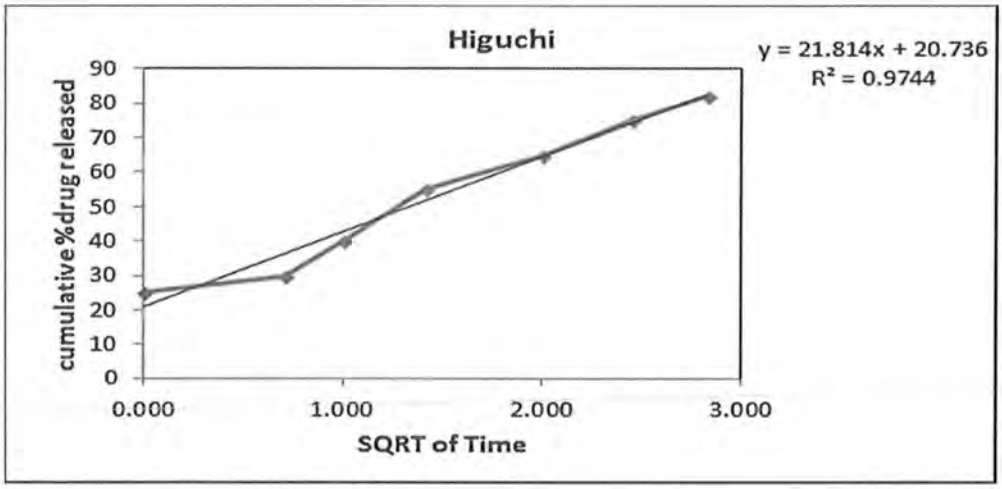

Figure 3.16: Best release model for drug release Film

3.7. Film Characterization

3.7.1. Swelling index

To indicate absorbing capacity of body secretions and fluids swelling index is an important indicator. Graph given below presents percent swelling of blank and drug loaded film at various intervals of time. Introduction of tacrolimus loaded NPs decreased the swelling capacity of film.

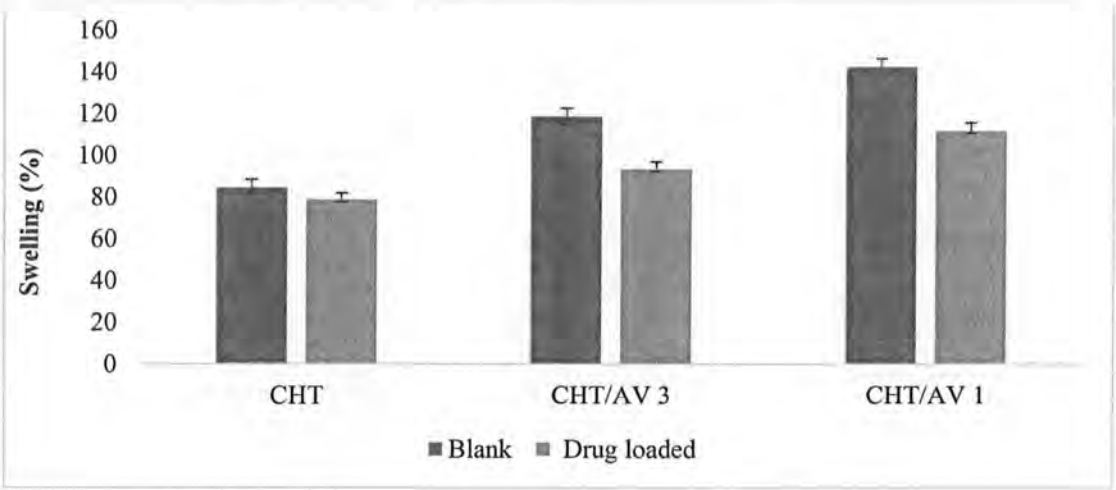

Figure 3.17: 24 hour swelling capacity of films

3.7.2. Erosion

To find the solubility of film at the body erosion test was performed. Results clearly showed that greater loss of mass was observed in the films containing higher percentage of aloe vera than films having less or no aloe vera content.

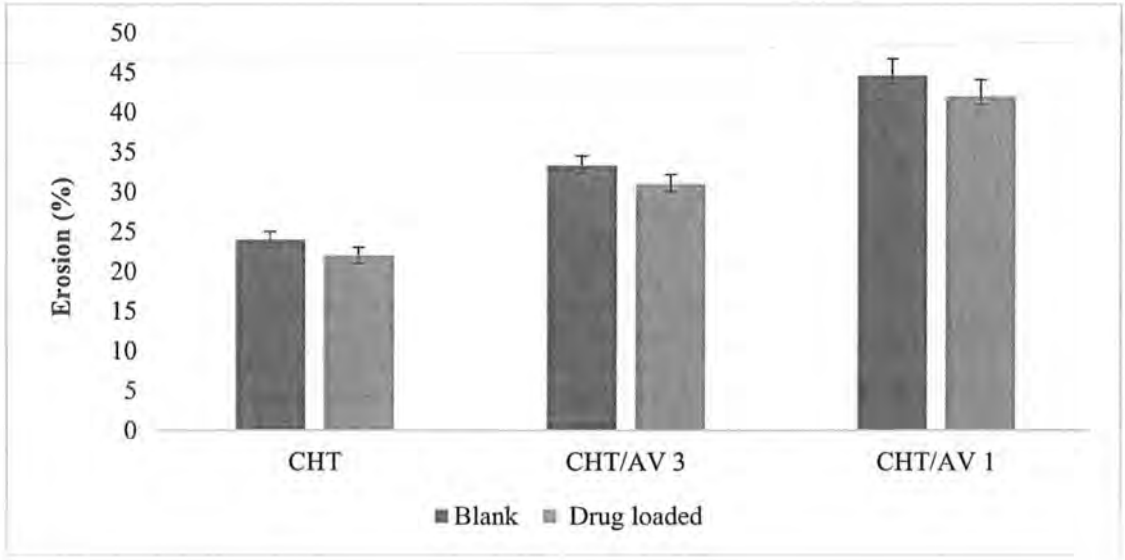

Figure 3.18: Erosion (% weight loss) of various films

3.7.3. Water vapor transmission test

For determination of moisture content via packaging material standard protocol was used established in USP 671. It can be used to quantify permeability of various films with a little modification. The graph given below showed us the quantity of water up take by desiccant through different films of blank and Tacrolimus loaded Nanoparticles at different days.

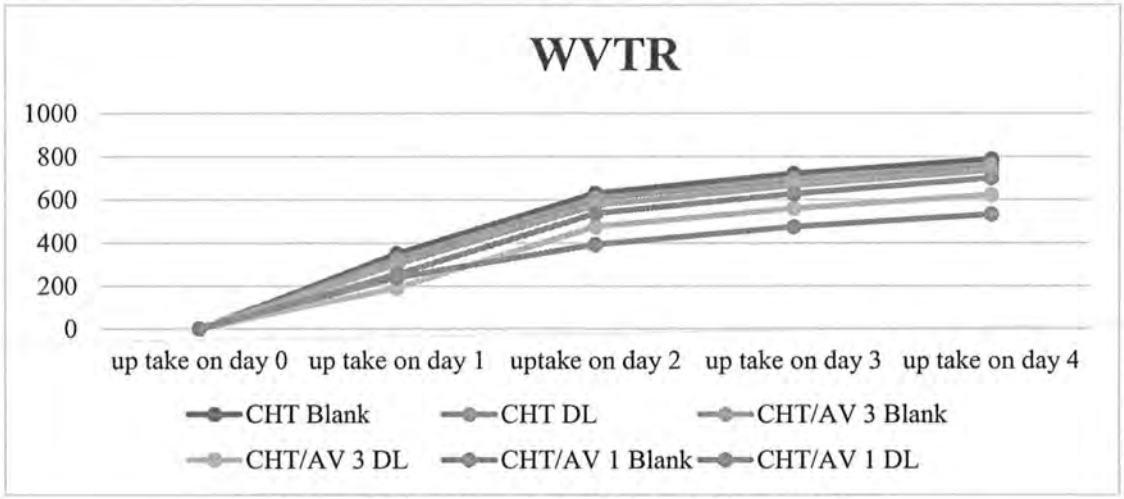

Figure 3.19: water gain (mg) by each film per day

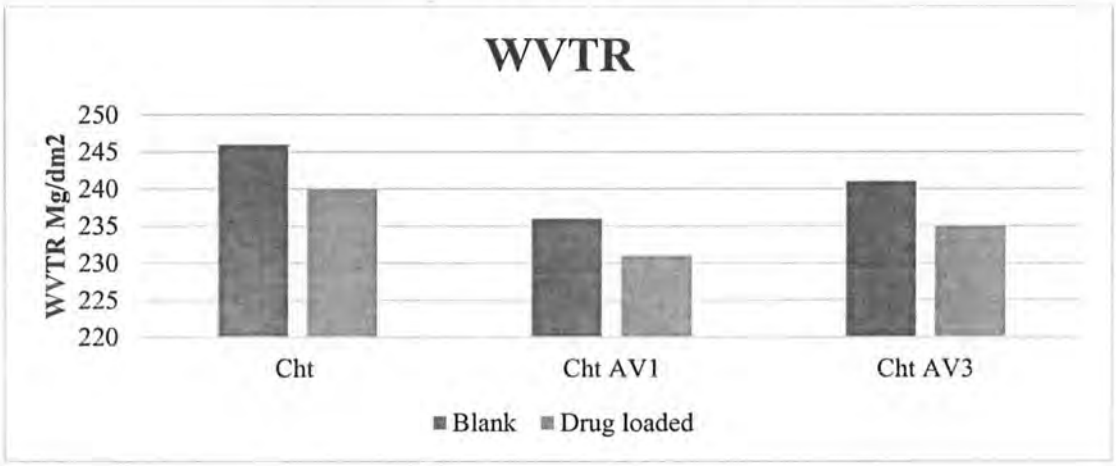

Figure 3.20: Effect of Tacrolimus nano particles and presence of AV on water vapor transmission rate.

Table 3.12: Permeance of various films and Water vapor transmission rate

	WVTR	WVP
Cht blank	246	677.5051
Cht DL	241	650.7094
Cht/AV 3 Blank	236	625.6599
Cht/AV 3 DL	231	579.6902
Cht/AV3 Blank	241	536.0000
Cht/AV1DL	235	500.5643

Chapter 4

Discussion

4. DISCUSSION

The main objective behind this research was to fabricate a nanocomposite system based on combination of Tacrolimus loaded polymeric nanoparticles, the smart polymer chitosan and natural ingredient aloe vera. The principal theme of this study was to overwhelm the short comings of marketed formulation additionally to get synergistic effect by combining different ingredients and to give an alternate dosage form. Tacrolimus was loaded into Eudragit E100-polymeric nanoparticles. The main purpose of utilizing aloe vera and chitosan was their natural source with benefits such as cost effectiveness, biodegradability, biocompatibility, anti-inflammatory and anti-bacterial properties (Nguyen *et al.*, 2014). Furthermore, this entire nanocomposite film system was organized at specific conditions without the use of cross linkers, undesirable toxic agents, solvents and chemicals. It enhanced the advantages provided by this novel nanocomposite film system.

Hypothesis, that the Tacrolimus loaded nano particles at the acidic pH of tissues effected with psoriatic disorders would provide the release of drug and will target the specific application site. To achieve desired purpose, Eu E100 was selected which is a positively charged polymer. The polymer had composition molar ratios of 2:1:1 dimethyl aminoethyl methacrylate, butyl methacrylate, and methyl methacrylate. It is widely utilized in pharmaceutical industry for various purposes such as release modification, coating, protection from moisture and for masking of taste as well as a excipient in several dosage forms (Gallardo *et al.*, 2008). Due to its dissolution properties at acidic medium Eu E100 is used as a base polymer for drug loading. It provides targeted drug delivery topically in dermatological diseases treatment by releasing drug through dissolution or diffusion of a polymeric matrix (Domínguez-Delgado *et al.*, 2011). Fast dissolution and targeted delivery can be achieved, when it reaches the acidic pH of the skin suffering with inflammations.

Poly vinyl alcohol is used as a formulation stabilizer which assists to provide stability to the formulation. The main function of PVA in the formulation is to reduce the tension at the interfaces between the organic and aqueous phase. Hence by reducing the particle size emulsion can be stabilized (Prajakta *et al.*, 2009). By combining polymers and stabilizers synergistic effect can be attained which helps in synthesizing

and stabilizing the nano particles. It also increases the drug solubility and penetration across the skin intrinsically (Huang *et al.*, 2016).

Tacrolimus (TAC), an immunosuppressant macrolide and isolated from Fungus, Streptomyces tsukubaensis, has been explored to be useful in the management of psoriasis by inhibition the expression of pro inflammatory mediators, cytokines and inflammation. It possess low solubility, high permeability and highly sensitive when uncovered to light, high temperature and oxidative environment (Honbo *et al.*, 1987). The marketed formulation has the draw back that it gives unpleasant sensation to the skin, greasy and oily appearance and make it difficult to wash off from the skin surface (Li *et al.*, 2012). Tacrolimus nano particles loaded into Eu E100 polymer by nanoprecipitation method or interfacial displacement were successfully fabricated first time in this study and PVA was used as formulation stabilizer. Additionally, by using combination of PVA and eudragit, tacrolimus nanoparticles have not been developed earlier by nanoprecipitation method. This technique is chosen for particles formulation, as it is too simple, easily applicable and consists on smaller number of instruments and steps (Anand *et al.*, 2010).

Blank and Tacrolimus loaded nano particles were formulated by defined method discussed in chapter 2. The ratio of the organic to aqueous phase was kept 1:4. The formulations were firstly visually analyzed for physical stability at room temperature. For characterization of nano formulation, particle size measurement is a significant parameter as it directs the permeability and solubility of particles via the cell membrane. The results of DLS are illustrated in figure 3.6 and 3.7 for blank and drug loaded nano particles formulations. Results indicated that the particles were in the nano range. After entrapment of drug into polymer increased particle size was observed as compared to simple blank formulations (Silva *et al.*, 2013).

Zeta potential (ZP) shows the physical stability of Nano system, it is measurement of the electric charge on the particle surface. Zeta potential of Tacrolimus loaded Nano formulation was found to be +31 mV. The positive charge is due to cationic nature of the polymer Eudragit E 100. This value indicates the stability of our formulation.

PDI is a significant tool to find the uniformity and homogeneity among different sizes of particles. Particles having narrow spread lie within range of 0-0.25. Particles having value above 0.5 show broad distribution. These widely distributed particles

cannot cross biological barriers easily. Range of our results was in between 0.1-0.25 which were in line with previous studies (Tang *et al.*, 2011).

SEM images of drug loaded nanoparticles are presented in fig 3.15, 3.16 at various resolution. It is clearly shown in figures that drug loaded nanoparticles had a round shape also analogous with size results that obtained through zeta sizer. The results of this present work also lie in correspondence with previously published data (Krishnakumar *et al.*, 2011).

To find out the total drug content or percentage of drug present in formulation indirect method was used for inferring the encapsulation and loading capacity of drug. These two variables are important as both have major role the release and absorption of drug across the barriers. Through this study, encapsulation efficiency and drug loading about 77+3.6%, and 21.33+4.16% respectively obtained. Percentage yield by nanoparticles of present work was 75.50+1.52%. The low yield value may be because of loss in processing, loss in handling and freeze-drying.

For optimization, various parameter's effect was evaluated for particle size and physical stability. All the stabilizers used in nanoparticles formulation have a prominent effect at stability of the nanoparticles (Lamprecht *et al.*, 1999). The stability of a formulation is mainly dependent concentration of stabilizer along with its nature. In formulation of oil in water emulsion, PVA is commonly used as a stabilizer it is a high molecular weight polymer (Lee *et al.*, 1999). From the results it can be seen that, nanoparticles that are formulated at low concentration were physically unstable. Beyond a certain limit, increase viscosity leads to physical instability and precipitation. Low concentration of PVA (0.5%) was selected as optimum concentration because high conc. leads to increased toxicity.

The effects of aqueous phase and organic phase on the particle size and stability were also studied. When volume of aq. Phase is kept high it becomes difficult to evaporate organic phase from formulation and it also takes too much time. It causes increased particle size due to aggregation of nano particles size (Mehrotra and Pandit, 2012). As suggested by the given data 40 ml volume was selected as optimum aqueous phase volume.

For determination of particle size injection rate is considered as a critical parameter for incorporation of organic phase into aqueous phase. As reported by the previous literature high speed of injection produces larger particle size which might be due to difficulty to achieve homogenous mixture or may be less intact time between two phases (Mora-Huertas *et al.*, 2011).

In our study, small particle size was obtained by maintaining the injection speed at 4.30 minutes for 1 ml while larger particles were produced at fast injection rate. It illustrates the fact that larger particles are produced when less time is given to form the homogenous mixture. When time of contact between aqueous phase and organic phase was higher it produced smaller particles. Optimum injection speed of 1 ml in 4.30 minutes was selected for formulation of nano particles.

Stirring speed (rpm) is one of the key parameters to give important properties to nano particles to form a homogenous mixture, in the form of energy imparting system. when high stirring is applied it breaks the particles into smaller one (Ibraheem *et al.*, 2014). In our findings the case is different the low speed tends to produce small particles comparatively, the reason behind it may be that at high speed the contact time between the two phases is less which may form particles of larger size. During our study 650 rpm was chosen the optimized speed.

FTIR results indicates the lack of any interaction in the Tacrolimus loaded nano particles formulation. Characteristics peaks of Tacrolimus i.e. phenol and carbonyl, aldehyde groups are maintained in all formulations. In the same way no appearance of any new group, as clearly perceived in figure permits the deficiency of interaction between the ingredients of formulations.

XRD analysis of drug, PVA and final formulation shows that drug was present in its free crystalline hydrated state but when it was loaded in to polymeric carrier it is converted into amorphous state.]Relative intensity of peak of E 100 (that is carrier of drug) is also low, indicating all drug is successfully encapsulated into polymer (Esfandiarpour-Boroujeni *et al.*, 2017).

The release of Tacrolimus from Nano particles and film occurred in a controlled manner at pH 5.4. It can be seen clearly from the figure 3.22 that all entrapped drug

nearly 70% was released within 60 hours from the nanoparticles and 65% was released from the film.

The mechanism by which drug releases from the Tacrolimus loaded nanoparticles and film was find out by applying several mathematical models including Higuchi, first order, zero order, Hixon crowel, korsmeyer peppas were fit to the release statistics. Kinetic model study clearly shows that drug release from both nanoparticles and film follows diffusion-based mechanism as model applied show higuchi kinetic model. The R2 value were 0.9958 and 0.9744 respectively.

Researchers have studied globally the potential of polymeric composite films in the management of diverse topical diseases. Chitosan films have been fabricated at various levels with different natural or synthetic polymers or ingredients. It has been incorporated with Alove Vera to treat various diseases (Silva *et al.*, 2013). In this study we take their work to a next level where such composite membranes were loaded with Tacrolimus nanoparticles to provide a synergistic effect for psoriasis. These films were prepared at different ratios of chitosan and Alove Vera extract. Film like structure was not achieved when higher proportion of aloe vera was used. This point has been supported by the fast claiming that the films forming properties are purely due to chitosan (Khoshgozaran-Abras *et al.*, 2012). As the concentration of Alove Vera when increases so pale color of the film decreases it shows that it is due to Alove Vera.

Swelling index of the blank and drug loaded Nano particles was measured in order to find out film capacity to absorb body fluids and secretions. The swelling capacity of blank Chitosan, Chitosan/AV3 and Chitosan/AV1 after 24 h of entanglement in PBS was 85%, 121% and 143% respectively. Reduced swelling capacity was observed while comparing with blank formulation when tacrolimus NPs were loaded into film. They were completely encapsulated into Eudragit (a hydrophobic polymer). This hydrophobic polymer causes interaction of water molecules and film thus reducing the swelling index.

Erosion studies shows direct quantification of the percent dry film matter loss after contact with biological fluids. The loss in the film mass is directly proportional to the Alove Vera concentration as it is increased, so film water solubility increases and so increase in the percentage loss in mass occurred after 24 h of immersion in PBS.

WVTR of formulation (films) calculated individually in triplicates in order to find out the WVP. It rests upon at the ability to diffuse and solubility of water in the film system. An increase in Aloe Vera content causes decrease in the permeability of the films as indicated by the results. Possible reason behind this effect may be there occurs increase in the hydrophilicity of the system and hence leads to increase water solubility. Retention of water vapors can be increased by increasing the water solubility of film as indicated by the swelling studies. It also causes interaction between film and water vapors that ultimately decreases water transmission. After incorporation of Tacrolimus NPs into WVTR of blank NPs were higher than the drug loaded nanoparticles. Hydrophobic polymer eudragit blocks transmission of water vapors across film leading to decreased diffusion of water vapors.

CONCLUSIONS

- ✓ Eudragit E100 nano particles loaded Film based on chitosan and Alove Vera was successfully prepared.
- ✓ Tacrolimus nano particles were fabricated to yield particles of an average nano size and formulation was optimized.
- ✓ SEM images showed the spherical morphology of the prepared nano particles.
- ✓ FTIR studies showed the lack of any interaction among different ingredients and XRD analysis showed successful loading of drug in to polymer.
- ✓ *In vitro* dissolution studies of nanoparticles and Film showed the capability of controlled release of drug up to 72 h.

FUTURE PERSPECTIVE

Animal model studies can be performed to evaluate the effectiveness of nanocomposite films. Hence short comings of marketed formulations can be overcome.

Other diseases like eczema, fungal diseases, and diabetic foot can be treated with nanocomposite films apart from psoriasis.

Different nanocomposite systems can be exploited for formulation of various dosage forms. Naturally occurring ingredients having intrinsic benefits can also be utilized

Aloe vera and chitosan two naturally occurring ingredients provide depot system for nanocarriers. They can also produce synergistic effect. Other antimicrobial agents can be encapsulated to achieve synergistic effect,

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