Formulation Development and Characterization of Nano Emulsion for Sirolimus Drug

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By

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DEDICATION

I would like to thank my parents for their love and support, who prayed for me at every turn, as well as my supervisor, who served as both my inspiration and my spirit, providing me with unwavering support and kind help without which this project would have remained an idle goal. Without them, this day would not have been possible.

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Muhammad Rizwan

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ABBREVIATIONS LIST

Abbreviations	Term
mTOR	Mammalian target of rapamycin
FTIR	Fourier Transform Infrared Spectroscopy
SRL	Sirolimus
ZS	Zeta Sizer
ZP	Zeta Potential
FDA	Food & Drug Administration

ABSTRACT

The mammalian target of rapamycin (mTOR)inhibitor i.e. everolimus and sirolimus widely used class as immunosuppressive in kidney and liver transplantation. Sirolimus drug is produced by fermentation of macrocyclic lactone. Sirolimus is immunosuppressant drug. Sirolimus has low water solubility drug and bioavailability .Sirolimus nano emulsion formulation was produced by sirolimus, propylene glycol, castrol oil, tween 20 & tween 80 and order of mixing is a key critical process parameter for development of nano emulsion formulation. Nano emulsion formulation characterized by UV spectroscopy, FTIR, zeta potential, zeta sizer and drug analysis. Maximum wave length absorption was at 277nm of drug formulation, the result of IR Spectrum has two strong stretching peaks at 1715.63 cm^{-1} and 1633.28 cm^{-1} in sirolimus nano emulsion. Zeta sizer of nanoparticles for drug formulations was 54.26nm and 15.54nm (≤ 200nm) respectively zeta potential of nanoparticles for drug formulations was -1.4mv and -0.8mv (Limit ± 10 mv). Drug analysis stated amount was 97 – 98 % of different formulation. Nano emulsion formulation of sirolimus was used to enhance solubility and permeability.

Key words: Sirolimus, immunosuppressant, mTOR, nano emulsion formulation and zeta sizer.

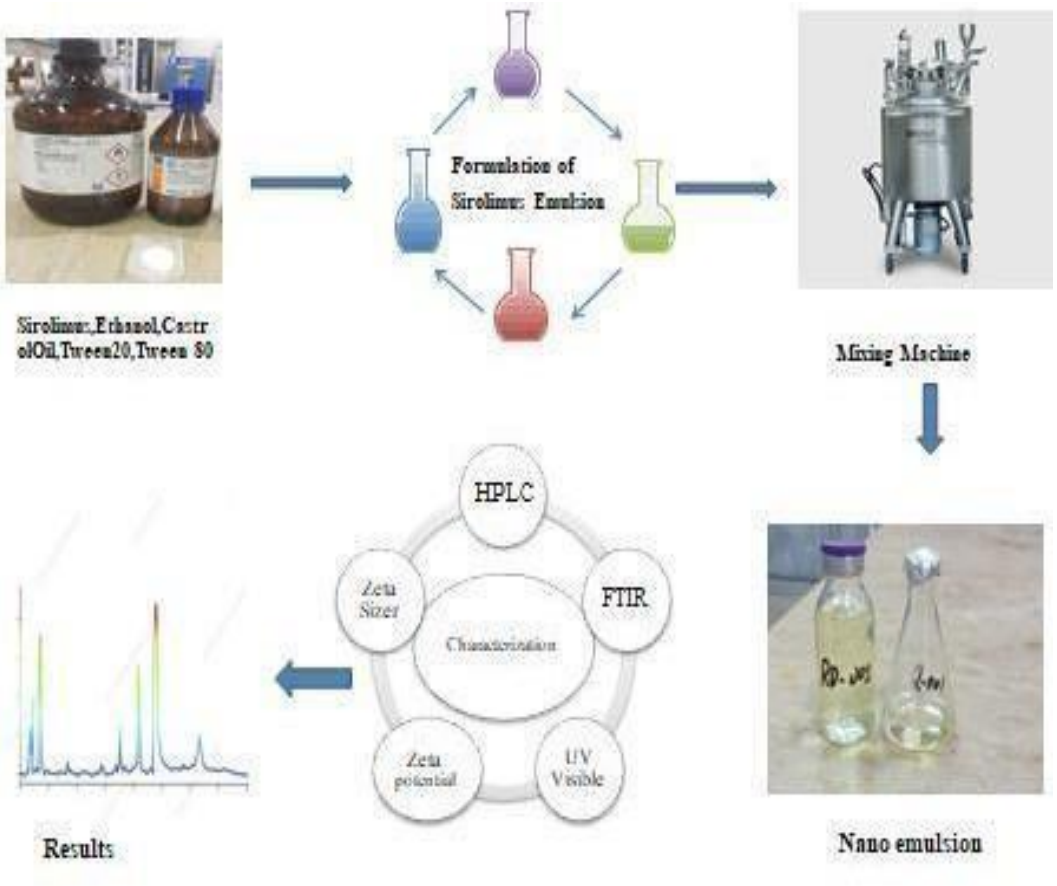

Figure 1 Scheme representation of sirolimus nano emulsion

CHAPTER 1

INTRODUCTION

Sirolimus is a drug derived from the fermentation of *Streptomyces hygroscopicus*, a macrocyclic lactone. It acts as an immunosuppressant and mTOR inhibitor, effectively preventing organ transplant rejections. Additionally, Sirolimus is utilized in the treatment of osteoarthritis, lymph system disorders, and perivascular epithelioid cell tumors in adult (1).

It indicates significant anticancer activity and has a substantial inhibitory impact on the development of T and B cells. The lipophilic nature of sirolimus, with a p Log value of approximately 4.35, renders it practically water insoluble (2.59 mg/mL) and lacking ionizable functional groups within the pH limit of one to ten. Consequently, formulating Sirolimus into intravenous or oral dosage forms proves challenging due to its low bioavailability and water insolubility. Nano-emulsions (NEs) are lipid-based formulations characterized by droplet sizes in the nano metric limit (≥200nm). Due to their ease of production, extraordinary long-term physical stability, and massive potential for a variety of medicinal uses, they have drawn considerable interest in pharmaceutical research. These self-emulsifying systems hold promise for enhancing the bioavailability of drugs that are less water soluble. Compared to simple micellar solutions, nano- emulsions possess a higher solubilization capacity and offer advantages through their long-term physical stability, which surpasses that of unstable dispersions. They are easy to prepare and enhance the mucosal permeability and solubility of compounds that are less water-soluble. Additionally, nano-emulsions provide protection to drugs against hydrolysis and enzymatic degradation under physiological conditions (2).

1.1. IMMUNE SYSTEM

It is a body defense system which response against any foreign body. Generally immune system is of two types: (1) specific (2) non-specific.

The specific immune system is further divided in two, In 1st antibodies are produced against a specific given antigen called humoral response while in other response is transferred by cells called cellular response(bursa) bone marrow cells are responsible for production of antibodies while intact thymus is needed for cellular immune

response via T-lymphocytes. Antigens are recognized through receptors which are present on both B & T-cells. B-cells usually generate antibodies while T- cells proceed as suppressor or helper as the case may be.

1.2. IMMUNOMODULATION

Change in the immune system either potentiation or suppression of immunity is called immunomodulation. Immune System potentiation is useful to control and treat many chronic infections and cancers. Cytokines, adoptive immune therapy and vaccination are the major sources potentiation of immune response.

Immunosuppression is achieved through depletion of T-cells. Several medicines are used for immunosuppression like corticosteroids, azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus, sirolimus etc.

1.3. ALLOGENEIC TRANSPLANT AND ITS IMMUNOLOGICAL ASPECTS

Solid organ transplant was a major breakthrough when 1st transplant was performed in 1954 at Peter Bent Brigham hospital by Dr. Joseph and his colleagues. It confirmed the hypothesis one kidney is sufficient for survival.

During transplant immunosuppression is a main target to prevent organ rejection. Immunosuppressive drugs are used for this purpose. These drugs play vital role to maintain and stabilize host-graft adaptation (3).

The matching of human leukocyte antigens (HLAs) of donor and recipients is of vital importance for clinical immunological tolerance. The short arm of chromosome 6 have a set of genes named as major histocompatibility complex (MHC). Peptides of MHC class I are forwarded to CD8+T-cells while class II MHC peptides are linked toCD4+T-cells. Each human have different pairs of class I and II MHCs. This process decides the donor graft acceptance or rejection during transplant.

In renal transplant success depends upon matching of donor and recipient MHC antigens while it is less critical in liver transplant. Many laboratory tests are performed before transplant like HLA and ABO type, cross matching and screening for preformed antibodies. Allograft rejection is prevented by suppression of immune system of host using immunosuppressive medicines.

1.4. IMMUNOSUPPRESSIVE DRUGS

These are the drugs which are used to suppress the immunity during transplant to prevent the allograph rejection. Table-1.1describes its classification.

Table 1.1 Classification of immunosuppressant drugs:

<table>
<tr><th colspan="7">Immunosuppressant drugs</th></tr>
<tr><td rowspan="2">Glucocorticoids</td><td colspan="2">cytostatic Agents</td><td colspan="2">Drugs acting on immunopillins</td><td rowspan="2">Antibodies</td><td rowspan="2">Other Drugs</td></tr>
<tr><td>Alkylating Agents</td><td>Antimetabolites</td><td>Calcinurin inhibitors</td><td>m-TOR Inhibitors</td></tr>
<tr><td>Dexamethasone</td><td>Cyclophosphamide (nitrogen mustard)</td><td>Methotrexate (Folic Acid Analogue)</td><td>Ciclosporin</td><td>Everolimus</td><td>Polyclonal Antibodies</td><td>Interferons</td></tr>
<tr><td>Hydrocortisone</td><td>Nitrosurease</td><td>Azathioprine (Purine Analogue)</td><td rowspan="4">Tacrolimus</td><td rowspan="4">Sirolimus</td><td>Monoclonal Antibodies</td><td>Opoids</td></tr>
<tr><td rowspan="3">Prednisone</td><td rowspan="3">Platinum compounds</td><td>Marcaptopurine (Purine Analogue)</td><td>T-Cell receptor directed antibodies</td><td>TNF Binding Protein</td></tr>
<tr><td>Flurouracil (Pyrimidine Analogue)</td><td rowspan="2">IL-2 receptor directed antibodies</td><td>MMF</td></tr>
<tr><td>Protein Synthesis Inhibitors</td><td>Small Biological Agents (Fingolimod, Myrion)</td></tr>
</table>

Corticosteroids have been used since 1960 as immunosuppressant. These are agonists of glucocorticoid receptors and acts by different mechanisms (4). Steroid-receptor complex produce immunosuppressive effects by DNA-binding.(5).

Alkylating agents prevent the cell division and protein synthesis that cause cell death when they tend to divide due to DNA alkylation. Cyclophosphamide inhibits B-lymphocyte proliferation but it promotes T-cell responses (6). Purine analogue like azathioprine inhibit T-Cells activation through blockage of gene proliferation (7)

Inosine monophosphate dehydrogenase inhibitors (IMPDH) like mycophenolate mofetil is more effective in combination, has less toxicity and increased the graft survival. Cyclosporine and tacrolimus corresponds to calcineurin inhibitors that acts by binding with immunophillin. Sirolimus and everolimus that belongs to mTORs (mammalian targets of rapamycin) by making a complex with FKBP12 protein. Other drugs for this purpose includes dihydroorotate dehydrogenase inhibitors and mono and polyclonal antibodies (8).

1.4.1. m-TOR Inhibitors

The mammalian target of rapamycin (mTOR) inhibitor i.e. everolimus and sirolimus widely used class as immunosuppressive in kidney and liver transplantation (9). These drugs creates complex by acting on FKBP12 protein which then block rapamycin target. The inhibition of rapamycin target prevents cytokine receptor to activate cell cycle by signal 3 blockage (10).

1.4.2. Everolimus

Everolimus is a derivative of sirolimus (rapamycin) having 2-hydroxy-ethyl chain substitution at position 40 on the rapamycin structure. It is more polar than its derivative. Enhancement of oral bioavailability was the purpose of its development. Both everolimus and sirolimus have an identical mechanism of action. Oral everolimus is rapidly absorbed from the oral route attaining peak plasma concentration within 1.3–1.8 hours. Like other immunosuppressant drugs monitoring of everolimus blood concentration is necessary (11).

1.4.3. Sirolimus

Sirolimus is a natural macrocyclic lactone produced by the bacterium streptomyces hygroscopic us consisting of a 29-membered ring containing 4 trans double bonds, three of which are conjugated. It is a macrolide, a cyclic acetal, a cyclic ketone, an ether, a lactam, a secondary alcohol, an organic heterotricyclic compound and an antibiotic antifungal drug.Its molecular formula is $C_{51}H_{79}NO_{13}$.Its molecular weight is 914.2 g/mol. Melting point is 183-185°C. It is soluble in ether, chloroform, acetone, methanol, and DMF; very sparingly sol in hexane and petro ether.octanol/water partition coefficient is 4.3 ATC Code.L04AA10 and as shown in figure 1.1.

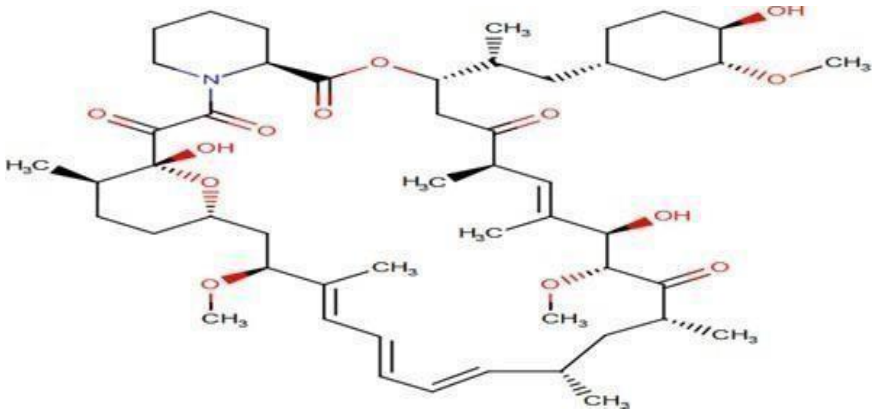

Figure 1.1 Structure of sirolimus

Sirolimus is a macrolide produced by *Streptomyces hygroscopicus.* Sirolimus deactivate the proliferation of T-lymphocytes and blocks the other T-cells receptors through immunophillin complex formation via FKBP12 protein. It then inhibits mTOR resulting in blockage of s cell-cycle progression at the G1 to S- phase transition. Bioavailability after oral administration is about 15% which is further reduced with fatty meals. Plasma protein binding is 40-50%. Metabolism occurs in liver by CYP3A4. Excretion is mainly through feces (91%). Urine excretion is only 2.5% (12) and as shown in figure 1.2.

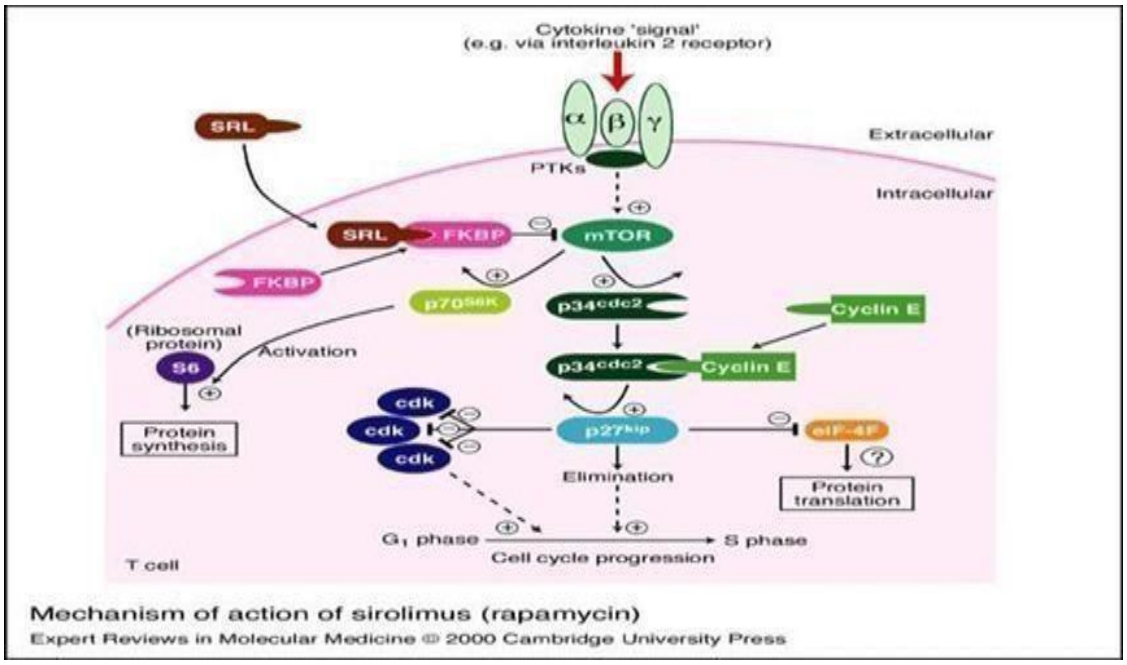

Figure 1.2 Mechanism of action of sirolimus (62).

1.5. DRUG CLASSIFICATION AND THE BASIS OF BIOPHARMACEUTICAL PROPERTIES

The BCS is a scientific way to classify a drug substance according to its aqueous solubility and intestinal permeability. Taking into account the in-vitro dissolution characteristics, BCS consider solubility, intestinal permeability, and dissolution rate parameters. According to BCS principal the drug substance is considered highly soluble if its highest strength is dissolved in maximum 250 ml of water in a pH range of 1.0-7.0 otherwise it will be a poor soluble molecule while permeability classification is based on extent of intestinal absorption. BCS classify the drugs in following four classes:

Class-I drugs possess high absorption and high dissolution.

Class-II drugs have high absorption and low dissolution.

Class-III drugs show low absorption and high dissolution properties.

Class-IV drugs are with low absorption and low dissolution properties.

Solubility of poorly soluble drugs can be increased by using different techniques e.g. increasing surface area, complexion method, by using different polymeric forms, use of surfactants (13).

1.6. DRUG RELEASE MECHANISM FROM POLYMERIC NANOPARTICLES

One of following three physico-chemical mechanisms work to release the polymeric drug at required site.

1. By hydration of polymer nanoparticles followed by swelling resulting in release through diffusion.
2. Through polymer rupture or cleavage or degradation by an enzymatic reaction at delivery site leading to drug release from the entrapped inner core.
3. By de-adsorption/release from the swelled nanoparticles by dissociation of the drug from the polymer (14).

1.7. NANO PARTICLES PREPARATION TECHNIQUES.

Different polymers are used to prepare the nanoparticles by entrapping, dissolving, encapsulating or attaching the drug. Nanoparticles serve as a good vehicle to deliver the drug at target site.(15).Size, medium and physico-chemical properties of nanoparticles describes growth mechanism and distribution function of nanoparticles (16). Several techniques are being used for nanoparticles preparation which are listed below: (17).

1.7.1. Methods for preparation of nanoparticles from dispersion of preformed polymer

a) Solvent evaporation

b) Nanoprecipitation

c) Emulsification/solvent diffusion

d) Salting out
e) Dialysis
f) Supercritical fluid technology (SCF)

1.7.2. Methods for preparation of nanoparticles from polymerization of monomers

a) Emulsion
b) Mini emulsion
c) Micro emulsion
d) Interfacial polymerization
e) Controlled/Living radical polymerization(C/LRP)

1.7.3. Ionic gelation or concentration of hydrophilic polymers solvent evaporation method:

It is the 1st method employed for the nanoparticles preparation. In this technique polymer is dissolved in volatile solvent which is then added in aqueous phase to formulate the emulsion. Organic solvents used includes dichloromethane, chloroform and ethyl acetate. Nanoparticle suspension is obtained by evaporation of organic solvent. Single or double emulsion process may be used for emulsion formulation by doing high speed homogenization or ultra-sonication. Solvent evaporation is done by continuous stirring at normal temperature or under reduced pressure. The solidified nanoparticles are collected by ultracentrifugation followed by lyophilization and as shown in figure 1.3.

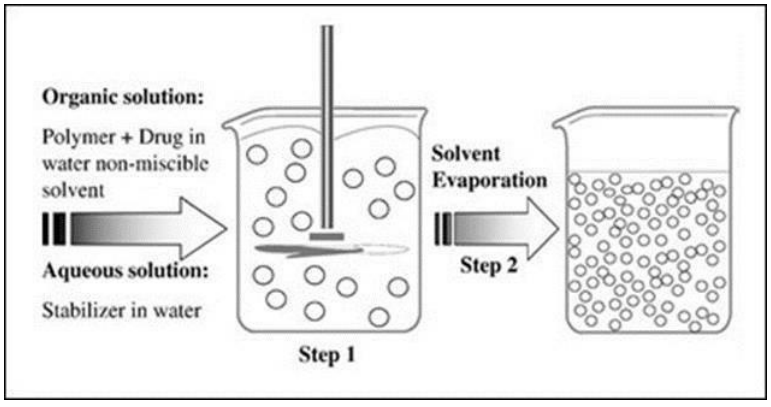

Figure1.3 Schematic representation of the solvent-evaporation technique (18).

1.8. APPLICATIONS OF NANOPARTICLES DRUG CARRIER

Nanoparticles promises the stability of volatile pharmaceuticals. These are becoming popular drug delivery system for the treatment of cancer, vaccines and other targeted drug delivery (19). Nanotechnology has good potential for use of nanoparticle uses in food science and food microbiology. Nanotechnology is offering good scope for in various areas like agriculture, food, and medicine (20) Nanotechnology provides good opportunity for cancer treatment and diagnosis through targeted delivery of the drug to the cancer cells leading to better efficacy and reduced toxicity. Similarly targeted delivery of an imaging agent provides the benefits of early stage cancer detection (21). Nanotechnology has changed the approaches for engineering or fabricating RNA into nanoparticles and exploration of RNA nanoparticles for therapeutic application and detection of pathogen and delivery of gene/drug (22).

Nanotechnology is playing a potential role in gastroenterology as gastrointestinal tract investigations and imaging as well as better drug delivery (23). Cancer nanotechnology due to its vast and diverse array of nanoparticles providing good solutions in comparison to current obstacles in cancer therapies. (24). Overall nanotechnology has a good future potential to serve the humanity.

1.9. CHITOSAN

Chitosan is natural polysaccharide having a similar structure to cellulose bearing hydroxyl, acetyl or free amino group (25). It is copolymer of glucosamine or D-glucosamine (deacetylated unit) and nacetylglucosamine or N-acetyl-D-glucosamine (acetylated units) connected at β-(1-4) position with biocompatible and biodegradable properties. Additionally it is low immunogenic, with good pharmacological and biomedical application without any lethal effects. Chitosan has an atomic weight of 3800-20000 daltons and it is usually deacetylated (66 to 95%). It has a pKa value of 6.5. Physicochemical and organic properties mainly are linked with atomic weight and deacetylation. Chitosan has the property to be modified in different structures like thiolated, carboxyalkyl (26) Chitosan has possibilities for mechanical chemical and structural alteration to produce unique attributes in different fields of life. Nano chitosan have advantageous features of high surface area, increased solvency (27) Because of cationic character and primary amino groups, chitosan bears many properties such as mucoadhesion,

controlled drug release, permeation enhancement and efflux pump inhibitory properties hence used in various drug delivery systems.(28).

1.9.1. Modified Chitosan

Attempts have been made to develop the chitosan with altered structures. Modification does not lead to change in basic structure but incorporate enhanced properties of solvency, targeted drug delivery etc. Modification of chitosan is usually caused by tailoring amine, hydroxyl or thiolated function. Alkylated chitosan are created through alkylation of essential amine group while methylation also lead to modification of chitosan. Examples includes trimethyl, diethylmethyl, triethyl, dimethyl ethyl chitosan's , N-trimethyl chitosan (TMC) (29). Carboxymethyl, dihydroxyethyl, sulfuryl and phosphoryl groups have been altered to chitosan to get its improved attributes (30).

1.9.2. Thiolated Chitosan

Thiolated chitosan is unique among other chitosan polymer because it is the only polymer which has cationic thiomer while others have COOH group in their skelton. It is soluble below pH 6.0. Thiol group is immobilized at 2- position of primary amino group. This immobilization results in improvement of mucoadhesive, cohesive (In situ gelling), permeation enhancing and efflux pump inhibition properties. Positively charged amino group of amino acid of polymer interacts with negatively charged sialic acid and sulfonic acid of the mucus which makes chitosan as mucoadhesive. The presences of thiol group over chitosan augmentin the mucoadhesive properties of thiolated chitosan. Oxidation of thiol group creates in situ gelling properties of thiolated chitosan at physiological pH. The permeation enhancing effect of chitosan is pH dependent through paracellular absorption mechanism by opening tight junctions by interaction between positively charged amino group of chitosan and negatively charged sites of cell membrane. Thiolated chitosan show increased muco adhesive attributes due to extra residence time. The other mechanism which enhances the permeation by using thiolated chitosan is inhibition of p-glycoprotein efflux pump. This inhibition occurs due to covalent linkage of thiol group with 6- mercaptonicotinamide (31).

1.10. APPLICATION OF CHITOSAN IN DRUG DELIVERY SYSTEM

Scientists are very much interested to evaluate the potential application of chitosan especially in medical and pharmaceutical field. It has unique attributes of susceptibility to enzymatic hydrolysis, biocompatibility and binding capability with many organic compounds. These properties have made the chitosan to have variety of application in drug delivery, targeting and nanotechnology. Drug delivery system with chitosan ranges from micro particles to nanoparticles.

Different modified forms of chitosan like thiolated, acetylated and carboxylate forms have been developed to overcome its weakness of poor solubility at physiological pH. Chitosan is the best choice for anionic drug delivery system. Immobilization of thiol group increases its mucoadhesive feature. Self branched chitosan possess gene transfer character without safety compromise. Chitosan is also reported for use in nasal drug delivery system. It also serves as injectable vehicle. In nanotechnology chitosan nanoparticles have a variety of application ranging from solubility enhancement to control of drug release with improved efficacy and safety features. Another era where chitosan have potential for drug delivery system is film preparation for oral drug delivery. (32).

CHAPTER 2

LITERATURE REVIEW

Sirolimus was a macrolide that inhibited the mTOR pathway by directly binding to the mammalian target of rapamycin (mTOR) complex, which blocked the activation of B and T cells. It showed promise in the treatment of various disorders such as RA, psoriasis, systemic lupus erythematosus, tuberous sclerosis, sjögren syndrome, and kaposi sarcoma. Sirolimus had the advantage of reduced renal toxicity unlike other medications. The bioavailability of sirolimus drug was approximately 14% and had very low bioavailability (31).

The nano emulsion technology was a tool that reduced the size of sirolimus drug and converted the crystalline form of the drug to an amorphous form. Sirolimus was completely insoluble in water and had a major issue with solubilizing the drug. The nano emulsion formulation was developed by using different types of oil phase excipients. The solubility of the drug was enhanced in the drug delivery system. A nano formulation method was utilized to create an emulsion of sirolimus by using triacetin oil, tween grade 20, 2-propanol, polyethylene glycol grade 400, and tween grade 80. The oil base solution was prepared in a separate vessel by stirring and sonication of the surfactant, a co-surfactant, and triacetin in different proportional ratios. Then, a specific quantity (1mg/ml) of sirolimus was added while continuously sonication, mixing, and stirring until a clear solution was obtained. The spontaneous emulsification technique was used to create a number of formulations that included 1 mg/ml of the medication (32).

These nano emulsions (NEs) described and put through stability tested over the period of 9 to 12 months at various temperatures and relative humidity condition. The chosen formulations tested used a dialysis sac during a 48-hour period to ascertain the cumulative medication released. HPLC Method was used for the rapamycin assay (33).

SRL-SMEDDS was prepared by nano emulsion SMEDDS system consisted of surfactant, oil and cosurfactant, so the drug solubilizing potential of these vehicles was the premise of optimum drug loaded while maintained an excellent emulsifying performance. Liquid sirolimus–self micro emulsifying pellets drug delivery system formulations were transformed into solidified pellets by used the extrusion-

spheronization method. An efficient SRL-SMEDDS formulation was screened by solubility tested. The ideal formulation consisted of 1 milligram pellets of SRL-SMEDDS. Scanning electron microscopy and visual observation were verified that the solid pellets had a good appearance. Analysis using IR (Infrared), XRPD (X-ray powder diffraction) and DSC (differential scanning colorimeter) techniques showed that the pellets contained no crystalline sirolimus and there was amorphous sirolimus (34).

To prepared the rapamycin-SMEDDS pellets, a mixture of crodamol (solubilize oil), oleic acid (oil), tween- 80, labrasol (surfactant), cremophor (non ionic emulsifier), transcutol, propylene glycol, PEG400, and glycerin (cosurfactant) stirred for several hours until a cleared solution was obtained (35). The liquid SMEDDS (self micro emulsifying pellets drug delivery system) formulation was then blended with solid adsorbents included cross- linked sodium carboxymethyl cellulose, crospovidone and talcum in a ratio of 1:2 by weight (36).The sirolimus -SMEDDS pellets characterized used various techniques such as DSC, XRPD, FTIR, redispersibility studied, scanning electron microscopy (SEM), pellet size distribution, friability, and assay of sirolimus drug (37).

Using the emulsion/solvent evaporation technique, nanoparticles (NPs) produced. 200 milligrams of poly(lactic-co-glycolic acid) PLGA should place in a 100 ml conical flask along with 20 milligrams of sirolimus, enough methylene chloride, and PVA grade 90 (poly vinyl alcohol 0.1 percentage weight/volume). The mixture should then been thoroughly mixed, stirred for 30 minutes, and then ultrasonically processed at 30 percent amplitude for 1 minute and 30 seconds until the solution became cleared (38). Then put the entire solution into the rotating flask and evaporate the entire methylene chloride solvent used the rotary evaporator device to produce the dispersible sirolimus nanoparticle. Polymeric drug delivery system played important role in solubility enhancement, rate of drug released, reduced degradation of drug and its toxicity and improved the therapeutic efficiency of a drug .(39).

Poly (lactic-co-glycolic acid) (PLGA) was the most widely used polymer for nanoparticle formulation. It was a biocompatible material (40). Many formulations based on PLGA and its homopolymer approved by FDA for medical used. PLGA nanoparticles be used for targeted drug delivery system. PLGA is converted to non-toxic products i.e. H_2O and CO_2 by biodegradation through hydrolysis. PLGA is

synthesized by polymerization of glycolic and lactic acid. The intrinsic attributes of PLGA totally depended upon the composition of its monomers. lactic/glycolic acid ratio in PLGA and its molecular weight determines degradation and drug released rate (41). PLGA nanoparticles could retain delivery of drugs by protected it from degradation in endolysosomes(42). EMA and FDA had approved PLGA as drug delivery system because of sustained released property, protection of drug from degradation and better interaction with biological material (43). Polymeric drug delivery system played important role in solubility enhancement, rate of drug released, reduced degradation of drug and its toxicity and improved the therapeutic efficiency of a drug (44). Poly (lactic-co-glycolic acid) (PLGA) is the most widely used polymer for nanoparticle formulation.

It was a biocompatible material (45). Many formulations based on PLGA and its homopolymer approved by FDA for medical used PLGA nanoparticles be used for targeted drug delivery system. PLGA converted to non-toxic products i.e. H_2O and CO_2 by biodegradation through hydrolysis. PLGA is synthesized by polymerization of glycolic and lactic acid. The intrinsic attributes of PLGA totally depended upon the composition of its monomers. Lactic/Glycolic acid ratio in PLGA and its molecular weight determines degradation and drug released rate (46). PLGA nanoparticles could retain delivery of drugs by protected it from degradation in ended lysosomes (47). EMA and FDA had approved PLGA as drug delivery system because of sustained released property, protection of drug from degradation and better interaction with biological material. (48).

The efflux pump capability to inhibit drug action has great potential to develop action plan for treatment (49). PGP is a protein having a molecular weight of 170kda which functions as a drug carrier and is known is efflux pump (50). PGP efflux pump is the 1st researched as efflux pump. Studies reflect that PGP play key role in drug binding and transportation and substrate behaving. PGP have many substrates including many drugs like chemotherapeutic agents, fluorescent dyes etc. During drug transportation, initially drug is moved to inside of membrane then get back to extracellular side leading to drug release. Usually lipophilic compounds are transported through PGP while its substrates are amphipathic in nature. Normally three action plans are used to coop the PGP namely evading (use of poor PGP substrate drugs), engaging (simultaneous administration of modulators) and exploiting (specific targeting of the

PGP molecule (31). PGP is important player of drug absorption and transportation system. In many cases it is evident that PGP may decrease the bioavailability of many drugs. Research shows that bioavailability can be increased by inhibition of PGP efflux pump (25). Efflux pump inhibitors (EPIs) are majorly classified in two groups namely small molecule inhibitors (SMIs) e.g. quinine, verapamil etc and polymeric inhibitors like tween 80, plutonic etc. There are many naturally occurring EPIs which includes polysaccharides, polypeptides and proteins. Polysaccharides are extensively used pharmaceutical excipients.

Literature review shows that among polysaccharides, starch, cellulose and chitosan perform the efflux pump inhibitory function. Instead of unmodified chitosan, thiomeric chitosan shows efflux pump inhibitory function (28).

The efflux pump capability to inhibit drug action has great potential to develop action plan for treatment (31). PGP is a protein having a molecular weight of 170kda which functions as a drug carrier and is known is efflux pump (27).

PGP efflux pump is the 1st researched as efflux pump. Studies reflect that PGP play key role in drug binding and transportation and substrate behaving. PGP have many substrates including many drugs like chemotherapeutic agents, fluorescent dyes etc. During drug transportation, initially drug is moved to inside of membrane then get back to extracellular side leading to drug release. Usually lipophilic compounds are transported through PGP while its substrates are amphipathic in nature. Normally three action plans are used to coop the PGP namely evading (use of poor PGP substrate drugs), engaging (simultaneous administration of modulators) and exploiting (specific targeting of the PGP molecule) (35). PGP is important player of drug absorption and transportation system. In many cases it is evident that PGP may decrease the bioavailability of many drugs. Research shows that bioavailability can be increased by inhibition of PGP efflux pump (51). Efflux pump inhibitors (EPIs) are majorly classified in two groups namely small molecule inhibitors (SMIs) e.g. quinine, verapamil etc and polymeric inhibitors like tween 80, plutonic etc. There are many naturally occurring EPIs which includes polysaccharides, polypeptides and proteins. Polysaccharides are extensively used pharmaceutical excipients. Literature review shows that among polysaccharides, starch, cellulose and chitosan perform the efflux pump inhibitory function. Instead of unmodified chitosan, thiomeric chitosan shows efflux pump inhibitory function (31).

Particle drug delivery system has emerged as potential research approach (52). This drug delivery system provides multiple advantages like more efficacies, less toxic and better patient compliance (53). Nanoparticles are defined as the polymeric colloidal system of natural, synthetic or semisynthetic polymers having size range of 10-1000nm (54). Nanotechnology is an emerging scientific technology which usually deals with nano delivery system is the system referred for working in nanometer range. It is an initiation of affecting the conventional delivery system. Nano drug delivery system possess a notable part of nano medicine (32). The key of nano drug delivery system is to design a planned function of molecules. Nanoparticles as drug delivery vehicle are used to improve solubility and bioavailability while reduces the toxicity (55). A good structural control leads nanoparticles ideal as drug delivery vehicle. Active drug may be incorporated as encapsulation, matrix or on the surfaces of nanoparticles through covalent bond or adsorption mechanism (56).

The future of nanotechnology lies in developing nano/micro production processes. From the pharmaceutical industry point of view, the objective of nanotechnology is to introduce such a formulation that would be useful for patient's treatment (57). Current research is emphasizing to merge the specific properties of nanomaterials like surface volume ratio (58).

CHAPTER 3

METHODOLGY

Sirolimus material was a gift from ALLMED Pharmaceutical and excipients castrol oil, propylene glycol was purchased from AL- Naseer trader. Sirolimus formulations were developed by castrol oil and ethanol. The emulsification method was developed to prepare two formulations.

Two different formulations were performed with 2 mg /ml drug for development of nano emulsion to enhance solubility and permeability of sirolimus drug. Order of mixing and excipients ingredients were different in both formulations. Both formulations were repeated three time for to get optimize results.

3.1-Self-emulsifying formulation

The formulation contained raw materials API (active pharmaceutical ingredient) sirolimus or rapamycin (Concord Biotech Limited India), Vitamin E (Alfa-tocopherol) Merck, castrol oil (Chromophore EL)CRODA, Tween 80 (UNI- CHEM), Tween 20 (UNI-CHEM), Propylene glycol (Pan ASIA Chemical Corporation), Ethanol 99.9% Merck, and purified water. These components play an important part in the formulation and as shown in table 3.1.

Formulation batch size: 100 ml **API stated amount:** 2mg/ml

Table 3.1 Formulation ingredients of self-emulsifying formulation

Sr No	Ingredient Name	Quantity	Safe Limit
01	Sirolimus	200 mg	2mg/ml
02	Castrol oil	20 ml	20-30%
03	Tween 20	5ml	NMT 5%
04	Tween 80	5ml	NMT 5%
05	PG	20 ml	20 – 30%
06	Ethanol 99.9%	30 ml	60 – 80%
07	Alfa-Tocopherol	0.5 ml	NMT 0.5%
08	Purified Water	QS	

The castrol oil emulsion, derived from the seed fam. Euphorbiaceae, was combined with 20 ml of pharmaceutical grade 99.90% pure propylene glycol and mixed for 2-3 hours at 4000 rpm. Sirolimus was weighed using an analytical balance with a weighing range of 0.1mg to 210 gm, and then dissolved in a solution of 30 ml of ethanol and 10 ml of purified water. The mixture underwent stirring and sonication for 3-5 hours to ensure thorough mixing of the co-surfactants and sirolimus solutions. The stirring was done at 4000 rpm until the solution became clear, indicating successful mixing and dissolution of the components.

Surfactants tween 80 (5ml) and tween 20 (5ml) were introduced and mixed at a rate of 4000rpm for 8-12 hours. Antioxidant alfa-tocopherol was then incorporated , was marked with water 100ml and mixed at the same speed for 2-3 hours. Following thorough stirring, a sirolimus emulsion was achieved, resulting in a clear, light yellow solution with a final volume of 100ml using purified water as needed.

After development of formulation, sample was collected for performing different characterization UV, IR, zeta testing and HPLC testing.

3.2- Nano size emulsion:

Formulation batch size: 100 ml API stated amount: 2mg/ml

Table 3.2 Formulation ingredients of nano emulsion formulation

Sr No	Ingredient Name	Quantity	Safe Limit
01	Sirolimus	200 mg	2mg/ml
02	Castrol oil	15 ml	20-30%
03	Tween 20	3ml	NMT 5%
04	Tween 80	3ml	NMT 5%
05	PG	20 ml	20 – 30%
06	Ethanol 99.9%	25 ml	60 – 80%
07	Alfa-Tocopherol	0.5 ml	NMT 0.5%
08	Purified Water	QS	

The extraction of castrol oil emulsion from the euphorbiaceae family of seeds utilized pharmaceutical-grade propylene glycol with a purity of 99.90% .A total of 15-20 hours were dedicated to stirring a mixture consisting of 15 milliliters of castrol oil and 20 milliliters of propylene glycol at 4000 rpm. Sirolimus was weighed using a sartorius Germany analytical balance (0.1 mg to 210 gm) and then dissolved in a 25ml ethanol. The combined mixture was agitated at 4000 rpm for 4 hours until the solution became clear. The co-surfactant and sirolimus solution were then combined, sonicated, and stirred for six hours.

The addition of surfactants (Tween 80 3ml and Tween 20 3ml) was followed by stirring at a speed of 4000rpm for 12 hours, while antioxidant alfa-tocopherol was added and 100ml was marked with water and stirred at the same speed for 4-5 hours. After stirring and mixing, a clear and light yellow sirolimus emulsion was successfully obtained, despite the varying amounts of excipient ingredients and different mixing orders and times in the two formulation. Following the preparation of the nano emulsion, a sample was extracted for further characterization tests.

Three sets of experiments were performed to enhance the formulations. Various characterizations were utilized to optimize the formulations, including physical

and chemical measurements. The physical characterizations included appearance and solubility, while the chemical characterizations involved UV, IR, zeta potential, zeta sizer, and drug potency using the HPLC Method.

The nano emulsion appeared as a solution ranging from light yellow to dark yellow. To test the solubility of the sirolimus-loaded, different solvent ratios, including castor oil: water (15:85 to 25:75) were selected to check the solubility of the loaded Sirolimus drug.

3.2. UV SPECTROPHOTOMETER METHOD:

Shimadzu 1700 UV Spectrophotometer was used to find out stated amount of sample by measuring the absorbance and identification of sample.

3.2.1. Reference preparation:

Sirolimus reference material was taken in methanol solvent and to make concentration of solution 0.2 mg /ml.

3.2.2. Drug sample preparation:

A 5 ml nano emulsion sample was transferred in a 50 ml volumetric flask and mixed with methanol as a solvent until reaching the 50 ml mark. The solution was then sonicated and stirred for 5 minutes, resulting in a sample concentration of 0.2 mg/ml. Both the sample and reference solutions at 200 μg/ml were prepared in methanol, and their maximum absorbance values were determined by scanning in the range of 200–400 nm against the reagent blank (methanol).To ensure the reliability of the results, the maximum absorbance was determined using spectrophotometry technique.

3.3. INFRARED SPECTROSCOPY:

Infra-red fourier transform instrument was used to check the functional groups present in the nano emulsion of sirolimus drug. The major functional groups C=C and C=O was in sirolimus. Major IR peaks was observed at 1750 and 1650 cm^{-1} in Sirolimus drug.

The Nicolet iS5 and ATR iD5 instruments from Thermo Scientific USA were utilized to analyze various functional groups present in a nano emulsion formulation. A small amount of sirolimus nano emulsion was placed on the ATR crystal, and the IR spectrum appeared on the screen in just 20 seconds. The IR spectrum of the sirolimus Nano emulsion was then compared with the reference IR spectrum of pure sirolimus drug. The presence of four C=C double bonds and six C=O double bonds in sirolimus was confirmed by observing characteristic peaks in the IR spectrum, such as the C=O stretch peak (1760–1670 cm^{-1}) and the C=C stretch peak (1680–1630 cm^{-1}).Finally, an IR spectrum analysis was conducted using the reference material of sirolimus for further comparison and verification.

3.4. PARTICLE SIZE MEASUREMENT BY ZETA SIZER:

Size of nanoparticles were measured with anton paar particle sizer analyzer (lite sizer DIA 500) by DLS technique

The zeta sizer nano range instruments are capable of measuring three key characteristics of particles or molecules in a liquid medium: particle size, zeta potential, and molecular weight. The zeta sizer is designed to measure particle sizes ranging from 0.3nm to 5µm in liquid samples. To prepare the sample, it can be used directly as a solution or diluted to 10% to 50% with a suitable solvent and sonicated for an appropriate amount of time. The sample should be placed in a 10 mm cell, the appropriate solvent added, and 50µl to 100µl of stock dispersion solution added. Parameters such as particle size, absorption, viscosity, and refractive index should be carefully selected for accurate measurements.

3.4.1. Sample Preparation:

To homogenize the solution, place a 10 ml sample of the nano emulsion in a 50 ml volumetric flask, add ethanol, mark 50 ml with ethanol, and sonicate for five minutes. Place the sample in a 10 mm cell, then add 50 µl of stock dispersions solution and 100 µl of ethanol.

3.5. ZETA POTENTIAL:

The potential of nano emulsion was assessed using the anton paar particle potential analyzer (lite sizer DIA 500). Zeta potential was utilized to determine the electrochemical balance at the particle-liquid interface, measuring the potential difference in mv of positive or negative ions in the nano emulsion sample. The presence of numerous charged ions in the formulation could lead to precipitation and affect the stability of the formulation. A stable formulation typically exhibited a minute quantity of negative charge. Zeta potential served as an instrument to measure the potential equilibrium within a range of ±150mv of the sample in the liquid phase through the zeta potential mode. The sample could be used directly as a solution or diluted to 10% to 50% with a suitable solvent, followed by sonication for an appropriate duration.

3.5.1. Sample Preparation:

Sample was taken in a 50-milliliter volumetric flask to make concentration 0.4 mg/ml and ethanol was used as solvent and sonicate the mixture for five minutes for homogenization. Pour 50 µl of stock dispersions solution and 100 µl of ethanol into a 10 mm cell containing the sample. Using the zeta potential mode and DLS approach.

3.6. HPLC METHOD:

The nano emulsion formulation was analyzed for sirolimus drug quantity using HPLC (Model No 1260 affinity II Agilent USA and Detector DAD at 277 nm). The HPLC method was utilized in various studies to quantify and evaluate the concentration of sirolimus in the nano emulsion formulation, as well as to assess thermal and photo stability. The reverse phase chromatography method was employed to determine the concentration of sirolimus in the nano emulsion formulation, with a flow rate of 2 ml/min and a column temperature of 60 ºC. The column used was C8 150 mm × 4.6 mm with a pore size of 5µm, and an injection volume of 20µl.

There were three isomers of sirolimus drug Isomer A, Isomer B and Isomer C.

3.6.1. Drug Concentration Method:

Preparation of Mobile phase:-

Acetonitrile: Water

55: 45

Then degas the solution for 10 minutes with ultrasonic bath.

Diluent: Acetonitrile: Water

30: 70

3.6.2. Standard Preparation:

Prepared a solution of 20 mg sirolimus working standard in a 50 ml volumetric flask by adding 15 ml of acetonitrile, mixing well, and then filling up to the mark with purified water. Sonicate for 5 minutes. Transfer 5 ml of the prepared solution into a 50 ml volumetric flask and adjust the volume with diluent to obtain the final sample solution.

3.6.3. Sample Preparation:

A sample of nano emulsion drug, equivalent to 20 mg of sirolimus, was placed in a 50 ml volumetric flask. 15 ml of acetonitrile was added, mixed well, and then the volume was made up to the mark with purified water before sonication for 5 minutes. Subsequently, 5 ml of the sample was transferred to a 50 ml volumetric flask and the volume was made up to the mark with diluent.

CHAPTER 4

RESULTS

Sirolimus nano emulsion characterization involved a set of tests to validate the successful formation of nano emulsions. The sampling strategy was carried out following the invention of the nano emulsion. Physical and chemical properties were investigated to optimize the nano emulsion formulation. It includes a visual inspection of nano emulsion, solubility, UV, FTIR, zeta sizer, zeta potential, and HPLC drug analysis.

The self-emulsifying formulation had a dark yellow solution appearance, whereas the nano size emulsion had a light yellow solution appearance. Pharmaceutically acceptable materials should be chosen for the creation of nano emulsion delivery systems. The drug's solubility in the oil phase was also a crucial factor in the creation of an effective NE formulation, as it improved the drug's ability to remain in a solubilized form in the NE. Low drug solubility leads to a decrease in drug association with the oily phase, which in turn calls for a larger inclusion of lipophilic and hydrophilic emulsifiers.

Table 4.1 Solubility of sirolimus drug in oil phase

Loaded Drug	Solvent System	Oil /water ratio	Concentration Sirolimus	Solubility of drug
Sirolimus drug	Castrol oil & Water	15 : 85	6 mg/ ml	Soluble
			8 mg/ml	Soluble
			10 mg/ml	Soluble
			12 mg/ml	Soluble
			14 mg/ml	Soluble
			16 mg/ml	Insoluble
		20: 80	12 mg/ml	Soluble
			14 mg/ml	Soluble
			16 mg/ml	Soluble
			18 mg/ml	Insoluble
		25:75	14 mg/ml	Soluble
			16 mg/ml	Soluble
			18 mg/ml	Insoluble

When compared to its Sirolimus water solubility (2.6 μg/mL) and sirolimus solubility of about 16 mg/mL in oil phase (20: 80) and (25:75) showed that the oil phase system was able to dissolve the medication. The solubility was enhanced

from 2.6 µg/mL to 16 mg / ml in oil phase system and as shown in table 4.1.

4.1. SPECTROPHOTOMETRY TECHNIQUE

Following the preparation of nano emulsion formulations, a sample was gathered to optimize the results and establish the maximum absorbance. The spectrophotometry technique was employed to measure the absorbance and identify the sample by scanning the sample solution, in order to determine the percentage of the sample.

4.1.1. Self-Emulsifying Formulation:

Surfactants, oil phases, and co-surfactants did not interfere with the scanning of sirolimus nano emulsions drug. To ensure reliability of the results, the maximum absorbance was determined using spectrophotometry technique. Sirolimus solutions at concentrations of 200 µg/mL were used as reference samples, along with nano emulsion samples, and scanned in scanning mode. The spectrum displayed a peak at 277.4 nm, indicating maximum absorbance in methanol as the solvent. A major peak of sirolimus was also detected at 277 nm, with an absorption of 1.260. No interference was observed from the oil phases (camphore EL), PG, tween 20, or tween 80 excipients and as shown in figure 4.1.

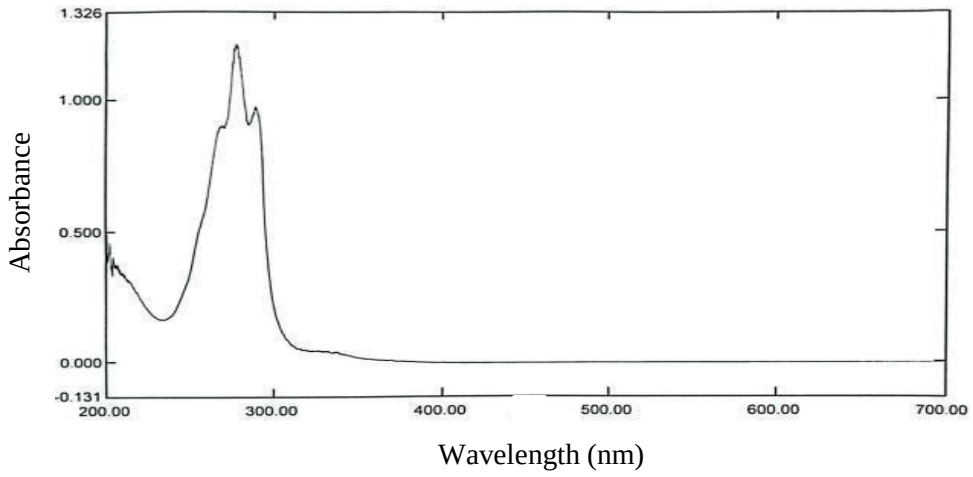

Figure 4.1 UV Scan of sirolimus in self-emulsifying formulation

Table 4.2 UV absorbance of sirolimus in self-emulsifying formulation

Sr No	Wavelength	Abs.
01	390.00	-0.006
02	381.80	-0.004
03	375.80	-0.002
04	370.00	0.003
05	356.80	0.008
06	326.80	0.043
07	318.00	0.045
08	288.40	0.973
09	277.40	1.206
10	200.80	0.489

4.1.2. NANO SIZE EMULSION:

A concentration of 200 µg/ml of sirolimus sample was prepared in methanol, and the highest absorbance readings were recorded by scanning these solutions within the 200–400 nm range compared to the reagent blank. Surfactants, oil phases, and co-surfactants did not interfere with the scanning process of sirolimus nano emulsions. Spectrophotometry was utilized to determine the maximum absorbance for result accuracy. The nano emulsion sample was scanned in scanning mode with a reference solution containing 200 µg/ml of sirolimus, revealing a maximum absorbance of sirolimus peak at 277 nm with a maximum absorption of 1.208 in methanol. No interference was observed with the oil phases (camphore EL), PG, tween 20, or tween 80 excipients and as shown in figure 4.2.

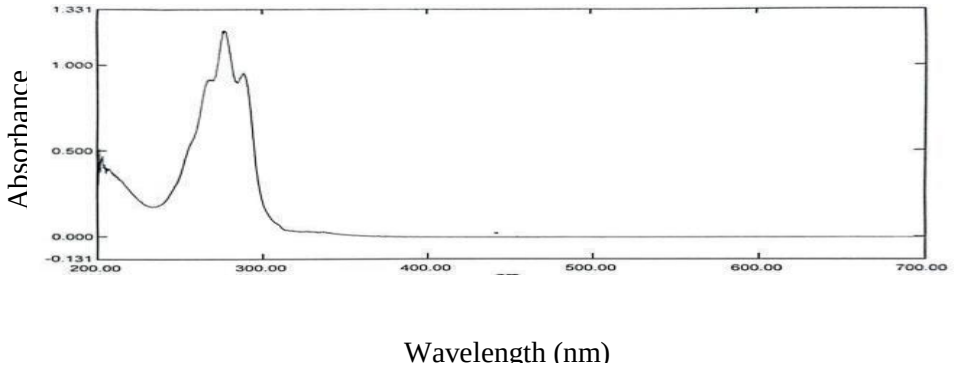

Figure 4.2 UV Scan of sirolimus in nano size emulsion

Table 4.3 UV absorbance of sirolimus in nano size emulsion

Sr No	Wavelength	Abs.
01	629.60	-0.005
02	393.40	-0.007
03	370.00	0.001
04	338.00	0.025
05	327.40	0.031
06	315.40	0.038
07	288.60	0.958
08	277.00	1.209
09	201.60	0.514

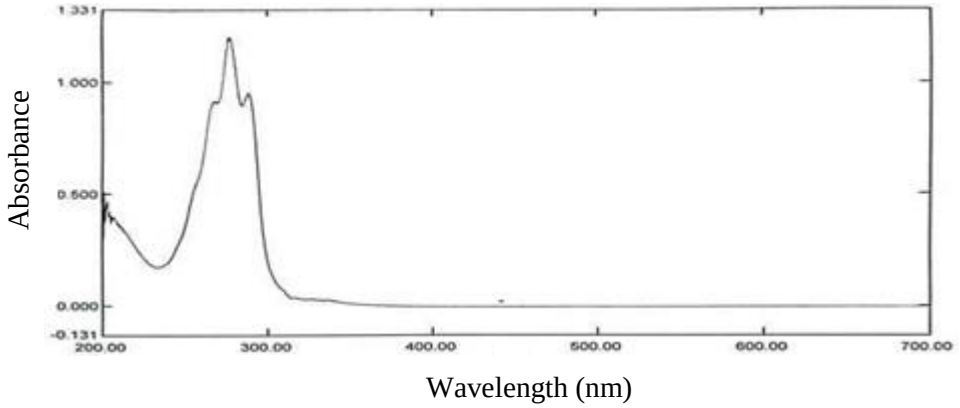

Figure 4.3 UV scan of sirolimus reference

Table 4.4 UV absorbance of sirolimus reference

Sr No	Wavelength	Abs.
01	430.60	-0.001
02	391.40	-0.007
03	366.00	0.010
04	330.00	0.021
05	318.30	0.045
06	315.40	0.038
07	286.60	0.910
08	277.00	1.301
09	205.60	0.534

4.2. FTIR

The nano emulsion formulation's functional groups were analyzed using infrared technique. After creating the nano emulsion, samples were collected meticulously in a clean, dry container. A small amount was placed on the ATR for IR spectrum analysis, which was then compared to a reference SRL material. Numerous peaks were detected in the IR spectrum of the nano emulsion, with prominent peaks observed around 1650 and 1750cm^{-1}.

4.2.1. Self-emulsifying formulation:

The sirolimus nano emulsion spectra displayed IR characteristic peaks for C=O stretch (1760 – 1670 cm^{-1}) and C=C stretch (1680–1630 cm^{-1}), showing similarity to the reference sirolimus spectrum. The FTIR spectrum of sirolimus nano emulsion exhibited strong stretching peaks at 1715.63 cm^{-1} and 1633.28 cm^{-1}, with minor peaks observed in the SRL-NE formulation that did not interfere with the major peaks at 1650 and 1750 cm^{-1} and as shown in figure 4.4.

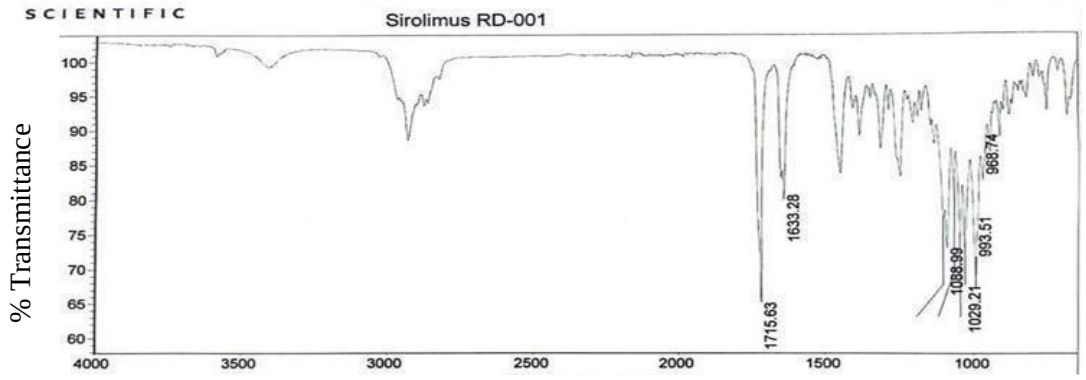

Figure 4.4 FTIR spectrum of self-emulsifying formulation

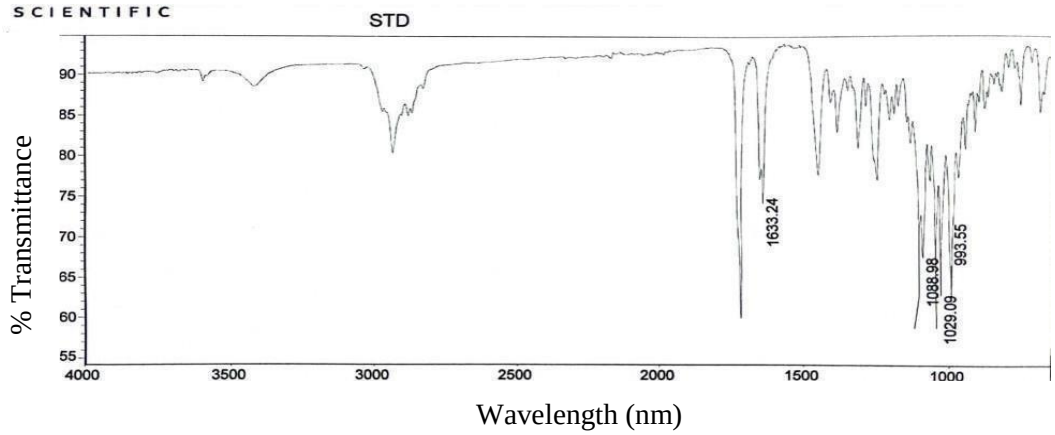

Figure 4.5 FTIR spectrum reference pure sirolimus

4.2.2. Nano size emulsion:

A comparison was made between the FTIR spectrum of the nano emulsion and the IR spectrum of the sirolimus reference material. Sirolimus, with its four C=C and six C=O double bonds, exhibited characteristic peaks in its nano emulsion spectra at 1760–1670 cm^{-1} and 1680–1630 cm^{-1} for C=O and C=C stretches, respectively. The similarity between the nano emulsion sirolimus FTIR spectrum and the reference sirolimus was evident, with two prominent stretching peaks observed at 1715.63 cm^{-1} and 1633.28 cm^{-1} in the FTIR spectrum data for the sirolimus nano emulsion and as shown in figure 4.6.

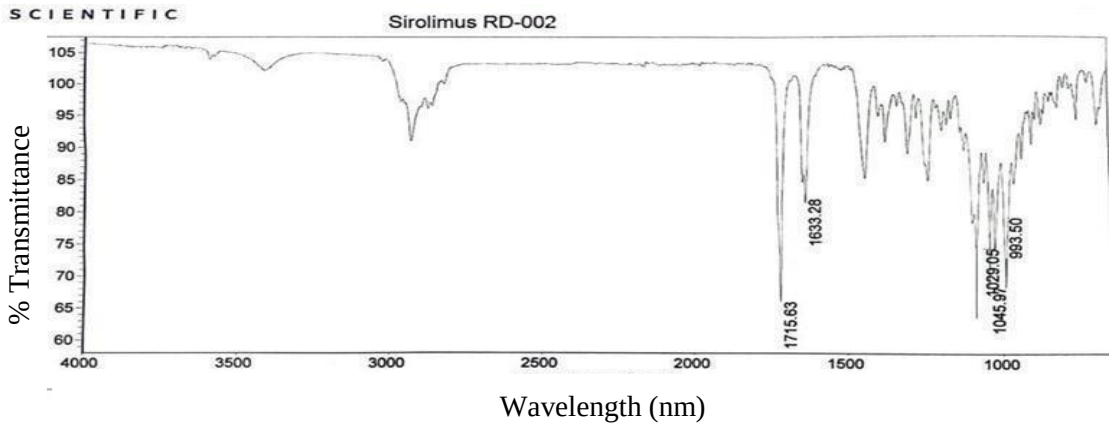

Figure 4.6 FTIR spectrum of nano size emulsion of sirolimus

4.3. PARTICLE SIZE MEASUREMENT BY ZETA SIZER:

The zeta sizer nano series of devices offers the capability to analyze three properties of particles or molecules in a liquid environment: particle size, zeta potential, and molecular weight. Following the creation of a nano emulsion formula, a sample was extracted to fine-tune the size of the nanoparticles.

4.3.1 Self-emulsifying formulation:

The size of the nanoparticles was determined using an anton paar particle sizer analyzer through the DLS technique, revealing a particle size of 54.26 nm (with a limit not more than 200nm) and a polydispersity index (PDI) of 51.7%. The viscosity and refractive index of the sample were measured at 0.0010393 pas and 1.3577, respectively and as shown in figure 4.7.

Particle size distribution (Intensity)

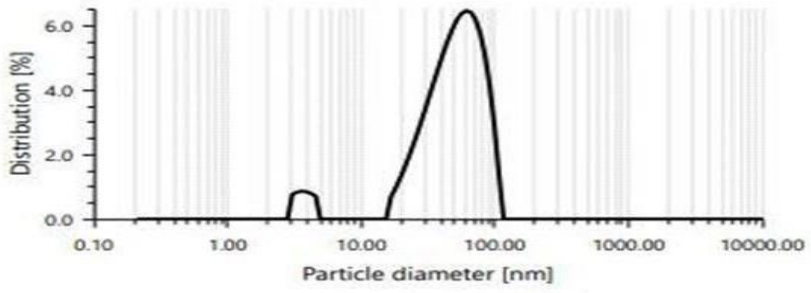

Figure 4.7 Particle size distribution of self-emulsifying formulation distribution vs particle diameter (nm)

Table 4.5 particle size distribution of nano size of self-emulsifying formulation

Hydrodynamic diameter	0.70 nm	**Mean Intensity**	192.9
Polydispesrity Index	51.7%	**Absolute Intensity**	617734.1
Diffusion coefficient	599.9	**Intercept g1º**	0.0164
Transmittance	79.0%	**Baseline**	1.002

Table 4.6 Size distribution of self-emulsifying formulation size Vs distribution %

Particle size distribution peaks (Intensity)			
Peak Name	**Size (nm)**	**Area %**	**Standard Deviation (nm)**
Peak 1	54.26	95.16%	27.55
Peak 2	3.74	4.84	0.48

4.3.2. Nano size emulsion:

The nanoparticle size was determined using the DLS approach with an anton paar particle sizer analyzer . The nano emulsion's particle size was measured to be 15.64 nm (limit NMT 200 nm) and its polydispersity index (PDI) was 46.4%. The sample's viscosity was 0.0010693 p.s. and its refractive index was 1.3592 and as shown in figure 4.8.

Particle Size distribution (Intensity)

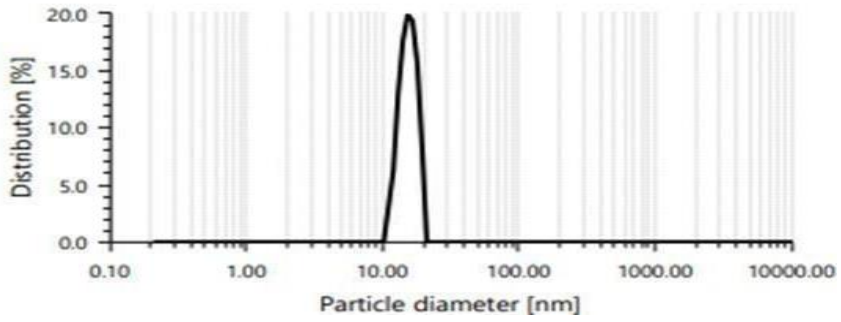

Figure 4.8 particle size distribution of nano size emulsion (distribution vs Particle diameter (nm)

Table 4.7 Particle size distribution of nano size of nano size emulsion

Hydrodynamic diameter	0.38 nm	**Mean Intensity**	200.8
Polydispesrity Index	46.4%	**Absolute Intensity**	35658.7
Diffusion coefficient	120.2	**Intercept g1º**	0.3631
Transmittance	8.9%	**Baseline**	0.958

Table 4.8 size distribution of nano size emulsion (size Vs distribution %)

Particle size distribution peaks (Intensity)			
Peak Name	**Size (nm)**	**Area %**	**Standard Deviation (nm)**
Peak 1	15.64	100 %	2.08
Peak 2	-	-	0.00

4.4. ZETA POTENTIAL:

The zeta potential was employed to assess the electrochemical balance at the interface of particle and liquid. It measured the potential difference in millivolts, indicating the presence of positive or negative ions in the nano emulsion sample. The formulation contained a significant amount of charged ions, which could lead to precipitation and disrupt the stability of the formulation. However, a small quantity of negative charge could enhance the stability of the formulation. Following the preparation of the nano emulsion, a sample was analyzed using potentiometric analysis to evaluate the stability of the formulation.

4.4.1 Self-emulsifying formulation:

The zeta potential of the nano emulsion was measured using the anton paar particle sizer analyzer by DLS technique and zeta potential mode, resulting in a mean zeta potential of -1.4mv (with a limit of ±10 mv). The electrophoretic mobility was measured at -0.0265µm*cm/Vs (with a limit of ±0.05 µm*cm/Vs). Additionally, the viscosity and refractive index of the sample were found to be 0.0021030pa.s and 1.3611, respectively and as shown in figure 4.9.

Zeta potential distribution

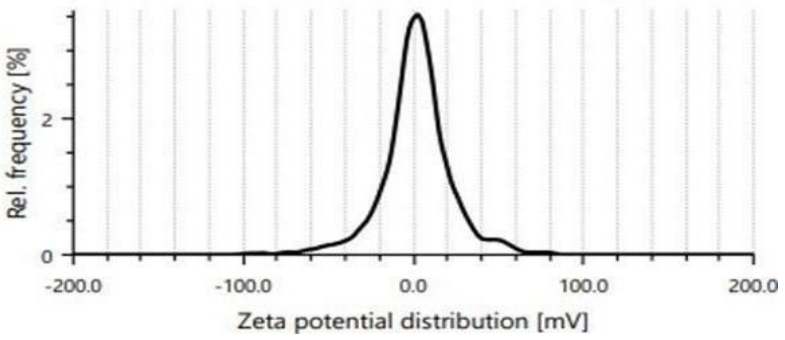

Figure 4.9 Zeta potential distribution of self emulsifying formulation ref.frequency vs zeta potential

Table 4.9 Zeta potential distribution result of self emulsifying formulation

Mean zeta potential	-1.4mv	**Mean Intensity**	741.3
Standard Deviation	0.7mv	**Filter optical density**	0.9035
Distribution Peak	2.5mv	**Conductivity**	0.038
Electrophoretic mobility	-0.0265	**Transmittance**	78.6%

4.4.2. Nano size emulsion:

The DLS approach in zeta potential mode was used to measure the zeta potential of the nanoparticle. The mean zeta potential of the nano emulsion was determined to be -0.8 mv (± 10 mv), and the electrophoretic mobility was found to be -0.0148 µm*cm/Vs (±0.05 µm*cm/Vs). The sample's viscosity was 0.0021030 pas, and its refractive index was 1.3611 and as shown in figure 4.10.

Zeta potential distribution

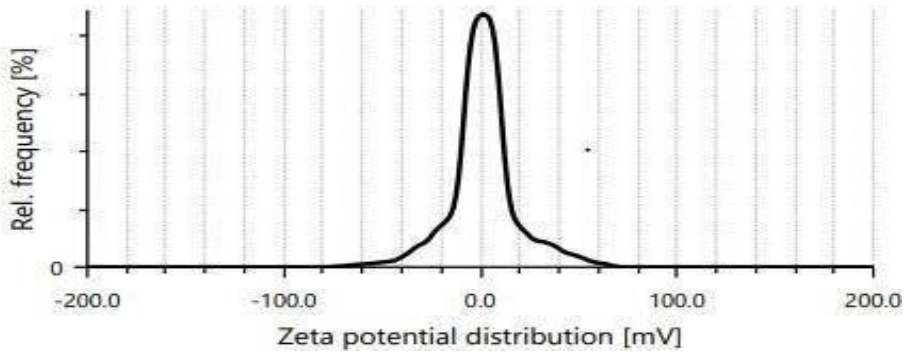

Figure 4.10 Zeta potential distribution of nano size emulsion ref. frequency vs zeta potential

Table 4.10 Zeta potential distribution of nano size emulsion

Mean zeta potential	-0.8mv	**Mean Intensity**	741.3
Standard Deviation	0.4mv	**Filter optical density**	1.7592
Distribution Peak	1.3mv	**Conductivity**	0.087
Electrophoretic mobility	-0.0148	**Transmittance**	8.8%

4.5. Drug analysis:

Various studies utilized the HPLC method to measure the sirolimus nano emulsion formulation's concentration, analyze its qualitative and quantitative aspects, as well as evaluate different formulations through thermal and photo stability studies. The specified drug amount in the nano emulsion formulation can be accurately determined using the HPLC method.

The drug concentration in the nano emulsion formulations varied slightly, with values ranging from 1.940 mg/ml to 1.972 mg/ml, despite the stated amount being 2mg/ml. The encapsulation efficiency percentage of sirolimus also showed variation, with values ranging from 97.02% to 98.60%. Similarly, the drug concentration in the nano-sized emulsion samples also showed slight differences, with values ranging from 1.974 mg/ml to 1.958 mg/ml. The encapsulation efficiency percentage of Sirolimus for these samples ranged from 97.90% to 98.73% and as shown in table 4.11.

Table 4.11 drug analysis summary of nano emulsion formulations of sirolimus

Sr No	Formulation	Stated amount Sirolimus 2mg/ml recovered	%age recovered	Standard deviation	Relative Standard deviation
01	Nano emulsifying formulation 1	1.940 mg /ml	97.02 %	0.80	0.82 %
02	Nano emulsifying formulation 2	1.972 mg/ml	98.50 %		
03	Nano emulsifying formulation 3	1.962 mg/ml	98.10 %		
04	Nano Size emulsion1	1.974 mg/ml	98.73 %	0.87	0.89 %
05	Nano Size emulsion2	1.961 mg/ml	98.00 %		
06	Nano Size emulsion3	1.958 mg/ml	97.90 %		

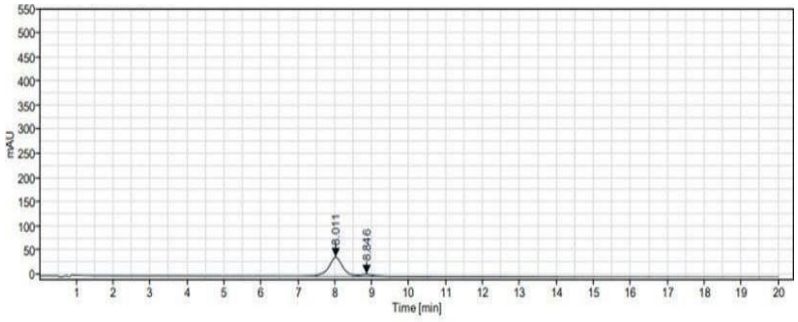

Figure 4.11 Chromatogram of sirolimus reference 1

The area under curved of sirolimus reference 1 of SRL Isomer B and C was 1001.88 and 112.79 respectively and total area of sirolimus reference was 1114.67.

Table 4.12 Chromatogram reading of sirolimus reference 1

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
STD 01	SRL Isomer B	89.88	8.011	1001.88	36.93	1.07636	2369.16779
	SRL Isomer C	10.12	8.846	112.79	4.22	1.74410	2378.68927
			Sum	1114.67			

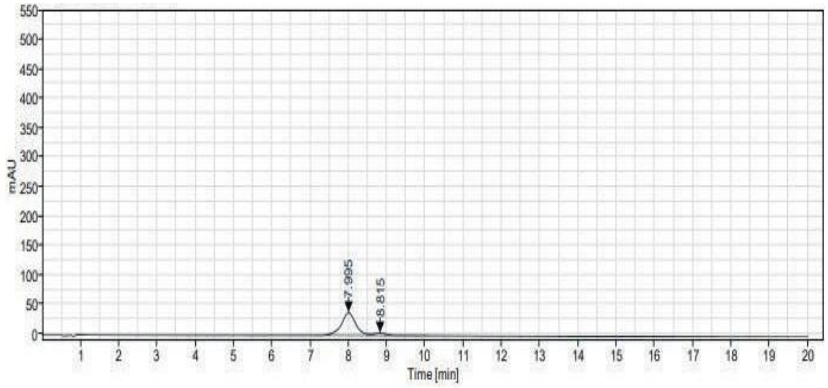

Figure 4.12 Chromatogram of sirolimus reference 2

The area under curved of sirolimus reference 2 of SRL Isomer B and C was 973.62 and 108.84 respectively and total area of sirolimus reference was 1082.46.

Table 4.13 Chromatogram reading of sirolimus reference 2

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
STD 02	SRL Isomer B	89.95	7.995	973.62	36.49	1.04501	2394.23850
	SRL Isomer C	10.05	8.815	108.84	4.04	1.58987	2263.43945
			Sum	1082.46			

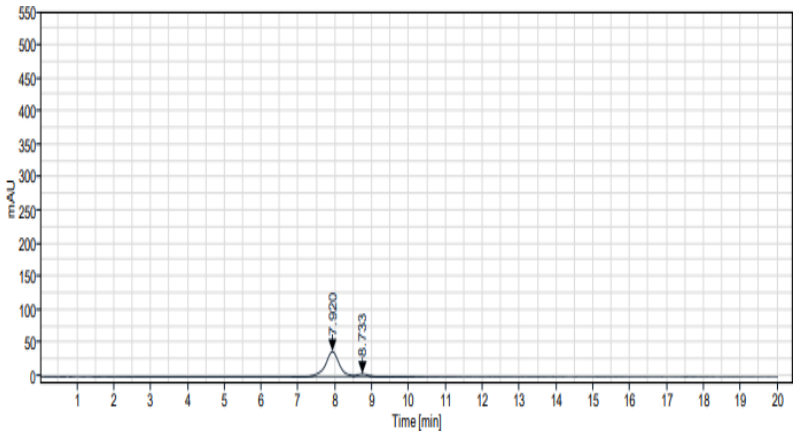

Figure 4.13 Chromatogram of sirolimus reference 3

The area under curved of sirolimus reference 3 of SRL Isomer B and C was 990.21 and 111.24 respectively and total area of sirolimus reference was 1101.44.

Table 4.14 Chromatogram reading of sirolimus reference 3

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
STD 03	SRL Isomer B	89.90	7.920	990.21	37.00	1.03569	2389.49849
	SRL Isomer C	10.10	8.733	111.24	4.36	1.95536	2488.54797
			Sum	1101.44			

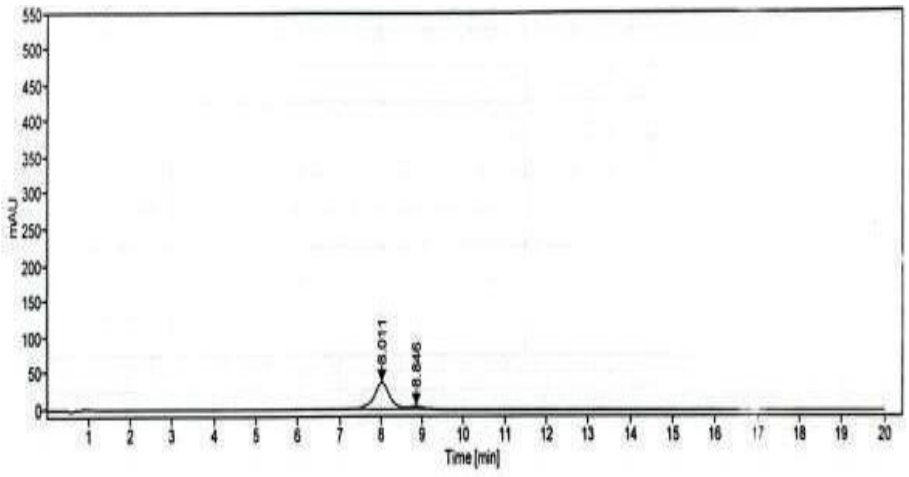

Figure 4.14 Chromatogram of sirolimus reference 4

The area under curved of sirolimus reference 4 of SRL Isomer B and C was 1001.88. and 112.79 respectively and total area of sirolimus reference was 1114.67.

Table 4.15 Chromatogram reading of sirolimus reference 4

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
STD 04	SRL Isomer B	89.88	8.011	1001.88	36.93	1.07636	2369.16779
	SRL Isomer C	10.12	8.846	112.79	4.22	1.74410	2378.68927
			Sum	1114.67			

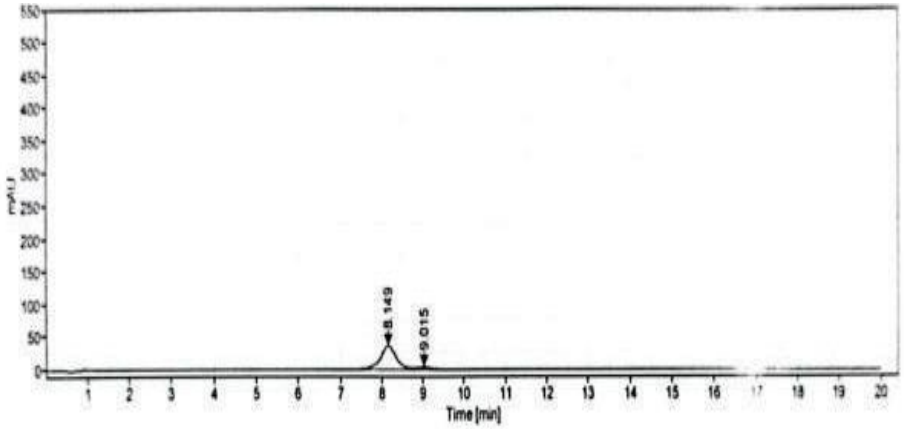

Figure 4.15 Chromatogram of sirolimus reference 5

The area under curved of sirolimus reference 5 of SRL Isomer B and C was 1004.97. and 106.45 respectively and total area of sirolimus reference was 1111.42.

Table 4.165 Chromatogram reading of sirolimus reference 5

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
STD 05	SRL-Isomer B	90.42	8.149	1004.97	36.73	1.05521	2422.60950
	SRL-Isomer C	9.58	9.015	106.45	4.15	1.69962	2375.83036
			Sum	1111.42			

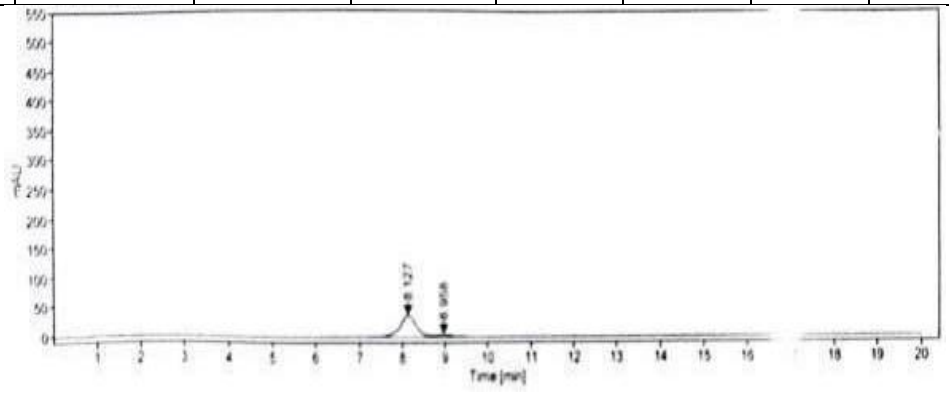

Figure 4.16 Chromatogram of sirolimus in self emulsifying formulation 1

The area under curved of sirolimus in self emulsifying formulation 1 of SRL Isomer B and C was 996.96 and 99.35 respectively and total area of sirolimus was 1096.30.

Table 4.17 Chromatogram reading of sirolimus in self emulsifying formulation 1

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 01	SRL-Isomer B	90.94	8.127	996.96	37.27	1.08155	2484.78262
	SRL-Isomer C	9.05	8.958	99.35	4.25	1.59334	2499.37934
			Sum	1096.30			

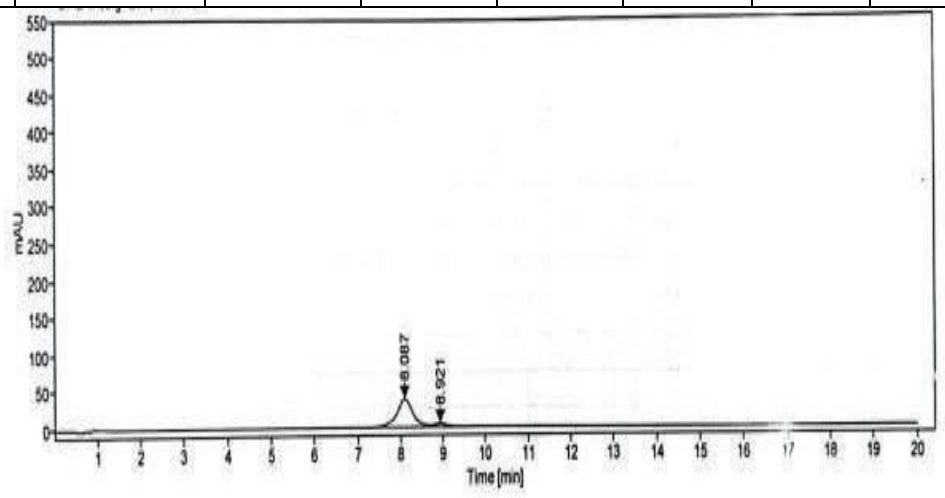

Figure 4.17 Chromatogram of sirolimus in self emulsifying formulation 2

The area under curved of sirolimus in self emulsifying formulation 2 of SRL Isomer B and C was 1013.94 and 109.46 respectively and total area of sirolimus was 1123.41.

Table 4.18 Chromatogram reading of sirolimus in self emulsifying formulation 2

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 02	SRL-Isomer B	90.26	8.087	1013.94	37.71	1.07916	2467.62743
	SRL-Isomer C	9.74	8.921	109.46	4.37	1.65897	2614.64679
			Sum	1123.41			

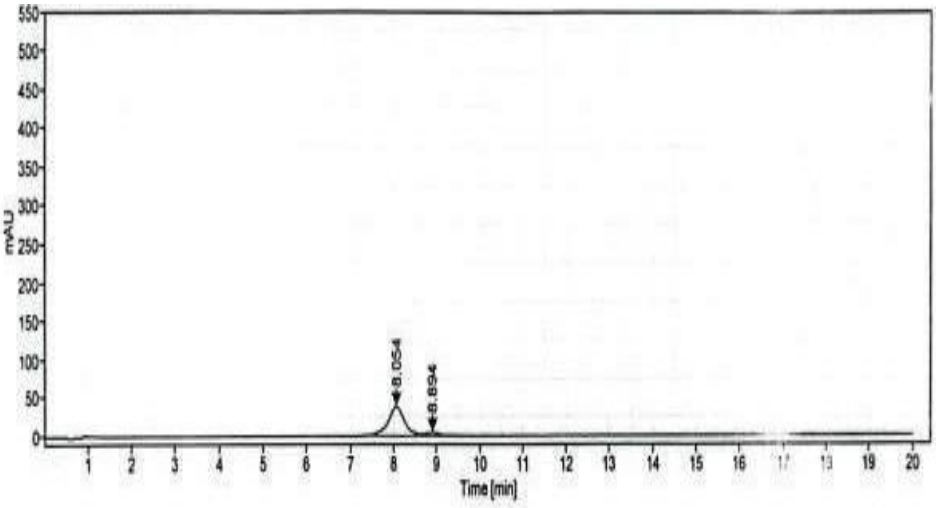

Figure 4.18 Chromatogram of sirolimus in self emulsifying formulation 3

The area under curved of sirolimus in self emulsifying formulation 3 of SRL Isomer B and C was 1027.32 and 104.55 respectively and total area of sirolimus was 1131.87.

Table 4.19 Chromatogram reading of sirolimus in self emulsifying formulation 3

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 03	SRL-Isomer B	90.76	8.054	1027.32	38.15	1.05431	2428.76984
	SRL-Isomer C	9.24	8.894	104.55	4.36	1.58708	2621.53531
			Sum	1131.87			

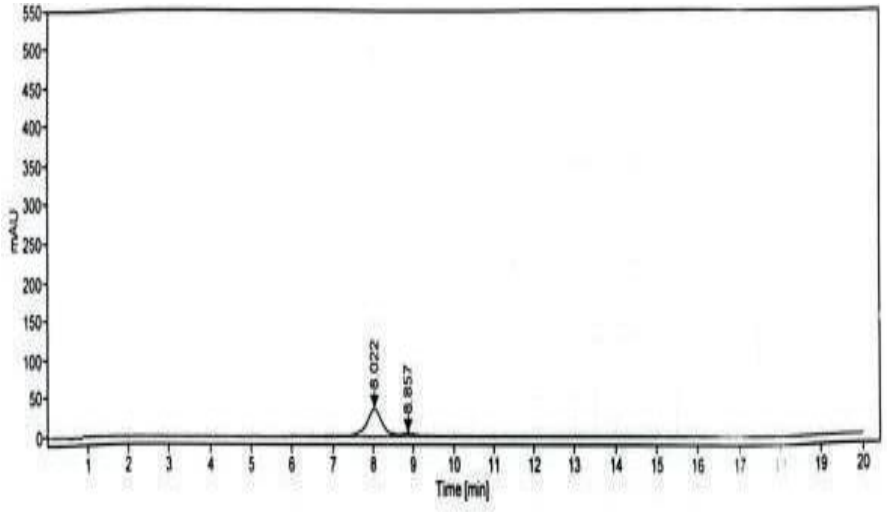

Figure 4.19 Chromatogram of sirolimus in nano size emulsion 1

The area under curved of sirolimus in nano size emulsion 1 of SRL Isomer B and C was 976.49 and 92.64 respectively and total area of sirolimus was 1069.22

Table 4.20 Chromatogram reading of sirolimus in nano size emulsion 1

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 04	SRL-Isomer B	91.34	8.022	976.49	36.14	1.05347	2392.07195
	SRL-Isomer C	8.66	8.857	92.64	3.99	1.58640	2602.46315
			Sum	1069.22			

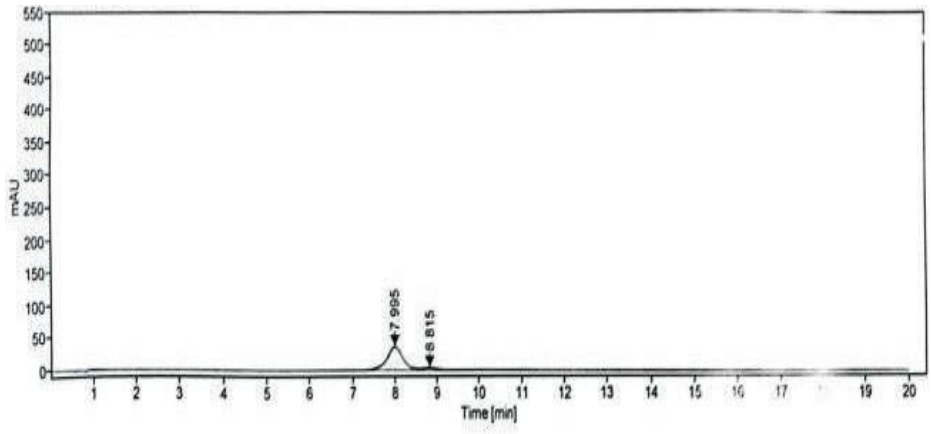

Figure 4.20 Chromatogram of sirolimus in nano size emulsion 2

The area under curved of sirolimus in nano size emulsion 2 of SRL Isomer B and C was 973.62 and 108.84 respectively and total area of sirolimus was 1082.46

Table 4.21 Chromatogram reading of sirolimus in nano size emulsion 2

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 05	SRL-Isomer B	89.95	7.995	973.62	36.49	1.04501	2394.23850
	SRL-Isomer C	10.05	8.815	108.84	4.04	1.58987	2263.43945
			Sum	1082.46			

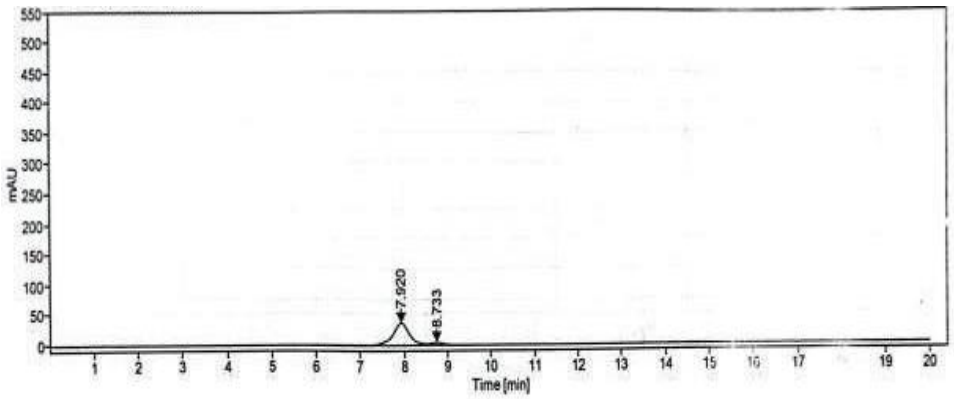

Figure 4.21 Chromatogram of sirolimus in nano size emulsion 3

The area under curved of sirolimus in nano size emulsion 3 of SRL Isomer B and C was 990.21 and 111.24 respectively and total area of sirolimus was 1101.44

Table 4.22 Chromatogram reading of sirolimus in nano size emulsion 3

Name	API Name	Peak Area Percent	RT (mint)	Area	Height	Tail Factor	Theoretical Plates (USP)
SMP 06	SRL-Isomer B	89.90	7.920	990.21	37.00	1.03969	2389.45849
	SRL-Isomer C	10.10	8.733	111.24	4.36	1.95536	2488.54797
			Sum	1101.44			

CHAPTER 5

DISCUSSION

Sirolimus nano emulsion was a macrolide compound that was used to coat coronary stents, prevent organ transplant rejection, treat a rare lung disease called lymphangioleio myomatosis, and treat perivascular epithelioid cell tumors. Sirolimus was practically insoluble in water and had low solubility and bioavailability. Nano emulsion formulation was prepared by excipient castrol oil phase, castor oil was the best options for solubilization of the sirolimus drug, castor oil was well known as a source of ricinoleic acid. Most fatty acids had a non-polar nature. Castor oil had alcoholic groups, polar and non-polar properties to solubilize the drug and reduce the size of the drug to nano size. The sirolimus drug was stable in castor oil-based formulations.

Although, the concentration of castor oil was different in both type of formulations, 20% and 15% respectively. The nano size of the emulsion was higher in formulation contains 20% castrol oil shown reading at 54 nm. On the other hand nano size of the emulsion lower in formulation contains 15% castrol oil shown reading at 15.64 nm. The sirolimus drug was soluble in both castrol oil concentrations, but the key factor for reducing the size of the sirolimus drug was the order of mixing and mixing time that were 15 to 20 hours for 20% castrol oil contained formulation and 25 to 30 hours for 15% castrol oil contained formulation. Previously the nano emulsion size was obtained at 108 nm by precirol ATO-5 and oleic acid oil (59).

Nano emulsion formulation was prepared at room temperature and anti-oxidant vitamin-E was used for the stability of sirolimus drug and obtained particle size was 15.64nm. The most outstanding feature of this study was the particle size of sirolimus formulation that was much smaller (15.64 nm) than the previously study nano emulsion formulations .The vitamin E was not only stabilized the nano drug but also not allow to increase the size of nano emulsion for 2 to 3 weeks after putting it into the stability chamber .Therefore due to smaller size nano particle it has the ability to enhance the permeability and bioavailability of sirolimus drug. The best nano size emulsion was created for enhancing solubility of drug. The solubility of the drug in oil phase was also an important criterion for the preparation of an efficient nano

emulsion formulation, because the maintenance of the drug in solubilized form in NE and was enhanced by the solubility of drug in the oil phase, As a consequence of low drug solubility, drug association to the oily phase decreases which in turn, necessitates a higher incorporation of hydrophilic and lipophilic emulsifiers.

The solubility of Sirolimus was 15.50 mg/mL, which in comparison to its water solubility (6μg/mL), it was indicated that oil phase system capable to better solubilize sirolimus drug. The solubility of sirolimus drug by triacetin oil base formulation was found to be 10.86 ±0.71 mg/ml and solubility in water was 2.6 μg/ml (33).

Solution of concentration (200 μg/mL) of sirolimus was scanned in overlay mode and the spectra showed peak at 277.40 nm in methanol and comparable with reference material sirolimus and maximum absorbance was at 277 nm in methanol solvent. The oil phase (castrol oil), surfactant, cosurfactant and tween80 when scanned over 200–400 nm exhibited no interference at wavelength 277nm. Two strong stretching peaks were observed at 1715.63 cm−1 and 1633.28 cm−1 in sirolimus. Nano emulsion was fully comparable with pure reference sirolimus material. Some minor peaks were observed in nano emulsion formulation at 1600 to 1650 cm-1 and 1700 to 1750 cm-1 but there was no major interference in major peaks at 1650 and 1750 cm-1. It was proved to be best sirolimus nano emulsion having no interference of excipients during compatibility study.

Zeta potential was a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles that affects the stability. Surface potential (zeta potential) formed by surfactants can produce repulsive/attractive electrical forces among approaching oil droplets and thus prevents their coalescence. Electrostatic charge potential of sirolimus emulsifying nano emulsion was almost zero. There was more negative and positive ion present in nano emulsion. It was to be proved that stable nano emulsion particles and there was no precipitation in nano emulsifying formulation. The mean zeta potential of the sirolimus nano emulsion were -0.8 mv and -1.4mv respectively (limit ± 10 mv) and was almost zero potential in formulation and it was comparable with selected RAP-loaded NEs, based on the release study (60).

The stated amount of content of sirolimus in nano size emulsion was 2mg / ml and previous developed nano emulsion formulation was of 1mg /ml. The spontaneous emulsification method was used to prepare formulation containing 1 mg/mL of the

drug sirolimus (61).

The recovered mean stated content of nano emulsion was 1.962mg/ml. On the basis of small nano emulsion droplet and polydispesrity index (PDI), optimum concentration of castrol oil and low concentrations of cosurfactant & surfactant, the best sirolimus nano emulsion formulation was developed. It was concluded that nano emulsion (15 nm) enhance the solubility and permeability of sirolimus drug. Zeta potential was also in negative mv (-0.8 mv) that shows it was a stable formulation. In the study of zeta sizer, the nano emulsion size was very low to enhance bioavailability of drug.

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