FORMULATION AND EVALUATION OF DUAL DRUG LOADED NANO-LIPID CARRIER BASED SYSTEM.

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Signature of the student ……Dishant……………

Name of student DISHANT SHARMA

Date 29-05-2015

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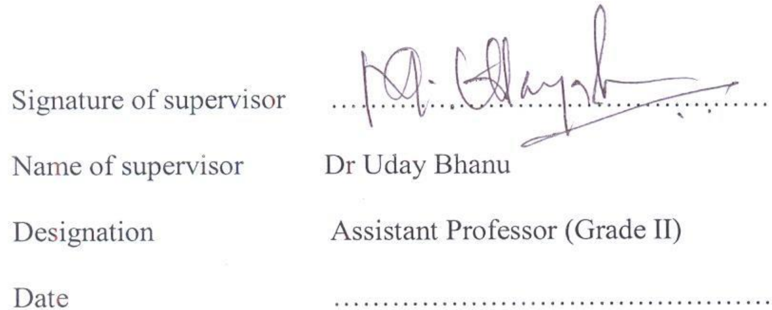
CERTIFICATE

This is to certify that the work entitled "**To prepare and evaluate dual drug loaded nano-lipid-carrier based formulation**" submitted by "**Dishant Sharma**" in partial fulfillment for the award of degree of B. Pharmacy of Jaypee University of Information and Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.



Signature of supervisor ....

Name of supervisor Dr Uday Bhanu

Designation Assistant Professor (Grade II)

Date ....

ABSTRACT

Background: Ciprofloxacin and Clotrimazole are used as antimicrobials. There are various diseases in which mechanism of disease causing microbe is unknown; therefore a formulation which acts by more than one mechanism of action might prove beneficial in the disease.Since both drugs have different mechanisms of actions. Thus attempts were made, to prepare a dual drug loaded formulation.

Objective: The aim of the research work is to prepare and evaluate dual drug loaded NLCs based formulation which has advantages of both drugs i.e. Ciprofloxacin and Clotrimazole.

Method:NLCs of dual drugs were prepared by emulsification-ultrasonicationmethod using stearic acid as lipid.

Results and discussion: The *in-vitro* studies were carried out at phosphate buffer pH 7.4 at 37±2º C. NLCs in all formulations were found to be spherical in shape; with size ranging from 200-800 nm. All formulations exhibited an *in-vitro* release of 65-85 % in 24hour. % Drug loading of (F4) was found to be highest. Therefore it was selected as best formulation.

Conclusion: The present study was carried out to develop the NLCs of dual drugs (ciprofloxacin and clotrimazole) using stearic acid. Thus to conclude dual drug loaded NLCs may provide advantages of both drugs.

Key words: NLCs, ciprofloxacin, clotrimazole, % drug loading and *in vitro* studies.

CHAPTER-1

INTRODUCTION

1.1 Nanostructured lipid carriers (NLCs):

These are alternative carrier system to liposomes and emulsions. Nanostructured lipid carrierexhibit many features for dermal application of cosmetics and pharmaceutics, i.e. controlled release of actives, drug targeting, occlusion and associated with it penetration enhancement and increase of skin hydration. Due to the production of nanostructuredlipid carrier from the physiological and/or biodegradable lipids, this carrier system exhibits an excellent tolerability. The lipid nanoparticles are a “nanosafe”carrier.Nanostructured lipid carrier was developed to overcome some potential limitations associated with Solid lipid nanoparticles. Compared to Solid lipid nanoparticles, nanostructured lipid carrier show a higher loading capacity for a number of active compounds, a lower water content of the particle suspension and avoid or minimize potential expulsion of active compounds during storage [2]. The overall solid content of nanostructured lipid carrier could be increased up to 95% than solid lipid nanoparticles[1].

Solid lipid nanoparticles (SLN) are distinguishable from nanostructured lipid carriers (NLC) by the composition of the solid particle matrix. Solid lipid nanoparticles were developed at the beginning of the 1990s as an alternative carrier system to emulsions, liposomes and polymeric nanoparticles. Solid lipid nanoparticles are produced by replacing the liquid lipid (oil) of an o/w emulsion by a solid lipid or a blend of solid lipids, i.e. the lipid particle matrix being solid at both room and body temperature.[1]

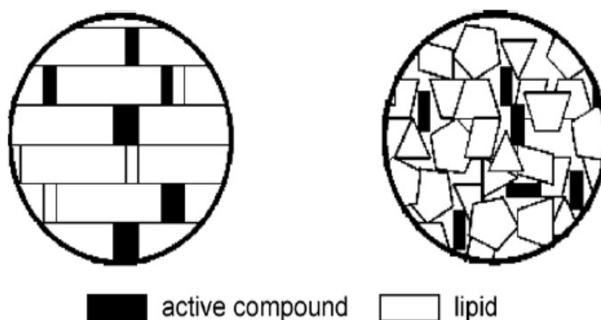


Figure 1:Formation of an almost perfect crystalline structure in SLN (left) by identically shaped molecules similar to a brick wall with limited loading capacity for actives. Formation of a solid particle matrix of NLC (right) with many imperfections

comparable to building awall from very differently shaped stones, the increased number of imperfections leads to an increased loading capacity for active compounds.

1.2 Different methods used for Nanostructured lipid carrierproduction:

Different production methods have been developed for lipid nanoparticles and most of the methods use two basic steps; emulsification and size reduction to nano size. Homogenization techniques are most frequently employed using hot and cold homogenization or ultrasonication for the production. On the other hand, few methods based on emulsification are also applied used earlier for polymeric nanoparticle production. Hot high pressure homogenization and ultrasonication are most commonly used method with scale up feasibility but costly equipment is biggest drawback. Other methods used to produce lipid nanoparticles are possible in a laboratory setup with no expensive equipments are needed but scale up is still a problem with such method along with regulatory problems associated with high surfactants concentrations in these formulations [3].

Methods of Manufacturing of Nanostructured lipid carrier:

1. Homogenization techniques.

- Hot high pressure homogenization technique.
- Cold high pressure homogenization technique.
- Melt emulsification ultrasound (ultrrasonication) homogenization technique.

2. Microemulsiontechnique.
3. Emulsification-solvent evaporation technique.
4. Solvent displacement or injection technique.
5. Emulsification-solvent diffusion; technique.
6. Phase inversion technique.
7. Film ultrasonication dispersion technique.
8. Multiple emulsion technique.
9. Membrane contactor technique.
10. Supercritical Particle from gas saturated solution technique.

1.2.1 Hot High Pressure Homogenization Technique:

For hot homogenization, a pre-emulsion of the drug loaded lipid melt and the emulsifier solutionthrough high pressure homogenization cycle at temperatures above the melting point of the lipid. Lipid nanoparticles are formed by the following cooling of the sample to room temperature or to temperatures below. The active compound-containing melted lipid is dispersed in the hot surfactant solution at the same temperature applying high-speed stirring. The obtained hot pre-emulsion is passed through a high pressure homogenizer applying number of homogenization cycles. A nanoemulsion is formed which is upon cooling yield aqueous dispersion of lipid nanoparticles. It can be used for the entrapment of lipophilic and insoluble drugs in the lipid. Temperature sensitive compounds can also be processed by hot high Pressure homogenization as exposure time to high temperatures is relatively short. However, for hydrophilic drugs this procedure is not the most appropriated one. During the homogenization of the melted lipid phase the drug will partition to the water phase resulting in a too low encapsulation rate [3]. Figure 2 describes the schematic procedure for the preparation of lipid nanoparticles by this method.

1.2.2 Cold High Pressure Homogenization Technique:

The cold homogenization is carried out with the solid lipid without melting as done in hot Homogenization process. Drug along with lipid in solid state is milled to form microparticles, and further dispersed in a solution containing emulsifier. The pre-suspension formed is then subjected to high pressure homogenization at or below room temperature [4] [5]. The cold high Pressure homogenization technique minimizes the thermal exposure to the drugs and active substances. Therefore, this technique may be applied for temperature sensitive compounds. Hydrophilic compounds can also be incorporated by this method which might partition from the liquid lipid phase to the water phase during the hot high Pressure homogenization. To further minimize the loss of hydrophilic compounds to the aqueous phase of the suspension, water can be replaced by liquids with low solubility for the drug, such as oils and polyethylene glycols of low molecular weight. Lipid particles prepared using the cold high Pressure homogenization technique possess a slightly higher PI and mean particle size compared to the ones

obtained by hot high Pressure homogenization technique. Homogenization cycles can be increase to further reduce the particle size and to minimize the poly dispersity [3]. Figure 2 has shown the schematic chart of this procedure.

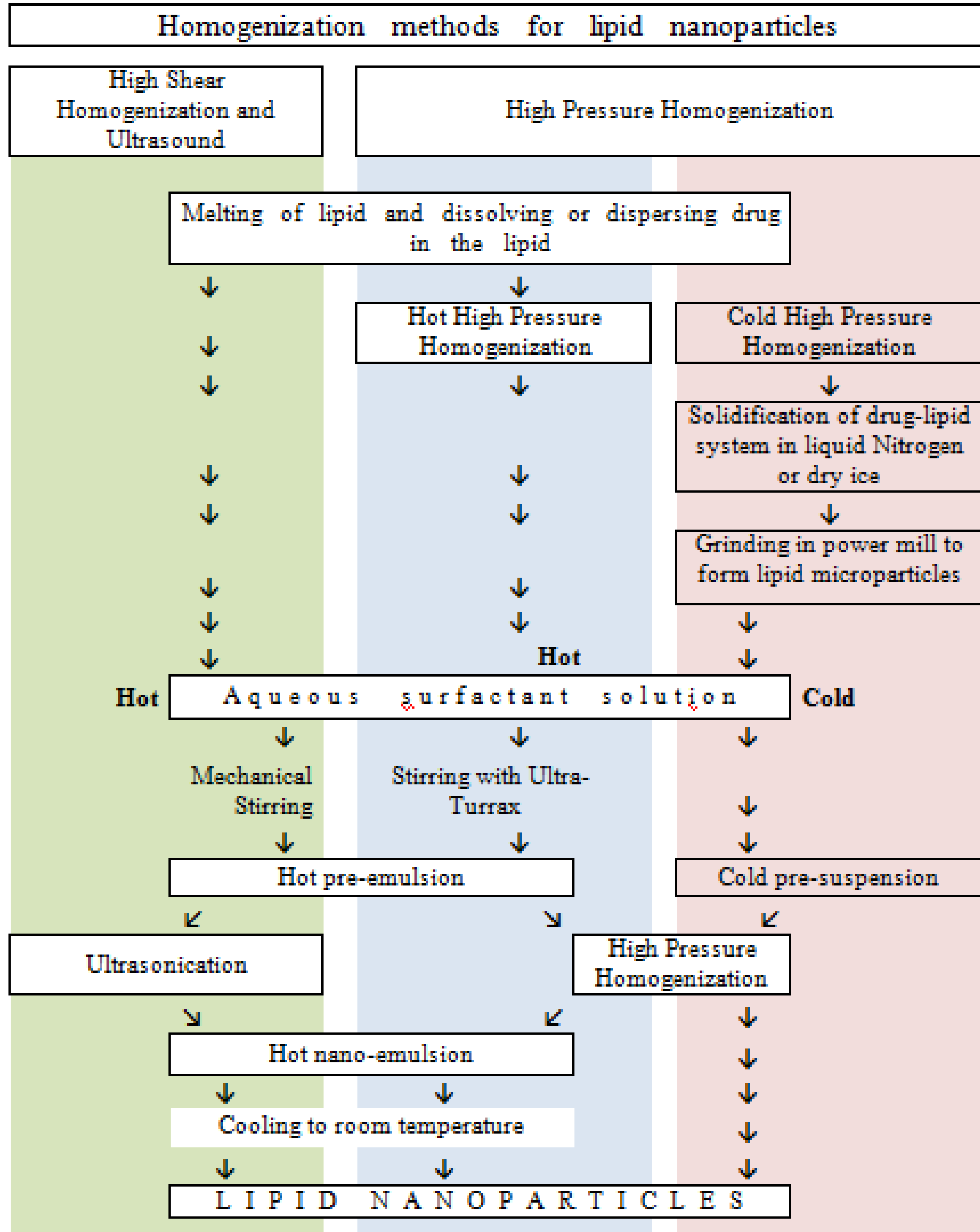


Figure 2: Schematic representation of the different homogenization techniques for the production of lipid nanoparticles

1.2.3 Melt Emulsification Ultrasound Homogenization Technique:

Ultrasonication instead of high pressure homogenization has been employed to produce lipid nanoparticles, this technology is based on the extreme conditions generated within the collapsing cavitational bubbles of the inner phase leading to size reduction. This method employs same procedure as hot high pressure homogenization except using ultrasonication device in place of homogenizer. Ultrasonic processing is fast and highly reproducible if the operating parameters are optimized. These parameters are operating temperature, ultrasonication time and power. Ultrasound probes are very easy to clean; sample losses are negligible and can be used for high scale production. However, it is believed that when applying high-shear homogenizers and ultrasonication, inhomogeneous power distributions are most likely to occur as compared with high pressure homogenizers which are characterized by a homogeneous power distribution due to the small size of the homogenizing gap [6] [7][8][9]. Figure 1 has shown the schematic chart of this procedure.

1.2.4 Microemulsion Technique:

In the microemulsion method, when excess amount of outer phase in cooling conditions added to a hot microemulsion the system has broken down and converting it into nanoemulsion which recrystallize internal oil or lipid phase forming particles. Briefly, the melted lipid containing drug mixed with surfactant, cosurfactant containing aqueous phase prepared at the same temperature as of the lipid in such a ratio to form microemulsion. The hot microemulsion is then diluted into excess of cold water. Sudden reduction in temperature causes breaking of the microemulsion, converting it into nanoemulsion, which upon recrystallization of lipid phase produces lipid particles. Break in microemulsion is supposed to be due to the dilution with water and the reduction in temperature narrowing the microemulsion region. The process variables affecting size and structure are microemulsion composition, dispersing device for the microemulsion dilution to the cold water, temperature condition and lyophilization of the product. This method has certain advantages which include no need for specialized equipment, energy for production is not required and scale-up production of lipid nanoparticles is possible. Disadvantage of the microemulsion technique is the dilution of the particles suspension

with water, thus removal of excess water need additional efforts. In addition, high concentrations of surfactants and co-surfactants, in the formulation raise regulatory concern. The removal of surfactants can further be performed using ultrafiltration, ultracentrifugation or dialysis adding one more step to the procedure which is time consuming and costly [10][11][12][13][14]. Different steps of the method are schematically represented in Figure 3.

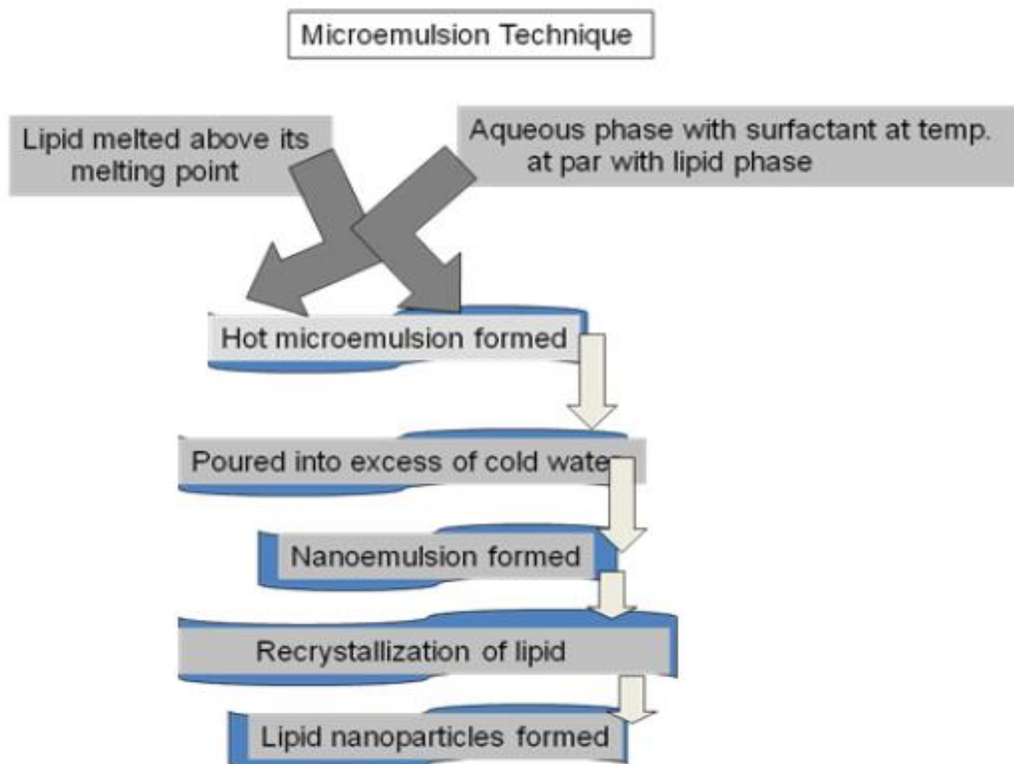


Fig 3:Schematic representation of the microemulsion technique for the production of lipid nanoparticles.

1.2.5 Emulsification-Solvent Evaporation Technique:

This is a method analogous to the production of polymeric nanoparticles and microparticles by solvent evaporation in o/w emulsions via precipitation. In the solvent emulsification-evaporation the lipid is dissolved in a water-immiscible organic solvent (e.g. toluene, chloroform) which is then emulsified in an aqueous phase before evaporation of the solvent under condition of reduced pressure. The lipid precipitates

upon evaporation of the solvent thus forming nanoparticles.This method is suitable for the incorporation of highly thermolabile drugs due to avoidance of heat during the preparation but presence of solvent residues in the final dispersion may create problems due to regulatory concern. Limited solubility of lipids in organic materials generally leads to dilute dispersions and need to concentrate by means of another process such as ultra-filtration, evaporation or lyophilization. On the other hand small particle size around 100 nm with narrow size distribution can be achieved by this method [15] [16].This procedure has schematically depicted in Figure 4.

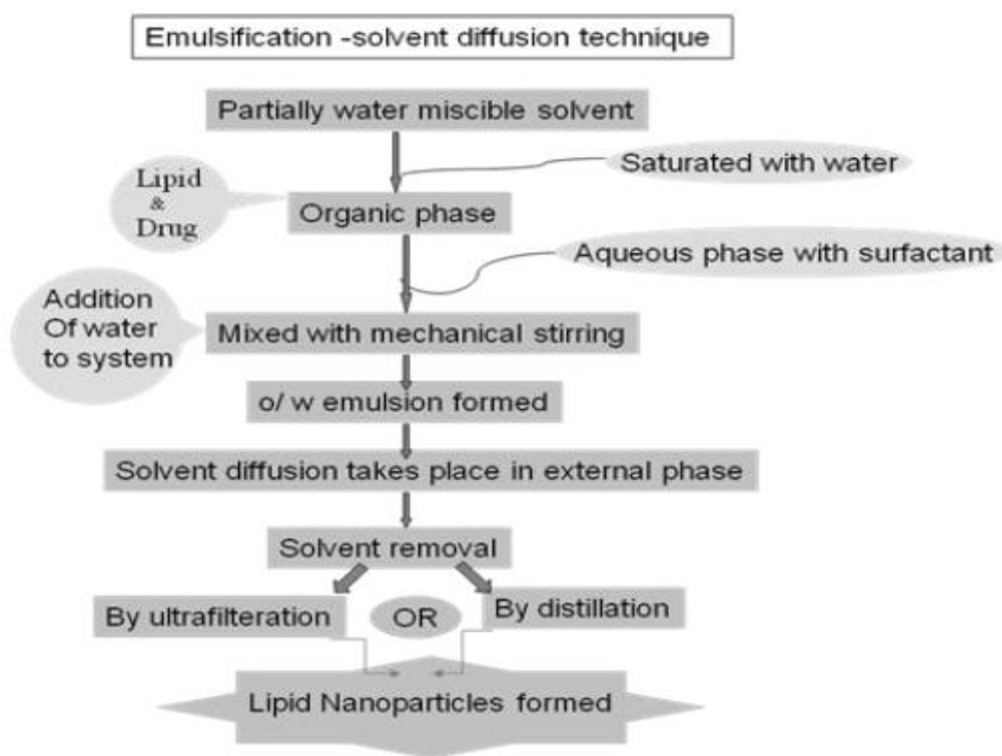


Fig 4:Schematic representation of the emulsification-solvent diffusion technique for the production of lipid nanoparticles

1.2.6 Phase Inversion Technique:

A novel phase inversion-based technique has been described for the preparation of lipid nanoparticles. It involves two basic steps, first is addition of formulation components with magnetic stirring and subsequent heating cooling cycles and second is dilution under

cooling conditions. The general procedure consists of magnetic stirring of all the components (lipid, surfactant and water) in the correct proportions optimized previously. Three cycles of heating and cooling from room temperature to 85ºC and back to 60ºC are subsequently applied at a rate of 4ºC/min (2). This thermal treatment (85ºC-60ºC-85ºC-60ºC-85ºC) will cause the inversion of the emulsion. It is followed by dilution with cold water. The system will break down due to an irreversible shock induced by dilution with cold water to the mixture maintained at the elevated temperature. This fast cooling dilution process with cold water leads to lipid particles in the nanometer range. Afterwards, a slow magnetic stirring is applied to avoid particle aggregation [17]. A general procedure has been schematically represented in Figure 5.

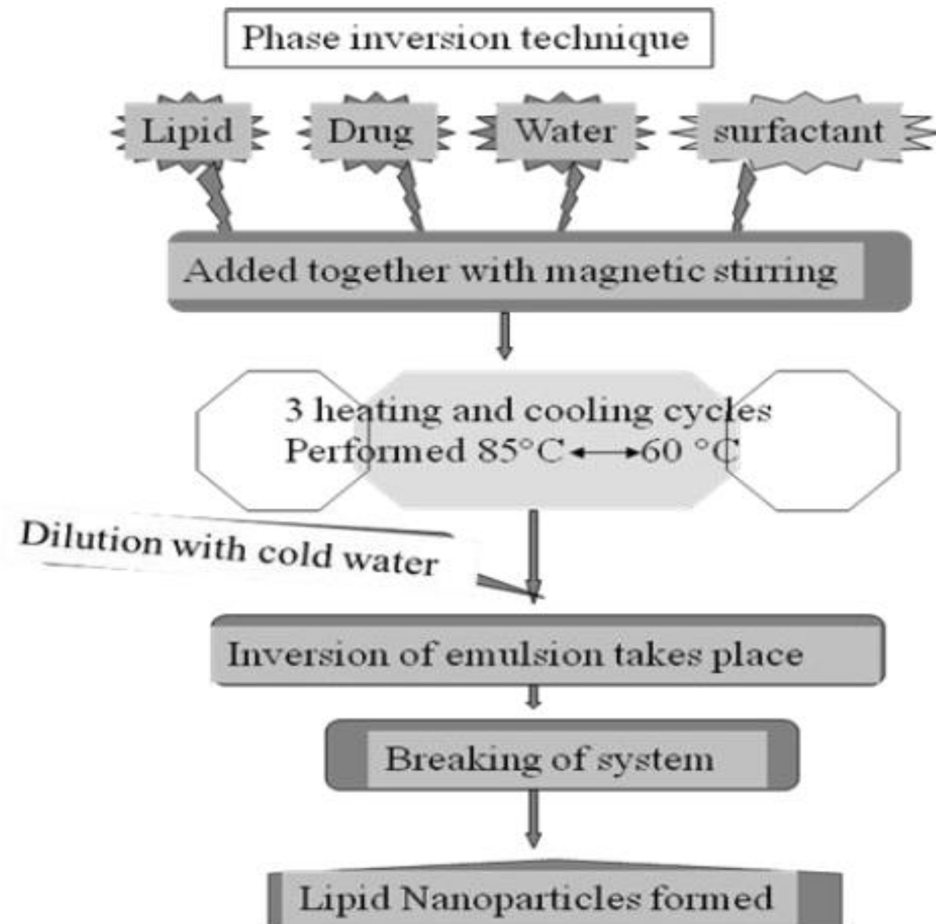


Fig 5:Schematic representation of the phase inversion-based technique for the production of lipid nanoparticles.

1.2.7 Film Ultrasonication Dispersion Technique:

Lipid nanoparticles can also be prepared by high speed stirring or sonication. In this method a thin film of lipid phase has been formed upon evaporation of solvent followed by ultrasonic dispersion in the presence of aqueous surfactant solution at elevated temperature; subsequent cooling of the system lead to the formation of lipid nanoparticles. The advantages of this method are use of common equipments that are available in every lab set up. Broader particle size distribution ranging into micrometer range is the biggest problem of this procedure and affects physical stability lead to particle growth upon storage. Contamination of metal during ultrasonication is also a major problem in this process. [18] [19]. Method of preparation has been schematically represented in Figure 6.

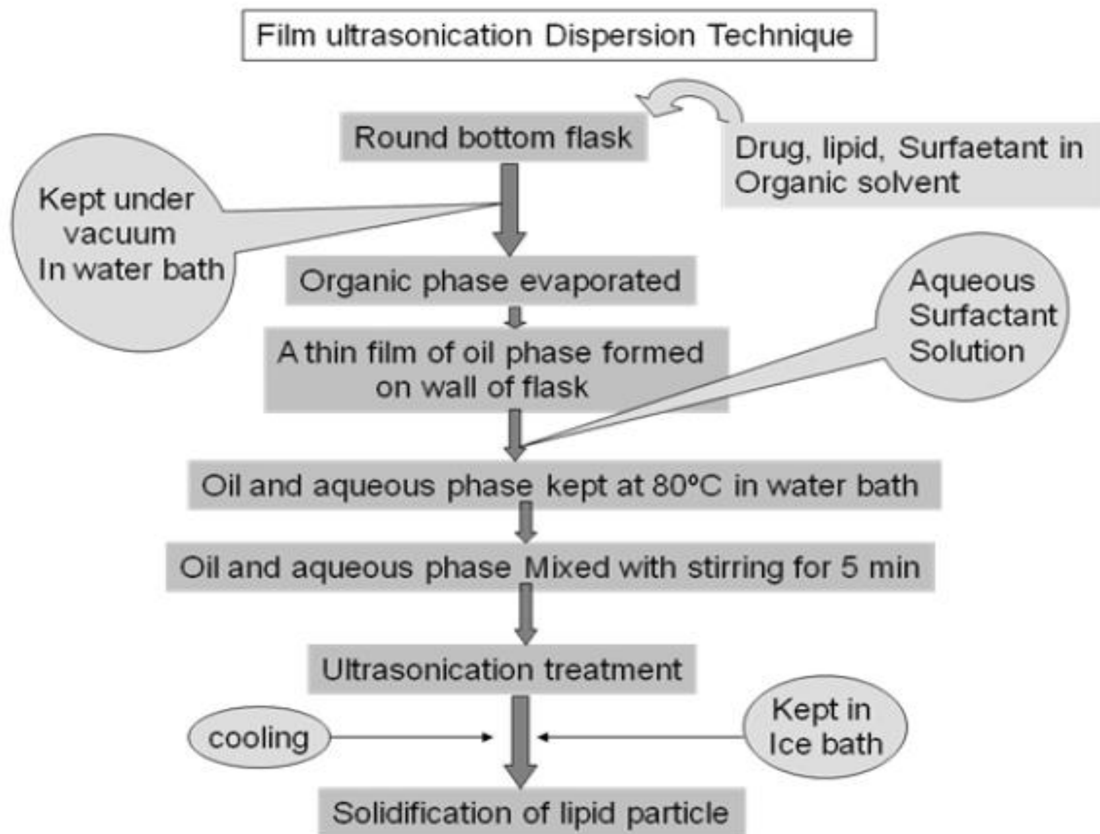


Fig. 6:Schematic representation of the film ultrasonication dispersion technique for the production of lipid nanoparticles.

1.2.8Multiple Emulsion Technique:

This is a modified solvent emulsification-evaporation method based on a w/o/w double emulsion. It applied emulsification followed by solvent evaporation for the preparation of hydrophilic drug substance loaded SLN. The drug is encapsulated with a stabilizer to prevent drug partitioning to external water phase during solvent evaporation in the external water phase of w/o/w double emulsion. It has advantages and limitation of previously described method of emulsification solvent evaporation technique but it can be applied for the incorporation of hydrophilic molecules such as peptides and proteins also. [20] [21]. A general procedure has been schematically represented in Figure 7.

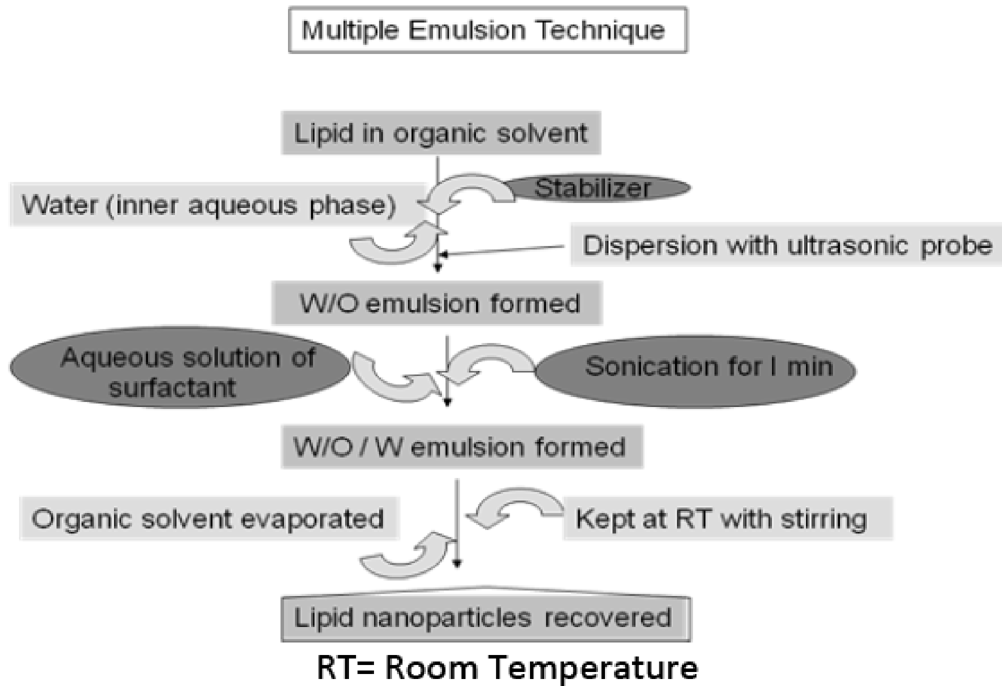


Fig .7:Schematic representation of the multiple emulsion technique for the production of lipid nanoparticles

1.2.9 Membrane Contactor Technique:

This method work on simple procedure of passing lipid from pores of a membrane with pressure keeping system above the melting temperature of lipid. The lipid phase is pressed through the membrane pores, at a temperature above the melting point of the

lipid, lead to formation of small droplets. On the other hand aqueous phase is circulated inside the membrane module, and droplets formed at the pore outlets are sweeps along with this aqueous phase. SLN are formed by the cooling of the preparation to room temperature. The velocity of aqueous phase flow, temperature of lipid and aqueous phase, membrane pore size and lipid phase pressure is the process variables which affect size and lipid flux of SLN [22]. Procedure has been represented schematically in Figure 8.

1.3 Nanoparticles can be classified into various categories:

1.3.1. Nanocrystals/nanosuspension:

Nanocrystals are crystals of poorly water-soluble drug in nanosize which when dispersed in water produce nanosuspension. Nanocrystalline drug suspensions have an advantage of higherloading (up to 90% of the crystalline particle is drug).

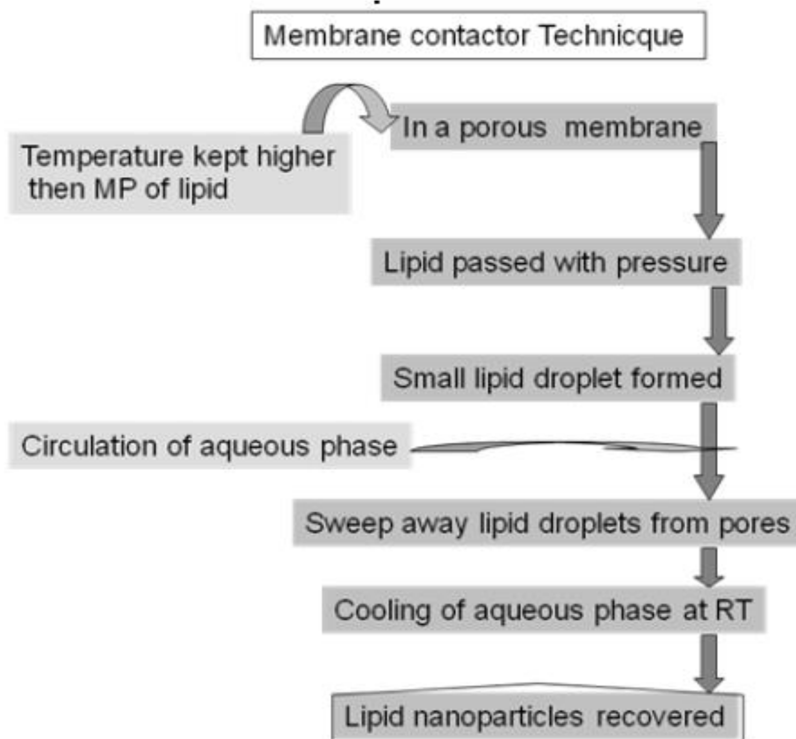


Fig 8:Schematic representation of the membrane contactor technique for the production of lipid nanoparticles.

1.3.2. Nanoeulsion/microemulsion:

Both nanoemulsion and microemulsion are clear, dispersed systems comprising of two immiscible liquids wherein the dispersed phase droplets are of nanosize but differ from each other in respects enlisted in Table 1 O/w nanoemulsions present the most important parenteral drug carrier systems where lipophilic drugs are dissolved in the inner phase of the emulsion. Self-emulsifying drug delivery systems (SEDDS) and self micro-emulsifying drug delivery systems (SMEDDS) can be described as isotropic solutions of oil and surfactant,which form o/w (micro) emulsions on mild agitation in the presence of water.

Table 1: Comparison between nanoemulsion and microemulsion

Nanoemulsion	Microemulsion
1.Kinetically stable	Thermodynamically stable
2.Form only after application of high shear	Form spontaneously
3.Contains less amount of surfactant	Contain more amount of surfactant

The dimension of macroemulsion,microemulsion/nanoemulsions and micelles are:

- Macro emulsions, R > 50nm (opaque and milky).
- Micellar solutions/microemulsions/nanoemulsions: 5-50 nm (5-10 nm is transparent, 10-50 nm is translucent).
- Micelles, R < 5 nm (they scatter little light and transparent).

1.3.3 Nanocapsule:

Oil – containing nanocapsule differ from o/w nanoemulsion in providing a barrier made from polymers between the core and the surrounding environment. Nanocapsules can be prepared by usual type A methods employed for preparing microcapsules.

1.3.4 Polymeric Nanoparticle/Nanospheres:

Polymeric Nanoparticle consists of drug dispersed in an amorphous form within a polymer matrix. Polymers suitable for the preparation of biodegradable nanoparticles include cellulose derivatives,poly(alkylcyanoacrylates),poly(methylidenemalonate)

polyorthoesters,polyanhydrides and polyesters such as poly(lactic acid), poly(glycolic acid) and poly(Є-caprolactone) and their co-polymers. It differs from nanocapsules in containing the drug in polymer matrix.

1.3.5 Solid-lipid Nanoparticles (SLN): Melt- emulsified nanoparticles based on lipids (or waxes) are solid at room temperature and generally prepared by hot high pressure homogenization. *Advantages* of these SLN are:-

- Use of physiological well-tolerable lipids.
- Avoidance of organic solvents in the preparation process.
- Wide potential application spectrum: dermal, *peroral* and intravenous.
- Improved bioavailability.
- Protection of sensitive drug molecules from the environment (water, light etc.).
- Controlled – release characteristics.

1.3.6.Nanostructured lipid carriers (NLC):

Nanostructured lipid carrier are oil- loaded solid lipid nanoparticles.Nanostructure lipid carriers are the new generation of lipid nanoparticles, attracting major attention as novel colloidal drug carriers for topical use. NLC were developed to overcome the limitations associated with the SLN. SLN consist of solid lipids, while NLC consist of a mixture of specially blended solid lipid (long chain) with liquid lipid (short chain), preferably in a ratio of 70:30 up to a ratio of 99.9:0.1. NLC offer several *advantages* over SLN such as:-

- Greater degree of drug loading.
- Reduced burst release of drug.
- Better control of drug release.

1.3.7 Liquid – drug conjugate (LDC) nanoparticles:

Covalent bonding or salt formation of a hydrophillic drug with lipid is done to-

- Enhance in *vivo*permeability.
- Improve membrane permeability.
- Control drug release.

1.4 Ciprofloxacin:

It is the most potent first generationfluroquinolones active against a broad range of bacteria, the most susceptible ones are the aerobic gram- negative bacilli, especially the *Enterobacteriaceae* and *Neisseria*. The MIC of ciprofloxacin against these bacteria is usually < 0.1 ug/ml, while gram postive bacteria are inhibited at relatively higher concentration.

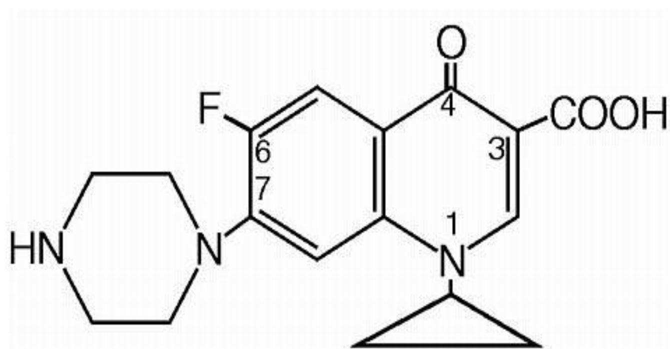


FIGURE 9 molecular structure of ciprofloxacin

1.4.1Pharmacokinetics:

Ciprofloxacin is rapidly absorbed orally, but food delays absorption, and first pass metabolism occurs. The most prominent feature of ciprofloxacin is high tissue penetrability: concentration in lung, sputum, muscle, bone, prostate and phagocytes exceeds that in plasma, but cerebrospinal fluid and aqueous levels are lower. It is excreted primarily in urine, both by glomerular filtration and tubular secretion. Urinary and biliary concentrations are 10-50 folds higher than plasma.

Table 2: Pharmacokinetic characteristics and doses of ciprofloxacin

Pharmacokinetic characteristic	Drug (ciprofloxacin)
Oral bioavailability (%)	60-80
Plasma protein binding (%)	20-35
Vol. o f distribution (L/kg)	3-4
Percent metabolized	2 0

Elimination t1/2 (hr)	3-5
Routes of administration	oral, i.v.
Dose (mg BD)	oral 250 - 750 iv 100 – 200

1.4.2 Antimicrobial spectrum:

- **Highly susceptible***against E. coli, K. pneumoniae, Enterobacter, Salmonella typhi , other Salmonella, Shigella, Proteus, Neisseria gonorrhoeae, N. meningitidis, H. influenzae, Vibrio cholerae, H. ducreyi, Campylobacter jejuni* and *Yersinia enterocolitica*.
- **Moderately susceptible***against Pseudomonas aeruginosa, Staph. Aureus, Staph. Epidermidis, Branhamellacatarrhalis, Legionella, Brucella, Listeria, Bacillus anthracis* and*Mycobacteriatuberculosis*.
- **Organisms which have shown low /variable susceptibility are:***Strep. pyogenes, Strep. faecalis,Strep. pneumoniae, Mycoplasma, Chlamydia, Mycobact. kansasii, Mycobact. avium.*
- **Notable resistant bacteria are***: BacteroidesJragilis, Clostridia, anaerobic cocci*

1.4.3 Adverse effect:

Ciprofloxacin has good safety record: side effects occur in - 10% patients, but are generally mild; withdrawal is needed only in 1 .5%. CNS: dizziness,headache, restlessness, anxiety, insomnia, impairment of concentration and dexterity (caution while driving), tremor. Seizures are rare; occur only at high doses or when redisposing factors are present: possibly reflect GABA antagonistic action of fluroquinolones. Skin/hypersensitivity: rash, pruritus, photosensitivity,urticaria, swelling of lips, etc. Serious cutaneous reactions are rare.

1.4.4 The remarkable microbiological features of ciprofloxacin are:

- Rapidly bactericidal activity and high potency: minimum bactericidal concentrations (MBCs) are close to minimial inhibitory concentrations (MICs).
- Relatively long post- antibiotic effect on *Enterobacteriaceae,pseudomonas*and *staph.*

- Low frequency of mutational resistance.
- Low propensity to select plasmid type resistant mutants.
- Protective intestinal sterptococci and anaerobes are spared.
- Active against many β-lactum and aminoglycoside resistant bacteria.
- Less active at acidic pH.

1.4.5 Interactions:

- Plasma concentration of theophylline, caffeine and warfarin are increased by ciprofloxacin due to inhibition of metabolism: toxicity of these drugs can occur.
- NSAIDs may enhance the central nervous system toxicity of ciprofloxacin; seizures are reported.
- Antacids, sucralfate and iron salts given concurrently reduce absorption of ciprofloxacin.

1.4.6 Mechanism of action:

The ciprofloxacin inhibit the enzyme bacterial DNA gyrase, which nicks double-stranded DNA, introduces negative supercoils and then reseals the nicked ends. This is necessary to prevent excessive positive supercoiling of the strands when they separate to permit replication or transcription. The DNA gyrase consists of two A and two B subunits: The A subunit carries out nicking of DNA, B subunit introduces negative supercoils and then A subunit reseals the strands. Ciprofloxacin bind to A subunit with high affinity and interfere with its strand cutting and resealing function. Recent evidence indicates that in gram-positive bacteria the major target of ciprofloxacin action is a similar enzyme topoisomerase IV which nicks and separates daughter DNA strands after DNA replication. Greater affinity for topoisomerase IV may confer higher potency against gram-positive bacteria. The bactericidal action probably results from digestion of DNA by exonucleases whose production is signalled by the damaged DNA. In place of ON A gyrase or tope isomerase IV, the mammalian cells possess an enzyme topoisomerase II (that also removes positive supercoils) which has very low affinity for ciprofloxacin hence the low toxicity to host cells.

1.5Clotrimazole:

Clotrimazole is effective in the topical treatment of infections like ringworm,athletes' foot, otomycosis. It is particularly favoured for vaginitis because of long lasting residual effect. It is also effective in skin infection caused by *corynebacteria.*Clotrimazole is well tolerated by most patients. Local irritation with stinging and burning sensation occurs in some. No systemic toxicity is seen after topical use. Topical clotrimazole cures dermatophyte infection in 60-100% of cases. The cure rates in cutaneous candidiasis are 80-100%. In a small fraction of recipients, clotrimazole on skin may cause stinging, erythema, edema, vesication, desquamation, pruritus, and urticaria

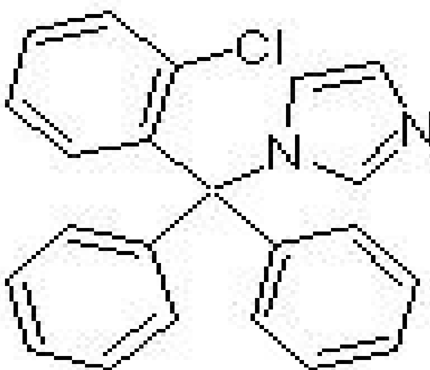


Fig. 10 Chemical structure of clotrimazole

1.5.1 Mechanism of action of clotrimazole:

The major effect of clotrimazole on fungi is inhibition of 14-α-sterol demethylase, a microsomal CYP. clotrimazolethu impair the biosynthesis of ergosterol for the cytoplasmic membrane and lead to the accumulation of 14-α-methylsterols.Thesemethylsterols may disrupt the close packing of acyl chains of phospholipids, impairing the functions of certain membrane-bound enzyme systems, thus inhibiting growth of the fungi.

1.5.2 Antifungal activity:

Clotrimazole have clinically useful activity against *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata, Cryptococcus neoformans,*

Blastomycesdermatitidis, Histoplasmacapsulatum, Coccidioides spp., paracoccidioidesbrasiliensis, and ringworm fungi(dermatophytes), Aspergillus spp., Scedosporiumapiospermum(Pseudallescheriaboydii), Fusarium, and *Sporothrixschenckii* are intermediate in susceptibility.

1.5.3 Interaction:

Clotrimazole is an inhibitor of the cytochrome P450 3A4, 2A6, 2C8/9, and 2E1 isoenzymes. Caution should be exercised and monitoring is suggested when concomitantly administering clotrimazole with drugs that have narrow therapeutic windows.

1.5.4 Adverse Effects:

- Topical oral therapy: abnormal liver function test results, nausea, vomiting, unpleasant mouth sensations, pruritus[23].
- Topical application to skin: blistering, erythema, edema, pruritus, burning, stinging, peeling, urticaria, general irritation of skin[24].
- Intravaginal therapy: vaginal burning, erythema, irritation, intercurrent cystitis

1.6 Tween 20:

TWEEN-20® is a non-ionic surfactant with hydrophobic alkyl side chains. In an aqueous environment the components form micelles above the critical micel concentration. This is based on hydrophobic interaction. When other hydrophobic components (proteins, gold conjugates etc.) are present these components are likely to get embedded in micellar structures. This has several effects: hydrophobic (background) interactions are largely suppressed, the added different molecules are slightly increased in size and previously existing hydrophobic complexes are broken up due to surface forces.[25]

- Density: 1.10 g/cm^3
- Formula: $C_{58}H_{114}O_{26}$
- Molar mass: 1,227.54 g/mol
- IUPAC name : Polyoxyethylene (20) sorbitanmonolaurate

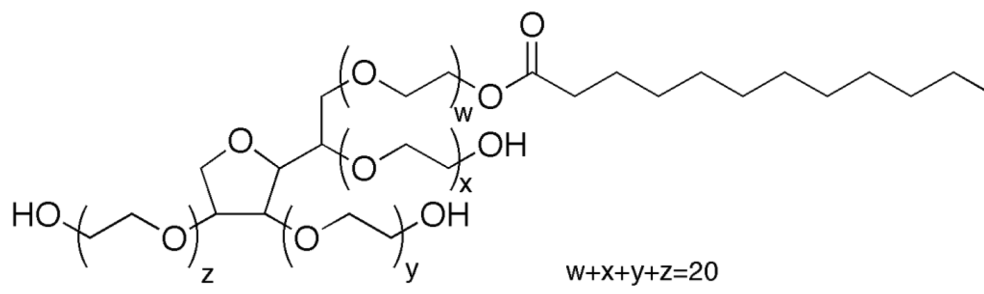


Fig 11: Molecular structure of tween 20.

1.7 Steric acid:

Stearic acid is a saturated fatty acid with an 18-carbon chain and has the IUPAC name octadecanoic acid. It is a waxy solid and its chemical formula is $C_{17}H_{35}CO_2H$. Its name comes from the Greek word στέαρ *"stéar"*, which means tallow. The salts and esters of stearic acid are called stearates. As its ester, stearic acid is one of the most common saturated fatty acids found in nature following palmitic acid. The triglyceride derived from three molecules of stearic acid is called stearin[26].

Fig 12: Molecular structure of stearic acid.

1.7.1 Production:

Stearic acid is obtained from fats and oils by the saponification of the triglycerides using hot water (above 200 °C). The resulting mixture is then distilled [27]. Commercial stearic acid is often a mixture of stearic and palmitic acids, although purified stearic acid is available.Fats and oils rich in stearic acid are more abundant in animal fat (up to 30%) than in vegetable fat (typically <5%). The important exceptions are cocoa butter and shea butter, where the stearic acid content (as a triglyceride) is 28–45%. [28].In terms of its biosynthesis, stearic acid is produced from carbohydrates via the fatty acid synthesis machinery wherein acetyl-CoA contributes two-carbon building blocks.[26]

1.7.2 Uses:

In general, applications of stearic acid exploit its bifunctional character, with a polar head group that can be attached to metal cations and a nonpolar chain that confers solubility in organic solvents. The combination leads to uses as a surfactant and softening agent. Stearic acid undergoes the typical reactions of saturated carboxylic acids, a notable one being reduction to stearyl alcohol, and esterification with a range of alcohols. This is used in a large range of manufactures, from simple to complex electronic devices [26].

AIM

The aim of this study was to prepare and evaluate nanostructured lipid carrier based topical gel of clotrimazole and ciprofloxacin. Stearic acid as the solid lipid, oleic acid as the liquid lipid, tween 20 as the surfactant, and polyvinylalcohol (PVA) as the co-surfactant were used. Nanostructured lipid carriers were prepared by sonication methods. Characterization of the nanostructured lipid carrier dispersion was carried out through particle size analysis, scanning electron microscopy (SEM), FTIR, and an *in vitro* release study.

CHAPTER 3

REVIEW OF LITERATURE

1. Lin YK et al 2010 developed nanostructured lipid carriers (NLCs) loaded with lipophilic calcipotriol and hydrophilic methotrexate as topical therapy. NLCs were composed of Precirol ATO 5 with various amounts of squalene as the liquid lipid. The particle size, surface charge, molecular environment, drug permeation, and skin irritation of the carriers were assessed. Hyperproliferative skin was also used as a permeation barrier in this study. The range of particle size of the NLC preparations was 270 to 320 nm, with vehicles containing a higher Precirol amount exhibiting a larger diameter. The methotrexate amount permeating the skin was 2.4 to 4.4-times greater using NLCs compared to that with the control. Dual drugloaded NLCs exhibited reduced skin permeation of calcipotriol but not methotrexate. These two drugs with extremely different polarities can successfully be combined in NLCs. Results suggest that NLCs may have the potential to serve as delivery carriers for antipsoriatic drugs because of enhanced drug permeation and limited skin irritation [27].

2. Fang Li et al 2010; evaluated safety, efficacy, in vitro cytotoxicity, pharmacokinetics, biodistribution, antitumor efficacy of bufadenolides loaded NLCs. Bufadenolides NLCs prepared by melt emulsification and ultrasonic method showed high plasma concentration and low clearance after IV administration as compared to bufadenolides solution. The biodistribution studies indicated that accumulation of bufadenolides in brain was higher and can be used for brain cancer [28].

3. Cirri M et al 2012 prepared Ketoprofen loaded NLCs, which showed improved therapeutic drug efficacy and safety, allowing an improvement in the dissolution stability, high tolerability, percutaneous and skin permeation properties of ketoprofen [29].

4. Pople PV et al formulatedTacrolimus loaded NLCs using high pressure homogenization method; lipid modification in formulation resulted in the formation of less perfect crystals offering space to accommodate dissolved drug leading to high entrapment efficiency and topical delivery [30].

5. Jia Yon Fang et al loaded Psoralens into NLCs using high pressure homogenization method; prepared NLCs showed improved skin permeation due to enhanced permeation and controlled release [31].

6. Paltolla RR et al 2010 have formulated celecoxib loaded NLCs using high pressure homogenisation; NLCs were able to release the drug in a controlled manner for a prolonged period of time and were able to deposit in alveolar region of the lungs of mice with an improved residence time in lungs [32].

7. Araujo J et al 2011 have used NLCs to increase ocular absorption and enhance prolonged drug residence time in the ocular surface and conjuctival sac, by sustained drug release from the delivery system; it also reduced the precorneal drug loss [33].

8. Chen CC et al 2010 formulated Lovastatin loaded NLCs using ultrasonication technique; more than 70 % of drug was entrapped in the NLCs. In vitro release kinetics demonstrated that the drug release could be reduced upto 60 % with NLCs. The oral bioavailability of lovastatin was enhanced [34].

9. LG Souza et al 2011, prepared NLCs loaded with anticancer drug topotecan by micro emulsion technique and prepared formulations were of satisfactory loading, entrapment and improved chemical stability and cytotoxicity [35].

10. Pardeika JS et al 2011 formulated itraconazole loaded NLCs using high pressure homogenization; prepared nanoparticles had an entrapment efficiency of 98.78 % [36].

11. Chi et al 2011 prepared NLCs based formulation to protect the entrapped lutein from simulated fluid; they were successful in protecting the drug as drug was slowly released in intestinal fluid in an *in vitro* study [37].

12. Shen J et al have prepared Cyclosporine loaded NLCs using melt emulsification having, mucoadhesive properties; NLC provided a promising system with prolonged residence time [38].

13. WenLZ et al 2010 used nanoprecipitation/solvent diffusion method to prepare tashinone loaded NLCs. Drug loading and stability was improved [39].

14. Oshima H et al prepared nifedipine loaded NLCs using high pressure homogenization; NLCs improved solubility and bioavailability of drug [40].

15. In a similar study Tsaia MJ et al 2012, developed baicalein loaded NLCs, intravenous delivery of formulated NLCs increased the plasma level and t1/2 compared to equivalent aqueous solution [41].

16. Silva AC et al 2011 developed Bromocriptine loaded NLCs; prepared NLCs had rapid onset of action and longer duration of action as compared to solution of plain drug[42].

17. Franciana MO et al 2010 prepared NLCs to slow down release of drug from lipid particles and improve stability of drug in contact with skin homogenates. They successfully developed NLCs to achieve slow release and enhanced stability [43].

4. MATERIAL AND METHODS

4.1.1 Materials

Test substance: Ciprofloxacin and Clotrimazole were obtained as gift samples from *Optimum Pharmaceuticals*Private Limited.

4.1.2Chemicals/ Reagent required

S.NO.	Name of Chemicals	Source/Company
1.	Stearic acid	Sigma Chemicals
2.	PVA	Sigma Chemicals
3.	Chloroform	SD fine chemicals
4.	Castor oil	Lobachemie
5.	Tween	Lobachemie
6.	Ethanol	Lobachemie
7.	Methanol	Lobachemie
8.	Disodium hydrogen phosphate	Sigma Chemicals
9.	Potassium dihydrogen phosphate	Sigma Chemicals
10.	Sodium chloride	Sigma Chemicals
11.	Tryptan blue	Sigma Chemicals

4.1.3Apparatus required:

S.NO	Apparatus and equipments
1.	Magnetic stirrer
2.	Weighing Balance
3.	Uv spectrophotometer
4.	Water bath sonicator

5.	Centrifuge

4.2. METHODS

4.2.1.Preformulation Studies

Preformulation testing is the first step in the rationale development of dosage forms of a drug. It can be defined as an investigation of physical and chemical properties of drug substance, alone and when combined with excipients. The overall objective of preformulation testing is to generate information useful to the formulator in developing stable dosage forms.

The goals of Preformulation studies are:

- To establish the necessary physicochemical characteristics of a new drug substance.
- To determine its kinetic release rate profile.
- To establish its compatibility with different excipients.

Preformulation studies of the obtained drug sample, includes its identificationby:

- Melting Point Determination
- UV spectrum studies
- Calibration curve of Clotrimazole and Ciprofloxacinin PBS pH 7.4
- FTIR spectras ofClotrimazole and Ciprofloxacin.

A. Melting Point determination

The Melting Point of Clotrimazole and Ciprofloxacinwere determined using Melting Point Testing Apparatus and method used was Capillary Fusion Method.

Capillary tube was taken and one end of this tube was sealed by gentle heating. Small amount of drug was filled in this capillary tube and the tube was then kept in the Melting Point Testing Apparatus. Melting point of the drug was noted at the point when it starts melting and was compared with the literature value.

B.λmax determination by UV:

Drugs were identified using uv spectrophotometer and the obtained λ max values were compared with reference.

C. Calibration Curve of Ciprofloxacin and Clotrimazole

The calibration curve of the ciprofloxacin was plotted in phosphate buffer (pH 7.4) as well as in distilled water. 10 mg drug was dissolved in 10ml of solvent (1000µg/ml). 1 ml of this solution was taken and again diluted to 10 ml with solvent (1000µg/ml). From this solution 0.5, 1, 1.5, 2, and 2.5 ml were pipette in different 10ml volumetric flasks and volume was made up to 10ml with solvent to get solutions having concentrations of 5, 10, 15, 20, and 25µg/ml. Then absorbance of these solutions was recorded in UV spectrophotometer at (260 nm for ciprofloxacin) and absorbance v/s concentration graph was plotted. Regression co-efficient and slope values were calculated from this graph. Similarly calibration curve of clotrimazole was plotted using methanol and phosphate buffer pH6.8 as solvents at 261 nm [44].

D. FTIR Spectrum of Drug, polymer and drug polymer mixture.

The infrared spectrums of drug samples were recorded and the spectral analysis was done. The dry sample of drug was directly placed after mixing and triturating with dry potassium bromide [45]. The FTIR spectra were recorded in the wavelength region between 4000 – 400 cm^{-1}. The spectra obtained for drugand physical mixture of drug with excipients was compared.

4.2.2.Formulation ofNLCs

NLCs were prepared following an emulsification-ultrasonication method with minor modifications [46-49]. Briefly, 100 mg of stearic acid was dissolved in 1 ml of chloroform, tween 20 and clotrimazole were added to this solution to prepare solution one. Similarly ciprofloxacin was dissolved in 1% aqeous PVA to prepare solution 2.Solution 2 was added to solution 1 to obtain a primary emulsion using a magnetic stirrer. This primary emulsion was added dropwise into 5 % PVA solution to obtain a secondary emulsion using magnetic stirring. After 5 min of emulsification, the coarse emulsion was sonicatedfor 15 minsby a water bath sonicator.

Table4.2.2: Formulation Design forNLCs

Formulation code	Stearic acid (mg)	Ciprofloxacin (mg)	Clotrimazole (mg)
F1	100	-	-
F2	100	25	-
F3	100	-	25
F4	100	25	25
F5	100	12.5	25
F6	100	25	12.5

4.3.Evaluation of nanoparticles

4.3.1 Particle size and size distribution analysis of formulation

Particle size and size distribution analysis offormulations were doneusing Microtac particle size analyzer.

4.3.2 Shape: SEM (Scanning electron microscopy) Studies

Surface characteristics of NLCs were characterized by SEM (Scanning Electron Microscopy). The SEM is also widely used to identify phases based on qualitative chemical analysis and/or crystalline structure. Precise measurement of very small features and objects down to 50 nm in size is also accomplished using the SEM [50].

4.3.3 Drug loading

1 ml of NLC formulation were taken and dissolved in 1ml of Methanol, this solution was further diluted 10 times, centrifuged for 20 minutes at 10000 rpm and the drug content in supernatant was recorded using UV-spectrophotometer (at 260nm and 261 nm). % drug loading was calculated using the following formula [51].

% drug loading $=(Wa-Ws/Wa-Ws+Wl) \times 100$ %

WhereWa, Ws and Wl were the weight of drug added in system, analyzed weight of drug in supernatant and weight of lipid added in system, respectively.

4.3.4 Entrapment efficiency:

1 ml of NLC formulation were taken and dissolved in 1ml of Methanol, this solution was further diluted 10 times, centrifuged for 20 minutes at 10000 rpm and the drug content in supernatant was recorded using UV-spectrophotometer (at 260nm and 261 nm).
Drug entrapment efficiency (EE) was calculated as below[51]:

EE (%) = (Wa-Ws/Ws) × 100%

WhereWa, Ws and Wl were the weight of drug added in system, analyzed weight of drug in supernatant and weight of lipid added in system, respectively.

4.3.5 In Vitro release studies

The drug release study NLC formulation was performed using a dialysis tube (10 kDa molecular cutoff). Pretreatment of a dialysis tube was done following the protocol mentioned by Sigma. The dialysis tube was soaked in the release media (10 mM phosphate buffer at pH 7.4 with 2% Tween R80) overnight prior to the experiment. First, one end of the dialysis tube was tightly tied. Then, 1mlNLC formulation was put in the dialysis tube. Finally, the other end of the tube was also tightlytiedandplacedintheglassbottle.Preheated(at37◦C) 10mlreleasemediawasthenpouredinthebottleandthebottle wasimmediatelykeptona magnetic stirrer at 100 rpm at 37◦C. To determine the amount of drug released from the formulation at differenttime points, 2 ml of the release media was withdrawn from the bottle at the predetermined time points and analyzed by UV. The withdrawn release media in the bottle was immediately replaced with 2 ml of fresh release media to maintain a constant volume of release media as well as to maintain the sink condition.Same procedure was followed for all the samples for 24 hours and percent drug release was calculated [52].

Finally, a graph was plotted between % CDR v/s time and the release profile of all the formulations was compared with each other.

4.3.6Cytotoxicity studies

For cell viability assay, cells (MDCK) were seeded at a density of 1×10^4 cells/well in 96 well plates in 0.2 ml of growth medium consisting of DMEM with 10% FBS and antibiotics. After that cells were incubated at 37 °C till confluence reached 70%. The cells were then treated with NLC blank and incubated for 12h, 24h and 48h. After desired hours of exposure MTT (20μl) was added to each well and further incubated at 37°C for three hours. Metabolically active cells were able to reduce MTT with the help of enzyme Succinate dehydrogenase and form purple coloured insoluble formazan. To dissolve these formazan crystals after three hours supernatant was discarded from each well and DMSO (200μl) was added. The absorbance was taken at 570 nm test wavelength and a 630nm reference wavelength to test the cell viability using a microplate reader (Bio-Rad, Model

680). Survival percentage was calculated as compared to mock treated cells (100% survival). All the experiments were performed in triplicates.

5. RESULTS

Sixformulations of ciprofloxacin-clotrimazole loaded NLCswere formulated using stearic acid as lipid and the composition of each formulation is shown in Table4.1.1. The formulations were subjected to evaluation parameters like % drug loading, entrapment efficiency,size distribution analysis, and *In Vitro* release studies.

5.1. Preformulation Studies

5.1.1. Melting point determination

Melting points of drugs were determined by capillary fusion method and the results are shown in Table 5.1.1.

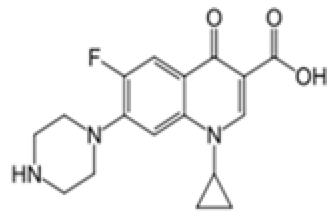
Table 5.1.1 Melting Point of Drugs

Drug	Literature value	Reference	Experimental value
Ciprofloxacin	250-252 °C	[53]	251°C
Clotrimazole	142.2-148.9 °C	[54]	146°C

5.1.2. Compatibility Studies

The infrared spectrums of drugsamples were recorded and the spectral analysis was done. The dry sample of drug was directly placed after mixing and triturating with dry potassium bromide. The FTIR graphs of drugs,stearic acid (lipid) and drug-lipid combinations are shown in Fig 5.1.2, Fig 5.1.3, Fig 5.1.4, Fig 5.1.5, Fig 5.1.6, Fig 5.1.7 and Fig 5.1.8 respectively.There was no significant change in these peaks when the drug was combined with lipid. This concluded that the drugs were compatible with the lipidused.

a.



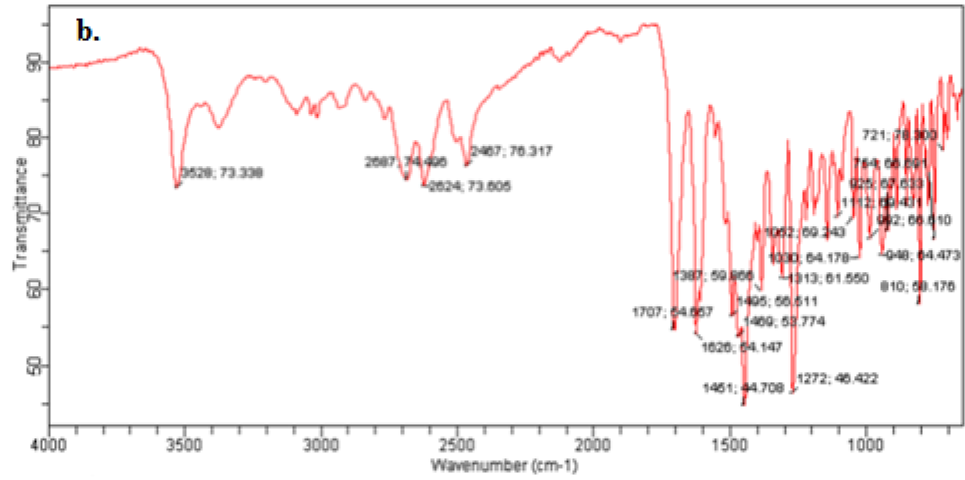


Figure 5.1.2: Ciprofloxacin Structure (a) and FTIR spectra (b)

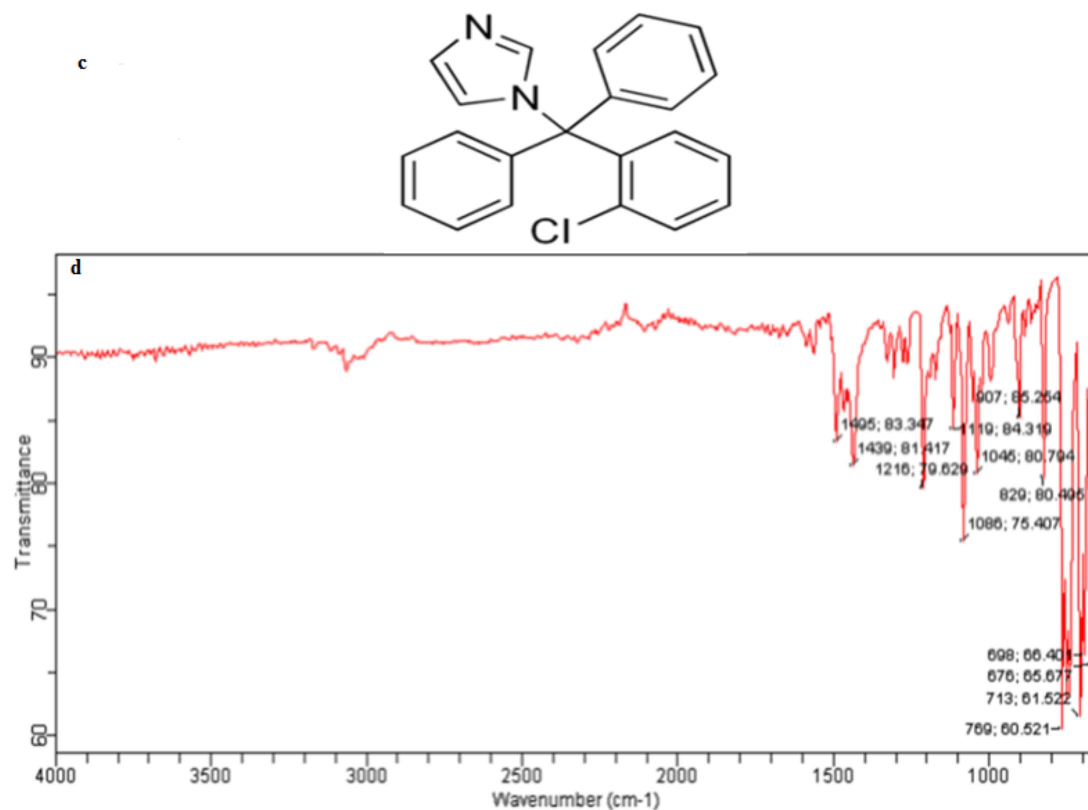


Figure 5.1.3Clotrimazole Structure (c) and FTIR spectra (d)

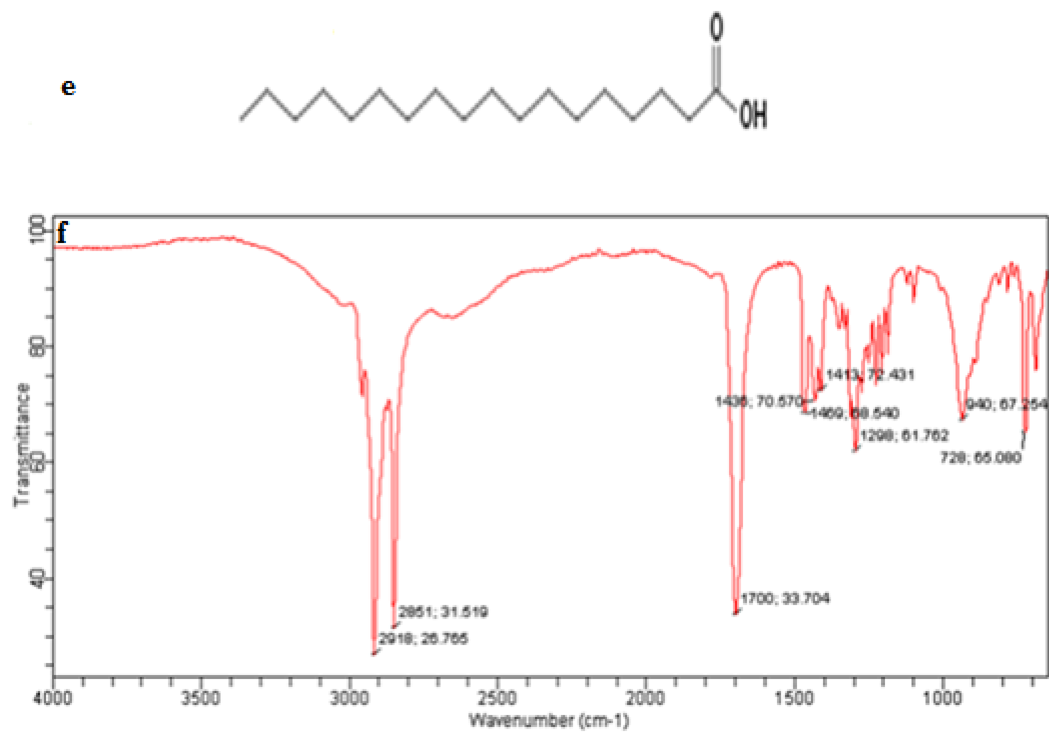


Figure 5.1.4:Stearic acid Structure (e) and FTIR spectra (f)

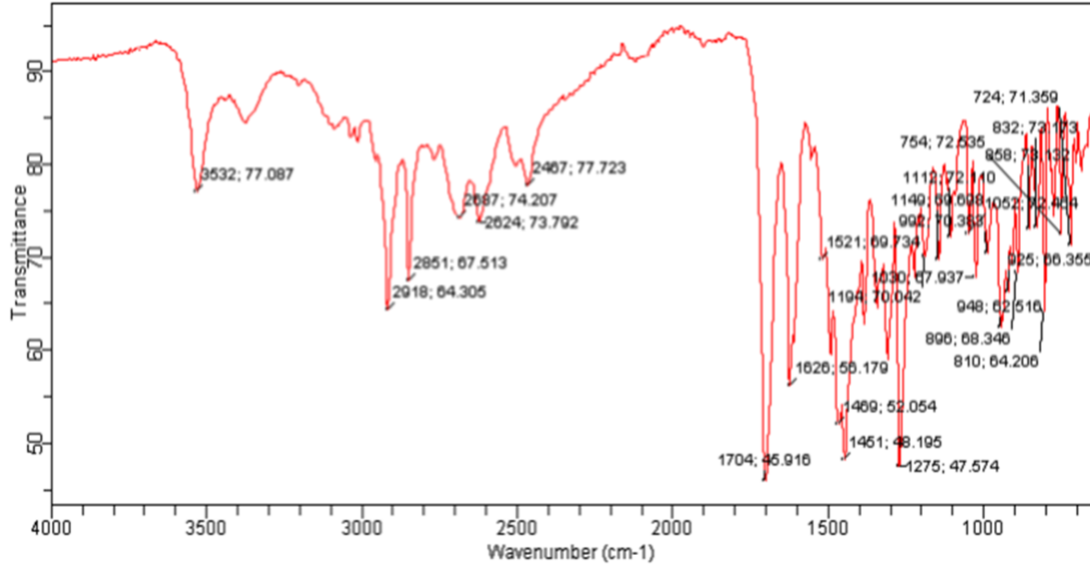


Figure 5.1.5 FTIR of Ciprofloxacin + Stearic acid

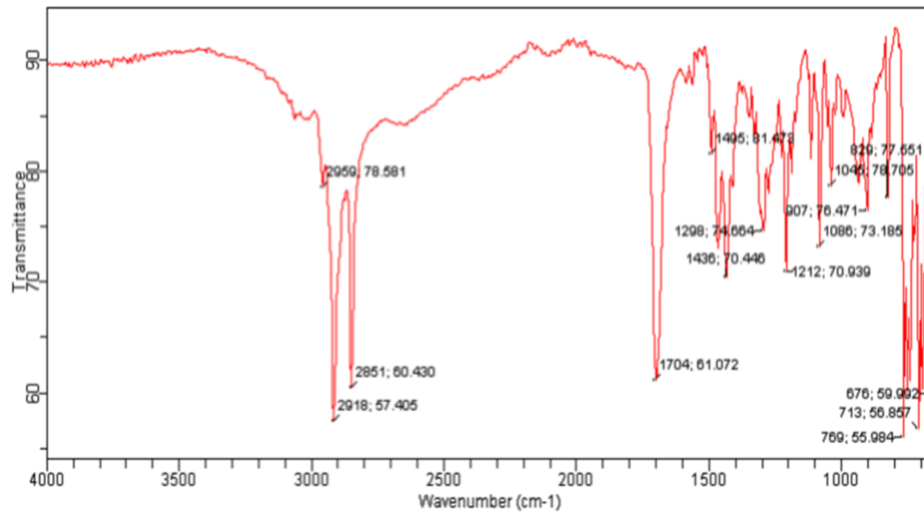


Figure 5.1.6 FTIR of Clotrimazole + Stearic acid

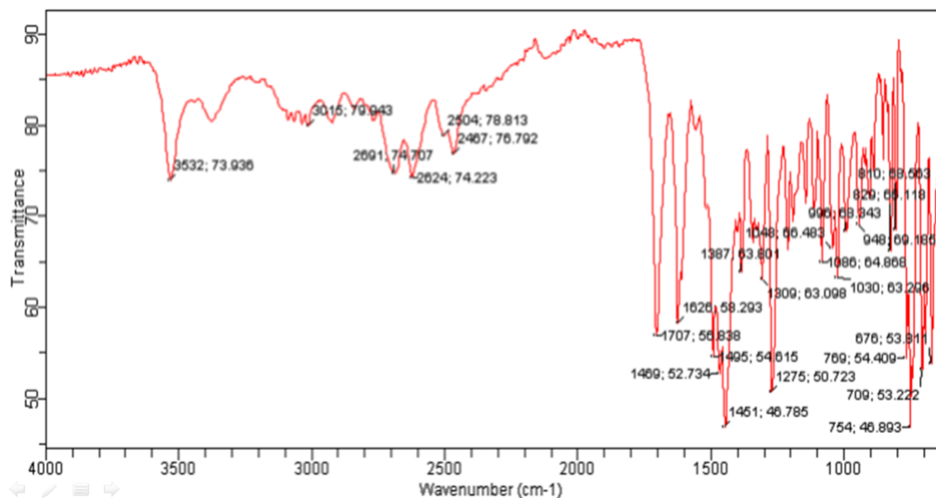


Figure 5.1.7 FTIR of Ciprofloxacin + Clotrimazole

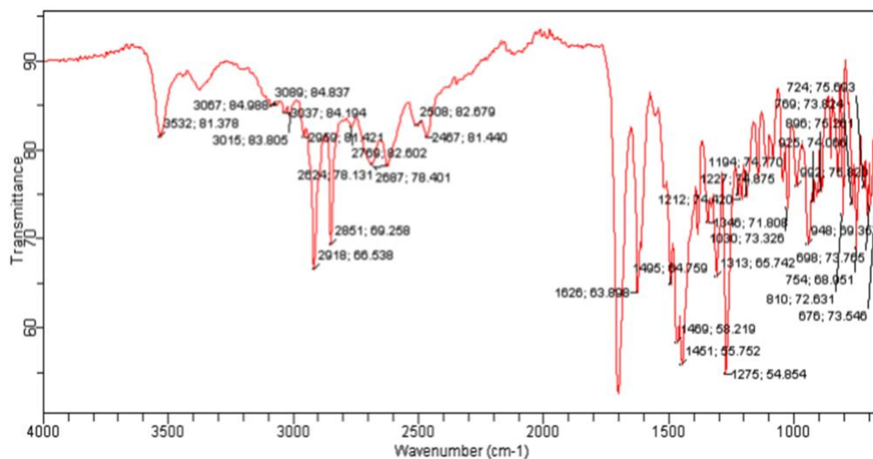


Figure 5.1.8 FTIR of Ciprofloxacin + Clotrimazole +Stearic acid

5.1.4. Identification of drug using UV

Drugs were identified by UV and the obtained λ max was compared with reference. Since there is negligible difference inlambda max values, therefore we can conclude that the given drugs are ciprofloxacin and clotrimazole.

Table 5.1.4: Identification of drug using UV.

Drug	Literature value λ max	Reference	Experimental value λ max

Ciprofloxacin	260.9	[56]	260
Clotrimazole	262	[57]	261

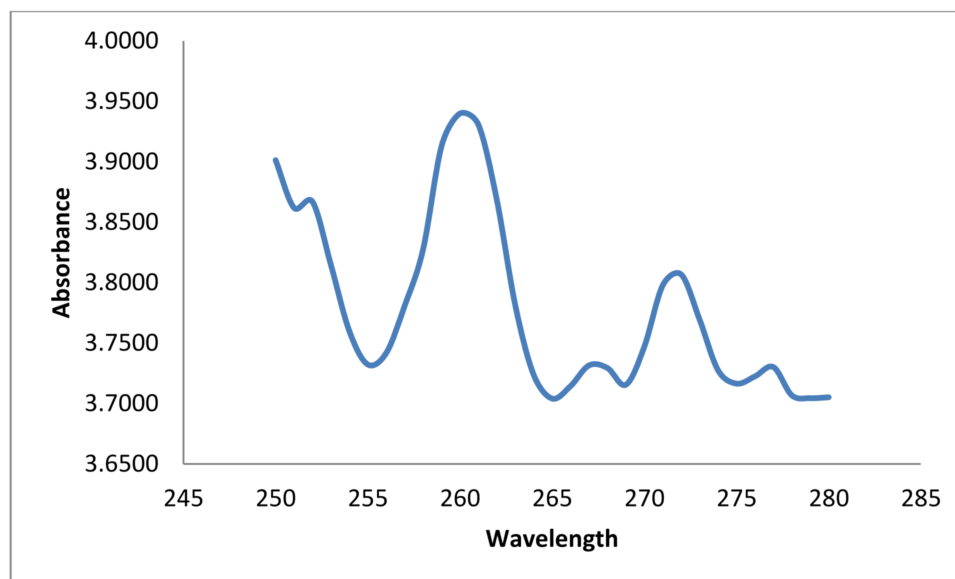


Figure-5.1.4: Spectrophotometric detection using UV (Ciprofloxacin)

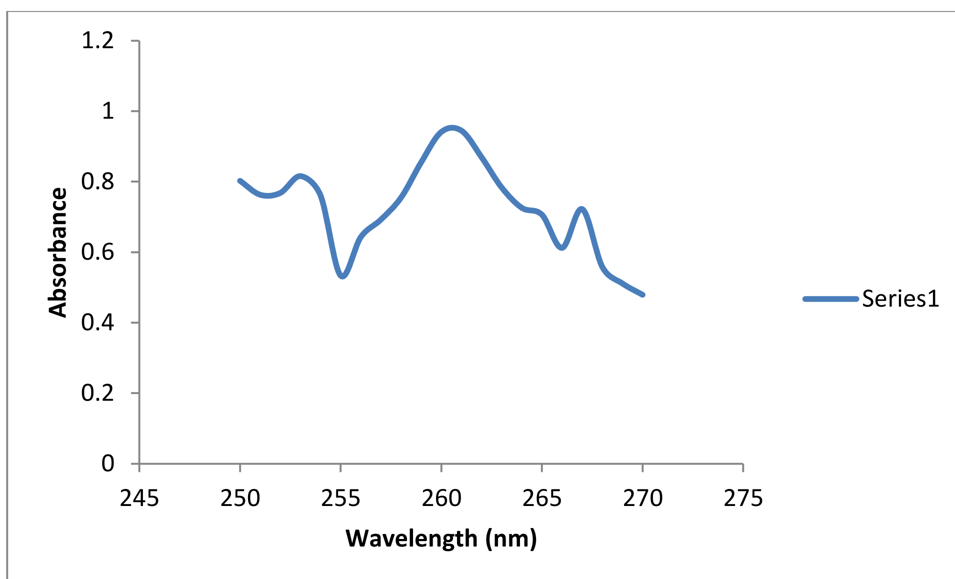


Figure-5.1.4a: Spectrophotometric detection using UV (Clotrimazole)

5.1.5. Calibration Curve

Calibration curve of Ciprofloxacinwas plotted in (water as well as phosphate buffer Ph 7.4)and similarly for clotrimazole in phosphate buffer (pH 6.8), the results are shown in Table 5.1.5. Stock solution 100µg/ml solution was prepared by dissolving 10 mg drug in 10 ml solvent. From this 5, 10, 15, 20, and 25 µg/ml solutions were prepared by taking 0.5, 1.0, 1.5, 2.0 and 2.5 ml of stock solution and making volume up to 10 ml withsolvent. These solutions were analyzed by UV spectrophotometer and absorbance for each solution was recorded. A graph was plotted between concentration and time.

Regression coefficient was calculated as 0.99 and slope was -0.008 (Ciprofloxacin) in distilled water.

Regression coefficient was calculated as 0.994 and slope was -0.030 (Ciprofloxacin) in phosphate buffer (pH 7.4).

Regression coefficient was calculated as 0.997 and slope was 0.007 (Clotrimazole) in methanol.

Regression coefficient was calculated as 0.997 and slope was 0.013 (Clotrimazole) in phosphate buffer (pH6.8).

Table 5.1.5: Calibration Curve of Ciprofloxacin in distilled water

S.No.	Conc(µg/ml)	Absorbance
1	5	0.0225
2	10	0.042
3	15	0.06
4	20	0.085
5	25	0.105

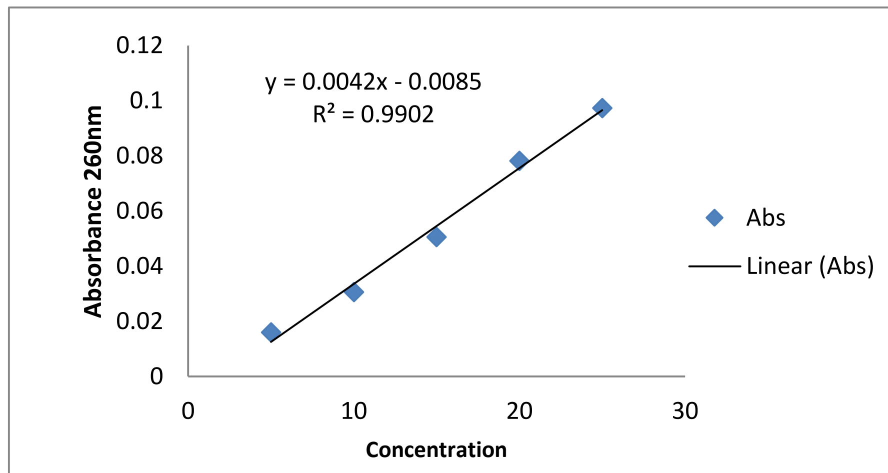


Figure 5.1.5Calibration curve of Ciprofloxacinin distilled water

Table 5.1.5a: Calibration Curve of Ciprofloxacin in Phosphate Buffer (pH 7.4)

S. No.	Conc(µg/ml)	Absorbance
1	5	0.0465
2	10	0.075
3	15	0.106
4	20	0.135
5	25	0.172

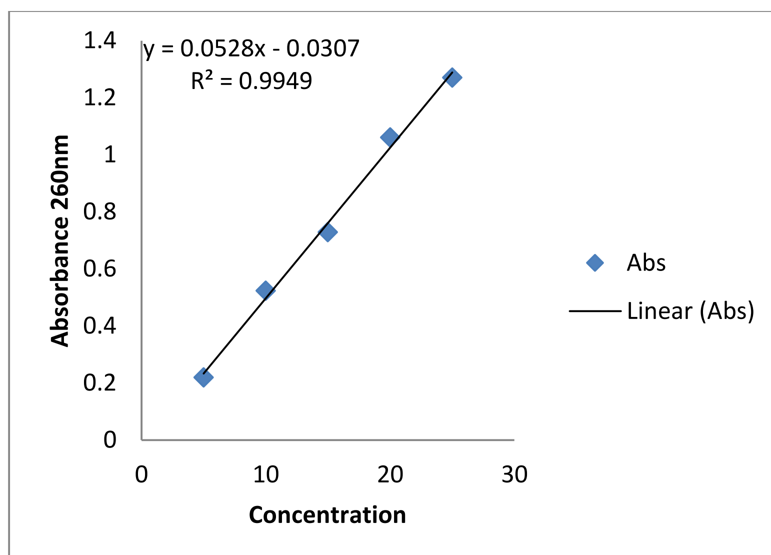


Figure 5.1.5a Calibration curve of Ciprofloxacin in Phosphate Buffer (pH 7.4)

Table 5.1.5b: Calibration Curve of Clotrimazole in Methanol

S.No.	Conc(µg/ml)	Absorbance (nm)
1	5	0.058
2	10	0.144
3	15	0.217
4	20	0.284
5	25	0.351

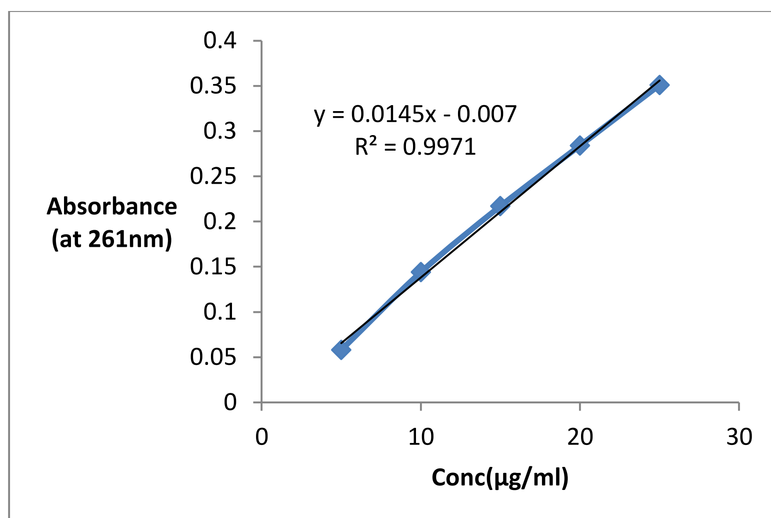


Figure 5.1.5b: Calibration curve of Clotrimazole in Methanol

Table 5.1.5c: Calibration Curve of Clotrimazole in Phosphate Buffer (pH 6.8)

S. No.	Conc(µg/ml)	Absorbance (nm)
1	5	0.0465
2	10	0.075
3	15	0.106
4	20	0.135
5	25	0.172

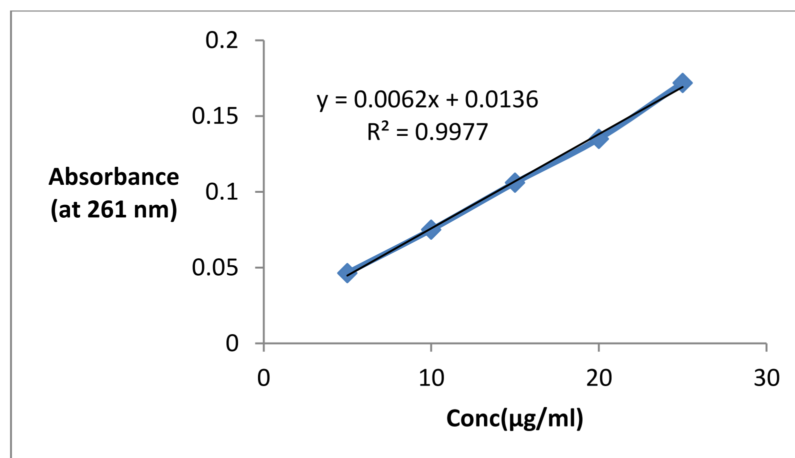


Figure 5.1.5c: Calibration curve of Clotrimazole in Phosphate Buffer (pH 6.8)

5.2. Evaluation Parameters

5.2.1. % Drug loading

Table 5.2.1: % drug loading

S.No	Formulation code	%drug loading (Ciprofloxacin)	%drug loading (Clotrimazole)
1	F1	0	0
2	F2	16.72	0
3	F3	0	15.73
4	F4	12.2	11.25
5	F5	6.26	12.64
6	F6	13.16	5.03

5.2.2. % entrapment efficiency (EE)

Table 5.2.2 % entrapment efficiency (EE)

S.No	Formulation code	% EE (Ciprofloxacin)	% EE (Clotrimazole)
1	F1	0	0
2	F2	83.6	0
3	F3	0	78.6
4	F4	61	56.26
5	F5	56.4	63.2
6	F6	65.8	45.33

5.2.3. Shape: SEM (Scanning Electron Microscopy).

Surface characteristics of nanoparticles were characterized by SEM (Scanning Electron Microscopy). NLCs were found to be smooth and spherical in shape.

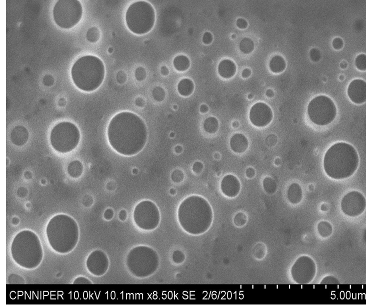


Figure 5.2.3a: Typical Scanning Electron Microphotographs of NLCs.

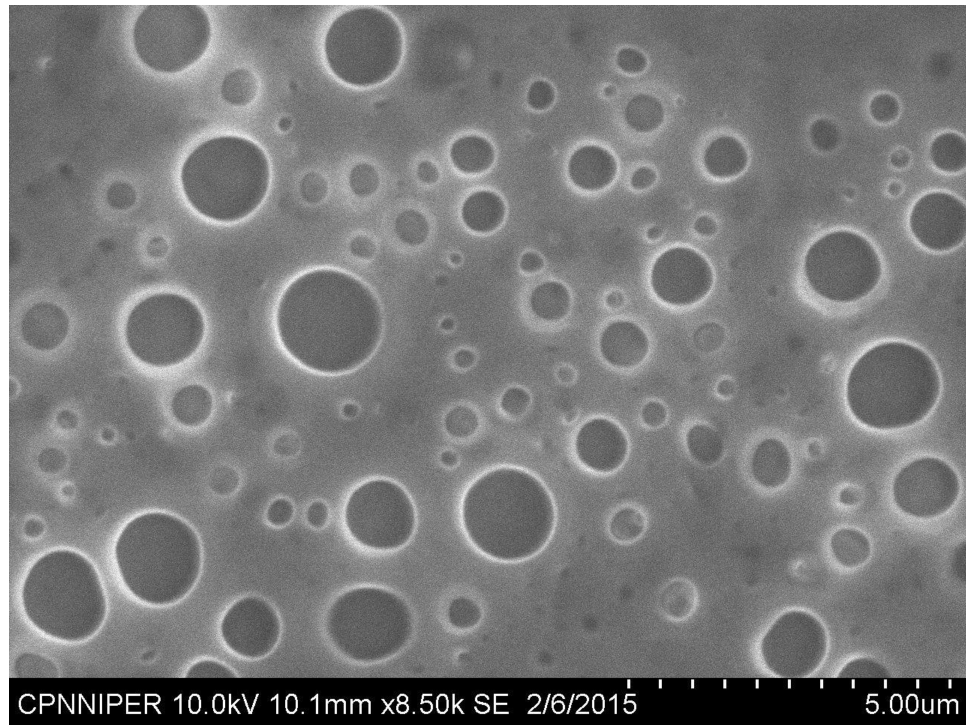


Figure 5.2.3b: Magnified Scanning Electron Microphotographs of NLCs.

5.2.4. Particle size

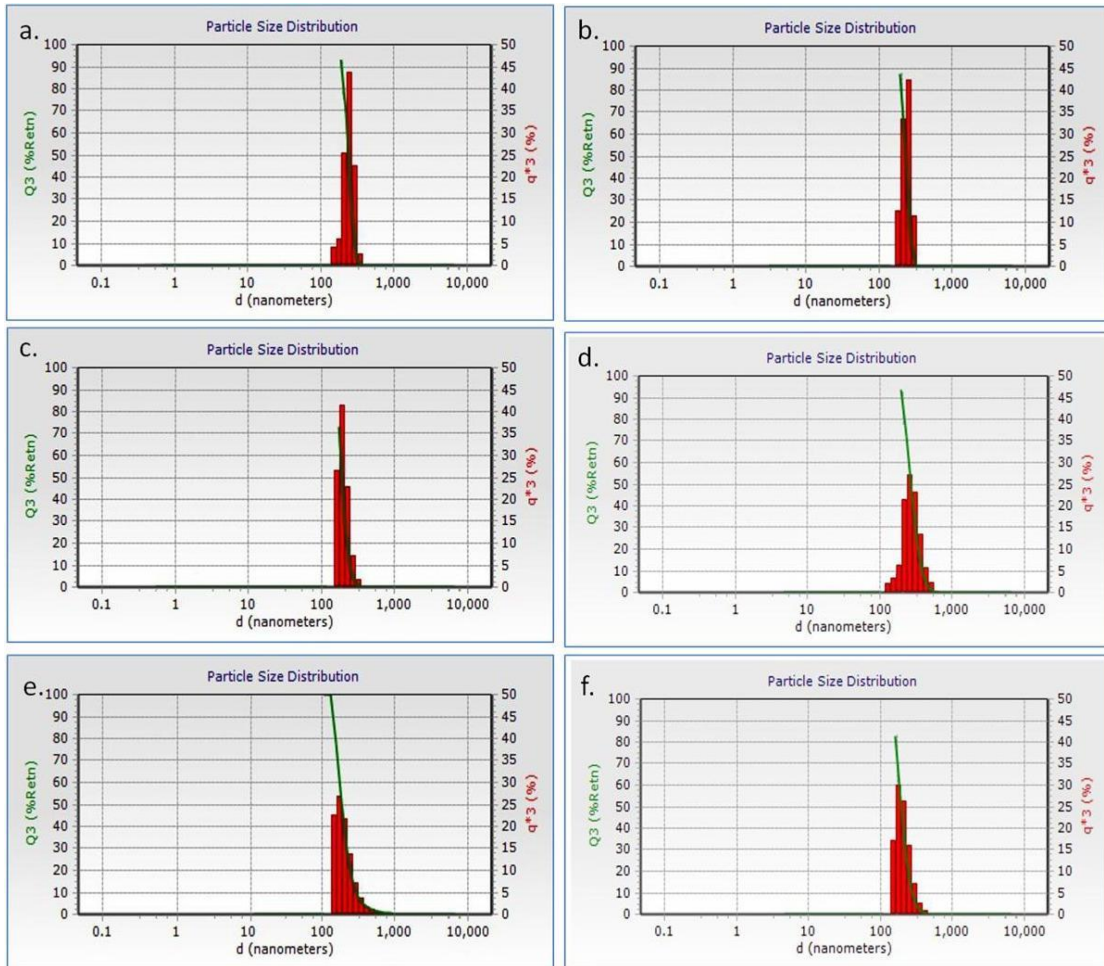


Figure 5.2.4: Particle size distribution of formulations F1 (a), F2 (b), F3 (c), F4 (d), F5 (e) and F6 (f).

5.2.5. *In Vitro* Release Studies

Amount of nanoparticles equivalent to 3mg of drug from each formulation was evaluated for *In Vitro* release and the results are shown in Figure 5.2.5.

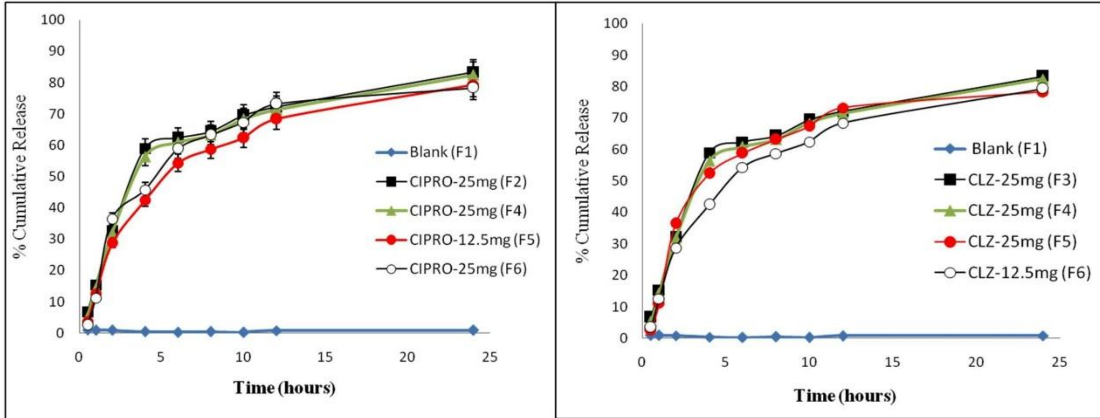


Figure 5.2.5: Dissolution profile of F1, F2, F3, F4, F5, F6 formulations.

5.2.6Cytotoxicity study results

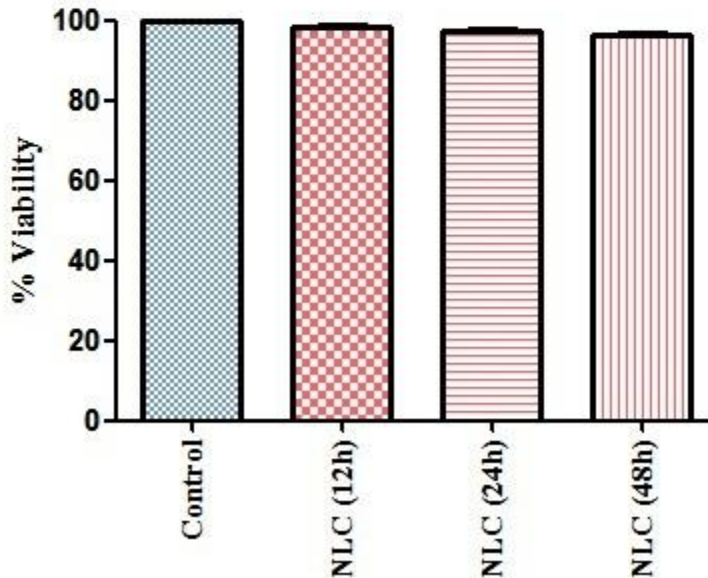


Figure 5.2.6: MTT (4, 5-dimethylthiazol-2`-yl)-2,5- dipheyltertazolium bromide assay.

CHAPTER 6

DISCUSSION AND CONCLUSION

In the present study we prepared ciprofloxacin and clotrimazole loaded NLCs by emulsification-ultrasonication method using stearic acid as lipidand evaluated them for % drug loading, % entrapment efficiency, particle size, shape, *in vitro* release studies and cytotoxicity studies. The *in-vitro* studies were carried out in phosphate buffer pH 7.4 at 37±2º C. NLCs in all formulations were found to be spherical with size ranging from 200-800 nm. All formulations exhibited an *in-vitro* release of 65-85 % in 24hour. % Drug loading of (F4) was found to be highest. Therefore it was selected as best formulation.Cytoxicity studies were carried out to evaluate toxic potential of formulation; blank formulation as well as drug loaded formulation was found to be non toxic towards cells, hence adding safety characters to formulation.

Thus to conclude dual drug loaded NLCs can be prepared easily meeting all evaluation parameters. This form of delivery system may provide advantages of both drugs i.e. ciprofloxacin as well as clotrimazole. Potential of such delivery systems should be exploited to treat microbial infections.

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