**THE THESIS**

**ENTITLED**

# "TECHNIQUES TO IMPROVE BIOAVAILABILITY OF SELECTIVE ESTROGEN RECEPTOR MODULATOR (SERM) FOR THE TREATMENT OF OSTEOPOROSIS"

**Submitted to**

**Sumandeep Vidyapeeth**

**In partial fulfillment of the requirement for**

**the award of**

## Doctor of philosophy

## In

## Pharmaceutical Sciences


**Guide:**
**Prof. & Head (Dr.) A K Seth**
**Dept. of Pharmacy, SV**

**Submitted by:**
**Mr. Nirmalkumar V Shah**
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**JULY 2015**

## SUMANDEEP VIDYAPEETH, VADODARA, GUJARAT

# Certificate

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This work has been carried out under my supervision and guidance. The matter compiled in this thesis has not been submitted earlier for the award of any other degree or fellowship and free from any kind of plagiarism.

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# Declaration

I hereby declare that the topic entitled **"TECHNIQUES TO IMPROVE BIOAVAILABILITY OF SELECTIVE ESTROGEN RECEPTOR MODULATOR (SERM) FOR THE TREATMENT OF OSTEOPOROSIS"** which is submitted herewith to Sumandeep Vidyapeeth, Vadodara for the partial fulfillment for the award of degree of **Doctor of Philosophy**, in **Pharmaceutical Sciences** is the result of work done by me in Department of Pharmacy, Sumandeep Vidyapeeth, under the guidance of **Dr. A K Seth**.

I further declare that the result of this work has not been previously been submitted for any degree or fellowship and free from any kind of plagiarism.

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**LIST OF PUBLICATIONS**

- **Accepted**

  **1.** Bioavailability enhancement of poorly soluble raloxifene by designing inclusion complex with β-cyclodextrin. (Scheduled to be published in Vol. 7, Issue 8, August 2015 in *International journal of pharmacy and pharmaceutical sciences***)**

- **Under review**

  **1.** Selective estrogen receptor modulator drug with improved bioavailability by designing microemulsion carrier system: *In vitro* and Pharmacokinetic parameters. (*Drug development and therapeutics)*

  **2.** Design and development of nanostructured lipid carriers for improvement in bioavailability of poorly soluble selective estrogen receptor modulator. (*Asian journal of pharmaceutical sciences*)

**PAPER PRESENTED**

- Poster presentation entitled "Bioavailability enhancement of poorly soluble raloxifene by designing inclusion complex with β-cyclodextrin" in seminar "*Challenges and opportunities on nanotechnology based drug delivery system*" held at Department of Pharmaceutical sciences, Saurashtra University, Rajkot sponsored by GUJCOST on 21$^{st}$ February, 2015.

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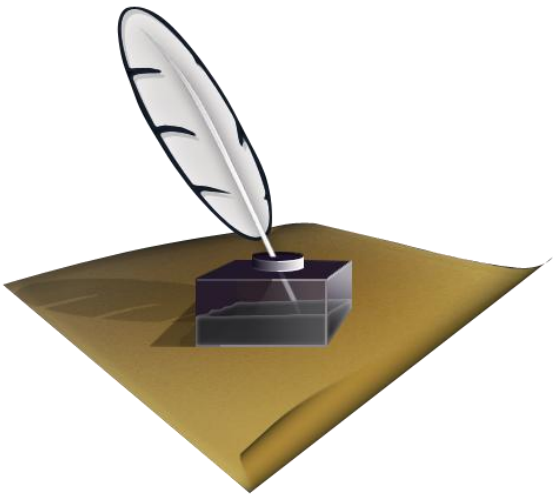
| | |
|---|---|
| SERMs | Selective Estrogen Receptor Modulators |
| HRT | Hormonal Replacement Therapy |
| RLX | Raloxifene Hydrochloride |
| BCS | Biological Classification System |
| NDDS | Novel Drug Delivery System |
| IC | Inclusion complex |
| ME | Microemulsion |
| NLCs | Nano structured lipid carriers |
| SLNs | Solid Lipid Nanoparticles |
| $S_{mix}$ | Surfactant – cosurfactant mixture |
| GMS | Glyceryl Monostearate |
| PVA | Poly Vinyl Alcohol |
| CDs | Cyclodextrins |
| β-CD | β-cyclodextrin |
| DMSO | Dimethyl sulphoxide |
| DMF | Dimethyl formamide |
| DCM | Dichloro methane |
| EDTA | Ethylene diamine tetra acetate |
| $NaHCO_3$ | Sodium Bicarbonate |
| Sodium CMC | Sodium Carboxy Methyl Cellulose |
| PEG | Poly ethylene glycol |
| HCl | Hydrochloric acid |
| % | Percentage |
| % T | Percentage Transmittance |
| °C | Degree Centigrade |
| µg | Microgram |
| µm | Micrometer |

| | |
|---|---|
| nm | Nanometer |
| ng | Nanogram |
| mM | Milli molar |
| Abs | Absorbance |
| API | Active Pharmaceutical Ingredient |
| CDR | Cumulative Drug Release |
| % CDR | Percent cumulative drug release |
| cm | Centimeter |
| % DC | Percent drug content |
| % EE | Percent entrapment efficiency |
| FTIR | Fourier transform infrared |
| SEM | Scanning Electron Microscope |
| TEM | Transmission Electron Microscope |
| DSC | Differential Scanning Calorimeter |
| XRD | X-ray diffraction |
| gm | Gram |
| h | Hour |
| hrs | Hours |
| ICH | International conference on harmonization |
| mg | Milligram |
| min | Minute |
| ml | Milliliter |
| L/kg | Liter per kilogram |
| mg/kg | Milligram per kilogram |
| mg/gm | Milligram per gram |
| mg/batch | Milligram per batch |
| pH | Hydrogen ion concentration |
| RH | Relative humidity |
| rpm | Revolution per minute |
| SD | Standard deviation |

| | |
|---|---|
| $t_{1/2}$ | Half life |
| $C_{max}$ | Maximum plasma concentration |
| $T_{max}$ | Time to achieve maximum plasma concentration |
| AUC | Area Under Curve |
| F | Relative bioavailability |
| USP | United State Pharmacopoeia |
| UV | Ultraviolet |
| $\lambda_{max}$ | Maximum wavelength |
| P-gp | P -glycoprotein |
| ER | Estrogen receptor |
| ANOVA | Analysis of Variance |
| $R^2$ | Correlation Coefficient |
| PCS | Photon Correlation Spectroscopy |
| µg/ml | Microgram per milliliter |
| µl | Micro liter |
| mW | Milli watt |
| cm/sec | Centimeter per Second |
| µs/cm | Microsecond per centimeter |
| cp | Centi poise |
| $cm^2$ | Centimeter Square |
| mV | Milli volt |
| % v/v | Percent volume by volume |
| % w/w | Percent weight by weight |



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## 1. INTRODUCTION

The oral route of drug administration is the most important route for administering drugs for systemic effects. It has been widely used for both the conventional as well as novel drug delivery. Despite the extensive research and success stories with other routes for drug delivery, the oral route is still the most preferred route because of its basic functionality and advantages.

The advantages of oral route of administration are:

- Most natural way of drug administration
- Safe
- Convenient
- Economical
- Self medication is possible
- Absorption is selective as per need (e.g. iron in anemia)[1]

Major limitations of oral route for drug administration are:

- Having poor aqueous solubility
- Having Improper partition coefficient
- Having deterioration of drug molecule in GIT
- P-glyco protein efflux of some drugs
- High first pass metabolism is the chief contributing aspect liable for inactivation of the drug before it goes to the blood circulation.
- Drugs that are given orally are absorbed in the systemic circulation by either portal circulation or lymphatic transport.

Drug that are transported through portal circulation or susceptible to the enzymes present in liver undergo first pass metabolism. Drugs from BCS class II and IV that have low aqueous solubility, are particularly prone to first pass metabolism[2,3]. Therefore, such drugs are required to be given through some other routes like ocular, rectal etc. As a result, for effective delivery of drugs most promising approach is to improve oral bioavailability.

Enhancement in oral bioavailability can be achieved by reducing the first pass metabolism. This can be achieved by prodrug approach or by novel drug delivery carriers like lipid nanoparticles (lovastatin SLN), microemulsion (lymphatic transport of halofantrine)[4], SMEDDS (Neoral® cyclosporine product)[5]. Even

though the complete reasons for their improved absorption rate are still not known. The probable reasons for the bioavailability enhancement are as follows[3,4].

1. Improved dissolution / solubilization:-
   - Enhance gall bladder and pancreatic secretions that increase solubilization of drugs.
   - An exogenous surfactants facilitates the Solubilization.
2. Increasing in gastric residence time:-
   - Lipid in gastric tract provokes holdup in gastric emptying.
3. Stimulation of lymphatic transport because of triglyceride core of chylomicrons.
4. Enhancement in intestinal permeability:-
   - Lipid preparations will change into fine emulsion droplets.
   - Exogenous surfactants facilitate absorption.
   - Reduced metabolism.

### 1.1 OSTEOPOROSIS

Osteoporosis is a chronic, progressive condition associated with micro architectural deterioration of bone tissue that results in low bone mass. As the condition progresses, there is an increase in bone fragility and a consequent increase in fracture risk.

**Sources to cause osteoporosis:-**

The many reasons of osteoporosis are (1) Short of physical stress on the bones because of immobility, (2) No production of protein matrix due to malnutrition, (3) deficiency of vitamin C that is require for the secretion of intercellular substances by all cells, like osteoid formation by the osteoblasts, (4) Shortage of estrogen secretion after menopause because it decrease the number and activity of osteoclasts and (5) Cushing's condition, since a huge quantities of glucocorticoids secreted in this condition cause decreased deposition of protein throughout the body and improve catabolism of protein and have the specific effect of reducing osteoblastic activity. Thus, many diseases of deficiency of protein metabolism can cause osteoporosis[6].

**Types of Osteoporosis:-**

Osteoporosis is a disease of bones that has emerged as an important metabolic bone disease globally. The disease is divided into two types, primary type I or type II and secondary osteoporosis. Type I osteoporosis is most frequent in women occur after

menopause. Primary type II (senile) osteoporosis developed at age 75 years or thereafter in both females and males with a 2:1 ratio. Secondary osteoporosis occurred at any age in equal proportion of men and women. This type of osteoporosis may be result of long-term use of some medications and the presence of susceptible medical troubles or disease conditions.

**Mechanism of Osteoporosis:-**

The underlying mechanism in all cases of osteoporosis is an disproportion between bone resorption and bone formation[7]. Bone resorption is condition in which osteoclasts break down bone and release the minerals that ends with moving of calcium from bone fluid to the blood. Hormonal factors stalwartly control the rate of bone resorption; Shortage of estrogen due to menopause, results in increases bone resorption that leads to low bone mass density, fragility and fractured too.

**Treatment of Osteoporosis:-**

- Pharmacological intervention:

1. Hormone replacement therapy (HRT): Estrogen, Androgens
2. Antiresorptive agents: Bisphosphonates ( Etidronate, Alendronate and Risedronate).
3. Selective estradiol receptor modulators (SERMs): Raloxifene
4. Bone- Forming agents: Fluoride
5. Vitamin D and Analogs
6. Miscellaneous: Thiazide Diuretics

- Non- Pharmacological intervention:

1. Hip protectors
2. Verbroplasty
3. Kyphoplasty

Early diagnosis and treatment of osteoporosis are the main steps to reduce fractures. Hormonal replacement therapy (HRT) had in the past been widely used in the prevention and treatment of postmenopausal osteoporosis[8]. The repletion of endogenous estrogen with the above therapy effectively prevents postmenopausal bone loss and reduces the risk of fragility fractures. While the benefit of HRT on the

prevention and treatment of postmenopausal osteoporosis and related fracture has been demonstrated, its effects on the health of other estrogen sensitive tissues such as the uterus and breast must be carefully considered. In many circumstances, estrogen induces proliferation of the uterine and breast tissues. In addition, postmenopausal women are at a higher risk for coronary heart disease (CHD) and breast cancer than their premenopausal counterparts, and the effects of HT may modify these risks[8,9].

**Selective Estrogen Receptor Modulators**

There is a need for an ideal estrogen, one that possesses the beneficial effects on the skeletal, cardiovascular, and central nervous systems with minimal to absent effects on the uterus and breast. On a clinical level, clinicians have become interestingly attracted to selective estrogen receptor modulators (SERMs) because these newer non-hormonal agents display both estrogen agonist and estrogen-antagonistic properties[10].

SERMs are compounds whose estrogenic activities are tissue-selective. The pharmocological goal of these drugs is to produce estrogenic actions in those tissues where these actions are beneficial (bone[11], brain, liver during post-menopausal hormone replacement) and to have either no activity or antagonist activity in tissues such as breast or endometrium where estrogenic actions (cellular proliferation) might be deleterious. So Selective Estrogen Receptor Modulator (SERM), a new form of antiresorptive therapy that reduces the risks of osteoporosis and also improves the cardiovascular profile in postmenopausal women[12].

**Mechanism of action of SERM:-**

It is believed that SERMs initially act via the estrogen receptor (ER). They are ligand inducible nuclear transcription factors. They are physiologically activated by steroids (eg, 17-b estradiol) and nonsteroidal compounds (eg, tamoxifen, raloxifene) [13]. There are two unique forms of the ER, α or β. Both forms are distributed throughout the body and bind with SERMs in differing affinities[14].In general, once the SERM crosses the cell membrane, it binds to the estrogen receptor (either α or β). The estrogen receptor next undergoes dimerization & further conformational change. In the dimerization process, the receptor may join either with a similar type of receptor or a completely different one. This receptor complex is subsequently acted upon by cellular proteins, which either enhance or inhibit the estrogen-ligand complex's

activity upon DNA transcription. Unlike estrogen- bound estrogen receptors, SERM-bound receptors do not activate the estrogen-responsive gene transcription via the estrogen response element.

**Raloxifene Hydrochloride (RLX)**

Raloxifene is a nonsteroidal, second generation SERM being developed by Eli Lilly and company primarily as an oral therapeutic agent for postmenopausal osteoporosis. Although raloxifene has been referred to as an "antiestrogen," a designation that includes tamoxifen and clomifene, the chemical structure of raloxifene is not closely related to these triphenylethylene analogs but is a benzothiophene derivative. Raloxifene is a poorly soluble but well absorbed BCS Class II drug (approximately 60% of the Evista® oral dose is absorbed) having an absolute bioavailability in humans of approximately 2%.

Many different types of SERMs have undergone large clinical trials. Among them, raloxifene may be the most attractive agent, because at least three large trials, including the Multiple Outcomes of Raloxifene Evaluation (MORE) [15–17], Continuing Outcomes Relevant to Evista (CORE) [18,19], and Raloxifene Use for the Heart (RUTH)[20], have shown practical and promising results when using raloxifene for the prevention and management of postmenopausal women with osteoporosis or osteopenia. In these trials, raloxifene not only decreased the incidence of osteoporosis-associated complications, such as vertebral fractures and possible non-vertebral fractures, but also offered benefits for breast cancer prevention, with a dramatic decrease in the incidence of all breast cancers[21].

## 1.2 TECHNIQUES TO IMPROVE BIOAVAILABILITY

**Bioavailability and its significance [22]**

The term bioavailability is defined as the rate and extent at which drug is available to systemic circulation from the dosage form. From the definition, it is clear that when drug is given by IV route then its bioavailability will be of 100%. However, some other route like oral shows decrement in bioavailability due many reasons. Bioavailability is one of the important tools in pharmacokinetics, so it must be carefully measured when calculating dosage for non-intravenous routes of administration.

The approaches to enhance oral bioavailability of the drugs are mentioned as follows:[22]

**A. Improvement of solubility followed by dissolution rate:**

Various approaches are available to enhance the solubility of poorly water soluble drugs. Some of the approaches for improvement of solubility are mentioned as below.

**1. Physical modification**

A. Particle size reduction
   a. Micronization
   b. Nanosuspension

B. Modification of the crystal habit

C. Drug dispersion in carriers
   a. Solid dispersion
   b. Eutectic mixtures
   c. Solid solution

D. Complexation

E. Solubilization by surfactants
   a. Micro emulsions

**2. Chemical modification**

**3. Other techniques**
   a. Co-crystallization
   b. Co-solvency
   c. Hydrotrophy

**B. Bypass of hepatic first pass metabolism**

1. Prodrugs to decrease presystemic metabolism
2. Concept of new drug carrier such as microemulsion, SMEDDS, nanoparticles

**1.2.1 Solubility enhancement by complexation technique:**

Complexation is the association is the association between two or more molecules to form a no bonded entity with a well-defined stoichiometry. The two types of complexation that are useful for increasing the solubility of drugs in aqueous media are stacking and inclusion. Stacking complexes are formed by the overlap of planar regions of aromatic molecules, while inclusion complexes are formed by the insertion of the non polar part of one molecule into the cavity of another molecule. Some

compounds that are known to form stretching complexes are as follows: Nicotinamide, Anthracene, etc.

An inclusion complex is a unique form of a chemical complex, in which one molecule, "the guest", is enclosed within another molecular structure, "the host". Covalent or ionic bonds are not necessary for the inclusion process between the host and guest molecules. Only weak van waals force or hydrogen bonds may play a role. Instead of the ordinary chemical bonds or the interaction between functional groups, the following requirements are of importance in inclusion compound formation: 1) The host molecule should be able to form void spaces of a certain molecular dimension. 2) The guest molecule has to fit or at least partially fit into these void spaces. 3) The stereo chemical structure and polarity of guest and host molecules may be important. Although the guest is not chemically modified by inclusion, the host molecule dominates its physico-chemical properties.

Cyclodextrins are mainly used to increase the aqueous solubility and dissolution rate of drugs. Cyclodextrins (CDs) are offering new hope of formulation scientists to develop an effective drug delivery system, with their ability to form inclusion complexes with substances, without affecting their pharmacological properties. Cyclodextrins and their derivatives have been employed as complexing agents to increase water solubility, dissolution rate and bioavailability of lipophilic drugs for oral or parenteral delivery.

**Approaches for making of inclusion complexes[23]**

1. Physical blending method
2. Kneading method
3. Co-precipitation technique
4. Solution/solvent evaporation method
5. Neutralization precipitation method
6. Milling/Co-grinding technique
7. Atomization/Spray drying method
8. Lyophilization/ Freeze drying technique
9. Microwave irradiation method
10. Supercritical antisolvent technique

### 1.2.2 Solubility enhancement by avoidance of hepatic first pass metabolism:[22]

**Use of Novel Drug carriers such as microemulsion and Nanoparticles:-**

To bypass first pass metabolism and improve oral bioavailability, use of lymphatic circulation is gaining more importance in current decade. The drugs with more lipophilicity, poor aqueous solubility and less oral bioavailability are suitable candidate for lymphatic targeting. These drugs could be efficiently transported by the intestinal lymphatics through thoracic lymph duct to the systemic circulation. This will bypass presystemic hepatic metabolism with improvement in the concentration of orally administered drugs in the systemic circulation. At the capillary level, the intercellular junctions between endothelial cells of lymphatic capillaries are more open compared with blood capillaries that results in molecular sieving of colloidal particles of large size directly into lymphatics, avoiding the blood capillaries[24]. Success of lymphatic targeting can be achieved by lipid-based carrier systems such as microemulsion, SMEDDS and solid lipid nanoparticles (SLNs).

#### 1.2.2.1 Microemulsion

Microemulsions are defined as single optically isotropic, thermodynamically stable system made up of oil, water and surfactant/cosurfactant. The globule size in a microemulsion is in the range of 1-100 nm in diameter. In microemulsions, dispersed phase have the diameter less than one-fourth ($1/4^{th}$) the wavelength of visible light (<120 nm). Therefore, they do not refract light and that is how microemulsions appear transparent to the naked eye. The crucial difference between emulsions and microemulsions are that emulsions show exceptional kinetic stability but they are thermodynamically unstable compared to microemulsion systems. Hoar & Schulman have developed the concept of microemulsion in the 1943. They have prepared clear single phase solution by titrating a milky emulsion with hexanol. They have introduced the term microemulsion for this system in 1959[25]. In current decade, microemulsions have shown sincere attention because of their biodegradability and compatibility, easiness in handling & preparation and having very good solubilization ability for both categories of drugs (lipophilic and hydrophilic drugs).

The rationale for developing and using medicated microemulsions is listed in table 1.1[26].

Table 1.1: Importance for development of medicated microemulsions

| Reason | Drug Examples |
|---|---|
| Improvement in solubility of poorly soluble compounds | Diazepam, Vitamin A, Vitamin E, Dexamethasone palmitate |
| Improvement in solubility of Hydro - lytically liable drugs | Lomustine, Physostigmine salicyalate |
| Improving patient compliance by decreasing pain, irritation or toxicity of intravenously administered compounds | Diazepam |
| To produce sustained availability of drugs | Barbiturates |
| Drug targeting delivery to different body organs | Cytotoxic drugs |

**Formulation of Microemulsions**

Microemulsions being optically clear and isotropic systems are difficult to prepare at some extent. Below mentioned components are required to manufacture the microemulsion:

a) Oil Phase: Esters of fatty acids, mineral or vegetable oils, Toluene etc, are extensively studied as oil part.

b) Aqueous phase: This phase may contain solution of some hydrophilic ingredients or some time buffers solutions may also tried for this purpose.

c) Primary surfactant: Ionic or nonionic surfactants are usually studied, but preferably non ionic surfactants are more utilized due to their good tolerability in application.

d) Co-surfactant: Co (secondary) - surfactants generally used were of short chain fatty alcohols (pentanol, benzyl alcohol). The main function of such surfactant is to maintain fluidity of bridge made between two phases namely the dispersed phase and dispersion medium.

**1.2.2.2 Nanostructured lipid carriers (NLCs)**

A promising approach to overcome failure of the conventional delivery systems is the development of feasible drug-delivery systems. During the past decades, some strategies have been studied to develop nano sized drug-carrier systems[27]. These

systems are basically divided into two groups: polymeric nanoparticles and lipid nanoparticles. Polymeric nano systems are solid colloidal particles consisting of non-biodegradable synthetic polymers or biodegradable macromolecular materials from synthetic, semi synthetic or natural resources. The drawbacks of polymeric nanoparticles are the cytotoxicity of polymers and the lack of suitable large-scale production techniques. Owing to the natural and biological origins of the materials, the toxicological risk associated with lipid nanoparticles is much less than the risk associated with polymeric nanoparticles. Lipid nanoparticles made with a solid matrix (solid lipid nanoparticles, SLNs) are derived from oil-in-water nanoemulsions formed by replacing liquid oil with a solid lipid.

Generally, drugs are incorporated between the fatty acid chains or in between lipid layers or in amorphous clusters in crystal imperfections within SLN matrix. However, SLNs prepared from one highly purified lipid can crystallize in a perfect crystalline lattice that allows very small space for the incorporation of drugs (Figure 1.1a). Lipids crystallize in high energetic lipid modifications, α and β′, immediately after preparation of SLN. However, the lipid molecules undergo a time-dependent restructuring process leading to formation of the low-energetic modifications, βi and β, during storage. Formation of this perfect lipid crystalline structure leads to expulsion of drug[28]. Therefore, despite SLNs being interesting delivery systems, relatively low drug-loading capacity and potential expulsion of the drug during storage led scientists to think about new strategies. As a result, NLCs have been developed, which in some extent can avoid the aforementioned limitations. In case of NLCs, spatially very different lipid molecules are mixed to create a lipid particle matrix as imperfect as possible (Figure 1.1b). Generally, solid lipid (long chain) and liquid (oil) lipid (short chain) are mixed in ratio of 70:30 up to a ratio of 99.9:0.1 to produce NLCs. Resultant matrix of lipid particles shows a melting depression compared to original solid lipid, even though that are still solid at room temperature as well as at body temperature[29]. Due to many imperfections in NLCs, drug-loading capacity is enhanced and drug expulsion during storage is minimized.

From this all conclusion NLCs can be defined as a second generation of SLNs, which has solid matrix mixed with liquid lipid (oil) to form an unstructured matrix that helps in increasing drug loading capacity of nanoparticles and avoids or reduces drug expulsion from the matrix during storage.[30,31]

**Advantages**:-[30,31]

1. NLC dispersions with higher solid content can be produced.
2. Drug-loading capacity is better than SLNs.
3. Drug release profile can be easily modulated.
4. Drug leakage during storage is lower than SLNs.
5. Production of final dosage forms (e.g., tablets, capsules) is feasible

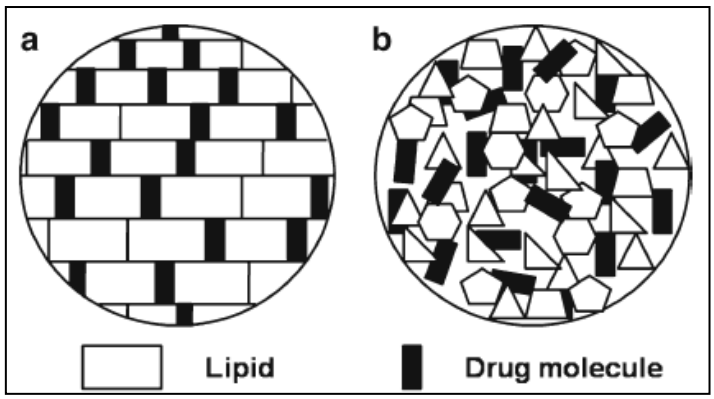


Figure 1.1:(a) Schematic structure of solid lipid nanoparticles and (b) Nanostructured lipid carriers

**Materials for NLCs**

The essential ingredients for NLCs include lipids, water and emulsifiers. Both solid and liquid lipids are included in NLCs for constructing the inner cores. These lipids are in a solid state at room temperature. They would melt at higher temperatures (e.g. > 80°C) during the preparation process. Liquid oils typically used for NLCs consist of digestible oils from natural sources. The medium chain triglycerides, such as Miglyol® 812, are often utilized as the constituents of liquid lipids because of their similar structures to Compritol®. Alternatively, the fatty acids, such as oleic acid, linoleic acid, and decanoic acid, are included in NLCs for their value as having oily components and as being penetration enhancers of topical delivery. In general, these lipids are already approved by European and American regulatory authorities for clinical applications and for their "generally recognized as safe" (GRAS) status. There is a need for novel and biocompatible oils that are cost-effective, non-irritating and capable of being sterilized before application. The emulsifiers have been used to stabilize the lipid dispersions. Most of the investigations employ hydrophilic emulsifiers, Lipophilic or amphiphilic emulsifiers such as Span 80 and lecithin for fabrication of NLCs if necessary.

**Formulation techniques**[32]

Various formulation techniques exist for the production of NLCs.

1. High-Pressure Homogenization
2. Emulsification-sonification
3. Microemulsion
4. Solvent emulsification-evaporation
5. Solvent diffusion
6. Solvent injection
7. Double emulsion

**1.3 REFERENCES**


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## 2. LITERATURE REVIEW

### 2.1 OSTEOPOROSIS AND SELECTIVE ESTROGEN RECEPTOR MODULATORS:

- **Ashajyoti V et al**, had reviewed that osteoporosis is brittleness of the bone due to low bone mineral density which changes the quality of life in patents. Osteoporosis is a foremost and growing public health problem for older women and men in western society. Bone is the major reservoir for the calcium and phosphate and is in constant state of remodelling during stress, many factor effects the bone resorption. Considering the physiology, pathophysiology and treatment of osteoporosis will direct the patients about precautions to be taken, to select the correct treatment regimen and to improve the quality of patient's life. The beneficial effects of treatments can be assessed by the outcome study using quality of life assessment tools[1].
- **Lopez FJ** had studied that maintenance of skeletal mass is the result of a tightly coupled process of bone formation and bone resorption under physiological conditions. Disease may arise when this delicate balance is disrupted such as in menopause, when estrogen levels decrease dramatically corresponding with the cessation of ovarian function. Current therapies for the treatment of osteoporosis, including estrogen replacement therapy, selective estrogen receptor modulators and bisphosphonates, are primarily based on blunting the resorption component of bone homeostasis. Although selective estrogen receptor modulators offer bone protection without the side effects of estrogen replacement therapy, there are some areas of improvement for the current generation of selective estrogen receptor modulators; particularly in reducing their antagonistic properties in the central nervous system that lead to vasomotor symptoms[2].
- **Hayashi T et al,** had studied the effects of selective estrogen receptor modulator treatment following hormone replacement therapy on elderly postmenopausal women with osteoporosis. A randomized prospective controlled trial was performed in a cohort of 32 elderly Japanese women with osteoporosis receiving HRT (estriol plus medroxy progesterone) for more than 1 year. SERM (Raloxifene) increased high-density-lipoprotein cholesterol levels and tended to decrease low-density-lipoprotein cholesterol levels ($p$ = 0.058) compared with baseline. Bone-mineral density was higher at baseline in the raloxifene and HRT groups than in the control group, and BMD increased 12 months after baseline in

the HRT and control group, SERM improved BMD and endothelial function in elderly postmenopausal women with osteoporosis who had received HRT, and these effects were comparable to or slightly stronger than those of HRT[3].

- **Delmas PD** had viewed the clinical interest in SERMs which is linked to the limitations of hormone replacement therapy (HRT). Long-term HRT is associated with an increase in bone mineral density and a decrease in skeletal fragility and with an improvement of the plasma lipoprotein profile that may result in a reduced incidence of coronary heart disease, although this is still controversial. The long-term compliance to HRT, however, is limited by side effects such as uterine bleeding and by the fear of breast cancer, the risk of which appears to increase after prolonged treatment. A SERM that would have an estrogen agonist activity on the skeleton and the cardiovascular system, without having some of the undesirable estrogen actions on tissues such as the endometrium and the breast, would represent a major advance for the management of postmenopausal women. There are several synthetic compounds, most of them nonsteroidal, that have both estrogen agonist and estrogen antagonist activities *in vitro* and in animal models. They can be classified as triphenylethylenes (tamoxifen, toremifene, droloxifene, and idoxifene), chroman (levormeloxifene) benzothiophenes (raloxifene), and others[4].
- **Albertazzi P and Purdie DW** had studied the current roles of oestrogen and selective oestrogen receptor modulators (SERMs) in the prevention and treatment of osteoporosis. Post-menopausal oestrogen replacement therapy (HRT) is the agent of choice for restraint of post-menopausal bone loss. In numerous observational, and in a few intervention studies, it has been shown to reduce osteoporotic fractures. Fear of breast cancer and refusal to tolerate resumption of cyclic withdrawal bleeding are the principal objections in HRT. Newer and more user-friendly forms of treatment are needed. Ideally, these should have an efficacy similar to oestrogen on the central nervous system, cardiovascular system and bone, without adverse effects on the breast and uterus. With this in mind, Selective oestrogen Receptor Modulators (SERMs) have been developed for the treatment of osteoporosis[5].

- **Lee WN et al**, had studied that among all SERMs, raloxifene may be the most attractive agent based on the evidence from five recent large trials (Multiple Outcomes of Raloxifene Evaluation [MORE], Continuing Outcomes Relevant to Evista [CORE], Raloxifene Use for the Heart [RUTH], Study of Tamoxifen and Raloxifene [STAR], and Evista Versus Alendronate [EVA]). The former three trials showed that raloxifene not only decreases the incidence of osteoporosis-associated fractures, but also has efficacy in breast cancer prevention. The head-to-head comparison with the anti-fracture agent alendronate (EVA trial) and the chemoprevention agent tamoxifen (STAR trial) further confirmed that raloxifene is a better choice[6].

**2.2 ENHANCEMENT OF DISSOLUTION RATE AND BIOAVAILABILITY:**

- **Yadav VB and Yadav AV** had reviewed the absorption rate of a poorly water-soluble drug (BCS class II), from the orally administered solid dosage form which is controlled by its dissolution rate in the fluid present at the absorption site. In the present work the BCS class II drug substances were formulated into granules by slugging and liquisolid compaction technique. The granules prepared by liquisolid compaction technique shows improvement in solubility, dissolution, wettability and other physicochemical properties comparative to granules by compaction (slugging) technique and raw crystals of drug substances. In conclusion, the results of this work suggest that liquisolid technique is a useful technique to enhance the solubility and dissolution rate of poorly water-soluble[7].
- **Thakkar H et al,** had reviewed various techniques for oral bioavailability enhancement of drugs. One approach to improve the systemic availability of the drug is to deliver it by alternative routes of administration such as parenteral, nasal, vaginal, rectal or transdermal. However, improvement of the oral bioavailability of the drug is the most realistic approach, as it is the most preferred and convenient route of administration. There are various techniques available to improve the solubility of poorly soluble drugs are 1) Physical modifications (Particle size reduction, solid dispersions etc) 2) Chemical Modifications (Salt formation, Change in pH of system etc) 3) Formulation Based Approaches (Co-solvency, Addition of solubilizers etc). The positive results obtained with the use of various delivery systems or different approaches of bioavailability enhancement seem to be promising[8].

- **Devalapally H et al,** had reviewed the role of nanotechnology in the pharmaceutical product development. In this review light has been thrown on optimization of the preformulation studies to develop a dosage form with proper drug delivery system to achieve desirable pharmacokinetic and toxicological properties can aid in the accelerated development of molecular entities into therapies. Nanoparticulate drug delivery systems show a promising approach to obtain desirable drug like properties by altering the biopharmaceutics and pharmacokinetics properties of the molecules.[9]
- **Shree NV et al,** had studied the role of nanoparticles for the treatment of osteoporosis. Although there is no cure for osteoporosis, a broad range of therapies have been approved by the Food and Drug Administration for postmenopausal women to prevent or treat osteoporosis by counter medications such as calcium and vitamin D, estrogen therapy, and newer medications such as "Antiresorptive agents" (i.e., Selective Estrogen-receptor modulators) and Anabolic therapy. As drug treatments have benefits as well as they also have possible side effects and risks. In the place of various drug therapies scientists as well as doctors prefer the patients to undergo nanoparticles treatment for osteoporosis. There are several experimental evidences given by researchers for treatment of osteoporosis by nanoparticles. This review summarizes about osteoporosis and the use of nanoparticles in treating osteoporosis[10].
- **Shakeel F et al,** had studied improvement of solubility and dissolution of the lipophilic drug (Aceclofenac) using three nanocarriers namely nanoemulsion, solid lipid nanosuspension and polymeric nanosuspension. The results of solubility and dissolution were highly significant in nanoemulsion as compared to lipid and polymeric nanosuspension ($P<0.01$). These results indicated that nanoemulsion is a promising nanocarrier as compared to lipid and polymeric nanosuspension for solubility and dissolution enhancement of aceclofenac[11].

## 2.3 INCLUSION COMPLEX (IC)

- **Patil JS et al,** had studied the inclusion complex system to improve the solubility and bioavailability of poorly soluble drugs. Solid dispersion, solvent deposition, micronization are some vital approaches routinely employed to enhance the solubility of poorly water soluble drugs. Among all, complexation technique has been employed more precisely to improve the aqueous solubility, dissolution rate, and bioavailability of poorly water soluble drugs. Cyclodextrins, the unique cyclic carbohydrates are successfully utilized as the potential complexing agents who form inclusion complex with insoluble drugs. A comprehensive literature survey was made collect the rightful utilization of cyclodextrins as complexing agents and permeation enhancers.[12]
- **Patil PH et al,** have carried out two methods to enhance solubility and dissolution of practically insoluble drug raloxifene HCL. In first method drug was kneaded with hydroxypropyl-β-cyclodextrin and in second method drug was co-grinded with modified guar gum. The solubility and dissolution study reveals that solubility and dissolution rate of raloxifene remarkably increased in both methods. In co-grinding mixture, a natural modified gum is use as surfactant and enhances the solubility and dissolution of RLX without requiring addition of organic solvent or high temperature for its preparation, thus process is less cumbersome and cost effective. But when both methods were compared hydroxypropyl-β-cyclodextrin complexation method showed significant enhancement of drug solubility[13].
- **Dhanaraju MD et al,** have formulated and evaluated solid dispersion of water insoluble drug raloxifene with improved bioavailability for the treatment of osteoporosis in postmenopausal women. Ten batches of RLX formulation were conducted to select prototype formula, batch S0010 was selected as optimized formulation which showed drastically increase in solubility in poorly soluble RLX. No substantial changes in its stability were observed[14].
- **Rangasamy M et al,** have prepared complexation of meloxicam with β-cyclodextrin. It was an attempted to enhance the solubility features of the drug. This work investigated the possibility of developing meloxicam tablets, allowing to get fast reproducible and complete drug disintegration by using β-cyclodextrin complexation. Stability study for the selected formulations exhibited no alteration in the physical appearance or content of the tablet. The complex prepared by

direct compression method was found to be yield full, very reliable and giving best results over that of the wet granulation method[15].

- **Tayade PT et al,** have studied the oral pharmacokinetics of ketoprofen in humans, following cyclodextrin complexation. The initial dissolution rate of ketoprofen in the inclusion complexes was 15 fold higher, than that of plain drug powder. The Cmax of ketoprofen followed by oral administration of inclusion complexes to human volunteers enhanced about 1.5 fold and there was no significant increase in area under concentration-time curve, $AUC_{0-5}$ compared to plain ketoprofen drug[16].
- **Uekama K et al,** have classified cyclodextrins (CyDs) into hydrophilic, hydrophobic, and ionic derivatives. This review provides information about the current application of CyDs in development of CyD based drug formulation, focusing on their ability to enhance the drug absorption across biological barriers, to control the rate and time profiles of drug release, and the ability to deliver a drug to a targeted site[17].
- **Singh R et al,** have studied that molecular structure of cyclodextrins derivatives revealing a hydrophilic exterior surface and a non polar cavity interior. The characterization of inclusion complexes was done with a purpose to determine the interaction of drug molecules with cyclodextrins which confirm the formation of inclusion complexes[18].
- **Rasheed A et al,** have studied applications of cyclodextrin (CD) and their derivatives in different areas of drug delivery. The objective of this contribution is to focus on the potential use of chemically modified cyclodextrins as high-performance drug carriers in drug delivery systems with emphasis on the more recent developments[19].
- **Sapkal NP et al,** have studied the effect of various parameters on the efficiency of Gliclazide inclusion complexes with β- cyclodextrin (β-CD) in solution and in solid state. The study was carried out to determine a suitable method for scaling up gliclazide-β-CD inclusion complex formation. Two methods namely kneading and co-precipitation methods in 1:2 molar ratios were found to be equally efficient in enhancing the drug solubility. The formation of inclusion complexes was evident in these formulations as shown by IR and XRD studies[20].
- **Venkatesh DN et al,** have studied that the influence of Aceclofenac/β-cyclodextrin (Ace/β-CD) complex on the solubility and dissolution rate of this

poorly soluble drug aceclofenac. Phase-solubility profile indicated that the solubility of aceclofenac was significantly increased in the presence of β-CD. Solid state characterization of the drug showed amorphous nature of drug in the formulation that leads to enhancement in dissolution rate[21].

- **Rao SK et al,** have attempted to prepare and characterize inclusion complexes of Glimepiride (GMP) with β-CD and HP-β-CD. The inclusion complex prepared with HP-β-CD by Kneading method exhibited greatest enhancement in solubility and fastest dissolution (97.41% GMP release in 60 min) of GMP[22].
- **Shiralashetti S et al,** have evaluated the effect of preparation methods on the solubility and dissolution of Simvastatin (SV)-β-cyclodextrin (β-CD) and hydroxypropyl β-cyclodextrin (HPβ-CD) inclusion complexes. Percent recovery of complexes in the kneading method was found to be 98.76% as compared to 92.05% in case of co-precipitation method[23].
- **Ventura CA et al,** have prepared the complex of 2,6-di-O-methyl-beta-cyclodextrin and the anti-inflammatory drug celecoxib (CCB) by kneading and freeze-drying methods. Water solubility and dissolution rate of CCB in a medium simulating gastric fluid, significantly increased after complexation. The increase of permeation observed was due to the fast dissolution rate of the included drug and to a destabilizing action exerted by the macrocycle on the biomembrane[24].
- **Sigurdsson HH et al,** have formulated the dorzolamide hydrochloride as 2% and 4% low viscosity solutions containing randomly methylated beta-cyclodextrin at pH 7.45. These formulations were studied in rabbits after topical administration. The topical availability of dorzolamide from the cyclodextrin containing eye drops appeared to be comparable to that from Trusopt (reference product) [25].
- **Newa J et al,** have prepared ibuprofen solid dispersions (SDs) in a relatively easy and simple manner. Quicker release of ibuprofen from SDs in rat intestine resulted in a significant increase in AUC and $_{Cmax}$, and a significant decrease in $_{Tmax}$ over pure ibuprofen. Preliminary results from this study suggested that the preparation of ibuprofen SDs could be a promising approach to improve solubility, dissolution, and absorption rate of ibuprofen[26].
- **Choi P et al,** have developed inclusion complex of nitrendipine with hydroxypropyl-β-cyclodextrin (HP-β-CD). The solubility of nitrendipine increased linearly as a function of HP-β-CD concentration. The *in vitro* dissolution rate of nitrendipine in pH 5.5 phosphate buffer was high in the order of

inclusion complex, physical mixture, and nitrendipine plain drug. The results indicated that the bioavailability of nitrendipine could be improved markedly by inclusion complexation[27].

- **Iacovino R et al,** had prepared inclusion complex of hydroxymethyl ferrocene (FeMeOH) with β-CD by different techniques such as physical mixture, co precipitation, kneading and freeze drying. The cytotoxic activity of FeMeOH ferrocene and its complexation product with β-CD was determined using the MTT assay on MDA-MB-231cell lines, showing that the inclusion complex has higher capability of inhibiting cell growth compared to that of pure FeMeOH[28].
- **Ghodke DS et al,** have been attempted to formulate inclusion complexes using methylated β-cyclodextrin. Kneading, ultrasonification and physical mixture method were used for preparation of inclusion complexes. The solubility and dissolution results revealed that there was a considerable increase in solubility and dissolution of all inclusion complexes as compared to pure drug. It was highest in case of methylated β-cyclodextrin in 1:1 molar ratio using ultrasonification method (USM1) [29].
- **Figueiras A et al,** have examined that the cyclodextrins, as a tool to form an inclusion complex with omeprazole (OME), a poorly water soluble drug. Solid binary systems between OME and MbCD were prepared experimentally in a stoichiometry 1:1 by different techniques (physical mixing, kneading, spray-drying and freeze-drying). The results obtained suggest that spray-drying and freeze-drying methods yield a higher degree of amorphous entities suggesting the formation of inclusion complexes between OME and MbCD[30].

## 2.4 MICROEMULSIONS (ME)

- **Cai-Xia He et al,** had studied the microemulsions extensively as potential drug delivery vehicles for poorly water-soluble drugs. An understanding of the physicochemical and biopharmaceutical characteristics of the microemulsions according to administration routes will provide guidance for designing the formulations of microemulsions. As the formulations of the microemulsion always include a great amount of surfactant and co-surfactant, they change the partition coefficient of poorly soluble drug and so solubility of same. Considering the potential in enhanced drug uptake/permeation and facing the limitations, their unique properties make microemulsions a promising vehicle for poorly water-soluble drugs[31].

- **Chaudhari et al**, had developed Raloxifene hydrochloride microemulsion by using Capryol 90 (oil), labrasol (surfactant) and ethanol (cosurfactant), which were selected based on maximum solubility of RLX. The result showed that microemulsion formulation of the RLX has higher bioavailability compared to the pure drug. Therefore it can be concluded that microemulsion may play vital role in improving bioavailability of poorly soluble drug [32].
- **Thakkar et al**, had prepared microemulsion and Self-Microemulsifying Drug Delivery System (SMEDDS) formulations of Raloxifene. Lipid-based formulations are reported to reduce the first-pass metabolism by promoting its lymphatic uptake. The results indicated that high drug loading, optimum size and desired zeta potential and transparency could be achieved with both SMEDDS and microemulsion. The *in vitro* intestinal permeability results showed that the permeation of the drug from the microemulsion and SMEDDs was remarkably more compared to drug dispersion and marketed product. From this study, It is confirmed that the oral bioavailability might be significantly enhanced by formulating microemulsion and SMEDDS[33].
- **Balakrishnan et al,** had tried to enhance the solubility and bioavailability of poorly water -soluble Co enzyme Q10 (CoQ10). The pharmacokinetic study in rats for the optimized formulation was performed and compared to powder formulation. SMEDDS have significantly increased the Cmax and area under the curve (AUC) of CoQ10 compared to powder ($p< 0.05$). Thus, this self-micro emulsifying drug delivery system should be an effective oral dosage form for improving oral bioavailability of lipophilic drug, CoQ10 [34].
- **Cheng et al,** were formulated W/O microemulsions by using Labrafac CC, Labrasol, Plurol Oleique CC 497 and saline (54/18/18/10%w/w). The w/o microemulsion showed a higher intestinal membrane permeability *in vitro* with more absorption and efficacy *in vivo* compared to control. The intra duodenal bioavailability of EFE-d for microemulsions was 208-fold more compared to control. Results shows that the w/o microemulsion may provide successful oral delivery system for hydrophilic bioactivity macromolecules[35].
- **Wu et al,** had studied SMEDDS of curcumin by using pseudo ternary phase diagrams. Solubility test showed that the formulation of SMEDDS composed of 20% ethanol, 60% Cremophor RH40, and 20% isopropyl myristate, in which the concentration of curcumin reached 50 mg/ml. Curcumin was released

completely from SMEDDS at 10 minutes. The developed SMEDDS formulation improved the oral bioavailability of curcumin significantly, and the relative oral bioavailability of SMEDDS compared with curcumin suspension was 12-13% [36].

- **Sarkar et al,** have developed microemulsion formulation containing Glipizide using capmul MCM (6.5%), cremophor EL (25%), transcutol P (7.5%) and distilled water for oral drug delivery. The optimized batch is showing higher *in vitro* drug release compared to plain drug suspension and the marketed drug. These results demonstrate the potential use of ME for improving the bioavailability of poor water soluble compounds [37].
- **Hwang et al,** had prepare parenteral phospholipids based microemulsion by using All- trans- retinoic acid and they have concluded that ME formulation of All-trans-retinoic acid showed improved solubility of All-trans-retinoic acid while maintaining its pharmacokinetic profile and anticancer efficacy [38].
- **Osmond et al,** had studied microemulsion gel formulation to improve the bioavailability through the vaginal/rectal mucosa of microbicidal drug substances against sexually transmitted diseases. From the result they have proved that spermicidal Gel-Mes have potential as dual function microbicidal contraceptives to improve vaginal bioavailability of poorly soluble antimicrobial agents without causing significant vaginal damage [39].
- **Solanki et al,** had made attempt to enhance the dissolution rate and bioavailability of Ampelopsin drug by developing a novel delivery system that is microemulsion (ME) and to study the effect of microemulsion (ME) on the oral bioavailability of ampelopsin. The optimized ME formulation showed higher *in vitro* drug release compared to plain drug suspension and the suspension of commercially available tablet. These results demonstrate the potential use of ME for improving the bioavailability of poor water soluble compounds, such as ampelopsin[40].
- **Patel et al,** were designed and developed a microemulsion formulation of clopidogrel for enhancing its solubility, and hence its oral bioavailability. Solubility of clopidogrel was successfully enhanced by 80.66 times compared with distilled water (pH = 7.4). 75.53% and 71.2 % of the drug content were found to be released within 9 h in the *in vitro* and *ex vivo* studies, respectively. Hence, by formulating into microemulsion, the solubility of clopidogrel was found to be significantly enhanced [41].

- **Piaoa et al,** aimed to enhance the solubility and bioavailability of poorly absorbable fexofenadine by preparing microemulsion system for intranasal delivery. The optimized microemulsion formulations showed higher solubilization of fexofenadine as compared to its intrinsic water solubility. These results suggested that microemulsion formulations could be used as an effective intranasal dosage form for the rapid-onset delivery of fexofenadine [42].
- **Nandi et al,** have evaluated the effect of alkanols and cyclodextrins on the phase behavior of an isopropyl myristate microemulsion system and to examine the solubility of model drugs. Triangular phase diagrams were developed for the microemulsion systems using the water titration method, and the solubility values of progesterone and indomethacin were determined using a conventional shake-flask method. In conclusion, microemulsion systems improve the solubility of pro-gesterone and indomethacin[43].
- **Ho et al,** have formulated an oral formulation of ciclosporin with more consistent intestinal absorption. In this prospective, open study, ciclosporin microemulsion was used to manage adolescent and adult patients with severe refractory atopic dermatitis. Ciclosporin microemulsion was found to be fast acting and effective in patients: disease severity score decreased by 63%; extent of disease score decreased by 46%; pruritus score decreased by 77%. It was concluded that ciclosporin microemulsion used as short course treatment was an effective therapy for severe refractory atopic dermatitis in adolescents and adults with acceptable side effects [44].
- **Anjali et al,** have investigated the anti-bacterial activity of refined sunflower oil, Tween 80, water micremulsion system. Kinetics studies showed inhibition of bacterial growth in all formulated microemulsions. Bacterial growth was enhanced in case of oil and surfactant alone[45].
- **Malakar et al,** have developed microemulsion using isopropyl myristate or oleic acid as the oil phase, Tween 80 as the surfactant, and isopropyl alcohol as the cosurfactant. The insulin-loaded microemulsion containing 10% oleic acid, 38% aqueous phase, and 50% surfactant phase with 2% dimethyl sulfoxide (DMSO) as permeation enhancer showed maximum permeation flux (4.93±0.12µg/cm2/hour) through goat skin. The preliminary data indicate the microemulsionas a promising formulation for transdermal delivery of insulin[46].

## 2.5 NANOSTRUCTURED LIPID CARRIERS (NLCs)

- **Kamble M S et al,** have given a review on Solid lipid nanoparticles (SLN) which is alternative system to emulsion, liposomes and polymeric microparticulate systems. This novel colloidal drug carrier system gained a lot of popularity among researcher due to its applicability for various routes such as oral, topical and parenteral. Also the properties of site specific and controlled drug delivery with reduced side effects. Along with their advantages, some challenges such as low drug loading and drug expulsion from SLN during storage were needed to be addressed. These limitations were overcome in Nanostructured lipid carriers (NLC), which are second generation SLN. NLC accommodate the drug because of their highly unordered lipid structures. NLC can be administered via oral, ocular, pulmonary and intravenous routes. The present reviews the types of NLC, preparation methods and characterization of SLN and NLC[47].
- **Fu-Qiang Hu et al,** had employed solvent diffusion method to produce NLC of Clobetasol propionate. As a result, monostearin solid lipid nanoparticles (without CT content, SLN) obtained at higher temperature (70◦C) exhibited slightly higher drug loading capacity than that of 0◦C ($p< 0.05$). The improved drug loading capacity was observed for NLC and it enhanced with increasing the CT content in NLC. NLC displayed a good ability to reduce the drug expulsion in storage compared to SLN. The *in vitro* release behaviors of NLC were dependent on the production temperature and CT content. NLC obtained at 70 ◦C exhibited biphasic drug release pattern with burst release at the initial 8 h and prolonged release afterwards, whereas NLC obtained at 0◦C showed basically sustained drug release throughout the release time. The drug release rates were increased with increasing the CT content. These results indicated that the NLC produced by solvent diffusion method could potentially be exploited as a carrier with improved drug loading capacity and controlled drug release[48].
- **Hejri et al,** have prepared β-carotene loaded nanostructured lipid carriers using the solvent diffusion method. Response surface methodology was employed in conjunction with a central composite design to evaluate the effect of the preparation variables on particle size and β-carotene stability to optimize the NLC formulation. The statistical evaluations revealed that the lipid phase concentration and the surfactant concentration had significant effect on particle size of NLC. In addition, the influence of the liquid lipid to total lipid ratio and temperature on β-

carotene degradation was more important. The optimum formulations with minimum particle size (8–15 nm) and low β-carotene degradation (0–3%) were derived from the fitted models and were experimentally examined which demonstrated a reasonable agreement between experimental and predicted values. Transmission electron microscopy (TEM) observations exhibited spherical morphology of β-carotene loaded NLC[49].

- **Hu FQ et al,** have prepared stearic acid (SA) nanostructured lipid carriers with various oleic acid (OA) content by solvent diffusion method in an aqueous system. The size and surface morphology of nanoparticles were significantly influenced by OA content. As OA content increased up to 30 wt%, the obtained particles showed pronounced smaller size and more regular morphology in spherical shape with smooth surface. Compared with solid lipid nanoparticles, NLC exhibited improved drug loading capacity, and the drug loading capacity increased with increasing OA content. These results were explained by differential scanning calorimetry (DSC) investigations. The addition of OA to nanoparticles formulation resulted in massive crystal order disturbance and less ordered matrix of NLC, and hence, increased the drug loading capacity. The drug *in vitro* release behavior from NLC displayed biphasic drug release pattern with burst release at the initial stage and prolonged release afterwards, and the successful control of release rate at the initial stage can be achieved by controlling OA content[50].
- **Shete et al,** had formulated Tamoxifen citrate (Tmx) in nanostructured lipid carrier system using long chain solid lipids and oils with the aim to target lymphatic system to improve its bioavailability in plasma and lymph node (initial sites for metastasis) and reduce its drug associated toxicity. Tamoxifen loaded NLC (Tmx-NLC) was formulated using solvent diffusion technique. Suitable storage condition below 30◦C could stabilize Tmx. Tmx-NLC with >90% entrapment efficiency and 215.60 ± 7.98 nm particle size were prepared and freeze dried. Dissolution profile of Tmx-NLC in various media showed sustained release pattern irrespective of pH of medium. No significant change in characteristics of Tmx-NLC was observed after 3 months of accelerated stability studies[51].
- **Zhuang et al,** have developed a nanostructured lipid carriers formulation for vinpocetine (VIN), to estimate the potential of NLC as oral delivery system for poorly water soluble drug. In this work, VIN–loaded NLC was prepared by a high

pressure homogenization method. The VIN–NLC showed spherical morphology with smooth surface under transmission electron microscope and scanning electron microscopic analysis. The average encapsulation efficiency was 94.9±0.4%. The crystallization of drug in NLC was investigated by powder X-ray diffraction and differential scanning calorimetry. In the *in vitro* release study, VIN–NLC showed a sustained release profile of VIN and no obviously burst release was observed. The oral bioavailability study of VIN was carried out using Wistar rats. The relative bioavailability of VIN–NLC was 322% compared with VIN suspension. In conclusion, the NLC formulation remarkably improved the oral bioavailability of VIN and demonstrated a promising perspective for oral delivery of poorly water-soluble drugs[52].

- **Li et al,** had developed an ocular drug delivery system based on Ibuprofen loaded nanostructured lipid carrier and investigated its *in vitro* and *in vivo* characteristics. Four different formulations of Ibuprofen nanostructured lipid carriers were prepared by melted-ultrasonic methods. The corresponding apparent permeability coefficients were 1.28 and 1.36 times more than that of the control preparation. Ibuprofen nanostructured lipid carriers displayed controlled-release property. The AUC of the optimized formulation of Ibuprofen nanostructured lipid carriers was 3.99 times more than that of Ibuprofen eye drops[53].
- **Fang CL et al,** have given an overview on Nanostructured Lipid Carriers (NLCs) for drug delivery and targeting. NLCs are drug-delivery systems composed of both solid and liquid lipids as a core matrix. It was shown that NLCs reveal some advantages for drug therapy over conventional carriers, including increased solubility, the ability to enhance storage stability, improved permeability and bioavailability, reduced adverse effect, prolonged half-life, and tissue-targeted delivery. In this article, the potential of NLCs for different administration routes (Parenteral and topical) is highlighted along with relevant issues for the introduction of NLCs to market, including pharmaceutical and cosmetic applications, are also discussed[54].
- **Uner M** had studied the benefits of NLC as colloidal drug carrier system. Modified SLN have been described which are NLC composed of liquid lipid blended with a solid lipid to form a nanostructured solid particle matrix. NLC combine controlled release characteristics with some advantages over SLN. This paper reviews the production techniques, characterization and physical stability of

these systems including destabilizing factors and principles of drug loading, then considers aspects and benefits of SLN and NLC as colloidal drug carriers[55].

- **Das et al,** have studied comparative evaluation between SLNs and NLCs. In this study SLNs and NLCs were developed using clotrimazole as model drug. Critical process parameters exhibited significant impact on the nanoparticles' properties size, zeta potential and entrapment efficiency of the developed SLNs and NLCs were <100 nm, < -22mv and >82% respectively. NLCs showed faster drug release than SLNs at low drug loading. NLCs showed better stability than SLNs at 25°C. Therefore it was concluded that NLCs have an advantages over SLNs[56].
- **Hu FQ et al,** have developed solvent diffusion method to overcome the disadvantages such as lower drug entrapment efficiency (EE) of lipid nanospheres prepared by conventional solvent diffusion method. Nimodipine was used as a model drug to incorporate into lipid nanospheres. The EE could be enhanced by the incorporation of liquid lipid (caprylic/capric triglycerides, CT) into SLN and the employing of drug saturated dispersion medium. *In vitro* drug release experiments indicated the present preparation method could delay the drug release rate from lipid nanospheres, and the drug release rate could adjust by the CT content in lipid nanospheres. The highest drug loading was reached up to 4.22 wt% when 8 wt% drug was charged in the preparation of lipid nanospheres[57].
- **Jia et al,** have studied potential of NLCs for the intravenous delivery of silybin, a poorly water-soluble antihepatopathy agent. Silybin-NLC showed higher AUC (area under tissue concentration–time curve) values and circulated in the blood stream for a longer time compared with silybin solution. The tissue distribution demonstrated a high uptake of silybin-NLC in RES organs particularly in liver. These results indicate that NLC is a potential sustained release and targeting system for silybin[58].
- **Sanad et al,** have studied oxybenzone loaded NLCs to enhance its sun screening efficacy and safety. Miglyol 812 and 30% liquid lipid were found to significantly decrease the particle size and increase the EE% when compared to oleic acid and 15% liquid lipid. NLCs prepared using Miglyol 812, 15% liquid lipid, and 10% oxybenzone showed slower drug release when compared to those prepared using oleic acid, 30% liquid lipid, and 5% oxybenzone, respectively. The incorporation of oxybenzone into NLCs greatly increased the *in vitro* sun protection factor and erythemal UVA protection factor of oxybenzone more than six- and eightfold,

respectively, while providing the advantage of overcoming side effects of free oxybenzone as evidenced by very low irritation potential[59].

- **Poovi G. et al,** have prepared repaglinide loaded chitosan polymeric nanoparticles by solvent evaporation technique. The prepared nanoparticles showed high drug loading capacity (11.22%w/w), encapsulation efficiency (97.0%) and nanoparticle recovery (86.40%) with nanosize. Based on the *in vitro* study, repaglinide released from prepared formulation was slow and sustained over 15 days. Application of the *in vitro* drug release data to various kinetic equations revealed first order release, swelling and diffusion mechanism from repaglinide nanoparticles[60].
- **Abdullah R. et al,** had developed NLCs stabilized with polysorbate 20 and polysorbate 80 by high pressure homogenisation technique. Zeta potentialof $NLC_{80}$ showed a more stable formulation than $NLC_{20}$. These findings suggest that polysorbate 80 was a better dispersing agent for NLC than polysorbate 20. The small size and superior particle surface to volume ratio would increase loading efficiency and bioavailability of drugs, thus making NLC a promising drug delivery system[61].
- **Gupta J et al,** had formulated SLNs of nateglinide using hot homogenization technique. It was revealed that increase in concentration of lipid content has increased the entrapment efficiency of SLN. It was concluded that SLNs with small particle size, excellent physical stability, high entrapment efficiency, good loading capacity for diabetic drug can maintain blood glucose level normally for a long period of time[62].
- **Park JS et al,** had developed NLCs of tacrolimus by the hot homogenization method followed by sonication technique. The particle size, zeta potential and entrapment efficiency (EE) were found to be 123±0.3nm,-24.3±6.2mV and 50 % respectively. *In vitro* penetration studies indicated that the tacrolimus-loaded NLCs have a penetration rate that is 1.64 times that of the commercial tacrolimus ointment, Protopic®[63].
- **Pardeike J et al,** had prepared itraconazole loaded NLC by hot high pressure homogenisation technique. Burst release of itraconazole from the developed carrier system was found. Itraconazole-loaded NLC possessed good storage stability. Nebulizing itraconazole-loaded NLC with a jet stream and an ultrasonic nebulizer had no influence on the particle size and the entrapment efficiency of

itraconazole in the particle matrix, being a precondition for pulmonary application[64].

- **Das S et al,** had studied SLN and NLC formulations by different formulation techniques. Stability of the formulations might increase due to the solid matrix of these lipid nanoparticles. Scaling up of the production process from lab scale to industrial scale can be easily achieved. Reasonably high drug encapsulation efficiency of the nanoparticles was documented. Oral absorption and bioavailability of several drugs were improved after oral administration of the drug loaded SLNs or NLCs[65].
- **Gokce EH et al,** had formulated resveratrol (RSV) loaded SLN and NLC by high shear homogenisation technique using compritol 888ATO, Myglycol, poloxamer 188 and tween 80. Entrapment efficiency was 18% higher in NLC systems. *Ex vivo* studies revealed that NLC are more efficient in carrying RSV to the epidermis. In conculsion, when two studies are compared, NLC penetrated deeper into the skin. RSV loaded NLC with smaller particle size and higher drug loading capacity appears to be superior to SLN for dermal applications[66].

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## 3. NEED AND OBJECTIVES OF THE STUDY

**Need of the Study:**

Osteoporosis is a common and significant health problem affecting all over the world, of which more than 75% are postmenopausal women. Osteoporosis is characterized by a deterioration of the skeleton leading to higher incidence of bone fractures. Under physiological conditions, skeletal mass is maintained by tightly coupled process of bone formation and bone resorption. Osteoporosis may arise when this delicate balance is disrupted such as in menopause, when estrogen levels decrease dramatically corresponding with the cessation of ovarian function.

Current therapies for the treatment of osteoporosis, including estrogen replacement therapy, selective estrogen receptor modulators (SERMs) and bisphosphonates, are primarily based on blunting the resorption component of bone homeostasis.

SERMs are popularly used as first line therapy for prevention and treatment of postmenopausal osteoporosis. SERMs are a new class of drugs that show great potential for the treatment of osteoporosis over estrogen replacement therapy which contributes to a significant increase in breast cancer. These compounds provide an excellent therapeutic profile. SERMs behave as estrogen receptor agonists in bone and the cardiovascular system, yet they exhibit antagonistic properties in tissues such as the uterus and the breast.

Raloxifene is a second generation benzothiophene derivative of SERMs that has an improved product profile compared with that of first generation tamoxifen in prevention and treatment of postmenopausal osteoporosis in women. Raloxifene is a poorly soluble BCS Class II drug. Nearby 60% oral dose is absorbed, but pre-systemic metabolism is extensive that limits its oral bioavailability in humans of approximately 2%.

Raloxifene being BCS Class II drug, it has poorly solubility but having good permeability rate. Drug with low solubility and stability in physiological environment constitutes a main hurdle in attaining the appropriate bioavailability. BCS class II drugs require innovative approaches to reach a sufficiently high bioavailability when administered by oral route. Poorly water soluble drugs can exhibit a number of negative clinical effects including potentially serious issues of inter patient variability and subsequent erratic absorption following dosing to individual patients. So the need of present study is to enhance the oral bioavailability of SERM'S by utilizing various

techniques such as formation of water soluble complexes, solid dispersion, reduction of particle size, solubilization in surfactant systems or use of pro-drugs to increase the dissolution rate of the drug.

One of the other prime strategies adopted by a formulation scientist for resolving a solubility and bioavailability problem includes changing the dosing vehicle. Several polymer-and lipid based nanotechnologies are being intended in order to optimize the technological (e.g., solubility, stability, bioavailability, etc.) aspects of drugs. Among them, polymeric nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers, liposomes, niosomes, nanoemulsion, solid, polymeric nanosuspension and microemulsions are also reported as potential drug delivery vehicles for poorly water-soluble drugs.

Therefore, the purpose of the study is to adopt various strategies to improve the water solubility & there by bioavailability of BCS class II raloxifene HCl (SERM) to formulate suitable formulation for better treatment of osteoporosis.

**Objectives of study:**

- Formulation of stable dosage form by suitable techniques (Drug carrier inclusion complex, Drug loaded microemulsion and nanostructured lipid carriers) to enhance solubility as well as bioavailability of raloxifene HCl.
- Optimization, characterization and in vitro evaluation of prepared formulations.
- In vivo evaluation of various pharmacokinetic parameters for optimized formulations.

## 4. PLAN OF WORK

1. Selection of drug, polymer and other excipients.
2. Preformulation study.
   - ❖ Preliminary study of drug
     - ■ Organoleptic properties
     - ■ Melting point determination
     - ■ Solubility studies
   - ❖ Analytical characterization of the drug.
   - ❖ Characterization of the excipients.
   - ❖ Drug - excipients compatibility studies
     - ■ Fourier Transform Infrared (FTIR) spectroscopy
     - ■ Differential Scanning Calorimetry (DSC) study
     - ■ Physical properties observation
   - ❖ Other appropriate parameters
3. Formulation of suitable dosage forms using bioavailability improvement technique such as physical modification (Inclusion complex) or novel drug delivery systems (Microemulsions and NLCs)
   - ❖ Optimization of the formulation variables by factorial design.
4. Evaluation of formulations for various parameters
   - ■ Solubility study
   - ■ Drug content
   - ■ Percentage yield
   - ■ Entrapment efficiency
   - ■ *In vitro* release study
   - ■ Particle size and zeta potential evaluation
   - ■ Surface morphological evaluation
     (Scanning electron microscopy, Transmission electron microscopy)
   - ■ Crystallinity by X-ray diffraction study
   - ■ Other appropriate parameters
5. *In vivo* studies for evaluation of pharmacokinetic parameters of the optimized formulation batch
6. Stability study of the optimized batch of the prepared formulations.

# 5. MATERIALS AND METHODOLOGY

## 5.1 MATERIALS:

### 5.1.1 List of raw materials

Following drug and excipients were used for the preparation of inclusion complex, microemulsions and nanostructured lipid carriers.

**Table 5.1: List of raw materials used in research work**

| Ingredients | Name of manufacturer |
|---|---|
| Raloxifene Hydrochloride | Aarti drugs, Mumbai, India |
| β-cyclodextrin | Triveni Interchem Pvt Ltd, Vapi, India |
| Capmul MCM C8 | Abitec corporation, Janesvillae, USA |
| Capmul PG 8 | |
| Labrafil ICM 1944 CS | |
| Labrafec CC | |
| Captex 300 | |
| Acconon C | Colorcon India Pvt Ltd, Goa, India |
| Transcutol | |
| Tween 80 | Sulab Laboratories , Vadodara, India |
| Tween 20 | |
| Tween 80 | |
| Polyethylene glycol (PEG) 200 | |
| PEG 400 | |
| Propylene glycol | |
| Dynasan 114 | Cremer Oleo GmbH & Co. KG**,** Germany |
| Dynasan 118 | |
| Glyceryl monostearate (GMS) | Loba Chemie, Mumbai, India |
| Methanol | |
| Acetone | |
| Chloroform | |

### 5.1.2 List of instruments

**Table 5.2: Instruments used in research work**

| Sr. No. | Name of Instruments | Model and name of Manufacturer |
|---|---|---|
| 1 | Electronic balance | Ohaush corporation, Pine Brook, NJ,USA |
| 2 | Magnetic stirrer | Macro Scientific Work Pvt Ltd, Delhi |
| 3 | pH Meter | Systonic, Ahmedabad (S-901) |
| 4 | Brookefield rheometer | Brookefield DV III , USA (LVDV-III U) |
| 5 | Conductometer | Macro Scientific Work Pvt Ltd, Delhi (MSW-554(A)) |
| 6 | UV Spectrophotometer | Shimadzu, Japan (UV-1800) |
| 7 | Cooling centrifuge | Remi Instrument Pvt Ltd , Mumbai (C-24 BL) |
| 8 | Zeta sizer | Malvern Instrument Ltd, UK (Nano ZS) |
| 9 | Stability chamber | Macro Scientific Work Pvt Ltd, Delhi (MSW-125) |
| 10 | Dissolution apparatus | Electrolab, Mumbai (TDT – 08L) |
| 11 | High Performance Liquid Chromatography (HPLC) | Shimadzu, Japan (UFLC) |
| 12 | Fourier Transform Infrared Spectroscopy (FTIR) | Shimadzu, Japan (IRAffinity-1) |
| 13 | Lyophilizer | Macro Scientific Work Pvt Ltd, Delhi (MSW-137) |
| 14 | Isothermal orbital shaker | Macro Scientific Work Pvt Ltd, Delhi (MSW-132) |
| 15 | Bath sonicator | Toshniwal instruments Pvt Ltd, Ajmer (SW - 4) |
| 16 | Mechanical stirrer | Remi Instrument Pvt Ltd , Mumbai (RQ-121/D) |
| 17 | Scanning Electron Microscope (SEM) | JEOL, England (JSM-5610LV) |
| 18 | Transmission Electron Microscope(TEM) | Philips (Tecnai– 20) |
| 19 | Differential Scanning Calorimeter (DSC) | Shimadzu, Japan (DSC TA – 60) |
| 20 | X-Ray Diffractometer (XRD) | Panalytical, The Netherlands (Xpert Pro MPD ) |

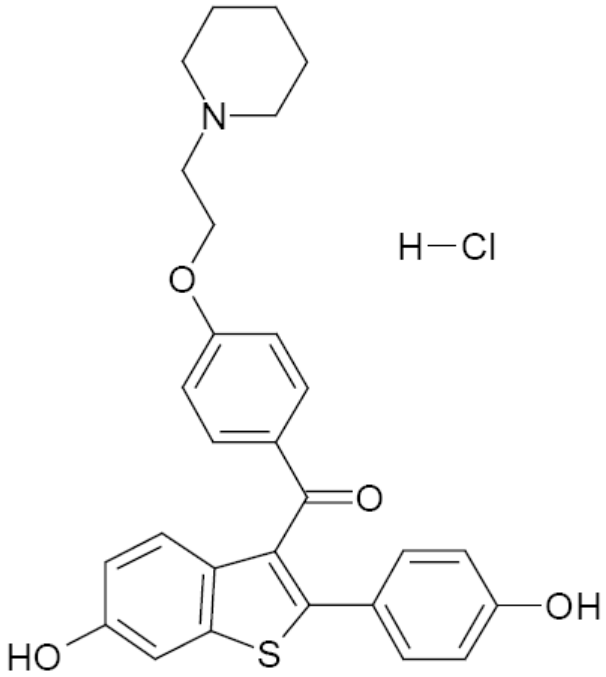
### 5.1.3 Drug and excipients profile [1-3]

#### 5.1.3.1 Raloxifene hydrochloride (RLX)

**IUPAC name:** [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl]-[4-(2-piperidin-1-ylethoxy)phenyl]methanone

**Description:** A second generation selective estrogen receptor modulator (SERM) used to prevent osteoporosis in postmenopausal women. It has estrogen agonist effects on bone and cholesterol metabolism but behaves as a complete estrogen antagonist on mammary gland and uterine tissue.

**Chemical structure:**




**Synonym:** Keoxifene

**Molecular formula:** $C_{28}H_{27}NO_4S \bullet HCl$

**Molecular weight:** 510.05 g/mol

**Appearance:** Light yellow solid

**Melting point:** 267.3-268.5 °C

**Solubility:** Raloxifene (hydrochloride) is sparingly soluble in aqueous buffers. It is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF).

**BCS class:** Class- II drug

**Protein binding:** 95%

**Half-life:** 27.7 h

**Bioavailability:** 2%

**Partition coefficient (Log P):** 6.09

**Pharmacology:-**

**Indication:**

- Used for the control and management of post menopausal induced osteoporosis in women.
- Sometimes also used for the decreasing the occurrence of invasive breast cancer in women having osteoporosis generated due to post menopause.

**Mechanism of action:**

Raloxifene produces estrogen-like effects on bone that result in reduction of bone resorption properties followed by improved bone mineral density. It binds to estrogen receptors that result in differential expression of multiple estrogen-regulated genes in different tissues. Preserving the bone mass by raloxifene and estrogens is because of the regulation of the gene-encoding transforming growth factor-β3 (TGF-β3), which is a bone matrix protein with bone formation characteristics. Raloxifene activates TGF-β3 by pathways which are estrogen receptor-mediated but involve DNA sequences distinct from the estrogen response element. The drug also binds to the estrogen receptor and acts as an estrogen agonist in preosteoclastic cells that result in the inhibition of cells multiplication capacity. This inhibition is thought to contribute to the drug's effect on bone resorption. Other mechanisms include the inhibition of the bone-resorbing cytokine interleukin-6 promoter activity.

**Pharmacodynamics:**

When estrogen binds to a ligand-binding domain of the estrogen receptor, biologic response is initiated as a result of a conformational change of the estrogen receptor that leads to gene transcription by specific estrogen response elements of target gene promoters. The subsequent activation or repression of the target gene is mediated through 2 distinct trans activation domains of the receptor: AF-1 and AF-2.

**Pharmacokinetic:**

**Absorption:**

Due to extensive presystemic glucuronide conjugation, nearly 60% of a dose is absorbed followed by oral administration. Therefore it has only 2.0% of oral bioavailability.

**Distribution:**

Following oral administration of single raloxifene doses ranging from 30 to 150 mg, the apparent volume of distribution is 2,348 L/kg and is not dose dependent. Raloxifene and the monoglucuronide conjugates are highly (95%) bound to plasma proteins. Raloxifene binds to both albumin and alpha-1-acid glycoprotein but not to sex steroid–binding globulin.

**Metabolism:**

Hepatic, raloxifene undergoes extensive first-pass metabolism to the glucuronide conjugates: raloxifene-4'-glucuronide, raloxifene-6-glucuronide, and raloxifene-6, 4'-diglucuronide. No other metabolites have been detected, providing strong evidence that raloxifene is not metabolized by cytochromeP450 pathways

**Excretion:**

Raloxifene is primarily excreted through the feces with less than 0.2% unchanged excretion in the urine. From the total raloxifene dose, nearly less than 6% of the dose is eliminated in the urine as glucuronide conjugates.

**5.1.3.2 β-cyclodextrin (β-CD)**

**Synonyms:** β-cycloamylose; β-dextrin

**Chemical formula:** $C_{42}H_{70}O_{35}$

**Molecular weight:** 1135

**Functional Category:** Solubilizing agent; stabilizing agent

**Applications in Pharmaceutical Formulation or Technology:** Cyclodextrins are crystalline, non-hygroscopic, cyclic oligosaccharides derived from starch. β-cyclodextrin has respectively 7 glucose units. Cyclodextrins are ‘bucket like’ or ‘conelike’ toroid molecules, with a rigid structure and a central cavity, the size of which varies according to the cyclodextrin type. Cyclodextrins may be used to form inclusion complexes with a variety of drug molecules, resulting primarily in improvements to dissolution and bioavailability owing to enhanced solubility and improved chemical and physical stability

It is the least expensive cyclodextrin; is commercially available from a number of sources; and is able to form inclusion complexes with a number of molecules of pharmaceutical interest.

**Melting point:** 255–265°C

**Solubility:** soluble 1 in 200 parts of propylene glycol, 1 in 50 of water at 20°C, practically insoluble in acetone, ethanol (95%), and methylene chloride.

### 5.1.3.3 Capmul MCM (C8)

Capmul MCM (C8) is mono/diglycerides of medium chain fatty acid mainly caprylic acid. It is an excellent solvent for many organic compounds including steroids. It is also a useful emulsifier for water-oil systems. According to 21 CFR 184.1505, mono- and diglycerides prepared from edible fats and oils or fat forming acids are confirmed GRAS.

**Chemical name:** 1, 2, 3-propanetriol

**Physical and Chemical properties:**

Physical state: soft solid or liquid

Odor: Fatty odor

Appearance: soft solid or liquid

Vapour pressure: <1 mm Hg at 25˚c

Vapour density: >1

Solubility: partially soluble in Water

**Application:**

• Carrier (vehicle).

• Solubilizer.

• Emulsifier / co-emulsifier.

• Bioavailability enhancer.

• Penetration enhancer (dermatological applications).

• Water / oil emulsifier.

• Recommended for creams, lotions, ointments and lipsticks.

### 5.1.3.4 Tween 20

Tween 20 is a polyoxyethylene sorbitol ester having molecular weight of 1,225 Daltons. Tween 20 has 20 ethylene oxide units, 1 sorbitol and 1 lauric acid as the primary fatty acid.

**Synonyms:** Polysorbate 20; PEG (20) sorbitan monolaurate,

Polyoxyethylene sorbitan monolaurate.

**IUPAC name:** Polyoxyethylene (20) sorbitan monolaurate

**Molecular formula:** $C_{58}H_{114}O_{26}$

**Molar mass:** 1227.54 g/mol

**Appearance:** Clear, yellow to yellow-green viscous liquid

**Density:** 1.1 g/ml (approximate)

**Boiling point:** > 100 °C

**Solubility:** Tween 20 is completely miscible with water (100 mg/ml) and giving a yellow colour solution. It is also miscible with alcohol, dioxane and ethyl acetate, but it is practically insoluble in liquid paraffin and fixed oils.

**Application:** These have been used as emulsifying agents for the preparation of stable o/w emulsions. It has been used in pre extraction of membranes to take out peripheral proteins. It is appropriate for using as a solubilizing agent of membrane proteins and as a blocking agent in Western blotting.

**5.1.3.5 Polyethylene glycol 400**

Polyethylene glycol 400(PEG 400) is a low molecular weight grade of Polyethylene glycol. They are prepared by the esterification of fatty acids with polyethylene glycols. It is a clear, colorless, viscous liquid. Due in part to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations

PEG 400 is strongly hydrophilic. The partition coefficient of polyethylene glycol 414 between hexane and water is 0.000015 (log$P$ = −4.8), indicating that when polyethylene glycol 414 is mixed with water and hexane, there are only 1.5 parts of polyethylene glycol 414 in the hexane layer per 100,000 parts of polyethylene glycol 414 in the water layer

**IUPAC name:** Polyethylene glycol

**Molecular formula:** $C_{2n}H_{4n+2}O_{n+1}$, n = 8.2 to 9.1

**Molar mass:** 380-420 g/mol

**Density:** 1.128 g/cm³

**Flash point:** 238 °C

**Viscosity:** 90.0 cSt at 25 °C, 7.3 cSt at 99 °C

**Solubility:** PEG 400 is soluble in water, acetone, alcohols, benzene, glycerin, glycols, and aromatic hydrocarbons and is slightly soluble in aliphatic hydrocarbons.

**Application:** Polyethylene Glycol (PEG) Esters are non-toxic and non-irritant nonionic emulsifiers. Polyethylene Glycol Esters are used as emulsifiers and in formulating emulsifier blends, thickener, resin plasticizer, emollient, opacifier, spreading agent, wetting and dispersing agent, and viscosity control agents. They also have application in the metalworking, pulp, paper, and textile and as defoamers for latex paints.

**5.1.3.6 Glyceryl mono stearate**

**Synonyms:** Capmul GMS-50, Cutina GMS

**Chemical Name:** Octadecanoicacid, monoester with 1,2,3-propanetriol

**Chemical formula:** $C_{21}H_{42}O_4$

**Molecular weight:** 358.6

**Applications in Pharmaceutical Formulation:** The many varieties of Glyceryl monostearate (GMS) are used as nonionic emulsifiers, stabilizers, emollients, and plasticizers in a variety of food, pharmaceutical, and cosmetic applications. It acts as an effective stabilizer, that is, as a mutual solvent for polar and nonpolar compounds that may form water-in-oil or oil-in-water emulsions. Glyceryl monostearate has also been used as a matrix ingredient for a biodegradable, implantable, controlled-release dosage form.

**Description:** Glyceryl monostearate is a white to cream-colored, wax like solid in the form of beads, flakes, or powder. It is waxy to the touch and has a slight fatty odor and taste. While the names Glyceryl monostearate and mono- and di-glycerides are used for a variety of esters of long-chain fatty acids, the esters fall into two distinct grades: 40–55 percent monoglycerides and 90 percent monoglycerides.

**Melting point:** 55–60°C

**HLB value:** 3.8

**Solubility:** Soluble in hot ethanol, ether, chloroform, hot acetone, mineral oil, and fixed oils. Practically insoluble in water, but may be dispersed in water with the aid of a small amount of soap or other surfactant.

## 5.2 METHODOLOGY:

### 5.2.1 PREFORMULATION STUDY OF DRUG:-[4-6]

Pre-formulation is a branch of pharmaceutical sciences that utilizes biopharmaceutical principles in the determination of physicochemical properties of a drug substance. The goal of pre-formulation studies is to choose the correct form of the drug pre-requisite for better formulation. Therefore in pre-formulation studies, various physical and chemical properties of drug with excipients are evaluated to generate a thorough understanding of the material's stability under various conditions, leading to the optimal drug delivery system. The pre-formulation study focuses on the physicochemical parameters that could affect the development of efficacious dosage form. A thorough understanding of these properties may ultimately provide a rationale for formulation design. Also it will help in minimizing problems in later stages of drug development, reducing drug development costs and decreasing product's time to market.

#### 5.2.1.1 Organoleptic properties of RLX

This study includes evaluation of drug sample for its colour, odour and appearance using descriptive terminology. Record of color is very useful in establishing appropriate specifications for production. Drugs generally have a characteristic odour and taste. Results are shown in table 7.1.

#### 5.2.1.2 Melting point determination of RLX

Melting point is a good indication of purity of the sample since the presence of relatively small amount of impurity can be detected by a lowering as well as widening in the melting point range. Melting point was determined by capillary method using melting point apparatus. The small amount of drug sample was taken in to the one end closed capillary tube. Capillary tube was kept in melting point apparatus and melting point of drug was recorded. Result is shown in table 7.2.

#### 5.2.1.3 Method for estimation of RLX

■ **Determination of UV absorption maxima ($\lambda_{max}$) for Drug:**

Weigh accurately 50 mg of drug and dissolved in 50 ml of methanol to obtain 1000 µg/ml of drug stock solution - I. From this stock solution – I, 5 ml of solution was transferred in 50 ml of solvent to obtain a 100 µg/ml of drug stock solution –II and from this stock solution – II, 10 µg/ml solution was prepared by proper dilution with solvent system. The solution was scanned in spectrum mode for absorbance between 200-800 nm using UV- visible spectrophotometer (Shimadzu 1800, Japan). Result is

shown in table 7.3.

■ **Preparation of Calibration curve of Drug:**

**Calibration curve of RLX for estimation in Methanol: DMSO (2:1)**

Calibration curve of drug was taken into Methanol: DMSO (2:1) solvent to determine the solubility study of drug in various solvents.

**Method:**

Weigh accurately 50 mg of drug and dissolved in portion of solvent system and diluted up to 50 ml with solvent system to make 1000 µg/ml of drug stock solution - I. From this stock solution - I, 5ml of solution was pipette out and diluted to 50 ml with solvent to make 100 µg/ml of drug stock solution - II. From stock solution - II, aliquots of 0.2 ml, 0.3 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml, 1.2 ml and 1.4 ml were pipette out and diluted up to 10 ml with solvent to prepare 2 µg/ml, 3 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml, 12 µg/ml and 14 µg/ml drug concentration solutions, respectively. The absorbance of each solution was measured at $\lambda_{max}$ 289 nm against solvent as a blank using UV- visible spectrophotometer. Same procedure was performed in triplicate and average of the absorbance was taken into consideration. Result is shown in table 7.4.

**Calibration curve of RLX for estimation in methanol**

Calibration curve of drug was taken into methanol to determine the amount of drug entrapped in the prepared nanoparticles formulation.

**Method:**

Weigh accurately 50 mg of drug and dissolved in portion of methanol and diluted up to 50 ml with methanol to make 1000 µg/ml of drug stock solution - I. From this stock solution - I, 5ml of solution was pipette out and diluted to 50 ml with solvent to make 100 µg/ml of drug stock solution - II. From stock solution – II, aliquots of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml, 1.2 ml and 1.4 ml were pipette out and diluted up to 10 ml with solvent to prepare 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml, 12 µg/ml and 14 µg/ml drug concentration solutions, respectively. The absorbance of each solution was measured at $\lambda_{max}$ 288 nm against solvent as a blank using UV-visible spectrophotometer. Same procedure was performed in triplicate and average of the absorbance was taken into consideration. Result is shown in table 7.5.

**Calibration curve of RLX for estimation in citro phosphate buffer pH 7.6 containing 1% of polysorbate 80**

Calibration curve of drug was taken into citro phosphate buffer pH 7.6 containing 1% of polysorbate 80 solvent system to determine the amount of drug released from prepared formulations.

**Method:**

Weigh accurately 25 mg of drug and dissolved in 3 ml of methanol followed by dilution with buffer (citro phosphate buffer pH 7.6 containing 1% of polysorbate 80) to make 100 ml stock solution – I having drug concentration of 250 µg/ml. From this stock solution - I, 10 ml of solution was pipette out and diluted to 25 ml with buffer to make 100 µg/ml of drug stock solution - II. From stock solution – II, aliquots of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml, 1.2 ml, 1.4 ml and 1.6 ml were pipette out and diluted up to 10 ml with buffer to prepare 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml, 12 µg/ml, 14 µg/ml and 16 µg/ml drug concentration solutions, respectively. The absorbance of each solution was measured at $\lambda_{max}$ 288 nm against buffer as a blank using UV-visible spectrophotometer. Same procedure was performed in triplicate and average of the absorbance was taken into consideration. Result is shown in table 7.6.

**Calibration curve of RLX for estimation in rat blood plasma**

Calibration curve of drug was taken into rat blood plasma to determine the amount of drug present in plasma from prepared formulations.

**Method:**

**1) Preparation of standard solution[7]**

A series of standard solutions of RLX were prepared in methanol, in the range of 0.1-10 µg/ml. Standard calibration samples were prepared by adding 50µl standard solution of drug in methanol and 200 µl acetonitrile to 100 µl of blank plasma in eppendorf tube. The mixture was then processed according to the sample extraction procedure described below. Final standard RLX concentrations in plasma were 0.05-5µg/ml.

**2) Sample extraction procedure[7,8]**

To the eppendorf tube containing 100 µl of plasma samples, 50 µl of standard solution of drug in methanol and 200 µl of acetonitrile solutions were added. The resulting solution was thoroughly vortex and mixed (Macro Scientific Work Pvt Ltd, Delhi) for 30 seconds. Centrifugation was carried out for the separation of denatured protein at 15,000 rpm for 10 min at -6°C (C-24 BL, Remi Instrument Pvt Ltd, Mumbai). After

centrifugation, the supernatant was transferred to new eppendorf tube, and aliquots of 20 µl of the filtered supernatant (0.45µm membrane filter) were injected into HPLC system and drug content was measured at 288 nm for analysis. Result is shown in table 7.7.

**5.2.1.4 Solubility determination[9-11]**

Solubility of RLX was checked in different solvents. Excess amount of drug was taken in stopper vials containing 5 ml of solvent and mixing was carried out on vortex mixer for 10 min. Vials were then kept at 25 ± 2.0$^{o}$C in an isothermal orbital shaker (MSW-132, Macro Scientific Work Pvt Ltd, Delhi) for 24 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 5000 rpm for 15 min using cooling centrifuge. Supernatant was separated and absorbance was measured at 289 nm using UV-VIS spectrophotometer to measure solubility profile of drug in various solvents. Result is shown in table 7.8.

**5.2.2 FORMULATION AND CHARACTERIZATION OF DRUG LOADED INCLUSION COMPLEXES:-**

**5.2.2.1 Preliminary studies**

Physicochemical incompatibility between drug and β-cyclodextrin (β-CD) was studied by drug excipients study as discussed underneath.

■ **Drug excipients compatibility studies:[12-14]**

Drug excipients compatibility study was carried out by Fourier Transform Infra Red spectrophotometer (FTIR) (IRAffinity-1, Shimadzu, Japan) for pure drug alone and with excipients. Drug was mixed with KBr in a ratio of 1: 300 and FTIR spectrum was recorded in the range of 4000 – 400 cm$^{-1}$ using FTIR spectrophotometer. Same procedure has been followed for β-Cyclodextrin and physical mixture of RLX & β-CD to study any interaction between them. Result is shown in table 7.9.

**5.2.2.2 Formulation of Inclusion complexes between RLX and β-CD**

**Method of preparation: [15,16]**

The inclusion complex of RLX with β-CD was prepared by three different methods using different ratios of RLX: β-CD (1:1, 1:2 & 1:3). For all batches amount of RLX was kept constant i.e.100 mg/batch.

■ **Physical mixture:**

Accurately weigh quantity of RLX and β-CD were mixed in a mortar and pestle for about 1 h with constant trituration. Mixture was passed through sieve no. 80 and

stored in desiccators over fused calcium chloride. Formulation codes for inclusion complexes are given in table 5.3.

■ **Co-precipitate method:**

Accurately weigh quantity of drug and β-CD were dissolved in methanol and distilled water, respectively. Drug solution was added slowly to β-CD solution and mixture was stirred for 1 h at room temperature followed by mild heating on a boiling water bath for complete evaporation of solvents. The product obtained was a drug loaded inclusion complex in a fine crystalline powder form which was pulverized and passed through sieve no. 80 and stored in a desiccator having fused calcium chloride. Formulation codes for inclusion complexes are given in table 5.3.

■ **Kneading method:**

Accurately weigh quantity of β-CD was triturated in the mortar containing small quantity of 50% methanol to get slurry like consistency. Drug was incorporated into the slurry and triturated further for 1 h. The mixture was air dried at 25°C for 24 h, pulverized and passed through sieve no. 80 and stored in desiccators over fused calcium chloride. Formulation codes for inclusion complexes are given in table 5.3.

Table 5.3: Formulation codes for inclusion complex of RLX & β-CD

| Formulation codes | Method of preparation | Drug: carrier ratio |
|---|---|---|
| IC-1 | Physical method | 1:1 |
| IC-2 | | 1:2 |
| IC-3 | | 1:3 |
| IC-4 | Co-precipitate method | 1:1 |
| IC-5 | | 1:2 |
| IC-6 | | 1:3 |
| IC-7 | Kneading method | 1:1 |
| IC-8 | | 1:2 |
| IC-9 | | 1:3 |

**5.2.2.3 Characterization of Inclusion complexes**

■ **Percentage yield:**

The yield was calculated by dividing the weight of the recovered inclusion complex by the weight of total amount of drug and carrier taken for development of the

inclusion complex. Results were carried out in triplicate. Results are shown in table 7.10.

$$\text{Percentage Yield} = \frac{\text{Weight of inclusion complex recovered}}{\text{Theoretical weight (drug + carrier)}} \text{ X } 100$$

■ **Drug content:**

Accurately weigh 5 mg of inclusion complex was transferred to 50 ml volumetric flask and volume was made to the required mark with citro phosphate buffer pH 7.6 containing 1% polysorbate 80. 1ml of above solution was transferred in 10ml volumetric flask and the volume was made with buffer system. The absorbance was measured at 288 nm against buffer system as blank using UV-Visible spectrophotometer. Results were carried out in triplicate. Results are shown in table 7.10.

■ **Solubility study in water:**[12]

Excess amount of inclusion complex was added to 10 ml water in stopper volumetric flask followed by sonication for 10 min. Flasks were kept at 25 ± 2.0°C in an isothermal orbital shaker for 24 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 5000 rpm for 15 min. The solubility profile of complex in water was determined from the supernatant by taking absorbance at 288 nm using UV-Visible spectrophotometer. Results were carried out in triplicate and shown in table 7.10.

■ ***In vitro* drug release:**

Dissolution rates from pure RLX and all nine RLX: β-CD inclusion complexes were determined in 900 ml citro phosphate buffer pH 7.6 containing 1% of polysorbate 80 at 37 ± 0.5°C with a stirrer rotation speed of 75 rpm using USP dissolution test apparatus type II (TDT – 08L, Electrolab, Mumbai). RLX: β-CD inclusion complex was taken equivalent to 60 mg of RLX in a capsule shell. Aliquots of 5 ml sample were withdrawn at predefined time interval with a pipette and replaced with fresh buffer at each time. The samples were filtered through cellulose acetate membrane filter (0.45µm) and analyzed by using UV-Visible spectrophotometer at 288 nm. Each dissolution test was carried out in triplicate. Result is shown in table 7.11.

■ **Drug excipients compatibility studies:**

Drug excipients compatibility study of drug loaded inclusion complex was carried out by FTIR and spectrum was recorded in same manner as discussed in section 5.2.2.1 to find out any incompatibility in prepared inclusion complex. Results are shown in table 7.12.

■ **Differential scanning calorimetry:**[17]

Differential scanning calorimetry (DSC) is widely used in thermal analysis to monitor endothermic processes (melting, solid-solid phase transitions and chemical degradation) as well as exothermic processes (crystallization and oxidative decomposition). It could be extremely useful in formulation development since it indicates the existence of possible drug-excipients or excipients-excipients interactions in formulation. Thermograms of RLX, β-CD & optimized inclusion complex (IC-6) were obtained by using Differential scanning calorimeter (DSC TA – 60, Shimadzu, Japan) as shown in figure 7.13, 7.14 and 7.15, respectively. Samples were weighed directly in pierced DSC aluminum pan and scanned in the temperature range of 50-300°C under an atmosphere of dry nitrogen. Heating rate of 10°C/min was used and thermogram obtained was observed for interaction between drug and excipients.

■ **X-Ray diffraction studies:**

The properties of a material can often be linked back to the arrangement of atoms in its crystal structure. X-ray diffraction (XRD) is a non-destructive analytical technique which can yield the unique fingerprint of Bragg reflections associated with a crystal structure.

X-ray diffraction study can become useful tool to find out amorphous or crystalline nature of formulations. XRD study of RLX, β – CD, physical mixture & optimized inclusion complex (IC-6) was carried out by as shown in figure 7.16, 7.17, 7.18 and 7.19, respectively using Panalytical Xpert PRO X Ray Diffractometer (Xpert Pro MPD, Panalytical, The Netherlands) where CuKα radiation wavelength of 1.5405 Å was used as X-ray source. For the analysis, samples were placed in the glass sample holders and scanned from 2° to 60° with a scan angular speed (2θ/min) of 2° / min at 40kv operating voltage and 30 mA current.

■ **Surface morphology study:[18-20]**

The surface morphology and shape of solid particles can be observed by scanning electron microscopy. Surface morphology of RLX and optimized inclusion complex (IC-6) was obtained by using scanning electron microscope (SEM) (JEOL JSM-5610LV, England) as shown in figure 7.20 and 7.21, respectively. SEM is an instrument that produces largely magnified image by using electrons instead of light. Electron gun produces a beam of electrons, which follows the vertical path through the microscope between electromagnetic fields and lenses towards the sample due to which electrons, and X-rays are ejected from sample. Prior to examination, the samples were mounted on to metal stubs using a double-sided adhesive tape under vacuum. Images were observed at various magnifications, operated with an acceleration voltage of 15 kV and working distance of 20 µm was maintained.

■ **Stability study:[19]**

The selected formulation was subjected to stability studies as per ICH guidelines. The sample were placed in vials and kept at $25^0$ C ± $2^0$ C /60% ± 5% RH ± SD and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber (MSW-125, Macro scientific work Pvt Ltd, Delhi) over period of six months. The samples were analyzed for physical appearance and the drug content at 0, 15, 30, 60, 120 & 180 days. Results are shown in table 7.13. Percentage cumulative drug release and drug excipients compatibility study was also carried out at the end of study for both stability conditions. Results were carried out in triplicate. Results of drug release and compatibility study are shown in table 7.14 and 7.15, respectively.

## 5.2.3 FORMULATION AND CHARACTERIZATION OF DRUG LOADED MICROEMULSION:-

### 5.2.3.1 Preliminary studies

■ **Screening of components:[9-11]**

The most important criterion for the screening of oil for microemulsion is the maximum solubility and compatibility with drug. Solubility of RLX was checked in different solvents. Excess amount of drug was taken in stopper vials containing 5 gm of selected oils (Soyabean oil, Isopropyl myristate, Castor oil, olive oil, Capmul MCM C8, Oleic acid & Seasame oil) and mixing was carried out on vortex mixer for 10 min. Vials were kept at 25 ± 2.0°C in an isothermal orbital shaker for 24 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 5000 rpm for 15 min using cooling centrifuge. Supernatant was separated and

absorbance was measured at 289 nm using UV-Visible spectrophotometer to measure solubility profile of drug in various solvents. Drug solubility was carried out in a same manner described above with different surfactants like Capmul PG 8, Captex 300, Labrafil IC M 1944 CS, Lebrafec CC, Acconon C, Tween 20, Tween 80 & Transcutol. Selection of co-surfactant is also playing important role as it gives flexibility to the interfacial film produced by surfactant to make stable emulsion. Therefore, co-surfactant having maximum drug solubility is used in formulation of microemulsion. Drug solubility was carried out in a same manner described above with different co-surfactants like Polyethylene glycol (PEG) 200, PEG 400 & Propylene glycol. Result is shown in table 7.16.

- **Drug excipients compatibility studies:**[11]

Drug excipients compatibility study was performed to check physical stability of formulation. Adequate quantity of drug mixed separately with each formulation components and physical mixture of all components were kept undisturbed for 15 days. Samples were checked at every third day for any precipitation, crystallization, phase separation and color change in order to determine their physical compatibility. Result is shown in table 7.17.

Drug excipients compatibility study was also carried out by FTIR for pure drug alone and with mixture of drug with each excipient. Spectrum was recorded in same manner as discusses in section 5.2.2.1 to find out any incompatibility between drug and excipients. Result is shown in table 7.18.

- **Pseudo ternary phase diagram:**[11,21,22]

Pseudo ternary phase diagram is a useful and important tool to study the phase behavior of microemulsions. Pseudo ternary phase diagram can be represented in a triangular format (triangle) which has three coordinates. Each coordinate represents one component of microemulsion system viz. (1) Oil phase, (2) Surfactant: co surfactant phase and (3) aqueous phase. Each coordinate also represents 0 to 100% concentration of each of the phases in the increment of 10%.

`Pseudo ternary phase diagram is constructed to obtain the appropriate concentration ranges that can result in large existence area of microemulsion. The microemulsion region is initially delineated by its transparency with less viscosity.

In this study, the Pseudo ternary phase diagrams of oil, surfactant/co-surfactant mixture ($S_{mix}$) and water were developed by using water titration method to obtain the components concentration ranges that can result in large existence area of

microemulsion. Different $S_{mix}$ ratios taken for study were 1:1, 2:1, 3:1, and 4:1. For each plot, the weight between oil to the $S_{mix}$ was taken as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 (w/w). For each $S_{mix}$ ratio, various compositions of $S_{mix}$ and oil were taken followed by drop wise addition of water with stirring till the clarity of the system persists in order to determine the maximum water uptake. Pseudo ternary phase diagrams of microemulsion were prepared by CHEMIX software to determine the microemulsion existence zone in which at any point (representing the concentration of different components), microemulsions can be prepared. Result is shown in figure 7.28.

**5.2.3.2 Formulation of RLX loaded microemulsion[23,24]**

ME formulations were prepared by water titration method. In this research work, ME was optimized using two dependent variables (concentration of oils and surfactant mixture) and keeping the concentration of RLX constant in each case as shown in table 5.4.

Accurately weigh quantities of surfactant and co-surfactant (Tween 20 & PEG 400) were added to oil (Capmul MCM C8) followed by addition of RLX with continuous stirring for 15 min on magnetic stirrer. The mixture was allowed to sonicate for 15 min on bath sonicator (SW-4, Toshniwal instruments Pvt Ltd, Ajmer) followed by heating at 65 ± 5°C for 10 min on magnetic stirrer with stirring. The mixture was allowed to cool at room temperature. At the end, required quantity of water was added drop wise from syringe to prepare stable and transparent ME.

Table 5.4: Formulation codes for RLX loaded microemulsions

| Sr. no. | Formulation Codes | Oil (% w/w) | $S_{mix}$ (% w/w) | Water content (% w/w) |
|---|---|---|---|---|
| 1 | RLX-1 | 8 | 30 | 62 |
| 2 | RLX-2 | 8 | 40 | 52 |
| 3 | RLX -3 | 8 | 50 | 42 |
| 4 | RLX -4 | 8 | 60 | 32 |
| 5 | RLX -5 | 10 | 30 | 60 |
| 6 | RLX -6 | 10 | 40 | 50 |
| 7 | RLX -7 | 10 | 50 | 40 |
| 8 | RLX -8 | 10 | 60 | 30 |
| 9 | RLX -9 | 12 | 30 | 58 |
| 10 | RLX -10 | 12 | 40 | 48 |
| 11 | RLX -11 | 12 | 50 | 38 |
| 12 | RLX -12 | 12 | 60 | 28 |
| 13 | RLX -13 | 14 | 30 | 56 |
| 14 | RLX -14 | 14 | 40 | 46 |
| 15 | RLX -15 | 14 | 50 | 36 |
| 16 | RLX -16 | 14 | 60 | 26 |
| Amount of RLX = 3mg/g for all batches | | | | |

### 5.2.3.3 Optimization of microemulsion

#### 5.2.3.3.1 Selection of microemulsions by checking primary parameters:- [25,26]

All formulations were selected based on their maximum transmittancy measured by UV- Visible spectrophotometer and visually checked by necked eyes.

■ **Percentage (%) Transmittance**: All sixteen batches of microemulsion formulations were diluted 10 times with distilled water. The % transmittance of various formulations was checked against distilled water using UV-visible spectrophotometer at 630 nm. Results were taken in triplicate. Result is shown in table 7.20.

■ **Visual transmittancy:** All sixteen batches of microemulsion formulations were kept at room temperature for 24 h in undiluted and diluted form (10 times dilution with distilled water) to check stability of microemulsions in form of any sign of phase

separation or precipitation. Result is shown in table 7.20.

**5.2.3.3.2 Optimization of stable microemulsion with augmented drug loading:-**

From the transmittancy study, six batches were selected which were further analyzed for optimization of stable microemulsion with increased drug loading (6mg/g of microemulsion).

■ **Preparation of microemulsion with augmented drug loading**

Selected six batches of microemulsion with increased drug loading were prepared in same manner as described earlier. Prepared microemulsions batches were evaluated further to prove their physical stability with loaded amount of RLX.

■ **Evaluation of maximum drug loaded microemulsion**

**Dilution test**:[25,26]

Dilution tests are based on the fact that the emulsion is only miscible with the liquid that forms its continuous phase. The system is diluted with either the oil or the aqueous phase, whichever is used in the microemulsion preparation as external phase. Hence in case of o/w system, the microemulsion is diluted with the aqueous phase while with w/o microemulsion, the system is diluted with the oil phase. In the present work o/w microemulsion was prepared. Therefore, all microemulsions batches were diluted 10 times with distilled water and kept for 24 h at room temperature to check physical stability. Result is shown in table 7.21.

**Centrifugation test:** [25,26]

This parameter was characterized to check the physical stability of microemulsions. Selected microemulsion systems were centrifuged at 10,000 rpm for 30 min using cooling centrifuge to determine sign of creaming or phase separation. The appearance of system was observed visually. Result is shown in table 7.21.

**Percentage Transmittance**: The % transmittance of prepared six microemulsion batches was tested in same manner as discussed earlier. Result is shown in table 7.21.

***In vitro* drug release:**

*In vitro* drug release study of selected microemulsions was carried out using dialysis membrane. (Himedia – Dialysis membrane 135, Mol.cut off 12000-14000 Da).

**Procedure for the activation of dialysis sac**

Cut 5 cm of dialysis sac and boiled for 20 min in 100 ml of 2% $NaHCO_3$ and 1mM EDTA solution in a beaker. Rinse the sac in hot water followed by boiling for 10 min in 1mM EDTA solution. Allow to cool. Dialysis sac was stored in 50% ethanol solution. Before the use, sac was washed with distilled water.

***In vitro* release method**[9,17,27,28]

An accurately measured amount of plain drug suspension (Drug powder in buffer system) and all six formulations equivalent to 5 mg of RLX were introduced into the sac separately and both ends of the sac were tied with the help of thread. The sac was suspended with the help of thread in beaker containing 200 ml of citro phosphate buffer pH 7.6 with 1% of polysorbate 80 kept on magnetic stirrer. The temperature of the receptor compartment was maintained at 37 ± 1°C. Aliquots of 5 ml sample were withdrawn at predefined time interval with a pipette and replaced with fresh buffer at each time. The samples were filtered through cellulose acetate membrane filter (0.45µm) and analyzed using UV-Visible spectrophotometer at 288 nm. Blank microemulsions were prepared and treated in same manner as discussed above. Blank formulation was taken for base correction by suitable dilution with buffer system in UV-Visible spectrophotometer to nullify any effect of ingredients other than drug in formulation. Each test was carried out in triplicate. Results are shown in table 7.22.

**Drug release kinetics:**[18]

To study the drug release mechanism from optimized microemulsion, the release data obtained from *in vitro* release studies were fitted to various kinetics models. The kinetics models used were Zero order equation ($Q_t = Q_0 – K_0t$), first order equation ($\ln Q_t = \ln Q_0 – K_1t$) and Higuchi's equation ($Q_t = K_2t^{1/2}$). The following plots were made, $Q_t$ vs. t (zero order kinetic model), ln ($Q_0 – Q_t$) vs. t (first order kinetic model) and $Q_t$ vs. $t_{1/2}$ (Higuchi model), where $Q_t$ is the percent of drug released at time t, $Q_0$ is the initial amount of drug percent in microemulsion and $K_0$, $K_1$ and $K_2$ were constant of the equation of zero order, first order and Higuchi model, respectively. Result is shown in table 7.23.

**5.2.3.4 Characterization of optimized microemulsion**[29-34]

Optimized microemulsion ME-8 selected from the above study is characterized further by following parameters other than studied earlier.

■ **Dye solubility test**:

This test was performed by considering fact that dye will be dispersed uniformly with the external phase. With an o/w emulsion, there is rapid incorporation of a water soluble dye into the system where as with w/o emulsion same dye forms microscopically visible clumps. The reverse happens on addition of an oil soluble dye. These tests essentially identify the continuous phase.

In present study, sample mixture consists of optimized microemulsion and water soluble methylene blue dye was taken on glass slide and observed by compound microscope under normal magnification to identify type of emulsion.

■ **Drug content:**

The loading efficiency of drug in optimized formulation was determined spectrophotometrically. Accurately measured quantity of microemulsion formulation was diluted suitably with methanol: DMSO (2:1) solvent system, filtered and measured at 289 nm using UV-Visible spectrophotometer. Each test was carried out in triplicate. Results are shown in table 7.24.

■ **Determination of pH**:

Determination of pH of the optimized microemulsion was carried out using pH meter (Systonic, S-901) and result is shown in table 7.24.

■ **Conductivity measurements:**

The solubilization of water phase in the selected oily mixture was monitored quantitatively by measuring the electrical conductivity(s). Conductivity of microemulsion was measured using a conductivity meter (MSW-554(a), Macro scientific works, Delhi). Each test was carried out in triplicate. Results are shown in table 7.24.

■ **Viscosity measurements:**

The viscosity of optimized microemulsion was determined using Brookfield viscometer (LVDV-III U), a rotational viscosity measuring device coupled with concentric cylinders, using spindle no. 63 with shear rate of 50 rpm. Each test was carried out in triplicate. Results are shown in table 7.24.

■ **Drug excipients compatibility study:**

Drug excipients compatibility study was carried out for optimized batch ME-8 by FTIR to study any interaction between drug and excipients. Same procedure was followed as discussed earlier in section 5.2.2.1. Results are shown in table 7.25.

■ **Globule size and zeta potential measurements:**

The globule size of optimize batch ME-8 was measured with Malvern zeta sizer (Nano ZS, Malvern Instruments, Worcestershire, UK) which able to measure sizes between 10 to 5000 nm. Helium-neon gas laser having intensity of 4mW was used as light source. The instrument is based on the principle of dynamic light scattering (DLS), also sometimes known as photon correlation spectroscopy (PCS) (which analyses the fluctuations in light scattering due to Brownian motion of the particles)

or quasi elastic light scattering. DLS is a technique of measuring the size of particles typically in the sub-micron region and is usually applied to the measurement of particles suspended within a liquid. The technique measures particle diffusion due to Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. The parameter calculated is defined as the translational diffusion coefficient. The particle size is then calculated from the translational diffusion coefficient using the Strokes-Einstein equation. Globule size of optimized batch is shown in figure 7.33.

Malvern zeta sizer was also used to measure the zeta potential of the globules based on the electrophoresis and electrical conductivity of the formed microemulsion. The electrophoretic mobility (μm/s) of the particles was converted to the zeta potential by in-built software based on Helmholtz-Smoluchowski equation. Measurements were performed using small volume disposable zeta cell. Latex dispersion having zeta potential -50mV ± 2.5 mV was used as a standard. Zeta potential of optimized batch is shown in figure 7.34.

■ **Surface morphology study:**

The surface morphology and shape of globules can be observed by electron microscopy. Surface morphology of optimized batch ME-8 was obtained using Transmission Electron Microscope (TEM) (Philips Tecnai - 20). One to two drops of 100 times diluted optimized ME was dropped on carbon coated copper grid followed by addition of 1% phosphotungstic acid solution and allow it to dry at normal environmental condition. This grid was mounted in the instrument and photographs were taken at various magnification. Results are shown in figure 7.35.

■ **Stability study:**[34]

The selected formulation was subjected to stability studies as per ICH guidelines. Stability of microemulsion was studied concerning the drug content, physical appearance and phase separation by centrifugation. The samples were placed in vials and kept at various stability conditions (Refrigerator condition 2-8$^0$ C, 25$^0$ C ± 2$^0$C / 60% ± 5% and 40$^0$ C ± 2$^0$ C / 75% ± 5% RH) using stability chamber over period of six months. The samples were analyzed at 0, 15, 30, 60, 120 &180 days for physical appearance (precipitation and colour change), % transmittance, drug content and centrifugation at 10,000 rpm for 30 min to inspect any phase separation. Results are shown in table 7.26, 7.27 and 7.28. Optimized batch was also studied for *in vitro* drug

release profile and drug excipients compatibility study at the end of stability period. Result for drug release and compatibility study is shown in table 7.29 and 7.30, respectively.

### 5.2.4 FORMULATION AND CHARACTERIZATION OF DRUG LOADED NANOSTRUCTURED LIPID CARRIERS (NLCs):-

#### 5.2.4.1 Preliminary studies

■ **Selection of solid lipid:-**[17,35-39]

Solid lipid was selected based on drug solubility to produce visibly clear solution in melted lipid. Visibility was checked with the naked eyes under normal light. Lipids used for this study were Dynasan 114 (Trimyristin), Dynasan 118 (Tristearin), stearic acid and Glyceryl monostearate (GMS). Weigh quantity of drug (50 mg) and various lipids (5 g each) were heated above melting point of lipid in temperature controlled water bath (Macro Scientific Work Pvt Ltd, Delhi) in 10 ml glass beaker. After melting of lipids, solubility of drug in each lipid was checked visually under normal light. Results are shown in table 7.31.

■ **Partition behavior of RLX in various solid lipids:-**[37,40]

Weigh quantity of drug (25 mg) was added in blend of melted lipid (5 g) and hot distilled water (5 g). Mixture was shaken on an isothermal orbital shaker for 24 h to reach equilibrium at 70 ± 2.0$^{o}$C. Aqueous phase was separated thereafter by centrifugation at 5000 rpm for 5 min and drug content was analyzed spectrophotometrically. Results are shown in table 7.32.

■ **Selection of liquid lipid:-**[9-11]

Liquid lipid was selected based on maximum solubility of drug in different liquid lipids. Various liquid lipid used for this study were Capmul MCM C8, Isopropyl myristate, oleic acid, Labrafil ILM 1944 CS and Lebrafec CC. Solubility study was carried out in same manner as described in section 5.2.3.1. Results are shown in table 7.33.

■ **Drug excipients compatibility studies:** [17,35,36]

Drug excipients compatibility study was carried out by FT-IR for pure drug alone, GMS & RLX, Capmul MCM C8 & RLX and physical mixture of RLX, GMS & Capmul MCM C8 to study any interaction between them. Procedure carried out to study compatibility was as same as discussed in section 5.2.2.1. Result is shown in table 7.34.

### 5.2.4.2 Formulation of RLX loaded NLCs

■ **Design of the experiment**

A complete $3^2$ factorial design was used to study the effect of solid to liquid lipid concentration and stabilizer concentration on the effect of entrapment efficiency of drug. Below mentioned table summarizes independent variables along with their levels. The total weight of solids (200 mg), concentration of drug (5% w/w to the total weight of drug and lipids), drug to lipid ratio (5:95), type of solid lipid (GMS), type of liquid lipid (Capmul MCM C8), organic solvent ratio (Ethanol:Acetone (1:1v/v), total 10ml volume), volume of aqueous phase (100 ml), type of stabilizer (polyvinyl alcohol, PVA) and stirring speed of mechanical stirrer (500 rpm) were kept constant and were selected based on preliminary studies. Table 5.5 summarizes various factors and levels for $3^2$ factorial design for RLX loaded NLCs.

Table 5.5: $3^2$ factorial design for RLX loaded NLCs

| **Factors** | **Levels** | | |
|---|---|---|---|
| | **-1** | **0** | **1** |
| **$X_1$** | 95:5 | 90:10 | 85:15 |
| **$X_2$** | 0.5 | 1.0 | 1.5 |

$X_1$= Solid: liquid lipid ratio (%w/w),

$X_2$ = Concentration of stabilizer (% w/v)

■ **Preparation of NLCS[37, 41-44]**

NLCs loaded with RLX were developed by solvent diffusion method in aqueous system with some modification [41]. Drug (5% w/w) and liquid lipid (Capmul MCM C8) were mixed in a 10 ml mixture of ethanol & acetone (1:1 v/v) followed by sonication for 10 min. The obtained mixture was kept on water bath maintained at 60°C with the addition of solid lipid (GMS) to make clear solution of lipid mixture and drug in organic solvent system. The resultant organic solution was dispersed quickly into 100 ml of an aqueous phase containing PVA as stabilizer kept on water bath at 70°C under continuous stirring at 500 rpm for 10 min using mechanical stirrer (RQ-121/D, Remi Instrument Pvt Ltd, Mumbai). The obtained dispersion was allowed to cool at room temperature on magnetic stirrer for 20 min for liberation of organic solvent to obtain RLX loaded NLCs as final product. NLCs were separated after centrifugation at -10°C for 17,000 rpm and

1h followed by freeze drying in lyophilizer to get dry product. Table 5.6 displays formulation code for preparation of different drug loaded NLCs batches.

Table 5.6: Formulation codes for RLX loaded NLCs

| Formulation codes | $X_1$ | $X_2$ |
|---|---|---|
| **NLC - 1** | -1 | -1 |
| **NLC - 2** | -1 | 0 |
| **NLC - 3** | -1 | 1 |
| **NLC - 4** | 0 | -1 |
| **NLC - 5** | 0 | 0 |
| **NLC - 6** | 0 | 1 |
| **NLC - 7** | 1 | -1 |
| **NLC - 8** | 1 | 0 |
| **NLC - 9** | 1 | 1 |

**5.2.4.3 Evaluation of RLX loaded NLCs**

■ **Percentage yield:**

The percentage yield was considered by dividing the weight of the recovered nanoparticles by the theoretical weight of drug and lipid utilized for development of the nanoparticles. Results are shown in table7.35.

$$\text{Percentage Yield} = \frac{\text{Weight of nanoparticles recovered}}{\text{Theoretical weight (drug + lipid)}} \text{ X } 100$$

■ **Drug loading and entrapment efficiency:**

**Indirect method:**

The prepared NLCs dispersion was transferred to centrifuge tubes equipped with cooling centrifuge and centrifugation was carried out at -10°C for 17,000 rpm and 1h. The supernatant was separated, diluted suitably with methanol and analyzed for RLX content spectroscopically at 288 nm using UV visible spectrophotometer. Results are shown in table 7.35.

Entrapment efficiency was calculated using following formula:[35,45]

$$\% \text{ Entrapment efficiency} = \frac{[\text{RLX}]\text{total} - [\text{RLX}]\text{supernatant}}{[\text{RLX}]\text{total}} \text{ X } 100$$

Where "[RLX] total" is the mass of total drug added and the "[RLX] supernatant" is the mass of free drug analyzed in supernatant

Drug loading was calculated using following formula: [35,46]

$$\% \text{ Drug loading } = \frac{\text{Amount of RLX entrapped in NLCs}}{\text{Amount of RLX and lipids added}} \text{ X } 100$$

■ ***In vitro* drug release:[17,27,28]**

*In vitro* drug release study of pure drug and NLCs was carried out using the dialysis bag method using dialysis membrane.

An accurately measured amount of plain drug suspension and NLCs formulations equivalent to 5 mg of drug were introduced into sac and both the end of the sac was tied with the help of thread. Remaining procedure was as same as discussed in section 5.2.3.3.2. Results are shown in table 7.36.

■ **Optimization of formulation:**

The optimization of prepared batches was done by considering % drug entrapment and by studying interaction between factors which are considered as follows.

**Interaction between the factors**

The statistical evaluation of all the obtained results data was carried out by analysis of variance (ANOVA) using Microsoft excel version 2007. The ANOVA results (p value) show the effect of the various independent variables on dependent parameter like % drug entrapment. Regression coefficients is statistically significant if $p < 0.05$. The significant factors in the equations were selected for the calculation of regression analysis. The terms of full model having non-significant p value have negligible contribution in obtaining dependent variables and thus neglected. The equations represent the quantitative effect of the formulation variables on responses.

**Construction of contour plots**

Two dimensional contour plots were established using reduced polynomial equation. Keeping one parameter stationary and varying others, contour plots were constructed by using sigma plot version 11.0. Plot is shown in figure 7.48.

**Response Surface Plots**

Response surface plots are very helpful in learning interaction effects of the independent variables. Plot is shown in figure 7.49.

**Evaluation of model / Check point analysis**

In order to assess the reliability of the model, two factors were conducted by varying the process variables at values other than that of the responses were estimated by using the equations and experimental procedure. The comparison between the experimental and predicted values of the responses for these experiments was reported.

**5.2.4.4 Characterization of optimized RLX loaded NLCs**

■ **Drug release kinetics:** [18]

Data obtain from the *in vitro* release studies were fitted to various kinetic models such as zero order, first order and Higuchi model. Equation and calculation for all kinetic models are as same as discussed in section 5.2.3.3.2. Results are shown in table 7.42.

■ **Drug excipients compatibility studies:** [17,35,36]

Drug excipients compatibility study of drug and optimize batch NLC-8 was carried out by FTIR and spectrum was recorded in the range of 4000 – 400 $cm^{-1}$ using FTIR spectrophotometer to find out any incompatibility in prepared NLCs. Results are shown in table 7.43.

■ **Particle size and zeta potential measurements:**

The particle size and zeta potential of optimize batch NLC-8 was measured with Malvern zeta sizer (Nano ZS, Malvern Instruments, Worcestershire, UK) as same as described in section 5.2.3.4. Measured particle size and Zeta potential of optimized batch is shown in figure 7.52 and figure 7.53, respectively.

■ **Differential scanning calorimetry:** [8,17,36]

Thermograms of RLX, GMS, physical mixture of drug, solid lipid & liquid lipid and optimize batch NLC-8 were obtained by using Differential scanning calorimeter (DSC TA – 60, Shimadzu, Japan). A procedure was as same as described in section 5.2.2.3. Result is shown in figure7.54, 7.55 and 7.56.

■ **X-Ray diffraction studies:**[8,17,36]

X-ray diffraction study can become useful tool to find out amorphous or crystalline nature of formulations. XRD study of RLX, GMS, physical mixture and optimize batch NLC-8 was carried out by using Panalytical Xpert PRO X Ray Diffractometer (Xpert Pro MPD, Panalytical, The Netherlands). A procedure was as same as described in section 5.2.2.3. Result is shown in figure 7.57, 7.58 and 7.59.

**Surface morphology study:** [10,11,41]

The surface morphology and shape of solid particles can be observed by electron microscopy. Surface morphology of optimized batch NLC-8 was obtained using Transmission Electron Microscope (TEM) (Philips Tecnai-20). NLCs were dispersed in distilled water and drop of dispersion was placed on carbon coated copper grid followed by drying. This grid was mounted in the instrument and photographs were taken at various magnifications. Result is shown in figure 7.60.

■ **Stability study:** [19,35,47]

The selected formulation in dried form was subjected to stability studies as per ICH guidelines. The samples were placed in vials and kept at $25^0$ C ± $2^0$ C / 60% ± 5% RH and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber (Macro scientific work Pvt Ltd, Delhi) over period of six months. The samples were analyzed for entrapment efficiency and physical appearance at 0, 15, 30, 60, 120 & 180 days. Result is shown in table 7.44. Cumulative drug release and drug excipients compatibility study was also carried out at the end of study as shown in table 7.45 and 7.46, respectively.

**5.2.5 STATISTICAL ANALYSIS:**

Statistical analysis of the obtained data was carried out by Graph Pad Instat program version 3.01 (Graph Pad Software, Inc. CA, USA) using student's *t*– test. The level of statistically significance between formulations was selected as less than $p<0.05$.

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## 6. *In vivo* study

To study the bioavailability of RLX, *in vivo* pharmacokinetic study was carried out in all three optimized formulations IC-6, ME-8 and NLC-8 as per the protocol discussed underneath.

### 6.1 Experimental animals[1-3]

The experimental protocol in the present study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC), SBKS medical college and research institute, Sumandeep Vidyapeeth, Vadodara bearing registration number (SVU/DP/IAEC/2014/03/18). The experiment was carried out on healthy female Wistar rats weighing 200-250g. Rats were housed in polypropylene cages, maintained under standardized condition (12 h light/dark cycle, 24°C, 35-60 % humidity) and allowed free access to diet (Nav Maharashtra oil mills ltd, Pune) and purified drinking water *ad libitium*.

### 6.2 Bioanalytical method [1,2,4,5]

In the present research work, HPLC (UFLC, Shimadzu corporation, Japan) prominence liquid chromatographic system was used for chromatographic separation which is controlled by LC solution software (Version 1.24 Sp1, Shimadzu corporation, Japan). The system was equipped with Binary pump (LC 20AD version 1.10, Shimadzu corporation, Japan), a manual injector, a column (C18 250mm × 4.6 mm, 5µm) (Luna, Phenominax, USA) and a photo diode array (PDA) detector (SPD 20A version 1.08, Shimadzu corporation, Japan). The mobile phase consisted of a 67% 0.05M ammonium acetate (pH was adjusted to 4.0 with glacial acetic acid) and 33% acetonitrile. The mobile phase was prepared daily and degassed by sonication and filtered through a 0.45µm membrane filter before use. The column was maintained at room temperature.

The mobile phase was delivered isocratically with a flow rate of 1 ml min−1, the injection volume was 20µl and the wavelength for UV detection was 288 nm.

### 6.3 Experimental design[5]

The animals were fasted at least 12 h prior to dose administrations and for 4 h after dosing with free access to water. Animals were divided into four groups each consisting of six animals. All animals were given different formulations group wise as described underneath.

Group I: Control group (Plain RLX suspension in 0.5% w/v sodium CMC[1,6], 15mg/kg, p.o.)

Group II: Formulation 1 (optimized Inclusion complex IC-6, 15mg/kg[2,4], p.o.)

Group III: Formulation 2 (optimized microemulsion ME-8, 15mg/kg, p.o.)

Group IV: Formulation 3 (optimized nanostructured lipid carrier NLC-8, 15mg/kg, p.o.)

Serial blood samples (0.5ml) were withdrawn through capillary inserted in to retro orbital plexus under mild ether anesthesia at a time interval of predose, 0.25, 1, 2, 4, 6, 8, 10, 12 and 24 h post dose. Blood samples were collected in micro centrifuge tubes containing anticoagulant (3.8% w/v sodium citrate[4]). The plasma samples were collected immediately from aforementioned samples after centrifugation at 5,000 rpm at 4°C for 10 min and stored immediately at -20°C until further analysis[1,7]. Samples were analyzed by standard HPLC method after sample extraction procedure as discussed in section 5.2.1.3.

**6.4 Pharmacokinetic data analysis:**

PK solver add-in program for Microsoft Excel (version 1.0, China) was used for the estimation of Pharmacokinetic parameters. Various parameters like maximum plasma concentration ($C_{max}$) and time for achieving maximum plasma concentration ($T_{max}$) were obtained directly from the graph between plasma concentration and time. Area under curve $[AUC]_{0-24}$ was measured by trapezoidal method up to last point of measurement. Relative bioavailability (F) was calculated by dividing $[AUC]_{0-24}$ of formulation with plain drug suspension[6]. Each experiment was carried out in triplicate and result is shown in table 7.46.

**6.5 Statistical analysis:**

Statistical analysis of the obtained data was carried out by one way analysis of variance (ANOVA) using student's *t*-test. Graph Pad Instat program version 3.01(Graph Pad Software, Inc. CA, USA) was utilized to determine the significance difference between formulations. The level of statistically significance was selected as less than $p<0.05$.

**6.6 References**

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# 7. RESULTS AND DISCUSSION

## 7.1 RESULTS:

### 7.1.1 Preformulation study of drug:

#### 7.1.1.1 Organoleptic properties of RLX

Table 7.1: Organoleptic properties of RLX

| Sr. no. | Parameter | Observation |
|---|---|---|
| 1 | Colour | Pale yellow |
| 2 | Odour | Odourless |
| 3 | Appearance | Fine powder |

#### 7.1.1.2 Melting point determination of RLX

Table 7.2: Melting point determination of RLX

| Reported Melting Point | Observed Melting Point |
|---|---|
| 267-269°c | 266°c-269°c |

#### 7.1.1.3 Method for estimation of RLX

- **Determination of UV absorption maxima for Drug:**

Table 7.3: Determination of $\lambda_{max}$ for RLX

| Drug | $\lambda_{max}$ (nm) |
|---|---|
| RLX | 288 |

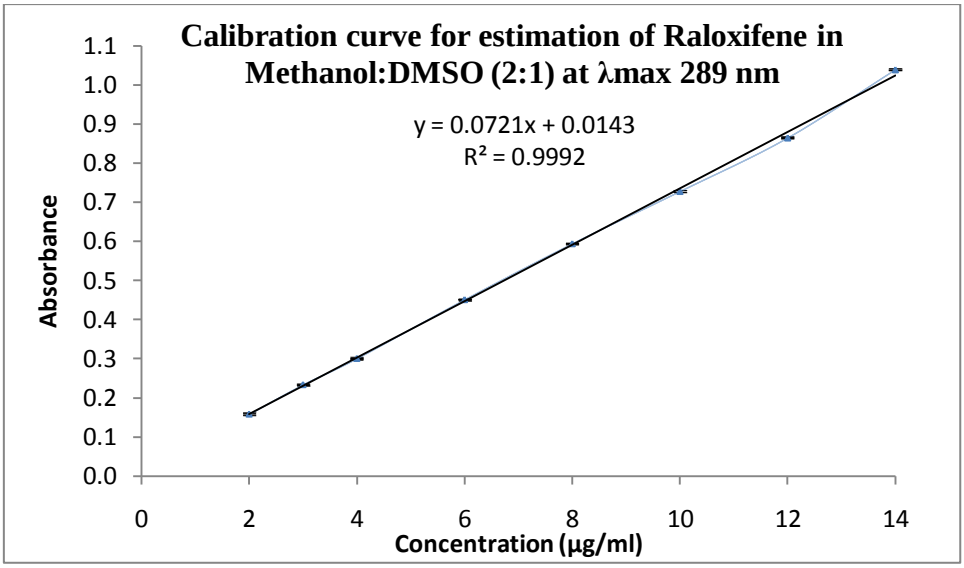
- **Preparation of Calibration curve of Drug:**

**Calibration curve of RLX for estimation in Methanol: DMSO (2:1)**

Table 7.4: Data for calibration curve of RLX for estimation in Methanol: DMSO (2:1)

| Sr. no. | Concentration (µg/ml) | Absorbance | | | Mean ± SD |
|---|---|---|---|---|---|
| | | I | II | III | |
| 1 | 2 | 0.158 | 0.156 | 0.161 | 0.158 ± 0.003 |
| 2 | 3 | 0.234 | 0.235 | 0.232 | 0.234 ± 0.002 |
| 3 | 4 | 0.301 | 0.299 | 0.302 | 0.301 ± 0.002 |
| 4 | 6 | 0.450 | 0.452 | 0.451 | 0.451 ± 0.001 |
| 5 | 8 | 0.594 | 0.595 | 0.593 | 0.594 ± 0.001 |
| 6 | 10 | 0.725 | 0.73 | 0.728 | 0.728 ± 0.003 |
| 7 | 12 | 0.866 | 0.864 | 0.863 | 0.864 ± 0.002 |
| 8 | 14 | 1.038 | 1.037 | 1.041 | 1.039 ± 0.002 |

Value are expressed as mean ±SD; n=3




*n=3

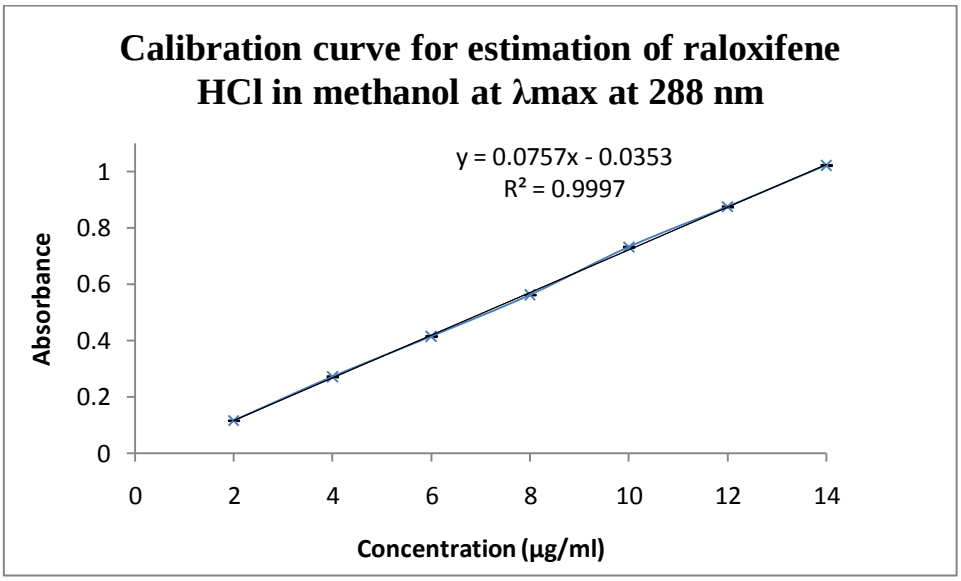
Figure 7.1: Calibration curve of RLX for estimation in methanol: DMSO (2:1)

**Calibration curve of RLX for estimation in Methanol**

Table 7.5: Data for calibration curve of RLX for estimation in methanol

| Sr. no. | Concentration (µg/ml) | Absorbance | | | Mean ± SD |
|---|---|---|---|---|---|
| | | I | II | III | |
| 1 | 2 | 0.115 | 0.117 | 0.117 | 0.116 ± 0.001 |
| 2 | 4 | 0.272 | 0.27 | 0.273 | 0.272 ± 0.002 |
| 3 | 6 | 0.413 | 0.416 | 0.417 | 0.415 ± 0.002 |
| 4 | 8 | 0.562 | 0.561 | 0.563 | 0.562 ± 0.001 |
| 5 | 10 | 0.731 | 0.733 | 0.731 | 0.732 ± 0.001 |
| 6 | 12 | 0.875 | 0.874 | 0.877 | 0.875 ± 0.002 |
| 7 | 14 | 1.022 | 1.021 | 1.024 | 1.022 ± 0.002 |

Value are expressed as mean ± SD; n=3




*n=3

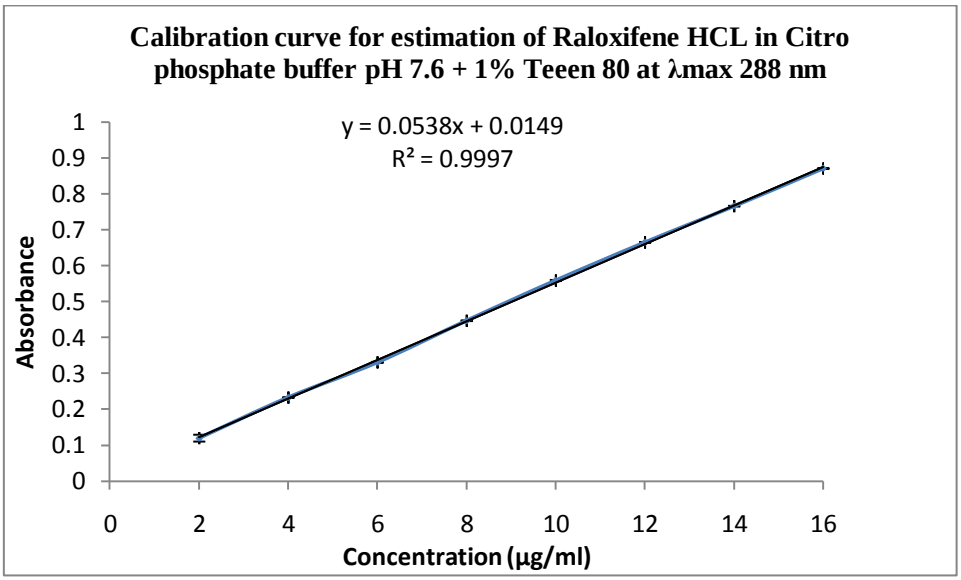
Figure 7.2: Calibration curve of RLX for estimation in methanol

**Calibration curve of RLX for estimation in citro phosphate buffer pH 7.6 containing 1% of polysorbate 80**

Table 7.6: Data for calibration curve of RLX for estimation in citro phosphate buffer pH 7.6 containing 1% of polysorbate 80

| Sr. no. | Concentration (µg/ml) | Absorbance | | | Mean ± SD |
|---|---|---|---|---|---|
| | | I | II | III | |
| 1 | 2 | 0.120 | 0.131 | 0.112 | 0.121 ± 0.010 |
| 2 | 4 | 0.233 | 0.234 | 0.233 | 0.233 ± 0.001 |
| 3 | 6 | 0.331 | 0.333 | 0.330 | 0.331 ± 0.002 |
| 4 | 8 | 0.447 | 0.446 | 0.448 | 0.447 ± 0.001 |
| 5 | 10 | 0.559 | 0.557 | 0.560 | 0.559 ± 0.002 |
| 6 | 12 | 0.665 | 0.663 | 0.665 | 0.664 ± 0.001 |
| 7 | 14 | 0.766 | 0.764 | 0.765 | 0.765 ± 0.001 |
| 8 | 16 | 0.871 | 0.872 | 0.872 | 0.872 ± 0.001 |

Value are expressed as mean ± SD; n=3




*n=3

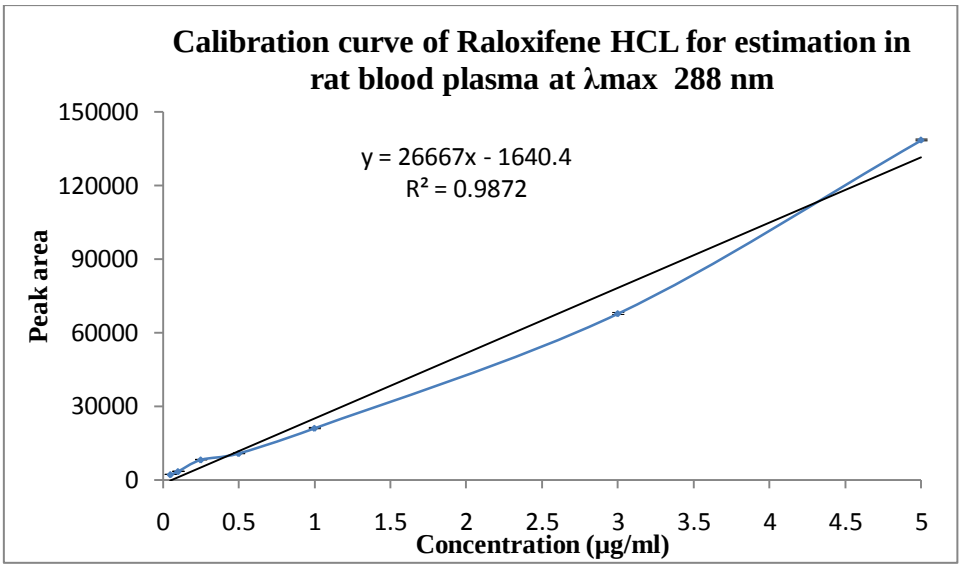
Figure 7.3: Calibration curve of RLX for estimation in citro phosphate buffer pH 7.6 containing 1% of polysorbate 80

**Calibration curve of RLX for estimation in rat blood plasma**

Table 7.7: Data for calibration curve of RLX for estimation in rat blood plasma

| Sr. no. | Concentration (µg/ml) | Peak area | | | Mean ± SD |
|---|---|---|---|---|---|
| | | I | II | III | |
| 1 | 0.05 | 2253.55 | 2299.08 | 2231.84 | 2261.49 ± 34.32 |
| 2 | 0.1 | 3465.38 | 3534.57 | 3545.44 | 3515.13 ± 43.43 |
| 3 | 0.25 | 8285.54 | 8112.22 | 8374.35 | 8257.37 ± 133.32 |
| 4 | 0.5 | 10865.79 | 11065.41 | 10668.14 | 10866.45 ± 198.64 |
| 5 | 1 | 20835.4 | 21344.96 | 21230.26 | 21136.87 ± 267.31 |
| 6 | 3 | 67420.66 | 67987.32 | 68197.33 | 67868.44 ± 401.75 |
| 7 | 5 | 138583.98 | 138255.14 | 138991.78 | 138610.30 ± 369.02 |

Value are expressed as mean ± SD; n=3




*n=3

Figure 7.4: Calibration curve of RLX for estimation in rat blood plasma

### 7.1.1.4 Solubility determination

Table 7.8: Solubility of RLX in different solvent systems

| Sr. no. | Solvent system | Solubility ± SD (mg/ml) |
|---|---|---|
| 1 | Water | 0.11 ± 0.02 |
| 2 | Dimethyl sulphoxide | >48 |
| 3 | Dichloro methane | 0.95 ± 0.05 |
| 4 | Dimethyl formamide | >42 |
| 5 | Methanol | 7.57 ± 0.64 |
| 6 | Chloroform | 0.32 ± 0.11 |
| 7 | Chloroform:Methanol (1:1) | 3.12 ± 0.51 |
| 8 | Chloroform:Methanol (1:2) | 5.34 ± 0.12 |
| 9 | Chloroform:Methanol (1:3) | 6.58 ± 0.95 |
| 10 | Phosphate buffer pH 6.8 | 0.13 ± 0.05 |
| 11 | Phosphate buffer pH 7.4 | 0.18 ± 0.08 |
| 12 | Phosphate buffer pH 7.4 + 1% Tween 80 | 0.25 ± 0.10 |
| 13 | Phosphate buffer pH 7.4 + 1% SLS | 0.28 ± 0.11 |
| 14 | Phosphate buffer pH 7.4 + 2% SLS | 0.51 ± 0.09 |
| 15 | Citro phosphate buffer pH 7.6 | 0.22 ± 0.10 |
| 16 | Citro phosphate buffer pH 7.6 + 1% cetrimide | 0.45 ± 0.13 |
| 17 | Citro phosphate buffer pH 7.6 + 1% Tween 80 | 0.35 ± 0.18 |

Value are expressed as mean ± SD; n=3

### 7.1.2 Formulation and characterization of drug loaded inclusion complexes:-

#### 7.1.2.1 Preliminary studies

Physicochemical incompatibility between drug and β-cyclodextrin was studied by drug excipients study as discussed underneath

**Drug excipients compatibility studies:**

Drug excipients compatibility study was carried out by FT-IR for pure drug alone, β – cyclodextrin and with physical mixture of drug & β-cyclodextrin.

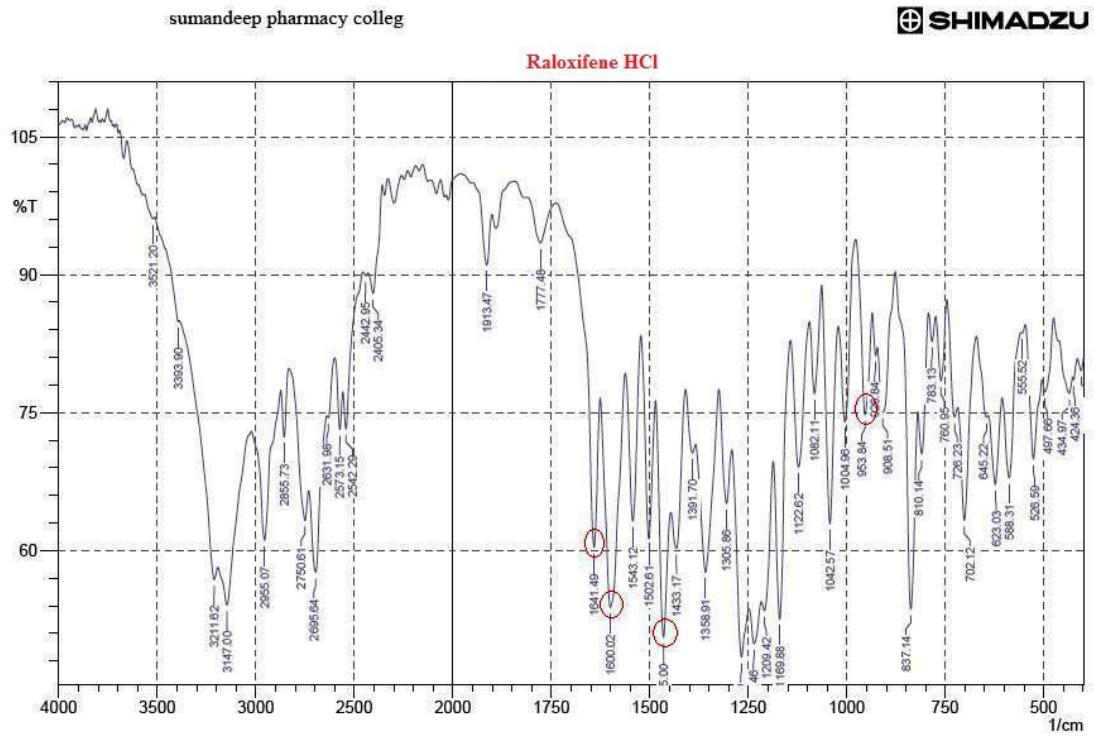



Figure 7.5: FTIR of RLX

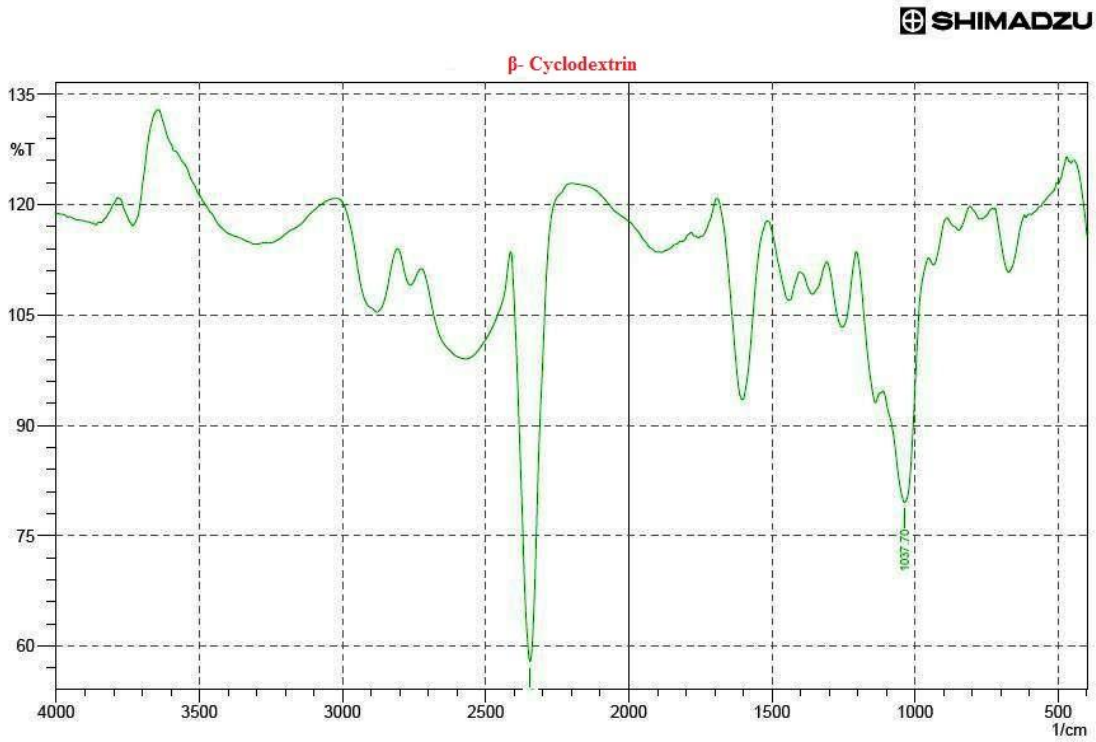



Figure 7.6: FTIR of β-CD

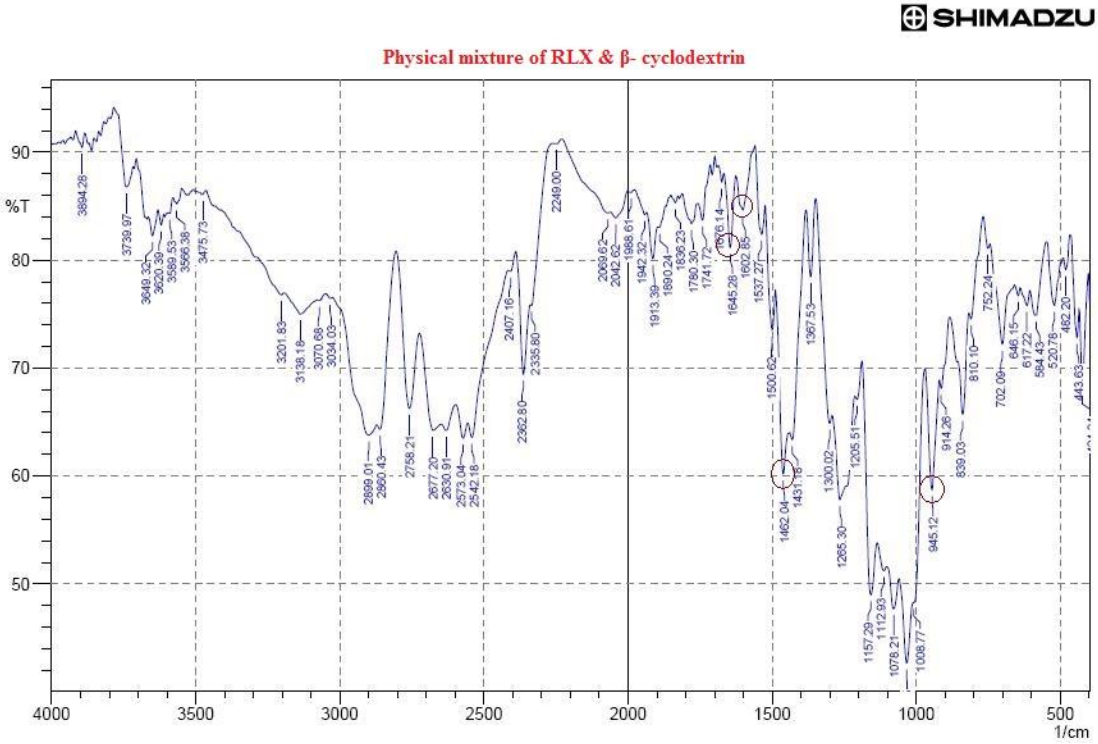



Figure 7.7: FTIR of Physical mixture of RLX & β-CD

Table 7.9: Comparison of characteristic peaks between RLX & β-CD physical mixture

| Functional group | Characteristic peaks ($cm^{-1}$) | |
|---|---|---|
| | Plain RLX drug | Physical mixture |
| Benzene ring | 953.84 | 945.12 |
| -S- benzothiophene | 1465.00 | 1462.04 |
| -C-O-C- stretching | 1600.02 | 1602.85 |
| C=O stretching | 1641.49 | 1645.28 |

### 7.1.2.2 Characterization of Inclusion complexes

**Practical yield, drug content and water solubility:**

Practical yield, drug content and water solubility of prepared inclusion complexes are shown in below mentioned table.

Table 7.10: Practical yield, drug content and water solubility of inclusion complexes

| Sr. no. | Batch code | Practical Yield ± SD (%) | Drug content ± SD (%) | Water solubility ± SD(mg/ml) |
|---|---|---|---|---|
| 1 | IC-1 | 99.08 ± 0.82 | 48.05 ± 1.34 | 0.17 ± 0.02 |
| 2 | IC-2 | 98.87 ± 0.98 | 32.10 ± 0.75 | 0.22 ± 0.02 |
| 3 | IC-3 | 98.95 ± 0.87 | 24.13 ± 0.54 | 0.29 ± 0.03 |
| 4 | IC-4 | 96.33 ± 1.76 | 47.19 ± 0.91 | 0.39 ± 0.02 |
| 5 | IC-5 | 96.12 ± 1.38 | 32.02 ± 1.18 | 0.50 ± 0.05 |
| 6 | IC-6 | 96.41 ± 2.05 | 24.11 ± 0.53 | 0.61 ± 0.04* |
| 7 | IC-7 | 97.61 ± 0.72 | 46.74 ± 1.08 | 0.28 ± 0.03 |
| 8 | IC-8 | 97.24 ± 1.03 | 31.62 ± 1.12 | 0.41 ± 0.04 |
| 9 | IC-9 | 96.68 ± 1.14 | 23.88 ± 0.24 | 0.48 ± 0.02 |
| Water solubility of pure drug | | | 0.11 ± 0.02mg/ml | |

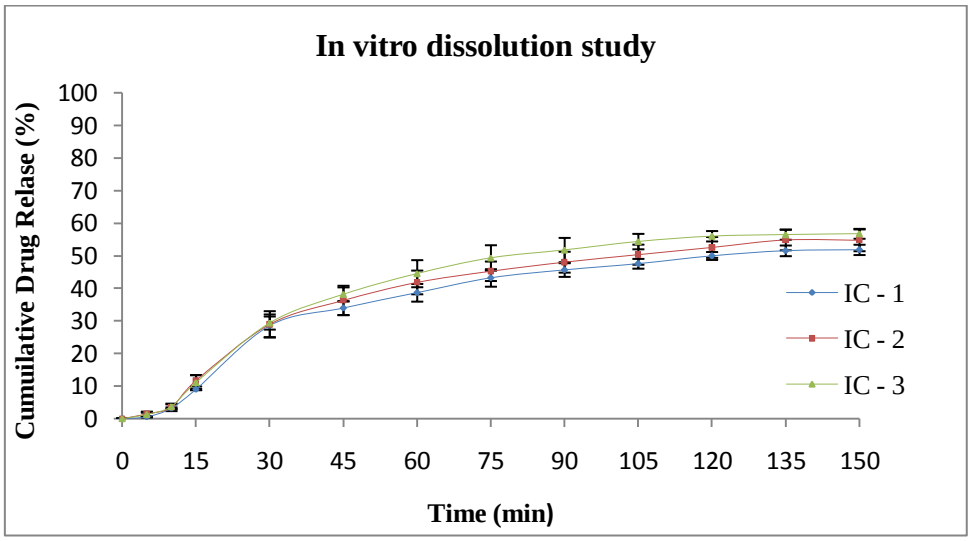
Value are expressed as mean ± SD; n=3, (*p< 0.05)

***In vitro* dissolution study:**

Table 7.11: *In vitro* dissolution profile of plain RLX and prepared inclusion complexes

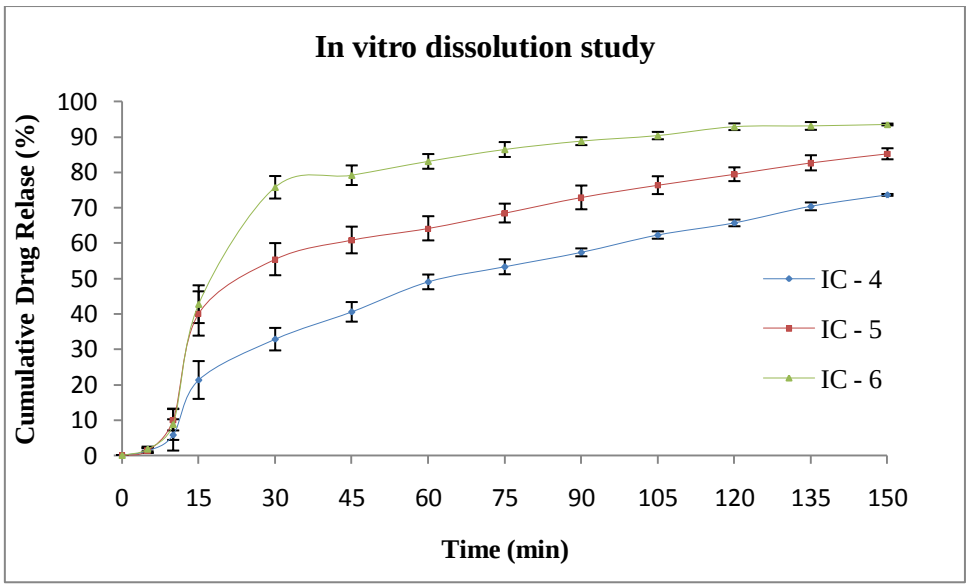
| Time (min) | Percent cumulative drug release (% CDR) ± SD | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Plain Drug** | **IC - 1** | **IC -2** | **IC - 3** | **IC – 4** | **IC -5** | **IC - 6** | **IC - 7** | **IC -8** | **IC - 9** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 1.12±0.85 | 0.55±0.19 | 1.45±0.71 | 1.29±0.54 | 1.31±0.52 | 1.38±0.70 | 1.77±0.66 | 1.09±0.14 | 3.24±1.26 | 4.94±0.72 |
| 10 | 3.10±1.10 | 3.17±0.30 | 3.47±1.15 | 3.70±0.69 | 5.74±0.90 | 10.02±3.02 | 8.75±4.42 | 2.56±0.85 | 11.29±1.59 | 16.42±2.85 |
| 15 | 10.72±3.77 | 8.96±0.18 | 11.64±1.76 | 11.05±2.29 | 21.26±2.99 | 40.05±6.26 | 42.68±5.34 | 11.37±1.36 | 25.82±4.06 | 42.24±5.39 |
| 30 | 28.12±4.31 | 28.54±3.48 | 28.97±4.05 | 29.37±2.01 | 32.80±2.52 | 55.40±4.54 | 75.71±3.18 | 29.52±262 | 37.22±5.45 | 55.23±2.72 |
| 45 | 36.32±2.56 | 33.96±2.12 | 36.31±4.52 | 38.18±2.16 | 40.51±2.86 | 60.82±3.77 | 79.12±2.77 | 38.04±2.58 | 48.59±5.72 | 59.70±1.03 |
| 60 | 41.03±2.13 | 38.67±2.72 | 41.85±3.64 | 44.54±4.16 | 48.98±1.67 | 64.12±3.42 | 83.02±2.08 | 43.24±2.07 | 54.23±3.40 | 63.39±2.93 |
| 75 | 44.22±1.06 | 43.23±2.69 | 45.28±2.99 | 49.37±3.91 | 53.25±2.33 | 68.43±2.66 | 86.38±2.11 | 48.18±1.40 | 59.42±2.50 | 67.78±0.95 |
| 90 | 46.34±0.45 | 45.66±2.10 | 48.07±3.21 | 51.80±3.73 | 57.32±1.89 | 72.85±3.36 | 88.75±1.12 | 52.52±2.51 | 63.86±2.81 | 72.10±0.55 |
| 105 | 47.92±0.91 | 47.58±1.50 | 50.38±3.08 | 54.40±2.37 | 62.22±1.15 | 76.31±2.52 | 90.32±1.04 | 54.60±2.73 | 68.31±4.39 | 75.46±1.28 |
| 120 | 49.18±1.15 | 49.98±1.22 | 52.60±3.20 | 56.04±1.60 | 65.64±0.86 | 79.40±1.95 | 92.82±0.94 | 56.73±1.08 | 71.74±4.08 | 78.46±1.03 |
| 135 | 50.05±0.55 | 51.56±1.64 | 54.90±3.18 | 56.51±1.54 | 70.32±2.73 | 82.62±2.14 | 93.05±1.10 | 57.29±1.30 | 75.63±3.74 | 81.39±0.81 |
| 150 | 50.39±0.39 | 51.85±1.58 | 54.79±3.35 | 56.79±1.52 | 73.56±2.15 | 85.18±1.56 | 93.46±0.26 | 58.26±1.13 | 78.30±3.15 | 82.66±0.33 |

Value are expressed as mean ±SD; n=3, $p<0.05$ compared with plain drug suspension



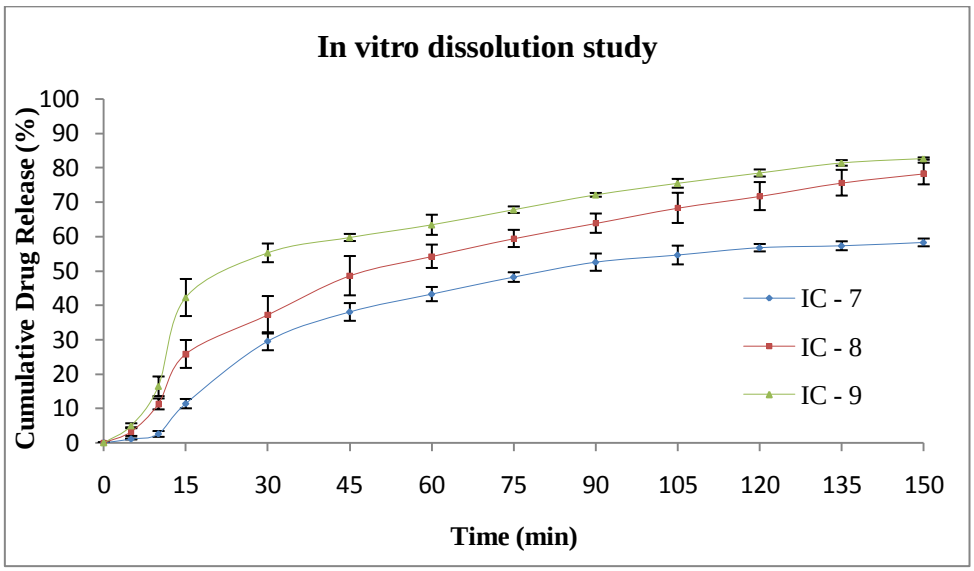

*n=3

Figure 7.8: *In vitro* dissolution profile of IC-1, IC-2 and IC-3



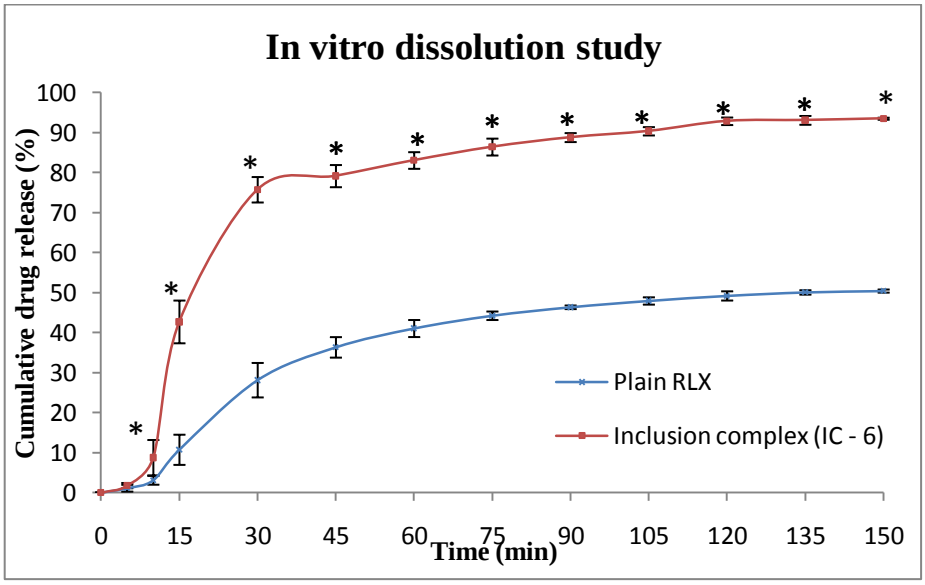

*n=3

Figure 7.9: *In vitro* dissolution profile of IC-4, IC-5 and IC-6




*n=3

Figure 7.10: *In vitro* dissolution profile of IC-7, IC-8 and IC-9




*n=3

Figure 7.11: *In vitro* dissolution profile of plain RLX and inclusion complex (IC - 6) (*p<0.05)

**Drug excipients compatibility studies:**

Drug excipients compatibility study was carried out by FTIR for optimized batch IC – 6 as shown below.

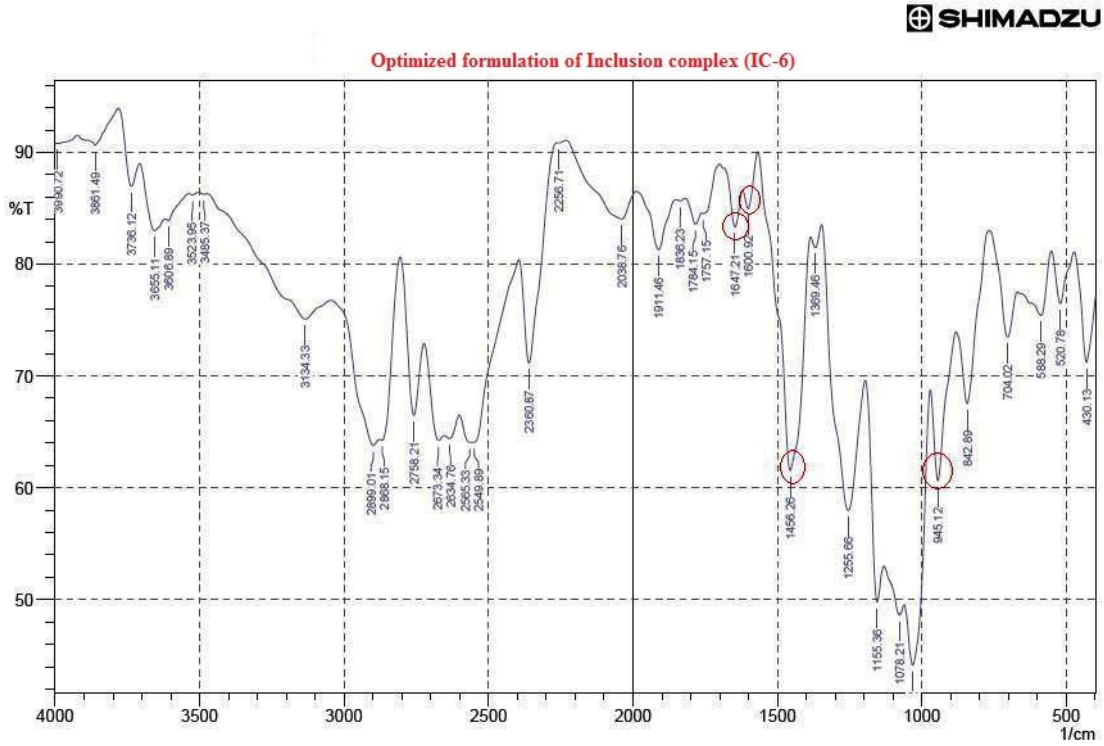



Figure 7.12: FTIR of inclusion complex of RLX with β-CD [IC – 6]

Table 7.12: Comparison of characteristic peaks between RLX & inclusion complex (IC- 6)

| **Functional group** | **Characteristic peaks ($cm^{-1}$)** | |
|---|---|---|
| | **Plain RLX drug** | **Inclusion complex (IC- 6)** |
| Benzene ring | 953.84 | 945.12 |
| -S- benzothiophene | 1465.00 | 1456.26 |
| -C-O-C- stretching | 1600.02 | 1600.92 |
| C=O stretching | 1641.49 | 1647.21 |

**Differential scanning calorimetry:**

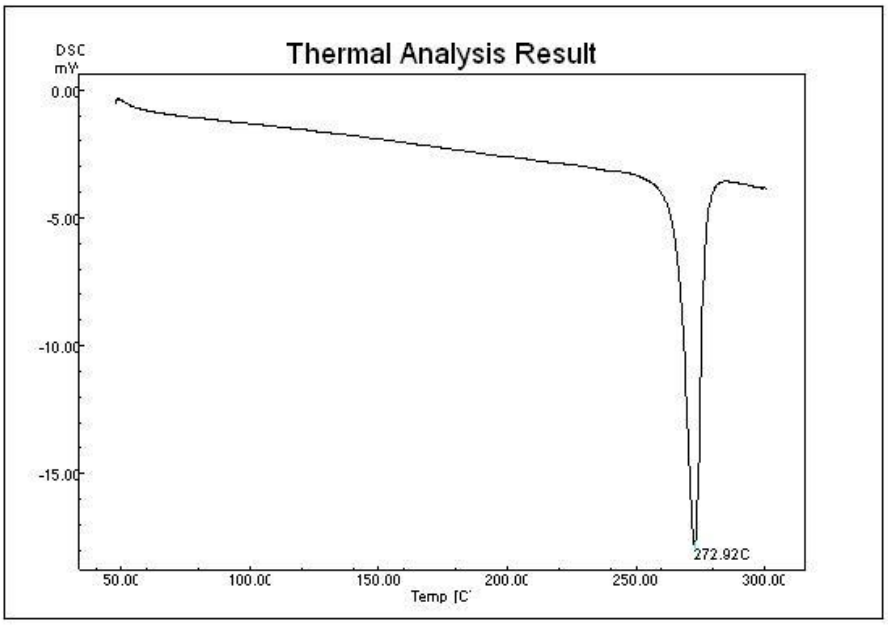



Figure 7.13: DSC thermogram of RLX

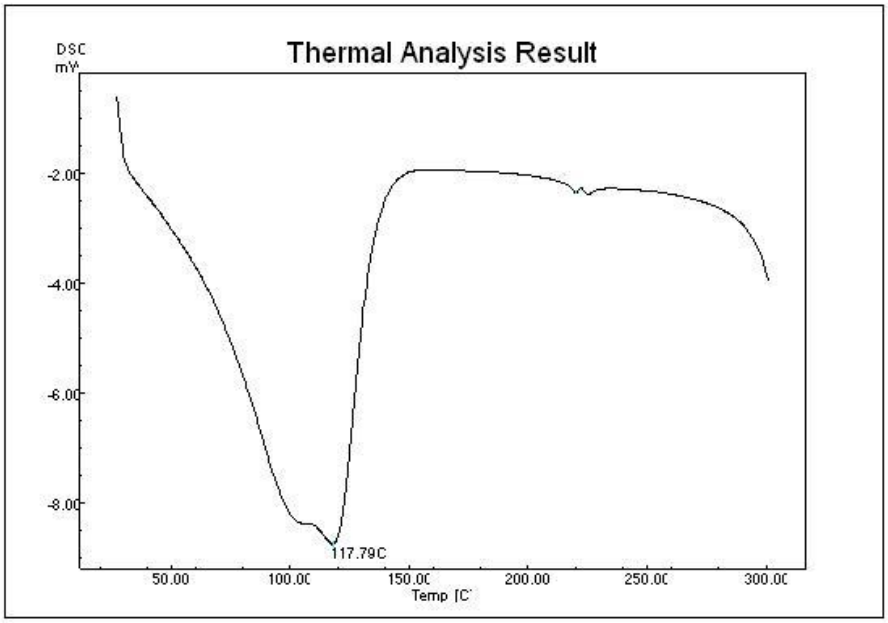



Figure 7.14: DSC thermogram of β-CD

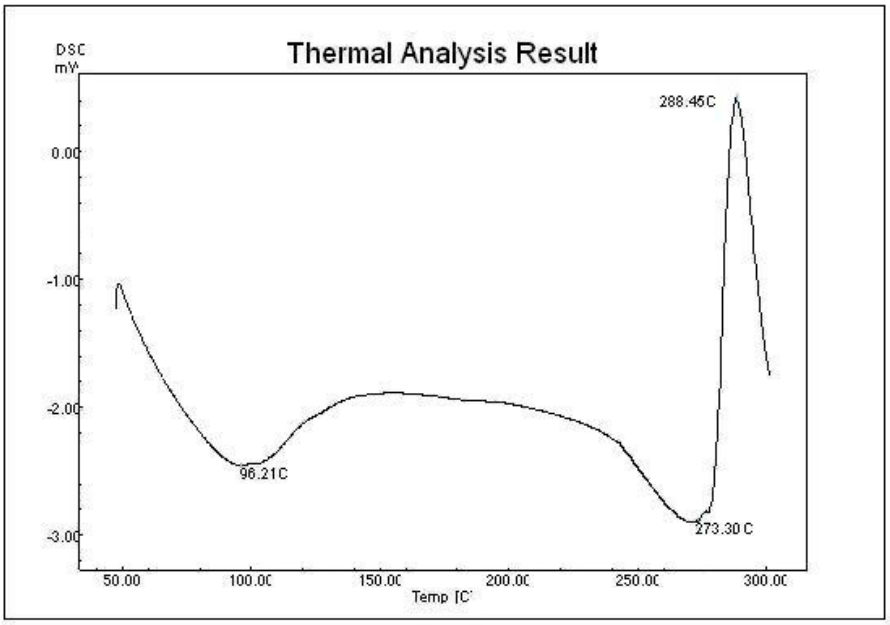



Figure 7.15: DSC thermogram of inclusion complex of RLX with β-CD [IC – 6]

**X-Ray diffraction studies:**

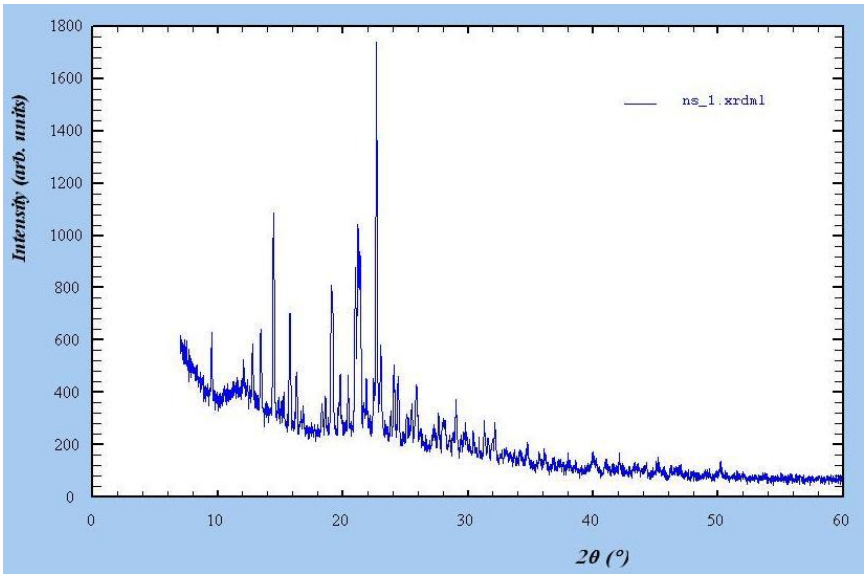



Figure 7.16: XRD pattern of RLX

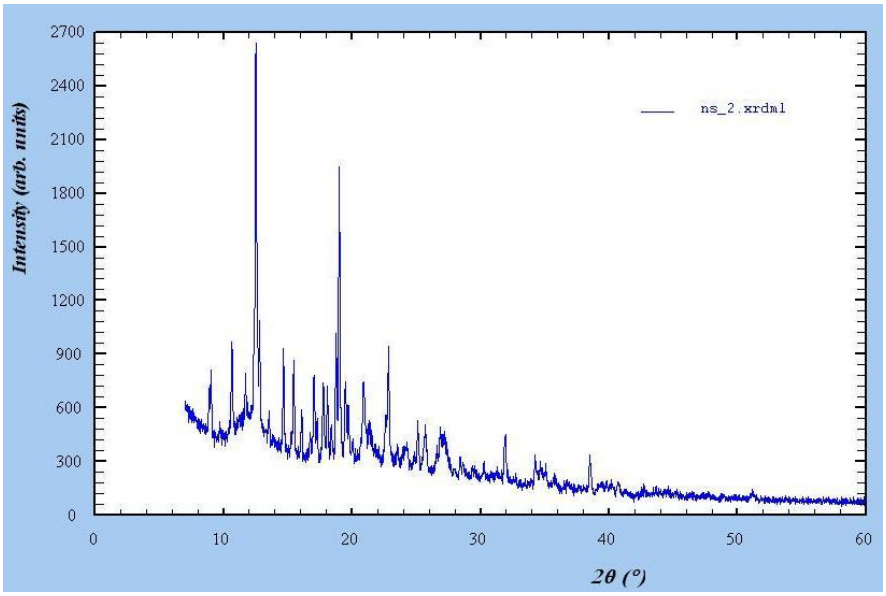



Figure 7.17: XRD pattern of β-CD

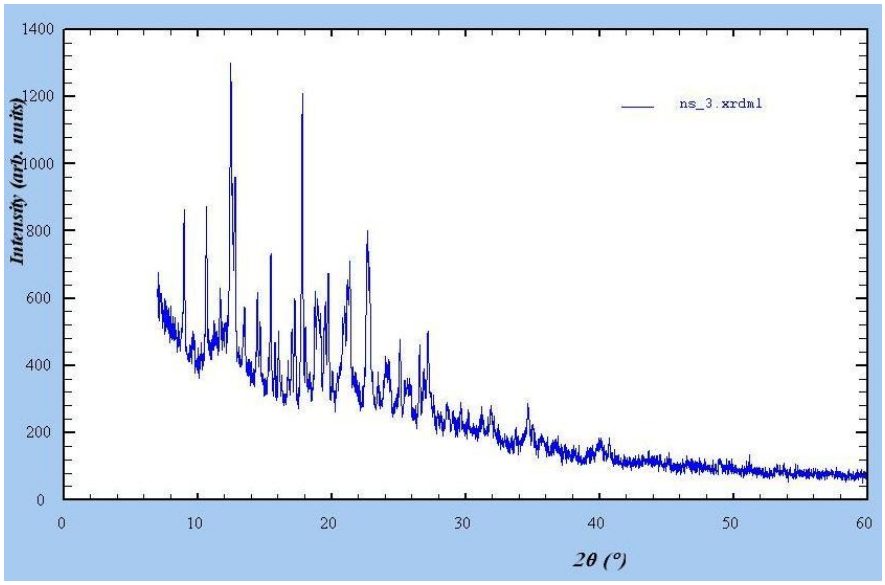



Figure 7.18: XRD pattern for physical mixture of RLX & β-CD

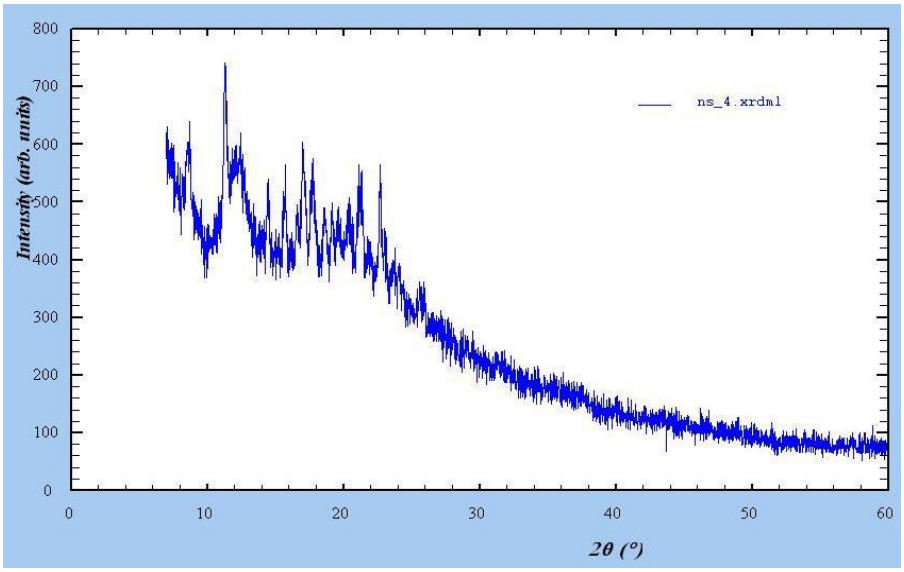



Figure 7.19: XRD pattern for inclusion complex of RLX with β-CD [IC – 6]

**Surface morphology study:**

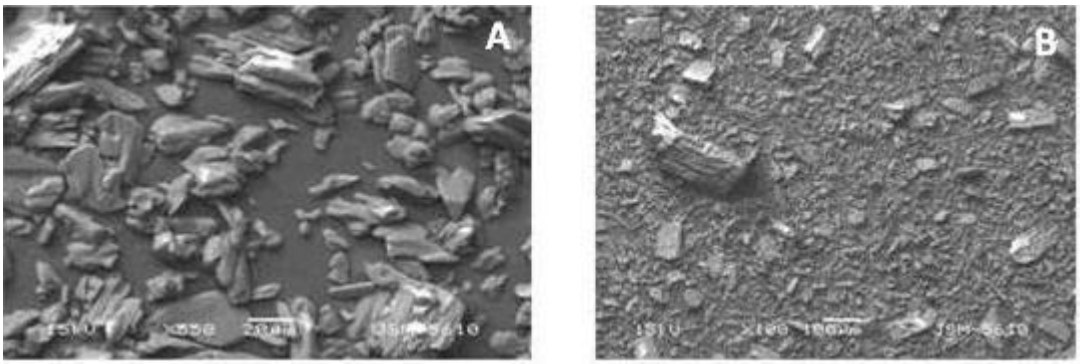



Figure 7.20: SEM image of RLX at different magnifications (A) 550 x (B) 100 x

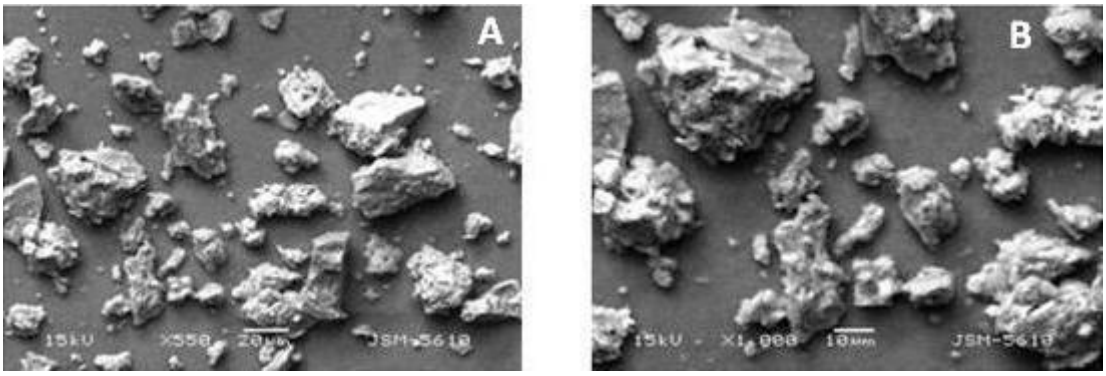



Figure 7.21: SEM image for inclusion complex of RLX with β-CD [IC – 6] at different magnifications (A) 550 x (B) 1000 x

**Stability study:**

The optimized batch was kept at $25^0$ C ± $2^0$ C /60% ± 5% RH and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber over period of six months. Physical appearance & drug content of IC -6 in stability study is given in table mentioned below.

Table 7.13: Physical appearance & drug content of IC -6 in stability study

| Sr. no. | Time (Days) | $25^0$C ± $2^0$C / 60% ± 5% RH | | $40^0$ C ± $2^0$C / 75% ± 5% RH | |
|---|---|---|---|---|---|
| | | Physical appearance | Drug content ± SD (%) | Physical appearance | Drug content ± SD (%) |
| 1 | 0 | Yellow free flowing Powder | 24.11 ± 0.53 | Yellow free flowing Powder | 24.11 ± 0.53 |
| 2 | 15 | | 24.10 ± 0.12 | | 24.10 ± 0.37 |
| 3 | 30 | | 24.08 ± 0.75 | | 24.12 ± 0.52 |
| 4 | 60 | | 24.09 ± 0.44 | | 24.06 ± 0.21 |
| 5 | 120 | | 24.05 ± 0.36 | | 23.98 ± 0.11 |
| 6 | 180 | | 24.00 ± 0.33 | | 23.95 ± 0.17 |

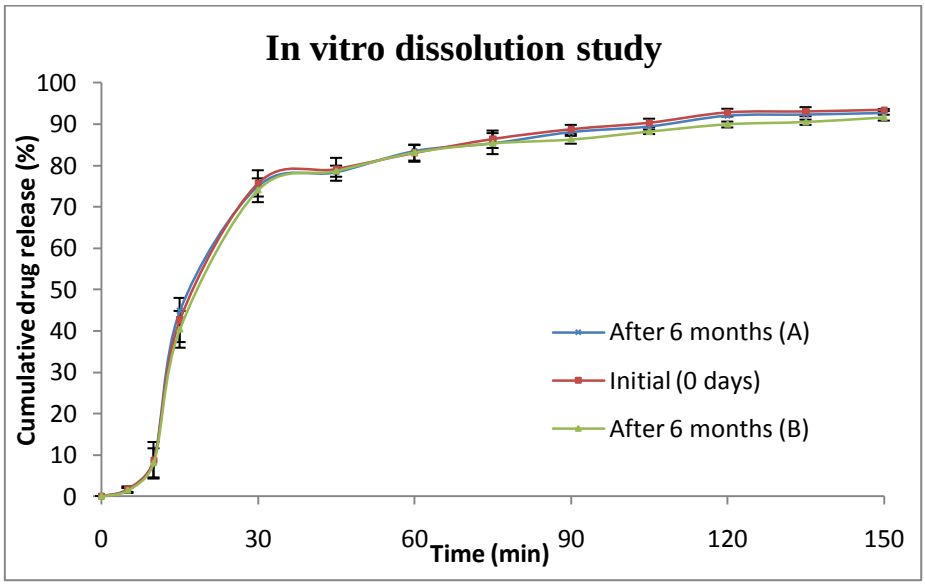
Value are expressed as mean ± SD; n=3, $p$>0.05

Percent cumulative drug release at initial stage and at the end of stability study is given in table mentioned below.

Table 7.14: *In vitro* drug release profile of IC – 6 after 6 months of stability period

| Time (min) | Percent cumulative drug release (% CDR) ± SD | | |
|---|---|---|---|
| | Initial (0 day) | After 6 months | |
| | | $25^0$C ± $2^0$C / 60% ± 5% RH | $40^0$ C ± $2^0$C / 75% ± 5% RH |
| 0 | 0 | 0 | 0 |
| 5 | 1.77 ± 0.66 | 1.72 ± 0.71 | 1.44 ± 0.61 |
| 10 | 8.75 ± 4.42 | 8.81 ± 3.99 | 8.13 ± 3.52 |
| 15 | 42.68 ± 5.34 | 44.86 ± 4.85 | 40.42 ± 4.45 |
| 30 | 75.71 ± 3.18 | 74.99 ± 3.54 | 74.05 ± 2.88 |
| 45 | 79.12 ± 2.77 | 78.35 ± 2.70 | 78.66 ± 1.34 |
| 60 | 83.02 ± 2.08 | 83.45 ± 1.95 | 83.11 ± 1.88 |
| 75 | 86.38 ± 2.11 | 85.33 ± 2.41 | 85.33 ± 2.54 |
| 90 | 88.75 ± 1.12 | 88.04 ± 1.22 | 86.26 ± 0.95 |
| 105 | 90.32 ± 1.04 | 89.41 ± 1.19 | 88.18 ± 0.54 |
| 120 | 92.82 ± 0.94 | 92.01 ± 1.06 | 89.95 ± 0.71 |
| 135 | 93.05 ± 1.10 | 92.28 ± 0.84 | 90.50± 0.63 |
| 150 | 93.46 ± 0.26 | 92.67 ± 0.15 | 91.61 ± 0.71 |

Value are expressed as mean ± SD; n=3, $p$>0.05




*n=3

Figure 7.22: *In vitro* release profile of IC - 6 after 6 months of stability period.
(A= $25^0$ C ± $2^0$ C / 60% ± 5% RH, B= $40^0$ C ± $2^0$ C / 75% ± 5% RH)

Compatibility study of optimized batch of inclusion complex carried out by FTIR is shown below.

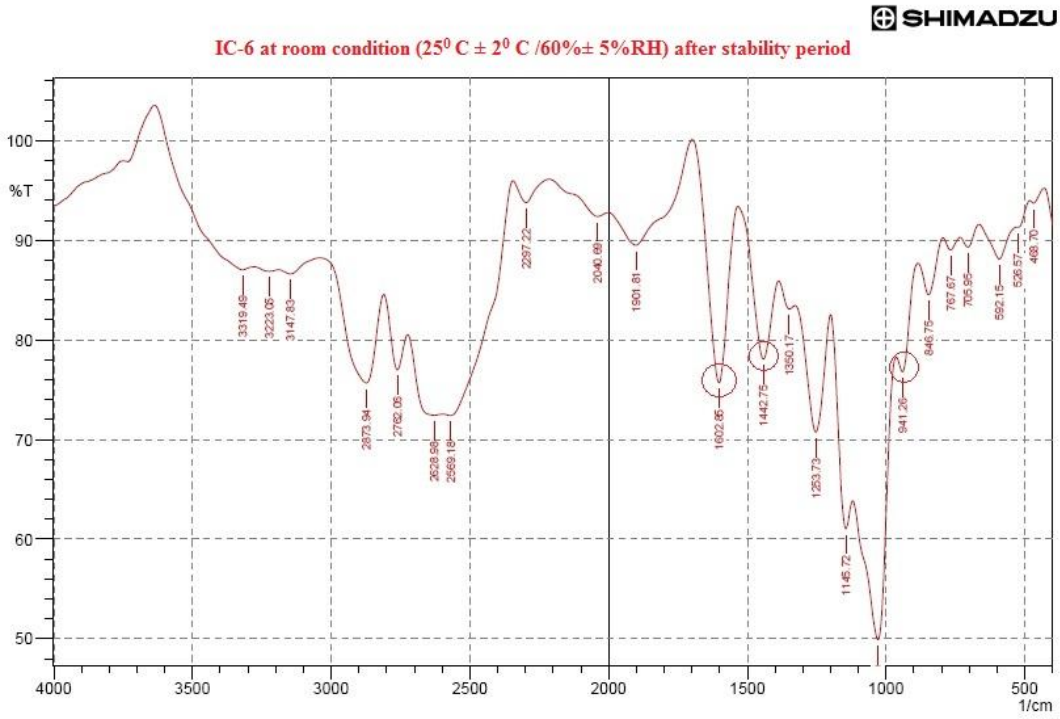



Figure 7.23: FTIR of IC-6 after six months of stability period at room condition

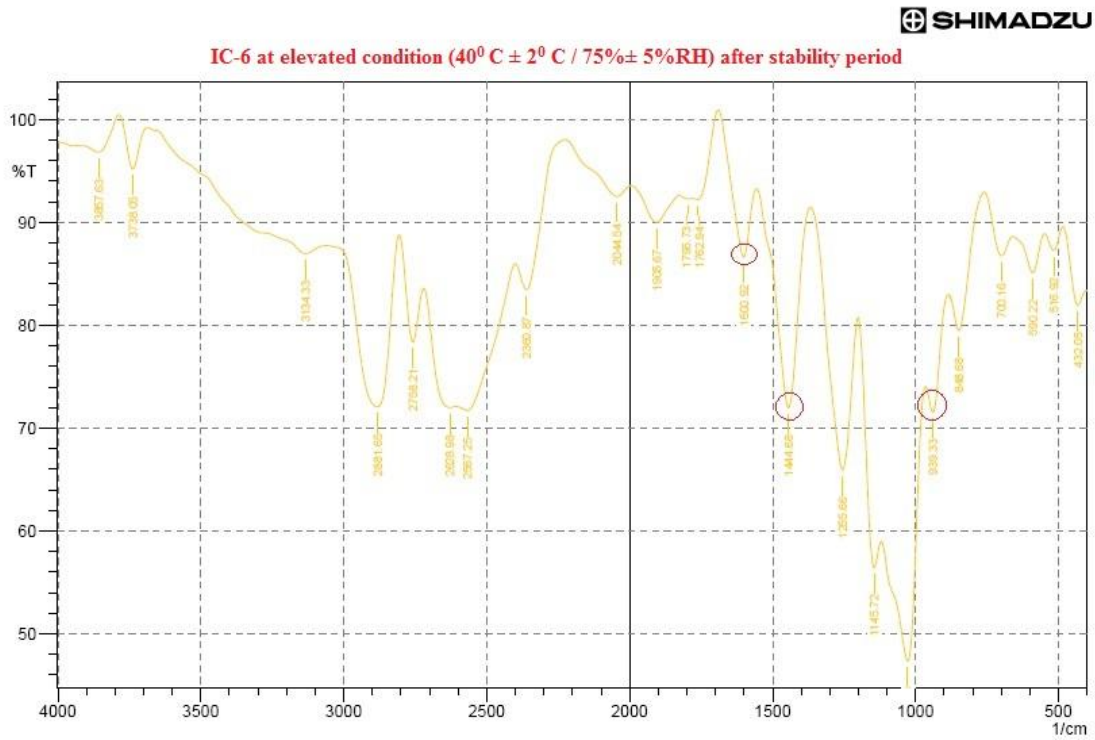



Figure 7.24: FTIR of IC - 6 after six months of stability period at elevated condition

Table 7.15: Comparison of characteristic peaks between RLX & optimized batch IC – 6 kept at different storage conditions

| Functional group | Characteristic peaks ($cm^{-1}$) | | |
|---|---|---|---|
| | Plain RLX drug | Optimized batch (IC- 6) | |
| | | A | B |
| Benzene ring | 953.84 | 941.26 | 939.33 |
| -S- benzothiophene | 1465.00 | 1442.75 | 1444.68 |
| -C-O-C- stretching | 1600.02 | 1602.86 | 1600.92 |

(A= $25^0$ C ± $2^0$ C /60% ± 5% RH, B= $40^0$ C ± $2^0$ C / 75% ± 5% RH)

### 7.1.3 Formulation and characterization of drug loaded microemulsion:-

#### 7.1.3.1 Preliminary studies

**Screening of components:**

Drug solubility was carried out in different oils, surfactants and co surfactants. Result is shown in below table**.**

Table 7.16: Solubility of RLX in various oils, surfactants and cosurfactants

| Category | Name of ingredients | Solubility ± SD (mg/g) |
|---|---|---|
| Oil | Soyabean oil | 1.21 ± 0.18 |
| | Isopropyl myristate | 1.14 ± 0.14 |
| | Castor oil | 0.73 ± 0.12 |
| | Capmul MCM C8 | 2.55 ± 0.96 |
| | Olive oil | 2.07 ± 0.12 |
| | Oleic acid | 2.08 ± 0.24 |
| | Seasame oil | 0.95 ± 0.10 |
| Surfactants | Capmul PG 8 | 2.29 ± 0.15 |
| | Captex 300 | 1.75 ± 0.21 |
| | Labrafil ICM 1944 CS | 1.24 ± 0.18 |
| | Lebrafec CC | 0.74 ± 0.07 |
| | Acconon C | 1.52 ± 0.13 |
| | Tween 20 | 7.25 ± 2.10 |
| | Tween 80 | 5.52 ± 1.05 |
| | Transcutol | 1.44 ± 0.12 |
| Cosurfactants | Polyethylene glycol (PEG) 200 | 6.14 ± 1.45 |
| | PEG 400 | 8.50 ± 1.94 |
| | Propylene glycol | 1.99 ± 0.25 |

Value are expressed as mean ± SD; n=3

**Drug excipients compatibility studies**

Table 7.17: Drug excipients compatibility study for microemulsion

| Sample | Precipitation or crystallization | | | | | Phase separation | | | | | Color change | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Days | | | | | | | | | | | | | | |
| | 3 | 6 | 9 | 12 | 15 | 3 | 6 | 9 | 12 | 15 | 3 | 6 | 9 | 12 | 15 |
| Drug+ oil | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |
| Drug + Tween 20 | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |
| Drug + PEG 400 | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |
| Physical mixture (oil and $S_{mix}$) | × | × | × | × | × | × | × | × | × | × | × | × | × | × | × |

(× showing no changes compared to initial '0' day observation)

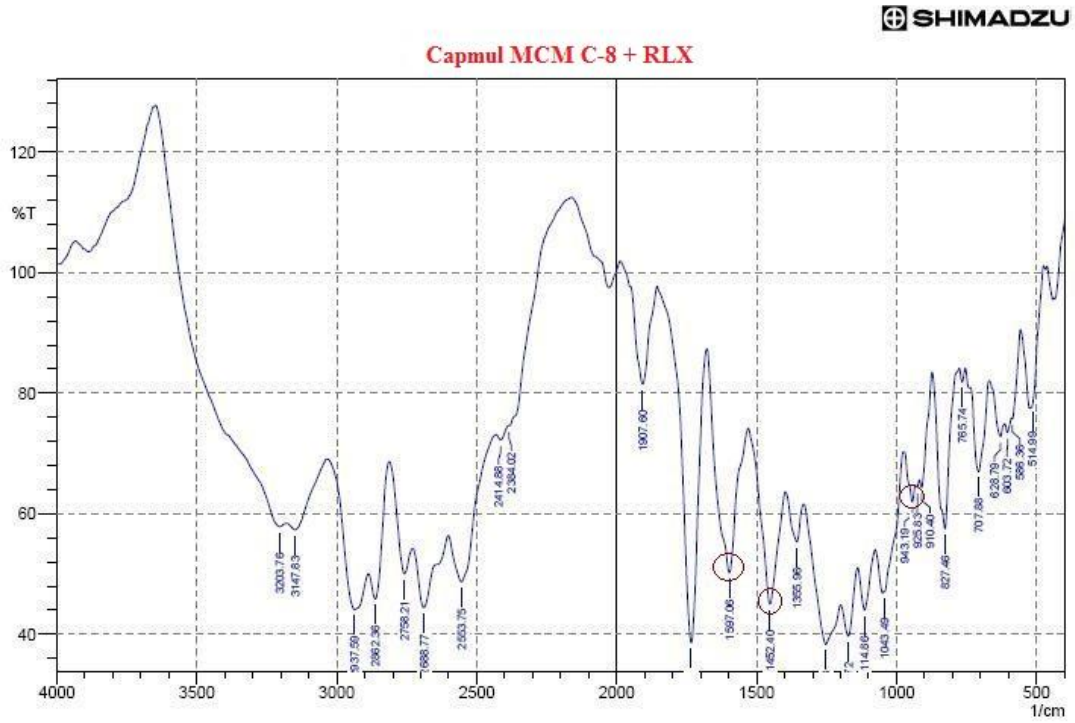



Figure 7.25: FTIR spectrum of Capmul MCM C8 + RLX

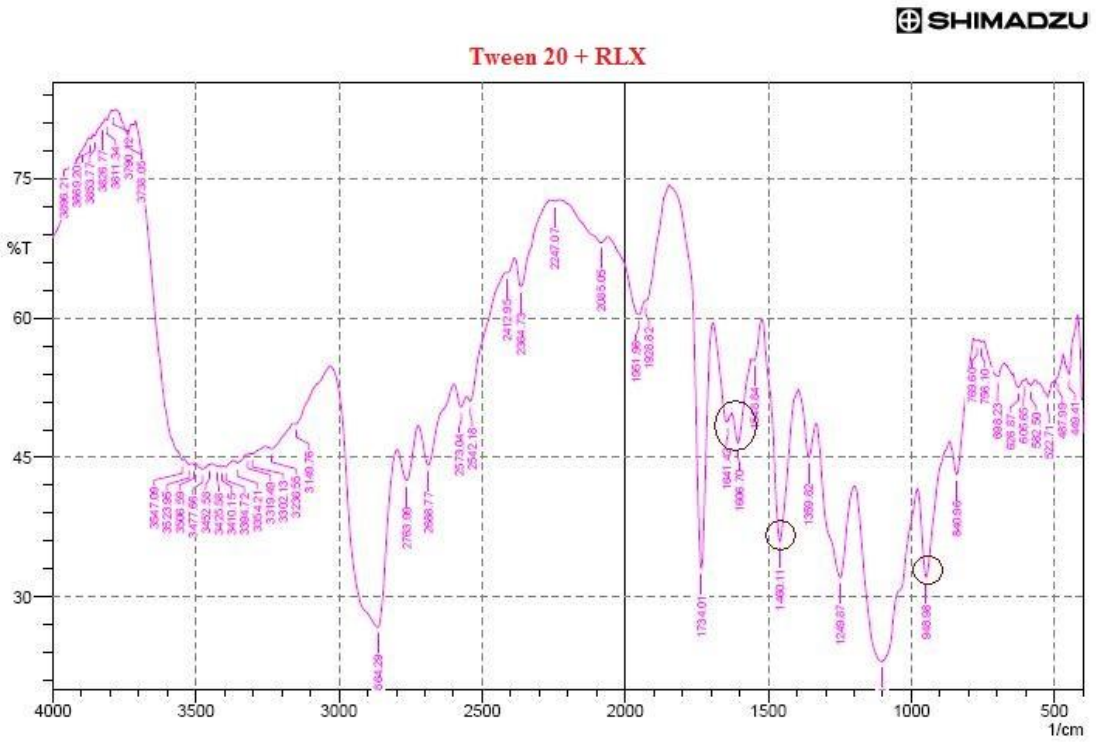



Figure 7.26: FTIR spectrum of Tween 20 + RLX

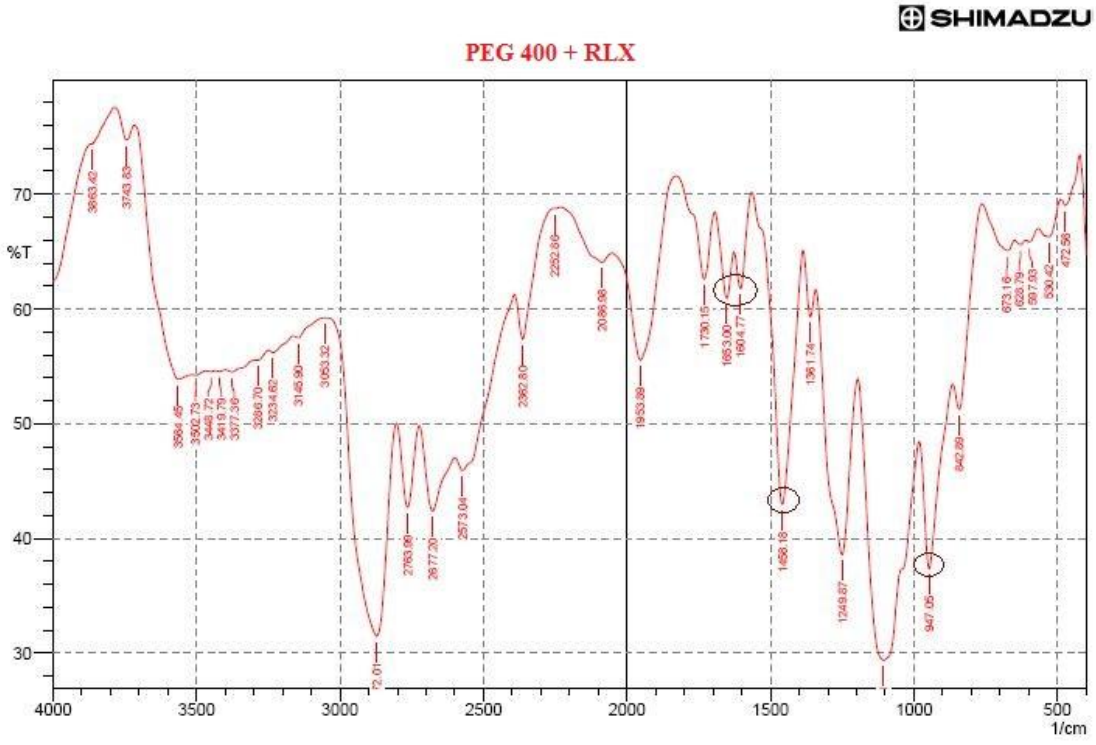



Figure 7.27: FTIR spectrum of PEG 400 + RLX

Table 7.18: Comparison of characteristic peaks between RLX & physical mixture with microemulsion excipients

| Functional group | Characteristic peaks ($cm^{-1}$) | | | |
|---|---|---|---|---|
| | Plain RLX drug | Capmul MCM C8 + RLX | Tween 20 + RLX | PEG 400 + RLX |
| Benzene ring | 953.84 | 943.19 | 948.98 | 947.05 |
| -S- benzothiophene | 1465.00 | 1452.40 | 1460.11 | 1458.18 |
| -C-O-C- stretching | 1600.02 | 1597.06 | 1606.70 | 1604.77 |
| C=O stretching | 1641.49 | --- | 1641.42 | 1653.00 |

**Pseudo ternary phase diagram:**

Table 7.19: Data for Pseudo ternary phase diagram

| Sr. No: | Oil :Smix (Ratio) | Oil :Smix (ml) | Dilution with water until microemulsion remains clear(ml) | | | |
|---|---|---|---|---|---|---|
| | | | 1:1 (s:cos) | 2:1 (s:cos) | 3:1 (s:cos) | 4:1 (s:cos) |
| 1 | 1 : 9 | 0.3 : 2.7 | Infinite | Infinite | Infinite | Infinite |
| 2 | 2 : 8 | 0.6 : 2.4 | 7.54 | 16.15 | Infinite | 40.15 |
| 3 | 3 : 7 | 0.9 : 2.1 | 0.63 | 5.24 | 7.65 | 6.25 |
| 4 | 4 : 6 | 1.2 : 1.8 | 0.58 | 0.70 | 1.45 | 1.05 |
| 5 | 5 : 5 | 1.5 : 1.5 | 0.51 | 0.62 | 0.95 | 0.62 |
| 6 | 6 : 4 | 1.8 : 1.2 | 0.55 | 0.56 | 0.75 | 0.60 |
| 7 | 7 : 3 | 2.1 : 0.9 | 0.48 | 0.52 | 0.70 | 0.50 |
| 8 | 8 : 2 | 2.4 : 0.6 | 0.47 | 0.48 | 0.68 | 0.50 |
| 9 | 9 : 1 | 2.7 : 0.3 | 0.44 | 0.47 | 0.62 | 0.49 |

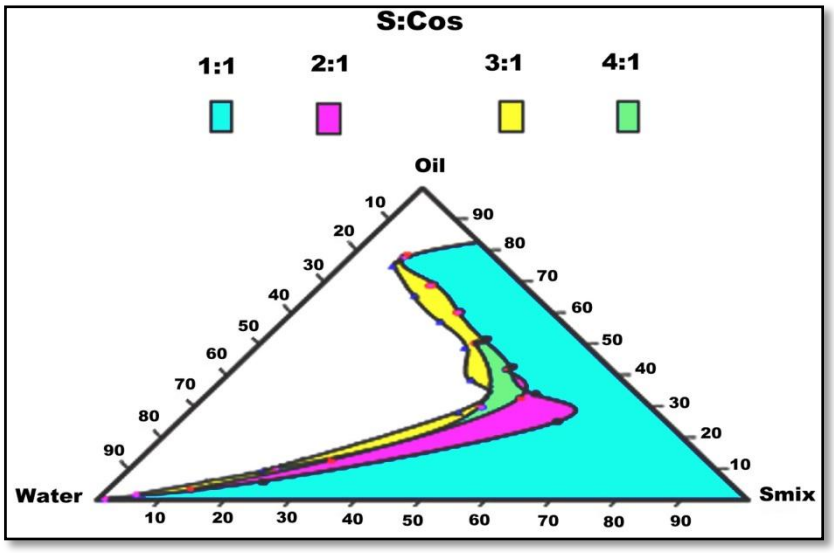



Figure 7.28: Pseudo ternary phase diagram of microemulsion composed of oil (Capmul MCM C8), $S_{mix}$ (Tween 20 : PEG 400) and water

### 7.1.3.2 Optimization of microemulsion

#### 7.1.3.2.1 Selection of microemulsions by checking primary parameters:-

All formulations were selected based on their maximum transmittancy measured by UV spectrophotometer and visually checked by necked eyes.

**Percentage Transmittance and visual transmittancy:**

Table 7.20: Data of primary parameters of all microemulsion batches

| Sr. no | Formulation code | % Transmittance ± SD ($\lambda_{max}$ 630nm) | Visual clarity (After 24 h) | | | |
|---|---|---|---|---|---|---|
| | | | Undiluted | | Diluted (10 times) | |
| | | | Phase separation | Precipitation | Phase separation | Precipitation |
| 1 | RLX-1 | 98.4 ± 0.20 | × | √√ | × | √√ |
| 2 | RLX-2 | 99.7 ± 0.31 | × | ×× | × | ×× |
| 3 | RLX-3 | 99.9 ± 0.16 | × | ×× | × | ×× |
| 4 | RLX-4 | 100.0 ± 0.10 | × | ×× | × | ×× |
| 5 | RLX-5 | 97.7 ± 0.23 | × | ×× | × | √√ |
| 6 | RLX-6 | 99.1 ± 0.15 | × | ×× | × | ×× |
| 7 | RLX-7 | 99.8 ± 0.08 | × | ×× | × | ×× |
| 8 | RLX-8 | 99.9 ± 0.24 | × | ×× | × | ×× |
| 9 | RLX-9 | 71.7 ± 1.24 | √ | √√ | √ | √√ |
| 10 | RLX-10 | 88.7 ± 0.78 | × | ×× | × | √√ |
| 11 | RLX-11 | 95.2 ± 0.11 | × | ×× | × | ×× |
| 12 | RLX-12 | 98.7 ± 0.25 | × | ×× | × | ×× |
| 13 | RLX-13 | 61.7 ± 2.72 | √ | √√ | √ | √√ |
| 14 | RLX-14 | 75.6 ± 1.38 | × | √√ | √ | √√ |
| 15 | RLX-15 | 93.8 ± 0.40 | × | ×× | √ | √√ |
| 16 | RLX-16 | 98.1 ± 0.24 | × | ×× | × | ×× |

(× & ×× indicates no phase separation and no precipitation respectively. √ & √√ indicates phase separation and precipitation respectively.)

**7.1.3.2.2 Optimization of stable microemulsion with augmented drug loading**

From the result of primary parameters studied earlier, six batches were selected which were further analyzed for optimization of stable microemulsion with increased drug loading (6mg/g of microemulsion). Following evaluation tests were carried out for the optimization of microemulsion.

**Dilution test, centrifugation test and % transmittancy**:

Table 7.21: Data for microemulsions with augmented drug loading

| Sr. no. | Formulation code | Dilution test | Centrifugation test | % Transmittance ± SD (λmax-630nm) |
|---|---|---|---|---|
| 1 | ME-3 | Clear & transparent | No Phase separation | 98.8 ± 0.21 |
| 2 | ME-4 | | | 99.8 ± 0.08 |
| 3 | ME-7 | | | 99.4 ± 0.07 |
| 4 | ME-8 | | | 99.8 ± 0.15 |
| 5 | ME-12 | Insignificant Haziness | Phase separation | 96.6 ± 0.33 |
| 6 | ME-16 | Haziness | Phase separation | 96.1 ± 0.14 |

Value are expressed as mean ± SD; n=3

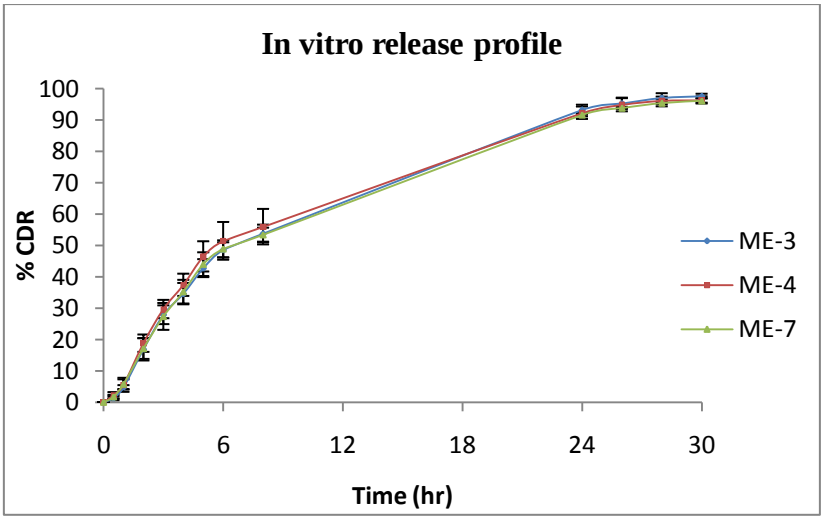
***In vitro* drug release:**

*In vitro* drug release study of selected microemulsions was carried out using dialysis membrane.

Table 7.22: *In vitro* drug release profile of plain RLX suspension and prepared microemulsions

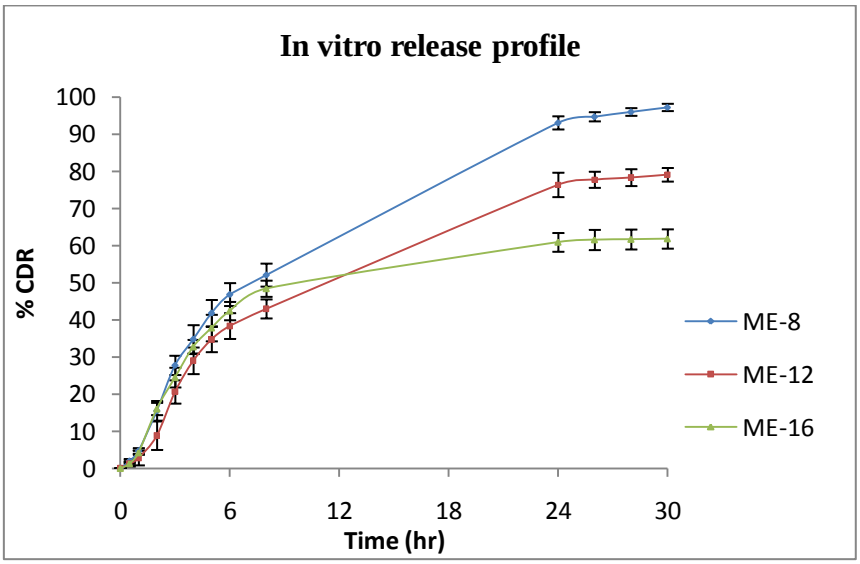
| Time (h) | Percent cumulative drug release (% CDR) ± SD | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Plain Drug suspension** | **ME-3** | **ME-4** | **ME-7** | **ME-8** | **ME-12** | **ME-16** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 0.15 ± 0.07 | 1.28 ± 0.63 | 2.23 ± 1.00 | 1.73 ± 0.63 | 1.96 ± 0.57 | 1.23 ± 0.73 | 1.27 ± 0.40 |
| 1 | 0.28 ± 0.09 | 4.74 ± 0.71 | 5.55 ± 2.24 | 5.76 ± 1.54 | 4.7 ± 0.76 | 2.81 ± 2.00 | 4.21 ± 0.56 |
| 2 | 0.58 ± 0.21 | 16.87 ± 3.56 | 18.82 ± 2.77 | 17.12 ± 3.31 | 15.5 ± 2.65 | 8.8 ± 3.84 | 15.99 ± 1.65 |
| 3 | 0.91 ± 0.24 | 27.89 ± 2.99 | 29.71 ± 2.92 | 27.36 ± 4.29 | 27.73 ± 2.59 | 20.57 ± 3.13 | 24.44 ± 2.65 |
| 4 | 1.44 ± 0.41 | 34.62 ± 3.41 | 37.44 ± 3.55 | 35.25 ± 3.77 | 34.75 ± 3.81 | 28.96 ± 3.58 | 32.69 ± 1.90 |
| 5 | 1.94 ± 0.33 | 42.75 ± 2.86 | 46.5 ± 4.82 | 44.02 ± 3.78 | 41.79 ± 3.55 | 34.69 ± 3.40 | 37.78 ± 3.57 |
| 6 | 2.26 ± 0.22 | 48.58 ± 2.41 | 51.45 ± 6.02 | 48.9 ± 2.72 | 46.8 ± 3.08 | 38.32 ± 3.47 | 42.35 ± 2.47 |
| 8 | 2.76 ± 0.27 | 53.8 ± 2.82 | 55.98 ± 5.66 | 53.42 ± 2.23 | 52.04 ± 3.09 | 42.93 ± 2.56 | 48.36 ± 2.18 |
| 24 | 9.59 ± 0.55 | 93.28 ± 1.58 | 92.3 ± 1.98 | 91.66 ± 0.53 | 93.02 ± 1.77 | 76.32 ± 3.28 | 60.85 ± 2.53 |
| 26 | 9.88 ± 0.70 | 95.32 ± 1.62 | 94.93 ± 2.18 | 93.84 ± 0.33 | 94.67 ± 1.24 | 77.7 ± 2.17 | 61.49 ± 2.72 |
| 28 | 10.18 ±0.84 | 97.1±1.42 | 96.23 ± 1.21 | 95.44 ± 1.11 | 95.96 ± 1.04 | 78.28 ± 2.27 | 61.62 ± 2.68 |
| 30 | 10.51 ± 0.87 | 97.65±0.70 | 96.39 ± 1.15 | 96.17 ± 0.67 | 97.18 ± 0.98 | 79.05 ± 1.83 | 61.76 ± 2.60 |

Value are expressed as mean ±SD; n=3, $p<0.05$ compared with plain drug suspension



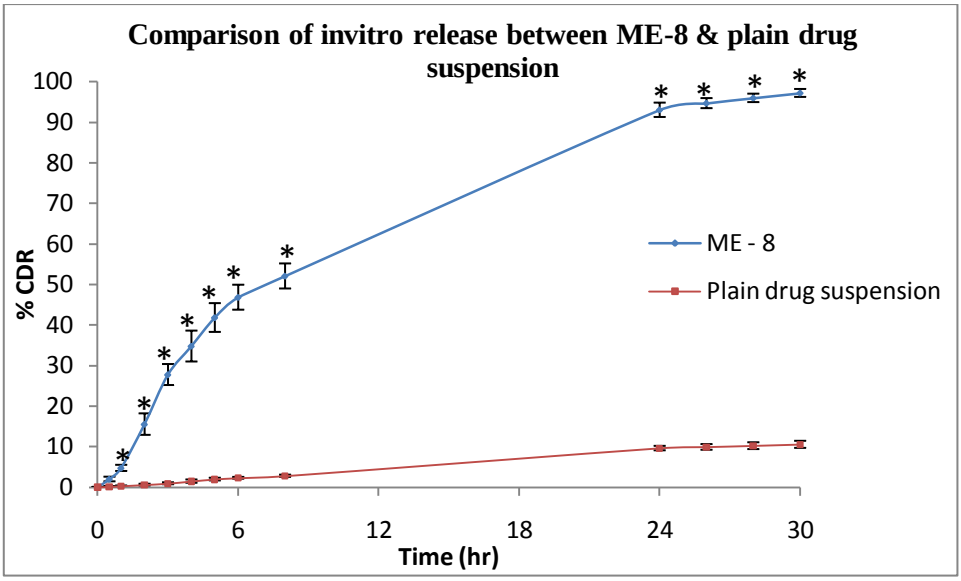

*n=3

Figure 7.29: *In vitro* release profile of ME-3, ME-4 and ME-7




*n=3

Figure 7.30: *In vitro* release profile of ME-8, ME-12 and ME-16




n=3

Figure 7.31: Comparison of *in vitro* release profile of ME-8 and plain drug suspension (* $p<0.05$)

**Drug release kinetics:**

Table 7.23: *In vitro* release kinetics of optimized batch ME-8

| Optimized batch | Regression coefficient ($R^2$) | | |
|---|---|---|---|
| | Zero order | First order | Higuchi model |
| ME - 8 | 0.925 | 0.586 | 0.981 |

### 7.1.3.3 Characterization of optimized microemulsion

Optimized microemulsion selected from the above study was characterized further by following parameters other than studied earlier.

**Dye solubility test**: Methylene blue dye was added to microemulsion sample and observed under microscope. It was found that dye was uniformly distributed throughout the microemulsion without any lump formation.

Table 7.24: Data for characterization of optimized batch ME-8

| Test | Optimized drug loaded microemulsion |
|---|---|
| Drug content ± SD (%) | 99.42 ± 0.22 |
| pH ± SD | 6.78 ± 0.17 |
| Conductivity ± SD (µS/cm) | 0.205 ± 0.24 |
| Viscosity ± SD (cp) | 84.57 ± 5.77 |
| Zeta potential (mV) | -5.05 |
| Globule size (nm) | 13.52 |

Value are expressed as mean ± SD; n=3

**Drug-excipients compatibility study:**

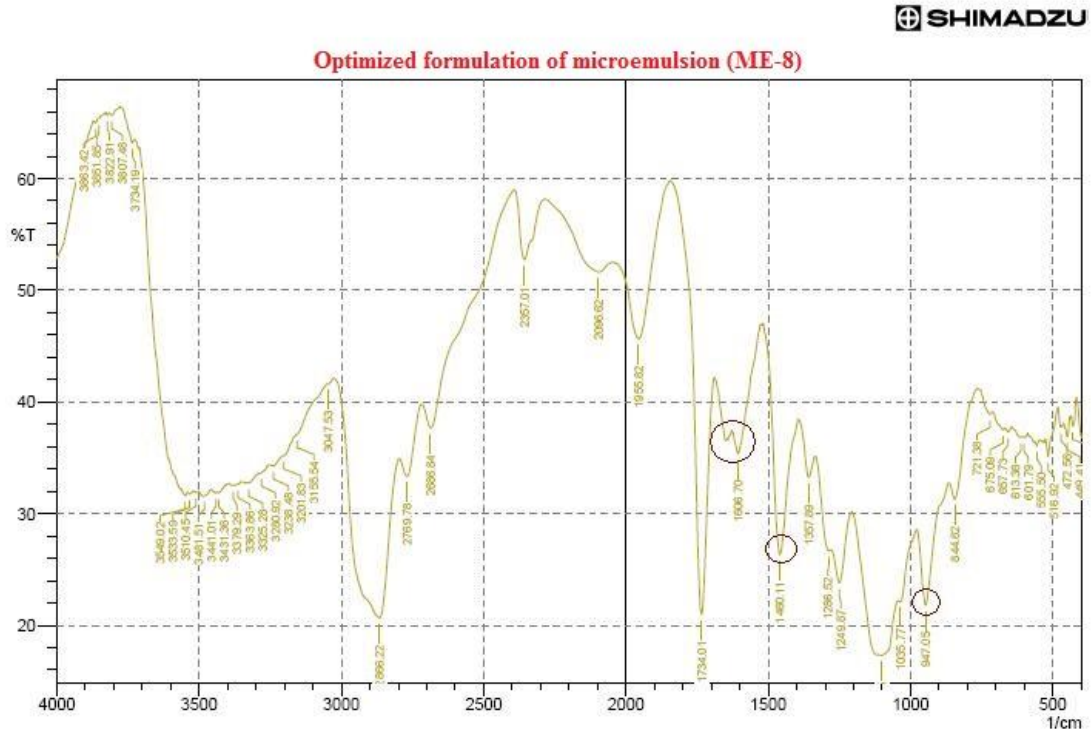



Figure 7.32: FTIR of optimized batch ME-8

Table 7.25: Comparison of characteristic peaks between RLX & optimized batch ME-8

| Functional group | Characteristic peaks | |
|---|---|---|
| | Plain RLX drug | Optimized batch ME - 8 |
| Benzene ring | 953.84 | 947.05 |
| -S- benzothiophene | 1465.00 | 1460.11 |
| -C-O-C- stretching | 1600.02 | 1606.70 |
| C=O stretching | 1641.49 | ≈1645.00 |

**Particle size and zeta potential measurement:**

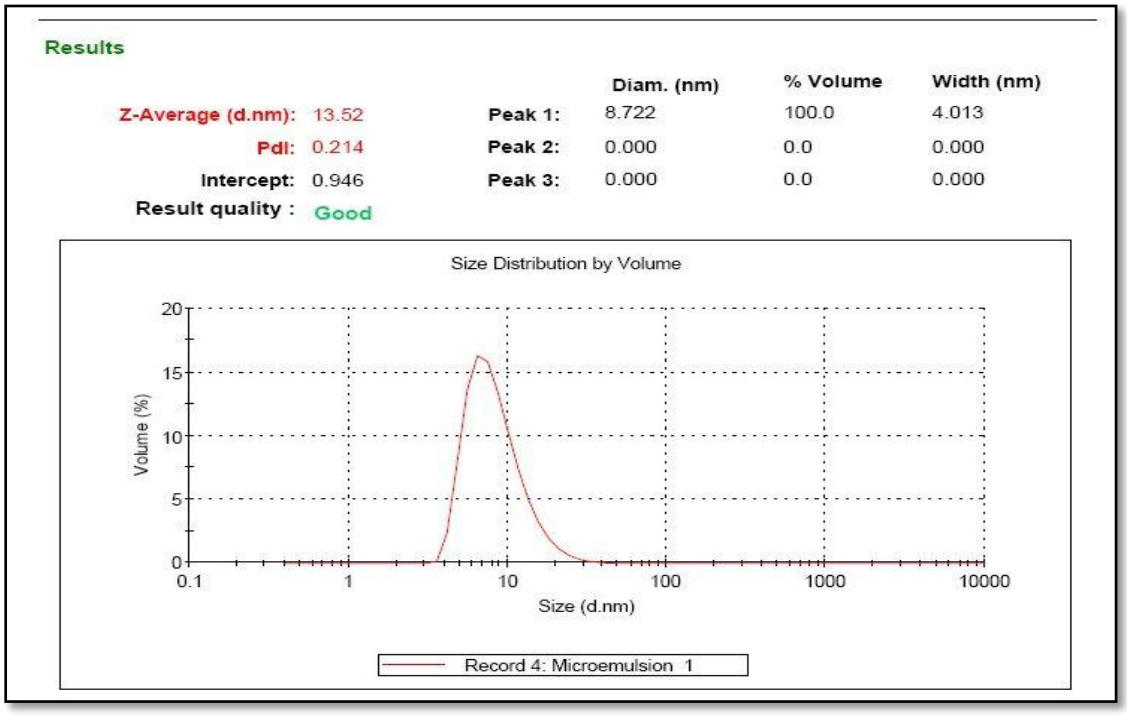



Figure 7.33: Particle size measurement of optimized batch ME-8

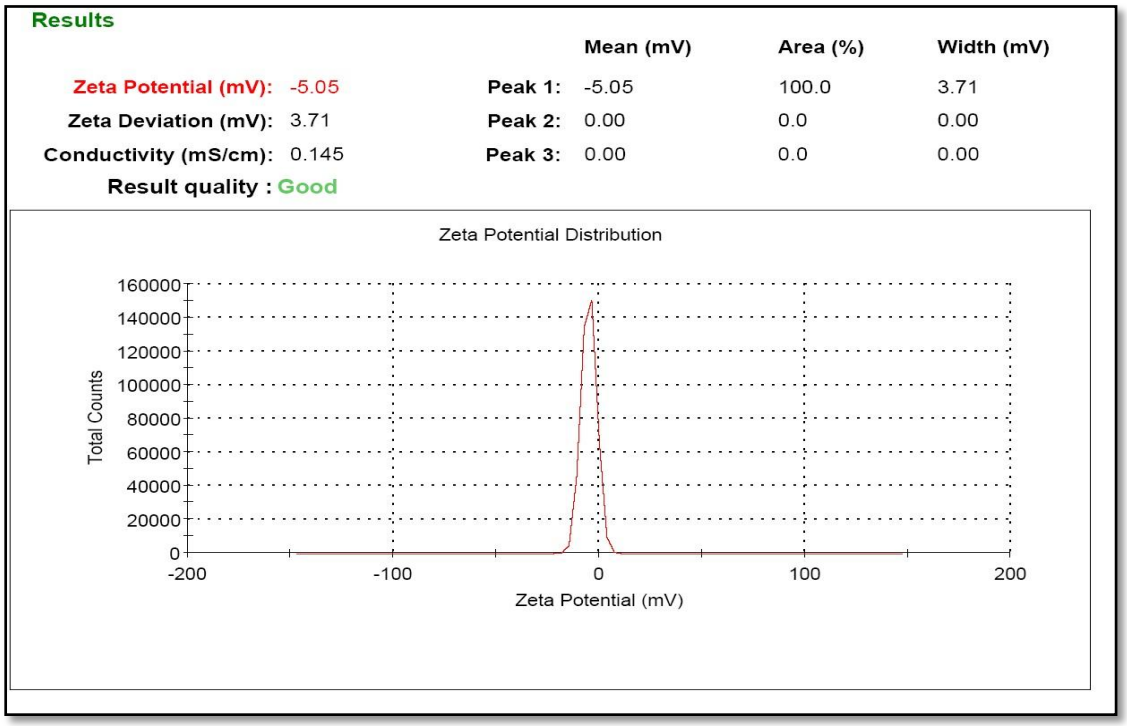



Figure 7.34: Zeta potential measurement of optimized batch ME-8

**Transmission electron microscopy:**

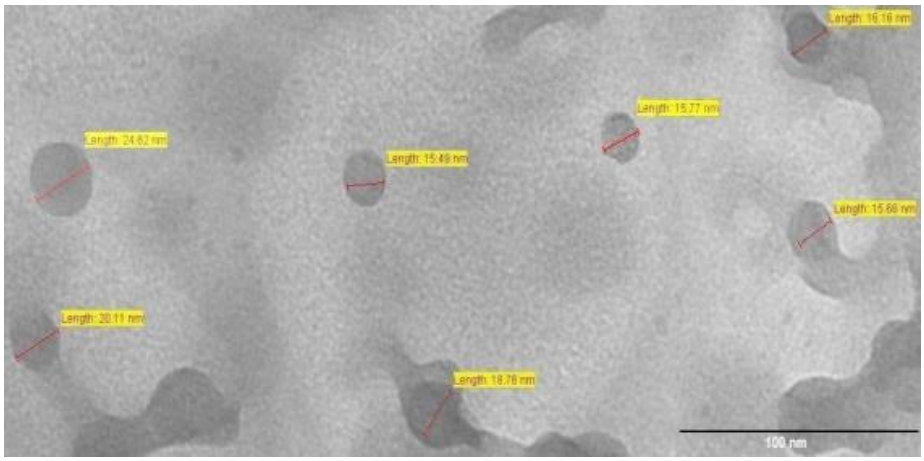



Figure 7.35: TEM image of optimized batch ME-8 (50,000 X)

**Stability study:**

The optimized batch was kept at refrigerator condition, $25^0$ C ± $2^0$ C/ 60% ± 5% RH and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber over period of six months. Data for Physical appearance, % transmittance, drug content and centrifugation of ME -8during stability study is given in table mentioned below.

Table 7.26: Data of optimized batch ME-8 kept at refrigerator condition during stability period

| Time (Days) | Refrigerator condition (2-8$^0$C) | | | | |
|---|---|---|---|---|---|
| | Physical appearance | | % Transmittance ± SD | Drug content ± SD (%) | Centrifugation |
| | Precipitation | Colour change | | | |
| 0 | No precipitation | Yellow liquid | 99.8 ± 0.15 | 99.42 ± 0.22 | No Phase separation |
| 15 | | | 99.9 ± 0.05 | 99.51 ± 0.07 | |
| 30 | | | 99.8 ± 0.10 | 99.34 ± 0.17 | |
| 60 | | | 99.6 ± 0.20 | 98.95 ± 0.28 | |
| 120 | | | 99.7 ± 0.05 | 98.70 ± 0.18 | |
| 180 | | | 99.6 ± 0.25 | 98.58 ± 0.39 | |

Value are expressed as mean ± SD; n=3, $p$>0.05

Table 7.27: Data of optimized batch ME-8 kept at $25^0$ C ± $2^0$ C/ 60% ± 5% RH during stability period

| Time (Days) | $25^0$ C ± $2^0$ C /60% ± 5% RH | | | | |
|---|---|---|---|---|---|
| | Physical appearance | | % Transmittance ± SD | Drug content ± SD (%) | Centrifugation |
| | Precipitation | Colour change | | | |
| 0 | No precipitation | Yellow liquid | 99.8 ± 0.15 | 99.42 ± 0.22 | No Phase separation |
| 15 | | | 99.8 ± 0.21 | 99.40 ± 0.12 | |
| 30 | | | 99.7 ± 0.14 | 99.47 ± 0.11 | |
| 60 | | | 99.7 ± 0.20 | 99.05 ± 0.19 | |
| 120 | | | 99.6 ± 0.25 | 98.95 ± 0.23 | |
| 180 | | | 99.6 ± 0.11 | 98.74 ± 0.28 | |

Value are expressed as mean ± SD; n=3, $p>0.05$

Table 7.28: Data of optimized batch ME-8 kept at $40^0$ C ± $2^0$ C/ 75% ± 5% RH during stability period

| Time (Days) | $40^0$ C ± $2^0$ C / 75% ± 5% RH | | | | |
|---|---|---|---|---|---|
| | Physical appearance | | % Transmittance ± SD | Drug content ± SD (%) | Centrifugation |
| | Precipitation | Colour change | | | |
| 0 | No precipitation | Yellow liquid | 99.8 ± 0.15 | 99.42 ± 0.22 | No Phase separation |
| 15 | | | 99.6 ± 0.09 | 99.55 ± 0.06 | |
| 30 | | | 99.5 ± 0.05 | 99.28 ± 0.24 | |
| 60 | | | 99.6 ± 0.15 | 98.35 ± 0.16 | |
| 120 | | | 99.5 ± 0.22 | 97.63 ± 0.34 | |
| 180 | | | 99.4 ± 0.05 | 97.11 ± 0.57 | |

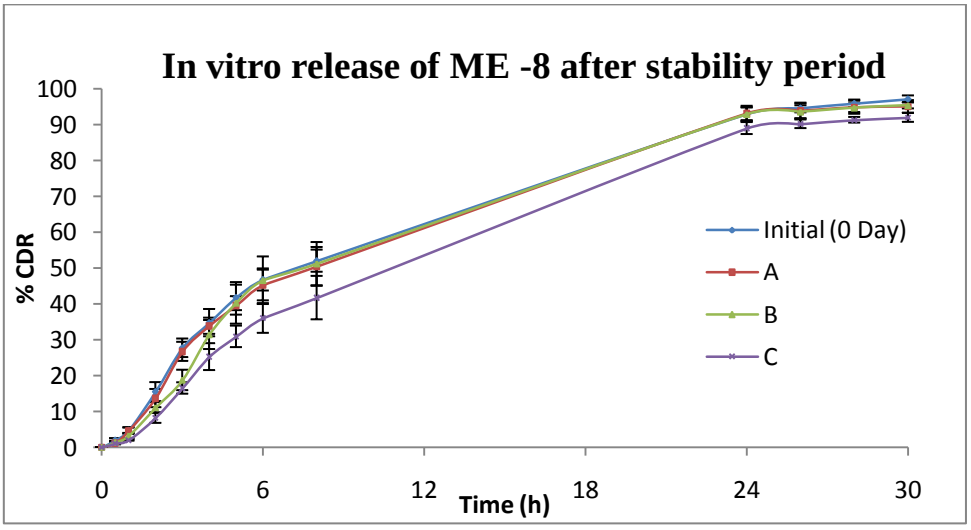
Value are expressed as mean ± SD; n=3, $p>0.05$

Percentage Cumulative drug release at initial stage and at the end of stability study is given in table mentioned below.

Table 7.29: *In vitro* drug release profile of ME-8 after 6 months of stability period

| Time (h) | Percent cumulative drug release (% CDR) ± SD | | | |
|---|---|---|---|---|
| | Initial (0 day) | After 6 months | | |
| | | Refrigerator condition (2-8$^0$C) | 25$^0$C ± 2$^0$C / 60%± 5% RH | 40$^0$C ± 2$^0$C / 75% ± 5% RH |
| 0 | 0 | 0 | 0 | 0 |
| 0.5 | 1.96 ± 0.57 | 1.18 ± 0.14 | 1.36 ± 0.44 | 1.04 ± 0.27 |
| 1 | 4.7 ± 0.76 | 4.64 ± 0.95 | 3.22 ± 0.76 | 2.00 ± 0.19 |
| 2 | 15.5 ± 2.65 | 13.70 ± 2.54 | 11.06 ± 1.51 | 8.26 ± 1.48 |
| 3 | 27.73 ± 2.59 | 26.73 ± 2.66 | 18.76 ± 2.85 | 16.51 ± 1.59 |
| 4 | 34.75 ± 3.81 | 33.88 ± 2.26 | 31.43 ± 4.05 | 25.27 ± 3.75 |
| 5 | 41.79 ± 3.55 | 39.56 ± 2.58 | 40.26 ± 5.80 | 30.92 ± 3.00 |
| 6 | 46.8 ± 3.08 | 45.25 ± 4.28 | 46.58 ± 6.64 | 36.05 ± 4.15 |
| 8 | 52.04 ± 3.09 | 50.44 ± 5.42 | 51.21 ± 6.03 | 41.74 ± 6.06 |
| 24 | 93.02 ± 1.77 | 93.17 ± 2.09 | 92.92 ± 2.00 | 89.05 ± 1.67 |
| 26 | 94.67 ± 1.24 | 93.91 ± 2.23 | 93.60 ± 1.80 | 90.24 ± 1.20 |
| 28 | 95.96 ± 1.04 | 94.86 ± 1.82 | 94.73 ± 1.21 | 91.36 ± 0.79 |
| 30 | 97.18 ± 0.98 | 95.10 ± 1.75* | 95.42 ± 0.89* | 92.02 ± 1.24** |

Value are expressed as mean ± SD; n=3, (*$p$>0.05, **$p$<0.05)




*n=3

Figure 7.36: *In vitro* release profile of ME-8 after 6 months of stability period.
(A= Refrigerator condition, B= 25$^0$ C ± 2$^0$ C / 60% ± 5% RH, C= 40$^0$ C ± 2$^0$ C / 75% ± 5% RH)

Compatibility study of optimized batch ME-8 carried out at different stability conditions by FTIR is shown below.

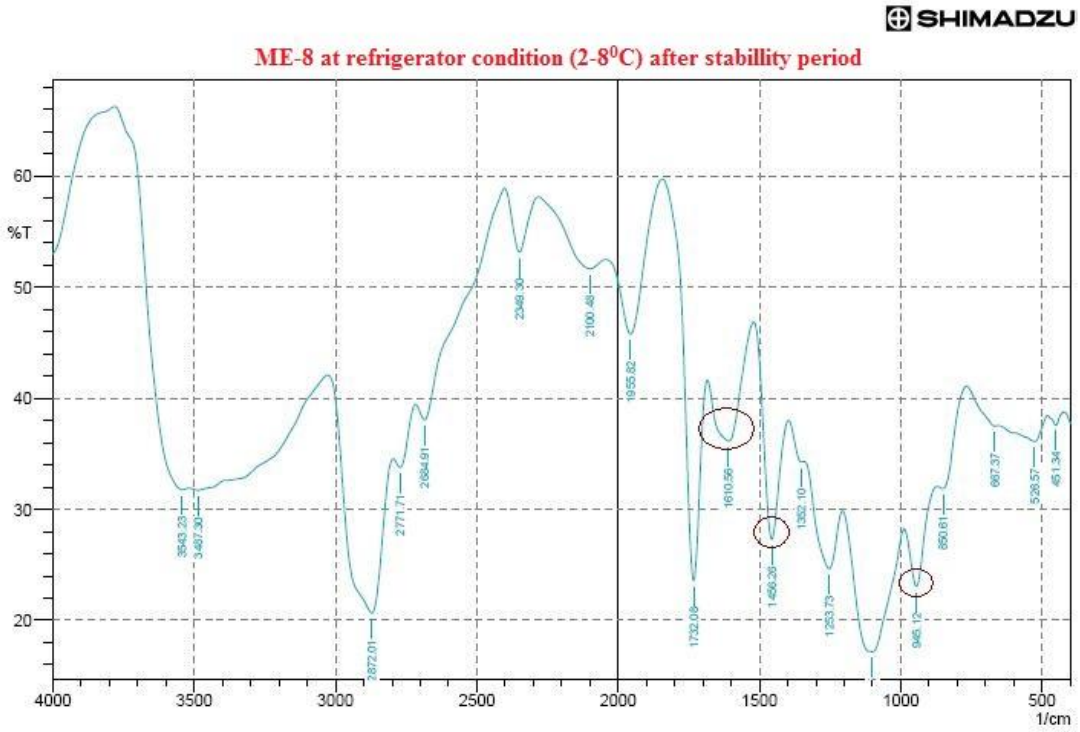



Figure 7.37: FTIR of ME-8 after 6 months of stability period at refrigerator condition

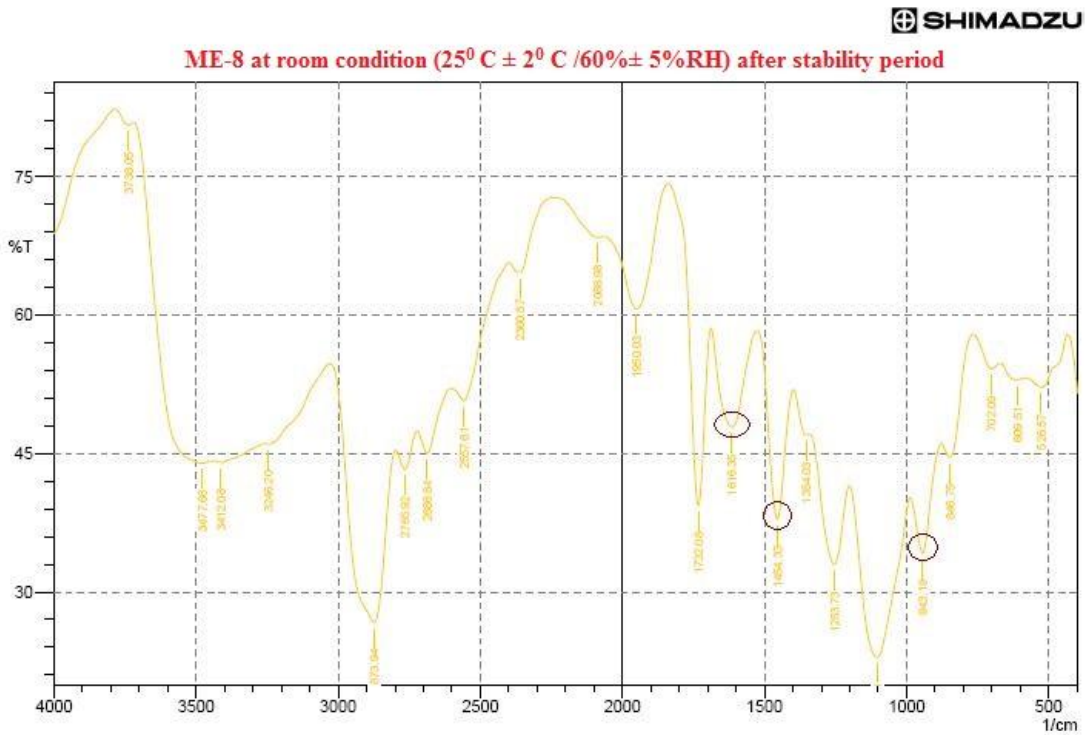



Figure 7.38: FTIR of ME-8 after 6 months of stability period at room condition

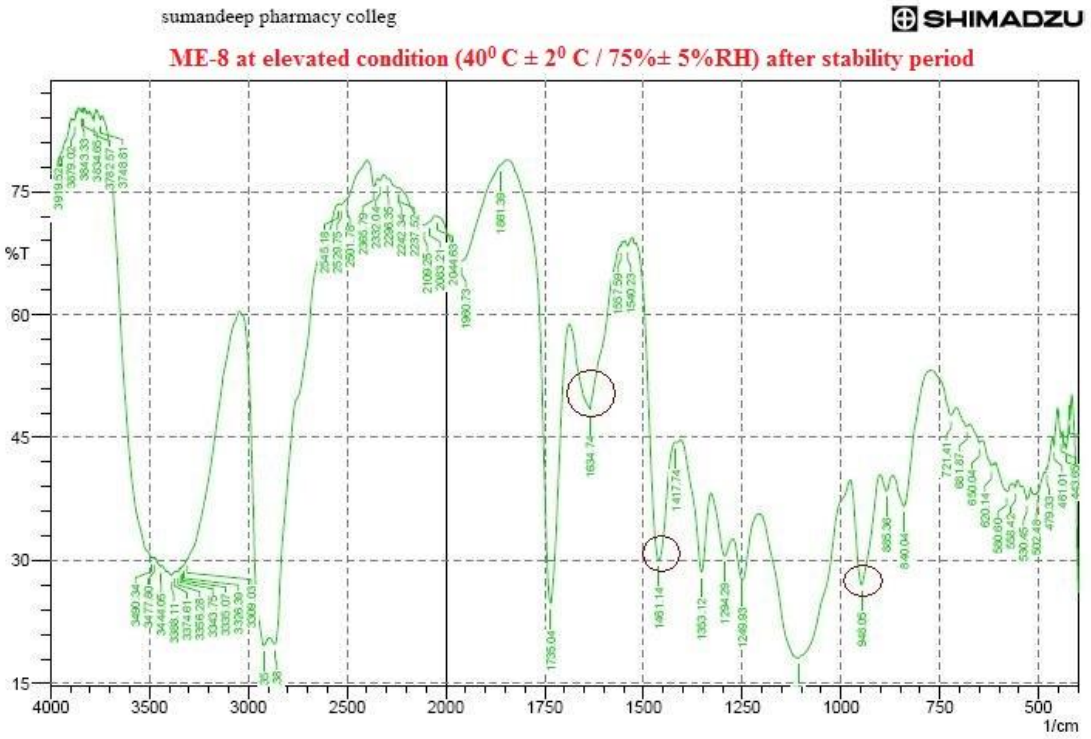



Figure 7.39: FTIR of ME-8 after 6 months of stability period at elevated condition

Table 7.30: Comparison of characteristic peaks between RLX & optimized batch ME-8 kept at different storage conditions

| Functional group | Characteristic peaks ($cm^{-1}$) | | | |
|---|---|---|---|---|
| | Plain RLX drug | Optimized batch ME - 8 | | |
| | | A | B | C |
| Benzene ring | 953.84 | 945.12 | 943.19 | 948.05 |
| -S- benzothiophene | 1465.00 | 1456.26 | 1454.33 | 1461.14 |
| -C-O-C- stretching | 1600.02 | 1610.56 | 1616.35 | 1634.74 |

(A= Refrigerator condition (2-8$^0$C), B= 25$^0$C ± 2$^0$ C /60% ± 5% RH, C= 40$^0$C ± 2$^0$ C / 75% ± 5% RH)

### 7.1.4 Formulation and characterization of drug loaded nanostructured lipid carriers

#### 7.1.4.1 Preliminary studies

**Selection of solid lipid:**

Table 7.31: Visibility data for solid lipid

| Sr. no | Name of lipid | Visibility |
|---|---|---|
| 1 | Dynasan 114 | Obscureness |
| 2 | Dynasan 118 | Obscureness |
| 3 | Stearic acid | Obscureness |
| 4 | Glyceryl monostearate | Fairly visible |

**Partition behavior of raloxifene HCl in various solid lipids:**

Table 7.32: Partition coefficient of RLX in various solid lipids

| Sr. no. | Name of lipid system | Apparent partition coefficient ± SD |
|---|---|---|
| 1 | Water / Dynasan 114 | 59.58 ± 3.69 |
| 2 | Water / Dynasan 118 | 72.89 ± 10.47 |
| 3 | Water / Stearic acid | 66.34 ± 5.41 |
| 4 | Water / GMS | 85.12 ± 9.48 |

Value are expressed as mean ± SD; n=3

**Selection of liquid lipid:**

Table 7.33: Solubility study of RLX in liquid lipids

| Sr. no | Name of lipid | Solubility ± SD (mg/g) |
|---|---|---|
| 1 | Capmul MCM C8 | 2.55 ± 0.96 |
| 2 | Isopropyl myristate | 1.14 ± 0.14 |
| 3 | Oleic acid | 2.08 ± 0.24 |
| 4 | Labrafil IC M 1944 CS | 1.24 ± 0.18 |
| 5 | Lebrafec CC | 0.74 ± 0.07 |

Value are expressed as mean ± SD; n=3

**Drug excipients compatibility studies:**

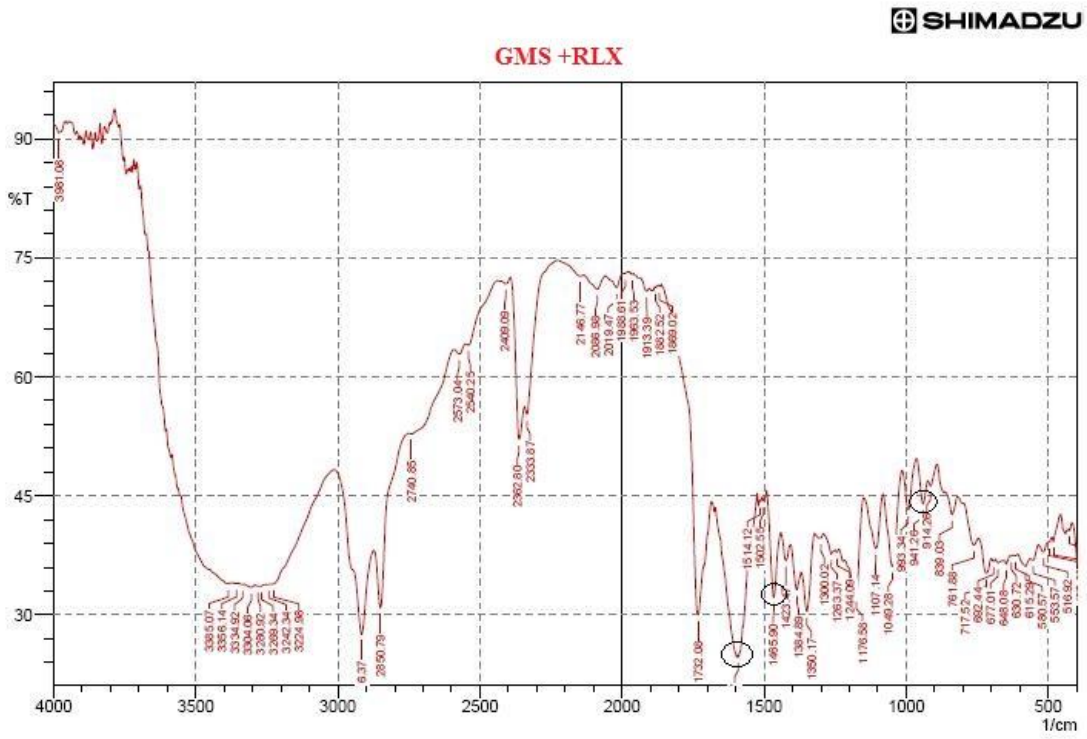



Figure 7.40: FTIR of GMS and RLX mixture

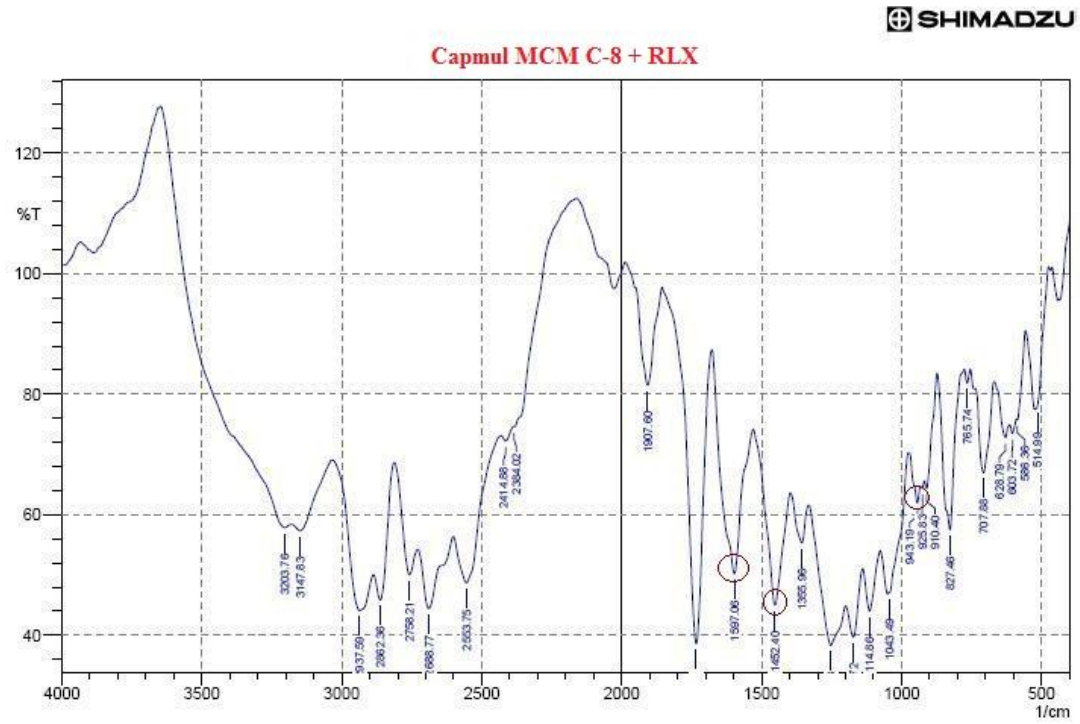



Figure 7.41: FTIR of Capmul MCM C8 and RLX mixture

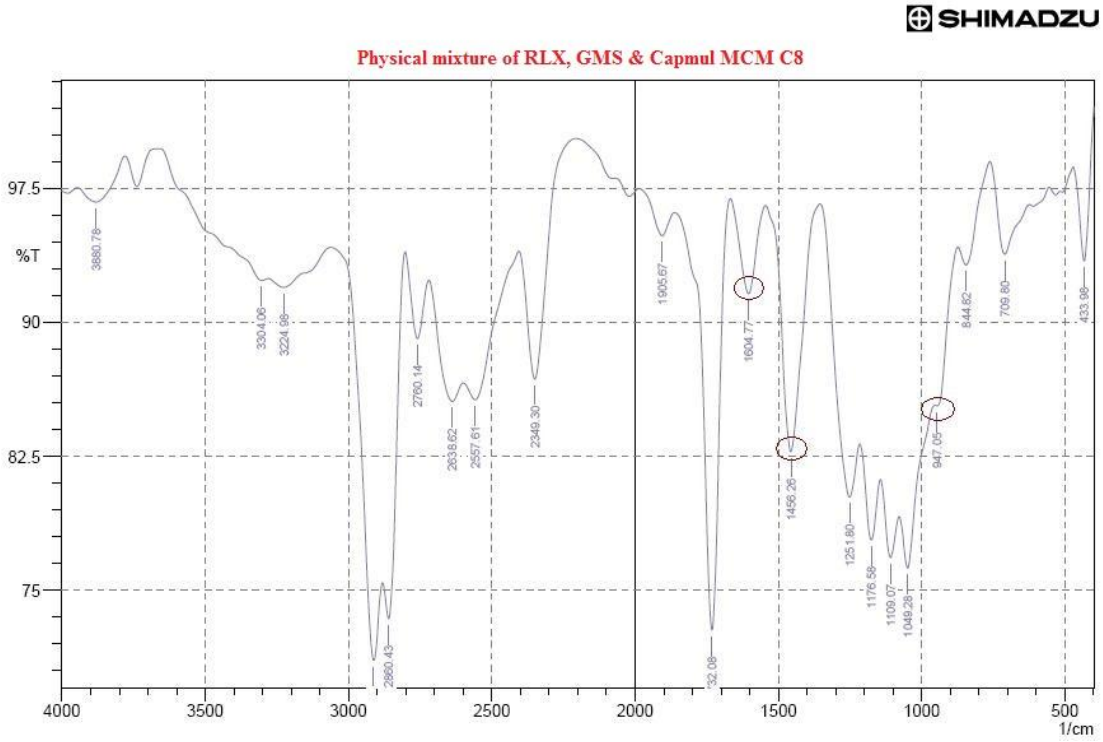



Figure 7.42: FTIR of physical mixture of RLX, GMS & Capmul MCM C8

Table 7.34: Comparison of characteristic peaks between RLX & its mixture with NLCs excipients

| Functional group | Characteristic peaks ($cm^{-1}$) | | | |
|---|---|---|---|---|
| | Plain RLX drug | GMS +RLX | Capmul MCM C8 + RLX | Physical mixture |
| Benzene ring | 953.84 | 941.26 | 943.19 | 947.05 |
| -S- benzothiophene | 1465.00 | 1465.90 | 1452.40 | 1456.26 |
| -C-O-C- stretching | 1600.02 | 1591.27 | 1597.06 | 1604.77 |

### 7.1.4.2 Evaluation of RLX loaded NLCs

**Practical yield, drug loading and entrapment efficiency:**

Practical yield, drug loading and entrapment efficiency of prepared NLCs are shown in below mentioned table.

Table 7.35: Practical yield, drug loading and entrapment efficiency of prepared NLCs

| Sr no. | Batch code | Practical Yield ± SD (%) | Drug loading ± SD (%) | Entrapment efficiency ± SD (%) |
|---|---|---|---|---|
| 1 | NLC - 1 | 80.15 ± 3.54 | 1.92 ± 0.12 | 30.83 ± 2.39 |
| 2 | NLC - 2 | 91.38 ± 1.29 | 2.11 ± 0.18 | 38.59 ± 3.61 |
| 3 | NLC - 3 | 91.29 ± 1.85 | 2.09 ± 0.10 | 38.18 ± 1.92 |
| 4 | NLC - 4 | 83.58 ± 2.18 | 2.64 ± 0.16 | 44.12 ± 3.11 |
| 5 | NLC - 5 | 92.59 ± 2.24 | 3.22 ± 0.13 | 59.58 ± 2.58 |
| 6 | NLC - 6 | 93.06 ± 1.67 | 3.14 ± 0.19 | 58.43 ± 3.79 |
| 7 | NLC - 7 | 84.05 ± 1.05 | 3.74 ± 0.11 | 62.81 ± 2.19 |
| 8 | NLC - 8 | 93.12 ± 2.88 | 4.02 ± 0.17 | 74.78 ± 3.34 |
| 9 | NLC - 9 | 93.85 ± 2.17 | 3.86 ± 0.64 | 72.41 ± 1.28 |

Value are expressed as mean ± SD; n=3

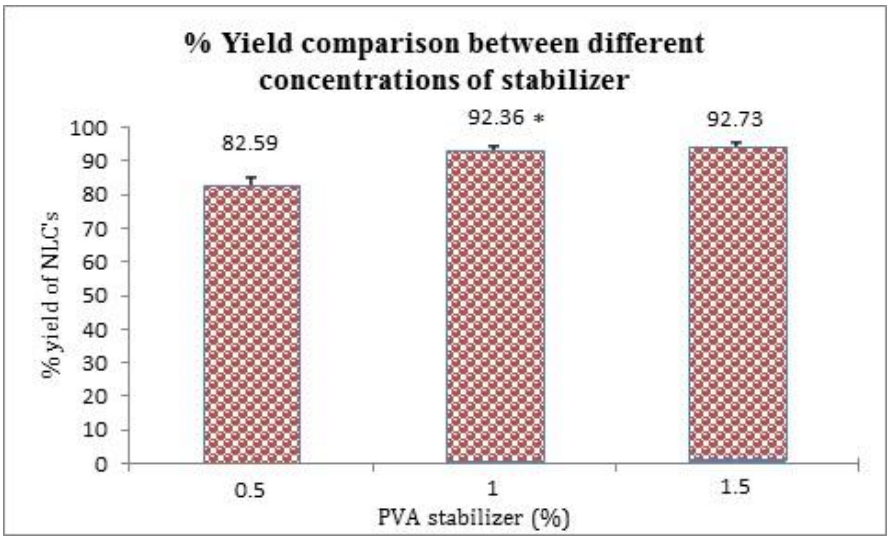



Figure 7.43: Graphical comparison of % yield between different concentrations of stabilizer (*$p$<0.05)

[Result of three columns represents average % yield of batches (NLC-1, NLC-4 & NLC-7), (NLC-2, NLC -5 & NLC-8) and (NLC-3, NLC-6 & NLC-9) respectively]

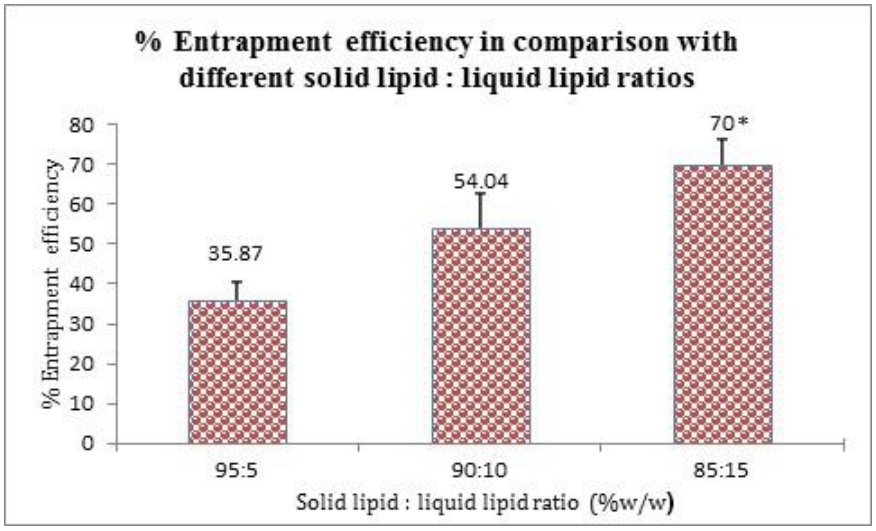



Figure 7.44: Graphical comparison of % entrapment efficiency with different solid lipid: liquid lipid ratios (*$p$<0.05)

(Result of three columns represents average % entrapment efficiency of batches NLC1-3, NLC 4-5 and NLC 7-9 respectively)

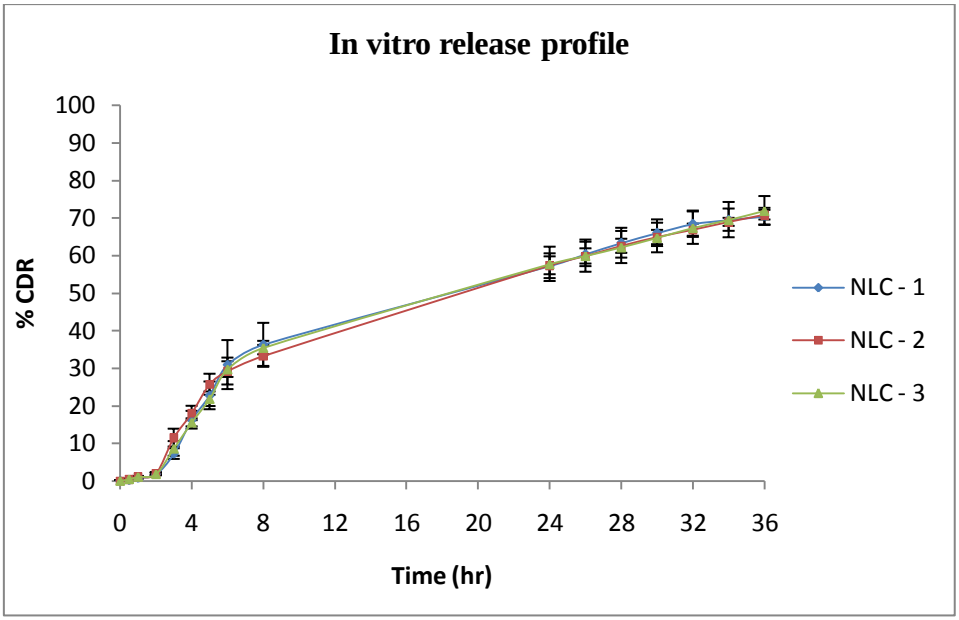
***In vitro* drug release:**

*In vitro* release profile was carried out in dialysis sac for drug suspension and all prepared NLC's batches.

Table 7.36: *In vitro* drug release profile of plain RLX suspension and prepared NLCs

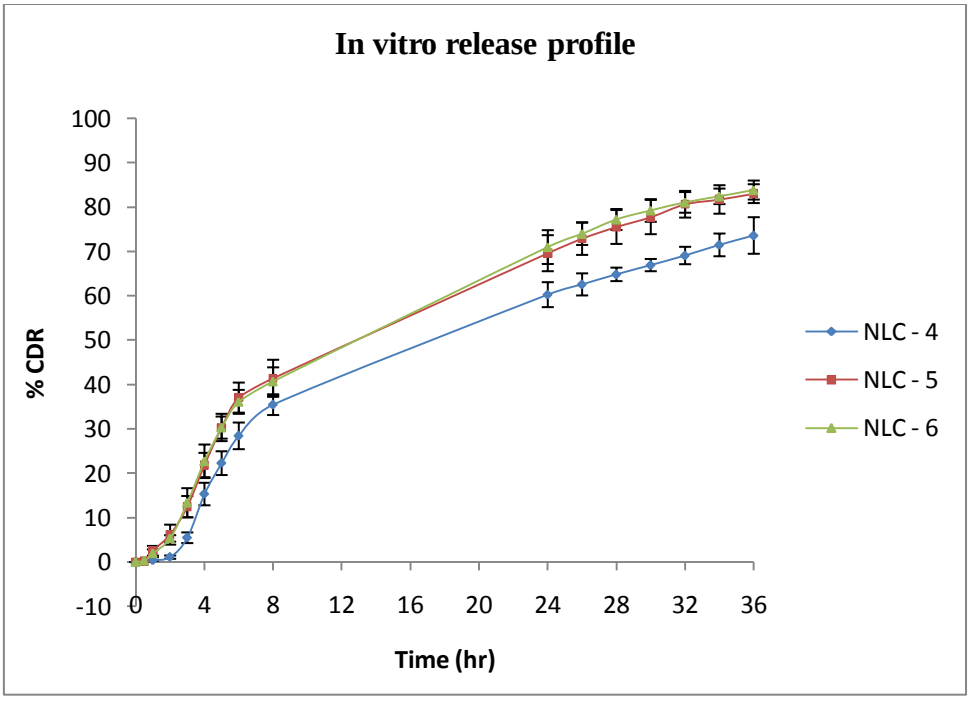
| Time (h) | Percent cumulative drug release (% CDR) ± SD | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Plain Drug suspension** | **NLC - 1** | **NLC -2** | **NLC - 3** | **NLC - 4** | **NLC -5** | **NLC - 6** | **NLC - 7** | **NLC -8** | **NLC - 9** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.5 | 0.15±0.07 | 0.17±0.08 | 0.40±0.08 | 0.46±0.11 | 0.16±0.11 | 0.22±0.2 | 0.29±0.07 | 0.35±0.07 | 0.51±0.29 | 0.41±0.13 |
| 1 | 0.28±0.09 | 0.75±0.20 | 1.07±0.20 | 1.08±0.16 | 0.36±0.09 | 2.54±1.09 | 1.96±0.89 | 2.71±0.60 | 2.33±0.44 | 2.20±0.36 |
| 2 | 0.58±0.21 | 1.78±0.33 | 1.94±0.35 | 1.79±0.32 | 1.10±0.37 | 6.17±2.25 | 5.32±0.73 | 7.57±1.96 | 11.24±4.97 | 12.86±2.61 |
| 3 | 0.91±0.24 | 7.21±1.42 | 11.47±2.37 | 8.59±1.95 | 5.47±1.20 | 12.47±2.40 | 13.36±3.28 | 15.83±4.06 | 25.82±4.68 | 27.94±6.69 |
| 4 | 1.44±0.41 | 16.23±2.36 | 18.03±1.92 | 15.54±1.07 | 15.32±2.54 | 21.86±2.74 | 22.7±3.78 | 28.81±6.57 | 33.08±5.09 | 33.68±3.27 |
| 5 | 1.94±0.33 | 22.72±3.70 | 25.68±2.80 | 21.82±1.94 | 22.28±2.68 | 30.32±3.08 | 30.29±2.49 | 37.08±3.12 | 38.94±2.71 | 38.85±3.37 |
| 6 | 2.26±0.22 | 30.92±6.53 | 29.2±3.58 | 29.71±2.09 | 28.43±3.00 | 37.05±3.39 | 36.09±2.70 | 41.94±3.06 | 43.19±2.14 | 41.48±2.62 |
| 8 | 2.76±0.27 | 36.30±5.74 | 33.24±2.92 | 35.45±1.79 | 35.46±2.34 | 41.40±4.18 | 40.69±3.18 | 45.74±2.91 | 46.55±2.81 | 44.71±3.16 |
| 24 | 9.59±0.55 | 57.25±3.30 | 57.36±2.38 | 57.75±4.57 | 60.25±2.81 | 69.59±4.06 | 70.97±3.81 | 75.23±1.02 | 76.80±2.62 | 75.81±2.66 |
| 26 | 9.88±0.70 | 60.39±3.25 | 59.86±2.03 | 59.93±4.29 | 62.56±2.49 | 72.89±3.68 | 73.95±2.50 | 77.94±1.66 | 79.76±1.22 | 79.55±1.72 |
| 28 | 10.18±0.84 | 63.38±3.97 | 62.55±1.88 | 62.23±4.28 | 64.82±1.52 | 75.50±3.82 | 77.19±2.36 | 80.76±1.54 | 82.82±1.36 | 82.27±2.36 |
| 30 | 10.51±0.87 | 66.04±3.55 | 64.89±1.91 | 64.76±3.94 | 66.91±1.38 | 77.73±3.86 | 79.22±2.55 | 82.86±1.56 | 85.85±1.51 | 85.47±1.77 |
| 32 | 10.76±0.95 | 68.43±3.51 | 66.85±1.62 | 67.39±4.32 | 69.07±1.96 | 80.62±3.01 | 81.04±2.33 | 84.06±1.03 | 88.33±1.78 | 87.52±2.02 |
| 34 | 11.05±1.04 | 69.51±2.97 | 68.93±1.07 | 69.53±4.70 | 71.46±2.56 | 81.70±3.21 | 82.40±1.75 | 86.03±1.67 | 89.59±2.54 | 89.05±2.12 |
| 36 | 11.30±1.02 | 70.45±2.22 | 70.84±1.28 | 71.96±3.85 | 73.58±4.12 | 83.01±2.12 | 83.82±2.14 | 86.95±1.84 | 90.82±2.40 | 89.93±1.94 |

Value are expressed as mean ± SD; n=3, $p<0.05$ compared with plain drug suspension



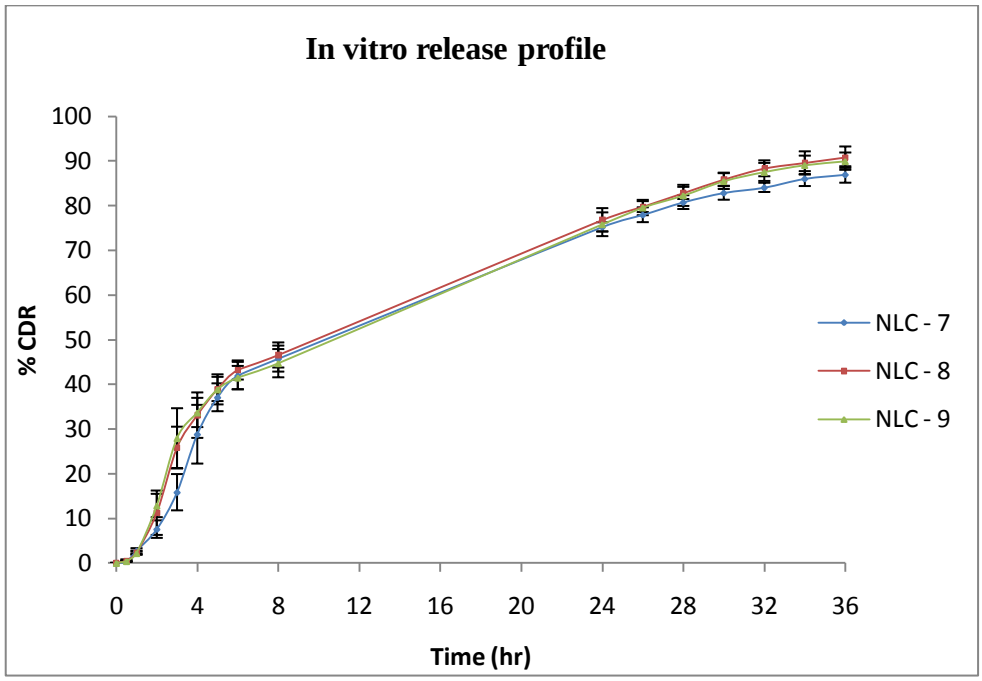

*n=3

Figure 7.45: *In vitro* drug release profile of NLC-1, NLC-2 & NLC-3




*n=3

Figure 7.46: *In vitro* drug release profile of NLC-4, NLC-5 & NLC-6




*n=3

Figure 7.47: *In vitro* drug release profile of NLC-7, NLC-8 & NLC-9

**Optimization of formulation:**

**Mathematical modeling**

Optimization of various formulation parameters were studied with the help of full factorial design. This serves as an excellent tool for the researcher to elucidate the different factors or conditions on the experimental results. Further it also helps to study the interrelationship between various factors and variables selected. In these designs, the most important variables which affect the important parameter are selected and systematic experiments are performed.

Based on the results obtained in the preliminary studies, the ratio of concentration of solid lipid to liquid lipid and concentration of stabilizer were found to be the variables influencing the entrapment efficiency (dependent variable). Thus these variables were selected at 3 levels to find out the optimized formula for higher entrapment efficiency using $3^2$ full factorial design.

From each experiment, a response (Y) was measured for each experiment and a quadratic model was generated by carrying out multiple regression analysis and F-statistics to identify statistically significant terms. The equation of the following form was generated for multiple regression analysis.

$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$ ..... (1)

Where Y is the dependent variable, while $b_0$ is the intercept; $b_i$ ($b_1$ and $b_2$) and $b_{ij}$ ($b_{12}$ and $b_{22}$) represent the regression coefficient for the second-order polynomial and $X_i$ represents the levels of independent formulation variables.

Coded value and actual values of varying two independent variables, the ratio of concentration of solid lipid to liquid lipid ($X_1$) and Stabilizer concentration ($X_2$) are represented in table 7.37. The response for each batch is also shown:

Table 7.37: Coded value and % entrapment of prepared NLCs

| **Batch No** | **$X_1$** | **$X_2$** | **$X_1$2** | **$X_2$2** | **$X_1X_2$** | **% Entrapment** |
|---|---|---|---|---|---|---|
| NLC - 1 | -1 | -1 | 1 | 1 | 1 | 30.83 |
| NLC - 2 | -1 | 0 | 1 | 0 | 0 | 38.59 |
| NLC - 3 | -1 | 1 | 1 | 1 | -1 | 38.18 |
| NLC - 4 | 0 | -1 | 0 | 1 | 0 | 44.12 |
| NLC - 5 | 0 | 0 | 0 | 0 | 0 | 59.58 |
| NLC - 6 | 0 | 1 | 0 | 1 | 0 | 58.43 |
| NLC - 7 | 1 | -1 | 1 | 1 | -1 | 62.81 |
| NLC - 8 | 1 | 0 | 1 | 0 | 0 | 74.78 |
| NLC - 9 | 1 | 1 | 1 | 1 | 1 | 72.41 |

Subsequently, a full model equation was established by putting values of regression coefficients for % EE in equation 1. The predicted values were calculated by using the mathematical model derived from the coefficients of the model as shown in table 6.39.The predicted values along with their observed values are shown in table 6.39.This gives information about the percentage of error obtained when the predicted value was compared with the observed values.

Table 7.38: Mathematical modeling of prepared NLCs

| Factor | Coefficients | t Stat | P-value |
|---|---|---|---|
| Intercept | 58.39 | 32.24439 | 6.56E-05* |
| $X_1$ | 17.07 | 17.20695 | 0.000428* |
| $X_2$ | 5.21 | 5.252823 | 0.013438* |
| $X_1^2$ | -1.11 | -0.64613 | 0.564219 |
| $X_2^2$ | -6.52 | -3.79526 | 0.032108* |
| $X_1X_2$ | 0.56 | 0.463054 | 0.674849 |

* ($p$<0.05)

Thus the full model for the factorial design is:

$Y = 58.39 + 17.07\ X_1 + 5.21\ X_2 - 1.11\ X_1^2 - 6.52\ X_2^2 + 0.56\ X_1X_2$ …… (2)

By considering very significant terms from full model, reduced model was established

$Y = 58.39 + 17.07\ X_1 + 5.21\ X_2 - 6.52X_2^2$ ……..…… (3)

Table 7.39: Observed and predicted value of drug entrapment for prepared NLCs

| Batch No. | Observed EE | Predicted EE | Residual | % Error |
|---|---|---|---|---|
| NLC - 1 | 30.83 | 29.04 | -1.79 | 6.16 |
| NLC - 2 | 38.59 | 40.21 | 1.62 | 4.03 |
| NLC - 3 | 38.18 | 38.34 | 0.16 | 0.42 |
| NLC - 4 | 44.12 | 46.66 | 2.54 | 5.44 |
| NLC - 5 | 59.58 | 58.39 | -1.19 | 2.04 |
| NLC - 6 | 58.43 | 57.08 | -1.35 | 2.37 |
| NLC - 7 | 62.81 | 62.06 | -0.75 | 1.21 |
| NLC - 8 | 74.78 | 74.35 | -0.43 | 0.58 |
| NLC - 9 | 72.41 | 73.6 | 1.19 | 1.62 |

From the above information, results of analysis of variance (ANOVA) of full model and reduced model were calculated and the F statistics was applied to check whether the non-significant terms can be omitted or not from the full model, which is shown in table 7.40.

Table 7.40: F statistics for prepared NLCs

| | | ***Df*** | ***SS*** | ***MS*** | ***F*** | ***$R^2$*** |
|---|---|---|---|---|---|---|
| Regression | FM | 5 | 1999.242 | 399.8483783 | 67.74141 | 0.991221 |
| | RM | 3 | 1995.512 | 665.1706889 | 155.1416 | 0.989371 |
| Error | FM | 3 | 17.70 | 5.9025 | | |
| | RM | 5 | 21.43753 | 4.287506667 | | |

DF: Degree of freedom, SS: Sum of square, MS: Mean sum of square, R: Correlation coefficient, $R^2$: Determination coefficient, Adj.$R^2$: Adjusted determination coefficient.

SSE2-SSE1=21.44-17.70=3.74

No. of terms omitted are 2

MS of Error of FM= 5.9025

$$\text{F Value} = \frac{\frac{\text{SSE 2} - \text{SSE 1}}{\text{No of nonsignificant terms omitted}}}{\text{MS of error of FM}}$$

Thus substituting the values in the above equation we get,

$$\text{F Value} = \frac{\frac{21.44 - 17.70}{2}}{5.9025} = 0.32$$

**Therefore, the F value (Calculated) = 0.32**

**Contour Plots**

Contour plots are diagrammatic representation of the values of the response. Two-dimensional contour plots were established using reduced polynomial equation 3 for % entrapment efficiency. Contour plots were established between $X_1$ and $X_2$ at fixed level of -1, 0 and 1 of both independent variables ($X_1$& $X_2$) as diagrammatically represented in figure 6.48. By establishment of two dimensional contour plots, the relationship between independent and dependent variables can be explained.

Contour plot for solid lipid : liquid lipid and concentration of stabilizer vs % entrapment

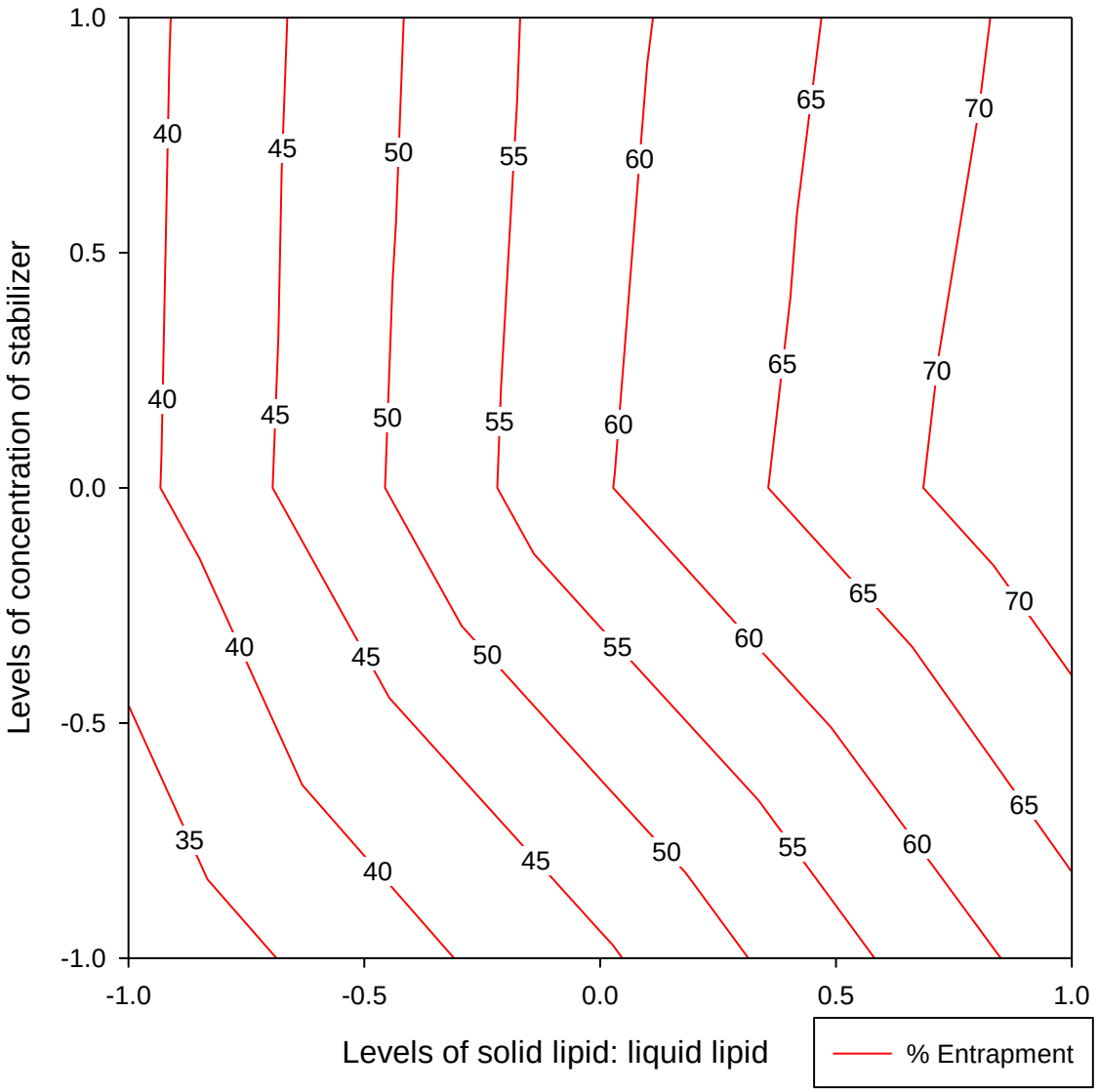



Figure 7.48: Contour plot for levels of solid lipid: liquid lipid and concentration of stabilizer with % entrapment of prepared NLCs

From the graph it is clear that the highest entrapment efficiency is found when the ratio of the concentration values of the solid lipid: liquid lipid is near to 1 and the stabilizer concentration is in range of 0 to 1.

**Response Surface Plots**

Response surface plots are very helpful in learning about both the main and interaction effects of the independent variables.

3D plot for solid lipid:liquid lipid and concentration of stabilizer vs % entrapment

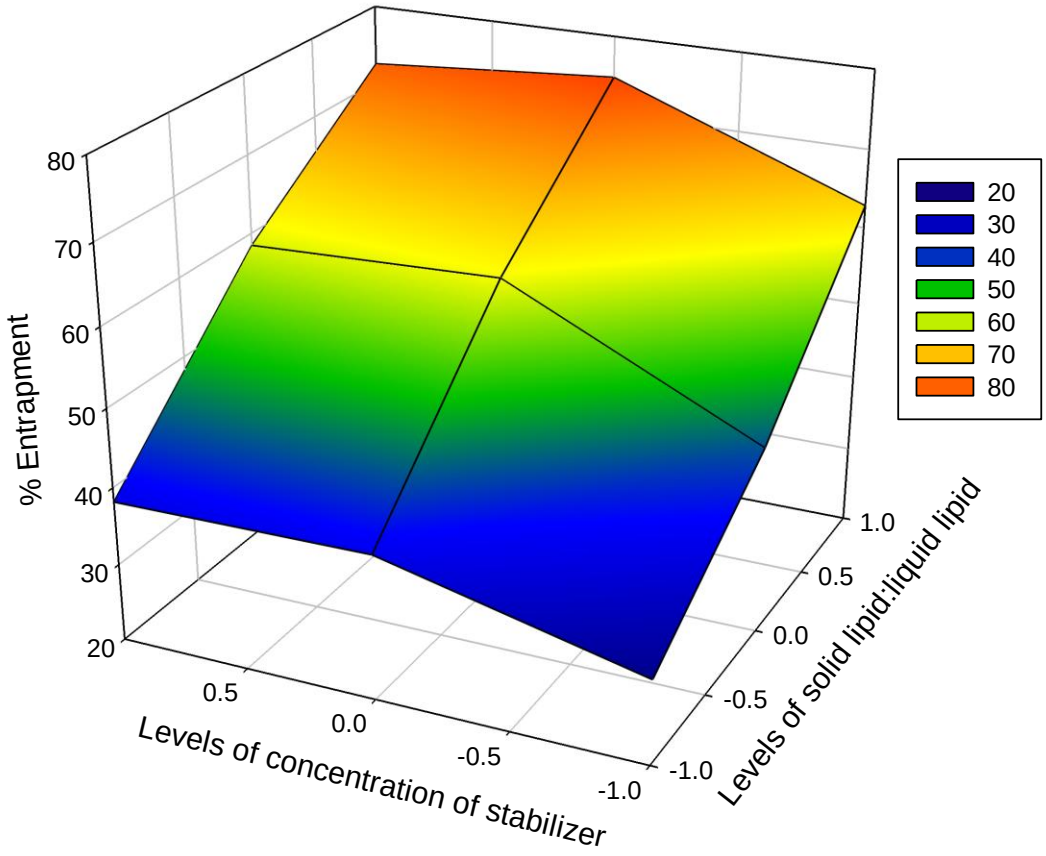



Figure 7.49: 3D plot for levels of solid lipid: liquid lipid and concentration of stabilizer with % entrapment of prepared NLCs

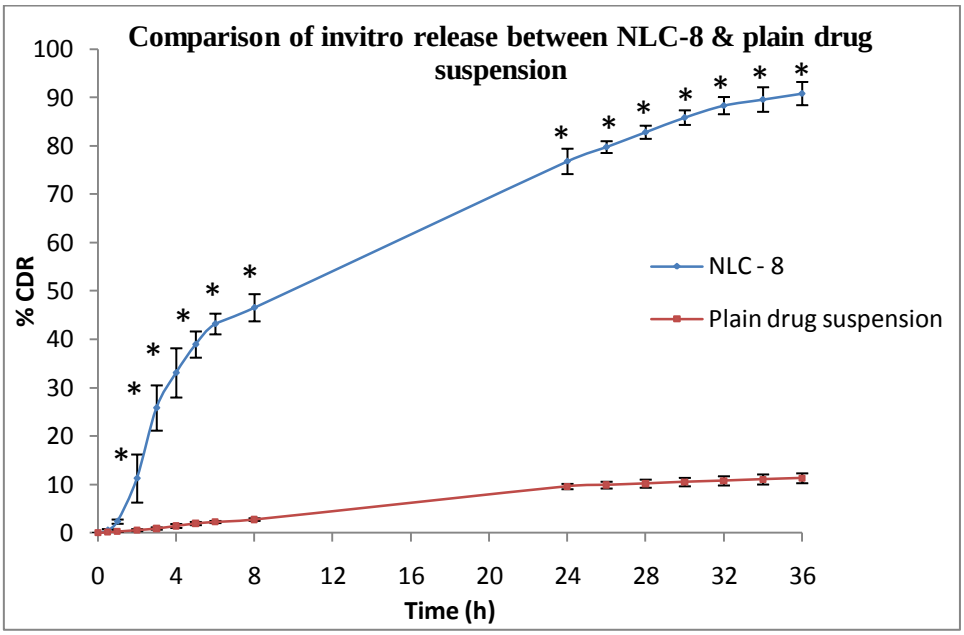
**Check point analysis**

Table 7.41: Check point analysis for NLC batch

| Sr. no | $X_1$ | $X_2$ | Observed EE | Predicted EE | Residual | % Error |
|---|---|---|---|---|---|---|
| 1 | 0.5 | 0.5 | 66.51 | 67.9 | 1.39 | 2.05 |

### 7.1.4.3 Characterization of optimized RLX loaded NLCs

**Drug release comparison of optimized batch with plain drug suspension:**




n=3

Figure 7.50: Comparison of *in vitro* drug release profile between plain drug suspension and NLC-8 (*$p$<0.05)

**Drug release kinetics:**

Table 7.42: *In vitro* release kinetics of optimized batch NLC-8

| Optimized batch | Regression coefficient ($R^2$) | | |
|---|---|---|---|
| | Zero order | First order | Higuchi model |
| NLC - 8 | 0.931 | 0.540 | 0.982 |

**Drug excipients compatibility studies:**

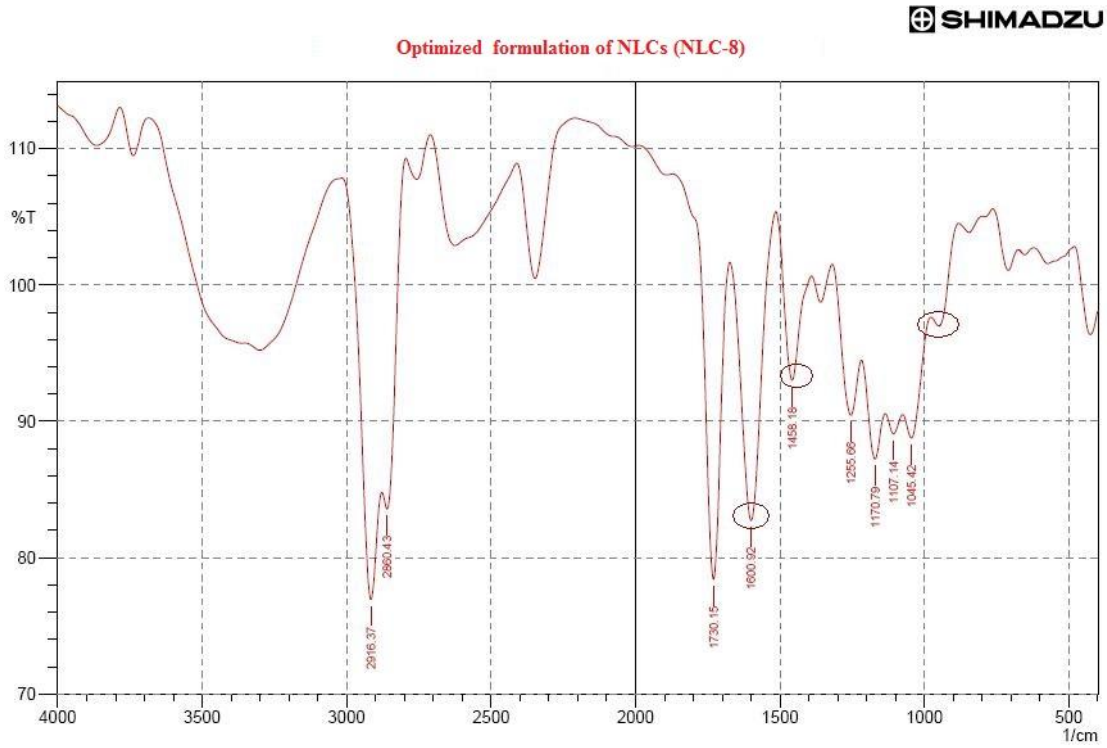



Figure 7.51: FTIR of optimized batch NLC-8

Table 7.43: Comparison of characteristic peaks between RLX & optimized batch NLC-8

| Functional group | Characteristic peaks | |
|---|---|---|
| | Plain RLX drug | Optimized batch NLC-8 |
| Benzene ring | 953.84 | ≈947.00 |
| -S- benzothiophene | 1465 | 1458.18 |
| -C-O-C- stretching | 1600.02 | 1600.92 |

**Results**

| | | Diam. (nm) | % Intensity | Width (nm) |
|---|---|---|---|---|
| Z-Average (d.nm): 27.3 | Peak 1: | 26.2 | 84.3 | 5.52 |
| PdI: 0.287 | Peak 2: | 1740 | 15.7 | 1160 |
| Intercept: 0.676 | Peak 3: | 0.00 | 0.0 | 0.00 |

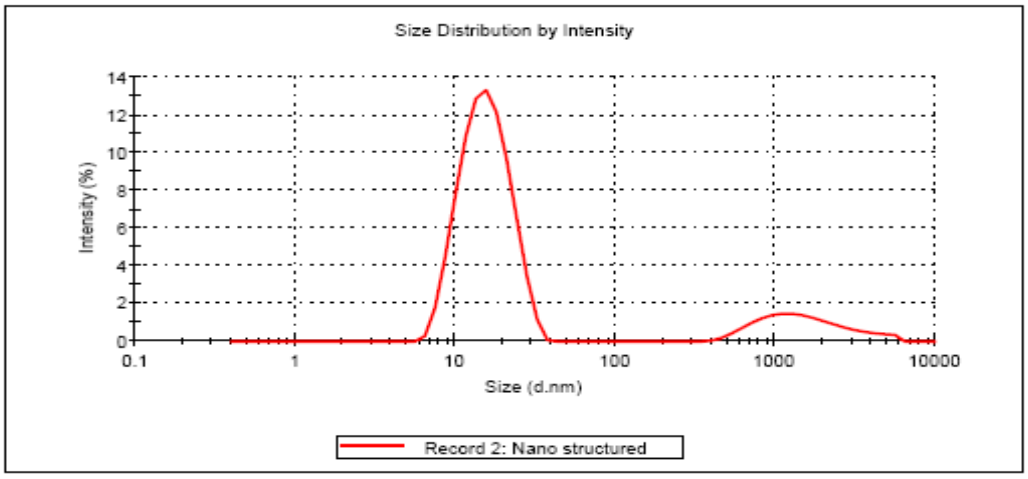



Figure 7.52: Graph for particles size measurement of optimized batch NLC-8

**Results**

| | | Mean (mV) | Area (%) | Width (mV) |
|---|---|---|---|---|
| Zeta Potential (mV): -13.6 | Peak 1: | -18.0 | 84.0 | 5.93 |
| Zeta Deviation (mV): 9.44 | Peak 2: | 2.21 | 16.0 | 2.09 |
| Conductivity (mS/cm): 0.0456 | Peak 3: | 0.00 | 0.0 | 0.00 |

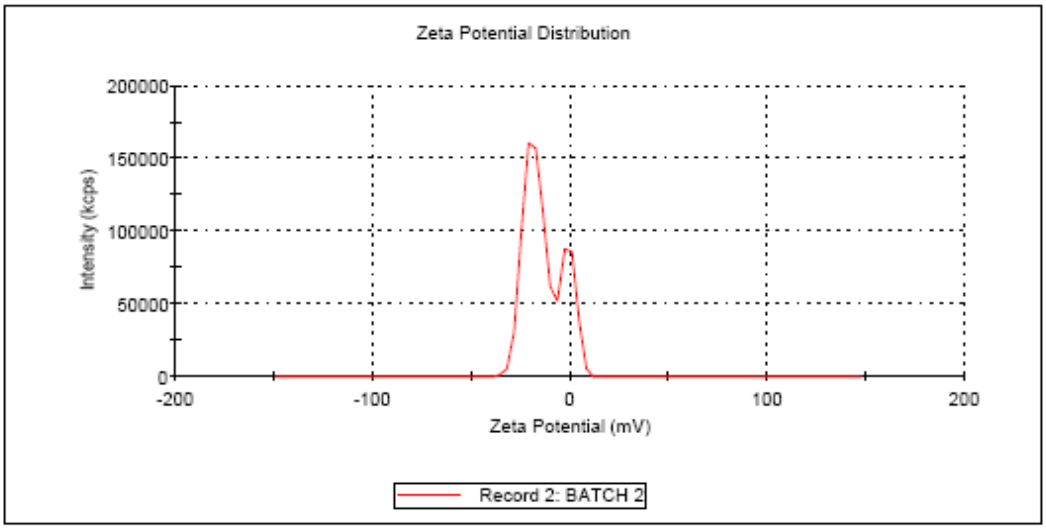



Figure 7.53: Graph for zeta potential measurement of optimized batch NLC-8

**Differential scanning calorimetry:**

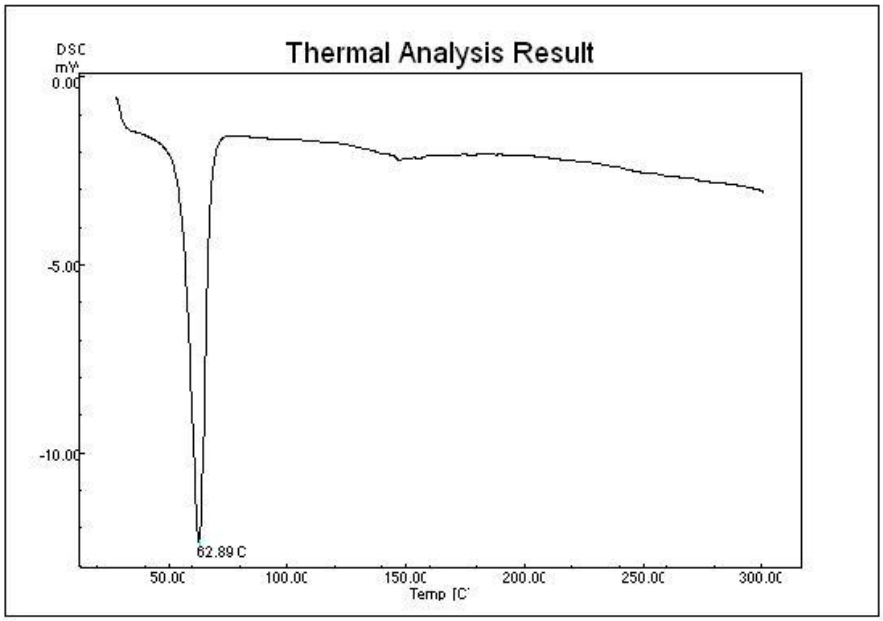



Figure 7.54: DSC thermogram of GMS

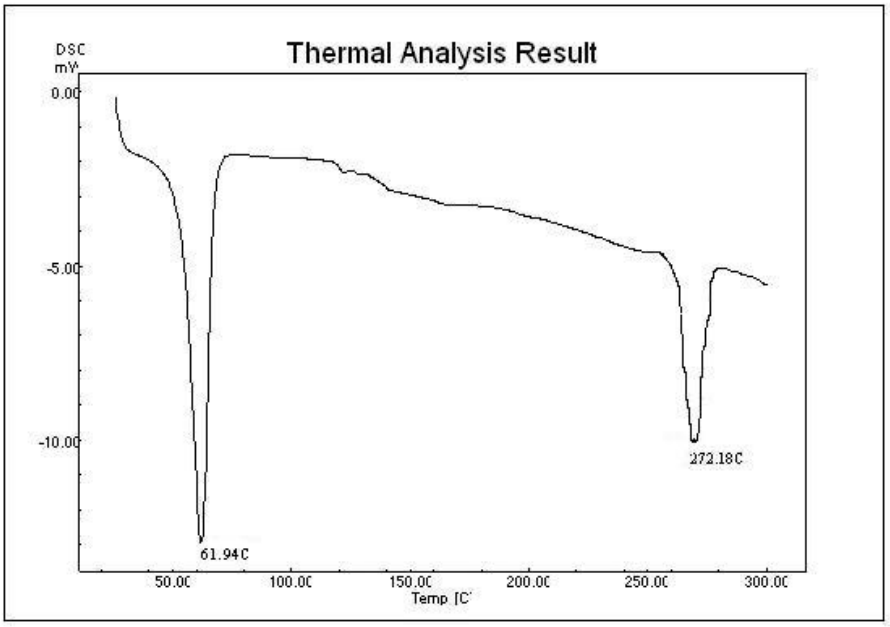



Figure 7.55: DSC thermogram of physical mixture (RLX, GMS & Capmul MCM C8)

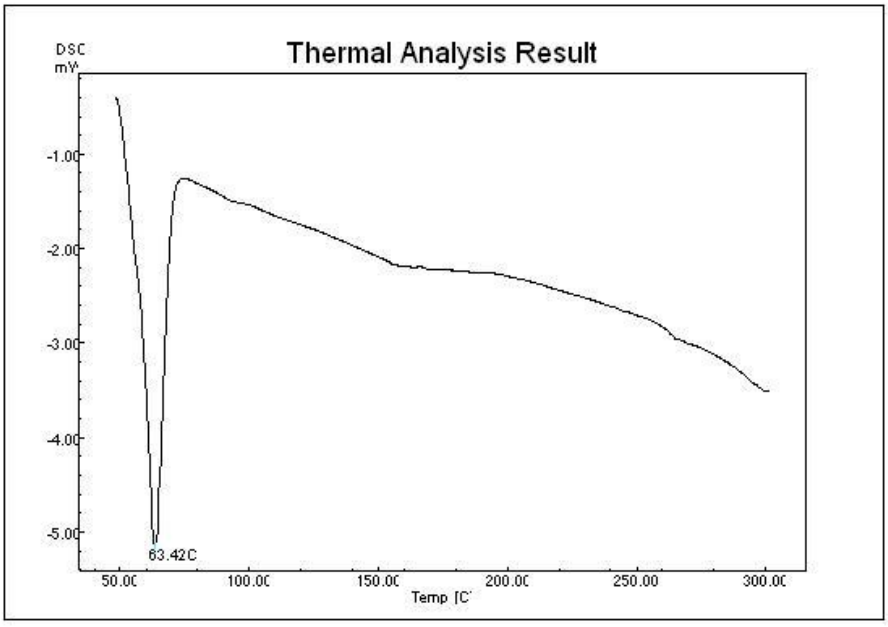



Figure 7.56: DSC thermogram of optimized batch NLC-8

**X-Ray diffraction studies:**

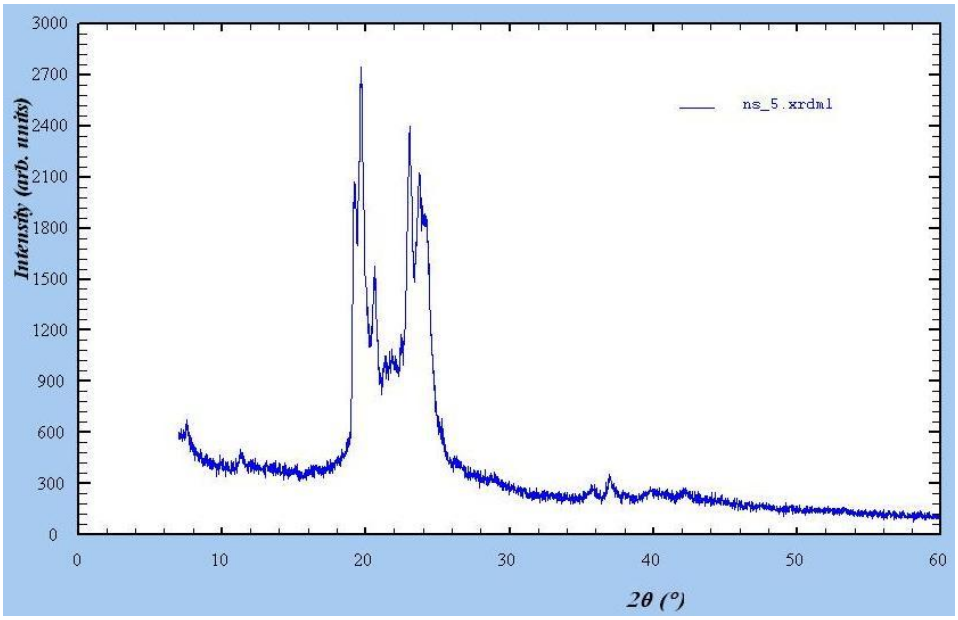



Figure 7.57: XRD pattern of GMS

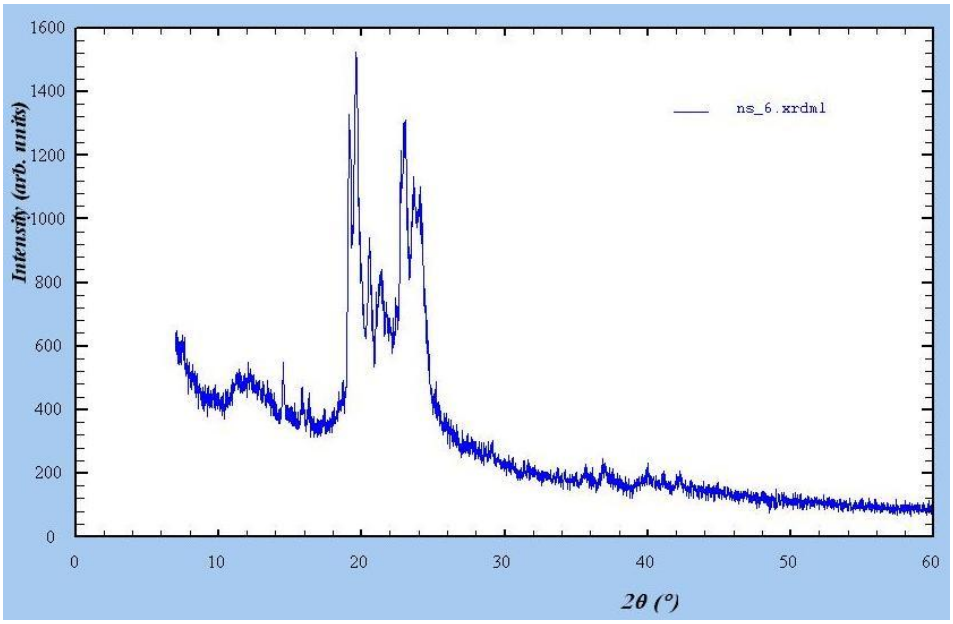



Figure 7.58: XRD pattern for physical mixture for NLCs

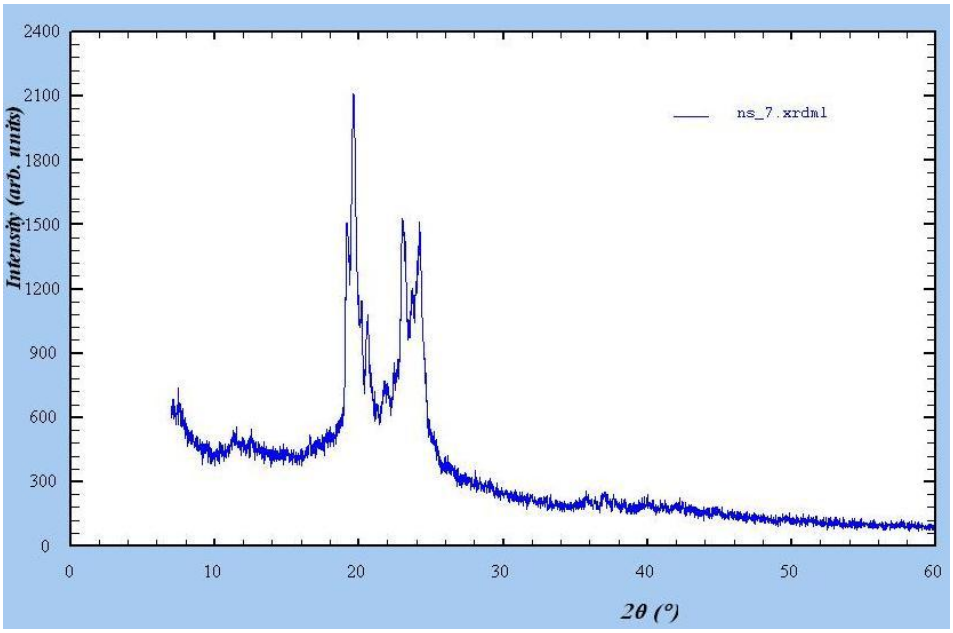



Figure 7.59: XRD pattern for optimized batch NLC-8

**Surface morphology study:**

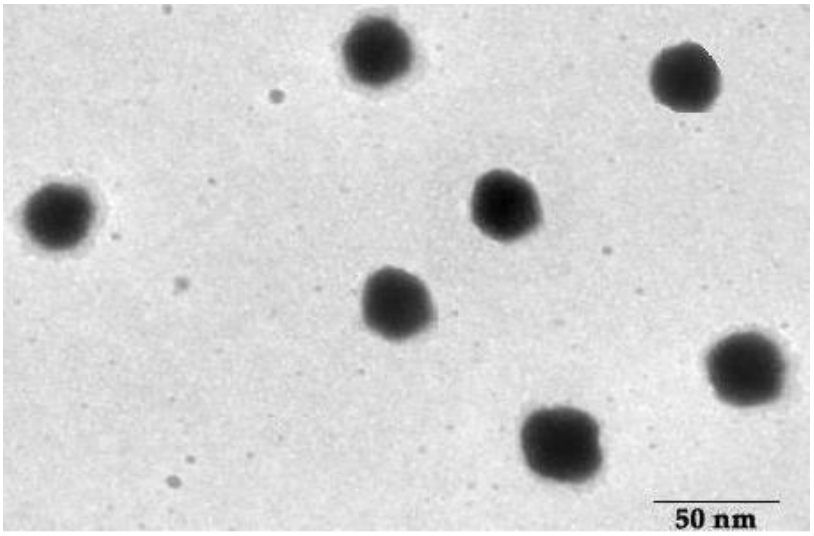



Figure 7.60: TEM image for optimized batch NLC-8 (65000 X)

**Stability study:**

The optimized batch was kept at $25^0$ C ± $2^0$ C /60% ± 5%RH and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber over period of six months. Physical appearance & entrapment efficiency of NLC - 8 during stability period is given in table mentioned below.

Table 7.44: Stability study data for NLC-8

| Sr. No | Time (Days) | $25^0$C ± $2^0$C / 60% ± 5% RH | | $40^0$ C ± $2^0$C / 75% ± 5% RH | |
|---|---|---|---|---|---|
| | | Physical appearance | Entrapment efficiency ± SD (%) | Physical appearance | Entrapment efficiency ± SD (%) |
| 1 | 0 | Yellow free flowing Powder | 74.78 ± 3.34 | Yellow free flowing Powder | 74.78 ± 3.34 |
| 2 | 15 | | 74.71 ± 3.18 | | 74.44 ± 2.25 |
| 3 | 30 | | 74.63 ± 2.10 | | 74.07 ± 2.44 |
| 4 | 60 | | 73.88 ± 3.52 | | 73.11 ± 0.85 |
| 5 | 120 | | 73.92 ± 2.47 | | 71.81 ± 1.64 |
| 6 | 180 | | 73.85 ± 1.18* | | 70.05 ± 1.26** |

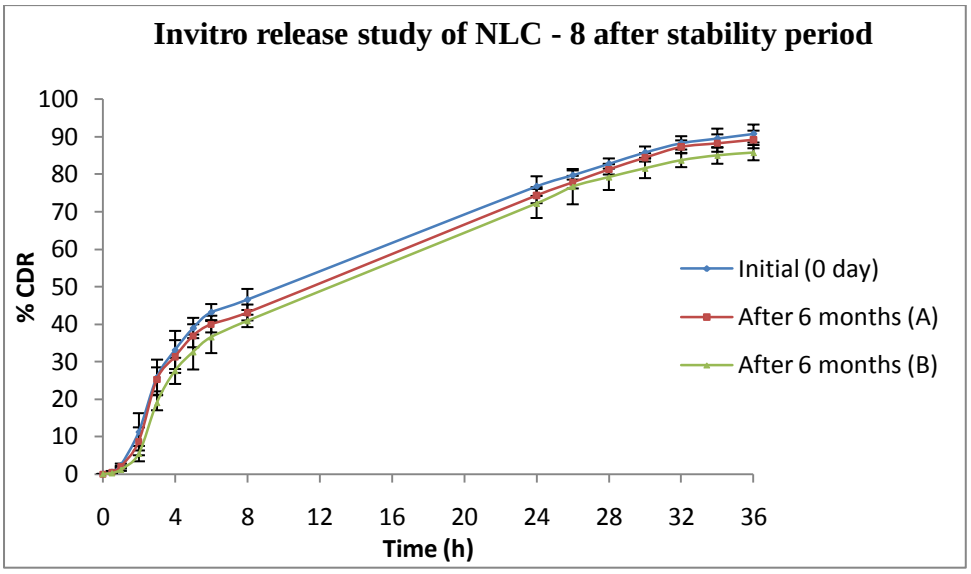
Value are expressed as mean ± SD; n=3, (*$p$>0.05, **$p$<0.05)

Percent cumulative drug release at initial stage and at the end of stability study is given in table mentioned below.

Table 7.45: *In vitro* drug release profile of NLC - 8 after 6 months of stability period

| Time (h) | Percent cumulative drug release (% CDR) ± SD | | |
|---|---|---|---|
| | Initial (0 day) | After 6 months | |
| | | $25^0C \pm 2^0C$ / 60% ± 5% RH | $40^0 C \pm 2^0C$ / 75% ± 5% RH |
| 0 | 0 | 0 | 0 |
| 0.5 | 0.51 ± 0.29 | 0.43 ± 0.27 | 0.23 ± 0.21 |
| 1 | 2.33 ± 0.44 | 1.97 ± 0.22 | 1.14 ± 0.32 |
| 2 | 11.24 ± 4.97 | 8.71 ± 3.72 | 5.42 ± 2.05 |
| 3 | 25.82 ± 4.68 | 25.24 ± 3.20 | 18.99 ± 2.00 |
| 4 | 33.08 ± 5.09 | 31.33 ± 4.37 | 27.50 ± 3.49 |
| 5 | 38.94 ± 2.71 | 36.87 ± 3.07 | 32.53 ± 4.66 |
| 6 | 43.19 ± 2.14 | 39.99 ± 2.23 | 36.52 ± 4.28 |
| 8 | 46.55 ± 2.81 | 43.08 ± 2.12 | 40.83 ± 1.65 |
| 24 | 76.80 ± 2.62 | 74.37 ± 2.13 | 72.16 ± 3.87 |
| 26 | 79.76 ± 1.22 | 77.85 ± 1.68 | 76.65 ± 4.71 |
| 28 | 82.82 ± 1.36 | 81.32 ± 1.43 | 79.19 ± 3.43 |
| 30 | 85.85 ± 1.51 | 84.46 ± 1.12 | 81.53 ± 2.61 |
| 32 | 88.33 ± 1.78 | 87.32 ± 1.67 | 83.69 ± 1.84 |
| 34 | 89.59 ± 2.54 | 88.28 ± 2.32 | 84.98 ± 2.22 |
| 36 | 90.82 ± 2.40 | 89.24 ± 2.35* | 85.72 ± 2.04** |

Value are expressed as mean ± SD; n=3,(*$p$>0.05, **$p$<0.05)




*n=3

Figure 7.61: *In vitro* release profile of NLC-8 after 6 months of stability period.
(A= $25^0$ C ± $2^0$ C /60%± 5%RH, B= $40^0$ C ± $2^0$ C / 75%± 5%RH)

Compatibility study of optimized batch NLC-8 carried out at different stability conditions by FTIR is shown below.

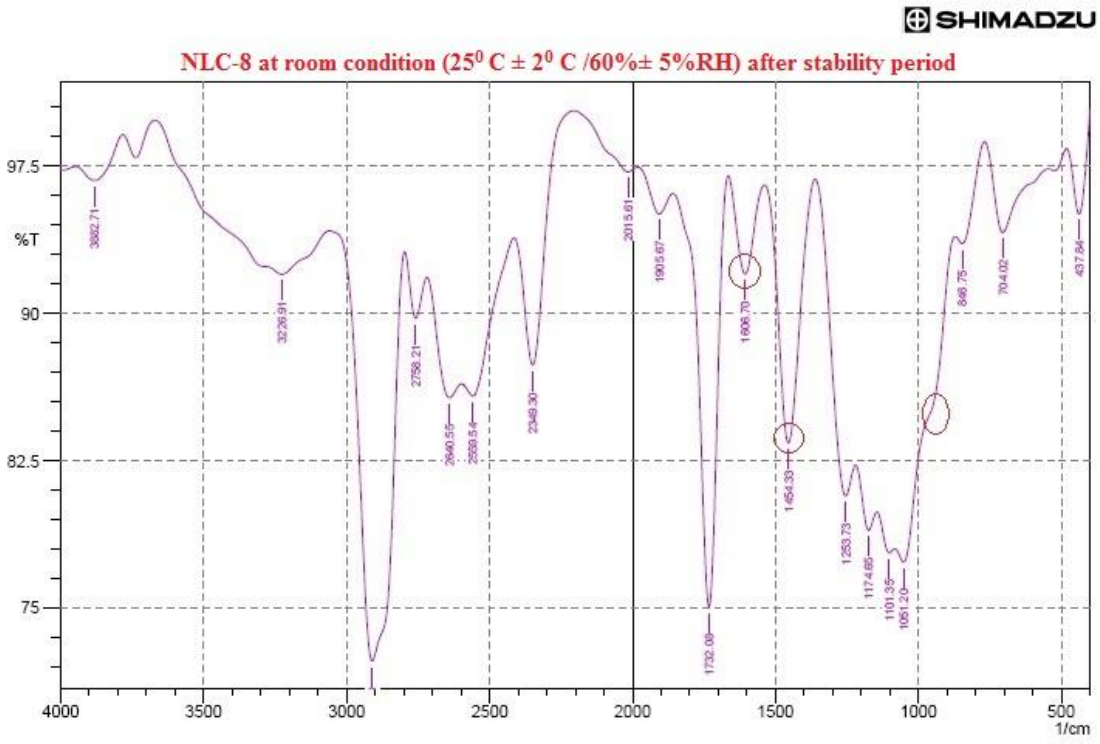



Figure 7.62: FTIR of NLC-8 after 6 months of stability period at room condition

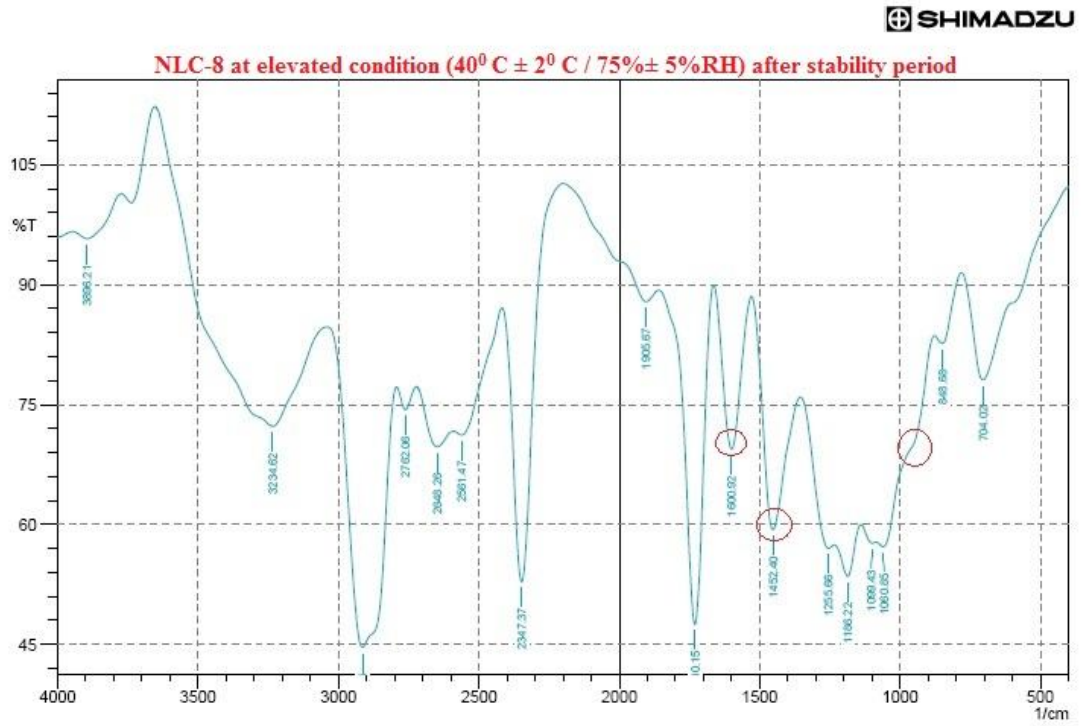



Figure 7.63: FTIR of NLC-8 after 6 months of stability period at elevated condition

Table 7.46: Comparison of characteristic peaks between RLX & optimized batch NLC-8 kept at different storage conditions

| **Functional group** | **Characteristic peaks ($cm^{-1}$)** | | |
|---|---|---|---|
| | **Plain RLX drug** | **Optimized batch NLC - 8** | |
| | | **A** | **B** |
| Benzene ring | 953.84 | ≈947.00 | ≈947.00 |
| -S- benzothiophene | 1465.00 | 1454.33 | 1452.40 |
| -C-O-C- stretching | 1600.02 | 1606.70 | 1600.92 |

(A=$25^0$ C ± $2^0$ C /60% ± 5% RH, B= $40^0$ C ± $2^0$ C / 75% ± 5% RH)

### 7.1.5 *In vivo* study

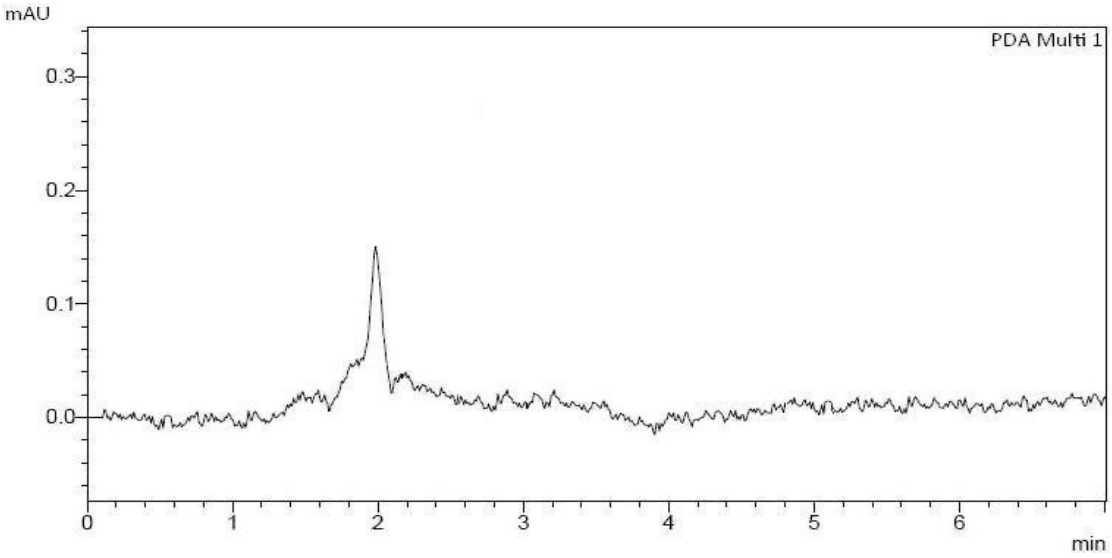



Figure 7.64: Chromatogram of rat plasma

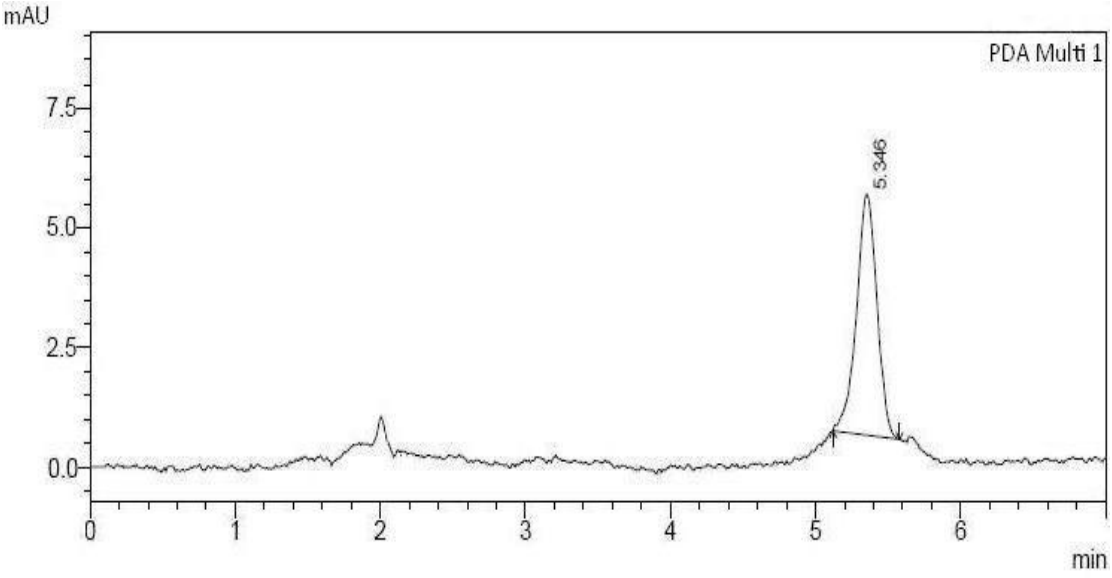
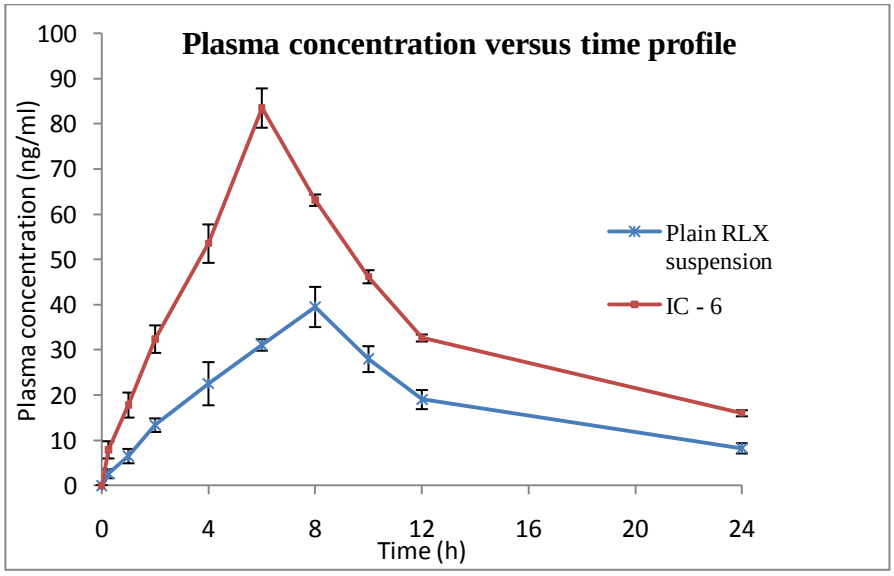



Figure 7.65: Chromatogram of RLX drug in rat plasma

Table 7.47: Comparative study of the pharmacokinetic parameters of optimized formulations and plain drug suspension

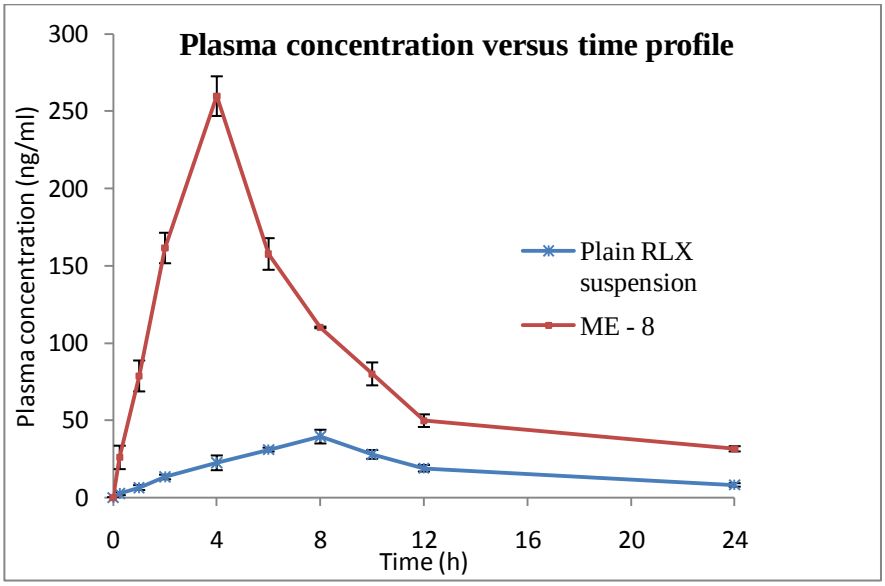
| Sample | Pharmacokinetic parameters | | | | |
|---|---|---|---|---|---|
| | **$C_{max}$ ± SD (ng/ml)** | **$T_{max}$ ± SD (h)** | **$[AUC]_{0-24}$ ± SD (ng*h /ml)** | **$t_{1/2}$ (h) ± SD** | **F** |
| Plain RLX suspension | 39.52 ± 4.44 | 8 | 452.46 ± 25.65 | 7.61 ± 0.34 | -- |
| IC-6 | 83.49 ± 4.35* | 6 | 878.64 ± 39.22* | 8.75 ± 0.14 | 1.94 |
| ME-8 | 259.77 ± 12.82* | 4 | 2078.19 ± 103.67* | 9.96 ± 0.14 | 4.59 |
| NLC-8 | 211.88 ± 16.92* | 4 | 1787.61 ± 107.64* | 9.64 ± 1.92 | 3.95 |

Value are expressed as mean ±SD; n=3, F – Relative bioavailability
*$p$< 0.05 compared with Plain RLX suspension



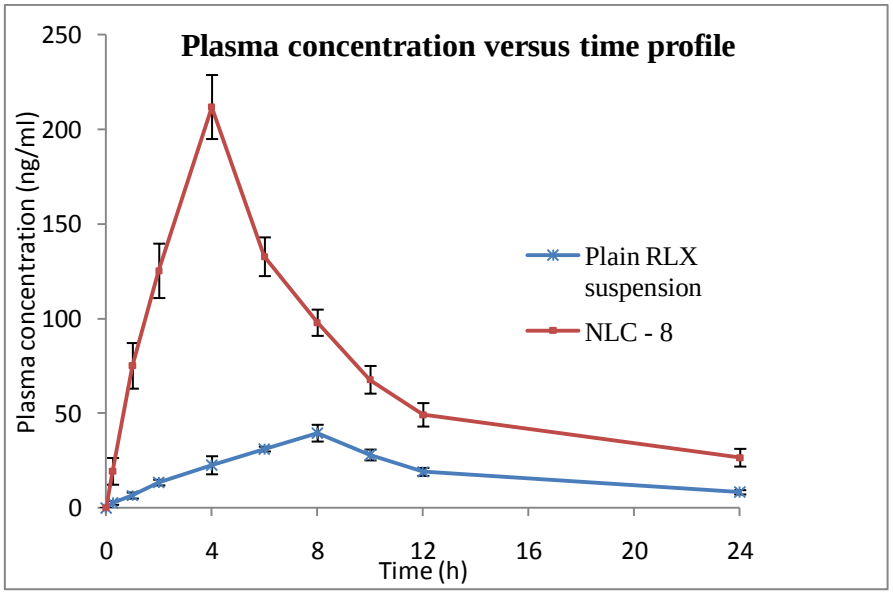

*n=3

Figure 7.66: Plasma concentration-time profile of the optimized inclusion complex IC-6 and plain drug suspension following oral administration to Wistar rats



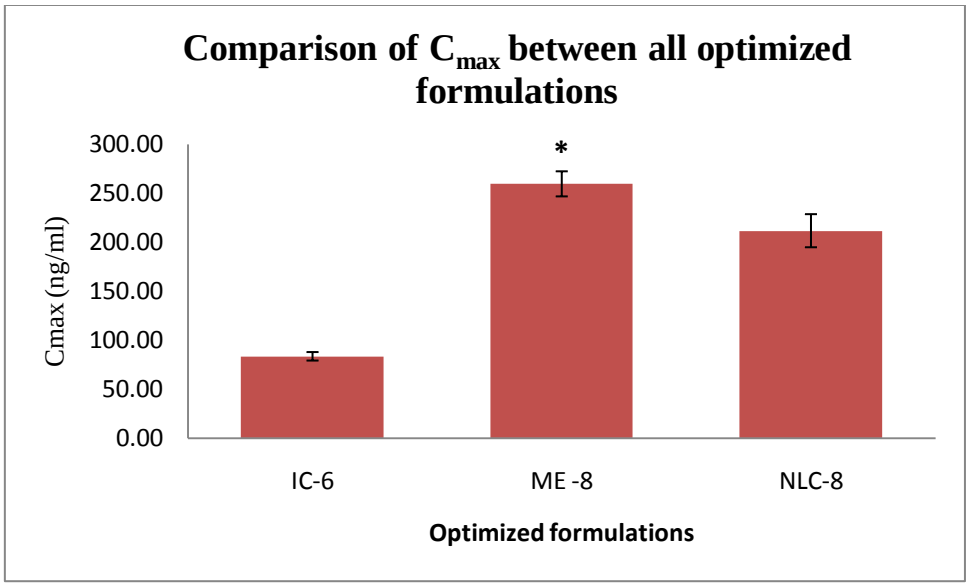

*n=3

Figure 7.67: Plasma concentration-time profile of the optimized microemulsion ME-8 and plain drug suspension following oral administration to Wistar rats



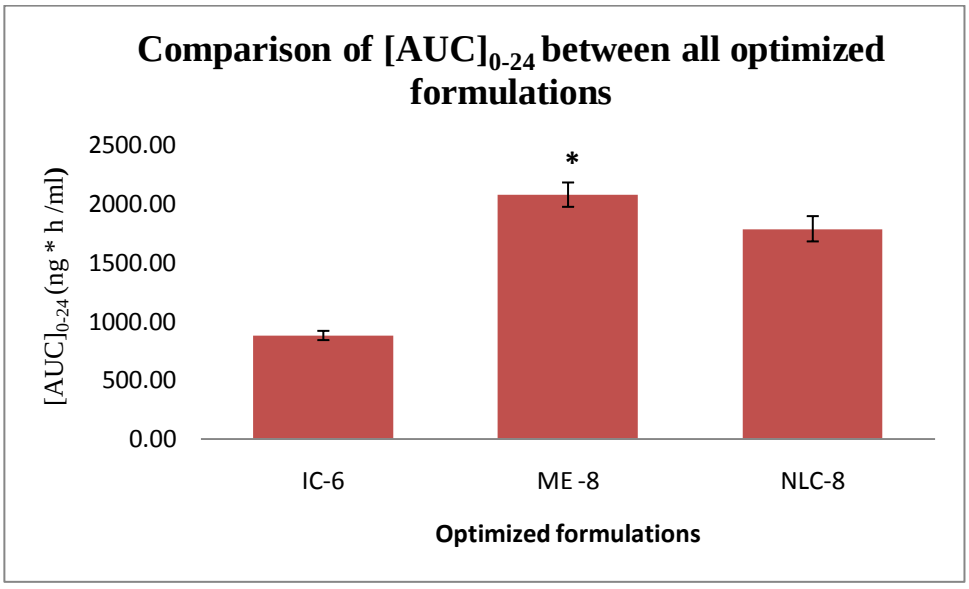

*n=3

Figure 7.68: Plasma concentration-time profile of the optimized NLC-8 and plain drug suspension following oral administration to Wistar rats




n=3

Figure 7.69: Comparison of maximum plasma concentration between all optimized formulations (*$p$<0.05)




n=3

Figure 7.70: Comparison of area under curve between all optimized formulations (*$p$<0.05)

## 7.2 DISCUSSION:

The objective of the present study was to prepare and characterize a stable RLX loaded solid dispersion, microemulsion and nanostructured lipid carriers.

### 7.2.1 Preformulation study of drug:

Before the development of various formulations, different preformulation parameters were carried out as discussed underneath.

#### 7.2.1.1 Organoleptic properties of RLX:-

From the organoleptic properties it was found that drug was in pale yellow colour odourless fine powder state.

#### 7.2.1.2 Melting point determination of RLX:-

After the study, it has been found that melting point of RLX was obtained in the range 266°C-269°C which met the standard specification of drug.

#### 7.2.1.3 Method for estimation of RLX:

- **Determination of UV absorption maxima for Drug:**

A maximum absorption maximum ($\lambda_{max}$) was determined for RLX in methanol. Result showed $\lambda_{max}$ at 288 nm that was accurately same to the earlier reported $\lambda_{max}$.

- **Preparation of Calibration curve of Drug:**

Calibration curve of RLX for estimation in Methanol: DMSO (2:1) was carried out. Graph showed linearity in the concentration range of 2-14 µg/ml with an equation Y= 0.0721 X + 0.0143 and regression coefficient of 0.9992 at $\lambda_{max}$ 289nm.

Calibration curve of RLX was carried out for estimation in Methanol. Graph showed linearity in the concentration range of 2-14 µg/ml with an equation Y= 0.0757 X - 0.0353 and regression coefficient of 0.9997 at $\lambda_{max}$ 288nm.

Calibration curve of RLX for estimation in Citro phosphate buffer pH 7.6 containing 1% of polysorbate 80 was also carried out. Graph showed linearity in the concentration range of 2-16 µg/ml with equation Y= 0.0538 X + 0.0149 and regression coefficient of 0.9997 at $\lambda_{max}$ 288nm.

Calibration curve of RLX was also carried out for estimation in rat blood plasma. Graph showed linearity in the concentration range of 0.05-5µg/ml with equation Y= 26667 X - 1640.4 and regression coefficient of 0.9872 at $\lambda_{max}$ 288nm.

### 7.2.1.4 Solubility determination

Solubility study of RLX was carried out in various non-aqueous and aqueous solvent systems. From the non-aqueous solvents DMSO, DMF and methanol had shown maximum drug solubility of >48, >42 and 7.57 ± 0.64 mg/ml, respectively. Water solubility of drug was found to be only 0.11 ± 0.02 mg/ml which proves its poor solubility in aqueous media. Drug solubility was also carried out in various buffer systems with or without surfactant as shown in table 7.8.

Below stated table shows summary of some important formulation and pharmacokinetic parameters for all three optimized batches. Each formulation is discussed separately as mentioned underneath.

Table 7.48: Summary table of three optimized formulations

| Evaluation parameters | Optimized formulations | | |
|---|---|---|---|
| | **IC-6** | **ME-8** | **NLC-8** |
| **Formulation related parameters** | | | |
| Drug content ± SD (%) | 24.11 ± 0.53 | 99.42 ± 0.22 | 4.02 ± 0.17 |
| % Cumulative drug release | 93.46 ± 0.26 (150 min) | 97.18 ± 0.98 (30 h) | 90.82 ± 2.40 (36 h) |
| Particle/globule size (nm) | -- | 13.52 | 27.3 |
| Zeta potential | -- | -5.05 | -13.6 |
| Entrapment efficiency ± SD (%) | -- | -- | 74.78 ± 3.34 |
| Practical yield ± SD (%) | 96.41 ± 2.05 | -- | 93.12 ± 2.88 |
| **Pharmacokinetic parameters** | | | |
| $C_{max}$ ± SD (ng/ml) | 83.49 ± 4.35 | 259.77 ± 12.82 | 211.88 ± 16.92 |
| $T_{max}$± SD (h) | 6 | 4 | 4 |
| $[AUC]_{0-24}$ ± SD (ng * h /ml) | 878.64 ± 39.22 | 2078.19 ± 103.67 | 1787.61 ± 107.64 |
| $t_{1/2}$ (h) ± SD | 8.75 ± 0.14 | 9.96 ± 0.14 | 9.64 ± 1.92 |
| Relative Bioavailability (F) | 1.94 | 4.59 | 3.95 |

Value are expressed as mean ± SD; n=3

### 7.2.2 Formulation and characterization of drug loaded inclusion complexes:-

The objective of the present study was to prepare and characterize the inclusion complex of RLX with β-CD. Inclusion complex was prepared by various methods such as physical mixture, co-precipitation and kneading method using β-CD as complexing agent.

### 7.2.2.1 Preliminary studies

**Drug excipients compatibility studies:**

Drug excipients compatibility study was carried out to check any physical and chemical interaction between drug and β-CD.

Drug excipients compatibility study was carried out by FTIR for pure drug alone and with β-CD. As shown in table 7.9, drug alone was showing its characteristics peaks at 953.84 $cm^{-1}$ (Benzene ring), 1465.00 $cm^{-1}$ (-S- benzothiophene), 1600.02 $cm^{-1}$ (-C-O-C- stretching) and 1641.49 $cm^{-1}$ (C=O stretching), which was found to be nearby similar when physical mixture of drug & β-CD were prepared i.e. 945.12$cm^{-1}$ (Benzene ring), 1462.04$cm^{-1}$ (-S- benzothiophene), 1602.85$cm^{-1}$ (-C-O-C- stretching) and 1645.28$cm^{-1}$ (C=O stretching). Characteristic peaks of drug were similar when mixture of drug and β-CD was studied. Therefore, it is indicating that there is no absence of any functional peaks in both spectra. Thus, it is revealed that there is no significant physiochemical interaction between drug and β-CD that indicate compatibility between them.

**7.2.2.2 Formulation of inclusion complexes**

The inclusion complex of RLX with β-CD was prepared by three different methods namely physical method, co-precipitation method and kneading method. Based on previously carried out preliminary trials, drug: β-CD was taken from 1:1 to 1:3 for all three methods. Furthermore increment in drug: carrier ratio beyond 1:3 did not give significant increment in drug content and water solubility. Therefore, drug: carrier ratio from 1:1 to 1:3 were considered for further optimization

**7.2.2.3 Characterization of inclusion complexes**

**Practical yield, drug content and water solubility:**

Practical yield of all batches were calculated as shown in table 7.10 and it was found that there is no significant difference in practical yield of all nine batches, but it was also observed that first three batches of physical mixture showed more yield compared to other two methods. The fact for this may be the easiness of physical mixture method to make inclusion complex compared to co-precipitation method and kneading method, as there might be chance of losing the product due to solidification of mixture.

Drug content of all batches were calculated as mentioned in table 7.10. It was observed that there was no significant effect of different methods of preparation for inclusion complexes on drug content. But from the study, it was noted that for a same method if ratio of drug to carrier was increased then there was significant increment in drug content which support the fact that more amount of carrier may accommodate larger amount of drug in a complex form.

From the solubility study shown in table 7.10, it was observed that plain drug has 0.11 ± 0.02 mg/ml solubility in water. Water solubility was drastically increased in a range from 0.17 ± 0.02 to 0.61 ± 0.04 mg/ml when plain drug was converted into complex form with β-CD. It was also noted that solubility of drug in physical method was significantly less while compared with other two methods. Maximum drug solubility was found in batch IC-6 prepared by co-precipitation method with drug: carrier ratio 1:3, which was found to be 5.5 fold more than plain drug. Therefore, based on solubility study batch IC-6 can be selected for further study, but it can be confirmed as optimized batch only after performing *in vitro* dissolution study.

***In vitro* drug release:**

*In vitro* drug release study was compared between plain drug and all nine batches of inclusion complexes as shown in table 7.11. From the release study of all batches it was observed that batch IC-6 was showing maximum drug release in 150 min owing to its high water solubility as discussed earlier. Release study of IC-6 batch was compared with plain drug and from that, it was found that there was pronounced enhancement in dissolution rate up to 93.46 ± 0.26% in RLX: β-CD inclusion complex compared to that of 50.39 ± 0.39 % by plain drug after 150 min. This is because of higher hydrophilicity and wetting property of β-CD. The results of the statistical analysis indicate significant dissolution rate enhancement of RLX from RLX: β-CD inclusion complex ($p< 0.05$) compared with plain RLX.

**Drug excipients compatibility study:**

Drug excipients compatibility study was carried out by FT-IR for optimized inclusion complex IC-6 as shown in figure 7.12. From the compatibility study it was observed that characteristic peaks of drug i.e. 945.12$cm^{-1}$ (Benzene ring), 1456.26$cm^{1}$ (-S- benzothiophene), 1600.92$cm^{-1}$ (-C-O-C- stretching) and 1647.21$cm^{-1}$ (C=O stretching) in inclusion complex IC-6 was found to be nearby similar with plain drug spectra. Therefore, it is indicating that there is no absence of any functional peaks in both spectra. Thus, it is revealed that there is no significant physiochemical interaction between drug and β-CD when formulating inclusion complex between them which indicate compatibility between drug and carrier.

**Differential scanning calorimetry:**

DSC measurements offer a close look at the crystallization and thermal behavior of the inclusion complex formulations. The main purpose of this study was to inspect whether prepared complex formulation is amorphous or crystalline in nature compared with raw

materials. The DSC thermograms of RLX, β-CD and inclusion complex of RLX & β-CD are shown in figure 7.13, 7.14 and 7.15, respectively. Thermograms of RLX and β-CD showed endothermic peaks at 272.92°C and 117.79°C corresponding to their melting points, respectively. Thermogram of optimized inclusion complex IC-6 showed the presence of β-CD peak in hydrated form at 96.21°C where broad peak of drug was observed at 273.30°C suggesting the conversion of RLX from crystalline form to amorphous form.

**X-Ray diffraction studies:**

XRD studies were performed in conjugation with DSC to verify the reduction of crystallinity of RLX. As shown in figure 7.16 to 7.19, the X ray diffraction patterns were recorded for pure drug, β-CD, physical mixture and inclusion complex of RLX & β-CD, respectively. Diffraction spectrum of drug showed distinct peaks at 2θ scale, which indicate crystalline nature of drug. Physical mixture of RLX & β-CD also showed distinct peaks at 2θ scale which also show crystalline nature of drug in physical mixture. In case of optimized batch IC-6 XRD pattern, there was a significant reduction in relative integrated intensity of all peaks and no distinct peak of drug was observed throughout the 2θ scale. This finding was supported with results obtained from DSC studies. Therefore, it can be concluded that RLX drug is completely converted into amorphous state when formulated in inclusion complex with β-CD that contribute enhancement in dissolution rate of RLX from inclusion complex.

**Surface morphology study:**

As shown in figure 7.20, scanning electron microscopic photograph of plain drug showed longer crystal with specific morphology, whereas figure 7.21 for RLX: β-CD inclusion complex showed decrease in crystallinity due to formation of drug: β-CD complex. This confirms complexation of drug with carrier β-CD attributed due to the conversion of amorphous form of drug in inclusion complex.

**Stability study:**

The optimized batch was kept at $25^0$ C ± $2^0$ C /60% ± 5% RH and $40^0$ C ± $2^0$ C / 75% ± 5% RH using stability chamber over period of six months. A result for stability study is shown in table 7.13. Throughout the study, insignificant change was observed in physical appearance and drug content of formulation in both stability conditions compared with initial observation of same batch. Dissolution rate for both stability conditions were satisfactory as shown in table 7.14. Compared to accelerated

conditions, normal condition showed more similar release rate with initial release rate of optimized formulation. As shown in table 7.15, FTIR study showed no difference between the characteristic peaks of RLX drug with the formulation kept at two different storage conditions. Thus, it is revealed that there is no significant physiochemical interaction between drug and excipients even after six months of storage period which indicate compatibility between them.

### 7.2.3 Formulation and characterization of drug loaded microemulsion:-

The objective of the present study was to prepare and characterize RLX loaded stable microemulsion prepared by water titration method.

#### 7.2.3.1 Preliminary studies

Before the formulation of drug loaded microemulsion, some preliminary studies like screening of components, drug excipients compatibility study and pseudo ternary phase diagram were carried out.

**Screening of components:**

The important criterion for selection of the materials was that all the components are pharmaceutically acceptable for oral administration and fall under GRAS (Generally recognized as safe) category. The relatively lowest solubility of the drug in the aqueous phase than surfactant and cosurfactant is important for the development of o/w microemulsion. Since, oily phase (internal phase) will act as drug reservoir it will provide controlled and sustained release of drug. Screening of components was carried out by performing solubility studies with different oils, surfactants and cosurfactants. Therefore, excipients having more drug solubility were selected for maximum drug loading. Result for solubility study is shown in table 7.16. Based on result for maximum solubility of drug Capmul MCM C8 (2.55 ± 0.96 mg/g), Tween 20 (7.25 ± 2.10 mg/g) and PEG 400 (8.50 ± 1.94 mg/g) were selected as oil, surfactant and cosurfactant, respectively. The selection of surfactant and cosurfactant mixture was done on the basis of HLB values, drug solubility, safety and stability profile. The non-ionic surfactant Tween 20 (HLB 16.7) has high chemical stability with less toxicity so it is more suitable for oral administration.

**Drug excipients compatibility studies:**

Compatibility study is an important step to prepare stable formulation. Compatibility between drug and excipients was checked physically and chemically.

Physical compatibility data are shown in table 7.17. Data were suggesting that there is no physical incompatibility between drug with oil and surfactant in terms of

precipitation, phase separation and color change when studied for the period of fifteen days. Data observed after the study were as same as at initial zero day. Therefore, it was suggesting that there is no any physical interaction take place between drug, oil and surfactant mixture.

Compatibility was checked at the end of fifteen days study for any chemical interaction using FTIR spectroscopy. Data is shown in table 7.18. Drug excipients compatibility study was carried out by FT-IR for pure drug and with excipients. The graph for pure drug was showing its characteristics peaks at 953.84 $cm^{-1}$ (Benzene ring), 1465.00 $cm^{-1}$ (-S- benzothiophene), 1600.02 $cm^{-1}$ (-C-O-C- stretching) and 1641.49 (C=O stretching), which was found to be nearby similar when physical mixture of drug each excipients was studied. Therefore it was indicating that there was no absence of any functional peaks in physical mixture of drug and individual excipients spectra. Thus, it was revealed that there was no significant physiochemical interaction between drug, oil and surfactants. From all these physicochemical compatibility studies it can be conclude that there is compatibility between all excipients with drug.

**Pseudo ternary phase diagram:**

A pseudo ternary phase diagram of the investigated quaternary system water/ Capmul MCM (C8)/ Tween 20/PEG 400 was prepared by water titration method and is depicted in figure 7.28. Formation of microemulsion systems (colored area) was observed at room temperature. Phase behavior investigations of this system demonstrated the suitable approach to determining the water phase, oil phase, surfactant concentration and cosurfactant concentration with which the transparent, one phase low-viscous microemulsion system was formed. The phase study revealed that the microemulsion region was found maximum when the surfactant-to-cosurfactant ratio was 3:1, so this ratio was taken for further study. From a formulation point of view, the increased oil content in microemulsion may provide a greater opportunity for the solubilization of RLX. Further, on the basis of phase diagram and the amount of drug to be incorporated in microemulsion, optimized formula will be developed.

**7.2.3.2 Optimization of microemulsion**

**7.2.3.2.1 Selection of microemulsions by checking primary parameters:-**

The drug loaded microemulsions were prepared and selected based on some primary parameters as mentioned in table 7.20. Sixteen batches were prepared by taking

different ratios of oil and surfactant mixture and evaluated for percentage transmittance, precipitation & phase separation in diluted and undiluted forms. The concentration of oil was selected between 8-14% w/w based on some previously done preliminary trials. Drug loaded microemulsion was showing turbidity beyond 14% w/w of oil concentration and below 8% w/w drug loading capacity of microemulsion was weakened. A wide range of percentage transmittance was observed from 61.7 ± 2.72 to 100.0 ± 0.10. RLX-9 and RLX-13 had shown very less transmittance 71.7 ± 1.24 and 61.7 ± 2.72, respectively. This may be attributed due to more amount of oil phase with respect to surfactant concentration in RLX-9 (12% W/W) and RLX-13 (14% W/W). From the visual transmittancy, it was observed that microemulsions formulation with low surfactant concentration or high oil content may leads to phase separation and precipitation problems. Therefore, from the study of these primary parameters, only six batches RLX-3, RLX-4, RLX-7, RLX-8, RLX-12 and RLX-16 were selected for further optimization of stable and maximum drug loaded microemulsion.

**7.2.3.2.2 Optimization of stable microemulsion with augmented drug loading:-**

Selected microemulsion batches were further analyzed for optimization of stable microemulsion with increased drug loading (6mg/g of microemulsion) as shown in table 7.21.

**Dilution test:**

All selected batches were diluted with water and kept for 24 h to check physical stability. At the end of study it was found that batches ME-3, ME-4, ME-7 and ME-8 were remained clear and transparent but batches ME-12 & ME-16 were showing haziness. Such type of insignificant haziness may be attributed due to high oil content in these two batches.

**Centrifugation test:**

To show any sign of creaming or phase separation, all batches were undergone highly stressful centrifugation test. Result for ME-12 & ME-16 was showing phase separation, which may attributed due to instability of dispersed phase at high speed.

**Percentage transmittance:**

Result of transmittancy study supported the data obtained in above mentioned two tests. Batches ME-12 & ME-16 were giving less transmittancy compared with other batches.

Data obtained for all selected six batches in aforementioned tests may point out the fact that microemulsion batches ME-12 and ME-16 were not remaining stable with augmented drug loading. Even though for final optimization all batches were studied for *in vitro* release.

***In vitro* drug release:**

*In vitro* drug release study of selected microemulsions and plain drug suspension were carried out using dialysis membrane as shown in table 7.22.

All six batches of microemulsion were showing sustained release of drug up to 30 h. At the end of study for the batches ME-12 and ME-16, it was found that microemulsion kept in dialysis membrane was become slight hazy. This results in less amount of drug release due to precipitation of drug within sac. All remaining four batches were showing comparatively similar sustained drug release (>95%) up to the period of 30 h. Further maximum drug loading study for more than 6mg/g was carried out in all these four batches. Based on this study, batch ME-8 was showing maximum drug loading (7mg/g) compared to other three batches. Therefore, based on aforementioned studies ME-8 was optimized for further characterization.

Release study of ME-8 batch was compared with plain drug as shown in figure 7.31 and from that, it was found that there was pronounced enhancement in release rate up to 97.18 ± 0.98% in optimized microemulsion compared to that of 10.51 ± 0.87% by plain drug suspension after 30 h. The results of the statistical analysis indicate significant release rate enhancement of RLX from optimize microemulsion ($p< 0.05$) compared with plain RLX suspension.

Various release kinetic models were fitted to determine release pattern of optimized batch as shown in table 7.23. From the regression analysis ($R^2$ value), release kinetics of optimized formulation had higher linearity for zero order and Higuchi model. Therefore, from all this discussion it can be concluded that optimized batch NLC-8 follows zero order kinetics where as it confirms diffusion controlled release mechanism from the Higuchi model.

**7.2.3.3 Characterization of optimized microemulsion**

Optimized batch ME-8 was characterized further by following parameters other than studied earlier.

**Dye solubility test**: Result of dye solubility stated that water soluble dye was uniformly distributed throughout the microemulsion without any lump formation. This conclusion identifies that the optimized microemulsion is o/w type.

**Drug content:** As shown in table 7.24, ME-8 showed 99.42 ± 0.22% drug content that indicates entrapment of entire amount of drug within microemulsion. High drug content also suggest that no amount of drug is degraded during the preparation of microemulsion at 65 ± 5°C.

**pH:** As shown in table 7.24, the pH value of the ME-8 was found to be 6.78 ± 0.17, which is ideal for oral administration of dosage form. Dosage form having pH value near to neutral pH would not irritate the mucosal membrane.

**Electro-conductivity**: Conductivity value of optimized ME-8 was found to be 0.205 ± 0.24 µS/cm as shown in table 7.24. From this it was also clear that the prepared microemulsion system was o/w type.

**Viscosity:** As shown in table 7.24, viscosity of ME-8 was found 84.57 ± 5.77 cp that is suitable for oral administration of liquid dosage forms.

**Drug-excipients compatibility study:**

Drug excipients compatibility study was carried out by FT-IR for optimized ME-8. From the compatibility study compared in table 7.25, it was observed that characteristic peaks of drug i.e. 947.05 $cm^{-1}$ (Benzene ring), 1460.11$cm^{-1}$ (-S- benzothiophene), 1606.70$cm^{-1}$ (-C-O-C- stretching) and 1645.00 (C=O stretching) in ME-8 was found to be nearby similar with plain drug spectra. Therefore, it is indicating that there is no absence of any functional peaks in both spectra. Thus, it is revealed that there is no significant physiochemical interaction between drug and excipients when formulating ME between them which indicate compatibility between drug, oil and surfactant mixtures.

**Globule size and zeta potential:** Result from figure 7.33 showed that globule size of ME-8 microemulsion have considerably smaller mean particle size of 13.52 nm with less polydispersity index of 0.214 which represent narrow distribution of globules within the system. The nano size of the globules was retained even after dilution with water that showed the microemulsion's suitability with more amount of water. Zeta potential is essential for evaluating storage stability of colloidal dispersion. The zeta potential of prepared optimized batch was found to be -5.05 mV as shown in figure 7.34 that impart good stability of microemulsion.

**Surface morphology study:**

Transmission electron microscopy was performed to study surface morphology and confirmation of globule size of optimized batch ME-8. TEM image of diluted sample of

microemulsion with distilled water is shown in figure 7.35. Image showed discrete globules of spherical shape with smooth surface. Aggregation of some globules was also found that because of overlapping of globules with each other. Globule size of optimized batch was found in between 15.49 to 24.62 nm that supports the data obtained in zetasizer.

**Stability study:**

Optimized batch was studied for six months stability period under three different conditions and results are reported in table 7.26, 7.27 & 7.28. Various stability evaluation tests were performed at different time intervals. No change was observed in physical appearance of formulations kept at different conditions that reveals physical stability at all three storage environments. Percentage transmittance of all batches was also found satisfactory. All batches were also studied for phase separation by applying high rate of centrifugal force, but no phase separation was reported for any batch. Drug content of all batches were showing satisfactory result up to one month stability period but thereafter little fall in drug content was observed. At the end of stability period, ME kept at accelerated stress showed insignificant reduction in drug content compared to other two conditions which attributed due to minute loss in drug due to high temperature.

Percentage Cumulative drug release was also carried out at the end of six month period and compared with drug release profile of initial day ME as shown in table 7.29. ME kept at refrigerator and room conditions showed satisfactory release profile with initial day ME release profile while ME kept at accelerated conditions showed significant less drug release profile. Such type of significant difference is might be because of instability of drug loaded ME at accelerated condition.

Therefore, from all these evaluation parameters, it can be concluded that prepared RLX loaded ME is giving satisfactory result at refrigerator and room conditions. Therefore it is suggested that ideal storage condition for RLX loaded ME is refrigerator and room conditions.

Drug excipients compatibility study carried out at the end of study shown in table 7.30 indicate no physicochemical interaction between drug and excipients in ME kept at all three conditions. This imparts stability between all drug, oil and surfactants for the period of six months.

### 7.2.4 Formulation and characterization of drug loaded nanostructured lipid carriers (NLCs):-

The objective of the present study was to prepare and characterize RLX loaded NLCs. In solid lipid nanoparticles, solid lipid turns in to perfect crystalline structure which leads to expulsion of drug. Therefore, despite SLNs being interesting delivery systems, relatively low drug-loading capacity and potential expulsion of the drug during storage led to development of new strategies in the form of NLCs. Hence NLCs were introduced as a new generation of lipid nanoparticles to overcome SLN shortfalls by blending solid lipid with liquid lipids. Because of NLCs' less ordered inner structure, NLC have higher drug loading capacity and they can also minimize drug expulsion during storage.

In a present work, NLCs were prepared by solvent diffusion method because it offers numerous advantages such as no need of using any special equipment or technique, easiness of handling and fast production.

#### 7.2.4.1 Preliminary studies

Before the formulation of drug loaded NLCs, some preliminary studies like selection of solid lipid, partition behavior of drug in various solid lipids, selection of liquid lipid and drug excipients compatibility study were carried out.

**Selection of solid lipid:**

To develop NLCs for poorly water soluble RLX, a selection of suitable lipids and other excipients is critical. All excipients under GRAS (Generally Regarded as Safe) category were selected for the formulation of NLCs. To keep drug in solubilization form, it is of prime important that drug has higher solubility in solid lipid. Solid lipids used under this study were Dynasan 114, Dynasan 118, stearic acid and GMS. From the necked eyes observation, it was found that drug solubility in Dynasan 114, Dynasan 118 and stearic acid was indistinct or indefinite. Where in case of GMS, solubility was found fairly satisfactory compared to others as shown in table 7.31.

**Partition behavior of RLX in various solid lipids:**

Drug entrapment efficiency and drug release from dosage forms are two important dependent factors for the development of any formulation. Determination of partition behavior of drug in selected lipid may also play a vital role in controlling these two parameters. Therefore, the success of development of NLCs is depending on selection of proper lipid for formulation of nanoparticles. For same purpose, partition of drug in various lipids was determined for better optimization of formulation. From the

result, it was found that raloxifene has higher partitioning in GMS (85.12 ± 9.48) compared to rest lipids. This finding also supported the high solubility of drug in GMS a discussed earlier. Therefore, GMS was selected as solid lipid for development of NLCs as it has good potential for solubilization and entrapment of more drug in NLC formulation. Results of partitioning of drug in lipid and aqueous phase are shown in table 7.32.

**Selection of liquid lipid:**

As discussed earlier for solid lipid, liquid lipid is also playing major role in entrapment of more amount of drug in case of NLC formulation. Various short chain liquid lipids like Capmul MCM C8, Isopropyl myristate, oleic acid, Labrafil ICM 1944 CS and Lebrafec CC were utilized for selection of best liquid lipid which can accommodate more amount of drug. From the result, it was found that Capmul MCM C8 has maximum drug solubility of 2.55 ± 0.96 mg/g compared with other lipids. Therefore, Capmul MCM C8 was selected as liquid lipid to make a matrix with GMS as solid lipid for the development of NLCs. Solubility of drug in various liquid lipids is shown in table 7.33.

**Drug excipients compatibility studies:**

Drug excipients compatibility study was carried out by FTIR for pure drug and with excipients as shown in table 7.34. The graph for pure drug was showing its characteristics peaks at 953.84 $cm^{-1}$ (Benzene ring), 1465.00 $cm^{-1}$ (-S-benzothiophene), and 1600.02 $cm^{-1}$ (-C-O-C- stretching), which was found to be nearby similar when physical mixture of drug, GMS & Capmul MCM C8 were prepared i.e. 947.05$cm^{-1}$ (Benzene ring), 1456.26$cm^{-1}$ (-S-benzothiophene) and 1604.77$cm^{-1}$ (-C-O-C-stretching). Characteristic peaks of drug were similar when mixture of drug and each excipient was studied. Therefore it is indicating that there was no absence of any functional peaks in physical mixture spectra. Thus, it can be said that there is no significant physiochemical interaction between drug and both lipids which indicate compatibility between them.

**7.2.4.2 Formulation of RLX loaded NLCs**

**Design of the experiment:-**

A complete $3^2$ factorial design was used to study the effect of two independent variables (solid lipid to liquid lipid concentration and stabilizer concentration) on the effect of entrapment efficiency of drug. Different ratios of solid lipid to liquid lipid (95:5, 90:10 & 85:15 % w/w) and concentration of PVA as stabilizer

(0.5,1.0 &1.5 % w/v) were used for optimization of NLCs. Based on solubility study of drug in various lipids, GMS and Capmul MCM C8 were selected as solid lipid and liquid lipid, respectively. Based on solubility of excipients in ethanol and rapid vaporization capacity of acetone, both in ratio of 1:1 (v/v) were selected as organic phase. PVA solution as aqueous phase was selected because of it reported stabilizing property in dispersion. Stirring speed of mechanical stirrer was kept fixed at 500 rpm based on finding obtained in preliminary trials. During preliminary study it was observed that uniform and distinct particles were not obtained when stirring speed of mechanical stirrer was kept below 500 rpm. It was also found that on increasing stirrer speed from 500-800 rpm, no significant difference was found in entrapment of drug and physical appearance of nanoparticles dispersion. In preliminary trials it was also observed that there is no any significant difference in drug entrapment when concentration of liquid lipid rises from 15% to 20% w/w. Therefore in present work, three different concentration of liquid lipid i.e. 5%, 10% and 15% w/w with respect to solid lipid were used for further optimization.

**7.2.4.3 Evaluation of RLX loaded NLCs**

**Percentage yield, drug loading and entrapment efficiency:**

Percentage yield, drug loading and entrapment efficiency of prepared NLCs are shown in table 7.35.

Practical yield of all NLCs batches were calculated and it was found that there was significant difference in percentage yield of all nine batches. Percentage yield of all batches were ranges from 80.15 ± 3.54 to 93.85 ± 2.17%. The observed significant difference in yield is because of stabilizer concentration. It was found that percentage yield of NLCs was increasing remarkably when concentration of stabilizer increased from 0.5% to 1.0% w/v ($p$<0.05) (shown in figure 7.43), but there was also noticeable fact found that no significant increment in percentage yield was obtained when concentration of stabilizer increased from 1.0% to 1.5% w/v as shown in figure 7.44. From this, it can be concluded that formulation with optimum 1.0% w/v PVA concentration may achieve maximum nanoparticles yield with good stability.

The drug entrapment efficiency and drug loading capacity of nanoparticles were increased from 30.83 ± 2.39 to 74.78 ± 3.34% and from 1.92 ± 0.12 to 4.02 ± 0.17%, respectively, with increasing percentage of Capmul MCM C8 from 5 to 15% w/w. Capmul MCM C8 being a Mono glycerides of caprylic acid form less perfect crystals with many imperfections offering space to accommodate the drug[1]. Liquid lipid

concentration of 15% was found to be significantly ($p$<0.05) increase the entrapment efficiency compared to 5% and 10% liquid lipid content. Increasing lipid content into the solid matrix (GMS) could increase solubility of the drug in lipid matrix, which increases its entrapment efficiency. Furthermore, it was noted that the incorporation of liquid lipid into solid lipid could led to many imperfections in the crystal lattice and left sufficient space to incorporate more amount of drug, thus leading to improved drug entrapment efficiency [2-4].

***In vitro* drug release:**

*In vitro* drug release study was carried out between plain drug suspension and various prepared NLCs as shown in table 7.36. From the result, it was observed that there is pronounced enhancement in releaser rate from 70.45 ± 2.22% to 90.82 ± 2.40% with respect to increment in entrapment efficiency of drug from 30.83 ± 2.39% to 74.78 ± 3.34%, respectively. From the obtained data, it was found that incorporation of more amount of liquid lipid which is prerequisite for the increment in entrapment efficiency plays a vital role for improvement of drug release rate from the NLCs. All prepared batches of NLCs have showed a biphasic drug release pattern. The burst drug release was found at initial 8 h followed by slow and sustained release up to 36 hr. Formulations (NLC-7, NLC-8 & NLC-9) containing high proportion of liquid lipid had shown fast release up to initial 8h compared to other formulations containing low proportion of liquid lipid.

Such type of drug release pattern was probably related with distribution of liquid lipid in nanoparticles. It was reported in earlier study[5] that when NLCs were prepared by solvent diffusion method at 70°C, liquid lipid was not allotted equivalently with solid lipid matrix. In such cases, more amount of liquid lipid was remain at the external shell of nanoparticles and very less liquid lipid was incorporated into the centre during cool process[6]. Therefore, the external part of particles was become soft and exhibited significantly more solubility for hydrophobic drugs[7] in which maximum drug can be loaded in NLCs with initially smoothly release profile. Therefore, NLC prepared at 70°C showed burst release in initial 8h followed by sustained release afterwards.

**Optimization of formulation:**

RLX loaded NLCs were prepared by using GMS, Capmul MCM C8 and PVA as the solid lipid, liquid lipid and stabilizing agent, respectively.

A $3^2$ full factorial design was employed in optimizing the formula. The concentration of GMS: Capmul MCM C8 ($X_1$) and concentration of PVA solution was taken as the independent variables whereas the entrapment efficiency was taken as the dependent variable. The maximum percent entrapment was found to be 74.78 at 1 level of $X_1$ and 0 level of $X_2$.

The entrapment efficiency was obtained by conducting systematic experiments at various levels and was subjected to regression analysis. Thus a polynomial equation of the full model was obtained. The entrapment efficiency for nine batches showed a variation in the range of 30.83% to 74.78% as shown in table 7.37.

Non-significant terms were rejected based on the P values (<0.05) to obtain reduced model followed by determination of regression of the reduced model as shown in table 7.38.

Significance of each coefficient was established by P value and T stat value. Larger the value of t stat, more significant is the factor whereas lower the magnitude of the P value, more significant is the corresponding coefficient.

Based on the P value, $X_1$, $X_2$ and $X_2^2$ factors were found to be significant. All the other factors were found to be insignificant.

The results of ANOVA for entrapment efficiency are given in table 7.39. F statistic of the ANOVA results of full and reduced models confirmed the omission of the significant and the non-significant terms. As the calculated F value was found to be less than the tabular F value ($\propto$ = 0.05, 2), we can conclude that the neglected terms do not significantly contribute in the prediction of the entrapment efficiency. The value for the coefficient of $X_1$ was found to be greatest. From this it was concluded that $X_1$ is the factor which affects the entrapment efficiency maximum. But at the same time the value of $X_2^2$ was found to be significant from this we can conclude that a slight increase in the value of concentration of surfactant also significantly influences our formulation. Therefore, the concentration of surfactant can also be considered a critical factor in formulation of RLX loaded NLCs.

The value of the adjusted coefficient $R^2$ for the full model and the reduced model was found to be 0.9766 and 0.9830. As these values are above 0.9, a high significance of the model is achieved.

The determination coefficient $R^2$ gives an excellent idea about the goodness of the fit of the model. The $R^2$ values for the full and the reduced model ware found to

be 0.9912 and 0.9894. From this we can conclude that above 90% of the variations are explained by the model.

Lastly, high values of the correlation coefficient for the full model and the reduced model implies a very good correlation between the selected independent variables.

**Contour plots**

Figure 7.48 showed the contour plot drawn at the selected values of the independent variables. The plots were found to be nonlinear and having curved segment for each prefixed values which signify nonlinear relationship between the selected variables. Further response plots were plotted (shown in figure 7.49) which gives information for learning about both the main and interaction effects of the independent variables. From the graph it is clear that the highest entrapment efficiency is found when the concentration values of the solid lipid: liquid lipid is near to 1(85:15) and the stabilizer concentration is in range of 0 to 1 (Between1 to 1.5%).

**Check point analysis**

Check point analysis was carried out to confirm the utility of established contour plot and reduced polynomial equation in preparation of raloxifene loaded NLC. Values of independent variables (X1 and X2) were taken from the contour plot and the value of % entrapment of efficiency was calculated by substituting the values in the reduced polynomial equation. Drug loaded NLC were prepared experimentally by taking the independent variables as decided earlier. Difference of theoretically calculated value of entrapment efficiency and experimentally obtained value were calculated and % error was found as shown in table 7.41. The percent error in the check point analysis was found to be low. This signifies the role of the reduced model, contour plots and the check point analysis in the mathematical modeling.

By studying full $3^2$ factorial design, it was concluded that batch NLC-8 was the optimized batch owing to its maximum entrapment efficiency of 74.78 ± 3.34%. Therefore NLC-8 was selected and proceeds further for characterization.

**7.2.4.4 Characterization of optimized RLX loaded NLC's**

**Drug release comparison of optimized batch with plain drug suspension:**

NLC-8 was compared for *in vitro* drug release rate with plain drug suspension as shown in figure 7.50. From the result, it was found that batch NLC-8 have shown biphasic sustained release of 90.82 ± 2.40% drug in 36 h while Plain drug suspension has shown only 11.30 ± 1.02% drug release in 36 h. The results of the statistical

analysis indicate significant *in vitro* release rate enhancement of RLX from NLC-8 ($p<0.05$) compared with plain RLX suspension.

**Drug release kinetics:**

Various release kinetic models were fitted to determine release pattern of optimized batch as shown in table 7.42. From the regression analysis ($R^2$ value), release kinetics of optimized formulation had higher linearity for zero order and Higuchi model. Therefore, from all this discussion it can be concluded that optimized batch NLC-8 follows zero order kinetics where as it confirms diffusion controlled release mechanism from the Higuchi model.

**Drug excipients compatibility study**

Drug excipients compatibility study was carried out by FTIR for optimized NLC-8. From the compatibility study shown in table 7.43, it was observed that characteristic peaks of drug i.e. 947.00$cm^{-1}$ (Benzene ring), 1458.18$cm^{-1}$ (-S- benzothiophene) and 1600.92$cm^{-1}$ (-C-O-C- stretching) in NLC-8 was found to be nearby similar with plain drug spectra. Therefore it was indicating that there is no absence of any functional peaks of drug in spectra of optimized batch of NLC. Thus, it can be revealed that there is no significant physiochemical interaction between drug and excipients when formulating NLC between them which indicate compatibility between drug and lipids.

**Particle size and zeta potential measurement**

Particle size and zeta potential of optimized batch NLC-8 were shown in 7.52 and 7.53, respectively. Particle size measurements were required to confirm the formulation of nanoparticles in nano range. Result showed that NLC-8 have considerably smaller mean particle size of 27.3 nm with less polydispersity index of 0.287 which represent narrow distribution of nanoparticles within the system. Zeta potential is essential for evaluating storage stability of colloidal dispersion. The zeta potential of prepared optimized batch was found to be -13.6 mV, which impart good stability of NLCs dispersion.

**Differential scanning calorimetry**

DSC measurements offer a close look at the crystallization and thermal behaviour of the nanoparticles. The main purpose of this study was to inspect change in amorphous or crystalline nature of prepared formulation compared with raw materials. The DSC thermograms of GMS, physical mixture of RLX, GMS & Capmul MCM C8 and optimized batch NLC-8 are shown in figure 7.54, 7.55 and 7.56, respectively.

Thermogram of RLX was shown in figure 7.13. Thermograms of RLX and GMS showed endothermic peaks at 272.92°C and 62.89°C corresponding to their melting points respectively. DSC plot of physical mixture showing existence of melting peak of drug and GMS both at their respective place. Thermogram of NLC-8 showed the presence of GMS peak at 63.42°C where no melting peak of drug was observed. This indicates either complete solubilization of drug in the lipid matrix or conversion of drug from crystalline form to amorphous form that has been dispersed in the lipid matrix.

**X-Ray diffraction studies**

XRD studies were performed in conjugation with DSC to verify the reduction of crystallinity of RLX. The X- ray diffraction patterns were recorded for GMS, physical mixture and optimized batch NLC-8 as shown in figure 7.57, 7.58 and 7.59. XRD pattern of drug was shown in figure 7.16. Diffraction spectrum of drug showed distinct peaks at 2θ scale which indicate crystalline nature of drug. Physical mixture also showed distinct peaks at 2θ scale which indicate crystalline nature of drug in physical mixture. In case of NLC-8 batch XRD pattern, there was a significant reduction in relative integrated intensity of all peaks and no distinct peak of drug was observed throughout the 2θ scale. This finding was supported with results obtained from DSC studies. Therefore, it can be concluded that RLX drug is completely in amorphous state when formulated in NLC with solid lipid and liquid lipid.

**Surface morphology study:**

The surface morphology of optimized NLC-8 was studied by transmission electron microscope. As shown in figure 7.60, the discrete NLC particles were observed with spherical shape and smooth surface. The spherical morphology of NLCs has been reported in previous studies[6,8]. In addition, TEM image also confirmed nano size of prepared NLCs which supports the result of particle size measurement by zetasizer.

**Stability study:**

Physical appearance & entrapment efficiency of NLC - 8 during stability period of six month is shown in table 7.44. At the end of study no change was observed in physical appearance of formulation in both stability conditions, where in case of entrapment efficiency there was significant reduction at accelerated condition compared with initial entrapment of drug. Percentage cumulative drug release was also carried out for both stability conditions at the end of study which is shown in figure 7.61. The release rate for the batch kept at room condition was satisfactory, but at accelerated

conditions there was significantly reduction in drug release rate. The result shown for entrapment efficiency and *in vitro* release rate at accelerated condition may attribute small degradation of drug at this condition which supports that fact that accelerated temperature is not suitable condition for the storage of lipid based formulation. To check any drug excipients interaction during both storage conditions, drug excipients compatibility study was also carried out as shown in table 7.46. Result showed no physicochemical interaction between drug and excipients in optimized batch kept at both conditions. This imparts stability between all drug, solid lipid and liquid lipid for the period of six months. But based on data obtained for percentage entrapment and release profile from both stability conditions, it can be concluded that room condition ($25^0C \pm 2^0C/60\% \pm 5\%$ RH) is a more favorable storage condition compared with accelerated condition for prepared lipid based NLC formulation for a longer period of time.

**7.2.5 *In vivo* study**

RLX was found to be well separated under used HPLC conditions. Retention time of drug was found to be 5.346 ± 0.21 min. As discussed in section 7.2.1.3, calibration curve of drug was found linear in rat plasma. In HPLC chromatogram of drug, well separated peak of RLX was found along with plasma peak as shown in figure 7.65.

The oral bioavailability of RLX is very much limited due to its poor water solubility and extensive first pass metabolism. Therefore, by keeping this point in mind an attempt was made to increase bioavailability of RLX using different techniques such as inclusion complex, particulate drug delivery and vesicular drug delivery systems. In the present work, plain drug suspension, drug loaded inclusion complex, microemulsion and NLCs were administered orally to female Wistar rats followed by determination of various pharmacokinetics parameters.

The plasma concentration verses time profile of plain drug suspension with optimized batches of IC, ME and NLC is shown in figure 7.66, 7.67 and 7.68, respectively. Pharmacokinetic parameters of all optimized batches was calculated and shown in table 7.47.

Figure 7.66 showed higher $C_{max}$ for optimized IC-6 batch (83.49 ± 4.35 ng/ml) at 6 h with respect to plain drug suspension (39.52 ± 4.44 ng/ml) at 8 h. The $[AUC]_{0-24}$ of prepared inclusion complex was found 878.64 ± 39.22 ng*h /ml in comparison of plain drug suspension 452.46 ± 25.65 ng*h/ml and was found

statistically significance ($p$<0.05). This result may be attributed due to more water solubility of optimized inclusion complexes than the plain drug. Elimination half-life ($t_{1/2}$) of inclusion complex was also found higher than drug suspension. All these result shows nearby two fold increments in bioavailability of drug in case of drug loaded inclusion complex.

Figure 7.67 and figure 7.68 showed higher $C_{max}$ for optimized batch ME-8 (259.77 ± 12.82 ng/ml) and NLC-8 (211.88 ± 16.92 ng/ml) at 4 h with respect to plain drug suspension (39.52 ± 4.44 ng/ml) at 8 h. The $[AUC]_{0-24}$ of ME-8 and NLC-8 was found 2078.19 ± 103.67 ng*h/ml and 1787.61 ± 107.64 ng*h/ml in comparison of plain drug suspension 452.46 ± 25.65 ng*h/ml and was found statistically significance ($p$< 0.05). This significance increment in $[AUC]_{0-24}$ for both ME and NLC may be due to their nano size and by the avoidance of first pass metabolism through lymphatic transport pathway. Elimination half-life ($t_{1/2}$) of drug was also found higher in both nano formulations than drug suspension. This result may be attributed due to their sustained release followed by slower elimination rate of drug form both nano size formulations than the plain drug. Overall nearby 4 fold increments in bioavailability with NLC-8 and 4.5 fold increments with ME-8 was obtained which confer their potential for improved oral delivery of RLX.

From overall comparison of pharmacokinetic parameters between all three optimized formulations inclusion complex, microemulsion and nano structured lipid carrier, optimized batch of microemulsion ME-8 showed greater improvement in $C_{max}$, $T_{max}$, $[AUC]_{0-24}$, $t_{1/2}$ with 4.5 fold increment in bioavailability compared to plain drug suspension as shown in figure 7.69 and figure 7.70. Several mechanisms might be working for enhancement of bioavailability in microemulsion such as (1) the reduction in efflux of RLX from intestinal membrane due to modulation of p-glycoprotein inhibitory function carried out by polysorbate 20 as primary surfactant (2) Improvement in dissolution rate of RLX due to effective surface area of nanosize globules of ME which may play important role in enhancement of absorption rate of drug owing to its higher concentration gradient (3) avoidance of first pass metabolism by lymphatic transport facilitate by fatty acids present in microemulsion formulation.

`In addition to this, microemulsion is very informal to formulate even at large scale production in a less time with cost effectiveness and loading capacity of entire amount of drug. Therefore from all the discussion, it can be said that ME have good potential as suitable carrier system for the improvement in oral bioavailability of poorly soluble RLX which can play major role in the better treatment of osteoporosis.

**7.2.6 References:**

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## 8. CONCLUSION

In the present work, an attempt was made to improve bioavailability of poorly soluble RLX by using different techniques such as physical modification of drug (inclusion complex) or novel drug delivery systems (microemulsion and nano structured lipid carrier).

Drug loaded inclusion complex was successfully prepared by co-precipitation method. Various solid characteristic studies like DSC, XRD and SEM shows transformation of crystalline nature of drug to amorphous nature which is an evidence for improving water solubility of RLX by 5.5 fold compared to plain drug that attribute the significant increment in dissolution rate of drug from optimized inclusion complex. No significant change in characteristics of optimized IC was observed after six months of stability studies.

Microemulsion was successfully prepared by water titration method. Particle size and TEM study shows nano sized discrete spherical globules with smooth surface which result in sustained release of drug from optimized batch up to the period of 30 h. Nano size imparts a model role in improvement of bioavailability owing to enhancement in dissolution rate. Stability study shows extremely stable formulation for the period of six months with no significant change in characteristics of optimized microemulsion was observed during this time.

NLCs were prepared by solvent diffusion method at 70°C which exhibit high entrapment efficiency with sustained release of drug up to the period of 36 h. DSC and XRD confirms transformation of crystal nature of drug into amorphous nature which plays an important role in enhancement of absorption rate followed by bioavailability. Particle size and TEM study confirms nano sized discrete spherical globules with smooth surface area. Stability study of optimized batch shows extremely stable formulation for the period of six months which support the fact that dried lyophilized nanocarriers may remain stable for long period of time that may helpful commercially too.

To draw a final conclusion, a pharmacokinetic study was carried out to find out improvement in bioavailability of optimized formulations. Result shows pronounced improvement in pharmacokinetic parameters like $C_{max}$, $T_{max}$, AUC and $t_{1/2}$ which is responsible for enhanced absorption, bioavailability and sustained release of drug (In case of nanocarriers).

# 8. CONCLUSION

Overall improvement in bioavailability from prepared formulations is near by two fold, four fold and 4.5 fold in case of inclusion complex, NLCs and microemulsion, respectively. From the aforementioned result, it is concluded that RLX loaded microemulsion is showing significant improvement in bioavailability of poorly soluble RLX than other formulations which confer its potential role as suitable carrier system for oral delivery of RLX in the treatment of osteoporosis.