# PREPARATION OF URSODEOXYCHOLIC ACID LIPOSOMAL FORMULATION FOR THE TREATMENT OF LIVER DISORDER

Thesis Submitted for the Partial Fulfilment of the
Degree of Master of Pharmacy in the
Faculty of Engineering & Technology
Jadavpur University

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## CERTIFICATE OF APPROVAL

*This is to certify that the project entitled* **"*Preparation of Ursodeoxycholic acid liposomal formulation for the treatment of liver disorder*"** *submitted by* **Ms. Itika Agarwal***, is absolutely based upon her work under my direct supervision at the Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, for the requirement of completion of M. Pharm. I am satisfied that she has completed her work with proper care & confidence to my entire satisfaction.*

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# DECLARATION OF ORIGINALITY
# AND
# COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that the thesis contains literature survey and original research work by the undersigned candidate, entitled **"*Preparation of Ursodeoxycholic acid liposomal formulation for the treatment of liver disorder*".**

All information in this document have been obtained and presented in accordance with academic rule and ethical conduct.

I also declare that as required by these rules and conduct, I have fully cited and referenced all materials that are not original to this work.

To the best of my knowledge the content of this thesis does not form a basis for the award of any previous degree to anyone else.

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# PREFACE

The present thesis entitled **"Preparation of Ursodeoxycholic acid liposomal formulation for the treatment of liver disorder"** comprises of the research work carried out under strict guidance, supervision and prior consultation of my guide Prof. (Dr.) Tapan Kumar Chatterjee, Pharmacology Research laboratory in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata for the degree of Master in Pharmacy.

The use of medicine has become the most common intervention in health care now a day. To promote safety and to reduce the toxicity of drugs, lots of research is initiated to minimize the harmful potentials of new drugs and maximize their bioavailability.

The present study deals with the preparation of Ursodeoxycholic acid liposomal formulation for the treatment of liver disorder. After preparation of liposomal formulation it was studied for different parameters and then the acute toxicity studies have been carried out to check the toxicity and the dose in animals. Hepatoprotective activity was carried out with this formulation.

In this thesis, the details covering the above mentioned studies are presented in a logical manner with related references attached to each chapter. The results of different studies are summarized in the form of table as well as figures indicating statistical significance level and conclusion drawn in a manner to justify the work scientifically.

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# CHAPTER 1

# INTRODUCTION

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1.LIPOSOME:

### 1.1.1. Introduction

Aiming of drug delivery at a rate dictated by the need of the body and at the targeted sites both selectively and efficiently lead to the development of micro particulate drug carriers[1]. These micro particulate drug carriers including liposome (lipid vesicles) are technologies aiming at effective medication by physicochemical modification of dosing formulations[2].

Liposome the lipid vesicles are enclosed simple, microscopic structures consisting of one (unilamellar) or more (multilamellar) concentric spheres of lipid bilayers separated by an aqueous compartment[3]. The liposomes are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar head groups of the lipids oriented towards the extravesicular solution and the inner cavity[4]. The encapsulation of both lipophilic and hydrophilic drugs occurs as the drug molecules can either be encapsulated in the aqueous space or intercalated into the lipid bilayers[3]. Here, the exact location of the drug depends upon its physicochemical characteristics and the composition of the lipids[1].

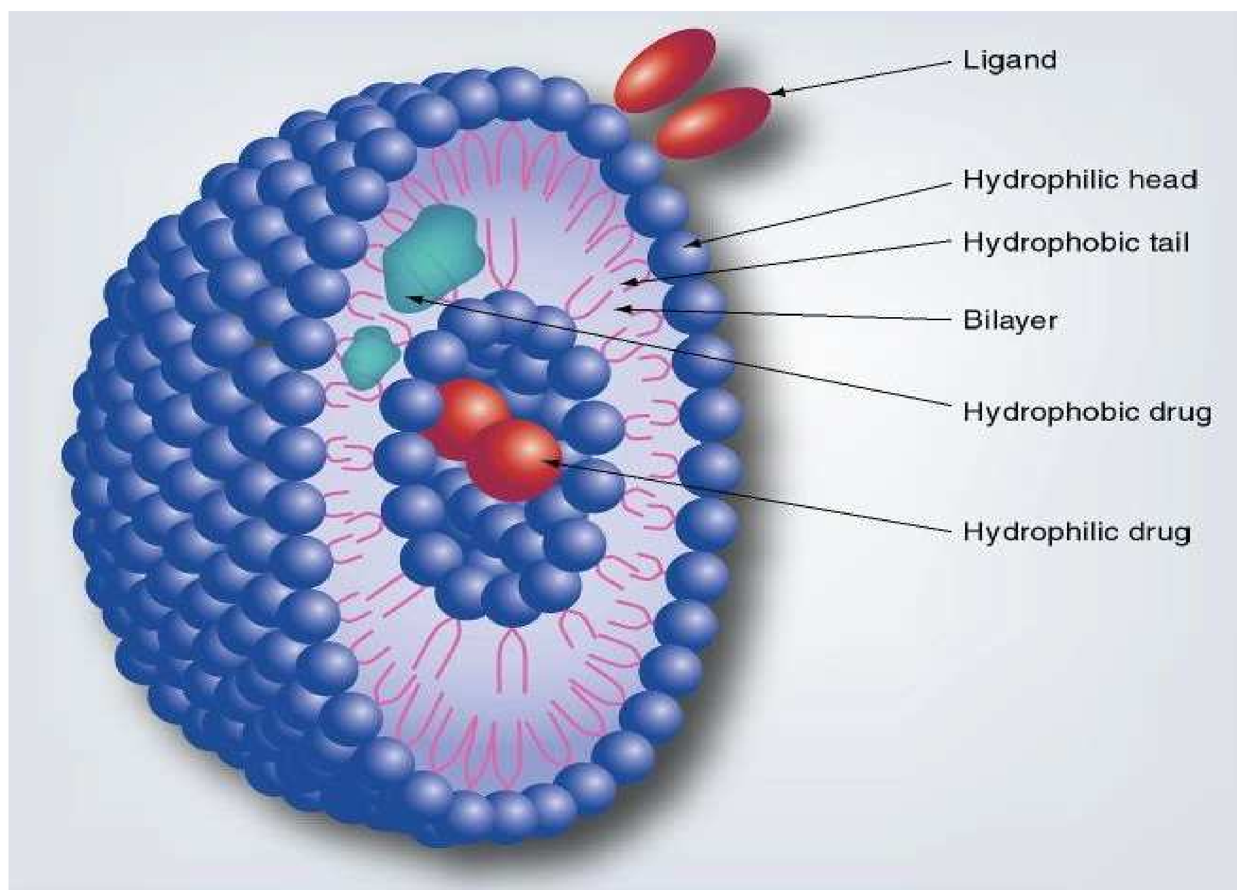



Figure1.1: Structure of liposome.

The lipid bilayer components of liposome can be utilized as the component of biological membrane therefore, making the liposome biologically inert, bio-compatible, biodegradable, non-toxic and non-immunological[5]. The usually high *in-vivo* tolerance of liposome is attributed by the fact that the structural components of the carrier, phospholipids and cholesterol, are no different than those of the membrane lipids. Therefore, inside the body they are acted upon by the enzyme systems into natural intermediates like glycerol phosphate, fatty acids, ethanolamine, choline and acyl-Co-A, and either metabolized further to provide energy or enter lipid pools which are drawn on to build new lipids, and which replace those that naturally turnover in biological membrane[3].

Liposomes were first described by Bangham in the early 1960s, and later due to its wide range of application it took a lot of advancement in technologies and introduced the concept of novel liposome. An example of the novel liposome or the second generation is the so-called stealth (sterically stabilized) liposomes, which have reduced toxicity during anticancer therapy, and these liposomes are the basic unit for the attachment of ligands for site-specific targeting. Another new area of liposome technology is their potential as vectors

to complex and delivers DNA, which has shown promise in delivery of genes in gene therapy[3].

Liposome carrier is amongst the few drug carriers which reached the stage of clinical trial showing strong potential as model membrane and as carrier of drugs, DNA, ATP enzymes and diagnostic agents [3].

### 1.1.2. ADVANTAGES OF LIPOSOME

Liposomes as drug delivery systems can offer several advantages over conventional dosage forms especially for parenteral (i.e. local or systemic injection or infusion), topical, and pulmonary route of administration. The preceding discussion shows that liposomes exhibit different bio-distribution and pharmacokinetics than free drug molecules. In several cases this can be used to improve the therapeutic efficacy of the encapsulated drug molecules. The limitations can be reduced bioavailability of the drug, saturation of the cells of the immune system with lipids and potentially increased toxicity of some drugs due to their increased interactions with particular cells. The benefits of drug loaded liposomes, which can be applied as (colloidal) solution, aerosol, or in (semi) solid forms, such as creams and gels, can be summarized into seven categories:

(i) Improved solubility of lipophilic and amphiphilic drugs. Examples include Porphyrins, Amphotericin B, Minoxidil, some peptides, and anthracyclines, respectively; furthermore, in some cases hydrophilic drugs, such as anticancer agent Doxorubicin or Acyclovir can be encapsulateded in the liposome interior at concentrations several fold above their aqueous solubility. This is possible due to precipitation of the drug or gel formation inside the liposome with appropriate substances encapsulated[6] .

(ii) Passive targeting to the cells of the immune system, especially cells of the mononuclear phagocytic system (in older literature reticuloendothelial system). Examples are antimonials, Amphotericin B, porphyrins and also vaccines, immunomodulators or (immuno)supressors;

(iii) Sustained release system of systemically or locally administered liposomes. Examples are doxorubicin, cytosine arabinose, cortisones, biological proteins or peptides such as vasopressin;

(iv) Site-avoidance mechanism: liposomes do not dispose in certain organs, suchas heart, kidneys, brain, and nervous system and this reduces cardio-, nephro-, and neuro-toxicity. Typical examples are reduced nephrotoxicity of Amphotericin B, and reduced cardiotoxicity of Doxorubicin liposomes;

(v) Site specific targeting: in certain cases liposomes with surface attached ligands can bind to target cells ('key and lock' mechanism), or can be delivered into the target tissue by local anatomical conditions such as leaky and badly formed blood vessels, their basal lamina, and capillaries. Examples include anticancer, antiinfection and antiinflammatory drugs;

(vi) Improved transfer of hydrophilic, charged molecules such as chelators, antibiotics, plasmids, and genes into cells; and

(vii) Improved penetration into tissues, especially in the case of dermally applied liposomal dosage forms. Examples include anaesthetics, corticosteroids, and insulin.

Among numerous studies which showed improved therapeutic index we shall mention only those which had significant impact and are also in various phases of preclinical and clinical studies in humans. In general, liposome encapsulation is considered when drugs are very potent, toxic and have very short life times in the blood circulation or at the sites of local (subcutaneous, intramuscular or intrapulmonary) administration.

### 1.1.3. TYPES OF LIPOSOMES

Liposomes are classified on the basis of the following properties:

- Structural parameters

- Method of preparation

- Composition and application

The varying size of the liposomes from the range of very small 0.25µm to large as 2.5µm helps classify them based on the structural parameters. The vesicle size of a liposome governs its circulating half-life and its number of bilayers controls the amount of drug encapsulation in the liposome. So, based on their size and number of bilayers the liposomes can be broadly classified into the following major groups: (a) unilamellar vesicles (ULV) (b) multilamellar vesicles (MLV) (c) oligolamellar vesicles (OLV) and (d) multivesicular vesicles. Further, the

ULV can be classified into (i) small unilamellar vesicles (ii) medium unilamellar vesicles (iii) large unilamellar vesicles and (iv) giant unilamellar vesicles.

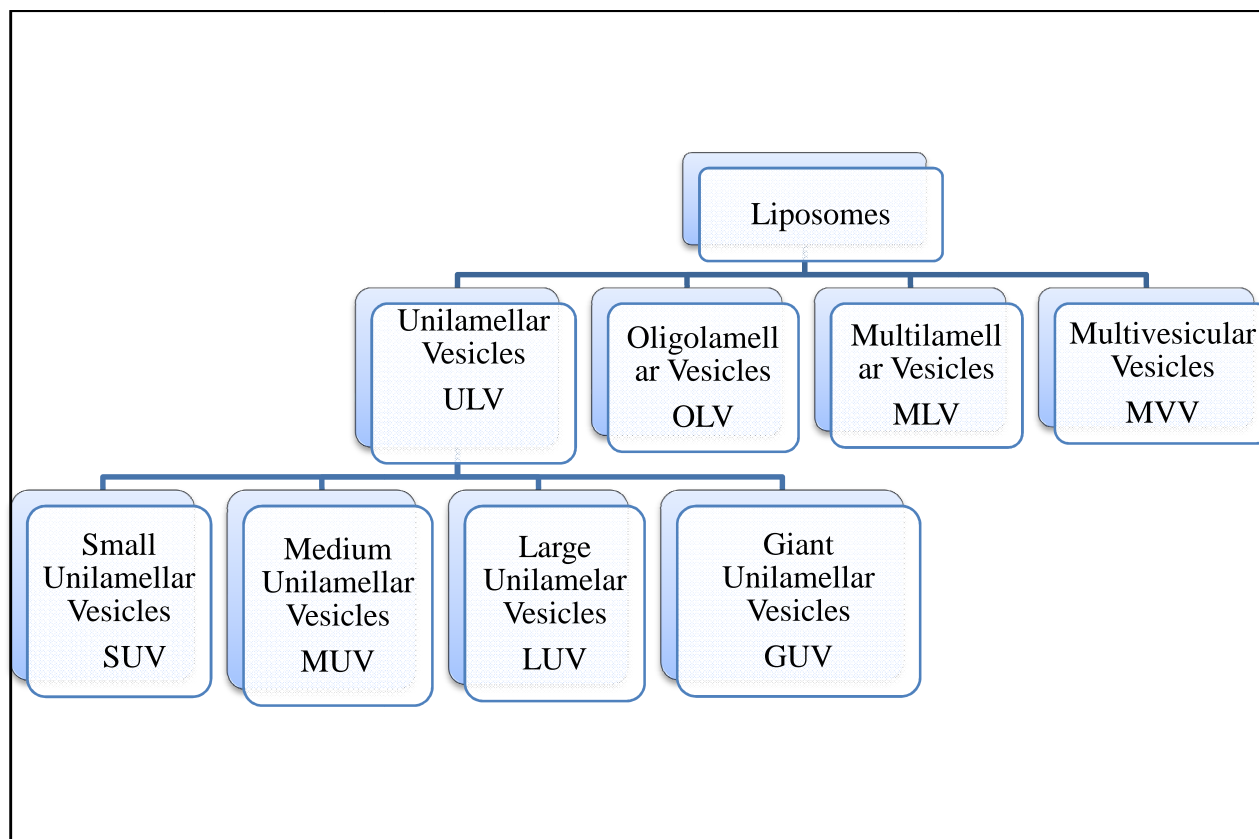



Figure 1.2: Classification of liposome based on Structural parameters.

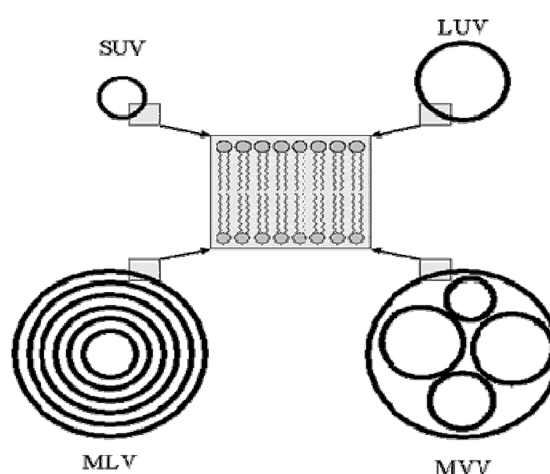



Figure 1.3: Schematic illustration of liposomes of different size and number of lamellae. SUV: Small unilamellar vesicles; LUV: Large unilamellar vesicles; MLV: Multilamellar vesicles; MVV: Multivesicular vesicles.

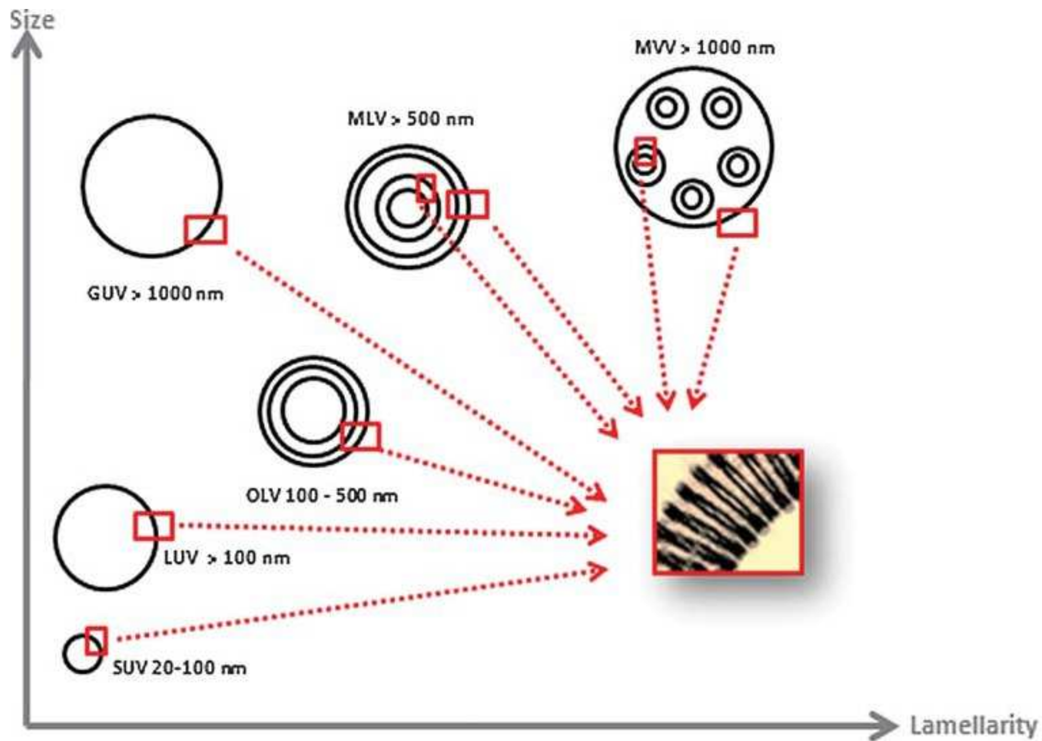



Figure 1.4: Liposomes classification based on size and lamellarity.

Based on the different methods of preparation the liposomes can be classified as given in the figure:

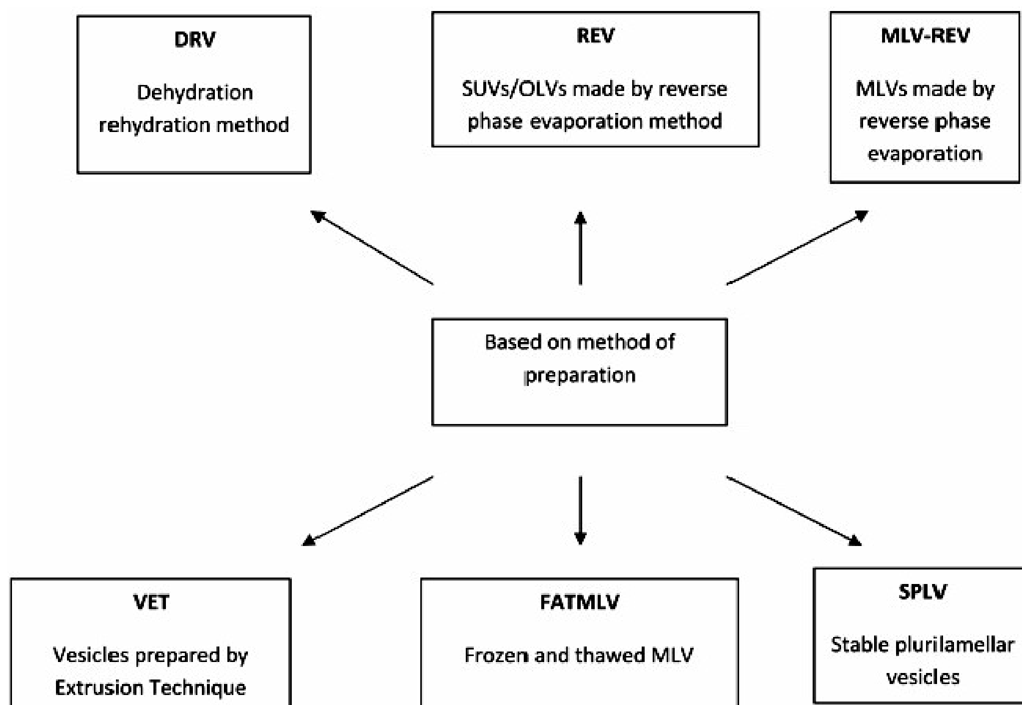



Figure 1.5: Classification of liposomes based on methods of preparation.

Based on the composition and application i.e., their mode of interaction the liposomes they can be broadly classified into the categories as (i) liposome with non-specific reactivity i.e., the conventional liposomes (ii) inert liposomes or sterically stabilized or Stealth ® liposomes (iii) liposomes with specific reactivity due to attached ligands i.e., the targeted liposomes (iv) reactive liposomes as they are capable of changing their structures upon interaction therefore are also called as the polymaorphs eg. The pH sensitive liposomes which upon change in pH undergoes phase transition from lamellar to hexagonl or lamellar to micellar and also the cationic liposomes which on complexation with the nucleic acid undergoes disintegration and restructures back[7]. A diagram (fig.1.6.) representing all the four types of liposomes and a table (table no.1.1.) briefly describing the different types of liposomes based on their composition and application focusing on their different characteristics is followed by:

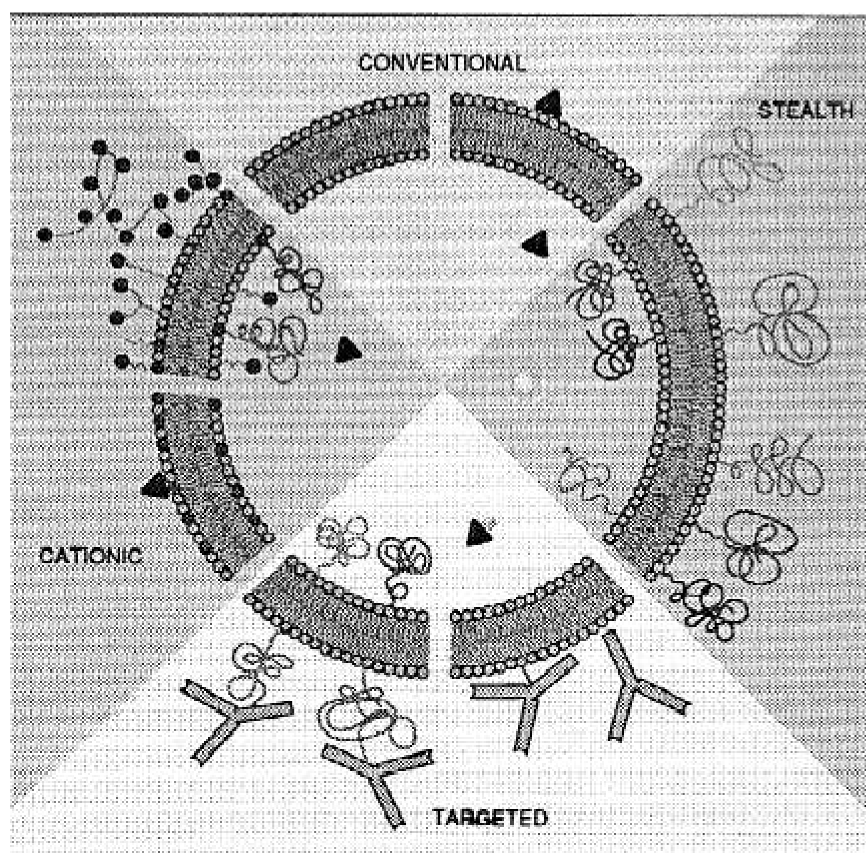



Figure 1.6: All the four types of liposomes in a schematic manner with different modes of drug association[8].

Table 1.1: Types of liposomes based on composition and application.

| TYPE | COMPOSITION | CHARACTERISTICS |
|---|---|---|
| Cationic liposomes | Cationic lipids | They possibly fuse with the cell or endosome membrane and are used to deliver negatively charged macromolecules (DNA,RNA). |
| Conventional liposomes | Neutral or negatively charged phospholipids and cholestrol | They subject to coated pit endocytosis contents ultimately delivered to lysosomes if they do not fuse from the endosome and are useful for RES targeting. |
| Immunoliposomes | Attached with monoclonal antibody or recognition sequence | They subject to receptor mediated endocytosis i.e., cell specific binding and can release contents extracellularly near the target tissues and drug diffuse through plasma membrane to produce their effects. |
| Long circulating liposomes or stealth liposomes | Neutral high transition temperature; lipid, cholesterol and 5-10% of PEG-DSPE | They have long circulating half-life. These have hydrophilic surface coating and low opsonisation and thus low uptake by RES. |
| Magnetic liposomes | PC, cholesterol, small amounts of linear chain aldehyde and colloidal particles of magnetic iron oxide. | They indigenously contain binding sites for attaching other molecules like antibodies on their exterior surface. These can be made use by an external magnetic |

| | | |
|---|---|---|
| | | field in their deliberate on-site and rupture immediately releasing their contents. |
| pH sensitive liposomes | Phospholipid such as PE, DOPE | They also subject to coated pit endocytosis, at low pH fuse with cell or endosome membrane and release their contents in the cytoplasm. |
| Temperature or heat sensitive liposomes | DPCC | Vesicles shows maximum release at 41˚C. Used for hyperthermia-based drug delivery. |

**A. Niosomes:**

The first niosome formulations were developed and patented in year of 1975. Nonionic surfactant vesicles (niosomes) result from the organized assembly of sufficiently insoluble surfactants in aqueous media. Due to the stability and the resultant ease of storage of niosomes, they are more alternative to use as nanocarrier than other micro and nanoencapsulated liposomes. They are formed from the hydrated mixtures of cholesterol, charge inducing substance, and nonionic surfactants such as monoalkyl or dialkyl polyoxyethylene ether. Basically, these vesicles do not form spontaneously. Thermodynamically stable vesicles form only in the presence of proper mixtures of surfactants and charge inducing agents.

**B. Transferosomes:**

Transferosome was first introduced in the early 1990s. It is an ultradeformable vesicle, elastic in nature. It can squeeze itself through a pore which is many times smaller than its size due to its elasticity. Transferosomes are applied to the skin and have been shown to permeate through the *stratum corneum* lipid lamellar regions as a result of the hydration or osmotic force in the skin. Transferosomes are made up of a phospholipids component along with a surfactant mixture. The ratio of individual surfactants and total amount of surfactants control

the flexibility of the vesicle. The uniqueness of this type of drug carrier system lies in the fact that it can accommodate hydrophilic, lipophilic as well as amphiphilic drugs. They can act as a carrier for low as well as high molecular weight drugs e.g. analgesic, anesthetic, corticosteroids, sex hormone, anticancer, insulin, gap junction protein, and albumin. Peripheral drug targeting, i.e. transdermal immunization can also be achieved with this type of drug delivery system.

**C. Ethosomes**:

Ethosomes are lipid vesicles containing phospholipids, alcohol (ethanol and isopropyl alcohol) in relatively high concentration and water. Ethosomes are soft vesicles made of phospholipids and ethanol (in higher quantity) and water. The size range of ethosomes may vary from tens of nanometers to microns ( $\mu$ ). Ethosomes permeate through the skin layers more rapidly and possess significantly higher transdermal flux in comparison to conventional liposomes.

**D. Proliposomes:**

Proliposomes are defined as dry free flowing particles that immediately form liposomal dispersion on contact with water body. Proliposomes are composed of water soluble porous powder as carrier upon which one may load phospholipids and drugs dissolved in organic solvent. The drugs and phospholipids are deposited in micro porous structure of the carrier materials, thus maintaining the free flowing surface characteristics of the carrier materials. Then their free flowing particulate properties permit the fabrication of proliposomes into solid dosage forms such as tablets and capsules, which are then converted to liposomes on contact with water or biological fluids. Proliposomes can be stored and sterilized in dry state and dispersed/dissolved to form an isotonic multilamellar liposomal suspension by addition of water as needed.

### 1.1.4. STRUCTURAL COMPONENTS:

Liposomes are basically, lipid vesicles consisting of mainly the phospholipid and the cholesterol. Also, depending upon the desirable properties such as thickness, fluidity, polarity and encapsulation there may be other additives.

### 1.1.4.1. Lipids

Phospholipids are the most commonly and majorly used lipids for the formation of liposomes as they are the major components of the biological membranes. They are amphiphathic molecules whose general chemical structure can be exemplified by phosphatidic acid. An overview of their structure is that it contains a backbone of glycerol bridge which holds together a pair of hydrophobic chains and a hydrophilic polar head group on opposite sides. Where, the hydrophilic polar head group consist of an phosphate group, whose hydroxyl group is esterified to phosphoric acid giving the name glycerolphospholipids. Also, on the phosphoric acid a oxygen molecule gets esterified further to give a wide variety of organic molecules like glycerol, choline, ethanolamine, serine and inositol giving rise to a wide variety of phospholipids namely, phosphotidylglycerol (PG), phosphotidylgcholine (PC), phosphotidylethanolamine (PE), phosphotidylserine (PS) and phosphotidylinositol (PI) respectively[3].

The hydrophobic part consist of two hydrocarbon chains of saturated and unsaturated fatty acids with 10-28 carbon atoms and both esterifying the same number of hydroxyl group of glycerol. The bilayer properties of the lipid like elasticity and phase behaviour are governed by the degree of unsaturation[10].

Table 1.2: Some common saturated and unsaturated fatty acids found in lipids used to form liposomes[3].

| COMMON NAME | MOLECULAR FORMULA | SYNTHETIC NAME |
|---|---|---|
| SATURATED FATTY ACIDS | | |
| Palmitic | $C_{16}H_{32}O_2$ | n-hexadecanoic acid |
| Stearic | $C_{18}H_{36}O_2$ | n-octadecanoic |
| UNSATURATED | | |
| Palmitoleic | $C_{16}H_{30}O_2$ | 9-hexadecenoic |
| Oleic | $C_{18}H_{34}O_2$ | *cis*-9-octadecenoic |
| Linoleic | $C_{18}H_{32}O_2$ | *cis,cis*-9,12-octadecadienoic |

(a) Phosphotidylcholine:

The most abundant phospholipid also known as lecithin originated from the Greek word "lekithos" meaning eggyolk. The polar hydrophilic head group in PC is a phosphocholine – $CH_2$-$CH_2$-$N(CH_3)^+$ which at physciological pH bears a positive charge at the quaternary ammonium and the phosphate group bearing a negative charge making it a dipolar molecule[11]. They can be naturally derived from sources like egg-yolk or soy and can also be prepared synthetically. Their insolubility in water and in aqueous media helps them to align closely in planar bilayer sheets forming liposome[1].

(b) Sphingolipids

These are an exception to the phospholipid species with a structural backbone of sphingosine or a related base other than the usual glycerol bridge. Structurally, sphingomyelin, the most abundant sphingolipid in higher animals, contains either phosphorylcholine or phosphorylethanolamine esterified at the 1-hydroxy group of cramide at its polar head group. In both plants and animals they are particularly found in brain and nerve tissue. Similar, to phospholipid they too form bilayer structure in aqueous media[3]. They are also zwitter ion at pH 7.

Glycosphingolipids

These are second class of sphingolipids specifically found in the gray matter of the brain tissue of higher animals. Structurally, their polar head group consist of a complex oligosaccharides with one or more sialic acid residue imparting it a net negative charge at pH 7. Therefore, they are used to give a layer of surface negative charged group in some liposome formulations[3].

### 1.1.4.2. CHOLESTROL

Another major and important component of the liposomal formulation. With the development of liposomes its properties such as flexibility, membrane permeability, stability or shorten shelf-life were required to be maintained which brought into consideration use of cholesterol. Cholestrol are derivatives of the tetracyclin hydrocarbon per hydrocyclopentanophenanthrene. Due to their structural similarity with lipids they get incorporated into the lipid bilayer with their hydroxyl groups oriented towards the aqueous surface and the aliphatic chains aligned parallel to the faty acid chains of lipids thereby,

filling in the empty spaces between the phospholipid molecules and anchoring them more tightly[1]. Therefore, the inclusion of cholesterol plays the following major role in the formulation of liposome.

Firstly, by filling in the spaces it decreases the flexibility of the surrounding lipid chain, increases the mechanical rigidity of the fluid bilayer.

Secondly, it reduces the permeability of the water soluble molecules through the membrane.

Lastly, and most importantly cholesterol improves the stability of liposome by reducing their interaction with the plasma proteins like albumin, m-transferrin and macroglobulin thereby overcoming the rapid clearance from systemic circulation by decreasing interaction with plasma opsonin responsible for the same[12]. Hence, liposome with improved shelf-life can be formed.

### 1.1.5. THE STRUCTURAL COMPONENTS *IN-VIVO*[3]

The structural components of liposome namely phospholipids and cholesterol contributes to its high bio-compatibility due to their similarity with the biological membrane lipids. Hence, also undergoes same metabolic fate as that of the biological lipids by the action of enzyme systems naturally occurring inside the body. Specific phospholipase (Phospholipase A, B, C, D) hydrolyses specific sites of phospholipids liberating fatty acid and glycerol phosphate. Where, the fatty acid enters the fatty acid pools and serves as a precursor for the generation for the generation of phospholipid or triglyceride or may also contribute in the generation of energy via the β-oxidation process. And the glycerol phosphate gets recycled to either new phospholipids or triglyceride as they cannot be broken further, being a good phosphate acceptor.

Likewise, cholesterol gets disposed of in liver which aids in digestion of fats. Also, a small portion of it gets excreted across the intestinal mucosa and acted upon by the intestinal bacteria to break into coprostanol. Cholesterol also serves as precursor of steroid hormones. Thus, liposomes have in *in-vivo* tolerability making it a suitable formulation of choice.

### 1.1.6. TARGETING OF LIPOSOMES

One of the greatest reasons for the development of formulation like liposome is the site specific delivery of drug reducing their toxic effects at the healthy tissues. These targeting of liposomes can be achieved in either of the two ways, the passive targeting particularly for the

conventional liposomes or the active targeting for those liposomes conjugated to ligands having affinity for the specific receptors[13].

#### 1.1.6.1. Passive Targeting

Passive targeting is the utilization of the existing physiological pathways and the knowledge about the various clearance mechanisms for the delivery of the encapsulated drug. As for example is the case of design of liposome encapsulated with the antifungal drug amphotericin B. Here, the intravesicular sites of fungal growth are targeted passively i.e., without any receptor-ligand interaction, by the utilization of the phagocytic uptake of the liposome by the same phagocytes which had engulfed the infected fungi[14].

Passive targeting is a boon to cancer therapy. Cancer is characterized by rapid angiogenesis leading to the formation of new vasculature which are usually leaky with improved permeability and also impaired drainage providing high retention together this effect is called as the enhanced permeability and retention effect (EPR). Thus, favouring the small non-targeted particles (<400 nm) to accumulate in the interstitial spaces of the cancerous tissues[14]. However, there are also certain loopholes in the future scope of using passive targeting for the treatment of cancer. The passive targeting is generally dependent on different factors of the tumor to effectively produce their desired action at the desired sites. These factors include the type of the tumor as the EPR effect is only applicable to the solid tumors which are larger than approximately 4.5 mm in diameter. Also the status of the tumor will govern the porosity and pore size of its vessels[15]. Additionally, the tumor location, the surrounding stroma, amount of infiltration by macrophages, patient characteristics and additional medications also govern EPR effect[16]. Nonvascularised and nascent tumors are at at no benefit with this EPR effect[14].

#### 1.1.6.2. Active targeting

The concept of active argeting was introduced in the year 1906 by Lehner *et al.*,[17]. Active targeting utilizes the surface modification of the liposome with a specific ligand so as to their accumulation at the targeted sites and releasing their contents by the receptor mediated-endocytosis[15]. Binding of targeting ligand which does not lose its function after getting conjugated with the liposome, stability, ease of preparation, scaling and effectiveness in binding to the targeted moiety are the vital lookouts in the design of such delivery systems [18].

For a hydrophilic targeting moiety conjugation to phospholipid or fatty acid will give a stable formulation[14]. But, for a hydrophobic anchor for the targeting moiety requires chemical conjugation.

Liposomes with active targeting can be obtained in the following three main approaches: (i) during liposome preparation, the desired targeting ligand bind to a lipid prior to mixing them with other lipid components[20]. (ii) immediately after preparation, liposomes are functionalized with the required targeting ligand[20] example, head group-modified lipids with a PEG spacer functionalized at the end with amine, carboxylicacid, thiol or maleimide groups[21] (iii) in preformed liposomes, the postinsertion of the functionalized lipid was proposed. This method is based on the spontaneous incorporation of functionalized lipids from the micellar phase into preformed and even drug-loaded liposomes[22]. Derivatization of the targeting molecule happens in a separated step, this approach prevents the interference of activated lipids with other liposomal components such as those present in the buffer[22].

## 1.1.7. Liposome preparation

Basically, the preparation of liposomes involves the drying down of the lipids from the organic solvent followed by the dispersion of the lipid in aqueous media and finally purification of the resultant liposomes for their analysis. Based on these basic steps the preparation methods of liposomes are classified below[1]:

1. A. Physical dispersion method
    a. Hand-shaken multilamelar vesicles (MLVs)
    b. Non-shaking vesicles
    c. Pro-liposomes
    d. Freeze-drying

B. Processsing of lipids hydrated by physical means
    a. Microemulsification liposomes (MEL)
    b. Sonicated unilamellar vesicles (SUVs)
    c. French Pressure Cell liposomes
    d. Membrane extrusion liposomes
    e. Dried Reconstituted vesicles (DRVs)
    f. Freeze Thaw Sonication (FTS)
    g. pH induced Vesiculation

    h. Calcium induced fusion
2. Solvent dispersion methods
    a. Ethanol injecton
    b. Ether injection
    c. Water in organic phase
    d. Double emulsion vesicles
    e. Reverse phase evaporation vesicles
    f. Stable plurilamellar vesicles (SPVs)
3. Detergent solubilisation

However, based on the convenience of preparation and their utility the different methods are discussed below:

#### 1.1.7.1. Conventional methods of liposome Preparation

a) Thin-Film Hydration Or The Bangham Method ,
b) Reversed Phase Evaporation,
c) Solvent-Injection techniques, and
d) Detergent Dialysis.

These are the most commonly used ones. Some techniques have been employed to help reduce the size of vesicles, for instance,

i. Sonication,
ii. High Pressure Extrusion and
iii. Microfluidization.

a) Thin Film Hydration method

Also called as the Bangham method as this method was pointed out by Alec Bangham in 1964 and was widely used for the preparation of multilamellar vesicles[23].The method involves two major steps where the first one is the preparation of thin film of lipid for hydration followed by the hydration of thin film for the formation of multilamellar vesicles. In the preparation of thin film a mixture of phospholipid and cholesterol are dispersed in organic solvent taken a completely dried round bottom flask .Then, the organic solvent is removed by means of evaporation by a Rotary Evaporator at reduced pressure. To ensure the complete removal of organic solvent used the film formed is maintained in vacuum overnight. Finally, the dry lipidic film deposited on the flask wall is hydrated by adding an

aqueous buffer solution under agitation at temperature above the lipid transition temperature. This method is widespread and easy to handle.

Disadvantage: Formation of a population of multilamellar liposomes (MLVs) heterogeneous both in size and shape (1–5 μm diameters). Also, there may not be complete removal of the organic solvent used.

b) Reverse-Phase Evaporation (REV) Technique[3]

This method was pioneered by Szoka and Papahadjopoulos in 1978 (36 no ref from lasics ) and is advantageous for the higher encapsulation of water soluble drugs. The LUVs can be prepared by this method. It involves the preparation of a water-in-oil emulsion of phospholipids and buffer in excess organic phase which is further removed at reduced pressure therefore the method called the reverse phase evaporation or REV technique. The phospholipids are first dissolved in organic solvents such as ethylether, isopropylether or mixture of two solvents such as isopropylether and chloroform. Then the aqueous phase containing the material to be trapped is directly added to the phospholipid-solvent mixture and the emulsification can be obtained either directly by sonication or other mechanical means. At reduced pressure the organic solvent is then removed which causes the phospholipid coated droplets of water to coalesce and eventually form a viscous gel which collapses into a smooth suspension of LUVs on removal of the final traces of the organic solvent. Up to 65% of entrapment efficiencies can be achieved by this method.

c) Solvent (Ether or Ethanol) Injection Technique.

The solvent injection methods involve the dissolution of the lipid into an organic phase (ethanol or ether), followed by the injection of the lipid solution into aqueous media, forming liposomes.

i) The ethanol injection method[3]

This method was described by Batzri and Korn. It is a simple method which involves the injection of lipids dissolved in ethanol into an excess of buffer solution forming SUVs.

The main advantage of the ethanol injection method is that a narrow distribution of small liposomes (under 100 nm) can be obtained by simply injecting an ethanolic lipid solution in water, in one step, without extrusion or sonication[24].

The major drawback of the method is complete removal of the ethanol because the ethanol forms an azeotrope with water thus, becomes difficult to be removed under vacuum or by distillation.

ii) The Ether Injection Method

This method was introduced by Deamer and Bangham in 1976[3]. SUVs are prepared by this method by the introduction of lipids dissolved in diethyl ether or ethanol/methanol mixture into an aqueous solution of material to be entrapped at 55˚C-65˚C or under reduced pressure. Subsequently the remaining ether is removed under vacuum and forms primarily unilamellar liposomes.

The basic difference between both the methods is that the ether is immiscible with the aqueous phase, which upon heating is removed from the liposomal product.

An advantage of this method compared to the ethanol injection method is the removal of the solvent from the product, enabling the process to be run for extended periods forming a concentrated liposomal product with high entrapment efficiencies.

d) Detergent Removal

This method involves the removal of detergent molecules from the aqueous dispersion of phospholipid/detergent mixed micelles. Where, on removal of detergent the micelles become rich in phospholipid leading to formation of closed single-bilayer vesicles. The removal of detergent can be achieved by the following three techniques:

a. Detergent Dialysis.
b. Column chromatography
c. Bio-beads

i) Sonication[1]

The MLVs prepared by the thin film hydration method can be reduced in size by the process called sonication. The sonicator reduces the particle size and imparts energy at a high level to a lipid suspension. This method is of two types (i) bath type and (ii) probe type. The bath type sonicators are used when the volume of the lipid suspension is high and where there is no need to reach any vesicle size limit. Secondly, the probe type sonicators are the ones which are used for small volume of lipid suspension. However, it has a disadvantage of

contaminating the preparation with metal tip of the probe which can further also degenerate the lipid.

ii) High Pressure Extrusion[3]

Another technique for the size reduction of MLVs to form SUVs also with the reduction in number of lamella is high pressure extrusion method. This method involves the passage of lipid suspension through very small diameter polycarbonate membrane (0.8-1.0 micron) under high pressure (up to 250 psi). The average diameter of the vesicles thereafter a passage of minimum 10 times is approximately reduced to around 60-80 nm. However this process has certain prerequisites which limit its usage. Firstly, in order to form the single-layered vesicles it is necessary that the aqueous core of the starting MLVs must be greater than 70nm. Secondly, to meet these size criteria the neutral vesicles or the ones with very few percentages of acidic lipids fail as the acidic lipids, such as PS or PG, are the ones with larger interbilayer distances and larger internal aqueous core due to the electrostatic repulsive force among the bilayers.

There are different drawbacks related to the conventional methods of liposome preparation which are listed below[25]

(1) The particle size of liposomes is too large or is not homogenous therefore requires size reduction.

(2) These processes utilises organic solvent which if remained in the final product may cause a serious issue since it not only affects the stability of some protein or polypeptide drugs, but also adversely affects clinical treatment.

(3) Sterilization of liposomal preparations can be a challenging as many of the lipids used are sensitive to temperature.

(4) In some procedures careful monitoring is needed and this subjective technique might influence reproducibility.

In order to overcome these problems, many novel preparation technologies have been developed for the preparation of liposomes.

### 1.1.7.2. Novel technologies for liposome preparation [25]

1. Supercritical Fluid Technology,
   a. Supercritical anti-solvent (SAS) method
   b. Supercritical reverse phase evaporation (SPER) method
2. Dual Asymmetric Centrifugation,
3. Membrane Contactor Technology,
4. Cross-Flow Filtration Technology And
5. Freeze Drying Technology.

Table 1.3: A brief insight into the novel technologies for the preparation of liposomes.

| Technique name | Particle Size (nm) | Characteristics (advantages and drawbacks) |
|---|---|---|
| Supercritical fluid Technology | 100-10,000 | a. Organic solvent can be excluded completely or even need not to be used.<br>b. Postal procedures should be utilized to achieve a narrow particle size distribution.<br>c. Efficiency of encapsulation has no significant improvement compared with Bangham method.<br>d. The recovery of raw material is usually raw. |
| Dual asymmetric centrifugation | 70-120 | a. The equipment of DAC is small in size and easy in operation with a good reproducibility.<br>b. Liposomes with small particle size can be achieved.<br>c. Water soluble drugs could have an efficient entrapment.<br>d. High phospholipids content should be in the formulation to obtain a sufficient viscosity. |
| Membrane contactor technology | ~100 | a. Homogeneous and small multilamellar liposomes with high encapsulation efficiency for lipophilic drugs can be obtained.<br>b. It is easy to scale-up.<br>c. The encapsulation for hydrophilic drugs still need further investigation. |
| Cross-flow filtration | ~50 | a. Liposomes of defined size, homogeneity and high stability can be prepared in a short time. |

| | | |
|---|---|---|
| detergent depletion method | | b. Sterile products can be obtained by the presented methods.<br>c. The waste filtrate can be recycled to minimize costs of production. |
| Freeze drying double emulsions method | <200 | a. Sterile preparation with good storage stability can be achieved.<br>b. Cryoprotectants are always carbohydrates, which limits the applications for patients with diabetes. |

**1.1.7.3. Large scale manufacturing techniques[26]**

1. Heating method
2. Spray drying
3. Freeze-drying
4. Super Critical Reverse Phase Evaporation (SCRPE)

**Heating method**

It is a new method for fast production of liposomes without the use of any hazardous chemical or process. This method involves the hydration of liposome components in an aqueous medium followed by the heating of these components, in the presence of glycerol (3% v/v), up to 120 ˚C. Glycerol being a water-soluble and physiologically acceptable chemical also having the capacity to increase the stability of lipid vesicles and does not need to be removed from the final liposomal product. The energy required for the formation of stable liposome is provided by the temperature and mechanical stirring. It is also reported that the lipids used do not degenerate at the applied temperature[27]. The particle size can be controlled by the phospholipid nature and charge, the speed of the stirring and the shape of the reaction vessel. Moreover, employment of heat abolishes the need to carry out any further sterilisation procedure reducing the time and cost of liposome production.

**Spray-Drying**

Since spray-drying is a very simple and industrially applicable method, the direct spray-drying of a mixture of lipid and drug was applied in the preparation of liposomes. The spray-drying process is considered to be a fast single-step procedure applied in the nanoparticles

formulation. Hence, liposomes can be prepared by suspending lecithin and mannitol in chloroform. The mixture is sonicated and subjected to spray-drying. The dried product is hydrated with different volumes of phosphate buffered saline (PBS; pH 7.4) by stirring for 45 min. The main factors influencing the liposomal size are the volume of aqueous medium used for hydration of the spray-dried product. However, mannitol plays an important role in increasing the surface area of the lipid mixture, enabling successful hydration of the spray-dried product.

**Freeze Drying**

This new method was described for the preparation of sterile and pyrogen-free submicron narrow sized liposomes. It is based on the formation of a homogenous dispersion of lipids in water-soluble carrier materials. Liposome-forming lipids and water-soluble carrier materials such as sucrose were dissolved in tert-butyl alcohol/water cosolvent systems in appropriate ratios to form a clear isotropic monophase solution. Then the monophase solution was sterilized by filtration and filled into freeze-drying vials. On addition of water, the lyophilized product spontaneously forms homogenous liposome preparation. The lipid/carrier ratio is the key factor affecting the size and the polydispersity of the liposome preparation. Therefore, TBA/water cosolvent system was used for economy concerns.

**Super Critical Reverse Phase Evaporation (SCRPE)**

The SCRPE is a one-step new method that has been developed for liposomes preparation using supercritical carbon dioxide. This method allowed aqueous dispersions of liposomes to be obtained through emulsion formation by introducing a given amount of water into a homogeneous mixture of supercritical carbon dioxide/LR-dipalmitoylphosphatidylcholine/ethanol under sufficient stirring and subsequent pressure reduction. Transmission electron microscopy observations revealed that vesicles are large unilamellar with diameters of 0.1– 1.2 m. The trapping efficiency of these liposomes indicated more than 5 times higher values for the water-soluble solute compared to multilamellar vesicles prepared by the Bangham method. The trapping efficiency for an oilsoluble substance, the cholesterol, was about 63%. Results showed that the SCRPE is an excellent technique that permits one-step preparation of large unilamellar liposomes exhibiting a high trapping efficiency for both water-soluble and oil-soluble compounds.

**Modified Ethanol Injection Method**

Novel approaches based on the principle of the ethanol injection technique such as the microfluidic channel method, the crossflow-injection technique, and the membrane contactor method were recently reported for liposome production.

a. The Crossflow Injection Technique

The concept of continuous crossflow injection is a promising approach as a novel scalable liposome preparation technique for pharmaceutical application. A cross flow injection module is made of two tubes welded together forming a cross. At the connecting point, the modules were adapted with an injection hole. The influencing parameters are the lipid concentration, the injection hole diameter, the injection pressure, the buffer flow rate, and system performance. A minimum of buffer flow rate is required to affect batch homogeneity and strongly influencing parameters are lipid concentration in combination with increasing injection pressures. After exceeding the upper pressure limit of the linear range, where injection velocities remain constant, the vesicle batches are narrowly distributed, also when injecting higher lipid concentrations. Reproducibility and scalability data show similar results with respect to vesicle size and size distribution and demonstrate the stability and robustness of the novel continuous liposome preparation technique.

b. Microfluidization

By using a microfluidic hydrodynamic focusing (MHF) platform, liposomes can be generated by injecting the lipid phase and the water phase into a microchannel. Microfluidic flow is generally laminar due to the small channel dimensions and relatively low flow rates. Well-defined mixing is then obtained by interfacial diffusion when multiple flow streams are injected in a microchannel. The size of the liposomes can mainly be controlled by changing the flow rate.

c. Membrane Contactor

In this method, a lipid phase (ethanol, phospholipid and cholesterol) was pressed through the membrane with a specified pore size. Nitrogen gas at pressure below 5 bar was sufficient for passing the organic phase through the membrane. At the same time, the aqueous phase flew tangentially to the membrane surface and swept away the formed

liposomes within the membrane device. The new process advantages are the design simplicity, the control of the liposome size by tuning the process parameters and the scaling-up abilities. As a result, these techniques lead from the conventional batch process to potential large scale continuous procedures.

### 1.1.8. Stability of liposomes

One of the greatest challenges in the development liposomal formulation was preservation of its stability. For any pharmaceutical product its stability is defined as the capacity of the formulation to maintain its defined limits for a predetermined period of time i.e., the shelf-life of the product[3]. The liposomal preparation undergoes mainly two types of changes with time. Where any changes related to the chemical structure of the molecules falls under the chemical stability and changes related to the size distribution and amount of membrane encapsulated comes under the category of physical stability. In details the different stability problems and the various methods to overcome them are discussed hereafter:

A. Physical Stability related problems and their solutions[1]

Liposomes are colloidal systems and unlike any other colloidal system undergo a common problem of aggregation leading to change in its size. Also, being a self-assembling colloid liposomes also undergoes changes such as fusion or phase change after the aggregation. Aggregation and sedimentation of neutral liposomes occurs due to the Van der Waals forces and is more common in larger vesicles, where with the increased planarity of the membrane the area of the membrane to come into interaction also increases. Also, factors such as the residual solvent and trace elements enhance this process for uncharged membranes. The simplest way to overcome this problem is by the introduction of small quantities of negative charge to the lipid mixtures.

The stability of liposomes can also be increased by cross-linking membrane component covalently using methods such as gluteraldehyde fixation, osmification or polymerization of alkyne-containing phospholipids. However, with these methods there may be an increase in the mechanical strength of the membrane but at the cost of decrease in susceptibility to disruption *in vivo*.

B. Chemical Stability problems and their solutions

Hydrolysis and oxidative reactions are the major chemical stability problems for liposomes. Where, the phospholipid undergoes hydrolysis and if it contains unsaturated fatty acids will undergo oxidation.

i. Lipid Peroxidation

The major component of liposomes i.e., the phospholipids chemically contains an unsaturated acyl chain in their structure which undergoes the process of lipid peroxidation (oxidative degradation). This deterioration of lipids leads to the generation of free radicals and forms cyclic peroxides and hydroperoxides. These changes can occur either during the preparation, storage or use. So as to reduce the oxidative degeneration of lipids the following steps must be followed:

a. Unsaturated fatty acids must not be used unless necessary.
b. To minimize the use of oxygen, nitrogen or argon must be used .
c. To minimize the exposure to light, light-resistant containers must be used.
d. Any trace of heavy metal should be removed.
e. Antioxidants such as α-Tocopherol or BHT can be used.

ii. Lipid Hydrolysis

Another problem of the component of liposome formulation. The hydrolysis of lecithin yields the most important degradation product lyso-lecithin (lyso-PC). This is formed by the hydrolysis of te ester bond at the C2 position of the glycerol moiety. Its formation has to be minimized during the storage as it enhances the permeability of the liposome.

### 1.1.9. APPLICATION OF LIPOSOMES

#### 1.1.9.1. Medical Application

***Liposomes in anticancer therapy***

Many anticancer agents are less selective therefore, being toxic to the normal cells. Liposome formulations of such anticancer agents were shown to be less toxic than the free drug [28]. For example, if we consider the drug Anthracyclines which acts by intercalating into the DNA of dividing cells and stop their growth and therefore kill predominantly quickly dividing cells. But such quickly diving cells are not only found in tumours, but also in gastrointestinal

mucosa, hair, and blood cells and therefore due to its non-selectivity this class of drugs is very toxic.

In another case of systemic lymphoma, the effect of liposome encapsulation showed enhanced efficacy due to the sustained release effect, i.e. longer presence of therapeutic concentrations in the circulation[29].

The main advantages of entrapping drug into liposomal formulation are:

(i) With the increase in circulation time of the drug, it gets internalized and tries to get deposited in the tissue.
(ii) The drug is protected from any metabolic degradation.
(iii) Increase in cell-selectivity and reduction in toxicity as the tissue uptake gets altered.

However, the efficacy in many cases gets compromised due to the reduced bioavailability of the drug, especially if the tumour was not phagocytic, or located in the organs of mononuclear phagocytic system.

In general, applications in man showed reduced toxicity, better tolerability of administration with not too encouraging efficacy. Several different formulations are in different phases of clinical studies and show mixed results[30]

***Liposome in oral treatment***

To preferably obtain the entry of drug through the portal circulation into the periphery the drug needs to be administered orally.

a. Arthritis

In arthritis the drug mainly used are steroids, which on oral administration gets destroyed by their peripheral effects and those administered locally, only transiently diffuse from their site of injection to the areas of inflammation. Therefore, liposomal formulations of steroids (e.g., cortisol palmitate) can be prepared to get the local effect.

b. Diabetes

Insulin can be entrapped into liposomal preparation to protect it against enzymatic degradation. Till date several studies have been conducted to prepare oral formulaion of

insulin. In a study a 1.3 units of insulin entrapped in dipalmitoyl-phosphatidylcholine/ cholesterol liposomes administered to normal rats was found to decrease blood glucose level in 4 h to about 77% of those before treatment. Higher doses (4.2 and 8.4 units) extended this effect over 24 h. 1.0 units of insulin entrapped in the same liposomes had an even more pronounced effect in diabetic rats: levels of blood-glucose were reduced to 57% of pre-treatment values after 4 h[31].In another study, liposome-entrapped insulin significantly reduced glucose and raised insulin in 54% of rats and 67% of the rabbits. Among the rats that responded, blood glucose fell from a basal of 318±21 mg/dl to a nadir of 186±22 mg/dl at 2 h. while insulin rose from 30±7 U/ml to a peak of 399±75 U/ml at 1 h[32].

***Liposomes in parasitic diseases and infections***

Since conventional liposomes are digested by phagocytic cells in the body after intravenous administration, they are ideal vehicles for the targeting of drug molecules into these macrophages. The best known examples of this 'Trojan horse-like' mechanism are several parasitic diseases which normally reside in the cell of mononuclear phagocytic system. They include leishmaniasis and several fungal infections.

***Liposome for ophthalmic delivery***

Liposomes being completely bio-degradable and non-toxic hold advantage over other conventional preparation for ophthalmic delivery. Also it outlooks the major disadvantage of the delivery of optimal concentration of drug at the site of action. Liposomes ability to intimately contact the corneal and conjunctivital surfaces enhances the drug absorption[1]. Megaw et al. (1981) was the first one to report targeting of liposome to the corneal surface.

***Liposome as immunological adjuvants***

The several advantages of using liposomes as immunological adjuvants are as followed:

i. It provides longer duration of functional activity.
ii. Reduction in toxicity and allergic reactions.
iii. Reconstitution of hydrophobic antigens.
iv. A non-immunogenic substance can be made into an immunogenic one.
v. A single liposome may be able to incorporate multiple antigens.

### 1.1.9.2. Application of liposomes in agro-food industry

The ability of liposomes to solubilize compounds with demanding solubility properties, sequester compounds from potentially harmful milieu, and release incorporated molecules in a sustained and predictable fashion can be used also in the food processing industry. Lipid molecules, from fats to polar lipids, are one of the fundamental ingredients in almost any food. For instance, lecithin and some other polar lipids are routinely extracted from nutrients, such as egg yolks or soya beans. Traditionally polar lipids were used to stabilize water-in-oil and oil-in-water emulsions and creams, or to improve dispersal of various instant powders in water. With the advent of microencapsulation technology, however, liposomes have become an attractive system because they are composed entirely from food acceptable compounds. The sustained release system concept can be used in various fermentation processes in which the encapsulated enzymes can greatly shorten fermentation times and improve the quality of the product. This is due to improved spatial and temporal release of the ingredient(s) as well as to their protection in particular phases of the process against chemical degradation. A classical example is cheese making. The first serious attempts to decrease the fermentation time using cell-wall-free bacterial extracts were encouraging enough to stimulate efforts to improve enzyme presentation[33]. In addition, due to the better dispersal of the enzymes the texture of cheeses was even and bitterness and inconsistent flavour due to the proteolysis of enzymes in the early phase of fermentation was much improved. In addition to improved fermentation, liposomes are being tried in the preservation of cheeses. Lysozyme is effective but quickly inactivated due to binding to cassein. Liposome encapsulation can both preserve potency and increase effectiveness because liposomes become localized in the water spaces between the casein matrix and fat globules of curd and cheese. This also happens to be where most of the spoilage organisms are located[34]. These applications of enhancing natural preservatives, including antioxidants such as vitamins E and C, will undoubtedly become very important due to recent dietary trends which tend to reduce the addition of artificial preservatives and ever larger portion of unsaturated fats in the diet. In other areas of the agro-food industry, liposomes encapsulated biocides have shown superior action due to prolonged presence of the fungicides, herbicides or pesticides at reduced damage to other life forms[35]. Liposome surface can be made sticky so that they remain on the leaves for longer times and they do not wash into the ground. In these applications inexpensive liposomes produced from synthetic lipids are used. The same liposomes are being tried in shellfish farms. These animals are susceptible for many parasitic infections. They are filter feeders and they pump

large amounts of water through their body. This seems to offset large dilutions of liposomes in the pool and the drug molecules as well as some essential nutrients needed in ppm to ppb quantities can be delivered.

### 1.1.9.3. Application of liposomes in cosmetics

The same properties of liposomes can be utilized also in the delivery of ingredients in cosmetics. In addition, liposomes as a carrier itself offers advantages because lipids are well hydrated and can reduce the dryness of the skin which is a primary cause for its ageing. Also, liposomes can act as a supply which acts to replenish lipids and, importantly, linolenic acid. In general the rules for topical drug applications and delivery of other compounds are less stringent than the ones for parenteral administration and several hundred cosmetic products are commercially available since Capture (C. Dior) and Niosomes (L'Or´eal) were introduced in 1987. They range from simple liposome pastes which are used as a replacement for creams, gels, and ointments for do-it-yourself cosmetical products to formulations containing various extracts, moisturizers, antibiotics, and to complex products containing recombinant proteins for wound or sunburn healing. Most of the products are anti-ageing skin creams. Unrinsable sunscreens, long lasting perfumes, hair conditioners, aftershaves and similar products, are also gaining large fractions of the market. Liposomes are a noninteractive, skin-nonirritating, water based matrix (without alcohols, detergents, oils and other non-natural solubilizers) for the active ingredients. In addition to the natural lipids, either phospholipids or 'skin lipids', which contain mostly sphingolipids, ceramides, oleic acid, and cholesterol sulphate, liposomes made from synthetic lipids are also being used. They include mostly liposomes made from nonionic surfactant lipids, which can be chemically more stable.

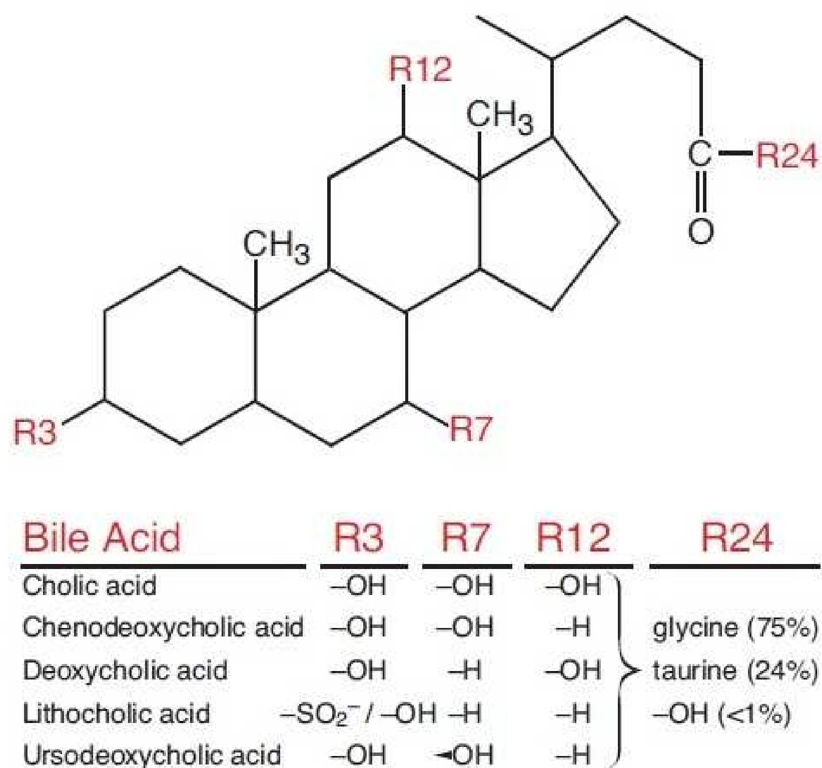
Table 1.4: Marketed liposomal products[36].

| Product | Drug | Indications |
|---|---|---|
| *Approved products* | | |
| AmBisome (Gilead) | Amphotericin B | Fungal infections Leishmaniasis, |
| Doxil/Caelyx (Johnson & Johnson) | Doxorubicin | Kaposi's sarcoma<br>Ovarian cancer<br>Breast Cancer<br>Multiple myeloma + Velcade |
| DaunoXome (Galen) | Daunorubicin | Kaposi's sarcoma |
| Myocet (Cephalon) | Doxorubicin | Breast cancer + cyclophosphamide |
| Amphotec (Intermune) | Amphotericin B | Invasive aspergillosis |
| Abelcet (Enzon) | Amphotericin B | Aspergillosis |
| Visudyne (QLT) | Verteporphin | Wet macular degeneration |
| DepoDur (Pacira) | Morphine sulfate | Pain following surgery |
| DepoCyt (Pacira) | Cytosine Arabinoside | Lymphomatous meningitis<br>Neoplastic meningitis |
| Diprivan (AstraZeneca) | Propofol | Anesthesia |
| Estrasorb (King) | Estrogen | Menopausal therapy |
| Lipo-Dox (Taiwan Liposome) | Doxorubicin | Kaposi's sarcoma, breast and ovarian cancer |
| Marqibo (Talon) | Vincristine | Acute lymphoblastic leukemia |
| *Products in clinical trials* | | |
| SPI-077 (Alza) | Cis-platin | Solid tumors |
| CPX-351 (Celator) | Cytarabine:daunorubicin | Acute myeloid leukemia |
| CPX-1 (Celator) | Irinotecan HCl:floxuridine | Colorectal cancer |
| MM-398 (Merrimack) | CPT-11 | Gastric and pancreatic cancer<br>Glioma and colon cancer |
| MM-302 (Merrimack) | ErbB2/ErbB3-targeted doxorubicin | ErbB2-positive breast cancer |
| MBP-436 (Mebiopharm) | Transferrin-targeted oxaliplatin | Gastric cancer and gastro-esophageal junction |
| Brakiva (Talon) | Topotecan | Relapsed solid tumors |
| Alocrest (Talon) | Vinorelbine | Newly diagnosed or relapsed solid tumors |
| Lipoplatin (Regulon) | cisplatin | Non-small cell lung cancer |
| L-annamycin (Callisto) | Annamycin | Adult relapsed ALL<br>Pediatric relapsed ALL and acute myelogenous leukemia<br>Doxorubicin-resistant breast cancer |
| ThermoDox (Celsion) | Thermosensitive doxorubicin | Primary hepatocellular carcinoma<br>Refractory chest wall breast cancer<br>Colorectal liver metastases |
| Endo-Tag-1 (Medigene) | Cationic liposomal paclitaxel | Pancreatic cancer<br>Triple negative breast cancer |
| ALN-TTR ALN-PCS ALN-VSP (Alnylam) | siRNA targeting transthyretin (TTR)<br>siRNA targeting PCSK9 RNAi targeting liver cancer | TTR amyloidosis<br>Hypercholesterolemia<br>Liver cancer and liver metastases |
| TKM-PLK1 TKM-ApoB (Tekmira) | RNAi targeting polo-like kinase 1 (POLO) RNAi targeting apoB | Liver tumors<br>High levels of LDL cholesterol |
| Stimuvax (Oncothyreon/Merck) | Anti-MUC1 cancer vaccine | Non-small cell lung cancer |
| Exparel (Pacira) | Bupivacaine | Nerve block<br>Epidural |

## 1.2. Ursodeoxycholic Acid

Bile acids and their conjugates are essential components of bile that are synthesized from cholesterol in the liver. Bile acids induce bile flow, feedback-inhibit cholesterol synthesis, promote intestinal excretion of cholesterol, and facilitate the dispersion and absorption of lipids and fat-soluble vitamins. After secretion into the biliary tract, bile acids are largely (95%) reabsorbed in the intestine (mainly in the terminal ileum), returned to the liver, and then again secreted in bile (enterohepatic circulation). Cholic acid, chenodeoxycholic acid, and deoxycholic acid constitute 95% of bile acids; lithocholic acid and ursodeoxycholic acid are minor constituents. The bile acids exist largely as glycine and taurine conjugates, the salts of which are called bile salts. Colonic bacteria convert primary bile acids (cholic and chenodeoxycholic acid) to secondary acids (mainly deoxycholic and lithocholic acid) by sequential deconjugation and dehydroxylation. These secondary bile acids also are absorbed in the colon and join the primary acids in the enterohepatic pool[37].

Dried bile from the Himalayan bear (Yutan) has been used for centuries in China to treat liver disease. In traditional Asian medicine, the Himalayan bear (Yutan) bile extracted from the gallbladder of *Ursus thibetanus* or *Ursus arctos* is used to treat liver diseases. With the development of modern medicine, it is found that the major active component in bear bile is ursodeoxycholic acid (UDCA)[38]. First reports on the effects of UDCA in patients with liver diseases came from Japan as early as 1961[39]. Since 1989, a number of controlled trials on the use of UDCA in primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) were published in the Western literature[40]. Nowadays, synthetic UDCA is a safe drug with no side effects and is widely used in the treatment of diseases, such as gallstones, primary cirrhosis, autoimmune hepatitis and colon cancer around the world[41]. To date, UDCA is widely used for the treatment of PBC for which it is the only drug approved by the U.S. Food and Drug Administration (FDA).




| Bile Acid | R3 | R7 | R12 | R24 |
|---|---|---|---|---|
| Cholic acid | –OH | –OH | –OH | |
| Chenodeoxycholic acid | –OH | –OH | –H | glycine (75%) |
| Deoxycholic acid | –OH | –H | –OH | taurine (24%) |
| Lithocholic acid | $–SO_2^-$ / –OH | –H | –H | –OH (<1%) |
| Ursodeoxycholic acid | –OH | –OH | –H | |

Figure 8: Different Bile Acids.

Ursodeoxycholic acid (UDCA) is a white crystalline powder that is poorly soluble in water and highly permeable[42], so it belongs to class II drug products in the Biopharmaceutical Classification System[43]. UDCA is a naturally occurring bile acid that is physiologically produced in the liver and present in in a low concentration of only about 3% of total bile acids.

**Chemical Structure:**

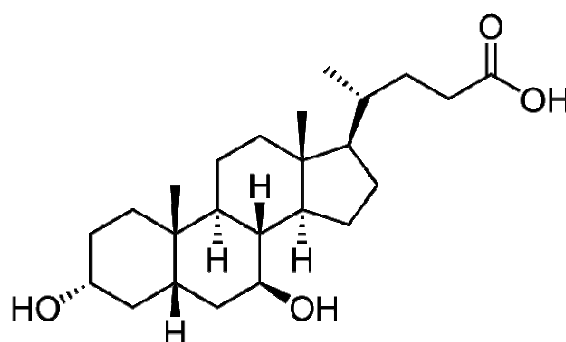



Figure 9: Chemical structure of UDCA

**Chemical Formula:** $C_{24}$ $H_{40}$ $O_4$

**IUPACName:**(4R)-4-[(1S,2S,5R,7S,9S,10R,11S,14R,15R)-5,9-dihydroxy-2,15-dimethyltetracyclo[$8.7.0.0^{2,7}.0^{11,15}$]heptdecan-14-yl] pentanoic acid.

### 1.2.1. Pharmacodynamics:

Ursodiol (also known as ursodeoxycholic acid) is one of the secondary bile acids, which are metabolic byproducts of intestinal bacteria. Primary bile acids are produced by the liver and stored in the gall bladder. When secreted into the colon, primary bile acids can be metabolized into secondary bile acids by intestinal bacteria. Primary and secondary bile acids help the body digest fats. Ursodeoxycholic acid helps regulate cholesterol by reducing the rate at which the intestine absorbs cholesterol molecules while breaking up micelles containing cholesterol. Because of this property, ursodeoxycholic acid is used to treat gall stones non-surgically.

### 1.2.2. Pharmacokinetics[44]:

UDCA capsules and tablets contain crystals of the acid form, which are poorly soluble at pH <7. The pKa of UDCA is 5.1, and the solubility of its protonated form is 9 µmol/L. After oral administration of pharmacologic doses (10-15 mg/kg/d), UDCA is absorbed by dissolution-limited passive nonionic diffusion mainly in the small intestine and to a small extent in the colon. Since the critical micellization pH for UDCA is close to pH 8, dissolution of UDCA in the proximal jejunum occurs by solubilization in mixed micelles of other bile acids.Thus, administration of UDCA with a meal may enhance absorption. In patients with cholestasis and decreased biliary secretion of endogenous bile acids, absorption of UDCA may be decreased. UDCA is taken up from the portal blood into the liver with a first pass extraction of about 50%, conjugated mainly with glycine and to a lesser extent with taurine, and actively secreted into bile. Although conjugates of UDCA appear to be the active species mediating the pharmacologic effects of UDCA in cholestatic liver disease, conjugation even in the cholestatic liver is so efficient that it apparently suffices to administer the unconjugated molecule. The degree of UDCA enrichment in bile following chronic ingestion correlates with the daily administered dose. A daily dose of 13 to 15 mg/kg UDCA causes an enrichment of approximately 40% to 50% in biliary bile acids of patients with PBC. Beyond a certain dose, which has not been adequately defined, additonal enrichment does not occur because of both the inability of UDCA to inhibit bile acid synthesis and the epimerization of UDCA to chenodeoxycholic acid. UDCA conjugates are absorbed mainly from the distal ileum, where they compete with endogenous bile acids for active transport, and undergo

enterohepatic circulation. Non-absorbed UDCA conjugates pass into the colon, are deconjugated, and converted to lithocholic acid by intestinal bacteria. Because of its low aqueous solubility, most of the lithocholic acid formed remains insoluble in the colonic content. The fraction of lithocholic acid returning to the liver undergoes sulphation that in turn leads to excretion via the feces. Even in patients with cholestatic liver disease, less than 5% of the dose of UDCA is found as conjugates and metabolites in the urine, showing that renal elimination represents a minor pathway of UDCA elimination[45].

### 1.2.3. Mechanisms of Action of UDCA

It suppresses the synthesis and secretion of endogenous cholesterol by the liver and inhibits intestinal absorption of cholesterol. It is a hydrophilic bile acid that solubilizes cholesterol, promoting dispersion into body fluids, reducing its viscosity and increasing bile flow. Consequently, UDCA reduces cholestasis, preventing the formation and promoting dissolution of cholesterol gallstones[42].

The mechanisms underlying the beneficial effects of UDCA in cholestatic disorders are increasingly being unraveled. Experimental evidence suggests three major mechanisms of action: (i) protection of cholangiocytes against cytotoxicity of hydrophobic bile acids, (ii) stimulation of hepatobiliary secretion, and (iii) protection of hepatocytes against bile acid–induced apoptosis. One or all of these mechanisms may be of relevance in individual cholestatic disorders and/or different stages of the cholestatic liver disease.

### 1.2.4. Therapeutic Uses and Efficacy[44]

***Primary Biliary Cirrhosis.***

This chronic cholestatic liver disease may be regarded as a model disease for UDCA therapy. It starts with an inflammatory lesion of interlobular bile ducts of unknown etiology, which results in bile duct destruction, fibrosis, and finally cirrhosis. Since the cause of the disease is unknown, therapy must aim at inhibiting the underlying pathogenetic processes to delay the progession of the disease.

***Primary Sclerosing Cholangitis.***

This chronic cholestatic liver disease of unknown cause is characterized by chronic periductal inflammation of intrahepatic and extrahepatic bile ducts leading to obliterative fibrosis, duct loss, and biliary cirrhosis.

***Intrahepatic Cholestasis of Pregnancy.***

This cholestatic disorder affecting pregnant women during the third trimester has been shown to respond to UDCA treatment.

***Liver Disease in Cystic Fibrosis.***

This genetic disorder is caused by mutations of the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, which result in the secretion of viscous bile. This may lead to the formation of bile duct plugs, biliary obstruction, focal biliary fibrosis, and focal biliary cirrhosis.

***Progressive Familial Intrahepatic Cholestasis.***

Progressive familial intrahepatic cholestasis (PFIC) represents a group of autosomal recessive inherited disorders of childhood in which cholestasis usually presents in the neonatal period or the first years of life and leads to death from liver failure at ages ranging from infancy to adolescence. They are caused by defective transporters of the canalicular membrane, namely FIC1 (PFIC 1), BSEP (PFIC 2), and MDR3 (PFIC 3). Although children with PFIC 1 or PFIC 2 are characterized by a normal serum γ-glutamyltranspeptidase (GGT), children suffering from PFIC 3 have high serum GGT.

***Chronic Graft-Versus-Host Disease.***

Graft-versu shost disease involving the liver may cause cholestasis.

***Drug- and Parenteral Nutrition–Induced Cholestasis.***

Small case series suggest that UDCA treatment may be beneficial in some of these disorders.

Although UDCA has been used for the treatment of cholestatic liver diseases in Western medicine for more than a decade, the underlying mechanisms of its anticholestatic effects are only now being unraveled. Future efforts will focus on definition of clinical uses of UDCA beyond those established so far, on optimized dosage regimens, as well as on further elucidation of potential mechanisms of action of UDCA.

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# CHAPTER 2

# LITERATURE REVIEW

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. Review of literature

**Feigenson *et al*., (1999)** studied a novel strategy for the preparation of liposomes i.e., by the rapid solvent exchange. Their study developed a new preparative method which entails the direct transfer of lipid mixtures between organic solvent and aqueous buffer. Also the liposomes prepared by this method required no more than a minute to prepare and manifest considerable entrapment volumes with a high fraction of external surface area.

**Mishra *et al*., (2005)** studied the enhancement of the tumour inhibitory activity, in vivo, of diospyrin, a plant-derived quinonoid, through liposomal encapsulation. The study revealed that liposomal delivery of diospyrin could significantly reduce EAC tumour growth and enhance the survival of tumour-bearing mice. Also, the biochemical assay of the glycolytic and liver function enzymes of the blood sera collected from these mice and histopathological studies on their liver tissues showed substantial restoration of the parameters to near normal levels.

**Deng *et al*., (2006)** studied the Preparation of submicron unilamellar liposomes by freeze-drying double emulsions. Their research work found out that the technique used was reproducible and simple technique can be used to prepare sterilized, submicron unilamellar liposomes with a relatively high encapsulation efficiency and excellent stability during long-term storage.

**Mukherjee *et al*., (2010)** studied Doxorubicin-loaded phosphatidylethanolamine conjugated nanoliposomes for *in-vitro* characterization and their accumulation in liver, kidneys, and lungs in rats. It was found that the PE-conjugated nanoliposomes released the drug in a sustained manner and was also seen to accumulate in different organs also. Thus, may be used for cell/ tissue targeting, attaching specific antibodies to PE.

**Kumar Nitesh *et al*., (2014)** studied the improvement of oral bioavailability of Silymarin liposomes besides targeting hepatocytes, and immune cells. The studies found that incorporating phytosomal form of silymarin in liposomal carrier system had better in vitro

and in vivo hepatoprotection besides showing better anti-inflammatory effects and improvement in histopathological changes as compared to silymarin suspension. These effects were further supported by increase in AUC and Cmax of silybin by silymarin-liposomes compared to silymarin suspension.

**Misran *et al*., (2015)** studied the preparation and characterization of liposomes coated withDEAE-Dextran. The studies found that the lecithin liposomes due to its less neg-ative zeta potential which encourages aggregation of particles, had significant increase in particle size after preparation as it exhibited DEAE-Dx coating give rise to a more positive zeta potential thus cor-responding to a more stable colloidal system. Also, DEAE-Dx coated liposomes have slightly enhanced the entrapment efficiency of cur-cumin. Thus, favouring the use of DEAE-Dx coated liposomes as an effective drug delivery carrier.

**Liu *e. al*., (2015)** studied the self-assembly and cytotoxicity of PEG-modified ursolic acid liposomes. The studies revealed that the PEGmodified ursolic acid liposomes possessed higher stability,slower release rate and low cytotoxic effect as compared to the conventional liposomes.

**Greige-Gerges *et al*., (2015)** studied the preparation and characterization of clove essential oil-loaded liposomes. Their studies revealed that liposomes exhibited nanometric oligolamellar and spherical shaped vesicles and protected eugenol from degradation induced by UV exposure; they also maintained the DPPH_-scavenging activity of free eugenol. Liposomes constitute a suitable system for encapsulation of volatile unstable essential oil constituents.

**2.2. Reference**


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# CHAPTER 3

# AIM OF THE RESEARCH WORK

# CHAPTER 3

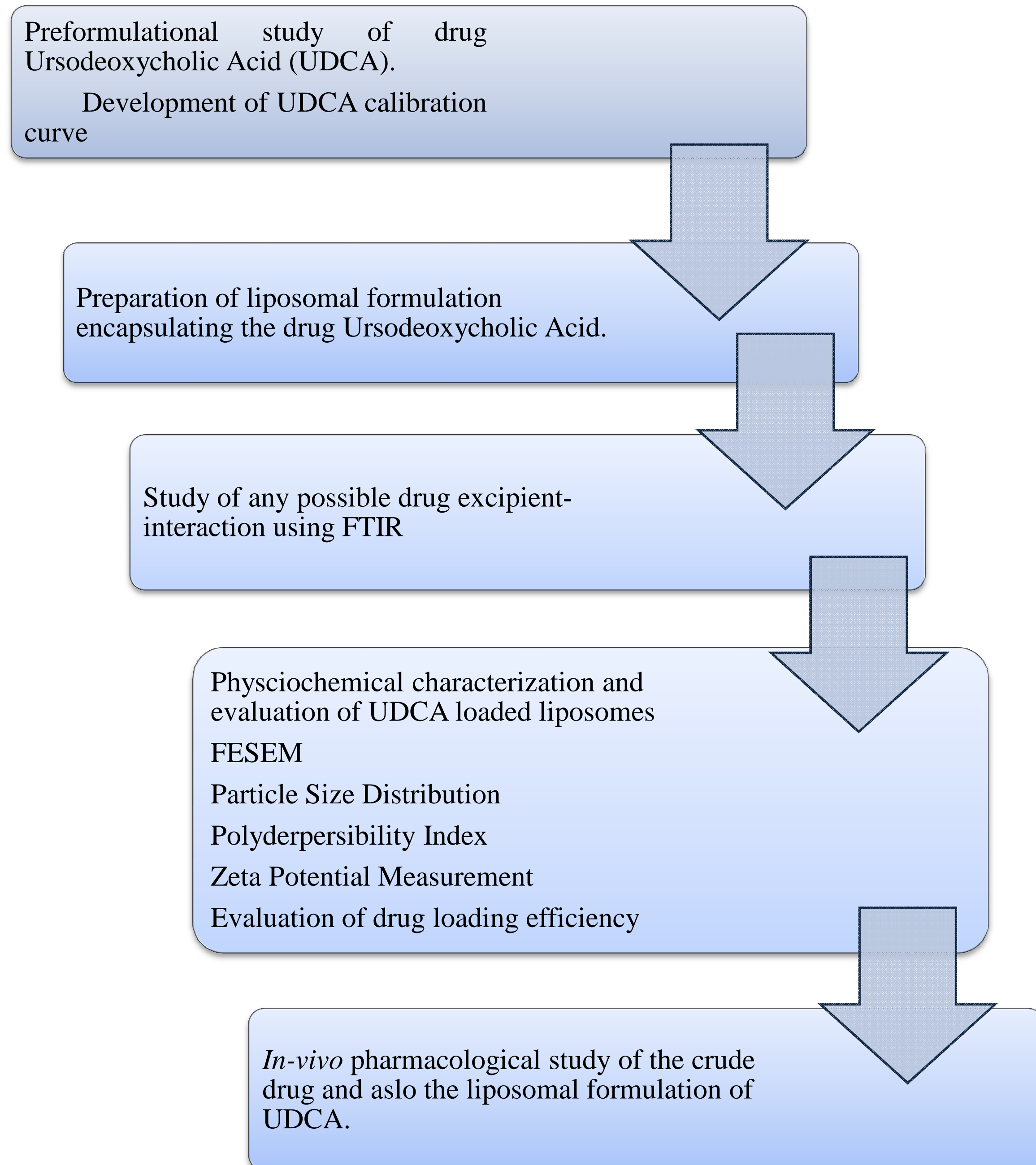
# AIM OF THE RESEARCH WORK

## 3.1. Aim of the research work

The primary objective of this research work is an attempt to develop a simple manufacturing approach of ursodeoxycholic acid loaded freeze-dried liposomes for intravenous administration to treat hepatic chirrosis possibly to reduce its rate of metabolism and increase its bioavailability *in-vivo*. After reconstitution of the freeze dried formulation, it could be directly injected into the vein.

In-order to meet the objectives the ursodeoxycholic acid loaded liposomes were formulated, characterized in terms of drug excipient interaction by FTIR spectroscopy, morphological study of liposomes by using field emission scanning electron microscopy (FESEM), particle size and particle size distribution, polydispersity index, zeta potential and drug loading. Finally, the liposomal preparations of ursodeoxycholic acid were studied for the hepatoprotective activity in comparision to free UDCA and a standard hepatoprotective drug.

## 3.2. Schematic Outline of work.


Preformulational study of drug Ursodeoxycholic Acid (UDCA).
Development of UDCA calibration curve
Preparation of liposomal formulation encapsulating the drug Ursodeoxycholic Acid.
Study of any possible drug excipient-interaction using FTIR
Physciochemical characterization and evaluation of UDCA loaded liposomes
FESEM
Particle Size Distribution
Polyderpersibility Index
Zeta Potential Measurement
Evaluation of drug loading efficiency
In-vivo pharmacological study of the crude drug and aslo the liposomal formulation of UDCA.

# CHAPTER 4

# MATERIALS & INSTRUMENTS

# CHAPTER 4

# MATERIALS & INSTRUMENTS

## 4.1. MATERIALS

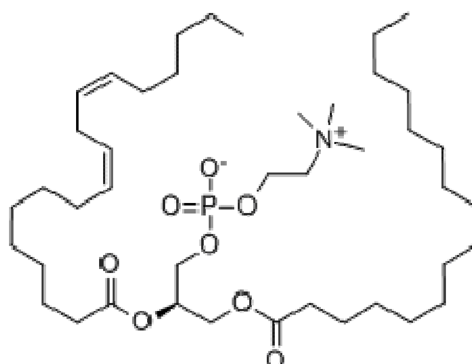
**Chemicals and Reagents used:**

Table 4.1: List of all the chemicals used for the preparation of ursodeoxycholic acid (UDCA) loaded liposomes.

| S. No. | Name | Source |
|---|---|---|
| 1 | Lecithin, Refined Solid | Alfa Aesar A Johnson Matthey Company |
| 2 | Cholesterol Reference standard grade | Sisco Research Laboratories Pvt. Ltd. |
| 3 | Butylated Hydroxy Anisole | Merck Life Science Private Limited. |
| 4 | Chloroform GR | Merck Life Science Private Limited. |
| 5 | Ursodeoxycholic Acid | Sigma-Aldrich |
| 6 | Methanol | Merck Limited |
| 7 | Potassium dihydrogen Phosphate | Merck Limited |
| 8 | Sodium Chloride | Merck Limited |
| 9 | Acetonitrile | Merck Limited |
| 10 | Orthophosphoric Acid | Merck Limited |

### 4.1.1. Soya-L-α–lecithin[1,2]

**Structure**



**Synonym**: 1, 2 diacyl-: ussn: ue-glycero-3-phosphocholine, Phosphatidyl Choline and Lecithin

**Molecular weight**: 760.09 g/mol.

**Molecular Formula**: $C_{42}H_{80}NO_8P$

**Solubility:** chloroform: 0.1 g/mL, slightly hazy, slightly yellow to deep orange

**Storage Temperature:** 2-8°C

**Source**

Lecithin is a combination of naturally-occurring phospholipids, which are extracted during the processing of soybean oil. The soybeans are tempered by keeping them at a consistent temperature and moisture level for approximately seven to 10 days. This process hydrates the soybeans and loosens the hull. The soybeans are then cleaned and cracked into small pieces and the hulls are separated from the cracked beans. Next, the soybean pieces are heated and pressed into flakes. Soybean oil is extracted from the flakes through a distillation process and lecithin is separated from the oil by the addition of water and centrifugation or steam precipitation[1].

**Functional properties**

Lecithin is utilized in a wide variety of food and industrial applications. The French scientist, Maurice Gobley, first discovered the substance in 1850, and named it "lekithos," the Greek

term for egg yolk. At the time, eggs provided a primary source of commercially-produced lecithin. Today, the majority of lecithin used in food applications is derived from soybeans.

Soy lecithin offers a multifunctional, flexible and versatile tool. It is probably best known for its emulsifying properties, which help promote solidity in margarine and give consistent texture to dressings and other creamy products. Lecithin is also used in chocolates and coatings and to counteract spattering during frying. Additionally, its unique lipid molecular structure makes lecithin useful for pharmaceutical and cosmetic applications and various industrial uses such as paints, textiles, lubricants and waxes.

**Health Benefits**

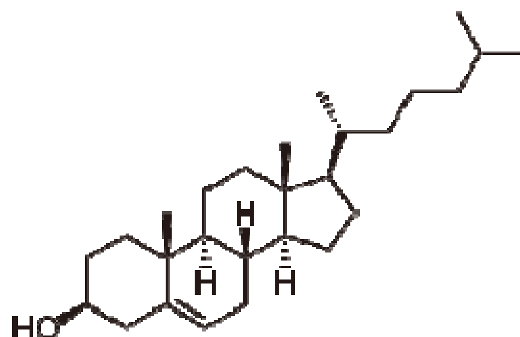
Lecithin provides an excellent source of choline, which is essential to every living cell in the body and is one of the main components of cell membranes. Not only is dietary choline important for the synthesis of the phospholipids in cell membranes, it is also necessary for methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid-cholesterol transport and metabolism[2]. Without choline, the cell membranes would harden, prohibiting important nutrients from entering and leaving the cell. Scientists believe lecithin and choline may aid in memory and cognitive function, cardiovascular health, liver function, reproduction and fetal development and physical and athletic performance.

In 1998, the Institute of Medicine (IOM) of the U.S. National Academy of Sciences identified choline as an essential nutrient and recommended daily intake amounts. And, in 2001, the U.S. Food and Drug Administration (FDA) approved a nutrient content claim for choline, enabling food manufacturers to inform their consumers via the food label.

Foods that contain over 110 mg of choline per serving may claim that they are an "excellent source of choline" and those with over 55 mg may claim that they offer a "good source of choline.

### 4.1.2. Cholesterol

**Structure**




**IUPAC name:** (10*R*,13*R*)-10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]henanthren-3-ol.

**Molecular formula:** $C_{27}H_{46}O$

**Solubility in water:** 0.095 mg/L (30 °C)

**Appearance:** white crystalline powder, usually acquires a yellow to pale tan colour on prolonged exposure to light or elevated temperature

**Solubility:**

Soluble in diethyl ether,  acetone.

Very slightly soluble in cold water.

Solubility in water: 0.2mg/100ml or 0.2%

Slightly soluble in alcohol; more soluble in hot alcohol.

Soluble in chloroform, pyridine, benzene, petroleum ether, oils, fats, aqueous solutions of bile salts.

Solubility in ether: 1 g/2.8 ml ether.

Solubilitiy in chloroform: 1 g/4.5 ml chloroform.

Solubility in pyridine: 1g/1.5 ml pyridine.

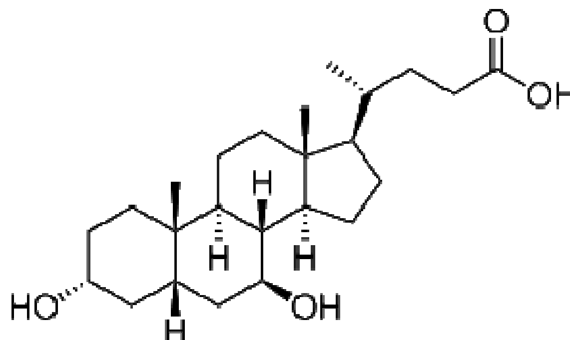
**Molecular weight:** 386.66

**Melting point:** 147 to 150°C

### 4.1.3. Ursodeoxycholic Acid[3]:

Ursodiol (ursodeoxycholic acid, UDCA) is a naturally occurring bile acid found in small quantities in normal human bile and in larger quantities in the biles of certain species of bears. It is a hydrophilic bile acid that is increasingly used for the treatment of various cholestatic disorders. To date, UDCA is widely used for the treatment of primary biliary cirrhosis (PBC) for which it is the only drug approved by the U.S. Food and Drug Administration (FDA).

**CHEMICAL STRUCTURE**




IUPAC name: (4R)-4-[(1S,2S,5R,7S,9S,10R,11S,14R,15R)-5,9-dihydroxy-2,15-dimethyltetracyclo[8.7.0.$0^{2,7}$.$0^{11,15}$]heptadecan-14-yl]pentanoic acid.

Molecular Weight: 392.56.

**Physical Properties:**

Taste: bitter-tasting

Colour: white powder consisting of crystalline particles

Solubility: freely soluble in ethanol and glacial acetic acid, slightly soluble in chloroform, sparingly soluble in ether, and practically insoluble in water.

**Mechanisms of Action of UDCA**

The mechanisms underlying the beneficial effects of UDCA in cholestatic disorders are increasingly being unraveled. When administered orally, ursodiol can alter relative concentrations of bile acids, decrease biliary lipid secretion, and reduce the cholesterol content of the bile so that it is less lithogenic. Ursodiol also may have cytoprotective effects on hepatocytes and effects on the immune system that account for some of its beneficial effects in cholestatic liver diseases. Experimental evidence suggests three major mechanisms of action:

i. protection of cholangiocytes against cytotoxicity of hydrophobic bile acids,

ii. stimulationof hepatobiliary secretion, and

iii. protection of hepatocytes against bile acid–induced apoptosis.

One or all of these mechanisms may be of relevance in individual cholestatic disorders and/or different stages of the cholestatic liver disease.

**CLINICAL PHARMACOLOGY**

Ursodiol (UDCA) is normally present as a minor fraction of the total bile acids in humans (about 5%). Following oral administration; the majority of ursodiol is absorbed by passive diffusion and its absorption is incomplete. Once absorbed; ursodiol undergoes hepatic extraction to the extent of about 50% in the absence of liver disease. As the severity of liver disease increases, the extent of extraction decreases. In the liver, ursodiol is conjugated with glycine or taurine, then secreted into bile. These conjugates of ursodiol are absorbed in the small intestine by passive and active mechanisms. The conjugates can also be deconjugated in the ileum by intestinal enzymes, leading to the formation of free ursodiol that can be reabsorbed and reconjugated in the liver. Non-absorbed ursodiol passes into the colon where it is mostly 7-dehydroxylated to lithocholic acid. Some ursodiol is epimerized to chenodiol (CDCA) via a 7-oxo intermediate. Chenodiol also undergoes 7-dehydroxylation to form lithocholic acid. These metabolites are poorly soluble and excreted in the feces. A small portion of lithocholic acid is reabsorbed, conjugated in the liver with glycine, or taurine and sulfated at the 3 position. The resulting sulfated lithocholic acid conjugates are excreted in bile and then lost in faeces.

**Therapeutic Uses and Efficacy:**

Primary Biliary Cirrhosis: Primary biliary cirrhosis is a chronic, progressive, cholestatic liver disease of unknown etiology that typically affects middle-aged to elderly women. Ursodiol (administered at 13-15 mg/kg per day in two divided doses) reduces the concentration of primary bile acids and improves biochemical and histological features of primary biliary cirrhosis, especially in early disease.

Ursodiol also has been used in a variety of other cholestatic liver diseases, including primary sclerosing cholangitis, and in cystic fibrosis; in general, it is less effective in these conditions than in primary biliary cirrhosis.

**Primary Sclerosing Cholangitis:** This chronic cholestatic liver disease of unknown cause is characterized by chronic periductal inflammation of intrahepatic and extrahepatic bile ducts leading to obliterative fibrosis, duct loss, and biliary cirrhosis.

**Intrahepatic Cholestasis of Pregnancy:** This cholestatic disorder affecting pregnant women during the third trimester has been shown to respond to UDCA treatment.

**Chronic Graft-Versus-Host Disease.** Graft-versushost disease involving the liver may cause cholestasis.

**Dosage and administration:** *Dose:* 450–600 mg daily in 2–3 divided doses after meals.

**Possible side effects:** Diarrhoea and hyper-transaminaemia are infrequent, but Gastric and esophageal mucosal resistance to acid is impaired favouring ulceration

## 4.2. Instruments:

Table 4.2: List of instruments used.

| Serial No. | Name | Source |
|---|---|---|
| 1 | Rotary vacuum evaporator | Hahnvapor rotary evaporator, by Hahnshin Scientific, made in Korea |
| 2 | Cold Centrifuge | 3K30, SIGMA, Shrewsbury, USA |
| 3 | Vacuum desiccators | Tarson, Kolkata, India |
| 4 | Bath Sonicator | Eumax digital ultrasonic cleaner |
| 5 | Lyophilizer | Instrumentation India, Kolkata-32, India |
| 6 | UV absorption spectroscopy | JASCO V-650, Spectrophotometer |
| 7 | Zeta sizer (nanosizer) | ZETASIZER, Nano ZS 90, MALVERN Instrument Ltd, Malvern, UK |
| 8 | Ultra low Freezer | New Brunswick Scientific, Freshwater Boulevard Enfield, U.S.A |
| 9 | FTIR Spectroscopy | JASCO Magna IR 750 series II FTIR instrument, JASCO Internatinal Co. Ltd. FTIR 4200 Tokye, Japan. |
| 10 | FE-Scanning Electron Microscope | JSM, JEOL, Tokyo, Japan |
| 11 | pH meter | Eutech Instruments, Haridwar, India |
| 12 | HPLC | Shimadzu LC-20 |

**References**

1. Food Allergy and Anaphylaxis Network. Adapted from Food Allergy News. 2001. Vol. 2, No. 1.

2. University of Illinois Functional Foods for Health Program, Regulatory Update: "Best Food Sources for Choline – FDA Authorizes New Content Claim for This Essential Nutrient.

3. Gustav Paumgartner, Ursodeoxycholic Acid in Cholestatic Liver Disease: Mechanism of Action and Therapeutic Use Revisited, Perspectives in Clinical Hepatology, 10, (2002), 36088.

# CHAPTER 5

# METHODOLOGY

# CHAPTER 5

# METHODOLOGY

## 5.1. HPLC method of drug assessment

As per the British Pharmacopoeia 2010, the chromatographic procedure was carried out using a stainless steel column packed with octadecylsilyl silica gel for chromatography maintained at 40˚. The mobile phase was with a flow rate of 1.5ml per mixture of 400 volumes of acetonitrile and 600 volumes of 0.001M potassium dihydrogen orthophosphate warned to room temperature and adjusting the pH to 2.0 with orthophosphoric acid (85%) and filtering and detection was carried out at a wavelength of 210 nm. For detection 50mg of ursodeoxycholic acid in 2ml of methanol which was dissolved with the aid of ultrasound for 10 minutes and diluted to 20ml with mobile phase was injected 10µl.

## 5.2. Development of calibration curve of Ursodeoxycholic Acid

### 5.2.1. Scanning of drug, Ursodeoxycholic Acid for the determination of absorption maxima

Drug was dissolved in phosphate buffer saline (PBS) pH 7.4 and methanol mixture (4:6). The drug solution was scanned from wavelength 200nm to 400nm using spectrophotometer taking the above solution as blank. Phosphate buffer saline and methanol mixture was used as a medium for drug release study.

### 5.2.2. Preparation of phosphate buffered saline (PBS), pH 7.4

1.7gm of sodium dihydrogen phosphate and 0.391gm of sodium hydroxide were weighed accurately and taken in a 250ml volumetric flask of which the volume was made upto 250 ml using double distilled water. Finally, the pH was adjusted at 7.4 using standardized pH meter.

### 5.2.3. Preparation of standard curve in PBS and methanol (4:6)

At first a stock solution of ursodeoxycholic acid with a concentration of 10mg/ml was prepared in previously prepared PBS and methanol (4:6) From the stock solution further dilutions were carried out to prepare 1mg/ml, 2mg/ml,3mg/ml, 4mg/ml, 5mg/ml, 6mg/ml and 7mg/ml concentrations. All the different dilutions prepared were then read against PBS:

methanol (4:6) as blank solution. The absorbance observed were noted and plotted to obtain the standard curve for ursodeoxycholic acid.

### 5.3. Preparation of Liposomes

Liposomes were prepared by lipid film hydration method. Weighed amounts of various combinations of soya L-α-lecithin and cholesterol (Table 5.1) containing BHA (2%w/w of lipid) and ursodeoxycholic acid were taken in a clean and dried 250ml round bottom flask (RB). The contents of the RB were then dissolved in chloroform by vigorous shaking. Then the chloroform was evaporated from the RB forming a thin film of lipid at the bottom of RB by a rotary vacuum evaporator (Hahnvapor rotary evaporator). The rotary vacuum evaporator was rotated at 120rpm at 40°C in a water bath. The flask was kept overnight in vacuum desiccators for complete removal of residue of chloroform. On the next day, the hydration was performed above the phase transition temperature (Tm) of the phospholipids (55 °C) i.e. at 60°C by pouring isotonic phosphate buffer pH 7.4 to the flask containing the thin dry lipid film and was hydrated in water bath fitted with a rotary vacuum evaporator and was rotated at 100rpm until the lipid film dispersed in the aqueous phase. The dispersion was sonicated for 30-40 min in a bath sonicator at room temperature to reduce the vesicle size. After sonication the preparation was kept at room temperature for about 1h for vesicle formation and then the preparation was kept at 4°C in an inert atmosphere for 24h. On the third day, the formulation was taken in centrifuge tube and was centrifuged for 1h at 15000rpm in a cold centrifuge. Then the suspended liposomal vesicles were separated from the supernatant and stored in deep freeze (-40°C) for standard pre-freezing overnight. Then the preparation was lyophilized in a lyophilizer (Instrumentation India, Kolkata-32, India) until the product was completely dry.

Table 5.1: Different composition

| Sl. NO. | Formulation Code | Composition in ratio PC:CH | Drug (mg) |
|---|---|---|---|
| 1 | L1 | 1.0:1 | 10 |
| 2 | L2 | 2.0:1 | 10 |
| 3 | L3 | 2.5:1 | 10 |
| 4 | L4 | 3.0:1 | 10 |

## 5.4. PHYSICOCHEMICAL CHARACTERIZATION AND EVALUATION OF THE URSODEOXYCHOLIC ACID LIPOSOME FORMULATION

### 5.4.1. Study of drug excipient-interaction using FTIR spectroscopy

The pure drug ursodeoxycholic acid, CHL, PC, and physical mixture of drug with CHL, drug with PC, drug with BHT, drug with PC and SPC, drug with PC, CH and BHT and lyophilized formulation with drug were mixed separately, with infrared (IR) grade potassium bromide (KBr) in the ratio of 1:100. Corresponding pellets were prepared by applying 5.5 metric ton pressure with a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4000–400 cm-1 in a FTIR instrument (JASCO magna IR 750 series II FTIR instrument).

### 5.4.2. Study of surface morphology of liposome by field emission scanning electron microscopy:-

The external morphology of ursodeoxycholic acid liposome of different formulations were analyzed by field emission scanning electron microscopy (FESEM). The freeze-dried particles were spread onto metal stubs and platinum coating was done by using ion-sputtering device. The coated particles were then vacuum dried and examined under field emission scanning electron microscope.

### 5.4.3. Particle size distribution study

The particle size distribution of the reconstituted lyophilized liposomes was determined by Dynamic Light Scattering (DLS) (ZETASIZER, NanoZS 90, Malvern Instrument Ltd. UK) and analyzed by DTS software. The principle of the particle size determination by DLS is the measurement of the rate of fluctuation of the intensity of scattered light due to Brownian motion of particles. Determination of these intensity fluctuations yields the value of Brownian motion of particles due to thermally induced collisions between the particles which are converted into particle-size by using DTS software. The mean particle diameter (Z average) was calculated by the software from the measured particle size distribution. The freshly prepared lyophilized formulations were suspended in phosphate buffer pH 7.4 and poured into the cuvette and analysed by the instrument.

### 5.4.4. Polydispersity index (PDI)

Polydispersity index is a number which is a measure of size distribution of particles in a given sample. This value may be equal to one or may be greater or less than one. Generally for the monodispersed sample the value is 0.05 and the values greater than 0.7 indicate that the sample has a very broad size distribution and is probably not suitable for the dynamic light scattering technique (Dynamic Light Scattering Common Terms Defined © 2011 Malvern Instruments Limited). Polydispersity index of the different formulation was measured by the instrument Zeta sizer nano ZS (Zetasizer, NanoZS 90, Malvern Instrument Ltd. UK).

### 5.4.5. Zeta potential measurement

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion or attraction between particles in a liquid suspension. This parameter ensures the dispersion stability of the nanoliposome formulation. Zeta potentials of the different formulations were determined by the instrument Zeta sizer nano ZS (Zetasizer, NanoZS 90, Malvern Instrument Ltd. UK).

### 5.4.6. Evaluation of drug loading and loading efficiency:

At first a homogeneous mixture of PBS, pH-7.4 and methanol in a ratio of (4:6) was prepared. 2mg of the lyophilised formulation was weighed accurately and taken in 2ml microcentrifuge tube to prepare 1mg/ml concentration in the previously mixed PBS:methanl (4:6). The preparation was then sonicated for few minutes and then centrifuged at 10,000 rpm for 15 minutes. Then the supernatant was separated and the absorbance of the supernatant was measured by using UV/VIS Spectrophotometer (JASCO V-650, Spectrophotometer.) against the blank at the wavelength of 210nm. The drug content was determined from the standard curve.

% Loading = [ (Weight of the drug in a formulation) X 100]/ Total weight of formulation

Percentage loading efficiency was determined using the following formula.

% Loading efficiency = [(Weight of the drug in 1mg formulation) X ( Total weight of formulation) X100]/ (Total amount of drug taken for each formulation)

# CHAPTER 6

# RESULTS & DISCUSSION

# CHAPTER 6

# RESULTS & DISCUSSION

## 6.1. Results

### 6.1.1. HPLC assessment of UDCA

The drug in its crude form was assessed by the high performance liquid chromatography method as mentioned in the British Pharmacopoeia and the retention time was found to be 15.820 mins. The details of the study are given below in figure:6.1&6.2.

Figure 6.1: Retention time for Ursodeoxycholic Acid

| Retention Time | Area | Area % | Name |
|---|---|---|---|
| 1.033 | 189 | 0.00 | |
| 1.333 | 205 | 0.00 | |
| 1.467 | 85 | 0.00 | |
| 1.760 | 719060 | 16.97 | |
| 2.187 | 330665 | 7.80 | |
| 2.547 | 14387 | 0.34 | |
| 2.687 | 92623 | 2.19 | |
| 3.040 | 37505 | 0.88 | |
| 3.400 | 1988 | 0.05 | |
| 8.100 | 368367 | 8.69 | |
| 15.820 | 2673281 | 63.07 | Ursodiol |
| Totals | 4238355 | 100.00 | |

Figure 6.2: Retention time values

## 6.1.2. PREPARATION OF CALIBRATION CURVE OF DRUG URSODEOXYCHOLIC ACID IN PHOSPHATE BUFFER SALINE (PBS) pH 7.4 AND METHAOL MIXTURE

### 6.1.2.1. Determination of absorption maxima of drug ursodeoxycholic acid in PBS.

The solution of drug ursodeoxycholic acid in PBS pH 7.4 and methanol (4:6) was scanned in the wavelength range of 200 nm to 400 nm in spectrophotometer (JASCO V-650, Spectrophotometer) to find out the absorption maxima. The absorption maxima was found to be at the wavelength of 210 nm.

### 6.1.2.2. Preparation of calibration curve.

Firstly, a stock solution of ursodeoxycholic acid of concentration 10 mg/ml was prepared in a mixture of PBS, pH 7.4 and methanol (4:6). From the stock solution, 3 sets each containing 7 different dilute solutions of varying concentrations were prepared. Calibration curve of the drug (figure no.1) was prepared by measuring the absorbencies of the solutions of varying concentrations. The mean absorbance (n=3) against different concentrations of the drug were plotted as shown in the given Table 6.1.

Table 6.1: Observation Table of Different Concentration of UDCA and its different absorbance

| Serial No. | Concentration (mg/ml) | Absorbance A1 | Absorbance A2 | Absorbance A3 | Average Absorbance |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 |
| 2 | 1 | 0.1159 | 0.116 | 0.118 | 0.116 |
| 3 | 2 | 0.2139 | 0.2239 | 0.2339 | 0.2239 |
| 4 | 3 | 0.311 | 0.334 | 0.35 | 0.331 |
| 5 | 4 | 0.476 | 0.48 | 4.676 | 0.4745 |
| 6 | 5 | 0.5465 | 0.5541 | 0.5561 | 0.5522 |
| 7 | 6 | 0.6233 | 0.6433 | 0.6249 | 0.6305 |

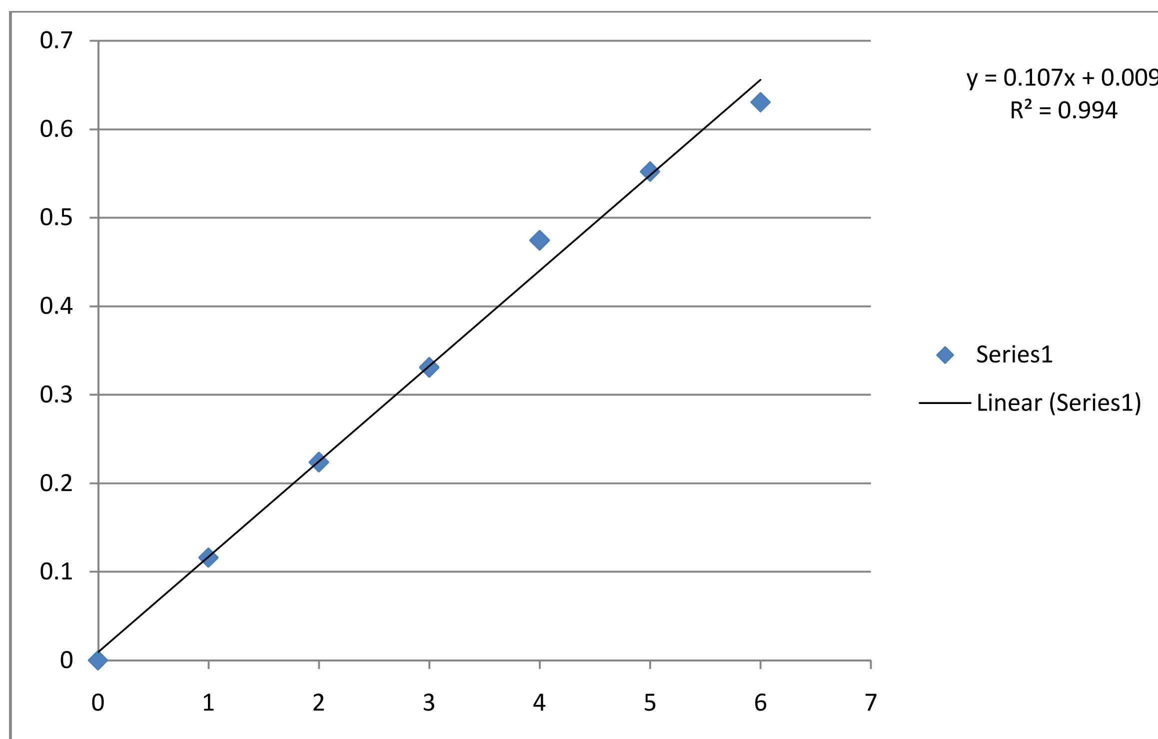



Figure 6.3: Calibration curve of UDCA.

### 6.1.3. STUDY OF DRUG-EXCIPIENT INTERACTION THROUGH FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY.

In the present study the drug-excipient interaction was evaluated by FTIR spectroscopy. FTIR spectra assess the drug-excipient interaction at the level of functional groups by determining their vibrational patterns. Here the spectra of pure drug ursodeoxycholic acid, CHL, PC, and physical mixture of drug with CHL, drug with PC, drug with BHT, drug with PC and SPC, drug with PC, CH and BHT and lyophilized formulation with drug are depicted from figure:6.4. to figure:6.11. When the spectra are compared it is found that there are some peaks at 2937 cm-1, 2867 cm-1 and 1714 cm-1 in the spectrum of drug as compared to liposome with drug where those peaks are not existing. Hence, the absence of those peaks may be due to the absence of functional groups of the drug at the surface of liposome and peak at 1737 cm-1 indicates the presence of lecithin on the surface forming the liposomal membrane. From the result no important interaction was observed and the drug was found to be compatible with the excipients in the liposome formulation.

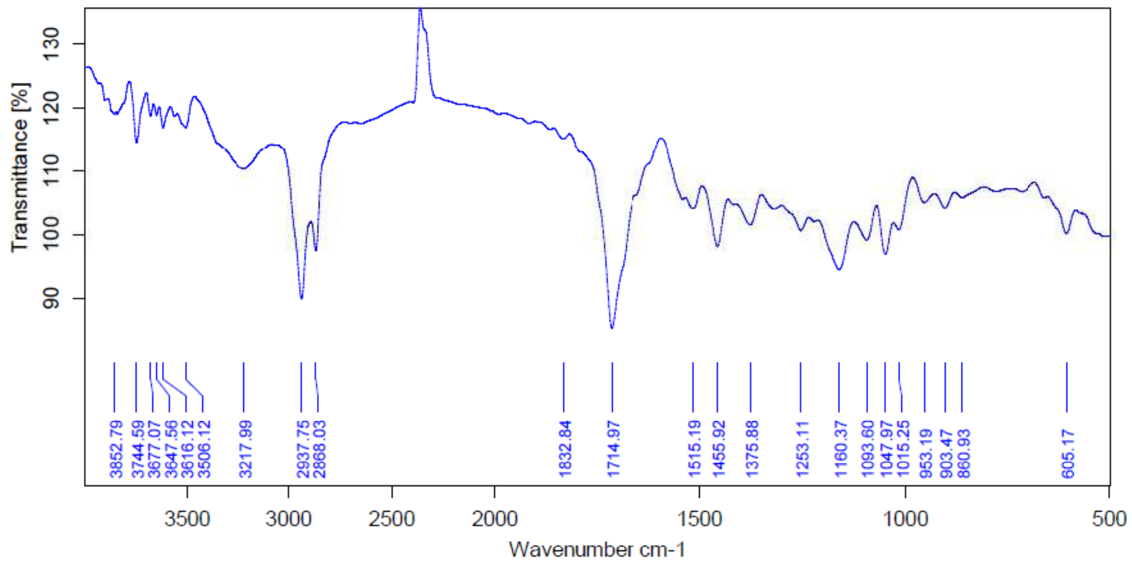



Figure 6.4: FTIR of drug

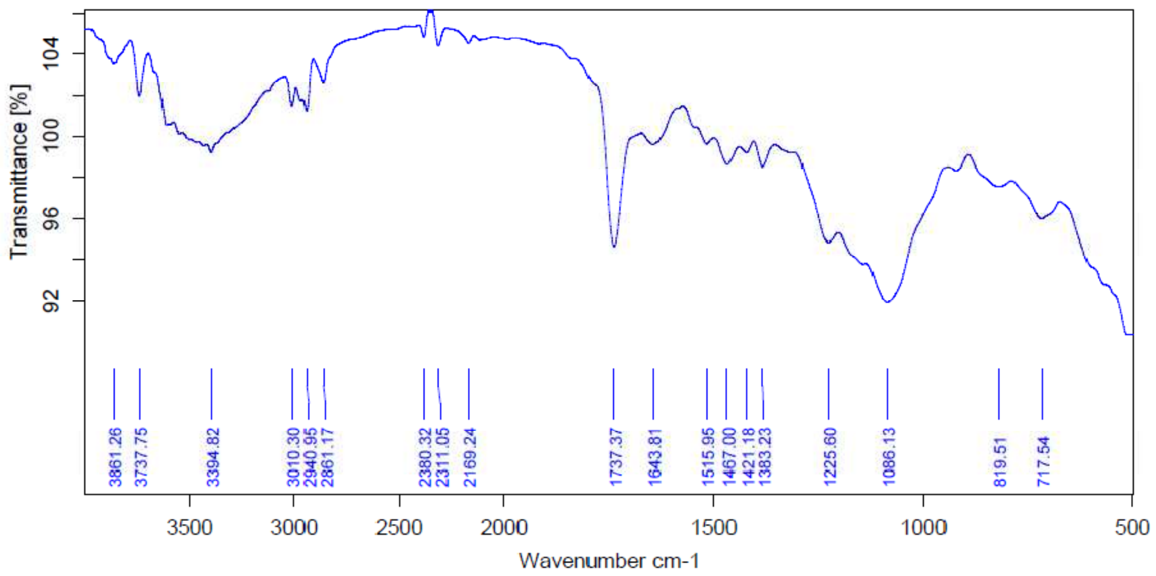



Figure 6.5: FTIR of Lecithin

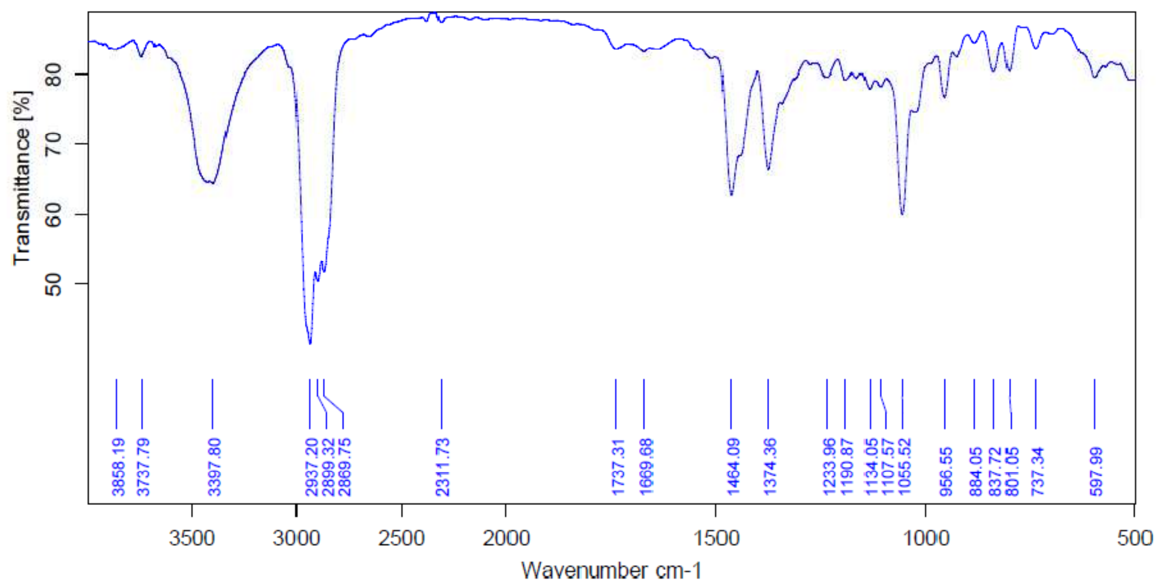



Figure 6.6: FTIR of Cholestrol

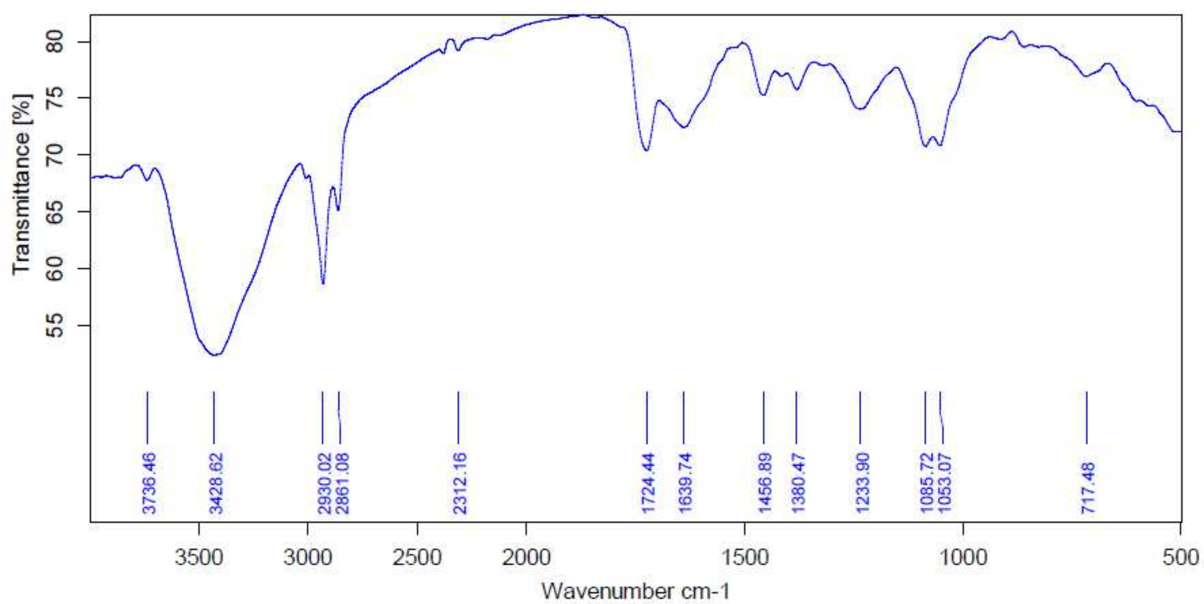



Figure: 6.7: FTIR of drug and lecithin

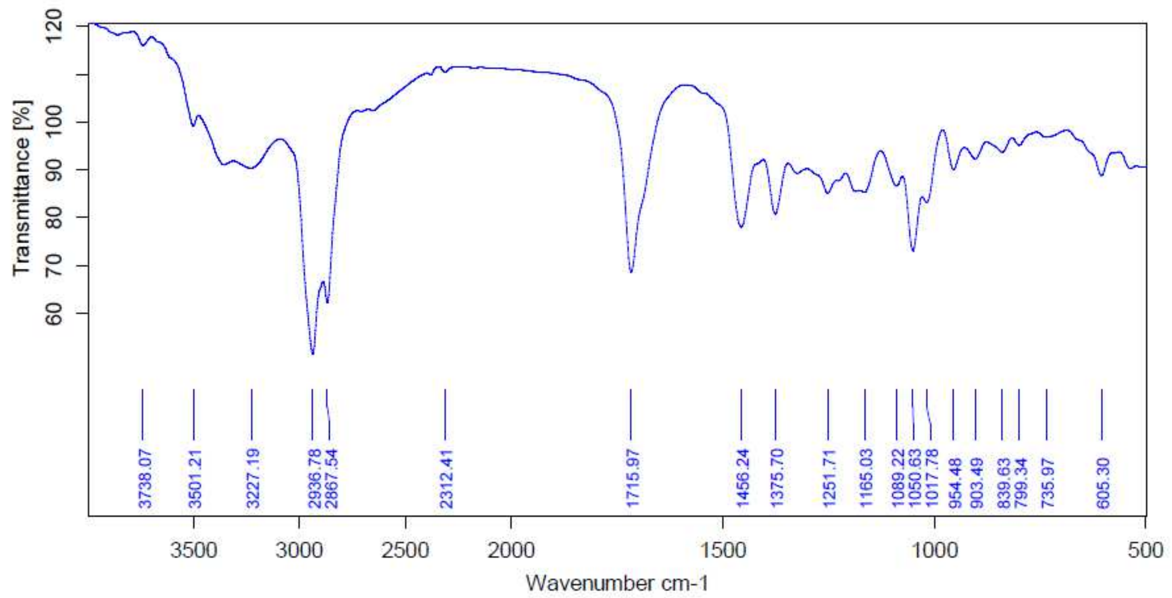



Figure 6.8: FTIR of dug and cholestro

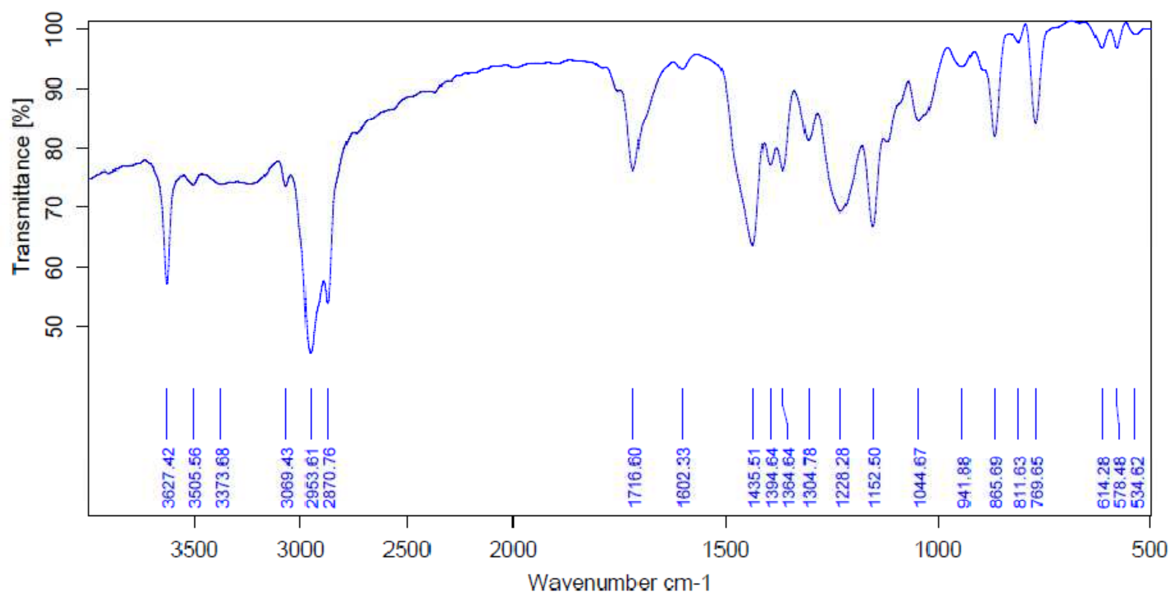



Figure 6.9: FTIR of drug and BHT

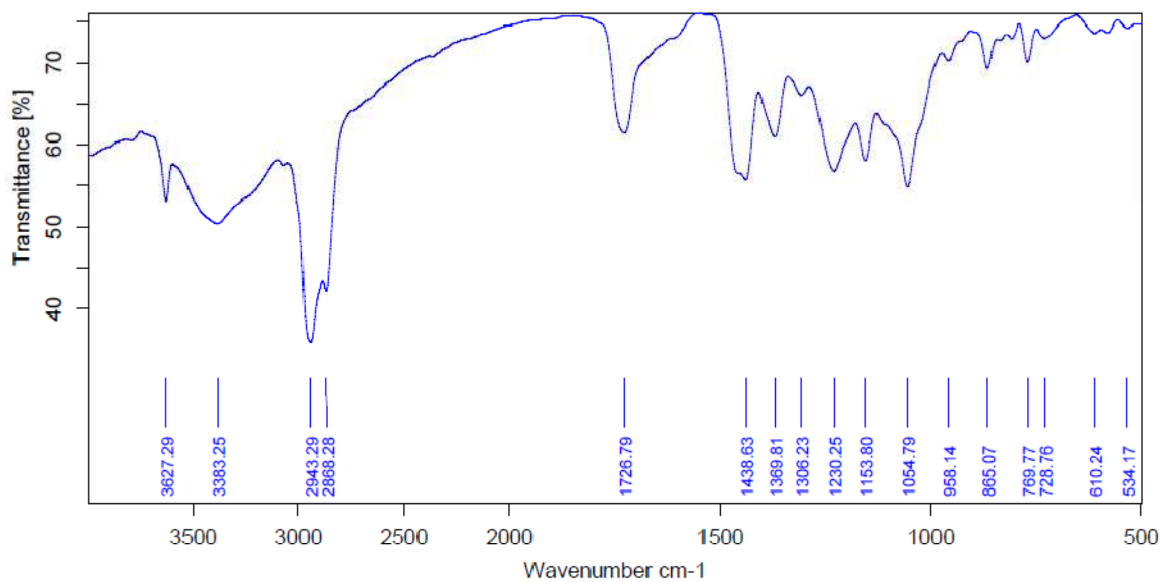



Figure 6.10: FTIR of drug with lecithin, cholestrol and BHT

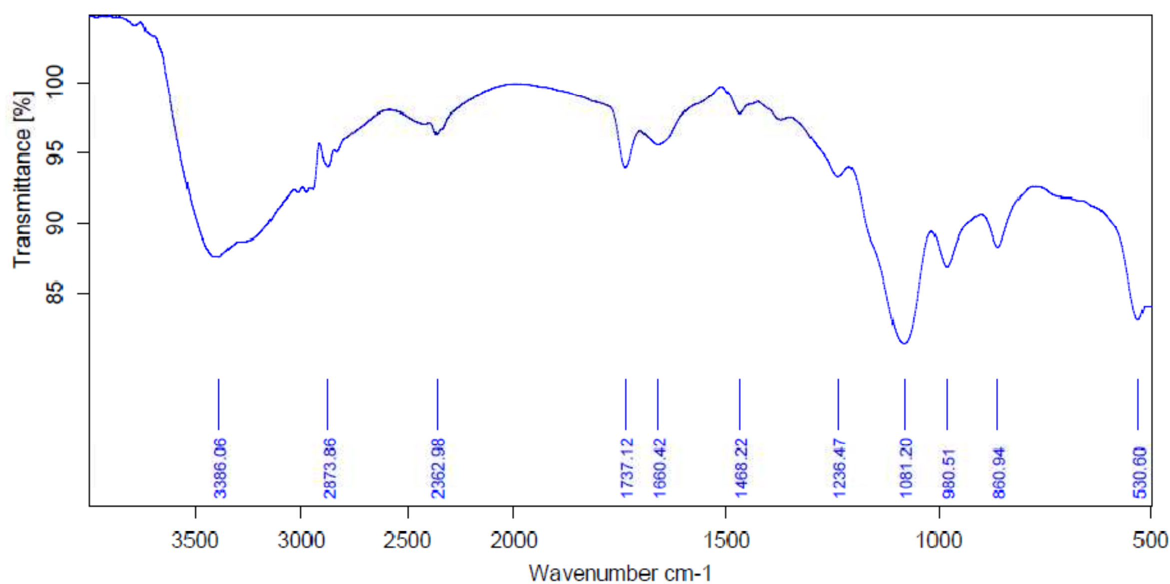



Figure 6.11: FTIR of liposome preparation

### 6.1.2. SURFACE MORPHOLOGY STUDY OF LYOPHILISED LIPOSOMES BY FIELD EMISSION SCANNING ELECTRON MICROSCOPY

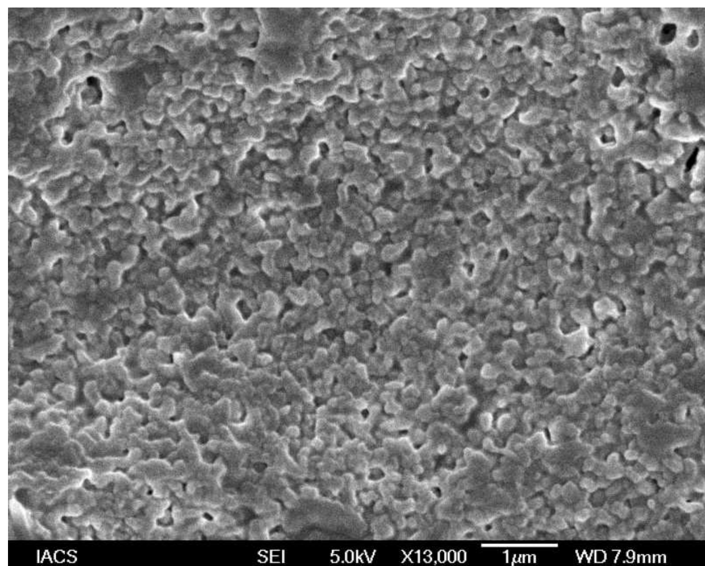



Figure 6.12: Scanning electron micrograph of freshly prepared lyophilised liposomes L1.

Scanning electron micrograph (Figure 6.12.) of freshly prepared lyophilized formulation L1 shows the drug loaded liposomes with diameters in the nanometric range. The freeze dried liposomes had smooth surface and were uniformly distributed.

Figure 6.13a: Scanning electron micrograph of freshly prepared lyophilized liposomes L3.

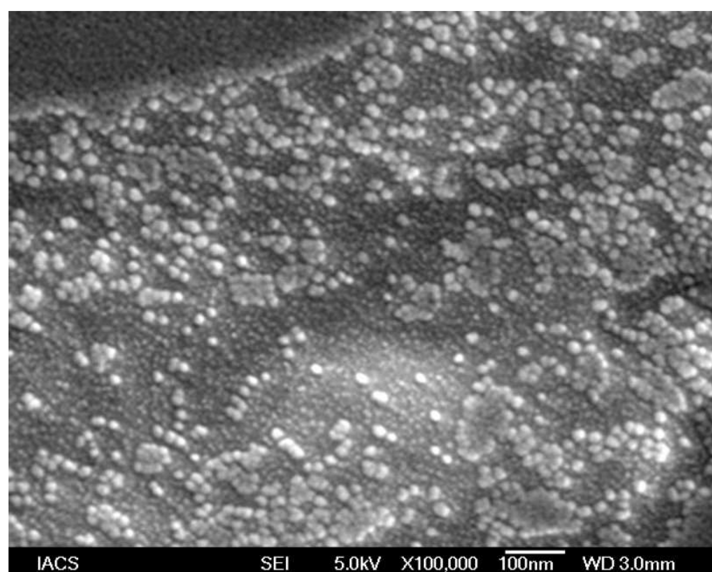



Figure 6.13b: Scanning electron micrograph of freshly prepared lyophilized liposomes L3.

Scanning electron micrograph (Figure 6.13a & 6.13b.) of freshly prepared lyophilized formulation L3 shows that the drug loaded liposomes were in the nanometric range like the previous formulation. They bear smooth surface and were uniformly distributed.

### 6.1.5. PARTICLE SIZE DISTRIBUTION STUDY

Particle size distribution curve for formulation L1

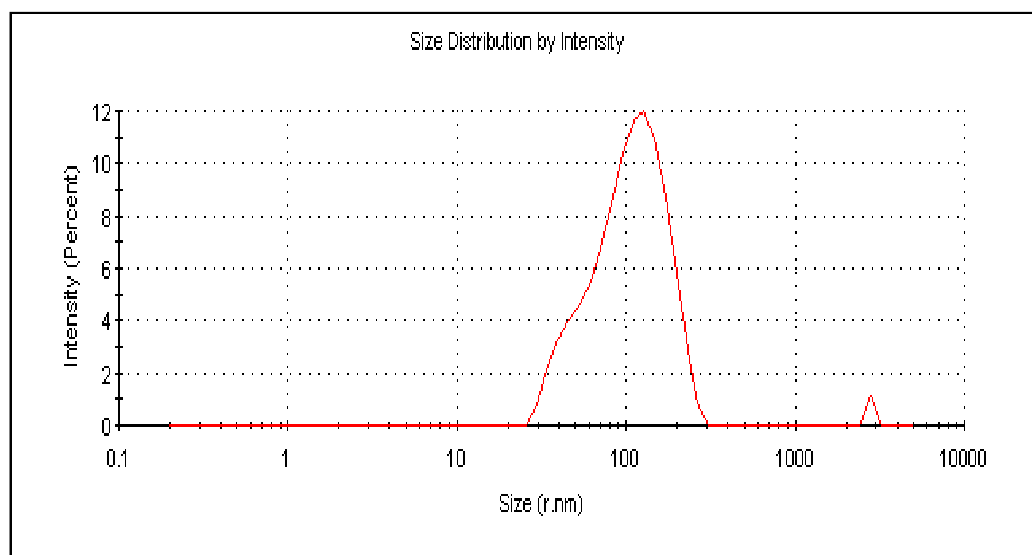



Figure 6.14: shows the particle size distribution pattern of lyophilized formulation L1 (1:1).

The average particle size (z-average) of the formulation L1 (Table 6.2) was 105.5 nm with PDI 0.364 (Table 6.3).

Particle size distribution curve for formulation L2 (2:1)

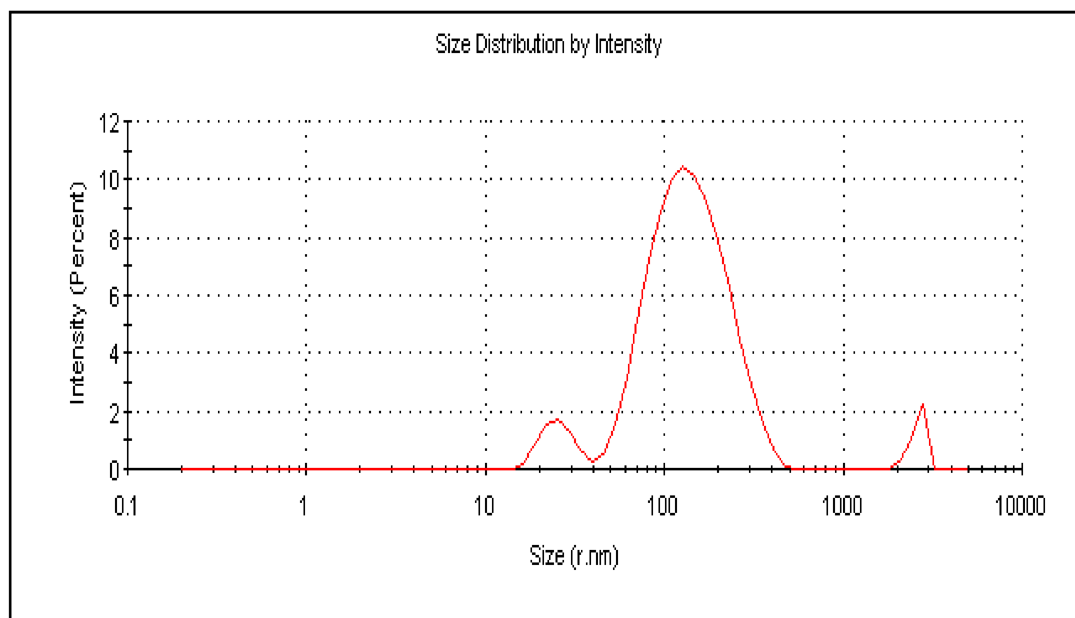



Figure 6.15: shows the particle size distribution pattern of lyophilized formulation L2 (2:1).

The average particle size (z-average) of the formulation (Table 6.2.) was 124.0 nm with PDI 0.384 (Table 6.3.).

Particle size distribution curve for formulation L3 (2.5:1)

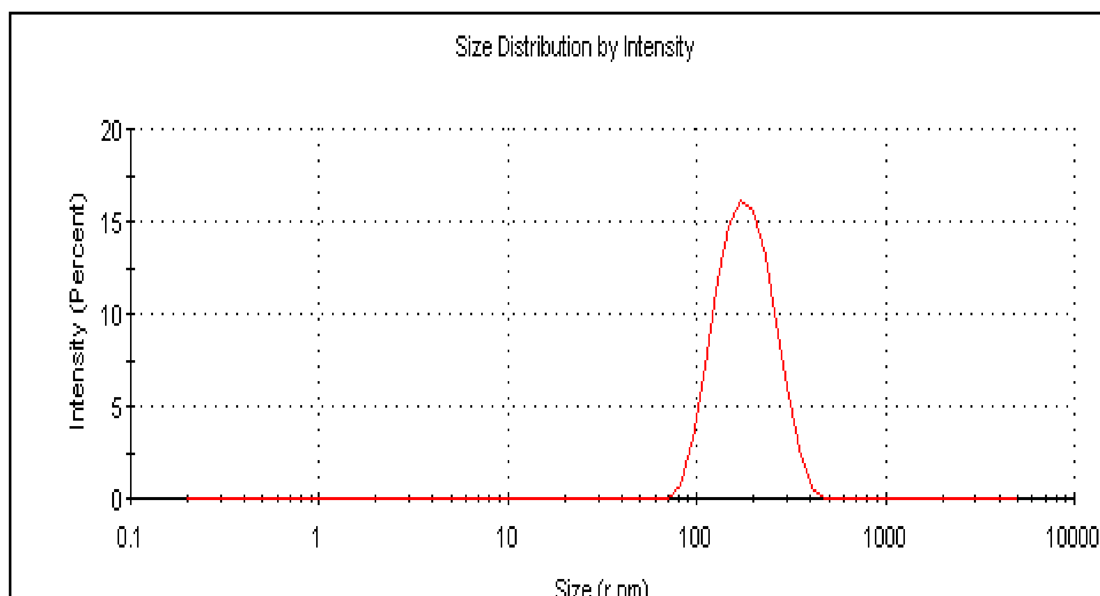



Figure 6.16: shows the particle size distribution pattern of lyophilized formulation L3 (2.5:1).

The average particle size (z-average) of the formulation (Table 6.2 ) was 153.5 nm with PDI 0.201 (Table 6.3.).

Particle size distribution curve for formulation L4 (3:1)

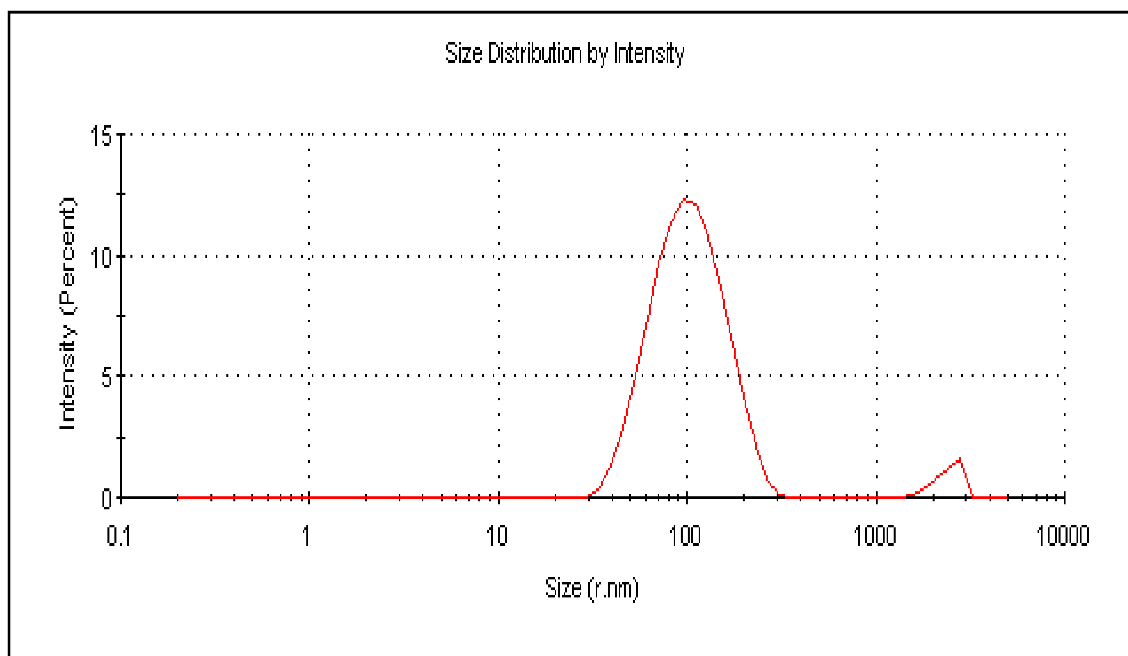



Figure 6.17: shows the particle size distribution pattern of lyophilized formulation F4 (3:1).

The average particle size (z-average) of the formulation (Table 6.2.) was 96.47 nm with PDI 0.276 (Table 6.3.).

## Z- AVERAGE VALUE OF THE EXPERIMENTAL FORMULATIONS

**Table 6.2:** shows the Z- average value of different lyophilized formulations.

| Formulation Code | Z-Average |
|---|---|
| L1 | 105.5 |
| L2 | 124.0 |
| L3 | 153.5 |
| L4 | 96.46 |

**POLYDISPERSITY INDEX OF THE EXPERIMENTAL FORMULATIONS**

**Table 6.3:** shows the polydispersity index of different formulations

| Formulation Code | Polydispersity Index |
|---|---|
| L1 | 0.364 |
| L2 | 0.384 |
| L3 | 0.201 |
| L4 | 0.276 |

Data of Table 6.2. and Table 6.3. indicates that all the lyophilized liposomes were in nano size range within a narrow distribution range, where L3 had the narrowest range of distribution with a PDI value 0.201.

**6.1.6. ZETA POTENTIAL STUDY**

Table 6.4: Zeta potential

| Formulation Code | Zeta potential |
|---|---|
| L1 | -29.6 |
| L2 | -28.3 |
| L3 | -34.8 |
| L4 | -31.5 |

### 6.1.7. DRUG LOADING STUDY OF DIFFERENT LYOPHILISED FORMULATIONS

Table 6.5: Constituents of liposomal formulations along with drug loading.

| Formulation Code | Composition PC:CH | Drug (mg) | Percentage Loading Efficiency |
|---|---|---|---|
| F1 | 1:1 | 10 | 8.122 |
| F2 | 2:1 | 10 | 9.57 |
| F3 | 2.5:1 | 10 | 9.69 |
| F4 | 3:1 | 10 | 9.43 |

## 6.2. DISCUSSIONS

### 6.2.1. DIFFERENT FACTORS CONSIDERED DURING THE LIPOSOME FORMULATION

Lecithin and cholesterol the components used for the preparation of liposome are liable to oxidation therefore to prevent so and enhance the stability of the formulation a suitable antioxidant, butylated hydroxyl anisole was used. Also regarding the stability of the liposomal membrane the concentration of cholesterol is an important factor. The size of the liposome is controlled by the process of sonication and its time. After bath sonication, the formulations were kept for minimum one hour, because after sonication, bilayers were fractured and required to regain into small vesicles, which needed some time. After that, they were kept at - 20˚C overnight and lyophilized for complete drying and kept inside the freeze. The entrapment of drug molecules within lipid vesicles depends upon physicochemical characteristics of drug, concentration of drug, ratio of drug to lipid, and temperature at which formulations were prepared.

### 6.2.2. SCANNING OF DRUG, URSODEOXYCHOLIC ACID

The purity of the drug ursodeoxycholic acid was another important factor taken into consideration for the development of the liposome formulation. The drug was at first assessed using the high performance liquid chromatographic method and then scanned which shows the $\lambda$ max of ursodeoxycholic acid, 210 nm, in phosphate buffer saline (PBS) and methanol mixture (4:6). The $\lambda$ max value of ursodeoxycholic acid in PBS:methanol was used for drug loading study.

### 6.2.3. DRUG – EXCIPIENTS INTERACTION STUDY

FTIR stands for Fourier Transform Infrared spectroscopy. The drug-excipient interaction is an important preformulation study which is also a critical factor considered during the liposome formulation. The stability of the drug in a formulation, the drug release pattern from it, and other physicochemical properties, such as surface charge, shape, size, etc. related to the formulation depends on drug-excipients interaction. Drugexcipient interaction may be studied by FTIR spectroscopy, IR spectroscopy and differential scanning calorimetry. In the present study we evaluated the drug-excipient interaction by FTIR spectroscopy. FTIR spectra assess the drug-excipient interaction at the level of functional groups by determining their vibrational patterns. Here the spectra of drug, the individual excipients (cholesterol and soya-l-$\alpha$-lecithin), mixture of cholesterol and soya-l-$\alpha$- lecithin, mixture of cholesterol and soya-l-$\alpha$-lecithin with drug and liposome with or without drug have been depicted in the results section. The results indicate that there was no chemical interaction between the drug and the excipients in the physical mixture and in the formulations. However, physical interactions such as weak hydrogen bond formation, dipoledipole interaction, Van der Waal ’s force of attraction were observed as some minor shifting of peaks were noticed. These physical interactions might help formation of the formulations.

### 6.2.4. LIPOSOME SHAPE AND MORPHOLOGY STUDY

Size, shape and external morphology of the liposome formulations were assessed by field-emission scanning electron microscopy (FESEM). From the result it was found that lyophilized liposomes had smooth surface and were in nano size range. The surface of the lipid vesicles had no leakage and the liposomes were uniformly distributed.

### 6.2.5. VESICLE SIZE AND SIZE DISTRIBUTION

Particle size and size distribution of the liposome play a crucial role in terms of its absorption through different biological barriers. Particle size and size distribution study of the liposome formulation was carried out by dynamic light spectroscopy. The principle of the particle size determination by DLS is the measurement of the rate of fluctuation of the intensity of scattered light due to Brownian motion of particles. Determination of these intensity fluctuations yields the value of Brownian motion of particles due to thermally induced collisions between the particles which are converted into particle-size by using DTS software. From the result it was observed that the particle size of the different formulations was within nano range and there was no predominant variation in the particle size distribution. Least variation in size distribution was observed for L3 (PI- 0.201) among the nanosize liposomes. This suggests that L3 had the narrowest range of distribution among the experimental formulations.

### 6.2.6. ZETA POTENTIAL STUDY

Zeta potential is a measure of the magnitude of the electrostatic charge repulsion or attraction between particles in a liquid suspension. This parameter ensures the dispersion stability of the nanoliposome formulation. Depending on the composition zeta potential of liposome can be positive, neutral or negative. It affects the physical stability (aggregation) and *in-vivo* behaviour of formulation. Generally zeta potential of liposomes is negative due to the presence of terminal carboxylic group in lipids. In the present study it was observed that the different liposome formulations had a high negative charge on their surface indicating the very high stability of the experimental liposome formulations when reconstituted. It was observed that due to increasing concentration of soya-l-$\alpha$-lecithin, zeta potential decreased as soya-l-$\alpha$-lecithin provided more positive zeta potential due to its amino group. The least negative value was found with cholesterol and soya-l-$\alpha$-lecithin (1:1) among the experimental formulations.

### 6.2.7. DRUG LOADING STUDY

The encapsulation efficiency of liposomes greatly depends on liposomal content, lipid concentration, method of preparation, and the drug used. By keeping the amount of cholesterol constant, four different liposome formulations, L1, L2, L3 and L4, were prepared with the varying concentrations of soya-l-$\alpha$-lecithin. From the result it was observed that

there was no major impact on drug loading due to the increase in the amount of soya-l-$\alpha$-lecithin.

# CHAPTER 7

# HEPATOPROTECTIVE ACTIVITY OF URSODEOXYCHOLIC ACID & ITS LIPOSOMAL PREPARATIONS

# CHAPTER 7

# PHARMACOLOGICAL ACTIVITY OF DRUG

**7.1. LIVER STRUCTURE AND FUNCTION[1,2] :** The liver is the largest organ of the body, weighing 1 to 1.5 kg and representing 1.5 to 2.5% of the lean body mass. The size and shape of the liver vary and generally match the general body shape—long and lean or squat and square. The liver is located in the right upper quadrant of the abdomen under the right lower rib cage against the diaphragm and projects for a variable extent into the left upper quadrant. The liver is held in place by ligamentous attachments to the diaphragm, peritoneum, great vessels, and upper gastrointestinal organs. It receives a dual blood supply; approximately 20% of the blood flow is oxygen-rich blood from the hepatic artery, and 80% is nutrient-rich blood from the portal vein arising from the stomach, intestines, pancreas, and spleen. The majority of cells in the liver are hepatocytes, which constitute two-thirds of the mass of the liver. The remaining cell types are Kupffer cells (members of the reticuloendothelial system), stellate (Ito or fat-storing) cells, endothelial cells and blood vessels, bile ductular cells, and supporting structures. Viewed by light microscopy, the liver appears to be organized in lobules, with portal areas at the periphery and central veins in the center of each lobule. However, from a functional point of view, the liver is organized into acini, with both hepatic arterial and portal venous blood entering the acinus from the portal areas (zone 1) and then flowing through the sinusoids to the terminal hepatic veins (zone 3); the intervening hepatocytes constituting zone 2. Hepatocytes perform numerous and vital roles in maintaining homeostasis and health. These functions include the synthesis of most essential serum proteins (albumin, carrier proteins, coagulation factors, many hormonal and growth factors), the production of bile and its carriers (bile acids, cholesterol, lecithin, phospholipids), the regulation of nutrients (glucose, glycogen, lipids, cholesterol, amino acids), and metabolism and conjugation of lipophilic compounds (bilirubin, anions, cations, drugs) for excretion in the bile or urine. Measurement of these activities to assess liver function is complicated by the multiplicity and variability of these functions. The most commonly used liver "function" tests are measurements of serum bilirubin, albumin, and prothrombin time. The serum bilirubin level is a measure of hepatic conjugation and excretion, and the serum albumin level and prothrombin time are measures of protein synthesis. Abnormalities of bilirubin, albumin, and prothrombin time are typical of hepatic dysfunction.

**7.2. LIVER DISEASES** While there are many causes of liver disease (Table 7.1), they generally present clinically in a few distinct patterns, usually classified as hepatocellular, cholestatic (obstructive), or mixed. In *hepatocellular diseases* (such as viral hepatitis or alcoholic liver disease), features of liver injury, inflammation, and necrosis predominate. In *cholestatic diseases* (such as gall stone or malignant obstruction, primary biliary cirrhosis, some drug-induced liver diseases), features of inhibition of bile flow predominate. In a mixed pattern, features of both hepatocellular and cholestatic injury are present (such as in cholestatic forms of viral hepatitis and many drug-induced liver diseases). Typical presenting symptoms of liver disease include jaundice, fatigue, itching, right upper quadrant pain, abdominal distention, and intestinal bleeding.

- Inherited hyperbilirubinemia
  - Gilbert's syndrome
  - Crigler-Najjar syndrome, types I and II
  - Dubin-Johnson syndrome
  - Rotor syndrome
- Viral hepatitis
  - Hepatitis A
  - Hepatitis B
  - Hepatitis C
  - Hepatitis D
  - Hepatitis E
  - Others (mononucleosis, herpes, adenovirus hepatitis)
  - Cryptogenic hepatitis
- Immune and autoimmune liver diseases
  - Primary biliary cirrhosis
  - Autoimmune hepatitis
  - Sclerosing cholangitis
  - Overlap syndromes
  - Graft-vs-host disease
  - Allograft rejection
- Genetic liver diseases
  - $\alpha_1$ Antitrypsin deficiency
  - Hemochromatosis
  - Wilson's disease
  - Benign recurrent intrahepatic cholestasis (BRIC)
  - Familial intrahepatic cholestasis (FIC), types I–III
  - Others (galactosemia, tyrosinemia, cystic fibrosis, Newman-Pick disease, Gaucher's disease)
- Alcoholic liver disease
  - Acute fatty liver
  - Acute alcoholic hepatitis
  - Laennec's cirrhosis
- Nonalcoholic fatty liver
  - Steatosis
  - Steatohepatitis
- Acute fatty liver of pregnancy
- Liver involvement in systemic diseases
  - Sarcoidosis
  - Amyloidosis
  - Glycogen storage diseases
  - Celiac disease
  - Tuberculosis
  - *Myobacterium avium intracellulare*
- Cholestatic syndromes
  - Benign postoperative cholestasis
  - Jaundice of sepsis
  - Total parenteral nutrition (TPN)– induced jaundice
  - Cholestasis of pregnancy
  - Cholangitis and cholecystitis
  - Extrahepatic biliary obstruction (stone, stricture, cancer)
  - Biliary atresia
  - Caroli's disease
  - Cryptosporidiosis
- Drug-induced liver disease
  - Hepatocellular patterns (isoniazid, acetaminophen)
  - Cholestatic patterns (methyltestosterone)
  - Mixed patterns (sulfonamides, phenytoin)
  - Micro- and macrovesicular steatosis (methotrexate, fialuridine)
- Vascular injury
  - Venoocclusive disease
  - Budd-Chiari syndrome
  - Ischemic hepatitis
  - Passive congestion
  - Portal vein thrombosis
  - Nodular regenerative hyperplasia
- Mass lesions
  - Hepatocellular carcinoma
  - Cholangiocarcinoma
  - Adenoma
  - Focal nodular hyperplasia
  - Metastatic tumors
  - Abscess
  - Cysts

Table 7.1: Liver Diseases

Cirrhosis is a pathologically defined entity that is associated with a spectrum of characteristic clinical manifestations. The cardinal pathologic features reflect irreversible chronic injury of the hepatic parenchyma and include extensive fibrosis in association with the formation of regenerative nodules. These features result from hepatocyte necrosis, collapse of the supporting reticulin network with subsequent connective tissue deposition, distortion of the vascular bed, and nodular regeneration of remaining liver parenchyma. The central event leading to hepatic fibrosis is activation of the hepatic stellate cell. Upon activation by factors released by hepatocytes and Kupffer cells, the stellate cell assumes a myofibroblast-like conformation and, under the influence of cytokines such as transforming growth factor α (TGF-α), produces fibril-forming type I collagen. Most types of cirrhosis may be usefully classified by a mixture of etiologically and morphologically defined entities as follows: (1) alcoholic; (2) cryptogenic and posthepatitic; (3) biliary; (4) cardiac; and (5) metabolic, inherited, and drug-related.

## 7.3. Materials and methods:

### 7.3.1. Animals

Male Wistar albino rats (100–120 mg), maintained under standard animal housing conditions (12 h light and dark cycle), and were used for all sets of experiments performed on six rats each. The rats were allowed standard laboratory feed and water *ad libitum*.

### 7.3.2. Chemicals

| Name | Source |
|---|---|
| Paracetamol | GlaxoSmithKline, B.No.ET139, Mfd.Nov.15, Exp.Oct.18 |
| Silymarin | Serum Institute of India Ltd. B.No. LR14.6, Mfd.Feb.2015, Exp.Jan.2017 |
| SGPT, SGOT, ALP, TB test kits | ARKRAY HEALTHCARE PVT. LTD. |

### 7.3.3. Acute toxicity

The ursodeoxycholic acid liposomal preparations were administered to the three different rats in a graded doses ranging from 10mg/kg body wt. up to 15mg/kg body wt. and the rats were observed for signs of toxicity and mortality for 48 h afterward.

### 7.3.4. Paracetamol-induced liver damage[3]

The rats were divided into 6 groups each containing 6 animals.

Group I: was administered normal saline (0.9% v/v, 5ml/kg body weight, orally).

Group II: was administered paracetamol (2gm/kg body weight, orally).

Group III: was administered silymarin (100mg/kg body weight, orally).

Group IV: was administered ursodeoxycholic acid (UDCA) ( 13mg/kg body weight, orally).

Group V: was administered UDCA liposomal preparation (10mg/kg body weight, orally).

Group VI: was administered UDCA liposomal preparation (15 mg/kg body weight, orally).

In case of paracetamol-induced hepatotoxicity, the ursodeoxycholic acid liposome (at doses of 10 and 15 mg/kg body wt.) and silymarin (100 mg/kg) were given orally to respective groups once daily for 5 days. On the fifth day, paracetamol at a dose of 2 g/kg body wt. was administered to all groups except for control, 30 min after the respective treatment. One group received only paracetamol to assist in assessing the severity of toxicity paracetamol administration, blood was collected from all groups, including control, and serum was separated and analyzed for various biochemical parameters.

### 7.3.5. Assessment of liver functions

Biochemical parameters, such as serum glutamic oxaloacetate transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), alkaline phosphate (ALP) and total bilirubin (TB) were analyzed according to the standard method using the respective test kits (ARKRAY HEALTHCARE PVT. LTD.).

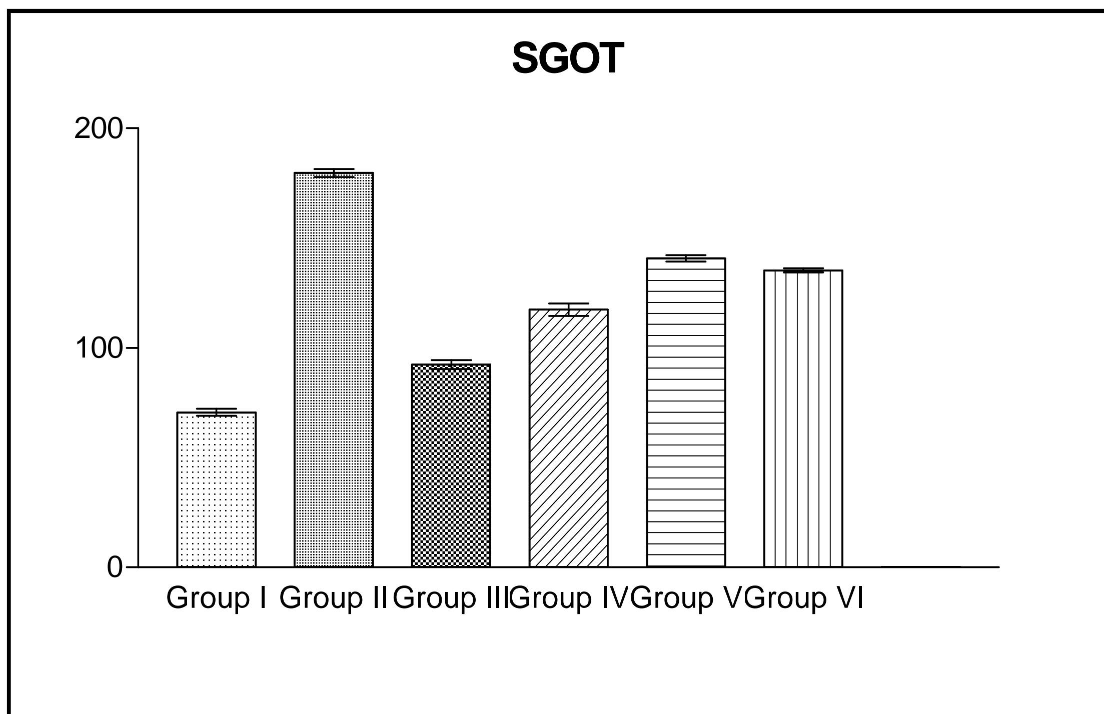
### 7.4. Results

The drug ursodeoxycholic acid was found to be practically nontoxic when administered orally to rats and its LD50 value was found to be higher than 1 g/kg body wt. Administration of paracetamol to rats caused significant liver damage, as evidenced by the altered serum biochemical parameters. Pretreatment of rats with ursodeoxycholic acid and its liposomal preparation exhibited marked protection against paracetamol induced hepatotoxicity, which is shown in Tables. The drug ursodeoxycholic acid and its liposomal preparation showed significant hepatoprotective activity against paracetamol, comparable with the standard silymarin.

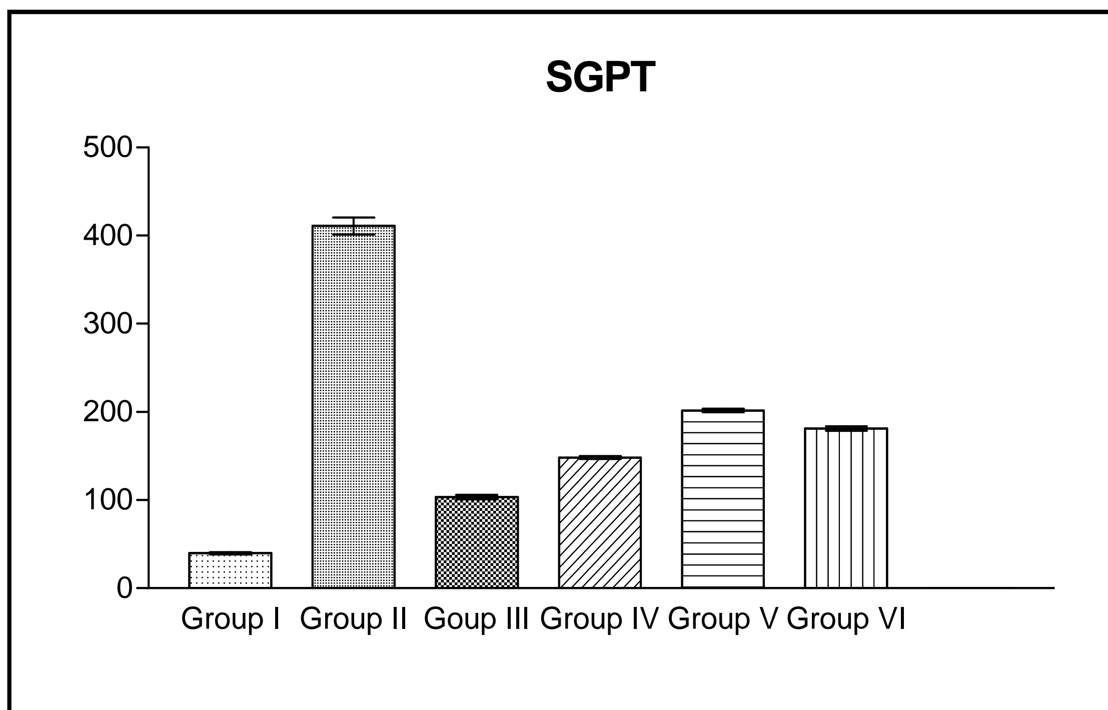
Table No: Effect of UDCA formulation in Paracetamol induced hepatic injury in rats

| Design of treatment | SGOT (IU/L) | SGPT (IU/L) | ALKP (IU/L) | Total. Bilirubin (mg/100ml) |
|---|---|---|---|---|
| Group I | 70.64±1.568** | 39.80±1.446** | 4.504±0.9009** | 0.3537±0.01138** |
| Group II | 179.7±1.758** | 411.2±9.572** | 54.92±1.902** | 2.695±0.1051** |
| Group III | 92.41±2.034** | 103.6±2.458** | 13.56±1.299** | 0.6624±0.01813** |
| Group IV | 117.4±2.772** | 148.3±1.367** | 26.13±0.4733** | 0.8584±0.01240** |
| Group V | 140.7±1.458** | 201.6±1.795** | 41.77±0.5905** | 1.335±0.06518** |
| Group VI | 135.3±0.9227** | 181.1±2.383** | 35.50±0.4361** | 1.008±0.009165** |

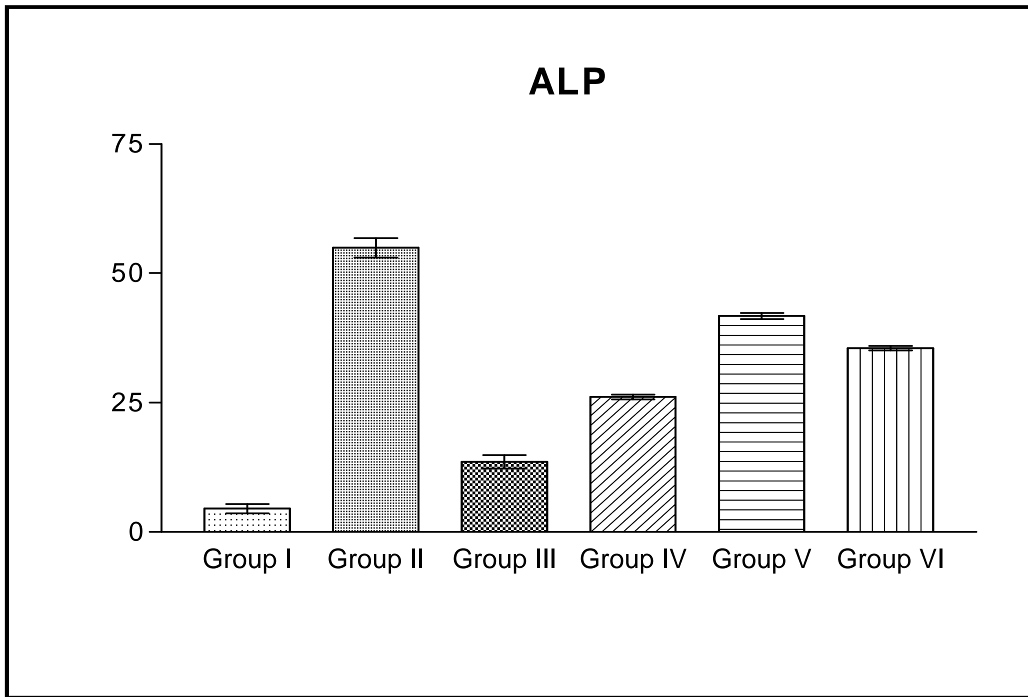
Values are mean±S.E; n=6; **p<0.01 compared to (paracetamol) Group II; *p<0.05compared to (paracetamol) Group II.



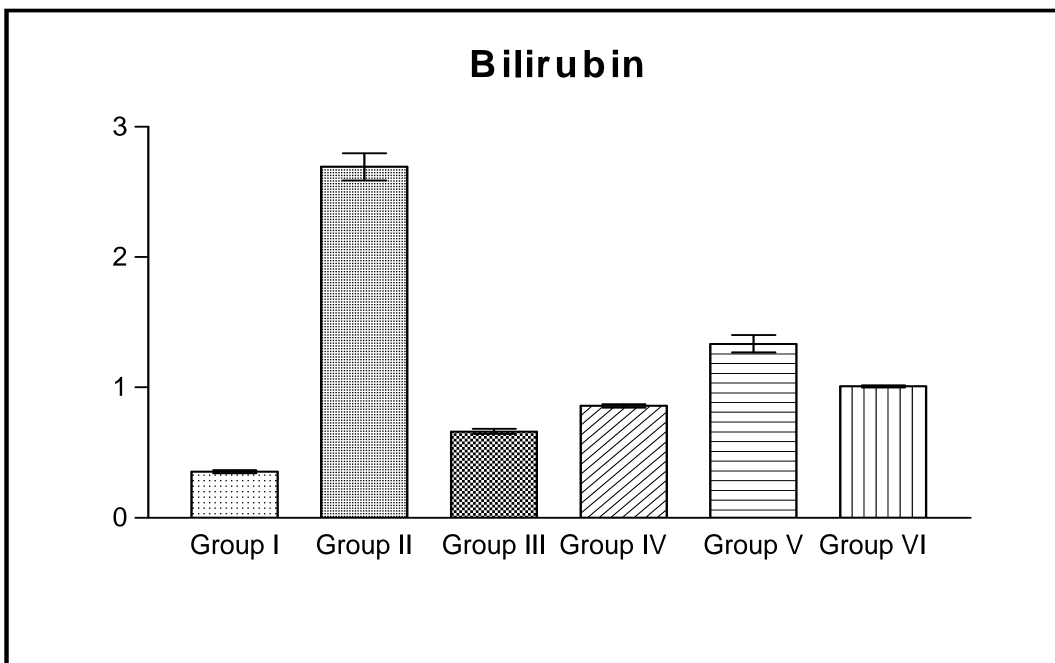

Graph 7.1: Effect of different drug on the the SGOT activity.




Graph 7.2: Effect of different drug on the the SGPT activity.




Graph 7.3: Effect of different drug on the the ALP activity.




Graph 7.4: Effect of different drug on the the total bilirubin concentration.

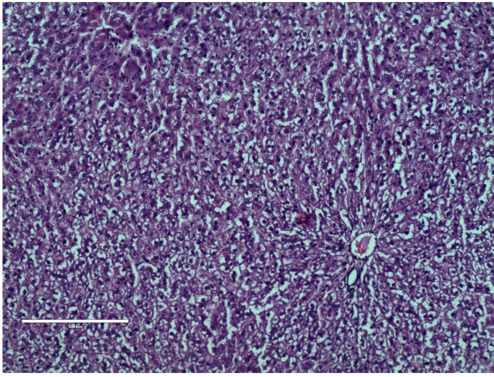


Fig.:7.1. Histopathology of Group I

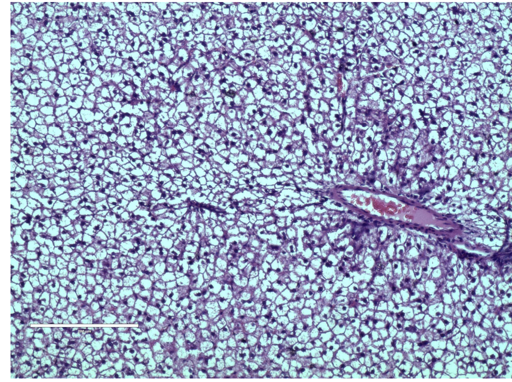


Fig.:7.2. Histopathology of Group III

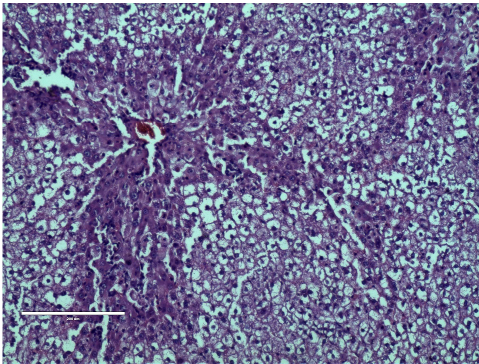


Fig.:7.3. Histopathology of Group IV

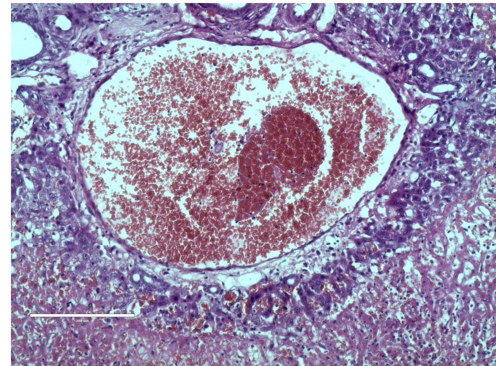


Fig.:7.4. Histopathology of Group II

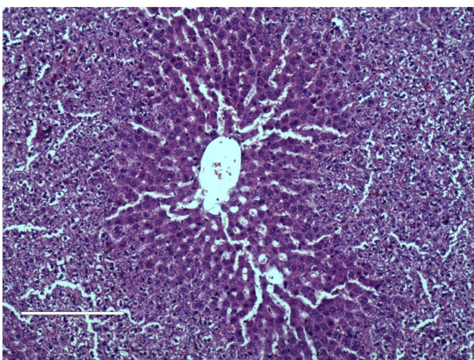


Fig.:7.5. Histopathology of Group V

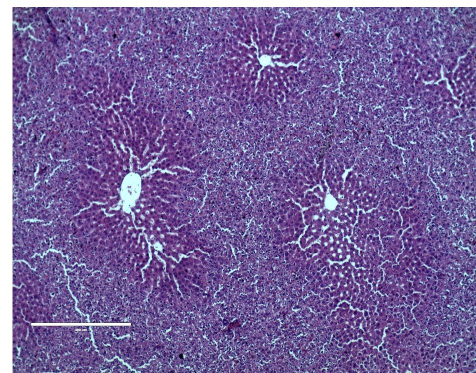


Fig.:7.. Histopathology of Group VI

### 7.5. Discussion

Silymarin was used as standard drug for the comparion of hepatoprotective activity of the crude drug ursodeoxycholic acid and its liposomal formulations entrapping different concentration of the drug. Upon treatment it was observed that the drug produced sufficient hepatic protection to the rat who were induced with liver cirhossis with paracetamol. The lowering of the elevated levels of SGPT, SGOT, TB and ALP was seen to be better in case of the drug encapsulated in the liposomal formulation. As seen in the Table 7.3. It is also observed that the activity is dose dependent as with the increase in the concentration of drug from 10mg/kg body wt. to 15mg/kg body wt. the elevated biochemical parameters were greatly reduced.

### 7.6. References

1. Brunton.L., Goodman & Gilman's The Pharmacological Basis of Therapeutics 12th Edition ,
2. Harrison's Principles of Internal Medicines 16th Edition, 1808-1880.
3. Ansari.S.H., et al.., Hepatoprotective activity of Abutilon indicum on experimental liver damage in rats., *Phytomedicine*, 12 (2005) 62–64

# CHAPTER 8

# CONCLUSION

# CHAPTER 8

# CONCLUSION

Liposome are one of the emerging drug carrying particles. They gain the advantage of being preferred for inclusion in the newer technologies of drug design as the components of this system are utilized in the body as its own components of the biological membrane. Hence, rendering it to be more compatible, non-toxic, non-allergic and biodegradable. Also, liposomes being taken up by the reticuloendothelial system of the body, directs the drug to the liver therefore providing the feature of drug targeting to the liver. Ursodeoxycholic acid (UDCA), a drug most commonly used for the various liver disorders is practically insoluble in water and also gets quickly metabolised in the body when present in free form. Liposomal formulation of UDCA thus, helps in overcoming both these two properties of the drug. By encapsulating the UDCA into the lipid membrane of the liposome its solubility can be increased and the drug can be administered parenterally with maximum efficiency without getting quickly metabolised . Being entrapped into the liposomal membrane the drug will also be released in a rate control manner proving longer duration of action. Liposomes with homogenous size in nano range were obtained. The effect of UDCA loaded liposome on the elevated levels of SGPT, SGOT, ALP and total bilirubin in paracetamol induced liver damage in rats showed that the drug was also released in the body thereby decreasing the elevation in activities of the biochemical parameters. Hence, liver the major organ of the body can be provided with better delivery system by the formulation of liposome and further encapsulating it with UDCA also provide protection against the liver damage.