DEVELOPMENT OF RIBAVIRIN NANOLIPOSOME FOR PERIPHERAL NERVE SPECIFIC DELIVERY

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Certificate of Approval

*This is to certify that the project entitled "**Development of ribavirin nanoliposome for peripheral nerve specific delivery**" was carried out by **Ms. Rhitabrita Chakraborty** based upon her work under my direct supervision at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, 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 this thesis contains literature survey and original research work by the undersigned candidate, as part of her Master of Pharmaceutical Technology studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

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

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Date:

Place:

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RHITABRITA CHAKRABORTY

DEDICATED TO

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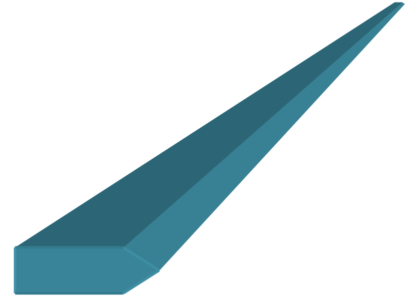


Chapter 1

Introduction

1. INTRODUCTION:

Targeted drug delivery is an effective method of delivering drugs to the patients comprises of the advantage of higher concentration of delivered drug to the targeted body part of interest which sequentially improves the therapeutic efficacy by reducing side effects of drug administration. Basically targeted therapy signifies the specific interaction between a drug and its receptor at the molecular level. Ehrlich's first introduced the drug targeting by finding targeted drugs dated back to the 1890s when he noticed that synthetic dyes could selectively stain specific pathogens and not their host cells. The intrinsic advantage of this technique leads to administration of required drug with its reduced dose and adverse effect. The strategies of drug targeting usually divided into “active targeting” and “passive targeting”. Active targeting demonstrates the interaction between drug and drug carriers to the target cells through ligand receptor interaction (You et al, 2011). Generally antibody, aptamers, peptides, sugars, vitamin, viral proteins, galactosamine are used as ligands for active targeting. Whereas passive targeting refers to the accumulation of drug at the targeted site. In case of tumors passive targeting is referred as “enhanced permeation and retention effect” (EPR) (Rani et al, 2014). Delivery of drugs to the nervous system is a challenge regardless advancement in the understanding of the mechanisms which are involved in the development of neurodegenerative disorders (Maysinger et al, 1997). In case of peripheral nerves, the scarring tissue formation, which represents a physical barrier to axon elongation, and not oriented outgrowth of neurites are the two main hindrances for the complete recovery of physiological nerve function (Caneva et al, 2004). Target specific delivery of drugs to specific cell types is critical in developing effective and safe treatments for neurodegenerative disorders. This challenge is particularly great during designing drugs for diseases of the peripheral nervous system (PNS), such as hereditary motor and sensory neuropathies (HMSNs). Because of the lengths of peripheral nerves, Effective drug delivery will likely require systemic drug administration. Different types of carriers are used in targeted drug delivery include liposomes, nanoparticles, nanogels, dendrimers, niosomes, resealed erythrocytes, immunoglobins etc. Ligand-mediated active targeting has emerged as a novel paradigm in targeting either vascular compartment (first-order), cellular (second-order), or intracellular (third-order) levels (Vyas et al, 2008).

1.1 DIFFERENT DRUG DELIVERY SYSTEM FOR TARGETING:

Nanoparticles: According to the definition from NNI (National Nanotechnology Initiative), nanoparticles are structures of sizes ranging from 1 to 100 nm in at least one dimension.

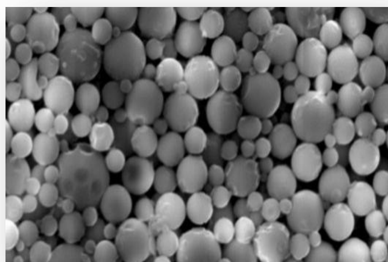


Figure 1.1: Scanning Electron Microscopic view of nanoparticles (www.in-pharmatechnologist.com)

Nanocarriers with optimized physicochemical and biological properties are taken up by the cells more easily than larger molecules, so they can be successfully used as delivery tools for currently available bioactive compounds. There are various nanoparticulate delivery system that successfully targets drugs '*in vivo*' to the sciatic nerve, opening novel avenues in the field of nanomedicine to the design of therapeutic strategies that enhance axonal regeneration (Suri et al, 2007). The controlled release of the dual synergistic growth factors by nanoparticles would improve the treatment of peripheral nerve injury to mimic the natural cellular microenvironments (Nadia et al).

Nanogels:

Nanogels are swollen nano-sized networks composed of hydrophilic or amphiphilic polymer chains, which can be non-ionic or ionic. They are developed as carriers for drug delivery and can be designed to spontaneously absorb biologically active molecules through formation of salt bonds, hydrogen bonds, or hydrophobic interactions.

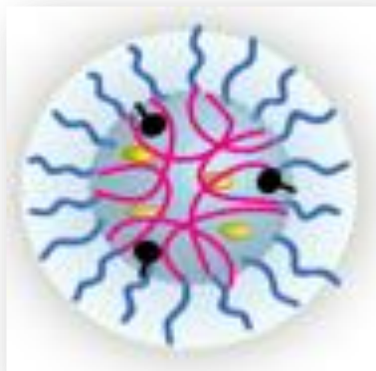


Figure 1.2: Schematic diagram of Nanogels (https://elements.chem.umass.edu)

Nanogel "surface" can be also decorated with biospecific targeting groups, which can enhance site-specific delivery of the nanogels in the body. These modifications generally concern the possibility of anchoring PEG moieties to the surface of nanogels by means of a chemical conjugation with polymeric materials (Alexander et al, 2009).

Dendrimer: Dendrimers are the emerging polymeric architectures that are known for their defined structures, versatility in drug delivery, high functionality and biomolecules resemblance. These nanostructured macromolecules have shown their potential abilities in entrapping or conjugating the high molecular weight hydrophilic/hydrophobic entities by host-guest interactions and covalent bonding.

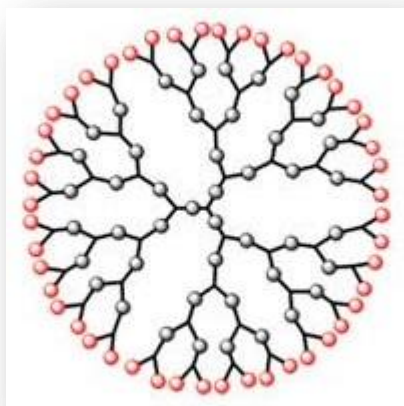


Figure 1.3: Schematic diagram of Dendrimer (http://dx.doi.org)

Moreover, high ratio of surface groups to molecular volume has made them a promising synthetic vector for gene delivery. Researchers have shown many therapeutic and biomedical applications of dendrimers (Tomalia et al, 1985).

Niosomes: Niosomes are formations of vesicles by hydrating mixture of cholesterol and nonionic surfactants.

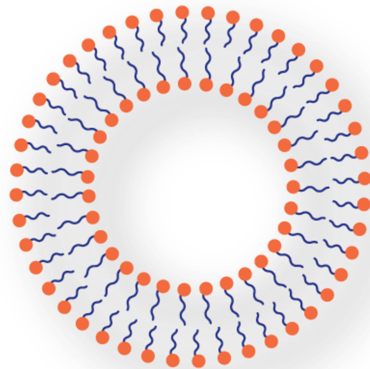


Figure 1.4: Schematic diagram of Niosome (www.niolip.com)

Niosomes are biodegradable, relatively nontoxic, more stable and inexpensive, an alternative to liposomes, the current deepening and widening of interest of niosomes in many scientific disciplines and, particularly are applied in medicine. (Malhotra et al, 1994)

Liposome: Liposomes are artificially constructed closed vesicles consist of one or more concentric bilayer membrane of discrete phospholipid molecules enclosing aqueous space, generally used to deliver both lipophilic and water-soluble microscopic substance to the body cells. They were first described by Alec Bangham in 1961 (published in the year 1965). The word liposome derives from two Greek words, 'lipos' means fat and 'soma' means body or structure that means a structure in which a fatty envelope encapsulates internal aqueous compartment(s). A liposome encapsulates a region of aqueous solution inside a hydrophobic membrane. Dissolved hydrophilic solutes cannot readily pass through the lipids. Hydrophobic chemicals can be dissolved into the membrane. By this way liposome can carry both hydrophobic molecules and hydrophilic molecules.

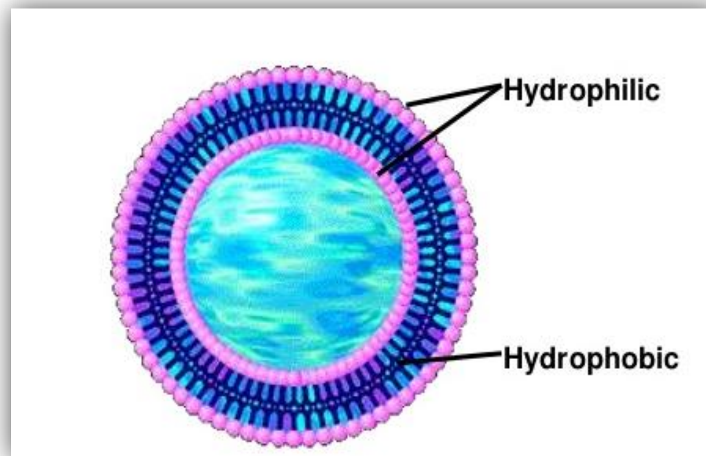
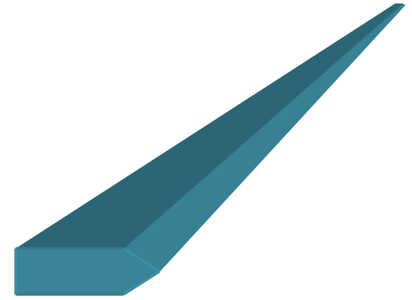


Figure 1.5: Schematic diagram Cross sectional view of liposome structure formed by phospholipids (Mukherjee et al., 2009)

Since the time of Bangham, several techniques were developed for preparation of different types of liposome and were utilized in pharmaceutical industry as novel drug delivery system. Several techniques were also developed for incorporation of single or multiple drug(s) into liposome, enhancement of drug loading, modification of liposome surface for attachment of site specific ligand, to produce steric hindrance, to protect them from degrading enzymes or to enhance hydrophilicity for prolongation of circulation in the system. The immediate uptake and clearance by reticulo endothelium system (RES) are the major problems of using liposome *in vivo* and low stability for *in vitro.* To overcome this problem poly ethylene glycol (PEG) may be added to the surface which can increase the circulation time from 200 to 1000 minutes. (Scot et al, 2008)

Liposomes are attractive options for delivery to the PNS as they can be optimized easily for specific cell types, have shown low toxicity *in vivo*, and can carry both hydrophobic and hydrophilic molecules. (Ashizawa et al, 2013)



Chapter 2

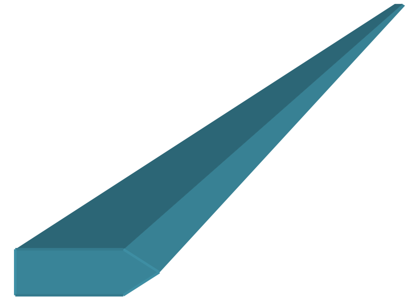
Aim of the Research Work

2. Aim of the Research Work:

Ribavirin is a broad spectrum antiviral drug effective against both DNA and RNA viruses without the undue toxicity (Crotty et al, 2002; Sidwell et al, 1972*)*. It is used specially for the treatment of Respiratory Syncytial Virus (RSV), Viral Haemorrhagic Fevers (VHFs), Hepatitis C Virus (HCV). It is also found efficacious against rabies virus *in vitro*. Viral infections specially rabies usually begins in the peripheral tissue and can then invade the central nervous system, thus spreading initially into PNS and more rarely the CNS. (Koyuncu et al, 2013; Rupprecht et al, 1996). Ribavirin by its own characteristics can penetrate the central nervous system (CNS) but is unable to enter into the peripheral nervous system (PNS). (Rhoades et al, 2011). Ribavirin encompasses an *in vitro* antiviral effect in rabies virus infected neuronal cells but stops working to offer benefit in rabies *in vivo* since it cannot invade PNS. (Appolinário et al, 2013)

The main drawback of ribavirin in the treatment of viral meningitis and rabies infection is that it does not reach inside the nerve cells due to its hydrophilic nature. If Ribavirin is supplied as liposomal drug delivery system and the size of the liposome is within the nanosize range, the targeting of drug to PNS can be achieved. More additionally, if Ribavirin is given by the liposomal drug delivery system- sustained release action can be achieved, systemic toxicity, side effect as well as dose can be reduced, and bioavailability of the drug can be increased. More specifically, if we attach this formulation with a ligand which can target the peripheral nerves, it can easily and selectively permeate through the peripheral nerves for better therapeutic efficacy.

Hence, the study was intended to develop Ribavirin loaded nanoliposome for peripheral nerve specific delivery and to characterize them *in vitro*.



Chapter 3

Liposome and drug delivery

3.1 Definition of Liposome:

Liposomes are artificially constructed closed vesicles consist of one or more concentric bilayer membrane of discrete phospholipid molecules enclosing an equal number of aqueous compartments, which generally used to deliver both lipophilic and water-soluble microscopic substance to the body cells. The bilayer is composed of two lamellae in which polar head groups of phospholipids (or sphingolipids) are arranged towards the aqueous phase and the lipophilic acyl chains are arranged towards themselves and attached side by side to form a tight packing (Laouini et al, 2012; Mansoori et al, 2012).

3.2 Mechanism of Liposome Formation:

Liposome forming materials like phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) etc. have a hydrophilic polar head group and a nonpolar tail group consisting of two fatty acid chains. These two fatty acid chains give the molecule an overall cylindrical shape. These phospholipid molecules when exposed to aqueous media, their polar head groups arrange themselves towards aqueous media and the hydrophobic tail portions attach to each other side by side to reduce oil-water interaction. Due to their tubular shape this type of arrangement results in formation of planar lamellae. When two such lamellae join to each other, facing both hydrophilic sides towards water phase, a bilayer is formed. In such a bilayer oil-water interaction is minimized and present only in the terminal regions. This interaction abolishes when the terminal regions of a bilayer joins to form a spherical vesicle, enclosing an aqueous compartment. This vesicle (liposome) may be further enclosed by another bilayer to form bilamellar vesicle. Further enclosing of this liposome by a number of bilayer results in formation of plurilamellar or multilamellar vesicles. However a number of vesicles may be enclosed in a large vesicle to form multivesicular vesicle (Albert et al, 2002).

3.3 Advantages of Liposomal Drug Delivery:

1) **Biocompatible ingredients:** The formulation ingredients (excipients) of liposome preparation are mainly phospholipids and cholesterol, which are the components of biological system, so they are easily accepted by the body.

2) **Improved pharmacokinetic profile:** Liposomal drug delivery increases circulation lifetime of therapeutic agents and reduces their elimination especially for PEGylated liposomes.

3) **Stability:** As drug is encapsulated into the vesicles (for hydrophilic drug) or stored into the bilayer (for hydrophobic drug) they are protected against degradative action of many enzymes.

4) **Targeted delivery:** It provides passive targeting to the selected tissues.

5) **Active targeting:** It is possible to attach several targeting ligands such as antibody, aptamer with liposome to achieve active targeting.

6) **Wide range of drug entrapment:** Both hydrophilic and lipophilic drugs can be incorporated into the liposome which allows delivery of a wide range of drug.

7) **Low drug requirement:** Liposomal delivery requires relatively very low amount of drug which increases therapeutic index & reduces adverse effects.

8) **Cellular drug delivery:** Liposome is capable of entering into the cell through endocytosis that allows intracellular drug delivery. Intracellular drug delivery is also possible through fusion of liposome i.e., merging of vescicular membrane with cellular membrane.

9) **Site avoidance effect:** Site avoidance effect can be achieved easily by liposomes (Loveleenpreet et al, 2013; Dua et al, 2012).

3.4 Limitation of Liposomal Drug Delivery:

1) **Low drug entrapment**: In most of the cases low entrapment of drug into liposome increases material consumption and labour.

2) **Leakage and fusion**: Leakage and fusion of encapsulated material requires special attention during formulation and also modification in method & material.

3) **Toxic effect**: Some liposomal ingredients exert toxic effect.

4) **Oxidation and hydrolysis**: Phospholipids are susceptible to oxidative and hydrolytic degradation which seeks attention in special storage condition for liposome.

5) **Instability**: Instability of liposomal carriers in biological system is another problem.

6) **Cost**: Large scale production of liposomal formulation is relatively costly (Loveleenpreet et al, 2013; Nayar et al, 2015).

3.5 Definition of Nanoliposome:

Nanoliposome or submicron bilayer lipid vesicle refers to the phospholipid vesicles having nano size range (Khosravi et al, 2010).

3.6 Advantages of Nanoliposome over Liposome:

1) The thermodynamic stability of nanoliposome is higher compared to liposome due to its smaller size.
2) It allows easier penetration into the cells.
3) In case of nanoliposome relatively lower amount of drug is required per equal volume of liposome which reduces drug toxicity and also gives economic advantage.

3.7 Limitation of Nanoliposome over Liposome:

Preparation of nanoliposome is more difficult than that of liposome and requires special methodology & instrumentation in most of the cases. Maintenance of particle size homogeneity and lamellarity are two major challenges of nanoliposome preparation.

3.8 Classification of liposomes:

Liposomes classification based on vesicle size:

(i) **Small unilamellar vesicles (SUV)**: 20–100 nm;
(ii) **Large unilamellar vesicles (LUV)**: > 100 nm;
(iii) **Giant unilamellar vesicles (GUV)**: > 1000 nm;
(iv) **Oligolamellar vesicle (OLV)**: 100–500 nm and
(v) **Multilamellar vesicles (MLV)**: > 500 nm

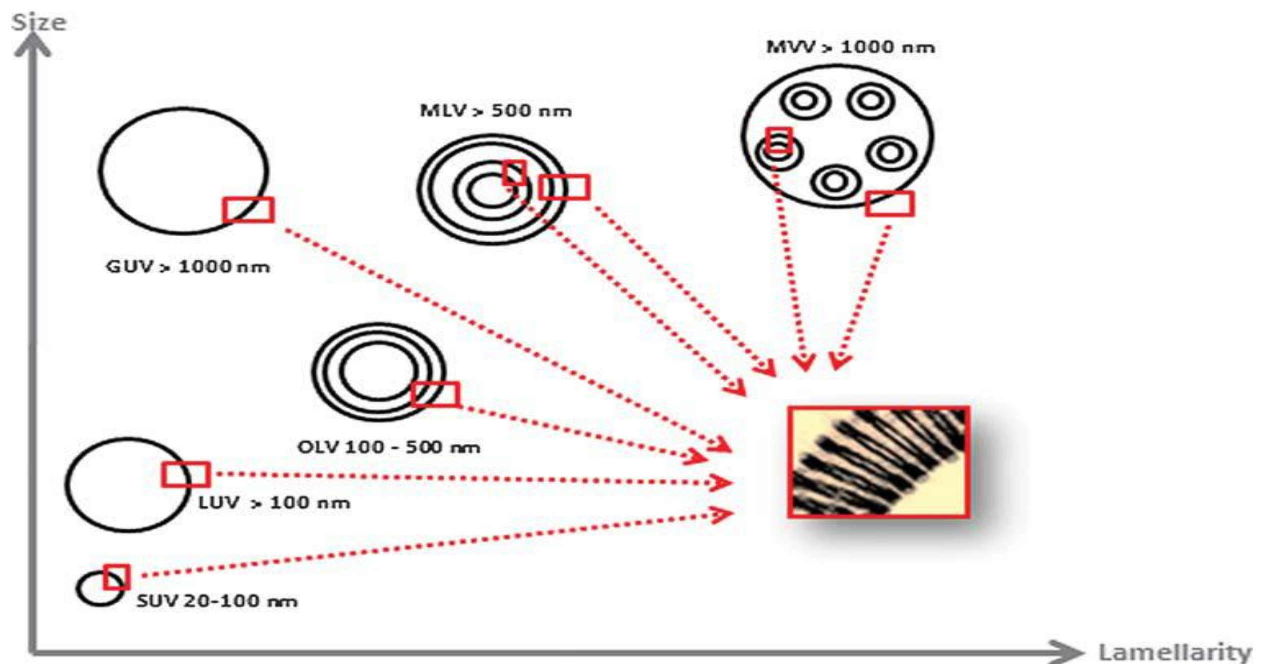


Fig 3.1: Liposomes classification based on size and lamellarity (Bordi et al, 2006; Laouini et al, 2012).

Liposome classification based on preparation methods:

(i) REV: Single or oligolamellar vesicle made by reverse phase evaporation method.

(ii) MLV / REV: Multilamellar vesicles made by reverse phase evaporation method.

(iii) SPLV: Stable plurilamellar vesicles.

(iv) FATMLV: Frozen and thawed multilamellar vesicles.

(v) VET: Vesicles prepared by extrusion method.

(vi) FUV: Vesicles prepared by fusion.

(vii) FPV: Vesicles prepared by french press method.

(viii) DRV: Dehydration- rehydration vesicles.

(ix) BSV: Bubblesomes.

(Kulkarni et al, 2012).

Liposome classification based on composition:

(i) Conventional liposome – Made of neutral or negatively charged phospholipid.

(ii) Fusogenic liposome - Reconstitute ultraviolet inactivated sendai virus envelop.

(iii) Cationic liposome - Cationic lipids make up the membrane of these liposomes.

(iv) Long circulatory liposome - The lipids used for this type of formulation are neutral lipids with a high transition temperature.

(v) pH sensitive liposomes - These liposomes fuse with cells when the pH is low, thus releasing its content into the cell cytoplasm.

(vii) Immuno liposome - Long circulatory liposome with attached monoclonal antibody.

(mansoori et al, 2012, Scholtz et al chapter).

3.9 Liposome Preparation Methods:

There are several methods for preparation of different types of liposomes. The choice of method depends on the following parameters:

1) The physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients.

2) The nature of the medium in which the lipid vesicles are dispersed.

3) The effective concentration of the entrapped substance and its potential toxicity.

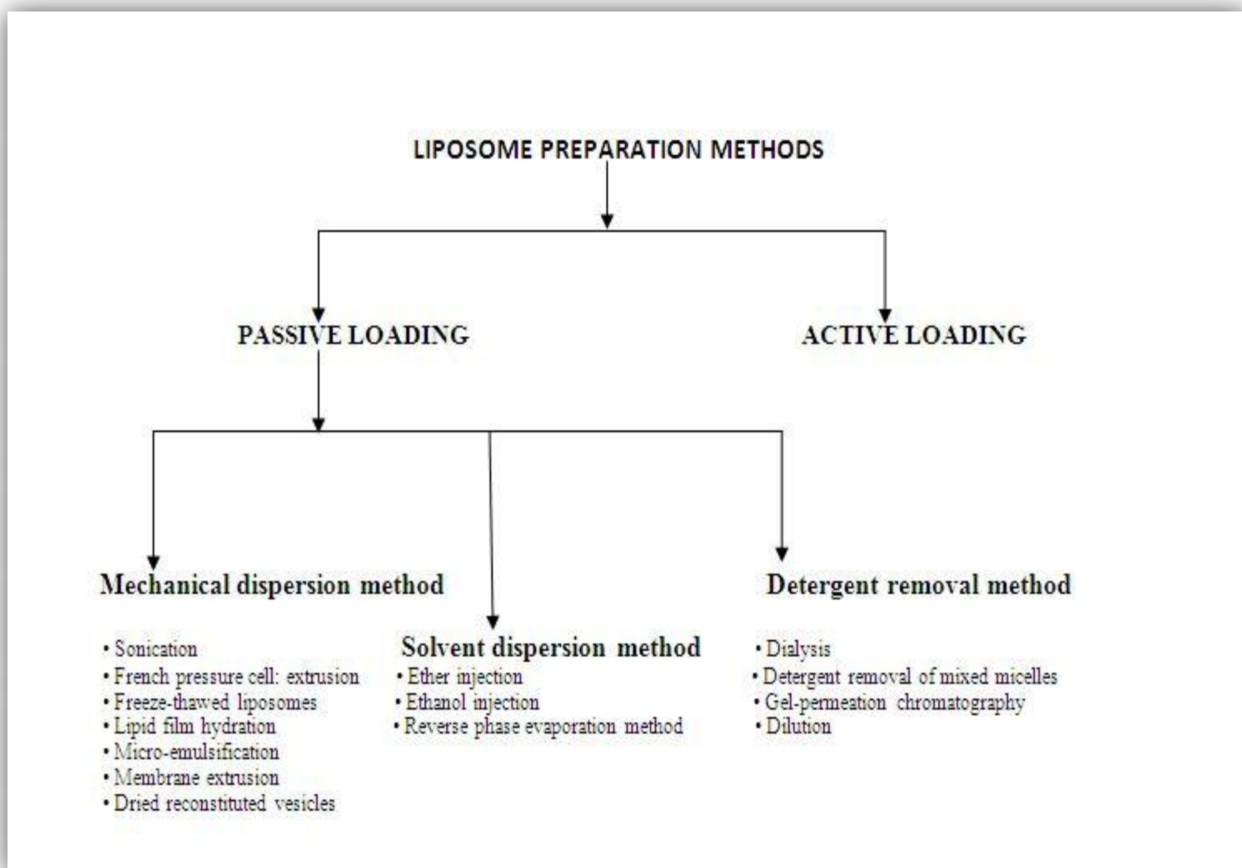
4) Additional processes involved during application/ delivery of the vesicles.

5) Optimum size, poly-dispersity and shelf-life of the vesicles for the intended application.

6) Batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products (Dua et al, 2012).

Depending upon the mode of dispersion liposome preparation methods are classified as

a) Mechanical dispersion method, b) Solvent dispersion method, and c) Detergent removal method. Here some methods under each category are described in short.



- **Mechanical dispersion methods:**
- **Sonication:** Sonication is mostly used technique for the preparation of SUV. MLVs are sonicated either with a bath type sonicator or a probe sonicator under a passive atmosphere. The main limitation with this method are very low internal volume or encapsulation efficacy, possible degradation of phospholipids and compounds to be encapsulated, elimination of large molecules, metal pollution from probe tip, and presence of MLV along with SUV(Riaz et al, 1996; Abolfazl et al, 2013). There are two types of sonication technique

a. Probe Sonication
b. Bath Sonication

- **French pressure cell extrusion:** This method involves the extrusion of MLV at 20,000 psi at 4°C through a small orifice. The method has several advantages over sonication method. The method is simple, rapid, and reproducible and involves gentle handling of unstable materials. The resulting liposomes are somewhat larger than sonicated SUVs (Dua et al, 2012).

- **Freeze-Thaw method:** In this method, SUVs are rapidly frozen and thawed slowly .The brief sonication disperses aggregated materials to LUV. The creation of unilamellar vesicles is as a result of the fusion of SUV throughout the processes of freezing and thawing. The encapsulation efficacies from 20% to 30% were obtained (Pick et al, 1981; Ohsawa et al, 1985; Liu et al, 1994).

- **Lipid film hydration:**

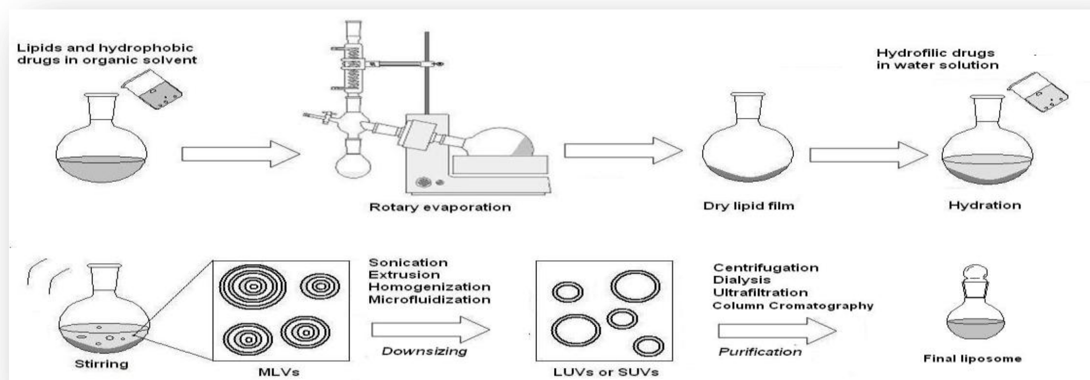


Fig no. 3.2 Schematic diagram of liposome preparation by lipid layer hydration method (http://www.intechopen.com)

In this method firstly lipids are dissolved in organic solvent to assure a homogeneous mixture of lipids and to obtain a clear lipid solution. Often it is carried out using chloroform or chloroform ethanol mixture. After the complete mixing, organic solvent is removed by evaporation under reduced pressure. After the evaporation, the dry lipid film is deposited in the flask wall. Then it is hydrated with aqueous buffer at standardized temperature above the transient temperature of lipid which results continuous

vesiculisation and entrapment of aqueous phase inside the vesicle (Laouini et al, 2012). After hydration procedure, sonication or extrusion is done to reduce the vesicle size. The drawback of this method is very low internal volume and encapsulation efficiency (Bangham et al, 1967; Sipai altaf et al, 2012).

- **Micro-emulsification**: In this method small vesicles are formed from concentrate lipid solution using micro fluidizer. The lipids are introduced into the fluidizer as a suspension of large MLV. Microfluidizer then pump the suspension at a very high speed through 5 micrometer screen. After that, it is forced to long micro channels where two streams collide with each other at a right angle. Then the fluid is collected and may be recycled to obtain the vesicle with optimum dimension (Kant sashi et al, 2012).

- **Membrane extrusion:** In this method vesicle size is reduced by passing through membrane filter of standard pore size. The tortuous path type membrane filter is used for sterile filtration. Liposomes that are bigger than channel diameter get struck in the membrane during passing. This method can be used for both LUV and MLV (Kant sashi et al, 2012).

- **Dried reconstituted vesicles:** Dried reconstituted vesicles (DRV) are liposomes that are formulated under mild conditions and have the capacity to entrap substantially high amounts of hydrophilic solutes (compared with other types of liposomes). This method is ideal for entrapment of peptide, protein or DNA vaccines and sensitive drugs (Deamer et al, 1978; Antimisiaris et al, 2010).

- ## Solvent dispersion methods:

- **Ether injection method:** Ether injection method involves dissolution of lipid in ether followed by injection of lipid solution in aqueous media. The method involves injection of ether-lipid solutions into warmed aqueous phases above the boiling point of the ether. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes (Deamer et al, 1978).

- **Ethanol injection method:** This method is very similar to ether injection method. In this method lipids are dissolved in ethanol solution and directly injected rapidly to aqueous medium or excess of saline buffer through a fine needle. Then ethanol is diluted in water and phospholipids dispersed evenly through the medium. High proportion of SUV having size about 25 nm is formed with this method (Kant sashi et al, 2012).

- **Reverse Phase evaporation method:**

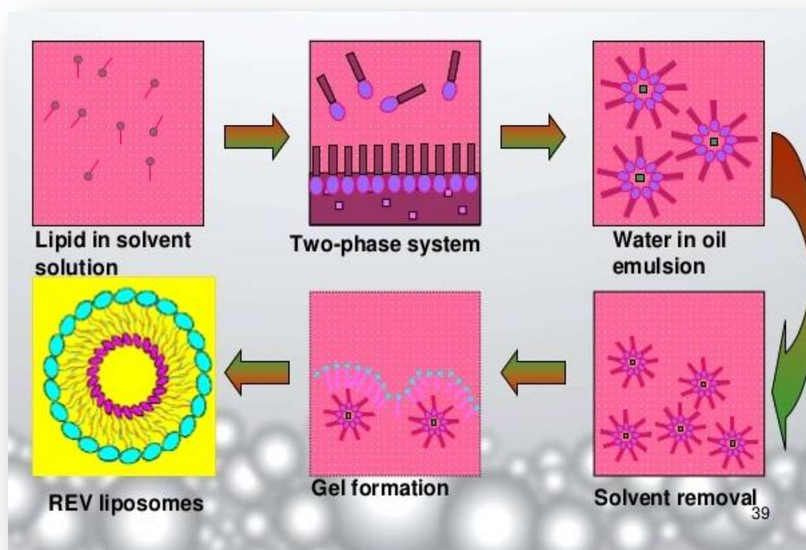


Fig no. 3.3 Schematic diagram of liposome preparation by reverse phase evaporation method (http://www.slideshare.net)

This is the most promising technique for the preparation of liposomes with hydrophilic drugs since; it allows high aqueous space-to-lipid ratio and a capability to entrap a large percentage of the aqueous material presented. This method is based on the creation of inverted micelles. These inverted micelles are shaped upon sonication of a mixture of a buffered aqueous phase, which contains the water-soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. When the organic solvents are slowly eliminated the inverted micelles converted into viscous state and gel form. Liposomes made by this method have aqueous volume to lipid ratio 4 times higher than hand shaken liposome (Himanshu et al, 2011; Kataria et al, 2011). Firstly water in oil emulsion is formed by brief

sonication of a two phase system containing phospholipid in organic solvent like chloroform, ether etc. or a mixture of solvent with aqueous buffer. If the drug is water soluble it should be added in aqueous buffer otherwise should be dissolved in organic solvent with lipid. After that the organic solvents are evaporated under reduced pressure and a viscous gel and subsequently an aqueous suspension is formed. Next, the excess buffer is added and the suspension is evaporated for additional 15 minutes to remove traces of solvent. Finally the preparation is centrifuged, dialyzed, or passed through Sepharose 4B column to remove non encapsulated material and residual organic solvent (Szoka et al, 1978).

3.10 Applications of liposomes:

Major applications of liposome is seen in the field of Pharmacology and Medicine and they are divided basically into two major parts –

- Diagnostic applications
- Therapeutic applications

Liposomes generally contain drugs, markers and they are used as a model, tool or reagent in the basic studies of cell interaction, cell recognition, mode of action etc.

1. Cancer Chemotherapy: There are different types liposomal formulation of anticancer drugs is available which show less toxicity when compared to free drugs (Fidler et al, 1989).

Anticancer drugs are less selective which results in toxicity of the healthy cells, entrapment of drug in liposomal carrier shows better results (Jain et al, 1997). Liposomal entrapment of the drugs eg. Doxorubicin, Danorubicin along with Vincristine showed reduced cardiotoxicity, dermal toxicity and better survival compared to free drug. Encapsulation of etoposide in liposomes significantly delayed tumor growth compared to non-liposomal etoposide. Liposomal formulation of adriamycin resulted in an increased drug concentration in the liver and lungs and a decreased concentration in the heart. Encapsulation of c-myb antisense oligodeoxynucleotides into immunoliposomes showed enhancement of their toxicity toward targeted cells while avoiding non-targeted cells from antisense effects and may be efficacious for the delivery of drugs with broad therapeutic applications to tumor cells (Goyal et al, 2005)

2. Parasitic disease and infection: Conventional liposomes are digested by phagocytic cells in the body after intravenous administration so they are ideal vehicles for the targeting of drug molecules into these macrophages. The best results shows in human therapy are probably liposomal Amphotericin B in antifungal therapies. Liposome encapsulated antiviral drugs such as acyclovir, ribavirin, or azide thymidines have also shown reduced toxicity compared to free drugs (Lasic et al).

3. **Liposomes as carrier of vaccines**: **(a) Immunological adjuvants**: There are several advantages of liposomes used as carrier of vaccines:

- Conversion of non- immunogenic substance to immunogenic one.
- Hydrophobic antigen may be reconstituted.
- Small amount of antigen can be used as immunogens.
- During liposomal delivery adjuvants may be incorporated with antigen.
- By inclusion in liposomes toxic or allergic reaction of antibody can be minimized.

Liposomised diphtheria toxoid is equally effective and shows same immune response.

(b) **Carrier of antigens**: Liposomes are widely used as an efficient system for the delivery of many biological substances. When liposomes are administered, a major fraction is taken up by the liver and spleen. Some methods can be adopted for delivery of liposomes to target cells are-

- The uptake of liposomes by Reticular Endothelial System (RES) can be reduced using small, neutral, unilamellar liposomes having cholesterol.
- By modifying the surface of liposomes which converts the liposome less recognizable by RES.
- Attaching specific ligand to the surface of liposome which can specifically bind to the receptors.

When cholera toxin has been formulated with DMPC, CH liposomes, the toxicity was completely eliminated and antigenicity is enhanced.

(c) **Antimicrobial therapy**: The liposomes are able to localize in the liver and spleen, especially the RES component, where many pathogenic microorganisms reside; they can be therefore used for targeting of antibiotics.

(d) **Gene therapy**: Cationic liposome formulation like lipofectin, lipofectamine, transfectace, transfectam can be easily used as non-viral human gene delivery system. Cationic liposomes formulated with dioleyloxypropyl trimethylammonium chloride (DOTMA) such as lipofectin is successful for *in-vitro* and *in-vivo* gene delivery. For transfecting genes plasmid liposome shows more efficiency than micelles of same composition (Goyal et al, 2005).

(e) **Radiochemotherapeutics:** Indium-111 ((111)In) labeled oxine when delivered by liposomes with a higher concentration of PEG (6 mol%) shows longer circulation time due to reduced phagocytic activity and enhanced tumor targeting efficiency (Chow et al, 2009). Rhenium-188 [(188)Re] labeled PEGylated liposomes loaded with anticancer drugs showed the potential advantages of tumor specific drug delivery and inhibition of cancer growth (Jian et al, 2013).

(f) **Oral delivery:** Liposomal oral delivery of drugs shows higher therapeutic efficiency compared to the other forms as it increases the solubility and absorption in gut. According to Segal et al, 1979, liposomal delivery of anti-arthritic drugs is very effective as it cannot be destroyed easily by peripheral effects. Liposome encapsulated insulin is a very potential system for oral delivery in diabetes.

(g) **Pulmonary delivery:** Many bronchodilators can be formulated as liposome for controlled release to the respiratory tract.

(h) **Ophthalmic delivery**: Many drugs such as iodoxuridine, adrenaline, triamcinolone acetonide, benzyl penicillin etc. can be formulated as liposome for their ocular delivery. The effectiveness sometime depends on size of liposome, drug distribution, and stability of liposomes.

(i) Food technology: Liposomes and nanoliposomes have been now extensively used in the food industry to deliver flavors, nutrients and recently, for their ability to incorporate antimicrobials that helps in the protection of food products against microbial contamination.

(j) **Cosmetics**: The lipids in liposome are well hydrated and able to reduce the dryness of the skin, which is a major cause of aging so liposomes are widely used in cosmetics (Jain et al, 1997).

3.11 Liposomal formulations available in market:

Table 3.1: Liposomal formulations available in market and under clinical trial

Liposomal Product	Trade name and manufacturers	Clinical trial phase	Therapeutic indication
Liposomal Doxorubicin	**Doxil**, JNJ	Approved	ovarian cancer, AIDS-related Kaposi's sarcoma, multiple myeloma
Liposomal Amphoteracin B	**AmBisome**, Astellas Pharma U.S.	Approved	Used against fatal fungal infections with fever and low white blood cells
Liposomal Daunorubicin	**DaunoXome**, NeXstar Pharmaceuticals	Approved	Used for treatment of Kaposi's sarcoma.
Liposomal Vincristine	**Marqibo,** CASI Pharmaceuticals	Phase III	Used for the treatment of metastatic malignant uveal melanoma.
Liposomal verteporfin	**Visudyne,** Bausch+Lomb	Approved	Used for treatment of age-related macular degeneration, pathologic myopia and ocular histoplasmosis.
Liposomal cytarabine	**DepoCyt**, Sigma-Tau Pharmaceuticals	Approved	Used by intrathecal administration for treatment of neoplastic meningitis and lymphomatous meningitis.

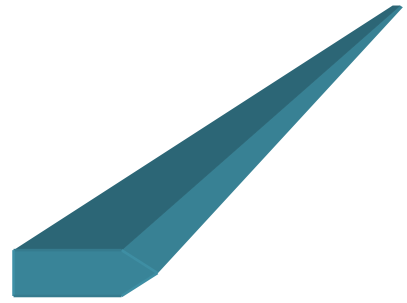
Liposomal morphine sulfate	**DepoDur**, Pacira Pharmaceuticals	Approved	Used by epidural administration for treatment of postoperative pain following major surgery.
Liposomal amikacin	**Arikace,** Insmed	Phase III	Used for treatment of lung infections due to susceptible pathogens.
Liposomal cisplatin	**Lipoplatin**, Regulon Inc.	Approved	Used for treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder and testicular cancers.
Liposomal Paclitaxel	**LEP-ETU**, Insys Therapeutics	Phase II	Used for treatment of ovarian, breast and lung cancer.
Hepatitis A vaccine	**Epaxal**, Crucell Spain S.A.	Approved	Used as a vaccine adjuvant in this formulation.
Influenza vaccine	**Inflexal V**, Berna Biotech Ltd**.**	Approved	Used as a vaccine adjuvant

3.12 PEGylation of liposomes:

When drugs or proteins are administered parenteraly, they are either quickly cleared off from the circulation by glomerular filtration or by reticulo endothelial system (RES), with the consequence of a rapid loss of their biological properties as well as therapeutic efficacy, so more frequent administrations are needed. Many studies are done to overcome this problem by enhancing the stability. The most successful approach is modification by linking one or more PEG molecule to the surface of the drug carrier eg. liposomes. The polymer most widely used for protein PEGylation is linear mPEG, end-capped on one side with a methoxyl group and terminated with a hydroxyl group. The most used method for preparation of clinically suitable liposomes is the anchoring of the PEG moiety to the liposomal membrane via a cross-linked lipid like PEG-distearoylphosphatidylethanolamine (PEG-DSPE).

. Rationale for using PEGylation of liposomes:

- PEGylation Improves Pharmacodynamic and Pharmacokinetic properties of drug by increasing water solubility.
- PEGylation shows increased half-life and decreased plasma clearance.
- PEGylation enhances circulation time in blood as well as increases the stability.
- PEGylation decreases immunogenic, pyrogenic and antigenic reactions.
- PEGylation acts as steric barrier for inhibiting liposome fusion (Nag et al, 2013; Pitrubhakta et al, 2012**;** Milla et al, 2012; Bru et al, 2002).



Chapter 4

Peripheral nervous system - a brief introduction

4. PERIPHERAL NERVOUS SYSTEM

4.1 Overview:

The peripheral nervous system refers to the parts of the nervous system outside the brain and spinal cord. It includes the cranial nerves, spinal nerves, their roots and branches, peripheral nerves and neuromuscular junctions. The anterior horn cells, although technically part of the central nervous system (CNS), are sometimes discussed with the peripheral nervous system because they are part of the motor unit.

In the peripheral nervous system, bundles of nerve fibers or axons conduct information to and from the CNS. The autonomic nervous system is the part of the nervous system concerned with the innervation of involuntary structures, such as the heart, smooth muscle, and glands within the body. It is distributed throughout the central and peripheral nervous systems.

Nerve cells are called neurons. A neurone consists of a cell body (with a nucleus and cytoplasm), dendrites that carry electrical impulses to the cell, and a long axon that carries the impulses away from the cell. The axon of one neurone and the dendrites of the next neurone do not actually touch. The gap between neurones is called the synapse.

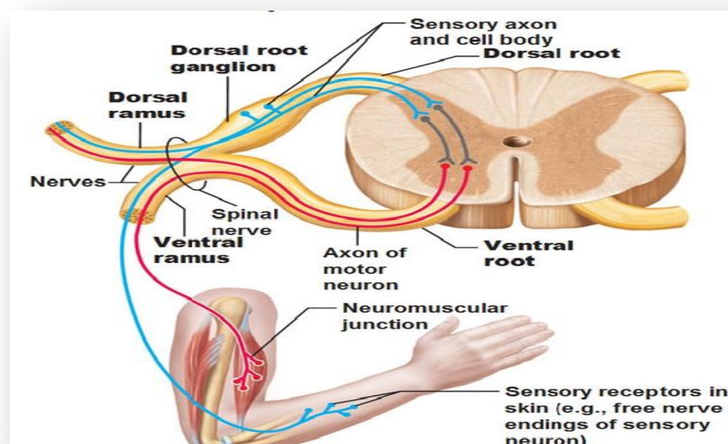


Fig no. 4.1: Overview of peripheral nervous system, spinal nerves and plexuses

(http://antranik.org/peripheral-nervous-system-spinal-nerves-and-plexuses)

Neuronal function is complex and involves numerous processes in nerve transmission. Generation of a nerve impulse (action potential) of a sensory neurone occurs as a result of a stimulus such as light, a particular chemical, or stretching of a cell membrane by sound. Conduction of an impulse along a neurone occurs from the dendrites to the cell body to the axon. Transmission of a signal to another neuron across a synapse occurs via chemical transmitter. This substance causes the next neurone to be electrically stimulated and keeps the signal going along a nerve.

Ganglia may be divided into sensory ganglia of spinal nerves (spinal or posterior root ganglia) and cranial nerves and autonomic ganglia. Sensory ganglia of spinal nerves are fusiform swellings situated on the posterior root of each spinal nerve just proximal to the root's junction with a corresponding anterior root. They are referred to as spinal or posterior root ganglia. Similar ganglia that are also found along the course of cranial nerves V, VII, VIII, IX, and X are called sensory ganglia of these nerve. Autonomic ganglia, which are often irregular in shape, are situated along the course of efferent nerve fibers of the autonomic nervous system. They are found in the paravertebral sympathetic chains, around the roots of the great visceral arteries in the abdomen, and close to, or embedded within, the walls of various viscera.

4.2 Gross anatomy:

- **The sensory (afferent) division** carries sensory signals by way of afferent nerve fibers from receptors in the central nervous system (CNS). It can be further subdivided into somatic and visceral divisions. The somatic sensory division carries signals from receptors in the skin, muscles, bones and joints. The visceral sensory division carries signals mainly from the viscera of the thoracic and abdominal cavities.

- **The motor (efferent) division** carries motor signals by way of efferent nerve fibers from the CNS to effectors (mainly glands and muscles). It can be further subdivided into somatic and visceral divisions. The somatic motor division carries signals to the skeletal muscles. The visceral motor division, also known as the autonomic nervous system, carries signals to glands, cardiac muscle, and smooth muscle. It can be further divided into the sympathetic and parasympathetic divisions (Snell et al, 2009; Blumenfeld et al, 2002; Blumenfeld et al, 2010; Brazis et al, 2011; Biller et al, 2011).

The sympathetic division tends to arouse the body to action. The parasympathetic divisions tend to have a calming effect.

Nerve fibers of the PNS are classified according to their involvement in motor or sensory, somatic or visceral pathways. Mixed nerves contain both motor and sensory fibers. Sensory nerves contain mostly sensory fibers; they are less common and include the optic and olfactory nerves. Motor nerves contain motor fibers.

- **Anatomy of nerves and ganglia**

A nerve is an organ composed of multiple nerve fibers bound together by sheaths of connective tissue. The sheath adjacent to the neurilemma is the endoneurium, which houses blood capillaries that feed nutrients and oxygen to the nerve. In large nerves, fibers are bundled into fascicles and wrapped in a fibrous perineurium. The entire nerve is covered with a fibrous epineurium.

A ganglion is a cluster of neuron cell bodies enveloped in an epineurium continuous with that of a nerve. A ganglion appears as a swelling along the course of a nerve. The spinal ganglia or posterior or dorsal root ganglia associated with the spinal nerves contain the unipolar neurons of the sensory nerve fibers that carry signals to the cord. The fiber passes through the ganglion without synapsing. However, in the autonomic nervous system, a preganglionic fiber enters the ganglion and in many cases synapses with another neuron. The axon of the second neuron leaves the ganglion as the postganglionic fiber.

- **Cranial nerves**

The cranial nerves emerge from the base of the brain and lead to muscles and sense organs in the head and neck for the most part. The twelve pairs of cranial nerves with their functions are as follows:

- Olfactory nerve (I): Sensory nerve that carries impulses for smell to the brain.
- Optic nerve (II): Sensory nerve that carries impulses for vision to the brain.

- Oculomotor nerve (III): Motor nerve that carries impulses to the extrinsic eye muscles, which help direct the position of the eyeball. This nerve also carries impulses to the muscles that regulate the size of the pupil.
- Trochlear nerve (IV): Motor nerve that carries impulses to one extrinsic eye muscle (the superior oblique muscle). Once again, this muscle helps regulate the position of the eyeball.
- Trigeminal nerve (V): A mixed nerve. The sensory fibers of this nerve carry impulses for general sensation (touch, temperature and pain) associated with the face, teeth, lips and eyelids. The motor fibers of this nerve carry impulses to some of the mastication muscles of the face.
- Abducens nerve (VI): A mixed nerve, but primarily a motor nerve. This nerve carries impulses to the lateral rectus muscle of the eye. This muscle is an extrinsic eye muscle that is involved in positioning the eyeball.
- Facial nerve (VII): A mixed nerve. The sensory fibers of this nerve carry taste sensations from the tongue. The motor fibers of this nerve carry impulses to many of the muscles of the face and they carry impulses to the lacrimal, submandibular, and sublingual glands.
- Vestibulocochlear nerve (VIII): A sensory nerve that carries impulses for hearing and equilibrium from the ear to the brain.
- Glossopharyngeal nerve (IX): A mixed nerve. The sensory fibers of this nerve carry basic sensory information and taste sensations from the pharynx and tongue to the brain. The motor fibers of this nerve carry impulses associated with swallowing to the pharynx.
- Vagus nerve (X): A mixed nerve. The sensory fibers of this nerve carry impulses from the pharynx, larynx, and most internal organs to the brain. The motor fibers of this nerve carry impulses to internal organs of the chest and abdomen and to the skeletal muscles of the larynx and pharynx.
- Accessory nerve (XI): A mixed nerve, but primarily motor. Carries impulses to muscles of the neck and back.
- Hypoglossal nerve (XII): Primarily a motor nerve. This nerve carries impulses to the muscles that move and position the tongue.

- **Spinal nerves**

  Thirty one pairs of spinal nerves exist: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal.

- Proximal branches: Each spinal nerve branches into a posterior root and an anterior root. The spinal or posterior root ganglion is occupied by cell bodies from afferent neurons. The convergence of posterior and anterior roots forms the spinal nerve. The cauda equina is formed by the roots arising from segments L2 to Co of the spinal cord.
- Distal branches: After emerging from the vertebral column, the spinal nerve divides into a posterior ramus, an anterior ramus, and a small meningeal branch that leads to the meninges and vertebral column. The posterior ramus innervates the muscles and joints of the spine and the skin of the back. The anterior ramus innervates the anterior and lateral skin and muscles of the trunk, plus gives rise to nerves leading to the limbs (see image below).

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- Nerve plexuses: The anterior rami merge to form nerve plexuses in all areas except the thoracic region

- **Autonomic nervous system**

  The visceral reflexes are mediated by the autonomic nervous system (ANS), which has two divisions (sympathetic and parasympathetic). The target organs of the ANS are glands, cardiac muscle, and smooth muscle: it operates to maintain homeostasis. Control over the ANS is, for the most part, involuntary. The ANS differs structurally from the somatic nervous system in that 2 neurons leading from the ANS to the effector exist, a preganglionic neuron and a postganglionic neuron.

- Anatomy of the sympathetic division: The sympathetic division is also called the thoracolumbar division because of the spinal nerve it uses. Paravertebral ganglia occur close to the vertebral column. Preganglionic ganglia are short, while postganglionic neurons, traveling to their effector, are long. When 1 preganglionic neuron fires, it can excite multiple postganglionic fibers that lead to different target organs (mass activation).

In the thoracolumbar region, each paravertebral ganglion is connected to a spinal nerve by 2 communicating rami, the white communicating ramus and the gray communicating ramus. Nerve fibers leave the paravertebral ganglia by gray rami communicantes and splanchnic nerves.

- Anatomy of the parasympathetic division: The parasympathetic division is also referred to as the craniosacral division because its fibers travel in some cranial nerves (III, VII, IX, X) and sacral nerves (S2-4). The parasympathetic ganglia (terminal ganglia) lie in or near the target organs. The parasympathetic fibers leave the brainstem by way of the oculomotor, facial, glossopharyngeal, and vagus nerves. The parasympathetic system uses long preganglionic and short postganglionic fibers.
- A motor unit consists of an anterior horn cell, its motor axon, the muscle fibers it innervates, and the connection between them (neuromuscular junction). The anterior horn cells are located in the gray matter of the spinal cord and thus are technically part of the CNS. In contrast to the motor system, the cell bodies of the afferent sensory fibers lie outside the spinal cord, in posterior root ganglia.
- Nerve fibers outside the spinal cord join to form anterior (ventral) motor roots and posterior (dorsal) sensory root nerve roots. The anterior and posterior roots combine to form a spinal nerve. Thirty of the 31 pairs of spinal nerves have anterior and posterior roots; C1 has no sensory root.
- The spinal nerves exit the vertebral column via an intervertebral foramen. Because the spinal cord is shorter than the vertebral column, the more caudal the spinal nerve, the further the foramen is from the corresponding cord segment. Thus, in the lumbosacral region, nerve roots from lower cord segments descend within the spinal column in a near-vertical sheaf, forming the cauda equina. Just beyond the intervertebral foramen, spinal nerves branch into several parts.
- Branches of the cervical and lumbosacral spinal nerves anastomose peripherally into plexuses, then branch into nerve trunks that terminate up to 1 μm away in peripheral structures. The intercostal nerves are segmental.

- The term peripheral nerve refers to the part of a spinal nerve distal to the nerve roots. Peripheral nerves are bundles of nerve fibers. They range in diameter from 0.3-22 μm. Schwann cells form a thin cytoplasmic tube around each fiber and further wrap larger fibers in a multilayered insulating membrane (myelin sheath).

- Peripheral nerves have multiple layers of connective tissue surrounding axons, with the endoneurium surrounding individual axons, perineurium binding axons into fascicles, and epineurium binding the fascicles into a nerve. Blood vessels (vasa vasorum) and nerves (nervi nervorum) are also contained within the nerve. Nerve fibers in peripheral nerves are wavy, such that a length of peripheral nerve can be stretched to half again its length before tension is directly transmitted to nerve fibers. Nerve roots have much less connective tissue, and individual nerve fibers within the roots are straight, leading to some vulnerability.

- Peripheral nerves receive collateral arterial branches from adjacent arteries. These arteries that contribute to the vasa nervorum anastomose with arterial branches entering the nerve above and below in order to provide an uninterrupted circulation along the course of the nerve.

- Individual nerve fibers vary widely in diameter and may also be myelinated or unmyelinated. Myelin in the peripheral nervous system derives from Schwann cells, and the distance between nodes of Ranvier determines the conduction rate. Because certain conditions preferentially affect myelin, they would be most likely to affect the functions mediated by the largest, fastest, most heavily myelinated axons.

- Sensory neurons are somewhat unique, having an axon that extends to the periphery and another axon that extends into the central nervous system via the posterior root. The cell body of this neuron is located in the posterior root ganglion or one of the sensory ganglia of sensory cranial nerves. Both the peripheral and the central axon attach to the neuron at the same point, and these sensory neurons are called "pseudounipolar" neurons.

- Before a sensory signal can be relayed to the nervous system, it must be transduced into an electrical signal in a nerve fiber. This involves a process of opening ion channels in the membrane in response to mechanical deformation, temperature or, in the case of

nociceptive fibers, signals released from damaged tissue. Many receptors become less sensitive with continued stimuli, and this is termed adaptation. This adaptation may be rapid or slow, with rapidly adapting receptors being specialized for detecting changing signals.

- Several structural types of receptors exist in the skin. These fall into the category of encapsulated or nonencapsulated receptors. The nonencapsulated endings include free nerve endings, which are simply the peripheral end of the sensory axon. These mostly respond to noxious (pain) and thermal stimuli. Some specialized free nerve endings around hairs respond to very light touch; also, some free nerve endings contact special skin cells, called Merkel cells.
- These Merkel cells (discs) are specialized cells that release transmitter onto peripheral sensory nerve terminals. The encapsulated endings include Meisner corpuscles, Pacinian corpuscles, and Ruffini endings. The capsules that surround encapsulated endings change the response characteristics of the nerves. Most encapsulated receptors are for touch, but the Pacinian corpuscles are very rapidly adapting and, therefore, are specialized to detect vibration. Ultimately, the intensity of the stimulus is encoded by the relative frequency of action potential generation in the sensory axon.
- In addition to cutaneous receptors, muscle receptors are involved in detecting muscle stretch (muscle spindle) and muscle tension (Golgi tendon organs). Muscle spindles are located in the muscle bellies and consist of intrafusal muscle fibers that are arranged in parallel with most fibers comprising the muscle (ie, extrafusal fibers). The ends of the intrafusal fibers are contractile and are innervated by gamma motor neurons, while the central portion of the muscle spindle is clear and is wrapped by a sensory nerve ending, the annulospiral ending. This ending is activated by stretch of the muscle spindle or by contraction of the intrafusal fibers (sees section V). The Golgi tendon organs are located at the myotendinous junction and consist of nerve fibers intertwined with the collagen fibers at the myotendinous junctions. They are activated by contraction of the muscle (muscle tension).

- Both the sympathetic and parasympathetic portions of the autonomic nervous system have a 2-neuron pathway from the central nervous system to the peripheral organ. Therefore, a ganglion is interposed in each of these pathways, with the exception of the sympathetic pathway to the suprarenal (adrenal) medulla. The suprarenal medulla basically functions as a sympathetic ganglion. The 2 nerve fibers in the pathway are termed preganglionic and postganglionic. At the level of the autonomic ganglia, the neurotransmitter is typically acetylcholine. Postganglionic parasympathetic neurons also release acetylcholine, while norepinephrine is the postganglionic transmitter for most sympathetic nerve fibers. The exception is the use of acetylcholine in sympathetic transmission to the sweat glands and erector pili muscles as well as to some blood vessels in muscle.

- Sympathetic preganglionic neurons are located between T1 and L2 in the lateral horn of the spinal cord. Therefore, sympathetics have been termed the "thoracolumbar outflow." These preganglionic visceral motor fibers leave the cord in the anterior nerve root and then connect to the sympathetic chain through the white rami communicans. This chain of connected ganglia follows the sides of the vertebrae all the way from the head to the coccyx. These axons may synapse with postganglionic neurons in these paravertebral ganglia. Alternatively, preganglionic fibers can pass directly through the sympathetic chain to reach prevertebral ganglia along the aorta (via splanchnic nerves).

- Additionally, these preganglionics can pass superiorly or inferiorly through the interganglionic rami in the sympathetic chain to reach the head or the lower lumbosacral regions. Sympathetic fibers can go to viscera by 1 of 2 pathways. Some postganglionic can leave the sympathetic chain and follow blood vessels to the organs. Alternatively, preganglionic fibers may pass directly through the sympathetic chain to enter the abdomen as splanchnic nerves. These synapse in ganglia located along the aorta (the celiac, aorticorenal, superior, or inferior mesenteric ganglia) with postganglionic. Again, postganglionics follow the blood vessels.

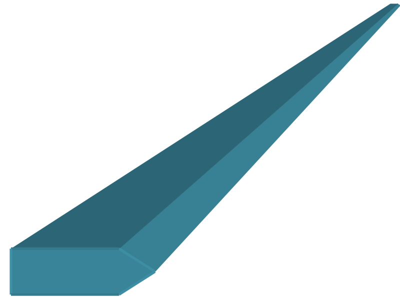
- Sympathetic postganglionics from the sympathetic chain can go back to the spinal nerves (via gray rami communicans) to be distributed to somatic tissues of the limbs and body walls. For example, the somatic response to sympathetic activation will result in

sweating, constriction of blood vessels in the skin, dilation of vessels in muscle and in piloerection. Damage to sympathetic nerves to the head results in slight constriction of the pupil, slight ptosis, and loss of sweating on that side of the head (called Horner syndrome). This can happen anywhere along the course of the nerve pathway including the upper thoracic spine and nerve roots, the apex of the lung, the neck or the carotid plexus of postganglionics.

- Parasympathetic nerves arise with cranial nerves III, VII, IX, and X, as well as from the sacral segments S2-4. Therefore, they have been termed the "craniosacral outflow." Parasympathetics in cranial nerve III synapse in the ciliary ganglion and are involved in pupillary constriction and accommodation for near vision. Parasympathetics in cranial nerve VII synapse in the pterygopalatine ganglion (lacrimation) or the submandibular ganglion (salivation), while those in cranial nerve IX synapse in the otic ganglion (salivation from parotid gland).
- The vagus nerve follows a long course to supply the thoracic and abdominal organs up to the level of the distal transverse colon, synapsing in ganglia within the organ walls. The pelvic parasympathetics, which appear as the pelvic splanchnic nerves, activate bladder contraction and also supply lower abdominal and pelvic organs.

4.3 Physiology

- The myelin sheath enhances impulse conduction. The largest and most heavily myelinated fibers conduct quickly; they convey motor, touch, and proprioceptive impulses. The less myelinated and unmyelinated fibers conduct more slowly; they convey pain, temperature, and autonomic impulses. Because nerves are metabolically active tissues, they require nutrients, supplied by blood vessels called the vasa nervorum (O'Brien et al, 2010).



Chapter 5

Literature Review

5. Literature Review

5.1. During 1960-1963, **Alec Bangham and Horne (1962)** discovered the liposomes when Bangham and R. W. Horne were testing the institute's new electron microscope by adding negative stain to dry phospholipids, observed that isolated, purified phospholipids of cellular origin dispersed in aqueous phases formed multilayered vesicles *(*Bangham, Horne, 1964*)*. These unique structures resembles with cellular organelles with lipid bilayers (Bangham, 1983).Bangham and his collaborates showed that a sequence of molecular rearrangement took place between lipid amphiphiles and aqueous phase. As a result, inner aqueous compartment was separated by bimolecular lipid membrane and outermost aqueous layer of whole structure was isolated from bulk aqueous phase (Bangham et al, 1965)

5.2. **Ken-ichirou et al. (1996)** developed unilamellar liposomes with diameters of 25-100nm in various physiological salt solutions, e.g.100 mM KCI plus 1 mM $CaCl_2$ by thin film hydration method. They identified the unilamellar vesicles by applying three methods

i) By observing under phase contrast microscope.

ii) By using lipophilic fluorescent dye, octadecyl rhodamine B.

iii) Micropipette aspiration test.

5.3. **Glavas-Dodov et al. (2005)** encapsulated 5-Fluorouracil (5-FU) into liposomes by modified lipid film hydration method and was lyophilized with or without saccharose as cryoprotectant. The effect of lyophilization on the stability of liposomes was evaluated by comparing the vesicle size, encapsulation efficiency and the drug release rate before and after lyophilization/rehydration. They observed that freeze-drying did not affect the particle size of liposomes containing saccharose as cryoprotectant.

5.4. **Francis et al. (1978)** described that large unilamellar and oligolamellar vesicles are formed during the introduction of aqueous buffer into a mixture of phospholipids and organic solvent and the organic solvent is subsequently removed by evaporation under reduced pressure. These vesicles can be made from various lipids or mixtures of lipids and have aqueous volume to lipid ratios that are 30 times higher than sonicated preparations and 4 times higher than multilamellar vesicles. This reverse phase evaporation offers great advantages for encapsulating various water-

soluble materials such as drugs, proteins, nucleic acids, and other biochemical reagents with great efficiency.

5.5. Jain et al. (2005) designed a depot delivery system of Acyclovir sodium using multivesicular liposomes (MLVs) to overcome the limitations of conventional therapies and to investigate its *in vivo* effectiveness for sustained drug delivery. They prepared MLVs of Acyclovir by the reverse phase evaporation method. The drug loading efficiency of the MLVs (45%- 82%) was found to be 3 to 6 times higher than conventional MLVs. The *in vitro* release of Acyclovir from MLV formulations was to be in a sustained manner and only 70% of drug was released in 96 hours, whereas conventional MLVs released 80% of drug in 16 hours.

5.6 Layek et al. (2010) developed stable sustained release liposomal drug delivery system for Tamoxifen citrate using soya phosphatidylcholine, cholesterol and span 20 as main ingredients by thin film hydration method. The main aim of this study was to develop a simple vesicular delivery system for Tamoxifen citrate which can deliver drug at a lower concentration over a prolong period of time and thereby reducing the potential dose related side effects. Stability study at different conditions was carried out. From this study they concluded that the liposomal formulation was a useful drug delivery system for sustaining the *in vitro* release of Tamoxifen citrate.

5.7 **Santra et al. (2010**) developed Dexmethasone loaded liposome. The objective of the study was to develop Dexmethasone-containing liposomes (DCL) based on different combonation of cholesterol and soya l-α-lecithin by lipid film hydration method with various standardized process parameters such as amount of drug loading, drug-release, liposome size, etc. They carried out the study of other different process parameters such as drug-excipients interaction (by FTIR study), surface morphology by scanning electron microscopy (SEM), particle size analysis and *in-vitro* drug release study. At the end of the study standard Dexamethasone loaded liposomes have been successfully developed based on cholesterol and soya-L-α-lecithin with a drug loading of around 1.5%.

5.8 **Ramana et al. (2010)** prepared Nevirapine liposome by lipid layer hydration method by using egg phospholipid to obtain spherical liposomes below 200 nm with a narrow polydispersity index. The prepared liposomes was characterized and optimized to select the best formulation. They observed that the size of the liposomes and the encapsulation efficiency of the drug increased concomitantly with the increasing ratio of drug and lipid and that maximum stability was observed at the physiological pH. They proposed that Nevirapine-loaded liposomal formulations could improve targeted delivery of the anti-retroviral drugs to select compartments and cells and alleviate systemic toxic side effects.

5.9. **Mukherjee et al. (2007)** designed the study to develop and compare Acyclovir containing nano-vesicular liposomes based on cholesterol, soya l-α-lecithin and non ionic surfactant, span 20. Liposomes and niosomes were prepared by lipid layer hydration method. They made effort to study *in vitro* whether Acyclovir loaded nanovesicles could sustain the release of the drug by increasing residence time and thus, Acyclovir could reduce its dose-related systemic toxicity. They found that the percentage of drug loading in case of niosome was higher and it processed more stability than liposomes and revealed that thus niosome could be a better choice for intravenous delivery of Acyclovir.

5.10 Cuyper et al. (2004) have found an advanced method for delivery of amino acid molecules through conjugation of amino acid with a hydrophilic cross linker molecule, attached to a nonpolar lipid molecule. In the first step, they have attached a hydrophilic polymer COOH-PEG2000- COOH with a lipid molecule DMPE (dimyristoylphosphatidylethanolamine) through attachment of a terminal COOH group of PEG-2000 molecule with the group of DMPE molecule. The synthesis protocol involves conversion of dicarboxylic PEG molecule into an intramolecular anhydride, and subsequent attachment of amino group gives DMPE-PEG2000-COOH molecule. In the second step the free COOH group of DMPE-PEG2000-COOH molecule was attached with an amino acid (tryptophan) with the help of carbodiimide mediated coupling reaction *e.g.*use of EDC and sulfo-NHS. This gives the DMPE-PEG2000-tryptophan molecule. This type synthesis strategy for attachment of ligand or protective molecule with the therapeutic moiety or formulation ingredient via intermediacy of a cross linker molecule is widely accepted and helpful in targeted drug delivery.

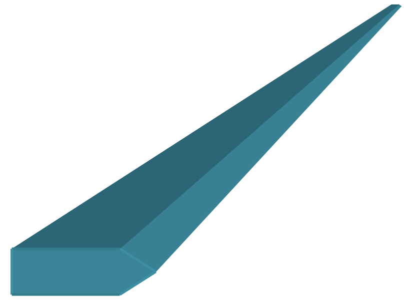
5.11 Rudra et al. (2010) developed phosphatidylethanolamine (PE)-conjugated nanoliposome by thin film hydration method and evaluated, characterized for their accumulation in liver, kidneys, and lungs in rats. They concluded that PE-conjugated nanoliposomes released the drug in a sustained manner and were capable of distributing them in various organs. So, this may be used for cell or tissue targeting therapy, attaching specific antibodies to PE.

5.12 Caneva et al. (2004) developed novel biocompatible constructs for the controlled release of anti-scarring antibody R-Phycoerythrin labelled Goat Anti-Mouse IgG by which the repair of peripheral nervous system injuries can be improved. The bioengineered constructs are made of an inner fibronectin (FN) mat surrounded by an outer HYAFF 11 tube (benzyl ester of the hyaluronic acid) for controlled release of antibody from the matrix. They demonstrate a good method for *in vitro* study of antibody conjugation which involves use of Aprotinin from bovine lung as protease inhibitor.

5.13 **Wang et al. (2012)** described that Poly(ethylene glycol) distearoylphosphatidylethanolamine (PEG-DSPE) block copolymers are biocompatible and amphiphilic polymers that are widely used in the preparation of liposomes, polymeric nanoparticles, polymer hybrid nanoparticles, solid lipid nanoparticles, lipid–polymer hybrid nanoparticles, and microemulsions. Specifically, the terminal groups of PEG can be activated and linked to various targeting ligands, which can increase the circulation time, improve the drug bioavailability, reduce undesirable side effects, and especially target specific cells, tissues, and even the intracellular localization in organelles.

5.14 **Ashizawa et al. (2013)** shows that cholesterol is critical for maximizing uptake into capillary endothelia and Schwann cells. They indicated by the pharmacological studies with isolated Schwann cells that, the DOPC/P188/Chol liposome internalization in the peripheral nerves occur mostly by caveolae and actin-mediated mechanisms. The minimal detection of DOPC/P188 and DOPC/P188/Chol liposomes within fibroblasts or muscle cells indicates that the formulation is not randomly absorbed by all cell types or tissues. The diffuse rather than punctate appearance of the particles within the BMECs as well as in the sciatic nerve supports

the point that these liposomes are suitable for the delivery and release of therapeutic agents within targeted cells of peripheral nerves.



Chapter 6

Materials

6. MATERIALS REQUIRED:

6.1 Chemicals: Various chemicals such as DSPE-PEG 2000, cholesterol, soya-L-α-lecithin (SPC), butylated hydroxy tolune (BHT), sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, chloroform, ethanol are used for the preparation of Ribavirin nanoliposome.

Table 6.1: List of chemicals used with their source.

Name	Source
DSPE-PEG2000	Lipoid
Cholesterol	Merck Specialities Private Limited, Mumbai, India
Soya-L-α-lecithin	HiMedia Laboratories Private Limited. Mumbai, India
Butylated Hydroxy Tolune (BHT)	Merck Specialities Private Limited, Mumbai, India
Disodium hydrogen phosphate	Merck Specialities Private Limited, Mumbai, India
Potassium dihydrogen phosphate	Merck Specialities Private Limited, Mumbai, India
Sodium Chloride	Merck Specialities Private Limited, Mumbai, India
Chloroform	Merck Specialities Private Limited, Mumbai, India
Ethanol	Merck Specialities Private Limited, Mumbai, India
Ribavirin	Swapnroop Drugs and Pharmaceuticals, Aurangabad, India

6.2 Equipments: Various instruments used in the preparation of Ribavirin nanoliposome and the company from which they are obtained are given below.

Table 6.2: List of equipments used in the liposome preparation.

Equipment Name	Name of Company
Rotary Vacuum Evaporator	ROTAVAP SUPERFIT (Model-PBU-6),Mumbai, India
Aspirator A3S	Eyela, Tokyo Rikakikai Co Ltd., Tokyo, Japan
Cold circulating bath	Spac N Service, Kolkata, India
Vacuum desiccator	Tarson, Kolkata, India
Bath type sonicator	TRANS-O-SONIC, Mumbai, India
FESEM	JSM, JEOL, Tokyo, Japan
Cold Centrifuge	3K30, SIGMA, Shrewsbury, USA.
FTIR Spectroscope	JASCO Magna IR 750 series II FTIR instrument, JASCO International Co. Ltd. FTIR 4200 Tokyo, Japan.
Deep freezer(-80 ºC)	New Brunswick Scientific, Freshwater Boulevard Enfield, U.S.A.
Lyophilizer	Instrumentation India, Kolkata-32, India
UV absorption spectroscopy	Advanced Microprocessor UV-VIS Single Beam, Intech-295, Andhrapradesh, India.
Zeta potential & particle size Analyzer	ZETASIZER, Nano ZS 90, MALVERN Instrument Ltd, Malvern, UK.
Magnetic stirrer	Remi Equipments, Mumbai, India.
Digital Balance	Sartoious, Goetingen, Germany
pH meter	Eutech Instruments, Haridwar, India

6.3 DESCRIPTION OF RIBAVIRIN:

Ribavirin is a broad spectrum antiviral drug and effective against both RNA and DNA viruses.

Structure:-

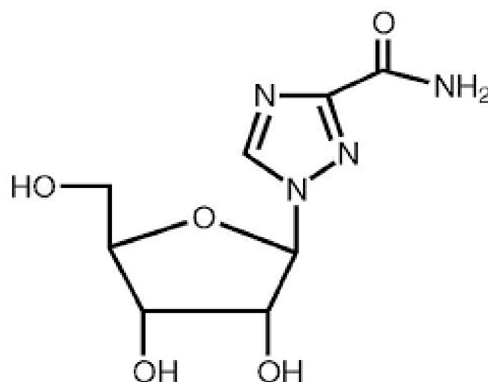


Figure 6.1 : Structure of Ribavirin

- **IUPAC NAME :** 1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1*H*-1,2,4-triazole-3-carboxamide.
- **Trade Name**: Copegus, Rebetol, Virazole
- **Routes of administration**: Oral, inhalation
- **Formula**: $C_8H_{12}N_4O_5$
- **Brand names**: Ribasphere, RibaPak, Rebetol, Copegus
- **Molecular Weight**: 244.20
- **Melting Point**: 174-176ºc
- **Half life:** 9.5 h. (32)

- **Pharmacokinetics of Ribavirin**:

  - **Absorption**: Ribavirin is absorbed from the GI tract probably by nucleoside transporters. Absorption is about 45%, and this is modestly increased (to about 75%) by a fatty meal. Once in the plasma, Ribavirin is transported through the cell membrane also by nucleoside transporters.
  - **Distribution**: Ribavirin is widely distributed in all tissues, including the CSF and brain. The pharmacokinetics of Ribavirin is dominated by trapping of the phosphate form inside cells, particularly red blood cells (RBCs) which lack the enzyme to remove the phosphate once it has been added by kinases, and therefore attain high concentrations of the drug. Most of the kinase activity which converts the drug to active nucleotide form is provided by adenine kinase. This enzyme is more active in virally infected cells. The volume of distribution of Ribavirin is large (2000 L/kg) and the length of time the drug trapped varies greatly from tissue to tissue.
  - **Metabolism**: Ribavirin is metabolized to 5'phosphates, de-riboside, and deriboside carboxylic acid.
  - **Excretion**: About one third of absorbed Ribavirin is excreted into the urine unchanged, and the rest is excreted into urine as the de-ribosylated base 1, 2, 4-triazole 3-carboxamide, and the hydrolysis product of this, 1, 2, 4-triazole 3-carboxylic acid.

- **Mechanism of action:**

  - **RNA viruses**:

Ribavirin's carboxamide group can make the native nucleoside drug resemble adenosine or guanosine, depending on its rotation. For this reason, when Ribavirin is incorporated into RNA, as a base analog of either adenine or guanine, it pairs equally well with either uracil or cytosine, inducing mutations in RNA-dependent replication in RNA viruses. Such hypermutation can be lethal to RNA viruses.

  - **DNA viruses**

Neither of these mechanisms explains effect of Ribavirin on many DNA viruses, which is more of a mystery, especially given the complete inactivity of Ribavirin's 2' deoxyribose analogue,

which suggests that the drug functions only as an RNA nucleoside mimic, and never a DNA nucleoside mimic. Ribavirin 5'-monophosphate inhibits cellular inosine monophosphate dehydrogenase, thereby depleting intracellular pools of GTP. (30)

- **Use :**

Ribavirin is a broad spectrum antiviral drug. It is effective against both RNA and DNA viruses.

i) Ribavirin has useful activity against many viruses of interest, including avian influenza, hepatitis B, polio, measles, and smallpox.

ii) Ribavirin has been found effective for the treatment of respiratory syncytial virus (RSV), infection in immunosuppressed and high-risk children and adults, viral haemorrhagic fever (VHF) caused by Arenaviridae and Bunyaviridae (Lassa, Junin, Crimean-Congo and Hantaan, a hanta virus) (as per FDA approval WHO), and hepatitis C virus (HCV) infection.

iii) The United States Food and Drug Administration (FDA) has approved the use of Ribavirin for treatment of respiratory syncytial virus and hepatitis C virus infection.

iv) It has been used (in combination with Ketamine, Midazolam, and Amantadine) in treatment of rabies.

- **Adverse effect**

1. Central nervous system: Fatigue, headache, insomnia, irritability/ anxiety/ nervousness, depression, concentration impairment, mood alternation.

2. Gastrointestinal: Nausea, anorexia,vomiting, abdominal pain, dry mouth, dyspepsia.

3. Hematologic: Anemia, lymphopenia, neutropenia, thrombocytopenia.

4. Musculoskeletal, Connective Tissue and Bone: Myalgia, arthralgia, back pain.

5. About<1% patient causes Hypotension, cardiac arrest, digitalis toxicity, rash, skin irritation, conjunctivitis, mild bronchospasm, worsening of respiratory function, apnea.

6.4 DESCRIPTION OF SOYA-L-α-LECITHIN (SPC):

Lecithin is a generic term to designate any group of yellow-brownish fatty substances occurring in animal and plant tissues composed of phosphoric acids, choline, fatty acids, glycerol, glycolipids, triglycerides and phospholipids (e,g., phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI)). The lecithin used by us mainly contains soya-l- α-lecithin and L- α-phosphatidylcholine.

- **Synonym:-** Plinopc; Azolectin; 16:0-18:2 PC;L-alpha lecithin; Lecithin (SOY), L- ; lecithin soybeans; Lecithin(EGG), L-; 2-linleoyl-1-palmitoyl; 2-linoleoyl-1-palmitoyl; 3-snphosphatidylcholine.

- **Structure: -**

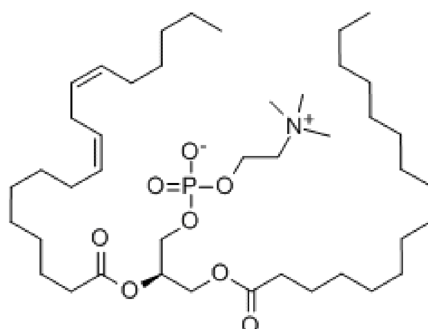


Figure 6.2 : Structure of Soya-L-α-Lecithin (SPC)

- **Molecular weight: -** 760.09 g/mol.
- **Molecular Formula: -** $C_{42}H_{82}NO_8P$
- **Solubility: -** Soluble in chloroform: 50 mg/ml, clear, very faintly yellow solution. Soluble in hexane-ethanol, methanol, ethanol, toluene, ether, mineral oils, fatty acids. Sparingly soluble in benzene. Insoluble in water (CMC < 0.001nM), cold acetone, cold vegetable and animal oils.

- **Sources of lecithin**

Lecithin can be obtained from plants, animals and marine sources. It is usually commercially available from sources such as soy beans, eggs, milk, marine sources, rapeseed, cottonseed, and sunflower. It is a complex mixture of acetone-insoluble phosphatides that consist chiefly of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, combined with various amounts of other substances such as triglycerides, fatty acids and carbohydrates. Among refined substances, especially concentrated sources of lecithin include dehydrated (powdered) egg yolk (14-20%), natural egg yolk (7-10%), wheat germ (2.82%), soy oil (1.8%) and butter fat (1.4%). Soy oil has the highest lecithin and phosphatide content of any known oil.

- **Pharmacokinetics**

Phosphatidylcholine is absorbed into the mucosal cells of the small intestine, mainly in the duodenum and upper jejunum, following some digestion by the pancreatic enzyme phospholipase, producing lysophosphatidylcholine (lysolecithin). Reacylation of lysolecithin takes place in the intestinal mucosal cells, reforming phosphatidylcholine, which is then transported by the lymphatics in the form of chylomicrons to the blood. Phosphatidylcholine is transported in the blood in various lipoprotein particles, including very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). It is then distributed to the various tissues of the body. Some phosphatidylcholine is incorporated into cell membranes.

- **Side Effects:**

It can cause some side effects including diarrhea, nausea, abdominal pain, or fullness. Lecithin is converted by gut bacteria into trimethylamine-N-oxide (TMAO), which is released into circulation, and may with time; contribute to atherosclerosis and heart attacks.

- **Uses:**

1) In the pharmaceutical industry, it acts as a wetting, stabilizing agent and a choline enrichment carrier, helps in emulsifications and encapsulation, and is a good dispersing agent.

2) Lecithin is used for treating memory disorders such as dementia and Alzheimer's disease. It is also used for treating gallbladder disease, liver disease, certain types of depression, high cholesterol, anxiety, and a skin disease called eczema. Some people apply lecithin to the skin as a moisturizer. Lecithin is used as an ingredient in some eye medicines to keep the medicine in contact with the eye's cornea.

3) In the paint industry, it forms protective coatings for surfaces with painting and printing ink. It has antioxidant properties, helps as a rust inhibitor, is a colour-intensifying agent, stabilizing and suspending agent.

4) Lecithin is used in a surprisingly large array of our daily foods. Perhaps most widely used in margarine (for anti-spatter and as an emulsifier), it is also used in chocolates, caramels and coatings (to control viscosity, crystallization, and sticking), in chewing gum (for its softening, plasticizing, and release effects), in instant foods such as cocoa powders, coffee creamer and instant breakfast (for wetting, dispersing, and emulsifying), in calf milk replacers (to add energy and aid digestibility and emulsification). It is also found in baked goods, cheeses, meat and poultry products, dairy and imitation dairy products, and still other products. Lecithin is applied in a thin film to a cooking utensil or a mold; it promotes release of food or other materials from that surface. It also serves as a stabilizer in ice creams and an antioxidant in oils and fats.

6.5 DESCRIPTION OF CHOLESTEROL:

Structure:-

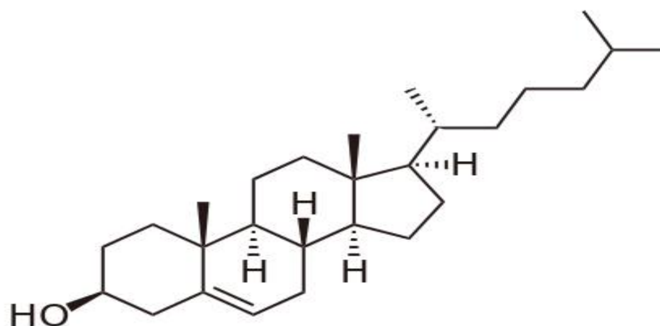


Figure 6.3 : Structure of cholesterol

- **IUPAC name: -** 10,13-dimethyl-17- (6-methylheptan-2-yl) -2,3,4,9,11,12,14,15,16,17-decahydro-1H-cyclopenta[a]phenanthren-3-ol.
- **Chemical Formula**: - $C_{27}H_{46}O$.
- **Appearance: -** white crystalline powder,
- **Molecular weight**:- 386.66
- **Melting point: -** 148–150°C.
- **Boiling point: -** 360°C (decomposes)
- **Solubility: -** Solubility in water 0.095 mg/L (30°C).Soluble in acetones, benzene, chloroform, ethanol, ethers, hexane, isopropyl myristate and methanol.
- **Dietary Source**: -

From a dietary perspective, cholesterol is not found in significant amounts in plant sources. In addition, plant products such as flax seeds and peanuts contain cholesterol-like compounds called phytosterols, which are believed to compete with cholesterol for absorption in the intestines. Animal fats are complex mixtures of triglycerides, with lesser amounts of phospholipids and cholesterol. As a consequence, all foods containing animal fat contain cholesterol to varying extents. Major dietary sources of cholesterol include cheese, egg yolks,

beef, pork, poultry, fish, and shrimp. Human breast milk also contains significant quantities of cholesterol.

- **Pharmacokinetics**

Cholesterol is found in all body tissues, especially in the brain, spinal cord, and in animal fats or oils. Cholesterol is distributed universally in all animal tissues. It can be derived either from intestinal absorption of dietary cholesterol or from synthesis de novo within the body. The fraction of dietary cholesterol absorbed is dependent on the intake. After reaching a plateau, the amount absorbed decreases with increased dietary intake. The primary site of absorption of dietary cholesterol is the proximal small intestine. Cholesterol is oxidized by the liver into a variety of bile acids. These, in turn, are conjugated with glycine, taurine, glucuronic acid, or sulfate. A mixture of conjugated and non-conjugated bile acids, along with cholesterol itself, is excreted from the liver into the bile. Approximately 95% of the bile acids are reabsorbed from the intestines, and the remainders are lost in the faeces. Cholesterol is eliminated from the body via the faeces, urine and skin surface. The cholesterol and cholesterol derivatives excreted in the faeces are in the form of bile salts. Cellular and membrane cholesterol from cells is sloughed from the intestine, unabsorbed dietary cholesterol and metabolic products of gut bacteria. Cholesterol and its derivatives excreted in the faeces accounts for about 50% of the total cholesterol in man. Cholesterol is also eliminated in the milk of lactating females, primarily in the membranes of milk fat globules.

- **Physiological Function**

Cholesterol is required to build and maintain membranes. It modulates membrane fluidity over the range of physiological temperatures. The structure of the tetracyclic ring of cholesterol contributes to the decreased fluidity of the cell membrane as the molecule remains in a trans-conformation making all but the side chain of cholesterol rigid and planar. In this structural role, cholesterol reduces the permeability of the plasma membrane to neutral solutes, protons (positive hydrogen ions) and sodium ions. Within the cell membrane, cholesterol also functions in

intracellular transport, cell signaling and nerve conduction. Cholesterol is the precursor molecule in several biochemical pathways. In the liver, cholesterol is converted to bile, which is then stored in the gallbladder. Bile contains bile salts which solubilize fats in the digestive tract and aid in the intestinal absorption of fat molecules as well as the fat-soluble vitamins, A, D, E and K. Cholesterol is an important precursor molecule for the synthesis of vitamin D and the steroid hormones including the adrenal gland hormones, cortisol and aldosterone, as well as the sex hormones progesterone, estrogen, testosterone, and their derivatives.

- **Pharmaceutical use**

Cholesterol is used as an emulsifying agent in the pharmaceutical formulations. It is also used as humectants. Liquid crystal properties of cholesterol and its derivatives (salt and ester) make them useful in the commercial applications of cosmetics and pharmaceuticals. Cholesterol is used in liposomes to encapsulate and deliver chemotherapeutic drugs to diseased tissues. Cholesterol-C14 is used clinically as an organ imaging agent and organs visualized by the technique include ovaries, adrenals, and spleen.

6.6 DESCRIPTION OF BUTYLATED HYDROXY TOLUENE (BHT):

Structure:

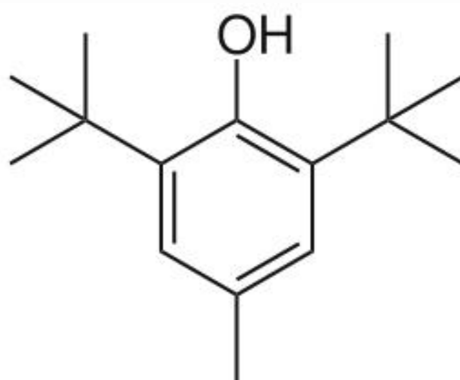


Figure 6.4 : Structure of BHT

- **IUPAC name: -** 2, 6-Bis (1, 1-dimethylethyl)-4-methylphenol
- **Synonyms: -** 2, 6-Di-tert-butyl-4-methylphenol; 2, 6-Di-tert-butyl-p-cresol; 3,5-Di-tert-butyl-4- hydroxytoluene (BHT)
- **CAS Registry Number: -** 128-37-0
- **Molecular formula: -** $C_{15}H_{24}O$
- **Molar mass: -** 220.35 g./mol
- **Appearance: -** White to yellow powder
- **Density: -** 1.048 g/cm^3
- **Melting point: -** 70°C
- **Boiling point: -** 265°C

BHT is primarily used as a food additive that exploits its antioxidant properties. BHT is also used as an antioxidant additive in diverse products such as cosmetics, pharmaceuticals, rubber, electrical transformer oil (at 0.35%), and embalming fluid. In the petroleum industry, where BHT is known as the fuel additive AO-29, it also finds uses in hydraulic fluids, turbine and gear oils, and jet fuels, among other applications. BHT is also used to prevent peroxide formation in diethyl ether and other laboratory chemicals.

6.7 DESCRIPTION OF DSPE-PEG 2000:

Structure:

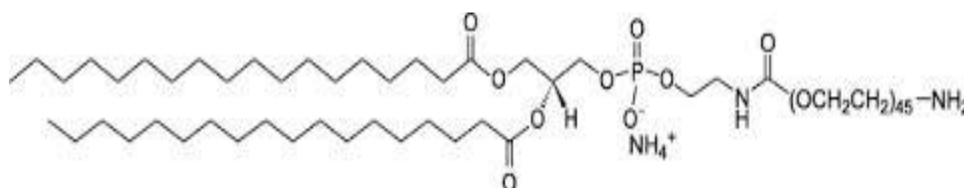
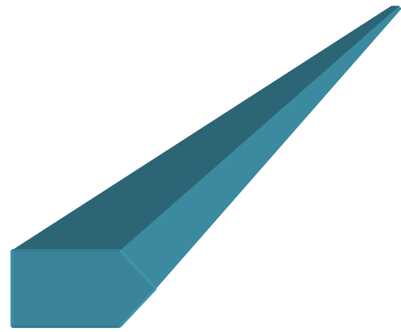


Figure 6.5 : Structure of DSPE-PEG 2000

This amphiphilic polymer is composed of a hydrophobic core (DSPE) and a hydrophilic shell (PEG).

- **IUPAC name:** 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000
- **Appearance:** White crystalline powder
- **Use**: Poly (ethylene glycol)–distearoylphosphatidylethanolamine (PEG-DSPE) block copolymers are biocompatible and amphiphilic polymers that can be widely utilized in the preparation of liposomes, polymeric nanoparticles, polymer hybrid nanoparticles, solid lipid nanoparticles, lipid–polymer hybrid nanoparticles, and microemulsions. Dos et al showed that only 0.5 mol% of PEG2000-DSPE can significantly increase the plasma circulation longevity of the liposomes when compared to 1, 2-distearoyl-*sn*-glycero-3-phosphatidylcholine (DSPC) (Dos et al, 2007)
- **Advantages:**
    1. It can be linked easily with targeted ligand.
    2. It can prolong the circulation time.
    3. It can improve bioavailability.
    4. It can reduce side effects.



Chapter 7

Methods

7. METHODOLOGY:

7.1 Development of calibration curve for ribavirin:

- **Preparation of PBS pH 7.4**

  Phosphate buffered saline (PBS), pH7.4, was prepared according to I.P. protocol. Disodium hydrogen phosphate (Na_2HPO_4) (2.38 g), 0.19 g potassium hydrogen phosphate (KH_2PO_4) and 8.0 g sodium chloride (NaCl) were dissolved in 1000 ml doubled distilled water and pH of the solution was adjusted at 7.4 using a precalibrated pH meter (Eutech Instruments, Kolkata.)

- **Determination of absorption maxima of Ribavirin:**

  Absorption maxima of Ribavirin were measured in two different media, one is in PBS (pH7.4) and another is in a mixture of PBS (pH 7.4): ethanol = 5:1. About 10 mg of drug was accurately weighed on a digital balance (Sartoious, Goetingen, Germany) and dissolved in 10 ml PBS in a volumetric flusk to produce a 1000 µg/ml solution. This solution was diluted 100 times with the same media to produce of 10 µg/ml solution concentration. This drug solution was scanned in the wavelength from 200 nm to 400 nm using PBS as blank solvent. The same process was repeated using a mixture of PBS and ethanol (5:1) as solvent. PBS (pH7.4) was used as medium for drug release study and PBS-ethanol mixture was taken as medium for drug loading study.

- **Preparation of calibration curve in PBS**

  About 10 mg of drug was accurately weighed using a digital balance and taken in a volumetric flask of 10 ml capacity. The previously prepared PBS (pH7.4) was added into the volumetric flask up to 10 ml mark. The flask was shaken well for proper dissolution of drug. This solution was 100 times diluted with the same media to prepare stock solution of Ribavirin having concentration of 10 µg/ml in PBS (pH 7.4). From the stock solution of drug 5 solutions of different concentrations were prepared. Absorbance of the above solutions was determined against PBS (pH7.4) as blank at a wavelength of 208 nm.

- **Preparation of calibration curve in PBS (pH 7.4)-ethanol mixture (5:1)**

  At first a homogenous mixture of PBS (pH7.4) and ethanol at a ratio of 5:1 was prepared. Then 10 mg of Ribavirin was accurately weighed on a digital balance and was taken in a volumetric flask of 10 ml capacity. The previously prepared PBS-ethanol mixture was added into it up to 10 ml mark. The flask was shaken well for proper dissolution of drug. This solution was 100 times diluted with the same media to prepare stock solution of Ribavirin having concentration of 10 µg/ml in PBS-ethanol mixture (5:1). From the stock solution five different concentrations were prepared. Absorbance of the above solutions was determined against PBS ethanol mixture prepared in the same way as mentioned above without the drug at a wavelength of 207.5 nm.

7.2 Preformulation study:

- **Drug-excipients interaction study by FTIR:**

  To study drug excipients interaction nine different specimens were taken as follows: 1) pure drug (Ribavirin), 2) cholesterol, 3)DSPE-PEG 2000, 4)SPC 5)BHT 6) a physical mixture of drug, DSPE-PEG 2000, SPC, BHT and cholesterol, 7) physical mixture of DSPE-PEG 2000, SPC, BHT and cholesterol, 8) drug formulation, and 9) formulation without drug. Each specimen was mixed separately with infrared (IR) grade potassium bromide (KBr) at the ratio of 1:100 and 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 an FTIR instrument (JASCO Magna IR 750 series II FTIR instrument, JASCO International Co. Ltd. FTIR 4200 Tokyo, Japan).

7.3 Preparation of liposome:

- **Lipid layer hydration method:**

  Liposomes were prepared by lipid layer hydration method (Rudra et al 2010; Mukherjee et al, 2007).Weighed amount of cholesterol, DSPE-PEG 2000 and BHT (2% of total lipid) *(Layek et al, 2010)* were taken in a round bottom flask of 250 ml capacity and

dissolved in chloroform by vigorous shaking. The mixture containing round bottom flask was placed in a rotary vacuum evaporator (Rotavap, Superfit, Model PBU-6) fitted with A3S aspirator (Eyela, Rikakikaic, Ltd.) and cold circulating water-bath (Spac N service, Kolkata-42, India) and rotated at 100 rpm at 30ºC in a water bath to evaporate the solvent. Then flask containing thin film was kept in vacuum desiccators overnight for complete removal of residual chloroform. On the next day, weighed amount of Ribavirin was dissolved in isotonic phosphate buffer (pH 7.4) and poured into the flask containing lipid film. It was then hydrated at 40ºC in a water-bath fitted with a rotary vacuum evaporator and was rotated at 150 rpm with the vacuum off, until the lipid film was dispersed in the aqueous phase. The milky white dispersion was sonicated in a bath sonicator at the same temperature for 45 min. After sonication, the dispersion was kept at room temperature for minimum 1h for the complete vesicle formation and then the preparation was kept at 8ºC in an inert atmosphere for overnight. Then it was centrifuged at 15,000 rpm for 1hr at 4ºC temperature to sediment the suspended liposomal vesicles. Then the supernatant was discarded and liposomal pellets were taken in a petri dish and stored in -20°C for standard pre-freezing overnight and lyophilized in a laboratory freeze dryer (Instrumentation India, Kolkata-32, India) until it became completely dry. To produce a blank formulation, the same process was done simultaneously using a different flask without adding the drug in buffer solution during hydration.

- **Reverse-phase evaporation method:**

  The lipid phase (weighed amount of cholesterol, DSPE-PEG 2000 and BHT and weighed amount of cholesterol, DSPE-PEG 2000, BHT and SPC) were dissolved in 15 ml of chloroform and mixed with 5 ml of drug solution in PBS (pH 7.4).The mixture was sonicated for 3 min in an ice bath. From the water-in-oil emulsion formed, the organic solvent was slowly removed by a rotary evaporator under reduced pressure at room temperature. Subsequently, PBS was added and the mixture was kept in the rotary evaporator for another 20 min at room temperature for removal of the residual solvent. Then the dispersion was sonicated in an ice bath and kept at room temperature for 1 h for vesicle formation. The preparation was centrifuged at 15000 rpm for 1 h and lyophilized after overnight storage at 4°C.

Table 7.1: Composition of liposome formulations containing Ribavirin

Formulation code	Composition	Amount of drug (mg)
F1	DSPE-PEG2000: Cholesterol (1:1)	10
F2	DSPE-PEG2000: Cholesterol (2:1)	10
F3	DSPE-PEG2000:SPC: Cholesterol (1:1:1)	10
F4	DSPE-PEG2000:SPC: Cholesterol (1:2:1)	10
F5	DSPE-PEG2000:SPC: Cholesterol (1:3:1)	10

7.4 Physicochemical characterization and evaluation of the formulations:

- **Surface morphology study by field emission scanning electron microscopy (FESEM):**
  Surface morphology of the lipid vesicles were determined by FESEM study. The freshly prepared lyophilized formulation was placed onto a metallic stub using adhesive carbon tape. Then it was undergone gold or platinum coating onto a thickness of 4 nm and the coated sample was vacuum dried and examined under FESEM.

- **Particle size distribution study**
  The size distribution of the reconstituted lyophilized liposomes was determined by dynamic light scattering (DLS) (ZETASIZER, NanoZS 90, MALVERN Instrument Ltd. Andhrapradesh, India) 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 average particle diameter (Z average) was calculated by the machine software from the measured particle size distribution. The freshly prepared lyophilized

formulations were suspended in double distilled water and poured into the cuvette and analyzed by the instrument.

- **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 less than one. Generally for the monodisperse 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 (DLS) technique (DYNAMIC LIGHT SCATTERING COMMON TERMS DEFINED © 2011 Malvern Instruments Limited). Polydispersity index of the different formulation was done by the instrument Zeta sizer nano ZS (ZETASIZER, NanoZS 90, MALVERN Instrument Ltd. Andhrapadesh, India).

- **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. It is one of the parameters, which affects dispersion stability. Zeta potentials of the different formulations were determined by the instrument Zeta sizer nano ZS (ZETASIZER, NanoZS 90, MALVERN Instrument Ltd. Andhrapradesh, India).

- **Evaluation of Ribavirin loading**

  At first a mixture of PBS (pH7.4) and ethanol at a ratio of 5:1 was prepared. About 2 mg of lyophilized formulation was weighed accurately and taken in a 2 ml of microcentrifuge tube. To it, 2 ml of PBS-ethanol mixture was added and formulation was suspended by vortex. Then it was sonicated for one hour and centrifuged at 10,000 rpm for 15 minutes. After centrifugation small aliquot of supernatant was collected and 10 times diluted with PBS-ethanol mixture. The absorbance of the supernatant solution was measured in UV/VIS Spectrophotometer (Advanced Microprocessor UV-VIS Single Beam, Intech-295, Andhrapradesh, India) against the blank at the wavelength of 207.5 nm. The drug content was determined from the calibration curve.

Percentage drug loading was calculated using the following formula:

$$\% \text{ Loading} = \frac{(\text{Weight of the drug in a formulation}) \times 100}{\text{Total weight of formulation}}$$

Percentage loading efficiency was determined using the following formula

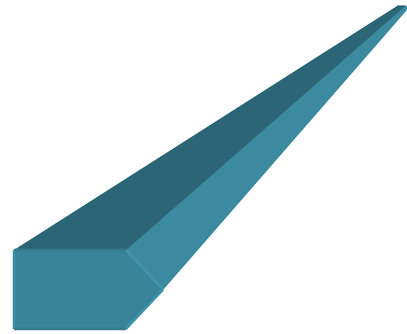
$$\% \text{ Loading efficiency} = \frac{(\text{weight of the drug in 1 mg formulation}) \times (\text{total weight of formulation})}{(\text{Total amount of drug taken for each formulation})}$$

- ***In vitro* drug release study from the formulation through dialysis membrane:**

In a 100 ml beaker, 50 ml of phosphate-buffered saline (PBS) was measured *(Mukherjee B et al, 2007).* A weighed amount of lyophilized liposomes (5 mg) was reconstituted in 1 ml PBS and was taken into a dialysis bag (HiMedia dialysis membrane-60, Mumbai, India). The two ends of the dialysis bag were tightly bound with cotton thread. The bag was hung inside the beaker with the help of a clamp-stand assembly so that a significant portion of the dialysis bag containing the formulation could dip into the buffer solution. The flask was kept on a magnetic stirrer. Stirring was maintained at 200 rpm with the help of a magnetic bead at room temperature. Sampling was done by withdrawing 1 ml from the released medium and 1 ml fresh medium was added. The samples were analyzed in a spectrophotometer at 208 nm. The concentration was calculated from the standard curve.

- **Study of drug release kinetics:**

  Drug release data from the different formulations was analyzed using studied several kinetic models such as Higuchi model, Hixson-Crowell model, Krosmeyer-Peppas model, zero order kinetics and first order kinetics. Comparative study was also done to find out which pathway or model is best followed by the formulation through graphical interpretation of each model. Different kinetic models represent different mechanism of drug release from formulation *e.g.,* Higuchi model represents drug released from formulation through diffusion only, Krosmeyer-Peppas model represents drug release through both diffusion and erosion, Zero order model represents that drug release does not depend on concentration of drug present etc.



Chapter 8

Results

8. Results

8.1 Preparation of calibration curve of ribavirin in PBS pH 7.4.

1. Determination of absorption maxima of ribavirin in PBS:

The solution of ribavirin in PBS pH 7.4 was scanned in the wavelength range from 200 nm to 400 nm using PBS as blank in spectrophotometer (Advanced Microprocessor UV-VIS Single Beam, Intech-295, Andhrapradesh, India) to find out the absorption maxima. The wavelength at 208 nm shows maximum absorption (Fig. 8.1).

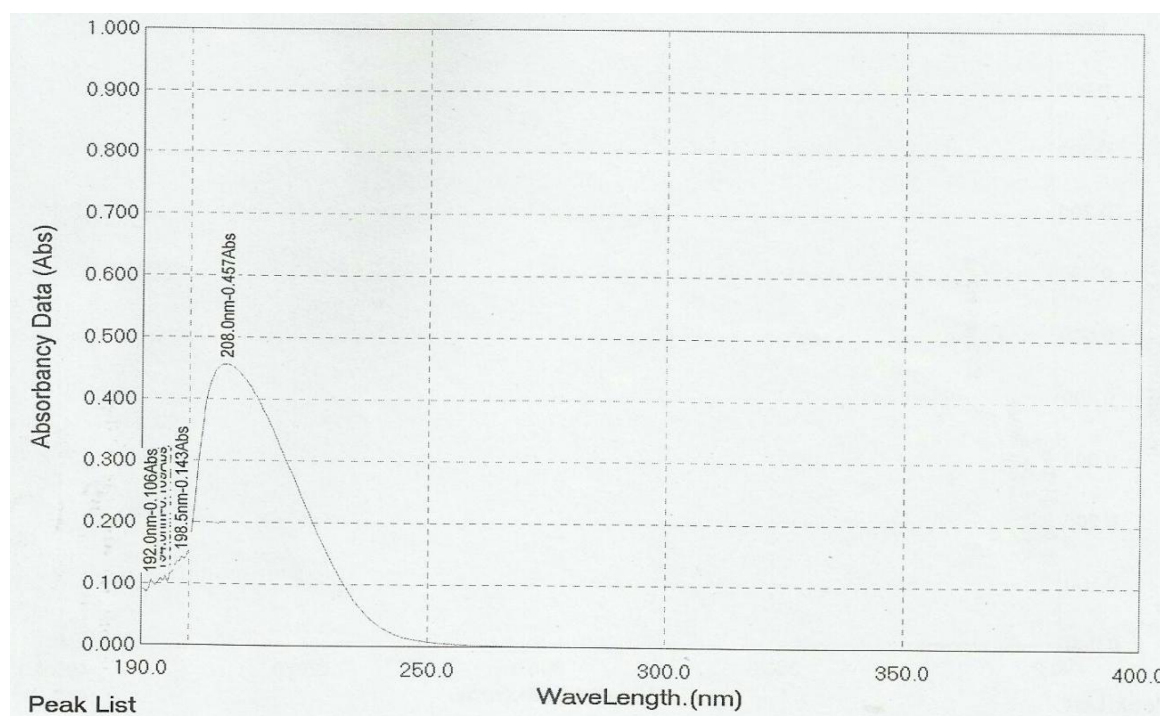


Figure 8.1: Wave length at 208 nm shows maximum absorption of ribavirin in PBS

2. Preparation of calibration curve:

A stock solution of concentration 10 µg/ml was prepared by dissolving ribavirin in PBS, pH 7.4. Aliquots were taken from this stock solution and solutions of different concentrations of ribavirin were prepared with PBS (pH-7.4). Calibration curve of the drug was prepared by measuring the absorbances of prepared solutions at 208 nm wavelength. The mean absorbances (n=3) of the

drug against different concentrations (Table 8.2) of the drug were plotted. (Fig. 8.3) The calibration curve was used for drug release study.

Table 8.1: Absorbance data of Ribavirin in different concentrations in PBS, pH 7.4

Sample No.	Concentration of drug (µg/ml)	Absorbance Set-1	Absorbance Set-2	Absorbance Set-3	Mean Absorbance (n=3)
1.	2	0.091	0.104	0.116	0.104
2.	4	0.178	0.198	0.209	0.195
3.	6	0.262	0.283	0.285	0.277
4.	8	0.351	0.378	0.372	0.367
5.	10	0.450	0.505	0.504	0.486
6.	20	0.886	0.932	0.928	0.915

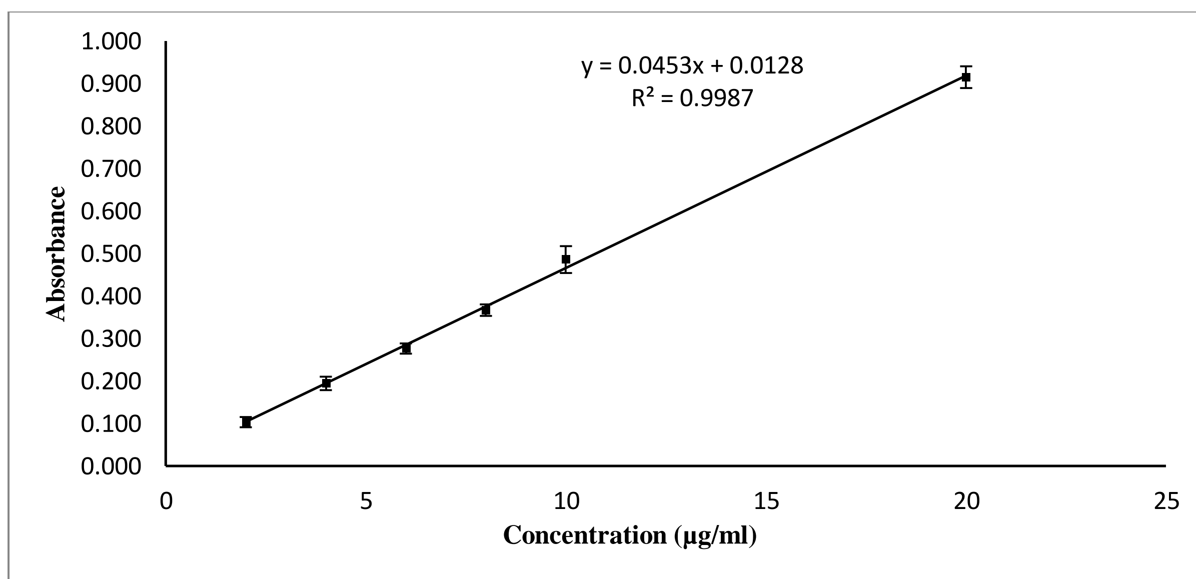


Figure 8.2: Calibration curve of Ribavirin in PBS, pH 7.4

Note: Values are represented as mean±SD (n=3)

8.2 Preparation of calibration curve of ribavirin in ethanol and PBS pH-7.4 mixture (1:5)

1. Determination of absorption maxima of Ribavirin in ethanol - PBS mixture (1:5)

The solution of Ribavirin in ethanol-PBS pH 7.4 mixture (1:5) was scanned in wavelength ranging from 200 nm to 400 nm using ethanol-PBS pH-7.4 mixture (1:5) as blank in spectrophotometer (Advanced Microprocessor UV-VIS Single Beam, Intech-295, Andhrapradesh, India) to find out the absorption maxima. The wavelength at 207.5 nm shows the maximum absorption. (Fig. 8.3)

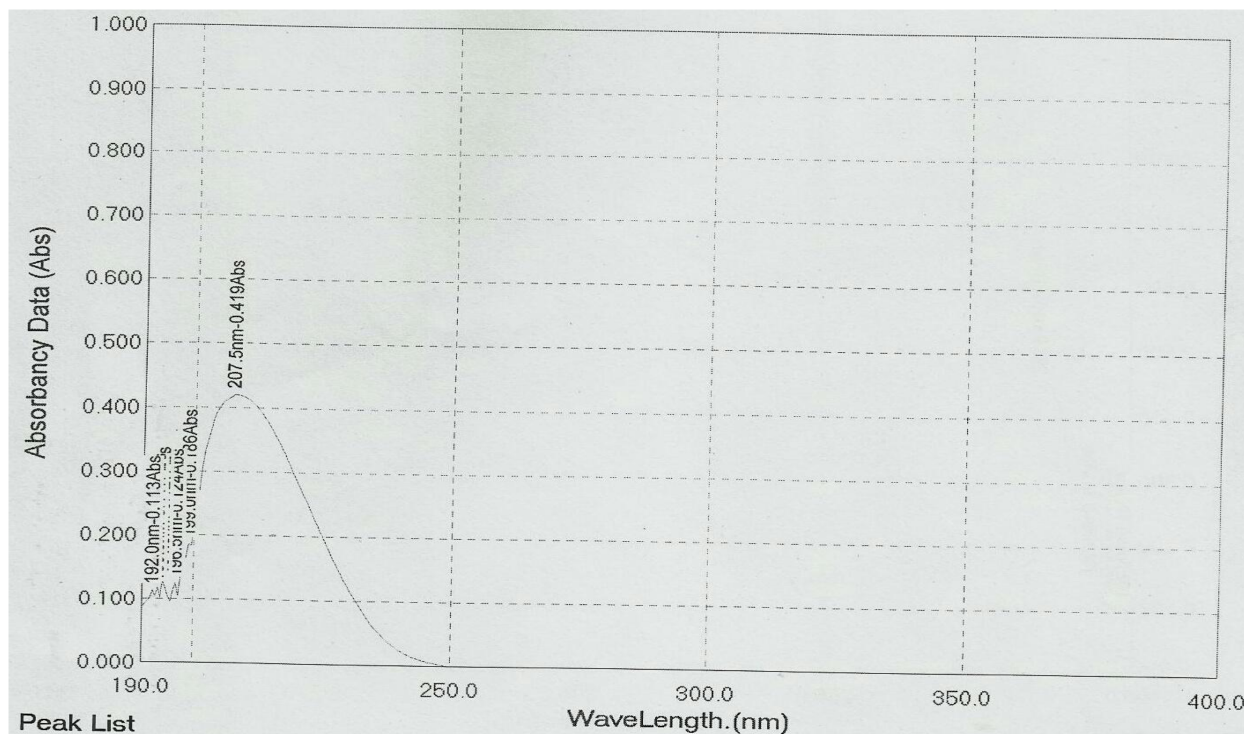


Figure 8.3: Wavelength at 207.5 nm shows absorption maxima.

2. Preparation of calibration curve:

A stock solution of concentration 10μg/ml was prepared by dissolving ribavirin in ethanol -PBS (pH 7.4) mixture (1:5). Aliquots were taken from this stock solution and solutions of different

concentrations of Ribavirin were prepared with ethanol-PBS (pH 7.4) mixture (1:5) (Table-8.2). Calibration curve of the drug was prepared by measuring the absorbance of prepared solutions at 207.5 nm wavelength. The mean drug absorbance (n=3) against different concentrations of the drug (Table-) were plotted as shown in Figure-8.4. The regression coefficient value as shown in the figure was 0.9995 showed the favorable accuracy standard of the experiments. The calibration curve was prepared to investigate drug loading in the formulations. The drug loading of the formulation was studied in ethanol-PBS (pH 7.4) mixture.

Table 8.2 – Absorbance data of Ribavirin at different concentration in ethanol PBS, pH 7.4 mixture

Sample No.	Concentration of drug (µg/ml)	Absorbance Set-1	Absorbance Set-1	Absorbance Set-1	Mean Absorbance(n=3)
1.	2	0.079	0.080	0.080	0.080
2.	4	0.166	0.172	0.174	0.171
3.	6	0.244	0.261	0.257	0.254
4.	8	0.331	0.345	0.343	0.340
5.	10	0.413	0.428	0.415	0.419
6.	20	0.808	0.822	0.821	0.817

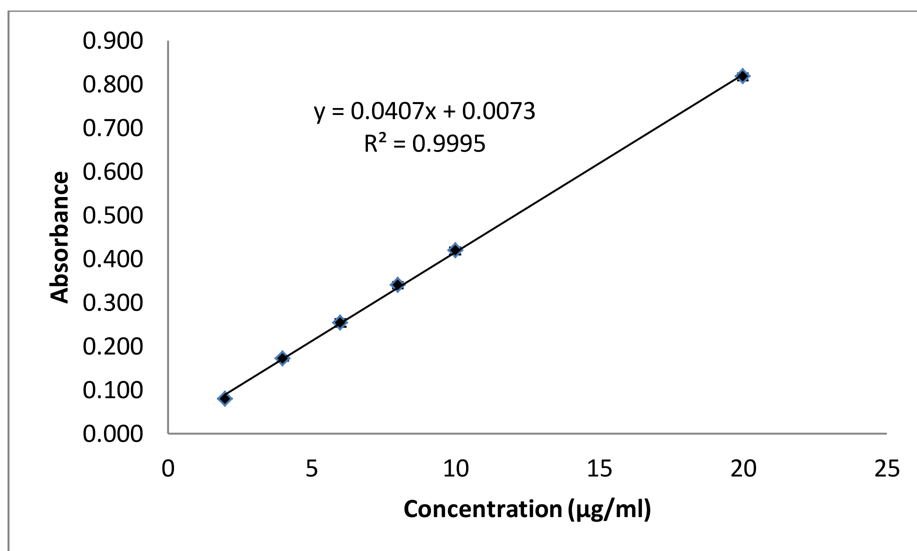


Figure 8.4: Calibration curve of Ribavirin in ethanol-PBS mixture (1:5)

Note: Values are represented as mean±SD (n=3)

8.3 Study of drug-excipient interaction through FTIR spectroscopy:

In the present study, the FTIR spectra of pure drug and excipients showed that the characteristic peaks of the drug and the individual excipients were present in their physical mixture. Ribavirin showed its characteristic peaks at the wave number 958.10 cm^{-1} and 1654.17 cm^{-1}. Ribavirin showed strong intensity bending vibration of C-H at 958.10 cm^{-1} and strong intensity stretching vibration of C=O at 1654.17 cm^{-1}. Soya-lecithin (SPC) has showed its characteristic peak at 2925.50 cm^{-1} whereas DSPE-PEG2000 and BHT showed their characteristic peaks at 1112.91 cm^{-1} and 1459.84 cm^{-1}, respectively. SPC gave its strong intensity C-H stretching at 2925.50 cm^{-1}. DSPE-PEG2000 provided medium intensity stretching vibration of C-N at 1112.91 cm^{-1}. BHT provided medium intensity bending vibration of CH_2 and CH_3 deformation at 1459.84 cm^{-1}. Cholesterol showed its characteristic peak at 1061.91 cm^{-1}. Cholesterol provided strong intensity stretching vibration of C-O at 1061.91 cm^{-1}. When the spectra of the lyophilized formulations were studied, the characteristic peaks of the excipients (DSPE-PEG2000, SPC, and cholesterol) were found to be present. However, minor shifting of some peaks in the range of 3580-3650 cm^{-1} (for OH), 970-1250 cm^{-1} (for C=O), 1710-1740 cm^{-1} (for NH_2) etc. is seen.

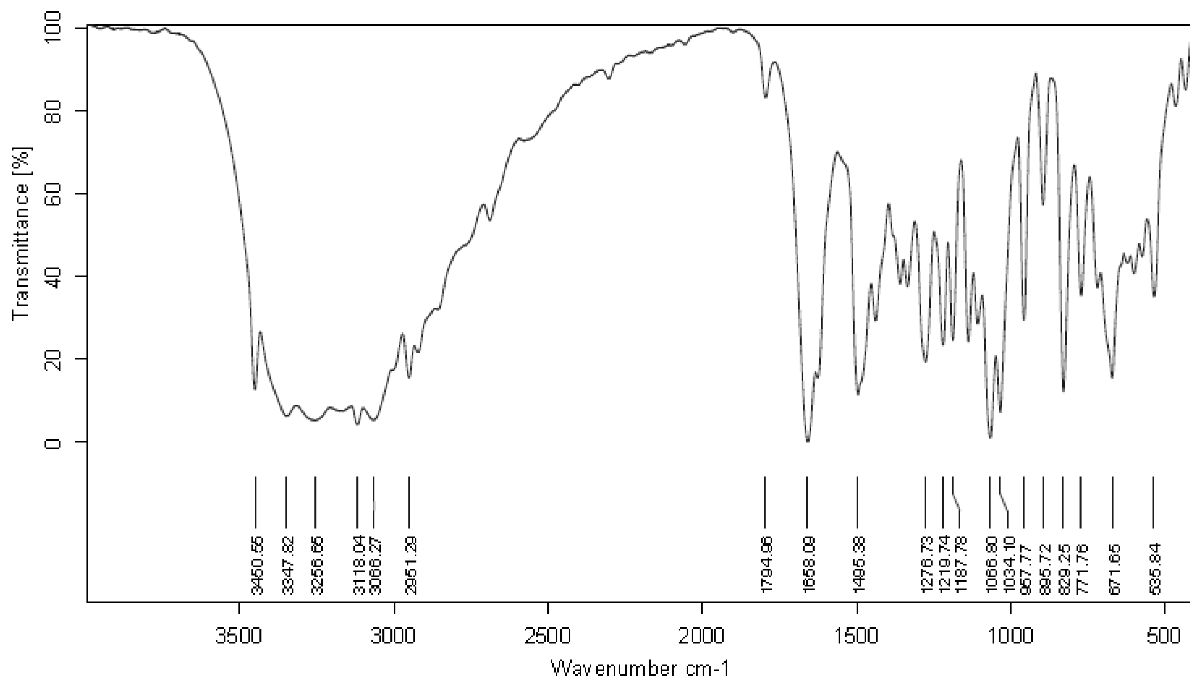


Figure 8.5: FTIR spectroscopic data/spectra of Ribavirin.

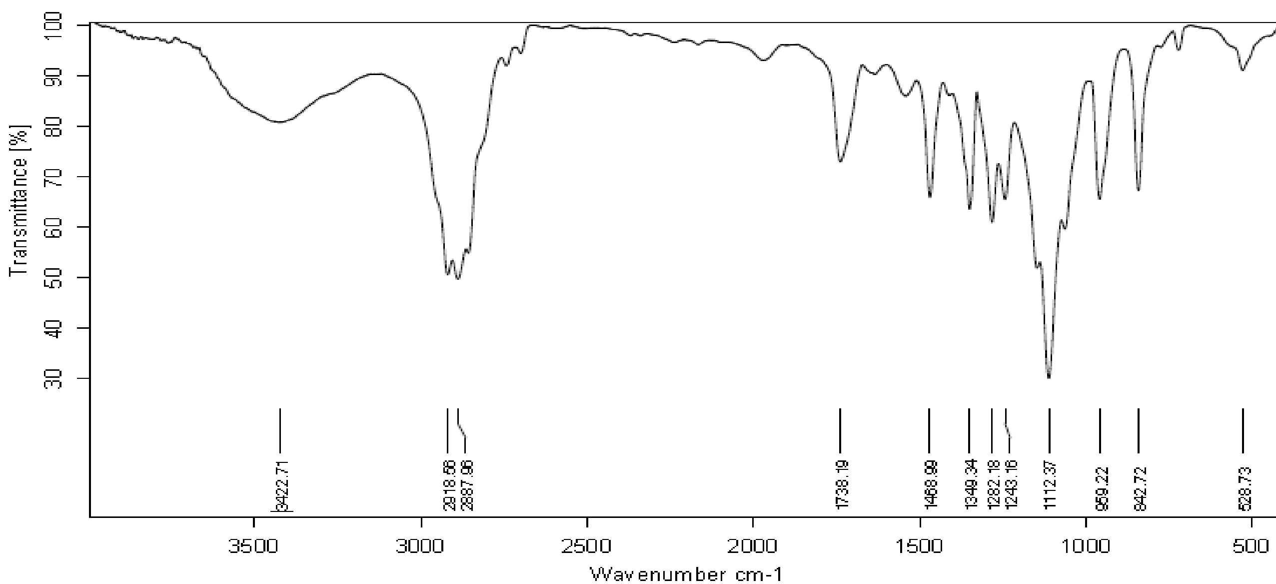


Figure 8.6: FTIR spectroscopic data/spectra of DSPE-PEG 2000.

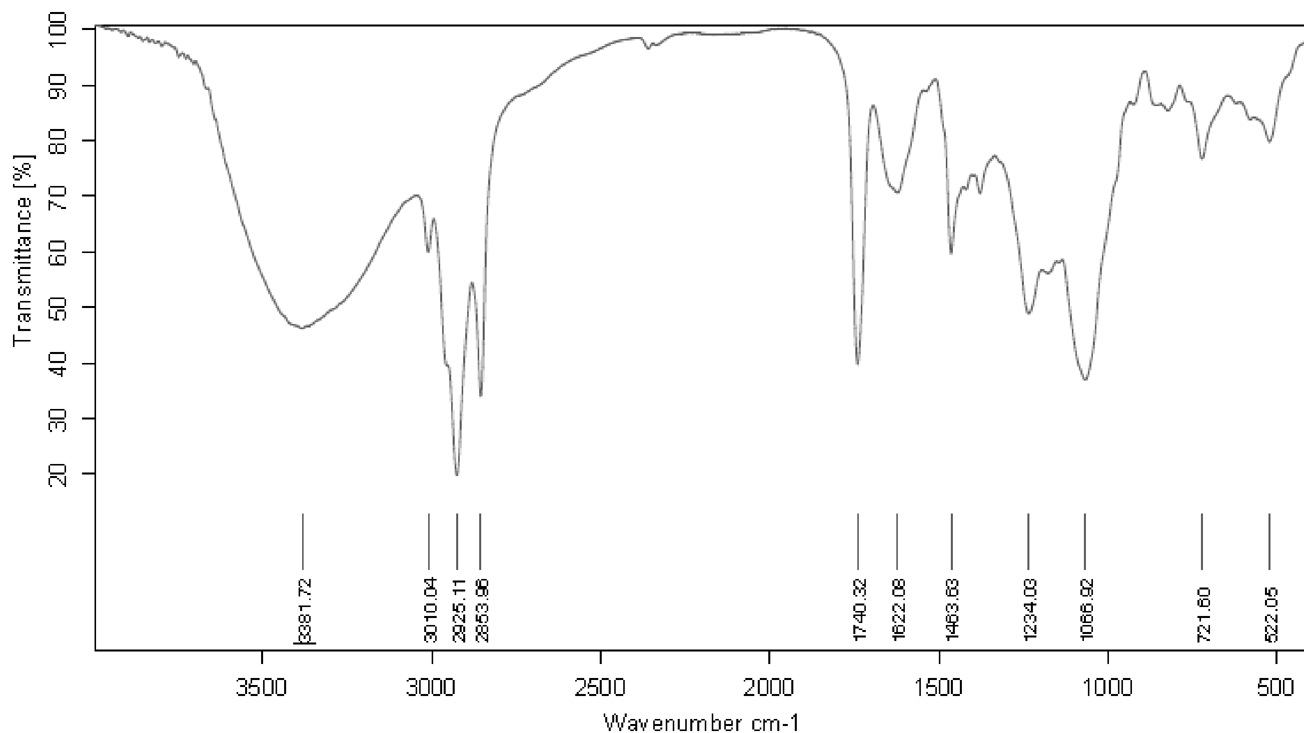


Figure 8.7: FTIR spectroscopic data/spectra of SPC.

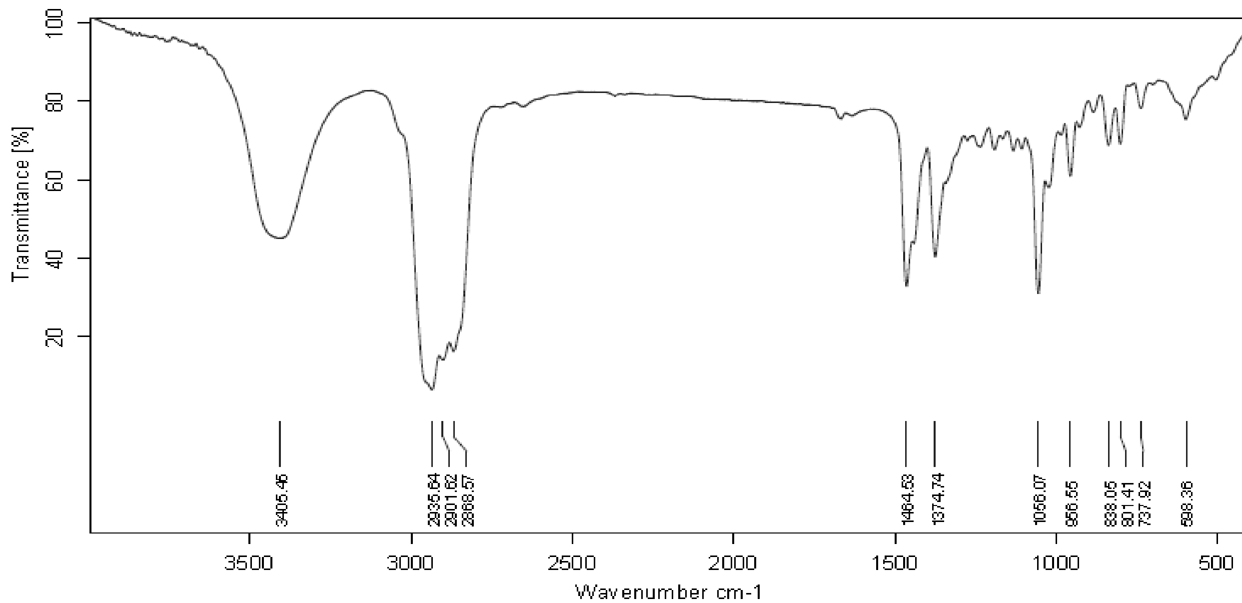


Figure 8.8: FTIR spectroscopic data/spectra of Cholesterol.

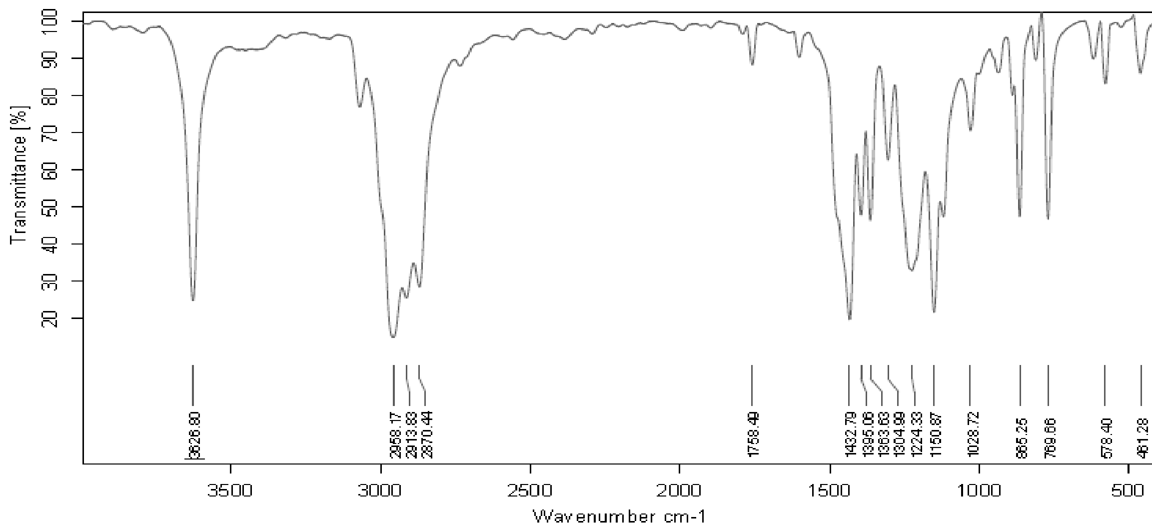


Figure 8.9: FTIR spectroscopic data/spectra of BHT.

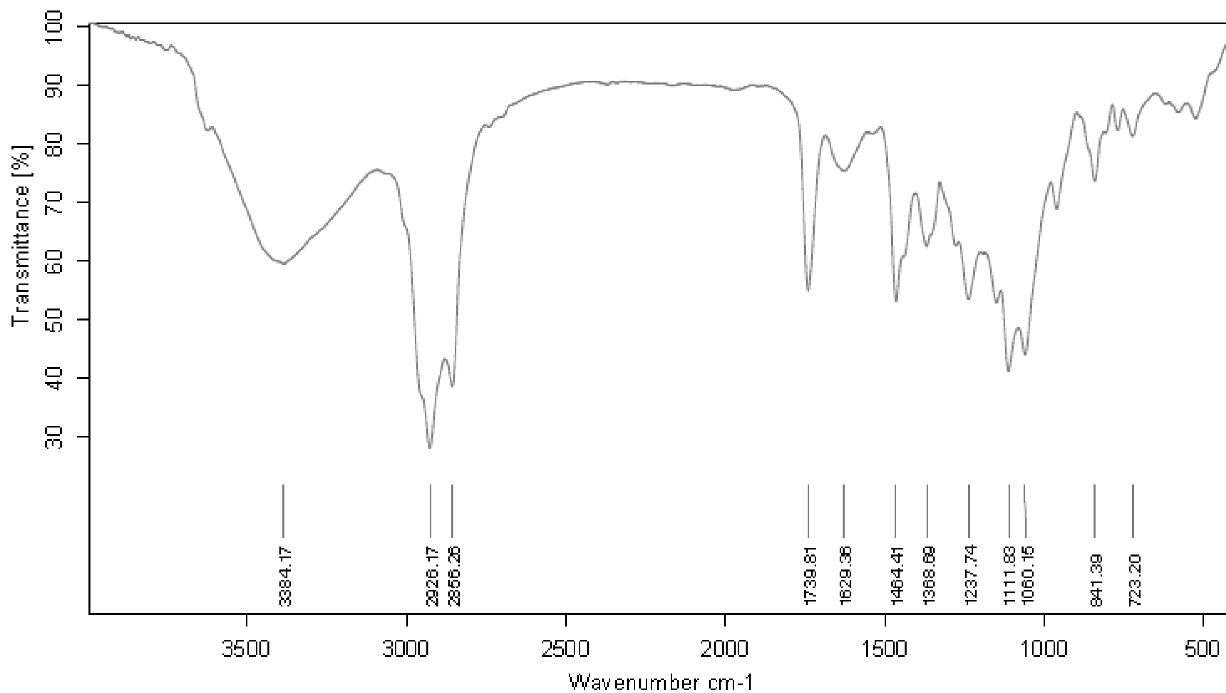


Figure 8.10: FTIR spectroscopic data/spectra of physical mixture without drug.

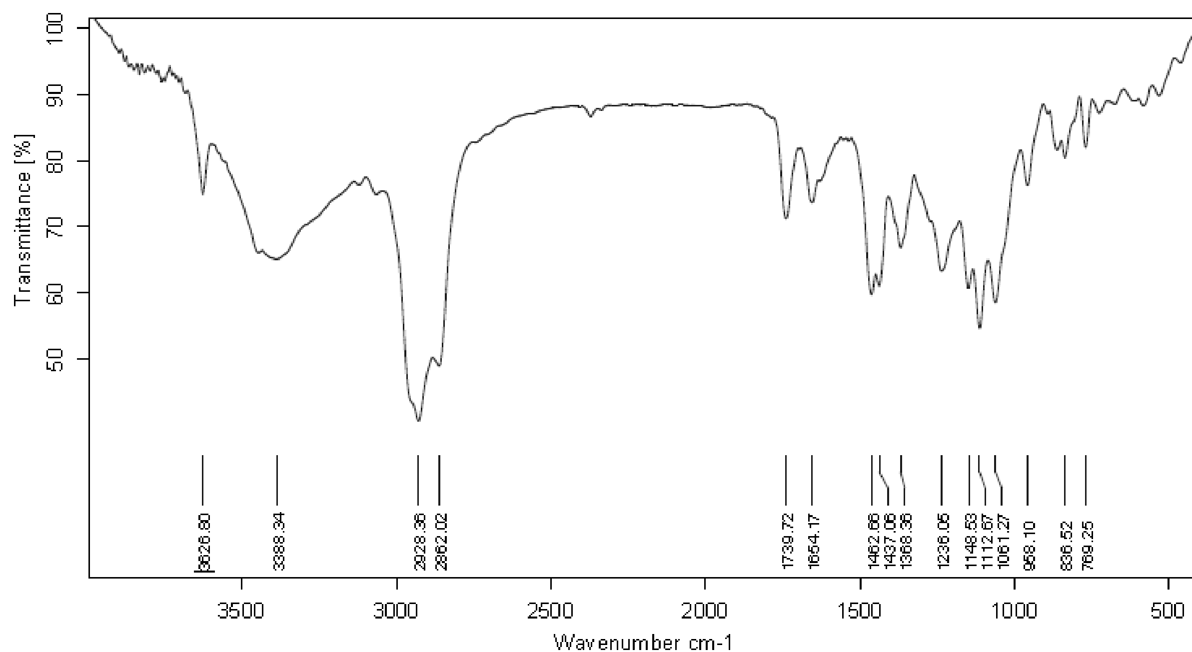


Figure 8.11: FTIR spectroscopic data/spectra of physical mixture with Ribavirin.

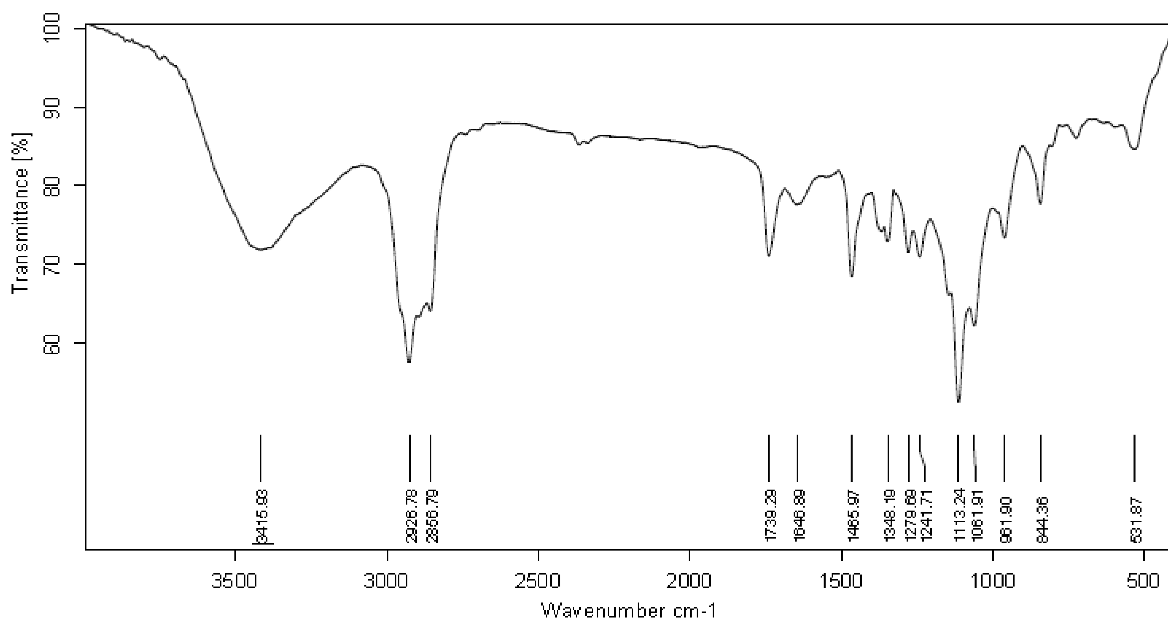


Figure 8.12: FTIR spectroscopic data/spectra of lyophilized formulation without Ribavirin.

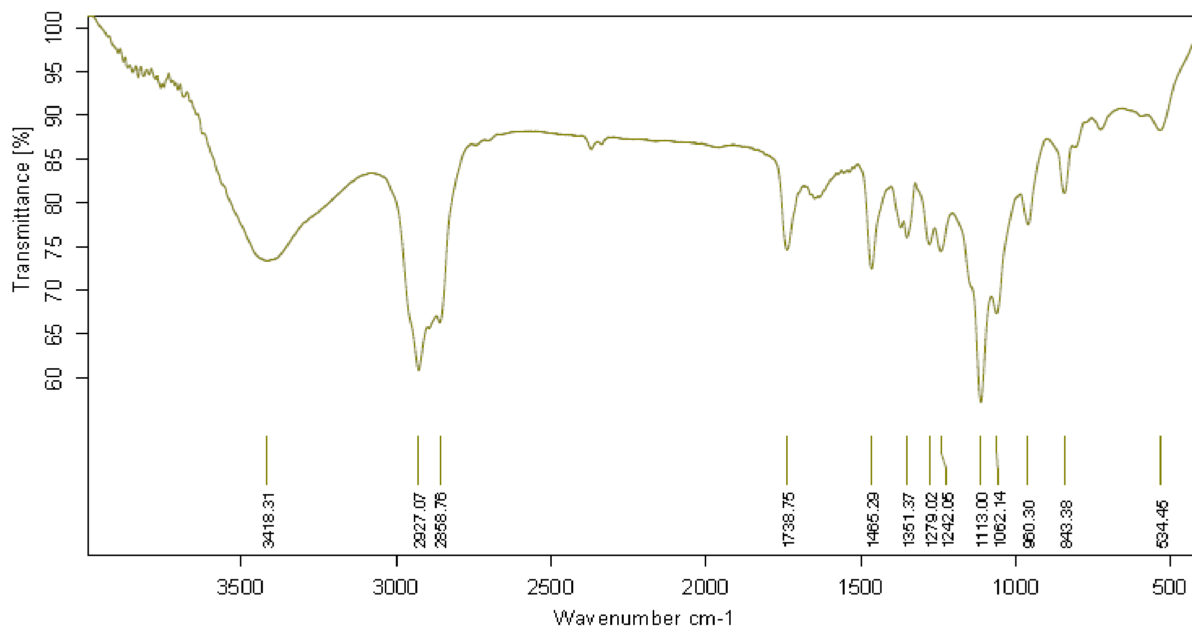


Figure 8.13: FTIR spectroscopic data/spectra of lyophilized formulation without Ribavirin.

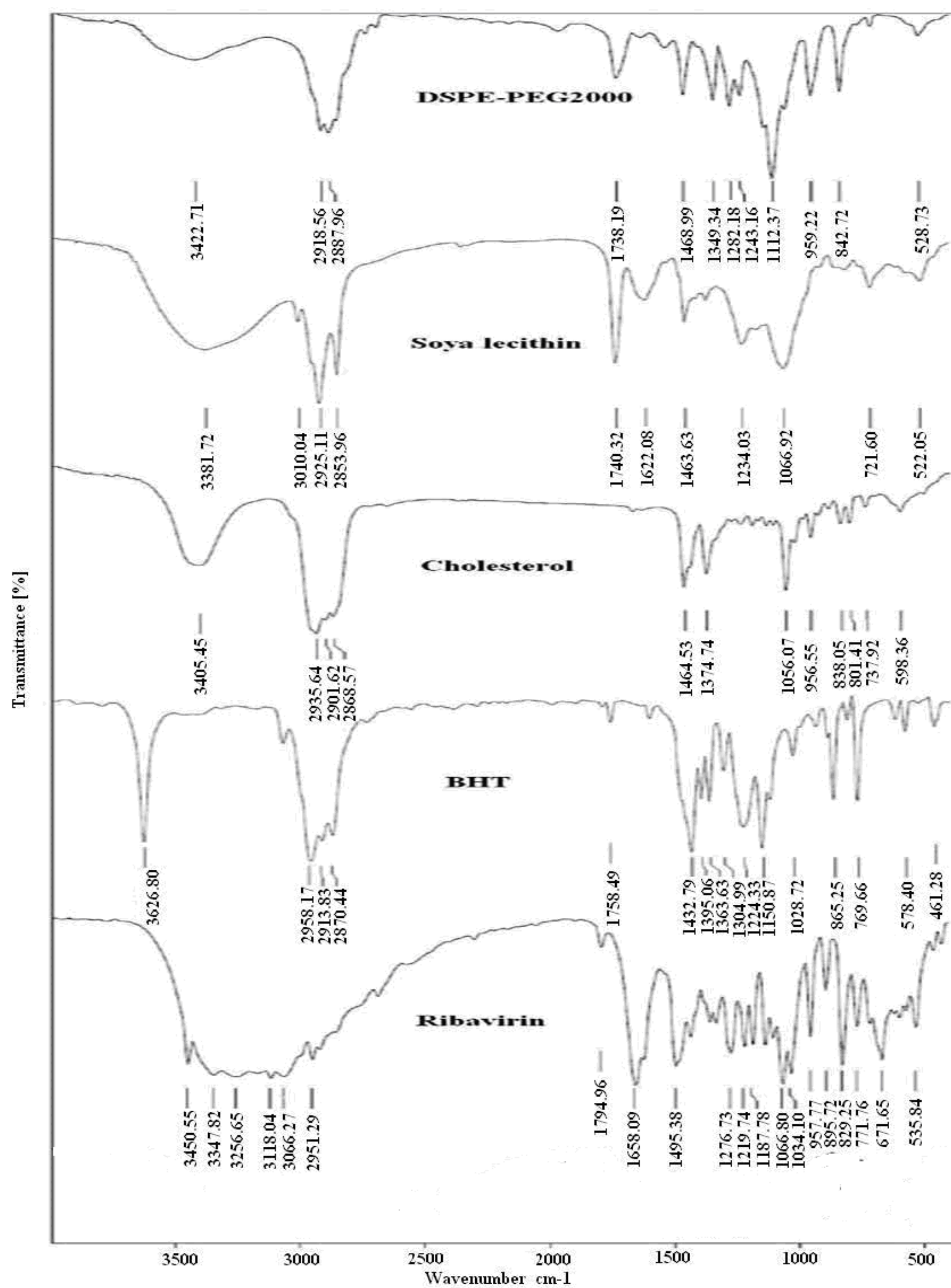


Figure 8.14: FTIR spectra of all individual components in a single figure.

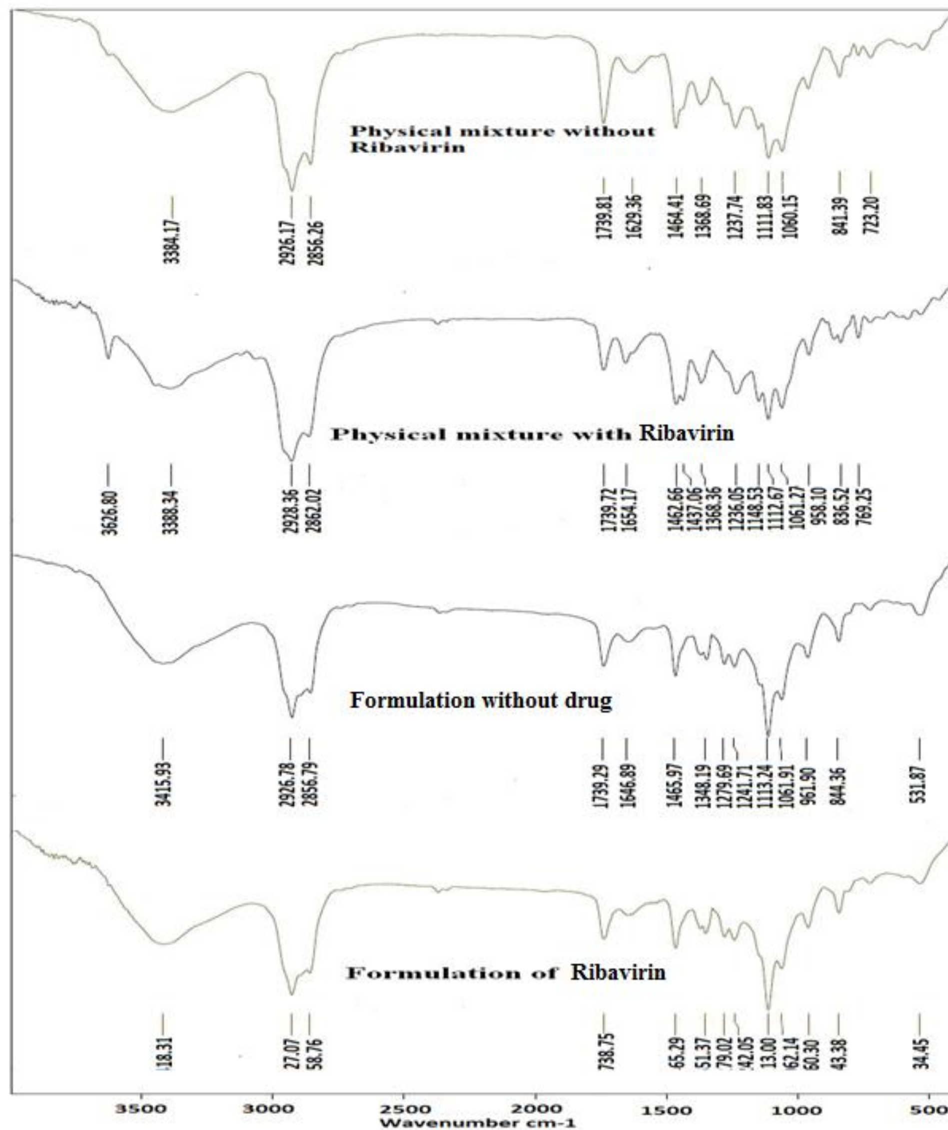


Figure 8.15: FTIR spectra of physical mixtures of the drug and the excipients and the experimental formulations.

8.4 Particle size distribution study:

1. Particle size distribution data of the formulation F1

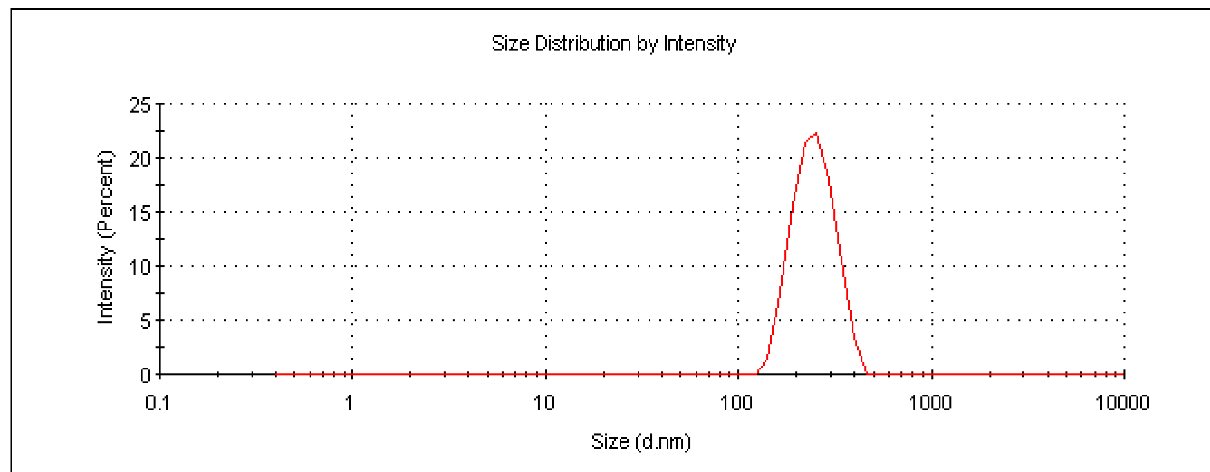


Figure 8.16: shows the particle size distribution pattern of the formulation F1. The average particle size of the formulation was 282.5 nm with PDI 0.384.

2. Particle size distribution data of the formulation F2

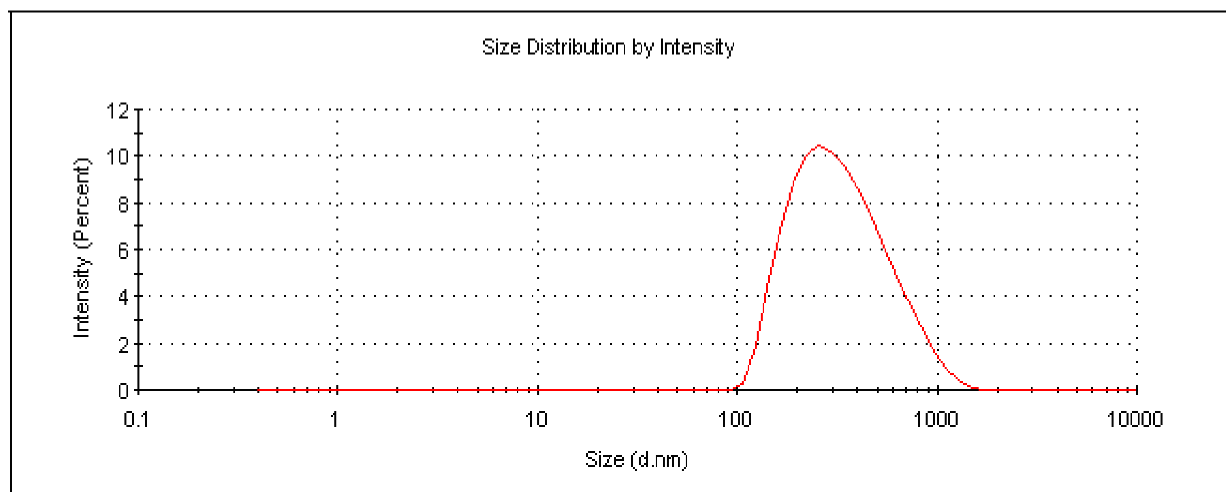


Figure 8.17: shows the particle size distribution pattern of lyophilized formulation **F2**. The average particle size of the formulation was 290.5 nm with PDI 0.212. The PDI value shows comparatively (with respect to F1) presence of wider size range of liposome.

3. Particle size distribution data of the formulation F3

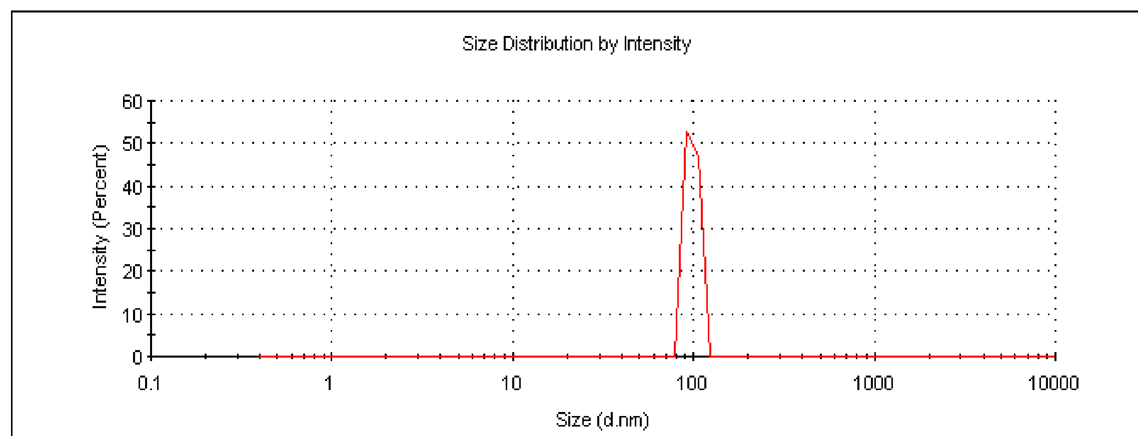


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Figure 8.18: shows the particle size distribution pattern of lyophilized formulation **F3**. The average particle size of the formulation was 98.07 nm with PDI 0.369. The PDI value shows narrower size distribution range.

Particle size distribution data of the formulation F4

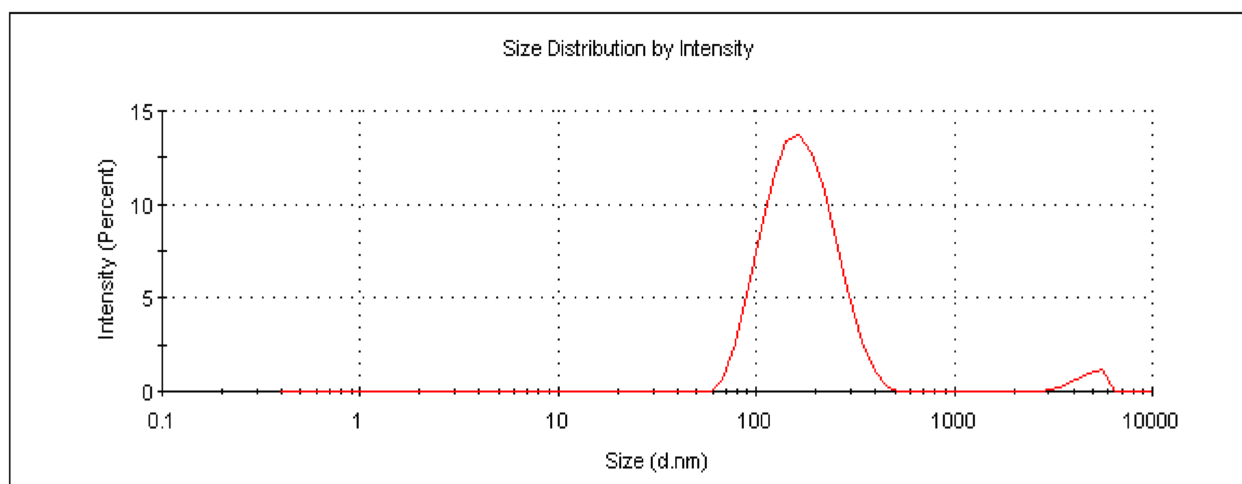


Figure 8.19: shows the particle size distribution pattern of lyophilized formulation F4. The average particle size of the formulation was 160.4 nm with PdI 0.219. The peak above 1000nm appears due to agglomeration of the particles.

Particle size distribution data of the formulation F5

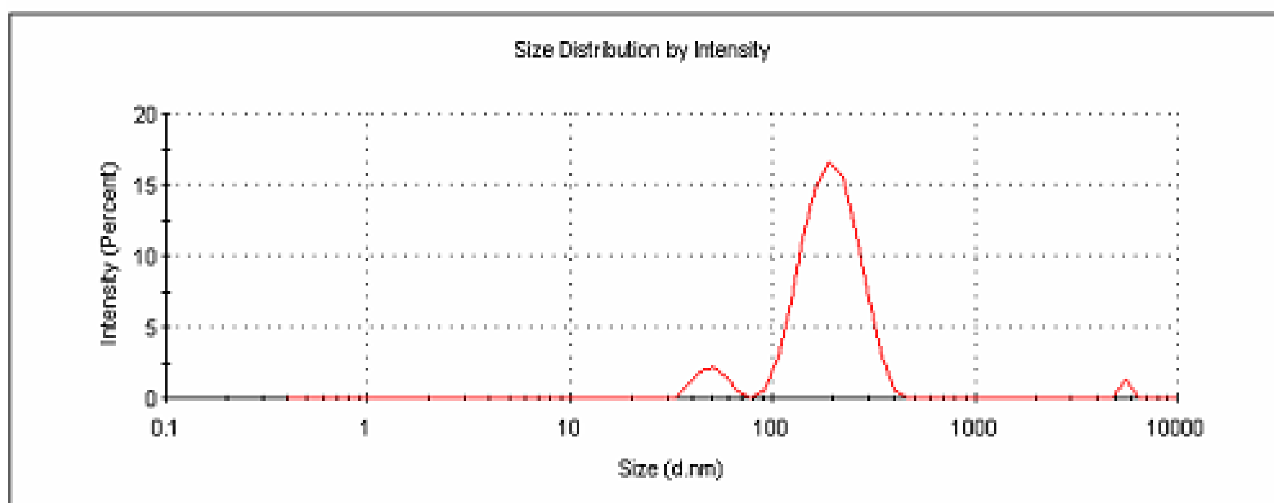


Figure 8.20: shows the particle size distribution pattern of the formulation F5. The average particle size of the formulation was 195.8nm with PdI 0.397. The figure shows the presence of some conglomerations.

□ **Z-Average:** The composition and the average size (z-average value) of all the experimental formulations are given in Table 8.3.

Table 8.3: - shows the Z- average value of the different formulations.

Formulation Code	Composition	Z – average (d.nm)
F1	DSPE-PEG2000**:** Cholesterol (1:1)	282.5
F2	DSPE-PEG2000**:** Cholesterol (2:1)	290
F3	DSPE-PEG2000:SPC**:** Cholesterol (1:1:1)	98.07
F4	DSPE-PEG2000:SPC**:** Cholesterol (1:2:1)	160.4
F5	DSPE-PEG2000:SPC**:** Cholesterol (1:3:1)	195.8

F3 shows lowest z-average value among the formulations studied here.

▢ Polydispersity index

Table 8.4: - shows the polydispersity index of different formulations

Composition	PDI
DSPE-PEG2000: Cholesterol (1:1)	0.384
DSPE-PEG2000: Cholesterol (2:1)	0.212
DSPE-PEG2000:SPC: Cholesterol (1:1:1)	0.348
DSPE-PEG2000:SPC: Cholesterol (1:2:1)	0.219
DSPE-PEG2000:SPC: Cholesterol (1:3:1)	0.397

Formulations F1, F3 and F5 had more or less similar narrower size range of particle size distribution was observed as compared to the other two formulations.

8.5 Zeta potential study:

Zeta potential data of formulation F1

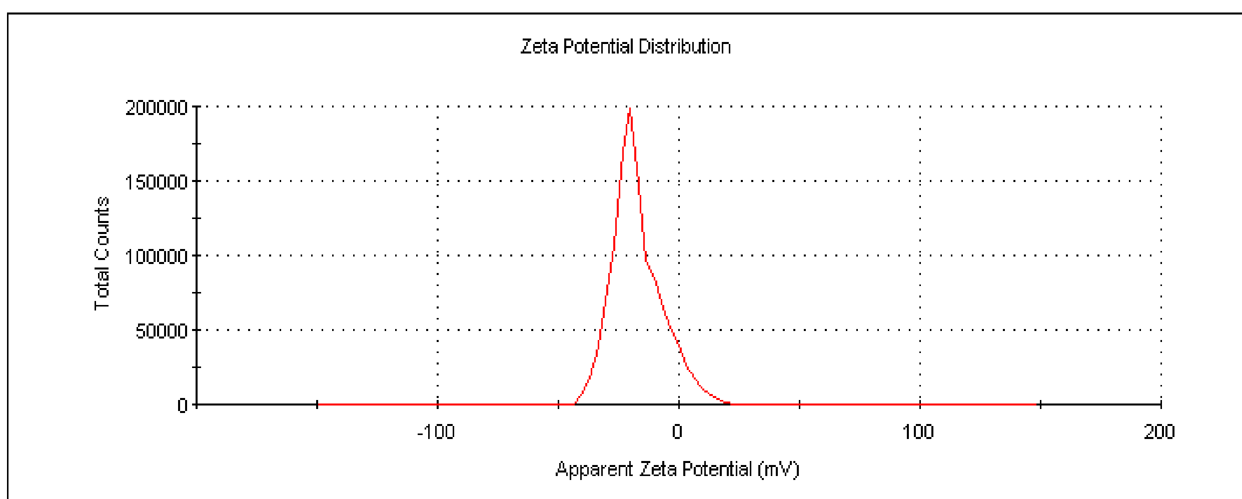


Figure 8.21: shows the zeta potential of formulation F1. The value of zeta potential was – 17.2 mV.

Zeta potential data of formulation F2

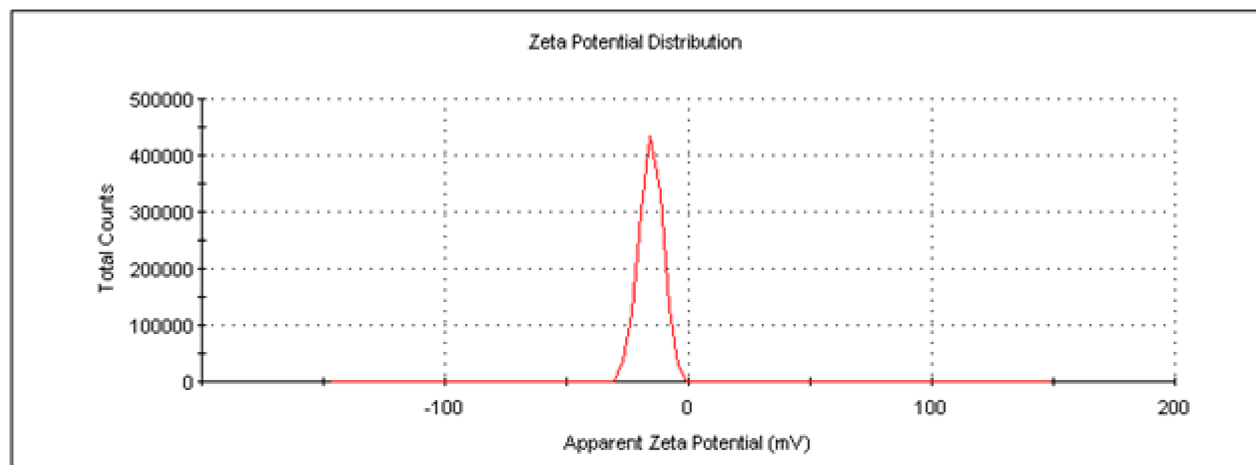


Figure 8.22: shows the zeta potential of formulation F2. The value of zeta potential was - 15.6 mV.

Zeta potential data of formulation F3

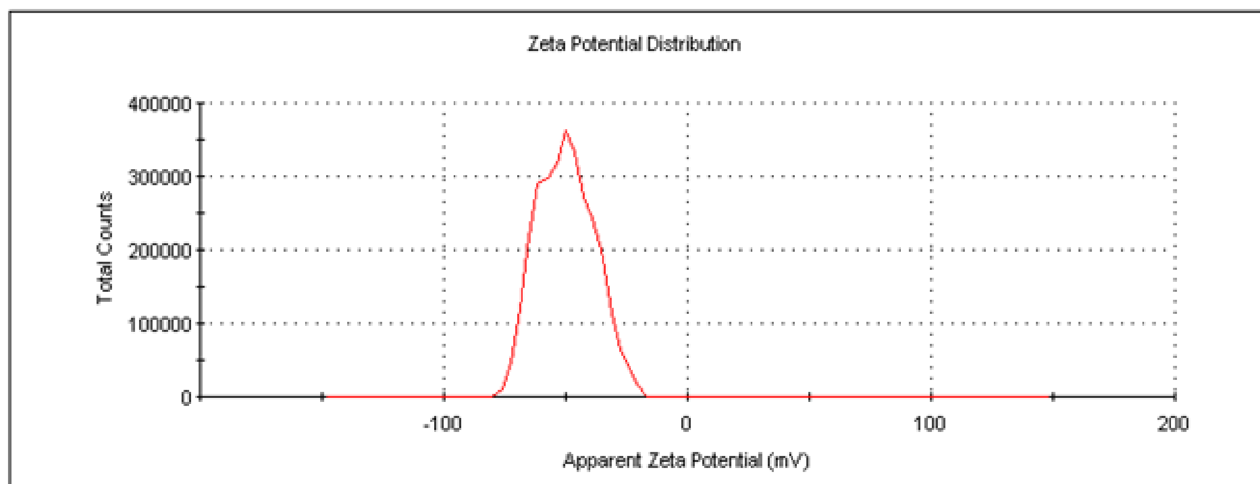


Figure 8.23: shows the zeta potential of formulation F3. The value of zeta potential was - 49.9 mV.

Zeta potential data of formulation F4

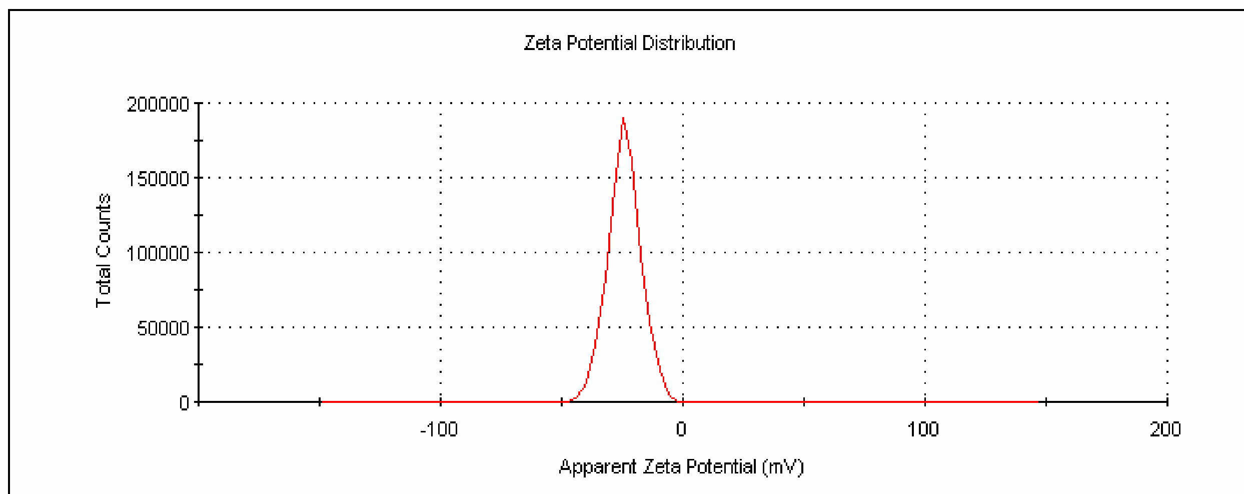


Figure 8.24: shows the zeta potential of formulation F4. The value of zeta potential was -24.1 mV.

Zeta potential data of formulation F5

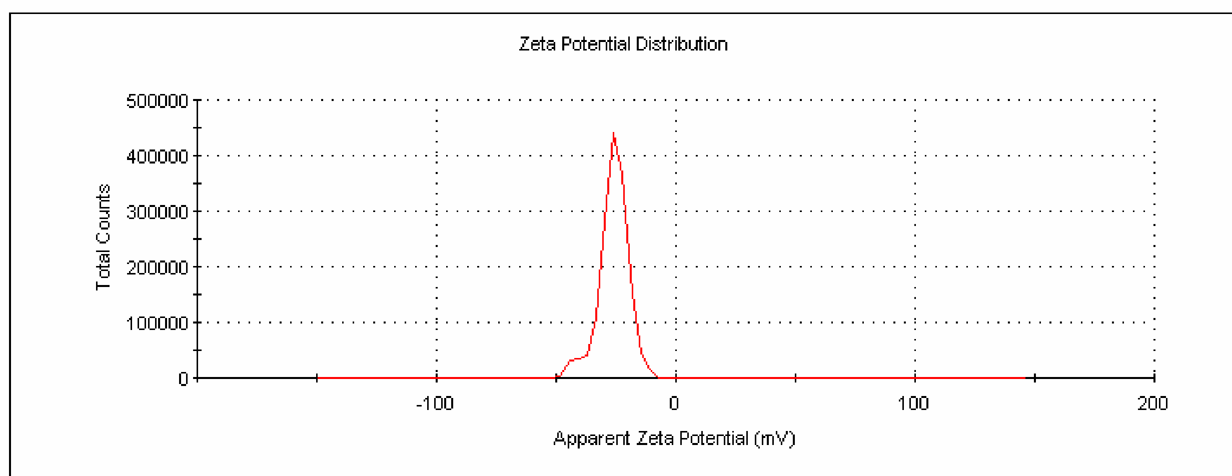


Figure 8.25: shows the zeta potential of formulation F5. The value of zeta potential was -26 mV.

The Zeta potential values varied from -17.2 mV to -49.9 mV, as shown in the figures (Fig 8.21-8.25). The lowest Zeta potential was seen in case of the formulation F3.

8.6 DRUG LOADING STUDY

Drug loading of the different formulations F1, F2, F3, F4, F5 are shown in the Table no 8.4 .So it is clear from the data that the formulation F3 shows maximum percentage of drug loading and maximum drug loading efficiency was observed with the same formulation. Keeping the amount of cholesterol fixed, the amounts of DSPE-PEG2000 and soya-L-α-lecithin were varied, and it was observed that the percentage of drug loading decreased with an increase of the amount of DSPE-PEG2000 and SPC.

Table 8.5: Constituents of liposomal formulations containing Ribavirin and drug loading

Sl. No.	Formulation Code	Composition	Drug loaded (mg)	Preparation Method	Percentage Yield (%)	Percentage Loading (%)	Percentage Efficiency (%)
1.	F	DSPE-PEG2000: Cholesterol (1:1)	10	Lipid thin film hydration	21	1.1±0.03	12.1±0.29
2.	F1	DSPE-PEG2000: Cholesterol (1:1)	10	Reverse Phase Evaporation	36.36	3.50±0.025	38.92±0.28
3.	F2	DSPE-PEG2000: Cholesterol (2:1)	10		12.12	1.09±0.321	17.54±5.14
4.	F3	DSPE-PEG2000: SPC: Cholesterol (1:1:1)	10		47.5	5.00±0.181	80.02±2.92
5.	F4	DSPE-PEG2000: SPC: Cholesterol (1:2:1)	10		13.33	0.76±0.06	16.03±1.28
6.	F5	DSPE-PEG2000: SPC: Cholesterol (1:3:1)	10		11.5	0.72±0.04	18.92±1.051

We have initially prepared different formulations. However based on their various physicochemical properties, we have further selected formulation F1 and F3 to report here.

8.7 Surface morphology of the experimental lyophilized liposomes (F1 and F3) by FESEM:

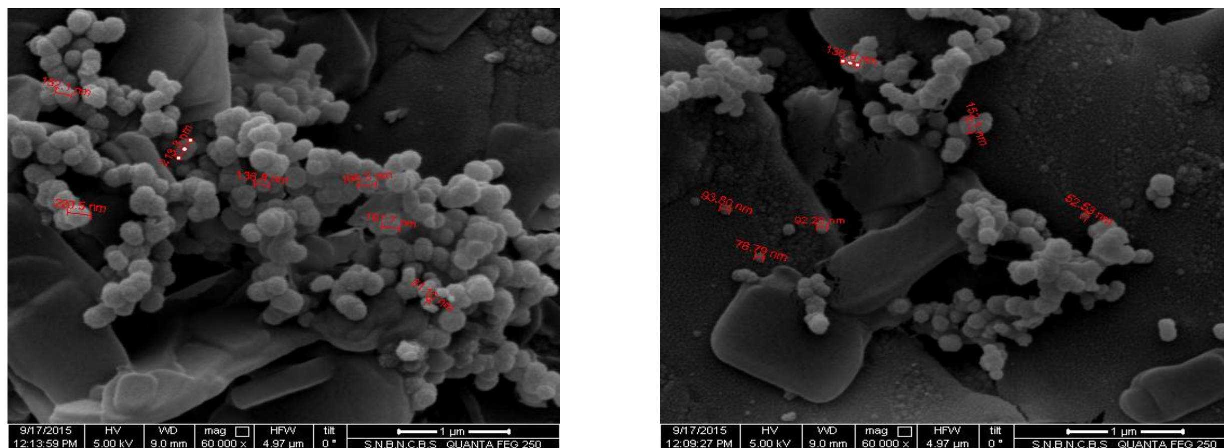


Figure 8.26: FESEM images of the lyophilized formulation, F1

FESEM photographs of the freshly prepared formulation show small lyophilized liposomes which were thickly dispersed mostly in clusters. The diameter of the liposomes was in nanometric size and the liposomes had smooth surface.

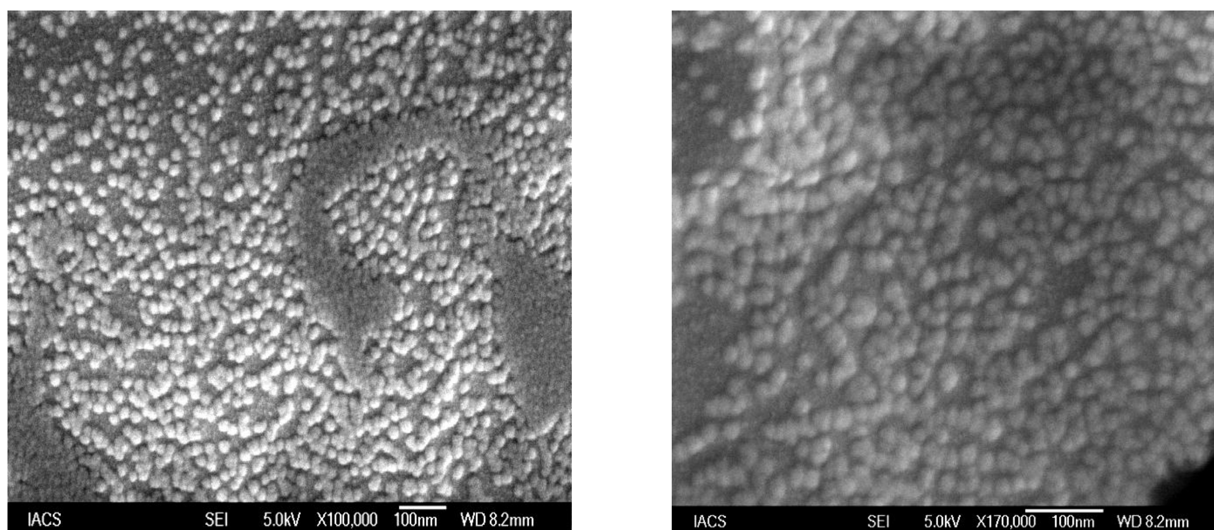


Figure 8.27: FESEM images of the lyophilized formulation F3

FESEM photographs of freshly prepared formulation (F3) show the lyophilized liposomes were of nanometric size with smooth surface and scattered throughout the field

8.8 *In vitro* drug release study:

Cumulative percentage of drug release were calculated and plotted against time. The *in vitro* cumulative release profile of the optimized formulation F3 was shown in Figure 8.28. From the formulation about 50 % of drug was released in a sustained manner over 24h of release study.

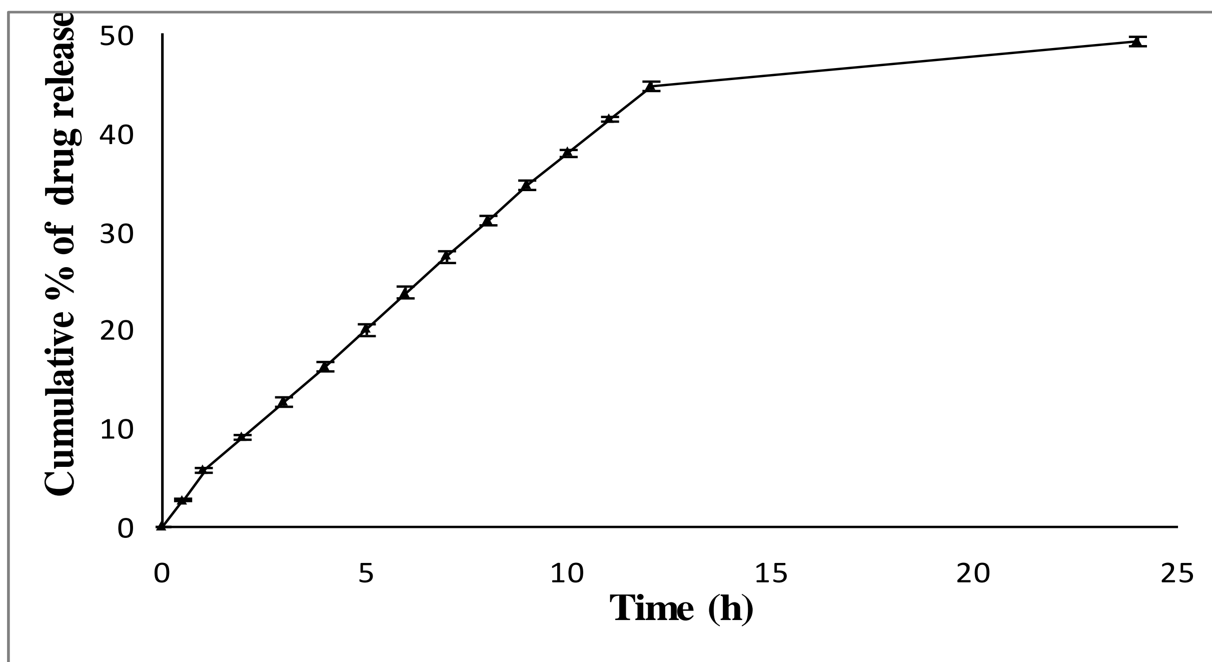


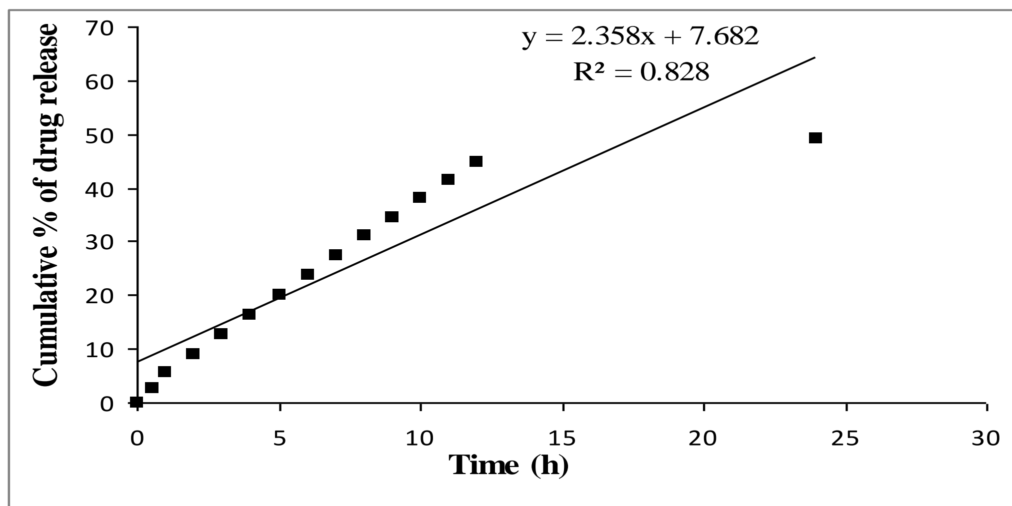
Figure 8.28: *In vitro* cumulative release of F3 formulation.

Drug was found to release steadily up to first 12 hours and then gradually drug release seemed to be slower.

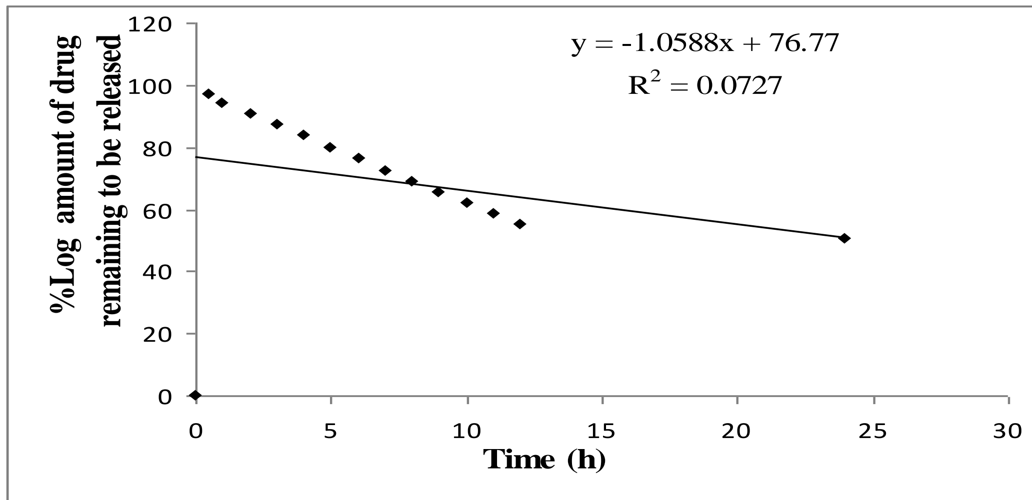
8.9 Drug Release Kinetics Study:

Table no 8.6: Drug release kinetic study of ribavirin loaded nanoliposome

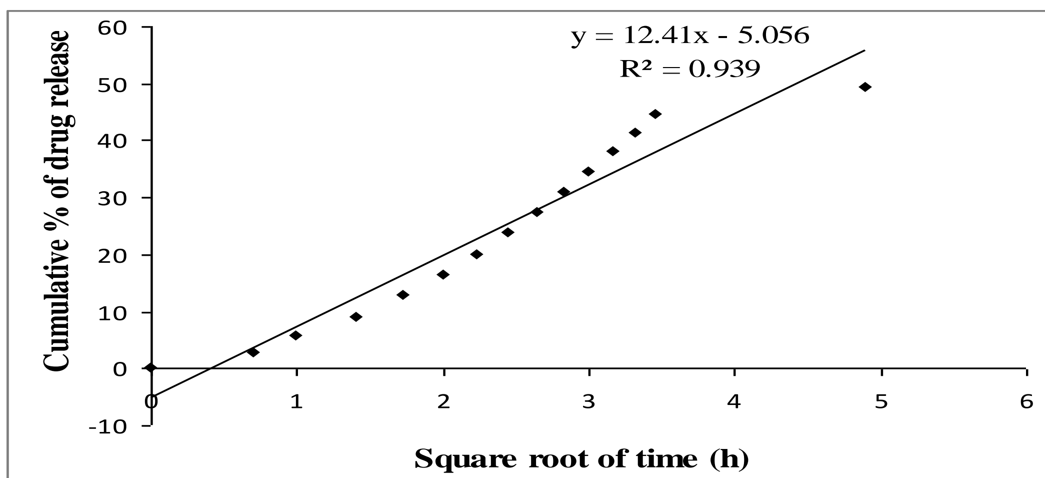
Kinetics Model	Formulation (F3)
Zero order kinetics	y = 2.358x+7.682 R^2 = 0.828
First order kinetics	y = -1.0588x+76.77 R^2 = 0.0727
Higuchi kinetics	y = 12.41x-5.056 R^2 = 0.939
Krosmeyer-Peppas kinetics	y = 0.9628x+0.5841 R^2 = 0.8613
Hixson-Crowell kinetics	y = 0.1007x+0.9887 R^2 = 0.7632



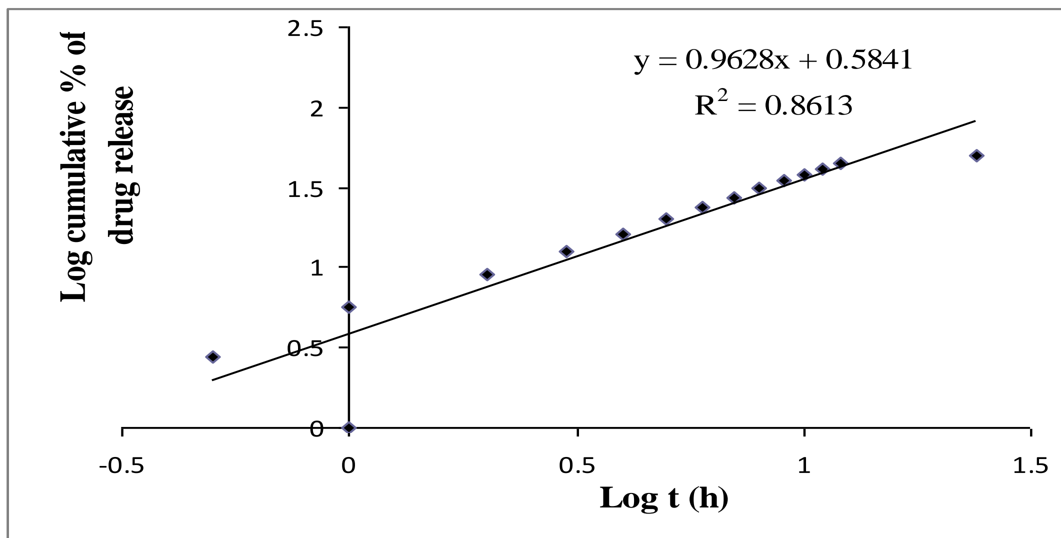
(A)



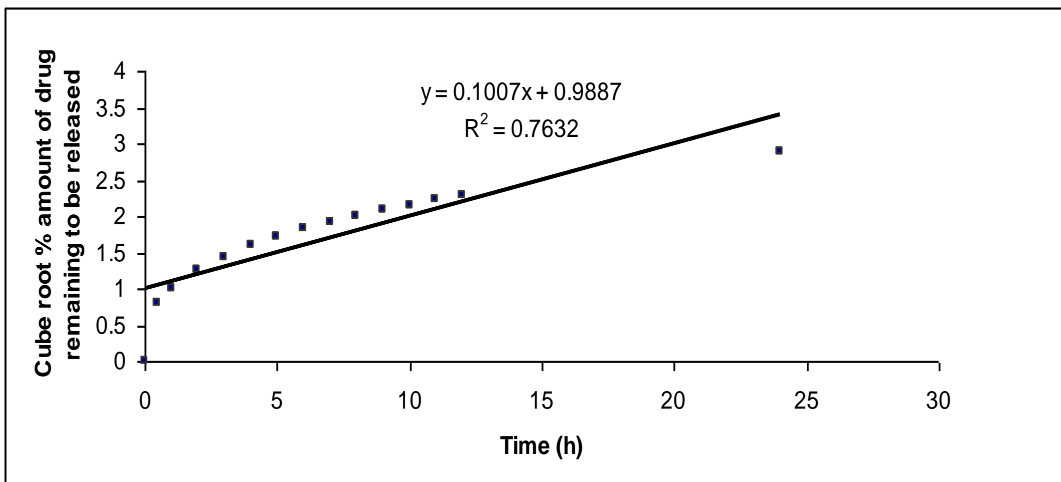
(B)



(C)

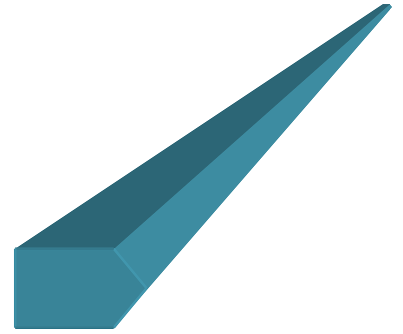


(D)



(E)

Figure 8.29: Different release kinetic model (A) zero order kinetics, (B) first order kinetics, (C) Higuchi kinetics (D) Korsmeyer-peppas kinetics (E) Hixson crowell kinetics



Chapter 9

Discussions

9. DISCUSSION:

9.1 Critical factors to be considered for selection of material and preparation of liposome formulation:

- **Selection of materials:**

  Based on the literature review, we have chosen DSPE-PEG2000 [sodium salt] as phospholipid because it has following advantages. DSPE is a synthetic phospholipid which possesses the advantage over natural phospholipids of being more stable due to inability of forming radicals (Archakov et al), PEGylated DSPE has the benefits of extending circulation time and for sustained release of the drugs *in vivo* (Wang et al, 2012). Soya-lecithin was taken as a natural phospholipid. Apart from using phospholipids, cholesterol is also used in the formulation because cholesterol by itself form bilayer structure and can be incorporated into phospholipid membranes in very high concentration up to 1:1 even 2:1 molar ratios of cholesterol to PC. Cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chained aligned parallel to acyl chains in the center of the bilayer *(Ladbrooke et al 1968).* Cholesterol fills in the gaps created by imperfect packing of lipid species and modulates membrane fluidity, elasticity, and permeability. Inclusion of cholesterol stabilizes liposomal phospholipid membranes against disruption by plasma proteins and results in decreased binding of plasma opsonins responsible for rapid clearance of liposomes from circulation. Also cholesterol is essential for improved uptake by PNS cells (Ashizawa et al, 2013).

- **Preparation of liposome formulation:**

  In this study **lipid layer hydration** method and **reverse phase evaporation** method were used to prepare liposome. In **lipid layer hydration** method DSPE-PEG 2000 and cholesterol were used to produce thin layer of lipid along the inner wall of a round bottom flask. Both DSPE-PEG 2000 and cholesterol are prone to oxidation. Hence an antioxidant, butylated hydroxyl toluene (BHT) was used to reduce the chance of oxidation. The lipid mixture was dissolved in minimum amount of chloroform to minimize residual chloroform after evaporation of organic solvent. The flask used should be completely dry during initial stage otherwise presence of moisture will emulsify the chloroform and may cause bubbling of lipid solution during evaporation which

ultimately hampers thin layer formation. It is necessary to shake the flask well for proper mixing of lipids in the chloroform solution.

The organic solvent was evaporated in a rotary vacuum evaporator rapidly with optimum rotation of flask. Temperature and rotation speed of the flask should be controlled in such a way that depending upon the amount of solvent present, it can help in formation of good layer of lipid without boiling or crystal formation (Mukherjee et al, 2007). The vacuum in a rotary evaporator is commonly insufficient for complete removal of chloroform. So, the flask was kept overnight in a vacuum desiccator for removal of residual chloroform. Hydration of lipid layer was carried out in an inert atmosphere. Hydration swells the lipid layer and helps to disperse it in aqueous media. The lipid layer, upon dispersion in aqueous media, forms bilayer structure as well as large multilamellar vesicles (LMVs). After hydration the lipid dispersion was sonicated in a bath sonicator. Sonication is the crucial step which controls size reduction and size distribution during preparation of liposome. Ice cooling of sonicator bath may be used to control overheating during sonication. Sonication breaks the bilayer and LMVs in small fragments. After sonication the dispersion was allowed to stand for minimum one hour without any agitation. During this time small bilayer fragments rejoins to form small unilamellar vesicles (SUVs). Entrapment of drug molecules into the liposome depends upon the physicochemical characteristics of drug, concentration of drug, drug to lipid ratio, and temperature at which formulations were prepared (Rudra et al, 2010). The liposome dispersion was then kept in a refrigerator at 4-8°C for overnight. After that it was centrifuged to separate liposomes as a precipitated pellet. The rotational speed and timing of centrifugation affect the nature and amount of sediment. The liposomal pellet was collected in a petridish then it was pre-freezed and lyophilized to obtain dry liposome powder or flakes which helps in long time storage of liposomes without affecting efficacy.

As ribavirin is hydrophilic in nature, the drug is loaded in the inner aqueous space of the liposome. It has been observed that liposome preparation by lipid layer hydration method does not show significant loading percentage due to less aqueous space formed during vesicle formation. But it has been observed that when liposome is prepared by reverse phase evaporation method it shows higher drug loading

compared to lipid layer hydration method as it offers more internal aqueous volume which is suitable for hydrophilic drug. So reverse phase evaporation method is chosen for preparation of ribavirin nanoliposome (Francis et al, 1978).

In **reverse phase evaporation** method, the formation of liposome is based on creation of inverted micelles. First, the water-in-oil emulsion is prepared by brief sonication of a two-phase system containing phospholipids in organic solvent with aqueous buffer. Two-phase system is sonicated briefly (2-5 min) in a bath-type sonicator until the mixture becomes either a clear one-phase dispersion or a homogeneous opalescent dispersion that does not separate for at least 30 min after sonication. The evaporation of organic solvent during initial step should be slow enough to convert the inverted micelles to the viscous state and gel form. This evaporation should be done in reduced pressure very carefully because the liposomes are shaped when residual solvent is detached during continued rotary evaporation under reduced pressure. After that, excess solvent is added and the suspension is evaporated for another 15 minutes to remove traces of solvent. Cholesterol is used in formulation, when lipid mixtures lacking cholesterol are used at low concentrations, the gel phase may not be apparent since the system rapidly reverts to a lipid-in-water suspension. (Francis et al, 1978)

9.2 Scanning of drug, Ribavirin to find absorption maxima:

The scanning of Ribavirin was carried out in two different solvent systems. Fig 8.1 shows the λ_{max} of Ribavirin in PBS and Fig 8.3 shows the λ_{max} of the drug in mixture of PBS and ethanol (5:1). It matches with the reported values of λ_{max} of this drug (208 nm) *(*Loregian et al, 2007*)*. This shows the purity of the compound. The λ_{max} value of Ribavirin in PBS was used for drug release study and the λ_{max} value of Ribavirin in ethanol-PBS mixture was used for drug loading study.

9.3 Preparation of calibration curve:

The regression coefficients (R^2) of Figure 8.2 and Figure 8.4 was 0.9987 and 0.9995 respectively. It means that deviation was minor. It was an indication of favorable accuracy of the adopted analytical methods.

9.4 Drug excipients interaction study:

Drug-excipients interaction study is an important preformulation study to prepare a stable 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 depend on drug-excipients interaction *(*Mukherjee et al, 2010*)*. This study can be done using various methods such as Differential Scanning Calorimetry (DSC), Fourier Transfer Infrared (FTIR) spectroscopy, Infrared (IR) analysis etc. Among the various methods we have selected FTIR spectroscopy to investigate interaction (if any) between the drug molecules and the selected excipients. FTIR spectroscopy generally assesses the interaction between the molecules at the level of their functional groups. Presence of the characteristic peaks of each individual component of the excipients and the drug suggests that there was no chemical interaction. However minor shifting of some peaks suggests that the presence of some physical interactions such as weak H-bonding, van der Waals' force of attraction, dipole-dipole interaction etc., which may be beneficial for the development of the formulation and sustained drug release from it. However, there were the absence of the peaks of the Ribavirin, Procaine and BHT, suggesting the complete encapsulation of those molecules in the formulation and there was no free drug molecule as well as BHT was present on the formulation surface.

9.5 Particle (vesicle) size and size distribution study:

For the effective drug delivery the vesicle size and size distribution of the vesicles are very important. Due to the tight endothelial junction in the blood-brain barrier only lipid soluble molecules with nano size are readily transported through the blood-brain barrier *(*Ito U et al, 1992*)*. Size distribution study has been done by dynamic light spectroscopy study. From the value of Z-average and polydispersity index it was clear that vesicles (F3) were within the nano size range (< 100 nm) which makes them suitable for endocytosis in the peripheral nerves with a narrow particle size distribution pattern from formulation to formulation. (Sooyeon et al, 2013)

9.6 Zeta potential study:

Zeta potential is a measure of net surface charge on the particle and potential distribution at the interface. Depending on the composition, zeta potential of liposome can be positive, neutral or negative. It affects the physical stability (aggregation) and *in-vivo* behavior of formulation. Generally zeta potential of liposomes is negative due to the presence of terminal carboxylic group in lipids *(*Mu et al 2002). In the present study zeta potential of the different formulations were evaluated and it was observed that F3 formulation had high negative charge (>-30 mV) on their surface. So, this formulation is stable at colloidal state. While others formulations (F1, F2, F4, F5) had zeta potential <-30 mV, indicates that the formulations are not stable at colloidal state. Zeta potential less than -30 mV or greater than +30 mV is considered to be stable at colloidal form for a prolong period and prevents settling down while in suspension *(Basu et al, 2012 and Yue et al, 2008)*

9.7 Drug loading study:

The encapsulation efficiency of liposomes greatly depends on liposomal content, lipid concentration, method of preparation, and the drug used. The formulation F3 shows maximum percentage of drug loading and maximum drug loading efficiency. It was observed when same formulation was prepared with thin film hydration method and reverse phase evaporation technique, reverse phase evaporation technique shows better loading efficiency. It may be due to the hydrophilic nature of the drug Ribavirin as this method offers larger aqueous space in the liposome which is very suitable for hydrophilic drug loading.

It was also observed that percentage of drug loading decreased with an increase in the amount of phospholipids (DSPE-PEG 2000 and SPC).

9.8 Liposome shape and morphology study:

The study of shape and morphology of the freshly prepared liposomes were carried out by FESEM. FESEM of lyophilized formulation F1 shows small lyophilized liposomes which were thickly dispersed in clusters. The diameter of the liposomes was in nanometric range. From the data of particle size and zeta potential study, formulation F3 was selected as the best among the experimental formulations and its FESEM study showed that lyophilized liposomes were in

nanometric range with smooth surface and scattered throughout the field more homogeneously and not in clustered forms.

9.9 *In vitro* drug release study:

On the basis of the results of drug loading study and particle size distribution study F3 formulation was considered for drug release study. It was observed that drug was released in a sustained manner from the formulation and about 50 % of drug was released from the formulation F3 in 24 h.

9.10 Kinetics study:

The release kinetics of drug from the formulations was evaluated according to zero-order kinetics, first-order kinetics, Higuchi's model, Krosmeyer-Peppas model and Hixson–Crowell's model.

Zero-order model

This method is applicable to dosage forms that do not disaggregate and release the drug slowly and can be represented by the equation:

$$Q_t = Q_o - k_o t$$

Where Q_t is the amount of drug remaining as a solid state at time t, Q_o is the initial amount of drug in the pharmaceutical dosage form and k_o is the zero-order release rate constant (Tanaka et al, 2005). To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as cumulative amount of drug released *versus* time. This relationship can be used to describe the drug dissolution of several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems, as well as matrix tablets with low soluble drugs in coated forms, osmotic systems, etc.

First order model

The release of the drug which followed first order kinetics can be expressed by the equation:

$$\ln Q_t = \ln Q_o - k_1 t$$

Where Q_t is the amount of drug remaining as a solid state at time t, Q_o is the initial amount of drug in the pharmaceutical dosage form and k_1 is the first-order release rate constant (Tanaka et al, 2005).

Higuchi model

The first example of a mathematical model aimed to describe drug release from a matrix system was proposed by Huguchi in 1961. This model is based on the hypotheses that (i) initial drug concentration in the matrix is much higher than drug solubility; (ii) drug diffusion takes place only in one dimension (edge effect must be negligible); (iii) drug particles are much smaller than system thickness; (iv) matrix swelling and dissolution are negligible; (v) drug diffusivity is constant; and (vi) perfect sink conditions are always attained in the release environment.

Accordingly, model expression is given by the equation:

$$Qt = k_H t^{1/2}$$

Where Qt is the amount of drug released at time t, and k_H is the Higuchi's (release) rate constant (Tanaka et al, 2005)

Hixson Crowell model

This method is represented by the equation:

$$Qo^{1/3} - Qt^{1/3} = K_s t$$

Where Q_t is the amount of drug remaining as a solid sate at time t, Q_o is the initial amount of drug in the pharmaceutical dosage form and Ks is the release rate constant (Tanaka et al, 2005).

The equation describes the release from systems where there is a change in surface area and diameter of particles or tablets. To study the release kinetics, data obtained from *in vitro cumulative* drug release studies were plotted as cube root of drug percentage remaining in matrix *versus* time.

Korsmeyer-Peppas model

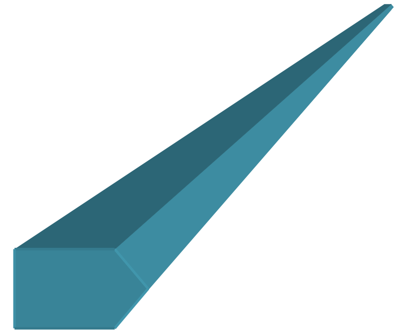
Korsmeyer derived a simple relationship which described drug release from a polymeric system equation.

$$M_t/M\alpha = k_t n$$

Where Mt/Mα is a fraction of drug released at time t, k is the release rate constant and n is the release exponent. The "n" value is used to characterize different release for cylindrical shaped matrices (Dash et al, 2010).

To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as log percentage of cumulative drug release *versus* log time.

From the values of regression coefficient (R^2) of different kinetic models in table 8.5 it is clear that **Higuchi model** fits best for F3 formulation (R^2 =0.939). This represents that drug possibly released from the formulations through diffusion.

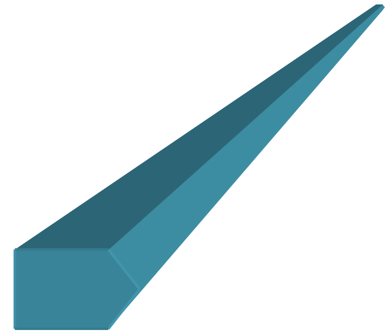


Chapter 10

Conclusions

9. CONCLUSION

In the present work, Ribavirin loaded PEGylated nanoliposomes were developed using DSPE-PEG 2000, SPC and cholesterol. Preformulation study performed by FTIR, showed no chemical interaction between the drug and the excipients. Nanoliposomes were prepared by lipid film hydration as well as by reverse phase evaporation method. The physico-chemical characterization of Ribavirin nanoliposomes was carried out *in-vitro* by particles size analysis, zeta potential measurement, % drug loading, FESEM analysis and finally *in-vitro* drug release study. Initially, the formulation F3 has been optimized. It was prepared by reverse phase evaporation method. The formulation F3 exhibited average vesicle size of 98.07 nm with PDI value 0.369 which reveals that nanoliposomes were within the nanosize range and showed narrower range of size distribution. Higher value of zeta potential (-49.9 mV) indicates the stability of the formulation and would not quickly settle after reconstitution. About 5.00±0.181% drug loading and 80.02±2.92% drug loading efficiency were observed. It has been observed that almost 50% of the drug released over a time period of 24 hours in a sustained manner. It has been found that, release kinetics has followed the Higuchi kinetic model, suggesting that the drug would be released by diffusion mechanism. In conclusion, PEGylated nanoliposomes containing Ribavirin by reverse phase evaporation method were successfully developed within nanosize range, having higher Zeta potential, good loading capacity and with satisfactory drug release profile.

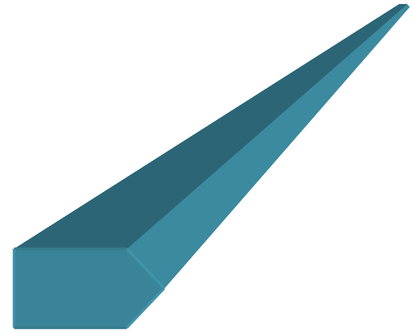


Chapter 11

Future prospective

11. FUTURE PROSPECTIVE:

In future, development of ligand attached PEGylated Ribavirin nanoliposomes may be carried out for better formulation development suitable for delivery of Ribavirin to the peripheral nervous system. Cellular studies will be done on neuronal cell line as comparative study between ligand attached liposome and liposome without ligand. Also those experimental nanoliposomes may be taken for *in-vivo* studies in suitable animal model to investigate their efficiency.



Chapter 12

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