FORMULATION AND EVALUATION OF LIPOSOMAL DRUG DELIVERY SYSTEM FOR DOXORUBICIN HYDROCHLORIDE

A dissertation submitted to

THE TAMILNADU Dr. M.G.R.MEDICAL UNIVERSITY, CHENNAI.

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY IN PHARMACEUTICS

BY

REG.NO: 26091390

Under the Guidance of

Prof. S.P.SENTHIL, M.Pharm., (Ph.D.,)

OCTOBER -2011

THE ERODE COLLEGE OF PHARMACY AND RESEARCH INSTITUTE

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DEDICATED

TO

MY

BELOVED FAMILY,

TEACHERS & FRIENDS

CERTIFICATES

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This work is original and has not been submitted in part or full to any other degree or diploma of this or any other university.

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ENDORSEMENT BY THE PRINCIPAL

This is to certify that the investigation in this thesis entitled **"FORMULATION AND EVALUATION OF LIPOSOMAL DRUG DELIVERY SYSTEM FOR DOXORUBICIN HYDROCHLORIDE"** submitted in partial fulfillment of the requirements for the Degree Of **MASTER OF PHARMACY in PHARMACEUTICS** were carried out in the laboratories of The Erode College Of Pharmacy and Research Institute, Erode by **Regd.No.26091390** under the guidance of **Prof.S.P.Senthil,M.Pharm.,(Ph.D.,)** The Erode College Of Pharmacy and Research Institute, Erode.

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DECLERATION

The research work embodied in this dissertation work entitled **"FORMULATION AND EVALUATION OF LIPOSOMAL DRUG DELIVERY SYSTEM FOR DOXORUBICIN HYDROCHLORIDE "** was carried out by me in the Department of Pharmaceutics, The Erode College of Pharmacy, Erode, under the direct supervision of **Prof. S.P.Senthil, M.Pharm.,(Ph.D.,) Dept. of Pharmaceutics, The Erode College of Pharmacy, Erode – 638 112.**

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The work is original and has not been submitted in part or full for the award of any other Degree or Diploma of this or any other University.

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Place : Erode **Reg. No. 26091390**

Date :

CONTENTS

CONTENTS

LIST OF ABBRIVIATIONS

1. AIDS Acquired immune deficiency syndrome
2. AUC Area under curve
3. AVE Artificial viral envelopes
4. CDR Cumulative Drug Release
5. DNA Deoxyribose nucleic acid
6. DSPC Distearoyl phosphatidyl choline
7. EE Encapsulation efficiency
8. FTIR Fourier Transform Infrared
9. GUV Gaint unilamellar vesicles
10. gm Gram
11. HPLC High performance liquid chromatography
12. IPA Iso propyl alchol
13. IR Infrared spectroscopy
14. KS Kaposis sarcoma
15. LUV Large unilamellar vesicles
16. MLV Multi laminar vesicles
17. MPS Mononuclear phagocytic system
18. MVV Multi vesicular vesicles
19. NDDS Novel Drug Delivery System
20. OLV Oligolamellar vesicles
21. PBS Phosphate buffer saline
22. PC Phosphatidyl choline
23. PDI Poly dispersive index
24. PEG Polyethylene glycol
25. RES Reticulo Endothelial system
26. RNA Ribonucleic acid
27. RPM Rotations per minute
28. SEM Scanning Electron Microscopy
29. SUV Small unilamellar vesicles
30. USP United States Pharmacopoeia

LIST OF TABLES

LIST OF FIGURES

LIST OF SPECTRA

LIST OF GRAPHS

LIST OF REPORT

INTRODUCTION

1. INTRODUCTION

The goal of any drug delivery system is to provide a therapeutic amount of drug to the proper site in the body, to achieve promptly and then maintain the desired drug concentration.

Conventional drug delivery system achieves as well as maintains the drug concentration with in the therapeutically effective range needed for treatment only when taken several times a day. This results in a significant fluctuation in drug level (Chien YM., 1992).

The concept of designing specified delivery system to achieve selective drug targeting has been originated from the perception of Paul Ehrlich, who proposed drug delivery to be as a "magic bullet".

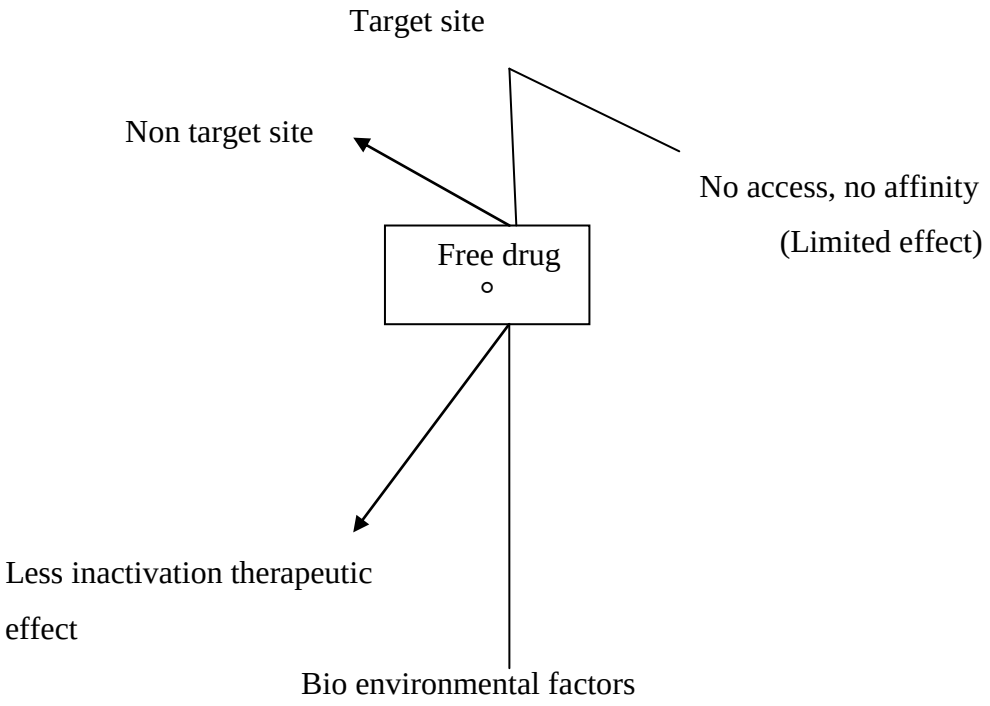
Controlled & Novel delivery envisages optimized drug in the sense that the therapeutic efficacy of a drug is optimized, which also implies nil or minimum side effects. It is expected that the 21st century would witness great changes in the area of drug delivery. The products may be more potent as well as safer. Target specific dosage delivery is likely to overcome much of the criticism of conventional dosage forms. The cumulative outcome could be summarized as optimized drug delivery that encompasses greater potency & greater effectiveness, lesser side effects and toxicity, better stability, low cost hence greater accessibility, ease of administration and best patient compliance (Jain N K., 2001).

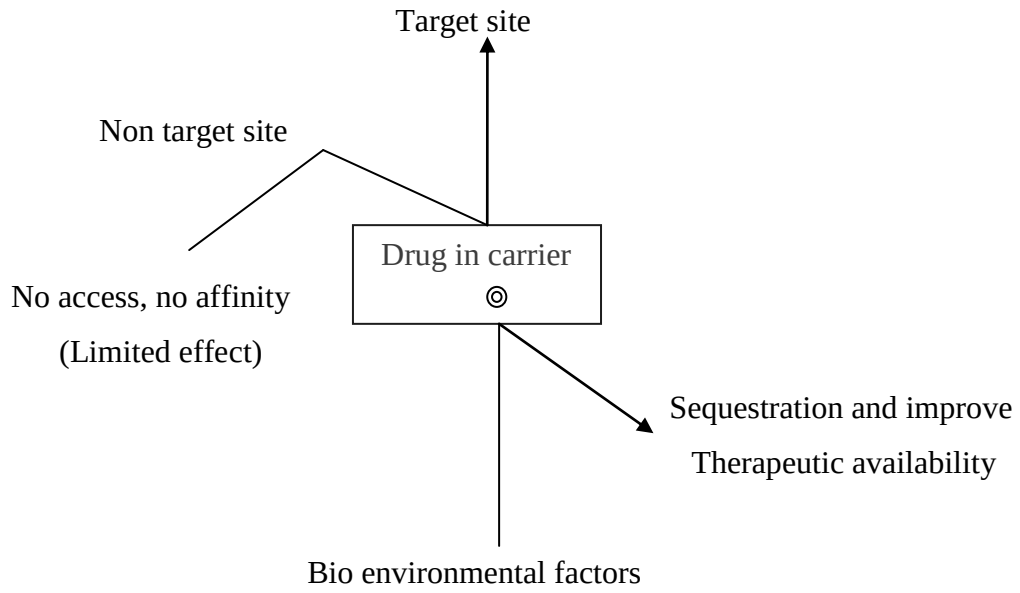
1.1 RATIONALE OF DRUG TARGETING (Jain N K., 2001)

The site-specific targeted drug delivery negotiates an exclusive delivery to specific pre identified compartments with maximum activity of drugs and concomitantly reduced access of drug to irrelevant non-target cells. The controlled rate & mode of drug delivery to pharmacological receptor and specific binding with target cells as well as bioenvironmental protection of the drug in route to the site of action are specific features of targeting. Invariably, every event stated contributes to higher drug concentration at the site of action and resultant lowers concentration at non-target tissue where toxicity might crop up. The high drug concentration at the target site is relative cellular result of the uptake of the drug vehicle, liberation and efflux of free drug from the target site.

Targeting is signified if the target compartment is distinguished from the other compartments, where toxicity may occur and also if the active drug could be placed predominantly in the proximity of target site. The restricted distribution of the parent drug to the non-target site(s) with effective accessibility to the target site(s) could maximize the benefits of targeted drug delivery.

Figure No: 1 Principle & Rationale of drug targeting:





1.2 APPROACHES (Vyas S P., 2001)

The various approaches of vectoring the drug to the target site can be broadly classified as:

- Passive targeting
- Inverse targeting
- Active targeting
- Dual targeting
- Double targeting
- Combination targeting

• PASSIVE TARGETING

System that target the systemic circulation are generally characterized as passive" delivery systems (i.e., targeting occurs because of the body's natural response to the physicochemical characteristics of the drug or drug-carrier system. It is a sort of passive process that utilizes the natural course of biodistribution of the carrier system through which, it eventually accumulation in the organ compartment(s) of the body. The ability of some colloids to be taken up by the RES especially in liver and spleen has made them as ideal vectors for passive hepatic targeting of drugs to these compartments.

This category of targetable devices includes drug bearing bi-layer vesicular systems as well as cellular of micron or sub micron size range.

• INVERSE TARGETING

It is essentially based on successful attempts to circumvent and avoid passive uptake of colloidal carriers by reticulo endothelial system (RES). This effectively leads to the reversion of biodistribution trend of the carrier and hence the process is referred to as inverse targeting. One strategy applied to achieve inverse targeting is to suppress the function of RES by pre-injection of a large amount of blank colloidal carriers or macromolecules like dextran sulphate. This approach leads to RES blockade and as a consequence impairment of host defense system. Alternate strategies include modification of the size, surface charge, composition, surface rigidity and hydrophilicity of carriers for desirable biofate.

- **ACTIVE TARGETING**

Conceptually, active targeting exploits modification or manipulation of drug carriers to redefine its biofate. The natural distribution pattern of the drug carrier composites is enhanced using chemical, biological and physical means, so that it approaches and is identified by particular biosites. The facilitation of the drug-carrier to target cells through the use of ligands or engineered homing devices to increase receptor mediated (or I some cases receptor independent but epitopes based) localization of the drug and target specific delivery of drug(s) is referred to as active targeting.

This target approach can further be classified into three different levels of targeting

First order targeting

It refers to restricted distribution of the drug-carrier system to the capillary bed of a predetermined target site, organ or tissue. Compartmental targeting in lymphatic, peritoneal cavity, cerebral ventricles, lungs, joints, eyes, etc., represents first order targeting (it could also be categorized as level of passive targeting).

Second order targeting

The selective delivery of drugs to a specific cell type such as tumor cells and not to the normal cells is referred as second order drug targeting. The selective drug delivery to the kupffer cells in the liver exemplifiers this approach.

Third order targeting

The third order targeting is defined as drug delivery specifically to the intracellular site of target cells. An example of third order targeting is the receptor based ligand-mediated entry of a drug complex into a cell by endocytosis, lysosomal degradation of carrier followed by release of drug intracellularly or gene delivery to nucleolus.

Ligand mediated targeting

Targeting components, which have been studied and exploited are pilot molecules themselves (bioconjugates) or anchored as ligands on some delivery vehicle (drug-carrier system). All the carrier systems, explored So far, in general, are colloidal in nature. They can be specifically functionalized using various biologically relevant molecular ligands including antibodies, polypeptides, oligosaccharides (carbohydrates), viral proteins and fusogenic residues. The ligands afford specific avidity to drug carrier. The engineered carrier constructs selectivity deliver the drug to the cell or group of cells generally referred to as target. The cascade of events involved in ligand negotiated specific drug delivery is termed as ligand driven receptor mediated targeting.

Physical targeting (triggered Release)

The selective drug delivery programmed and monitored at the external level (*ex vivo*) with the help of physical means is referred to as physical targeting. In this mode of targeting, some characteristics of the bioenvironmental are used either to direct the carrier to a particular location or to cause selective release of its content.

- **DUAL TARGETTING**

  This classical approach of drug targeting employs carrier molecules, which have their own intrinsic antiviral effect thus synerging the antiviral effect of the loaded active drug. Based on this approach, drug conjugates can be prepared with the fortified activity profile against the viral replication. A major advantage is that the virus replication process can be attacked at multiple points, excluding the possibilities of resistant viral strain development.

- **DOUBLE TARGETING**

  For a new future trend, drug targeting may be combined with another methodology, other than passive and active targeting for drug delivery systems. The combination is made between spatial control and temporal control of drug delivery.

The temporal control of drug delivery has been developed in terms of control drug release prior to the development of drug targeting. If spatial targeting is combined with temporal control results in an improved therapeutic index by the following two effects. First, if drug release or activation is occurred locally at therapeutic sited, selectively is increased by multiplication of the spatial selectively with the local release/activation. Second, the improvement in the therapeutic index by a combination of a spatially selective delivery and a preferable release pattern for a drug, such as zero order release for a longer time period of the drugs. When these two methodologies are combined, it may be called "Double targeting".

- **COMBINATION TARGETING**

Petit and Gombtz., 1998 have suggested the term combination targeting for the site specific delivery of proteins and peptides. These targeting systems are equipped with carriers. Polymers and homing devices of molecular specificity that could provide a direct approach to target site. Modification of proteins and peptides with natural polymers, such as polysaccharides, or synthetic polymers, such as poly (ethylene glycol), may alter their physical characteristics and favor targeting the specific compartments, organs or their tissues within the vasculature.

1.2.3 LIMITATIONS OF TARGETED DRUG DELIVERY SYSTEMS (Cheiny M., 1992)

Several problems have been identified which require alterations in targeting strategies particularly, in vivo. These include:

- Rapid clearance of targeting systems especially antibody targeting carriers

- Immune reactions against intravenous administered carrier systems.
- Target tissue heterogenecity.
- Problems of insufficient localizations of targeted systems into tumor cells.
- Down regulation and sloughing of surface epitopes.
- Diffusion and redistribution of released drug leading to no-specific accumulation.

1.2.4 CARRIERS USED IN TARGETING DRUG DELIVERY SYSTEMS

Carrier is one of the most important entities essentially required for succ essful transportation of the loaded drug(s). They are drug vectors, which sequester, transport and retain drug en route. While eluting or delivering it within or in vicinity of target.

Colloidal carriers:

- Vesicular systems: Liposomes; pharmacosomes; virosomes; immunoliposomes.
- Micro particulate systems: Nanoparticles; Microparticles; Magnetic Microspheres; Nanocapsules.
- Cellular carriers: Resealed erythrocytes; Serum albumin; Antibodies; Platelets; Leukocytes.

Supramolecular delivery system:

- Micelles; reverse micelles; mixed micelles; polymeric micelles; liquid crystal: lipoproteins.

Polymer based systems:

- Signal sensitive; muco-adhesive; biodegradable; bioerodable; solute synthetic polymeric carriers.

Macromolecular carriers:

Proteins, glycoproteins, neo glycoproteins and artificial viral envelops (AVE); Glycosylated water-soluble polymers (poly-L-lysine).

Mabs; Immunological Fab fragments; antibody enzyme complex & bispesific Abs; Toxins, immunotoxin & rCD4 toxin conjugates

Lecithins (Con A) & polysaccharides.

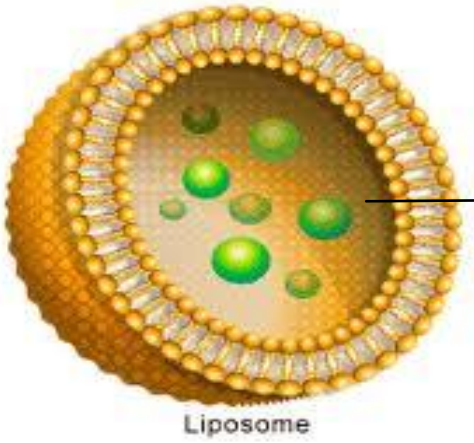
1.3 INTRODUCTION TO LIPOSOMES

Liposomes (marc J. Ostro., 1987) have reached the clinical only recently, but they are not a new invention Alec D. Bangham of the Agricultural Research Council's institute of Animal physiology in Cambridge, England, inadvertently produced the first liposome in 1961, while evaluating the effect of phospholipids on blood clotting. When Bangham put water in a flask containing a phospholipid film, the water molecules to arrange themselves in to what he discovered. He found vesicles composed of a bilayered phospholipids membrane surrounding water entrapped from the environment.

Phospholipids form closed, fluid-filled spheres when they are mixed with water in part because the molecules are amphipathic; they have a hydrophobic "tail" and a hydrophilic or polar "head". Two fatty acid chains, each composed of 10 to 24 carbon atoms, make up the hydrophobic tail of most naturally occurring phospholipids molecules. Phosphoric acid bound to any of several water soluble molecules composes the hydrophilic head. When a high enough concentration of phospholipids is mixed with water, the hydrophobic tails spontaneously herd together to exclude water, whereas the hydrophilic heads bind to water.

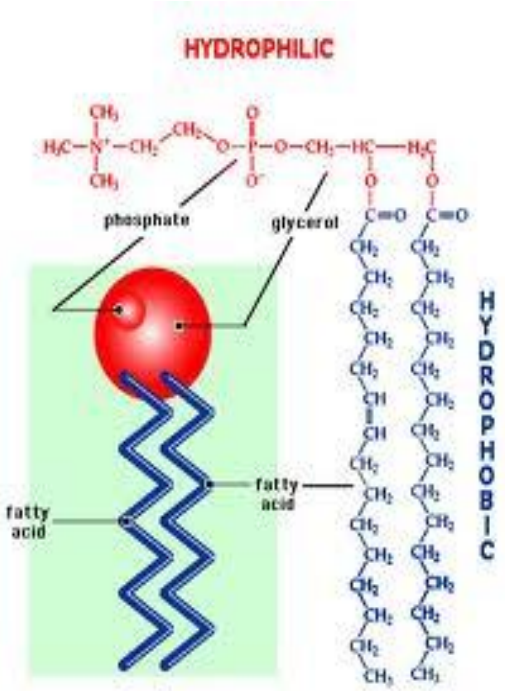
The result is a bilayer in which the fatty acid tails in to the membrane's interior and the polar head groups point outward the polar groups at one surface of the membrane point toward the liposome's interior and those at the other surface point toward the external environment. It is this remarkable reactivity of phospholipids to water that enables workers to load medications in to liposomes. In a liposome form, any water soluble molecules that have been added to the water are incorporated in to the aqueous spaces in the interior of the spheres, whereas any lipid soluble molecules added to the solvent during vesicle formation are incorporated in to the lipid layer.

Liposomes employed for drug delivery typically range in diameter from 250 angstrom units to several micrometers and are usually suspended in a solution. They have two standard forms: "onion-skinned" multilamellar vesicles (MLVs) made up of several lipid bilayers separated by fluid, and unilamellar vesicles, containing of a single bilayer surrounding an entirely fluid core. The unilamellar vesicles are typically characterized as being small (SUVs) or large (LUVs).



Liposome structure formed by phospholipids

Figure No. 2 Structure of liposome



Shape of phospholipids molecule

Figure No. 3 Shape of phospholipids molecule.

1.4 STRUCTURAL COMPONENTS OF LIPOSOMES:

The main components of liposome are

- Phospholipids
    - Phosphoglycerides
    - Sphingolipids
- Cholesterol

1.5 TYPES OF LIPOSOMES (Marc J Ostro., (1987) & Amarnath Sharma (1997)

1.5.1 Classification based on structural parameters

1. Multilamellar Large vesicles (MLV 0.1-6µm)
2. Small unilamellar vesicles (SUV 0.02-0.05 µm)
3. Large unilamellar vesicles (LUV>0.06)
4. Oligolamellar vesicles (OLV 0.1-1 µm)
5. Unilamellar vesicles (UV- wide range)
6. Gaint unilamellar vesicles (GUV –cell size vesicle s>1 µm)
7. Medium Unilamellar vesicles
8. Multivesicular vesicle (>1 µm)

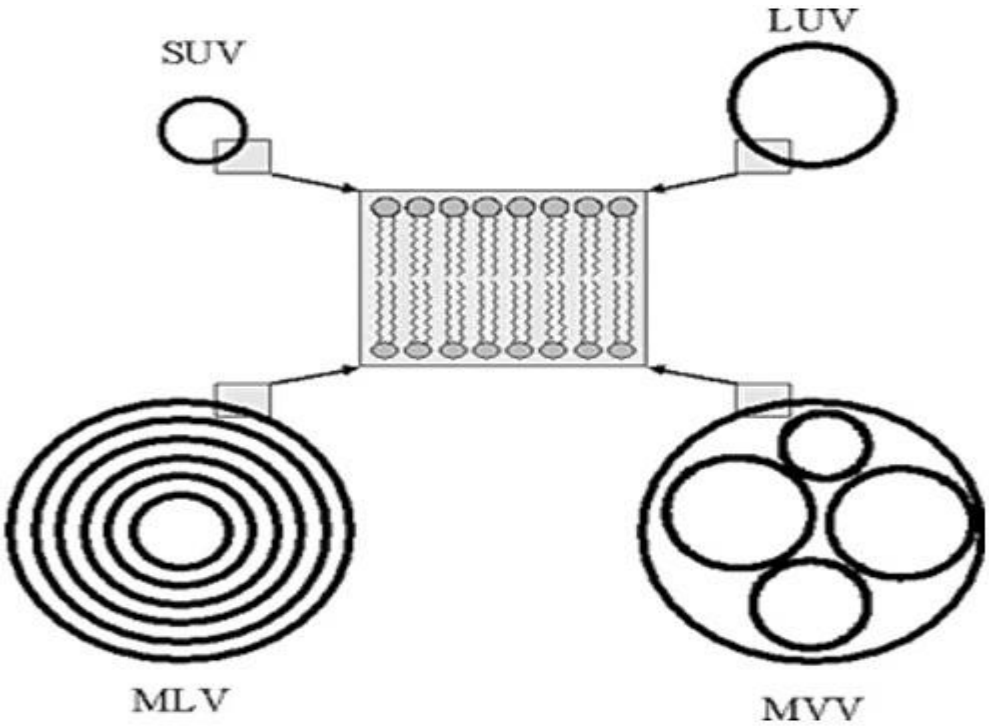


Figure No: 4 Schematic illustrations of liposomes of different size and number of Lamellae. SUV: Small unilamellar vesicles; LUV: Large unilamellar vesicles; MLV: Multilamellar vesicles; MVV: Multivesicular vesicles.

1.5.2 Classification

The Liposomes are classified as vesicular systems from biological origin and non biological origin. Specialized for cellular drug targeting are grouped separately (Vyas and Dexit, 1998).

Biological origin

1) Liposomes

Vesicles can be formed from adverse range of amphiphillic building blocks, which are prepared mainly with lecithin (Bangham et al., 1965: grogoriadis, 1972) and synthetic phospholipids.

2) Polymerizable Liposomes (Fendler, 1982)

This type of Liposomes mainly contains phospholipid derivatives of polymerizable group in their fatty acyl chains. The diacetylenic lipids, methacryloyl lipids, dienoyl lipids are used as building blocks for this type.

3) Polymer capped Liposomes (Regen et al., 1984)

Here the cationic phospholipids are ion-paired with a polymerizable anion like methacrulate ion. The elute is dispersed in Liposomes followed by polymerization using irradiation or chain initiator. In this the polymeric network encases the bilayer of Liposomes without covalent linkage.

4) Polymerized phospholipid Liposomes (Weber et al., 1987)

Phospholipid polymerized with 1, 2-bis-(2-mercaptohexadecanoyl) 3-glycerol-3-phosphocholine could readily assemble to form this type of vesicles.

5) Redox Liposomes (Samvel et al., 1985)

This type of vesicles based on thiol-disulfide redox cycle.

6) Polymer grafted Liposomes (Allen et al., 1991)

These types of Liposomes are formed by natural or synthetic phospholipids with covalently linked PEG polymer.

7) Virosomes (Al-Ahdal et al., 1994)

Virosomes, like Liposomes are spiked with virus glycoprotein like Sendai virus. They mainly contain reconstituted viral spiked glycoprotein.

8) Emulsomes (Amselem et al., 1994)

Emulsomes were constructed by phospholipids which have high concentration along with high molecular weight fatty acids.

Non-biological origin

1) Niosomes (Handijani-vila et al., 1979)

The single chain non-ionic surfactants of low HLB values were contributed mainly in construction of Liposomes.

2) Discomes (Vyas et al., 1997)

Solubilization of niosomes with non-ionic surfactant (Polyoxy ethylene acetyl ether class) forms discomes.

3) Pharmacosomes (Vizaglou et al., 1986)

The Pharmacosomes are formed by mesogenic drug which itself acts as a building block in combination with a lipid.

4) Ufasomes (Gebicks et al., 1973)

The single chain unsaturated fatty acids (Oleic, linoleic acid) are the main building blocks in this type.

5) Cryptosomes (Blume et al., 1990)

Combination of natural phospholipids with suitable poly-oxythylene derivatives of PE (Phosphatidylethanolamine) gives Cryptosomes.

Specialized Liposomes

1) Glycoprotein bearing Liposomes

Incorporation of glycoprotein in bilayer membrane gives this type of vesicles. The major glycoprotein, the glycoprotein of the human erythrocyte plasma membrane has been incorporated in to Liposomes.

2) Glycolipid bearing Liposomes

Glycolipid (like monosialo-gangliaside) appended liposome are prepared and tried to target lymphocytes.

3) Glycoside bearing Liposomes

The plant glycosides, asiaticiside and corhorusin D containing rhamnose and glucose in terminal sugar respectively have been grafted on the liposomal that are tried against *M.leprae* and *M.tuberculosis* by Medda et al., (1995)

4) Protein coated Liposomes (Longaman and co-workers, 1995)

Liposomes with protein immobilized on the surface are investigated to target extra vascular spaces by using biotinylated antibody and streptavidin.

5) Peptide carrying Liposomes (Zalipsky et al., 1995)

In this type the peptide conjugated with PWG (DSPE) which was incorporated in liposome for systemic drug delivery.

6) Polysaccharide bearing Liposomes

Polysaccharide coated Liposomes used for targeting to *Legionella pneumophilia* infected human monocyte and guinea pig.

7) Bacteriosomes, Proteoliposomes

Lectinization of Liposomes was used for targeting to Hela cells and streptococcus etc.,

8) Bioreactive Liposomes (Gregoriadis *et al.*, 1983)

Enzymes were encapsulated in Liposomes and were called as Bioreactive Liposomes. Eg. Streptokinase containing Liposome.

9) Immunosomes

Immunosomes are liposomal constructs engineered by employing immunoglobulins as pilot molecules anchored on or as a structural part of vesicles to confer specificity to a wide range of target sites. These are classified in to

a) Immunoliposomes

b) Haptenated Liposomes

c) Immunotoxin anchored Liposomes

d) Immunoprotective Liposomes

e) Immunoadjuvant Liposomes

10) Transferin based Liposomes (Wagner *et al.*, 1994)

Transferin is an important glycoprotein which is used for coating Liposomes and selectively targeted to proliferating neoplastic cell lines.

11) Enzymosomes

In this type enzymes are covalently immobilized or coupled to the surface of liposome and if a prodrug is administered simultaneously, it can convert to active form at target site by immobilized enzyme.

1.6 Liposomes delivered intercellularly

Liposomes deliver their contents to the cytoplasm of cells in culture either by fusion with the outer cell membrane or by endocytosis where upon they are concentrated in acidic lysozymal sacs. (Ostro *et al.,* 1987)

1) Inter membrane transfer

Inter membrane transfer of lipid component can take place upon close approach of the two phospholipid bilayers without the need for disruption of the liposome. Only specific phospholipids exchange (PC &PE) via intermediate of a specific cell surface exchange protein. Similarly Liposomes and lipoproteins (HDL) interactions is important liposomal stability in circulation. Addition of cholesterol retards immediate destabilization of Liposomes.

2) Contact release

The liposome content with cell causes an increase in permeability on the entrapped content through bilayer membrane, curiously, cell induced leakage of solutes have been observed to be greater in membranes with cholesterol concentration above 30%. This process strengthened by means of receptor/ligand between the two membranes.

3) Adsorption

The adsorption of Liposomes takes place either as a result of physical attractive forces, or as a result of binding by specific receptors to ligands on the vesicle membranes. The attraction depends on the specific cell surface protein. This interaction was more in case of gel phase of liposome.

4) Fusion

Close approaching of Liposomes and cell membranes can lead to fusion of the two resulting in complete mixing of liposomal lipids with those of the plasma membrane of the cell and the liposomal content released in to cytoplasm. This process takes place after phagocytosis and endocytosis of Liposomes. Inside the Endosome, Liposome were affected by acidic pH. There by this will completely fuse and deliver the drug content in to cytoplasm.

1.7 Methods of liposome preparation

1. Passive loading techniques

a. Mechanical dispersion methods

- Lipid film hydration by hand shaking non-hand shaking or freeze drying.
- Micro emulsification
- Sonication
- French pressure cell
- Membrane extrusion
- Dried reconstituted vesicles
- Freeze thawed Liposomes

b. Solvent dispersion methods

- Ethanol injection
- Ether injection
- Double emulsion vesicles
- Reverse phase evaporation vesicles
- Stable plurilamellar vesicles

c. Detergent removal methods

- Detergent removal from mixed micelles
- Dialysis
- Column chromatography
- Dilution
- Reconstituted sandal virus enveloped vesicles

2. Active loading techniques

ADVANTAGES OF LIPOSOMES

The pharmaceutical and pharmacological justification of the use of liposomes as drug carriers is as follows:

1. Liposomal supply both a lipophilic environment and aqueous "milleu interne" in one system and are therefore suitable for the delivery of hydrophobic, amphipatic and hydrophilic drugs and agents.
2. Liposomes are chemically and physically well characterized entities.
3. The biological fate of liposomes after their administration is related to their composition and physical properties.
4. Liosomes are biocompatible due to their biodegradability, low toxicity and lack of immunogenicity.
5. Liposomes can serve as device for controlled release of drugs in body fluids (micro reservoir concept) and inside cells (after endocytic uptake).

6. Liposomes help to reduce exposure of sensitive tissues to toxic drugs.
7. Liposomes can be administered through mostnroutes of administration including ocular, pulmonary, nasal, oral, intramuscular, subcutaneous, topical and intravenous.
8. Pharmacokinetics and *in-vivo* distribution of liposomes can be controlled by their port of entry combined with their lipid composition and size.

DISADVANTAGES OF LIPOSOMES

1. Aggregation, fusion and drug leakage during storage.
2. Chemically instable i.e., degradable by oxidation and hydrolysis.
3. In physiological environment they are destabilized by high density lipoproteins(HDL)
4. Purity of natural phospholipids and cost of production.
5. They undergo complete mediated phagocytosis and lipid exchange reactions.

1.8 FACTORS EFFECTING DRUG ENTRAPMENT AND RELEASE CHARACTERISTICS

Factors effecting drug entrapment and release characteristics of Liposomes include,

1. Charge
2. Lipid content
3. partition coefficient
4. Method of preparation of Liposomes

Charge

The presence of negatively charged lipid such as Phosphotidyl serine, Phosphatidicacid, Phosphotidyl inositol and Phosphotidyl glycerol or positively charged detergents such as stearylamine will tend to increase the intercellular distance

between successive bilayers in the MLV structure and thus lead to greater overall entrapped volume.

Lipid content

The total amount of liposomal lipid used and thee internal volume of the Liposomes will affect the total amount of loading of non-polar and polar group in to Liposomes. Efficient capture will depend on the use of drugs at concentration which do not exceed the saturation limit of the drug in the aqueous compartment or the lipid bilayers.

Partition coefficient

The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayer and the maximum amount of drug that can be entrapped within Liposomes in dependent on its total solubility in each phase.

Method of preparation of Liposomes

The method of preparation of Liposomes can also affect drug location and overall entrapment efficacy. Several methods are now available for preparation of Liposomes. The cast film method is simple but the major drawback with MLVs prepared is the relatively low encapsulation in terms of aqueous space per mole of lipid. Dilute preparations of Liposomes with a low encapsulation efficiency is obtained, when solvent injection method is used.

1.9 APPLICATIONS OF LIPOSOME

During the past 40 years Liposomes have received attention from the scientific community and from the industry, due to the possibility of being a pharmaceutical carrier for numerous problematic drugs. This success to drive the present interest on

the field with more than 2000 papers and reviews per year, of which most of them are related to anticancer, anti-inflammatory and anti-microbial.

1. Dinitroanilines when administered in the form of liposome shows better antimicrobial activity against leishmania than that of free drug. The improved antimicrobial activity is due to the engulfing of Liposomes by MPS cells (Mononuclear Phagocytic System) which is type of passive targeting.
2. Rifamycin in the liposomal formulation has superior effect against tuberculi when compared to that of free drug.
3. Doxorubicin can be encapsulated in liposome to reduce the serious side effects of the drug.

Hence liposome can be used as the safer and more efficient drug delivery for many problematic entities.

1.10 STABILITY OF LIPOSOMES

The stability of Liposomes is of major concern in their development for pharmaceutical applications. A drug containing Liposomes can be unstable because of physical or chemical stability. The stability studies could be broadly covers under two main sections.

Stability *invitro* mainly covers the stability aspects prior to the administration of the formulation and with regard to the stability of the constituted lipids.

Stability *invivo*, which covers the stability aspects once the formulation, is administered via various routes of biological fluids.

Stability *invitro* mainly covers :

I. Chemical degradation

[a] Oxidation (Hunt C *et al.*, 1981)

The oxidative degradation of liposome can be prevented by following the below mentioned precautions

1. Start with freshly purified lipids and freshly distilled solvent
2. Avoid procedures which involves high temperature
3. Carryout the manufacturing process in the absence of oxygen
4. De-oxygenate aqueous solution in an inert atmosphere

[b] Hydrolysis (Frfkjaer *et al.*, 1984)

1. Using lipid containing ether linkage instead of ester linkage
2. Sphingomyelin prevents *invivo* hydrolyses

II. Physical degradation (Wong M *et al.*, 1982)

Sedimentation, leaching of drugs aggregation or fusion. High manufacturing temperature and many other factors can induce this.

Physical stability can be achieved by

1. Manufacturing and storing at temperature below its transition temperature
2. Adding 10% of P.A [Phosphatidic acid] or P.O to neutral liposome for providing negative pH.
3. By cross linking membrane components covalently using gluteraldehyde fixation or polymerization of alkyne containing phospholipid.

Approaches that can be used to increase liposome stability involve efficient formulation and lyophilization. Formulation involves the selection of the appropriate lipid composition and concentration of bilayer, in addition to the aqueous phase ingredients such as buffer, antioxidants, metal-chelators and cryoprotectants. Charge inducing lipids such as Phosphotidyl glyceride be incorporated in to the liposome bilayer to decrease fusion, while cholesterol and sphingomyelin can be incorporated in formulation, in order to decrease the permeability and linkage of encapsulated drugs.

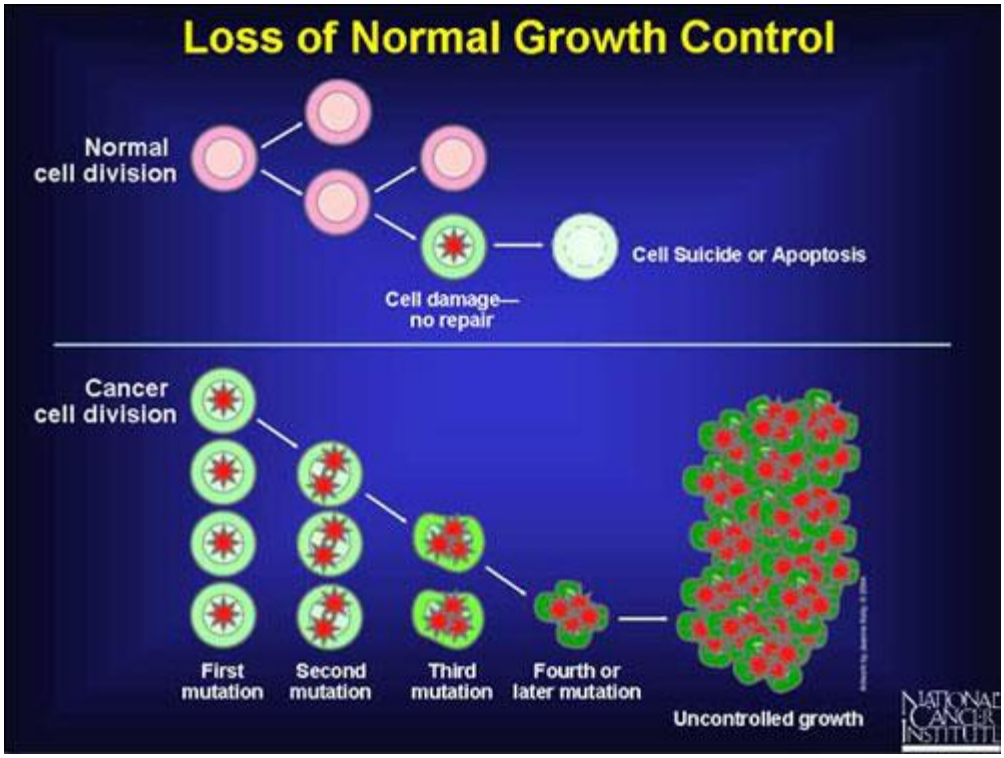
1) Buffers at neutral pH can decrease hydrolysis
2) Addition of antioxidants such as sodium ascorbate, can decrease oxidation
3) Freeze-dried liposome formulations should incorporate a lipoprotectant like non-reducing disaccharides such as trehaloes and sucrose.

1.11 CANCER CHEMOTHERPHY

Cancer (Sydney. Basic clinical pharmacology (3^{rd} edition)) is a group of neoplastic diseases that occur in human of all age groups and races as well as in all animals species. The incidence, geographic distribution and behavior of specific types of cancer are related to multiple factors include sex, age, race, genetic predisposition and exposure to environmental carcinogen.

Cancer is a disease of uncontrolled cell division, invasion and metastatic. It is generally considered to be due to the clonal expansion of a singe neoplastic cell. However there may be additional somatic leading to heterogeneous cell population.

Fig No:5 Understanding series of Cancer and normal Cell division



Cancer chemotherapy has been under intensive development for the past 30 years, resulting in cures of certain types of disseminated cancers that were previously fatal. Even patients with advanced disease have improved dramatically with chemotherapy.

Mode of action of chemotherapeutic agents

Most antineoplastic agents are regarded as "cell-cycle specific". They act specifically on processes such as DNA synthesis, transcription, or the function of mitotic spindle.

All slow growing and fast growing tumor cells display a similar pattern during the division. This may be characterized as follows:

1. There is presynthetic phase
2. The synthesis of DNA occurs
3. An interval follows the termination of DNA synthesis, the post-synthetic phase.

4. Mitosis ensures, the G2 cell, containing a double complement of DNA, divides in to 2 daughter GI cells, may immediately re-enter to the cell cycle.

Antitumor drugs are better at killing cells during DNA synthesis and active division. When a tumor is young, most of its cells are making DNA. This is defined as large growth function in this state, tumors are destroyed by drugs because the majority of their cells are making DNA and dividing.

The major problems in cancer chemotheraphy are the toxic drugs effects on normal cells and the rapid clearance of the drug from the tumor cells. Usefull drugs without side effects do not at exist. Rapidly dividing normal cell such as hair follicles, cells lining the gastrointestinal tract and bonemarrow cells involved in the immune defence system are also destroyed by the present day chemotheraphy. Nausea, hair loss, increased susceptibility to infection and many others comprising a discouraging list.

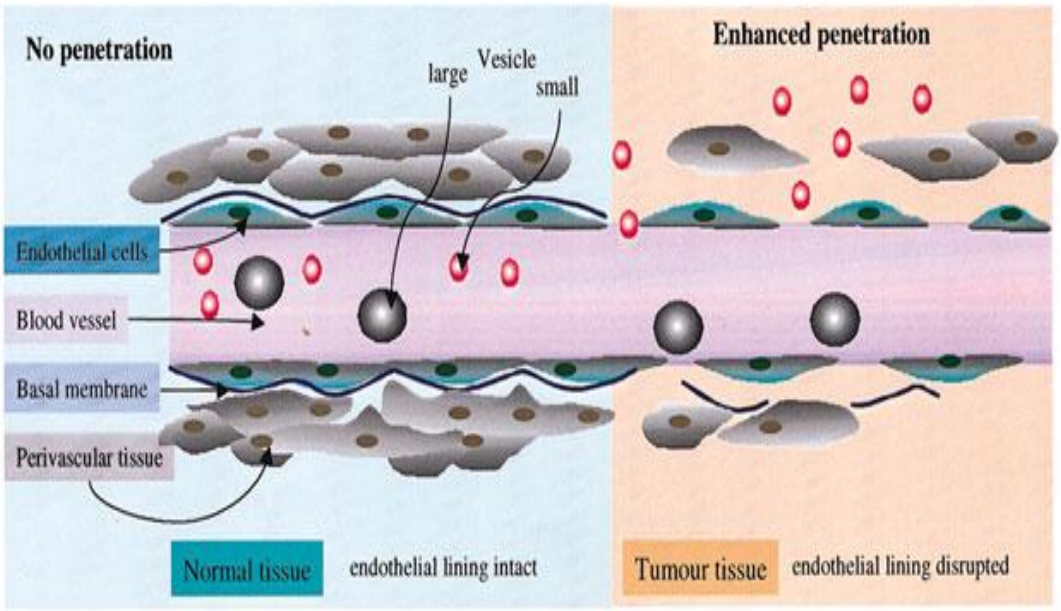
Liposomes: An ideal "Drug Carrier" for anticancer drugs

Anticancer drugs (Sayed S. Daoud (1989)) are known to produce serious side effects to other healthy tissues. The more serious effects are myocardiopathy and pulmonary toxicity. Therefore targeting such type drugs to the cancerous cell is essential because these drugs are new for the treatment of different type of carcinomas effectively. Usually a therapeutically profitable target agent relationship is far from ideal and undesirable side effects are usually observed. The alternative is to use simple functional molecules which transport the drug to the specific site and release it to perform task. Liposomes are non-toxic, biodegradable microcapsule made up of one or multiple lipid bilayers membranes.

Chemicals of interest can be entrapped inside the aqueous compartment of liposomes or can be incorporated in to the lipid bilayer. Covalent attachment of functional group to lipid molecules adds flexibility to liposomes. Liposomes have been proved a suitable vehicle for selective drug delivery and controlled drug release.

Important characteristics of drug carrier include protection of the encapsulated compound. Selective delivery of the entrapped material to specific tissues with minimal losses of drug during transit, regulation of the drug delivery rate, reduction of toxicity and removal of unused drug. All these function can co-exist in a single liposome preparation which makes it an ideal carrier of drug. Liposomes have proved to be suitable vehicles for antitumor drugs.

Figure No: 6 Accumulations of Lip osomes within Solid Tumors



For example, doxorubicin which attacks dividing cells rapidly is used in the treatment of malignant tumors. Doxorubicin's most serious side effect is progressive and irreversible damage to the heart. In addition the drug attacks hair follicles, intestinal cells and cells of immune system suppression. Laboratories in U.S, Canada and Israel have demonstrated in rodent, dogs that liposomal doxorubicin is an effective as the free drug but is several time less toxic to the heart.

If liposomes carrying a drug are exposed to oxygen radicals, the hydro peroxides formed will affect the permeability, behavior of the vesicles, thus causing premature release of the drugs targeting to a specific site. The targeting of compounds attached to proteins of polymers, which may be modified by radical attack, could provoke an adverse response thus amplifying the initial damage. Eg: In microbial infections with excessive phagocyte action or in disease involving redistribution and decompartmentalisation of iron. However optimization of a particular function is possible by modifying liposome composition, charge and size.

LITERATURE REVIEW

2. LITERATURE REVIEW

Amarnath Sharma *et al.,* described that liposomes are microparticulate lipoidal vesicles which are under extensive investigation as drug carriers for improving the delivery of therapeutic agents. In this they discussed the potential applications of liposomes in drug delivery with examples of formulation approved for clinical use, and the problems associated with further exploitation of these drug delivery systems.

Antoneta *et al.,* studied about cholesterol and other sterols are important components of biological membranes and are known to strongly influence the physical characteristics of lipid bilayers. Although this has been studied extensively in fully hydrated membranes, little is known about the effects of cholesterol on the stability of membranes in the dry state.

Chieny *et al.,* described the concept of designing specified delivery systems to achieve selective drug targeting. In this they compared between the conventional and targeting drug delivery systems. They described about advantages and limitations of target drug delivery systems. They described that the principle and rationale of drug targeting.

Eugenia *et al.,* reported the advantages and application of liposome with respect to anti-cancer, anti-inflammatory and anti-microbial agents. In this they described the advantages of the encapsulation macromolecules like enzymes into liposomes. In this study they incorporated the enzymes into liposomes by dried film hydration method and studied the therapeutic activity of enzymes.

Fresta *et al.,* described the preparation of various kinds of loaded of 5FU and reported on encapsulation efficacy, storage stability and fusogenic properties. They concluded that the most suitable liposome preparation was the SPL Vs that showed both better drug loading and stability parameters than others.

Ganesh *et al.,* studied Inclusion of docetaxal in liposomal formulation has proved to be a good approach to eliminating the vehicle and improving the drug's antitumor activity. We formulated docetaxel Liposomes containing phosphatidylcholine (soybean lecithin), cholesterol and various stabilizers by the dried thin film hydration method. Particle size analysis, drug content and entrapment efficiency in charged Liposomes were strongly affected by the different stabilizers, the stability of the lyophilized docetaxel Liposomes were evaluated after stored at 40C and the room temperature for 3 months. The Liposomes stored at 40C were found to be stable for duration of 3months. Hence it can be concluded that stabilizers like Stearylamine and Dicetylphosphate along with cholesterol were suitable carrier for the preparation of liposomal docetaxel.

Gautam Vinod *et al.,* invented a long circulating non-pegylated liposomal doxorubicin hydrochloride composition for parenteral administration and a process for its preparation. The circulation time in Swiss albino mice is at least 25 times longer than conventional non-liposomal formulations. The non-pegylated liposomes are stable, exhibit low toxicity and have been found to be efficacious in different tumor models.

Gregoiadis *et al.,* reported the method of liposome formulation with lecithin (egg phosphatidly choline). In this they described about method to find out the percentage of drug loading and the usage of Triton-X 100 for the percentage of drug loading in liposome. They found that Triton-X 100 can be used to disrupt the lipid bilayer in liposome. Hence thereby the bound drug liberated out of the liposome.

Harrington *et al.,* described the slow process involved in the development of clinically relevant liposomal therapies and has show that, after concerted effort, a number of formulations are entering routine clinical practice. Indeed, in the near future, it is likely that more formulations of existing or novel cytotoxic agents will appear. In addition to this work, a number of approaches with the aim of enlarging the therapeutic repertoire of liposomal agents and improving their targeting potential are under investigation. Strategies employing liposomes to delivery radiosensitizers,

cytokines and immunomodulators have been described, as have attempts to increase tumour targeting with the use of hyperthermia and antibody-coated liposomes. Leptosomes are also receiving increased attention as potentially useful vehicles for the delivery of recombinant DNA constructs in the emerging science of gene therapy, although most of this work has focused on cationic liposomes which have very different pharmacokinetic profiles to the liposomes discussed in this review article.

Jain *et al.,* described the target drug delivery systems were likely to overcome much of the criticism of conventional dosage forms. They summarized that the optimized drug delivery that encompasses greater potency & greater effectiveness, lesser side effects and toxicity, better stability, low cost hence greater accessibility, ease of administration and best patient compliance. They also described about carriers used in targeted drug delivery systems.

Jorge *et al.,* reported the methods of liposomal formulation for encapsulating the enzyme (L-Asparaginase). In this study they formulated liposomes with natural phospholipids (egg yolk lecithin). They also described the method to conjugated the enzyme with palmitic acid to form palmitoyl-enzyme complex, which can further prolongs the circulation time of enzyme in general circulation without affecting the enzymatic activity i.e. from 2.88 hr to 23.7 hr.

Lasic *et al.,* described about the recent discoveries in the field of liposomes and latest application of liposomes. They described the usage of different types of liposomes and their advantages. In this they studied the efficacy of DNA encapsulation in liposomes. And in the treatment of colon carcinoma and AIDS related Kaposi sarcoma. From this study they confirmed the liposomes can be used as carrier for biological like RNA, DNA, etc.

Luigi Cattel *et al.,* synthesized a series of increasingly lipophilic pro drugs of gemcitabine by linking the 4-amino group with valeroyl, heptanoyl, lauroyl and stearoyl linear acyl derivatives. They studied their stability at storage, in plasma and with the lysosomal intracellular enzyme cathepsins and also studied incorporation of these lipophilic prodrugs in liposomes, where their encapsulation efficiency (EE)

closely depends on the length of the saturated 4-(N)-alee chain, the phospholipids chosen and the presence of cholesterol. A maximum EE for 4-(N)-steroyl-gemcitabine incorporated in DSPC/DSPG 9:1. This formulation was correlated with the highest stability in vitro and in vivo. Cytotoxicity of Gemcitabine prodrugs, free or encapsulated in liposomes, was between two- and sevenfold that of free gemcitabine.

Marc Ostro *et al.,* described the methods to reduce the dosage of drug with the help of liposomes and their potential advantages in different types of diseased states. In this they confirmed that liposomes are better dosage form than conventional dosage forms. They have also stated the drug can be targeted by active and passive targeting and the uses of both passive and targeting of liposomes.

Mirant Ahmad *et al.,* developed a well characterized novel lyophilized liposome-based paclitaxel (LEP-ETU) formulation that is sterile, stable and easy-to-use. The mean particle size of the liposomes is about 150 nm before and after lyophilization, and the drug entrapment efficiency is greater than 90% stability data indicated that the lyophilized LEP-ETU was physically and chemically stable for at least 12 months at 2-8 and 25 8C. Moreover the formulation can diluted to about 0.25 mg/ml without drug precipitation or change in particle size. In vitro drug release study in phosphate-buffered saline (PBS, pH 7.4) showed that less than 6%of the entrapped paclitaxel was released after 120 h, indicating that the drug is highly stable in an entrapped form at physiologic temperature.

Monostoi *et al.,* [2004] studied the stability and transdermal absorption of topical amphotericin B liposome formulation and found that the positively charged liposome might be the best formulation for AmB, dne to its higher stability than other formulations.

Sayed Daoud *et al.,* described the challenge of chemotherapy in this they discussed about liposomes in cancer therapy and liposomes: an ideal “drug carrier” for anti cancer drugs. They described the preclinical studies, clinical trials-phase 1 and 2 and drug resistance of anthracyclines and also described alkylating agents and platinum compounds.

Soleiman Mohammadi-Samani *et al.,* Described Cyproterone acetate (CA) has been loaded to liposome by solvent evaporation and thin film formation technique. The effects of some formulation variables such as temperature of organic solvent evaporation, rotary evaporator speed, volume of organic solvent, volume of balloon and temperature of hydrating buffer has been evaluated. Finally percutaneous absorption of CA from simple gel and liposomal formulations was assessed. The results showed that liposomal formulation has better penetration potential than conventional CA formulation (simple gel).

Sydney *et al.,* described the cancer chemotherapy in this they discussed incidence, geographic distribution and behavior of specific types of cancer. In that they described the mode of action, adverse effects, clinical use and dose administration of different chemotherapeutic agents.

Tyrrell *et al.,* described the general consideration and method of preparation of liposomes and also described about liposome- protein interaction and protein entrapment liposomes. They summarized about uptake of liposomes in vivo and interaction of liposomes with cell culture and immunological aspects of liposomes.

Uchegbu *et al.,* attempted at anti-cancer drug targeting with doxorubicin (DOX), a DOX N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer showing tumour tropism in animals and designed to release DOX following intracellular cleavage by lysosomal enzymes is now in early clinical development. This macromolecular prodrug targets tumours by an enhanced penetration and retention (EPR) effect in which the leaky vasculature and decreased lymphatic drainage within tumours results in high intra tumoural levels of the drug which may be elevated further by increasing the polymer molecular weight. To reduce renal clearance of PKI and thus increase tumour accumulation, a non-ionic surfactant vesicle (niosome) formulation of PKI has been developed. Here we have studied the effect of method of preparation on PKI loading noisome, size, stability and DOX release.

Wollina *et al.,* studied the usage of two drugs namely, doxorubicin and daunorubicin in liposomal formulation for treatment of skin cancer like cutaneous T-cell lymphoma, malignant melanoma, AIDS related Kaposi's sarcoma. They formulated liposomes with synthetic phospholipids and stabilizing agents like stearylamine and cholesterol. They also found that the efficacy of drug is improved in liposomal formulation than that of free drug.

Xue Ming *et al.,* studied site specific delivery of drugs and therapeutics can significantly reduce drug toxicity and increase the therapeutic effect. Transferring is one suitable ligand to conjugated to drug delivery systems to achieve site specific targeting, due to specific binding to transferring receptors, Administration of Tf-DOX to tumor-bearing mice could be used to deliver DOX effectively to the targeted site. This study indicated that the Tf-coupled PEG Liposomes could be as the targeted carriers to facilitate the delivery of the encapsulated anticancer drugs in to tumor cells by receptor-mediated way.

Yousefi *et al.,* An improved pegylated liposomal formulation of docetaxel has been developed with the purpose of improving the docetaxel solubility without any need to use tween80 that is responsible for hypersensitivities following administration. Liposomes all had spherical shape with size of 130–160 nm. The most important finding of this study is that pegylated Liposomes were prepared with significant increase in docetaxel encapsulation efficiency and stability of the formulation in comparison with last reports on docetaxel Liposomes. In vitro release studies revealed that such a formulation could be stable in the blood circulation and meet the requirements for an effective drug delivery system.

AIM AND PLAN OF WORK

3. AIM AND PLAN OF WORK

Liposomes have been used to target drug to specific organs, delay the loss of rapidly cleared, drugs, enhances therapeutic potency and offer a host of the other advantages.

Doxorubicin hydrochloride is one of the most commonly used cytotoxic anthracycline antibiotics used in cancer chemotherapy and has been shown to have activity against a wide variety of neoplasms.

Conventional compositions of doxorubicin hydrochloride are available as freeze-dried product (or) as a solution of doxorubicin hydrochloride in water. Both these products have been associated with a number of toxicities when administered intravenously. Severe myelosupression, nausea, vomiting, alopecia, mucosistis & cardio toxicity, limits the use of Doxorubicin Hcl. It also causes extravasations & necrosis at the site of injection.

To overcome these problems, an alternative approach is needed. In the present study doxorubicin Hcl liposomes are formulated using various biolipids and Stabilizers (Positive and Negative) to check effect of drug loading and particle size. Several approaches has taken in an effort to increase the circulation time of liposome by coating the liposomal surface with a hydrophilic polymer such as polyethylene glycol (PEG) to prevent adsorption of various blood plasma proteins to the liposome surface. These liposomes appeared to reduce some of the toxic effects caused by the release of their contents, but have new toxic effects appeared like skin toxicity generally known as "Hand-Foot Syndrome" and the presence of large molecules (PEG) on the liposomal surface may reduce the interaction of liposomal with cells & hinder entry of liposomes in to tumor tissue.

Thus, these remains a need for stable, long circulating liposomes that do not cause such deleterious effects such as the "Hand-Foot Syndrome" as well as methods of manufacturing such liposomes & composition based on them. The present formulation meets this need, and testing the effect of stabilizers on particle size analysis, percent free drug, Assay, *In-vitro* drug release studies, release kinetics & stability studies.

IT WAS PLANNED TO CARRY OUT THE PRESENT STUDY AS FOLLOWS:

STAGE 1:

1. Preformulation studies
    a. Standard calibration curve of Doxorubicin hydrochloride in UV
    b. Compatibility studies

STAGE 2:

1. Preparation of Plain Liposomes with Ammonium sulphate and stabilizers.
2. Preparation of drug loaded liposomes with Stabilizers by Dried Thin Lipid Film Hydration Technique.

STAGE 3:

1. Physical characterization of liposomes
    a. Particle size analysis
    b. Zeta potential
    c. Scanning Electron Microscopy
    d. Polydispersity index

STAGE 4:

1. *In vitro* characterization
    a. Percent free drug
    b. Assay
    c. Study on in vitro drug release.
    d. Release kinetics

STAGE 5:

1. Short term stability studies

PROFILES

4. PROFILE

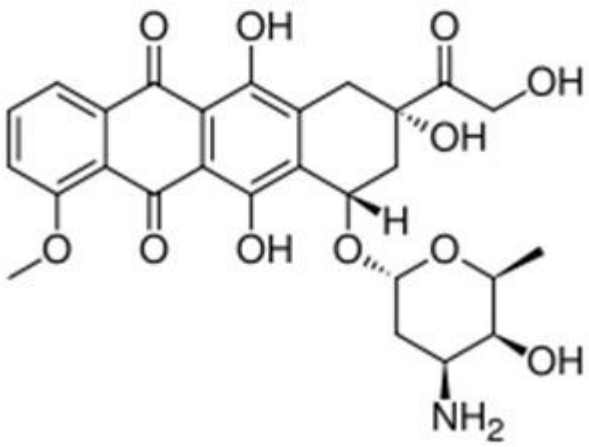
4.1DRUG PROFILE:

Doxorubicin hydrochloride:

Doxorubicin is a drug used in cancer chemotherapy, it is an anthracycline topoisomerase inhibitor isolated from *streptomyces peucetius var. caesius.*

Doxorubicin is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas (Wikipedia).

Chemistry



STRUCTURE OF DOXORUBICIN HYDROCHLORIDE

Systemic (IUPAC) name
(8*S*, 10S)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2*H*-pyran-2-yloxy) -6, 8, 11-trihydroxy-8-(2hydroxyacetyl) -1-methoxy-7, 8, 9, 10-tetrahydrotetracene -5, 12-dione

Molecular formula: $C_{27}H_{29}NO_{11}$

Molecular weight: 579.98gm.

Melting point: 204-205^{o} C.

Physics description: Red crystalline powder**.**

Solubility: Sparingly soluble in cold water and Insoluble in diethyl ether.

Bioavailability: 5% (Oral).

Half-life:12–18.5 hours

Mechanism of action:

The exact mechanism of action of doxorubicin is complex and still somewhat unclear, though it is thought to interact with DNA by intercalation. Doxorubicin is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes super coils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures.

Pharmacokinetics

The plasma pharmacokinetics of Doxorubicin Hydrochloride were evaluated in 42 patients with AIDS relative Kaposi's sarcoma (KS) who received single doses of 10 or 20mg/m2 administered by a 30-minute infusion. Twenty –three of these

patients received single doses of 10 and 20 mg/m2 with a 3-week wash-out period between doses.

Doxorubicin Hydrochloride linear pharmacokinetics is over the range of 10 to 20 mg/m2. Disposition occurred in two phases after Doxorubicin hydrochloride administration, with a relatively short first phase (5 hours) and a prolonged second phase (55 hours) that accounted for the majority of the area under the curve (AUC).

Distribution:

In contrast to the pharmacokinetics of doxorubicin, which displays a large volume of distribution, ranging from 700 to 1100 L/m2, the small steady state volume of distribution of Doxorubicin Hydrochloride shows that Doxorubicin Hydrochloride is confined mostly to the vascular fluid volume. Plasma protein binding of Doxorubicin Hydrochloride has not been determined; the plasma protein binding of doxorubicin is approximately 70%.

Metabolism:

Doxorubicinol, the major metabolites of doxorubicin, was detected at very low levels (range: of 0.8 to 26.2 ng/mL) in the plasma of patients who received 10 or 20 mg/m2 Doxorubicin Hydrochloride.

Excretion:

The plasma clearance of Doxorubicin Hydrochloride was slow, with a mean clearance value of 0.041 $L/h/m^2$ at a dose of 20 mg/m^2. This is in contrast to doxorubicin, which displays a plasma clearance value ranging from 24 to 35 $L/h/m^2$.

Because of its slower clearance, the AUC of Doxorubicin Hydrochloride, primarily representing the circulation of liposome-encapsulated doxorubicin, is approximately two to three orders of magnitude larger than the AUC for a similar dose of conventional doxorubicin HCL as reported in the literature.

Tissue distribution in patients with Kaposi's sarcoma:

Kaposi's sarcoma lesions and normal skin biopsies were obtained at 48 and 98 hours pos infusion of 20 mg/m^2 Doxorubicin hydrochloride in 11 patients. The concentration of Doxorubicin Hydrochloride in KS lesions was a median of 19 (range, 3-53) times higher than in normal skin at 48 hours post treatment; however, this was not corrected for likely difference in blood content between KS lesions and normal skin. The corrected ratio may lie between 1 and 22 times. Thus, higher concentrations of Doxorubicin Hydrochloride are delivered to KS lesions than to normal skin.

CLINICAL STUDIES

Ovarian cancer:

Doxorubicin Hydrochloride was studied in three open-label, single-arm, clinical studies of 176 patients with metastatic ovarian cancer. One hundred forty-five (145) of these patients were refractory to both paclitaxel- and platinum-based chemotherapy regimens. Refractory ovarian cancer is defined as disease progression while on treatment, or relapse within 6 months of completing treatment. Patients in these studies received Doxorubicin Hydrochloride at 50 mg/m^2 infused over one hour every 3 or 4 weeks for 3-6 cycles or longer in the absence of dose-limiting toxicity or progression of disease.

Side effects:

The following adverse reactions are discussed in more detail in other sections of the labeling.

- Cardiac Toxicity
- Infusion reactions
- Myelosuppression

The most common adverse reactions observed with Doxorubicin Hydrochloride are

- Asthenia
- Fatigue
- Fever
- Nausea
- Stomatitis
- Vomiting
- Diarrhea
- Constipation
- Anorexia
- Hand-foot syndrome
- Rash and neutropenia
- Thrombocytopenia and anemia.

DOSAGE AND ADMINISTRATION

Liposomal encapsulation can substantially affect a drug's functional properties relative to those of the unencapsulated drug. Therefore do not substitute one drug for the other.

Do not administer as a bolus injection or an undiluted solution. Rapid infusion may increase the risk of infusion-related reactions. Doxorubicin Hydrochloride must not be given by the intramuscular or subcutaneous route.

Doxorubicin Hydrochloride should be considered an irritant and precautions should be taken to avoid extravasations. With intravenous administration of Doxorubicin Hydrochloride, extravasation may occur with or without an accompanying stinging or burning sensation, even if blood returns well on aspiration of the infusion needle. If any signs or symptoms of extravasation have occurred, the infusion should be immediately terminated and restarted in another vein. The

application of ice over the site of extravasations for approximately 30 minutes may be helpful in alleviating the local reaction.

Ovarian Cancer:

Doxorubicin Hydrochloride should be administered intravenously at a dose of 50 mg/m2 at an initial rate of 1 mg/min to minimize the risk of infusion reactions.

Kaposi's sarcoma:

Doxorubicin hydrochloride should be administered intravenously at a dose of 20 mg/m2. An initial rate of mg/min should be used to minimize the risk of infusion-related reactions.

Multiple Myeloma:

Bortezomib is administered at a dose of 1.3 mg/m^2 as intravenous bolus on days 1, 4, 8 and 11, every three weeks. Doxorubicin Hydrochloride 30 mg/m^2 should be administered as a 1-hr intravenous infusion on day 4 following bortezomib.

Dose Modification Guidelines:

Doxorubicin Hydrochloride exhibits nonlinear pharmacokinetics at 50 mg/m^2; therefore, dose adjustments may result in a non-proportional greater change in plasma concentration and exposure to the drug.

Patients should be carefully monitored for toxicity. Adverse reactions, such as HFS, hematologic toxicities, and stomatitis may be managed by dose delays and adjustments. Following the first appearance of a grade 2 or higher adverse reactions, the dosing should be adjusted or delayed as described in the following tables. Once the dose has been reduced, it should not be increased at a later time.

4.2 LIPID PROFILE:

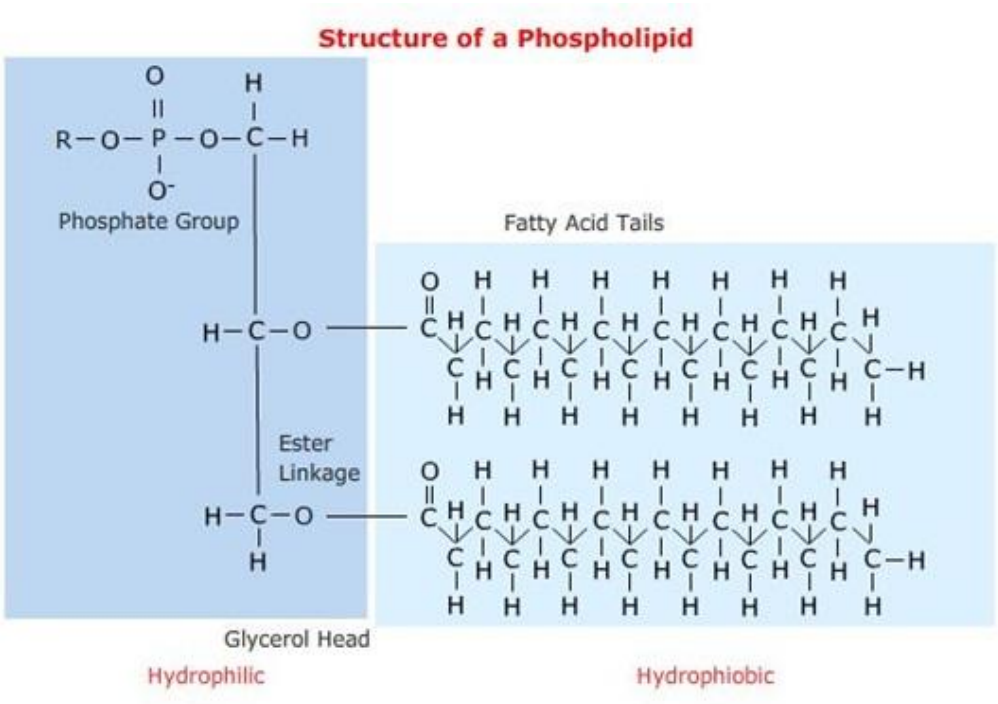
Soy lecithin

Lecithin (Wikipedia. Org/wiki/lecithin) is a group of yellow-brownish fatty substance occurring in animal and plant tissues, and in egg yolk, composed of phosphoric acid, choline, fatty acids, glycerol, glycolipids, triglycerides, and phospholipids (e.g., Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol). However, lecithin is sometimes used as a synonym for pure phosphatidylcholine, a phospholipids that is the major component of its phosphatide fraction. It may be isolated either from egg yolk (in Greek lekithos or from soy beans, from which it is extracted chemically (using hexane)) or mechanically.

Lecithin is used as a food supplement and for medical uses.

Chemistry:

Lecithin used for the study is composed of different type of phospholipids like phosphatidylcholine, phosphatidyletanolamine and phoaphatidyllinositol and lysophophatidylcholine cholesterol. The structure of basic phospholipids molecule is given below.



Description:

Colour: Yellowish brown

Molecular Formula: $C_{36}H_{72}NO_8P$

Molecular Weight: 677.93gm

Consistency: Agglomerates

Iodine value: 85-95

Peroxide value: n.m.t 3

Solubility:

Lecithin is soluble in both aqueous and organic phase. Hence it can be used as emulsifier in food industry and it is also capable of forming vesicles thereby it is used in pharmaceutical industry. It gives clear or slightly opalescent solutions with both phases.

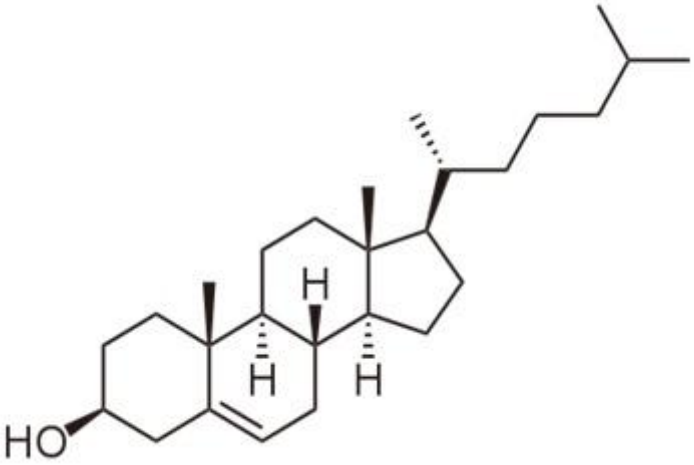
4.3 Cholesterol:

Cholesterol is a waxy steroid of fat that is manufactured in the liver or intestines. It is used to produce hormones and cell membranes and is transported in the blood plasma of all mammals. It is an essential structural component of mammalian cell membranes. It is required to establish proper membrane permeability and fluidity. In addition cholesterol is an important component for the manufacture of bile acids, steroid hormones and vitamin D.

Description:

White or faintly yellow, almost odorless, pearly leaflets, needles, powder and granules. On prolonged exposure to light and air cholesterol acquires a yellow to tan color.

CHEMISTRY:



IUPAC name

(3β)-Cholest-5-en-3-ol

Synonyms: Cholestrin

Molecular Formula: $C_{27}H_{46}O$

Molar mass: 386.65 g/mol

Appearance: white crystalline powder 111

Density: 1.052 g/cm^3

Melting point: 148-150^{o}C

Boiling point: 360^{o}C (decomposes)

Solubility in water: 0.095 mg/L (30^{o}C)

Solubility: Soluble in acetone, benzene, chloroform, ethanol, ether, hexane, isopropyl myristate and methanol.

Metabolism and Excretion:

Cholesterol is oxidized by the liver in to a variety of bile acids. These in turn are conjugated with glycine, taurine, glucuronic acid, or sulphate. A mixture of conjugated and non-conjugated bile acids along with cholesterol itself is excreted from the liver in to the bile. Approximately 95% of the bile acids are reabsorbed from the intestine and the remainder lost in the feces. The excretion and reabsorption of bile acids forms the basis of the Entero-hepatic circulation which is essential for the

digestion and the absorption of dietary fats. Under certain circumstances, when more concentrated, as in the gallbladder, cholesterol crystallizes and is the major constituent of most gallstones, although lecithin and bilirubin gallstones also occur less frequently.

Function:

Cholesterol is required to build and maintain cell membranes; it regulates membrane fluidity over a wide range of temperatures. The liver produces about 1 gram of cholesterol per day, in bile. The hydroxyl group on cholesterol interacts with the polar. Head groups of the membrane phospholipids and Sphingolipids, while the bulky steroid and the hydrocarbon chain is embedded in the membrane, alongside the nonpolar fatty acid chains of the other lipids. Bile which is stored in the gallbladder and helps digest fats is important for the absorption of the fat soluble vitamins, vitamins A, D, E and K. It also reduces the permeability of the plasma membrane. In myelin, it envelops and insulates nerves, helping greatly to conduct nerve impulses. It also reduces the permeability of the plasma membrane to protons (Positive hydrogen ions) and sodium ions.

Stability and storage Condition:

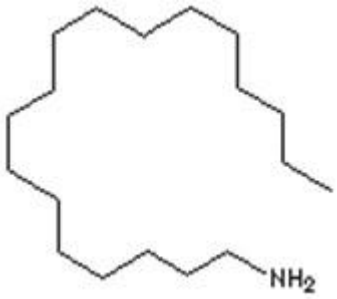
Cholesterol is stable and should be stored in a well closed container, protected from light.

EXCEPIENTS PROFILE:

4.4 STEARYL AMINE:

Synonyms : 1-Amonooctadecane, Octadecylamine, n-Stearylamine, 1-Octadecanamine, Octadecylamine, Monooctadecylamine, n- Octadecylamine.

Structure:



Molecular formula: $CH_3(CH_2)_{17}NH_2$

Molecular weight: 269.52gm.

Classification: Amines / Surfactants

Physical state: White to off-white solid

Melting Point: 47-53^{0}C

Boiling Point: 232^{0}C

Solubility in water: Practically insoluble

Stability: Stable under ordinary conditions

Applications:

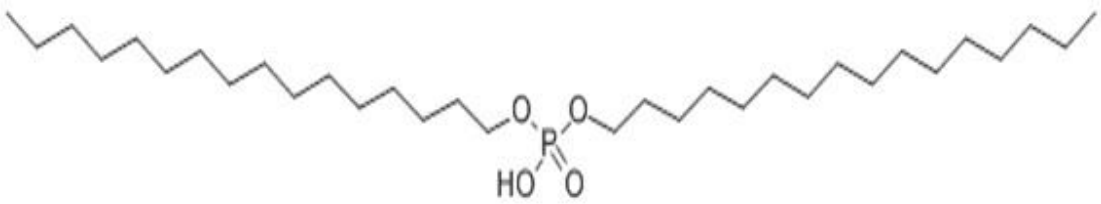
- Cationic surfactants (disinfectants, fungicides, germicide, leveling agents, hair rinse bases, wood preservatives, textile softeners, dyeing auxiliaries, ore flotation. pigment grinding aids. anticaking agents)
- Amphoteric surfactants and Amine oxides (antistatic agent, textile scouring agent, ingredient for low irritation shampoo, liquid detergent, foam booster, oil recovery agent)
- Corrosion inhibitors and asphalt emulsifier
- Dispersants, lubricants, water treatment agents.

- Fatty amine products are used as a dispersing agent or internal/external lubricant for benefits in polymer production of to facilitate and stabilize the dispersion of solid compounding materials to enhance processability, to decrease friction and abrasion of the polymer surface, and to contribute color stability and corrosion prevention.

4.5 DICETYL PHOSPHATE:

Synonyms: 1-Hexadecanol, hydrogenphosphate; bis (hexadecyl) phosphate; Dicetylhydrogenphosphate; di-n-hexadecylphosphate; phosphoric acid Dihexadecyl ester; dihexadecyl hydrogen phosphate; dihexadecyl phosphate; Dicetyl phosphate.

Structure:



Molecular Formula: $C_{32}H_{67}O_4P$

Molecular Weight: 546.85

Physical state: White powders

Melting Point: 74-75^{0}C

Storage temp: -20^{0}C

Applications:

- Anionic surfactant.
- Corrosion inhibitors and asphalt emulsifier
- Dispersants, lubricants, water treatment agents.

MATERIALS AND INSTRUMENTS

5. MATERIALS AND INSTRUMENTS

Materials

The materials used in the present investigation were either AR/LR grade or the best possible pharma grade.

Table No:1 List of Chemicals

S.No	Ingredients	Manufactures
1.	Doxorubicin hydrochloride	Sterling biotech, china.
2.	Soybean lecithin	Lipoid Pvt. Ltd., Mumbai.
3.	Cholesterol	Lipoid Pvt. Ltd., Mumbai.
4.	Dicetyl phosphate	Sigma Aldrich, Mumbai.
5.	Stearylamine	Sigma Aldrich, Mumbai.
6.	Ammonium sulphate	Triveni chemicals, Mumbai.
7.	Sucrose	Triveni chemicals, Mumbai.
8.	Histidine	Merck chemicals, Mumbai.
9.	Chloroform	Fisher scientific, Mumbai. .
10.	Sodium hydroxide	Merck chemicals, Mumbai.
11.	Triton X-100	Merck chemicals, Mumbai.
12.	Acetonitrile	Merck chemicals, Mumbai.
13.	Methanol	Merck chemicals, Mumbai.
14.	Sodium lauryl sulphate	Merck chemicals, Mumbai.
15.	Isopropyl alcohol	Merck chemicals, Mumbai.

Equipments and Instruments:

1. UV- Visible spectrophotometer (PerkinElmer's)
2. Infra Red spectroscopy (Bruker)
3. Rotary vacuum evaporator (Buchi)
4. Homogenizer (Panda)
5. Peristaltic pump (Electro lab)
6. Electronic balance (Sartorius)
7. Centrifuge (Remi Instruments)
8. Bath Sonicator
9. Electronic Microscope (Motic)
10. HPLC (LC20AD, Shimadzu)
11. Magnetic stirrer (Remi Instruments)
12. Zeta Sizer version 6.00(Malvern)
13. Scanning Electron Microscopy(Field Instruments)

METHODOLOGY

6. METHODOLOGY

6.1STANDARD CALIBRATION CURVE

Standard calibration curve of doxorubicin hydrochloride was developed using phosphate buffer pH 7.4 and estimated by UV-Visible spectrophotometer at 254nm.

6.1.1 General Procedure for the preparation of calibration curve by UV

A stock solution of (1mg/ml) of standard drug was prepared, later required dilutions were made with a phosphate buffer pH 7.4. To a series of 10ml volumetric flasks aliquots standard solutions were taken and the volume was made up using a phosphate buffer pH 7.4. The absorbance of these solutions was measured at respective wave length of maximum absorbance, using 1cm quartz cuvette in UV-Visible spectrophotometer. Absorbance values were plotted against respective concentration to obtain standard calibration curve.

6.2 COMPATIBILITY STUDIES

IR spectroscopy can be used to investigate and predict any physicochemical interactions between different components in a formulation and therefore it can be applied to the selection of suitable chemically compatible excipients.

The aim of the present study was to test, wether there is any interactions between the carriers and drug; The following IR spectroscopy were recorded

- Doxorubicin hydrochloride.
- Soybean lecithin.
- Cholesterol.
- Mixture of phospolipid and cholesterol.
- Mixture of phospolipid, cholesterol, drug and Stearylamine.
- Mixture of phospolipid, cholesterol, drug and Dicetyl phosphate.

One part of the sample and three parts of potassium bromide were taken in a mortar and triturated. A small amount of triturated sample was taken in to a pellet maker and was compressed at 10kg/cm^2 using hydraulic press. The pellet was kept on to the sample holder and scanned from 4000cm^{-1} to 400cm^{-1} in Bruker IR spectrophotometer. Then it was compared with original spectra.

IR spectra was compared and checked for any shifting in functional peaks and non-involvement of functional group. From the spectra it is clear that there is no interaction between the selected carriers, drug and mixtures. Hence the selected carrier was found to be compatible in entrapping the selected doxorubicin hydrochloride with carriers without any mutual interactions.

6.3 PREPARATION OF DOXORUBICIN LIPOSOMES

6.3.1 Procedure for the preparation of doxorubicin liposome (Xue Ming Li *et al.*, 2009)

The preparation of liposomes with Soybean lecithin was prepared by dried thin film hydration technique using rotary evaporator.

Accurately weighed quantities of Soy lecithin, cholesterol, Stearylamine and Dicetylphosphate are dissolved in chloroform and rotated in a rota-vap by applying vaccum of about 25mmHg at 25^{0}c, until it forms a thin film. Required quantities of ammonium sulphate and sucrose (0.3%) are dissolved in W.F.I and it is added to the above thin film in R.B flask and rotated until it forms a milky white suspension. The above solution is homogenized for 15 cycles to reduce particle size of liposomes. The above solution is undergone for 25 cycles of dialysis, by using sucrose solution (10%) to remove free ammonia and sulphate from the lipid solution. Drug solution is prepared by adding the required quantities of Drug and Histidine in a W.F.I and pH is adjusted to 6.4 to 6.7 and this drug solution is added to the solution in a R.B flask (lipid solution) and rotated for 1hr.

In-process Checks:

RPM: 65-70rpm (Film formation), 50-55rpm (Hydration), 60-65rpm (Drug Loading).

Temperature: 40-45°C (Film formation), 65-70°C (Hydration), 65°C (Drug Loading).

The composition and ratios of lecithin, cholesterol and stabilizers for different types of Liposomes were mentioned in Table No: 7 and 8.

6.4 PHYSICAL CHARACTERIZATION OF LIPOSOMES

All the liposomal formulation was evaluated by studying their physicochemical properties like

- Particle size analysis
- Polydispersity index
- Zeta potential analysis
- SEM analysis

6.4.1 Determination of particle size distribution

Determination of average vesicle size of doxorubicin hydrochloride liposomes with carrier was very important characteristic. It was carried out by using MALVERN INSTRUMENTS, STARTECH LABS PVT. LTD.

6.4.2 Polydispersity Index:

Polydispersity was determined according to the equation,

Polydespersity = D (0.9) - D (0.1) / D (0.5)

Where,

D (0.9) corresponds to particle size immediately above 90% of the sample.

D (0.5) corresponds to particle size immediately above 50% of the sample.

D (0.1) corresponds to particle size immediately above 10% of the sample.

6.4.3 SCANNING ELECTRON MICROSCOPY

Determination of surface morphology (roundness, smoothness and formation of aggregates) of doxorubicin hydrochloride Liposomes with carrier was carried out by scanning electron microscopy (**SEM**). Samples for by **SEM** were mounted on metal studs and were magnified to X 2000.

6.4.4 ZETA POTENTIAL ANALYSIS

Zeta potential is a physical property which is exhibited by any particle in suspension. It can be used to optimize the formulations of suspensions and emulsions. Knowledge of the zeta potential can reduce the time needed to produce trial formulation. It is also an aid in predicting long-term stability. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repeal each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values then there will be no force to prevent the particles coming together and flocculating.

The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or

flocculate. A value of 25mV (positive or negative) can be taken as the arbitrary value that separates low-charged surfaces from high-charged surfaces. The zeta potential was analyzed by MALVERN ZETASIZER in INDIAN INSTITUTE OF CHEMICAL TECHNOLOGY.

6.5 *IN VITRO* CHARACTERIZATION

6.5.1Estimation of free ammonia:

Added 1ml of lipid solution to 1ml of barium chloride solution and mixed together in a centrifuge tube and centrifuged for 10 minutes at 5000rpm, it forms a precipitate of barium sulphate, the formed precipitate is dried and weight of barium sulphate is noted.

Free ammonia = weight of barium sulphate ×0.56590

6.5.2 Percent free drug: (Howard G *et al.*, 1977)

Measure the absorbance of solution at 590nm using sucrose Histidine solution as blank.

1. Transferred 0.1ml of sample to a 20ml stoppered test tube, add 8ml of Sucrose-Histidine solution to it, mix well, measure the absorbance at 590nm using calibrated UV spectrophotometer. Transfer the solution from the cell to test tube (A_1).
2. To the above test tube containing solution, added 1ml sodium hydroxide solution, mix well measure the absorbance at 590 nm using UV transfer the solution from the cell to test tube (A_2)

3. To the above test tube containing solution, add 1ml of Triton X-100 solution, mix well measure the absorbance at 590 nm using calibrated UV (A_3)

Percent Free Doxorubicin Hcl = $[(A_2 \times 1.125) - A_1/A_3 \times 1.25] \times 100$

6.5.3 Doxorubicin Hcl Assay (Howard G *et al.*, 1977)

A standard and sample solution were prepared, Inject separately 20 microlitre of the standard and sample solution in chromatographic condition and record the chromatogram. Calculate the content of drug per ml in liposomal injection as follows.

Assay= A/B×W/200×5/50×C/100×100-D/100×50/5×100/5

Where,

A = Area corresponding to Doxorubicin Hcl in sample.

B = Area corresponding to Doxorubicin Hcl in working standard.

C = % purity of Doxorubicin Hcl in working standard.

D = % water content of working standard.

W = Weight of working standard in mg.

Chromatographic conditions:

Column	:	C_{18} BDS (250×4.6mm)
Mobile phase	:	Buffer + Acetonitrile + Methanol (47ml+48ml+ 5ml)
Buffer	:	2.8% w/w sodium lauryl sulphate + 2.3% w/v Phosphssoric Acid.
Wave length	:	254nm.
Flow rate	:	1.7sml/mn.

Solvent : Acidified IPA (90mlIPA+ 0.68ml Hcl+ Make up to 100ml with water)

6.5.4 *In vitro* dissolution studies of Doxorubicin hydrochloride liposome

The *in vitro* release of drug from the liposomal formulation was carried out by using dialysis membrane employing in two sides open ended cylinder.

4 ml of liposomal suspension containing known amount of drug was placed in a dialysis membrane previously soaked overnight. The two sides open cylinder was placed in 200ml of PBS (pH 7.4), maintained at 37^{o} C and stirred with the help of a magnetic stirrer. Aliquots (4ml) of release medium were withdrawn at different time intervals and the sample was replaced with fresh PBS (pH 7.4) to maintain constant volume. 1 ml of acetonitrile was added to each aliquot to precipitate the lipids and dissolve the entrapped Doxorubicin hydrochloride and then the samples were analyzed by UV spectrophotometry at a λ max of 254nm.

6.5.5 Release kinetics (Harris shoaib *et al.*, 2006)

To analyze the *in vitro* release data various kinetic models were use to describe the release kinetics. The zero order rate Eq. (2) describes the systems where the drug release rate is independent of its concentration. The first order Eq. (3) describes the release from system where release rate is concentration dependent. Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

The results of *in vitro* release profile obtained for all the formulations were plotted in modes of data treatment as follows:

1. Zero - order kinetic model – Cumulative % drug released versus time.
2. First – order kinetic model – Log cumulative percent drug remaining versus time.
3. Higuchi's model – Cumulative percent drug released versus square root of time.
4. Korsmeyer equation / Peppa's model – Log cumulative percent drug released versus log time.

a. Zero order kinetics:

Zero order release would be predicted by the following equation:

$$A_t = A_0 - K_0 t$$

Where

A_t = Drug release at time't'

A_0 = Initial drug concentration.

K_0 = Zero- order rate constant (hr^{-1})

When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys Zero – order kinetics and its slope is equal to Zero order release constant K_0**.**

b. First order kinetics:

First - order release could be predicted by the following equation:

$$\text{Log } C = \log C_0 - K_t / 2.303$$

Where,

C = Amount of drug remained at time‘t’

C_0 = Initial amount of drug.

K = First - order rate constant (hr^{-1}).

When the data plotted as log cumulative percent drug remaining versus time, yields a straight line, indicating that the release follow first order kinetics. The constant ‘K_1’ can be obtained by multiplying 2.303 with the slope value.

c. Higuchi’s model:

Drug release from the matrix devices by diffusion has been described by following

Higuchi’s classical diffusion equation:

$$Q = [D\epsilon / \tau(2A - \epsilon Cs)\, Cst]^{1/2}$$

Where,

Q = Amount of drug release at time‘t’

D = Diffusion coefficient of the drug in the matrix.

A = Total amount of drug in unit volume of matrix.

Cs = Solubility of drug in the matrix.

Є = Porosity of the matrix.

τ = Tortuosity.

t = Time (hrs at which q amount of drug is released.

Above equation can be simplified as if we assumes that 'D', 'Cs' and 'A' are constant. Then equation becomes:

$$Q = Kt^{1/2}$$

When the data is splitted according to equation i.e. cumulative drug release versus square root of time yields a straight line, indicating that the drug was released by diffusion mechanism. The slope is equal to 'K' (Higuchi's 1963).

d. Korsmeyer equation / Peppa's model:

To study the mechanism of drug release from the liposomal solution, the release data was also fitted to the well-known exponential equation (Korsmeyer equation/ Peppa's law equation), which is often used to describe the drug release behavior from polymeric systems.

$$M_t / M_\alpha = Kt^n$$

Where,

M_t / M_α = The fraction of drug released at time't'.

K = Constant incorporating the structural and geometrical characteristics of the drug / polymer system.

n = Diffusion exponent related to the mechanism of the release.

Above equation can be simplified as follows by applying log on both sides,

$$\text{Log } M_t / M_\alpha = \text{Log } K + n \text{ Log } t$$

Mechanism of drug release

Table 2: Diffusion exponent and solute release mechanism for cylindrical shape

S.No	Diffusion	Exponent (n) Overall solute diffusion mechanism
1.	0.45	Fickian diffusion
2.	0.45<n<0.89	Anomalous (non-Fickian) diffusion
3.	0.89	Case-II transport
4.	n>0.89	Super case-II transport

6.6 SHORT TERM STABILITY STUDIES

The stability of a pharmaceutical delivery system may be defined as the capability of a particular formulation, in a specific container. The short-term stability was conducted to monitor physical and chemical stabilities of the liquid form of doxorubicin hydrochloride liposomal formulations at 40^{o}C and room temperature for up to three months. The stability parameter, such as Assay was determined as function of the storage time.

RESULTS

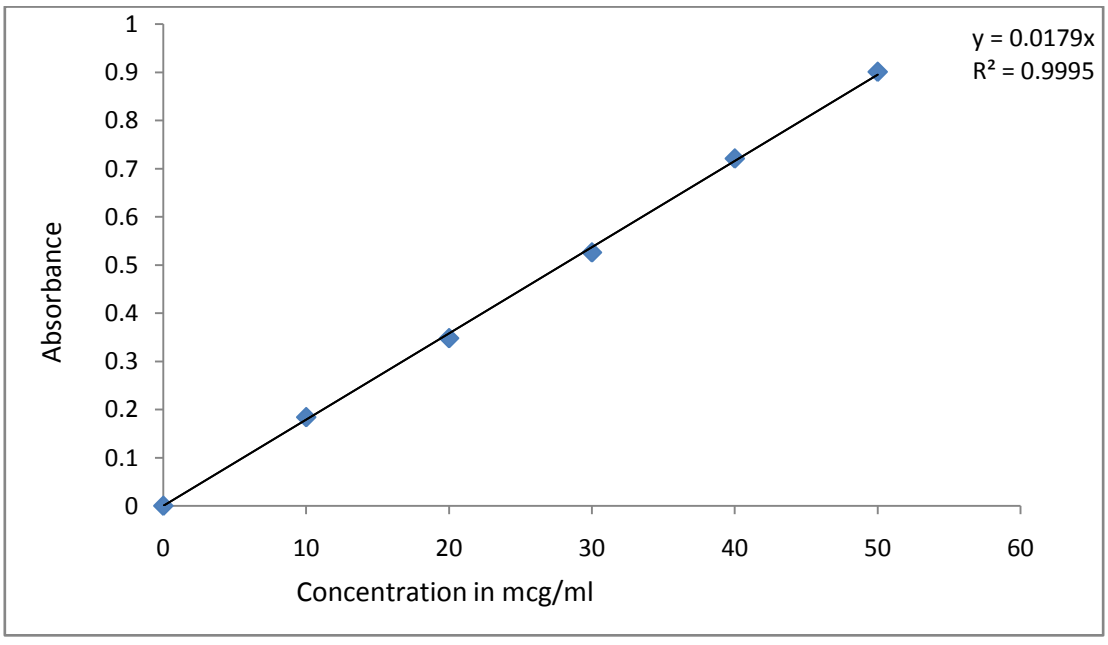
7. RESULTS

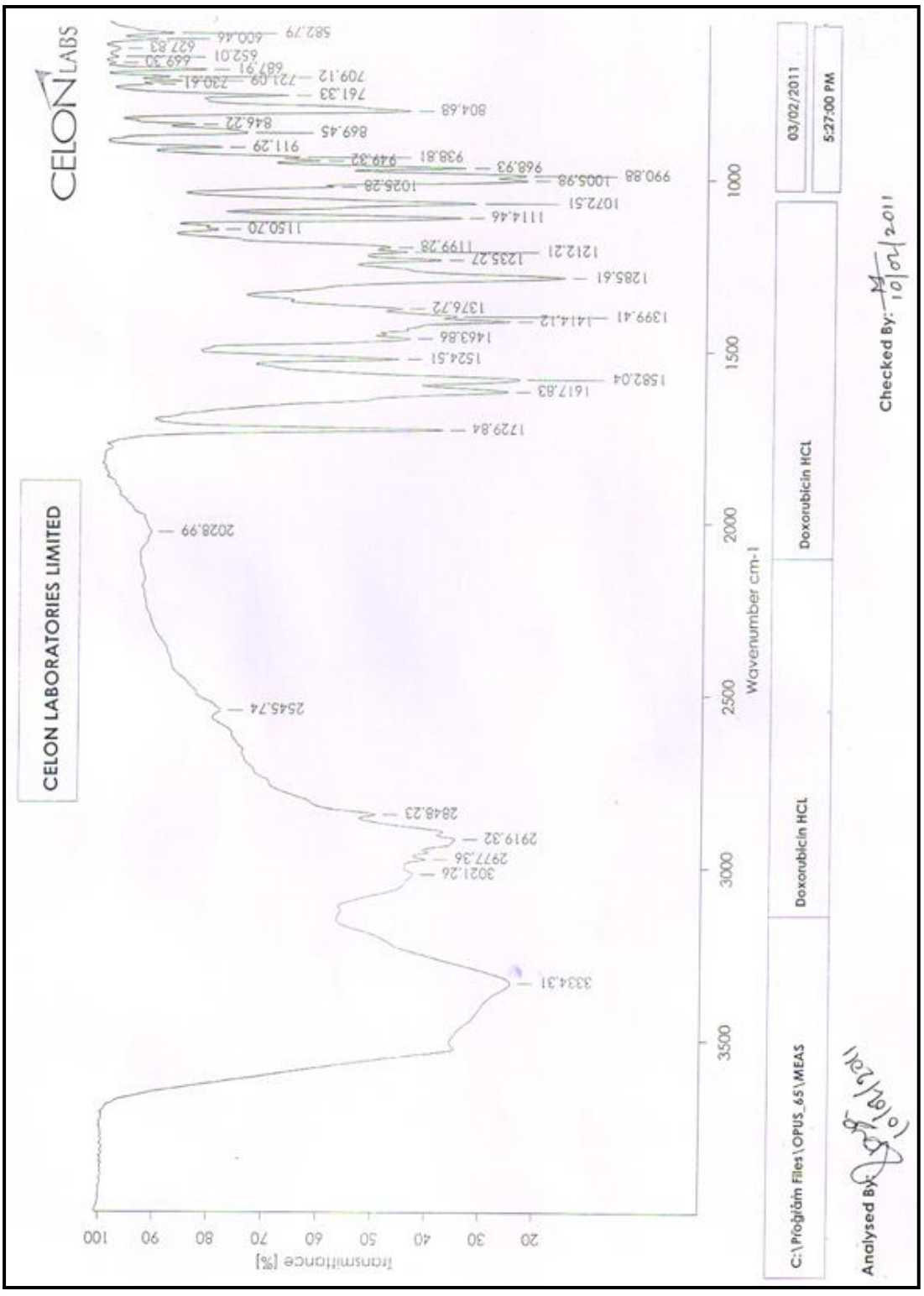
TABLSES

Table No: 3 Standard readings of Doxorubicin hydrochloride in UV

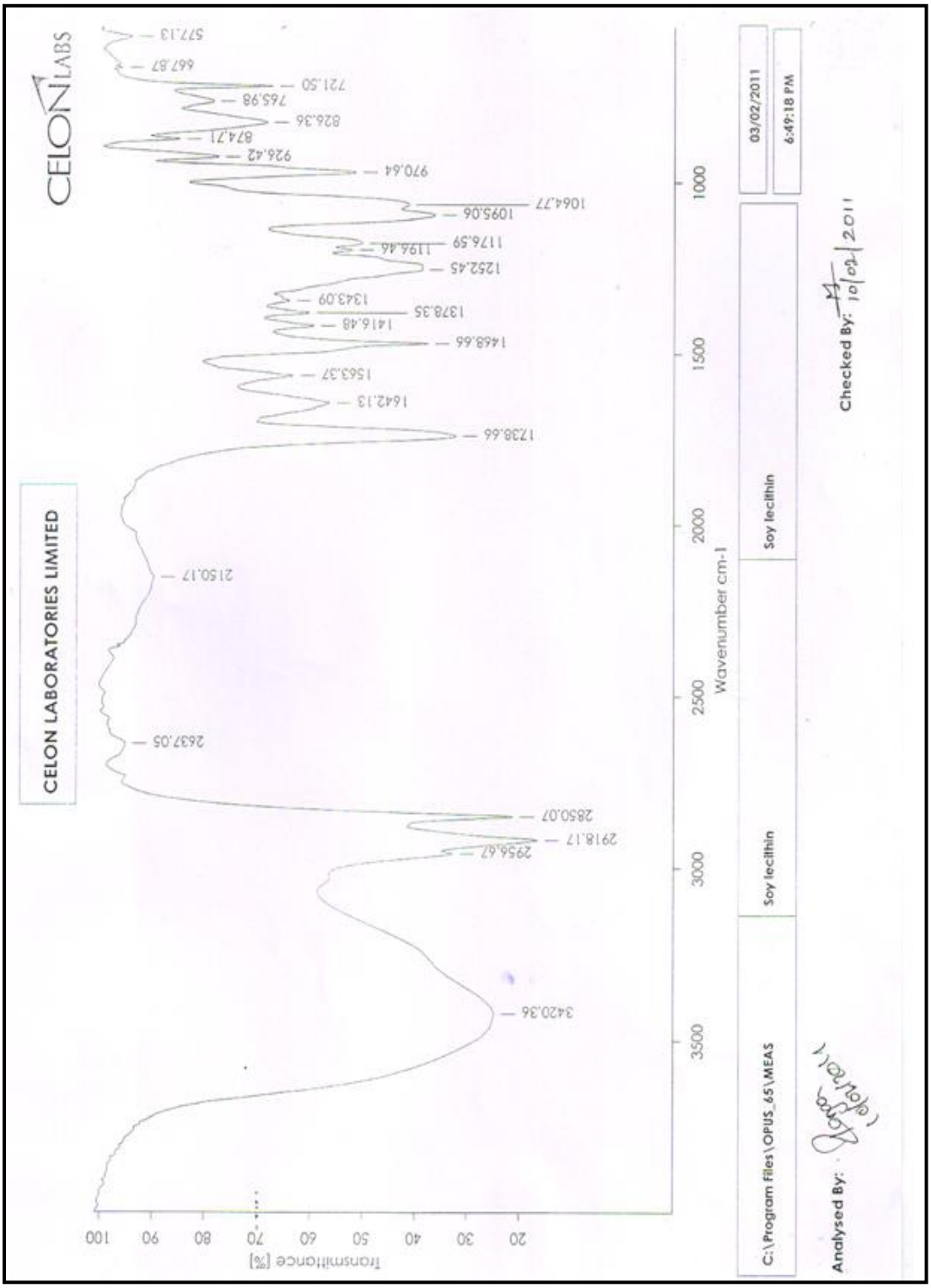
S. No.	Concentration (µg/ml)	Absorbance at 254nm
1.	0	0
2.	10	0.184
3.	20	0.348
4.	30	0.526
5.	40	0.721
6.	50	0.901

Graph No: 1 Standard graph of Doxorubicin hydrochloride in phosphate buffer of pH 7.4.

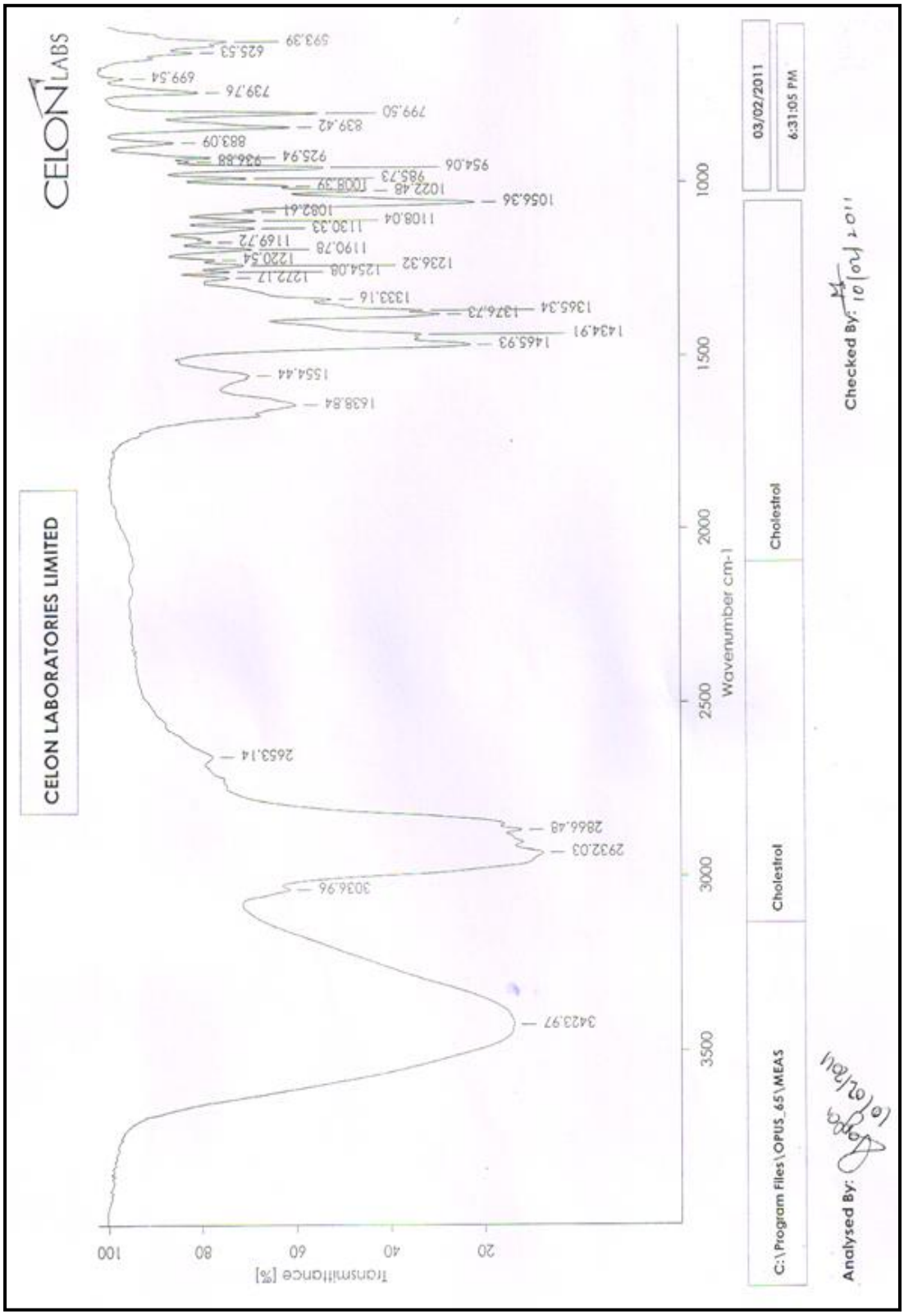




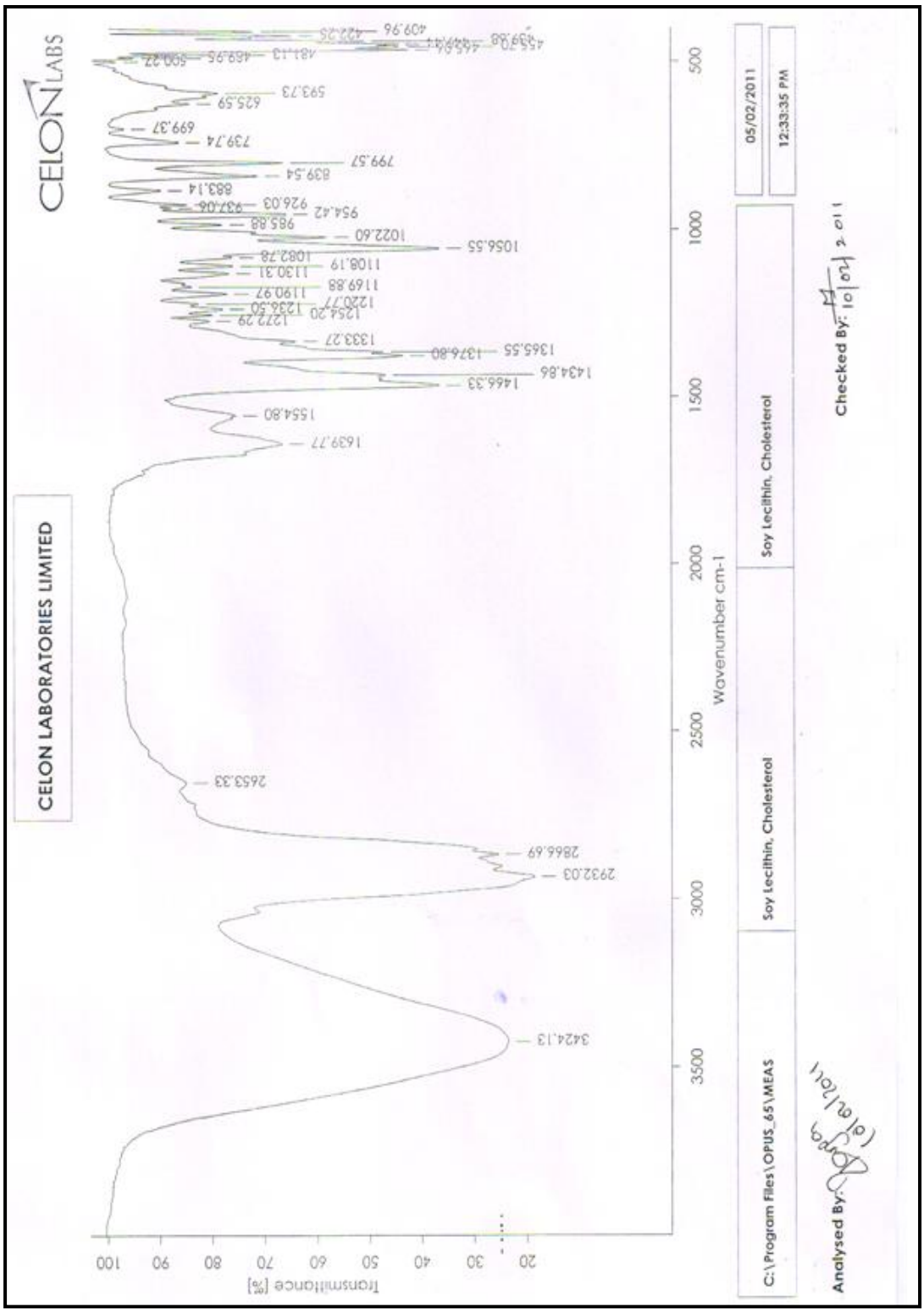
Spectra No: 1 FTIR of Doxorubicin Hcl



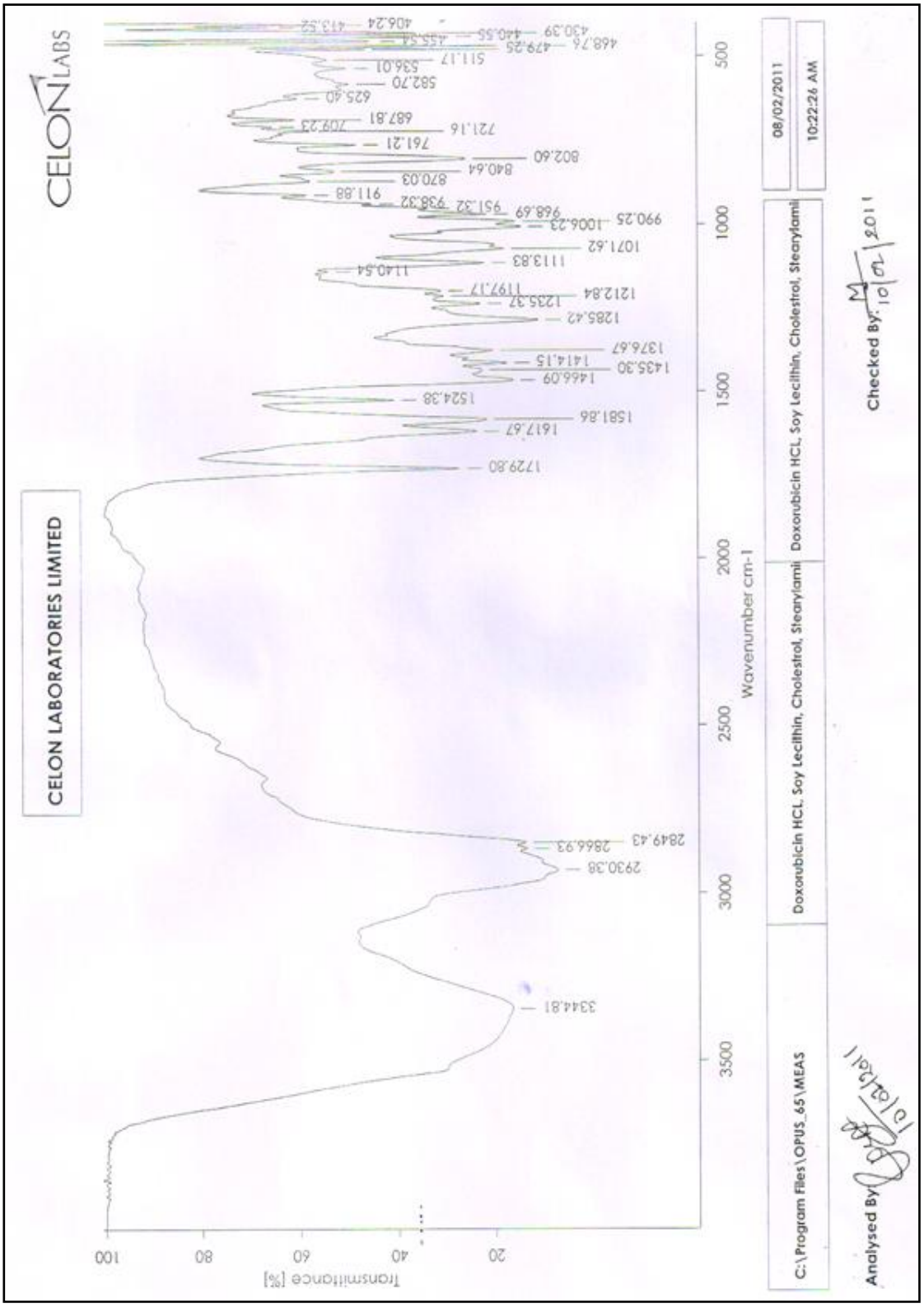
Spectra No: 2 FTIR of Soy lecithin



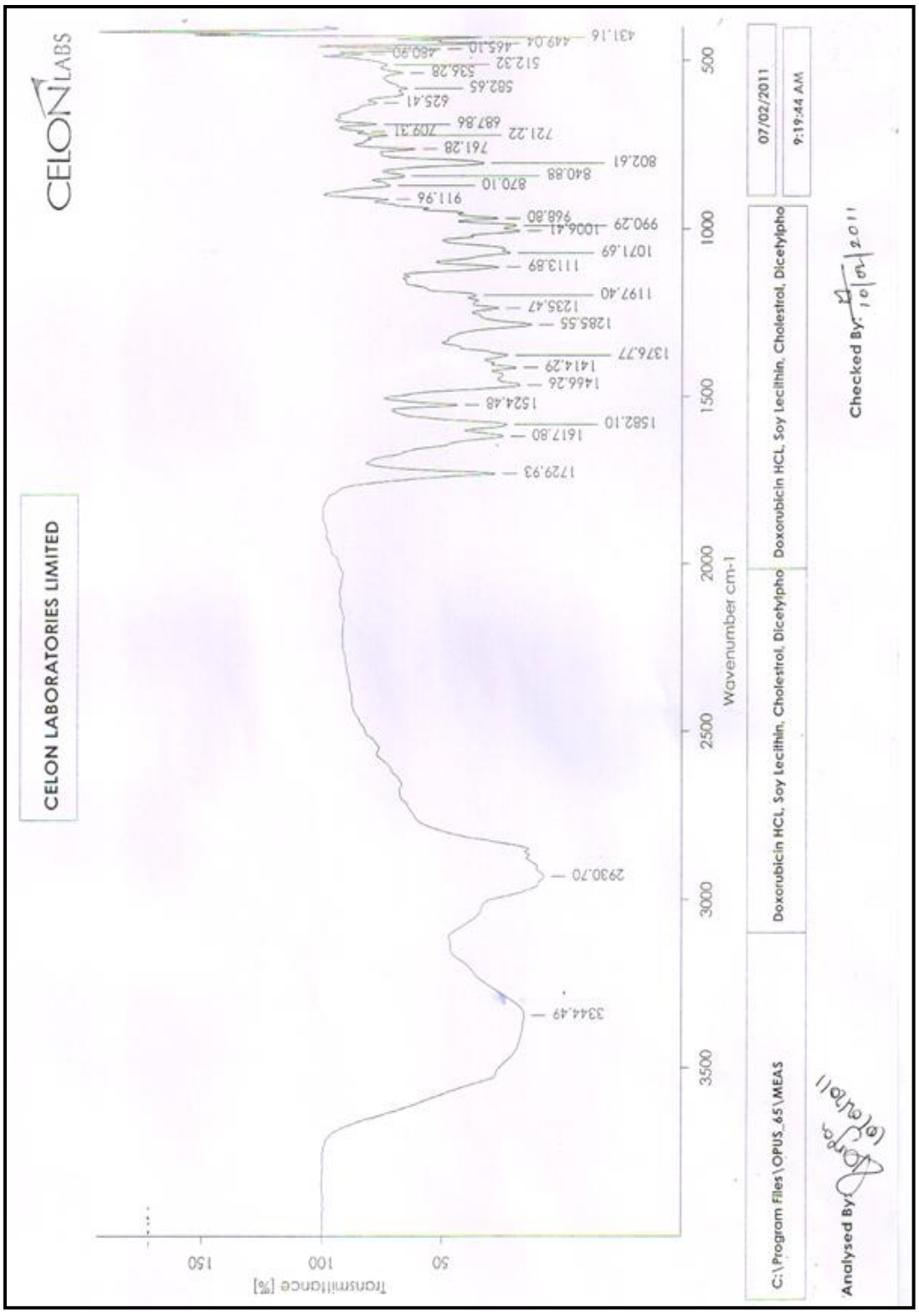
Spectra No: 3 FTIR of Cholesterol



Spectra No: 4 FTIR of Soy lecithin, Cholesterol.



Spectra No:5 FTIR of Doxorubicin Hcl, Soy lecithin, Cholesterol, Stearyl amine.


CELON LABS
CELON LABORATORIES LIMITED
C:\Program Files\OPUS_65\MEAS
Doxorubicin HCL, Soy Lecithin, Cholestrol, Dicetylpho
Doxorubicin HCL, Soy Lecithin, Cholestrol, Dicetylpho
07/02/2011
9:19:44 AM
Checked By:
Analysed By:
Wavenumber cm-1
Transmittance [%]
3500
3000
2500
2000
1500
1000
500
150
100
50
3344.49
2930.70
1729.93
1617.80
1582.10
1524.48
1466.26
1414.29
1376.77
1285.55
1235.47
1197.40
1113.89
1071.69
1006.41
990.29
968.80
911.96
870.10
840.88
802.61
761.28
721.22
709.31
687.86
625.41
582.65
536.28
512.32
480.90
465.10
449.04
431.16

Spectra No:6 FTIR of Doxorubicin Hcl, Soy lecithin, Cholesterol, Dicetyl phosphate.

Table No: 4 Interpretations of FTIR Spectra for pure drug.

S.No.	FUNCTIONAL GROUPS	ASSESMENT PEAK OF PURE DRUG CM^{-1}	RANGE OF GROUPS CM^{-1}
1.	C=C Streching (Aromatic)	1463.86 1524.51	1450 – 1600
2.	O-H Bending (Alcohol)	1072.51	1050 - 1150
3.	C=O Streching	1729.84	1705 - 1735
4.	N-H Bending	1617.83	1500 – 1650
5.	C-O Streching (6-Membered cyclic)	1114.46	1100 - 1120

Table No: 5 Interpretations of FTIR Spectra for pure drug and Spectra-5

S.No.	FUNCTIONAL GROUPS	ASSESMENT PEAK OF PURE DRUG CM^{-1}	ASSESMENT PEAK OF SPECTRA-5 CM^{-1}
1.	C=C Streching(Aromatic)	1463.86 1524.51	1466.09 1524.38
2.	O-H Bending (Alcohol)	1072.51	1071.62
3.	C=O Streching	1729.84	1729.80
4.	N-H Bending	1617.83	1617.67
5.	C-O Streching (6-membered cyclic)	1114.46	1113.83

Table No: 6 Interpretations of FTIR Spectra for pure drug and Spectra-5

S.No.	FUNCTIONAL GROUPS	ASSESMENT PEAK OF PURE DRUG CM^{-1}	ASSESMENT PEAK OF SPECTRA-1 CM^{-1}
1.	**C=C Streching (Aromatic)**	1463.86 1524.51	1466.26 1524.48
2.	**O-H Bending (Alcohol)**	1072.51	1071.69
3.	**C=O Streching**	1729.84	1729.93
4.	**N-H Bending**	1617.83	1617.80
5.	**C-O Streching (6-Membered cyclic)**	1114.46	1113.89

Table No: 7 The composition and ratios of Soy lecithin, Cholesterol, Stearylamine, Dicetylphosphate and Ammonium Sulphate for different types of liposomes.

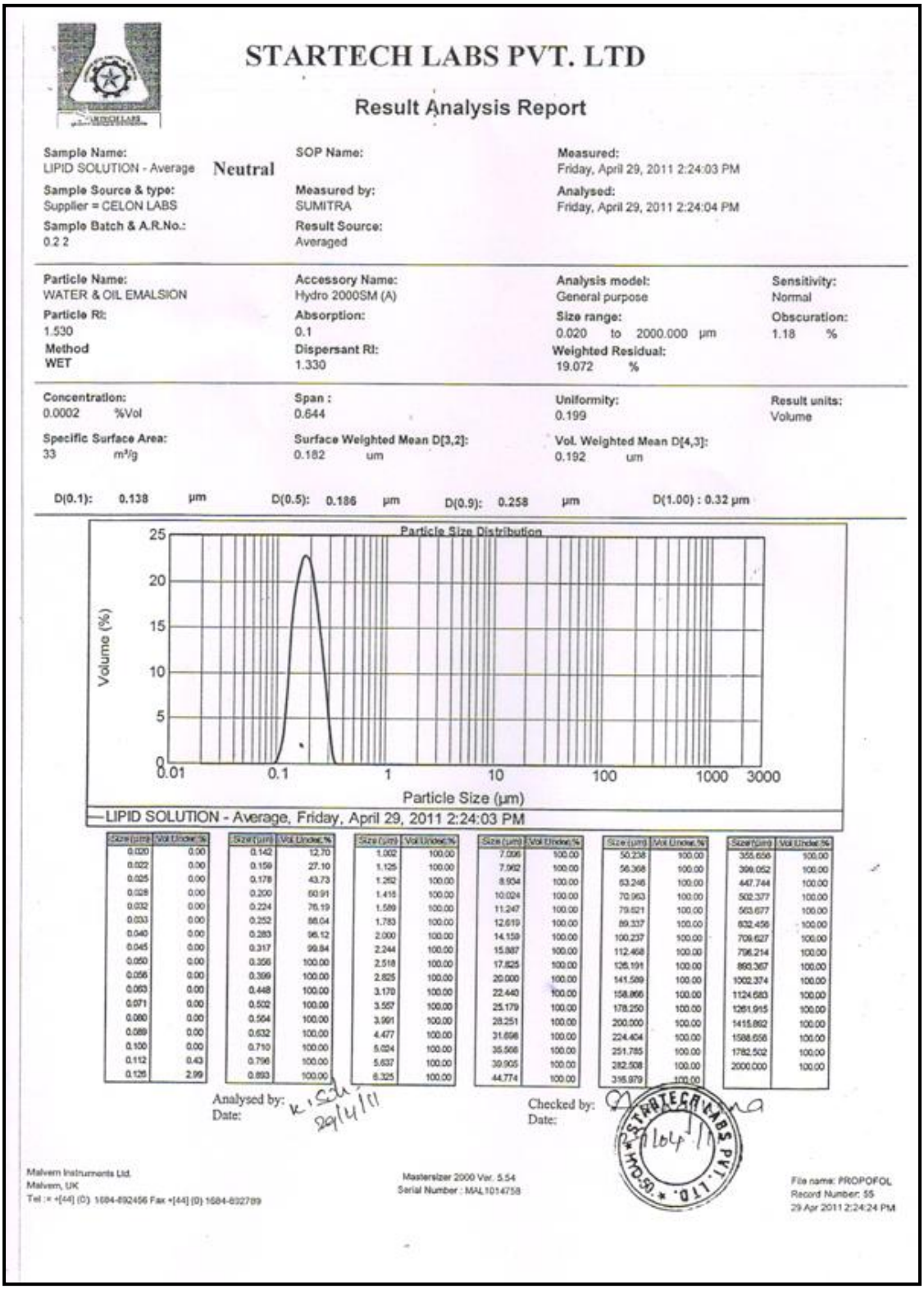
Ratio of ingredients	Tipes of liposomes		
	Neutral	**Positive**	**Negative**
Lecithin : cholesterol : Stearyl amine : Diacetylphosphate : Ammonium Sulphate	5:5:0:0:30	5:5:1:0:30	5:5:0:1:30
	5.5:4.5:0:0:30	5.5:4.5:1:0:30	5.5:4.5:0:1:30
	6:4:0:0:30	6:4:1:0:30	6:4:0:1:30
	6.5:3.5:0:0:30	6.5:3.5:1:0:30	6.5:3.5:0:1:30
	7:3:0:0:30	7:3:1:0:30	7:3:0:1:30
	7.5:2.5:0:0:30	7.5:2.5:1:0:30	7.5:2.5:0:1:30
	8:2:0:0:30	8:2:1:0:30	8:2:0:1:30
	4.5:5.5:0:0:30	4.5:5.5:1:0:30	4.5:5.5:0:1:30
	4:6:0:0:30	4:6:1:0:30	4:6:0:1:30
	3:7:0:0:30	3:7:1:0:30	3:7:0:1:30

Table No: 8 The composition and ratios of Drug, Soy lecithin, Cholesterol, Stearylamine, Dicetylphosphate and Ammonium sulphate for optimized batches.

Formulation code	**Drug** (mg/ml)	**Soy lecithin** (mg/ml)	**Cholesterol** (mg/ml)	**Stearylamine** (mg/ml)	**Dicetyl phosphate** (mg/ml)	**Ammonim Sulphate** (mg/ml)
F1	2	7	3	-	-	30
F2	2	7.5	2.5	-	-	30
F3	2	7	3	1	-	30
F4	2	7.5	2.5	1	-	30
F5	2	7	3	-	1	30
F6	2	7.5	2.5	-	1	30

Table No: 9 Physicochemical characteristics of doxorubicin hydrochloride Liposomes for Optimized Batches.

S.No.	**Formulation code**	**Average vesicular size (nm)**	**Zeta Potential (mV)**	**Poly dispersive index (Pdi)**
1.	F2	356nm	5.21	0.635
2.	F4	564nm	24.66	0.762
3.	F6	317nm	-23.4	0.645



STARTECH LABS PVT. LTD

Result Analysis Report

Sample Name: LIPID SOLUTION - Average Neutral
Sample Source & type: Supplier = CELON LABS
Sample Batch & A.R.No.: 0.2 2
SOP Name:
Measured by: SUMITRA
Result Source: Averaged
Measured: Friday, April 29, 2011 2:24:03 PM
Analysed: Friday, April 29, 2011 2:24:04 PM

Particle Name: WATER & OIL EMALSION
Particle RI: 1.530
Method WET
Accessory Name: Hydro 2000SM (A)
Absorption: 0.1
Dispersant RI: 1.330
Analysis model: General purpose
Size range: 0.020 to 2000.000 µm
Weighted Residual: 19.072 %
Sensitivity: Normal
Obscuration: 1.18 %

Concentration: 0.0002 %Vol
Specific Surface Area: 33 m²/g
Span : 0.644
Surface Weighted Mean D[3,2]: 0.182 um
Uniformity: 0.199
Vol. Weighted Mean D[4,3]: 0.192 um
Result units: Volume

D(0.1): 0.138 µm D(0.5): 0.186 µm D(0.9): 0.258 µm D(1.00) : 0.32 µm

Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %
0.020	0.00	0.142	12.70	1.002	100.00	7.006	100.00	50.238	100.00	355.656	100.00
0.022	0.00	0.159	27.10	1.125	100.00	7.962	100.00	56.368	100.00	399.052	100.00
0.025	0.00	0.178	43.73	1.262	100.00	8.934	100.00	63.246	100.00	447.744	100.00
0.028	0.00	0.200	60.91	1.415	100.00	10.024	100.00	70.953	100.00	502.377	100.00
0.032	0.00	0.224	76.19	1.589	100.00	11.247	100.00	79.621	100.00	563.677	100.00
0.033	0.00	0.252	88.04	1.783	100.00	12.619	100.00	89.337	100.00	632.456	100.00
0.040	0.00	0.283	96.12	2.000	100.00	14.159	100.00	100.237	100.00	709.627	100.00
0.045	0.00	0.317	99.84	2.244	100.00	15.887	100.00	112.468	100.00	796.214	100.00
0.050	0.00	0.356	100.00	2.518	100.00	17.825	100.00	126.191	100.00	893.367	100.00
0.056	0.00	0.399	100.00	2.825	100.00	20.000	100.00	141.589	100.00	1002.374	100.00
0.063	0.00	0.448	100.00	3.170	100.00	22.440	100.00	158.866	100.00	1124.583	100.00
0.071	0.00	0.502	100.00	3.557	100.00	25.179	100.00	178.250	100.00	1261.915	100.00
0.080	0.00	0.564	100.00	3.991	100.00	28.251	100.00	200.000	100.00	1415.892	100.00
0.089	0.00	0.632	100.00	4.477	100.00	31.698	100.00	224.404	100.00	1588.656	100.00
0.100	0.00	0.710	100.00	5.024	100.00	35.568	100.00	251.785	100.00	1782.502	100.00
0.112	0.43	0.796	100.00	5.637	100.00	39.905	100.00	282.508	100.00	2000.000	100.00
0.126	2.99	0.893	100.00	6.325	100.00	44.774	100.00	316.979	100.00		

Analysed by: K. Selvi
Date: 29/4/11

Checked by:
Date:

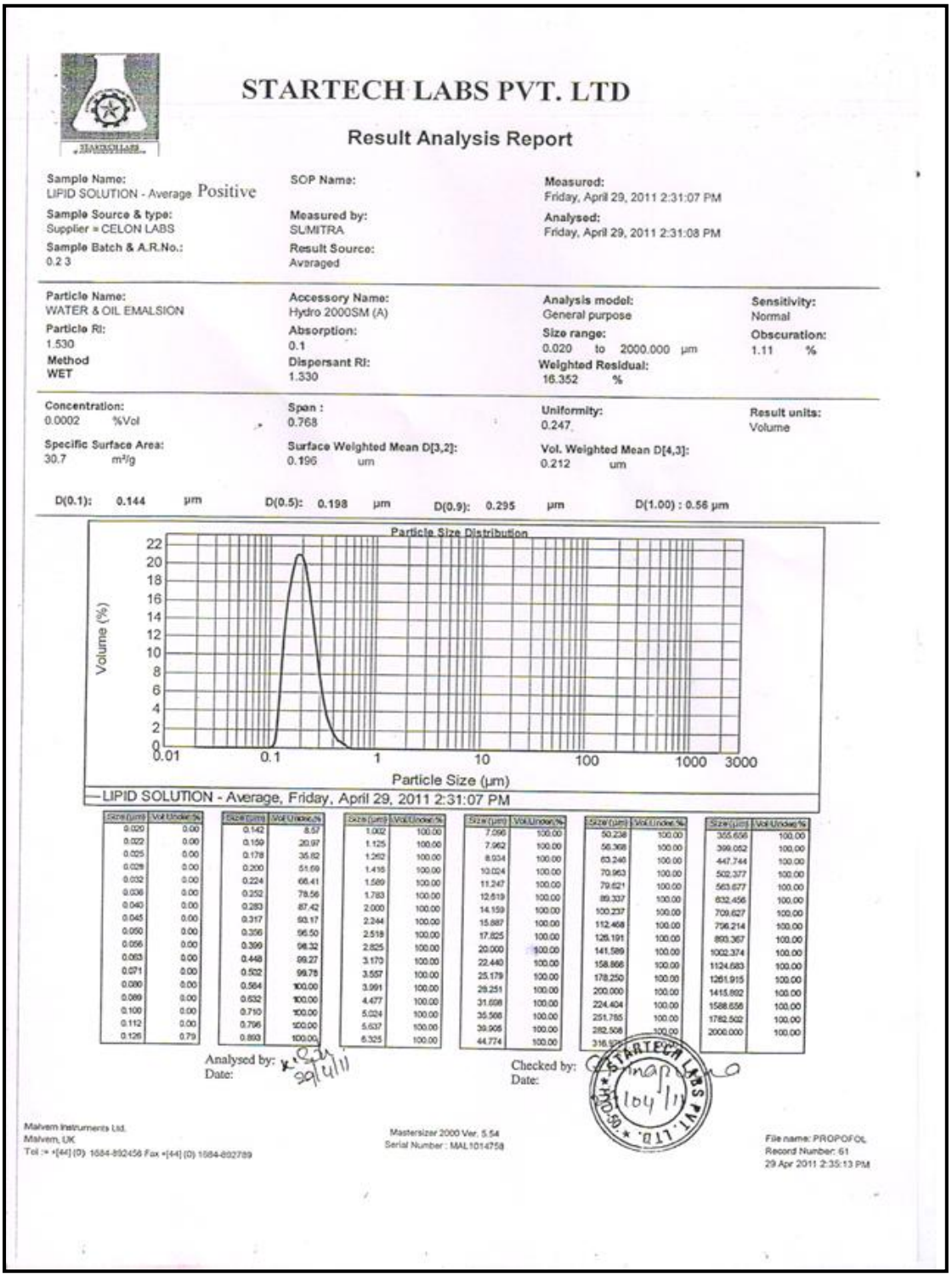
STARTECH LABS PVT. LTD. 164

Malvern Instruments Ltd.
Malvern, UK
Tel : + +[44] (0) 1684-892456 Fax +[44] (0) 1684-892789

Mastersizer 2000 Ver. 5.54
Serial Number : MAL1014758

File name: PROPOFOL
Record Number: 55
29 Apr 2011 2:24:24 PM

Report No: 1 Particle size distribution by wet method of Doxorubicin Hcl liposomal solution for F2 formulation.



STARTECH LABS PVT. LTD

Result Analysis Report

Sample Name: LIPID SOLUTION - Average Positive
Sample Source & type: Supplier = CELON LABS
Sample Batch & A.R.No.: 0.2 3
SOP Name:
Measured by: SUMITRA
Result Source: Averaged
Measured: Friday, April 29, 2011 2:31:07 PM
Analysed: Friday, April 29, 2011 2:31:08 PM

Particle Name: WATER & OIL EMALSION
Particle RI: 1.530
Method WET
Accessory Name: Hydro 2000SM (A)
Absorption: 0.1
Dispersant RI: 1.330
Analysis model: General purpose
Size range: 0.020 to 2000.000 µm
Weighted Residual: 16.352 %
Sensitivity: Normal
Obscuration: 1.11 %

Concentration: 0.0002 %Vol
Specific Surface Area: 30.7 m²/g
Span : 0.768
Surface Weighted Mean D[3,2]: 0.196 um
Uniformity: 0.247
Vol. Weighted Mean D[4,3]: 0.212 um
Result units: Volume

D(0.1): 0.144 µm D(0.5): 0.198 µm D(0.9): 0.295 µm D(1.00) : 0.56 µm

Analysed by:
Date: 29/4/11

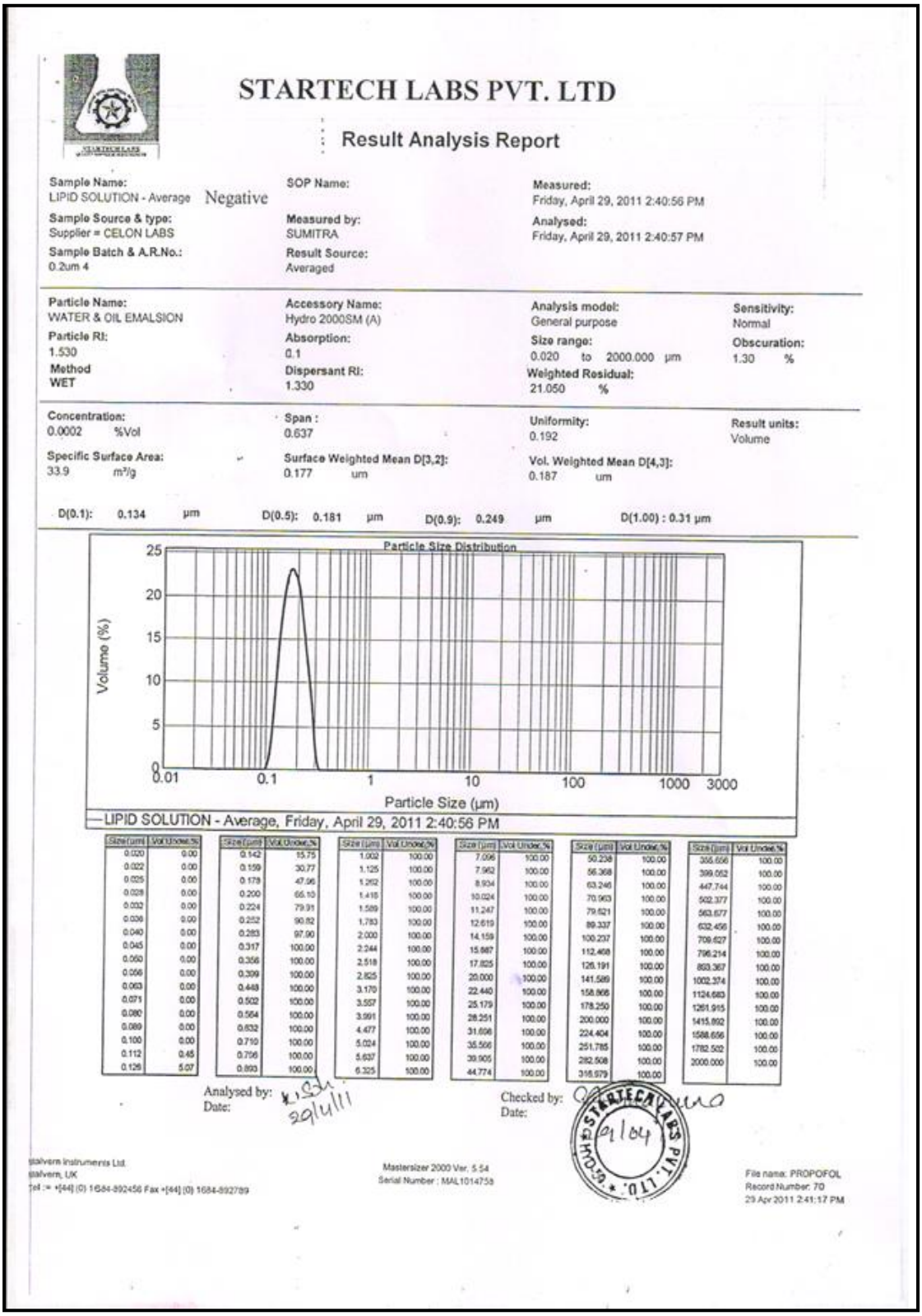
Checked by:
Date:

Malvern Instruments Ltd.
Malvern, UK
Tel := +[44] (0) 1684-892456 Fax +[44] (0) 1684-892789

Mastersizer 2000 Ver. 5.54
Serial Number : MAL1014758

File name: PROPOFOL
Record Number: 61
29 Apr 2011 2:35:13 PM

Report No:2 Particle size distribution by wet method of Doxorubicin Hcl liposomal solution for F4 formulation.



STARTECH LABS PVT. LTD

Result Analysis Report

Sample Name: LIPID SOLUTION - Average Negative
Sample Source & type: Supplier = CELON LABS
Sample Batch & A.R.No.: 0.2um 4

SOP Name:
Measured by: SUMITRA
Result Source: Averaged

Measured: Friday, April 29, 2011 2:40:56 PM
Analysed: Friday, April 29, 2011 2:40:57 PM

Particle Name: WATER & OIL EMALSION
Particle RI: 1.530
Method WET

Accessory Name: Hydro 2000SM (A)
Absorption: 0.1
Dispersant RI: 1.330

Analysis model: General purpose
Size range: 0.020 to 2000.000 µm
Weighted Residual: 21.050 %

Sensitivity: Normal
Obscuration: 1.30 %

Concentration: 0.0002 %Vol
Specific Surface Area: 33.9 m²/g

Span : 0.637
Surface Weighted Mean D[3,2]: 0.177 um

Uniformity: 0.192
Vol. Weighted Mean D[4,3]: 0.187 um

Result units: Volume

D(0.1): 0.134 µm D(0.5): 0.181 µm D(0.9): 0.249 µm D(1.00) : 0.31 µm

Size (µm)	Vol Under,%	Size (µm)	Vol Under,%	Size (µm)	Vol Under,%	Size (µm)	Vol Under,%	Size (µm)	Vol Under,%	Size (µm)	Vol Under,%
0.020	0.00	0.142	15.75	1.002	100.00	7.096	100.00	50.238	100.00	355.656	100.00
0.022	0.00	0.159	30.77	1.125	100.00	7.962	100.00	56.368	100.00	399.052	100.00
0.025	0.00	0.179	47.96	1.262	100.00	8.934	100.00	63.246	100.00	447.744	100.00
0.028	0.00	0.200	66.10	1.416	100.00	10.024	100.00	70.963	100.00	502.377	100.00
0.032	0.00	0.224	79.91	1.589	100.00	11.247	100.00	79.621	100.00	563.677	100.00
0.036	0.00	0.252	90.82	1.783	100.00	12.619	100.00	89.337	100.00	632.456	100.00
0.040	0.00	0.283	97.00	2.000	100.00	14.159	100.00	100.237	100.00	709.627	100.00
0.045	0.00	0.317	100.00	2.244	100.00	15.887	100.00	112.468	100.00	796.214	100.00
0.050	0.00	0.356	100.00	2.518	100.00	17.825	100.00	126.191	100.00	893.367	100.00
0.056	0.00	0.399	100.00	2.825	100.00	20.000	100.00	141.589	100.00	1002.374	100.00
0.063	0.00	0.448	100.00	3.170	100.00	22.440	100.00	158.906	100.00	1124.683	100.00
0.071	0.00	0.502	100.00	3.557	100.00	25.179	100.00	178.250	100.00	1261.915	100.00
0.080	0.00	0.564	100.00	3.991	100.00	28.251	100.00	200.000	100.00	1415.892	100.00
0.089	0.00	0.632	100.00	4.477	100.00	31.696	100.00	224.404	100.00	1588.656	100.00
0.100	0.00	0.710	100.00	5.024	100.00	35.566	100.00	251.785	100.00	1782.502	100.00
0.112	0.45	0.796	100.00	5.637	100.00	39.905	100.00	282.508	100.00	2000.000	100.00
0.126	5.07	0.893	100.00	6.325	100.00	44.774	100.00	316.979	100.00		

Analysed by: Date: 29/4/11

Checked by: Date: 29/4/11

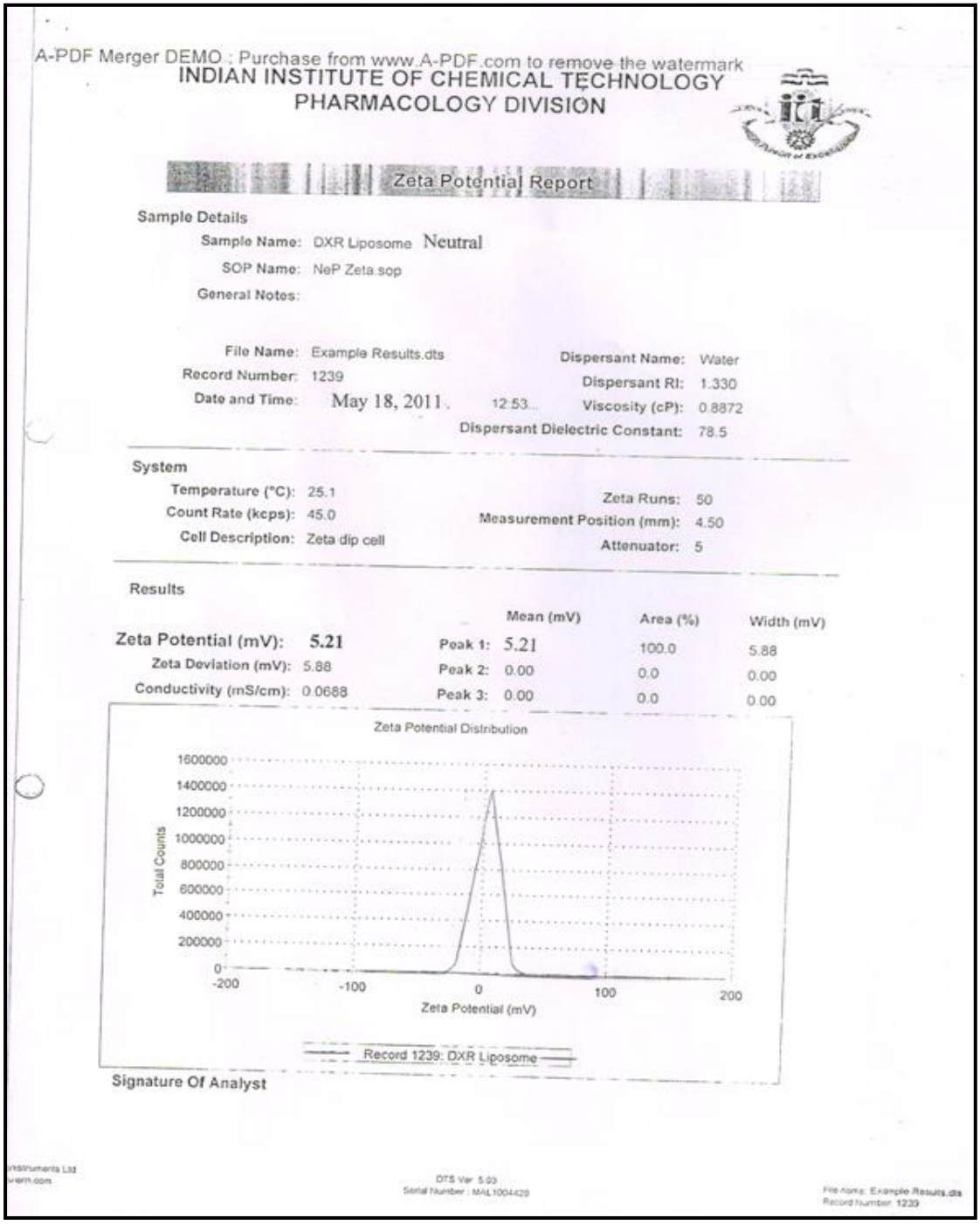
STARTECH LABS PVT. LTD.

Malvern Instruments Ltd
Malvern, UK
Tel := +[44] (0) 1684-892456 Fax +[44] (0) 1684-892789

Mastersizer 2000 Ver. 5.54
Serial Number : MAL1014758

File name: PROPOFOL
Record Number: 70
29 Apr 2011 2:41:17 PM

Report No: 3 Particle size distribution by wet method of Doxorubicin Hcl liposomal solution for F6 formulation.



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INDIAN INSTITUTE OF CHEMICAL TECHNOLOGY
PHARMACOLOGY DIVISION

Zeta Potential Report

Sample Details

Sample Name: DXR Liposome Neutral
SOP Name: NeP Zeta.sop
General Notes:

File Name: Example Results.dts
Record Number: 1239
Date and Time: May 18, 2011 12:53

Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (cP): 0.8872
Dispersant Dielectric Constant: 78.5

System

Temperature (°C): 25.1
Count Rate (kcps): 45.0
Cell Description: Zeta dip cell

Zeta Runs: 50
Measurement Position (mm): 4.50
Attenuator: 5

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 5.21	Peak 1:	5.21	100.0	5.88
Zeta Deviation (mV): 5.88	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0688	Peak 3:	0.00	0.0	0.00

Signature Of Analyst

DTS Ver 5.03
Serial Number : MAL1004429

File name: Example Results.dts
Record Number 1239

Report No: 4 Zeta potential for Doxorubicin Hcl Liposomal solution for F2 formulation.

INDIAN INSTITUTE OF CHEMICAL TECHNOLOGY
PHARMACOLOGY DIVISION

Zeta Potential Report

Sample Details

Sample Name: DXR Liposome Positive
SOP Name: NeP Zeta sop
General Notes:

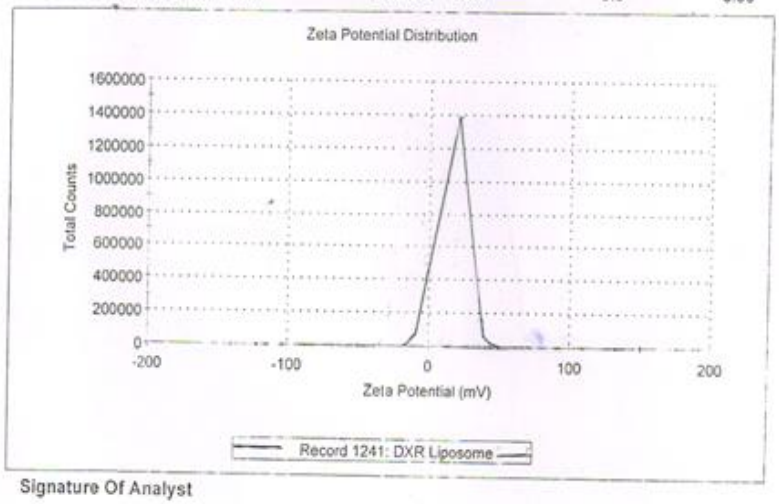
File Name: Example Results.dts
Record Number: 1241
Date and Time: May 18, 2011 1:02:...
Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (cP): 0.8872
Dispersant Dielectric Constant: 78.5

System

Temperature (°C): 25.0
Count Rate (kcps): 137.6
Cell Description: Zeta dip cell
Zeta Runs: 50
Measurement Position (mm): 4.50
Attenuator: 6

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	**24.66**	Peak 1: 24.66	100.0	7.13
Zeta Deviation (mV):	7.13	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm):	0.127	Peak 3: 0.00	0.0	0.00



Signature Of Analyst

DTS Ver. 5.03
Serial Number : MAL1004428

File name: Example Results.dt
Record Number: 1241

Report No: 5 Zeta potential for Doxorubicin Hcl Liposomal solution for F4 formulation.

INDIAN INSTITUTE OF CHEMICAL TECHNOLOGY
PHARMACOLOGY DIVISION

Zeta Potential Report

Sample Details

Sample Name: DXR Liposome Neutral
SOP Name: NeP Zeta sop
General Notes:

File Name: Example Results.dts
Record Number: 1242
Date and Time: May 18, 2011 1:07:...

Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (cP): 0.8872
Dispersant Dielectric Constant: 78.5

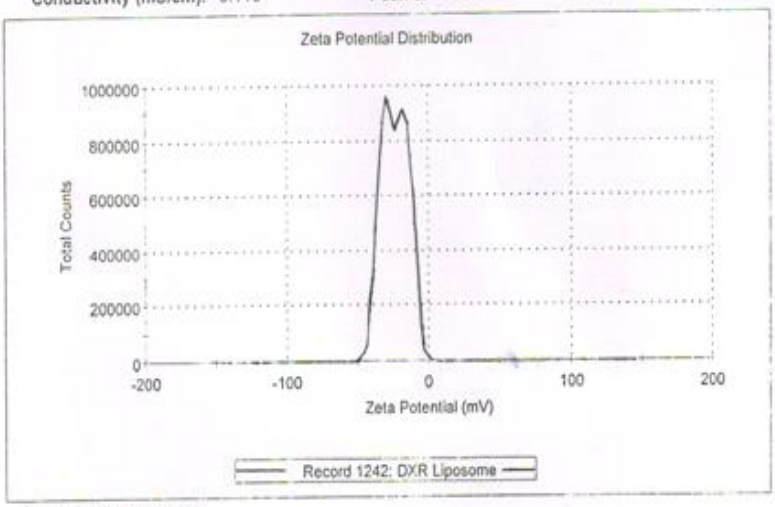
System

Temperature (°C): 25.0
Count Rate (kcps): 71.3
Cell Description: Zeta dip cell

Zeta Runs: 50
Measurement Position (mm): 4.50
Attenuator: 6

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -23.4	Peak 1:	-17.0	51.3	5.58
Zeta Deviation (mV): 9.27	Peak 2:	-30.3	48.7	5.35
Conductivity (mS/cm): 0.110	Peak 3:	0.00	0.0	0.00



Signature Of Analyst

Report No: 6 Zeta potential for Doxorubicin Hcl Liposomal solution for F6 formulation.

SCANNING ELECTRRON MICROSCOPY

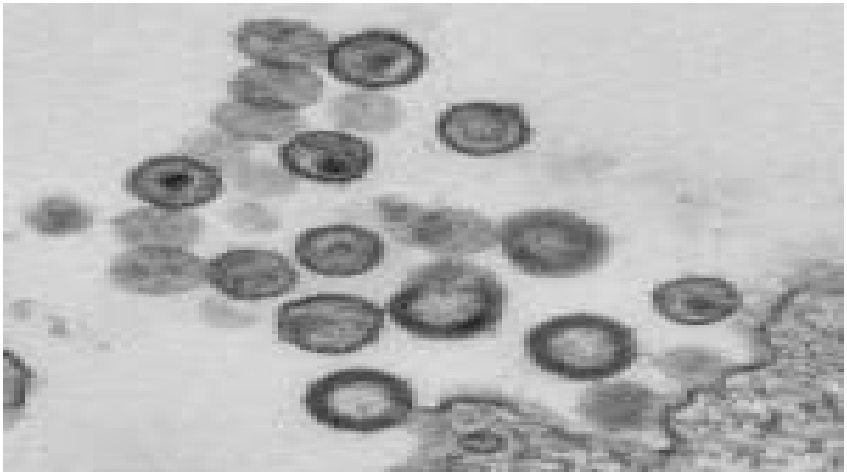


Fig No: 7 SEM photography of Liposomal solution for F2 formulation.

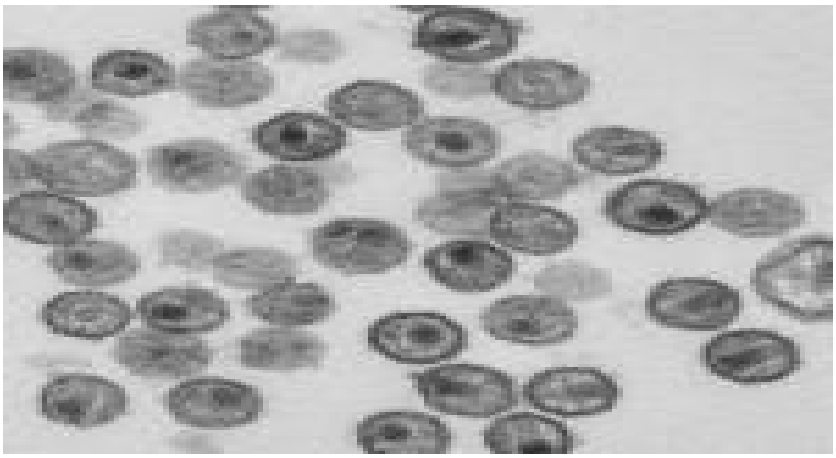


Fig No: 8 SEM photography of Liposomal solution for F6 formulation.

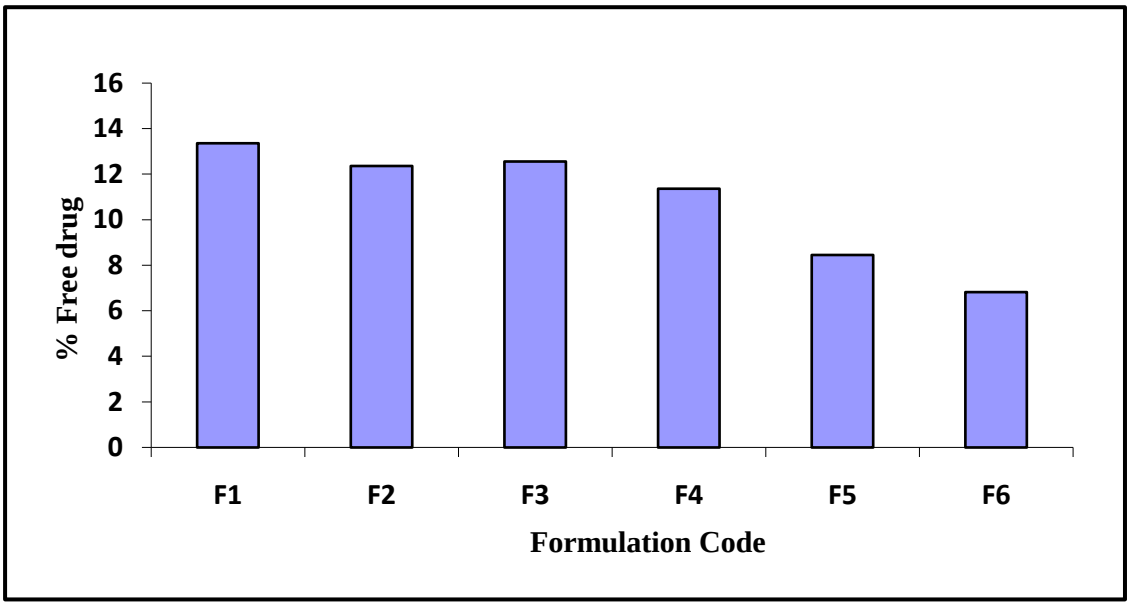
Table No: 10 Percent free drug of Doxorubicin liposomal solution for F1, F2, F3, F4, F5 and F6 Formulations. (n=3)

S. No.	Formulation code	Percentage of free drug
1.	F1	13.36±1.1%
2.	F2	12.36±1.1%
3.	F3	12.56±4.6%
4.	F4	11.36±1.3%
5.	F5	8.45±5.9%
6.	F6	6.83±0.8%

Table No: 11 Assay of Doxorubicin liposomal solution for F1, F2, F3, F4, F5, F6 Formulations. (n=3)

S. No.	Formulation code	Doxorubicin Hcl Assay
1.	F1	98.8±0.32%
2.	F2	99.7±0.65%
3.	F3	95.6±0.65%
4.	F4	97.3±0.96%
5.	F5	97.2±0.58%
6.	F6	98±1.32%

Graph N0:2 Percent free drug plot for F1, F2 F3, F4, F5, F6 Formulations



Graph N0:3 Assay plot for F1, F2 F3, F4, F5, F6 Formulations

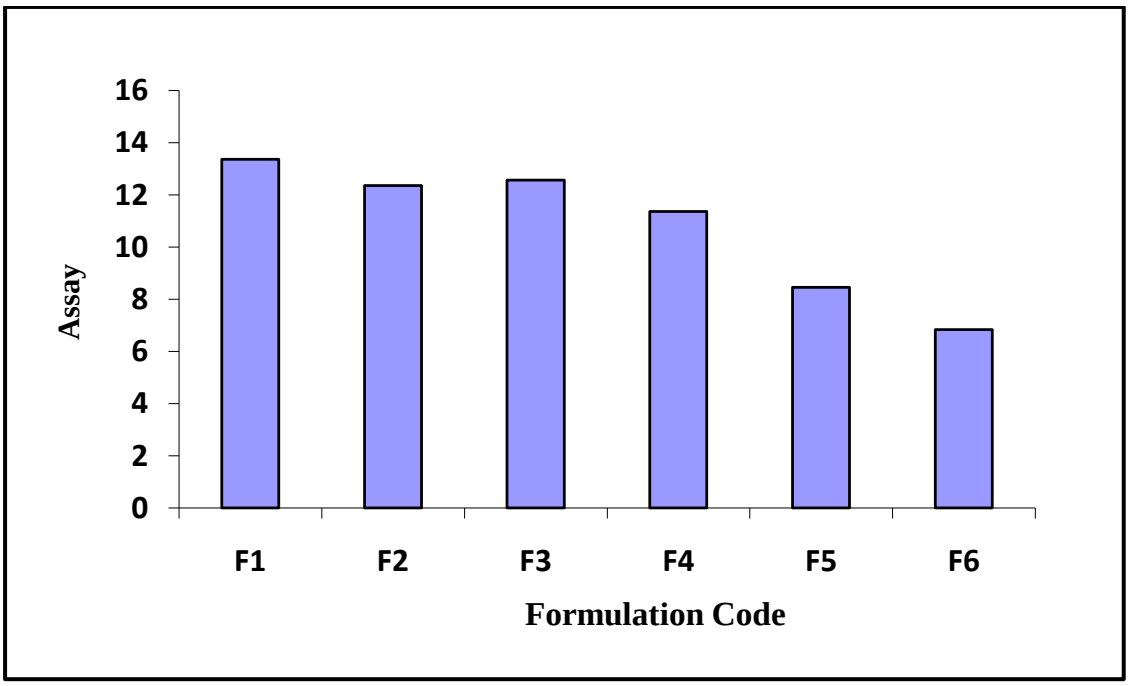
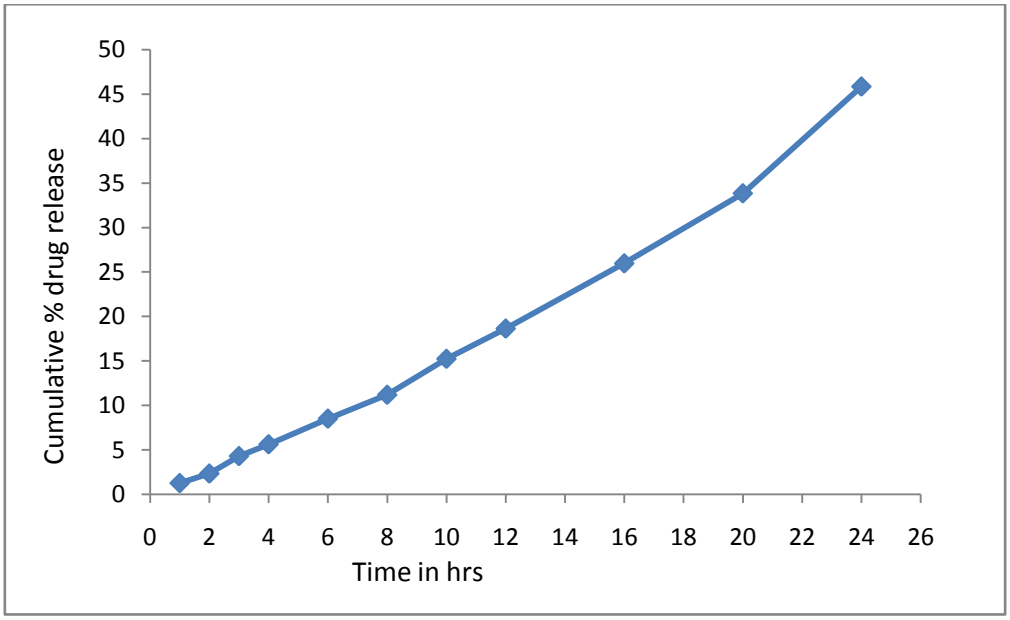


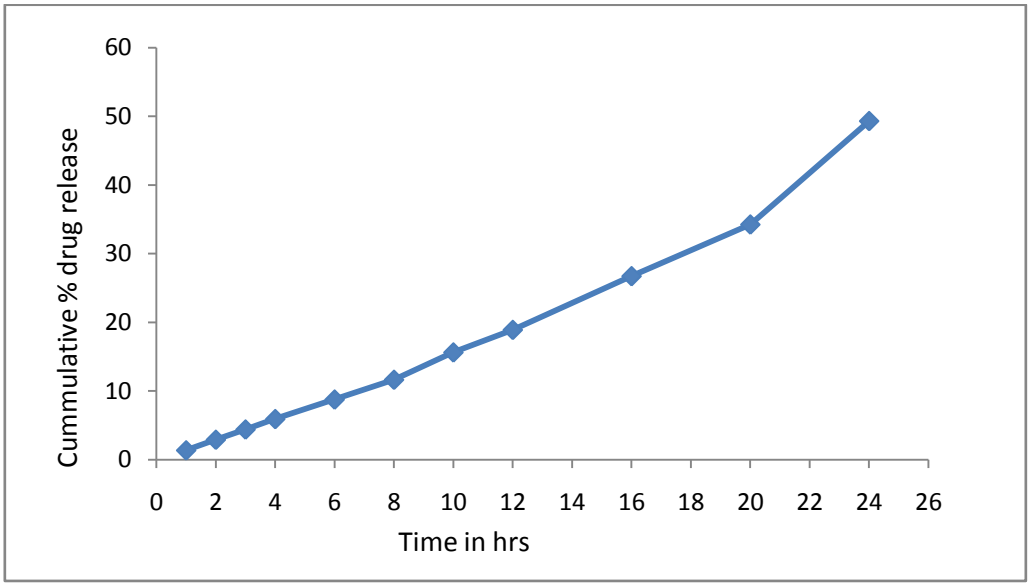
Table No: 12 *In vitro* cumulative % drug release profile of doxorubicin hydrochloride liposomal formulations.

Time (hrs)	Cumulative % drug release					
	F1	**F2**	**F3**	**F4**	**F5**	**F6**
1	1.29	1.38	1.65	1.74	2.12	2.15
2	2.36	2.92	3.21	3.37	4.02	4.07
3	4.32	4.41	5.01	5.11	6.12	6.19
4	5.65	5.93	6.56	6.82	8.32	8.43
6	8.53	8.77	9.85	10.5	12.92	13.01
8	11.21	11.65	13.56	13.9	17.53	17.62
10	15.25	15.63	17.04	17.38	21.45	21.62
12	18.65	18.91	20.53	20.86	25.35	25.56
16	25.98	26.73	29.62	29.82	35.95	36.47
20	33.85	34.25	36.56	36.78	44.86	45.07
24	45.85	49.31	49.59	52.84	55.25	58.67

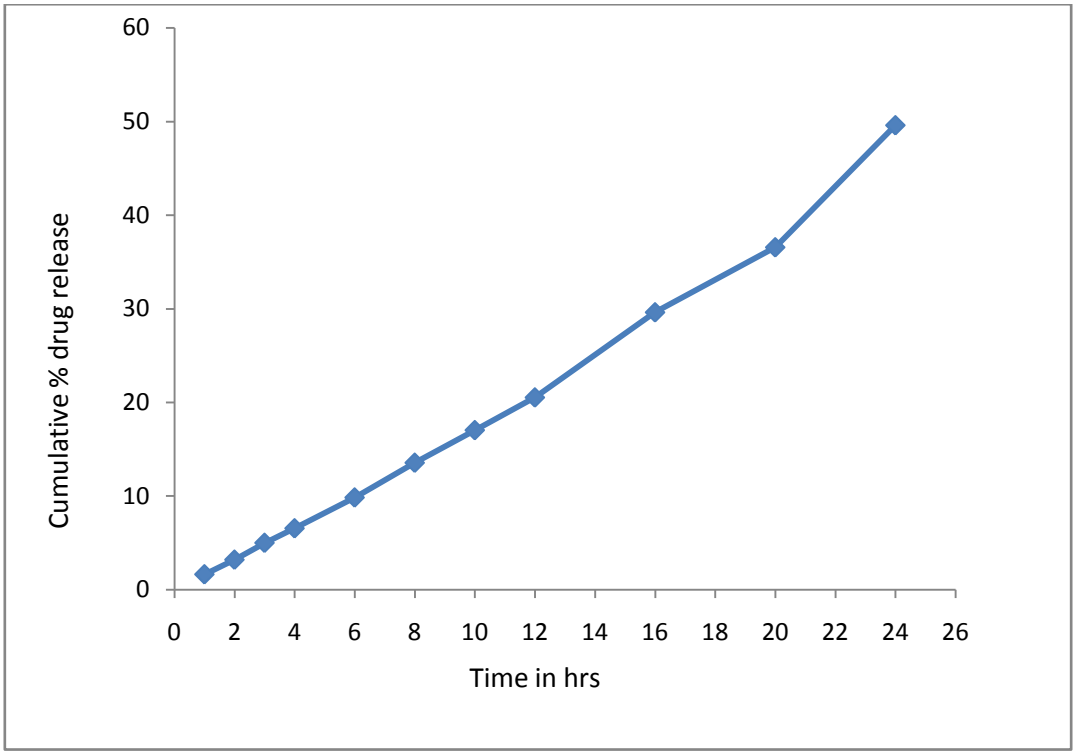
Graph N0:4 *In vitro* release study for F1 formulation.



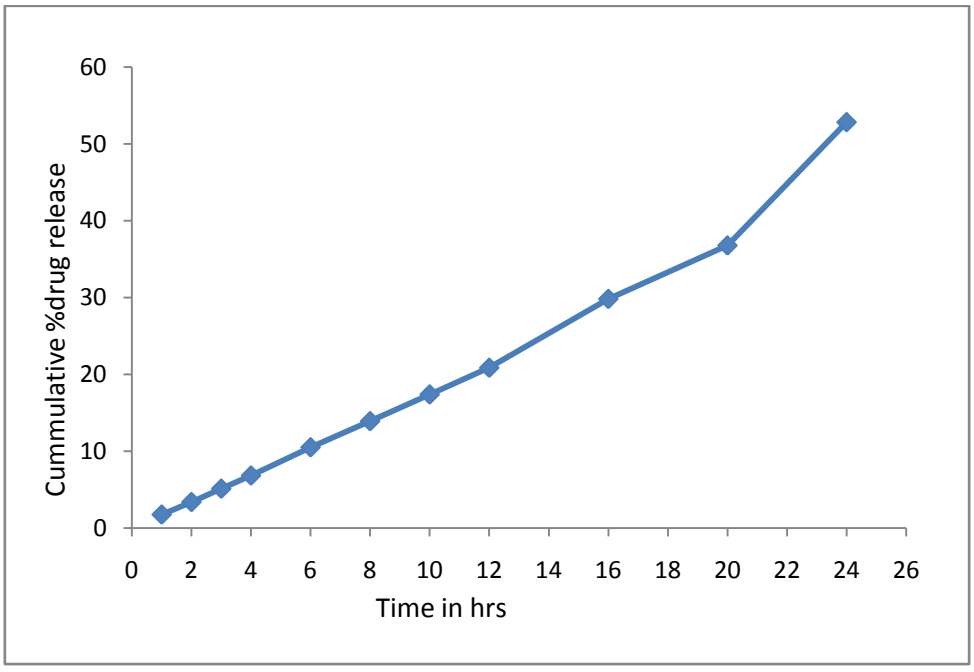
Graph No: 5 *In vitro* release study for F2 formulation.



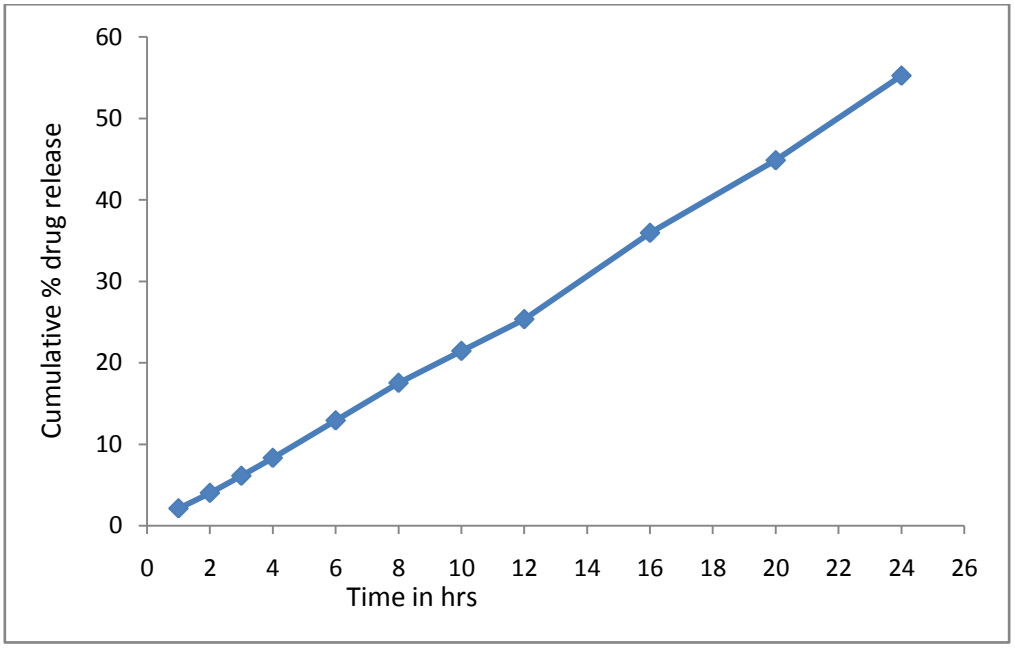
Graph No: 6 *In vitro* release study for F3 formulation.



Graph No: 7 *In vitro* release study for F4 formulation.



Graph No: 8 *In vitro* release study for F5 formulation.



Graph No: 9 *In vitro* release study for F6 formulation.

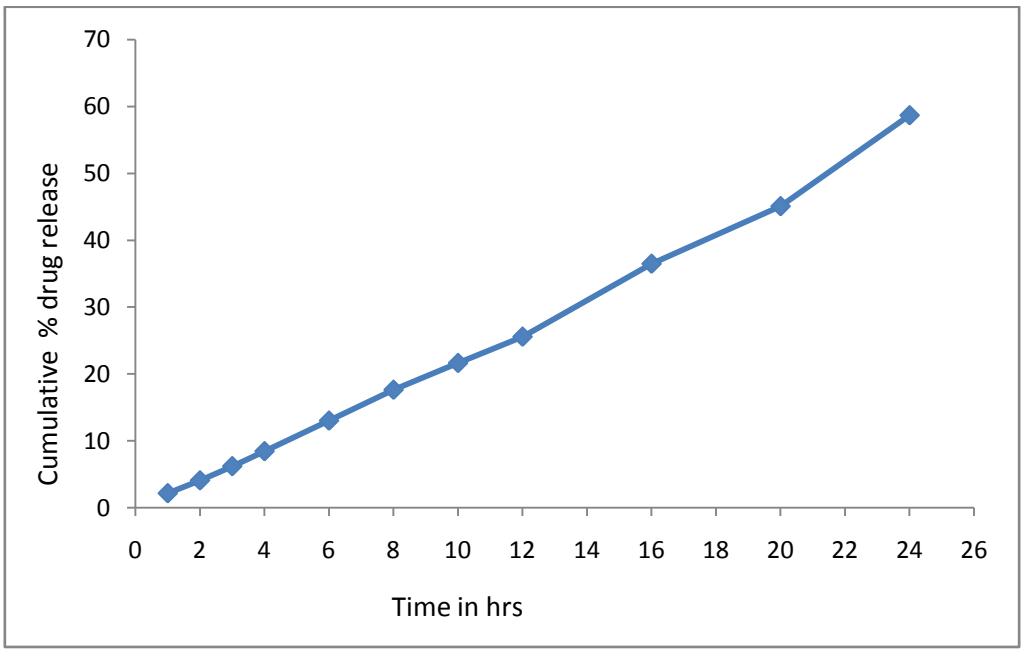


Table No: 13 Zero order release model of doxorubicin hydrochloride liposomal Optimized formulations.

Time (hrs)	Cumulative % drug release		
	F2	**F4**	**F6**
1	1.38	1.74	2.15
2	2.92	3.37	4.07
3	4.41	5.11	6.19
4	5.93	6.82	8.43
6	8.77	10.5	13.01
8	11.65	13.9	17.62
10	15.63	17.38	21.62
12	18.91	20.86	25.56
16	26.73	29.82	36.47
20	34.25	36.78	45.07
24	49.31	52.84	58.67

Table No: 14 First order release model of doxorubicin hydrochloride liposomal Optimized formulations.

Time (hrs)	Log Remaining % drug release		
	F2	**F4**	**F6**
1	1.993	1.992	1.990
2	1.987	1.985	1.981
3	1.980	1.977	1.972
4	1.973	1.969	1.961
6	1.960	1.951	1.931
8	1.946	1.935	1.915
10	1.926	1.917	1.896
12	1.908	1.898	1.871
16	1.864	1.846	1.802
20	1.817	1.800	1.739
24	1.745	1.717	1.665

Table No: 15 Higuchi release model of doxorubicin hydrochloride liposomal Optimized formulations.

$\sqrt{t}$	Cumulative % drug release		
	F2	**F4**	**F6**
1	1.38	1.74	2.15
1.414	2.92	3.37	4.07
1.732	4.14	5.11	6.19
2	5.93	6.82	8.43
2.449	8.77	10.5	13.01
2.828	11.65	13.9	17.62
3.162	15.63	17.38	21.26
3.464	18.91	20.86	25.56
4	26.73	29.82	36.47
4.472	34.25	36.78	45.07
4.898	49.31	52.84	58.67

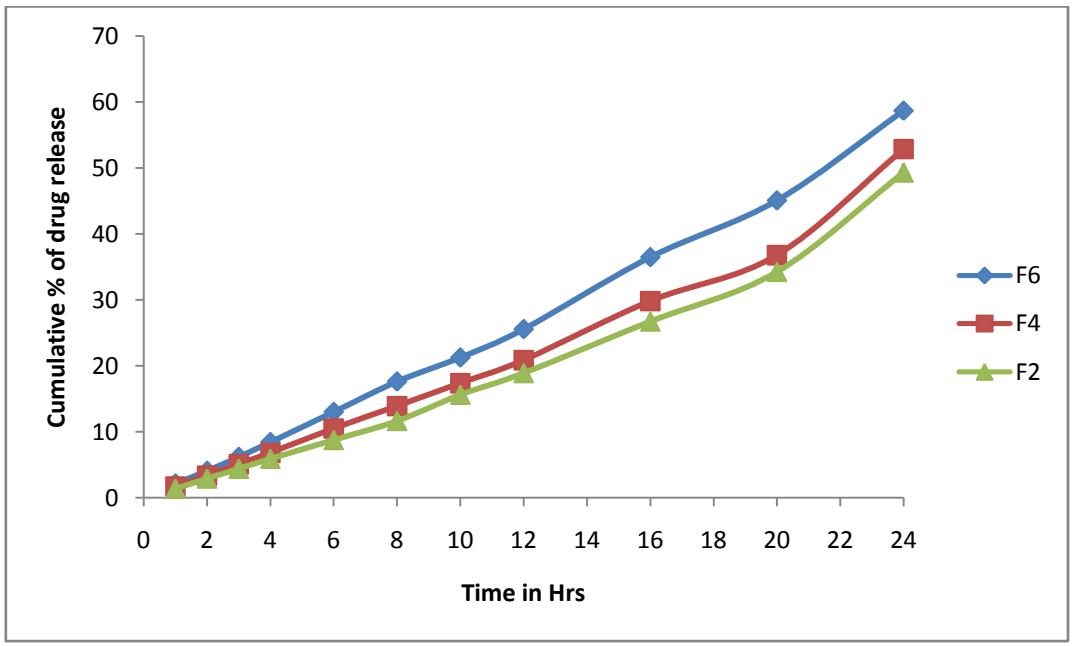
Table No: 16 Korsmeyer-Peppas model for mechanism of drug release.

Log time	Log Cumulative % drug release		
	F2	**F4**	**F6**
0	0.139	0.240	0.332
0.301	0.465	0.527	0.609
0.477	0.644	0.708	0.719
0.602	0.773	0.833	0.925
0.778	0.942	1.021	1.114
0.903	1.066	1.143	1.246
1	1.193	1.240	1.334
1.079	1.276	1.319	1.407
1.204	1.426	1.474	1.561
1.301	1.534	1.565	1.653
1.380	1.692	1.722	1.768

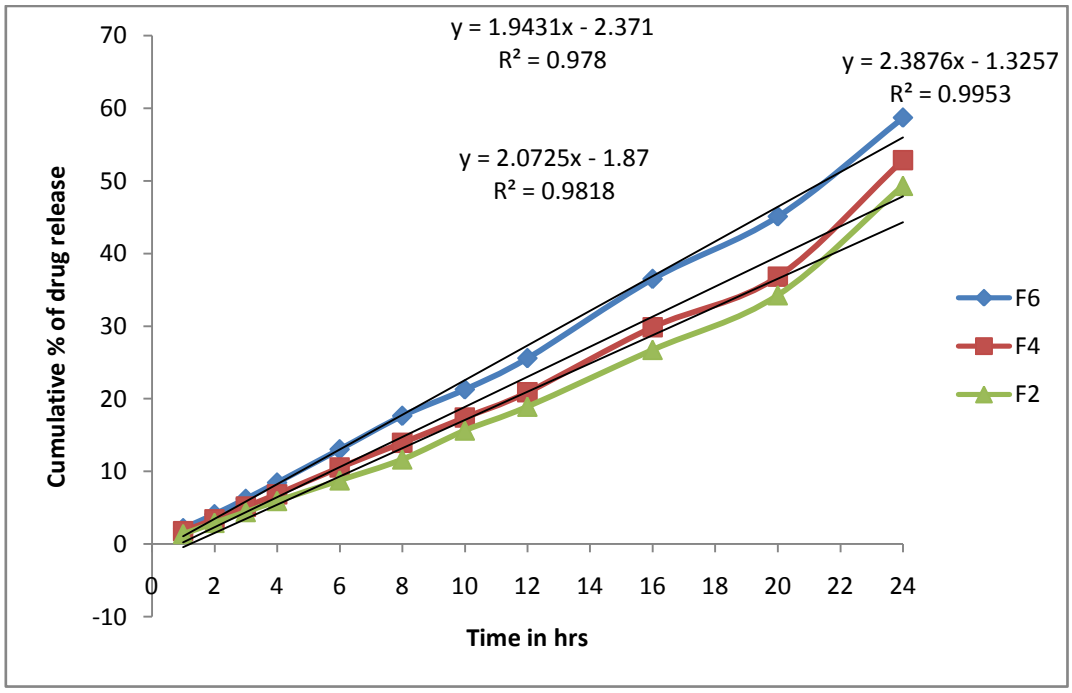
Table No: 17 Curve fitting data of release rate profile of Formulatons F2, F4, F6.

Type of Formulation	Zero-order (R^2)	First-order (R^2)	Higuchi (R^2)	Korsmeyer – Peppas (n)
F2	0.978	0.9758	0.9053	1.0890
F4	0.9818	0.9761	0.9162	1.0483
F6	0.9953	0.9869	0.9437	1.0502

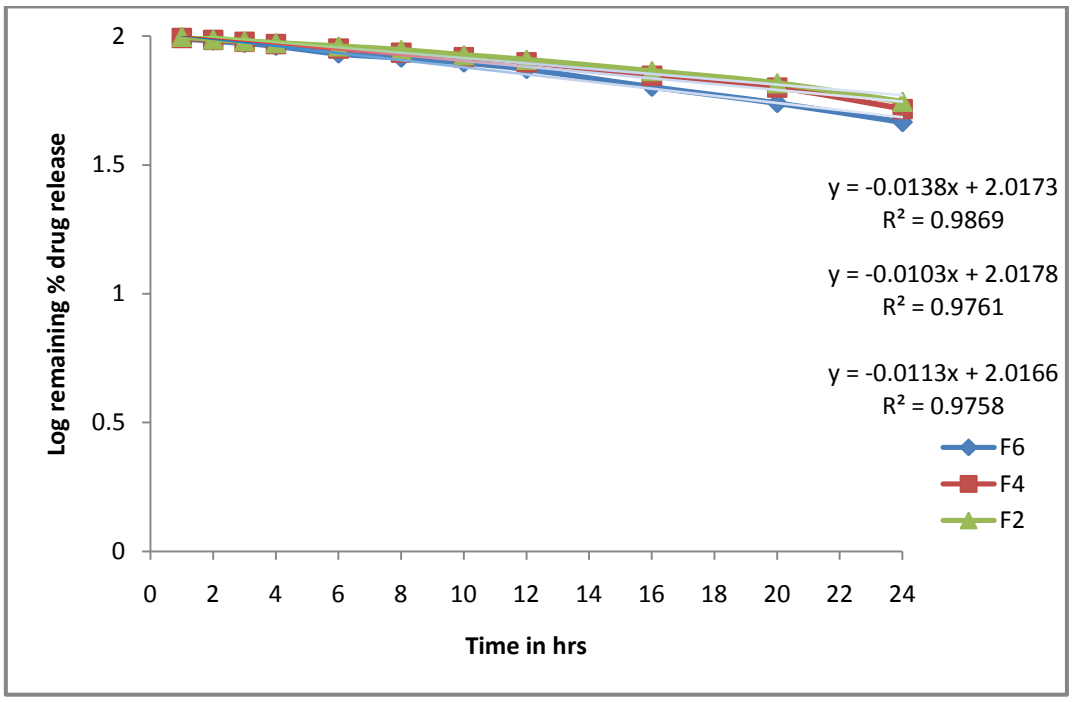
Graph no: 10 Comparison of *in vitro* release studies for optimized formulations F2, F4, F6.



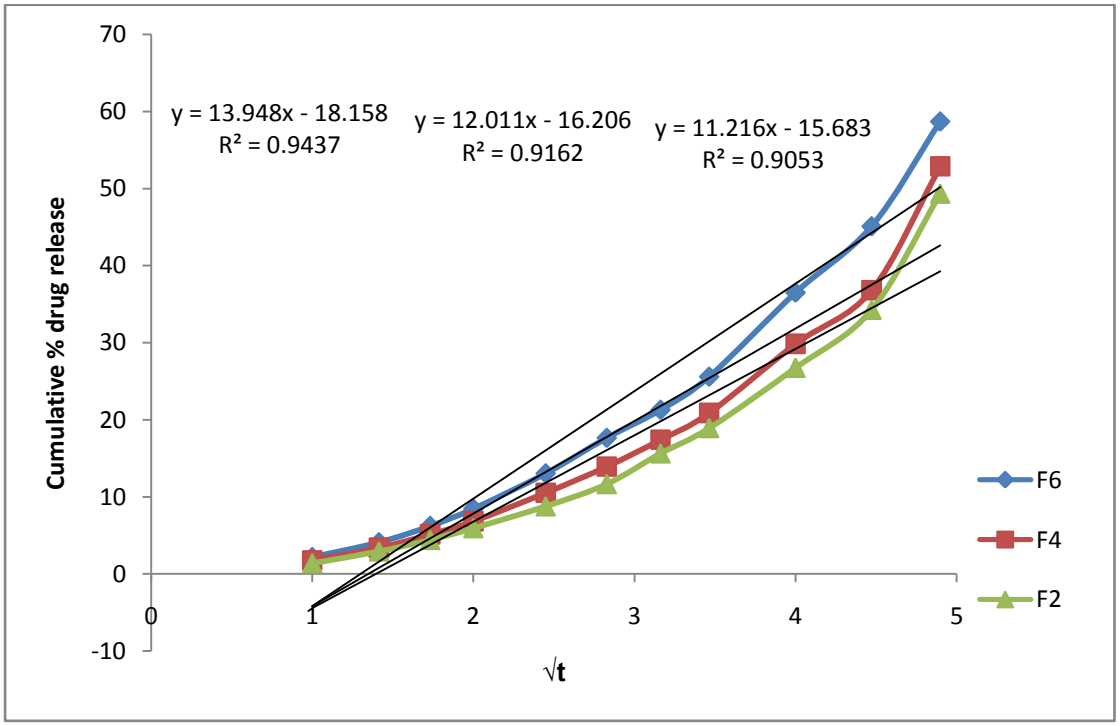
Graph No: 11 Comparison of Zero order release studies for optimized formulations F2, F4, F6.



Graph No: 12 Comparison of First order release studies for optimized formulations F2, F4, F6.



Graph No: 13 Comparison of Higuchi's order plot for optimized formulations F2, F4, F6.



Graph No: 14 Comparison of Korsmeyer –Peppa's model for optimized formulations F2, F4, F6.

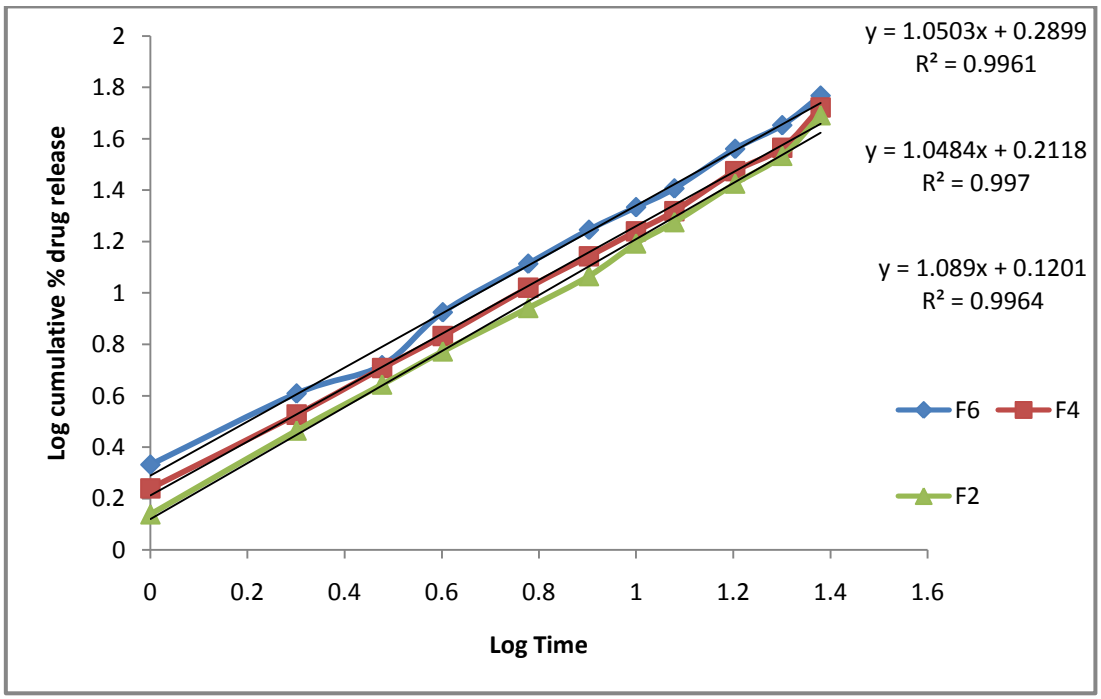


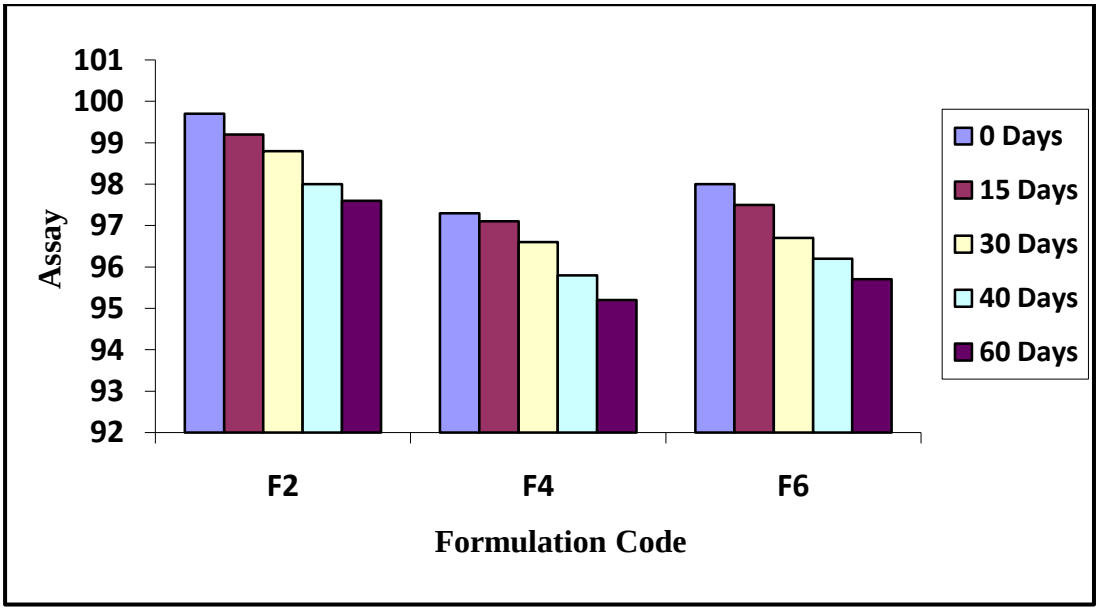
Table No: 18 Effect of temperature on Assay of doxorubicin HCl at 4^{o}c.

Formulation code	Effect of stability on Assay at 4^{o}c				
	0day	**15 days**	**30 days**	**40 days**	**60 days**
F2	99.7±0.65%	99.2±0.47%	98.8±0.72%	98±1.0%	97.6±0.65%
F4	97.3±0.96%	97.1±0.73%	96.6±1.12%	95.8±0.43%	95.2±0.96%
F6	98±1.3%	97.5±1.0%	96.7±0.9%	96.2±0.63%	95.7±1.3%

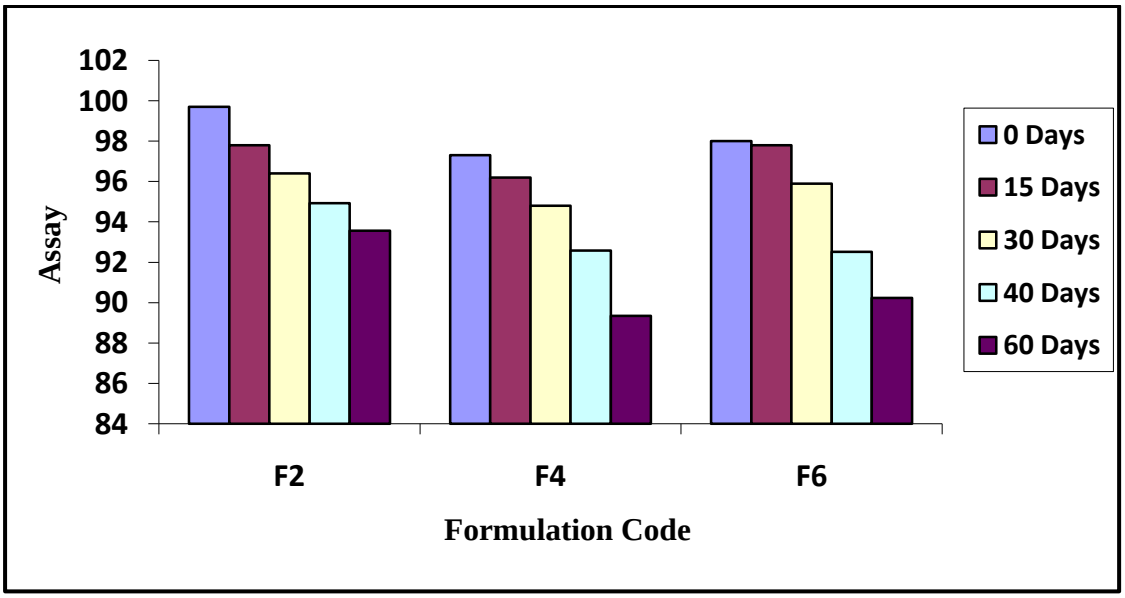
Table No: 19 Effect of temperature on Assay of doxorubicin HCl at room temperature.

Formulation code	Effect of stability on Assay at room temperature.				
	0day	**15 days**	**30 days**	**40 days**	**60 days**
F2	99.7±0.65%	97.8±0.48	96.4±0.54	94.92±0.32	93.56±0.85
F4	97.3±0.96%	96.2±0.92	94.8±0.62	92.58±0.58	89.35±0.53
F6	98±1.3%	97.8±0.65	95.9±0.69	92.52±0.86	90.24±0.74

Graph N0:15 Stability plot for F1, F2 F3, F4, F5, F6 Formulations at 4^0c



Graph N0:16 Stability plot for F1, F2 F3, F4, F5, F6 Formulations at Room temperature.



DISCUSSION

8. DISCUSSION

8.1 Standard calibration curve of Doxorubicin hydrochloride in UV spectrophotometer:

The UV absorbance's of Doxorubicin hydrochloride standard solution in the range of 10-50 μg/ml of drug in buffer, pH 7.4 showed linearity at λ max 254nm. The linearity was plotted for absorbance against concentration with R2 value 0.9995 and with the slope equation y=0.0179x-0.003. The absorbance values and standard curve shown in Table No:1 and Graph No:1

8.2 Compatibility studies:

The compatibility between the drug and the selected lipid and other excipients was evaluated using FTIR peak matching method. There was no appearance or disappearance of peaks in the drug-lipid mixture, which confirmed the absence of any chemical interaction between the drug, lipid and other chemicals. The results shown in Table No: 4, 5 and 6 and Spectra No: 1, 2, 3, 4, 5 and 6.

8.3 Doxorubicin liposomal formulation:

The Liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug and carrier (soybean lecithin)**.**

The formulation containing Doxorubicin were prepared with different stabilizers like Dicetylphosphate and Stearylamine and all other parameters like temperature, vacuum and RPM were kept constant. The composition and ratios of compounds showed in Table No: 7. among those compositions 6 Formulations are selected as optimized batches for further evaluation, 2 from each of neutral, positive and negative as showed in Table No: 8

8.4 Physicochemical characterization

8.4.1 Particle size distribution:

The particle size distribution was analyzed for F2, F4, F6 formulations of doxorubicin Liposomes by wet method. The particle size was optimum in F6 Formulation, When compared to F2 and F4, The results were shown in Table No: 9 and Report No: 1, 2 and 3.

8.4.2 Scanning Electron Microscopy:

The Morphology and surface appearance of Liposomes were examined by using SEM. The SEM photographs of F2 and F6 formulation showed that the particles have smooth surface. The SEM images were shown in Figure No: 6 and 7.

8.4.3 Zeta Potential analysis:

The zeta potential report of liposomal solution for F2, F4, F6 formulations are 5.21mV, 24.66mV, -23.4 which lies near to the arbitary value. The report shows good stability value for formulated liposomal solution, the results were shown in Table No: 9 and Report No: 4, 5 and 6.

8.5 *IN VITRO* CHARACTERIZATION

8.5.1 Percent free drug:

The percent free drug is determined for all the formulations F1to F6. The percent free drug was optimum in F6 formulation, which is within the limit (10%), the percent free drug was as shown in the Table No: 10 and Graph No: 2

8.5.2 Assay:

The assay value is determined for all the formulations from F1to F6. The assay value is within the limit (90%) for all the formulations, the results were shown in the Table No: 11 and Graph No: 3

8.5.3 *In vitro* Dissolution data:

The *in vitro* dissolution profile of prepared formulations was determined by membrane diffusion method. The dissolution was carried out for a period of 24 hrs in 7.4 pH phosphate buffer.

The cumulative percent release of F1 to F6 formulations at various time intervals was calculated and tabulated in Table No: 12 The cumulative percent drug release in all formulations was plotted against time in Graph No: 4, 5, 6, 7, 8, and 9. The Maximum percent of drug release was found in F6 formulation which contains maximum drug entrapment.

8.5.4 Release Kinetics:

The release kinetics of F2, F4, F6 formulations were studied. All formulations follow Zero order release kinetics and follow case II transport when it applied to the Korsmeyer-Peppa's Model for mechanism of drug release. F6 formulation has better kinetic results when compared to F2 and F4 formulations. The results are shown in Table N0: 13, 14, 15 and 16 and Graph No: 10, 11, 12, 13 and 14.

8.6 Stability data:

The stability of the Doxorubicin Liposomes was evaluated after storage at 4^0c and room temperature for 60 days. The assays of the samples were determined as a function of the storage time. The Liposomes stored at 4^0c were found to be stable for duration of 60 days. The results were showed in Table No: 18 and 19 and Graph No: 15 and 16.

SUMMARY AND CONCLUSION

9. SUMMARY AND CONCLUSION

The main objective of this work was designed to prepare and evaluate the Doxorubicin Hcl Liposomes. This formulation will target the site of action with effect of various stabilizers on drug entrapment efficiency, and to reduce the side effects by formulating non-pegylated Liposomes. This liposomal formulation was formulated using the soyabeanlicithin and cholesterol which has lesser toxicity.

The Liposomes were prepared by dried thin film hydration technique using rotary evaporator with drug, carrier, ammoniumsulphate and stabilizers. The parameters like temperature, vacuum and RPM were maintained accordingly. After preparation, the Liposomes were stored in freezed condition, and given for further evaluation.

The prepared Liposomes of F2, F4 and F6 formulations were evaluated for physical and chemical characteristics like average vesicle size, shape and zeta potential. The evaluated batches showed good physicochemical characteristics in F6 formulation (Negative) when compared to the F2 (Neutral) and F4 (Positive) formulations.

The prepared Liposomes of F1 to F6 were evaluated for % free drug and Assay, The % Free drug was optimum in F6 (Negative) formulation when compared to other formulations of F1, F2, F3, F4 and F5. The Assay was optimum in F2 (Neutral) formulation when compared to other formulations of F1, F3, F4, F5 and F6.

This developed liposomal drug delivery system was also evaluated for dissolution study by pH 7.4 phosphate buffer using membrane diffusion method. The release of drug from F6(Negative) formulation was found to be sustained to certain extent when compared to F1, F2, F3, F4 and F5 formulations.

The release kinetics of F2, F4 and F6 Formulations were studied. All formulations follow Case II transport when it applied to the Korsmeyer – Peppa's

model for mechanism of drug release. F6 (negative) formulation has better kinetic results when compared to F2 and F4 formulations.

The stability of the Doxorubicin Hcl Liposomes was evaluated after stored at 4^{o}c and room temperature for 60 days. The assay of the samples was determined as a function of the storage at different time intervals. The Liposomes stored at 4^{o}c were found to be stable for duration of three months.

From the results of physical characterization, *in-vitro* evaluation, release kinetics and stability studies, it was found that charged Liposomes containing Doxorubicin might be used for the treatment of a Kaposi's sarcoma when compared to the normal drug and neutral Liposomes.

CONCLUSION:

From the executed experimental results, it could be concluded that the stabilizers like Stearylamine and Dicetylphosphate along with Soy lecithin and cholesterol were suitable carrier for the preparation of Doxorubicin Liposomes. Though the preliminary data based on *in-vitro* dissolution profile, release kinetics and stability studies proved that the suitability of such formulations, Still a thorough experiment will be required based on the animal studies. There after we can find the actual mode of action of this kind of dosage form. Therefore, a future work will be carried out as follows,

- ✓ Long term stability studies
- ✓ *Invitro* Cytotoxicity studies
- ✓ *Invivo* Pharmacological work on animals.
- ✓ *Invivo* pharmacokinetic studies on animals.

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