Formulation and Evaluation of Self Nano-Emulsifying Drug Delivery System (SNEDDS) for Tamoxifen

A Consultancy Project

Submitted to

Inception Source Pvt Ltd – Hyderabad

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Tilte: Formulation and Evaluation of Self Nano-Emulsifying Drug Delivery System (SNEDDS) for Tamoxifen

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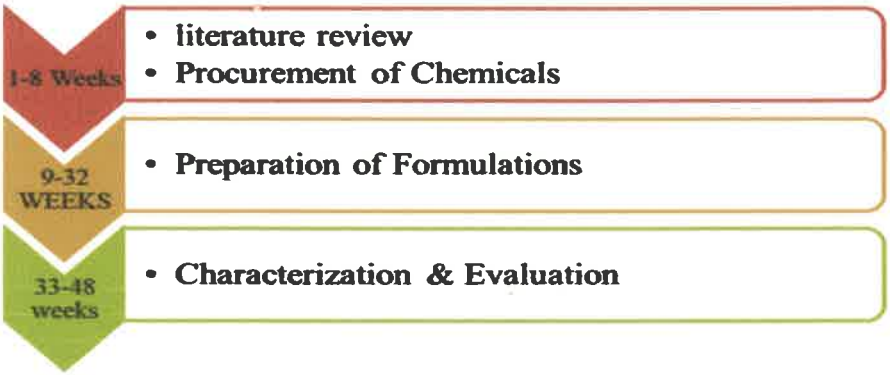
Co-Investigator: Dr.Ayesha Sultana M. Pharm PhD

Stages of Work



Cost Analysis

S.No	Parameter	Amount in Rupees
1	Man power	56221
2	Consumables	128779
4	Contingencies	25000
5	Overhead Charges	15000
Total		225,000

Principal

Principal Investigator

Co-Investigator

Principal
St. Paul's College of Pharmacy
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INCEPTION SOURCE PVT. LTD. HYDERABAD.

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Abstract:

Nanotechnology has become a buzzword for scientific experts, and efforts are ongoing to extend its applications in various medical and pharmaceutical aspects. The nanoscale technologies can be generally categorized into lipid-based nanocarriers, polymeric nanocarriers, inorganic nano-carriers, and drug nanoparticles or nanosuspensions . Self-nanoemulsifying drug delivery systems (SNEDDS) are anhydrous homogeneous liquid mixtures, composed of oil, surfactant, drug, and/or co-solvents, which spontaneously form transparent nano-emulsion (20–200 nm droplet size) upon aqueous dilution with gentle agitation and Tamoxifen is used to treat breast cancer that has spread to other parts of the body in men and women. It is used to treat early breast cancer in women who have already been treated with surgery, radiation, and/or chemotherapy. Self-nano emulsifying drug delivery systems of Tamoxifen will be prepared and in-vitro studies were done

KEY WORDS: nanoparticles, Tamoxifen, homogenous liquid mixtures

1. Introduction

1. Introduction

1.1 Lipid based drug delivery system

Nano carriers have come long way from its birth with various nano-carriers being attempted as drug delivery system, undergoing clinical trials and also commercially available. Various biomaterials such as albumin, gelatin for nanoparticles, phospholipids for liposomes, polymers have been reinvented as excipients without any toxicity issues are used to prepare these tiny wonders with particle size in nanometers. The drug to be delivered can be absorbed or conjugated to the surface or encapsulated in lipid or polymers or dissolved in matrix. **(Bamrungsap S, 2012)** We decided to work upon solid lipid nanoparticles and liposomes for their characteristics and suitability for the anticancer drugs selected.

1.2 Solid Lipid nanoparticles, a particulate nanocarrier for commercial use.

Lipid nanoparticles are aqueous dispersions of biocompatible solid lipids of nanosize ranging from 50-200 nm. These drug delivery systems score over the others such as liposomes, polymeric nanoparticles and nanoemulsions as they overcome specific disadvantages such as memberane stability , drug leaching, biodegradation and toxicity.**(Muller RH, Shegaonkar R, 2011)** Emulsification and solvent evaporation solvent diffusion **(Westesen K, 1996)** are many techniques used to prepare solid lipid nanoparticles.High pressure homogenization (HPH) is highly recommended method as its industrially feasible. In this technique melted lipid is dispersed in hot surfactant solution by high speed stirring. The emulsion is further homogenized at 5°C above the melting temperature of the lipid and then cooled and crystallized slowly to obtain SLN. The excipients used in formulation of SLN such as oils, lipids (Long chain and medium chain triglycerides) and surfactants (lecithin, tween 80, poloxamer and PVP) have been

accepted as Generally regarded as safe (GRAS) status **(FDA,2010)** and also are accepted for injectables.

1.2.1 Fate of SLN *in vivo* and on storage

The toxicity of SLN is required to be checked, as the physicochemical properties change in nanodimension and nanoparticles can be internalized by macrophages and can be taken up by all cells by endocytosis if less than 100 nm. Another area of concern is bio-persistency in body as well as in environment. The fate of nanoparticle will depend upon absorption of blood proteins on the surface. If opsonin gets adsorbed the uptake is more by the macrophages of mononuclear phagocytic system (MPS), mainly liver and spleen. If dysopsonin get absorbed, the lipid particles keep circulating for a longer period of time, thus releasing drug slowly. Similar exchanges are expected to happen between i.v. injected SLN and chylomicrons such as LDL, HDL in the blood. The surfactant plays important role in interaction with MPS as well as blood cells such as leukocytes and proteins. **(Kristl J et al, 2003)**

1.2.2 Parenteral use and Storage stability of SLN

SLN formulations for parenteral use have been already developed for drugs such as clozapine, cisplatin **(Doijad RC,2008; Tian J,2008)**, etoposide **(Reddy HL,2005),** doxorubicin **(Zara GP,2010)**, camptothecin **(Yang SC,1999)**, tashinone IIA **(Liu J,2005)**, buprenorphine **(Wang JJ,2009),** chlorambucil **(Sharma P,2009)**, methotrexate **(Rukmani K,2006)** etc.

The physicochemical characterization such as particle size, zeta potential measurement, XRD and DSC analysis are used to get optimized SLN. The storage stability of SLN is improved by lyophilization or spray drying. The cryoprotectants used are selected from different saccharides

which decrease the osmotic activity of water of crystallization and favor glassy state of the frozen samples.

1.2.3 Surface modification and anticancer drug entrapment of SLN

Solid lipid Nanoparticles can be surface modified with the ligands to improve targeting to specific organ of interest. Studies with ligand such as Transzumab ,Pertuzumab ,Transferrin (Tf)-conjugated solid lipid nanoparticles were investigated for their ability to deliver quinine dihydrochloride to the brain for the management of cerebral malaria. **(Gupta Y,2007)**Attempts have been made by Lemieux to deliver transferrin doxorubicin conjugate to breast cancer cell lines. **(Lemieux P, 1994)** Sahoo and Labhasetwar improved antiproliferative activity of transferrin conjugated paclitaxel loaded nanoparticles. **(Sahoo SK, Labhasetwar P,2005)**

1.3 Liposome, a vesicular option for encapsulation of anticancer agent

Encapsulation of active ingredient into liposome can enhance selectivity of drug and increase the therapeutic effect by improving bioavailability, reducing systemic and organ toxicity and longer circulation time **(Torchilin VP, 2007)**.Chemotherapeutic drugs can be delivered to tumors by long circulating liposomes.There is a positive correlation between the circulation time of liposomes and their localization in tumors. The drug is protected against the metabolic degradation. This shows enhanced uptake in organs rich in mononuclear phagocytic cells such as liver, spleen and bone marrow and decreased uptake in the kidney, myocardium and brain.

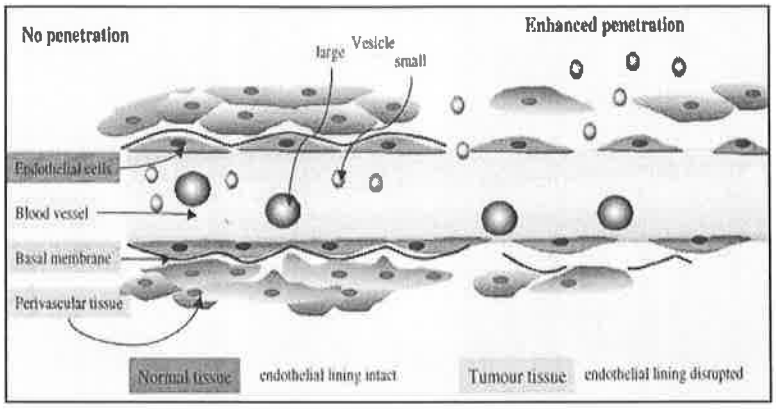


Fig 1.1: Leaky vasculature of tumor tissue

1.3.1 Liposome similarity to biological membrane

Liposomal vesicles are formed by phospholipids, the amphipathic lipid molecules containing polar heads and hydrophobic hydrocarbon tails suspended in an aqueous medium associate spontaneously to form bilayer vesicles. Liposome which consists of Phospholipids and Cholesterol is similar to biological cell membranes. (Fig 1.2, 1.3)

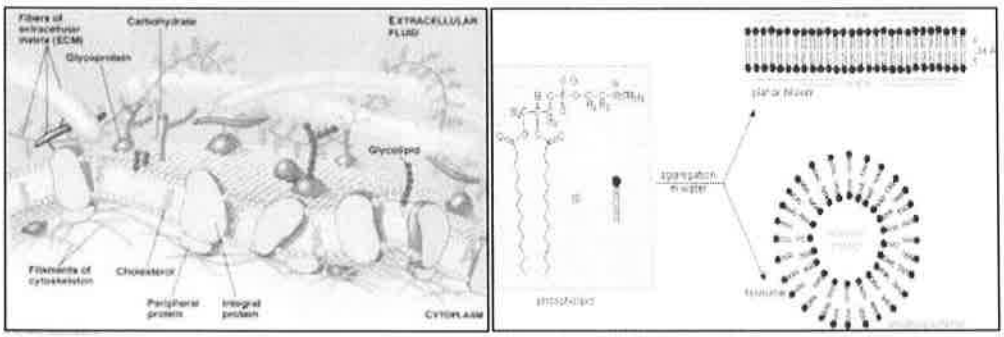


Fig1.2 Liposome structure

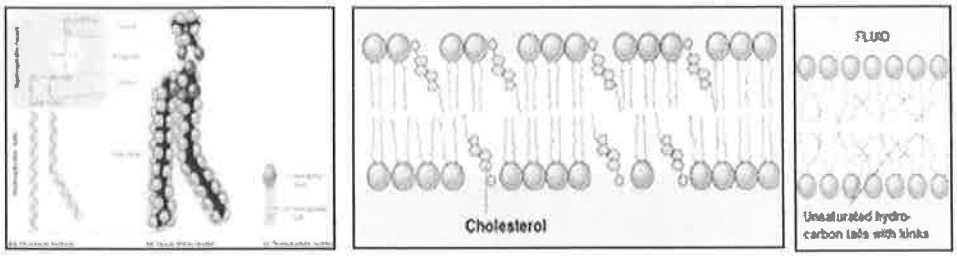


Fig 1.3 Composition of Liposome: Phospholipid and role of cholesterol

Cholesterol improves the fluidity of the lipid bilayer membrane, reduces the permeability of water soluble molecules through the membrane, and improves the mechanical rigidity of fluid bilayers. Liposomes without cholesterol tend to react with the blood proteins such as albumin, m- transferrin, and macroglobulin which may destabilize liposomes. When lipids are dispersed in water, lipid sheets get hydrated, swollen and when agitated, they self close to large multi-lamellar vesicles. The size of formed vesicles can be reduced by sonication, extrusion etc. (Fig 1.4)

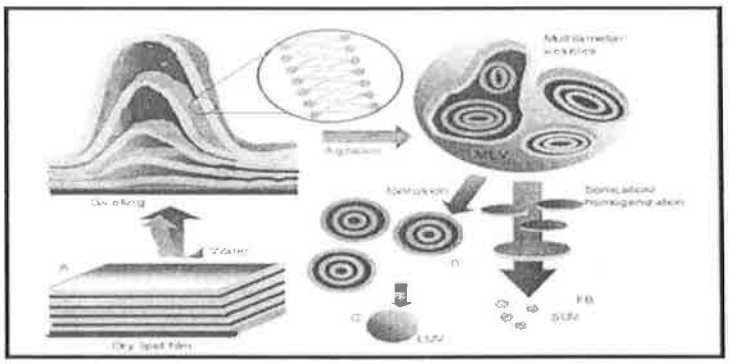


Fig1. 4 Formation of liposomes

1.3.2 Stealth liposomes

The sterically stabilized liposomes, are formulated by hydrophilic long-chain polymers, such as polyethylene glycol (pegylation) in the bilayer, forming a coated surface on the liposome

that repels opsonin penetration and adsorption. **(Woodle MC, 1998; Esfahani MKM,2014)** The long-circulating liposomes have been prepared using poly [N-(2hydroxypropyl) methacrylamide **(Whiteman KR et al, 2001)** poly- N-vinyl pyrrolidones, **(Torchilin VP et al, 2001)** l-amino acid-based, biodegradable, polymer–lipid conjugates **(Metselaar JM, 2003)** and polyvinyl alcohol **(Takeuchi H, Kojima H, 2001)**. The relative role of the liposome charge and protective polymer molecular size was investigated

(Levchenko TS et al, 2002), showing that opsonins with different molecular sizes may be involved in the clearance of liposomes containing differently charged lipids. Interactions of plasma proteins with liposomes of different composition may be shielded differently by PEG **(Chiu GN et al, 2001)**. In the present research work, long circulating, `sterically stabilized (Stealth) liposomes were prepared using two different phospholipids viz. 1,2-distearoyl phosphatidyl choline (DSPC) and DSPG Na.

1.3.3 Liposome stability and the gel-to-liquid crystalline phase transition

At a higher temperature, phosphatidylcholines undergo an abrupt transition from gel state (stable), a highly ordered, tightly packed arrangement, to a less ordered and less tightly packed, liquid-crystalline state (unstable or metastable), where the freedom of movement of each molecule is high. This phase transition happens as the fatty acid chain adopts a new confirmation other than the all Trans straight chain confirmation such as gauche confirmation. This is called gel to liquid crystalline phase transition. Saturated lipids with hydrocarbon chains comprising more than 14 carbon atoms

e.g. 1, 2-distearoylphosphatidylcholine (DSPC) is in the gel state at room temperature. The stability of liquid-crystalline bilayers can be improved by incorporation of cholesterol.

The various methods of preparation of liposome are Lipid Hydration Method, Solvent Spherule Method **(Kim et al., 1981)**, solvent injection method e.g. ether infusion. In the present research work liposomes were developed by Ethanol Injection method in which a lipid solution of ethanol is rapidly injected to a vast excess of vehicle (Fig 1.5) to form multi lamellar vesicles (MLVs). For further reduction in size, sonication method or French pressure cell method were used.

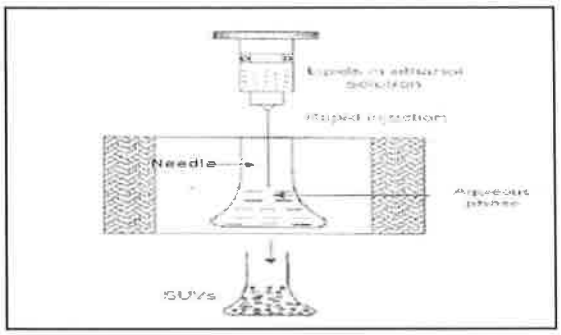


Fig1. 5 Ethanol injection method for preparation of liposomes

Drugs can be loaded using passive loading that is, the drug is incorporated in the liposomes during formation process, either dissolved in the aqueous phase used to hydrate the lipid or included with the lipids, depending on the solubility of the compound. In active loading drug is loaded in the preformed liposomes using different techniques such as ion gradient or pH gradient technique. Another method is remote loading consists of forced entrapment of drug molecules upon establishing certain concentration gradients between liposome interior and exterior.

1.3.4 Stability of liposomes

Lyophilisation is an excellent method to extend the shelf life of compounds with stability problems in aqueous media. Both physical and the chemical properties were evaluated to check the stability of liposomes. ICH guidelines Q1A (R2) were followed for liposome stability studies.

1.3.5 Liposomes behaviour *in vivo* and its clearance

In vivo fate of liposomes depends upon physicochemical properties such as size, surface charge, bilayer packing, fluidity, surface hydration as well as on other factors such as dose and route of administration, method of preparation. Surface modification of liposomes by various means is preferred approach for the enhancement of uptake by tumor cells.

Immediately after i.v. injection, liposomes become coated by proteins circulating in the blood. Some of these proteins compromise the integrity of the lipid bilayer causing rapid leakage of liposome contents. Others promote recognition and subsequent elimination of liposomes from the blood. Proteins called opsonins, mark liposomes for removal through phagocytic cells. Examples of opsonins include components of the complement system (C3b, iC3b), IgG-2 glycoprotein 1 and fibronectin. The removal of liposomes is carried out by the mononuclear phagocyte system (MPS), in particular the resident macrophages of the liver (Kupffer cells), spleen, lung and bone marrow.

Table no .1. 1Approved liposomal formulations in cancer therapy

Product	Company	Drug	Particle size	Composition/size	Therapeutic indication
DaunoXome®	Nextar/ Glead	Daunorubicin	60 nm	DSPC/Chol liposomes	Advanced Kaposi`s sarcoma
Doxil®/ Caelyx®	Alza corporation	Doxorubicin	80 – 120 nm	HSPC/Chol/PEG-DSPE- liposomes,	Metastatic ovarian cancer, advanced Kaposi`s sarcoma
Myocet™	Liposome Company/ Elan	Doxorubicin	100 nm	EPC/Chol liposomes,	Metastatic breast cancer

1.4 Receptors theory

Receptor is a protein molecule that recognises and responds to endogenous chemical signals. Each receptor is linked to a specific cellular biochemical pathway. **(Lynch JW, 2004)** Eventhough numerous receptors are found in most of the cells, each receptor will only bind with ligands of a particular structure, similar to lock that accepts specifically shaped keys to open. When a ligand binds to its corresponding receptor, it activates or inhibits the receptor's associated biochemical pathway.

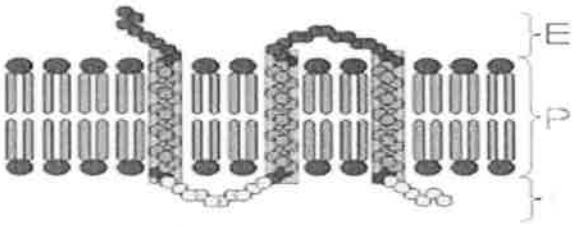


Fig 1.6 Trans membrane Receptors E: Extracellular space, P: Plasma Membrane, I: Intracellular space.

Ionotropic receptors are the targets of fast neurotransmitters and show extracellular ligand-binding domain, G protein coupled Receptors (metabotropic) includes the receptors for several hormones and slow transmitters with extracellular and intracellular domains. Kinase linked receptors possess an extracellular domain containing the ligand binding site and an intracellular domain, often with enzymatic function whereas nuclear Receptor **(Chawla A et al, 2001)** are located in the cytosol and migrate to the nucleus after binding with their ligands. These proteins sense steroid and thyroid hormone. These receptors work with other proteins to regulate the expression of specific genes, thereby

Controlling the development, homeostatis and metabolism of the organism. There are different theories of drug receptor or ligand receptor conjugation. Occupation theory of drugreceptor or receptor-ligand binding suggests, effect of drug is directly proportional to

number of receptors occupied, rate theory suggests activation of receptors is proportional to total number of encounters whereas induced fit theory says the receptor alters the conformation of its binding site to produce drug receptor complex as the drug approaches the receptor. Cells can increase/ upregulate or decrease / downregulate the number of receptors to a given hormone or neurotransmitter, to alter its sensitivity to drug or ligand. Drug can be either agonist, antagonist, inverse agonist or allosteric modulaor. **(Yixin Liu et al, 2009)**

Ligand is a molecule that binds to the receptor to trigger response e.g. Peptide, pharmaceutical drug, toxin, neurotransmitter, hormone. When a ligand binds to its corresponding receptor, it activates or inhibits the biochemical pathway associated with receptors and shows the effect.

1.4.1 Cell cycle

To understand the behavior of rapidly growing cancer cells, we must understand the mechanism of cell cycle. In normal cells, growth, division, and differentiation are highly regulated processes with signaling molecules. Growth factors promoting cell division whereas other signaling molecules cause cells to stop growth. Cancer cells can alter signaling pathways and stimulate growth. Increased levels of certain proteins in a pathway, or genetic mutations that alter these proteins, may cause the pathway to transmit growth signals on its own. Alternatively, other mutations may keep cells from receiving or transmitting signals which control cell growth. Another way of controlling growth is apoptosis or cell death which routinely removes diseased, malfunctioning, irreversibly damaged cells or aged cells. Within every cell there are signaling pathways that favor cell survival and others that favor apoptosis. Apoptosis can be initiated from outside the cell and within the cell and there are signaling pathways that favor cell survival or apoptosis **(Byrne JD 2008)** Angiogenesis is the creation of new blood vessels. On a cellular level, angiogenesis occurs when a nearby cells are in need of nutrients or oxygen with help of

Matrix mellanoproteins, endothelial cells migrate in the direction of the cell in need and form new blood vessels. Once the tumor reaches a certain size, it requires blood supply to continue growing. Thus formation of incomplete irregular shaped, dilated and tortuous tumor blood vessels occurs. This ability of tumors to progress from a nonangiogenic to angiogenic phenotype, called the "angiogenic switch" is important in progression of cancer and allows the dissemination of cancer cells throughout the body, leading to metastasis.

1.4.2 Role of receptors in therapies of breast cancer

HER family receptors (HER 1/EGFR, HER2/ ErbB2, HER3/ErbB3, HER4) exist on the surface of cells and contain extracellular, transmembrane and intracelluar tyrosine kinase domains. The extracellular domain binds to ligands, the intracellular tyrosine kinase domain activates downstream signaling pathways. Receptor activation and cellular signaling are tightly regulated in normal cells. There may be growth and spread of cancer cells, when HER signaling pathways are inappropriately activated. Broader blockade of multiple HER family receptors may be therapeutically important in HER2 amplified breast cancer. **(Molina MA 2001).**

The chemokine receptors, CXCR4 and CCR7 play an important role in cancer invasion and metastasis. Chemokine ligand-21 (CCL21) - the ligand for chemokine receptor-7 (CCR7) is highly expressed in the lymph nodes of breast cancer patients. Transferrin receptor(TfR) is a cell membrane associated receptor for glycoprotein involved in the cellular uptake of iron and the regulation of cell growth. There is elevated expression levels of TfR on cancer cells compared with normal cells. It is expressed on rapidly dividing cells, with 10,000 to 100,000 molecules per cell commonly found on tumor cells or cell lines in culture. In contrast, in nonproliferating cells,

expression of TfR1 is low or frequently undetectable. **(Megumi Kawamoto 2011)** The vitamin folic acid (folate) has also been used for cancer targeting because folate receptors (FRs) are frequently overexpressed in a range of tumor cells including ovarian, endometrial and kidney cancer. **(Yixin Liu 2010)**

Hormone Receptor associated with breast cells are estrogen receptors (ER) and progesterone receptors (PR; also called PgR). Cancer cells with these receptors depend on estrogen and related hormones to grow. Estrogen and progesterone also play important role in many hormonal functions in women. Arginine–glycine–aspartic acid (RGD), which is the ligand of the cell adhesion integrin $\alpha v\beta 3$ on endothelial cells results in increased intracellular drug delivery in different murine tumour models. RGD also binds to other integrins such as $\alpha 5\beta 1$ and $\alpha 4\beta 1$ and therefore is not specific to cancer cells, which may limit its use.

1.5 Mechanism of Passive Targeting

Nanoparticles such as biological macromolecules or synthetic polymers bigger than 30–40 kDa and liposomes up to a diameter of 200 – 400 nm are able to diffuse out of the leaky tumor blood vessels and accumulate in the tumor tissues, but not in healthy organs and tissues.**(Cohen MH 2004)** Extensive angiogenesis and hyper vasculature, lack of smooth muscle layer, defective fenestrations, irregular blood flow, inefficient lymphatic drainage that leads to enhanced retention in the interstitium of tumors. The slow venous return also leads to accumulation from the interstitial lumen. This chain of events is popularly known as Enhanced permeability and retention effect. (EPR) Passive targeting takes advantage of the unique pathophysiological characteristics of tumor vessels, enabling nano drugs to accumulate in tumor tissues. The 'leaky' vascularization, which refers to the EPR effect, allows migration of macromolecules up to 400 nm in diameter into the surrounding tumor region. **(Kwangjae Cho 2008)** The random approach of passive targeting

cells may not be feasible in all tumors as some drugs cannot diffuse efficiently, certain tumors do not exhibit an EPR effect, and the permeability of vessels may not be the same throughout a single tumor.

1.6 Tumor uptake by active targeting

To overcome the limitations of passive targeting, affinity ligands are attached to the surface of the nanocarriers by a variety of conjugation mechanisms. These ligands bind to specific receptors on the cell surface e.g. antibodies, peptides, aptamers, small molecules. Thus

Nanocarriers will recognize and bind to target cells through ligand receptor interactions. To achieve high specificity, those receptors should be highly expressed on tumor cells, but not on normal cells. The receptors should express homogeneously. Internalization of targeting conjugates can also occur by receptor mediated endocytosis after binding to target cells, facilitating drug release inside the cells. In the receptor-mediated endocytosis mechanism, targeting conjugates bind with their receptors first, followed by plasma membrane enclosure around the ligand–receptor complex to form an endosome. The endosome is transferred to specific organelles, and drugs could be released by acidic pH or enzymes. Nano carrier based drugs currently approved for clinical use and under clinical trials are relatively simple and lack active targeting or triggered drug release components. **(Byrne 2008)** Active targeting of drugs to specific receptors is better therapy than random passive targeting to treat cancer. These receptor usually present on normal cells which are overexpressed in cancer cells. Hence normal cells can be saved from harmful effects of chemotherapeutic drugs. Direct drug towards site of action can kill the cancer cells more effectively with less dose.

The main advantage of receptor targeted drug therapy is reduced side effects and precise action.

Table no 1.2 Targeted drug delivery in cancer treatment

Drug	Mechanism	Indication
Alemtuzumab (Campath1H)	Humanised monoclonal antibody against CD52 antigen (expressed on lymphocytes)	B- cell chronic lymphocytic Leukemia
Bevacizumab (Avastin)	Humanized monoclonal antibody against VEGF(Vascular Endothelial growth factor)	First-line treatment for metastatic colorectal cancer
Bortezomib (Vecade)	Proteasome inhibitor	Multiple myeloma relapsed after two prior treatments
Cetuximab (Erbitux)	Chimeric monoclonal antibody against epidermal growth factor receptor (EGFR)	EGFR-positive, irinotecan-refractory metastatic colorectal carcinoma
Gefitinib (Iressa®)	Tyrosine kinase inhibitor	Third-line treatment of non-small cell lung cancer
Gemtuzumab Mylotarg®)	Cytotoxic antibiotic calicheamicin linked to a humanized monoclonal antibody against CD33 antigen (expressed on myeloid cells)	CD33-positive acute myeloid Leukemia
Imatinib	Inhibitor of Bcr-Abl and c-kit	Chronic myelogenous leukemia

(Gleevec)	tyrosine kinases	and gastrointestinal stromal tumors
Tositumomab (Bexxar)	Radioisotope iodine 131 linked to a chimeric monoclonal antibody against CD20 antigen	Follicular non Hodgkin Lymphoma
Trastuzumab (Herceptin)	Humanized monoclonal antibody against HER2	Metastatic breast cancer expressing HER2

1.7 The selection of right drug for Breast cancer treatment

In the present research work, nanocarriers of two different anticancer drugs were developed. Both the drugs act in breast cancer therapy at different levels viz. at the site of action and on metastasized tumor cells. Thus would be giving duel effect if used as a combination therapy in the treatment of breast cancer.

Tamoxifen citrate is a hydrophobic, selective estrogen receptor modulator (SERM) which acts as anticancer by selectively binding the estrogen receptors on cancer cell to reduce prevalence of cancer and subsequent spread of cancer. It is being administered as tablets orally with 10 mg daily dose. Tamoxifen exhibits numerous biopharmaceutical and toxicological issues such as high susceptibility to liver metabolism and precipitation as free in acidic environment of stomach that contributes to low Tamoxifen bioavailability. Owing to its hydrophobic nature it was encapsulated in solid lipid nanocarrier and further modified to accumulate at cancer cells by active targeting.

2. NEED TO STUDY

2. Need of study :

An increase in the proportion of poorly aqueous soluble drugs that exhibit problems in oral bioavailability is one of the major problems in formulation development. Formulation of lipid-based nanocarriers, such as self-nanoemulsifying drug delivery systems (SNEDDS) and self-microemulsifying drug delivery systems (SMEDDS) have received a lot of attention in recent years as an approach for overcoming poor solubility and oral bioavailability of drugs. SNEDDS are isotropic mixtures of oil, surfactant (HLB>12) and cosurfactant.

These systems are spontaneously emulsified in situ when exposed to gastrointestinal tract (GIT) fluids, forming oil-in-water nanoemulsions with droplet size of 100–250 nm. Self-microemulsifying drug delivery systems (SMEDDS), in comparison, contain a higher content of hydrophilic surfactants and cosurfactants, wherein lipid content is reduced. After the dispersion in aqueous media, SMEDDS form homogeneous, transparent, isotropic, and thermodynamically stable microemulsions, with droplet size of less than 100 nm. However, SNEDDS are normally prepared as liquids that have some disadvantages, e.g., high production costs, low stability and portability, low drug loading, and different dosage forms. Irreversible drug/excipient precipitation may also be problematic.

More importantly, the large quantity of surfactants in the formulations can induce gastrointestinal irritation. In order to avoid these problems, numerous approaches have been established for transforming liquid SMEEDS/SNEEDS into solid dosage forms, such as adsorption onto porous carriers, spray-drying, freeze drying, melting granulation, extrusion, etc. Solid self-emulsifying drug delivery systems combine the advantages of liquid formulation (i.e., enhanced solubility and bioavailability) with those of solid dosage forms (e.g., low production cost, convenience of process control, high stability and reproducibility, and better patient compliance). This chapter provides a review of the current state and recent progress in SNEEDS formulation development, with particular emphasis on the approaches for overcoming their limitations and enabling wider commercial application of these lipid nanocarriers.

3. Aim and Objectives

3. Aim and Objectives

The present project aims at development of nanocarriers for treatment of breast cancer with improved activity and decreased side effects or toxicity.

The aim was to develop, characterize and evaluate anti-cancer drug loaded surface modified drug delivery formulations for targeting Breast cancer.

Finally to assess and compare pharmacokinetic profiles of the developed formulations to establish the clinical benefits of using surface modified antitumor drugs for targeting Breast cancer.

3.1 .Plan of work

- Literature search.
- Procurement of the drugs and other excipients.
- Analytical method development for estimation of drug in formulations.
- Analytical method development for estimation of tamoxifen citrate in plasma during pharmacokinetic studies.
- Forced degradation studies.
- Drug - excipient compatibility studies.
- Fabrication of solid lipid nanoparticles.
- Fabrication of surface modified nanoparticles for target specificity.
- Characterization of anticancer agent loaded nanoparticles.
- *In vivo* pharmacokinetic study of anticancer agent loaded nanoparticles.
- *In vivo* cancer model studies, of the developed nanoparticles, to prove target specificity.
- Fabrication of stealth liposomes using pegylation.
- Characterization of anticancer agent loaded liposomes.
- Stability studies of designed nanoparticles and liposomes preparations.

3A.1 Introduction

Preformulation testing is the first step in the rational development of dosage forms. It can be defined as an investigation of physical and chemical property of a drug substance alone and when combined with excipients. To develop a stable, safe and efficacious formulation, it is essential to take into account drug-excipient interactions as well as the interactions of excipients with each another. Data obtained from preformulation studies plays a significant role in anticipating formulation problems and identifying their solutions and also helps in thoroughly understanding the properties of a drug candidate which forms the basis for

Formulation design **(Fiesse, 1991)**

Selection and procurement of drug:

The two selected drugs for the treatment of estrogen sensitive breast cancer are Tamoxifen citrate (CAS NO.10540-29-1), a selective estrogen receptor modulator and Tamoxifen citrate is obtained as a generous gift sample from Neon Laboratories and Biochem Pharmaceuticals India Pvt. Ltd. Tamoxifen citrate is given as post operative hormonal therapy for breast cancer patients to minimize recurrence of breast cancer. It competitively binds to the estrogen receptors and inhibits the growth of breast cancer cells. It is given as an oral therapy at daily dose of 10-20 mg per day. In the present work we are planning to encapsulate the drug in solid lipid nanoparticles and further surface modify the same using ligand to achieve target specificity to the receptors present on cancer cells. This will decrease the dose, decrease toxicity to other organs, minimize dosing intervals etc.

3A.2 Experimental Work: Characterization of selected drug.

3A.2.1 Colour

Color of the drug and excipients is inspected against dark background.

3A.2.2 Solubility assessment

About 100 mg of the test sample was weighed and transferred to 10 ml test tube. Solubility of drug in different lipids, combination of lipids and combination of lipids and surfactant was checked. 5 ml of lipid or mixture of lipids and surfactant was added to the drug in test tube. The test tube was then covered with aluminum foil and sonicated for 10 min in bath sonicator. 100 mg of drug was added to the solution and kept for 24 hrs. The procedure was continued for 3 days till saturation solubility was achieved. The solution was centrifuged at 5000 rpm and 4 ml of buffer was added to 1 ml of supernatant and absorbance was checked on UV Spectrophotometer at 268 nm wavelength.

3A.2.3 Infrared Spectroscopy (IR)

IR spectrum of the drug, polymer and excipients were obtained by using Infrared spectrophotometer. Approximately 200 mg of KBr , previously dried and cooled was ground into fine powder using mortar 2 mg material was mixed with fine KBr by grinding together. A thin transparent disc was prepared using the mixture and IR spectrum was recorded by placing the disc in the pathlength of IR beam. The IR spectrum was recorded at 4000 cm^{-1} to

400 cm^{-1}using Shimadzu Infrared Spectrophotometer. (Fig 2A.3)

3A.2.4 Powder X Ray Diffraction (XRD)

X ray diffraction study was performed for drug, excipients and combination of drug and excipients to ascertain crystalline behavior of the drug.10 mg sample of materisl was sprinkled and sprayed over vacuum grease coated glass slide to form 0.5 mm thick layer. The samples were exposed to Cu k α radiation (λ = 0.15418 nm) as target filter having voltage/ current of 40KV/30mA. The samples were scanned at speed of 4^{0}/min and the

radiation diffraction angle(2θ)ranged from 5^0 to 70^0 .The study was conducted at TIFR using X ray diffractometer.(Philips Expertpro MPD diffractometer, PAN Analytical Inc Germany) with resolution of 0.001A°) XRD analysis was carried out for the pure drug (Fig 2A.4) and physical mixture of drug with each of the lipids (L2 and L3). (Fig 2A.22)In case of ligand conjugated solid lipid nanoparticles, the compatibility of drug and lipids were checked with the ligand Transferrin.

3A.2.5 Differential scanning colorimetry (DSC)

Identification of compounds was confirmed using DSC analysis. DSC analysis of Tamoxifen citrate, Vinorelbine tartarate, was performed on S11 nanotechnology (Seiko) DSC 6220. DSC analysis measures the heat loss or heat gain resulting from physical or chemical changes within a sample as a function of temperature. DSC analysis was carried out for pure drug, bulk lipids and the two lyophilized solid lipid nanoparticles formulations and modified nanoformulations to study the polymorphic transitions exhibited by them to ascertain the compatibility. 3-5 mg of lyophilized sample was weighed in standard aluminum pans and subjected to heating under nitrogen purging at the rate of 10^0C per minute in the temperature range of 25-300°C.The thermograms were recorded. (Fig 2A.2)

3A.2.6 Identification by UV spectrophotometer

Jasco UV spectrophotometer was used to get the UV spectrum of the drugs. 10 ml solution of drug with 20 ppm (0.002% w/v) solution of active in methanol was prepared in methanol and scanned in the UV range of 200 – 400 nm to understand λ_{max} of Tamoxifen citrate.(Fig 2A.5)

3A.2.7 Identification by HPLC

HPLC analysis of 1 ppm Tamoxifen citrate solution in methanol was done using methanol:

Water (90:10) as mobile phase. (Fig 2A.6)

3A.2.8 Forced Degradation studies

For forced degradation studies of the drug Tamoxifen citrate, the compound was heated with solutions of 0.1 N NaOH, 0.1 N HCl, 30% H_2O_2 w/v and double distilled water for 1 hour, analysed by HPLC for any degradation which will be denoted by shift in the peak.

3A.3 Results and discussion

3A.3.1Characterization of hormonal agent: Tamoxifen citrate

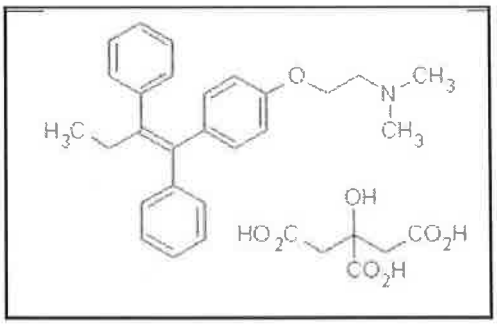


Fig 2A.1: Structure of Tamoxifen citrate

Tamoxifen citrate is official in Indian Pharmacopoeia, European Pharmacopoeia 2016 and USP 29. It is evaluated for the parameters given in Table 2A.1 and found to be complying with Indian Pharmacopoeial standards.

3A.3.2 Identification of Tamoxifen citrate

Tamoxifen citrate was characterized for its functional groups through IR Spectrophotometry (Fig 2A.3)The FTIR spectrum of drug shows peak at 3027 cm^{-1} (C-H sp3)and 1731cm^{-1} is characteristic of C=O stretching and at 1438cm^{-1} shows characteristic –OH bending. Two peaks at 1240 (C–O ether) and 1280 show carboxylate ion stretching. Characteristic peak at

1599 (C=C Alkanes), 1506 and 1476 show –C=C- stretching of aromatic rings.

Powder X ray diffraction pattern reveals the nature of the compound. Tamoxifen citrate showed characteristic and sharp peaks at 2θ values of 19 and 21 indicative of highly crystalline nature of drug. (Fig 2A.4)

Tamoxifen citrate is an unsaturated molecule with the presence of electronegative atoms like nitrogen and oxygen. The carbon-carbon double bonds (–C=C), carbonyl (–C=O) groups and hydroxyl group (-OH) involved in its structure act as chromophores and absorb in the ultra- violet region of the electromagnetic spectrum **(Mahajan SS, 2010.)** As per the Indian Pharmacopoeia, a 20 ppm (0.002% w/v) solution of selected drug in methanol shows absorption maxima at 237 and 275 nm when scanned in the UV range of 200 - 360nm. It is matching with absorbance maxima of our sample at 237.2 and 275.2 nm. This confirms the purity of our sample drug. (Fig 2A.5)

The Differential scanning colorimetry shows a sharp exotherm peak at 148.46 °characteristic

of the model drug confirming the crystalline structure of the drug. (Fig 2A.2)

An HPLC method was developed for identification of compound with further determination of its purity. The chromatographic conditions for the study were wavelength 268 nm and mobile phase, methanol: water (90:10) at pH 5. This gives sharp peak at 5.1 min (Fig 2A.6)

Table 2A.1: Evaluation of Tamoxifen citrate

Sr. No.	Test	Specifications	Results
1	Description	White or almost white crystalline powder.	White crystalline powder
2	Solubility	Slightly soluble in water, acetone. Soluble in methanol.	Complies
3	Identification	I. R. Absorption*	Complies
		U.V. Absorption**	Complies
		TLC	Complies
		Chemical test	Complies
4	E-Isomer and related substances (By HPLC)	The area of peak is not greater than 0.3 times that of the principal peak in the chromatogram	Complies
5	Heavy metals	N.M.T. 10 ppm	< 10 ppm
6	Sulphated ash	N.M.T. 0.1%	0.088 % w/w
7	Residue on ignition	N.M.T 0.2%	0.60 % w/w

8	Related substances (By HPLC)	The area of peak less than 0.05 times the area of the principal peak in the chromatogram	Complies
9	Impurities A,B,C,D,E,F,G	N.M.T 0.3%	Complies
10	Sum of impurities other than A	N.M.T 0.5%	Complies
11	Loss on drying	N.M.T 0.5%	0.339 % w/w
12	Assay	N.L.T 99.0% and N.M.T. 101 on dried basis (As per I.P.)	99.88% on dried basis.

Storage: Stored at room temperature in amber coloured light resistant container.

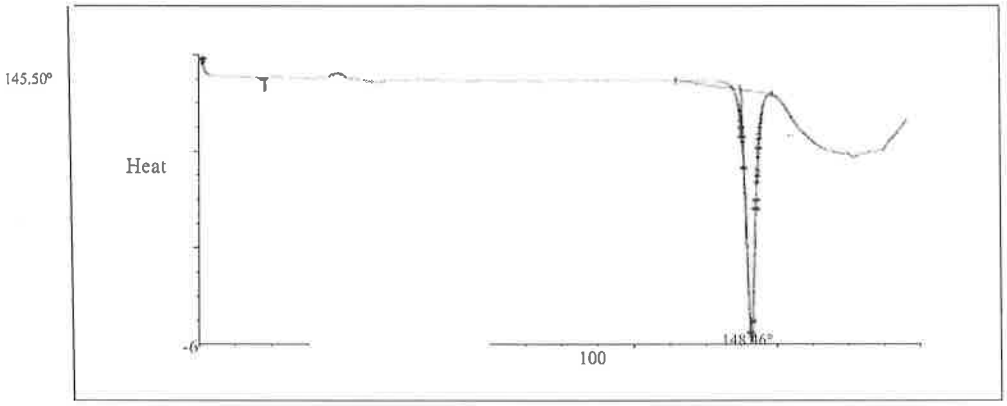


Fig 2A.2 DSC thermogram of Tamoxifen citrate

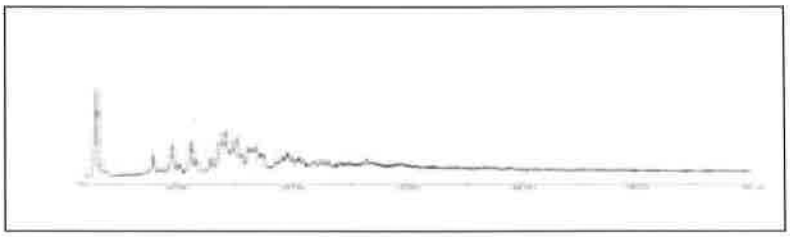


Fig 2A.3 XRD of Tamoxi fen citrate

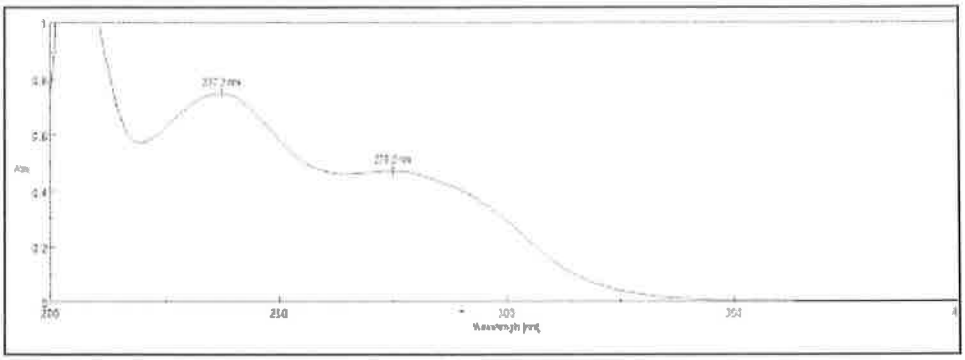


Fig 2A.4 : UV spectrum of Tamoxifen citrate

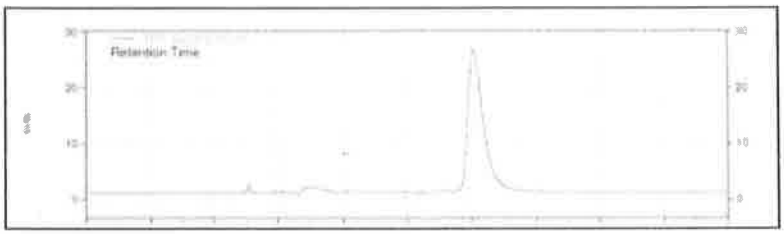
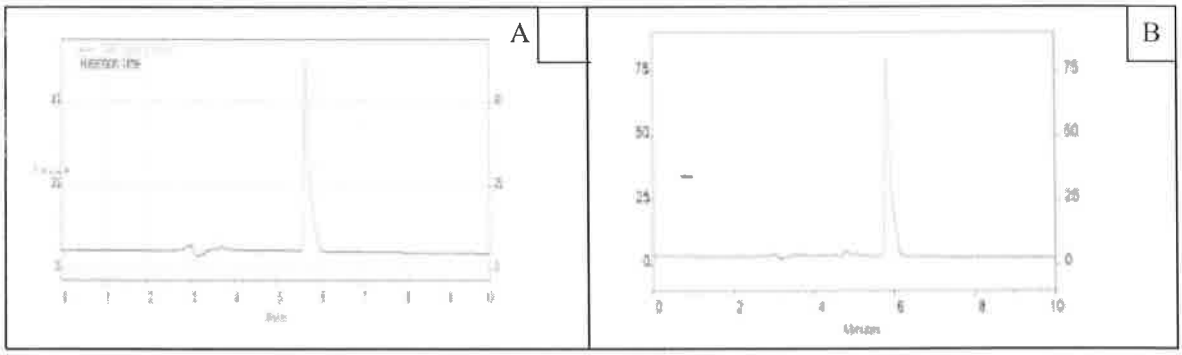


Fig 2A.5 : HPLC of Tamoxifen citrate

Forced degradation studies



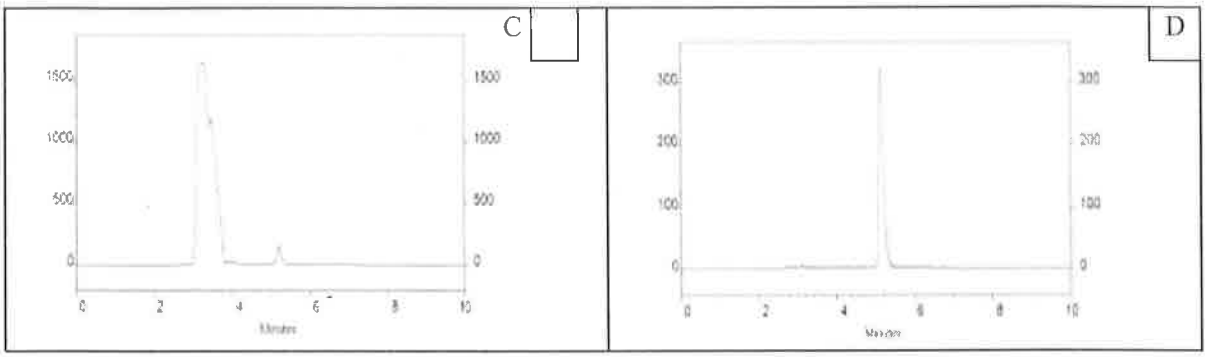


Fig 2A.6 Degradation of drug in (A)NaOH, (B)HCl, (C)H_2O_2 and (D)water Forced degradation studies were carried out to determine the stability of the drug in the presence of acidic , alkaline and oxidizing agents. The degradation of drug with 0.1 N NaOH was carried out and the solution was analysed by HPLC with UV detector at 265nm. The drug shows a shift of peak at higher concentration. In acidic condition, there is a shift in the RT but percent drug content remains unchanged. When exposed to H_2O_2, the drug shows degradation with drug peak at 3 min. In wet and dry hydrolysis no degradation of Tamoxifen citrate is seen. The drug was found to be stable in acidic or alkaline conditions but slight shift in the RT was recorded due to change in pH conditions. It was found to be unstable in the presence of hydrogen peroxide. (The method developed for detection of Tamoxifen citrate by HPLC is explained in Chapter 3)

Materials used for preparation of solid lipid nanoparticles are listed in Table 2A.2

Table 2A.2: List of Ingredients

Sr. No	List of Chemicals	Source
1	Tamoxifen citrate	Neon Laboratories Ltd, Andheri, Mumbai, India.(Gift sample)
2	Solutol HS, Cremophor EL, Poloxamer 188	BASF Chemical India Pvt. Ltd., Turbhe , Mumbai, India.(Gift sample)
3	Glyceryl behenate, Glyceryl palmitostearate, Tristearin	Gattefosse, France. (Gift samples)
4	Tween 80, Span 80, Sodium hydroxide pellets, Potassium dihydrogen orthophosphate,	S. D. fine Chemicals India Pvt. Ltd., India.

5	Stearic acid	S. D. Fine Chemicals India Pvt. Ltd, India.
6	Cetyl palmitate	Bajaj Organics Pvt Ltd, India.
7	Dynasan 118,114	IOI Oleochemicals , hamburg. (Gift sample)
8	Transferrin	M.P. Biologicals, India.

3A.3.3 Selection and procurement of excipients:

The model drug is an antiestrogen, nonsteroidal derivative of triphenylethylene. It is a BCS Class II drug having low solubility and a high lop P value between 6 and 7 which indicates the fact that it is a highly hydrophobic compound and thus a good candidate for lipid based carrier systems.

The solid lipids used for development of nanoparticles were selected from a range of triglycerides such as tri-stearin (m.p.68-75°C), trimyristine (m.p.55-58°C), Glyceryl behenate (m.p.65-77°C), glyceryl palmitostearate (m.p.52-55°C) , partial glycerides such as Glyceryl stearate or Imwitor 900 (m.p.54-64°C), fatty acids e.g. stearic acid (m.p.67-72°C) and waxes, cetyl palmitate (m.p.54°C).The selection of lipids is based on solubility of drug in lipid and combination of lipids and surfactants. More the solubility more is the entrapment of drug in SLN. Fig 2A.7 to 2A.11 show the comparison of solubility of tamoxifen in the mentioned lipids which comprises the oil phase (expressed in mg/ml), in surfactant and in combination of lipids and surfactant. Each bar represents the mean of 3 samples ± SD **(Wissing , 2004)**

Fig 2A.8 Solubility of drug in lipids

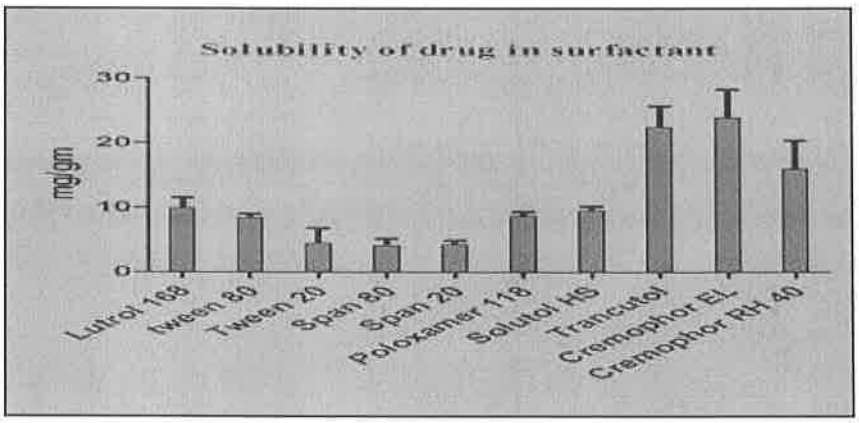


Fig 2A.9 Solubility of dug in surfactant

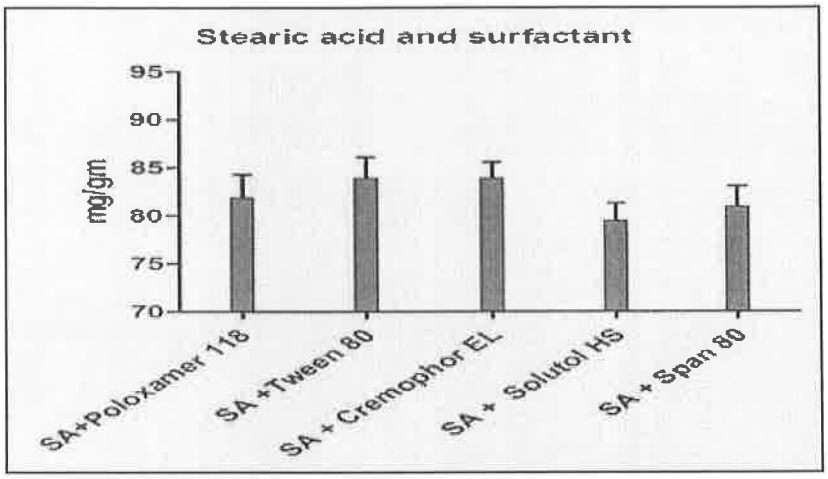


Fig 2A.10 Solubility of drug in stearic acid and surfactant

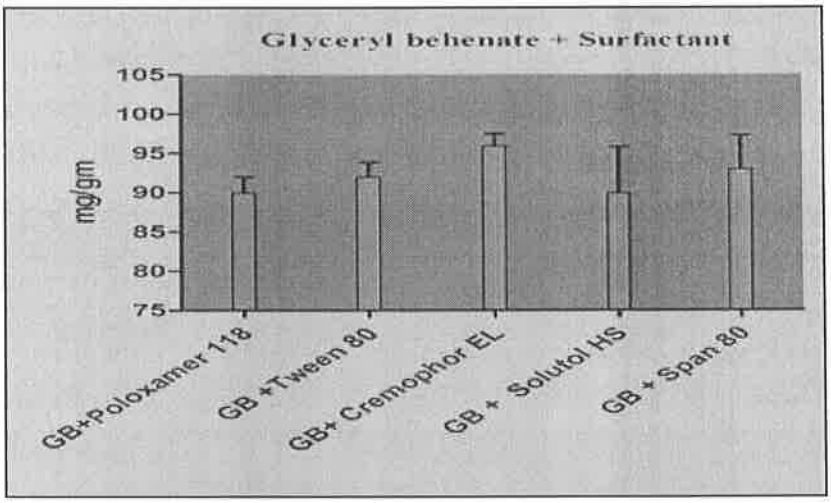


Fig 2A.11 Solubility of drug in Glyceryl behenate and surfactant

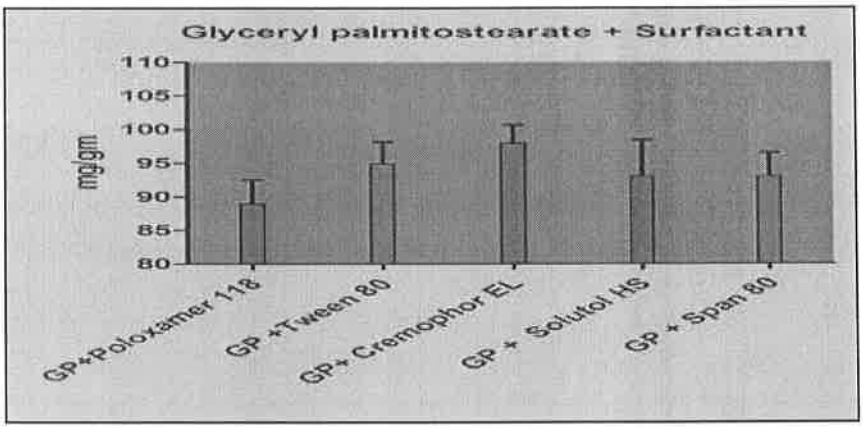


Fig 2A.12 Solubility of drug in Glyceryl palmitostearate and surfactant

3A.3.4 Lipids (Rowe RC, 2005)

Tamoxifen citrate is very less soluble or almost insoluble in Trimyristine, cetyl palmitate and Imwitor900 and showed more solubility in stearic acid, glyceryl behenate and glyceryl palmitostearate. The selection of the oily phase depends on the drug solubility. Lipids are used as carriers for the drug. The entrapment efficiency of the active in the SLN depends on the affinity between the active and lipid. Tamoxifen citrate has high log P value. When solubility was determined, direct correlationship was seen between drug solubility and carbon chain length. Drug showed solubility in the fatty acid with more than C 16 carbon atoms and also in the mixture of triglycerides. **(Monteagudo , 2012)(Shah, 2016)**

Solubility of TMX in the five co-surfactants and in three selected lipids is depicted in Fig. 2A.8 to Fig 2A.12 The most important factor that contributes to the final solubilizing capacity in poorly water soluble drugs is the solubility in the lipid internal phase. On the basis of solubility and melting point, following lipids were selected for the preparation of solid lipid nanoparticles.

Stearic acid:

It is a mixture of stearic acid ($C_{18}H_{36}O_2$) and palmitic acid ($C_{18}H_{32}O_2$), (Fig 2A.13) containing not less than 40.0% stearic acid and sum of the two acids is not less than 90.0%. Its chemical name is octadecanoic acid (CAS Registry number 57-11-4). Stearic acid is white or faint yellow colored glossy crystalline solid with 53°C melting point and specific gravity 0.53-0.82g/cm^3. It is used as an emulsifying and solubilizing agent in various pharmaceutical dosage forms. It shows acid value and saponification value as 208 and 209 It is freely soluble in benzene, carbon tetrachloride , chloroform, ether and soluble in ethanol

(95%), hexane, propylene glycol, practically insoluble in water.

Storage: Stored at room temperature in a tightly fitted light resistant container.

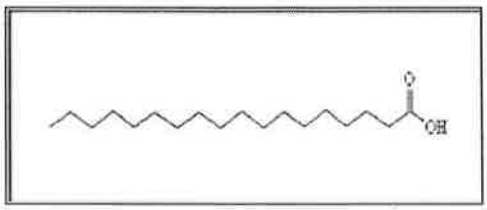


Fig. 2A.13 : Structure of stearic acid(Lipid1)

Glyceryl behenate:

Glyceryl behenate is a mixture of glycerides of fatty acids mainly behenic acid (Fig 2A.14). The chemical name of glyceryl behenate is docasanoic ester with glycerine. It shows acid value less than 4mg KOH / gm and saponification value 145-165mg of KOH/gm. It shows free glycerol content 0.8% (≤ 1.0%) and water content 0.03% (≤ 1.0%) Glyceryl behenate is used in the preparation of oral pharmaceuticals, cosmetics and has been widely investigated in the preparation of aqueous colloidal dispersions such as solid lipid microparticles, and nanostructured lipid carriers for encapsulation of lipophilic drugs.

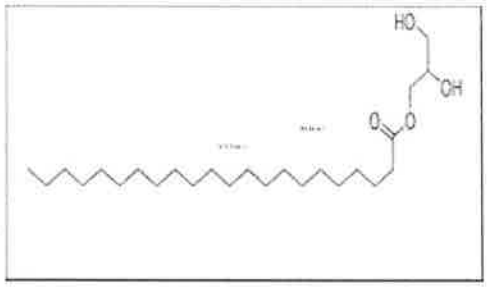


Fig 2A.14 Structure of glyceryl behenate (Lipid 2)

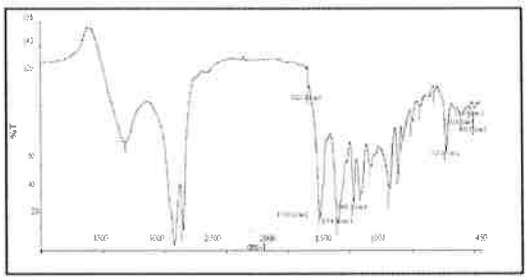


Fig 2A.15 IR spectrum of glyceryl behenate

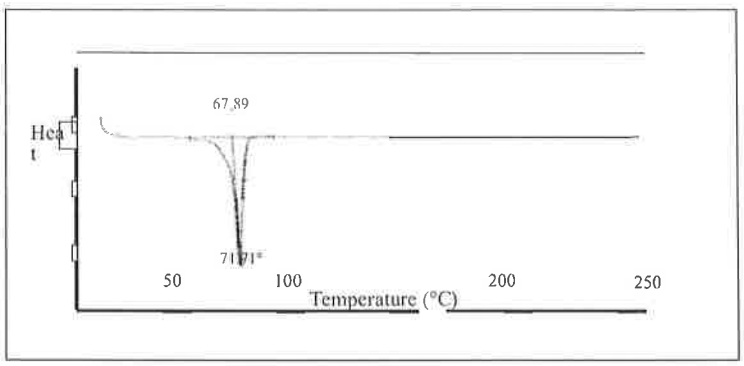


Fig 2A.16 DSC thermogram of glyceryl behenate

Storage: Stored at room temperature in a tightly fitted light resistant container.

Glyceryl palmitostearate:

Glyceryl palmitostearate (CAS No. 8067-32-1)is a mixture of mono-, di-, and triglycerides of C_{16} and C_{18} fatty acid. Its chemical name is Octadecanoic acid-2, 3-dihydroxypropyl ester with glycerine. Glyceryl pamlitostearate is used as a biodegradable material, release modifying agent and in the preparation of microparticulate systems.

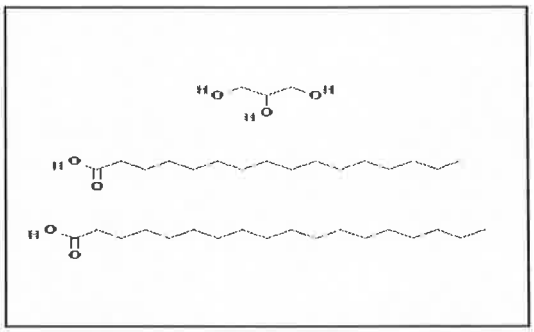


Fig 2A.17 Structure of glyceryl palmitostearate (Lipid 3)

Glyceryl behenate:

Glyceryl behenate is a mixture of glycerides of fatty acids mainly behenic acid (Fig 2A.14). The chemical name of glyceryl behenate is docasanoic ester with glycerine. It shows acid value less than 4mg KOH / gm and saponification value 145-165mg of KOH/gm. It shows free glycerol content 0.8% (≤ 1.0%) and water content 0.03% (≤ 1.0%) Glyceryl behenate is used in the preparation of oral pharmaceuticals, cosmetics and has been widely investigated in the preparation of aqueous colloidal dispersions such as solid lipid microparticles, and nanostructured lipid carriers for encapsulation of lipophilic drugs.

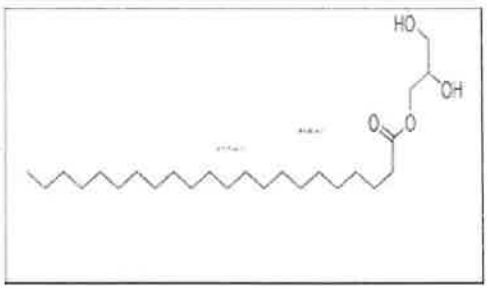


Fig 2A.14 Structure of glyceryl behenate (Lipid 2)

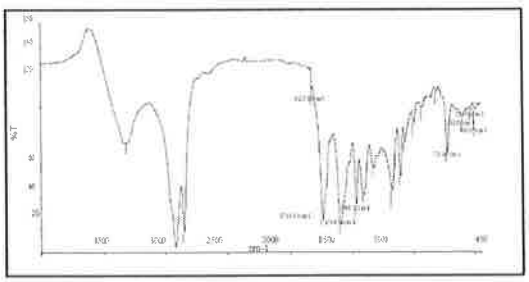


Fig 2A.15 IR spectrum of glyceryl behenate

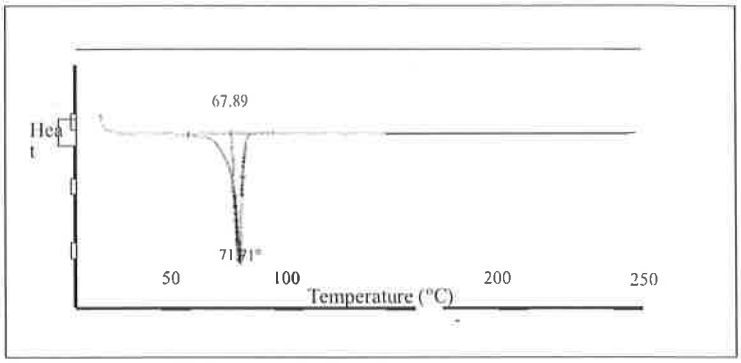


Fig 2A.16 DSC thermogram of glyceryl behenate

Storage: Stored at room temperature in a tightly fitted light resistant container.

Glyceryl palmitostearate:

Glyceryl palmitostearate (CAS No. 8067-32-1)is a mixture of mono-, di-, and triglycerides of C_{16} and C_{18} fatty acid. Its chemical name is Octadecanoic acid-2, 3-dihydroxypropyl ester with glycerine. Glyceryl pamlitostearate is used as a biodegradable material, release modifying agent and in the preparation of microparticulate systems.

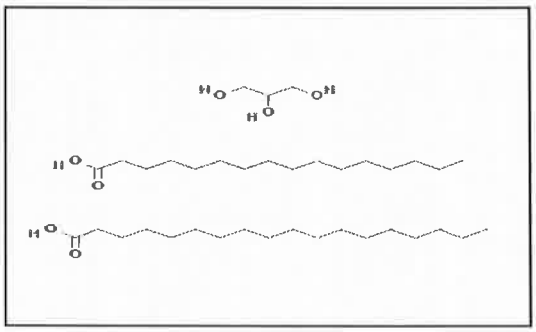
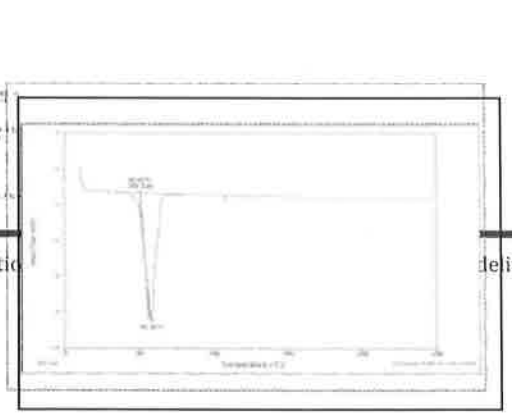


Fig 2A.17 Structure of glyceryl palmitostearate (Lipid 3)



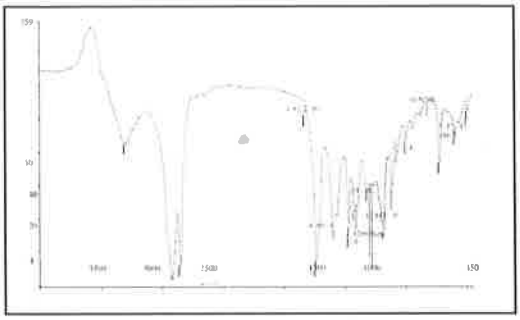


Fig 2A.18 DSC thermogram of Glyceryl palmitostearate

Fig 2A.19 IR Spectrum of Glyceryl palmitostearate

Surfactants (Rowe, 2009)

Various surfactants were screened for the preparation of nanoparticulate formulation depending upon their category, HLB value and applications. All other materials used for formulation development and analysis were of analytical grade and standardized as per pharmacopoeial specifications for the development of a stable dosage form. The surfactants screened are non-ionic in nature and act as surfactants as well as solubilizing and emulsifying agents. Suitable combination of hydrophilic and hydrophobic surfactants helps in stabilizing the solid lipid nanoparticulate formulation. All other ingredients used in the study were of analytical grade and Generally recognized as safe (GRAS).

The highest solubilizing capacity was achieved when combination of lipids with tween 80 and cremophor EL were used. Both compounds were selected to act as coemulsifiers in the forthcoming screening. However, TMX showed a considerable solubility in solutol HS and poloxamer 188. (Fig 2A.9- Fig 2A.12)

Table 2A.3: Selection of surfactants

Name	Tween 80	Poloxamer 188	Span 80	Solutol HS	Cremophor EL
Chemical Name	Polyoxy-ethylene20 Sorbitan Monooleate	α-Hydro-ω-hydroxypoly-(oxyethylene)-poly-(oxypropylene) poly-(oxyethylene) blockcopolymer	Sorbitan mono-9-octadecanoate	2Hydroxyethyl-12-hydroxy octadecanoate	Polyethoxylated-35- castor oil
CAS No	9005-65-6	9003-11-6	1338-43-8	70142-34-6	61791-12-6
Category	Non-ionic surfactant	Non-ionic emulsifying and solubilizing agent	Non-ionic surfactant	Non-ionic surfactant and solubilizing agent	Non-ionic emulsifying & solubilizing agent
HLB value	15	29	4.3	14-16	12-4

Storage: Stored at room temperature in a tightly fitted light resistant container.

Transferrin

Transferrinis a large iron carrier protein molecule with molecular weight 78- 81 KDa (CAS No. 11096-37-0) present in the human serum. The ingredient procured from M.P.Biologicals has to be stored at 2-8°C. Since it is lyophilised Transferrin being a

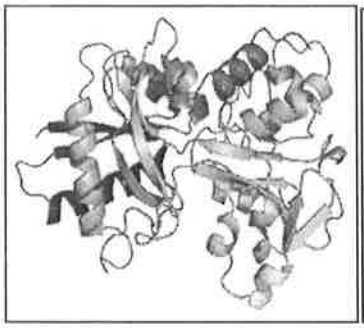


Fig 2A.20 Structure of TrasferrinTrasferrin

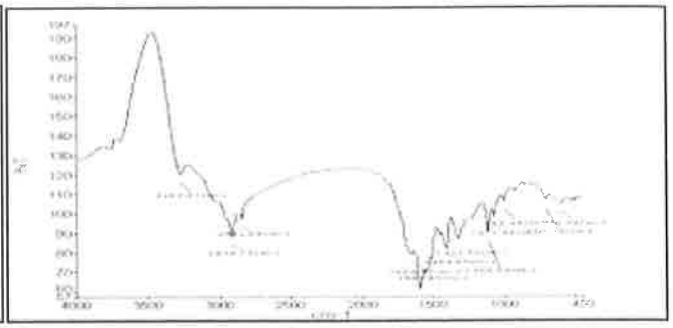
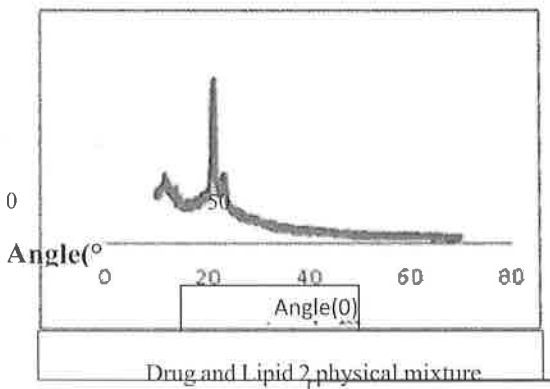


Fig 2A.21 IR spectrum of

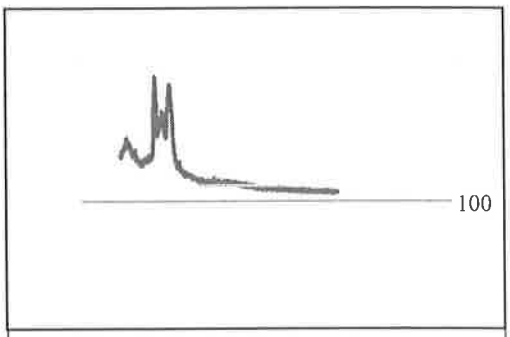
XRD Studies

Sharp peaks were obtained at 9.256, 11.418, 13.571, 15.968, 17.495, 21.071° (2θ) of pure drug **(Hiremath,2011)**. The physical mixtures of Drug and transferrin, Drug and lipid 2, Drug and lipid 3, drug transferrin and lipid 2 and drug transferrin and lipid 3 showed almost similar pattern of peaks without much interference by lipid or transferrin. Crystalline materials display many diffractions bands, whereas amorphous compounds present show more or less regular baseline.

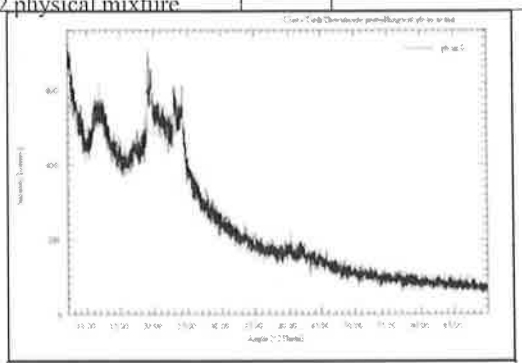
Compatibility studies by X-Ray Diffraction



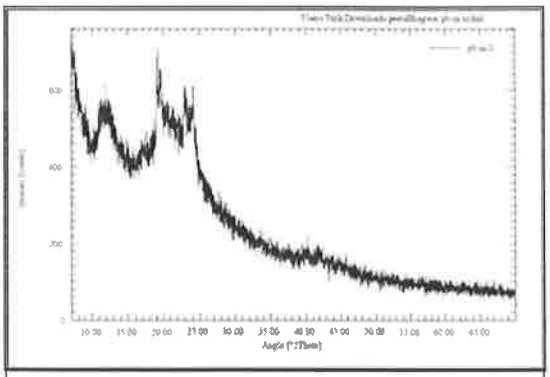
Drug and Lipid 2 physical mixture



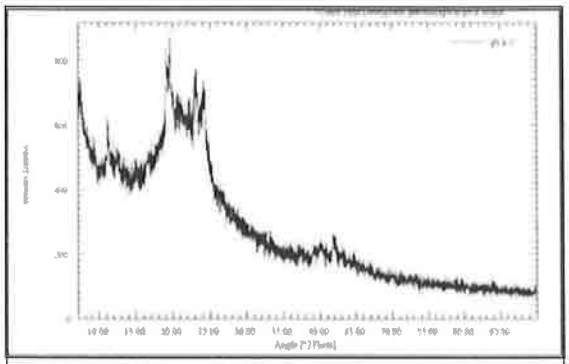
Drug and lipid 3 Physical mixture



Transferrin and Lipid 2 physical mixture



Drug, Lipid 2 and Transferrin physical



Drug, Lipid 3 and Transferrin physical

Fig 2A.22 XRD studies of physical mixture combination of drug, lipids and transferrin

3A.4 Conclusion

Standardization of selected drug and other formulation excipients were carried out using UV analysis, DSC and XRD studies. The lipids selected to be used to prepare solid lipid nanoparticles, based on the study of physicochemical properties of the drug and lipids. The lipids and surfactants were selected based on the solubility and stability characteristics. Drug excipient compatibility studies confirmed suitability of their use in the formulation.SLN of Tamoxifen citrate were developed by using lipids stearic acid, glyceryl palmitostearate and

large protein molecule shows many amino acids C and N terminal for conjugation with lipids. It is used as a carrier

for Tamoxifen by actively binding to the lipids encapsulating the drug.

glycerylbehenate.

4. Review of Literature

Literature review

Breast cancer represents a major ongoing public health problem as the most common non-cutaneous malignancy among U.S. women *(Grobmyer et al. 2012)*. It is the second leading cancer worldwide after lung cancer, about one fifth of the cancer patient suffers from the breast cancer, and it is the leading cause of cancer death in women. The global burden of breast cancer exceeds all other cancers and the incidence rates of breast cancer are increasing *(Jemal et al. 2010)*.

Over the last several decades the incidence of breast cancer has increased globally *(Hortobagyi et al. 2005; Anderson et al. 2008; Porter et al. 2008)*. The incidence rate of breast cancer greatly increased in Asian countries *(Green et al. 2008)*. In Asia, breast cancer incidence peak shows among women in their forties *(Agarwal et al. 2007)*, whereas in the United States and Europe, it shows among women in their sixties. In India about 50% of all breast cancer patients belong to premenopausal *(Agarwal et al. 2007)*.

Tamoxifen was approved by the FDA in 1977 for the treatment of metastatic breast cancer. The recommended daily dose of Tamoxifen is 20-40 mg (*http://www.pdrhealth.com/drugs/tamoxifen-citrate./)*

Tamoxifen is widely used in the treatment of breast cancer and as a preventative agent after its surgery *(Owens et al. 2002)*. Tamoxifen has both a pro- and antiestrogenic effect on the nuclear estrogen receptors (ER), modifies the function of the plasma membrane, the microsomes, the proliferative and antiproliferative factors as TGF or cyclins, et cetera *(Kangas et al. 1986; Noguchi et al. 1993; Maas et al. 1995; Clemons et al. 2002)*. As a "selective ER modulator" (SERM), Tamoxifen significantly influences the activity of ER *(Lonard et al. 2002)*.

It has been used in the management of breast cancer for over 30 years. Since its introduction for the treatment of advanced breast cancer, its indications have increased to include the treatment of early breast cancer, ductal carcinoma in situ, and more recently for breast cancer chemoprevention *(Clemons et al. 2002)*. Tamoxifen has a good tolerability profile and moreover, unlike many other endocrine therapies, it is efficacious in both pre- and postmenopausal women. It is the combination of efficacy and tolerability that allows Tamoxifen to maintain its position as the hormonal treatment of choice for most patients with oestrogen-receptor positive breast cancer.

Cho et al. *(2008)* and Chidambaram et al. *(2011)* have reported in their review article that cancer nanotherapeutics are rapidly progressing and are being implemented to solve several limitations of conventional drug delivery systems such as nonspecific biodistribution and targeting, lack of water solubility, poor oral bioavailability, low therapeutic indices and multidrug resistance. To improve the biodistribution of cancer drugs, nanoparticles have been designed for optimal size and surface characteristics to increase their circulation time in the bloodstream. They are also able to carry their loaded active drugs to cancer cells by selectively using the unique pathophysiology of tumors, such as their enhanced permeability and retention effect and the tumor microenvironment. In addition to this passive targeting mechanism, active targeting strategies using ligands or antibodies directed against selected tumor targets amplify the specificity of these therapeutic nanoparticles. Drug resistance, another obstacle that impedes the efficacy of both molecularly targeted and conventional chemotherapeutic agents, might also be overcome, or at least reduced, using nanoparticles. Nanoparticles have the ability to accumulate in cells without being recognized by P-glycoprotein, one of the main mediators of multi drug resistance, resulting in the increased intracellular concentration of drugs. Multifunctional and multiplex nanoparticles are now being actively investigated and are on the horizon as the next generation of nanoparticles, facilitating personalized and tailored cancer treatment.

Various chemotherapeutic agents are used for the treatment of cancer which may be used alone or in combination with other forms of therapy. Multidrug resistance is a challenge in cancer chemotherapy which can be significantly reversed by delivering drug through solid lipid nanoparticles, polymeric nanoparticles, mesoporous silica nanoparticles, nanoparticulated chemosensitizer, nanoparticluated poloxamer and magnetic nanoparticles. Hydrophobic nature of chemotherapeutics leads to poor aqueous solubility and low bioavailability which can be overcome by nanocrystals, albumin based nanoparticles, liposomal formulation, polymeric micelles, cyclodextrin and chitosan based nanoparticles.

Nanotechnology has been firmly focusing in to the area of drug delivery. Drug delivery through the nanotechnology are continuously improved and maximize therapeutic activity and minimize undesirable side-effects and toxicities. Safari et al. *(2014)* in their review article described the advanced drug delivery systems based on micelles, polymeric nanoparticles, and dendrimers. Polymeric nanoparticles, carbon nanotubes and many others demonstrate a broad variety of useful properties.

Engineered nano materials ranges between 1 and 100 nm have novel optical, electronic, and structural properties that are not available either in individual molecules or bulk solids such as, clusters of atoms, molecules, and molecular fragments into small particles. The concept of nanoscale devices has led to the development of biodegradable self-assembled nanoparticles, which are being engineered for the targeted delivery of anticancer drugs and imaging contrast agents. Nanoconstructs should serve as customizable, targeted drug delivery vehicles capable of delivering large doses of chemotherapeutic agents or therapeutic genes into malignant cells while sparing healthy cells. Such „„smart"" multifunctional nanodevices hold out the possibility of radically changing the practice of oncology, allowing easy detection and then followed by effective targeted therapeutics at the earliest stages of the disease. Sinha et al. *(2006)* in their article briefly discussed about the use of bioconjugated nanoparticles for the delivery and targeting of anticancer drugs.

Researchers have are developed polymeric nanoparticles to deliver various kinds of anticancer drugs such as docetaxel, paclitaxel, doxorubicine etc. to the specific site of action to reduce the side-effects *(Pradhan et al. 2013; Koopaei et al. 2014; Gupta et al. 2014; Guo et al. 2015).*

A variety of methods have been used to prepare polymeric nanoparticles. Those methods include solvent evaporation, nanoprecipitation and multiple emulsifications etc *(Sinha et al. 2013; Rudra et al. 2010; Sahana et al. 2010; Astet et al. 2006; Horn et al. 2001; Birnbaum et al. 2000).*

Memisoglu-Bilensoy et al. *(2005)* developed nanospheres and nanocapsules of β-CDC6, (amphiphilic β-cyclodextrin modified on the secondary face with 6C aliphatic esters) were prepared by nanoprecipitation technique directly from inclusion complexes of TMX and β-CDC6 (1:1 molar ratio). Blank and loaded nanospheres and nanocapsules were characterized by particle size distribution, zeta potential, drug loading and in vitro drug release. Particle sizes were between 250 nm and 300 nm for different formulations of nanospheres and nanocapsules. Zeta potential which was around −18 mV for blank particles and between +12 and +15 mV for Tamoxifen-loaded particles. Average entrapped drug quantity was found to be around 150 μg/mL for particles prepared from inclusion complexes and this was double the loading value for conventionally prepared particles. Pre-loaded formulations showed a significantly slower drug release profile extended up to 6 h while formulations loaded conventionally displayed rapid and complete drug release within an hour. Cytotoxic efficacy of TMX loaded nanospheres and nanocapsules were determined against MCF-7 cells and TMX incorporated in amphiphilic β-cyclodextrin nanoparticles was found to be cytotoxic and effective against this cell line.

Ravikumara et al. *(2011)* reported in their study they had developed TMX-loaded chitosan nanoparticles (TMXL-ChtNPs) and TMX-free chitosan nanoparticles (TMXF-ChtNPs) were prepared by an ionic gelation (IG) method. The physicochemical properties of the nanoparticles were analyzed for particle size, zeta potential, and other characteristics using photon correlation spectroscopy (PCS), zeta phase analysis light scattering (PALS), scanning electron microscopy (SEM), Fourier transform infrared (FTIR), and differential scanning calorimetry (DSC). The variation in particle size was assessed by changing the concentration of chitosan, pentasodium tripolyphosphate (TPP), and the pH of the solution. The optimized TMXL-ChtNPs showed mean diameter of 187 nm, polydispersity of 0.125, and zeta-potential of +19.1 mV. The encapsulation efficiency (EE) of TMX increased at higher concentrations, and release of TMX from the chitosan matrix displayed controlled biphasic behavior. Those TMXL-ChtNPs tested for chemosensitivity showed dose- and time-dependent antiproliferative activity of TMX. Further, TMXL-ChtNPs were found to be hemocompatible with human red blood cells (RBCs) and safe by in vitro cytotoxicity tests, suggesting that they offer promise as drug delivery systems in therapy.

Patel et al. *(2011)* formulated and evaluated chitosan nanoparticles of TMX for cancer therapy. Nanoparticles of TMX were prepared with chitosan using ionic gelation method. The concentration of the polymer chitosan was selected based on the results on preliminary screening. The nanoparticles prepared were evaluated for morphology, drug loading efficiency, in vitro drug release and in vitro anticancer activities. The particle shape and morphology of the prepared TMX nanoparticles was determined by SEM analysis. The amount of TMX entrapment in the nanoparticles was calculated by the difference between the total amount of drug added to the nanoparticle and the amount of non-entrapped drug remaining in the aqueous supernatant. A diffusion cell was used to monitor TMX release from the nanoparticles. The formulations FM-1 showed good drug release from the polymer. The percentages of cumulative drug release after 3, 4, 5, 6, 7 and 8 hours were 40.54, 48.68, 56.26, 65.84, 71.42 and 78.03% respectively.

Sarmah et al. *(2012)* studied the antiproliferative action of TMX, on a Jurkat (human T-cell leukemia cell line), by comparing the free drug and TMX loaded guar gum nanoparticles. They have developed a new formulation containing chemically cross-linked guar gum nanoparticles (GG NPs) loaded with TMX by single step (oil in water) emulsion and in-situ polymer cross linking technique was employed to prepare spherical and smooth surfaced nanoparticles in the size range of 200-300nm. Nanoparticle size and shape was confirmed by transmission electron microscope (TEM) analysis. Cytotoxicity on Jurkat (human T-cell leukemia) cell lines as

determined by cell growth inhibition after 48 h of incubation has indicated that TMX loaded guar gum nanoparticles were as efficient as the free drug when applied to the cancer cells. From this study the researchers further concluded that the crosslinked guar gum nanoparticles loaded with TMX exhibited sustained release of the drug and delayed apoptosis over a long period of time making it suitable for cancer treatment.

Sarmah et al. *(2014)* have prepared, characterized and studied the biodistribution of TMX loaded cross-linked guar gum (GG) nanoparticles. Nanoparticles were prepared via a single step emulsion process and particle size was evaluated. The extent of tissue distribution and retention following oral administration of TMX loaded GG Nanoparticles and TMX tablet in female albino mice was analyzed over a period of 48 hours. Till 48 hours, the particles remained detectable in both mammary and ovary tissue (estrogen receptors). Uptake and retention of TMX from nanoparticles and tablets in mammary gland and ovary tissue changed with time. Results showed that the uptake and retention of nanoparticles were more in the mammary gland between 24 - 48 hours (11.2% at 24 h; 4.65% at 48 h). As mammary gland is the target organ in breast cancer therapy, it may be concluded that the cross-linked GG nanoparticles are capable of releasing drug at the target and minimize the uptake and retention in non-target tissue, the ovary (7.98% at 24 h; 1.9% at 48 h). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with time were measured. Further they reported that no abnormal changes in the liver enzymes were observed. GG nanoparticles may be used as a drug carrier system for treating cancer.

Avgoustakis *(2004)* has reported that pegaylated biodegradable nanoparticles made of PLA, PLGA evaded phagocytosis and thus extended the range of sites within the body where the nanoparticles reached.

Sahana et al. *(2010)* prepared TMX nanoparticle, a loaded PLGA (85:15) by multiple emulsification (w/o/w) and solvent evaporation technique. Different parameters such as drug-polymer ratio, polyvinyl alcohol concentrations, and homogenizing speeds were varied at different stages of preparation to optimize the desired size and release profile of drug. The characterization of particle morphology and shape was performed by field emission scanning electron microscope (FE-SEM) and particle size distribution patterns were studied by direct light scattering method using zeta sizer. In vitro drug release study showed that release profile of TMX from biodegradable nanoparticles varied due to the change in speed of centrifugation for separation. Drug loading efficiency varied from 18.60% to 71.98%. The FE-SEM study showed that biodegradable nanoparticles were smooth and spherical in shape. The stability studies of

TMX in the experimental nanoparticles showed the structural integrity of TMX in PLGA nanoparticles up to 60°C in the tested temperatures. So by performing the various in vitro characterization studies they have confirmed that nanoparticles containing TMX could be useful for the controlled delivery of the drug for a prolonged period.

Cirpanli et al. *(2010)* were formulated Tamoxifen in nanoparticulate carrier systems in the form of PLGA, poly-- caprolactone (PCL) and chitosan nanoparticles. The PLGA and PCL nanoparticles were prepared by a nanoprecipitation technique whereas the chitosan nanoparticles were prepared by the ionic gelation method. Mean particle sizes were under 260 nm for PLGA and PCL nanoparticles and around 400 nm for chitosan nanoparticles. Polydispersity indices were less than 0.4 for all formulations. Zeta potential values were positive for TAM loaded nanoparticles because of the positive charge of the drug. Drug loading values were significantly higher for PCL nanoparticles when compared to PLGA and chitosan nanoparticles. All nanoparticle formulations exhibited controlled release properties. These results indicate that TAM loaded PLGA; PCL and chitosan nanoparticles may provide promising carrier systems for tumor targeting.

5. EXPERIMENTAL METHODS

METHODS:

Following Chemicals & Excepients were used in our in-vitro study

S.No.	Material	Manufacturer
1	Tamoxifen	Pharmadeep Remedies, HYD
2	Tween® 20	Qualigens, Fischer Scientifics, India
3	Tween® 80	Qualigens, Fischer Scientifics, India
4	Span® 60	Qualigens, Fischer Scientifics, India
5	CremophoreRH40	Baris pharma Pvt Ltd, Hyderabad
6	Oleic acid	Qualigens, Fischer Scientifics, India
7	Soy bean oil	Qualigens, Fischer Scientifics, India
8	Ethyl alcohol	Qualigens, Fischer Scientifics, India
9	Cotton seed oil	Pharmadeep Remedies, HYD
10	Aerosil® 200	Pharmadeep Remedies, HYD
11	PEG 400	Qualigens, Fischer Scientifics, India
12	Span® 80	Qualigens, Fischer Scientifics, India

Equipments:

S.No.	Equipment	Make	Model
1	Weighing balance	SHIMADZU	AX200
2	Orbital Shaker	REMI ELECTROTECH LTD, India.	CL 24
3	Magnetic stirrer	REMI Equipment, Mumbai, India	5ML
4	UV–Visible Spectrophotometer	LABINDIA, India.	3200
5	Dissolution test apparatus II USP	LABINDIA , India	DS 8000
6	Zeta sizer	HORIBA, JAPAN & Malvern Instruments, UK	Nano 90& Nano ZS
7	pH meter	LABINDIA , India	Phan
8	Conductivity meter	LABINDIA, India	Pico+
9	Ultrasonicator	CITIZEN ,India	CD 4820
11	Viscometer	BROOKFIELD,USA	LVDV-II+ pro
12	FTIR	BRUKER, Germany	ALPHA T
13	Cyclo mixer	REMI Equipments, Mumbai, India	CM 101DX
14	DSC	SIIO,Japan	6300

15	HPLC-PDA	SHIMADZU,Japan	SPD20A Detector, LC-20AD Pumps, DGU-20A3 Degasser
16	XRPD	PHILIPS, Netherlands	PW 1729
17	TEM	HITACHI, Tokyo, Japan	H-7500

Experimental Work:

Preparation of L-SNEDDS (Long time self nanoemulsifying drug delivery system) of Tamoxifen

10 mg of Tamoxifen was dissolved in 1g of the mixture of oil and Smix respectively. The prepared mixture was vortexed using vertex mixer (Remi India) to obtain a clear homogeneous formulation. Various regions in phase systems at lower, medium and higher concentration of oil and Smix were selected to load the drugs in to plain nanosuspensions then the final drug content of the formulation was 1 % and 0.2 % w/w for Tamoxifen. The final formulations of suspension were examined for signs of turbidity and thermodynamic stability or phase separation after 72 hours prior to self-emulsification and droplet size determination studies.

Table 1: Formulations of Tamoxifen selected from the Region of phase diagram

Sr.no.	Oil :Smix	Surfactant: Co-surfactant	Cotton seed oil (%)	Tween® 80 (%)	PEG 400 (%)
FC1	1:9.00	1:1	10.00	45.00	45.00
FC2	1:4.00	1:1	20.00	40.00	40.00
FC3	1:2.33	1:1	30.00	35.00	35.00
FC4	1:1.50	1:1	40.00	30.00	30.00
FC5	1:9.00	2:1	10.00	60.00	30.00
FC6	1:4.0	2:1	20.00	53.44	26.66
FC7	1:2.33	2:1	30.00	46.66	23.33
FC8	1:1.50	2:1	40.00	40.00	20.00
FC9	1:9.00	3:1	10.00	67.50	22.50
FC10	1:4.00	3:1	20.00	60.00	20.00
FC11	1:2.33	3:1	30.00	52.50	17.50
FC12	1:1.50	3:1	40.00	45.00	15.00

Screening of the suspension formulations for physical & Thermodynamic Stability

All the 12 formulations for Tamoxifen was employed to heating & cooling, centrifugation and freeze thaw analysis to observe thermodynamic stability.

Heating, cooling cycle: The 12 formulations for Tamoxifen was taken in microtubes and six cycle between freezing temperature 4° C and 45°C with storage at each temperature of not less than 48 hours was studied. The formulations those are constant at both the temperature, were selected for centrifugation test.

Centrifugation test: The twelve formulations for Tamoxifen was taken in 2 mL microtubes. All the formulations were centrifuged at 3000 rpm at least for 30 minutes using micro centrifuge (Remi India).The formulations did not show any phase separation was taken for freez thaw stress test .

Freeze-thaw cycle: The formulations for Tamoxifen was taken in 2 mL microtube and three freez thaw cycles between -4° C & 40°C for 24 hours was done for the formulations. The observations made for phase separation and drug precipitation. The formulations those are stable and passed thermodynamic stress test were further chosen for optimization of suspension.

Optimization of Tamoxifen suspension using droplet size and polydispersity index

The diameter of nanosuspensions globules and polydispersity index of the formulation selected was determined by dynamic light scattering particle size analyzer (Nano ZS, Malvern, UK) at 635 nm wavelength of 90° scattering angle at 25° C.0.1 mL formulation was added to 200 mL beaker containing 100 mL of distilled water and shaken gently using magnetic stirrer to form fine and transparent nanosuspensions and kept at 25° C for 12 hours (47,61, 62 and 56). The z-average diameter were recorded. The z-average diameter also as the harmonic intensity weighed average hydrodynamic diameter of droplets. The z-average diameter of droplets obtained from cumulated examination by the auto measured software tool (Malvern Instruments, UK).The final and optimized formulations were shown in table 2 based on particle size and PDI.

Table 2: Optimized formulation of Tamoxifen

Code	Drug	%OIL	%Surfactant	%Cosurfactant
FC 9	Tamoxifen	10	67.5	22.5

Characterization of optimized Tamoxifen nanosuspension

Transmission electron microscopy (TEM)

The structure and morphological examination of the oil droplets loaded with the drug in nanosuspensions were observed with transmission electron microscopy (56 & 75).TEM is also important to observe drug precipitation upon addition of aqueous phase. The optimized formulation

(FC 9) was diluted with distilled water in 1:50 ratio and mixed gentle; a drop of diluted sample was placed over the formwar coated grid. The diluted sample droplets were stained negatively for 10 minutes with phosphotungstic acid (1% w/v) solution. Excess liquid is blotted with whatmann filter paper. The samples were then examined with TEM (HITACHI, H-7500, Japan) operated at 80 kV.

Determination of viscosity of formulation

Viscosity of the optimized suspension of Tamoxifen was investigated using LVDV II T Brook field viscometer using spindle number 64 attached with UL small sample adopter at 25 ^{0}C .

Determination of Refractive index of nano suspension

A drop of the optimized suspension of Tamoxifen was placed in the lenses of the Abbe's type of Refractometer (Bellingam+ Stanley Ltd, USA)

Invitro release studies of nanosuspension

The drug release studies from optimized fornulation were performed using USP type II dissolution test apparatus (LABINDIA, India) rotating speed of 100 rpm at 37^{0} C ± 0.5^{0} C. 5 mg and 1 mg equivalent amount of the suspension for Tamoxifen were placed into the 0 size hard gelatin capsule shells and sealed manually. The sealed capsules were dropped in to the 900 mL of phosphate buffer pH 7.4 and 7.0 for Tamoxifen respectively. The 5 mL of the sample was withdrawn using cannula attached with 0.4 micron membrane filter at 0, 5, 10, 15, 30, 45 and 60 minutes time intervals. A 5 mL quantity of the dissolution medium was replaced to maintain sink conditions of the dissolution study. The samples withdrawn were diluted with fresh dissolution medium if necessary. The samples were analyzed using RP-HPLC attached with PDA (73)

Preparation of the S-SNEDDS (Short time self nanoemulsifying drug delivery system) from optimized nanosuspension

The nanosuspension of Tamoxifen formulated, adsorbed onto Aerosil® 200 (1:1 ratio) by physical mixing in a small motor and pestle for 5 minutes to form a free flowing and dry homogenous mass(47 & 48). The free flowing powder was passed through a sieve number 30 and the S-SNEDDS powder placed into the 0 size hard gelatin capsule shells and sealed manually. The resulting S-SNEDDS was a free flowing powder that was subsequently subjected to solid state characterization and dissolution studies.

Table 3 Composition of an optimized nano suspension

Formula	Components in S-SNEDDS	Proportions in mg	% Drug in S-SNEDDS
FC 9	Tamoxifen	10	0.5% w/w
	Cotton seed oil	100	
	Tween® 80	675	
	PEG 400	225	
	Aerosil® 200	1000	

Characterization of S-SNEDDS of Tamoxifen

Estimation of drug content in nanosuspension

The nano suspension containing 10 mg equivalent amount of Tamoxifen was dispersed in corresponding mobile phase in 100 mL volumetric flask by adding 20 ml of mobile phase and sonicated using bath sonicator (Citizen,India) for 10 minutes and made up to the volume with corresponding mobile phase to extract Tamoxifen and glimepiride totally , and centrifuged at 3000 rpm for 20 minutes separate un dissolved excipients. The supernatant was taken and was passed through a 0.45 micron membrane filter (PALL, USA). The samples were analyzed using RP-HPLC attached with PDA at a max of 228 nm.

Reconstitution properties of nanosuspension

The time required for self-emulsification of nanosuspension was determined using USP type II dissolution rate apparatus. 100 mg nanosuspension was taken in to 500 mL of distilled water in a dissolution vessel at 37◦ C under gentle agitation at 50 rpm. The emulsification time of suspension assessed visually. All the studies performed triplicate to obtain accurate results.

Droplet size determination of reconstituted suspension

The z-average diameter of nanosuspensions droplets formed after reconstitution of 100 mg of nanosuspension into 50 ml of deionized water was determined by dynamic light scattering particle size analyzer (Nano ZS, Malvern, UK) at 635 nm wavelength of 90° scattering angle at 25° C . The z-average diameter of droplets obtained from cumulated examination by the auto measured software tool (Malvern Instruments, UK).

Differential scanning calorimetry (DSC)

DSC curves for pure drug and optimized Suspension were obtained in a differential scanning calorimeter (SIIO, 6300, Japan) using platinum pans with 2 mg of sample purged with nitrogen gas at a flow rate of 50 mL/min with heating rate of 10^0 C/min over a temperature range of 25-250^0 C.

Scanning electron microscopy (SEM)

The samples of drug (Tamoxifen), colloidal silica and suspension samples were mounted on a double adhesive tape. The samples mounted were sputtered with thin gold palladium layer by VG-microtechsputter coater unit. The surface microphotographs were analyzed with an S- 120 Sterioscan scanning electron microscope (Cambridge, UK).

X-ray powder diffraction (XRPD) study

The diffraction patterns of plain drugs (Tamoxifen), colloidal silica and Suspension were obtained on Philips PW 1729 X-ray diffractogram, with monochromatized Cu Kα radiation (1.542 A^0),The samples were analyzed between 2^0 and 50^0 angles of 2θ, voltage of 30 kV and current of 30mA (47).

Stability of nano suspension in simulated gastric fluid (SGF)

10 mg of Suspension of Tamoxifen were introduced into 50 ml of simulated gastric fluid containing pepsin at pH of about 1.2 for 3 hours. The structure and morphological examination of the oil droplets loaded with the drug in nanosuspensions were observed with transmission electron microscopy mentioned in chapter 4.8.1. A drop of sample was placed over the formwar coated grid. The sample droplets were stained negatively for 10 minutes with phosphotungstic acid (1& w/v) solution. Excess liquid is blotted with whatmann filter paper. The samples were then examined with TEM (HITACHI, H-7500, Japan) operated at 80 kV. The TEM images of droplets observed for the drug precipitation, coalescence and break down of nanosuspension globules.

Stability studies at accelerated conditions

The optimized formulation powder placed into the 0 size hard gelatin capsule shells and sealed manually. Furthermore the capsules were sealed in amber colour glass bottle and placed in stability chambers maintained at 40+ 2^0 C/75 % + 5 % (accelerated conditions as per ICH). The studies were conducted for 3 months.The samples were collected for 0, 1, 2, and 3 months and evaluated for Tamoxifen assay using RP-HPLC method. The size of droplets of suspension was determined by method depicted to determine effect of storage conditions on size of droplets of suspension

Results & Discussion:

Solubility studies of Tamoxifen in oils, Surfactants and co surfactants

The SUSPENSION is prepared by one or more surfactants and drug dissolved in oil. At room temperature the mixture should be an opaque, monophasic liquid and supposed to have fine solvent characters to permit solubilisation of drug in solution. During solubility experiments cotton seed oil showed the highest solubility for Tamoxifen compared to other oils like isopropymyristate, soy bean oil, sunflower oil and oleic acid. In the vicinity of triglyceride chains of the cotton seed oil supports absolute solubilization of Tamoxifen. PEG 400 as cosurfactants , cotton seed oil as lipid and Tween® 80as surfactant were selected for the construction of ternary phase diagrams to identify the nanosuspension domains such that at particular concentration of oil and surfactant co -surfactant ratios a stable nanosuspension formulation is formed.

The lipophilic surfactant promote emulsification of oil but it produces crude suspension with large globule size as the lipophilic surfactants have HLB value less than 10.Hydrophilic surfactants HLB > 10 are superior at giving fine and uniform suspension droplets which are more likely to empty quickly from the stomach. Large surface area helps in faster and complete absorption. In most cases it is the right blend of low and high HLB surfactants leads to the formation of stable nanosuspension upon exposure to water. Based on the efficiency of self-emulsification, Tween® 80 with HLB value of 15 was selected for the formulation of Tamoxifen SUSPENSION. PEG 400 selected as cosurfactant correspondingly and cottonseed oil was selected as an oil phase.

Table 4 : Solubility of Tamoxifen in vehicles

Oil vehicle	**Solubility (mg/mL)**
Cottonseed oil	6.37±0.075719
Oleic acid	2.42±0.1253
Sunflower oil	2.09±0.179536
Soy bean oil	1.25±0.155349
Isopropyl myristate	0.36±0.045826
Miglyol® 812	1.09±0.020817
Surfactant (HLB)	**Solubility(mg/ml)**
Tween® 80 (15.0)	22.77±0.452
Cremophor RH40 (13)	17.23±0.351
Span® 20 (8.6)	4.36±0.511
Span® 80 (4.3)	1.30±0.220
PEG400 (13.1)	9.90±0.458
Propylene glycol	0.69±0.03

Construction of the ternary phase diagram based on solubility data of Tamoxifen

Ternary phase diagrams presents the information about the concentration range of the oil (Cotton seed oil), surfactant (Tween® 80) and cosurfactant (PEG 400) concentrations to form clear nanosuspension after titration with water (spontaneous emulsification method).The shaded area for SUSPENSION region were chosen based on percentage transmission after spontaneous emulsification method. The area shaded by the points in the phase diagram displays the concentration mixture components that resulted in a clear nanosuspension out of all the trial concentrations. All the combinations under test impulsively formed a nanosuspension in particular concentrations which are located within the shaded area of phase diagram. This is possibly due to spontaneous stabilization of oil droplets by surfactant owing to their high concentration. When cotton seed oil concentration increases more than 40% the percentage transmittance decreased resulted from low accessibility of Smix to emulsify the cotton seed oil to form nanosuspension and the turbid suspension out of shaded area of phase diagrams were shown in figure 5.6.The emulsification efficiency was found to be capable, when the Smix amount of concentration was supplementary more than 60% of formulation

Preparation of nanosuspension

Tamoxifen showed maximum solubility in Tween 80 and PEG 400 among surfactants and cosurfactants correspondingly. There are more chances of drug precipitation when the drug concentration is more than its solubility. Only 10 mg/gm i.e 1% w/w drug is loaded into the plain suspension formulations. Upon aqueous dilution, the drug should not precipitate and is established by spontaneous emulsification method. After loading the drug, all the formulation (FC1 to FC12) presented.

Screening of the SUSPENSION for physical and thermodynamic stability:

Thermodynamically stable nanosuspension shown in table 5 formed at a particular concentration of oil, Smix and water, with spontaneous emulsification with no phase separation, creaming or cracking. The formulations selected subjected to thermodynamic stability test. All the formulation (FC1 to FC12) presented do not show any drug precipitation after thermodynamic stability test and results. All the formulations emulsified within 1 minute

Table: 5: Thermodynamic Stability of SUSPENSION formulations

Sno	Oil :Smix	Surfactant: Co-surfactant	Cotton seed oil (%)	Tween 80 (%)	PEG 400 (%)	Thermo dynamic Stability
FC1	1:9.00	1:1	10.00	45.00	45.00	Stable
FC2	1:4.00	1:1	20.00	40.00	40.00	Stable
FC3	1:2.33	1:1	30.00	35.00	35.00	Stable
FC4	1:1.50	1:1	40.00	30.00	30.00	Stable
FC5	1:9.00	2:1	10.00	60.00	30.00	Stable
FC6	1:4.0	2:1	20.00	53.44	26.66	Stable
FC7	1:2.33	2:1	30.00	46.66	23.33	Stable
FC8	1:1.50	2:1	40.00	40.00	20.00	Stable
FC9	1:9.00	3:1	10.00	67.50	22.50	Stable
FC10	1:4.00	3:1	20.00	60.00	20.00	Stable.
FC11	1:2.33	3:1	30.00	52.50	17.50	Stable
FC12	1:1.50	3:1	40.00	45.00	15.00	Stable

Optimization of Tamoxifen SUSPENSION using droplet size and Polydispersity Index

Tamoxifen SUSPENSION formulations (FC1-FC12) presented in Table 5 were further tested for their droplet diameter and polydispersity index(PDI) determined by dynamic light scattering particle size analyzer at 635 nm wavelength of 90° scattering angle at 25° C. The size and PDI significantly affected with percentage of cottonseed oil and Smix. The z-average diameter and zeta potential confirmed that increased in concentration of cottonseed oil proportionately increased the suspension droplet diameter whereas the increase in surfactant percentage-the droplet diameter was decreased. The surfactant Tween 80 adsorbed at interface of oil and water to form thin film and decrease the oil droplet diameter and also helped to stabilize the nanosuspension. SUSPENSION formulation FC 9 containing cotton seed oil yielded a particle diameter of 143 ±2 nm with a PDI of 0.251. Formulation FC 9 was therefore chosen for formulation of S-SUSPENSION of Tamoxifen because of its desirable particle size and low PDI value. The final composition of SUSPENSION preparation FC 9 containing 10 mg of Tamoxifen was therefore as follows: Cotton seed oil (10%), Tween 80 (67.50 %) and PEG 400 (22.50%). Zeta potential was found to be −14.9 shown in Figure 5.8, The results indicate that the drug loaded oil droplet are stable and well distributed without coalescence (83).The cosurfactant PEG 400 play potential role for forming nanosuspension due to hydrophilic nature (84).The optimized SUSPENSION formulation FC 9.

Table 6: Droplet size and PDI of SUSPENSION of Tamoxifen

S. no.	Oil :Smix	Cotton seed oil(%)	Tween 80(%)	PEG 400(%)	Z-Avg size(d nm)	PDI
FC1	1:19	10.00	45.00	45.00	98.2	0.725
FC2	1:9	20.00	40.00	40.00	258	0.610
FC3	1:4	30.00	35.00	35.00	670	0.810
FC4	1:2.33	40.00	30.00	30.00	3942	0.422
FC5	1:19	10.00	60.00	30.00	85.50	0.603
FC6	1:9	20.00	53.44	26.66	199.8	0.554
FC7	1:4	30.00	46.66	23.33	805.1	0.493
FC8	1:2.33	40.00	40.00	20.00	2666	0.613

FC9	1:19	10.00	67.50	22.50	143	0.251
FC10	1:9	20.00	60.00	20.00	306.2	0.220
FC11	1:4	30.00	52.50	17.50	722.2	0.432
FC12	1:2.33	40.00	45.00	15.00	918.5	0.744

Characterization of optimized Tamoxifen SUSPENSION

Transmission electron microscopy (TEM)

The microphotograph of the optimized SUSPENSION (FC9) observed as dark globules with bright surrounding. The TEM image demonstrates that nanosuspension come into viewed as spherical oil droplets after dilution with aqueous phase, attributable to nanosize of cotton seed oil droplets loaded with Tamoxifen.

Viscosity determination

Formulation FC9 SUSPENSION of Tamoxifen was yielded a viscosity of 104.7± 0.3 cps. The viscosity evaluation confirming that the liquid formulation FC 9 behaves as Newtonian fluid .

Refractive index Determination

Formulation FC 9 SUSPENSION of Tamoxifen was yielded a Refractive index of 1.419±0.01819.The refractive index value was nearly closer to water value at 25° C. Furthermore the result of RI represents transparent homogenous nature of SUSPENSION.

Characterization of S-SUSPENSION of Tamoxifen

Estimation of drug content in SUSPENSION

The SUSPENSION containing 10 mg equivalent amount of Tamoxifen was dispersed in corresponding mobile phase in 100 mL volumetric flask by adding 20 ml of mobile phase and sonicated using bath sonicator (Citizen, India) for 10 minutes and made up to the volume with corresponding mobile phase to extract Tamoxifen totally , and centrifuged at 3000 rpm for 20 minutes separate un dissolved excipients.The supernatant was taken and was passed through a 0.45 micron membrane filter (PALL, USA). The samples were analyzed using RP-HPLC attached with PDA at a max of 228 nm (73).The experiments were performed in triplicate (n=3) and Tamoxifen content present in S-SUSPENSION

In-vitro drug release studies

For both liquid and dry nanosuspension formulations show greater than 85% drug liberate after 15 min and in case of marketed product it was less than 15 %. Finally from these comparative dissolution studies it was noticed that liquid nanosuspension and dry nanosuspension has shown greater drug release than marketed product .*In vitro* drug release profile of SUSPENSION showed complete drug release within 30 min but marketed formulation released only 20 percentage of

Tamoxifen and the time verses drug release. The SUSPENSION rapidly hydrated during *invitro* dissolution to form oil in water suspension. The nanosized droplets produce in high specific surface are of delivery system results in improved dissolution rate compared to pure Tamoxifen. The *in vitro* dissolution reports on S-SUSPENSION exposed that Tamoxifen release from porous carriers (Aerosil® 200) was slow when compared to SUSPENSION. This could be because of extra steps involved during dissolution such as disintegration of Solid structure of S-SUSPENSION and desorption of SUSPENSION from the voids of porous carriers. The SUSPENSION when exposed to dissolution medium, leads to desorption of the SUSPENSION from the silica surface due to stronger interaction between silica and dissolution medium than those between silica and SUSPENSION. Drug freed from SUSPENSION was initially slow when compared to SUSPENSION. This could be because of increase in diffusion path length and capillary forces for adsorbed liquid formulation in the matrix of porous Aerosil® 200 carrier. Wicking properties of liquid filled porous carrier upon contact with dissolution medium, could also be responsible for slower drug release. *In vitro*dissolution, studies confirmed that the self-nanoemulsifying formulations can improve the release profile of Tamoxifen.

Table No:7 % Drug Release of Formulations FC1 to FC 6

Time	FC1	FC2	FC3	FC4	FC5	FC6
0	0	0	0	0	0	0
5	29.02	28.04	18.87	27.86	27.73	29.02
10	35.70	35.43	27.19	36.35	35.63	35.70
15	60.92	58.89	57.13	60.24	75.41	60.24
20	66.08	64.53	63.63	66.73	83.84	66.73
30	70.44	69.43	69.71	71.34	102.80	76.34
45	80.90	79.98	79.27	80.17		88.52
60	87.27	83.98	89.02	93.28		98.67

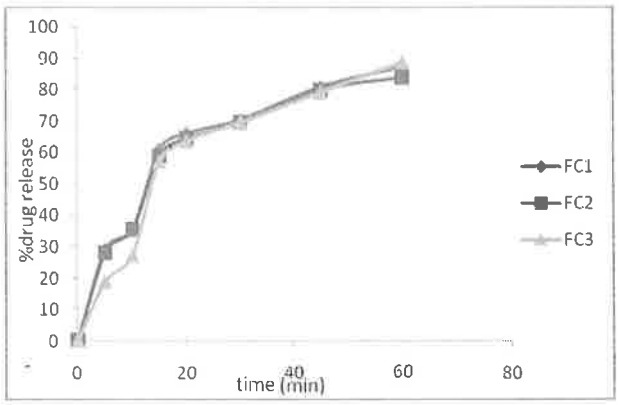


Fig : 1 % drug release of formulation (FC1,FC2,FC3)

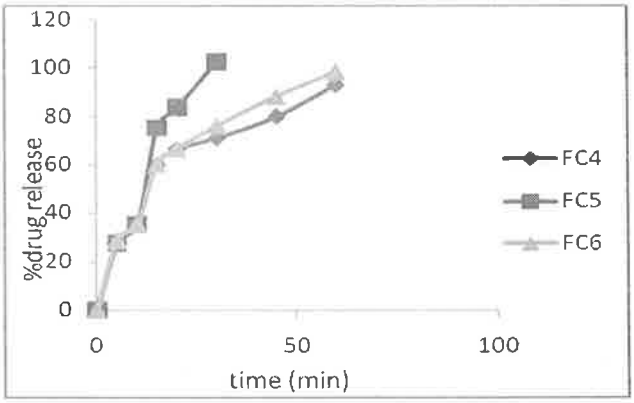


Fig 2 : % drug release of formulation (FC4,FC5,FC6)

Table No:8 % Drug Release of Formulations FC7 to FC 12

Time	FC7	FC8	FC9	FC10	FC11	FC12
0	0	0	0	0	0	0
5	27.86	30.23	37.47	18.42	19.62	19.89
10	36.35	44.9	59.93	27.73	27.86	28.04
15	41.45	50.87	65.85	35.63	36.35	43.81
20	55.25	66.37	89.55	42.04	41.45	58.89
30	60.24	70.84	96.67	64.33	71.34	79.98
45	79.01	76.56	77.88	75.41	78.52	83.52
60	84.23	90.64	86.6	83.84	80.17	88.65

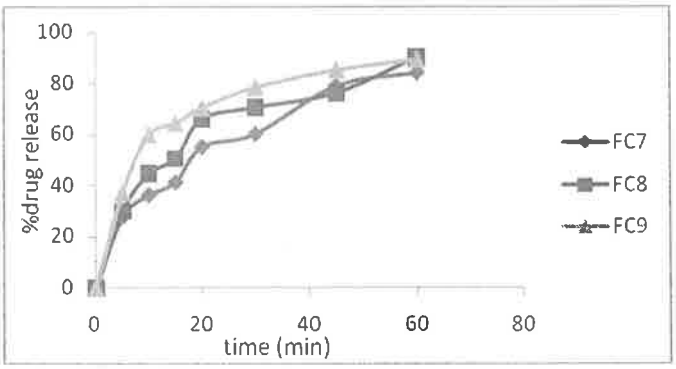


Fig 3 : % drug release of formulation (FC7,FC8,FC9)

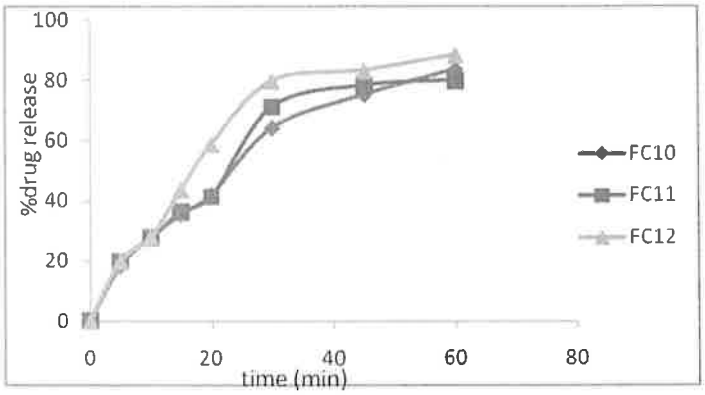


Fig 4 : % drug release of formulation (FC10,FC11,FC12)

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -32.4	Peak 1:	-33.1	98.5	16.4
Zeta Deviation (mV): 17.7	Peak 2:	15.3	1.0	2.62
Conductivity (mS/cm): 1.27	Peak 3:	48.9	0.5	1.58

Result quality : See result quality report

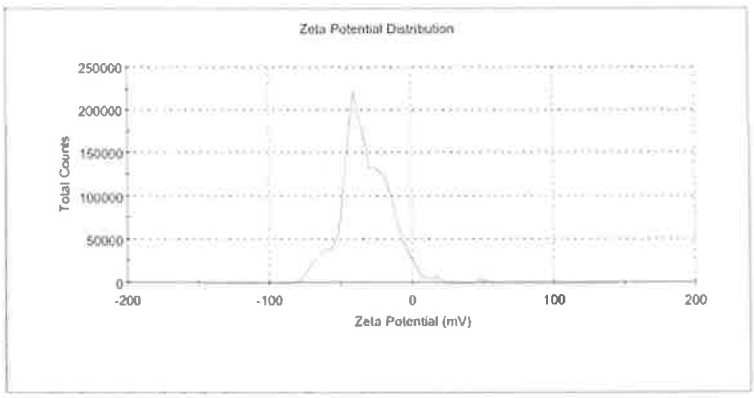


Fig No 5 : Particle Size Distribution in zeta

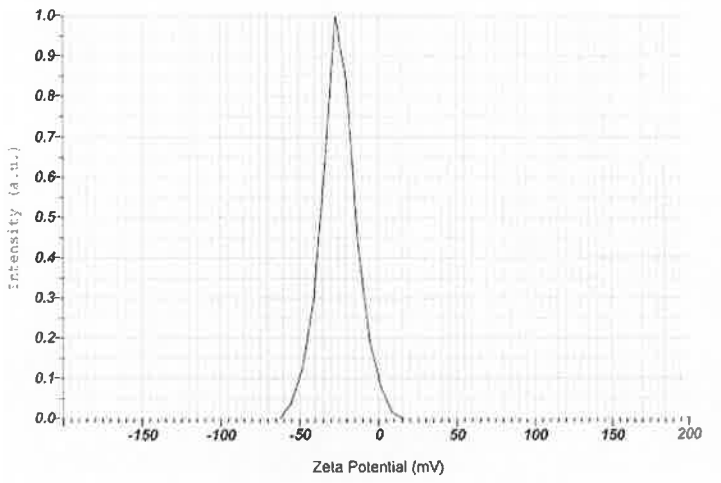


Fig No 6: Zeta Potential Results of nanosuspension

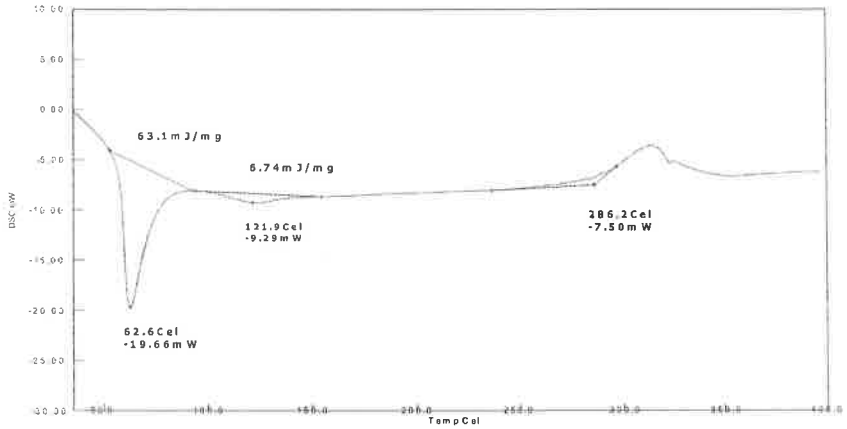


Fig no: 7 Differential Scanning Calorimetry (DSC) of nanosuspension.

CALCULATION RESULTS

Table No:9 Zeta Potential Values

Peak.No	Zeta potential	Electrophoretic Mobility
1	-24.9mV	- 0.000203 cm2/Vs
2	---mV	---cm2/Vs
3	---mV	---cm2/Vs

Zeta Potential (mean) : -24.9Mv

Electrophoretic mobility mean : -0.000203 cm2/Vs

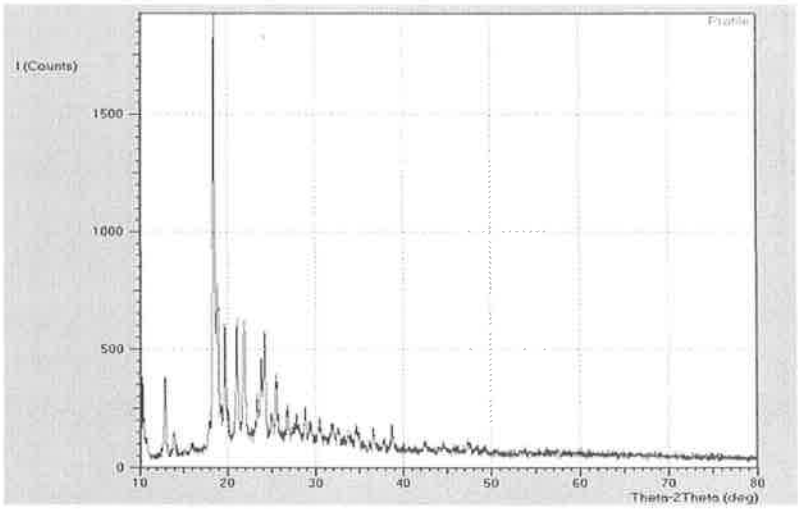


Fig No 8 : X –Ray Diffraction of Nanosuspension

Stability:

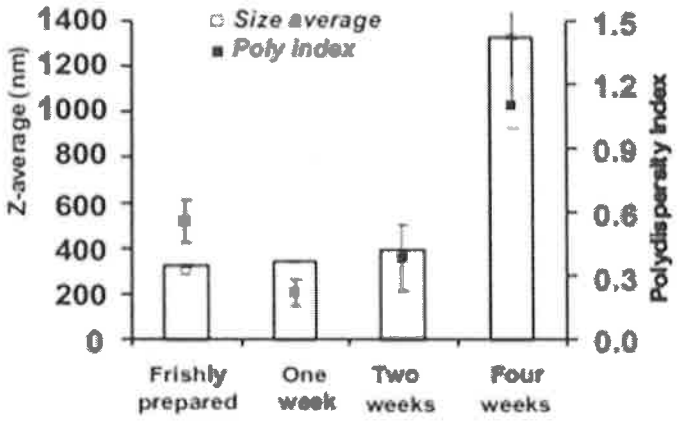


Fig No. 9: Showing One month stability of Suspension

Physical stability of the medicated nanosuspensions was intensively evaluated over 30 days period. Figure shows particle size distribution of the Tamoxifen nanosuspensions over 30 days storage. According to the obtained results, the Tamoxifen nanosuspension could retain the particle size distribution in the nanometer range within 14 days. Afterwards, the particle size began to increase and within 30 days the particle size of the Tamoxifen nanosuspensions had grown rapidly to be very big particles. The particle growth was due to the existence of aggregated particles which follows Ostwald ripening phenomenon

Conclusion:

It could be concluded that it was possible to obtain Tamoxifen nanosuspensions with fine solubility and dissolution properties, and the nanosuspensions possessed a high drug- loading (10%), which could reduce the dosage administration and gastrointestinal side effects. High pressure homogenization can be employed to produce aqueous drug nanosuspensions that are stable up to one month. The zeta potential values are about -30 mv or higher, i.e. in the range for a long-term stable suspension. Aqueous nanosuspension can be converted to dry nanocrystals by lyophilization. Dried Tamoxifen nanocrystals offer superior physicochemical properties. The very fine particles of the dried nanocrystals re- disperse completely in water. This characteristic is critical in improving the kinetic solubility and the dissolution behavior of drugs

Table 1: Formulations of Tamoxifen selected from the Region of phase diagram

Sr.no.	Oil :Smix	Surfactant: Co-surfactant	Cotton seed oil (%)	Tween® 80 (%)	PEG 400 (%)
FC1	1:9.00	1:1	10.00	45.00	45.00
FC2	1:4.00	1:1	20.00	40.00	40.00
FC3	1:2.33	1:1	30.00	35.00	35.00
FC4	1:1.50	1:1	40.00	30.00	30.00
FC5	1:9.00	2:1	10.00	60.00	30.00
FC6	1:4.0	2:1	20.00	53.44	26.66
FC7	1:2.33	2:1	30.00	46.66	23.33
FC8	1:1.50	2:1	40.00	40.00	20.00
FC9	1:9.00	3:1	10.00	67.50	22.50
FC10	1:4.00	3:1	20.00	60.00	20.00
FC11	1:2.33	3:1	30.00	52.50	17.50
FC12	1:1.50	3:1	40.00	45.00	15.00

Table 2: Optimized formulation of Tamoxifen

Code	Drug	%OIL	%Surfactant	%Cosurfactant
FC 9	Tamoxifen	10	67.5	22.5

Table 2: Optimized formulation of Tamoxifen

Table 3 Composition of an optimized nano suspension

Formula	Components in S-SNEDDS	Proportions in mg	% Drug in S-SNEDDS
FC 9	Tamoxifen	10	0.5% w/w
	Cotton seed oil	100	
	Tween® 80	675	
	PEG 400	225	
	Aerosil® 200	1000	

Table 4: Solubility of Tamoxifen in vehicles

Oil vehicle	Solubility (mg/mL)
Cottonseed oil	6.37±0.075719
Oleic acid	2.42±0.1253
Sunflower oil	2.09±0.179536
Soy bean oil	1.25±0.155349
Isopropyl myristate	0.36±0.045826
Miglyol® 812	1.09±0.020817
Surfactant (HLB)	**Solubility(mg/ml)**
Tween® 80 (15.0)	22.77±0.452
Cremophor RH40 (13)	17.23±0.351
Span® 20 (8.6)	4.36±0.511
Span® 80 (4.3)	1.30±0.220
PEG400 (13.1)	9.90±0.458
Propylene glycol	0.69±0.03

Table: 5: Thermodynamic Stability of SUSPENSION formulations

S.No.	Oil :Smix	Surfactant: Co-surfactant	Cotton seed oil (%)	Tween 80 (%)	PEG 400 (%)	Thermo dynamic Stability
FC1	1:9.00	1:1	10.00	45.00	45.00	Stable
FC2	1:4.00	1:1	20.00	40.00	40.00	Stable
FC3	1:2.33	1:1	30.00	35.00	35.00	Stable
FC4	1:1.50	1:1	40.00	30.00	30.00	Stable
FC5	1:9.00	2:1	10.00	60.00	30.00	Stable
FC6	1:4.0	2:1	20.00	53.44	26.66	Stable
FC7	1:2.33	2:1	30.00	46.66	23.33	Stable
FC8	1:1.50	2:1	40.00	40.00	20.00	Stable
FC9	1:9.00	3:1	10.00	67.50	22.50	Stable
FC10	1:4.00	3:1	20.00	60.00	20.00	Stable
FC11	1:2.33	3:1	30.00	52.50	17.50	Stable
FC12	1:1.50	3:1	40.00	45.00	15.00	Stable

Table 6: Droplet size and PDI of SUSPENSION of Tamoxifen

S. no.	Oil :Smix	Cotton seed oil(%)	Tween 80(%)	PEG 400(%)	Z-Avg size(d nm)	PDI
FC1	1:19	10.00	45.00	45.00	98.2	0.725
FC2	1:9	20.00	40.00	40.00	258	0.610
FC3	1:4	30.00	35.00	35.00	670	0.810
FC4	1:2.33	40.00	30.00	30.00	3942	0.422
FC5	1:19	10.00	60.00	30.00	85.50	0.603
FC6	1:9	20.00	53.44	26.66	199.8	0.554
FC7	1:4	30.00	46.66	23.33	805.1	0.493
FC8	1:2.33	40.00	40.00	20.00	2666	0.613
FC9	1:19	10.00	67.50	22.50	143	0.251
FC10	1:9	20.00	60.00	20.00	306.2	0.220
FC11	1:4	30.00	52.50	17.50	722.2	0.432
FC12	1:2.33	40.00	45.00	15.00	918.5	0.744

Table No:7 % Drug Release of Formulations FC1 to FC 6

Time	FC1	FC2	FC3	FC4	FC5	FC6
0	0	0	0	0	0	0
5	29.02	28.04	18.87	27.86	27.73	29.02
10	35.70	35.43	27.19	36.35	35.63	35.70
15	60.92	58.89	57.13	60.24	75.41	60.24
20	66.08	64.53	63.63	66.73	83.84	66.73
30	70.44	69.43	69.71	71.34	102.80	76.34
45	80.90	79.98	79.27	80.17		88.52
60	87.27	83.98	89.02	93.28		98.67

Table No:8 % Drug Release of Formulations FC7 to FC 12

Time	FC7	FC8	FC9	FC10	FC11	FC12
0	0	0	0	0	0	0
5	27.86	30.23	37.47	18.42	19.62	19.89
10	36.35	44.9	59.93	27.73	27.86	28.04
15	41.45	50.87	65.85	35.63	36.35	43.81
20	55.25	66.37	89.55	42.04	41.45	58.89
30	60.24	70.84	96.67	64.33	71.34	79.98
45	79.01	76.56	77.88	75.41	78.52	83.52
60	84.23	90.64	86.6	83.84	80.17	88.65

Table No: 9 Zeta Potential Values

Peak.No	Zeta potential	Electrophoretic Mobility
1	-24.9mV	- 0.000203 cm2/Vs
2	---mV	---cm2/Vs
3	---mV	---cm2/Vs

Zeta Potential (mean) : -24.9Mv
Electrophoretic mobility mean : -0.000203 cm2/Vs

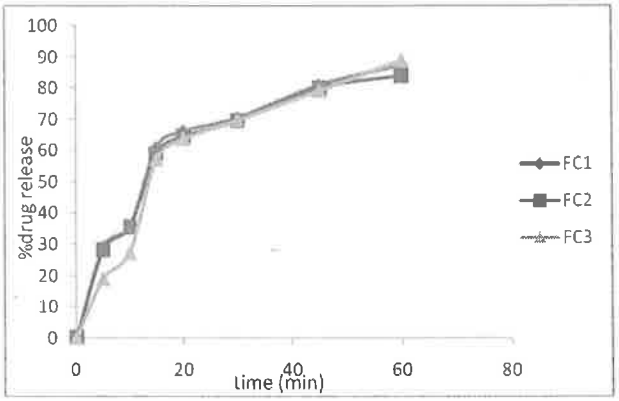


Fig : 1 % drug release of formulation (FC1,FC2,FC3)

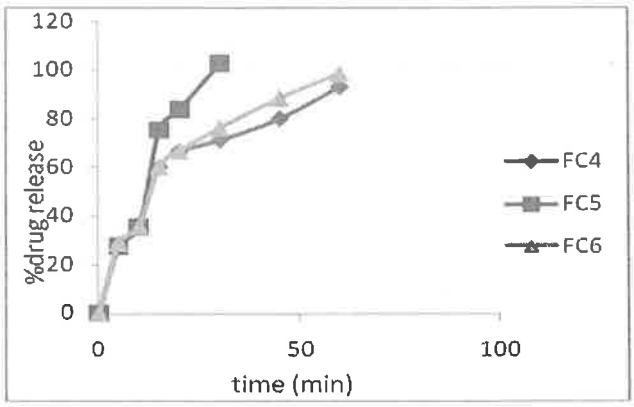
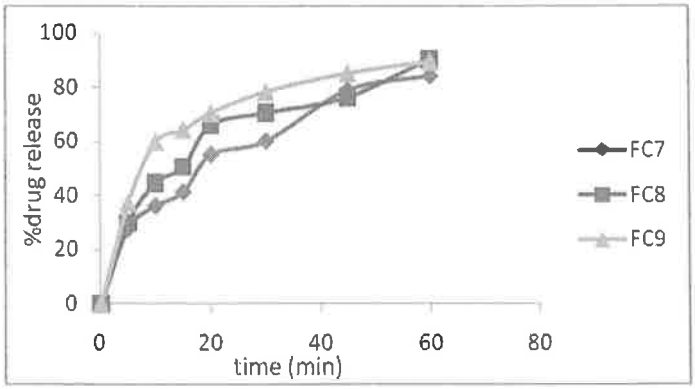


Fig 2 : % drug release of formulation (FC4,FC5,FC6)



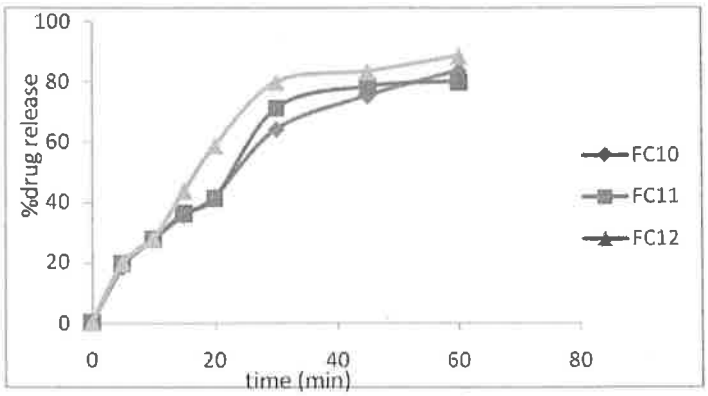


Fig 3 : % drug release of formulation (FC7,FC8,FC9)

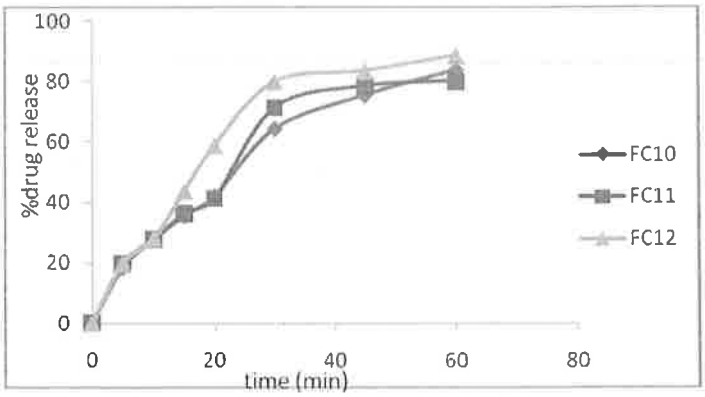


Fig 4 : % drug release of formulation (FC10,FC11,FC12)

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-32.4	Peak 1:	-33.1	98.5	16.4
Zeta Deviation (mV):	17.7	Peak 2:	15.3	1.0	2.62
Conductivity (mS/cm):	1.27	Peak 3:	48.9	0.5	1.58

Result quality : See result quality report

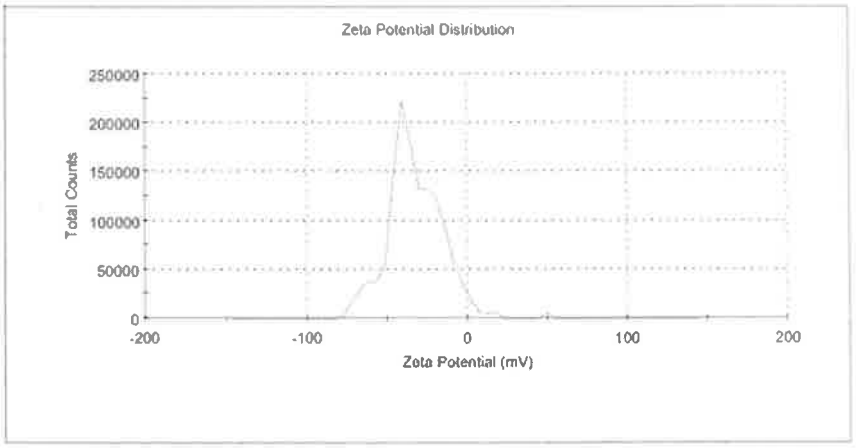


Fig No 5 : Particle Size Distribution in zeta

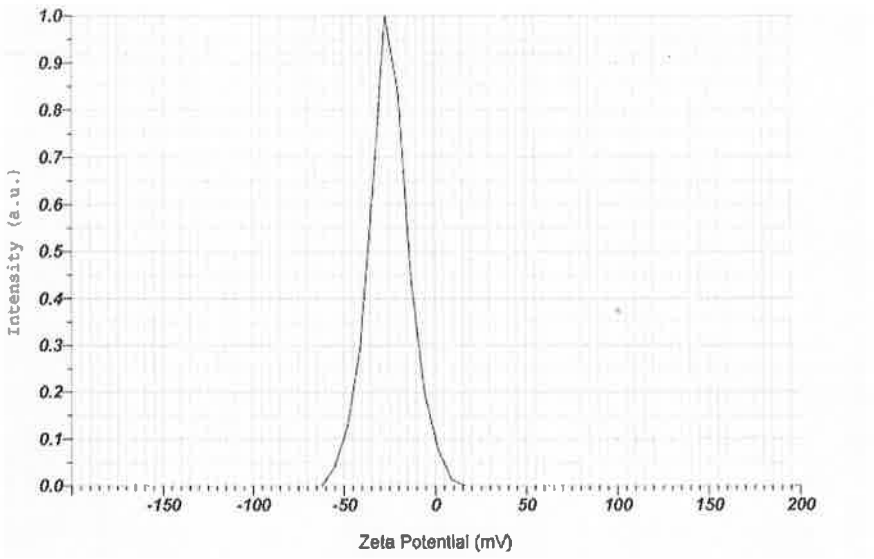


Fig No 6: Zeta Potential Results of nanosuspension

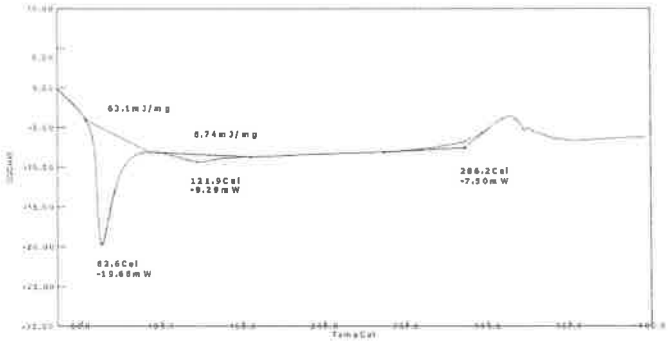


Fig no: 7 Differential Scanning Calorimetry (DSC) of nanosuspension

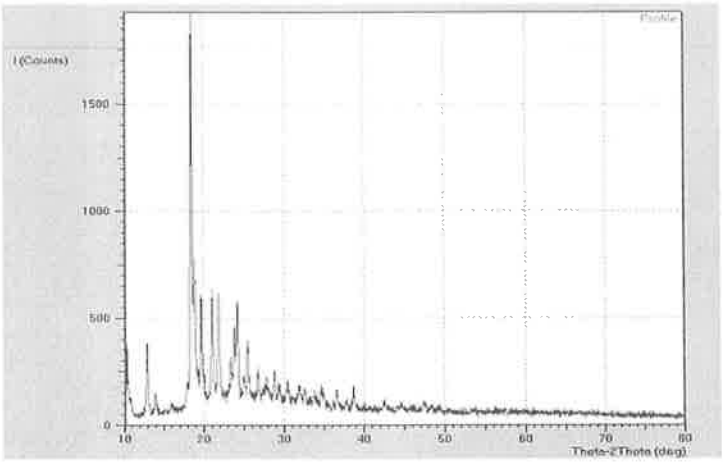


Fig No 8 : X –Ray Diffraction Of Nanosuspension

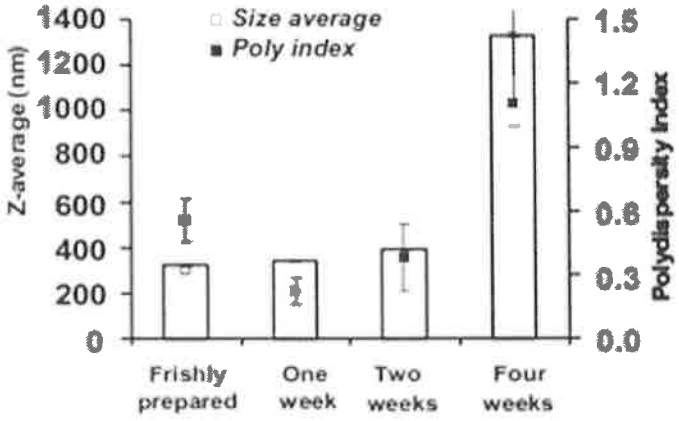


Fig No. 9: Showing one month stability of suspension

6. Conclusion

Conclusion

The work presented in this thesis describes the development, characterization, *in vitro, in vivo* evaluation of nanoparticles and liposomes for the treatment of breast cancer.

The study was initiated with the hypothesis of building a platform technology for encapsulating hydrophobic drug in lipid matrix and improving the reach ability of drug at the tumor site and uptake of the drug by tumor cells. The passive targeting technique makes use of altered morphology of tumor cells to accumulate the nanocarriers carrying drug. The tumor morphology shows irregularly formed cells with weak endothelium and cluster of blood vessel formation. The encapsulated drug releases slowly in the circulation making itself available for longer time without degradation. Lipid nanoparticles go to highly perfused organs causing toxicity problem which can be overcome by target specificity. The tumor shows angiogenesis and the requirement of oxygen is much higher than normal cells, expressing more number of transferrin receptors on the cell surface. The ligand attached to the surface of nanocarrier, orients the drug towards tumor. The solubility, bioavailability and toxicity issues with conventional anticancer agents are overcome in the developed formulations.

Hydrophilic drug encapsulated within the lipid core of liposome is shielded from degradation and the release is controlled through liposome core. The surface modification with Polyethylene glycol improves the circulation time and therapeutic index. The targeting of anticancer cytotoxic drug would decrease the unwanted side effects.

Estimation of the formulations by developing suitable analytical method

Formulation development for entrapment of lipophilic drug

Statistical method of 3^2factorial design was used to optimize concentration of lipids and surfactants used in the formulation. It is observed that as the concentration of lipid increases, size of the nanoparticles increases and as the concentration of the surfactant increases, the size of nanoparticles decreases. The Lower concentration of lipid had more surfactant available on the newly formed surfaces, which resulted in the formation of small-sized particles during hot emulsification. In another term, increasing the lipid content results in poor emulsifying efficiency and increase in particle agglomeration In the method of preparation, process variables and formulation variables were altered to get final optimized formulation which can be easily scaled up for the industrial production. The lipid based nanocarriers are prone to agglomeration during stability of the formulation. Lyophilization was found to be best option with 3% w/v trehalose as a choice of cryoprotectant, to improve long term stability of the formulation. Compritolis a mixture of glycerol tribehenate, glycerol dibehenate, and glycerol monobehenate and it possesses amphiphilic properties due to the presence of partial acyl glycerol.Tween-80 belongs to the polysorbate family and is used as water based surfactant. Although possessing a high zeta potential value -20mV is essential in developing a highly dispersed suspension of nanoparticles, steric hindrance property of polysorbates further helps

maintaining the SLNs stability by keeping the particles apart. Lipid forming a highly crystalline state with a perfect lattice would lead to drug expulsion. Any imperfection (lattice defects) in the lipid structure could offer space to accommodate the drugs. Thus, improving the drug loading and entrapment efficiency. Glyceryl behenate having high melting point showed much slower drug release initially as compared to lower melting Glyceryl palmitostearate.

The DSC thermograms of Tamoxifen citrate, glyceryl behenate and glyceryl palmitostearate exhibit melting endotherms at 148.5°C, 71.71°C and 56.1°C respectively. These peaks indicate that all components exist as highly ordered crystalline materials.DSC thermograms of optimized formulations showed complete elimination of drug peak decreasing the melting range of glyceryl behenate and glyceryl palmitostearate to 69.51°C and 47°C and the

drug peak is diminished. This indicates complete solubilization of drug in the lipids. Another reason for a decrease in melting enthalpy is the small particle size of the formulations and increase in the surface area which made the heat flow through the material slower compared to larger crystals. DCS thermograms of SLNs showed slight broadening of glyceryl behenate endothermic peak. This might be due to excipients undergoing a heating and cooling cycle, smaller particle size resulting in higher surface area, and the presence of impurities in surfactant used. This indicates physical homogeneity and partial drug entrapment of formulations It can be suggested that during SLNs formation, crystallization of drug was inhibited due to entrapment of drug in the molten lipid structure. Thus the drug showed higher energy in amorphous state with better solubility, dissolution, and bioavailability. The melting point decrease of colloidal systems can be assigned to the colloidal dimensions of the particles in particular to their large surface to volume ratio and not to recrystallisation of the lipid matrices in a metastable polymorph possessing a lower melting point.Transmission electron microscopy images of batch SLN1 and SLN2 revealed spherical nature of lipid nanoparticles and confirmed the mean particle size of 20–250 nm respectively. The particle size obtained through TEM confirms with the size obtained by zetasizer.

Stability studies were carried as per ICH guidelines for new formulations. It was observed that the particle size was maintained at 5°C and at accelerated

condition it was increased by 15% as compared to initial particle size, after reconstitution showing slight aggregation of partiles at accelerated condition on stability. The zeta potential values did not change much and the drug content and was decreased SLN1 and from 94 to 91 % and from 91 to

89% at 5°C whereas drug release within the range at 5°C accelerated conditions.

Development of Formulation of surface modified, targeted nanoparticles

Transferrin protein loaded with iron encounters the transferrin receptor on the surface of the cell, it gets bound and transported into the cell in a vesicle by receptor mediated endocytosis. The need to import iron into the cell is regulated in response to intracellular iron concentration. When tumor volume reaches 1–2 mm^3, it starts formation of new blood vessels in order to bring oxygen and nutrients to the growing cells **(Feron 2004).** As requirement of iron by tumour cell is much higher, more amount of Tf is required by tumor cells. The exponentially higher expression of TF

receptors on tumor cells than that on normal cells makes it a target for surface modified SLN. Transferrin used the same mechanism to transport conjugated solid lipid nanoparticles encapsulating drug, towards the cancer cell.

DCC (dicyclohexylcarbodiimide) and EDC are used to crosslink carboxylic acids on the lipids to primary amines and alcohols. DCC is soluble in non- aqueous solvents, hence used as NHS-ester crosslinkers.

Transferrin was covalently coupled by its carboxyl group to the hydroxyl group present in the lipid on the surface of preformed Tamoxifen loaded SLNs using DCC-NHS as a coupling agent. Cellular uptake occurs via several pathways such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis or phagocytosis.The particle size of nanoparticles has increased upto 390 nm and 430 nm respectively after surface modification for Lipid 2 and Lipid 3 nanoparticles respectively(Fig 4B.19,20)

The same is reflected in the TEM images (Fig 4B 23,24) as surface of nanoparticle is looking hazy. After lyophilization and reconstitution the particle size is slightly increased to 440 nm and 485 nm. After modification the zeta potential values were increased upto -3.2and -7.0, respectively, as transferrin is a glycoprotein containing many amino acids of which carboxyl groups wer

conjugated to lipids. The anionic NH_4^- impart positive charge to the modified nanoparticles and the charge on nanoparticles is increased. It is important to lyophilize the nanoparticles to get better stability. The reconstituted batches show change in zeta potential.

Stability studies were carried out at 5°C and 25°C/ 65% RH A stable solid lipid nanoparticles based drug delivery system was developed, optimized and evaluated for anti-cancer agent. However, limitations like low drug content were observed which can be improved by change in type and concentration of lipids. Glyceryl behenate (m.p.69 - 74°C) is a solid lipid based on glycerol esters of behenic acid (C22), with more than 85% behenic acid and other fatty acids (C16-C20). Glyceryl palmitostearate (m.p.52.0-56.0°C) is composed of palmitic acid (C16) and stearic acid (C18) more than 90%. High melt temperature resulting in higher viscosity plus the long hydrocarbon chain length of Glyceryl behenate results in larger particle size of Solid lipid nanoparticles compared to glyceryl palmitostearate.

Formulation development of Liposome

Liposomes composed of gel state phospholipids and cholesterol are one of the preferred drug carrier systems for *in vivo* applications. These optimised batches containing lipid to cholesterol ratio of 80: 20 and 70: 30 with phospholipids DSPC and DSCGNa. The particle size and zeta potential of developed liposomes were found to be 65±5nm, - 20±4 mV and 90±5nm, - 12.5±4 mV respectively. The uniformity in size would lead to more uniform pharmacokinetic characteristics liposomes and the drug clearance rates are found to be dependent on the physiochemical properties of the liposome .The developed liposomes show more stability at 5°C than accelerated stability conditions.

7. OUTCOME AND RECOMMENDATIONS

OUTCOME AND RECOMMENDATIONS

- ***In vitro* cell line studies**

  Pharmacokinetics &

- ***in vivo* studiesFuture**

  scope.

- ◈ The conjugation of ligand to receptor can be depicted by *in silico* approach. Theplatform technology developed for BCS class II drugs to be used as anticancer.
- Post sterilization characterization for free radical determination of developed nanoparticles can be done to assess scale up feasibility.
- ◈ The toxicity studies for developed liposomes and stealth liposomes can be done and usefulness can be established via *in vitro* cell line studies and *in vivo* study.

Neoplasm or tumor is a mass of tissue formed as a result of abnormal, excessive, uncoordinated, autonomous and purposeless proliferation of cells. The term cancer was coined due to its resemblance with the nature of crab to stick to the part of the body stubbornly. Malignant tumors are cancerous and can be localized or metastasized to otherparts of the body. The microanatomy of Breast reveals presence of stromal tissues, comprising of fatty and fibrous connective

tissues and 10% epithelial tissues comprising of lobules and collecting duct system.[1] The term breast cancer refers to a malignant tumor that has developed from cells in the breast either in the lobules, the milk-producing glands or the ducts, the passages that drain milk from the lobules to the nipple.[2]

Breast cancer is the most common malignancy diagnosed in women worldwide which makes it the second leading cause of cancer deaths among women.[3,4] In India , breast cancer has replaced cervical cancer as the leading cause of cancer deaths among women.[5]Depending on the origin and etiology, breast cancer can be differentiated into many types such as ductal carcinoma insitu, invasive lobular carcinoma, tubular breast carcinoma, medullary breast carcinoma, mucinous breast carcinoma, pappilary carcinoma, phallods tumor, intraductal papilloma etc. Invasive ductal Carcinoma is the most commonly encountered cancer of breast. In spite of extensive clinical and experimental research including epidemiologic studies, its exact etiology remains elusive. Some risk factors that are considered significant are genetic factor, dietary factors, prolonged reproductive life etc. Amongst all excess endogenous estrogen or exogenously administered estrogen is an important factor in the development of Breast cancer[6].

Estrogen plays an important role in regulating the growth and differentiation of normal as

well as malignant breast epithelial cells, through interaction with two nuclear estrogen receptor i.e.ER α and ER β. They also play an active role in the different steps of breast carcinogenesis including cancer progression to

metastasis and in prevention, prognosis and treatment of the disease. Estrogen Receptor positive cancer cells depend on estrogen for their growth, so they can be treated with drugs that block estrogen effects.[8]

Progesterone receptor is a protein found inside cells and is activated by the steroid hormone

progesterone.[9]G protein-coupled receptor (GPR30) binds to 17β-estradiol. Human Epidermal Receptor, HER2 is encoded by *ERBB2*, a known proto oncogene. Epidermal growth factor receptor (EGFR) is one of the first identified important targets of the novel antitumor agents. Approximately half of cases of triple-negative breast cancer and inflammatory breast cancer over express EGFR.[10]

There are several additional Nuclear Receptors present in the biology of breast cancer with potential importance. These include receptors for steroid hormones including androgens and glucocorticosteroids, fat-soluble vitamins A and D, fatty acids, and xenobiotic lipids derived from diet. It is now clear that after Nuclear Receptors activation, both genomic and nongenomic Nuclear Receptors pathways can co-ordinately activate growth factor signaling pathways. Understanding of both Nuclear Receptors functional networks and epithelial cell growth factor signaling pathways have revealed a frequent interplay between Nuclear Receptors and epithelial cell growth factor family signaling that is clinically relevant to breast cancer. [11]

Transferrin receptors are generally found on cell surface, but are over expressed by proliferating malignant cells. Thus these receptors are potential sites for delivering anticancer agents, proteins and genes.[12]

Current treatment of Breast Cancer

Today also the main treatment of breast cancer is surgery when the tumor is localized, followed by chemotherapy, radiation therapy and adjuvant hormonal therapy for Estrogen receptor positive tumors and targeted therapy.

The estrogen sensitive breast cancer can be treated with drugs having various mechanisms of action for blocking estrogen effect. The ovaries are the main source of estrogen so by blocking ovarian function or ovarian ablation, estrogen levels can be reduced by surgically removing the ovaries called oophorectomy or by treatment with radiation or by treatment with drugs called as leutinising hormone releasing hormone

agonists. e.g. Goserelin (Zoladex®), leuprolide (Lupron®). Blocking estrogen production by aromatase inhibitors can be used to block the activity of an enzyme called aromatase, used to make estrogen in the ovaries and in other tissues. e.g. Anastrazole (Arimidex®), letrozole (Femara®), both of which temporarily inactivate aromatase, and exemestane (Aromasin®), that permanently inactivates the enzyme. Selective estrogen receptor modulators (SERMs) bind to estrogen receptors, preventing estrogen from binding. e.g. Tamoxifen citrate (Nolvadex), Raloxifene (Evista), and Toremifene (Fareston). Tamoxifen has been used for more than 30 years to treat hormone receptor-positive breast cancer. Fulvestrant (Faslodex®), attaches to the estrogen receptor and functions as an estrogen antagonist. However, does not show estrogen agonist effects. It is a pure antiestrogen.[13]

HER2+ cancer cells respond to drugs such as the monoclonal antibody trastuzumab in combination with conventional chemotherapy, and this has improved the prognosis significantly. Combining two drugs that target the HER2 protein, trastusumab (Herceptin®) and pertuzumab (Perjeta™), with chemotherapy is a new treatment option for women with HER2-positive Metastatic Breast Cancer [14]

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