"FORMULATION DEVELOPMENT AND CHARACTERIZATION OF IRINOTECAN LIPOSOME"

A Thesis Submitted to

NIRMA UNIVERSITY

in Partial Fulfillment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICS

BY

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May 2019

Certificate

This is to certify that the dissertation work entitled **"FORMULATION DEVELOPMENT AND CHARACTERIZATION OF IRINOTECAN LIPOSOME"** submitted by **Ms. Krupali Thacker with Regn. No. (17MPH107)** in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutics" is a bonafide research work carried out by the candidate at the Department of Pharmaceutics, Institute of Pharmacy, Nirma University under my/our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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Certificate of Originality of Work

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I hereby declare that the dissertation entitled **"FORMULATION DEVELOPMENT AND CHARACTERIZATION OF IRINOTECAN LIPOSOME"**, is based on the original work carried out by me under the guidance of Dr. Mohit Shah Assistant Professor, Department of Pharmaceutics, Institute of Pharmacy, Nirma University and Dr. Hiren Patel, General Manager, R&D, Emcure Pharmaceuticals Ltd.. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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"Words are tools of expressing the feelings but they might be failed miserably when it comes to thanks giving."

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List of Abbreviations

QbD	Quality by Design
QTPP	Quality target Product Profile
DoE	Design of Experiment
OGD	Office of Generic Drug
BE	Bioequivalence
AUC	Area under curve
L	Litre
Kg	Kilogram
%	Percentage
mg	milligram
USP	United States Pharmacopoeia
RPM	Revolution per Minute
CQA	Critical Quality Attributes
PK	Pharmacokinetic
FDA	Food and Drug Administration
°C	Degree Celsius
API	Active Pharmaceutical Ingredient
XRD	X-ray Diffraction
NLT	Not Less than
Min	Minimum
Max	Maximum
MLV	Multi Lamellar Vesicles
NA	Not Applicable
HPLC	High Performance Liquid Chromatography
R&D	Research & Development
No.	Number
nm	Nanometer

UV	Ultraviolet
PSD	Particle size Distribution
qty	Quantity

1.1 Liposome: a general overview

In 90's, Paul Ehrlich (A Germany scientist), declared the term "magic bullet," means chemical carriers which possess the property of choosiness in killing damaged cells without any effect on the living cells. [1] Many of the approaches which are depending on the number of chemical and physical properties were adopted To improve the specificity through drug delivery technology,. [2]

Liposomes is very small bilayer of round shape which are formed from natural phospho lipids, other lipids and cholesterol. Due to their hydrophilic and hydrophobic character and, size of liposomes are favorable mechanism for drug delivery system. Characteristics of liposomes may vary with formulation of lipid, particle size, zeta potential and the method of manufacturing. In addition, the main component of bilayer components like phospholipids and cholesterol regulates the 'fluidity' or 'rigidity' and the zeta of the bilayer of liposome.

Un-saturated phospholipids origin from super natural sources like soybean or egg phosphatidyl choline are far more penetrable and less steady bilayers, whereas the saturated phospho lipids, like dipalmitoylphosphatidylcholine, HSPC, DSPG, DSPC form a intact, rather resistant bilayer Structure [3 - 5]

Table 1.1 Merits and Demerits of liposome [6]

Merits	Demerits
May increase the effectiveness and therapeutic index of drug	In-vivo Little solubility
May increase stability via encapsulation and less free drug	Little half-life of liposome
Are less-toxic, biodegradable, biocompatible, non-	phospholipid have tendency

immunogenic and very flexible for any route of administrations	for oxidation and hydrolysis
Liposomes may reduce the toxicity of the encapsulated active ingredients	Leakage and fusion of encapsulated drug/molecules
Liposomes reduce the exposure to sensitive tissues of toxic drugs	Production cost is very high compare to normal injection
Liposome have Site escaping effect	Only Less molecules stables
Elasticity to couple with site-specific ligands like albumin to achieve active targeting	

When phospholipid hydrated in aqueous solutions, they form closed structure with vesicles because of inherent property of Phospholipids. Any of the hydrophilic or hydrophobic drugs can travel/transport/encapsulate in side these liposomes generate with one or more phospholipid vesicle membranes. as lipids are amphipathic (hydro phobic and hydrophilic) in flora with aqueous medium, the thermo dynamic phase properties and self-inherent features of closing will impact entropically focused impounding of their hydrophobic units into spherical bilayers. These films are called as lamellar. [7].

Liposomes are convinced as a sphere-shaped vesicle having size between 30 nm to some micrometers. Liposomes contains the one or more lipidic bilayer in which polar head parts are arranged in a way to inner and exterior part of aqueous phase. Along with this, self-accumulation of polar heads are not incomplete to conservative bilayer structures which may be governed by temperature, shape, and ecological and preoperational conditions but may self- closed into several kinds of colloidal small particles [8]

In cosmetic and pharmaceutical industries liposomes are widely utilized as carriers for many drugs and materials. Use of liposomes in food and farming productions for encapsulating to produce drug formulation that can capture unsteady compounds (like anti-cancers, antioxidants, bio-active elements, antimicrobials and flavors) and protect their nature is broadly studied.

Both hydrophilic and hydrophobic compounds can entrap in Liposomes, to avoid degradation of the encapsulated drug/molecules and %release of the encapsulated at defined objectives [9-11].

Due to liposome's non-toxicity, biocompatibility, biodegradability and skill to entrap lipophilic and hydrophilic drugs [12] and abridge targeted drug formulation to Cancerous cells [13], it is superior as a new investigated system and commercialized as a drug-formulation system. Severe studies has been performed and continuing on liposomes with the aim of decreasing drug adverse effect and/or site precise delivery. [14-16].

Encapsulation of Liposome technology is the latest method adopted by medicinal detectives to transport drugs that action as healing organizers to the certain body organs. This type of formulation system proposes the site specific delivery of vital amalgamations to the body. Microscopic foams known as liposomes are generated by method called encapsulation, this method encapsulate numerous materials and drugs inside the liposome.

Liposome may form a layer around their molecules, to protect it from the enzymes in stomach, neck, mouth, digestive juices basic solutions, intestinal flora, bile and other components and protect them from all such body fluids. The composition of the liposomes like drugs are shielded from the some of the degradation like oxidation and degradation in normal tissues. This protective phospholipid layer remains intact till the composition of the liposomes is transported to the appropriate specific system, organ or tissue, where the substances need to be consumed [17, 18].

1.1.1 Liposome Classification based on size

Vesicles of liposomes have spherical size which may differ from small (0.025 μm) to large (2.5 μm) bilayer. Furthermore, they are made of one or more bilayer vesicles.

For determination of half-life during circulation of liposome, size of vesicle is a critical parameter. And percentage of drug entrapment inside the liposome is affected by both vesicle size and number of vesicles.

Considering the number of vesicles and particle size of vesicles in liposome, it may also classified as: (1) Multi-lamellar bilayer (MLV) and (2) uni-lamellar vesicle/Bilayer. Uni-lamellar vesicles/bilayer also classified into two sub parts: (1) large uni-lamellar bilayer (LUV) and (2) small uni-lamellar bilayer (SUV) [16-19]. The vesicles with one phospho lipid bilayer sphere enfolding the aqueous solution are uni-lamellar bilayer. The vesicles which have an onion type structure are multi-lamellar liposomes.

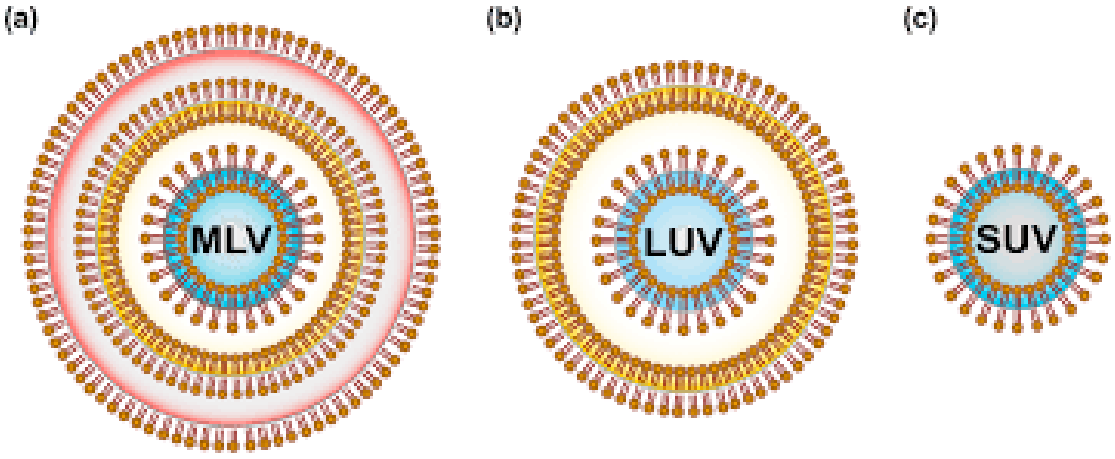


Figure 1.1 (a) Multi-lamellar vesicles, (b) Large uni-lamellar vesicle, (c) Small Uni-lamellar vesicles

1.1.2 Different Method of Liposome "Preparation"

Liposomes are prepared by many different approaches, which contain the usage of power-driven liposome preparation, solvent evaporation, detergent removal from phosphor lipid/detergent vesicle mixtures. "

For preparation of liposome, quantities and classes of phosphor lipid, time of hydration of vesicles and ionic and zeta potential properties of aqueous medium, are key features that regulate the final liposome structure." [20]

Multi-lamellar vesicles preparation

Multi-lamellar vesicles have the easiest method of preparation in all liposome manufacturing methods. For this type of method, liposome generation can be done using solvent for dissolving of phospho lipid and drying/evaporation of the resulted mixture. Amalgamation of phospho lipids such as cholesterol, phosphatidyl choline /phosphatidyl glycerol, egg lecithin in a ratio of molar 0.99:0.89:0.1 are used respectively. In a typical ratio of 2:1 and 1:1 chloroform or a mixture of chloroform and methanol/ethanol are used respectively.

Primarily, each and every lipid constituent needs to be solubilized in the solvent mixture individually, after that mixing them in suitable amount with the other solubilized lipid compound to check the even mixing of the lipids in mixture. Subsequently, nitrogen gas to be used to make/generate a thin film from the mixture. Likewise, in above to eradicate any residue of solvent mixture, the thin film of lipidic compound is adequate to dry entirely in a closed chamber till complete evaporation. [21]

Uni-lamellar vesicles preparation

In liposomes uni-lamellar vesicle/bilayer are the one widely used and most popular type. Uni-lamellar liposome permits a even delivery of encapsulated molecules within a specific internal aqueous medium.

Uni-lamellar liposome can be prepared by many methods like extrusion through membrane filters, ethanol injection, ultra-sonication, freeze-thaw and detergent method. Many scientist has used combination of diverse small uni-lamellar vesicles/bilayer (SUVs) populations to achieve ternary Giant Uni-lamellar Vesicle/bilayer with even property. [22]

Giant Uni-lamellar vesicles manufacturing

Giant liposomes can be prepared by several methods using, non-electrolyte, distilled water and/or zwitterions. The attendance of ions imparting a surface charge causing

attraction between members and preventing the separation of the membrane vesicles during the re-hydration and swelling process. There are many literatures for manufacturing of giant liposomes, using physio logical strength buffers/media (Table 1.2). GUV can be prepared and manufactured using many techniques like, electro formation, giant uni-lamellar liposomes prepared in quick manufacturing, by physio logical buffer/media for preparation of giant uni-lamellar liposomes/vesicles and osmotic shock method.[23] Also, many scientists have used microfluidic as size reduction and reparation of GUV and mechanical characterization.[24]

Table 1.2 Merits and Demerits of Unilamellar Liposome Preparation Methods

Liposomes Manufacturing Method	Demerits	Merits
"Electro formation"[25]	To spread over in little ionic strength buffers/media	Preparation of im-mobilized giant liposomes/vesicles
Rapid preparation of giant liposomes[26]	Buffer/media with ionic little strength (a maximum of 50mM)	Easy, firm single – step manufacturing procedure
Giant unilamellar manufactured in biological buffer[27]	Time unbearable and tedious procedure	By many physiological salt solutions, such as 100mM KCl plus 1mM CaCl2

1.1.3 Drug loading inside the liposomes

There is a two technique for drug loading, which attained either actively (i.e., after liposome formation) or passively (the drug/molecules gets entrapped during liposome/vesicle formation). Hydrophobic and water insoluble drugs/molecules like Amphotericin and anamycin could be straight entrapped into vesicle during lamellar creation, quantity of encapsulation and preservation is directed by drug to lipid ratio. 100 % encapsulation of drug inside the liposome is not attainable, but mostly reliant on the solubility of the drug in the liposome membrane.

Passive drug loading of hydrophobic molecules can be governed by the capability of vesicles to encapsulate aqueous media comprising a dissolved molecule during lamellar formation.

Encapsulation efficiency less than 30% is inadequate with entrapped volume enclosed in the vesicles and molecule solubility. pH gradient is the technique in active loading, where 100 % entrapment can achieve via hydrophilic drugs which have protonizable amine functions" [28],[29].

Table 1.3 Aids of drug loading in liposome

Aids of drug loading in liposome	Cases
Solubility improvement in lipophilic and amphiphilic drugs"	Minoxidil, Peptides, Anthracyclines, Amphotericin B"
Passive loading and specific targeting to the immune system's mono-nuclear phagocytic" cell	Amphotericin B, Antimonials, vaccine, immune-modulators, porphyrins
Extended release delivery of locally and systemically administered liposomes	Cytosine, Doxorubicin, cortisones
Site-avoidance and targeting mechanism	Amphotericin B and Doxorubicin
Targeted Site-specific	Anti-cancer, Anti inflammatory molecules, anti infectious agents
Better transfer of water soluble and surface charge molecules	Chelators, Genes and plasmids
Enhanced penetration into cell and tissues	Anesthetics, Corticosteroids, insulin

1.1.4 Liposomes for anti-cancer therapy

Liposome formulation of anticancer drug is less toxic than the available free drug of anti-cancer molecule [30-34]. Anthracyclines drugs working with the principle of stoping the

growing of separating tissues with interposing into the DNA and because of that destroy mainly rapidly separating tissues. Hair, gastrointestinal mucosa, and blood cells are also having these cells. So, class of molecule is highly toxic.

Maximum applicable, utilized and examined is Adriamycin (Doxorubicin hydrochloride; Ben Venue Labs., Bedford, Ohio). Dosage of drug is also restricted by its increasing cardio toxicity along with above-mentioned acute toxicities with various formulations were tried and studied. In every cases, the high toxicity and adverse effect was decreased up to only 50%. But liposome can decrease the acute and chronic toxicities due to encapsulation of anti-cancer drugs inside the liposome. For the similar aim, the entrapment efficacy is in various cases negotiated due to the decreased bioavailability of the molecule, mainly if the cancer is not located in the organs of mono-nuclear phagocytic system or phagocytic.

In most of the cases like systemic lymphoma, the result of liposome entrapment efficiency displayed better efficacy due to the continuous release effect, i.e., extensive attendance of therapeutic concentration in the blood stream, whereas in many of other cases, the confiscation of the drug/molecule into tissues of mono-nuclear phagocytic system actually decreased its efficiency."

1.2 Irinotecan Hydrochloride tetrahydrate

1.2.1 Pharmacopeial status

“Irinotecan is official in USP, and not official in BP, EP, JP, Chinese Pharmacopoeia and IP.”

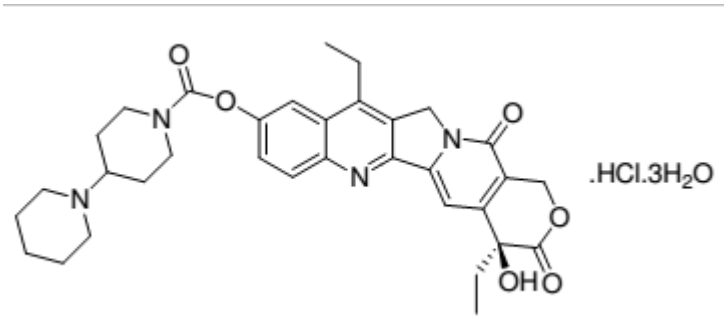
1.2.2 Description [1, 2,3,4]

“Irinotecan is an antineoplastic enzyme inhibitor primarily used in the treatment of colorectal cancer. It is a derivative of camptothecin that inhibits the action of topoisomerase I. Irinotecan prevents religation of the DNA strand by binding to topoisomerase I-DNA complex, and causes double-strand DNA breakage and cell death. It is a derivative of camptothecin. Irinotecan was approved for the treatment of advanced pancreatic cancer in October, 2015 (irinotecan liposome injection, trade name Onivyde).”

IUPAC name; “(S)-[1,4’-bipiperidine]-1’-carboxylic acid, 4, 11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo-1H-pyrano [3’, 4’ :6,7] indolizino [1,2-b] quinolin-9-yl ester monohydrochloride trihydrate”

“Chemical formula; C33H38N4O6. HCl. 3H2O”

“Molecular weight; 677.18 g/mol”



1.2.3 Physical properties [5,6,7,8]

- **Description;** Pale yellow to yellow crystalline powder.
- **“Solubility;** Slightly soluble in water, Methanol and Ethanol”

- **Polymorphism;** X ray diffraction pattern of the test preparation shows significant characrteristic 2 theta values of form B at 7.5, 8.16, 9.42, 10.88 and 12.28º.
- **Stability;** Irinotecan is stable at each conditions. Forced degradation data suggest that degradation of Irinotecan is in 0.1 M NaOH. Also minor degradation with 1% H_2O_2, photolytic and thermal treatments.

1.2.4 Pharmacokinetics [9,10]

1.2.4.1 Absorption: The maximum plasma concentration (Cmax) in patients with solid tumors is 1660 ng/mL when a dose of 125 mg/m^2 is given. The AUC (0-24) is 10,200 ng·h/mL. The Cmax is 3392 ng/mL when a dose of 340 mg/m^2 is given to patients with solid tumors is. The AUC (0-24) is 20,604 ng·h/mL .

1.2.4.2 Metabolism: The metabolism is by Liver. The active metabolite SN-38 is formed by metabolism of Irinotecan which is mediated by carboxylesterase enzymes and primarily occurs in the liver. SN-38 is afterwards conjugated principally by the enzyme UDP-glucuronosyl transferase 1A1 (UGT1A1) to form a glucuronide metabolite .

1.2.4.3 Volume of Distribution: The volume of distribution is 110 L/m^2 when a dose of 125 mg/m^2 is given to patients with solid tumors during of terminal elimination phase. The volume of distribution when a dose of 340 mg/m^2 given to patients with solid tumors is 234 L/m^2 during terminal elimination phase. The drug is 30%-68% bound to protein, mainly to albumin .

1.2.4.4 Route of elimination: The cumulative biliary and urinary excretion of Irinotecan and its metabolites (SN-38 and SN-38 glucuronide) over a period of 48 hours following administration of irinotecan in two patients ranged from approximately 25% (100 mg/m2) to 50% (300 mg/m2).

1.2.4.5 Half-life: 6 - 12 hours is the half-life of Irinotecan, but the active metabolite SN-38 is having terminal elimination half-life, of 10 - 20 hours .

1.2.5 Clinical pharmacology [11,12,13,14]

1.2.5.1 Mechanism of action: Irinotecan is a topoisomerase I inhibitor. Irinotecan prevents demotion of the DNA strand by binding to topoisomerase I-DNA complex. The ternary complex formed of this interferes with the moving replication fork, induces reproduction arrest and lethal double-stranded breaks in DNA. As a result, DNA damage is not efficiently restored and apoptosis (programmed cell death) occurs

1.2.5.2 Indications and usage: It is first-line therapy for the treatment of metastatic colorectal cancer with 5-fluorouracil and leucovorin. Irinotecan is also used in combination with cisplatin for the treatment of wide spreading small cell lung cancer. Irinotecan is presently under research for the treatment of metastatic or recurrent cervical cancer. Also used in combination with fluorouracil and leucovorin for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy

1.2.5.3 Adverse effects: severe diarrhea and extreme suppression of the immune system is the most noteworthy adverse effects of Irinotecan [8]

Diarrhea: Diarrhea associated with Irinotecan is very severe and clinically significant, sometimes leading to severe dehydration requiring hospitalization or intensive care unit admission. This side-effect of Irinotecan is managed with the aggressive use of antidiarrheal such as loperamide or co-phenotrope with the first loose bowel movement

Immunosuppression: Irinotecan adversely affects the immune system. As there is affectedly lowered white blood cell counts in the blood, in particular the neutrophils. The patient also undergo phase suffering from neutropenia (it is a clinically significant decrease of neutrophils in the blood) while the bone marrow increases white cell production to recompense

Aim of present work:

Cancerous cells are likely to nurture rapidly, and chemotherapeutic drugs are acting by killing the fastest growing cells. As these ant cancer drugs are free, so it travel throughout the human body and acting and killing the other normal fastest growing cell also. Damage to normal cells causes severe adverse effects. Here adverse effects are not always bad as it is an effect of chemotherapeutic drug but to normal cells. The normal living cells of the body which are fastest growing and likely to be damaged and affected by chemo drugs are:

1. bone marrow (Blood-forming cells)
2. Hair on the body
3. Normal cells in the digestive tract, mouth and reproductive system

Some of the chemo drug/molecules can harm to the cells in bladder, heart, lungs, kidneys and nervous system. Along with this patient can take some of the medicines which can protect the adverse effect of chemo drug to the normal cell.

Almost all the anti-cancer drug/ molecule used in chemotherapy are highly cyto-toxic to both cancerous as well as the normal cells. Therefore, specific site-targeting the tumor vasculatures is crucial for tumor decrement. In this project work, encapsulation of anti-neoplastic molecules inside the liposome delivery offers protected podia for the site targeting formulation of anti-neoplastic molecules for the action on tumor. Because of encapsulation of Anti-cancer drugs will help to decrease the cytotoxic side effect of drug to normal cell with target specific delivery. Drug inside Liposome delivery offer the opportunity of growing effectiveness while decreasing the toxic adverse effects of anti-neoplastic agents. Liposome with encapsulation of drug can impact the tissue distribution of drug with its pharmacokinetic behaviors.

Irinotecan is the active ingredients used for the chemotherapy with lots of adverse events. But in 2014, Onivyde has launched the liposomal formulation of Irinotecan with more than 10% free drug which again causes the toxicity to normal cell and not able to provide maximum dose to tumor. Also onivyde is patented, so none can enter into market with

using same formulation to give better therapeutic effect with less free drug. So, following are the objective:

1. To develop an Irinotecan liposome, which is having very less toxic effect compare to marketed products in terms of % free drug in the formulation.
2. To Develop an Irinotecan liposome by non-infringing formula and bypassing the patent of onivyde.
3. To develop and characterize the Irinotecan liposome formulation for physicochemical parameter and all in vitro testing.
4. To develop an Irinotecan liposome which is better than marketed formulation (in terms of % free drug) with same efficacy and other parameters.

3.1 Review of work done on Liposome

Zuzanna D; et al,; [1] has proposed the management of different physicochemical properties of liposomes allows the design of particular carriers with the desired pharmacokinetic and pharmacodynamic properties. Most studies regarding liposomal antibiotics deal with aminoglycosides, quinolones, polypeptides, and betalactames. Some of the studies focused on improving pharmacokinetics and reducing toxicity, while others involved enhancing antibacterial activity. In an era of an inundation of increasing bacterial resistance and severe problems in treating bacterial infections, the application of liposomal antibiotic carriers could be useful, but the high cost of liposome preparation and treatment should also be considered

Burkhard V; et al.; [2] has primed liposomes comprising of amikacin and of SPC which is soy phosphatidylcholine, HSPC i.e hydrogenated SPC and PG phosphatidylglycerol or cholesterol were formed with the use of extrusion and evaluated w.r.t content of drug, efficiency of encapsulation of drug, retention of drug in lung lavage fluid, aerosolization stability, and in vitro efficacy against MAI in murine AM. Separation of encapsulated amikacin was carried out by dialysis and ion-exchange adsorption which was

comparatively if taken into consideration was fast, complete and less burdensome than TFF. Content of drug was increasing linearly with the concentration of drug for a fixed lipid concentration from for SPC 1-12% , and for SPC/PG around 5-21% in 7:3 molar ratio liposomes, while the amikacin content remained constant with SPC at 0.5-1% and SPC/PG with 1.5-2% above a concentration of lipid in the array of 20-160 mg/ml for a stable concentration of amikacin

Sriram V; et al.; [3] have studied various characteristics of process development work relevant to aseptic process techniques for liposomes. This article also has discussed the bilayer properties of liposomes and showed the nomenclature used to classify the liposomes. Discussed is the pH gradient method to load liposomes. Issues and challenges involved in prolonging the shelf-life of liposomes is presented. This review covered the importance of complete removal of organic solvent that is used in the process. Finally the authors presented an HPLC method for quick identification and assay of various phospholipids in a mixture of phospholipids

Wassim A.; et al.; [4] has been evaluated freeze-drying as a good technique to improve the long-term stability of colloidal nanoparticles. The poor stability in an aqueous medium of these systems forms a real barrier against the clinical use of nanoparticles. This article reviews the state of the art of freeze-drying nanoparticles. It discusses the most important parameters that influence the success of freeze-drying of these fragile systems, and provides an overview of nanoparticles freeze-drying process and formulation strategies with a focus on the impact of formulation and process on particle stability

Shutao G.; et al.; [5] has shown that drug formulations with use of nanoparticles have been widely in investigation and industrialized in the arena of drug delivery systems as a resources to resourcefully deliver unsolvable drugs to cancer cells. Via these strategies of the improved penetrability and retaining effect, the drug formulations with nanoparticles are skillful of significantly increasing the safety, pharmacokinetic profiles and bioavailability of the directed cure. At this point, the advancement of various nanoparticle

inventions in both investigation and medical presentations is exhaustive with emphasis on the improvement of gene and delivery systems. Specifically, the irreplaceable gains and minuses of liposomes, solid lipid nanoparticles, polymeric nanoparticles, lipid-coated nanoparticles, nanocrystals for battered drug delivery will be studied in detail.

Huguette P.; et al.; [6] have examined new and recent advances for snowballing the bioavailability of anti-bacterial drugs by recent and trending drug delivery systems which are liposomes and other form of nanoparticles. But liposomes are trending drug delivery system these days. Liposomes contain excipients which are phospholipids in the outer layer therefore they are not immediately washed out from the body as they are not considered as foreign material for the body. Also the use of polymers which are biodegradable for body are now a days widely used for anti-microbial therapy and also for site-specific delivery of such drugs reducing the side- effects to other organs. The administration route of the carriers is responsible for their action and therapeutic effect in the body i.e. IV or IM etc. They target the phagocyte system with using antibiotics intravenously. This review majorly focuses on the use and targeting antibiotics using different carriers. From the studies carried out during last years it clearly indicates that liposomes and other Nano particulate drug delivery systems are promising treatment for increasing the targeting of drug at specific site and reducing the toxicity.

Mohammad R.; et al.; [7] has informed about various methods of liposome preparation (including the large scale manufacture) has been reviewed. The advantages and disadvantages of the methods have been described in terms of size distribution and encapsulation efficiency

Katie A.; et al.; [8] Different qualitative and quantitative parameters are to be considered for the action of liposomes. The size and polydispersity index, encapsulation of the drug, drug and lipid ratio distribution in lamellar are the important analytical and bio analytical uses of liposomes. This review is focusing on advantages and disadvantages of liposomes in various ways considering their route, cost, efficacy, availability, manufacturing complexity etc.

Andreas F. et al.; [9] this study reviews about a change in the loading method of the liposomes i.e. doxorubicin liposomes. The past studies showed that doxorubicin HCl can be loaded into liposomes up to 95-98% that too in to large unilamellar vesicles with concentration of 7:3 phosphatidylcholine and cholesterol respectively. This concentration creates a Trans membrane gradient inside the liposomes. Ammonium and sodium salts i.e. acetate, citrate, sulfate, phosphate can be used as remote loading agents. pH of the solution, the buffer used all parameters play a crucial role in loading of drug inside the liposomes. The newly developed method for drug loading is based on the pH difference between the inside and outside environment of the liposomes. The entrapment of doxorubicin inside the liposomes is feasible at physiological pH of the body well as the release is achieved at pH near to 5.2-5.4.

Tatsuhiro I.; et al.; [10]. Doxorubicin liposomes are used for the curative treatment of B cell lymphoma. These liposomes are stealth and pH-sensitive liposomes. The study emphasis on two major aspects one is the release mechanism of liposomes from pH sensitive liposomes. And secondly on retaining the drug inside the liposomes for longer time and reducing the free drug as well as the toxicity. This study have shown various experimental data on change in the molar ratios of various lipids and its effect on the properties of liposomes and its action, available drugs and the free drug. DOPE and cholesterol HSPCC and MPEG 2000 are the various lipids used in the formulation with different concentrations. The acidic environment is very preferable for the release of drug inside the tumor cells.

Donatella P; et al.; [11] have established the in-vitro chemotherapeutic activity of gemcitabine, an anti-neoplastic agent promptly delaminated to the inactive metabolite 2′,2′-difluorodeoxyuridine, entrapping it into unilamellar PEGylated liposomes made up of DSPG / CH / MPEG-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (6:3:1 molar ratio). This work is focusing on use of gemcitabine liposomes for the use of anaplastic thyroid carcinoma with dose 5 mg/kg the treatment is given for 4 weeks. The method is so effective and it contains API less than10 times in concentration; The

formulation can increase the concentration of drug within the tumor cells and with enhanced half- life. The formulation is not showing any adverse effect of blood toxicity

3.2 Review of work done on Irinotecan

Danial E.; et al.; [1] Irinotecan liposomes are used promising drug delivery systems in combination as second line therapy for metastatic pancreatic cancer with leucovorin and 5 –fluorouracil with drug of choice gemcitabine delivering anti-neoplastic drugs to site of action. The liposome are promising drug delivery systems for tumor cells. According to this experiment direct study comparing two different Irinotecan formulations have not been conducted. Severe diarrhea and other hematologic effects As Irinotecan is having side effect of the patient needs to be monitored.

Danielle C.; et al.; [2] the study have demonstrated nano formulation of topoisomerase inhibitor the effectiveness of with other. Anti-neoplastic drugs for synergistic effects with the results for this combination therapy are limited.

Andrea W.; et al.; [3] the study is based on the history of patients treated with gemcitabine and Irinotecan plus fluorouracil as second line treatment for adenocarcinoma. The survival rates have increased in patients after this combination of therapy showing synergistic effects.

Wonhee W.; et al.; [4] the study focuses on the nano particulate formulation of topoisomerase inhibitor i.e Irinotecan the treatment is highly effective with reduced side effects and toxicity in treated patients. This combination of anti cancer drugs have shown promising results in phase 3 trials.

Annette K.; et al.; [5] this study shows combination of irinotecan with other anti-cancer drugs other than fluorouracil to improve the effectiveness and to overcome the resistance. More studies are still under investigation.

Corrie L.; et al.; [6] “this study reviews the change in the loading mechanism of Irinotecan into liposomes. The liposomes are formulated using phospholipid that is DSPG

and the use of cholesterol. The mechanism of drug loading is based on the pH gradient mechanism. Where the internal and external pH difference enhances the drug loading mechanism. The in vivo studies have been conducted for this formulation using IV administrative route. The results obtained have enhanced therapeutic efficacy for metastatic pancreatic cancer. This concludes that the loading method can affect the therapeutic efficacy of the liposomal formulation to great extent.

Section	Title
4.1	Raw materials utilized during work
4.2	Equipment utilized during work

4.1 Materials used in present investigation

S.N.	Materials	Vendor
1.	Irinotecan	Laurus Labs
2.	Hydrogenated soy phosphatidyl choline	Lipoid, Germany
3.	MPEG DSPE 2000	Lipoid, Germany
4.	Cholesterol	Dishman Netherland
5.	Ammonium Sulfate	Merck / Avntor / Pharmonics
6.	Sucrose	Merck / Avntor / Pharmonics
7.	Hydrochloric Acid	Merck / Avntor / Pharmonics
8.	Sodium Hydroxide	Merck / Avntor / Pharmonics
9.	Water for Injection	IN house
10.	Disodium hydrogen phosphate heptahydrtae	Merck / Avntor / Pharmonics
11.	Sodium dihydrogen phosphate monohydrate	Merck / Avntor / Pharmonics
12.	EDTA disodium salt dihydrate	Merck / Avntor / Pharmonics
13.	Copper sulfate	Merck / Avntor / Pharmonics
14.	DSPC	Lipoid, Germany
15.	sodium chloride	Merck / Avntor / Pharmonics
16.	HEPES	Biospectra, Bangor, PA
17.	sucrose octasulfate salt	Merck / Avntor / Pharmonics
18.	Ammonium dihydrogen phosphate	Merck / Avntor / Pharmonics

4.2 Equipment utilized during work

S. N.	Instrument name	Role
1	Overhead stirrer	Stirring / dissolving
2	Magnetic stirrer	Stirring / dissolving
3	HSH-High speed homogenizer	homogenization
4	HPH-High pressure homogenizer	Particle size reduction
5	Extruder	Particle size reduction
6	Rota evaporator	Solvent evaporation
7	Masterflex peristaltic pump	Solution transfer
8	Water bath	Heating
9	Weighing balance	Weighing
10	pH meter	pH measurement
11	Dialysis assembly	Dialysis/ ultrafiltration
12	Hollow fibre assembly	Dialysis/ ultrafiltration
13	Lyophilizer	Lyophilization
14	Spray dryer	Spray drying
15	Zeta sizer	Particle size measurement
16	XRAY diffraction instrument	XRD analysis
17	U V	Analysis
18	HPLC	Analysis
19	SPE Cartridges for free drug measurement	Free drug measurement
20	Bottle rotating apparatus	% in vitro release
21	Osmometer	Osmolality measurement
22	Chiller	Cooling

5.1 Reference listed drug characterization

5.1.1 General details of reference listed product

- Name of Reference : Onivyde®
- Dosage form : Liposomal Intravenous injection for Infusion
- Dosage : Each mL contains
  Irinotecan (in Liposome) …4.3 mg,
  DS Phosphatidyl Choline …..6.81 mg,
  Cholesterol base… 2.22 mg,
  MPEG DSPE 2000….0.12 mg,
  HEPES…4.05 mg,
  Sodium Chloride… 8.42
- Manufacturer : IPSEN Inc

5.1.2 Physical characterization of Reference listed product (Onivyde)

Table 5.1.1 Physical characterization of reference product

Parameters	**Details**
Dosage form	Intravenous Injection
Strength	4.3 mg/mL
Batch no. / lot no	B1848/01
Packing	Single Dose Vial (43mg/10 mL)
Description	• ONIVYDE is a liposomal sterile, white to slightly yellow milky isotonic suspension • Uni-lamellar lipidic bilayer vesicles • Approx. 110 nm (Z-avg) in diameter • Gelated/ precipitated Irinotecan inside the liposome
Storage condition	At 2ºC - 8ºC (36ºF - 46ºF)

Preparation	• Take defined volume of ONIVYDE from the vial. Add 500 mL 5% Dextrose Injection, USP or 0.9% Sodium Chloride Injection, US to ONIVYDE suspension to get desired concentration. And mix gently. • It is a light sensitive, so protect it. • Administration of diluted solution should be completed within 4 hours of preparation at room temperature or within 24 hours of preparation when stored at 2ºC - 8ºC (36ºF - 46ºF). • Diluted solution should come to room temperature before administration.
Administration	Infusion of diluted solution with intra venous injection over 90 minutes. Do not use any filters and throw the unused dispersion.

5.1.3 Chemical analysis of reference product (Onivyde)

Table 5.1.2 Chemical analysis of reference product

Sr. No.	**Testname**	**Results**
1.	Description	white to slightly yellow milky isotonic suspension
2.	Assay of Irinotecan	99.4%

3.	Assay of DSPC	92.1%
4.	Assay of MPEG DSPE 2000	90.5%
5.	Assay of Cholesterol	91.8%
6.	pH of liposome	5.7
7.	%drug Entrapment	88.4%
8.	% Free Irinotecan	11.6% (more than 10%) NOT AS PER FDA requirements
9.	Particle Size (z-avg)	122 nm
10	Zeta Potential	- 24 mV

Remarks: As per USFDA guidance, % free drug for any liposomal formulation must be below 10%. But reference product formulation contains the more than 10% free drug, which may generate the toxicity of free drug inside the body.

5.1.4 Stability study of reference product (Onivyde)

Reference formulation was charged for short term stability study to evaluate the behavior during storage period. As product is stable at 2-8 C only, reference formulation was charged at refrigerated condition only.

Table 5.1.3 Stability data of reference listed drug

Sr. No.	Test	Specification	Initial	1M 2-8ºC	3M 2-8ºC

1.	Description	white to slightly yellow milky isotonic suspension			
2.	Assay of Irinotecan	Between 90.0 % to 110.0%	99.4%	97.4%	95.5%
3.	Assay of DSPC	Between 85.0% to 115.0%	92.1%	90.8%	90.8%
4.	Assay of MPEG DSPE 2000	Between 85.0% to 115.0%	90.5%	90.6%	90.8%
5.	Assay of Cholesterol	Between 85.0% to 115.0%	91.8%	90.6%	89.1%
6.	pH of liposome	Between 5.0 to 7.0	5.7	5.62	5.64
7.	%drug Entrapment	NLT 90%	88.4%	85.4%	83.0%
8.	% Free Irinotecan	NMT 10.0%	11.6%	14.6%	17.0%
9.	Particle Size (z-avg)	NMT 150 nm	122 nm	130 nm	138 nm
10	Zeta Potential	Between -10 to -40 mV	- 24 mV	- 26 mV	- 27 mV

Stability study of reference formulation suggest, % free drug and particle size are increasing during storage. So, for formulation development, % free drug and particle size of formulation needs to control during stability study.

5.2 Preliminary evaluation and Finalization

5.2.1 Objective

For development of non infringing formulation of Irinotecan liposome injection for infusion, which is stable and equivalent to Onivyde, Ipsen Inc, currently approved and being marketed in USA.

Qualitative and Quantitative formulation should be derived by different trials to keeping a test product having identical physico chemical properties as that of the reference product.

5.2.2 Selection of excipient

5.2.2.1 Qualitative

Excipient to be used for Irinotecan liposome injection for infusion were finalized based on the data gathered during the literature survey and Pre-formulation study. The qualitative and quantitative formulation composition of the excipient were same as that used by the reference product considering the guidelines for maintaining the qualitative and quantitative (Q1/Q2) same composition for ANDA (application 505 J) filling. Also innovator has patent for usage of sucrose octa sulfate, remote loading agent will be selected based on different trials.

Table 5.2.1 Selection of Excipients

Ingredients	**RLD**	**Test**
Irinotecan	√	To be established
sucrose octasulfate salt	√	To be replaced by other remote loading agent
DSPC	√	6.81 mg/mL
MPEG-2000-DSPE	√	0.12 mg/mL
cholesterol	√	2.22 mg/mL
HEPES buffer	√	To be established
Sodium chloride	√	To be established

5.2.2.2 Sourcing

All of the excipients needs to be used during development were procured from skilled vendors as mentioned in chapter 4.

5.2.3 “Quality Target Product Profile for Irinotecan Liposome Injection”

“The quality target product profile (QTPP) is a potential summary of the quality features for a drug product that preferably will be attained to confirm the anticipated quality, taking into account efficacy and safety of the drug product”

“The QTPP is the basis of design of the product and includes all product attributes that are needed to ensure equivalent safety and efficacy to the Reference Standard. Based on the label and physicochemical properties of Reference Standard, a quality target product profile was defined and justified as shown in the below table, to guide the development of generic irinotecane Liposome Injection, that is equivalent to the Reference Standard”

Table 5.2.2 Quality Target Product Profile for Irinotecan Liposome Injection		
Quality Attribute of Drug Product	**Target**	**Justification**
Dosage form	Injection	Pharmaceutical equivalence requirement: same dosage form
Route of administration	Intravenous infusion	Pharmaceutical equivalence requirement: same route of administration
Dosage strength	4.3 mg/ML or other	Pharmaceutical equivalence requirement: same strength or new strength for 505 B2
Stability	At least 18-month shelf-life at 2°C-8°C temperature (Intended Storage Condition)	Equivalent to or better than Reference Standard shelf-life
Composition	Qualitatively and quantitatively same as Reference Standard or non infringing formulation	Pharmaceutical equivalence to Reference Standard with 505 B2 application
Drug Product Quality Attributes	Description	Physico chemical equivalence: Must meet the same specification or other Compendial quality standards
	pH at 25°C	
	Assay of Irinotecan	
	Content of Free Irinotecan	
	Content of Entrapped Drug	
	Lipid content: Cholesterol MPEG2000-DSPE sodium DSPC	
	Particle size	
	Zeta Potential	

5.2.4 Identification of critical quality attributes

"A critical quality attribute (CQA) is a chemical, physical, microbiological or biological property or characteristic that should be within a specification limit, range, or distribution to confirm the anticipated product quality. Below table summarizes the quality attributes of generic Irinotecan Liposome Injection and specifies which aspects were classified as

drug product critical quality attributes (CQAs). For this product pH, Assay, Related Substances and other chemical attributes are investigated and discussed in detail in subsequent formulation and process development studies"

Table 5.2.3 Critical Quality Attributes (CQAs) of Proposed Irinotecan Liposome Injection

Quality Attribute of Drug Product	**Target**	**Is this CQA?**	**Justification**
Description	white to slightly yellow opaque isotonic liposome suspension	**Yes**	Description of product is an indirect measure of the quality of the product. Any change in color may impact on product
pH at 25°C	Between 5.0 to 7.0	**Yes**	
Assay of Irinotecan	Between 90.0 to 110.0%	**Yes**	Variability in Assay (API and lipids), free drug and entrapment will affect safety and efficacy; therefore critical.
Content of Free Irinotecan	NMT 10.0%	**Yes**	
Content of Entrapped Drug	NLT 90%	**Yes**	
Lipid content: Cholesterol MPEG2000-DSPE DSPC	Between 85.0 to 115.0%	**Yes**	

Particle size	NMT 150 nm	**Yes**	Variability in particle size will affect pharmacokinetic of product and so safety and efficacy; therefore particle size is critical.
Zeta Potential	Between -10 to -40 mV	**Yes**	Variability in zeta potential will affect aggregation behavior of liposome

5.2.5 Preliminary screening for Drug (API) loading process.

The aim was to develop a stable formulation of Irinotecan Liposome Injection, which is pharmaceutically equivalent (qualitative and quantitatively) to Onivyde, Applicant Ispen. Following pharmaceutical excipients and quantity were used in prototype formulation along with API. For liposome preparation, Active loading and passive loading technique is widely used for manufacturing.

During active loading of drug, initial dummy liposome (without API) to be manufactured with controlling particle size and pH gradients to generate active loading of API inside the liposome. Whereas during passive loading liposome is initially formed with API and then size reduction and dialysis to be performed. Normally for the soft liposome, Active loading process is to be utilized for manufacturing. But considering complexity of development both method was evaluated for deciding the drug loading process.

5.2.5.1 Formulation and method of drug loading: following is the composition and method of drug loading for initial evaluation parameters.

Table 5.2.4 Preliminary screening for Drug (API) loading process

S. N.	**Ingredients**	**Quantity used (mg/mL)**			
		001*	**002***	**003***	**004***
Drug Loading Method		**Active loading**		**Passive Loading**	
Instrument used during drug loading		**Extruder**		**Spray dryer**	**Rota evaporator**

1	Irinotecan Free Base	4.3	4.3	4.3	4.3
2	Sucrose Octasulfate salt	250 mM	-	250 mM	-
3	Sucrose	-	250 mM	-	250 mM
4	DSPC	6.81	6.81	6.81	6.81
5	Cholesterol	2.22	2.22	2.22	2.22
6	MPEG-2000-DSPE	0.12	0.12	0.12	0.12
7	2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulphonic acid (HEPES)	4.05	4.05	4.05	4.05
8	Sodium Chloride	8.42	8.42	8.42	8.42
9	Chloroform	-	-	50.0	50.0
10	Ethanol	79.80	79.80	100.0	100.0
11	Water for Injection	1 mL	1 mL	1 mL	1 mL
*** Quantity in mg/mL**					

5.2.5.2 Manufacturing process

For Batch 1 and 2:

- Dispense all the materials as per requirements.
- Take all the lipids in ethanol and dissolve it at 65ºC till clear solution obtained.
- Take sucrose octasulfate/sucrose in water and dissolve till clear solution and heat at 65ºC.
- Add solvent phase in to aqueous phase to form multivesicular liposome.
- Pass the liposomal dispersion through extruder till desired particle size (below 150 nm).
- Complete the diafiltration to remove the outer sucrose octasulfate using 750 KD dialysis cassette to generate pH gradient.
- Use this dummy liposome for further drug loading.
- Take the drug in to water for injection and dissolve at 50ºC and add in to dummy liposomal dispersion for drug loading.
- After completion of drug loading complete the volume make up and send the sample for analysis.

For batch 3&4:

- Dispense all the materials as per requirements. Take all the lipids and Irinotecan in ethanol chloroform mixture and dissolve it at 65ºC till clear solution obtained.
- Spray dry/ Rota evaporate the solution to generate the powder of liposome.
- Hydrate the powder with sodium octasulfate/sucrose solution at 65ºC and reduce the particle size through HPH/Extruder till desired particle size.
- Complete the volume make up and send the sample for analysis.

5.2.5.3 Evaluation Parameters: Prepared batch was evaluated for the % assay of Irinotecan, % free drug in final liposome, % drug entrapment and particle size of liposome.

5.2.5.4 Results and discussion: Batches were send for analysis and following are the compilation of all results. Data suggest that % free drug is very less in active loading process compare to passive loading process. Due to % free drug, particle size of liposome is also coming high in passive loaded liposome. So, for further batches, active loading of API was selected for manufacturing process.

Table 5.2.5 Results of Preliminary screening for Drug (API) loading process

Batch No.	%Assay	% Free drug	% Entrapment	Particle size (nm)
001*	96.8	18.1	81.9	125
002*	99.0	32	68.0	136
003*	94.8	74.6	25.4	134
004*	96.6	55.3	44.7	156

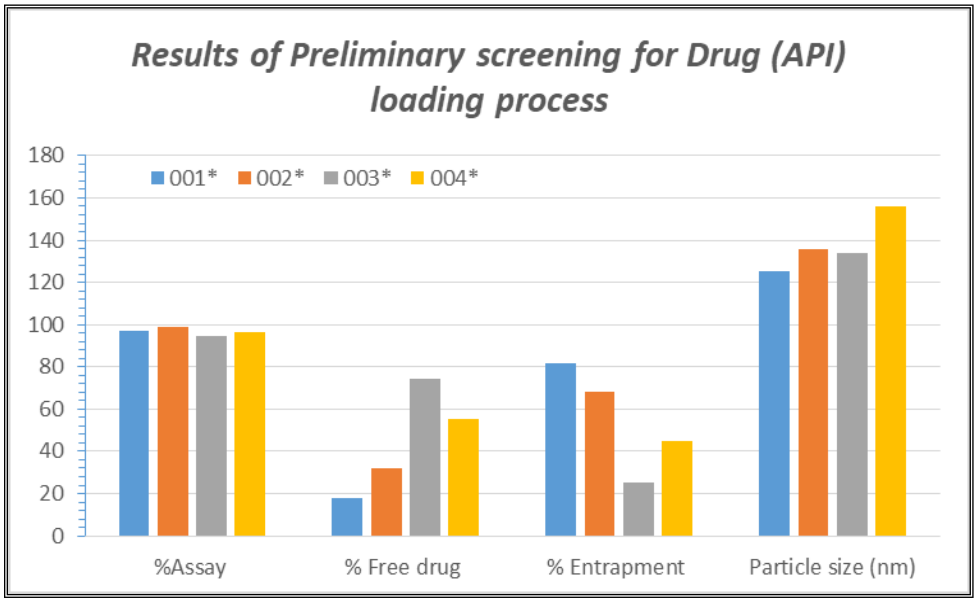


Figure 5.3.1 Results of Preliminary screening for Drug (API) loading process

5.2.6 Preliminary screening for particle size reduction process.

Aim of the trials to optimize and select the particle size reduction method for Irinotecan liposome. Particle size reduction can be done by Extruder, High pressure homogenization at different pressure. Extruder is the instrument which generate less pressure compare to HPH. Following batches were prepared and evaluated for the % free drug.

5.2.6.1 Formulation and method of drug loading: following is the composition and method of drug loading for initial evaluation parameters.

Table 5.2.6 Preliminary screening for for particle size reduction process

S. N.	**Ingredients**	**Quantity used (mg/mL)**			
		005*	**006***	**007***	**008***
Instrument used for particle size reduction		**Extruder**		**HPH**	
Pressure during size reduction (PSI)		**300**	**800**	**15000**	**25000**
1	Irinotecan Free Base	4.3	4.3	4.3	4.3
2	Sucrose Octasulfate salt	250 mM	-	250 mM	-

3	Sucrose	-	250 mM	-	250 mM
4	DSPC	6.81	6.81	6.81	6.81
5	Cholesterol	2.22	2.22	2.22	2.22
6	MPEG-2000-DSPE	0.12	0.12	0.12	0.12
7	2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulphonic acid (HEPES)	4.05	4.05	4.05	4.05
8	Sodium Chloride	8.42	8.42	8.42	8.42
9	Chloroform	-	-	50.0	50.0
10	Ethanol	79.80	79.80	100.0	100.0
11	Water for Injection	1 mL	1 mL	1 mL	1 mL
*** Quantity in mg/mL**					

5.2.6.2 Manufacturing process

- Dispense all the materials as per requirements.
- Take all the lipids in ethanol and dissolve it at 65ºC till clear solution obtained.
- Take sucrose octasulfate/sucrose in water and dissolve till clear solution and heat at 65ºC.
- Add solvent phase in to aqueous phase to form multivesicular liposome.
- Pass the liposomal dispersion through extruder/ HPH at defined pressure (mentioned in above table) till desired particle size (below 150 nm).
- Complete the diafiltration to remove the outer sucrose octasulfate using 750 KD dialysis cassette to generate pH gradient.
- Use this dummy liposome for further drug loading.
- Take the drug in to water for injection and dissolve at 50ºC and add in to dummy liposomal dispersion for drug loading.
- After completion of drug loading complete the volume make up and send the sample for analysis.

5.2.6.3 Evaluation Parameters: Prepared batch was evaluated for the % assay of Irinotecan, % free drug in final liposome, % drug entrapment and particle size of liposome.

5.2.6.4 Results and discussion: Batches were send for analysis and following are the compilation of all results.

Table 5.2.7 Results of Preliminary screening for particle size reduction method

Batch No.	%Assay	% Free drug	% Entrapment	Particle size (nm)
005*	95.8	17.1	82.9	132
006*	97.0	18.4	81.6	116
007*	101.8	29.6	70.4	104
008*	102.3	35.3	64.7	86

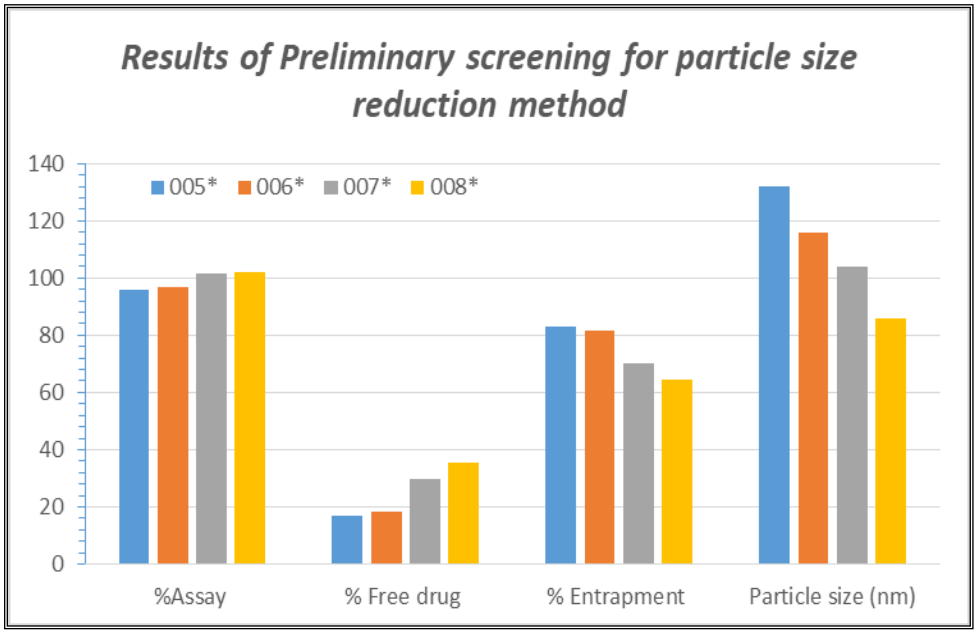


Figure 5.3.2 Results of Preliminary screening for Particle size reduction method

Data suggest that % free drug is very less in batches prepared with Extruder for particle size reduction compare to HPH at high pressure. During HPH, liposome is breaking with uneven particle size, which generate the more % free drug compare to very uniform

liposome with extrusion process. So, Extrusion was selected for particle size reduction method for further trials.

5.2.7 Preliminary screening for Active/remote drug loading agents.

Active loading process with extrusion was selected for the final formulation. But active loading agent for pH gradient and % entrapment of Irinotecan is most important to achieve the higher entrapment. Also, innovator has taken the patent for sucrose octasulfate as a remote loading agent. So formulation should be free from sucrose octa sulfate and with new remote loading agent to get more % drug entrapment with low % free drug. Followings are the different trials taken with different remote loading agents to check the efficacy with respect to % free drug.

5.2.7.1 Composition and method of drug loading: following is the composition with different remote loading agent.

Table 5.2.8 Preliminary screening for Active/remote drug loading agents

S. N.	Ingredients	Quantity used (mg/mL)								
		009*	**010***	**011***	**012***	**013***	**014***	**015***	**016***	**017***
1	Irinotecan Free Base	4.3	2.0	4.3	4.3	2.0	4.3	2.0	4.3	2.0
2	Sucrose Octasulfate salt	250 mM	250 mM	-	-	-	-	-	-	-
3	Sucrose	-	-	250 mM	-	-	-	-	-	-
4	Ammonium sulfate	-	-	-	250 mM	250 mM	-	-	-	-
5	Copper sulfate	-	-	-	-	-	250 mM	250 mM	-	-
6	Ammonium dihydrogen phosphate	-	-	-	-	-	-	-	250 mM	250 mM
7	DSPC)	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81
8	Cholesterol	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22
10	MPEG-2000-DSPE	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
11	2-[4-(2-hydroxyethyl) piperazin-1-	4.05	4.05	4.05	4.05	4.05	4.05	4.05	4.05	4.05

	yl]ethanesulphonic acid (HEPES)									
12	Sodium Chloride	8.42	8.42	8.42	8.42	8.42	8.42	8.42	8.42	8.42
13	Ethanol	79.80	79.80	79.80	79.80	79.80	79.80	79.80	79.80	79.80
14	Water for Injection	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
*** Quantity in mg/mL**										

5.2.7.2 Manufacturing process

- Dispense all the materials as per requirements.
- Take all the lipids in ethanol and dissolve it at 65ºC till clear solution obtained.
- Take remote loading agent (as per above formula table) in water and dissolve till clear solution and heat at 65ºC.
- Add solvent phase in to aqueous phase to form multivesicular liposome.
- Pass the liposomal dispersion through extruder till desired particle size (below 150 nm).
- Complete the diafiltration to remove the outer sucrose octasulfate using 750 KD dialysis cassette to generate pH gradient.
- Use this dummy liposome for further drug loading.
- Take the drug in to water for injection and dissolve at 50ºC and add in to dummy liposomal dispersion for drug loading.
- After completion of drug loading complete the volume make up and send the sample for analysis.

5.2.7.3 Evaluation Parameters: Prepared batch was evaluated for the % assay of Irinotecan, % free drug in final liposome, % drug entrapment of liposome.

5.2.7.4 Results and discussion: Batches were send for analysis and following are the compilation of all results. Data suggest that batches with an Ammonium dihydrogen phosphate shows better drug entrapment compare to all other remote loading agent. Even Ammonium dihydrogen phosphate shows better drug entrapment with less free drug

compare to sucrose octa sulfate, which is used by innovator. So, formula was finalized with Ammonium dihydrogen phosphate and other ingredients are same as innovator.

Table 5.2.9 Results of Preliminary screening for Active/remote drug loading agents			
Batch No.	**%Assay**	**% Free drug**	**% Entrapment**
009*	97.4%	16.1%	83.9%
010*	98.4%	14.2%	85.8%
011*	99.5%	38.4%	71.6%
012*	100.2%	19.6%	80.4%
013*	101.1%	20.4%	79.6%
014*	103.2%	11.6%	88.4%
015*	102.4%	10.4%	89.6%
016*	100.3%	7.3%	92.7%
017*	98.4%	6.4%	93.6%

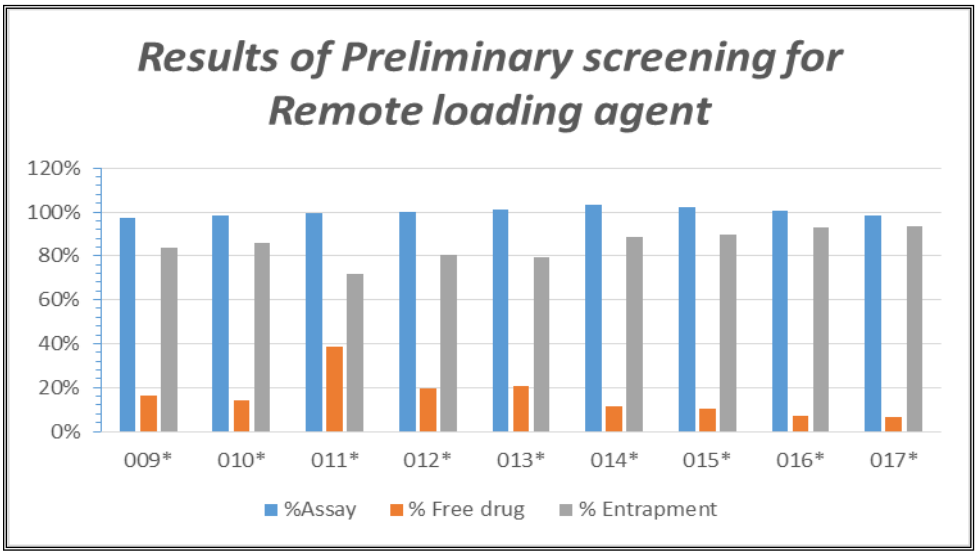


Figure 5.3.3 Results of Preliminary screening for Remote loading agent

Conclusion: For better entrapment of drug inside the liposome and less free drug, formula was finalized with ammonium dihydrogen phosphate as a remote loading agent. Further process optimization and design space for process parameters will be generated using risk assessment approach.

5.3 Process Optimization, Results and Discussion

After formulation development, Process optimization activities should be evaluated. To meet all product specification with a uniformity, Process optimization activity should be carried out to define the process parameters. Formula and process optimization includes, evaluation of key process ingredients that affect the product attributes and evaluation of generic and specific equipment that may require. After finalizing the prototype formulation, different critical process parameters were taken for the process optimization.

Following are the other attributes that were identified during product development having potential influence on product quality, performance and manufacturing:

1. Attribute of drug substance in the drug product.
2. Effect of process parameters (e.g.Hydration time, Temperature, Extrusion parameters etc.)

Figure 5.3.1 Process Flow of Irinotecan Liposome Injection

A good formulation must be manufacturable, chemically and physically stable throughout the manufacturing process and product shelf life and bioequivalent to the Reference Standard. The formulation was evaluated for robustness around the prototype formulation. In the formulation development studies, acceptable range for the high risk attributes have been established and are included in the control strategy.

The Critical Material Attributes (CMA) have been identified during development which may affect the Critical Quality Attributes (CQAs) of the drug product.

5.3.1 Preliminary Risk Assessment of the Manufacturing Process Variables

“The initial risk assessment includes prior knowledge and experience with related formulations and information about drug substances from published literature and

characterization. The outcome of the initial risk assessment of the manufacturing process variables are presented in below table and the explanation for risk assessment is presented in below table"

Table 5.3.1 Preliminary Risk Assessment of the Manufacturing Process Variables

Quality attributes of the drug product	Manufacturing Process Variables								
	Lipids Concentration in ethanol	Hydration temperature	Hydration time	Extruder temperature	Extruder pressure	Drug loading heating temperature	Drug loading heating time	Drug loading cooling temperature	Drug loading cooling time
Content of Free Irinotecan	Low*	Low*	Low*	Low*	Low*	High*	High*	High*	High*
% Assay of Irinotecan	Low*	Low*	Low*	Low*	Low*	High*	High*	High*	High*
Particle size	High*	High*	High*	High*	High*	High*	High*	High*	High*

The justification of the preliminary risk assessment is provided in below table.

Table 5.3.2 Justification for Initial Risk Assessment for the Process Variables

Formulation Variables	Drug Product CQAs	Justifications
Lipids Concentration in ethanol	Particle size	Lipid concentration is directly proportional to initial particle size after hydration, the risk is high.
	Content of Free Irinotecan	Lipids Concentration in ethanol is unlikely to affect any of these CQAs of drug product. The risk is low.
	% Assay of Irinotecan	
Hydration temperature	Particle size	Hydration temperature can affect the initial particle size after hydration, the risk is high.
	Content of Free Irinotecan	Hydration temperature is unlikely to affect any of these CQAs of drug product as API is not incorporated in this step. The risk is low.
	% Assay of Irinotecan	

Hydration time	Particle size	Hydration time can affect the initial particle size after hydration, the risk is high.
	Content of Free Irinotecan	Hydration time is unlikely to affect any of these CQAs of drug product as API is not incorporated in this step. The risk is low.
	% Assay of Irinotecan	
Extruder temperature	Particle size	Extruder temperature can affect the particle size reduction process, the risk is high.
	Content of Free Irinotecan	Extruder temperature is unlikely to affect any of these CQAs of drug product as API is not incorporated in this step. The risk is low.
	% Assay of Irinotecan	
Extruder pressure	Particle size	Extruder pressure is direcly proportional to particle size reduction process, the risk is high.
	Content of Free Irinotecan	Extruder pressure is unlikely to affect any of these CQAs of drug product as API is not incorporated in this step. The risk is low.
	% Assay of Irinotecan	
Drug loading heating temperature	Content of Free Irinotecan	Content of Free Irinotecan is dependent on the temperature provided during drug loading process, the risk is high.
	% Assay of Irinotecan	Drug loading heating temperature directly affect the %Assay of the drug product, the risk is high.
	Particle size	Particle size can be affected by the temperature provided during the drug loading as Irinotecan salt formation can be affected by temperature provided,

		the risk is high.
Drug loading heating time	Content of Free Irinotecan	Content of Free Irinotecan is combinely dependent on the temperature and time provided during drug loading process, the risk is high.
	% Assay of Irinotecan	Drug loading heating temperature as well as temperature directly affect the % Assay of the drug product, the risk is high.
	Particle size	Particle size can be affected combinely by the temperature and time provided during the drug loading as Irinotecan Salt formation can be affected by temperature and time provided, the risk is high.
Drug loading cooling temperature	Content of Free Irinotecan	Content of Free Irinotecan is dependent on the cooling temperature provided during drug loading process, the risk is high.
	% Assay of Irinotecan	Drug loading cooling temperature directly affect the % Assay of the drug product, the risk is high.
	Particle size	Particle size can be affected by the temperature provided during the drug loading cooling temperature as Irinotecan Salt formation can be affected by temperature provided, the risk is high.
Drug loading cooling time	Content of Free Irinotecan	Content of Free Irinotecan is combinely dependent on the temperature and time provided during drug loading process, the risk is high.
	% Assay of Irinotecan	Drug loading cooling temperature as well as time directly affect the % Assay of the drug product, the risk is high.
	Particle size	Particle size can be affected combinely by the

		temperature and time provided during the drug loading as Irinotecan Salt formation can be affected by temperature and time provided, the risk is high.

5.3.2 Manufacturing Process Optimization Studies

Manufacturing process optimization concentrated on assessment of the high risk process variables as identified in the preliminary risk assessment. Initial drug product development trials were performed based on above mentioned excipient selection. Manufacturing process optimization studies were conducted at laboratory scale. Details of Instrument and the related process parameters utilized in these studies are listed in following table.

Table 5.3.3 Instrument and Process Parameters utilized in manufacturing process optimization Studies

Process Step	Equipment
Hydration	• Stirred Water Bath • Over head Stirrer (Model: RQG-121D) • Magnetic Stirrer • Glass beaker
Size reduction by Extrusion	• Thermobarrel Extruder (Make: Northern Lipid) • Compressed Nitrogen Supply
Ultrafiltration	• Ultrafiltration Assembly containing 300 KD membrane (Make: Sartorius)
Drug Loading and Volume make up	• Stirred Water Bath • Over head Stirrer (Model: RQG-121D) • Magnetic Stirrer • Glass beaker
Filtration and filling	• Lab scale filtration assembly

5.3.3 Manufacturing process optimization Study #1: Optimization of processing variables using factorial design for Hydration and Extrusion process

Formulation optimization study was accompanied to estimate the effect of Lipids Concentration in ethanol, Hydration temperature, Hydration time, Extruder temperature and Extruder pressure on Particle size. The goal of manufacturing process optimization study was to understand if there were any interaction of these variables on studied responses. A $2^{(5-1)}$ fractional factorial Design of Experiments (DOE) with two centre points was used to study the impact of these process factors on the response variables listed in below table. Table summarizes the factors and responses studied

Table 5.3.4 Formulation and process variables of Irinotecan Liposome Injection

Formulation/ Process variables		**Levels**	
		-1	**+1**
A	Lipids Concentration in ethanol (mg/g)	100.00	300.00
B	Hydration temperature (°C)	60.00	70.00
C	Hydration time (min)	10.00	50.00
D	Extruder temperature (°C)	60.00	70.00
E	Extruder pressure (psi)	300.00	800.00
Responses		**Acceptance criteria**	
R1	PSD D90 after hydration	500-4000 nm	
R2	PSD D90 after extrusion	100-175 nm	

The concentration of the lipids in ethanol may play a major role in the formation of liposomes upon injecting into the ammonium sulfate solution. In order to check the effect of concentration of lipids on the CQAs of liposomes formed, various concentration of lipids in ethanol were evaluated. The liposomes will be formed at a temperature just above its glass transition temperature of the lipids used. In order to check the effect of temperature on the hydration of the liposomes, different hydration temperatures were used and the effect on CQAs like particle size were evaluated.

After injection of lipid solution into the buffer, the liposomes formed were allowed to hydrate in the buffer at higher temperature for certain period of time and the effect of hydration time at higher temperature on the CQAs like particle size etc were studied. The temperature of the liposomal suspension shows a main character in the ease of extrusion. The temperature to be used will depend on the lipid composition of the liposomes. The operational temperature for extrusion should be above the glass transition temperature of the liposomal suspension for easy size reduction. Different temperatures were used for checking the effect on CQAs like particle size during extrusion process.

The size reduction of the liposomes is based on the pressure at which it is pushed into the extruder containing Nucleopore membranes. In general, more the pressure applied, faster the extrusion process. Experiments were carried out by performing extrusion at various pressures to check the effect of pressure on CQAs.

5.3.3.1 Composition and Manufacturing process: Composition and Manufacturing process parameters for Irinotecan liposome injection was similar as labelled in section 5.2.7.

5.3.3.2 Evaluation parameter: As these were optimization batches, particle size after hydration and extrusion were the parameters for assessment.

5.3.3.3 Results and Discussion: The experimental results for particle size after hydration and extrusion are presented in below Table.

Table 5.3.5 Analytical result of DoE batches of Irinotecan Liposome Injection

Batch No.	Formulation Process Variables (Independent Variables)	Responses (Dependent Variables)

	A: Lipid Concentration (mg/g)	B: Hydration Temperature (°C)	C: Hydration time (min)	D: Extrusion Temperature (°C)	E: Extrusion Pressure (PSI)	Particle size - D90 After hydration (nm)	Particle size - D90 After Extrusion (nm)
18	100.0	70.0	10.0	60.0	300.0	1050	160
19	100.0	70.0	10.0	70.0	800.0	591	156
20	100.0	70.0	50.0	70.0	300.0	746	154
21	100.0	60.0	10.0	60.0	800.0	839	161
22	300.0	60.0	50.0	70.0	300.0	2240	164
23	300.0	60.0	10.0	60.0	300.0	2420	160
24	300.0	70.0	50.0	70.0	800.0	3090	148
25	100.0	60.0	50.0	70.0	800.0	727	147
26	200.0	65.0	30.0	65.0	550.0	3750	150
27	300.0	60.0	50.0	60.0	800.0	2550	148
28	100.0	60.0	10.0	70.0	300.0	708	146
29	300.0	70.0	10.0	70.0	300.0	2750	157
30	200.0	65.0	30.0	65.0	550.0	1380	157
31	300.0	60.0	10.0	70.0	800.0	2270	152
32	100.0	70.0	50.0	60.0	800.0	793	151
33	300.0	70.0	10.0	60.0	800.0	3360	150
34	100.0	60.0	50.0	60.0	300.0	1040	179
35	300.0	70.0	50.0	60.0	300.0	3170	164

5.3.3.3.1 Effects of independent variables on D90 after hydration:

Pareto chart: The Pareto chart for the effect of selected independent variables on the D90 after hydration is shown in below figure

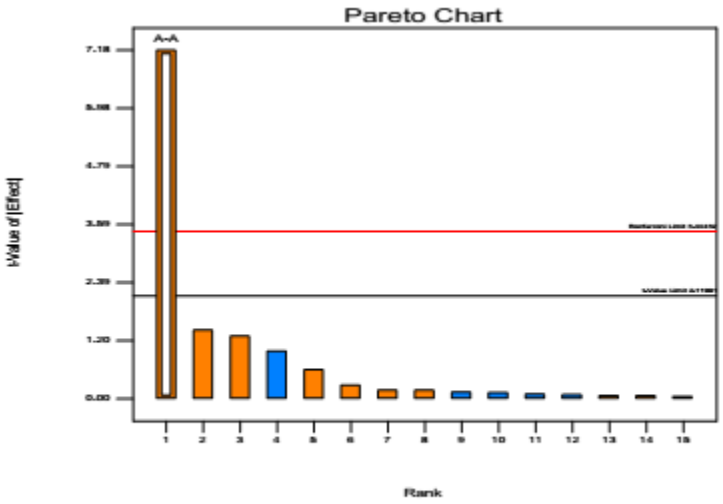


Figure 5.3.24 Pareto Chart for selection of significant effects on D90 after hydration

ANOVA table: The selected factors were statistically analyzed and the results of ANOVA analysis are represented in below table.

Table 5.3.6 ANOVA analysis of D90 after hydration step

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1.474E+007	1	1.474E+007	43.59	< 0.0001
A-Lipid concentration	1.474E+007	1	1.474E+007	43.59	< 0.0001
Residual	5.409E+006	16	3.381E+005	-	-
Lack of Fit	2.601E+006	15	1.734E+005	0.062	0.9989

The Model F-value with 43.59 suggests the model is significant. Here only a 0.01% chance and that could happen due to noise. Values of Prob > Fless than 0.0500 suggest model terms are significant. The Lack of Fit F-value of 0.06 specify the Lack of Fit is not significant virtual to the pure error. There is a 99.89% chance that a lack of Fit F-value this large could occur due to noise. Furthermore, Adeq Precision was found to be 9.904 which indicates an adequate signal

Response plots: The response plots including contour plots and 3D surface plots of all the significant model terms are depicted in succeeding section

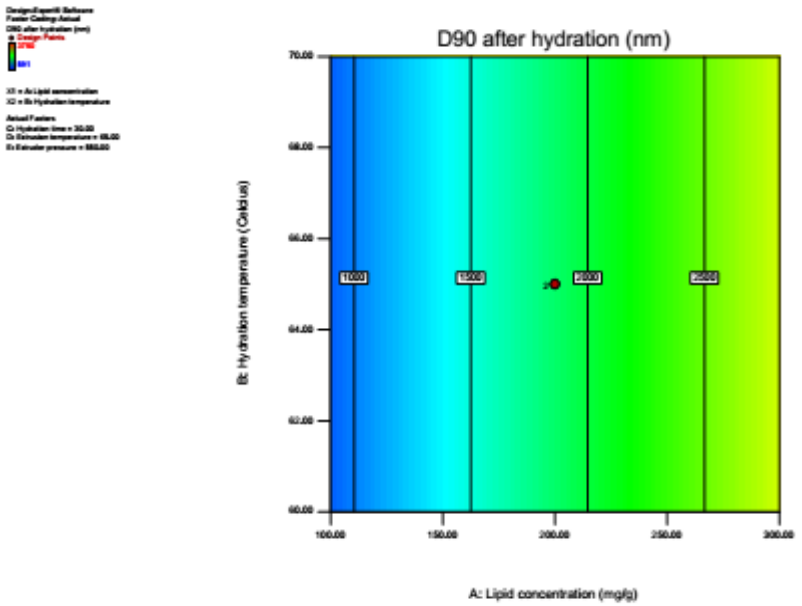


Figure 5.3.35 Contour plot for effect of lipid concentration and hydration temperature on D90 after hydration

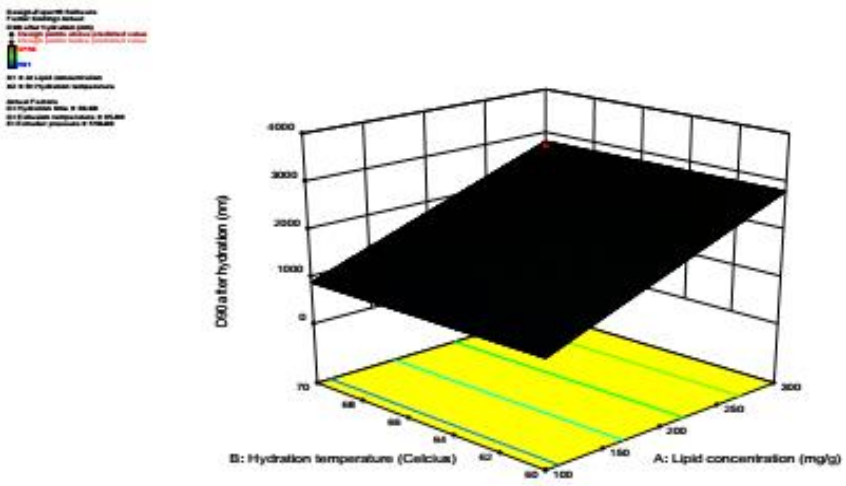


Figure 5.3.46 3D surface plot for effect of lipid concentration and hydration temperature on D90 after hydration

5.3.3.3.2 Effect of factors on D90 after extrusion:

Pareto chart: **"**The Pareto chart for the effect of selected independent variables on the D90 after extrusion is shown in below figure"

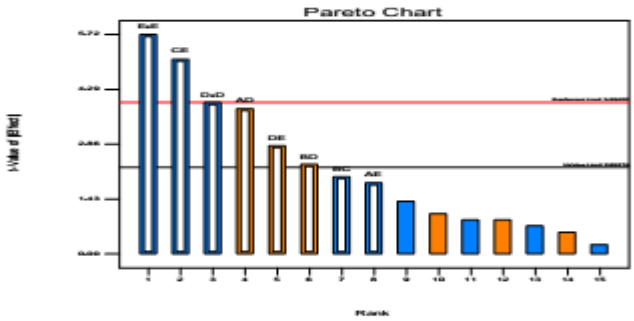


Figure 5.3.5 Pareto Chart for selection of significant effects on D90 after extrusion

ANOVA Table: The selected factors were statistically analysed and the results of ANOVA analysis are represented in below table.

Table 5.3.7 ANOVA analysis of D90 after Extrusion step

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1052.50	8	131.56	13.36	0.0004
D-Extrusion temperature	150.06	1	150.06	15.24	0.0036
E-Extruder pressure	315.06	1	315.06	32.00	0.0003
AD	138.06	1	138.06	14.02	0.0046
AE	33.06	1	33.06	3.36	0.1001
BC	39.06	1	39.06	3.97	0.0776
BD	52.56	1	52.56	5.34	0.0462
CE	248.06	1	248.06	25.20	0.0007
DE	76.56	1	76.56	7.78	0.0211
Residual	88.61	9	9.85		
Lack of Fit	64.11	8	8.01	0.33	0.8815
Pure Error	24.50	1	24.50		
Cor Total	1141.11	17			

“F-value of 13.36 suggests the model is significant. Here only a 0.04% chance that and that occur due to noise. Values of "Prob > F" less than 0.0500 suggest model terms are significant. The Lack of Fit F-value of 0.33 specify the Lack of Fit is not significant relative to the pure error. There is a 88.15% chance that a Lack of Fit F-value this large could occur due to noise. Furthermore, Adeq Precision was found to be 13.972 which indicates an adequate signal”

Response plots: The response plots including contour plots and 3D surface plots of all the significant model terms are depicted in succeeding section.

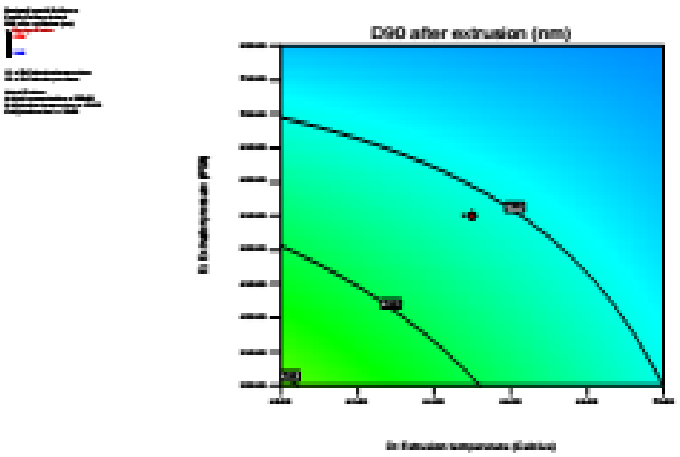


Figure 5.3.6 Contour plot for effect of extruder pressure and extrusion temperature on D90 after extrusion

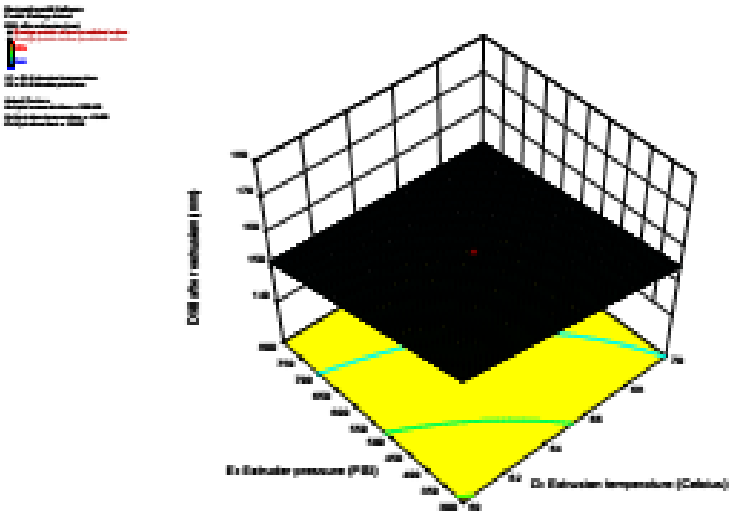


Figure 5.3.7 3D surface plot for effect of extruder pressure and extrusion temperature on D90 after extrusion

The response parameter D90 after extrusion is dependent on extruder pressure and extrusion temperature, which have inverse relationship on particle size and thus D90 decrease with increase in the above mentioned factors.

Conclusion for optimization study I: The overlay contour plots of selected independent variable upon the response under study are shown in preceding section. The yellow zone indicates the design space, where all selected response were estimated to be within desired acceptance criteria. The overlay contour plots demonstrated that all the center point of the selected design were found to be within the design space and hence chosen as an optimized formula or process parameters as per below table.

Table 5.3.8 Optimized Process Parameters for Manufacturing process optimization Studies of Irinotecan Liposome Injection

Sr. No.	Formulation/ Process variables	Optimized Process parameters
1	Lipids Concentration in ethanol (mg/g)	200.00
2	Hydration temperature (°C)	65.00
3	Hydration time (min)	30.00
4	Extruder temperature (°C)	65.00
5	Extruder pressure (psi)	300.0 – 800.0

5.3.4 Manufacturing process optimization Study #2: Optimization of processing

variables using factorial design for Active Drug loading process

As studied in extrusion process, the temperature of liposomal suspension plays a major role in the drug loading into liposomes. The drug enters into the liposome and forms a crystalline gel structure. The loading of the drug into the liposome should be done at a temperature above the glass transition temperature of the lipids used. In order to check the effect of loading temperature on the CQAs like particle size, free drug at different temperatures were evaluated.

The drug solution is added to the liposomal suspension and it was allowed to incubate for certain period of time at a higher temperature in order to load the drug inside the liposomes. As the loading is done at higher temperature, it is necessary to perform the loading for an optimum period of time, so that complete loading is achieved without degradation of the lipids.

In order to check the effect of loading time on the CQAs like particle size, free drug at different loading times were evaluated. After loading of drug into the liposomes, the liposomal suspension is cooled down to make the lipid bilayer rigid so that drug leakage from inside can be avoided. In order to check the effect of cooling temperature on the CQAs like particle size, free drug at different cooling temperatures along with time were evaluated.

Considering drug loading process, Manufacturing process optimization study was accompanied to estimate the effect of Drug loading heating temperature, Drug loading heating time, Drug loading cooling temperature and Drug loading cooling time on Particle size and Free drug. The goal of manufacturing process optimization study was to recognize if there were any interface of these variables on deliberated responses.

A $2^{(4-1)}$ fractional factorial Design of Experiments (DOE) with one center points was utilized to evaluate the effect of these formulation features on the response variables listed in below table. Table summarizes the factors and responses studied.

Table 5.3.9 Formulation process variables for active drug loading process of Irinotecan Liposome Injection			
Formulation process variables (Independent variables)		**Levels**	
		-1	**+1**
A	Drug loading heating temperature	60.00	70.00
B	Drug loading heating time	30.00	90.00
C	Drug loading cooling temperature	2.00	15.00
D	Drug loading cooling time	10.00	50.00
Responses		**Acceptance criteria**	
R1	Particle size (Z- Avg)	50-150 nm	
R2	% Free drug (Irinotecan)	0-5 %	

Here, 2 level, 4 factor fractional factorial design $2^{(4-1)}$ with 10 trials including 2 center points was selected for optimization study.

5.3.4.1 Composition and Manufacturing process: Composition and Manufacturing process parameters for Irinotecan liposome injection was similar as depicted in section 5.2.7.

5.3.4.2 Evaluation parameter: As these were optimization batches, particle size after (z-avg) and % free drug (Irinotecan) were the factors for assessment.

5.3.4.3 Results and Discussion: The experimental results for particle size after (z-avg) and % free drug (Irinotecan) are presented in below Table.

Table 5.3.10 Results of DOE trials for optimizing process of Active drug Loading

Batch No.	Formulation process variables (Independent variables)				Responses (Dependent Variables)	
	Drug loading heating temperature (ºC)	Drug loading heating time (ºC)	Drug loading cooling temperature (ºC)	Drug loading cooling time (ºC)	Particle size (nm)	%Free drug
36	70.00	30.00	2.00	50.00	89.9	0.6
37	70.00	90.00	2.00	10.00	91.3	0.7
38	70.00	30.00	15.00	10.00	89.7	20.8
39	60.00	30.00	2.00	10.00	90.5	3.3
40	65.00	60.00	8.50	30.00	89.2	0.64
41	60.00	30.00	15.00	50.00	89	1.52
42	70.00	90.00	15.00	50.00	90.8	0.65
43	60.00	90.00	15.00	10.00	90.1	0.4
44	65.00	60.00	8.50	30.00	90.8	0.7
45	60.00	90.00	2.00	50.00	90.5	0.5

Following is the outcome for each process variable with respect to different process parameters and response.

5.3.4.3.1 Effect of independent variables on particle size (Z-average):

Pareto chart: "The Pareto chart for the effect of selected independent variables on the particle size Z-average is shown in below figure"

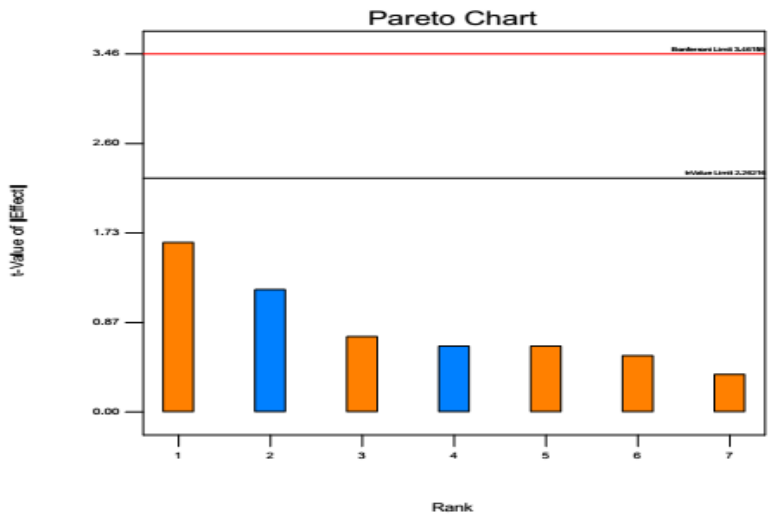


Figure 5.3.8 Pareto Chart for selection of significant effects on particle size Z-average

The particle size of final formulation was controlled in desired range using extrusion process, thus particle size of the final formulation was found to be independent of all the processing parameters during active drug loading of Irinotecan liposome Injection, concluding that the selected factors have insignificant effect on response.

5.3.4.3.2 Effect of independent variables on free drug content:

Pareto chart: "The Pareto chart for the effect of selected independent variables on the total impurity is shown in below figure"

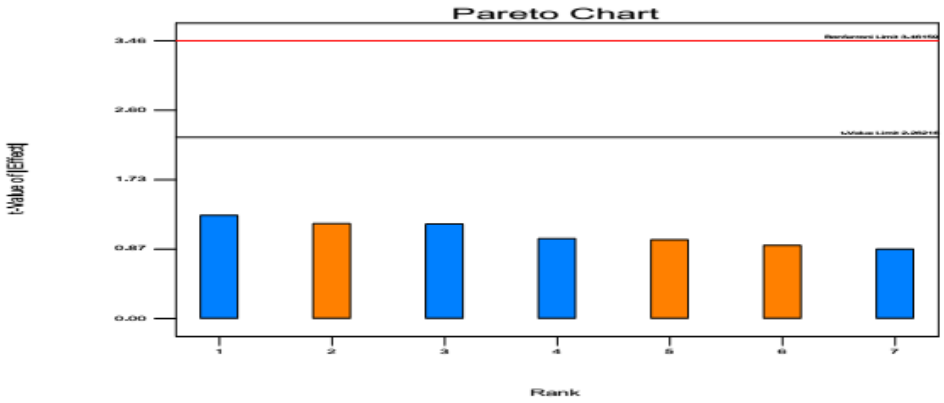


Figure 5.3.9 Pareto Chart for selection of significant effects on free drug content

Irinotecan is highly prone to entrapped inside the liposome because of pH gradient, so within a specified process variables, it is able load inside the liposome. Due to loading mechanism of Irinotecan, free drug content of final formulation were found insignificant in studied range of all the processing parameters (independent variables).

5.3.4.3.3 Summary of manufacturing process optimization Study #2- Optimization of processing variables using factorial design for Active Drug loading process

The design space for processing parameters during active drug loading step of Irinotecan Liposome Injection is shown in figures below.

5.3.4.3.3.1 Design space for Drug loading heating temperature and Drug loading cooling temperature

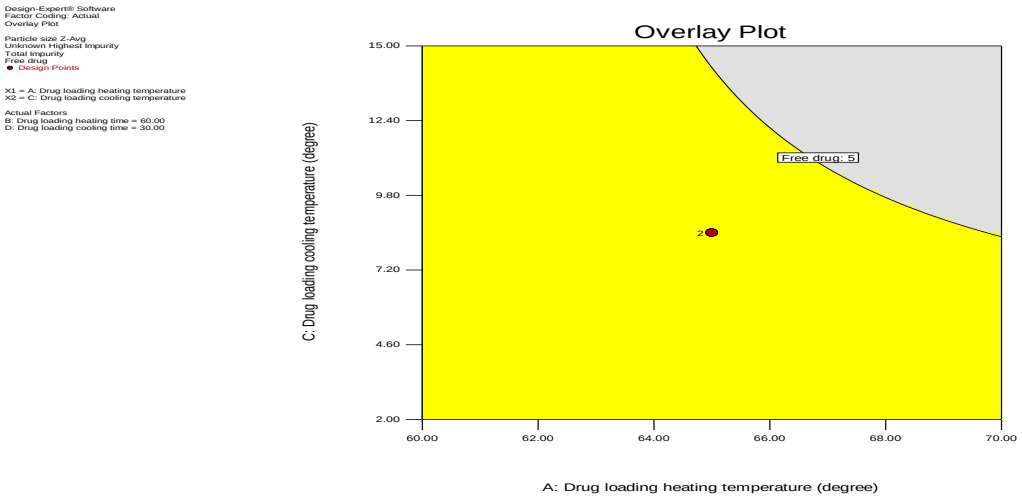


Figure 5.3.10 Design space for heating temperature and cooling temperature

Remarks: Less drug loading cooling temperature with optimized drug loading heating temperature generate very less free drug. High drug loading cooling temperature with high drug loading heating temperature generate high amount of free drug.

5.3.4.3.3.2 Design space for Drug loading heating temperature and Drug loading cooling time

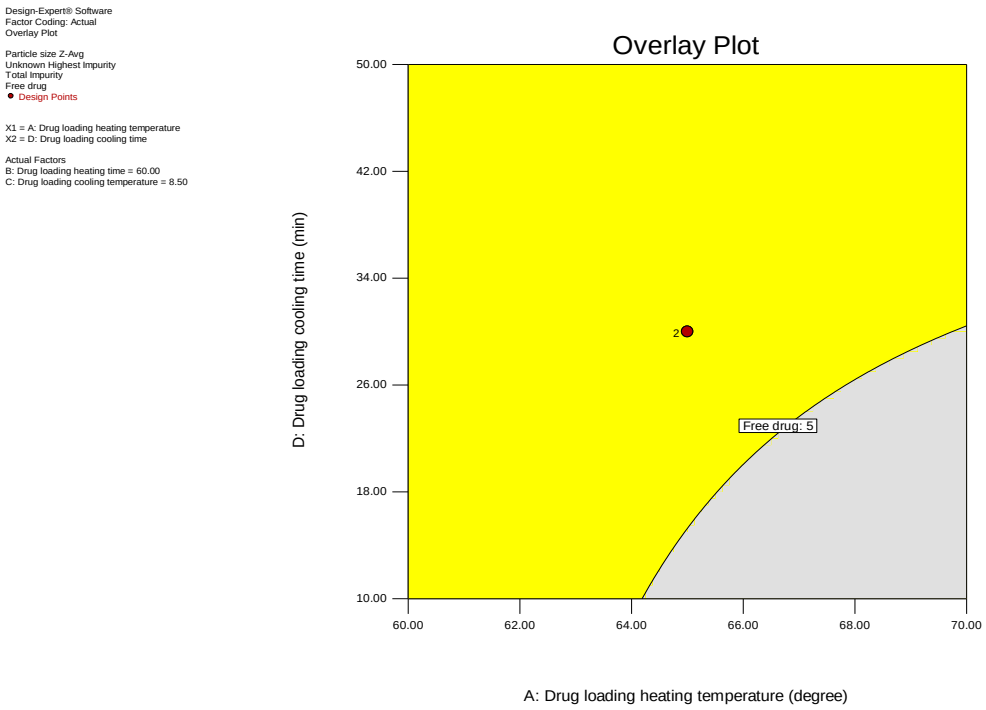


Figure 5.3.11 Design space for heating temperature and cooling time

Remarks: Optimized drug loading heating temperature with specified drug loading cooling time generate very less free drug. High drug loading heating temperature with low drug loading cooling time generate high amount of free drug.

5.3.4.3.3.3 Design space for Drug loading heating time and Drug loading cooling temperature

Remarks: Less drug loading cooling temperature with optimized drug loading heating time generate very less free drug. High drug loading cooling temperature with low drug loading heating time generate high amount of free drug.

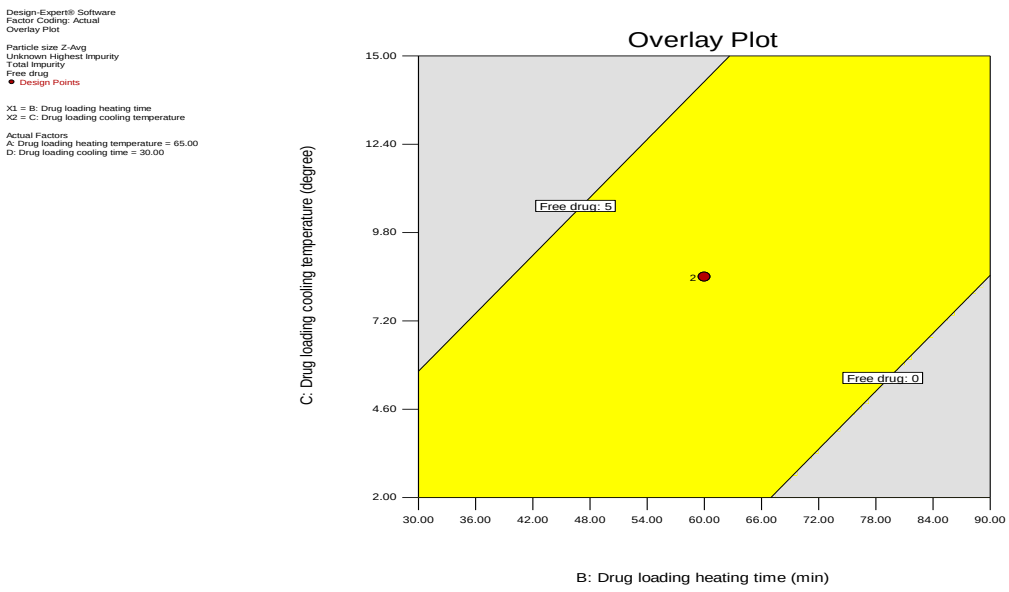


Figure 5.3.12 Design space for heating time and cooling temperature

5.3.4.3.3.4 Design space for Drug loading heating time and Drug loading cooling time

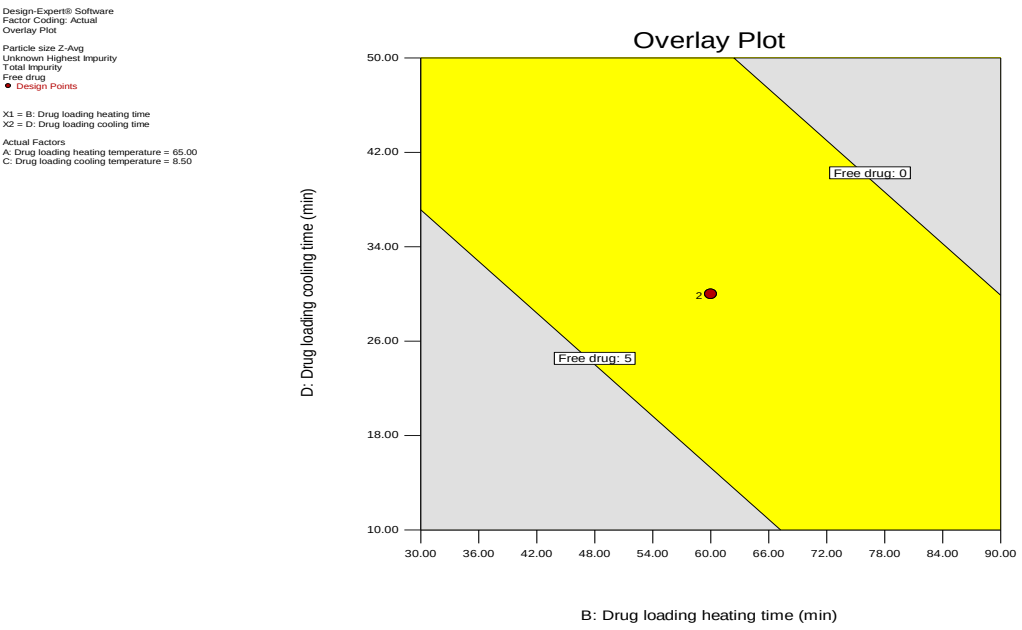


Figure 5.3.13 Design space for heating time and cooling time

Remarks: High drug loading heating time with high drug loading cooling time generate

very less free drug. Less drug loading heating time with less drug loading cooling time generate high amount of free drug.

Conclusion for optimization study II: The overlay contour plots of selected independent variable upon the response under study are shown in preceding section. The yellow zone indicates the design space, where all selected response were estimated to be within desired acceptance criteria. The overlay contour plots demonstrated that all the centre point of the selected design were found to be within the design space and hence chosen as an optimized formula. Drug loading heating temperature, Drug loading heating time, Drug loading cooling temperature and Drug loading cooling time were finalized as shown in below table.

Table 5.3.11 Optimized Process Parameters for Manufacturing process optimization Studies of Irinotecan Liposome Injection

Sr. No.	**Formulation/ Process variables**	**Optimized Process parameters**
1	Drug loading heating temperature	65°C
2	Drug loading heating time	60 min
3	Drug loading cooling temperature	2°-8°C
4	Drug loading cooling time	30 min

5.3.5 Updated Risk Assessment of the Manufacturing Process Variables

Acceptable ranges for the high risk formulation variables have been established during optimization study I and II and are included in the control strategy. During optimization study, all the process parameters were evaluated for its impact on DP-CQA and finalized to control the DP-CQA.

Based on the results of the Manufacturing process optimization studies, the risk assessment of the formulation process variables was updated and is presented in below

table.

Table 5.3.12 Updated Risk Assessment for the Manufacturing Process Variables

Quality attributes of the drug product	Formulation Process Variables								
	Lipids Concentration in ethanol	Hydration temperature	Hydration time	Extruder temperature	Extruder pressure	Drug loading heating temperature	Drug loading heating time	Drug loading cooling temperature	Drug loading cooling time
Content of Free Irinotecan	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*
% Assay of Irinotecan	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*
Particle size	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*	Low*

The justification for the assigned level of updated risk assessment for manufacturing process variables is provided in below table.

Table 5.3.13 Justification for Updated Risk Assessment for the Manufacturing Process Variables

Formulation Variables	Drug Product CQAs	Justifications
Lipids Concentration in ethanol	Particle size	The risk is reduced from high to low by optimising the lipid concentration in ethanol within range studied.
Hydration temperature	Particle size	Risk is reduced from high to low by controlling the temperature during process in specific range studied.
Hydration time	Particle size	Hydration time was found insignificant for the particle size achieved after hydration, so the risk is reduced from high to low.
Extruder	Particle size	Particle size after extrusion is significantly affected

temperature		by extruder temperature. The risk is reduced from high to low by fixing the extruder temperature range in the process within the range studied.
Extruder pressure	Particle size	Particle size after extrusion is significantly affected by extruder pressure. The risk is reduced from high to low by fixing the extruder pressure range in the process within the range studied.
Drug loading heating temperature	Content of Free Irinotecan	Content of Free Irinotecan is not dependent on the temperature provided during drug loading process. The risk is reduced from high to low.
	% Assay of Irinotecan	Drug loading heating temperature directly affect the % Assay of the drug product. The risk is reduced from high to low by fixing the drug loading heating temperature range in the process within the range studied.
	Particle size	Particle size was not affected by the temperature provided during the drug loading, the risk is reduced from high to low.
Drug loading heating time	Content of Free Irinotecan	Content of Free Irinotecan is not dependent on the heating time provided during drug loading process. The risk is reduced from high to low.
	% Assay of Irinotecan	Drug loading heating time directly affect the % Assay of the drug product. The risk is reduced from high to low by fixing the drug loading heating time range in the process within the range studied.
	Particle size	Particle size is not affected by the time provided during the drug loading, the risk is reduced from

		high to low.
Drug loading cooling temperature	Content of Free Irinotecan	Content of Free Irinotecan is not dependent on the temperature range studied during drug loading cooling process. The risk is reduced from high to low by fixing the temperature range.
	% Assay of Irinotecan	Drug loading cooling temperature did not affect the % Assay of the drug product. The risk is reduced from high to low.
	Particle size	Particle size is not affected by the temperature provided during the drug loading cooling step, the risk is reduced from high to low.
Drug loading cooling time	Content of Free Irinotecan	Content of Free Irinotecan is not dependent on the cooling time range studied during drug loading cooling process. The risk is reduced from high to low by fixing the cooling time.
	% Assay of Irinotecan	Drug loading cooling time did not affect the % Assay of the drug product. The risk is reduced from high to low.
	Particle size	Particle size is not affected by the cooling time provided during the drug loading cooling step, the risk is reduced from high to low.

5.3.6 Stability study of final formulation

Stability study of final formulation of Irinotecan liposome Injection was performed for 3 months at 2-8ºC condition. Stability study final formulation was performed for mainly reason of its degradation and content of % free drug, % assay, % lipid content and particle size. Stability study of optimized Irinotecan liposome injection was carried out as

per the ICH guideline. As this is a liposomal formulation containing lipids with lower TG (less than 50ºC), stability study at higher temperature is not possible. So batch was charged for stability study at refrigerated condition. The results stability study after 3 months with initial were shown in below Table.

Table 5.3.14 Stability study of Final formulation

Sr. No.	Test	Specification	Initial	1M 2-8ºC	3M 2-8ºC
1.	Description	white to slightly yellow opaque isotonic liposome suspension			
2.	Assay of Irinotecan	Between 90.0 % to 110.0%	100.3%	99.6%	100.5%
3.	Assay of DSPC	Between 85.0% to 115.0%	98.9%	92.2%	91.8%
4.	Assay of MPEG DSPE 2000	Between 85.0% to 115.0%	96.2%	95.7%	94.8%
5.	Assay of Cholesterol	Between 85.0% to 115.0%	95.8%	95.6%	95.1%
6.	pH of liposome	Between 5.0 to 7.0	5.65	5.62	5.64
7.	%drug Entrapment	NMT 10.0%	92.7%	92.4%	92.0%
8.	% Free Irinotecan	NLT 90%	7.3%	7.6%	8.0%
9.	Particle Size (z-avg)	NMT 150 nm	114 nm	117 nm	110 nm
10	Zeta Potential	Between -10 to -40 mV	- 25 mV	- 22 mV	- 27 mV

Conclusion: Stability study of Final formulation suggest no any degradation of impurity and no any % free drug is increasing with respect to stability time. Data suggest, there is no any % free drug increment in final formulation, whereas in reference formulation, free drug is increasing with respect to time.

So, based on above data, it can be concluded that final formulation is comparable and more stable than the reference formulation.

5.3.7 Characterization of Irinotecan Liposome Injection

As per USFDA draft guidance for Liposome Injection, various physicochemical properties of Irinotecan liposome injection are required to be determined and compare with the Reference product.

As per the guidance, in-vitro liposome characterization should be conducted on at least one batches of the ANDA and the RLD or reference standard products. Attributes that should be included in the characterization of ANDA's claiming equivalence to the RLD or reference standard are:

1. Liposome composition
    i. Lipid content
    ii. Free and encapsulated drug
2. State of encapsulated drug
3. Internal Environment
    i. Internal Volume
    ii. Internal pH
4. Liposome morphology and number of lamellae
5. Lipid bilayer phase transition
6. Liposome size distribution
7. Grafted PEG at the liposome surface
8. Electrical surface potential or charge
9. In vitro leakage under multiple conditions

To execute the above listed parameters by FDA, some studies were conducted by Emcure at in-house facility while some studies were performed at outsourced laboratory on test product and reference standard.

All above characterization were performed with specific instrument which is mentioned in below table.

Table 5.3.15 Liposome Characterization and Methodology		
Sr. No.	**Evaluation Parameter**	**Methodology**
1	**Liposome composition**	Lipid Content by HPLC
		Free and encapsulated drug by SPE cartridge
2	**State of encapsulated drug**	Fluorescence studies
		X-ray diffraction (small angle)
		Cryo-TEM
3	**Internal environment**	Internal Volume
		Internal pH
4	**Liposome morphology and number of lamellae**	Atomic force microscopy (AFM)
		Cryo-TEM
5	**Lipid bilayer phase transition**	Differential scanning calorimetery (DSC)
		Thermal Gravimetric Analysis (TGA)
		Differential Thermal Analysis (DTA)
6	**Liposome size distribution**	Dynamic light scattering (DLS)
		Size-exclusion chromatography (SEC-MALS)
		Static light scattering and field flow fractionation [FFF)
7	**Grafted PEG at the liposome surface**	NMR spectroscopy
		Cryo-TEM
		Fixed aqueous layer thickness (FALT)
8	**Electrical surface potential or charge**	Zeta potential
		Electrophoretic mobility distribution
9	**In vitro leakage under multiple conditions**	In Vitro Drug Leakage using bottle rotating apparatus

All above characterization was performed on final formulation and all data is comparable with reference formulation. Emcure has restricted to provide all the data of characterization, so data is not presented in the present work. Only cryo TEM images

were provided, so following are the comparison of cryo TEM images of reference product and Irinotecan liposome injection.

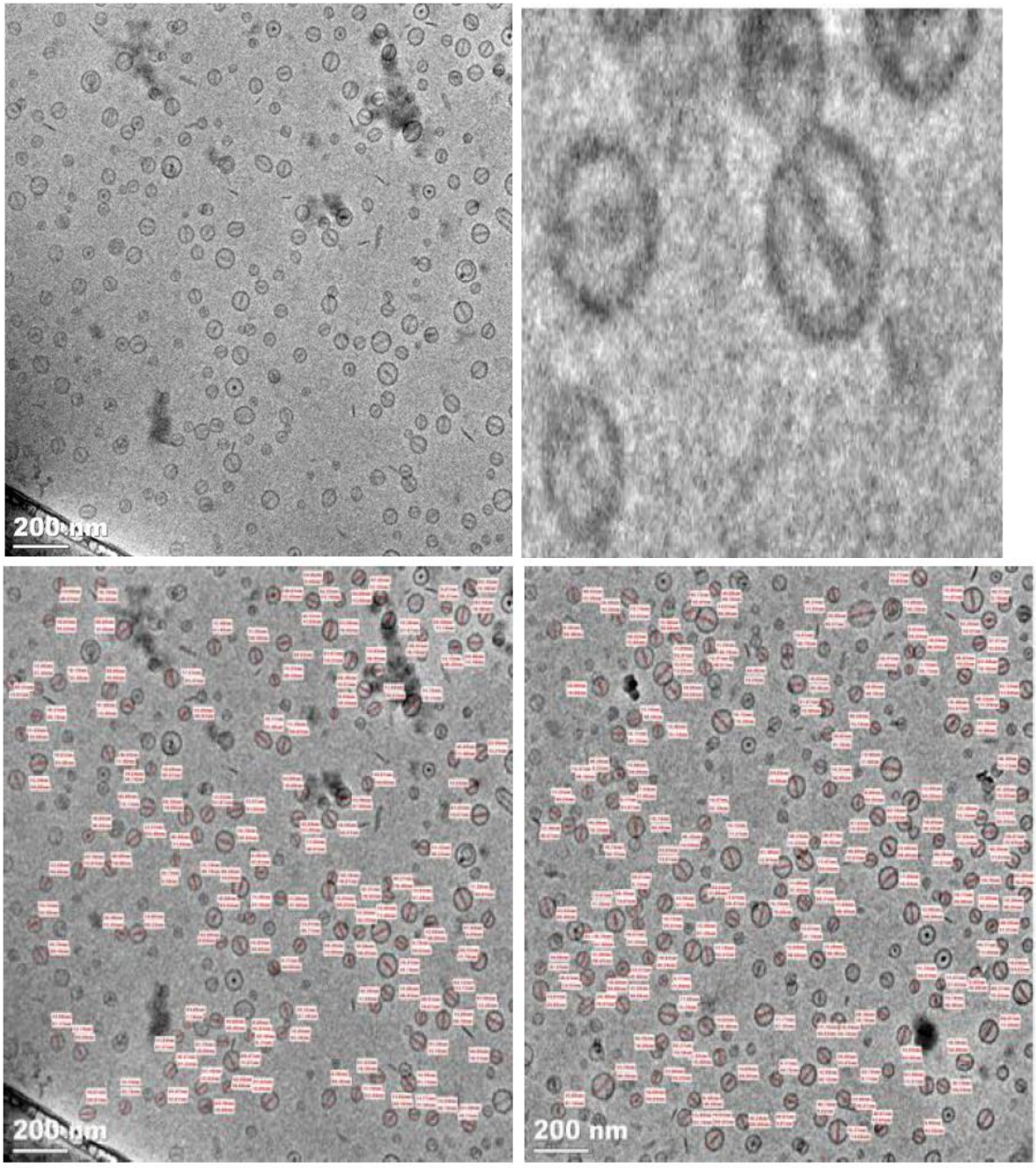


Figure 5.3.14 Cryo TEM images of Onivyde (reference product)

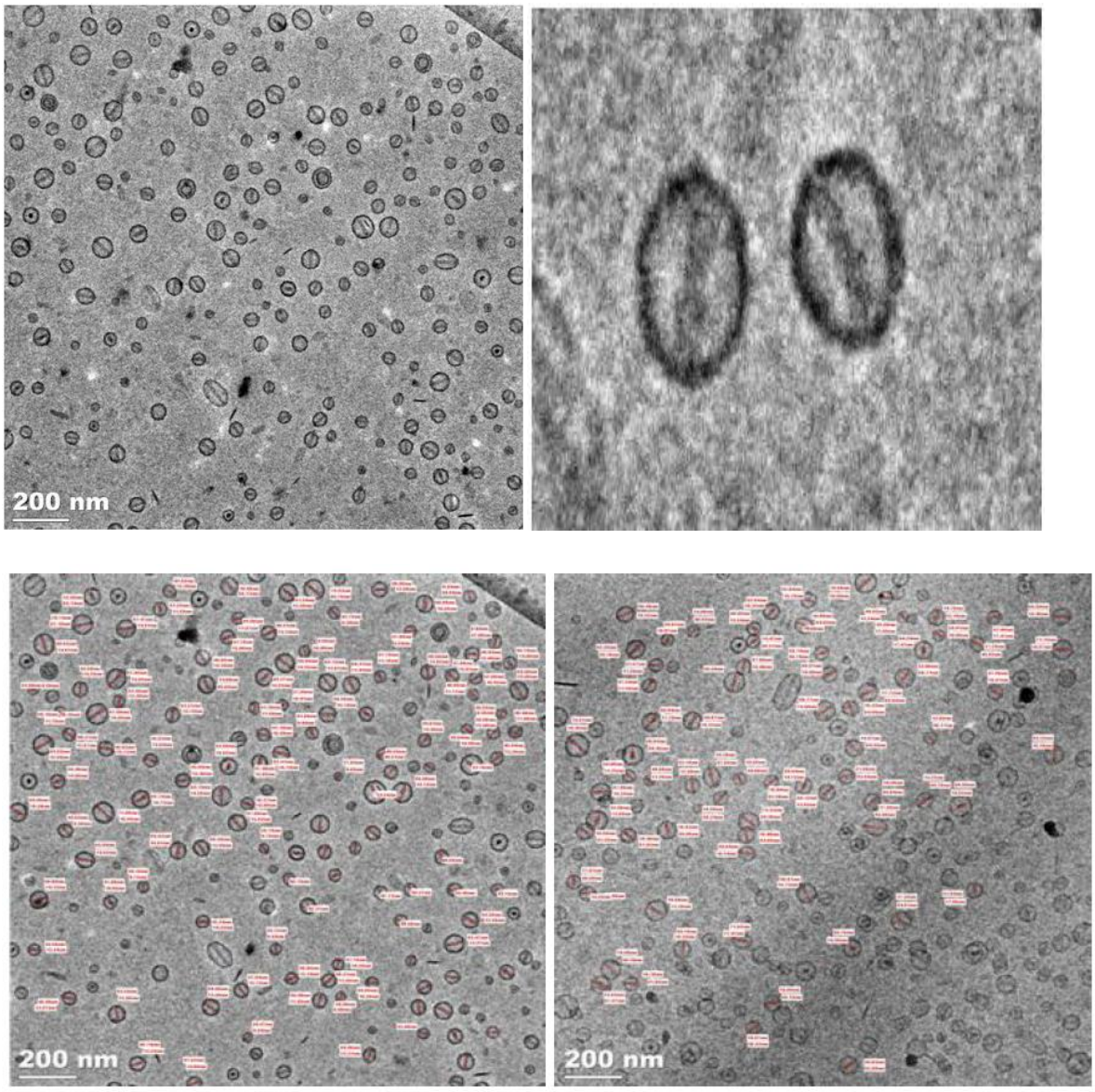


Figure 5.3.14 Cryo TEM images of Final formulation (Test product)

Cryo TEM images also confirms that % free drug is very less in Test product compare to reference product. Also Cryo TEM images confirms the sphericity, particle size, Pegylation and State of encapsulated drug inside the liposome.

So, based on above all results, it can be concluded that Test product is more stable than Reference product with very less % free drug in Irinotecan liposome Injection.

The present investigation work summarizes the development of Irinotecan Liposome Injection for intravenous infusion, 4.3 mg/25mL, a generic equivalent of Reference Product Onivyde Irinotecan Liposome Injection for intravenous infusion, 4.3 mg/25mL, which is indicated for colorectal cancer dosing for Camptosar. Quality by Design (QbD) approach has been used to develop generic Irinotecan Liposome Injection and manufacturing process that ensures the quality, safety and efficacy of Irinotecan Liposome Injection.

Onivyde (refrence product) characterization suggest that the % free drug in final formulation is more than 10.0%, which is not as per the requirements of USFDA for liposomal formulation. Also % free drug is increasing during the stability. So, due to higher % free drug, Refrence product may generate more toxicity because of free drug.

Initially, the Quality Target Product Profile (QTPP) was defined based on the properties of the drug substance, characterization of the Reference Standard and consideration of the Reference product label and intended patient population. Development of Irinotecan Liposome Injection was designed to achieve all of the attributes defined in the QTPP along with less % free drug (less than 10.0%) compare to reference formulation. Along with this, reference formulation is patented with respect to sucrose octa sulfate as remote loading agent. So, during development, sucrose ocat sulfate need sto be remove and other remote loading agent with same quality and efficacy needs to evaluated and utilized for liposome preparation for drug entrapment and less % free drug.

Preliminary screening trials for drug (API) loading suggest that active loading process is more relevant and useful compare to passive loading for producing liposome with higher entrapment and less free drug.

Preliminary screening trials for particle size reduction suggest that Extruder is more relevant and useful compare to high pressure homogenization for producing soft liposome with higher entrapment and less free drug.

Preliminary screening trials for Active/remote drug loading agents suggest that ammonium dihydrogen phosphate is best remote loading agent compare to sucrose octasulfate, sucrose, copper sulfate and ammonium sulfate. Data suggest that batches with an Ammonium dihydrogen phosphate shows better drug entrapment compare to all other remote loading agent. Even Ammonium dihydrogen phosphate shows better drug entrapment with less free drug compare to sucrose octa sulfate, which is used by innovator. So, formula was finalized with Ammonium dihydrogen phosphate and other ingredients are same as innovator.

Considering the complexicity and critical manufacturing process, initial risk assesment was performed and all critical parameters were kept on high risk to mitiagte the risk with optimized and defined process parameters. Optimization study I was conducted to evaluate the effect of Lipids Concentration in ethanol, Hydration temperature, Hydration time, Extruder temperature and Extruder pressure on Particle size. A $2^{(5-1)}$ fractional factorial Design of Experiments (DOE) with two centre points was used to study the impact of these process factors on the response variables. Data suggest that all the parameters have impact on particle size of liposome. So, based on design space, optimized process parameters were defined for maintaining the required particle size.

Optimization study II was conducted to evaluate the effect of Drug loading heating temperature, Drug loading heating time, Drug loading cooling temperature and Drug loading cooling time on Particle size and free drug. A $2^{(4-1)}$ fractional factorial Design of Experiments (DOE) with one center points was used to study the impact of these formulation factors on the response variables. Data suggest that all the parameters have impact on particle size of liposome and % free drug. So, based on design space, optimized process parameters were defined for maintaining the required particle size and % free drug.

Stability study of Final formulation suggest no any degradation of impurity and no any % free drug is increasing with respect to stability time. Data suggest, there is no any % free drug increment in final formulation, whereas in reference formulation, free drug is

increasing with respect to time. So, it can be concluded that final formulation is comparable and more stable than the reference formulation.

All characterization were performed on final formulation and all data is comparable with reference formulation. Emcure has restricted to provide all the data of characterization, so data is not presented in the present work. Only Cryo TEM images were provided. Cryo TEM images also confirms that % free drug is very less in Test product compare to reference product. Also Cryo TEM images confirms the sphericity, particle size, Pegylation and State of encapsulated drug inside the liposome.

So, based on above all results, it can be concluded that Test product is more stable than Reference product with very less % free drug in Irinotecan liposome Injection.

- Emcure has taken the Patent Application for this investigation work.
- **Strict instruction by Emcure pharmaceuticals for no any publication of review article or research article for this m. Pharm Work. Otherwise, they can take strict action as patent application has been applied from Emcure.**