"Development of Ligand Appended Lipid Nanoparticulate Systems of Anti-Cancer Agent for the Treatment of Colorectal Cancer"

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BY

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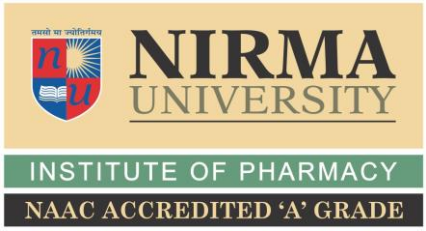

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CERTIFICATE

*This is to certify that the dissertation work entitled "**Development of Ligand Appended Lipid Nanoparticulate Systems of Anti-Cancer Agent for the Treatment of Colorectal Cancer**" submitted by Mr. Neel Nilesh Shah with Regn. No. (14MPH116) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Technology and Biopharmaceutics" is a bonafide research work carried out by the candidate at the Department of Pharmaceutics, Institute of Pharmacy, Nirma University under 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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DECLARATION

*I hereby declare that the dissertation entitled "**Development of Ligand Appended Lipid Nanoparticulate Systems of Anti-Cancer Agent for the Treatment of Colorectal Cancer**", is based on the original work carried out by me under the guidance of Dr. Mayur M. Patel, Associate professor, Department of Pharmaceutics and Dr. Bhoomika M. Patel, Assistant Professor, Department of Pharmacology, Institute of Pharmacy, Nirma University. 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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ABSTRACT

Development of Ligand Appended Lipid Nanoparticulate Systems of Anti-Cancer Agent for the Treatment of Colorectal Cancer

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Colorectal cancer remains the third most common cancer causing death in majority of the population worldwide. Chemotherapeutical treatment generally includes treatment by administration of chemotherapeutical formulations mostly by intravenous route of administration which is painful, toxic, time consuming and costly for the patients. Chemotherapy also causes toxicity and cell death to other normal cells in the body apart from cancerous cells. The aim of the present investigation was to formulate an orally administrable nano-particulate drug delivery system which causes lower exposure to normal body cells by higher efficacy of targeting the cancerous cells, producing lower toxicity rates and avoiding maximum possible side effects. Henceforth, an anti-neoplastic agent has been used in order to prepare Nano-structured Lipid Carriers (NLCs) by High Pressure Homogenizer which can be administered orally and thereby exert its anti-cancer activity on the affected cancerous region. Oral application of the NLCs can be made possible by modifying the surface of the NLCs by PEGylating them and thereby its uptake by Gastrointestinal Lymphatic System of the body. Also PEGylation helps the NLCs to escape from RES uptake which can cause NLCs to degrade in the systemic circulation providing long circulation and exerting the effect. Henceforth, PEGylated NLCs were formulated and optimized using Box-Behnken Design and in-vivo study on tumour induced animal model was performed in order to evaluate the efficacy and toxicity of the formulation.

Colorectal cancer (CRC) is the third most common cancer in the world and the second most common cause of cancer-related deaths. In the USA, other than skin cancer, CRC was diagnosed as the third most common cancer in both men and women. It has been reported that estimated new CRC cases and deaths in the USA in 2014 will be 136,830 and 50,310, respectively[1]. Currently depending on the stage of cancer, CRC is been treated either by chemotherapy, surgery, radiation therapy or by immunotherapy. Further, it is a well-known fact that the conventional dosage form when used for the treatment of CRC delivers the drug to both normal and cancerous tissues, thus leading to undesirable adverse effects.

In order to overcome the drawbacks associated with available conventional chemotherapy, a system needs to be developed which is able to deliver the drug to systemic circulation. Recently oral chemotherapy is attaining tremendous interest among scientific community for improving quality of life of patients.

Lipid Nanoparticles have been discovered to be one of the most efficient and bio-degradable drug delivery system for treating a number of diseases[2]. There are generally two types of lipid based nano-particulate systems which includes Solid Lipid Nano-particles (SLNs) and Nano-Structured Lipid Carriers (NLCs). The advantage of NLCs over SLNs is the increased % Drug Loading and stability on-storage.

For oral chemotherapy, it is necessary for the drug delivery system to maintain its stability in the gastric environment of the body and deliver the drug to the cancer affected sites. The intestinal lymphatic system is one such route of drug delivery which deliver drugs into the systemic circulation by avoiding hepatic first pass metabolism.

It has been reported that PEGylation of nanoparticles offer several benefits with respect to enhanced oral bioavailability and targetability of drugs[3], viz. prevention of degradation in gastrointestinal tract, reduction of elimination by the reticuloendothelial system (RES) and enhanced permeability and retention (EPR) in the tumor tissue (i.e. passive targeting of drugs to tumors). Although, the concept of PEGylation has been extensively used to improve the in-vivo blood circulation time (and thereby half-life) of parenterally administered nanoparticles, till date very few findings on orally administered PEGylated nanoparticlulate system have been reported. Further, folic acid (FA) targeting is an interesting approach for cancer therapy because it offers several advantages over the use of monoclonal antibodies. More importantly, folate receptors are overexpressed on tumors of various organs such as colon, lung, prostate, ovaries, mammary glands, and brain[4].

In lieu of this, we propose for the first time development of Ligand appended PEGylated-NLCs as vehicles for oral delivery of NNS for the treatment of CRC.

2.1. Introduction to Colorectal Cancer

Cancer has been described as a global burden and the number of deaths has been expected to rise by 13.2 million in 2030[1]. Among the malignant diseases affecting men and women, colorectal cancer (CRC) is the third most leading cause of deaths by cancer in United States where 101,340 new cases are expected to be diagnosed in 2011 and 49,380 patient death by CRC in the same year. The most common age group getting affected by this disease has a median age of 71 years. Colon and rectal cancer generally arise from benign and adenomatous polyps. The adenoma is basically asymptomatic and is generally discovered during the routine screening. It is the main premalignant lesion leading to colon cancer. It is a multistep process that results from accumulation of acquired genetic and epigenetic changes leading in to pathologic transformation to invasive adenocarcinoma. Around 5% of individuals are affected due to genetic syndromes familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC), whereas 25% occurs due to family history of the disease. Other specific factors assessed to be associated with CRC are age, gender, obesity, smoking, diet, and inflammatory-bowel disease such as ulcerative colitis, alcohol consumption and sedentary life style.

Significant advances has been made in the treatment of CRC with about 200% increase in survival rate over the past few years. The current management parameters for CRC includes surgery, chemotherapy, radiation therapy, and most recently immunotherapy (targeted molecular therapies)[5]. Two or more types of treatments are combined for the therapy depending upon the stage of the CRC. The most ideal therapeutic approach is to avoid the occurrence of CRC. Long-standing inflammation of the colon and rectal region in ulcerative colitis and Crohn's disease has been widely accepted for its association with the occurrence of subsequent dysplasia and CRC. Hence, it can be aforesaid that the drugs used for treating inflammatory bowel disease can be able to help decrease the risk of CRC. 5-aminosalicylic acid (5-ASA) with its anti-inflammatory property used in the treatment of inflammatory bowel disease has also been studied for it cancer inhibiting activity as it seems to reduce the risk of colorectal cancer incidence[6].

The conventional dosage forms used in the treatment of CRC not only delivers the drug to the necessary sites for drug action but also to the unwanted sites i.e. the normal cells[1]. This results into several unwanted adverse side effects. The mechanism of chemotherapeutic agents is to kill cells that are rapidly dividing in the body. This not only kills the rapidly dividing cancerous cells but also the rapidly dividing healthy cells resulting into damage to the normal active and healthy cells of the body such as the linings of the gastro-intestinal tract, the mouth membrane linings, hair follicles and the bone marrow. Some common and expected adverse effects associated with CRC therapies includes anaemia, neutropenia, diarrhoea, gastrointestinal toxicity, hand-foot syndrome, mucositis, vomiting, nausea, fatigue, liver toxicity and haematological disorders. Targeted therapies provides increased drug concentration at the affected areas and also reduces the incidence of side effects[7].

2.1.1. Stages and Treatment of CRC

It is important to find out whether cancer has spread within the colon to other parts of the body in order to plan for the treatment of CRC. Depending on the extent of cancer spreading, CRC is classified as Stage 0, Stage I, Stage II, Stage III, and Stage IV. In stage 0, abnormal cells are found in the mucosa (innermost layer of the colon wall). These abnormal cells may become cancer and spread. Stage 0 is also called carcinoma *in situ*. The surgical option is to remove the abnormal cells by excision or polypectomy. Colon resection may be carried out for larger lesions not amenable to local excision. Several reports have indicated the beneficial effects of cancer preventive agents in the prevention of CRC. Thus, the pharmacotherapy option may be to use site-specific delivery of cancer preventative agents such as celecoxib and 5-ASA, locally to the colon to prevent the transformation of abnormal or inflamed cells to turn into cancer cells[1].

Several formulations have been developed and evaluated for possible use in the prevention of CRC. However, this formulation approach has not been tested for their clinical benefit in humans. In stage I CRC, cancer has formed in the mucosa (innermost layer) of the colon and has spread to the layer of tissue under the mucosa. Cancer may have spread to the muscle layer of the colon wall. The surgical option of treating stage

I CRC is usually resection and anastomosis. Because of its localized nature, the cure rate is considered high. The cure rate of stage I CRC may be improved, probably with the use of colon-targeted delivery of orally active chemotherapeutic agents such as 5-FU and capecitabine. The targeted delivery of 5-FU and capecitabine to locally in colon provides high drug concentration to cancer cells of mucosa as well as to the cancer cells of the issue layer under the mucosa[8]. In stage II CRC, cancer has spread through the muscle layer of the colon wall to the serosa (outermost layer) of colon wall and spreads from there to the nearby organs. The treatment option for stage II CRC is resection and anastomosis. Following surgery, patients should be considered for entry into carefully controlled clinical trials evaluating the use of systemic or regional chemotherapy, radiation therapy, or biologic therapy (bevacizumab, cetuximab, and panitumumab). Invasive cancers that are confined within the wall of the colon (tumour - nodemetastasis stages I and II) are curable, but if untreated, they spread to regional lymph nodes (stage III) and then metastasize to distant sites (stage IV). In stage III CRC, cancer may have spread through the mucosa of the colon wall to the submucosa and muscle layer, and as spread to nearby lymph nodes or tissues near the lymph nodes. The treatment option for stage III CRC is resection and anastomosis followed by chemotherapy or clinical trials of chemotherapy after surgery. In stage IV CRC, cancer has spread through the blood and lymph nodes to other parts of the body, such as the lung, liver, abdominal wall, or ovary. Treatment of stage IV and recurrent CRC may include resection with or without anastomosis, surgery to remove parts of other organs such as liver, lungs and ovaries where the cancer may have spread or recurred, radiation therapy or chemotherapy as palliative therapy relieve symptoms and improve quality of life, and chemotherapy and targeted therapy with a monoclonal antibody. Surgical resection is highly effective for early stage colon cancers, providing cure rates of over 90% in stage I and 75% in stage II disease, and up to 73% of cases of stage III disease are curable by surgery combined with adjuvant chemotherapy. Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable. The presence of nodal involvement (stage III) predicts for a 60% likelihood for recurrence.

Treatment of these high-risk individuals with a postsurgical course of 5-FU-based chemotherapy reduces the recurrence rate to 40%, increasing overall survival to 60%, and is now the standard of care for stage III patients; however, newer adjuvant regimens

are clearly needed to reduce the still substantial failure rate. Furthermore, the traditional parenteral chemotherapy in treating stage II, III and IV CRC after surgery results in several adverse effects because of the nonspecific action on rapidly developing healthy cells.

2.2. Introduction to Oral Chemotherapy:

Cancer is defined as a multi-step cascade of diseased condition caused by continuous stagnant tissue injury and host – environment interactions[9]. The constant and continuous exposure of carcinogens such as tobacco, ultraviolet light and infections leads to various genetic (mutations), epigenetic (loss of heterozygosity) and global transcriptome changes (via inflammation pathways) and is associated with increased risk of cancer. Owing to increased occurrence of cancer and worldwide prevalence during the last decade, it has posed a great challenge to the health care professionals. The latest WHO statistics suggests about 45% increase in the global cancer deaths by 2030, of which 70% would be contributed from developing countries like India. With continuous upgradation in the field of science and technology, the need for addressing the practical problems associated with the drug therapies increased proportionately[10]. The major portion of cancer therapy till the last couple of decades was based on parenteral route of administration. However, looking at the quality of life and need of follow-up therapy after the diagnosis of the disease, oral route has gained major focus as compared to the parenteral route. Oral route is considered as one of the most abundant and traditional ways of drug delivery; main advantage being greatest safety, convenience and patient compliance[11]. The possibility of tailor-made design as per physicochemical properties of the drug substances further increases the attraction of the scientific community. However, diverse properties of drug substances, limitations in the choice of excipients and principally, physiological barriers pose great challenge for design and development of oral drug delivery system.

The use of oral anticancer therapy affects many clinically relevant aspects such as the following[11]:

1. An appropriate plasma drug concentration can be maintained to achieve a prolonged exposure of drugs to cancerous cells. This will increase the efficacy and decrease the side effects of the anticancer drugs.

2. Modulation of drug release from the dosage forms also provides an added advantage compared to that in other routes of administration.

3. It further facilitates the use of more chronic treatment regimens. This is especially important for cell cycle specific agents, especially those of predominantly cytostatic effect such as angiogenesis inhibitors and signal transduction inhibitors. For these agents, prolonged exposure to the drug may lead to pharmacodynamic advantages over intermittent intravenous administration.

4. Oral chemotherapy avoids the discomfort of injection and can be conducted at home. This approach may enhance the patient cooperation and their quality of life, which is an important issue and thus deserves high attention for any medical treatment.

5. The risks of infection and extravasations associated with intravenous infusion lines is avoided.

6. The treatment cost for the patient can be highly reduced due to avoidance of hospitalization, sterile manufacturing and trained personnel assistance.

7. Apart from the therapeutic applications, oral therapy can also be explored in the segment of prophylactics due to high level of ease in administration. An interesting study has been carried out to evaluate the patient's preference for route of administration and it was found that almost 78.7% wanted themselves to be treated by oral route for recurring breast cancer disease, whereas nearly 2.7% preferred parenteral route while 18.6% landed with no preference.

Synchronizing with these results, the current scenario for development of new drug molecules has also rapidly shifted towards oral delivery. Approximately 20 molecules are already present in market for oral therapy of cancer, whereas a number of them are pipeline. This clearly indicates the developer's insight and intentions for oral delivery. However, oral delivery of anticancer drugs is a great challenge owing to their peculiar physicochemical properties, and physiological barriers such as pre-systemic metabolism and gastrointestinal instability[12]. Upon oral administration of such drugs, only a fraction of dose is available to systemic circulation for execution of therapeutic response e.g. oral bioavailability of paclitaxel, docetaxel, doxorubicin, tamoxifen, etc. is in the range of 5–20%. Broadly, this could be attributed to low aqueous solubility, poor intestinal permeability, high level of P-glycoprotein (P-gp) efflux and pre-systemic metabolism. The P-gp efflux also has a key role in the execution of multidrug

resistance in the tumour cells and thereby needs special consideration while designing the formulation of poor biopharmaceutical properties, as the amount which is required to achieve the therapeutic response might be very high ultimately leading to multidrug resistance[12].

Furthermore, cost of manufacturing novel formulations of the existing parenteral drugs and limited therapeutic window of the anticancer drugs leading to sub-therapeutic or toxic dose, also restricts the developability for oral route of administration. However, recent advances in nanotechnology based drug delivery system posed potential advantages in overcoming these limitations. This includes lipid nanoparticles, polymeric nanoparticles, polymeric micelles, micro-emulsion, self-emulsifying drug delivery systems (SEDDS), carbon nanotubes, layersomes, liposomes, lipid–drug conjugates, nanocrystals, etc[13]. The therapeutic efficacy of the formulation depends upon its capability to deliver the drug at the right place and at the right time in amount adequate enough to yield a therapeutic response. Comparative therapeutic equivalence of oral and intravenous routes has been studied for wide variety of drugs and promising results were observed in most of the cases[14].

2.3. Introduction to Lipid Nanoparticles:

Lipid nanoparticles with a solid matrix are generally two types: solid lipid nanoparticle (SLN) and nanostructured lipid carrier (NLC). Solid Lipid Nanoparticle SLNs are prepared from lipids which are solid at room temperature as well as at body temperature. Different solid lipids are exploited to produce SLNs, such as, tripalmitin/ Dynasan® 116 , cetyl alcohol , cetyl palmitate, Compritol® 888 ATO , Glyceryl monostearate , Precirol® ATO5, trimyristin/Dynasan® 114, tristearin/Dynasan® 118 , stearic acid , Imwitor® 900[15].

There are several advantages of SLN formulations, such as[16]:

(a) Photosensitive, moisture sensitive, and chemically labile drug molecules can be protected from degradation in external environment (during storage) and in the gut (following oral administration).

(b) Bioavailability of highly lipophilic molecules can be improved.

(c) Biodegradable and physiological lipids are used to prepare SLNs.

(d) Scaling up of the formulation technique to industrial production level is feasible at low cost and in a relatively simple way.

(e) Use of organic solvents can be avoided to produce SLNs.

In contrary, several disadvantages are also associated with SLNs, such as[16]:

(a) SLN dispersions contain high amount of water.

(b) Drug-loading capacity of SLNs are limited due to crystalline structure of solid lipid.

(c) Expulsion of encapsulated drug may take place during storage due to formation of a perfect crystalline lattice especially when SLNs are prepared from one highly purified lipid.

(d) Drug release profile may change with storage time.

(e) Polymorphic transitions are possible.

(f) Particle growth is possible during storage, and

(g) Gelation of the dispersion may take place during storage.

In, Nanostructured Lipid Carrier generally, drugs are incorporated between the fatty acid chains or in between lipid layers or in amorphous clusters in crystal imperfections within SLN matrix. However, SLNs prepared from one highly purified lipid can

crystallize in a perfect crystalline lattice that allows very small space for the incorporation of drugs. Lipids crystallize in high energetic lipid modifications, α and β′, immediately after preparation of SLN. However, the lipid molecules undergo a time-dependent restructuring process leading to formation of the low-energetic modifications, βi and β, during storage. Formation of this perfect lipid crystalline structure leads to expulsion of drug. Therefore, despite SLNs being interesting delivery systems, relatively low drug-loading capacity and potential expulsion of the drug during storage led scientists to think about new strategies. As a result, NLCs have been developed, which in some extent can avoid the aforementioned limitations. In case of NLCs, spatially very different lipid molecules are mixed to create a lipid particle matrix as imperfect as possible. Generally, solid and liquid (oil) lipids are mixed to produce NLCs that are still solid at room temperature as well as at body temperature. Due to many imperfections in NLCs, drug-loading capacity is enhanced and drug expulsion during storage is minimized[13].

NLCs have several advantages, such as:

(a) NLC dispersions with higher solid content can be produced.

(b) Drug-loading capacity is better than SLNs.

(c) Drug release profile can be easily modulated.

(d) Drug leakage during storage is lower than SLNs, and

(e) Production of final dosage forms (e.g., tablets, capsules) is feasible.

2.3.1 Formulation Techniques:

Various formulation techniques exist for the production of SLNs and NLCs. Among them, high-pressure homogenization (HPH) and micro-emulsion techniques have demonstrated strong potential for scaling up to industrial production scale. The following sections describe different existing approaches for SLN and NLC formulations. However, in some instances combination of different methods has been utilized to prepare the nanoparticles.

2.3.1.1 High-Pressure Homogenization[14]

HPH is a reliable and suitable technique for and the drug is dissolved or homogeneously dispersed in the melted lipid(s). Then a hot aqueous surfactant solution (preheated at the same temperature) is added to the drug–lipid melt and homogeneously dispersed (pre-emulsion) by a high shear mixing device. Subsequently, this hot pre-emulsion is subjected to a high-pressure homogenizer at the same temperature. This homogenization process is repeated till the nano-emulsion of desired average particle size is obtained. The obtained nano-emulsion is then cooled down to room temperature. During this cooling down, lipid droplets of the nano-emulsion re-crystallize and form lipid nanoparticles with solid matrix. Cold high-pressure homogenization. Similar to hot HPH, the lipid(s) is/are melted at 5–10°C above its/their melting points and the drug is dissolved or homogeneously dispersed in the melted lipid(s) in the cold HPH technique. Then the drug-lipid melt is rapidly cooled down by means of liquid nitrogen or dry ice and subsequently milled to micro-particles by means of a ball mill or mortar. These micro-particles are suspended in a cold aqueous surfactant solution and then homogenized at or below room temperature forming lipid nanoparticles. This cold HPH technique is suitable for hydrophilic or thermo-labile drugs as this method is expected to avoid temperature-induced drug degradation and drug distribution into aqueous phase during homogenization. However, complete avoidance of drug exposure to high temperature is impossible as the drug needs to dissolve or disperse in the molten lipid and some heat is generated during the homogenization process. Generally, scaling up of a process encounters several problems. Nevertheless, usage of the larger scale machines during HPH leads to an even better quality of the product with regard to a smaller particle size and its homogeneity. Additionally, HPH technique is widely used and well-established technique in pharmaceutical and food industry. SLN prepared by HPH can also be produced in non-aqueous dispersion media as long as the dispersion medium does not dissolve the lipid, e.g., liquid polyethylene glycol or oils (e.g., mineral oil) the preparation of lipid nanoparticles. There are two types of HPH, hot HPH and cold HPH.

2.3.1.2 Hot High-Pressure Homogenization

In this technique, first the lipid(s) is/are melted at 5–10°C above its/their melting point(s) and the drug is dissolved or homogeneously dispersed in the melted lipid(s). Then a hot aqueous surfactant solution (preheated at the same temperature) is added to the drug–lipid melt and homogeneously dispersed (pre-emulsion) by a high shear mixing device. Subsequently, this hot pre-emulsion is subjected to a high-pressure homogenizer at the same temperature. This homogenization process is repeated till the nano-emulsion of desired average particle size is obtained. The obtained nano-emulsion is then cooled down to room temperature. During this cooling down, lipid droplets of the nano-emulsion re-crystallize and form lipid nanoparticles with solid matrix[14].

2.3.1.3 Cold High-Pressure Homogenization

Similar to hot HPH, the lipid(s) is/are melted at 5–10°C above its/their melting points and the drug is dissolved or homogeneously dispersed in the melted lipid(s) in the cold HPH technique. Then the drug-lipid melt is rapidly cooled down by means of liquid nitrogen or dry ice and subsequently milled to micro-particles by means of a ball mill or mortar. These micro-particles are suspended in a cold aqueous surfactant solution and then homogenized at or below room temperature forming lipid nanoparticles. This cold HPH technique is suitable for hydrophilic or thermo-labile drugs as this method is expected to avoid temperature-induced drug degradation and drug distribution into aqueous phase during homogenization. However, complete avoidance of drug exposure to high temperature is impossible as the drug needs to dissolve or disperse in the molten lipid and some heat is generated during the homogenization process. Generally, scaling up of a process encounters several problems. Nevertheless, usage of the larger scale machines during HPH leads to an even better quality of the product with regard to a smaller particle size and its homogeneity. Additionally, HPH technique is widely used and well-established technique in pharmaceutical and food industry. SLN prepared by HPH can also be produced in non-aqueous dispersion media as long as the dispersion medium does not dissolve the lipid, e.g., liquid polyethylene glycol or oils (e.g., mineral oil)

2.3.1.4 Emulsification-Sonification

The first part of this method is similar to HPH. Briefly, the lipid(s) is/are melted at a temperature of 5–10°C above its/their melting point(s) and the drug is dissolved/dispersed in the melted lipid(s). Then a hot aqueous surfactant solution (preheated at the same temperature) is added to the drug-lipid melt and homogeneously dispersed by a high shear mixing device. Coarse hot oil-in-water emulsion obtained is ultra-sonicated using probe sonicator till the desired sized nano-emulsion is formed. Finally, lipid nanoparticles are obtained by allowing hot nano-emulsion to cool to room temperature. However, metallic contamination of the product may happen during sonication by probe sonicator[14].

2.3.1.5 Micro-Emulsion

Micro-emulsion method for the preparation of SLNs was developed by Gasco et al., which has been adapted and/or modified by other researchers. In this method, first the solid lipid(s) are melted and the drug is dissolved/dispersed in the molten lipid(s). After in non-aqueous dispersion media as long as the dispersion medium does not dissolve the lipid, e.g., liquid polyethylene glycol or oils (e.g., mineral oil) that, aqueous surfactant–cosurfactant solution (pre-heated above the melting temperature of solid lipid) is added to the lipid melt with mild agitation to obtain transparent micro-emulsion. Subsequently, the micro-emulsion is dispersed in cold water (2–10°C) with mild agitation, where the micro-emulsion breaks into ultrafine nano-emulsion droplets which immediately crystallize to form SLNs. Strong dilution of the particle suspension due to usage of large volume of water (ratio of hot micro-emulsion to cold water=1:25–1:50) is the main concern of this technique. Thus, the excess water needs to remove either by ultra- filtration or by lyophilization to obtain a concentrated dispersion. Another disadvantage of this method is the necessity of high concentrations of surfactants and co-surfactants, which is not desirable. Industrial scale production of lipid nanoparticles by the micro-emulsion technique is possible. In the large-scale production, a large temperature controlled tank is used to prepare the micro-emulsion. Subsequently, the micro-emulsion is pumped into a cold water tank for the precipitation step. The temperature of the micro-emulsion and water, temperature flow in the aqueous medium, and hydrodynamics of mixing are the critical process parameters in

the large-scale production. Solvent Emulsification-Evaporation In this technique, first the lipid(s) is/are dissolved in a water-immiscible organic solvent (e.g., cyclohexane, chloroform) and then emulsified in an aqueous phase containing surfactants under continuous stirring. The organic solvent evaporates during emulsification, which results in lipid precipitation. As the whole formulation procedure can be conducted in room temperature, this technique is highly suitable for thermo-labile drugs. However, the major concern is the production of very dilute dispersion that needs to be concentrated by means of ultra-filtration or evaporation. Another concern is the use of organic solvent, some of which may remain in the final preparation. In contrary to solvent emulsification–evaporation technique, partially water-miscible organic solvents (e.g., benzyl alcohol, ethyl formate) are used in solvent-diffusion technique. In this case, organic solvents are mutually saturated with water to ensure initial thermodynamic equilibrium of both liquids. The transient oil-in-water emulsion is passed into water under continuous stirring, which leads to solidification of dispersed phase forming lipid nanoparticles due to diffusion of the organic solvent. However, similar to micro emulsion technique, dilute nanoparticle dispersion is produced, which needs to be concentrated by ultra-filtration or lyophilization. Usage of organic solvent is also a concern as some of it may remain in the final preparation. Solvent Injection The basic principle of the solvent injection method is similar to the solvent diffusion method. In case of solvent injection method, lipids are dissolved in a water-miscible solvent (e.g., acetone, isopropanol, and methanol) or water. Das and Chaudhary miscible solvent mixture and quickly injected into an aqueous solution of surfactants through an injection needle. The advantages of this method are the easy handling and fast production process without technically sophisticated equipment (e.g., high-pressure homogenizer). However, the main disadvantage is the use of organic solvents.

2.3.1.6 Double Emulsion

The double emulsion (w/o/w) method[17] is based on solvent emulsification–evaporation method. This method is mainly for the production of lipid nanoparticles loaded with hydrophilic drugs. In this case, the drug and stabilizer are encapsulated in the inner aqueous phase of the w/o/w double emulsion. A stabilizer is necessary to prevent drug partitioning to the outer aqueous phase during solvent evaporation. This type of

formulations is usually named as 'lipospheres' due to their comparatively larger particle size than SLNs.

2.3.2 Characterization

Characterization of the lipid nanoparticles is critical due to complexity of the system and colloidal size of the particles. Nevertheless, proper characterization of the formulations is necessary to control the product quality, stability, and release kinetics. Hence, accurate and sensitive characterization methods should be used. There are several important characterization techniques as follows[14].

2.3.2.1 Particle Size

The precise determination of the particle size is very important. Particle size less than 500 nm are advisable for the intestinal transport. Photon correlation spectroscopy (PCS) and laser diffraction (LD) are the most powerful and widely used techniques for the particle size measurement of lipid nanoparticles. PCS is also known as dynamic light scattering. The fluctuation of the intensity of the scattered light, caused by particles movement, is measured by this technique. It is relatively accurate and sensitive method. However, only size range from few nano-meters to about 3 μm can be measured by PCS. This size range is enough to characterize lipid nanoparticles. On the other hand, LD can measure bigger particle sizes (>3 μm). LD covers a broad size range from the nano-meter to the lower millimetre range. This method is based on the dependence of the diffraction angle on the particle radius. Smaller particles lead to more intense scattering at high angles than the larger particles. However, it is always recommended to use both PCS and LD method simultaneously as both methods do not directly measure particle sizes, rather particle sizes are calculated from their light scattering effects. This is because particles are non-spherical in many instances.

2.3.2.2 Polydispersity Index

As SLNs/NLCs are usually poly-disperse in nature, measurement of poly-dispersity index (PI) is important to know the size distribution of the nanoparticles to be in a narrow range. The lower PI value signifies the mono dispersity of the formulation. Most

of the researchers accept PI value less than 0.3 as optimum value. PI can be measured by PCS.

2.3.2.3 Zeta Potential

The zeta potential (ZP) indicates the overall charge of the particle. Stability of the nano dispersion during storage can be predicted form the ZP value. The ZP indicates the degree of repulsion between the particles in the dispersion. High ZP indicates highly charged particles. Generally, high ZP (negative or positive) prevents aggregation of the particles due to electric repulsion and electrically stabilizes the nanoparticle dispersion. On the other hand, in case of low ZP, attraction exceeds repulsion and the dispersion coagulates or flocculates. However, this assumption is not applicable for all colloidal dispersion, especially the dispersion which contain steric stabilizers. The ZP value of −30 mV is enough for good stabilization of a nano dispersion. The ZP of the Nano dispersions can be determined by PCS.

2.3.2.4 Shape and Morphology

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) are very useful techniques to determine the shape and morphology of lipid nanoparticles. These techniques can also determine the particle size and size distribution. SEM utilizes electron transmission from the sample surface, whereas TEM utilizes electron transmission through the sample. In contrast to PCS and LD, SEM and TEM provide direct information on the particle shape and size. Several SEM and TEM study showed spherical shape of the lipid nanoparticles. Although normal SEM is not very sensitive to the nano meter size range, field emission SEM (FESEM) can detect nano meter size range. However, sample preparation (e.g., solvent removal) may influence the particle shape. Cryogenic FESEM might be helpful in this case, where liquid dispersion is frozen by liquid nitrogen and micrographs are taken at the frozen condition. AFM technique is also gaining popularity for nanoparticle characterization. AFM provides a three-dimensional surface profile unlike electron microscopy which provides two-dimensional image of a sample. AFM directly provides structural, mechanical, functional, and topographical information about surfaces with nanometer- to angstrom-scale resolution. In this technique, the force

acting between a surface and a probing tip results in a spatial resolution of up to 0.01 nm for imaging. Direct analysis of the originally hydrated, solvent-containing samples is possible as no vacuum is needed during operation and the sample does not need to be conductive. Zur Muhlen compared AFM with SEM and reported same particle size of the nanoparticles by both methods[14].

2.3.2.5 Crystallinity and Polymorphism

Determination of the crystallinity of the components of SLN/NLC formulations is crucial as the lipid matrix as well as the incorporated drug may undergo a polymorphic transition leading to a possible drug leaching during storage. Lipid crystallinity is also strongly correlated with drug incorporation and release rates. Thermodynamic stability and lipid packing density increase, whereas drug incorporation rates decrease in the following order: Supercooled melt, α-modification, β′-modification, and β-modification[13]. However, lipid crystallization and modification changes might be highly retarded due to the small size of the particles and the presence of emulsifiers. Differential scanning calorimetry (DSC) and X-Ray diffractometry (XRD) are two widely used techniques to determine the crystallinity and polymorphic behaviour of the components of the SLNs/ NLCs. DSC provides information on the melting and crystallization behaviour of all solid and liquid constituents of the particles, whereas XRD can identify specific crystalline compounds based on their crystal structure. DSC utilizes the fact that different lipid modifications possess different melting points and melting enthalpies. In XRD, the monochromatic beam of X-ray is diffracted at angles determined by the spacing of the planes in the crystals and the type and arrangement of the atoms, which is recorded by a detector as a pattern. The intensity and position of the diffractions are unique to each type of crystalline material. XRD pattern can predict the manner of arrangement of lipid molecules, phase behaviour, and characterize and identify the structure of lipid and drug molecules. However, best results are observed when SLN dispersions are investigated directly as solvent removal may change the modification. Another two techniques, infrared and Raman spectroscopy are also useful to investigate structural properties of lipids. However, they have not been extensively used to characterize SLNs/ NLCs.

2.3.2.6 Assessment of Alternative Colloidal Structures

In several cases, lipid nanoparticles coexist with other colloidal structures (e.g., micelles, liposomes, mixed micelles, super cooled melts, and drug nanoparticles). However, characterization and quantification of these colloidal structures are difficult due to the similarities in size, low resolution of PCS to detect multimodal distributions, modification of the equilibrium of the complex colloidal system during sample preparation. Furthermore, dilution of the original nanoparticle dispersion with water might cause the removal of surfactant molecules from the particle surface and induce further changes. Hence, the methods which are sensitive to the simultaneous detection of different colloidal species and which do not require preparatory steps should be used. Nuclear magnetic resonance (NMR) and electron spin resonance (ESR) techniques are suitable for this purpose. These techniques are useful for investigating dynamic phenomena and the characteristics of the nano-compartments in colloidal lipid dispersions[18]. Detection of super-cooled melts due to the low line widths of the lipid protons is possible by 1 H-NMR spectroscopy. This technique is based on the different proton relaxation times in the liquid and semisolid/solid state. NMR also can characterize liquid nano-compartments in NLCs. ESR requires a paramagnetic spin probes to investigate SLN dispersions. Direct, repeatable, and non-invasive characterization of the distribution of the spin probe between the aqueous and the lipid phase can be performed by ESR. However, despite the great potential, NMR and ESR have been rarely applied to characterize SLNs and NLCs.

2.4 Introduction to Lipids

2.4.1 Dynasan 118

2.4.1.1 Description:

DYNASAN 118® microcrystalline triglycerides are glycerin esters of selected saturated, even numbered and unbranched fatty acids of plant origin. They are free from antioxidants and other stabilizers.

2.4.1.2 Synonyms:

Tristearin, Glyceryl Tristearate.

2.4.1.3 IUPAC Name and CAS Number:

IUPAC Name: 2,3-di(octadecanoyloxy)propyl octadecanoate.

CAS No: 555-43-1.

2.4.1.4 Application:

In tablets as lubricants having a very low influence on disintegration.

In suppositories, vaginal ovula and pharmaceutical/cosmetic sticks as crystallization accelerators and seeding agents to improve the solidification process.

2.4.1.5 Toxicology:

$LD_{50(rats)}$ = 2000 mg/kg.

2.4.2 Glyceryl Mono Oleate[19]

2.4.2.1 Nonproprietary Names BP:

Glycerol mono-oleates PhEur: Glyceroli mono-oleates USPNF: Glyceryl monooleate

2.4.2.2 Synonyms:

Aldo MO; Atlas G-695; Capmul GMO; glycerol-1-oleate; glyceryl mono-oleate; Kessco GMO; Ligalub; monolein; Monomuls 90-O18; mono-olein; a-mono-olein glycerol; Peceol; Priolube 1408; Stepan GMO; Tegin.

2.4.2.3 Chemical Name and CAS Registry Number:

9-Octadecenoic acid (Z), monoester with 1,2,3-propanetriol

CAS: [25496-72-4]

2.4.2.4 Empirical Formula and Molecular Weight:

C21H40O4

356.55

2.4.2.5 Structure:

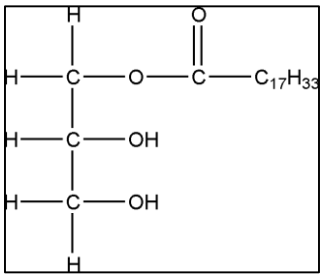

Fig 2.1: Structure of GMO.

Glyceryl monooleate is a mixture of the glycerides of oleic acid and other fatty acids, consisting mainly of the monooleate;

2.4.2.6 Functional Category:

Emollient; emulsifying agent; emulsion stabilizer; gelling agent; muco-adhesive; non-ionic surfactant; sustained release agent.

2.4.2.7 Regulatory Status:

GRAS listed. Included in the FDA Inactive Ingredients Guide (oral capsules, oral powder, oral tablets; creams, controlledrelease transdermal films). Included in nonparenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Non-medicinal Ingredients.

2.4.3 Soya Lecithin[19]

2.4.3.1 Non-Proprietary Name:

USPNF: Lecithin

2.4.3.2 Synonym:

E322; egg lecithin; LSC 5050; LSC 6040; mixed soybean phosphatides; ovolecithin; Phosal 53 MCT; Phospholipon 100 H; soybean lecithin; soybean phospholipids; Sternpur; vegetable lecithin.

2.4.3.3 Empirical Formula and Molecular Weight:

The USPNF 23 describes lecithin as a complex mixture of acetone-insoluble phosphatides that consists chiefly of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, combined with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates as separated from a crude vegetable oil source.

The composition of lecithin (and hence also its physical properties) varies enormously depending upon the source of the lecithin and the degree of purification. Egg lecithin, for example, contains 69% phosphatidylcholine and 24% phosphatidylethanolamine, while soybean lecithin contains 21% phosphatidylcholine, 22% phosphatidylethanolamine, and 19% phosphatidylinositol, along with other components.

2.4.3.4 Structural Formula:

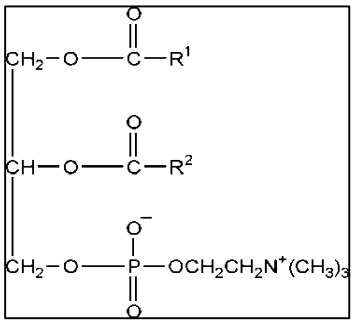

Fig 2.2: Structure of Soya Lecithin.

2.4.3.5 Functional Category:

Emollient; emulsifying agent; solubilizing agent.

2.4.3.6 Regulatory Status:

GRAS listed. Accepted for use as a food additive in Europe. Included in the FDA Inactive Ingredients Guide (inhalations; IM and IV injections; otic preparations; oral capsules, suspensions and tablets; rectal, topical, and vaginal preparations). Included in non-parenteral and parenteral medicines licensed in the UK. Included in the Canadian List of Acceptable Non-medicinal Ingredients.

3. Literature Review:

3.1 Literature Review on Oral NLC:

Fang et al.[20] Developed (NLC) for oral delivery of docetaxel (DTX) and investigated the absorption mechanism in vivo. Docetaxel-loaded nanostructured lipid carriers (DNLCs) were prepared by emulsification-ultrasonication and their physicochemical properties were characterized Differential scanning calorimetry (DSC) demonstrated that the drug was present in amorphous state and FTIR analysis suggested that the interaction of DTX and lipids involved hydrogen bonding and hydrophobic interactions. DNLCs were found to be stable in simulated gastrointestinal fluids and in vitro drug release studies revealed that the formulation exhibited sustained drug release for 48 h by Fickian diffusion. The drug absorption in the intestine was markedly improved by NLCs. An in vivo pharmacokinetic study demonstrated that the AUC for DNLCs (536.18 ± 91.21 ng/mLh) was increased 4.31-fold compared with that of docetaxel solution (DTX-Sol). DNLCs could be absorbed into the enterocytes through both endocytosis and passive transport. The oral bioavailability of DNLCs was significantly reduced after the lymphatic transport pathway was blocked. The overall results showed that the NLCs were a very effective method for increasing the oral absorption of docetaxel.

Tran et al.[21] investigated the potential of nanostructured lipid carriers (NLCs) in improving the oral bioavailability of a lipid lowering agent, fenofibrate (FEN). FEN-loaded NLCs (FENNLCs) were prepared by hot homogenization followed by an ultrasonication method using Compritol 888 ATO as a solid lipid, Labrafil M 1944CS as a liquid lipid, and soya lecithin and Tween 80 as emulsifiers. NLCs were characterized in terms of particle size and zeta potential, surface morphology, encapsulation efficiency, and physical state properties. Bioavailability studies were carried out in rats by oral administration of FEN-NLC. NLCs exhibited a spherical shape with a small particle size (84.9±4.9 nm). The drug entrapment efficiency was 99% with a loading capacity of 9.93±0.01% (w/w). Biphasic drug release manner with a burst release initially, followed by prolonged release was depicted for in vitro drug release studies. After oral administration of the FEN-NLC, drug concentration in plasma and AUC was fourfold higher, respectively, compared to the free FEN

suspension. According to these results, FENNLC could be a potential delivery system for improvement of loading capacity and control of drug release, thus prolonging drug action time in the body and enhancing the bioavailability.

Rahman et al[21]. developed Zerumbone, a natural dietary lipophilic compound with low water solubility (1.296 mg/L at 25°C) was used in this investigation. The zerumbone was loaded into nanostructured lipid carriers using a hot, high-pressure homogenization technique. The physicochemical properties of the zerumbone-loaded nanostructured lipid carriers (ZER-NLC) were determined. The ZER-NLC particles had an average size of 52.68 ± 0.1 nm and a polydispersity index of 0.29 ± 0.004 µm. Transmission electron microscopy showed that the particles were spherical in shape. The zeta potential of the ZER-NLC was -25.03 ± 1.24 mV, entrapment efficiency was 99.03%, and drug loading was 7.92%. In vitro drug release of zerumbone from ZER-NLC was 46.7%, and for a pure zerumbone dispersion was 90.5% over 48 hours, following a zero equation. Using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in human T-cell acute lymphoblastic leukemia (Jurkat) cells, the half maximal inhibitory concentration(IC) of ZER-NLC was 5.64 ± 0.38 µg/mL, and for free zerumbone was 5.39 ± 0.43 µg/mL after 72 hours of treatment. This study strongly suggests that ZER-NLC have potential as a sustained release drug carrier system for the treatment of leukemia.

Lamprecht et al.[22] The aim of the present work was to investigate the preparation of nanoparticles (NP) as potential drug carriers for proteins. The hydrophilic protein bovine serum albumin (BSA) was chosen as the model drug to be incorporated within NP. Owing to the high solubility of the protein in water, the double emulsion technique has been chosen as one of the most appropriate method. In order to reach submicron size we used a micro fluidizer as a homogenization device with a view to obtaining NP with a very high grade of monodispersity. Two different biodegradable polymers, poly [D,L-lactic-co-glycolic acid] 50:50 (PLGA) and poly [o-caprolactone] (PCL) has been used for the preparation of the NP. The drug loading has been optimized by varying the concentration of the protein in the inner aqueous phase, the polymer in the organic phase, the surfactant in the external aqueous phase, as well as the volume of the external aqueous phase. The BSA encapsulation efficiency was high (80%) and release profiles were characterized by a substantial initial burst release for both PLGA and PCL NP. A

higher release was obtained at the end of the dissolution study for PLGA NP (92%) compared with PCL NP (72%).

Bhandari et al.[23] Developed the constitution of the lipid matrix to achieve significantly high entrapment of hydrophilic drugs within solid lipid nanoparticles (SLNs). Isoniazid was selected as a representative hydrophilic drug with a high solubility of 230 mg/ml and a log P of -0.402 at 25°C (determined as per OECD TG 105 and 107 respectively). Three lipids/fatty acids (Glyceryl monostearate, Compritol 888 ATO and stearic acid) were evaluated out of which Compritol 888 ATO® and stearic acid showed favourable interactions (FTIR and DSC studies) with isoniazid. The two lipids were used alone or in combination for preparing SLNs. Formulation of SLNs by micro-emulsification, method involved pouring the hot micro-emulsion into cold water under constant stirring, which may result in expulsion of the hydrophilic drug from the lipid matrix; hence, partitioning of isoniazid from the hot lipid melts into cold water was also determined. Results indicate that combining stearic acid with Compritol 888 ATO® in certain ratio (1:4) led to significant entrapment efficiency (EE) of 84.0±1.1%. The formulations were subjected to morphological, physiochemical and *in vitro* drug release studies. Developed SLNs were found to be stable for 1 year at 4 °C. The study demonstrated the benefit of excipient screening techniques in improving entrapment efficiency of a hydrophilic drug.

Ranpise et al.[24] developed Lercanidipine hydrochloride is a calcium channel blocker used in the treatment of hypertension. It is a poor water soluble drug with absolute bioavailability of 10%. The aim of this study was to design lercanidipine hydrochloride-loaded nanostructured lipid carriers to investigate whether the bioavailability of the same can be improved by oral delivery. Lercanidipine hydrochloride nanostructured lipid carriers were prepared by the method of solvent evaporation at a high temperature and solidification by freeze drying. The nanostructured lipid carriers were evaluated for particle size analysis, zeta potential, entrapment efficiency, in vitro drug diffusion, ex vivo permeation studies and pharmacodynamics study. The resultant nanostructured lipid carriers had a mean size of 214.97 nm and a zeta potential of -31.6 ±1.5 mV. More than 70% lercanidipine hydrochloride was entrapped in the NLCs. The SEM studies indicated the formation of type 2 nanostructured lipid carriers. The in vitro release studies demonstrated 19.36% release in acidic buffer pH 1.2 indicating that the drug

entrapped in the nanostructured lipid carriers remains entrapped at acidic pH. The ex vivo studies indicated that the drug release was enhanced from 10% to 60.54% at blood pH in 24 h. The in vivo pharmacodynamic study showed that NLCs released lercanidipine hydrochloride in a controlled manner for a prolonged period of time as compared to plain drug. These results clearly indicate that nanostructured lipid carriers are a potential controlled release formulation for lercanidipine hydrochloride and may be a promising drug delivery system for the treatment of hypertension.

Li et al. showed[25] (5-fluorouracil, irinotecan, oxaliplatin) and folinic acid (a vitamin B derivatives reducing the side effect of 5-fluorouracil), has proved to be effective in the treatment of pancreatic cancer, and is more efficacious than the long-term reference standard, gemcitabine. However, the FOLFIRINOX is associated with high-grade toxicity, which markedly limits its clinical application. Encapsulation of drugs in nano-carriers that selectively target cancer cells promises to be an effective method for co-delivery of drug combinations and to mitigate the side effects of conventional chemotherapy. Here we reported the development of multiple layer-by-layer lipid-polymer hybrid nanoparticles with targeting capability that show excellent biocompatibility and synergistically combine the favourable properties of liposomes and polymer nanoparticles. Relative to nanoparticles consisting of polymer alone, these novel nano-carriers have a long half-life in vivo and a higher stability in serum. The nano-carriers were loaded with the three active anti-tumour constituents of FOLFIRINOX regimen. Little drugs were released from the nanoparticles in phosphate buffered saline (PBS) solution, but the cargoes were quickly released after the nanoparticles were taken up by tumour cells. These innovative drug-loaded nanoparticles achieved higher anti-tumour efficacy and showed minimal side effects compared with the FOLFIRINOX regimen alone. Our study suggested that the multiple layer-by-layer hybrid nanoparticles have great potential for improving the chemotherapeutic efficacy for the patients with pancreatic cancer. This platform also provides new opportunities for tailored design of nanoparticles that may offer therapeutics benefits for a range of other tumour s.

Mendes et al.[25] Miconazole is a widely used antifungal agent with poor aqueous solubility, which requires the development of drug delivery systems able to improve its therapeutic activity. For this purpose, a miconazole-loaded nanostructured lipid carriers

(NLC) dispersion was prepared and characterized. Further, the dispersion was used to prepare a NLC-based hydrogel formulation proposed as an alternative system to improve the local delivery of miconazole to the oral mucosa. NLC dispersion showed particles in the nanometer range (˜200 nm) with low poly dispersity index (<0.3), good physical stability and high encapsulation efficiency (>87%). A controlled miconazole release was observed from NLC and NLC-based hydrogel formulations, in contrast to a commercial oral gel formulation, which demonstrated a faster release. Additionally, it was observed that the encapsulation of miconazole in the NLC improved its antifungal activity against Candida albicans. Therefore, it was demonstrated that the encapsulation of miconazole in NLC allows for obtaining the same therapeutic effect of a commercial oral gel formulation, using a 17-fold lower dose of miconazole.

Zheng et al.[26] demonstrated after oral administration in rodents, triptolide (TP), a diterpenoid triepoxide compound, active as anti-inflammatory, immunosuppressive, anti-fertility, anti-cystogenesis, and anticancer agent, is rapidly absorbed into the blood circulation (from 5.0 to 19.5 minutes after dosing, depending on the rodent species) followed by a short elimination half-life (from about 20 minutes to less than 1 hour). Such significant and rapid fluctuations of TP in plasma likely contribute to its toxicity, which is characterized by injury to hepatic, renal, digestive, reproductive, and haematological systems. With the aim of prolonging drug release and improving its safety, TP-loaded nanostructured lipid carriers (TP-NLCs), composed of Compritol 888 ATO (solid lipid) and Capryol™ 90 (liquid lipid), were developed using a micro-emulsion technique. The formulated TP-NLCs were also characterized and in vitro release was evaluated using the dialysis bag diffusion technique. In addition, the pharmacokinetics and toxicology profiles of TPNLCs were compared to free TP and TP-loaded solid lipid nanoparticles (TP-SLNs; containing Compritol 888 ATO only). Results demonstrate that TP-NLCs had mean particle size of 231.8 nm, increased drug encapsulation with a 71.6% efficiency, and stable drug incorporation for over 1-month. TP-NLCs manifested a better in-vitro sustained-release pattern compared to TPSLNs. Furthermore, TP-NLCs prolonged mean residence time (MRT) (P,0.001, P,0.001), delayed T max (P,0.01, P,0.05) and decreased max 0–t (P,0.01, P,0.05) compared to free TP and TP-SLNs, respectively, which was associated with reduced sub-acute

toxicity in male rats. In conclusion, our data suggest that TP-NLCs are superior to TP-SLNs and could be a promising oral delivery system for a safer use of TP.

Zhuang et al.[27] developed an optimized nanostructured lipid carriers (NLC) formulation for vinpocetine (VIN), and to estimate the potential of NLC as oral delivery system for poorly water-soluble drug. In this work, VIN–loaded NLC (VIN–NLC) was prepared by a high pressure homogenization method. The VIN–NLC showed spherical morphology with smooth surface under transmission electron microscope (TEM) and scanning electron microscopic (SEM) analysis. The average encapsulation efficiency was 94.9 ±0.4%. The crystallization of drug in NLC was investigated by powder X-ray diffraction and differential scanning calorimetry (DSC). The drug was in an amorphous state in the NLC matrix. In the in vitro release study, VIN–NLC showed a sustained release profile of VIN and no obviously burst release was observed. The oral bioavailability study of VIN was carried out using Wistar rats. The relative bioavailability of VIN–NLC was 322% compared with VIN suspension. In conclusion, the NLC formulation remarkably improved the oral bioavailability of VIN and demonstrated a promising perspective for oral delivery of poorly water-soluble drugs.

3.2 Literature Review on Oral Chemotherapy:

Guerrero et al.[9] showed the increase in the therapeutic arsenal in the last 20 years, has given rise to changes in treating colorectal cancer (CRC) with only pyrimidines to combine several cytotoxic drugs. However, the present question is to determine the optimal sequence of this combination. This review presents an update of data on chemical and clinical features of chemotherapy used for colorectal cancer and the mechanisms of cellular resistance and potential predictive and prognostic biomarkers, which may contribute to a better selection of a therapeutic strategy.

Sharma et al.[11] showed A number of novel oral chemotherapeutic agents are entering practice or are under development in the United States. Many of these agents display significant clinical activity against colorectal cancer. Many classes of compounds, including fluoropyrimidine analogs, dihydropyrimidine dehydrogenase (DPD) inhibitors, topoisomerase inhibitors, farnesyl transferase inhibitors, and others, are being developed for oral administration. This manuscript describes the progress of clinical development of these agents and also explores the relative merits and challenges of these approaches. Economic issues, patient preference, and patient selection issues surrounding oral chemotherapy for colorectal cancer will also be discussed.

Golla et al.[28] showed apodoxonano and lactodoxonano, the number of neoplastic nodules was significantly lower than that of rats administered with saline or with doxo. Apodoxonano and lactodoxonano did not exhibit decrease in mean body weight, which was markedly reduced by 22% in the case of doxo administered rats. In rats treated with nanoformulations, the number of liver nodules was found reduced by >93%. Both nanoformulations showed significantly high localization in liver compared to doxo.

Zhou et al.[29] showed the therapeutic use of this compound is limited by its poor solubility and low bioavailability. Here a novel biotin-modified nanostructured lipid carrier (NLC) was developed to enhance the bioavailability of Ori. The effect of ligand (biotin) modification on oral absorption of Ori encapsulated in NLCs was also explored. Ori-loaded NLCs (Ori-NLCs) were prepared by the melt dispersion-high pressure homogenization method. Biotin modification of Ori-NLCs was achieved by EDC and NHS in aqueous phase. The obtained biotin-decorated Ori-NLCs (Bio-Ori-NLCs) were

144.9 nm in size with an entrapment efficiency of 49.54% and a drug load of 4.81%. Oral bioavailability was enhanced by use of Bio-Ori-NLCs with a relative bioavailability of 171.01%, while the value of non-modified Ori-NLCs was improved to 143.48%. Intestinal perfusion showed that Ori solution unexpectedly exhibited a moderate permeability, indicating that permeability was not a limiting factor of Ori absorption. Ori could be rapidly metabolized that was the main cause of low bioavailability. However, there was a difference in the enhancement of bioavailability between Bio-Ori-NLCs and conventional NLCs. Although severe lipolyses happened both on Bio-Ori-NLCs and non-modified NLCs, the performance of Bio-Ori-NLCs in the bioavailability improvement was more significant. Overall, Bio-Ori-NLCs can further promote the oral absorption of Ori by a ligand-mediated active transport. It may be a promising carrier for the oral delivery of Ori.

Cho et al. [30] showed Drug resistance, another obstacle that impedes the efficacy of both molecularly targeted and conventional chemotherapeutic agents, might also be overcome, or at least reduced, using nanoparticles. Nanoparticles have the ability to accumulate in cells without being recognized by P-glycoprotein, one of the main mediators of multidrug resistance, resulting in the increased intracellular concentration of drugs. Multifunctional and multiplex nanoparticles are now being actively investigated and are on the horizon as the next generation of nanoparticles, facilitating personalized and tailored cancer treatment.

3.3 Literature Review on PEGylated Nano-Particles

Yuan et al.[31] Evaluated the potential of PEGylated solid lipid nanoparticle (pSLN) as mucus penetrating particles (MPP) for oral delivery across gastrointestinal mucus. The SLN was prepared by an aqueous solvent diffusion method, subsequently modified with PEG2000-stearic acid (PEG2000-SA) as hydrophilic groups. Surface properties, cytotoxicity, cellular uptake, and transport across Caco-2/HT29 coculture cell monolayers, intestinal absorption, and pharmacokinetics of pSLN were studied compared with that of SLN. Quantitative cellular uptake showed that the internalization of SLN and pSLN was an active transfer process, which would be restrained by several inhibitors of cell activity. Compared with SLN, the permeation ability of pSLN decreased through Caco-2 cell monolayer while it increased through a mucus-secreting Caco-2/HT29 coculture cell monolayer, which indicated that the mucus layer has a significant impact on determining the efficiency of oral nanoformulations. In addition to increasing permeation ability, the stability of the nanoparticles in simulated intestinal fluids was also increased by the PEGylation. Moreover, in vitro everted gut sac technique and the ligated intestinal loops model in vivo also demonstrated that pSLN can rapidly penetrate mucus secretions, whereas the SLN were strongly trapped by highly viscoelastic mucus barriers. The pharmacokinetic studies presented that pSLN exhibited improved absorption efficiency and prolonged blood circulation times with a 1.99-fold higher relative bioavailability compared with SLN. In conclusion, PEGylated solid lipid nanoparticles had advantages in enhancing the bioavailability of oral administration.

Sharma et al.[32] developed It was envisaged to develop surface modified Guar Gum Nanoparticles (GGNP) with Folic acid (FA) charged with methotrexate (MTX) to target the colon specifically. The MTX loaded FA functionalized GGNP (MTX-FA-GGNP) have been prepared by emulsion crosslinking method. These surface modified nanoparticles were compared with unmodified MTX loaded GGNP (MTX-GGNP). The developed formulations were evaluated for size and size distribution, zeta potential, Differential Scanning Calorimetry (DSC), release profile and uptake studies. The nanoparticles have been found to have average size of 325 nm in diameter having polydispersity index (PDI) 0.177 indicating mono-disperse particles. The zeta potential

of the particles was found to be -36.9 mV. The percent growth inhibition of Caco 2 cells with MTX-FA-GGNP was found to be better than MTX-GGNP indicating folate receptor mediated uptake. The MTX-GGNP protects the release of MTX in upper gastrointestinal tract while maximum release of MTX occurred in simulated colonic fluids of pH 6.8. The in vivo uptake studies revealed preferential uptake of nanoparticles formulation in the colon. These studies provide evidences that MTX-FA-GGNP holds promise to address colorectal cancer over-expressing folate receptors. This prototype formulation enjoys dual advantage of having propensity to release the drug in the colon and in the conditions of colorectal carcinoma; it could be better localized and targeted with improved therapy due to over-expression of folate receptors.

Semete et al.[33] Elucidated the effect of surface coating with various concentrations of polymeric surfactants (PEG and Pluronics F127) on the in vitro protein binding as well as the tissue biodistribution, post oral administration, of PLGA nanoparticles. The in vitro protein binding varied depending on the polymeric surfactant used. However, in vivo, 1% PEG and 1% Pluronics F127 coated particles presented similar biodistribution profiles in various tissues over seven days. Furthermore, the percentage of PEG and Pluronics coated particles detected in plasma was higher than that of uncoated PLGA particles, indicating that systemic circulation time can also be increased with oral formulations. The difference in the in vitro protein binding as a result of the different poloxamers used versus similar in vivo profiles of these particles indicates that in vitro observations for nanoparticles cannot represent or be correlated to the in vivo behaviour of the nanoparticles. Our results therefore suggest that more studies have to be conducted for oral formulations to give a better understanding of the kinetics of the particles.

Zhang et al.[34] Optimized the formulations of dihydroartemisinin nanostructured lipid carrier (DHA-NLC). The CCRD consisting of three-factored factorial design with three levels was used in this study. The drug encapsulation efficiency (EE), drug loading (DL) percentage and particle size of DHA-NLC were investigated with respect to three independent variables including DHA concentration (X1), lipid concentration (X2) and ratio of liquid lipid to total lipid (X3). The result showed that the optimal formulation could be obtained from this response surface methodology. The optimal formulation for DHA-NLC was composed of DHA concentration (X1) of 1 g/l, lipid concentration

(X2) of 1% and ratio of liquid lipid to total lipid (X3) of 0.1:1. DHA-NLC under the optimized conditions gave rise to the EE of (98.97) %, DL of (15.61) %, mean diameter of (198) nm, polydispersity index (PI) of 0.146 and zeta potential value of (- 21.6) mV. TEM of the optimized NLC showed spherical particles.

Fuentes, et al.[3] reported that Colloidal drug carriers prepared from solid triglycerides have been presented as a promising alternative to polymer nanoparticles. The present work is aimed at developing surface-modified lipid nanoparticles intended to encapsulate peptides within their structure and to study their physicochemical properties and in vitro stability in gastrointestinal fluids. The final goal is to explore their potential as oral delivery vehicles for macromolecules. The W/O/W multiple emulsion technique was originally applied and conveniently modified for the production of tripalmitin nanoparticles. This technique was selected because its makes the encapsulation of peptides feasible. Additionally, the surface of the particles could be modified through the incorporation of Poloxamer 188 or the lipid derivative PEG 2000-stearate into the formulation. This modification led to a reduction in the zeta potential values, varying from -34 mV for the non-coated particles to -20 mV for those prepared with PEG-stearate. Results of the stability of the nanoparticles in gastric and intestinal media indicate that the low pH of the gastric medium and the pancreatic enzymes in intestinal medium are responsible for the extensive aggregation and degradation of the non-coated lipid nanoparticles (80% degradation in 4 h). In contrast, PEG-stearate coated nanoparticles were more stable, as their polymer coating layer totally prevented aggregation in both media and significantly reduced pancreatin-induced degradation (40% approximately in 4 h). Preliminary studies showed that insulin, chosen as a model peptide, could be associated and released from PEG-stearate coated nanoparticles. Nevertheless, further work is required in order to optimize the release behaviour of the entrapped peptide.

4.1 Materials:

Dynasan 118 was obtained as a gift sample from Cremer Oleo, GmBH, Germany, Glyceryl Mono Oleate was obtained as a gift sample from Hallstar, GmBH, Germany, Soya Lecithin, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) was purchased from Himedia, Mumbai, MONEPEG S™ 401 (Polyethylene Glycol Stearate) was obtained as a gift sample from Mohini Organics Pvt Ltd., Mumbai, Stearyl Amine and Tween 80 was purchased from CDH, Sephadex G-50 was purchased from MPBio, USA. Dichloromethane, Methanol was purchased from CDH.

Phospholipon 90 H, Phospholipon 90 G, Phospholipon 90 S, Lipo-PGO was a kind gift from Lipoid, GmBH, Germany. Span 80 was purchased from CDH. TPGS was a kind gift from Antares Pharma, USA.

Creatinine Test Kit, Urea Berthelot Test Kit, SGPT Test Kit, SGOT Test Kit, Total Protein Test Kit, Albumin Test Kit were purchase from Accucare, Mumbai.

4.2 Methodology

4.2.1 Identification of NNS

4.2.1.1 Melting Point Determination of NNS

The Thiel's tube method for the determination of melting point in the presence of liquid paraffin was applied for determining the melting point of NNS. The capillary was filled with drug crystals and placed in the melting point apparatus. The temperature at which the solid drug crystals started melting was marked as the melting point of the drug.

4.2.1.2 UV Determination of Drug

40 µg/ml solution of NNS was prepared in distilled water and was analysed using Shimadzu-1800 double beam UV-Visible Spectrophotometer in the range of 200 nm to 800 nm.

4.2.2 Establishment of Calibration Curve of NNS

4.2.2.1 Establishment of Calibration Curve of NNS in Phosphate Buffer pH 7.4:

A stock solution was prepared by accurately weighing 10 mg of NNS in 10 ml volumetric flask and volume was made up by PBS (pH 7.4). Further dilutions in the range of 20 µg/ml to 120 µg/ml was prepared and analysed under double beam UV-Visible Spectroscopy. The graph of concentration vs. absorbance was prepared.

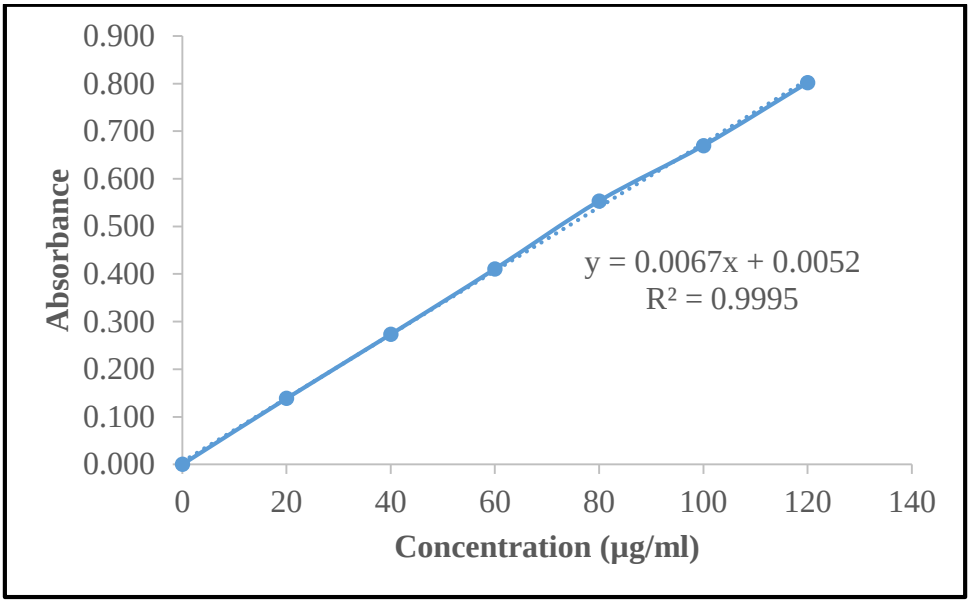

Fig 4.2: Calibration Curve of NNS in Phosphate Buffer pH 7.4

Table 4.3: Regression Analysis of NNS in PBS 7.4

Regression Parameter	Value
Correlation Coefficient (R^2)	0.9995
Slope	0.0067
Intercept	0.0052

4.2.2.2 Establishment of Calibration Curve of NNS in Distilled Water:

A stock solution was prepared by accurately weighing 10 mg of NNS in 10 ml volumetric flask and volume was made up by Distilled Water. Further dilutions in the range of 20 µg/ml to 120 µg/ml was prepared and analysed under double beam UV-Visible Spectroscopy. The graph of concentration vs. absorbance was prepared.

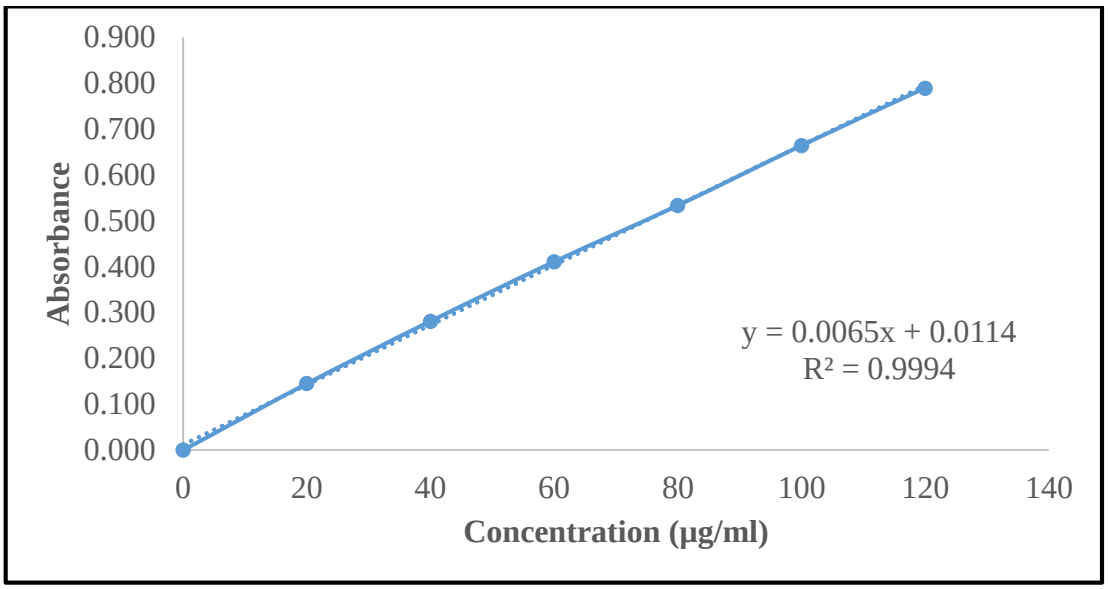

Fig 4.4: Calibration Curve of NNS in Water

Table 4.5: Regression Analysis of NNS in Water

Regression Parameter	Value
Correlation Coefficient (R^2)	0.9994
Slope	0.0065
Intercept	0.0114

4.2.2.3 Establishment of Calibration Curve of NNS in Solvent System:

Dichloromethane and Methanol was chosen as the solvent system in the ratio of (1:1). A stock solution of 1000 ppm was prepared by dissolving 10 mg of drug in 10 ml of solvent system. Further dilutions in the range of 20µg/ml to 120µg/ml was prepared and analysed under double beam UV-Visible Spectroscopy. The graph of concentration vs. absorbance was prepared.

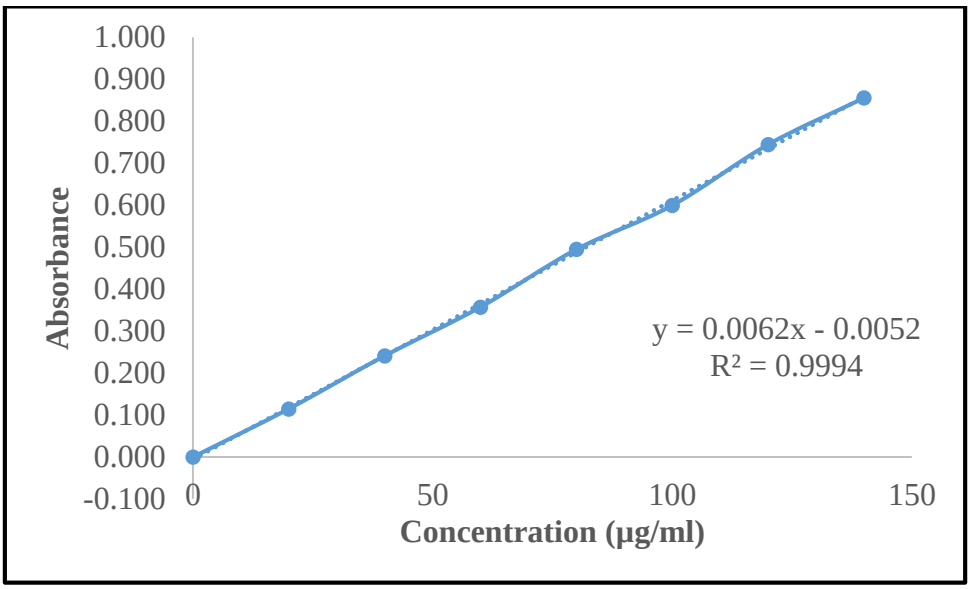

Fig 4.6: Calibration Curve of NNS in Solvent System

Table 4.7: Regression Analysis of NNS in Solvent System

Regression Parameter	Value
Correlation Coefficient (R^2)	0.9994
Slope	0.0062
Intercept	0.0052

4.2.2.4 Establishment of Calibration Curve of NNS in Phosphate Buffer pH 6.8:

A stock solution was prepared by accurately weighing 10 mg of NNS in 10 ml volumetric flask and volume was made up by PBS (pH 6.8). Further dilutions in the range of 20 µg/ml to 120 µg/ml was prepared and analysed under double beam UV-Visible Spectroscopy. The graph of concentration vs. absorbance was prepared.

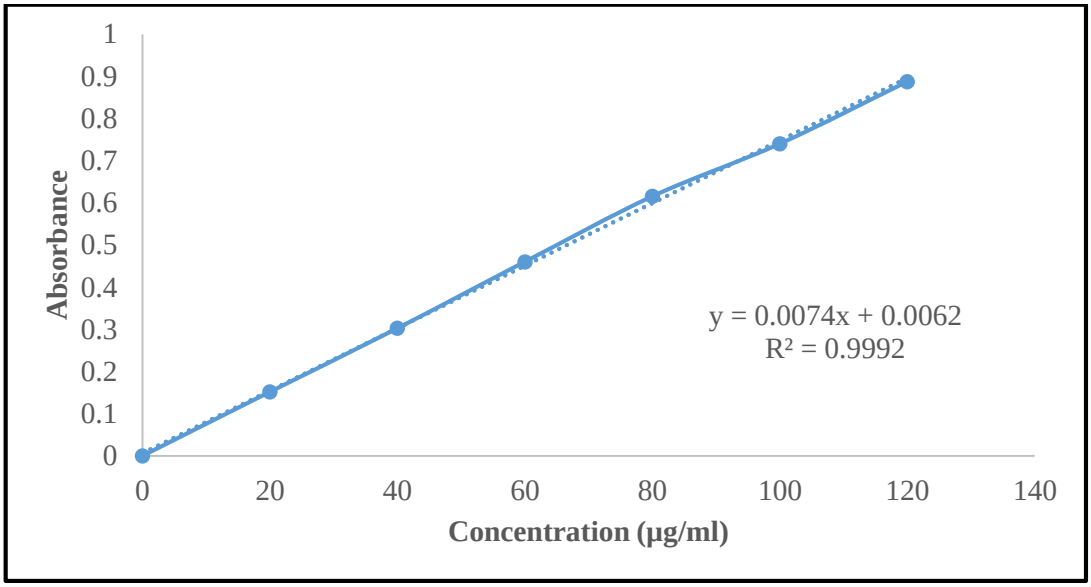

Fig 4.8: Calibration Curve of NNS in Phosphate Buffer pH 6.8.

Table 4.9: Regression Analysis of NNS in Solvent System

Regression Parameter	Value
Correlation Coefficient (R^2)	0.9992
Slope	0.0074
Intercept	0.0062

4.2.2.5 Establishment of Calibration Curve of NNS in 0.1 N HCL pH 1.2:

A stock solution was prepared by accurately weighing 10 mg of NNS in 10 ml volumetric flask and volume was made up by 0.1 N HCL (pH 1.2). Further dilutions in the range of 20 µg/ml to 120 µg/ml was prepared and analysed under double beam UV-Visible Spectroscopy. The graph of concentration vs. absorbance was prepared.

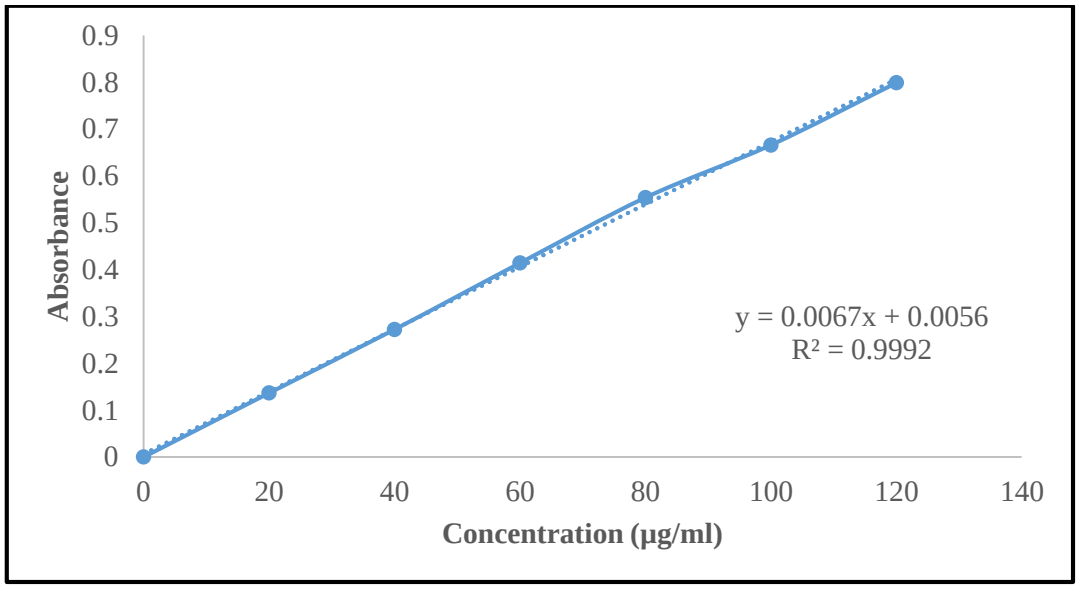

Fig 4.10: Calibration Curve of NNS in 0.1 N HCL pH 1.2.

Table 4.11: Regression Analysis of NNS in Solvent System

Regression Parameter	Value
Correlation Coefficient (R^2)	0.9992
Slope	0.0067
Intercept	0.0056

4.2.4 Development of Nano-Structured Lipid Carrier Systems NLCs

4.2.4.1 Preparation of Non-PEGylated NLCs

The binary lipid mixture was allowed to melt at 5˚C above its melting point. A surfactant solution was prepared separately. Further, the pre-dispersion was added into the surfactant solution & shearing it at 8000 rpm under mechanical stirrer. The micro-emulsion prepared was then allowed to undergo further size reduction using High Pressure Homogenizer.

4.2.5 Characterization of Nano-Structured Lipid Carriers:

4.2.5.1 Determination of Particle Size Distribution (Z-Avg) and Polydispersity Index (PDI)[14]

The particle size distribution and poly-dispersity index was measured under photon correlation spectroscopy using Malvern Zetasizer NS. Briefly, the sample was diluted 10x with water for injection and placed in glass cuvette for particle size distribution and poly-dispersity index analysis.

4.2.5.2 Determination of Zeta-Potential[14]

The electrophoretic mobility (zeta potential) measurements has been made using Malvern Zetasizer NS. The sample was diluted 10x with water for injection and injected into the Zeta-Cell, thereby placing it under Malvern Zetasizer for the analysis of zeta potential of the sample.

4.2.5.4 Differential Scanning Calorimetry Analysis[35]

DSC thermogram was recorded by heating the samples from 35 C to 300 C at a heating rate of 10 C/min. using an empty aluminium pan as the reference. Powdered samples were accurately weighed and subjected to DSC using Shimadzu DSC-60 and analysed using the software TA-60.

4.2.5.5 Transmission Electron Microscopy Analysis[35]

The morphology of NLCs were determined using Tecnai-20 (Philips, Holland). A drop of NLC dispersion (1mg/ml) was placed on copper grid, followed by addition of 2% (w/v) uranyl acetate. After 3 min incubation at room temperature, the grid was air-dried and images were captured using iTEM software.

4.2.5.6 Fourier Transform Infrared Analysis[35]

The FTIR spectra of freeze dried NLCs without the presence of cryoprotectant were analysed using Shimadzu 8400 S FTIR. The freeze-dried NLC were mixed with well dried Potassium Bromide (KBr) as the diluent. A total of 100 scans were run over the range of 4000-400 cm^{-1}.

4.2.6 In-Vitro Characterization of NLCs

4.2.6.1 In-Vitro Release Study of NLCs[35]

In-vitro drug release study was carried out by taking 5ml of nano-particulate suspension and suspended into dialysis membrane with molecular weight cut-off of 12kDa. The dialysis membrane was soaked in buffer, 12 h prior to the experiment to activate the pores and removal of preservatives. The bag was suspended individually in 50 ml of HCL pH 1.2, PBS pH 6.8, and PBS pH 7.4 Buffers at 37˚C in water bath at 100 rpm. Aliquots were taken at the time points of 1h, 2h, 3h, 4h, 6h, 8h, 12h, 24h, 48h and 72h and replenished by equal amount of the buffer in order to maintain the sink condition and subjected to analysis for the presence of drug in the respective buffer by UV-Vis spectrophotometry.

4.2.10 In-Vivo Characterization of Nano-Structured Lipid Carriers

4.2.10.11 Ethics Committee Approval

Protocol of the experiment was approved by institutional animal ethics committee in accordance with the guidance of committee for the purpose of control and supervision of experiments on animals (CPCSEA), held under Ministry of Forests, Environment and Climate Change, Government of India.

4.5 Biochemical Parameters

4.5.1 Albumin

Method:

Colorimetric end point test.

Principle:

The measurement of serum albumin is based on its quantitative binding to the indicator 3, 3', 5, 5' - tetrabo-m-cresol sulphophthalein (bromocresol green, BCG). The albumin-BCG complex absorbs maximally at 578 nm.

Sample:

Serum or Plasma

Reagents:

Reagent I : BCG Reagent

Reagent II : Albumin standard : 4 g/dl (store at 2 - 8°C)

Manual Procedure:

PIPETTE INTO TEST TUBE

	BLANK	STD	SAMPLE
SAMPLE	-	-	5 µl
STANDARD	-	5 µl	-
REAGENT	1000 µl	1000 µl	1000 µl

Mix well and wait for 5 min at 20 - 25°C. Measure the absorbance of the sample (Ac) and standard (As) against reagent blank.

Calculation And Linearity:

Ac/As x Conc. Std. = g/dl Albumin

4.5.2 Creatinine

Method:

Colorimetric kinetic test.

Principle:

Creatinine in alkaline solution reacts with picrate to form a coloured complex which absorbs at 500 - 520 nm. The amount of complex formed is directly proportional to the creatinine concentration.

Sample:

Serum, Urine 24h diluted 1 : 20 with distilled water.

Reagents:

Reagent I : Buffer Reagent

Reagent II : Picrate reagent

Reagent III : Creatinine Standard 2 mg/ dl

Reagent Preparation:

Mix equal volumes of solution 1 and 2 and let stand for 30 min.

Manual Procedure:

PIPETTE INTO TEST TUBE

REAGENT 1 µl	BLANK	STD	Sample
Sample	-	-	100 µl
Standard	-	100 µl	-
Reagent	1000 µl	1000 µl	1000 µl

Mix and after 30 secs at R.T., read initial absorbance and start timer simultaneously. Read again after 2 min. determine p A min. of standard (As) and sample (Ac) against reagent blank.

Calculation and Linearity:

P Ac/p As x C = mg / dl Creatinine Serum
p Ac/p As x C x 20 = mg / dl Creatinine Urine

4.5.3 Blood Urea Nitrogen (BUN)

Method:

Colorimetric end point test.

Principle:

The Berthelot reaction has long been used for the measurement of urea and ammonia. The present method is a modified Berthelot Method. The urea colorimetric procedure is a modification of the Berthelot reaction. Urea is converted to ammonium by the use of urease. Ammonium ions then react with a mixture of salicylate, sodium nitroprusside and hypochlorite to yield a blue-green chromophore. The intensity of the color formed is proportional to the urea concentrtion in the sample.

Sample:

Serum or Plasma.

Reagents:

Reagent I : Urea Enzyme reagent

Reagent II : Urea Color developer

Reagent III : Urea Std : 50 mg/dl

Reagent Preparation:

Reagent I : Dissolve contents of Reagent I with the Volume of distilled water as specified on the vial

Reagent II : Ready to use

Manual Procedure:

	BLANK	STD	SAMPLE
SAMPLE	-	-	10µl
STANDARD	-	10µl	-
REAGENT I	1000µl	1000µl	1000µl

Incubate all tubes for 5 mts at 37°C or 10 mts at rt.

REAGENT II	1000µl	1000µl	1000µl

Incubate all tubes for 5 mts at 37°C or 10 mts at rt. Zero sectrophotometer with the reagent blank at 578 nm. Read and record absorbance of samples of all tubes.

Calculation and Linearity:

Abs of Unknown / Abs of Std. X Conc. of Std. = mg/dl Urea

4.5.4 SGPT (Glutamic – pyruvic transaminase)

Method:

Kinetic UV test

Principle:

Glutamic-pyruvic Transaminase (GPT - ALT) catalyses the reaction between alpha - ketoglutaric acid and alanine giving L-glutamic acid and Pyruvic acid. Pyruvic acid, in the presence of lactate dehydrogenase (LDH) reacts with NADH giving lactic acid and NAD. The rate of NADH consumption is determined photometrically and is directly proportional to the GPT activity in the sample.

Sample:

Serum or plasma.

Reagents:

Reagent I : Buffer reagent

Reagent II : Enzyme reagent

Reagent Preparation:

Dissolve one vial of enzyme reagent with amount of buffer reagent as specified on the enzyme vial.

Manual Procedure:

PIPETTE INTO TEST TUBE

STANDARD	100 µl
REAGENT	1000 µl

Mix well and let stand for 1 min. at 37°C. Measure absorbance decrease per minute during 3 minutes (p A/min)

Calculation and Linearity:

p A/min. x 1746 = U/l ALT

4.5.5 SGOT (Glutamic – oxaloacetic transaminase)

Method:

Kinetic UV test

Principle:

Aspartate transaminase (GOT - AST) catalyses the reaction between alpha - ketoglutaric acid and L-aspartate giving glutamate and oxaloacetate. Oxaloacetate, in the presence of malate dehydrogenase (MDH) reacts with NADH giving malate and NAD. The rate of NADH decrease is determined photometrically and is directly proportional to the GOT activity in the sample.

Reagents:

Reagent I : Buffer Reagent

Reagent II : Enzyme reagent

Sample:

Serum or plasma.

Reagent Preparation:

Dissolve one vial of enzyme reagent with amount of buffer reagent as specified on the enzyme vial.

Manual Procedure:

PIPETTE INTO TEST TUBE

SAMPLE	100 µl
REAGENT	1000 µl

Mix well and let stand for 1 min. at 37°C. Measure absorbance decrease per minute during

3 minutes and determine the p A/min

Calculation and Linearity:

p A/min. x 1746 = U/l AST

5.1 Size Reduction by High Pressure Homogenization

It is purely mechanical process, passing the fluidic media through a narrow slit under high pressure. The liquid media is subjected to very high shear stress causing the formation of very fine emulsion droplets. Origin of this shear is the sudden restriction of flow under high pressure through a restrictive valve. Therefore, extreme shear and high energy input reduces the particles size from micro to nano scale. The size reduction achieved by high pressure homogenizer is indeed one of its own kind and reproducible. A uniform reduction in the size of the particles with narrow particle size distribution can be achieved by this method of size reduction. A number of articles has been published related to production of lipid nanoparticles using high pressure homogenizer describing the efficiency and reproducibility of this technique.

5.2.1 Selection of Solid Lipid

The selection of solid lipid was based on maximum solubility of drug in it. A number of lipids were chosen for the study.

5.2.2 Selection of Liquid Lipid

As mentioned above in the selection of solid lipid, the criteria for selection of liquid lipid remained the same.

5.2.4 Selection of Surfactant

Various types of non-ionic surfactants were evaluated. The formulation of NLCs with Poloxamer 407 and 188 produced a lot of aggregation whereas the sphericity of the particles was compromised. In case of Cremophor EL, Cremophor RH and Solutol HS, the batches prepared were failed to get properly emulsified. No sphericity was observed in the particles whereas there was aggregation of the particles although the aggregation was less compared to poloxamer but then again there was no sphericity of the particles. Tweens were far better compared to all the other surfactants so we had to evaluate the selection of surfactants between the tweens. Although being better than other surfactants, there was a distinguishable difference between Tween 80 and Tween 20. The sphericity of the particles obtained in Tween 80 were better than in Tween 20 whereas even the aggregation was very less in Tween 80 compared to Tween 20. Tween 80 appeared to be an eligible candidate for the selection of surfactant for the system.

The selection of the surfactant had to be evaluated at such a concentration of 5% because of the failure in the formulation of the batches. At lower concentration such as 2%, 3% and 4%, the batches formulated did not showed acceptable physical appearance. There were floating particles which failed to get emulsified completely. Even at a 5% there were a few non-emulsified particles but comparing with other concentrations, it was better and showed a positive direction for the further formulation.

The failure in achievement of a well emulsified batch raised up several questions and parameters to be controlled upon while formulating the batches. For this purpose one such parameter of the speed of homogenization drew our attention. Also the size reduction achieved primarily helps better size reduction at high pressure homogenization step.

5.2.5 Effect of Speed of Homogenization and Number of HPH Cycles

The batches SH1-SH4 were formulated by keeping the concentration of surfactant constant at 3%, keeping the amount of drug constant at 5%, and pressure of HPH at 1000 bar. The batches were evaluated for the effect of number of cycles of homogenization and speed of homogenization by varying the value at 10 and 20 respectively for number of cycles for HPH and the speed for homogenization at 5000 and 7000. The results obtained in these batches were not satisfactory in terms of D90 and PDI which reflected very poor quality of the formulation. Since the evaluation was performed at 3% surfactant concentration, the batches SH5-SH8 were formulated increasing the concentration of the surfactant to 9% and keeping the other values as mentioned for batches SH1-SH4. Since the same results appears even on increasing the concentration of the surfactant in terms of PDI and D90 that reflected poor quality of batches.

The results obtained as such may also be due to the pressure of HPH and in order to terminate that particular dilemma, we carried out the same procedure but varying the pressure of HPH this time. The batches mentioned in Table 5.6 were formulated in order to evaluate the effect of pressure of HPH by keeping the speed of homogenization constant.

5.2.6 Effect of Pressure and Number of Cycles of Homogenization

The evaluation of effect of pressure of HPH and number of cycles of HPH were evaluated by preparing the batches SH9-SH16 in which the amount of drug and speed of homogenization cycle was kept constant at 5% and 9000 rpm respectively. In batches SH9-SH12 the concentration of surfactant was kept constant at 3%, evaluation for the effect of pressure of HPH and number of cycles for HPH were performed at two different levels 700 bar - 1000 bar and 10 cycles – 20 cycles, respectively.

From the results obtained, it was clear the batches represented poor formulation in terms of PDI and D90. Henceforth, the concentration of the surfactant was increased and the same protocol as mentioned above was performed in batches SH13-SH16.

Also at higher concentration of 9% of surfactant in batches SH13-SH16 there was no significant difference than it appeared in 3% surfactant concentration.

So from these particular studies we were at a conclusion of addition of co-surfactant in the system. In order to evaluate different types of co-surfactant batches shown in Table 5.7 were performed.

5.3 Effect of Formulation and Process Parameters

5.3.1 Selection of Concentration of Surfactant

Based on preliminary trial batches, Tween 80 was selected as best surfactant for the preparation of NLCs. Further in order to achieve better particle size, PDI, D90 value, entrapment efficiency and better physical stability of the batches, the concentration of surfactant was optimized. The batches mentioned in Table 5.11 were performed in order to select the concentration of the surfactant.

At lower concentration of surfactant in batches CST1 and CST2, the batches could not be prepared properly due to poor emulsification of the system. Floating particles were observed on the top layer and on the walls of beaker through naked eyes even upon the addition of the co-surfactant. Further 9% surfactant concentration in CST5 resulted in improved Z-AVG and increased PDI but the D90 value was slightly more than that of CST4. Although there was a very minor difference in the entrapment efficiency between CST3 and CST4, there was a difference of entrapment efficiency in CST5 than in CST4 also the PDI was increasing. Apart from this, better entrapment efficiency and acceptable Z-Avg, D90 and PDI was obtained at 5% surfactant concentration in batch CST3. Henceforth the surfactant concentration at 5% (w/v) with respect to aqueous media was selected for the formulation. High concentration of surfactant resulted in decreased particle size which further decreases the entrapment efficiency. This could be due to the leaching of the drug while subjecting under shear also a high concentration of the surfactant results into increased solubility of the drug into the aqueous media. Therefore a low value with acceptable results was chosen for the formulation.

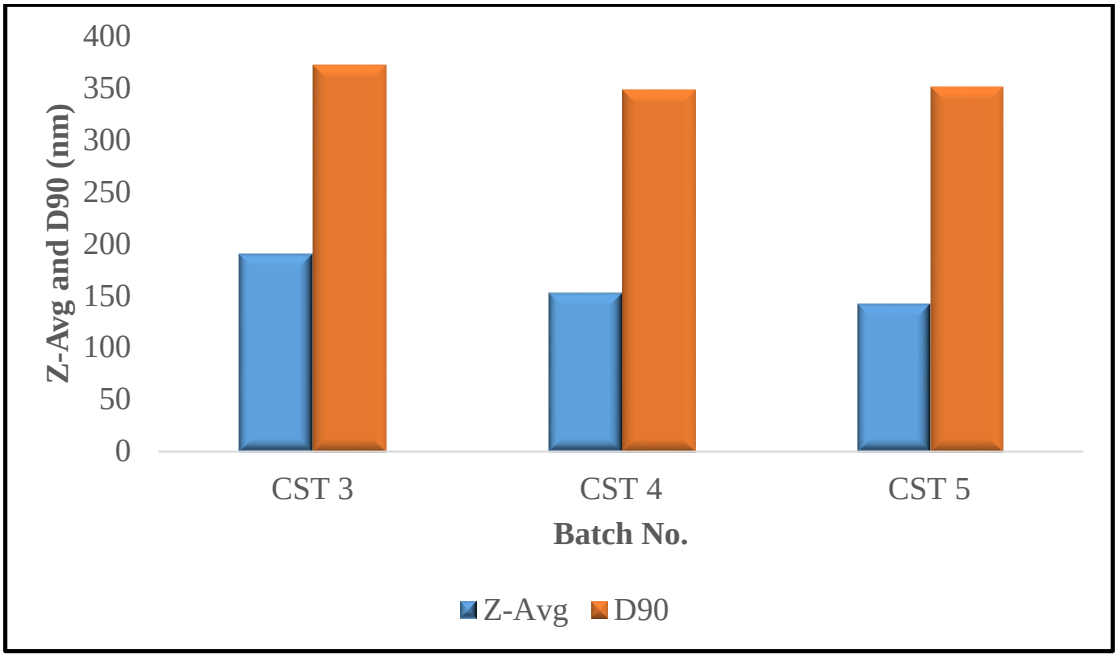

Fig 5.5(a): Effect of Concentration of Surfactant on Z-Avg, D90 and PDI.

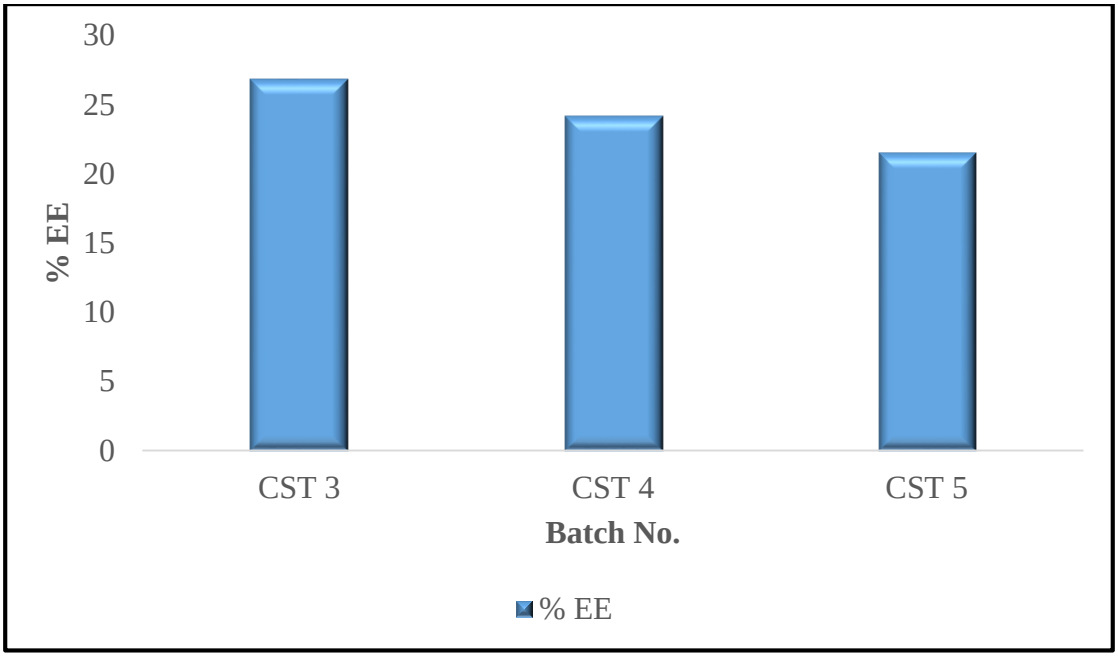

Fig 5.5(b): Effect of Concentration of Surfactant on % EE and % DL.

5.3.3 Selection of Pressure and Cycles for High Pressure Homogenization

Being a process parameter, the selection of pressure and number of cycles for High Pressure Homogenizer was mandatory in order to achieve a desired particle size distribution, PDI and %EE of the formulation. Table 5.12 describes batches evaluated in order to select the pressure and number of cycles for HPH.

Selection of pressure and number of cycles for HPH was made by keeping the composition of the formulation constant and varying the process parameters. The selection was made keeping a higher limit and lower limit of pressure i.e., 700 bar and 1000 bar respectively. The number of cycles were screened at 3 different levels 8, 12 and 16 cycles.

While carrying out the protocol at 1000 bar pressure, the resultant Z-Avg, D90 and PDI at 8 cycles in batch HPS4 were 231.4nm, 808 and 0.385 respectively. Upon increase in the number of cycle to 12 in batch HPS5, the Z-Avg, D90 and PDI were, 180.9nm, 438 and 0.319. As expected, the increase in number of cycles resulted in decrease in particle size, D90 value and improved PDI of the formulation. Further increasing the cycles to 16 in batch HPS6, showed no difference in Z-Avg compared to batch HPS5 whereas D90 decreased to 420 and more improved PDI at 0.247 was observed. Also the peaks in Zeta-Potential were consistent with 100% area of peak intensity. Therefore selecting on the basis of better entrapment efficiency of batch HPS5 than in batch HPS6, and considering the achievement of acceptable result in fewer cycles than in high number of cycles, the pressure and number of cycles were selected at 1000 bar and 12 cycles respectively.

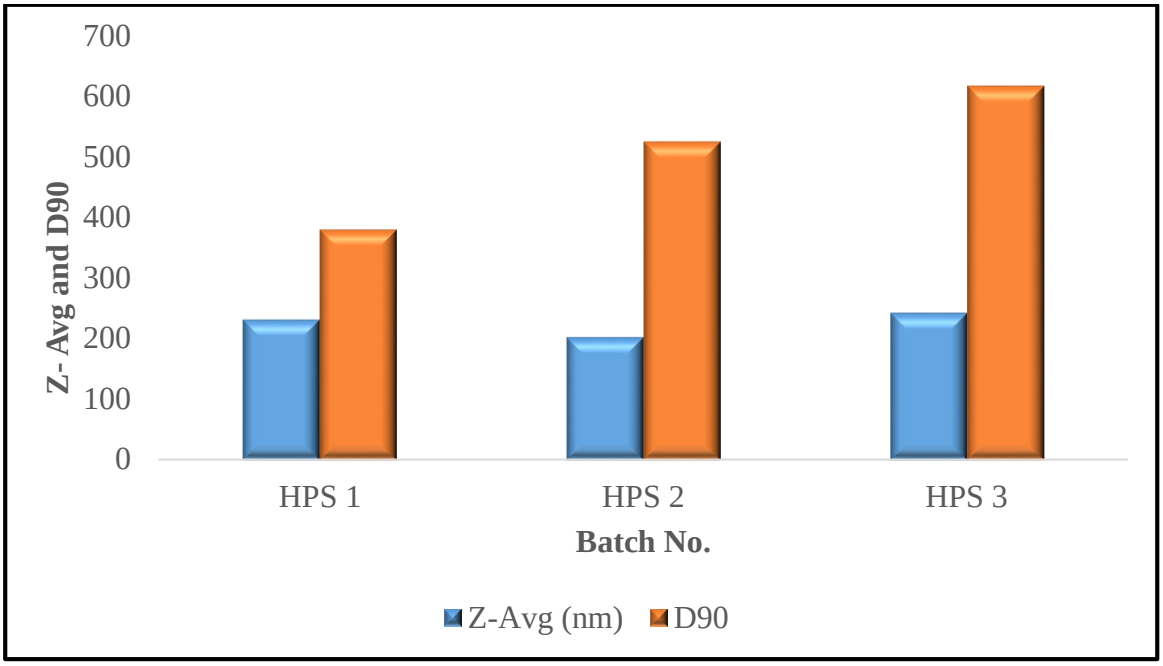

Fig 5.6(b): Effect of Pressure and Number of Cycles of HPH on Z-Avg, D90 and PDI.

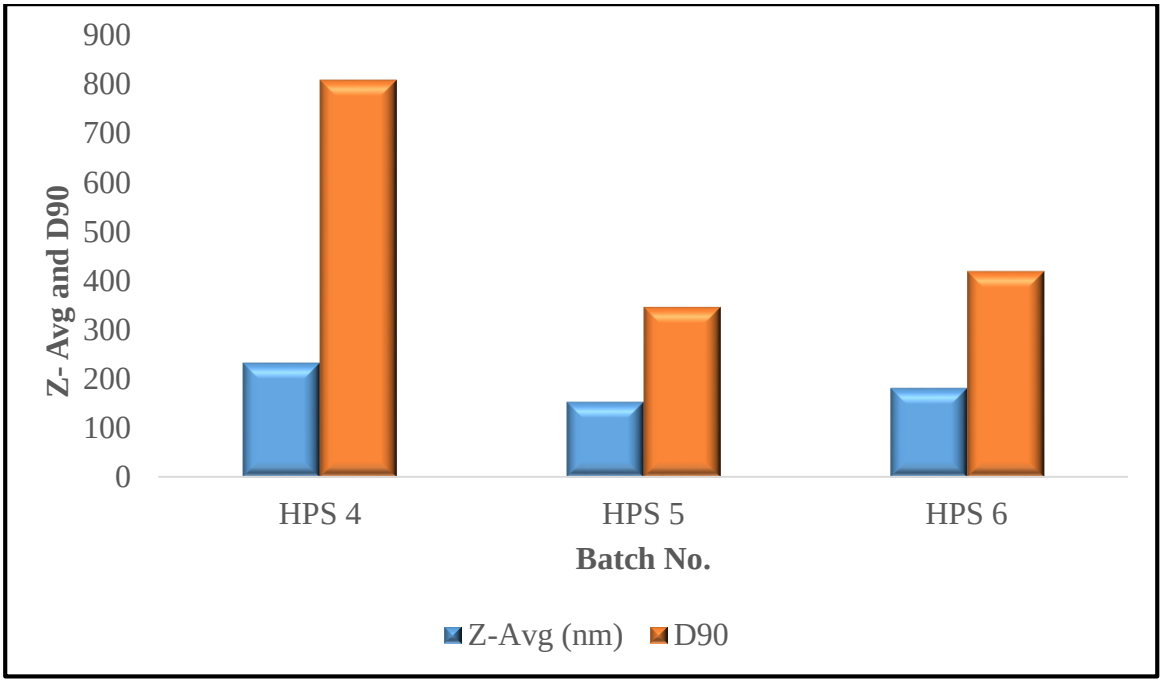

Fig 5.6(c): Effect of Pressure and Number of Cycles of HPH on Z-Avg, D90 and PDI.

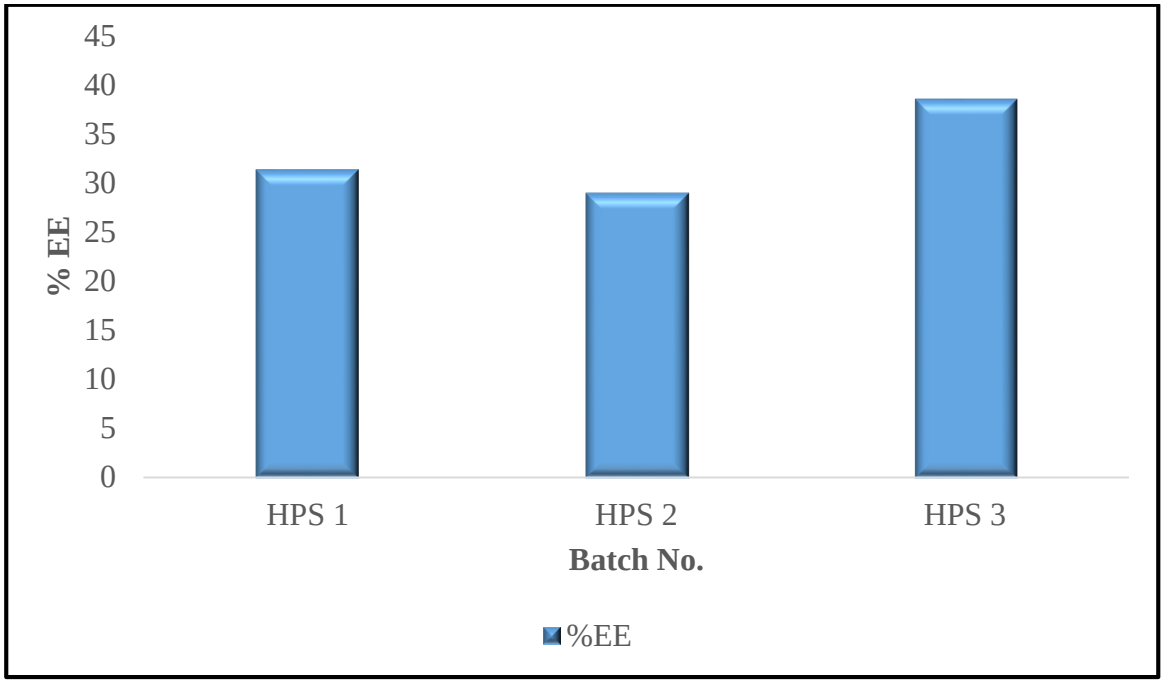

Fig 5.6(d): Effect of Pressure and Number of Cycles of HPH on % EE and % DL.

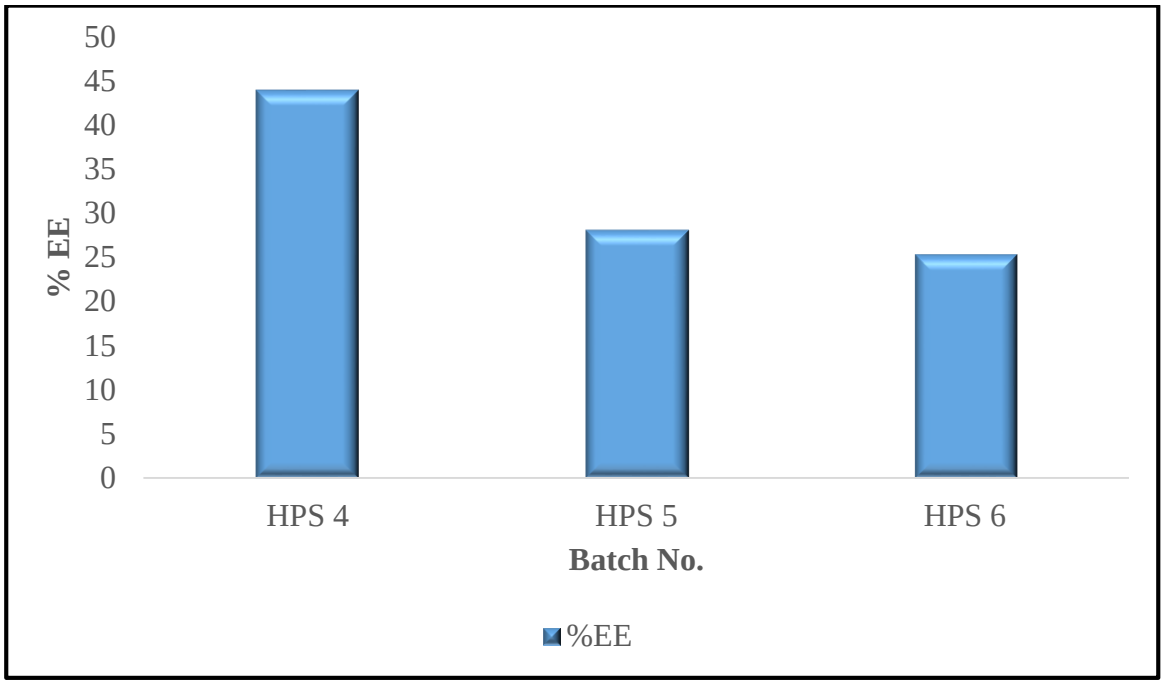

Fig 5.6(e): Effect of Pressure and Number of Cycles of HPH on % EE and % DL.

5.3.4 Effect of Drug Concentration

The concentration of drug was varied by keeping other ingredients and process parameters constant. Henceforth, it was preferable to add a high amount of drug in the formulation as such the maximum amount of drug entrapped was 169.74 mg out of 300 making a %EE of 56.58 %, it was clear to further optimize within this range of drug concentration.

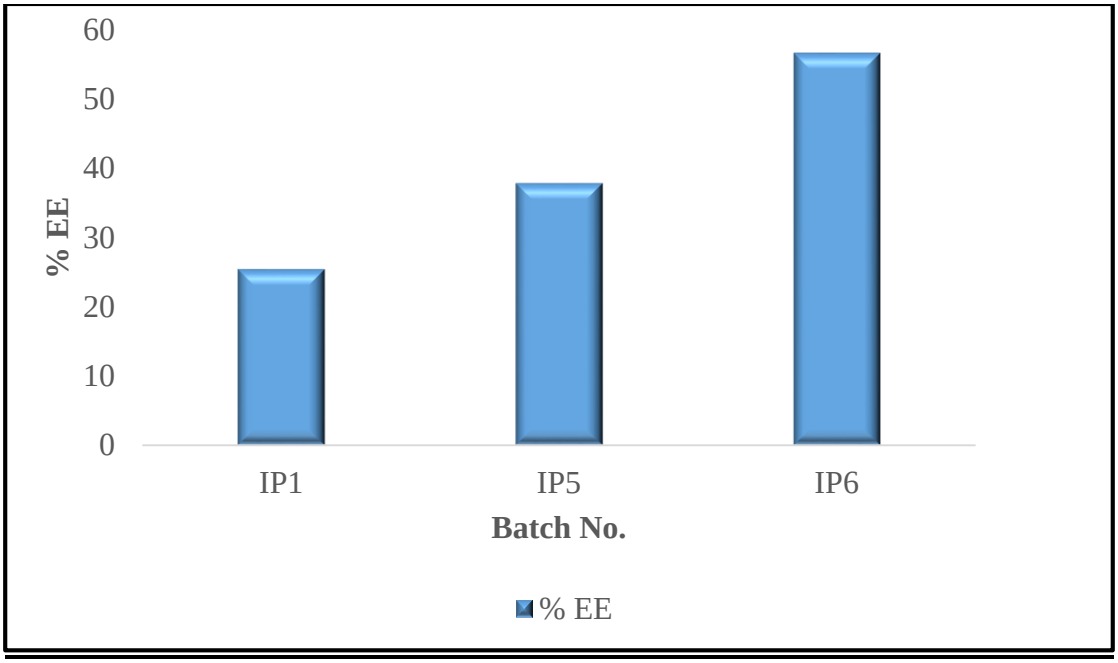

Fig 5.7(a): Effect of Drug Concentration on % EE and % DL.

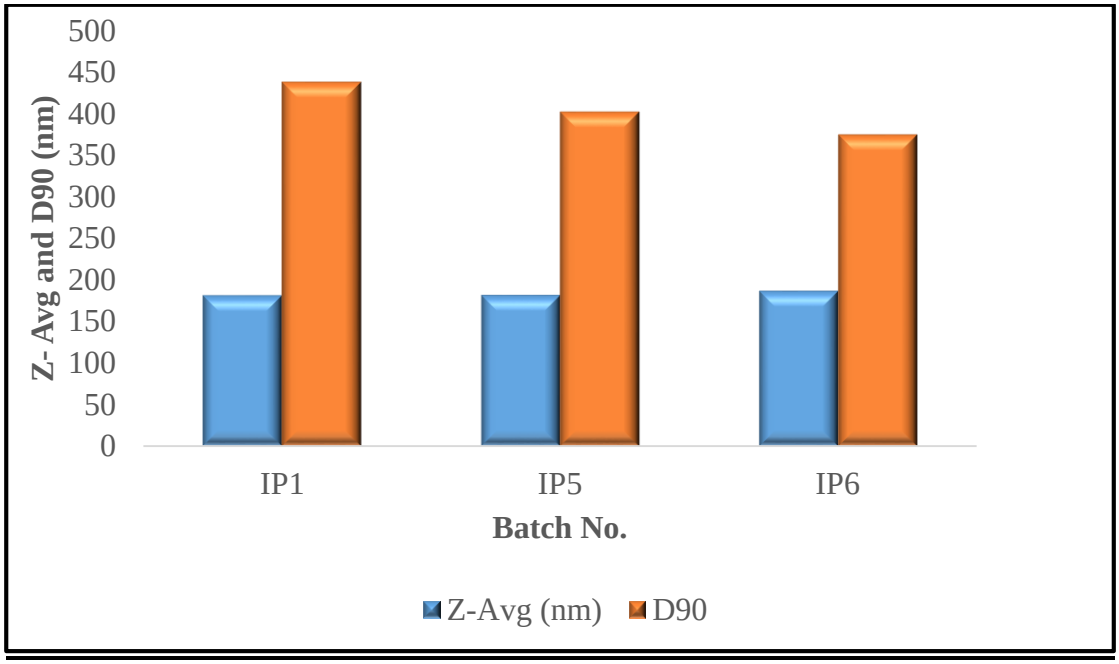

Fig 5.7(b): Effect of Drug Concentration on Z-Avg, D90 and PDI.

5.5 Optimization of Formulation by DoE

5.5.1 Optimization of Formulation by Box-Behnken Design (BBD)

In general, BBD of *k* factors are composed of $k(k-1)/2$ factorial designs 2^2, taking each point of factor in turn, and keeping the other factors at the level coded zero. The number of experiments increases with the increase in number of factors. The experimental domain is spherical, radius $\sqrt{2}$, whatever the number of factors, but with the experimental points arranged in a (cubic) pattern with only 3 levels for each factor. Thus, there may be an advantage in using BBD when a spherical domain is required for prediction, but where there are limitations to the number of levels to which certain factors can be set. BBD allows to optimize the formulation on 3 different levels with minimal number of 15 runs with 2 checkpoint batches as compared to 3^3 full factorial design with 27 runs. Henceforth, the optimization of the batches is achieved with low number of experimental runs. The coded value of the runs has been described in Table 5.15(a). The statistical equation for BBD is as follows:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_4 X_1 X_2 + b_5 X_1 X_3 + b_6 X_2 X_3 + b_7 X^2_1 + b_8 X^2_2 + b_9 X^2_3. \quad (5.1)$$

Where, $b_{(0\text{-}9)}$ represents constants.

$X_{(1\text{-}3)}$ represents variables. Y represents responses

5.5.3 Differential Scanning Calorimetry

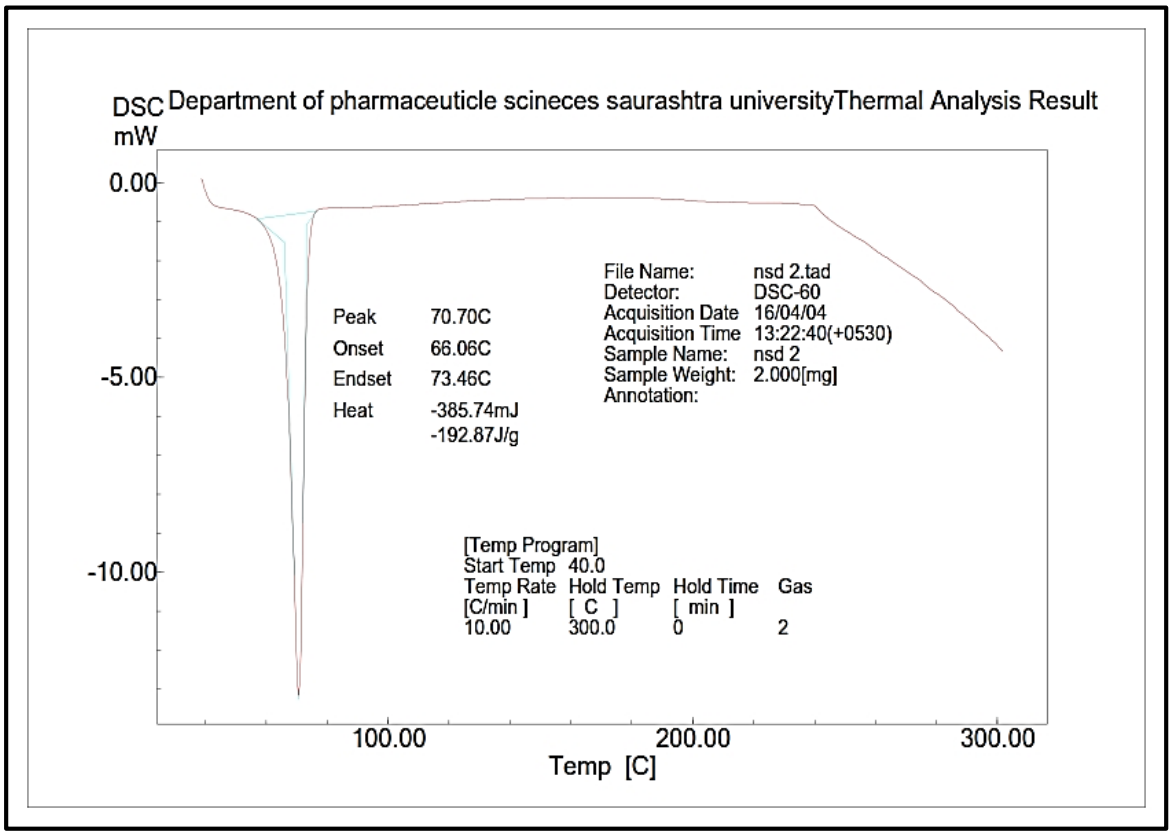

Fig 5.16(b): DSC Analysis of Physical Mixture of Lipids.

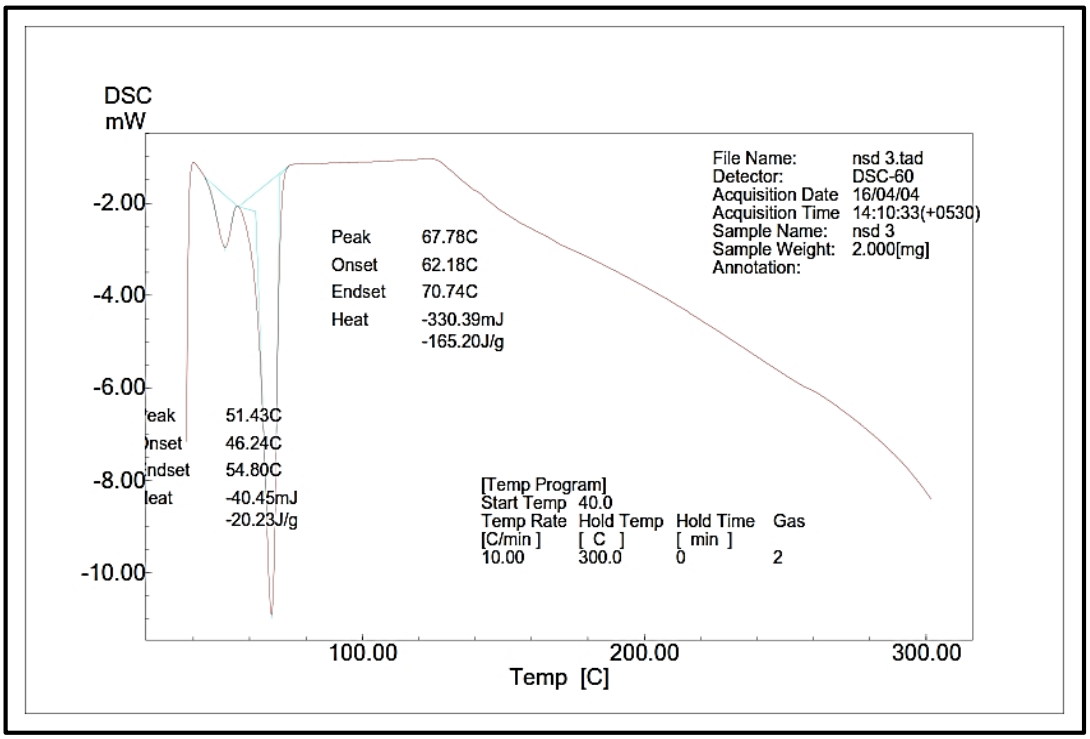

Fig 5.16(c): DSC Analysis of Blank Batch without drug.

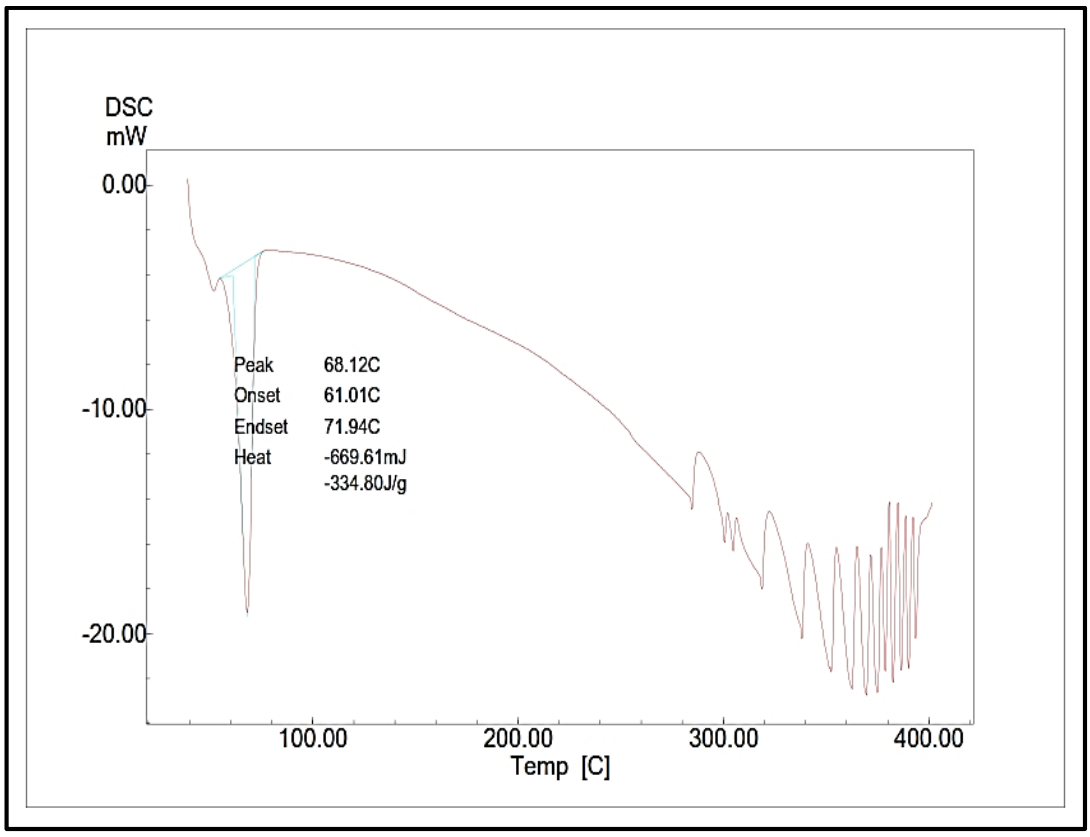

Fig 5.16(d): DSC Analysis of Drug Loaded Batch.

Colorectal cancer has been discovered as the third most common cancer causing death to the individuals worldwide. Cancer basically comprises of uncontrolled growth of non-functionalizing cells which tend to occupy into the tissues and keeps growing. Further they feed on external nutrient sources present in the body fluid as well as take up the newly formed blood vessels for their growth creating several complications. Thus the unorganized cellular growth occurring in the colon and the rectal region of the body leading to several complications has been termed as colorectal cancer.

Conventional dosage forms available in the market for its treatment are generally administrable via i.v route which is painful for the patient and costly. Also the dosage regimens are not single drug dependent instead they are administered in combination. Such high doses leads to several adverse effects and toxicity to the normal cells of the body apart from the cancer affected cells.

Nanoparticulate drug delivery systems has several advantages over the conventional dosage forms in terms of higher efficacy, novel routes of uptake and which can provide better results than the available dosage forms. In order of which Nano-structured lipid carries (NLCs) were prepared which can be orally administered and are able to target the cancer affected cells by making them recognizable to receptor based cell mediated endocytosis and thereby causing minimal toxicity to the normal cells and henceforth show efficacious result in terms of treatment and lower toxicity.

The NLCs were prepared using high pressure homogenization technique. The purpose of using high pressure homogenizer was due to its distinct quality of size reduction which uniformly reduces the size of the particles as well as easy for industrial scale-up.

A variety of lipids were chosen for the drug incorporation of which Dynasan 118 and Glyceryl Mono Oleate were found to be capable of achieving the desired quality of product. The lipid was selected based on a number of studies that were conducted prior to the lipid selection.

In order to achieve a desired quality of the product, preliminary trials were conducted to evaluate the effect of surfactant on the product, effect of speed of homogenization, pressure and cycles of HPH, concentration of co-surfactant, effect of drug concentration on the % entrapment efficiency and the drug loading capacity of the product. Based on the preliminary trials, the concentration of drug, concentration of co-surfactant and number of cycles of HPH were evaluated of having an impact on the final quality of the product. In regards of which, these factors were evaluated using Box-Behnken Design of optimization and the parameters

such as particle size, D90, PDI, % entrapment efficiency and % drug loading of the formulation were studied.

In-vivo characterization of the formulation was done using chemical induced colorectal cancer in rat model. Several parameters including histopathology of the organs, tumor volumes, body weights:length ratio and biochemical parameters such as TNF-α, IL-1β, CEA levels, albumin, creatinine, urea, SGPT and SGOT were evaluated in order to assess the efficacy and toxicity of the developed formulation.

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