*A PhD thesis*

# "DEVELOPMENT AND EVALUATION OF NOVEL DRUG DELIVERY SYSTEMS CONTAINING ACTIVE HERBAL CONSTITUENT"

*Submitted to*

**SUMANDEEP VIDYAPEETH**

*in partial fulfillment of the requirements*
*for the award of the degree of*

# Doctor of Philosophy
# in
# Pharmaceutical Sciences

**Research guide**


**Dr. A. K. SETH**

**M. Pharm., Ph. D.**

Dean, Faculty of Allied Health Sciences &
Research Director, Sumandeep Vidyapeeth


**Submitted by**


**GIRISH UTTAMBHAI SAILOR**

(Registration No: PhD 013 2011)

**DEPARTMENT OF PHARMACY,**
**SUMANDEEP VIDYAPEETH,**
**PO- PIPARIA, TA- WAGHODIA, VADODARA-391760, GUJARAT**


**JULY-2015**

# SUMANDEEP VIDYAPEETH

## PIPARIA, VADODARA

# CERTIFICATE

This is to certify that, the thesis entitled **"DEVELOPMENT AND EVALUATION OF NOVEL DRUG DELIVERY SYSTEMS CONTAINING ACTIVE HERBAL CONSTITUENT"** submitted by **Mr. GIRISH UTTAMBHAI SAILOR (Registration No. PhD 013 2011)** in partial fulfillment for the award of degree of **DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES** to Sumandeep Vidyapeeth, Piparia. This work has been carried out under my supervision and guidance. The matter compiled in this thesis has not been submitted earlier for the award of any other degree or fellowship and free from any kind of plagiarism.

Date:

Place:

**Dr. A. K. SETH**
**M. Pharm., Ph. D.**
Dean, Faculty of Allied Health Sciences &
Research Director, Sumandeep Vidyapeeth

**SUMANDEEP VIDYAPEETH, VADODARA, GUJARAT**

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Date:

Place:

**GIRISH UTTAMBHAI SAILOR**

Department of Pharmacy,

Sumandeep Vidyapeeth, Piparia

**SUMANDEEP VIDYAPEETH, VADODARA, GUJARAT**

# SUMANDEEP VIDYAPEETH

## PIPARIA, VADODARA

# DECLARATION

I hereby declare that the topic entitled **"DEVELOPMENT AND EVALUATION OF NOVEL DRUG DELIVERY SYSTEMS CONTAINING ACTIVE HERBAL CONSTITUENT"** which is submitted herewith to Sumandeep Vidyapeeth, Piparia for the partial fulfillment for the award of degree of Doctor of Philosophy in Pharmaceutical Sciences is the result of work done by me in Department of Pharmacy, Sumandeep Vidyapeeth, under the guidance of Dr. A. K. Seth.

I further declare that the results of this work have not been previously been submitted for any degree or fellowship and free from any kind of plagiarism.

Date:

Place:

**GIRISH UTTAMBHAI SAILOR**

Department of Pharmacy,

Sumandeep Vidyapeeth, Piparia

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# ABBREVIATION

| | |
|---|---|
| P-gp | P-Glycoprotein |
| SEDDS | Self-Emulsifying Delivery System |
| BCS | Biopharmaceutical Classification System |
| LFCS | Lipid Formulations Classification System |
| TAG | Triacylglycerol |
| MCT | Medium chain TAG |
| LCT | Long chain TAG |
| SLN | Solid Lipid Nanoparticles |
| SMEDDS | Self Micro-Emulsifying Drug Delivery Systems |
| SNEDDS | Self Nano-Emulsifying Drug Delivery Systems |
| PDA | Photodiode Array |
| BER | Berberine |
| CHOL | Cholesterol |
| SPC | Soyphosphotidylcholine |
| FTIR | Fourier Transform Infrared |
| CV | Coefficients of Variation |
| RSD | Relative Standard Deviation |
| EE | Entrapment Efficiency |
| DL | Drug Loading |
| XRD | X-ray diffraction |
| TEM | Transmission electron microscopy |
| PEG | Polyethylene Glycol |
| HBSS | Hank's Balanced Salt Solution |
| ER | Efflux Ratio |
| LY | Lucifer Yellow |
| Caco-2 | Human colonic carcinoma cell line |
| TC | Total Cholesterol |
| TG | Triglyceride |
| HDL-C | High-Density Lipoprotein Cholesterol |
| LDL-C | Low-Density Lipoprotein Cholesterol |
| US FDA | United States Food and Drug Administration |
| TG | Triglycerides |
| FA | Fatty Acids |
| DG | Diglycerides |
| PC | Phosphotidylcholine |
| SPC | Soyphosphatidylcholine |
| GRAS | Generally Recognized As Safe |
| PVA | Polyvinyl Alcohol |
| SA | Stearic Acid |
| PCS | Photon Correlation Spectroscopy |
| ICH | International Conference on Harmonization |

# 1. INTRODUCTION

## 1.1. Background

Chemical substances derived from animals, plants and microbes have been used to treat human disease since the dawn of medicine. Since last two decades, the usage of dietary supplements has gradually increased worldwide marked from an increased in herbal medicine market.[1] This emergent popularity has been particularly notable in Western countries, where almost 20% of consumers report regular usage of such supplements.[2] Even though, demonstrable efficacy of many of these dietary supplements has remained mysterious.[3, 4]

Generally, administration of drugs by oral route is the most easiest and convenient way by most consumers. Different bio-molecules obtained from plants are known to possess several pharmacological activities, but a severe limitation exists in oral absorption of this active constituents. Two principal factors underlie the poor oral bioavailability demonstrated for many phytochemicals: poor water solubility and extensive presystemic metabolism. In addition, due to their specific chemical structures, the biological uptakes of many nutraceuticals are incomplete, which results in a large percentage of bioactive compounds remaining unabsorbed and being excreted out of body. With diverse environmental conditions within the human GI tract, the orally consumed bioactives are facing much more challenges to sustain the original chemical structure than other administration routes.[5]

To become accessible for absorption, the compound must be solubilized or dispersed in the aqueous intestinal lumen. The hydrophobicity of phytoconstituents is the major cause for inadequate dissolution of the oral formulation into gastric or intestinal fluids, and subsequent poor bioavailability. Conversely, highly water-soluble phytochemicals, which are primary constituents of aqueous extractions, often

exhibit excellent dissolution profiles. Yet, in many instances, they too exhibit poor oral bioavailability. The reason for this are explained as, after intestinal absorption, bioactives subjected to extensive metabolic activities, which may change their chemical structure and lead to functionality alteration.

**Table 1.1. Novel oral formulation technologies for phytochemical delivery[6]**

| Technology | Phytochemicals | Botanical Species |
|---|---|---|
| **Liposome** | (+)-Catechin | *Camellia sinensis* (green tea) |
| | Curcumin | *Curcuma longa* (turmeric) |
| | Silibinin | *Silybum marianum* (milk thistle) |
| | Quercetin | (Various plant species) |
| **Phytosome** | Catechin polyphenols | *Camellia sinensis* (green tea) |
| | Curcumin | *Curcuma longa* (turmeric) |
| | Ginkgolides, bilobalide | *Ginkgo biloba* |
| | Grape seed proanthocyanidins | *Vitis viniferia* (grape seeds) |
| | Milk thistle flavonolignans | *Silybum marianum* |
| **Nanoparticles** | Catechin polyphenols | *Camellia sinensis* (green tea) |
| | Curcumin | *Curcuma longa* (turmeric) |
| | Milk thistle flavonolignans | *Silybum marianum* |
| | Quercetin | (Various plant species) |
| | Resvertarol | (Various plant species) |
| **Self-emulsifying microemulsions** | Curcumin | *Curcuma longa* (turmeric) |
| | Ginkgolides, bilobalide | *Ginkgo biloba* |
| | Milk thistle flavonolignans | *Silybum marianum* |
| | Schisandrins, schisandrols | *Schisandra chinensis* |
| **Natural CYP inhibitors (Piperine)** | Curcumin | *Curcuma longa* (turmeric) |
| | Catechin polyphenols | *Camellia sinensis* (green tea) |

In short, due to various factors, such as presystemic metabolism and the diverse physicochemical properties, phytochemicals may have poor bioavailability, which causes insufficient concentration of bioactives to produce meaningful

therapeutic functionalities. Recognizing these limitations, researchers and supplement manufacturers have begun to incorporate new extract-formulation technologies in an effort to achieve a marked improvement in phytochemical oral absorption (Table 1.1). Such innovative technologies include the use of liposomes/proliposomes, phytosomes, nanoparticles, self-emulsifying microemulsions, and incorporation of plant-based inhibitors and efflux transporters.[7, 8] The possible mechanisms liable for enhancements of oral drug delivery observed with nanocarriers have been shown in Figure 1.1.[9]

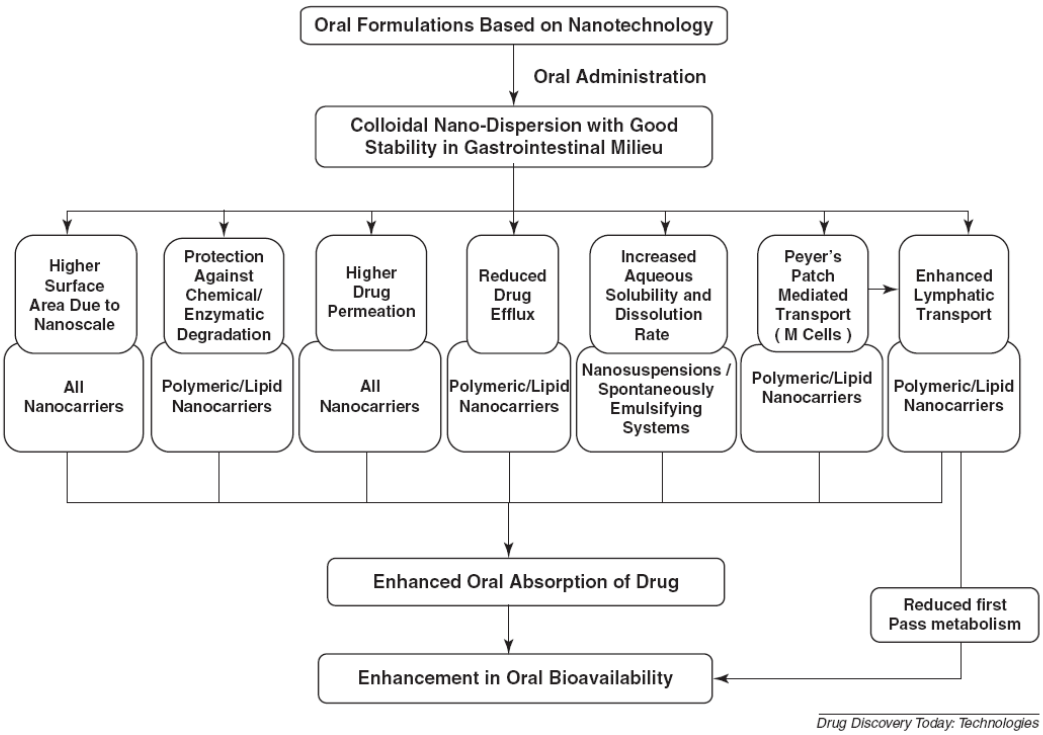



**Figure 1.1. Overview of nanocarriers-mediated mechanisms leading to enhanced oral drug delivery**

Development of Novel dosage form of herbal drug have a number of advantages, including solubility and bioavailability enhancement, toxicity reduction, enhancement of pharmacological activity, protection from physical and chemical degradation, increasing tissue macrophages distribution, sustained delivery etc.[10-12] Therefore, the novel formulations of phytoconstituents could be used to overcome the

problem associated with it which ultimately leads to improvement in therapeutic. However, the formulation of phytoconstituents into novel dosage forms is slow owing to the complexity of the active constituents. Even though several formulations for phytoconstituents have been developed possesses similar efficacy to that of chemically synthesized modern drugs, a lot more investigation still needs to be done.[7]

### 1.1.1. Lipid and lipid based formulation

In recent years, lipid based drug delivery system of herbal drug emerged as a challenging and one of the most successful approach for improving bioavailability and therapeutic efficacy of a number of poorly soluble, highly lipophilic plant constituents. It may be due to the unique properties of lipids such as physiochemical diversity, biocompatibility and selective lymphatic uptake which made lipid very attractive candidates as carriers for oral formulations. With the above promises, the emerging field of oral lipid-based drug delivery system has attracted considerable academic attention.[13-16]

### 1.1.2. Herbal medicine formulation

Unlike fruits and vegetables, for which the degree of phytoconstituents exposure is dependent on the quantity ingested, exposure to plant secondary metabolites (PSMs) in botanical supplements is dependent on the dose and type of preparation ingested. The majority of botanical dietary supplements available today in most retail outlets are either capsule or tablet formulations containing single or multiple plant extracts. As a general rule, plant extracts are prepared by exposing dried plant parts (e.g., leaves, stems, roots, seeds, flowers, and fruits) to various solvents, evaporating the solvents, and then collecting the dried residue. The extraction process concentrates the

purported "active" phytochemicals into a powder or paste that can be easily formulated into an oral dosage form. The type of solvent used in the extraction process (aqueous or nonaqueous) often determines a finished product's PSM profile and content.[17]

Plant extracts are not created equally, therefore "standardized" extracts to contain known quantities of specific phytochemicals may differ significantly than "nonstandardized" components.[17] Still, quantities of phytoconstituents may vary into various dosage forms prepared by different manufacturer by using standardized extracts. Such inconsistencies in the formulations can affect the rate and extent to which phytoconstituents concentrations reach the intestinal lumen. This is demonstrated by an examination of nine separate milk thistle formulations whose 1 h percent release rates of the phytochemical silibinin into an aqueous buffered solution (pH 7.5, 37°C) ranged from 0 to 85%.[18] Because many phytochemicals are highly lipophilic, the inadequate dissolution of the oral formulation into gastric or intestinal fluids, and subsequent poor bioavailability, can be traced to their hydrophobicity. This problem is often evident with phytochemicals of plant extracts generated using nonpolar organic solvents. Conversely, highly water-soluble phytochemicals, which are primary constituents of aqueous extractions, often exhibit excellent dissolution profiles. Yet, in many instances, they too exhibit poor oral bioavailability. The polarity and molecular weight of such compounds (aglycones) are often augmented by conjugation *in planta* with one or more natural sugars (glycosides), which may impede absorption across lipophilic cell membranes via simple passive diffusion processes.

In short, presystemic metabolism and the diverse physico chemical properties of phytochemicals are two after maths of "plant–animal warfare" that negatively

impact the efficacy of botanical supplements. Recognizing these limitations, researchers and supplement manufacturers have begun to incorporate new extract-formulation technologies in an effort to achieve a marked improvement in phytochemical oral absorption. Liposomes/proliposomes, phytosomes, nanoparticles, self-emulsifying microemulsions, and incorporation of plant-based inhibitors of mammalian XME and efflux transporters are some of the innovative technologies use to overcome the limitations of phytochemicals.[7, 8] In all cases, oral bioavailability of certain phytochemicals can be improved drastically when compared with conventional formulations.

### 1.1.3. Liposome

Liposomes are artificial vesicles composed of phospholipid membranes surrounding an aqueous core. Because polar and nonpolar molecules can be incorporated into the vesicle, liposomes can improve the aqueous solubility and cellular permeability of various drugs and phytochemicals. As parenteral formulations, liposomes hold many advantages for drug delivery, but physicochemical instability (e.g., aggregation, sedimentation, and hydrolysis) is one disadvantage that can often limit their utility and shelf life.

Proliposomes, on the other hand, are dry, free-flowing particles that immediately form a liposomal suspension when in contact with water.[7] Because proliposomes exhibit properties of solids, shelf stability is less of a concern. However, vesicle instability within the gastrointestinal tract as a result of acidic and enzymatic hydrolysis remains a problem for orally delivered liposomes and proliposomes. Nevertheless, the bioavailability of phytochemicals exhibiting varying degrees of lipophilicity, including silibinin from milk thistle,[19, 20] curcumin from turmeric,[21] (+)-catechin from green tea,[22] and the ubiquitous plant flavonoid quercetin,[23] has

been significantly enhanced by these technologies. To date, oral phytochemical liposomal formulations have been evaluated in animal models only, although the improvements in bioavailability and efficacy are certainly promising, their clinical utility still remains unknown.

### 1.1.4. Solid lipid nanoparticles (SLN)

Nanoparticle delivery is an alternative to using liposomes as drug carriers, and nanotechnology is a promising area of drug-delivery research. Nanoparticles are solid, colloidal particles that range from 10 to 1000 nm in size.[22] Several types of formulations, including nanospheres, nanocapsules and solid lipid nanoparticles, fall under the broad definition of "nanoparticles". A nanosphere is biosynthetic polymer matrix containing dispersed active ingredient throughout the particle, whereas nanocapsules have a polymeric membrane and an active ingredient within the core. Polymeric nanocapsules, unlike liposomes, are held together by strong covalent bonds, which make them particularly robust. Solid lipid nanoparticles are similar to nanospheres in that they have an active ingredient dispersed within a matrix produced from physiologically compatible lipids, remains solid at both room and body temperature.

Because of their large surface area relative to size, nanoparticles provide many advantages for oral delivery of phytochemicals, such as enhancement of solubility and bioavailability as well as improved stability and therapeutic efficacy.[7] Most compelling, perhaps, has been the impact of nanoparticle technology on the absorption of oral curcumin. Curcumin, a phenylpropanoid isolated from *Curcuma longa* (turmeric), possesses multitude pharmacological activities *in vitro*, yet its bioavailability is virtually nil.[24] Using a solid lipid nanoparticle formulation, Kakkar *et al.* improved the bioavailability of a 1 mg/ kg dose of curcumin by a factor of

155.[25] An excellent review of nanoparticles as oral delivery systems for nutraceuticals was done by Nair *et al.*[26] Nanoparticulate technology has been shown to greatly improve the bioavailability of a host of orally administered phytochemicals, including silymarin, curcumin[27], quercetin, resveratrol, and epigallocatechin gallate; however, as with liposomes, these findings have been limited to animal models only and therefore require clinical study in humans.[7, 8, 26]

### 1.1.5. Self emulsifying drug delivery system (SEDDS)

Self-emulsifying drug delivery systems are a vital tool to overcome the low bioavailability problems associated with poorly soluble drugs. In these systems, hydrophobic drugs can be dissolved, which made them a unit dosage form to be administered orally. When such a system is released in the lumen of the gastrointestinal tract, it disperses to form a fine emulsion (micro/nano) with the aid of GI fluid. This leads to *in situ* solubilization of drug that can subsequently be absorbed by lymphatic pathways, bypassing the hepatic first-pass effect.

Self-emulsifying drug delivery systems (SEDDS) is a broad term, usually producing emulsions with a droplet size ranging from a few nanometers to several microns.[27] It is an isotropic mixture of drug/phytochemicals, lipids and surfactants, typically with one or more hydrophilic cosolvents or coemulsifiers.[28] These systems can instantly form fine emulsion (O/W) upon mild agitation followed by dilution with aqueous media. The motility of the stomach and intestine provides the agitation necessary for self-emulsification *in vivo*. The factors that control the *in vivo* performance of these microemulsions include their ability to form small (<5 μm) droplets of oil, thereby providing a large interfacial area for enzymatic hydrolysis of the oil droplets and formation of micelles containing the phytochemical. Surfactants used in these formulations are known to improve bioavailability by improving

phytochemical dissolution, increasing intestinal epithelial and tight-junction permeability, and inhibiting efflux transporter activity.[27] Several FDA-approved drug products as well as some nutraceuticals (e.g., coenzyme Q-10) utilizing this technology are currently on the market. Botanical extracts and individual phytochemicals whose bioavailability has been improved when formulated as self-emulsifying microemulsions include *Ginkgo biloba*,[29]*Schisandra chinensis*,[30] curcumin,[31] and silymarin.[32]

### 1.1.6. Selection of drug candidate for lipid based formulation

Berberine (BER) is a quaternary isoquinoline alkaloid obtained from root and the stem bark of *Hydrastis canadensis* (goldenseal), *Berberis aquifolium* (Oregonegrape), *Berberis aristata* (tree turmeric), *Berberis vulgaris* (barbery), and many other plants.[33] It occurs as a yellowish crystalline powder that is odorless, or has a faint characteristic odor, with a bitter taste. It is sparingly soluble in methanol, slightly soluble in ethanol, and very slightly soluble in water; however, the salt forms are relatively more soluble and contribute to the tissue coloring.[34] It has been historically used as an anti-diarrheal, anti-protozoal, and anti-microbial agent in Ayurvedic and Chinese medicine. Berberine has demonstrated significant antimicrobial activity towards a variety of organisms including bacteria, fungi, protozoans, viruses, chlamydia, and helminthes and is used to treat several skin and eye ailments.[35, 36] It has been mainly used for the treatment of diarrhea and gastroenteritis for centuries in traditional Eastern medicine.[37] It has also been reported to have a multitude of biological effects, including anti-malarial,[38] anti-hypertensive,[39] anti-arrhythmic,[40, 41] anti-hyperglycemic,[42] anti-tumor,[43, 44] anti-inflammatory,[45] anti-oxidative,[46] and cerebro-protective[47] activities. Recently, it has been reported that berberine helps in reducing cholesterol and lipid accumulations in both the plasma and in the liver.[48]

Although berberine has wide-ranging therapeutic potential, *in vivo* pharmacokinetic studies demonstrate that its apparent permeability co-efficient ($P_{app}$) across the intestinal tissue is in the order of only $10^{-7}$ cm/s.[49] The poor absorption characteristic is probably because of the P-glycoprotein (P-gp) expressed in intestinal cells and the significant first-pass metabolism by CYP 450-dependent processes.[50, 51] Literature precedents suggest that its metabolism in humans is primarily based on phase I demethylation and phase II glucuronidation and/or sulfate conjugation.[52] Higher levels of berberine chloride in the plasma are essential when the drug is intended for treating systemic disorders.

## 1.2. Need For Study

Berberine is a quaternary isoquinoline alkaloid obtained from various plants of Berberis species. It has been historically used as an anti-diarrheal, anti-protozoal, and anti-microbial agent in Ayurvedic and Chinese medicine. It also possesses multitude of biological effects, including anti-inflammatory, antidiabetic, lipid peroxidation, and neuroprotective activity.[53-57] However, quarternary amine cation of BER causes poor water solubility, resulting in low bioavailability. In addition, BER also induce the activity of multidrug efflux transporter P-glycoprotein (P-gp) in the intestine, responsible for active efflux of drug from cells, cause its own ejection resulting in 90% reduction in BER transport.[58-60] Moreover, intramuscular and intravenous administration may leads to risk of adverse reactions, such as drug rash and anaphylactic shock.

Recently, lipid based formulations are widely used for the oral administration of phytoconstituents. Nevertheless, lipid-based formulation can also be formulated in different dosage form like self-emulsifying systems, multiple emulsions,

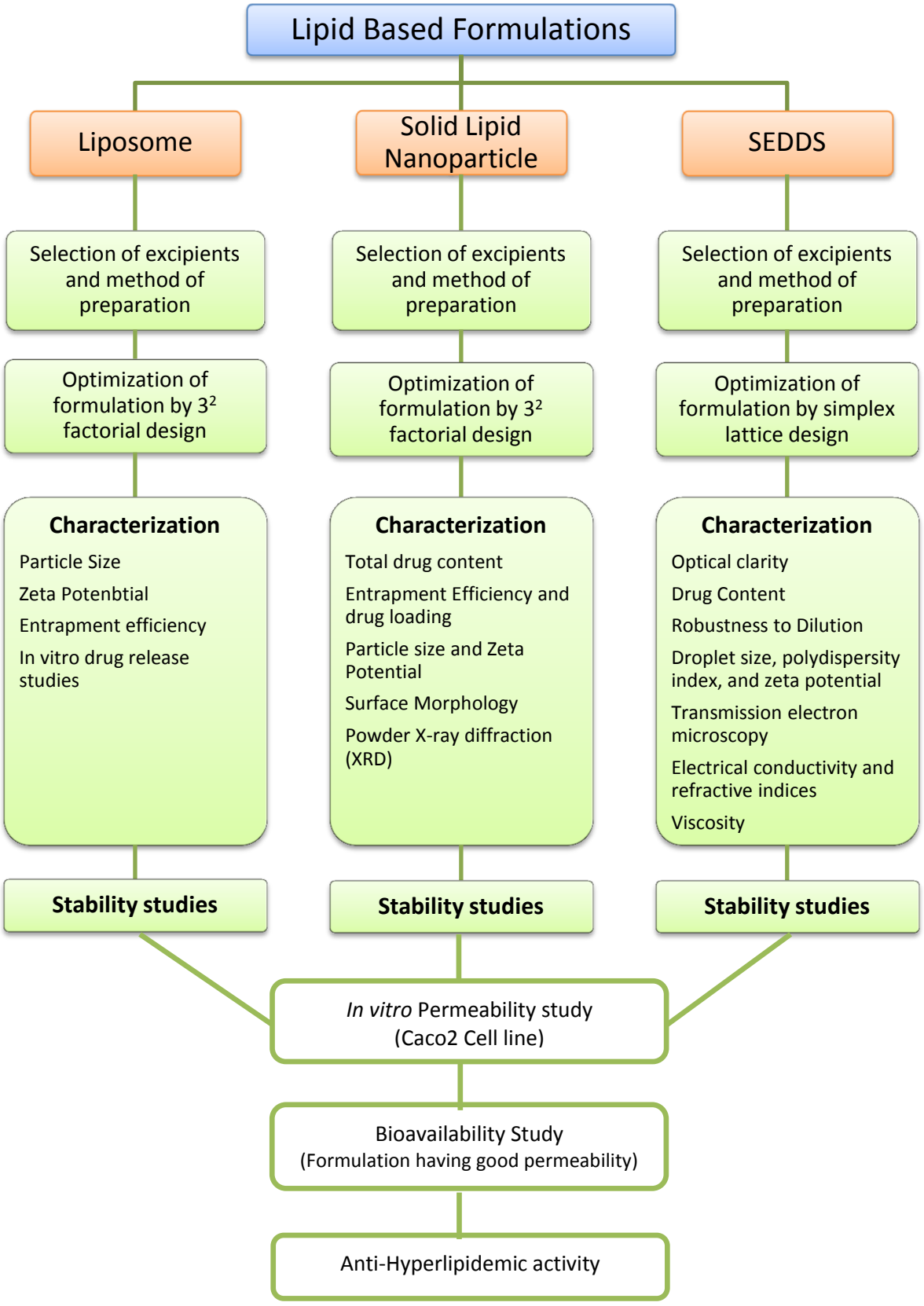
microemulsions, liposomes, and solid lipid nanoparticle. There are various mechanisms responsible for the absorption enhancement of drug from lipid based formulation for instance, altering the intestinal environment, interacting with enterocyte-based transport, stimulation of lymphatic transport, and active ingredients release modification. Furthermore, degradation of active ingredient in gastrointestinal tract can be protected by phospholipids.[61]

## 1.3. Objectives

On the basis of this background, the present study was aimed:

- To prepare and characterize novel drug delivery system such as liposome, solid lipid nanoparticles and self emulsifying drug delivery system
- To evaluate *in vitro* intestinal permeability of optimized formulation
- To investigate *in vivo* bioavailability studies of optimized formulation and compared with pure drug suspension
- To evaluate anti-hyperlipidemic activity of optimized formulation

## 1.4. Plan of Work



# 2. LITERATURE REVIEW

## 2.1. Lipid Based Formulation

A drug is classified as 'poorly soluble' when its dissolution rate is considered so slow that dissolution takes longer than the transit time past the prime absorptive region in the GIT. When administered in conventional solid dose formulations, these compounds have a tendency to exhibit low bioavailability as their absorption is described as dissolution rate limited. The dissolution rate of poorly soluble drugs can be extremely low under physiological conditions leading to poor oral bioavailability and nonlinear exposure with increasing dose.[62] The bioavailability of certain lipophilic drug can also increases due to the strong food effect in which lipids present in food solubilized the drug and food also cause excretion of bile which further increase the solubility.[63, 64] However, sometime food can also interfere with certain drug absorption which cause decrease in bioavailability. In lipid-based formulations drugs are present in solubilized form, which made them potential formulation to increase bioavailability and eliminate the food effect. However, lipid based formulations have generated some important reservations within the pharmaceutical industry. This discretion is due to various factors such as physicochemical complexity, stability, inadequate lipid solubility of certain poorly water-soluble drugs, gastrointestinal (GI) handling before absorption, a lack of information about the *in vivo* behavior and influence of co-administered drugs/lipids and finally, the lack of predictive *in vitro* and *in vivo* testing methodologies. In spite of these limitations, clinical and commercial successes of lipid-based formulations of some drugs - namely cyclosporine marketed as Sandimmune™ and Neoral™ makes the lipid based formulation a potential drug delivery system for enhancing drug bioavailability of poorly soluble drug.[65]

### 2.1.1. Role of lipids in bioavailability enhancements

Normally, oral absorption of drug requires high solubility and permeability. However, many drugs known to possesses poor and variable bioavailability due to high dose to solubility ratio.[66]

Crounse[67] reported that the co-administration of food was significantly enhanced the bioavailability of many poorly water soluble drugs. In view of these, several studies found that, lipid part of food is mainly important among other food constituents in stimulating the absorption of poorly soluble or lipophilic drug.[68-72] However, this food dependant drug absorption leads to major concern about the sub-therapeutic plasma drug concentration when co-administered without food. In addition, it is also serious problem for drug with narrow therapeutic index, where increased bioavailability may leads to serious unwanted effects. Hence, food intake control or/and monitoring is required when dosing such drugs.[73]

Moreover, Martin[74] stated that *in vivo* behavior of such lipophilic drugs depends on its physical state. The crucial advantage of lipid based formulation is that, the drug is in solubilized form, but it is not enough if solubilization capacity is lost upon aqueous dilution and dispersion. These cause precipitation of drugs and re-dissolution of such drugs takes longer times compared to the intestinal transit time which ultimately leads to incomplete drug absorption.[74] Ideally, presence of lipid based excipients of maintains adequate solubilization sufficient for drug absorption during the passage of formulation in gastrointestinal tract by promoting the supersaturation. Additionally, there are various mechanisms by which lipid based formulations enhance the oral bioavailability. Potential effects of lipid based formulation on oral absorption are shown in Figure 2.1.

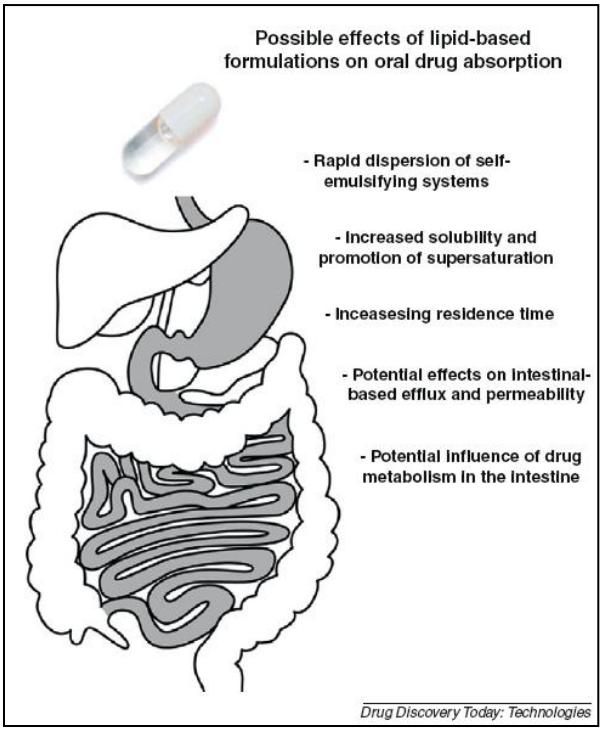



**Figure 2.1. Potential effects of lipid-drug delivery systems on oral drug absorption[74]**

Aungst *et al.*[75] found that several lipid based excipients like glycerides, fatty acids and non-ionic surfactants are permeability enhancers. The mechanism responsible for these includes membrane fluidity enhancement, or alternatively, tight junction opening by lipid excipients. In other study, Pang *et al.*[76] reported other mechanism of permeability enhancement by interaction with efflux transporter, P-glycoprotein (P-gp), at the apical membrane of human intestine. The various groups such as anticancer compounds, HIV-protease inhibitors, hormones, cardiovascular drugs contains P-gp substrate drug. The permeability of these drugs is increased via inhibition of efflux pump by lipid based excipients such as medium chain glycerides, polyethylene glycols, polysorbate, polyethoxylated castor oil.[77]

The US FDA in December 2002 issued a guidance entitled, "Food-Effects Bioavailability and Fed Bioequivalence Studies".[78] FDA recommended high-fat (800–1000 cal; 50–65% from fat, 25–30% from carbohydrates, and15–20% protein) diet for food effect studies. These conditions are significant effect on GI physiology

which ultimately changes the drug availability. The bioavailability of certain drugs is increased when co-administered with food. Though, many drug molecules when co-administered with food exhibits an opposite effect on the extent of bioavailability and efficacy.[79]

As per the biopharmaceutical classification system (BCS), numerous drug bioavailability can be influenced by food have been reported in the literature in correlation to class of the drug (Table 2.1). Class I drug, high solubility and permeability, mainly transport by passive diffusion and capable of saturating efflux as well as absorptive trasporter. In view of the domination of passive diffusion, there is a minimal interaction of Class I drug with food cause no significant effect on the extent of bioavailability. Likewise, Class II drugs with lipophilicity and high permeability are primarily absorbed by passive diffusion. However, poor solubility of drug can prevent saturation of efflux transporters leads to increase in the extent of oral bioavailability and the rate of absorption of these drugs in the presence of food.[80] Several researchers showed that, though good solubility of Class III drugs makes it adequately present in the gut lumen, it is poorly permeable and metabolized and therefore majorly reliant on on cellular uptake transporter for penetration in the intestine. These drugs could show lower bioavailability with high fat diet, due to inhibition of uptake transporter in the intestine.[63, 81, 82] Class IV drugs may behave similarly as Class III drugs in solubility improvement characteristics with high fat food, though it is difficult to predict the *in vivo* behavior of such drugs.

**Table 2.1. Biopharmaceutical Classification System (BCS) and potential advantage of Lipid-based systems.**

| BCS Class | Aqueous Solubility | Membrane Permeability | Potential Advantage of Lipid Based Systems |
|---|---|---|---|
| I | High | High | Enzymatic degradation<br>Gut wall efflux |
| II | Low | High | Solubilization<br>Bioavailability |
| III | High | Low | Enzymatic degradation<br>Gut wall efflux<br>Bioavailability |
| IV | Low | Low | Solubilization<br>Enzymatic degradation<br>Gut wall efflux<br>Bioavailability |

### 2.1.2. Lipid excipients

For the purpose of drug delivery formulation, lipids component has a great influence on absorption enhancement. Thus, it is necessary to know about the characteristics of various excipients.[14] Kalepu[73] suggested that, the choice of lipid excipients depend upon various factors includes solvent capacity; miscibility; self-dispersibility and ability to promote formulation self dispersion; digestability and fate of digested product; regulatory issues- irritancy, toxicity, purity, chemical stability; melting point; capsule compatibility and cost.

Lipids for oral drug administration, can be divided roughly into digestible and non-digestible in the GI tract.[65] Non-digestible lipids comprise of mineral oil, sucrose polyesters and others, are not absorbed from the gut lumen and can decrease drug absorption by holding a fraction of the co-administered drug.[83, 84] On the other hand, digestible lipids comprises of triglycerides (TG), diglycerides (DG), fatty acids (FA), phospholipids, cholesterol, as well as several synthetic derivatives. In general, these lipids are defined according to their carbon chain length, i.e. long chain triglyceride (LCT) or medium chain triglyceride (MCT), lipid class such as TG, DG, MG or FA,

degree of saturation and their interaction with water. The extent and rate of digestion, amount of digestion products formed during the digestion process and degree of dispersion of these products are the factor governing the potential of lipid component to enhance absorption.[85]

Generally, it is difficult to tell about the bioavailability enhancement of poorly soluble drug by particular lipids. For example, Bloedow[86] examine the decrease in griseofulvin bioavailability in all lipids during the study on lipid effects. On the other hand, acetyl sulfisoxazole showed increase in bioavailability with digestible lipids, unlike with nondigestible lipids. Furthermore, formulation used MCT shows higher serum concentration level than that of the formulation containing non-digestible lipids, might be due to the rapid digestion of the MCT. Conversely, long chain triglyceride (LCT) shows low bioavailability due to the slower and incomplete digestion.

Barnwell *et al.*[87] found that co-administration of oleic acid increases the bioavailability of propranolol, a lipophilic drug, by modulating biochemical processes. Based on their results, the authors proposed two possible explanations. First, oleic acid may promote lymphatic absorption of propranolol since it is known to activate lymph production. The avoidance of hepatic first-pass metabolism, therefore, led to increased bioavailabilty. Second, oleic acid may reverse the inhibition of lymph production caused by propranolol. This hypothesis was consistent with the previous findings that several lipophilic drugs, including propranolol, inhibit protein kinase C (a key enzyme involved in cellular signal transduction which controls secretary events, including lymph production) and that oleic acid reverses inhibition of protein kinase C by propranolol.

Lipid excipients have been reviewed by Hauss in context of their applications in lipid-based formulations[88] and application of phospholipids in oral drug delivery has been reviewed by Fricker *et al.*[61] Medium chain TAG (MCT) and longchain TAG (LCT) are commonly used for lipid-based formulations. Several major differences exist between MCT and LCT with respect to their *in vivo* fate, such as lipolytic products, differences in the modulation on gastric emptying [71] and the contraction of the gallbladder in humans.[72] Long chain lipolytic products delay gastric emptying and facilitate the contraction of the gallbladder to a larger extent than the medium chain lipolytic products.[72]

Likewise, several other studies have demonstrated that certain lipidic formulations can act as effective modulators of P-gp counter transport systems,[89-93] which offers a qualitative explanation for enhanced drug transport in independently examined cases, for instance, increased bioavailability of cyclosporine when co-administered with water-soluble vitamin E.[94, 95]

### 2.1.3. Mechanism of improved oral bioavailability

It is difficult to predict the resulting biopharmaceutical effect of lipid based excipients due to several mechanisms involved in enhancement of oral bioavailability of poorly soluble drugs. However, it is believed that, there are three primary mechanisms by which lipids and lipophilic excipients affect drug absorption, bioavailability and disposition after oral administration. These are the alteration of the composition and character of the intestinal environment, the recruitment of intestinal lymphatic drug transport, and the interaction with enterocyte-based transport processes (Fig. 2.2).[96]

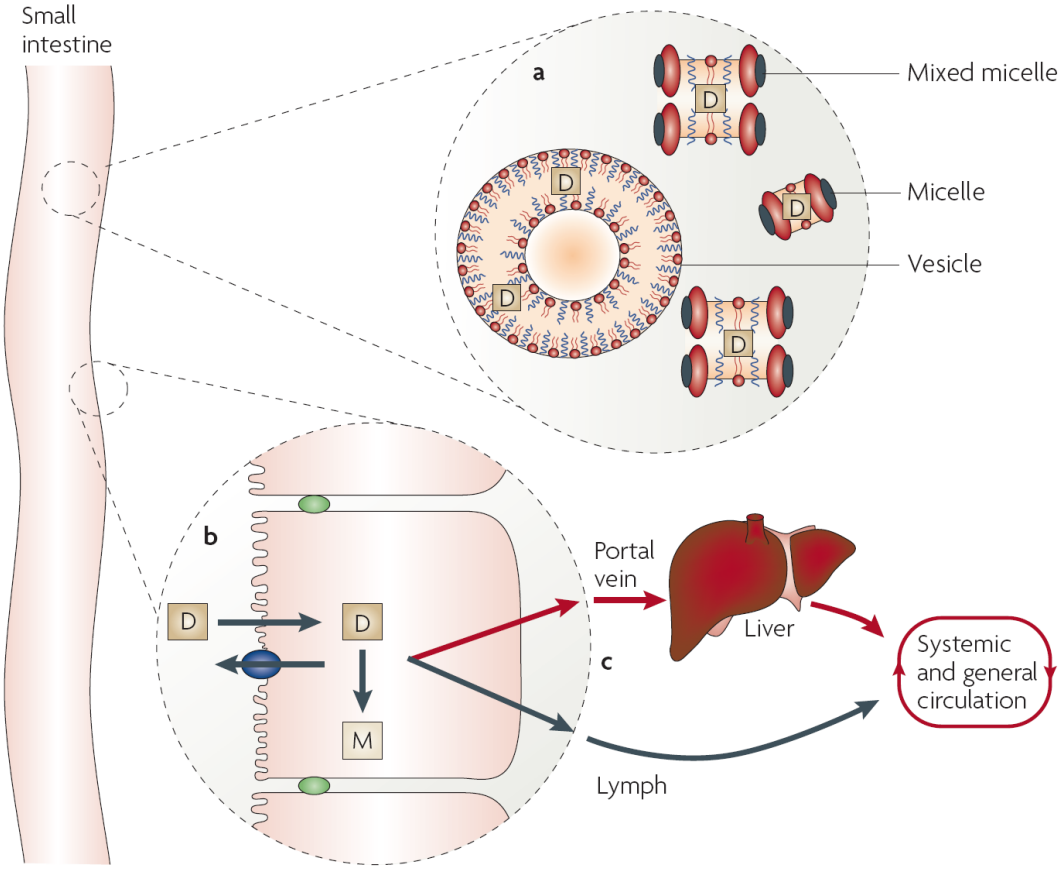



**Figure 2.2. Potential effect of lipids and lipidic excipients on drug absorption.** [96]

Lipids can affect drug absorption in three ways: by enhancing drug (D) solubilization in the intestinal milieu through alterations to the composition and character of the colloidal environment — for example, vesicles, mixed micelles and micelles (a); by interacting with enterocyte-based transport and metabolic processes, thereby potentially changing drug uptake, efflux, disposition and the formation of metabolites (M) within the enterocyte (b); or by altering the pathway (portal vein versus intestinal lymphatic system) of drug transport to the systemic circulation — which in turn can reduce first-pass drug metabolism as intestinal lymph travels directly to the systemic circulation without first passing through the liver (c). Cellular junctions are represented by green ovals, and a representative transport protein is depicted by a blue oval.

### 2.1.4. *In vivo* lipid digestion process

The environment of GI system is one of the most important aspects to consider while developing oral lipid based drug delivery system. This includes both the gastric juice and the digestion processes. The GI environment will also depend upon the nutritional state, whether fasted or fed.

Kleberg *et al.*[97] found that, in the fasted intestine dispersed lipid based formulation encounters a relatively small concentration of bile salt and presumably a

low level of enzymes. However, Kossena *et al.*[98] observed that as little as 2 mL of long chain fatty acids are able to induce gall bladder contraction in humans and thus increase the level of bile salt/phospholipid (BS/BL) micelles in the small intestine.

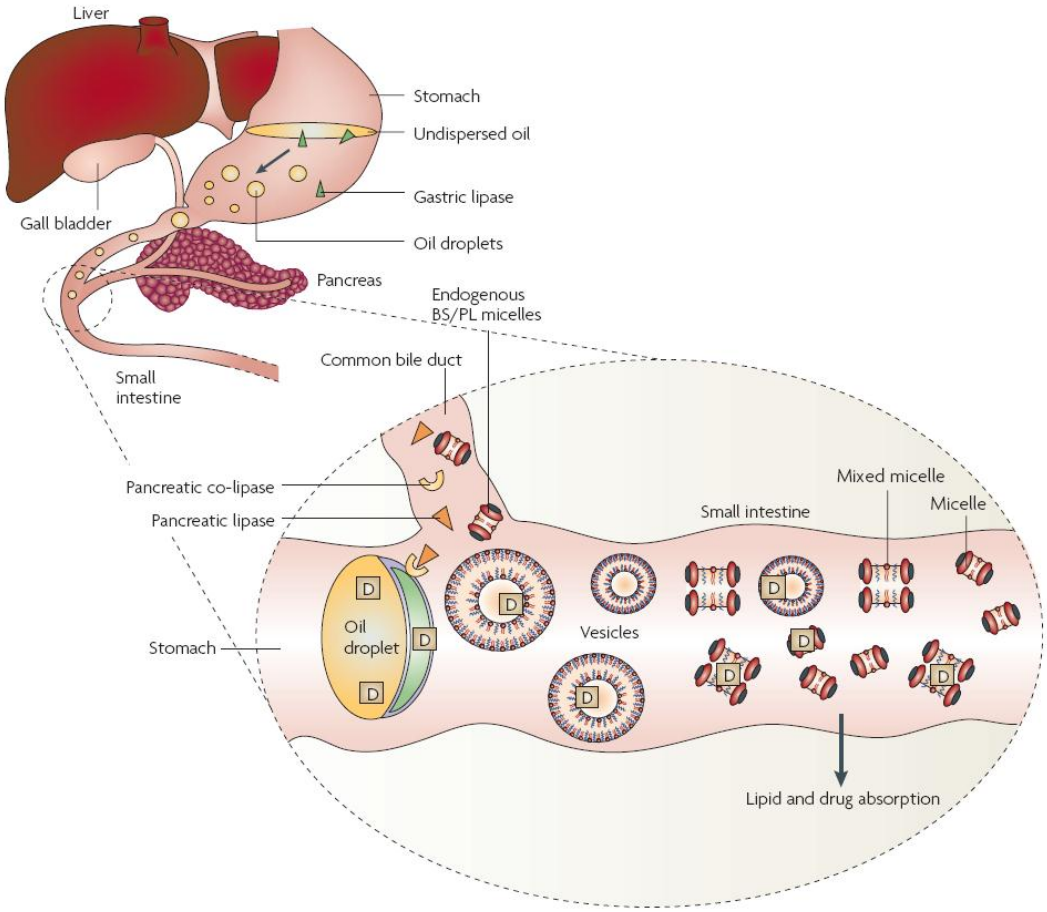



**Figure 2.3. Various mechanisms of enhancement of drug bioavailability in the presence of lipids: [96]**

(a) solubilization of drug in the intestinal fluid by formation of colloidal species viz., vesicles, mixed micelles and micelles; (b) interference with enterocyte-based transport and metabolic processes, thereby potentially changing drug uptake, efflux, disposition and the formation of metabolites (M) within the enterocyte; (c) by selective lymphatic uptake which reduces first-pass drug metabolism as intestinal lymph travels directly to the systemic circulation

Food intake induces secretion of gastric lipase in the stomach which digest lipid into diglycerides and fatty acids.[83, 84] Triglyceride lipid further hydrolyzed to form 2-monoglyceride and two fatty acids by pancreatic lipase. This hydrolyzed product induces the secretion of biliary and pancreatic fluids, causing a significant change in the intestinal environment. Moreover, the digested products are more water-

soluble than the parent lipids, and they can be solubilized within bile salt mixed micelles (Fig. 2.3).

Excipients used in formulation play an important role in determining the rate and extent of absorption of drugs from the GI tract.[99] Therefore, in-depth knowledge of the digestive process in gastro intestinal environment is required for understanding of the biopharmaceutical properties of lipid-based oral formulations. In addition, appropriate design of *in vitro* tests is also essential which can predict the *in vivo* behavior of formulation into gastro intestinal environment. For these, researcher are in search of a biorelevant dissolution media as well as to understand the *in vivo* colloidal behavior of the lipid based formulations in the presence of endogenous solubilizing species viz., bile salts (BS), phosphotidylcholine (PL) and cholesterol (CL) and enzymes (lipase).

### 2.1.5. Lipid based drug delivery formulations

Dug candidate with high therapeutic activities frequently have poor aqueous solubility due to the lipophilic nature which leads to poor absoption and bioavailability after oral administration. On the contrary, intravenous injection of such drugs may cause embolization of blood vessels by aggregation of insoluble drugs and may produce a local toxicity due to high drug concentration at site of deposition.[100] To solve these, excipients including surfactant, ethanol, and polyethoxylated castor oil (Cremophore EL) have been used for parentral administration.[101-103] Nevertheless, such excipients require special manufacturing and packaging condition because of toxic substance extraction from devices like infusion tubing and containers. In addition, precipitation of drug on dilution with physiological fluids is also concern for drug dissolved in such excipients. Surfactants are also not able to retain the solubilized drug at concentration lower than critical micelle concentration (CMC) values particularly for low molecular

surfactant with high CMC value. Additionally, co-solvent or surfactants administration may cause unwanted side effects such as respiratory distress and venous irritation.[104-106] In view of these, there is a need for novel formulations which are biocompatible, aqueous stable and cost-effective. It may allow use of range of poorly soluble drugs, provide a satisfactory shelf-life under storage conditions, and avoid the use of toxic solvents. The lipid based formulations such as emulsions,[107, 108] solid lipid nanoparticles,[109, 110] liposomes, and polymeric micelles is a recent approaches for solubilization of poorly soluble drugs.

**Table 2.2. The lipid formulation classification system[14]**

| Type I | Type II | Type IIIA | Type IIIB (microemulsion) | Type IV |
|---|---|---|---|---|
| Oils without surfactant | Oils and water insoluble surfactants | Oils, surfactant, cosolvents (both water-insoluble and water-soluble excipients | Oils, surfactant, cosolvents (both water-insoluble and water-soluble excipients | Water-soluble surfactants and cosolvents (no oil) |
| Non-dispensing | Emulsion (SEDDS) | SEDDS/SMEDDS formed with water-soluble components | SEDDS/SMEDDS formed with water-soluble components and low oil content | Disperses typically to form a micellar solution |
| Requires digestion | Will be digested | Digestion may not be necessary | Digestion may not be necessary | Limited digestion |

Pouton[14, 111] proposed a Lipid Formulations Classification System (LFCS) and categorized lipid-based formulations into four different types according to their compositions (Table 2.2). The *in vivo* behavior of the formulation can be interpreted easily by LFCS. It can be helpful in predicting suitable formulation for drugs based on their physico-chemical properties. The most of the product available in market are Type III systems, which are diverse with wide range of oil and water soluble substances. Hence, Type III has been further divided into Type IIIA (oils) and Type IIIB (water soluble) based on the proportion of oils and water soluble substances.

**Table 2.3. Commercially available Lipid-based products for oral administration[112]**

| Trade name | Molecule | Therapeutic use | Company |
|---|---|---|---|
| **Agenerases®** | Amprenavir | HIV antiviral | GlaxoSmithKline |
| **Rocaltrol®** | Calcitriol | Calcium regulator | Roche |
| **Cipro®** | Ciprofloxacin | Antibiotic | Bayer |
| **Neoral** | CyclosporinA/I | Immuno-suppressant | Novartis |
| **Gengraf®** | CyclosporinA/III | Immuno-suppressant | Abott |
| **Accutane®** | Isotretinoin | Anti-comedogenic | Roche |
| **Kaletra®** | Lopinavir and Ritonavir | HIVantiviral | Abott |
| **Norvir®** | Ritonavir | HIV antiviral | Abott |
| **Lamprene®** | Clofazamine | Treatment of Leprosy | Alliance laboratories |
| **Sustiva®** | Efavirenz | HIV antiviral | Bristol-Meyers |
| **Fenogal®** | Finofibrate | Anti hyperlipproteinomic | Genus |
| **Restandol®** | Testosterone undecanoate | Hormone replacement therapy | Organon laboratories |
| **Convulex®** | Valproic acid | Antiepileptic | Pharmacia |
| **Juvela®** | Tocopherolnicotinate | Hypertension, hyperlipidemic | EisaiCo. |

Strickley[112] has reviewed the commercially available lipid based formulation listed in Table 2.3. Lipid-based drug delivery systems can be developed successfully by careful consideration of the formulation objectives. The systematic approach includes pre-selection of excipients based on their melting point, fatty acid composition, HLB value, digestibility and disposability; screening of selected excipients for solubility, dissolution/dispersion properties, stability and compatibility; identification of a formulation technique which is suitable for the intended dosage form; design of appropriate animal models to predict the *in vivo* performance of the chosen formulation; and optimization of the formulation considering the drug loading and dissolution profile.

## 2.2. Liposome

In the 1970s, oral formulation of liposome was developed to protect the labile drugs from the acidic milieu which reduced the degradation rate and increased the extent of uptake. Liposomes are spherical-shaped vesicle consisting of one or several phospholipid bilayers separated by aqueous inner compartments and are nontoxic, biocompatible and biodegradable. These vesicles have ability to incorporate hydrophobic, hydrophilic and ampiphilic substances. It has also been demonstrated that liposomes can improve solubility, stability and encapsulation efficiency, and drug protection against degradation. Many researchers indicated that bioavailability of oral administered drug with poor solubility and permeability was obviously enhanced after encapsulation with liposomes and changes the *in vivo* distributions of entrapped drugs. [113-118]

Among the lipid based systems, liposome seems to be the most promising system for its ability to enhance the permeability of drug across the enterocyte, to stabilize drugs, and provide the opportunity of controlled release.[119]

Ling *et al.* showed that bioavailability and intestinal lymphatic uptake of several drugs were significantly increased after the incorporation of drug in liposome.[120] Generally, drug must pass through the liver for first pass metabolism before going into the systematic circulation. However, drug loaded liposome was pass through the lymphatic system into systematic circulation by avoiding first pass metabolism.

For oral administration of liposome formulations, intestinal absorption and stability of liposome are the prime formulation concerns. Hashida *et al.*[121] studied the *in vivo* absorption characteristics of carboxy fluorescein loaded liposome, and

compared the concentration of carboxy fluorescein in plasma and lymph to that of the free dye. The results indicated insignificant difference between the two formulations suggested poor absorption of drug loaded liposomes across the intestinal mucosa. Conversely, when liposome was co-administration with lipid-surfactant, permeability of drug was greatly improved which might be due to the interaction between the lipid-surfactant micelle and the lipid bilayer of the intestinal cell membrane.

There are several hydrophilic drugs possesses poor lymphatic transport due to their low intestinal bioavailability, limiting their use in the clinic. Ling *et al*.[120] prepared oral liposomal formulation of cefotaxime, poorly bioavailable hydrophilic drug, in a rat model by comparing the liposomal formulation with free drug and physical mixture of drug with blank liposome. The data shown a 2.7-fold increase in its oral bioavailability of liposomal formulation compared to free drug, and a 2.3-fold increase for the physical mixture. Furthermore, they also reported a significant enhancement of the lymphatic localization of the drug encapsulated liposome relative to the other two formulations. Therefore, liposome systems may be useful carriers for poorly bioavailable hydrophilic drugs, promoting their lymphatic transport in the intestinal lymph as well as their systemic bioavailability.

## 2.3. Solid Lipid Nanoparticles

In addition, solid lipid nanoparticles (SLN) have attracted a lot of attention as a tool to increase the solubility of poorly soluble drugs.[122] Solid lipid nanoparticles are similar to nanospheres in that they have an active ingredient dispersed within a matrix produced from physiologically compatible lipids that remain in a solid state at both room and body temperature. Therefore, SLNs can improve stability and provide controlled release and also improve drug targeting. In addition, size of formulation

allows efficient uptake of drugs into the intestine, particularly via the lymphatic route.[123]

Shah *et al.*[124] were prepared Carvedilol-loaded solid lipid nanoparticles (SLNs) using solubility parameter (δ) to select the lipid, and hot homogenization to fabricate SLNs. Higher carvedilol uptake from SLNs compared to drug solution in the Caco-2 cell line exhibited a potential prolonged drug release. Moreover, upon cellular uptake, SLNs could then enter the lymphatic system which will avoid first pass metabolism and hence higher oral bioavailability.[124]

Shah *et al.*[125] also documented enhanced oral bioavailability of poorly aqueous soluble drugs encapsulated in solid lipid nanoparticles (SLNs) via lymphatic delivery has been documented. The study suggests that main absorption mechanism of prepared SLNs could be endocytosis and, more specifically, clathrin-mediated endocytosis. When Transwell permeable supports were used for the cells, carrier-mediated mechanism for prepared SLNs and passive absorption mechanism (transcellular and paracellular) for SLNs physical mixture and drug solution were concluded.[125]

Clozapine SLNs administered intravenously and intraduodenally showed increased bioavailability with an increase in (AUC) of 3 and 4.5 times, respectively, as compared to clozapine suspension. This increased AUC for SLNs could be due to avoidance of first pass hepatic metabolism by SLNs driven intestinal lymphatic transport.[126]

Tobramycin, a drug that is not absorbed through the GI tract and is administered parenterally, was administered to rats duodenally in the form of SLNs resulting in 100 and 20 times higher AUC than IV administered tobramycin SLNs and

tobramycin solution, respectively. This difference between the two administration routes can be attributed to the transmucosal transport of SLN to lymph instead to blood. These results indicate that SLNs could be a useful drug delivery system that improves the bioavailability of lipophilic drugs.[127]

## 2.4. Self Emulsifying Drug Delivery System

Among the lipid-based formulations, self (micro) emulsifying drug delivery systems (S[M]EDDS) have been characterized more systematically from a physicochemical point of view. They are mixtures of lipids, surfactants (hydrophilic or/and lipophilic), co-solvents and the poorly soluble drug substance, forming stable micro-emulsions on dilution in water. When SEDDS form emulsion particles in the nanometer range, they can be referred to as self nano-emulsifying drug delivery systems (SNEDDS). Development and characterization of SEDDS and SMEDDS has been extensively reviewed.[14, 128-130] Recently new types of self micro-emulsifying drug delivery systems have been developed. Namely the supersaturated-self micro-emulsifying drug delivery systems (S-SMEDDS)[131, 132] and the U-type microemulsions[133, 134] S-SMEDDS formulations are designed to contain a reduced amount of a surfactant, but a water-soluble cellulosic polymer (or other polymers) is added to prevent precipitation of the drug by generating and maintaining a supersaturated state *in vivo*.[131, 132] In the development of SMEDDS, it is important to consider the dispersion of the SMEDDS that will be occurring in the GI tract; an optimal formulation is expected to be a one-phase system with no precipitation of drug under all dilutions.[135] Dry emulsions have been suggested as one way to circumvent disadvantages like the instability such as creaming, flocculation, coalescence, and phase separation of conventional emulsions.[136, 137] Dry emulsions are stabilized emulsions that are dried, often by spray drying. This forms a powder that can be

tableted, which is often considered an advantage in the pharmaceutical industry. Tablets containing lipid formulations have been described recently.[138] Dry emulsions have been successfully applied as oral drug delivery systems for lipophilic and poorly soluble drug substances.[139]

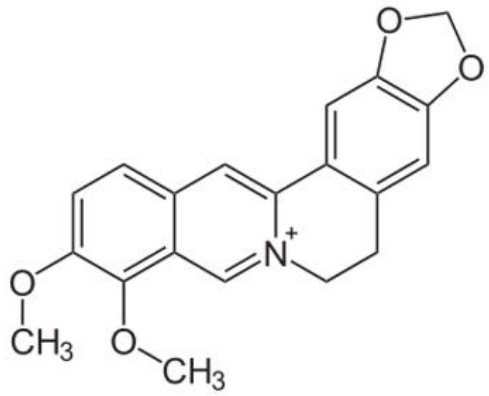
Several FDA-approved drug products as well as some nutraceuticals (e.g., coenzyme Q-10) utilizing this technology are currently on the market. Botanical extracts and individual phytochemicals whose bioavailability has been improved when formulated as self-emulsifying microemulsions include *Ginkgo biloba*,[29] *Schisandra chinensis*,[30] curcumin,[31] and silymarin.[32]

Porter and colleagues summarized lipid delivery systems with focus on self-emulsifying delivery system (SEDDS) and assessment of lipid-based formulations using *in vitro* lipolysis[15], and provided a good overview on lipid digestion and drug solubilisation in the small intestine as well as lymphatic transport.[96]

Singh *et al.*[140] have made a general review covering SEDDS and solid dispersions. In a recent review a rational strategy for the development of lipid and surfactant based drug delivery system was suggested.[141] In connection to this the LFCS proposed by Pouton was evaluated and considerations suggested in particular reflecting the differences between triglycerides (TG) and partial glycerides.

# 3. DRUG AND EXCIPIENTS PROFILE

## 3.1. Drug Profile (Berberine)



| | |
|---|---|
| **Molecular formula** | $C_{20}H_{18}NO_4^+$ |
| **Molecular Mass** | 336.36122 g/mol |
| **Appearance** | Yellow solid |
| **Melting Point** | 145 $^oC$ |
| **Solubility in Water** | Slowly soluble |

**Figure 3.1. Drug summary[142]**

### 3.1.1. Background

Berberine (*2,3-methylenedioxy-9,10-dimethoxy-protoberberine*) is a bitter-tasting, yellow, plant alkaloid with a long history of medicinal use in Ayurveda and Traditional Chinese Medicine as an anti-microbial, anti-protozoal, and anti-diarrheal agent.[143] Berberine is present in the roots, rhizomes and stem bark of various plants including *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric).[144] Berberine has also been used historically as a dye, due to its yellow color.

It has shown efficacy against various bacteria strains such as cholera, giardia, shigella, and salmonella; potentially also staphylococcus, streptococcus, and clostridium.[143, 145] Its actions against protozoa extend to *Giardia lamblia*, *Trichomonas vaginalis*, *Leishmania donovani*, and Malaria.[146-148] Surprisingly, crude extracts are more potent than isolated berberine in these anti-protozoan effects suggesting synergistic or additive effects with other compounds in these plants.[149]

### 3.1.2. Pharmacodynamics/Kinetics

#### *3.1.2.1. Absorption*

Overall bioavailability of Berberine is quite low at 'less than 5%'[150, 151] with 0.68% having been reported in rats.[152]

Berberine appears to be subject to P-Glycoprotein mediated efflux from the intestines[50, 153] and liver.[154] Due to low intestinal uptake rate, large doses (1g) are associated with constipation.[155] This constipative effect is also due to some properties of berberine in the colon, and can be useful to reducing watery diarrhea at 400 mg, four 100 mg doses.[156]

Low absorption may precede intestinal side-effects with high doses, due to large colonic levels

#### *3.1.2.2. Distribution*

Berberine binds to both Bovine and Human Serum Albumin relatively well and in a 1:1 ratio (indicating a single binding site) and has slightly higher affinity than does the related structure Palmatine;[157] Berberine may also bind to the β-Trp37 residue on Hemoglobin.[158]

#### *3.1.2.3. Metabolism*

Berberine can have its structure metabolized into four possible metabolites known as Thalifendine, Jatrorrhizine, Berberrubine, and Demethyleneberberine; Berberrubine may passively isomerize between two molecules.[159, 160]

Berberine can be possibly metabolized into four different metabolites, with all four metabolites both being active on the same mechanisms as Berberine but to a lesser potency.

In rats, all four metabolites have been detected in serum following ingestion of 40mg/kg Berberine[33] and when measuring Berberine concentrations 3 hours after

ingestion (rat liver) most Berberine appears to not be metabolized but a small increase in Thalifendine is noted relative to other metabolites.[161]

Berberine appears to be somewhat preserved in the parent form after oral administration

After incubation with intestinal bacteria for 7 days (human and rat), no visible metabolism of Berberine by intestinal bacteria was noted and the tested metabolites were similarly not metabolized further; it is thought that intestinal bacteria does not play a role in the metabolism of Berberine.[33]

### *3.1.2.4. Excretion*

Orally ingested Berberine (chloride) at 900 mg daily for 3 days was metabolized into three different urinary metabolites, with one (thought to be Jatrorrhizine-3-Sulfate) being the primary metabolite being excreted at 15-125 times more than the other two metabolites (Demethyleneberberine-2-sulfate and Thalifendine-10-sulfate, Berberrubine being undetectable in urine).[162]

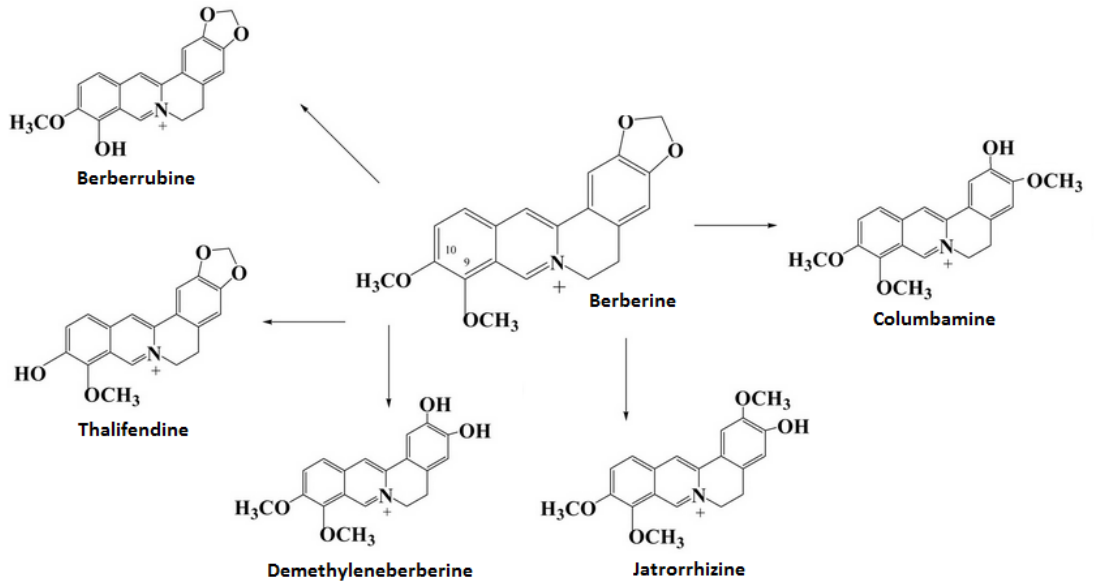



**Figure 3.2. Different metabolites of berberine**

### 3.1.3. Safety and Toxicology

#### *3.1.3.1. General*

One meta-analysis of 14 trials (1068 diabetic patients) given a variable range of 0.5-1.5g Berberine for an average period of 12 weeks with or without standard oral hypoglycemic therapy noted that Berberine appeared to be associated with more gastrointestinal/abdominal discomfort and mixed effects on stool (with reports of both diarrhea and constipation), most of which were alleviated with dose reduction or dividing the dose into multiple servings a day; no significant differences were noted between placebo/control and Berberine for hypoglycemia in these diabetics.[163]

Despite inhibition of the CYP1A1 enzyme, and reports of plants containing berberine to exert toxic effects, berberine in itself is considered to have low toxic potential.[164, 165] In most persons, Berberine appears to be relatively safe.

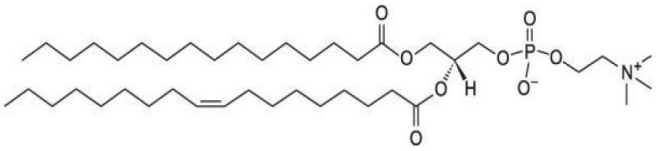
#### *3.1.3.2. Contraindications*

Due (partially) to inhibition of the CYP3A4 enzyme, berberine can adversely interact with Cyclosporin A and increase bioavailability of the latter, which necessitates a lower dosage.[166, 167] Berberine can also adversely interact with warfarin, thiopental, and tolbutamide by displacing them from their sites of action and increasing blood toxicity potential.[168]

It also displaces bilirubin from albumin at a very high rate, which may be a factor for reported green stools alongside berberin's natural coloration as yellow. Because of the former reactions, however, it should not be used in jaundiced neonates and pregnant women.[168, 169]

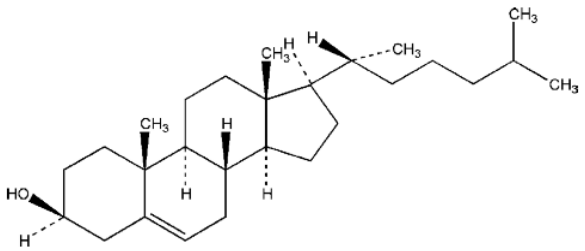
## 3.2. Excipients Profile

### 3.2.1. Soyphosphatidylcholine (SPC)[170]

| | |
|---|---|
| **Nonproprietary Names** | Lecithin |
| **Synonyms** | soybean lecithin; soybean phospholipids; Lectithol; |
| **Chemical Name** | 2-linoleoyl-1-palmitoyl; 3- SN-phosphatidylcholine. |
| **Structural formula** |  |
| **Empirical formula** | $C_{42}H_{82}NO_8P$ |
| **Molecular weight** | 760.09 g/mol |
| **Melting point** | $-5^{o}C$ |
| **Solubility** | Soluble in chloroform: 50 mg/ml, clear, very faintly yellow. Soluble in hexane-ethanol, methanol, ethanol, toluene, ether, mineral oils, fatty acids. Sparingly soluble in benzene. Insoluble in water (CMC < 0.001nM), cold acetone, cold vegetable and animal oils. |
| **Description** | Lecithin is a generic term to designate any group of yellow brownish fatty substances occurring in animal and plant tissues composed of phosphoric acid, choline, fattyacids, glycerol, glycolipids, triglycerides and phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol).<br>Lecithins vary greatly in their physical form, from viscous semiliquids to powders, depending upon the free fatty acid content. They may also vary in color from brown to light yellow, depending upon whether they are bleached or not. lecithin as a complex mixture o 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 |

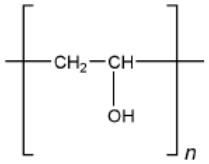
| | |
|---|---|
| | carbohydrates as separated from a crude vegetable oil source soybean lecithin contains 21% phosphatidylcholine, 22% phosphatidylethanolamine, and 19% phosphatidylinositol, along with other components |
| **Functional Category** | Emollient; emulsifying agent; solubilizing agent |
| **Stability and storage** | Stored in cool, dry, ventilated location in a well-closed container, protected from extreme heat and strong oxidizing agent |

### 3.2.2. Cholesterol[170]

| | |
|---|---|
| **Nonproprietary Names** | BP: Cholesterol; JP: Cholesterol; PhEur: Cholesterolum; USPNF: Cholesterol |
| **Synonyms** | Cholesterin; cholesterolum. |
| **Chemical Name** | Cholest-5-en-3b-ol |
| **Structural formula** |  |
| **Empirical formula** | $C_{27}H_{46}O$ |
| **Molecular weight** | 386.67 |
| **Solubility** | Practically insoluble in water and soluble in acetone and vegetable oils at 20°C. |
| **Boiling point** | 360°C |
| **Melting point** | 147–150°C |
| **Density** | 1.052 g/cm$^3$ (anhydrous form) |
| **Specific rotation** $[\alpha]_D^{20}$ | 39.5°(2% w/v solution in chloroform); 31.5° (2% w/v solution in ether). |
| **Dielectric constant D$^{20}$** | 5.41 |
| **Description** | Cholesterol occurs as white or faintly yellow, almost odorless, pearly leaflets, needles, powder, or granules. On prolonged exposure to light and air, cholesterol acquires a yellow to tan color |

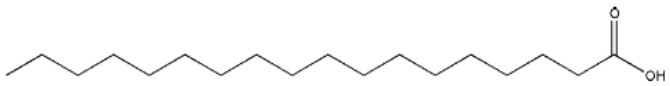
| | |
|---|---|
| **Functional Category** | Emollient; emulsifying agent |
| **Stability and storage** | Cholesterol is stable and should be stored in a well-closed container, protected from light. |

### 3.2.3. Polyvinyl alcohol[170]

| | |
|---|---|
| **Nonproprietary Names** | PhEur: Poly (vinylisacetas), USP: Polyvinyl alcohol |
| **Synonyms** | Airvol; Alcotex; Elvanol; Gelvatol; Gohsenol; Lemol; Mowiol; Polyvinol; PVA; vinyl alcohol polymer. |
| **Chemical Name** | Ethenol, homopolymer |
| **Structural formula** |  |
| **Empirical formula and Molecular weight** | $(C_2H_4O)_n$ 20000–200000<br>Polyvinyl alcohol is a water-soluble synthetic polymer represented by the formula $(C_2H_4O)_n$. The value of n for commercially available materials lies between 500 and 5000, equivalent to a molecular weight range of approximately 20000–200000<br>*Commercially available grades of polyvinyl alcohol*<br>High viscosity (≈200 000, MW)<br>Medium viscosity (≈130 000, MW)<br>Low viscosity (≈20 000, MW) |
| **Solubility** | Soluble in water; slightly soluble in ethanol (95%); insoluble in organic solvents. |
| **Melting point** | 228°C for fully hydrolyzed grades<br>180–190°C for partially hydrolyzed grades |
| **Density** | 1.19–1.31 for solid at 25°C;<br>1.02 for 10% w/v aqueous solution at 25°C. |
| **Viscosity (dynamic) [mPas (cP)]** | High viscosity 40.0–65.0<br>Medium viscosity 21.0–33.0<br>Low viscosity 4.0–7.0 |

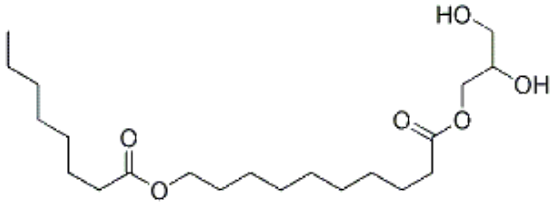
| | |
|---|---|
| **Refractive index $n_D^{25}$** | 1.49–1.53 |
| **Description** | Polyvinyl alcohol occurs as an odorless, white to cream-colored granular powder |
| **Functional Category** | Coating agent; lubricant; stabilizing agent; viscosity-increasing agent |
| **Stability and storage** | Polyvinyl alcohol is stable when stored in a tightly sealed container in a cool, dry place. Aqueous solutions are stable in corrosion-resistant sealed containers. Preservatives may be added to the solution if extended storage is required. Polyvinyl alcohol undergoes slow degradation at 100°C and rapid degradation at 200°C; it is stable on exposure to light. |

### 3.2.4. Stearic acid[170]

| | |
|---|---|
| **Nonproprietary Names** | BP: Stearic acid; JP: Stearic acid; PhEur: Acidumstearicum; USPNF: Stearic acid |
| **Synonyms** | Cetylacetic acid; Crodacid; E570; Edenor; Emersol; Hystrene; Industrene; Kortacid 1895; Pearl Steric; Pristerene; stereophonic acid; Tegostearic |
| **Chemical Name** | Octadecanoic acid |
| **Structural formula** |  |
| **Empirical formula** | $C_{18}H_{36}O_2$ |
| **Molecular weight** | 284.47 (for pure material) |
| **Solubility** | Freely soluble in benzene, carbon tetrachloride, chloroform, and ether; soluble in ethanol (95%), hexane, and propylene glycol; practically insoluble in water. |
| **Melting point** | 554°C |
| **Moisture content** | Contains practically no water. |
| **Density** | Density (bulk): 0.537 g/cm$^3$<br>Density (tapped): 0.571 g/cm$^3$<br>Density (true): 0.980 g/cm$^3$ |
| **Saponification value** | 200–220 |

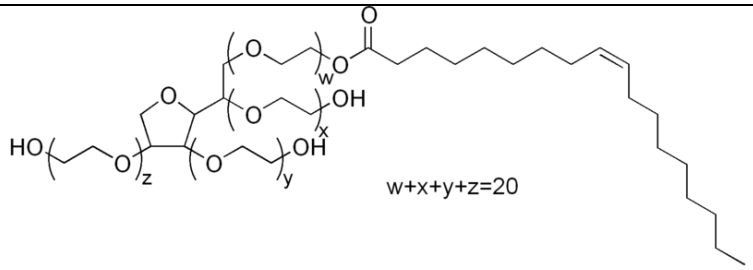
| | |
|---|---|
| **Acid value** | 200–212 |
| **Description** | Stearic acid is a hard, white or faintly yellow-colored, somewhat glossy, crystalline solid or a white or yellowish white powder. It has a slight odor and taste suggesting tallow. |
| **Functional Category** | Emulsifying agent; solubilizing agent; tablet and capsule lubricant. |
| **Stability and storage** | Stearic acid is a stable material; an antioxidant may also be added to it. The bulk material should be stored in a well-closed container in a cool, dry place. |

### 3.2.5. Capmul MCM C8[170]

| | |
|---|---|
| **Nonproprietary Names** | Glyceryl monocaprylate |
| **Synonyms** | Glyceryl monocaprylate; Medium chain mono- & diglycerides; Glyceryl mono- & dicaprylate |
| **Chemical Name** | Glyceryl caprylate |
| **Structural formula** |  |
| **Empirical formula** | $C_{21}H_{40}O_6$ |
| **Molecular weight** | 388.53 |
| **Solubility** | Partially soluble in water |
| **Flash point** | >150$^{o}$C |
| **Acid value** | ≤2.5 |
| **Description** | Capmul MCM C8 is composed of mono and diglycerides of medium chain fatty acids (mainly caprylic). It is an excellent solvent for many organic compounds, including steroids. It is also a useful emulsifier for water-oil systems. It appear as a waxy mass, paste or liquid at 25$^{o}$C |
| **Functional Category** | Carrier (vehicle); Solubilizer; Emulsifier / co-emulsifier; Bioavailability enhancer; Penetration enhancer |

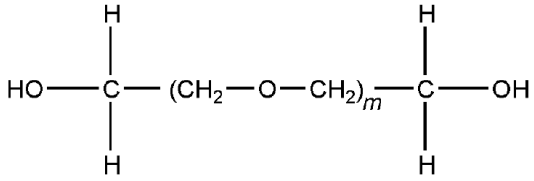
| | |
|---|---|
| | (dermatological applications) |
| **Stability and storage** | Store in a dry location at ambient temperature.<br>Retest/requalify 20 months after date of manufacture.<br>Contents of package must be heated slightly with agitation to ensure uniformity prior to use (maximum of 6 heat cycles) |

### 3.2.6. Tween 80[(170)]

| | |
|---|---|
| **Nonproprietary Names** | BP: Polysorbate 80; JP: Polysorbate 80; PhEur: Polysorbatum 80; USPNF: Polysorbate 80 |
| **Synonyms** | Atlas E; Armotan PMO 20; Capmul POE-O; Cremophor PS 80; Crillet 4; Crillet 50; Drewmulse POE-SMO; Durfax 80K; E433; Hodag PSMO-20; Montanox 80; polyoxyethylene 20 oleate; Protasorb O-20; Ritabate 80; (Z)-sorbitan mono-9-octadecenoate poly(oxy1,2-ethanediyl) derivatives; Tego SMO 80; Tego SMO 80V; Tween 80. |
| **Chemical Name** | Polyoxyethylene 20 sorbitan monooleate |
| **Structural formula** |  |
| **Empirical formula** | $C_{64}H_{124}O_{26}$ |
| **Molecular weight** | 1310 |
| **Solubility** | soluble in water, ethanol, cottonseed oil, corn oil, ethyl acetate, methanol, toluene |
| **Boiling point** | > 100°C |
| **Moisture content** | 3% |
| **Specific gravity** | 1.06–1.09 g/mL, oily liquid at 25°C |
| **Viscosity** | 300–500 centistokes at 25°C |
| **Acid value** | 2 |
| **Saponification value** | 45-55 |
| **HLB value** | 15 |
| **Description** | Polysorbates have a characteristic odor and a warm, |

| | |
|---|---|
| | somewhat bitter taste. It is yellow oily liquid at 25$^{o}$C |
| **Functional Category** | Emulsifying agent; nonionic surfactant; solubilizing agent; wetting, dispersing/suspending agent |
| **Stability and storage** | Polysorbates are stable to electrolytes and weak acids and bases; gradual saponification occurs with strong acids and bases. The oleic acid esters are sensitive to oxidation. Polysorbates are hygroscopic and should be examined for water content prior to use and dried if necessary. Also, in common with other polyoxyethylene surfactants, prolonged storage can lead to the formation of peroxides.<br>Polysorbates should be stored in a well-closed container, protected from light, in a cool, dry place. |

### 3.2.7. PEG 400[(170)]

| | |
|---|---|
| **Nonproprietary Names** | BP: Macrogols; JP: Macrogol 400;mPhEur: Macrogola; USPNF: Polyethylene glycol |
| **Synonyms** | Carbowax; Carbowax Sentry; Lipoxol; Lutrol E; PEG; Pluriol E; polyoxyethylene glycol |
| **Chemical Name** | α-Hydro-ω-hydroxypoly(oxy-1,2-ethanediyl) |
| **Structural formula** |  |
| **Empirical formula** | $HOCH_2(CH_2OCH_2)_mCH_2OH$ where m represents the average number of oxyethylene groups. For PEG 400 m=8.7 |
| **Molecular weight** | 380–420 |
| **Solubility** | All grades of polyethylene glycol are soluble in water and miscible in all proportions with other polyethylene glycols (after melting). Liquid polyethylene glycols are soluble in acetone, alcohols, benzene, glycerin, and glycols. Solid polyethylene glycols are soluble in acetone, dichloromethane, ethanol (95%), |
| **Moisture content** | Liquid polyethylene glycols are very hygroscopic, although |

| | |
|---|---|
| | hygroscopicity decreases with increasing molecular weight |
| **Flash point** | 238$^{o}$C |
| **Freezing point** | 4–8$^{o}$C |
| **Density at 25°C** | 1.11–1.14 g/cm$^{3}$ for liquid PEGs; |
| **Viscosity (Dynamic) [mPas (cP)]** | 105–130 |
| **Refractive index $n_D^{25}$** | 1.465 |
| **Description** | Polyethylene glycol is an addition polymer of ethylene oxide and water. Polyethylene glycol grades 200–600 are liquids; grades 1000 and above are solids at ambient temperatures.<br>Liquid grades (PEG 200–600) occur as clear, colorless or slightly yellow-colored, viscous liquids. They have a slight but characteristic odor and a bitter, slightly burning taste. |
| **Functional Category** | Ointment base; plasticizer; solvent; suppository base; tablet and capsule lubricant. |
| **Stability and storage** | Polyethylene glycols are chemically stable in air and in solution, although grades with a molecular weight less than 2000 are hygroscopic. Polyethylene glycols do not support microbial growth, and they do not become rancid.<br>Polyethylene glycols and aqueous polyethylene glycol solutions can be sterilized by autoclaving, filtration, or gamma irradiation. Oxidation of polyethylene glycols may also be inhibited by the inclusion of a suitable antioxidant.<br>The temperature must be kept to the minimum necessary to ensure fluidity; oxidation may occur if polyethylene glycols are exposed for long periods to temperatures exceeding 50$^{o}$C. However, storage under nitrogen reduces the possibility of oxidation.<br>Polyethylene glycols should be stored in well-closed containers in a cool, dry place. Stainless steel, aluminum, glass, or lined steel containers are preferred for the storage of liquid grades. |

# 4. PRE-FORMULATION STUDY

Pre-formulation studies were carried out to validate the drug information provided by supplier in accordance with the standard specification. These can be useful in the development of formulation. In this study, we investigated some physicochemical properties that may influence the formulation design. Drug excipient compatibility was also check to show the stability of drug with excipients. Following tests were carried out as a preformulation of the drug:

## 4.1. Methodology

### 4.1.1. Materials

All the analytical solvent such as methanol, chloroform, dichloromethane, toluene, ethyl acetate, formic acid used in the study was purchased from Loba Chemie Pvt. Ltd., Mumbai. HPLC grade acetonitrile and triethylamine were procured from Merck India Ltd., Mumbai

### 4.1.2. Organoleptic character

Sufficient quantity of drug was taken to evaluate the color and odour of drug. It is a first line indication for purity of drug.

### 4.1.3. Physicochemical characterization

#### *4.1.3.1. Melting point*

Melting point was determined by open capillary method using melting point testing apparatus. Drug was taken in glass capillary whose one end was sealed by flame. The capillary containing drug was put inside the melting point apparatus and the temperature at which drug started to melting was reported as observed in the attached thermometer.

#### *4.1.3.2. Solubility*

Solubility of berberine in various solvents like distilled water, ethanol, methanol, dichloromethane, chloroform, phosphate buffer pH 6.8, and in buffer pH 1.2 was determined by using saturation solubility method. In this, excess amount of drug was dissolve in 5 mL of each solvent in a test tube. The resulting solution was centrifuge at 10000 rpm for 30 min and supernatant was taken. UV spectrophotometer was used to analyze the sample for dissolved drug after suitable dilution, if necessary.

### 4.1.4. HPTLC fingerprinting

The HPTLC was performed on 10 x 10 cm pre-coated silica gel $60F_{254}$ HPTLC plates. (E.Merck, Germany). Samples were applied as 8 mm bands using Linomat 5 applicator (Camag, Switzerland). The plate was developed in a glass twin trough chamber with toluene: ethyl acetate: formic acid: methanol (9:9:3:1, v/v/v/v) as a mobile phase. The plate was developed at a distance of 8 cm followed by air drying. The plate was scan at 350 nm using densitometer (Camag TLC scanner) in absorbance reflectance mode operated by winCAT software.

### 4.1.5. Compatibility study

Compatibility study of drug was done to check the stability of drug with excipients used in formulation. Fourier transform infrared spectra (FTIR) of berberine and physical mixture of berberine with excipients were traced at 400 to 4000 $cm^{-1}$ on FTIR spectrometer. All spectras were compared to know that excipients affect the drug molecule or not. The spectra were analyzed for the absence or shift on the wave number of the characteristic peaks and reported.

### 4.1.6. Analytical method

#### *4.1.6.1. Determination of λmax*

First of all λmax of berberine was determined by scanning of 10 µg/mL drug solution prepared in different solvent i.e in methanol, phosphate buffer pH 6.8 and buffer pH 1.2 at UV range 200-400 nm.[171]

#### *4.1.6.2. Calibration curve of berberine*

Standard stock solution was prepared by dissolving 10 mg of berberine in methanol and make upto 100 mL. Suitable aliquots of the stock solutions were pipette out into 10 mL volumetric flasks and the volume was made upto 10 mL with methanol to give final concentration of 4-12 µg/mL. The absorption of all the prepared solutions was then measured at the absorbance maxima, 350 nm against the reagent blank. The readings were recorded in triplicate. Mean value (n=3) along with the standard deviation (SD) are recorded. The average values of absorption were plotted graphically against the concentrations. Similar procedure was followed to prepare calibration curve in phosphate buffer pH 6.8 and buffer pH 1.2. Standard concentrations (4, 8 and 12 µg/mL) were subjected to estimation of accuracy and precision.

Stability of the berberine in distilled water was ascertained by observing the changes in the absorbance of the solution at the analytical wavelength, over a period of 48 h at room temperature. No change in λmax and absorbance was observed over period of 48 h.

### 4.1.7. Bioanalytical method

Several HPLC methods are reported for the estimation of berberine in biological samples.[154, 172-174] In this study reverse phase HPLC method developed by Gui *et al.*[175] was used for the estimation of berberine in plasma.

#### *4.1.7.1. HPLC Condition*

The HPLC system consisted of a LC-20 AD pump (Shimadzu, Japan) and SPD M 20A photodiode array (PDA) detector (Shimadzu, Japan). The mobile phase consisted of acetonitrile/0.05 M $KH_2PO_4$/triethylamine (50:50:0.5, v/v/v) and the wavelength was 350 nm. Separation was achieved by Enable $C_{18}$ G reverse phase column (250 x 4.6 mm, 5 μm) with a flow rate of 1.0 mL/min. The method was found to be suitable for detection of berberine.

#### *4.1.7.2. Calibration of berberine in plasma*

Briefly, blood samples were taken from rat and plasma was separated by centrifugation at 4000 rpm for 10 min. Plasma sample (10 mL) was mixed with acetonitrile (50 mL) for precipitation of protein and vortexed for 1 min using vortex mixer. After 15 min, the mixture was centrifuge at 4000 rpm for 10 min to remove precipitated proteins. The obtained organic phase (acetonitrile solution) was evaporated to dryness. The residue was dissolve in mobile phase, sonicated for 1 min and filtered through a 0.45 μm membrane filter. The filtrate was kept for injection.

Stock solution of berberine (1 mg/mL) was prepared in methanol. Standard solution of berberine at concentration of 0.5, 2.5, 5, 10, 15, 20, 25, 30, and 35 μg/mL were prepared by serial dilution of the stock solution with methanol. The samples for calibration curves were prepared by spiking the 100 μL blank plasma with 20 μL appropriate standard solutions. The sample concentration for standard calibration curves were 0.1, 0.5, 1, 2, 3, 4, 5, 6, and 7 μg/mL. The samples were subjected to HPLC analysis by injection 20 μL of sample into injection port. The area under the curve for each peak obtained was plotted against concentration to make the calibration. Precision of the method was assessed by analyzing the plasma samples spiked with berberine at different concentration (0.1, 3 and 7 μg/mL).

## 4.2. Results and Discussion

The objectives of preformulation studies are to develop a portfolio of information about the drug substance which can be useful in the development of formulation. It is the first step in the rational development of dosage form of a drug substance. It can be defined as investigation of physical and chemical properties of drug substance alone and when combined with excipients. In preformulation study, all the characteristics of drug were observed like physical appearance, melting point, solubility in different solvents and compatibility study with other excipients used for the formulation development. These characteristics may influence the formulation design, method of manufacture, and pharmacokinetic-biopharmaceutical properties of the resulting product. Analytical methods were also performed to determine the amount of drug content in formulation.

### 4.2.1. Organoleptic character

Berberine occurs as bright yellow color that is odourless. The organoleptic characters were changed due to deterioration of sample on storage. This characteristics may sometime use for the identification of sample quality.

### 4.2.2. Physicochemical characterization

#### *4.2.2.1. Melting point*

Melting point is an indicator of sample quality as it is changed by presence of impurities in sample. Melting point of pure drug was found to be 148-150$^{o}$Cm which is near to the reported value 145$^{o}$C. Hence, it can be said that drug does not contains any impurities.

#### *4.2.2.2. Solubility*

Solubility is a basic property that should be evaluated early in the formulation development. Solubility ofberberine was determined in various solvent like distilled

water, ethanol, methanol, dichloromethane, chloroform, phosphate buffer pH 6.8, and in buffer pH 1.2 (Table 4.1). It indicates that berberine is freely soluble in ethanol, methanol, dichloromethane and chloroform whereas it is soluble in distilled water and buffer solution. This solubility data can be useful in estimation of drug in formulation as well as for drug release study.

**Table 4.1. Solubility of berberine in various solvents**

| Solvents | Solubility± SD (mg/mL) |
|---|---|
| **Distilled water** | 1.54 ± 0.21 |
| **Ethanol** | 5.31 ± 0.68 |
| **Methanol** | 10.21 ± 0.08 |
| **Dichloromethane** | 15.75 ± 1.68 |
| **Chloroform** | 21 ± 1.39 |
| **Phosphate buffer pH 6.8** | 4.8 ± 0.17 |
| **Buffer pH 1.2** | 2.41 ± 0.26 |

### 4.2.3. HPTLC fingerprinting

The $R_f$ value of berberine was 0.57 in a toluene: ethyl acetate: formic acid: methanol (9:9:3:1, v/v/v/v) with good resolution (Fig. 4.1). The $R_f$ value was found to be in close agreement with standard $R_f$ value of berberine indicates the good quality of drugs.

winCATS Planar Chromatography Manager

| Peak | Start Rf | Start Height | Max Rf | Max Height | Max % | End Rf | End Height | Area | Area % | Assigned substance |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | -0.01 | 3.2 | 0.02 | 646.8 | 43.63 | 0.10 | 101.2 | 17467.2 | 44.43 | unknown * |
| 2 | 0.16 | 74.2 | 0.16 | 77.5 | 5.22 | 0.22 | 61.6 | 2783.8 | 7.08 | unknown * |
| 3 | 0.28 | 68.3 | 0.29 | 86.6 | 5.84 | 0.30 | 68.0 | 1486.8 | 3.78 | unknown * |
| 4 | 0.32 | 67.0 | 0.33 | 76.7 | 5.17 | 0.35 | 58.5 | 1430.3 | 3.64 | unknown * |
| 5 | 0.36 | 58.7 | 0.37 | 67.9 | 4.58 | 0.40 | 50.2 | 1618.2 | 4.12 | unknown * |
| 6 | 0.40 | 50.3 | 0.44 | 78.0 | 5.26 | 0.46 | 67.1 | 2896.6 | 7.37 | unknown * |
| 7 | 0.57 | 64.0 | 0.75 | 334.5 | 9.11 | 0.79 | 155.2 | 10329.6 | 8.49 | unknown * |
| 8 | 0.78 | 26.9 | 0.79 | 38.5 | 2.60 | 0.80 | 35.8 | 644.4 | 1.64 | unknown * |
| 9 | 0.81 | 34.7 | 0.85 | 90.1 | 6.08 | 0.87 | 42.1 | 2767.1 | 7.04 | unknown * |
| 10 | 0.89 | 34.3 | 0.91 | 60.8 | 4.10 | 0.93 | 52.3 | 1412.9 | 3.59 | unknown * |
| 11 | 0.95 | 46.6 | 0.96 | 64.4 | 4.34 | 0.97 | 61.1 | 963.8 | 2.45 | unknown * |
| 12 | 0.97 | 61.5 | 1.01 | 117.4 | 7.92 | 1.04 | 2.4 | 3205.3 | 8.15 | unknown * |

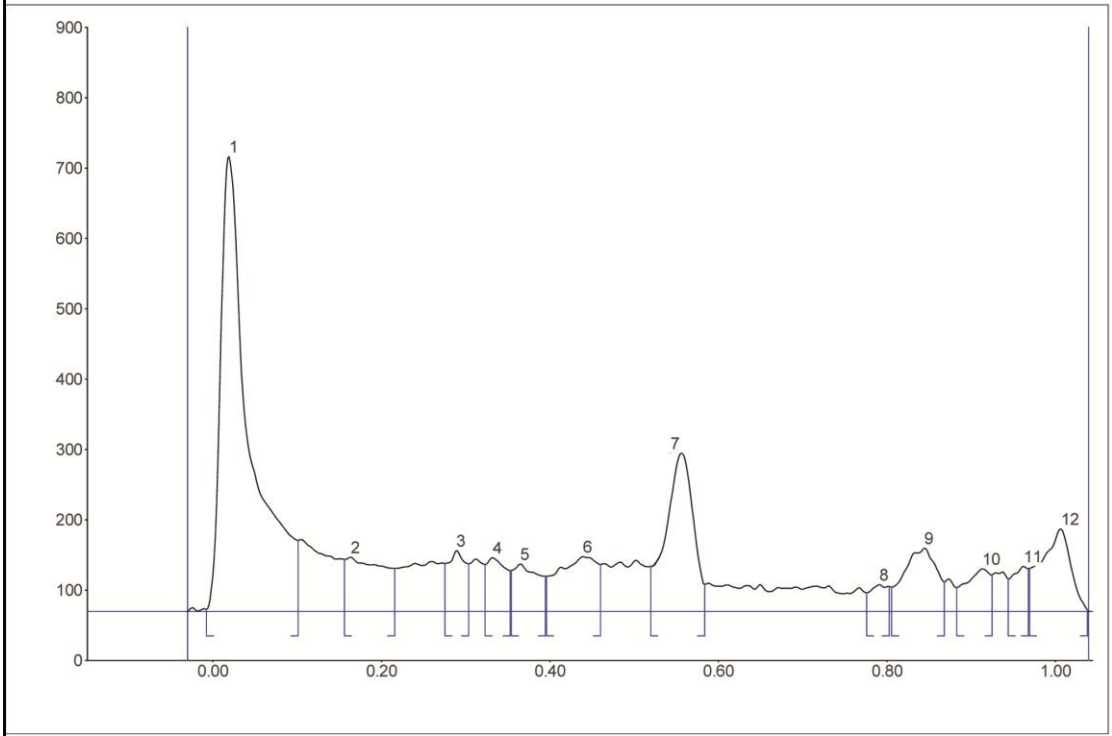



**Figure 4.1 Berberine HPTLC**

### 4.2.4. Compatibility study

Assessment of possible incompatibilities between drug and excipients is a key element of preformulation. Physical and chemical interaction between drug and excipients may affect chemical nature, stability and bioavailability of drug product, and subsequently their therapeutic efficacy and safety.[176]

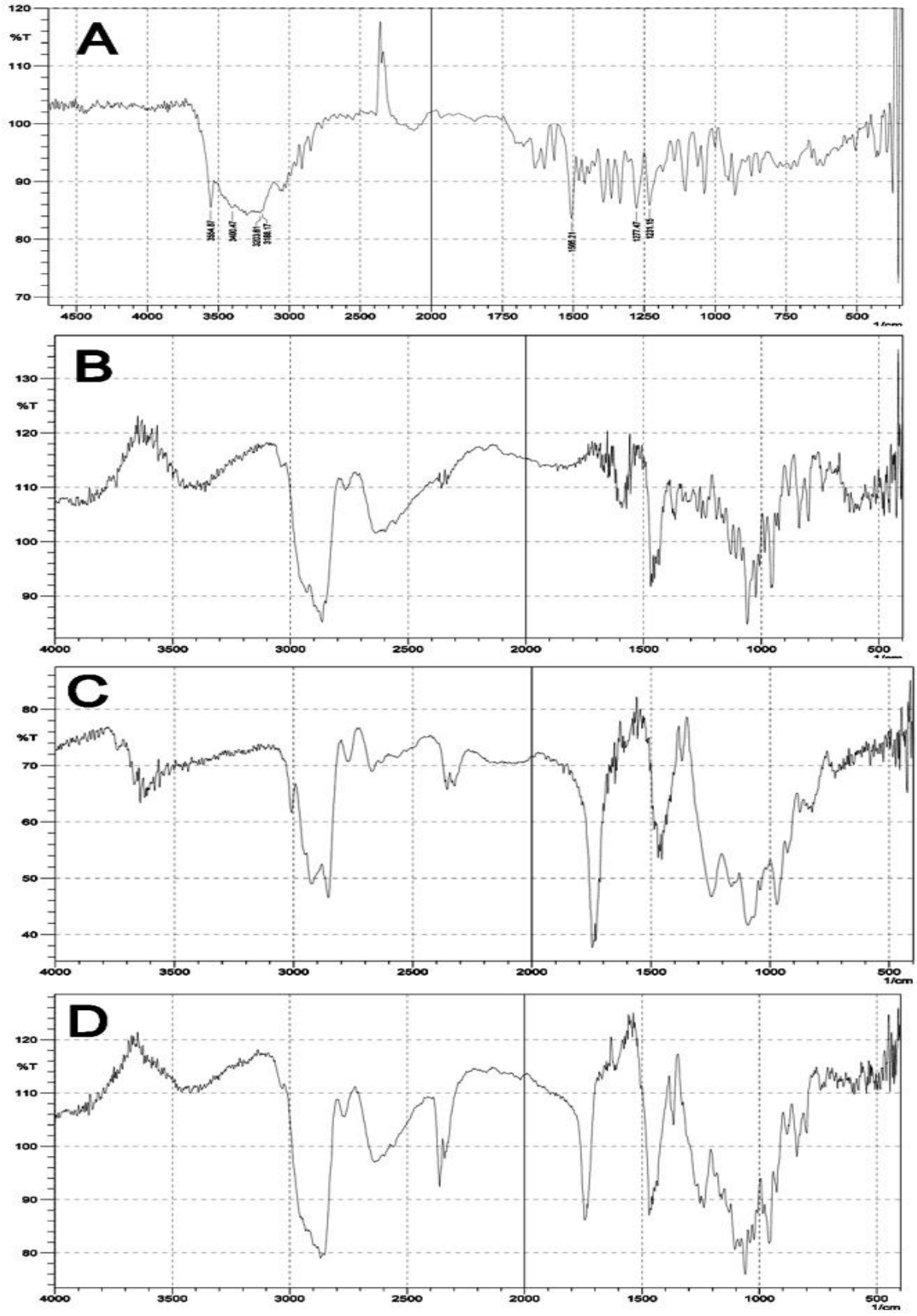



**Figure 4.2. FTIR spectra of drug with excipients used in preparation of liposome; (A) BER, (B) CHOL, (C) SPC (D) BER+CHOL+SPC.**

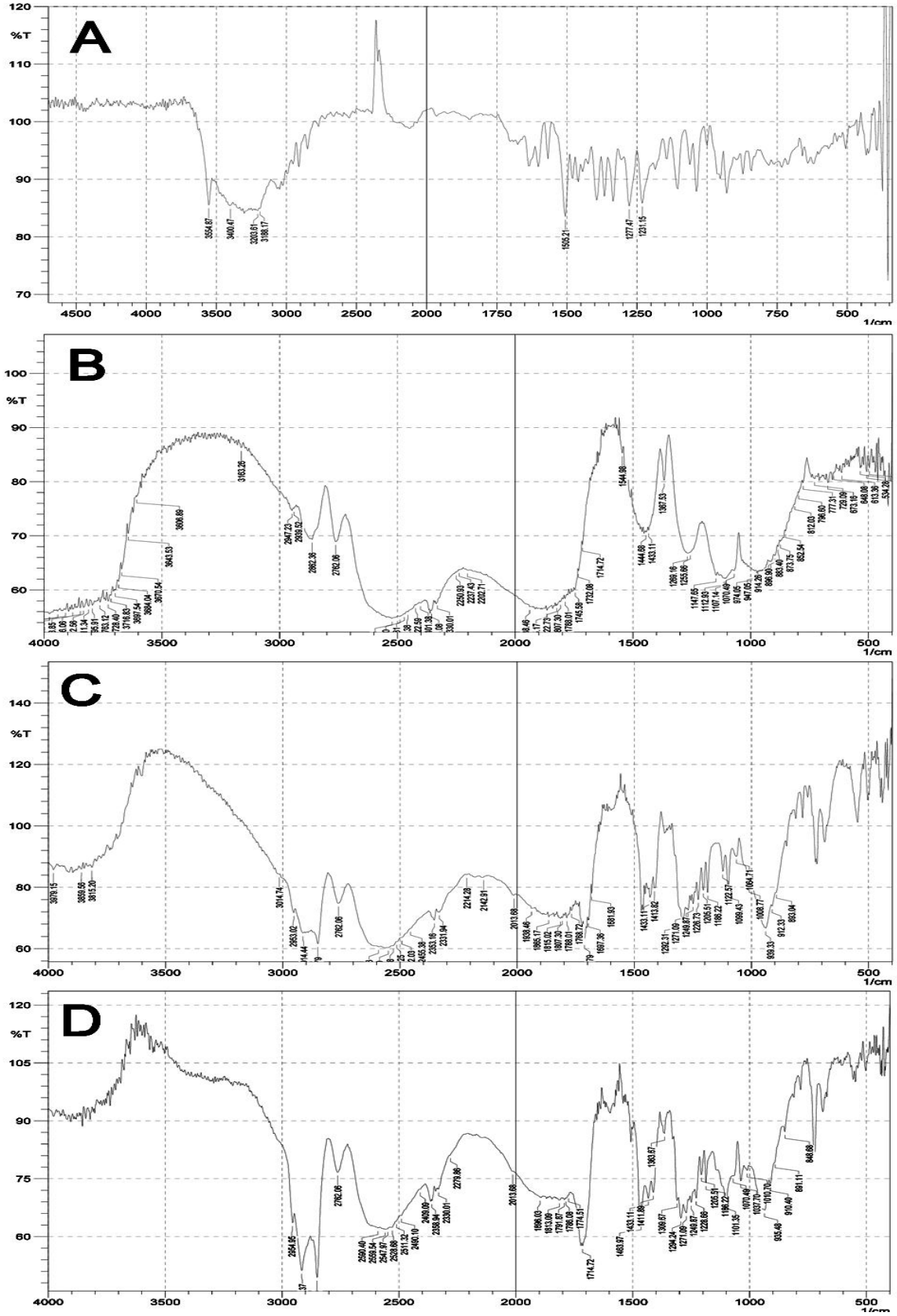



**Figure 4.3. FTIR spectra of drug with excipients used in preparation of SLN;**

**(A) BER, (B) PVA, (C) SA, (D) BER+PVA+SA**

FTIR spectra of berberine and physical mixture of berberine with excipients used in preparation of liposome and SLN were compared (Fig 4.2, 4.3). Spectrum containing BER + CHOL + SPC blend did not exhibit any characteristic peak of the drug suggesting an interaction. However definite conclusion cannot be made on compatibility of excipients based on this observation, because all other specific peak of the drug could be possibly overshadowed by the peak of corresponding excipients.

### 4.2.5. Analytical method

#### *4.2.5.1. Calibration curve in methanol*

Berberine in methanol showed absorption maximum at 229, 265, 350 and 428 nm. From this, 350 nm was chosen as the analytical wavelength based on previous literature. Beer's law was obeyed between 4-12 µg/mL (Table 4.2).

**Table 4.2. Calibration data for berberine in methanol**

| CONC (µg/ml) | ABSORBANCE | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Mean ± SD |
| 2 | 0.1088 | 0.1125 | 0.1023 | 0.1079 ± 0.0052 |
| 3 | 0.1627 | 0.1725 | 0.1608 | 0.1653 ±0.0063 |
| 4 | 0.2276 | 0.2304 | 0.2297 | 0.2292 ±0.0015 |
| 5 | 0.3125 | 0.3282 | 0.2982 | 0.3130 ±0.0150 |
| 8 | 0.5093 | 0.5036 | 0.5144 | 0.5091 ±0.0054 |
| 10 | 0.6153 | 0.6355 | 0.6206 | 0.6238 ±0.0105 |
| 12 | 0.7677 | 0.7703 | 0.7632 | 0.7671 ±0.0036 |

Regression analysis was performed on experimental data. Regression equation for standard curve was Y= 0.065 X - 0.024 (Fig. 4.4). Correlation coefficient for developed method was found to be 0.998 signifying that linear relationship existed between absorbance and concentration of the drug. Parameters indicating linearity for the used UV spectrometric method of analysis for berberine are shown in Table 4.3.

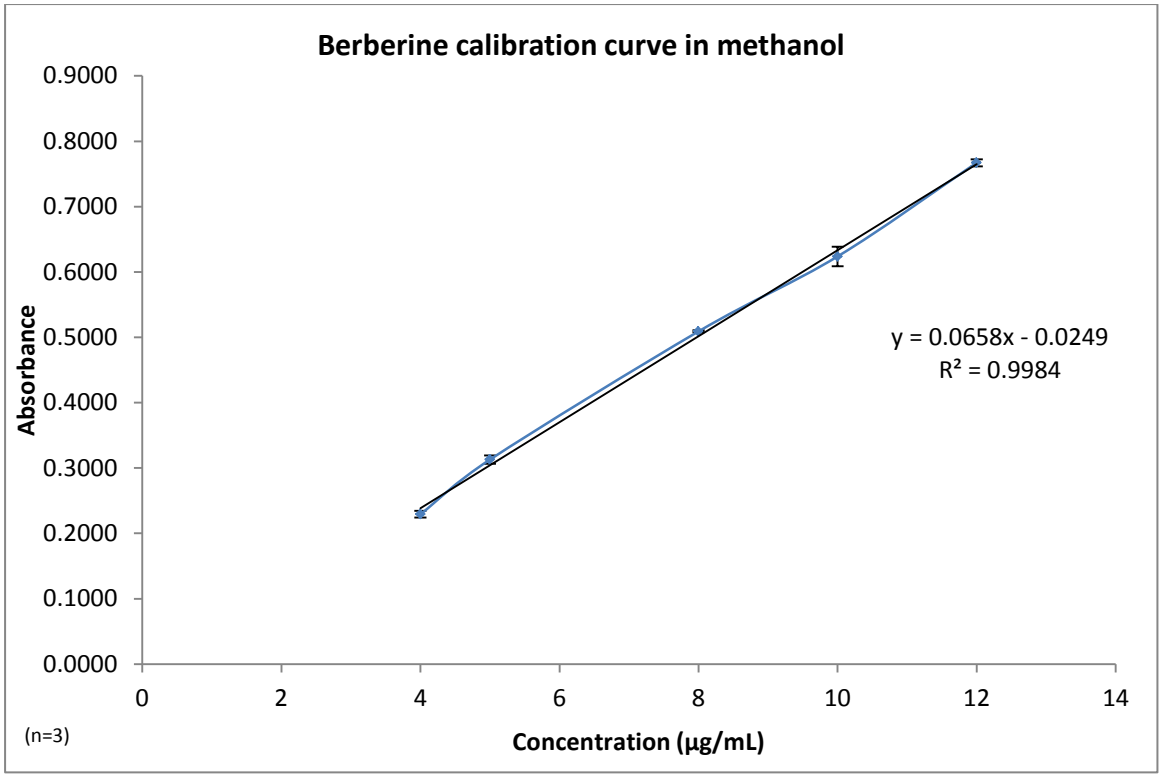



**Figure 4.4. Standard curve of berberine in methanol**

**Table 4.3. Parameters for estimation of berberine in methanol by UV spectroscopy**

| Parameters | Results |
|---|---|
| λmax | 350 nm |
| Linearity range | 4-12 µg/mL |
| Regression equation | Y =0.065 X-0.024 |
| Correlation coefficient | 0.998 |

**Table 4.4. Precision and Accuracy for estimation of berberine in methanol by UV spectroscopy**

| Standard concentration (µg/mL) | | Precision (%)* | Accuracy (%)# |
|---|---|---|---|
| Actual | Observed | | |
| 4 | 3.88 | 0.64 | 97.06 |
| 8 | 8.12 | 1.06 | 101.53 |
| 12 | 12.03 | 0.47 | 100.26 |

* Expressed as a relative standard deviation, RSD

RSD = (Standard deviation/Mean concentration) x 100

# Expressed as (mean observed concentration/actual concentration) x 100

Table 4.4. shows precision and accuracy for the berbeirne assay by UV spectroscopy. The low % RSD value indicated precision of the method. No significant difference between amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method.

### *4.2.5.2. Calibration curve in phosphate buffer pH 6.8*

Berberine in phosphate buffer showed absorption maximum at 228, 263, 345 and 421 nm. The variation in absorption maximum may occur due to change in solvent. From this, 345 nm was chosen as the analytical wavelength based on previous literature. Beer's law was obeyed between 5-18 µg/mL (Table 4.5).

**Table 4.5. Calibration data for berberine in phosphate buffer pH 6.8**

| CONC (µg/ml) | ABSORBANCE | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Mean ± SD |
| **5** | 0.2252 | 0.2209 | 0.2238 | 0.2233 ± 0.0022 |
| **7** | 0.3465 | 0.3494 | 0.336 | 0.3440 ± 0.0071 |
| **10** | 0.4777 | 0.4706 | 0.4751 | 0.4745 ± 0.0036 |
| **12** | 0.5911 | 0.5883 | 0.5946 | 0.5913 ± 0.0032 |
| **15** | 0.7378 | 0.7388 | 0.7406 | 0.7391 ± 0.0014 |
| **18** | 0.9003 | 0.8996 | 0.9051 | 0.9017 ± 0.0030 |

Regression analysis was performed on experimental data. Regression equation for standard curve was Y= 0.051 X - 0.030 (Fig.4.5). Correlation coefficient for developed method was found to be 0.998 signifying that linear relationship existed between absorbance and concentration of the drug. Parameters indicating linearity for the used UV spectrometric method of analysis for berberine are shown in Table 4.6.

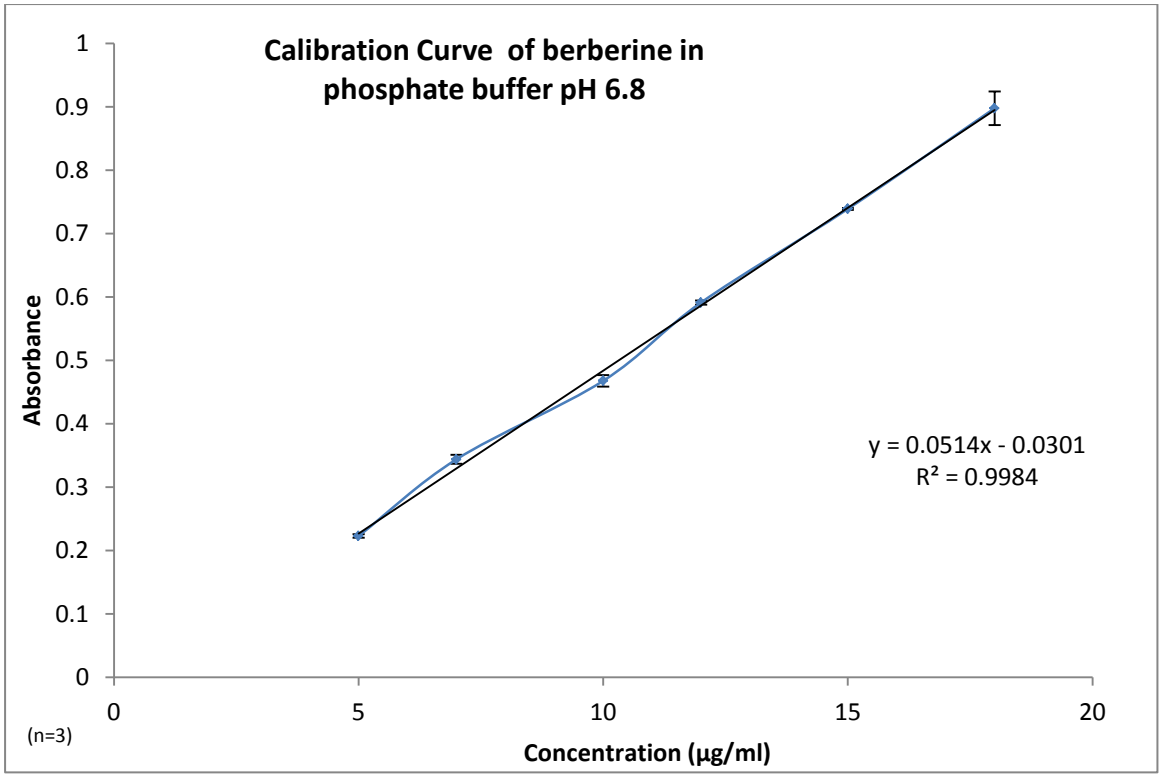



**Figure 4.5. Standard curve of berberine in phosphate buffer pH 6.8**

**Table 4.6. Parameters for estimation of berberine in phosphate buffer pH 6.8 by UV spectroscopy**

| Parameters | Results |
|---|---|
| λmax | 345 nm |
| Linearity range | 5-18 µg/mL |
| Regression equation | Y =0.051 X-0.030 |
| Correlation coefficient | 0.998 |

**Table 4.7. Precision and Accuracy for estimation of berberine in phosphate buffer pH 6.8 by UV spectroscopy**

| Standard concentration (µg/mL) | | Precision (%)* | Accuracy (%)# |
|---|---|---|---|
| Actual | Observed | | |
| 5 | 4.97 | 0.98 | 99.33 |
| 12 | 12.18 | 0.53 | 101.53 |
| 18 | 18.27 | 0.33 | 101.49 |

* Expressed as a relative standard deviation, RSD

RSD = (Standard deviation/Mean concentration) x 100

# Expressed as (mean observed concentration/actual concentration) x 100

Table 4.7 shows precision and accuracy for the berbeirne assay by UV spectroscopy. The low % RSD value indicated precision of the method. No significant difference between amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method.

### *4.2.5.3. Calibration curve in buffer pH 1.2*

Berberine in phosphate buffer showed absorption maximum at 228, 263, 345 and 421 nm. From this, 345 nm was chosen as the analytical wavelength based on previous literature. Beer's law was obeyed between 5-18 µg/mL (Table 4.8).

**Table 4.8. Calibration data for berberine in buffer pH 1.2**

| CONC (µg/ml) | ABSORBANCE | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Mean ± SD |
| 5 | 0.171 | 0.1704 | 0.1736 | 0.1717 ± 0.0017 |
| 7 | 0.2455 | 0.2409 | 0.2469 | 0.2444 ± 0.0031 |
| 10 | 0.3608 | 0.3596 | 0.3678 | 0.3627 ± 0.0044 |
| 12 | 0.4701 | 0.4756 | 0.4728 | 0.4728 ± 0.0028 |
| 15 | 0.6076 | 0.6097 | 0.6065 | 0.6079 ± 0.0016 |
| 18 | 0.7135 | 0.7165 | 0.7126 | 0.7142 ± 0.0020 |

Regression analysis was performed on experimental data. Regression equation for standard curve was Y = 0.043 X-0.050 (Fig. 4.6). Correlation coefficient for developed method was found to be 0.996 signifying that linear relationship existed between absorbance and concentration of the drug. Parameters indicating linearity for the used UV spectrometric method of analysis for berberine are shown in Table 4.9.

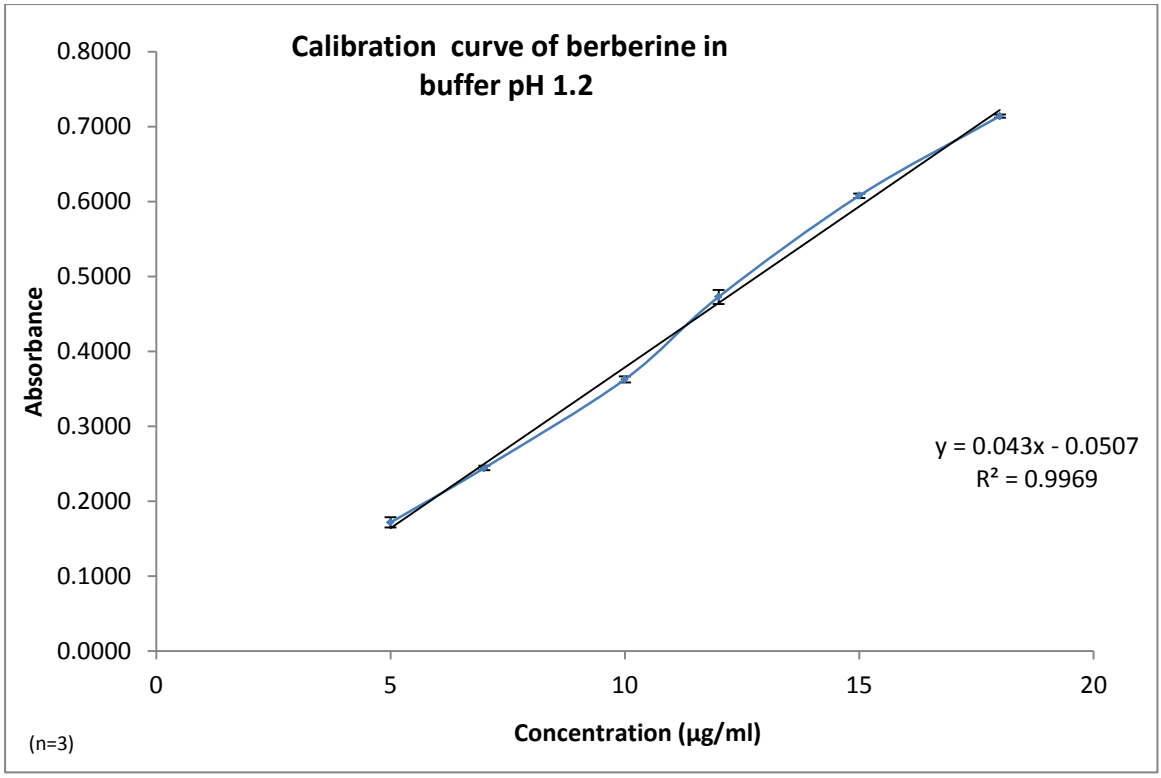



**Figure 4.6. Standard curve of berberine in buffer pH 1.2**

**Table 4.9. Parameters for estimation of berberine in buffer pH 1.2 by UV spectroscopy**

| Parameters | Results |
|---|---|
| λmax | 345 nm |
| Linearity range | 5-18 µg/mL |
| Regression equation | Y =0.043 X-0.050 |
| Correlation coefficient | 0.996 |

**Table 4.10. Precision and Accuracy for estimation of berberine in buffer pH 1.2 by UV spectroscopy**

| Standard concentration (µg/mL) | | Precision (%)* | Accuracy (%)# |
|---|---|---|---|
| Actual | Observed | | |
| 5 | 5.23 | 0.99 | 104.60 |
| 12 | 12.40 | 0.58 | 103.34 |
| 18 | 18.15 | 0.29 | 100.82 |

* Expressed as a relative standard deviation, RSD

RSD = (Standard deviation/Mean concentration) x 100

# Expressed as (mean observed concentration/actual concentration) x 100

Table 4.10 shows precision and accuracy for the berberine assay by UV spectroscopy. The low % RSD value indicated precision of the method. No significant difference between amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method.

### 4.2.6. Bioanalytical method

The retention time for berberine was found to be 6.374 min. The calibration curve of berberine in plasma is shown in Fig.4.7 and Table 4.11. Fig.4.8 shows a typical chromatogram of berberine in plasma. The calibration graph for berberine in plasma was linear over the range of 1-7 µg/mL. The data for calibration graph of berberine in plasma by HPLC was fitted to a linear equation Y = 83.53 X + 10.79 with correlation coefficient of $R^2$=0.997, which indicated the linearity of the plot (Table 4.12). Precision of the method was assessed by analyzing the plasma samples spike with berberine at different concentration (1, 3 and 7µg/mL).

**Table 4.11. Calibration data for berberine in rat plasma**

| CONC (µg/mL) | Area under curve (mAU) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Mean ± SD |
| 1 | 95.23 | 94.67 | 97.09 | 95.66 ± 1.27 |
| 2 | 187.7 | 185.09 | 186.15 | 186.31 ± 1.31 |
| 3 | 248.76 | 249.48 | 249.31 | 249.18 ± 0.38 |
| 4 | 351.87 | 352.46 | 349.24 | 351.19 ± 1.71 |
| 5 | 423.01 | 420.63 | 421.89 | 421.84 ± 1.19 |
| 6 | 509.76 | 498.24 | 506.78 | 504.93 ± 5.98 |
| 7 | 598.54 | 609.54 | 607.92 | 605.33 ± 5.94 |

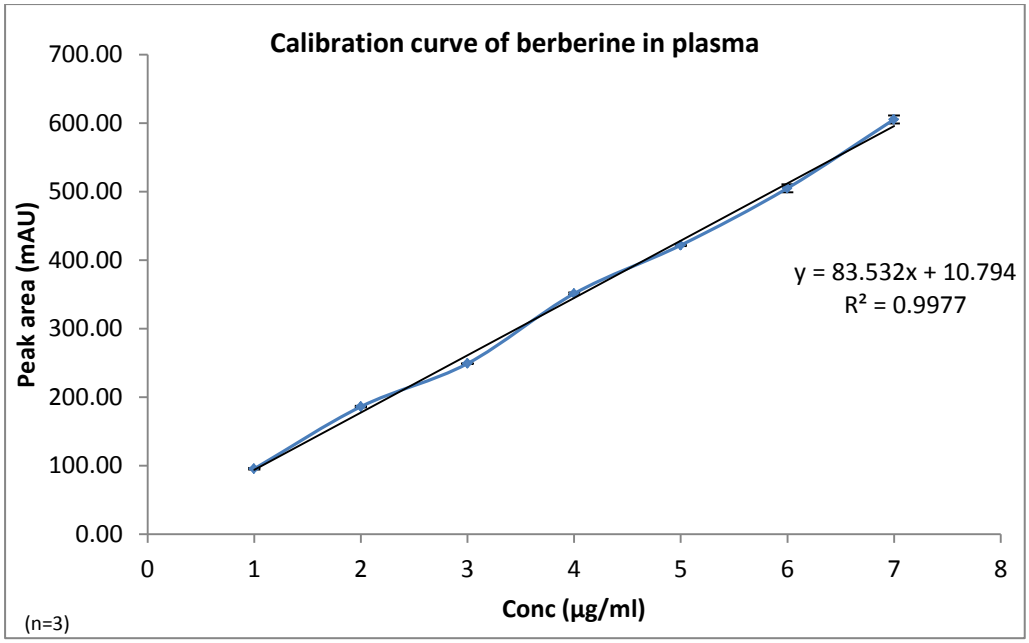



**Figure 4.7. Standard curve of berberine in rat plasma**

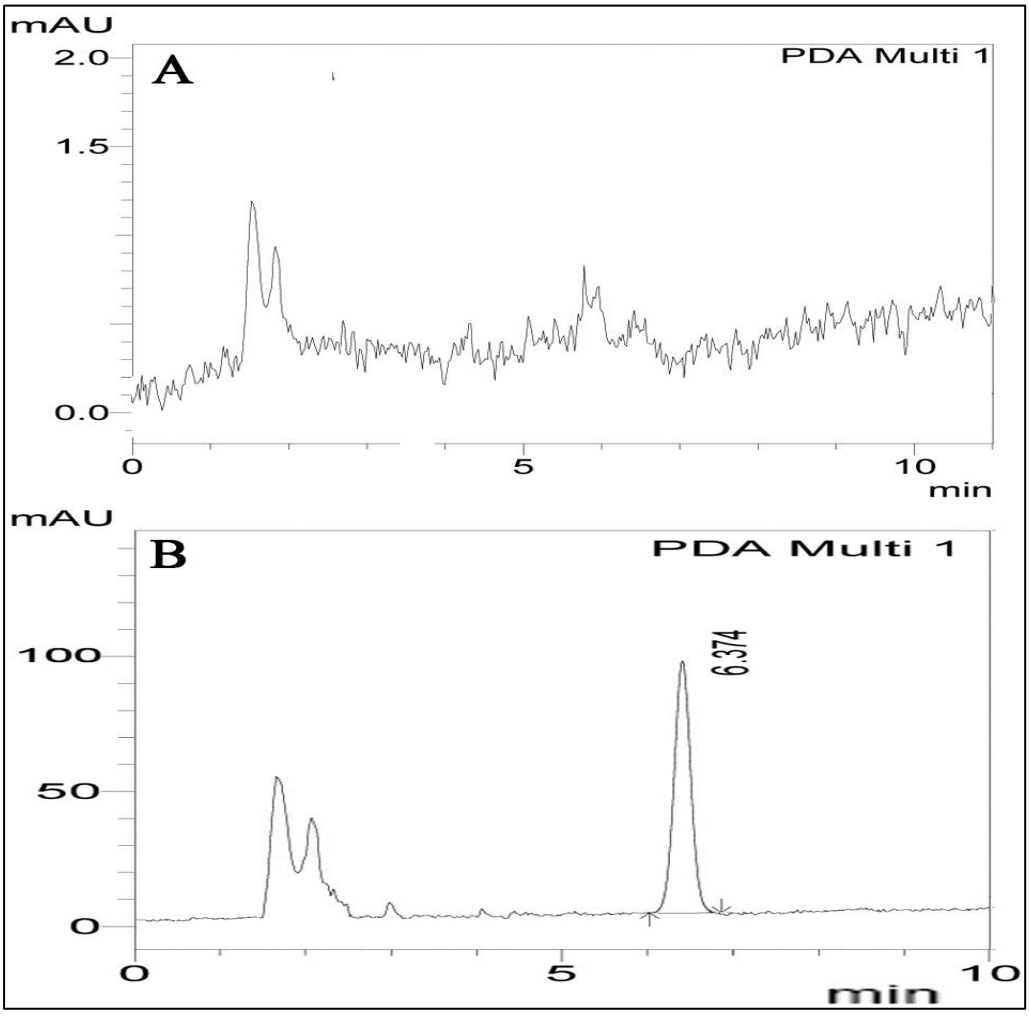



**Figure 4.8. Chromatogram of blank plasma (A), berberine in plasma (B)**

**Table 4.12. Parameters for estimation of berberine in rat plasma by HPLC**

| Parameters | Results |
|---|---|
| λmax | 345 nm |
| Linearity range | 1-7 µg/mL |
| Regression equation | Y =83.53 X + 10.97 |
| Correlation coefficient | 0.997 |

To evaluate precision, the mean values and the % RSD values were calculated for each concentration. Table 4.13 shows precision and accuracy for the berbeirne assay by UV spectroscopy. The low % RSD value indicated precision of the method. No significant difference between amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method.

**Table 4.13. Precision and Accuracy for estimation of berberine in rat plasma by HPLC**

| Standard concentration (µg/mL) | | Precision (%)* | Accuracy (%)# |
|---|---|---|---|
| Actual | Observed | | |
| 1 | 1.04 | 1.32 | 104.09 |
| 4 | 4.08 | 0.49 | 102.03 |
| 7 | 7.10 | 0.98 | 101.50 |

* Expressed as a relative standard deviation, RSD

RSD = (Standard deviation/Mean concentration) x 100

# Expressed as (mean observed concentration/actual concentration) x 100

# 5. PREPARATION AND CHARACTERIZATION OF LIPOSOME

## 5.1. Introduction

The name liposome is derived from two Greek words: 'Lipos' meaning 'fat' and 'Soma' meaning 'body'. Liposomes were first produced in England in 1961 by Alec D. Bangham, who was studying phospholipids and blood clotting.[177] Alec Bangham first described how membrane molecules, e.g. phospholipids, interact with water to form unique structures now recognized as liposomes[178] and found that phospholipids combined with water immediately formed a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble. Water soluble medications added to the water trap inside the aggregation of the hydrophobic ends; fat soluble medications are incorporated into the phospholipids layers (Fig. 5.1).

Among the lipid based systems, liposome seems to be most promising system for its ability to enhance the permeability of drug across the enterocyte, to stabilize drugs, and provide the opportunity of controlled release.[119] Liposomes are spherical-shaped vesicle consisting of one or several phospholipid bilayers separated by aqueous inner compartments and are nontoxic, biocompatible and biodegradable. These vesicles have ability to incorporate hydrophobic, hydrophilic and ampiphilic substances. It has been demonstrated that liposomes can improve solubility, stability and encapsulation efficiency, and drug protection against degradation. Many researchers indicated that bioavailability of oral administered drug with poor solubility and permeability was obviously enhanced after encapsulation with liposomes and changes the *in vivo* distributions of entrapped drugs.[113-118]

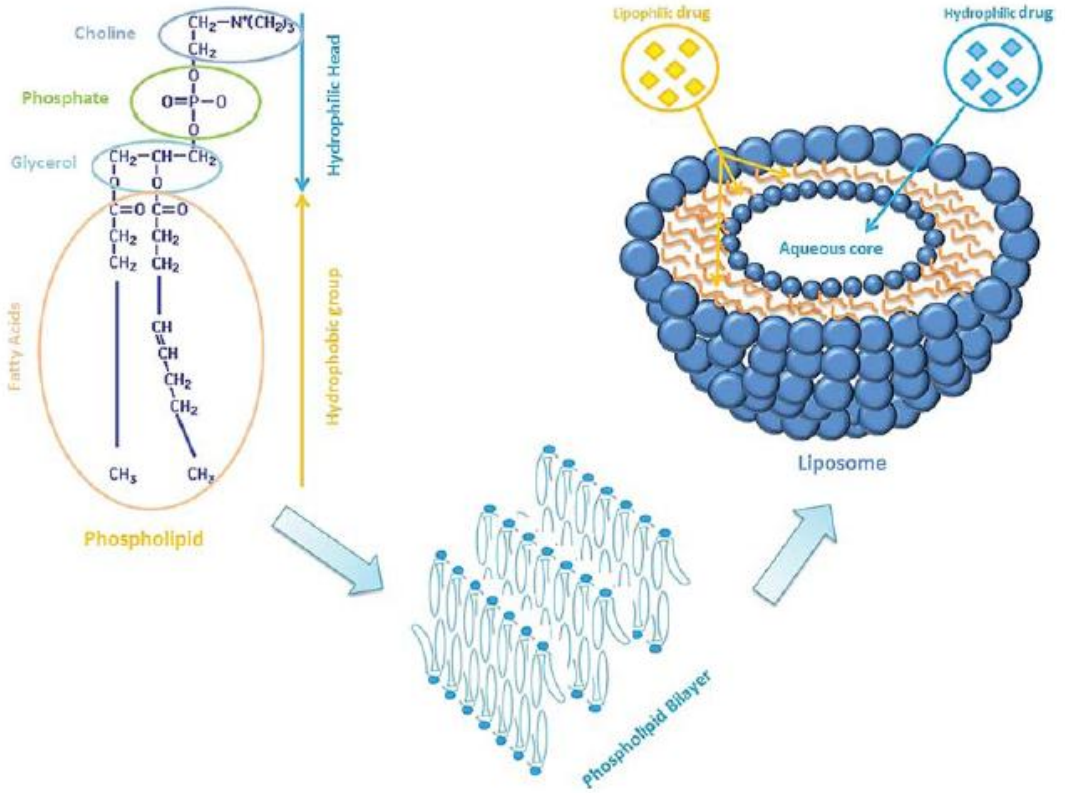



**Figure 5.1. Basic liposome structure and hydrophilic or lipophilic drug entrapment model**

### 5.1.1. Composition of liposome

The number of components of the liposomes is varied; however phospholipids and cholesterol are the main components. The most commonly used phospholipids include phosphatidylcholine (PC). Apart from that some synthetic phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phsophatidylinositol are also used for liposome preaparation. PC is an amphipathic molecule in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar headgroup, phospho choline. Phosphatidylcholine, also known as “lecithin”, can be derived from natural and synthetic sources. Lecithin membranes can exist in different phases at various temperatures. At elevated temperatures lipid membranes pass from tightly ordered gel to liquid crystal phase where freedom of movement of individual molecules is higher. In general, increasing

the chain length, or increasing the saturation of the chains, increases the transition temperature. Incorporation of sterols (cholesterol) in liposome bilayer can bring about major changes in the preparation of these membranes. Cholesterol does not by itself form bilayer structure, but can be incorporated in to phospholipid membranes in very high concentrations up to 1:1 or even 2:1 molar ratios of cholesterol to PC. Cholesterol inserts in to the membrane with its hydroxyl group oriented towards aqueous surface and aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. Cholesterol incorporation increases the separation between the choline head groups and eliminates the normal electrostatic and hydrogen bonding interactions. The interaction of PC and cholesterol is represented in Fig.15. The high solubility of cholesterol in phospholipid liposomes has been attributed to both hydrophobic and specific head group interactions.

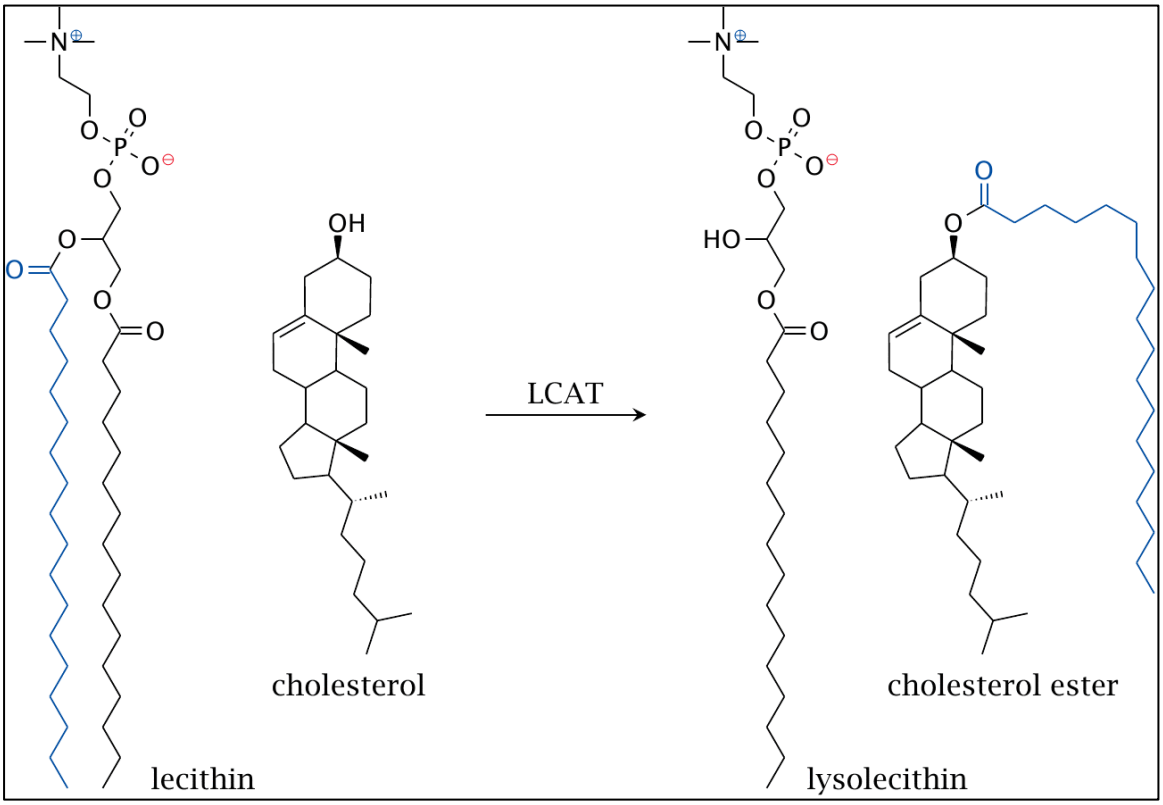



**Figure 5.2. Phosphotidylcholine and cholesterol interaction[179]**

### 5.1.2. Types of liposomes:

#### *5.1.2.1. Niosomes:*

Analogous to liposomes, niosomes are formed from the self-assembly of non-ionic amphiphiles in combination with other lipidic surfactants in aqueous medium.[180] The primary difference between the two types of vesicle includes the superior chemical stability and relatively low cost of niosomes in compared with liposomes. During dispersion, both niosomes and liposomes are at risk of aggregation, fusion, and leakage of encapsulated drug.[181]A promising product, called the proniosome, is a dry granular product that dissolves to form niosome suspension with the addition of water. Proniosomes have several advantages over niosomes, including the minimization of physical instability problems, such as aggregation, fusion and leakage or hydrolysis of encapsulated drug. In addition, it provides ease of transportation, distribution, storage, and dosage. Proniosomes have shown equal or greater efficacy in drug release performance when compared with conventional niosomes.[181]

#### *5.1.2.2. Transfersomes:*

Transfersomes are ultradeformable hydrophilic lipid vesicles that purportedly cross the skin under the influence of a transepidermal water activity gradient.[182] These vesicles are up to 105 times more deformable than un-modified liposomes. This characteristic allows transfersomes up to 200-300 nm in size to squeeze through pores in the stratum corneum. These pores are less than one-tenth of liposome diameter.[182, 183]

#### *5.1.2.3. Ethosomes:*

Ethosomes are multi-lamellar vesicles composed of phospholipids (soy phosphatidylcholine), ethanol and water. Ethosome is known to be an efficient enhancer of permeability.[184, 185] Experiments using fluorescent probes and

ultracentrifugation have shown that ethosomal systems have a higher entrapment capacity for molecules of various lipophilicities, e.g. acyclovir, minoxidil and testosterone.[186, 187]

#### *5.1.2.4. Proliposomes:*

Proliposomes are defined as dry free-flowing particles that immediately form liposomal dispersion on contact with water in body. Proliposomes are composed of water soluble porous powder as carrier upon which one may load phospholipids and drugs dissolved in organic solvent. The drug and phospholipids are deposit in the micros porous structure of the carrier materials, thus maintaining the free-flowing surface characteristics of the carrier materials. Their free-flowing particulate properties permit the fabrication of proliposomes into solid dosage forms such as, tablets and capsules, which then converted to liposomes on contact with water or biological fluids. Proliposomes can be stored sterilized in dry state and dispersed/dissolved to form an isotonic multi-lamellar liposomal suspension by addition of water as needed.[181, 188]

### 5.1.3. Classification of liposomes

Liposomes can be classified on the basis of composition and mechanism of intracellular delivery into five types.[189]

1. Conventional liposomes
2. pH-sensitive liposomes
3. Cationic liposomes
4. Immunoliposomes
5. Long circulating liposomes

Half-life of liposomes is critically based on vesicle size. In addition, size and number bilayers can influence the amount of drug encapsulation within liposomes. Thus, liposomes were classified on the basis of their size and number of bilayers into (Table 5.1, Fig. 5.3):

**Table 5.1. Vesicle types with their size and number of lipid layers**

| Vesicle type | Diameter | No of lipid bilayer |
|---|---|---|
| Unilamellar vesicle (UV) | All size range | One |
| Small Unilamellar vesicle (SUV) | 20-100 nm | One |
| Large Unilamellar vesicle (LUV) | More than 100nm | One |
| Giant Unilamellar vesicle (GUV) | More than 1 µm | One |
| Oligolamellar vesicle (OLV) | 0.1-1 µm | Approx. 5 |
| Multilamellar vesicle (MLV) | More than 0.5 | 5-25 |
| Multi vesicular vesicle (MV) | More than 1 micro meter | Multi compartmental structure |

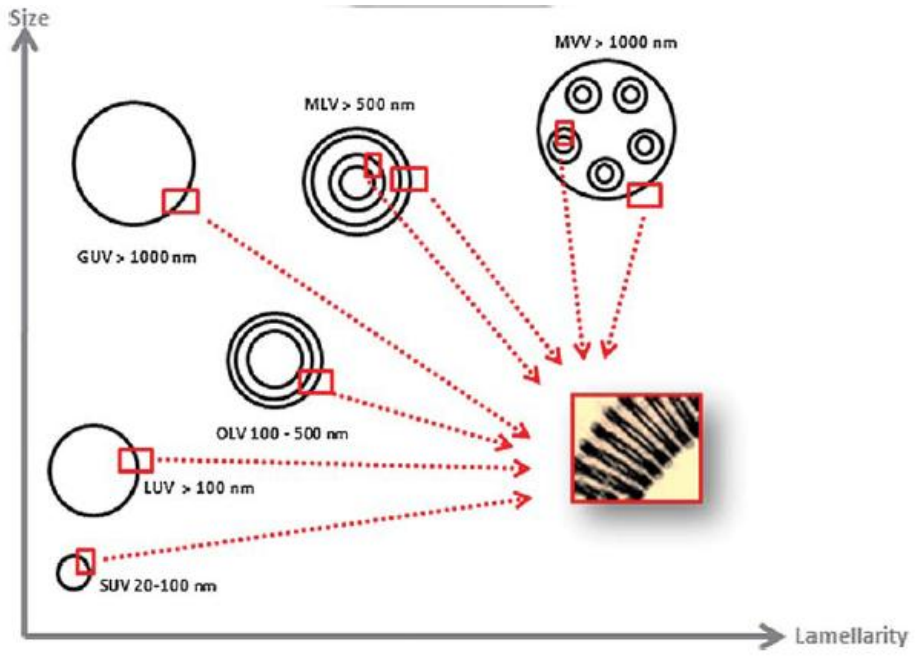



**Figure 5.3. Types of liposome based on size and lamellarity**

### 5.1.4. Liposome preparation

There are mainly four conventional methods were used for the preparation of liposome. The difference between these methods is the way in which lipid drying from organic phase and then re-dispersed in aqueous phase.[190]

#### *5.1.4.1. Thin lipid film hydration method*

This method is also called as Bengham's method because it was initially used for preparation of liposome.[191] A mixture of phospholipid and cholesterol were dispersed in organic solvent. Then, the organic solvent was removed by means of evaporation using a rotary evaporator at reduced pressure. Finally, the dry lipidic film deposited on the flask wall was hydrated by adding an aqueous buffer solution under agitation at temperature above the lipid transition temperature.

This method is widespread and easy to handle, however, dispersed-phospholipids in aqueous buffer yields a population of multilamellar liposomes (MLVs) heterogeneous both in size and shape (1–5 $\mu$m diameter). Thus, liposome size reduction techniques, such as sonication for SUVs formation or extrusion through polycarbonate filters forming LUVs.[192, 193] were useful to produce smaller and more uniformly sized population of vesicles.

#### *5.1.4.2. Reverse phase evaporation method*

Historically, this method provided a breakthrough in liposome technology, since it allowed for the first time the preparation of liposomes with a high aqueous space-to-lipid ratio and able to entrap a large percentage of the aqueous material presented. Reverse phase evaporation is based on the formation of inverted micelles. These inverted micelles are formed upon sonication of a mixture of a buffered aqueous phase, which contains the water soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized.

The slow removal of the organic solvent leads to transformation of these inverted micelles into a gel like and viscous state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The excess of phospholipids in the environment contributes to the formation of a complete bilayer around the remaining micelles, which results in formation of liposomes. Liposome made by this method can be made from various lipid formulations and have aqueous volume to lipid ratios that are four time higher than multi lamellar liposomes or hand shaken method.

#### *5.1.4.3. Freeze dried rehydration method*

Freeze dried liposomes are formed from preformed liposomes. Very high encapsulation efficiencies even for macromolecules can be achieved using this method. During the dehydration the lipid bilayers and the material to be encapsulated into the liposomes are brought into close contact. Upon reswelling, the chances for encapsulation of the adhered molecules are much higher. The rehydration is a very important step and should be done very carefully. The aqueous phase should be added in very small portions with a micropippete to the dried materials. After each addition the tube should be vortexed thoroughly. As a general rule the total volume used for rehydration must be smaller than the starting volume of the liposome dispersion.

#### *5.1.4.4. Solvent (Ether or Ethanol) injection technique.*

This method involves lipid dissolution into organic solvent followed by lipid solution injection into an aqueous solvent.[194] The ethanol injection method was first described in 1973. Liposome prepared by ethanol injection method is of less than 100 nm size without extrusion or sonication. The ether injection method differs from the ethanol injection method since the ether is immiscible with the aqueous phase, which is also heated so that the solvent is removed from the liposomal product. The method

involves injection of ether-lipid solutions into warmed aqueous phases above the boiling point of the ether. The ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms primarily unilamellar liposomes.[195] An advantage of the ether injection method compared to the ethanol injection method is the removal of the solvent from the product, enabling the process to be run for extended periods forming a concentrated liposomal product with high entrapment efficiencies.

Besides this methods, there are various other methods also have been used for liposomes preparation such as: calcium induced fusion,[196] nanoprecipitation,[197] and emulsion techniques.[198, 199]

However, these classical techniques require large amounts of organic solvent, which are harmful both to the environment and to human health, requiring complete removal of residual organic solvent. Furthermore, conventional methods consist of many steps for size homogenization and consume a large amount of energy which is unsuitable for the mass production of liposomes.

Since industrial scale production of liposomes has become reality, the range of liposome preparation methods has been extended by a number of techniques such as Heating Method, Spray drying, Freeze Drying, Super Critical Reverse Phase Evaporation (SCRPE), and several modified ethanol injection techniques which are increasingly attractive.

### 5.1.5. Behavior of liposomes *in-vivo*:

Every conceivable oral and parenteral route has carried out administration of liposomes into animals and humans. However, most of knowledge concerning liposomal behavior in-vivo has been obtained by injecting a variety of formulations intravenously. This is probably because not only it is easier to monitor liposomes in

the blood and their possible extravasations and uptake by tissues; it is also the most important route for rank of therapeutic application. The oral route, which is more convenient to the patient, is problematic since many liposomes formulations are rapidly destabilized in the gut following interaction with bile salts. Intravenous injection of liposomes is normally followed by interaction with at least two distinct groups of plasma proteins, probably simultaneously. These are (i) the so called opsonin which by, adsorbing on to the surface of vesicles, mediates their endocytosis by the fixed macrophages of reticuloendothelial system (RES) and circulating monocytes and (ii) high density lipoproteins (HDL) which remove the phospholipids molecules from the vesicle bilayers, leading to varying degree of vesicle disintegration and release of encapsulated solutes at rates dependent on the extent of bilayer damage. The RES, presumably through the opsonin on the bilayer surface, intercepts destabilized liposomes and solutes still entrapped.[200]

Prolonged residence time of liposomes in the circulation is required when these are designed to act on non-RES tissues within the vascular system, extravascularly through leaky capillaries or as circulating drug reservoir. All such function would be optimal with longlived small liposomes, especially if the lipid to drug mass ratio can be reduced by using lipid-drug conjugates or by using newly developed techniques that ensure substantial passive drug in aqueous phase.[201]

### 5.1.6. Parameter influence in-vivo behavior of liposomes:

The composition of the lipid bilayer is critically important in determination the pharmaceutical properties of liposomes, mainly through influences on membrane fluidity, permeability and surface properties. Membrane fluidity refers to existence of thermal phase transitions in phospholipid aggregates. As temperature increases these lipids move from a relatively ordered gel stat to a more disordered, fluid like

crystalline state. In gel state, liposomal membrane are more stable, less permeable to solutes and less likely to interact with destabilizing macromolecules than in the lipid crystalline state. The maximum bilayer permeability occurs at the transition temperature (Tm).[202] Lipids have a characteristic phase transition temperature and they exist in different physical states above and below the Tm. The lipids are in a rigid well-ordered arrangement (solid gel-like phase) below the Tm and in a liquid-crystalline (fluid) phase above the Tm. The fluidity of liposome bilayer can be altered by using phospholipids with different Tm which in turn can vary from -20 to 90$^{o}$C depending upon the length and the nature of the fatty acid chain.

Presence of high Tm lipids (Tm > 37$^{o}$C) makes the liposome bilyaer membrane less fluid at physiological temperature and less leaky in contrast liposomes composed of low (Tm < 37$^{o}$C) are more susceptible to leakage of encapsulated in aqueous phase at physiological temperature.[189] The length and degree of saturation of the alkyl chains mainly determine the transition temperature of the membrane.[203]

A wide range of phospholipids and lipids extracted from biological membranes can be used to prepare liposomes or other lipid-based vesicles. Depending on the gel-liquid crystalline transition temperature (Tm) of phospholipids (i.e. the temperature at which the acyl chains melt), liposomes membrane can attain various degree of fluidity at ambient temperature.

This fact can be controlled quite accurately to achieve a wide range of Tm values by using appropriate mixture of two or more phospholipids.[200]

## 5.2. Methodology

### 5.2.1. Materials

Berberine (BER) was purchased from Yucca Enterprise, Mumbai. Soyphsophatidylcholine (SPC, purity, 98%) was provided as a gift sample from Lipoid GmbH Company (Ludwigshafen, Germany). Cholesterol (CHOL) and all other solvents and reagents used were analytical grade and purchased from S D Fine-Chem Ltd (Mumbai, India).

### 5.2.2. Preparation of liposome

Thin film hydration method was used to prepare berberine loaded liposome.[204-206] In this method, SPC (Lipoid S 100), CHOL and BER were firstly dissolved in chloroform in different molar ratio (Table 20). The chloroform was evaporated at 60$^{o}$C for 1 h under vacuum at 150 rpm by rotary evaporator (Remi Instruments, Mumbai, India) to form a thin lipid film. The dried thin lipid film was hydrated by adding phosphate buffer saline (PBS) pH 6.8 at 45$^{o}$ C in rotary vacuum evaporator rotated at 100 rpm until the dispersion of all the lipids in the aqueous phase. For vesicle size reduction, the dispersion was subjected to bath sonication (Toshniwal Instruments, Ajmer) for 20-30 min at a frequency of about 30±3KHz at 40°C. Thereafter, the mixture was kept for 1 h at room temperature for the formation of vesicle followed by 4°C for 24 h in an inert atmosphere. The formulation was taken in centrifuged tube and was centrifuged for 1 h at 15000 rpm in a cooling centrifuge (Remi Instruments, Mumbai, India). Then the supernatant containing the vesicles in each case was separated and was taken for further studies as a suspended formulation for further studies.

### 5.2.3. Experimental design

#### *5.2.3.1. $3^2$ factorial designs*

The formulations were optimized by $3^2$ factorial designs consisting of drug: lipid molar ratio ($X_1$) and SPC: cholesterol ($X_2$) as a dependant variables while vesicle size ($Y_1$) and entrapment efficiency ($Y_2$) as response (Table 5.2). Nine formulations were prepared and evaluated for response. The obtained data were fitted into Design Expert software (Design Expert 9.0.4, Stat-Ease, Minneapolis, MN). Analysis of variance (ANOVA) was used to validate design.

**Table 5.2. Variables in $3^2$ Factorial designs for liposome**

| Factor | Levels [Coded (Actual)] | | |
|---|---|---|---|
| | **Low (-1)** | **Medium (0)** | **High (+1)** |
| **Independent variables** | | | |
| $X_1$ =Drug: Lipid (Molar ratio) | -1 (1:5) | 0 (1:10) | +1 (1:15) |
| $X_2$ = SPC: Cholesterol (% of total lipid) | -1 (70:30) | 0 (60:40) | +1 (50:50) |

#### *5.2.3.2. Response surface plot*

Contour plot and (3D) response surface plots were constructed to establish the understanding of relationship of variables and its interaction.

#### *5.2.3.3. Optimization using desirability function*

The formulations were optimized by keeping the $X_1$ and $X_2$ within the range used in present work while $Y_1$ at minimum and $Y_2$ at maximum using Design-Expert software. On the basis of these assigned goals software determines the possible formulation composition with maximum desirability value.

#### *5.2.3.4. Checkpoint analysis*

According to desirability value and composition of variables, formulation was prepared and evaluated for response. The predicted and observed response was

compared and percentage prediction error was calculated to confirm the validity of design for optimization

### 5.2.4. Characterization of Liposome

#### *5.2.4.1. Morphology of liposome*

Shape and lamellarity of vesicle was observed by placing the suspension under optical microscope (Olympus BX 41, USA). Photomicrographs were taken by a camera attached to the optical microscope in 10 x100 magnifications.

#### *5.2.4.2. Vesicle size*

The optimized formulation, serially diluted 100-fold with double distilled, was used to determine mean vesicle size and polydispersity index (PDI) using Zetasizer HAS 3000 (Malvern instrument Limited, UK).

#### *5.2.4.3. Zeta potential*

Zeta potentials of the optimized formulations was measured by Zetasizer HAS 3000 (Malvern instrument Limited, UK) at 25$^{o}$C.[206]

#### *5.2.4.4. Entrapment efficiency*

Liposome suspension was centrifuge at 15000 rpm to separate un-entrapped drug. Free drug present in supernatant was determined using UV spectrophotometer at 345 nm. EE% was calculated by following equation 5.1:

$$\mathbf{EE\ (\%) = [(C_{total} - C_{free})/C_{total}]x100} \quad \textbf{(Eq. 5.1)}$$

Where, $C_{total}$ = total drug added, $C_{free}$ = unentrapped drug

#### *5.2.4.5. In vitro diffusion study*

Membrane diffusion technique was used to determine release of BER from plain drug suspension and formulation. Liposomal suspension (1.5 mL) with known amount of drug was filled in semi-permeable membrane bag (previously soaked in distilled water for 24 h). The bag was placed in 25 mL of phosphate buffer saline (PBS, pH

6.8), continuously stirred by magnetic stirrer, maintained at 37°C. Samples (1 mL) were withdrawn at specified time interval and substituted with fresh PBS (pH 6.8). UV spectrophotometer was used to determine drug from sample at 345 nm.

### 5.2.5. Stability Study

Berberine loaded liposomes were stored in glass vials and kept at 4-8°C, 25±2°C and 37±2°C for one month. The samples were taken after one month and entrapment efficiency was determined as described earlier.

## 5.3. Results and Discussion

### 5.3.1. Experimental design

The purpose of the factorial design was to identify variables that have significant effect on the dependent variables analyzed. The choice of independent variables was based on previous studies that showed the influence of lipid and cholesterol on the characterization of liposome.[207-209] The three level two factor design is an effective approach for investigating variables at different levels with a limited number of experimental runs (Table 5.3).

**Table 5.3. $3^2$ Factorial designs of independent variables with measured responses**

| Batch | Independent Variables | | Dependent Variables | |
|---|---|---|---|---|
| | $X_1$ | $X_2$ | $Y_1$(nm) | $Y_2$ (%) |
| **BL1** | 1 | 1 | 876 | 82.38 |
| **BL2** | -1 | -1 | 982 | 56.08 |
| **BL3** | 0 | 1 | 642 | 77.13 |
| **BL4** | -1 | 0 | 854 | 67.4 |
| **BL5** | 1 | 0 | 1104 | 80.24 |
| **BL6** | 1 | -1 | 1105 | 75.76 |
| **BL7** | 0 | -1 | 1021 | 69.08 |
| **BL8** | 0 | 0 | 995 | 74.51 |
| **BL9** | -1 | 1 | 571 | 69.24 |

$X_1$ = Drug: Lipid (Molar ratio), $X_2$ = SPC: Cholesterol (% of total lipid)
$Y_1$ = Vesicle size (nm), $Y_2$ = Entrapment efficiency (%)

### *5.3.1.1. Fitting the model to data*

Response data of all formulations were fitted to cubic, linear and quadratic model. According to Design Expert software, best-fitted model was linear for response $Y_1$ (Table 5.4) and quadratic for response $Y_2$ (Table 5.5).

**Table 5.4. Model summary statistics for vesicle size**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| Linear | 83.24 | 0.8572 | 0.8097 | 0.6954 | 88726.01 | |
| 2FI | 81.60 | 0.8857 | 0.8171 | 0.7240 | 80397.37 | |
| **Quadratic** | **34.87** | **0.9875** | **0.9666** | **0.8809** | **34700.17** | **Suggested** |
| Cubic | 45.33 | 0.9929 | 0.9436 | -0.2860 | 3.745E+005 | |

**Table 5.5. Model summary statistics for entrapment efficiency**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| **Linear** | **2.41** | **0.9316** | **0.9089** | **0.8170** | **93.59** | **Suggested** |
| 2FI | 2.20 | 0.9525 | 0.9241 | 0.7165 | 145.01 | |
| Quadratic | 1.47 | 0.9874 | 0.9664 | 0.8563 | 73.49 | |
| Cubic | 1.03 | 0.9979 | 0.9833 | 0.6196 | 194.60 | |

Polynomial equations 5.2 and 5.3 representing the individual main effects and interaction effects of independent factors on each dependent variable are as follows:

**Vesicle size:**

$$Y_1 = 964.78 + 113.X_1 - 169.83X_2 + 45.50X_1X_2 - 29.33X_1^2 - 118.17X_2^2 \quad \text{(Eq. 5.2)}$$

**Entrapment efficiency:**

$$Y_2 = 75.20 + 7.61X_1 + 4.64X_2 - 1.64X_1X_2 - 1.72X_1^2 - 2.44X_2^2 \quad \text{(Eq. 5.3)}$$

The positive coefficients before independent variables of quadratic equation indicate a favorable effect on the responses, while negative coefficient indicates an unfavorable effect on the responses. The quadratic equation for $Y_1$ shows that the

SPC: CHOL ratio ($X_2$) has largest coefficient indicated that the SPC: CHOL ratio was the most influential factor and had a significant and negative effect on $Y_1$. However, there were no significant difference between the $X_1$ and $X_2$ in the entrapment efficiency indicates both term were useful in optimization of entrapment efficiency of formulation. Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert ®software. The results of analysis for observed response is shown in Table 5.6 and 5.7. The effect of each factor was tested using ANOVA test with a corresponding p value. The model is significant for probability > F less than 0.05, while model is not significant for probability > F greater than 0.05. The smaller p value and larger F-value were desired for more significant corresponding coefficients.

The model F-value of 47.31 for $Y_1$ and 47.02 for $Y_2$ indicates the model is significant ($p<0.05$). The resulted $R^2$ for $Y_1$=0.9875 and $Y_2$=0.9874, indicates good correlation. Further Adj-$R^2$ of 0.9666 and Pred-$R^2$ of 0.8809 for $Y_1$, and for $Y_2$ Adj-$R^2$ of 0.9664 and Pred-$R^2$ of 0.8563, were in reasonable agreement, i.e. difference is less than 0.2, indicating that the data were described adequately by the mathematical model. Adequate precision is a measure of the range of a predicted response relative to its associated error, that is, signal to noise ratio. The ratio greater than 4 is desirable for navigating the design space. The ratio of "adequate precision" for $Y_1$ (19.869) and $Y_2$ (20.470) indicating an adequate signal. Values of ''p'' less than 0.05 indicated that model terms were significant except for responses $Y_1$, model terms $X_1^2$ was at $p>0.05$ (p value:0.3197), and for $Y_2$, model term $X_1X_2$, $X_1^2$ and $X_2^2$ was at $p>0.05$ (p value: 0.1119, 0.1949, 0.1001, respectively) indicated necessary model reduction to improve the model. Hence, the reduced polynomial equations for $Y_1$ (Eq. 5.4) and $Y_2$ (Eq. 5.5) were generated by omitting the least contributing factors.

**Vesicle Size: $Y_1 = 984.33 + 113.00\ X_1 - 169.83\ X_2 + 45.50\ X_1 X_2 - 118.17\ X_2^2$ ..... (Eq. 5.4)**

**Entrapment efficiency: $Y_2 = 72.42 + 7.61\ X_1 + 4.64\ X_2$ ..... (Eq. 5.5)**

Further analysis using ANOVA indicated significant effects of the independent factors (p>F) on response $Y_1$ and $Y_2$. The insignificant terms were omitted in the reduced model but no major improvement was observed in entrapment efficiency evident from decrease in F-value from 47.02 to 40.88. Adequate precision value was also lowered in reduced model for entrapment efficiency. Therefore, full quadratic model was chosen for optimization of response. F-value for $Y_1$=53.25 while resulted $R^2$ for $Y_1$=0.9875 indicates more significant model than full model. The p value for reduced model was also less as compared to the full model, which favors the model for optimization of formulation. Therefore, quadratic model was chosen for the select design based on the ANOVA results using model F-value, p value, $R^2$, PRESS and adequate precision confirmed the excellent goodness of fit.

### *5.3.1.2. Contour plot and response surface analysis*

The obtained results can be observed visually in the response surface (3D) and contour plots. Response surface graph of $Y_1$ (Fig.5.4) shows that vesicle size of liposome was decreased with decreasing SPC concentration because phospholipids constitute the liposome membrane. With increasing total lipid (SPC:CHOL) concentration more drug could be incorporate into liposome. In addition, response surface graph of $Y_2$ (Fig.5.5) shows that the increase in SPC:CHOL ratio significantly increased the drug entrapment efficiency. These results supported by the fact that, movement of fatty acids hydrophobic tails was reduced by incorporation of a bulky molecule of cholesterol in the lipid bilayer of liposome. It leads to permeability reduction of liposome membrane via resistance of exchange of phospholipids with apoprotein. These ultimately improve the drug retention in liposome by prevention of drug leakage from lipid bilayer.

**Table 5.6. ANOVA results for full and reduced quadratic model for vesicle size**

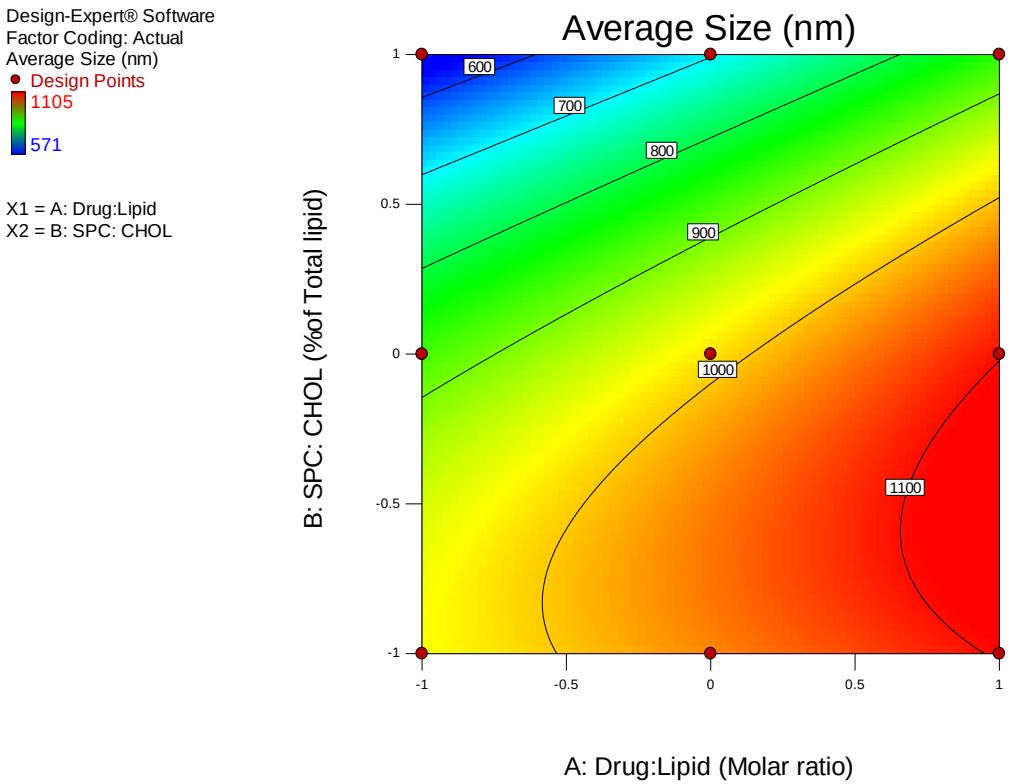
| Source | SS | Df | Mean Square | F Value | Prob > F | SD | Mean | C.V. | PRESS | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adequate precision |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Full Model** | | | | | | | | | | | | | |
| Model | 287600 | 5 | 57520.56 | 47.31 | 0.0047 | 34.87 | 905.56 | 3.85 | 34700.17 | 0.9875 | 0.9666 | 0.8809 | 19.869 |
| $X_1$-Drug:Lipid | 76614.00 | 1 | 76614.00 | 63.01 | 0.0042 | | | | | | | | |
| $X_2$-SPC:CHO | 173100 | 1 | 173100 | 142.34 | 0.0013 | | | | | | | | |
| $X_1X_2$ | 8281.00 | 1 | 8281.00 | 6.81 | 0.0797 | | | | | | | | |
| $X_1^2$ | 1720.89 | 1 | 1720.89 | 1.42 | 0.3197 | | | | | | | | |
| $X_2^2$ | 27926.72 | 1 | 27926.72 | 22.97 | 0.0173 | | | | | | | | |
| Residual | 3647.44 | 3 | 1215.81 | | | | | | | | | | |
| Cor Total | 291300 | 8 | | | | | | | | | | | |
| **Reduced Model** | | | | | | | | | | | | | |
| Model | 285900 | 4 | 71470.47 | 53.25 | 0.0010 | 36.63 | 905.56 | 4.05 | 36504.94 | 0.9816 | 0.9631 | 0.8747 | 20.716 |
| $X_1$-Drug:Lipid | 76614.00 | 1 | 76614.00 | 57.09 | 0.0016 | | | | | | | | |
| $X_2$-SPC:CHO | 173100 | 1 | 173100 | 128.95 | 0.0003 | | | | | | | | |
| $X_1X_2$ | 8281.00 | 1 | 8281.00 | 6.17 | 0.0679 | | | | | | | | |
| $X_2^2$ | 27926.72 | 1 | 27926.72 | 20.81 | 0.0103 | | | | | | | | |
| Residual | 5368.33 | 4 | 1342.08 | | | | | | | | | | |
| Cor Total | 291300 | 8 | | | | | | | | | | | |

SS=sum of squares; df=degree of freedom; MS=mean of squares; Prob>F=probability; SD=standard deviation; C.V.=coefficient of variation; Adj-$R^2$= adjusted $R^2$; Pred- $R^2$=predicted $R^2$; Adeq=adequate; PRESS=predicted residual error sum of squares.

**Table 5.7. ANOVA results for full and reduced quadratic model for entrapment efficiency**

| Source | SS | Df | Mean Square | F Value | Prob > F | SD | Mean | C.V. | PRESS | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adequate precision |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Full Model** | | | | | | | | | | | | | |
| Model | 505.08 | 5 | 101.02 | 47.02 | 0.0047 | 1.47 | 72.42 | 2.02 | 73.49 | 0.9874 | 0.9664 | 0.8563 | 20.470 |
| $X_1$-Drug:Lipid | 347.47 | 1 | 347.47 | 161.75 | 0.0010 | | | | | | | | |
| $X_2$-SPC:CHO | 129.08 | 1 | 129.08 | 60.09 | 0.0045 | | | | | | | | |
| $X_1X_2$ | 10.69 | 1 | 10.69 | 4.98 | 0.1119 | | | | | | | | |
| $X_1^2$ | 5.94 | 1 | 5.94 | 2.76 | 0.1949 | | | | | | | | |
| $X_2^2$ | 11.89 | 1 | 11.89 | 5.54 | 0.1001 | | | | | | | | |
| Residual | 6.44 | 3 | 2.15 | | | | | | | | | | |
| Cor Total | 511.53 | 8 | | | | | | | | | | | |
| **Reduced Model** | | | | | | | | | | | | | |
| Model | 476.56 | 2 | 238.28 | 40.88 | 0.0003 | 2.41 | 72.42 | 3.33 | 93.59 | 0.9316 | 0.9089 | 0.8170 | 17.575 |
| $X_1$-Drug:Lipid | 347.47 | 1 | 347.47 | 59.62 | 0.0002 | | | | | | | | |
| $X_2$-SPC:CHO | 129.08 | 1 | 129.08 | 22.15 | 0.0033 | | | | | | | | |
| Residual | 34.97 | 6 | 5.83 | | | | | | | | | | |
| Cor Total | 511.53 | 8 | | | | | | | | | | | |

SS=sum of squares; df=degree of freedom; MS=mean of squares; Prob>F=probability; SD=standard deviation; C.V.=coefficient of variation; Adj-$R^2$= adjusted $R^2$; Pred- $R^2$=predicted $R^2$; Adeq=adequate; PRESS=predicted residual error sum of squares.



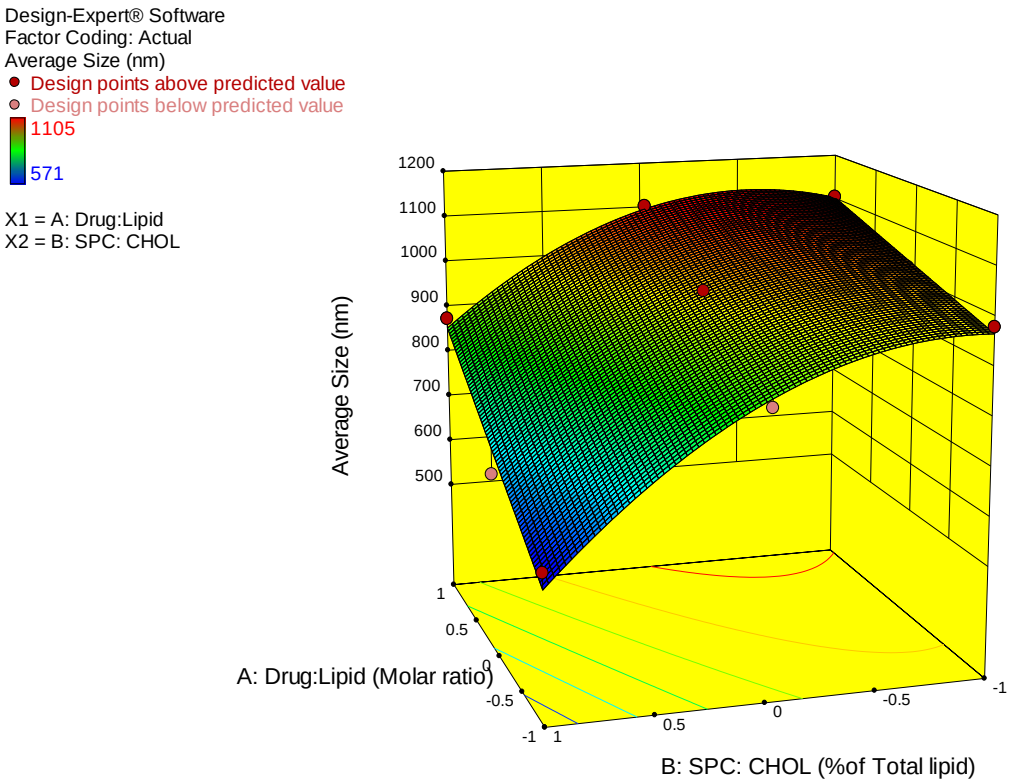




**Figure 5.4. Contour plot and its Response surface shows effect of $X_1$ and $X_2$ on vesicle size**

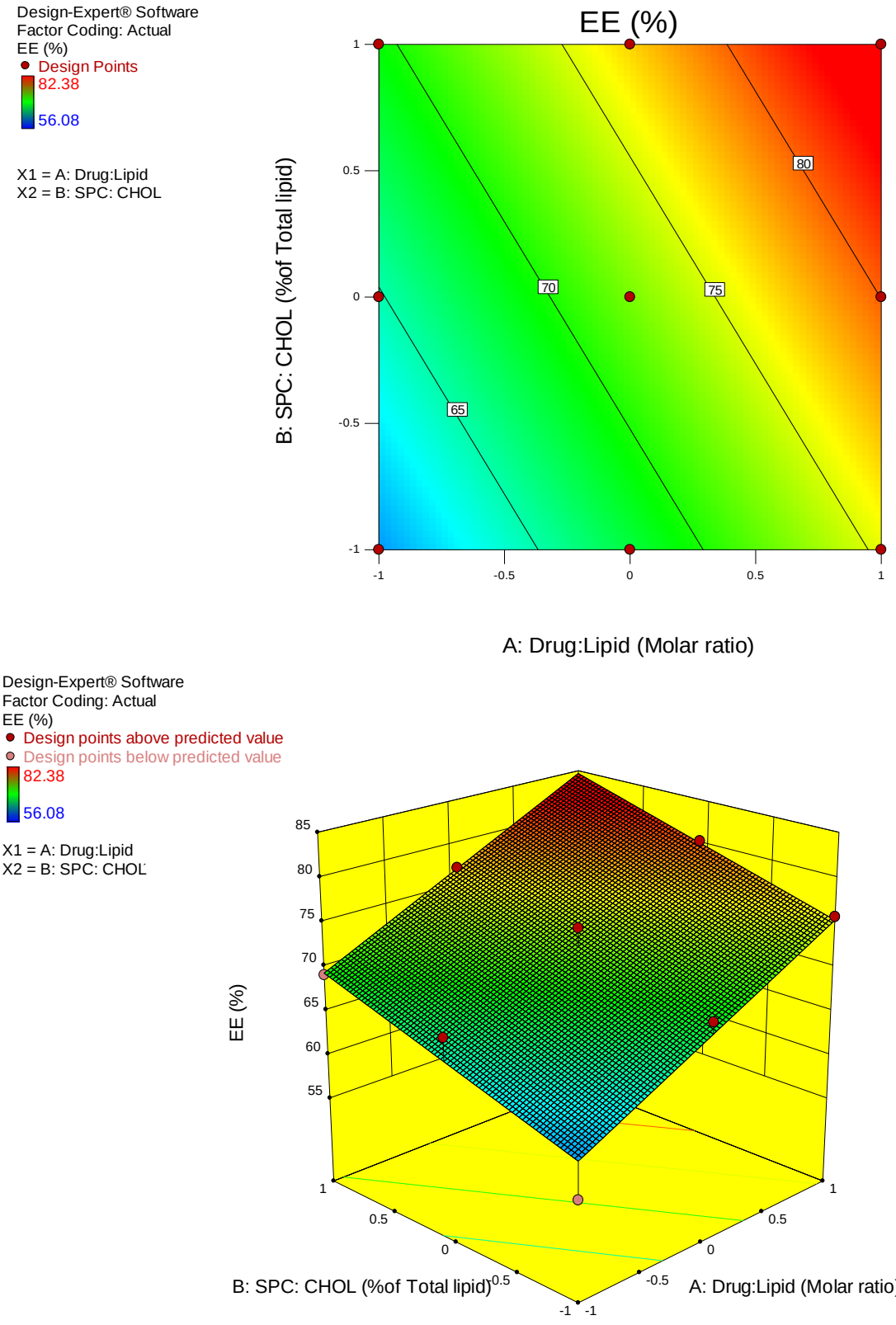



**Figure 5.5. Contour plot and its Response surface shows effect of $X_1$ and $X_2$ on Entrapment efficiency**

### *5.3.1.3. Optimization of formulation*

The search for the optimized formulation composition was carried out using the desirability function approach with Design expert software, criterion being one having the maximum desirability value. The optimization process was performed by setting the $Y_1$ at minimum and $Y_2$ at maximum while $X_1$ and $X_2$within the range obtained. The optimized formulation was achieved at $X_1$=1:9.56, $X_2$=50:50 with the corresponding desirability *(D)* value of 0.782 (Fig.5.6). This factor level combination predicted the responses $Y_1$=654 nm, $Y_2$= 75.68%.

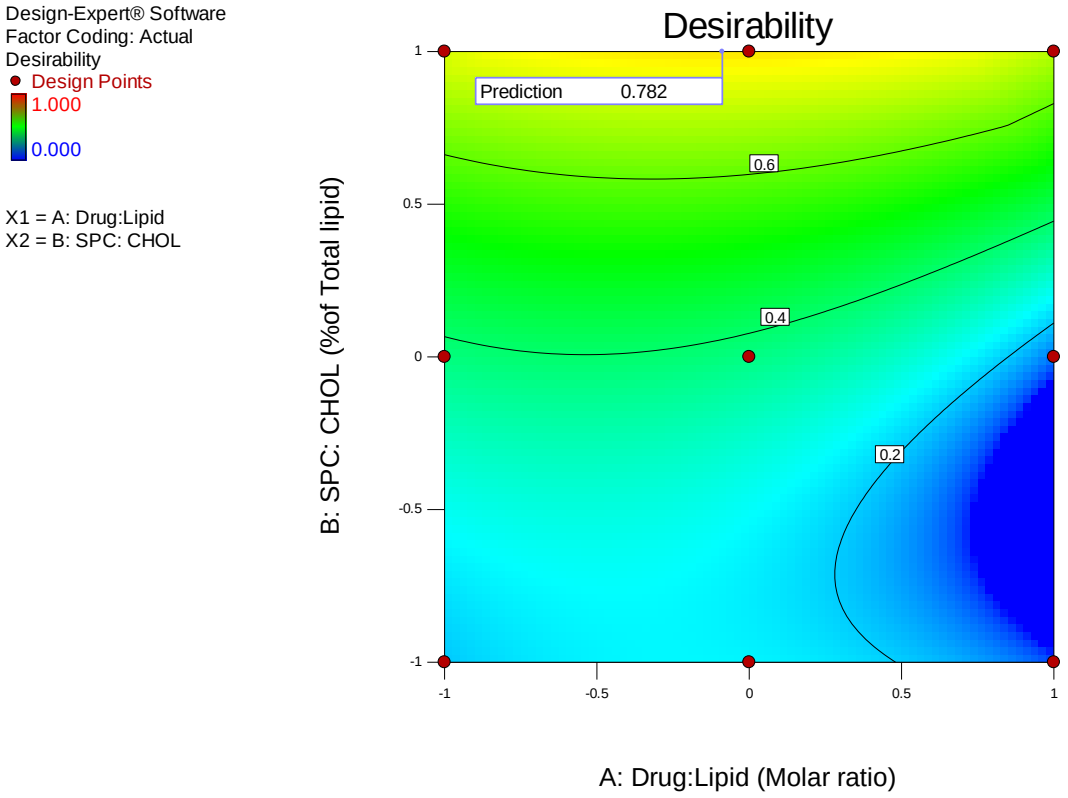



**Figure 5.6. Contour plot for overall desirability of liposome as a function of $X_1$ and $X_2$**

#### *5.3.1.4. Checkpoint Analysis*

The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of liposome formulations (Table 5.8).

**Table 5.8. Checkpoint batch with their predicted and observed value of responses**

| | Independent Variables | | Vesicle size ($Y_1$) (nm) | | Entrapment efficiency ($Y_2$) (%) | |
|---|---|---|---|---|---|---|
| **Batch** | **$X_1$** | **$X_2$** | **Observed** | **Predicted** | **Observed** | **Predicted** |
| BL10 | -0.089 (1:9.56) | +1 (50:50) | 648 | 654 | 77.91 | 75.68 |
| **Percentage prediction error (%)** | | | -0.92 | | +2.86 | |

### 5.3.2. Characterization of Optimized Formulation

#### *5.3.2.1. Vesicle size and shape*

Vesicle size determination is essential parameters for application of liposome.[210] Several methods are available for preparation of liposome with different size, composed of one or more lipid bilayer. Generally, lipid film hydration is used for preparation of multilamellar vesicles. Sonication was done to produce small unilamellar vesicle. The optimized liposome (BL 10) was spherical in shape and found to be unilamellar to multilamellar (Fig. 5.7). The average vesicle size was found to be 0.823 nm with 0.354 polidispersity index (Fig. 5.8).

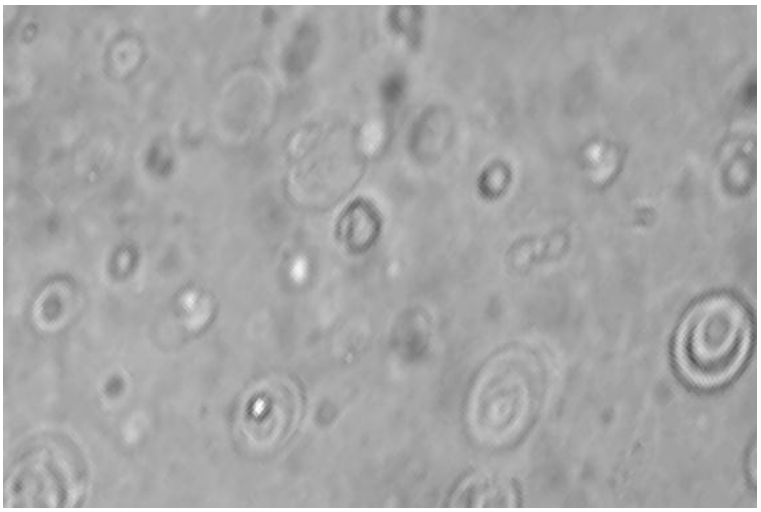


**Figure 5.7. Microscopy of optimized liposome (BL10)**

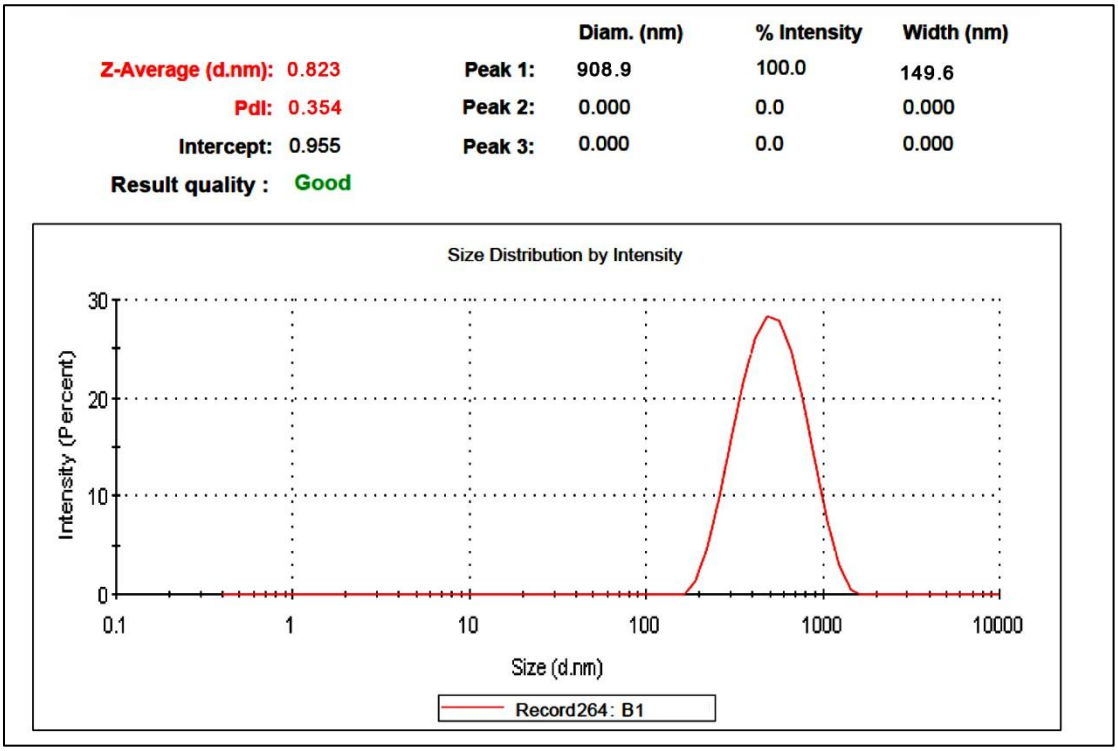



**Figure 5.8. Particle size of optimized liposome (BL10)**

### *5.3.2.2. Zeta potential*

Zeta potential of liposome ensures stability and entrapment efficiency and also used to predict *in vivo* behavior.[210] Entrapment efficiency was increased due to electrostatic attraction between charged molecule and liposomes. Any subsequent modifications of the

liposomal surface, such as cholesterol incorporation, also influence zeta potential. The higher values of zeta potential enhance the stability of liposome by increasing the repulsion of vesicle, and thereby preventing aggregation. Liposome prepared by using different lipids acquires different surface charge. Liposome employing pohosphatidylserine, stearylamine or dioleoyl trimethylammonium propane and phosphatidylcholine get negative, positive and neutral charge respectively.[211] On the contrary, in present study liposome prepared with phosphatidylcholine possess slightly negative charge (-1.93) (Fig. 5.9). It may be due to the effect of cholesterol on surface charge.

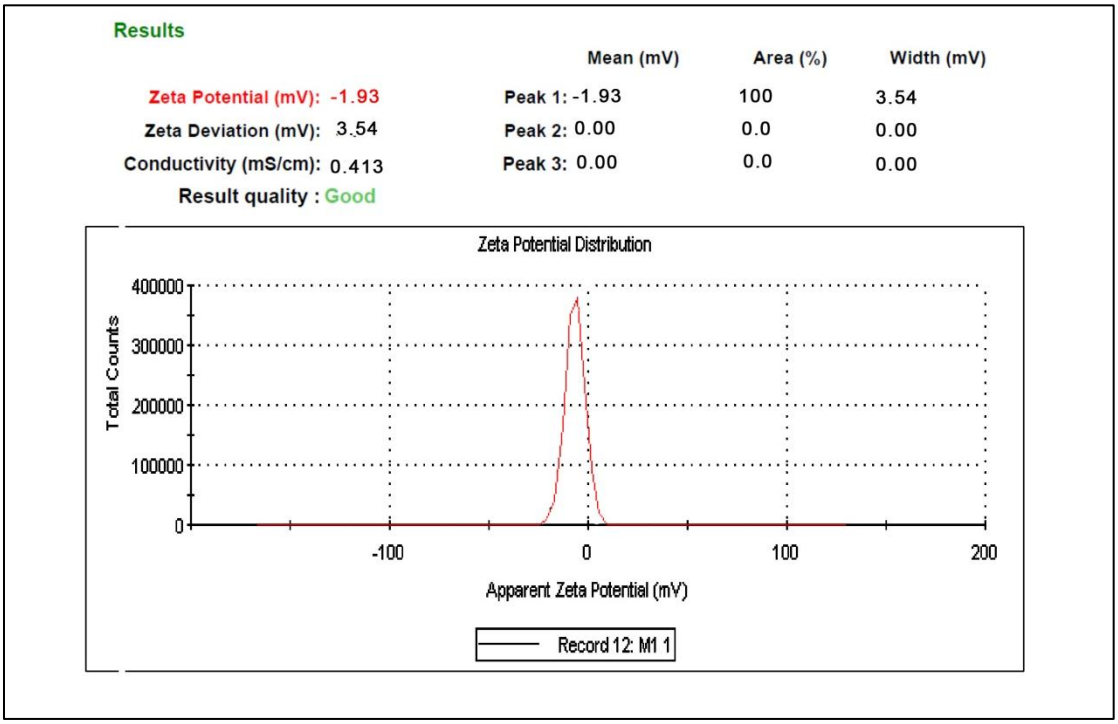



**Figure 5.9. Zeta potential of optimized liposome**

### *5.3.2.3. Entrapment efficiency*

Drug can be incorporated into liposome by several ways depending on various properties like polarity and solubility. It can be adsorbed on surface of membrane, entrapped in lipid

bilayer, encapsulated in inner aqueous core, attached between polar head or supported by a hydrophobic tail.[212] Method of preparation and composition of lipid can also influence the entrapment efficiency. The present study shows 78.43% entrapment efficiency indicating good electrostatic interaction between bioactive agent and liposome.

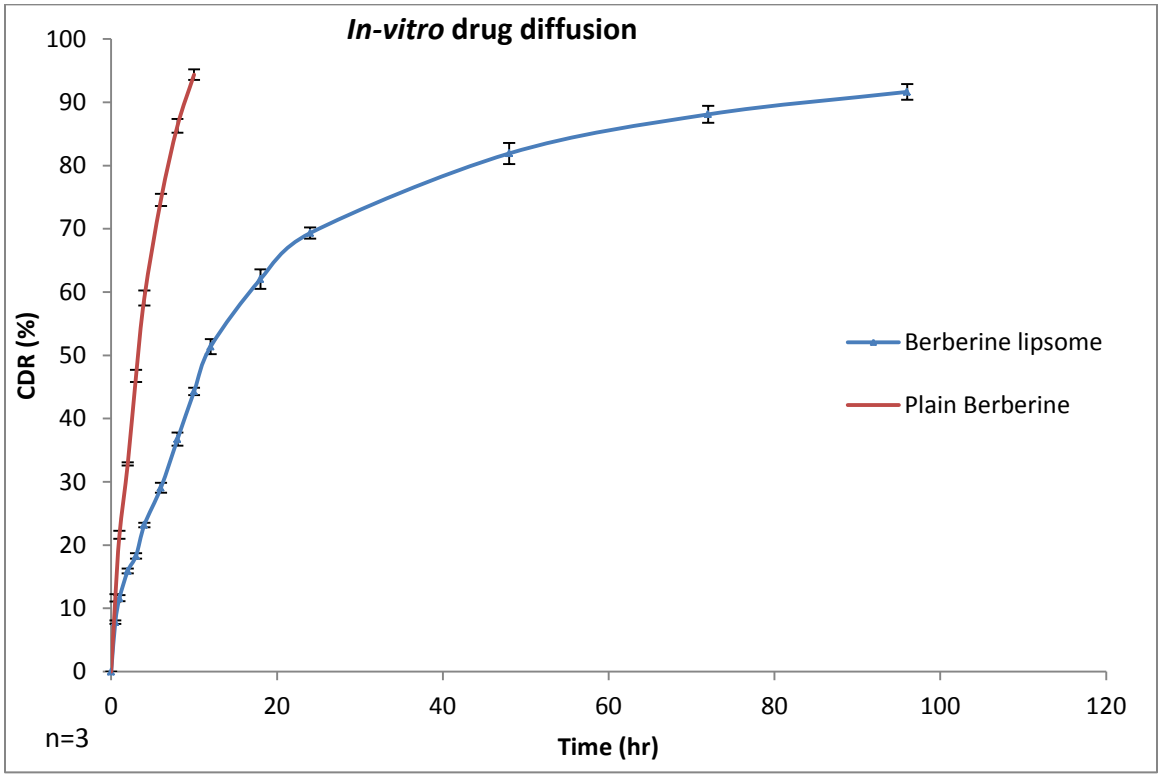



**Figure 5.10. *In vitro* drug diffusion of berberine loaded liposome and plain drug**

### *5.3.2.4. In vitro diffusion study*

Release characteristics of BER from liposome was evaluated *in vitro* and compared to that of pure drug. The result of release study showed that release of BER suspension was completed within 10 h while for liposomal formulations release was continued up to 24 h (Fig. 5.10). About 70% of drug was released within 24 h. This results supported support by the fact that the layer of drug-encapsulated liposomes attached to the semi-permeable membrane breaks and leaches its contents slowly before another layer replaces the

leached vesicles. Due to this mechanism controlled release of drug in liposomes can be expected over a prolonged period of time.

### 5.3.3. Stability Study

Stability study reveals considerable drug loss (approx 12%), was marked from formulation storage at high temperature, i.e., 37±2°C. On contrary, formulation stored at 4-8°C and 25±2°C, could retain 93% and 97% of the entrapped drug, respectively. Substantial loss of drug at high temp may be due to the deprivation of phsopholipids leads to disturbance in packing of membrane. In addition, high temperature also cause change in gel to liquid transition of lipid bilayer. Thus, the study showed that development of liposome with BER can overcome the limitation of the molecule related to poor oral absorption and can enhance the bioactivity of the BER.

# 6. PREPARATION AND CHARACTERIZATION OF SOLID LIPID NANOPARTICLE

## 6.1. Introduction

Oral delivery of drugs incorporated in solid lipid nanoparticle (SLN) has gained considerable interest since last two decades. As they are derived from physiologically compatible lipids, SLN represent a safe and effective alternative in comparison to the conventional polymeric nanoparticles. Solid lipid nanoparticles generally are spherical in shape and are comprised of a solid lipid core stabilized by a surfactant interfacial region. In theory, solid lipid nanoparticles combine the advantages of lipid emulsion systems and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that plague the aforementioned approaches. Utilizing biological lipids is theorized to minimize carrier cytotoxicity, and the solid state of the lipid is theorized to permit more controlled drug release due to increased mass transfer resistance.

Oral delivery of drugs incorporated in SLN has gained considerable interest since last two decades. As they are derived from physiologically compatible lipids, SLNs have been reported as an alternative drug delivery system to traditional polymeric nanoparticles. In theory, solid lipid nanoparticles combine the advantages of lipid emulsion systems and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that plague the aforementioned approaches. The use of solid lipids was introduced to lower drug mobility observed with liquid lipids. Reduction in mobility inhibits drug leakage and also counteracts drug migration into the emulsifier film. The solid core of the colloidal carrier provides better physicochemical stability. A

few of the advantages that the solid lipid imparts to the carrier system are outlined in Table 6.1.

**Table 6.1. Advantages of using a solid lipid in the manufacture of colloidal drug carriers**[213]

| |
|---|
| **Solid core** |
| Enhanced physicochemical stability of colloidal carrier |
| Enhanced chemical stability of encapsulated drug molecules |
| **Reduced mobility of drug molecules** |
| Enhanced mechanical stability |
| Increase in electrochemical stability due to reduction in diffusing drug molecules (which otherwise decrease stability) |
| Prevention of drug leakage |
| Sustained-release of drugs |
| **Static emulsifier-particle interface** |
| Facilitates surface modification |
| Facilitates drug targeting |

Solid lipid nanoparticles typically are spherical with average diameters between 50 to 500 nanometers. Solid lipid nanoparticles possess a solid lipid core matrix that can solubilize lipophilic molecules. The lipid core is stabilized by surfactants (emulsifiers). The core lipids can be fatty acids, acylglycerols, waxes, and mixtures of the same. Biological membrane lipids such as phospholipids, sphingomyelins, bile salts such as sodium taurocholate, sterols such as cholesterol, and mixtures of the same are utilized as surfactant stabilizers. Polyethylene glycol incorporation can provide stearic stabilization and inhibit immune clearance.[122] Ligands can be conjugated to nanoparticles to promote tissue targeting.[214] For pharmaceutical applications, all formulation excipients must have Generally Recognized as Safe (GRAS) status.[215] Figure 6.1 illustrates the theoretical

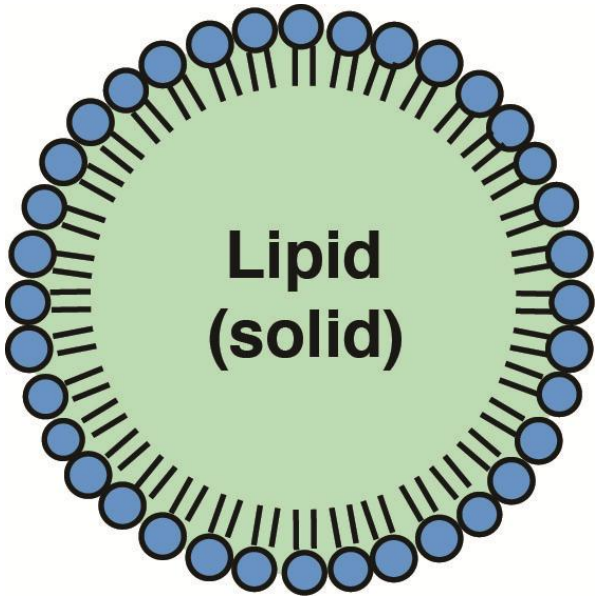



**Figure 6.1. Structure of Solid Lipid Nanoparticle**

structure of a single solid lipid nanoparticle. In this schematic, solid lipid forms the core, surfactant stabilizes the interface, and the model drug is solubilized by the lipid core. SLNs potentially emphasize the benefits of colloidal carriers while reducing the probable shortcomings associated with them. Several potential advantages associated with SLNs are listed in Table 6.1. SLNs have been widely studied for delivery of drugs through dermal, peroral, parenteral, ocular, pulmonary and rectal routes.[213]

### 6.1.1. Composition of SLNs

To achieve and maintain a solid lipid particle upon administration, the lipid nanoparticles melting point must exceed body temperature (37°C). High melting point lipids investigated include triacylglycerols (triglycerides), acylglycerols, fatty acids, steroids, waxes, and combinations thereof. Surfactants investigated include biological membrane lipids such as lecithin, bile salts such as sodium taurocholate, biocompatible nonionics such as ethylene oxide/propylene oxide copolymerrs, sorbitan esters, fatty acid ethoxylates, and mixtures thereof.[122] Table 6.2 identifies the types of lipids and surfactants reported in solid lipid nanoparticles formulations.

**Table 6.2. Lipids and surfactants used in solid lipid nanoparticle production**[122]

| Lipids | Surfactants |
|---|---|
| *Triacylglycerols* | *Phospholipids* |
| Tricaprin | Egg lecithin |
| Trilaurin | Soy lecithin |
| Trimyristin | Phosphatidylcholine |
| Tripalmitin | *Ethylene oxide/propylene oxide copolymers* |
| Tristearin | Poloxamer 188 |
| *Acylglycerols* | Poloxamer 182 |
| Glycerol monostearate | Poloxamer 407 |
| Glycerol behenate | Poloxamine 908 |
| Glycerol palmitostearate | *Sorbitan ethylene oxide/propylene oxide copolymers* |
| *Fatty acids* | Polysorbate 20 |
| Stearic acid | Polysorbate 60 |
| Palmitic acid | Polysorbate 80 |
| Decanoic acid | *Alkylaryl polyether alcohol polymers'* |
| Behenic acid | Tyloxapol |
| *Waxes* | *Bile salts* |
| Cetyl palmitate | Sodium cholate |
| *Cyclic complexes* | Sodium glycocholate |
| Cyclodextrin | Sodium taurocholate |
| *para*-acyl-calix-arenes | Sodium taurodeoxycholate |
| | *Alcohols* |
| | Ethanol |
| | Butanol |

### 6.1.2. Structure of solid lipid nanoparticles

Many different drugs, mainly lipophilic, have been incorporated into solid lipid nanoparticles such as immune-suppressants, corticosteroids, anticancer etc. Incorporated

drug molecules interact in specific way with carrier system according to its properties. The drug loading capacity (the amount of incorporated drug related to content of matrix lipid or to the content of dispersed material) of solid lipid nanoparticles depend upon suitability of drug molecule with carrier system.

Factor affecting loading capacity of drug in the lipid are:[216]

- Solubility of drug in melted lipid
- Miscibility of drug melt and lipid melt
- Chemical and physical structure of solid matrix lipid
- Polymorphic state of lipid material

The prime concern for high loading capacity is the solubility of drug substance in the lipid melts. Conversely, enhancement in aqueous solubility of drug leads lower to entrapment efficiency. For this reason, Müller *et al.*[217, 218] reported a cold homogenization technique which is performed at room temperature or below (0°C). Therefore, solubility of the drug is also an important factor for choosing the production method of SLN. While the hot homogenization technique is much more suitable for lipophilic drugs, the cold homogenization technique is employed for hydrophilic drugs in order to reach the highest payload and to prevent drug partition to the aqueous phase during SLN production.

In general, solid lipid nanoparticles have three different models based on the location of the incorporated drug molecule (Fig. 6.2).

- Solid solution model
- Drug enriched shell model
- Drug enriched core model

### *6.1.2.1. Solid solution model*

A solid solution model, also referred to as the homogenous matrix model, is obtained when the drug is homogenously dispersed within the lipid matrix in molecules or amorphous clusters. This model is usually described for SLN prepared by the cold homogenization techniques and using no surfactant or drug solubilizing surfactant.

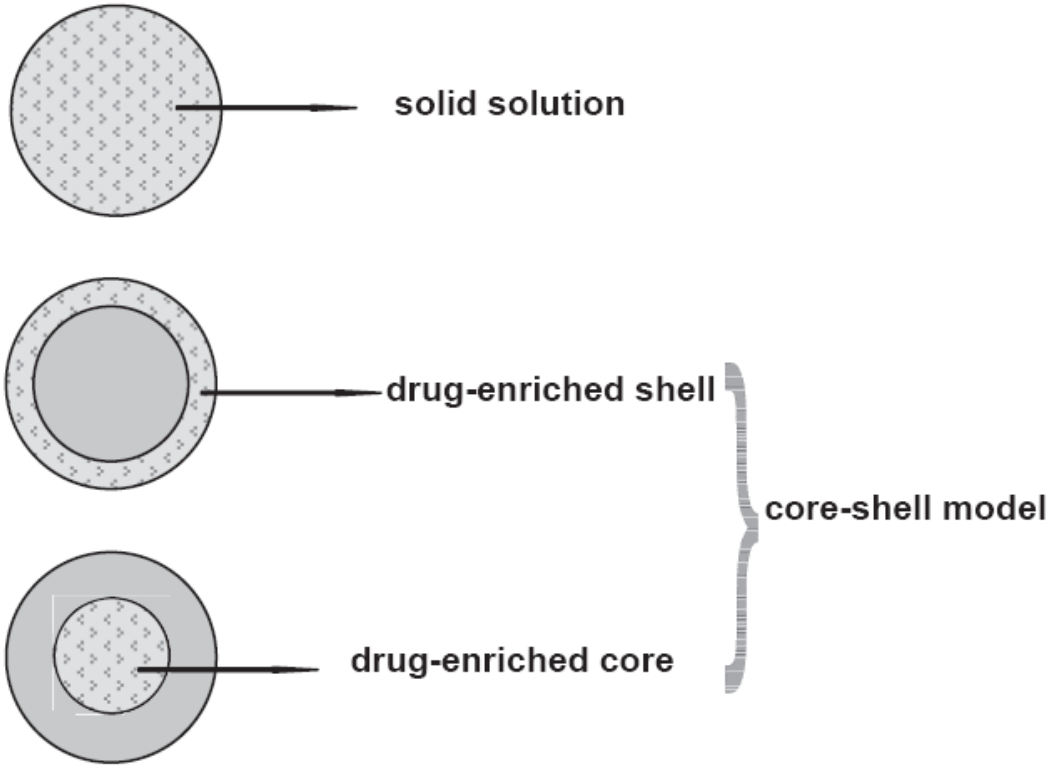



**Figure 6.2. Models of drug incorporation into SLN:** homogeneous matrix of solid solution (upper), drug-free core with drug-enriched shell (middle), drug-enriched core with lipid shell (lower).

### *6.1.2.2. Drug-enriched shell model*

The drug enriched shell is a lipid core enclosed by a drug-enriched outer shell. Such a structure is obtained when there is re-partitioning of the drug to the lipid phase during cooling of the obtained nanoemulsion in the hot homogenization techniques.

#### *6.1.2.3. Drug-enriched core model*

The drug-enriched core model is obtained when the drug precipitates first, before recrystallization of the lipids. The drug is solubilized in the lipid melt at or close to its saturation solubility. Subsequent cooling of the lipid emulsion cause super-saturation of the drug in the lipid melts and ultimately leads to the drug crystallization prior to lipid crystallization. Further cooling will finally lead to lipid recrystallization that forms a membrane around the already crystallized drug-enriched core. This structural model is suitable for drug that requires prolonged release over a period of time, governed by Fick's law of diffusion.[219]

### 6.1.3. Production techniques

Several approaches for the preparation solid lipid nanoparticles have been reported since early 1990s when it was first described. The techniques for the preparation of formulation has significant role in its performance and it may be influenced by:

- Physicochemical properties of drug to be incorporated
- Stability of drug to be incorporated
- Desired particle characteristics of formulation
- Stability of formulation
- Availability of production techniques

A brief description of several methods extensively used for the preparation of SLN is described in the literature:

- High pressure homogenization[220-222]
- Breaking of o/w microemulsion[223-225]
- Solvent emulsification- evaporation or diffusion[226-229]

- Solvent injection[230]
- High shear homogenization[231] and/or ultrasound dispersion[232]
- Double emulsification (w/o/w)[226, 233]
- Membrane contactor[234]

## 6.2. Methodology

### 6.2.1. Materials

All the analytical solvent such as methanol, chloroform, dichloromethane used in the study was purchased from Loba Chemie Pvt. Ltd., Mumbai. Stearic acid and polyvinyl alcohol was also procured from Loba Chemie Pvt. Ltd., Mumbai. Dialysis bag (Mw cut-off = 12000-14000) used for *in vitro* drug release was obtained from Himedia laboratories, Mumbai.

### 6.2.2. Preparation of solid lipid nanoparticle (SLN)

The SLN were fabricated using solvent injection method. Stearic acid (SA) and drug (20 mg) were dissolved in 5 mL solvent mixture consisting of dichloromethane: methanol (2:3). This organic phase was rapidly injected through an injection needle into stirred (1500 rpm) aqueous phase (100 mL) with PVA solution containing 0.125% v/v Tween 80. The dispersion was continuously stirred for 2 h at room temperature (25°C) and kept overnight for complete evaporation of solvent. Finally, the dispersion was filtered with a paper filter to remove any excess lipid and used as such for further analysis.

### 6.2.3. Experimental design

#### *6.2.3.1. $3^2$ factorial designs*

The SLN were prepared with stearic acid by solvent injection method. The formulations were optimized by $3^2$ factorial designs consisting of polyvinyl alcohol (PVA)

concentration ($X_1$) and amount of lipid ($X_2$) as a independant variables while particle size ($Y_1$) and entrapment efficiency ($Y_2$) as response (Table 6.3). Nine formulations were prepared and evaluated for response. The obtained data were fitted into Design Expert software (Design Expert 9.0.4, Stat-Ease, Minneapolis, MN). Analysis of variance (ANOVA) was used to validate design.

**Table 6.3. Variables in $3^2$ Factorial designs for SLN**

| Factor | Level used, Actual (Coded) | | |
|---|---|---|---|
| | Low (-1) | Medium (0) | High (+1) |
| **Independent variables** | | | |
| $X_1$ =PVA concentration (%w/v) | 0.25 (-1) | 0.5 (0) | 1 (+1) |
| $X_2$ = Amount of lipid (mg) | 200 (-1) | 300 (0) | 400 (+1) |

### *6.2.3.2. Response surface plot*

The models were presented as contour plot and three dimensional (3D) response surface graphs. These plots were used to establish the relationship between independent variables and dependent variables (responses).

### *6.2.3.3. Optimization using desirability function*

All the responses were simultaneously optimized by a desirability function using Design-Expert software. In the desirability function approach, the formulations were optimized by keeping the $X_1$ and $X_2$ within the range used in present work while $Y_1$ at minimum and $Y_2$ at maximum. On the basis of these assigned goals software determines the possible formulation composition with maximum desirability value.

### *6.2.3.4. Checkpoint analysis*

According to desirability value and composition of variables, formulation was prepared and evaluated for response. The predicted and observed response was compared and

percentage prediction error was calculated to confirm the validity of design for optimization.

### 6.2.4. Characterization of optimized formulation

#### *6.2.4.1. Total drug content*

Total amount of drug in formulation was determined by dissolving 1 mL of suspension in 10 mL of methanol. The amount of berberine in each sample was determined by UV spectrophotometer. Formulation without drugs was treated similarly for the preparation of blank for UV absorbance. The total drug content was calculated by using the equation 6.1 given below.

**Total Drug Content= Conc. x dil. factor x vol. of formulation ……. (Eq. 6.1)**

#### *6.2.4.2. Entrapment efficiency and drug loading*

The entrapment efficiency (EE) was determined by determining the free drug content in supernatant obtained after centrifuging of SLN suspension in high speed centrifuge at 16000 rpm for 30 min at 0°C using Remi cooling centrifuge (Remi Instruments, Mumbai, India). The %EE and drug loading (%DL) were calculated using equation 6.2 and 6.3 respectively, which are as follows:

$$EE\ (\%) = \frac{\text{Total drug (assay)-free drug}}{\text{Total Drug}} \times 100 \quad \text{..... (Eq. 6.2)}$$

$$DL\ (\%) = \frac{\text{Total drug-free drug}}{\text{Total lipid}} \times 100 \quad \text{..... (Eq. 6.3)}$$

#### *6.2.4.3. Particle size and zeta potential*

The particle size and surface charge (zeta potential) of the formulation were determined by photon correlation spectroscopy using Zetatrac (Microtrac Inc., USA).

#### *6.2.4.4. FTIR and X-ray diffraction (XRD)*

The interaction between the lipids and drug was identified from the fourier transform-infrared (FTIR) studies. FTIR spectra of BER, BER loaded SLN, PVA and stearic acid were recorded in infrared spectrophotometer (IR affinity-1, Shimadzu, Japan). The samples were mixed with dry powdered potassium bromide and compressed under pressure. The disk was scanned over the range of 4000-400 $cm^{-1}$.

X-ray diffraction study was done to check the crystalline properties of formulation using X-ray diffractometer. The analysis of blank and drug loaded solid lipid nanoparticle was performed at ambient temperature. The sample was filled in a copper holder and exposed to Cu K-α radiation (40 KV x 40 mA) in X-ray diffractometer (Xpert PRO MPD, Panalytical, Netherland). The sample was scanned between the angular ranges of 5 to $40^{o}$ two theta.

#### *6.2.4.5. Surface morphology*

The shape and surface morphology of optimized formulation was observed by scanning electron microscopy (Zeiss Ultra 55 SEM, Carl Zeiss, Germany). Briefly, sample was suspended in distilled water and the dispersion was mounted on a metal strip. The sample was dried on hot plate and examined under different magnification. The images obtained were recorded.

#### *6.2.4.6. In vitro drug release*

Dialysis bag methods were used for studying *in vitro* release studies were performed in pH 6.8 phosphate buffer. The suspension (5 mL) was placed inside the dialysis bag, tied at both ends and immersed in the dissolution medium with continuous stirring at 100 rpm using magnetic bead at 37 ± 0.2°C. An aliquot (2 mL) were withdrawn at predetermined

time intervals (0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24 h) and substituted by an equal volume of fresh dissolution medium. The samples were analyzed for drug content by UV spectrophotometer after suitable dilution. The concentration of berberine in test samples was calculated by using the regression equation of the calibration curve.

### 6.2.5. Stability study

The stability study for optimized formulation was done as per ICH guidelines. The formulation was kept in screw capped glass container at subfreezing temperature 2-8°C, at room temperature 25°C ± 2°C (60% ± 5% RH) and at an elevated temperature 40 °C ± 2°C (75% ± 5% RH) for a period of 30 days. The samples were analyzed for physical appearance, drug content and drug release at regular interval of 15 days.

## 6.3. Results and Discussion

### 6.3.1. Preparation of SLN by solvent injection method

The solvent injection method is a well-established technique modified from solvent diffusion techniques.[235, 236] The BER loaded SLNs were successfully prepared using stearic acid as a lipid, Tween 80 as a surfactant, PVA as a stabililizer and dichloromethane: methanol as an organic solvent. The advantages of this method are the use of pharmaceutically acceptable organic solvents, avoidance of high pressure homogenization, easy handling and less time consuming techniques without use of sophisticated equipments.[230]

### 6.3.2. Experimental design

The purpose of the factorial design was to identify variables that have significant effect on the dependent variables analyzed. The choice of independent variables was based on previous studies that showed the influence of PVA and stearic acid on the lipid

nanoparticle characterization. The three level two factor design is an effective approach for investigating variables at different levels with a limited number of experimental runs. The vesicle size and entrapment efficiency were found to be in the range of 385 to 867 nm and 70.5% to 87.71%, respectively (Table 6.4).

**Table 6.4. $3^2$ Factorial designs of independent variables with measured responses**

| Batch | Independent Variables | | Dependent Variables | |
|---|---|---|---|---|
| | $X_1$ | $X_2$ | $Y_1$ (nm) | $Y_2$ (%) |
| **BN1** | -1 | -1 | 51.31 | 584 |
| **BN2** | 0 | -1 | 64.73 | 413 |
| **BN3** | 1 | -1 | 73.18 | 377 |
| **BN4** | -1 | 0 | 74.86 | 732 |
| **BN5** | 0 | 0 | 78.45 | 495 |
| **BN6** | 1 | 0 | 83.98 | 417 |
| **BN7** | -1 | 1 | 75.28 | 867 |
| **BN8** | 0 | 1 | 77.84 | 536 |
| **BN9** | 1 | 1 | 81.46 | 432 |

$X_1$ = PVA concentration (%w/v), $X_2$ = Amount of lipid (mg)

$Y_1$ = Particle size (nm), $Y_2$ = Entrapment efficiency (%)

### *6.3.2.1. Fitting Data to Model*

All the responses observed for 9 formulations prepared were simultaneously fitted to linear, cubic and quadratic models using Design Expert software. It was observed that the best-fitted model was quadratic for response $Y_1$and $Y_2$ (Table 6.5, 6.6).

**Table 6.5. Model summary statistics for particle size**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| Linear | 69.75 | 0.8656 | 0.8208 | 0.6515 | 75719.22 | |
| 2FI | 56.92 | 0.9254 | 0.8807 | 0.7457 | 55255.57 | |
| **Quadratic** | **16.03** | **0.9965** | **0.9905** | **0.9579** | **9145.29** | **Suggested** |
| Cubic | 7.33 | 0.9998 | 0.9980 | 0.9549 | 9801.00 | |

**Table 6.6. Model summary statistics for entrapment efficiency**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| Linear | 6.02 | 0.7247 | 0.6329 | 0.3174 | 539.89 | |
| 2FI | 5.59 | 0.8025 | 0.6840 | 0.1606 | 663.94 | |
| **Quadratic** | **2.06** | **0.9839** | **0.9571** | **0.8138** | **147.25** | **Suggested** |
| Cubic | 1.30 | 0.9979 | 0.9830 | 0.6116 | 307.21 | |

Polynomial equations 6.4 and 6.5 representing the individual main effects and interaction effects of independent factors on each dependent variable are as follows:

**Particle size:**

$$Y_1 = 490.11 - 159.50X_1 + 76.83\,X_2 - 57.00X_1\,X_2 + 86.83X_1^2 - 13.17X_2^2 \quad \text{(Eq. 6.4)}$$

**Entrapment efficiency:**

$$Y_2 = 79.32 + 6.20X_1 + 7.56X_2 - 3.92X_1\,X_2 - 0.33\,X_1^2 - 8.64X_2^2 \quad \text{(Eq. 6.5)}$$

Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert ®software. The results of analysis for observed response is shown in Table 6.7 and 6.8. The positive coefficients before independent variables of quadratic equation indicate a favorable effect on the responses, while negative coefficient indicates an unfavorable effect on the responses. The effect of each factor was tested using ANOVA test with a corresponding p value. The model is significant for probability

> F less than 0.05, while model is not significant for probability > F greater than 0.05. The smaller p value and larger F-value were desired for more significant corresponding coefficients.

The model F-value of 168.45 for $Y_1$ and 36.66 for $Y_2$ indicates the model is significant ($p<0.05$). The resulted $R^2$ for $Y_1$=0.9949 and $Y_2$=0.9836, indicates good correlation. Further Adj-$R^2$ of 0.9905 and Pred-$R^2$ of 0.9579 for $Y_1$ and for $Y_2$ Adj-$R^2$ of 0.9571 and Pred-$R^2$ of 0.8138, were in reasonable agreement, i.e. difference is less than 0.2, indicating that the data were described adequately by the mathematical model. Adequate precision is a measure of the range of a predicted response relative to its associated error, that is, signal to noise ratio. The ratio greater than 4 is desirable for navigating the design space. The ratio of "adequate precision" for $Y_1$ (36.108) and $Y_2$ (19.221) indicating an adequate signal. Values of ''p'' less than 0.05 indicated that model terms were significant except for responses $Y_1$, model terms $X_1^2$ was at $p>0.05$ (p value:0.3295), and for $Y_2$, model term $X_2^2$ was at $p>0.05$ (p value: 0.8362) indicated necessary model reduction to improve the model. Hence, the reduced polynomial equations for $Y_1$ (Eq. 6.6) and $Y_2$ (Eq. 6.7) were generated by omitting the least contributing factors.

**Particle Size: $Y_1 = 481.33-159.50\ X_1+76.83\ X_2-57.00\ X_1\ X_2+86.83\ X_1^2$ ..... (Eq. 6.6)**

**Entrapment efficiency: $Y2 = 79.10+6.20\ X1+7.56\ X2-3.92\ X1\ X2-8.46\ X_2^2$ ..... (Eq. 6.7)**

The results of reduced model showed higher F-value for $Y_1$ (193.35) and $Y_2$ (60.07) indicates more significant model than full model. The p value for reduced model was also less as compared to the full model, which favors the model for optimization of formulation. Therefore, quadratic model was chosen for the select design based on the

ANOVA results using model F-value, p value, R2, PRESS and adequate precision confirmed the excellent goodness of fit.

The quadratic equation for $Y_1$ shows that the PVA concentration ($X_1$) has largest coefficient. It indicated that the PVA concentration was the most influential factor and had a significant and negative effect on $Y_1$. In addition, PVA concentration ($X_1$) and amount of lipid ($X_2$) was found to be significant factor that contributing to the variation in $Y_2$. The variable $X_1$ and $X_2$ showed the positive effect on entrapment efficiency (Y2) suggesting that an increase in value of $X_1$ and $X_2$ will cause an increase in value of entrapment efficiency ($Y_2$).

**Table 6.7. ANOVA results for full and reduced quadratic model for particle size**

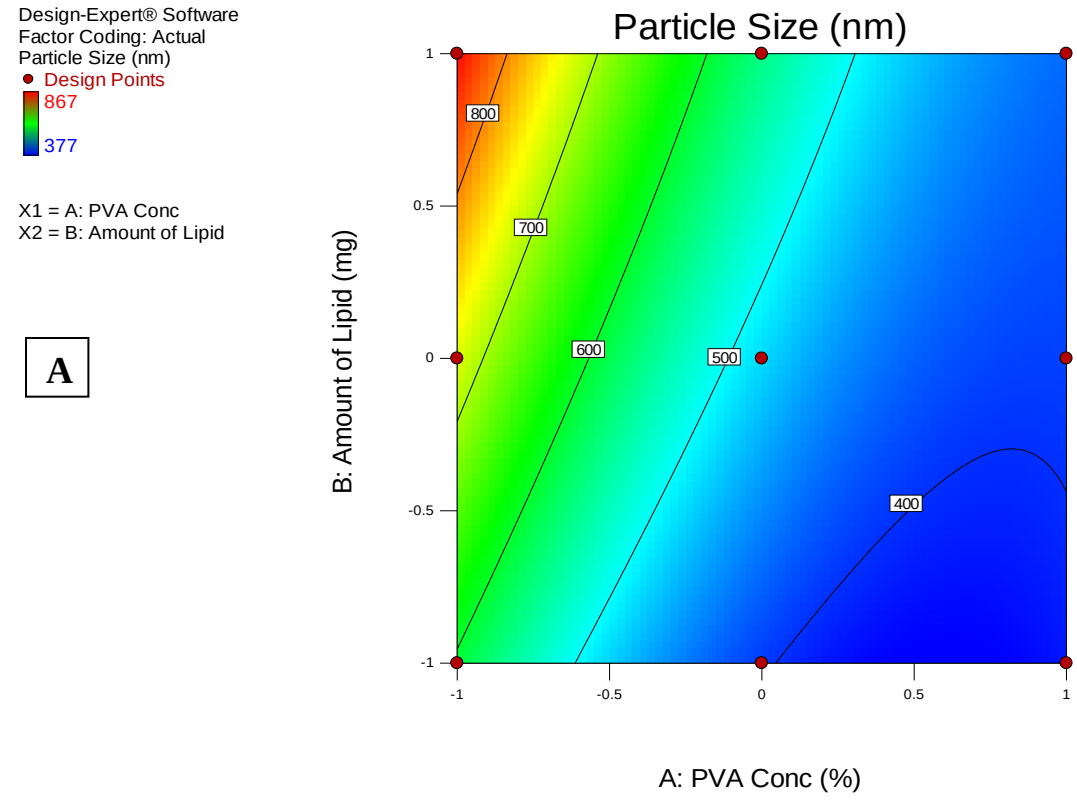
| Source | SS | Df | Mean Square | F Value | Prob > F | SD | Mean | C.V. | PRESS | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adequate precision |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Full Model** | | | | | | | | | | | | | |
| Model | 216500 | 5 | 43296.89 | 168.45 | 0.0007 | 16.03 | 539.22 | 2.97 | 9145.29 | 0.9965 | 0.9905 | 0.9579 | 36.108 |
| $X_1$-PVA Conc | 152600 | 1 | 152600 | 593.85 | 0.0002 | | | | | | | | |
| $X_2$-Amt. of Lipid | 35420.17 | 1 | 35420.17 | 137.80 | 0.0013 | | | | | | | | |
| $X_1X_2$ | 12996.00 | 1 | 12996.00 | 50.56 | 0.0057 | | | | | | | | |
| $X_1^2$ | 15080.06 | 1 | 15080.06 | 58.67 | 0.0046 | | | | | | | | |
| $X_2^2$ | 346.72 | 1 | 346.72 | 1.35 | 0.3295 | | | | | | | | |
| Residual | 771.11 | 3 | 257.04 | | | | | | | | | | |
| Cor Total | 217300 | 8 | | | | | | | | | | | |
| **Reduced Model** | | | | | | | | | | | | | |
| Model | 216100 | 4 | 54034.43 | 193.35 | < 0.0001 | 16.72 | 539.22 | 3.10 | 7340.75 | 0.9949 | 0.9897 | 0.9662 | 37.934 |
| $X_1$-PVA Conc | 152600 | 1 | 1.52600 | 546.20 | < 0.0001 | | | | | | | | |
| $X_2$-Amt. of Lipid | 35420.17 | 1 | 35420.17 | 126.75 | 0.0004 | | | | | | | | |
| $X_1X_2$ | 12996.00 | 1 | 12996.00 | 46.50 | 0.0024 | | | | | | | | |
| $X_1^2$ | 15080.06 | 1 | 15080.06 | 53.96 | 0.0018 | | | | | | | | |
| Residual | 1117.83 | 4 | 279.46 | | | | | | | | | | |
| Cor Total | 217300 | 8 | | | | | | | | | | | |

SS=sum of squares; df=degree of freedom; MS=mean of squares; Prob>F=probability; SD=standard deviation; C.V.=coefficient of variation; Adj-$R^2$= adjusted $R^2$; Pred- $R^2$=predicted $R^2$; Adeq=adequate; PRESS=predicted residual error sum of squares.

**Table 6.8. ANOVA results for full and reduced quadratic model for entrapment efficiency**

| Source | SS | Df | Mean Square | F Value | Prob > F | SD | Mean | C.V. | PRESS | $R^2$ | Adj-$R^2$ | Pred-$R^2$ | Adequate precision |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Full Model** | | | | | | | | | | | | | |
| Model | 778.21 | 5 | 155.64 | 36.66 | 0.0068 | 2.06 | 73.45 | 2.80 | 147.25 | 0.9839 | 0.9571 | 0.8138 | 19.221 |
| $X_1$-PVA Conc | 230.27 | 1 | 230.27 | 54.24 | 0.0052 | | | | | | | | |
| $X_2$-Amt. of Lipid | 342.92 | 1 | 342.92 | 80.78 | 0.0029 | | | | | | | | |
| $X_1X_2$ | 61.54 | 1 | 61.54 | 14.50 | 0.0318 | | | | | | | | |
| $X_1^2$ | 0.22 | 1 | 0.22 | 0.051 | 0.8362 | | | | | | | | |
| $X_2^2$ | 143.26 | 1 | 143.26 | 33.75 | 0.0102 | | | | | | | | |
| Residual | 12.74 | 3 | 4.25 | | | | | | | | | | |
| Cor Total | 790.94 | 8 | | | | | | | | | | | |
| **Reduced Model** | | | | | | | | | | | | | |
| Model | 777.99 | 4 | 194.50 | 60.07 | 0.0008 | 1.80 | 73.45 | 2.45 | 95.35 | 0.9836 | 0.9673 | 0.8138 | 24.110 |
| $X_1$-PVA Conc | 230.27 | 1 | 230.27 | 71.12 | 0.0011 | | | | | | | | |
| $X_2$-Amt. of Lipid | 342.92 | 1 | 342.92 | 105.91 | 0.0005 | | | | | | | | |
| $X_1X_2$ | 61.54 | 1 | 61.54 | 19.01 | 0.0121 | | | | | | | | |
| $X_2^2$ | 143.26 | 1 | 143.26 | 44.25 | 0.0027 | | | | | | | | |
| Residual | 12.95 | 4 | 3.24 | | | | | | | | | | |
| Cor Total | 790.94 | 8 | | | | | | | | | | | |

SS=sum of squares; df=degree of freedom; MS=mean of squares; Prob>F=probability; SD=standard deviation; C.V.=coefficient of variation; Adj-$R^2$= adjusted $R^2$; Pred- $R^2$=predicted $R^2$; Adeq=adequate; PRESS=predicted residual error sum of squares.



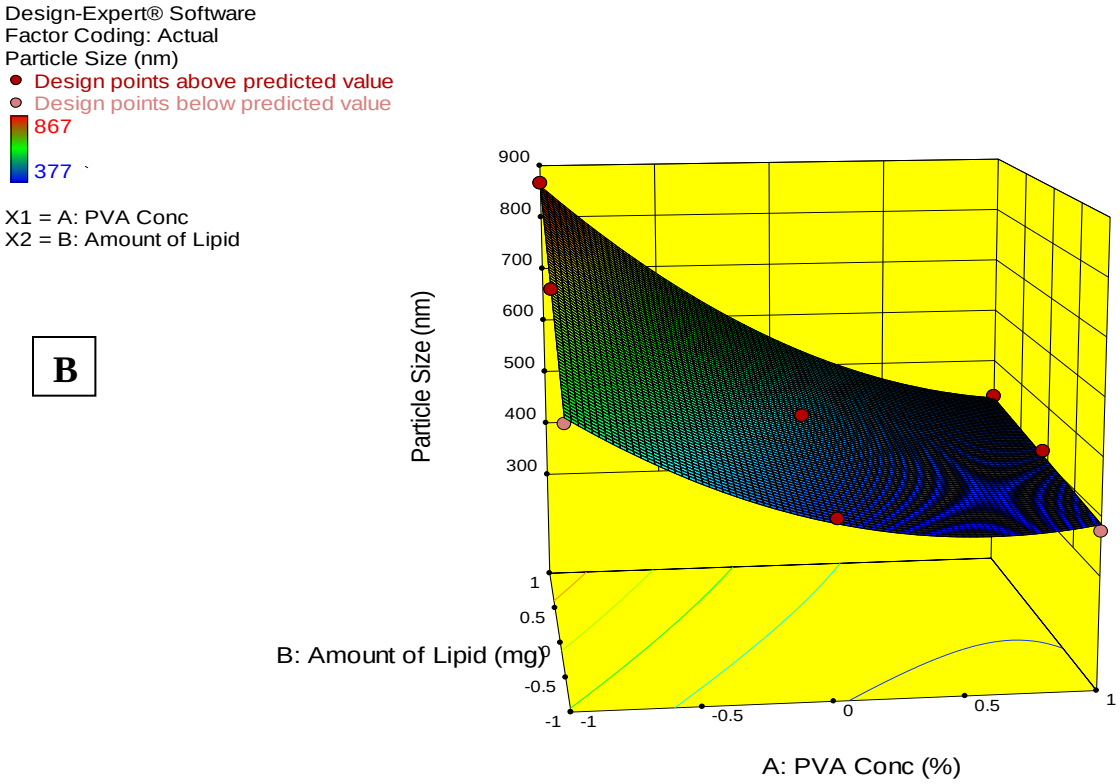




**Figure 6.3. Contour plot and its Response surface shows effect of $X_1$ and $X_2$ on particle size**

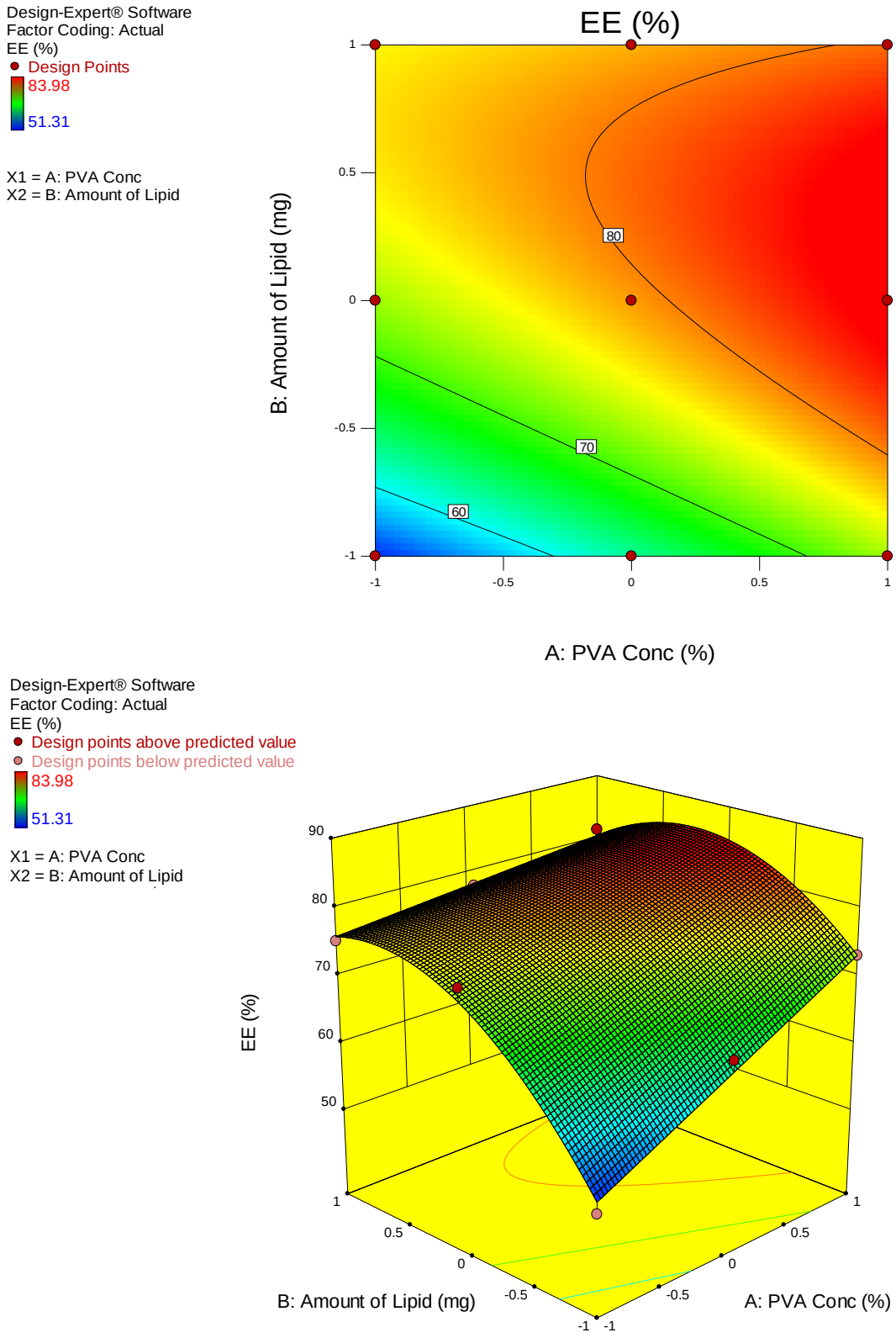



**Figure 6.4. Contour plot and its Response surface shows effect of $X_1$ and $X_2$ on Entrapment efficiency**

#### *6.3.2.2. Contour plot and response surface analysis*

The obtained results can be observed visually in the contour plots and surface plots. Response surface graph of $Y_1$ (Fig. 6.3) shows that particle size of SLN was significantly influenced by PVA concentration. The particle size was found to decrease with increase in the PVA concentration at a constant amount of lipid. The effect is mainly attributed to the increasing viscosity of the PVA solution. During the process of emulsification, the droplet size reduces under the influence of high shear. At the same time they even have a tendency to aggregate in order to reduce their surface energy. However, the presence of surfactant molecules stabilizes the emulsion by forming a thick protective layer around the droplets which prevent the coalescence of the droplets. The particle size was found to be significantly affected with increase in lipid amount at lower PVA concentration. In addition, response surface graph of $Y_2$ (Fig. 6.4) shows that EE was found to be significantly affected by both, PVA concentration and the lipid amount at every level studied. The EE was increased with increase in PVA concentration and lipid amount which may be due to more availability of lipid to encapsulate the drug.

#### *6.3.2.3. Optimization of formulation*

The optimization process was performed by setting the $Y_1$ at minimum and $Y_2$ at maximum while $X_1$ and $X_2$ within the range obtained. The optimized formulation was achieved at $X_1$=0.99%, $X_2$=279 mg with the corresponding desirability *(D)* value of 0.972 (Fig. 6.5). This factor level combination predicted the responses $Y_1$=403.902 nm, $Y_2$= 83.98%.

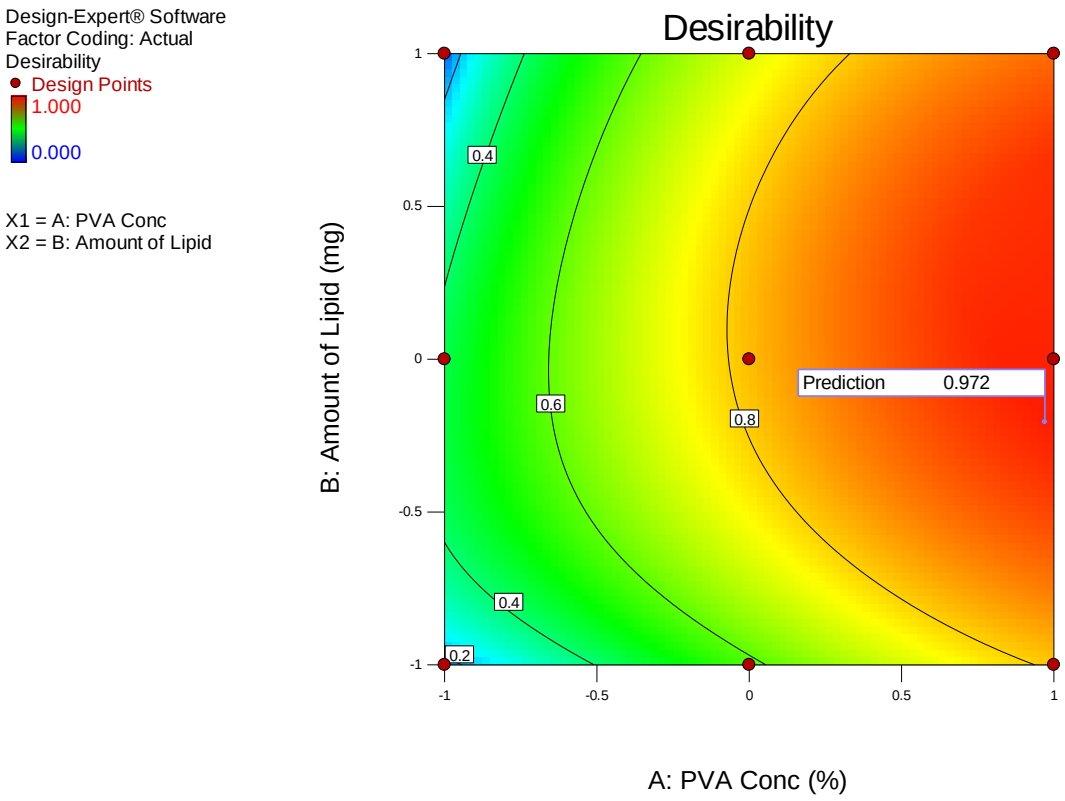



**Figure 6.5. Contour plot for overall desirability of SLN as a function of $X_1$ and $X_2$**

### *6.3.2.4. Checkpoint Analysis*

All of the responses were evaluated for each optimized formulation. The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of SLN formulations (Table 6.9).

**Table 6.9. Checkpoint batch with their predicted and observed value of responses**

| | Independent Variables | | Particle size ($Y_1$) (nm) | | Entrapment efficiency ($Y_2$) (%) | |
|---|---|---|---|---|---|---|
| **Batch** | **$X_1$** | **$X_2$** | **Observed** | **Predicted** | **Observed** | **Predicted** |
| BN10 | 0.971 (0.99) | -0.206 (279) | 395 | 403.90 | 82.44 | 83.98 |
| **Percentage prediction error (%)** | | | -2.25 | | -1.85 | |

### 6.3.3. Characterization of optimized formulation

#### *6.3.3.1. Total drug content*

Total drug content of optimized formulation was found to be 19.085 mg indicates smaller amount of drug loss during the preparation of formulation. It may be explained by low aqueous solubility of berberine leads to decrease in drug loss during the preparation of formulation.

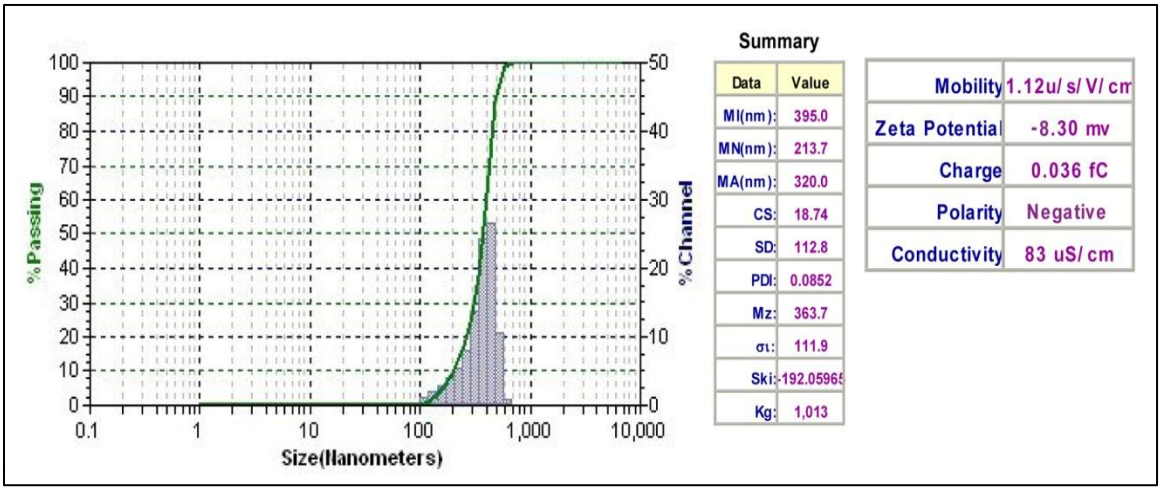
#### *6.3.3.2. Entrapment efficiency and drug loading*

Drug can be incorporated into SLN by several ways like adsorbed on surface, entrapped in lipid matrix, encapsulated in inner core. As explained in contour plot and 3D surface graph, entrapment efficiency was found to be significantly affected by PVA concentration and amount of stearic acid. PVA is a polymer responsible for particle coating and viscosity. The optimized batch shows highest amount of PVA which cause increase in viscosity of external phase. It facilitate higher amount of drug to incorporate into lipid matrix. The entrapment efficiency (EE) of optimized batch was found to be 82.44% while drug loading was 5.67%. The results confirmed that the drug dissolved in lipid matrix remained associated with matrix and there was no drug diffusion.

#### *6.3.3.3. Particle size and zeta potential*

Particle size is a key factor that may influence the fate of nanoparticles in the biological system.[(210)] Photon correlation spectroscopy is the most widely used method for the particle size measurement of solid lipid nanoparticles. The optimized formulation BN10 shows particle size of 395 nm 0.0852 polydispersity index (Fig. 6.6). The polydispersity index is a sign of homogeneity of size distribution. The low polydispersity index of SLNs indicated that method and optimal composition could be used for preparation of stable

SLN with narrow size distribution. Zeta potential is also an important factor that may influence the stability and *in vivo* behavior of solid lipid nanoparticles. The formulation shows negative zeta potential of -8.30 mV (Fig. 6.6). The higher values of zeta potential enhance the stability of SLN by increasing the repulsion of vesicle, and thereby preventing aggregation.

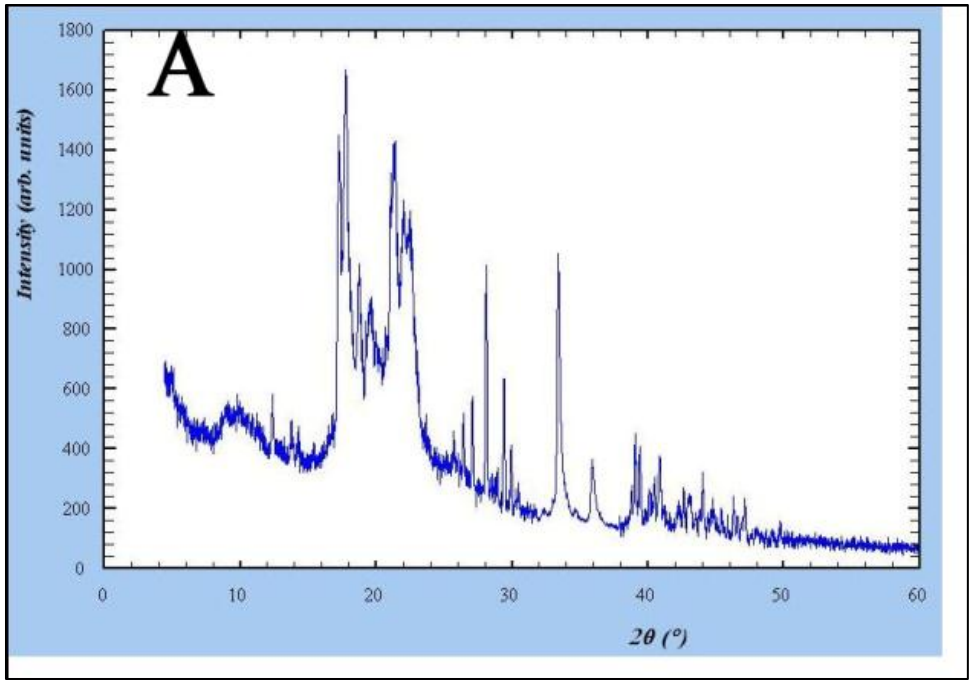



**6.6. Particle size, polydispersity index and zeta potential of optimized SLN**

### *6.3.3.4. FTIR and Powder X-ray diffraction (XRD)*

FTIR spectra of BER loaded nanoparticles showed peaks resulting from simple superimposition of infra-red spectrum of their separated components (Fig. 4.2). From this it can be concluded that no strong chemical interaction occurred between the drug and lipid.

The solid state of lipid particles affects the release properties of SLNs. Crystalline structure of component of solid lipid nanoparticles formulations as well as incorporated drug is a crucial factor to decide in determining whether a drug will be expelled or firmly incorporated during storage.[237] XRD is the widely used techniques for determination of



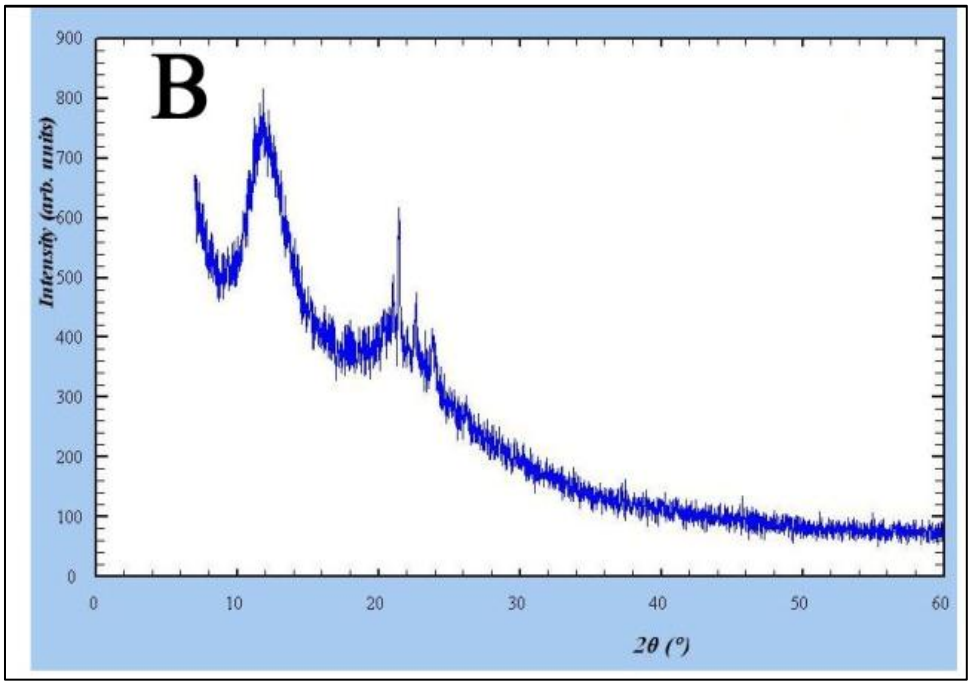




**Figure 6.7. XRD of plain berberine (A) and berberine loaded SLN (B)**

crystallinity and polymorphic behavior of the component of SLNs. Fig. 6.7 shows the X-ray diffraction patterns of BER and BER loaded SLN. The XRD patterns of BER showed the sharp peaks at a different diffraction angle reveals that the drug is present in

crystalline form. The XRD pattern of SLN was showed the diffuse spectra and no characteristic sharp peaks of drug indicate the conversion of crystalline form of drug to amorphous form in SLNs. X-ray diffraction of formulation confirms the successful wrapping of drug in lipid carrier.

### *6.3.3.5. Surface morphology*

Morphology of BER loaded SLN was determined by SEM. The microphotographs of formulation reveal that the particles are roughly spherical in shape with different size distribution (Fig. 6.8). Generally, spherical particle have smallest specific surface area and hence are stabilized with small amount of surfactant. In addition, spherical particles provide controlled release of incorporated drugs due to the longest diffusion pathway.[213]

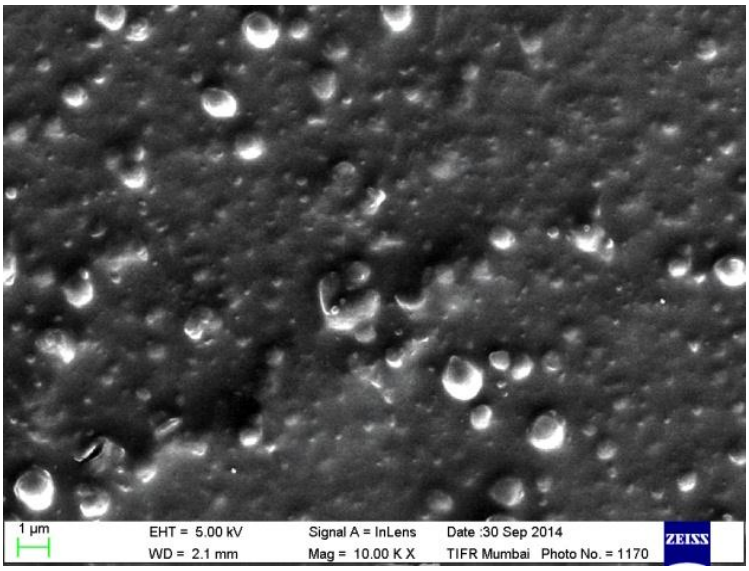



**Figure 6.8. SEM of berberine loaded SLN**

### *6.3.3.6. In vitro drug release*

Dissolution study was performed to show the release of BER from formulation. *In vitro* release study show initial burst release during the first 4 h followed by slow and continuous release (Fig. 6.9). Drug release from SLN is dependent on the diffusion of the drug molecule through lipid matrix and *in vivo* degradation of lipid matrix. The initial burst release can be attributed to the presence of unincorporated drug in the external

phase and drug adsorbed on the surface of particles while the slow release was due to slow diffusion of encapsulated drug through the lipid matrix.[238, 239]

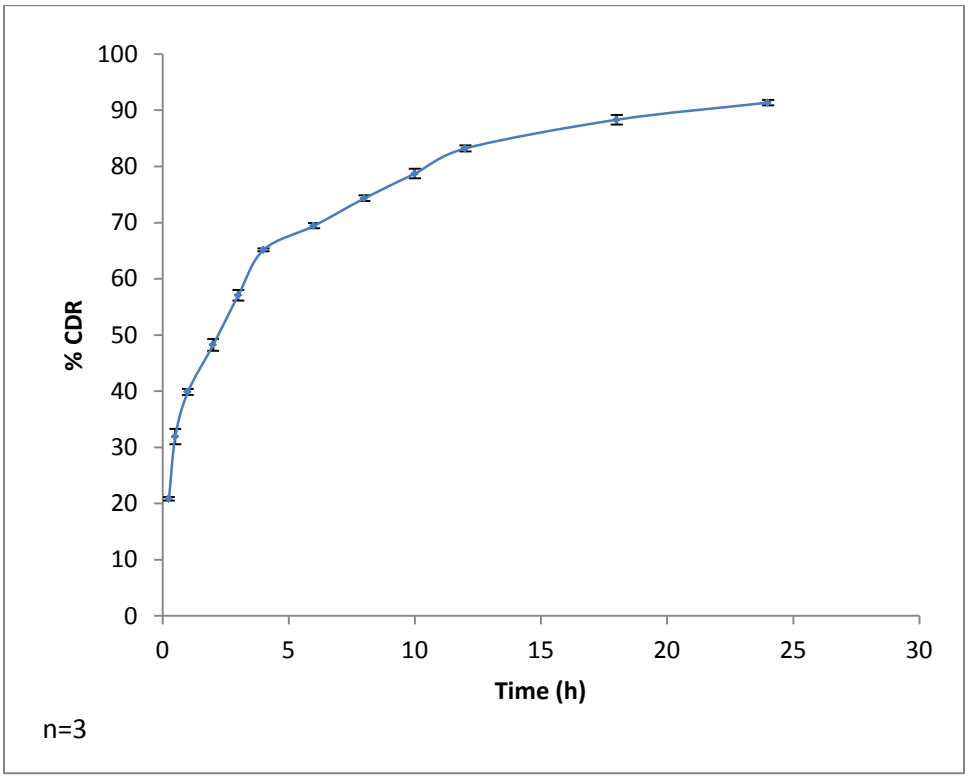
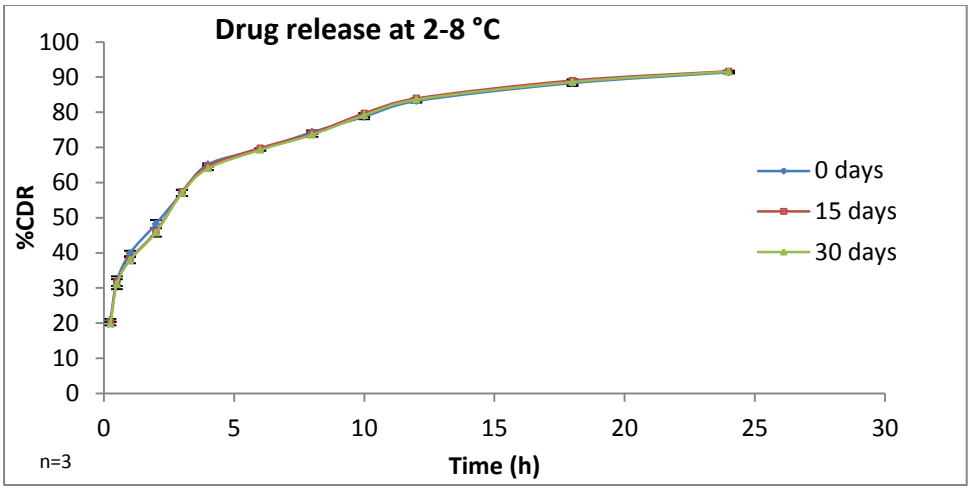
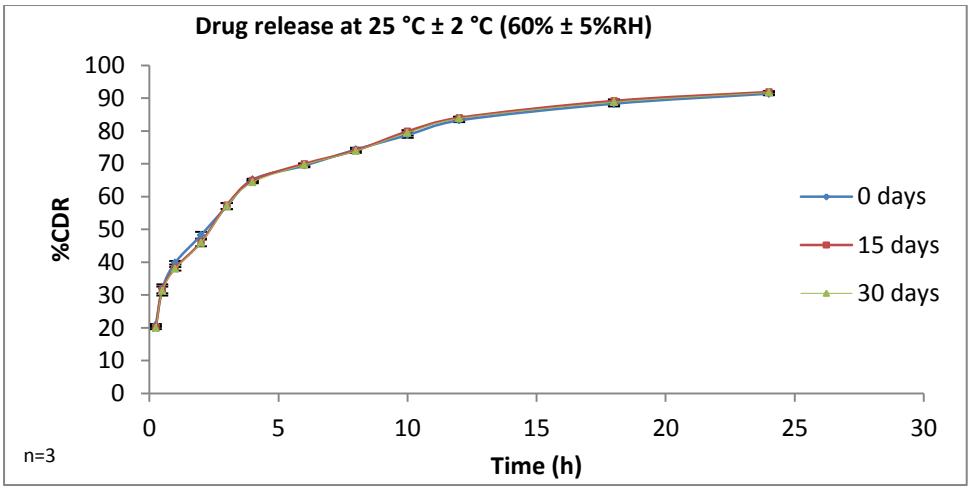



**Figure 6.9 *In vitro* drug release from berberine loaded SLN**

### 6.3.4. Stability study

The results of the stability study revealed that the formulation remains stable at different condition of temperature and relative humidity (RH) for 1 month (Table 6.10). The *in vitro* release study shows close resembles with initial formulation (Fig. 6.10). These results indicated that the BER loaded SLN could potentially be exploited as a delivery system with improved drug entrapment efficiency and controlled drug release.



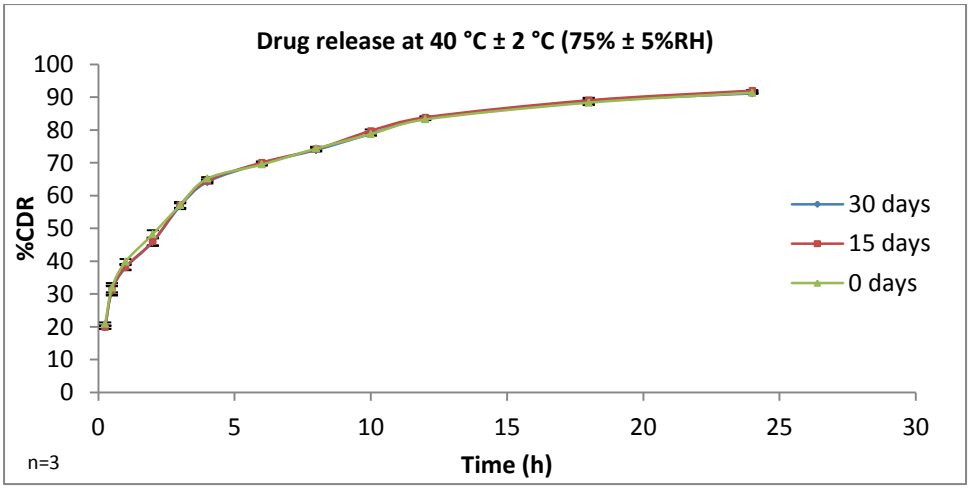




**Figure 6.10. Drug release study at different temperature conditions**

**Table 6.10 Stability study of optimized formulation**

| No of Days | 2-8ºC (Subfreezing Temperature) | | 25ºC ± 2ºC/60% RH ± 5% RH | | 40ºC ± 2ºC/75% ± 5% RH | |
|---|---|---|---|---|---|---|
| | **Physical Appearance** | **Drug Content (mg)** | **Physical Appearance** | **Drug Content (mg)** | **Physical Appearance** | **Drug Content (mg)** |
| 0 | No Change | 19.085±0.09 | No Change | 19.085±0.09 | No Change | 19.085±0.09 |
| 15 | No Change | 19.18±0.06 | No Change | 19.018±0.05 | No Change | 19.005±0.04 |
| 30 | No Change | 19.018±0.06 | No Change | 19.012±0.05 | No Change | 18.99 ± 0.03 |

# 7. PREPARATION AND CHARACTERIZATION OF SELF EMULSIFYING DRUG DELIVERY SYSTEM (SEDDS)

## 7.1. Introduction

Self-emulsifying drug delivery systems has arisen as a prospective tool with great promise in enhancing and enabling oral bioavailability of low aqueous solubility molecules, which unless otherwise may not be delivered orally. SEDDS are composed of a defined mixture of several lipid excipients including oils, surfactants and co-surfactants. Since SEDDS do not include the aqueous phase, the drawbacks of ready-to-use microemulsions are overcome. However, one of the obstacles for the development of self-emulsifying drug delivery systems and other lipid-based formulations is the lack of good predicative *in vitro* models for assessment of the formulations. In addition to this, chemical instabilities of drugs and high surfactant concentrations in formulations (approximately 30-60%) which irritate GIT are also concerned. Several mechanisms that are believed to play major role is digestion of emulsion droplets in the intestine, drug solubilization into drug the digestion process, and alterations in intestinal permeability and lymphatic transport.

In a SEDDS, insoluble drugs are dissolved in the oil phase in the presence of surfactants and co-surfactants. When SEDDS are diluted with water or gastrointestinal fluid, fine O/W emulsions are formed under gentle agitation such as digestive mobility of stomach and intestine. SEDDS typically produce an emulsion with droplet size between 100 and 300 nm. When the droplet size of emulsion is less than 25% of the wavelength of visible light, the emulsion becomes transparent, therefore self-microemulsifying drug delivery systems (SMEDDS) which form transparent microemulsion have the droplet size

of less than 150 nm.[240] When the droplet size of emulsion is less than 50 nm, it is referred as self-nanoemulsifying drug delivery systems (SNEDDS). Because the drug remains in the solution, the drug dissolution step is avoided. In addition, such small particle sizes yield an extremely large interfacial area. As a result, the drug can be quickly released into the external phase during the process of drug absorption. With the examples of commercial success, high interest has been generated in studying SEDDS.[111, 130, 241, 242] One successful example is Neoral®. After oral administration of Neoral®, a marked SMEDDS of cyclosporine, to healthy volunteers, the absorption of cyclosporine was increased by 49% compared to reference soft gelatin capsules. This improvement in drug absorption can be attributed to the greater solubility of the lipophilic cyclosporine in the SMEDDS.[243]

### 7.1.1. Self-emulsifying drug delivery systems

Self-emulsifying drug delivery systems is an isotropic and thermodynamically stable mixture of oil, surfactants and co-solvent or co-surfactant which when introduced into an aqueous media, under gentle agitation, forms fine oil-in-water micro/nano-emulsions (Fig. 7.1).

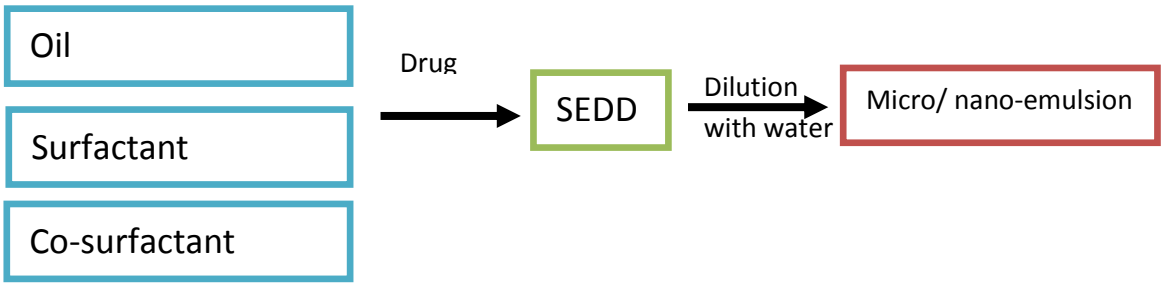



**Figure 7.1. Schematic flowchart on the general strategy of formulating self-emulsifying systems and their subsequent conversion to micro/nano emulsion**

These systems when incorporated with a drug compound, it is distributed in the aqueous solution entrapped inside oil droplets.[241] Figure 7.2 shows a schematic of a

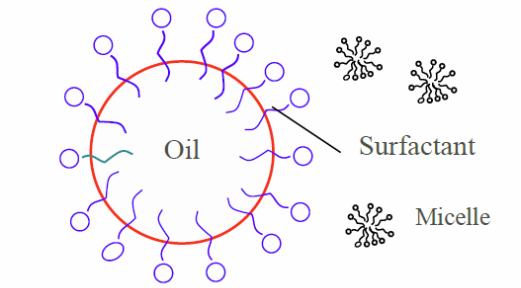



**Figure 7.2. Schematic of an emulsion system in which an oil droplet is stabilized by a surfactant which also forms micelles in the free solution**

SEDDS. SEDDS enable distribution of hydrophobic drug component in the aqueous media and creates a drug solubilization in the gastrointestinal environment. Distribution of drug inside oil droplets prevents drug from being an undisolved substance, precipitating and being excreted from body. However, their mechanisms of action in the body are not limited to solubilization enhancement and also include other processes such as intestinal permeability and lymphatic transport enhancement. The pathway of herbal drug transport from SEDDS is presented in Figure 7.3.

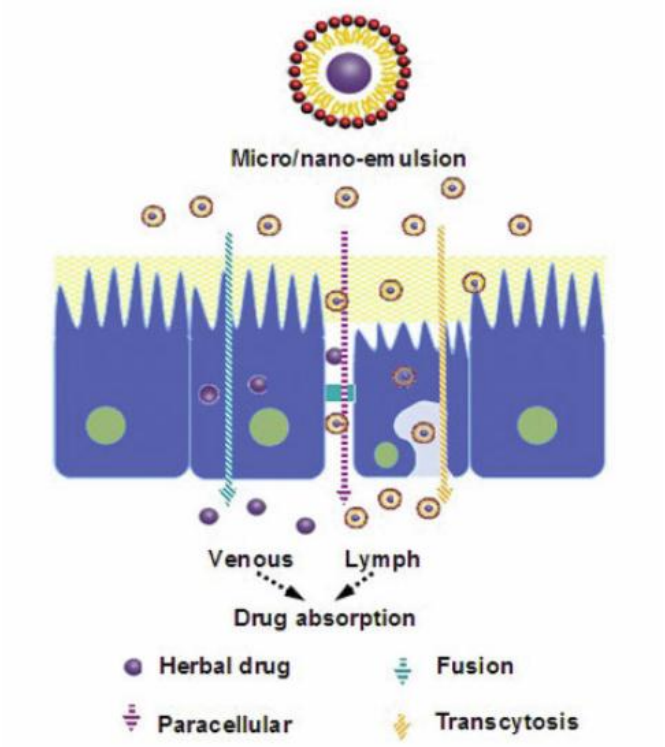



**Figure 7.3. The pathway of herbal drug transport from self-emulsifying drug delivery system**

SEDDS by nature are thermodynamically stable emulsions compared to unstable regular emulsions with high solubilization capacity for lipophilic drugs, and also can be filled directly into soft or hard gelatin capsules for convenient oral administration. Their stability is thought to be dependent on their relatively small dispersed oil droplet size and narrow range of droplet distribution.[244] SEDDS are typically composed of emulsion droplets having a diameter of 50 to 500 nm whereas systems having droplet size less than 50 nm are called self-nano emulsifying drug delivery systems (SNEDDS).[28]

### 7.1.2. Mechanism of self-emulsification

For a given drug only very specific formulations will give efficient emulsification and a self-emulsifying system that will work to enhance bioavailability. Efficiency of SEDDS therefore, as explained in detail by Gursoy *et al.*,[28] is governed by surfactant concentration, oil/surfactant ratio, polarity of the emulsion, droplet size and charge of the droplets. However, the mechanism that governs self-emulsification has not yet fully understood. It is suggested that water penetrates through the gel and liquid crystal (LC) phases that occur at the surface of the droplets. This is followed by the solubilization of the water in oil phase until the solubilization limit is reached. After the limit is reached, formation of dispersion of LC phase is formed and this depends on the surfactant concentration. With this formation, SEDDS become resistant to coalescence.[245]

Emulsion stability is governed by a variety of factors such as physical nature of the interfacial film, presence of electrostatic or steric barriers on the droplet, viscosity of the continuous phase, droplet size distribution, oil to water ratio, temperature and the amount of surfactant that is absorbed on the surface of the oil droplet. The more surfactant is absorbed on the surface, the more decreased the interfacial tension between

oil and water which consequently yields delayed coalescence of droplets by electrostatic and steric repulsion.[246, 247] Although with the addition of high amounts of drug, which is common case for potential oral dosage forms, it is harder to have stabilized emulsions. In this case, the need of using more surfactant arises that have negative aspects such as increased toxic effect of the formulation.

The total interfacial area that is generated is proportional to the work done to the system, as described with equation 7.1 below;[248]

$$W = \gamma(\Delta A) \quad \text{..... (Eq. 7.1)}$$

where W is the amount of work put into the system, γ is the interfacial tension and A is the change in the interfacial area. Therefore systems with smaller droplets have a decreased interfacial tension

### 7.1.3. Drug candidate for SEDDS

The Lipid Formulation Classification System as shown in Table 7.1 is a fairly new classification system and is being used to facilitate the identification of the most appropriate formulations for drugs with reference to their physicochemical properties.[249] It is a framework for comparing the performance of lipid-based formulations and for interpreting *in vivo* studies more efficiently. Type I formulations comprise of drug in solution in triglycerides and/or mixed glycerides. The inclusion of a lipophilic surfactant may improve the solvent capacity as well as provide dispersion such as in Type II formulations. Type IIIA and IIIB include water soluble components that may not depend on digestion. Type II and III are termed as self-emulsifying drug delivery systems (SEDDS) due to their ability to self emulsify spontaneously in aqueous solutions on mild agitation. Type IIIB may also be referred to as self-microemulsifying systems (SMEDDS) due to their optical clarity and ability to produce very fine droplets. Type IV

contains no lipophilic component and suffers from risk of precipitation which is generally higher whenever a higher proportion of hydrophilic components are incorporated.

**Table 7.1. Characteristic features, advantages and disadvantages of the various types of 'lipid' formulations**

| Formulation | Type I Oil | Type II SEDDS | Type III | | Type IV Oil Free |
|---|---|---|---|---|---|
| | | | Type IIIA SEDDS | TYPE IIIB SMEDDS | |
| **Performance Criteria** | No Surfactant | No water soluble component | Includes water soluble surfactants and co-solvents | | Comprise only water soluble surfactants and co-solvents |
| **Advantages** | GRAS status; simple; excellent capsule compatibility | Unlikely to lose solvent capacity on dispersion | Clear or almost clear dispersion; drug absorption without digestion | Clear dispersion; drug absorption without digestion | Good solvent capacity for many drugs; disperses to micellar solution |
| **Disadvantages** | Formulation has poor solvent capacity unless drug is highly lipophilic | Turbid o/w dispersion (0.25-2 μm particle size) | Possible loss of solvent capacity on dispersion; less easily digested | Likely loss of solvent capacity on dispersion | Loss of solvent capacity on dispersion; may not be digestible |

The selection of a suitable lipid-based system depends on the physicochemical properties of the drug. For many lipophilic drugs, formulating as SEDDS (Type II and Type III) is an advantageous approach as it maximizes the chance of maintaining the drug in solution after dispersion in the stomach. This strategy ensures that the drug remains in the solubilized form during as well as after the digestion process.[250, 251]

### 7.1.4. Composition of SEDDS

SEDDS are composed of a defined mixture of several lipid excipients including oils, surfactants and co-surfactants. There are large varieties of liquid or waxy excipients available which can be used to formulate the drug loaded colloidal emulsions. In formulation of self-emulsifying sytems, the following points should be considered: (i) solubility of the drug in different oil, surfactants and cosolvents and (ii) selection of oil, surfactant and cosolvent based on the solubility of the drug and the preparation of the phase diagram. The right concentration of the lipids, surfactants and cosolvents decides the self-emulsification and particle size of the oil phase in the emulsion formed. These ingredients are discussed below.

#### *7.1.4.1. Lipids*

These are major excipient in the SEDDS formulation as they can help solubilize the required dose of the lipophilic drug, facilitate self- emulsification and more importantly increase the fraction of lipophilic drug transported via the lymphatic system thereby increasing the absorption from the GIT.[129, 130, 240, 252] Both long and medium chain triglycerides (LCT and MCT) have been used in SEDDS formulations.[253] The chain length and saturation degree of the lipid have an effect on the solvent capacity and the digestibility of the formulation. Natural triglyceride vegetable oils are advantageous as they are commonly ingested in food, fully digested and absorbed. Therefore, they do not present any safety issues and are generally regarded as safe (GRAS).[254] Few examples of the natural lipids used in SEDDS are com oil, olive oil, palm oil and soybean oil. Edible natural oils are not commonly used due to their limited dissolution ability. Synthetic and semi-synthetic lipids commonly described as amphiphilic compounds are quickly

replacing the natural lipids. They have both lipid as well as emulsifying properties. Examples of such lipids include glyceryl monocaprylocaprate (Capmul MCM); glyceryl monostearate (Geleol™, Imwitor® 191, Cutina™ GMS or Tegin™ ); glyceryl distearate (Precirol™ ATO 5); glyceryl monooleate (Peceol™); glyceryl monolinoleate (Maisine™ 35-1); glyceryl dibehenate (Compritol®888 ATO). They may be composed of unsaturated long chain fatty acids like oleyl polyoxylglycerides (Labrafil® M1944CS) and linoleyl polyoxylglycerides (Labrafil® M2125CS) or saturated medium chain fatty acid esters like lauroyl polyoxylglycerides (Gelucire® 44/14). [242, 255]

### *7.1.4.2. Surfactants*

It has been reported that a surfactant concentration of 30 - 60 % is required for self emulsification and quick dispersion of SEDDS. Nonionic surfactants with high hydrophilic-lipophilic balance (HLB) values are mostly used in the formulation of SEDDS. Surfactants with high HLB help in immediate formation of o/w droplets and rapid spreading of the formulation in the aqueous environment, providing good dispersion and emulsification. They also reduce the risk of drug precipitation following dilution in the GI fluids. Commonly used surfactants in SEDDS formulations are Span 80, Tween 80, Cremophor RH40®, Labrafil®, Labrasol®.[256] A high quantity of surfactant may irritate the GI membrane and could even be toxic. For these reasons natural surfactants such as lecithin, medium chain monoglycerides (MCM) are preferred over synthetic ones but provide less efficient emulsification. Nonionic surfactants are safer and provide higher emulsion stability over a wide range of pH and ionic strength than their ionic counterparts. In addition, nonionic surfactants can produce reversible changes in intestinal mucosal permeability and facilitate absorption of the drug. [240, 256]

### *7.1.4.3. Co-solvents*

Traditional co-solvents like ethanol, propylene glycol, polyethylene glycol, polyoxyethylene and newer ones such as transcutol HP® are used in SEDDS to dissolve large amounts of the hydrophobic drugs in the lipid.[254]However to enhance the solvent capacity significantly, the co-solvent must be present at high concentration and this has its own disadvantages. Co-solvents lose their solvent capacity following dilution, which may cause drug precipitation. High amount of co-solvents can be immiscible with the oil components and low molecular weight co-solvents could be incompatible with the capsule shells. For many drugs the relationship between co-solvent concentration and solubility is near to logarithmic.[257, 258] Another reason for inclusion of co-solvents is to aid in the dispersion of the SEDDS which contain a high proportion of water-soluble surfactants.

### *7.1.4.4. Additives*

*Co-surfactants:* Sometimes a co-surfactant is also used in the formulation of SEDDS. A co-surfactant of usually HLB of 10-14 is used to lower the oil water interfacial tension, fluidize the hydrocarbon region of the interfacial film and help form a spontaneous emulsion. The choice of surfactant and co-surfactant is crucial for formation of emulsion and also for solubilization of the emulsion. Medium chain length alcohols (C3-C8) are commonly added as co-surfactants.[259, 260]

*Antioxidants:* Antioxidants that are lipid soluble such as a-tocopherol, p-carotene, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate may also be incorporated to prevent the oxidation of excipients especially of lipids.

*Polymers:* In many cases, the solvent capacity of SEDDS is lost which causes drug precipitation. The use of supersaturated systems that maintain drug solubilization above equilibrium solubility without precipitation for a long time is a new approach to enhance drug absorption. Hydrophilic polymers such as hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) 4000 have been used in SEDDS formulations as precipitation inhibitors to form supersaturable selfemulsifying systems (S-SEDDS). It is suggested that the adsorption of the polymer on to the crystal surface of the drug may play a role in inhibition of crystallization and precipitation by hydrogen bonding.[261]

## 7.2. Methodology

### 7.2.1. Materials

Tween 80 was purchased from Loba Chemie Pvt. Ltd., Mumbai. Capmul MCM C8 was provided as a gift sample from Abitec Corporation Ltd. (Columbus, Ohio, USA). PEG 400 was obtained from Himedia laboratories, Mumbai. All other solvent were used of analytical grade.

### 7.2.2. Solubility studies

The solubility of berberine in various excipients (surfactant, co-surfactant and oil) was determined from a calibration curve of berberine in methanol using UV spectrophotometer. 500 mg of berberine was added to 4 mL of each excipient in a micro centrifuge tube and vortexed for 5 minutes. Drug-excipient mixtures were heated to 40°C in a water bath to facilitate solubilization followed by continuous shaking on an orbital mixer for 48 hours at ambient room temperature (~ 25°C). The mixtures were centrifuged at 3000 rpm for 20 min. Aliquots of supernatants were prepared and diluted using

methanol and the drug content was quantitatively determined using UV spectrophotometer (Shimadzu, Japan).

### 7.2.3. Construction of pseudo-ternary phase diagrams

To determine optimum concentration of oil, surfactant, and co-surfactant, phase diagrams were constructed. The formulations were prepared by mixing surfactant and co-surfactant in ratios 1:1, 1:2 and 1:3 (Smix, w/w). Ternary mixtures with varying compositions of Smix, and oil were prepared. Nine different combinations of oil and Smix, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, were made so that maximum ratios were covered to define the boundaries of phase formed in the phase diagrams.

### 7.2.4. Preparation of SNEDDS

SNEDDS was prepared by taking variable proportions of oil, surfactant and co-surfactant. All the components were taken into 10 mL beaker and mixed by gentle stirring. Thereafter, a clear and transparent solution was obtained after appropriate mixing. On the bases of these results, the contents of surfactant, co-surfactant and oil were chosen at the range of 30–60%, 30–60% and 10–40%, respectively. These concentration ranges can further used to prepare optimum formulation.

### 7.2.5. Experimental design

#### *7.2.5.1. Simplex lattice design*

A simplex lattice design was used to optimize the compositions of formulation. In these design concentration of independent variables such as surfactant ($X_1$), co-surfactant ($X_2$) and oil ($X_3$), were change to observe the effect on response variables. The droplet size ($Y_1$) of diluted microemulsion and solubility of BER in SNEDDS ($Y_2$) were taken as

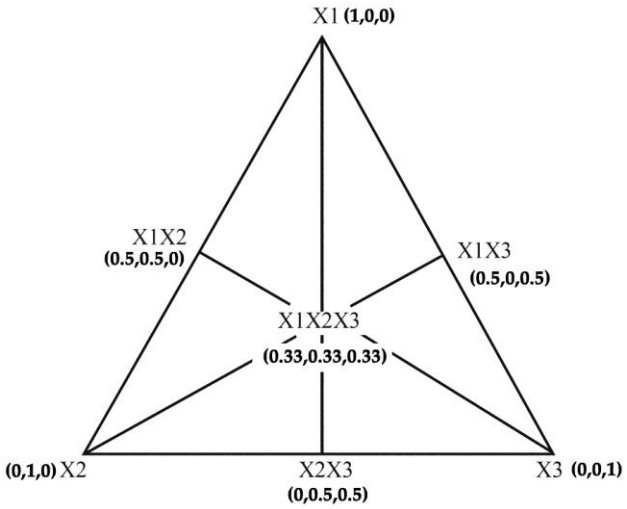



**Figure 7.4. Simplex lattice design for three component of SNEDDS**

responses, respectively. However, the total concentration of components was constant for all the formulation. In this design, seven formulations were prepared as follows: three vertexes ($X_1$, $X_2$, $X_3$), three halfway points between vertices ($X_1X_2$, $X_2X_3$, $X_1$) and the center point ($X_1X_2X_3$) (Fig. 7.4, Table 7.2).

**Table 7.2. Variable levels used in simplex lattice design**

| Batch | Points | Level used, Actual (Coded) | | |
|---|---|---|---|---|
| | | $X_1$ | $X_2$ | $X_3$ |
| **BM1** | $X_1$ $X_2$ | 45 (0.5) | 45 (0.5) | 10 (0) |
| **BM2** | $X_1$ $X_3$ | 45 (0.5) | 30 (0) | 25 (0.5) |
| **BM3** | $X_1$ $X_2X_3$ | 40 (0.33) | 40 (0.33) | 40 (0.33) |
| **BM4** | $X_1$ | 60 (1) | 30 (0) | 10 (0) |
| **BM5** | $X_2$ | 30 (0) | 60 (1) | 10 (0) |
| **BM6** | $X_3$ | 30 (0) | 30 (0) | 40 (1) |
| **BM7** | $X_2$ $X_3$ | 30 (0) | 45 (0.5) | 25 (0.5) |

($X_1$) Surfactant, ($X_2$) Co-surfactant, ($X_3$) Oil

The equation 7.2 for simplex lattice design is described as follows:

$$\mathbf{Y= b_1X_1+ b_2X_2+ b_3X_3+ b_{12}X_1X_2+ b_{13}X_1X_3+ b_{23}X_2X_3 + b_{123}X_1X_2X_3} \quad \textbf{..... (Eq. 7.2)}$$

Where Y is the dependent variable and $b_i$ is the estimated coefficient for the factor $X_i$

#### *7.2.5.2. Response surface plot*

The models were presented as contour plot and three dimensional (3D) response surface graphs. These plots were used to establish the relationship between independent variables and dependent variables (responses).

#### *7.2.5.3. Optimization using desirability function*

All the responses were simultaneously optimized by a desirability function using Design-Expert software. In the desirability function approach, individual responses are assigned goals. According to the simultaneously assigned goals for all responses, the Design-Expert software determines the maximum desirability value.

#### *7.2.5.4. Checkpoint analysis*

The optimized formulation was prepared to confirm the validity of the optimal parameters and predicted responses calculated. All of the responses were evaluated for optimized formulation.

### 7.2.6. Characterization of SNEDDS

#### *7.2.6.1. Spectroscopic characterization of optical clarity*

The optical clarity of aqueous dispersions of SNEDDS formulation was measured spectrophotometrically. A total of 1 mL of the SNEDDS was diluted to 100 times with distilled water, 0.1 N HCl and phosphate buffer pH 6.8. The % transmittance of solution was measured at 650 nm, using distilled water as a reference.[262-264]

#### *7.2.6.2. Drug content*

SNEDDS formulation equivalent to 25 mg of berberine was taken and diluted in methanol. Volume was made up to 25 mL with methanol (1mg/mL). From the above stock solution, 0.2 mL (200 μg/mL) was withdrawn and diluted up to 10 mL with

methanol (20 µg/mL). Samples were prepared in triplicate and absorbance measured at 345 nm using UV-Visible Spectrophotometer.[265, 266] Methanol was used as a reference solution.

#### *7.2.6.3. Robustness to Dilution*

Robustness of SNEDDS to dilution was studied as per Date and Nagarsenker's method with slight modification.[267] SNEDDS was diluted to 10, 100, and 1,000 times with various dissolution media, viz., water, pH 1.2 buffer and pH 6.8 buffer. The diluted microemulsions were stored for 12 h and observed for any signs of phase separation or drug precipitation.

On the basis of the above test, diluted SNEDDS was used for assessment of various *in vitro* parameters. Diluted SNEDDS was prepared by diluting 25 µL of SNEDDS with 25 mL of water.

#### *7.2.6.4. Determination of droplet size, polydispersity index, and zeta potential*

The particle size and zeta potential of the microemulsion were measured by dynamic light scattering (DLS) utilizing a Zetasizer HAS 3000 (Malvern instrument Limited, UK). DLS is a non-invasive, well-established technique for measuring the size of globule. The formulation was diluted 10 times with distilled water for measurement of globule size and zeta potential.

#### *7.2.6.5. Transmission electron microscopy (TEM)*

The morphology of SNEDDS was observed using a transmission electron microscope (TEM) (Phillips Tecnai 20, Netherland). The optimized liquid SNEDDS formulation was diluted with distilled water 1:25 and mixed by slight shaking. A drop of microemulsion samples were applied to a carbon film-covered copper grid to form a thin film, which was

then stained with 1% phosphotungstic acid for 30s and visually observed under microscope.

#### *7.2.6.6. Electrical conductivity*

Electrical conductivity of optimized formulation was measured using a conductivity meter (Macro scientific works ltd., Delhi, India). Based on electrical conductivity, the phase systems of the microemulsion were determined.

#### *7.2.6.7. Viscosity determination*

The viscosity of optimized SNEDDS was determined using Brookfield DVIII ultra rheometer (Brookfield engineering laboratory, USA). The SNEDDS (0.5 g) was diluted 10 times and 100 times with distilled water in a beaker with constant stirring on magnetic stirrer. Viscosity of the resultant microemulsion and initial SNEDDS was measured using spindle LV-3 96 at 25 ± 0.5$^{o}$C temperature.

#### *7.2.6.8. In vitro dissolution studies*

Dissolution studies were performed for berberine loaded SNEDDS and plain berberine in dissolution media containing pH 1.2 or pH 6.8 buffer.[268-270] The SNEDDS formulations containing 20 mg of berberine or 20 mg plain berberine were put into hard gelatin capsules (0 sizes) and introduced into 500 mL of a dissolution medium and maintained at 37$^{o}$C. The revolution speed of the paddle was kept constant at 100 rpm. During the release studies, a 5 mL sample of medium was taken out at different interval and filtered through 0.45μm membrane filter. The berberine content was determined using UV spectrophotometer at 354 nm. The removed volume was replaced each time with 5 mL of fresh medium.

### 7.2.7. Stability

Berberine loaded SNEDDS was tightly sealed in a vial for storage under different storage conditions (refrigerated 4°C/75% RH), real time (room temperature) storage (30°C/75 % RH) and accelerated (40°C/75% RH) according to ICH guidelines for one month. The stability was assessed by analysing the physical appearance, droplet size and drug content at day 0, 15 and 30.

Thermodynamic stability of optimized formulation was assesses by applying stress condition. The formulations were subjected to 3 to 4 freeze-thaw cycles, which included freezing at -4°C for 24 h followed by thawing at 40°C for 24 h. The formulations were then centrifuged at 3000 rpm for 5 min observed for phase and separation.

## 7.3. Result and Discussion

### 7.3.1. Solubility Studies

The solubility of berberine in various oils, surfactant and co-surfactant were shown in Table 7.3. Based on the preliminary solubility study, Capmul MCM C8 was selected as oil for its better solubility than others. Although Capmul MCM C8 was screened as a surfactant, we used it as an oil phase because all the oil shows poor solubility of BER. Even though glycerol showed better solubility of BER than other co-surfactants, PEG 400 was selected as co-surfactants because of the P-gp inhibition property. This could be helpful in increasing the absorption of P-gp substrate drugs. Furthermore, Tween 80 was selected as a hydrophilic surfactant for its good compatibility with the w/o emulsions.

**Table 7.3. Solubility of Berberine in various surfactant, co-surfactant and oils at room temperature**

| Vehicle | Solubility ± SD (mg/mL) |
|---|---|
| **Surfactant** | |
| Tween 80 | 1.55 ± 0.5 |
| Tween 60 | 1.27 ± 0.4 |
| Tween 20 | 1.0 ± 0.2 |
| Cremophore EL | 0.87 ± 0.09 |
| Labrasol | 4.94 ± 1.1 |
| Pluronic F 127 | 14.3 ± 2.3 |
| Capmul MCM | 26.35 ± 2.6 |
| Capmul MCM C8 | 35.08 ± 2.9 |
| **Co-surfactant** | |
| Glycerol | 162.33 ± 16.25 |
| Ethanol | 5.31 ± 0.68 |
| Isopropanol | 16.88 ± 3.1 |
| Propylene glycol | 53.27 ± 4.3 |
| PEG-400 | 67.62 ± 5.67 |
| **Oil** | |
| Ethyl linoleate: Oleic acid (2:1) | 1.06 ± 0.4 |
| Soyabean oil | 0.6 ± 0.3 |
| Oleic acid | 1.89 ± 0.4 |
| Acrysol K-150 | 16.29 ± 2.5 |

### 7.3.2. Construction of Pseudo-Ternary Phase diagrams

It was observed during these experiments that high concentration of oil forms poor emulsion with entrapment of very less amount of water upon dilution. Another

observation was that as concentration of S/CoS increases, the time estimated to form

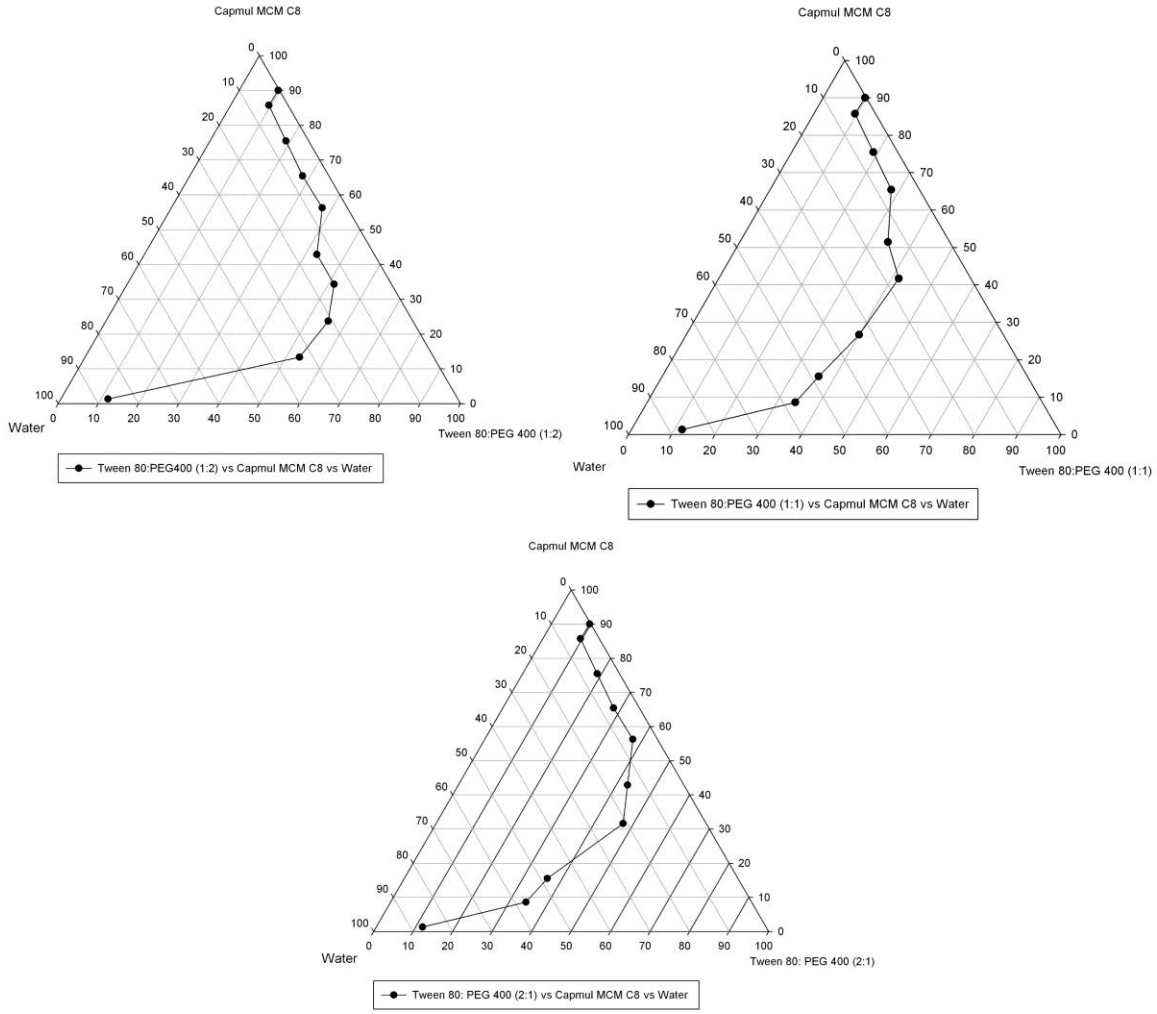



**Figure 7.5. Ternary diagram of SNEDDS containing Capmul MCM C8, Tween 80 and PEG 400**

microemulsion decreases. A series of microemulsions were prepared at different concentrations of oil and S/CoS, but concentration of oil was found to be a rate-limiting factor, and in all cases, high oil concentration resulted in poor emulsion region. Other important factors affecting microemulsions were concentration and ratio of S/CoS. In the present study, three S/CoS ratios, 2:1, 1:1, and 1:2, were tried. The phase diagram clearly shows that formulation prepared with S/CoS ratio of 1:1, cover maximum self-microemulsifying region as compared to other two ratios (Fig. 7.5). Based on the results

of above experiment and the reported concentration scope of three ingredients forming SNEDDS, the contents of surfactant, co-surfactant and oil were chosen at the range of 30–60%, 30–60% and 10–40%, respectively, in order to obtain the optimal formulation.

### 7.3.3. Experimental Design

The simplex lattice design for a three component was represented by and equilateral triangle in two dimensional space. The formulations were prepared and evaluated for their droplet size and solubility (Table 7.4).

**Table 7.4. Simplex lattice designs of independent variables with measured responses**

| Batch | Independent Variables | | | Dependent Variable | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $Y_1$ (nm) | $Y_2$ (mg/g) |
| BM1 | 45 (0.5) | 45 (0.5) | 10 (0) | 86.08 | 16.24 |
| BM2 | 45 (0.5) | 30 (0) | 25 (0.5) | 41.31 | 22.7 |
| BM3 | 40 (0.33) | 40 (0.33) | 40 (0.33) | 154.74 | 15.94 |
| BM4 | 60 (1) | 30 (0) | 10 (0) | 18.16 | 18.23 |
| BM5 | 30 (0) | 60 (1) | 10 (0) | 171.22 | 12.53 |
| BM6 | 30 (0) | 30 (0) | 40 (1) | 380.43 | 17.64 |
| BM7 | 30 (0) | 45 (0.5) | 25 (0.5) | 204.12 | 14.35 |

($X_1$) Surfactant, ($X_2$) Co-surfactant, ($X_3$) Oil ; $Y_1$=droplet size (nm), $Y_2$= solubility (mg/g)

#### *7.3.3.1. Fitting data to model*

Seven different formulations of BER loaded SNEDDS were prepared according to simplex lattice design and selected concentration range for surfactant, co-surfactant and oil. The responses for seven formulations (Table 7.4) were used to fit an equation for simplex lattice model which then can predict the properties of all possible formulations. With the aid of Design Expert software, the model equation was developed to be the best

explanation for the relationship between the solubility and particle size characteristics. The fitted results are shown in equation 7.3 for $Y_1$ and 7.4 for $Y_2$ are as follows:

**Droplet size:**

$$Y_1 = 18.23\ X_1 + 12.53\ X_2 + 17.64\ X_3 - 3.44\ X_1X_2 - 19.06\ X_1X_3 - 2.94\ X_2X_3 - 63.90\ X_1X_2X_3 \quad \text{(Eq. 7.3)}$$

**Solubiity:**

$$Y_2 = 18.16\ X_1 + 171.22\ X_2 + 380.43\ X_3 - 34.44\ X_1X_2 - 631.94\ X_1X_3 - 286.82\ X_2X_3 + 1909.29\ X_1X_2X_3 \quad \text{(Eq. 7.4)}$$

These equations show positive values for the three components indicating their positive effect on the globule size as well as solubility.

### *7.3.3.2. Contour plot and response surface analysis*

Based on the two equations (Y1 and Y2), contour plots of solubility and mean droplet size were constructed (Fig. 7.6). The contour plot of droplet size could be useful for preparing SNEDDS with various droplet sizes. To prepare the SNEDDS with small droplet size, the percentage of oil in the SNEDDS should be low while high level of S/CoS ratio is desired for small particle size. In addition, drug content and loading capacity of the SNEDDS could also be increase by increase in S/CoS ratio. However, the solubility of drug in the formulation relies on the solubilization capacity of overall system. The SNEDDS containing high amount of surfactant enables to incorporate large amount of drugs in the formulations.[271] The response surface of solubility and droplet size indicated that low level of oil and medium level of S/CoS resulted in stable formulation with desired responses.

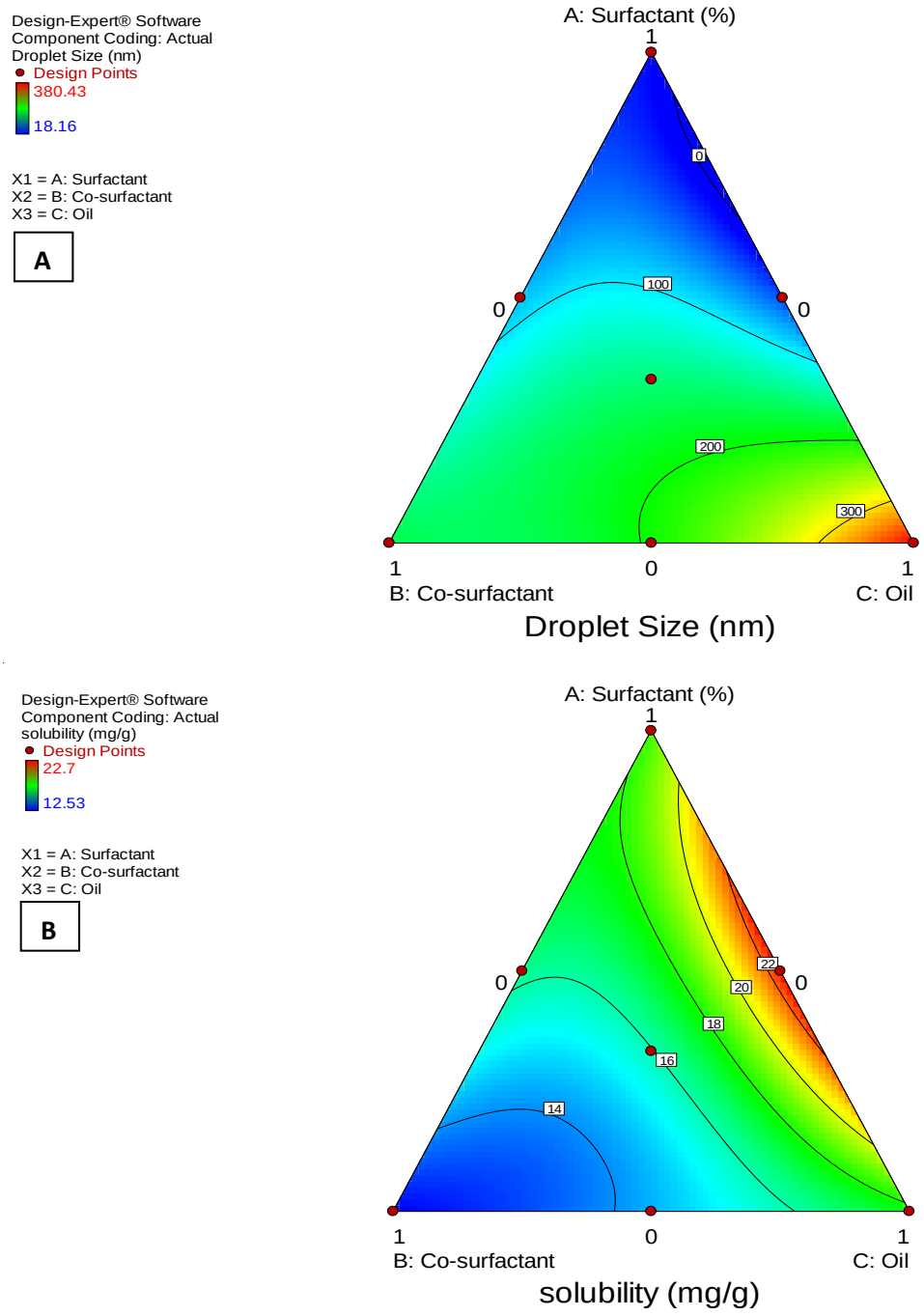



**Figure 7.6. Contour plot shows effect of $X_1$, $X_2$ and $X_3$ on and droplet size (A) solubility (B)**

### *7.3.3.3. Optimization of formulation*

The search for the optimized formulation composition was carried out using the desirability function approach with Design expert software, criterion being one having

the maximum desirability value. The optimization process was performed by setting the $Y_1$ at minimum and $Y_2$ at maximum while $X_1$, $X_2$ and $X_3$within the range obtained. The optimized formulation was achieved at $X_1$=56.2, $X_2$=30 and $X_3$=22.81 with the corresponding desirability (*D*) value of 0.997 (Fig. 7.7). This factor level combination predicted the responses $Y_1$=18.16 nm, $Y_2$= 22.64 mg/g.

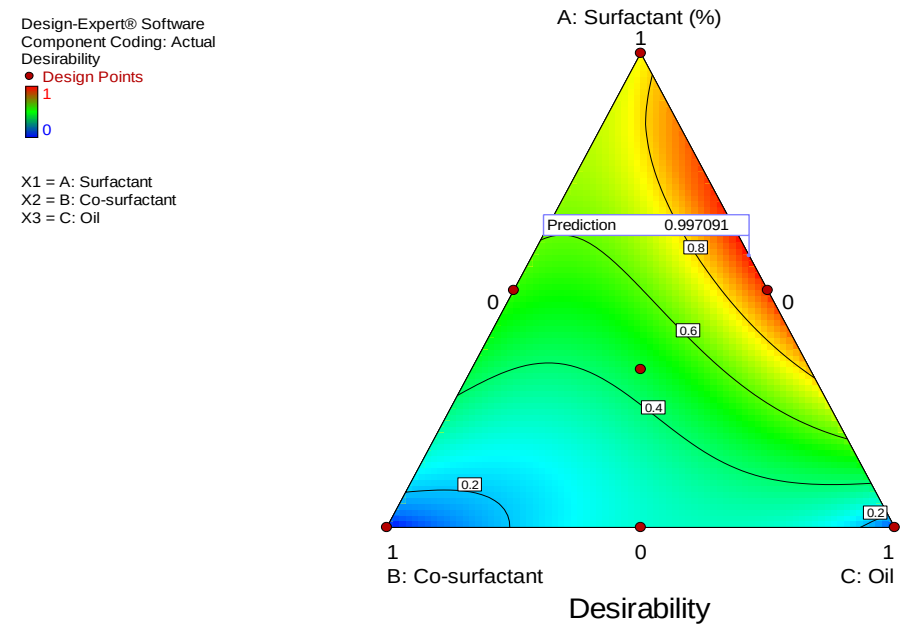



**Figure 7.7. Contour plot for overall desirability of SNEDDS as a function of $X_1$, $X_2$ and $X_3$**

### *7.3.3.4. Checkpoint analysis*

Finally, to confirm the validity of the optimal parameters and predicted responses calculated, optimized formulations were prepared and evaluated for responses. The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of SNEDDS formulations (Table 7.5). The composition of optimized formula was also given in Table 7.5.

**Table 7.5. Checkpoint batch with their predicted and observed value of responses**

| | Independent Variables | | | Droplet size ($Y_1$) (nm) | | Solubility ($Y_2$) (mg/g) | |
|---|---|---|---|---|---|---|---|
| **Batch** | **$X_1$** | **$X_2$** | **$X_3$** | **Observed** | **Predicted** | **Observed** | **Predicted** |
| BM10 | 0.573 (56.2) | 0 (30) | 427 (22.81) | 20.54 | 22.64 | 21.54 | 18.16 |
| **Percentage prediction error (%)** | | | | -10.22 | | 15.69 | |

### 7.3.4. Characterization of Optimized Formulation

#### *7.3.4.1. Spectroscopic characterization of optical clarity*

SNEDDS was diluted with various media to confirm the formation of microemulsion with the external phase of the system without phase separation. A clear o/w micro-emulsion was formed in dilution media. On 100 fold dilution percent transmittance of the studied aqueous dispersion of BER SNEDDS was found to be 97.307 with distilled water, 96.821 with 0.1N HCl and 96.356 with phosphate buffer pH (6.8).

#### *7.3.4.2. Drug content*

Assay of berberine SNEDDS was carried out by UV-visible spectrophotometer. The amount of drug content in the optimized formulation was found to be in the range of 98.81-101.33% indicating the suitability of the system for high entrapment in the internal phase. The higher entrapment of BER may be attributed to the solubilization capacity of Capmul MCM C8, which dissolves the BER to a greater extent than conventional vegetable oils. The addition of PEG may also responsible for improvement of BER solubility in the lipid vehicle.

#### *7.3.4.3. Robustness to Dilution*

Diluted SNEDDS did not show any precipitation or phase separation on storage in various dilution media. This reveals that all media were robust to dilution. The optimized

formulations spontaneously formed self-emulsion of small globule size, which influence the absorption of BER. This may be due to the Capmul MCM C8 and PEG 400 which increased the solubilization capacity of BER and high kinetic stability of SNEDDS on dilution.

#### *7.3.4.4. Determination of droplet size, polydispersity index, and zeta potential*

The droplet size of the formulation is a critical factor for self-emulsification process as it determines the rate and extent of drug release as well as absorption.[242, 272] Photon correlation spectroscopy (PCS) is used to determine the droplet size of SNEDDS specifically for the emulsion which properties do not change upon dilution.[273-276] In the present study, the average droplet size of optimized formulation (BM10) was found to be 20.54 nm with polydispersity index of 0.269 (Fig. 7.8) and zeta potential of -40.20 mV (Fig.7.9).

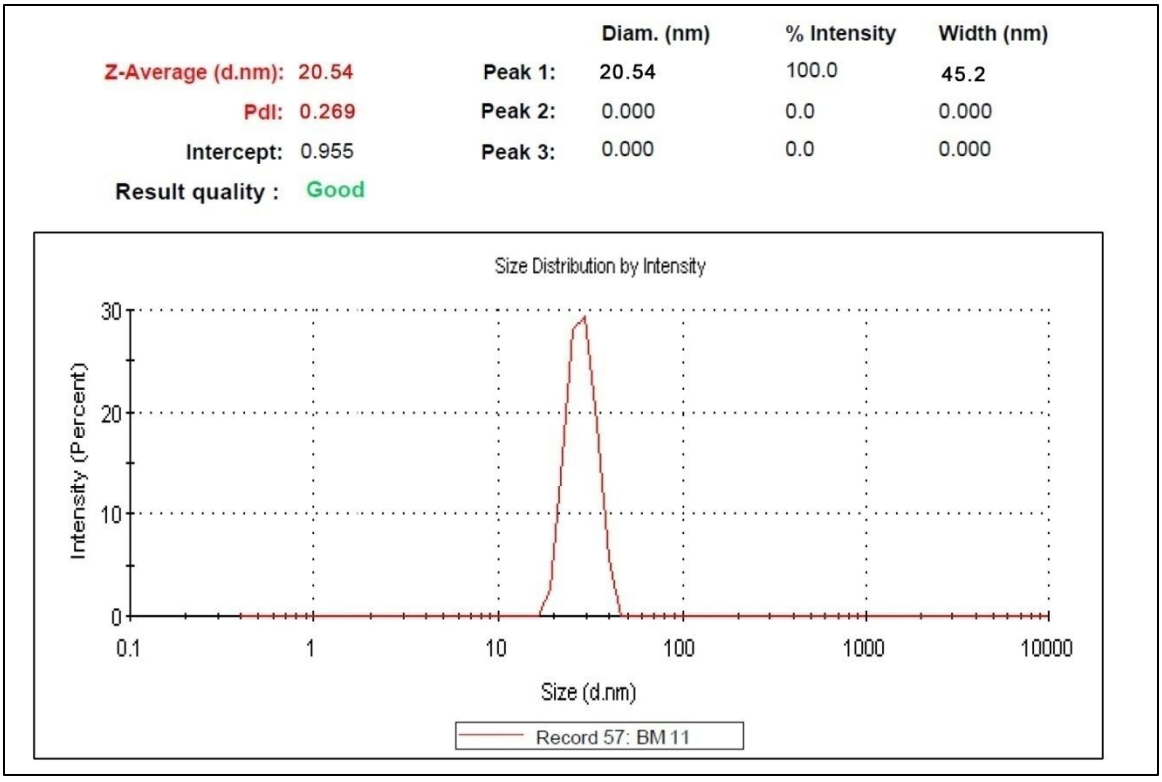



**Figure 7.8. Particle size and polydispersity index of optimized formulation (BM10)**

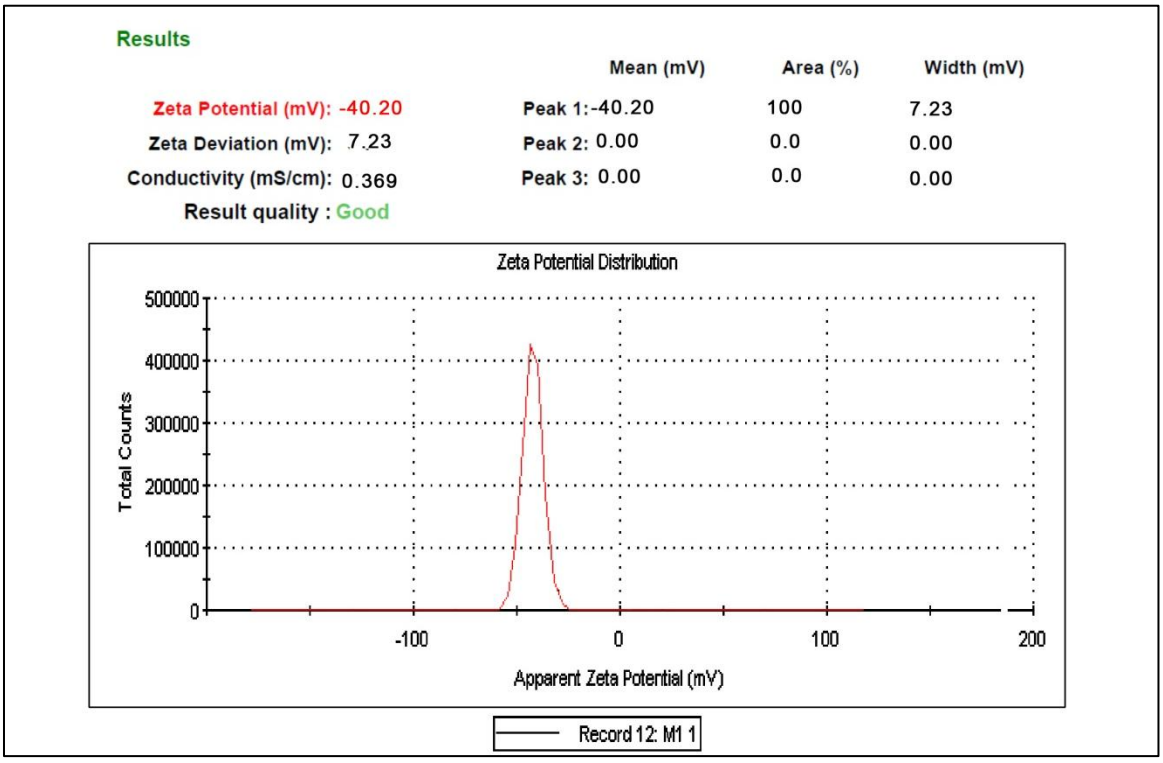



**Figure 7.9. Zeta potential of optimized formulation (BM10)**

### *7.3.4.5. Transmission electron microscopy (TEM)*

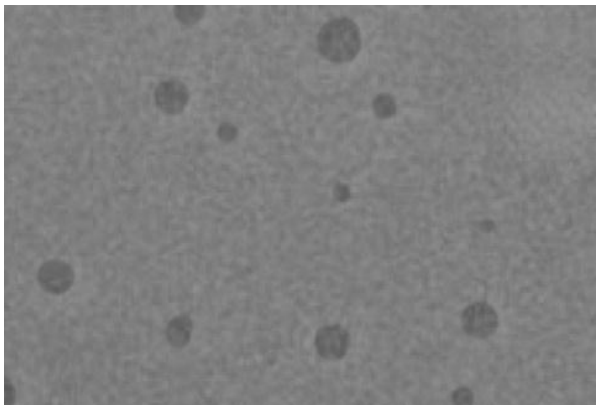


**Figure 7.10. TEM of berberine loaded SNEDDS (BM10)**

Morphology of the microemulsions formed from optimized SNEDDS-containing BER (BM10) was viewed under a TEM, the microemulsion vesicles appeared as perfect round shape without aggregation (Fig. 7.10). Smaller globule size may be attributed to high zeta potential of formulation which prevents coalescence of globule.

### *7.3.4.6. Electrical conductivity*

Electrical conductivity of emulsion was increased significantly after loading BER (BM10). Based on the difference in electrical conductivity of micro emulsification from SNEDDS before and after loading BER, it can be concluded that o/w microemulsion could be formed when SNEDDS was diluted with distilled water.

### *7.3.4.7. Viscosity determination*

Viscosity is crucial in determining its ability to be filled in hard or soft gelatin capsules. The viscosity of undiluted liquid SNEDDS at room temperature was 978 cP. Since this value was less than 10 000 cP, it implied that the developed SNEDDS can be filled in hard-gelatin capsules by commercial liquid filling equipments without leaking problem. When SNEDDS was diluted 10 times and 100 times with water, viscosity of SNEDDS was decreased indicates that when formulation will be diluted with the stomach fluid its viscosity will be decreased and therefore absorption from stomach will be fats.

### *7.3.4.8. In vitro dissolution studies*

*In vitro* release of BER from optimized SNEDDS and drug suspension in buffer of pH 1.2 and 6.8 was determined by using dialysis bag method. Dissolution studies revealed remarkable increase in dissolution of the drug as compared to plain drug in buffers of pH 1.2 and 6.8 (Fig. 7.11). The data shows that release of BER was similar in both media, at 10 min about 84% of BER from SNEDDS was dissolved in medium, and more than 90% was release after 30 min. The results indicated that SNEDDS could form quickly clear and transparent solution under the dissolution conditions. It was also evident that pH has no significant effects on release patterns of BER.

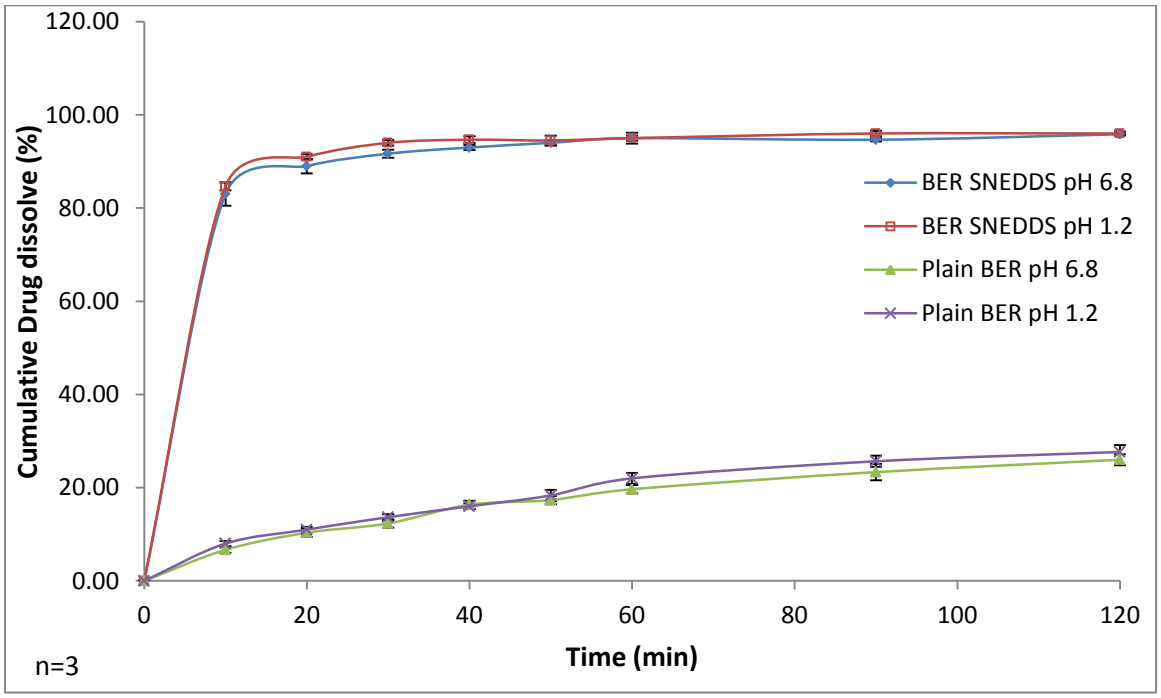



**Figure 7.11. *In vitro* release of plain berberine and berberine loaded SNEDDS at pH 6.8 and pH 1.2**

### 7.3.5. Stability Studies

The stability was assessed by analyzing the physical appearance and drug content at day 0, 15 and 30. The formulation does not show any change in the physical appearance as

well as drug content indicated stable formulation (Table 7.6). The optimized formulation was also subjected to thermodynamic stability tests in different stress conditions such as centrifugation, heating cooling cycle and freeze-thaw cycle. The formulation did not show any phase separation in all stress condition indicates the stable system.

**Table 7.6. Stability study of optimized formulation (BM10)**

| | **4 °C/75 % RH** | | **30 °C/75 % RH** | | **40 °C/75 % RH** | |
|---|---|---|---|---|---|---|
| | **Physical Appearance** | **Drug content (%)** | **Physical Appearance** | **Drug content (%)** | **Physical Appearance** | **Drug content (%)** |
| **0 days** | No change | 98.81 | No change | 98.81 | No change | 98.81 |
| **15 days** | No change | 98.67 | No change | 98.79 | No change | 98.78 |
| **30 days** | No change | 98.61 | No change | 98.76 | No change | 98.75 |

# 8. *IN VITRO* PERMEABILITY AND *IN VIVO* BIOAVAILABILITY

## 8.1. Introduction:

The body contains many biological barriers that serve to protect its interior from a variety of external invaders and toxins. The skin is the largest such obstacle, while the blood-brain barrier forms the tightest barrier to penetration of molecules from the blood stream to the brain. Similarly, for a drug molecule to be orally bioavailable, it has to traverse the epithelial layer of the gastrointestinal tract. Thus, many factors for enhancing the delivery of molecules through this intestinal mucosal barrier must be considered.[(277)] The intestine is the most important site for drug absorption and regulates the extent of orally administered drug that reaches the circulation.

Several different obstacles must be overcome for the delivery of drugs through the intestinal mucosa or the blood-brain barrier. These obstructions to drug delivery can be categorized as physiological, biochemical, and chemical barriers. The physical barrier arises from cell membranes and the intercellular junctions between the cells (e.g. tight junctions). Permeation of drugs across the intestinal epithelium is restricted to paracellular and transcellular pathways depending on their physicochemical properties (e.g. size, charge, lipophilicity, and conformation). Most large molecules that are hydrophilic are prevented from passing across the cell membranes unless some specific membrane proteins are involved to serve as channels, carriers, or transporters. Only lypophilic molecules may directly pass across the lipid bilayer of the cell membranes by passive diffusion. In addition to the physical barrier, the intestinal epithelium also possesses various metabolic enzymes (e.g. intestinal peptidases, cytochrome P450) and polarized efflux systems (e.g. *p*-glycoprotein, *P*-gp) which act as biochemical barriers

further limiting drug absorption in the intestine. Consequently, many drug candidates are restricted from oral dosing in clinical development owing to this biological barrier. Finally, the drug has to have optimal physiochemical properties for its permeation across the biological barriers. Thus, these various barriers have to be taken into account when designing drugs with improved absorption characteristics.[278]

Lipid based formulation is an effective approach for optimization of oral drug delivery. For commercial success and enhance the development potential of lipid based formulation, it is essential to develop confidence amongst the industry for these delivery system.[279] To fulfill this objective, it is necessary to established *in vitro/ in vivo* correlations that shortened drug development period and improved product quality. Determining the dissolution, solubility, lipolysis of lipid excipients, intestinal membrane techniques (isolated animal tissue and cell culture models) are various *in vitro* techniques that can be used to asses lipid based formulations.[280] These techniques provide information about specific aspects of the formulation only not about *in vivo* interaction and performance of these systems.

On the other hand, *in vivo* studies performed with humans and laboratory animals are expensive, time consuming and often even unethical, *in vitro* methods, as accurate as possible, are needed in screening of new drug candidates. Immortalized, often of cancer origin, animal and human cell cultures have been used for estimation and prediction of human drug absorption. Several possible *in vitro* human cell models are available for this purpose, one of which is the Caco-2 cell model, a well characterized cell line. According to Biopharmaceutics Classification System (BCS) and FDA approval, Caco-2 cells can be used as a screening method for new drug candidates during drug discovery and

development.[81, 281, 282] For the suitability and reliability of the method, permeability of several model compounds with known intestinal absorption in humans has to be demonstrated. FDA recommends the use of compounds with high, low, and zero permeability, passive and active transport, and use of efflux markers for this purpose. The simultaneous use of model compounds requires that they do not express cytotoxicity, do not interact with each other during permeation, and that they are easily detected. Therefore, the use of different sets of model compounds has to be validated before the actual experiments with drug candidates can be performed.

### 8.1.1. *In vitro* Caco-2 method

Various *in vitro* methods are listed in United States FDA guidelines, acceptable to evaluate the permeability of a drug substance, includes monolayer of suitable epithelial cells. One such epithelial cell line that has been widely used as a model system of intestinal permeability is the Caco-2 cell line. Since most drugs are known to absorb via intestines without using cellular pumps, passive permeability models have came into the limelight. In the 1990s membrane-based drug assays led to the passage of drugs through the intestinal mucosa and an important Caco-2 assay emerged in pharmaceutical research.[283]

In a typical Caco-2 experiment, a monolayer of cells is grown on a filter separating two stacked micro well plates. The permeability of drugs through the cells is determined after the introduction of a drug on one side of the filter. The entire process can be automated, and when used in conjunction with chromatography and/or mass spectroscopy detection, it enables any drug's permeability to be determined.

The Caco-2 cell line, which exhibits a well-differentiated brush border on the apical surface and tight junctions, and which expresses typical small-intestinal microvillus hydrolases and nutrient transporters, has proven to be the better *in vitro* model for the following reasons: (a) to rapidly assess the cellular permeability of potential drug candidates (b) to elucidate pathways of drug transport (e.g., passive versus carrier mediated) (c) to assess formulation strategies designed to enhance membrane permeability (d) to determine the optimal physicochemical characteristics for passive diffusion of drugs,(e) to assess potential toxic effects of drug candidates or formulation components on this biological barrier.

Since differentiated Caco-2 cells express various cytochrome P450 isoforms and phase II enzymes such as UDP-glucuronosyltransferases, sulfotransferases and glutathione-S-transferases, this model could also allow the study of presystemic drug metabolism.

The Caco-2 cell model has the advantages of simplicity and reproducibility. US FDA recognizes Caco-2 to measure permeability as part of the bioequivalence waiver process.

### 8.1.2. *In vivo* method

In spite of tremendous innovations in the field of drug delivery and the acquisition of detailed knowledge about promising alternative routes of administration, it is estimated that 90% of all medicine usage is in oral form and oral drug delivery systems comprise more than half the drug delivery market.[(284)] Thus, oral bioavailability plays an imperative role for successful therapy by this route. Oral bioavailability depends on number of factors like aqueous solubility, dissolution rate, residence time, drug

permeability, presystemic metabolism, first pass metabolism and susceptibility to efflux mechanisms. In addition, different characteristics of drugs such as size, density, pH, diffusion, swelling, adhesion, and degradation can also be modified to enhance the oral bioavailability. Thus, only *in vitro* evaluation will not be able to predict exact role of nanoparticles in improving bioavailability. The impact of excipients on the bioavailability and pharmacokinetic profile of drug can be estimated by designing appropriate *in vivo* studies.

Bioavailability is the ratio of the area under curve (AUC) after administration by the route of interest and after administration of the same amount of drug direct into the systemic circulation, usually by intravenous injection. Bioavailability is one of the principal pharmacokinetic properties of drugs. It is a subcategory of absorption and it is the processes that are involved in transferring the drug in solution from the site of administration to the venous blood. Relative bioavailability or bioequivalence is the most common measure for comparing the bioavailability of one formulation of the same drug to another. The mean responses such as $C_{max}$ and AUC are compared to determine relative bioavailability. The AUC refers to the extent of bioavailability while $C_{max}$ refers to the rate of bioavailability

## 8.2. Methodology

### 8.2.1. *In vitro* Intestinal Permeability

Possible intestinal absorption enhancement of drug incorporated with liposome, SLN and SNEDDS were assessed with drug transport studies. Test system for permeability study is shown in Figure 8.1.

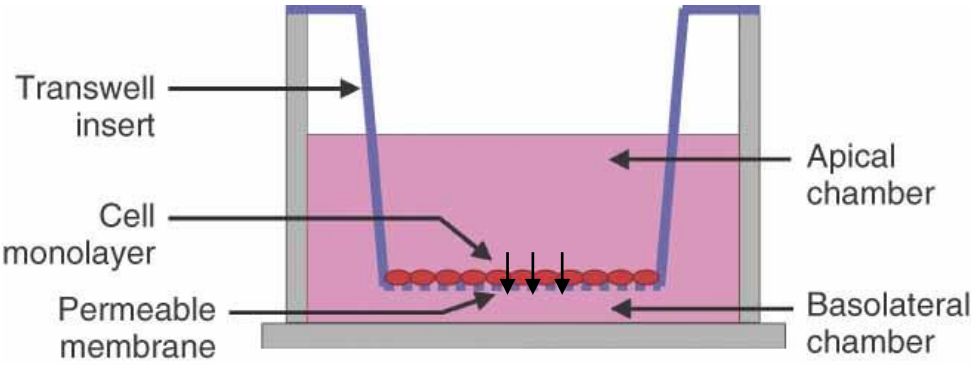



**Figure 8.1. Permeability test system in Transwell plate**

### *8.2.1.1. Drug transport measurements across Caco-2*

Transport across intestinal epithelial cells was tested using model drug compounds Berberine. Cells were seeded at 2 x $10^6$ cells/mL and cultured on 24 well plates Transwell® permeable supports (0.4 um pore size) for 27 days. Cell culture medium was changed every other day. On day 27, cell culture medium was removed from both the apical and basolateral compartments, and cells were rinsed once with HBSS. For the experiment with Caco-2 monolayers, berberine and its formulation with and without berberine in HBSS were added to the apical compartment of Transwell plate. The formulations were added at a dose equivalent to 100 µM berberine. The basolateral compartment solution was replaced with HBSS. The cells were exposed to formulation for 2 hours inside the incubator at 37$^o$C. After three hours, samples were taken from basolateral compartment and analyzed for drug content using a HPLC. All the experiments were conducted in triplicate. Apparent permeability coefficient, $P_{app}$, of drug for each formulation was calculated according to the following equation 8.1:

$$\boldsymbol{P_{app} = (dQ|dt)(1|AC_0 * 60)} \quad \textbf{..... (Eq. 8.1)}$$

where dQ/dt is the cumulative transport rate (µmol/min, nmol/min or µg/min) defined as the slope obtained by linear regression of cumulative transported amount as a function of time (min), A is the surface area of

the monolayers, $C_0$ is the initial concentration of the compounds on the donor side (µmol/mL, nmol/mL or µg/mL), and 60 is the coefficient when minutes are converted to seconds.

The concentration of the transported drug was measured from A-B and B-A, i.e., $P_{app}$ (AB) and $P_{app}$ (BA), respectively, and the efflux ratio (ER) was calculated from the following Eq. 8.2.

$$\mathbf{ER} = \frac{P_{app(AB)}}{P_{app\ (BA)}} \quad \textbf{..... (Eq. 8.2)}$$

Absorption enhancement ratio (*R*) was calculated by eqation 8.3:

$$\mathbf{R} = \frac{P_{app(Sample)}}{P_{app(Control)}} \quad \textbf{..... (Eq. 8.3)}$$

### *8.2.1.2. Monolayer Integrity test*

At the end of the experiment, the monolayer integrity test was done by analyzing the concentration of Lucifer Yellow (LY) in the apical and basolateral compartments. An initial stock solution of LY (50 mM) was prepared and diluted to 100 µM working solution. Four hundred microliter of the 100 µM working solution of LY was added to the apical side of Caco-2 cell monolayer (in the wells in which drug transport study was performed), and 800 µL of HBSS buffer was added to the basolateral side. The plate was then kept in an incubator at 37$^{o}$C. After 120 min, 700 µL and 300 µL of the samples were withdrawn from the basolateral side and apical side, respectively. The samples were analyzed by fluorescence spectroscopy at an excitation wavelength ($\lambda_{ex}$) of 485 nm and emission wavelength ($\lambda_{em}$) of 535 nm using a Spectrofluorophotometer (RF-5301-PC, Shimadzu, Kyoto, Japan).

### 8.2.2. Bioavailability Study

Thirty six male Wistar rats weighing 220–240 g were fasted overnight for at least 12 h, with free access to water, and randomly divided into three groups for oral administration. The drug suspension (control), formulation I (SLN) and formulation II (SNEDDS) were administered by oral gavage at a dose of 50 mg/kg. The rats will be anaesthetized using ether anesthesia. Blood samples (approximately 0.3 mL) will be withdrawn from the retro-orbital plexus at 0 (pre-dose), 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 24 and 36 h. The blood was collected into a 2 mL heparinized Eppendrof tubes and centrifuged at 4000 rpm for 10 min. The supernatant plasma was collected and stored at -20$^{o}$C for later analysis. The analysis of samples of pharmacokinetic studies was performed as per the HPLC method given in section 4.1.7.

### 8.2.3. High fat diet induced hyperlipidemia

Twenty four Wistar rats of either sex (200-250 g), will be placed in four groups (n=6). Negative control, toxic control, standard and test will receive distilled water, cholesterol, drug suspension along with cholesterol and formulation along with cholesterol, respectively. Hyperlipidemia will be induced by the use of high fat diet containing 200mg of cholesterol suspended in 2 mL of coconut oil for 14 days. Treatment will be given orally, using 18-gauge oral feeding needle, 2 h after the administration of high fat diet. After fourteen days of treatment, the rats will be anaesthetized using light ether anesthesia and blood samples (0.5 mL, once) will be withdrawn from retro orbital plexus. The biochemical parameters such as serum lipid level like total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density

lipoprotein cholesterol (LDL-C) level was were estimated by standard diagnostic kit (SPAN Diagnostic and Crest Biosystem, India).

## 8.3. Results and Discussion

### 8.3.1. *In vitro* Intestinal Permeability

Intestinal absorption enhancement of drug incorporated with liposome, SLN and SNEDDS were assessed with *in vitro* transport studies (Fig. 8.2). It was observed that the permeability coefficient for plain BER was $0.74\times10^{-6}$ cm/s in the absorptive direction (A→B), whereas it was $2.84\times10^{-6}$ cm/s in the secretory transport (B→A). The efflux ratio was 3.84 indicates the low absorption of BER from intestine suggesting the P-gp efflux of drug. However, permeability coefficient for BER loaded liposome, SLN and SNEDDS were higher than for plain BER in both direction. This is consistent with the presence of several excipients in lipid based formulations indirectly inhibit P-gp through effects on the lipid membrane and thus enhance the intestinal membrane permeability and oral absorption of the substrate drug.Increased drug absorption through the intestinal mucosa is often associated with damage caused to the intestinal cells and to their barrier function. The effect of different formulations on the monolayer integrity was examined by measuring the permeability of the paracellular leakage marker, Lucifer yellow across the monolayers. The apparent permeability coefficient ($P_{app}$) for Lucifer yellow was more than $1\times10^{-6}$cm/s for formulations while it was less in plain BER. This implied that formulations may affect the paracellular route through the opening of tight junctions and thus reduce the cell integrity of Caco-2 cells. However, $P_{app}$ values measured 48 h after transport experiment (recovery) revealed that all the monolayers fully recovered. This

indicated that although the formulations affected the tightness of the cell monolayer, it reversibly recovered after the experiment.

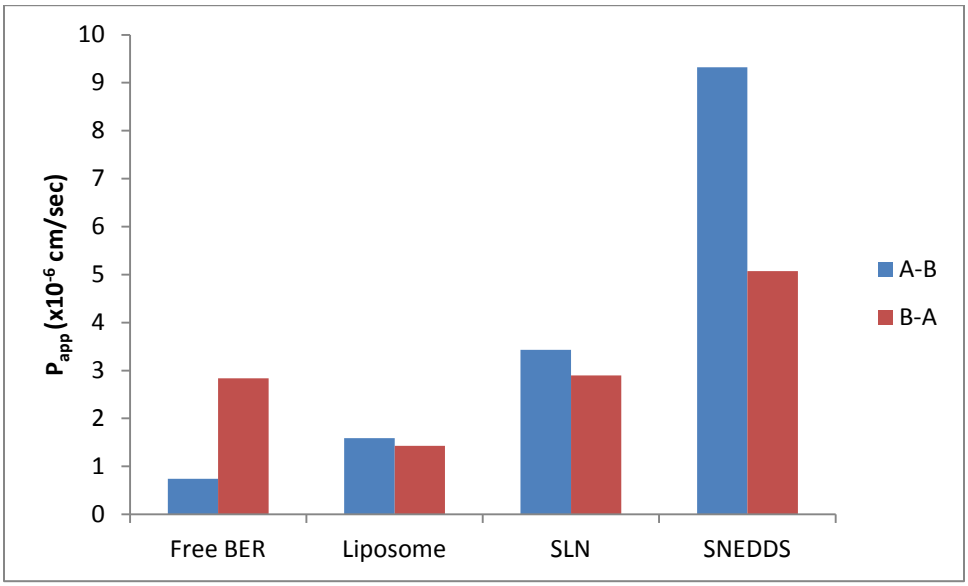



**Figure 8.2. $P_{app}$ of plain berberine and berberine loaded liposome, SLN and SNEDDS**

### 8.3.2. Bioavailability Study

Based on the permeability study, SLN and SNEDDS were selected for *in vivo* bioavailability study.The results of single dose bioavailability studies showed $C_{max}$ for SLN and SNEDDS was found to be 192.32 ± 5.25 ng/mL and 391.12 ± 22.64 ng/mL respectively, which was significantly higher than the plain drug solution 66.88 ± 2.15 ng/mL (Table 8.1). A higher $C_{max}$ for formulations could be achieved as drug loaded in SLN and SNEDDS was capable to bypass hepatic first pass metabolism and able to reach directly tosystemic circulation by virtue of size and surface properties of nanocarrier system. $T_{max}$ for SLN and SNEDDS were found to be 2 and 1.5 h respectively, while for plain drug solution was found to be 2 h (Fig. 8.3). $AUC_{0-36}$ for SLN and SNEDDS were found to be 1383.44 ± 139.68 ng.h/mL and 2921.74 ± 319.9ng.h/mL, which is significantly ($P<0.05$) higher than $AUC_{0-36}$ for plain drug solution; 334.41 ± 44.35

ng.h/mL (Table 8.1). Improvement in bioavailability could be attributed to ability of lipid based formulations to reach the oral lymphatic region after absorption and reaching to systemic circulation. Thus, lipid based formulations could play important role inenhancement of its bioavailability. From this data it can be concluded that the SNEDDS are effective tool for enhancing bioavailability of BER.

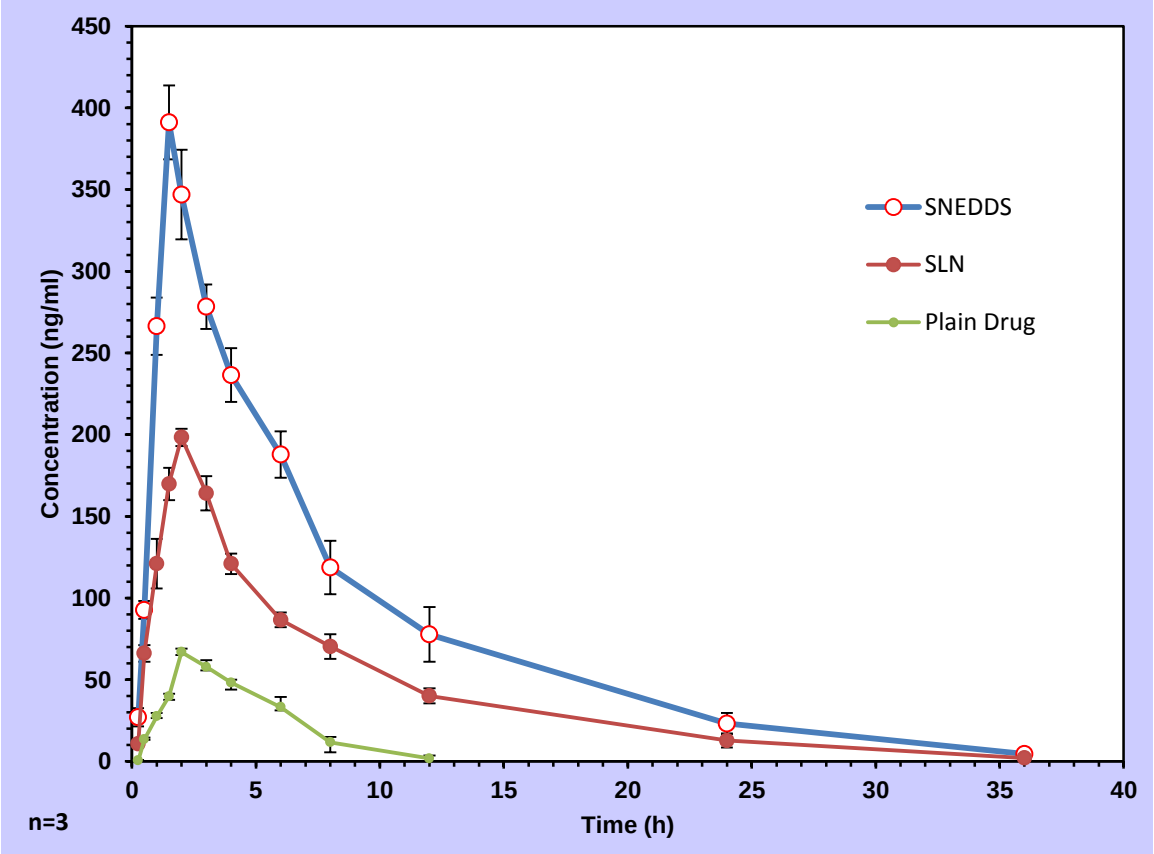
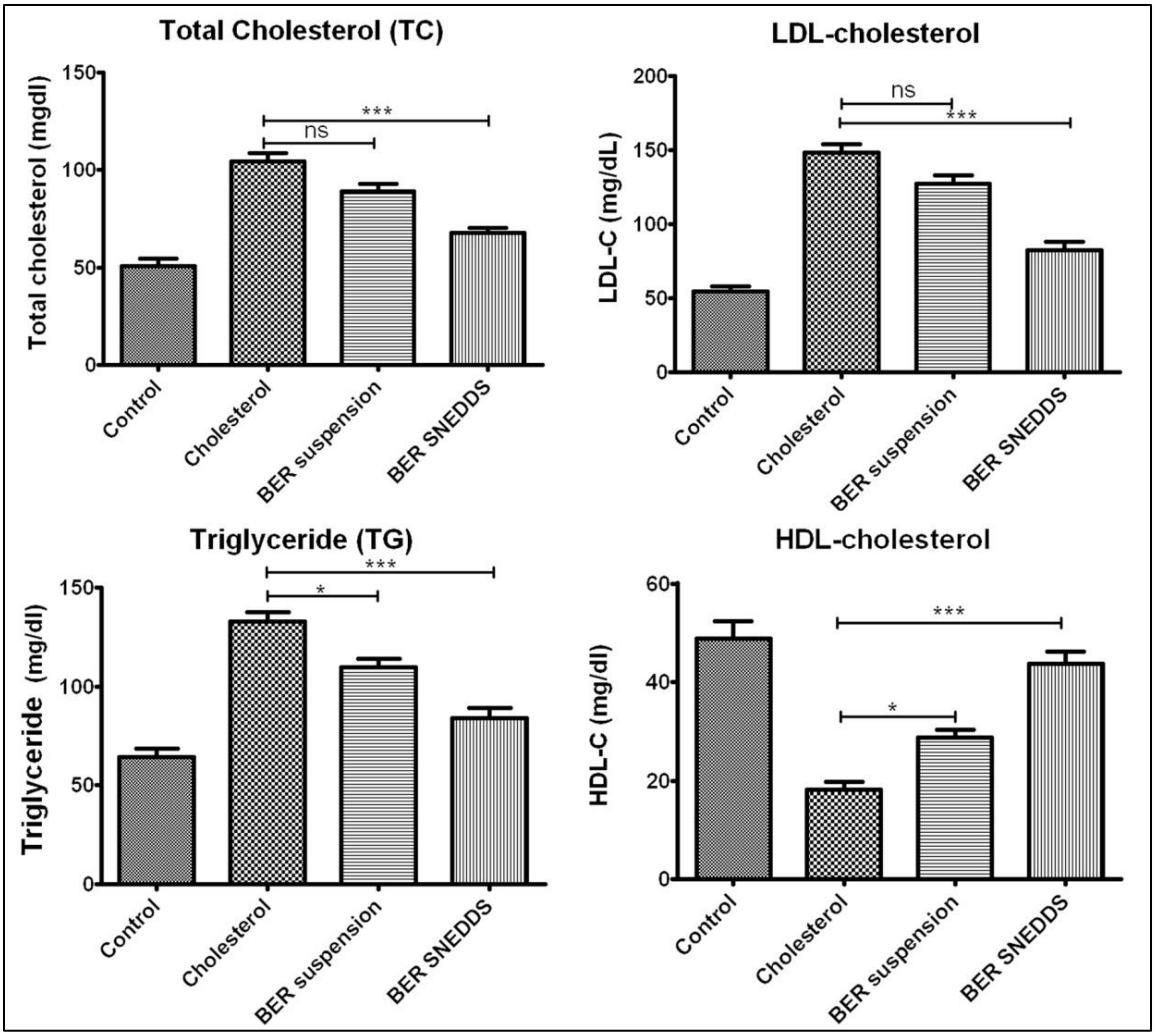



**Figure 8.3. Bioavailability study of plain BER, SLN and SNEDDS**

**Table 8.1. Pharmacokinetic parameters for single dose oral bioavailability of plain BER, SLN and SNEDDS**

| | $C_{max}$± SD (ng/mL) | $T_{max}$ (h) | $AUC_{0-36}$± SD |
|---|---|---|---|
| **Plain Drug** | 66.88±2.15 | 2 | 334.41±44.35 |
| **SLN** | 192.32±5.25** | 2 | 1383.44±139.68** |
| **SNEDDS** | 391.12±22.64 ** | 1.5 | 2921.74±319.9** |

**P<0.01




*P<0.05, ***P<0.0001, ns=non significant

**Figure 8.4.Effect of berberine loaded SNEDDS on plasma lipid levels of high-fat diet induced hyperlipidemic rats**

### 8.3.3. Anti-hyperlipidemic activity

The selected formulation was used to check the effect of formulation on high fat diet induced hyperlipidemia in rats. Induction of hyperlipidemia was confirmed from the increase in TC, TG, LDL and decreased in level of HDL in control. Treatment with BER loaded SNEDDS (100 mg/kg) significantly ameliorate the level of TC, TG, HDL and LDL compared to hyperlipidemic control (Fig. 8.4). These results indicate that the prepared SNEDDS was more efficient in controlling hyperlipidemia as compared to plain

drug and this can be attributed to enhance bioavailability. Hence BER loaded SNEDDS can be exploited as an antihyperlipidemic therapeutic agent or adjuvant in existing therapy for the treatment of hyperlipidemia.

# 9. SUMMARY AND CONCLUSION

With the therapeutic potential of natural health products, botanicals have a major role to play in the management of varied diseases. Development of valuable drug delivery system from natural resources is very much necessary because of the beneficial role of herbal drug in the management of varied diseases. Untiring efforts have been given to invent useful delivery system of potent herbal molecules which can maximize their healing property.

Different bio-molecules obtained from plants are known to possess several pharmacological activities, but a severe limitation exists in oral absorption of these active constituents. However, most drugs of plant origin possess poor solubility and hydrophobic property lead to lower bioavailability and increased systemic clearance, requiring repeated administration or increased dose, as a result of which the clinical use of herbal medicines is limited and cannot be solved by classical formulations. So, extensive research in the field of herbal drug delivery system to improve the therapeutic indices of these phytoconstituents is of prime importance.

Berberine, a quaternary protoberberine isoquinoline alkaloid, reported to have a multitude of biological effects. Recently, it has been reported that berberine helps in reducing cholesterol and lipid accumulations in both the plasma and in the liver. Although berberine has wide-ranging therapeutic potential, poor absorption characteristic and the significant first-pass metabolism limits its bioavailability. The present study was aimed to develop lipid based formulation of berberine to enhance therapeutic efficacy. Lipids and lipophilic excipients can have significant and beneficial effects on the absorption and exposure of co-administered drugs.

Pre-formulation studies were carried out to validate the drug information provided by supplier in accordance with the standard specification. These can be useful in the development of formulation. Previously developed analytical and bioanalytical methods were employed to estimate berberine. The method was performed in house and validate by precision and accuracy. The methods were found to be simple, accurate and precise.

Liposome, solid lipid nanoparticle (SLN) and self emulsifying drug delivery system (SEDDS) were prepared and optimized by factorial design.

The liposome was prepared by thin film hydration method and optimized by $3^2$ factorial designs consisting of drug: lipid molar ratio ($X_1$) and SPC: cholesterol ($X_2$) as a dependant variables while vesicle size ($Y_1$) and entrapment efficiency ($Y_2$) as response.

The SLN were prepared with stearic acid by solvent injection method. The formulations were optimized by $3^2$ factorial designs consisting of polyvinyl alcohol (PVA) concentration ($X_1$) and amount of lipid ($X_2$) as a dependant variables at 3 different levels as low (-1), medium (0) and high (1), while particle size ($Y_1$) and entrapment efficiency ($Y_2$) as response.

A simplex lattice experiment design was adopted to optimize SEDDS. The concentrations of surfactant ($X_1$), co-surfactant ($X_2$) and oil ($X_3$) were chosen as the independent variables. The droplet size ($Y_1$) of formed microemulsion by diluting SNEDDS with distilled water and solubility of BER in SNEDDS ($Y_1$) were taken as responses, respectively.

All the responses were simultaneously fitted to linear, cubic and quadratic models using Design Expert software. Polynomial equations were established according to best-

fitted model. Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert® software. Statistical models were generated for each response parameter and tested for significance. The obtained results can be observed visually in the contour plots and surface plots. Finally, the search for the optimized formulation composition was carried out using the desirability function approach with Design expert software, criterion being one having the maximum desirability value. The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of formulations.

Characterizations of optimized formulations were carried out for different parameters. Finally, the stability study of formulation was performed at different condition. All the formulations were found to be stable at the condition used in present investigations. However, long term stability should be necessary for further evaluation of product.

In this study, possible intestinal absorption enhancement of drug incorporated with liposome, SLN and SNEDDS were assessed with drug transport studies. It was observed that the permeability coefficient for plain berberine was $0.74\times10^{-6}$ cm/s in the absorptive direction (A→B), whereas it was $2.84\times10^{-6}$ cm/s in the secretory transport (B→A). The efflux ratio was 3.84 indicates the low absorption of berberine from intestine suggesting the P-gp efflux of drug. However, permeability coefficient for berberine loaded liposome, SLN and SNEDDS were higher than for plain berberine in both direction.

Single dose oral bioavailability studies showed significant improvement in $C_{max}$ and $AUC_{0-36}$ for SLN and SNEDDS compared to plain drug solution. However $T_{max}$ for SNEDDS was found to be reduced from plain drug which was unchanged for SLN. Improvement in bioavailability could be attributed to ability of lipid based formulations to reach the oral lymphatic region after absorption and reaching to systemic circulation. Thus, lipid based formulations could play important role in enhancement of its bioavailability. From the bioavailability data it can be concluded that the SNEDDS are better than SLN for enhancing bioavailability of BER.

The selected formulation was used to check the effect of formulation on high fat diet induced hyperlipidemia in rats. Induction of hyperlipidemia was confirmed from the increase in TC, TG, LDL and decreased in level of HDL in control. Treatment with berberine loaded SNEDDS (100 mg/kg) significantly ameliorate the level of TC, TG, HDL and LDL compared to hyperlipidemic control. These results indicate that the prepared SNEDDS was more efficient in controlling hyperlipidemia as compared to plain drug and this can be attributed to enhance bioavailability.

In conclusion, this work could be a contribution towards the enhancement of bioavailability of BER used in treatment of hyperlipidemia. By improving the systemic availability of purportedly healthful phytochemicals, lipid based formulation strategies can dramatically improve the efficacy of botanical dietary supplements. Lipid based formulation significantly enhance the permeation through intestinal barrier by inhibiting P-gp leading to enhance bioavailability. Thus, lipid based formulation of BER could be used as an oral hypolipidemic drug, benefiting the patients by avoiding repeated and high dose administration. Future studies that verify the clinical efficacy of such dosage forms

may not only bolster the public's confidence and use of nutraceuticals but also quell the scientific community's current skepticism about these products.

# 10. REFERENCES


1. Solecki RS. Shanidar IV, a Neanderthal Flower Burial in Northern Iraq. Science. 1975;90(4217):880-1.
2. Bailey RL, Gahche JJ, Lentino CV, Dwyer JT, Engel JS, Thomas PR, et al. Dietary supplement use in the United States, 2003–2006. The Journal of nutrition. 2010:jn. 110.133025.
3. Biesalski H-K, Dragsted LO, Elmadfa I, Grossklaus R, Müller M, Schrenk D, et al. Bioactive compounds: Safety and efficacy. Nutrition. 2009;25(11):1206-11.
4. Yeung KS, Gubili J, Cassileth B. Evidence-based botanical research: applications and challenges. Hematology/oncology clinics of North America. 2008;22(4):661-70.
5. Ting Y, Jiang Y, Ho C-T, Huang Q. Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. Journal of Functional Foods. 2014;7:112-28.
6. Gurley B. Emerging technologies for improving phytochemical bioavailability: benefits and risks. Clinical Pharmacology & Therapeutics. 2011;89(6):915-9.
7. Musthaba SM, Baboota S, Ahmed S, Ahuja A, Ali J. Status of novel drug delivery technology for phytotherapeutics. Expert opinion on drug delivery. 2009;6(6):625-37.
8. Ajazuddin, Saraf S. Applications of novel drug delivery system for herbal formulations. Fitoterapia. 2010;81(7):680-9.
9. Desai PP, Date AA, Patravale VB. Overcoming poor oral bioavailability using nanoparticle formulations–opportunities and limitations. Drug Discovery Today: Technologies. 2012;9(2):e87-e95.
10. Kumar CS. Nanotechnology tools in pharmaceutical R&D. Materials Today. 2010;12:24-30.
11. Raffa V, Vittorio O, Riggio C, Cuschieri A. Progress in nanotechnology for healthcare. Minimally Invasive Therapy & Allied Technologies. 2010;19(3):127-35.
12. Khan J, Alexander A, Saraf S, Saraf S. Recent advances and future prospects of phyto-phospholipid complexation technique for improving pharmacokinetic profile of plant actives. Journal of Controlled Release. 2013;168(1):50-60.
13. Porter CJ, Charman WN. In vitro assessment of oral lipid based formulations. Advanced drug delivery reviews. 2001;50:S127-S47.
14. Pouton CW, Porter CJ. Formulation of lipid-based delivery systems for oral administration: materials, methods and strategies. Advanced Drug Delivery Reviews. 2008;60(6):625-37.
15. Porter CJ, Pouton CW, Cuine JF, Charman WN. Enhancing intestinal drug solubilisation using lipid-based delivery systems. Advanced Drug Delivery Reviews. 2008;60(6):673-91.
16. Trevaskis NL, Charman WN, Porter CJ. Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. Advanced drug delivery reviews. 2008;60(6):702-16.
17. van Breemen RB, Fong HH, Farnsworth NR. Ensuring the safety of botanical dietary supplements. The American journal of clinical nutrition. 2008;87(2):509S-13S.
18. Schulz H, Schurer M, Krumbiegel G, Wachter W, Weyhenmeyer R, Seidel G. Investigation of dissolution and bioequivalence of silymarin products. ARZNEIMITTEL-FORSCHUNG/DRUG RESEARCH. 1995;45(1):61-4.

19. El-Samaligy MS, Afifi NN, Mahmoud EA. Evaluation of hybrid liposomes-encapsulated silymarin regarding physical stability and in vivo performance. International Journal of Pharmaceutics. 2006;319(1):121-9.
20. Yan-Yu X, Yun-mei S, Zhi-peng C, Qi-neng P. Preparation of silymarin proliposome: a new way to increase oral bioavailability of silymarin in beagle dogs. International Journal of Pharmaceutics. 2006;319(1):162-8.
21. Takahashi M, Uechi S, Takara K, Asikin Y, Wada K. Evaluation of an oral carrier system in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. Journal of agricultural and food chemistry. 2009;57(19):9141-6.
22. Huang Y-B, Tsai M-J, Wu P-C, Tsai Y-H, Wu Y-H, Fang J-Y. Elastic liposomes as carriers for oral delivery and the brain distribution of (+)-catechin. Journal of drug targeting. 2011;19(8):709-18.
23. Ghosh A, Mandal AK, Sarkar S, Panda S, Das N. Nanoencapsulation of quercetin enhances its dietary efficacy in combating arsenic-induced oxidative damage in liver and brain of rats. Life sciences. 2009;84(3):75-80.
24. Kumar A, Ahuja A, Ali J, Baboota S. Conundrum and therapeutic potential of curcumin in drug delivery. Critical Reviews™ in Therapeutic Drug Carrier Systems. 2010;27(4).
25. Kakkar V, Singh S, Singla D, Kaur IP. Exploring solid lipid nanoparticles to enhance the oral bioavailability of curcumin. Molecular nutrition & food research. 2011;55(3):495-503.
26. Nair HB, Sung B, Yadav VR, Kannappan R, Chaturvedi MM, Aggarwal BB. Delivery of antiinflammatory nutraceuticals by nanoparticles for the prevention and treatment of cancer. Biochemical pharmacology. 2010;80(12):1833-43.
27. Singh B, Bandopadhyay S, Kapil R, Singh R, Katare OP. Self-emulsifying drug delivery systems (SEDDS): formulation development, characterization, and applications. Critical Reviews™ in Therapeutic Drug Carrier Systems. 2009;26(5).
28. Neslihan Gursoy R, Benita S. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. Biomedicine & Pharmacotherapy. 2004;58(3):173-82.
29. Tang J, Sun J, Cui F, Zhang T, Liu X, He Z. Self-emulsifying drug delivery systems for improving oral absorption of ginkgo biloba extracts. Drug delivery. 2008;15(8):477-84.
30. Shao B, Tang J, Ji H, Liu H, Liu Y, Zhu D, et al. Enhanced oral bioavailability of Wurenchun (Fructus Schisandrae Chinensis extracts) by self-emulsifying drug delivery systems. Drug development and industrial pharmacy. 2010;36(11):1356-63.
31. Wu X, Xu J, Huang X, Wen C. Self-microemulsifying drug delivery system improves curcumin dissolution and bioavailability. Drug development and industrial pharmacy. 2011;37(1):15-23.
32. Wu W, Wang Y, Que L. Enhanced bioavailability of silymarin by self-microemulsifying drug delivery system. European journal of pharmaceutics and biopharmaceutics. 2006;63(3):288-94.
33. Zuo F, Nakamura N, Akao T, Hattori M. Pharmacokinetics of berberine and its main metabolites in conventional and pseudo germ-free rats determined by liquid chromatography/ion trap mass spectrometry. Drug Metabolism and Disposition. 2006;34(12):2064-72.

34. Dostál J, Man S, Sečkářová Pn, Hulová D, Nečas M, Potáček M, et al. Berberine and coptisine free bases. Journal of Molecular Structure. 2004;687(1):135-42.
35. Hayashi K, Minoda K, Nagaoka Y, Hayashi T, Uesato S. Antiviral activity of berberine and related compounds against human cytomegalovirus. Bioorganic & medicinal chemistry letters. 2007;17(6):1562-4.
36. Birdsall TC, Kelly GS. Berberine: therapeutic potential of an alkaloid found in several medicinal plants. Altern Med Rev. 1997;2(2):94-103.
37. Taylor CT, Winter DC, Skelly MM, O'Donoghue DP, O'Sullivan GC, Harvey BJ, et al. Berberine inhibits ion transport in human colonic epithelia. European journal of pharmacology. 1999;368(1):111-8.
38. Le Tran Q, Tezuka Y, Ueda J-y, Nguyen NT, Maruyama Y, Begum K, et al. In vitro antiplasmodial activity of antimalarial medicinal plants used in Vietnamese traditional medicine. Journal of Ethnopharmacology. 2003;86(2):249-52.
39. Ko W-H, Yao X-Q, Lau C-W, Law W-I, Chen Z-Y, Kwok W, et al. Vasorelaxant and antiproliferative effects of berberine. European journal of pharmacology. 2000;399(2):187-96.
40. Tsai P-L, Tsai T-H. Hepatobiliary excretion of berberine. Drug Metabolism and Disposition. 2004;32(4):405-12.
41. Sánchez-Chapula J. Increase in action potential duration and inhibition of the delayed rectifier outward current IK by berberine in cat ventricular myocytes. British journal of pharmacology. 1996;117(7):1427-34.
42. Pan G-Y, Huang Z-J, Wang G-J, Fawcett JP, Liu X-D, Zhao X-C, et al. The antihyperglycaemic activity of berberine arises from a decrease of glucose absorption. Planta medica. 2003;69(7):632-6.
43. Jantová S, Košťálová D. Effect of berberine on proliferation, cell cycle and apoptosis in HeLa and L1210 cells. Journal of pharmacy and pharmacology. 2003;55(8):1143-9.
44. Kettmann V, Košťálová D, Jantova S, Čerňáková M. In vitro cytotoxicity of berberine against HeLa and L1210 cancer cell lines. Die Pharmazie-An International Journal of Pharmaceutical Sciences. 2004;59(7):548-51.
45. Yeşilada E, Küpeli E. Berberis crataegina DC. root exhibits potent anti-inflammatory, analgesic and febrifuge effects in mice and rats. Journal of Ethnopharmacology. 2002;79(2):237-48.
46. Misik V, Bezáková L, Máleková L, Kostalova D. Lipoxygenase inhibition and antioxidant properties of protoberberine and aporphine alkaloids isolated from Mahonia aquifolium. Planta medica. 1995;61(4):372-3.
47. Ma L, Xiao P, Guo B, Wu J, Liang F, Dong S. [Cerebral protective effects of some compounds isolated from traditional Chinese herbs]. Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica. 1999;24(4):238-9, 56-inside back cover.
48. Doggrell SA. Berberine-a novel approach to cholesterol lowering: KONG W, WEI J, ABIDI P et al.: Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat. Med.(2005) 12: 1344-1351. 2005.

49. Baird AW, Taylor CT, Brayden DJ. Non-antibiotic anti-diarrhoeal drugs: factors affecting oral bioavailability of berberine and loperamide in intestinal tissue. Advanced drug delivery reviews. 1997;23(1):111-20.
50. Pan Gy, Wang GJ, Liu XD, Fawcett JP, Xie YY. The Involvement of P-Glycoprotein in Berberine Absorption. Pharmacology & toxicology. 2002;91(4):193-7.
51. Gurley BJ, Swain A, Hubbard MA, Williams DK, Barone G, Hartsfield F, et al. Clinical assessment of CYP2D6-mediated herb–drug interactions in humans: Effects of milk thistle, black cohosh, goldenseal, kava kava, St. John's wort, and Echinacea. Molecular nutrition & food research. 2008;52(7):755-63.
52. Ye M, Fu S, Pi R, He F. Neuropharmacological and pharmacokinetic properties of berberine: a review of recent research. Journal of pharmacy and pharmacology. 2009;61(7):831-7.
53. Lee I-A, Hyun Y-J, Kim D-H. Berberine ameliorates TNBS-induced colitis by inhibiting lipid peroxidation, enterobacterial growth and NF-κB activation. European journal of pharmacology. 2010;648(1):162-70.
54. Liu X, Li G, Zhu H, Huang L, Liu Y, Ma C, et al. Beneficial effect of berberine on hepatic insulin resistance in diabetic hamsters possibly involves in SREBPs, LXRα and PPARα transcriptional programs. Endocrine journal. 2009;57(10):881-93.
55. Zhou J, Zhou S. Berberine regulates peroxisome proliferator-activated receptors and positive transcription elongation factor b expression in diabetic adipocytes. European journal of pharmacology. 2010;649(1):390-7.
56. Wu M, Wang J, Liu L-t. Advance of studies on anti-atherosclerosis mechanism of berberine. Chinese journal of integrative medicine. 2010;16(2):188.
57. Zhao L, Li W, Han F, Hou L, Baillargeon J-P, Kuang H, et al. Berberine reduces insulin resistance induced by dexamethasone in theca cells in vitro. Fertility and sterility. 2011;95(1):461-3.
58. Shan Y-Q, Ren G, Wang Y-X, Pang J, Zhao Z-Y, Yao J, et al. Berberine analogue IMB-Y53 improves glucose-lowering efficacy by averting cellular efflux especially P-glycoprotein efflux. Metabolism. 2013;62(3):446-56.
59. Di Pierro F, Villanova N, Agostini F, Marzocchi R, Soverini V, Marchesini G. Pilot study on the additive effects of berberine and oral type 2 diabetes agents for patients with suboptimal glycemic control. Diabetes, metabolic syndrome and obesity: targets and therapy. 2012;5:213.
60. Zhang X, Qiu F, Jiang J, Gao C, Tan Y. Intestinal absorption mechanisms of berberine, palmatine, jateorhizine, and coptisine: involvement of P-glycoprotein. Xenobiotica. 2011;41(4):290-6.
61. Fricker G, Kromp T, Wendel A, Blume A, Zirkel J, Rebmann H, et al. Phospholipids and lipid-based formulations in oral drug delivery. Pharmaceutical research. 2010;27(8):1469-86.
62. Hörter D, Dressman JB. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. Advanced Drug Delivery Reviews. 1997;25(1):3-14.
63. Fleisher D, Li C, Zhou Y, Pao L-H, Karim A. Drug, meal and formulation interactions influencing drug absorption after oral administration. Clinical pharmacokinetics. 1999;36(3):233-54.

64. Charman WN, Porter CJ, Mithani S, Dressman JB. Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. 1997.
65. Humberstone AJ, Charman WN. Lipid-based vehicles for the oral delivery of poorly water soluble drugs. Advanced drug delivery reviews. 1997;25(1):103-28.
66. Chakraborty S, Shukla D, Mishra B, Singh S. Lipid–an emerging platform for oral delivery of drugs with poor bioavailability. European Journal of Pharmaceutics and Biopharmaceutics. 2009;73(1):1-15.
67. Crounse RG. Human Pharmacology of Griseofulvin: The Effect of Fat Intake on Gastrointestinal Absorption1. Journal of Investigative Dermatology. 1961;37(6):529-33.
68. Karen M. Cunningham RJB, Michael Horowitz, Anne F. Maddox, Michela A.L. Edelbroek, and Barry E. Chatterton. Use of technetium-99m (V) thiocyanate to measure gastric emptying of fat. Journal of Nuclear Medicine. 1991;32:878-81.
69. Feinle C, Rades T, Otto B, Fried M. Fat digestion modulates gastrointestinal sensations induced by gastric distention and duodenal lipid in humans. Gastroenterology. 2001;120(5):1100-7.
70. Stone BG, Ansel HJ, Peterson FJ, Gebhard RL. Gallbladder emptying stimuli in obese and normal-weight subjects. Hepatology. 1992;15(5):795-8.
71. Hunt J, Knox M. A relation between the chain length of fatty acids and the slowing of gastric emptying. The Journal of physiology. 1968;194(2):327-36.
72. Ladas S, Isaacs P, Murphy G, Sladen G. Comparison of the effects of medium and long chain triglyceride containing liquid meals on gall bladder and small intestinal function in normal man. Gut. 1984;25(4):405-11.
73. Kalepu S, Manthina M, Padavala V. Oral lipid-based drug delivery systems–an overview. Acta Pharmaceutica Sinica B. 2013;3(6):361-72.
74. Kuentz M. Lipid-based formulations for oral delivery of lipophilic drugs. Drug Discovery Today: Technologies. 2012;9(2):e97-e104.
75. Aungst BJ, Saitoh H, Burcham DL, Huang S-M, Mousa SA, Hussain MA. Enhancement of the intestinal absorption of peptides and nonpeptides. Journal of controlled release. 1996;41(1):19-31.
76. Pang KS, Liu L, Sun H. Interaction of Drug Transporters with Excipients. Role of Lipid Excipients in Modifying Oral and Parenteral Drug Delivery: John Wiley & Sons, Inc.; 2006. p. 1-31.
77. Bogman K, Erne-Brand F, Alsenz J, Drewe J. The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins. Journal of pharmaceutical sciences. 2003;92(6):1250-61.
78. Food and Drug Administration, Guidance for Industry: Food-Effect Bioavailability and Fed Bioequivalence Studies. Food and Drug Administration, Rockville, Maryland, USA2002. http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm
79. Winstanley P, Orme M. The effects of food on drug bioavailability. British journal of clinical pharmacology. 1989;28(6):621-8.

80. Benet L, Cummins C, Wu C. Unmasking the dynamic interplay between efflux transporters and metabolic enzymes. International journal of pharmaceutics. 2004;277(1):3-9.
81. Food and Drug Administration, Guidance For Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System, (2000). http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064964.htm
82. Custodio JM, Wu C-Y, Benet LZ. Predicting drug disposition, absorption/elimination/transporter interplay and the role of food on drug absorption. Advanced drug delivery reviews. 2008;60(6):717-33.
83. Liao TH, Hamosh P, Hamosh M. Fat digestion by lingual lipase: mechanism of lipolysis in the stomach and upper small intestine. Pediatric research. 1984;18(5):402-9.
84. Hamosh M, Scanlon JW, Ganot D, Likel M, Scanlon KB, Hamosh P. Fat digestion in the newborn: characterization of lipase in gastric aspirates of premature and term infants. Journal of Clinical Investigation. 1981;67(3):838.
85. Dahan A, Hoffman A. Rationalizing the selection of oral lipid based drug delivery systems by an in vitro dynamic lipolysis model for improved oral bioavailability of poorly water soluble drugs. Journal of Controlled Release. 2008;129(1):1-10.
86. Bloedow DC, Hayton WL. Effects of lipids on bioavailability of sulfisoxazole acetyl, dicumarol, and griseofulvin in rats. Journal of pharmaceutical sciences. 1976;65(3):328-34.
87. Barnwell S, Burns S, Higginbottom S, Whelan I, Corness D, Hay G, et al. Demonstration of the importance of biphasic oleic acid delivery for enhancing the bioavailability of propranolol in healthy volunteers. International journal of pharmaceutics. 1996;128(1):145-54.
88. Hauss DJ. Oral lipid-based formulations: enhancing the bioavailability of poorly water-soluble drugs: CRC Press; 2007.
89. Buckingham LE, Balasubramanian M, Safa AR, Shah H, Komarov P, Emanuele RM, et al. Reversal of multi-drug resistance in vitro by fatty acid PEG fatty acid diesters. International journal of cancer. 1996;65(1):74-9.
90. Nerurkar MM, Burton PS, Borchardt RT. The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system. Pharmaceutical research. 1996;13(4):528-34.
91. Wacher VJ, Silverman JA, Zhang Y, Benet LZ. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. Journal of pharmaceutical sciences. 1998;87(11):1322-30.
92. Buckingham LE, Balasubramanian M, Emanuele RM, Clodfelter KE, Coon JS. Comparison of solutol HS 15, Cremophor EL and novel ethoxylated fatty acid surfactants as multidrug resistance modification agents. International journal of cancer. 1995;62(4):436-42.
93. Bates SE, Currier SJ, Alvarez M, Fojo AT. Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate. Biochemistry. 1992;31(28):6366-72.

94. Chang T, Benet LZ, Hebert MF. The effect of water-soluble vitamin E on cyclosporine pharmacokinetics in healthy volunteers. Clinical Pharmacology & Therapeutics. 1996;59(3):297-303.
95. Pan SH, Lopez RR, Sher LS, Hoffman AL, Podesta LG, Makowka L, et al. Enhanced Oral Cyclosporine Absorption With Water-Soluble Vitamin E Early After Liver Transplantation. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. 1996;16(1):59-65.
96. Porter CJ, Trevaskis NL, Charman WN. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nature Reviews Drug Discovery. 2007;6(3):231-48.
97. Kleberg K, Jacobsen J, Müllertz A. Characterising the behaviour of poorly water soluble drugs in the intestine: application of biorelevant media for solubility, dissolution and transport studies. Journal of Pharmacy and Pharmacology. 2010;62(11):1656-68.
98. Kossena GA, Charman WN, Wilson CG, O'Mahony B, Lindsay B, Hempenstall JM, et al. Low dose lipid formulations: effects on gastric emptying and biliary secretion. Pharmaceutical research. 2007;24(11):2084-96.
99. Brogård M, Troedsson E, Thuresson K, Ljusberg-Wahren H. A new standardized lipolysis approach for characterization of emulsions and dispersions. Journal of colloid and interface science. 2007;308(2):500-7.
100. Lukyanov AN, Torchilin VP. Micelles from lipid derivatives of water-soluble polymers as delivery systems for poorly soluble drugs. Advanced drug delivery reviews. 2004;56(9):1273-89.
101. Alkan-Onyuksel H, Ramakrishnan S, Chai H-B, Pezzuto JM. A mixed micellar formulation suitable for the parenteral administration of taxol. Pharmaceutical research. 1994;11(2):206-12.
102. Kawakami K, Miyoshi K, Ida Y. Solubilization behavior of poorly soluble drugs with combined use of Gelucire 44/14 and cosolvent. Journal of pharmaceutical sciences. 2004;93(6):1471-9.
103. Chambin O, Jannin V. Interest of multifunctional lipid excipients: case of Gelucire® 44/14. Drug development and industrial pharmacy. 2005;31(6):527-34.
104. Constantinides PP, Tustian A, Kessler DR. Tocol emulsions for drug solubilization and parenteral delivery. Advanced drug delivery reviews. 2004;56(9):1243-55.
105. Dye D, Watkins J. Suspected anaphylactic reaction to Cremophor EL. British medical journal. 1980;280(6228):1353.
106. Hassan S, Dhar S, Sandström M, Arsenau D, Budnikova M, Lokot I, et al. Cytotoxic activity of a new paclitaxel formulation, Pacliex, in vitro and in vivo. Cancer chemotherapy and pharmacology. 2005;55(1):47-54.
107. Constantinides PP, Lambert KJ, Tustian AK, Schneider B, Lalji S, Ma W, et al. Formulation development and antitumor activity of a filter-sterilizable emulsion of paclitaxel. Pharmaceutical research. 2000;17(2):175-82.
108. Park K-M, Lee M-K, Hwang K-J, Kim C-K. Phospholipid-based microemulsions of flurbiprofen by the spontaneous emulsification process. International journal of pharmaceutics. 1999;183(2):145-54.

109. Straubinger RM, Balasubramanian SV. Preparation and characterization of taxane-containing liposomes. Methods in enzymology. 2005;391:97-117.
110. STRAUBINGER RM, ARNOLD RD, ZHOU R, MAZURCHUK R, SLACK JE. Antivascular and antitumor activities of liposome-associated drugs. Anticancer research. 2004;24(2A):397-404.
111. Pouton CW. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and 'self-microemulsifying'drug delivery systems. European journal of pharmaceutical sciences. 2000;11:S93-S8.
112. Strickley RG. Currently Marketed Oral Lipid-Based Dosage Forms: Drug Products and Excipients. Oral Lipid-Based Formulations. p. 1-32.
113. Jain S, Kumar D, Swarnakar NK, Thanki K. Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. Biomaterials. 2012;33(28):6758-68.
114. Jain S, Patil SR, Swarnakar NK, Agrawal AK. Oral delivery of doxorubicin using novel polyelectrolyte-stabilized liposomes (layersomes). Molecular pharmaceutics. 2012;9(9):2626-35.
115. Niu M, Lu Y, Hovgaard L, Guan P, Tan Y, Lian R, et al. Hypoglycemic activity and oral bioavailability of insulin-loaded liposomes containing bile salts in rats: the effect of cholate type, particle size and administered dose. European Journal of Pharmaceutics and Biopharmaceutics. 2012;81(2):265-72.
116. Moutardier V, Tosini F, Vlieghe P, Cara L, Delpero J, Clerc T. Colloidal anticancer drugs bioavailabilities in oral administration models. International journal of pharmaceutics. 2003;260(1):23-38.
117. Deshmukh DD, Ravis WR, Betageri GV. Improved delivery of cromolyn from oral proliposomal beads. International journal of pharmaceutics. 2008;358(1):128-36.
118. Gradauer K, Barthelmes J, Vonach C, Almer G, Mangge H, Teubl B, et al. Liposomes coated with thiolated chitosan enhance oral peptide delivery to rats. Journal of Controlled Release. 2013;172(3):872-8.
119. Charman W, Stella V. Estimating the maximal potential for intestinal lymphatic transport of lipophilic drug molecules. International journal of pharmaceutics. 1986;34(1):175-8.
120. Sheue Nee Ling S, Magosso E, Abdul Karim Khan N, Hay Yuen K, Anne Barker S. Enhanced oral bioavailability and intestinal lymphatic transport of a hydrophilic drug using liposomes. Drug development and industrial pharmacy. 2006;32(3):335-45.
121. HASHIDA N, MURAKAMI M, YOSHIKAWA H, TAKADA K, MURANISHI S. Intestinal absorption of carboxyfluorescein entrapped in liposomes in comparison with its administration with lipid-surfactant mixed micelles. Journal of pharmacobio-dynamics. 1984;7(3):195-203.
122. Mehnert W, Mäder K. Solid lipid nanoparticles: production, characterization and applications. Advanced drug delivery reviews. 2001;47(2):165-96.
123. Yuan H, Chen J, Du Y-Z, Hu F-Q, Zeng S, Zhao H-L. Studies on oral absorption of stearic acid SLN by a novel fluorometric method. Colloids and Surfaces B: Biointerfaces. 2007;58(2):157-64.

124. Shah MK, Madan P, Lin S. Preparation, in vitro evaluation and statistical optimization of carvedilol-loaded solid lipid nanoparticles for lymphatic absorption via oral administration. Pharmaceutical development and technology. 2013;19(4):475-85.
125. Shah MK, Madan P, Lin S. Elucidation of intestinal absorption mechanism of carvedilol-loaded solid lipid nanoparticles using Caco-2 cell line as an in-vitro model. Pharmaceutical development and technology. 2014(0):1-9.
126. Manjunath K, Venkateswarlu V. Pharmacokinetics, tissue distribution and bioavailability of clozapine solid lipid nanoparticles after intravenous and intraduodenal administration. Journal of Controlled Release. 2005;107(2):215-28.
127. Cavalli R, Zara GP, Caputo O, Bargoni A, Fundarò A, Gasco MR. Transmucosal transport of tobramycin incorporated in SLN after duodenal administration to rats. Part I—a pharmacokinetic study. Pharmacological research. 2000;42(6):541-5.
128. Pouton CW. Formulation of self-emulsifying drug delivery systems. Advanced drug delivery reviews. 1997;25(1):47-58.
129. Gershanik T, Benita S. Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs. European journal of pharmaceutics and biopharmaceutics. 2000;50(1):179-88.
130. Gursoy N, Garrigue JS, Razafindratsita A, Lambert G, Benita S. Excipient effects on in vitro cytotoxicity of a novel paclitaxel self-emulsifying drug delivery system. Journal of pharmaceutical sciences. 2003;92(12):2411-8.
131. Gao P, Morozowich W. Development of supersaturatable self-emulsifying drug delivery system formulations for improving the oral absorption of poorly soluble drugs. 2006.
132. Gao P, Rush BD, Pfund WP, Huang T, Bauer JM, Morozowich W, et al. Development of a supersaturable SEDDS (S-SEDDS) formulation of paclitaxel with improved oral bioavailability. Journal of pharmaceutical sciences. 2003;92(12):2386-98.
133. Garti N, Amar I, Yaghmur A, Spernath A, Aserin A. Interfacial modification and structural transitions induced by guest molecules solubilized in U-type nonionic microemulsions. Journal of dispersion science and technology. 2003;24(3-4):397-410.
134. Garti N, Yaghmur A, Aserin A, Spernath A, Elfakess R, Ezrahi S. Solubilization of active molecules in microemulsions for improved environmental protection. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2003;230(1):183-90.
135. Spernath A, Aserin A. Microemulsions as carriers for drugs and nutraceuticals. Advances in colloid and interface science. 2006;128:47-64.
136. Christensen K, Pedersen G, Kristensen H. Physical stability of redispersible dry emulsions containing amorphous sucrose. European journal of pharmaceutics and biopharmaceutics. 2002;53(2):147-53.
137. Shively ML, Thompson DC. Oral bioavailability of vancomycin solid-state emulsions. International Journal of Pharmaceutics. 1995;117(1):119-22.
138. Nazzal S, Khan MA. Controlled release of a self-emulsifying formulation from a tablet dosage form: Stability assessment and optimization of some processing parameters. International Journal of Pharmaceutics. 2006;315(1):110-21.

139. Dollo G, Le Corre P, Guérin A, Chevanne F, Burgot JL, Leverge R. Spray-dried redispersible oil-in-water emulsion to improve oral bioavailability of poorly soluble drugs. European journal of pharmaceutical sciences. 2003;19(4):273-80.
140. Singh A, Worku ZA, Van den Mooter G. Oral formulation strategies to improve solubility of poorly water-soluble drugs. Expert opinion on drug delivery. 2011;8(10):1361-78.
141. Müllertz A, Ogbonna A, Ren S, Rades T. New perspectives on lipid and surfactant based drug delivery systems for oral delivery of poorly soluble drugs. Journal of pharmacy and pharmacology. 2010;62(11):1622-36.
142. Berberine [last visited Mar. 31, 2015]. Available from: http://en.wikipedia.org/w/index.php?title=Berberine&oldid=651732751.
143. Vuddanda PR, Chakraborty S, Singh S. Berberine: a potential phytochemical with multispectrum therapeutic activities. Expert opinion on investigational drugs. 2010;19(10):1297-307.
144. Monograph Berberine. Alternative Medicine Review. 2000;5(5):175-7.
145. Čerňáková M, Košťálová D. Antimicrobial activity of berberine—a constituent ofMahonia aquifolium. Folia microbiologica. 2002;47(4):375-8.
146. Sheng WD, Jiddawi M, Hong X, Abdulla S. Treatment of chloroquine-resistant malaria using pyrimethamine in combination with berberine, tetracycline or cotrimoxazole. East African medical journal. 1997;74(5):283-4.
147. Ghosh AK, Bhattacharyya FK, Ghosh DK. Leishmania donovani: Amastigote inhibition and mode of actior of berberine. Experimental parasitology. 1985;60(3):404-13.
148. Kaneda Y, Torii M, Tanaka T, Aikawa M. In vitro effects of berberine sulphate on the growth and structure of Entamoeba histolytica, Giardia lamblia and Trichomonas vaginalis. Annals of tropical medicine and parasitology. 1991;85(4):417-25.
149. Kaneda Y, Tanaka T, Saw T. Effects of berberine, a plant alkaloid, on the growth of anaerobic protozoa in axenic culture. The Tokai journal of experimental and clinical medicine. 1990;15(6):417-23.
150. Pan G, Wang G, Sun J, Huang Z, Zhao X, Gu Y, et al. [Inhibitory action of berberine on glucose absorption]. Yao xue xue bao= Acta pharmaceutica Sinica. 2003;38(12):911-4.
151. Maeng HJ, Yoo HJ, Kim IW, Song IS, Chung SJ, Shim CK. P-glycoprotein–mediated transport of berberine across Caco-2 cell monolayers. Journal of pharmaceutical sciences. 2002;91(12):2614-21.
152. Chen W, Miao Y-Q, Fan D-J, Yang S-S, Lin X, Meng L-K, et al. Bioavailability study of berberine and the enhancing effects of TPGS on intestinal absorption in rats. Aaps Pharmscitech. 2011;12(2):705-11.
153. Chae H-W, Kim I-W, Jin H-E, Kim D-D, Chung S-J, Shim C-K. Effect of ion-pair formation with bile salts on the in vitro cellular transport of berberine. Archives of pharmacal research. 2008;31(1):103-10.
154. Tsai P-L, Tsai T-H. Simultaneous determination of berberine in rat blood, liver and bile using microdialysis coupled to high-performance liquid chromatography. Journal of Chromatography A. 2002;961(1):125-30.

155. Zhang Y, Li X, Zou D, Liu W, Yang J, Zhu N, et al. Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid berberine. The Journal of Clinical Endocrinology & Metabolism. 2008;93(7):2559-65.
156. Khin-Maung-U M-K, Nyunt-Nyunt-Wai A-K. Clinical trial of berberine in acute watery diarrhoea. British Medical Journal (Clinical research ed). 1985;291(6509):1601.
157. Khan AY, Hossain M, Kumar GS. Binding of plant alkaloids berberine and palmatine to serum albumins: a thermodynamic investigation. Molecular biology reports. 2013;40(1):553-66.
158. Hazra S, Hossain M, Kumar GS. Binding of isoquinoline alkaloids berberine, palmatine and coralyne to hemoglobin: structural and thermodynamic characterization studies. Molecular BioSystems. 2013;9(1):143-53.
159. Wang Y-X, Kong W-J, Li Y-H, Tang S, Li Z, Li Y-B, et al. Synthesis and structure–activity relationship of berberine analogues in LDLR up-regulation and AMPK activation. Bioorganic & medicinal chemistry. 2012;20(22):6552-8.
160. Li Y, Ren G, Wang Y-X, Kong W-J, Yang P, Wang Y-M, et al. Bioactivities of berberine metabolites after transformation through CYP450 isoenzymes. J Transl Med. 2011;9(1):62.
161. Qiu F, Zhu Z, Kang N, Piao S, Qin G, Yao X. Isolation and identification of urinary metabolites of berberine in rats and humans. Drug Metabolism and Disposition. 2008;36(11):2159-65.
162. Pan J-F, Yu C, Zhu D-Y, Zhang H, Zeng J-F, Jiang S-H, et al. Identification of three sulfate-conjugated metabolites of berberine chloride in healthy volunteers' urine after oral administration. Acta Pharmacologica Sinica. 2002;23(1):77-82.
163. Dong H, Wang N, Zhao L, Lu F. Berberine in the treatment of type 2 diabetes mellitus: a systemic review and meta-analysis. Evidence-Based Complementary and Alternative Medicine. 2012;2012.
164. Imanshahidi M, Hosseinzadeh H. Pharmacological and therapeutic effects of Berberis vulgaris and its active constituent, berberine. Phytotherapy research. 2008;22(8):999-1012.
165. Chan E. Displacement of bilirubin from albumin by berberine. Neonatology. 1993;63(4):201-8.
166. Wu X, Li Q, Xin H, Yu A, Zhong M. Effects of berberine on the blood concentration of cyclosporin A in renal transplanted recipients: clinical and pharmacokinetic study. European journal of clinical pharmacology. 2005;61(8):567-72.
167. Xin H, Wu X, Li Q, Yu A, Zhong M, Liu Y. The effects of berberine on the pharmacokinetics of ciclosporin A in healthy volunteers. Methods and findings in experimental and clinical pharmacology. 2006;28(1):25-30.
168. Cao J-W, Luo H-S, Yu B-P, Huang X-D, Sheng Z-X, Yu J-P. Effects of berberine on intracellular free calcium in smooth muscle cells of Guinea pig colon. Digestion. 2001;64(3):179-83.
169. Affuso F, Mercurio V, Ruvolo A, Pirozzi C, Micillo F, Carlomagno G, et al. A nutraceutical combination improves insulin sensitivity in patients with metabolic syndrome. World journal of cardiology. 2012;4(3):77.
170. Rowe RC, Sheskey PJ, Quinn ME, Association AP, Press P. Handbook of pharmaceutical excipients: Pharmaceutical press London; 2009.

171. Banerjee M, Dash TK, Kumari A, Khatua S. Optimized UV-Vis spectrophotometric method for estimation of anastrozole in pharmaceutical solid dosage form. Der Pharma Chemica. 2014;6(3).
172. Kheir MM, Wang Y, Hua L, Hu J, Li L, Lei F, et al. Acute toxicity of berberine and its correlation with the blood concentration in mice. Food and chemical toxicology. 2010;48(4):1105-10.
173. Gupta PK, Hubbard M, Gurley B, Hendrickson HP. Validation of a liquid chromatography–tandem mass spectrometric assay for the quantitative determination of hydrastine and berberine in human serum. Journal of pharmaceutical and biomedical analysis. 2009;49(4):1021-6.
174. Lu T, Liang Y, Song J, Xie L, Wang GJ, Liu XD. Simultaneous determination of berberine and palmatine in rat plasma by HPLC–ESI-MS after oral administration of traditional Chinese medicinal preparation Huang-Lian-Jie-Du decoction and the pharmacokinetic application of the method. Journal of pharmaceutical and biomedical analysis. 2006;40(5):1218-24.
175. Gui SY, Wu L, Peng D, Liu Q, Yin B, Shen JZ. Preparation and evaluation of a microemulsion for oral delivery of berberine. Die Pharmazie-An International Journal of Pharmaceutical Sciences. 2008;63(7):516-9.
176. Bharate SS, Bharate SB, Bajaj AN. Incompatibilities of pharmaceutical excipients with active pharmaceutical ingredients: a comprehensive review. Journal of Excipients and Food Chemicals. 2010;1(3):3-26.
177. Bangham A. Liposomes: the Babraham connection. Chemistry and physics of lipids. 1993;64(1):275-85.
178. Bangham AD, Horne RW. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. Journal of Molecular Biology. 1964;8(5):660-IN10.
179. [cited 2015 13/07]. Available from: http://watcut.uwaterloo.ca/webnotes/Metabolism/cholesterolTransport.html.
180. Uchegbu IF, Vyas SP. Non-ionic surfactant based vesicles (niosomes) in drug delivery. International Journal of Pharmaceutics. 1998;172(1):33-70.
181. Hu C, Rhodes DG. Proniosomes: a novel drug carrier preparation. International Journal of Pharmaceutics. 1999;185(1):23-35.
182. Cevc G, Blume G. New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2001;1514(2):191-205.
183. Cevc G, Blume G, Schätzlein A. Transfersomes-mediated transepidermal delivery improves the regio-specificity and biological activity of corticosteroids in vivo. Journal of controlled release. 1997;45(3):211-26.
184. Valjakka-Koskela R, Kirjavainen M, Mönkkönen J, Urtti A, Kiesvaara J. Enhancement of percutaneous absorption of naproxen by phospholipids. International Journal of Pharmaceutics. 1998;175(2):225-30.

185. Kirjavainen M, Urtti A, Valjakka-Koskela R, Kiesvaara J, Mönkkönen J. Liposome–skin interactions and their effects on the skin permeation of drugs. European journal of pharmaceutical sciences. 1999;7(4):279-86.
186. Horwitz E, Pisanty S, Czerninski R, Helser M, Eliav E, Touitou E. A clinical evaluation of a novel liposomal carrier for acyclovir in the topical treatment of recurrent herpes labialis. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 1999;87(6):700-5.
187. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M. Ethosomes—novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. Journal of controlled release. 2000;65(3):403-18.
188. Song K-H, Chung S-J, Shim C-K. Preparation and evaluation of proliposomes containing salmon calcitonin. Journal of controlled release. 2002;84(1):27-37.
189. Sharma A, Sharma US. Liposomes in drug delivery: progress and limitations. International Journal of Pharmaceutics. 1997;154(2):123-40.
190. Mozafari MR. Liposomes: an overview of manufacturing techniques. Cellular and Molecular Biology Letters. 2005;10(4):711.
191. Bangham AD, De Gier J, Greville G. Osmotic properties and water permeability of phospholipid liquid crystals. Chemistry and physics of lipids. 1967;1(3):225-46.
192. Olson F, Hunt C, Szoka F, Vail W, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1979;557(1):9-23.
193. Mui B, Chow L, Hope MJ. Extrusion technique to generate liposomes of defined size. Methods in enzymology. 2003;367:3-14.
194. Szebeni J, Breuer JH, Szelenyi JG, Bathori G, Lelkes G, Hollan SR. Oxidation and denaturation of hemoglobin encapsulated in liposomes. Biochimica et Biophysica Acta (BBA)-General Subjects. 1984;798(1):60-7.
195. Deamer DW. Preparation of solvent vaporization liposomes. Liposome Techn. 1984;1:29-35.
196. Papahadjopoulos D, Nir S, Düzgünes N. Molecular mechanisms of calcium-induced membrane fusion. Journal of bioenergetics and biomembranes. 1990;22(2):157-79.
197. Cauchetier E, Fessi H, Boulard Y, Deniau M, Astier A, Paul M. Preparation and physicochemical characterization of atovaquone-containing liposomes. Drug development research. 1999;47(4):155-61.
198. Nii T, Ishii F. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. International journal of pharmaceutics. 2005;298(1):198-205.
199. Shum HC, Lee D, Yoon I, Kodger T, Weitz DA. Double emulsion templated monodisperse phospholipid vesicles. Langmuir. 2008;24(15):7651-3.
200. Gregoriadis G, Florence A. Liposomes in Drug Delivery. Drugs. 1993;45(1):15-28.
201. Gregoriadis G, da Silva H, Florence AT. A procedure for the efficient entrapment of drugs in dehydration-rehydration liposomes (DRVs). International Journal of Pharmaceutics. 1990;65(3):235-42.

202. Fielding RM. Liposomal drug delivery. Clinical pharmacokinetics. 1991;21(3):155-64.
203. Brandl M. Liposomes as drug carriers: a technological approach. Biotechnology annual review. 2001;7:59-85.
204. Szoda FCaP, D. Liposomes: preparation and characterization. Knight CG, editor. Amsterdam: Elsevier; 1981.
205. Fresta M, PANICO A, BUCOLO C, GIANNAVOLA C, PUGLISI G. Characterization and In-vivo Ocular Absorption of Liposome-encapsulated Acyclovir. Journal of pharmacy and pharmacology. 1999;51(5):565-76.
206. Law S, Hung H. Properties of acyclovir-containing liposomes for potential ocular delivery. International journal of pharmaceutics. 1998;161(2):253-9.
207. Lee S-C, Lee K-E, Kim J-J, Lim S-H. The effect of cholesterol in the liposome bilayer on the stabilization of incorporated retinol. Journal of liposome research. 2005;15(3-4):157-66.
208. Tadakuma T, Yasuda T, Tamauchi H, Saito K, Tsumita T, Kinsky SC. Effect of lipid A incorporation on characterization of liposomal model membranes as thymus-independent type 1 or type 2 immunogens. The Journal of Immunology. 1982;128(1):206-10.
209. Mady MM, Darwish MM. Effect of chitosan coating on the characteristics of DPPC liposomes. Journal of Advanced Research. 2010;1(3):187-91.
210. Maherani B, Arab-Tehrany E, Kheirolomoom A, Reshetov V, Stebe MJ, Linder M. Optimization and characterization of liposome formulation by mixture design. Analyst. 2012;137(3):773-86.
211. Brgles M, Jurašin D, Sikiric MD, Frkanec R, Tomašic J. Entrapment of ovalbumin into liposomes-Factors affecting entrapment efficiency, liposome size, and zeta potential. Journal of liposome research. 2008;18(3):235-48.
212. Maherani B, Arab-Tehrany E, R Mozafari M, Gaiani C, Linder M. Liposomes: a review of manufacturing techniques and targeting strategies. Current Nanoscience. 2011;7(3):436-52.
213. Shah R, Eldridge D, Palombo E, Harding I. Lipid Nanoparticles: Production, Characterization and Stability: Springer; 2015.
214. Hussain N. Ligand-mediated tissue specific drug delivery. Advanced drug delivery reviews. 2000;43(2):95-100.
215. Food and Drug Administartion, Guidance for Industry: Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients that Are Color Additives, (2014).
216. MuÈller RH, MaÈder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery–a review of the state of the art. European journal of pharmaceutics and biopharmaceutics. 2000;50(1):161-77.
217. Müller R, Mehnert W, Lucks J-S, Schwarz C, Zur Mühlen A, Meyhers H, et al. Solid lipid nanoparticles (SLN): an alternative colloidal carrier system for controlled drug delivery. European journal of pharmaceutics and biopharmaceutics. 1995;41(1):62-9.
218. Muller R, Hildebrand G. Pharmazeutische Technologie: Moderne Arzneiformen, Lehrbuch fur Studierende der Pharmazie-Nachschlagewerk fur Apotheker in Offizin, Krankenhaus und Forschung 2. Erweiterte Aufl. Wissenschaftliche Verlagsgesellschaft, Stuttgart; 1998.

219. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Advanced Drug Delivery Reviews. 2002;54:S131-S55.
220. Jores K, Mehnert W, Drechsler M, Bunjes H, Johann C, Mäder K. Investigations on the structure of solid lipid nanoparticles (SLN) and oil-loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy. Journal of Controlled Release. 2004;95(2):217-27.
221. Üner M, Wissing S, Yener G, Müller R. Skin moisturizing effect and skin penetration of ascorbyl palmitate entrapped in solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) incorporated into hydrogel. Die Pharmazie-An International Journal of Pharmaceutical Sciences. 2005;60(10):751-5.
222. Benita S. Submicron emulsions in drug targeting and delivery: CRC Press; 1998.
223. Gasco MR. Method for producing solid lipid microspheres having a narrow size distribution. Google Patents; 1993.
224. Igartua M, Saulnier P, Heurtault B, Pech B, Proust J, Pedraz J, et al. Development and characterization of solid lipid nanoparticles loaded with magnetite. International journal of pharmaceutics. 2002;233(1):149-57.
225. Cavalli R, Caputo O, Carlotti ME, Trotta M, Scarnecchia C, Gasco MR. Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. International Journal of Pharmaceutics. 1997;148(1):47-54.
226. Cortesi R, Esposito E, Luca G, Nastruzzi C. Production of lipospheres as carriers for bioactive compounds. Biomaterials. 2002;23(11):2283-94.
227. Sjöström B, Bergenståhl B. Preparation of submicron drug particles in lecithin-stabilized o/w emulsions I. Model studies of the precipitation of cholesteryl acetate. International Journal of Pharmaceutics. 1992;88(1):53-62.
228. Shahgaldian P, Da Silva E, Coleman AW, Rather B, Zaworotko MJ. Para-acyl-calix-arene based solid lipid nanoparticles (SLNs): a detailed study of preparation and stability parameters. International journal of pharmaceutics. 2003;253(1):23-38.
229. Quintanar-Guerrero D, Tamayo-Esquivel D, Ganem-Quintanar A, Allémann E, Doelker E. Adaptation and optimization of the emulsification-diffusion technique to prepare lipidic nanospheres. European journal of pharmaceutical sciences. 2005;26(2):211-8.
230. Schubert M, Müller-Goymann C. Solvent injection as a new approach for manufacturing lipid nanoparticles–evaluation of the method and process parameters. European journal of pharmaceutics and biopharmaceutics. 2003;55(1):125-31.
231. Kržič M, Šentjurc M, Kristl J. Improved skin oxygenation after benzyl nicotinate application in different carriers as measured by EPR oximetry in vivo. Journal of controlled release. 2001;70(1):203-11.
232. Mei Z, Chen H, Weng T, Yang Y, Yang X. Solid lipid nanoparticle and microemulsion for topical delivery of triptolide. European journal of pharmaceutics and biopharmaceutics. 2003;56(2):189-96.

233. Morel S, Terreno E, Ugazio E, Aime S, Gasco MR. NMR relaxometric investigations of solid lipid nanoparticles (SLN) containing gadolinium (III) complexes. European journal of pharmaceutics and biopharmaceutics. 1998;45(2):157-63.
234. Charcosset C, El-Harati A, Fessi H. Preparation of solid lipid nanoparticles using a membrane contactor. Journal of controlled release. 2005;108(1):112-20.
235. Batzri S, Korn ED. Single bilayer liposomes prepared without sonication. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1973;298(4):1015-9.
236. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. International journal of pharmaceutics. 1989;55(1):R1-R4.
237. Souto E, Mehnert W, Müller R. Polymorphic behaviour of Compritol® 888 ATO as bulk lipid and as SLN and NLC. Journal of microencapsulation. 2006;23(4):417-33.
238. Misra R, Acharya S, Dilnawaz F, Sahoo SK. Sustained antibacterial activity of doxycycline-loaded poly (D, L-lactide-co-glycolide) and poly (ε-caprolactone) nanoparticles. Nanomedicine. 2009;4(5):519-30.
239. Esmaeili F, Hosseini-Nasr M, Rad-Malekshahi M, Samadi N, Atyabi F, Dinarvand R. Preparation and antibacterial activity evaluation of rifampicin-loaded poly lactide-co-glycolide nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine. 2007;3(2):161-7.
240. Tenjarla S. Microemulsions: an overview and pharmaceutical applications. Critical Reviews™ in Therapeutic Drug Carrier Systems. 1999;16(5).
241. Charman SA, Charman WN, Rogge MC, Wilson TD, Dutko FJ, Pouton CW. Self-emulsifying drug delivery systems: formulation and biopharmaceutic evaluation of an investigational lipophilic compound. Pharmaceutical research. 1992;9(1):87-93.
242. Shah N, Carvajal M, Patel C, Infeld M, Malick A. Self-emulsifying drug delivery systems (SEDDS) with polyglycolyzed glycerides for improving in vitro dissolution and oral absorption of lipophilic drugs. International Journal of Pharmaceutics. 1994;106(1):15-23.
243. Noble S, Markham A. Cyclosporin. Drugs. 1995;50(5):924-41.
244. Leal-Calderon F, Schmitt V, Bibette J. Emulsion science: basic principles: Springer Science & Business Media; 2007.
245. Rang M-J, Miller CA. Spontaneous emulsification of oils containing hydrocarbon, nonionic surfactant, and oleyl alcohol. Journal of Colloid And Interface Science. 1999;209(1):179-92.
246. Becher P. Emulsions: theory and practice. 1965.
247. Rosen MJ, Kunjappu JT. Surfactants and interfacial phenomena: John Wiley & Sons; 2012.
248. Tadros TF, Vincent B, Becher P. Encyclopedia of emulsion technology. Vol 1Dekker, NY. 1983:129.
249. Pouton CW. Self-emulsifying drug delivery systems: assessment of the efficiency of emulsification. International Journal of Pharmaceutics. 1985;27(2):335-48.
250. Pouton CW. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. European journal of pharmaceutical sciences. 2006;29(3):278-87.

251. Mohsin K, Long MA, Pouton CW. Design of lipid-based formulations for oral administration of poorly water-soluble drugs: Precipitation of drug after dispersion of formulations in aqueous solution. Journal of pharmaceutical sciences. 2009;98(10):3582-95.
252. Holm R, Porter CJ, Müllertz A, Kristensen HG, Charman WN. Structured triglyceride vehicles for oral delivery of halofantrine: examination of intestinal lymphatic transport and bioavailability in conscious rats. Pharmaceutical research. 2002;19(9):1354-61.
253. Reddy S, Katyayani T, Navatha A, Ramya G. Review on self micro emulsifying drug delivery systems.
254. Constantinides PP. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. Pharmaceutical research. 1995;12(11):1561-72.
255. Kommuru T, Gurley B, Khan M, Reddy I. Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q< sub> 10</sub>: formulation development and bioavailability assessment. International Journal of Pharmaceutics. 2001;212(2):233-46.
256. Khamkar Ganesh S. SELF MICRO EMULSIFYING DRUG DELIVERY SYSTEM (SMEED) O/W MICROEMULSION FOR BCS CLASS II DRUGS: AN APPROACH TO ENHANCE AN ORAL BIOAVAILABILITY. International Journal of Pharmacy & Pharmaceutical Sciences. 2011;3.
257. Yalkowsky SH. Solubility and solubilization in aqueous media. 1999.
258. Cole ET, Cadé D, Benameur H. Challenges and opportunities in the encapsulation of liquid and semi-solid formulations into capsules for oral administration. Advanced drug delivery reviews. 2008;60(6):747-56.
259. Bose S, Kulkarni P. Self emulsifying drug delivery systems: A review. Indian Journal of Pharmaceutical Education. 2002;36:184-90.
260. Lawrence MJ, Rees GD. Microemulsion-based media as novel drug delivery systems. Advanced drug delivery reviews. 2000;45(1):89-121.
261. Chen Z-Q, Liu Y, Zhao J-H, Wang L, Feng N-P. Improved oral bioavailability of poorly water-soluble indirubin by a supersaturatable self-microemulsifying drug delivery system. International journal of nanomedicine. 2012;7:1115.
262. Wu Z, Guo D, Deng L, Zhang Y, Yang Q, Chen J. Preparation and evaluation of a self-emulsifying drug delivery system of etoposide-phospholipid complex. Drug development and industrial pharmacy. 2011;37(1):103-12.
263. Cirri M, Mura P, Mora PC. Liquid spray formulations of xibornol by using self-microemulsifying drug delivery systems. International Journal of Pharmaceutics. 2007;340(1):84-91.
264. Subramanian N, Ray S, Ghosal SK, Bhadra R, Moulik SP. Formulation design of self-microemulsifying drug delivery systems for improved oral bioavailability of celecoxib. Biological and Pharmaceutical Bulletin. 2004;27(12):1993-9.
265. Anbazhagan S, Indumathy N, Shanmugapandiyan P, Sridhar SK. Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. Journal of pharmaceutical and biomedical analysis. 2005;39(3):801-4.
266. Sarkar M, Khandavilli S, Panchagnula R. Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative estimation of

antiretroviral drugs in pharmaceutical dosage forms. Journal of Chromatography B. 2006;830(2):349-54.

267. Date AA, Desai N, Dixit R, Nagarsenker M. Self-nanoemulsifying drug delivery systems: formulation insights, applications and advances. Nanomedicine. 2010;5(10):1595-616.
268. Borhade V, Nair H, Hegde D. Design and evaluation of self-microemulsifying drug delivery system (SMEDDS) of tacrolimus. AAPS PharmSciTech. 2008;9(1):13-21.
269. Paradkar A, Ambike AA, Jadhav BK, Mahadik K. Characterization of curcumin–PVP solid dispersion obtained by spray drying. International Journal of Pharmaceutics. 2004;271(1):281-6.
270. Patel AR, Vavia PR. Preparation and in vivo evaluation of SMEDDS (self-microemulsifying drug delivery system) containing fenofibrate. The AAPS journal. 2007;9(3):E344-E52.
271. Pappinen S, Urtti A. Microemulsions in topical drug delivery. Percutaneous penetration enhancers. 2006;2:109-16.
272. Tarr BD, Yalkowsky SH. Enhanced intestinal absorption of cyclosporine in rats through the reduction of emulsion droplet size. Pharmaceutical research. 1989;6(1):40-3.
273. Gershanik T, Benita S. Positively charged self-emulsifying oil formulation for improving oral bioavailability of progesterone. Pharmaceutical Development and Technology. 1996;1(2):147-57.
274. Gershanik T, Benzeno S, Benita S. Interaction of the self-emulsifying lipid drug delivery system with mucosa of everted rat intestine as a function of surface charge and droplet size. Pharm Res. 1998;15(6):863-70.
275. Wakerly M, Pouton C, Meakin B. Evaluation of the self-emulsifying performance of a non-ionic surfactant-vegetable oil mixture. J Pharm Pharmacol. 1987;39(6).
276. Craig D, Barker S, Banning D, Booth S. An investigation into the mechanisms of self-emulsification using particle size analysis and low frequency dielectric spectroscopy. International Journal of Pharmaceutics. 1995;114(1):103-10.
277. Pauletti GM, Gangwar S, Siahaan TJ, Aubé J, Borchardt RT. Improvement of oral peptide bioavailability: Peptidomimetics and prodrug strategies. Advanced drug delivery reviews. 1997;27(2):235-56.
278. Lehr C-M. Cell culture models of biological barriers: in vitro test systems for drug absorption and delivery: CRC Press; 2003.
279. O'driscoll C, Griffin B. Biopharmaceutical challenges associated with drugs with low aqueous solubility—the potential impact of lipid-based formulations. Advanced drug delivery reviews. 2008;60(6):617-24.
280. Seeballuck F, Ashford MB, O'Driscoll CM. The effects of Pluronic® block copolymers and Cremophor® EL on intestinal lipoprotein processing and the potential link with P-glycoprotein in Caco-2 cells. Pharmaceutical research. 2003;20(7):1085-92.
281. Artursson P, Borchardt RT. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. Pharmaceutical research. 1997;14(12):1655-8.
282. Rubas W, Cromwell M, Shahrokh Z, Villagran J, Nguyen TN, Wellton M, et al. Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. Journal of pharmaceutical sciences. 1996;85(2):165-9.

283. Karlsson J, Artursson P. A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers. International journal of pharmaceutics. 1991;71(1):55-64.
284. Torchilin V. Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example. In: Schäfer-Korting M, editor. Drug Delivery. Handbook of Experimental Pharmacology. 197: Springer Berlin Heidelberg; 2010. p. 3-53.

# ANNEXURE-I

# LIST OF INSTRUMENTS

| INSTRUMENTS | MANUFACTURER |
|---|---|
| **UV SPECTROPHOTOMETER** | UV-1800, SHIMADZU, JAPAN |
| **HPTLC** | CAMAG, SWITZERLAND |
| **FTIR** | IR AFFINITY-1, SHIMADZU, JAPAN |
| **HPLC** | SHIMADZU, JAPAN |
| **SONICATOR** | SW-4, TOSHNIWAL INSTRUMENTS, AJMER |
| **ROTARY VACUUM EVAPORATOR** | REMI INSTRUMENTS, MUMBAI, INDIA |
| **OPTICAL MICROSCOPE** | OLYMPUS BX 41, USA |
| **PARTICLE SIZE ANALYZER** | ZETASIZER HAS 3000, MALVERN INSTRUMENT LIMITED, UK |
| **MAGNETIC STIRRER** | REMI INSTRUMENTS, MUMBAI, INDIA |
| **MECHANICAL STIRRER** | REMI ELEKTROTECHNIK LTD., MUMBAI |
| **COOLING CENTRIFUGE** | REMI INSTRUMENTS, MUMBAI, INDIA |
| **PARTICLE SIZE ANALYZER** | ZETATRAC, MICROTRAC INC., USA |
| **X-RAY DIFFRACTOMETER** | XPERT PRO MPD, PANALYTICAL, NETHERLAND |
| **SCANNING ELECTRON MICROSCOPY** | ZEISS ULTRA 55 SEM, USA |
| **VORTEX SHAKER** | MACRO SCIENTIFIC WORKS LTD., DELHI, INDIA |
| **TRANSMISSION ELECTRON MICROSCOPE** | PHILLIPS TECNAI 20, NETHERLAND |
| **CONDUCTIVITY METER** | MACRO SCIENTIFIC WORKS LTD., DELHI, INDIA |
| **BROOKFIELD VISCOMETER** | DVIII ULTRA RHEOMETER, BROOKFIELD ENGINEERING LABORATORY, USA |

# ANNEXURE-II

# PAPER PUBLICATION AND PRESENTATION

## (A) Publication

**Sailor G,** Seth A K, Parmar G, Chauhan S, Javia A. Formulation and *in vitro* evaluation of berberine containing liposome optimized by $3^2$ full factorial designs. Journal of Applied Pharmaceutical Science. 2015;5(7): XXX-XXX. **(Accepted)**

## (B) Presentation

- Poster presented on "Formulation and *in vivo* evaluation of self-nanoemulsifying systems of berberine" in National Conference on ***'Frontiers and Avenues in the field of herbal drug research'*** organized by L. M. College of Pharmacy, Ahmedabad, India on $6^{th}$ -$7^{th}$ Feb 2015.
- Poster presented on "Preparation and characterization of berberine loaded solid lipid nanoparticles" in GUJCOST sponsored one day national seminar on **'*Challenges and Oppourtunities on Nanotechnology based drug delivery system*'**, organized by Department of Pharmaceutical Sciences, Saurashtra University, Rajkot on $25^{th}$ Feb 2015.

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# Formulation and *in vitro* evaluation of berberine containing liposome optimized by $3^2$ full factorial designs

Girish Sailor*, A K Seth, Ghanshyam Parmar, Sachin Chauhan, Ankur Javia

Department of Pharmacy, Sumandeep Vidyapeeth, Vill. Piparia, Tal. Waghodia, Vadodara-391760, Gujarat, India.

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***Key words:***
Berberine; $3^2$ full factorial design; desirability function; lipid based formulation .

**ABSTRACT**

The present study demonstrates the application of $3^2$ full factorial design for optimization of berberine loaded liposome for oral administration. Thin film hydration method was used to prepare liposome and optimization was done by $3^2$ full factorial designs combined with desirability function. Nine formulations were prepared by using different drug : lipid and soyphosphatidylcholine : cholesterol (SPC:CHOL) ratios and evaluated for entrapment efficiency and vesicle size. The statistical validity of model was done by analysis of variance (ANOVA). Response surface graph and contour plots were used to understand the effect of variables on responses. The optimized formulation with 0.782 desirability value was prepared and evaluated for responses. The results of entrapment efficiency and vesicle size were found to be very close with the predicted values. In addition, an optimized formulation was also characterized for zeta potential, *in vitro* drug release and morphology. The formulation was found to be spherical shape with an average diameter of 0.823 nm and -1.93 mV zeta potential and also shows sustained release pattern. These results support the fact that $3^2$ full factorial designs with desirability function could be effectively used in optimization of berberine loaded liposome.

## INTRODUCTION

Berberine (BER) is a quaternary isoquinoline alkaloid obtained from various plants of Berberis species. It has been historically used as an anti-diarrheal, anti-protozoal, and anti-microbial agent in Ayurvedic and Chinese medicine. It also possesses multitude of biological effects, including anti-inflammatory, antidiabetic, lipid peroxidation, and neuro-protective activity (Liu *et al*., 2009; Lee *et al*., 2010; Wu *et al*., 2010; Zhou *et al*., 2010; Zhao *et al*., 2011). However, quaternary amine cation of BER causes poor water solubility, resulting in low bioavailability. In addition, BER also induce the activity of multidrug efflux transporter P-glycoprotein (P-gp) in the intestine, responsible for active efflux of drug from cells, cause its own ejection resulting in 90% reduction in BER transport (Zhang *et al*., 2011; Di Pierro *et al*., 2012; Shan *et al*., 2013). Moreover, intramuscular and intravenous administration may leads to risk of adverse reactions, such as drug rash and anaphylactic shock.

* *Corresponding Author*
*E-mail: sailorgirish @ gmail.com*

Oral route is the most easiest and convenient way for administration of drugs. However, some of the drugs have a very low oral bioavailability because of poor aqueous solubility and permeability, multidrug resistance protein (MRP) efflux and metabolic stability (Choi *et al*., 2004). Recently lipid based formulations are widely used for the oral administration of phytoconstituents. Nevertheless, lipid-based formulation can also be formulated in different dosage form like self-emulsifying systems, multiple emulsions, microemulsions, liposomes, and solid lipid nanoparticle. There are various mechanisms responsible for the absorption enhancement of drug from lipid based formulation for instance, altering the intestinal environment, interacting with enterocyte-based transport, stimulation of lymphatic transport, and active ingredients release modification. Furthermore, degradation of active ingredient in gastrointestinal tract can be protected by phospholipids (Fricker *et al*., 2010).

Among the lipid based systems, liposome seems to be the most promising system for its ability to enhance the permeability of drug across the enterocyte, to stabilize drugs, and provide the opportunity of controlled release (Charman *et al*., 1986). Liposomes are spherical-shaped vesicle consisting of one or several phospho-lipid bilayers separated by aqueous inner compartments and are

nontoxic, biocompatible and biodegradable. These vesicles have ability to incorporate hydrophobic, hydrophilic and ampiphilic substances. It has also been demonstrated that liposomes can improve solubility, stability and encapsulation efficiency, and drug protection against degradation. Many researchers indicated that bioavailability of orally administered drug with poor solubility and permeability was obviously enhanced after encapsulation with liposomes and changes the *in vivo* distributions of entrapped drugs.(Moutardier *et al.*, 2003; Deshmukh *et al.*, 2008; Jain *et al.*, 2012a; Jain *et al.*, 2012b; Niu *et al.*, 2012; Gradauer *et al.*, 2013). In the present investigation, we prepared a BER loaded liposome using thin film hydration technique, and was optimized using $3^2$ full factorial design. They were further characterized for their entrapment efficiency, vesicle size and zeta potential, *in vitro* drug release and morphology.

## MATERIALS AND METHODS

### Materials

Berberine (BER) was purchased from Yucca Enterprize, Mumbai. Soyphsophatidylcholine (SPC, purity, 98%) was provided as a gift sample from Lipoid GmBH Company (Ludwigshafen, Germany). Cholesterol (CHOL) and all other solvents and reagents used were analytical grade and purchased from S D Fine-Chem Ltd (Mumbai, India).

### Preparation of liposome

Thin film hydration method was used to prepare berberine loaded liposome (Szoda, 1981; Law *et al.*, 1998; Fresta *et al.*, 1999). In this method, SPC (Lipoid S 100), CHOL and BER were firstly dissolved in chloroform in different molar ratio (Table 1).

**Table 1**: $3^2$ Factorial designs of independent variables with measured responses.

| Batch | Independent Variables | | Dependent Variables | |
|---|---|---|---|---|
| | $X_1$ | $X_2$ | $Y_1$ (nm) | $Y_2$ (%) |
| BL1 | 1 | 1 | 876 | 82.38 |
| BL2 | -1 | -1 | 982 | 56.08 |
| BL3 | 0 | 1 | 642 | 77.13 |
| BL4 | -1 | 0 | 854 | 67.4 |
| BL5 | 1 | 0 | 1104 | 80.24 |
| BL6 | 1 | -1 | 1105 | 75.76 |
| BL7 | 0 | -1 | 1021 | 69.08 |
| BL8 | 0 | 0 | 995 | 74.51 |
| BL9 | -1 | 1 | 571 | 69.24 |

$X_1$ = Drug: Lipid (Molar ratio), $X_2$ = SPC: Cholesterol (% of total lipid)
$Y_1$ = Vesicle size (nm), $Y_2$ = Entrapment efficiency (%)

The chloroform was evaporated at 60 $^oC$ for 1 h under vacuum at 150 rpm by rotary evaporator (Remi Instruments, Mumbai, India) to form a thin lipid film. The dried thin lipid film was hydrated by adding phosphate buffer saline (PBS) pH 6.8 at 45$^oC$ in rotary vacuum evaporator rotated at 100rpm until the dispersion of all the lipids in the aqueous phase. For vesicle size reduction, the dispersion was subjected to bath sonication (Toshniwal Instruments, Ajmer) for 20-30 min at a frequency of about 30±3KHz at 40°C. Thereafter, the mixture was kept for 1 h at room temperature for the formation of vesicle followed by 4°C for 24h in an inert atmosphere. The formulation was centrifuged for 1h at 15000 rpm in a cold centrifuge (Remi Instruments, Mumbai, India). Then, the supernatant containing the vesicles in each case was separated and taken for further studies in a suspended form.

### Experimental design

#### *$3^2$ factorial designs*

The formulations were optimized by $3^2$ factorial designs consisting of drug: lipid molar ratio ($X_1$) and SPC: cholesterol ($X_2$) as a independent variables while vesicle size ($Y_1$) and entrapment efficiency ($Y_2$) as response (Table 1). Nine formulations were prepared and evaluated for response. The obtained data were fitted into Design Expert software (Design Expert 9.0.4, Stat-Ease, Minneapolis, MN). Analysis of variance (ANOVA) was used to validate design.

#### *Response surface plot*

Contour plot and (3D) response surface plots were constructed to establish the understanding of relationship of variables and its interaction.

#### *Optimization using desirability function*

The formulations were optimized by keeping the $X_1$ and $X_2$ within the range used in present work while $Y_1$ at minimum and $Y_2$ at maximum using Design-Expert software. On the basis of these assigned goals, software determines the possible formulation composition with maximum desirability value.

#### *Checkpoint analysis*

According to desirability value and composition of variables, formulation was prepared and evaluated for response. The predicted and observed response was compared and percentage prediction error was calculated to confirm the validity of design for optimization.

### Characterization of Liposome

#### *Morphology of liposome*

Shape and lamellarity of vesicle was observed by placing the suspension under optical microscope (Olympus BX 41, USA). Photomicrographs were taken by a camera attached to the optical microscope in 10x100 magnifications.

#### *Vesicle size*

The optimized formulation, serially diluted 100-fold with Double distilled water, was used to determine mean vesicle size and polydispersity index (PDI) using Zetasizer HAS 3000 (Malvern instrument Limited, UK).

#### *Zeta potential*

Zeta potentials of the optimized formulations was measured by Zetasizer HAS 3000 (Malvern instrument Limited, UK) at 25$^oC$.(Law *et al.*, 1998)

***Entrapment efficiency***

Liposome suspension was centrifuge at 15000 rpm to separate unentrapped drug. Free drug present in supernatant was determined using UV spectrophotometer at 345 nm. EE(%) was calculated by following equation:

$$EE\ (\%) = [(C_{total} - C_{free})/C_{total}]x100$$

Where, $C_{total}$ = total drug added, $C_{free}$= unentrapped drug

***In vitro diffusion study***

Membrane diffusion technique was used to determine release of BER from plain drug suspension and formulation. Liposomal suspension (1.5 mL) with known amount of drug was filled in dialysis bag (Mw cut-off = 12000-14000, Hi-media laboratories, Mumbai), previously soaked in distilled water for 24h. The bag was placed in 25mL of phosphate buffer saline (PBS, pH 6.8), continuously stirred by magnetic stirrer, maintained at 37°C. Samples (1 mL) were withdrawn at specified time interval and substituted with fresh PBS (pH 6.8). UV spectrophotometer was used to determine drug from sample at 345 nm.

**Stability Study**

Berberine loaded liposomes were stored in glass vials and kept at 4-8°C, 25±2°C and 37±2°C for one month. The samples were taken after one month and entrapment efficiency was determined as described earlier.

**RESULTS AND DISCUSSION**

**Experimental design**

The three level two factor design is an effective approach for investigating variables at different levels with a limited number of experimental runs (Table 2). The vesicle size and EE of total 9 batches showed a wide variation from 571 to 1105 nm and 56 to 82%, respectively.

**Table 2** Variables in $3^2$ Factorial designs for liposome

| Variable | Levels [Coded (Actual)] | | |
|---|---|---|---|
| | **Low (-1)** | **Medium (0)** | **High (+1)** |
| Independent variables | | | |
| $X_1$ =Drug: Lipid (Molar ratio) | -1 (1:5) | 0 (1:10) | +1 (1:15) |
| $X_2$ = SPC: Cholesterol (% of total lipid) | -1 (70:30) | 0 (60:40) | +1 (50:50) |

***Fitting the model to data***

Response data of all formulations were fitted to cubic, linear and quadratic model. According to Design Expert software, best-fitted model was linear for response $Y_1$ and quadratic for response $Y_2$. All the responses were fitted to model to establish full model (FM) polynomial equation.

$$Y_1 = 964.78 + 113.\ X_1 - 169.83\ X_2 + 45.50\ X_1 X_2 - 29.33\ X_1^2 - 118.17\ X_2^2$$

$$Y_2 = 75.20 + 7.61\ X_1 + 4.64\ X_2 - 1.64\ X_1 X_2 - 1.72\ X_1^2 - 2.44\ X_2^2$$

Statistical validity of the polynomials was established on the basis of ANOVA provision in the Design Expert ®software. Further analysis using ANOVA indicated significant effects of the independent factors (p>F) on response $Y_1$ and $Y_2$. F-value for $Y_1$=53.25 and $Y_2$=40.88, while resulted $R^2$ for $Y_1$=0.9875 and $Y_2$=0.9876. Statistical models were generated for each response parameter and tested for significance. Further Adj-$R^2$ and Pred-$R^2$ values for all responses were in reasonable agreement, indicating that the data were described adequately by the mathematical model. Values of ''p'' less than 0.05 indicated that model terms were significant except for responses $Y_1$, two model terms $X_1^2$ and $X_1X_2$ were at p>0.05 (p value: 0.3197, 0.0797, respectively), and for $Y_2$, model term $X_1^2$, $X_2^2$and $X_1X_2$ were at p>0.05 (p value: 0.1949, 0.1001,0.1119, respectively) indicated necessary model reduction to improve the model (Table 3).

**Table 3** Analysis of Variance of the factorial models for the responses.

| | **Source** | **Sum of squares** | **df** | **Mean square** | **F value** | **p-value Prob>F** |
|---|---|---|---|---|---|---|
| Vesicle size (nm) | Model | 287600 | 5 | 57520.56 | 47.31 | 0.0047 |
| | A-Drug:Lipid | 76614.00 | 1 | 76614.00 | 63.01 | 0.0042 |
| | B-SPC:CHO | 173100 | 1 | 173100 | 142.34 | 0.0013 |
| | AB | 8281.00 | 1 | 8281.00 | 6.81 | 0.0797 |
| | A2 | 1720.89 | 1 | 1720.89 | 1.42 | 0.3197 |
| | B2 | 27926.72 | 1 | 27926.72 | 22.97 | 0.0173 |
| | Residual | 3647.44 | 3 | 1215.81 | | |
| | Cor Total | 291300 | 8 | | | |
| Entrapment efficiency (%) | Model | 505.08 | 5 | 101.02 | 47.02 | 0.0047 |
| | A-Drug:Lipid | 347.47 | 1 | 347.47 | 161.75 | 0.0010 |
| | B-SPC:CHO | 129.08 | 1 | 129.08 | 60.09 | 0.0045 |
| | AB | 10.69 | 1 | 10.69 | 4.98 | 0.1119 |
| | A2 | 5.94 | 1 | 5.94 | 2.76 | 0.1949 |
| | B2 | 11.89 | 1 | 11.89 | 5.54 | 0.1001 |
| | Residual | 6.44 | 3 | 2.15 | | |
| | Cor Total | 511.53 | 8 | | | |

***Response surface (3D) and Contour plot analysis***

The obtained results can be observed visually in the response surface (3D) and contour plots (Fig.1, 2). Response surface graph of $Y_1$ shows that vesicle size of liposome was decreased with decreasing SPC concentration because phospholipids constitute the liposome membrane. With increasing total lipid (SPC:Cholesterol) concentration more drug could be incorporate into liposome. In addition, response surface graph of $Y_2$ shows that the increase in SPC:Cholesterol ratio significantly increased the drug entrapment efficiency. These results supported by the fact that, movement of fatty acids hydrophobic tails was reduced by incorporation of a bulky molecule of cholesterol in the lipid bilayer of liposome. It leads to permeability reduction of liposome membrane via resistance of phospholipids exchange with apoprotein. These ultimately improve the drug retention in liposome by prevention of drug leakage from lipid bilayer.

***Optimization of formulation***

The search for the optimized formulation composition was carried out using the desirability function approach with

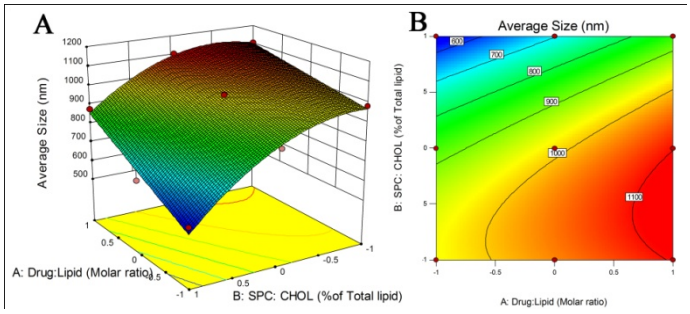


**Fig. 1** Response surface (A) and its Contour plot (B) shows effect of $X_1$ and $X_2$ on vesicle size.

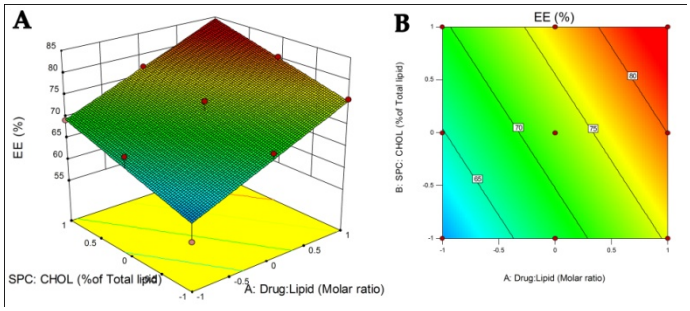


**Fig. 2** Response surface (A) and its Contour plot (B) shows effect of $X_1$ and $X_2$ on Entrapment efficiency.

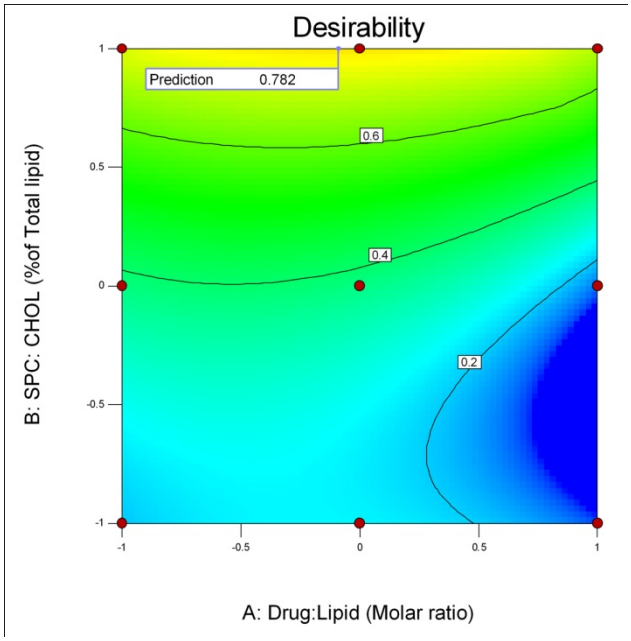


**Fig. 3** Contour plot for overall desirability of liposome as a function of $X_1$ and $X_2$.

Design expert software, criterion being one having the maximum desirability value. The optimization process was performed by setting the $Y_1$ at minimum and $Y_2$ at maximum while $X_1$ and $X_2$ within the range obtained. The optimized formulation was achieved at $X_1$=1:9.56, $X_2$=50:50 with the corresponding desirability *(D)* value of 0.782 (Fig.3). This factor level combination predicted the responses $Y_1$=654 nm, $Y_2$= 75.68%.

### *Checkpoint Analysis*

The comparisons of predicted and experimental results shows very close agreement, indicating the success of the design combined with a desirability function for the evaluation and optimization of liposome formulations (Table 4).

**Table 4.** Checkpoint batch with their predicted and observed value of responses

| | Independent Variables | | Vesicle size($Y_1$) | | Entrapment efficiency ($Y_2$) | |
|---|---|---|---|---|---|---|
| **Batch** | **$X_1$** | **$X_2$** | **Observed** | **Predicted** | **Observed** | **Predicted** |
| BL10 | -0.089 (1:9.56) | +1 (50:50) | 648 | 654 | 77.91 | 75.68 |
| **Percentage prediction error (%)** | | | -0.92 | | +2.86 | |

## Characterization of Optimized Formulation

### *Vesicle size and shape*

Vesicle size determination is essential parameters for application of liposome (Maherani *et al.*, 2012). Several methods are available for preparation of liposome with different size, composed of one or more lipid bilayer. Generally, lipid film hydration is used for preparation of multilamellar vesicles. Sonication was done to produce small unilamellar vesicle. The optimized liposome (BL 10) was spherical in shape and found to be unilamellar to multilamellar (Fig. 4). The average vesicle size was found to be 0.823 nm with 0.354 polydispersity index (Fig. 5).

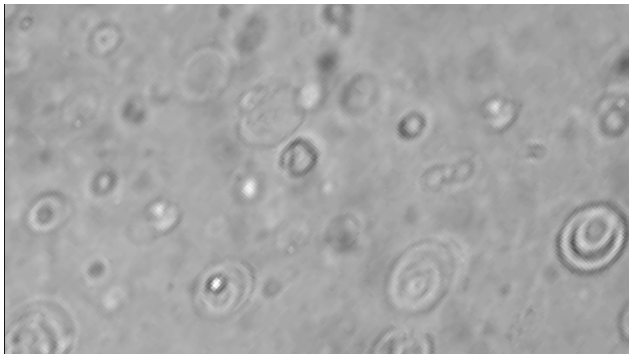

**Fig. 4.** Microscopy of optimized liposome (BL10).

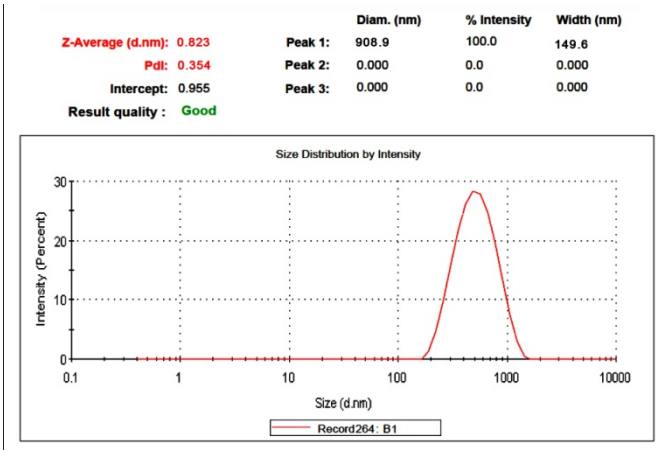


**Fig. 5.** Particle size of optimized liposome (BL10).

***Zeta potential***

Zeta potential of liposome ensures stability and entrapment efficiency and also used to predict *in vivo* behavior (Maherani *et al.*, 2012). Entrapment efficiency was increased due to electrostatic attraction between charged molecule and liposomes. Any subsequent modifications of the liposomal surface, such as cholesterol incorporation, also influence zeta potential. The higher values of zeta potential enhance the stability of liposome by increasing the repulsion of vesicle, and thereby preventing aggregation. Liposome prepared by using different lipids acquires different surface charge. Liposome employing pohosphatidylserine, stearylamine or dioleoyltrimethylammonium propane and phosphatidylcholine get negative, positive and neutral charge respectively (Brgles *et al.*, 2008). On the contrary, in present study liposome prepared with phosphatidylcholine possess slightly negative charge (-1.93 mV) (Fig. 6). It may be due to the effect of cholesterol on surface charge.

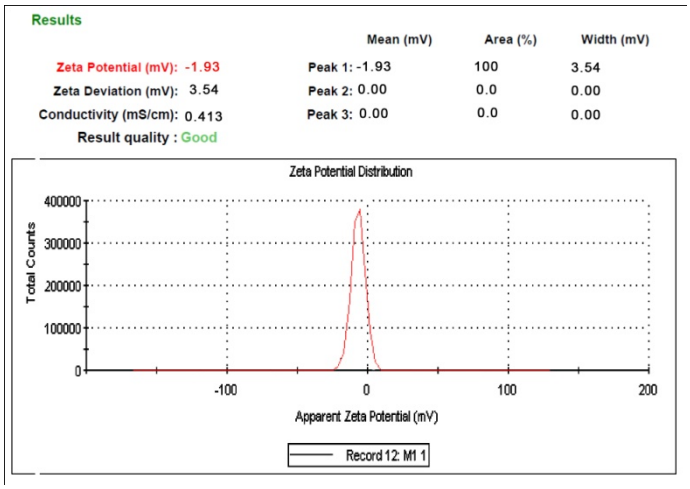


**Fig. 6** Zeta potential of optimized liposome (BL10).

***Entrapment efficiency***

Drug can be incorporated into liposome by several ways depending on various properties like polarity and solubility. It can be adsorbed on surface of membrane, entrapped in lipid bilayer, encapsulated in inner aqueous core, attached between polar head or supported by a hydrophobic tail (Maherani *et al.*, 2011). Method of preparation and composition of lipid can also influence the entrapment efficiency. The present study shows 78.43% entrapment efficiency indicating good electrostatic interaction between bioactive agent and liposomes.

***In vitro diffusion study***

Release characteristics of BER from liposome was evaluated *in vitro* and compared to that of pure drug. It was observed that the release of BER suspension was completed within 10 h while liposomal formulations shows 70% release within 24 h (Fig. 7). This results supported support by the fact that the layer of drug-encapsulated liposomes attached to the semi-permeable membrane breaks and leaches its contents slowly before another layer replaces the leached vesicles. Due to this mechanism controlled release of drug in liposomes can be expected over a prolonged period of time.

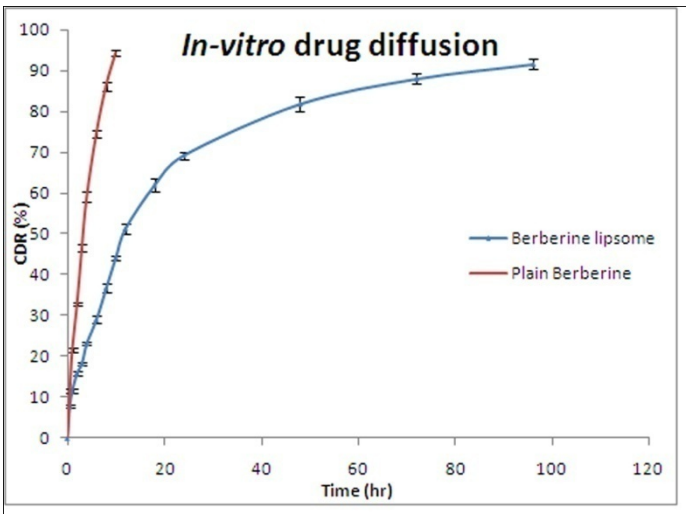


**Fig. 7** *In vitro* drug diffusion of berberine loaded liposome and plain drug.

**Stability Study**

Stability study reveals considerable drug loss (approx. 12%), was marked from formulation storage at high temperature, i.e., 37±2°C. On contrary, formulation stored at 4-8°C and 25±2°C, could retain 93% and 97% of the entrapped drug, respectively. Substantial loss of drug at high temp may be due to the deprivation of phsopholipids leads to disturbance in packing of membrane. In addition, high temperature also cause change in gel to liquid transition of lipid bilayer. The results of the study indicate that the development of BER loaded liposome can overcome the limitation of the molecule related to poor oral absorption and can enhance the bioactivity of the BER.

**CONCLUSION**

In this study, $3^2$ full factorial designs were used for predicting the optimum condition for preparation of liposome. The formulations were successfully prepared by thin film hydration method to observe the effect of drug:lipid and soyphosphatidylcholine:cholesterol ratio on vesicle size and entrapment efficiency. Increase in lipid concentration was found to produce liposome with highest entrapment efficiency. On the other hand, decrease in SPC concentration produce smaller vesicle. These effects were fitted into polynomial model to identify the significant effects of independent variables on response and visually observed by contour plot and response surface (3D) plots. The effectiveness of experimental design was confirmed by close agreement of experimental value with estimated value of optimized formulation prepared in accordance with desirability value. Thus, $3^2$ full factorial design with desirability function is an effective means to optimize berberine loaded formulations.

## REFERENCES

Brgles M, Jurašin D, Sikiric MD, Frkanec R, Tomašic J. Entrapment of ovalbumin into liposomes-Factors affecting entrapment efficiency, liposome size, and zeta potential. J Liposome Res, 2008;18(3):235-48.

Charman W, Stella V. Estimating the maximal potential for intestinal lymphatic transport of lipophilic drug molecules. Int J Pharm, 1986;34(1):175-8.

Choi J-S, Jo B-W, Kim Y-C. Enhanced paclitaxel bioavailability after oral administration of paclitaxel or prodrug to rats pretreated with quercetin. Eur J Pharm Biopharm, 2004;57(2):313-8.

Deshmukh DD, Ravis WR, Betageri GV. Improved delivery of cromolyn from oral proliposomal beads.Int J Pharm, 2008;358(1):128-36.

Di Pierro F, Villanova N, Agostini F, Marzocchi R, Soverini V, Marchesini G. Pilot study on the additive effects of berberine and oral type 2 diabetes agents for patients with suboptimal glycemic control. Diabetes, MetabSyndrObes: Targets Ther, 2012;5:213.

Fresta M, Panico A, Bucolo C, Giannavola C, Puglisi G. Characterization and In-vivo Ocular Absorption of Liposome-encapsulated Acyclovir. J Pharm Pharmacol, 1999;51(5):565-76.

Fricker G, Kromp T, Wendel A, Blume A, Zirkel J, Rebmann H, et al. Phospholipids and lipid-based formulations in oral drug delivery. Pharmaceut Res, 2010;27(8):1469-86.

Gradauer K, Barthelmes J, Vonach C, Almer G, Mangge H, Teubl B, et al. Liposomes coated with thiolated chitosan enhance oral peptide delivery to rats. J Controlled Release, 2013;172(3):872-8.

Jain S, Kumar D, Swarnakar NK, Thanki K. Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. Biomaterials. 2012;33(28):6758-68.

Jain S, Patil SR, Swarnakar NK, Agrawal AK. Oral delivery of doxorubicin using novel polyelectrolyte-stabilized liposomes (layersomes).Mol Pharmaceutics, 2012;9(9):2626-35.

Law S, Hung H. Properties of acyclovir-containing liposomes for potential ocular delivery.Int J Pharm, 1998;161(2):253-9.

Lee I-A, Hyun Y-J, Kim D-H.Berberine ameliorates TNBS-induced colitis by inhibiting lipid peroxidation, enterobacterial growth and NF-κB activation. Eur J Pharmacol, 2010;648(1):162-70.

Liu X, Li G, Zhu H, Huang L, Liu Y, Ma C, et al. Beneficial effect of berberine on hepatic insulin resistance in diabetic hamsters possibly involves in SREBPs, LXRα and PPARα transcriptional programs. Endocr J, 2009;57(10):881-93.

Maherani B, Arab-Tehrany E, Kheirolomoom A, Reshetov V, Stebe MJ, Linder M. Optimization and characterization of liposome formulation by mixture design. Analyst. 2012;137(3):773-86.

Maherani B, Arab-Tehrany E, R Mozafari M, Gaiani C, Linder M. Liposomes: a review of manufacturing techniques and targeting strategies. CurrNanosci, 2011;7(3):436-52.

Moutardier V, Tosini F, Vlieghe P, Cara L, Delpero J, Clerc T. Colloidal anticancer drugs bioavailabilities in oral administration models. Int J Pharm, 2003;260(1):23-38.

Niu M, Lu Y, Hovgaard L, Guan P, Tan Y, Lian R, et al. Hypoglycemic activity and oral bioavailability of insulin-loaded liposomes containing bile salts in rats: the effect of cholate type, particle size and administered dose. Eur J Pharm Biopharm, 2012;81(2):265-72.

Shan Y-Q, Ren G, Wang Y-X, Pang J, Zhao Z-Y, Yao J, et al. Berberine analogue IMB-Y53 improves glucose-lowering efficacy by averting cellular efflux especially P-glycoprotein efflux. Metabolism, 2013;62(3):446-56.

SzodaFCaP, D. Liposomes: preparation and characterization. Knight CG, editor. Amsterdam: Elsevier; 1981.

Wu M, Wang J, Liu L-t. Advance of studies on anti-atherosclerosis mechanism of berberine. Chin J Integr Med, 2010;16(2):188.

Zhang X, Qiu F, Jiang J, Gao C, Tan Y. Intestinal absorption mechanisms of berberine, palmatine, jateorhizine, and coptisine: involvement of P-glycoprotein. Xenobiotica, 2011;41(4):290-6.

Zhao L, Li W, Han F, Hou L, Baillargeon J-P, Kuang H, et al. Berberine reduces insulin resistance induced by dexamethasone in theca cells in vitro. Fertil and steril, 2011;95(1):461-3.

Zhou J, Zhou S. Berberine regulates peroxisome proliferator-activated receptors and positive transcription elongation factor b expression in diabetic adipocytes. Eur J Pharmacol, 2010;649(1):390-7.

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