4.1. Introduction

Preformulation studies are a group of preliminary trials/experiments conducted before initiating formulation development process. These studies provide basic fundamentals of the behaviour/nature of the different classes of materials/chemicals to be used in the preparation of formulation. Scientists can also record the influence of various process parameters influencing the product quality attributes. The scientific knowledge gained by the preformulation studies help in reducing waste of time and resources during formulation development. The present chapter describes identification, compatibility studies and preliminary experiments conducted to gain knowledge regarding behaviour of various formulation components.

4.2. Materials and Equipment

Paclitaxel (PTX) and Carboplatin (CBP) was obtained as a gift sample from Sun Pharma Advanced Research Centre, Vadodara, India. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and L-α-phosphatidylcholine, Egg (EggPC) was obtained from Avanti Polar Lipids, Inc Alabaster, Alabama. 1,2-Diacyl-sn-glycero-3-phosphocholine, hydrogenated (HSPC) and 1,2-Diacyl-sn-glycero-3-phosphocholine, Soy (SPC) were obtained as gift samples from Lipoid GmbH, Germany. Sodium Deoxycholate was purchased from Loba Chemie Pvt Ltd, Mumbai, India. HPLC grade methanol (MeOH) and acetonitrile (ACN) were procured from Fisher Scientific (Vadodara, Gujarat) to carry out chromatographic analysis. Double distilled water used in the study was filtered using 0.22 micron nylon filter, Nylon N66 membrane filters 47 mm, Rankem, India. All other reagents were purchased from S.D. finechem Ltd, India and were of analytical grade.

Equipment

- Electronic weighing balance (ATX 224, Shimadzu, Japan)
- Rotary evaporator (IKA RV10, Karnataka, India)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)

- Fourier Transform Infrared spectrophotometer (Bruker, USA)
- Fourier Transform Infrared spectrophotometer (Shimadzu, Japan)
- Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan)
- Cooling Centrifuge (CPR-30, Remi Equipment, Mumbai, India)
- Melting point apparatus (VMP-PM, Veego, India)
- Probe Sonicator (LabsonicM, Sartorius Ltd, Mumbai, India)
- Nikon H600L Microscope (DS-Fi2, Nikon, Japan)
- Zeta sizer (Nano ZS Malvern Instruments, UK)

4.3. Preformulation

4.3.1. Methods

4.3.1.1. Fourier Transform Infrared spectroscopy (FTIR)

PTX was mixed with IR grade anhydrous Potassium bromide (KBr) in a ratio of 1:100 and pellets were prepared by applying 10 metric ton of pressure in a hydraulic press. The pellets were then scanned over a range of 4000 – 400 cm^{-1} in FTIR instrument (Bruker, USA).

CBP was triturated individually in mortar and pestle to remove lumps, if any. A small amount of fine powder of drug was kept in sample holder and the spectra was recorded by scanning in the wavelength region of 4000 – 600 cm^{-1} using FTIR spectrophotometer (IR Affinity-1, Shimadzu, Japan).

The IR spectrum of individual drugs were compared with that of their respective reported IR values. The major peaks obtained in the FTIR spectra corresponding to the functional groups present were studied carefully in comparison with the reported values of the individual drugs for their identification.

4.3.1.2. Melting point determination

Melting point of both the drugs was determined using capillary method. Drug sample powder was filled in one end sealed capillary up to 3 - 4 mm length. The capillary was then inserted in hole provided on heater stand on melting point apparatus (Veego-VMP-PM) and the melting point was recorded for both the drugs.

4.3.1.3. Differential scanning calorimetry (DSC)

Differential scanning calorimeter (DSC-60, Shimadzu, Japan) equipped with an intra-cooler and a refrigerated cooling system was used to analyse the thermal behaviour of drugs. Approximately, 2 to 3 mg of drug sample was heated, in aluminium pan over the range of 30 to 300 °C at a heating rate of 10 °C per min with Nitrogen supplied at 50 mL per min and 100 mL per min through cooling unit.

4.3.1.4. Drug-Excipients compatibility study

A drug-excipient compatibility study was carried out with potential formulation excipients for assessment of possible drug – excipients interaction. The drug-excipients compatibility study was carried out by visual observation and by using DSC. The mixture of drugs with different excipients to be used in formulation (DSPC, EggPC, HSPC, SPC and SDC) were subjected to Differential Scanning Calorimetric (DSC) studies (Shimadzu, Japan) as described above. The results were compared to that of DSC endotherms of the pure drugs.

4.3.2. Results and Discussion

4.3.2.1. Fourier Transform Infrared spectroscopy (FTIR)

FTIR spectra of PTX and CBP pure drug are shown in **Fig. 4-1** and **Fig. 4-2** respectively. When analysing the spectrum of PTX pure drug, the strong peaks obtained in the region of 1735-1710 and 1647 cm^{-1} might be due to the -C=O stretching of ester/keto groups and amide group respectively while peak at 709 cm^{-1} could be due to out of plane bending of aromatic C-H present in the molecule.

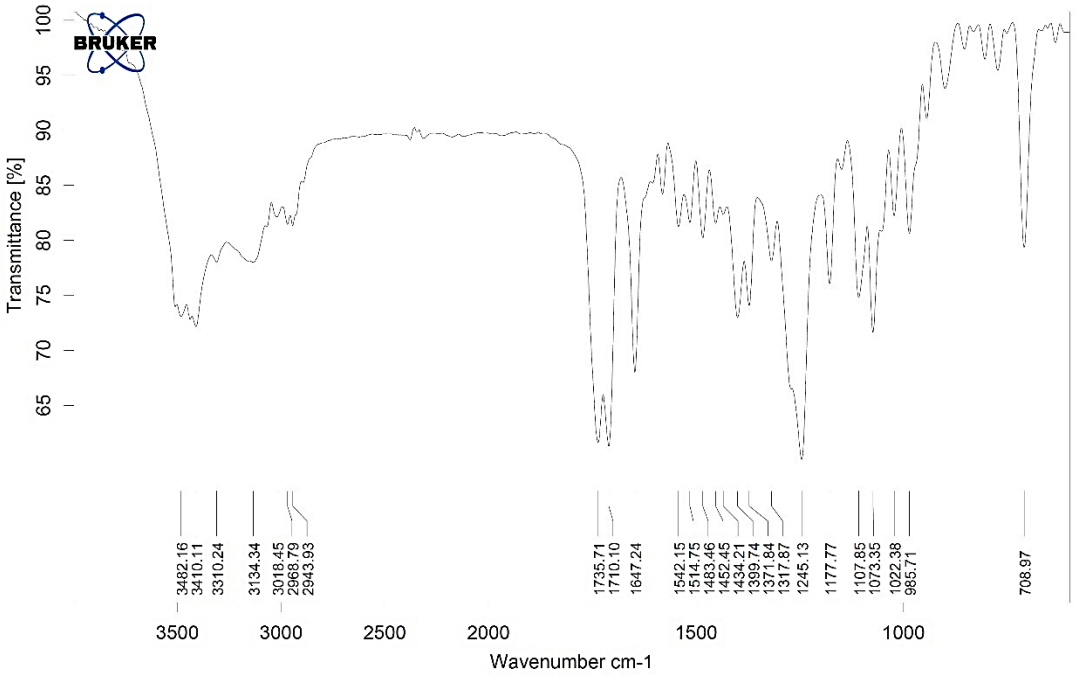

Fig. 4-1: FTIR spectrum of PTX

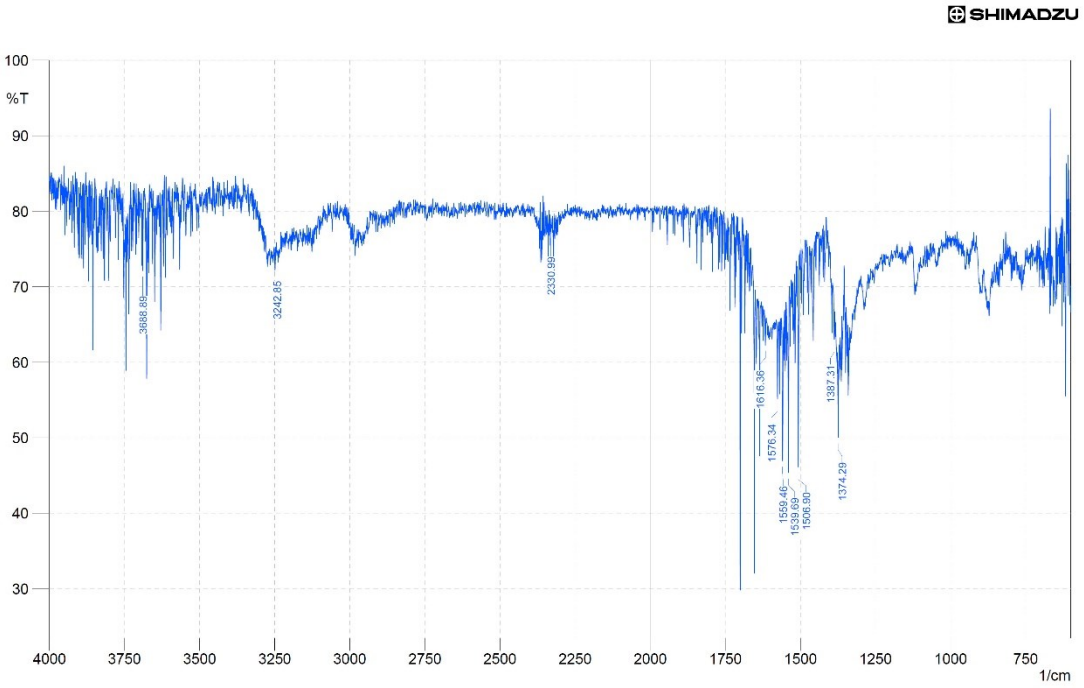

Fig. 4-2: FTIR spectrum of CBP

The most intense peaks in the spectrum of CBP pure drug may be attributed to ester group carbonyl stretch (-C=O) around 1730-1715 cm^{-1} and -C–O stretches appear as

two or more bands in the region 1300-1000 cm^{-1}. The band obtained at around 3242 cm^{-1} may be attributed to the associated –NH group. The bands at around 2900 and 2800 cm^{-1} correspond to asymmetric and symmetric stretching vibrations of -CH respectively. Characteristic peaks for Pt-NH_2 bond appear at 1374 and 1387cm^{-1}. The peaks at 1616 cm^{-1} may be attributed to the bending vibrations of -NH group.

4.3.2.2. Melting point determination

Melting point of PTX and CBP was obtained experimentally and was compared with that reported in Literature. Experimental melting point of PTX was obtained in the range of 213-215°C which was comparable with the reported range of 213-216°C [1]. The experimental melting point of CBP was observed in the range of 238-245°C which was comparable with the reported range of 235-245°C [2].

4.3.2.3. Differential scanning calorimetry (DSC) and Drug-Excipients compatibility study

DSC thermograms were obtained for both pure drugs PTX and CBP. The drug excipient mixtures for both the formulation were also run in DSC to obtain thermograms. The thermograms were compared and depicted in **Fig. 4-3** and **Fig. 4-4**.

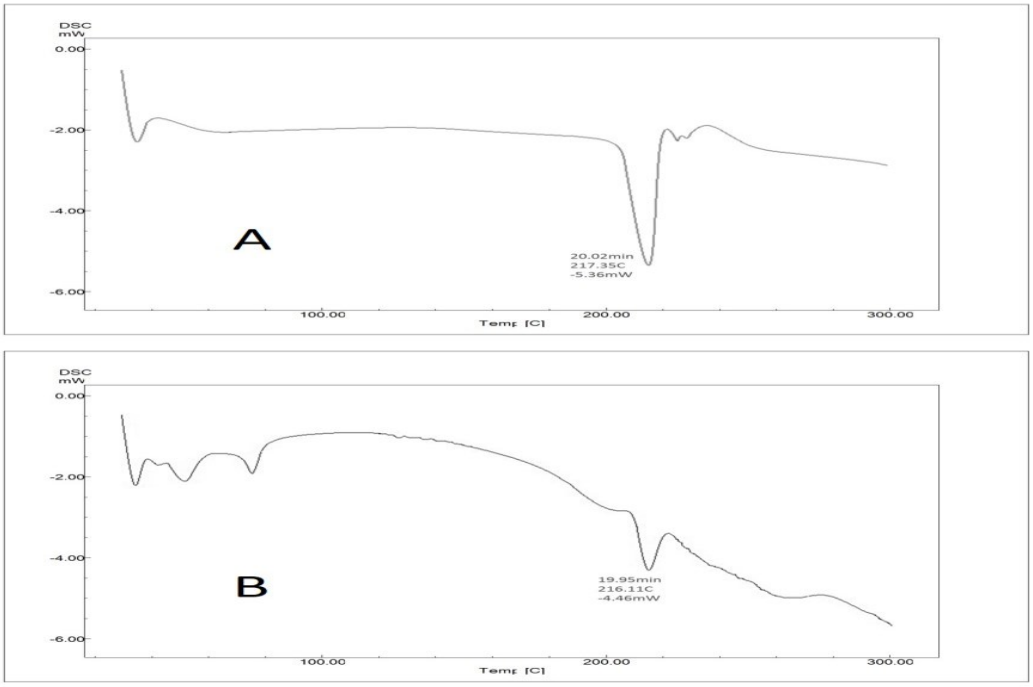

Fig. 4-3: DSC thermograms of A) pure PTX and B) PTX-excipients mixture

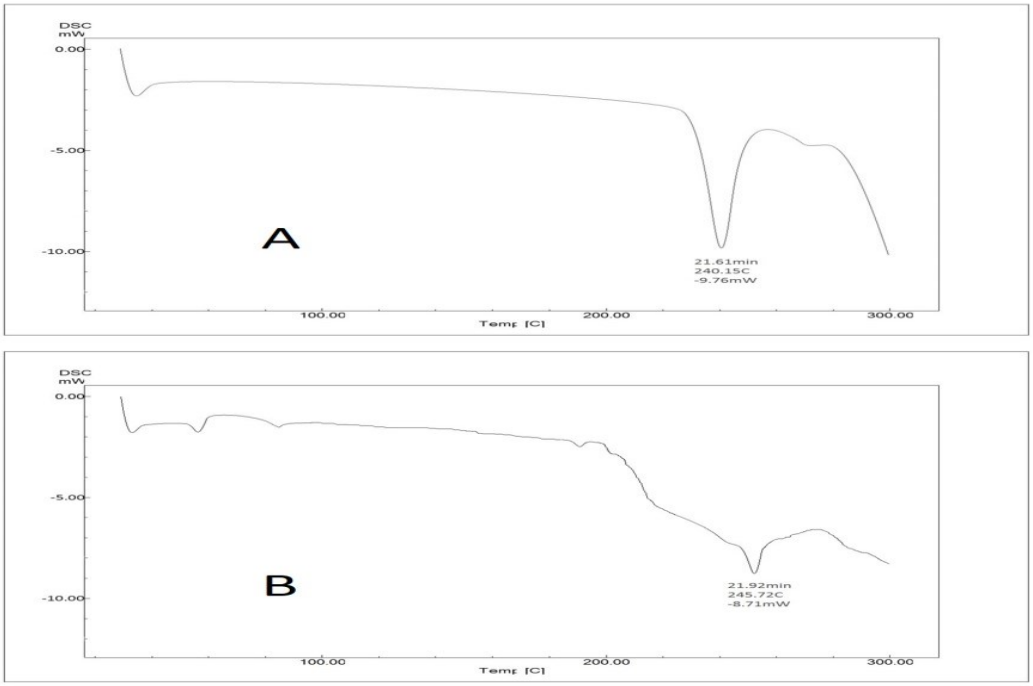

Fig. 4-4: DSC thermograms of A) pure CBP and B) CBP-excipients mixture

The thermograms of pure drugs showed their characteristic endothermic peaks corresponding to their respective melting point values. The drug molecule peaks in the thermograms of the drug excipients mixture of both the drugs were not shifted significantly from the peak positions obtained in the DSC thermograms of pure drugs. The results indicate that there is no chemical interactions between drug and excipient. It can be concluded from the results that there is no incompatibilities between individual drug and excipients and hence indicates that the selected excipients are suitable for use further in drug formulations.

4.4. Feasibility trials of the formulation

Formulation feasibility trials were taken initially to understand the process and experimental set up. After literature search, HSPC and SDC were selected as lipid and edge activator respectively in ratio of 8:2 and dissolved in MeOH:DCM (7:3) mixture to prepare blank formulation. A thin film of lipid was obtained using rotary evaporator (IKA RV10, Karnataka, India) at 55°C under vacuum. After complete removal of organic solvent, 10 ml of distilled water was added to the flask as hydration media and the thin film was allowed to hydrate for 30 min. The vesicle size of the dispersion was measured

using size analyser (Zeta Sizer Nano Series, Malvern Instruments, UK). The size of the vesicular dispersion was found to be 679.2 nm. Sonication cycle with 80 % amplitude, 0.8 s pulse rate and 1 min cycle time was run for 3 times to obtain vesicular dispersion of size 91.29 nm. The physical appearance of the vesicular dispersion of two different size was observed (as shown in **Fig. 4-5**). The pre-sonicated dispersion appeared as a hazy dispersion whereas after sonication it appeared clear with slight blue tint due to the dissimilarity in diffraction pattern of the light by different sized vesicles. Also a drop of pre-sonicated dispersion was put on a slide and observed for formation of vesicles under the microscope (Nikon H600L Microscope (Nikon, Japan) as shown in **Fig. 4-6**. The vesicles formed were observed as smooth and spherical in shape.

Fig. 4-5: Appearance of dispersion before and after sonication

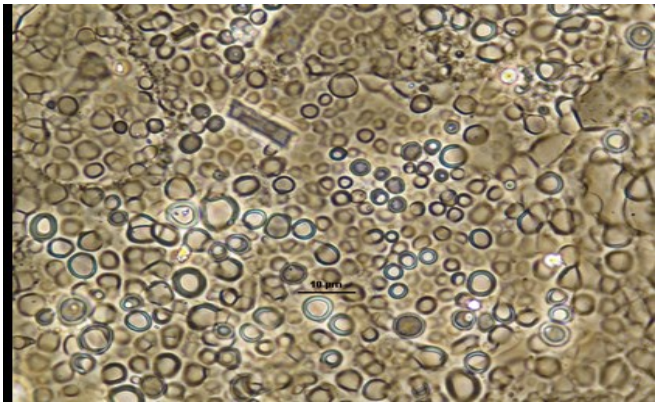

Fig. 4-6: Pre-sonicated blank vesicular dispersion under microscope

Selection of Lipids

The differences in phospholipid aliphatic chains, head groups, and alcohols leads to the wide variety of phospholipids. Moreover, various categories of phospholipid are available based on their source of origin or method of synthesis. Several phospholipids, such as soybean-phosphatidylcholine, egg-phosphatidylcholine, or synthetic-phosphatidylcholine, hydrogenated-phosphatidylcholine, are generally used in different kinds of formulations and can offer numerous choices. However, it is now crucial to select suitable lipids to design drug delivery system to accomplish the therapeutic goals [3]. Hence, after review of literatures, four different types of lipids were tried for the formulations i.e. SPC, HSPC, DSPC and EggPC. DSPC was selected for PTX UDNVs on the basis that it possess longer alkyl chain length i.e. 18 carbon chain length which furnishes DSPC with high capabilities of entrapment of lipophilic drugs like PTX. EggPC was used in combination with DSPC and not used as single lipid since phase transition temperature of the Egg PC is as low as −5 to −15 °C whereas phase transition temperature corresponding to DSPC is 55 °C. DSPC alone can form rigid phospholipid bilayer and can exert positive effect on drug release as well as on clearance of the vesicle by mononuclear phagocyte system (MPS) [4]. EggPC also possess special characteristics of its behaviour as surface active agent which stabilizes lipid based systems. For CBP UDNVs formulation, a combination of Hydrogenated SPC (HSPC) and SPC natural lipid was selected. HSPC is a saturated while SPC is an unsaturated phospholipid. A combination of both the lipids was used to achieve higher loading in vesicular formulations [5-7]. HSPC exhibits phase transition temperature of 52 °C whereas SPC exhibits very low phase transition temperature in the range of -20 to -30 °C. Hence the combination of lipids in the bilayer that resulted in to two separate phases, a gel phase and a liquid-crystalline phase. The coexistence of these two immiscible phases in the membrane was said to create discontinuous regions in the bilayer, which reduced the movement and aggregation of the drug, resulting in increased stability of the formulation [6]. Moreover, SPC is a natural lipid which is less

costly than the synthetic lipids and produced using less solvents and chemicals which makes HSPC and SPC as a choice of lipid for CPB UDNVs [8].

4.5. Preliminary optimization of PTX-UDNVs formulation

From the literature and trials conducted in the lab, the following parameters were found to affect the formation:

Process Parameters

Thin film hydration step-

- ✓ Hydration medium
- ✓ Hydration Volume
- ✓ Hydration Time
- ✓ Hydration Temperature

Size reduction step-

- ✓ Time
- ✓ Amplitude
- ✓ Pulse rate

Preliminary trials:

Thin film hydration step-

Initial trial batches were prepared using lipids DSPC : EggPC (1:1) and sodium deoxycholate (SDC) as edge activator (EA). Lipids and EA were dissolved in Methanol : chloroform (1:9) as organic solvent. Drug was added and dissolved in lipid-EA solution and thin film obtained in RBF using thermostatic water bath at 50 ± 2°C at the speed of 150 RPM. 10 ml of hydration media was added directly into the RBF and the thin film was allowed to hydrate at 60°C. After 30 minutes of continuous hydration process, the dispersion turned hazy and needle shaped crystals settled at the bottom. The analysis of supernatant revealed only 36.40% drug entrapment. The settled particles were dissolved in methanol and analysed by HPLC. It was confirmed that the particles

were of PTX. The observation might be due to nature of PTX to get crystallized when it comes into direct contact with high humidity conditions or when comes into contact with aqueous environment [9]. Thus a study was undertaken to understand the crystallization behaviour of PTX when in contact with water.

Crystallization of PTX

- PTX can exist in at least two crystalline forms. One form is known as highly insoluble anhydrous crystalline PTX. Mechanism that explains this form is Head to tail and Head to head "DIMER" formation of PTX molecules by Hydrogen bonding when comes into contact with relatively high humidity conditions i.e. 30 to 70%RH. This form when comes into contact with very high RH conditions i.e. 90 to 100% RH, or when comes in contact with aqueous environment, it transforms to *Di-, Tri- or Tetra-hydrate* forms. These forms have even less solubility than anhydrous form. These crystalline forms are biologically inactive thus must be prevented during formulation process [10].
- The property of PTX to get crystallized in presence of water was studied by adding varying volumes of water to PTX solution in methanol. The saturated solution of PTX in methanol was prepared at room temperature. 1 ml of the saturated solution of PTX was added into four different microcentrifuge tubes.
- Different volumes of triple distilled water i.e. 50µl, 100µl, 150µl and 200µl was added gradually with micropipette to each microcentrifuge tube containing saturated solution of PTX.
- A drop from each microcentrifuge tube was placed on four different glass slides and observed for the pattern of crystallization under microscope (Nikon H600L Microscope (Nikon, Japan) in all four solutions listed in **Table 4-1**.

Table 4-1: Results of PTX crystallization study

Solution	Anti-solvent (µl)	Results of microscopy
A	50	Needle shape crystals seen immediately
B	100	Long needle shaped crystals showing homogenous growth
C	150	Spherulites formation- heterogeneous crystal growth
D	200	Axialite hydrates formation

Fig. 4-7: Solution A - Needle shaped crystals of PTX at initiation

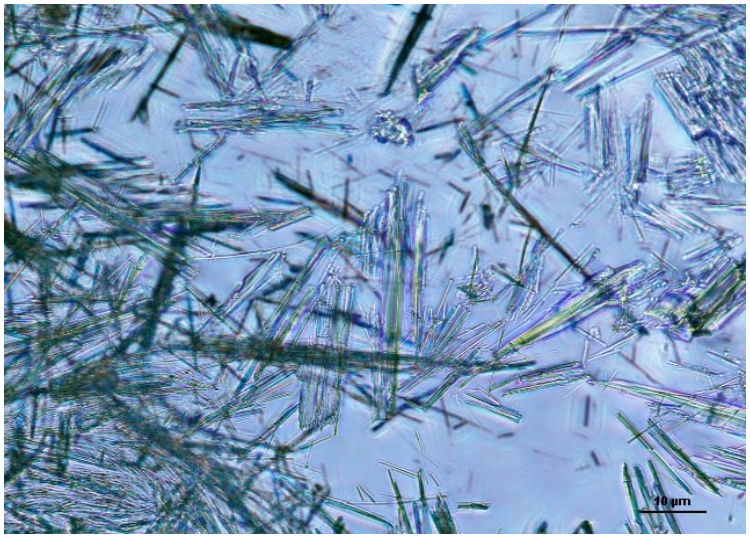

Fig. 4-8: Solution B - Intermediate homogeneous growth of PTX crystals

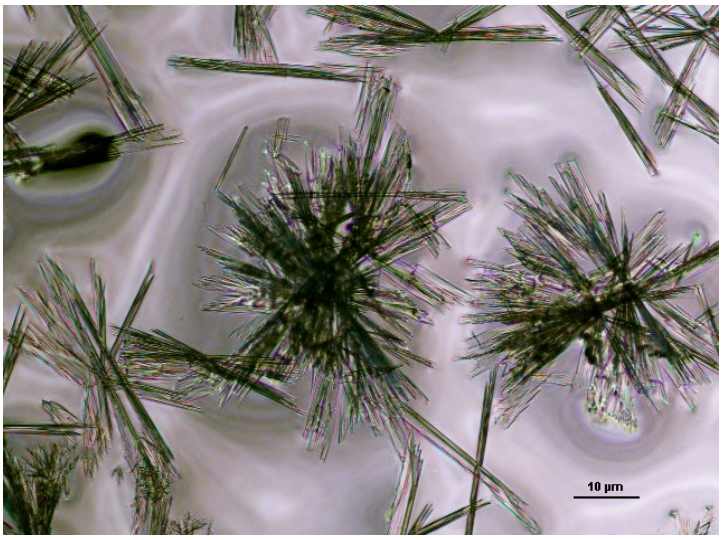

Fig. 4-9: Solution C - spherulite formation/heterogenous crystallization of PTX

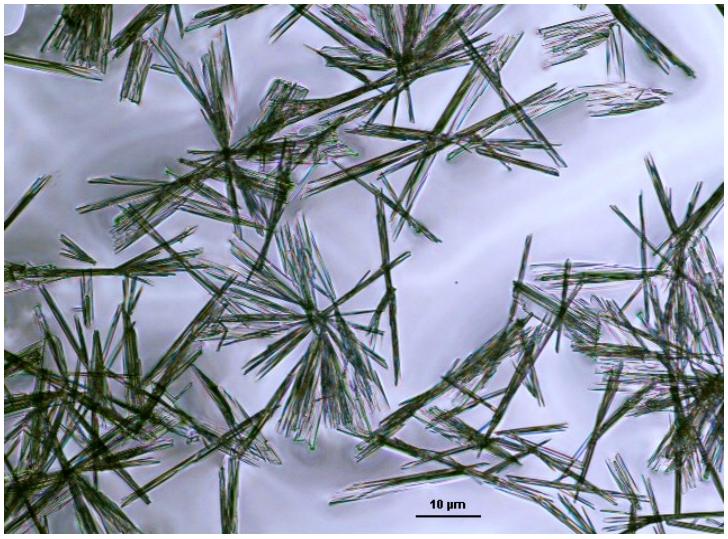

Fig. 4-10: Solution D - Di-/Trihydrate axialite formation of PTX

As the amount of water increased in the saturated solutions of PTX, the hydrates of the drug started forming and aggregating as per the nature of the molecule. Water played role as an anti-solvent in the saturated solution of the drug. As the amount of anti-solvent increased, the drug favourably could not remain in the dissolved form in the solution. Crystals formed after completion of hydration step in the formulation preparation resembled morphologically to the axialites formed in Solution D. This hydrate form of PTX was not able to get incorporated into phospholipid bilayers resulting in very low entrapment although being lipophilic drug molecule. Thus, crystallization of drug must be avoided to get higher entrapment efficiency.

It was clear from the experiments and literature that the three main parameters affecting crystallization of PTX are as follows:

- ✓ Volume of hydration media
- ✓ Contact time with aqueous environment
- ✓ Temperature

So as to prevent crystallization of PTX during hydration step, some modifications were made in thin film hydration process.

Thin film hydration process parameters were optimized that can better avoid crystallization of PTX and results in higher entrapment efficiency. Three different levels for each parameter were set as shown in **Table 4-2**.

Table 4-2: levels of hydration step process parameters

Process Parameter	Low	Medium	High
Hydration Volume aliquotes (ml)	1	2.5	5
Hydration Time (min)	10	20	30
Hydration Temperature (°C)	40	50	60

Hydration medium

The prepared thin film was hydrated by triple distilled water instead of any buffer as the presence of ions in hydration solution lead to reduced capture volume of vesicles and decreased trans-membrane uptake and fluidity. Use of buffers/ionic solutions as hydration media may adversely affect vesicle size and may reduce zeta potential which ultimately affects stability of the dispersion [11, 12].

Hydration Volume

Exposure of PTX to large quantities of hydration fluid leads to rapid formation of crystals as PTX Di or Tri-hydrates during hydration process. Also insufficient hydration volume leads to improper swelling of phospholipid bilayers, results in low entrapment efficiency. Three batches were prepared with different aliquots of hydration media (Total volume = 10 mL). Pre-heated (50°C) hydration media was added in aliquots (**Table 4-3**) followed by shaking RBF manually for 2-3 minutes after each addition of

aliquot into RBF. After addition of total volume of hydration, the thin film was further allowed to hydrate at 50 °C for 30 min.

Table 4-3: Effect of hydration volume aliquots on entrapment efficiency

Total Volume of Hydration	Aliquot volume (ml)	Number of Aliquots	Entrapment Efficiency (%) (n=3)
10 mL	1.0	10	61.37 ± 1.72
	2.5	4	69.04 ± 1.52
	5.0	2	52.11 ± 1.03

The results of entrapment efficiency after addition of different aliquot volumes of hydration media are tabulated in **Table 4-3**. The results indicate that addition of water in aliquots of 2.5 ml gives highest entrapment efficiency than that of addition by 1.0 ml and 5 ml aliquots of water. Therefore to avoid rapid crystallization and to allow proper hydration and swelling of lipid bilayer, hydration volume aliquots of 2.5 ml was found suitable.

Hydration Time

Longer exposure intervals of un-entrapped PTX to aqueous environment hasten crystallization of the drug. Crystallised drug does not get incorporated between lipid bi-layers ultimately leading to lower entrapment efficiency. So, to prevent formation of Di or Tri-hydrates of PTX, the hydration time should be optimum which also sufficient to hydrate lipid bilayers in step. Three batches with different hydration times were prepared keeping 50°C as hydration temperature and 10 ml hydration volume in single addition.

Table 4-4: Effect of hydration time on entrapment efficiency

Hydration time (min)	Entrapment Efficiency (%) (n=3)
10	63.69 ± 2.82
20	60.66 ± 1.33
30	49.32 ± 0.76

The results showed that less amount of drug entrapped as hydration time increases. This might be due to prolonged exposure of drug with aqueous environment at

hydration temperature which results in increased rate of crystallization of drug and hence less entrapment. Moreover, as the results suggests significant increase in entrapment efficiency was found when hydration time was reduced from 30 min to 20 min. Highest entrapment efficiency was achieved at 10 min hydration time hence found suitable for the process.

Hydration Temperature

It is reported in the literatures that the size and number of crystals in the solution can be strongly affected by the concentration of PTX and the temperature [10]. It is also well known fact that the free drug with limited solubility in the anti-solvent media exhibits rate of crystallization inversely proportional to the temperature of the anti-solvent media. Transition temperature of the lipid DSPC is 55°C and for Egg PC, it is -10°C. Three different temperature conditions were tried.

Table 4-5: Effect of hydration temperature on entrapment efficiency

Hydration Temperature (°C)	Entrapment Efficiency (%) (n=3)
40	65.02 ± 0.99
50	68.20 ± 2.91
60	59.87 ± 2.24

Results indicate that the entrapment efficiency was reduced at higher temperature of hydration. The observed effect might be due to over exposure of free PTX to unfavourable conditions in the hydration media. Also, low temperature conditions are not preferable as lipids exhibit highest drug entrapment efficiency at its glass transition temperature. Out of the various temperatures studied, highest entrapment was found at 50°C which could be selected as hydration temperature to avoid rapid morphological changes in drug molecule.

Size reduction step-

Formulation batch with most favourable processing conditions i.e. hydration by 2.5 ml aliquots at 50°C temperature for 10 min was prepared. The goal of the vesicle size reduction step was to reduce vesicle size which could help to achieve maximum tissue

permeation without compromising the entrapment of the drug. Amongst various combinations of processing parameters, sonication cycle with 80% amplitude in three runs at constant pulse rate of 0.8 s for 20 second cycle duration was found suitable for the formulation to reduce vesicle size with minimal impact on entrapment efficiency. Application of 100% intensity of sonication was not done as there was possibility of hydrolysis of the phospholipids due to production of heat and •OH radical because of friction arising within the bulk solution from the mixing effect and friction between the bulk solution and the stationary boundary layer adjacent to the side of the vessel [13].

4.6. Preliminary optimization of CBP-UDNVs formulation

Experiments were conducted to understand behaviour of the drug and the parameters affecting development of the formulation. The method of preparation was affecting significantly on various characteristics of the formulation. The process parameters were explored thoroughly during preliminary studies and practically finalized for further development.

Among the methods available for preparation of nano vesicular formulation, the most commonly employed method is thin layer hydration (TLH), which was first proposed by Bangham et al. [14]. The disadvantage for hydrophilic small molecules like CBP is that usually very low encapsulation efficiency is obtained because the drug must be dissolved in the hydration medium to be encapsulated in the central aqueous compartment; hence, a large quantity of the drug rests un-encapsulated in the outside aqueous medium [15]. According to JO Eloy et al. [16], the following methods are reported to improve the encapsulation efficiency of hydrophilic molecules inside nano vesicular formulation: the cycles of freeze–thaw (FT) [17], reverse phase evaporation (REV) [15, 18] and dehydration–rehydration of preformed empty vesicles (DRV) [19]. For the small hydrophilic molecules, as reported by M Wehbe et al. [20] for drugs that cannot be loaded remotely, the alternative has been passive encapsulation where the compound of interest is added to dried lipid films or lipids solubilized in compatible solvents such as ethanol. The drug solution is processed with the lipids which, as indicated above, carry inherent risks. Further, the trapping efficiency of these methods

can be very poor and is limited by the aqueous trapped volume of the vesicles, the size and the lipid concentration. Therefore, the loading efficiencies are low assuming the selected compound does not interact with the lipids used to prepare the vesicles [21]. Additionally, because of issues related to non-equilibrium solute distribution, when using methods involving hydration of dried lipids, the concentration of solute in the aqueous space inside vesicles can be substantially lower than the concentration in the external solution [17, 22]. Since there is substantial waste of often expensive drugs, this method can be uneconomical [23].

Hence, the method of preparation was suitably modified to achieve high encapsulation of CBP. Trials were conducted to optimize three major process parameters found affecting entrapment of the drug.

- ✓ Hydration volume
- ✓ Hydration Temperature
- ✓ Hydration Time

Initially, formulation prepared using HSPC & SPC (1:1) and SDC as edge activator dissolved in 10 ml of Methanol: Chloroform (1:9) mixture in round bottom flask (RBF) and thin film was obtained. The film was hydrated using 10 ml double distilled water containing accurately weighed amount of CBP. The lipid thin film was allowed to hydrate for three different hydration times 10 min, 20 min and 30 min to allow the drug present in aqueous media to passively equilibrate between external compartment and inner aqueous core of the vesicles formed and the entrapment of the drug was measured. The results of %EE were 22.89 %, 28.77% and 30.15% for 10 min, 20 min and 30 min of hydration time respectively.

After extensive literature search it was found that the lower encapsulation of the drug might be due to passive encapsulation phenomenon only contributing for the CBP entrapment inside vesicles. Passive encapsulation of water-soluble drugs depends on the ability of liposomes to trap aqueous media containing a dissolved drug during vesicle formation. Trapping effectiveness (generally <30%) is limited by the trapped

volume enclosed in the liposomes and drug solubility [23]. M Wehbe et al. [20]; described passive equilibration method which relies on addition of candidate drugs to pre-formed liposomes as an alternative method for preparing liposome encapsulated drugs. The main difference between passive encapsulation and passive equilibration is that for passive equilibration, loading is completed after the liposomes have been prepared. Hence, to harvest advantages of both the loading technologies, in the present investigation, hybrid technique for preparation of CBP UDNVs was employed.

During the equilibration in hydration step, the vesicular dispersion was allowed to incubate at 55 °C for 20 min. The initial volume of hydration media (10 ml) was reduced to three different residual volume of hydration i.e. 2 ml, 4 ml and 6 ml under vacuum at 55 °C. The results tabulated in **Table 4-6**.

Table 4-6: Effect of residual hydration volume on entrapment efficiency

Initial Volume of Hydration	Residual Hydration Volume (mL)	Entrapment Efficiency (%) (n=3)
10 mL	2	49.44 ± 3.34
	4	50.32 ± 3.08
	6	39.56 ± 2.98

The entrapment efficiency of the formulation was found to be highest when initial volume of hydration was reduced to 4 mL after evaporation under vacuum. 2 mL residual volume of hydration resulted into insignificantly lower entrapment of CBP hence 4 mL of residual hydration volume was found suitable which could also maintain proper hydration conditions of the lipids in the dispersion.

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