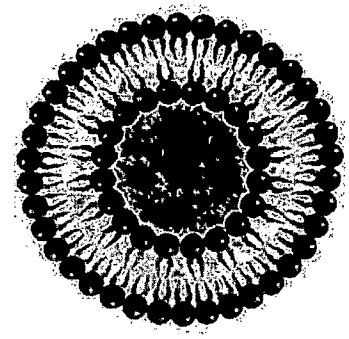
Chapter - 4

Preparation of Conventional Liposomes



4.1. Introduction

Liposomes have been widely considered as potential drug delivery systems ever since the published observation of Bangham and co-workers (Bangham et al., 1965). Liposomes are colloidal vesicles ranging from few nanometers to several micrometers in diameter with one or more lipid bilayers surrounding aqueous compartments (Vemuri and Rhodes, 1995; Chatterjee and Banerjee, 2002). They are prepared from natural or synthetic phospholipids and cholesterol, however, other lipids or derivatives can also be incorporated as needed (Patel and Sprott, 1999; Chatterjee and Banerjee, 2002). Liposomes are biodegradable and biocompatible, non-toxic, non-immunogenic (Voinea and Simionescu, 2002). They can entrap a wide variety of therapeutic drugs (Abraham et al., 2005; Bakker-Woudenberg et al., 2005; Salem et al., 2005) and genetic material. Hydrophilic drugs can be entrapped in aqueous compartments of liposomes, whereas hydrophobic drugs incorporated in their lipid bilayer (Voinea and Simionescu, 2002). A few liposomal formulations are in clinical practice and some in preclinical trials.

Many methods are exist for preparing liposomes and loading them with foreign substances of interest, most of which methods involve forming the liposome vesicles within an aqueous carrier lipid containing said substances distributed therein. During liposome formation, a portion of said carrier liquid becomes entrapped within the vesicles, together of course, with a small amount of the desired substances to be encapsulated. This technique is called "passive entrapment". The efficiency of loading liposomes with passively entrapped aqueous phases is often quite low because it strongly depends on the nature of the carrier phase and, practically, the concentration of the substance dissolved therein which may affect the yield of liposome formation. However, for drug delivery purpose, the loading efficiency (which is generally defined as the weight of material entrapped over the total weight of material involved in entrapment) is usually not critical because the non-entrapped material can generally be recovered and reused afterwards; hence, the important factor is rather the ratio of useful entrapped material versus the weight of the lipids used for entrapment, i.e., the lipids involved in forming the liposomes membrane.

The ratio of the weight of encapsulated material over the weight of encapsulating lipids is in direct relation with the so-called captured volume, i.e. the volume of the aqueous phase entrapped in the liposome core per weight of liposome lipids (µl/mg) (Schneider, 2004). In classical passive entrapment described by Bangham et al., (Bangham et al., 1965), the aqueous phase containing the compound of interest is put into contact with a film of dried phospholipids deposited on the walls of a reaction vessel. Multilamellar vesicles (MLVs) are formed spontaneously when phospholipids are hydrated into aqueous solution with mechanical agitation due to swelling of the lipids. Large unilamellar vesicles (LUVs) can be prepared from MLVs, for example by extrusion. The most common way to manufacture small unilamellar vesicles (SUVs) is to sonicate MLVs with probe sonicator.

The captured volume of MLVs is low, typically near 2 to 4µl/mg of lipids. By sonication, the MLVs can be converted to small unilamellar vesicles whose captured volume is even smaller, e.g., near 0.5-14µl/mg. The Reverse Phase Evaporation (REV) method described

(Szoak and Papahdjopoulos, 1978) in which a solution of lipids in water insoluble organic solvent is emulsified in an aqueous carrier phase and the organic solvent is subsequently removed under reduced pressure gave liposomes with captured volume of 8 to 15µl/mg of lipids.

Improved passive entrapment has been achieved by subjecting liposomes to successive dehydration and rehydration treatment, or freezing and thawing; dehydration was carried out by evaporation or freeze-drying (Kirby and Gregoriadis, 1984). The other method described (Shew and Deamer, 1985) wherein, liposomes were prepared by sonication are mixed in aqueous solution with the solute to be encapsulated. Further attempts to increase the amount of the drugs entrapped in liposomes by using higher concentrations thereof in the carrier liquid reduced the captured volume and had a detrimental effect on captured volumes.

Docetaxel (trademarked as Taxotere® by Rhone-Poulenc Rorer now it is Sanofi Aventis) was approved by the FDA for the treatment of advanced ovarian cancer in April 1994 and in December 1999 for the treatment of patients with locally advanced or metastatic non-small cell lung cancer. Docetaxel is obtained by semisynthesis from 10-deacetylbaccatin III, non-cytotoxic precursor extracted from the needles of the European yew, Taxus baccata (Zamir et al., 1992). Taxotere is approximately twice as potent as taxol in inhibiting cold and calcium-induced depolymerization of microtubules (Gueritte et al., 1993). The clinical application of DTX is limited by the poor aqueous solubility (7µg/mL) (Du et al., 2007; Lili et al., 2011), low bioavailability and high toxicity. Presently used Taxotere® and Duopafei® in clinical contain high concentration of non-ionic surfactant tween-80. The adverse reactions due to either the drug itself or the solvent system have been reported in patients (e.g., hypersensitivity, fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia) (Chu et al., 2000). In order to eliminate the tween-80-based vehicle and increase the drug solubility, alternative dosage forms have been developed, such as microparticulate lipoidal vesicles (liposomes) (Naik et al., 2010; Zhai et al., 2010), cyclodextrins (Grosse et al., 1998), polymeric nanoparticles (Hwang et al., 2008), micelles (Li et al., 2008), solid lipid nanoparticles (SLN) (Xu et al., 2009) and nanostructured lipid carriers (NLC) (Li et al., 2009). Among these forms, liposomes, NLC and SLN belong to lipid-based nanocarriers which have such favourable characteristics as: (a) improved drug dispersibility; (b) enhanced drug solubilisation; (c) enhanced drug transmembrane transport capability and (d) increased therapeutic efficacy and reduced toxicity. In the present study, anionic liposomes composed of mixture of phospholipids (HSPC, DPPC and DPPG), as combination of more than one lipid can increase the drug loading, were developed. We studied the effect of various process and formulation variables during the preparation of docetaxel conventional liposomes and optimized them for maximum docetaxel loading in minimum quantity of phospholipids.

4.2. Materials and Methods

Docetaxel Trihydrate, 7-epidocetaxel, and 10-oxo-7-epidocetaxel were obtained as gift sample from Fresenius Kabi Oncology Limited, Gurgaon, India. Fully hydrogenated soya

phosphatidylcholine (HSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DDPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylglecerol (DPPG), and DSPE-mPEG$_{2000}$, were kindly gifted by Lipoid GMBH (Ludwigshafen, Germany). Cholesterol Extra Pure, Citric acid, Tartaric acid, Maleic acid, Ascorbic acid, and Vitamin E were purchased from Sigma Aldrich, Mumbai, India. All other reagents used were of analytical reagent grade and were used without further purification.

4.3. Preparation of liposomes by thin film hydration method

The negatively charged liposomes composed of HSPC/DPPC/DPPG/CHOL (0.08991:0.08991:0.0116:0.0522mM) and anticancer drug docetaxel (0.0058mM; 5mg) and degradation inhibitor tartaric acid (400µg) were prepared by Thin Film Hydration Technique as described by Bangham (Bangham et al., 1965). ***Formation of Thin Lipid Film:*** The lipid phase (HSPC, DPPC, DPPG, and Cholesterol), DTX, and tartaric acid were weighed accurately and dissolved in chloroform and methanol solvent mixture (2:1 ratio, v/v). The round bottom flask was then attached to the rotary evaporator (BUCHI Rotavapor R-200), evacuated and rotated at 120 rpm in a thermo stated water bath maintained at 45±2 °C. The process was allowed to continue until all the solvent had evaporated and a dry thin lipid film had deposited on the walls of the flask. The flask was rotated under vacuum for additional 30 minutes after the dry residue was first appeared. Subsequently, the flask was kept overnight under vacuum to remove the residual solvents. ***Hydration of Lipid Film:*** The dry lipid film was hydrated with double distilled water (5mL) at above glass transition temperature (85±1 ^{0}C) for 30 minutes. Then, the liposomal dispersion was transferred to volumetric flask, diluted with double distilled water and annealed by keeping in a water bath maintained 85±1 ^{0}C for about 1 hour. ***Production of Small Unilamellar Vesicles (SUVs):*** To make small liposomes, the obtained multilamellar liposomes were probe-sonicated (Sartorius, LABSONIC® M) for 1 minute (6 cycles of 10 seconds each at 80 % amplitude and 0.6 duty cycle). After each cycle the dispersion was stand at 85±1 ^{0}C temperature for 2 minutes that helps to reform liposomes after probe sonication. The sonicated liposomal dispersion was centrifuged at 6000 rpm for 10 minutes to separate the unloaded drug and the supernatant was analyzed for % docetaxel content, % impurity content, average mean particle size, and zeta potential.

4.3.1. Optimization of formulation variables by applying 3^3 factorial design

Based on the results obtained in preliminary experiments, drug/lipid ratio, HSPC/DPPC ratio, and hydration volume were found to be the major variables affecting the % drug content (PDC) and mean particle size (MPS). Hence, 3^3 full factorial design was applied to find the optimized condition for higher drug content and lower particle size. Twenty-seven batches of different combinations were prepared by taking values of selective variables X1, X2, and X3 at different levels as shown in Table 4.1. All other formulation and process variables were kept invariant throughout the study (Cholesterol concentration: 0.011602 mM (4.489 mg); DPPG concentration: 0.011602mM (8.643 mg); hydration time: 1 hr; annealing time: 2 hr; hydration temperature: 65±2 ^{0}C). The

prepared batches were evaluated for drug content and particle size, as dependent variables, and the results are recorded in Table 4.2. Table 4.2 summarizes the experimental runs and the factor combinations employed, along with the translation of their coded levels into the units used in the study.

4.3.2. Optimization of process variables

The optimised formulation was used to study the effect of some process variables such as hydration temperature, annealing time, and probe sonication time on % docetaxel content and mean particle size of anionic liposomes. The above optimized parameters were kept constant and these process variables were varied one by one to study their individual effect.

4.3.3. Optimization of cholesterol concentration

At the above optimized conditions for formulation and process variables we prepared anionic liposomes containing different concentrations of cholesterol (0.011602mM; 4.489mg, 0.0232mM; 8.979mg, 0.0348mM; 13.469mg, and 0.0464mM; 17.959mg) to determine the effect of cholesterol concentration on mean particle size and % drug content and loaded drug retention character.

1mL of liposomal formulations prepared with different concentrations of cholesterol were transferred to nitrogen purged screw capped glass vials (in triplicate) and stored at 2-8 ^{0}C for a period of two weeks. After two weeks the formulations were centrifuged at 6000 rpm for 10 minutes to separate the liposomes from leaked DTX. The supernatant liposomal dispersion was separated from the sediment and analysed for mean particle size and % drug retained.

4.3.4. Characterization of prepared liposomes

4.3.4.1. Docetaxel content

Docetaxel entrapped within the liposomes was estimated after removing the unentraped drug by centrifugation in a cooling centrifuge (Remi Equipments, Mumbai, India) at 6000 rpm for 10 minutes at a temperature of 25 °C. An aliquot of supernatant liposomal dispersion was treated with methanol to extract the loaded drug and lipids, suitably diluted and analysed using UV-visible spectrophotometer (Shimadzu 1601, Japan) at 230nm against blank (methanol containing lipid concentration similar to formulations tested). The concentration of drug was calculated from the standard calibration curve. The percentage docetaxel loaded in the anionic liposomes was determined using the following formula.

$$\%\ \text{Drug content} = \frac{\text{Actual amount of drug entraped in liposomes}}{\text{Actual amount of drug used for liposome preparation}} \times 100$$

4.3.4.2. Particle size analysis

The mean particle size (z-average) and polydispersity index (PDI) of the liposomes were analysed by photon correlation spectroscopy (PCS) using Malvern Zetasizer Nano (NanoZS, Malvern Instruments, UK). 0.2mL of liposomal suspension was diluted to 2.0mL with distilled water and measured after equilibration time of 2 minutes. The Zetasizer Nano is operating with a 4mW He-Ne-Laser at 633nm and non invasive back-scatter technique (NIBS) at a constant temperature of 25 ^{0}C. The measurements were conducted in the manual mode using 20 sub runs of 10 seconds. The size distribution by intensity and volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software version 4.0 (Malvern, Herrenberg, Germany). Thereby, the resulting size distributions show the hydrodynamic diameter. The average particle size and PDI was calculated after performing the experiments in triplicate. The PDI of 0.0 represents a homogenous particle population while 1.0 indicates a heterogeneous size distribution of liposomes.

4.3.4.3. The zeta (ς) potential analysis

The zeta potential of the liposomal suspensions prepared was measured by microelectrophoresis using Malvern Zetasizer NanoZS (Malvern Instrument, U.K.). 0.2mL of the liposomal suspension was diluted to 2.0mL with distilled water for zeta potential analysis. Zetasizer NanoZS offers the highest ever sensitivity, accuracy and resolution of zeta potential. The instrument works on the principal of Brownian motion and measured the light by Phase Analysis Light Scattering (PALS). The zeta potential was measured at 25 ^{0}C using standard sample cell. The measurements were performed in triplicate.

4.4. Preparation of impurity free docetaxel liposomes

4.4.1. Introduction

In acidic media or in the presence of electrophilic agents, D ring as well as B ring opening and/or rearrangement will occur with docetaxel (Figure 4.1A) depending on the conditions employed. In basic media, the cleavage of ester groups at positions 2, 4, and/or 13 will occur. One of the principal paths of degradation is the epimerization of the hydroxyl group at position 7 which results in the formation of 7-epi-docetaxel by way of retro aldol reaction. The degradation of docetaxel can results in products which have reduced activity or are completely inactive. They also demonstrate pharmacological and toxicological profiles completely different from the active drug (Machado et al., 2009).

Bornique and Lemarie (Bornique and Lemarie 2002) investigated the interactions of docetaxel and its epimer 7-epidocetaxel with recombinant human cytochrome P450 1B1 (hCYP1B1) which is present in various human tumors and is postulated to be responsible for the development of resistance of tumor cells toward chemotherapeutic agents, including docetaxel. The authors observed that at a concentration of 10μM, the 7-epidocetaxel increased the activity of hCYP1B1 by more than 7 fold, confirming that it is a potent inducer of this enzyme. Hence, the presence of 7-epidocetaxel in the

pharmaceutical formulations is responsible for the development of resistance of tumor cells to the active drug, docetaxel and/or its trihydrate. Therefore, it is desirable to minimize or eliminate the presence of 7-epidocetaxel in pharmaceutical formulations containing docetaxel and/or its trihydrate.

Taxotere one compartment composition containing a taxane derivative in association with ethanol and polysorbate 80 is proved to be fairly unstable (Sandoz, 2008). This composition shows a significant degradation, expressed in the formation of 7-epidocetaxel when exposing it to heating. The 7-epidocetaxel is a well known degradation product of the docetaxel (Vasu Dev et al., 2006). It would be therefore highly desirable to develop alternative compositions having an improved stability.

As per the invention of Sandoz (Sandoz, 2008), it has surprisingly found that, the addition of an organic and/or inorganic acid induces a significant increase in stability of the composition even after heating at higher temperature. According to the invention the acid is selected in the group consisting of citric acid, acetic acid, formic acid, ascorbic acid, aspartic acid, benzoic acid, hydrochloric acid, sulphuric acid, phosphoric acid, tartaric acid, glutamic acid, lactic acid, maleic acid or succinic acid. Most preferred acid is citric acid. Especially preferred acid is citric acid anhydrous as defined in the European Pharmacopoeia 2007. Therefore, we tested different concentration of citric acid as degradation inhibitor in liposomes prepared at high temperature. We also compared the efficiency of other organic acids with citric acid as degradation inhibitors in order to prepare impurity free DTX liposomes.

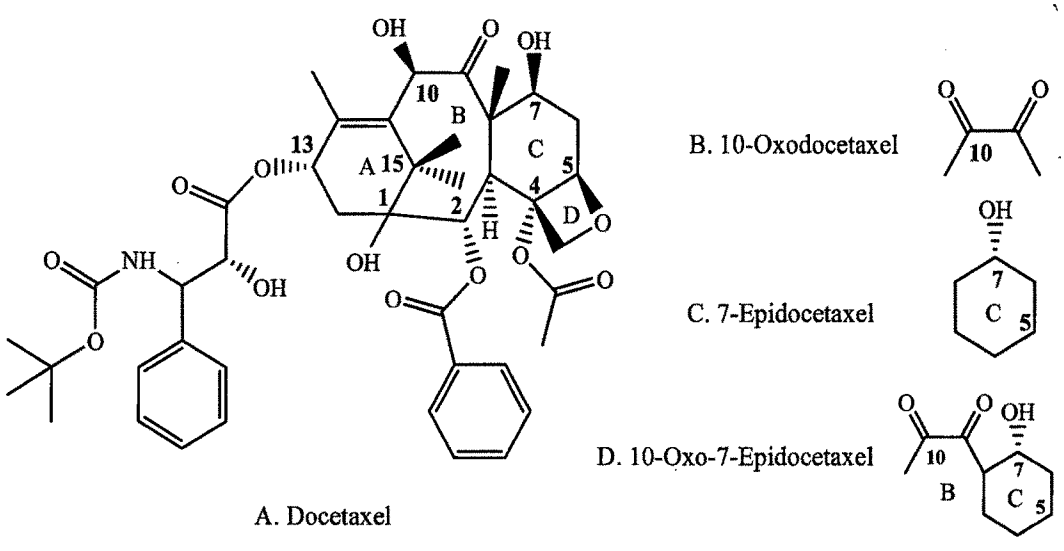


Figure 4.1. Chemical structure of A. Docetaxel and its degradation impurities B. 10-Oxodocetaxel, C. 7-Epidocetaxel and D. 10-Oxo-7-Epidocetaxel

4.4.2. Identification of docetaxel impurities formed at different temperature

We carried out the preliminary experiments (above discussed optimization experiments) containing no organic acid as degradation inhibitor to optimize some basic formulation and process variables. The optimized final liposomal formulation prepared in the absence of organic acid showed significant amounts of degradation impurities (10-oxodocetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel were identified as common impurities at our optimized experimental conditions). We are unable to identify these impurities in the preliminary optimization using UV-visible spectroscopy at 230nm. To identify and quantify the impurities formed during the process, we prepared liposomal formulations containing no organic acid at 3 different temperatures of hydration and annealing (60, 75 and 85 ^{0}C), and drug to lipid ratio of 1:30. The Cholesterol concentration of 0.0522mM was used and the formulation was exposed to above said temperatures for a total period of 1.5hr (includes hydration, annealing and during probe sonication) during the preparation. Prepared formulations were analysed for actual docetaxel and its degradation impurities loaded and total number and % of impurities generated using RP-HPLC method as discussed in chapter analytical methods.

$$\% \text{ Impurity content} = \frac{\text{Actual amount of impurity entrapped in liposomes}}{\text{Actual amount of DTX used for liposome preparation}} \times 100$$

4.4.3. 3^3 factorial design-2

Based on the results obtained in preliminary experiments, cholesterol concentration (X1), hydration temperature (X2) and citric acid concentration (X3) were found to be the major variables affecting the % drug loading and % impurity generation. Hence, the 3^3 full factorial design was applied to find the optimized condition for higher drug content and minimum and/or absence of degradation impurities. Twenty-seven batches of different combinations were prepared by taking values of selective variables X1, X2, and X3 at different levels as shown in Table 4.6. All other formulation and process variables were kept invariant throughout the study. The prepared batches were evaluated for % drug content, % impurities generated and pH as dependent variables, and the results are recorded in Table 4.6. Table 4.6 summarizes the experimental runs and the factor combinations employed.

4.4.4. Comparison of different organic acids as degradation inhibitors

At the optimized concentration of citric acid we tested the efficiency of other organic acids such as ascorbic acid, maleic acid, and tartaric acid in controlling degradation impurities. We also tested the impurity controlling efficiency of Vitamin-E as antioxidant by replacing citric acid. The best suitable organic acid, one which better controls the impurity formation and improves and/or retains the drug loading was identified and used in the final formulation.

4.5. Results and Discussion

4.5.1. Preparation of conventional liposomes (CLs)

Conventional liposomes were prepared by thin film hydration method. Phospholipid DPPC was selected as trapping efficiency of liposomes increases with increase in fatty acid carbon chain length from C_{12} (Dilauroyl Phosphatidylcholine) to C_{16} (Dipalmitoyl phosphatidylcholine) (Louis 1997). DPPC is a natural phospholipid and is most common components of biological membranes. DPPC has low transition temperature (Tc) of 41 ^{0}C at physiological pH. Liposomes composed of DPPC results in fusion of liposomes with cell plasma membrane which may further lead to endocytosis by the cell having the endocytic activity (Gerd et al., 1988). The use of more than one phospholipid in the preparation of liposomes will increase the hydrophobic drug loading (Chen et al., 2006; Kan et al., 2011). Hence, we selected HSPC and DPPG phospholipids in combination with DPPC. The use DPPG, negatively charged phospholipid, will enhance the suspension stability by inducing negative charge over liposomes and it also reduces the liposomal size to nanometer during probe sonication. In our experiments hydration of phospholipid was carried out using double distilled water, instead of any buffer, because the presence of ionic solute in the hydration media can interact with negatively charged liposome and might alter the physical character of liposomes (increase in particle size and decrease in zeta potential) during preparation and storage.

Indu Javeri (Indu Javeri et al, 2011) invented docetaxel liposomes composed of 500mg docetaxel, 6mg of sodium oleate, L-α-phosphatidyl choline (soya), and containing no cholesterol. They prepared liposomes loaded with docetaxel (5mg/mL) and having size less than 100nm. The presence of cholesterol has one of the most important roles in the maintenance of membrane bilayer stability and long circulation time *in vivo* (Gregoriadis and Davis, 1979; Senior and Gregoriadis, 1982; Senior, 1987). In the absence of cholesterol, conventional liposomes are destabilized by high density lipoprotein (HDL) particles (Chobanian et al., 1979; Damen et al., 1980) and release their components, which upon readily eliminated from the circulation. Hence we used cholesterol as one of the major components of the liposomes. The chemical and *in vivo* stability of liposomes prepared with saturated phospholipids was more as compared to liposomes prepared with unsaturated phospholipids (Maurer et al, 2001). The phospholipid component also plays a prominent role in the maintenance of high plasma levels of liposomes. DSPC/Cholesterol and sphingomyelin (SM)/Cholesterol liposomes have higher $T_{1/2}$ values in the circulation compared with more fluid liposomes containing unsaturated eggPC (Senior, 1987). To be most effective, the phosphatidyl choline component must have a phase transition that is significantly above 37°C. The gel-to-liquid-crystalline phase transition (T_g) for eggPC is below 37°C, whereas DPPC has a T_g value of only a few degrees above body temperature (42 °C). However, the HSPC have T_g value of 15-17°C higher than 37°C. Thus, at 37°C, HSPC and DPPC containing liposomes have a considerably more rigid membrane bilayer that resists penetration of serum opsonins than do eggPC containing formulations. It is no surprise, then, that these liposomes tend to be the most stable in the circulation and display the longest circulation lifetimes. Hence, in the present study we prepared the liposomes using combination of HSPC and

DPPC as these phospholipids have T_g above body temperature and also this combination would enhance the docetaxel loading.

Using 3^3 factorial design as shown in Table 4.2, 27 batches of DTX loaded anionic liposomes were prepared varying three independent variables as shown in Table 4.1. The % DTX content and mean particle size are recorded as dependent variable in Table 4.2. Our objective of this factorial design was to optimize basic parameters like drug to lipid ratio, HSPC:DPPC ratio and hydration volume. We also optimized cholesterol concentration and some basic process variables.

The increase in drug:lipid ratio shows increase in drug loading and mean particle size. At 1:20 drug to lipid ratio we observed maximum DTX loading and minimum particle size. With increase in drug:lipid ratio beyond this level (i.e. 1:30) we observed similar amount of drug loading but increased mean particle size than 1:20. Hence, we considered drug to lipid ratio of 1:20 as optimal condition. The HSPC:DPPC ratio was optimized (1:1) for maximum drug content and minimum average particle size by preparing liposomes at different ratio of HSPC and DPPC by keeping DPPG (0.011602mM) and cholesterol (0.011602mM) concentration as constant. The decrease in DTX loading and increase in mean particle size was observed when the HSPC:DPPC ratio was 2:1 as compared to 1:1 and 1:2 ratio. This might be due to high concentration of HSPC (having T_g of 60 ^{0}C) and that might require hydration and annealing temperature more than what we used (65±2^{0}C) as compared to DPPC having T_g of just 42 ^{0}C. The hydration volume of 3-5mL was considered as optimal. We observed increase in DTX loading and decrease in mean particle size with increase in hydration volume from 1.5mL to 5mL and in between 3-5mL we observed similar results. The decrease in DTX loading and increase in mean particle size at hydration volume of 1.5mL might be due to insufficient water which is unable to completely hydrate the total phospholipids. As expected, the presence of negatively charged phospholipid (DPPG) decreased the size during probe sonication to nanometer and also enhanced the suspension stability at 4 ^{0}C.

The % DTX loading of 87.7±2.4% and mean particle size of 92±1nm (PDI: 0.258±0.015) was observed at drug to lipid ration of 1:20, HSPC:DPPC ratio of 1:1, hydration volume of 3mL, hydration and annealing time of 3hr, hydration and annealing temperature of 65 ±2 ^{0}C, and probe sonication time of 1minute (6 cycle at an interval of 10 sec each).

Table 4.1. Independent variables and their corresponding levels

Variables	Levels		
	-1	0	1
X1 (Drug:Lipid Ratio)	01:10	01:20	01:30
X2 (HSPC:DPPC Ratio)	01:02	01:01	02:01
X3 (Hydration Volume)	1.5 ml	3 ml	5 ml

Table 4.2. 3^3 Full factorial design consisting of experiments for the study of three experimental factors in coded and actual levels with experimental results

Formulation	Actual value variables			Response value	
	X1	X2	X3	% Drug content	Mean particle size with PDI
AL-1	1:10	1:1	1.5 mL	30.6±2.4	113±3(0.380±0.085)
AL-2	1:10	1:1	3 mL	41.8±1.4	93±2(0.326±0.062)
AL-3	1:10	1:1	5 mL	43.7±1.3	99±1(0.339±0.044)
AL-4	1:10	1:2	1.5 mL	28.6±3.2	101±5(0.247±0.011)
AL-5	1:10	1:2	3 mL	36.7±2.3	88±3(0.243±0.009)
AL-6	1:10	1:2	5 mL	37.7±1.1	86±2(0.257±0.019)
AL-7	1:10	2:1	1.5 mL	26.8±3.2	113±5(0.316±0.044)
AL-8	1:10	2:1	3 mL	33.1±2.5	108±3(0.303±0.019)
AL-9	1:10	2:1	5 mL	32.6±2.4	97±2(0.321±0.022)
AL-10	1:20	1:1	1.5 mL	71.4±2.6	99±2(0.235±0.008)
AL-11	1:20	1:1	3 mL	87.7±2.4	92±1(0.258±0.015)
AL-12	1:20	1:1	5 mL	87.4±1.6	92±2(0.282±0.013)
AL-13	1:20	1:2	1.5 mL	75.3±4.7	98±3(0.291±0.012)
AL-14	1:20	1:2	3 mL	86.6±3.6	88±4(0.227±0.008)
AL-15	1:20	1:2	5 mL	86±3.3	92±2(0.277±0.022)
AL-16	1:20	2:1	1.5 mL	66.6±4.5	95±1(0.248±0.006)
AL-17	1:20	2:1	3 mL	76.2±3.7	90±3(0.257±0.014)
AL-18	1:20	2:1	5 mL	75.1±2.9	95±2(0.261±0.014)
AL-19	1:30	1:1	1.5 mL	92.3±1.6	105±6(0.414±0.011)
AL-20	1:30	1:1	3 mL	92.4±1.4	94±3(0.33±0.019)
AL-21	1:30	1:1	5 mL	89±2.6	110±5(0.424±0.011)
AL-22	1:30	1:2	1.5 mL	80.4±5	95±3(0.251±0.021)
AL-23	1:30	1:2	3 mL	90.8±1.2	92±3(0.261±0.018)
AL-24	1:30	1:2	5 mL	90.2±1.6	92±2(0.367±0.014)
AL-25	1:30	2:1	1.5 mL	85.4±2.4	151±8(0.461±0.031)
AL-26	1:30	2:1	3 mL	89.5±1.4	113±5(0.381±0.021)
AL-27	1:30	2:1	5 mL	88.4±1.8	115±3(0.341±0.018)

Values are Mean±SD, n=3. AL: Anionic Liposomes

4.5.2. Optimization of process variables

The some important process variables such as hydration temperature, annealing time and probe sonication time were optimized for maximum DTX loading and minimum average particle size (Table 4.3). The experiment was carried out at the above optimized experimental conditions by varying these variables one by one. We observed increase in DTX loading with increase in hydration and annealing temperature (from 55 °C to 75 °C) and annealing duration from zero (immediately after hydration of lipid film for 1hr) to 3hr. No significant change in mean particle size was observed. As we increases the probe sonication time we observed decrease in DTX loading and mean particle size. We observed maximum DTX loading (93±3%) and minimum particle size (95±5nm; PDI: 0.249±36) at optimized condition of hydration temperature (70-75 °C), annealing time (2-3hr), and probe sonication time (1min).

Table 4.3. Effect of some process parameters on % drug loading and mean particle size of optimized formulation AL-11

Process parameters		% Drug content	Mean particle size
Hydration temperature	55 °C	20.52±5.6	107±7 (0.364±0.022)
	65 °C	85.5±3.4	89±3 (0.241±0.015)
	75 °C	91.4±1.5	94±2 (0.248±0.011)
Annealing time	Zero*	42.6±6.5	96±4 (0.244±0.014)
	1 hr	68.14±3.2	92±3 (0.241±0.015)
	2 hr	85.5±3.4	89±3 (0.243±0.011)
	3 hr	86.2±2.8	89±2 (0.248±0.012)
Probe Sonication time	1 min	85.5±3.4	89±3 (0.241±0.015)
	2 min	84.1±2.9	88±2 (0.242±0.003)
	4 min	81.3±3.5	80±1 (0.248±0.004)

* After hydration of lipid film for 1hr. Values are Mean±SD, n=3.

4.5.3. Optimization of cholesterol concentration

Anionic liposomes containing different concentrations of cholesterol (0.011602mM; 4.489mg, 0.0232mM; 8.979mg, 0.0348mM; 13.469mg, and 0.0464mM; 17.959mg) were prepared and analysed for % DTX loading, mean particle size, and drug retention property (Table 4.4). The maximum % DTX loading (93.4±2.2) and minimum mean particle size (84±4nm) was observed at cholesterol concentration of 0.0116mM. As the cholesterol concentration was increased from 0.0116mM to 0.0464mM the decrease in % DTX loading and increase in mean particle size was observed.

After 2 weeks of storage at 2-8 °C all formulations were analysed for mean particle size and % DTX content in order to determine the effect of cholesterol concentration on % DTX and mean particle size retention behaviour of liposomes. The formulation with lowest cholesterol content (0.0116mM) having lowest mean particle size and highest %

drug content showed more leakage of loaded drug and increased mean particle size as compared to other formulations (Table 4.4). This increased leakage might be attributed to the low membrane rigidity at low cholesterol content (Sulkowski et al., 2005; Hong et al., 2001). Similarly, the formulation with highest cholesterol content (0.0464mM) having highest mean particle size and lowest docetaxel content also failed to retain the same like formulation with lowest cholesterol concentration. This might be due to increased rigidity of bilayer membrane with increased cholesterol concentration that leads to more leakage of drug from the liposomes. At cholesterol concentration of 0.0348milimos (13.469mg), we observed about 83.6±2.6 of % DTX loading and mean particle size of 107±6nm at day 1. After 2 weeks of storage at 2-8 °C the drug content of this formulation was found to be maximum (72.8±2.8%) and mean particle size was minimum (145±2nm) as compared to other formulations. Hence, the cholesterol concentration of 0.0348milimos was considered optimal because at this cholesterol concentration the formulation showed minimum drug leakage (10±2%) as compared to other concentrations.

Table 4.4. Effect of cholesterol concentration on % drug loading and mean particle size

Formulation	Cholesterol concentration (mM)	Formulations at day 1		Formulations after 2 weeks	
		% Drug content	Mean size with PDI	% Drug content	Mean size with PDI
AL -11.1	0.0116	93.4±2.2	84±4nm (0.259±0.008)	29.3±3.8	193±8nm (0.368±0.016)
AL - 11.2	0.0232	87.3±3.7	95±2nm (0.242±0.022)	30.8±2.2	187±5nm (0.429±0.045)
AL - 11.3	0.0348	83.6±2.6	107±6nm (0.275±0.024)	72.8±2.8	145±2nm (0.288±0.011)
AL - 11.4	0.0464	74.2±4.4	120±2nm (0.346±0.022)	33.8±4.3	155±4nm (0.383±0.021)

Values are Mean±SD, n=3. PDI: Polydispersity Index

4.5.4. Preparation of impurity free liposomes

4.5.4.1. Identification of docetaxel impurities in liposomes

The HPLC analysis of above optimised liposomal suspension shows the degradation of DTX into impurities, which are identified as 10-oxodocetaxel (formed at 85 ^{0}C of hydration), 7-epidocetaxel and 10-oxo-7-epidocetaxel by running standard impurities at the same analytical condition as used for docetaxel. The retention time and relative retention time of docetaxel and impurities are shown in (Figure 4.2). The docetaxel elute was first followed by 10-oxodocetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel under the condition of analysis which indicated that the impurities are more lipophilic than the docetaxel. The order of lipophilicity of the eluted constituents was 10-oxo-7-epidocetaxel > 7-epidocetaxel > 10-oxodocetaxel > docetaxel. These results are in accordance with the sequence of lipophilicity reported previously by Reddy et al. (Reddy et al., 2010). Particularly, the impurity separated just after docetaxel and before 7-epidocetaxel has showed RT (5.51±0.073minutes) and RRT (1.143) values which are in conformity for 10-oxodocetaxel as per their report (Reddy et al., 2010). The types and percentage of impurities formed at different temperature of hydration and annealing were shown in (Figure 4.3 and Table 4.5). As the temperature increases the percentage and number of impurities also increased. Therefore, the formation of DTX impurities is temperature and time dependent. From the results obtained it is very clear that the DTX loading increases with increase in temperature and annealing time. Hence, the compromise in temperature will reduce the impurity generation but will also reduce the DTX loading. The preparation of liposomes with phospholipids having T_g above body temperature such as DPPC (42 ^{0}C) and HSPC (60 ^{0}C) will enhance the *in vivo* circulation time and also reduces the *in vivo* drug leakage as compared to unsaturated phospholipids and phospholipids having T_g below body temperature. Hence, it is need to use these saturated phospholipids and prepare liposomes at higher temperature of hydration and annealing (Drummond et al., 2011). The use of degradation inhibitors such as organic and inorganic acids in the docetaxel injection will prevent the DTX degradation when heated at higher temperature (Sandoz, 2008). Therefore in the present research we prepared liposomes using HSPC and DPPC at high temperature of hydration and annealing by adding organic acid, citric acid, as degradation inhibitor.

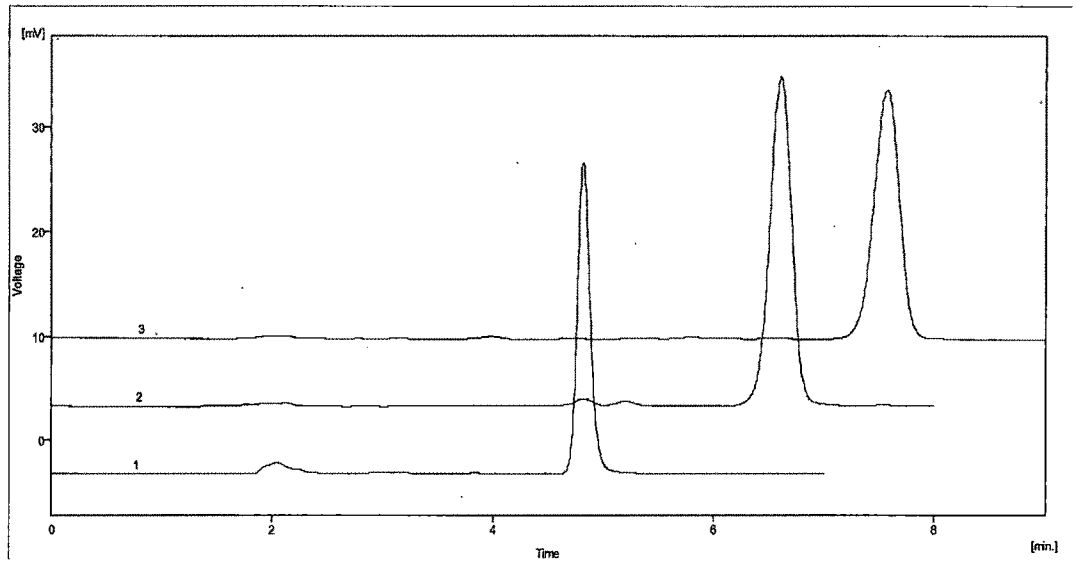


Figure 4.2. Characteristic peaks of (1). DTX (RT: 4.820±0.076 minute; RRT: 1) (2). 7-epidocetaxel (RT: 6.587±0.054 minute; RRT: 1.366) and (3). 10-oxo-7-epidocetaxel (RT: 7.573±0.042 minute; RRT: 1.571).

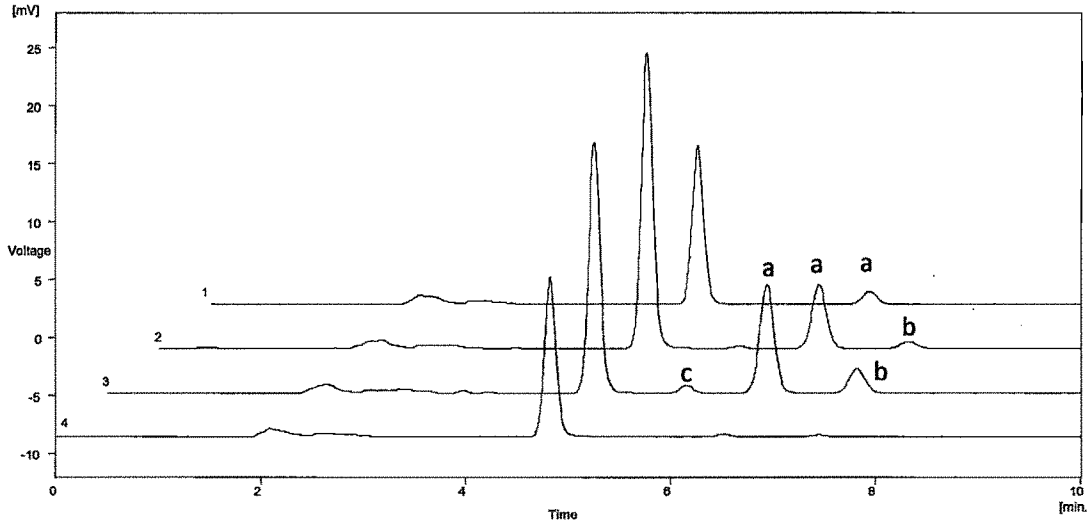


Figure 4.3. Identification of types of impurities formed in liposomes prepared at different hydration temperature in the absence of organic acid (1). Liposomes prepared at 60 ^{0}C (a: 7-epidocetaxel) (2). At 75 ^{0}C (a: 7-epidocetaxel; b: 10-oxo-7-epidocetaxel) (3). At 85 ^{0}C (a: 7-epidocetaxel; b: 10-oxo-7-epidocetaxel; c: 10-oxodocetaxel) (4). Liposomes prepared at 85 ^{0}C in the presence of tartaric acid (0.4mg).

Table 4.5. Comparison of % docetaxel and impurities loading after preparation of liposomes at different hydration temperature in the absence of organic acid

Temp (°C)	% DTX	% 10-oxo docetaxel	% 7-epidocetaxel	% 10-oxo-7-epidocetaxel	% Total loading
60±1	33.9±3.4	--	2.96±0.23	--	36.86±3.6
75±1	61.64±2.6	--	14.78±1.4	1.602±0.3	78.022±4.3
85±1	63.54±1.9	2.34±0.47	25.66±1.1	6.6±0.5	95.1±3.9

Values are Mean±SD, n=3. The docetaxel loading increased highly significantly at 75 °C, and 85 °C as compared to 60 °C (***p<0.0001). Similarly, the % 7-epidocetaxel loading decreased highly significantly at 60 °C as compared to 75 °C, and 85 °C (***p<0.0001).

4.5.4.2. 3^3 factorial design

Based on the results obtained in preliminary experiments, cholesterol concentration (X1), hydration and annealing temperature (X2), and citric acid concentration (X3) were found to be major variables affecting % impurity formation (Y1) and % docetaxel loading (Y2). Hence, 3^3 full factorial design was applied to find the influence of these variable parameters (X1, X2, X3) on the response parameters (Y1 & Y2) and to optimize conditions for preparing DTX liposomes with maximum drug loading and No/minimum impurity. Liposomes were prepared adopting procedure discussed earlier using drug lipid ratio of 1:30 and the organic acid (citric acid) was added during film formation to avoid the chances of degradation of docetaxel during film formation. In total 27 batches of DTX loaded liposomes were prepared and their details are given in Table 4.6.

Table 4.6. 3^3Full factorial design consisting of experiments for the study of three experimental factors in coded and actual levels with experimental results

Formulation	Actual value variables			Response value		
	X1 (mM)	X2 (°C)	X3 (mg)	% Drug content	% 7-EDTX formed	Formulation pH
CA-AL-1	0.0232	75 °C	0.4	42.58±3.76	--	5.4
CA-AL-2	0.0232	75 °C	1	26.74±1.93	--	4.65
CA-AL-3	0.0232	75 °C	2	11.44±1.46	--	4.01
CA-AL-4	0.0232	80 °C	0.4	51.82±2.81	1.676±0.143	5.55
CA-AL-5	0.0232	80 °C	1	26.1±2.74	1.023±0.124	4.52
CA-AL-6	0.0232	80 °C	2	26.92±1.0	--	3.93
CA-AL-7	0.0232	85 °C	0.4	57.62±1.92	1.786±0.234	5.27
CA-AL-8	0.0232	85 °C	1	49.36±3.24	1.462±0.098	4.45
CA-AL-9	0.0232	85 °C	2	37.41±2.67	--	3.76
CA-AL-10	0.0348	75 °C	0.4	40.12±1.11	--	5.55
CA-AL-11	0.0348	75 °C	1	24.16±2.29	--	4.41
CA-AL-12	0.0348	75 °C	2	21.9±2.22	--	3.82
CA-AL-13	0.0348	80 °C	0.4	40.52±1.87	1.243±0.057	5.4
CA-AL-14	0.0348	80 °C	1	28.8±2.26	0.923±0.034	4.56
CA-AL-15	0.0348	80 °C	2	21.92±2.98	--	3.88
CA-AL-16	0.0348	85 °C	0.4	46.62±3.78	1.388±0.121	5.23
CA-AL-17	0.0348	85 °C	1	38.44±3.33	1.116±0.099	4.42
CA-AL-18	0.0348	85 °C	2	35.58±1.67	--	3.75
CA-AL-19	0.0522	75 °C	0.4	27.08±1.74	--	5.01
CA-AL-20	0.0522	75 °C	1	20.98±2.44	--	4.35
CA-AL-21	0.0522	75 °C	2	14.61±2.78	--	3.71
CA-AL-22	0.0522	80 °C	0.4	31.1±0.98	0.434±0.034	5.22
CA-AL-23	0.0522	80 °C	1	21.22±1.66	--	4.36
CA-AL-24	0.0522	80 °C	2	13.12±2.55	--	3.72
CA-AL-25	0.0522	85 °C	0.4	49.64±3.12	0.558±0.079	5.12
CA-AL-26	0.0522	85 °C	1	37.86±2.45	--	4.37
CA-AL-27	0.0522	85 °C	2	36.14±3.87	--	3.91

Values are Mean±SD, n=3. CA-AL: citric acid containing anionic liposomes; EDTX: 7-epidocetaxel

Table 4.6a: 2*3 factorial design to study the influence of the three experimental factors (X_1, X_2 and X_3) in coded levels on % drug loaded (Y_2) in liposomal batches.

Formulation code	X1	X2	X3	X1.X2	X1.X3	X2.X3	X1.X2.X3	Y2*
CA-AL-1	-1	-1	-1	+1	+1	+1	-1	42.6
CA-AL-19	+1	-1	-1	-1	-1	+1	+1	27.1
CA-AL-7	-1	+1	-1	-1	+1	-1	+1	57.6
CA-AL-25	+1	+1	-1	+1	-1	-1	-1	49.6
CA-AL-3	-1	-1	+1	+1	-1	-1	+1	11.4
CA-AL-21	+1	-1	+1	-1	+1	-1	-1	146
CA-AL-9	-1	+1	+1	-1	-1	+1	-1	37.4
CA-AL-27	+1	+1	+1	+1	+1	+1	+1	36.1
Calculated Effect # (%)	-3.67	+49.27	-40.73	+0.07	+4.73	+0.49	-1.04	

*Values rounded to one decimal point; # Calculated by the method of Yetes.

Results of the factorial was first analysed based on the influence of the variable parameter on the Y1 response (% impurity formation) since the ideal formulation expected should contain no/minimum concentration of the impurity formed by the degradation of docetaxel. Basically, increase in cholesterol and citric acid concentration decreased the % of impurity formation while, temperature of hydration and annealing increased the impurity formation. All batches (CA-AL-1, 10, 19) prepared with 0.4 mg of CA and varied concentration of cholesterol at 75°C showed no impurity formation while many batches prepared at 80 °C and 85 °C showed impurities. Similar observation were recorded with all batches (CA-AL-2, 11, 20) prepared with 1 mg CA at 75 °C and batches prepared with 1 mg CA and 0.0522mM of cholesterol at 80 °C and 85 °C. All batches prepared with 2mg of CA irrespective of cholesterol concentration and temperature of hydration and annealing showed no impurity formation. These results conclude that (1) all batches prepared at 75 °C irrespective of the concentration of cholesterol and CA and (2) batches prepared with highest concentration of cholesterol (0.0522mM) with 1mg/2mg CA showed no impurity formation.

Analysis of the influence of variable parameters on the % drug loading (Y_2 Response), showed positive response with increase in temperature of hydration and annealing (X2) but negative response with increase in the concentration of cholesterol (X1) and citric acid (X3).

Reduced factorial design with 2 levels (Lowest and highest) of all the three factors (2*3) was conducted to study the magnitude of main and combined effect of the factors using the selected data from Table 4.6. Details are given in Table 4.6a.

Main effects and combined effect of factors on the % drug loading calculated show temperature of hydration and annealing has maximum positive contribution (+49.27%) followed by remarkable negative contribution of CA concentration (-40.73%). Cholesterol

contribution on the % drug loading is also negative but not very significant (-3.67). Combined effects of two factors are positive but negligible in case of cholesterol conc.: Temp. (X1.X2) and Temp.: CA conc, (X2.X3) while combined effect of cholesterol conc.: CA conc. (X1.X3) was quite higher (+4.73) in comparison to others. Combined effect of all the three factors (X1.X2.X3) is also negligible (-1.04). These results clearly indicate that the two negative factors, cholesterol and CA concentration contradicts positive effect of temperature of hydration and annealing on % drug loading. Hence, liposomal batch prepared with high temperature of hydration and annealing with low cholesterol and CA concentration could yield high % drug loading as evidenced by batch number CA-AL-7 (57.62±1.92%).

However, high temperature of hydration and annealing increases docetaxel degradation as evidenced by batch number CA-AL-7 where the % 7-epidocetaxel estimated is maximum (1.786±0.234%) among the 27 batches tested (Table 4.6). Looking in to batches of liposomes showing no formation of 7-epidocetaxel, it is interesting to note that those batches prepared at 75 ^{0}C with (1) 0.0232 mM of cholesterol and varied concentrations of CA (CA-AL-1, 2 & 3); (2) 0.0348 mM cholesterol and varied concentrations of CA (CA-AL-10, 11 & 12) and (3) 0.0522 mM of cholesterol and varied concentrations of CA (CA-AL-19, 20 & 21) are included. However, it is also noted that, docetaxel degradation due to exposure to higher temperature of hydration and annealing was prevented by increasing the concentration of citric acid. For example from Table 4.6, batches prepared with 2 mg CA (irrespective of cholesterol content & temperature of hydration and annealing) showed no evidence of docetaxel degradation. Similarly, increasing cholesterol content to 0.0522 mM protected docetaxel from degradation in batches prepared at 80 ^{0}C with 1mg and 2 mg CA (CA-AL-23 & 24) and at 85^{0}C with 1mg & 2mg CA (CA-AL-26 & 27).

The liposome batch, CA-AL-25, prepared at 85 ^{0}C of hydration & annealing with cholesterol (0.0522mM) and citric acid (0.4 mg) showed evidence of docetaxel degradation (0.558±0.079%) within the Indian pharmacopoeial limit (should be less than 1%) and significant drug loading (49.64±3.12%) was optimized. Also, this batch comprises high cholesterol concentration so that we can expect decreased *in vivo* docetaxel leakage and enhanced *in vivo* liposomal circulation time.

The pH of the formulation prepared at 0.4, 1, and 2mg citric acid at all cholesterol and temperature levels was found to be 5.305±0.184, 4.454±0.102, and 3.832±0.105, respectively. As the CA concentration increases, the pH of the final formulation decreases which then decreases the impurity generation even after heating the formulation at higher temperature (85 ^{0}C) during hydration and annealing for a period of 1.5-2hr. Hence, we can conclude that the pH of formulation also plays a pivotal role in controlling the degradation of docetaxel and the docetaxel remain more stable at pH near 4.0 (marketed Taxotere having pH of 4.0 was adjusted with citric acid) than at higher pH. Optimized liposomal formulation described above has the pH of 5.1 (with 0.4 mg CA) with substantial drug loading and minimum 7-epidocetaxel.

4.5.4.3. Comparison of different organic acids as degradation inhibitors

At the optimized level of cholesterol (0.0522mM), temperature (85 ^{0}C), hydration and annealing time of 1.5hr, and probe sonication time of 1 minute we have tested the efficiency of other organic acids (ascorbic acid, maleic acid, and tartaric acid), as degradation inhibitor, at the same optimized concentration of citric acid (0.4mg). The effect of these acids on % docetaxel loading and % impurity generation was shown in Figure 4.4 and Table 4.7. No significant change in the % docetaxel loading with these acids (except maleic acid) was observed as compared to citric acid. We observed very less amount of impurities in liposomes prepared with tartaric acid (0.144±0.128%) as compared to citric acid (0.558±0.079%), maleic acid (0.609±0.0997%) and ascorbic acid (1.559±0.666%). This significant decrease in impurity generation is due to better control of liposomal pH by tartaric acid (pH 4.408±0.11) as compared to other acids (Table 4.7). The decrease in the pH of formulation was better correlated with pKa of these acids used in the formulation. The pKa of tartaric acid was less than the other acids used (Table 4.7), and hence it better controlled the pH and thereby degradation of docetaxel in liposome after heating at high temperature during hydration and annealing for a long period time.

Vitamin E has anti-oxidant property and might prevent oxidative degradation of docetaxel to form 10-oxodocetaxel and 10-oxo-7-epidocetaxel. Hence, we also tested vitamin E as degradation inhibitor at the same concentration of citric acid (0.4mg). Our results reveal that vitamin E is unable to prevent oxidation of docetaxel at our experimental conditions. The liposomal formulation prepared with vitamin E (pH 6.71±0.24; which is similar to formulation containing no acids) shows all three possible impurities (10-oxodocetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel) at our experimental conditions (Figure 4.4). Hence, we used tartaric acid, compared citric acid and other acids, as degradation inhibitor in the liposomal formulation as it better control the impurity generation without significantly affecting the docetaxel loading. The final optimized conventional liposomes showed mean particle size of 111±5nm (PDI: 0.266±0.101) (Figure 4.5) and zeta potential of -40.2±2.4mV (Figure 4.6).

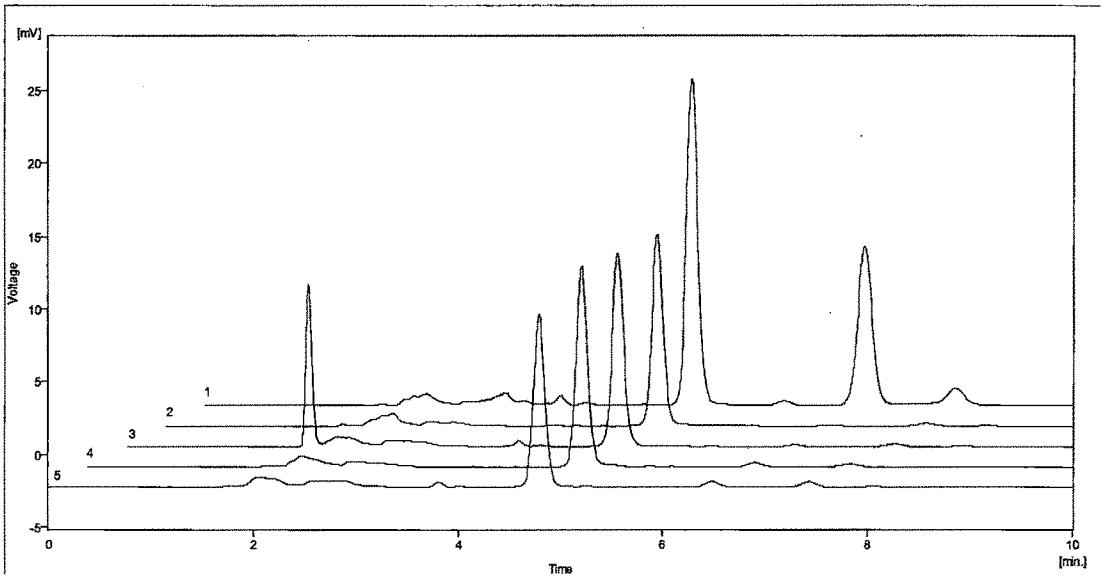


Figure 4.4. Comparison of liposomal formulations containing Vitamin E (1) and organic acids as degradation inhibitors [2: Tartaric acid (0.4mg); 3: Maleic acid (0.4mg); 4: Citric acid (0.4mg); 5: Ascorbic acid (0.4mg)].

Table 4.7. Comparison of vitamin E and organic acids as degradation inhibitors.

Organic acid	pH values	% DTX loaded	% 7-EDTX loaded
CA (pKa;3.12, 4.76 & 6.39)	5.305±0.184	47.566±2.59	0.558±0.079
AA (pKa; 4.17 & 11.57)	5.43±0.22	42.273±5.13	1.559±0.666
MA (pKa;3.4 & 5.2)	5.043±0.121	30.486±5.18	0.609±0.0997
TA (pKa; 2.93 & 4.23)	4.408±0.11	45.72±4.43	0.144±0.128
Vitamin E (as anti-oxidant)	6.71±0.24	56.373±2.10	27.113±2.052

Values are Mean±SD, n=3. DTX: docetaxel; EDTX: 7-epidocetaxel. Significant decreased docetaxel loading was observed with maleic acid as compared to citric acid (*$p<0.05$). No significant difference in % 7-epidocetaxel loading was observed between citric acid and other acids but found significant increase in % 7-epidocetaxel loading with Vitamin E (***$p<0.0001$) as compared to all other acids.

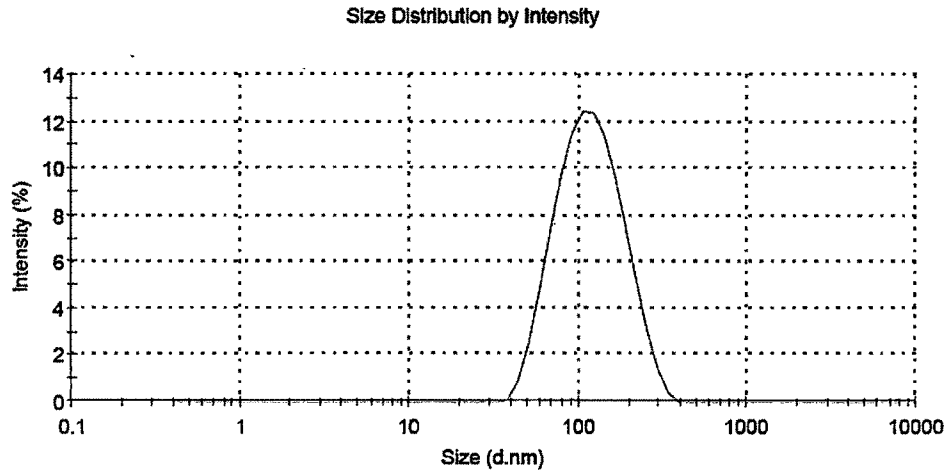


Figure 4.5. Mean particle size of prepared conventional liposomes

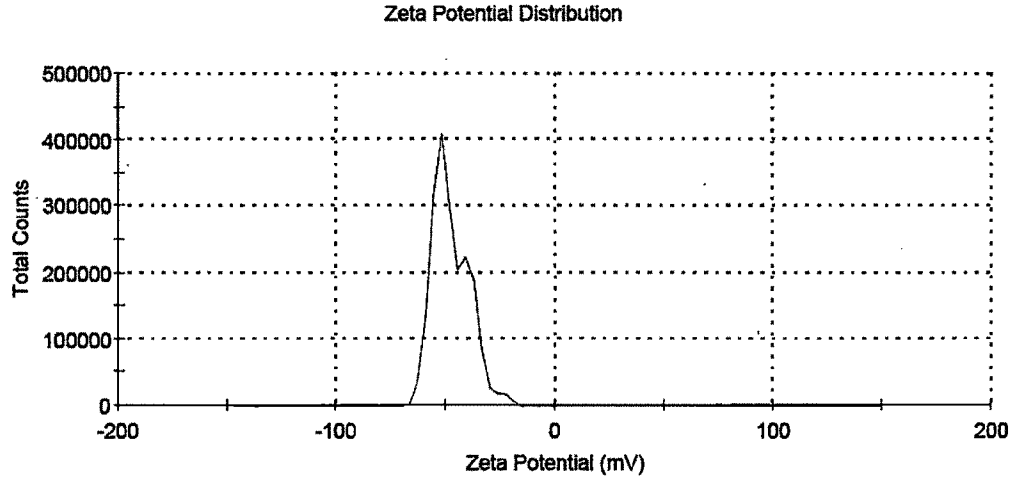


Figure 4.6. Zeta potential of prepared conventional liposomes

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