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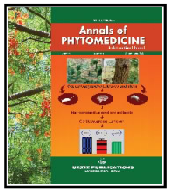

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Augumentation and evaluation of betasitosterol based liposomes

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Abstract

The combination of liposomes and betasitosterol (BS) may alter the current state of drug delivery technology. Although, liposomes are widely considered as a promising model for drug delivery of bioactive compounds, there are still significant barriers to widespread drug use. Two ways to overcome the factors associated with the effectiveness of liposomes in drug delivery are suggested. The primary involves the preparation of the liposome containing active components, while the second involves the synthesis of liposomes pre-loaded with drugs. This seeks to provide intelligent solutions to the limitations of normal liposomes such as short half-life of plasma, toxicity, stability, and poor control of long-term drug release. This review describes significant developments in integrated technologies combining the concepts of depot polymeric scaffolds with liposome technologies to bypass the limits of standard pharmaceutical liposomes.

1. Introduction

Decades ago, because of their unique features, such as hydrophilic and hydrophobic properties, good biocompatibility, low toxicity, systemic deficits, and targeted delivery of bioactive compounds into the workplace, liposomes gained a lot to be considered as a network system of active therapeutic compounds (Chen *et al*., 2010; Mastrobattista *et al*., 2002; Schnyder *et al*., 2005). In addition, some of the achievements since liposomes have been determined by size from microscale to nanoscale as well as engineered polymer adhesives that act on peptide, protein, and antibody (Bangham *et al*., 1974; Yousefi *et al*., 2009). Although, liposomes are widely considered as promising carriers of an effective therapeutic combination, a number of the main effects of medical liposomes are that rapid deterioration due to systemic (RES) and inability to detect ongoing drug delivery over a long period of time (Torchilin *et al*., 2005). New ways are needed to overcome these challenges. Two polymeric methods have been suggested at this point. The primary method involves the surface conversion of liposomes by hydrophilic polymers such as polyethylene glycol (PEG) while the other is to synthesize pre-loaded drug liposomes within polymer depot-based systems (Chen *et al*., 2010). A study by Stenekes and colleagues (Stenekes *et al*., 2000; Vishvakrama *et al*., 2014) reported the success of using a temporary depot of polymeric materials to manage the extraction of loaded liposomes of pharmaceutical applications. This breakthrough results in new applications, which require research into interactions between drugs, biomaterials, chemistry, molecular, and cell biology. Numerous studies in this context are reported within the interim depot delivery system to manage the release of pre-loaded drug liposomes (Chung *et al*., 2006; Hara *et al*., 2001; Wallace *et al*., 2003). This method was developed to combine benefits while avoiding your imperfections in both liposome and polymeric based systems. Liposome-based systems are known to have limitations such as instability, half-life, and rapid specification. However, they are more biocompatible than polymer-based systems (Bangham *et al*., 1974; Yousefi *et al*., 2009). On the other hand, polymer-based systems are known to be stable and provide continuous improved delivery compared to liposome-based systems. However, one of the main obstacles is biocompatibility impairment associated with bioactive losses (*i.e*., drug) during the formation of conditions such as sonication, temperature or exposure to organic solvents (Mulik *et al*., 2009; Mudshinge *et al*., 2011). The benefits of a composite system, however, include improved liposome stability, liposome ability to manage drug release in the short term of your life, and preserving drug bio activeness in polymeric-based technologies. Additionally, improved efficiency can also be achieved in this integrated delivery system by setting aside that of polymeric-based or liposome-based systems. Therefore, the purpose of this article is to review existing liposome and polymeric based technologies, similarly because the integration of liposome-based technologies within technology based on a polymeric depot for continuous drug extraction. The interview will guide different types of liposome-based technologies, different ways to embed drug-depleted liposomes within the depot, and various reported methods to control the speed of continuous drug delivery within depot systems over a long period of time. Liposomes of the vesicle are concentrated when the fluid phase is completely absorbed by the lipid layer (Dwivedi *et al*., 2014; Immordino *et al*., 2006). The outer lipid layer is sometimes produced in phospholipids with hydrophilic head and hydrophobic tail (Oussoren *et al*., 1999; Poste *et al*., 1982; Shivhare *et al*., 2009). Liposomes cancombining a hydrophilic drug into its aqueous interior with a lipophilic drug by a lipid bilayer (Demanty *et al*., 2009; Elbialy and Mady, 2015;

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Engin *et al*., 2007). Liposome binds to the drug, and thus reduces its toxicity, and protects against rapid deterioration within the blood circulation (Mastrobattista *et al*., 2002). Liposome also helps to reduce volume and side effects (Abumweis *et al*., 2008; Katan *et al*., 2003). In interaction with the plasma membrane, liposomes enter the cell through the distribution process and release the drug into the cell. In addition, liposomes are attacked by the RES system which ends up rapidly absorbing liposomes (Poste *et al*., 1982). Phytosterols and tocopherols are organic (plant metabolites) with many health applications. The first health benefit of phytosterols is to lower lipoprotein (LDL) cholesterol levels in plasma (Plat *et al*., 2005; Ryan *et al*., 2007; Heale *et al*., 1994). As a result of this health claim, the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) has recommended phytosterols as adjuvant treatment for statins in hypercholesterolemia (Frei *et al*., 1994; Niki *et al*., 2004; Taylor *et al*., 2002). Phytosterols compete with cholesterol to be synthesized in salt mammals, preventing the absorption of cholesterol in the blood (Frank *et al*., 2005; Vivekananthan *et al*., 2003; Ryan *et al*., 2007). Tocopherols, on the other hand, are great scavengers and natural antioxidants (Taylor *et al*., 2002), abundant oilseed crop in Canada (Schwartz *et al*., 2008). It is an upscale source of 4 phytosterols, namely; betasitosterol, campesterol, stigmasterol, brassicasterol and 4 tocopherols (alpha, beta, gamma and delta (Maguire *et al*., 2004; Verleyen *et al*., 2001). Oil loses a number of its valuable components during the refining process (Wallace *et al*., 2003). Significant amount of phytosterols and tocopherols are transferred to the waste stream, termed oil deodorizer distillate (CODD) (Vivekananthan *et al*., 2003) which offers a perfect source of those components.

2. Materials and Methods

2.1 Materials

Betasitosterol (BS), cholesterol (Ch), phosphatidylcholine (PC), polyethylene glycol (PEG), methanol and chloroform were purchased from Shubh Scientific Corporation, Lucknow. All other chemicals used in this study were analytical grade.

2.2 Methodology

2.2.1 Preformulation studies

With the help of this study, pre-formulation is providing information about the physicochemical and biopharmaceutical study properties like drug molecule, non-drug substance, and materials used for packaging and also about the compressibility.

2.2.2 Identification of the drug

2.2.2.1 Melting point

With the help of the melting point, we can get the purity of the sample. If the melting point decreases, then it, means the presence of undesirable substances. The point of melting was determined by the use of melting tools by filling the drug samples on one closed side of the capillary tube, and detection was recorded

2.2.2.2 λmax of the drug

With the use of phosphate buffer, high absorption of betasitosterol was obtained. For this, we make solutions at a range of (2-10 µg/ml) and are scanned using a UV spectrophotometer. UV absorption using the Shimadzu double beam UV-VIS spectrophotometer (UV 1601, Shimadzu, Japan) betasitosterol spectrum (10 µg/ml) was extracted with ethanol, a distance of 200-600 nm. The wavelength of high absorption is determined and recorded.

2.2.2.3 Solubility analysis

Betasitosterol solubility studies were performed on solvents with a wide range of non-polarity, as well as in different aqueous buffer systems at different. The melting of betasitosterol in various solvents including ethanol, chloroform, *etc*., was also measured. 10 mg of betasitosterol was added to a test tube containing approximately 0.5 ml of solvents, and allowed to be shaken to 37 ± 0.5°C and the concentration of betasitosterol was determined by UV spectroscopy.

2.2.2.4 Physical drug excipient compatibility

At 75% humidity and 40°C**,** the formulation was kept closed for 30 days. Then, it was compared with the initial state of any co-occurrence.

2.2.2.5 Fourier transforms infrared (FTIR) studies

With the help of infrared absorption spectral analysis (FTIR), betasitosterol suppression with a non-pharmacological component and a body composition of large doses were produced. Spectral analysis using IR helped to identify any alterations in the formation of compounds when combined with a non-pharmaceutical substance.

2.2.3 Methods

2.2.3.1 Thin film hydration method

Phospholipids and cholesterol in various molar concentrations were dissolved in chloroform in a pear-shaped flask by a mild shock. In this way, the chloroform was easily absorbed by the rotating flask above the changing temperature of the lipids at 120 rpm to obtain a thin film on the flask. The film was then stored overnight in a vacuum to remove chloroform particles, if any. After this, the film is soaked in water with the help of phosphate buffer saline (pH 7.4). And this hydrated solution was then rotated with an evaporator without a vacuum above the changing temperature of the phospholipid until a white suspension was obtained at 40°C**.** To further reduce the size of the liposomes, dispersing was sonicated for 1 min, using a wash sonicator (Imeco Ultrasonics). After sonication, the suspension was subjected to vesicle size testing and PDI. The rotational speed, hydration time and sonication time employed in this method are based on the results of the initial studies.

Figure 1: Liposomes prepared by thin film hydration method.

2.2.3.2 Ether injection method

In the course of ether injection, phospholipids and cholesterol in various molar concentrations were dissolved in diethyl ether. First in the box the phosphate buffer saline (10 ml, pH 7.4) was constantly stirred by a magnetic storm and its temperature was maintained between 55-60°C. With the help of a syringe, the phospholipid and cholesterol solution was then injected slowly to disperse the solution to the phosphate buffer saline (pH 7.4). Beaker was stirred in a magnetic stirrer at 55-60°C until the ether was completely evaporated and a milky dispersion was detected. The dispersion was less than 1 min sonication. The vesicle size and PDI of liposomes were then evaluated.

Effect of molar phospholipid dosage and cholesterol on the size of vesicles and PDI.

Table 1: Formulation of various batches of liposome betasitosterol

Formulation code	Soy lecithin	Cholesterol	Drug	Comments
F1	1 mg	2 mg	1 mg	Liposomes were not formed
F2	2 mg	1 mg	1 mg	Liposomes were not formed
F3	10 mg	5 mg	1 mg	Liposomes were not formed
F4	5 mg	5 mg	1 mg	Liposomes were formed
F5	5 mg	10 mg	1 mg	Liposomes were formed

2.3 Characterization of betasitosterol liposomes

2.3.1 Vesicular size evaluation microscopic evaluation

A small amount of betasitosterol liposomes were dissolved in 10-12 ml of phosphate buffer (pH 7.2). The distribution of liposomes was interrupted from time-to-time. When a decrease in scattering was observed with a microscope, circular vesicular segments were observed in a microscope.

2.3.2 Liposomes formulation

Projection images demonstrate the formation of vesicle in Figure 2.

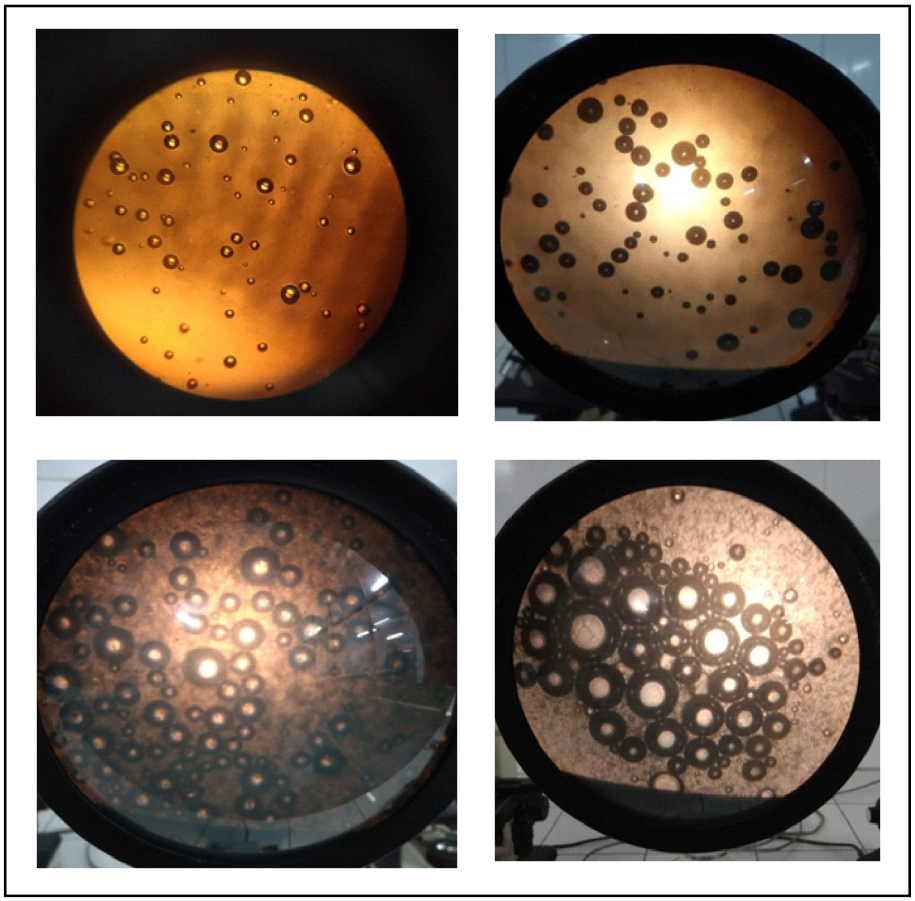

Figure 2: Projection images of liposomes.

2.3.3 Entrapment efficiency

The effective penetration of liposomes was determined by centrifugation of liposomes ware centrifuge and 3000 rpm at 5ºC for 45 min. To separate the free drug from the solution, a clear supernatant solution of liposomes was obtained. The supernatant was collected containing untreated drug and absorption was measured using UV-VIS spectroscopy (Shimadzu, Japan) at 213 nm. Login efficiency is calculated using the following calculation:

$$\text{Entrapment efficiency} = \frac{\text{Practical weight of drug}}{\text{Theoretical weight of drug}} \times 100$$

2.3.4 Determination of pH

Determination of the pH of the test liposome correction was performed using a pH meter Cyber lab India using a glass electrode. pH meter measurement is done using a computer in 7.4 about half an hour. After pH meter measurement, the pH analysis of the liposome correction was performed at temperatures varying in 37ºC, 20ºC, 25ºC, 40ºC and 45ºC.

2.3.5 Betasitosterol content analysis

Betasitosterol in liposomes was measured using a spectrophotometer based on correction. First, a mass of 0.1 g of liposomes was diluted in 10 ml of anhydrous ethanol and centrifuged at 2000 rpm for 5 min at 4ºC. The supernatant was transferred to a volumetric flask, and centrifugation was repeated until betasitosterol was not available in the upper extremity. Betasitosterol concentration was calculated from absorption values at 213 nm using a spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA). Pure water-soluble ethanol has been used as a neutral. The bioactive compound was measured using the analytical curve of pure betasitosterol (≥94%) in ethanol anhydrous (R^2 = 0.9996).

2.3.6 Percentage drug release

To quantify the release of the *in vitro* drug liposomal BS, a dialysis bag was used. After separating the free drug, 50 mg of liposomes were measured and placed directly in a dialysis bag and closed at both ends and obtained at 1000 ml fresh PBS buffer medium (pH 7.4) at 37ºC at 90 rpm under perforated sink conditions. At pre-determined times, 1ml of medium was taken for further UV exposure. The concentration of BS throughout the output time was measured using the equation. The recorded results are the average number of three runs performed on each liposome concentration.

2.3.7 Pharmacokinetic study

Animals used in pharmacokinetic research were adult SD mice of any gender, weighing 150-200 g. All animals were purchased from CSIR and approved by the institution's ethics committee, Integral University Lucknow. The animals were housed in polypropylene cages, 3 in each cage, and free access to standard laboratory food and water was obtained. Mice stored under normal laboratory conditions at 26 ± 2ºC, relative humidity of 45 ± 15% and normal image time (12 h black/12 h light) were used for testing.

2.3.8 Linearity of detector response for betasitosterol standard

Solutions containing betasitosterol in different combinations of 10, 20, 30, 40, 50, 60, 70, 80, 90,100 µg/ml were prepared with chloroform each of these solutions (10 µl) was used and different concentrations were measured.

Male SD mice starve for 18 h before taking betasitosterol. An empty sample (0 h) of 2 ml of blood was collected in the venous sinus before taking the dose and the mouse was fed orally with 20 g/kg body weight of the suspended mice in 10 ml distilled water using a 10gauge needle number. After suspension treatment, blood samples at 1, 2, 3, 4, 6, 8, 12, 24, and 48 h were collected in eppendorf tubes.

2.3.9 Extraction from plasma

Blood samples were centrifuged at 1500 rpm for 15 min. then 0.5 ml of plasma is transferred to standing and clean test tubes. Then diluted with a phosphate buffer to obtain the desired concentration.

$$\text{Glucose conc. (mg/dl)} = \frac{\text{Absorbance (Sample)}}{\text{Absorbance (Standard)}} \times 100$$

2.3.10 Statistical analysis

Statistical analysis of the obtained results was performed on the SD calculator. All analyzes are done three times with average values unless otherwise stated, and the error bars displayed represent the ± standard deviation.

3. Results

3.1 λmax of the drug

The λmax of the drug was found to be 213 nm, and it was in line with the official standard. The above parameters as a melting point, λmax, and FTIR showed that it was compliant with the reference and, hence the pure sample of BSI.

3.2 Solubility of the drug

As a pure drug is a steroid. Therefore, the solubility of pure wood is more soluble in non-environmental solvent compared to polar solvent. It also shows that pure medicine is highly soluble in acetone, ether, and ethanol (95%) and is insoluble in water.

3.3 Fourier transforms infrared (FTIR) studies

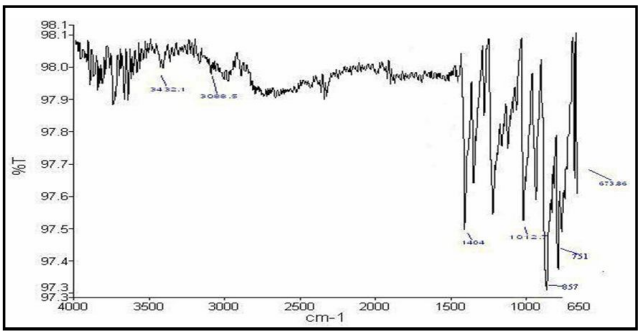

Figure 3: FTIR spectrum of betasitosterol.

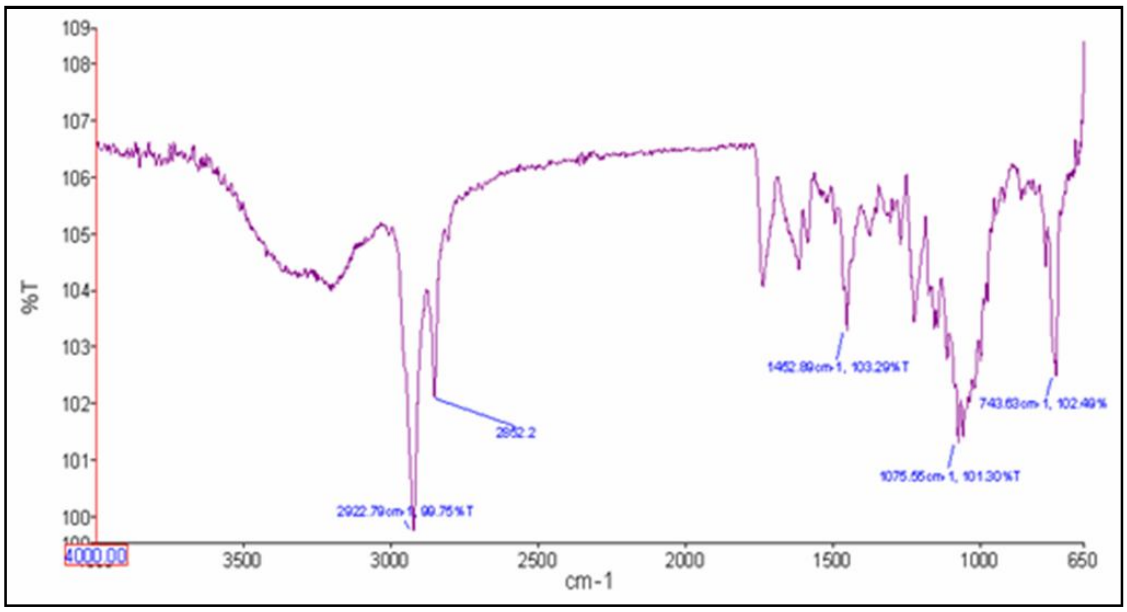

Figure 4: FTIR spectrum of soy lecithin.

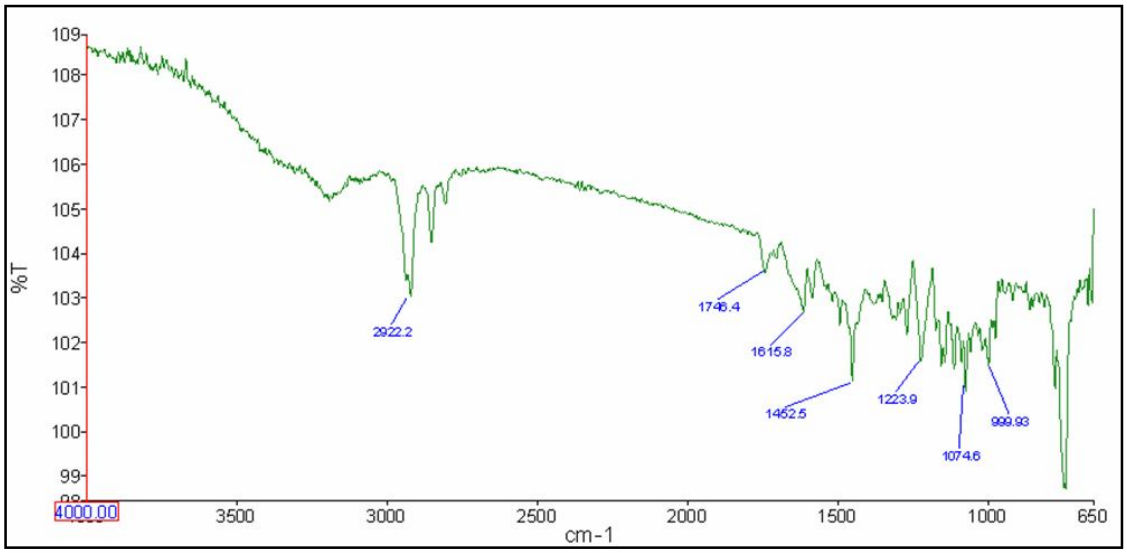

Figure 5: FTIR spectrum of cholesterol.

Table 3: FTIR characterization of betasitosterol, soy lecithin and cholesterol

Sr. No.	Group	Observed peak
1	-OH	3424 cm^{-1}
2	C-H (Stretching in aliphatic)	2872 cm^{-1}
3	C=C	1643 cm^{-1}
4	C-O stretching	1056 cm^{-1}
5	C-H (Bending in CH_2)	1486 cm^{-1}
6	C-H (Bending in gem group)	1347 cm^{-1}
7	C=C-H	965 cm^{-1}
8	C-C (Stretching)	1243cm^{-1}
9	C_6H_6	725 cm^{-1}

The FTIR spectroscopy studies were conducted for betasitosterol, soy lecithin and cholesterol.

Table 4: Physical characterization of betasitosterol

S.No.	Identification parameters	Observed value	Reported/standard value (Reference)	Remarks
1	Physical appearance	Solid , white color powder	Solid, white powder (HMDB)	Complies
2	Melting point	142-147°C	142°C (HMDB)	Complies
3	Solubility	More soluble in acetone, ether, ethanol, chloroform, dichloromethane and insoluble in water	Same as observed value (HMDB)	Complies
4	UV scan, λ_{max}	In ethanol: 425 nm	In ethanol: 425 nm	Complies

3.4 Physical drug excipient compatibility

In the physical drug excipient, there were found no incompatibilities or interaction between drug and excipients.

3.5 Analytical method

Construction of calibration curve (Figure 2) in phosphate buffer pH 7.4 shows the absorption reading of standard drug solution containing 2-10 µg/ml of drug in phosphate buffer (pH 7.4) at a wavelength of 213 nm. The following equation from the UV result was obtained Y= 0.009 x + 2.794 where Y is the area under curve and X is the concentration of BS, the regression line of R^2 = 0.997 was obtained as well. Based on this standard curve (Figure 2), the calculations of drug contents and *in vitro* drug release are studied.

Table 5: Absorbance maxima at different concentration of betasitosterol

Sr. No.	Concentration	Absorbance
1	10	2.699
2	20	2.608
3	30	2.511
4	40	2.397
5	50	2.324
6	60	2.225

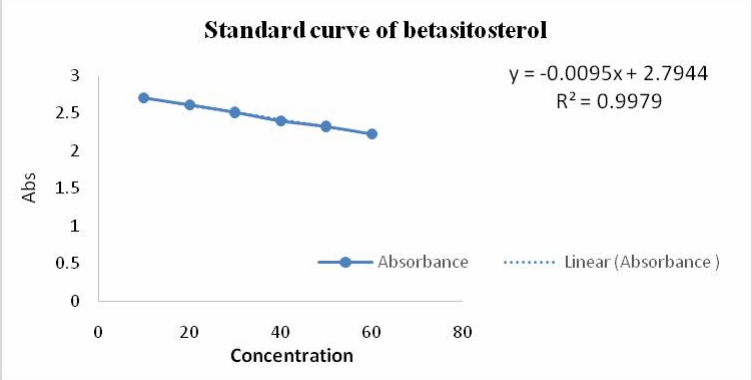

Figure 6: Standard curve of betasitosterol.

3.6 Entrapment efficiency and *in vitro* drug release

3.6.1 Betasitosterol entrapment efficiency

The efficacy of entrapment is a study conducted to evaluate the amount of BS bound to liposomes. The effectiveness of the entrapment of liposomes is achieved by a different combination of solvents. Due to the presence of a sufficient number of stabilizing stabilizers, there has been an increase in the efficiency of entrapment which helps to bind the drug and stabilize the drug-binding molecule. In the given design, the LF5 showed great efficiency of entry (Table 6).

Table 6: % Entrapment efficiency of liposome formulations

Sr. No.	Formulation code	% Entrapment efficiency
1	LF1	40
2	LF2	44
3	LF3	60
4	LF4	65
5	LF5	85

As can be seen in Table 6, the formulation, liposomes contain a high efficiency of about 100% incorporation. And Figure 7 shows BS *in vitro* release within 24 h. Where both the formation of liposomes exhibits a stable and continuous extraction profile. Since both formulations include the same ingredient that differs only in PC value, they also have a comparable release pattern with slight variations. At the start of 5-6 h, the LF5 shows a faster release rate compared to the others (Figures 8-10).

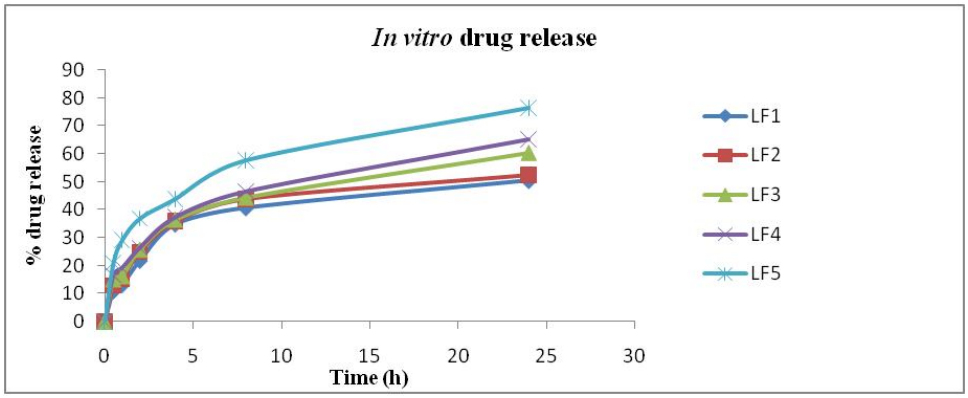

Figure 7: *In vitro* drug release of various batches.

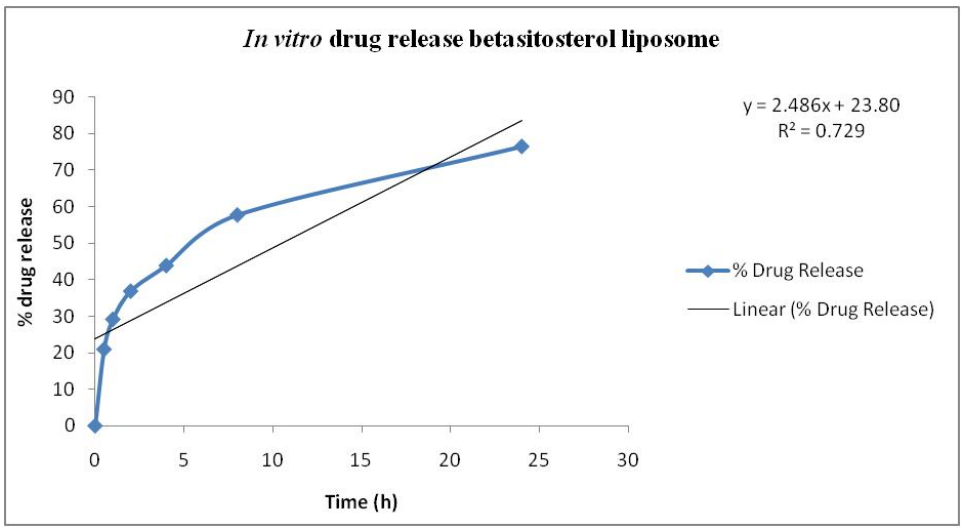

Figure 8: *In vitro* drug release of optimized batch (LF5).

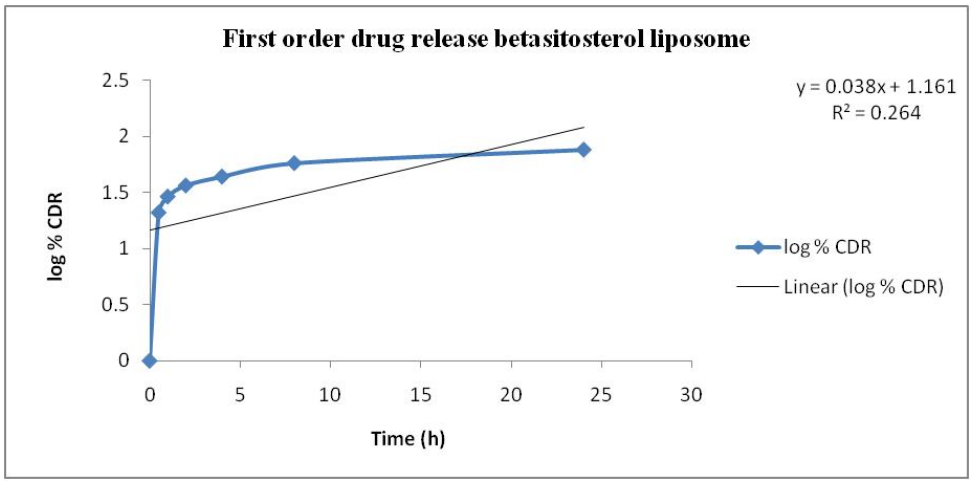

Figure 9: First orderdrug release of optimized batch (LF5).

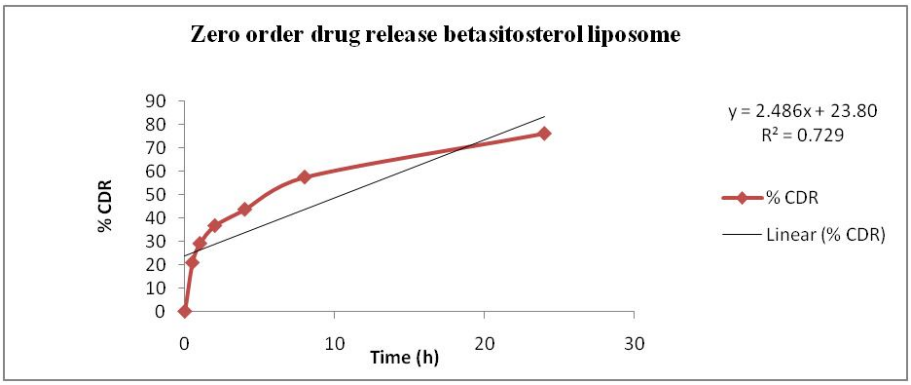

Figure 10: Zero order drug release of optimized batch (LF5).

4. Discussion

Firstly, a comparison of thin film hydration and ether ethanol injection method was done and no major differences were seen between the different methods. The film method was thus chosen as the method for further liposome preparation. Further, bath sonication and direct probe sonication were compared in terms of preparation of liposomes. The latter method gave satisfied size reduction for our purpose and was used during the study.

For separation of liposomes from BS a centrifugation approach was used. Centrifugation at 3000 rpm for 15 min was not found sufficient for removal of BS from the supernatant. Upon changing the duration to 20 min, all BS were settled in the pellet and no BS was visible. 20 min centrifugation was concluded to be the appropriate centrifugation condition for our study.

Second, BS-liposomes with different lipid compositions were prepared and evaluated for incorporation capacity and retention ability. The optimum preparation was F5. It was observed that PC and cholesterol affect the entrapment efficiency and lipid vesicles size. PC and cholesterol increased the entrapment efficiency, although, PC had a more positive effect on encapsulation efficacy than cholesterol. High cholesterol content generally decreased percentage encapsulation. The particle size of lipid vesicles enhanced as phosphatidylcholine and cholesterol content increases.

Third, in the preliminary PEGylation studies, the PEG containing liposomes showed lower incorporation capacity compared to the liposomes without PEG, while better retention and slower leakage from the liposomes.

In summary, we have made progress towards establishing a method for producing and testing of BS-liposomes as well as identifying a formulation that effectively can incorporate and retain the drug in circulation. However, we have yet to arrive at the ideal formulation, and, as we are still evaluating elements that affect all areas of incorporation and retention. Further studies are needed in order to reach the goal of a formulation appropriate for *in vivo* studies. The liposomal formulations containing BS were lyophilized to improve long term stability during storage.

5. Conclusion

In the present study, the BS liposome was prepared using different methods with different moving speeds and tested for particle size,% efficiency of penetration, % release of drugs such as *in vitro*, and *in vivo* release were studied. A major achievement of this study was the development and testing of betasitosterol liposomes to improve bioavailability and structural stability. The main reason for this study was that they were developed considering the medical field and industry requirements such as scale up, validation, low cost, *etc*. BS liposomes are successfully prepared by thin film and ether / ethanol injection method. BS liposomes showed an improvement in the rate of release of *in vitro* and *in vivo* drugs.

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Ethical approval for animal use

The work proposal is approved by IAEC Integral University. Approval no. is IU/IAEC/20/24.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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