Formulation Development, Characterization and In-vitro Evaluation of Tamoxifen Loaded Liposomes

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Authors’ contributions

This work was carried out in collaboration among all authors. Author MAZ designed the entire study. Author MH coordinated the results with statistical analysis and wrote the manuscript. Author MMH carried out the experiments. Authors AHMNH and IJ participated in its design, literature search and technical support. All authors read and approved the final manuscript.

ABSTRACT

Background: The study was aimed to prepare and evaluate tamoxifen loaded controlled release liposomes to reduce the side effects of tamoxifen during cancer treatment.

Methods: Different tamoxifen loaded liposomes were prepared by modified ether injection (MEIM) and thin film hydration method (TFHM) under prescribed conditions. The prepared liposomes were characterized by using optical microscopy, evaluating encapsulation efficiency, in-vitro and ex-vivo diffusion studies by using dialysis membrane and chicken intestinal sac respectively.

Results: The data revealed that all of the liposomes were spherical in shape and stable under three physical conditions i.e. 4, 25 and 37 ± 2°C temperatures and 60 ±5% relative humidity. Additionally most of the liposomes followed zero order and class II release kinetics. It was also observed that with the increase of phospholipids and cholesterol, entrapment efficiency of liposome vesicles increased thus giving a controlled release drug delivery system but further increase reduced this efficiency at a certain level.

Conclusion: The formulated control release liposomes might be a good drug delivery system for target oriented drug delivery with minimum side effects of tamoxifen during cancer treatment.
Keywords: Tamoxifen; liposomes; ether injection release; in-vitro drug diffusion study.

1. INTRODUCTION

Tamoxifen is used very commonly for the treatment of estrogen receptor positive breast cancer which is considered a pioneering drug because of its ubiquitous application in both treatment and chemoprevention of breast cancer [1] and also reduction of the chances of breast cancer in high-risk patients [2]. It is generally administered through oral and parenteral route to treat patients but several potential problems arise uniquely because of the use of oral therapy and oncologists need to be aware about the drug interactions and bioavailability of tamoxifen [3]. Whitfield et al. has reported that tamoxifen has a relatively high biological half-life which is 5-7 days and 99% protein binding after administration which reduces the effectiveness of the drug. So there is a strong clinical need and market potential for a new drug delivery system [4].

Bozzuto and Molinari [5] proposed that liposomal drug delivery system is a controlled targeted drug delivery system and has benefits like patient compliance, avoiding multiple dosing, increased bioavailability, fewer side effects these will help to overcome the problems associated with conventional system. Liposomes are considered the almost ideal drug-carrier system due to their similar morphology to cellular membranes and have ability to encapsulate both hydrophilic and lipophilic substances because of unique structural characteristics [6,7]. It is reported that a number of drugs have already been successfully encapsulated in liposomes, from antibacterials [8] and interferons to antitumor drugs such as doxorubicin [9] where it is proven that liposomes are useful in terms of biocompatibility, biodegradability, low toxicity, and can control bio distribution by changing the size, lipid composition, and physical characteristics [10,11,12].

In our study, modified ether injection method [13] and thin film hydration method [14] were used to prepare tamoxifen loaded liposomes where cholesterol was used to improve the liposomal delamination and to improve membrane fluidity, stability of the layers and reduce the permeability of water-soluble molecules through membranes. The presence of cholesterol in the lipid layers increased the stability and results in the formation of a hard membrane with similar liquid properties [15,16].

The purpose of this work was to formulate and evaluate a modern drug delivery system, tamoxifen loaded liposome to improve bioavailability, therapeutic effectiveness as well as reduce drug resistance, cytotoxicity and other unwanted effects.

2. MATERIALS AND METHODS

2.1 Materials

Materials used for the preparation and characterization of tamoxifen loaded liposomes were listed in Table 1.

2.2 Preparation of Tamoxifen Loaded Liposomes by Modified Ether Injection Method (MEIM)

Cholesterol and phospholipids were dissolved in 8 mL diethyl ether and mixed with 2 mL methanol containing weighed quantity of tamoxifen. The resulting solution was injected using a micro syringe at a rate of 1 mL/min into 10 mL phosphate buffer (pH 7.4) hydrating solution at constant temperature (50-55°C) with continuous stirring on magnetic stirrer at 300 RPM (MS 300 Hot plate Magnetic Stirrer, BANTE instruments, USA). As the lipid solution was injected slowly into the aqueous phase, the difference in temperature between phases caused rapid vaporization of ether, resulting in spontaneous vesicle formation of liposomes. All the formulations as per experimental design were prepared using similar procedure by addition of various quantities of phospholipids and cholesterol [17].

2.3 Preparation of Tamoxifen Loaded Liposomes by Thin Film Hydration Method (TFHM)

Cholesterol and phospholipids were dissolved in 8 mL chloroform and mixed with 2 mL methanol containing weighted quantity of tamoxifen and mixed together in a round bottom flask. Using the rotary flash evaporator, the organic solvents were removed at 45-50°C at 120 RPM which leaves a thin layer of solid mixture on the wall of the flask. The dried film is then rehydrated with 20 mL of pH 7.4 phosphate buffer solution at the temperature of 60 ± 2°C for a specified period of time (about 2-2.5 hours) with gentle agitation.
The dispersion was left undisturbed at room temperature for 2-3 hours to allow complete swelling of the lipid film and hence to obtain vesicular suspension. Finally the liposome dispersion was stabilized by keeping at 2-8°C for 24 hours [18].

2.4 Formulation Design
For the preparation of tamoxifen loaded liposomes using central composite design of user defined factorial design was adopted to optimize the formulation parameters and to study the influence of independent formulation variables on dependent variables. Percent of drug entrapment efficiency and drug release were considered as dependent variables while phosphatidylcholine and cholesterol were taken as independent variables for liposomal formulations.

Nine experimental runs were designed (design Export® software-Trial Version 11 Stat-Ease Inc., MN) by selecting two parameters phosphatidylcholine and cholesterol amount at three levels each (low, medium and high) as independent variables where the amount of drug (10 mg) was kept constant for each batch shown in Table 2.

2.5 Preparation of Standard Curve
Standard curve of tamoxifen was prepared by dissolving 10 mg pure drug (hydrated with 3-4 drops of ethanol) up to 100 mL with phosphate buffer (pH 7.4 & 6.8) and acidic buffer (0.1N & 0.02N HCl) respectively. Afterwards these standard primary solutions were diluted with respective media to obtain varying concentration of stock solutions and absorbance was obtained at 237 nm by using UV spectrophotometer (UV mini-1240, Shimadzu Corporation, Japan).

2.6 Quantitative Assay of Liposomes Containing Tamoxifen

2.6.1 Determination of percentage of drug encapsulated in the liposomes
Entrapment efficiency was measured by measuring the un-entrapped free drug. The free drug was determined by subjecting the liposomal formulation to centrifugation (Model 400, Shimadzu Corporation, Japan) at 4000 RPM for 2 hours to separate the free drug, after centrifugation the supernatant was collected. The collected supernatant was analyzed spectrophotometrically to determine drug content at 237 nm. The % entrapment was determined by following formula: [19]

%DEE = (Amount of entrapped drug/Total amount) x100

2.6.2 Stability studies
The ability of vesicles to retain the drug (i.e., drug retentive behavior) was assessed by keeping the liposomal suspensions at three different temperature conditions, i.e., 4-8°C (refrigerator; RF), 25±2°C (room temperature; RT), and 37±2°C at 60 ± 5% relative humidity (RH) for a period of 3 weeks according to the recommendations of International Conference on Harmonization (ICH) guidelines. The liposomal suspensions were kept in sealed ampoules (10 mL capacity). Samples were withdrawn periodically and analyzed for the drug content, in the manner described under drug entrapment studies [20].

2.7 Characterization of Physical Parameters of Liposomes
Prepared liposomal batches were monitored for their morphological attributes using optical microscope (at suitable magnification). Size distribution profile of liposomes was determined by light scattering based on laser diffraction method [21].

2.8 Ex-vivo Permeability Study by Using Chicken Intestinal Sac
Ex-vivo permeability studies were carried out through dissolution study conducted in a dissolution machine using USP II paddle apparatus by using chicken intestinal sacs. Total nine cleaned intestinal segments (each six cm length) were ringed with a phosphate buffer solution (pH 7.4) and 2 mL of liposomes suspension was placed in the sac which was then sealed at both ends. The sacs were dipped into the receptor compartment containing 1000 mL of phosphate buffer (pH 7.4) at 100 RPM and temperature was maintained at 37°C [22]. 10 mL of the sample was withdrawn at predetermined intervals from each basket, filtered and vessel was replenished with fresh medium. Absorbance was taken by using spectrophotometer at 237 nm and the permeability study was checked for eight hours.
Table 1. List of materials

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Source and country of origin</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td>Beacon Pharmaceutical LTD, Bangladesh</td>
<td>Active pharmaceutical ingredients</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>ALFA Aesar, UK</td>
<td>Bilayer forming agent</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>ALFA Aesar, UK</td>
<td>Phospholipid</td>
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<td>Solvent</td>
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<tr>
<td>Ethanol</td>
<td>MERCK, Germany</td>
<td>Solvent</td>
</tr>
<tr>
<td>Di-ethyl ether</td>
<td>MERCK, Germany</td>
<td>Solvent</td>
</tr>
</tbody>
</table>

Table 2. Design layout of experiments as per user defined factorial design

<table>
<thead>
<tr>
<th>Formulation Code for MEIM</th>
<th>Formulation Code for TFHM</th>
<th>Phosphatidylcholine (mg)</th>
<th>Levels</th>
<th>Cholesterol (mg)</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
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<td>TEI-1</td>
<td>TTFH-1</td>
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<td>Low</td>
<td>0</td>
<td>Low</td>
</tr>
<tr>
<td>TEI-2</td>
<td>TTFH-2</td>
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<td>Medium</td>
<td>0</td>
<td>Low</td>
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<tr>
<td>TEI-3</td>
<td>TTFH-3</td>
<td>150</td>
<td>High</td>
<td>0</td>
<td>Medium</td>
</tr>
<tr>
<td>TEI-4</td>
<td>TTFH-4</td>
<td>50</td>
<td>Low</td>
<td>10</td>
<td>Medium</td>
</tr>
<tr>
<td>TEI-5</td>
<td>TTFH-5</td>
<td>100</td>
<td>Medium</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>TEI-6</td>
<td>TTFH-6</td>
<td>150</td>
<td>High</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>TEI-7</td>
<td>TTFH-7</td>
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<td>20</td>
<td></td>
</tr>
<tr>
<td>TEI-8</td>
<td>TTFH-8</td>
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<td>Medium</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>TEI-9</td>
<td>TTFH-9</td>
<td>150</td>
<td>High</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

2.9 In-vitro Permeability Study by Using Cellulose Dialysis Membrane

In vitro permeability study was done using cellulose dialysis membrane (Spectrapor, USA) in USP II paddle method. Dialysis membrane was cut into nine cm in length and soaked in 500 mL distilled water at room temperature for 30 minutes to 1 hour to remove the sodium azide preserving agent. Then the membrane was rinsed thoroughly in distilled water and 2 mL of liposomes was placed in the membrane which was then sealed at both ends. The membrane was dipped into the receptor compartment containing 1000 mL of phosphate buffer (pH 7.4) dissolution medium. The dissolution was carried out based on the specification followed in ex vivo permeability study [23].

2.10 Interpretation of Dissolution Profile of Liposomes

2.10.1 Interpretation of dissolution profile

Percent release of drug was obtained from the formulation of liposomes by comparing the absorbance value with the standard curve of tamoxifen [24].

2.10.2 Release kinetics

Data obtained from in vivo release studies were fitted to various kinetic equations to find out the mechanism of drug release from the liposomes.

2.10.3 Zero-order kinetic model

Zero order described the system where the release rate of drug was independent of its concentration where data obtained from the in-vitro drug release studies were plotted as cumulative amount of drug released versus time [25].

This can be represented by the equation:

\[ Q_t = Q_0 + k_0 t \]

Here,

\[ Q_t = \text{Release of drug at time} 't' \]
\[ Q_0 = \text{Initial amount of drug in the solution at } t=0 \]
\[ k_0 = \text{First order release constant} \]

2.10.4 First order kinetic model

This model was used to describe the absorption and elimination of drugs from liposomes where obtained data were placed as log cumulative percentage drug remaining versus time, which yields a straight line with slope = (K / 2.303) [26].

The drug release which followed the first order kinetic can be expressed by the equation:

\[ \log C = \log C_0 - (kt / 2.303) \]

Here,

\[ C = \text{Concentration of drug} \]
C_0= Initial concentration of drug 
\( k \) = First order constant

### 2.10.5 Higuchi kinetic model

This model was used to describe drug release from matrix system where data obtained were plotted as cumulative percentage of drug release versus square root of time [27].

A form of Higuchi square root law was given by equation:

\[
Q = kH \sqrt{t}
\]

Here,

- \( Q \) = Amount of drug dissolved at time \( t \)
- \( k_H \) = Higuchi dissolution constant

### 2.10.6 Korsmeyer-peppas model

This model described drug release from a polymeric system equation where first 60% drug release data were fitted in Korsmeyer-Peppas model [28].

\[
\frac{M_t}{M_\infty} = k_t^n
\]

Here,

- \( M_\infty \) = Amount of drug release after infinite time
- \( k_t \) = Korsmeyer release rate constant
- \( n \) = Diffusion exponent

### 2.10.7 Successive fractional dissolution time

To characterize the drug release in different experimental conditions, T25%, T50% (mean dissolution time) and T80% were calculated from dissolution data according to the following equations, [29]

- \( T25\% = (0.25/k)^{1/n} \)
- \( T50\% = (0.5/k)^{1/n} \)
- \( T80\% = (0.8/k)^{1/n} \)

Mean dissolution time can also be calculated by the following equation:

\[
\text{MDT} = \frac{n}{n+1} k
\]

Here,

- \( K \) = Kinetic constant
- \( n \) = Exponent that characterizes the mechanism of drug release.

### 3. RESULTS AND DISCUSSION

The liposomal formulation containing tamoxifen were prepared by modified ether injection method (MEIM) and thin film hydration method (TFHM) using phosphatidylcholine and cholesterol with the purpose of evaluating the effect of phospholipids and cholesterol and their different level on physical and structural properties of liposomes.

#### 3.1 Standard Curve of Tamoxifen

Standard curve of tamoxifen was prepared in acidic buffer (0.1N and 0.02N HCl) and phosphate buffer (pH 6.8 and pH 7.4) respectively. Absorbance of tamoxifen for different media was cited in Table 3. It was observed that \( R^2 \) value was highest in phosphate buffer (pH 7.4) shown in Fig 1.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance (0.1N HCl)</th>
<th>Absorbance (0.02N HCl)</th>
<th>Absorbance (pH 6.8)</th>
<th>Absorbance (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.071</td>
<td>0.138</td>
<td>0.083</td>
<td>0.089</td>
</tr>
<tr>
<td>6</td>
<td>0.128</td>
<td>0.191</td>
<td>0.139</td>
<td>0.176</td>
</tr>
<tr>
<td>9</td>
<td>0.19</td>
<td>0.272</td>
<td>0.203</td>
<td>0.245</td>
</tr>
<tr>
<td>12</td>
<td>0.285</td>
<td>0.352</td>
<td>0.238</td>
<td>0.334</td>
</tr>
<tr>
<td>15</td>
<td>0.338</td>
<td>0.453</td>
<td>0.282</td>
<td>0.367</td>
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<td>18</td>
<td>0.394</td>
<td>0.51</td>
<td>0.341</td>
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</tr>
<tr>
<td>21</td>
<td>0.464</td>
<td>0.59</td>
<td>0.392</td>
<td>0.485</td>
</tr>
<tr>
<td>24</td>
<td>0.515</td>
<td>0.682</td>
<td>0.471</td>
<td>0.538</td>
</tr>
<tr>
<td>27</td>
<td>0.594</td>
<td>0.775</td>
<td>0.535</td>
<td>0.61</td>
</tr>
<tr>
<td>30</td>
<td>0.641</td>
<td>0.86</td>
<td>0.632</td>
<td>0.697</td>
</tr>
</tbody>
</table>
3.2 Percent Drug Entrapment Efficiency (%DEE) of Liposomes

Total nine liposomal formulations of tamoxifen were prepared by following MEIM where percent of drug entrapment efficiencies of different formulations were in range of 60.12% to 86.83% and TFHM in range of 59.19% to 86.03% respectively. It was seen that formulation TEI-1 and TTFH-1 showed lowest tamoxifen entrapment efficiency due to the lowest level of phosphatidylcholine and cholesterol in the formulation. On the other hand, TEI-9 and TTFH-9 revealed highest tamoxifen entrapment efficiency because of highest amount of phosphatidylcholine and cholesterol present in the formulation for both methods [30].

Additionally, having very low phospholipids contents in liposomal formulation was found to cause low entrapment efficiency but it was also observed that increase in phospholipids contents along with the cholesterol amount leads to higher entrapment efficiency referred in Table 4. The liposomal formulation having a high phospholipids concentration has the higher entrapment efficiency but depends upon the cholesterol amount. It can also be seen that increasing cholesterol amount for a given amount of phospholipids increases drug entrapment efficiency. This may be due to the increased stability of phospholipids bi-layers by cholesterol [31].

3.3 In vitro Drug Release Studies of Liposomes Prepared by MEIM and TFHM Method

The release kinetics and the plots of nine formulations appeared from Fig. 2 (A, B & C) showed that the percentage release of the drug increased with time. It was observed that amount of phospholipids and cholesterol affected the drug release of the liposomes for both methods. Additionally, the drug release was decreased with increase in phospholipids and it was seen by the percent of drug release of formulation TEI-3 to TEI-6 (98.83% to 94.98%) and TTFH-3 to TTFH-6 (96.83% to 93.98%) respectively. Formulation TEI-1 & TEI-2 by MEIM and TTFH-1 & TTFH-2 by TFHM revealed percentage of drug release (99.78% to 85.32%) and (97.78% to 88.42%) respectively but an increase release was seen from TEI-2 to TEI-3 (83.32% to 98.83%) and TTFH-2 to TTFH-3 (88.42% to 96.63%). These observations can also be seen for other formulations where increase of phospholipids decreased drug release rate, but further increase in phospholipids increased drug release. From percentage drug released, it was
observed that by increasing the cholesterol ratio the drug release decreased because cholesterol acted as retardant barrier [32].

3.4 Ex Vivo Drug Diffusion Studies of Liposomes Formed by MEIM and TFHM Methods

In ex-vivo permeability studies, Fig. 3 showed that the percentage of drug release from liposomes was increased with time. It was also observed that ratio of phospholipids and cholesterol greatly influenced the drug release profile from liposomes in both methods. Drug release pattern of the liposome formulations showed that the drug release decreased with the increase of the amount in cholesterol and phospholipids but further increase in phospholipids increased drug release [33].

Table 4. Percent of drug entrapment efficiency (%DEE) of tamoxifen loaded liposomes

<table>
<thead>
<tr>
<th>Formulation Code for MEIM</th>
<th>% DEE</th>
<th>Formulation Code for TFHM</th>
<th>% DEE</th>
<th>Level of Phosphatidylcholine/Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEI-1</td>
<td>60.12</td>
<td>TTFH-1</td>
<td>59.19</td>
<td>low/low</td>
</tr>
<tr>
<td>TEI-2</td>
<td>66.02</td>
<td>TTFH-2</td>
<td>63.67</td>
<td>medium/low</td>
</tr>
<tr>
<td>TEI-3</td>
<td>75.39</td>
<td>TTFH-3</td>
<td>71.21</td>
<td>high/low</td>
</tr>
<tr>
<td>TEI-4</td>
<td>61.85</td>
<td>TTFH-4</td>
<td>60.19</td>
<td>low/medium</td>
</tr>
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<td>TEI-5</td>
<td>70.33</td>
<td>TTFH-5</td>
<td>68.64</td>
<td>medium/medium</td>
</tr>
<tr>
<td>TEI-6</td>
<td>81.95</td>
<td>TTFH-6</td>
<td>75.12</td>
<td>high/medium</td>
</tr>
<tr>
<td>TEI-7</td>
<td>69.65</td>
<td>TTFH-7</td>
<td>68.25</td>
<td>low/high</td>
</tr>
<tr>
<td>TEI-8</td>
<td>76.67</td>
<td>TTFH-8</td>
<td>75.12</td>
<td>medium/high</td>
</tr>
<tr>
<td>TEI-9</td>
<td>86.83</td>
<td>TTFH-9</td>
<td>86.03</td>
<td>high/high</td>
</tr>
</tbody>
</table>
A) Zero order plot
B) First order plot

- Log % remaining of drug vs. Time (hour) for TFHM method
- Cumulative % release vs. Square root of time (hour) for MEIM method
C) Higuchi plot
D) Korsmeyer-peppas plot

Fig. 2. Release kinetic plot of tamoxifen loaded liposomes prepared by MEIM&TFHM method, a) zero order, b) first order, c) higuchi & d) korsmeyer-peppas plot
Fig. 3. Ex vivo release kinetic plot of tamoxifen loaded liposomes formed by a) MEIM and b) TFHM respectively

3.5 Mechanism of Drug Release of Tamoxifen Loaded Liposomes

Four mathematical models such as zero order, first order, Higuchi and Korsmeyer-Peppas were used to characterize the release mechanism of tamoxifen liposomes. The kinetic constant of release models were described by $K_0$, $K_1$, $K_H$ and $n$ for zero order, first order, Higuchi and Korsmeyer-Peppas respectively where highest correlation coefficient ($R^2$) was considered to express the best drug release pattern from liposomes. From Table 5 it was seen that most of the formulations (13 out of 18) followed zero order kinetics [34].

It was reported that when $n$ is below 0.45, the Fickian diffusion phenomenon dominates whereas $n$ between 0.45 and 0.89 is an anomalous transport often termed as first order release. Purely matrix relaxation or erosion mediated release occurs for $n=0.89$ (zero order kinetic). After the $n$ value reaches 0.85 and above, the release can be characterized by class II and super class II transport, which means the drug release rate does not change over time and the release is characterized by zero order release. In this case, the drug release was dominated by the erosion and swelling of the polymer [35,36]. The release exponent ‘$n$’ was the slope of log fraction of drug release versus log time curve Fig. 2 (C). The value of diffusion exponent ($n$) that was shown in Table 5 proved that the formulations were following super class II transport because of the diffusion exponent ($n$) was more than 0.85.

3.6 Successive Fractional Dissolution Time of Liposomes Formed by MEIM and TFHM Method

Successive fractional dissolution time was used to characterize the drug release rate from the liposomes and the retarding efficiency of the surfactant and cholesterol. A higher value of mean dissolution time (MDT) indicated a higher retaining ability of the surfactant and vice-versa [37]. In case of ether injection method, formulation TEI-9 (Fig. 4-A) showed highest drug retarding ability (MDT value 3.698) which possessed highest ratio of phospholipids and cholesterol where cholesterol acted as the liposomal bilayer stabilizing factor [38].
Table 5. Drug release mechanism of tamoxifen loaded liposomes based on release rate constants and $R^2$ values for different release kinetics

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Higuchi</th>
<th>Korsmeyer-Peppas</th>
<th>Release Mechanism</th>
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<tr>
<td>code</td>
<td>$K_0$</td>
<td>$R^2$</td>
<td>$K_1$</td>
<td>$R^2$</td>
<td>$K_H$</td>
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<td>TEI-1</td>
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<td>-0.103</td>
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<td>0.963</td>
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<td>0.959</td>
<td>36.23</td>
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<td>-0.18</td>
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<td>0.95</td>
<td>33.13</td>
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<td>0.806</td>
<td>41.1</td>
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<td>0.837</td>
<td>40.14</td>
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<td>11.02</td>
<td>0.944</td>
<td>-0.109</td>
<td>0.963</td>
<td>33.80</td>
</tr>
<tr>
<td>TFHM-3</td>
<td>12.31</td>
<td>0.976</td>
<td>-0.152</td>
<td>0.829</td>
<td>36.31</td>
</tr>
<tr>
<td>TFHM-4</td>
<td>10.89</td>
<td>0.986</td>
<td>-0.141</td>
<td>0.98</td>
<td>34.45</td>
</tr>
<tr>
<td>TFHM-5</td>
<td>12.48</td>
<td>0.989</td>
<td>-0.17</td>
<td>0.923</td>
<td>37.20</td>
</tr>
<tr>
<td>TFHM-6</td>
<td>12.77</td>
<td>0.906</td>
<td>-0.16</td>
<td>0.978</td>
<td>38.90</td>
</tr>
<tr>
<td>TFHM-7</td>
<td>10.33</td>
<td>0.801</td>
<td>-0.202</td>
<td>0.976</td>
<td>34.58</td>
</tr>
<tr>
<td>TFHM-8</td>
<td>11.65</td>
<td>0.969</td>
<td>-0.153</td>
<td>0.963</td>
<td>35.61</td>
</tr>
<tr>
<td>TFHM-9</td>
<td>13.33</td>
<td>0.963</td>
<td>-0.211</td>
<td>0.898</td>
<td>39.79</td>
</tr>
</tbody>
</table>
Additionally, for thin film hydration method, drug retarding ability increased from least to highest from formulation TTFH-1 (MDT= 0.603) to TTFM-5 (MDT=3.646) showed in Fig. 4-B. Overall MDT values showed that for high amount of phospholipids and cholesterol increased the retarding affinity of formulations for a certain level. After an optimum level, increase in amount of surfactant and cholesterol resulted in decreased drug retarding affinity because cholesterol starts to break the bilayer of the vesicle which has to be controlled by the amount of phospholipids [39,40].

3.7 Stability Studies of Liposomes

The intermediate stability study for different formulations was performed for 21 days according to the ICH guide lines where drug entrapment was fixed as physical parameter for the test. Liposomal formulations TEI-9 and TTFH-9 were selected for this study due to having highest tamoxifen entrapment efficiency (Table 4). From Table 6, it was revealed that the formulations were stable when stored at 4±2°C, 25±2°C and 37±2°C at 60± 5% RH. Throughout the study period (0 to 21 days), the selected formulations from MEIM and TFHM methods were stable due to the optimum amount of phosphatidylcholine and cholesterol in liposomes which stabilized the liposomal bilayer [41].

Table 6. Stability studies of liposomes

<table>
<thead>
<tr>
<th>Formulation code (MEIM)</th>
<th>TEI-9 (RH = 60 ± 5%)</th>
<th>TTFH-9 (RH = 60 ± 5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>4 ± 2°C</td>
<td>25 ± 2°C</td>
</tr>
<tr>
<td>7 days</td>
<td>86.83</td>
<td>86.73</td>
</tr>
<tr>
<td>14 days</td>
<td>86.13</td>
<td>85.96</td>
</tr>
<tr>
<td>21 days</td>
<td>85.75</td>
<td>84.87</td>
</tr>
</tbody>
</table>

3.8 Physical Appearance of Liposomes under Optical Microscopy

The morphology of all the liposomal formulations were determined by optical microscope (1000X) equipped with digital camera. These photomicrographs confirmed that the liposomes were spherical vesicles Fig. 5.
3.9 Comparison of Marketed Product with Liposomes

The comparison of cumulative % release of drug of an available marketed product of tamoxifen was performed with two of the formulated (TEI-9 and TTFH-9) liposomes which showed negligible deviation of drug entrapment efficacy and optimum stability during stability test entitled in Tables 5 and 6. Fig. 6 expressed that the prepared liposomes had a sustained release characteristics because after one hour percent release of marketed product was 85.56% whereas the formulated liposomes TEI-9 and TTFH-9 revealed 81.41% and 89.41% release respectively after 6 hours. From this observation it can be said that the prepared liposomes will release for a prolonged period of time and sustained release characteristics was obtained [42].
Total nine tamoxifen loaded controlled release liposome formulations were prepared by ether injection and thin film hydration method where ratio of phospholipids and cholesterol present in liposomes affect all the evaluation parameters significantly. *In vitro* dissolution study showed the controlled release of drugs from the liposomes for about 8 hours. It has been established that the drug dissolution profile could be slowed down by increasing the amount of phospholipids and cholesterol in the formulation. Optical images of different formulations also proved the vesicular...
structures of liposomes. Hence the prepared tamoxifen loaded liposomes might be a potential candidate for safe and effective controlled drug delivery system for the treatment of breast cancer. It may also reduce the risk of severe side effects of tamoxifen tablet.

CONSENT
It is not applicable.

ETHICAL APPROVAL
It is not applicable.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

REFERENCES


