PREPARATION AND CHARACTERIZATION OF CALCIUM PHOSPHATE COATED VINBLASTINE SULFATE LIPOSOMES

HARESH PATEL, HIREN PATEL, NITESH PATEL, Dr. AMIT GUPTA

Mahatma Gandhi college of Pharmaceutical sciences, Jaipur.

Abstract

Vinblastine sulfate loaded liposomes were prepared with an aim to improve stability, reduce drug leakage during systemic circulation, and increase intracellular uptake. Liposomes were prepared by the thin-film hydration method, followed by coating with calcium phosphate, using the sequential addition approach. Prepared formulations were characterized for size, zeta potential, drug-entrapment efficiency, morphology by transmission electron microscopy (TEM). Effect of formulation variables, such as drug: lipid ratio as well as nature and volume of hydration media, were found to affect drug entrapment. Size, zeta potential, and TEM images confirmed that the liposomes were effectively coated with calcium phosphate.

Accepted Date: 13/04/2013
Publish Date: 27/04/2013

Keywords
Liposomes, TEM,
INTRODUCTION

Various methods have been reported for preparation of liposomes. There are at least fourteen major reported methods (Ostro, 1987 and Martin, 1990). The difference between various methods of manufacture lies in the manner the membrane components are dispersed in aqueous media before being allowed to coalesce in the bilayer sheets form. From pharmaceutical point of view, the three most important aspects to be evaluated before selecting the method of preparation are the trapping efficiency, drug retention property and drug/lipid ratio (Betagiri et al., 1993).

Apart from above factors, other factors which need to be considered in selection of the methods for preparation of liposomes include choosing of methods which would avoid the use of organic solvents and detergents which are difficult to remove, yield well-defined and reproducible liposomes and which are rapid and feasible for scale up procedures. Selection of the appropriate method is also dependent on applications of the liposomes. TFH method was selected for the preparation of liposomes in this investigation due to non-tediousness and feasible at lab scale compared to other techniques.

Many lipid compositions can be employed for liposomal delivery system; however stability and cost are important determinants. The optimum drug/lipid ratio of liposomal formulation will likely be dictated by the biological efficacy and toxicity of the preparation. From a pharmaceutical point of view high drug/lipid ratios are obviously more economical.

In summary, optimum liposomal formulations will exhibit higher drug trapping efficiencies, employ inexpensive and relatively saturated lipids and cholesterol and using highest possible drug/lipid ratio results in consistent and maintained efficacy of the preparation. Thus, from the viewpoint of % entrapment, cost, availability and stability, Hydrogenated Soya Phosphatidylcholine (HSPC), Dipalmitoyl glycerophosphocholine (DPPC) negatively charged lipid Dipalmitoyl glycerophosphatic acid (DPPA) and cholesterol were used in this investigation.

Separation of unentrapped or unincorporated drug (Betagiri et al, 1993)
from liposomes can be achieved either by ‘gel filtration’ (Sephadex mini-column centrifugation), ultra centrifugation, Protamine aggregation, dialysis or controlled centrifugation at low speed. Gel filtration was found to be very tedious method with limited capacity and was not feasible for the entire formulation purification. Dialysis method was time consuming and it was observed that drug leaks during the dialysis period. Protamine aggregation was destructive approach and its use is restricted for the determination of the drug entrapment and could not be used for the separation of the liposomal dispersion.

In this investigation, SephadexG-50 gel chromatography has been used for separation of free drug so that separated liposomal suspension can be used for calcium phosphate coating while protamine aggregation was method used for determination of percent drug entrapment. Above mentioned methods have been selected due to feasibility at lab scale and non availability of higher speed ultracentrifuge (beyond 25000 rpm).

For the coating of liposome by calcium phosphate, three types of methods have been reported as given below:

1) Drop wise method relied on manual titrations of calcium and phosphate salts

2) One step super saturation method is performed by simultaneous addition of all ingredients to the reaction

3) Stepwise super saturation method is a hybrid of the two methods and utilizes the strengths of both methods.

The major challenges in coating of liposome with calcium phosphate involves control over size, thickness of coat and aggregation after aging. Stepwise super saturation method was selected for the coating of liposomes in this investigation because it requires convenient reaction condition, provides more accurate size control and reproducible results compared to other methods.

This chapter demonstrates the preparations of liposomes by TFH technique followed by coating with calcium phosphate using stepwise super saturation method. Prepared liposomes and calcium phosphate coated liposomes were characterized for size and size distribution, zeta potential, and percent drug Entrapment (PDE). The various optimization parameters are also discussed.
1. MATERIALS AND METHODS:

1.1 PREPARATION OF LIPOSOMES BY TFH:

Multilamellar vesicles comprising HSPC, DPPC, DPPA and CHOL with entrapped Vinblastine Sulfate (VBS) were prepared by Thin Film hydration (TFH) technique. Briefly, the lipids were dissolved in a mixture of chloroform and methanol in a 100 ml round bottom flask in different molar ratios. The solvent was evaporated in the rotary flask evaporator under vacuum. The thin dry lipid film thus formed was hydrated using different types of hydration media of different pH at 58 ± 3°C i.e. above phase transition temperature of lipid (Tg). The formed liposomal dispersion was sonicated in probe sonicator. The sonicated liposomes were then allowed to stand undisturbed for about 60 min, for annealing. Resultant Liposomes were subjected to centrifugation at 3,000 RPM, 4°C for 10 minutes using Remi centrifuge to remove unhydrated lipid, if any.

A flowchart depicting the process of preparation of liposome is shown below:

1.2 METHOD FOR SEPARATION OF UNENTRAPPED DRUG:

For separation of free drug and liposome ‘gel exclusion’ chromatography was followed as reported in literature (New R.R.C., 1990). Briefly, Sephadex G-50 column was prepared by soaking Sephadex G-50 into 0.15 M sodium chloride overnight. Then column of 2 cm was prepared by pouring sephadex G-50 slurry into a 2 ml syringe. The syringe was put into 10ml centrifuge tube and centrifuged at 1000 rpm for 10 min. to remove excess solvent in Remi cooling
centrifuge. The column was pre-equilibrated with 0.15M sodium chloride by three consecutive passes and each time centrifuged to remove excess 0.15M sodium chloride. Then 1ml liposomal suspension was applied at top of column and elute was collected which contains vinblastine loaded liposome while the free drug was retained into the column.

Thus separated liposomal suspension was then characterized for vesicle size, zeta potential and percent drug entrapment (PDE), optimized batch was coated with calcium phosphate.

1.3 ESTIMATION OF VINBLASTINE IN LIPOSOMES:

The vinblastine sulphate loaded liposomes were subjected to Protamine Aggregation method for determination of free drug and entrapped drug.

PROTAMINE AGGREGATION METHOD
(New R.R.C., 1990)

(1) 0.5 ml of liposomal suspension was placed in 2 ml centrifuge tube.

(2) To the liposomal suspension 0.5 ml of Protamine solution (10 mg/ml, in distilled water) was added. Mixed it on vortex mixer and allowed to stand for 10 minutes at room temperature.

(3) Then it was centrifuged at 3000 rpm for 10 min using Remi Centrifuge at room temperature.

(4) The supernatant I was collected, diluted 20 times with distilled water, absorbance was measured at 296.8 nm using distilled water as blank on UV-Visible Spectrophotometer (UV-1601, Shimadzu). The amount present in supernatant was calculated using regression equation of VBS in distilled water.

(5) The sediment (pellet) was dissolved in 2 ml Methanol by vortexing vigorously to break liposome and then centrifuged at 3000 rpm for 10 minutes. Then supernatant II (containing extracted drug from liposome) was 20 times diluted with methanol, and absorbance was taken at the wavelength of 297.3 nm using methanol as blank on UV-Visible Spectrophotometer (UV-1601, Shimadzu) to find out percentage drug entrapment.

(6) The mass balance was established to ensure accuracy of determination.

1.4 PROCEDURE OF COATING:
Coating of liposome with calcium phosphate (Ca-Pi) was carried out by the following method (Schmidt and Ostafin, 2002). 1ml of liposomal suspension was diluted to 10 ml with distilled water in 50 ml two necked round bottom flask. The pH was maintained and continuously monitored after adjustment with NaOH to 7.8-8.0 throughout the process using pH meter. 100 µl of different millimolar concentration of Ca$^{2+}$ precursor (Calcium chloride) and Po$_4^{-3}$ precursor (Potassium dihydrogen phosphate) were added alternatively using micropipette keeping in mind that ratio of Ca$^{2+}$ to Po$_4^{-3}$ remains 1.67 which corresponds to hydroxyapatite coating (kemenade and bruyan, 1987). The procedure was carried out at room temperature.

After the addition of both solutions, calcium phosphate coated suspension was allowed to stir for 2 hours and finally kept aside overnight (12 hrs.) for effective aging of calcium phosphate. The CPCL were passed through the 0.45 µm polycarbonate filters to remove calcium phosphate crystal and larger aggregates of coated liposome while allowing monodispersed coated liposome to pass through filter.

A flowchart depicting the process of coating of liposome is shown below:

1.5 Characterization

The liposomes and coated liposomes were characterized for the following physico-chemical properties.

2.5.1 Size and size distribution

The size of Liposomes and coated liposomes was measured by dynamic light scattering with a Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). Diluted liposome suspension was added to the sample cuvette and then cuvette is place in zetasizer. Sample is stabilized for two minutes and reading was measured. The average particle size
was measured after performing the experiment in triplicate.

2.5.2 Zeta Potential

The zeta potential of developed liposomes and coated liposomes was determined using Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The zeta potential was calculated by Smoluchowski’s equation from the electrophoretic mobility of liposomes at 25 °C (Mu and Feng 2001).

2.5.3 Microscopy

(a) Optical microscopy:

Prepared liposomal suspension (MLVs) was observed under Olympus microscope (Olympus Optical Co. Ltd., Japan) to study the shape and morphology of the liposomes.

(b) TEM microscopy:

1-2 µl drop of shaken solution was placed in the center of carbon coated 300-mesh copper grids with a formvar support grid and allowed to air dry. The sample was visualized using TEM microscope operated at 200kV and image was obtained on Kodak electron microscopy film.

2.5.4 Percent Drug Entrapment

PDE is expressed as:

\[
PDE = \frac{\text{drug entrapped}}{\text{drug entrapped} + \text{free drug}} \times 100
\]

- Separation of Free Drug From Drug Entrapped In Liposomes

Protamine aggregation method was used for separation of free drug from liposomes. In this method 1 ml of liposomes was transferred in micro centrifuge tube and to that 1 ml of Protamine sulphate solution (10 mg/ml in distilled water) was added. The micro centrifuge tube was kept for 3 minutes at room temperature then centrifuged at 3000 rpm for 10 minutes. Liposomes settled down and formed cake at the bottom of the centrifugation tube. By this way we can separate unentrapped drug from the formulation.

- Analysis of Sediment For Drug Content

Sediment was obtained by centrifugation at 3000 rpm for 10 minutes. The sediment was dissolved in methanol and transferred to 10 ml volumetric flask and volume made up to 10 ml with methanol.
The content was then analyzed for VBS content using UV-spectrophotometric Method.

2. RESULTS AND DISCUSSION:

Liposomes of VBS were prepared by the selected TFH method and are optimized to achieve maximum PDE and desired size range. Optimized liposomal suspension was subjected to coating with calcium phosphate using step by step synthesis method. First of all, the optimization and selection of various process and formulation variables were carried out followed by the selection of suitable method for the particle size reduction and separation of un-entrapped drug. The results are summarized and discussed in the following sections.

3.1 Optimization of method

Optimization of the method has been achieved by optimizing variables of the process as well as of the formulation.

(a) Optimization of Process Parameters

The various process parameters studied for optimization of the method were:

- Hydration time
- Sonication

(b) Optimization of formulation parameters

The various Formulation parameters studied for optimization of the method were:

- Solvent system

Hydration time

An optimal hydration time is required for complete conversion of planner bilayers to spherical liposomes. The lipid film was hydrated from 30 minutes to 90 minutes before size reduction. Lower hydration time led to a non-uniform shape and size of the liposomes and also the unhydrated part posed difficulty in size reduction. The hydration time beyond 75 min. resulted in no further improvement. Hence, 75 min. hydration time was found to be optimum.

Sonication

Sonication is required to convert the micro-ranged liposomal suspension into nano range. Sonication for 1 min. was not able to convert liposomes into nano size range while sonication for 3 to 4 minutes resulted in lesser entrapment possibly due to drug leakage from the liposomes. So optimum time considered was 2 min (30 seconds x 4 times).
Solvent system

The solvent system composition should be such that it prevents precipitation of formulation components during solvent stripping process. The organic solvent system of chloroform: methanol (3:1) was used for dissolving the formulation components like HSPC, DPPC, CHOL, DPPA and drug for preparation of liposomes.

Drug : Lipid

Increase in the lipid proportion relative to drug led to the increase in the drug entrapment from 10.69±0.85 % to 39.83 ± 2.32%, with increase in quantity of lipids, more number of liposomes per ml of the liposomal dispersion was formed, resulting into increased drug entrapment (Schneider et al, 1994). Beyond 1:30 drug to lipid ratio, not much larger increment in entrapment was noted. Hence, drug: lipid ratio of 1:30 was selected.

Hydration Media

Three different types of hydration media were used for liposomal vesicle formation but distilled water was found to be optimum hydration media due to higher entrapment of VBS compared citrate buffer and PBS. Lesser PDE in case of PBS is probably due to improper hydration of phospholipids while slight precipitation of phospholipids results in case of Acetate buffer.

Drug to lipid ratio (1:30), Phospholipids ratio (7:4)

3.2 Optimization of Calcium chloride concentration

Optimization of calcium chloride concentration for effective coating purpose, was carried out with respect to vesicles size and zeta potential of coated liposomes. During the coating process, it was found that above 50mM concentration of calcium chloride used for coating results in aggregation after overnight as well as size above 200 nm. Hence 50mM calcium chloride was optimized with respect to desired size, zeta and stability after overnight.

Comparative study of uncoated and coated liposome with respect to size, and zeta potential indicates that calcium phosphate coating has taken place. Before coating the size and zeta was 116nm and -43.1 mV while after coating size increased, up to 160.3nm and zeta...
decreased to -3.31 mV. TEM image further confirm that coating has been done.

3.3 Liposome Characterization

3.3.1 Liposome size and Zeta Potential

Liposome prior to sonication had a greater mean size and broader size distribution, however, upon sonication it acquired a narrower range of distribution and a mean liposomal size is below 150 nm. Zeta potential was found to be -43.1mV.

Fig.4: Liposomal Zeta Potential

3.3.2 Optical Microscopy

Distinct Liposomal images were viewed into the OLYMPUS microscope. Below given images were viewed under 40 X magnification.

Fig. 5: Optical Microscopy of MLV’s prepared by TFH

3.3.3 Percent drug entrapment:

The mean PDE obtained during the optimization of liposomal VBS by THF method were reported in Table 8. Batches with higher PDE were selected for the coating process.
3.4.1 Coated Liposome size and Zeta Potential:

Liposome prior to coating had a lesser mean size and narrow size distribution however, upon coating it acquired a comparatively broader range of distribution and a mean liposomal size is above 150 nm. Reduction in Zeta potential was found most probably due to neutralization of negative surface charge present on liposome by calcium attachment. After coating, the zeta potential was found to be -3.1mV. This indicates the possibility of aggregation of particles in dispersed state. However the dispersion is further converted into a dry powder form by lyophilization to increase its stability.

Fig. 6: Liposomal size after coating

3.4.2 TEM Analysis:

TEM image indicate that both thin and thick coating of liposomes have taken place.

Fig. 7: Zeta Potential after coating

Fig. 8: TEM image of coated liposomes

Table 1: Effect of Hydration time
### Table 2: Effect of Sonication

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time(min.)</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydration time</td>
<td>30</td>
<td>Poor hydration</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>Improper hydration</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>Proper hydration</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>No further improvement</td>
</tr>
</tbody>
</table>

### Table 3: Effect of Solvent system

<table>
<thead>
<tr>
<th>Solvent system selected</th>
<th>Ratio</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform: MeOH</td>
<td>1:1</td>
<td>Incomplete removal of lipid film</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>Incomplete removal of lipid film</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>Improper hydration</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>Suitable removal of lipid film</td>
</tr>
</tbody>
</table>
**Table 4: Effect of Drug: Lipid ratio**

<table>
<thead>
<tr>
<th>SR NO.</th>
<th>DRUG:LIPID</th>
<th>PDE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:10</td>
<td>10.69±0.85</td>
</tr>
<tr>
<td>2</td>
<td>1:20</td>
<td>22.14±3.12</td>
</tr>
<tr>
<td>3</td>
<td>1:30</td>
<td>39.83±2.32</td>
</tr>
<tr>
<td>4</td>
<td>1:40</td>
<td>36.50±1.95</td>
</tr>
</tbody>
</table>

*Mean ± SEM (n = 3)

Phospholipids ratio (7:4), Hydration media (Distilled water)

**Table 5: Effect of Hydration Media**

<table>
<thead>
<tr>
<th>SR NO</th>
<th>HYDRATION MEDIA</th>
<th>PDE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Citrate buffer 4.0</td>
<td>20.25±0.63</td>
</tr>
<tr>
<td>2</td>
<td>PBS 7.4</td>
<td>32.75±1.57</td>
</tr>
<tr>
<td>3</td>
<td>Distilled water</td>
<td>40.30±1.58</td>
</tr>
</tbody>
</table>

*Mean ± SEM (n = 3)

**Table 6: Optimization of calcium concentration**

<table>
<thead>
<tr>
<th>Calcium conc. (mM)</th>
<th>Avg. size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Polydispersivity index (PDI)</th>
<th>Inference (after over night)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>294.3</td>
<td>+12.5</td>
<td>0.378</td>
<td>Aggregated</td>
</tr>
<tr>
<td>100</td>
<td>267.5</td>
<td>+9.5</td>
<td>0.316</td>
<td>Aggregated</td>
</tr>
<tr>
<td>75</td>
<td>193.7</td>
<td>+5.4</td>
<td>0.289</td>
<td>Aggregated</td>
</tr>
<tr>
<td>50</td>
<td>160.3</td>
<td>-3.31</td>
<td>0.233</td>
<td>Stable</td>
</tr>
<tr>
<td>30</td>
<td>135.4</td>
<td>-19.8</td>
<td>0.227</td>
<td>Stable</td>
</tr>
</tbody>
</table>
Table 7: Comparative study of uncoated and coated liposomes

<table>
<thead>
<tr>
<th>Optimized batch</th>
<th>Avg. size (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
<th>PDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome</td>
<td>116.4</td>
<td>-43.1</td>
<td>0.185</td>
<td>39.56±1.15</td>
</tr>
<tr>
<td>Calcium phosphate coated liposome</td>
<td>160.3</td>
<td>-3.31</td>
<td>0.233</td>
<td>37.87±0.78</td>
</tr>
</tbody>
</table>

Table 8: Characterization of Liposomal suspension prepared by TFH

<table>
<thead>
<tr>
<th>Characterization parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Drug Entrapment</td>
<td>39.56 ± 1.15</td>
</tr>
<tr>
<td>Particle size (PDI)</td>
<td>116.4nm (0.185)</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-43.1 mV</td>
</tr>
</tbody>
</table>
3. REFERENCES:


10. Hope M.J., Cullis P.R., Bally M.B., Madden T.D., Mayer L.D., Janoff A.S. and Ostro M.J. in “Liposomes from Biophysics to Therapeutics” NY, Marcel Dekker, 1990; 39


19. Martin, F.J., Pharmaceutical Manufacturing of Liposomes, in


