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### DEVELOPMENT OF STABILIZED CALCIUM PHOSPHATE COATED LIPOSOME

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**Abstract:** Multilamellar vesicles comprising HSPC, DPPC, DPPA and CHOL with entrapped Vinblastine Sulfate (VBS) were prepared by Thin Film hydration (TFH) technique. Multilamellar vesicles were converted to Unilamellar using Probe sonication technique. To improve stability, reduce drug leakage during systemic circulation, and increase intracellular uptake calcium phosphate coating was done. The problem of instability is one of the limiting factors in commercial development of liposome products. Nevertheless, limited success was attained to improve the long-term retention of encapsulated solutes by freeze-drying. Freeze-drying is often used to stabilize various pharmaceutical products including viral vaccines, protein and peptide formulations, liposome, and small chemical drug formulations. Cryoprotection is needed during lyophilization and sugars such as sucrose, lactose, and trehalose are used to protect the liposomes during freezing stage of lyophilization cycle.

**Keywords:** Multilamellar vesicles, Thin Film Hydration.

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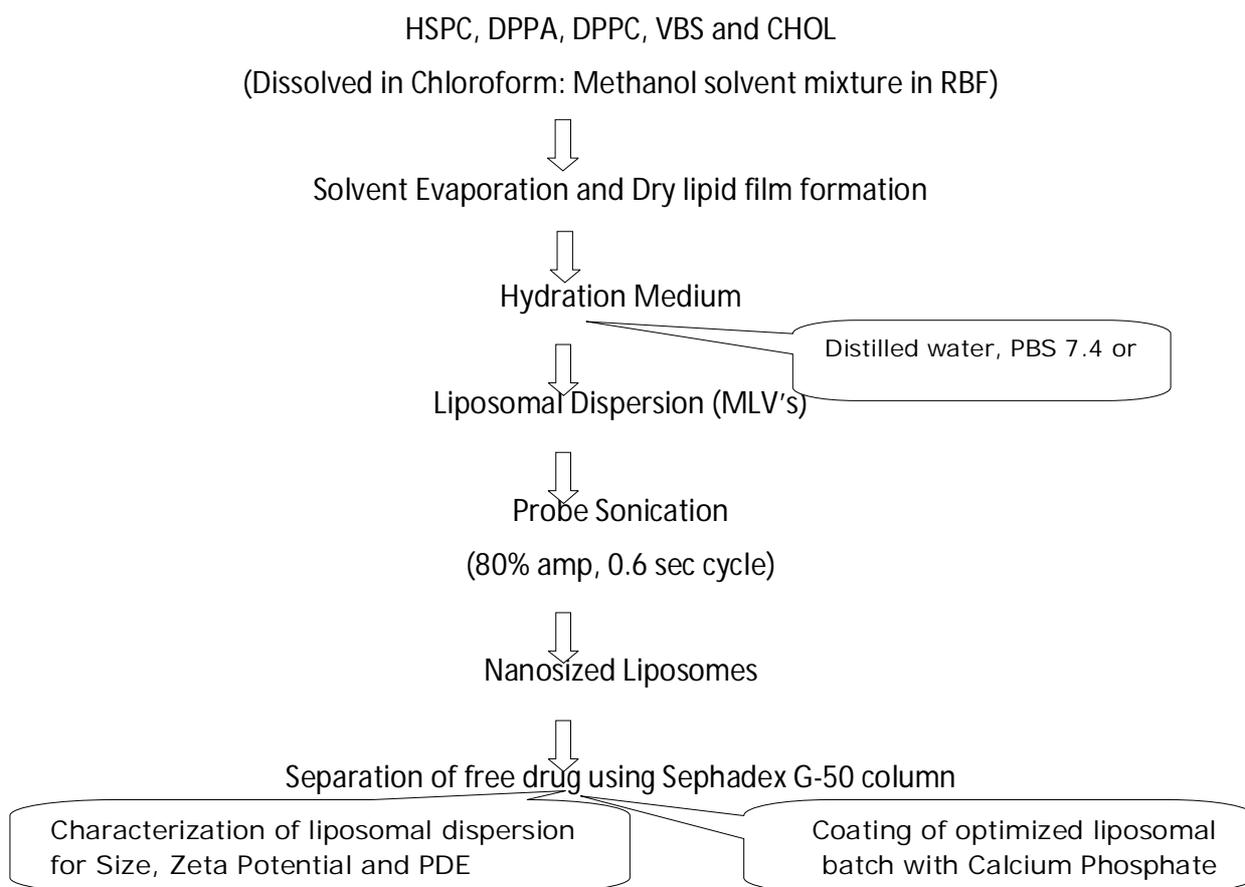
**INTRODUCTION**

**FORMULATION PARAMETERS:**

**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  $58 \pm 3^\circ\text{C}$  i.e. above phase transition temperature of lipid ( $T_g$ ). 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,  $4^\circ\text{C}$  for 10 minutes using Remi centrifuge to remove unhydrated lipid.

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



**Fig 1 Flow chart showing an overview of formulation development**

### **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.

### **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.

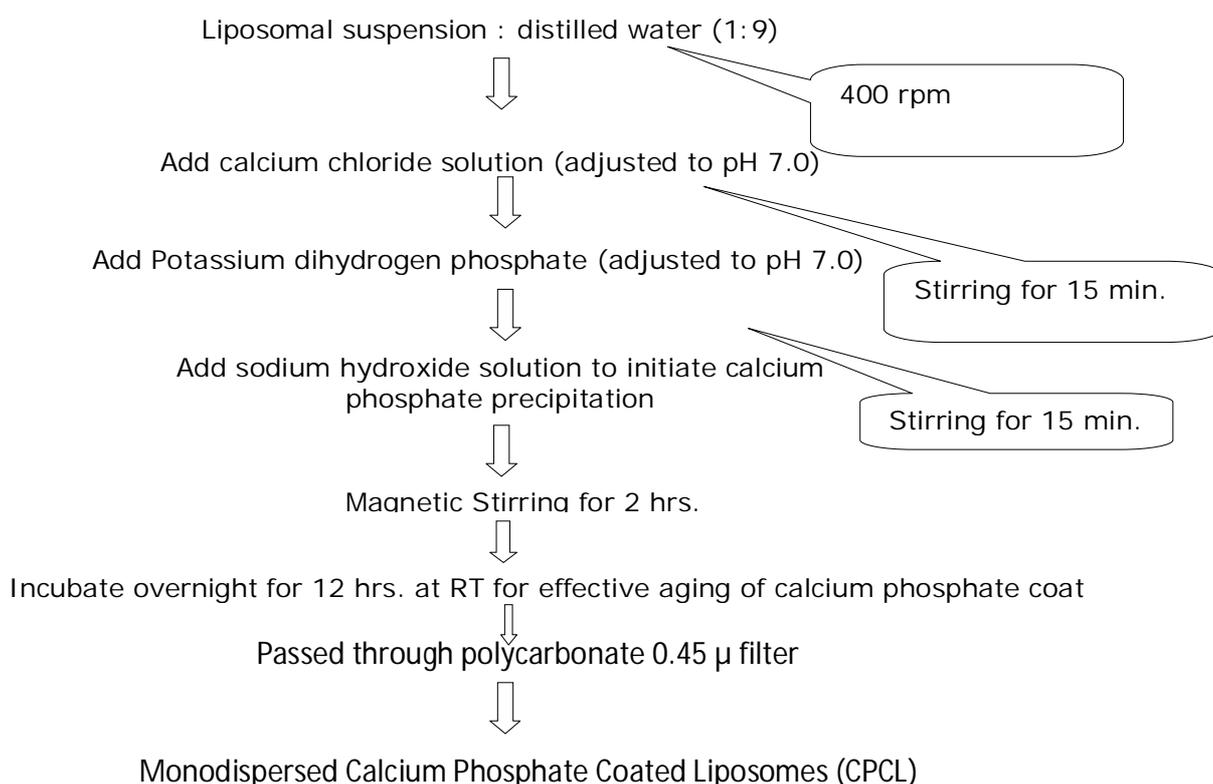
### **PROCEDURE OF COATING**

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  $\mu$ l of different millimolar concentration of  $\text{Ca}^{+2}$  precursor(Calcium chloride) and  $\text{Po}_4^{-3}$  precursor (Potassium dihydrogen phosphate) were added alternatively using micropipette keeping in mind that ratio of  $\text{Ca}^{+2}$  to  $\text{Po}_4^{-3}$  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  $\mu$ m 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:



**Fig 2. Flow chart for coating of liposomes by step by step method**

## CHARACTERIZATION

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

### Liposome size, size distribution and zeta potential

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, size distribution and zeta potential was measured after performing the experiment in triplicate.

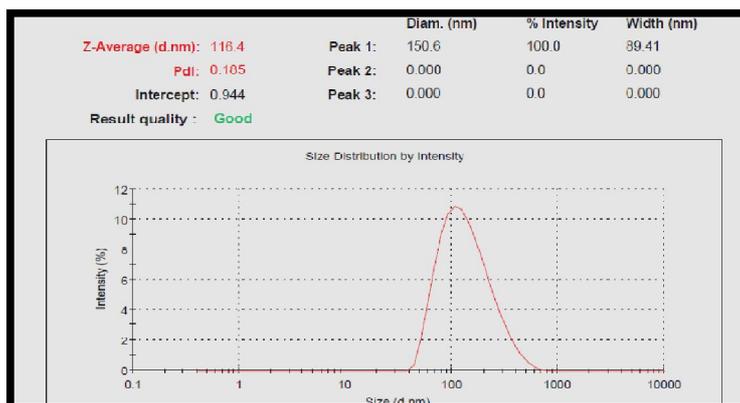


Fig 3. Liposomal size of uncoated Liposome



Fig.4. Liposomal size of coated Liposome

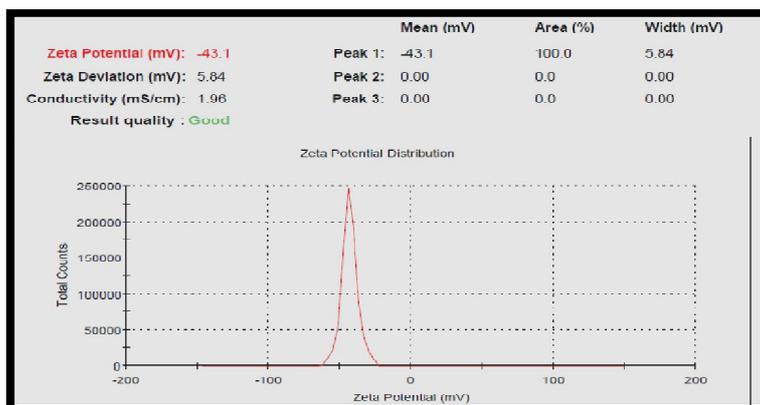


Fig 5. Liposomal Zeta Potential of uncoated Liposome

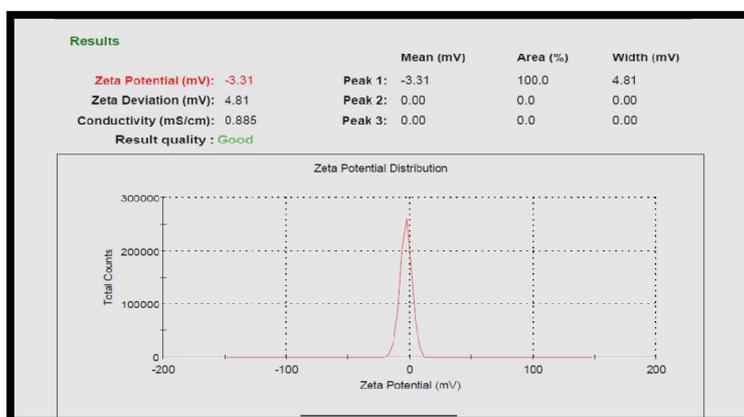


Fig 6. Zeta Potential of coated Liposome

### 1.5.2 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.

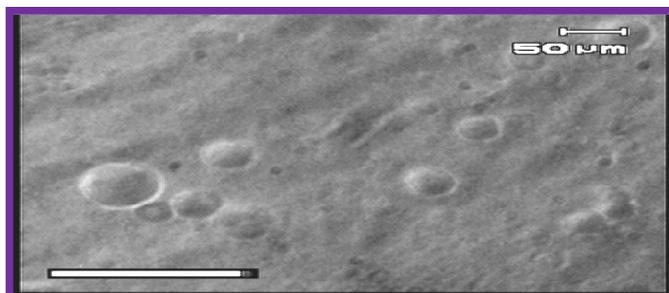
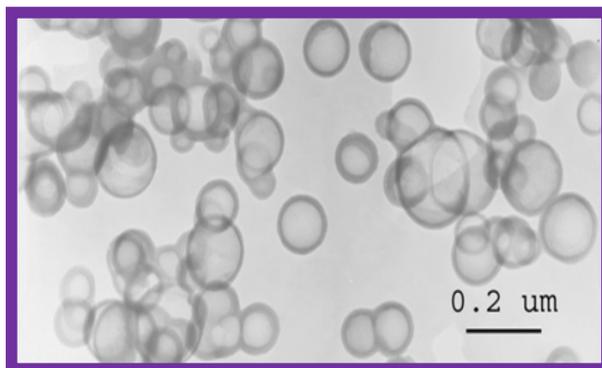


Fig 7. Optical Microscopy of MLV's prepared by TFH

**(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.



**Figure 8 TEM image of coated liposomes**

**Table 1 Comparative result of uncoated and coated liposomes**

Optimized batch	Avg. size (nm)	Zeta potential (mV)	PDI	PDE
Liposome	116.4	-43.1	0.185	39.56±1.15
Calcium phosphate coated liposome	160.3	-3.31	0.233	37.87±0.78

**LYOPHILIZATION:**

Freeze-drying is often used to stabilize various pharmaceutical products including viral vaccines, protein and peptide formulations, liposome, and small chemical drug formulations. Cryoprotection is needed during Lyophilization and sugars such as sucrose, lactose, and trehalose are used to protect the liposomes during freezing stage of Lyophilization cycle. Upon rehydration water molecules quickly replace the sugars and liposomes appear to reseal before significant leakage occurs. Lyophilization also makes the product sterile thus there no need to add the antimicrobial agent to the product going for the Lyophilization. It has been proposed that sugars preserve membrane structure (Cryoprotection) by hydrogen bonding to the phospholipids' head group and effectively replacing the bound water. Sugars when added to the liposome dispersion form a glassy matrix during freezing. This prevents fusion of the vesicles and provides protection against ice formation.

An important feature of phospholipids' membrane is the existence of temperature-dependent reversible phase transition, where the hydrocarbon chains of the phospholipids undergo a transition from an ordered (gel) to a more disordered fluid (liquid crystalline) state. The physical state of the bilayer affects the permeability, leakage, and overall stability of the liposomes. Freeze-dried phospholipids material, and dry phospholipids' films absorb water and swell. The degree of water absorption is a function of the hydrophilic moiety in the phospholipids' head group and the composition of the hydrocarbon chain. Dry lipids are in gel phase at temperatures at which they would be in liquid crystalline phase if they were hydrated.

## MATERIALS AND REAGENTS

**Table 2 List of Materials and Reagents with their Source**

COMPOUNDS	SOURCE
VBS loaded Liposomal suspension (Drug: Lipid 1:30), (HSPC:DPPC:CHOL:DPPA- 7:4:1:1)	Prepared in Laboratory
Calcium Phosphate Coated Liposome(CPCL)	Prepared in Laboratory
Trehalose	Spectrochem, India.

## EQUIPMENT AND APPARATUS

**Table 3 List of Equipments and Apparatus with their Source**

EQUIPMENT/APPARATUS	SOURCE
USP Type I Glass Vials	Borosil Glasswares, India.
Digital Analytical Balance	Schimdu SCS, Switzerland.
Deep Feezer	Amancio Lab, Mumbai.
Heto Freeze-dry system	Hetodry, Denmark.
UV-Visible Spectrophotometer	Shimadzu UV-160, Japan
Malvern zeta sizer (Nano ZS)	Zeta Sizer, Malvern, UK.

## PROCEDURE OF LYOPHILIZATION

Freeze drying technique was used for stabilization and to prevent the leakage of entrapped Vinblastine Sulphate (VBS). Optimized Batch of VBS loaded Liposomal suspension composed of (Drug: Lipid 1:30), (HSPC: DPPC: CHOL: DPPA- 7:4:1:1) and optimized calcium phosphate coated

liposome (CPCL) were freeze dried using trehalose as cryoprotectant to preserve the vesicular size and shape, hence the PDE. In selected uncoated liposomal and coated liposomal suspension of VBS batch containing the total lipids equivalent to 120 mg, 360 mg (3 times) and 600 mg (5 times) of trehalose cryoprotectant were dissolved. This uncoated liposomal and coated liposomal dispersion then subjected to two stage of freeze drying.

1. The resultant dispersion was deep freezed at  $-70^{\circ}\text{C}$  for 24 hr in Deep Freezer, to form dry ice cake.
2. The formed dry ice cake containing vials transferred to the Heto Freeze-dry system (Heto dry, Denmark) and lyophilized at the  $-70^{\circ}\text{C}$  and for 24 hours.

The porous cake thus formed was sized successively through #120, Size and PDR of freeze dried uncoated and coated liposomes were determined following dehydration-rehydration cycle. Thus effect of lyophilization on vesicle size and PDR was studied.

## CHARACTERIZATION

### Percent Drug Retained

Percent drug retained (PDR) is the percentage of drug retained in the liposomes after lyophilization cycle or in stability samples to initially drug entrapped in the liposomal dispersion. 10 mg of powder was rehydrated with 1ml of distilled water with gentle, occasional agitation for 30 minutes. The liposomal dispersion thus obtained was separated from the drug leaked during lyophilization cycle by sephadexG-50 gel chromatography method.

### Size of rehydrated liposomal vesicles

The vesicle size of rehydrated liposomes was determined by laser diffraction using Malvern Zeta sizer Nano ZS (Malvern, UK.). The Liposomal formulations were rehydrated and diluted with distilled water. Dispersion was filled in sample cuvette and analyzed for vesicle size and zeta potential.

The effect of trehalose (cryoprotectant) on PDR and vesicle size shown in **Table 4**.

The mean percent drug remained entrapped with its standard error of mean, for three observations were determined.

## RESULTS AND DISCUSSION

Effective cryoprotectant would be one that provide the dry free flowing powder of the liposomal formulation with easily resuspendable formulation with higher percent drug retained and lesser increase in size. Effect of Trehalose (cryoprotectant) on uncoated and coated liposomal formulation during lyophilisation is shown below:

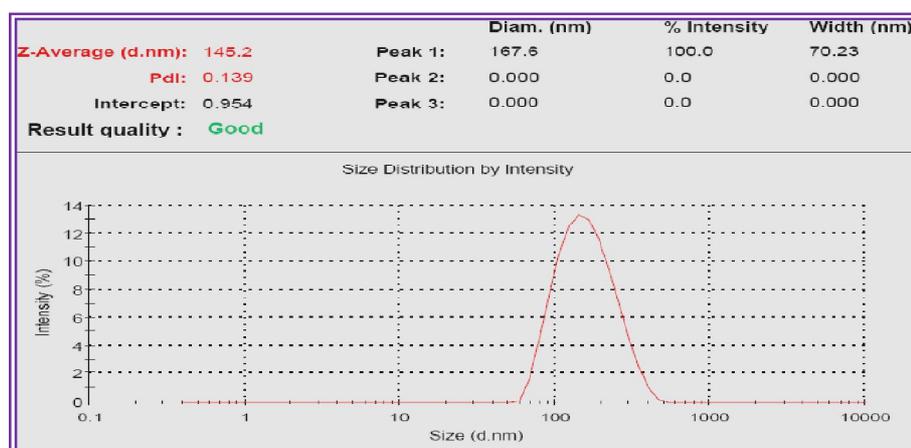
**Table 4. Influence of Cryoprotectants on freeze dried Liposomes.**

BEFORE LYOPHILIZATION			
Type of formulation		Size(nm)*	% Drug content*
Uncoated liposomes		116.4±4.78 nm	39.56 ± 1.15
Calcium phosphate liposome(CPCL)	Coated	160.3±5.98 nm	37.87 ± 0.78
AFTER DEHYDRATION-REHYDRATION			
Type of formulation	Trehalose to total lipid ratio (w/w)	Size(nm)*	% Drug retained (PDR)*
Uncoated liposomes	3 times	145.2±1.8 nm	38.45±0.69
	5 times	167.5±2.8 nm	36.42±0.80
Calcium phosphate coated liposome(CPCL)	3 times	196.9±4.5 nm	35.14±0.95
	5 times	233.7±3.8 nm	33.73±0.35

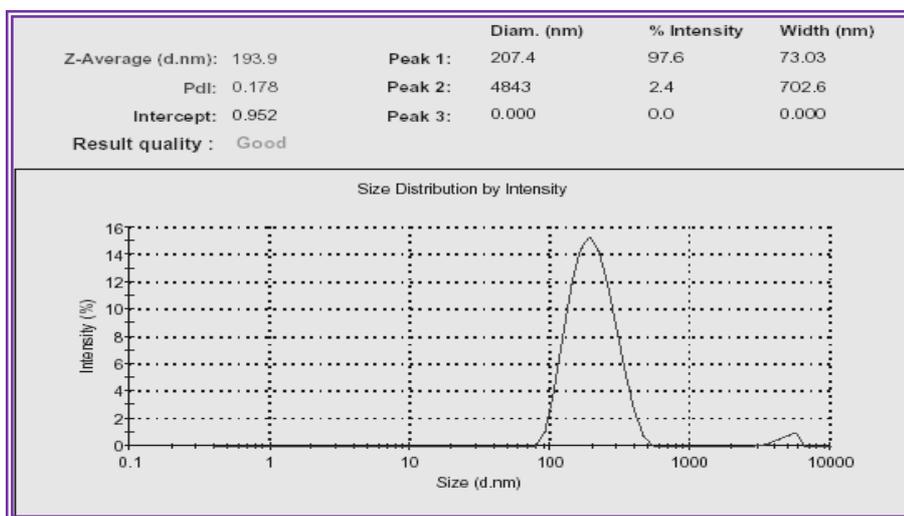
\*Mean ± SEM (n = 3)

From the above observation table, it is clear that :

- ❖ Trehalose (3times) to total lipid ratio, show higher PDR compared to Trehalose (5times) to total lipid ratio for both uncoated (38.45±0.69) and coated (35.14±0.95) liposomal formulation.
- ❖ Trehalose (3 times) to total lipid ratio, show not so much increase in vesicle size for both uncoated (145.2±1.8 nm) and coated liposomal (196.9±4.5 nm) formulation compared to 5 times ratio.



**Fig 9 Vesicle size of UNCOATED LIPOSOME after Lyophilization**



**Fig 10 Vesicle Size of CPCL after Lyophilization**

Although there is no significant difference for both uncoated and coated liposomal formulations with respect to PDR using either 3 times or 5 times Trehalose to total lipid ratio but there is significant difference with respect to size using 3times and 5 times ratio. Hence we can conclude that Trehalose (3 times) to total lipid ratio is optimum for both uncoated and coated liposomal formulation.

**ANALYTICAL PARAMETERS:**

**ANALYTICAL METHOD DEVELOPMENT:**

**Preparation of Buffer (IP 1996)**

Phosphate Buffer pH 7.4

50.0 ml of 0.2 M potassium dihydrogen phosphate was taken in 200 ml volumetric flask, to it 39.1 ml of 0.2 M sodium hydroxide was added, mixed well and volume was made up to the mark with distilled water.

Acetate Buffer pH 4.5:

Dissolve 5.4 g of sodium acetate in 50 ml of distilled water, add 2.4 ml of glacial acetic acid and dilute with distilled water to 100 ml. Adjust the pH, if necessary.

**Preparation of stock solution**

5 mg accurately weighed quantity of VBS was transferred to 50 ml volumetric flasks containing the small volume of respective solvent or buffer in which absorption maxima have to be determined. The volume was made up to the mark with respective solvent or buffer. Thus 100 µg/ml stock solution of VBS was prepared in respective solvent or buffer.

### Determination of Absorption maxima ( $\lambda_{\max}$ )

The absorption maximum ( $\lambda_{\max}$ ) in various solvent or buffer systems, was determined by scanning 10  $\mu\text{g/ml}$  solutions of VBS using respective systems as blank on UV-Visible Spectrophotometer (UV-1601, Shimadzu).

### Construction of Calibration plot

From the above stock solutions, aliquots of 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 ml were accurately withdrawn with the help of pipette and transferred to 10ml volumetric flasks and the volume was made up to the mark with respective solvent or buffer to give the final concentration of 10, 15, 20, 25, 30, 35  $\mu\text{g/ml}$  of VBS. Thus prepared test solutions of VBS in various solvents or buffers were used for absorption maxima and calibration plot in respective solvent or buffer systems.

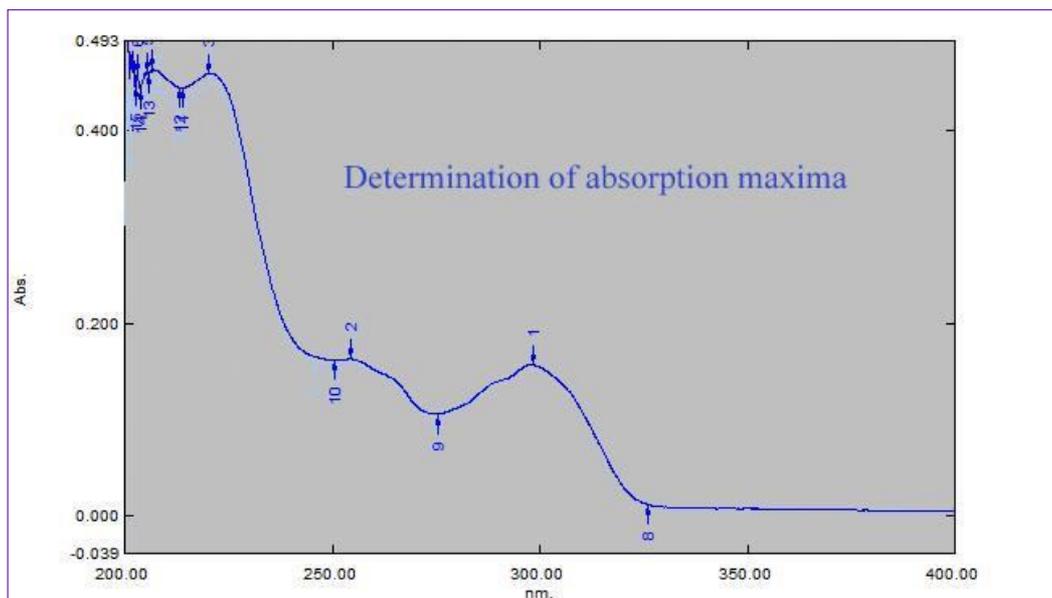
The absorbance of all the prepared solutions was then measured at the absorbance maxima (which was previously determined in respective system) using respective solvent or buffer system as blank. The readings were recorded in triplicate. Mean value ( $n=3$ ) along with the standard error of mean (SEM) were recorded. The regressed values of absorbance were plotted graphically against the concentrations. Regression coefficient ( $R^2$ ) and the regression equation were determined in respective systems.

### RESULT AND DISCUSSION

The absorption maximum ( $\lambda_{\max}$ ) in various solvents or buffer systems is found to be approximately 297 nm which is reported in IP 1996.

Medium	$\lambda_{\max}$
Methanol	297.3 nm
Distilled water	296.8 nm
PBS pH 7.4	296.6 nm
Acetate Buffer pH 4.5	297.5 nm

Table 5  $\lambda_{\max}$  of VBS in various solvent or buffer systems



**Fig 11 UV spectrum  $\lambda$  max determination in Methanol**

Vinblastine was estimated UV spectrophotometrically at 297.3 nm in methanol and at 296.8 nm in distilled water. Absorbance values in respective solvent system is shown below-

**Table 6 Data for calibration curve of VBS in Methanol and D.W.**

Medium	Range	Regression equation	Regression Coefficient ( $R^2$ )
<b>Methanol</b>	10-35 $\mu\text{g/ml}$	$Y = 0.016x - 0.014$	0.998
<b>Distilled water</b>	10-35 $\mu\text{g/ml}$	$Y = 0.016x - 0.022$	0.998

**Table 7 Optical characteristics of VBS**

Concentration ( $\mu\text{g/ml}$ )	* Mean Absorbance $\pm$ S.E.M.	
	In methanol	In distilled water
<b>10</b>	$0.155 \pm 0.0011$	$0.144 \pm 0.0015$
<b>15</b>	$0.240 \pm 0.0034$	$0.224 \pm 0.0025$
<b>20</b>	$0.326 \pm 0.0045$	$0.292 \pm 0.0090$
<b>25</b>	$0.401 \pm 0.0059$	$0.392 \pm 0.0138$
<b>30</b>	$0.482 \pm 0.0078$	$0.470 \pm 0.0094$
<b>35</b>	$0.585 \pm 0.0096$	$0.547 \pm 0.012$

\* n = 03

The  $R^2$  value for calibration curve of Vinblastine sulphate was found to be 0.998 in methanol as well as in distilled water. This indicates the linearity of analytical method in the concentration range  $10\mu\text{g/ml}$ - $35\mu\text{g/ml}$ .

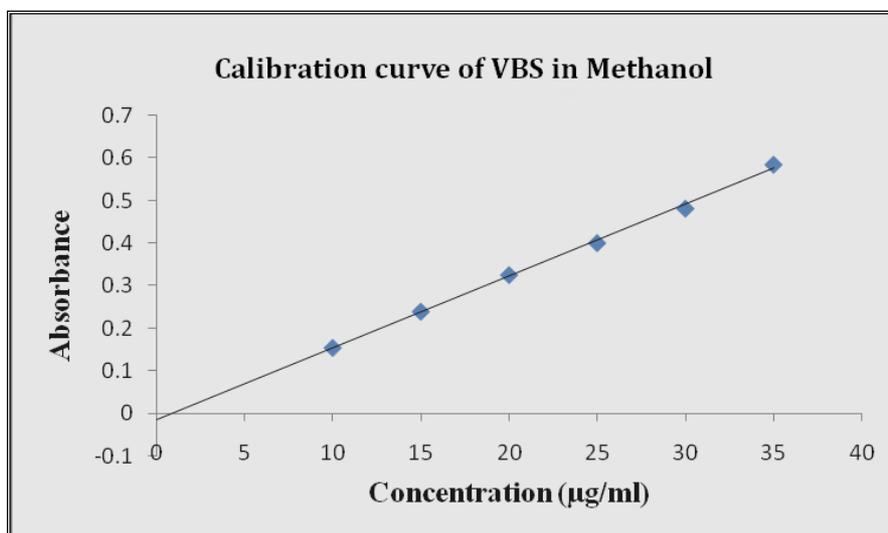


Fig 12 Calibration curve of VBS in Methanol

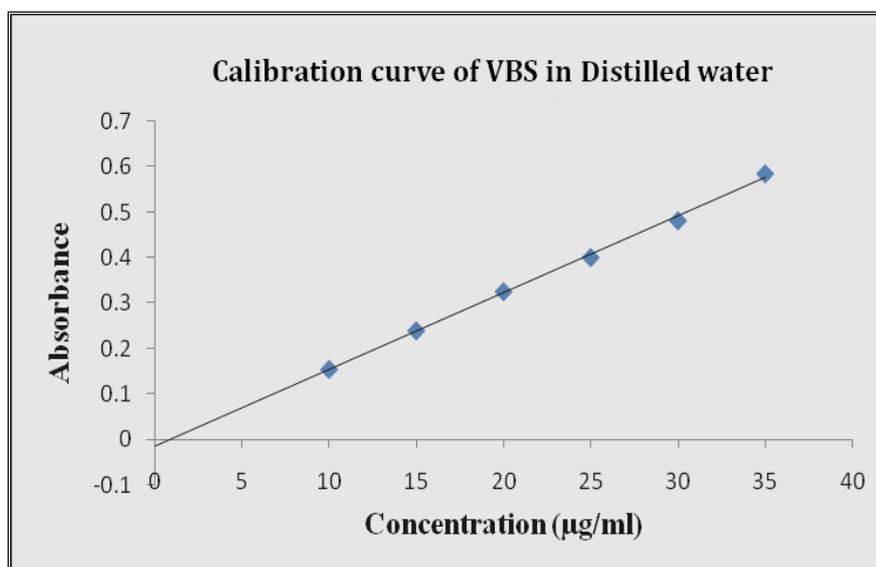


Fig 13 Calibration curve of VBS in Distilled water

For Diffusion studies the samples of the diffusion media was analyzed at 296.6 nm and 297.5 nm using PBS pH7.4 and Acetate Buffer pH4.5 as blank respectively. Absorbance values in respective solvent system is shown below-

**Table 8 Data for calibration curve of VBS in PBS 7.4 and Acetate buffer 4.5**

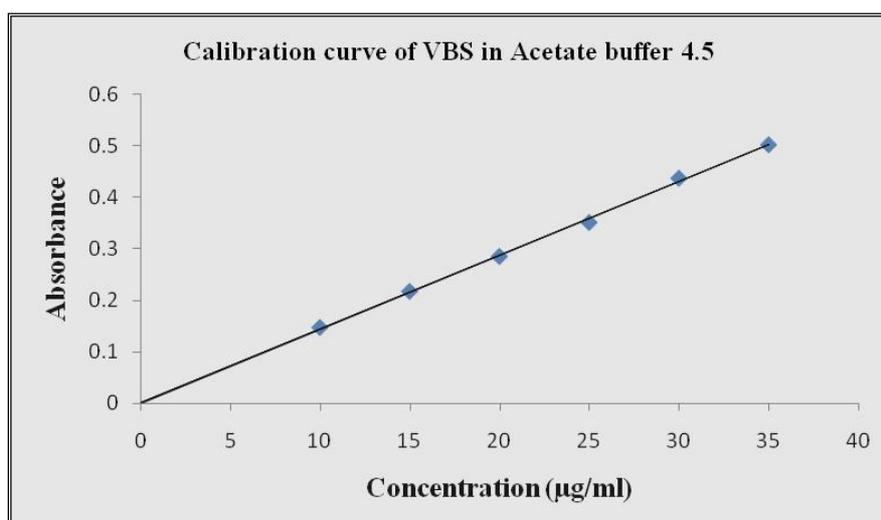
Concentration (µg/ml)	*Mean Absorbance ± S.E.M.	
	In PBS 7.4	In Acetate buffer
10	0.148 ± 0.0023	0.147 ± 0.0026
15	0.232 ± 0.0045	0.217 ± 0.0092
20	0.302 ± 0.0056	0.285 ± 0.0058
25	0.394 ± 0.091	0.351 ± 0.0045
30	0.466 ± 0.0012	0.437 ± 0.0120
35	0.542 ± 0.0078	0.502 ± 0.0051

\* n=03

The R<sup>2</sup> value for calibration curve of VBS was found to be 0.999 and 0.998 in diffusion medium PBS 7.4 and Acetate Buffer pH 4.5 respectively, indicating the linearity of analytical method in the concentration range 10µg/ml-35 µg/ml and showing that method obeyed the Beer-Lambert law.

**Table 9 Optical characteristics of VBS**

Medium	Range (µg/ml)	Regression equation	Regression coefficient (R <sup>2</sup> )
PBS 7.4	10-35	Y = 0.015x - 0.008	0.999
Acetate buffer 4.5	10-35	Y = 0.014X + 0.001	0.998



**Fig 14 Calibration curve of VBS in Acetate buffer 4.5**

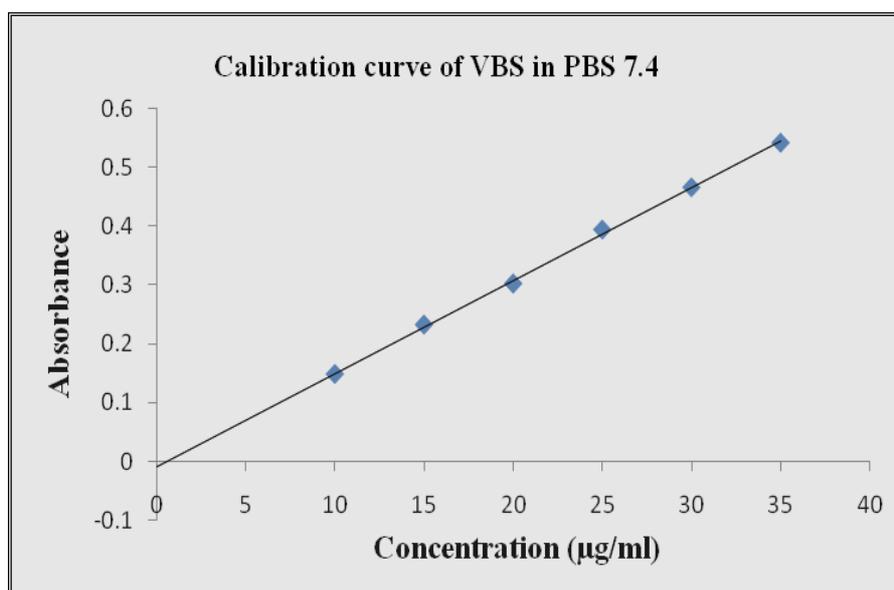


Fig 15 Calibration curve of VBS in PBS 7.4

### IN-VITRO RELEASE PROFILE

The dialysis bag diffusion technique is widely used to evaluate drug release from micro and nanosized carriers. A small volume of the concentrated drug-carrier suspension is contained in dialysis bag, which is immersed in larger volume of continuous-phase acceptor fluid. Preferably, both compartments are stirred, and the drug then diffuses out of particulate carrier into its local continuous phase, and then through the dialysis membrane into the acceptor phase, which is periodically sampled and assayed. The measured rate of drug appearance in the external sink depends largely on the partition coefficient of a drug between its carrier and the local acceptor phase. This technique allows the comparison of different formulations.

### EXPERIMENTAL SETUP

#### 1. Reagents

Disodium hydrogen phosphate, Potassium dihydrogen phosphate, Sodium acetate, glacial acetic acid of analytical reagent grade (S.D fine chemicals Ltd., Boisar)

#### 2. Solutions (I.P 1996)

##### Phosphate buffer, pH 7.4 (PBS)

50.0 ml of 0.2 M potassium dihydrogen phosphate was taken in 200 ml volumetric flask, to it 39.1 ml of 0.2 M sodium hydroxide was added, mixed well and volume was made up to the mark with distilled water.

### Acetate buffer pH 4.5

Dissolve 5.4 g of sodium acetate in 50 ml of distilled water, add 2.4 ml of glacial acetic acid and dilute with distilled water to 100 ml. Adjust the pH, if necessary.

### 3. Apparatus

Magnetic stirrer (Remi scientific equipments, Mumbai); Teflon coated bar magnet; open ended dialysis tubing made of cellulose (retains substances with molecular weight greater than 12,000 daltons) having flat width 35 mm and inflated diameter 21 mm (Sigma Diagnostics, USA).

#### Selection of diffusion medium

- Receptor compartment containing 50 ml of PBS pH 7.4, with constant stirring simulated in vivo systemic circulation condition.
- Receptor compartment containing 50 ml of Acetate buffer pH 4.5, with constant stirring simulated tumor interstitium condition.

### 4. Preparation of dialysis sac and dialysis set up

A 5 cm long portion of the dialysis tubing was made into a dialysis sac by folding and tying up one end of the tubing with thread, taking care to ensure that there would be no leakage of the contents from the sac. Plain drug solution, liposomes and calcium phosphate coated liposomes containing drug equivalent to 1 mg of VBS were accurately transferred into the sac, which thus became the donor compartment. The sac was once again examined for any leaks and then was suspended in a glass beaker containing 50 ml of buffer PBS (pH7.4) and acetate (pH4.5), which acted as a receptor compartment.

The contents of the beaker were stirred using Teflon coated bar magnet and the beaker was closed with the aluminium foil to prevent any evaporative losses during the experiment run. The temperature of the bulk of the solution was maintained at  $37 \pm 0.5^\circ\text{C}$ .

### 5. Sampling

At predetermined intervals of time, 2 ml aliquots were withdrawn from the receptor compartment and subjected to analysis using the analytical method described in chapter 3. Fresh buffer was used to replenish the receptor compartment. Analysis was carried out immediately after withdrawal. The study was continued up to 48 hr. samples were withdrawn after 0.25, 0.50, 1, 2, 4, 6, 8, 12, 24, 28, and 48 hrs. Fresh buffer was used to replenish the receptor compartment.

The study was carried out for plain VBS, Liposomes and Calcium phosphate coated liposome and results obtained were compared. The diffusion studies and sample analysis were carried out three times and recorded.

Table 10 Diffusion study data

Time (hr)	Plain drug solution % Release*		Liposomes % Release*		CP coated liposomes % Release*	
	PBS 7.4	Acetate buffer 4.5	PBS 7.4	Acetate buffer 4.5	PBS 7.4	Acetate buffer 4.5
0.25	8.21±1.55	6.92±1.25	5.21±1.39	4.41±1.66	2.14±0.76	3.5±0.92
0.5	21.25±2.45	17.39±2.12	9.35±2.31	7.35±2.24	5.21±1.22	8.23±1.67
1	45.35±2.38	33.08±3.16	19.28±2.36	14.12±3.21	15.24±2.59	18.74±2.57
2	52.62±2.22	45.12±1.98	25.65±3.36	21.54±3.45	20.09±2.33	24.67±3.52
4	68.21±3.11	59.62±2.48	34.55±3.65	27.42±2.56	26.51±3.83	34.97±2.75
6	85.67±2.56	75.35±3.45	38.24±3.65	33.65±3.16	32.19±2.64	39.02±1.98
8	92.25±2.37	86.91±3.87	52.29±4.12	39.57±2.54	37.11±1.57	45.70±3.42
12	<b>95.12±1.27</b>	<b>91.2±2.47</b>	68.79±3.89	62.76±3.5	52.93±2.25	68.82±2.45
24			85.66±2.45	79.86±2.9	68.71±3.35	83.90±3.14
48			95.25±1.29	87.24±1.15	<b>76.29±1.89</b>	<b>94.38±2.27</b>

\* Mean±SEM (N = 3)

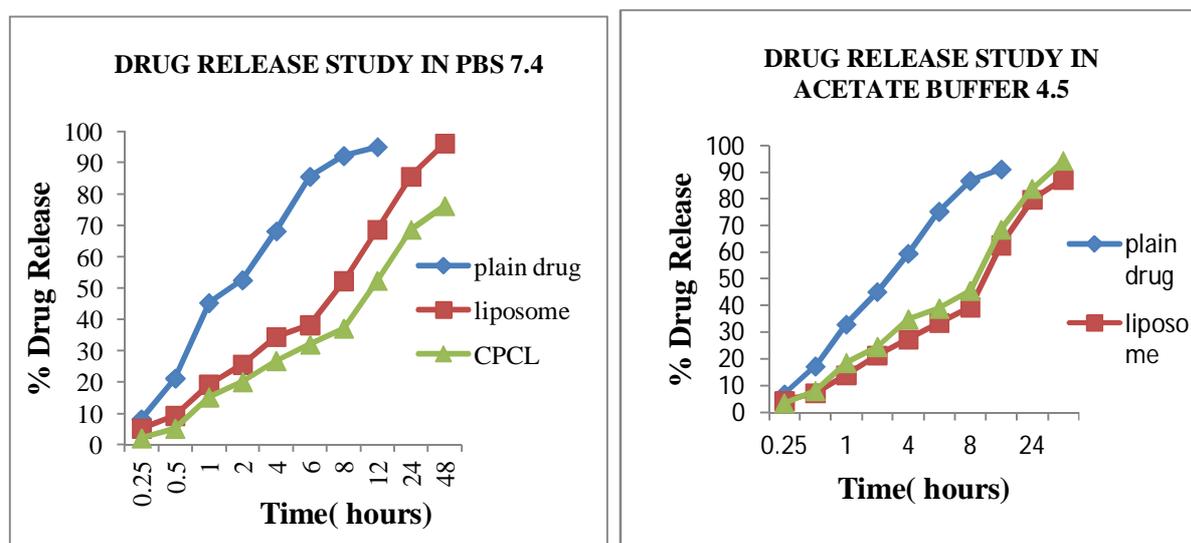
## RESULTS AND DISCUSSION

Comparative diffusion studies were carried out for plain VBS solution, uncoated and coated VBS liposomes using dialysis membrane. The results of these studies are shown below.

The results of **linear correlation coefficient (R<sup>2</sup>)** after fitting the drug diffusion data of VBS liposome and calcium phosphate coated liposomal formulation are shown below.

Table 11 Linear correlation coefficient (R<sup>2</sup>) values

Formulation		Zero order	First order	Higuchi
Plain Drug	PBS 7.4	0.968	0.971	0.970
	Acetate Buffer 4.5	0.990	0.930	0.990
Liposome	PBS 7.4	0.963	0.766	0.966
	Acetate Buffer 4.5	0.937	0.807	0.937
CPCL	PBS 7.4	0.960	0.869	0.961
	Acetate Buffer 4.5	0.959	0.770	0.959



**Fig 16 Drug release plot from plain drug and formulations in PBS 7.4 and Acetate buffer 4.5**

From the table, it is cleared that after 48 hours drug release from liposome is 95.25% (PBS 7.4) and 87.24% (Acetate buffer 4.5) while in case of calcium phosphate coated liposomes % drug release is 76.29% (PBS 7.4) and 94.38% (Acetate buffer 4.5). Hence, the diffusion data indicates that coating reduces the drug release rate in PBS 7.4 because calcium phosphate coating is less soluble in neutral and basic conditions. While at acidic condition, coat dissolves and release is almost similar to liposome. All these observations lead us to the conclusion that calcium phosphate coating on liposomal formulation has a potential to release the drug preferentially at tumor interstitium having acidic pH (4.5 to 5.5) compared to systemic circulation. Thus, calcium phosphate coating on liposome could overcome the unwanted side effects arising from drug leakage during systemic circulation.

There are three barriers that can influence the drug diffusion to the receptor compartment, one is the liposomal membrane, other is the dialysis membrane and last one is calcium phosphate coating. But from the table 6.1, the percentage drug diffusion of encapsulated drug is found to be dependent upon the type of formulations (coated and uncoated liposome). Moreover, the plain drug diffusion is very fast indicating that the liposomal membrane and coating of calcium phosphate act as rate-controlling barriers to the drug diffusion. The dialysis membrane acts only as physical barrier preventing the liposomes and coated liposomes to diffuse into the donor compartment and not regulating the drug diffusion to the receptor compartment.

In vitro diffusion study was carried out up to 48 hr and the results were compared with each other after applying different kinetic model. Among the three different models regression coefficient of the plot %drug diffused Vs square root of time was found to be nearest to 1 (i.e.

0.966 and 0.937 for the liposome and 0.961 and 0.959 for calcium phosphate coated liposome in PBS 7.4 and Acetate buffer 4.5 respectively) indicating that diffusion from both liposomes and calcium phosphate coated liposomes obey Higuchi's Diffusion Model.

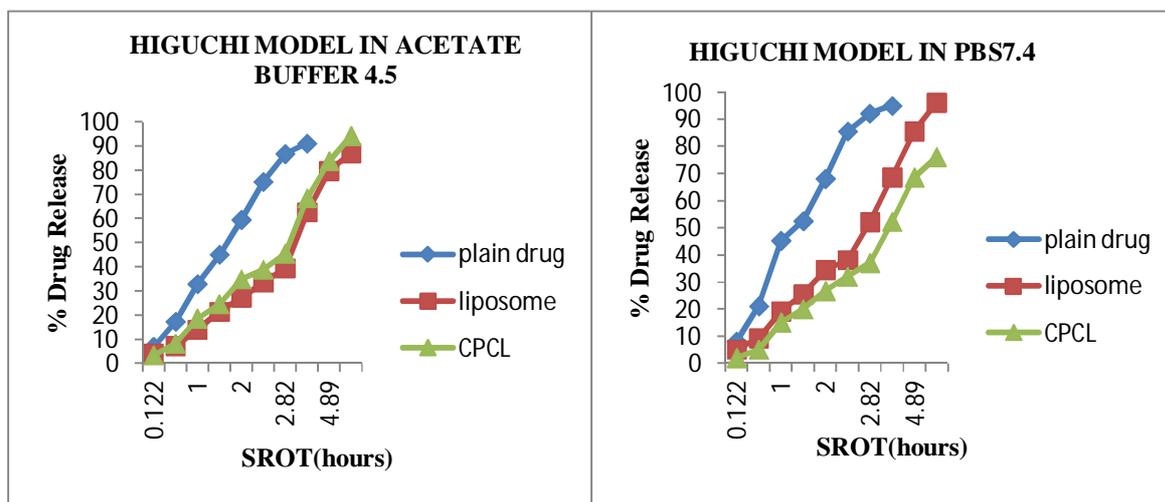


Fig 17 Higuchi square root of time plot for VBS loaded liposomes and CPCL

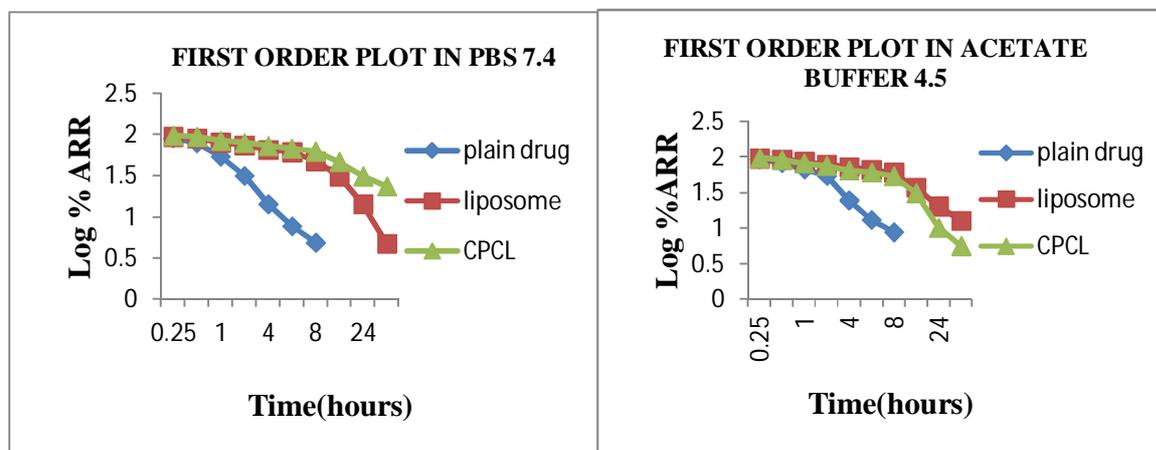


Fig 18 First order plot of VBS from liposome and calcium phosphate coated liposome formulations

### CONCLUSION

Coating the liposomes with materials as calcium phosphate leads to improvement in shelf stability of the product, indicated by stability studies. The in-vitro release studies indicate the possibility of drug leakage reduction during its systemic circulation. The pH - dependent

solubility of calcium phosphate plays an important role in targeting the drug preferentially to tumor site. The in-vitro cell cytotoxicity studies indicate the enhanced intracellular uptake and subsequently increased cytotoxic effect. The formulation seems to be promising and further work needs to be done by attaching specific ligands or antibodies for tumor targeting.

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