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SELECTION OF PHOSPHOLIPID AND METHOD OF FORMULATION FOR OPTIMUM ENTRAPMENT AND RELEASE OF LAMIVUDINE FROM LIPOSOME

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ABSTRACT

The present investigation was aimed to compare various phospholipids and different methods that would be appropriate to produce liposomes having vesicle size in the range of 200-300 nm, PDI less than 0.500, maximum entrapment and delayed release of lamivudine. The phospholipids employed were Phospholipon® 90G, Phospholipon® 90H, 1,2-Dimyristoyl-sn-glysero-3-phosphocholine (DMPC) and 1,2-Dipalmitoyl-sn-glysero-3-phosphocholine (DPPC). They were used in various molar ratio with cholesterol and blank liposomes were prepared initially by thin film hydration and ether injection method. The thin film hydration method was only found to be appropriate to produce liposome of desired size and PDI. Hence, the molar ratios of employed phospholipids:cholesterol that produced liposomes as per the expected parameters was then used to load lamivudine. The method of preparation, phospholipid: cholesterol molar ratio, hydrations above transition temperature and hydration time were showed direct influence on vesicle size, PDI, percentage encapsulation and *in-vitro* release. Phospholipon® 90H: cholesterol: lamivudine in the molar ratio 1:2:1 produced liposomes having desired vesicle size and PDI with maximum drug entrapment and sustained release. The encapsulation efficiency of drug in liposomes was in the order of Phospholipon® 90H> Phospholipon® 90G > DPPC > DMPC.

Keywords: Liposomes, Lamivudine, Phospholipid, Thin film hydration method, Ether injection method, encapsulation efficiency

INTRODUCTION

Lamivudine (3TC) is an antiretroviral agent, used either alone or in combination for a prolonged period in the treatment of AIDS (Acquired immunodeficiency syndrome). HIV mainly found in CNS, lymphatic system, liver, lungs, T-lymphocytes and macrophages etc[1]. It is difficult for majority of drug to reach and maintain its therapeutic concentration at the HIV localized site by conventional therapy and short half life[2], which lead to non compliance to the therapy, and increase in viral load. AIDS requires lifetime treatment and development of resistance demands further rise in the dose[3]. High dose of 3TC is required to maintain minimum effective concentration at the HIV reservoir to control the viral load. Frequent administration results in

to higher interaction even with uninfected tissues, leading to untoward side effects.

Therefore, to restrict its stay in free form, earlier workers have attempted to incorporate it in special formulations such as nanoparticles[4-7], ethosomes[8], liposomes[9], dendrimers[10], neosomes[11] etc, so that control and targeted delivery can be achieved. Among these, nanoparticles tend to aggregate due to small size, and that limits the release of entrapped drug or some time leads to burst release. Multiple molecules attach to dendrimers leading to inconsistent batches during large scale production. Other issues related to dendrimers are deterioration of material homogeneity[12], biocompatibility and cytotoxicity[13,14]. Neosomes suffers stability problems[15-16].

Liposomes are the concentric vesicles in which an aqueous phase is entirely entrapped by a lipid layer[17]. The outer lipid layer is usually made of phospholipids having a hydrophilic head and hydrophobic tail[18]. Liposomes are able to encapsulate hydrophilic drug in their interior aqueous compartment and lipophillic drug with in lipid bilayer[19,20]. Liposome encapsulates the drug and thereby reduces its toxicity, and protects it against rapid degradation in the blood circulation[21]. Liposome helps to increase therapeutic index of antiviral agent[22-25]. Also help to reduce the dose and consequent side effects[26]. On interaction with the cell membrane, the liposomes enter the cell by diffusion process and release the drug to the cell. Moreover, liposomes are taken up by RES system which leads to rapid uptake of liposomes[27], where HIV normally harbors[1,28]. Therefore, liposomal formulation is preferred for the reason that water soluble 3TC is entrapped in the centre, and it is surrounded by concentric phospholipid layer/s. Engulfment of such liposomes by microphages will ensure targeted delivery of 3TC to the cells with high viral load. Moreover, liposomes being made up of phospholipids are well accepted by the body and have no clinical reports of allergic reactions when administered by parenteral route. Therefore it was thought worthwhile to prepare 3TC loaded liposome for targeting the macrophages by parenteral route.

The literature documents a long list of phospholipids. Interestingly, except the common chain structure, there exist diversity in their single unit forming the chain and hence their ability to accommodate lipophilic or hydrophilic agents differs extensively. In the preparation of liposome the earlier workers used wide range of phospholipids such as Phospholipon® 90G, Phospholipon® 90H, 1,2-Dimyristoyl-sn-glysero-3-phosphocholine (DMPC) and 1,2-Dipalmitoyl-sn-glysero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) etc. However, due to isolated studies it is difficult to compare which phospholipid can produce 3TC loaded liposomes with the desired characteristics. Thin film hydration and ether injection method are the most employed methods in the preparation of liposomes. Therefore, we initially compared these methods by using various concentrations of phospholipids and cholesterol. The batches of liposomes produced by each method were later compared for vesicle size and PDI. Many literatures documented that uptake of liposomes by MPS cells was improved with increased size[29-30]. Therefore, the method and phospholipid that produced vesicles of size in the range of 200-300 nm with PDI below 0.500 was then employed to prepare 3TC loaded liposomes. These 3TC loaded liposomes were further evaluated for drug entrapment and *in-vitro* release.

MATERIALS AND METHODS

Materials

Phospholipon® 90G, Phospholipon® 90H, DMPC and DPPC were the gift samples from Lipoid GmbH Germany, and 3TC was the gift sample from Cipla Limited, Kurkumbh India. All other used chemicals were of HPLC grade. Fresh double distilled water was used throughout the experiment.

Preparation of empty liposomes

1. Thin film hydration method

Phospholipid and cholesterol in various molar ratios were dissolved in chloroform in pear shape flask with gentle shaking. Chloroform was evaporated in a rotary flask evaporator (Buchi type) above the transition temperature of lipids at 120 rpm to get a thin film in the flask. This film was kept overnight under vacuum to remove traces of solvents if any. The casted film was then hydrated by adding 10 ml of phosphate buffer saline (pH7.4). To disperse the film in the solution, the flask was again rotated on the rotary flask evaporator without vacuum above the transition temperature of phospholipid until a white milky suspension was obtained[31]. To further reduce the size of liposomes, the dispersion was sonicated for 1 min, by using bath sonicator (Imeco Ultrasonics). After sonication, the suspension was subjected to the evaluation of vesicle size and PDI.

The rotational speed, time period for hydration and period of sonication employed in this method is based on the results of preliminary studies. The influence of the molar ratio of Phospholipid: cholesterol on vesicles size and PDI is as shown in Table 1.

2. Ether injection method

Phospholipid and cholesterol in various molar ratios were dissolved in diethyl ether. In a beaker phosphate buffer saline (10 ml, pH7.4) was continuously stirred with a magnetic stirrer and its temperature was maintained between 55-60°C. With the help of syringe the solution of phospholipid and cholesterol was then slowly injected to disperse the solution in phosphate buffer saline (pH7.4). Beaker was continuously stirred on magnetic stirrer at 55-60°C until the ether got completely evaporated and a milky dispersion was obtained[32,33]. The dispersion was subjected to sonication for 1 min. The vesicle size and PDI of the liposomes was then evaluated. The influence of the molar ratio of Phospholipid: cholesterol on vesicles size and PDI is as shown in Table 2.

Evaluation of empty liposomes

Appearance

The appearance of the liposomal suspension was assessed by naked eyes.

Vesicle size and PDI

Aliquots (1ml) of the samples were serially diluted with phosphate buffer saline (pH7.4) to obtain 1000-fold dilution, and the globule size and size distribution was accessed by using Malvern particle size analyzer (Malvern Nano ZS90, Malvern, UK). Each study was carried out in triplicate (Mean±SD, n=3).

Table 1: Liposome formulations prepared by thin film hydration method

Sr. No.	Batch code	Phospholipid	Molar Ratio of phospholipid: cholesterol	Appearance	Vesicle size (nm)	PDI
1.	LH1	Phospholipon®90H	0.5: 1.5	Milky white dispersion	276.0±21.6	0.492±0.043
2.	LH2	Phospholipon®90H	1.0: 1.0	Milky white dispersion	259.0±10.3	0.362±0.042
3.	LH3	Phospholipon®90H	1.0: 2.0	Milky white dispersion	268.0±13.5	0.267±0.028
4.	LH4	Phospholipon®90H	1.5: 0.5	Milky white dispersion	289.1±15.6	0.620±0.022
5.	LH5	Phospholipon®90H	2.0: 1.0	Milky white dispersion	374.1±14.5	1.000±0.045
6.	LG1	Phospholipon®90G	0.5: 1.5	Milky white dispersion	302.1±09.6	0.500±0.021
7.	LG2	Phospholipon®90G	1.0: 1.0	Milky white dispersion	264.0±25.2	0.450±0.037
8.	LG3	Phospholipon®90G	1.0: 2.0	Milky white dispersion	231.5±22.8	0.308±0.051
9.	LG4	Phospholipon®90G	1.5: 0.5	Milky white dispersion	299.6±19.7	0.590±0.032
10.	LG5	Phospholipon®90G	2.0: 1.0	Milky white dispersion	392.0±25.6	0.281±0.061
11.	LP1	DPPC	0.5: 1.5	Milky white dispersion	278.2±26.1	0.220±0.045
12.	LP2	DPPC	1.0: 1.0	Milky white dispersion	327.0±24.8	0.263±0.081
13.	LP3	DPPC	1.0: 2.0	Milky white dispersion	368.2±31.2	0.436±0.073
14.	LP4	DPPC	1.5: 0.5	Milky white dispersion	317.1±29.3	0.890±0.048
15.	LP5	DPPC	2.0: 1.0	Milky white dispersion	431.0±31.7	0.420±0.081
16.	LM1	DMPC	0.5: 1.5	Milky white dispersion	321.0±37.9	0.800±0.039
17.	LM2	DMPC	1.0: 1.0	Milky white dispersion	352.0±20.9	0.434±0.091
18.	LM3	DMPC	1.0: 2.0	Milky white dispersion	280.1±21.2	0.590±0.034
19.	LM4	DMPC	1.5: 0.5	Milky white dispersion	321.2±26.8	0.212±0.082
20.	LM5	DMPC	2.0: 1.0	Milky white dispersion	301.1±41.8	0.362±0.029

(Mean ±SD, n=3)

Preparation of 3TC loaded liposomes

The preliminary studies revealed that thin film hydration method produced the vesicle of 200-300 nm size with the PDI below 0.500. The molar ratio of phospholipid: cholesterol which produced liposomes of such size and PDI is as shown in Table 3. In ether injection method, none of the molar ratio of phospholipid: cholesterol could produce vesicles in the desired range, and the PDI was above 0.500. Therefore, thin film hydration method was selected as the carrier to load 3TC. The molar ratio of phospholipid to cholesterol as shown in Table 3 was used for this purpose. 3TC was incorporated in the liposomes by the following procedure.

The test drug was dissolved in phosphate buffer saline (10ml, pH7.4), and was then added to a pear shape flask,

the wall of which had a deposit of a film left over after the total evaporation of the solvent. The flask containing drug solution in phosphate buffer saline (pH7.4) and the film on the wall was then slowly rotated on the rotary flask evaporator without vacuum above the transition temperature of phospholipid so that the film gets converted in to the liposomal vesicles entrapping the drug containing solution. To further reduce the size of liposomes, the dispersion was sonicated for 1 min, by using bath sonicator (Imeco Ultrasonics). The 3TC loaded liposomes were then subjected to the evaluation of vesicle size, PDI, % encapsulation efficiency, TEM study, zeta potential, entrapment efficiency and *in-vitro* release. The formulation details of various batches of 3TC loaded liposomes are as shown in Table 3.

Table 2: Liposomal formulations prepared by ether injection method

Sr. No.	Batch code	Phospholipid	Molar Ratio of phospholipid: cholesterol	Appearance	Vesicle size (nm)	PDI
1.	EH1	Phospholipon®90H	0.5: 1.5	Milky white dispersion	342.7±072.5	0.394±0.035
2.	EH2	Phospholipon®90H	1.0: 1.0	Milky white dispersion	313.3±053.8	0.281±0.048
3.	EH3*	Phospholipon®90H	1.0: 2.0	----	----	----
4.	EH4*	Phospholipon®90H	1.5: 0.5	----	----	----
5.	EH5*	Phospholipon®90H	2.0: 1.0	----	----	----
6.	EG1	Phospholipon®90G	0.5: 1.5	Milky white dispersion	476.6±039.2	1.000±0.039
7.	EG2	Phospholipon®90G	1.0: 1.0	Milky white dispersion	401.0±041.5	0.392±0.057
8.	EG3*	Phospholipon®90G	1.0: 2.0	----	----	----
9.	EG4*	Phospholipon®90G	1.5: 0.5	----	----	----
10.	EG5*	Phospholipon®90G	2.0: 1.0	----	----	----
11.	EP1	DPPC	0.5: 1.5	Milky white dispersion	347.9±051.4	0.445±0.038
12.	EP2*	DPPC	1.0: 1.0	----	----	----
13.	EP3*	DPPC	1.0: 2.0	----	----	----
14.	EP4*	DPPC	1.5: 0.5	----	----	----
15.	EP5*	DPPC	2.0: 1.0	----	----	----
16.	EM1*	DMPC	0.5: 1.5	----	----	----
17.	EM2	DMPC	1.0: 1.0	Milky white dispersion	456.8±029.7	0.854±0.074
18.	EM3	DMPC	1.0: 2.0	Milky white dispersion	473.0±024.7	0.643±0.091
19.	EM4*	DMPC	1.5: 0.5	----	----	----
20.	EM5*	DMPC	2.0: 1.0	----	----	----

*Phase separation occurred in these batches hence further evaluation was not possible. (Mean ±SD, n=3)

Table 3: Formulation and evaluation of 3TC loaded liposomes

Formulation code	Phospholipid	Molar Ratio of phospholipid: cholesterol: drug	Appearance	Vesicle size	PDI	Zeta potential	% entrapment
TCLH3	Phospholipon®90H	1.0:2.0:01	Milky white dispersion	273.0±2.04	0.267±0.019	-13.7±0.3	63.64±03.29
TCLG3	Phospholipon®90G	1.0:2.0:01	Milky white dispersion	241.5±2.12	0.208±0.024	-11.3±0.2	57.07±01.54
TCLP1	DPPC	0.5:1.5:01	Milky white dispersion	286.2±3.28	0.220±0.036	-11.6±0.4	49.13±02.90
TCLM3	DMPC	1.0:2.0:01	Milky white dispersion	261.2±2.82	0.212±0.068	-10.5±0.2	37.35±02.56

(Mean ±SD, n=3)

Evaluation of 3TC loaded liposomes

1. Percent encapsulation efficiency

The liposomal suspension (2 ml) was centrifuged at 13,000 rpm at 4°C (Remi cooling centrifuge) for 30 min. Clear supernatant was decanted, and after suitable dilutions, the amount of unentrapped drug was estimated on UV spectrophotometer at 270 nm using phosphate buffer saline (pH7.4) as blank. Each study was carried out in triplicate (Mean±SD, n=3). The amount of drug entrapped in liposomes was calculated by following formula[34].

% Encapsulation efficiency =

$$\frac{\text{Total amount drug} - \text{Amount of drug in supernatant}}{\text{Total amount drug}} \times 100$$

2. Transmission electron microscopy study

The size and the shape of 3TC loaded liposomes were confirmed by transmission electron microscopy (TEM) (Make Jeol Model JM 2100, accelerating Voltage 200kV, resolution 0.24nm). In brief, the liposomes were suspended in nanopure water and ultrasonicated to disperse the particles. A drop of the suspension was

placed on carbon-coated grids of 200 mesh, and allowed to dry. The size and shape was measured.

3. Zeta potential

The formulation stability is directly related to the magnitude of the surface charge. The liposomes were suspended in 10 ml distilled water and further diluted serially to obtain ratio of 1:1000 (v/v). Zeta potential was determined using Zetasizer (Malvern equipment). Each study was carried out in triplicate (Mean±SD, n=3).

4. *In-vitro* release of 3TC

The prepared 3TC loaded liposomal dispersion was centrifuged. The supernatant was discarded to eliminate untrapped 3TC. The deposited liposomes were reconstituted in 10 ml of phosphate buffer saline (pH7.4), and sonicated for 30 sec. An accurately measured volume of drug loaded liposomal suspension equivalent to 2 mg of drug was transferred in cellulose dialysis bag (a tube having 6.3 mm diameter with one end closed and molecular weight cut off between 12000-14000, Himedia, Mumbai) which was pre soaked in distilled water for 24 h before dialysis to ensure complete wetting of membrane and removal of preservative. After closing the open end of the bag with a thread, it was vertically suspended in 250 ml beaker containing 200 ml phosphate buffer saline (pH7.4) at $37\pm2^{o}C$, which was continuously being stirred at 300 rpm with a magnetic stirrer. A care was taken to suspend the bag in such a manner that the whole bag remained immersed in the phosphate buffer saline[35]. The release of 3TC from liposome was compared with free drug by dissolving 2 mg 3TC in phosphate buffer saline (pH7.4) in similar bag.

For *in-vitro* release of 3TC, 5ml of phosphate buffer saline was withdrawn at predetermined intervals and subjected to estimate the amount of 3TC by spectrophotometer at 270 nm. The withdrawal of sample was replaced with same volume of fresh phosphate buffer saline (pH7.4). Each study was carried out in triplicate (Mean±SD, n=3) (Figure 3).

5. Fourier Transfer Infra-Red spectroscopy

Drug, cholesterol and phospholipid interaction study were carried out using FTIR Spectrophotometer (Shimadzu, Tokyo, Japan). The spectrum was recorded in the wavelength region of 4000-400 cm^{-1}.

The procedure involves dispersing individually the samples of 3TC, cholesterol and Phospholipon$^{®}$ 90H, and compressing into discs by applying a pressure of 5 t

for 5 min in a hydraulic press. The pellets were placed in the light path and the spectrum was recorded. Initially a baseline correction was carried out using dry potassium bromide pellet. The FTIR spectrum of mixture of 3TC, cholesterol and Phospholipon$^{®}$ 90H were also taken by following same procedure.

RESULTS AND DISCUSSION

The objective of the present work was to find out the appropriate molar ratio of various phospholipid:cholesterol and the appropriate method for preparing liposome having vesicle size in the range 200-300 nm with PDI below 0.500 and having maximum entrapment with delayed release of 3TC.

The preliminary studies revealed that by employing different phospholipid:cholesterol molar ratio, thin film hydration method produced the vesicles in the size range of 231.5-431.0 nm and PDI from 0.212-1.00 and few phospholipid:cholesterol molar ratio produced liposomes of desired size and PDI (Table 1). Ether injection method produced the vesicles in the size range of 313.3-476.5 nm and PDI from 0.281-1.000 (Table 2). However, by ether injection method these phospholipids produced liposomes of very large size and none of the phospholipid: cholesterol molar ratio could produce vesicles in desired range, and PDI was above 0.500. Also, sonication of the dispersion could not lower the size to desired range and PDI remain higher than 0.500.

Therefore, it was thought worthwhile to prepare 3TC loaded liposomes by thin film hydration method using the phospholipids and cholesterol in the molar ratios revealed by the primary study. However, the variables those can influence the size of vesicle in thin film hydration method were first optimized. This optimization study revealed that a relatively thinner film was obtained when the rotational speed of flask was 120 rpm as compared to 80 rpm and 100 rpm. This may be due to higher speed might have favored even spreading and faster evaporation of chloroform. The thin film was hydrated with phosphate buffer saline (pH7.4) for 2, 4 and 6 h. It was observed that 6 h of hydration resulted in to the vesicles of desired size and PDI. The final dispersion was subjected to sonication for 1 min. Sonication leads to rearrangement of lipid molecules and breakdown of multi-lamellar structure of vesicles. It was observed that 1 min sonication decreased the vesicle size and dispersion was less heterogeneous as compare to dispersion before sonication. Figure 1 is a prototype figure to represent the average vesicle size and PDI.
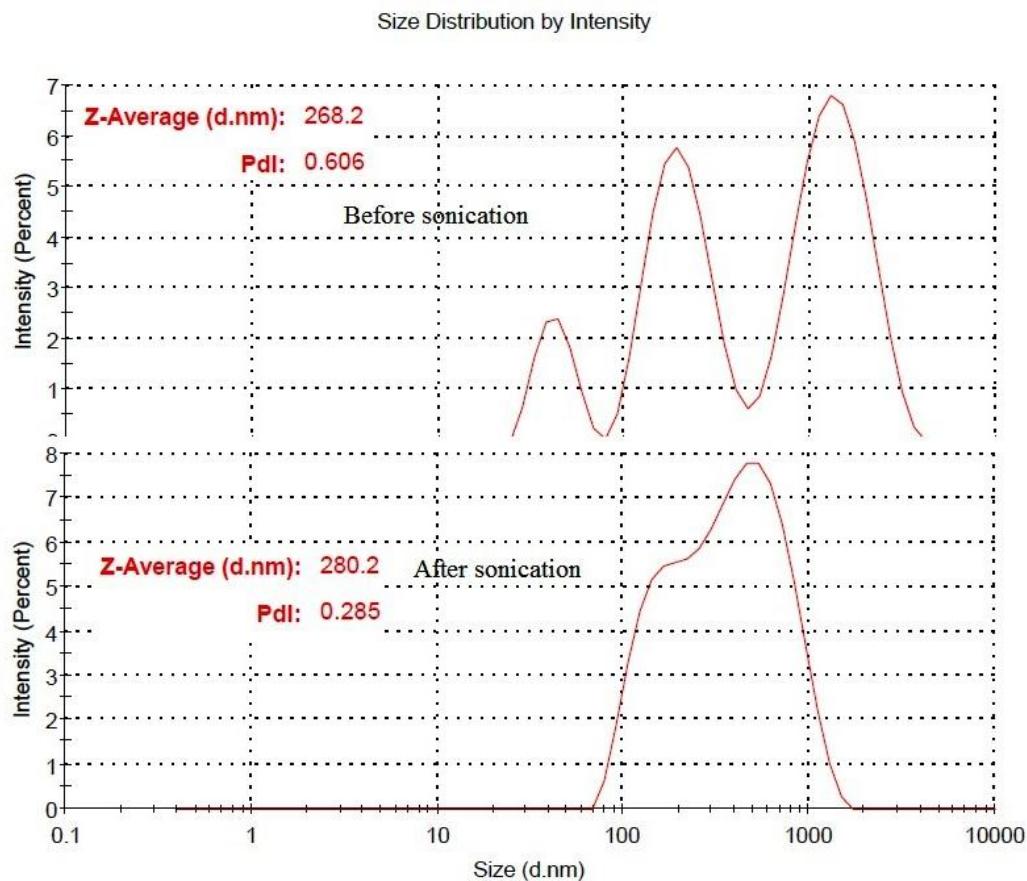
Figure 1: Representative figure of vesicle size and PDI of the 3TC loaded liposomes

1. Appearance

Liposomes prepared by thin film hydration method were found to be milky in appearance. Phase separation was occurred in some formulations of liposomes prepared by ether injection method. Thin film hydration method generated superior liposomes compared with ether injection method.

2. Vesicle size and PDI

It was observed that as the concentration of lipid was increased, vesicle size was increased. Liposomes prepared by thin film hydration method produced the vesicles of size ranging from 231.5-431.0 nm and PDI from 0.212-1.000. Variation in vesicle size and PDI was observed, which may be due to the phospholipid:cholesterol molar ratio, preparation method and operating parameters which affects the size of the vesicles.

3. Percentage encapsulation efficiency

Encapsulation efficiency of liposomes was influenced by many factors such as, molar ratio of phospholipid:cholesterol, vesicle size and method of preparation[36]. To evaluate the effect of lipids on the encapsulation efficiency of drug, four different lipids viz. Phospholipon® 90H, Phospholipon® 90G, DPPC and DMPC were selected for study. The entrapment efficiency was found to be maximum with a formulation containing Phospholipon® 90 H and cholesterol in the molar ratio 1:2. Cholesterol has marked effect on

encapsulation efficiency. It improves the fluidity and stability of bilayer membrane. Increasing the concentration of cholesterol imparts rigidity and increases the lipophilic properties of lipid bilayer, hence, increased in the entrapment of drug[37]. Encapsulation efficiency of the drug in liposomes was decreased in order of Phospholipon® 90H> Phospholipon® 90G > DPPC > DMPC from 39.0±2.60 - 63.80± 2.06 (Table 3 and Figure 2).
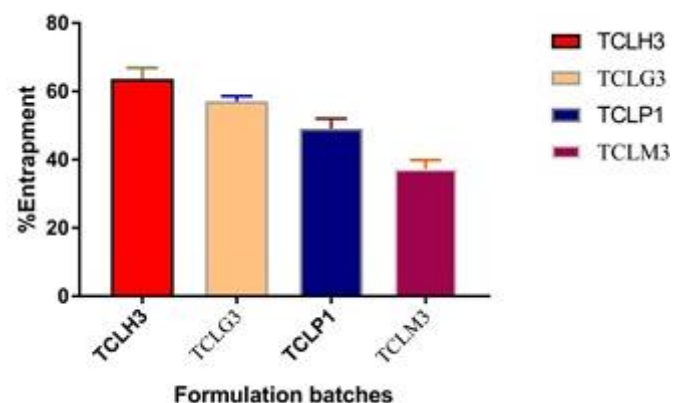

Figure 2: Percentage encapsulation efficiency of liposomal formulations

4. Zeta potential determination

The zeta potential measured for 3TC loaded liposomes was found to be -10.5±0.2 to   -13.7±0.3 mV, which indicated the liposomes having negative charge, which

is closer to range. Results demonstrated a low scope of coalescence of the vesicle systems and the potential for acceptable physical stability of formulated liposomes.

5. In-vitro drug release study

The drug release from liposomes depends on many factors including the composition of liposomes and the type of drug encapsulated. Once released, drug that normally crosses the membrane of a cell will enter the cell. At the end of 8 h study maximum 96.85±1.38% drug release was observed from liposomes. Thus, the release of 3TC from the liposomes could be contributed to the breaking of liposome structure. The leakage of liposome could be accounted by either liquefaction of liposomes or the force generated by stirring. Thus, liposomes containing 3TC gives steady release for longer period of time (Figure 3).

It was observed that liposomal formulation TCLH3 containing Phospholipon[®] 90H:cholesterol:drug in 1:2:1 molar ratio produced milky white dispersion having vesicle size 273.0±2.04 nm and PDI 0.267±0.019. The entrapment efficiency was 63.64±3.29% which is highest amongst the studied lipids. Also, formulation TCLH3 released 96.85±1.38% drug, and showed extended release up to 8 h. The extended release may be contributed to concentration of cholesterol (Kirby et al., 1980)[38], and the transition temperature of lipid which retard the releases of drug.

Hence, TCLH3 having Phospholipon[®] 90H with better entrapment efficiency and extended *in-vitro* release was considered to be the best liposomal formulation for incorporation of 3TC.
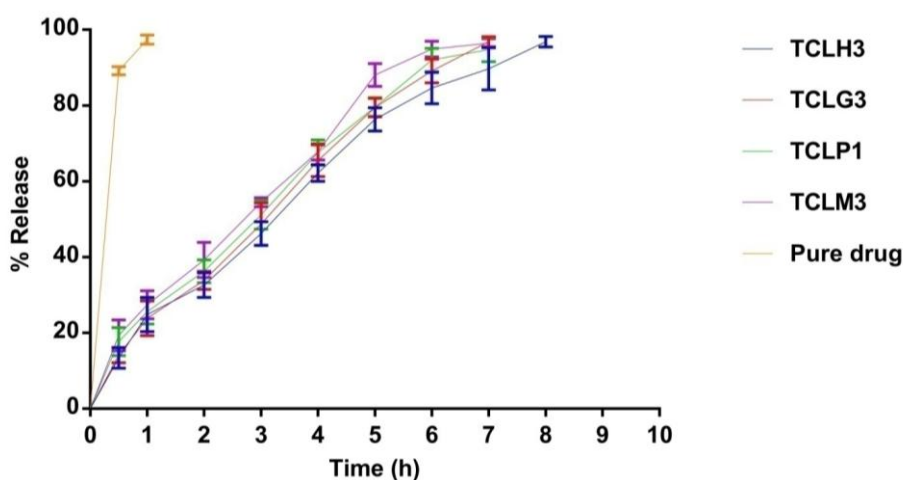

Figure 3: *In-vitro* release of 3TC at various time interval

6. TEM study

Liposome formation was confirmed by TEM photograph. The grid scan showed the spherical vesicles confirming the formation of liposomes (Figure 4).
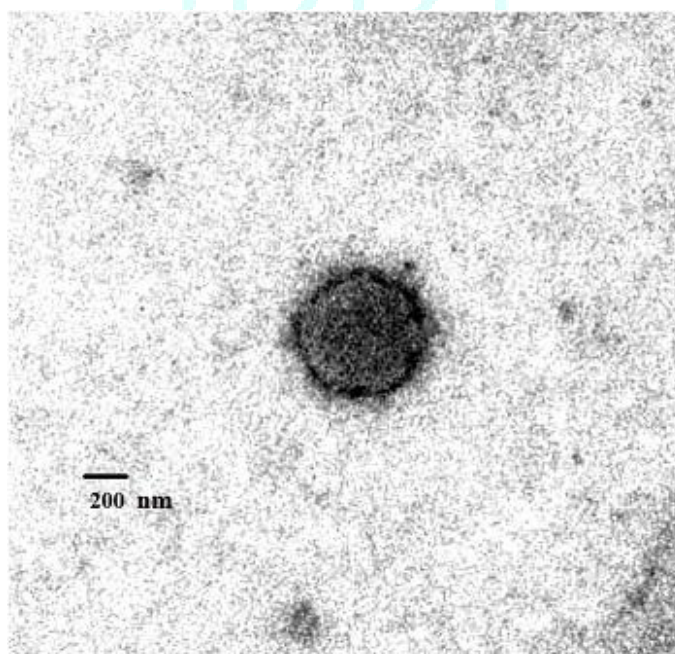

Figure 4: Representative TEM image of 3TC loaded liposome

7.   FTIR study

In FTIR spectrum of 3TC, cholesterol and Phospholipon® 90H physical mixture no significant difference in the characteristic peaks were observed. Indicating that there is no interaction between 3TC, cholesterol and Phospholipon® 90H. (Figure 5).
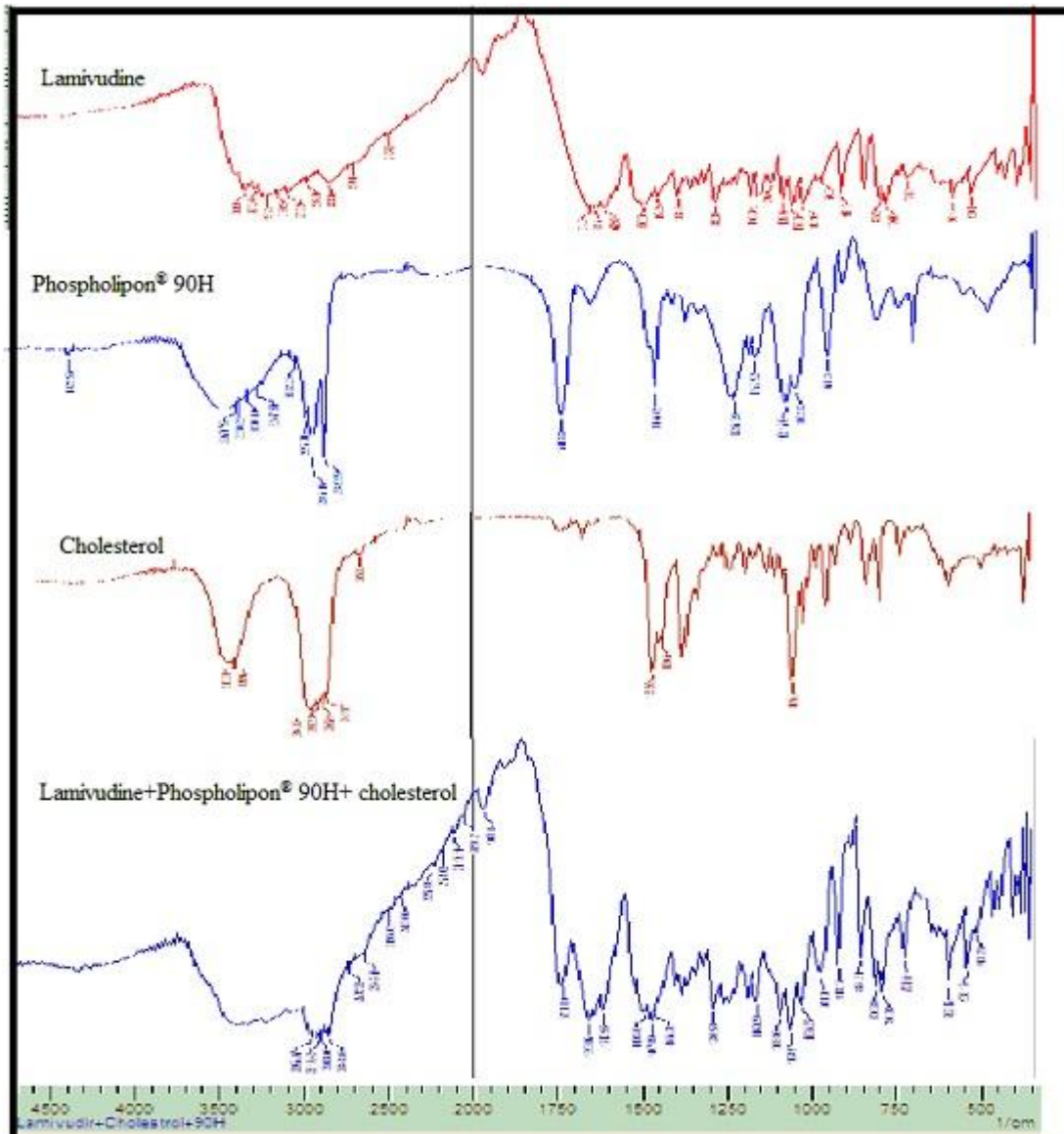

Figure 5: FTIR graph of 3TC, Phospholipid and their physical mixture

CONCLUSIONS

The conventional rotary vacuum evaporation method was found to be simple and suitable for the formation of 3TC liposomal dispersion. Phospholipid:cholesterol molar ratio, method of preparation, hydration above transition temperature and hydration time were showed direct influence on vesicle size, PDI, percentage encapsulation and *in-vitro* release. The formation of liposomes was confirmed by TEM photography. Higher 3TC encapsulation in liposomes will have long dosing intervals, and greater flexibility in dosing which ultimately will improve patient compliance and reduction in manufacturing cost. From the results it can be concluded that prepared liposomal formulation could serve as useful dosage form for the long term 3TC delivery to reduce the toxic effect during the therapy.

DECLARATION OF INTEREST: None

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