WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 9, 755-768. Research Article ISSN 2277 – 7105

# PREPARATION AND OPTIMIZATION OF DICLOFENAC ENCAPSULATED LIPOSOMES USING LIPID HYDRATION TECHNIQUE

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Article Received on
24 Aug 2014,

Revised on 18 Sept 2014,
Accepted on 13 Oct 2014

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## ABSTRACT

The aim of the study is to encapsulate, optimize and characterize the liposomal preparations of various formulations of Diclofenac sodium (DS) along with phosphatidylcholine, cholesterol, stearylamine and dicetylphosphate. Rotary evaporator is set at a temperature of $40^0$C with constant rotation speed. Liposomes were prepared by Lipid-Hydration technique using rotary evaporator (RE-300). The prepared liposomes were analyzed for size, zeta potential, percentage of drug encapsulated, in-*vitro* drug release and stability studies. Particle size of the drug loaded liposome was decreased when compared to that of the drug free. Encapsulation efficiency of the drug loaded liposomes with PC shows increase in the percentage of drug encapsulated to that of the lower concentrated vesicles and positive charge inducer have revealed elevated encapsulation efficiency. Liposomes composed of PC: CHOL: SA observed to be released at high rate and stability studies confirms that PC: CHOL: SA is supreme stable at varied temperatures. Phosphatidylcholine, cholesterol and stearylamine based preparations posses the suitable % drug encapsulated and release rate. The composition PC: CHOL: SA at a concentration of 16:8:4 µmoles proved as a stable suspension. From the study it can be concluded that cholesterol and stearylamine based phosphatidylcholine liposomes are most suitable to encapsulate the Diclofenac sodium.

**KEYWORDS:** Liposomes, Diclofenac sodium (DS), phosphatidylcholine (PC), entrapment, lipid, cholesterol (CHOL), stearylamine (SA), dicetylphosphate (DCP).

## INTRODUCTION

The liposome is made up of bilayer of hydrophilic head and lipophilic tail. Entrapment of hydrophilic, lipophilic and amphiphilic drugs can be possible. Polyethylene glycol acts as a

protective layer against the immune destruction[1]. The water soluble drugs are entrapped in the hydrophilic core and the lipid soluble drug is entrapped in the phospholipids bilayer.[2-5]

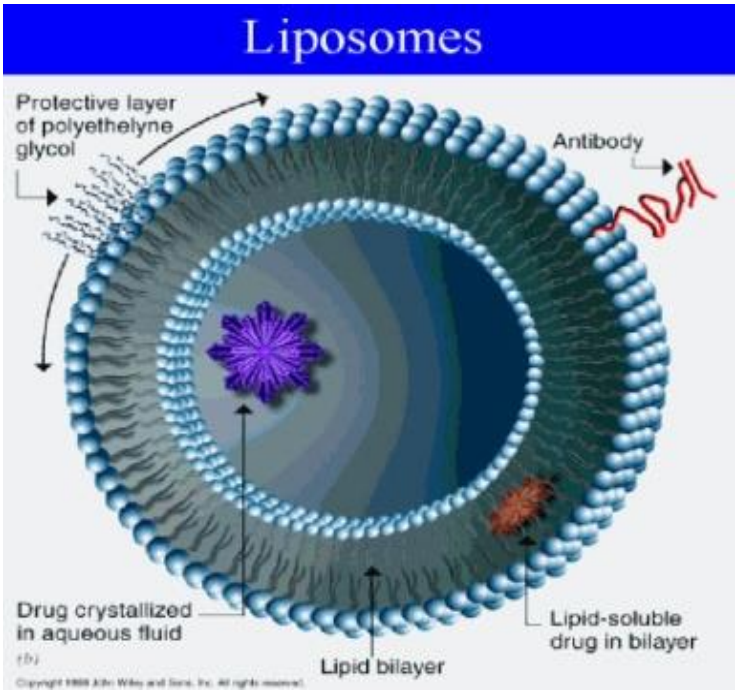


**Fig-1: Liposome encapsulates both hydrophilic and lipophilic drugs. The water soluble drugs occupy the inner core and the lipid based drugs are located between the acyl chains[5-9].**

**The mechanisms involved in the drug release at the target tissue**

1. Clathrin Coated pit endocytosis of conventional, pH-sensitive and cationic liposomes[10]
2. Release of pH sensitive liposome in the form of acidic endosome.
3. Long circulating liposomal drug release intravascular or extracellular.
4. Endocytosis of immune-liposomes through receptors.
5. Cationic liposomes fusion with plasma membrane.

A liposome encapsulates a region of aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily pass through the lipids [11]. Hydrophobic chemicals can be dissolved into the membrane, and in this way liposome can carry both hydrophobic molecules and hydrophilic molecules. To deliver the molecules to sites of action, the lipid bilayer can fuse with other bilayer such as the cell membrane, thus delivering the liposome contents. By making liposomes in a solution of DNA or drugs (which would normally be unable to diffuse through the membrane) they can be (indiscriminately) delivered

past the lipid bilayer. A liposome does not necessarily have lipophobic contents, such as water, although it usually does.[12-15]

Liposomes are used as models for artificial cells. Liposomes can also be designed to deliver drugs in other ways. Liposomes that contain low (or high) pH can be constructed such that dissolved aqueous drugs will be charged in solution (i.e., the pH is outside the drug's pI range). As the pH naturally neutralizes within the liposome (protons can pass through some membranes), the drug will also be neutralized, allowing it to freely pass through a membrane. These liposomes work to deliver drug by diffusion rather than by direct cell fusion.[16]

Liposome has been known as drug carrier for wide variety of compounds, to improve therapeutic effects and to reduce the side effects. The mucus which covers the surface epithelium of the GI tissue poses an adsorbed layer of phospholipids, provides a hydrophobic layer between epithelium and luminal contents.[17] There are a number of lipid species which reminds the surface active phospholipids among which phosphatidyl choline (PC) is suitable to incorporate DS. It has been suggested that presence of ionic surfactants DS shield the changes which are pH-dependant and the complex remains lipophilic. [18-20]

In the present work, drug is encapsulated into the liposomes along with the positive and negative surfactants and the effect of drug encapsulation on the size and zeta potential is studied. The samples were being stores at three different temperatures and the size and zeta potential were measured at an interval of 7 days. Size, zeta potential, percentage of drug release and encapsulation efficiency was observed.

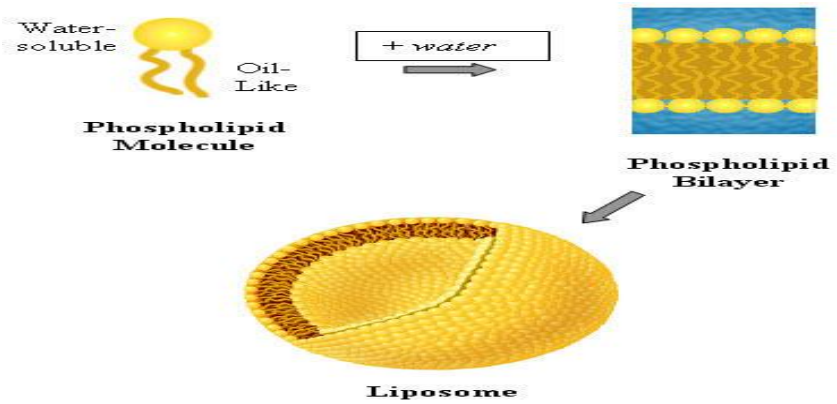


**Fig-2: Liposomes are made of water-soluble head and lipid-soluble tail; these are arranged as bilayer in presence of water. The bilayer will form a liposome which incorporates the drug and releases at the target tissue.**

## MATERIALS AND METHODS

### Materials

Diclofenac Sodium, Cholesterol, Phosphatidylcholine, Stearylamine, Dicetylphosphate, Chloroform and methanol are procured from chandra labs.

### Method of preparation

Stock solutions were prepared by using 9:1 ratio of chloroform and methanol and stored at $-20^0$C

Drug (DS): 1mg/ml

Phosphatidyl choline (PC): 100mg/ml

Cholesterol (CHOL): 15mg/ml

Stearyl amine (SA): 5mg/ml

Dicetyl phosphate (DCP): 5mg/ml

Phosphate buffer saline (PBS): 0.01M of PBS was prepared by dissolving one tablet into 200ml of distilled water.

Lipid hydration technique was used in the preparation of drug loaded and drug free liposomes. The above stock solutions were used at different compositions. Appropriate volumes were transferred by using a micro pipette into a dry 100ml round bottom flask. The rotary evaporator (RE-300)[1] was set to temperature of 40$^o$C, rotation speed was kept constant for all the preparation and the whole system. Round bottom flask was detached from the system after the solvent completely evaporated by releasing the vacuum. The film was hydrated by using PBS solution by vortexing (Whirl mixer) until the lipid film dissolved. The resultant milky suspension was transferred into a fresh bijou and tested for it size and zeta. The method was followed for all the other preparations.

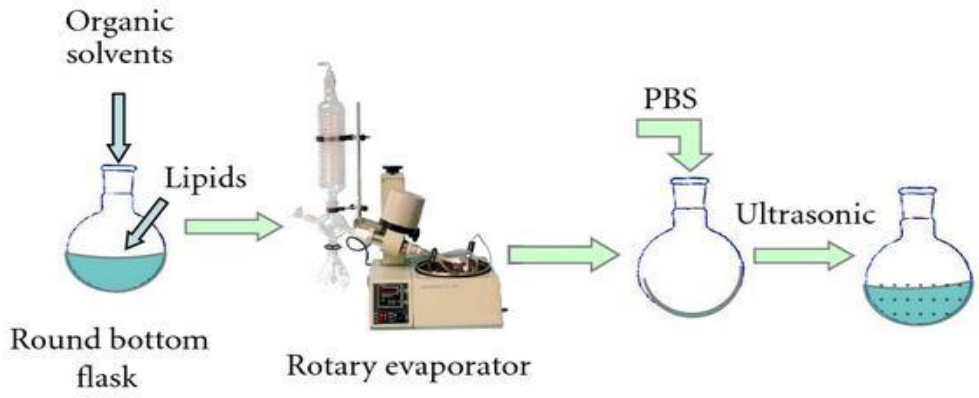


**Fig-3: Preparation of Liposomes using Rotary Evaporator.**

## RESULTS AND DISCUSSION

### Particle Size determination

In the present work, liposomes prepared with hydrogenated PC, cholesterol, stearylamine (positive charge inducer) and dicetylphosphate (negative charge inducer). The size of the drug-loaded liposomes is compared with that of the drug free liposome, and significant difference can be seen between drug free and drug loaded liposome. The interaction of DS with PC: CHOL shows the decrease in the diameter. Vesicular size decrease in presence of DS and the charge inducers, this would be due to penetration of charge inducers into phospholipids hydrocarbon chain. DS practically interacts with PC, affecting its polar head and acyl chain regions. The hydration of PC results filling water at polar inter-bilayer spaces with the resulting width increase. This may allow incorporation of DS into PC bilayer, which results in disruption of PC bilayer. The amphiphilic nature of DS locates into PC bilayer in such a way that its negatively charged carboxyl group interacts electrostatically with the positively charged terminal +N $(CH_3)^3$ group of phosphatidylcholine, while the non-polar ring sets in the hydrophobic acyl chain. The results are shown in the table 2 given below.

**Table-1:Compositions of drug loaded liposomes**

| Formulations | Phosphatidyl Choline (µmoles/ml) | Cholesterol (µmoles/ml) | Stearyl amine (µmoles/ml) | Dicetyl phosphate (µmoles/ml) | DS (mg/ml) |
|---|---|---|---|---|---|
| PC | 8 | 0 | 0 | 0 | 1 |
| PC | 16 | 0 | 0 | 0 | 1 |
| PC:CHOL | 16 | 8 | 0 | 0 | 1 |
| PC:CHOL | 16 | 16 | 0 | 0 | 1 |
| PC:CHOL:SA | 16 | 8 | 4 | 0 | 1 |
| PC:CHOL:SA | 16 | 8 | 8 | 0 | 1 |
| PC:CHOL:DCP | 16 | 8 | 0 | 4 | 1 |
| PC:CHOL:DCP | 16 | 8 | 0 | 8 | |

Note: *Phosphatidyl choline (PC), cholesterol (CHOL), stearylamine (SA), dicetylphosphate (DCP) and Diclofenac sodium (DS) are formulated in the above mentioned compositions*.

**Table-2: Particle size of the liposomes**

| Composition | Drug free (µm) | Drug-loaded (µm) |
|---|---|---|
| PC ( 8µ moles) | 8.74±1.17 | 8.5±1.2 |
| PC ( 16µ moles) | 9.09±0.34 | 7.7±0.3 |
| PC:CHOL ( 16:8µ moles) | 11.26±1.42 | 6.4±0.3 |
| PC:CHOL ( 16:16µ moles) | 10.52±2.6 | 7.7±0.8 |
| PC:CHOL:SA ( 16:8:4µ moles) | 6.78±0.34 | 5.8±0.1 |
| PC:CHOL:SA (16:8:8µ moles) | 29.07±10.82 | 20.7±14 |
| PC:CHOL:DCP ( 16:8:4µ moles) | 38.35±5.49 | 5.8±0.6 |
| PC:CHOL:DCP ( 16:8:8µ moles) | 42.69±13 | 22.3±3.8 |

**Zeta potential**

Zeta potential of the drug free and drug loaded liposomes did not prove much variation as shown in table 3, but the formulations with stearylamine and dicetylphosphate shows an increase charge and else than the other formulations, zetapotential of the charge induced liposomes have demonstrated increased zetapotential ranging from 55 to 60 which indicates enhanced stable suspension compared to the other formulations.

**Table-3: zeta potential of drug free liposomes**

| Zeta potential | | |
|---|---|---|
| **Composition** | **Drug free** | **Drug –loaded** |
| PC ( 8µ moles) | -2.59±0.74 | -4.1±0.6 |
| PC ( 16µ moles) | -2.00±1.1 | -2.7±0.7 |
| PC:CHOL ( 16:8µ moles) | -1.00±0.92 | -1.5±0.1 |
| PC:CHOL ( 16:16µ moles) | -0.77±0.9 | -18.7±1.3 |
| PC:CHOL:SA ( 16:8:4µ moles) | 54.83±3.26 | 59.4±1.7 |
| PC:CHOL:SA (16:8:8µ moles) | 60.27±1.22 | 53.1±2.4 |
| PC:CHOL:DCP ( 16:8:4µ moles) | -61.67±4.76 | -57.2±1.8 |
| PC:CHOL:DCP ( 16:8:8µ moles) | -58.50±2.59 | -52.6±1.3 |

**Encapsulation efficiency**

Encapsulation efficiency as shown in table 4 depends on the concentration ratio of the lipids. Percentage of drug encapsulated of PC (16µ moles) is higher than PC (8µ moles), which may be due to increased concentration. The vesicular dispersions of different encapsulating efficiencies are reported. The liposomes prepared with PC shows 20% increase in the % encapsulated to that of the lower concentrated vesicles. This is probably due to higher concentration which allows superior packing of the lipids producing more stable drug loading, this could also be observed with PC: CHOL compositions. Encapsulation efficiency of the formulations with positive and negative charge inducers are decreased when compared to other liposomes, presence of the penetration enhancer produced a de-stabilization of the liposomal bilayer and consequent partial aggregation and fusion of the vesicles which causes drug leakage from the bilayer. Presence of either SA or DCP produced decrease in % of drug encapsulated which may be due to formation of mixed micelles that leads to vesicle destruction; SA could be more suitable than the DCP formulations due to the advantageous structural similarity of PC and SA. The % drug encapsulated can be related as PC: CHOL > PC: CHOL: SA >PC: CHOL: DCP.

**Table-4 : Encapsulation efficiency (%) of drug loaded liposomes**

| Composition-Drug loaded | Encapsulation efficiency |
|---|---|
| PC ( 8µ moles) | 69.81±1.43 |
| PC ( 16µ moles) | 83.5±1.68 |
| PC:CHOL ( 16:8µ moles) | 44.37±3.31 |
| PC:CHOL ( 16:16µ moles) | 77.7±2.38 |
| PC:CHOL:SA ( 16:8:4µ moles) | 67.93±3.09 |
| PC:CHOL:SA (16:8:8µ moles) | 63.93±1.46 |
| PC:CHOL:DCP ( 16:8:4µ moles) | 32.34±0.87 |
| PC:CHOL:DCP ( 16:8:8µ moles) | 46.4±6.24 |

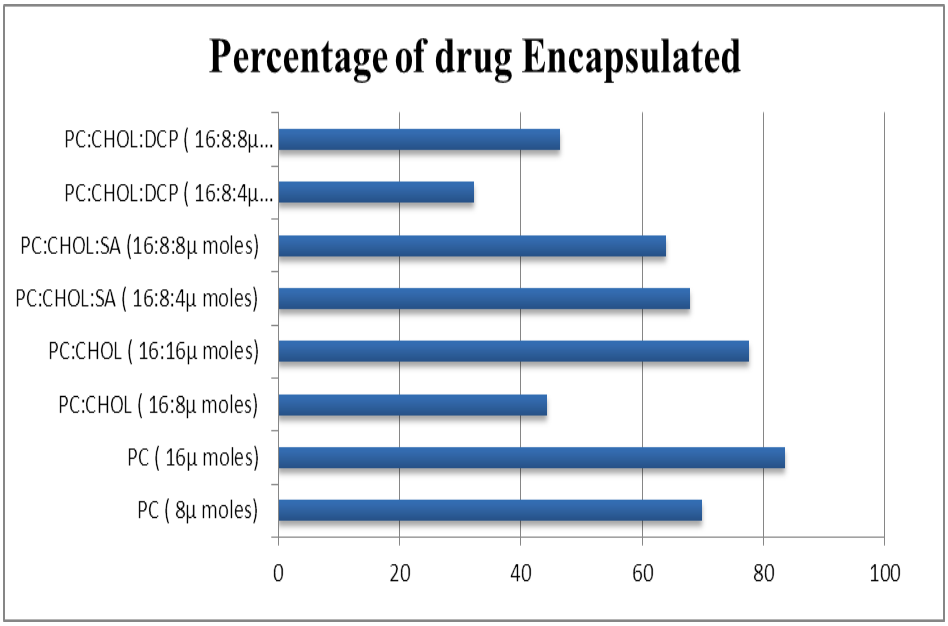


**Fig-4: Percentage of drug (Diclofenac sodium) encapsulated of drug loaded liposomes containing phosphatidyl choline (PC), cholesterol (CHOL), stearylamine (SA), and dicetylphosphate (DCP) was drawn against compositions.**

***In-vitro* Drug release study**

The results are analysed by single factor Analysis of Variance (ANOVA). *F-value* for PC: CHOL: DS and PC: CHOL: SA: DS was found to be 24.99 which is greater than *F-crit* (1.5), thus there is a significant difference between the results. *F-value* for PC: CHOL: DS and PC: CHOL: DCP: DS was found to be was found to be 32.08 which is greater than *F-crit* (1.5), thus there is a significant difference between the results. The *F-value* for PC: CHOL: SA: DS and PC: CHOL: DCP: DS was found to be was found to be 0.92 which is less than *F-crit* (1.5), thus there is no significant difference between the results.

The percentage of drug released as shown in table 5 of the liposomal formulations composed of PC: CHOL observed to be released at high rate than the formulations composed of

stearylamine and dicetylphosphate. This could be due to presence of the charged inducers, which reduces the leakage or permeability of the encapsulating membrane by decreasing the fluidity of the membrane, that the composition with high encapsulation efficiency has the low release rate. However, cholesterol significantly increases the release of DS and that could be explained by the fact that at certain concentration cholesterol can disrupt the regular linear structure of the liposomal membrane. Cholesterol at the particular ratio produces hydrophobicity, which decrease transient hydrophilic holes formation by decreasing the membrane fluidity, which obviously releases the drug though the liposomal bilayer. The SA and DCP formulated composition have slow rate of release when compared to cholesterol formulation, which release minimal amount of the drug 35.5 % and 36.7% of DCP and SA respectively after 24 hours period. This might be due to incorporation of drug into the charged vesicles and rapid ionization upon suspending in to the positively and negatively charged inducers. PC:CHOL compositions can be designed for rapid release of drug while PC:CHOL:SA and PC:CHOL:DCP can be considered for slow and controlled release of Diclofenac sodium, which is an advance stage for the development of liposomal preparations of Diclofenac sodium.

The percentage of drug of the compositions can be related as,

PC: CHOL > PC: CHOL: SA >PC: CHOL: DCP.

**Table-5: In-vitro drug release of drug loaded liposomes**

| **Sample time** | ***PC:CHOL:DS*** **% Drug release** | ***PC:CHOL:SA:DS*** **% Drug release** | ***PC:CHOL:DCP:DS*** **% Drug release** |
|---|---|---|---|
| 0.5 | 32.46±7.2 | 3.46±0.8 | 0.62±1.3 |
| 1 | 40.86±2.9 | 8.96±1 | 4.1±1.6 |
| 2 | 53.1±3.5 | 14.7±1.2 | 7.96±1.9 |
| 4 | 70.1±5.2 | 22.3±0.9 | 14.13±2.1 |
| 6 | 77.3±7.5 | 23.9±1.4 | 15.25±2.3 |
| 8 | 78.2±8.1 | 26.2±1.6 | 17.9±2.4 |
| 24 | 76.86±9.75 | 36.7±4.2 | 35.5±4.8 |

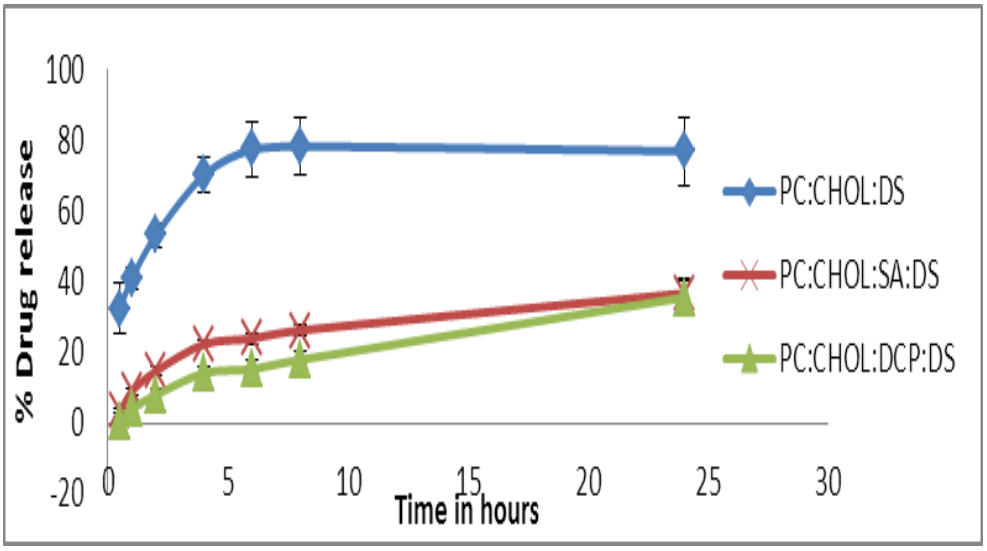


**Fig-5: The drug loaded liposomes of the compositions PC:CHOL:DS, PC:CHOL:SA:DS, PC:CHOL:DCP:DS were being studied for the amount of drug released, and the graph was plotted by taking time in hours on X-axis and % drug released on Y-axis.**

**Stability studies**

It can be observed from table 6 and 7 that the size of the vesicles was not much varied upon storing them for a period of 21 days at three different temperatures. But the variation in the zeta potential is clearly noticeable in case of PC: CHOL formulation. The formulations with SA and DCP have the zeta potential not with great difference. This is more likely because lack of surface charge can reduce the physical stability of the liposomes by increasing their aggregation. The charged surfaces not only increase the stability but also increase the intracellular uptake of liposome by target cells, and the positively charged liposomes deliver the drug contents to cells by fusion with cell membranes.

The preparation, lipid hydration also plays an important role in the physical stability of the liposomes. Water molecules bind with the PC polar head group by forming a hydration shell through hydrogen bonding with the polar group. In the presence of DS the orientation of water molecules may be affected, thus, the carboxylic group of the DS which is located near by the phosphate region could compete with water molecules to form hydrogen bonds, which weakens the affinity of PC to water, resulting in the less stable vesicle which aggregate upon storage. This could be overcome by incorporating DS along with the stearylamine and dicetylphosphate. The formulations with SA and DCP have the zeta potential not with great difference. The increase in surface charge can increase the physical stability of the liposomes

by decreasing their aggregation. It is evident that inducing charge amplifies zetapotential which leads to formation of extremely stable suspension.

The stability of the formulations can be related as,

PC: CHOL > PC: CHOL: DCP > PC: CHOL: SA

**Table-6 :Standard zetapotential values and their related stability behaviour**

| Zeta potential (mV) | Stability behaviour of the colloid |
|---|---|
| From 0 to ±5 | Rapid flocculation |
| From ±10 to ±30 | Incipient instability |
| From ±30 to ±40 | Moderate stability |
| From ±40 to ±60 | Good stability |

**Table-7: Data of the particle size and zeta potential during stability studies**

| **Particle size** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **$0^0$C temperature** | | | | **$25^0$C temperature** | | | | **$40^0$C temperature** | | | |
| Composition | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day |
| PC:CHOL:DS | 6.4±0.3 | 6.4±0.4 | 6.8±0.5 | 8.8±1.2 | 6.4±0.3 | 7.0±0.8 | 13.2±2 | 30.9±7 | 6.4±0.3 | 8.6±5.6 | 10.6±6 | 8.9±1.5 |
| PC:CHOL:SA:DS | 5.8±0.1 | 5.7±0.3 | 5.5±0.1 | 5.6±0.3 | 5.8±0.1 | 5.8±0.4 | 5.6±0.2 | 6.3±1.1 | 5.8±0.1 | 5.5±0.1 | 5.83±0.5 | 6.2±0.8 |
| PC:CHOL:DCP:DS | 5.7±0.6 | 5.6±0.4 | 5.8±0.1 | 5.7±0.5 | 5.6±0.6 | 5.7±0.6 | 6.7±1.7 | 10.2±4 | 5.7±0.6 | 6.5±3.2 | 7.6±2 | 6.6±0.2 |
| **Zeta potential** | | | | | | | | | | | | |
| | **$0^0$C temperature** | | | | **$25^0$C temperature** | | | | **$40^0$C temperature** | | | |
| Composition | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day | $0^{th}$ day | $7^{th}$ day | $14^{th}$ day | $21^{st}$ day |
| PC:CHOL:DS | -1.5±2.2 | -2.83±0.4 | -15.7±4.2 | -24.2±1.0 | -1.5±0.2 | -18.8±3.3 | -33.5±0.8 | -34.5±3.3 | -1.5±0.2 | -29.3±4.3 | -38.2±1.8 | -24.6±7.0 |
| PC:CHOL:SA:DS | 59.3±1.8 | 52.2±2.1 | 51.4±2.6 | 51.3±3.5 | 59.3±1.8 | 48.1±5.9 | 49.6±4.0 | 47.2±4.5 | 59.3±1.8 | 44.4±1.4 | 39.7±5.6 | 42.8±1.8 |
| PC:CHOL:DCP:DS | -57.2±1.8 | -59.7±2.9 | -63.5±4.0 | -61.6±2.3 | -57.2±0.8 | -57.2±4.2 | -60.8±3.0 | -66.7±4.9 | -57.2±1.8 | -61.5±5.8 | -68.5±2.5 | -67.8±2.5 |

## CONCLUSION

The main aim of the study was to prepare the liposomal suspension, and the characterization. The size of the drug loaded liposomes was decreased when compared to the drug free formulations. The entrapment efficiency and the release rate of cholesterol based Phosphatidylcholine liposomes were higher than that of the formulation composed with positive and negative charge inducers. Though the charge induced liposomes do not form the aggregates because of the charged bilayer, they were having the lower % of drug encapsulated and lower release rate but stearylamine acted as an exception by showing encapsulation efficiency of 67.93%. The initial preparations of the cholesterol based phosphatidylcholine liposomes were shown less charged bilayer, though upon storage they had the increased charge, the value is still less compared to positive and negative charge inducer. From table 3 the standard values indicate the DCP and SA formulated liposomes are extremely stable, these values are not altered during storage, which indicated that charge induced formulations, are highly stable. Drug release profile studies illustrated that PC:CHOL:DS based preparations can be formulated for rapid action and PC:CHOL:SA and PC:CHOL:DCP based preparations can be formulated for sustained or controlled release of drug. It can be concluded that the cholesterol based phosphatidylcholine liposomes with stearylamine at a concentration of 16:8:4 µmoles are most suitable to encapsulate the Diclofenac sodium.

## FUTURE DIRECTIONS

The liposomes have been proved to be the best drug carriers for a wide variety of drug and it has a lot of scope for incorporating highly toxic anti-inflammatory, anti-infective, anti-cancer and anti-AIDS drugs. The charge inducer produces the charged liposomes which are highly stable, but at the same situation they are poor for the drug encapsulation and releasing the drugs, thus much more studies have to be done to obtain an optimum concentration of the charge inducers to be used for formulating, which yield the best liposomal suspensions. Until now there were only a few tests conducted *in-vivo*, so there is a need to improve the studies *in-vivo* in-order to prove the pharmacological and toxicological investigations.

## ACKNOWLEDGEMENT

The authors are grateful to our respected Mr. Devendar reddy, the chairman of St.john College of Pharmacy for providing all necessary things to do this work.

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