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Preparation, Optimization and Evaluation of Liposomes Encapsulating Diclofenac Sodium and Charge Inducers to Enhance Stability Using Lipid Hydration Method

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ABSTRACT

The present study was aimed at preparation and characterization of Diclofenac Sodium encapsulating liposomes along with negative and positive charge inducers in order to increase the stability of liposomes. The liposomes consisting of phosphatidylcholine and cholesterol have been proven to be the best suitable formulation which provides amphiphilic nature and flexibility. The stability was increased by the addition of the charge inducers which improve the Zeta potential. Liposomes were prepared by Lipid-Hydration technique using rotary evaporator (RE-300) and Rotary evaporator is set at a temperature of 40^0C with constant rotation speed Instruments used during the study includes Whirl mixer, Hellos software, Zetasizer, Ultracentrifugation, Dialysis tube and UV Spectrophotometer. 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 Phosphatidylcholine shows increase in the percentage of drug encapsulated to that of the lower concentrated vesicles and positive charge inducer have exposed elevated encapsulation efficiency. Liposomes composed of PC: CHOL: SA observed to be released at high rate and stability studies confirm that PC:CHOL:SA is supreme stable at diverse temperatures. The composition PC: CHOL:SA at a concentration of 16:8:4 µmoles proved as a stable suspension with regard to zeta potential. From the outcome, it can be accomplished that cholesterol and stearylamine based phosphatidylcholine liposomes are most appropriate to encapsulate Diclofenac sodium.

1. INTRODUCTION

Since 1974 Diclofenac sodium (DS) has been used as a potent non-steroidal anti-inflammatory drug (NSAID) for its antipyretic and analgesic effects. DS is associated with inhibiting the activity of cyclooxygenase (COX) which produces prostaglandins. Similar to other NSAID, DS is also associated with serious gastrointestinal (GI) side effects like ulceration when administered orally and cutaneous lesions by intramuscular injection and it also undergoes first pass metabolism when administered orally. As the amphiphilic drugs have the tendency to form aggregates with celluloses, other different techniques have been followed to reduce the side effects. Incorporation of drug with organized structures is of great interest on drug absorption and targeting. Encapsulation by phospholipids has been suggested one of the ways to improve the condition and the drug causes the structural modification of phospholipids forming the surface active monomers. Recently, lipid-based formulations have been proven to improve the bioavailability of hydrophobic drugs like DS when compared with conventional dosage forms (L.B.Lopesa., et al 2004; L.B.Lopesa., et al 2006; Hongyu Piao., et al 2004).

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. There are a number of lipid species which reminds the surface active phospholipids among which phosphatidylcholine (PC) is suitable to incorporate DS. It has been suggested that presence of ionic surfactants DS shields the changes which are pH-dependant and the complex remains lipophilic (Masahiro Yamauchi., et al 2006).

Since 1970 liposomes have been widely used as drug carriers for improving the drug delivery to specific sites in the body. Liposomes are biocompatible and biodegradable. In nature, liposomes are made up of bilayer phospholipids; hydrophilic head faces the surface and the lipophilic tail away from the surface. Liposomes have proven to increase therapeutic effects and to reduce the side effects (D.D. Lasic., 1997).

Cholesterol also used as one of the ingredients which plays an important role in stabilising the liposomes, it increases the rigidity, thus improves encapsulation efficiency and increase in concentration decreases the release rate.

Stearylamine and Dicetylphosphate are the positive and negative charge inducers, which creates an electrostatic repulsion between the adjacent vesicles thus prevents from the formation of micelles and forms a highly stable suspension (Tze-Wen Chung., et al 2000; M. Manconib., et al 2009).

In present work, DS encapsulated into the liposomes are prepared by lipid hydration method along with the positive and negative surfactants and the effect of drug encapsulation on the size and zeta potential were experimented. The samples were being stored 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.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Diclofenac sodium, phosphatidylcholine, cholesterol, Dicetylphosphate, stearylamine was obtained from Chandu pharmaceuticals. Phosphate buffer saline of pH 7.4 was used as a blank. Chloroform and methanol were used as solvents for the stock solutions preparation and distilled water was used as a solvent for buffer.

2.2. Instrumentation

Rotary evaporator (RE-300) for liposome preparation, Whirl mixer, Hellos software (size analysis), Zetasizer (zeta potential), Ultracentrifugation, Dialysis tube (*in-vitro* studies) and UV Spectrophotometer.

2.3. Stock solutions

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

Drug (DS): 1mg/ml

Phosphatidylcholine (PC): 100mg/ml

Cholesterol (CHOL): 15mg/ml

Stearylamine (SA): 5mg/ml

Dicetylphosphate (DCP): 5mg/ml

Phosphate buffer saline (PBS) – pH 7.4: 0.01M of PBS was prepared by dissolving one tablet into 200ml of distilled water.

2.4. Preparation of standard solution

To prepare standard curve 10mg of the drug (DS) was dissolved in 10ml of phosphate buffer saline (pH–7.4), which gives a concentration of 1mg/ml. This was treated as the stock solution and the serial dilutions 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 30µg/ml and 40µg/ml and their absorbance were measured by UV spectroscopy at 275nm wavelength. The calibration points were estimated by UV method.

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2.5. Preparation and characterisation

Liposomes were prepared by Lipid-Hydration technique. These are Phosphatidylcholine (PC) based liposomes, which composed of cholesterol (CHOL), Stearylamine (SA) and Dicetylphosphate (DCP) at different compositions as mentioned in table 1. 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 micropipette into a dry 100ml round bottom flask. The rotary evaporator (RE-300) was set to a temperature of 40^{o}C, rotation speed was kept constant for all the preparation and for the whole system. Round bottom flask was detached from the system by releasing the vacuum after the solvent is completely evaporated (Malcolm n. Jones., et al 1997). The film was hydrated by using PBS solution by vortexing (Whirl mixer) until the lipid film is dissolved. The resultant milky suspension was transferred into a fresh bijou and tested for its size and zeta. The method was followed for all the other preparations (Manjanna K.M., et al 2009).

The resultant suspension is characterised for Size (Hellos software), Zeta Potential (Zetasizer), encapsulation efficiency by measuring the absorbance at 275nm of the supernatant of the suspension, percentage of drug release (Dialysis tube) was measured and the drug stability studies were conducted by storing in a stability chamber at 0^{o}C, 25^{o}C, and 45^{o}C to observe the behaviour of properties like size and zeta potential for a period of 21 days, by measuring the size and zeta potential at an interval of 7 days for a period of 21 days, by measuring the size and zeta potential at an interval of 7 days.

Table 1: Compositions of drug loaded liposomes: Phosphatidyl choline (PC), cholesterol (CHOL), stearylamine (SA), dicetylphosphate (DCP) and diclofenac sodium (DS) are formulated in the above mentioned compositions

Formulations	Phosphatidyl Choline (µmoles/ml)	Cholesterol (µmoles/ml)	Stearyl amine (µmoles/ml)	Dicetyl phosphate (µmoles/ml)	Diclofenac Sodium (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	1

Standard curve

Absorbance of the prepared standard solutions is obtained from UV spectrophotometer at 275nm wavelength (Figure 1).

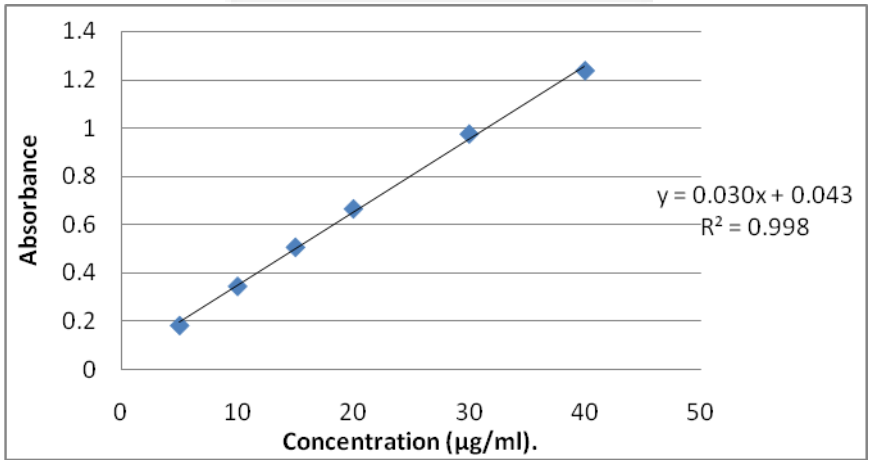

Figure 1: Calibration curve; standard curve was drawn by taking concentration on X-axis and absorbance on Y-axis, the absorbance was measured by UV spectroscopy.

3. RESULTS AND DISCUSSION

3.1. Particle size of the drug loaded liposomes was observed to be decreased when compared to that of the drug free. This might be due to the amphiphilic nature of the DS, which locates it 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 table 3.

3.2. Zeta potential of the drug free and drug loaded liposomes did not prove much variation (table 3), but the formulations with stearylamine and dicetylphosphate show an increased charge and else than the other formulations, zeta potential of the charge induced liposomes have demonstrated increased zeta potential ranging from 55 to 60 which indicates enhanced stable suspension compared to the other formulations (table 2).

Table 2: Standard zeta potential values and their related stability behaviour

Zeta potential (mV)	**Stability behaviour**
0 to ±5	Swift flocculation
±10 to ±30	Initial instability
±30 to ±40	Modest stability
±40 to ±60	Fine stability
More than ±61	Tremendous stability

Table 3: Particle size and Zeta potential of the drug free and drug loaded liposomes.

Composition	Particle size		Zeta potential	
	Drug free (µm)	Drug-loaded (µm)	Drug free	Drug - loaded
PC (8µ moles)	8.74±1.17	8.5±1.2	-2.59±0.74	-4.1±0.6
PC (16µ moles)	9.09±0.34	7.7±0.3	-2.00±1.1	-2.7±0.7
PC:CHOL (16:8µ moles)	11.26±1.42	6.4±0.3	-1.00±0.92	-1.5±0.1
PC:CHOL (16:16µ moles)	10.52±2.6	7.7±0.8	-0.77±0.9	-18.7±1.3
PC:CHOL:SA (16:8:4µ moles)	6.78±0.34	5.8±0.1	54.83±3.26	59.4±1.7
PC:CHOL:SA (16:8:8µ moles)	29.07±10.82	20.7±14	60.27±1.22	53.1±2.4
PC:CHOL:DCP (16:8:4µ moles)	38.35±5.49	5.8±0.6	-61.67±4.76	-57.2±1.8
PC:CHOL:DCP (16:8:8µ moles)	42.69±13	22.3±3.8	-58.50±2.59	-52.6±1.3

3.3. Encapsulation efficiency of the drug loaded liposomes prepared with PC proves an increase in the percentage of drug 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 negative inducers was decreased when compared to rest of formulations of liposomes, the presence of the negative charge inducer destabilises the liposomal bilayer and consequent partial aggregation and fusion of the vesicles which leaks out the drug from the bilayer. Presence of DCP produced decrease in % of drug encapsulated which may be due to the formation of mixed micelles that leads to vesicle destruction (Table 4) (Figure 2). Presence of positive charge inducer have demonstrated increased entrapment efficiency which is 67.93%, but this assessment is less compared to PC and PC:CHOL compositions.

Table 4: percentage of drug encapsulated.

Composition-Drug loaded	% Drug Encapsulated
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

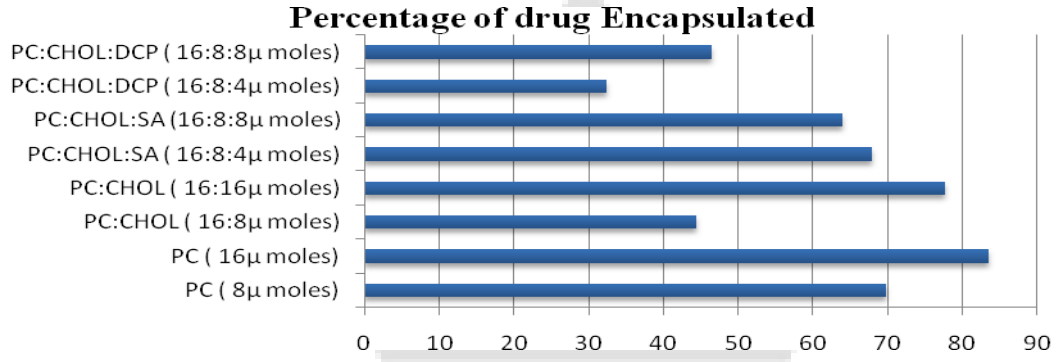

Figure 2: Graphical representation of Percentage of drug (Diclofenac sodium) encapsulated

3.4. In-vitro Percentage drug release studies of the liposomal formulations were conducted and the liposomes 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 charge inducers, which reduces the leakage or permeability of the encapsulating membrane by decreasing the fluidity of the membrane. However, cholesterol significantly increases the release of the drug and that could be explained by the fact that at certain concentration CHOL can disrupt the regular linear structure of the liposomal membrane. The SA and DCP formulated composition have slow rate of release when compared to cholesterol formulation, which releases 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 into the positively and negatively charged inducers (Table 5) (Figure 3). PC:CHOL compositions can be designed for rapid

release of the drug while PC:CHOL:SA and PC:CHOL:DCP can be considered for slow and controlled release of Diclofenac sodium, which is an advanced stage for the development of liposomal preparations of Diclofenac sodium.

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

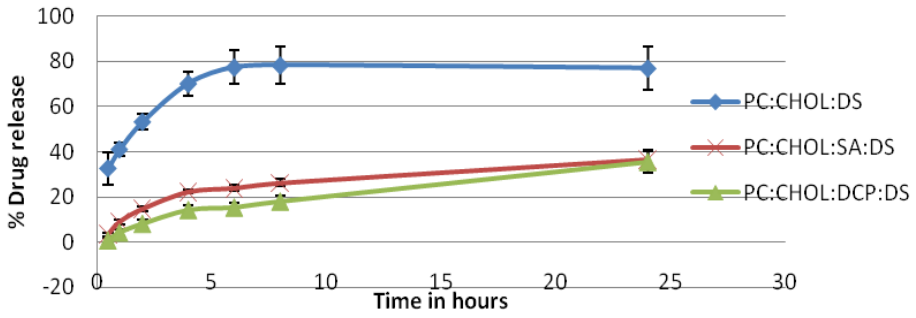

Figure 3: The drug loaded liposomes of the compositions were being studied for the amount of drug released and the graph was plotted by taking time in hours on x-axis and % drug released on y-axis.

3.5. Stability studies release the data of accelerated studies. It was observed that the size of the vesicles was not much varied upon storing them for a period of 21 days at three different temperatures in a stability chamber (Table 6). But the variation in the zeta potential is clearly noticeable in the case of PC: CHOL formulation that is due to increased negative charge of the vesicles. 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 (Table 7). From the table 7 and table 3, it is evident that inducing charge amplifies zeta potential, which leads to formation of extremely stable suspension. Graphical representation of stability storage variations of particle size and zeta potential is illustrated in figure 4.

Table 6: Data of the particle size during stability studies

PARTICLE SIZE (µm)				
	COMPOSITION/ NO.OF DAYS	PC:CHOL:DS	PC:CHOL:SA:DS	PC:CHOL:DCP:DS
0^0C	0^{th} Day	6.4±0.3	5.8±0.1	5.7±0.6
	7^{th} Day	6.4±0.4	5.7±0.3	5.6±0.4
	14^{th} Day	6.8±0.5	5.5±0.1	5.8±0.1
	21^{st} Day	8.8±1.2	5.6±0.3	5.7±0.5
25^0C	0^{th} Day	6.4±0.3	5.8±0.4	5.6±0.6
	7^{th} Day	7.0±0.8	5.8±0.4	5.7±0.6
	14^{th} Day	13.2±2	5.6±0.2	6.7±1.7
	21^{st} Day	30.9±7	6.3±1.1	10.2±4
$4O^0$C	0^{th} Day	6.4±0.3	5.8±0.1	5.7±0.6
	7^{th} Day	8.6±5.6	5.5±0.1	6.5±3.2
	14^{th} Day	10.6±6	5.83±0.5	7.6±2
	21^{st} Day	8.9±1.5	6.2±0.8	6.6±0.2

Table 7: Data of the zeta potential during stability studies

ZETA POTENTIAL				
	COMPOSITION/ NO.OF DAYS	PC:CHOL:DS	PC:CHOL:SA:DS	PC:CHOL:DCP:DS
0^0C	0^{th} Day	-1.5±2.2	59.3±1.8	-57.2±1.8
	7^{th} Day	-2.83±0.4	52.2±2.1	-57.2±1.8
	14^{th} Day	-15.7±4.2	51.4±2.6	-63.5±4.0
	21^{st} Day	-24.2±1.0	51.3±3.5	-61.6±2.3
25^0C	0^{th} Day	-1.5±0.2	59.3±1.8	-57.2±0.8
	7^{th} Day	-18.8±3.3	48.1±5.9	-57.2±4.2
	14^{th} Day	-33.5±0.8	49.6±4.0	-60.8±3.0
	21^{st} Day	-34.5±3.3	47.2±4.5	-66.7±4.9
$4O^0$C	0^{th} Day	-1.5±0.2	59.3±1.8	-57.2±1.8
	7^{th} Day	-29.3±4.3	44.4±1.4	-61.5±5.8
	14^{th} Day	-38.2±1.8	39.7±5.6	-68.5±2.5
	21^{st} Day	-24.6±7.0	42.8±1.8	-67.8±2.5

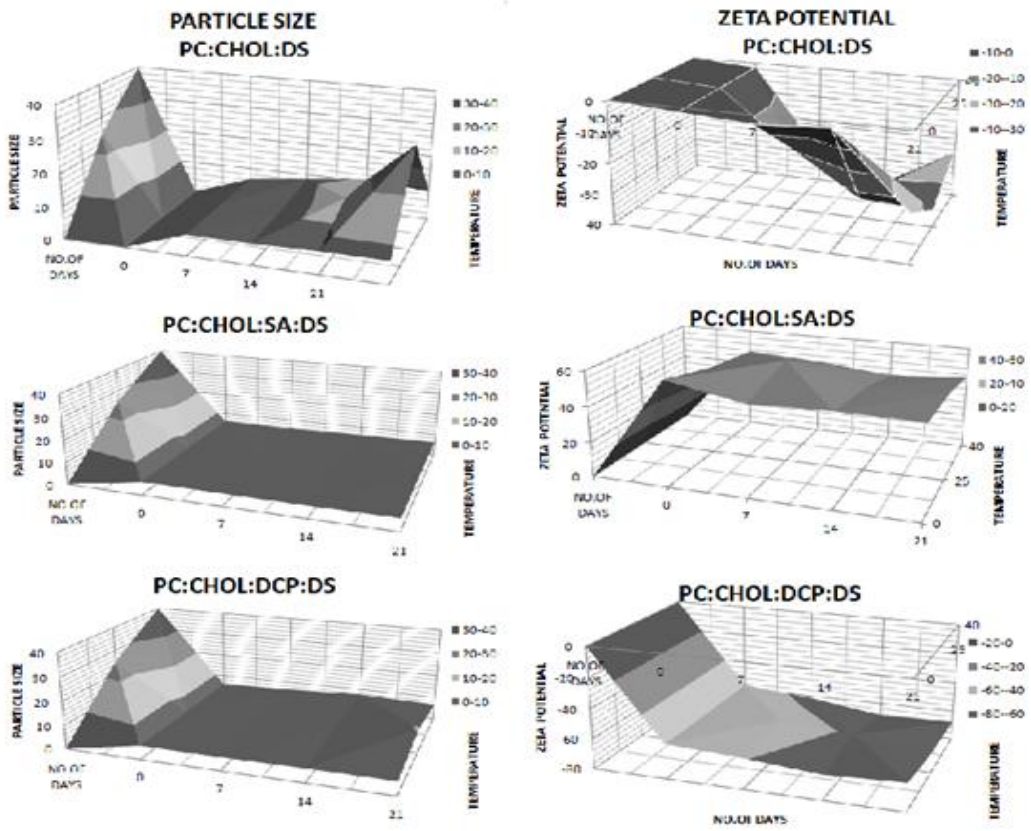

Figure 4: Particle size and zeta potential of various compositions of drug loaded liposomes which are stored in the stability chamber at specified temperature (storage conditions).

4. 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 2 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.

5. ACKNOWLEDGMENT

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