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Celecoxib – encapsulated liposomes of long alkyl chain lipids: Formulation, characterization and *in vitro* performance

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ABSTRACT

The objective of the present study was to develop liposomal drug delivery system for a hydrophobic non steroidal anti-inflammatory drug, celecoxib. The celecoxib liposomes were prepared using different long alkyl chain lipids, DPPC and DSPC. All the formulations were characterized for their vesicle size profile and drug entrapment. The influence of cholesterol on drug entrapment and release pattern was analyzed. The present work showed that liposome with less amount of cholesterol (lipid/cholesterol mole ratio of 4:1) could produce good drug entrapment as well as retention behavior. Thirty days stability study showed that drug entrapment and size profile were stable in both refrigerated and room temperature. SEM analysis confirmed that liposomal samples were spherical shaped and showed concentric lamellae. Release profile showed that it follows zero order kinetics and mechanism of drug release is of diffusion. Thus, new, reproducible liposomes of celecoxib with good stability and appreciable controlled drug release with good retention of the drug for more than a day were prepared successfully.

Keywords: Celecoxib, DPPC, DSPC, Scanning electron microscopy, Stability.

INTRODUCTION

Directing drugs to particular cells continues to be almost as great a challenge as it was shortly after the turn of the century, when Paul Ehrlich first proposed the concept of a drug delivery system that would target only diseased cells the “magic bullet.” Drugs that are most potent are also the most toxic because often less than 1% of the injected drug reaches its target while the rest damages healthy cells [1]. For parenteral administration of drugs that might be targeted to specific cells, small particulate carriers are required. Emulsions, micro emulsions, micelles, microspheres have all been investigated, but by far the most widely studied approach makes use of liposomes. Small spherical vesicles called liposomes form naturally when lipids or phospholipids are dispersed in an aqueous solution. Thus, liposomes are biocompatible and biodegradable [2]. Liposomes can be made commercially to precise specifications, and drugs can be carried by them. With appropriate surface molecules incorporation, liposomes can be targeted to specific cell type. So they are a possible answer to the question of how to increase the delivery of the drugs to diseased cells without increasing toxic effects on healthy cells. Avoiding recognition and destruction by the immune system, a major problem for any drug delivery system has only recently been achieved.

The celecoxib that is selected here as the model for hydrophobic drug [3], is the first candidate of the coxibs, a specific cyclo oxygenase - 2 inhibitor [3], a NSAID family that is frequently used for treating arthritis comparable to other NSAIDs [4]. It is also used in the treatment of familial adenomatous polyposis and in colorectal adeno carcinomas [5, 6]. The COX inhibitors are usually been associated with adverse cardio vascular side effects due to an imbalance in the production of prostacyclin (vasodilator) and thromboxane (vaso constrictor) [7, 8]. Since celecoxib has large volume of distribution (455±166 liters) [9, 10] and high plasma protein binding (97%) [11], oral formulations of celecoxib are administered at high doses, increasing risks of cardiovascular side effects further [12]. To get rid of all the systematic side effects associated with celecoxib and to enhance the therapeutic activity we decided to incorporate the same in liposome, which can passively target the inflammatory area by extravascularization in inflammatory arthritis. In the present study, celecoxib encapsulated liposomes were prepared using longer alkyl chain lipids to avoid poor encapsulation and retention of drug which is one of the usual draw backs of liposomal drug delivery system prepared using natural phospholipids. Further in this study we optimized the cholesterol content in liposome composition *in vitro*, because cholesterol is the important component which will give rigidity to the membrane structure by controlling permeability and enhancing plasma stability [13]. We investigated the effect of cholesterol on percentage drug encapsulation, vesicle size and stability. In addition we investigated the release profile of prepared formulations to attain controlled as well as prolonged delivery of drug and there by reducing the potential dose related side effects.

MATERIALS AND METHODS

Materials

Celecoxib was a generous gift from aurobindo Pharma, Hyderabad. Dipalmitoyl phosphatidyl choline, (DPPC) and distearoyl phosphatidyl choline, (DSPC) were kindly gifted by lipoid, Switzerland. High purity cholesterol, sigma membrane (12000 MW cutoff) were obtained from Sigma Aldrich, Hyderabad. Pottassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, chloroform, methanol, ethanol were purchased from commercial source.

Preparation of celecoxib liposomes [14, 15]

Celecoxib liposomes were prepared using the thin film hydration technique. A drug-lipid solution phase was prepared by dissolving accurately weighed quantities of drug, DPPC or DSPC with or without cholesterol in a solvent mixture of chloroform and methanol (2:1, v/v) in a 250ml round bottom flask. A layer of thin lipid film was formed in the wall of flask by evaporating the organic solvent using rotary evaporator (HS 3001 NS) under reduced pressure. During the process, the instrumental conditions such as temperature (55°C) and speed (150rpm) were maintained constant. The flask was left overnight in a vacuum desiccator to ensure the complete removal of residual solvents. Phosphate buffer saline pH7.4 was used as the hydration media to hydrate the lipid film at 55±2° temperature. The suspension thus obtained was vortexed for about 2min followed by a stand rest for 2-3 hours to allow complete swelling of the lipid film. The resultant suspension was sonicated for 3min using probe sonicator. Five formulations (CLB1 – CLB5) were prepared using different lipid – cholesterol ratio and characterized with respect to vesicle size and its distribution, shape, % drug encapsulation and % drug release. (Table 1)

Estimation of percentage drug encapsulation [16, 17]

The percentage of drug encapsulation was determined by centrifugation. The prepared liposomal formulations were subjected to centrifugation using refrigerated centrifuge at 10,000rpm at 4°c twice each of 30min duration with 10min gap to separate free drug from entrapped drug. Thus the clear supernatant was collected and analyzed by UV spectrophotometer for drug content after appropriate dilution. The pellet obtained after centrifugation was lysed in absolute alcohol and sonicated for 10min. The concentration of celecoxib was determined in alcohol spectro photometrically at 251.2nm using a UV visible spectrophotometer. Percentage drug encapsulation of the liposomes was calculated as follows: [(T-C)/T] *100, where as T is the total amount of drug that is calculated both in supernatant and sediment and C is the amount of drug found only in the supernatant. Percentage drug encapsulation was calculated for 3 formulations of each formulation code and average was tabulated (Table 1)

Fourier transform, infrared (FTIR) study

All the excipents such as DPPC, DSPC, cholesterol individually, physical mixture of excipients, pure drug celecoxib, physical mixture of excipients as well as drug were mixed separately with infrared (IR) grade KBr in the ratio of 1:100 and corresponding pellets were prepared by applying 15000 lb of pressure in a hydraulic press. The

pellets were scanned in an inert atmosphere over a wave number range of 4000-400 cm^{-1} in Magna IR 750 series II (Nicolet, USA) FTIR instrument.

Vesicle size distribution profile [14, 18]
All the prepared liposomal batches were monitored for their morphological attributes with the help of binocular compound microscope (optics) at suitable magnification. Aggregation and precipitation were detected using the same. Then the selected formulations were studied with the help of scanning electron microscopy for their morphology. (Figure 6)

Stability analysis [19, 20]
The stability profile of the vesicles was assessed by storing the liposomal suspension at 4 different temperatures i.e. (4-8ºC (refrigerator RF), 25 ±2ºC (room temperature RM), 37±2ºC and 45 ±2ºC for a period of 30 days. During the study, the liposomal suspension were kept in sealed vials of 10ml capacity. The drug content of the vesicles was determined periodically every 10 days as given in estimation of % drug encapsulation.

In vitro drug release [21]
Modified USP XXI dissolution rate model was used for the determination of drug release from liposomic preparation. This model consists of a beaker (250ml) and a plastic tube of diameter 17.5mm opened from both the ends. Sigma membrane (Sigma 12000 MW cutoff) was tied at one end of the tube & the other end left free. This assembly was dipped in to the beaker containing 90ml of dissolution medium. The temperature was maintained at 37 ±1ºC. 10ml of liposomal suspension was added in to the tube and a paddle type stirrer was placed in the center of the beaker. The speed of the stirrer was maintained at 100rpm. Dissolution sample of 1ml was withdrawn periodically every one hour up to 24 hours and analyzed spectrophotometrically at 251.2nm. With the help of the standard curve prepared earlier, drug concentration was measured.

RESULTS AND DISCUSSION

A highly hydrophobic molecule, celecoxib was encapsulated in multi lamellar liposomal vesicles composed of longer alkyl chain lipids such as DPPC and DSPC without cholesterol. (Table – 1) Lipid analysis showed that more than 98.9% (≈ 99%) of the lipids were pelleted after centrifugation of the hydrated films and therefore they were incorporated in to multilamellar liposomal vesicles. In our study first two formulations were prepared using 2 different longer alkyl chain lipids, DPPC (16 alkyl chain length), DSPC (18 alkyl chain length) without cholesterol to find out the influence of alkyl chain length on characterization of liposomes. 10mg of the drug was incorporated in to each formulation which was optimized as per previous studies (results not shown). As shown in the results (Table -1), it was found that liposomes prepared with DSPC, (CLB2) has shown greater drug entrapment i.e. 93.6% ±1.11% with good retention behavior of the drug during release studies even after 24 hours. (60.57%) Therefore CLB2 formulation was selected to find the influence of cholesterol on characteristics of liposomes, because cholesterol is one of the components which will give rigidity to the membrane structure, in addition to the major lipid component, DSPC. For this purpose 3 different formulations were prepared using different DSPC/cholesterol mole ratio (4:1, 2:1, 1:1). Along with 100mg of DSPC, 12mg, 24mg & 50mg of the cholesterol respectively was used in these three formulations (Table – 1). It was found that presence of cholesterol reduces % drug encapsulation in the following order CLB2>CLB3>CLB4>CLB5 interestingly, although DSPC/ cholesterol composition of CLB5 routinely used as starting composition regarding conventional liposomes. Later when we came across some literature, we came to know that high content of cholesterol may decrease the percentage encapsulation of hydrophobic molecules. Some of them are given below: liposomal MLVs prepared with egg phosphatidyl choline could encapsulate 29.5% of ibuprofen, when ibuprofen encapsulation reduced to 23.2% with 30% of cholesterol and even to 17.1% with 50% of cholesterol [22]. In a study of developing cremophor – EL free liposomal paclitaxel formulation, it was seen that increasing content of cholesterol decreased the drug loading efficiency dramatically from 99.3 to 6.2% respectively [23]. However in some cases increasing amount of cholesterol showed increasing drug encapsulation efficiency, for example Bhatia et all reported 30% cholesterol addition lead to increased drug entrapment efficiency from 45.2% – 57.5% [14]. So there is variation on the encapsulation efficiency of hydrophobic molecules due to the increasing or decreasing amount of cholesterol. These effects may be due to molecular interaction between the phospholipids, cholesterol and drug. But in general, cholesterol increases the hydrophobicity of the bilayered membrane which may favor the inclusion of hydrophobic molecules [24]. On the other hand, considering the conflicting fact that cholesterol may prefer to accommodate it in the hydrophobic bilayered structure which is of having the limited space. So there might be the competition between cholesterol and

drug in getting aligned them for this space between the alkyl chains of phospholipids resulted in lower encapsulation with increasing cholesterol content.

Drug, excipients interaction was studied before developing the formulation by using FTIR-spectroscopy, which is one of the most important analysis to describe about the stability of formulation, presence of drug & drug release. Fig. 3 shows minor shifting of some peaks compared with individual excipients (Fig. 1 and Fig. 2), like aliphatic alcoholic O-H stretch (3420.26 to 3410.59), C=O stretch of ester (1740.48 to 1739.78), C-O stretching of hydroxyl group. (1054.90 to 1058.49) Minor shifts were observed when the fig. 5 compared with spectrum of pure drug (Fig. 4) and excipients (Figure 1 and Figure 2) like, aliphatic N-H stretching (3410-3342), C-O stretch of ester. These shifts observed may be due to the formation of hydrogen bonds, vanderwaal attractive forces or dipole moment which are weak forces seen in the polar functional groups of drugs and excipients. The frequency of absorption due to the corbonyl group depends mainly on the force constant which in turn depends upon inductive effect, conjugative effect, field effect, steric effects. The shifts seen due to the above mentioned interaction may however support the formation of favorable vesicle shape, structure with good stability and sustained drug release.

The vesicle size of the liposome was found to be in the range of 5.7±0.8- 6.2±0.3µm with 90% population of the liposomes equal or below 6µm. Most of the vesicle was found to be spherical in shape. Log-size distribution curve confirms the normal size distribution of the vesicles. Size analysis was repeated for 3 formulations of each formulation code and vesicle size data was compared. Data was found to be highly reproducible every time. The SEM photograph of CLB3 liposomal formulation showed that vesicles are homogeneous and spherical in shape. (Figure 6), (Table 1)

There is no evident for aggregation, fusion or disruption of the vesicles during the studied period and it was found that the prepared formulations were able to retain their multilamellar nature and shape uniformity to an appreciable extent. The bar diagram shows the % drug leakage from the lipid vesicles over the period of 30 days at different storage temperature. It was found that samples stored at elevated storage temperatures, i.e. 37º ± 2ºC and 45º ±2ºC showed that the % drug leakage of the samples varied from 6% - 18%. On the other hand liposomes stored at lower temperatures i.e. room temperature and refrigerated temperature showed that they could retain 95% and 97% of the encapsulated drug respectively. (Fig. 7)

Among the formulations prepared, CLB1 liposomes prepared with DPPC alone could release the highest amount of the drug than CLB2, which is prepared with DSPC alone. Among the four composition used in the present study using DSPC, liposomes prepared with DSPC without cholesterol could release the highest amount of drug followed by DSPC/cholesterol groups of CLB3, CLB4 & CLB5 in decreasing order. DSPC liposomes could retain 54% of the initial drug content where as the formulations CLB3 containing 12mg of cholesterol could retain 65% of their drug content respectively. When the % of drug retained in MLVs were normalized to lipid content of each samples, CLB2 liposomes (DSPC alone) was seen to retain the drug remarkably lower where as other liposomes could retain the drug at higher percentage, supporting the result that highest extent of drug release occurred in cholesterol free formulation.(Fig. 8) This trend of drug release may be due to the fact that cholesterol might locate itself to the glycerol back bone of the membrane pushing celecoxib to the inner core which may explain the decreased release rates with increasing concentrations of cholesterol. The drug release was not of the sudden burst in any of the prepared formulation. Drug release from all the prepared formulation followed zero order kinetics and release mechanism was of diffusion. This was conformed by regression values of the respective plots. (Table -2)

Table 1: Formulae for different batches and physical characteristics of prepared liposomes of celecoxib

S. NO	Drug (mg)	CH (mg)	DPPC (mg)	DSPC (mg)	% EE	% Release	Particle Size (µm)
CLB1	10	--	100	--	91.47±1.02	48.24	5.9±0.2
CLB2	10	--	--	100	93.6±1.11	46.43	6.1±0.8
CLB3	10	12	--	100	90.7±1.21	35.45	6.2±0.3
CLB4	10	24	--	100	78.06±1.55	31.05	5.8±0.1
CLB5	10	50	--	100	65.9±1.31	22.62	5.7±0.8

Abbreviations Used: CH-Cholesterol, DPPC-Dipalmitoyl phosphatidylcholine, DSPC-Distearoylphosphatidylcholine, %EE- Percentage encapsulation efficiency.

Fig. 1: FTIR Spectrum of Pure DSPC

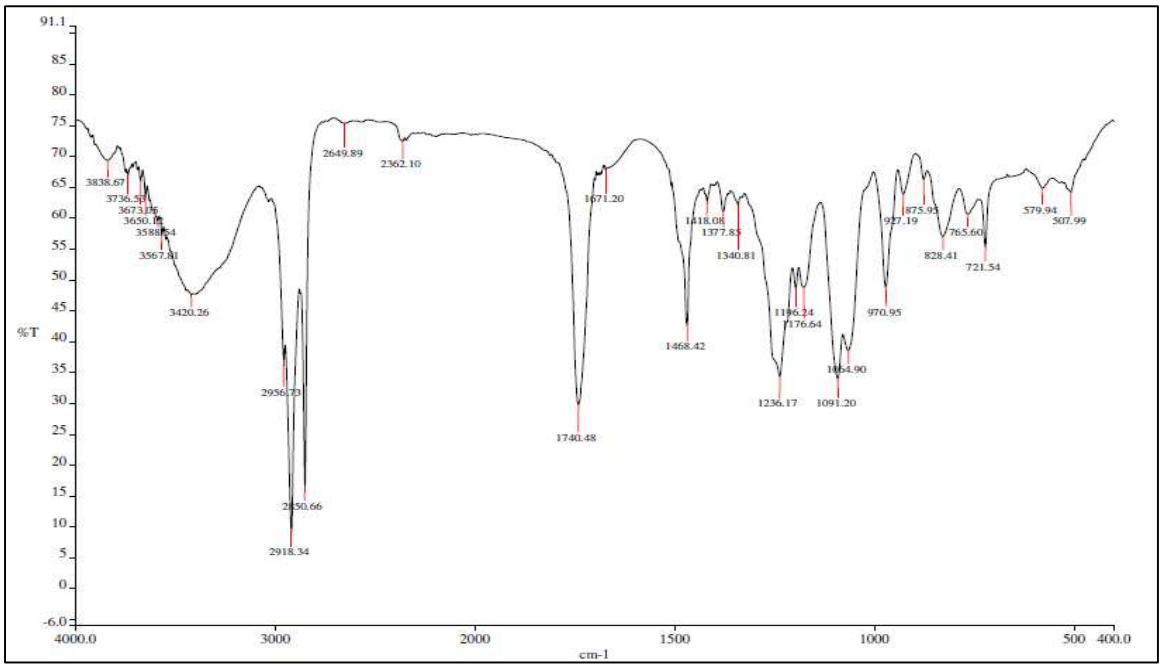


Fig. 2: FTIR Spectrum of Pure cholesterol

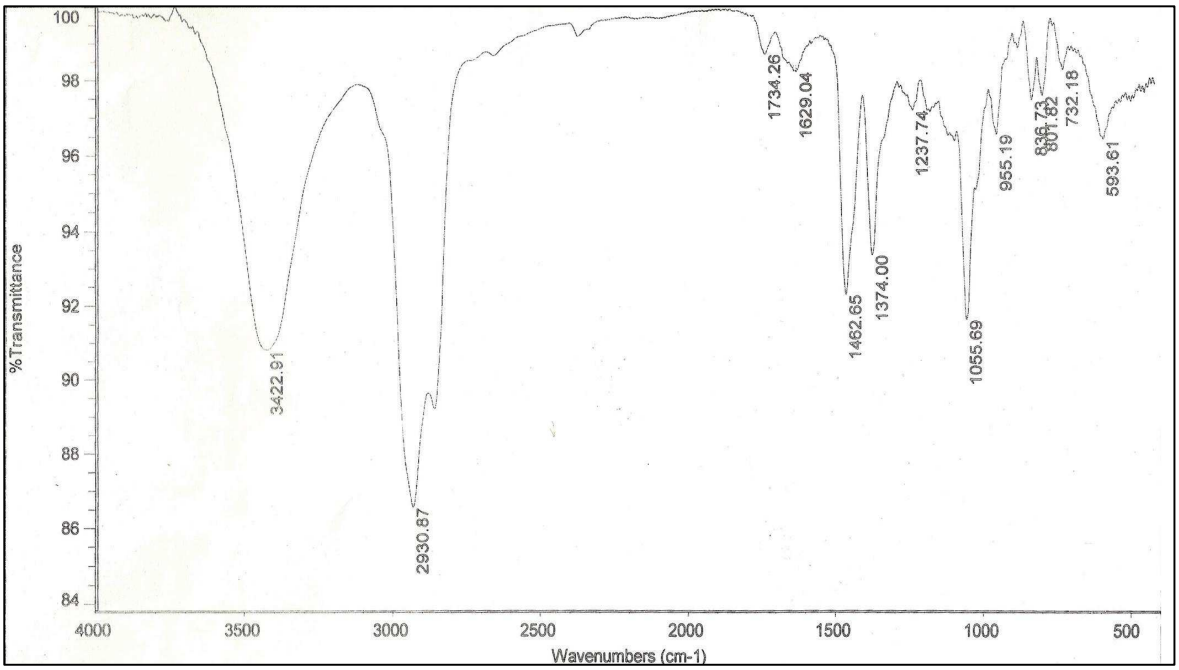


Fig. 3: FTIR Spectrum of physical mixture of excipients

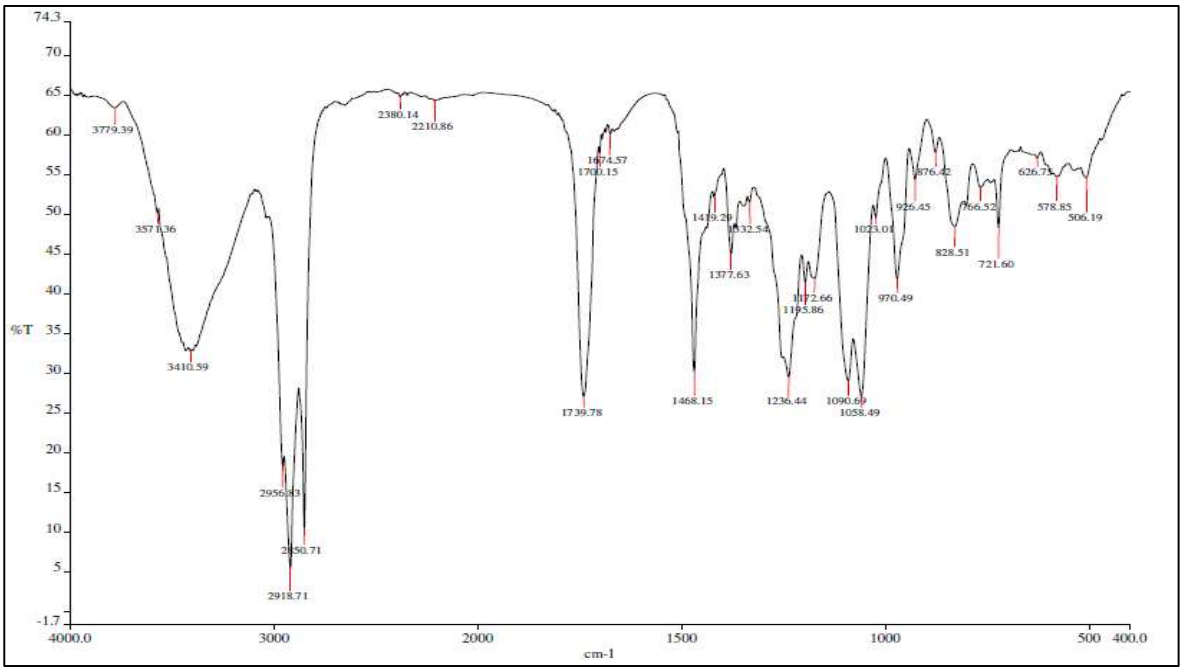


Fig. 4: FTIR Spectrum of Pure celecoxib

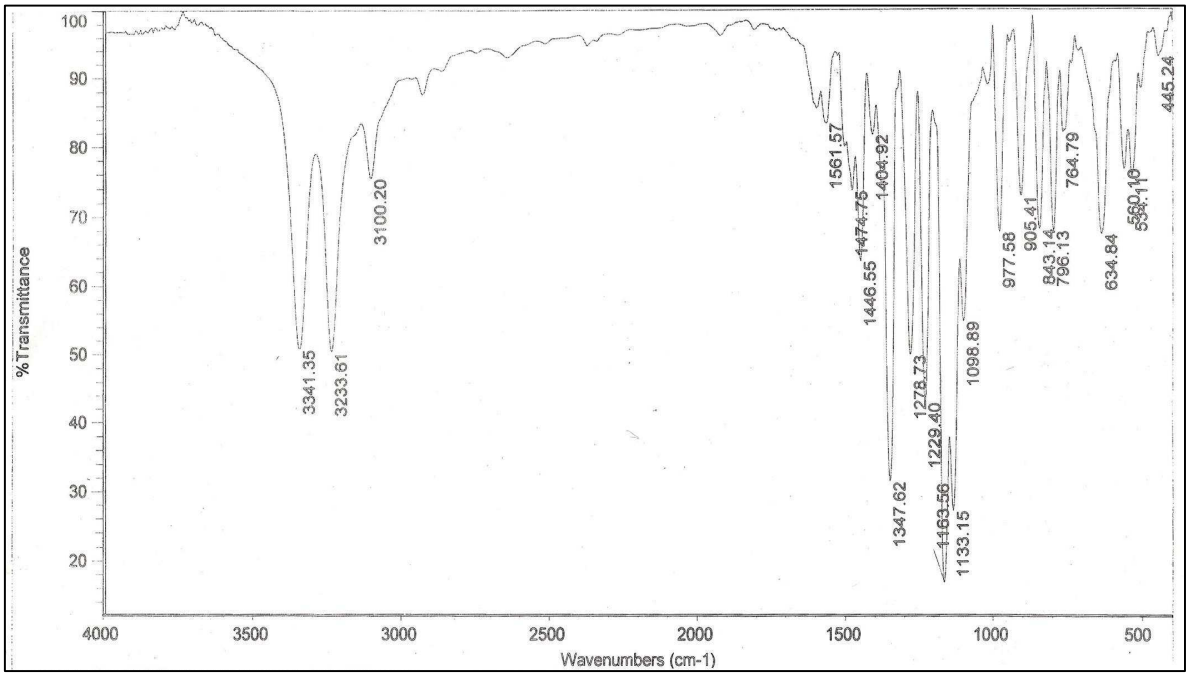


Fig. 5: FTIR Spectrum of physical mixture of celecoxib and excipients

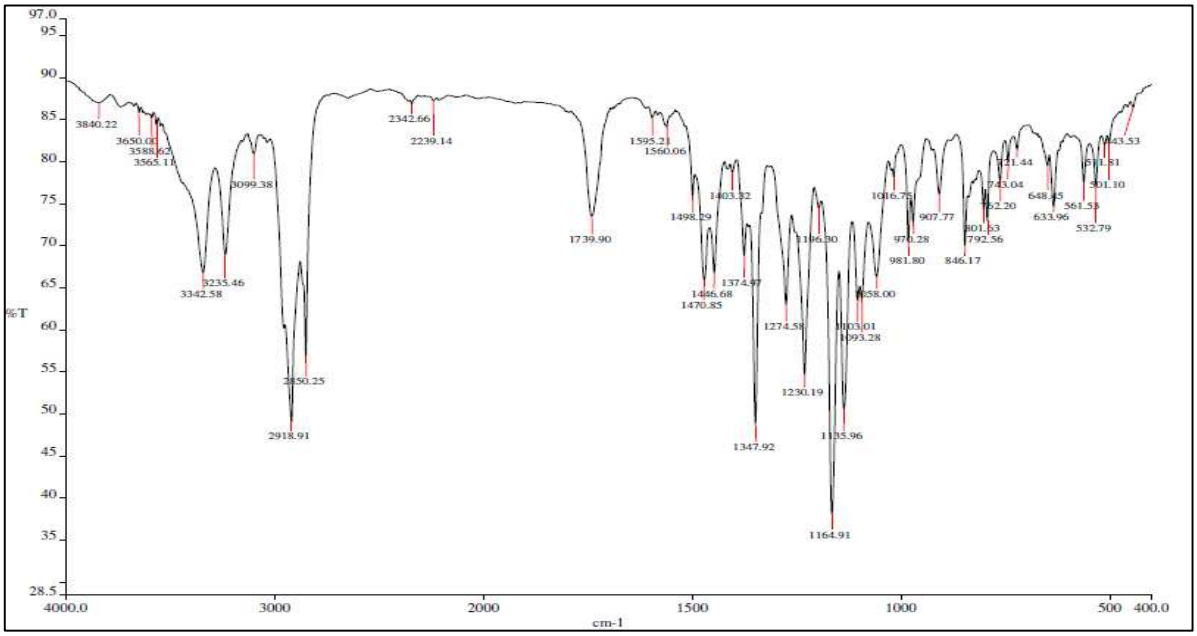


Table 2: Mathematical model showing order and mechanism of drug release

Formulation code	Zero order plot		First order plot	Higuchi'splot
	Regression	Slope% /hrs	Regression	Regression
CLB1	0.9965	1.6678	0.7307	0.9846
CLB2	0.9965	1.6678	0.8359	0.9101
CLB3	0.993	1.5159	0.8609	0.9303
CLB4	0.9941	1.3265	0.8883	0.9497
CLB5	0.9933	0.9734	0.8466	0.9218

Fig. 6: SEM Photograph of CLB3

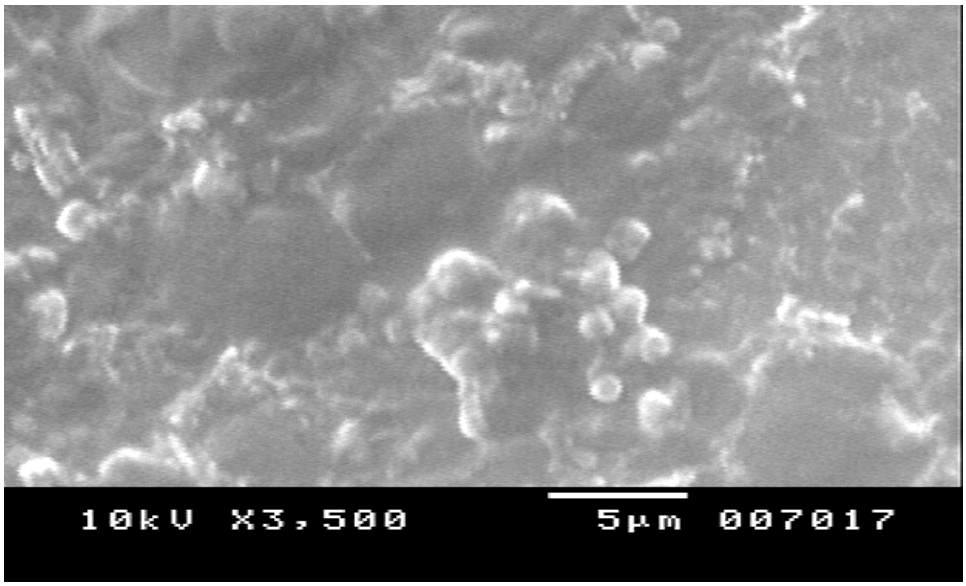
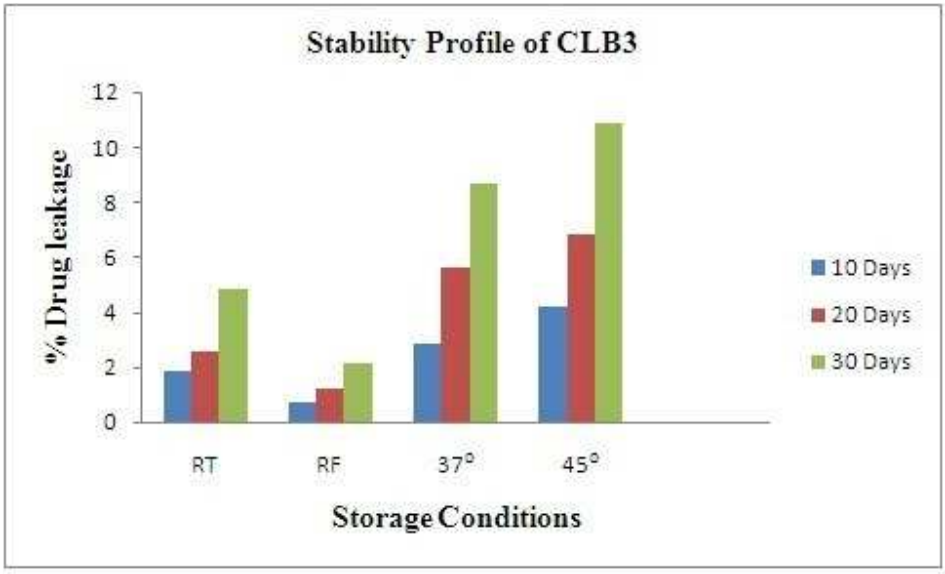
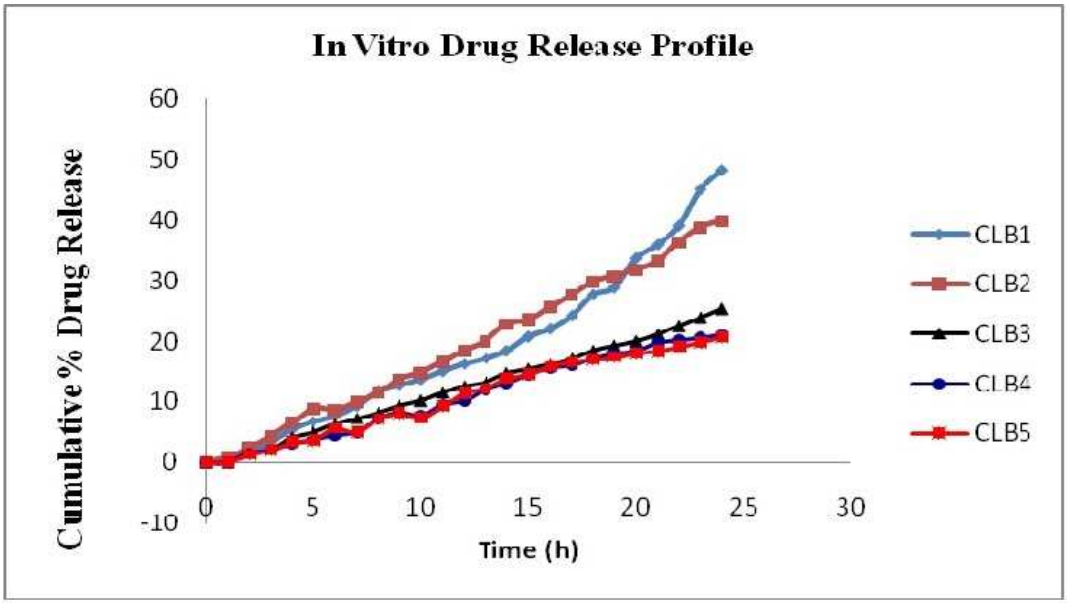


Fig. 7: Extent of drug leakage from CLB3 liposomes at different storage temperatures.



Abbreviations Used: RT- Room temperature, RF- Refrigerated temperature

Fig. 8: Plots of *in vitro* cumulative percentage drug released vs. time for different formulations of celecoxib Liposomes



CONCLUSION

In the present study, highly hydrophobic molecule colecoxib was successfully incorporated in to liposomes composed of different lipids DPPC & DSPC with desired qualities. DSPC liposomes were found to have greater drug entrapment and to have influenced by bilayer cholesterol content. Increasing amount of cholesterol was found to decrease drug entrapment and drug release from liposomes. On the other hand liposomes prepared with DSPC with zero cholesterol showed the rapid release pattern with lowest drug retention after 24hours. So the present study

has given us knowledge that liposome with low amount of cholesterol is the better candidate for celecoxib liposomes.

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