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CELECOXIB LOADED LIPOSOMES: DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* EVALUATION

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ABSTRACT

CLX (celecoxib) is a highly hydrophobic non-steroidal anti-inflammatory drug with high plasma protein binding. We describe here the encapsulation of CLX in MLVs composed of SPC and variable amounts of cholesterol. The influence of drug – lipid ratio was studied and amount of the drug could be encapsulated was optimized. The effect of cholesterol and other process parameters were studied to obtain the liposomal vesicles with desired quality. All the prepared formulations were characterized for their physico chemical properties such as appearance, vesicle size, vesicle size distribution and percentage drug entrapment. Stability of the liposomes in terms of their drug leakage and drug retention behaviour was studied by storing the liposomal formulations under different conditions for the period of 30 days. The optimized formulation parameters and process parameters resulted the liposomes with mean vesicle diameter of 4.81µ. The maximum percentage drug entrapment was achieved with the formulation CL3 which contains the drug – lipid ratio of 1:10%W/W and the percentage drug entrapment is equal to 72.33±0.64 (%). In vitro release data showed that release profile follows zero order kinetics. Celecoxib liposomes with good stability and appreciable controlled drug release with good retention of the drug even after 24 hours were prepared successfully.

INTRODUCTION: Celecoxib (CXB) is a highly selective COX-2 inhibitor and primarily inhibits the isoform of cyclooxygenase (and thus causes inhibition of prostaglandin production which involves in inflammatory response) [1]. CXB is more selective for COX-2 (IC50=0.04µM) inhibition over COX-1 (IC50=15µM).[2] It binds with its polar sulfonamide side chain to a hydrophilic side pocket region close to the active COX-2 binding site [1]. Non specific NSAIDS inhibits both cyclooxygenase 1 and 2 (COX-1 & COX-2) which are important for the regulation of homeostasis in many tissues. The inhibition of COX-1 activity results in number of side effects such as gastrolesivity [3] and interference with platelet function [4].

CXB is highly effective in the treatment of osteoarthritis and rheumatoid arthritis when compared to other NSAIDS, for example Naproxen and Diclofenac [5, 6]. CXB is also used to treat pain and inflammation associated with ankylosing spondylitis, as well as to treat menstrual cramps and colonic polyps [1].

CXB (4- [5- (4- methylphenyl)-3- (trifluromethyl)-1H-pyrazol-1-yl]Benzene sulfonamide), as a consequence of it's chemical structure, shows high apolar characteristics [7]. The amide group present in its structure is weakly acidic with a pKa value of about 11. For this reason CXB is insoluble at physiological pH. No liquid formulation exists and only available oral dosage form is capsule [7].

The absorption of CXB given in capsule is delayed by food, although systemic exposure increased by 3-5 fold. [8] CXB exhibits poor flow properties and compressibility [9]. CXB is highly hydrophobic and is almost completely absorbed after oral administration. However, much of the drug is metabolized by liver during its first passage through portal circulation [10]. CXB has high volume of tissue distribution of 455± 166 liters indicating extensive permeation into number of organs [11, 12]. Since it is 97% plasma protein bound [13], oral CXB formulations require to be administered at high daily doses, thereby increasing concerns about cardiovascular side effects [14].

Thus the problems associated with oral administration made us to design the novel drug delivery system for CXB with an alternate route of administration. Novel drug delivery carriers such as liposomes are very versatile to suit the delivery of various drug molecules [15]. Thus, liposomes have been selected for the present work assuming that incorporation of CXB in to liposomes may reduce the side effects associated with it by reducing the availability of the drug in systemic circulation and increasing accumulation of drug in the sites of inflammation, possibly by extravascularization through the gaps formed between the endothelial cells of the vasculature.

The current work includes the preparation of CXB liposomes by examining the influence of various formulation and process parameters. Further various characteristics such as percent drug encapsulation, vesicle size, vesicle size distribution, drug leakage profile, and *in vitro* drug release pattern was assessed.

MATERIALS AND METHODS:

Materials: CXB was obtained from Aurobindo Pharma, Hyderabad, as the gift sample. Soyphosphatidyl choline (SPC) was generously gifted by lipoid, Germany. High purity cholesterol was purchased from local source which is of analytical grade. Chloroform, methanol, ethanol, sodium chloride, potassium-dihydrogen-phosphate, disodium hydrogen phosphate were of analytical grade. Semi permeable membrane was obtained from commercial source. Double beam UV-Visible spectrophotometer (SL 164 ELICO) was used for analysis of all the samples. Double distilled water was used throughout the experiment.

Preparation of calibration curve of CXB [16, 17]: 100mg of the drug was dissolved in little amount of solution of 50:50%V/V of acetonitrile and phosphate buffer pH 5.5 and volume was made up to 100ml with the same. 10ml of this solution was pipetted out and made up to 100ml. Then aliquots were further diluted to 2-12µg/ml. Absorbance was found out spectrophotometrically and standard curve of CXB was plotted at λ_{max} 251.2nm

Method of preparation of CXB liposomes [18, 19]: CXB multilamellar liposomal vesicles were prepared by using thin film hydration technique. Seven formulations were prepared by using SPC as a lipid component with or without cholesterol. Accurately weighed quantities of drug, SPC with or without cholesterol were transferred to 250ml round bottom flask and dissolved in solvent mixture of chloroform and methanol (2:1, v/v). Thin layer of lipid film was formed by evaporating the solvent system under reduced pressure using rotary evaporator (HS-3001 NS).

During this process, the conditions of the instrument such as temperature (45±2^{o}C) and speed (60rpm) were kept constant. Residual solvents were removed by storing the thin film overnight in vacuum desiccator. Then the thin lipid film was hydrated with phosphate buffer saline [PBS] pH7.4 using vortex mixture about 2min to form MLVs. The suspension was allowed to stand at room temperature for an optimized period of 2h to achieve the complete swelling of the lipid film and to obtain the liposomal suspension. Then the suspension obtained was sonicated for 3min in ultrasonic homogenizer (ultrasonic 3000).

Fourier Transform Infrared (FTIR) Study: All the excipients such as SPC, cholesterol individually, physical mixture of excipients, pure drug Celecoxib, physical mixture of excipients and 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.

Determination of percentage encapsulation efficiency [20]: 10ml of liposomal suspension were placed in centrifuge tubes and they were centrifuged at 4500rpm for 10 min. The supernatant was removed and the drug content of supernatant was analyzed. The resulted pellet was dissolved in ethanol and it was diluted suitably and analyzed for the drug content.

Percentage encapsulation efficiency (EE %)

$$= \frac{\text{Amount of drug in pellet}}{\text{Total drug}} \times 100$$

(EE %) was calculated for 3 formulations of each formulation code and average was tabulated (**Table 1**).

Microscopy and Vesicle Distribution Profile [18]: The entire prepared liposomes were observed under binocular compound microscope (optics) at 45x10 magnification for studying the vesicle size and shape. Formulations showing precipitated drug and undispersed lipid film were removed. Scanning electron microscopic analysis was carried out on selected formulations for their morphology.

Stability analysis [15]: The property of the liposome to retain the drug was studied by storing the liposome at 4 different temperature conditions, i.e., 4-8^{o}C (refrigerator RF), 25±2^{o}C (room temperature RT), 37±2^{o}C and 45±2^{o}C for a period of 1 month. The liposomal preparations were kept in sealed vials. Periodically samples were with drawn and analyzed for the drug content following the same method described in % encapsulation efficiency (Table 1).

Determination of Residual Solvents in Liposomal CXB [21]: The prepared liposomal formulations were analyzed for the presence of residual solvents using gas chromatography (Shimadzu24-17A GC) with flame ionization detector and column DB 624. The column pressure was 19 kPa, column flow rate was 2mL/min, linear velocity was 21cm/sec, the total flow was 93mL/min and the split ratio was 1:22. The injection volume was 1µl and the column temperature was maintained at 200ºC. This was chosen based on the boiling point of solvents to be analysed (chloroform 62ºC and methanol 64.7^{o}C). After diluting the formulations with dimethyl sulfoxide they were injected.

The retention time and the area under chromatograph of the formulations were compared with chromatograph of pure chloroform and methanol.

Differential Scanning Calorimetric Analysis: Differential scanning calorimetric studies were carried out using differential scanning calorimeter (model number TA-60, Shimadzu, Japan) for the samples such as pure drug CXB, SPC, cholesterol and CXB incorporated multilamellar vesicles. For this purpose 5mg of samples of each were sealed thermatically in standard aluminium pans. Thermograms were obtained at a scanning rate of 10^{o}C/min. Each sample was scanned between 30 - 300^{o}C using nitrogen as the purge gas. For calibrating enthalpy indium was sealed in aluminum pan with sealed empty pan as a reference.

***In-vitro* Drug Release Studies** [22]: Drug release was determined with the help of modified USP XXI dissolution rate model. The model comprises of a beaker (250 ml) and a plastic tube of diameter 17.5 mm opened from both the ends. The tube was tied at one end with treated cellophane membrane and dipped into the beaker containing dissolution medium. The beaker was filled with 90 ml phosphate buffer (pH 7.4) and temperature was maintained at 37±1°C. Liposomal preparation (10 ml) was added in the tube. The dissolution medium was stirred using magnetic stirrer. The speed was maintained at 100 rpm. Dissolution sample (1 ml) was withdrawn at every one hour up to 24 h and analyzed spectrophotometrically. The results were tabulated.

RESULTS AND DISCUSSION:

Preparation of CXB liposomes: Various factors that influence the product such as vacuum, speed of rotation , hydration medium, and hydration time were studied in order to prepare liposome encapsulated CXB with desired qualities. Thickness and uniformity of lipid film were found to be influenced by rotational speed of the flask. The speed of 150rpm was found to be optimum, since; the same resulted in a uniform, thin film on the flask and responded the homogenous lipid vesicles after hydration. The lipid film was kept under vacuum overnight to remove the presence of residual solvents if any and to attain complete drying. Further this may avoid formation of emulsion which may result

due to the presence of solvent residuals during hydration. Hydration of the lipid film was achieved in two minutes vortexing, as this was found to be optimum in obtaining the liposomes free from aggregation. Further it was found that percent drug entrapment was not affected by the process of vortexing, confirmed by drug entrapment studies done before and after vortexing. Seven formulations were prepared using varying drug-lipid ratio, with or without cholesterol.

TABLE 1: COMPOSITION AND PHYSICOCHEMICAL PROPERTIES OF CXB LIPOSOMES

Drug (mg)	Soy Phosphatidyl Choline (mg)	Cholesterol (mg)	Encapsulation Efficiency (%)	Drug Release (%)	Vesicle Size (µ)
5	100		60.07 ± 0.92	67.66	4.7±0.6
7.5	100		68.22±0.76	68.16	4.6±0.2
10	100		72.33±0.64	68.19	5.2±0.1
15	100		52.44±0.65	65.06	4.5±0.3
10	100	12	66.2±0.8	62.68	5.5±0.7
10	100	24	59.23±0.84	53.89	4.8±0.4
10	100	50	47.27±0.85	51.27	4.7±0.3

First four formulations CL1 to CL4 were prepared without cholesterol to study the effect of drug - lipid ratio on percent drug entrapment of the vesicles. It was found that encapsulation efficiency % (EE %) of liposomes was dependent on the drug - lipid ratio used in the preparation of the vesicles. Increasing amount of the CXB from 5mg to 10mg increases the EE% considerably from 60.07±0.92% to 72.33±0.64%. However the PDE value was found to be decreased with further increase in quantity of the drug used. In all the four formulations the amount of the lipid used was fixed i.e. 100mg and drug - lipid ratio of 1:10% w/w showed the greater drug entrapment (Table 1).

Fourier Transform Infrared (FTIR) Study: Drug, excipient 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. **Figure 3** shows minor shifting of some peaks compared with individual excipients (**Figure 1 & 2**), like aliphatic alcoholic O-H stretch (3422.91 to 3421.68), C-H stretch (2920.16 to 2918.71), carbonylic C=O stretch of ester (1738.98 to 1739.88), C-O stretch of ester (1237.74 to 1237.60).

Minor shifts were observed when the **figure 5** compared with spectrum of pure drug (**Figure 4**) and excipients (Figure 1 and Figure 2) like, aliphatic O-H stretch (3341.35 to 3413.69), C-H stretch (2955.75 to 2920.08), carbonylic C=O stretch of ester (1739.88 to 1738.87), C-O stretch of ester (1237.60 to 1236.43). These shifts observed may be due to the formation of hydrogen bonds, Vanderwalls attractive forces or dipole moment which are weak forces seen in the polar functional groups of drug and excipients. The frequency of absorption due to the carbonyl group depends mainly on the force constant which in turn depends upon inductive effect, conjugative effect, field effect, stearic 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.

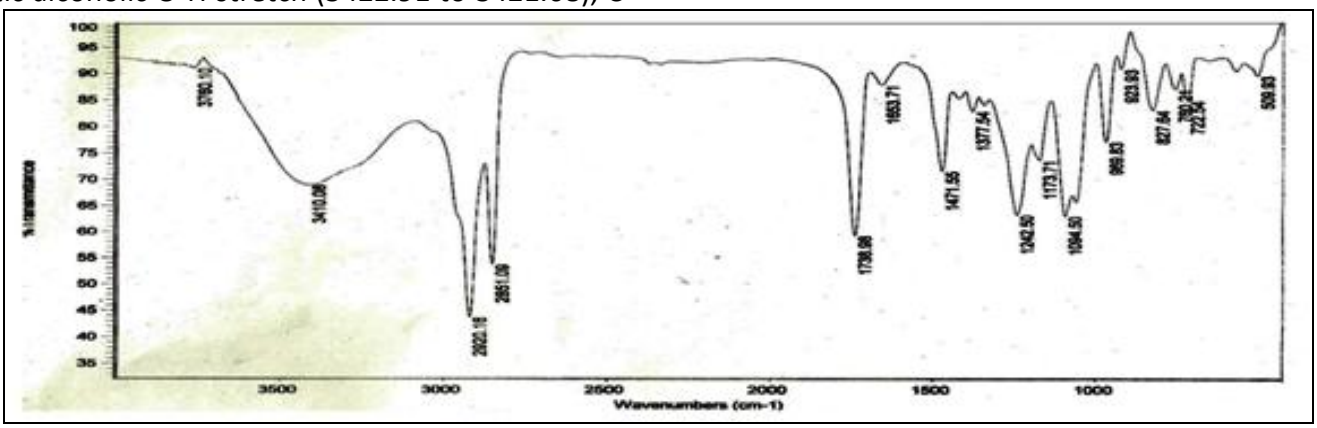

FIG. 1: FTIR SPECTRUM OF PURE SPC

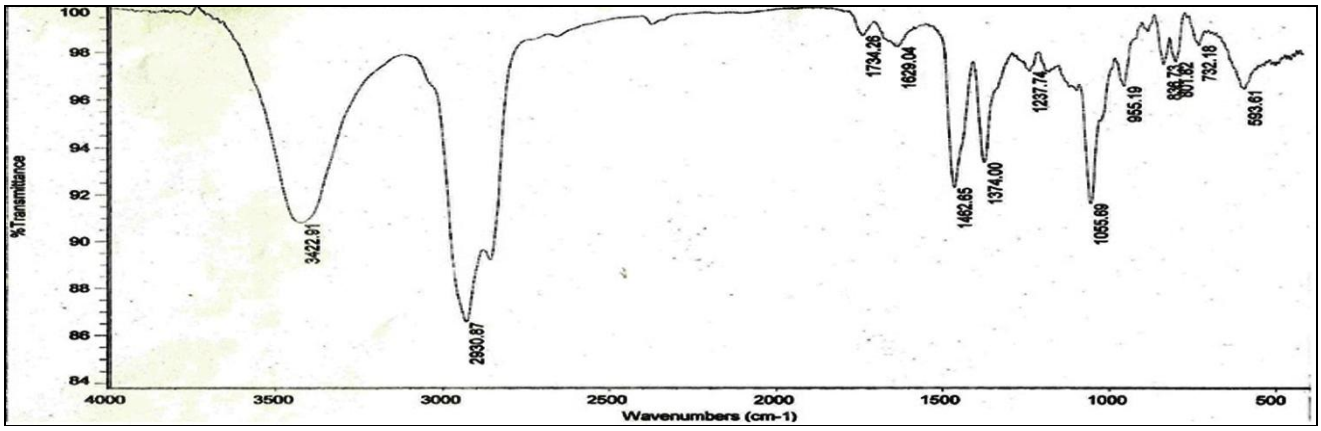

FIG. 2: FTIR SPECTRUM OF PURE CHOLESTEROL

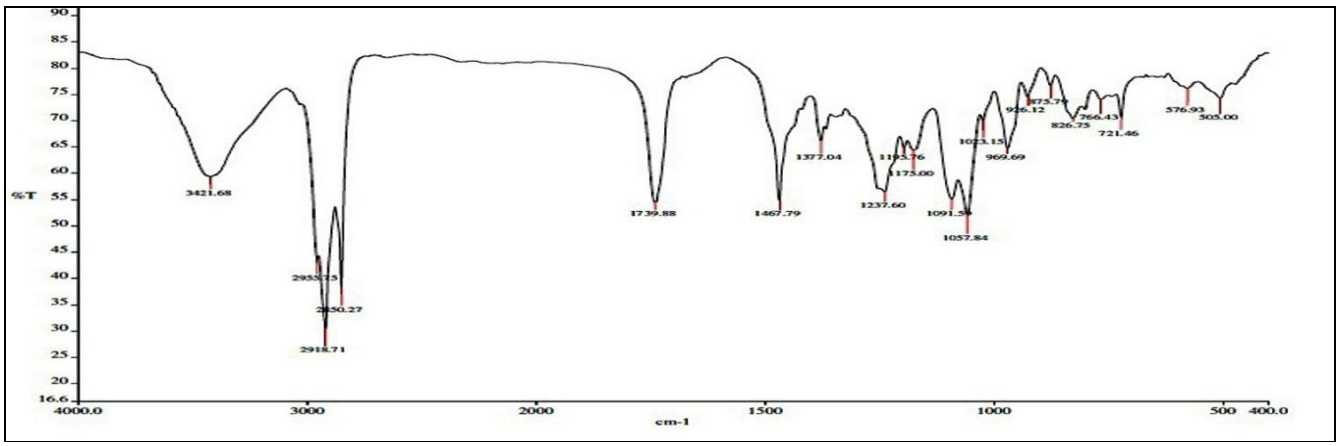

FIG. 3: FTIR SPECTRUM OF PHYSICAL MIXTURE OF EXCIPIENTS

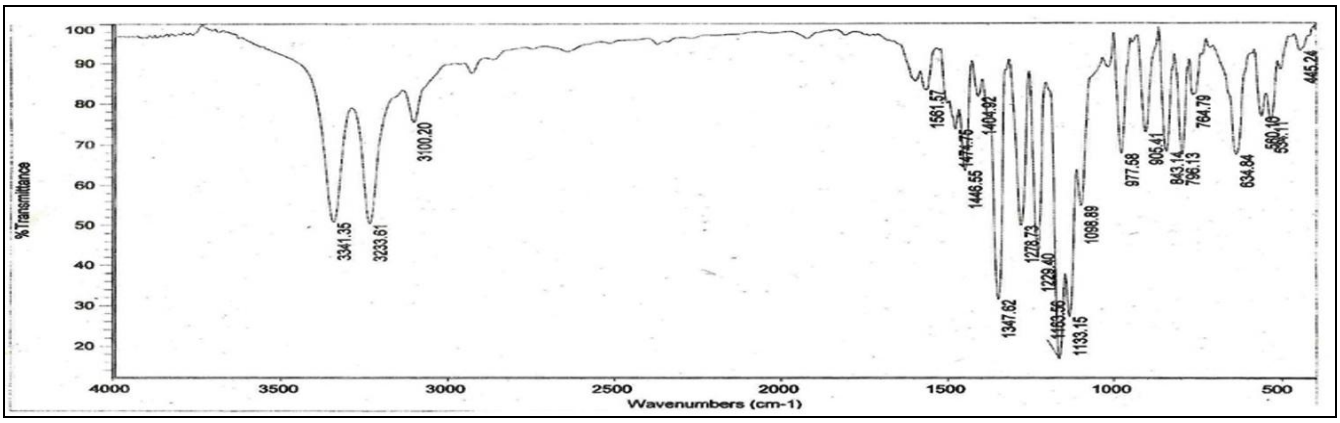

FIG. 4: FTIR SPECTRUM OF PURE CXB

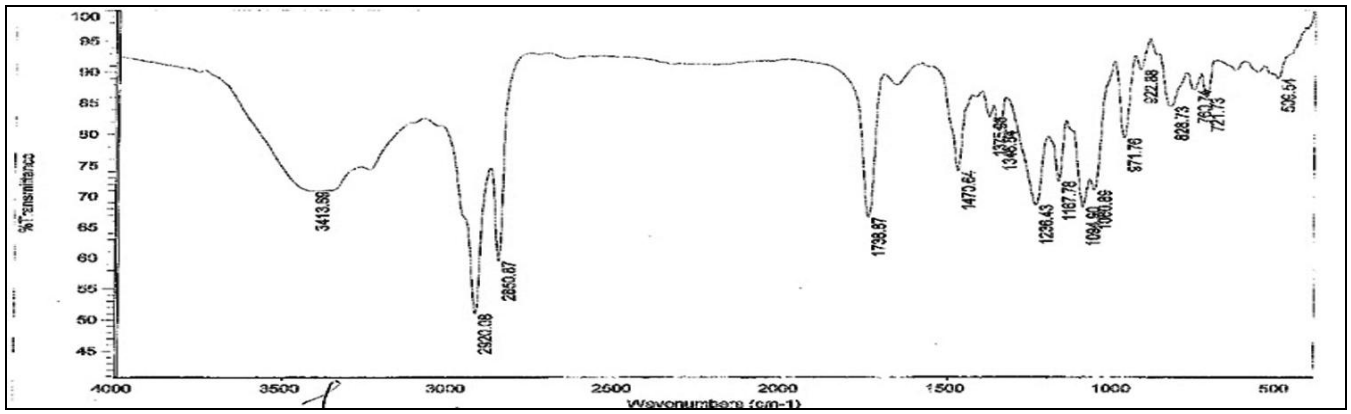

FIG. 5: FTIR SPECTRUM OF PHYSICAL MIXTURE OF CXB AND EXCIPIENTS

The influence of Cholesterol content of Liposomes on CXB Entrapment: Three formulations (CL5, CL6, and CL7) were prepared to find out the influence of cholesterol on encapsulation efficiency of CXB into liposomes. It was found that EE% of liposomes is dependent on the amount of cholesterol in their structure. Liposomes prepared with SPC only had the highest percent drug entrapment, where as liposomes prepared with cholesterol have shown the drug entrapment in the following order CL5 >CL6 >CL7. [SPC/Cholestrol mole ratio of 4:1 (CL5), 2:1 (CL6), 1:1 (CL7)] (Table 1)

Vesicle Size and its Distribution: The vesicle size of the liposome was found to be in the range of 4.5±0.3 - 5.5±0.7µm with 90% population of the liposomes equal or below 4.81µm. 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 fig. 6 shows SEM photograph of liposomal formulation CL5 (**Figure 6**), (Table 1).

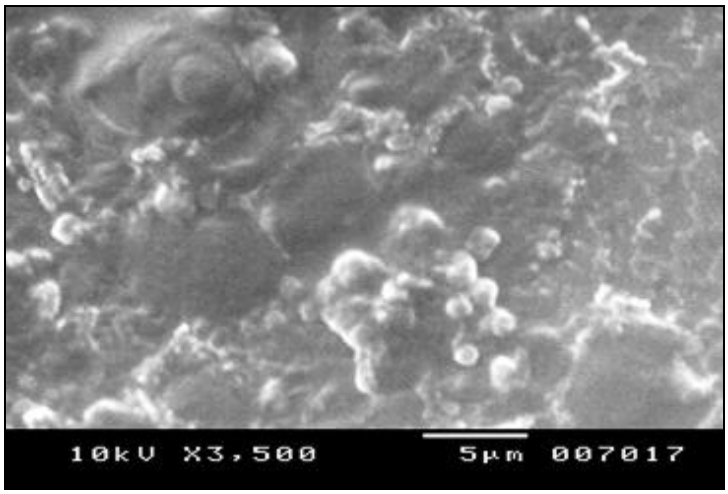

FIG. 6: SEM PHOTOGRAPH OF CL5

Stability Profile: 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. 37ºC ± 2ºC and 45ºC ± 2ºC showed the % drug leakage of the samples varied from 7%-20%. On the other hand liposomes stored at lower temperatures i.e. room temperature (RT) and refrigerated temperature (RF) showed that they could retain 96%-98% of the encapsulated drug respectively (**Figure 7**).

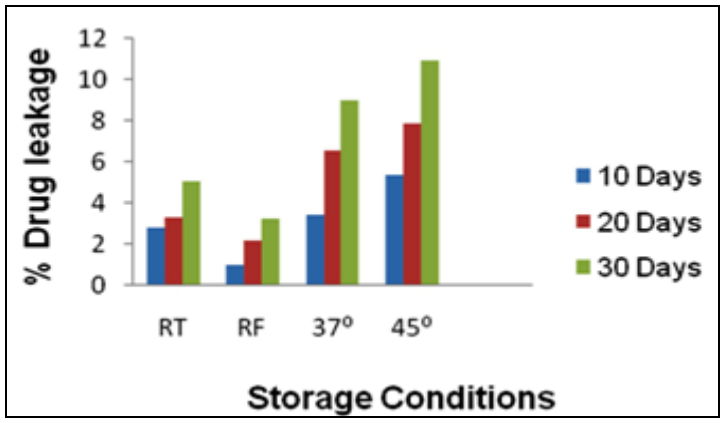

FIG. 7: EXTENT OF DRUG LEAKAGE FROM CL5 LIPOSOMES AT DIFFERENT STORAGE TEMPERATURES

Determination of Residual Solvents and Lipid Content in Liposomes: The chromatogram for the mixture of chloroform and methanol showed the retention time of 2.036 and 2.693 min with a peak area of 6.03 and 2.02 for methanol and chloroform respectively. It was found that the gas chromatographic method is very sensitive for the detection of these solvents at very low concentration. The formulation CL5 did not show the presence of chloroform and ethanol.

Differential Scanning Calorimetry Analysis: DSC analysis was carried out for CXB, SPC, cholesterol, CXB bearing MLVs using the method described in previous section. DSC thermogram of CXB showed endotherm at 163.24ºC. DSC thermogram of SPC and cholesterol showed endotherms at 130.59^{o}C and 148.23^{o}C respectively. CXB liposomes composed of SPC and cholesterol (CL5) showed thermogram at 143.46^{o}C, showed disappearance of melting endotherm of CXB at 163.24^{o}C. It was observed that the melting peak of SPC shifted from 130.59^{o}C to 143.46^{o}C, signifying that the entire lipid component interacts with each other to a great extent while forming the lipid bilayer. The incorporated CXB associated with lipid bilayers and interacted physically to a large extent. In view of our

observations, absence of CXB endotherm and shift in endotherm of lipid component SPC in the endotherm of drug loaded MLVs suggested that there could be significant physical interaction of CXB with bilayered lipid components leading to increased drug entrapment and sustained drug release (**Figure 8**).

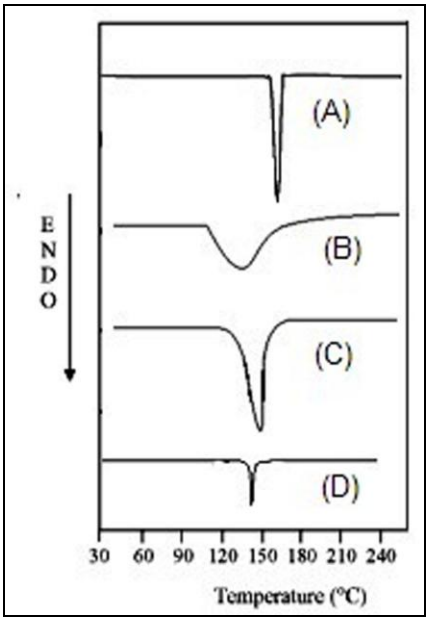

FIG. 8: DSC THERMOGRAMS OF CXB (A), SPC (B), CHOLESTEROL (C), CXB LOADED MLVS

***In-vitro* Drug Release:** Among the formulations prepared, CL3 containing 1:10% w/w of drug, SPC and zero cholesterol could release in 24 hours, the highest amount of CXB i.e.,68.19% followed by CL5, CL6 and CL7 containing 4:1, 2:1 and 1:1 mole ratios of SPC and cholesterol in decreasing order (62.68 %, 53.89% and 51.27%). The drug release was not of the sudden burst in any of the formulations. Cholesterol is normally incorporated in lipid/cholesterol ratio of 2:1 in liposomal preparations. We have shown that cumulative release profile of CXB is similar for liposomes with low cholesterol as well. Considering the significant reduction in encapsulation at high cholesterol concentrations, 4:1 mole ratio (CL5, 100mg of SPC and 12mg of cholesterol) would be suitable for liposomal formulations of CXB.

Release profile of liposomes were normalized to the lipid content of each sample, liposomes with only SPC were shown to retain lower CXB, where as other liposomes with cholesterol could retain the drug at higher percentages. This may be due to the fact that cholesterol may locate itself close to the glycerol backbone of the membrane, confining CXB to the inner core, which may explain the lower release rates with increasing concentration of cholesterol and this also support the result that highest extent of drug release occurred in cholesterol free liposomes [23, 24]. Drug release from all the prepared formulation followed zero order kinetics and release mechanism was of diffusion. This was confirmed by the regression values of the respective plots (**Table 2**, **Figure 9**).

TABLE 2: MATHEMATICAL MODEL SHOWING ORDER AND MECHANISM OF DRUG RELEASE

Formulation Code	Zero Order Plot		First Order Plot Regression	Higuchi's Plot Regression
	Regression	Slope (%/Hour)		
CL1	0.9987	2.8921	0.8651	0.9377
CL2	0.9974	2.9095	0.8682	0.9399
CL3	0.9985	2.8995	0.8647	0.9395
CL4	0.9929	2.872	0.8845	0.9476
CL5	0.9785	2.5999	0.9163	0.9611
CL6	0.979	2.3415	0.9111	0.9565
CL7	0.9942	2.2332	0.8169	0.9052

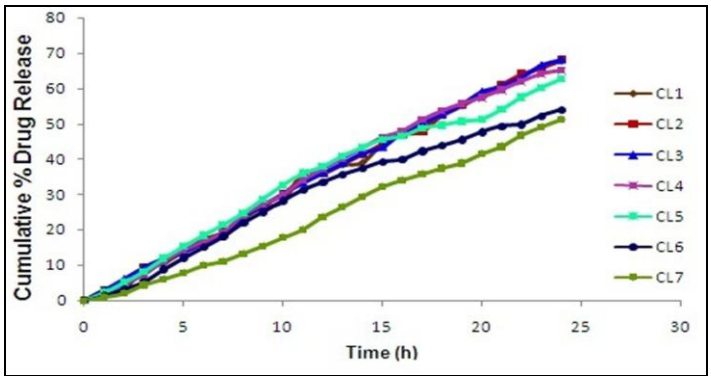

FIG. 9: *IN VITRO* DRUG RELEASE PATTERN OF CELECOXIB LIPOSOMES

CONCLUSION: In the present study, hydrophobic drug celecoxib was successfully incorporated in to liposomes with desired qualities. Prepared liposomes were shown to be influenced by drug-lipid ratio and bilayer cholesterol content. Presence of optimum amount of cholesterol (12mg) was found to enhance encapsulation efficiency and increasing amounts of cholesterol were found to decrease the encapsulation as well as drug release. Thus, the present study has given us knowledge that liposomes with low amount of cholesterol are better candidates for liposomes of celecoxib.

Author's statement:

Competing interest: The authors declare no conflicts of interest.

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