

Ketorolac Tromethamine Loaded Liposomes of Long Alkyl Chain Lipids: Development, Characterization and *In Vitro* Performance

M.Yasmin Begum^{1*}, Mahammed Rafi Shaik², K.Abbulu³, M.Sudhakar⁴

^{1*,2,4} Malla Reddy College of Pharmacy, Maisammaguda, Secunderabad,India.

³Malla Reddy Institute of Pharmaceutical Sciences, Maisammaguda, Secunderabad,India.

*Corres.author: yaminimp47@gmail.com
+919030851697

Abstract: Ketorolac is a nonsteroidal anti inflammatory drug with high plasma protein binding. We describe here the encapsulation of ketorolac in liposomes composed of DPPC or DSPC and with optimum amount of cholesterol. FTIR study was used to determine the molecular interaction between drug, lipid and cholesterol. All the batches of liposomes were characterized for their vesicle size profile, drug encapsulation and drug release. Three month stability studies conducted on liposomal dispersion and lyophilized form of liposomes showed that later is more stable for more than two months. 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 ketorolac with good stability and appreciable controlled drug release with good retention of the drug for more than a day were prepared successfully.

Key words: Ketorolac tromethamine, DPPC, DSPC, FTIR, SEM analysis, Freeze drying.

INTRODUCTION

Ketorolac is a non-steroidal anti-inflammatory drug with a potent analgesic and anti inflammatory activity due to prostaglandin related inhibitory effect of drug¹. It is a non selective cyclo oxygenase (COX) inhibitor. The drug is currently used orally and intra muscularly in multiple divided doses clinically for the management of cancer pain, post surgical pain and in the treatment of migraine pain^{1, 2, 3}. Ketorolac tromethamine is also used in the treatment of arthritis. An open label, uncontrolled ED study was conducted by shrestha et al in 1995 proved that intra muscularly administered ketorolac and oral indomethacin have

similar effects on the pain of acute gouty arthritis.⁴ So IM ketorolac is an alternate to the oral indomethacin if a parenteral medication is required.⁴ Ketorolac is one of the NSAIDs which is approved for parenteral administration.⁵ Another study showed that ketorolac is 36 times more potent than phenyl butazone, approximately twice as potential as indomethacin and three times more potent than naproxen in suppressing carrageenan induced paw edema in rat.⁶ It has been found through previous studies that analgesic efficacy of ketorolac is greater than that of other NSAID's and that of Morphine in acute pain models.^{7, 8, 9} So ketorolac has certain advantages in comparison with opioid analgesic drugs that it is non sedating, it is not

associated with pruritis, nausea, respiratory depression, urinary retention and it has opioid sparing qualities. Since the drug is a non selective cyclo oxygenase inhibitor of arachidonic acid with no increase of lipoxygenase pathway, the adverse effects associated with this is very much severe, and the drug is implicated as a contributing cause of increased post operative bleeding¹⁰, renal failure and gastritis.⁵ The severity of these side effects is dose related.^{11, 12} ketorolac has short biological half life of 4- 6 hours, which necessitates frequent dosing to retain the action¹³. The frequent occurrence of gastro intestinal bleeding, perforation, peptic ulceration and renal failure lead to the development of other drug delivery strategies for the appropriate delivery of ketorolac. The ideal solution would be to target the drug only to the cells or tissues affected by the disease.¹⁴ Selected carriers such as liposomes, niosomes, micro and nanospheres, erythrocytes and polymeric and reverse micelles were studied, but by far the most widely studied approach makes use of liposomes.¹⁴ Their attraction presents in their composition, which makes them biodegradable and biocompatible.¹⁵ Liposomes consists of an aqueous core enveloped by one or more bilayers of natural or synthetic phospholipids.¹⁵ These are the carriers which are suitable for encapsulation of drugs with different lipophilicities such as strongly lipophilic drugs, strongly hydrophilic drugs and drugs with intermediate log P.¹⁵ Liposomes can protect the encapsulated drug or drugs and can target the organ or tissue passively.¹⁵

In the present investigation our aim was to develop a simple vesicular delivery system for ketorolac tromethamine which can deliver the drug at a lower concentration over the prolonged period of time to the target site and thereby reducing the potential dose related side effects.

MATERIALS AND METHODS

MATERIALS

Ketorolac tromethamine was a generous gift from FDH Ltd., Mumbai. Dipalmitoyl phosphatidyl choline, (DPPC) and distearoyl phosphatidyl choline, (DSPC) were kindly gifted by lipid, Germany. High purity cholesterol, sigma membrane (12000 MW cutoff) were obtained from Sigma Aldrich, Hyderabad. Pottassium dihydrogen phosphate, disodium hydrogen phosphate, sodium choride, chloroform, methanol, ethanol were purchased from commercial source.

METHOD OF PREPARATION OF KETOROLAC LIPOSOMES^{16, 17}

Ketorolac multilamellar liposomal vesicles were prepared by using thin film hydration technique. Five formulations were prepared by using DSPC or DPPC as lipid component with or without cholesterol. Accurately weighed quantities of drug, DSPC or DPPC with or without cholesterol and with or without charge inducing agents 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 ($55\pm 2^\circ\text{C}$) and speed (150rpm) 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).

INCORPORATION OF CHARGED SPECIES

Inclusion of negatively charged lipids such as dicetyl phosphate or positively charged lipid such as stearyl amine tend to increase the interlamellar distances between the successive bilayers in the MLV swelling the structure with the greatest proportion of the aqueous phase. These effects lead to greater overall entrapped volume. For this purpose two batches of liposomes were prepared to find out the influence of DCP and its content on %EE.

FOURIER TRANSFORM, INFRARED (FTIR) STUDY

All the excipients such as DPPC, DSPC, cholesterol individually, physical mixture of excipients, pure drug ketorolac, 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.

DETERMINATION OF PERCENTAGE ENCAPSULATION EFFICIENCY¹⁸

10ml of liposomal suspension were placed in centrifuge tubes and they were centrifuged at 4500rpm after balancing the tubes on the other side with equivalent weight. The centrifugation was continued for 10min. The supernatant was removed and the drug content of supernatant was analyzed. The pellet resulted was dissolved in methanol and it was diluted suitably and analyzed for the drug content.

Percentage encapsulation efficiency (EE %) = Amount of drug in pellet/ Total drug x 100

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

VESICLE SIZE DISTRIBUTION PROFILE & MICROSCOPY^{19, 20}

All the batches of ketorolac loaded liposomes were examined for their morphological attributes using binocular compound microscope (optics) at suitable magnification. The batches containing non dispersed lipid film, drug precipitate or aggregates were detected and discarded. Scanning electron microscopic analysis was carried out on selected formulations for their morphology.

STABILITY ANALYSIS¹⁷

The behavior of the liposome to retain the drug was studied by storing the liposome at 4 different temperature conditions, i.e., 4 - 8⁰ (refrigerator RF), 25±2⁰ (room temperature RT), 37±2⁰ and 45±2⁰ for a period of 1 month. The liposomal preparations were

kept in sealed vials. Periodically samples were withdrawn and analyzed for the drug content following the same method described in % drug encapsulation efficiency.

FREEZE DRYING (LYOPHILLIZATION)

For freeze drying, liposomal suspension was prepared with cryoprotectant (lactose ; 1:5 lipid – carbohydrate ratio). The freshly prepared liposomal suspension was enriched with lactose solution and quickly frozen with iced acetone, stored at -80⁰C overnight and lyophilized for 48h using freeze dryer. Before measurements the lyophilized samples were re-suspended in double distilled water. Rehydration process is completed in 5min by vortexing.

IN VITRO DRUG RELEASE²¹

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 100ml of dissolution medium. The temperature was maintained at 37±1⁰C. 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 24h and analyzed spectrophotometrically at 323nm. With the help of the standard curve prepared earlier, drug concentration was measured.

Table 1. Composition and Physicochemical properties of Ketorolac Liposomes

S.No	DRUG (mg)	CH	DPPC	DSPC	SA	DCP	EE%	Drug Release %	Vesicle Size (μ)
KL1	10	0	100	78.35	75.2	4.91±0.5
KL2	10	0	100	84.75	72.48	5.27±0.7
KL3	10	24	...	100	83.2	66.4	5.48±0.7
KL4	10	24	100	17	89.39	59.2	5.72±0.4
KL5	10	24	100	17	86.08	63.52	5.36±0.3

Abbreviations Used: CH-Cholesterol, DPPC-Dipalmitoyl phosphatidylcholine, DSPC-

Distearoylphosphatidylcholine, SA- Stearyl amine, DCP- Dicetyl phosphate, EE%- Encapsulation efficiency percentage.

RESULTS AND DISCUSSION

PREPARATION OF KETOROLAC 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 the 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.

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 (KL2) has shown greater drug entrapment i.e. 84.75% with 27.52% of drug retention behavior during release studies after 24 hours. Therefore KL2 formulation was selected for the further studies. To improve the retention behavior of liposomes, we decided to incorporate cholesterol as one of the component, because cholesterol will give rigidity to the membrane structure, in addition to the major lipid component, DSPC. For this purpose, a liposomal batch was prepared using DSPC/cholesterol 4:1, %w/w. (It was optimized as per our earlier studies. Results not shown.) It was found that incorporation of cholesterol increases the drug encapsulation % and increases the drug retention behavior even after 24hrs. Literature review showed that there are variations on the encapsulation efficiency of 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 molecules. 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 themselves for this space between the alkyl chain of phospholipids resulted in lower encapsulation with increasing cholesterol content. Presence of cholesterol was known to influence vesicle stability positively and permeability reversely.^{22,23} This may be due to increased bilayer stability, while cholesterol content is increased. (Table 1)

INFLUENCE OF CHARGED SPECIES

Surface potential plays an important role in the behavior of liposomes *in vivo* and *in vitro*. In general charged liposomes were found to be more stable against aggregation and fusion than uncharged liposomes as well as it increases the EE%, since it tends to increase the interlamellar distances between the successive bilayers in the MLV, swelling the structure with the greatest proportion of the aqueous phase and hence lead to the greater overall entrapped volume. Two formulations were prepared to find out the influence of charged species such as dicetyl phosphate and stearyl amine on the EE% of ketorolac in to liposomes. It was observed that incorporation of DCP increased the PDE considerably from 83.2-86.08%, where as incorporation of stearylamine increased the PDE from 83.2-89.39%. (Table 1)

FOURIER TRANSFORM, INFRARED (FTIR) STUDY

Drug excipient interaction studied before preparing the formulation by using FTIR-spectroscopy, which is one of the most important analysis described about the stability of formulation. Presence of drug & drug release. Fig 3 shows minor shifting of some peaks compared to excipients, 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 and excipients like acidic O-H stretch (3352.28 to 3358.12), aromatic C=C stretch (1468.42 to 1470.96), C-H bending 1275.53 to 1277.09), C-O stretch of-OH of acid (1050.75 to 1052.15). These shifts observed may be due to the formulation of hydrogen bonds vanderwall 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 carbonyl 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 formulation of favorable vesicle shape, structure with good stability and sustained drug release.

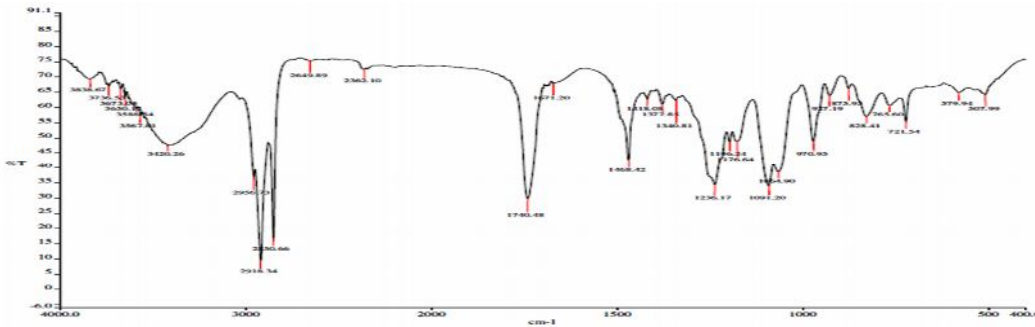


Fig. 1: FTIR Spectrum of Pure DSPC

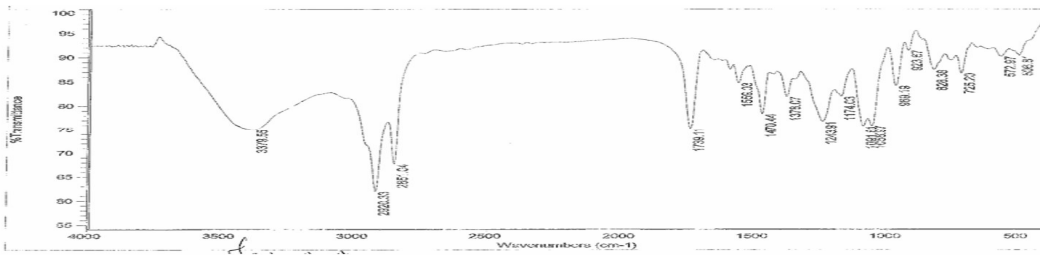


Fig. 2: FTIR Spectrum of Pure cholesterol

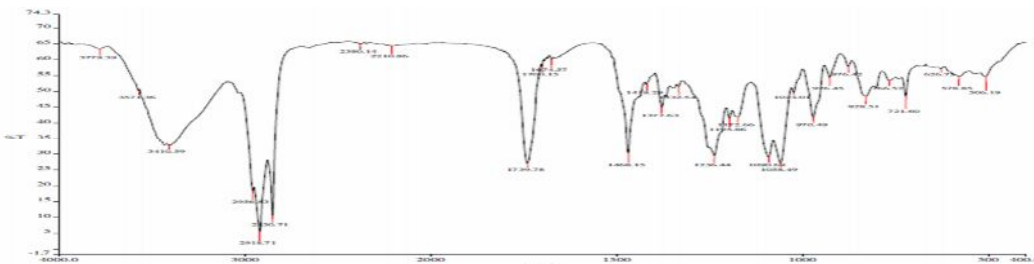


Fig. 3: FTIR Spectrum of physical mixture of excipients



Fig. 4: FTIR Spectrum of Pure ketorolac

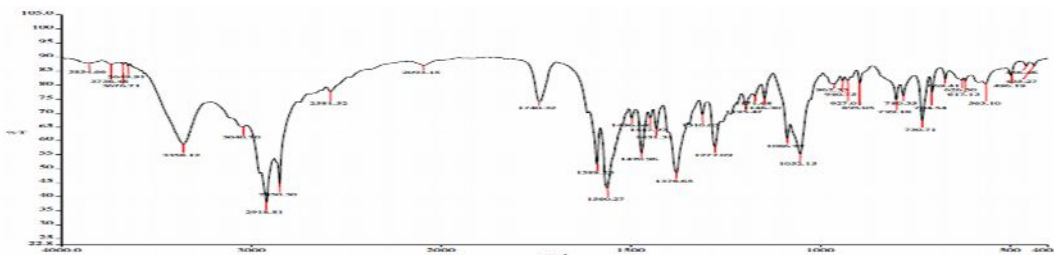


Fig. 5: FTIR Spectrum of physical mixture of ketorolac and excipients

MICROSCOPY AND VESICLE SIZE DISTRIBUTION PROFILE

The vesicle size of the liposome was found to be in the range of 4.91 ± 0.5 - $5.72 \pm 0.4 \mu\text{m}$ with 90% population of the liposomes equal or below $5 \mu\text{m}$. Most of the vesicle was found to be spherical in shape. Log-size distribution curve confirms the normal size distribution of the vesicles. The liposomes were photographed using scanning electron microscope. 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 figure 6 shows SEM photograph of liposomal formulation KL4. (Fig 6), (Table 1)

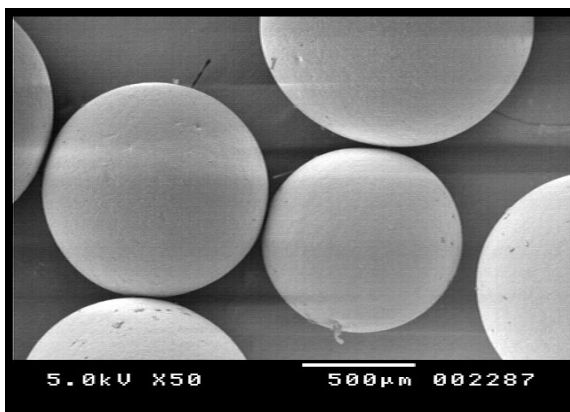


Fig. 6: SEM Photograph of KL4

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. $37^\circ\text{C} \pm 2^\circ\text{C}$ and $45^\circ\text{C} \pm 2^\circ\text{C}$ showed the % drug leakage of the samples varied from 8%-10%. On the other hand liposomes stored at lower

temperatures i.e. room temperature (RT) and refrigerated temperature (RF) showed that they could retain 96%-99% of the encapsulated drug respectively. (Fig 7)

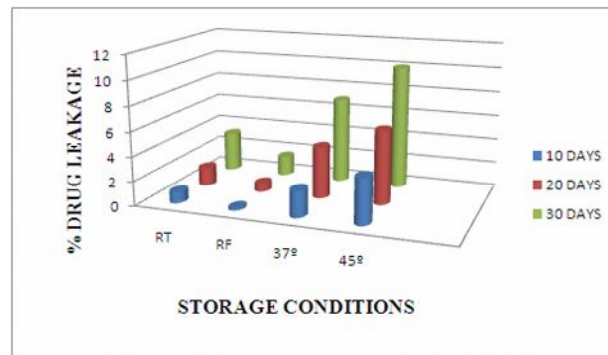


Fig. 7: Extent of drug leakage from KL4 liposomes at different storage temperatures

FREEZE DRYING

To compare the stability of liposomal suspension and freeze dried form of liposomes, stability studies were extended for 3 months at refrigerator storage condition. At the end of one month, negligible drug loss and vesicle size change were observed without any change in color in both the form of liposomes. At the end of 2nd month %drug loss was increased (18.22%) for liposomal suspension comparatively with freeze dried form (1.5%) with marked increase vesicle size, and there was change in colour only for liposomal suspension. (from milky white to yellow). After 3rd month, the liposomal suspension has shown drastic increase in drug loss (32.22%) and vesicle size ($12.34 \mu\text{m}$) when compared to the freeze dried form. But there was a noticeable colour change observed for freeze dried form of liposomes also at the end of the 3 months. (Table 2)

IN VITRO DRUG RELEASE

Among the liposomal batches prepared with long alkyl chain lipids such as DPPC and DSPC, formulation prepared with DPPC alone could release the highest amount of the drug.

Table 2. Comparitive 3Months Stability Profile for Liposomal Suspension and Lyophilized Form of KL4 Liposomes.

Name of the sample	Additive	I month			2 month			3 month		
		Drug loss	Vesicle size(μ)	Colour change	Drug loss	Vesicle size(μ)	Colour change	Drug loss	Vesicle size(μ)	Colour change
Liposomal suspension		1.63%	6.44μ	No	18.22%	8.12μ	Yes (yellow)	32.22%	12.34μ	Yes (yellow)
Freeze dried form	Lactose 5%	0.98%	5.02μ	No	1.5%	5.4μ	no	6.80%	6.02μ	Yes (yellow)

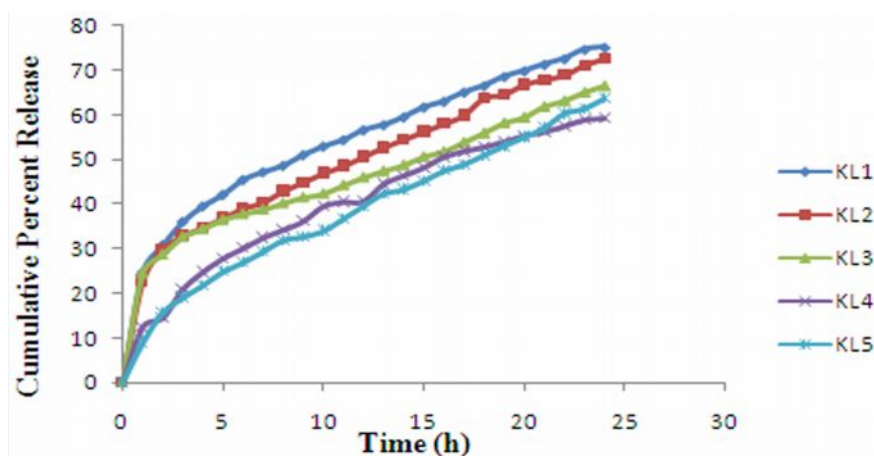


Fig 8. Plots of *in vitro* cumulative percentage drug released vs. time for different Ketorolac liposomes.

Table 3. Mathematical Model showing Order and Mechanism of Drug Release

Formulation code	Zero order plot Regression	slope (%/Hour)	First order plot Regression	Higuchi's plot Regression
KL1	0.874	2.263	0.383	0.982
KL2	0.913	2.242	0.417	0.983
KL3	0.884	1.901	0.383	0.962
KL4	0.934	2.112	0.529	0.997
KL5	0.972	2.270	0.59	0.987

Among the four composition used in the present study using DSPC, liposomes prepared with DSPC without cholesterol could release the highest amount of the drug followed by liposomes prepared using DSPC and optimized amount of cholesterol. This could be due to decreased leakage and permeability of the formulation at this weight ratio in presence of cholesterol. It has been reported that cholesterol increases the hydrophobicity which decreases the formation of transient hydrophilic holes by decreasing membrane fluidity, responsible for drug release through the bilayers.²⁴ (Fig 8) 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 3)

CONCLUSION

Concluding the above said results, EE% of the ketorolac in to liposomes prepared by the thin film

hydration technique was a function of formulation and processing variables such as drug lipid ratio, cholesterol content, vacuum, speed of rotation, hydration medium and hydration time. The electrostatically induced change in bilayer packing and electrostatic interaction between drug and charged head groups of SA and DCP could influence the incorporation ketorolac in to liposomes. Incorporation of optimum amount of cholesterol increased the drug entrapment and reduces the drug permeability. On the other hand liposomes prepared with DSPC alone exhibited more rapid release profile and lower drug retention in 24h. Freeze dried form of liposome was found to be stable than liposomal dispersion for more than 3 months.

Author's Statement:Competing Interest-

The authors declare no conflicts of interest.

REFERENCES

1. Radwan M.A., et al. Pharmacokinetics of ketorolac loaded to Polyethyl cyanocrylate nanoparticles using UPLC MS/MS for its determination in rats. *International journal of pharmaceutics*. 2010; 397:173-178.
2. Ibrahim A.A., et al. Proniosomes as drug carrier for transdermal delivery of ketorolac. *European journal of pharmaceutics and biopharmaceutics*. 2005; 59:485-490.
3. Basu S.K., Kavitha K. and Kumar M.R., Evaluation of Ketorolac tromethamine microspheres by chitosan/Gelatin B complex coacervation. *Scientia pharmaceutica*. 2010; 78: 79-92.
4. Shrestha M., et al. Randomised double blind comparison of the analgesic efficiency of intra muscular ketorolac and oral indomethacin in the treatment of acute gouty arthritis. 1995; 26(6):682-686.
5. Bhaskaran S. and Suresh S., Biodegradable microspheres of ketorolac tromethamine for parenteral administration. *J.Microencapsulation* 2004; 7: 743-750.
6. Gem C.L., et al. Micro encapsulation of ketorolac tromethamine by means of a co – acervation – phase separation technique induced by the addition of non solvents. *J.Microencapsulation* 1998;15(1):45-53.
7. Yae J.P., et al. Comparison of intramuscular ketorolac tromethamine and morphine sulphate for analgesia of pain after major surgery. *Clin.pharmacol. Ther.* 1986; 237: 253-261.
8. Brown C.R., et al. Comparison of intravenous ketorolac tromethamine and morphine sulphate in the treatment of post operative pain. *Pharmacotherapy*. 1990; 10: 116S-121S.
9. Goodman E., Use of ketorolac in sickle cell disease and vaso occlusive crisis. *Lancet*. 1991; 338: 641-642.
10. Rusy L.M., et al. A double blind evaluation of ketorolac tromethamine versus acetaminophen in pediatric tonsillectomy: analgesia and bleeding. *Anesth. Analg.* 1995; 80: 226-229.
11. Corelli R.L. and Gericke KR. Renal insufficiency associated with intramuscular administration of ketorolac tromethamine. *Ann.pharmacother.* 1993; 27: 1055-1057.
12. Hyers T.M., Tricomi S.M. and Liao J.J., Inhibition of some human neutrophil functions by the cyclo oxygenase inhibitor, ketorolac tromethamine. *J.Leukoc. Biol.* 1992; 51: 490-495.
13. Green D., et al. Interaction of low molecular weight heparin with ketorolac. *J.Lab. Clin. Med.* 1996; 127: 583-587.
14. Danilo DL., Liposomes. *Science and medicine*. 1996; 34-43.
15. Immordino M.L., Dosio F. and Cattel L., Stealth liposomes: review of the basic science, rationale and clinical applications existing and potential. *Int J Nanomedicine*. 2006; 1(3): 297- 315.
16. Hathout R.M., et al. Liposomes as an ocular delivery system for acetazolamide: *Invitro* and *Invivo* studies. *AAPS pharm sci tech*. 2007; 8(1): E1-E12.
17. Pai R.S. and Devi K.V., Lamivudine Liposomes for Transdermal Delivery-Formulation, Characterization, Stability, and *Invitro* Evaluation. *Int. J. Pharm. Sci. Nanotechnol.* 2009; 1: 317- 326.
18. Bonepally C.R. and Aukunuru J.V., Preparation and characterization of piperine multilamellar vesicular liposomes intended for testing anticonvulsant activity in rats. *Indian Drugs*. 2007; 44: 458-465.
19. Kumar R. and Katare O.P., Tamoxifen in topical liposomes: development, characterization and *in vitro* evaluation. *J Pharm Pharmaceut Sci*. 2004; 7: 252-259.
20. Buddhadev L. and Biswajit M., Tamoxifen citrate encapsulated sustained release liposomes: preparation and evaluation of physico chemical properties. *Sci pharm*. 2010; 76: 507-515.
21. Rathode S. and Deshpande S.G., Design and evaluation of liposomal formulation of pilocarpine nitrate. *Indian J Pharm Sci*. 2010; 72(2): 155-160.
22. Rogerson A., Cummings J. and Florence AT., Adriamycin loaded liposomes drug entrapment, stability and release. *J.Microcapsul* 1987;4:321 – 328.
23. Gregoriadis G, *Liposomes Technology*, 2nd ed CRC Press Boca Raton FL.,
24. Lecora M, et al. Permeability investigations of phospholipids liposomes by adding cholesterol. *Colloids surf.A : Physicochem.Engg Aspects*. 2003; 221: 9 – 17.
