

INVESTIGATION OF COCHLEATES AS CARRIERS FOR TOPICAL DRUG DELIVERY

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ABSTRACT

Objective: Cochleates which are tubular or cockle shaped structures derived from liposomes were discovered by Papahadjopoulos in 1975. Although investigated for several applications to be administered by oral and parenteral route, the potential of cochleates in topical drug delivery remains unexplored. The present study is a comprehensive report aimed at ascertaining the role of cochleates in topical delivery of drugs.

Methods: It involved development and evaluation of phosphatidylserine based cochleate formulation of ketoconazole, the model drug of the study. A 3² factorial design was utilized to optimize cochleate formulation and to study effect of phosphatidylserine and drug on the properties and performance of cochleates. Cochleates were also characterized by FTIR and DSC. The antifungal activity and stability of KCZ cochleates too was investigated.

Results: Cochleates of size 0.282 $\mu\text{m} \pm 0.05$ to 72.52 $\mu\text{m} \pm 2.2$ and entrapment efficiency of 57.86% $\pm 4.55\%$ to 97.27% ± 2.77 were obtained. The variables of the 3² factorial design significantly affected the cochleate size. Cochleates demonstrated promising role in topical delivery of drugs as the small sized cochleates caused significant release across the skin while the larger ones were retained in the skin leading to drug accumulation therein. Antifungal activity testing confirmed the preservation of antifungal activity by the encocchleated drug. Nevertheless, the prepared cochleate formulations possessed stability profile similar to liposomal counterpart.

Conclusion: The study concluded that cochleates are promising carriers for topical drug delivery.

Keywords: Lipidic, Vesicular, Topical, Cochleate, Skin deposition, Antifungal, Factorial design, Liposome

INTRODUCTION

Lipids, surfactants, polymers and proteins comprise the field of 'soft materials' science or 'complex fluids' [1, 2]. Lipid based drug delivery system now-a-days is experiencing resurgence due to new drug applications, such as gene therapy and novel self-assembly approaches to more complex structures. Liposomes, one of the earliest lipid based drug delivery systems, have been exploited heavily for the purpose of topical delivery of drugs [3-5]. For the purpose of drug delivery via topical administration it is required that a large enough amount of drug should successfully penetrate the SC and reach the target in order to obtain therapeutic effects [6]. This is especially true in case of fungal and bacterial infections of skin. These infections present different clinical manifestations such as scaling, fissures, maceration of skin, hyperkeratosis and vesiculation. A typical change occurs in the skin thickness, further increasing the barrier effect of the skin and challenging the penetration of drugs which accounts for the success of the therapy. In such circumstances vesicular systems like liposomes, niosomes, ethosomes etc. have proved advantageous [7-9].

A distinct group of lipidic vesicular carriers called as cochleates was first identified by Demetrios Papahadjopoulos and co-workers while studying interactions of divalent cations with negatively charged lipid bilayers. They reported that the addition of multivalent cations (calcium ions) to small phosphatidylserine vesicles caused them to collapse, fuse into long sheets of lipid, and roll up to jelly-roll like structures. These discrete liposome derived structures were termed as "cochleate" cylinders, after the Greek name for a snail with a spiral shell. The lipid bilayers were associated with each other via the positively charged calcium which interacted with negative headgroups on the opposing lipid bilayers. The interaction further stabilized the structure and removed the water of hydration. Interestingly, chelation of calcium with EDTA resulted in unrolling or unwinding of cochleates and spontaneously forming large single-bilayer, unilamellar vesicles [10].

Liposomes at physiological temperature are comprised of fluid bilayer membrane with aqueous space contained within compartments bounded by the lipid bilayers. The liposomal

bilayer is susceptible to harsh environmental conditions like extremes of pH, or enzymes that digest lipid. However, cochleates which are spiral, elongated, tubular and multilamellar stable structures devoid of aqueous space can withstand all the harmful conditions. The intrinsic properties of cochleates have led to advantages in the important areas of safety, stability, efficacy, immune response targeting, combining vaccines to multiple infectious agents, alternate routes of administration (including oral and intranasal), and generation of mucosal immunity. Cochleates have been investigated for the delivery of drugs like tobramycin, doxorubicin, cyclosporine A, nelfinavir [11, 12]. It is worthwhile to report at this point that cochleate delivery of Amphotericin B, adopted and patented by BioDelivery Sciences Inc. was a huge success [13]. Although cochleates have been investigated for oral and mucosal routes, their application in skin delivery systems remains unexplored. Liposomes have attracted enormous research efforts as a cross membrane drug delivery vehicle because of their structural resemblance with cell membrane [14-22]. They find application in topical delivery of important drugs like triamcinolone acetonide, acyclovir, lidocaine, econazole etc. [23-26]. Since cochleates are liposome based structures, it was hypothesized that they too might have prospective for topical delivery of drugs. Hence the present study was undertaken to investigate the potential of cochleate in topical drug delivery. For this purpose an antifungal drug, ketoconazole (KCZ) was used as the model drug.

KCZ is a drug with a wide antifungal spectrum and is usually prescribed for topical fungal infections such as athlete's foot, ringworm, and candidiasis. For an antifungal drug, criteria for successful therapy would be maximum skin residence. There are previous reports on formulation development of KCZ such as β -CD complexes, solid dispersion, liposome, cream, niosomes, solid-lipid nanoparticle [27-32]. Thus the present study describes a novel topical formulation of KCZ.

Acidic phospholipids that may be useful in preparing cochleates are phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, and phosphatidyl glycerol, phosphatidylserine [11]. Phosphatidylserine (PS) is an essential component of mammalian cell membranes. Apparently, formulations based on phosphatidylserine would exhibit

excellent compatibility for human use [13]. Marone et al. [33] investigated effect of various phospholipids on the formation of cochleate cylinders through small-angle X-ray scattering (SAXS) and cold field emission scanning electron microscopy. Briefly, it was concluded that dioleoylphosphatidylserine (DOPS)-Ca⁺⁺ forms stable, homogenous rolled cylindrical structures while soya PS and palmitoyl-oleoyl phosphatidylserine (POPS) formed spherical and planar sheets respectively. Zarif et al. [34] too reported that DOPS-Na formed efficient cochleate cylinders. Thus, DOPS was chosen as the negatively charged cochleate forming lipid.

Divalent cations Ca⁺⁺, Mg⁺⁺, Ba⁺⁺ and Zn⁺⁺ can be used for preparing cochleates [35]. It has been reported that Ca⁺⁺ forms a more tightly packed, highly ordered and less hydrated structure than does Mg⁺⁺ with phospholipids. Also it is required in much lower concentration than Mg⁺⁺ [36]. It is well documented that Ca⁺⁺ plays a vital role in natural membrane fusion phenomena while other cations listed above are ineffective in most such systems. Hence it is most compatible with the body. Thus calcium is the most suitable divalent cation reported for preparing cochleates and hence used in the present work.

The present study included development of liposomal and cochleate formulation of KCZ. The 3² factorial design approach was adopted to derive a formulation with optimum amount of DOPS and KCZ (the selected variables of the design) possessing the desired characteristics viz. vesicular size, entrapment efficiency and the skin deposition. The study also included antifungal activity and stability testing. On comparing the performance of liposomal KCZ to that of encocleated KCZ, it was concluded that encocleation facilitated topical delivery of KCZ to skin. Cochleates pose a new and valuable alternative to liposomes for topical delivery of drugs.

MATERIALS AND METHODS

Dioleoylphosphatidylserine (sodium salt, molecular weight 810.3) was a generous gift from Lipoid (Germany). Ketoconazole was a gift from Khandelwal Lab. Mumbai (India). Chloroform, sodium acetate disodium EDTA, sodium hydroxide and other reagents were of analytical grade. Marketed KCZ cream was procured from the local market.

Preparation of cochleates

Cochleates were prepared by trapping method. To form cochleates, calcium chloride solution (3mM) was added drop-wise to 1ml of liposomal SUV dispersion under constant vortexing. A precipitate was formed which was refrigerated at 2–8° C.

Factorial design

A 3² design factorial design was applied to establish the inter-relationship between the selected variables. The variables studied were the amount of drug (X₁) and amount of lipid (X₂) at three different levels. The coded and the actual values of the variables of experimental design are given in Table 1. The responses measured were particle size, entrapment efficiency and skin permeation and deposition of drug. The response values obtained from various batches were subjected to multiple regression analysis using "PCP Disso V3" software (IIPC, PCP, Pune, India). The equation fitted was,

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 + \beta_{12}X_1X_2 \dots\dots\dots (1)$$

Where, Y is the dependent variable, β_0 is the arithmetic mean response of the nine runs, β_1 and β_2 are the estimated coefficient for the independent factors X₁ and X₂ respectively. β_{11} , β_{22} and β_{12} are the estimated coefficients for the interaction X₁X₁, X₂X₂ and X₁X₂ respectively. The main effects terms (X₁ and X₂) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X₁X₂) show how the response changes when two factors are simultaneously changed. The polynomial terms (X₁X₁, X₂X₂) are included to investigate non linearity. The effects of the variables are interpreted considering the magnitude of coefficient and the mathematical sign it carries (i.e. positive or negative).

Table 1: 3² factorial design with the batch codes and actual values of the levels of the variables in formulation of KCZ cochleate

Batch code	Batch name	Amount of drug (X ₁) mg	Amount of lipid (X ₂) mg
-1,-1	C1	2	9
-1,0	C2	2	12
-1,+1	C3	2	15
0,-1	C4	4	9
0,0	C5	4	12
0,+1	C6	4	15
+1,-1	C7	6	9
+1,0	C8	6	12
+1,+1	C9	6	15

Calibration of KCZ in sodium acetate buffer, pH 5.0

Appropriate amount of KCZ was dissolved in ethanol to obtain the final concentration of 1000µg/ml. Dilutions were further carried out with sodium acetate buffer, pH 5.0 in order to obtain concentrations of 20 - 300 µg/ml solutions. The absorbance was recorded at 289 nm using UV-spectrophotometer (Jasco V630, Japan). This method was calibrated and it yielded the following equation with R²=0.9969,

$$y = 0.003 x + 0.007 \dots\dots\dots (2)$$

Evaluation and characterization of cochleates

Particle size determination

The mean particle size of the liposomal dispersion and cochleates dispersion was determined by laser diffraction technique using Malvern 2000SM (Malvern, UK). Analysis was carried out at 30±2°C temperature keeping angle of detection 90°. The mean vesicle size was expressed in terms of volume mean diameter D [4, 3] which is the average diameter of a sphere having volume same as that of the particle under measurement.

Determination of entrapment efficiency (EE) of cochleates

One hundred micro liters of cochleates was aliquoted into centrifugation tubes. To each tube 60 µl pH 9.5 EDTA and 1ml of ethanol were added while vortexing. The resulting solution is clear and colorless [37]. The samples were suitably diluted and absorbance determined at 289 nm to calculate entrapment efficiency as per equation 3.

$$\text{Entrapment Efficiency} = \frac{\text{amount of drug present in cochleates}}{\text{total amount of present}} \times 100 \dots\dots (3)$$

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR measurements of KCZ, DOPS-Na and drug loaded freeze dried cochleates were obtained on JASCO FTIR 4100 (Japan) equipped with Spectra manager version 2. Samples were prepared by mixing with KBr and placing in the sample holder. The spectra were scanned over the wave number range of 3600–400 cm⁻¹ at ambient temperature.

Differential Scanning Calorimetry (DSC)

Thermograms of KCZ, DOPS-Na and freeze dried cochleates were obtained using a Mettler-Toledo DSC 821e (Switzerland) instrument equipped with an intra cooler. Instrument was calibrated for DSC temperature and enthalpy using Indium standard. The samples were hermetically sealed in perforated aluminium pans and heated at constant rate of 10°C/min over the temperature range of -10 to 180 °C. The system was purged with nitrogen gas at the rate of 100 mL/min to maintain inert atmosphere.

In vitro drug deposition studies

The in vitro deposition of KCZ from the vesicles into the skin was studied using artificial cellophane membrane (Membra - Cel MD 3414, cut-off 14kD). For this experiment a vertical Franz diffusion cell having a surface area of 2.54 cm² and a reservoir capacity of 32 ml was used. The artificial membrane was securely placed between the two halves of the diffusion cell. The receptor fluid consisted of a

mixture of phosphate buffer pH 7.4 and ethanol in the ratio 4:1, its temperature maintained at $37 \pm 1^\circ\text{C}$ and stirred continuously using magnetic stirrer. The prepared KCZ cochleates dispersion was added to the donor compartment. One ml of the sample was withdrawn from the receptor compartment at definite time intervals and replaced with equal volume of fresh receptor fluid. At the end of 24 hours, the diffusion assembly was dismantled and the artificial membrane was carefully removed from the cell. Drug present on the surface was removed by gentle scraping and washing the surface of the membrane 10 times with ethanol-water mixture. The percent drug deposition was calculated as follows

% drug deposition = $100 - [\% \text{ cumulative release at the end of 24 hours} + \% \text{ remaining in the donor compartment} + \% \text{ drug extracted by washing}] \dots\dots (4)$

Ex-vivo skin deposition studies

The ex-vivo skin deposition study was performed on excised Wistar rat skin according to the study protocol approved by the Institutional Animal Ethics Committee constituted under Committee for the Purpose of Control and Supervision on Experimental Animals, India. The abdominal skin of rat was shaved, carefully excised and defatted to remove the subcutaneous fat. Further procedure was similar to that mentioned in the previous section. The rat skin was placed on the Franz diffusion cell with the epidermal side facing the donor compartment and the dermal side in contact with the receptor solution. The experiment was run for Carbopol® 934 P gel containing cochleates dispersion of batch C3 and C7 and liposomal dispersion of the same. KCZ dissolved in ethanol acted as the control. In all the experiments an amount of gel equivalent to $500\mu\text{g}$ of KCZ was applied to the skin in the donor compartment.

Antifungal Activity

Antifungal activity of KCZ loaded liposome and cochleates was tested on *Aspergillus Niger*. The media used for this purpose was Saboraud's dextrose agar media. The activity was carried out by using cup and well method of detecting antifungal activity. Appropriate dilution ($500\mu\text{g}/\text{ml}$) cochleate suspension (blank as well as drug loaded) were made and filled in the cavities. The plates were incubated at 37°C over a period of four days. Antifungal activity of liposomal KCZ and cochleate KCZ was compared against equivalent concentration of standard KCZ by measuring the zone of inhibition of each sample at the end of four days.

Stability study

For this study, the cochleates dispersions was kept at $2-8^\circ\text{C}$ and $25 \pm 2^\circ\text{C}/60\% \text{ RH}$ for a period of 3 months. The stability of the vesicles in terms of change in particle size and percent entrapment efficiency was investigated.

RESULTS AND DISCUSSION

The present investigation was focused on exploring the possible potential of cochleates for topical drug delivery. Cochleates were prepared from DOPS liposomes by the folding action of Ca^{++} . In the preliminary study, a 3mM concentration of calcium was found

optimum to convert liposomes into cochleates and was kept constant throughout the study.

Morphological characterization of cochleates

The optical micrograph of cochleates as obtained by using Nikon plane polarized microscope at 45X is shown in Figure 1. The film hydration method yielded multilamellar vesicles which on sonication produced small unilamellar vesicles. These liposomes, on addition of calcium chloride, transformed into overlapping long filamentous or tubular structures, identified as cochleates and reported as comprising of no internal aqueous space.

Evaluation of particle size

The implementation of a 3^2 factorial design aided the understanding of the roles and extent to which the negatively charged phospholipid and the drug affected the properties and performance of liposomes and cochleates.

As shown in Table 2, the particle size (volume mean diameter D [4, 3]) of cochleates ranged from $0.282 \mu\text{m} \pm 0.05$ to $72.52 \mu\text{m} \pm 2.2$. It was found that variables of the factorial design did not affect the size of the cochleates.

Evaluation of entrapment efficiency (E.E)

The E.E of the cochleates ranged from $57.86\% \pm 4.55\%$ to $97.27\% \pm 2.77$ and it was fairly affected by the variables of the study ($R^2 = 0.8816$).

The multiple regression analysis yielded following equation,

$$\% \text{ Entrapment Efficiency}_{(\text{Cochleates})} = 76.11 - 9.00X_1 + 11.63X_2 \dots\dots\dots (5)$$

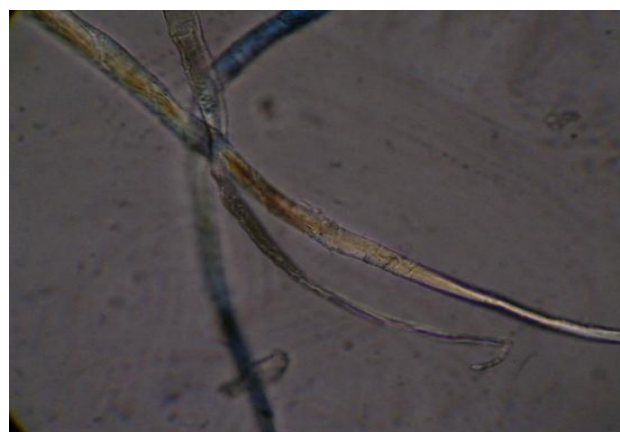


Fig. 1: Optical photomicrograph of Cochleates

The phospholipid (Figure 2) predominantly affected the entrapment efficiency in a positive manner. As the concentration of lipid was augmented the lipid domains to which the drug could bind increased leading to greater entrapment.

Table 2: Summary of responses of cochleates

Batch	Particle size $\pm \text{S.D}^* (\mu\text{m})$	Entrapment efficiency (%) $\pm \text{S.D}^*$	Cumulative release (%) $\pm \text{S.D}^*$	Drug Deposition (%) $\pm \text{S.D}^*$
C1	0.407 ± 0.18	68.51 ± 5.2	19.07 ± 2.6	35.87 ± 2.1
C2	1.052 ± 0.32	83.60 ± 1.7	45.52 ± 1.7	39.14 ± 3.2
C3	0.368 ± 0.20	97.27 ± 2.77	63.19 ± 3.1	23.11 ± 4.9
C4	0.282 ± 0.19	69.90 ± 4.7	9.95 ± 2.4	72.70 ± 2.6
C5	3.379 ± 0.21	81.13 ± 5.2	16.79 ± 1.3	62.87 ± 3.7
C6	7.307 ± 0.18	89.25 ± 2.6	37.70 ± 2.1	65.71 ± 2.8
C7	15.360 ± 0.28	57.86 ± 4.55	4.51 ± 2.4	78.1 ± 6.9
C8	72.520 ± 0.41	57.95 ± 3.9	13.06 ± 3.1	58.71 ± 3.8
C9	21.357 ± 1.5	79.54 ± 7.1	22.48 ± 3.5	50.95 ± 4.2

* Results are expressed as mean \pm standard deviation of 3 determinations

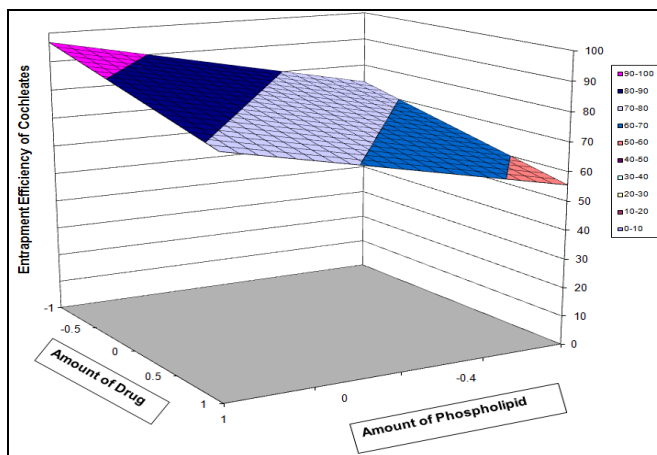


Fig. 2: Effect of variables on entrapment efficiency of cochleates

Evaluation of *in-vitro* Cumulative Release

The cumulative release of KCZ from cochleates is shown in Table 2. When this data was subjected to multiple regression analysis following equation was obtained,

$$\% \text{ cumulative release}_{(\text{Cochleates})} = 25.80 - 14.62X_1 + 14.93X_2 \dots\dots\dots (6)$$

The regression co-efficient for the above equation (0.8960) suggests that the *in-vitro* cumulative release was reasonably affected by the variables; as also reflected by Figure 3. The influence of the variables on the cumulative release is probably a consequence their effect on the entrapment efficiency.

The *in-vitro* drug deposition from cochleates varied from 23.11% ± 4.9 to 78.1% ± 6.9. Multiple regression analysis yielded the following equation (R²= 0.7815)

$$\% \text{ drug deposition}_{(\text{Cochleates})} = 67.09 + 14.94X_1 - 19.44 X_1X_1 \dots\dots\dots (7)$$

The term X₁X₁ had significant effect on drug deposition (Figure 4). This probably could be correlated to the size of cochleates. Overall, it could be said that higher concentration of drug lead to cochleates of bigger size which simply formed a film over the stratum corneum and did not penetrate to cause deposition. As compared to liposomes, higher drug deposition was demonstrated by cochleate formulation.

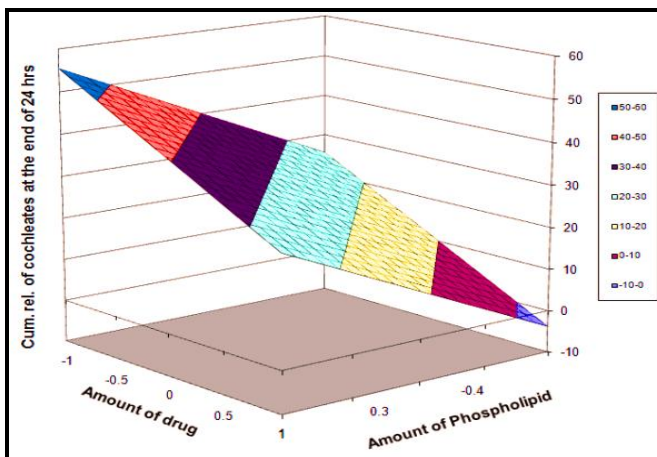


Fig. 3: Cumulative release of cochleate entrapped KCZ at the end of 24 hrs.

***In-vitro* drug deposition study**

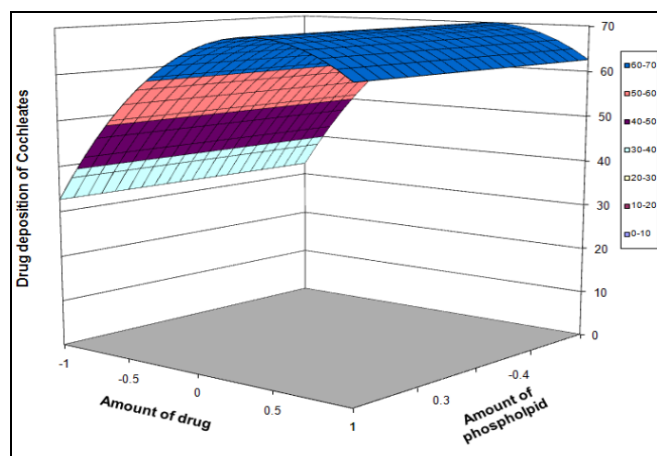


Fig. 4: Drug deposition of cochleate entrapped KCZ at the end of 24 hrs.

Ex-Vivo Skin Deposition Study

Liposomal and cochleate formulation demonstrated higher flux as well as deposition in skin as compared to the pure drug. The ex-vivo drug deposition and drug release observed in liposomes as well as cochleates was once again related with particle size. As noted in Table 4, it was found that batch C3 possessing less particle size displayed better drug release while batch C7 possessing particle size much greater than C3 exhibited better deposition in skin. Similar results were obtained when the cochleate formulations were compared to the corresponding liposomal formulations. Thus, cochleate formulation hold promise for both dermal and

transdermal delivery of the drugs. The mechanism of skin penetration probably is similar to that of liposomes which involves fusion of vesicles. However, detailed study of the interaction of cochleates with the skin structure is needed to confirm the mechanism.

Fourier Transform Infra-red Spectroscopy

The FT-IR spectra [Figure (5a)] of pure drug showed aliphatic C-H stretching at 3000-2800 cm^{-1} , CONH stretching vibration at 1644 cm^{-1} and C=C aromatic vibrations between 1500-1400 cm^{-1} while the fingerprint region marked C-O-C and N-C=N.

Table 3: Ex-Vivo Skin Deposition Study

Batch code	Particle size \pm S.D. μm	% EE \pm S.D.	% Cumulative release \pm S.D.	% drug deposition \pm S.D.
L3 gel	1.28 \pm 0.10	44.06 \pm 3.6	17.79 \pm 1.5	68.40 \pm 2.3
C3 gel	0.36 \pm 0.20	97.27 \pm 2.7	58.95 \pm 1.2	20.08 \pm 4.1
L7 gel	1.14 \pm 0.07	56.10 \pm 5.2	36.43 \pm 2.1	29.42 \pm 1.4
C7 gel	15.36 \pm 0.28	57.86 \pm 4.5	8.93 \pm 3.6	64.06 \pm 2.3
Pure drug	-	-	32.32 \pm 1.9	50.73 \pm 1.8

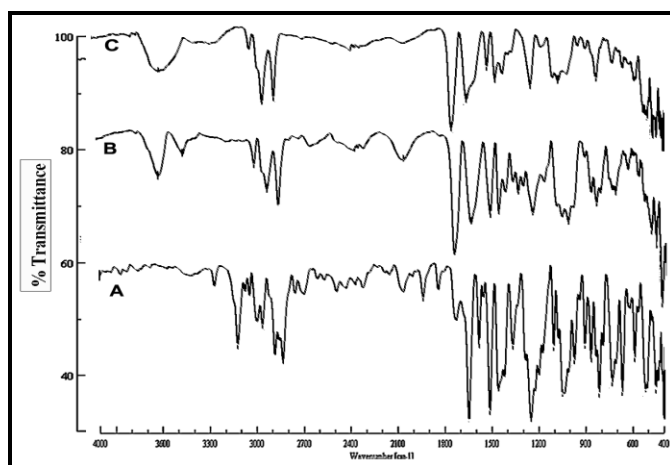


Fig. 5: FTIR spectra of (a) Pure drug (b) Phospholipid (DOPS) (c) Freeze dried cochleates

The spectra of DOPS-Na showed presence of moisture peak at 3600 cm^{-1} which may have occurred due to storage conditions. Aliphatic C-H stretching was observed between 3000-2800 cm^{-1} . The Fig. 5 also shows C=O vibrations at 1741 cm^{-1} . The spectrum of freeze dried cochleates shows considerable interaction between lipid and drug as the peaks at 1800-600 cm^{-1} are absent. Also there is a shift in the peak of the phospholipid in the cochleate.

Differential Scanning Calorimetry (DSC)

Crystallinity of pure drug was revealed from a sharp melting point endothermic peak at 149.46 $^{\circ}\text{C}$ in its DSC scan [Figure 6 (a)]. Absence of this characteristic sharp peak in DSC scans of cochleates structure revealed possible interaction between the drug and phospholipid. The sharp melting peak in the cochleate structure might be due some impurities present in the phospholipid.

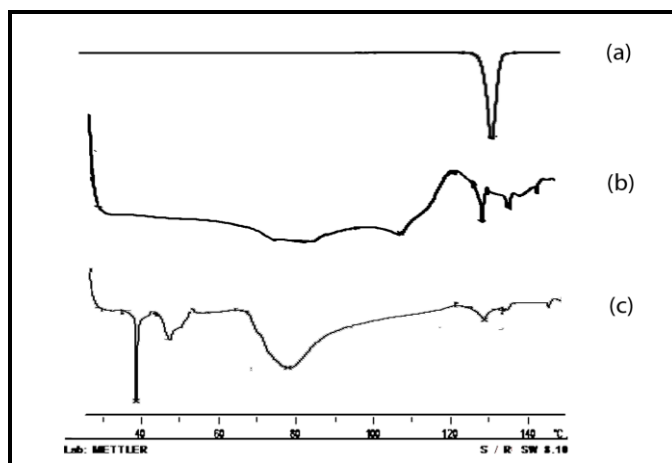


Fig. 6: DSC thermograms of (a) KCZ, (b) DOPS and (c) freeze dried cochleates

Table 4: Zone of Inhibition of Liposome and Cochleates

Name of formulation	Zone of inhibition (mm \pm S.D*)	
	After 24 hours	After 48 hours
KCZ	13 \pm 3	13 \pm 3
KCZ in liposome	10 \pm 3	13 \pm 2
KCZ in cochleate	10 \pm 2	13 \pm 1

* Results are expressed as mean \pm S.D of 3 observations

Table 5: Stability study testing of cochleates

Batch name	Parameter	Initial (0 day)	25 \pm 2°C/60% RH, 8 weeks	2-8°C, 12 weeks
C3	Vesicle size (μ m) \pm S.D*	0.368 \pm 0.23	0.371 \pm 0.08	0.362 \pm 0.10
	%E.E \pm S.D*	97.27 \pm 2.7	94.52 \pm 2.34	95.86 \pm 1.52

* Results are expressed as mean \pm S.D of 3 observations

Antifungal Activity

The antifungal activity results reveal that the zone of inhibition of both liposomes and cochleates were analogous and hence cochleates can be said to be an alternative drug delivery system to liposomes topically.

Stability study

The results of the 3 months stability study conducted on batches C3 is shown in Table 5. It was observed that the prepared cochleates were stable at room temperature (25 \pm 2°C) for 8 weeks and at 2-8°C for 12 weeks as there was no significant change in the mean vesicle size and % E.E of both the systems on storage at the respective conditions. The encouraging results of the skin permeation study in conjunction with the evaluation of antifungal activity and stability are persuasive to proclaim cochleates as alternate lipidic carriers to liposomes.

CONCLUSION

The present study successfully entrapped KCZ in a novel lipidic carrier, termed as cochleates. This novel structure, reported to be more stable than liposomes, causes dermal and transdermal release. Alongside, the KCZ loaded cochleates retained antifungal activity. The results concluded that cochleates hold promise for the said application and should be promoted as novel lipid carriers for dermal and transdermal delivery of drugs. Ongoing in-vivo studies undertaken by us shall further the prospective of cochleates drug delivery.

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