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Research Article

Formulation Design and Characterization of Rifapentine Nano Liposome by Lipid Film Hydration Method Using a Constitutive Lipids (Sunflower Lecithin: Cholesterol) For the Improvement of Drug Permeability

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Abstract
Rifapentine is considered a first-line drug for treating tuberculosis (TB), but its absorption can be inconsistent when administered alongside other anti-TB medications, often necessitating higher doses. To address this issue, Nano-liposomal formulations of Rifapentine were developed by introducing calcium ions to interact with the negatively charged phospholipids. The preparation involved a two-step method: initially, liposomes of the drug were formed using a film hydration technique with a rotary evaporator. Following this, calcium ions were added drop wise to the liposomal suspension under continuous stirring, resulting in the formation of Nano-liposomes.
The liposomes were then assessed for various characteristics such as vesicle size and drug entrapment efficiency. The most promising formulations were selected for further processing. The resulting Cochleates were subjected to particle size analysis, size distribution studies, and scanning electron microscopy (SEM), which revealed that the particles had an elongated, rod-like shape at sub-micron dimensions.
To evaluate the potential of the Nano-liposomal Rifapentine for enhanced absorption, permeability studies were conducted using an ex-vivo rat intestine model. The apparent permeability of Rifapentine from the standard formulation was compared to that of Rifapentine-loaded Nano-liposomes. The results showed a notable increase in the apparent permeability of Rifapentine when delivered in the Nano-liposomal form (7.78 × 10.6 cm/sec) compared to the conventional drug (3.56 × 10.6 cm/sec). This enhancement suggests that Nano-liposomal Rifapentine can significantly improve drug absorption across the small intestine, offering a potential strategy for optimizing Rifapentine therapeutic efficacy.

Keywords: Rifapentine, Nano liposomes, Cochleates, apparent permeability, liposomes.
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INTRODUCTION

Cochleates are unique, elongated, rolled microstructures composed of lipid bilayers that are formed through the condensation of small, unilamellar negatively charged liposomes. These structures were initially described by Papahadjopoulos and Wilschut. They are solid particles made from continuous lipid bilayer sheets that curl into spiral shapes due to their interaction with multivalent cations such as Ca^{2+}. These cochleates lack any internal aqueous space. They are stable phospholipid-cation complexes made from naturally occurring phospholipids, such as dioleylphosphatidylserine, or a mixture of phospholipids including phosphatidylserine, phosphatidylinositol, phosphatic acid, and phosphatidylglycerol, often with up to 75% by weight. The formulation can also include phosphatidylcholine, phosphatidylethanolamine, and other less prevalent phospholipids. Due to their distinctive structure, cochleates can encapsulate both hydrophilic and hydrophobic substances of various shapes and sizes, making them highly versatile carriers for delivering a wide range of drugs, proteins, and peptides. Additionally, cochleates provide protection for the entrapped molecules against adverse conditions such as changes in pH, temperature, and degradation by lipases1.

Rifapentine is a semisynthetic antibiotic with bactericidal properties, derived from *Streptomyces mediterranei*. It has a broad spectrum of antibacterial activity, particularly against Mycobacterium species. Rifapentine is a key drug in the treatment of tuberculosis (TB), often used as part of a multi-drug regimen alongside isoniazid, ethambutol, and pyrazinamide for a treatment duration of 4-6 months. Rifapentine is classified as a BCS class II drug, meaning it has low solubility but high permeability. It has an apparent permeability of approximately $5.79 \pm 0.053 \times 10^{-6}$ cm/s, which is above the critical limit of 2×10^{-6} cm/s, suggesting a bioavailability above 90%. However, clinical observations reveal that TB patients often experience low Rifapentine concentrations, and the drug's permeability can vary across different areas of the intestinal mucosa. Suboptimal drug concentrations can contribute to the development of resistant strains of *Mycobacterium tuberculosis*. Low drug levels may result from inadequate dosing, irregular drug intake, or conditions like malabsorption caused by impaired intestinal function during treatment, or when the drug is taken with food or antacids2.

The absorption of a drug depends significantly on the absorptive capacity of the intestinal mucosa, which can be influenced by various clinical factors. For example, TB patients co-infected with HIV often exhibit reduced serum concentrations of Rifapentine and ethambutol. One potential solution to address Rifapentine's variable absorption is the formulation of nano-liposomes. These nano-sized liposomes can effectively encapsulate the hydrophobic Rifapentine molecule, protecting it from external environmental factors. This approach helps increase the drug's permeability across the intestinal mucosa, potentially lowering the required dosage of Rifapentine while also minimizing gastrointestinal side effects like abdominal cramps and diarrhea. Thus, nano-liposomal formulations of Rifapentine may offer a promising strategy for improving its absorption and therapeutic efficacy in TB treatment3, 4.

MATERIALS & METHODS

Rifapentine, used in this study, was kindly provided by Modern Laboratories, Indore, India. Sunflower lecithin, sourced from Lipoids, Germany, served as the vesicle-forming phospholipid, while cholesterol (Sigma-Aldrich) was included to enhance the flexibility of the liposomal vesicles, thereby maintaining their shape. Chloroform (Loba Chem, India) was employed as the organic solvent to dissolve both sunflower lecithin and cholesterol. Bovine serum albumin (Sigma-Aldrich) acted as an aggregation inhibitor during the nanoliposomal formation, preventing the particles from aggregating into clumps. Calcium chloride (Loba Chem, India) was used as a cochleating agent, as the Ca^{2+} ions are necessary for rolling up the lipid bilayer sheets into cochleate structures. All solvents used in the preparation were of analytical grade.

Preparation of Small Unilamellar Vesicles via Lipid Film Hydration Method05

Negatively charged liposomes were prepared using the lipid film hydration method. Sunflower lecithin and cholesterol were mixed in different molar ratios (5:5, 6:4, 7:3, 8:2, 9:1) and dissolved in a minimal volume of chloroform. Rifapentine (100 mg), dissolved in chloroform, was added to the lipid solution. The organic solvent was then removed using a rotary evaporator (Lab India) at 37 ± 2°C to leave behind a thin lipid film on the walls of the flask. The film was left overnight to allow for the evaporation of any remaining organic solvent. The lipid film was then hydrated with phosphate-buffered saline (PBS, pH 7.4) to reach a final phospholipid concentration of 20 mg/ml. Mechanical agitation was applied by wrist shaking for 3 hours, and the resulting liposomes were further subjected to two cycles of ultrasonication for 10 minutes each to form small unilamellar vesicles.

Vesicle Size and Drug Entrapment Analysis of Small Unilamellar Vesicles06, 07

The formed small unilamellar vesicles were analyzed for vesicle size, drug entrapment efficiency, and external morphology. The vesicle size was determined using a Nanotrac-150 (USA), which utilizes advanced power spectrum analysis of Doppler shifts to calculate particle size distributions based on volume, mass, and intensity. The suspension was analyzed by passing light through a fiber-optic cable to a single detector, and advanced software was used to process the data. Drug entrapment was measured by lysing the vesicles with 0.1% Triton X-100 and centrifuging the mixture at 13,000 rpm for 15 minutes at 4°C. The supernatant was analyzed using UV spectrophotometry at 334 nm to quantify Rifapentine.

Preparation of Cochleate Cylinders08, 09, 10

Bovine serum albumin was added to the liposomal preparation at a final concentration of 1% to prevent aggregation during the cochleation process. Cochleate cylinders were formed by adding a calcium chloride solution to the liposomal preparation under continuous stirring. The final concentration of Ca^{2+} was 10 mM. The cochleate size was controlled by subjecting the preparation to two cycles of ultrasonication for 10 minutes each. After the evaporation of water, the solid cochleates were extracted.

Size Measurement of Cochleate Particles11, 12, 13

The size and distribution of the cochleate particles were determined using a Malvern Zetasizer 2000 (Malvern, UK). The vesicular suspensions were mixed with PBS (pH 7.4), and measurements were taken in multimodal mode at 25°C, following a 5-minute thermal equilibration. The Zetasizer detects backscattering at an angle of 173° to accurately size larger particles, even at higher concentrations. Assumptions made for viscosity (0.89 mPas) and refractive index (1.33) of the medium were used in the analysis.

Scanning Electron Microscopy (SEM) Studies

The surface morphology of the cochleates was observed using a scanning electron microscope (SEM, Model JSM 840A, JEOL, Japan). The samples were first dried in a vacuum desiccator and then mounted on brass specimens for observation. They were coated with a thin layer of gold-palladium alloy (1200 Å) using a sputter coating unit (Model E5 100, Polaron, UK) under an argon atmosphere. The sputtering process lasted for approximately 5 minutes to achieve a uniform coating, ensuring high-quality SEM images. The SEM was operated at a low accelerating voltage of 10 kV and a load current of 80 µA, with a working distance of 39 mm14, 15, 16.

Permeability Studies Using Ex-Vivo Everted Gut Sac Model

For the permeability studies, male Albino Wistar rats (150-200 g) were euthanized by cervical dislocation, and the abdomen was opened to remove the small intestine, which was then washed with a 0.9% NaCl solution containing 0.3% glucose. The intestine was everted using a glass rod, and the everted segment was placed in glucose saline at room temperature. A 5 cm portion of the everted intestine was mounted in a permeability apparatus. This setup was placed in a 250 ml beaker containing a 1000 µg/ml solution of Rifapentine, as well as an identical setup containing Rifapentine-loaded Nano Liposomes at the same concentration. The mucosal side of the intestine was perfused with Krebs' solution, and the assembly was maintained at 37°C with continuous stirring. Samples were collected every 5 minutes for 1 hour and analyzed using HPLC to assess the drug permeability across the intestinal mucosa17, 18.

$$Papp = \frac{dQ}{dt} * \frac{1}{A * C0} = v * \frac{dC}{dt} * \frac{1}{A * C0}$$

The apparent permeability (Papp) was calculated by the equation19, 20.Where, Papp: Apparent permeability co-efficient dQ/dT: Cumulative amount of drug (Q) appearing inthe acceptor compartment as a function of time, andwas obtained from the slope of the linear portion of the amount transported- versus-time plot A: Surface area of the intestine (cm2), taking 0.18 cm as radius, C0: Initial concentration of drug in the donor compartment (µg/ml)

V: Volume of sample (ml)

RESULTS AND DISCUSSION

Characterization of vesicles formed [21, 22]

The vesicles produced were spherical and unilamellar in structure. However, the size ofthe vesicles varied depending on the molarratio of the structural lipids used.Additionally, the entrapment efficiency alsofluctuated, with the highest observedentrapment efficiency being 39.86 ± 0.38%.The results are compiled under table 1.

Table1:Characterization of small unilamellarvesicles

S. No.	Composition (molar ratio)	Vesicle size (nm)	Entrapment (%)
1.	Sunflower lecithin: Cholesterol (5:5)	401	27.63 ± 0.15
2.	Sunflower lecithin: Cholesterol (6:4)	435	34.73 ± 0.52
3.	Sunflower lecithin: Cholesterol (7:3)	431	39.86 ± 0.38
4.	Sunflower lecithin: Cholesterol (8:2)	451	26.95 ± 0.19
5.	Sunflower lecithin: Cholesterol (9:1)	506	25.29 ± 0.44

Size and size distribution of Nano Liposomes formed[23]

The Nano Liposomes underwent particle size analysis, and the particle size distribution was evaluated.

The average diameter (Z-Average) of the particles was determined to be 1005 nm, with a peak size observed at 522.6 nm.

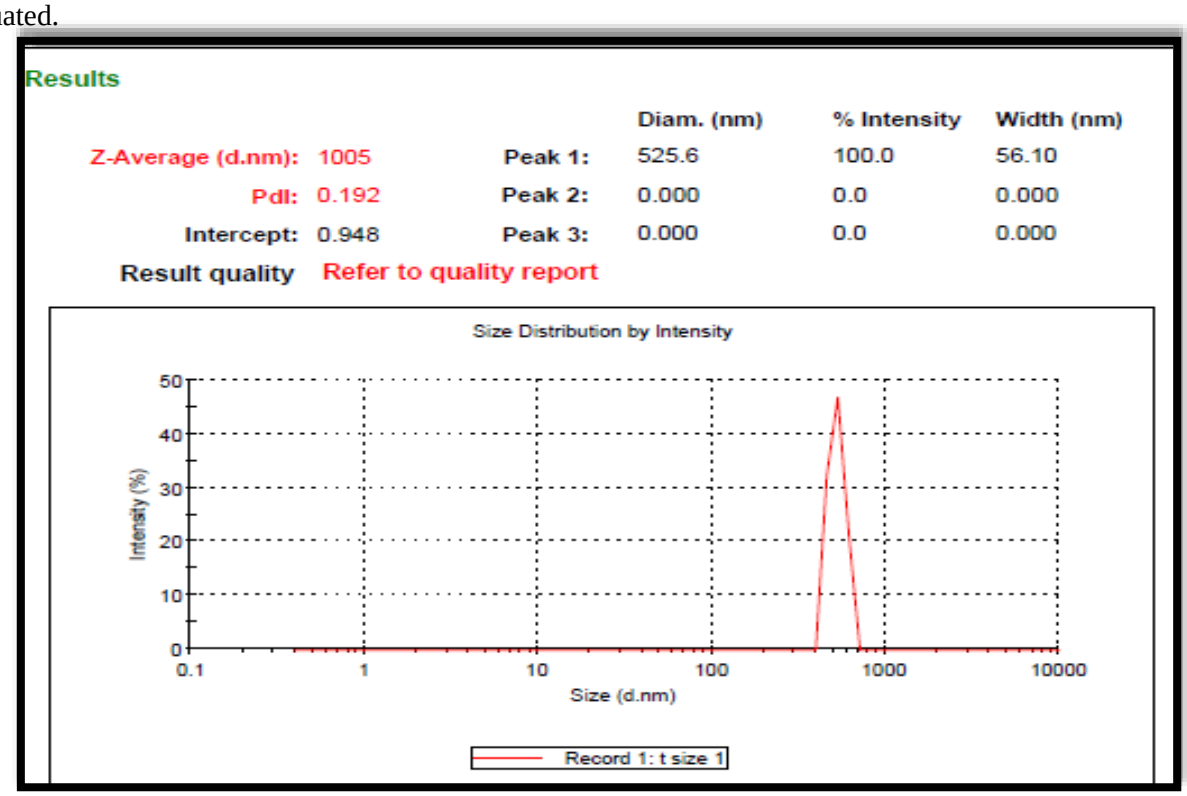


Figure 1: Particle size of cochletaes by Malvernzetasizer

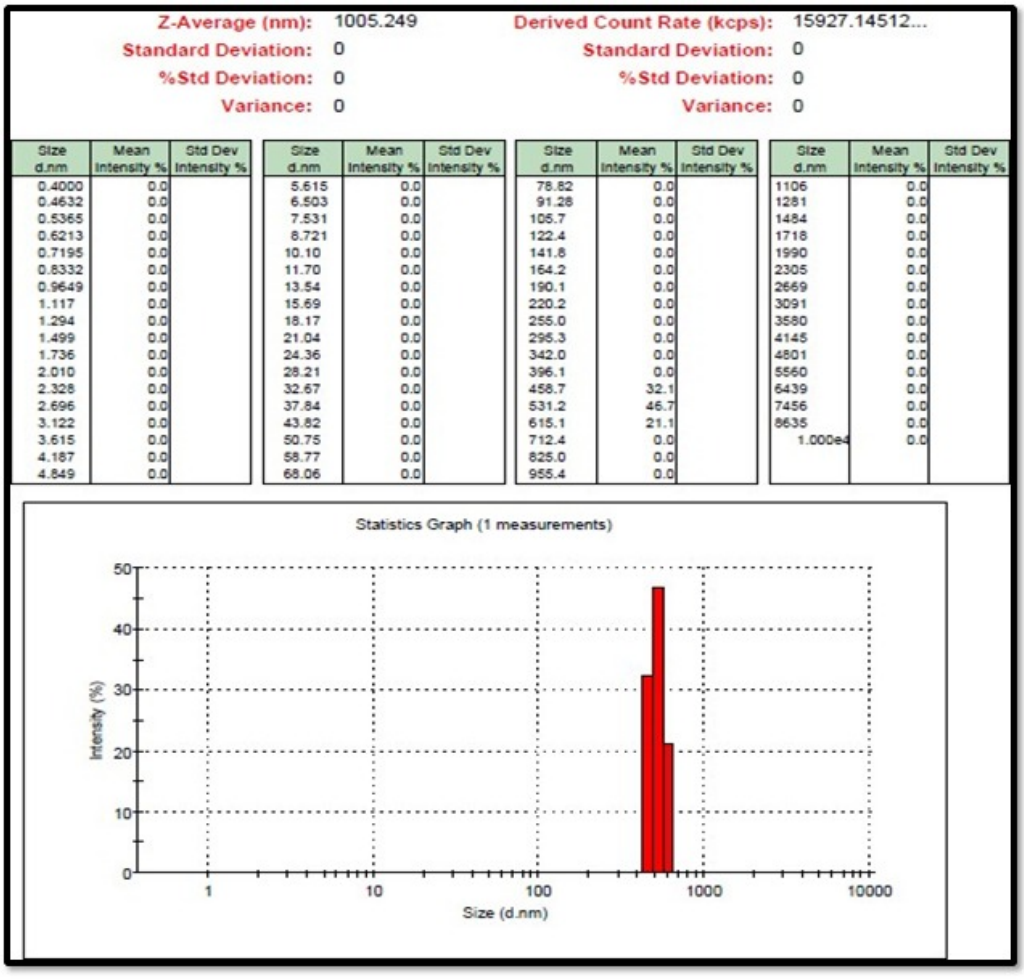
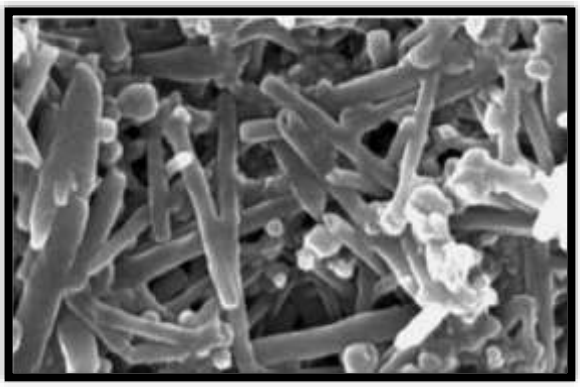


Figure2:Size Distribution of Cochleates by Malvern Zetasizer

Surface morphology of Cochleates particles (SEM studies)[24].

The images are shown in Figure 3. The micro size rods and clusters of such elongated rod shaped Cochleates were observed.

Figure 3: SEM images of Cochleates particles



Permeability estimation[25]

The absorption rate, represented by the cumulative concentration at various timeintervals across the intestinal segment, is shownin Figure 4 for both Rifapentine and Rifapentine- loaded Nano Liposomes. Both formulations exhibited a linear absorption after an initial lag phase, followed by a plateau. The slope of thelinear phase was used to calculate the rate ofchange in drug concentration (dQ/dt), fromwhich the apparent permeability coefficient (Papp) was determined. The Papp values werefound to be 3.56×10^{-6} cm/sec for Rifapentineand 7.78×10^{-6} cm/sec for Rifapentine -loaded Nano Liposomes.

Table 2: Absorption kinetics of Rifapentine and Rifapentine loaded Nano liposomes by everted rat intestine method

S. No.	Time (sec)	Cumulative Concentration (µg)	
		Rifapentine	Rifapentine loaded Nano Liposomes
1.	300	36.075 ± 4.5	96.135 ± 7.5
2.	600	51.870 ± 5.2	238.485 ± 17.6
3.	900	70.590 ± 8.3	292.110 ± 21.3
4.	1200	109.394 ± 9.6	365.235 ± 23.3
5.	1500	187.590 ± 12.3	437.580 ± 24.6
6.	1800	243.490 ± 23.4	524.745 ± 26.7
7.	2100	300.300 ± 22.1	587.925 ± 21.2
8.	2400	302.835 ± 21.4	737.100 ± 20.2
9.	2700	327.210 ± 23.7	771.615 ± 27.8
10.	3000	369.525 ± 23.4	807.495 ± 21.9
11.	3300	376.155 ±24.4	828.750 ± 23.3
12.	3600	381.030 ± 21.2	842.945 ± 25.6

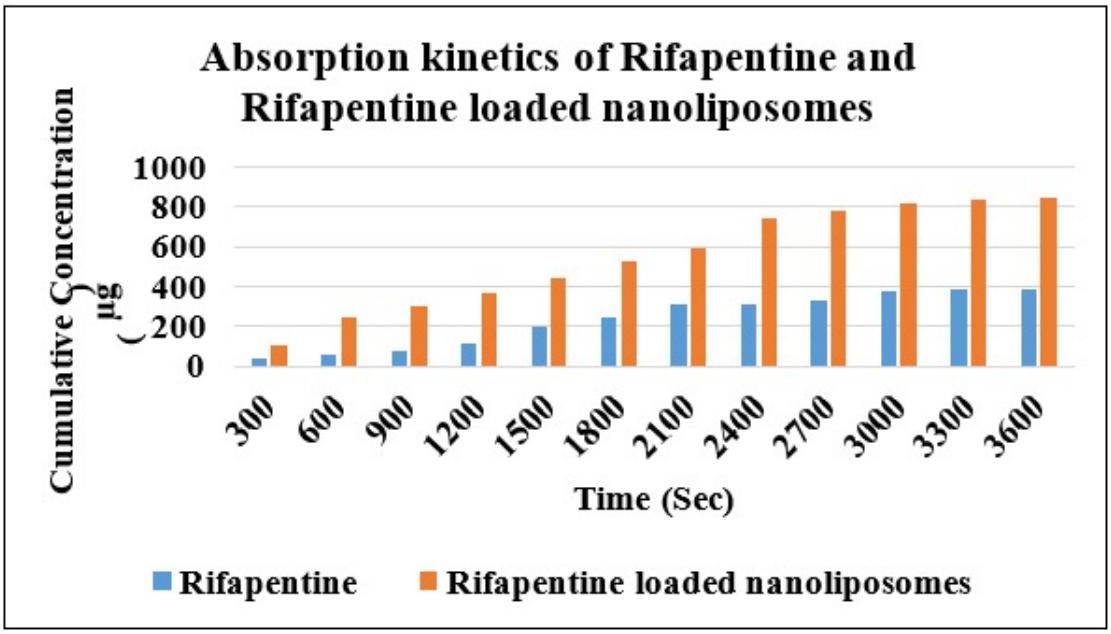


Figure4: Absorption kinetics of Rifapentine and Rifapentine loaded Nano Liposomes usinge verted intestine apparatus

CONCLUSION

The formulation of Rifapentine Nano Liposomes presents a promising approach for enhancing its absorption through the intestinal mucosa. It was found that the Cochleates formulation of Rifapentine significantly improved its apparent permeability. The Cochleates structures are formed by the interaction of divalent Ca^{2+} ions with small unilamellar vesicles of Rifapentine. The formulation process began by selecting an appropriate lipid-cholesterol composition for the preparation of small unilamellar vesicles (SUV). Sunflower lecithin was chosen as the structural phospholipid due to its availability and composition, which includes phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)—all ideal for creatingnegatively charged vesicles for Cochleatesformation. Initial experiments aimed at optimizing liposome formation using the film hydration methodfocused on vesicle size and drug entrapmentefficiency. The best results were obtained whensunflower lecithin and cholesterol were combinedin a 7:3 molar ratio, achieving a maximum drugentrapment of 39.86 ± 0.38%. This highentrapment can be attributed to the lipophilicnature of Rifapentine, as drug entrapmentdepends on the lipid-to-aqueous phase ratio.The vesicle size across different formulations variedbetween 400–500 nm, with larger vesiclesobserved when the phospholipid content increased. Morphologically, the vesicles werespherical and unilamellar. The liposomal formulation with the highestRifapentine entrapment was then used forcochleate formation. Initial trials with varying concentrations of Ca^{2+} ions indicated that a higherconcentration of Ca^{2+} (10 mM) was necessary toachieve optimal cochleate formation. Microscopic analysis of the cochleates revealedaggregation into larger clumps, which couldaffect absorption kinetics. To address this, bovineserum albumin (1% w/v of the total cochleateformulation) was added as an anti-aggregation agent prior to the addition of Ca^{2+} ions. Characterization of the Nano Liposomes was conducted to evaluate particle size,distribution, and morphology. The average diameter (Z-Average) of the cochleates was determined to be1005 nm, with a peak size of 522.6 nm. This difference can be attributed to the elongated shape of the cochleate particles. The size distribution also revealed the highest particle intensity in the 450-620 nm range. Scanning electron microscopy (SEM) confirmed theelongated, rod-shaped structure of the cochleates. Permeability studies demonstrated that the cochleate formulation of Rifapentine resulted in more than a two fold increase in apparent permeability compared to normal Rifapentine.

The apparent permeability of Rifapentine was3.56×10^{-6} cm/s, whereas the nanoliposome formulation increased this value to 7.78×10^{-6}cm/s. This enhanced absorption through the intestinal mucosa suggests that Nano Liposomes significantly improve Rifapentine'sbio availability. This approach can help address the issue of Rifapentine malabsorption, especially when taken with other anti-TB drugs, food, orantacids, potentially reducingthe required dosage and side effects. Further clinical studies are needed to confirm these findings.

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