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Original Article

Statistically Optimized Facile Development, Characterization and Evaluation of Niosomal Nasal Drug Delivery System of Ropinirole Hydrochloride: *In vitro* Drug Release, Cytotoxicity and *ex vivo* Permeability Studies

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ABSTRACT

Introduction: Nasal route is an alluring route for direct drug delivery to the central nervous system (CNS), owing to its avoidance of the hepatic first-pass metabolism and deciphering the blood–brain barrier passage issues. ROP-HCl has a lower experimental BBB permeability therefore ROP-HCl freighted niosomes were fabricated by ethanol injection method and served two purposes one being a novel Niosomal formulation impacting the permeability and other obtaining a sustained release property which likely would lead to an improved bioavailability of ROP-HCl. **Objectives:** The present study is aimed to develop a stable non-ionic surfactant vesicle; Niosomes embodying Ropinarole Hydrochloride (ROP-HCl) for ameliorated treatment of Parkinson's disease (PD) by statistical optimization employing a 3-level factorial design using Design Expert® software. **Materials and Methods:** All the formulations were characterized physiochemically and morphologically. Additionally, the drug and excipients interaction studies were evaluated using Differential scanning calorimetry (DSC), X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). Further, *in vitro* release kinetics using DD solver excel add-in and mechanism was studied for developed Niosomal formulation. Cytotoxicity study was studied in Raw 264.7 cells. in a concentration dependent manner and *ex vivo*, permeability was done using sheep mucosa. Toxicity was studied by histological examination. **Results:** The IC_{50} value of developed Niosomes was lower than drug itself and further the permeability of developed Niosomes was considerably enhanced compared to ROP-HCl alone. Histological examination revealed safe nature of developed formulation. **Conclusion:** These results conveyed that, Niosomes can be a valuable carrier for the nasal delivery of ROP-HCl to CNS.

Keywords: Ropinirole hydrochloride, Statistical approach, Niosomes, Nasal drug delivery, cytotoxicity, Release kinetics, *Ex-vivo* permeability, Toxicity study.

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INTRODUCTION

Central nervous system (CNS) is the mainstay of human body and any disorders arising or associated with it will negatively impact the memory, sensory and daily activities. A myriad range of diseases associated with CNS viz; Parkinson's, Alzheimer's, Huntington's, schizophrenia, migraine, brain tumor and meningitis need targeted delivery of drugs to the brain for treatment and surpassing the Blood Brain Barrier (BBB) is a major obstacle in this regard.[1] The BBB which is a network of blood vessels and tissues; a lipophilic barrier plays a major role in preventing the harmful substances from reaching Brain with exceptions to water, oxygen, carbon dioxide and some anesthetics. Another important aspect is presence of drug efflux transporters which prevent drug accumulation in the Cerebrospinal fluid (CSF)[2] Parkinson's disease is progressive neurodegenerative disorder pervaded by retrogression of dopamine producing cells in the substantia niagra.[3] Apart from the early diagnosis, it is necessary to employ delivery systems which aid in enhanced penetration and increased targeting of therapeutics to Brain. Some of the carriers successful in achieving this include Liposomes,

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Niosomes, Microspheres immunoglobulin, serum proteins, synthetic polymers and erythrocytes, each with their own share of pros and cons.[4] But the major advantage is their capability in providing targeted delivery which leads to nil or fewer side effects associated with drugs and enhancing their Bioavailability and therapeutic efficacy. Liposomes can engulf various types of drugs in controlled or sustained manner for targeted drug delivery and are more advantageous over other carriers, but their high production costs and limited shelf life are major barriers leading to thrust in researching their alternatives like vesicular systems which can conquer drawbacks of liposome.

Niosomes are bilayer systems that contain Non-ionic surfactants as well as cholesterol. They have a longer shelf life, greater stability, and the ability to deliver drugs to the target site in a controlled or sustained manner, resulting in increased bioavailability. Non-ionic surfactants are used because of their ability to increase the solubility and bioavailability of poorly water-soluble drugs. Another significant benefit of using Niosomes is that they can entrap both hydrophilic and lipophilic drugs, either in an aqueous layer or in a vesicular membrane made of lipid materials. Niosomes are thought to be suitable colloidal carrier systems for drug delivery via the nose because they are composed of biocompatible and biodegradable phospholipids that are similar to the components of the nasal mucous membrane. The niosomes are coherent in reducing the mucociliary clearance in the nose and in providing sustained release properties.[5] Their distinctive feature over liposomes includes less expensiveness due to low production costs involved, chemically more stable and rugged, preparation from single chain surfactant, no special storage or handling required due to absence of any phospholipid. Last but not the least, is the most important aspect is their safety margin is higher attributed to their non-ionic nature.

Because of its precedence in various aspects such as BBB circumvention, first pass metabolism (FPM) avoidance, safety, practicability, ease of administration, and non-invasive nature,[6-8] the nasal route is a preferred route for targeting Brain and related diseases. Nose-to-brain delivery has proven to be one of the most sought-after areas for research in improving treatment for neurodegenerative diseases such as Alzheimer's and Parkinson's, which are most likely conciliated via the olfactory or trigeminal neuron pathway. Nasal mucosa offers advantages to deliver drugs to brain via olfactory route thus providing a rapid onset of drug action and hence a faster therapeutic effect. Developing new treatment modalities is desperately needed in this regard. The limitations of the nasal route like ciliary clearance, enzymatic degradation and low permeability can be conquered by colloidal carrier systems like niosomes, which offer advantages like rapid permeability, better retention of the drug in the nasal mucosa and better absorption by enhanced penetration. The ideal combination of equitable delivery system and the appropriate route of absorption may cause enhanced penetration into CNS and a sustained presence of the drug in the brain. A summary of the recent studies,[6-13] involving Niosomes applications as drug delivery carriers is summarized in Table 1.

Ropinirole Hydrochloride is a non-ergoline dopamine agonist used to treat Parkinson's disease and restless legs syndrome. It works as a dopamine substitute by binding to and activating dopamine D2 and D3 receptors in the brain's caudate putamen, thereby improving motor performance. The medication is taken orally three times a day at a dose of 0.25 mg for Parkinson's disease. ROP-HCl possesses relatively low permeability toward BBB.[14] There has been lot of work done on ROP-HCl so far with Ethosomal formulations, Hydrogel based nanoparticles, polymer-lipid nanoparticles and chitosan coated Nanoparticles for controlled release and intranasal delivery.[15-20] The details are summarized in Table 2. The present study is unique in that it is the first instance of a Niosomal formulation of ROP-HCL being reported based on a statistically optimized method and researched for its potential Intranasal delivery and overcoming drug permeability limitations as well as investigating feasibility of nose to brain delivery route for this formulation, thereby opening new avenues in Parkinson's disease treatment.

MATERIALS AND METHODS

Materials

ROP-HCl Active Pharmaceutical Ingredient (API) was received as a sample gratis from MSN Laboratories Pvt. Ltd., Hyderabad, India. DPSM (Egg Sphingomyelin) along with Cholesterol was procured from Lipoid Pvt. Ltd., Germany. Span 80, Oleic acid, Potassium dihydrogen phosphate, Triethyl amine, Orthophosphoric acid, chemicals were purchased from S.D Fine Chemicals (Mumbai, India). Dialysis membrane 60 (Make: HiMedia, Mumbai, India, with cut-off Molecular weight of 12000D). MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), trypsin, EDTA (Ethylenediamine tetraacetic acid), PBS (Phosphate Buffer Solution) and RAW 264.7 macrophage cells (ECACC, Salisbury, UK) were maintained in Dulbecco's Modified Eagle Medium (DMEM) were procured from Sigma Chemicals, Mumbai. DMEM and FBS was procured from Thermo Fischer Scientific, Gibco. 96-well plate purchased from Eppendorf India. Analytical grade solvents were used, all chemicals were used without further purification and double distilled (DD) water was used throughout the experiment unless mentioned otherwise.

Methods

Experimental Design for Optimization of Niosomes

A full factorial design was used as design matrix to fabricate an optimized formulation of Niosomes. The input parameters included SPAN 80 and cholesterol in varied levels and the Critical quality attributes were 5 in number: drug content, % entrapment, Particle size, Zeta Potential and % Cumulative drug release. The

Table 1: Niosomes as drug delivery systems- recent researches.

Sl. No.	Formulation	Type of Surfactant	Target Site	Technique	Reference
1	Intranasal Niosomal formulation	Span 20, Span 40, Span80 and Span 85	Nasal mucosa	Thin-film hydration method	6
2	Niosomes for intranasal drug delivery	Tween 20	Nose to brain	Thin film hydration method	7
3	Spanlastics formulations	Span 60	Brain targeting via intranasal route	Ethanol injection method	8
4	Nose-to-brain noisome drug delivery formulation	Span 60	Olfactory pathway	Ethanol injection method	9
5	Niosomal sustained release formulation	Span 20	Multi route delivery for instance, intravenous and pulmonary routes and via nasal aerosol pump	Ethanol injection method	10
6	Pro-niosomal gels	Tween 80	Ocular irritation	Coacervation phase separation method	11
7	Surface modified niosomes	Span 20, Span 40, Span60 and Span 80	Brain targeting via nasal route	Thin film hydration technique	12
8	Dual drug-loaded niosomes for nasal delivery	Span 20	Nose-to-brain delivery	Ethanol Injection method	13

Table 2: Ropinirole Hydrochloride with different Carriers used for Drug Delivery.

Type of carrier / formulation	Type of Chemicals and reagents	Technique	Conclusion	Reference
Ethosomal formulations	Soyaphosphatidyl-choline, Ethanol	QbD approach	The formulation variations in responses can easily found by using desirability function.	15
Nanoparticles enriched with hydrogel	Propylene glycol monocaprylate, tripalmitin, Tween20, haloperidol, glutathione reductase, 5-5-dithio-bis-2- nitrobenzoic acid, thiobarbituric acid, ethylene-diamine tetra acetic acid.	Hot melt emulsification coupled with ultra-wave sonication method	RP loaded lipid nanoparticles and hydrogel formulations were successfully developed and optimized. The obtained results were demonstrated that the enriched formulations were successfully administrated with alternative route i.e., oral delivery for parkinsonism.	16,17
Polymer-lipid microparticles	1,2-dipalmitoyl-sn-glycero-3-phosphocholine), PLGA, L-leucin Alpha-lactose monohydrate, Chitosan	Spray-dried	The fabricated formulation was successfully developed with the PLGA and Chitosan (different molecular weights. These formulations are proving the no excipient interactions, *ex-vivo* studies and explains the no cytotoxicity on Calu-3 cell line.	18
Intranasal delivery	Chitosan, tripolypho-sphatetannous chloride dihydrate	Ionic gelation	The RH-CSNPs showed Controlled release profiles for up to 18 hr. Gamma scintigraphy imaging in rats was accomplished to ascertain the localization of drug in the brain following intranasal administration of formulations. The novel formulation showed the advantage of nose to brain delivery of RH using mucoadhesive nanoparticles compared with other delivery routes reported earlier.	19
Solid-lipid nanoparticles	Glycerol monostearate, Soya lecithin and tween 80 Chloroform	Double emulsion process	While increasing the concentration of soya lecithin desired particle size of SLNs was obtained as well as sonication time of primary emulsion. The burst release at the initial phase followed by sustained release was observed compared to pure drug solution ROP-SLN obtained *in vitro* and *ex vivo* release experiments, exhibited a biphasic release pattern. However, *in vivo* studies for ROP-SLN should be performed to determine its brain delivery efficacy.	20

3-D contour plots obtained helped in studying the relationship between input variables and CQAs. The Quadratic based non-linear model was used for in the study design. ANOVA was found significant and lack of fit insignificant in all models. The equation generated involved interaction between all variables A and B and squared terms represent quadratic term of the equation. Desirability factor was the criteria for selection of optimized formulation.

Synthesis of Vesicles and Drug Loading into Niosomes

The major components of a niosomal system includes drug, non-ionic surfactant, lipid and stabilising agent. Niosomes were formulated as per the literature reported method. Smaller vesicles and higher entrapment efficiencies have been reported to be achieved by Ethanol Injection method.[6,21,22] Out of the various experiments conducted it was found suitable for encapsulation of ROP-HCl. As per the quantity of ingredients Span 80 and cholesterol ratio was optimized statistically and others fixed as 20 mg DPSM and 10 mg Oleic acid taken in a 100 mL beaker and dissolved in sufficient quantity of ethanol with heating required temperature on a hot plate. Simultaneously, 50 mg ROP-HCl was dissolved in 10 mL of SNF pH 6.8 by Remi Magnetic Stirrer which was maintained at (1000 to 1200 rpm) at room temperature using teflon coated bead. The lipid mixture was injected into the drug solution kept under stirring at the rate of 0.25 mL/min using the 14-gauge needle. The aqueous phase immediately turned milky indicating the vesicle formation. The system was kept under stirring for up to 1.5-2 h, to facilitate the removal of ethanol. The vesicular dispersion was made up to 20 mL with SNF pH 6.8 and the dispersion was filtered through sterile graded filter (0.22µm PVDF filter, Pall Corporation, Pall India Pvt. Ltd., Mumbai, India) to obtain sterilized vesicles. The vesicles were then transferred to 10 CC vials and transfer was then followed by nitrogen sparging and sealing. The prepared niosomes as suggested from statistical runs (14 runs with duplications for combinations); batch numbers from ROP-HCl F1 to ROP-HCl F6 were stored at 2-8°C, until analysis.

Determination of Percentage Entrapment Efficiency (%EE)

The percentage of drug entrapped in the Niosomal formulation was determined by measuring the concentration of the drug in the aqueous phase by ultra-filtration method using centrisart devices (Sartorious) equipped with a filter membrane (molecular wt. cut off 20,000 daltons) at the base of the sample recovery chamber. About 1 mL of undiluted sample is placed in the outer chamber on the top of the sample holder and kept in the centrifuge. The unit is centrifuged at 20000 rpm for 1hr. The niosomes along with the encapsulated drug remain in the outer chamber and the aqueous phase is moved into the sample recovery chamber through the membrane. The amount of drug in the aqueous phase is estimated by HPLC at 250 nm by using the below equation.

$$\text{Entrapment Efficiency}\,(\%) = \left[\frac{\text{Amount of ROP} - \text{HCl entrapped}}{\text{Total ROP} - \text{HCl}}\right] \times 100 \quad (1)$$

Characterization and Analysis

The pH of the niosomal dispersions was measured by a pH meter of model wensar LMPH-12 by following 5-point calibration using standards. The custom functional groups were identified by Fourier transform Infra-red spectroscopy (FT-IR). The IR spectra of the samples were recorded on a Bruker FTIR spectrophotometer equipped with Opus software. Appropriate amounts of ROP-HCl pure drug, span 80, DPSM, cholesterol, Oleic acid physical mixture (1:1) and ROP-HCl niosomal formulation were studied by FTIR to elucidate the functional group positions, and any interaction between the drug and the other used excipients. The IR spectrum of drug, excipients and test products were recorded in the ambit of 400 to 4000 cm^{-1}. The thermal analysis was recorded for drug ROP-HCl, placebo mixture and Niosomal formulation by using DSC, Shimadzu TA instruments. The thermograms were recorded at a heating rate of 10°C /min and in the temperature of 0-300°C using nitrogen as a carrier gas. Surface morphology of the Niosomes was studied by Field emission scanning electron microscope. Before observation, the formulations were placed on a double adhesive carbon tape, which was stuck on aluminum stubs and then coated with gold under an argon atmosphere. The samples were visualized by FESEM with an accelerating voltage of 8-20 kV The mean particle size of the niosomal dispersion was measured by using dynamic light scattering technique using zetasizer (Nano ZS 90; Malvern Instruments, Malvern, UK). Analysis was carried out at 25°C temperature keeping the angle of detection at 90°. The prepared niosomal solution and ROP-HCl solutions were diluted (1:100) with 0.1M sodium chloride solution. All measurements were carried out in triplicate to ensure accuracy. The mean vesicle size is expressed in terms of diameter in nanometers (average of the vesicle size). The size distribution of vesicles is expressed in terms of poly dispersity index (PDI). The zeta potential is expressed in terms of surface charge of the system (mV). HPLC was used to measure the quantity of ROP-HCl in the samples, % of EE, % of drug content, and *in-vitro* drug release. HPLC system (Water Alliance model: 2695e separation module with 2996 photodiode array detector) with a reverse - phase Kromasil C_{18} (250x4.6 mm, 5µ) column was used in the analysis. 0.01 M Phosphate buffer containing 2mL of triethylamine (pH 3.00±0.05) and acetonitrile (85:15 w/v) used as a mobile phase. 20 µL samples were injected. Flow rate of 1.5 mL/min was maintained and 250 nm (λ_{max}) used for analysis. The standard calibration curve was established in the range of (0.054 µg/mL to 500.25µg/mL) used for the determination of drug concentration in the sample. The method linearity was confirmed $Y = 0.104x + 0.034$ with *R* value of 0.999. For determining % drug loaded; a weighed amount of the prepared niosomes, equivalent to 2.5 mg of ROP-HCl, was taken in a test tube and was lysed with 9 mL of methanol. Subsequent dilutions (10 mL) were made with the SNF pH 6.8 and the absorbance was measured at a λ_{max} of 250 nm using a UV-VIS spectrophotometer. The study was performed in triplicate.

In vitro Release Study by Dialysis and Release Kinetics

The *in vitro* drug release studies of Niosomal formulation were conducted in SNF stimulated pH 6.8 buffer by dialysis process using cellulose dialyzing membrane (dialysis membrane 60 from Hi-Media, Mumbai, India, whose molecular cut-off is 12000D-14000D). 2.5 mg equivalent dose of niosomal formulations were taken and one end of the dialysis membrane tied and hanged into three different beakers containing each 500 mL of SNF pH 6.8 buffer solutions maintained at 37±0.5°C on a temperature controlled magnetic stirrer and stirred at 100 rpm with teflon coated bead. 5 mL aliquots were withdrawn at predetermined time intervals from the beaker and were replaced with equal volume of fresh buffers solution to maintain sink conditions. The samples were analyzed through HPLC at 250 nm for ROP-HCl. These *in vitro* drug release profiles were subjected for order of release kinetic models (zero order, cumulative % released vs. time) and first order (log % drug remaining vs. time) and mechanism of drug release using Higuchi's model (cumulative % drug released vs. square root of time) and Korsmeyer-Peppas model (log % drug released vs. log time). The model with the highest correlation coefficient of determination (r), highest Model Selection Criteria (MSC) and lowest AIC (Akaike coefficient) was considered as the most appropriate model for the diffusion data. The calculations were done using an excel add-in DD solver.

In vitro Cell Cytotoxicity Study (MTT Assay)

Raw 264.7 (ATCCR TIB-71TM) cells were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics (Pen-strep) and used in a colorimetry-based MTT assay to assess the cytotoxicity of prepared Niosomes. The cells were screened for contamination on a regular basis. The cells were cultured at low passage and seeded with 2×104 cells per well in 96-well microtiter plates in DMEM medium before being incubated overnight at 37°C in a 5 percent carbon dioxide (CO_2) environment. After the cells in each well had reached 70-80 percent confluence, the medium was removed and 100 µL of fresh DMEM medium was added to each well. In triplicate, the test products of Niosomal formulation and ROP-HCl solution of various concentrations were added. Following that, the plates were thoroughly mixed and incubated for 24 hr at 37°C in a 5 percent CO_2 environment. After 24 hr of incubation the medium was removed and washed with 200 µL PBS per well twice to remove any unbound formulations and serum. Then to each well, 100 µL of MTT reagent prepared with DMEM medium (i.e. 25 µL of MTT reagent (stock conc. 2 mg/mL) + 75 µL of DMEM medium without FBS was added and incubated at 37°C for 3 hr under 5% CO_2 environment. After 3 hr of incubation the MTT reagent was removed and 100 µl of DMSO/methanol (1:1 v/v) added into each well and incubated for 15 min on shaker and the optical density recorded at A540 nm excitation.

Cell viability was determined by below mentioned equation

$$\text{Cell viability}\,(\%) = \frac{\text{I (sample)} - \text{I Blank}}{\text{I (control)} - \text{I Bank}} \times 100 \qquad (2)$$

where I sample, I control, and I blank represent absorbance intensity at 540 nm for cells treated with different samples (positive control), untreated cells+MTT+DMSO (negative control), and a blank well devoid of cells (Media+MTT+DMSO), respectively.

Ex vivo Nasal Permeation Studies

Ex vivo permeation studies of selected formulations were performed using a Franz diffusion cell with a capacity of 10 ml. The nasal mucosa of a sheep was used in this study. Before beginning the experiment, the nasal mucosa was equilibrated in PBS for about 30 min. The mucosa was placed in a Franz diffusion cell between the donor and receptor chambers, with the donor chamber facing the mucosal surface. Different volumes of niosomal solution bearing fixed concentrations of ROP-HCl were poured into the donor chamber. At predefined time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, and 24 hr), samples were extracted from the receptor chamber. The removed quantities were replaced with equal proportions of new medium to maintain sink conditions and avoid saturation.

Toxicity study: Histopathological Examination

Nasal mucosa tissue was assessed for Toxicity by Histopathological examination. Under a microscope, the histological sections were stained with Hematoxylin and Eosin stain (H&E). The purpose of this study was to determine the *ex vivo* toxicity of optimized ROP-HCl Niosomal formulation.

RESULTS

Optimization of Formulation

In various studies, various statistical tools have been used to optimize the delivery system. For this purpose, we used the computer-assisted process with Design-Expert® software. The provision of data about selected dependent variables of the formulation during the optimization process allows for the prediction of the amount of those variables as well as other process parameters for the preparation of optimized product. The process also predicted the outcomes of various dependent factors such as particle size, EE, zeta potential, % drug content and %CDR (Cumulative drug release). Using the given variables, the statistically suggested optimised formulation was prepared and analysed for morphology, different physio-chemical properties, and drug release kinetics modelling. For descriptive purpose data of selected trials and finalized formulation is given in Table 3. ROP-HCl-F3 is the final optimized formulation. Table 4. Gives a detailed comparison of software predicted final formulation and practically obtained values for optimized formulation denoted as ROP-HCl-F3. Figure 1(a-f) depicts the 3-D contour plots for niosomal formulation.

Table 3: Physicochemical properties of ROP-HCl loaded niosomes based on Span 80: cholesterol ratio from factorial trials.

Batch Code	Span 80(µL)	Cholesterol (mg)	Drug Content (%)	Size (nm)	PDI	%EE	Zeta Potential (mV)	Cumulative % *in vitro* drug release
ROP-HCl F_1	75	25	98.1±0.5	186.1±4.8	0.112±0.02	38.1±1.2	-16.4±0.8	48.2±2.2
ROP-HCl F_2	75	35	98.9±0.9	238.2±2.6	0.131±0.08	54.0±1.8	-21.6±1.1	76.9±1.2
ROP-HCl F_3	100	35	99.3±0.1	136.6±2.1	0.102±0.06	92.1±1.4	-42.2±0.6	96.3±0.8
ROP-HCl F_4	100	45	97.8±0.3	251.2±3.1	0.214±0.01	55.1±1.2	-19.1±0.4	62.5±0.8
ROP-HCl F_5	125	55	99.8±0.8	351.4±3.5	0.186±0.05	68.2±2.2	-14.3 ±0.8	51.2±0.8
ROP-HCl F_6	125	65	101.2±0.6	381.1±3.2	0.104±0.06	45.2±1.6	-26.7±0.6	58.9±0.8

Table 4: Comparison of statistically predicted values and practically obtained.

Span 80	Cholesterol	Drug content	Entrapment	Size	ZP	%CDR	Desirability
Predicted Formulation							
100.12	35.05	99.46	92.34	136.5	41.50	96.80	0.9
Actual optimised formulation							
100	35	99.3	92.1	136.6	42.2	96.3	----

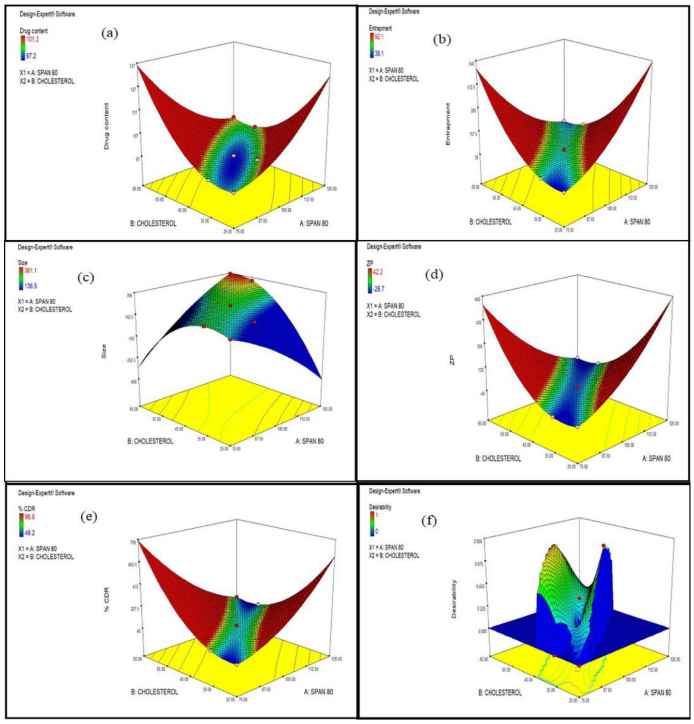

Figure 1: 3-D Contour plots for CQAs and their variability with Span 80 and cholesterol concentrations. (a)% drug content (b)% Entrapment (c) particle size (d) Zeta potential (e) % *in vitro* CDR and (f) Desirability contour plot.

Characterization of the Niosomes FESEM analysis

The SEM images obtained is shown in Figure 2. A smooth surfaced, spherical and unilamellar vesicles were observed in the morphological study through SEM. A distinct homogeneous spherical shape was clearly visible in the images exhibiting the successful completion of synthesis and loading of ROP-HCl Niosomes.

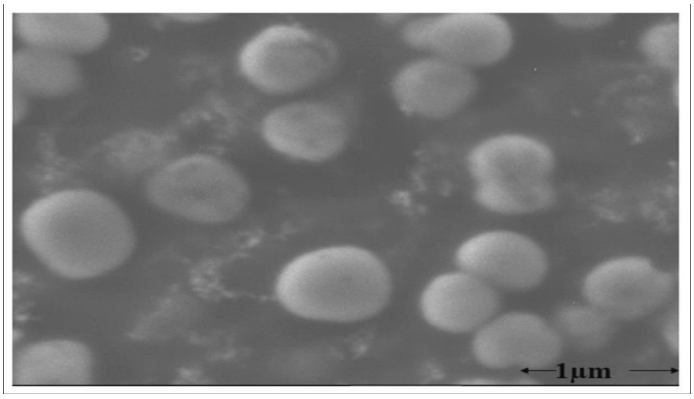

Figure 2: SEM analysis of ROP-HCl-F3 optimised noisome formulation.

FT-IR Spectroscopy

The FTIR spectrum of the pure drug shows the characteristic FTIR peaks at 3416 cm^{-1} (N-H stretching), 1626 cm^{-1} (C=C stretching), 3076 cm^{-1} (aromatic, C H stretching), 2938 cm^{-1} and 2881 cm^{-1} (aliphatic C-H stretching), 1312 cm^{-1} and 1347 cm^{-1} (C-N stretching), 1759 cm^{-1} (C=O stretching. It was concluded, basing on the FTIR results (Figure 3), that there were no drug excipients interactions, as the above-mentioned peaks at specific wave numbers, were also observed in physical mixtures and formulations developed, using span 80, DPSM and cholesterol. The FT-IR spectra of the pure drug, Placebo, ROP-HCl Niosomal Formulation, and the excipients used in the study revealed no interaction between the drug and the excipients used in the study. demonstrating the compatibility of the drug and the excipients used in the current study.

Differential Scanning Calorimetry (DSC)

The DSC thermograms of native materials (ROP-HCl and physical mixture of placebo and ROP-HCl F_3 are presented in

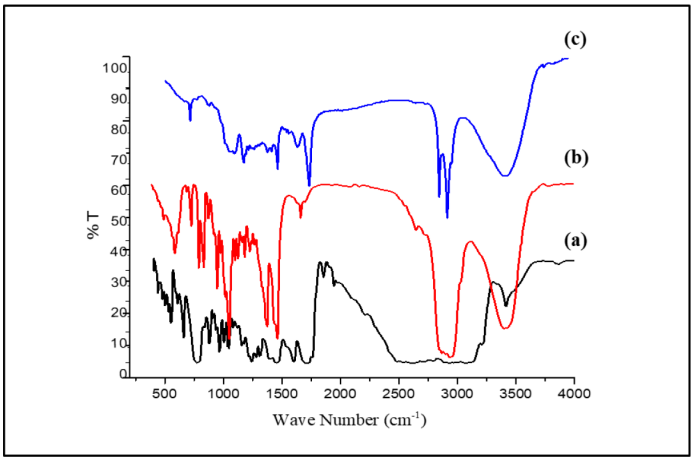

Figure 3: IR Spectra of (a) ROP-HCl, (b) Placebo, and (c) ROP-HCl-F3 Niosomal Formulation.

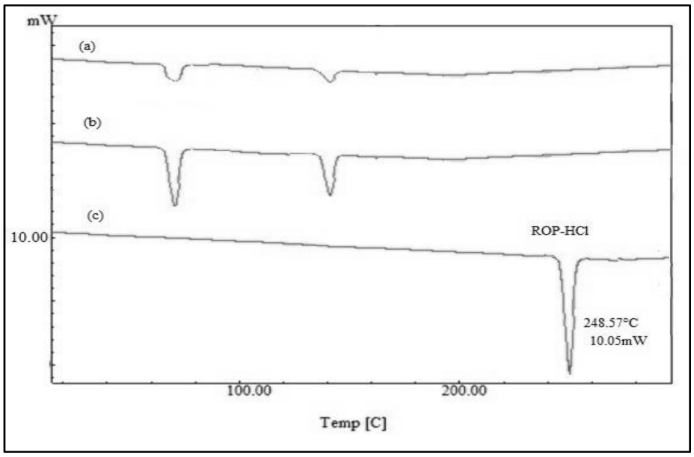

Figure 4: DSC Thermograms of (a) ROP-HCl Niosomal formulation, (b) Placebo physical mixture and (c) ROP-HCl.

Figure 4. The crystalline ROP-HCl exhibited a sharp peak at 248.57°C indicating its melting point and that the active is in crystalline form (Figure 4 C). The placebo mixture exhibited two small endothermic humps which might be attributed to Span 80 and Cholesterol. Notably, DSC thermogram of ROP-HCl F3 was devoid of any crystalline peak of drug indicative of conversion to amorphization of ROP-HCl in the Niosomes and considerable interaction of bilayer with active.

X-Ray Diffraction Studies

The X-Ray powder diffraction pattern of ROP-HCl, placebo mixture and ROP-HCl niosomes are shown in Figure 5. ROP-HCl showed a characteristic of a crystalline form with intensity maxima appearing at 2theta at 6.820, 20.020, 21.700, and 22.860. The diffraction obtained from the physical mixture of placebo shown a broad hump which indicating the amorphous state which evident that the encapsulation technique used had no effect on the lipid and surfactant characteristics. The pattern obtained for formulation ROP-HCl F3 (2.5 mg ROP- HCl) showed the

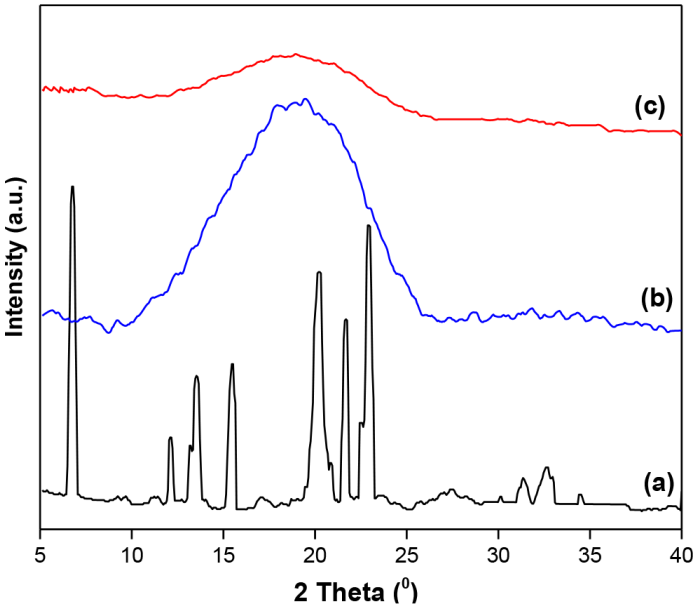

Figure 5: XPRD of (a) ROP-HCl (b) Placebo mixture, and (c) ROP-HCl Niosomal formulation.

absence of peaks characteristic of ROP-HCl due to conversion from crystalline to amorphous form.

pH and % Drug Content

The pH of the prepared ROP-HCl loaded niosomes was found to be around 6.8±1.3. There was no significant difference in pH of the niosomal formulations. This is because the dilution of the final vesicular dispersions was made with SNF of pH 6.8. This result indicates that all the formulations were uniform with respect to their pH.

A weighed amount of the prepared niosomes, equivalent to 2.5 mg of ROP-HCl, was taken in a test tube and was lysed with 9 mL of methanol. Subsequent dilutions (10 mL) were made with the SNF pH 6.8 and the absorbance was measured at a λ_{max} of 250 nm using a UV-VIS spectrophotometer. The study was performed in triplicate.

The prepared ROP-HCl loaded niosomes were characterized for percent drug content. The percent drug content values for ROP-HCl F_1 to ROP-HCl F_6 niosomal formulations were found to be in the range of 97.8±0.3% to 101.2±0.6% respectively. Percent drug content values indicated that the ROP-HCl was uniformly distributed in the aqueous core of the vesicular dispersions and the percent drug content was found to be near to 100%. This result indicated that there was no loss of drug during the development of niosomes. Figure 1a describes contour plot for Variation of % drug content with Span80: Cholesterol.

$$\text{Drug content: } Y = +97.38 - 0.45A + 1.75B - 17.15AB + 7.58A^2 + 11.80B^2 \quad (3)$$

% Entrapment Efficiency

The percent entrapment efficiency (% EE) of vesicular systems is an important parameter to assess the drug delivery potential of the system. The %EE of the vesicles was determined by using ultra centrifugation method. The niosomal %EE value varied from 38.1±1.2% to 92.1±1.4% respectively. Figure 6 depicts entrapment efficiency of developed niosomal formulations. From the results of optimized formula of lipids, it was assumed that the inner core of the vesicle is large enough to accommodate the hydrophilic drug ROP-HCl. A high zeta potential value frequently leads to increase in the repulsion forces of the bilayer structures of the vesicles, which consequently increases the size of the inner core of the niosomes. Since ROP-HCl is a hydrophilic compound, increasing the aqueous core compartment contributes to increasing the amount of ROP-HCl in the vesicles. Figure 1b describes contour plot for Variation of % EE with Span80: Cholesterol. The addition of cholesterol inhibits the surfactant's gel to liquid phase transition by embedding itself in the bilayer, providing rigidity to the vesicles and preventing active leakage.[9]

$$\text{Entrapment: } Y = +55.38+10.80A-7.30B-236.85AB+91.18A^2+132.10B^2 \quad (4)$$

Zeta Potential and Size

The zeta potential values of ROP-HCl loaded niosomal dispersions were found to be in the range of -14.3±0.8 mV to - 42.2±0.6 mV respectively, Surface charge is essential for the stability of any dispersed system. This result is probably due to the surface charge imparting nature of DPSM. The values of zeta potential showed that vesicles had sufficient charge to inhibit aggregation of vesicles due to electric repulsion. Figure 7 depicts zeta potential and size variations of developed formulations.

The mean vesicle size of ROP-HCl loaded niosomes was found to be in the range of 136.6±2.1 nm to 381.1±3.2 nm and PDI was in the range of 0.102±0.6 to 0.112±0.2 respectively. These results indicate the predominant effect of Span 80 and cholesterol ratio on the niosomal vesicle size. The concentration of cholesterol determines the size and flow behaviour of the niosomes. At higher levels of Span 80 (125 mg) and cholesterol (55 mg), the prepared niosomes showed larger vesicle size (381±.2nm) and with the amount of Span 80 (100) and medium level of cholesterol (35 mg), resulted in vesicles with 102.6±2.1 nm in size respectively.

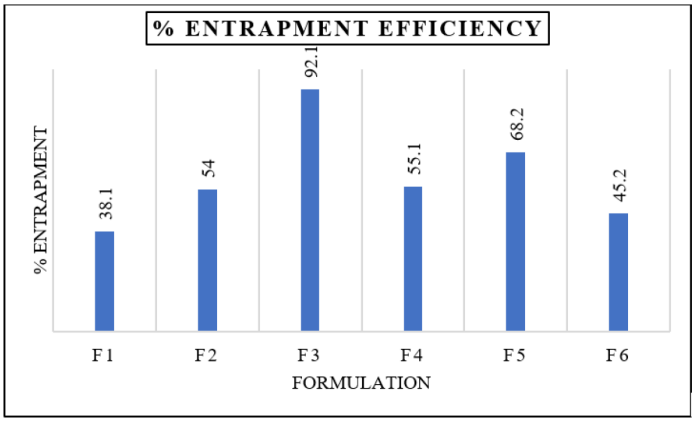

Figure 6: % Entrapment efficiency of developed Niosomal Formulations (F1-F6), Optimised formulation is ROP-HCL-F3.

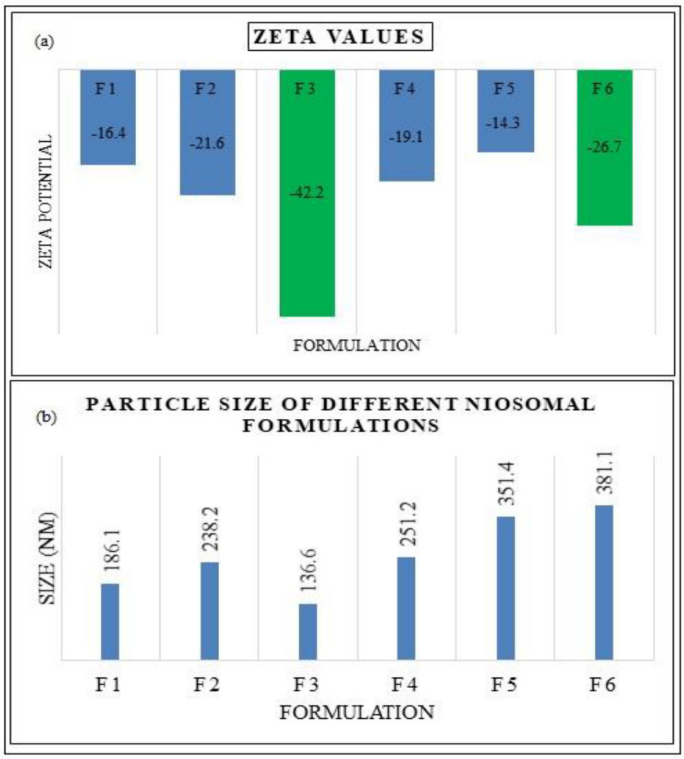

Figure 7: (a) & (b) Zeta Potential and particle size of developed Niosomal Formulations (F1-F6), Optimised formulation is ROP-HCl-F3.

$$\text{Size: } Y = +249.65+15.08A+82.65B+407.30AB-86.93A^2-287.10B^2 \quad (5)$$

$$\text{Zeta Potential: } Y = -19.02+12.52A-17.65B-315.45AB+107.45A^2+205.60B^2 \quad (6)$$

In vitro Drug Release Studies

The *in vitro* drug release was determined by dialysis bag method. A range of niosomal formulations were prepared to evaluate the effect of various formulation parameters on the *in vitro* drug release profile. As per theory drugs encapsulated in the niosomal systems are released possibly by the following mechanisms: a) passive diffusion b) vesicle erosion. The ROP-HCl drug release from niosomal formulations was found to be slow, gradual and extended over 24 hr. After 24 hr, there was no further rise in the values of the cumulative percent drug release. The *in vitro* drug release profiles of all the ROP-HCl loaded niosomes are shown in Figure 8 which represents the cumulative percent drug release

of ROP-HCl from F_1 to F_6 niosomal formulations. Among these six batches of niosomes, F_3 formulation showed maximum cumulative percent drug release, i.e., 96.3±0.8% respectively. The developed niosomes were able to release the drug up to 24 hr, in a sustained manner, whereas, drug release from ROP-HCl solution was rapid and, 99.96±1.3% of drug was released within 2 hr.

$$\%CDR: Y = +62.48 - 30.45A + 35.55B - 331.65AB + 115.73A^2 + 207.40B^2 \quad (7)$$

In vitro Drug Release Kinetics

A critical aspect of formulation characterization is determining the release kinetics of the encapsulated drug from the niosomes. The use of a mathematical model to describe a drug release profile allows for the elucidation of the drug release mechanism and can be used to guide formulation development efforts. Zero order models describe a release rate that is independent of drug concentration and has a constant amount of drug released per unit time over the entire period of drug release. The first order model describes a release process that is proportional to the drug concentration in the vesicle. Higuchi's model suggests drug release mechanisms such as diffusion by erosion, non-Fickian diffusion, Fickian diffusion, and super case transport, whereas Korsmeyer-model Peppas explains drug release mechanisms such as diffusion by erosion, non-Fickian diffusion, Fickian diffusion, and super case transport. The release mechanism is a function of the diffusion exponent 'n', according to Korsmeyer-Peppas equation. Typically, the n value varies with the geometrical shape of the dosage form. If the vesicles have a spherical shape and the value of n is ≤0.43, the release mechanism is Fickian diffusion. If n is between 0.43 and 0.85, the mechanism follows non-Fickian (anomalous) diffusion; if *n*=0.85, it is non-Fickian case II transport; and if n>0.85, it is non-Fickian Super Case II transport. The drug release kinetics of *in vitro* drug release data for niosomes were studied by fitting them to various kinetic equations such as zero order, first order, Huguchi, and Korsmeyer- Peppas for all vesicles from 0 to 24 hr. Table 5 displays the correlation coefficient 'r', release rate constants, and n values. The korsmeyer-peppas model was found to be the best fit, and the diffusion exponential value (n) was used to determine the type of release mechanism involved in the diffusion of ROP-HCl-F_3 from the Niosomes. The n value for ROP-HCl-F_3 being 0.359 indicated a Fickian release mechanism involved and also possessed highest MSC and lowest AIC amongst all models.

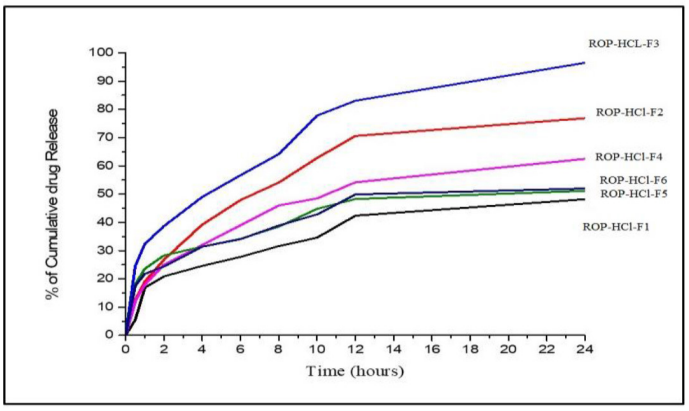

Figure 8: *In vitro* Cumulative % drug release vs time for Niosomal formulations.

In vitro Cell Cytotoxicity: MTT Assay

Figure 9 shows that cell-viability reduces by raising the ROP-HCl and ROP-HCl loaded Niosomal formulation concentration-levels. ROP-HCl loaded niosomal formulation shows better activity when compare to the free drug under identical concentration. These annotations imply that intra-cellular concentration of ROP-HCl delivered through Niosomal formulation is higher than that of free ROP-HCl and proved ROP-HCl loaded Niosomal formulation is a better carrier to deliver the drug into the cells. Further, IC_{50} of formulation is 0.15 µM and free ROP-HCl is 0.45 µM, this indicates ROP-HCl loaded Niosomal formulation has greater cytotoxicity when compared to ROP-HCl alone.

Ex vivo Nasal Permeation Studies

Figure 10 represents *ex vivo* permeation profile of ROP-HCl solution and ROP-HCl loaded niosomes formulation across an excised sheep nasal mucosa as a permeation barrier. The plain drug solution permeated within 4 hr (73.1±1.67%). The ROP-HCl loaded niosomes showed sustained *ex vivo* transmucosal transport, (89±2.6%) respectively. This could be attributed to the drug embedded within the lipid, causing stronger adhesion

Table 5: *In-vitro* release kinetics for ROP-HCl loaded niosomes.

Batch code	Zero order				First order				Higuchi			Korsmeyer-Peppas			
	K_0	R	MSC	AIC	K_1	R	MSC	AIC	R	MSC	AIC	R	n	MSC	AIC
ROP-HCl F_1	2.756	0.9337	-0.4100	67.115	0.042	0.9724	0.1781	61.8185	0.9837	1.9619	45.7644	0.9865	0.365	3.1769	94.8293
ROP-HCl F_2	4.544	0.8930	-0.0222	75.2070	0.103	0.9927	1.9695	57.2822	0.9734	2.5431	52.1194	0.994	0.863	2.7682	50.0932
ROP-HCl F_3	5.528	0.9221	-0.5255	80.3511	0.165	0.991	1.4977	62.1429	0.9868	1.8576	58.9036	0.9935	0.359	3.8936	40.9757
ROP-HCl F_4	3.664	0.9253	-0.4946	72.6598	0.068	0.9882	0.4593	64.0744	0.9875	1.9184	50.9422	0.9937	0.356	3.9252	32.8813
ROP-HCl F_5	3.171	0.8957	-1.2440	73.5167	0.056	0.9621	-0.6549	68.2156	0.9681	0.4744	58.0514	0.9800	0.268	2.7858	37.2486
ROP-HCl F_6	3.359	0.9538	-0.6330	71.3330	0.058	0.9924	0.1005	64.7314	0.9923	1.5760	51.4525	0.9900	0.338	3.4596	34.4997

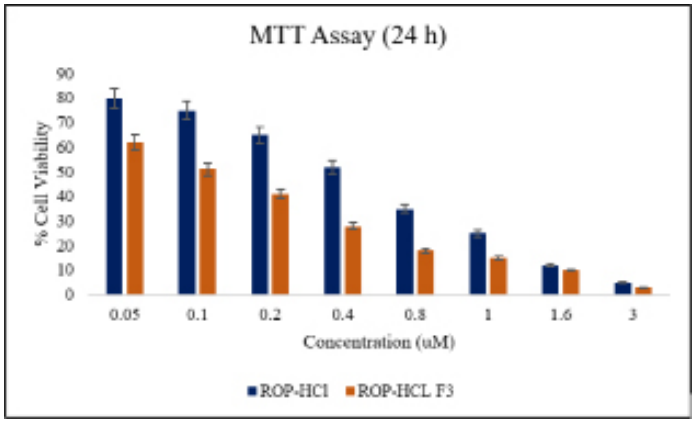

Figure 9: *In vitro* cell viability of (a) ROP -HCl, (b) ROP- HCl Niosomal formulation.

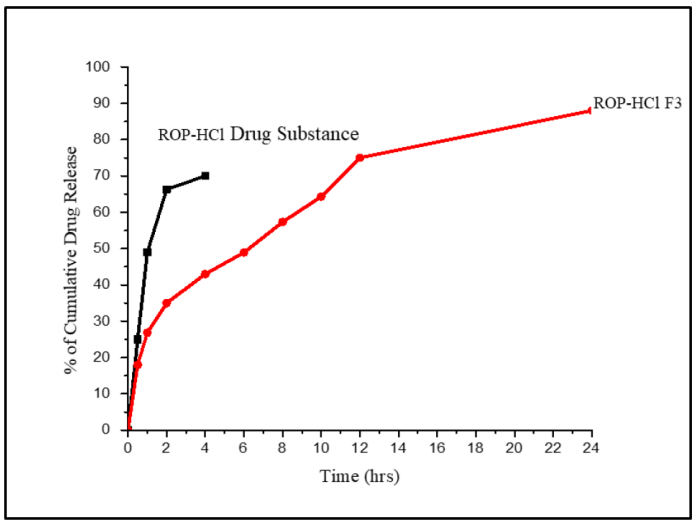

Figure 10: *Ex vivo* permeation studies of optimized Niosomal formulation of ROP-HCl and ROP-HCl Drug substance.

Table 6: *Ex vivo* drug release kinetics of the formulations.

Formulation	Zero order		First order		Higuchi	Korsmeyer-Peppas	
	K_0	R	K_1	r	R	r	n
ROPHCl Solution	15.365	0.827	0.130	0.908	0.960	0.950	0.348
ROP-HCL F_3	3.202	0.889	0.044	0.984	0.992	0.989	0.387

of the formulation to the mucosal surface. The results of the *ex vivo* drug release kinetics are shown in Table 6 and depicted in Figure 10. The ROP-HCL F_3 formulation showed sustained *ex vivo* transmucosal transport which could be due to the drug embedded within the lipid, causing stronger adhesion of the formulation to the mucosal surface. The *ex vivo* cumulative percent drug release studies revealed, that, the percent drug release values of the test formulations were decreased when compared with *in vitro* drug release values of the corresponding formulations. The e*x vivo* drug release kinetics study revealed, that the tested products followed

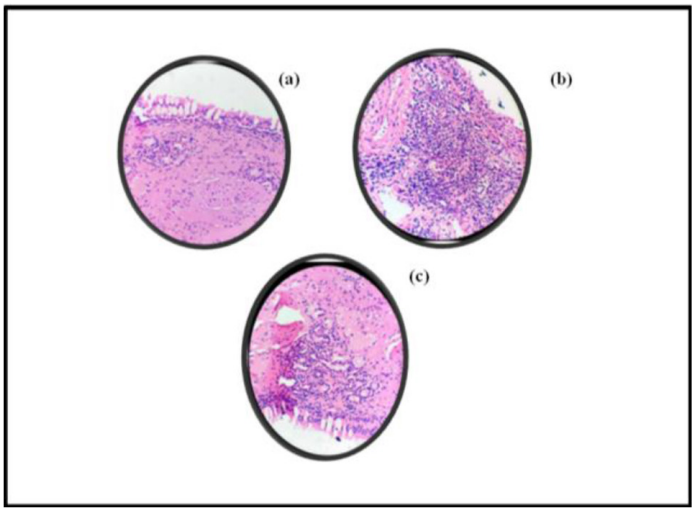

Figure 11: Histopathological images of the sheep nasal mucosal skin treated with A) SNF pH 6.8 B) 10 % neutral carbonate buffered formalin solution C) ROP-HCl F3.

Higuchi and korsmeyer peppas (comparable values) kinetics in their drug release. The drug release mechanism was observed to be Fickian mechanism. There was about 1.3 times enhancement in permeability of ROP-HCl-F3 Niosomes as compared to plain drug solution.

Toxicity Study: Histopathological Examination

Histopathological studies were performed on sheep nasal mucosa, and the results showed that the prepared niosomal formulation had no irritation or toxic effect on the nasal mucosal tissue. These findings imply that the prepared formulations can be used safely without causing irritation or damage to the nasal mucosa. Figure 11 depicts histopathological examination of nasal mucosa.

DISCUSSION

The focus of the study revolves around developing a therapy for Parkinson's disease that is effective and avoids the drawbacks of conventional routes and medicines. Therefore, Ropinirole Hydrochloride a widely used drug for neurodegenerative disorder such as PDs was used as a model drug and incorporated into niosomes. The niosomes were synthesized by Ethanol injection method; employing various ratios of ingredients in varied proportions. Statistically developed and optimized formulations developed ROP-HCl-F1, ROP-HCl-F2, ROP-HCl-F3, ROP-HCl-F4, ROP-HCl-F5 and ROP-HCl-F6 were characterized by various techniques. Egg sphingomyelin (DPSM) has a Sphingomyelin is an essential component of both the phospholipid monolayer of lesional lipoproteins and of membranes of lesional cells. Cholesterol was included in the formulation to improve fluidity and stability of the vesicles. The prepared ropinirole hydrochloride (ROP-HCl) loaded niosomes were evaluated for percent drug content, % entrapment efficiency, particle size, zeta potential, *in vitro* drug release, *in vitro* cell viability, *ex vivo and*

stability studies. And the drug and excipients interaction studied using FTIR, XRD, and DSC.

Incorporation of lipids, charged positively or negatively, such as, stearylamine or phosphoglycerol (DPSM), phosphatidyl serine, or diacetylphosphate modified the net surface charge of niosomes. The presence of positive or negative charge leads to larger overall volume for aqueous entrapment and reduces probability of aggregation after preparation of Niosomes. Another additive used in the preparation of niosomes is cholesterol. It is added to reduce leakage, to impart membrane fluidity and to stabilize the niosomes. Stability here refers to retention of entrapped drug within the niosomal vesicles. In the present work, DPSM was selected as lipid, span 80 was selected as a surfactant and cholesterol was used as a stabilizing agent. Simulated nasal fluid (SNF) of pH 6.8 was used as the dispersion medium.

The mean vesicle size of the particles was small. This indicates that the surface area of the particles is high and so the ROP-HCl was easily encapsulated into the aqueous core of the niosomal vesicles. As a general observation medium concentration of span 80 and cholesterol showed lowest vesicle size distributions, whereas, when the concentration of span 80 was increased, it led to an increase in the vesicle size for the niosomes. It was also observed that the presence of negatively charged DPSM also influenced the vesicle size. The results were suggesting that the vesicle size and their polydispersibility index were strongly affected by the selected variables span 80 and cholesterol. The mean vesicle size of the niosomes was found to be in the range of 82.6±2.1 nm to 381.1±3.2 nm and the PDI was in the range of 0.102±0.6 to 0.3450.4 respectively. These results indicate that the Span 80 and cholesterol have a significant effect on the niosomal vesicle size.

The observed responses of vesicle size, PDI and zeta potential indicated that the niosomal dispersion approached a mono disperse stable system. It may be concluded that it could deliver the drug effectively, owing to the large surface area of its nano-sized vesicles.

The vesicle size and %EE are the basic parameters of vesicular systems based on which the formulations were optimized. An inverse relation between the % EE and the vesicle size of ROP-HCl was observed. As the cholesterol concentration increased, there was a significant decrease in %EE and increase in vesicle size. This result may be due to high concentrations of cholesterol, leading to rigidity in the vesicles, which in turn decreases the % EE and increases the vesicle size. Basically, ROP-HCl is, freely soluble in water. It was observed that higher level of cholesterol restricts the penetration of the aqueous medium into the core of the noisome. Due to this reason, entrapment was decreased. Cholesterol beyond certain level (55 mg), starts disrupting the regular bi-layered structure leading to the escape of the drug and results in minimum drug entrapment. The optimum levels of cholesterol and span 80 produced higher percentage entrapment by showed lower vesicle size. The low size may be helpful in providing a large surface area to encapsulate ROP-HCl in the niosomal vesicular aqueous core. The use of negatively charged lipid leads to improved entrapment efficiency by increasing entrapped volume and hinders the drug leakage through the stabilization of bilayers. Statistically optimized Formulation ROP-HCL F_3 proved to be the best among all the prepared formulations.

The vesicular drug release mechanism is a complex process; it depends on many factors like nature of drug, surfactant composition, drug-lipid matrix interactions, lamellarity, dispersion medium and the method of preparation ROP-HCl F_3. *In vitro* drug release studies were conducted in order to determine the effect of surfactant: cholesterol ratio on drug release. The stability and drug release properties of formulations of ROP-HCl loaded niosomes were determined by their surfactant: cholesterol ratio. The release pattern from niosomes extended over 24 hr, depending upon the proportion of surfactant and cholesterol. Formulations having higher concentration of cholesterol gave decreased rate of drug release. This result might be due to the disorder of the niosomal vesicular bilayers. Percent drug release showed dependence on the levels of surfactant and levels of cholesterol. Cholesterol makes the lipid bilayers more rigid and retards the release of the drug. When the combined effect of surfactant and cholesterol was studied, it was observed that, at the medium level of these components, the percent drug release was the maximum. It indicated that the lipid composition in niosomes determines its membrane fluidity, which in turn influences the rate of drug release.

From both the formulations the drug release studies reveal that typical biphasic release pattern was observed with niosomal formulations, with an initial rapid burst release (for 2 hr) followed by a sustained release for a period of 24 hr.

It was observed from the results, that the drug release kinetics The best fitted model was found to be Korsmeyer Peppas model. The conclusion was based on the parameters of highest R^2 value, Highest MSC (Model selection criterion) and lowest AIC value (Akaike coefficient). N value was 0.338.

The results of the *ex vivo* drug release kinetics are shown in Table 6 and depicted in Figure 10. It was observed that all the test formulations followed Korsmeyer peppas model and the drug release followed Fickian diffusion mechanism.

The significantly 3.2 times lower IC_{50} for developed optimized ROP-HCl formulation over ROP-HCl alone is indicative of its effectiveness. The enhancement of permeability 1.3 times obtained is encouraging over ROP-HCl alone. The safety is supported by the images of histopathological studies.

CONCLUSION

When compared to ROP-HCl nasal solution, ROP-HCl niosomal formulation demonstrated higher permeability; this could be attributed to the presence of DPSM in the niosomal formulation. By interacting with the lipophilic portion of the nasal membrane, the DPSM has the ability to increase the fluidity of the nasal mucosal membrane. When compared to the free drug in solution form, the niosomal formulation achieved better absorption following intranasal administration. It is possible to conclude that the formulated ROP-HCl niosomes, when administered nasally. By avoiding the BBB, these niosomes can improve drug bioavailability and reach the brain more effectively. The current studies show that ROP-HCl niosomes were successfully designed and optimised using the ethanol injection method, and that they were thoroughly biopharmaceutically evaluated. The optimised formulation's intranasal administration is likely to show improved bioavailability when compared to the orally administered drug solution as well as the nasally administered drug solution. As a result, it is possible to conclude that the developed formulation has a higher potential for reaching the brain. This treatment with nasally administered ROP-HCl niosomes may be considered to be cost effective, and acceptable to patients because of its convenience along with its primary advantage of the potential for enhanced bioavailability and enhanced access to the brain.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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