Available online on 15.07.2018 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Research Article

FORMULATION AND EVALUATION OF TOPICAL FORMULATION FOR CUTANEOUS TUBERCULOSIS

Rao Monica*, Kadam Manali, Rao Shivani

Department of Pharmaceutics, AISSMS College of Pharmacy, near RTO, Kennedy Road, Pune -411001, India

ABSTRACT

Cutaneous Tuberculosis is also known as dermal tuberculosis or tuberculosis cutis (extrapulmonary tuberculosis) which can occur in any age group and patients who may or may not be suffering from pulmonary tuberculosis. The current treatment given for the disease is oral therapy of anti-tubercular drugs which has many side effects such as hepatotoxicity, headache, anxiety, euphoria, insomnia, eosinophilia, hepatitis. Hence to avoid these side effects and to increase efficiency of current therapy a topical proniosomal gel of isoniazid was formulated. Coacervation phase separation method was used and proniosomal gel was formulated by using Span 20, soya lecithin, and cholesterol. Optimum concentration of 3 factors Span 20, soya lecithin, and cholesterol were determined using Box Behnken design with at 2 levels and vesicle size and entrapment efficiency as responses. The optimized proniosomal gel was characterized by vesicle size, entrapment efficiency, transmission electron microscopy (TEM), in vitro drug release, skin retention studies, skin irritation studies and stability studies. The optimised batch showed vesicle size of 2.27±1.82 µ, and entrapment efficiency of 98.15±0.25 %. The optimised formulation was stable under refrigeration condition (5°C) in amber coloured bottle, was non-irritating and showed 98.10±1.28 % release and 85±1.53 % permeation after 6 h and 436±12 µg drug was retained in the skin after 3 hrs.

Keywords: Cutaneous tuberculosis, isoniazid, proniosomal gel, stability

Article Info: Received 23 May, 2018; Review Completed 10 July 2018; Accepted 12 July 2018; Available online 15 July 2018

Cite this article as:

Rao M, Kadam M, Rao S, Formulation and evaluation of topical formulation for cutaneous tuberculosis, Journal of Drug Delivery and Therapeutics. 2018; 8(4):102-116 **DOI:** http://dx.doi.org/10.22270/jddt.v8i4.1723

***Address for Correspondence:**

Dr. Monica RP Rao, AISSMS College of Pharmacy, Kennedy road, Near R.T.O, Pune-411001, M.H. (India).

INTRODUCTION

Mycobacterium tuberculosis is a worldwide problematic, communicable pathogen causing both pulmonary as well as skin tuberculosis. Cutaneous tuberculosis (CTB) also known as dermal tuberculosis or tuberculosis cutis refers to pathological lesions of the skin caused by any one of the following: *Mycobacterium tuberculosis*, *Mycobacterium bovis* or the BCG vaccine[1]. Most often CTB is an airborne transmissible disease with skin manifestations presenting as a result of haematogenous spread or direct extension from latent or active foci of infection. However, primary inoculation may occur as a direct introduction of the mycobacterium into the skin or mucosa of a susceptible individual by trauma or injury. Drug addicts and patients suffering from AIDS, diabetes, malignancies and end stage renal disorders are at high risk of acquiring this disease [2]. CTB is frequently misleading as it mimics a wide differential diagnosis and also evades microbiological confirmation despite recent advances in sophisticated techniques. Although rare (0.7% worldwide) it is important for clinicians to recognize the many clinical variants of CTB to prevent missed or delayed diagnoses.

CTB is treated with a combination of rifampicin, ethambutol, pyrazinamide, isoniazid and streptomycin that is tailored to individual needs [3]. Currently the treatment given for the CTB is the same as that for pulmonary TB and consists of long term two phase multidrug therapy i.e. isoniazid, rifampicin, ethambutol and pyrazinamide for 2 months in initial phase and then isoniazid and rifampicin for the next 4 months as second phase of treatment [2, 4]. The antitubercular drugs (isoniazid, rifampicin, ethambutol and pyrazinamide)

are administered until complete clearance of the condition is seen. The drawback of oral antitubercular therapy includes adverse effects in the patient such as insomnia, eosinophilia, hepatitis, hepatotoxicity, peripheral neuropathy, psychosis, seizures, toxic encephalopathy or coma. Hence a topical preparation is required to overcome the drawbacks of current therapy and provide an efficacious treatment to patient [5] No current topical treatment for CTB has been found in the literature. Although topical therapy would not be a substitute for conventional treatments, it may be used concurrently and possibly aid in improving treatment outcomes [3].

Isoniazid (INH) is a first line antitubercular drug for the treatment of pulmonary as well as cutaneous tuberculosis. It is white crystalline powder, odourless and slowly affected by exposure to air and light. INH gets converted to isonicotinic acid due to radical-mediated chain reaction (Bhutani et al).It comes under borderline of BCS Class I and Class III drugs with good aqueous solubility of (140000 mg/L at 25°C), Log P -0.71(hansch, ceta1995) and pKa of 1.82 at 20°C(Perrin, DD 1965). It is a bactericidal drug and acts by inhibiting biosynthesis of mycolic acid which is major component of bacterial cell wall [6]. The drug faces various stability problems like hydrolysis, oxidation and photolysis, forming isonicotinic acid and isonicotinamide. INH is stable to dry heat at 50-60°C whereas degradation is increased in presence of metal ions like Cu, Mg etc.[7].

Proniosomal gel (PNG) preparations are semisolid liquid crystal products of non-ionic surfactants prepared by dissolving the surfactant in a minimum amount of organic solvent and aqueous phase [9]. PNG offers a great potential to reduce the side effects of drugs and increase therapeutic effectiveness. These can entrap both hydrophilic and hydrophobic drugs[10]. Liposomes are formulated using lipids which may cause vesicle instability. Niosomes are more prone to oxidation and hydrolysis as it consists of greater proportion of aqueous solvent as compared to proniosomal gel. PNG was better candidate for formulating INH topical preparation as it consists of non-ionic surfactant which stabilises vesicles and contains very less amount of aqueous solvent. Hence PNG was selected as it is reported to prevent hydrolysis and oxidation of encapsulated drug[11]. PNG show controlled and sustained release of drugs due to depot formation. These formulations are biodegradable, biocompatible and non-immunogenic to the body[12].

The aim of the present study was to develop a topical formulation which would be effective against CTB as well as overcome the drawbacks of current oral therapy. The PNG formulationwas prepared by coacervation phase separation method reported by Perret et al with some modifications. Theformulation was optimised by Box Behnken design with 3 factors at 2 levels using Design Expert software version 11. The formulations were further evaluated for its vesicle size, entrapment efficiency (EE), viscosity, spreadability, skin retention, skin permeation, skin irritation and stability.

MATERIALS AND METHODS

Materials:

INH was gifted by Amsal Pharmaceuticals Pvt. Ltd, Mumbai, India. Soya lecithin (SL), cholesterol, Span 20 was purchased from S.D. Fine Pvt. Ltd Mumbai. Methanol (AR grade) was purchased from Loba chemicals, Mumbai. All other chemicals and reagents used were of analytical grade.

Methods:

Characterisation of Drug:

The thermal behaviour of drug was checked by differential scanning calorimeter (Make: Mettler Toledo DSC, Model: 823e).Characterisation of drug was also done by fourier transfer infrared spectroscopy (FT-IR) (Make: Brukers alpha, Software: Opus) of INH was performed.

High Performance Liquid Chromatography (HPLC) analytical method:

Weighed quantity of drug was dissolved in Distilled Water (DW) (HPLC grade) to obtain 1000µg/mL solution. From this stock solution 5, 10,15,20,25 µg/mL solutions were prepared and analysed by RP HPLC (Make: Agilent, Model: 1120 Compact LC) equipped with EZ- Chrome Elite software with UV detector set at 263nm. The areas of the dilutions were observed and plotted against concentration (Fig 2). The mobile phase used was according to IP [13] with some modifications. The mobile phase was prepared by dissolving 1.4 g disodium hydrogen phosphate and 1 mL of trimethylamine in 1000 mL with DW (HPLC grade). The pH was adjusted 6.0 with orthophosphoric acid and mixed with acetonitrile in the ratio of 90:10. The sample (20µl) was injected in the loop injector with flow rate of 1 mL/min and the column used was C_{18} (Make: Agilent TC C18 (250 mm x 4.6 mm i.d., 5 µm particle size). The method was validated for linearity (5-25µg/mL), precision (interday and intraday), accuracy, limit of detection (LOD), limit of quantification (LOQ).

Drug Excipient compatibility studies:

The drug and potential excipients were kept in 1:1 ratio at 5°C in refrigerator (Make: Bluestar) in amber coloured vials to check any reaction which may take place between drug and excipients. The samples were analysed by FT-IR after 1 month along with standard drug and excipients as the reference.

Preliminary Stability Testing:

Preliminary stability studies were conducted in two stages according to ICH[14] guidelines as follows:

Solution state stability:

INH (10mg) was dissolved in 10 mL of DW and kept at accelerated conditions (40°C/75 % RH), room temperature (RT) (30°C/65 % RH), refrigeration conditions (5°C) in stability chamber (Make: Thermolab, Model: TH 200S) in amber coloured vials for 1 month. The solutions were analysed for drug content by HPLC (Table 1)

Table 1: Layout of excipients for selection of surfactant

Batch	Span 20 (mg)	Span 60	Span 80	SL	Cholesterol (mg)	Ethanol	0.1% glycerol solution
B_1	450	-	-	50	50	0.25	0.8
B_2	-	450	-	50	50	0.25	0.8
B_3	-	-	450	50	50	0.25	0.8

Solid state stability:

Solid INH was kept at RT and refrigeration conditions in amber coloured vial for 1 month. The samples were subjected to FT-IR studies and drug content.

Stability in cream formulation:

The INH o/w cream was formulated with excipients such as white petrolatum (5g), cetostearyl alcohol (5g), propylene glycol (2.4g), tween 80 (0.2g) and DW (17.4mL) [15]. The cream was prepared by mixing oil phase (white petrolatum, cetosteryl alcohol, and propylene glycol) and aqueous phase (water, tween 80, drug) when both the solutions acquired same temperature (70°C) while continuous stirring at mechanical stirrer for 10 min at 800 rpm to obtain white homogenised cream. The cream was analysed to check if the drug remains stable in the formulation as no reported data was obtained regarding topical formulation of INH. The cream was stored at accelerated conditions, RT in stability chamber and refrigeration for 1 month in amber coloured wide mouth bottle. The formulation was evaluated for drug content at an interval of 7 days by HPLC.

Stability in INH plain gel:

The INH plain gel was formulated with excipients such as Carbopol 934 (0.1 g), glycerine (0.5 g), propylene glycol (1 g) and DW (3.348 mL) [16]. The gel was prepared by adding a hot mixture (60-70°C) of propylene glycol, glycerine and drug into a beaker containing carbopol 934 and warm water. The gel was analysed to check if the drug remains stable in the formulation. Based on the results obtained from the drug content of the cream formulation, the gel was stored at RT in stability chamber and refrigerationfor 1 month in amber coloured bottle. The formulation was evaluated for drug content at an interval of 7 days by HPLC.

Stability in Proniosomes:

The INH proniosomes were prepared with cholesterol, SL and different surfactants like Span 20, Span 60 and Span 80 singly. Required quantities of surfactant, cholesterol and SL were mixed with ethanol in a beaker. Beaker was covered to prevent loss of solvent and the mixture was heated in a water bath (Make: Metalab) at 30-40°C for 10 min until surfactant dissolved completely. To this, 0.1% aqueous glycerol solution was added and heated again at 30-40°C for 10 min[17]. Then mixture was allowed to cool to RT. The proniosomes were checked for drugs stability in the formulation. The proniosomes were stored at RT in stability chamber and refrigeration for 1 month in amber coloured wide mouth bottle. The formulation was evaluated for drug content at an interval of 7 days by HPLC.

Preliminary studies:

Selection of surfactant:

Blank proniosomes were prepared with similar procedure as mentioned above without adding drug (Table 1).The proniosomes were analysed for vesicle size and polydispersity index (PDI) by Zeta Sizer (Make: Malvern, Model: nano Zs-90) by diluting 0.1g of blank proniosomes with glycerine. The surfactant producing least vesicle size and PDI was selected for further studies.

Experimental Design:

Based on results of preliminary studies, the Box-Behnken experimental design was selected to investigate the effect of 3 independent variables at 2 levels on responses. These variables included concentration of Span 20 (A), cholesterol (B) and SL (C) with vesicle size (Y1) and EE (Y2) as responses. The 2 levels (Table 2) of Span 20 (A) in the formulation were varied from 0.9 and 3.6, while levels of cholesterol (B) varied were 0.1 and 0.4 g and levels of SL (C) varied were 0.1 and 0.4 g. Based on 2 levels and one centre point given by the software, fifteen trial batches (R1-R15) were generated by Design Expert software (Design Expert 10, Stat-Ease, Minneapolis, MN) (Table 3). Optimized batch was selected from solutions given by software on the basis of constraints provided to software and 3D response curves for both the responses. The responses of all the 15 runs were fitted in the quadratic polynomial model. The appropriate fitting model for each response was selected based on the difference of numerous statistical parameters such as R^2, sequential model sum of squares and partial sum of square given by the analysis of variance (ANOVA). The optimised batch of proniosomes so obtained was added into the Carbopol 934 gel (5%) in the ratio 3:0.5 to obtain optimised 1% INH PNG.

Table 2: Box-Behnken design layout for optimization of proniosomes.

Factors	**Levels**			**Responses**
	-1	0	+1	
Factor A: Span 20 (g)	0.9	2.25	3.6	% Entrapment efficiency and vesicle size
Factor B: Cholesterol (g)	0.1	0.25	0.4	
Factor C: SL (g)	0.1	0.25	0.4	

Evaluation of optimisation batches of proniosomes:

Vesicle Size:

The vesicle size of the proniosomal gel prepared was measured by Zeta Sizer. Proniosomes (0.1 g) were taken and diluted with glycerine and analysed for vesicle size [19, 20].

Entrapment efficiency (EE):

In a glass tube weighed (Make: Shimadzu, Model: AW 220) quantity of proniosomes (0.1g) were taken and made up the volume up to 10 mL with phosphate buffer pH 5.5 [23]. The solution was centrifuged at 6,000 rpm at 30°C for 90 min in an ultra-centrifuge (Make: Remi, Model: RM-12C BL). The supernatant was assayed by validated HPLC method [21, 22]. The percentage of drug entrapped (EE (%)) was calculated by the following Eq. 1:

$$Entrapment\ efficiency = \frac{Total\ amout\ of\ drug - unentrapped\ drug}{Total\ amount\ of\ drug} * 100$$

Evaluation of Proniosomes:

The factorial batches were analysed for vesicle size and EE and the optimised batch of proniosomes was analysed for optical microscopy at 40X, zeta potential (ZP) [18] and optimised PNG was evaluated for pH, spreadability, viscosity, *in vitro* release, *ex vivo* permeation, skin irritation, *in vitro* retention, and stability studies.

Evaluation of optimisedproniosomal gel

Determination of pH:

The pH of the optimised PNG was determined by a digital pH meter (Make: Deluxe pH meter, Model: Deluxe 101). Optimised PNG (1 g) was dissolved in 20 mL of DW and the electrode was then dipped into gel formulation and constant reading was noted. The measurements of pH of formulation were replicated three times [24].

Determination of viscosity:

The viscosity determination of the formulation is important as it affects spreadability of the formulation and in turn its performance [25]. Brookfield digital viscometer (Make: Brookfield, Model: DV II + Pro viscometer) was used for the viscosity determination. The quantity of 100 g of optimised PNG was taken in a beaker, spindle S 07 was dipped into it and the viscosity in centipoises (cp) was measured at 20 rpm after 30 sec at ambient temperature and in triplicate. The % torque (τ) was in the range of 10-100 %.

Spreadabiliy:

The spreadability of PNG was determined by using Texture Analyser (Make: Brookfield, Model: CT 100). Optimised PNG (20 g) was taken in the cup of the texture analyser, previously aligned with the probe (TA3/100). The trigger load was 0.5 g and test speed was 1 mm/sec. The hardness value obtained was recorded [26].

Determination of drug content:

Optimised PNG (0.1 g) was accurately weighed and diluted to 10mL with DW and vortexed (Make: Remi Laboratories CM101) to produce stock solution. From this stock solution 1 mL of the solution was taken and was further diluted to 10 mL with mobile phase (same as given above for HPLC analytical method). The solution was filtered using Whatman filter paper (0.45 µm) and HPLC analysis was conducted[27].

***In vitro* release studies and *ex vivo* skin permeation studies:**

In vitro release studies of optimised PNG formulation was performed by Franz diffusion cell (Make: Orchid Scientifics Model: FDC03) using cellophane membrane previously soaked in phosphate buffer pH 5.5 [24] for 24 h. The cellophane membrane (Make: Pal life sciences ltd. pore size=0.45µm) was mounted between the donor and receptor compartment both containing phosphate buffer pH 5.5. Weighed quantity of optimised PNG (0.3 g containing 4.4 mg of drug) was applied on donor side of the cellophane membrane. The assembly was maintained at 37±1°C with constant stirring using magnetic beads. At sampling intervals of 30 min for 6 h, 1 mL sample was withdrawn and replaced by equal volumes of fresh medium to maintain sink condition. The samples were diluted to10 mL with phosphate buffer pH 5.5 and filtered using Whatman filter paper (0.45 µm) and analyzed by HPLC[28, 29].

Ex vivo skin permeation studies were performed using same procedure and analytical method as used for *in vitro*release studies. The cellophane membrane was replaced with subcutaneous skin of male albino Wistar rats with diffusion area of 2.26 cm^2.The skin was properly cleaned to remove the subcutaneous fat and other blood vessels.The cumulative amount of drug permeated across the rat skin was calculated and plotted against time and the flux was calculated as drug permeated per cm^2 per hour [30].

***In vivo* skin irritation studies:**

Protocol approved by institutional animal ethics committee with approval no: CPCSEA/IAEC/PT-01/01-2K18 at AISSMS College of pharmacy. The albino Wistar rats were housed in polypropylene cages, with free access to standard laboratory diet and water. Animals were acclimatized for at least 7 days before experimentation. The dorsal abdominal skin of rats was depilated (approx. 10 cm^2) 24 h before study. Optimised PNG was applied and site of application was covered with a non sensitizing microporous tapes. Formalin (0.8 % w/v) solution was applied as standard skin irritant. Blank PNG was applied as placebo. After washing the applied gel, the resulting reaction was evaluated by visual observation of the skin for any change such as erythema at 24, 48 and 72 h after the application of the formulations. The mean erythemal scores were recorded (ranging from 0-4), depending on the degree of erythema, as follows: no erythema=0; slight erythema (barely perceptible-light pink) =1; moderate erythema (dark pink) =2; moderate to severe erythema (light red) =3; and severe erythema (extreme redness) =4 grade[31].

Skin retention studies:

Protocol approved by institutional animal ethics committee with approval no: CPCSEA/IAEC/PT-0+1/01-2K18 AISSMS College of Pharmacy. The optimised PNG was applied to the depilated skin of the male albino Wistar rats, and after a predetermined time interval (3 h), the animals were euthanized and the skin was surgically removed for analysis of drug retained in the skin. For drug analysis, the skin was cut into small pieces and then vortexed in methanol for drug extraction. The skin tissue was also homogenized by using a homogenizer (Make: Biolab instruments Model: BL244). Drug quantification was performed by using validated HPLC method [32].

Stability studies:

The optimised PNG formulation was analysed for stability studies according to ICH guidelines (Q1A(R2)) [14]. The optimised PNG was stored at RT as well as at refrigeration conditions. The % assay was carried out in order to determine the degradation of drug and in turn shelf life. The assay procedure as well as method of analysis was same as that for the drug content.

RESULT AND DISCUSSION

Characterisation of drug:

INH was characterised for identity and purity by determination of thermal behaviour of drug by DSC and FTIR spectra. The characteristic IR peaks observed for INH included NH stretch at 3346.61 cm^{-1},C-H aromatic ring stretch at 3027.38 cm^{-1}, C-H stretch at 2857.64 cm^{-1},C=O stretch at1724.42 cm^{-1}, C=C stretch at 1573 cm^{-1} andC-N stretch at1208.44 cm^{-1}(Fig 1A).The melting endotherm of INH was observed at 171.94°C (Fig 1B) which conformed to the reported value (171-173ºC) thus, confirming its purity and identity.

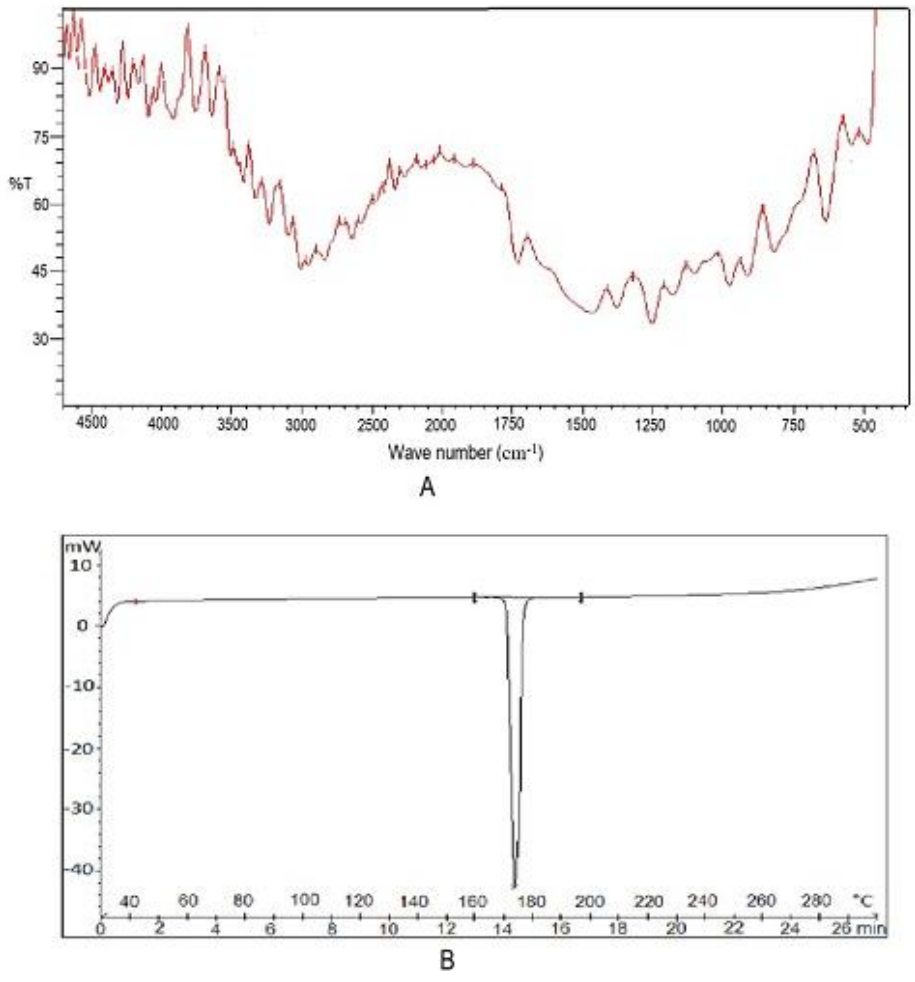

Figure 1: A: FT-IR spectra of INH, B: DSC thermogram of INH

High Performance Liquid Chromatography (HPLC) Analytical method:

The calibration curve showed good linearity in the Beer's law limit of 5-25 µg/mL in mobile phase with correlation coefficient (r^2) of 0.9946 (Fig 2). The method was validated by different parameters which include precision (intra-day and inter-day precision), accuracy, limit of detection (LOD) and limit of quantification (LOQ). The percent relative standard deviation (% RSD) values of interday and intraday precision were found to be 1.18 and 1.39 for 5 µg/mL. The mean recovery (n= 3) was found to be 98.70% (% RSD =0.60) showing the accuracy of the method. Limit of detection (LOD) and limit of quantification **(**LOQ) was found to be 0.47 µg/mL and 1.42 µg/mL respectively.

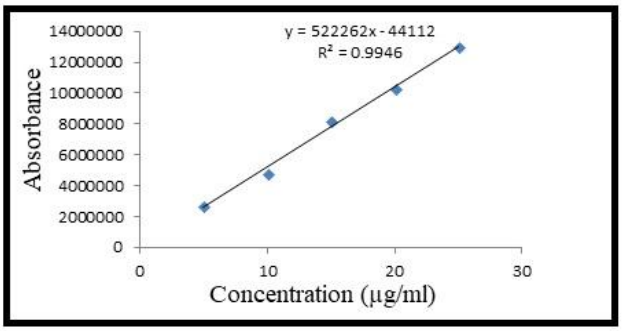

Figure 2: Calibration curve of INH by HPLC (*n=3, mean ± SD)

Drug excipient compatibility studies:

This study was carried out to check the interaction of drug and excipients. The FT-IR spectral analysis of INH showed the principal peaks of NH stretchat wave number of 3346.61 cm^{-1},C-H aromatic ring stretch at 3027.38 cm^{-1}, C-H stretch at 2857.64 cm^{-1},C=O stretch at 1724.42 cm^{-1}, C=C stretch at 1573 cm^{-1} and C-N stretch at 1208.44 (Fig 3) confirming the purity of drug. The FT-IR spectra of binary mixture and physical mixture of drug with cholesterol, SL, Span 20 and Carbopol 934 showed no significant changes which indicated that there was no interaction between INH and other excipients.

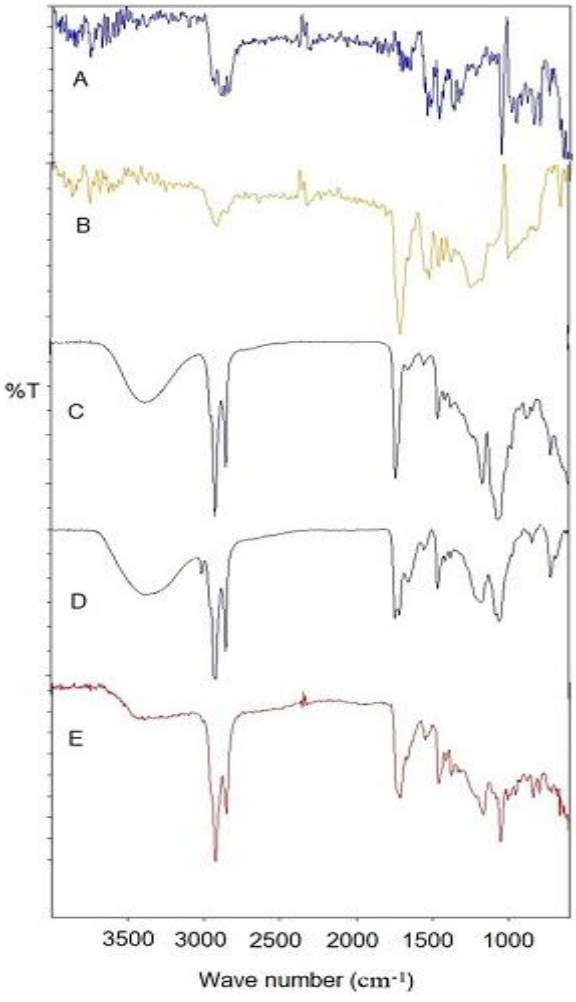

Figure 3: Compatibility studies of binary mixtures of drug and excipients (1:1 ratio) with A: cholesterol, B: Carbopol 934, C: Span 20, D: SL and E: physical mixture.

Preliminary Stability studies:

Solution state stability studies:

Solution state stability studies of 1 % aqueous solution of INH revealed significant degradation with time. The results indicated that the degradation was strongly temperature-dependent with significant decrease in drug content (Table 2). INH was found to have maximum stability at refrigeration. Kakemiand co-workers had studied the degradation of INH in aqueous solution under aerobic conditions. Alkaline hydrolysis under aerobic conditions yields a mixture of isonicotinic acid, isonicotinamide and 1, 2 di isonicotinoyl hydrazine plus small amounts of unidentified products. Under anaerobic conditions isonicotinic acid and 1, 2 di isonicotinoyl hydrazine were the principal products. Hald found that INH underwent slow oxidation in aqueous solution. Pawelczyk and co-workers found that as long as conditions were kept anaerobic the decomposition of INH in the pH range 3-7 followed first order kinetics. They reported that 1% solution of the drug was 37 times more stable at pH 6 than at pH 3 [33].Razak et al [34] reported that INH in solution form degrades 2 folds at 40°C than that of the RT due to high humidity and temperature conditions leading to hydrolysis and oxidation. They also mentioned about degradation of INH solution due to light at both conditions, exposure to UV light as well as at room light. They discussed that INH absorbed photons at certain wavelengths and there is an increased in its energy which leads to INH decomposition. Also, it is reported that INH is stable at RT for more than 14 days in aqueous solution and more than 6 weeks when stored at about 4°C [35], thus justifying our findings.

Solid state stability studies:

The solid state stability of INH was investigated at RT, accelerated conditions and refrigeration for 1 month in stability chamber and evaluated for its drug content at weekly intervals and FT-IR spectra was recorded after 1 month. The FT-IR spectra (Fig 4) of the drug kept in both amber and transparent vials at RT conditions and accelerated conditions showed a prominent peak of NH stretch at 3458.48 cm^{-1}. Besides this the C=O peak was shifted from1663.66 cm^{-1} to 1659.80 cm^{-1}. Bhutani et al [7] confirmed that the degradation compound of INH due to photolysis showed peaks at 3432 cm^{-1} (NH); 1682 cm^{-1} and 1660 cm^{-1} (C=O). This indicated that the degradation in solid drug at accelerated conditions and RT condition in amber and transparent vials was due to photolysis. Stets et al [36] estimated that the INH degradation occurs with a relevant participation of O_2, with non-negligible contributions of both OH and H^+. They also concluded that INH could be effectively degraded by UV-A radiation in TiO_2-mediated photocatalysis and homogeneous photo-Fenton processes; both the processes allowed degradation around 80% of the initial pharmaceuticals content within 60 min. Bhutani et al [7] reported that degradation of INH followed SRN1 type radical mediated chain reaction involving three steps. First step involves transfer of an electron to INH in the presence of light, leading to the

formation of a radical anion. The radical ion is assumed to disintegrate through a unimolecular process into a 4-pyridoyl radical and hydrazine anion. In the second step, 4-pyridoyl radical would abstract a proton from the NH_2 group of INH to form an anion of isoniazid and hydrazine. The nucleophilic attack by anion of isoniazid on 4-pyridoyl radical. Third step leads to radical anion that finally transfers an electron to INH to produce the end product (Isonicotinic acid). In the process, radical anion is released in first step, which continues to propagate the chain reaction. The drug kept at refrigeration did not show any significant changes in FT-IR spectra as compared to drug, thus confirming that the solid drug was stable at refrigeration. The drug content of the solid INH at weekly intervals for 1 month (Table 3) showed significant degradation at accelerated condition and RT conditions indicating that the drug was unstable in these conditions. Whereas, the drug was stable at refrigeration as no significant change in the drug content was observed.

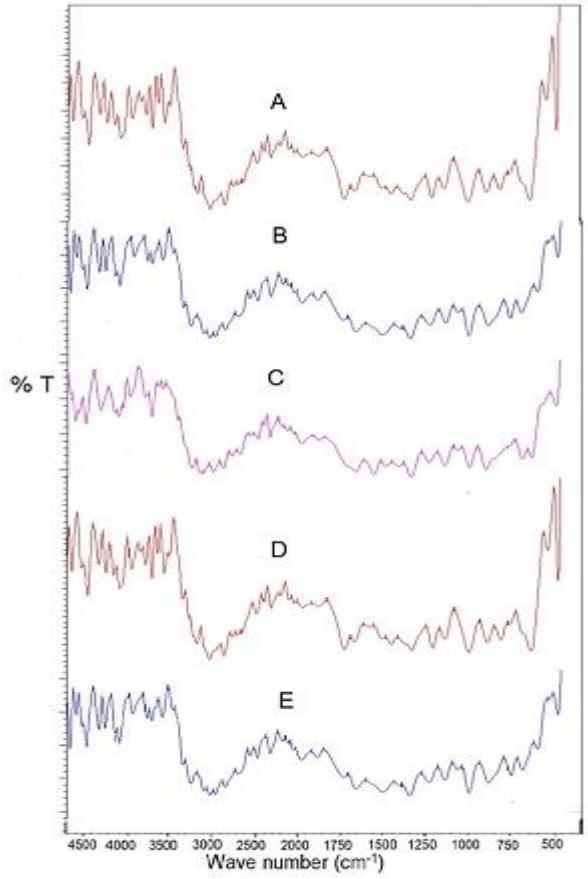

Figure 4: FT-IR spectra of drug A: at 0 days, B: in transparent vial at 30°C/75 %RH C: in amber coloured vial at 30°C/75, D: at 5°C amber coloured vial, E: at 40°C/75%RH in amber coloured vial.

Stability studies of INH cream:

The INH cream was prepared and kept at accelerated, RT and refrigeration conditions. The results for drug content of cream showed significant degradation at all the three storage conditions. Maximum degradation was observed at accelerated conditions followed by RT and refrigeration. This confirmed that INH degradation is highly temperature dependent in presence of water. Lee et al [37] stated that the INH intravenous solution was more stable at refrigeration condition than that of RT, supporting the findings. This suggests storage of INH topical preparation at lower temperatures (Table 4). There was significant degradation seen even at refrigeration, this indicated that INH require additional protection from hydrolysis and oxidation. No literature was found regarding semisolid formulation of isoniazid.

Table 3: Results of drug content of INH solution (*n = 3, mean ± SD)

Condition	Week	%Assay
40°C/75%RH. (Amber coloured bottle)	1	102%±1.93
	2	90.35%±0.27
	3	80.39%±1.84
	4	75%±2.37
30°C /65% RH (Amber coloured bottle)	1	103%±1.82
	2	101%±2.60
	3	96%±2.70
	4	85.59%±1.65
5°C (Amber coloured bottle)	1	102%±1.11
	2	96.02%±1.24
	3	98.92%±2.67
	4	97.54%±1.44
	5	94.23%±0.32
	6	90.40%±1.21

Table 4: Results of drug content of solid drug (*n = 3, mean ± SD)

Condition	Week	%Assay
40°C/75%RH	0	100%±0.49
	1	95.36%±1.45
	2	87.31%±1.40
	3	82.15%±128
	4	71.53%±0.47
30°C /65% RH (Amber coloured bottle)	0	100%±1.99
	1	98%±1.41
	2	95%±1.20
	3	89%±2.11
	4	83%±1.44
5°C (Amber coloured bottle)	1	102%±0.89
	2	100%±0.70
	3	101%±1.23
	4	99.58%±1.46
	5	98%±1.38
	6	99.37%±1.79

Stability studies of INH plain gel:

The INH plain gel was prepared and kept for stability studies for 1 month in amber coloured bottle. From the results obtained of stability of INH in solution state, solid state and in cream, it was confirmed that the drug was unstable at accelerated conditions. Hence the gel

formulation was stored at RT and refrigeration conditions. The results indicated that the drug in gel form degraded significantly at RT as well as refrigeration (Table 5). The extent of degradation was greater in gel than cream which was obviously due to higher water content in gels. These results suggested that carriers may aid in protection of INH from hydrolysis, oxidation and photodegradation.

Table 5: Results of drug content of INH cream (*n = 3, mean ± SD)

Condition	Week	% Assay
40°C/75%RH. (Amber coloured bottle)	0	98.25%±1.35
	1	84.55%±1.40
	2	66.62%±0.89
	3	54.62%±1.39
	4	49.46%±1.91
30°C /65% RH (Amber coloured bottle)	0	97.27%±1.61
	1	94%±1.49
	2	90.45%±1.38
	3	85.39%±2.10
	4	81.61%±1.44
5°C (Amber coloured bottle)	0	98.39%±1.50
	1	92.54%±2.18
	2	90.61%±1.47
	3	85.62%±1.18
	4	83.82%±1.33

Stability studies of INH proniosomal gel:

The results of stability studies of INH in cream and plain gel formulation indicated its high degree of instability. Hence further stability studies were conducted using proniosomes as carriers. The results for drug content of PNG at refrigeration conditions showed good stability as compared to RT. This indicated that INH was stable in PNG at refrigeration condition (Table 6). The greater stability of the drug in the PNG might be due to the protective barrier properties of proniosomes. Shukla and Tiwari [38] confirmed that PNG formulation prevents hydrolysis of encapsulated drugs. Mokhtar *et al*. [39] stated that liposomes and niosomes are dispersed aqueous systems and have a problem of degradation by hydrolysis or oxidation but there is no such condition with use of proniosomal gel. Hence PNG was selected as a carrier for topical INH preparation with storage condition at 5°C in amber coloured bottle. The reported literature also revealed that PNG is more stable at refrigeration conditions than at RT followed by accelerated conditions, thus supporting the findings [18].

Selection of surfactant:

The trial batches of proniosomes were prepared using different surfactants. The blank proniosomes prepared using Span 20, Span 60 and Span 80 with other excipients such as SL, cholesterol and ethanol were analysed for vesicle size as well as PDI. The proniosomes prepared using Span 20 showed least vesicle size as well as low PDI as compared to Span 60 and Span 80as PDI >0.3 indicate heterogenicity of vesicles [40]. The vesicle size is strongly influenced by the chain length of the surfactant with smaller chain length surfactants forming smaller vesicles. Span 20 (C12) [41] have shorter chain length as compared to Span 60 (C15) and Span 80 (C18). Hence Span 20 was selected among the other surfactants (Table 7).

Table 6: Results of drug content of plain gel (*n = 3, mean ± SD)

Condition	**Week**	**% Assay**
30°C /65 % RH (Amber coloured bottle)	0	97%±0.50
	1	91%±1.39
	2	85%±2.15
	3	71%±1.30
	4	65%±1.13
5°C (Amber coloured bottle)	0	103.23%±1.27
	1	98.33%±1.13
	2	86.47%±1.30
	3	82.99%±1.55
	4	74.11%±1.13

Table 7: Results of drug content of proniosomal gel (*n = 3, mean ± SD)

Condition	Week	% Assay
30°C /65 % RH (Amber coloured bottle)	0	101%±1.21
	1	98±1.34
	2	90.62±1.78
	3	86.14±2.19
	4	80.29±1.13
5°C (Amber coloured bottle)	0	101.74±1.28
	1	103.28±1.69
	2	101.19±1.27
	3	92.91±1.50
	4	95.57±1.67

Experimental design:

Effect of 3 independent variables at 2 different levels on various responses was evaluated using Box-Behnken design. These independent variables included Span 20, cholesterol and SL with vesicle size and percent EE as responses. Based on 2 levels and one centre point, 15 batches (F_1-F_{15}) generated by Design Expert Software (Design Expert 11, Stat-Ease, Minneapolis, MN) were prepared. The measured responses were used to construct 3D response surface plots to establish the relationship between variables and their interaction. Analysis of variance (ANOVA) was used to validate the design.

1. Vesicle size:

Vesicle size is an important evaluation parameter to prevent its aggregation and maintain stability of vesicles. Smaller the vesicle size greater is the stability of the vesicles and permeation through the skin barriers. The mean vesicle size (Y_1) of all factorial batches of proniosomes was found to be between 1-9µm.The significant ($p < 0.05$) quadratic effect of Span 20 was found to be negative (Eq.2) which indicated that the increase in concentration of Span 20 (A) decreased vesicle size. This could be explained by the easier formation of the vesicle and/or better accommodation of the surfactant in the vesicle structure with increase in surfactant concentration (Shatabdi et al., 2010) [42]. Also, pankaj et al [43] reported that increase in the concentration of surfactant increases the hydrophobicity and in turn decreases the surface energy of the vesicles thus decreasing the particle size. The significant ($p < 0.05$) quadratic effect of concentration of SL on vesicle size was found to be negative which might be due to increased hydrophobicity with increase in concentration of SL. Thus, decreases water intake in the vesicles which could lead to reduced vesicle size [44]. A non-significant ($p > 0.05$) interaction was observed between all the other variables. The above hypothesis could be proved with the response surface plot (Fig 5).

$$\mathbf{Y_1 = 9.05 - 0.1539A + 1.19B - 0.3869C - 0.6720AB + 0.5047AC + 1.28BC - 3.02A^2 - 1.06B^2 - 3.41C^2} \quad \text{(Eq.2)}$$

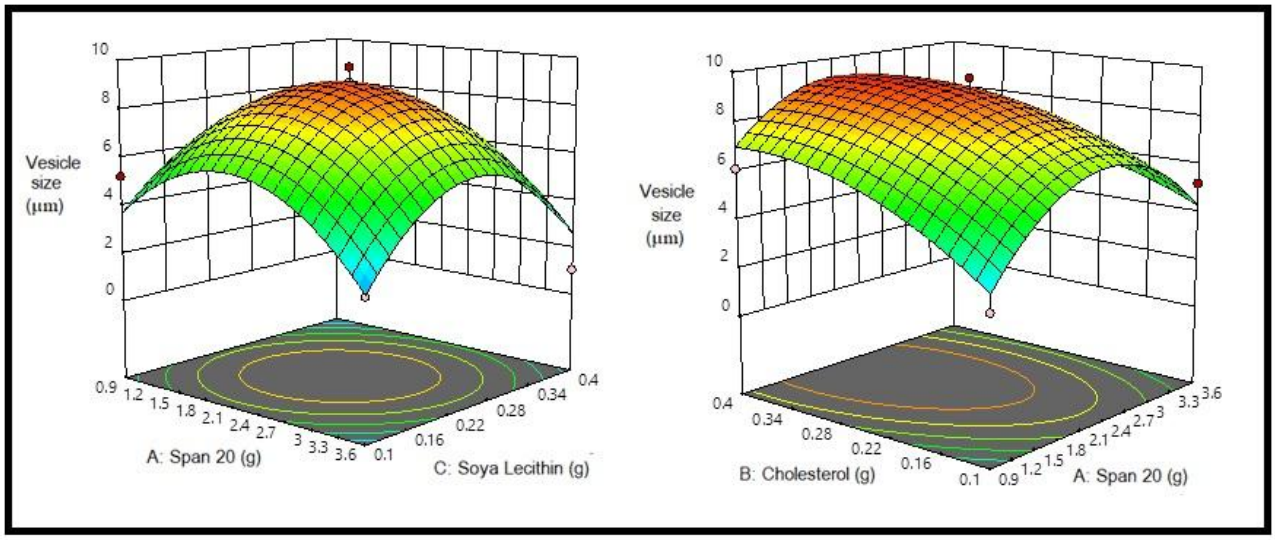

Figure 5: Response surface curve plot for vesicle size (Y_1)

2. Entrapment efficiency:

The EE of drug in any carrier system is an important parameter as it affects the amount of carrier required and also performance of drug delivery form. The EE (Y_2) of all factorial batches of proniosomes was found to be between 88-98 %. The polynomial equation (Eq.3) indicated that concentration of Span 20 (A) had positive effect on EE ($p<0.05$). Since the non-ionic surfactant is the principal component responsible for the bilayer structure formation, an increase in surfactant concentration resulted in increase in EE. Increased concentration of surfactant could be forming more number of niosomes leading to higher entrapment [31]. Besides this, it may also be proposed that the volume of the hydrophobic bilayer of the vesicles increases due to higher number of surfactant molecules getting interspersed in these bilayers leading to greater internal volume available for drug entrapment [45]. In case of SL(C) increase in its concentration was found to increase entrapment up to a certain concentration followed by decrease in EE with further increase in concentration.This may be attributed to increased rigidity and hydrophobicity of the vesicles which restricts the entrapment of a hydrophilic drug like INH. Also, low solubility of the drug in SL could be the reason for decreased EE at higher concentrations of SL [40]. All the other factors were found to have insignificant effect on EE ($p > 0.05$). The above hypothesis could be proved with the response surface graphs (Fig 6).

$$\mathbf{Y_2 = 98.06+2.97A-0.3788B-2.97C+0.4425AB-0.7100AC-1.29BC+2.33A^2+0.8087B^2-2.79C^2} \quad \text{(Eq.3)}$$

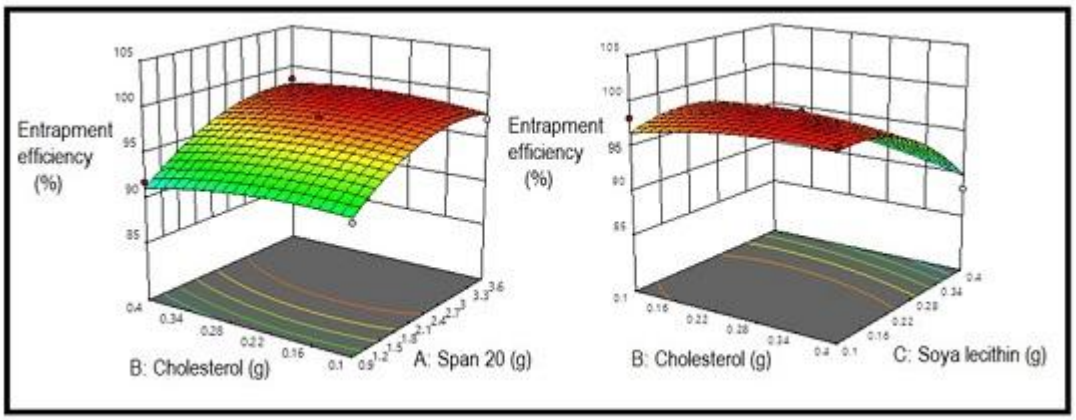

Figure 6: Response surface curve plot for Entrapment efficiency (Y_2)

Selection of optimised batch:

The selection of optimised batch for PNG was done by making various trials with the goal of least particle size (1 - 9 µm) and highest EE (88 - 98 %). The selection of batch was done by numeric optimisation with desirability function. The constraints for vesicle size (Y_1) was1-3µm and for EE (Y_2) it was 98 %. Different optimised batches (3) given by software were prepared and analysed and one batch was selected based on the desirability function. The optimised batch consisted of Span 20 (A=3.576 g), cholesterol (B=0.395 g), and SL (C=0.1 g) with vesicle size predicted to be 2.747µm and EE predicted to be 98.58 % with desirability 1. Vesicle size observed was 2.27 µm and EE of 98.15 % with standard error of 1.82 for vesicle size and 0.25 for EE.Thus, the batch giving minimum vesicle size and maximum EE was selected as optimised batch.

Evaluation of optimised proniosomes:

Besides the two responses (Y_1 and Y_2) the optimised proniosomes were also evaluated for its ZP. The ZP of the optimised batch was determined by Zeta sizer and was found to be -5.40mV. Normally the ZP in the range of -25 to +25 mV is considered to be good and the formulation to be stable [18]. The ZP outside the range is indicative of weak repulsive forces between the vesicles which causes aggregation and instability of the proniosomes.

The optimised proniosomes were also evaluated by optical microscopy. The proniosomes were mounted on glass slides and viewed under a microscope (Make: Olympus, Model: DSZ 77) with a magnification of 40X for morphological observation after suitable dilution [47]. The image (Fig 7) revealed that the proniosomes of INH formed from Span 20, cholesterol and SL was spherical in shape and no agglomeration or aggregation of the proniosomes was seen.

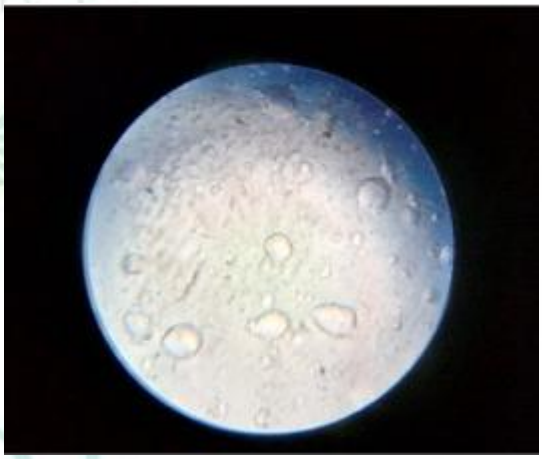

Figure 7: Microscopic image of proniosomal gel.

Evaluation of optimised proniosomal gel:

The optimised proniosomes when dispersed in carbopol 934 gel formed PNG. This optimised PNG was evaluated for various evaluation parameters such as *in vitro* release studies, *ex vivo* permeation studies, skin irritation studies and *in vivo* skin retention studies.

Table 8: Results of trial batches for selection of surfactant (*n = 3, mean ± SD)

Batch	Surfactant	Vesicle size (µm)	Polydispersibility index
B_1	Span 20	3.117±2.3	0.365±0.128
B_2	Span 60	4.309±3.8	1±0.01097
B_3	Span 80	8.080±2.4	1±0.153575

Table 9: Results of batches for optimization of formulation (*n = 3, mean ± SD)

Std	Factor 1	Factor 2	Factor 3	Response 1 Y_1	Response 2 Y_2
	A: Span 20	B: Cholesterol	C: Soya Lecithin	Vesicle Size	Entrapment Efficiency
	g	g	g	(µm)	%
F1	0.9	0.1	0.25	2.535±2.4	92.24±1.26
F2	3.6	0.1	0.25	5.191±3.5	97.18±1.37
F3	0.9	0.4	0.25	6.087±2.125	91.78±2.12
F4	3.6	0.4	0.25	6.055±3.348	98.49±2.13
F5	0.9	0.25	0.1	5.211±2.11	91.15±1.30
F6	3.6	0.25	0.1	2.274±2.218	98.64±1.17
F7	0.9	0.25	0.4	1.961±1.129	88.67±1.32
F8	3.6	0.25	0.4	1.043±2.129	93.32±1.4
F9	2.25	0.1	0.1	4.230±1.35	98.13±1.3
F10	2.25	0.4	0.1	3.23±2.495	98.77±1.9
F11	2.25	0.1	0.4	2.355±3.5	92.74±1.07
F12	2.25	0.4	0.4	7.491±2.8	88.22±1.59
F13	2.25	0.25	0.25	9.011±1.7	98.07±1.21
F14	2.25	0.25	0.25	8.47±2.1	97.99±1.72
F15	2.25	0.25	0.25	9.66±1.92	98.12±1.52

Table 10: Results of drug content of optimised proniosomal gel (*n = 3, mean ± SD)

Week	Refrigeration condition in amber coloured bottle	Room temperature condition in amber coloured bottle
	% Assay	%Assay
1	101.74±14	100.12
2	103.28±17	98.36
3	101.19±33	93.88
4	92.91±12	89.11
5	95.57±16	82.57
6	97.33±22	78.19
7	101.12±26	75.64
8	99.67±23	70.16
9	110.99±11	-
10	97.37±32	-
11	95.62±15	-
12	91.30±17	-

1. pH:

The pH is an important evaluation as the drug shows degradation in acidic and basic environment [7]. Also, acidic or basic pH may cause irritation on the skin. Hence the pH of the optimised PNG was determined by digital pH meter in ambient temperature in triplicate. The pH of the optimised PNG was found to be 7.3±0.19. These results indicated that the optimised PNG was of neutral pH. As the optimised PNG showed neutral pH, the pH dependent degradation of drug was avoided.

2. Viscosity:

Viscosity of the optimised PNG was determined by Brookfield digital viscometer at ambient temperature and at fixed rpm (20). The viscosity of the optimised PNG was found to be 60250±250 cp and optimised proniosomes was found to be 628±33 cp. Viscosity of optimized PNG was due to the concentration of Carbopol 934 (5%) present in formulation. The viscosity of 5 % Carbopol gel was found to be 59650±409.26 cp. This indicated that incorporation of proniosomes into the Carbopol gel increases the viscosity of the formulation. This might be due to proniosomes being incorporated into the gel matrix of the Carbopol gel. The results indicate that the optimised PNG has adequate viscosity and hence will have good residence time on skin as well as good spreadability.

3. Spreadability:

The spreadability of optimised PNG was determined by texture analyser with trigger load of 0.5 g and test speed of 1 mm/sec by measuring force required to spread the formulation. This property depends onhardness of PNG, as hardness increases spreadability of PNG decreases. The hardness value of the optimised PNG was found to be 37.82 g which indicate good spreadability of the formulation. The spreadability of the formulation is inversely proportional to the viscosity. Hence it could be concluded that the formulation is having good spreadability and will evenly distribute on skin surface covering larger skin surface area.

4. *In vitro* release studies and *ex vivo* permeation studies:

The *In-vitro* release profile of INH from the optimised PNG as well as INH plain gel was investigated for 6 h using cellophane membrane by Franz diffusion cell at 37°C in phosphate buffer pH 5.5. *In-vitro* release studies will be predictive of *In-vivo* product behaviour. The results (Fig 8A) indicated that during the first hour, the drug release was slow probably because of the slow diffusion of drug from vesicles then it increased till the 6th hour. The % of INH released from optimised PNG was found to be 98.10±1.28% and from plain gel it was

found to be 71.28±1.66 % after 6 h. Span 20 has a relatively lower phase transition temperature (16) and the proniosomes get converted to niosomes due to hydration by skin moisture at skin temperature. This also makes the vesicle bilayers more fluid and leaky resulting in release of the drug [30]. Rahman et al [32] stated that niosomes exhibit an alkyl chain length-dependent release and the higher the chain length, the lower the release rate. As Span 20 has smaller chain length relatively faster release was observed from optimised PNG. Also, Shehata et al [48] reported that negatively charged proniosomes showed better release of the drug than that of the neutral or the positively charged proniosomes. This could be another possible explanation for higher release of drug from the proniosomes, as the ZP of the proniosomes was found to be negative.

Ex vivo permeation studies help in predicting *in vivo* permeation behaviour of the drug. *Ex vivo* permeation studies were performed similar to that of release study by replacing cellophane membrane with the subcutaneous skin of male albino rat. The results (Fig 8 B) showed the percentage drug release of INH through rat skin (phosphate buffer pH 5.5). The results showed 85±1.53 % and 56.36±1.69 % permeation of the drug from the rat skin within 6 h with flux of 293.80 and 190.55 µg/cm^2/h for the optimised PNG and plain gel respectively. Ruela et al [32] stated that drugs with low melting point (<200 ºC), which is related to an appropriate solubility in water and oils is required to achieve a high concentration gradient and increase the diffusion force across the skin. INH has a melting point 171ºC (<200 ºC) which contributes to the increased permeation of the drug through the skin [32].PNG permeates through transcellular route (a polar pathway) by swelling of the intracellular protein matrix, alteration of protein structure within the corneocytes which enhances drug delivery [49].Prasad et al [30], reported that increased permeation flux may be due to the non-ionic surfactant present in it, which modifies the structural composition of stratum corneum and increases the thermodynamic activity of the drug as well as skin vesicular partitioning. Also, presence of SL in the formulation acts as permeation enhancer besides its role as vesicle stabilizer. The absence of surfactant may be the reason for less permeation in plain gel as compared to optimised PG. Also, the PNG has the composition similar to stratum corneum lipids which enhances its penetration, once applied to the skin surface in contrast with that of plain gel which deposits the drug over the surface of the skin, being hydrophilic in nature. Ibrahim et al [50] also stated that the release of the drug from PNG is more than that of its permeation indicating the barrier properties of the skin. This justifies the decrease in permeation of the drug through animal skin as compared to the release of the drug from PNG.

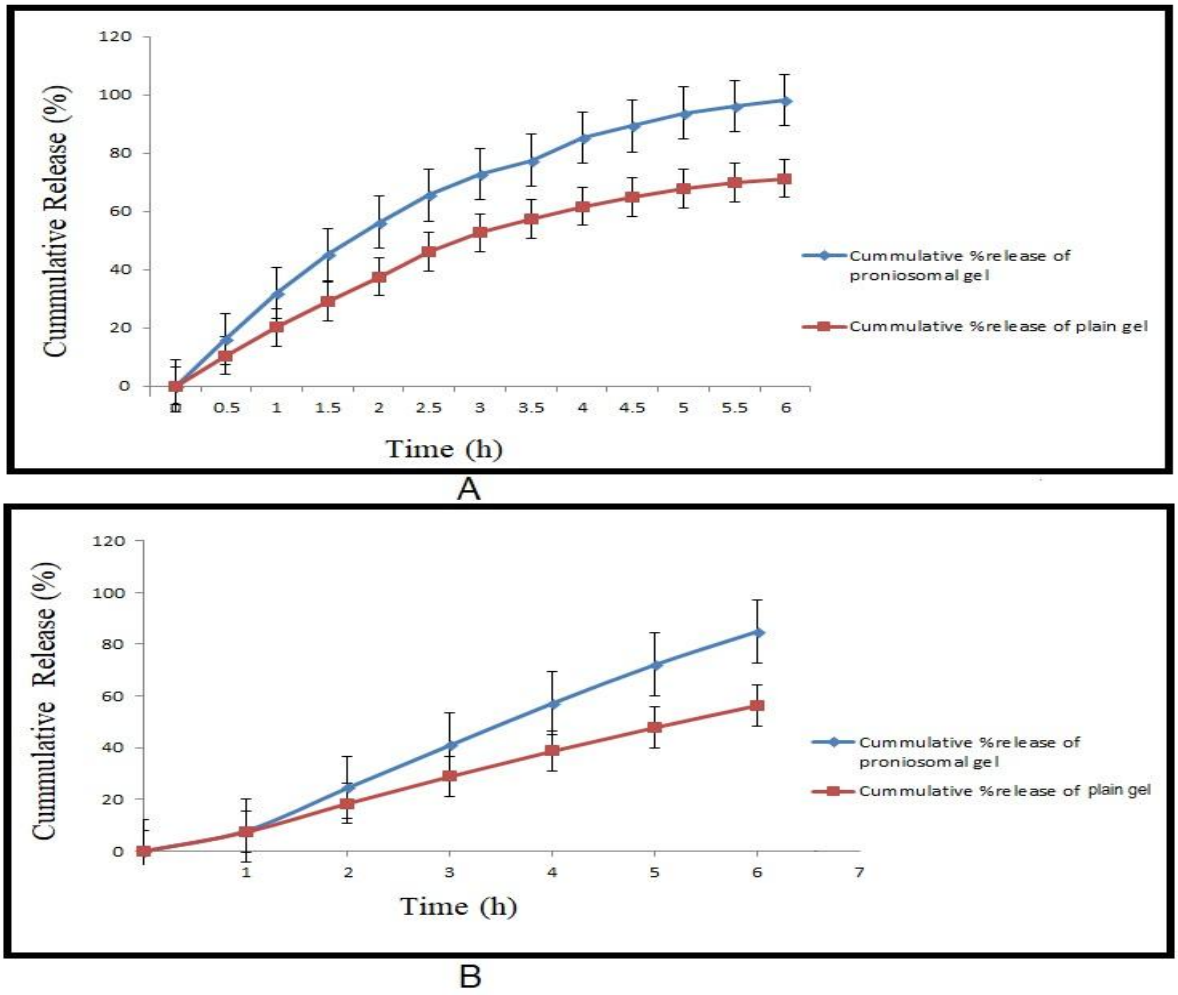

Figure 8: A: The *in vitro* release studies and B: *ex vivo* permeation of optimised proniosomal gel

5. *In vitro* skin irritation studies:

The *in vitro* skin irritation studies were carried out on male albino Wistar rats with standard approved protocol. The skin was observed for any visual change such as erythema at 24, 48 and 72 h after the application of the optimised PNG to the shaved rat skin. The results (fig 9) showed that the rat skin to which standard irritant (formalin 0.8 %) was applied showed score 4 (extreme erythema). There was no difference in skin score of control rat (no application of formulation or irritant) and the rat skin on which optimised PNG or blank PNG was applied. The mean erythemal score for rat on which optimised formulation was applied was 0, which indicated absence of irritation to the rat skin. This is points to the safety and non-irritant nature of the formulation.

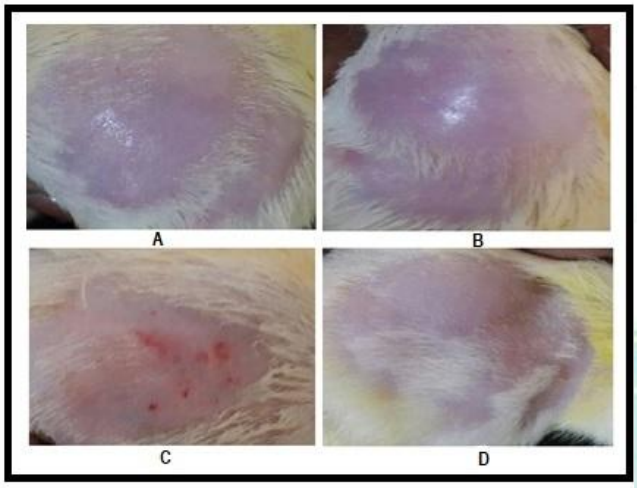

Figure 9: Skin irritation studies results showing A: control, B: blank proniosomal gel, C: standard irritant D: optimised Proniosomal gel formulation.

6. *In vivo* retention studies:

In retention study, the amount of INH accumulated into the viable skin after 3 h of applying the optimised PNG in *vivo* to the rat skin was recorded. The result showed that 436±12 µg of drug was retained in the skin. The higher amount of drug retained in the skin could be contributed to small vesicle size, the interaction between optimised PNG having similar lipidic composition as that of stratum corneum lipids resulted into providing a deposit effect of the drug in the skin which may prolong the drug residence time. Moreover, surfactant present in the optimised PNG may be responsible for the higher drug retention which may also integrate as well as mix with skin lipids to loosen their structure by disturbing the lamellar arrangement of the lipids[31]. The ideal physicochemical properties of a drug selected for cutaneous administration are low molecular weight (<600 Da) [32]. The molecular weight of INH is 137.05 Da, which is much less than the ideal requirement which in turn suggested increased retention of drug into the skin. Apart from these facts the results of *in vitro* and *ex vivo* release studies showed that there is decrease in drug release when used rat skin than that of cellulose membrane which suggested that the various barriers present in the skin retain the drug for the longer time. Also, Udasi et al[51] stated that PNG shows sustained and control release by depot formation. These all factors together contribute to higher amount of drug retained in the skin.

7. Stability studies:

The formulation was kept for stability study at refrigeration, RT conditions for 3 months. The optimised PNG was analysed for drug content after every week by HPLC as analytical method. The results (Table 9) of drug content showed degradation in the sample kept at RT in amber coloured bottle as well as in transparent bottles. The sample kept at refrigeration conditionsin amber coloured bottle showed insignificant degradation. This indicated that the formulation kept at refrigeration conditions is stable. The results showed that the drug is stable in optimised PNG formulation atrefrigeration conditions. According to Udasi et al [51], PNG decreases the hydrolysis of the drug and also decreases the problem of physical instability like aggregation, leakage, sedimentation during storage. The drug is entrapped inside the bilayer, thus making it less exposed to the external environment like oxygen, light and water. This makes the drug stable against hydrolysis, oxidation and photodegradation. This might be the reason for stability of the drug in the provesicular approach (PNG). Also, as reported before the ZP of the formulation being very less, it in turn contributes to the increased stability of the formulation. Various literature reports that PNG is more stable at refrigeration conditions than that of the room temperature thus supporting our results[30,31,32,52,53].

CONCLUSION

Oral therapy of anti tubercular drugs for treatment of cutaneous tuberculosis is beset with problems related to inadequate quantity of drug reaching the peripheral site. No literature was found regarding topical therapy for cutaneous tuberculosis hence there was a need to develop a topical preparation to enhance the efficiency of current therapy. The current research provides an alternative drug delivery of INH to the skin affected by tuberculosis by provesicular approach. The PNG of INH was formulated which showed high release, permeation through the skin as well as significant retention in the skin. Also, there was insignificant degradation of the drug in the formulation when stored at refrigeration conditions indicating stability of the drug in the formulation.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Ashwini Madgulkar Principal, AISSMS College of Pharmacy, Pune-01, Maharashtra (India) for providing necessary help and facilities to carry out the research work and also thankful to Amsal pharmaceuticals, Mumbai (India) for providing drug as gift sample.

DECLARATION OF INTEREST

There is no declaration of interest, financial or otherwise associated with this project.

REFERENCES

1. Frankel A, Penrose C, Emer J, Cutaneous Tuberculosis A Practical Case Report and Review for the Dermatologist, Am J Clin Aesth Derm, 2009; 2:19-27.
2. Caona T, Camposb CEM, Simõesa CMV, Silvaa MAS, Novel perspectives in the tuberculosis treatment: Administration of isoniazid through the skin, Int J Pharm, 2015; 494 (1):463–470.
3. Zy LV, du Plessis J, Viljoen J, Cutaneous tuberculosis overview and current treatment regimens. Tuberculosis, 2015; 95:629-638.
4. Loukia, A., Joseph, S., Brian, B., Nicole, A., & Zacharie, K. Multidrug resistant tuberculosis in children in the democratic republic of Congo: First experience with a short treatment course in a university hospital. Journal of Drug Delivery and Therapeutics, 2018; 8(2):123-128. doi:10.22270/jddt.v8i2.1682
5. Chen S, MQin, Han Y, Zhao L, Fu Y, Shang Y et al, Assessment of the efficacy of drug transdermal delivery by electro-phonophoresis in treating tuberculous lymphadenitis, Drug Deliv, 2016,; 23(5):1588–1593.
6. Chen S, Han Y, Yu D, Huo F, Wang F, Li Y et al, Transdermal delivery of isoniazid and rifampin in guinea pigs by electro-phonophoresis, Drug Deliv, 2017; 24(1):467–470.
7. Bhutani H, Singh S, Vir S, Bhutani KK, Kumar R, Chakraborti AK et al, LC and LC-MS study of stress decomposition behaviour of isoniazid and establishment of validated stability-indicating assay method, J Pharm Biomed Anal, 2007; 43:1213–1220.
8. Bolmal U.B, Pandey C.K, Phatarpekar V, Dhople N.G, and Kotha Rajkumar, Preparation and Evaluation of Isoniazid and Rifampicin Dispersible Tablets Prepared by Direct Compression and Sublimation Method. Int J Pharm Sci Nanotech,2013; 6(4):2225-2236.
9. Chang R, Raw A, Lionberger R and Yu L, Generic Development of Topical Dermatologic Products: formulation Development, Process Development, and Testing of Topical Dermatologic Products, AAPS J,2013; 15:41-52.
10. Desai D, Shah M, A Review: Validated Analytical Methods Developed on Antitubercular Drug, Rifampicin. J of Pharm Sci Biosci Res, 2015; 5:254-265.
11. Mishra A, Kapoor A, Bhargava S, Proniosomal Gel as a Carrier for Improved Transdermal Drug-Delivery, Asian J Pharm Life Sci,2011; 1(4):370-379.
12. Mehta M, Garg M. Proniosomal Gel: A Promising Drug Carrier for Boswellic Acids, J Med Sci. 2015; 15(3):130-134.
13. Indian Pharmacopoeia,The Indian Pharmacopoeia Commission Ghaziabad. 2007; 2:629-630.
14. ICH Harmonised Tripartite Guideline, Stability Testing of New Drug Substances and Products Q1A (R2). Step 4 Version Dated 6 Feb 2003.
15. Krilla MA, Das D, John G. Augustine, Semisolid Formulation, Development: The CRO Approach. SP formulations, Wareham 2009; 1-12.
16. Doaa A. Helal, Dalia Abd El-Rhman, Sally A. Abdel-Halim, Mohamed A. El-Nabarawi, Formulation and Evaluation of Fluconazole Topical Gel, Int J Pharmacy Pharm Sci. 2012; 4:176-183.
17. Perrett, S., M. Golding and W.P. Williams, A simple method for the preparation of liposomes for pharmaceutical applications: Characterization of the liposomes, J. Pharm. Pharmacol, 1991; 43:54-161.
18. Singh G, Dwivedi H, Saraf SK, Saraf SA, Niosomal Delivery of Isoniazid - Development and Characterization, Trop J Pharm Res, 2011; 10(2):203-210.
19. Patel KK, Kumar P, Thakkar HP, Formulation of Niosomal Gel for Enhanced Transdermal Lopinavir Delivery and Its Comparative Evaluation with Ethosomal Gel, AAPS Pharm SciTech,2012; 13(4):1502-1510.
20. Asthana GS, Asthana A, Singh D, Sharma PK, Etodolac Containing Topical Niosomal Gel: Formulation Development and Evaluation, Drug Deliv, 2016; 1-9.
21. Sandeep G, Vasavi Reddy D, Srinivas Reddy Devireddy, Formulation and Evaluation of Fluconazole Pro-Niosomal Gel for Topical Administration, J App Pharm Sci,2014; 4(7):98-104.
22. Mishra S, Vasistha P, Sachdeva M, Sara U V S, Formulation, Optimization and Characterization of Proniosomal Gel For Transdermal Delivery Of Naproxen, Int J Uni Pharm Life Sci. 2013; 3(3):145-161.
23. Budhiraja A, Dhingra G, Development and characterization of a novel antiacne niosomal gel of rosmarinic acid, Drug Deliv, 2015, 22(6):723–730.
24. Rajabalaya R, Leen G, Chellian J, shrikumar chakravarthi, sheba. R. David, Tolterodine tartarate Proniosomal gel transdermal delivery for overactive bladder, Pharmaceutics, 2016; 8(27):2-15.
25. Kamboj S, Saini V, Bala S, Sharma G, Formulation and Characterization of Drug Loaded Niosomal Gel for Anti-Inflammatory Activity, Int J Pharmacol and Pharm Sci. 2013; 7(12):877-881.
26. Qumbar M, Ameeduzzafar, Imam SS, Javed Ali, J Ahmad, Ali A, Formulation and optimization of lacidipine loaded niosomal gel for transdermal delivery: In-vitro characterization and in-vivo activity, Biomed &Pharmacother,2017; 93:255–266.
27. Patil HN, Hardikar SR, Bhosale AV, Formulation Development & Evaluation of Proniosomal Gel of Carvedilol, Int J Pharm Pharma Sci.2012; 4(1):191-197.
28. Vasistha P, Ram A, Proniosomes, as a Drug Carrier for Transdermal Delivery of Hydrochlorothiazide and Lisinopril Combination. Int J of Pharm and Pharma Sci. 2013; 5:142-149.
29. Prasad V, Chaurasia S, Performance evaluation of non-ionic surfactant based tazarotene encapsulated proniosomal gel for the treatment of psoriasis, Materials Sci and Eng, 2017; C(79):168–176.
30. Sambhakara S, Paliwala S, Sharmaa S, Singh B, Formulation of risperidone loaded proniosomes for effective transdermal delivery: An in-vitro and in-vivo study, Bulletin Fac Pharma. 2017; 55(2):1-9.
31. Rahman SA, Abdelmalak NS, Badawi A, Elbayoumy T, Sabry N, El Ramly A, Formulation of tretinoin-loaded topical proniosomes for treatment of acne: in-vitro characterization, skin irritation test and comparative clinical study, Drug Deliv, 2015; 22(6):731–739.
32. Ruela ALM, Perissinato AG, Monica Esselin de Sousa Lino, Paula Silva Mudrik, Gislaine Ribeiro Pereira, Evaluation of skin absorption of drugs from topical and transdermal formulations, Brazilian J Pharm Sci, 2016; 52:527-544.
33. Brewer GA, Isoniazid, in: K. Florey (Ed 1), Analytical Profiles of Drug Substances, London:Academic; 1976; 8:184–258.
34. Razak SA, Yaacob SFFS, Abdullah JM, Adnan R. Isoniazid and β-cyclodextrin complexes, A stability study in aqueous solution, J ChemPharm Res, 2015; 7(7):346-355.
35. https://pubchem.ncbi.nlm.nih.gov/compound/isoniazid#section=LogS (2018) Accessed 12th March 2018.
36. Sandra Stets, Bianca do Amaral, Jéssica Tamara Schneider, Ivan Ricardo de Barrosa, Marcus Vinícius de Liz, Ronny Rocha Ribeiro et al, Antituberculosis drugs degradation by UV-based advanced oxidation processes, J of Photochem and Photobio A: Chem, 2018; 353:26–33.
37. http://www.ajhp.org/content/75/10/622(2018) Accessed on 6th July 2018.
38. Shukla, N.D. and M. Tiwari, Proniosomal drug delivery system-clinical applications, Int. J. Res. Pharm. Biomed. Sci. 2011; 2:880-887.
39. Mokhtar, M., O.A. Sammour, M.A. Hammad and N.A. Megrab, Effect of formulation parameters on flurbiprofen encapsulation and release rates of niosomes prepared from proniosomes, Int J Pharm,2008,361,104-111.
40. Benipal G, Design, Development and Evaluation of Proniosomal Gel of an Antifungal Drug – Ketoconazole, Int J Pharm Sci RevRes, 2015; 31(2):265-272.

41. Varshosaz J, Pardakhty A, Hajhashemi V, Najafabadi AR, Development and Physical Characterization of Sorbitan Monoester Niosomes for Insulin Oral Delivery, Drug Deliv, 2003; 10:251–262.
42. Shatalebi MA, Mostafavi SA, Moghaddas A. Niosome as a drug carrier for topical delivery of N-acetyl glucosamine, Res PharmSci, 2010; 5:107–17.
43. Pankaj S, Rini T, Dandagi PM, Formulation and Evaluation of Proniosome Based Drug Delivery System of The Antifungal Drug Clorimazole, Int J of Pharm SciNanosci,2013; 6:1945-1951.
44. Lather V, Sharma D, Pandita D, Proniosomal Gel-Mediated Transdermal Delivery of Bromocriptine: In Vitro And Ex Vivo Evaluation, J of Exp Nanosci,2016; 11(13):1044-1057.
45. El-Laithy HM, Shoukry O, Mahran LG, Novel sugar esters proniosomes for transdermal delivery of vinpocetine: Preclinical and clinical studies, Europ J PharmBiopharm, 2011; 77(1):43-55.
46. El Maghraby GM, Ahmed AA, Osman MA, Penetration enhancers in proniosomes as a new strategy for enhanced transdermal drug delivery, Saudi Pharm J. 2015; 23(1):67–74.
47. Solanki AB, Parikh JR, Parikh RH, Formulation and Optimization of Piroxicam Proniosomes by 3-Factor, 3-Level Box-Behnken Design, AAPS Pharm SciTech, 2007; 8(4):1-7.
48. Shehata TM, Abdallah MH, Ibrahim MM, Proniosomal Oral Tablets for Controlled Delivery and Enhanced Pharmacokinetic Properties of Acemetacin, AAPS PharmSciTech, 2015,16; 375-383.
49. Alli MS, Srilakshmi.Ch, Ganesan G. Proniosome Gel: An Effective Novel Therapeutic Topical Delivery System, Int J of PharmTech Res, 2013; 5:1754- 1764.
50. Ibrahim MMA, Sammour OA, Hammad MA, Megrab NA, In Vitro Evaluation of Proniosomes as a Drug Carrier for Flurbiprofen, AAPS PharmSciTech, 2008; 9:782-790.
51. Udasi TA, Wankhade VP, Ingle LM, Atram S, Tapar KK, Proniosome: A Novel Approach to Vesicular Drug Delivery System, Int J Pharm and Pharm Sci Res. 2013; 3(1):1-6.
52. Radha G.V., Veerendranath Chowdary CH., Formulation and Evaluation of Ornidazole Proniosomal Gel, Indo Am J of Pharm Res,2014; 4:2657-2664.
53. Mehta S.K., Jindal N, Formulation of Tyloxapol niosomes for encapsulation, stabilization and dissolution of anti-tubercular drugs, Colloids and Surfaces B: Biointerfaces, 2013; 101:434– 441.