Annexure-IX

UNIVERSITY GRANTS COMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI- 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. TITLE OF THE PROJECT :

Development and Characterization of Vitamin E TPGS based Nanoemulsion Formulation for Sustained and Targeted Delivery of Anti-fungal Agent

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR :

Dr. Subheet Kumar Jain

Professor

Department of Pharmaceutical Sciences

Guru Nanak Dev University,

Amritsar 143 005, Punjab.

3. NAME AND ADDRESS OF THE INSTITUTE :

Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar.

4. UGC APPROVAL NO. AND DATE :

No. F.42-673/2013 (SR) , Dated 25th March 2013

5. DATE OF IMPLEMENTATION : 01-04-2013

6. TENURE OF PROJECT : 01-04-2013 to 31-03-2017

7. TOTAL GRANT ALLOCATED : Rs. 11.59 LAKH

8. TOTAL GRANT RECEIVED : Rs, 11.20 LAKH

9. FINAL EXPENDITURE : Rs. 10,90,727

(Rs. Ten lac ninety thousand seven huandred twenty seven only)

Unspent balance of rs. 28,273 have been refunded in UGC account No. 8627101002122 on 18/12/2017 by RTGS mode

10. TITLE OF THE PROJECT:

Development and Characterization of Vitamin E TPGS based Nanoemulsion Formulation for Sustained and Targeted Delivery of Anti-fungal Agent

11. **OBJECTIVES OF THE PROJECT**

1. The goal of the proposed study is to prepare and evaluate Sodium deoxy cholate free Vitamin E TPGS based nanoemulsion formulation of Amphotericin B (AmB) for its sustained and targeted delivery
2. To study the effect of different formulation and process variables on drug loading and stability of nanoemulsion formulation
3. To prepare, characterize and optimize the nanoemulsion based nanogel formulation of AmB.
4. In vitro skin permeation and deposition study of nanoemulsion based gel formulation in comparison to marketed AmB formulation.
5. To carry out the vesicle-skin interaction study using FT-IR, CLSM, SEM, TEM and fluorescence microscopy techniques to determine the possible mechanism of better skin permeation of nanoemulsion formulation.
6. To compare the skin irritation and hemolytic toxicity of nanoemulsion gel formulations in comparison to commercial marketed AmB cream.
7. To compare the biological anti-fungal activity of nanoemulsion gel formulations in comparison to commercial marketed AmB formulation.

12. WHETHER OBJECTIVES WERE ACHIEVED (GIVE DETAILS)

Yes all the objectives were achieved. Nanoemulsion based nanogel formulation using Vitamin E TPGS as surfactant was successfully developed and characterized extensively in vitro, ex vivo and in vivo

The study lead to the preparation and evaluation of the Sodium deoxy cholate free Vitamin E TPGS based nanoemulsion formulation of AmB for its sustained and targeted delivery. To study the effect of different formulation and process variables, AmB nanoemulsion formulations were prepared by high energy method in which aqueous and oil phases were prepared and heated separately. The oil phase containing olive oil, oleic acid, transcutol and DMSO was then added to aqueous phase consisting of hydrophilic surfactants and co-surfactants under continuous stirring. Oil phase was selected based on the solubility of drug in various oils and their mixtures, wherein maximum solubility with a value of 12.50±0.08 mg/ml was found in mixture of olive oil: oleic acid: DMSO (2:2:1) and same was selected as oil phase. Surfactant and co-surfactant were selected based on their miscibility with oil phase. Vitamin E TPGS NF (TPGS, D-a-tocopheryl polyethylene glycol 1000 succinate) was selected as one of surfactant for

the preparation of nanoemulsion formulation as its chemical properties have suggested its wide use as a solubilizer, an emulsifier, an intestinal absorption enhancer for increasing the oral bioavailability of poorly water soluble drugs.

The prepared formulation was characterized and optimised for globule size (nm) and shape, polydispersity index, entrapment efficiency, % transparency, drug content, viscosity, thermodynamic stability and dispersibility.

In vitro skin permeation and deposition studies were performed using rat, pig and human cadaver skin. The profused skin penetration and deposition potential of developed nanogel formulation was confirmed by CLSM study. Penetration of nanogel formulation into skin and fluorescence intensity was 2.5 fold higher, with higher skin deposition as compared to marketed gel of enhancement ratio to 3.5., as compared to simple gel formulation.

To compare the skin irritation study of the developed formulation, the nanogel treated skin showed no sign of erythema and oedema after 1, 24, 48, 72 h of application in acute toxicity testing. Similar results were found after repeated application for 28 days period in sub-acute toxicity testing. Finding of both these studies demonstrated that developed nanogel formulation has better safety profile.

The optimized nanoemulsion formulation was compared for biological anti-fungal activity. The formulation showed higher antifungal activity against *A. niger* and anticandidal activity against *C. albicans.*

Overall, it is evident from the present study that the developed topical formulations could be a better alternative for safe and effective delivery of AmB.

13. ACHIEVEMENTS FROM THE PROJECT

Three international publications with cumulative impact factor of approx 6.0 and one research presentation in international conference were made

Publications

1. Kaur, L., Singh, K., Paul, S., Singh, S., Singh, S., and **Jain, S.K.** Mechanistic insight to determine the structural similarities between artificial membrane Strat-M^{TM} and biological membranes and its application to carry out skin permeation study of Amphotericin B nanoformulations. AAPS Pharm. Sci. Tech. 2018, 1-19, DOI :10.1208/s/12249-018-0959-6 (**Impact Factor 2.451**)

2. Kaur, L., **Jain, S.K.** and Singh, K. Vitamin E TPGS based nanogel for skin targeting of high molecular weight anti-fungal drug: Development, in vitro and in vivo assessment. ***RSC Advances***. 2015; 5: 53671-53686 (**Impact Factor 3.84**)
3. Mahajan, M., Hhurana, R.K., Singh, H., Dhatwalia, A., Kumar, C., Rampal, A., Puri, R., Lakha, A., Suman, Rana, D., Kaur, L., Kumar, D., and **Jain, S. K.** An overview of current applications of nanotechnology in biomedical research: A patent survey. *Recent Patents on Nanomedicine* 2014, 4, 46-56,

Presentations

1. Recent advances in delivery of antifungal drugs. International Conference on Advances in Pharmaceutical Nanotechnology and Nanomedicine (ICAPNN-2015), Held on 6-8 Feb 2015 at ISF College of Pharmacy, Moga

14. SUMMARY OF THE FINDINGS

Amphotericin B (AmB), a membrane-active polyene is considered as the most effective gold standard antifungal and is clinically used for the treatment of systemic and local fungal infections. This drug also has antiprotozoal activity and is used for the treatment of both visceral and cutaneous leishmaniasis. AmB can be administered both intravenously and topically. Presently, the preferred route for administration of AmB for above mentioned disease conditions is parenteral.

Recently, topical route has also been explored for safe and efficacious delivery of AmB for the treatment of surface fungal infections, for instance; skin and nail infections caused by dermatophytes, burn related fungemia and *Cutaneous leishmaniasis*. Also, in the case of patients with decreased immunity and ongoing treatments with antiretroviral and other immunosuppressant drugs, it is quite difficult to treat them with systemic antifungal therapy; wherein topical therapy seems to be better. Almost 90% of people suffering from AIDS develop at least one fungal infection over the course of disease, out of which 10–20% of infections prove fatal. Thus, effective topical delivery of antifungal drug is required clinically for the effectual management of multiple disease conditions and at the same time to increase the patient compliance. Currently, two types of topical formulations of AmB have been developed which are commercially available (Fungizone^{TM} cream/lotion and Fungisome^{TM} gel). However, these formulations suffer from the drawbacks of very poor skin permeation, due to which they possess limited clinical effectiveness. The reason behind the ineffectiveness of topical therapy of AmB is its high molecular weight and bulky structure, which comprises hydrophobic heptane chains and a hydroxyl rich hydrophilic chain that is responsible for its poor solubility in both aqueous and hydrophobic systems thus leading to the poor skin partitioning resulting in poor permeation of

drug. As a result, with existing conventional formulation, it is difficult to effectively treat topical fungal infections which provide a rationale to explore the potential of novel carrier based formulation for effective topical delivery of AmB.

Nanocarrier based formulations are increasingly gaining importance in terms of topical drug delivery for effective management of skin diseases. Therefore, in order to enhance the topical effectiveness of AmB, nanoemulsion based nanogel formulation was prepared and characterized.

AmB nanoemulsion formulations were prepared by high energy method in which aqueous and oil phases were prepared and heated separately. The oil phase containing olive oil, oleic acid, transcutol and DMSO was then added to aqueous phase consisting of hydrophilic surfactants and co-surfactants under continuous stirring. Oil phase was selected based on the solubility of drug in various oils and their mixtures, wherein maximum solubility with a value of 12.50±0.08mg/ml was found in mixture of olive oil: oleic acid: DMSO (2:2:1) and same was selected as oil phase. Surfactant and co-surfactant were selected based on their miscibility with oil phase. Vitamin E TPGS NF (TPGS, D-a-tocopheryl polyethylene glycol 1000 succinate) was selected as one of surfactant for the preparation of nanoemulsion formulation as its chemical properties have suggested its wide use as a solubilizer, an emulsifier, an intestinal absorption enhancer for increasing the oral bioavailability of poorly water soluble drugs like paclitaxel and vancomycin, as skin permeation enhancer for drugs like griseofulvin and estradiol and also as stabilizer. Pseudoternary phase diagrams were mapped in order to optimize the surfactant to co-surfactant ratio and type of co-surfactant that could result in large existence area of nanoemulsion. The prepared nanoemulsion was then mixed with blank Carbopol 934P and Carbopol 940P gel base under mechanical stirring to form the nanogels.

Prepared nanoemulsion based topical formulations were characterized and optimized with respect to different process and formulation variables and were characterized for globule size (nm) and shape, polydispersity index, entrapment efficiency, % transparency, drug content, viscosity, thermodynamic stability and dispersibility. Vesicle size and polydispersity index was determined and optimized formulation exhibited uniform globule size in nanometric range of 21.64±0.8 nm. Shape was analyzed under transmission electron microscope. All the nanoemulsion formulations were found to have the Grade A dispersibility which may be attributed to lesser interfacial tension between oil and aqueous phase and formation of thermodynamic stable emulsion. They remained stable and no physical changes were observed after subjecting them to stress conditions of heating, cooling and freeze thaw cycle in

thermodynamic stability testing followed by centrifugation. Based on the various *in vitro* characterization studies, the nanoemulsion composed of S_{mix} (42% v/v), oil (11% v/v), water (45.9% v/v) and drug (0.1% w/v) was optimized depending upon its superior properties such as small globule size, polydispersity index, *in vitro* dispersibility, good flow properties, thermodynamic stability etc. for preparation of nanoemulsion based nanogel formulation.

The prepared nanogels were then characterized for characteristic quality control test of topical gels like drug content, extrudability, pH, rheology, thixotrophy and textural analysis with respect to gel former concentration ranging from 0.25 to 1.75% and were optimized based on better extrudability, yield stress, thixotrophic behavior, adhesiveness and cohesiveness as compared to marketed gel. Rheological analysis was performed using Rheometer (Rheolab QC, Anton parr, Germany) and mechanical properties like cohesiveness, adhesiveness and hardness of nanogel was determined using texture analyzer (TA/TX2-plus, Stable Micro Systems, Surrey, UK).

In case of nanoemulsion based nanogel, two gel formers: Carbopol 934P and Carbopol 940P in concentration range of 0.5 to 1.75% w/w were selected. Viscosity determined at constant shear rate and variable shear rate at a temperature of 25ºC suggested non-newtonian shear thinning flow behavior of nanogels characterized by decrease in viscosity with increase in shear rate. While evaluating viscosities, Carbopol 934P and 940P based formulation showed the comparable viscosity to that of marketed gel at the concentration of 1.25 % and 1.5 % w/w, respectively. Nanogel containing 1.25% Carbopol 940P(ANG14) showed requisite results in case of all tested parameters like thixotrophy, yield stress, adhesivessness, cohesiveness hardness and viscosity and so was optimized. During thixotrophic analysis, the percent recovery for optimized nanogel was found to be 99.80±1.6%, whereas marketed gel showed a value of 89.84±1.1%. The yield stress value for nanogel and marketed gel was 109.51±2.1 Pa and 73.84±1.7 Pa, respectively. Adhesiveness of gel increased with increase in gel former concentration reflecting constant contact of the preparation with human skin and was found to be -0.317±0.002 g. sec for nanogel and -0.3±0.002 g. sec for marketed gel, respectively. Cohesiveness and hardness values for nanogel and marketed gel were found to be 0.94±0.03, 0.045±0.0005N and 0.896±0.01, 0.0459±0.0004 N, respectively, indicating better results with optimized nanogel.

In vitro skin permeation and deposition study was performed using optimized nanogel formulation in comparison to commercial gel formulation. The optimized nanogel showed higher skin deposition as compared to marketed gel which enhancement ratio of 3.5 and 3.9 fold,

respectively. The profused skin penetration and deposition potential of developed nanoethogel and nanogel formulation was confirmed by confocal laser scanning microscopy (CLSM) study. CLSM study has inimitable advantage in determination of quantitative estimation of depth and extent of penetration of a marker. The intensity graph resulted from CLSM study showed the highest fluorescence intensity with deepest skin permeation with nanogel formulations. Penetration of nanogel into skin and fluorescence intensity was 2.5 fold as compared to simple gel formulation. This marked the improved skin penetration potential and depot forming properties of examined nanogel formulation. The values of enhancement ratio found in CLSM study were well correlated with results of *ex vivo* skin deposition study measurement.

Further scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analyses were done to shed some insight into the mechanism of better skin permeation of nanogel formulation. Prepared formulations were found to affect the compact surface of skin creating pathway through pores formation for their better penetration. The SEM photomicrographs of control rat skin exhibited uniformity with smooth and compact surface. However, nanogel treated skin showed loosening and creation of pores which is indicated as the basic mechanism for better permeation with nanogel. In case of FTIR analysis of treated and control rat skin samples, there were presence of distinguishing peaks of hydrocarbon chains of SC at 2950 cm-1 and 2850 cm-1 owing to asymmetric and symmetric C-H vibration in case of control rat skin. But, the application of nanogel formulation considerably abridged the extent of these peaks demonstrating extraction of skin lipids and perturbation effects. It is reported that the extraction of skin lipids and lipid perturbation effects can be described by variations in the height, area and displacement of these two peaks. These outcomes clearly indicate the permeation enhancement effect of Vitamin E TPGS and other components of nanogel formulation.

In order to further determine the effectiveness of prepared formulations, antifungal activity was carried out against *Aspergillus niger* and anticandidal activity against *Candida albicans.* MIC of both nanogel was found to be 3.12 µg and 6.25 µg against *Aspergillus niger* and *Candida albicans*, respectively and in comparison plain drug solution and marketed gel of AmB showed the value of 6.25 µg and 12.5 µg against *Aspergillus niger* and *Candida albicans*, respectively. However, zone of inhibition against *Aspergillus niger* and *Candida albicans* was found to be significantly ($p<0.05$) higher in case of nanogel as compared to marketed formulation and drug solution. To be more precise, this antifungal activity of developed formulation was also carried out *in vivo* using *Candida albicans* induced mycosis model. The

animals treated with nanogel showed significant reduction in *Candida* induced infections (cutaneous candidiasis) as compared to drug solution. In nanogel treated group rapid recovery of infection was observed and no animal out of six treated animals exhibited positive culture test, whereas in case of drug solution and marketed cream treated groups, 4 and 1 animal out of six showed positive culture test, respectively. This result of *in vivo* performance study confirmed the better antifungal activity of nanogel due to its enhanced skin permeation and retention ability at affected site which indicated better recovery of topical fungal lesions in short period of time via use of developed topical formulations as compared to marketed gel.

AmB is clinically recommended for long term repeated administration as fungicidal and leishmanicidal. So, the skin irritation study was carried out as the prepared formulations are for topical use. Nanogel treated skin showed no sign of erythema and oedema after 1, 24, 48, 72 h of application in acute toxicity testing. Similar results were found after repeated application for 28 days period of time in sub-acute toxicity testing. Finding of this study demonstrated that developed nanogel formulation has better safety profile. Overall, it is evident from the conducted studies and results, that the developed topical formulations could be a better alternatives for efficient topical delivery of AmB.

15. CONTRIBUTION TO THE SOCIETY

Intensifying incidences of skin mycosis caused by different species of yeast, fungi and dermatophytes in both immunodeficient and immune-competent patients remain an imperative and adequately addressed medical problem. It affects more than 25% of the world's population. It may be life-threatening and can have debilitating effects on a patient's quality of life especially in immunocompromized patients suffering from diseases like AIDS, diabetes and cancer or may become invasive (systemic) and in some cases it may also spread to other people. Cutaneous fungal infections (*Cutaneous candidiasis,* burn fungemia, *cutaneous leishmaniasis*) are more rampant in sub-tropical countries like India, Africa and Srilanka where the climatic conditions are warm and humid that further accelerate the occurrence, growth and spread of dermatomycosis. Candida species is one of the major causes associated with skin membranes (cutaneous candidiasis) and fingernails (candidiasis or thrush) related infections. These infections are generally difficult to diagnose and treat because they are often misguided for other disorders, such as eczema or psoriasis.

New safe and effective topical formulation for treatment of topical fungal infections was developed in the present study.

16. WHETHER ANY PH.D. ENROLLED/ PRODUCED OUT OF THE PROJECT

NA (No research fellow/JRF sanctioned in the project)

17. NO. OF PUBLICATIONS OUT OF THE PROJECT
(Please attach)

Three international publications with cumulative impact factor of 6.0 and one research presentation made

Publications : 03

4. Kaur, L., Singh, K., Paul, S., Singh, S., Singh, S., and **Jain, S.K.** Mechanistic insight to determine the structural similarities between artificial membrane Strat-M^{TM} and biological membranes and its application to carry out skin permeation study of Amphotericin B nanoformulations. AAPS Pharm. Sci. Tech. 2018, 1-19, DOI :10.1208/s/12249-018-0959-6 (**Impact Factor 2.451**)
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6. Mahajan, M., Hhurana, R.K., Singh, H., Dhatwalia, A., Kumar, C., Rampal, A., Puri, R., Lakha, A., Suman, Rana, D., Kaur, L., Kumar, D., and **Jain, S. K.** An overview of current applications of nanotechnology in biomedical research: A patent survey. *Recent Patents on Nanomedicine* 2014, 4, 46-56,

Presentation

1. Recent advances in delivery of antifungal drugs. International Conference on Advances in Pharmaceutical Nanotechnology and Nanomedicine (ICAPNN-2015), Held on 6-8 Feb 2015 at ISF College of Pharmacy, Moga

Principal Investigator
(Signature with Seal)

Registrar
(Signature with Seal)