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5. SUMMARY AND CONCLUSIONS

Interest in pharmaceutical research related to the area of topical delivery has been escalating owing to existing challenges of dermatological disorders, while skin per se being the target organ to be accessed. Hitherto, this area remains majorly unexplored because of the deficient approach of formulating the drug using conventional approaches. But now with the advanced understanding of the requirements of a formulation for the delivery drug to a particular site, the focus has shifted to the external mode of administration rather than using the systemic way of reaching the skin site. The approach, if successful, is likely to save the sensitive organs and tissues against the unnecessary exposure of the toxic drugs while patient compliance would be one of very high order. With these benefits in sight, the present work was conceived to develop the dermal drug delivery systems using the carrier concept in order to make the two very important drugs viz. methotrexate (MTX) and cyclosporin A (CysA) useful on external application.

In fact, the previous research efforts could not lead to the fruitful results because of the limitations associated with respect to the physicochemical characteristics of the investigated drugs. Henceforth, in the present approach efforts were made to modify the innate properties of the molecule as well as of the skin milieu to facilitate their transport into the deeper layers of skin.

Depending upon the nature of drug candidates, different carrier systems were selected for their topical delivery. Specifically for topical delivery of methotrexate, the microemulsion (ME) system was chosen. The hypothesis behind selecting the ME as a delivery systems lies in its composition with high amphiphile content which would provide better solubility at the molecular level and partitioning with the skin tissue that will improve the topical delivery. Herein, the vesicular systems were not preferred because of the Log P value which provide unfavorable entrapment, the essential requirements for the transport across the stratum corneum. In case of cyclosporin A, three major classes of drug delivery approaches viz., ME (a micellar system), vesicular system and particulate system, have been studied in literature. It was found that the former two were more preferred for topical delivery because they opt multiple mechanisms to help the molecules to permeate across the horny skin layer, the stratum corneum. However, the latter (i.e., particulate system) has the major restriction in terms of size to act as vehicle for the topical delivery. For the molecules like cyclosporine A,
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whose clinical failure on topical application is because of its high molecular weight and unfavorable Log P, micellar systems and particulate systems would not be a good option. The carrier system (like vesicles) which can hold this molecule within its structure and permeate along with the molecule, could be the appropriate choice. Therefore, the organosomes, a type of vesicles out of reverse micellar system, was selected here. This is a different approach which generates the vesicles on dilution with water. These vesicles are formed as a result of micellar transformation and in the process lead to the entrapment of lipophilic drug within vesicles. This system provides a conducive micro-interior for such type of molecules.

Preliminary studies with both drugs:

The obtained gift samples of MTX and CysA, respectively from M/s Ipca Laboratories Ltd., Mumbai, India, and M/s Lifecare Innovations Pvt. Ltd., Gurgaon, India, were first confirmed for their respective physical and chemical identities using techniques like macroscopic observation, FTIR and \(^1\)H NMR spectroscopic studies. These characters were subsequently matched with the specification listed in the USP (2009) and CoA provided by the supplier. To analyze the drug during various stages of the experimental work, a suitable, simple and reproducible analytical techniques based on UV and HPLC were developed and validated in the laboratory conditions for linearity, linearity range, accuracy, inter-day and intra-day precession, etc. Besides, taking phospholipid as important to include in the objectives, taking as these excipients are functional in nature with respect to delivery as well as pharmacodynamic action, its Steward assay was reproduced in the laboratory condition.

Studies on microemulsion for methotrexate

To develop ME-based MTX formulations, solubility studies were performed in a number of oils, organic solvents, surfactants of different strengths of solution and buffers at 30 °C. Out of the varied components, IPP, PBS (pH 7.4) and Tween 80 were selected as oil, aqueous and surfactant phases, respectively. Phospholipid (phospholipon 90G) and ethanol were selected as the co-surfactants. Different pseudo-ternary phase diagrams were constructed to find out the maximum area relating to ME phase. A series of pseudo-ternary phase diagrams were prepared by titrating three phases, i.e., aqueous, oil and surfactant-mix (Smix: Surfactant + Co-surfactant) in different ratios. Initially, changes were made by using different ratios of ethanol to phospholipid, and then ratios of surfactant to co-surfactant. The pseudo-ternary phase diagram, which was chosen to
find 1:2 (w/w) ratio of surfactant to co-surfactant and 4:1 (w/w) ratio of ethanol to phospholipid. From this phase diagram, ranges of all three phases (i.e., oil, aqueous and Smix) were found, wherein the ME get formed. Further, these ranges were used to systematically optimize the formulation for the desired characteristics employing rational approach of FbD in two successive steps. In the first step, Taguchi design was used to screen the critical formulation variables (CFVs) amongst the range of materials used to formulate ME. And in the second more vital step, the selected CFVs were further optimized by employing IV optimal mixture design to get the formulation with desired multiple responses.

With 7 input variables, usage of Taguchi design suggested 8 different combinations of formulations. All these formulations were prepared and characterized for desired 3 important responses i.e., skin retention, transmittance and Q6. ANOVA-based Pareto, half-normal and normal charts were used to study the statistical significance of each response variable. By two limit lines, the Pareto chart establishes t value of effect to know the degree of significance. These limit lines are Bonferroni limit line (t value of effect = 6.580) and t limit line (t value of effect = 3.182). The response coefficient with t value above 6.580 (i.e., above Bonferroni limit line) was designated as indeed significant having more contribution in the generated responses and the response coefficient with t value in between 6.580 and 3.182 was considered as likely to be significant. All these charts indicated phospholipid, aqueous phase and co-surfactant as CFVs because of their significant impact on the respective responses. Also, Shapiro-Wilk test performed with p value >0.10 in case of all three responses, indicated that there is no deviation from assumption of normality for these points.

Application of Taguchi design could not find the absolute and precise mathematical data to formulate the system with the desired attributes. The detailed mathematical data, however, were obtained by selective mixture design, i.e., IV optimal design. In this, less influential factors were kept constant at their best level and most influential variables were optimized. A total number of 16 ME systems were prepared as suggested by the model design of Mixture IV optimal and responses were noted in terms of skin retention, flux and transmittance. Based on the result outcomes, used design was tested by assessing minimal requirements in different plots. In plots of residuals between Normal Percent Probability vs. Internally Studentized Residuals and plots of response values between predicted response vs. actual response have been found to follow the linear relationships. Closeness of all the points to the diagonal line, in case of all three
responses, indicated that the model is well-equipped to analyze the data accurately. In plots of Residual Response vs. Ascending Predicted Response values, Residuals vs. Experimental Run Order and Internally Studentized Residuals vs. Factor randomly scattered pattern of all points between limit lines, across the graph were found. This indicated that model is able to provide good analysis with no further transformation required. In power law transformation plots between Ln Residual vs. Lambda, value of lambda at the 95% confidence interval was around 1 for percent transmittance and drug skin retention. In case of permeation flux lambda value was ‘0’ and model was transformed by natural log. The plot between Externally Studentized Residuals vs. Number of Runs served as a tool to identify abnormal run(s) and guide that the data of particular run (point) should remain in the data set or not. To judge the abnormality, the "control limits" were ascertained on the externally studentized residuals plot, with each point outside the limit considered as an outlier. All the points were found to be positioned well within the limits, i.e., no outlier was found in any of the cases. Also, in plots between Dffits vs. Runs and in DFBETAS vs. Run all the generated points remained within the specified limits. The plots between Cook’s distance vs. Run number also furnished information about the outlier points. Here, a point that has relatively very high distance value from the other may be an outlier. In all the three cases studied no outlier point was found, further satisfying the aptness of the employed model.

After analyzing the model, effects of CFVs on final responses were studied using RSM technique under holistic approach of FbD. It was observed that co-surfactant and, to some extent phospholipid, showed its positive influence on permeation flux, whereas for skin retention, it was phospholipid which showed marked effect. And amongst all three CFVs, only co-surfactant was found with positive effect on percent transmittance of the ME. Finally, the optimized formulation with desired quality attributes was found out from the design space by numerical and graphical optimization. To construct the design space, constraints were set and a total of 10 validation formulations were located, out of which 6 were having desirability of ‘1’. The model was validated by taking different formulations from design space well within the knowledge space, and percent prediction error was calculated. The overall percent prediction error was found to be -0.895 ± 6.667.

The selected phase diagram was further characterized by studying the behavior of ME with different compositions along the aqueous dilution line. All the studied ME formulation were found to be optically clear. Isotropic behavior was studied using
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polarized microscope, which revealed that no light crossed under cross-polarization. In TEM and Cryo-SEM studies, globules of the developed ME were found to be spherical and below 100 nm in size. In the studies pertaining to refraction of MEs, RI was increased from 1.369 to 1.460 on increasing the aqueous content to indicate the conversion of oily continuous phase to that of aqueous continuous phase. Simultaneously, transmittance was also increased with aqueous content from 68 to 86%. On dilution, density of the system was increased from 0.837 to 0.954 which means its closeness to that of water. This suggests a majority of ME systems belongs to aqueous phase as continuous phase. On pH assessment, ME pH was found to be in the range of 5.3 to 6.4, so as it be tolerated well by the skin.

During a study relating to conductivity on dilution with water, the studied ME composition showed low conductivity with low volume fraction of aqueous phase. As the aqueous volume fraction increased, the conductivity also increased. On analyzing the graph plotted between conductance and weight percentage of aqueous phase, it was found to exist in three different phases on three lines of fitness in linearity. Initial linear points ($r^2 = 0.998$) till 5.66% (w/w) of aqueous phase seemed to have low conductivity, therefore, confirming oil as the continuous phase in ME. Next few points showed increased conductivity but were not showing linearity ($r^2 = 0.977$ and 0.981), therefore were confirmed as bicontinuous phase of ME. This increase conductivity relates to newly developed aqueous channels. On further increment in aqueous content from 26.47% (w/w) conductivity abruptly improved in linear fashion ($r^2 = 0.991$), confirming as aqueous continuous phase in the ME systems. Overall, the measurement of conductivity has been very useful in understanding the changes in the internal state of matter so as to find which type of microemulsion phase existed in the prevailing conditions and compositions.

During the studies to understand the micromeritics of the systems, size of all studied ME was found within 100 nm with very high uniformity, PDI < 1. However, on increasing the aqueous phase, viscosity of ME was found to be increased, which is ascribable on the basis of swelling of micellar structure to an extent on the increased availability of aqueous phase for the hydration of polar head group of the constituting surfactants like phospholipids. In the studies related to drug loading determination, interestingly, it was noted to be increased on increasing the dilution. This may be attributed to the solubility effect by virtue of improved micellization. With dilution, drug loading increased from 0.739 to 2.537 mg/mL. The increased area provides extra space for the homing of drug.
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molecule apart from solubility in aqueous phase, oil phase and in \( S_{\text{mix}} \). At last, all the ME systems were checked for physical stability. No phase separation was observed at 2026 xg for 30 min (three cycles), which is in conformity with their thermodynamic state to provide high physical stability.

For better topical application, the optimized ME was incorporated in a number of gels having range of hydrophilicity and lipophilicity. On the basis of one month of storage at higher temperature and high speed centrifugation study, ME with 20% poloxamer 407 (w/w), 10% and 15% GMS (w/w) were found to be stable. The rheological studies indicated the shear-thinning nature of all of these. And textural profile showed that these developed systems exhibited good gel strength to maintain its structure on prolonged storage, extrudability from tube, ease of spreading, thus finding it patient compliable on application, and sufficient bio-adhesion that would facilitate its longer availability on the application site. Hence, all the parameters were chosen for further exploratory studies.

Further, all the gelled systems were studied for \textit{in vitro} drug release characterization and compared with drug suspension (in PBS pH 7.4) and conventional cream. Here, poloxamer-based ME gel showed better release. However, gelling hampered drug release initially to significant extent. During \textit{ex vivo} skin permeation studies, performed on pig ear pinna, poloxamer-based ME gel attained maximum cumulative drug permeation (38.14 µg) in 48 h with maximum permeation coefficient 4.76E-04 (cm².h⁻¹) and permeation flux 0.496 (µg/cm² per h). Its enhancement ratio was found to be 13.41 with respect to the conventional cream. This is maximum out of all in studied group. However without gelling, enhancement ratio for optimized ME was found to be 18.43. The enhanced permeation could be accounted to the favorable alteration in the lipid and the aqueous polar pathways to allow the penetration of drug. Similar observations were made in skin drug retention study, poloxamer-based ME gel showed 2.625 (µg cm⁻²) drug concentration in whole skin that is more than 50 folds higher in comparison to other active comparators (i.e., drug dispersion in poloxamer gel, in suspension and in conventional cream). Overall, more than 97% of drug was recovered during \textit{ex vivo} studies to account for permeation and retention both.

Further, efficacy of the developed formulation (M-ME Polox-20), with most desirable characteristics was determined on psoriatic cell lines, imiquimod induced psoriatic model, allergic contact dermatitis model and rat tail model. In all of these studies,
optimized ME as well as its gel formulation showed appreciable anti-psoriatic activity. Skin safety studies were also performed using Draize test and the developed formulation was found to be quite safe for topical application.

Chemical stability study was also performed before and after gelling of the developed system at three different conditions (i.e., 5 ± 2°C, 30 ± 2°C /65% ± 5% RH and 40 ± 2°C/75% ± 5% RH) as per the ICH guidelines. The studies revealed that the developed MTX formulation was stable at all the temperature conditions. However, its storage at room temperature should be preferred because of likely change in viscosity at other temperatures.

**CysA-Organosomal Systems**

In order to develop organosomes-based CysA formulation after having accomplished the analytics, solubility studies was initiated in different solvents viz. ethanol, propylene glycol, surfactant solutions and in buffers at 30 °C. Amongst all the studied solvent systems 10% PG was selected as the sink medium to maintain conditions for *in vitro* and *ex vivo* studies. This could achieve 33.0 mg/mL of drug’s solubility to provide sink condition. Other solvent systems like, different percent solutions of Solutol HS, Tween 20 and Tween 80 were also showed good solubilization capacity, but was observed that while analyzing drug in low concentration range chromatogram gets distorted, therefore could not be considered in the study.

To formulate organosomes, different constituents as bilayer builders, membrane stabilizer and edge activators were studied, in different percentages. Amongst the studied materials, Phospholipon 90G and Poloxamer 407 were selected as bilayer builders, cholesterol as membrane stabilizer and Span 80 and 85 the as edge activators. Organosomes were prepared by a simple three-step procedure involving mixing of the selected ingredients in a specific sequence. Foe systematic FbD based optimization, Plackett-Burman design was employed for screening the CFVs out of various potential variables including constituents and process variables used in the development of organosomes. An L12-array for 11 factors at 2 level Plackett-Burman Design was adopted for the current studies. Percent drug entrapment, skin retention, size, Q6 and flux were the key response variables investigated thoroughly for selecting the significant formulation and process variables. After analyzing the generated design using ANOVA, pareto, half-normal and normal plots were studied resulting in final identification of CFVs as percentage of phospholipid, poloxamer and span. These three CFVs were further
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systematically FbD optimized using Box-Behnken design. A total of 17 formulations of Org-CysA were prepared, as per the model design and responses were noted in terms of percent drug entrapment, size, skin retention and flexibility index. ANOVA was used to judge the significance of the terms generated by the design.

The predictive ability of the design was diagnosed by verifying the limits in different plots. In normal plots of residuals and in plots of response values, all the points were found to follow the linear relationship, which is indicative of normal distribution of data while the design is able to predict response values. In plots of response values and plots of residuals vs. experimental run order, points were randomly scattered under the constant range, between the limit lines, across the graph. This indicated that the design was able to study all the responses quite effectively. The plots between Ln Residual vs. Lambda were also studied to know whether further transformation was required or not in the design. The calculated values of lambda for vesicular size and skin retention were found to be ‘0’, hence the data were transformed to their natural logs for both of these two cases. Rest two (i.e., percent drug entrapment and flexibility index) were found to observe lambda value ‘1’ that indicated no transformation was required. Plots between Externally Studentized Residuals vs. Number of Runs and Cook’s distance vs. Run number were used to adjudge any abnormality in the design in terms of outlier points. All the points were noticed to be positioned well within the limit, i.e., no outlier was found with any of the responses studied. All the generated values remained within the desirable limits in plots between Dffits vs. Runs and DFBETAS vs. Run, thus ruling out any plausibility of analyzing any erroneous data.

The influence of CFVs on varied responses (i.e., percent drug entrapment, vesicular size, skin retention and flexibility index), 3D response surface plots, 2D contour plots and perturbation charts were analytically studied and interpreted. In case of percent drug entrapment, increasing the quantities of Span and phospholipid resulted in increases of drug entrapment, whereas, Poloxamer showed opposite effect on percent drug entrapment. In case of the size, moderate levels of all the three components (i.e., phospholipid, Poloxamer and Span), yielded vesicles with maximum size. And smaller sized vesicles were found to be located near lower and higher values of the components. Effects of phospholipid and Span were found to be favorable for the drug skin retention. As the amount increased, drug skin retention also improved. On the contrary, increase in the amounts of poloxamer resulted in the formulations yielding lower values of skin retention. Increase in amounts of phospholipid and Span increased the flexibility. Effect
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of poloxamer on flexibility was found to be negligible at all the levels of Span, whereas in presence of phospholipid, improved flexibility was observed at higher levels of both poloxamer and phospholipid. Finally, the optimized formulation (BBDOPT-1) with desired quality attributes was found out from the design space by numerical as well as graphical optimization. To get the design space, constrains were set and design application yielded a total of 37 prediction formulations, out of which 5 were having desirability of ‘1’. The model was validated by taking different formulations from the chosen design and knowledge space, and the percent prediction error was calculated. The overall percent prediction error (± SD) was found to be –1.291 ± 5.995.

For the purpose of topical application, it is necessary to bring the delivery system in appropriate semisolid form, hence the selected system, BBDOPT-1 was gelled using Carbopol gel in three different concentrations i.e., 0.5, 1.0 and 1.5% w/w. On screening, only 1.0% gel (CysA-Org Cbp-1.0) was found to be better on the basis of their physical appearance and stability, while rest of the two formulations were found to be stable to some extent.

In rheological study, the developed gel system (CysA-Org Cbp-1.0) showed shear-thinning nature. And textural profile showed that the developed systems exhibited good gel strength to maintain its structure on prolonged storage, extrudable from tube, ease of spreading which make it patient-compliant. Also, a sufficient level of bioadhesion was recorded to facilitate the longer availability of medication on the application site.

Further, the developed gel systems were studied for their in vitro release behaviour and compared with that of the corresponding drug suspension and conventional cream. Here, the developed gel system showed better release; however, gelling hampered drug release up to a certain extent. On testing the various release models for the best fit, first-order release kinetics was observed with organosomal systems in its dispersion and as well as with the gel form. Ex vivo skin permeation studies were performed on pig ear pinna and the developed gel system was compared with traditional liposomal system (with and without gel), drug dispersion in Carbopol gel, drug suspension and conventional cream. Developed gel system attained maximum cumulative drug permeation (111.64 μg) in 24 h with maximum permeation coefficient 1.47 E-04 (cm². h⁻¹) and permeation flux 4.43 (μg/cm² per h). Its enhancement ratio was found to be 5.34 for gel and 4.61 for dispersion, with respect to traditional liposomal gel and liposomal dispersion, respectively, which is maximum in the under study group. The improved
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Permeation of drug by organosomal system may be ascribed on the basis of phenomenon wherein the vesicles with flexible membranes could find the way into the skin as against the naturally occurring transdermal osmotic gradient across the SC. It is the pliability of vesicles which could be beneficial to retain its integrity while traveling through intercellular channels of skin.

In drug skin retention study, the developed gel system showed 1.225 (µg cm⁻²) drug concentration in the whole skin that is higher other than 12 folds in comparison to the gel liposomal system. However, in comparison to other active comparators (i.e., carbopol gel, drug suspension and drug’s conventional cream), it showed more than 20 folds higher drug skin retention. A total of 97% drug was recovered, which accounted for the drug retained in the skin, permeated through skin as well as assayed drug concentration of the formulation applied in the donor compartment.

Further, efficacy of the developed formulation was observed by cell line studies, imiquimod induced psoriatic model, allergic contact dermatitis model and rat tail model. In all of these studies the FbD optimized organosomal systems as well as its gel formulation showed significant and distinct anti-psoriatic activity. Skin safety studies were also performed using Draize test and developed formulation was found to be safe for topical application.

Stability study was also performed before and after gelling of the developed system at three different conditions (i.e., 5 ± 2°C, 30 ± 2°C /65% ± 5% RH and 40 ± 2°C/75% ± 5% RH). Overall, the stability studies corroborated the stability of the developed CysA formulation at all the studied temperature, while increased temperature noticeably reduced the gel characteristics and assay. Thus, its storage at room temperature is recommended.

In a nutshell, diverse studies in all the possible directions and dimensions have been undertaken and investigated to accomplish the varied set objectives to reach to the carrier based-formulations of MTX and CysA with optimum performance attributes. Utmost care and deep thinking have been undertaken in drawing decisions at each and every level of the envisioning of experimental protocol plan, design and execution. In case of MTX, ME-based system and in case of CysA, organosomal-based system could achieve the desired results explicitly, as per the conceived hypothesis and projections. For methotrexate, it is the size, surface area and solubility by way of combination of surfactants and other excipients as well as their supramolecular association, which have
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been held as causative and responsible factors. And for CysA, it is majorly the unique vesicular characteristics like membrane-flexibility coupled with aqua-lipoidal compartmentalization of drug molecules, which has made it possible for the skin penetration, permeation and retention and interactions with molecular targets as the desired site. In organosomes, the novelty lies in generating the vesicles out of the micellar systems by choosing the right form of composition as well as experimental and processing conditions. In both the carriers, remarkable changes can be noted on different planes of working to favor the delivery and pharmacodynamics. Both the drugs, in fact, do suffered from their odd physicochemical characteristics, such as unfavourble solubility, permeability, penetration, and surface properties, while skin barrier (in the normal and diseased state) is generally the opposing factor. But the carriers, i.e., ME for MTX and organosomes for CysA have brought the desirable modifications in the said properties of molecules as well as in the barrier function of skin, as reflected in the final outcomes. Besides, the efficacy related aspects in other critically important aspects of all the drug delivery systems like safety, stability and scalability of process have been proved to be acceptable with satisfaction.

Conclusively, the extensive and intensive efforts within the framework of the plan could be able to accomplish the final goal of the optimized delivery of MTX and CysA in successful and satisfactory manner. And, the work, on evolutionary path, further proposes for immense potential for commercial exploitation and extension, implying its transfer of technology (TOT) on the shop floor of market facility and multilevel testing in clinics so as to realize the dream of commercial level application for the suffering patients.